



ALKALs are in vivo ligands for ALK family receptor tyrosine kinases in the neural crest and derived cells

Andrey Fadeev^{a,b}, Patricia Mendoza-Garcia^{c,d}, Uwe Irion^a, Jikui Guan^c, Kathrin Pfeifer^c, Stephanie Wiessner^e, Fabrizio Serluca^e, Ajeet Pratap Singh^{a,e}, Christiane Nüsslein-Volhard^{a,1}, and Ruth H. Palmer^{c,1}

^aMax-Planck-Institut für Entwicklungsbiologie, 72076 Tübingen, Germany; ^bMax-Planck-Institut für Infektionsbiologie, 10117 Berlin, Germany; ^cDepartment of Medical Biochemistry and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy, Gothenburg University, Gothenburg, Sweden; ^dDepartment of Molecular Biology, Umeå University, Umeå, Sweden; and ^eNovartis Institutes for Biomedical Research, Cambridge, MA 02139

Contributed by Christiane Nüsslein-Volhard, December 9, 2017 (sent for review November 2, 2017; reviewed by Marianne Bronner and Robert Kelsh)

Mutations in anaplastic lymphoma kinase (ALK) are implicated in somatic and familial neuroblastoma, a pediatric tumor of neural crest-derived tissues. Recently, biochemical analyses have identified secreted small ALKAL proteins (FAM150, AUG) as potential ligands for human ALK and the related leukocyte tyrosine kinase (LTK). In the zebrafish *Danio rerio*, DrLtk, which is similar to human ALK in sequence and domain structure, controls the development of iridophores, neural crest-derived pigment cells. Hence, the zebrafish system allows studying Alk/Ltk and Alkals involvement in neural crest regulation in vivo. Using zebrafish pigment pattern formation, *Drosophila* eye patterning, and cell culture-based assays, we show that zebrafish Alkals potentially activate zebrafish Ltk and human ALK driving downstream signaling events. Overexpression of the three DrAlkals cause ectopic iridophore development, whereas loss-of-function alleles lead to spatially distinct patterns of iridophore loss in zebrafish larvae and adults. *alkal* loss-of-function triple mutants completely lack iridophores and are larval lethal as is the case for *ltk* null mutants. Our results provide in vivo evidence of (i) activation of ALK/LTK family receptors by ALKALs and (ii) an involvement of these ligand-receptor complexes in neural crest development.

ALK | ALKAL | FAM150 | Ltk | iridophore

Receptor tyrosine kinases (RTK) constitute a large family of surface receptors involved in a wide range of cellular processes, both in normal development and carcinogenesis. RTK inhibitors show strong promise as a targeted patient-specific therapy (1). Despite intense interest in RTKs, two of them remain obscure in terms of their biochemistry and native function— anaplastic lymphoma kinase (ALK) and the related leukocyte tyrosine kinase (LTK), which constitute a subgroup of receptor tyrosine kinases (ALK RTKs) involved in human cancers (2).

Human ALK (HsALK) has a unique extracellular domain (ECD) composition among RTKs containing two MAM domains (after meprin, A-5 protein and receptor protein-tyrosine phosphatase μ), an LDLa domain (low-density lipoprotein), and a glycine-rich domain (GR). In comparison, the ECD of human LTK (HsLTK) is smaller and lacks the LDLa and both MAM domains (Fig. 1A) (2). Although ALK ligands have been identified in invertebrates, no evidence has been presented describing ligand activation of vertebrate ALKs in vivo.

While knowledge of endogenous ALK and LTK function in humans is limited, ALK, as a result of protein fusion, overexpression, or activating mutations, is involved in the development of various cancer types (2). Mice lacking ALK exhibit defects in neurogenesis and testosterone production but are viable, as are *ALK;LTK* double mutants (3–5). The *Drosophila melanogaster* and *Caenorhabditis elegans* genomes each contain a single ALK RTK: *Dalk* and *sdc-2*, respectively (6, 7). Zebrafish (*Danio rerio*) has two members of the ALK RTK family—Ltk (DrLtk) and Alk (DrAlk) (8, 9). DrLtk possesses two MAM domains, while DrAlk has a smaller ECD that lacks one MAM domain (Fig. 1A) (8). Thus, based on overall domain structure,

DrLtk displays many similarities to HsALK. Mammalian *LTK* is expressed in pre-B and B lymphocytes and brain (10, 11), whereas zebrafish *alk* is expressed in the developing central nervous system (9). Zebrafish *ltk* is expressed in neural crest-derived iridophores and mammalian *ALK* in the central nervous system as well as in peripheral ganglia that are neural crest derived (8, 12, 13).

In zebrafish, Ltk has a firmly established role in the development of iridophores in pigment pattern formation: mutants in *shady* (*shd*), the gene encoding DrLtk, lack iridophores and display patterning defects (8, 14, 15) (Fig. 1B). Zebrafish develops two pigment patterns throughout its life. The first is a larval pattern that derives directly from migrating neural crest cells during early embryogenesis. In the body dorsal to the yolk sac, this simple pattern is comprised of relatively evenly distributed yellow xanthophores and three stripes of black melanophores, accompanied by silvery iridophores lining the dorsal and ventral stripes. Dense sheets of iridophores cover the iris of the eye and form lateral patches on the swim bladder (Fig. 2C) (16, 17). The adult zebrafish pattern of longitudinal light and dark stripes develops during metamorphosis (3–6 wk after fertilization). Adult iridophores arise from stem cells located in the dorsal root ganglia; these stem cells are multipotent and produce all three pigment cell types, as well as peripheral neurons and glia (18, 19). Iridophores appear in the skin along the horizontal

Significance

Neuroblastoma is a pediatric tumor arising from the neural crest. Dysregulation of the receptor tyrosine kinase ALK has been linked to neuroblastoma, making it important to understand its function in native conditions. In zebrafish, a related receptor—Ltk—is also expressed in neural crest and regulates development of specific pigment cells—iridophores. Ligands activating human ALK were recently identified as the ALKAL proteins (FAM150, AUG) by biochemical means. Our data show that this ligand-receptor pair functions in vivo in the neural crest of zebrafish to drive development of iridophores. Removal of Ltk or all three zebrafish ALKALs results in larvae completely lacking these cells. Using *Drosophila* and human cell lines, we show evolutionary conservation of this important interaction.

Author contributions: A.F., P.M.G., U.I., C.N.-V., and R.H.P. designed research; A.F., P.M.G., U.I., J.G., K.P., S.W., F.S., A.P.S., and R.H.P. performed research; A.F., P.M.G., U.I., S.W., F.S., and A.P.S. contributed new reagents/analytic tools; A.F., P.M.G., U.I., J.G., K.P., S.W., F.S., A.P.S., C.N.-V., and R.H.P. analyzed data; and A.F., C.N.-V., and R.H.P. wrote the paper.

Reviewers: M.B., California Institute of Technology; and R.K., University of Bath.

The authors declare no conflict of interest.

This open access article is distributed under [Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 \(CC BY-NC-ND\)](https://creativecommons.org/licenses/by-nc-nd/4.0/).

¹To whom correspondence may be addressed. Email: christiane.nuesslein-volhard@tuebingen.mpg.de or ruth.palmer@gu.se.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1719137115/-DCSupplemental.

