

Slit1b-Robo3 Signaling and N-Cadherin Regulate Apical Process Retraction in Developing Retinal Ganglion Cells

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When neurons exit the cell cycle after their terminal mitosis, they detach from the apical surface of the neuroepithelium. Despite the fact that this detachment is crucial for further neurogenesis and neuronal migration, the underlying mechanisms are still not understood. Here, taking advantage of the genetics and imaging possibilities of the zebrafish retina as a model system, we show by knockdown experiments that the guidance molecule Slit1b and its receptor Robo3 are required for apical retraction of retinal ganglion cells (RGCs). In contrast, N-cadherin seems to be responsible for maintenance of apical attachment, as expression of dominant-negative N-cadherin causes RGCs to lose apical attachments prematurely and rescues retraction in *slit1b* morphants. These results suggest that Slit-Robo signaling downregulates N-cadherin activity to allow apical retraction in newly generated RGCs.

Introduction

Upon neurogenesis in the vertebrate CNS, progenitors undergo divisions at the apical surface of the neuroepithelium, which in the final round of division gives rise to postmitotic neurons (Götz and Huttner, 2005). These newly generated neurons often first adopt a neuroepithelial morphology but then quickly detach their process from the apical surface and migrate toward the basal side of the neuroepithelium (Nadarajah et al., 2001; Miyata et al., 2004). Recent studies in the mouse cortex disagree on whether integrin function (Loulie et al., 2009) or adherens junctions (Cappello et al., 2006; Imai et al., 2006; Loulie et al., 2009) regulate retraction of apical processes. It is also not known whether extrinsic signals regulate this event.

In a study focused on axon initiation in transgenically labeled *ath5:gapGFP* retinal ganglion cells (RGCs), Zolessi et al. (2006) made the unexpected observation that *slit1b* morphants showed inhibition of apical retraction. They used this finding to demonstrate that axonogenesis, which happened on schedule, did not depend on apical retraction. However, Zolessi et al. (2006) did

not investigate the mechanism of retraction. Slit proteins act as repulsive guidance ligands for axonal growth cones expressing Robo receptors and it has been reported that activated Robo or addition of purified Slit can inhibit N-cadherin-mediated cell adhesion in chick retinal cell cultures (Rhee et al., 2002, 2007). In *Drosophila*, Slit-Robo signaling negatively regulates cadherin-mediated adhesion on cardioblasts to enable proper lumen formation (Medioni et al., 2008; Santiago-Martinez et al., 2008). These results raised the possibility that a similar mechanism mediates the retraction of apical processes in the neuroepithelium. We found that Slit1B or Robo3 downregulation in transgenically labeled RGCs leads to inhibition of apical retraction. Expression of dominant-negative N-cadherin in RGCs results in the opposite phenotype—premature apical detachment. N-cadherin dominant-negative overexpression rescues apical retraction in *slit1b* morphants. These results are consistent with a model in which Slit/Robo signaling downregulates N-cadherin-based adhesion allowing apical retraction.

Materials and Methods

Animals. Wild-type and transgenic zebrafish were bred and kept at 26.5°C. Embryos obtained from natural mating were raised in 0.003% phenylthiourea at 28.5°C to prevent pigment formation. Transgenic lines [Tg(*ath5:gapGFP*) and Tg(*ath5:gapRFP*)] were from Zolessi et al. (2006).

Constructs and antibodies. Constructs used for embryo injection: *ath5:gapGFP* (Zolessi et al., 2006), *hsp70:mCherryCAAX*, and *hsp70:ncadhΔEC-mCherry*. *pDONR-ncadhΔEC* was subcloned from *pEGFP-N1-ncadhΔEC*, a gift from Dr. James Jontes (Ohio State University, Columbus, OH), using primers to add *att* sites at the 5' and 3' ends of the N-cadherin sequence. *hsp70:mCherryCAAX* and *hsp70:ncadhΔEC-mCherry* constructs were made using the Tol2kit as published (Kwan et al., 2007). Entry clones, except *pDONR-ncadhΔEC*, were gifts from Drs. Kate Lewis (Syracuse University, Syracuse, NY) and Chi-bin Chien (University of Utah, Salt Lake City, UT).

