INTRODUCTION

The vertebrate retina contains six major classes of neurons organized into three laminae: the outer nuclear layer (ONL), which contains photoreceptor nuclei; the inner nuclear layer (INL), which contains amacrine, bipolar and horizontal cells; and the retinal ganglion cell (RGC) layer. Between the inner and outer nuclear layers, the outer plexiform layer (OPL) contains connections between the photoreceptors and bipolar and horizontal cells. The inner plexiform layer (IPL) is positioned between the INL and the ganglion cell layer and contains the dendrites of RGCs and processes of bipolar and amacrine cells. Spanning all layers of the retina are the radially oriented Müller glia.

The neural retina is initially composed of a single layer of proliferating cells that extend processes to both apical and basal surfaces of the neuroepithelium. Nuclei of the proliferative cells translocate along the apicobasal axis with cell divisions occurring when nuclei are located at the apical surface. After exiting the cell cycle, post-mitotic cells migrate basally to differentiate as retinal neurons or glia. Cell fate decisions in the developing retina are independent of cell lineage and so it seems likely that multipotent progenitor cells change their competence to generate different retinal cell-types in response to position and stage-dependent environmental cues (Livesey and Cepko, 2001; Marquardt and Gruss, 2002).

Although the mechanisms that regulate retinal lamination are poorly understood, there has been some recent progress in identifying genes involved. For example, genetic approaches in zebrafish have shown that retinal lamination is severely perturbed in fish carrying mutations at the *mosaic eyes* (*moe*), *oko meduzy* (*ome*), *heart and soul* (*has*), *nagie oko* (*nok*) and *glass onion* (*glo*) loci (Malicki and Driever, 1999; Horne-Badovinac et al., 2001; Jensen et al., 2001; Pujic and Malicki, 2001; Peterson et al., 2001; Wei and Malicki, 2002). Cloning has identified Has as an atypical protein kinase C (aPKC) and Nok as a member of the membrane associated guanylate kinase (MAGUK) family that is localized to adherens junctions.
apical ends of the retinal neuroepithelial cells (Horne-Badovinac et al., 2001; Peterson et al., 2001; Wei and Malicki, 2002). In nok mutant retinas, adherens junctions are not maintained and dividing cells are detached from the apical surface of the neural retina, leading to severe disorganization of retinal laminae (Wei and Malicki, 2002). Stardust, a fly homolog of Nok, and an interacting protein, Crumbs, are essential for formation of adherens junctions and epithelial polarity (Tepass, 2002), suggesting that a vertebrate Crumbs/Stardust pathway is involved in retinal lamination. aPKC is required for maintenance of cell polarity in a number of biological systems (reviewed by Ohno, 2001) and in has mutants, junctional complexes in the retina are disrupted (Horne-Badovinac et al., 2001; Peterson et al., 2001).

In vitro studies have again implicated proteins that modulate cell adhesion in the establishment of retinal lamination. For example, function-blocking N-cadherin (Ncad; Cdh2 – Zebrafish Information Network) antibodies disrupt retinal lamination although the local application of high concentrations of antibodies leaves the issue of specificity open (Matsunaga et al., 1988a). Mice lacking Ncad function show defects in development of brain, heart, somites and pancreas but retinal phenotypes have not been reported (Radice et al., 1997; Esni et al., 2001; Luo et al., 2001). A large number of studies also implicate Ncad in the regulation of axon growth. For example, in vitro, RGC axon outgrowth is promoted on Ncad-expressing cells (Matsunaga et al., 1988b), and expression of dominant-negative Ncad in vivo disrupts RGC axon outgrowth (Riehl et al., 1996). Genetic studies in flies (Clandinin and Zipursky, 2002), worms (Broadbent and Pettitt, 2002) and fish (Lele et al., 2002) have all confirmed roles for Ncad in establishing axon trajectories.

A simple model of how Ncad modulates axon extension is that homophilic binding mediates adhesive interactions between cells, and that differential adhesion directly modulates axonal growth. However, the mechanisms underlying regulation of axonal growth by Ncad and other cell adhesion molecules may be more complex. For example, soluble versions of cell adhesion molecules containing only the extracellular domains can still stimulate axonal growth (Doherty et al., 1995), suggesting that their function may be mediated by activating second messenger cascades. In particular, several studies have suggested that Ncad-mediated outgrowth may be regulated through direct interaction of Ncad with Fgf receptors (Fgrfs) (Williams et al., 2001).

In this study, we demonstrate that the zebrafish ncad (Bitzur et al., 1994; Lele et al., 2002; Liu et al., 2001) mutant parachute (pac) shows defects in retinal lamination, amacrine cell process outgrowth, confinement of cells to appropriate forebrain compartments, RGC axon guidance, closure of the choroid fissure and maturation of lens fiber cells. Null mutant pac embryos fail to maintain adherens junctions between retinal neuroepithelial cells and exhibit abnormal localization of dividing cells and severe disorganization of retinal laminae. In a weaker pac allele, proliferation appears to be unaffected, but RGCs and amacrine cells are nevertheless frequently intermingled and IPL forms in ectopic locations. In these retinas, amacrine cell processes are over-elaborated and mis-positioned, suggesting that Ncad modulates the outgrowth and targeting of these neurites. In addition to these retinal phenotypes, pac embryos also show defects in closure of the choroid fissure and in guidance of RGC axons towards their central targets. These guidance defects are primarily due to a failure to restrict cells to their appropriate CNS compartments, as many retinal neurons and reticular astrocytes invade the ventral brain along the trajectory normally taken by the RGC axons. Disrupting Fgfr function in the retina does not phenocopy pac, suggesting that most aspects of Ncad function do not require Fgfr activity.

**MATERIALS AND METHODS**

**Fish**

Zebrafish (*Danio rerio*) were maintained according to standard procedures (Westergaard, 1995). RIKEN wild type, WIK, pac<sup>crw95</sup> (Lele et al., 2002), pac<sup>crw95</sup> and a transgenic fish strain carrying green fluorescent protein (GFP) under an ath5 retinal enhancer were used in this study.