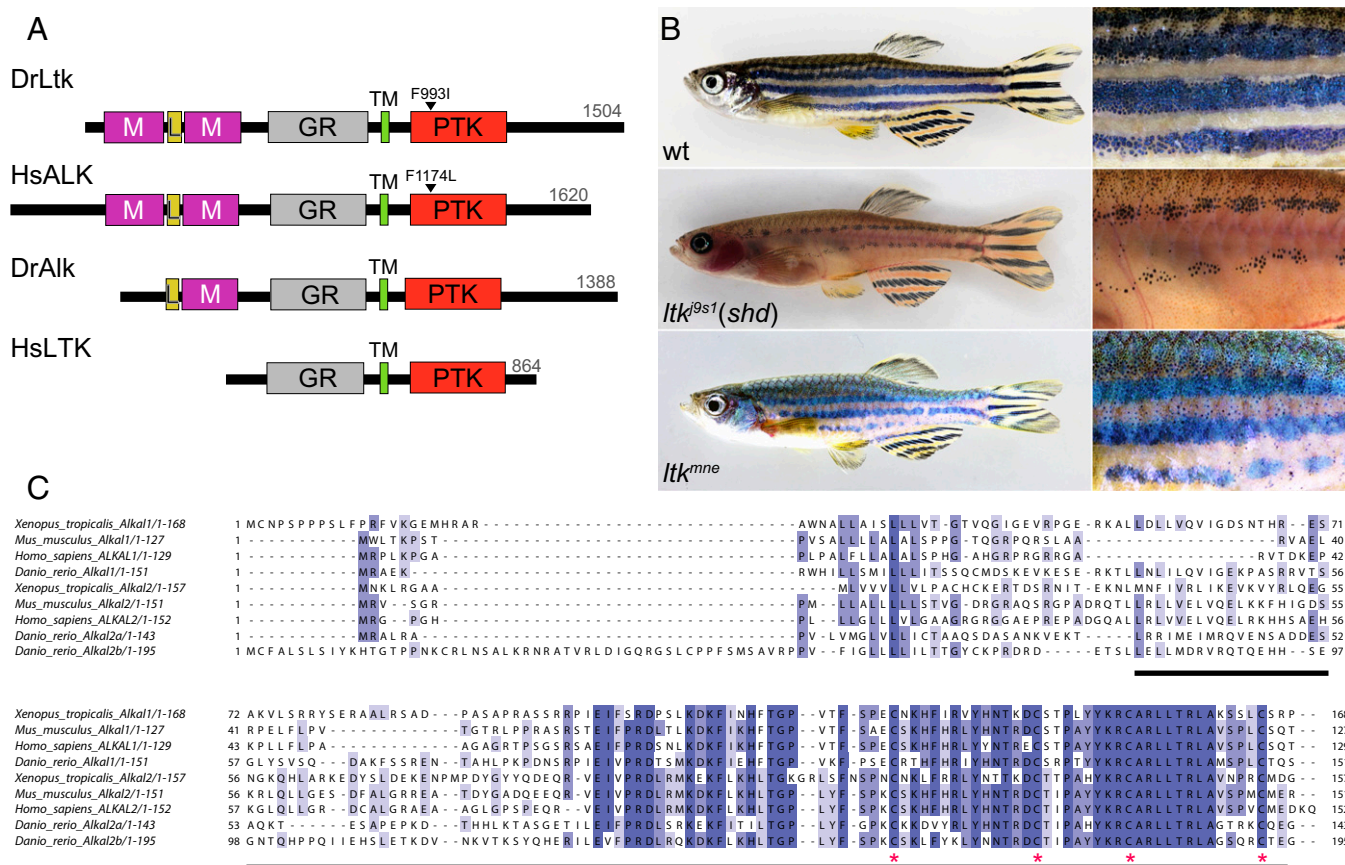


Fig. 1. Ectopic expression of ALKs and overactivation of DrLtk leads to supernumerary iridophores in *D. rerio*. (A) Domain structure of the human and zebrafish ALK/LTK RTK family. GR, glycine rich; L, LDLA; M, MAM; PTK, kinase domain; TM, transmembrane domain. The activating F993I (DrLtk) and F1174L (HsALK) mutations within the kinase domain are indicated. (B) *ltk* loss-of-function *shady* mutants (*ltk*^{9s1}) lack iridophores, while gain-of-function *moonstone* (*ltk*^{mne}) exhibit increased numbers of iridophores. (C) Alignment of ALKAL proteins from different species. Underlined, the FAM150 domain; red asterisks, conserved Cys. Note high conservation of the C-terminal half of the FAM150 domain.

myoseptum where they proliferate and spread dorso-ventrally to successively form the light stripes by patterned aggregation (15, 17, 18, 20, 21). Numerous iridophores cover the iris of the eyes, the operculum of the gills, and the exposed margins of the scales. Proliferation and spreading of iridophores along the anteroposterior axis is controlled by homotypic competition (22). Interestingly, while iridophores are indispensable for striped pattern formation in the trunk, they are not required for stripe formation in the fins (15, 18–20).

In *shd* mutants, iridophores are missing; complete loss of function in *ltk* leads to larval lethality, whereas mutants carrying weaker alleles are adult viable (Fig. 1B) (8, 15, 21, 23). *ltk* is expressed in the early neural crest and gradually becomes restricted to iridophores (8). Chimeras, obtained by blastomere transplantations, revealed that *ltk* is autonomously required in iridophores (8, 15). Taken together, these findings lead to the conclusion that *ltk* is required for the establishment and proliferation of iridophores and their progenitors from multipotent neural crest cells (8).

Recently, a gain-of-function zebrafish mutant *moonstone* (*ltk*^{mne}), harboring a hyperactive point mutation in Ltk (F993I), analogous to the human ALK-F1174 hotspot mutation observed in neuroblastoma patients, was described (14). Point mutations are the most common cause of ALK activation in both familial and sporadic neuroblastoma, and the corresponding mutation in HsLTK, F568L, has been shown to cause ligand-independent

activity (24–30). Fish carrying the *ltk*^{mne} mutation display ectopic iridophores in the trunk as larvae, and an increased number of iridophores on scales and fins as adults, giving them a strong blue-green sheen (Fig. 1B). Moreover, treatment with the ALK inhibitor TAE684 during metamorphosis decreases the number of iridophores, suggesting that Ltk activity is continually required for iridophore development and maintenance. When presented with a wild-type environment in chimeric animals, *ltk*^{mne} iridophores can massively overgrow in the surrounding skin, suggesting that Ltk is involved in control of iridophore proliferation by homotypic interactions (14, 22).

The identity of the ligand(s) for vertebrate ALK and LTK has been elusive for many years, and although ligands for ALK have been identified in invertebrates—Jeb in *Drosophila* and HEN-1 in *C. elegans*—homologous ligands in vertebrates remain unidentified (7, 31–34). Recently human secreted small proteins ALKAL1 and 2 (for “ALK And LTK ligand”, HsALKALs), which were previously reported as family-with-sequence-similarity-150 (FAM150) or Augmentor (AUG), have been shown to activate human ALK and LTK in cell culture and when coexpressed in *Drosophila* eyes (35–37). Characterized by the highly conserved FAM150 domain at their C termini and four conserved cysteines (Fig. 1C), HsALKALs bear no obvious similarities to Jeb and HEN-1. In contrast to the two HsALKALs, the zebrafish genome harbors three ALKAL homologs (DrAlkals), encoded by *alkal1*, *alkal2a*, and *alkal2b*. Hereafter we will refer to HsALKALs

