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Supplemental Information

Cas Adaptor Proteins Organize the Retinal Ganglion Cell Layer Downstream of Integrin Signaling

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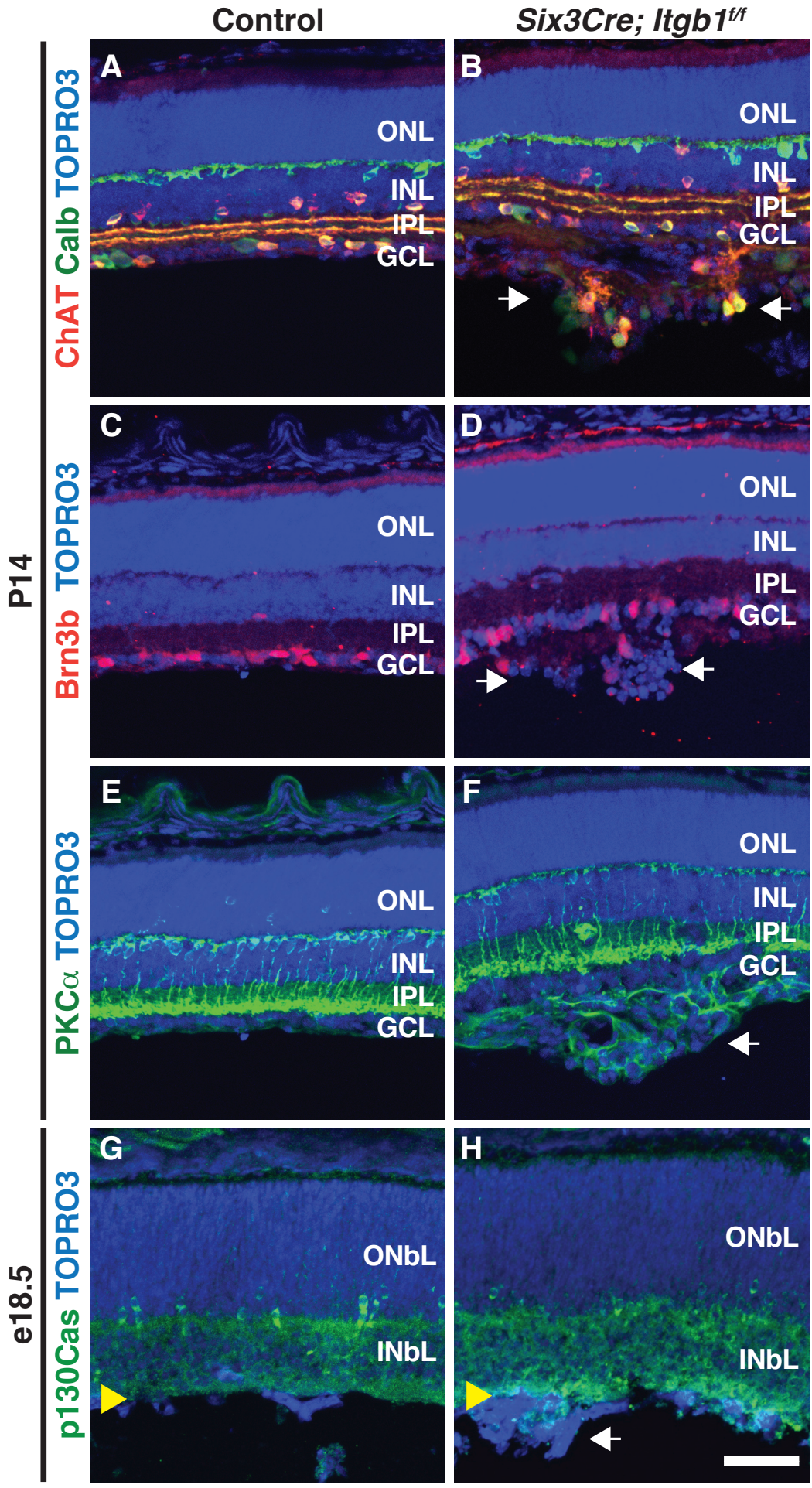
Control
Six3Cre; Itgb1f/f
P14
e18.5
ChAT Calb TOPRO3
Brn3b TOPRO3
PKCα TOPRO3
p130Cas TOPRO3
A
B
C
D
E
F
G
H
ONL
INL
IPL
GCL
ONbL
INbL

Figure S1

Figure S1, related to Figure 1. Ectopic Rosette Structures in *Six3Cre; Itgb1$^{f/f}$* Retinas Contain Multiple GCL Cell Types

(A-F) Immunostaining of Control (A, C and E) and *Six3Cre; Itgb1$^{f/f}$* (B, D and F) retina sections using antibodies for the AC and RGC marker calbindin (Calb, green A and B), the starburst AC marker ChAT (red A and B), the RGC marker Brn3b (C and D), and the Rod ON BC marker PKCα (E and F). Ectopic aggregates in *Six3Cre; Itgb1$^{f/f}$* (B, D and F) retinas contain both RGCs (A-D) and ACs (A and B), and they are innervated by BCs (E and F). (G and H) p130Cas total protein expression in the retina is identical in control (G) and *Six3Cre; Itgb1$^{f/f}$* (H) mice. Yellow arrowheads mark the ILM; white arrows mark the ectopic aggregates. Scale bar, 50 μm.

