SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES

Figure S1, related to Figure 1: Growth cones form a highly polarized epithelial-like structure before they extend to their targets.
(A) Z-projection of lamina (8μm) shown as an *en face* view (bottom) and a digitally rotated side view at 28% apf; R4 cells were labeled with *mδ-Gal4 UAS-mtdTomato* (green) and all R cells were labeled with mAb24B10 (magenta). White arrows denote orientation of R4 growth cones; white T's demark the location of the R4 targets. (B) Schematic reconstruction of R cell growth cones in the lamina, based on EM reconstructions (Meinertzhagen and Hanson, 1993), showing the arrangement of several R cell bundles (one bundle labeled in magenta).

Figure S2, related to Figure 2: Analysis of growth cone polarity.
(A) Single growth cones of subtypes R1, R2 and R3 labeled with *GFPmyr*, from 0% apf until 33% apf; ventral hemisphere, equator to the right, anterior up. **(B)** Unit circles showing that growth cone polarity in wild-type at 28% apf (blue) and at 33% apf (green) correlates with angle of extension at 40% apf (magenta). R cells were labeled with *GFPmyr* and counterstained with mAb24B10. Arrows indicate mean polarity vector for the population. Individual dots represent individual R cells. Grey areas show the angles of each R cell's target LMCs.

Figure S3, related to Figure 3: Testing RNAi and Gal4 constructs for genetic interactions; Generation and testing of the Fmi Bac rescue construct.
(A) *Ncad* or *fmi RNAi* expression in R cells using the early driver *ey3.5-Flp actin-FRT-y-FRT-Gal4* phenocopied previously published mutant phenotypes, demonstrating that all RNAi constructs are functional. In all three mutants, cartridges were disorganized and contained varying numbers of R cells. *Ecad RNAi* expressing R cells could not be imaged, as the construct causes lethality

when expressed with this driver. Laminas of adult flies labeled with Csp2a (green) are shown. **(B)** Expression of Ncad RNAi using *gmr-Flp actin-FRT-y-FRT-Gal4* resulted in a moderate reduction of Ncad protein in R cells in the retina. **(C)** Expression of fmi RNAi using the same driver also resulted in a moderate (and somewhat variable) reduction of Fmi protein in the lamina. **(D)** Genomic region spanned by the Bac rescue construct *CH321-66D09*, which includes the *fmi* ORF, as well as the up- and down-stream regions. **(E)** The Bac construct *66D09* completely rescued lethality of a *fmi* trans-heterozygous mutant. **(F)** *Fmi* MARCM on 2L using the Bac rescue construct phenocopied PCP defects within the *fmi* mutant clone, while there were no PCP defects outside the labeled clone, demonstrating that the Bac construct rescued the *fmi* mutant PCP phenotype. Within the *fmi* mutant clone (labeled by *CD8GFP*, green) many ommatidia were incorrectly polarized (arrows). R cells labeled with mAb24B10 (magenta); hooked arrows indicate ommatidial polarity; white arrows label ommatidia with wild-type R3 and R4; green arrows label ommatidia with *fmi* mutant R3 and/or R4. **(G)** Genetic interaction between *Ncad* and *fmi*. *Fmi* mutant clones were generated using MARCM such that mutant cells co-expressed *Ncad RNAi*. Both *fmi* and *Ncad* single mutants displayed only very weak phenotypes, while double mutant growth cones displayed highly penetrant defects, mostly failure to extend. All groups were significantly different from each other with $p<0.001$, or $p<0.05$ for *control* vs. *fmi*, except *Ncad* vs. *fmi RNAi* (n.s.); Fisher's exact test, adjusted for multiple comparisons.

Figure S4, related to Figure 4: Driver characterization and R cell subset-specific loss of *Ncad* and *fmi*.