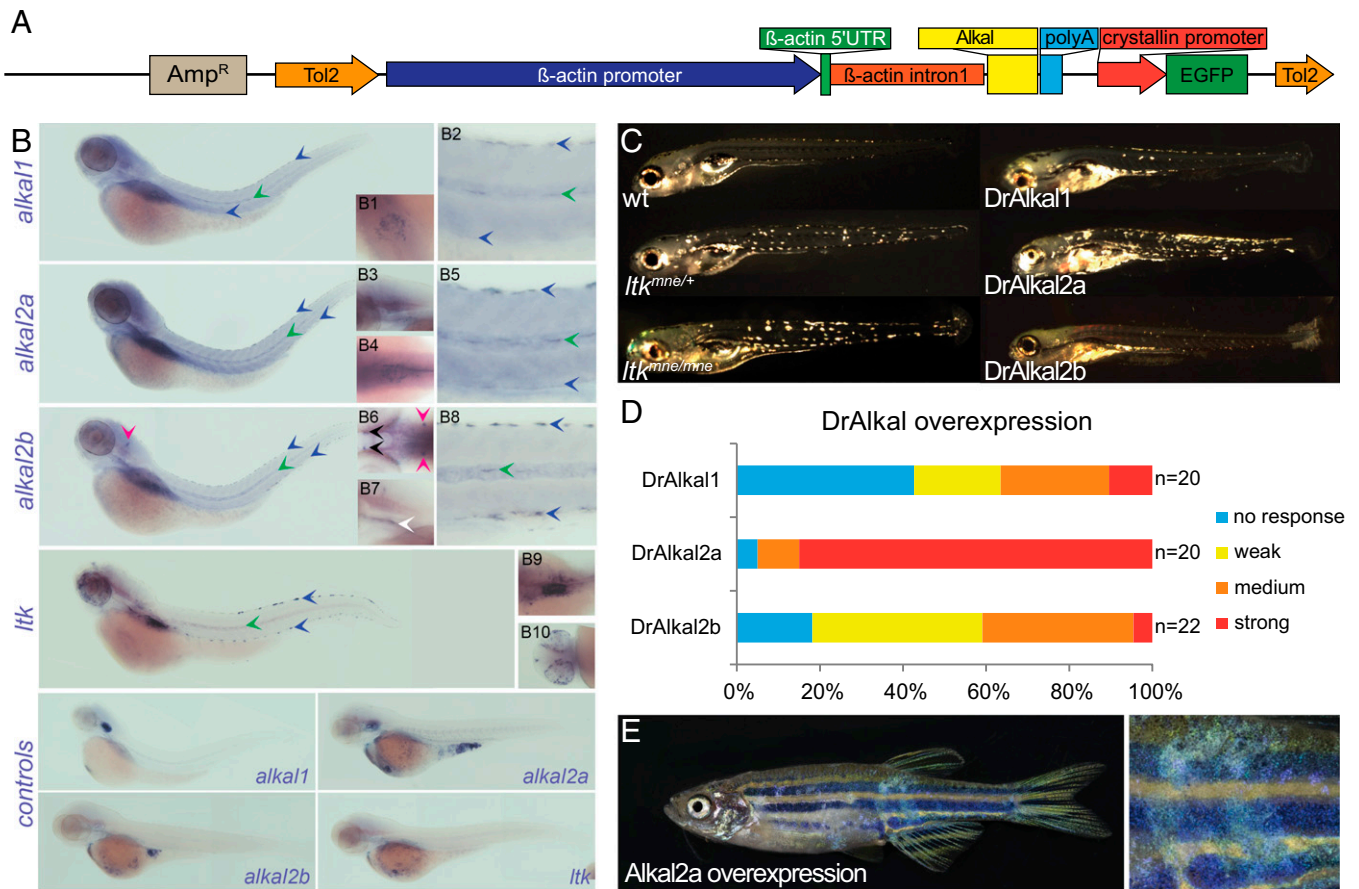


Fig. 2. Expression of DrAlkals is sufficient for ectopic production of iridophores. (A) Schematic of ALKAL-expression constructs. Amp^R, ampicillin resistance; Tol2, medaka Tol2 transposase recognition sequence. (B) *alkal1* expression in 72 hpf *albino* larvae. Weak *alkal1* signal can be observed in the head, swim bladder (B1), notochord (B2; green arrowheads), and iridophores in dorsal and ventral stripes (B2; blue arrowheads). *alkal2a* mRNA is detected in the head (B3), swim bladder (B4), notochord (B5; green arrowheads), and iridophore stripes (B5; blue arrowheads). *alkal2b* mRNA is detected in swim bladder, a row of cells at the ventral aspect of the head (B7; white arrowhead), bilateral clusters in the head (B6; red arrowheads), behind the eyes (B6; black arrowheads), notochord (B8; green arrowheads), and iridophore stripes (B8; blue arrowheads). *ltk* expression is visible in swim bladder (B9), eyes (B10), notochord (green arrowheads), and iridophores (blue arrowheads). Negative expression, corresponding sense probes. (C) Supernumerary iridophores were observed in 5 dpf larvae upon ectopic expression of indicated DrAlkals in F₀-injected fish. *ltk^{mme/+}* larvae are used as positive controls. (D) Distribution of phenotypes of C. Fisher exact probability test showed significant differences ($P < 0.001$) in phenotype distribution in the following comparisons: any Alkal overexpressing fish against uninjected control and Alkal2a overexpressing fish against Alkal1 or Alkal2b overexpressing fish. No significant difference was found between Alkal1 and Alkal2b overexpressing fish ($P = 0.2$). (E) Mosaic overexpression of *alkal2a* produces patches of supernumerary iridophores in adults.

and DrAlkals together as ALKALS. Physiological function ascribed to ALKALS has been lacking in any model organism, as has in vivo genetic evidence supporting activation of ALK RTK signaling by ALKALS. The structural similarities together with the shared neural crest pattern of tissue expression between zebrafish *Ltk* and human ALK offer an opportunity to define the role of the ALKAL ligands in activation of the ALK family of RTKs in vivo.

In this study, we present evidence for an in vivo interaction between ALKALS and DrLtk, a member of the vertebrate ALK RTK family. Local overexpression of the DrAlkals results in excess iridophore production in larvae, and an iridophore overgrowth in the adult striped pattern. We show in mammalian cell cultures and in *Drosophila* eyes that DrLtk and HsALK are activated by zebrafish and human ALKALS. HsALK activation by the zebrafish Alkal2a in human neuroblastoma cells demonstrates evolutionary conservation of this interaction. Similar to *ltk* loss-of-function mutants, knockout of the zebrafish *alkal* genes reduces iridophore numbers, indicating that these ligands act in a spatially specific, partially redundant manner. The triple mutant, in which all three Alkals are knocked out using the CRISPR/Cas9 system, is larval lethal and displays a complete loss of iridophores as does the *ltk* null allele. This demonstrates

that all three Alkals are necessary to fully activate DrLtk in target tissues. The activation of DrLtk and HsALK by human and zebrafish ALKALS provides genetic evidence for the interaction of ALKALS and ALK/LTK RTKs as an evolutionarily conserved functional signaling unit in the neural crest and neural crest-derived tumors.

Results

DrAlkals and DrLtk Have Similar Expression Domains. The zebrafish genome harbors three FAM150 domain containing ALKAL homologs (DrAlkals), encoded by *alkal1*, *alkal2a*, and *alkal2b* (Fig. 1C). We employed in situ probes against *alkal1*, *alkal2a*, and *alkal2b* to characterize their larval expression patterns (Fig. 2B). *alkal1* signal was weak but detectable in the notochord and iridophore stripes, the eye, and the swim bladder. *alkal2b* expression was wider but strong in the swim bladder and single cells of unknown identity in the head. The strongest signal was observed for *alkal2a*, expressed in the notochord and iridophore stripes of the trunk, as well as in the eye and swim bladder. In agreement with previous data (8), we observed *ltk* mRNA in the head and iridophores of the trunk and eyes, as well as in the swim

bladder (Fig. 2B). Thus, *alkals* appear to be expressed in a pattern overlapping with that of *ltk*.

Overexpression of ALKALs Results in Ectopic Iridophores in Zebrafish.

To investigate the effect of ALKALs on iridophore development, we used a genome integration cassette driving Alkal expression under the ubiquitous β -*actin* promoter to drive mosaic overexpression (Fig. 2A). Clonal expression of all three zebrafish Alkals resulted in the appearance of ectopic iridophores in 5 d post fertilization (dpf) larvae, similar to *ltk^{mne}* mutants (Fig. 2C). The phenotypes were classified into four classes by the number of ectopic iridophore clusters (or single cells): (i) no response, (ii) weak (<10), (iii) medium (10–30), and (iv) strong (>30) phenotypes (Fig. S1A). While Alkal1- and Alkal2b-overexpressing larvae exhibited mostly no, weak, or medium phenotypes, expression of Alkal2a produced predominantly strong responses (Fig. 2D, shown are the results of one of the two experiments performed). Wild-type controls did not exhibit ectopic iridophores. As adults, Alkal2a overexpressing fish exhibited patches of supernumerary iridophores on scales, fins and eyes—a phenotype similar to that of *moonstone* mutants (Fig. 2E). Strikingly, patches of dense clusters of tumor-like disorganized iridophore overgrowth appeared in the skin resembling the iridophore clusters in chimeras in which *ltk^{mne}* iridophores develop in juxtaposition with *ltk⁺* iridophores. Ectopic iridophores could also be induced by human and mouse ALKALs (Fig. S1B). Taken together, these results suggest that overexpression of ALKALs leads to the activation of endogenous ALK family RTKs in vivo.

Human and Zebrafish ALKALs Activate Zebrafish Ltk in the *Drosophila* Eye.

We next analyzed the activation of ALK/Ltk receptors by Alkal proteins by coexpressing them in *Drosophila* eyes, taking advantage of the clear readout of this system. In brief, ligands and receptors were ectopically expressed in the fly eye using the *GAL4-UAS* system (38) employing *pGMR-GAL4* to drive expression in the developing fly eye. Activation of the ALK/LTK receptors in this assay results in a rough eye phenotype (35, 39). Both human ALKALs activated HsALK and lead to a rough eye phenotype, in agreement with previous data (35) (Fig. 3A). As with the HsALK receptor, both HsALKALs robustly activated DrLtk, killing flies raised at 25 °C. Weaker transgene expression, achieved by rearing flies at 18 °C, resulted in severe rough eye phenotypes (Fig. 3A). No phenotype was observed when wild-type Ltk was expressed alone (Fig. 3A). In contrast, expression of the constitutively active *Ltk^{mne}* led to a rough eye phenotype (Fig. S2A). Ectopic expression of either of the zebrafish Alkals together with DrLtk (but not alone) resulted in rough eyed flies, thus all three DrAlkals are able to activate DrLtk (Fig. 3B). These data suggest the ability of ALKAL proteins to activate ALK/LTK receptors is conserved across vertebrates.

