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Leukocyte Tyrosine Kinase Functions in Pigment Cell Development

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Abstract
A fundamental problem in developmental biology concerns how multipotent precursors choose specific fates. Neural crest cells (NCCs) are multipotent, yet the mechanisms driving specific fate choices remain incompletely understood. Sox10 is required for specification of neural cells and melanocytes from NCCs. Like sox10 mutants, zebrafish shady mutants lack iridophores; we have proposed that sox10 and shady are required for iridophore specification from NCCs. We show using diverse approaches that shady encodes zebrafish leukocyte tyrosine kinase (Ltk). Cell transplantation studies show that Ltk acts cell-autonomously within the iridophore lineage. Consistent with this, ltk is expressed in a subset of NCCs, before becoming restricted to the iridophore lineage. Marker analysis reveals a primary defect in iridophore specification in ltk mutants. We saw no evidence for a fate-shift of neural crest cells into other pigment cell fates and some NCCs were subsequently lost by apoptosis. These features are also characteristic of the neural crest cell phenotype in sox10 mutants, leading us to examine iridophores in sox10 mutants. As expected, sox10 mutants largely lacked iridophore markers at late stages. In addition, sox10 mutants unexpectedly showed more ltk-expressing cells than wild-type siblings. These cells remained in a premigratory position and expressed sox10 but not the earliest neural crest markers and may represent multipotent, but partially-restricted, progenitors. In summary, we have discovered a novel signalling pathway in NCC development and demonstrate fate specification of iridophores as the first identified role for Ltk.

Introduction
Understanding mechanisms determining the selection of specific fate choices by multipotent precursors is of fundamental importance in developmental and stem cell biology. Neural crest cells (NCCs) are a favoured model for investigation of fate specification mechanisms, being multipotent precursors of diverse cell-types, including craniofacial cartilage, peripheral neuronal and glial cell-types and pigment cells [1]. The mechanism driving specification of multipotent progenitors in the neural crest (NC) to fate-restricted cell types is controversial. Multipotent NG stem cells with broad potential have been isolated from embryos, even from post-migratory locations, leading to the hypothesis of direct fate restriction, whereby local signals instruct multipotent stem cells to adopt specific fates [reviewed in [2]]. In contrast, numerous studies indicating that NCCs include partially-restricted cells has suggested progressive fate restriction as an alternative model (reviewed in [3,4]). Thus, multipotent precursors gradually lose the potential to generate certain derivative cell-types, forming partially-restricted precursors before eventually becoming specified to an individual fate. The number and character of these intermediate precursors in vivo remains largely undefined.

The molecular mechanisms underlying fate restriction also remain poorly understood. Genetic analysis in mouse and zebrafish identifies key transcription factors required for specification of several or individual fates. Perhaps the best characterised example is that of Microphthalmia-related transcription Factor (Mitf), which is pivotal for melanocyte specification [5,6,7]. Sox10 is required in multipotent NCCs to drive transcription of Mitf and other transcription factors (e.g. [8,9,10,11,12]) (reviewed in [13]), while Pax3 acts synergistically with Sox10 to regulate the mouse Mitf promoter [11,14].

Extracellular signals are also important in NCC fate specification. For example, Wnt signals are required for melanocyte...
specification via transcriptional activation of Mitf [15,16,17]. Signalling by these pathways acts together with intrinsic factors such as Sox10 and Pax3 to induce specific cell-fates. Further signals remain to be identified and for some NC-derived fates, including other pigment cell-types, no fate specification factors have yet been identified. Surprisingly, even where key factors have been identified, in most cases the receptors mediating these signals are unknown.

Leukocyte tyrosine kinase (LTK) was first identified as an insulin receptor-like receptor tyrosine kinase (RTK) expressed in mouse haematopoietic cells [18]. Within the insulin receptor superfamily, LTK is most closely-related to anaplastic lymphoma kinase (ALK) [19]. In mammals LTK’s function remains unknown, although it is expressed in pre-B and B lymphocytes and in the adult brain [18,20]. It is widely expressed in human leukaemias [21] and is a candidate locus contributing to the multigenic autoimmune disease, systemic lupus erythematosus (OMIM#152700)[22].

Mutations in the zebrafish shady (shd) locus were identified in a large-scale mutagenesis screen [23]. Iridophores, an iridescent pigment cell widespread in anamniotes, are reduced in number in shd mutants. Here we show that other NC derivatives are not affected in shd mutants. We demonstrate that shd encodes the zebrafish orthologue of LTK and functions cell-autonomously within the NC. We show that strong shd mutants lack iridoblast lineage markers, including btk which is highly reduced from the very earliest stage in pre-migratory NCs. Later some NCs die by apoptosis, after failing to become specified to iridophore or other fates. Zebrafish sox10 (also known as colourless) mutants share the strong iridophore phenotype of shd mutants and show consistent defects in fate specification of non-ectomesenchymal NC derivatives [9,24,25,26,27]. We have previously proposed that fate specification defects may underlie the sox10 mutant iridophore defect [13,26]. As expected, late iridophore markers are absent. At earlier stages btk expression is detected in an increased number of cells compared with wild-type (WT) siblings. Our data indicates that these btk-expressing cells are most likely partially-restricted precursors. Together, these observations identify the first loss of function mutants for this poorly characterised RTK and suggest that Ltk mediates iridophore fate specification from multipotent NCs.

**Results**

**Shd mutants have reduced iridophores and form a broad allelic series**

shd mutant embryonic phenotypes formed a clear allelic series; homozygotes for strong alleles (e.g. shd<sup>j9s1</sup>) have very few (<3) iridophores and die as larvae, those for weaker alleles (e.g. shd<sup>j9e2</sup>) show reduced numbers of differentiated iridophores in the embryo and are adult viable, but phenotypically normal (Figure 1A, 1B, 1D, 1E, 1G, 1H). In all cases, and in contrast to all iridophore mutant phenotypes identified before [23], remaining iridophores in shd mutants were invariably normally pigmented and hence appeared normally differentiated. Independent screens for adult pigment pattern mutants identified further mutants with reduced iridophores in the body and eyes (j9s1, j9e1 and j9e2). The most severe of these, j9s1, also lacked late stripe melanophores, and appeared similar to the ro<sup>z10985</sup> phenotype [28]; SLJ unpublished(Figure 1C, 1F, 1I). Mapping and complementation testing showed these to be allelic to shd (data not shown). These adult viable alleles had no detectable abnormal embryonic phenotype as homozygotes, although in transheterozygous combination with shd<sup>j9e2</sup> they showed an embryonic iridophore phenotype of intermediate strength (data not shown); thus the adult viable alleles are also hypomorphic alleles.