Primary antibodies used were diluted in blocking solution as follows: Zn5, 1:500 (Zebrafish International Resource Center); anti-N-cadherin antibody, 1:200 (ab12221; Abcam); anti-GFP, 1:200 (11814460001; Roche); and anti-GFP-FITC-conjugated, 1:500 (ab6662; Abcam). Sec-

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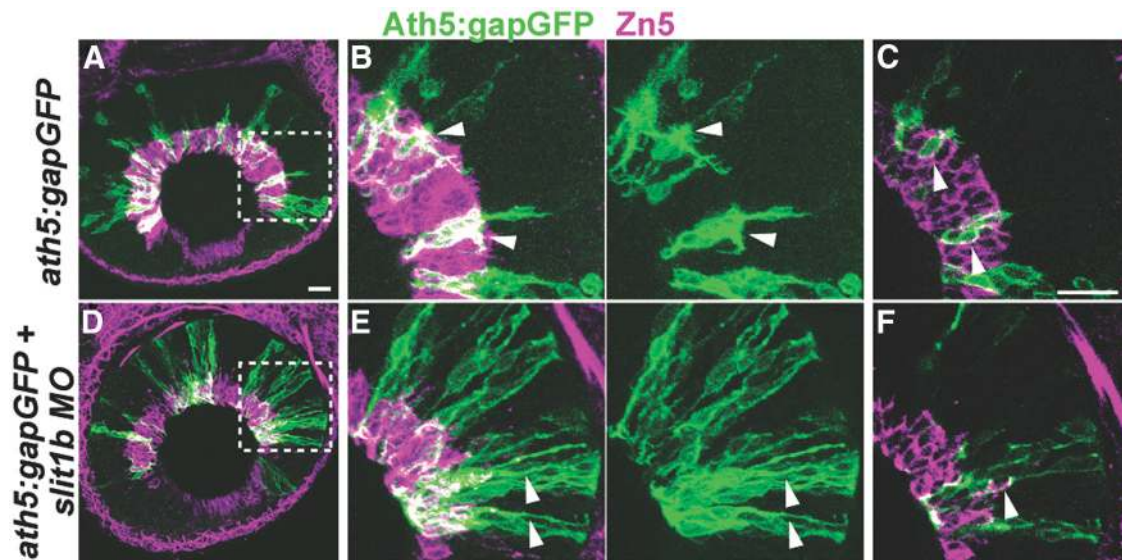


Figure 1. Apical processes retraction of RGCs is delayed in *slit1b* morphants. Analysis was performed on *ath5:gapGFP* DNA-injected embryos at 48 hpf. **A, D**, Extended-focus confocal images of the retinas of a control embryo (**A**) or a *slit1b* morphant (**D**). Boxed regions in **A** and **D** are enlarged in **B** and **E**, respectively. Arrowheads point to the retracted apical processes in the control retina (**B**) and un-retracted apical processes in the *slit1b* morphant retina (**E**). Zn5 (magenta) labels the ganglion cell soma and axon. **C, F**, Single optical section images of the boxed regions in **A** and **D**, respectively, showing that the *Ath5:gapGFP*+ cells express Zn5 and are thus RGCs (arrowheads). Scale bars, 20 μ m.

ondary antibodies used were as follows: donkey or goat anti-mouse IgG conjugated to Alexa 488, 594, or 647 fluorophores (Invitrogen), diluted to 1:1000 in blocking solution.

Image analyses. Optical sections of whole-mount embryos were obtained with a confocal microscope (SP2; Leica) at ~ 0.6 μ m intervals, as described previously (Poggi et al., 2005). Images were analyzed using Volocity software (Improvision). Using Zn5 (or *ath5:gapGFP*; see Fig. 3) as a marker for the differentiated ganglion cells, a differentiated RGC with retracted apical process had both its soma and apical process located within the labeled ganglion cell layer, while a RGC with an un-retracted apical process was defined as one with a positive cell body that retained an apical process outside of the ganglion cell layer. Counting was performed manually.

