Sonic hedgehog is indirectly required for intraretinal axon pathfinding by regulating chemokine expression in the optic stalk

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SUMMARY
Successful axon pathfinding requires both correct patterning of tissues, which will later harbor axonal tracts, and precise localization of axon guidance cues along these tracts at the time of axon outgrowth. Retinal ganglion cell (RGC) axons grow towards the optic disc in the central retina, where they turn to exit the eye through the optic nerve. Normal patterning of the optic disc and stalk and the expression of guidance cues at this choice point are necessary for the exit of RGC axons out of the eye. Sonic hedgehog (Shh) has been implicated in both patterning of ocular tissue and direct guidance of RGC axons. Here, we examine the precise spatial and temporal requirement for Hedgehog (Hh) signaling for intraretinal axon pathfinding and show that Shh acts to pattern the optic stalk in zebrafish but does not guide RGC axons inside the eye directly. We further reveal an interaction between the Hh and chemokine pathways for axon guidance and show that cxcl12a functions downstream of Shh and depends on Shh for its expression at the optic disc. Together, our results support a model in which Shh acts in RGC axon pathfinding indirectly by regulating axon guidance cues at the optic disc through patterning of the optic stalk.

KEY WORDS: Axon guidance, Tissue patterning, Hh signaling, Shh, Cxcl12, Zebrafish

INTRODUCTION
The first steps for correct RGC axon pathfinding to the optic tectum are axon extension towards the optic disc and turning into the optic stalk to exit the eye through the optic nerve. When RGC axons fail to turn at the optic disc, they project within the eye and become trapped. Several molecules have been implicated in axon pathfinding out of the eye, acting either through ocular tissue patterning, direct axon guidance or modulation of guidance cues (Deiner et al., 1997; Schauerte et al., 1998; Dakubo et al., 2003; Li et al., 2005). Shh is one factor that regulates ocular patterning in multiple species. A gradient of Shh along the proximodistal axis, formed by shh expression in the floorplate and notochord, has been implicated in specifying early eye tissue into optic stalk and retina through regulation of Pax2 and Pax6 expression domains (Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003). Additionally, Shh expressed by RGCs is required for normal formation of astrocytes at the optic disc and stalk in mouse, with subsequent effects on RGC axon guidance (Wallace and Raff, 1999; Dakubo et al., 2003; Dakubo et al., 2008). However, there is also evidence for direct axon guidance by Shh. Both in vitro and in vivo studies in mouse and chick have implicated Shh in RGC axon guidance by signaling through non-canonical Hh pathways independent of target gene transcription (Trousse et al., 2001; Kolpak et al., 2005; Kolpak et al., 2009; Sánchez-Camacho and Bovolenta, 2008; Fabre et al., 2010; Gordon et al., 2010).

In this study, we used zebrafish to determine whether Shh regulates intraretinal axon pathfinding indirectly through tissue patterning or directly as a guidance molecule. Our results strongly suggest an indirect requirement for Hh signaling in intraretinal pathfinding through patterning of the optic stalk and argue against a direct requirement for Shh in intraretinal axon guidance in zebrafish. We show further that Shh regulates the expression of several genes at the optic stalk and disc. One of these, cxcl12a, has previously been implicated in intraretinal axon guidance in zebrafish (Li et al., 2005). We show that the Hh and chemokine signaling pathways interact genetically for axon guidance out of the eye. Altogether, our data lead us to propose that Hh signaling during early optic vesicle development is required for proper optic stalk patterning and correct expression of downstream guidance molecules, specifically cxcl12a, at the optic disc that direct RGC axons out of the eye.

MATERIALS AND METHODS
Mutant and transgenic lines
Fish were of Tü or TL strains. Embryos were raised at 28.5°C in 0.1 mM phenylethiourea. Mutant alleles used were: shha1747 (Schauerte et al., 1998), smoo419157 (Chen et al., 2001), cxcl12a19664 (Valentin et al., 2007) and cxcr4b52603 (Knaut et al., 2003). Transgenic lines used were: Tg(–17.6isl2b:GFP)zc7 (Pittman et al., 2008), Tg(–17.6isl2b:tagRFP)zc80, Tg(hsp70l:GFP)zc112, and Tg(hsp70l:cxcl12a-2A-EGFP)zc85. The Tg(hsp70l:cxcl12a-2A-EGFP)zc85 line was generated using the Tol2 kit (Kwan et al., 2007). The cxcl12a full-length middle clone was generated using primers with attB1F and attB2R sites flanking the cxcl12a coding sequence, omitting the stop codon.

