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28. The four-subunit human pol α complex was purified from coinfected Sf9 cells by immuno-affinity chromatography as described (*25*). Depletion of pol α resulted in diminished DNA replication relative to mock-depleted extracts [12% (±3%) of mock-depleted extract] and addition of recombinant pol α restored DNA replication in pol α–depleted extracts to 96% (±6%) the level measured in the mock-depleted extract.
29. Antibodies used for immunoblotting were against the p70 subunit of human pol α (gift of T. Wang, Stanford University), *Xenopus* RPA, human PCNA (Santa Cruz Biotechnology, Santa Cruz, CA), *Xenopus* RCC1, and *Xenopus* Cdc45.
30. *Xenopus* Cdc45 protein (*18*) was expressed as a six-histidine tagged protein in insect Sf9 cells and was purified on nickel–nitrilotriacetic acid (nickel-NTA) agarose (*9*). Antibodies to the recombinant protein were produced (*9*) and used for immunoblotting.
31. The Chk1 ΔKD coding sequences were subcloned into a bacterial expression vector and expressed as a six-histidine tagged protein. Purified protein was added to extracts at 50 ng/μl. The protein was detected by immunoblotting with a T7-tag monoclonal antibody (Novagen, Madison, WI).
32. Recombinant p180 and p70 subunits were purified from coinfected Sf9 cells as described for the pol α complex (*25*). Primase proteins were produced through overexpression, in *Escherichia coli*, of a bicistronic expression vector [pETHis-Hp58-Hp48 (*26*)] encoding p58 and either wild-type or R304A p48 subunit. The p58/p48 complexes were purified on nickel-NTA agarose by virtue of a six-histadine tag fused to the p58 subunit.
33. We thank T. Wang for pol α antibodies, J. Walter for recombinant Cdc45, L. Kong for purified SJK-132, H.-P. Nasheuer for pETHis-Hp58-Hp48, and R. Kuchta for antibodies to p48 and for very helpful discussions. Supported by a postdoctoral fellowship from the Cancer Research Fund of the Damon Runyon–Walter Winchell Foundation (DRG-1460) to W.M.M., NIH grant RO1GM33523-16 to J.N., and NIH grant 52948 to E.F.

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Patterning of the Zebrafish Retina by a Wave of Sonic Hedgehog Activity

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The *Drosophila* retina is patterned by a morphogenetic wave driven by the Hedgehog signaling protein. Hedgehog, secreted by the first neurons, induces neuronal differentiation and *hedgehog* expression in nearby uncommitted cells, thereby propagating the wave. Evidence is presented here that the zebrafish Hedgehog homolog, Sonic Hedgehog, is also expressed in the first retinal neurons, and that Sonic Hedgehog drives a wave of neurogenesis across the retina, strikingly similar to the wave in *Drosophila*. The conservation of this patterning mechanism is unexpected, given the highly divergent structures of vertebrate and invertebrate eyes, and supports a common evolutionary origin of the animal visual system.

The vertebrate neural retina develops from a layer of pseudostratified epithelium lining the inside of the optic cup, whereas the pigmented retina (RPE) develops from cells on the outside of the cup. The ganglion cell layer (GCL) forms part of the neural retina, and ganglion cells are the first neurons to be born in the retina. Neurogenesis proceeds in a wave from the central to the peripheral retina (*1*). Sonic Hedgehog (Shh) is expressed in the GCL and RPE and directs proliferation and differentiation of several late arising cell types, such as photoreceptors and glia (*2–5*). Here we show that at earlier stages, Hedgehog (Hh) signaling drives a wave of *shh* expression and neurogenesis across the GCL.

To investigate early functions of Shh in retinal neurogenesis, we constructed a zebrafish strain harboring a green fluorescent protein (GFP) transgene under the control of the Shh promoter (*6*). Two ShhGFP transformant lines faithfully recapitulate many aspects of *shh* RNA expression (*7*, *8*). In contrast to the observations of Stenkamp *et al.* (*5*), we detect zebrafish *shh* RNA and ShhGFP not only in the RPE, but also in the GCL (Fig. 1, A and E) (*8*), which is in agreement with the data from other vertebrates (*2–4*). ShhGFP and *shh* RNA expression is activated at 28 to 30 hours in a patch of cells ventral and nasal to the optic disc (Fig. 1B) (*8*). These cells are the first retinal ganglion cells (RGCs) to differentiate and express the RGC marker Zn5 (Fig. 1B) (*9*). ShhGFP and *shh* RNA expression then spreads from this point, together with Zn5 immunoreactivity, and fills the central retina by 52 hours (Fig. 1, A to E) (*8*). Only a subset of the RGCs express ShhGFP (Fig. 1I).