(A) Left panels: expression of *R25B08-Gal4* driving *CD8GFP* (green), counterstained with mAb24B10 (magenta) in the ventral retina, anterior is up, at 28% apf. R1, R3, R4, and R6 were labeled. Middle and right panels: expression of *R25B08-Gal4 mδ-Gal80*. At 28% apf only R1 and R6 expressed GFP, while at 0% the driver was also expressed in some R2 and R5 cells (white arrows). Schematic insets in top panels show summary of expression, insets in bottom

panels show GFP expression in a single, representative ommatidium. **(B)** Expression of *fmi* or *Ncad+fmi* RNAi using *R25B08-Gal4 mδ-Gal80* did not induce any significant planar cell polarity defects; dorsal retina at 28% apf, labeled with mAb24B10 (anterior is down). Ommatidial polarity is indicated for the left half of each panel by a white arrow; inset shows the wild-type positions of R3 and R4. **(C)** Graphical representation of R cell targeting as observed at 40% apf, after expression of RNAi against *Ncad*, *fmi* or both using *R25B08-Gal4 mδ-Gal80*. Each oval represents one cartridge. Pie charts display the percentage with which the R1, R2, or R3 cells targeted to a specific cartridge in the different genetic backgrounds. The home cartridge is shown in grey and the wild-type target cartridge with a bold outline. **(D)** Single cells mutant for *Ncad* and *fmi* (magenta) vs. control (blue) using Bac 2L MARCM did not have any polarity defects, as measured at 28% apf. Shown are mean polarity vectors and standard ellipses for each growth cone subtype. No significant differences in the mean vectors between wild-type and double mutant R cells were found using ANOVA with Newman-Keuls post hoc test, except for R1 ($p<0.05$); no significant differences in variance between wild-type and mutant R cells were found using bootstrap and Z-test, except for R5 ($p<0.001$; see Supplemental Methods).

Figure S5, related to Figure 5: Subset specific loss of Ncad and fmi did not have strong effects on filopodial numbers or lengths. Growth cone targeting angles correlate with early polarity angles.

Ncad- and/or *fmi RNAi* expression in an R cell subset using *R25B08-Gal4 mδ-Gal80.* **(A)** Average number of filopodia sorted by growth cone type at 28% apf, n=17-24 for each type and genotype. **(B)** Average filopodial length for each growth cone type at 28% apf, n=156-457 filopodia. **(A,B)** Significant differences between wild type and mutant genotypes are indicated by brackets, *$p<0.05$, **$p<0.01$, ***$p<0.001$; one-way ANOVA with Dunnett's post hoc test. **(C)** Polar plots of *Ncad* and *fmi* subset double knockdown at 28% apf. Plots for R1, R2 and R3 with growth cone polarity angles at 28% (blue) and growth cone extension angle at 40% apf (magenta) are shown.

Figure S6, related to Figure 6: Fmi, but not Ncad is differentially expressed in R cells. Description of a sparse R2, R5 driver.

(A) *Ncad RNAi* expression in LMCs does not affect polarity of growth cones. Shown is a polar plot with the lines representing the mean polarity vector and the ovals the standard ellipse (akin to standard deviation), blue: control, magenta: *gcm-Gal4* driving *Ncad RNAi* expression, growth cones were quantified at 28% apf. No significant differences in the mean vectors between wild-type and mutant R cells were found using ANOVA with Newman-Keuls post hoc test; no significant differences in variance between wild-type and mutant R cells were found using bootstrap and Z-test (see Supplemental Methods). **(B)** Expression of RNAi against *Ncad* in LMCs using *gcm-Gal4* results in a specific knockdown of Ncad protein in LMC processes. Ncad staining in R cells was not affected. Retina and lamina at 28% apf are shown, all R cells were labeled with mAb24B10 (magenta), and Ncad protein (green); Z-stacks of 10-14μm.

(C) Ncad and Fmi partially colocalize within R cell growth cones. Single growth cones labeled with *GFPmyr*, Ncad was depleted in LMCs by *gcm-Gal4* driven expression of *Ncad RNAi*. Top row displays growth cones; bottom three rows display colocalization signal between the growth cone and Ncad (green in merge) or Fmi (red in merge); ventral hemisphere, equator to right, anterior up. Only the colocalization signal is shown for Ncad and Fmi. Please note that the colocalization signal for Ncad is restricted to the main body of the growth cone and not found in filopodia as in Figure 6. This is due to thresholding and because the main growth cone body has the highest signal for both Ncad and GFP staining. **(D)** Quantification of Fmi protein levels as the number of colocalized voxels between the growth cone and Fmi. Levels of Fmi are significantly different between growth cones with $p<0.001$, except R3 vs. R4 (n.s.), R2 vs. R5 (n.s.) and R1 vs. R6 ($p<0.05$). **(E)** Quantification of Ncad protein levels as the number of colocalized voxels between the growth cone and Ncad protein, while Ncad was depleted in LMCs, reveals no significant differences between any of the groups. **(F)** R1- R6 growth cones were not significantly different in size. **(D-F)**