ALKALs Activate Zebrafish Ltk and Human ALK. To investigate activation of DrLtk by the ALKAL proteins in more detail and in a more controlled environment, we employed a rat PC12 cell neurite outgrowth assay. Expression of zebrafish Alkals or DrLtk alone did not lead to neurite differentiation, unlike *Ltk^{mne}*, which promoted neurite extension and ERK activation downstream of Ltk in the absence of ALKALs (Fig. 3 C–E). This effect was abrogated by the addition of the ALK inhibitor lorlatinib, shown to inhibit the activity of human ALK-F1174 activating mutations, analogous to *ltk^{mne}* (40, 41) (Fig. 3 C, D, and F). Coexpression of zebrafish Alkals with DrLtk also led to robust induction of neurite outgrowth, phosphorylation of the Ltk receptor, and ERK activation, in agreement with our findings in the *Drosophila* eye assay (Fig. 3 C–E). Furthermore, DrLtk was activated by both human ALKALs (Fig. S2 B and C), whereas DrAlkal2a (but not DrAlkal1 or DrAlkal2b) led to robust

neurite outgrowth when expressed together with HsALK (Fig. S2D). Thus, this independent assay also supports ALK family RTK activation by ALKALs in a manner dependent on the tyrosine kinase activity of the receptor.

Endogenous HsALK Signaling in Neuroblastoma Cells Is Activated by Zebrafish Alkal2a.

To address whether endogenous levels of human ALK respond to DrAlkal proteins, we employed DrAlkal-conditioned medium to stimulate IMR-32 neuroblastoma cells that express wild-type ALK, using phosphorylation of ALK and ERK as a readout. As shown previously, ALK was robustly activated by both HsALKALs (Fig. 3G and Fig. S2D) (35). In agreement with our results in PC12 cells, endogenous HsALK was visibly activated by Alkal2a, but not Alkal1 or Alkal2b, suggesting that these proteins may be less effective in activating human ALK as well as the DrLtk receptor in larvae (Fig. 3G). Similar results were obtained in the NB1 neuroblastoma cell line, where *ALK* lacks exon 2–3 (Fig. 3H). In summary, using multiple approaches, we demonstrate that ALKALs are able to activate zebrafish Ltk and human ALK.

Regulation of Iridophores by DrAlkal Requires Ltk.

To test whether DrLtk is critical for DrAlkal-dependent induction of iridophores in zebrafish, we investigated the effect of DrAlkal2a overexpression (performed as described above, Fig. 2) in fish lacking *ltk*. To do this, we generated fish carrying knockout frameshift mutations in *ltk* using the CRISPR/Cas 9 system to introduce 2-bp and 22-bp insertions in the first exon. *ltk* knockout (*ltk^{ko}*) larvae did not develop any iridophores (Fig. 4A). Similarly, larvae treated with the ALK inhibitor lorlatinib did not develop iridophores, consistent with the results of neurite outgrowth assays (Fig. 4B). When overexpressed in *ltk^{ko}* mutants, DrAlkal2a was no longer able to produce ectopic iridophores, whereas expression of DrAlkal2a in heterozygous or wild-type siblings led to increased number of iridophores as observed earlier (Fig. 4A). This indicates that DrAlkal2a acts upstream of DrLtk to generate iridophores. The *ltk^{ko}* mutant fish did not survive past 6 dpf, and lethality may be in part due to failure of the swim bladder to fill with air. Interestingly, the adult viable *shady* allele *ltk^{js91}* was still able to produce ectopic iridophores in response to DrAlkal2a overexpression, suggesting the production of Ltk^{js91} protein that is still ligand-responsive in these *ltk* (*shd*) mutants (Fig. 4A). We also observed that the hyperactive *ltk^{mne}* allele can be further activated by ectopic expression of DrAlkal2a, which shows dense iridophore patches in the striped pattern, overgrowing the dark melanophore stripe, a phenotype never seen in *ltk^{mne}* individuals (Fig. 4C). These results support the role of DrAlkals as effectors of iridophore development acting specifically through the DrLtk receptor.

Loss of DrAlkals Leads to Spatially Specific Loss of Iridophores.

To better understand the function of endogenous DrAlkals and further strengthen evidence of ALK RTK activation by ALKALs in vivo, we knocked out DrAlkals utilizing CRISPR/Cas9 technology. Many F₀ mosaic loss-of-function fish (especially those mutant for *alkal2a*) exhibited patches devoid of iridophores during metamorphosis, which in most cases disappeared by adulthood, presumably due to proliferation of residual iridophores successfully filling the space devoid of iridophores (22). Since presence of iridophores during metamorphosis is crucial for correct stripe formation in the trunk (but not fins), we investigated the iridophores and stripe pattern of adult mutants completely lacking functional Alkals. *alkal1* mutants exhibited strong reduction of iridophore number exclusively on the operculum with no detectable phenotype in the trunk (Fig. 4D). *alkal2a* mutant fish showed reduced dense iridophores in the light stripes of the trunk, particularly in the anterior region, resulting in irregular interruptions of melanophore stripes (Fig. 4D). *alkal2b* mutant fish, in contrast, did not exhibit any detectable trunk phenotype