Iridophores are derived from the NC [29]. However, both of the other pigment cell-types, melanophores and xanthophores, as well as peripheral neuronal, glial and skeletogenic derivatives examined with specific markers showed comparable numbers and patterns in shd mutants and WT siblings (Figure S1). We conclude that the shd mutant phenotype is restricted within the NC to the iridophore lineage.

**Shd acts cell-autonomously within the NC**

We then used genetic chimaeras formed by transplanting WT cells, labelled with rhodamine and biotin-conjugated dextran beads, into shd<sup>j9e2</sup> mutants to ask whether the shd iridophore phenotype resulted from cell-autonomous function within the NC [27]. Of 291 host embryos receiving WT cells, 188 survived the procedure to 3 dpf and could be scored for iridophore phenotype. Of these, 40 embryos (21.2%) were identified as shd mutant hosts by the general absence of iridophores and 22 were chimaeric, containing some rhodamine fluorescent cells. Of these, seven embryos had iridophore counts above normal shd<sup>j9e2</sup> mutant levels (Table S1). In these individuals, most or all iridophores exhibited biotin tracer and were derived from WT donor cells, consistent with cell-autonomous shd gene function (Figure S1H, S1I, and S1O). As expected, some, but importantly not all, of these embryos also had up to two unlabelled iridophores in the expected sites (dorsal stripe or lateral patch) for the occasional ‘escaper’ iridophores seen in shd<sup>j9e2</sup> mutants. Thus, these studies demonstrated that shd acts cell-autonomously in iridophore lineage development.

**Positional cloning identifies shd as encoding zebrafish leukocyte tyrosine kinase**

In the absence of candidate genes, we utilised a positional cloning approach to identify shd. Linkage analysis mapped shd within 0.1 cM of marker z10985 on linkage group 17 (Figure 2A). Assuming average recombination frequencies, we reasoned that this marker likely lay within c. 70 kb of the shd gene and that both might be contained within the insert of a single genomic PAC. We isolated three PACs containing z10985 from the PAC706 library [30]. Pulse field gel electrophoresis analysis of the PAC inserts defined an overlapping contig spanning 207.5 kb of genomic
sequence (Figure 2B). Microinjection of PAC DNA into 1- or 2-cell stage zebrafish embryos demonstrated that PAC3, but not the others, partially rescued the \textit{shd} \textsuperscript{b02} mutant iridophore phenotype (Figure 2C), indicating that PAC3 contained a functional \textit{shd} gene. Analysis of the PAC contig sequence (Sanger Centre zebrafish genome project) using the NIX gene prediction package identified one gene fully contained within PAC3. This gene encodes an RTK, of the insulin receptor class and most similar to human \textit{ALK} and LTK (Figure 2E).

Sequencing cDNAs for this \textit{ALK/LTK}-like gene from AB WT embryos identified distinct isoforms generated by alternative splicing (Figure S2). BLAST searches identified closely-related RTKs from the zebrafish XM\textunderscore 686872, XM\textunderscore 001342889 and XM\textunderscore 687805 and other vertebrates (Table S2) in the NCBI databases. We used multiple sequence alignments and phylogenetic estimation protocols to determine the likely relationships between our cDNAs and these genes (Figure 2H, Figure S3). Our phylogenetic analyses identified: i) the invertebrate genes as a clear outgroup to the vertebrate homologues; ii) zebrafish sequence XM\textunderscore 686872, on chromosome 17 but physically distant from \textit{shd}, as the zebrafish \textit{ALK} orthologue, which we name \textit{alk}; iii) our cDNAs and XM\textunderscore 001342889 and XM\textunderscore 687805 as zebrafish \textit{Ltk} sequences. These latter genes resolve in build 7 of the zebrafish genome (Zv7) to one locus, the zebrafish orthologue of \textit{LTK}, which we name \textit{lk}. We note that while all mammalian \textit{Ltk} proteins are predicted to lack MAM (meprin/A5/A6) domains, thought to mediate protein-protein interactions [31], the predicted chicken and zebrafish \textit{Ltk} proteins, and all \textit{Alk} proteins, possess them (Figure 2E).

These data strongly suggested that the \textit{shd} mutations identified the \textit{lk} gene. Injection of WT embryos with a translation-blocking \textit{lk} morpholino oligonucleotide generated strong morphant phenotypes of \textit{shd} mutants in a dose-dependent manner (Figure 2D; data not shown). Embryos injected with double doses (18.4 or 36.8 ng) of a 5 bp mismatch morpholino showed no phenotype (0/406 injected), whereas siblings injected with single doses (9.2 or 18.4 ng) of \textit{lk} morpholino showed a substantial proportion of embryos with a severe loss of iridophores (27/264 injected), as well as many with weaker phenotypes. The knockdown phenotype precisely phenocopied the \textit{shd} mutant allelic series, supporting the conclusion that \textit{shd} is \textit{lk}.

