

# Retinal pattern and the genetic basis of its formation in zebrafish

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## Abstract

The vertebrate nervous system contains an immense diversity of distinct cellular components that are organized into precise spatial patterns. The importance of accurate neuronal architecture is particularly obvious in the retina, where it is necessary for the formation of visual images. The retina is structured in a distinct layered pattern that is remarkably conserved in evolution, including phyla as diverse as primates and teleost fish. Genetic analysis in zebrafish reveals mechanisms that are essential for the formation of this architecture. © 2003 Elsevier Ltd. All rights reserved.

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The retina shares its embryonic origins with other regions of the central nervous system but owing to its physical isolation, relatively large size, and a distinct and uniform architecture, it is particularly accessible to experimental investigations. Among lower vertebrates, teleosts in particular, the zebrafish has become a powerful model system to study the genetic mechanisms that underlie retinal development, including the basis of pattern formation.

## 1. The pattern

A quick glance at a simple histological section of the retina allows one to appreciate its regular structure. Cells of the retina are arranged in layers that arch around the vitreal cavity that harbors the lens. The three main layers: the ganglion cell layer, the inner nuclear layer, and the photoreceptor cell layer, are immediately obvious. The outermost layer of the retina is formed by radially arranged elongated bodies of photoreceptor cells while the innermost one consists of ganglion neurons. In between these two layers, four classes of interneurons, including amacrine, horizontal, bipolar, and interplexiform cells, form the inner nuclear layer. These too are stratified: the oblong bodies of horizontal cells are arranged tangentially in the outermost tier of the inner nuclear layer, while the amacrine cells occupy the vicinity of the inner perimeter, and the bipolar cells are located in between. In addition to neurons, the inner nuclear layer contains so-

mata of Muller glia. The processes of these cells extend radially from the inner nuclear layer into the ganglion and photoreceptor cell layers, and their extremities contribute to the formation of so-called inner and outer limiting membranes (Fig. 1). The outer limiting membrane, contrary to its name, is an array of cell junctions that bind photoreceptors and Muller glia and presumably provides mechanical support for the outer retina [1]. The inner limiting membrane, on the other hand, is a somewhat atypical basement membrane that forms on the surface of glial cells—mostly termini of Muller glia that closely adhere to each other [2].

The subdivision of retinal cells into six major classes reflects only the most prominent features of their position, morphology, and function. Most of them, if not all, consist of many distinct subpopulations that differ in subtle aspects of their function and appearance. The ganglion and amacrine cells are the most diverse. In the rabbit retina, over 20 types of amacrine cells and more than 10 types of ganglion neurons have been distinguished on the basis of morphology [3,4]. Although zebrafish retinal neurons have not been studied in as much detail as their mammalian counterparts, several distinct amacrine cell populations can be identified based on the presence of neurotransmitters or neuropeptides, such as GABA, serotonin, acetylcholine transferase, neuropeptide Y, substance P, and others (Fig. 1) [5,6]. Within the plane of the amacrine cell layer, the distribution of many cell types is non-random; cells are less likely to localize near each other than one would predict based on theoretical estimates of random distribution [6]. At least three mechanisms may account for this phenomenon: inhibitory cell–cell signaling during neurogenesis, translocation of cell somata early in development, and/or death of cells located in inappropriate

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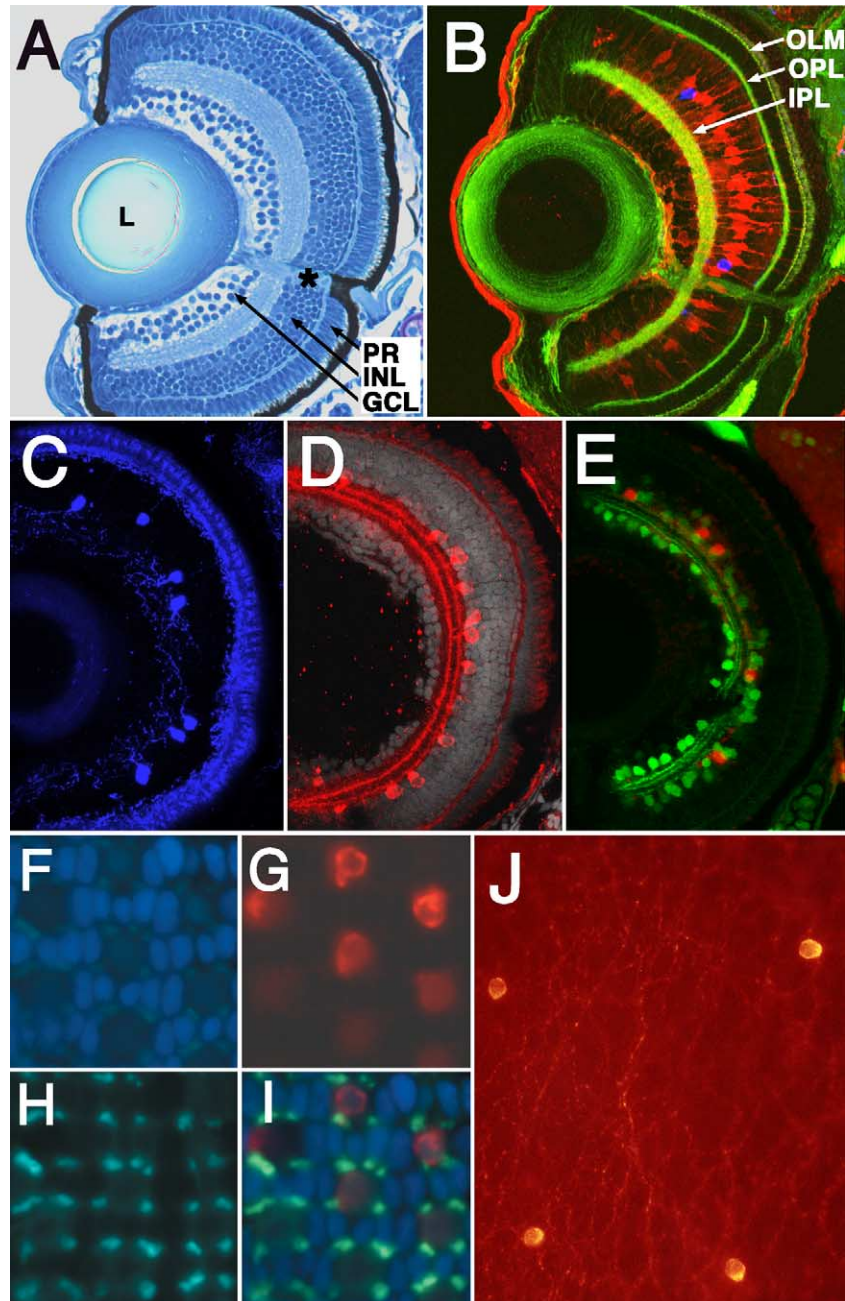


