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., 1995; 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 cues at 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.

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 phenylthiourea. Mutant alleles used were: shha¹⁸¹,¹⁸² (Schauerte et al., 1998), smoh¹⁵⁶ (Chen et al., 2001), cxcl12a⁰⁸¹ (Valentin et al., 2007) and cxcr4b¹²⁶ (Knaut et al., 2003). Transgenic lines used were: Tg(–17.6isl2b:GFP)¹⁷ (Pittman et al., 2008), Tg(–17.6isl2b:tagRFP)¹⁷¹, Tg(ksp70l:GFPP)¹⁸⁴ and Tg(ksp70l:cxcl12a-2A-EGFP)²⁸².

The Tg(ksp70l:cxcl12a-2A-EGFP)²⁸² line was generated using the Tol2kit (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 Thissen and Thissen (Thissen and Thissen, 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.
**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://rsbweb.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 an Olympus SZX16 fluorescence stereomicroscope. Embryos with stalled RGC wave progression and no visible axon outgrowth were not included in the analysis.

**Cell transplants**

Embryos were anesthetized with tricaine, mounted in 1% low-melting point (LMP) agarose and covered with fish ringer. Roughly 20 retinal precursors were transplanted at 24 hpf. For ectopic cxi12a expression: hsp70l:EGFP or hsp70l:cxi12a-2A-EGFP embryos were used as donors. shha or wild-type (wt) embryos were used as hosts. About 50 anterior retinal cells were transplanted at 24 hpf into the anterior eye of host embryos. Hosts and donors were subjected to three rounds of heat-shock (40°C) at 28, 32 and 36 hpf.

**Morpholino injections**

**ath5MO** (4 ng) was injected at the 1-cell stage as described previously (Pittman et al., 2008). Inhibition of RGC differentiation in morphants was monitored using isl2b-GFP embryos. Morphants were used as host embryos for transplant experiments as described above. netrin1asBMO (5.6 ng) was co-injected with 2 ng p53MO (Langheinrich et al., 2002). p53MO morphants served as controls. Inhibition of netrin1a mRNA splicing was tested by RT-PCR (Wilson et al., 2006). shhbMO (formerly known as rwhh; 2 ng) (Hammond et al., 2003) with 2 ng p53MO was injected at the 1-cell stage.

**Focal lipophilic dye injections**

DiI or DiO was injected as previously described (Poulain et al., 2010). Briefly, microinjection needles were coated with melted dye crystals. Needles were quickly inserted into RGC layer of fixed embryos embedded laterally in 1% LMP agarose. Dye was allowed to diffuse overnight (RT). Embryos were washed in PBS and cleared with glycerol for imaging.

**Statistical analysis**

Fisher’s exact test was calculated using the VassarStats website (http://faculty.vassar.edu/lowry/fisher.html). Student’s t-test was calculated using Microsoft Excel. For the Mann-Whitney U test, embryos from three experiments were pooled for each genotype and ranked into categories. The percentage of embryos with delayed or no RGC layer differentiation was calculated using the Fisher’s exact test. The analysis was performed on the VassarStats website (http://faculty.vassar.edu/lowry/ustest.html).

**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. In wild-type (wt) embryos, RGC axons exit through the optic nerve and become trapped within the eye, projecting posteriorly or anteriorly within the retina (Fig. 1A,B,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 mutants 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 sham 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 (i, 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.](image-url)
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 (Ptc) 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., 2008), 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.
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 atoh7 (atoh7 – Zebrafish Information Network) MO injections. By transplanting donor RGCs into atoh7 morphants, we analyzed axon pathfinding of donor RGCs in a host RGC-free environment. 