Confirmation that \textit{shd} is \textit{lk} came from identification of \textit{lk} point mutations in three \textit{shd} mutant alleles by sequencing the \textit{Ltk} coding region from RNA extracted from \textit{shd} homozygotes (Figure 2E, Figure S2). The \textit{shd} \textsuperscript{b02} mutation 2356A\textsuperscript{T} (taking the A in the translation initiation codon as +1) results in a premature STOP codon, generating a truncated predicted protein lacking the tyrosine kinase domain, consistent with the strong mutant phenotype. This mutation fortuitously generates an RFLP allowing us to confirm that segregation of this mutation correlated perfectly with the embryonic phenotype (Figure 2F). \textit{shd} \textsuperscript{b02} mutates a splice donor site, resulting in a transcript with an in-frame deletion of exon 26 encoding a variant of \textit{Ltk} in which the tyrosine kinase domain activation loop [32] is deleted (Figure 2G). It is likely that this variant protein will be less readily activated, consistent with the weak mutant phenotype. \textit{shd} \textsuperscript{b11} mutation, 2275C\textsuperscript{T}, results in a P759S substitution in the extracellular region. This nucleotide change is the only difference between the \textit{shd} \textsuperscript{b11} allele and the cDNA sequence for the WT C32 allele that the mutation was isolated on (data not shown). Moreover, this nucleotide change is not found on the 3 sequenced BACs or PACs, each derived from a different haplotype, that span this region, further supporting that the P759S substitution generates a mutant protein. Interestingly, this proline residue is conserved in chicken and mammalian LTKs, as well as in the LTK orthologue \textit{Drosophila Alk}, but not in the corresponding tetrapod ALKs. Mutations affecting this residue in \textit{Drosophila Alk} have not been reported to date [33].

Together, our data unambiguously identify \textit{shd} as zebrafish \textit{lk}, showing that the \textit{shd} mutant iridophore phenotype results from loss of \textit{Ltk} signalling within the developing NC. This is the first time that a vertebrate \textit{Ltk} loss of function phenotype has been defined.

\textbf{shd expression is prominent in NCCs and iridophores}

To clarify the role of \textit{lk} in iridophore development, we determined the spatiotemporal pattern of \textit{lk} gene expression by whole-mount mRNA in situ hybridisation [ISH]. Our cell-autonomy studies predicted NC expression of \textit{lk}, but expression might be restricted to differentiated iridophores or be found at earlier stages in NC development. Here we focus on expression in NCCs and their derivatives, but we also saw \textit{lk} expression in notochord from 18–24 hpf (Figure 3A–3C) and prominently in brain and swim bladder from 3 dpf (data not shown).

From 48 hpf onwards, \textit{lk}-expressing cells formed a series of spots along the dorsal and ventral stripes, as well as on the eye, a pattern strikingly reminiscent of differentiated iridophores (Figure 1A and 1B, Figure 3J, 3O; 3XY and RNK, unpub. data). Consistent with this, the pattern was identical to that of \textit{edabhl}, the
only characterised iridophore marker (Figure 3O, 3U, 3AA, and 3AE) [34]. To test definitively if iridophores express \( \text{ltk} \), we photographed the dorsal stripe iridophore pattern of individual WT embryos at 72 hpf, processed the embryos for \( \text{ltk} \) expression and then photographed the \( \text{ltk} \) pattern; \( \text{ltk} \)-expressing cells (Figure 3E) showed an excellent correlation. Thus, at least in these late stages, \( \text{ltk} \) expression in NC derivatives is restricted to iridophores.

Initial \( \text{ltk} \) expression in a subset of NCCs was seen near the eye at 18–24 hpf (Figure 3A–3C). Between 26 and 30 hpf, these cells spread over the pigmented retinal epithelium from the dorsal surface of the eye (Figure 3F, 3K, and 3Q). This widespread scattered distribution was then maintained, but the density in a ring around the lens increased (data not shown), consistent with the WT pattern of corneal iridophores (Figure 1A). Plastic sections showed cells on the eye were superficial to the pigmented retinal epithelium (Figure 3F, 3K, and 3Q). For example, in the dorsal stripe, cells were medially positioned and somewhat regularly spaced along the posterior trunk and tail. Hence, we interpreted these cells as iridoblasts and suggest that \( \text{ltk} \)-expression marks the iridoblast lineage throughout their development. However, NC expression of \( \text{ltk} \) is seen very early in a subset of premigratory NCCs, which may include a subset of multipotent NCCs.

\( \text{shd} \) mutants lack iridophore markers

\( \text{shd}^{\text{mos}} \) mutants usually showed no iridophores and hence a primary role for \( \text{ltk} \) in iridoblast proliferation was unlikely, since phosphohistone H3 as a marker for proliferating cells, we were unable to detect a significant effect on NCC proliferation (Figure S4). To address a role in iridophore differentiation, we examined both known iridoblast markers, \( \text{ednrb1} \) and \( \text{ltk} \), reasoning that if \( \text{ltk} \) function was required only for iridophore differentiation these early markers would still be expressed normally in \( \text{shd} \) mutants.
However, no ednr1- or ltk-expressing cells were seen in shdty82 mutants at 50–72 hpf (Figure 3P, 3V, 3AB, and 3AF) and only reduced numbers in the weaker shdty9 mutants (data not shown). We were unable to use ednr1 as an iridophore marker at earlier stages since it is expressed in cells of multiple pigment cell lineages prior to 48 hpf [34]. Instead, we examined ltk expression in earlier embryos. At these stages shdty82 mutants could not be directly distinguished, but, from c. 20 hpf onwards, the expected 25% of embryos showed a consistent phenotype of severely reduced numbers of ltk-expressing cells. That these were shdty82 homozygotes was confirmed by RFLP genotyping of 20–24 hpf embryos prior to whole-mount in situ analysis. All homozygous WT (n = 68) showed normal ltk expression (Figure 3B, 3C, 3I, 3K, 3M, 3N, and 3O; data not shown), whereas all shdty82 homozygotes (n = 67) showed the reduced pattern (Figure 3L, 3N, 3T, 3Z, and 3AD; data not shown). Mutants consistently showed three main features: i) ltk-expressing cells failed to spread across the eye and remained low in number (Figure 3G, 3L, and 3R); ii) strong ltk expression was absent, with expression restricted to at most a few faintly expressing cells (Figure 3I, 3N, and 3T), except iii) from 35 hpf, a variable but always greatly reduced number of strongly expressing ‘escaper’ cells in the anterior trunk ventral stripe (i.e. residual lateral patch cell clusters) (data not shown). In summary, throughout the stages when NCCs in WT are specified to individual fates and begin to differentiate, shdty82 mutants showed a consistent phenotype of highly reduced numbers of ltk-positive cells, with ‘escaper’ cells with normal ltk expression restricted to a few cells on the dorsal eye and in the residual lateral patches. Both the presence of these escaper cells and the similar strong reduction in numbers of ltk-expressing cells in ltk morphants (JM and RNK, data not shown) argue against the possibility that absence of ltk-expressing neural crest cells reflects nonsense-mediated decay of ltk transcripts in this mutant. Thus, specification of almost all iridoblasts fails in shdty82 mutant embryos.
**shd mutants do not show precocious melanoblast or xanthoblast specification**