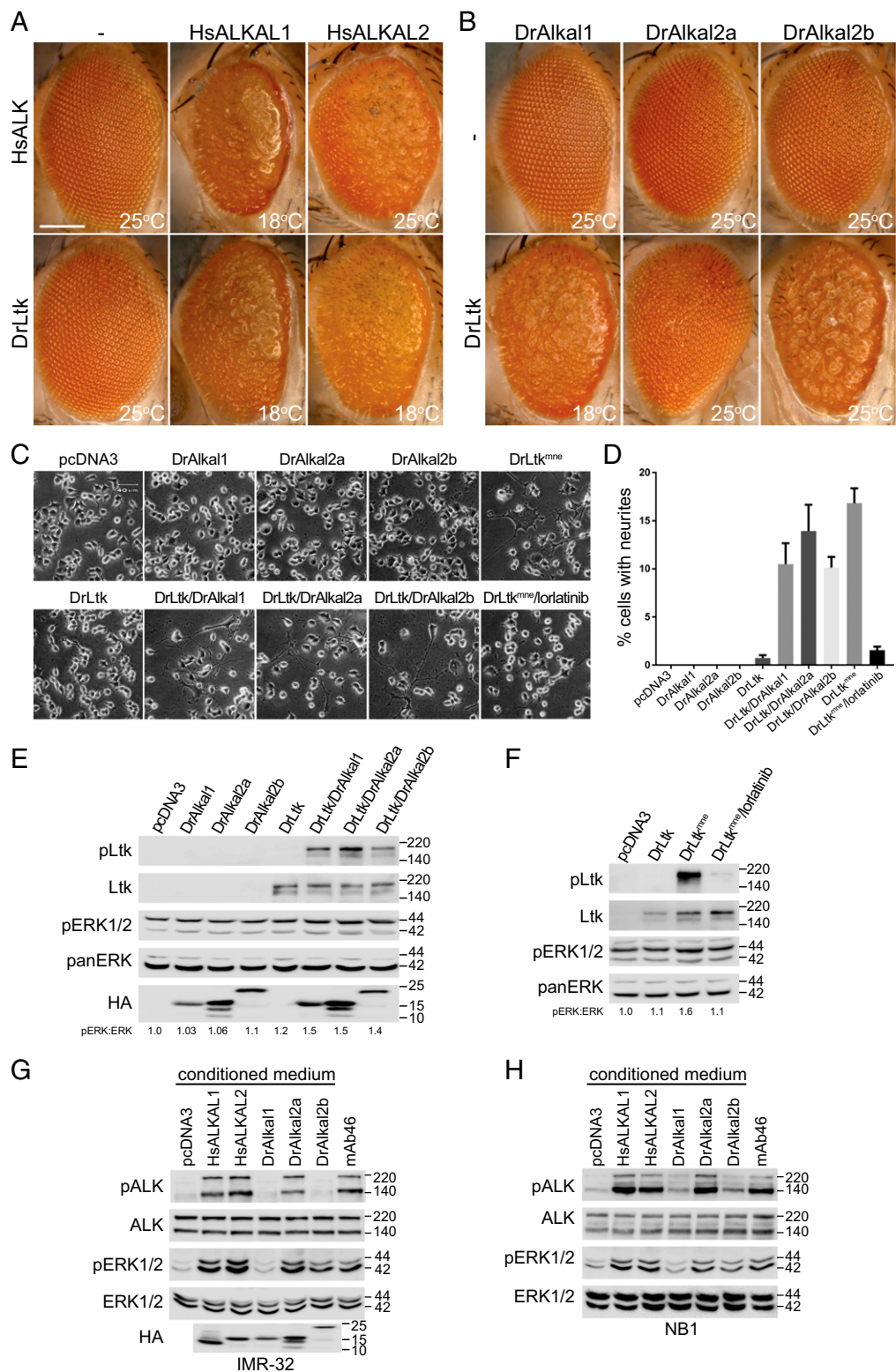


Fig. 3. Human and zebrafish ALKs activate DrLtk and HsALK. (A) Ectopic expression of either HsALK or DrLtk in combination with HsALKs, but not alone, leads to a disorganization of the ommatidial pattern in *Drosophila* eye. (B) Expression of DrAlkals and DrLtk together, but not alone, results in rough eye phenotypes. Temperatures at which flies were grown are indicated. (C) Neurite outgrowth in PC12 cells expressing either *pcDNA3* vector control, DrAlkals or DrLtk alone, combinations of DrLtk and DrAlkals, or DrLtk^{mne} in the presence or absence of the ALK tyrosine kinase inhibitor lorlatinib as indicated. (D) Quantification of neurite outgrowth in PC12 cells as indicated in C. Error bars: SD. (E) Immunoblot of whole-cell lysates from PC12 cells expressing DrLtk and DrAlkals alone or in combination as indicated. (F) Immunoblot of whole-cell lysates from PC12 cells expressing wild-type DrLtk and DrLtk^{mne} in the presence or absence of lorlatinib as indicated. (G and H) Activation of endogenous HsALK by ALKAL-containing medium in neuroblastoma cell lines: IMR-32 (G) and NB1 (H). Antibodies against ALK and ERK were used as loading controls. α -myc, total DrLtk protein; HA antibodies, ALKAL proteins; pALK-Y1278, phosphorylated HsALK or DrLtk. pERK1/2 indicates activation of downstream signaling. Agonist mAb46 ALK was employed as a positive control.

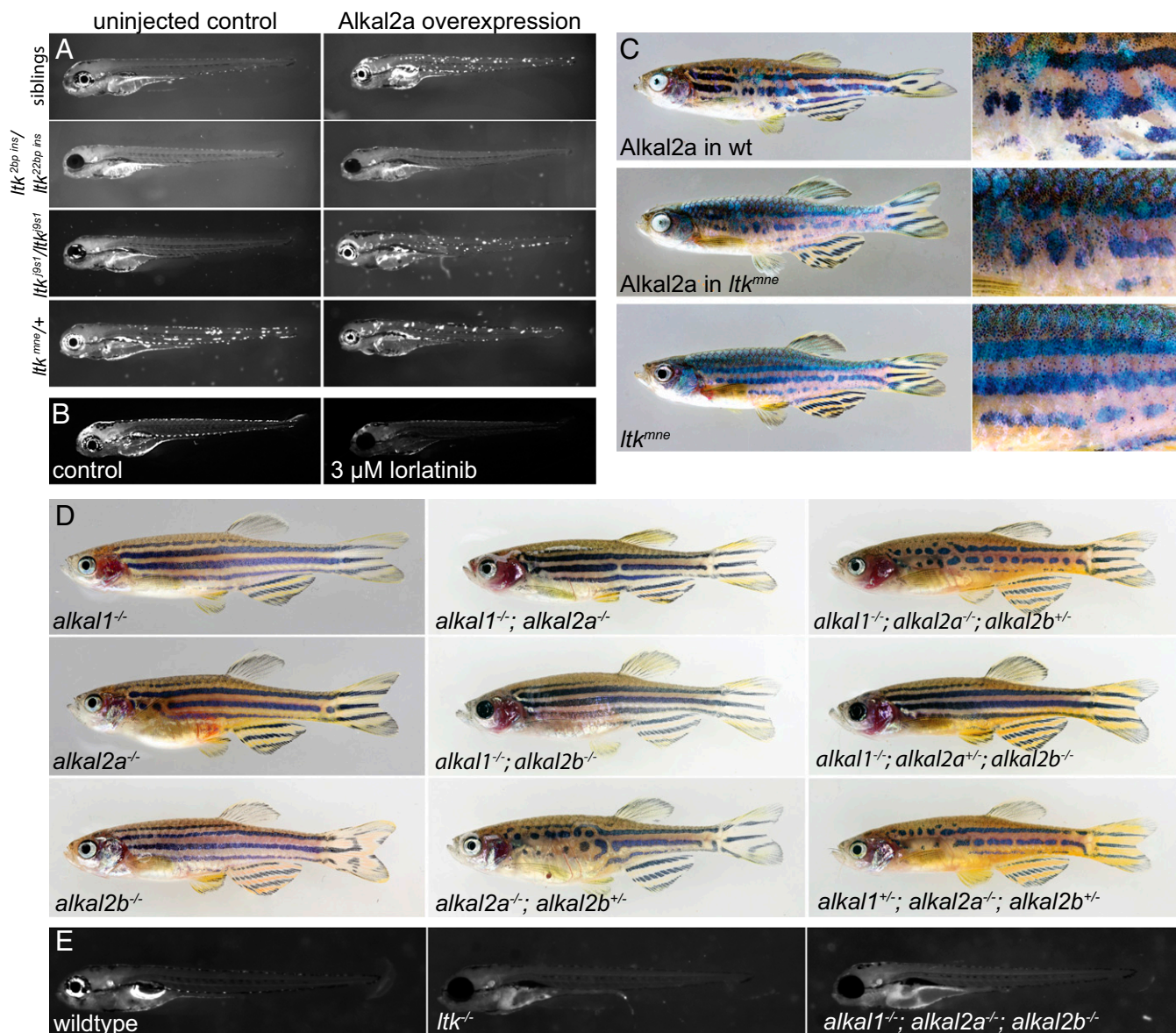


Fig. 4. DrAlkals affect iridophore development via DrLtk. (A) Ectopic expression of DrAlkal2a does not rescue loss of iridophores in 4 dpf transheterozygous *ltk* knockout zebrafish larvae (2 bp insertion/22 bp insertion, $n = 9/9$), whereas in heterozygous or wild-type siblings and *ltk*^{9s1} larvae, it leads to overproduction of iridophores ($n = 29/29$ and $34/35$). Overexpression of DrAlkal2a in *ltk*^{mne} mutant slightly enhances the phenotype. (B) Treatment with lorlatinib results in diminished iridophore numbers in 4 dpf larvae. (C) Mosaic overexpression of DrAlkal2a produces patches of supernumerary iridophores in wild-type and *ltk*^{mne} adults. Overexpression of DrAlkal2a in *ltk*^{mne} mutants enhances production of supernumerary iridophores compared with uninjected *ltk*^{mne}. (D) Zebrafish *alkal* mutants display defects in iridophore development. *alkal1*^{ko} mutants have reduced iridophores in eyes and operculum, and removal of both *alkal1* and *alkal2b* results in a complete loss of eye iridophores. *alkal2a*^{ko} mutants show the strongest phenotype, with reduced numbers of iridophores in the trunk, especially anteriorly. This phenotype displays increased penetrance in *alkal2a;alkal2b* double mutants. (E) Triple mutant for all *alkal* genes is embryonic lethal and displays total loss of iridophores, similar to *ltk* transheterozygous knockout.

in adults. The *alkal1* mutant phenotype was further enhanced by mutation in either *alkal2a* or *alkal2b*. For example, mutants in both *alkal1* and *alkal2b* lacked iridophores on the operculum and in the eye. The *alkal2a;alkal2b* double mutant was larval lethal with mutants failing to inflate the swim bladder. The *alkal1;alkal2a;alkal2b* triple mutant was also larval lethal and displayed a larval phenotype indistinguishable from *ltk* knockout alleles (Fig. 4E). The trunk phenotype of *alkal2a* mutants was enhanced by additionally removing one copy of *alkal2b*, which on its own did not show a detectable phenotype. Interestingly, of the six copies representing the three *alkal* genes, one copy of *alkal2a* or *alkal2b* alone was sufficient to produce

viable fish with a reduced number of iridophores and some patterning defects, indicating that the requirement of Alkal protein for activation of DrLtk is very low (Fig. 4D). In summary, we observe overlapping and partially redundant spatial functions of the three Alkals in zebrafish.

