

Zebrafish N-cadherin, encoded by the *glass onion* locus, plays an essential role in retinal patterning

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Received for publication 23 January 2003, accepted 12 March 2003

Abstract

Genetic screens in zebrafish identified several loci that play essential roles in the patterning of retinal architecture. Here, we show that one of them, *glass onion*, encodes the N-cadherin gene. The *glo*^{m117} mutant allele contains a substitution of the Trp2 residue known for its essential role in the adhesive properties of classic cadherins. Both the *glo*^{m117} and *pac*^{m101b} mutant N-cadherin alleles affect the polarity of the retinal neuroepithelial sheet and, unexpectedly, both result in cell-nonautonomous phenotypes in retinal patterning. The late onset of mutant N-cadherin phenotypes may be due to the ability of classic cadherins to substitute each other's function.

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Keywords: Zebrafish; Eye; Retina; Cadherin; Neuroepithelium; Adhesion

Introduction

The six major neuronal cell classes of the vertebrate retina are organized into distinct layers that fulfill specific functions. Photoreceptor cells detect light. The interneurons of the inner nuclear layer convey visual information from the photoreceptor cells to ganglion neurons. These, in turn, convey visual input to the brain. Most, if not all, of the major retinal cell classes consist of many distinct cell types (reviewed in Masland, 2001). Despite a thorough understanding of cellular architecture in the retina, the genetic basis of retinal pattern formation is poorly understood. One important source of insights into the genetic bases of retinal architecture is the studies of zebrafish mutations that affect the organization of retinal neurons. Mutations in the zebrafish loci *glass onion* (*glo*), *heart and soul* (*has*), *mosaic eyes* (*moe*), *oko meduzy* (*ome*), and *nagie oko* (*nok*) lead to a severe disruption of retinal patterning and thus most likely affect key components of mechanisms involved in the formation of retinal architecture (Jensen et al., 2001; Mal-

icki and Driever, 1999; Malicki et al., 1996; Pujic and Malicki, 2001). These loci play essential and global roles in retinal pattern formation as evidenced by the observation that their mutant phenotypes affect all cellular laminae. Previous studies indicated that most of these loci also function in the retinal neuroepithelium before the onset of neurogenesis. Molecular cloning of *heart and soul* and *nagie oko* revealed that they encode an atypical protein kinase C and a MAGUK-family scaffolding factor, respectively (Horne-Badovinac et al., 2001; Peterson et al., 2001; Wei and Malicki, 2002). The molecular nature of the remaining zebrafish retinal patterning loci remains, so far, unknown.

The integrity of cellular architecture is likely to require the presence of adhesion molecules. Classical cadherins are calcium-dependent transmembrane homophilic adhesion factors that play a role in numerous developmental processes, including the formation of the central nervous system. In mammals alone, the cadherin superfamily includes over 80 members (reviewed in Yagi and Takeichi, 2000). The classic cadherins, which include E-, N-, R-, and P-cadherin among others, contain three distinct structural features: five ectodomains involved in adhesive binding, a transmembrane segment, and a cytosolic domain associated with catenins (reviewed in Yagi and Takeichi, 2000). A number of studies, including crystallographic analysis, have

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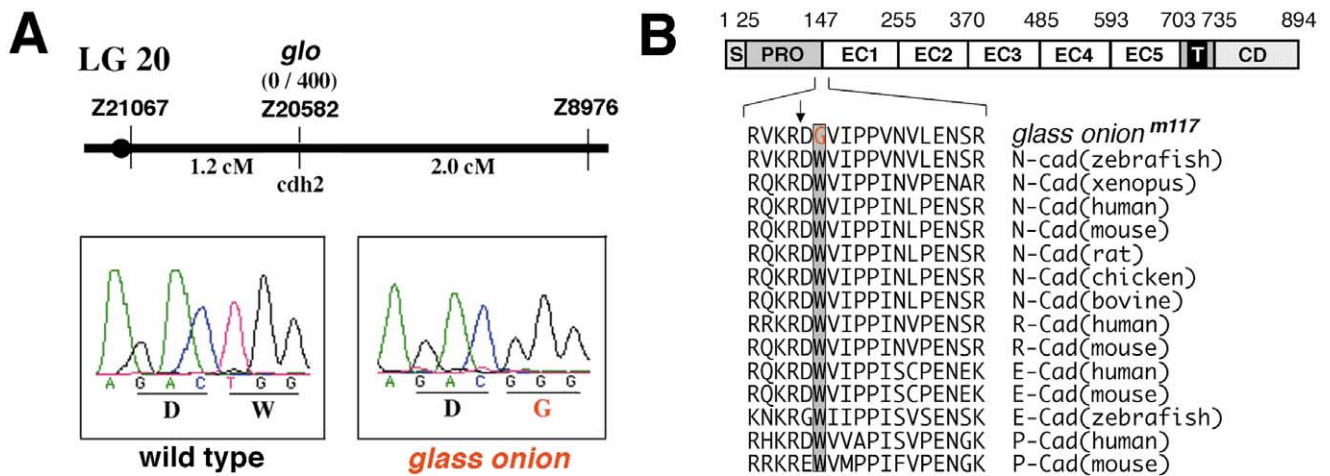


Fig. 1. Molecular cloning of the *glass onion* gene. (A) Genetic map of the *glass onion* genomic region (top) and identification of the mutation site (bottom). Initial analysis using half-tetrad embryos showed a strong linkage between the *glass onion* locus and Z21067. Further mapping with Z20582 that colocalized with N-cadherin (*cdh2*) revealed no recombination events in 400 meioses. (B) Schematic diagram of the zebrafish N-cadherin structure and amino acid sequence comparison of vertebrate cadherins in the region that contains the *glo*^{m117} W-to-G substitution. The conserved tryptophan residues are shaded. Arrow indicates endoproteolytic cleavage site.

been conducted to reveal the molecular basis of adhesive interactions that characterize cadherins. Classic cadherins are believed to form homophilic interactions both in *cis* with cadherin molecules on the same cell surface and in *trans* with cadherins on the surface of neighboring cells. Most models assume that the *trans* interactions are mediated predominantly or exclusively by the first ectodomain of the cadherin polypeptide. In particular, several lines of evidence indicate that the Trp2 residue of ectodomain 1 is essential for the *trans* interaction and consequently for the adhesive properties of classic cadherins (Boggon et al., 2002; Pertz et al., 1999; Tamura et al., 1998). A recent crystal structure study of the whole extracellular domain of C-cadherin provided evidence that the Trp2 residues of cadherin molecules interact symmetrically with hydrophobic pockets on cadherin polypeptides from the opposing cell surface (Boggon et al., 2002).

The role of classic cadherins, N-cadherin (cadherin-2) in particular, in nervous system development has been postulated ever since the discovery of this class of adhesion molecules (Hatta et al., 1985). Numerous experiments have also focused specifically on the role of N-cadherin in the retina. Due to a lack of suitable loss-of-function alleles, these studies relied on indirect ways of blocking gene function, such as antibody interference or the use of dominant negative constructs. In the chicken retina, antibody-blocking experiments suggested that N-cadherin plays a role in maintaining the overall integrity of the undifferentiated retina and at later developmental stages in the integrity of the photoreceptor cell layer (Matsunaga et al., 1988). In contrast, overexpression of a dominant negative N-cadherin construct in *Xenopus* did not reveal a patterning role for N-cadherin either in the neuroepithelium or in the differentiated retina. Instead, these studies indicated that N-cadherin

may be necessary for axon outgrowth in retinal ganglion cells (Riehl et al., 1996). Although a targeted gene knockout of N-cadherin has been generated in the mouse, its usefulness in the study of retinal development has been limited by the early lethality of homozygous mutant embryos (Radice et al., 1997). Thus, the function of N-cadherin in the development of the retina is complex and remains only partly understood.