We then investigated the fate of NCCs that failed to become specified as iridoblasts. In mitfa/nacre mutants, melanophore fate specification fails and increased iridophore numbers are seen, perhaps due to multipotent melanophore precursors adopting an iridophore fate in elevated numbers (Lister et al. 1999). Hence we considered whether some shd mutant iridoblast precursors might adopt another pigment cell fate. The late melanophore pattern in shd<sup>ty82</sup> mutants is overtly normal (see Figure S1C, S1D, S1E, and S1H), and counts of melanophores in the dorsal stripe of 3 dpf shd<sup>ty82</sup> mutants (mean±s.d. = 87.4±2.06, n = 33) and their WT siblings (82.4±2.19, n = 31) showed no significant difference (Student's t-test, p = 0.100)(Figure 4B). We then considered the possibility that an overproduction of melanoblasts or xanthoblasts (Student's t-test, p = 0.100)(Figure 4B). We then considered the possibility that an overproduction of melanoblasts or xanthoblasts at early stages might later be compensated by regulative processes. Hence, we asked whether shd<sup>ty82</sup> homozygotes showed elevated melanoblast or xanthoblast numbers at 30 hpf compared with homozygous WT siblings (Figure 4C and 4D). Embryos were genotyped prior to mRNA whole mount ISH for, respectively, dopachrome tautomerase (dct), [35] or guanine cyclohydrolase (gch), [36]. Whilst dct is expressed exclusively in the melanophore lineage, gch expression, like other characterised xanthophore markers, is seen transiently in the melanophore lineage, as well as being strongly upregulated in xanthophore lineage cells [36]. In order to ensure that our counts focused as much as possible on the xanthophore lineage, we confined our attention to gch-expressing cells on the lateral pathway, since xanthoblasts do not utilise the medial pathway [29]. Counts of dct-positive melanoblasts in shd<sup>ty82</sup> homozygotes (128.0±3.77, n = 35) and homozygous WT siblings (125.3±3.42, n = 39) were indistinguishable (Student's t-test, p = 0.598). Similarly, counts of gch-positive xanthoblasts in shd<sup>ty82</sup> homozygotes (97.8±5.63, n = 23) and homozygous WT siblings (90.5±4.50, n = 23) were indistinguishable (Student t-test, p = 0.315). Thus we found no evidence for a shift of iridoblast precursors to either a melanophore or xanthophore fate.

**shd mutants show elevated NCC death**

In sox10 mutant, neural and pigment cell precursors that fail to become fate-specified are later [35–45 hpf] lost by apoptosis [26]. We explored whether any NCCs in shd<sup>ty82</sup> mutants were lost by apoptosis. We generated shd<sup>ty82</sup> fish carrying the 7.2sox10;egfp transgenic line (J.R. Dutton and R.N.K., in prep.), in which a 7.2 kb fragment of the zebrafish sox10 transgene [9] drives expression of GFP, robustly labelling all NCCs. We combined the TUNEL technique with immunofluorescent detection of GFP expression of GFP, robustly labelling all NCCs. We combined the TUNEL technique with immunofluorescent detection of GFP expression of GFP, robustly labelling all NCCs. We then investigated the fate of NCCs that failed to become iridoblast precursors to either a melanophore or xanthophore fate.

We sorted such fish and genotyped them by iridophore phenotype at 3 dpf (Figure 4A). This data confirmed that apoptosis of NCCs at 35–50 hpf embryos from crosses of shd<sup>ty82</sup> heterozygotes carrying this transgene, scoring TUNEL+ cells, consistent with the idea that NCC death was a feature of shd homozygotes. To test directly the hypothesis that NCC death was characteristic of shd mutants, we counted dying NCCs in the trunk and tail of live embryos from such crosses. Approximately 25% (20/89) showed one double-labelled cell, consistent with the idea that NCC death was a feature of shd homozygotes. We then investigated the fate of NCCs that failed to become iridoblast precursors to either a melanophore or xanthophore fate.
ltk expression in sox10 mutants reveals NCCs trapped in an intermediate phase of NC or iridoblast development

In sox10 mutants iridophores are almost invariably absent, whereas in sox10 mutants occasional, normally differentiated escaper iridophores are seen in the dorsal and ventral stripes [27]; the iridophore phenotype is thus directly comparable with that of shd mutants (Figure 5A and 5B). Furthermore, our previous studies of the sox10 mutant neural crest phenotype showed several features shared with the shd phenotype, specifically the absence of fate-switching and late death of neural crest cells[26]. Given the general failure of fate specification of non-ectomesenchymal derivatives in sox10 mutant fish and mice [8,9,10,11,12,13,24,25,37,38,39,40], we expected iridoblast specification markers to be absent from the earliest stages in sox10 mutants. Hence, we examined ltk expression in sox10 mutants and their WT siblings (Figure 5C–5H). Unlike WT’s, but just like in shd mutants, sox10 mutants almost entirely lacked ltk expression on the eye and in the dorsal, ventral and yolk sac stripes at 48 hpf, although a few escaper cells were seen in the anterior trunk ventral stripe (Figure 5G and 5H), as expected since unspecified pigment cell precursors undergo apoptosis [26]. In contrast, a striking, but unexpected, ltk phenotype was seen at earlier stages in these mutants. In WT siblings at 24 and 30 hpf, ltk expressing cells are seen in the trunk and tail in premigratory NCCs, on migration, in the ventral stripe or clustered behind the otic vesicle (Figure 5C–5F). In contrast, in sox10 mutants, ltk-expressing cells were increased in number compared with WT siblings, and were restricted to premigratory NC (Figure 5D and 5E).