In situ hybridization
Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, washed in PBS, dehydrated through a methanol series and stored at –20°C. Whole-mount in situ hybridization staining was performed according to Thisse and Thisse (Thisse and Thisse, 2008). For sectioning, embryos were prepared as described previously (Pittman et al., 2008) and sectioned at 15 μm on a Reichert-Jung 2050 Supercut microtome with a glass knife. Images were taken on an Olympus BX51WI compound microscope using a SPOT RT3 camera. Images were processed using Adobe Photoshop CS2.

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Immunohistochemistry
For whole-mount immunohistochemistry, embryos were fixed (4% PFA, overnight), washed in PBS, dehydrated in methanol and stored at -20°C, then rehydrated and washed in PBST (PBS + 0.1% Tween 20), permeabilized with 0.1% collagenase [15 minutes, room temperature (RT)]. Embryos were blocked (2 hours, RT) with 10% newborn calf serum with 0.1% Tween 20 (NCST), incubated in primary antibodies (overnight, 4°C), washed in PBST, incubated in secondary antibodies plus ToPro3 (1:1000, Invitrogen; 4 hours, RT) and washed in PBST. Primary antibodies used were: mouse anti-GFP (1:200, Millipore), rabbit anti-GFP (1:200, Invitrogen), mouse anti-tagRFP (1:200; Evrogen) and rabbit anti-Pax2a (1:300, gift of Dr Michael Brand, Center for Regenerative Therapies Dresden, Technische Universität Dresden, Germany). Secondary antibodies used were: goat anti-mouse 488 (1:200; Invitrogen), goat anti-rabbit 488 (1:200, Invitrogen), goat anti-mouse Cy3 (1:200, Jackson ImmunoResearch) and goat anti-rabbit Cy3 (1:200, Jackson ImmunoResearch).

Confocal microscopy
Embryos were cleared in 50% glycerol/H2O (3 hours, 4°C) and stored at 4°C in 80% glycerol/H2O. Heads were dissected and embedded between two #0 cover slips separated by two layers of black electrical tape. Images were taken on a FV1000-XY Olympus confocal microscope using a 40× water objective. Maximum-intensity projection images were generated using ImageJ (http://rswebw.nih.gov//ij/index.html).

SANT75 treatment
Twenty isl2b:GFP embryos were incubated in 40 μM SANT75 (gift of Dr Shuo Lin, University of California Los Angeles, CA, USA) in 1% DMSO in E3 or E2/GN (E2+10 μg/ml gentamycin sulfate) bath-applied in 6-well plates. For the 1-24 hours post-fertilization (hpf) treatment, SANT75 was washed off with E3 (three 15 minute washes). Phenotypes were scored using Microsoft Excel. For the Mann-Whitney U test, embryos from three batches were included in the analysis.

RESULTS
Hh mutant RGC axons make intraretinal pathfinding errors
In wild-type (wt) zebrafish, RGC axons project towards the optic disc in the central retina and exit the eye through the optic nerve. RGCs and their projections were visualized using embryos carrying the isl2b transgene, which is expressed in RGCs and photoreceptors inside the eye and extra-retinal expression of which includes the trigeminal ganglion (Pittman et al., 2008) (Fig. 1A,D,G,I). In sonic hedgehog a (shha) mutants, many RGC axons fail to exit through the optic nerve and become trapped within the eye, projecting posteriorly or anteriorly within the retina (Fig. 1B,E,H,J). In addition, RGC axons that exit the eye through the optic nerve in shha mutants often make mistakes further along the pathway and project to the ipsilateral optic tectum instead of crossing at the chiasm (Fig. 1B). Hh pathway mutations lead to retinal cell proliferation defects (Neumann and Nusslein-Volhard, 2000), which result in decreased eye size compared with wt (Fig. 1D-F).