DNA, RNA, and morpholino injections. Plasmid DNA encoding the fluorescent proteins were injected into the cytoplasm of one-cell stage zebrafish embryos. *ath5:gapGFP* construct was injected together with meganuclease *I-SceI* at a concentration of 10 ng/ μ l (Zolessi et al., 2006). To improve integration and expression of the transgenes, all the Tol2 constructs were injected with the transposase RNA in a 1:1 ratio (Kwan et al., 2007). RNA and morpholinos (MOs; Genetools) were injected into the yolk of one- to four-cell stage embryos. MOs used were as follows: standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3'); 0.2 ng of anti-*slit1b*, translation blocking (5'-GCTCGGTGCCGGCATCTCCAAAAG-3') (Zolessi et al., 2006); 2 ng of anti-*robo2*, translation blocking (5'-GTAAGGTTGTGTTAAAGGACCCAT-3'); and a combination of 1 ng of anti-*robo3var1*, translation blocking (5'-CCCTAAAA GCGCTACAATCCACCTG-3') and 1 ng of anti-*robo3var2*, translation blocking (5'-TCTTTATCAGGAACGCAGCATCTC-3') (Challa et al., 2005; Devine and Key, 2008).

Blastomere transplantation. Embryos were dechorionated with Pronase (30 mg/ml; Sigma). Blastomeres from transgenic donor embryos, Tg(*ath5:gapRFP*), injected with *H2B-YFP* mRNA, and morpholinos were transplanted into the animal poles of unlabeled host blastulas using a glass micropipette.

Heat shock experiments. Embryos injected with the heat-shock promoter-driven constructs were raised at 28.5°C until 20 or 24 h post-fertilization (hpf). The embryos were then incubated at room temperature for 30–60 min, transferred to a tube of prewarmed medium, and heat-shocked at 37°C for 1 h.

RNA synthesis. *pCS2FA-transposase* was a gift from Dr. Brian Link (Medical College of Wisconsin, Milwaukee, WI). The transposase RNA was transcribed using the mMessage machine *in vitro* transcription kit (Ambion) from the SP6 promoter of *pCS2FA-transposase*, after linear-

ization with NotI. The mRNA was subsequently purified using RNeasy RNA purification kit (Qiagen).

In situ hybridization. *slit1b* and *robo3* antisense probes were generated by digesting *pCR2.1-TOPO-slit1b-2C* and *pCR2.1-TOPO-robo3* with BamHI and HindIII, respectively, then transcribing with T7 RNA polymerase. *pCR2.1-TOPO-slit1b-2C* and *pCR2.1-TOPO-robo3* were gifts from Dr. Chi-bin Chien. Whole-mount *in situ* hybridization of *slit1b* mRNA was performed on wild-type embryos as previously described (Shimamura et al., 1994), hybridized embryos were subsequently sectioned for image acquisition. *In situ* hybridization for *robo3* mRNA was performed on 20 μ m cryosections as previously described (Butler et al., 2001).

Statistical analysis. The Mann–Whitney *U* test was used to compare the percentage of *Ath5:gapGFP*-expressing cells with unretracted apical processes in WT and *slit1b* morphants per retina, using InStat software (GraphPad). Binomial test was used in all other experiments to assess statistical significance.

Results

Slit1b and Robo3 regulate apical process retraction of RGCs

To validate and extend the findings of Zolessi et al. (2006) on the role of Slit1b in apical retraction, embryos were injected with *ath5:gapGFP* with or without *slit1b* morpholino and fixed at 48 hpf. As *Ath5:gapGFP* is expressed by both the differentiated RGCs and their progenitors, Zn5, which labels the axon and soma of RGCs in zebrafish, was used as a definitive marker for the differentiated ganglion cells (Schmitt and Dowling, 1996). An *Ath5:gapGFP*+ RGC was judged to have an un-retracted apical process if its cell body was in the RGC layer and positive for Zn5 yet retained an apical process that extended outside of the ganglion cell layer (Fig. 1*E, F*, arrowheads). At 48 hpf, only few RGCs in control retinas had un-retracted apical processes, while a significant number of RGCs in the *slit1b* morphant retina retained un-retracted apical processes (Fig. 1). The reason that the RGC layer is thinner in *slit1b* morphants is probably that many RGC somas have trouble migrating basally when still attached apically (Zolessi et al., 2006). To check that this phenotype is not a result of a general developmental delay, we compared the relative timing of apical retraction to another RGC developmental event—axonogenesis using time-lapse analysis of *Ath5:gapGFP*-expressing RGCs