To determine whether *shh* expression might be regulated by Shh itself, we examined ShhGFP expression in *sonic you* (*syu*) mutants, in which the zebrafish *sonic hedgehog* gene is disrupted (*10*). In *syu* mutants, ShhGFP expression is initiated in the first RGCs, but then fails to spread further (Fig. 1, F to H). This is very similar to the *Drosophila* eye, where Hh signaling is required for the spread, but not the induction, of the first Hh-expressing neurons, which instead requires *decapentaplegic* signaling (*11*). In contrast, Zn5 immunoreactivity and RGC differentiation do spread in *syu* mutants, but this spread is retarded (Fig. 1, G and H), and the RGCs are disorganized and reduced in number (Fig. 1, I and J). The reduction of RGCs correlates well with the observation that the optic nerve is thinner in *syu* mutants (*7*). In addition, the layering of *syu* mutant eyes is not as pronounced as in wild-type eyes (Fig. 1, K and L). At 76 hours, there are many apoptotic cells in *syu* eyes (Fig. 1L) (*7*), but elevated cell death is not observed until after 50 hours (*7*), indicating that cell death is not responsible for the reduced ShhGFP expression, which is already evident well before this (Fig. 1G).

Since Shh is necessary for its own expression, we asked whether it might also be sufficient to induce itself. We injected a *shh* cDNA under the control of a heat shock–inducible promoter (*12*) into *syu* RNA null mutants (*13*) carrying ShhGFP. DNA injection into zebrafish embryos leads to mosaic expression of the transgene (*14*). We activated expression by heat shock at 28 hours and examined the effect on ShhGFP at 52 hours. Patches of cells expressing *shh* RNA were found to induce ShhGFP expression in the GCL (Fig. 2A), indicating that Shh is sufficient to activate its own expression. Consistent with this observation, wild-type cells transplanted into *syu* eyes are able nonautonomously to induce ShhGFP expression in mutant cells located in the vicinity (Fig. 2B). It is interesting that wild-type clones do not rescue ShhGFP expression if they do not

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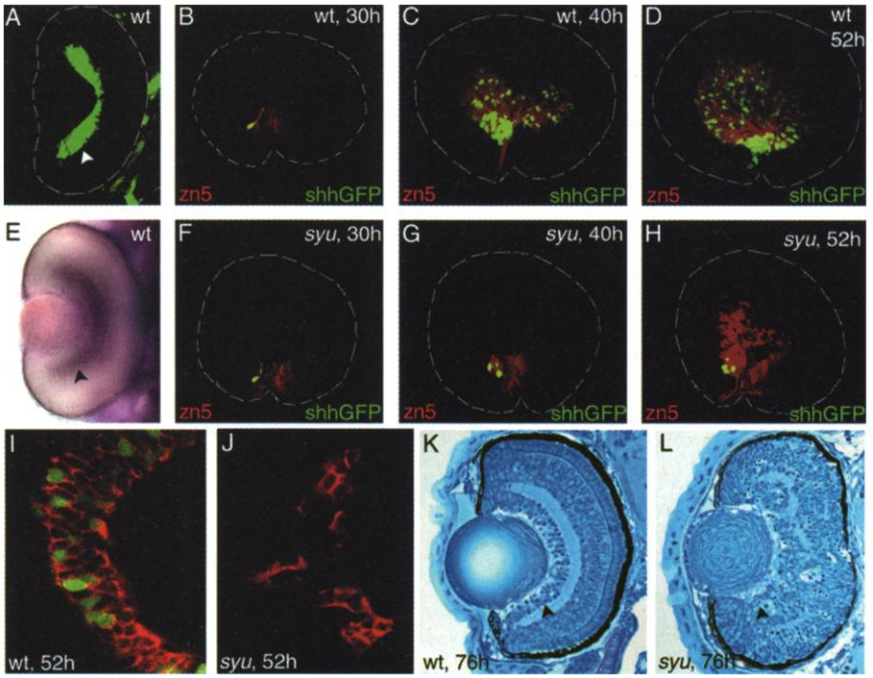

Fig. 1. The *shh* gene is expressed in a wave in the neural retina. (**A** to **D** and **F** to **J**) Confocal micrographs of eyes showing ShhGFP expression (green) and Zn5 staining (red) (*32*). (**E**) *shh* RNA (*33*). (**K** and **L**) Methylene blue–stained sections (*33*). Anterior is to the left and ventral is down in (B) to (D) and (F) to (J), which are side views of eyes. The broken line demarcates the eye outline. Anterior is up in (A), (E), (K), and (L), which are ventral views of eyes. (A, D, E, H, I, and J) 52 hours; (B and F) 30 hours; (C and G) 40 hours; and (K and L) 76 hours. (A to E, I, and K) Wild type; (F to H, J, and L) *syu* mutant (*13*). (I and J) Confocal sections through the GCL, taken at five times the magnification of the other panels. Arrowheads point to the GCL.