**Mutagenesis**

Mutations were induced in male gametes of RIKEN wild-type fish using N-ethyl-N-nitrosourea (ENU). Subsequent breeding and screening for recessive mutations followed standard protocols (Solnica-Krezel et al., 1994). To isolate retinal mutants, embryos from F2 pairwise crosses were screened by labeling with anti-acetylated α-tubulin antibody.

**Mapping mutant loci**

pac<sup>crw95</sup> embryos were collected from parents derived from a pac<sup>crw95</sup>/RIKEN wild type × WIK/WIK cross and stored in methanol. Genomic DNA was extracted from individual embryos at 1 day post-fertilization (dpf). PCR analysis with SSLP markers mapped to each linkage group was performed on DNA from pools of 20 wild-type and 20 mutant embryos. Map positions were refined by testing additional SSLP markers on individual embryos.

**Isolation and sequencing of Ncad mutant cDNAs**

Total RNA was extracted from 1 dpf pac<sup>crw95</sup> embryos using Ultraspec RNA (Biotecx laboratory). After purification of mRNA with an oligo-dT<sub>TM</sub>-MAG mRNA purification kit (TaKaRa Biochemicals), ncad cDNA was amplified with the RNA LA PCR<sup>TM</sup> kit (TaKaRa Biochemicals), using two primers, 5′-GGTTTCTTTTATA-CAGAACGGAATT-3′ (5′-untranslated region prior to the first Met) and 5′-TCTCTCCGGTTTACTTGAGTTCA-3′ (3′-untranslated region after the stop codon). PCR products corresponding to full-length cDNA were amplified in at least two independent reactions, subcloned into the TA cloning vector, pCR2.1 (Invitrogen), and sequenced using a ABI PRISM<sup>®</sup> 310. To exclude nucleotide changes derived from polymorphisms, genomic DNA from male grandparents of the family containing the pac<sup>crw95</sup> mutation was also sequenced.

**Sectioning**

Embryos were fixed, dehydrated to 100% ethanol, embedded in JB4 resin (Agar Scientific), and sectioned (5 μm) using a Microtome Rotatif, HM335E (MICROM International GmbH). Sections were stained in 0.1% Toluidine Blue (MERCK). For cryosectioning, fixed embryos were transferred to 30% sucrose in phosphate buffer, mounted in OCT compound, and sectioned at 8 μm on a Microtome cryostat, HM550M-OM (MICROM International GmbH).

**Antibody labeling and in situ hybridization**

Standard procedures were used (Wilson et al., 1990) with anti-Pax6 antibody (Macdonald and Wilson, 1997) at 1:400; zn-5 antibody (Oregon Monoclonal Bank) at 1:50; zpr-1 (formerly FRET43) antibody (Larson and Bremiller, 1999) at 1:100;
anti-acetylated α-tubulin antibody (Sigma) at 1:1000; anti-protein kinase C (PKC) antibody (sc-209, Santa Cruz Biotechnology) at 1:300; anti-γ-tubulin antibody (Sigma) at 1:200; and anti-phosphorylated histone H3 antibody (Upstate biotechnology) at 1:500. Sytox Green (Molecular probes) was used at 1:50,000. In situ hybridization to RNA probes was carried out by standard procedures (Xu et al., 1994).

Staining with rhodamine-phalloidin and BODIPY-ceramide
Rhodamine-conjugated phalloidin (Molecular Probes; 10−7M) was applied to cryosections of 28 hour post-fertilization (hpf) retinae. After washing with phosphate buffer, slides were mounted with 70% glycerol and scanned with a LSM 510 laser-scanning microscope (Carl Zeiss). BODIPY-ceramide staining of live embryos was carried out as previously described (Lele et al., 2002).

Cell transplantation
Cell transplantation at late blastula stage was carried out according to Westerfield (Westerfield, 1995). Approximately 5-20 cells were transplanted to host embryos from donor embryos labeled at the one to two cell stage with a mix of rhodamine- and biotin-conjugated dextrans in a 1 to 1 ratio (Molecular Probes; 3–5% w/v in 100 mM KCl). The genotypes of hosts and donors were inferred by assessing defects in brain morphology at 1 dpf. For analyses of IPL formation, mosaic embryos were fixed at 3 dpf in 4% paraformaldehyde (PFA) in PBS. For analyses of RGC axon trajectories, an ath5:GFP transgene was used to visualize donor retinal cells. As an alternative method to generate mosaic embryos, retinal cells were directly removed from host optic cups at 24 hpf with glass capillary needles and transplanted into recipient retinae. Using this protocol, retinal cells did not form columns of neurons in the host neural retina, but RGCs did differentiate and extend axons into the brain.

Visualization of retinal cells with GFP
Genomic fragments (14 kb) covering the zebrafish ath5 transcription unit (Masai et al., 2000) were cloned from a genomic library. About 7 kb 5′- and 3′-genomic fragments containing untranslated regions were inserted into multi-cloning-sites upstream or downstream of the GFP-coding region in the expression vector pEGFP (Clontech). Injection of one-cell stage embryos with the linearized construct leads to GFP expression in small numbers of cells in the developing retinae.

dil and diO injection
Embryos (60 hpf and 4 dpf) were fixed in 4% PFA, dil and diO (C-18, Molecular Probes; each 2 mg/ml in N, N-dimethylformamide (DMF) or chloroform) were injected into eyes of fixed embryos using glass capillaries and scanned by confocal laser microscopy LSM510 (Zeiss).

SU5402 treatment
Previous studies have indicated that median inhibiting concentration (IC50) values for in vitro kinase inhibition and for inhibition of neurotogenic effects of Fgf2 are 10 μM and 25 μM SU5402, respectively (Mohammadi et al., 1997; Skaper et al., 2000). Therefore, for pharmacological inhibition of Fgfr activity, wild-type embryos were treated with 10 μM-1mM SU5402 (Calbiochem) in the dark. We also confirmed that this range of SU5402 concentration effectively blocks expression of sef, a downstream target of Fgf8 signaling (Tsang et al., 2002) (data not shown).