Shown are mean values + SEM; p values were determined by one- way ANOVA with Newman-Keuls post hoc test. **(G)** At 28% apf *R49A06-Gal4* alone drives expression of *UAS-mCD8GFP* (green, counterstained with mAb24B10 in magenta) sparsely in R2, R4, R5 and R8. Addition of *mδ-Gal80* represses R4 expression and further sparsens expression to 1-2 cells per ommatidia, which are R2, R5 and/or R8. **(H)** Fmi protein localizes to the growth cone surface. 28% apf pupal brains were either treated with or without Triton detergent before and during incubation with primary antibodies against the extracellular portion of Fmi and the intracellular portion of mCD8GFP (expressed by mδ-Gal4). mCD8GFP is not labeled in absence of Triton, while Fmi staining remains. The Fmi staining is weaker in the absence of detergent, most likely because of reduced permeability of the brains, yet the overall Fmi pattern remains unaltered.

Supplemental Table 1 (relates to Figures 1-7)

List of all fly strains used in experiments for main Figures, (> indicates FRTs)

Figure	Panel	Genotype
1	B	*w-132* *w-133* *Oregon-R*
	C	*hs-Flp;; mδ-Gal4, UAS-mtdTomato/ gmr>white>GFPmyr*
2	A,B	*hs-Flp;; mδ-Gal4, UAS-mtdTomato/ gmr>white>GFPmyr*
	C	*hs-Flp; gmr>white>GFPmyr; UAS-mtdTomato* *hs-Flp;; mδ-Gal4, UAS-mtdTomato/ gmr>white>GFPmyr (33% apf)*
3	A	*gmr-Flp; actin>y>Gal4/ UAS-myrEGFP (control)* *gmr-Flp; actin>y>Gal4/ UAS-Ncad RNAi* *gmr-Flp; actin>y>Gal4/ UAS-fmi RNAi* *gmr-Flp; actin>y>Gal4/ UAS-Ecad RNAi* *gmr-Flp; actin>y>Gal4/ UAS-fmi RNAi, UAS-Ncad RNAi* *gmr-Flp; actin>y>Gal4/ UAS-fmi RNAi; UAS-Ecad RNAi*
	B	*elav-Gal4^{C155}, hs-Flp, UAS-mCD8GFP; gmr:RFPmyr, FRT40A/ Ncad, tub-Gal80, FRT40A*
	E,F	*elav-Gal4^{c155}, hs-Flp, UAS-mCD8GFP; Bac(fmi), tub-Gal80, FRT40A, fmi^{192}/ FRT40A (control)* *elav-Gal4^{c155}, hs-Flp, UAS-mCD8GFP; Bac(fmi), tub-Gal80, FRT40A, fmi^{192}/ FRT40A, fmi^{e59}* *elav-Gal4^{c155}, hs-Flp, UAS-mCD8GFP; Bac(fmi), tub-Gal80, FRT40A, fmi^{192}/ Ncad^{Δ14}, FRT40A* *elav-Gal4^{c155}, hs-Flp, UAS-mCD8GFP; Bac(fmi), tub-Gal80, FRT40A, fmi^{192}/ Ncad^{Δ14},* *FRT40A, fmi^{e59}* *elav-Gal4^{c155}, hs-Flp, UAS-mCD8GFP; Bac(fmi), gmr-RFP, FRT40A, fmi^{192}/ Ncad^{Δ14}, tub-Gal80, FRT40A, fmi^{e59} (reverse MARCM, 2x)*
4	A-D	*hs-Flp; gmr>white>GFPmyr; R25B08-Gal4, mδ-Gal80/ UAS-mtdTomato (control)* *hs-Flp; gmr>white>GFPmyr/ UAS-Ncad RNAi; R25B08-Gal4, mδ-Gal80* *hs-Flp; gmr>white>GFPmyr/ UAS-fmi RNAi; R25B08-Gal4, mδ-Gal80* *hs-Flp; gmr>white>GFPmyr/ UAS-Ncad RNAi, UAS-fmi RNAi; R25B08-Gal4, mδ-Gal80*
5	A-C	*same as Figure 4*