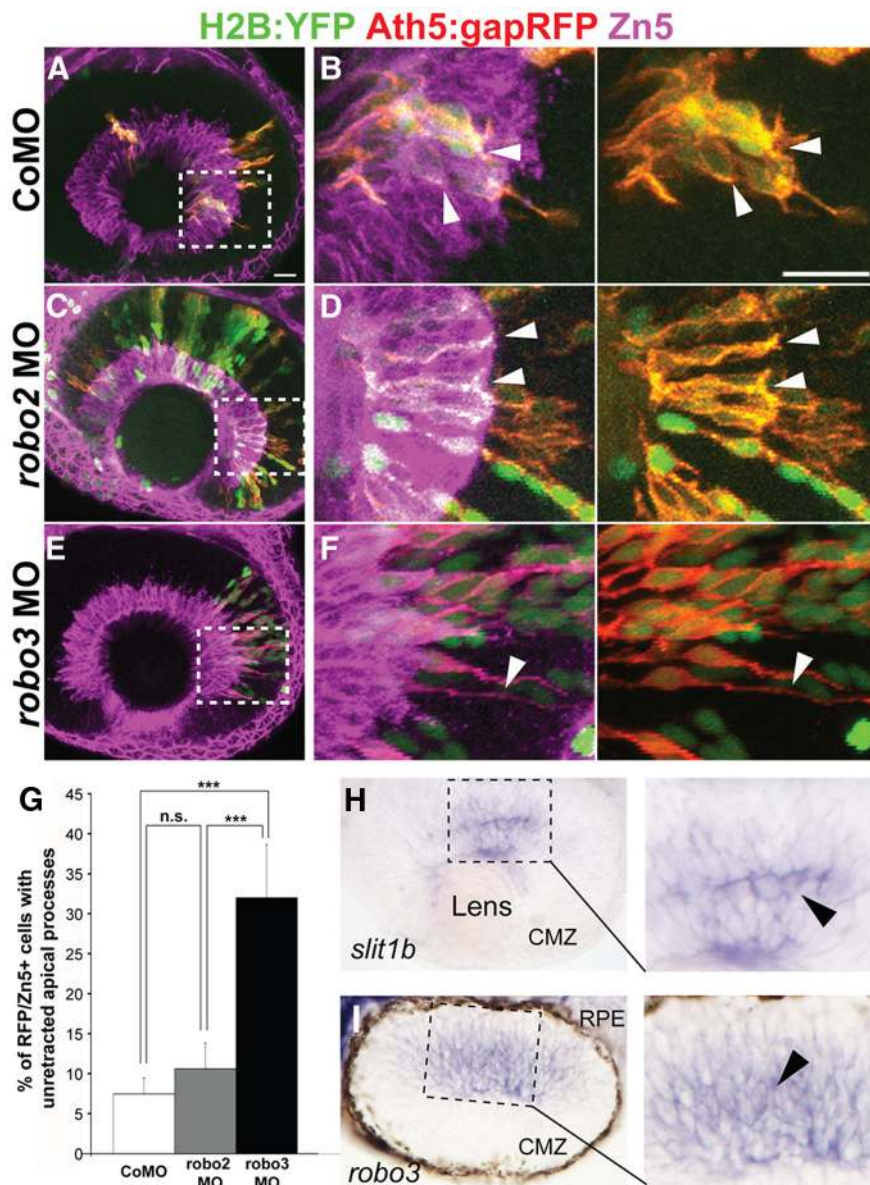


Figure 2. Knockdown of *robo3* but not *robo2* inhibits apical process retraction. **A–F**, Confocal images from wild-type hosts transplanted with blastomeres from Tg(*ath5:gapRFP*) embryos injected with control morpholino (CoMO; **A, B**), *robo2* morpholino (**C, D**), or a combination of *robo3v1* and *v2* morpholinos (**E, F**) at 48 hpf. Zn5 (magenta) labels the ganglion cell layer. Boxed regions in **A, C**, and **E** are enlarged in **B, D**, and **F**, respectively. Arrowheads in **B** and **D** indicate the retracted apical processes of transplanted Ath5:gapRFP+ RGCs from control or *robo2* morpholinos-injected embryos; arrowheads in **F** indicate the un-retracted apical process of a transplanted RGC from a *robo3v1* and *v2* double-morphant. Scale bars, 20 μ m. **G**, Percentage of Ath5:gapRFP and Zn5 double-positive cells with un-retracted apical processes at 48 hpf ($n = 187$ cells from control morpholino-injected embryos; $n = 94$ cells from *robo2* morphants; $n = 50$ cells from *robo3* morphants). Error bars indicate SEs ($***p < 0.001$). **H, I**, A retinal section from a 38 hpf whole-mount *in situ* hybridized embryo, showing that *slit1b* and *robo3* mRNAs are expressed in the developing zebrafish retina. **I**, *In situ* hybridization image from a similarly staged retinal section showing that *robo3* mRNA is expressed in subsets of cells in the developing retina. Note that pigments were allowed to form and the retinal pigmented epithelium (RPE) is thus visible. CMZ, Ciliary marginal zone.