Fig. 1. Intraretinal axon pathfinding defects in Hh pathway mutants. (A-J) Retinal projections at 2 dpf in wt, shha and smo zebrafish embryos with isl2b:GFP (green) or isl2b:tagRFP (pseudocolored green in C,F) transgene; nuclei, ToPro3 (magenta). Ventral (A,C) or lateral views (D,F) of maximum-intensity projections and schematics of wt and mutant axon projections showing lateral (G,H) and ventral views (I,J) are shown. In wt embryos (A,D,G,I), RGC axons converge at the optic disc (arrow), where they turn and pass through the optic nerve (asterisk). In shha (B,E,H,J) and smo (C,F,H,J) mutants, some axons fail to exit the eye, projecting posteriorly or occasionally anteriorly within the eye (arrowheads). Hh mutants also exhibit misprojections to the ipsilateral optic tectum (asterisk in B). D, dorsal; V, ventral; A, anterior; P, posterior. Scale bars: 100 μm.
Zebrafish carry two paralogs of shh: shha and shhb. However, although both variants are expressed in RGCs (Neumann and Nuesslein-Volhard, 2000), only the loss of shha results in intraretinal pathfinding errors. Knockdown of shhb in wt embryos using a translation blocking morpholino (MO) did not induce intraretinal axon guidance errors, whereas MO injections into shha embryos led to severe midline patterning defects similar to smo mutants (Varga et al., 2001) without an increase in the severity of the intraretinal pathfinding phenotype (data not shown).

Focal lipophilic dye injections in the RGC layer revealed that RGC axons from all quadrants of the shha retina project towards the optic disc, where some axons fail to turn and misproject within the eye, with a predominance of posterior over anterior projections (supplementary material Fig. S1).

The ligand Shh binds to Patched (Ptch) receptors, which signal through Smoothened (Smo) to activate the Hh signaling pathway (Ingham and McMahon, 2001). Zebrafish carry one smo gene and mutations in smo therefore lead to complete inhibition of the Hh signaling pathway. smo embryos exhibit intraretinal guidance errors and ipsilateral projections analogous to those seen in shha mutants (Fig. 1C,F,H,J). Smo embryos show strong midline patterning defects, but are rescued from complete cyclopia by maternally expressed smo (Varga et al., 2001). In summary, Hh signaling is required for correct intraretinal axon pathfinding in zebrafish.

**Hh pathway components are expressed during eye patterning and RGC axon outgrowth**

To determine how Hh signaling regulates intraretinal axon pathfinding, we analyzed the spatial and temporal expression patterns of both ligand and receptor components. We performed in situ hybridization for shha, ptch2 and smo at 16 hours post fertilization (hpf), after the optic vesicle has formed (Li et al., 2000), at 28 hpf, just as RGCs start to differentiate (Laessing and Stuermer, 1996), and at 48 hpf, when most RGCs have differentiated and several axons have reached the optic tectum (Laessing and Stuermer, 1996). At 16 hpf, shha, ptch2 and smo mRNAs are expressed in anterior midline neurectoderm (Fig. 2A,D,G). Whereas the expression of shha and ptch2 is very specific, smo shows a broader expression throughout the head region. At 28 hpf, shha is strongly expressed at the midline (Fig. 2B), whereas ptch2 is expressed at the midline and strongly in the optic stalk (Fig. 2E) and smo shows broad expression throughout the brain and optic stalk (Fig. 2H). At 48 hpf, shha midline expression is still strong (Fig. 2C) and all three Hh pathway genes are expressed in RGCs (Fig. 2C,F,I). Thus, Hh pathway components are expressed both during ocular tissue patterning, as well as during RGC axon outgrowth, consistent with a potential role in both processes.

**Shha and Smo act non-cell-autonomously in RGC axon pathfinding**

We next used cell transplants to test functionally for cell autonomy of Hh pathway components. If Hh signaling directly regulates the guidance of RGC axons, we expect Shha to act non-cell-autonomously, whereas receptor components would act cell autonomously in RGCs. By contrast, if Hh signaling patterns the eye and optic stalk to ensure the correct cellular environment for intraretinal axon pathfinding, we expect both ligand and receptor to act non-cell-autonomously.

We used wt, shha and smo embryos to transplant retinal precursor cells (RPCs) at 24 hpf from donor into host embryos and analyzed donor RGC axon projections at 54 hpf. To visualize RGC projections, donor embryos carried the isl2b transgene (Pittman et al., 2008). When wt RPCs were transplanted into wt hosts, all donor RGC axons exited the host eye through the optic nerve (100% of transplants) (Fig. 3A,H). However, when wt cells were transplanted into shha hosts, donor RGC axons were misguided (50% of transplants) (Fig. 3B,H; P=0.015). Similarly, when wt cells were transplanted into smo hosts, we found misguided donor RGC axons (62% of transplants) (Fig. 3C,H; P=0.006). When shha RPCs were transplanted into wt hosts all donor axons exited the eye normally (Fig. 3D,H), whereas axons were misguided (55% of transplants) when shha cells were transplanted into shha hosts (Fig. 3E,H; P=0.003). Similarly, donor axons from smo into wt transplants always exited the eye normally (Fig. 3G,H), whereas smo into smo transplants resulted in misguided donor axons (56% of transplants) (Fig. 3I,H; P=0.003). Altogether, we find that wt and mutant RGC axons are equally able to exit the eye in wt hosts but exhibit pathfinding errors when transplanted into shha or smo embryos. This indicates that both Shha and Smo act non-cell-autonomously in intraretinal axon pathfinding.