Cell sorting/intermingling assay using zebrafish animal caps
Detailed procedures are described previously (Mellitzer et al., 1999). Capped-RNA encoding wild-type and mutant (pacW059) Ncad was injected into one cell-stage embryos with Alexa-488 conjugated dextran (Molecular Probes). Animal caps were dissected and fused with animal caps from non-injected embryos labeled with Rhodamine-conjugated dextran. The fused cell masses were cultured in L15 medium at 28.5°C under a coverslip and scanned by confocal microscopy. Cell sorting/intermingling was quantified by counting the total number of isolated cells that had crossed the boundary from either territory into the other.

RESULTS

labyrinth is a novel allele of ncad/pac
To elucidate mechanisms that underlie retinal lamination, we screened zebrafish mutants by visualizing retinal layers with anti-acetylated α-tubulin antibody (Fig. 1A). Through this screen, we identified labyrinth (lyr), a mutant in which the IPL is disrupted (Fig. 1B). Mapping of the lyr mutation using SSLP revealed close linkage to the ncad gene. We found no recombination between the lyr mutation and the microsatellite marker z9343 in 290 meioses (0.35 cM; Fig. 1C) and as the genetic distance between z9343 and ncad is less than 0.04 cM, it raised the possibility that the lyr gene encodes Ncad. parachute (pacW72) (Jiang et al., 1996; Lele et al., 2002) is a likely null allele of the zebrafish ncad gene (Lele et al., 2002) (Fig. 1F) and although eye phenotypes in pac embryos have not been reported, lyr embryos show similar defects in the dorsal midbrain and hindbrain to pac embryos (data not shown). Complementation tests between carriers of lyr and pac resulted in about one quarter of the embryos (65/239) with defects in the dorsal midbrain and hindbrain suggesting that lyr is indeed an allele of pac (Fig. 1D-E).

Sequencing of ncad from lyr embryos revealed a miss-sense mutation resulting in an amino acid change Tyr558Cys (amino acid number is based upon GenBank Accession Number AF418565) (Fig. 1F). As Tyr558 is a highly conserved amino acid in the b-sheet of EC4 (Overduin et al., 1995; Shapiro et al., 1995), this mutation is likely to alter Ncad activity. To elucidate if Ncad adhesive activity is reduced in lyr, we assessed Ncad-mediated cell sorting/intermingling using a zebrafish animal cap assay. Wild-type Ncad expressing cells do not intermingle with non-expressing cells (Fig. 1G). The lyr mutation compromises this ability of Ncad to segregate cells (Fig. 1H-I), suggesting that the cell adhesive activity of Ncad is reduced in lyr mutants.

Injection with Ncad morpholino-antisense oligonucleotides (MO-Ncad) mimics the brain defects of pac (Lele et al., 2002). ncad morphants show similar IPL defects to lyr (data not shown), suggesting that downregulation of Ncad can mimic the retinal phenotype of lyr. From complementation testing, mapping, sequencing data, functional analyses of lyr-mutated Ncad and, finally, MO-Ncad injection, we conclude that lyr is an allele of pac and call this mutation pacW059 from here onwards.

pac mutants show defects in retinal lamination
To elucidate the role that Ncad plays during eye development, we examined the morphology of pac retinae. In order to identify subtle phenotypic features that might be masked by the strong adhesion defects of the pacW72 null allele, we also examined retinae in trans-heterozygous pacW059fr7 embryos and embryos homozygous for the weak allele pacW059. In 3 dpf wild-type eyes, the three nuclear and two plexiform layers are established (Fig. 2A) and the IPL forms a continuous layer of neuropil between the RGC and amacrine cell layers (Fig. 2E).
In both pacrw95 and pacrw95/fr7 retinas, the RGC and amacrine cell layers are partially fused, resulting in disruption to the IPL (Fig. 2B,C). In mutant eyes, patches of IPL are surrounded by disorganized RGCs, amacrine and bipolar cells (Fig. 2F,G). Although different classes of retinal cells are distinguishable by their cell type-specific morphology in pacrw95 retinae, histological differences between the retinal neurons are less distinct in pacrw95/fr7 eyes (Fig. 2C,G) and are absent altogether in pacfr7 eyes (Fig. 2D,H).

In the ONL of pacrw95 retinae, some cells lack the columnar morphology of wild-type photoreceptors (Fig. 2I,J), and appear to be detached from the OPL. Although the OPL is intact in pacrw95 eyes, it is locally disrupted in pacrw95/fr7 retinae (Fig. 2K) and completely indistinguishable in pacfr7 eyes, which consist entirely of rosettes of morphologically indistinct cells surrounding patches of neuropil (Fig. 2L).

To elucidate whether disrupted lamination in pac mutants is due to defective formation of retinal layers or alternatively due to a failure in their maintenance, we examined early steps of IPL formation in living eyes. In 2 dpf wild-type eyes, the first processes of the IPL accumulate at the interface between RGC and amacrine cell layers from the outset (Fig. 2M). By contrast, in pacrw95 and to a greater extent in pacrw95/fr7 eyes, the initial aggregation of neurites occurs at many positions in the amacrine layer (Fig. 2N,O). Over time these initial neuritic foci enlarge until they form the rosettes characteristic of later stages (data not shown). Reflecting later phenotypic severity, initial association of neurites occurs randomly throughout the eyes of pacfr7 embryos (Fig. 2P). Together, these data indicate that Ncad is required for establishment of both retinal cell type morphology and retinal lamination and that inner layers of the retina are most sensitive to reduced levels of Ncad activity.