Our previous single NCC labelling studies showed that a large proportion of NCCs in sox10 mutants fail to migrate, a defect also detected by whole-mount ISH for sox10 [26]. We asked whether these sox10 mutant NCCs might be trapped in an early NCC state, but two very early NC markers, snail2 and foxd3 [41,42], showed identical expression in sox10 mutants and their WT siblings (Figure 6A–6H). In addition, cells expressing these early NC markers were located more posteriorly than the ltk-expressing cells in both sox10 mutants and WT siblings (Figure 6E–6J). We conclude that ltk is not expressed in the developmentally youngest NCCs, but only in those at a slightly more mature stage.

Early sox10 expression is widespread in NCCs [26]. Furthermore, sox10 expression is associated with multipotency of NC stem cells [39]. We asked whether ltk-expressing cells in sox10 mutants showed sox10 expression (Figure 7). For these experiments we used sox10 embryos, which also show NCCs trapped in a premigratory position, since sox10 transcripts are apparently destabilised in sox10 mutant embryos [26]. Interestingly, many of these sox10-expressing cells showed ltk-expression at 30 hpf (Figure 7C and 7D). In contrast, WT siblings had largely mutually exclusive sox10 and ltk expression, suggesting that ltk-expressing cells were specified iridoblasts by this stage in WT embryos (Figure 7A and 7B). Together these data show that ltk expression comes on in NCCs after the early premigratory NC markers snail2 and foxd3, and that it labels cells that have developed beyond the initial premigratory NC state, but which may retain multipotency.

Discussion

We present a combination of genetic mapping, morpholino-mediated knockdown, and molecular lesion data that unambiguously identifies the shd locus as encoding zebrafish ltk. In mammals, ALK and LTK are distinguished by the presence or absence respectively of MAM domains. However, our phylogenetic analysis shows that presence of MAM domains is the ancestral
Figure 6. Early NC markers are unaffected in sox10 mutants. WT (A,C,G,I) and sox10<sup>10210</sup> mutants (B,D,F,H,J) showing foxd3 (A,B,E,F) or snai2 (C,D,G,H) or ltk expression at 24 (A–D) and 30 hpf (E–J).
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Figure 7. Co-expression of sox10 in ltk-expressing cells in sox10 mutants. Dorsal views of posterior trunk of 30 hpf sox10<sup>m618</sup> (C,D) and WT sibling (A,B) embryos double-labelled for sox10 (red) and ltk (purple, arrows). Autofluorescence from red sox10 signal shown in panels B and D.
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condition in the ALK/LTK subfamily. Their loss is unique to mammalian Lks, and not generally diagnostic of the LTK family. Our phylogenetic analysis also suggests that vertebrate Alk and Ltk arose by a gene duplication event early in the vertebrate lineage and are thus co-orthologues of the *Drosophila* Alk and *C. elegans* T10F9.2 genes.

The functions of this subfamily of RTKs remain poorly understood. Human LTK is expressed in pre-B lymphocytes and various other tissues, but its endogenous function remains entirely unknown. In *Drosophila*, Alk signalling specifies visceral muscle pioneers [43,44] and regulates axonal guidance [45] and in *C. elegans* it functions in synapse differentiation [46]. Mouse Ltk knockout mice have not been described. Thus we identify the first vertebrate model for studying Ltk gene function.

We demonstrate a key role for *ltk* in NC-derived pigment cell development. We see initial low level *ltk* expression in a subset of premigratory NCcs followed by persistent robust expression in the iridophore lineage. Furthermore, iridophore lineage markers are absent from *shdltk* mutants, suggesting a very early role in iridophore development, most likely fate specification of iridoblasts from multipotent neural crest cells. Previously, there has been no data concerning the timing when iridophore fate specification occurs, although an informative comparison can be made with melanophore development for which fate specification can be defined precisely as the time when *mitfa* is first expressed. The timing of this varies along the antero-posterior axis, but at approximately 21 hpf has just begun in the posterior trunk [47]. Two other very early markers of melanoblasts, *det* and *kit*, also begin to be expressed in this region from approximately 21 hpf [35,47,48]. Thus, in the case of the melanophore, specification begins in the posterior trunk approximately 21 hpf and this is also reflected by expression of two other very early melanoblast marker genes. We show that *ltk* is already expressed more broadly, throughout the posterior trunk, at this stage. In an *mitfa* mutant, defects in melanoblasts are seen from 23 hpf at least [47]; in contrast, defects in melanoblast markers in strong mutants for the *kit* gene, an RTK important for melanoblast survival, are absent at 24 hpf, but detectable at 36 hpf [35]. In addition, the severity of the *shdltk* mutant phenotype, with iridophores totally absent, fits well with the *mitfa* mutant phenotype, but contrasts with that of the survival mutant, *kit*. Thus, using the best analogy available, the nature and timing of the defects in *shdltk* mutants, being already visible at 20 hpf, best reflects a defect in iridoblast specification. Previous identification of numerous mutants affecting iridophore development shows many in which iridophore differentiation is not expressed in these cells in *shdltk* mutants, being already visible at 20 hpf [35,47].