**Fig. 2. Hh pathway genes are expressed in the zebrafish optic stalk and RGC layer.** Whole-mount in situ hybridizations (15 μm sections) for shha, ptch2 and smo mRNA. A,D,G are dorsal views; B,C,E,F,I,J are frontal views. (A) 16 hpf, shha expression in anterior midline neurectoderm (arrow). (B) 28 hpf, shha expression at the midline (arrowhead) but not optic stalk (arrow). (C) 48 hpf, shha strongly expressed at the midline (arrowhead) and in RGC layer (arrow). mRNA also detected in the optic nerve (asterisk). (D) 16 hpf, ptch2 expressed at the anterior midline (arrow). (E) 28 hpf, ptch2 strongly expressed in the optic stalk (arrow) and midline (arrowheads). (F) 48 hpf, ptch2 detected in the RGC layer (arrow) and optic nerve (asterisk). (G) 16 hpf, smo expressed throughout head region. (H) 28 hpf, smo broadly expressed, including optic stalk (arrow). (I) 48 hpf, smo localized in the RGC layer (arrow) and optic nerve (asterisk). Dashed line in A,D,G outlines the optic vesicle. A, anterior; P, posterior; D, dorsal; V, ventral; L, lateral; M, medial. Scale bar: 100 μm.
Previously, we found that pioneer RGCs are necessary to guide later-born axons out of the eye (Pittman et al., 2008). Thus, although we found a non-cell-autonomous effect for Smo, this result could be explained by axon-axon interactions, for which donor RGC axons simply follow host pioneers, thereby masking a cell-autonomous effect of Smo in intraretinal axon guidance. To prevent such pioneer-follower interactions, we inhibited RGC differentiation in host embryos until at least 54 hpf with ath5 (atoh7 – Zebrafish Information Network) MO injections. By transplanting donor RGCs into ath5 morphants, we analyzed axon pathfinding of donor RGCs in a host RGC-free environment. Isl2b:GFP embryos were injected with 4 ng ath5 MO at the 1-cell stage. Isl2b:tagRFP donor RPCs were transplanted at 24 hpf into ath5 morphants and RGC axon projections analyzed at 54 hpf. Isl2b:GFP expression in the trigeminal ganglion was used as a control to ensure successful inhibition of RGC differentiation in transgenic embryos (Fig. 4A, arrow). When wt RPCs were transplanted into ath5 morphants, donor RGC axons exited the eye in most cases; only 18% of the transplants showed intraretinal pathfinding errors (Fig. 4B,E). Similarly, smo axons exited the eye in most ath5 morphants; 31% of the transplants exhibited errors (Fig. 4C,E; $P=0.44$). By contrast, wt RGC axons were misrouted in smo mutant ath5 morphant host eyes in 96% of transplants (Fig. 4D,E; $P=3.9E-8$). These results demonstrate that Smo is required in the environment to ensure correct RGC axon pathfinding, but not in RGCs themselves.

**Hh signaling is not required during axon pathfinding for correct axon outgrowth**

We used pharmacological inhibition of Hh signaling during embryogenesis to determine when Hh pathway activity is necessary for intraretinal pathfinding. We applied the small molecule compound SANT75 (Smoothened antagonist 75), which specifically inhibits Smo (Yang et al., 2009). We chose SANT75 rather than cyclopamine because of better solubility and stability properties. Because the genes encoding Ptch receptors and Gli transcription factors are themselves target genes of the Hh pathway, expression levels of ptch2 and gli1 can be used as readouts for
pathway inhibition. SANT75 treatment inhibits ptc2 and gli1 expression in a dose-dependent manner (Yang et al., 2009). SANT75 application (40 μM) resulted in downregulation of ptc2 expression in the brain at 24 hpf (Fig. 5A,B, arrowheads) but no cyclopaia. Bath application of 40 μM SANT75 from 1-54 hpf induced a strong intraretinal pathfinding phenotype (79% of isl2b:GFP transgenics) (Fig. 5C,F). DMSO-control embryos never showed pathfinding errors (Fig. 5D,F).