Discussion

This work provides in vivo evidence that the ALKAL (FAM150/Augmentor) class of ligands bind and activate the ALK receptor tyrosine kinase. We show that overexpression of DrAlkals produces responses in zebrafish similar to overactivation of DrLtk (*moonstone*)—namely supernumerary and ectopic iridophores.

Likewise, loss of function of these proteins results in a severe reduction of iridophore numbers, both in larvae and in adults. Human and zebrafish ALKs activate zebrafish Ltk signaling in both *Drosophila* eye and neurite outgrowth assays, in a manner dependent on the tyrosine kinase activity of the receptor. In addition, zebrafish Alkal2a and human ALKs strongly activate exogenous human ALK in a neurite outgrowth assay and the endogenously expressed ALK receptor in neuroblastoma cells.

We demonstrate the involvement of a ligand–receptor interaction in the control of neural crest-derived iridophores. Importantly, this Ltk–Alkal interaction controls both larval iridophores directly differentiating from neural crest, and metamorphic iridophores, which come from multipotent stem cells and produce the adult pigmentation pattern (17–19). The three ligands display slightly different spatial requirements, but with the exception of the iridophores on the operculum, which absolutely require Alkal1, other regions of the body are devoid of iridophores only when the function of more than one Alkal is eliminated by mutation. The complete loss of function of all three Alkals results in the total absence of iridophores and in larval lethality, a phenotype indistinguishable from that of Ltk mutants. This indicates that the three Alkals cooperate to fully activate the receptor and ensure the full repertoire of iridophore development.

Our expression analysis shows that in the zebrafish embryo, *alkals* are expressed in the regions where iridophores develop, and where also DrLtk is expressed (8). This raises the possibility that in this system, ligand and receptor are expressed in the same cells—in the iridophores themselves. Other ligand–receptor pairs have already been suggested to play important roles in zebrafish pigmentation. *Endothelin receptor b1* (*rose*) and *endothelin 3b* are suggested to function in iridophore cell shape transitions (14, 42); *kita* (*sparse*) and *kit ligand a* (*kitla* or *sparse-like*) are necessary for establishing embryonic and most adult melanophores. Xanthophores are controlled by homotypic competition and depend on Csf signaling; however, *kitla* and *csf1* ligands are not expressed in corresponding pigment cells, i.e., melanophores and xanthophores, respectively (21, 22, 43). The differences in expression pattern of different ligand–receptor pairs may have implications on the dynamics of color pattern formation in zebrafish and related *Danio* species. Our analysis suggests that Alkal/DrLtk-mediated signaling triggers iridophore proliferation in zebrafish, where this signaling pathway might link homotypic competition (known to regulate iridophore proliferation) to cell cycle machinery. Coexpression of ligand and receptor in the same cells may lead to autocrine/paracrine activation of Ltk, resulting in the high rate of proliferation of iridophores observed during metamorphosis in zebrafish. The function of Ltk signaling therefore might be to stimulate cell proliferation, differentiation, and survival rather than providing guidance cues for directed migration, e.g., as a morphogen gradient. However, it is likely that there are additional constraints on iridophore proliferation during development—while metamorphic iridophores tend to spread where possible and cover the whole skin, larval iridophores do not do so. Ectopic expression of Alkal ligands in zebrafish causes increased numbers of iridophores in larvae as well as adult fish, suggesting that Alkal/DrLtk signaling drives iridophore proliferation. In summary, color pattern formation in zebrafish offers an exciting opportunity to investigate this question valuable to our understanding of the fundamental role of ALK family RTKs in cell proliferation and cancer.

It appears that zebrafish Ltk displays many similarities to human ALK. These similarities are not only in the ECD structure, but importantly also include expression in neural crest-derived structures. This raises an intriguing question of orthology relationship between human and zebrafish genes. The original designation of Ltk in zebrafish was based upon similarities in the

kinase domains; however, it is feasible that HsALK and DrLtk are orthologs, and that DrAlk possibly arose by duplication from DrLtk and that a more correct designation may be DrLtk → DrAlk1, and DrAlk → DrAlk2. This is a complex question that requires future characterization of the zebrafish ALK family members to help resolve this at the functional level. In the meantime, this work provides not only genetic and biochemical evidence for the ALKAL/ALK family RTK interaction, but also demonstrates conservation of this interaction in vertebrates, since human ligands are able to activate zebrafish receptors and vice versa. Furthermore, we show the importance of this interaction for the regulation of neural crest-derived cells. This becomes especially significant when the role of HsALK-F1174 gain-of-function mutations in human neuroblastoma is compared with the effect of the gain-of-function mutation in exactly the same position in DrLtk^{mne} (F993), which leads to ectopic differentiation of neural crest-derived iridophores. Our findings, and their significance, are further corroborated by complementary work recently published by ref. 44. We hope that the present study will facilitate further investigations in the role of ALKs in neuroblastoma. Outstanding questions remain, such as the individual contribution of the zebrafish Alkals in terms of spatial and temporal regulation of Ltk signaling, that should be addressed in future studies. In summary, this work confirms a conserved role for the ALKAL ligands in activation of ALK family RTKs in the vertebrates.

Materials and Methods

Zebrafish Maintenance and Image Acquisition. The following fish lines were bred and maintained as described (45): Tü wild-type, *shady*^{js1} (8), *ltk*^{mne} (14), *albino*^{b4} (46). All experiments with zebrafish were performed in accordance with the guidelines of the Max Planck Society and approved by the Regierungspräsidium Tübingen, Baden-Württemberg, Germany (Aktenzeichen: 35/9185.46). Anesthesia was performed as described previously (18). Canon 5D Mk II and Leica M205FA were used to obtain images. Adobe Photoshop and Illustrator CS6 and 4 were used for image processing. For adult fish photos multiple RAW camera images were taken in different focal planes and auto-align and auto-blend functions of Photoshop were used. Blemishes on the background were removed using the brush tool, without affecting the image of the fish.

Multiple Alignments and Trees. Multiple protein alignment was obtained using MUSCLE (47) and visualized using Jalview (48). The following GenBank sequences were used for analysis: LTK - ACA79941.1 (*D. rerio*), P29376 (*H. sapiens*); ALK - XP_691964.2 (*D. rerio*), NP_004295.2 (*Homo sapiens*); ALKAL1 - NP_001182661.1 (*Mus musculus*), NP_997296.1 (*H. sapiens*), XP_012820744.1 (*Xenopus tropicalis*), XP_005166986.1 (*D. rerio*); ALKAL2: NP_001153215.1 (*M. musculus*), NP_001002919.2 (*H. sapiens*), XP_004914540.1 (*X. tropicalis*), XP_015140421.1 (*Gallus gallus*), XP_002665250.2 (Alkal2b, *D. rerio*), XR_659754.3 (Alkal2a, *D. rerio*, translated from 1,771 to 2,202 bp).

Injections. Microinjections into zebrafish eggs were performed as described (45). Zebrafish Injection Pipettes OD 20 μ m (BioMedical Instruments) and Pneumatic PicoPump SYS-PV820 (World Precision Instruments) were used with the following parameters: pressure 30 psi, period 1.0 s.

Overexpression and Knockout. The following mixes were used to inject yolk of one cell stage embryos: for knockout—350 ng/ μ L Cas9 protein, 15 ng/ μ L sgRNA, and 5% Phenol Red (P3532; Sigma-Aldrich); for ectopic expression of DrAlkal: 12.5 ng/ μ L Tol2 mRNA (49), 25 ng/ μ L of a plasmid, and 5% Phenol Red. ALKAL-overexpression plasmids were provided by F.S. and S.W. Freeman—Halton extension of the Fisher exact probability test (50) was used for pairwise comparisons of overexpression phenotype distribution. Cas9 protein was expressed in *E. coli* (BL21 DE3 pLysS) with an amino-terminal Twin-Strep-Tag and 6xHis tag. The protein was purified according to the manufacturer's instructions (IBA Lifesciences, "Expression and purification of proteins using double tag [Strep-tag/6xHistidine-tag]" vPR36-0001) and dialyzed into PBS + 300 mM NaCl + 100 mM KCl. sgRNAs targeting zebrafish *alkal* coding sequences were prepared as described before (51), using the following primers: *alkal1*_sgRNA_for TAGGTCTGCTGTTGCCGGCT, *alkal1*_sgRNA_rev AAACAA-GCCGACAACAGCAGA, *alkal2a*_sgRNA_for TAGGACACGCCATCTCAAAA,

alkal2a_sgRNA_rev AAACCTTTGAGATGGTGCCTGT, *alkal2b_sgRNA_for* TAGGACCCCTATGAGACAGGT, *alkal2b_sgRNA_rev* AACACCTGTCTTCATAGGGCT.

