

## N-cadherin mediates retinal lamination, maintenance of forebrain compartments and patterning of retinal neurites

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### SUMMARY

The complex, yet highly ordered and predictable, structure of the neural retina is one of the most conserved features of the vertebrate central nervous system. In all vertebrate classes, retinal neurons are organized into laminae with each neuronal class adopting specific morphologies and patterns of connectivity. Using genetic analyses in zebrafish, we demonstrate that N-cadherin (Ncad) has several distinct and crucial functions during the establishment of retinal organization. Although the location of cell division is disorganized in embryos with reduced or no Ncad function, different classes of retinal neurons are generated. However, these neurons fail to organize into correct laminae, most probably owing to compromised adhesion between retinal cells. In addition, amacrine cells exhibit exuberant and misdirected

outgrowth of neurites that contributes to severe disorganization of the inner plexiform layer. Retinal ganglion cells also exhibit defects in process outgrowth, with axons exhibiting fasciculation defects and adopting incorrect ipsilateral trajectories. At least some of these defects are likely to be due to a failure to maintain compartment boundaries between eye, optic nerve and brain. Although *in vitro* studies have implicated Fgf receptors in modulating the axon outgrowth promoting properties of Ncad, most aspects of the Ncad mutant phenotype are not phenocopied by treatments that block Fgf receptor function.

Key words: Axon guidance, Cell adhesion, Coloboma, *Danio rerio*, Polarity, Zebrafish

### 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 at the

apical ends of the retinal neuroepithelial cells (Horne-Badovinac et al., 2001; Peterson et al., 2001; Wei and Malicki, 2002). In *nok* mutant retinæ, 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 (Fgfrs) (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 retinæ, 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 (Westerfield, 1995). RIKEN wild type, WIK, *pac*<sup>li7</sup> (Lele et al., 2002), *pac*<sup>rw95</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  $\alpha$ -tubulin antibody.

### Mapping mutant loci

*pac*<sup>-/-</sup> embryos were collected from parents derived from a *pac*<sup>rw95</sup>/RIKEN wild type  $\times$  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>rw95</sup> <sup>-/-</sup> embryos using Ultraspec RNA (Biotex laboratory). After purification of mRNA with an oligo-dT<sup>TM</sup>-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'-GGTTTCTTATA-CAGAACGGAATTT-3' (5'-untranslated region prior to the first Met) and 5'-TCCTCCGGTCTTACTTGTAGTTCA-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>TM</sup> 310. To exclude nucleotide changes derived from polymorphisms, genomic DNA from male grandparents of the family containing the *pac*<sup>rw95</sup> mutation was also sequenced.

### Sectioning

Embryos were fixed, dehydrated to 100% ethanol, embedded in JB4 resin (Agar Scientific), and sectioned (5  $\mu$ m) 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  $\mu$ m 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 (Larison and Bremiller, 1990) (Oregon Monoclonal Bank) at 1:100;

anti-acetylated  $\alpha$ -tubulin antibody (Sigma) at 1:1000; anti-protein kinase C (PKC) antibody (sc-209, Santa Cruz Biotechnology) at 1:300; anti- $\gamma$ -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;  $10^{-7}$ M) 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.

#### diI and diO injection

Embryos (60 hpf and 4 dpf) were fixed in 4% PFA. diI 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 ( $IC_{50}$ ) values for in vitro kinase inhibition and for inhibition of neurotogenic effects of Fgf2 are 10  $\mu$ M and 25  $\mu$ M SU5402, respectively (Mohammadi et al., 1997; Skaper et al., 2000). Therefore, for pharmacological inhibition of Fgfr activity, wild-type embryos were treated with 10  $\mu$ M-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 (*pac*<sup>rw95</sup>) 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  $\alpha$ -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* (*pac*<sup>fr7</sup>) (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).

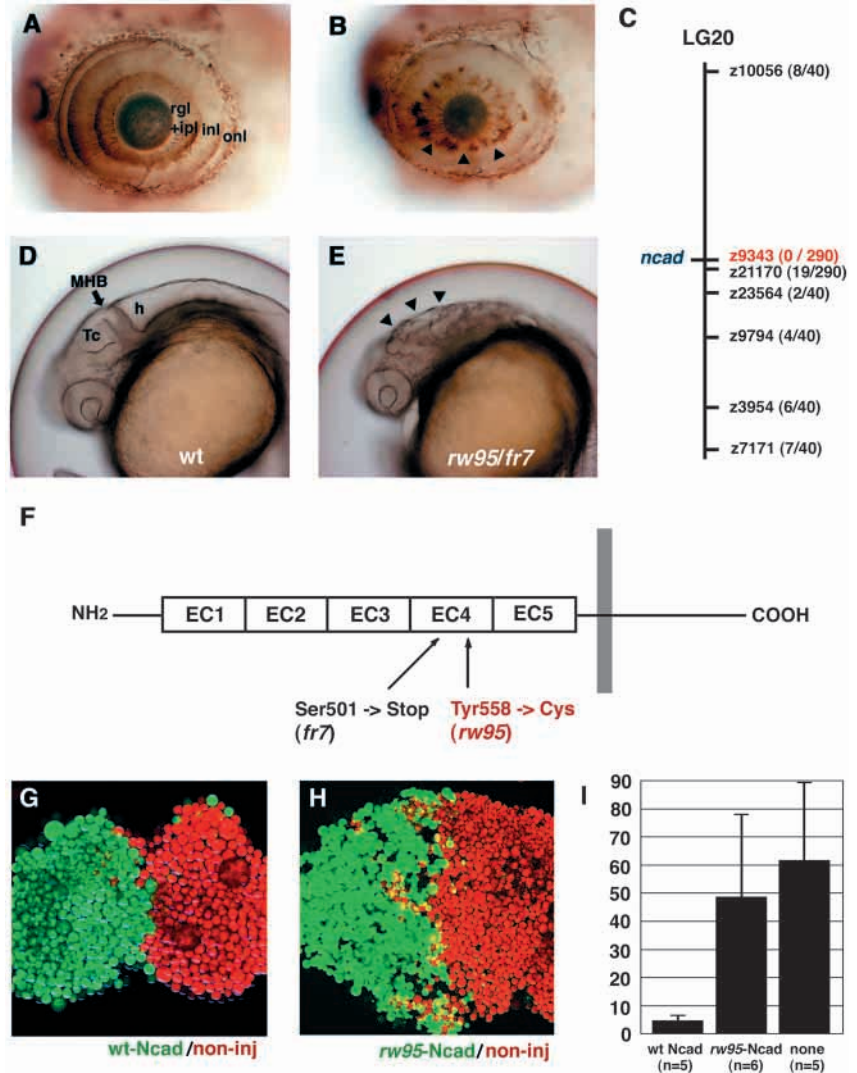
Sequencing of *ncad* from *lyr* embryos revealed a mis-sense mutation resulting in an amino acid change Tyr558Cys (amino acid number is based upon GenBank Accession Number AF418565) (Fig. 1F). As Tyr558 is a highly conserved amino acid in the  $\beta$ -sheet of EC4 (Overduin et al., 1995; Shapiro et 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 *pac*<sup>rw95</sup> 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 *pac*<sup>fr7</sup> null allele, we also examined retinae in trans-heterozygous *pac*<sup>rw95/fr7</sup> embryos and embryos homozygous for the weak allele *pac*<sup>rw95</sup>. 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).