Fig. 1. Pattern in the retina. (A) A transverse plastic section through the retina at the exit point of the optic nerve. A layered arrangement of neurons is obvious. (B) A transverse cryosection through the retina showing the distribution of carbonic anhydrase-positive Muller glia (red) and tyrosine hydroxylase-positive interplexiform neurons (blue). F-actin staining (green) highlights the plexiform layers and the outer limiting membrane. (C–E) The diversity of cell types in the amacrine cell layer is evidenced by the expression patterns of neurotransmitters, neuropeptides, and various other antigens: neuropeptide Y (C), choline acetyltransferase (D), serotonin (E, red), and parvalbumin (E, green). In addition to amacrine cells, photoreceptors are stained in panel C. (F–I) Within the plane of photoreceptor layer, cells display a particularly regular pattern. Cone nuclei are arranged in rows (F). Within each row, short single cones (G) are separated from each other by other photoreceptors. Rods are also aligned in rows that run parallel to the rows of cones (H). Four rods, equidistant from each other, surround each short single cone (I, overlay of F–H). The distribution of somatostatin-positive cells within the plane of the inner nuclear layer is less regular than the photoreceptor pattern (J). In panel A–E, lens is left, dorsal is up. GCL, ganglion cell layer; INL, inner nuclear cell layer; IPL, inner plexiform layer; L, lens; OLM, outer limiting membrane; OPL, outer plexiform layer; PR, photoreceptor cell layer. Asterisk indicates the optic nerve. Panels F–I provided courtesy of Jim Fadool. Panel J provided courtesy of David Cameron.

positions (reviewed in Ref. [7]). While cells of the same identity appear to influence each other's position, different subpopulations of amacrine cells are randomly distributed with respect to each other [6].

The precision of cell arrangement is by far the most evident within the photoreceptor cell layer. The zebrafish retina contains five types of photoreceptor cells: rods, short single cones, long single cones, and the red and green members of double cone pairs. They can be distinguished from each other by their morphology and the type of the visual pigment they express. In adult retinæ, the cone photoreceptors form parallel rows that consist of double cones separated from each other by alternating blue cones and short single cones [8]. In contrast to the cones, the pattern of rod distribution is more variable. In regions of the adult retina that are characterized by a lower rod density, rods are evenly spaced, forming rows that run parallel to cone arrays [9]. Rods from neighboring rows are aligned with each other. The distance between rods located within a row is the same as the distance between neighboring rows, creating the impression that they are located at the intersections of grid lines (Fig. 1H). Short single cones neatly fit in this pattern, each is surrounded by four evenly spaced rods (Fig. 1I). Rod photoreceptors are generated continuously in adult teleost retinæ, and in the areas of high density their distribution is less regular; they appear to form compact arrays that separate rows of cones [8]. During embryogenesis, rods are initially generated at a low density and do not appear to form a regular pattern until approximately 10 days postfertilization (dpf) [9]. As the density of cones in embryonic retinæ is higher and more uniform, their regular distribution becomes noticeable between 2 and 3 dpf [8,10].