starting at 35 hpf. We found that only $31.6 \pm 10.6\%$ of normal RGCs sent out axons before retracting their apical processes ($n = 19$), but in *slit1b* morphants, although axonogenesis was not premature, $68.4 \pm 9.1\%$ of RGCs extended an axon while retaining an apical process ($n = 26$). This significant difference ($p < 0.01$) indicates that apical retraction is inhibited compared with axonogenesis by the knocking down Slit1b.

Robo proteins are receptors for Slit ligands. Therefore, we wondered whether newborn RGCs express Robo, which could

mediate the Slit1b-regulated apical retraction. Among the four zebrafish *robo* genes, only *robo2* and *robo3* mRNA are detected in the developing retina (Lee et al., 2001). In zebrafish, two forms of *robo3* exist: *robo3v1* and *robo3v2* (Challa et al., 2005). Therefore, to test whether Robo2 or 3 in RGCs is involved in apical process retraction, we injected Tg(*ath5:gapRFP*) embryos with control, *robo2*, or a combination of *robo3v1* and *robo3v2* morpholinos and H2B-YFP mRNA as a nuclear marker of donor cells. Blastomeres from injected embryos were then transplanted into uninjected wild-type hosts of the same stage. We found that Ath5:gapRFP-positive cells from control and *robo2* morpholino-injected embryos retracted their apical processes normally, while Ath5:gapRFP-positive cells from *robo3* morphants retained apical processes (Fig. 2*B, D, F*). Thus, *robo3* knockdown, but not *robo2* knockdown, phenocopies *slit1b* morphants, suggesting that Robo3 is the receptor downstream of Slit1b during apical retraction.

An important question is why Slit1b and Robo3 do not cause neuroepithelial cells to detach before their final division. RGCs of the embryonic zebrafish retina go through their final S-phase between 30 and 40 hpf (Hu and Easter, 1999) and finish mitosis a few hours later (Poggi et al., 2005). Using *in situ* hybridization at a variety of time points between 24 and 40 hpf, we first detected a clear signal for both *slit1b* and *robo3* mRNA in scattered cells at ~ 36 – 38 hpf (Fig. 2*H, I*). Thus, the timing of expression of this receptor–ligand pair may provide part of the answer to this question.

Expression of dominant-negative N-cadherin leads to premature apical process retraction

Immunohistochemistry reveals that N-cadherin is expressed strongly in the developing zebrafish retina (Liu et al., 2001), consistent with a possible role in apical retraction. As N-cadherin is expressed in the apical processes of Ath5:gapGFP-positive RGC progenitors (Fig. 3*A*), we investigated whether it is involved in apical process attachment using a dominant-negative construct lacking the extracellular N-cadherin domain (Niemann et al., 1999; Jontes et al., 2004). To bypass the severe defects that result from disrupting N-cadherin in early development (Pujic and Malicki, 2001), we controlled the expression of the dominant-negative protein by a heat-shock-inducible promoter. Expression was induced by a 1 h heat-shock at 37°C at 20 hpf in Tg(*ath5:gapGFP*) embryos either injected with *hsp70:ncadherin Δ EC-mCherry* or a control construct (*hsp70:mCherryCAAX*). The injected embryos were fixed at 36 hpf. As most RGCs have just exited cell cycle at 36

hpf, we speculated that most of the newly born RGCs would still retain apical processes (Hu and Easter, 1999). As expected, the majority of the Ath5:gapGFP + cells expressing *hsp70:mCherryCAAX* had un-retracted processes attached to the apical surface. However, Ath5:gapGFP + cells expressing the dominant-negative N-cadherin protein had fewer un-retracted apical processes (Fig. 3D). Thus, N-cadherin function appears to be essential for the attachment of newborn RGC apical processes.