**Fig. 1.** *lyr* is a new allele of the Ncad mutant, *pac*. (A,B) Lateral views of 3 dpf wild-type (A) and *lyr*<sup>-/-</sup> (B) eyes labeled with anti-acetylated  $\alpha$ -tubulin antibody. Arrowheads indicate disruption of the IPL. (C) Map of microsatellite markers used to map *lyr* to the same position on LG20 as *ncad*. Recombination rate is indicated by parenthesis. (D,E) Lateral views of wild-type (D) and mutant (E) embryos produced by crossing *lyr* and *pac*<sup>fr7</sup> carriers. Morphological defects are evident in the tectum and hindbrain of the mutant (E, arrowheads). (F) Mutation sites in the two *pac* alleles used in this study. A nonsense mutation present in *pac*<sup>fr7</sup> (Lele et al., 2002) and a mis-sense mutation (Tyr→Cys) found in *lyr*<sup>-/-</sup> embryos both occur in EC4 domain of Ncad. (G,H) Confocal images of aggregates of cells in which animal caps labeled with Alexa-488 conjugated dextran (green) and expressing either wild-type Ncad RNA (G) or *lyr*-mutated Ncad RNA (H) were juxtaposed to non-injected animal caps labeled with rhodamine-conjugated dextran (red). The sharp boundary between the Ncad-expressing and non-expressing cell masses is disrupted by the *lyr* mutation. (I) Quantification of cell intermingling. The bars indicate the average number of cells isolated within the apposing animal cap. Numbers of animal caps examined are indicated under the columns. h, hindbrain; inl, inner nuclear layer; ipl, inner plexiform layer; MHB, midbrain-hindbrain boundary; onl, outer nuclear layer; rgl, retinal ganglion cell layer; Tc, optic tectum.

In both *pac*<sup>rw95</sup> and *pac*<sup>rw95/fr7</sup> retinæ, 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 *pac*<sup>rw95</sup> retinæ, histological differences between the retinal neurons are less distinct in *pac*<sup>rw95/fr7</sup> eyes (Fig. 2C,G) and are absent altogether in *pac*<sup>fr7</sup> eyes (Fig. 2D,H).

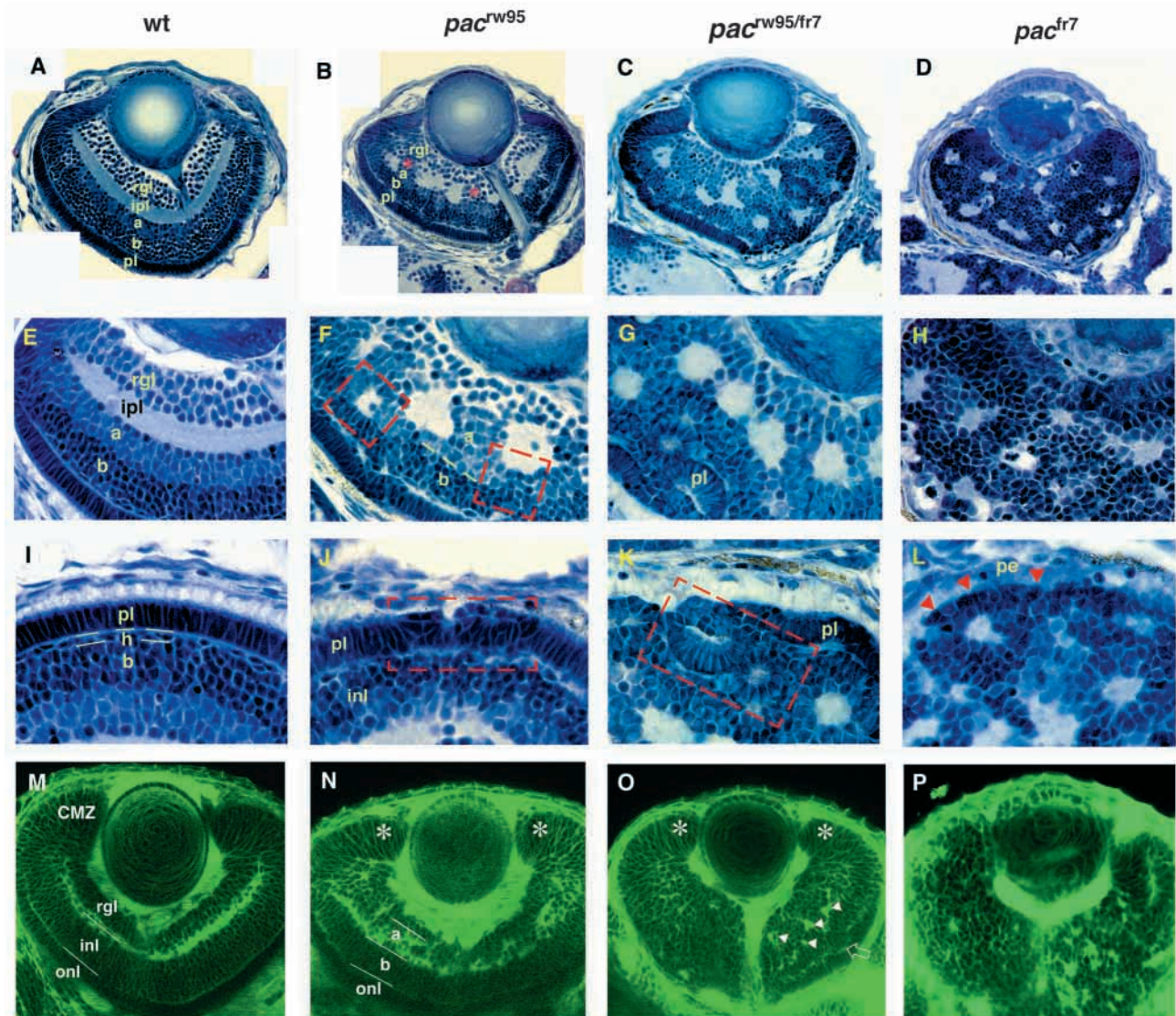
In the ONL of *pac*<sup>rw95</sup> retinæ, some cells lack the columnar morphology of wild-type photoreceptors (Fig. 2I,J), and appear to be detached from the OPL. Although the OPL is intact in *pac*<sup>rw95</sup> eyes, it is locally disrupted in *pac*<sup>rw95/fr7</sup> retinæ (Fig. 2K) and completely indistinguishable in *pac*<sup>fr7</sup> 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 *pac*<sup>rw95</sup> and to a greater extent in *pac*<sup>rw95/fr7</sup> 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 *pac*<sup>fr7</sup> 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, retinæ were labeled with retinal neuron-specific antibodies. In both *pac*<sup>rw95</sup> and *pac*<sup>rw95/fr7</sup> 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