Neuronal identity is established but patterning is perturbed in pac mutants

To determine if neuronal specification or patterning is perturbed in pac embryos, retinae were labeled with retinal neuron-specific antibodies. In both pacrw95 and pacrw95/fr7 eyes, the ciliary marginal zone (CMZ) at which neurons are generated is initially indistinguishable from wild-type (Fig. 2M-O). Indeed, in all mutant allele combinations, differentiated RGCs, Müller glia, amacrine, bipolar and photoreceptor cells are present (Fig. 3; data not shown). However, their distribution is progressively more disturbed...
from mild to severe alleles. Thus, in \(\text{pac}^{\text{rw95}}\) retinas, the position of photoreceptors and bipolar cells is normal and most amacrine cells are positioned between RGC and bipolar cell layers (Fig. 3B,F). In \(\text{pac}^{\text{rw95fr7}}\) and to a greater extent in \(\text{pac}^{\text{fr7}}\) eyes, the different retinal neuron classes are much more intermingled (Fig. 3C,D,G,H,K,L). Photoreceptors are the neuronal subtype least affected by \(\text{ncad}\) mutations but even they are mis-positioned in \(\text{pac}^{\text{rw95fr7}}\) and \(\text{pac}^{\text{fr7}}\) eyes (Fig. 3K,L).

Ncad mutations disrupt maintenance of adherens junctions and localization of cell divisions in the neural retina

The retinal lamination defects of \(\text{pac}^{\text{fr7}}\) embryos are reminiscent of those in \(\text{has, one, glo}\), and \(\text{nok}\) mutants in which junctional complexes are disrupted. To elucidate if junctions are disrupted in \(\text{pac}^{\text{fr7}}\) embryos, we examined several markers that reveal the integrity of adherens junctions and cell polarity of neuroepithelial cells in the eye. In wild-type 28 hpf retinal
epithelium, dense foci of actin microfilaments are associated with the apical cell termini (Malicki and Driever, 1999) (Fig. 4A), while in pac<sup>fr7</sup> eyes, actin foci spread further basally within the ventral retina (Fig. 4B). The retinal neuroepithelium is also polarized with respect to the arrangement of centrosomes, which are localized near the apical pole of the cells (Malicki and Driever, 1999) (Fig. 4C). The localization of centrosomes in pac<sup>fr7</sup> retinae is also abnormal with a pattern similar to that of actin microfilaments, suggesting that retinal epithelial cell polarity is disturbed in the absence of Ncad function (Fig. 4D).

To elucidate if the disruption of adherens junctions and polarity affects cell division, pac<sup>fr7</sup> retinae were labeled with an antibody that reveals proliferating cells (Wei and Allis, 1998). In the ventral retina of 1 dpf pac<sup>fr7</sup> eyes, dividing cells are positioned far from their normal location at the apical surface (Fig. 4E,F). In mutant eyes, the morphology of the cells is also abnormal in the ventral retina with retinal cell nuclei losing their elongated shape (Fig. 4F). Surprisingly, dorsal retina in pac<sup>fr7</sup> eyes initially shows none of these defects. However, by 2 dpf, dividing cells spread throughout the retina of pac<sup>fr7</sup> mutants (Fig. 4G,H). These data suggest that disruption to adherens junction integrity begins ventrally and spreads dorsally in the retinae of pac mutants and this is accompanied by mis-localization of dividing cells and loss of neuroepithelial polarity. pac<sup>rw95</sup> eyes show normal localization of actin foci and dividing cells (data not shown), suggesting that low levels of Ncad activity are sufficient to regulate cell polarity and movements associated with cell proliferation.

**Ncad is required to regulate outgrowth of amacrine cell processes**

In pac<sup>rw95</sup> eyes, the IPL is severely disrupted despite the normal establishment of cell polarity, development of the CMZ and localization of proliferating cells. This suggests that Ncad regulates additional events during the formation of retinal laminae. The detection of mispositioned neuropil during formation of the IPL raised the possibility that outgrowth of

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**Fig. 3.** Neuronal patterning is affected in pac mutants. (A-D) Two dpf wild-type (A), pac<sup>rw95</sup> (B), pac<sup>rw95/fr7</sup> (C) and pac<sup>fr7</sup> (D) retinae labeled with zn5 antibody (red), which stains axons and cytoplasm of RGCs. Nuclei of retinal cells are counter-stained green with Sytox Green Nucleic Acid Stain. RGCs differentiate in all mutants (white arrowheads) but their localization is progressively more disrupted in pac<sup>rw95</sup>, pac<sup>rw95/fr7</sup> and pac<sup>fr7</sup> eyes. (E-H) Four dpf wild-type (E), pac<sup>rw95</sup> (F), pac<sup>rw95/fr7</sup> (G) and pac<sup>fr7</sup> (H) retinae labeled with anti-Pax6 antibody, which stains amacrine cells (red). (I-L) Four dpf wild-type (I), pac<sup>rw95</sup> (J), pac<sup>rw95/fr7</sup> (K) and pac<sup>fr7</sup> (L) retinae labeled with zpr-1 antibody, which stains double-cone photoreceptors (red). In pac<sup>rw95</sup>, the photoreceptor cell layer is normal. In pac<sup>rw95/fr7</sup>, the photoreceptor cell layer is locally disrupted and in the pac<sup>fr7</sup> retina, zpr-1-positive photoreceptors do not form an outer layer, but instead are located sparsely near the scleral region of the eye (white arrowheads). a, amacrine cell layer; pl, photoreceptor layer; rgl, retinal ganglion cell layer.
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amicrène cell processes may be directly perturbed by ncad mutations. To assess if this is the case, we examined the morphology of individual retinal neurons in pacfr7 embryos by labeling small numbers of cells with GFP. In wild-type retinae, neuroepithelial progenitor cells produce columns of neurons in which specific neuronal classes differentiate at appropriate apicobasal positions (Fig. 5A,C). Prior to 52 hpf, there is relatively little outgrowth of amacrine cell processes, and those that do form are small. In striking contrast, large and irregularly branched neurites are frequently observed throughout the presumptive INL of pacfr7 embryos (Fig. 5B). However, wild-type cells at the edge of clones are sometimes mis-positioned between RGC and amacrine cell layers (Fig. 5K), suggesting that local Ncad deficiency can disrupt wild-type cell patterning. Complementing these conclusions, pacfr7 cells aggregate and make rosette-like structures in wild-type retinae leading to fusion of the RGC and amacrine cell layers (Fig. 5L). However, isolated mutant amacrine cells normally extend their processes to wild-type IPL, suggesting that local Ncad activity can rescue neurite patterning of pacfr7 amacrine cells (Fig. 5L). These observations show that Ncad is essential for local cell-cell interactions that mediate formation of the IPL.
Ncad function is required for guidance of RGC axons