The zebrafish model offers the possibility of generating mutant alleles via the chemical mutagenesis approach. Mutagenesis screens led to the isolation of numerous mutations affecting the development of the retina (reviewed in Malicki, 2000), including the *glass onion* (*glo*^{m117}) allele (Malicki et al., 1996; Pujic and Malicki, 2001). Here, we show that the zebrafish *glass onion* locus encodes N-cadherin and that mutations in this gene result in a dramatic disruption of retinal patterning. The *glo*^{m117} allele contains a substitution of the tryptophan 2 by glycine and thus provides *in vivo* evidence for the importance of this residue in the function of classic cadherins. The *glo*^{m117} phenotype is similar to the effects of N-cadherin function knockdown but involves stronger defects in comparison to those reported for other zebrafish N-cadherin alleles, including *pac*^{tm101b} that results in a truncation of the N-cadherin polypeptide. Unexpectedly, the retinal phenotypes of both *glo*^{m117} and *pac*^{tm101b} alleles are cell-nonautonomous. Cell-nonautonomy of the N-cadherin phenotype may explain inconsistencies in the previous studies of the N-cadherin function. Studies of the *glo*^{m117} and *pac*^{tm101b} alleles suggest that the patterning phenotype of N-cadherin in the retina is at least partially caused by a defect in neuroepithelial polarity and argue against a requirement for N-cadherin for adhesive interactions between photoreceptor cells.

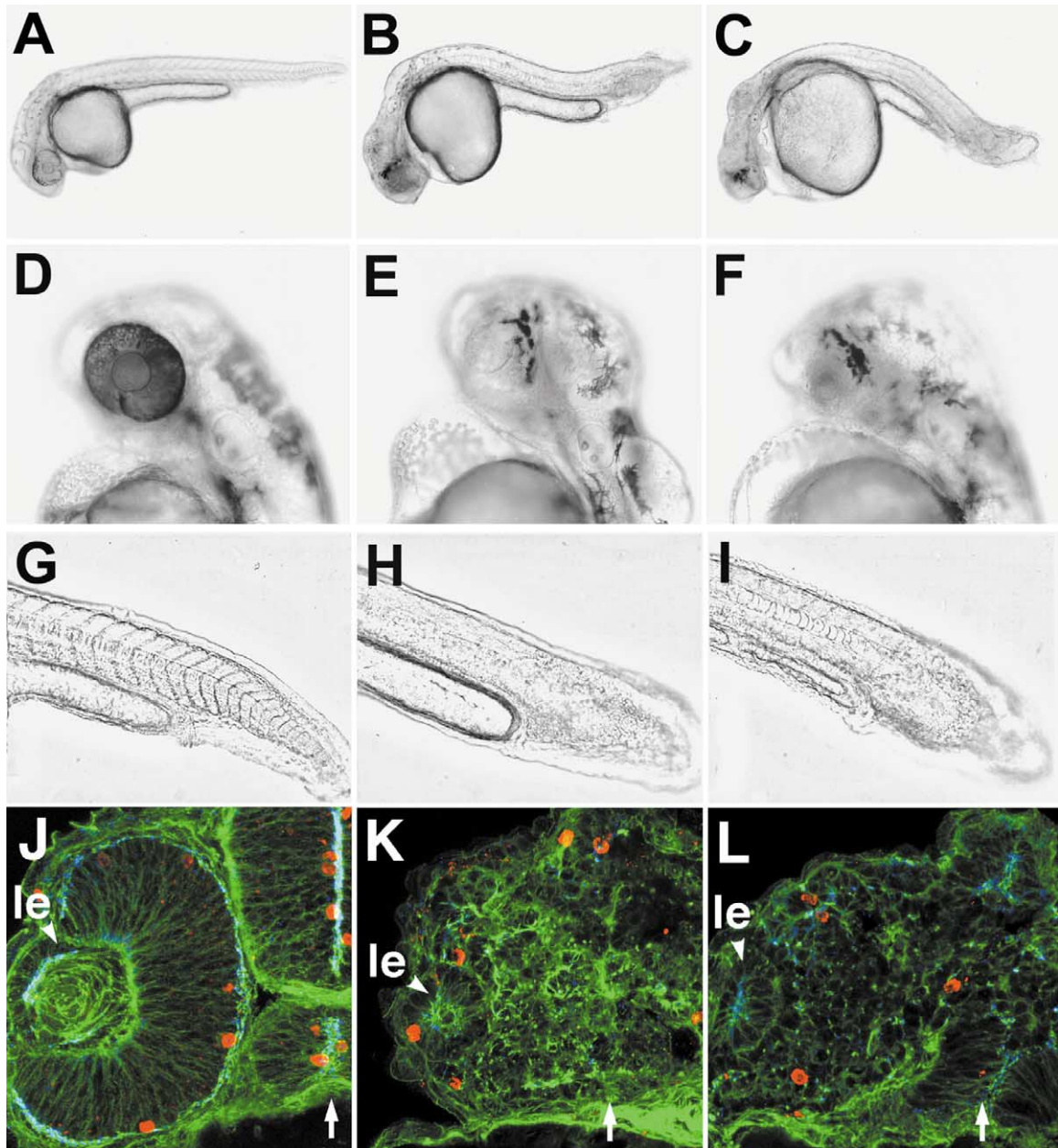


Fig. 2. Knockdown phenotype of N-cadherin. Anti-N-cadherin morpholino-modified antisense oligonucleotides reproduce the *glass onion* phenotype. Gross morphology of the wild type (A), *glo^{m117}* (B), and anti-N-cadherin morpholino-treated animals (C) at 36 hpf. Eye pigmentation of the wild type (D), *glo^{m117}* (E), and morpholino-treated animals (F) at 48 hpf. Tail and somites of the wild type (G), *glo^{m117}* (H), and morpholino-treated animals (I) at 30 hpf. Somite boundaries are poorly defined in both mutant and morpholino-treated embryos. Analysis of cryosections indicates that retinal neuroepithelium is severely disorganized at 28 hpf in morpholino-treated animals (K, L compare with the wild type in J). In (J–L), centrosomes are stained with anti- γ -tubulin antibody (blue), adherens-junction associated actin foci are visualized with Alexa-488-conjugated phalloidin (green), while M-phase nuclei are identified by anti-phosphohistone antibody staining (red). Embryos in (K) and (L) were treated with anti N-cadherin morpholinos 2 and 3, respectively. In (G–I) anterior is right and dorsal is top. “le” in (J–L) indicates lens. Arrows indicate the midline.

Materials and methods

Strains of zebrafish

The *glo^{m117}* and *pac^{m101b}* alleles were originally recovered independently in the course of large-scale chemical mutagenesis screens (Jiang et al., 1996; Malicki et al.,

1996). The *glo^{m117}* phenotype has been previously described in detail (Pujic and Malicki, 2001). Previous studies of the *pac^{m101b}* allele did not report patterning defects in the retina (Jiang et al., 1996; Lele et al., 2002). Staging of embryos was performed as described previously (Kimmel et al., 1995). Detailed phenotypic observations of whole embryos were conducted by using a Zeiss Axioscope micro-

scope, and images were recorded by using a Zeiss Axiocam digital camera (Carl Zeiss, Inc.) and processed with Photoshop software (Adobe, Inc.).

Mapping and characterization of the glo^{m117} allele

The glo^{m117} mutation was assigned to a linkage group using a panel of F₂ gynogenetic diploid embryos obtained using F₁ females from a cross between glo^{m117} AB carriers with WIK strain animals (Johnson et al., 1996; Streisinger et al., 1986; Wei and Malicki, 2002). Subsequent mapping was performed by using a panel of diploid F₂ embryos from the same cross. To localize the genetic defect responsible for the glo^{m117} phenotype, total RNA from heads of wild-type and mutant embryos was isolated at 12–24 h postfertilization (hpf) and reverse transcribed using an oligo(dT) primer. The resulting cDNA was used as a template for PCR. Amplification products were cloned and sequenced to identify the mutation site. Our sequencing data of the wild-type N-cadherin gene are consistent with those published by Lele et al. (2002).

Morpholino knockdown and phenotypic rescue

Morpholino-modified oligonucleotides (morpholinos) were reconstituted in 0.12 M KCl and injected into the yolk of 2–8 blastomere wild-type embryos by using a glass needle as described previously (Malicki et al., 2002; Westerfield, 2000). Embryos were reared at 28.5°C and processed for immunolabeling as below. The following morpholinos were used in this analysis: N-Cadherin MO1 (5'-GTTGAGTGAA GTTGATGTGT ACCAT-3'); N-Cadherin MO2 (5'-TGCAGAGCGG CGAGAAGCCC CAGCA-3'); N-Cadherin MO3 (5'-TCTGTATAAA GAA-ACCGATA GAGTT-3'); E-Cadherin (5'-ATCCACAGT TGTTACACAA GCCAT-3'); control morpholino (5'-CCTCTTACCT CAGTTACAAT TTATA-3'). All morpholinos were provided by GeneTools Inc.