6	A-E	*R49A06-Gal4, UAS-mCD8GFP/ mδ-Gal80*
	F	*hs-Flp; Ncad-V5(FSF); lexAoP-mtdTomato*
7	A-C	*hs-Flp; gmr>white>GFPmyr; UAS-Ncad /mδ-Gal4, UASmtdTomato* *hs-Flp; gmr>white>GFPmyr; mδ-Gal4, UASmtdTomato (control)*

Supplemental Table 2 (relates to Figures S1-6)

List of all flystrains used in experiments for Supplementary Figures (> indicates FRTs)

Figure	Panel	Genotype
S1	A	*mδ-Gal4, UAS-mtdTomato*
S2	A	*hs-Flp;; mδ-Gal4, UAS-mtdTomato/ gmr>white>GFPmyr*
	B	*hs-Flp; gmr>white>GFPmyr; UAS-mtdTomato* *hs-Flp;; mδ-Gal4, UAS-mtdTomato/ gmr>white>GFPmyr (33% apf)*
S3	A	*$ey^{3.5}$-Flp; actin>y>Gal4/ Oregon-R (control)* *$ey^{3.5}$-Flp; actin>y>Gal4/ fmi RNAi* *$ey^{3.5}$-Flp; actin>y>Gal4/ Ncad RNAi*
	B	*gmr-Flp; actin>y>Gal4 (control)* *gmr-Flp; actin>y>Gal4/ Ncad RNAi*
	C	*gmr-Flp; actin>y>Gal4 (control)* *gmr-Flp; actin>y>Gal4/ fmi RNAi*
	E	*FRT42D, fmi^{e59}/ FRT40A, tub-Gal80, fmi^{192}* *FRT42D, fmi^{e59}/ 66D09, FRT40A, tub-Gal80, fmi^{192}*
	F	*$elav\text{-}Gal4^{c155}$, hs-Flp, UAS-mCD8GFP; Bac(fmi), tub-Gal80, FRT40A, fmi^{192}/ FRT40A, fmi^{e59}*
	G	*$elav\text{-}Gal4^{c155}$, hs-Flp, UAS-mCD8GFP; FRT42D, tub-Gal80/ FRT42D (control)* *$elav\text{-}Gal4^{c155}$, hs-Flp, UAS-mCD8GFP; FRT42D, tub-Gal80/ FRT42D, fmi^{e59}* *$elav\text{-}Gal4^{c155}$, hs-Flp, UAS-mCD8GFP; FRT42D, tub-Gal80/ FRT42D, Ncad RNAi* *$elav\text{-}Gal4^{c155}$, hs-Flp, UAS-mCD8GFP; FRT42D, tub-Gal80/ FRT42D, fmi^{e59}, Ncad RNAi*
S4	A	*R25B08-Gal4/ UAS-mCD8GFP* *R25B08-Gal4, mδ-Gal80/ UAS-mCD8GFP*

	B,C	*hs-Flp; gmr>white>GFPmyr; R25B08-Gal4, mδ-Gal80/ UAS-mtdTomato (control)* *hs-Flp; gmr>white>GFPmyr/ UAS-Ncad RNAi; R25B08-Gal4, mδ-Gal80* *hs-Flp; gmr>white>GFPmyr/ UAS-fmi RNAi; R25B08-Gal4, mδ-Gal80* *hs-Flp; gmr>white>GFPmyr/ UAS-Ncad RNAi, UAS-fmi RNAi; R25B08-Gal4, mδ-Gal80*
	D	*same as S3G*
S5	A-C	*same as S4B,C*
S6	A,B	*hs-Flp; gcm-Gal4, gmr>white>GFPmyr (control)* *hs-Flp; gcm-Gal4/ Ncad RNAi, gmr>white>GFPmyr*
	C,E	*hs-Flp; gcm-Gal4/ Ncad RNAi, gmr>white>GFPmyr*
	D,F	*hs-Flp;; mδ-Gal4, UAS-mtdTomato/ gmr>white>GFPmyr*
	G	*R49A06-Gal6/ UAS-mCD8GFP* *R49A06-Gal4, UAS-mCD8GFP/ mδ-Gal80*
	H	*mδ-Gal4, UASmCD8GFP*