Previous studies have shown that in the absence of Ncad function, RGC axons exit the eye but may not form a normal optic chiasm (Lele et al., 2002). To elucidate RGC axon guidance defects, we determined the trajectories of RGC axons in pac mutants by combined diI-diO labeling. This analysis revealed RGC axon defects at many positions along their pathway (Fig. 6A,B). Within the optic nerve, RGC axons were sometimes defasciculated and some axons exited the nerve before reaching the midline to adopt ipsilateral trajectories (Fig. 6B). Intermingling of axons from the two eyes at the optic chiasm was often observed (Fig. 6D). Within the diencephalon, axons occasionally deviated from the optic tract despite usually maintaining a course towards the tectum (Fig. 6B). As RGC axons approached their target zones, they formed terminal arbors within the tectum. At the stages examined, both ipsilateral and contralateral axons formed overlapping terminal arbors within the tecta (Fig. 6B). pac^{rw95/fr7} and pac^{fr7} embryos showed similar defects although phenotypic penetrance was lower in pac^{rw95/fr7} (Table 1). By contrast, pac^{fr7} embryos show normal projections (Table 1), suggesting that low Ncad activity is sufficient to rescue these trajectory defects.

In order to facilitate the analysis of RGC axon trajectories, we crossed pac^{fr7} to a transgenic line in which GFP is expressed in retinal cells under the control of ath5/lak promoter/enhancer elements (Fig. 6E). Many retinal cells were not confined to the retina in pac^{fr7} eyes and instead extensively invaded the ventral forebrain (Fig. 6F). This phenotype even occurred in chimaeric embryos in which pac^{fr7} cells incorporated into wild-type eyes (Fig. 6G). These results indicate that the environment through which RGC axons extend in both pac^{fr7} mutants and chimaeras is highly abnormal and may contribute to the pathfinding defects of the pac mutant axons. Indeed, in some cases (n=2/5), we observed axons being deflected from their normal trajectories in the vicinity of...
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ectopic retinal cells (Fig. 6H,I). Despite the highly abnormal patterns of cell migration in pac mutants, the overall morphology of the ventral brain is not severely disrupted and at least one putative RGC axon guidance protein, Slit3 (Hutson and Chien, 2002; Plump et al., 2002), is expressed relatively normally (Fig. 6K,L).

In order to address if pathfinding errors by pac mutant RGC axons are alleviated if the diencephalic environment is normal, we transplanted mutant cells into wild-type eyes at 24 hpf. These late transplanted cells did not migrate out of the eye and in cases where they differentiated as RGCs, axons formed tightly fasciculated contralateral projections (n=5/5; Fig. 6J). These data add support to the conclusion that local environmental defects contribute significantly to the axon guidance defects of Ncad deficient RGC axons.

Ncad is required for closure of the choroid fissure

Subsequent to outgrowth of RGC axons, ventral nasal and ventral temporal retinal tissue fuses to close the choroid fissure (Fig. 7A,C). This process is disrupted resulting in severe coloboma in pacfr7 and pacfr7rw95 eyes (Fig. 7B; data not shown). In these mutants, retinal neurons stream out of the back of the eye towards or even into the diencephalon (Fig. 6F, Fig. 7D). The choroid fissure in pacrw95 embryos is macroscopically indistinguishable from wild type but sectioning reveals some retinal cells detached from the neural retina through the exit point of the optic nerve (data not shown).

Mutations in the paired box transcription factor Pax2 lead to coloboma in fish, mice and humans (Sanyanusin et al., 1995; Torres et al., 1996; Macdonald et al., 1997) and so we examined expression of this gene in pac mutants. Initiation of expression is normal, even in the severe pacfr7 allele, but by the 20-somite stage, pax2.1/noi expression is expanded along the AP axis of optic vesicle (Fig. 7E,F), suggesting defects in pax2.1 regulation or in morphogenesis of the optic stalks. After formation of the optic cup, pax2.1 expression is expanded in ventronasal and ventrotemporal cells.

Table 1. Four dpf projection pattern of RGC axons to the optic tectum

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<th>Ipsilateral</th>
<th>Mixed (ipsi+contra)</th>
<th>Contralateral (normal)</th>
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<tr>
<td>pacfr7</td>
<td>0/12</td>
<td>5/12</td>
<td>7/12</td>
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<td>pacfr7rw95</td>
<td>0/19</td>
<td>2/19</td>
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<td>pacrw95</td>
<td>0/17</td>
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<tr>
<td>SU5402(8-96h)</td>
<td>6/10</td>
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<tr>
<td>SU5402(30-96h)</td>
<td>0/25</td>
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*Seventeen out of 25 show a different phenotype (i.e. retinal axons fail to enter tectal target areas; Fig. 9E,F).
around the choroid fissure of pacfr7 eyes (Fig. 7G,H). By 48 hpf pax2.1 expression remains disorganized in the ventral eye and at the junction of the optic nerve with the brain (Fig. 7J,K). Indeed some pax2.1+ cells were located in ectopic locations within the ventral forebrain, suggesting that prospective astrocytes as well as retinal neurons inappropriately invade the brain in pac mutants.

Ncad is required for lens fiber formation

In addition to retinal phenotypes lens fiber formation is also perturbed in pac mutants. In pacfr7, degeneration of lens fibers occurs, suggesting that Ncad is required for proper differentiation of the lens (Fig. 8A-C). To elucidate how lens fiber formation is perturbed in pac mutants, we analyzed lens cell morphogenesis. Until at least 37 hpf, the lens developed normally in pacfr7 (31 hpf; Fig. 8D,E). However, at around 48 hpf, differentiating fiber cells fail to elongate and remain on the basal side of the lens sphere (Fig. 8F,G). These data suggest that N-cadherin is required for maturation of lens fibers.