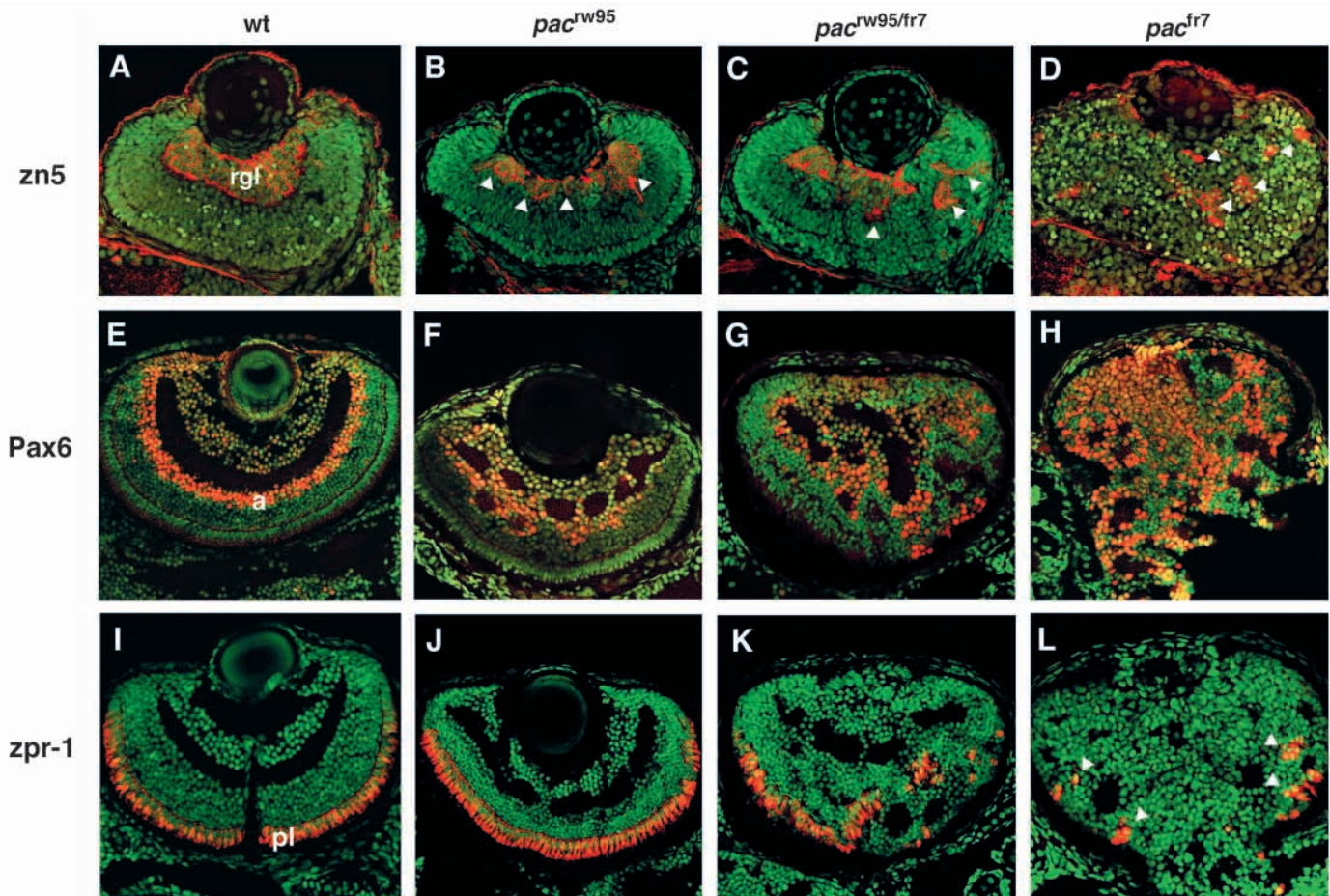
**Fig. 2.** *Ncad* is required for lamination and structural integrity of retinal cells. (A–D) Plastic sections of 3 dpf wild-type (A), *pac<sup>rw95</sup>* (B), *pac<sup>rw95/fr7</sup>* (C) and *pac<sup>fr7</sup>* (D) retinas. The partial fusion of the RGC and amacrine cell layers is indicated in the *pac<sup>rw95</sup>* retina (B, red asterisks). (E–H) High magnification of the INL in wild type (E), *pac<sup>rw95</sup>* (F), *pac<sup>rw95/fr7</sup>* (G) and *pac<sup>fr7</sup>* (H). A patch of the IPL adjacent to the bipolar cell layer is indicated in the *pac<sup>rw95</sup>* retina (F, broken red square). (I–L) High magnification of the ONL in wild type (I), *pac<sup>rw95</sup>* (J), *pac<sup>rw95/fr7</sup>* (K) and *pac<sup>fr7</sup>* (L). Some photoreceptors show irregular shape and abnormally aggregate in *pac<sup>rw95</sup>* (J, red broken parentheses). The photoreceptor layer is wavy and forms rosette structures in *pac<sup>rw95/fr7</sup>* (K, broken red square). In *pac<sup>fr7</sup>*, cells adjacent to the pigmented epithelium do not display photoreceptor-specific columnar shape (L, red arrowheads). (M–P) BODIPY-ceramide labeled living 48 hpf wild-type (M), *pac<sup>rw95</sup>* (N), *pac<sup>rw95/fr7</sup>* (O) and *pac<sup>fr7</sup>* (P) retinas. In wild type, the IPL forms at the interface between the RGC layer and INL. By contrast, patches of IPL neuropil are observed in the amacrine cell layer of *pac<sup>rw95</sup>* eye. In the *pac<sup>rw95/fr7</sup>* retina (O), patches of INL (white arrowheads) are more irregular and the OPL is also wavy (open white arrow). White asterisks show the CMZ. a, amacrine cell layer; b, bipolar cell layer; CMZ, ciliary marginal zone; h, horizontal cell layer; inl, inner nuclear layer; ipl, inner plexiform layer; pe, pigmented epithelium; pl, photoreceptor layer; rgl, retinal ganglion cell layer.

from mild to severe alleles. Thus, in *pac<sup>rw95</sup>* 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,J). In *pac<sup>rw95/fr7</sup>* and to a greater extent in *pac<sup>fr7</sup>* 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 *ncad* mutations but even they are mis-positioned in *pac<sup>rw95/fr7</sup>* and *pac<sup>fr7</sup>* 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 *pac<sup>fr7</sup>* embryos are reminiscent of those in *has*, *ome*, *glo* and *nok* mutants in which junctional complexes are disrupted. To elucidate if junctions are disrupted in *pac<sup>fr7</sup>* 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





**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) retinæ 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) retinæ 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) retinæ 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.