To rescue the glo defect and the E-cadherin knockdown phenotype, the N-cadherin ORF was cloned into the pXT7 vector (Malicki et al., 2002; Tang et al., 1995) and transcribed by using the mMessage mMachine transcription kit (Ambion Inc.). The resulting RNA was injected into embryos at the one- to four-cell stage. In glo rescue experiments, we needed to distinguish rescued homozygous mutants from wild-type animals. This was accomplished by PCR genotyping of the Z20582 polymorphism tightly linked to the N-cadherin gene (data available in public databases).

Immunohistochemistry

Embryos (12–84 hpf) were fixed in 4% paraformaldehyde (pH 7.2) for 2 h at room temperature, washed in PBST (PBS with 0.1% Tween 20), infiltrated in 30% (w/v) sucrose in PBST overnight, and embedded in TBS tissue freezing

medium (Polysciences, Inc.) Sections of 14 μ m were thaw-mounted on Superfrost Plus slides (Fisher Scientific Inc.) and stored at room temperature for 1 h. Sections were rehydrated in PBS (5 min) and blocked for 1 h in 10% (v/v) normal goat serum, 0.5% Triton X-100 (v/v) in PBST. The primary antibody was diluted in block solution and applied for 2 h. Sections were washed in PBST (3 times, 5 min each), stained for 1 h at room temperature with the secondary antibody diluted 1:1000 in block solution, washed as described previously, mounted in 50% glycerol (v/v), 2% n-propyl gallate (w/v), 200 mM Tris-HCl (pH 8.0), and viewed by using a Leica SP2 confocal microscope.

For whole-mount immunolabeling, embryos were dechorionated and fixed in 4% paraformaldehyde (pH 7.2) overnight at 4°C and then stored in methanol at –20°C for at least 12 h. Embryos were then rehydrated in PBST and blocked for 1 h as above. Following incubation in primary antibody, embryos were washed four times, 15 min each, in PBST, incubated in secondary antibody, washed again, and embedded in TBS tissue freezing medium. Antibody staining and washes were performed as above. Cryosections of 14 μ m were prepared as above and viewed by using confocal microscopy.

The following antibodies and dilutions were used for immunostaining: Zpr-1 (formerly Fret 43) for red–green double cones (1:200, Oregon Monoclonal Bank); anti-carbonic anhydrase for Muller glia (1:100, gift from Paul Linser); anti- γ -tubulin for centrosomes (1:200, Sigma Chemical Co. Inc.); mouse anti-HuC/D (1:25, Molecular Probes Inc.); mouse anti-parvalbumin (1:500, Sigma Inc.); rabbit anti-N-Cadherin (1:500, gift from Pamela Raymond). The primary antibodies were detected by using anti-mouse or anti-rabbit secondary monoclonal antibodies conjugated to Alexa 488 (Molecular Probes Inc.), Cy3, or Cy5 (Jackson ImmunoResearch Inc.) at a dilution of 1:500.

For F-actin staining, sections were prepared as above, washed in PBS (5 min), blocked in 1% (w/v) BSA in PBS for 1 h, incubated for 1 h in Alexa 488- or rhodamine-conjugated phalloidin (1:40, Molecular Probes Inc.), washed with PBS (3 times, 10 min each), mounted, and viewed by using confocal microscopy as above.

Immunoblotting

Embryos were homogenized in Laemmli loading buffer with a cocktail of protease inhibitors (Roche Inc.) at 1 embryo/5 μ L of buffer. The homogenate was separated by using conventional SDS-PAGE (7.5% acrylamide), transferred onto PVDF membrane (Millipore, Inc.) by electroblotting, and blocked in 3% Carnation brand non-fat milk (Nestle Inc.) in PBST. Membranes were incubated overnight at 4°C in rabbit anti-N-cadherin antibody (Liu et al., 2001), washed in PBST, and then incubated for 1 h at room temperature in goat anti-rabbit-HRP antibody at 1:30,000. After washing, membranes were developed for enhanced

chemiluminescence by using ECL Plus detection reagents (Amersham Inc.).

Mosaic analysis

Blastomere transplantations were performed as described previously (Ho and Kane, 1990; Malicki, 1999; Westerfield, 2000). Donor embryos were injected at the 2- to 8-cell stage with a 2.5% mixture of biotin- and Texas Red-conjugated dextrans in a 9:1 ratio (Molecular Probes, Inc.). At the blastula stage, 10–40 cells were removed from a labeled donor with a glass pipette and transferred to an unlabeled host. Host embryos were cryosectioned at a desired stage and processed for immunofluorescence as described above, with the additional modification that dextran tracer signal was detected by using Alexa Fluor 488-conjugated streptavidin (1:500; Molecular Probes Inc.) during the secondary antibody step. Confocal analysis of mosaic clones was carried out as above. To score for patterning defects, embryos were reared until 3 dpf, cryosectioned, and stained to visualize F-actin (using fluorophore-conjugated phalloidin), donor-derived clones, and Zpr-1 as described above. Donor-derived clones were considered normal if they did not disrupt the photoreceptor cell layer. Zpr-1 staining was used to aid in the evaluation of the photoreceptor cell pattern. Clones that contained ectopic Zpr1-positive cells or were located in the photoreceptor cell layer but did not display elongated photoreceptor morphology were considered defective. Clone sizes were evaluated on single sections by counting the number of donor-derived cells within the boundaries of the photoreceptor cell layer. As no effort was made to reconstruct clones on consecutive sections, this method tended to underestimate the size of large clones. A clone was defined as a group of cells staining positive for biotin, with each cell being separated by no more than one biotin-negative cell from other cells in the clone. Biotin-positive cells separated from each other by two or more biotin-negative cells were considered to belong to separate clones. Efforts were made to evaluate each clone only once. Data were plotted as a scattergram with mean \pm s.d. A Whitney–Mann *U* test was performed for both *pac* and *glo* transplant experiments to determine statistical significance of clone size differences.

Results

The glass onion^{m117} allele contains a mutation in a critical amino acid in N-cadherin

We have previously shown that mutation of the *glass onion* (*glo^{m117}*) locus leads to a loss of neuroepithelial integrity in many regions of the central nervous system followed by severe neuronal patterning defects in the retina and brain (Pujic and Malicki, 2001). In addition to neuronal patterning defects, the *glo* phenotype is characterized by a

tail blister, the loss of posterior somites, and defects in the heart and circulation, indicating that the *glo* gene plays a role in various biological contexts. To localize *glo* in the zebrafish genome, we employed half-tetrad analysis (Johnson et al., 1995; Streisinger et al., 1986) and mapped the *glass onion* locus to linkage group 20. Subsequent analysis of an F₂ mapping panel comprising 400 meioses localized *glo* distal to Z21067 and proximal to Z8976. By comparing our mapping results with data available in public databases, we noticed that *cdh2*, zebrafish N-cadherin, is located within this region (Fig. 1A). A previous study reported that zebrafish N-cadherin was expressed in the somites, neural keel, brain, and developing eye primordia (Bitzur and Geiger, 1994). This expression pattern corresponds to phenotypic defects in the *glo* mutant. In addition, in chicken embryos, incubation with antibodies that perturb N-cadherin-mediated cell adhesion leads to defects in retinogenesis and somitogenesis (Linask et al., 1998; Matsunaga et al., 1988), and N-cadherin knockout in the mouse results in defects in heart and somites (Radice et al., 1997).

The above observations led us to believe that the *glo* locus might encode zebrafish N-cadherin. To investigate this possibility, we compared cDNA sequences of N-cadherin from wild-type and *glo* embryos. Sequence analysis revealed that, in the *glo* mutant strain, the N-cadherin gene carries a T-to-G transversion that leads to a Trp-to-Gly substitution in the second amino acid in the ectodomain 1 (EC1) of the mature polypeptide. Several lines of evidence suggest that Trp2 plays a crucial role in adhesive properties of cadherins. First, this residue is well conserved in vertebrate classic cadherins, such as N-, E-, R-, and P-cadherins (Fig. 1B). Second, electron microscopic studies of cadherin oligomers indicate that Trp2 is necessary for adhesive contacts between oligomers (Pertz et al., 1999). Third, the substitution of Trp2 with alanine completely blocks calcium-dependent aggregation of cadherin-transfected cells (Tamura et al., 1998). Finally, a recent crystallographic study provided evidence that Trp2 interacts with a hydrophobic pocket in another cadherin molecule, thus providing a basis for homophilic adhesion (Boggon et al., 2002). These observations strongly suggest that the Trp2 substitution in *glo^{m117}* is responsible for the mutant phenotype of this allele.