Lorlatinib Treatment. Lorlatinib (PF 06463922; Tocris, Bio-Techne) was dissolved in dimethylsulphoxide (DMSO; Sigma-Aldrich) to a final concentration of 0.1 mM. Standard E2 embryonic medium (45) was supplemented with 50 µg/mL gentamycin (NH09, Carl Roth GmbH + Co. KG) and 0.03% DMSO with 3 µM lorlatinib or without for controls. Thirty embryos were manually dechorionated at 9 hpf and treated in 50 mL of the medium in a 30 mm 1% agarose-coated Petri dish with daily changes of medium for fresh one until 4 dpf.

Genotyping of Zebrafish. DNA from embryos or adult caudal fin biopsies was prepared as described in ref. 52. The regions surrounding CRISPR targets were amplified with RedTaq DNA Polymerase (D4309; Sigma-Aldrich) and sequenced using Big Dye Terminator v3.1 kit (4337455; Thermo Fisher Scientific) with the following primers: *alkal1_for* AGCAAGGAGGTAAGGAGTC, *alkal1_rev* CACTCTTTGATCTACAGAGGG, *alkal2a_for* TGGGCTCGTATTGTAATC, *alkal2a_rev* GCATAACAGAGTACACCCCA, *alkal2b_for* TGCTTTGCGTTATCGTTA-TCA, *alkal2b_rev* AGACTAGCAGGGAGTCAGCG.

Whole-Mount in Situ Hybridization. Partial cDNA sequences (Ensembl v10) of zebrafish *alkals* and *ltk* cloned into pcDNA3 were used as templates to generate sense and antisense probes using SP6- and T7-Megascript Kits (Ambion/Lifescience). For detailed sequences, see [Supporting Information](#). The whole-mount in situ hybridization was carried out after (53). Embryos were treated with 10 µg/mL Proteinase K for 10 min.

Expression of Ltk and Alkal in Drosophila Eye. *Drosophila* were maintained on a potato-mash diet under standard husbandry procedures. Crosses were performed at 25 °C unless otherwise stated. *UAS-ltk*, *UAS-ltk^{mne}*, *UAS-alkal1a*, *UAS-alkal2a*, and *UAS-alkal2b* were synthesized (GenScript), and transgenic flies were obtained by injection (BestGene Inc.). *white¹¹¹⁸* (*w¹¹¹⁸*) flies were employed as control. *GMR-Gal4* fly stock (reference no. 1104) was from the Bloomington Drosophila Stock Center at Indiana University (NIH P40OD018537). *UAS-ALK*, *UAS-ALK^{F1174L}*, *UAS-ALKAL1*, and *UAS-ALKAL2* were generated previously (35). Fly eye samples were analyzed under Zeiss Axio Imager.Z2 and AxioZoom.V16 microscopes. Images were acquired with an Axiocam 503 color camera.

Generation of DrLtk and DrAlkal Expression Constructs. Zebrafish *ltk*, *ltk^{mne}*, *alkal1*, *alkal2a*, and *alkal2b* constructs were synthesized in pcDNA3 vector (GenScript). *ltk* constructs were tagged C-terminally with myc, while Alkal constructs were tagged C-terminally with HA. Human ALK and ALK-F1174L constructs have been described previously (39).

Neurite Outgrowth Assay. PC12 cells (2×10^6) were cotransfected with 0.5 µg of pEGFP-N1 vector (Clontech) together with one or more of the following constructs: 0.5 µg of pcDNA3-*HsALK*, 1.0 µg of pcDNA3 empty vector, 1.0 µg

of pcDNA3 vector containing zebrafish *alkal1*, *alkal2a*, *alkal2b*, *ltk*, *ltk^{mne}*, or human *HsALKs*. Cells were transfected using an Amaxa Nucleofector electroporator (Amaxa GmbH) with Ingenio electroporation solution (Mirus Bio LCC) and transferred to RPMI 1640 medium (HyClone, GE Healthcare Bio-Sciences Austria GmbH) supplemented with 7% horse serum (Biochrom AG) and 3% FBS (Sigma-Aldrich Chemie GmbH). Approximately 5% of cells were seeded into 12-well plates for neurite outgrowth assays. The remaining cells were seeded in six-well plates for immunoblotting assays. The ALK inhibitor lorlatinib was used at a final concentration of 30 nM to inhibit the activity of *Ltk^{mne}* (40). Two days after transfection, the percentage of GFP-positive and neurite-carrying cells versus GFP-positive cells was analyzed under a Zeiss Axiovert 40 CFL microscope. Cells with neurites longer than twice the length of the cell body were considered neurite carrying. Experiments were performed in triplicate, and each sample within an experiment was assayed in duplicate.

Stimulation of Endogenous HsALK with Human and Zebrafish ALKs. ALKAL-containing conditioned medium was generated as described previously (35). In brief, HEK293 cells in complete DMEM (HyClone, GE Healthcare Bio-Sciences Austria GmbH) were seeded into 10-cm dishes and transfected with 6 µg of pcDNA3 empty vector, ALKAL1, ALKAL2, *alkal1*, *alkal2a*, or *alkal2b*-coding plasmid using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific). Medium was changed to neuroblastoma cell medium (RPMI 1640, 10% FBS) 6 h after transfection, and conditioned medium was collected after another 30–40 h. Human neuroblastoma IMR-32 or NB1 cells seeded in 12-well plates were stimulated with conditioned medium or the agonist monoclonal antibody mAb46 for 30 min before lysis (54).

Cell Lysis and Immunoblotting. Electroporated PC12 cells seeded in six-well plates as described above were cultured in RPMI 1640 medium supplemented with 7% horse serum and 3% FBS for 24 h and then starved for 36 h. Both PC12 cells and human neuroblastoma cells were lysed directly in 1× SDS sample buffer. Precleared lysates were run on SDS/PAGE. DrLtk or HsALK phosphorylation was analyzed with pALK-Y1278 antibodies (Cell Signaling Technology) and activation of downstream components was detected with pERK1/2 antibodies (Cell Signaling Technology). Pan-ERK (BD Biosciences) was employed as loading control. Total Ltk was detected with anti-Myc tag antibody (ab9132; Abcam). Total ALK was detected with ALK (D5F3) antibody (Cell Signaling Technology). ALKs were detected with anti-HA.11 antibodies (Clone 16B12; BioLegend).

ACKNOWLEDGMENTS. This work has been supported by Swedish Cancer Society Grant (to R.H.P.), Swedish Childhood Cancer Foundation Grants 15/0096 (to R.H.P.) and 16/0011 (to J.G.), Swedish Research Council Grant 621-2015-04466 (to R.H.P.), Swedish Foundation for Strategic Research Grant RB13-0204, the Göran Gustafsson Foundation (R.H.P.), the Max Planck Society, and a European Research Council advanced grant (to C.N.-V.).