The distribution and morphology of most cell classes is uniform throughout the plane of retinal layers. In some vertebrate species, however, the composition of the photoreceptor cell layer varies dramatically between different regions of the retina [11]. Except for the differences in rod density, the zebrafish photoreceptors display a uniform distribution [8,10]. Dorso-ventral and temporal-nasal differences are obvious in the ganglion cell layer since cells from different regions produce projections to different parts of the optic tectum [12]. The axonal trajectories of ganglion cells follow precise routes to their targets in the brain. Within the retina, ganglion cell axons first extend along the inner limiting membrane to a common target in the ventral region of the central retina, the optic nerve head, and from there they exit as a single bundle—the optic nerve. Within the optic nerve, the spatial relationships that characterize ganglion cell bodies in the plane of the ganglion cell layer are transformed so that axons of peripheral cells are found in the center of a crescent-shaped cross-section of the nerve [13]. The optic nerve is the only route of information flow between the retina and the rest of the central nervous system.

The retina is largely a self-contained structure. Prior to neurogenesis, it connects to the brain via a narrow tubular extension, the optic stalk. Later in development, the op-

tic stalk is replaced by the optic nerve. This limited connection between the retina and other regions of the central nervous system restricts cell migration during embryogenesis, making it easier to interpret developmental events. The rapid development of the retina, its physical isolation, uniform structure, and relatively large size are important assets in developmental and genetic studies.

## 2. Embryonic origins

Cells of the vertebrate retina and their intricate architecture originate from a single sheet of epithelium. By morphological criteria, from the formation of the optic lobe at approximately 12-h postfertilization (hpf) to the onset of neurogenesis at 28 hpf, the retina consists of a single population of neuroepithelial cells [14–16]. The neuroepithelium of the retina is very similar to the ones that give rise to the neuronal formations of the brain and spinal cord. Its nuclei display apico-basal movements during the cell cycle that are also typical of other neuroepithelia [17]. The nuclei of cells that are about to divide translocate apically and cytokinesis takes place at the apical surface. Until recently, retinal neuroepithelial cells were believed to lose contact with the basal surface during cell divisions [17,18]. Two-photon imaging of small clones in living zebrafish retinæ have demonstrated, however, that dividing neuroepithelial cells retain a thin process that connects them to the basal surface [19]. This is an interesting realization that may have implications for the understanding of cell fate decisions in the retina; a neuroepithelial cell during the final mitotic division no longer seems exclusively immersed in the apical environment and may also be influenced by factors that are deposited at the basal surface of the neuroepithelium.

Despite its uniform morphology, the retinal neuroepithelium is a site of dynamic transformations that are evident in rapid changes of gene expression profiles. Transcripts involved in the specification of the eye field, such as *rx3* and *six3*, become downregulated or absent in the neuroepithelium while other genes are upregulated [20–22]. Even in neighboring groups of cells, gene expression profiles frequently differ from each other and some of these variations presage events of neurogenesis. *ath5*, for example, is first expressed at 25 hpf exclusively in a small group of cells in the ventral retina, and only later does its expression spread into the central, and subsequently dorsal eye [22]. This gradual advance of expression towards the dorsal region of the optic cup is biased towards the nasal areas, which express *ath5* before the temporal ones. The spatio-temporal profile that characterizes *ath5* expression is noteworthy because many processes in retinal development follow it. Shortly after the onset of *ath5* expression, cells exit the cell cycle following a similar profile and the expression of *shh*, *isl1*, and *lim3* spreads similarly [15,22,23]. Even later, the expression of differentiation markers, such as neuroilin or opsins, follows a similar pattern [24]. This gradual spread of developmen-

tal events through the neuroepithelial sheet of the retina has earned the name of “the neurogenic wave”. It brings to mind the morphogenetic furrow in the *Drosophila* eye imaginal disc—perhaps not a trivial similarity given that it involves homologous genetic loci: *ath* and *shh*.

The mode of cell divisions in the developing retinal neuroepithelium has recently been given much attention. A dividing neuroepithelial cell can produce daughter cells either of the same or of different identity. In the first case, a cell division is called symmetric, in the latter, asymmetric. Prior to the onset of neurogenesis, all cell divisions produce mitotically active neuroepithelial cells and thus are symmetric, at least by the criteria of daughter cell morphology. After the onset of neurogenesis, some cell divisions produce an asymmetric outcome: a postmitotic neuron, and a mitotically active neuroepithelial cell [25]. In the developing cortex, asymmetric outcomes of cell divisions correlate with the apico-basal orientation of the mitotic spindle and involve an asymmetric inheritance of the *notch* gene product by daughter cells [26]. These observations suggest that the mitotic spindle orientation relative to the apico-basal cell axis influences the fate of daughter cells; cells that are generated by apico-basal divisions tend to exit the cell cycle and differentiate [25,26].

The orientation of the mitotic spindle in a dividing neuroepithelial cell is variable. In the two extreme cases, it either aligns with the apico-basal axis of neuroepithelial cells or, alternatively, in planar cell divisions, it runs parallel to the surface of the neuroepithelial sheet. Intermediate orientations are also common and, in fact, no clear tendency to assume two specific orientations has been observed in the retinae of several species [19,27,28]. As the surface of the retina approximates a hemisphere, two types of planar cell divisions can be distinguished in addition to apico-basal divisions: central-peripheral and circumferential [19]. In the first case, the mitotic spindle axis is oriented longitudinally from the center of the hemisphere to its periphery; in the second, it parallels the lines of latitude that run around of the retinal hemisphere.