Expression of *mitfa* in a subset of multipotent neural crest cells. Previously, there has been no data concerning the timing when iridophore fate specification occurs, although an informative comparison can be made with melanophore development for which fate specification can be defined precisely as the time when *mitfa* is first expressed. The timing of this varies along the antero-posterior axis, but at approximately 21 hpf has just begun in the posterior trunk [47]. Two other very early markers of melanoblasts, *det* and *kit*, also begin to be expressed in this region from approximately 21 hpf [35,47,48]. Thus, in the case of the melanophore, specification begins in the posterior trunk approximately 21 hpf and this is also reflected by expression of two other very early melanoblast marker genes. We show that *ltk* is already expressed more broadly, throughout the posterior trunk, at this stage. In an *mitfa* mutant, defects in melanoblasts are seen from 23 hpf at least [47]; in contrast, defects in melanoblast markers in strong mutants for the *kit* gene, an RTK important for melanoblast survival, are absent at 24 hpf, but detectable at 36 hpf [35]. In addition, the severity of the *shdltk* mutant phenotype, with iridophores totally absent, fits well with the *mitfa* mutant phenotype, but contrasts with that of the survival mutant, *kit*. Thus, using the best analogy available, the nature and timing of the defects in *shdltk* mutants, being already visible at 20 hpf, best reflects a defect in iridoblast specification. Previous identification of numerous mutants affecting iridophore development shows many in which iridophore differentiation is clearly abnormal, with cells looking duller or whiter than in WT development shows many in which iridophore differentiation is not expressed in these cells in *shdltk* mutants, being already visible at 20 hpf [35,47].

The alternative interpretation of our data, that *ltk* expression is restricted to, and indeed defines, specified iridoblasts would lead to the conclusion that iridoblast fate specification occurs in *sox10* mutants, but that further development fails. Whilst plausible, we do not favour this model because our data is fully consistent with the specification model shown to have general applicability to all other fates examined to date. Furthermore, we have previously shown that *crestin*, a general and early marker of differentiated lineages [50], is not expressed in these cells in *sox10* mutants [25]. Since in *sox10* mutants all pigment cell precursors fail to migrate, whereas neural precursors migrate normally [26], we speculate that the *ltk*-expressing cells may be multipotent pigment cell progenitors [1,51]. A definitive test of our proposal will require development of zebrafish neural crest cell culture or of tools to definitively fate map *ltk*-expressing cells.

Our data identifies a novel RTK pathway mediating NC development. RTKs have diverse roles in development, including in fate specification. Indeed, *Drosophila* Alk functions in specification of visceral muscle pioneers [43,44]. However, in pigment cell development RTK function has been shown to be important for proliferation, survival and migration, but not fate specification [36,48,52–56]. For example, zebrafish *kit* mutants show a partial reduction in melanoblast numbers from approximately 36 hpf [35,48] and *fms* mutants show a failure of xanthoblast migration approximately 28 hpf [36]. In contrast, *shdltk* mutants show a phenotype that is both earlier and much more severe than in these other RTK mutants. Indeed, our data reveal that iridophore fate specification occurs very early, with
LTK in Pigment Cell Fate Specification

Figure 8. Model for iridophore development from neural crest. We propose that early NCCs are initially highly multipotent (indicated by the rainbow shading) and express Sox10, but not Ltk. During progressive fate restriction, a subset of partially-restricted, but still multipotent (fewer colours), cells are formed, marked by expression of Ltk; we propose that these cells’ fates include multiple pigment cell-types (Other partially-restricted cell-types are expected to exist, but are not shown here for simplicity). Ltk signalling, acting together with Sox10 function, initiates iridoblast development (specification) in some cells (indicated by blue colour). In contrast cells adopting other fates, e.g. melanophores, extinguish Ltk expression, but may express other characteristic lineage-specific RTKs, e.g. Kit). Specified iridoblasts maintain Ltk expression as they differentiate into pigmented iridophores (blue). The early mutant phenotype precludes direct examination of the role of Ltk in these differentiating cells, although we favour cell proliferation as the likely late function. In ltk mutants (ltk	extsuperscript{-}), iridoblast fate specification fails. Cells survive for sometime (although Ltk expression is lost), before apoptosing; we cannot rule out the possibility that some precursors may transfate to other cell-types, although our data indicates most do not become melanoblasts or xanthoblasts, perhaps because of an intrinsic order of fate-specification in multipotent progenitors. In sox10 mutants (sox10	extsuperscript{-}) fate specification is also prevented; since in this mutant fate specification to all other fates is also prevented, these ltk-expressing precursors accumulate in a premigratory position, before they also eventually die.

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Materials and Methods

Fish strains

shd	extsuperscript{b92}, shd	extsuperscript{b9} and shd	extsuperscript{b20} have been described [23]. shd	extsuperscript{b91} was identified as a spontaneous mutation in AB stocks, and shd	extsuperscript{b92} in an early pressure screen for adult phenotypes [59]. shd	extsuperscript{b2} was identified in a non-complementation screen with shd	extsuperscript{b91}. WIK11 WT was used to generate the reference mapping crosses. WT cDNA was amplified from the AB line. All studies conformed to local and UK national ethical guidelines.

Timing and LTK expression in iridophores

Figure 8 shows that the timing of Ltk expression is equivalent to that of melanocyte fate specification [5], despite the later differentiation of iridophores (c. 42 hpf for iridophores, c. 23 hpf for melanophores).

We show that in zebrafish Ltk is crucial for specification of a particular pigment cell type, the iridoilobe, from NCCs. Our data contributes to understanding how pigment cell fate specification from these multipotent cells occurs. The challenge for the future will be to identify the genetic interactions between ltk, sox10 and other genes determining pigment cell fate choice. The ubiquitous nature of iridophores in fish, amphibians and reptiles suggests that Ltk function in NC is likely to be widespread. At least some birds, including doves, show iridophore-like cells in their iris [57], but their embryological origin is unclear, so examination of Ltk expression in appropriate avian embryos will be revealing. Iridophores have been lost in mammals, yet Ltk has been evolutionarily conserved. Strong shd mutant alleles are homozygous lethal [23], but this lethality cannot be attributed to the iridophore phenotype, and perhaps results from conserved functions in brain [20]. Further characterisation of defects in shd mutants will allow identification of any conserved roles. Finally, our data suggest simple, visual in vivo screens for LTK inhibitors which may be of utility considering the growing links of these RTKs to autoimmune disease [22,58].

Embryos were imaged on an Eclipse E800 (Nikon) using a U-III or DS-U1 camera (Nikon) or an LSM Meta confocal (Zeiss) microscope.

Whole-mount ISH

Embryos were processed as previously [60].