EXTENDED EXPERIMENTAL PROCEDURES

Fly Stocks

The following fly stocks were used: *w-132, w-133* (San Diego Stock Center), *UAS-mtdTomato* (gift from L. Luo), *hs-Flp* and *fmi*^{192} (Bloomington Stock Center), *gmr-FRT-white+-FRT-GFPmyr* (gift from P. Garrity), *gmr-Flp* (Pignoni et al., 1997), *ey*$^{3.5}$*-Flp* (Bazigou et al., 2007), *actin-FRT-yellow+-FRT-Gal4* (gift from G. Struhl), *Ncad*$^{\Delta 14}$ *FRT40A* (Prakash et al., 2005), *FRT42D fmi*^{e59} (Usui et al., 1999), *gcm-Gal4* (Chotard et al., 2005), UAS-Ncad -7b-13b-18a (Yonekura et al., 2006), conditionally tagged *Ncad-V5 lexAoP-myr-tdTomato* (Pecot et al., 2013), *Oregon-R, mδ-Gal4* (Chen and Clandinin, 2008). The RNAi lines *fmi 107993, Ncad 1092* and *Ecad 27081* were all obtained from the VDRC (Dietzl et al., 2007). The *fmi* and *Ncad* RNAi constructs phenocopied the mutant phenotypes of these genes when expressed using the strong R cell driver *ey*$^{3.5}$*-Flp actin-FRT-yellow+-FRT-Gal4* (Figure S3). The *Ecad* RNAi construct driven by the

same Gal4 line or by *tub-Gal4* (Lee and Luo, 1999) induced lethality showing that it is functional. For MARCM analysis the following strains were used: *elav-Gal4^{c155} hs-Flp UAS-mCD8GFP, FRT42D tub-Gal80, tub-Gal80 FRT40A, FRT40A* and *FRT42D* (Lee and Luo, 1999), *FRT40A gmr-RFPmyr* (Chen and Clandinin, 2008). The lines *R25B08-Gal4* and *R49A06-Gal4* were obtained from G. Rubin (Pfeiffer et al., 2010). *R25B08-Gal4* was generated using a 3.9kb DNA fragment of the *Delta* gene, while *R49A06-Gal4* was generated using a 3.4kb DNA fragment of the *beat III-c* gene.

Generation of Transgenic lines

To generate *mδ-Gal80* flies, the mδ-enhancer was cloned by nested PCR (primer pair 1: tgatctcagaaacctcccccacga, cttcggatcttcgcccgagctagt; primer pair 2: aagcttgccatcagatgtcagc, ggatccttttggcgcacagt; (Cooper and Bray, 1999), including HindIII and BamH1 restriction sites. The *hsp70* promoter was cloned by PCR from the *pIB-GFP* vector (ggatccggcgaaaagagcg, gaattcaggcattgtgtgtgag; Bateman et al., 2006), including BamH1 and EcoR1 restriction sites. Both the *mδ* enhancer fragment and the *hsp70* fragment were triple- ligated into *pUAST-attB*, cut by HindIII and EcoR1, generating the *p-mδ-attB* vector. *Gal80* was cloned from *pCaspr-tubGal80* (Lee and Luo, 1999) into the *p-mδ-attB* vector by Not1 and Xba1 digest. Transformants were generated using standard protocols for phiC31- mediated insertions into the VK31 and attP2 landing sites (Bischof et al., 2007; Groth et al., 2004).

To generate the Bac rescue construct, a full-length Bac covering the entire Fmi locus (*CH321-66D09,* BacPac Resources; Venken et al., 2009) was inserted into the VK37 phiC31 landing site.

UAS-myrEGFP was generated by fusing a myristoylation signal (atg ggg agc agc aag agc aag ccc aag gac ccc agc cag cgc) to EGFP via PCR (primer1: caaacatggggagcagcaagagcaagcccaaggaccccagccagcgcagttcatctgtgagcaagggcg aggag, primer2: ctcgagttacttatacagctcgtc). The resulting product was sub-cloned into pCR-TOPO2.1 (Invitrogen), and inserted into pUAST via EcoR1 and Xho1

(Brand and Perrimon, 1993). Transgenic flies were generated by P element insertion according to standard procedures.