Blocking Fgfr signaling disrupts RGC axon guidance but not retinal lamination

In vitro studies have suggested that neurite outgrowth induced by Ncad requires the presence of functional Fgfrs (Williams et al., 2001). To address whether aspects of Ncad function during eye development require Fgfr function, we assayed whether blocking Fgf signaling phenocopied pac eye defects. We used the pharmacological Fgfr inhibitor SU5402 to block Fgf signaling (Mohammadi et al., 1997) given that there is no straightforward genetic approach to severely inhibit Fgf signaling at the relevant sites and stages.

In embryos treated with SU5402 from 8 to 96 hpf, retinal axons frequently projected to ipsilateral optic tecta (Table 1; Fig. 9A,B) or in some cases failed to extend to the midline (Fig. 9C). Fgf signaling is required for patterning of midline tissue where the optic axons normally decussate (Shanmugalingam et al., 2000) and the domains of slit3 expression that normally delineate the retinal axon pathway are perturbed by SU5402 treatment (Fig. 9D), suggesting that midline defects are likely to contribute to retinal misguided in SU5402 embryos. When SU5402 was applied at stages after midline patterning had occurred then retinal axons did project contralaterally. However, they often failed to enter the tecta (Table 1; Fig. 9E-G), consistent with previous observations that blocking Fgf signaling slows the extension of retinal axons and can lead to failure to enter the tecta in frog (McFarlane et al., 1996; Lom et al., 1998). From these data, it is likely that environmental cues required for crossing the midline are affected by abrogation of Fgf signaling as is the ability of retinal axons to enter their termination zones.

Treatment with SU5402 had no significant effect on the
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Localization of dividing cells to the apical surface of the retina (Fig. 9H), to neuronal differentiation (Fig. 9J), to the initial establishment of the IPL (Fig. 9J,K) or to the closure of the choroid fissure (Fig. 9L,M). In some cases, dying cells were apparent in the eyes at later stages suggesting a requirement for Fgf signals for survival of retinal neurons (Fig. 9N). Indeed with the exception of the CMZ, the entire neural retina degenerated when treated with very high doses of SU5402 (Fig. 9O). Altogether, these studies indicate that the retinal defects observed in pac embryos are not phenocopied by Fgfr blockade, suggesting that Ncad functions are largely independent of Fgfr function during retinal development.

DISCUSSION

In this paper, we show that zebrafish Ncad/Pac is required for several discrete events during the development of the retina and differentiation of retinal neurons. There is an initial requirement for Ncad in localizing cell division to the apical side of the neural retina and for establishment of retinal laminae. Ncad subsequently modulates the outgrowth of amacrine cell processes during formation of the IPL. Ncad is also required for the epithelial fusion events that close the choroid fissure, for maturation of lens fiber cells, and for restricting cell movement between retina, optic nerve and brain. Finally, Ncad is non-autonomously required for guidance of RGC axons to their tectal targets. These activities of Ncad appear to be largely independent of Fgfr function. Our data provide genetic evidence that Ncad plays diverse and crucial roles during development of the vertebrate eye.

Adhesive activity of Ncad is reduced in pac alleles

As the retinal phenotype in pac<sup>rw95</sup> is weaker than in the likely null pac<sup>fr7</sup> allele, then Ncad may retain some activity in pac<sup>rw95</sup> embryos. Antibody labeling shows that Ncad is translated in pac<sup>rw95</sup>, and the correct localization of catenin and actin (M.Y. and I.M., unpublished) suggests that it is able to assemble apical cytoplasmic protein complexes. These data suggest that the intracellular domain of Ncad is still active in pac<sup>rw95</sup>. Nevertheless, pac<sup>rw95</sup>-Ncad has reduced cell adhesive activity, consistent with phenotypic severity being related to the dose of the pac<sup>rw95</sup>-Ncad allele. As Ncad is expressed broadly in CNS, this hypomorphic allele will continue to be useful to elucidate various other roles of Ncad that may be masked in the null allele.

Biochemical studies have reported that soluble truncated forms of cell adhesion molecules may function in a dominant-negative manner (Doherty et al., 1995). In pac<sup>fr7</sup>, a stop codon occurs in the extracellular region, raising the possibility that a soluble, and hence interfering, form of Ncad may be produced in pac<sup>fr7</sup>. However, retinal phenotypes are only observed in embryos homozygous for pac<sup>fr7</sup> mutations, strongly suggesting that pac<sup>fr7</sup> eye phenotypes result from loss-of-function of Ncad. Indeed, Ncad morphants exhibit the same retinal defects as pac<sup>fr7</sup> mutants, supporting previous data (Lele et al., 2002) that pac<sup>fr7</sup> is a null mutation.
Ncad is required for localizing apical proteins and cell division in the neural retina

A feature common to all of the zebrafish mutants affecting retinal lamination (ome, nok, has, glo and pac) is the disruption to proteins associated with junctional complexes and apicobasal polarity of retinal cells (Malicki and Driever, 1999; Horne-Badovinac et al., 2001; Pujic and Malicki, 2001; Peterson et al., 2001; Wei and Malicki, 2002) (this study). In nok, ome and glo retinae, adherens junctions are not maintained in the retinal neuroepithelium, and it is thought that this contributes to the abnormal localization of dividing cells (Malicki and Driever, 1999; Pujic and Malicki, 2001; Wei and Malicki, 2002).

Although the cadherin-catenin complex is a major component of adherens junctions, it is the maintenance and not the initial establishment of these junctions that is disrupted in pac 

retinae. Perhaps reflecting the late requirement for Ncad activity, organization of proliferative neuroepithelial cells at the CMZ of pac mutants is relatively normal compared with more mature central retina. These observations raise three possibilities. The first is that cadherins are involved in establishment of adherens junctions and that other cadherins compensate for the loss of Ncad function at early stages of retinal maturation. R-cadherin is one candidate as it heterodimerizes with Ncad in vitro (Shan et al., 2000) and is expressed in the eye, although perhaps at stages too late to regulate early neuroepithelial junctions (Liu et al., 2001).