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> retinæ 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> retinæ 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

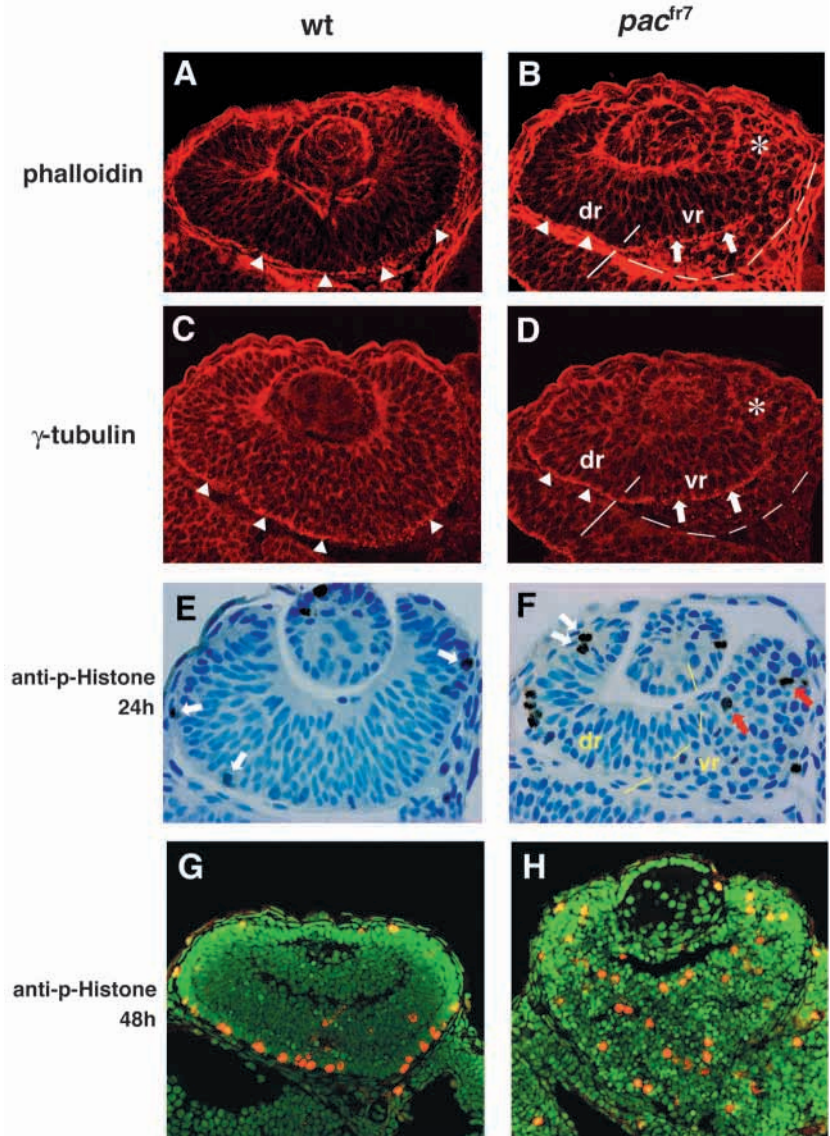
retina in *pac*<sup>fr7</sup> eyes initially shows none of these defects. However, by 2 dpf, dividing cells spread throughout the retina of *pac*<sup>fr7</sup> mutants (Fig. 4G,H). These data suggest that disruption to adherens junction integrity begins ventrally and spreads dorsally in the retinæ of *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



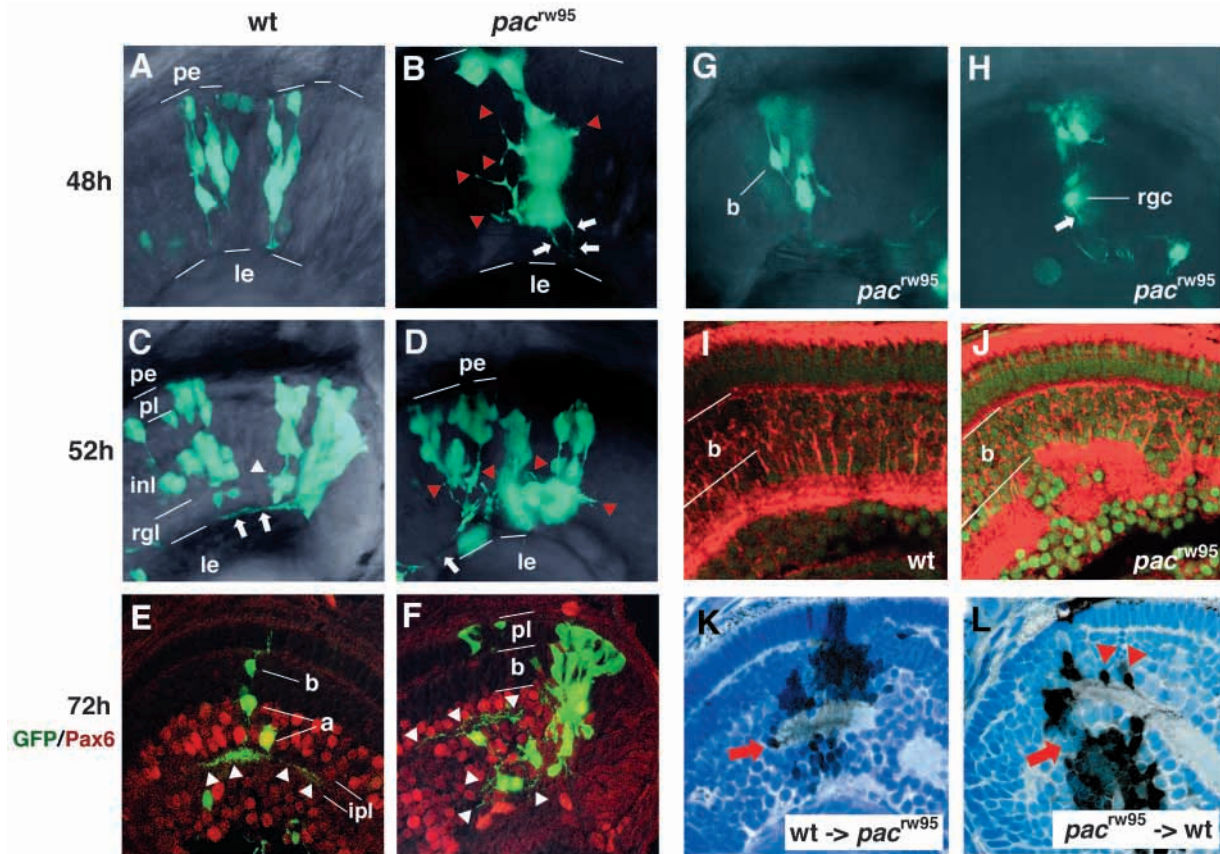
**Fig. 4.** Ncad is required for maintenance of adherens junctions in the retina. (A,B) Twenty-eight hpf wild-type (A) and *pac<sup>tr7</sup>* (B) retinæ labeled with rhodamine-conjugated phalloidin, which stains actin microfilaments (red). In wild type, strongly stained actin foci are visible in the apical termini of retinal cells, closely associated with the pigmented epithelium (A, white arrowheads). In the *pac<sup>tr7</sup>* eye, actin foci are normally localized in the dorsal retina (white arrowheads), while the line of actin foci (white arrows) is detached from the pigmented epithelium (broken lines) in the ventral retina and localization of actin foci is completely disrupted in the most ventral cells (B, white asterisk). (C,D) Twenty-eight hpf wild-type (C) and *pac<sup>tr7</sup>* (D) retinæ labeled with anti- $\gamma$ -tubulin antibody, which stains centrosomes. Sections shown in B,D are serial sections. In the wild-type retina, the centrosomes are localized in the apical termini of retinal neuroepithelial cells (white arrowheads). In *pac<sup>tr7</sup>*, the centrosomes fail to be localized apically in the ventral retina (white arrows) and their localization is completely random in the most ventral region (D, white asterisk). In the dorsal region, position of the centrosomes seems to be normal (white arrowheads). (E,F) Plastic sections of 24 hpf wild-type (E) and *pac<sup>tr7</sup>* (F) retinæ labeled with anti-phosphorylated histone H3 antibody, which stains proliferating cells in late G2 and M-phase. In the wild-type retina, dividing cells are localized to the apical surface of the neural retina (white arrows). In *pac<sup>tr7</sup>*, proliferating cells are positioned far from the apical surface in the ventral retina (F, red arrows), but normal in the dorsal retina (F, white arrows). (G,H) Forty-eight hpf wild-type (G) and *pac<sup>tr7</sup>* (H) retinæ labeled with anti-phosphorylated histone H3 antibody. The location of dividing cells is random throughout the *pac<sup>tr7</sup>* retina. dr, dorsal retina; vr, ventral retina.