The presence of apico-basal cell divisions in the retina and their relevance to the symmetry of cell division has been a contentious issue. In the rat retina, up to one fifth of cell divisions display an apico-basal spindle orientation and their frequency peaks around birth [28]. It has been reported that while planar cell divisions tend to produce daughter cells of the same identity in the rat retina, apico-basal divisions produce cells of different identities [29]. In contrast to that, in the chicken retina apico-basal cell divisions are rare and their frequency does not change during development [27]. The apico-basal divisions are as likely to produce an asymmetric outcome as the planar ones [27]. Finally, in the zebrafish retina apico-basal cell divisions have been reported to be either rare or absent [19,30]. Detailed analysis of planar cell divisions revealed, however, that the frequency of circumferential divisions relative to central-peripheral ones increases during neurogenesis [19]. This change of orientation may

generate an asymmetry relative to the neurogenic wave that sweeps across the retina and thus it may affect cell fate.

### 3. Genetic basis of pattern formation

What are the genetic mechanisms that regulate the appearance of multiple cell classes in correct ratios and subsequently orchestrate their assembly into a multilayered structure characterized by precise spatial cell arrangements? Genetic screens in zebrafish have revealed defects in several loci that cause severe abnormalities of neuronal architecture. The majority of these fall into two groups. The first includes genes that are involved in the specification of neuronal identities. Most of them appear related to hedgehog signaling, either as components of the hedgehog pathway or its downstream targets, and include the loci *sonic you* (*syu*), *slow muscle omitted* (*smu*), *lakritz* (*lak*), and *young* (*yng*). The second group contains loci involved in the organization of neurons with relatively little, if any, contribution to the specification of cell identities. *nagie oko* (*nok*), *heart and soul* (*has*), and *ncad* are best characterized in this category. Mutations in these genes first affect the polarity of the zebrafish retinal neuroepithelium and at later stages cause a severe disorganization of retinal neurons. In addition to these two groups of genes, mutations or knockdowns of several other loci also disrupt the patterning of retinal neurons, but their function has been characterized much less extensively.

#### 3.1. Specification of neuronal identities and the sonic hedgehog pathway

The hedgehog genes, *sonic hedgehog* (*shh*) and *tiggy winkle hedgehog* (*twhh*), are important regulators of retinal patterning in zebrafish. Both are orthologous to mammalian *sonic hedgehog* and play a role at multiple stages of eye development. Early during embryogenesis, *shh* is involved in optic stalk specification. The absence of *shh* signaling results in an expansion of the retinal field and a loss of optic stalk identity, evidenced by the increased size of the Pax-6 expression domain and the loss of Pax-2.1 and Fgf-8 expression, respectively [31,32]. The opposite effect is produced by the overexpression of *shh*: a reduction of the Pax-6 expression domain, and the expansion of the optic stalk region [22,33]. Later in embryogenesis, hedgehog signaling is necessary for the differentiation of retinal neurons. In *sonic you* (*syu*<sup>t4</sup>) or *slow muscle omitted* (*smu*<sup>b641</sup>) mutant animals, which lack *sonic hedgehog* and *smoothed* functions, respectively, the retina frequently arrests at the neuroepithelial stage, or its differentiation is inhibited as indicated by a delayed expression or the absence of ganglion and photoreceptor cell-specific genes [23,31,34]. A similar phenotype is produced by a morpholino double knockdown of the *shh* and *twhh* genes, which display distinct, although most likely partially redundant, roles in the retina. The loss of *shh* alone does not affect the *ath5* expression and causes a delay in

ganglion cell differentiation, whereas a simultaneous interference with both *shh* and *twhh* activity completely blocks the *ath5* expression in a subset of embryos, and subsequently stops differentiation of ganglion neurons [23,31,35].

Several lines of evidence indicate that the *shh* function in retinal differentiation is at least partially mediated by the activation of the MAP kinase pathway. First, the activation of the ERK kinase occurs shortly after the onset of *shh* expression, and it spreads across the retina in the same wave-front fashion [23]. Second, *shh* mutations as well as the inhibition of hedgehog signaling with cyclopamine, block ERK activation. Finally, blocking the MAP kinase pathway using pharmacological means results in the inhibition of retinal differentiation [36]. The role of the MAP kinase signaling in retinal differentiation is further demonstrated by the phenotype of the zebrafish *young* (*yng*) locus. *yng* encodes *brahma-related gene* (*brg1*), a component of the SWI/SNF chromatin remodeling complex [36]. In the absence of *yng* function, activated ERK is not present in differentiating retinae. It has not been investigated so far whether *yng* is necessary for the induction of ERK activity or only for its maintenance. The normal expression of *ath5* in *yng* retinae suggests that the latter may be the case.