Immunohistochemistry and Confocal Imaging

Pupal and adult brains were dissected in 2% Paraformaldehyde in 0.1 M L-lysine containing 0.05M phosphate buffer, and washed in PBS containing 0.5% Triton X-100. Pupal brains were fixed for 55 min and adult brains for 70 min at room temperature. For immunolabeling, the following antibodies were used: anti-RFP (rabbit 1:100) and anti-GFP (chicken 1:1000) from Abcam, anti-V5-alexa647 (mouse 1:10) from Serotec, anti-mAb24B10 (mouse 1:10), anti-Csp2a (6D6, ms 1:10), anti-Ncad (rat DN-Ex8, 1:100), anti-Fmi (mouse 1:50) and anti-Ecad (DCAD2, rat 1:10) from Developmental Studies Hybridoma Bank. For immunofluorescence, the following secondary antibodies were used: goat anti-chicken Alexa-488, goat anti-mouse Cy3, goat anti-mouse 546 IgG1, goat anti-rat Cy5, goat anti-rat Alexa-633, goat anti-mouse Alexa-633 IgG2a, goat anti-rabbit Cy3, goat anti-mouse Alexa-488 (all at 1:200, Invitrogen).

For staining of pupal brains without detergent, washes and primary antibody incubation was performed in saline solution without Triton-X100. Subsequent washes and secondary antibody incubation were performed in PBS with 0.5% Triton-X100. It was also confirmed that the chicken anti-GFP antibody was able to penetrate the brain in the absence of Triton-X100 (data not shown).

To stochastically label single R cells, an FRT-flanked stop cassette of *gmr-FRT-white-FRT-GFPmyr* was excised by activating Flipase expression using a 4’20” heat shock ca. 24-26 hours prior to 0% apf. For single cell MARCM experiments, third instar larvae were heat shocked at 37°C for 18-22 minutes 30-34 hours (at 25°C) prior to 0% apf. For generating larger clones, larvae were heat shocked about 72 hours prior to 0% apf for 40-50 minutes. For random labeling of single R cells using conditional *Ncad-V5*, third instar larvae were heat shocked at 37°C for 4-5 minutes, 24-27 hours prior to 0% apf.

Cryosectioning and SIM imaging

Pupal brains were dissected in 2% Paraformaldehyde in 0.1 M L-lysine containing 0.05M phosphate buffer (PB), fixed for 55 min at room temperature and washed in PB. Brains were cryoprotected in 5, 10, and finally 20% sucrose in PB overnight at 4°C and embedded in NEG 50 (Thermo Scientific). 10μm thin sections were cut on a cryotome, collected onto poly-L-lysine (Sigma) coated no. 1.5 coverslips and rehydrated. For immunohistochemistry, brains were washed in PB and PBS, followed by blocking in PBS with 0.5% Triton-X100 and 10% normal goat serum (NGS). Incubation in primary and secondary antibodies was performed according to our standard procedures (chicken anti-GFP at 1:5000, mouse anti-Fmi at 1:200, chicken alexa-488 and mouse Cy3 at 1:1000). Sections were then rinsed in water and mounted in antifade mounting medium for imaging.

Positions of thin sections on the slide were determined using a Zeiss Axioscope with Axiovision Software and coordinates were converted between systems using the Mosaic Planner Software. Superresolution imaging was performed on an OMX V4 structured illumination microscope (Applied Precision) with a 60x N.A. 1.42 lens. Images were acquired using API DeltaVision OMX Master acquisition software and processed using OMX softWoRx.

Image Analysis

Measuring targeting errors in adults

To measure the targeting error rate of R cells, adult brains were stained with Csp2a to outline all R cell terminals. The number of terminals per cartridge was manually counted, excluding all equatorial and the three peripheral-most rows of cartridges. Presence of one cartridge with seven terminals and a neighboring cartridge with five terminals, or presence of only one cartridge with seven terminals counted as one targeting error. Presence of one cartridge with five terminals only was counted as an error in cell differentiation or death.