Immunofluorescent studies

Embryos for antibody staining were processed as previously described [61]. Antibodies used: mouse anti-Hu C/D (Molecular Probes); rabbit anti-phospho-Histone H3 (Upstate Biotechnology, Cat.#06-570); mouse anti-GFP, goat anti-mouse Alexa488 and anti-rabbit Alexa546 (Molecular Probes). TUNEL assays were carried out using an ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Cat.#S7100) according to manufacturer’s instructions.

Cell transplantation

Assessment of cell-autonomy was performed as described before [27]. Labelled cells were detected by rhodamine fluorescence in the live embryo and by peroxidase detection of biotinylated tracer in embryos fixed after photographing the iridophore pattern.

Mapping and PAC library screen

Heterozygous F1 fish from the mapping cross were incrossed and separate pools of F2 homozygous shd mutants and their WT siblings were used for simple sequence length polymorphism analysis [62]. Linkages from the pools were confirmed and refined by genotyping 1000 individual mutant embryos. The PAC 706 genomic library (RZPD) was screened with the marker z10985 by PCR; three positive PAC clones, BUSMP706P14181Q2 (PAC1), BUSMP706N10265Q2 (PAC2) and BUSMP706O16107Q2 (PAC3), were provided by RZPD.
Phenotypic rescue
450 pg of PAC DNA was injected in 2-cell stage embryos from \(\text{shd}^{\text{g2074}}\) carrier cross. Morpholino antisense oligonucleotides (Gene Tools) were injected into fertilized WT eggs at the one-cell stage, at concentrations up to 35 ng per embryo and incubated at 28.5°C until 72 hpf. \(\text{shd}\) morpholino sequence, 5'-aggtgtcagtagtaatataacct-3'; mismatch control, 5'-actttctcaataattatgtcag-3'.

Sequencing and gene identification
Three PACs containing z10985 were sequenced by the Danio rerio Sequencing Project at the Wellcome Trust Sanger Institute (Sequences of PACs 1–3 have accession numbers BUSM1-181P14, BUSM1-265N10 and BUSM1-107O16 respectively and can be accessed from http://www.sanger.ac.uk/Projects/D_rerio/). Predicted genes were identified using Nucleotide Identify X (NIX) software (UK Human Genome Mapping Project Resource Centre).

Mutation identification
WT cDNAs were isolated using SMART RACE PCR (Clontech) and long PCR using the Herculase enhanced DNA polymerase (Stratagene), cloned in Zero Blunt TOPO (Invitrogen) and sequenced commercially (Oswel). \(\text{shd}\) cDNAs were amplified from \(\text{shd}^{\text{g2074}}\) and \(\text{shd}^{\text{p}0}\) embryos and sequenced directly. RFLP analysis of \(\text{shd}^{\text{g2074}}\) and siblings was performed on PCR fragments amplified from genomic DNA prepared from single embryos. Sequences were aligned using ClustalW multiple sequence alignment software. WT and mutant cDNA sequences have been deposited in Genbank under accession numbers 1051419 and 1057253 respectively.

Phylogenetic analysis
The cloned sequences were compared with homologous sequences in a phylogenetic analysis using a Bayesian method [63] implemented in MrBayes (v3.1.2) and a maximum likelihood analysis applied in TREEFINDER [64] applied to each of four alignments. See Table S2 and Figure S3.

Supporting Information
Figure S1 NG derivatives other than iridophores are overtly normal in \(\text{shd}^{\text{g2074}}\) mutants. WT siblings (A,C,D,F,J,M,P,R) and \(\text{shd}^{\text{p}0}\) mutants (B,E,G,H,K,N,Q,S) are shown. A,B) Alcian blue staining of cartilage. C,H) Melanophores (*) and xanthophores (x) in head; iridophores in dorsal head indicated by arrowhead. D,E) Melanophores and xanthophores of posterior trunk. F,G) Enteric nervous system precursors (stained for \(\text{phox2b}\) mRNA); J,K) Glia of posterior lateral line nerve (sox10). M,N) Enteric neurons of posterior gut (anti-Hu). P,Q) Sensory neurons of tail dorsal root ganglia (anti-Hu immunostaining). R,S) Schwann cells of posterior lateral line nerve (eGFP from \(4.9\text{sox10}:\text{egfp}\) transgene). Similarly, visual inspection of fin mesenchyme at 4 dpf, anti-Hu immunofluorescence labelling of sympathetic neurons and fosd3-labelled posterior lateral line glia showed no defects in \(\text{shd}\) mutants (data not shown). I,LO) Transplants of WT cells into \(\text{shd}^{\text{g2074}}\) mutants rescued iridophore formation (see Table 1). Part of ventral stripe of a 5 dpf WT→\(\text{shd}^{\text{g2074}}\) chimaera to show rescued iridophores (I, incident light). Note that rescued iridophores all show both lineage tracers (L, rhodamine dextran; O, biotinylated dextran). Stages as indicated. Embryos are shown in lateral view, except in A and B (ventral view) and C, F-H (dorsal view). Found at: doi:10.1371/journal.pgen.1000026.s001 (6.51 MB TIF)

Figure S2 Structure of the zebrafish \(\text{ltk}\) gene. Zebrafish \(\text{ltk}\) gene is at least 86047 bp long and includes 29 exons, represented as horizontal boxes, shown to scale and numbered in bold. Genomic numbering based on the sequence of PAC3 (GenBank accession number BUSM1-107O16) is given in brackets, with the A of the translation initiation codon defined as +1. Introns, indicated by angled lines, are not drawn to scale, but sizes in base pairs are indicated above. Exons are colour-coded, grey representing untranslated regions, coloured portions correspond to regions encoding protein domains as per Figure 3E; numbers below the box indicate the first cDNA base pair (upper) and the amino acid number (lower, underlined) for each exon. First and last nucleotide positions within the cDNA of the coding region are also given. Our cDNA sequencing has identified two \(\text{ltk}\) splice variants (dashed lines above boxes), both of which generate distinct protein variants. Thus, removal of exon 10 produces a frame shift leading to a truncated protein, and an inclusion of intron 18 (striped box) does not alter the frame, but adds 26 amino acids. In the text and phylogenetic figures clones 1 and 3 refer respectively to the variants with intron 18 not spliced out and spliced out respectively; both have exon 10. The location of three identified mutations are shown, nomenclature as recommended in [63]. \(\text{shd}^{\text{g2074}}\) is a g.67908A>T substitution resulting in a nonsense mutation at amino acid 706. \(\text{shd}^{\text{p}0}\) is a g.32532G>T substitution (asterisk) and results in the skipping of exon 26 (dotted lines); although the reading frame remains intact, this results in a 34 amino acid deletion (A1073_W1106del) within the tyrosine kinase domain (red) (see Figure 2h); \(\text{shd}^{\text{g2074}}\) is a g.63750C>T substitution resulting in substitution of a Proline with a Serine at amino acid 759. Found at: doi:10.1371/journal.pgen.1000026.s002 (2.04 MB TIF)