Dominant-negative N-cadherin rescues the retraction phenotype in *slit1b* morphants

As we found that blocking Slit1b/Robo3 signaling inhibits apical retraction, whereas interfering with N-cadherin function leads to precocious retraction in developing RGCs. One possible explanation for these opposing phenotypes is that Slit/Robo signaling normally downregulates N-cadherin-mediated apical adhesion. If this was the case, the dominant-negative N-cadherin phenotype should be epistatic to the Slit1b phenotype. To test this notion, embryos were coinjected with control morpholino and *hsp70:mCherryCAAX* or *hsp70:ncadh Δ EC-mCherry*, or with *slit1b* morpholino and *hsp70:mCherryCAAX* or *hsp70:ncadh Δ EC-mCherry*. Embryos were then heat-shocked at 24 hpf and fixed at 48 hpf. Knockdown of Slit1b was confirmed to inhibit apical retraction, as shown by a significantly higher proportion of Hsp70:mCherryCAAX RGCs with un-retracted apical processes in *slit1b* morphants compared with those in the control morphants (Fig. 4A–F). The Hsp70:Ncadh Δ EC-mCherry-expressing RGCs, however, retracted their apical processes efficiently in these morphants (Fig. 4G), showing that blocking N-cadherin function in the morphant cells rescues apical retraction. These results suggest a model in which Slit-Robo signaling in newborn RGCs downregulates N-cadherin-mediated attachment at the apically located adherens junction, allowing apical retraction upon cell cycle exit.

Discussion

In this report, we define a putative molecular pathway underlying the retraction of apical processes in newborn RGCs. We first show that knocking down Slit1b inhibits apical process retraction, corroborating previous data (Zolessi et al., 2006). We identified Robo3 as the likely receptor for Slit1b in this process. As work in other systems has shown that Slit/Robo signaling is capable of modulating cadherin function (Rhee et al., 2002; Medioni et al., 2008; Santiago-Martínez et al., 2008), we next investigated whether N-cadherin, which is known to be part of the adherens junctions at the apical complex in neuroepithelial cells, is also involved in the process of RGC apical retraction. We