amacrine 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 *pac<sup>rw95</sup>* embryos by labeling small numbers of cells with GFP. In wild-type retinæ, 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 *pac<sup>rw95</sup>* embryos (Fig. 5B) and sorting of different cell-types into laminae is less distinct (Fig. 5D). Expression of high levels of Pax6 at 72 hpf confirmed that the neurons with exuberant and ectopic processes in *pac<sup>rw95</sup>* eyes are indeed amacrine cells (Fig. 5E,F) (Macdonald and Wilson, 1997). Despite the amacrine cell defects, the morphology of cell somata of RGCs and bipolar cells in *pac<sup>rw95</sup>* eyes resembles wild type at all stages (Fig. 5G,H). In contrast to amacrine cell processes, anti-PKC labeled bipolar cell axons are oriented relatively normally towards the

patches of IPL in *pac<sup>rw95</sup>* eyes (Fig. 5I,J). These data indicate that the irregularly branched neurites belong to amacrine cells, and suggest that abnormal outgrowth of amacrine cell processes results in disruption of the IPL in *pac<sup>rw95</sup>*.

To determine if Ncad acts locally to regulate retinal lamination, we created chimaeric retinæ consisting of both wild-type and mutant cells. Columns of wild-type cells in *pac<sup>rw95</sup>* retinæ resemble wild-type with IPL formation rescued (Fig. 5K). 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, *pac<sup>rw95</sup>* cells aggregate and make rosette-like structures in wild-type retinæ 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 *pac<sup>rw95</sup>* amacrine cells (Fig. 5L). These observations show that Ncad is essential for local cell-cell interactions that mediate formation of the IPL.



**Fig. 5.** Ncad regulates outgrowth of amacrine cell dendrites. (A–D) Confocal images of GFP<sup>+</sup> cell columns in wild-type (A, C) and *pac<sup>rw95</sup>* (B, D) retinas. White arrows and arrowheads show retinal axons and amacrine cell dendrites, respectively. Irregularly branched neurites extend from the INL in *pac<sup>rw95</sup>* (B, D, red arrowheads). (E, F) GFP (green) and anti-Pax6 antibody (red) labeling of wild-type (E) and *pac<sup>rw95</sup>* (F) retinas. In the wild-type retinae, amacrine cell dendrites spread horizontally at the interface between RGCs and amacrine cells (white arrowheads). By contrast, Pax6-positive amacrine cells extend abnormally arborized neurites in the amacrine and RGC layers in *pac<sup>rw95</sup>* (white arrowheads). There is no equivalent arborization in the bipolar cell layer. (G, H) Confocal images showing morphology of bipolar cells (G) and a RGC (H) in 72 hpf *pac<sup>rw95</sup>* eyes. White arrows indicate axons of RGCs. (I, J) Anti-PKC labeling (red) of 4 dpf wild-type (I) and *pac<sup>rw95</sup>* (J) retinae. (K, L) Section of a 3 dpf *pac<sup>rw95</sup>* eye in which wild-type cells have incorporated into the retina. The wild-type column of cells (brown) forms a normal IPL. The red arrow indicates a wild-type cell positioned within the mixed RGC/amacrine cell layer. (L) Section of a 3 dpf wild-type eye incorporating *pac<sup>rw95</sup>* cells. The red arrow shows disruption of IPL formation by mutant cells. The red arrowheads indicate isolated mutant amacrine cells showing normal neurite morphology. a, amacrine cells; b, bipolar cells/cell layer; inl, inner nuclear layer; ipl, inner plexiform layer; le, lens; pe, pigmented epithelium; pl, photoreceptor layer; rgc, retinal ganglion cells; rgl, retinal ganglion cell layer.

### Ncad function is required for guidance of RGC axons

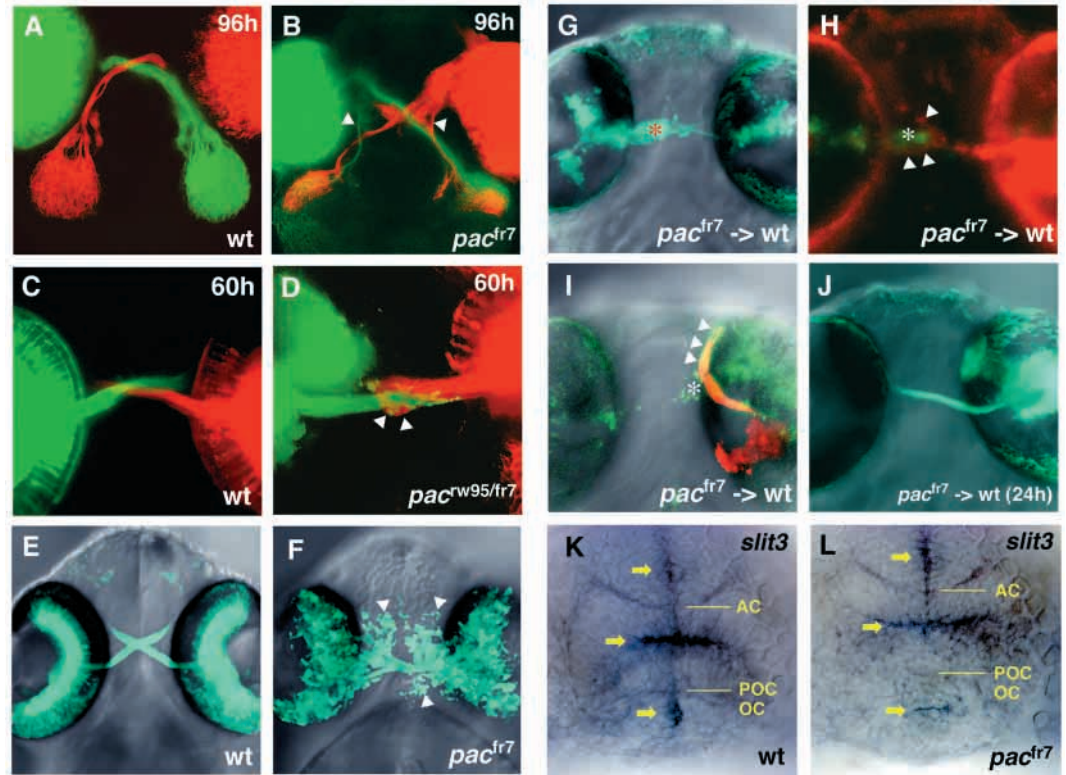
Previous studies have shown that in the absence of Ncad function, RGC axons exit the eye but may not form a normal optic chiasm (Lele et al., 2002). To elucidate RGC axon guidance defects, we determined the trajectories of RGC axons in *pac* mutants by combined diI–diO labeling. This analysis revealed 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<sup>rw95/fr7</sup>* and *pac<sup>fr7</sup>* embryos showed similar defects although phenotypic penetrance was lower in *pac<sup>rw95/fr7</sup>* (Table 1). By contrast, *pac<sup>rw95</sup>* 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<sup>fr7</sup>* 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<sup>fr7</sup>* embryos and instead extensively invaded the ventral forebrain (Fig. 6F). This phenotype even occurred in chimaeric embryos in which *pac<sup>fr7</sup>* cells incorporated into wild-type eyes (Fig. 6G). These results indicate that the environment through which RGC axons extend in both *pac<sup>fr7</sup>* 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