- Lin JJ, Riely GJ, Shaw AT (2017) Targeting ALK: Precision medicine takes on drug resistance. *Cancer Discov* 7:137–155.
- Hallberg B, Palmer RH (2013) Mechanistic insight into ALK receptor tyrosine kinase in human cancer biology. *Nat Rev Cancer* 13:685–700.
- Bilsland JG, et al. (2008) Behavioral and neurochemical alterations in mice deficient in anaplastic lymphoma kinase suggest therapeutic potential for psychiatric indications. *Neuropsychopharmacology* 33:685–700.
- Weiss JB, et al. (2012) Anaplastic lymphoma kinase and leukocyte tyrosine kinase: Functions and genetic interactions in learning, memory and adult neurogenesis. *Pharmacol Biochem Behav* 100:566–574.
- Witek B, et al. (2015) Targeted disruption of ALK reveals a potential role in hypogonadotropic hypogonadism. *PLoS One* 10:e0123542.
- Loren CE, et al. (2001) Identification and characterization of DALK: A novel Drosophila melanogaster RTK which drives ERK activation in vivo. *Genes Cells* 6:531–544.
- Reiner DJ, Ailion M, Thomas JH, Meyer BJ (2008) C. elegans anaplastic lymphoma kinase ortholog SCD-2 controls dauer formation by modulating TGF-β signaling. *Curr Biol* 18:1101–1109.
- Lopes SS, et al. (2008) Leukocyte tyrosine kinase functions in pigment cell development. *PLoS Genet* 4:e1000026.
- Yao S, et al. (2013) Anaplastic lymphoma kinase is required for neurogenesis in the developing central nervous system of zebrafish. *PLoS One* 8:e63757.
- Ben-Neriah Y, Bauskin AR (1988) Leukocytes express a novel gene encoding a putative transmembrane protein-kinase devoid of an extracellular domain. *Nature* 333:672–676.
- Bernards A, de la Monte SM (1990) The *ltk* receptor tyrosine kinase is expressed in pre-B lymphocytes and cerebral neurons and uses a non-AUG translational initiator. *EMBO J* 9:2279–2287.
- Iwahara T, et al. (1997) Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. *Oncogene* 14:439–449.
- Vernersson E, et al. (2006) Characterization of the expression of the ALK receptor tyrosine kinase in mice. *Gene Expr Patterns* 6:448–461.
- Fadeev A, Krauss J, Singh AP, Nüsslein-Volhard C (2016) Zebrafish Leucocyte tyrosine kinase controls iridophore establishment, proliferation and survival. *Pigment Cell Melanoma Res* 29:284–296.
- Frohnhofer HG, Krauss J, Maischein H-M, Nüsslein-Volhard C (2013) Iridophores and their interactions with other chromatophores are required for stripe formation in zebrafish. *Development* 140:2997–3007.
- Kelsh RN, et al. (1996) Zebrafish pigmentation mutations and the processes of neural crest development. *Development* 123:369–389.
- Singh AP, Nüsslein-Volhard C (2015) Zebrafish stripes as a model for vertebrate colour pattern formation. *Curr Biol* 25:R81–R92.
- Singh AP, Schach U, Nüsslein-Volhard C (2014) Proliferation, dispersal and patterned aggregation of iridophores in the skin prefigure striped colouration of zebrafish. *Nat Cell Biol* 16:607–614.
- Singh AP, et al. (2016) Pigment cell progenitors in zebrafish remain multipotent through metamorphosis. *Dev Cell* 38:316–330.
- Fadeev A, Krauss J, Frohnhofer HG, Irion U, Nüsslein-Volhard C (2015) Tight junction protein 1a regulates pigment cell organisation during zebrafish colour patterning. *Elife* 4:e06545.
- Patterson LB, Parichy DM (2013) Interactions with iridophores and the tissue environment required for patterning melanophores and xanthophores during zebrafish adult pigment stripe formation. *PLoS Genet* 9:e1003561.
- Walderich B, Singh AP, Mahalwar P, Nüsslein-Volhard C (2016) Homotypic cell competition regulates proliferation and tiling of zebrafish pigment cells during colour pattern formation. *Nat Commun* 7:11462.

23. Parichy DM, et al. (2000) Mutational analysis of endothelin receptor b1 (rose) during neural crest and pigment pattern development in the zebrafish *Danio rerio*. *Dev Biol* 227:294–306.
24. Carén H, Abel F, Kogner P, Martinsson T (2008) High incidence of DNA mutations and gene amplifications of the ALK gene in advanced sporadic neuroblastoma tumours. *Biochem J* 416:153–159.
25. Chand D, et al. (2013) Cell culture and *Drosophila* model systems define three classes of anaplastic lymphoma kinase mutations in neuroblastoma. *Dis Model Mech* 6: 373–382.
26. Chen Y, et al. (2008) Oncogenic mutations of ALK kinase in neuroblastoma. *Nature* 455:971–974.
27. George RE, et al. (2008) Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature* 455:975–978.
28. Janoueix-Lerosey I, et al. (2008) Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature* 455:967–970.
29. Mossé YP, et al. (2008) Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* 455:930–935.
30. Roll JD, Reuther GW (2012) ALK-activating homologous mutations in LTK induce cellular transformation. *PLoS One* 7:e31733.
31. Englund C, et al. (2003) Jeb signals through the Alk receptor tyrosine kinase to drive visceral muscle fusion. *Nature* 425:512–516.
32. Lee H-H, Norris A, Weiss JB, Frasch M (2003) Jelly belly protein activates the receptor tyrosine kinase Alk to specify visceral muscle pioneers. *Nature* 425:507–512.
33. Ishihara T, et al. (2002) HEN-1, a secretory protein with an LDL receptor motif, regulates sensory integration and learning in *Caenorhabditis elegans*. *Cell* 109:639–649.
34. Weiss JB, Suyama KL, Lee HH, Scott MP (2001) Jelly belly: A *Drosophila* LDL receptor repeat-containing signal required for mesoderm migration and differentiation. *Cell* 107:387–398.
35. Guan J, et al. (2015) FAM150A and FAM150B are activating ligands for anaplastic lymphoma kinase. *Elife* 4:e09811.
36. Reshetnyak AV, et al. (2015) Augmentor α and β (FAM150) are ligands of the receptor tyrosine kinases ALK and LTK: Hierarchy and specificity of ligand–receptor interactions. *Proc Natl Acad Sci USA* 112:15862–15867.
37. Zhang H, et al. (2014) Deorphanization of the human leukocyte tyrosine kinase (LTK) receptor by a signaling screen of the extracellular proteome. *Proc Natl Acad Sci USA* 111:15741–15745.
38. Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401–415.
39. Martinsson T, et al. (2011) Appearance of the novel activating F1174S ALK mutation in neuroblastoma correlates with aggressive tumor progression and unresponsiveness to therapy. *Cancer Res* 71:98–105.
40. Guan J, et al. (2016) The ALK inhibitor PF-06463922 is effective as a single agent in neuroblastoma driven by expression of ALK and MYCN. *Dis Model Mech* 9:941–952.
41. Infarinato NR, et al. (2016) The ALK/ROS1 inhibitor PF-06463922 overcomes primary resistance to crizotinib in ALK-driven neuroblastoma. *Cancer Discov* 6:96–107.
42. Krauss J, et al. (2014) Endothelin signalling in iridophore development and stripe pattern formation of zebrafish. *Biol Open* 3:503–509.
43. Dooley CM, Mongera A, Walderich B, Nüsslein-Volhard C (2013) On the embryonic origin of adult melanophores: The role of ErbB and kit signalling in establishing melanophore stem cells in zebrafish. *Development* 140:1003–1013.
44. Mo ES, Cheng Q, Reshetnyak AV, Schlessinger J, Nicoli S (2017) Alk and Ltk ligands are essential for iridophore development in zebrafish mediated by the receptor tyrosine kinase Ltk. *Proc Natl Acad Sci USA* 114:12027–12032.
45. Nüsslein-Volhard C, Dahm R (2002) *Zebrafish: A Practical Approach* (Oxford Univ Press, New York).
46. Dooley CM, et al. (2013) Slc45a2 and V-ATPase are regulators of melanosomal pH homeostasis in zebrafish, providing a mechanism for human pigment evolution and disease. *Pigment Cell Melanoma Res* 26:205–217.
47. Edgar RC (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32:1792–1797.
48. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ (2009) Jalview Version 2–A multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25: 1189–1191.
49. Kawakami K, Shima A (1999) Identification of the Tol2 transposase of the medaka fish *Oryzias latipes* that catalyzes excision of a nonautonomous Tol2 element in zebrafish *Danio rerio*. *Gene* 240:239–244.
50. Freeman GH, Halton JH (1951) Note on an exact treatment of contingency, goodness of fit and other problems of significance. *Biometrika* 38:141–149.
51. Irion U, Krauss J, Nüsslein-Volhard C (2014) Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. *Development* 141:4827–4830.
52. Meeker ND, Hutchinson SA, Ho L, Trede NS (2007) Method for isolation of PCR-ready genomic DNA from zebrafish tissues. *Biotechniques* 43:610–614.
53. Pfeifer K, Dorresteyn AWC, Fröbius AC (2012) Activation of Hox genes during caudal regeneration of the polychaete annelid *Platynereis dumerilii*. *Dev Genes Evol* 222: 165–179.
54. Moog-Lutz C, et al. (2005) Activation and inhibition of anaplastic lymphoma kinase receptor tyrosine kinase by monoclonal antibodies and absence of agonist activity of pleiotrophin. *J Biol Chem* 280:26039–26048.