The role of the hedgehog genes in retinal neurogenesis is closely associated with another important patterning locus: *lakritz* (*lak*). *lak* encodes the helix-loop-helix transcription factor, *ath5*, and its loss of function in zebrafish as well as in other species produces the absence of ganglion cells [37–39]. This phenotype is highly specific as only minor changes are detectable in other cell classes. Interestingly, the *lak* phenotype is associated with a cell cycle exit delay. While wild-type retinal cells become postmitotic at approximately 29 hpf, in *lakritz* retinae, the first cell cycle exit is retarded to approximately 43–48 hpf [37]. This delay is reminiscent of much more severe defects seen in a subset of *sonic you* mutant animals in which the retina remains an undifferentiated neuroepithelial sheet until even later stages [34]. This similarity of phenotypes may reflect a regulatory relationship between hedgehog signaling and *ath5* function. Indeed, *shh* appears to regulate *ath5* during at least two phases of eye development. First, it appears to be essential for the initiation of *ath5* activity; in one fifth of the *shh/twhh* morpholino double knockdown animals, the *ath5* expression is never activated [31]. This inductive activity most likely originates in the prechordal plate because *shh* is not expressed in the eye until approximately 28 hpf, and the absence of the prechordal plate correlates with the loss of *ath5* expression [22,23]. Second, hedgehog activity may play a role in the propagation of *ath5* expression as evidenced by double knockdown of *shh/twhh* function during early stages of neurogenesis [31]. Thus, the delay or absence of retinal differentiation that are associated with hedgehog pathway defects may be partially mediated by the loss of *ath5* activity.

A gradual advance of expression across the retinal surface that characterizes both *sonic hedgehog* and *atonal* is an intriguing aspect of eye neurogenesis. The spread of *shh* ex-

pression involves autoregulation [23]. In the *syu*<sup>t4</sup> mutants which lack a functional *shh* polypeptide, *shh* expression is initiated in a ventral group of cells but it does not spread further, indicating that *shh* is necessary for its own expression (Fig. 2A and B). Moreover, mosaic analysis indicates that *shh* can activate its own expression in neighboring cells that do not contain a functional *shh* gene. These observations suggest that the spread of the neurogenic wave through the retina is sustained by the diffusion of *shh*, followed by the upregulation of its own activity. The same mosaic studies indicate that the spread of the neurogenic wave is regulated differently at its origin, in comparison to later stages of development; wild-type cell clones upregulate *shh* activity in *syu* mutant embryos only if they include the origin of the neurogenic wave in the ventral retina. The ventral retina must therefore contain a unique combination of factors that are necessary to activate *shh*.

The mechanisms that underlie the advance of the *ath5* wave of expression have also been studied in some detail and share many similarities with *shh*. *ath5* expression also requires optic stalk-proximal regions for its expression in more distal areas [22]. Even more specifically, a small area of the retina at the optic stalk/retina interface is both necessary and sufficient to induce *ath5* expression in the dorsal retina. Although it has been originally suggested that cells of optic stalk identity are necessary for the expression of retinal *ath5*, this conclusion now appears questionable because in *smu* mutant animals, *ath5* is expressed in the absence of optic stalk specification. Accordingly, it has been suggested that *shh* activity in the prechordal plate may activate the retinal *ath5* expression via a pathway that does not require the optic stalk [31]. The initiation of neurogenesis in the retina thus appears to involve a series of inductive interactions that originate in the prechordal plate, spread to progressively more lateral regions, and eventually advance throughout the entire eye field.

### 3.2. Epithelial polarity genes

Patterning defects of a different category are produced by mutations in *nagie oko* (*nok*) and related genes. In contrast to *sonic you* and *lakritz*, defects in *nok*-related genes do not produce major changes of cell identity. Instead, neurons do not form a stratified pattern that characterizes wild-type retina. In addition to *nok*, mutations in four other loci produce similar phenotypes: *heart and soul* (*has*), *mosaic eyes* (*moe*), *ncad*, and *oko meduzy* (*ome*). *ncad* alleles, originally uncovered independently by several groups, were originally referred to as *glass onion*, *parachute*, and *lyra* [40–42]. Mutant phenotypes of these loci share several features. The most striking phenotype is a dramatic disorganization of retinal lamination. Photoreceptors, for example, do not form a layer next to the retinal pigment epithelium (RPE) but are scattered throughout the retina and frequently found near the lens (Fig. 2H). Similarly, ganglion neurons frequently localize to the vicinity of the RPE (Fig. 2D). Instead of