**Fig. 6.** Ncad is required for guidance of RGC axons. (A) Dorsal view of 4 dpf wild-type embryos labeled with diI (red) and diO (green) in the left and right eyes, respectively. RGC axons project to contralateral optic tectum. (B) Trajectories of diI/diO labeled RGC axons in *pac<sup>fr7</sup>*. Retinal axons project to both ipsilateral and contralateral tecta. White arrowheads indicate mis-routed retinal axons. (C,D) Ventral view of 60 hpf diI/diO-labeled wild-type (C) and *pac<sup>fr7/rw95</sup>* embryo (D). In *pac<sup>fr7/rw95</sup>*, RGC axons change outgrowth direction by following retinal axons that come from the opposite eye (D, white arrowheads). (E,F) Expression of *ath5:GFP* in 3 dpf wild-type (E) and *pac<sup>fr7</sup>* embryos (F). In *pac<sup>fr7</sup>*, retinal cells have migrated out from the optic cups and invaded the ventral brain (white arrowheads). There is variability in the phenotype and this embryo is more severe than most. (G-I) Three dpf chimaeric eyes in which *pac<sup>fr7</sup>* cells (green) were transplanted into wild-type embryos. Asterisks indicate retinal cells that escape from the eye cups. (H) Red indicates diI-labeled wild-type axons that bifurcate at ectopically positioned retinal cells (white arrowheads). (I) *pac* (green, GFP) and wild-type (red, diI) retinal axons (white arrowheads) turn in an inappropriate direction at the interface with retinal cells (white asterisk) and project to the telencephalon. (J) Three dpf wild-type embryos, in which *pac<sup>fr7</sup>* cells were transplanted to the retinae at 24 hpf. *pac<sup>fr7</sup>* retinal axons adopt a normal contralateral trajectory. (K,L) Ventral view of 30 hpf wild-type (K) and *pac<sup>fr7</sup>* (L) forebrains labeled with a *slit3* RNA probe. Arrows indicate three domains of *slit3* expression, which bound the anterior commissure, post-optic commissure and the optic chiasm. AC, tract of anterior commissure; OC, position of optic chiasm; POC, post-optic commissure



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 *pac<sup>fr7</sup>* and *pac<sup>fr7/rw95</sup>* 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 *pac<sup>rw95</sup>* embryos is macroscopically indistinguishable from wild type but sectioning reveals some

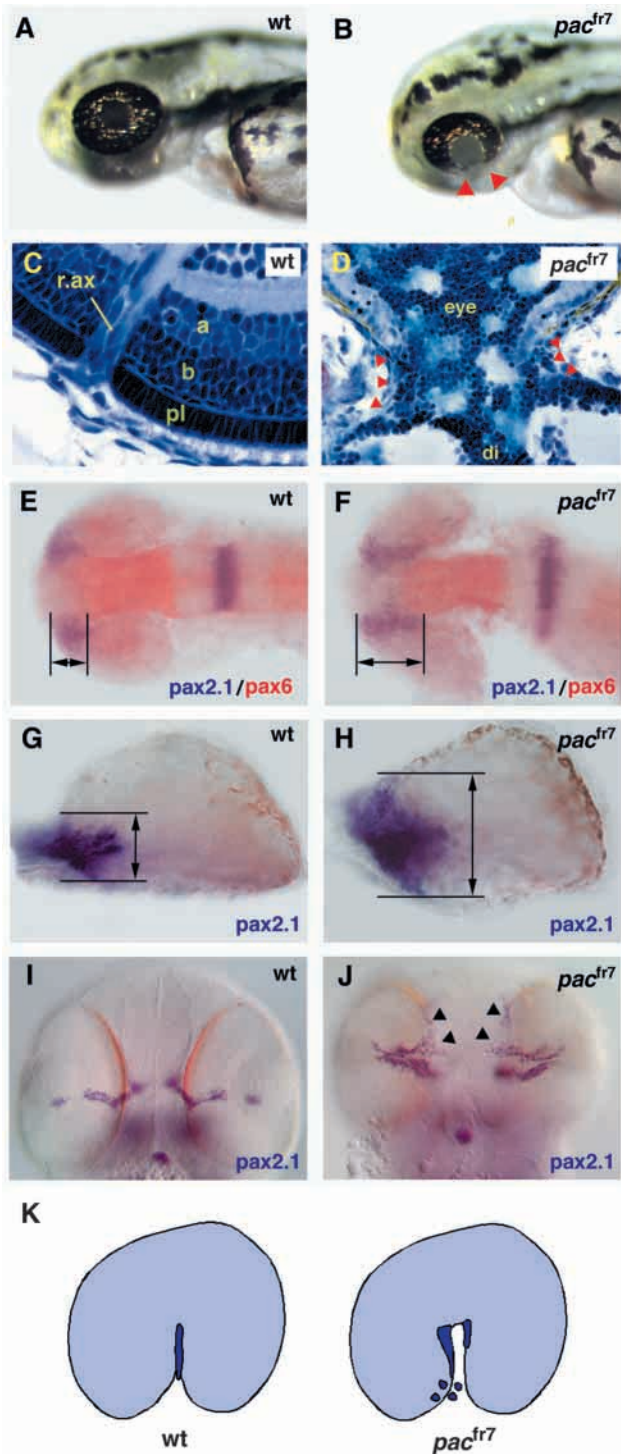
**Table 1.** Four dpf projection pattern of RGC axons to the optic tectum

	Ipsilateral	Mixed (ipsi+contra)	Contralateral (normal)
<i>pac<sup>fr7</sup></i>	0/12	5/12	7/12
<i>pac<sup>fr7/rw95</sup></i>	0/19	2/19	17/19
<i>pac<sup>rw95</sup></i>	0/17	0/17	17/17
SU5402(8-96h)	6/10	2/10	2/10
SU5402(30-96h)	0/25	0/25	25/25*

\*Seventeen out of 25 show a different phenotype (i.e. retinal axons fail to enter tectal target areas; Fig. 9E,F).