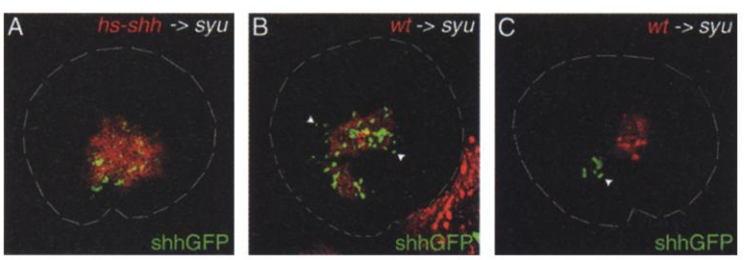

Fig. 2. Shh induces its own expression in the GCL. (**A** to **C**) Confocal micrographs of *syu; shhGFP* eyes, anterior to the left, ventral to the bottom. The broken line demarcates the eye outline. (A) *shh* RNA (red) driven by *hs-shh* (*12*) induces ShhGFP-expression (green) in the GCL of *syu* mutants. *hs-shh* was injected into the RNA null allele of *syu* (*13*). (B) Wild-type *shhGFP* cells labeled with rhodamine (red) and transplanted into *syu; shhGFP* embryos (*34*) induce ShhGFP-expression (green) in mutant cells in the vicinity (arrowheads). (C) Wild-type *shhGFP* cells (red) do not express ShhGFP (green), nor do they rescue ShhGFP-expression in mutant cells if they do not include the point of origin of the neurogenic wave (arrowhead).

include the region where the wave of neurogenesis starts (Fig. 2C), suggesting that Shh signaling in this area is a prerequisite for the subsequent spread.

These results show that Shh is both necessary and sufficient to control a wave of its own expression that sweeps through the GCL (*15*). This is strikingly similar to the function of Hh in controlling the morphogenetic furrow of the *Drosophila* eye (*16*–*18*). In contrast to *Drosophila*, neurogenesis per se is only partially dependent on Shh in the zebrafish retina. As several other Hh genes are known in the zebrafish (*19*, *20*), it is possible that one of these might be responsible for the Shh-independent neurogenesis. Consistent with this possibility, we find that *tiggywinkle hedgehog* (*twhh*) is expressed in the GCL, and that this expression is detectable, though reduced, in *syu* eyes (Fig. 3, A and B). To further address this issue, we treated embryos with cyclopamine, which inhibits signaling by both Shh and other Hh family members (*21*–*23*). Treatment of embryos with cyclopamine from 26 to 52 hours blocks both the spread of ShhGFP and the spread of neurogenesis (Fig. 3C), indicating that several Hh genes cooperate to drive the wave of neurogenesis in the zebrafish retina.

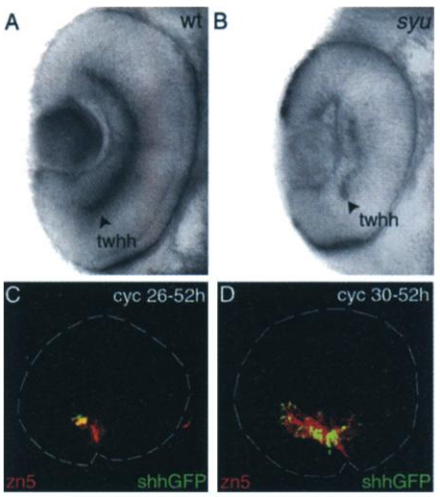

Fig. 3. Contribution of *twhh* to retinal neurogenesis. (**A** and **B**) *twhh* RNA (*33*), ventral view of eyes, anterior to the top. (A) Wild type; (B) *syu* mutant. The arrowheads point to *twhh* expression in the GCL. (**C** and **D**) Confocal micrographs of cyclopamine-treated eyes, ShhGFP (green), zn5 (red) (*32*), anterior to the left, ventral to the bottom. The broken line demarcates the eye outline. (C) Treatment with cyclopamine from 26 to 52 hours (*13*) blocks both the spread of ShhGFP-expression and neurogenesis. (D) Treatment with cyclopamine from 30 to 52 hours blocks the spread of ShhGFP and neurogenesis after it fills the ventral retina.