Fig. 3. Shh and Smo act non-cell-autonomously in intraretinal axon pathfinding in zebrafish. (A-G) Representative images of host eyes at 54 hpf after cell transplants at 24 hpf. Lateral views of maximum-intensity projections. Wt RGCs axons exit the eye through the optic disc (asterisk) in wt hosts (A), but often misproject (arrowheads) in shha (B) and smo (C) hosts. Shha RGC axons always exit the eye in wt hosts (D), whereas many misproject in shha hosts (E). Similar results found with smo RGCs in wt (F) or smo (G) hosts. Transplanted RGCs are isl2b:GFP (green) or isl2b:tagRFP (pseudocolored green in F, G); nuclei, ToPro3 (magenta). D, dorsal; V, ventral; A, anterior; P, posterior. Scale bar: 100 μm. (H) Percentage of embryos with misrouted donor RGCs. Numbers of embryos shown at base of bars. *P<0.05, **P<0.01, ***P<0.001, Fisher’s exact test.

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). 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 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.

Fig. 4. Transplants into RGC-free hosts confirm non-cell-autonomous effect of Smo in intraretinal axon pathfinding. (A-D) Maximum-intensity projections of 54 hpf isl2b:GFP or isl2b:tagRFP zebrafish embryos injected with ath5 MO at 1-cell stage (A) and transplanted with donor cells at 24 hpf (B-D). (A) wt No RGC differentiation in ath5 morphants; trigeminal ganglion as control for transgene expression (arrow). (B) Wt RGC axons (isl2b:tagRFP, red) in isl2b:GFP (green) ath5 morphants rarely make errors. (C) smo (isl2b:tagRFP) RGC axons in isl2b:GFP ath5 morphants are rarely misguided. (D) Wt (isl2b:GFP) RGC axons in smo ath5 morphants make errors (arrowhead). Asterisk indicates optic disc. Nuclei, ToPro3 (blue). D, dorsal; V, ventral; A, anterior; P, posterior. Scale bar: 100 μm. (E) Percentage of embryos with misrouted axons. Numbers of embryos shown at base of bars. **P<0.005, Fisher’s exact test.
pathway inhibition. SANT75 treatment inhibits *ptch2* and *gli1* expression in a dose-dependent manner (Yang et al., 2009). SANT75 application (40 μM) resulted in downregulation of *ptch2* expression in the brain at 24 hpf (Fig. 5A, B, arrowheads) but no cyclopia. 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 *ptch2* 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 *sf11a*) and its homolog *cxcl12b* are expressed in the optic stalk and *Cxl12b* 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.

**Cxl12a 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 *Cxl12a*, exhibit intraretinal axon pathfinding errors (Li et al., 2005). Analysis of intraretinal RGC projections in *Cxl12a* mutants revealed the same highly penetrant pathfinding errors (Fig. 7A,B). The intraretinal axon guidance phenotypes in *Cxl12a* and *Cxc4b* 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 Cxl12b has an attractive effect on RGC axons inside the eye (Li et al., 2005). To determine whether Cxl12a has a
showed anterior RGC projections (Fig. 7F,I, P = 22% of wt embryos transplanted with Cxcl12a-expressing cells did not lead to any anterior RGC axon projections (Fig. 7E,I), whereas an attractive effect of Cxcl12a. Control cells expressing EGFP did score host embryos for anterior axon projection, as indication for subjected the embryos to three rounds of heat-shock. At 54 hpf, we magnified optic stalk region, Pax2a staining only. (Fig. 6). Whole-mount in situ hybridizations (28 hpf). netrin1a at the optic fissure (arrowheads) is decreased in shha (E) and lost in smo (F) compared with wt (D). (G-I) Coronal sections of whole mount in situ hybridizations (28 hpf). cxcl12a expression in optic stalk (arrowheads) is reduced in shha (H) and lost in smo (I) compared with wt (G). D, dorsal; V, ventral; A, anterior, P, posterior. Illustration below shows plane of views for panels above. Adapted with permission (Kimmel et al., 1995). Scale bars: 100 μm.

similar effect, we induced ectopic cxcl12a expression and analyzed whether RGC axons show attraction towards this chemokine. We made an expression construct with the heat-shock promoter driving full-length cxcl12a (hsp70:cxcl12a-2A-EGFP). Global overexpression of Cxcl12a after three heat-shocks (at 28, 32 and 36 hpf) led to intraretinal axon guidance errors similar to cxcl12a loss of function (Fig. 7D). A control line expressing enhanced green fluorescent protein (EGFP) under the heat-shock promoter did not show any axon guidance errors (Fig. 7C).