A second possibility is that cadherin function is not essential for the initial establishment of adherens junctions. Indeed, adherens junctions can form in the absence of Hmr-1 cadherin,
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Defects in localization of cell divisions and adhesive interactions between neuroepithelial cells are very likely to contribute to the lamination defects in pac and other retinal lamination mutants. However, in the hypomorphic pac^{rw95} allele, there are no obvious defects in early neuroepithelial cell organization or in the localization of cell divisions. Despite this, RGCs and amacrine cells fail to segregate and consequently differentiate at inappropriate apicobasal positions. This phenotype suggests that Ncad is also involved in the guidance of RGCs and amacrine cells to their correct sites of differentiation. The fact that RGC and amacrine cell organization is disrupted to a greater extent than outer retinal neurons adds further support to the notion that this phenotype is distinct from early steps of retinal patterning. In other zebrafish mutants in which cell division and/or junctional complexes are disrupted, it is generally the outer retina that is most severely affected, with RGCs showing the least degree of disorganization (Malicki and Driever, 1999; Horne-Badovinac et al., 2001; Jensen et al., 2001; Pujic and Malicki, 2001; Peterson et al., 2001; Wei and Malicki, 2002).

Little is known of the signals that position different neuronal classes after they are born in the retina. From genetic analyses of nok and moe zebrafish mutants, it has been speculated that guidance cues exist on neuroepithelial cells (Wei and Malicki, 2002) and that positioning signals pass between pigmented epithelium and neural retina (Jensen et al., 2001). Furthermore, the absence of Müller glia is closely associated with formation of photoreceptor rosettes, suggesting that Müller glia may also be a source of guidance signals (Wang et al., 2002). However, in all cases, the identity of the actual guidance cues is unknown. We suggest that Ncad contributes to the ability of cells to sort into laminae containing discrete neuronal classes, perhaps by promoting cell class specific homotypic interactions.

There is extensive literature showing that cadherins contribute to cell type specific sorting (reviewed by Redies, 2000) and recent studies have provided compelling evidence that cadherins mediate sorting of pools of different classes of motoneurons in the lateral motor column (Price et al., 2002). Indeed, our cell sorting/intermingling assays suggest levels of Ncad influence cell affinities. This suggests that even though ncad is widely expressed (Liu et al., 2001), subtle cell-type specific differences in the activity of Ncad protein may contribute to sorting of different neuronal classes.

Ncad modulates outgrowth of amacrine cell dendrites

A surprising phenotype in the hypomorphic pac^{rw95} allele is the exuberant and undirected extension of amacrine cell processes within the INL. With the exception of interplexiform cells, the only neurites elaborated from all classes of amacrine cells are horizontally directed processes within one or more laminae of the IPL. The mechanisms controlling the initial directed outgrowth of amacrine processes are unknown but are unlikely to depend upon adjacent RGCs as an IPL forms in the zebrafish lakritz mutant which lacks RGCs (Kay et al., 2001). In pac mutants, amacrine cell morphology is more severely disrupted than other neuronal cell types, suggesting that Ncad activity has specific roles in directing the outgrowth of amacrine cell processes. As pac amacrine cells can adopt more normal morphologies in a wild-type environment, then it is likely that Ncad is a local environmental signal that is read by the growth cones of the amacrine processes. This is consistent with the observation that Ncad is expressed at high levels in the IPL (Liu et al., 2001) and that neurites expressing Ncad preferentially elongate along substrates that express the highest levels of Ncad (Redies, 2000).

Perhaps analogous to the amacrine cell phenotype, in flies, mosaic analyses have revealed roles for Ncad in the selection by retinal axons of appropriate target laminae within the optic lobe (reviewed by Clandinin and Zipursky, 2002). However, in this case, over-elongation of processes is not observed and the morphology of terminals is relatively normal (Lee et al., 2001). Similarly, application of function blocking Ncad antibodies to avian retinal axons and their tectal targets disrupts lamina selection by RGC growth cones (Inoue and Sanes, 1997). Recent studies using cultured vertebrate hippocampal neurons have also implicated cadherin function in modulating dendritic morphology (Togashi et al., 2002) but even in situations where all cadherin activity is likely to be blocked, overall organization and length of dendritic processes are not significantly affected. The alterations in amacrine cell processes in fish pac mutants are considerably more severe than these other situations.

Ncad is required for epithelial fusion of the choroid fissure

Although there are few, if any, studies on the cellular basis of choroid fissure closure, it almost certainly involves a series of events similar to those that characterize other epithelial fusions (Martin and Wood, 2002). The initial interactions between leading edge epithelial cells may be mediated by a variety of different proteins that include, at least in vertebrates, Eph receptors and ephrins (reviewed by Holmberg and Frisén, 2002). Subsequent to these events, adherens junctions stabilize the connections between the apposing epithelia (Simske and Hardin, 2001). Within the ventral eye, the abrogation of Ncad
function may compromise the stabilization of any interactions between ventronasal and ventrotemporal retinal cells leading to a failure of epithelial fusion.

It is likely that the epithelial cells that mediate choroid fissure closure are Pax2+ prospective astrocytes that line the choroid fissure (Sanjuan et al., 1995; Torres et al., 1996; MacDonald et al., 1997). The exodus of Pax2.1+ cells from the optic nerve into the brain of pac mutants suggests that the normal adhesive interactions that maintain the cohesion of the prospective astrocytic network of the nascent optic nerve are compromised. Indeed, the reduced convergence of ventronasal and ventrotemporal retinal cells is likely to compromise further the ability of such cells to undergo fusion. Together, these observations strongly suggest that the mis-localization of Pax2.1+ prospective astrocytes coupled with compromised adhesive interactions/junction formation between these cells is the cause of the coloboma in pac mutants.

Ncad maintains boundaries between retina, optic nerve and ventral brain

Perhaps the most dramatic of all the eye phenotypes after abrogation of Ncad activity is the invasion of the brain by retinal neurons and prospective optic nerve astrocytes. This phenotype implies that retinal neurons no longer respect the tissue compartment boundaries between retina, optic stalk/nerve and ventral brain. Even in chimaeric embryos in which mutant cells are in a predominantly wild-type environment, pac mutant retinal neurons invade the ventral brain.