In *Drosophila*, Hh is continuously required for furrow progression (*17*). To determine whether the same might be true in the zebrafish eye, we treated embryos with cyclopamine at later time points. Treatment of embryos from 30 to 52 hours results in eyes in which the spread of ShhGFP and neurogenesis is blocked after it fills a small domain in the ventral anterior retina (Fig. 3D), revealing a continuous requirement for Hh signaling for the neurogenic wave in the zebrafish retina.

In the *Drosophila* retina, activation of the Ras/MAP-kinase pathway spreads together with the morphogenetic furrow (*24*), and signaling through the Ras pathway is necessary for retinal neurogenesis, and depends on Hedgehog activity (*25*). To explore this scenario in the zebrafish retina, we stained eyes with an antibody against the activated form of mitogen-activated protein kinase (dp-ERK) (*24*). We find that dp-ERK is detectable at 32 hours in the same domain where ShhGFP expression is first activated, and then spreads from this point parallel to ShhGFP expression and neurogenesis (Fig. 4, A to C), as in *Drosophila*. The spread of dp-ERK occurs in *syu* eyes, although its domain is smaller (Fig. 4D), whereas it is blocked in embryos treated with cyclopamine from 26 to 52 hours (Fig. 4E).

Analysis of the Pax6/Eyeless gene has indicated that the mechanism of eye induc-

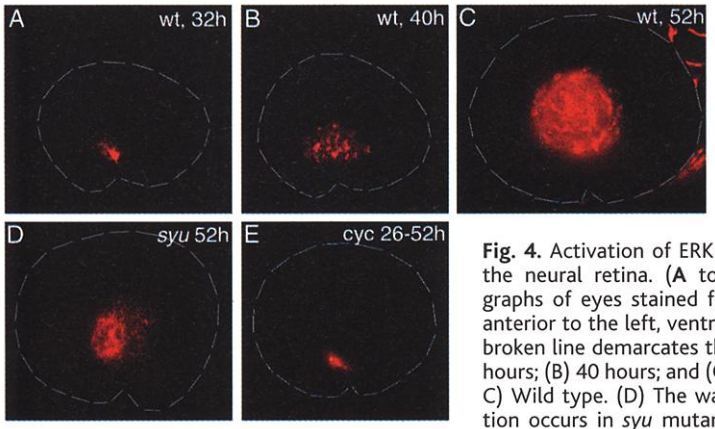

Fig. 4. Activation of ERK spreads in a wave in the neural retina. (**A** to **E**) Confocal micrographs of eyes stained for dp-ERK (red) (*32*), anterior to the left, ventral to the bottom. The broken line demarcates the eye outline. (A) 32 hours; (B) 40 hours; and (C to E) 52 hours. (A to C) Wild type. (D) The wave of dp-ERK activation occurs in *syu* mutants, although the domain is reduced. (E) The wave of dp-ERK activation is blocked in embryos treated with cyclopamine from 26 to 52 hours (*13*).

tion may be conserved across the animal kingdom (*26*). However, the dramatic variation of eye structure not only between vertebrates and invertebrates, but also within the vertebrate lineage, has suggested that events downstream of eye induction may have evolved independently. Our results now show that the role played by Hh signaling in retinal differentiation is conserved between flies and fish. This suggests that Hh was already used to pattern a primordial eye structure before vertebrate and invertebrate lineages diverged, and thus supports a common evolutionary origin of the animal eye.

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13. The *syu* null mutant (*10*) syu^{t4} was used for all experiments. Cyclopamine treatment was performed as described (*23*). Embryos were either treated from 26 to 52 hours (Figs. 3C and 4E), or from 30 to 52 hours (Fig. 3D). Embryos were treated with 0.003% phenylthiourea from 22 hours onward to inhibit pigmentation.
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33. In situ hybridization (*23*) and histological sections (*30*) were performed as described.
34. Transplantation was performed as described (*31*). Five percent fixable rhodamine was injected into eggs from a *shhGFP/shhGFP* wild-type cross, and cells from these embryos were transplanted into the animal pole of embryos from a *syu/+; shhGFP/shhGFP* cross at the shield stage.

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