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 *pac<sup>fr7</sup>* allele, but by the 20-somite stage, *pax2.1/noi* (Brand et al., 1996) 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



around the choroid fissure of *pac*<sup>fr7</sup> 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*<sup>+</sup> 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

**Fig. 7.** *pac* mutants exhibit severe coloboma. (A,B) Lateral views of 4 dpf wild-type (A) and *pac*<sup>fr7</sup> (B) heads. The choroid fissure does not close (red arrowheads) in *pac*<sup>fr7</sup>. (C,D) High magnification of the optic nerve exit point/choroid fissure in 4 dpf wild-type (C) and *pac*<sup>fr7</sup> (D). Retinal cells spill out of the open fissure (D, red arrowheads) and spread to the diencephalon in *pac*<sup>fr7</sup>. (E-J) *pax2.1* labeling of optic stalks in wild-type and *pac* mutants. (E,F) Dorsal views of 19.5 hpf embryos showing *pax2.1* (blue) and *pax6* expression (red). Broader expression of *pax2.1* in the optic vesicles of the *pac* mutant is indicated (arrows/lines) (G,H) Lateral views of 24 hpf optic cups showing expanded *pax2.1* expression in the ventral retina of the *pac* mutant. (I,J) Ventral view of 48 hpf heads. *pax2.1* expression is present in prospective astrocytes of the optic nerve. Additionally in the *pac* mutant, *pax2.1*-expressing cells are present in the ventronasal and ventrotemporal retina and spread from the optic fissure into the brain (arrowheads). (K) Lateral views of the choroid fissure in wild-type and *pac*<sup>fr7</sup> eyes. Light blue indicates the neural retina and dark blue shows *pax2.1*<sup>+</sup> prospective astrocytes. a, amacrine cell layer; b, bipolar cell layer; di, diencephalon; r.ax, retinal axon; pl, photoreceptor layer.

perturbed in *pac* mutants. In *pac*<sup>fr7</sup>, 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 *pac*<sup>fr7</sup> (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.

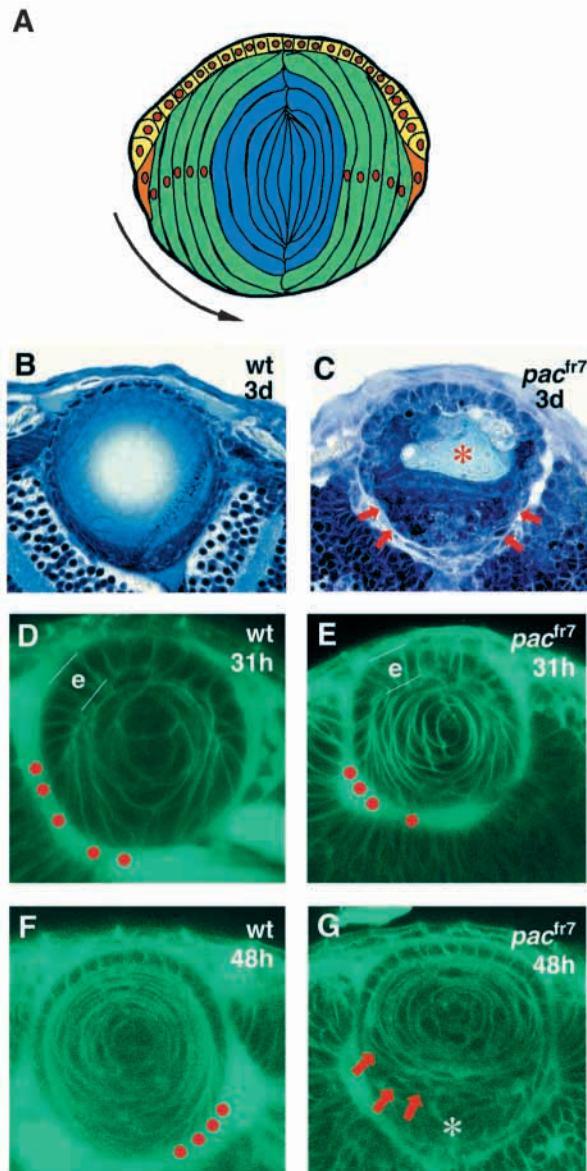
#### Blocking Fgfr signaling disrupts RGC axon guidance but not retinal lamination

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 misguidance 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





**Fig. 8.** Lens differentiation is perturbed in *pac*<sup>-/-</sup> embryos. (A) The developing lens. Proliferating apical epithelial cells (yellow) move to the equatorial zone (orange) where they start to differentiate as lens fiber cells. The apical and basal ends of the elongating lens fiber cells (green) migrate medially along the apical surface of proliferative epithelium (yellow) and basal lens capsule, respectively. The thinning fiber cells consequently cover the core of older lens fibers (blue) like the layers of an onion. The arrow indicates the progression of lens fiber cell maturation. (B,C) Plastic sections of 3-day-old wild-type (B) and *pac*<sup>fr7</sup> lens (C). In *pac* the apical proliferating cells are irregularly shaped and cells on the basal side of the lens do not display the elongate morphology of differentiating lens fibers (red arrows). Red asterisk indicates degenerated lens fibers. (D-G) Wild-type (D,F) and *pac*<sup>fr7</sup> lenses (E,G) labeled with BODIPY-ceramide. (D,E) Thirty-one hpf lens. Cuboidal proliferating cells form a superficial single layer on the lens surface. Differentiating cells are forming at the equatorial zone and elongating (red circles) in both wild-type and *pac*<sup>fr7</sup> lenses. (F,G) Forty-eight hpf lens. In wild type, lens fibers have differentiated (red circles), whereas they fail to elongate (red arrows) and remain on the basal side of the lens in *pac* mutants (white asterisk).

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.