At 10 hpf, the eye field is specified and optic vesicle evagination is commencing. At 16 hpf, the optic vesicle is formed but eye patterning is still ongoing. At 24 hpf, basic eye patterning is completed and at 28 hpf, the first RGCs start to differentiate. Applying SANT75 at these specific time points allowed us to determine whether Hh signaling is required before optic vesicle specification (1-10 hpf), for optic vesicle specification and basic eye patterning (10-24 hpf), or during RGC axon outgrowth (after 28 hpf). Treatment with 40 μM SANT75 from 10-54 hpf resulted in 78% of isl2b:GFP embryos with intraretinal axon pathfinding errors (Fig. 5F). Similarly, when we started treatment at 1 hpf and washed out SANT75 at 24 hpf, we found intraretinal axon pathfinding errors in 70% of embryos (Fig. 5F). These results are not significantly different from continuous treatment (1-54 hpf), for which 79% of embryos showed errors. Starting treatment at 16 hpf or 24 hpf, by contrast, resulted in significantly fewer embryos with pathfinding errors, 40% (P=0.002) and 33% (P=0.0001), respectively (Fig. 5F). When treatment began at 28 hpf, just as RGCs differentiation begins, only 5% of embryos showed intraretinal pathfinding errors (Fig. 5F; P=4.4E-6). Treatment starting at 1 hpf; 24 hpf and 28 hpf resulted in comparable knockdown of ptc2 mRNA. Additionally, treatment starting at 1 hpf and 24 hpf resulted in loss of Pax2 expression by 54 hpf, but returned to control Pax2 expression levels by 54 hpf after wash-off at 24 hpf (data not shown). Therefore, the significantly weaker pathfinding phenotype with treatment starting at 24 and 28 hpf indicates that Hh signaling is not necessary at the time of RGC axon pathfinding out of the eye. In addition, our wash-off experiment showed that early inhibition of Hh signaling causes intraretinal pathfinding errors similar to those seen with continuous treatment. Our results show that inhibition of Hh signaling during optic vesicle patterning is sufficient to induce intraretinal axon pathfinding errors later during development.

**Optic stalk markers are downregulated in Hh pathway mutants**

The strong indication for a role of Hh signaling in eye patterning prompted us to analyze the expression of several optic stalk markers in shha and smo mutants. Pax2, a transcription factor expressed in the developing optic stalk (Macdonald et al., 1995), was downregulated at 28 hpf in shha and lost in smo embryos compared with wt (Fig. 6A-C). netrin 1a, which encodes a known axon guidance molecule, is expressed along the optic fissure (Macdonald et al., 1997). netrin 1a mRNA levels were decreased in shha mutants and no expression was found in smo eyes at 28 hpf (Fig. 6D-F). chemokine ligand 12a (cxcl12a, previously known as sdf1a) and its homolog cxcl12b are expressed in the optic stalk and Cxcl12b was proposed to have an attractive effect on RGC axons inside the eye in zebrafish (Li et al., 2005). cxcl12a mRNA levels were strongly downregulated at 28 hpf in the optic stalk in shha embryos and not expressed in smo mutants (Fig. 6G-I). Thus, loss of Hh signaling leads to downregulation of known transcription factors and axon guidance molecules in the stalk region.

**Cxcl12a has an attractive effect on shha RGC axons**

Our results suggest a model in which Hh signaling during eye specification regulates optic stalk/disc expression of guidance molecules necessary for correct RGC axon pathfinding out of the eye. Although netrin 1 mutants show intraretinal axon guidance errors in mouse (Deiner et al., 1997), we observed no errors using morpholino knockdown in zebrafish (supplementary material Fig. S2). By contrast, zebrafish mutants for cxcr4b, a receptor for Cxcl12a, exhibit intraretinal axon pathfinding errors (Li et al., 2005). Analysis of intraretinal RGC projections in Cxcl12a mutants revealed the same highly penetrant pathfinding errors (Fig. 7A,B). The intraretinal axon guidance phenotypes in Cxcl12a and Cxcr4b mutants are strikingly similar to Hh pathway mutant phenotypes. Therefore, we tested whether downregulation of chemokine signaling at the optic disc in Hh pathway mutants might be responsible for their intraretinal axon pathfinding phenotype. It was reported that Cxcl12b has an attractive effect on RGC axons inside the eye (Li et al., 2005). To determine whether Cxcl12a has a
showed anterior RGC projections (Fig. 7F,I), whereas posterior projections were more common in these embryos (Fig. 7G, arrow). Here, anterior and posterior projections represent pathfinding errors due to the loss of shha and not due to an attractive effect of EGFP (Fig. 1B; supplementary material Fig. S1). By sharp contrast, Cxcl12a expression in the anterior eye of shha mutants led to anterior projections in 100% of transplants (Fig. 7H,I; $P=0.0003$). These results indicate that Cxcl12a has an attractive effect on RGC axons in both wt and shha embryos. In addition, ectopic Cxcl12a expression in shha mutant eyes resulted in more embryos with anterior projections than in wt, possibly owing to the decreased endogenous Cxcl12a at the optic disc in shha mutants.