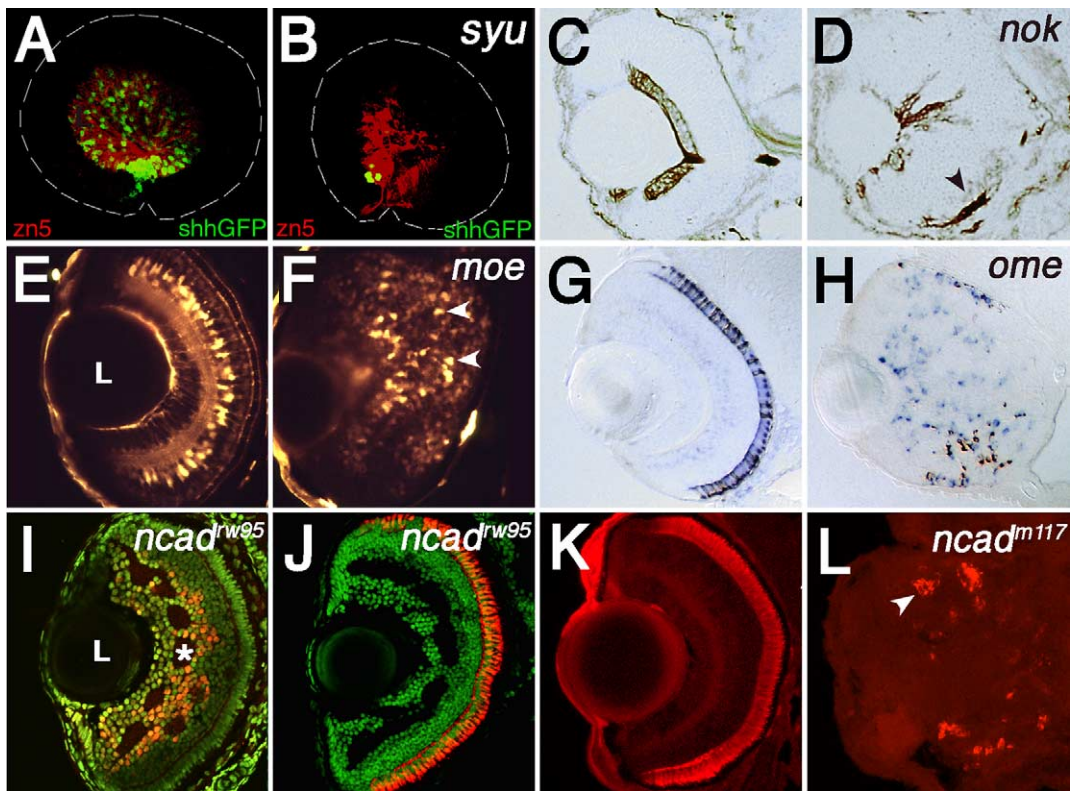


Fig. 2. Patterning phenotypes in the zebrafish retina. A gradual advance of the *sonic hedgehog* expression as monitored with a reporter transgene has spread nearly throughout the entire retina by 52 hpf (green in panel A). The reporter construct contains the GFP gene under the control of *shh* upstream regulatory sequences. Differentiating ganglion cells are visualized by neuroilin staining (red in panel A). By contrast, *shh* expression is confined to a small patch of ventral cells in the *sonic you* mutant animals and ganglion cell differentiation is inhibited (B). A compact appearance of the ganglion cell layer that characterizes the wild type (C) is disrupted in *nok* (D). Some ectopic ganglion cells localize near the RPE (D, arrowhead). The somata of wild-type Muller glia form a regular array parallel to the inner plexiform layer (E). In contrast to that, they are dispersed in *moe* mutants (arrowheads in panel F). Both rods (brown) and blue cones (blue) are scattered throughout the retina in *ome* mutant larvae (H). Wild-type siblings of the *ome* mutants are shown in panel G. A hypomorphic mutant allele of the zebrafish *ncad* locus, *ncad<sup>rw95</sup>*, disrupts the inner plexiform layer so that it has a patchy appearance (asterisk in panel I). As evidenced by the Pax-6 staining, the amacrine cells are also affected (red in panel I) but the photoreceptor cell layer is intact (red in panel J). A wild-type sibling of *ncad<sup>rw95</sup>* mutant animals is shown in panel K. A stronger *ncad* allele, *ncad<sup>m117</sup>*, also disrupts the photoreceptor layer (L). An ectopic cluster of photoreceptors is indicated (arrowhead in L). A wild-type sibling of *ncad<sup>m117</sup>* mutant animals is shown in panel K. Panels A and B show parasagittal confocal sections through the zebrafish retina. Panels C–L show transverse sections. Panels A and B reprinted with permission from [23], copyright 2000 AAAS. Panels E and F courtesy of Abbie Jensen [43]. Panels I and J courtesy of Ichiro Masai [42]. In all panels, ventral is down. L, lens.

forming plexiform layers, neuronal processes form patches scattered throughout the retina. Are all cell classes specified in these mutant retinæ? Analysis of cell class-specific markers indicate that this is the case, although the relative frequencies of different cell types may be affected [43–45].

The second major phenotypic feature that is shared by mutants in this category is a loss of neuroepithelial polarity that precedes neurogenesis. This phenotype is less apparent than the previous one and requires the use of markers to monitor polarity of the neuroepithelium. Centrosomes, M-phase nuclei, and several polypeptides localize to the apical termini of wild-type neuroepithelial cells. By contrast, the same markers are displaced to the center of the retinal neuroepithelial sheet in mutant retinæ. In *ncad* mutants, neuroepithelial integrity is initially normal and the mutant phenotype appears gradually later in development. Although it has not been thoroughly investigated, this is most likely also the case for other mutants in this cate-

gory because the appearance of their eyes is normal until at least 20 hpf. Another phenotype common to mutants in this class is a retinal pigmented epithelium defect, visible as a patchiness in eye pigmentation in mutant embryos. The RPE phenotype has not been the subject of a thorough investigation but is likely to have originated from a common neuroepithelial defect: a loss of epithelial integrity and polarity, especially since both neural retina and RPE originate as a single outpocketing of the neural tube. Finally, nearly all mutant phenotypes that have been investigated in this group of loci are cell non-autonomous, indicating an involvement in cell–cell interactions [40,43–46].