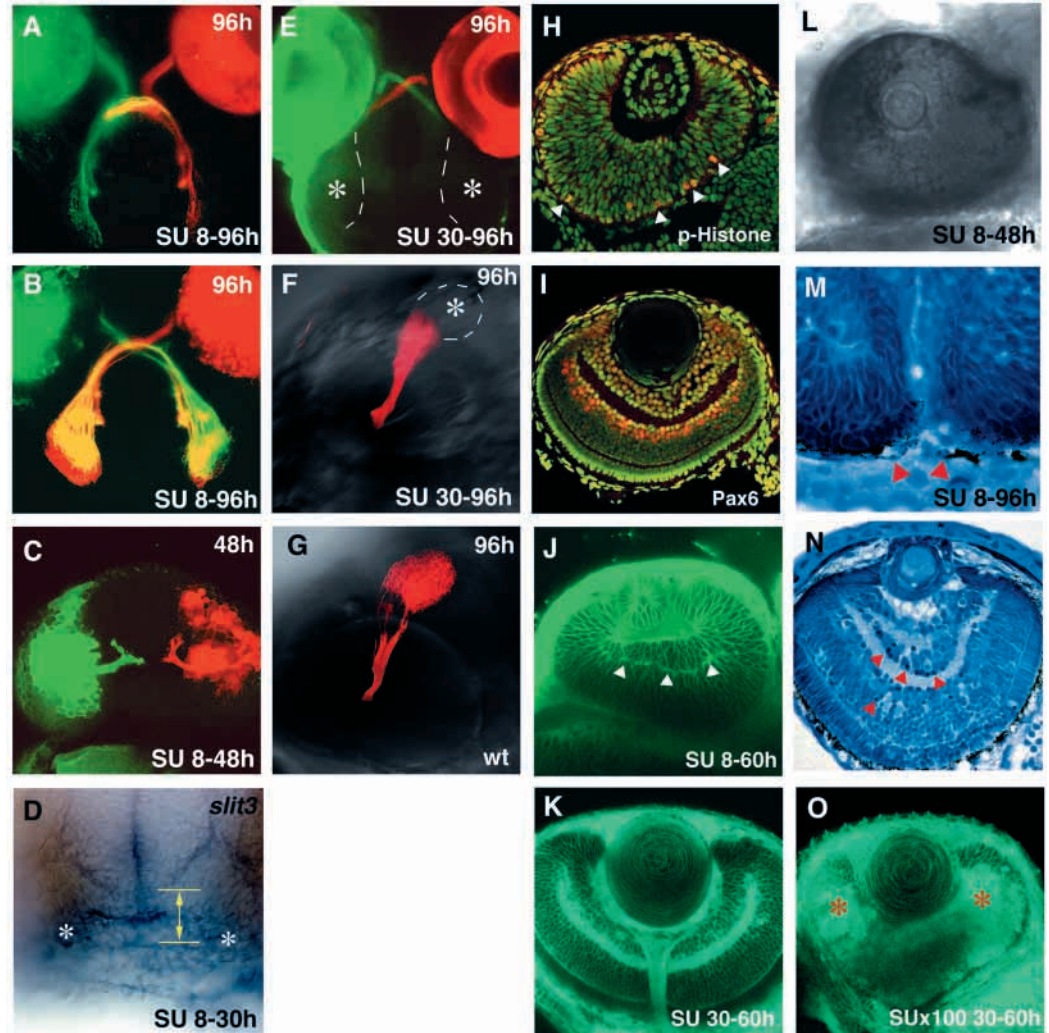
Biochemical studies have reported that soluble truncated forms of cell adhesion molecules may function in a dominant-negative manner (Doherty et al., 1995). In *pac*<sup>fr7</sup>, a stop codon occurs in the extracellular region, raising the possibility that a soluble, and hence interfering, form of 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.

localization of dividing cells to the apical surface of the retina (Fig. 9H), to neuronal differentiation (Fig. 9I), 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

**Fig. 9.** SU5402 treatment induces abnormal RGC axon trajectories but does not affect retinal lamination. (A-C,E-G) diI/diO-labeled RGC axons in SU5402-treated (A-C,E,F) and wild-type (G) embryos at 2 dpf (C) or 4 dpf (A,B,E-G). (A,B) Dorsal view showing ipsilateral projections (A) and mixed ipsi- and contra-lateral projections (B) (10  $\mu$ M SU5402, 8-96 hpf). (C) Frontal view showing RGC axons extending ipsilaterally before reaching the optic chiasm (10  $\mu$ M SU5402, 8-48 hpf). (D) *slit3* expression in SU5402-treated 30 hpf embryos. The distance between the anterior commissure and retinal axons is narrow (arrows/lines). White asterisks indicate ectopic *slit3* expression at positions where retinal axons enter the midline area (compare with Fig. 6K). (E) Dorsal view showing retinal axons which fail to enter target areas in the tectum (white asterisks) (10  $\mu$ M SU5402, 30-96 hpf). (F,G) Lateral views. Wild-type retinal axons project and arborize in the optic tectum, but SU5402-treated axon fails to enter the tectum (white asterisk). (H) SU5402-treated 60 hpf retinae (10  $\mu$ M, soaked from 8 hpf) labeled with anti-phosphorylated-histone H3 antibody. The locations of dividing cells (arrowheads) resemble wild-type (compare with Fig. 4G). (I) SU5402-treated 3 dpf retinae (10  $\mu$ M, soaked from 8 hpf) labeled with anti-Pax6 antibody. (J) SU5402-treated 60 hpf retinae (10  $\mu$ M, soaked from 8 hpf) labeled with BODIPY-ceramide. Arrowheads indicate the IPL (compare with Fig. 2M). (K) SU5402-treated 60 hpf retinae (10  $\mu$ M, soaked from 30 hpf) labeled with BODIPY-ceramide. (L) Lateral view of a 48 hpf living SU5402-treated eye (10  $\mu$ M SU5402, 8-48 hpf). (M) Plastic section through the closed choroid fissure (red arrowheads) of a 4 dpf SU5402-treated retina. (N) Plastic section of 4 dpf SU5402-treated retina (10  $\mu$ M, soaked from 8 hpf). Retinal lamination is globally normal, but some dying cells are observed as dark-stained nuclei (red arrowheads). (O) SU5402-treated 60 hpf retinae labeled with BODIPY-ceramide. Embryos were soaked with 1mM SU5402 from 30 hpf. No patches of IPL neuropil are observed, but instead large areas of cell degeneration are evident (red asterisks). Such severe cell death was not observed with treatment of 100  $\mu$ M SU5402 (data not shown).



### 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*<sup>fr7</sup> 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,



the only classic cadherin in *C. elegans* (Costa et al., 1998) and although DE-cadherin/Shotgun is important for morphogenesis of epithelial cells in flies, adherens junctions form in zygotic null *shotgun* mutants (Uemura et al., 1996; Tepass et al., 1996). It is possible that other protein complexes compensate for the absence of cadherin-catenin based cell interactions during adherens junction formation. For example, a complex containing Nectin and Afadin is concentrated at adherens junctions and interacts with the actin cytoskeleton (Mandai et al., 1997; Takahashi et al., 1999) and both the Par/aPKC and Crumbs/Stardust pathways have key roles in establishment of the zonula adherens (reviewed by Tepass, 2002; Johnson and Wodarz, 2003). Altogether, these data are consistent with our observation that initial formation of adherens junctions seems to be normal in the null allele of *pac*.

A third possibility is that Ncad regulates the renewal of adherens junctions that may occur following cell divisions. Disruption of adherens junction integrity begins ventrally and spreads dorsally in *pac<sup>fr7</sup>* retina with junctional fragments eventually scattering throughout the retina. This progressive degeneration of adherens junctions is reminiscent of that seen in the neural tube after Ncad blockage (Gänzler-Odenthal and Redies, 1998), suggesting similar Ncad-dependent regulation of junctional complex renewal may occur both in the brain and eye.

### Ncad function is required for retinal lamination

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<sup>rw95</sup>* 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<sup>rw95</sup>* 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-elaboration 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<sup>+</sup> prospective astrocytes that line the choroid fissure (Sanyanusin et al., 1995; Torres et al., 1996; Macdonald et al., 1997). The exodus of Pax2.1<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells defining the boundaries. We have previously shown that these Pax2.1<sup>+</sup> cells provide barriers to axonal navigation (Macdonald et al., 1997). Our current studies indicate that Pax2.1<sup>+</sup> 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 (data not shown). 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<sup>+</sup> 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 epidermal 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 *pac*<sup>fr7</sup>. 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., 1995; Overduin et al., 1995), whereas the one in EC4 is implicated in the interaction with Fgfrs (Williams et al., 2001). As the *pac*<sup>rw95</sup> 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 Fgfrs 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.

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### Note added in proof

Recent studies have shown that *glass onion* is allelic with *parachute* (Malicki et al., 2003).

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