Figure S3 Alternative phylogenies for \(\text{Alk/Ltk}\) family. A) Maximum likelihood analysis of amino acid alignment 2. The same topology was also found, for example, using a likelihood analysis of the nucleotide version of alignment 1. B) Maximum likelihood analysis of amino acid alignment 4. For sequence accession numbers, see Figure 2H and Table S2. As seen here and in Figure 2H, although our zebrafish clones consistently cluster with the other vertebrate Ltk, in some instances chicken Ltk appears more distant from the mammals Ltk cluster (e.g. Supp. Fig. 3A). In only a minority does the zebrafish cluster appear as sister to the chicken/mammal clade (e.g Figure S3B) as should be expected. In all cases, however, support values for the relevant clades are relatively low suggesting that the data is consistent with any of the three possibilities. Either way, this zebrafish cluster appears to be best interpreted as LTK-like, not ALK-like. Two versions of the cloned sequence were compared with homologous sequences in a phylogenetic analysis. Putative
homologs were identified by BLAST analysis against all non-redundant sequences at NCBI using E = 0.1 cut off. Representative Alk and Ltk sequences (from Human and mouse) were further blasted against individual genome assemblies. A total of 23 further homologous sequences were identified from species other than zebrafish (see Table S2) as well as related sequences from zebrafish. No hits were found for Fugu (assembly 4) (using blast at http://genome.jgi-psf.org/Takru4/Takru4.home.html). For each GenBank file the coding sequences were extracted by reference to the annotations in the GenBank files using gbbparse (http://sunflower.bio.indiana.edu/~wfischer/Perl_Scripts/). Alignment was performed using MUSCLE on the translated sequences (Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1792-1797). The nucleotide alignment was reconstructed from the protein alignments using AA2NUC.tcl (a tcl script written by L.D.H., available on request). Phylogenetic estimation was implemented using a Bayesian method (Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754-755) implemented in MrBayes (Jobb G, von Haeseler A, Strimmer K (2004) TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol Biol 4: 18) applied to each of four alignments (each both as nucleotide and protein). Four alignments of Alk/Ltk sequences generated by MUSCLE were employed. First the raw MUSCLE derived alignment (alignment 1). This was parsed by Gblocks (Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17: 540-552) under three settings to provide three further filtered alignments: a near default setting (differing from default only in permitting gaps) which retained 27% of codons (alignment 2), a more stringent alignment that retained 19% (alignment 3) and an extremely stringent alignment that retained only 1080 codons, 14% (alignment 4). This approach was taken as, with the sorts of evolutionary distances under consideration, no single alignment can be considered a priori to be optimal. The less stringent alignments may permit non-orthologous codons to be present in the alignment, so losing phylogenetic signal, while the more stringent ones by necessity may strip the alignment to such highly conserved parts that phylogenetic signal can be lost. Phylogenetic estimation was implemented using two protocols applied to each alignment and each alignment was considered both as nucleotide and protein. The two protocols were a Bayesian method (Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754-755) implemented in MrBayes (v3.1.2) and a maximum likelihood analysis implemented in TREEFINDER (Jobb G, von Haeseler A, Strimmer K (2004) TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol Biol 4: 19). For each Bayesian analysis two independent simulations were performed. In each, one million generations were simulated resulting in 10,000 trees. Concordance between the independent simulations was found in all runs. For nucleotide alignments site-specific rates with sites partitioned by codon position were assumed. For protein alignments a mixed model was employed. The last 3000 of the 10000 all have approximately the same likelihood and represent the 3000 most likely topologies. From these a 50% majority rule consensus tree was reconstructed. For the likelihood method sites were partitioned by position in the codon and the HKY model assumed. For the protein alignments the JTT model was assumed. Found at: doi:10.1371/journal.pgen.1000026.s003 (0.93 MB TIF)

**Figure S4** Proliferation of NCCs is not distinguishable in *shd* mutants and WT siblings. Counts of pH3+ GFP+ cells in the trunk and tail were made in 25 - 45 hpf embryos from incrossed *shd*+/-; *sox10*?/?; *zgf*+/- heterozygotes, before embryos were genotyped by RFLP. Counts were expressed as mean±s.d. per embryo. Counts within each age class are indistinguishable between WTs and *shd* mutants (two tailed t-test; p>0.05). Found at: doi:10.1371/journal.pgen.1000026.s004 (0.21 MB TIF)

**Table S1** Counts of iridophores in rescued chimaeric embryos. Found at: doi:10.1371/journal.pgen.1000026.s005 (0.03 MB DOC)

**Table S2** ALK/LTK-like sequences employed from species other than zebrafish. Found at: doi:10.1371/journal.pgen.1000026.s006 (0.04 MB DOC)

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**Author Contributions**

Conceived and designed the experiments: SL XY TC PH RG SLJ AW RNK. Performed the experiments: SL XY JM TC AA GJR AJ LDH MDM. Analyzed the data: SL XY JM TC AA GJR AJ LDH MDM SLJ RNK. Performed the experiments: SL XY JM TC AA GJR AJ LDH MDM. Wrote the paper: SL XY TC LDH SLJ AW RNK.

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