To confirm that *glo* indeed encodes N-cadherin, we first carried out knockdown experiments (Nasevicius and Ekker, 2000) by injecting a morpholino oligonucleotide targeted to the 5' region of the published sequence (Bitzur and Geiger, 1994). Contrary to our expectations, the phenotypes of the morpholino-injected embryos were not consistent with those of *glo* animals, and expression of N-cadherin mRNA failed to rescue the *glo* phenotype (data not shown). Upon closer inspection of the cDNA sequence, we realized that, although the published sequence contained the intact mature protein, it lacked the pro- domain including the signal peptide sequence, which is essential for membrane targeting and is present in N-cadherins of all other species. This

finding prompted us to look for a full-length cDNA. Sequence analysis of an EST clone revealed that the zebrafish N-cadherin contained a pro-domain of 146 amino acids with the first amino acids providing a signal peptide. Injection of morpholino oligonucleotides targeted to the 5' UTR of the full-length cDNA led to the characteristic *glo* phenotypes, including patchy eye pigmentation, tail blisters, and somite defects. These experiments were performed by using two morpholinos in two different genetic backgrounds (Fig. 2, and Table 1). To confirm that morpholino antisense oligonucleotides fully phenocopy the *glo^{m117}* phenotype, we sectioned retinæ of morpholino-treated animals. These studies indicated that the knockdown of the N-cadherin function leads to a severe disorganization of retinal neuroepithelium

prior to the onset of neurogenesis (Fig. 2K and L). This phenotype is very similar to that previously described in *glo^{m117}* mutants (Pujic and Malicki, 2001). The N-cadherin knockdown also produces a defect in the brain (Fig. 2K and L). Similar to the results of previous analysis, this defect is less severe in the ventral-most and the dorsal-most regions of the neural tube (Fig. 2L).

To further ascertain that an N-cadherin mutation was responsible for the *glo* phenotype, we rescued the *glo* defect by injecting N-cadherin mRNA. To ensure that rescued embryos are genotypically homozygous mutant, we genotyped them using the Z20582 SSLP polymorphism located in the vicinity of the N-cadherin gene (Fig. 1). N-cadherin mRNA expression restored eye pigmentation and largely

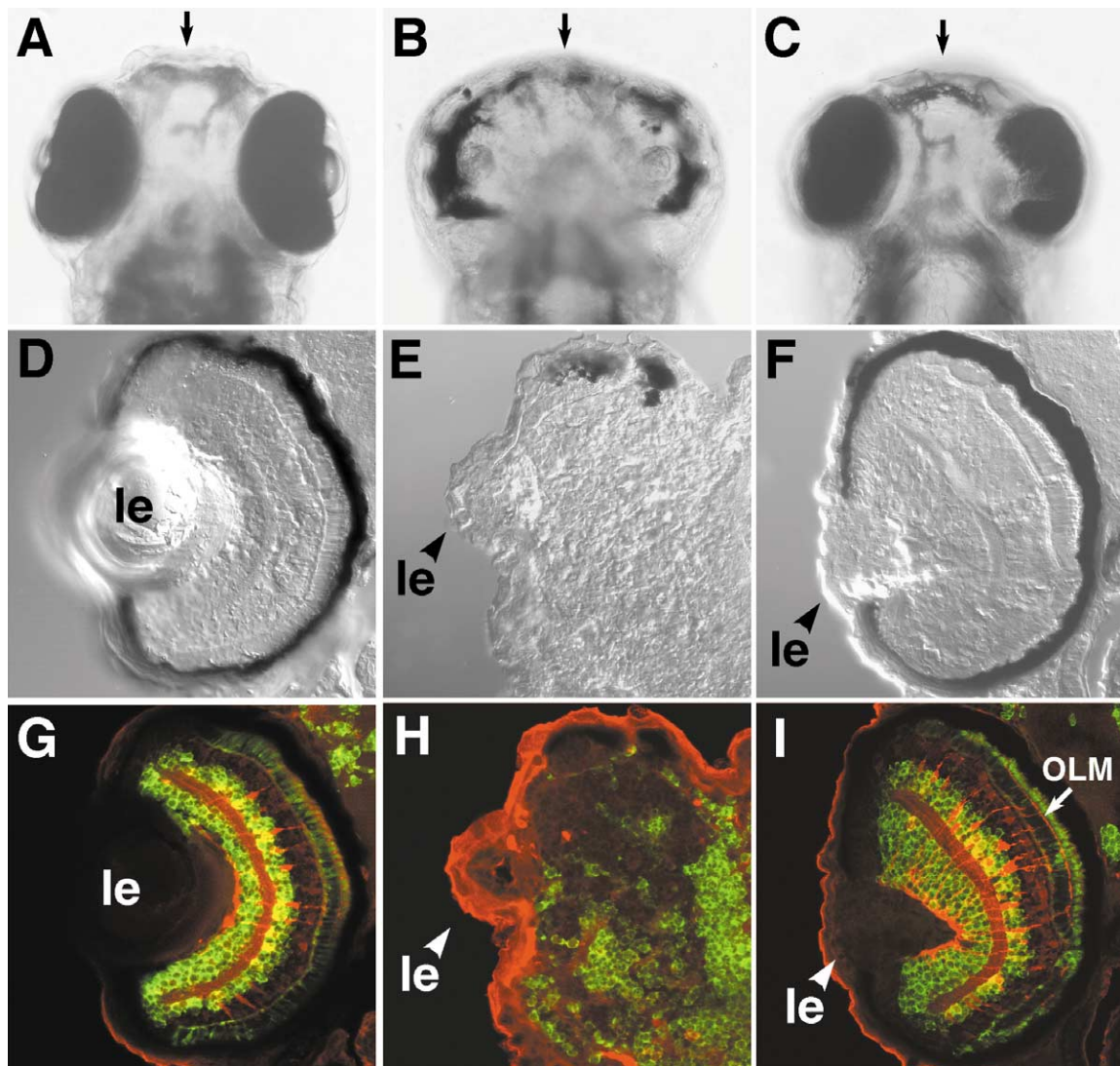


Fig. 3. Rescue of the *glo^{m117}* phenotype by N-cadherin mRNA expression. Eye pigmentation and retinal architecture of the wild type (A, D, G), *glo^{m117}* (B, E, H), and rescued *glo^{m117}* embryos (C, F, I) at 4 dpf. (A–C) Ventral views of whole embryos. (D–F) Images of transverse sections through retinæ. In (G–I), transverse sections of the retina were costained with anti-Hu (green, stains ganglion and amacrine cells), Zpr-1 (green, stains cone photoreceptor cells), and anti-carbonic anhydrase (red, stains Muller glia) antibodies. Note that the architecture of Muller glia and the outer limiting membrane (OLM, arrow) are restored in rescued animals. For rescued embryos, mutant genotype was confirmed by using a polymorphic marker linked to the N-cadherin locus. In (D–J), “le” indicates lens, dorsal is up. Arrows in (A–C) indicate the midline.

Table 1
Knockdown phenocopy of the *glo* phenotype

Morpholino/Strain	Concentr. (mg/mL)	Tail blister at 30 hpf	Somite defects at 30 hpf	Patchy eye pigmentation ^a
MO#2/AB	4.5	85/95 (89%)	83/95 (87%)	79/95 (83%)
MO#2/WIK	4.5	81/92 (88%)	77/92 (84%)	76/92 (83%)
MO#2/AB	5	40/46 (87%)	33/46 (72%)	30/46 (65%)
MO#3/AB	5	30/35 (86%)	29/35 (83%)	28/35 (80%)
MO#2 + #3/AB	5+5	19/19 (100%)	19/19 (100%)	16/19 (84%)
Control MO/AB	5	3/75 (4%)	4/75 (5%)	4/75 (5%)

Note. The effect of anti-N-cadherin morpholino injection (see Materials and methods) was evaluated by scoring three phenotypes: tail shape, somite defects, and eye pigmentation.

^a Assayed at 48 hpf.

rescued the neuronal patterning defect in the eye of *glo*^{m117} mutant animals (Fig. 3, Table 2). Taken together, these experiments provide strong evidence that a defect in the N-cadherin gene is responsible for the *glo* mutant phenotype.