It is widely believed that classical cadherins contribute to the separation of cell compartments during embryonic development (Redies, 2000). For example, within the telencephalon, R-cadherin and cadherin 6 are thought to contribute to the sorting of cells between pallial and sub-pallial territories (Inoue et al., 2001). Ncad may, therefore, contribute to the maintenance of boundaries between neural retina, optic stalk and ventral brain. However, how it would achieve this is uncertain as no differences in Ncad expression have been reported between these different regions. A common feature of the retina/optic nerve and optic nerve/ventral brain interfaces is the presence of Pax2.1+ cells defining the boundaries. We have previously shown that these Pax2.1+ cells provide barriers to axonal navigation (Macdonald et al., 1997). Our current studies indicate that Pax2.1+ cells fail to tightly associate with each other in pac mutants, suggesting that the optic stalk tissue may play a more comprehensive role in restricting both axonal extension and neuronal migration.

One further possibility is that retinal neurons are drawn out of the eye by exiting RGC axons in pac mutants. Indeed, there appears to be little invasion of the brain by retinal cells prior to outgrowth of the optic nerve. If neuronal contacts within the retina are weakened, then perhaps there is less anchoring of the neurons within the neural retina and a greater propensity to be drawn out of the eye.

Ncad activity is required non-autonomously for guidance of RGC axons

pac mutants exhibit severe retinotectal pathfinding defects with axons adopting ectopic trajectories both ipsilaterally and within the optic tract. Axons from the two eyes are frequently intermingled at the optic chiasm, no doubt contributing to the ipsilateral axon guidance defects. Although many other studies have suggested that Ncad may be required for navigation of RGC axons (Matsunaga et al., 1988b; Riehl et al., 1996), we found that pac mutant RGC axons could establish a tightly fasciculated contralateral projection in a wild-type background. This suggests that the majority of the severe guidance defects can be attributed to pathway defects within the pac optic nerve, chiasm and tract. Indeed the ectopic distribution of Pax2.1+ cells and retinal neurons along the RGC axon pathway are very likely to disrupt growth cone navigation. Our studies do not discount the possibility that Ncad is cell-autonomously required within RGCs for more subtle aspects of guidance, such as selective fasciculation (Treubert-Zimmerman et al., 2002) or target selection (Lee et al., 2001).

Ncad is required for lens fiber differentiation

Lens fiber differentiation consists of a series of tightly coordinated events that include the spatially regulated proliferation of superficial lens epithelial cells, elongation and migration of lens fiber cells, and fusion of maturing fiber cells at the medial lens suture (Fig. 8A). Although it has been suggested that cell adhesion molecules including Ncad are involved in morphogenesis of the lens (Bassnett et al., 1999; Leong et al., 2000), early steps in lens vesicle formation proceed normally in pacfr7. It is the elongation and differentiation of lens fiber cells that requires Ncad function. The late appearance of lens defects is consistent with the fact that, as within the retina, adherens junctions initially form in the lens vesicle of pac embryos (M.Y. and I.M., unpublished). As adherens junctions form at the apical ends of lens fiber cells (Lo et al., 2000), absence of Ncad may reduce the ability of the elongating lens fiber cells to absorb mechanical stresses with the consequence that they lose apical attachments and accumulate basally within the lens.

Ncad function is largely independent of Fgfr function during eye development

Although it was originally thought that cadherins regulate adhesion solely through homophilic interactions, it is likely that the situation is more complex. For example, there is now ample evidence of heterophilic interactions between different cadherins and, in flies, a receptor-type protein tyrosine phosphatase called DLAR is intimately associated with Ncad activity (reviewed by Redies, 2000; Clandinin and Zipursky, 2002).

An interaction between Ncad and Fgfrs was initially suggested as they share HAV binding motifs (Williams et al., 1994), previously thought only necessary to mediate homophilic interactions. The HAV motif in the EC1 domain of Ncad is involved in homophilic interactions (Shapiro et al., 1994; Overduin et al., 1995), whereas the one in EC4 is implicated in the interaction with Fgfrs (Williams et al., 2001). As the pacfr7 mutation is within the EC4 domain, it is likely to disrupt any interactions of this domain. Given these findings, we attempted to address whether Ncad function requires Fgfr function by comparing Ncad mutant phenotypes to Fgfr blockade phenotypes.

Within the eye, none of the pac mutant phenotypes was phenocopied by Fgfr inhibition, suggesting that Ncad and Fgfr function independently during retinal development. Furthermore, although there are superficial similarities
between the RGC axon phenotypes in pac mutants and SU5402-treated embryos, in both cases the major defects are likely to be due to problems within the environment through which the RGC axons extend. It remains possible that more subtle aspects of Ncad function during RGC axon extension do require Fgfr function and indeed for motor axons, we observe similar Ncad and Fgfr phenotypes (Z.L. and S.W.W., unpublished). However, it is reasonable to conclude that Ncad functions are largely independent of Fgfr activity with respect to most/all of the roles we have revealed for Ncad during eye development.

In conclusion, through the use of severe and weak pac mutant alleles we have revealed a variety of crucial functions for Ncad during the formation of the vertebrate eye. Indeed, given the complexity of the pac mutant phenotypes, it is very likely that there will be further roles for Ncad in this region of the CNS. Our work identifies Ncad activity as being absolutely central to the establishment of a functional visual system.

We thank Jarema Malicki and Margret Moré for communication of unpublished data. We thank all technical staff involved in the zebrafish mutagenesis project in H.O.'s laboratory. We also acknowledge all members of the I.M., S.W. and H.O. laboratories for advice and suggestions on this research. This work was supported by grants from RIKEN and PRESTO, JST (I.M.); and from RIKEN BSI and CREST, JST (H.O.); and by the BBSRC and Wellcome Trust (S.W.). S.W. is a Wellcome Trust Senior Research Fellow.

Note added in proof
Recent studies have shown that glass onion is allelic with parachute (Malicki et al., 2003).

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