To determine whether Cxcl12a has an attractive effect on RGC axons, we transplanted retinal cells from hsp70l:cxcl12a-2A-EGFP embryos into the anterior eye of wt or shha hosts at 24 hpf and subjected the embryos to three rounds of heat-shock. At 54 hpf, we scored host embryos for anterior axon projection, as indication for an attractive effect of Cxcl12a. Control cells expressing EGFP did not lead to any anterior RGC axon projections (Fig. 7E,I), whereas 22% of wt embryos transplanted with Cxcl12a-expressing cells showed anterior RGC projections (Fig. 7F,I, P = 0.04). In 33% of shha embryos with anteriorly placed EGFP-expressing cells we observed anterior projections (Fig. 7I), 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, Cxcl112a 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 Cxcl112a 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 Cxcl112a 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+/+;isl2b:GFP fish to shha+/-;cxcl12a-2A-isl2b: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;cxcl112a/+ 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;cxcl112a/+ embryos compared with shha (P = 0.001). As cxcl112a heterozygous embryos exhibit no intraretinal axon guidance errors, our finding that cxcl112a 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; Charon 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; Trouse et al., 2001; Kolpak et al., 2005; Kolpak et al., 2009; 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 motorneurons. X irradiation

**Fig. 7.** Cxcl12a acts as an RGC axonal attractant in wt and shha and interacts genetically with the Hh pathway in zebrafish. (A-H) Maximum-intensity projections of ventral (A,C,D) and lateral (B,E-H) views at 2 dpf. (A,B) Cxcl12a mutants exhibit intraretinal axon guidance errors (arrowheads). Isl2b:GFP (green); nuclei, ToPro3 (magenta). (C) Normal axonal projections in hsp70l:EGFP embryos after heatshock. (D) Global Cxcl12a-2A-EGFP overexpression induces intraretinal axon guidance errors (arrowheads). α-tubulin (pseudocolored green); nuclei, ToPro3 (magenta), EGFP not shown. (E-H) Cxcl12a-expressing cells attract RGC axons in wt and shha embryos. Transplanted EGFP- or Cxcl12a-2A-EGFP-expressing cells (green), α-tubulin (red); nuclei, ToPro3 (blue). (F) Anterior EGFP-expressing cells in wt embryos do not affect RGC outgrowth. (F’) Anterior projections in wt embryos with anterior Cxcl12a-expressing cells. (F”) Substack of boxed region in F with misguided axons (red arrowhead). (G) Rare anterior projections in shha embryos with EGFP-expressing cells. (H) Shha embryos always show anterior projections with anterior Cxcl12a-expressing cells. Optic disc, asterisk. D, dorsal; V, ventral; A, anterior; P, posterior. Scale bars: 100 μm. (I) Percentage of host embryos with anterior RGC projections. Number of embryos shown at base of bars. *P<0.05, ***P<0.001, Fisher’s exact test. (J) Analysis of genetic interaction between shha and Cxcl12a. Percentage of embryos per category (0-3, illustrated below graph) of RGC axon projection phenotype in shha (light gray) and shha;Cxcl12a/+(dark gray). Error bars represent s.e.m. n=3 experiments. Mann-Whitney U test, P=0.00103, of embryos ranked in four categories in shha (n=107) and shha;Cxcl12a+/+(n=57) populations.
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 netrin 1 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 netrin 1 expression at the optic disc due to the loss of astrocyte precursor cells in this region (Dakubo et al., 2003). Similar to mouse netrin 1 (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 netrin 1 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 cxcr4 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 cxcr4b 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