Retinal phenotype of glass onion mutant animals

pac^{m101b} is a previously described mutant allele of the N-cadherin gene that produces hindbrain and tail defects

(Lele et al., 2002). Compared with the *glo*^{m117/m117} and *pac*^{m101b/m101b} homozygotes, *glo*^{m117}/*pac*^{m101b} transheterozygotes display an intermediate phenotype. Are retinal defects present in *pac*^{m101b/m101b} mutants? To answer this question, we stained *pac*^{m101b/m101b} mutant retinæ with an antibody to γ -tubulin that recognizes centrosomes of neuroepithelial cells. In wild-type retinæ, all centrosomes localize to the apical surface of the neuroepithelium at 26 hpf (arrows in Fig. 4A). In contrast, centrosomes localize to ectopic positions in the ventral retina of *pac*^{m101b/m101b}

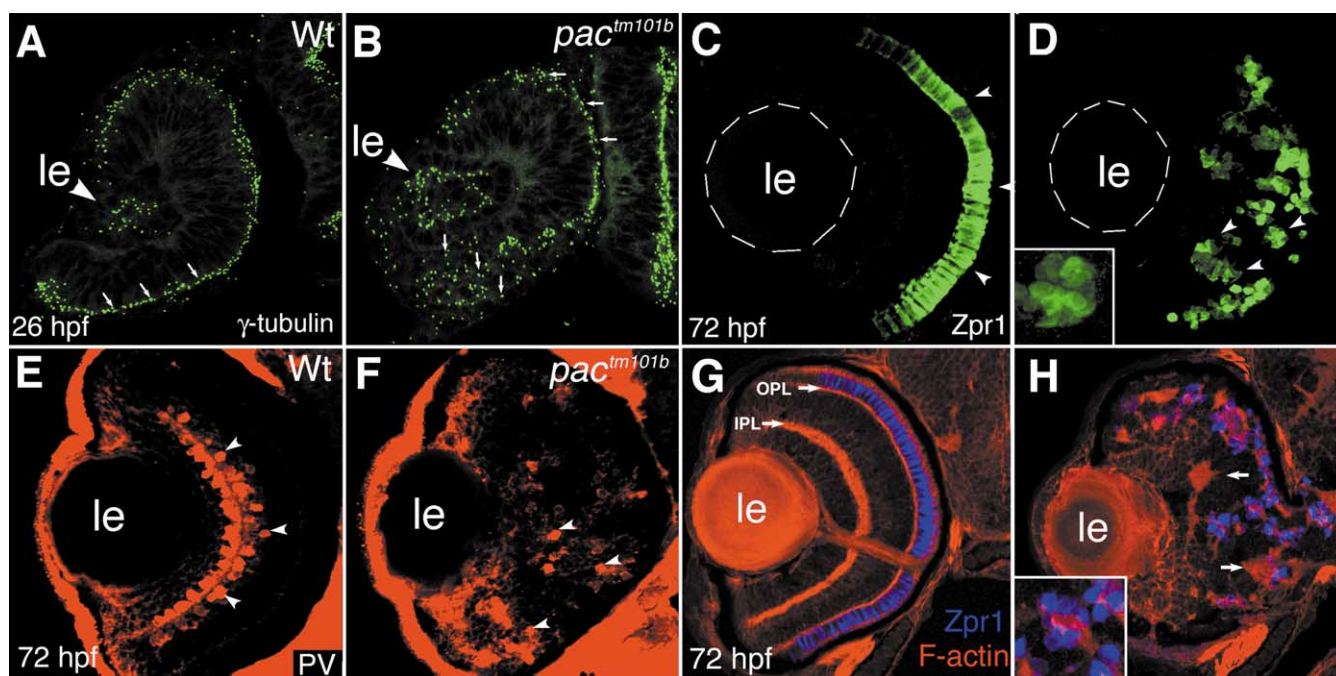


Fig. 4. Phenotype of the *pac*^{m101b} mutant allele. *pac*^{m101b} produces defects in the retinal neuroepithelium and in the organization of retinal neurons. In wild-type retinal neuroepithelium, all centrosomes are positioned apically at 26 hpf (A). By contrast, the centrosomes of *pac*^{m101b} mutant retina are scattered along the apicobasal axis of the neuroepithelium in the ventral portion of the eye cup (vertical arrows in B), while the dorsal retina appears unaffected (horizontal arrows in B). At 3 dpf, photoreceptor cells of *pac*^{m101b} mutant animals are scattered throughout most of the retina (arrowheads in D), while their wild-type counterparts form a uniform layer (arrowheads in C). Ectopic photoreceptors adhere to each other forming clusters, rosettes (inset in D). Parvalbumin-positive amacrine cells localize to ectopic positions in *pac*^{m101b} retina (arrowheads in F). In wild-type zebrafish larvae, these cells form a layer in the center of the retina (arrowheads in E). Neuronal processes of the zebrafish retina form two uniform layers that strongly stain with phalloidin: outer plexiform layer (OPL) and inner plexiform layer (IPL) (arrows in G). In the retinæ of *pac*^{m101b/m101b} mutants, neuronal processes form disorganized patches instead of layers (arrows in H). The lens, “le”, is outlined with dashes in (C) and (D). All panels show transverse cryosections through the retina stained with the following reagents: anti- γ -tubulin antibody (A, B), Zpr-1 antibody (C, D, G, H), anti-parvalbumin antibody (E, F), and phalloidin (G, H). In all panels, dorsal is up.

Table 2
Rescue of eye pigmentation in *glo* mutants by N-cadherin mRNA

mRNA injected	Mutant embryos	Partially rescued embryos ^a	Fully rescued embryos ^b
GFP	13	0	0
N-cadherin (wt) ^c	64	27	12
N-cadherin (mt)	14	0	0

Note. Embryos were treated with wild-type N-cadherin (wt), or *glo* mutant N-cadherin (mt). The genotype of rescued embryos was determined by PCR.

^a 50–75% restoration of eye pigmentation in one or both eyes at 3 dpf.

^b 75%–complete restoration of eye pigmentation in one or both eyes at 3 dpf.

^c Numbers represent a total of four independent experiments.

mutants (Fig. 4B, arrows). Unlike in *glo*^{m117/m117} mutant animals, however, the dorsal neuroepithelium of the *pac*^{m101b/m101b} retinae is normal at this stage. Following this experiment, we decided to determine whether *pac*^{m101b/m101b} mutant retinae are affected at later stages of development. To address this question, we stained wild-type and mutant retinae with antibodies to specific cell types. Staining with the Zpr-1 antibody (Larison and Bremiller, 1990) indicated that double cones are mispositioned in *pac*^{m101b/m101b} mutant retinae (Fig. 4D, compare with C). The ectopic mutant photoreceptors form clusters, also called rosettes, away from the retinal pigment epithelium (Fig. 4D, inset). Similarly, parvalbumin-positive amacrine cells also localize to abnormal positions (arrowheads in Fig. 4F, compare with E). To visualize the architecture of retinal plexiform layers, we stained sections with fluorophore-conjugated phalloidin. In wild-type retina, phalloidin strongly stains both inner and outer plexiform layers (Fig. 4G), whereas the plexiform areas of the *pac*^{m101b/m101b} mutant retinae are grossly disorganized (Fig. 4H). Some ectopic plexiform patches, presumably remnants of outer plexiform layer, are found in the vicinity of ectopic photoreceptor cells (Fig. 4H, inset). The *pac*^{m101b/m101b} eye phenotype is weaker than the one observed in *glo*^{m117/m117} mutant animals in that it does not affect the lens and produces defects only in the ventral portion of the retinal neuroepithelium and the RPE.

Expression pattern of mutant N-cadherin polypeptides

Zebrafish N-cadherin is expressed in the retinal neuroepithelium and at later stages of development in plexiform layers and axonal projections of ganglion cells (Bitzur and Geiger, 1994; Liu et al., 2001). Since the *glo*^{m117} mutant allele leads to a severe disruption of retinal architecture, we asked whether the *glo*^{m117} mutant polypeptide is characterized by the same tissue distribution as the wild-type polypeptide. Antibody staining revealed that N-cadherin subcellular distribution is similar, if not identical, in wild-type and *glo*^{m117/m117} mutant animals at 24 hpf (Fig. 5A and B). At later stages of development, similar to wild-type

staining, the *glo*^{m117} mutant polypeptide localizes to ganglion cell projections (arrowhead in Fig. 5E, compare with D) and forms staining foci that presumably correspond to ectopically located plexiform patches (Fig. 5E, arrow). Consistent with this result, Western blotting of embryonic extract indicated that the N-cadherin mutant polypeptide is at least partially intact in *glo*^{m117/m117} homozygotes, although a presumptive degradation product is also observed (Fig. 5J). Similar studies performed on the *pac*^{m101b} allele revealed that the mutant polypeptide is undetectable in mutant retinal neuroepithelium (Fig. 5C), and at later stages of development, it does not localize to ganglion cell axons or ectopic plexiform patches (Fig. 5F). On a Western blot, *pac*^{m101b} mutant polypeptide, or its truncated mutant form, are not detectable (Fig. 5I).