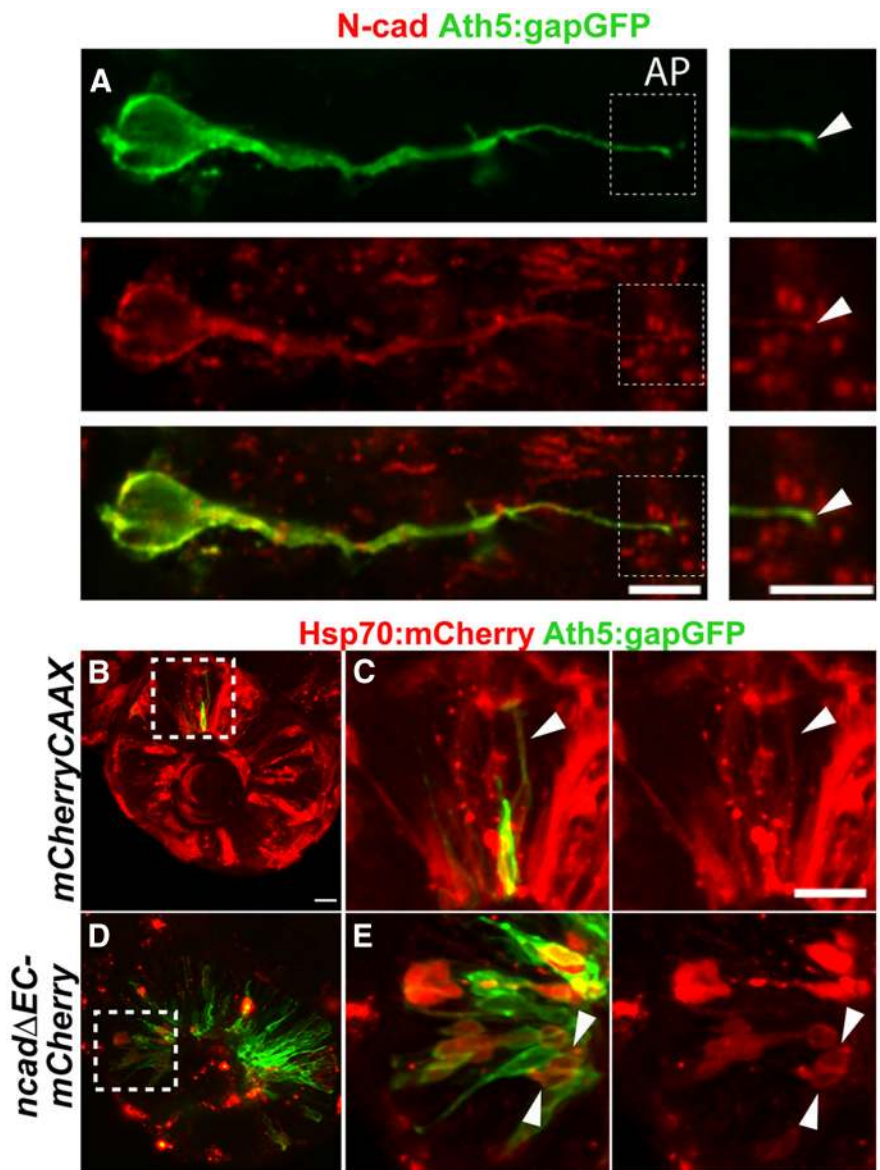


Figure 3. Expression of heat-shock-induced dominant-negative N-cadherin leads to premature apical processes retraction in RGC progenitors. **A**, Confocal images of the apical region (AP) of the retina of a 36hpf Tg(*ath5:gapGFP*) embryo stained with anti-N-cadherin and anti-GFP antibodies. N-cadherin (red) expression is detected in the apical processes of Ath5:gapGFP + cells (arrowheads). **B–E**, Confocal images of the retinas of Tg(*ath5:gapGFP*) embryos injected with *hsp70:mCherryCAAX* (**B**, **C**) or *hsp70:ncadh Δ EC-mCherry* (**D**, **E**) at 36 hpf. Boxed regions in **B** and **D** are enlarged in **C** and **E**, respectively. Arrowheads in **C** indicate the un-retracted apical process of an Ath5:gapGFP-positive cell expressing Hsp70:mCherryCAAX. Arrowheads in **E** indicate Ath5:gapGFP-positive cells expressing Hsp70:Ncadh Δ EC-mCherry with retracted apical processes. Scale bars, 20 μ m.

found that the expression of a dominant-negative N-cadherin in RGCs leads to premature detachment of their apical processes and rescues the apical detachment phenotype in *slit1b* morphants.

Our results are thus consistent with the possibility that N-cadherin is downregulated by Slit-Robo signaling, leading to the loss of apical adhesion. To establish further details of this molecular pathway, it will be necessary to investigate the links between Slit/Robo signaling and N-cadherin. Epithelial/mesenchyme transition has been found to be mediated by Rab5-dependent endocytosis of E-cadherin (Fujita et al., 2002; Palacios et al., 2005; Emery and Knoblich, 2006), and E-cadherin internalization can be induced by soluble factors (Kamei et al., 1999; Ulrich et al., 2005; Bryant et al., 2007). Preliminary experiments