The Hh and chemokine pathways interact genetically for intraretinal axon guidance

To investigate further the interaction between the Hh and chemokine pathways for intraretinal axon guidance, we analyzed the axon pathfinding phenotype in shha mutants that are either wt or heterozygous for cxcl12a. We crossed shha/+;is12b:GFP fish to shha/+;cxcl12a+/+;is12b:GFP carriers. At 2 dpf, we scored the intraretinal pathfinding phenotypes and grouped them into four categories: ‘0’, no errors, all axons exit the eye; ‘1’, few errors, most axons leave the eye; ‘2’, most axons make errors inside the eye, few exit; ‘3’, all axons make errors, no axons leave the eye (Fig. 7J). The shha and shha;cxcl12a/+ embryos were separated after genotyping and the severity of the intraretinal axon pathfinding phenotype for both groups was analyzed using Mann-Whitney U statistics. Our analysis showed a significant increase in the severity of the pathfinding phenotype in shha;cxcl12a/+ embryos compared with shha ($P=0.001$). As cxcl12a heterozygous embryos exhibit no intraretinal axon guidance errors, our finding that cxcl12a heterozygosity increases the severity of intraretinal axon pathfinding phenotype in shha mutants indicates that the Hh and chemokine pathways interact genetically for RGC axon guidance inside the eye.

Chemokine signaling acts directly on RGC axons for correct intraretinal axon guidance

To test whether chemokine signaling has a direct role in RGC axon pathfinding, we performed cell transplants using cxcr4b mutants, which lack the receptor for cxcl12a in RGCs (Li et al., 2005), as donor or host embryos. Because transplanted RGC axons tend to follow existing axon pathways, we used both unmanipulated and ath5 morphants that lack RGCs as hosts. Wt cells transplanted into wt embryos exited the eye normally (100% of transplants) (Fig. 8A,G) but when transplanted into cxcr4b mutants, transplanted wt cells made errors (66.6% of transplants) (Fig. 8B,G; $P=0.003$), probably because they followed aberrant axon pathways laid out by earlier born RGCs. Along the same line, cxcr4b mutant axons rarely made errors in wt eyes with a full complement of RGCs (15% of transplants) (Fig. 8C,G) but when transplanted into ath5MO-injected wt embryos that lack previously formed axon pathways, we observed misguided cxcr4b axons in 94.7% of transplants (Fig. 8D,G). Consistently, 33.3% of the transplants exhibited errors when wt cells were transplanted into ath5 morphants regardless of whether these hosts were wt (Fig. 8E,G; $P=0.0014$) or cxcr4b mutant (Fig. 8F,G). These results show that Cxcr4b acts cell autonomously in RGCs for correct axon pathfinding and supports our hypothesis that chemokine signaling acts directly in intraretinal axon guidance in zebrafish.
DISCUSSION
Previous research has shown involvement of Shh both in tissue patterning and in direct axon guidance, such as in spinal cord patterning and commissural axon guidance across and along the midline (Ericson et al., 1997; Briscoe and Ericson, 1999; Charron et al., 2003; Bourikas et al., 2005; Okada et al., 2006; Yam et al., 2009; Domanitskaya et al., 2010), as well as in optic stalk and retina patterning and RGC axon guidance along the retinotectal pathway (Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003; Trousse et al., 2001; Kolpak et al., 2005; Sánchez-Camacho and Bovolenta, 2008; Fabre et al., 2010; Gordon et al., 2010). We determined that Hh signaling acts non-cell-autonomously for intraretinal axon pathfinding in zebrafish and that Hh pathway activity is required during early eye patterning for correct intraretinal pathfinding later in development. Additionally, we revealed a genetic interaction of the Hh and chemokine signaling pathways for intraretinal axon guidance and showed that Cxcl12a acts as an attractant for RGC axons inside the eye.