Cell-nonautonomous behavior of the glass onion phenotype

Our previous studies indicated that the *glass onion* phenotype is cell-nonautonomous in the eye and brain neuroepithelia (Pujic and Malicki, 2001). To extend these studies, we asked whether cell-nonautonomy of the *glo* phenotype persists until later stages of development, when retinal lamination is fully formed. Cells were transplanted from donor to host embryos at the blastula stage and the integrity of the photoreceptor cell layer was evaluated by using antibody staining on frozen sections at 3 dpf. In control retinae, wild-type donor-derived photoreceptors in wild-type host retinae always localize to the correct position and the overall architecture of the photoreceptor cell layer is well preserved ($n = 9$). When mutant clones of cells develop in the context of wild-type retinae, the position of donor-derived mutant photoreceptors depends on clone size. In small clones (≤ 8 cells in the photoreceptor cell layer), photoreceptors consistently localize to their correct positions in the photoreceptor cell layer ($n = 19$, Fig. 6A and F). By contrast, larger mutant clones (≥ 10 cells in the photoreceptor cell layer) frequently induce abnormalities in the photoreceptor cell layer architecture (11 out of 13 clones produced abnormalities; Fig. 6B and F). Thus, the phenotype of mutant clones in a wild-type environment strongly depends on clone size ($P < 0.0001$). These results indicate that the *glo* phenotype is cell-nonautonomous and suggest that its cell-nonautonomy is mediated by short-range cell–cell interactions.

This conclusion is somewhat surprising because N-cadherin is a homophilic adhesion molecule and thus it is expected to produce cell-autonomous loss of cell adhesion. This, in fact, has been observed in tissue culture studies (Tamura et al., 1998). Although the mutations of Trp2 in the N-cadherin polypeptide are known to lead to the loss of adhesion in vitro, it is possible that the mutant *glo*^{m117} polypeptide retains some ability to interact with wild-type N-cadherin molecules on neighboring cells, which could account for the cell-nonautonomy of its phenotype. To

exclude this possibility, we performed mosaic analysis using the *pac*^{m101b} allele. This allele contains a premature termination codon in the fourth ectodomain of the N-cadherin polypeptide and is presumably a functional null because it produces a protein product lacking the transmembrane domain (Lele et al., 2002). Consistent with its lack of function, the *pac*^{m101b} protein product is not detectable on Western blots (Fig. 5I). In *pac*^{m101b} mutant animals, the majority of photoreceptor cells localize to ectopic positions (Fig. 4D). To investigate whether this phenotype is cell-autonomous, we generated mosaic animals. These experiments revealed that *pac*^{m101b} mutant photoreceptor cells almost always localize to normal positions in the environment of the wild-type retina and do not produce defects in photoreceptor cell layer architecture ($n = 22$; Fig. 6D and F). Rare exceptions to this rule are seen in particularly large cell clones ($n = 2$, Fig. 6E and F). Thus, similar to *glo*^{m117}, the *pac*^{m101b} phenotype is cell-nonautonomous in the retina.

Functional equivalence of N- and E-cadherin

N-cadherin is expressed in the zebrafish embryo starting at the shield stage as indicated by Northern and Western blotting (Bitzur and Geiger, 1994). It is therefore surprising that in *glo*^{m117/m117} mutant animals, phenotypic defects are not observed until 12 hpf and in *pac*^{m101b/m101b} homozygotes until even later. One possible explanation for this is functional redundancy with other cadherins. Several other cadherins are expressed in the zebrafish embryo and may account for the late onset of the *glass onion* phenotype. E-cadherin, for example, is expressed in the early embryo and its transcript is downregulated in the midline between 9 and 10.5 hpf (Babb et al., 2001). Since the downregulation of E-cadherin transcript coincides with the appearance of the *glo* phenotype, we decided to investigate whether E- and N-cadherins can functionally substitute for each other. To test this, we performed a morpholino knockdown of E-cadherin function, which we then attempted to rescue using the N-cadherin transcript. As expected from studies in other organisms (Larue et al., 1994), knockdown of the E-cadherin function in zebrafish leads to early embryonic lethality. Following the injection of anti-E-cadherin morpholinos at the concentration of 3 mg/ml into zebrafish embryos, 100% of embryos die between 50 and 90% of epiboly (0/42 embryos survived). Given this severe lethality, we lowered the concentration of anti-E-cadherin morpholino to 0.1 mg/ml. Although this lower concentration decreases lethality at 50% epiboly, all embryos injected die by 24 hpf. Coinjection of N-cadherin transcript together with anti E-cadherin morpholino significantly increases survival so that 50% of embryos survive to 24 hpf (Table 3). This result indicates that N-cadherin expression can partially substitute for the loss of E-cadherin function and supports our hypothesis that different classic cadherin genes are at least partially functionally interchangeable. It is thus likely that the late

onset of the *glo* phenotype may be caused by the presence of other cadherins at earlier stages of development.

Discussion

In addition to *glo*^{m117}, N-cadherin alleles were isolated independently by other groups. The zebrafish *parachute* (*pac*) locus, originally identified independently in a large-scale mutagenesis screen, has been recently reported to encode the N-cadherin gene (Jiang et al., 1996; Lele et al., 2002). Surprisingly, several defects present in *glo*^{m117} mutant animals (Pujic and Malicki, 2001) are either weaker, are absent, or have not been described in *pac* mutants. These include defects in the retina, RPE, lens, somites, and heart. This is rather surprising because the *pac*^{m101b} and *pac*^{tr7} alleles cause truncations of the cadherin polypeptide upstream of the transmembrane domain and thus are expected to produce a null phenotype (Lele et al., 2002).

The differences between *glo* and *pac* can be explained in several ways that are not mutually exclusive. First, the *pac* and *glo* alleles have been maintained in different genetic backgrounds, which may account for some of their phenotypic differences. Second, the *pac* alleles may produce a small amount of functional protein product due to alternative splicing. Finally, the *glo*^{m117} allele may produce a weak dominant phenotype by interference with other cadherin molecules. Indeed, a partially penetrant late onset dominant phenotype has been observed in some *glo*^{m117} adult heterozygotes (Pujic and Malicki, 2001; and J.M., unpublished results). Apart from the above, two lines of evidence suggest that the *glo*^{m117/m117} homozygotes display a null phenotype: first *glo*^{m117} somite, heart, and lens defects are reminiscent of phenotypes reported following mouse N-cadherin gene knockout analysis and in vitro studies of N-cadherin function (Ferreira-Cornwell et al., 2000; Frenzel and Johnson, 1996; Luo et al., 2001; Radice et al., 1997); and second, morpholino knockdown of N-cadherin quite closely reproduces *glo*^{m117} phenotype, including somite, and RPE defects.

The *glo*^{m117} allele involves a substitution of tryptophan 2 in the first ectodomain of N-cadherin. This residue is well conserved in evolution, and its importance for adhesive properties of classic cadherins has been documented in at least two types of experiments. First, electron microscopic studies of oligomerized E-cadherin revealed that Trp2 is necessary for interactions between oligomers (Ahrens et al., 2002; Pertz et al., 1999). Second, a parallel line of studies indicated that a substitution of Trp2 with alanine abolishes the ability of N-cadherin-transfected cells to undergo aggregation (Tamura et al., 1998). Although the importance of Trp2 for homophilic adhesion has been evident for quite some time, the exact role of Trp2 in N-cadherin structure was a subject of controversy. Competing models proposed that the Trp2 moiety fits in a hydrophobic pocket on another cadherin molecule on the surface of the same cell (Shapiro