Identification of R cell subtypes

Randomly labeled single R cell subtypes were identified by their stereotyped position within the ommatidium in the retina and subsequently in the

lamina based on both axon tracing and the preservation of spatial relationships between labeled R cells in both structures. At 0 and 20% apf, R cells were identified by their position within the ommatidium relative to R3 and R4 cells, which were simultaneously labeled using *mδ-Gal4* driven expression of *UAS-mtdTomato*.

Measuring growth cone polarity

We used the mean alignment of filopodia as a measure of growth cone polarity. Filopodia were defined as filamentous protrusions from the growth cone; lamellipodia were not observed. As the majority of filopodia extended primarily along the plane of the lamina, extension across the depth of the lamina was disregarded. Measurements were made in straight lines only, from the growth cone base to the filopodial tip. Thus, filopodial length was defined as the distance covered within the plane of the lamina. The angles of all filopodia were registered to the line bisecting the anterior from the posterior half of the lamina, which runs perpendicular to the equator. To obtain this line, we measured either the parallel alignment of neighboring R4 growth cones labeled by *mδ-Gal4 UAS-mtdTomato*, or the alignment of R cell bundles just a couple of microns distal to the lamina. As growth cones from the dorsal and ventral lamina hemispheres are mirror-symmetric to each other, growth cones measured from the ventral hemisphere were DV flipped ("dorsalized") and their data was merged with data from all dorsal growth cones.

For each growth cone, a mean filopodial orientation vector consisting of mean length $\bar{l}$ and angle $\bar{\theta}$ was calculated using standard trigonometry equations (see below):

$$\bar{x} = \sum l_i \cos\theta_i \qquad \bar{y} = \sum l_i \sin\theta_i$$

$$\bar{\theta} = \tan\left(\frac{\bar{y}}{\bar{x}}\right) \quad \text{if } \bar{x} > 0 \qquad \bar{\theta} = \tan\left(\frac{\bar{y}}{\bar{x}}\right) + \pi \quad \text{if } \bar{x} < 0$$

$$\bar{l} = \sqrt{\overline{(x^2 + \overline{y^2})}}$$

Data are shown in polar plots with a standard ellipse that was calculated according to Batschelet (1981). In short, the axes lengths of the ellipse are $2s_x$ and $2s_y$ (s= standard deviation); its center is the mean vector and roughly 40% of sample points fall inside the ellipse.

Because circular data follows a Rayleigh instead of a Gaussian distribution, standard tests for variance could not be applied to the polarity data. Instead we performed statistics using a bootstrap method to obtain the population distribution of the long axis of the standard ellipse for each R cell type and each genotype. These distributions were then compared using the Z-test.

Measuring angles of R cell targets (Figure 2 and S2)

LMCs were labeled using *GH146-Gal4* expression of *UAS-myrEGFP* and R cells were simultaneously labeled with mAb24B10 at 28% apf. Target angles were measured from the center of the R cell bundle visualized by mAb24B10 to the boarders of LMCs labeled by myrEGFP.

Quantification of targeting defects

Growth cone targeting phenotypes were scored at 38 or 40% apf, about six to eight hours after growth cones have extended to their targets. R cells within ommatidia with planar cell polarity defects were not included in the analysis. For all MARCM single cell analysis, intra-ommatidial two-cell clones were also excluded from analysis, except for combinations consisting of one outer (R1-R6) with one inner R cell (R7, R8).

Quantification of protein expression levels

To quantify colocalization of Ncad or Fmi with single GFP labeled growth cones, thresholds were set manually in Imaris, and the number of colocalizing voxels was recorded for each growth cone and subsequently averaged.

To measure growth cone volume, the growth cone signal was separated from the background by thresholding, and the number of voxels above the threshold was counted and converted to μm^3.

3D reconstructions of SIM images

Growth cone outlines were manually traced in Fiji/ Image J and used as a binary 3D mask. Using Imaris, only Fmi protein within the mask was selected using colocalization between the mask and Fmi protein. Both the colocalization channel and the CD8-GFP channel were then reconstructed using iso-surfacing in Imaris. Three growth cones imaged from different angles were reconstructed.

SUPPLEMENTAL REFERENCES

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