Shh regulates optic stalk patterning but not direct RGC axon guidance in zebrafish
Shh expressed by notochord and floorplate cells forms a gradient along the proximodistal axis of the embryo, which is necessary for the specification of optic stalk and retina tissue in both *Xenopus* and zebrafish (Perron et al., 2003; Ekker et al., 1995; Macdonald et al., 1995). High levels of Shh induce Pax2 expression in proximal tissue, whereas distal optic tissue exposed to low levels of Shh expresses Pax6 (Ekker et al., 1995; Macdonald et al., 1995). Overexpression of Shh leads to an expansion of the Pax2-positive
Hh pathway receptors are also expressed in murine RGCs and ganglion-cell-autonomous Hh signaling has been shown to regulate intraretinal axon guidance in mice (Sánchez-Camacho and Bovolenta, 2008). Similarly, inhibition of Hh signaling using cyclopamine in chick suggested a role for Shh as direct axon guidance molecule for intraretinal pathfinding (Kolpak et al., 2005). Although we detected expression of both Hh pathway ligand and receptors in the RGC layer at the time of axon outgrowth, we showed an RGC-non-autonomous role for Hh signaling in intraretinal axon pathfinding. Instead, we observed a requirement for Hh pathway activity during early eye development for subsequent retinal pathfinding in zebrafish. The early requirement for Hh pathway activity is supported by a previous study, using cyclopamine treatment (Kay et al., 2005). We propose that in zebrafish Hh signaling acts in optic stalk patterning early during eye development but not in direct axon guidance at the time of axon outgrowth to regulate correct intraretinal pathfinding. This role in optic stalk patterning corresponds well with previous findings that Hh signaling regulates axon pathfinding at the zebrafish midline indirectly by determining glial cell position (Barresi et al., 2005). Therefore, although Hh signaling has a conserved role in vertebrate retinal axon pathfinding, the mechanisms appear to be distinct in different model systems.

**Axon guidance at the optic disc**

In both mouse and zebrafish, Hh pathway mutants show intraretinal axon pathfinding errors where axons fail to turn at the optic disc and instead project within the eye (Sánchez-Camacho and Bovolenta, 2008; Schauerte et al., 1998). Additionally, mouse netrin1 mutants exhibit intraretinal guidance errors (Deiner et al., 1997). Intraretinal pathfinding errors in a conditional mouse model with loss of shh in RGCs have been explained by the lack of netrin1 expression at the optic disc due to the loss of astrocyte precursor cells in this region (Dakubo et al., 2003). Similar to mouse netrin1 (Deiner et al., 1997), zebrafish netrin1a is expressed in the optic disc/stalk region during RGC axon outgrowth (Park et al., 2005) and we show that loss of Hh signaling leads to downregulation of netrin1a expression in the stalk. But in zebrafish, MO-mediated knockdown of netrin1a failed to induce any retinal pathfinding errors. None of the other zebrafish netrin paralogs is expressed in the eye during development (Park et al., 2005), thus making gene compensation unlikely. We found that the chemokine cxcl12a is similarly transcriptionally regulated by Hh signaling. Additionally, cxcl12a and cxcr4b mutants exhibit intraretinal axon pathfinding errors very similar to Hh mutants. At this point, it cannot be excluded that other guidance molecules affect pathfinding at the optic disc in addition to cxcl12a, but the high penetrance of intraretinal axon pathfinding errors in cxcl12a and cxcr4b mutants as well as our transplants showing a cell-autonomous effect for cxcr4b in intraretinal axon guidance support our view of this ligand-receptor pair as a crucial cue at the optic disc in zebrafish. Therefore, we propose that whereas netrin1 is the main guidance cue inducing turning at the optic disc in mice, this role is taken by cxcl12a in zebrafish.

**Chemokine signaling in axon guidance**

A few studies have implicated chemokine signaling in axon guidance, demonstrating both attractive (Li et al., 2005; Arthur et al., 2009) and repulsive effects (Xiang et al., 2002) of Cxcl12 on axons, as well as a modulatory effect on other guidance cues (Chalasani et al., 2003; Chalasani et al., 2007; Lieberam et al., 2005). In zebrafish, cxcl12a is expressed in the distal optic stalk.
and MO-mediated knockdown of cxcl12a induced intraretinal axon pathfinding errors (Li et al., 2005). Additionally, we showed that cxcl12a mutants exhibit a highly penetrant intraretinal axon pathfinding phenotype.