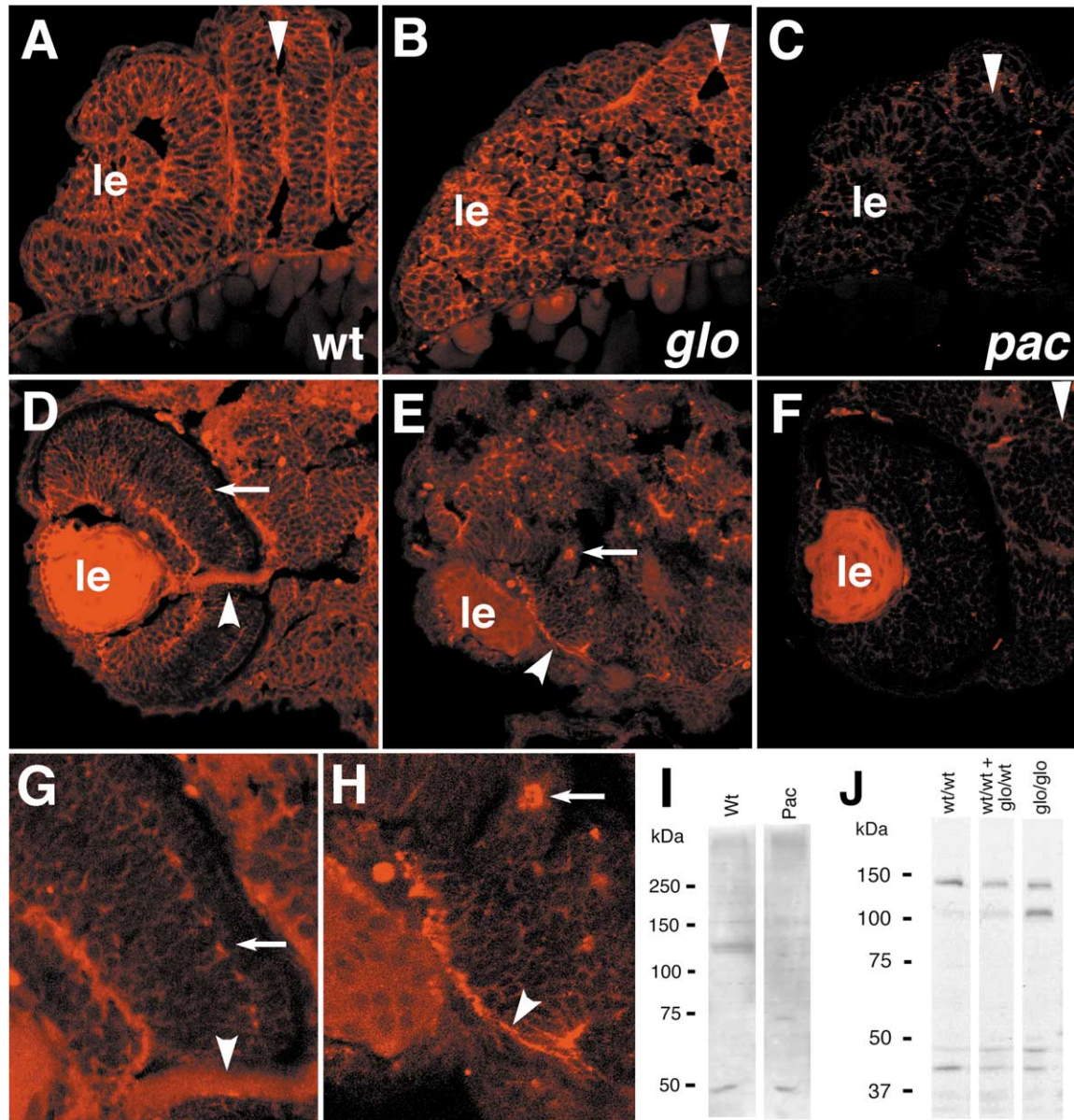


Fig. 5. Protein product localization in mutant retinas. At 24 hpf, N-cadherin polypeptide is present in wild-type (A), *glo^{m117}* (B) but not in *pac^{m101b}* (C) retinas. This situation persists at later stages of development as shown for the wild-type (D), *glo^{m117}* mutants (E), and *pac^{m101b}* mutants (F). Residual staining present in (C) and (F) is most likely due to background fluorescence. As reported previously in wild-type animals, N-cadherin is expressed in plexiform layers (arrows in D and G) and in the optic nerve (arrowheads D in G). The *glo^{m117}* mutant polypeptide appears to retain this distribution. It is present both in the optic nerve (arrowheads in E and H) and in ectopically localized plexiform patches (arrows in E and H). Western blotting indicates that N-cadherin polypeptide is absent in *pac^{m101b/m101b}* mutant embryos (I) but it persists in *glo^{m117/m117}* animals (J) at 3 dpf. (D, E, G, H) Retinae at 50 hpf. Retina in (F) is at 72 hpf. "le" indicates lens. Arrowheads in (A–C) indicate the midline. In (A–H) dorsal is up.

Fig. 6. Mosaic analysis of the *glass onion* phenotype at 3 dpf. The phenotype of *glo^{m117/m117}* mutant photoreceptor cell clones in the environment of the wild-type retina depends on clone size. In small clones, mutant double cones (yellow) display proper morphology and localize to the photoreceptor cell layer (A). In larger clones, the photoreceptor cell layer is frequently disrupted and ectopic photoreceptors are sometimes observed (B). This is never seen in control experiments when wild-type clones develop in the environment of wild-type retina regardless of clone size (C). When *pac^{m101b}* mutant clones develop in the environment of the wild-type retina, the photoreceptor cell layer remains normal (D). Rare exceptions to this are seen in the case of particularly large clones (E). The correlation of photoreceptor cell layer abnormalities with clone size is quantitated for mutant to wild-type transplants in (F). Each dot in (F) represents a donor-derived clone that was physically separate from other donor-derived clones. Clone size is plotted on the vertical axis. Larger clones have a clear tendency to cause patterning defects in the retina. Data for *glo^{m117}* and *pac^{m101b}* alleles are plotted in separate columns. Donor-derived cell clones are in green, while double cones are stained with the Zpr-1 antibody and are depicted in red. Donor-derived double cones appear yellow. Dashes in (E) outline a large mutant clone in otherwise wild-type retina.