Cloning experiments revealed that *nok* encodes a MAGUK-family scaffolding factor, *has* an atypical protein kinase C, and *ncad* a zebrafish cadherin-2 (N-cadherin, Ncad) ortholog [30,42,45,47]. Interestingly, all three genes have *Drosophila* homologs that are involved in the polarity of embryonic epithelia: *nok* is homologous to the fly *stardust*

(*std*), *has* to *DaPKC*, and *ncad* to *shotgun* (*shg*). Similar to zebrafish *nok* and *has*, *std* and *DaPKC* both localize to the apical surface of embryonic epithelia. The subcellular distribution of *shg* is somewhat different from *ncad*. While *shg* localizes mainly to cell junctions, anti-Ncad antibodies appear to stain the entire surface of neuroepithelial cells [48,49]. Mutant phenotypes of the zebrafish and fly genes also display common features that indicate a loss of cell polarity: rounding of cell shape and a displacement of polypeptides and organelles from the apical surface. Particularly close similarities exist between the *nok* and *std* phenotypes: both genes produce a relatively late epithelial defect that involves an abnormal position of cell junctions in baso-lateral regions [45,50]. The close structural and phenotypic similarities between the zebrafish genes identified so far and *Drosophila* loci suggest that homologs of other fly epithelial polarity genes may also play a role in the patterning of the vertebrate retina.

Epithelial polarity in the fly embryo has been a subject of extensive studies. Numerous other loci are involved in fruit fly epithelial development (reviewed in Refs. [51,52]). In addition to *std* and *DaPKC*, at least four other *Drosophila* genes display apical distribution in embryonic epithelia: *crumbs* (*crb*), *discs lost* (*dlt*), *bazooka* (*baz*), and *DmPAR-6*. Mammalian homologs of these loci have also been identified, and similar to their invertebrate counterparts localize to the apical regions in cultured epithelial cells [53,54]. Both *Drosophila* and mammalian polypeptides have been tested for binding interactions with each other and with other proteins (Fig. 3). The *nok* homolog in *Drosophila*, *std*, interacts with a conserved ERLI motif in the *crb* cytoplasmic tail

through its PDZ domain [48], while the mouse *nok* homolog, *pals1*, is thought to interact with polypeptides encoded by the mammalian homologs of *par-6*, *dlt*, and *lin-7*, through its N-terminus, the L27N domain, and the L27C domain, respectively [53–55]. A potential link between *nok* and *ncad* is provided by the finding that *lin-7* binds to the C-terminus of  $\beta$ -catenin [56]. Similar to *nok*, the polypeptides encoded by the *has* homologs in the fly and in mammals are also thought to bind several other proteins, including PAR-6, Mlg1, and bazoooka [57–59]. Thus, *nagie oko*, *heart and soul*, and *ncad* protein products are potentially involved in a complex network of binding interactions (Fig. 3B). As these have been demonstrated outside the context of the nervous system so far, it remains to be investigated whether they occur in the retinal neuroepithelium.

How do defects in these loci affect the patterning of neurons in the developing retina? Their mutant phenotypes affect all layers in the retina and therefore are likely to disrupt a global patterning mechanism that is relevant to all cell classes. One obvious possibility is that the loss of retinal architecture is a consequence of a neuroepithelial defect. Shortly after the exit from the cell cycle, cells of the developing retina migrate in the context of a neuroepithelial sheet [18,19], and most likely utilize positional cues that are deposited either on the surface of neuroepithelial cell or in the extracellular matrix that surrounds them. In the absence of correct polarity, these cues are likely to be mispositioned and produce abnormal migration patterns. This idea is consistent with the cell non-autonomy of the *nok* phenotype. In mosaic retinæ, *nok* mutant photoreceptor or ganglion cells that are surrounded by wild-type tissue localize to normal positions, indicating that the *nok* mutations do not affect intrinsic mechanisms that are necessary for cell motility or the directionality of migration [45]. As the molecular cues that guide the positioning of retinal neurons have not yet been identified, it is not possible to test directly whether they are affected in zebrafish retinal patterning mutants.

In addition to their function in epithelial polarity, loci in this category may be involved in local patterning events. In *nok* and *ome* mutant retinæ, for example, photoreceptor cells are entirely dissociated from each other (*ome* is shown in Fig. 2H), suggesting that these genes play a local role in the integrity of the photoreceptor cell layer. This hypothesis is supported by the observation that *nok* is expressed in the vicinity of the outer limiting membrane but not in other parts of the differentiated retina [45]. Similarly, in addition to its global role in neuroepithelial integrity, *ncad* may also play a local role in the integrity of the inner plexiform layer. This is suggested by the phenotype of a hypomorphic allele, *ncad<sup>w95</sup>*, which disrupts the inner plexiform layer and produces an excessive neurite outgrowth in amacrine cells but not in bipolar or ganglion neurons [42]. Such a defect is consistent with antibody staining experiments that reveal the enrichment of *ncad* in the inner plexiform layer. Thus, both *nok* and *ncad* may function at multiple stages of development:

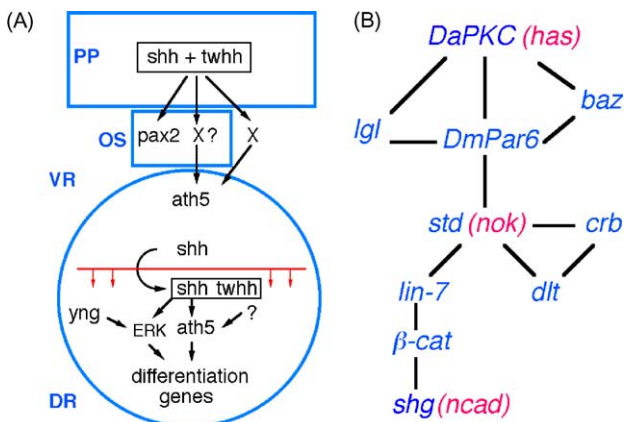


Fig. 3. Genetic mechanisms of retinal patterning in zebrafish. (A) The genetic basis of the neurogenic wave in the zebrafish retina. Horizontal red line represents the front of the neurogenic wave. Arrows indicate regulatory relationships that have been demonstrated in the zebrafish retina so far [22,23,31,33,36,37]. PP, prechordal plate; OS, optic stalk; DR, dorsal retina; VR, ventral retina. (B) Protein products of the *Drosophila* and mammalian homologs of the MAGUK factor, *nagie oko* (*nok*), and an atypical protein kinase C, *heart and soul* (*has*), are thought to engage in binding interactions with other proteins [48,53–59,64,65]. Some of the potential binding partners of *has* and *nok* are illustrated. *baz*, bazoooka; *crb*, crumbs; *dlt*, discs lost; *Igl*, lethal giant larvae; *shg*, shotgun; *std*, stardust.

first in the polarity of the retinal neuroepithelium, and later in the patterning of specific cell populations.

### 3.3. Other patterning loci

In addition to the two groups of loci that are discussed above, mutagenesis screens revealed other genes that play a role in retinal patterning. Some of them have been cloned, while others still await characterization. The *foggy* locus, for example, encodes an Spt5-related transcription elongation factor that is thought to regulate the rate of transcription elongation [60]. The *fog*<sup>m806</sup> mutant allele produces cell fate changes in the diencephalon: the number of dopamine-containing cells decreases, while the number of serotonin-containing cells is elevated. The same mutation inhibits cell differentiation in both the inner nuclear layer and the photoreceptor cell layer, while the number of ganglion cells may be increased. It remains to be investigated whether *fog* functions in the specification or in the differentiation of retinal neurons. Mutations in two other loci, *bashful* (*bal*) and *chameleon* (*con*), have been reported to produce ganglion cell patterning defects but their molecular nature still awaits characterization [61].

The involvement of many key loci in retinal patterning has been determined using chemically induced mutant alleles. Alternative, reverse genetic approaches have recently become available. One productive way to search for patterning genes is through in situ screening of randomly selected cDNAs [62]. Genes that display expression patterns confined to single cell classes or cell types at early stages of neurogenesis can be subsequently tested for function in neurogenesis using antisense compounds, morpholino-modified oligonucleotides, for example [35]. This approach can also be used to test the function of candidate genes. The knock-down approach has been used, for example, to show that RPTP $\alpha$  is necessary for normal retinal lamination [63].

## 4. Summary

The zebrafish retina has turned out to be a treasure chest of fascinating discoveries. Advances have come from descriptive studies of cell morphology, from the analysis of mutant genes, and from increasingly sophisticated tests of gene function during advanced stages of embryogenesis. Recent experiments demonstrated that dividing neuroepithelial cells retain a process that connects them to the basal surface of the neuroepithelial sheet, and that their mitotic spindles change their orientation preference during neurogenesis. Furthermore, the decisions to exit the cell cycle and to initiate differentiation take place at a different time in different regions of the retina: first in a ventral group of cells, and then gradually in more and more dorsal regions. At least one locus that is involved in this process, *sonic hedgehog*, is required to propagate its own expression, and is sufficient to activate itself in a cell non-autonomous manner, suggesting a mechanism that

may propel the advance of neurogenesis from the ventral to the dorsal regions of the retina. Finally, genetic screens have identified loci that play key roles in the specification of individual cell classes and revealed that epithelial polarity genes are crucial components of retinal patterning mechanisms.

Much remains to be done. Gene networks that regulate the acquisition of cell class identities are described only partially and nearly nothing is known about the mechanisms that specify the numerous cell type identities within a cell class. Genetic pathways that regulate the assembly of retinal cells into a precisely organized stratum or into a precise mosaic pattern within the plane of a cell layer also need to be investigated. Finally, the morphogenesis of photoreceptor structure, dendritic arbors of retinal interneurons, and the retinotectal projection will be a source of rewarding genetic studies. Undoubtedly, the zebrafish retina will continue to be a source of tantalizing new findings, and as it is usually the case, the most interesting of these will be unanticipated.

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