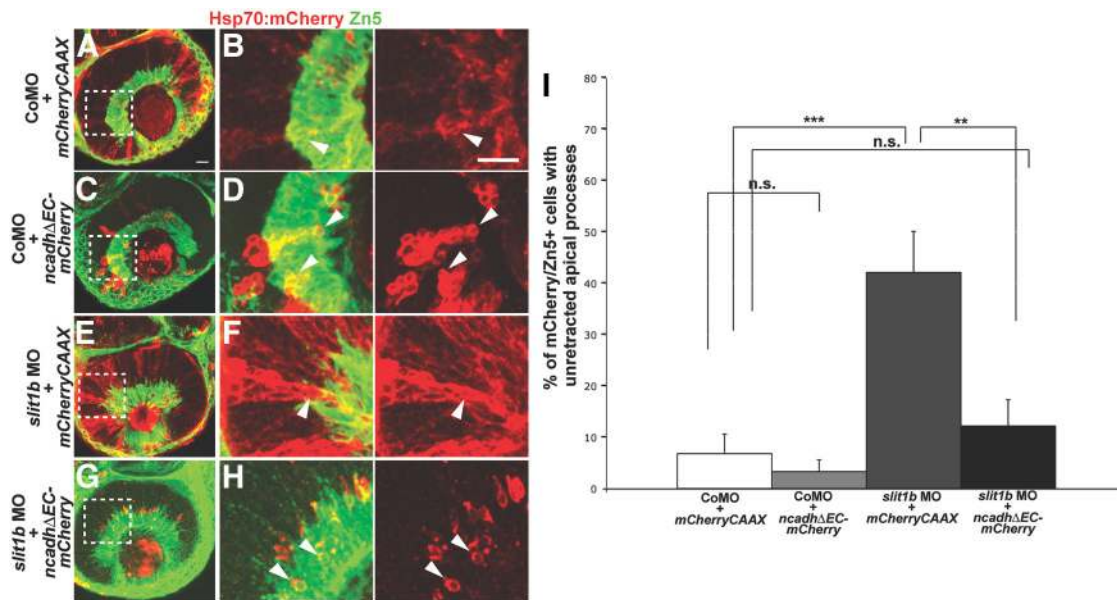


Figure 4. Expression of dominant-negative N-cadherin rescues apical retraction in *slit1b* morphant RGCs. **A–H**, Confocal images from control morpholino-injected (CoMO) embryos expressing Hsp70:mCherryCAAX (**A, B**) or Hsp70:NcadhΔEC-mCherry (**C, D**); confocal images from *slit1b* morphants expressing Hsp70:mCherryCAAX (**E, F**) or Hsp70:NcadhΔEC-mCherry (**G, H**) at 48 hpf. Zn5 (pseudocolored in green) labels the ganglion cells. Boxed regions in **A, C, E**, and **G** are enlarged in **B, D, F**, and **H**, respectively. Arrowheads in **F** indicate the un-retracted apical process of an Hsp70:mCherryCAAX-expressing RGC (defined as cells that are Zn5+) in a *slit1b* morphant; arrowheads in **B, D, F**, and **H** indicate mCherry-positive RGCs with retracted apical processes. Scale bars, 20 μ m. **I**, Percentage of mCherry-positive RGCs with un-retracted apical processes at 48 hpf ($n = 44$ Hsp70:mCherryCAAX-expressing cells from control morpholino-injected embryos; $n = 61$ Hsp70:NcadhΔEC-mCherry-expressing cells from control morpholino-injected embryos; $n = 38$ Hsp70:mCherryCAAX-expressing cells from *slit1b* morphants; $n = 41$ Hsp70:NcadhΔEC-mCherry-expressing cells from *slit1b* morphants). Error bars indicate SEs (** $p < 0.01$, *** $p < 0.001$).

with endocytosis inhibitors and the expression of dominant-negative Rab5, however, failed to interfere with apical retraction of RGCs in our system (data not shown), suggesting that other effectors might be at play. Likely candidates include those discovered in cell culture systems by Rhee et al. (2002, 2007), who found that Slit activation of Robo leads to the phosphorylation of β -catenin by Abl; this causes a decrease in the binding affinity of β -catenin for N-cadherin and reduces N-cadherin-mediated adhesion by severing its link to the cytoskeleton.

Transient disruption of integrin functions by injection of blocking antibody in mouse ventricular zone leads to detachment of apical processes from the ventricular surface (Loulrier et al., 2009); this suggests that integrin signaling could also play a role in the apical attachment of neuroepithelial cells in the developing mouse cortex. Our results, though more consistent with a role for adherens junctions in apical attachment as suggested by Cappello et al. (2006) and Imai et al. (2006), do not rule out the possibility that both cadherins and integrins are involved in the attachments of apical processes, and that neither of them alone is sufficient. Given the expression of Slits, Robos, and Cadherin in different developing tissues (Bitzur et al., 1994; Challa et al., 2001; Lee et al., 2001; Hutson et al., 2003), we propose that Slit/Robo signaling and cadherin downregulation may be a common mechanism of apical processes retraction.

Precocious detachment affects the proliferation potential (Cappello et al., 2006; Imai et al., 2006), and failure to retract would inhibit the temporally orchestrated migration to distant sites, such as the cerebral cortex. Obviously a key issue for future research is what regulates the timing of this signaling. One attractive possibility in the retina is that Robo3 is only expressed when cells have completed their final mitosis. Our *in situ* results on the timing and localized expression of *robo3* mRNA in subsets of retinal cells are consistent with this idea. Interestingly, Slit1b expression is also turned on just at this stage, so, even if retinal cells

prematurely express Robo3, they cannot detach until Slit1b is expressed and vice versa. We hope this work will stimulate further research into these relatively unexplored questions of developmental cell biology.

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