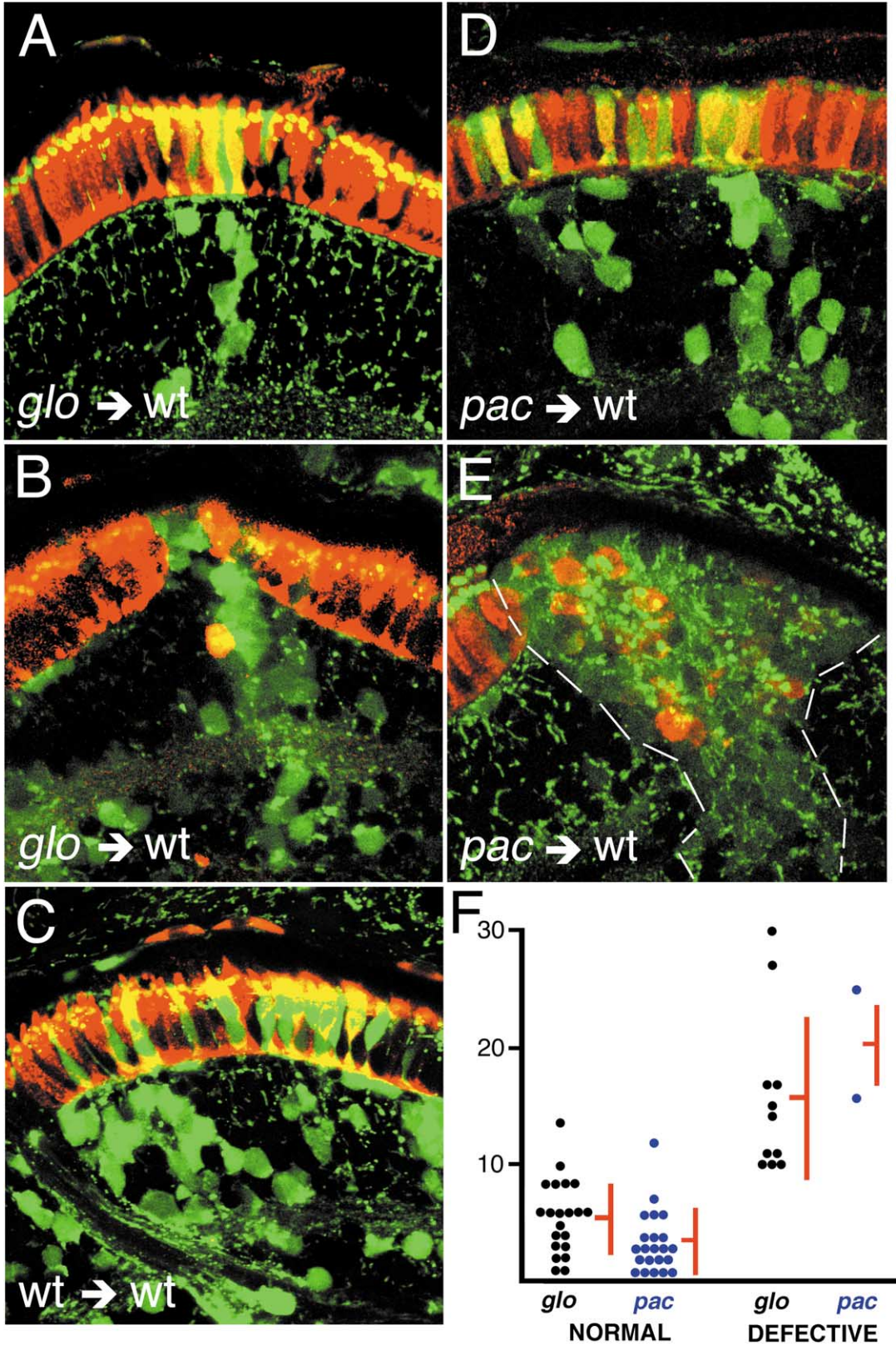


Table 3
Rescue of morpholino-induced E-cadherin phenotype

Method of treatment	1k	50%	24 hpf
Control MO	30	27 (90%)	27
Anti-E-cadherin MO	211	174 (82%)	0
Anti-E-cadherin MO + GFP	60	54 (90%)	0
Anti-E-cadherin MO + N-cad (wt) ^a	488	404 (83%)	229 (47%)
Anti-E-cadherin MO + N-cad (mt)	174	119 (68%)	0

Note. Following injections, healthy embryos were counted at the 1000-cell stage (1k), and their survival was evaluated at 50% epiboly and at 24 hpf. Numbers of surviving embryos are provided.

^a Data from six independent experiments.

et al., 1995) or, on the contrary, that it fits in a hydrophobic pocket on the surface of its own polypeptide (Pertz et al., 1999). The most recent, and presumably the most complete so far, crystallographic study of the entire N-cadherin extracellular fragment provided evidence for a third model, that Trp2 fits in a hydrophobic pocket of a cadherin molecule that projects from the surface of an opposing cell. In this model, Trp2 is directly involved in interactions that presumably form the basis for cadherin-mediated adhesion. Given the attention that Trp2 has received in numerous *in vitro* studies, it is fortuitous that the *glo*^{m117} allele contains a substitution of this amino acid. Our studies of this allele provide the first evidence that Trp2 is indeed of key importance for the function of classic cadherins *in vivo*.

Given that cadherins are homophilic adhesion molecules, their loss-of-function phenotypes should be cell-autonomous. What then is the reason that the *glo* phenotype is cell-nonautonomous? Several scenarios could explain this observation. One possibility is that an interaction of the mutant *glo*^{m117} polypeptide with a wild-type polypeptide produces a sufficient level of adhesion. Although this possibility is compatible with structural studies of N-cadherin, it appears inconsistent with our analysis of the *pac*^{tm101b} allele, which most likely does not produce a stable protein product. Also, cell aggregation studies argue against this possibility. Cells transfected with N-cadherin that contains a substitution of Trp2 by alanine do not form aggregates with wild-type N-cadherin-expressing cells (Tamura et al., 1998). Thus, in contrast to the *glo*^{m117} defect, the *in vitro* phenotype of Trp2 mutant cells is cell-autonomous. The cell-nonautonomy of the *glo*^{m117} phenotype could also be explained by interactions with other adhesion molecules (Karecla et al., 1996), the FGF signaling pathway (Williams et al., 2001), or a community effect. In the latter scenario, the absence of N-cadherin-mediated cell–cell adhesion could be hypothetically rescued in a small clone of cells by another, perhaps weaker, type of interaction. Cell-nonautonomous behavior of the N-cadherin phenotype may also explain discrepancies in the previous studies of N-cadherin function. While Matsunaga et al. (1988) proposed that N-

cadherin plays a role in the patterning of retinal neurons, the studies of Riehl et al. (1996) did not detect any functional involvement of N-cadherin in retinal lamination. This inconsistency may have resulted from the cell-nonautonomous behavior of retrovirus-induced phenotype in the Riehl et al. studies. At a later stage of development, however, Riehl et al. demonstrated that N-cadherin may function in axonal outgrowth cell-autonomously.

The *glo* early embryonic phenotypes do not correlate very well with the N-cadherin expression pattern. Although N-cadherin expression starts at the shield stage, the *glo*^{m117} phenotype is not evident until much later in development. Similarly, although N-cadherin is fairly uniformly distributed throughout the neural tube (Bitzur and Geiger, 1994; Lele et al., 2002), the severity of the *glo*^{m117} phenotype markedly varies in different regions of this tissue. We have previously pointed out, for example, that the spinal cord and both the dorsal and ventral regions of the brain are relatively unaffected in *glo* mutant animals (Pujic and Malicki, 2001). Functional redundancy with other cadherins is one likely explanation for the lack of the *glo* phenotype in some tissues that express N-cadherin. Ventral Neural Cadherin, for example, is expressed in the ventral neural tube (Franklin and Sargent, 1996) and may account for the absence of the *glo*^{m117} phenotype in ventral brain regions. Other, so far uncharacterized, cadherins may be responsible for the lack of the *glo* phenotype in other tissues. The idea that cadherins can functionally substitute each other is supported by several experiments. Expression of N-cadherin restores aggregation of E-cadherin deficient cells (Larue et al., 1996), and the expression of E-cadherin from a transgene rescues a cardiovascular phenotype in N-cadherin-deficient mice (Luo et al., 2001). Our experiments in the zebrafish embryo corroborate the conclusions of these studies and provide additional evidence that the functions of classic cadherins are at least partially interchangeable.

glass onion is one of five zebrafish loci known to play essential roles in the patterning of the retina (Horne-Badovinac et al., 2001; Jensen et al., 2001; Malicki and Driever, 1999; Malicki et al., 1996; Peterson et al., 2001; Pujic and Malicki, 2001; Wei and Malicki, 2002). In addition to neuronal patterning defects, mutations in all five of these loci cause a loss of polarity in the retinal neuroepithelium. Two of them were previously characterized on the molecular level. *nagie oko* encodes a MAGUK-family factor that is associated with cell junctions both in the retinal neuroepithelium and in the photoreceptor cell layer (Wei and Malicki, 2002), while *heart and soul (has)* encodes an atypical protein kinase C (Horne-Badovinac et al., 2001; Peterson et al., 2001). This work provides molecular characterization of the third locus from this group, *glass onion*. Although the *glass onion* phenotype in the retina is similar to the ones produced by other zebrafish retinal patterning mutants, one difference is of note. Unlike *oko meduzy*, *nagie oko*, or *mosaic eyes*, the *glo* mutant photoreceptor cells retain the ability to adhere to each other and form rosettes.

This observation indicates that the N-cadherin function is not necessary for adhesive interaction between photoreceptors. Finally, it is noteworthy that homologues of *glo*, *nok*, and *has* in *Drosophila*—*shotgun*, *stardust*, and *DaPKC*—play a role in the polarity of fruit fly embryonic epithelia (Bachmann et al., 2001; Hong et al., 2001; Tepass et al., 1996; Uemura et al., 1996; Wodarz et al., 2000). The conservation of both structure and function in this group of genes indicates that embryonic insect epithelia and the neuroepithelium of the vertebrate eye share similar developmental mechanisms.

Note added in proof. Following discussions with the authors of papers on the zebrafish *parachute* and *labyrinth* N-cadherin alleles, the zebrafish N-cadherin locus will be referred to as *ncad* in all future publications.

Acknowledgments

We thank the Tuebingen Stock Center for the *pac^{tm101b}* allele, Dr. P. Linser for anti-carbonic anhydrase antibody, and Dr. P. Raymond for anti-N-cadherin antibody. This manuscript has greatly benefited from comments of Drs. William Harris, Katherine Strissel, and Jacek Topczewski. Funding was provided by grants from Research to Prevent Blindness (to J.M.), March of Dimes Birth Defects Foundation (to J.M.), the National Eye Institute RO1 EY11882 (to J.M.), Knights Templar Eye Foundation (to Z.P.), and NEI postdoctoral training program (to H.J.).

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