cxcl12b is localized in the proximal stalk region but knockdown of cxcl12b alone did not induce intraretinal pathfinding errors (Li et al., 2005). These findings show that cxcl12a is necessary for intraretinal axon pathfinding and demonstrate that cxcl12b cannot compensate for this role. Previously, it was shown that Cxcl12b misexpression in the eye has an attractive effect on RGC axons (Li et al., 2005). We showed that Cxcl12a exhibits a similar attractive effect on both wt and shha mutant RGC axons inside the eye. This demonstrates that Shh is not required as a competence factor for the attractive effect of Cxcl12a. By contrast, we found that shha mutant axons showed a stronger attraction towards misexpressed Cxcl12a in the anterior eye compared with wt axons. This could be explained by the stark downregulation of cxcl12a in the shha mutant optic stalk. Whereas endogenous Cxcl12a at the optic disc attracts wt axons into the stalk, thereby counteracting the effect of misexpressed Cxcl12a in the anterior eye, the low level of endogenous Cxcl12a in shha mutants could allow for a stronger attraction of RGC axons towards the misexpressed Cxcl12a.

Genetic interaction between the Hh and chemokine pathways

Hh signaling was shown to promote the expression of chemokine pathway components at the level of cxcl12 and ccr4 in cholangiocytes, endothelial progenitor cells and medulloblastoma (Omenetti et al., 2009; Yamazaki et al., 2008; Yoon et al., 2009). However, the relation between chemokine signaling and the Hh signaling pathway for axon pathfinding was not known. We showed that although cxcl12a heterozygosity by itself does not lead to intraretinal pathfinding errors, the loss of one allele of cxcl12a in shha mutants significantly increases the intraretinal pathfinding phenotype compared with shha mutants. Intraretinal pathfinding errors are seen in nearly 100% of cxcl12a and ccr4b mutants, whereas Hh pathway mutants only show pathfinding errors in ~50% of the embryos. We observed a low level of cxcl12a expression at the optic disc in shha mutants, and this residual expression might be sufficient to partly rescue the pathfinding phenotype in shha mutants. Using pharmacological Hh pathway inhibition starting at 1 hpf, we induced pathfinding errors in up to 80% of the embryos. This might indicate that maternal smo mRNA deposition (Varga et al., 2001) is sufficient to partly rescue optic stalk patterning in smo mutants during early eye development even though cxcl12a expression was undetectable at 28 hpf in these embryos using in situ hybridization. Therefore, we propose that the increased pathfinding phenotype in shha embryos with only one allele of cxcl12a compared with shha mutants might be explained by a further downregulation of Cxcl12a levels at the optic disc. Decreased levels of cxcl12a expression at the disc in shha mutants can either be explained by loss of specific gene expression or by a failure of cells differentiation in the optic stalk. Studies in mice showed that Hh signaling is necessary for astrocyte differentiation and maintenance in the optic stalk (Wallace and Raff, 1999; Dakubo et al., 2003; Dakubo et al., 2008). Our genetic interaction experiment, however, supports the model that axon pathfinding errors in shha mutants are due to the loss of cxcl12a. This identifies the Hh signaling pathway as a regulator of guidance cue expression in the zebrafish optic stalk. A similar indirect requirement for Hh signaling has been observed at the zebrafish chiasm, where Shh regulates the expression of Slit guidance cues at the midline (Barresi et al., 2005).

We were unable to determine whether expression of cxcl12a in the optic stalk in shha mutants is sufficient to rescue the pathfinding phenotype in these embryos owing to technical limitations. First, polystyrene beads coated with Cxcl12a protein placed into the optic fissure at 24 hpf were pushed out of the eye during optic stalk closure and eye rotation movements. Second, hsp70:cxcl12a-2A-GFP cells transplanted into the fissure could similarly not be detected by 54 hpf. Third, the expression of a cxcl12a:GFP construct resulted in GFP expression in the wt optic stalk but this expression could not be detected in shha embryos. The lack of transgene expression in mutant optic stalk cells supports our finding of the regulation of cxcl12a expression by Hh signaling. Instead, we used cell transplants to show that Cxcr4b acts cell autonomously in RGCs for correct retinal axon pathfinding. This result clearly supports our hypothesis that Cxcl12a expressed at the optic disc acts as direct guidance cue for RGC axon pathfinding inside the retina, in contrast to Hh signaling, which acts indirectly on axon pathfinding through patterning of the eye. It will be interesting to assess whether this mechanism holds true in other organisms also.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


Intraretinal axon pathfinding