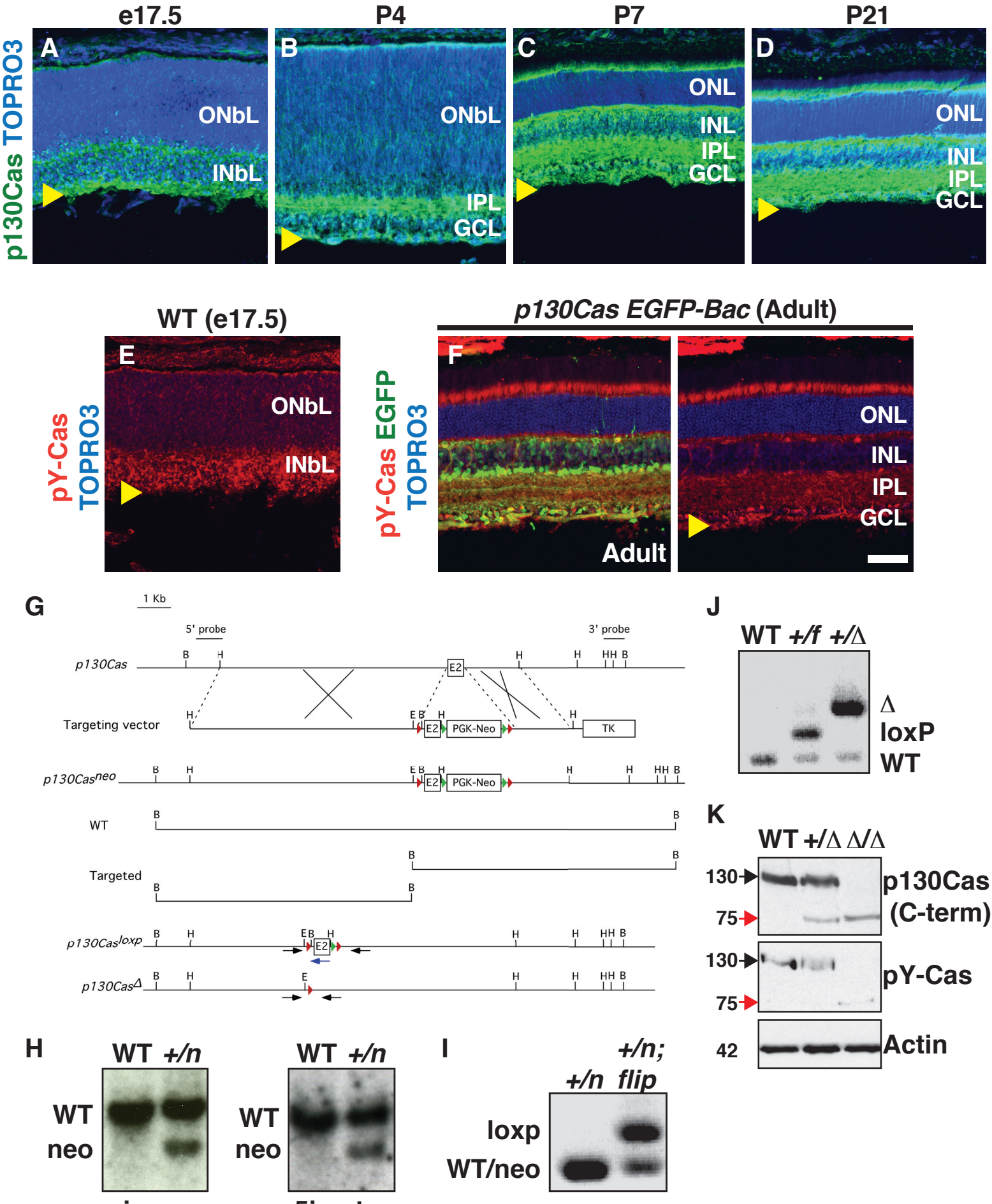
e17.5
P4
P7
P21
p130Cas TOPRO3
A
B
C
D
ONbL
INbL
IPL
GCL
ONL
INL
WT (e17.5)
p130Cas EGFP-Bac (Adult)
E
F
pY-Cas
TOPRO3
pY-Cas EGFP
Adult
G
1 Kb
5' probe
3' probe
p130Cas
Targeting vector
p130Casneo
WT
Targeted
p130Casloxp
p130CasΔ
E2
PGK-Neo
TK
J
WT +/f +/Δ
Δ
loxP
WT
K
WT +/Δ Δ/Δ
130
75
42
p130Cas
(C-term)
pY-Cas
Actin
H
WT +/n
WT
neo
inner
5' outer
I
+/n;
+/n flip
loxp
WT/neo

Figure S2

Figure S2, related to Figure 2. Expression of Endogenous p130Cas Protein in the Retina, and Generation of a Targeted Conditional *p130Cas/Bcar1* Allele

(A-D) Expression profile of p130Cas protein in the mouse retina during development at e17.5 (A), P4 (B), P7 (C) and P21 (D) in WT animals. (E) Expression of phosphorylated-p130Cas (PY-Cas) in e17.5 WT retinas is indistinguishable from PY-Cas expression in *p130Cas EGFP-Bac* animals (compare to Figure 2B). (F) Immunohistochemistry for EGFP (green) and PY-Cas (red) in adult *p130Cas EGFP-Bac* retinas. (G-K) Generation of a targeted, conditional, *p130Cas* allele. (G) Schematic representation of the targeting strategy to generate the $p130Cas^{\Delta}$ mutant allele. (H) Southern blot analysis of WT and targeted ES cells. Genomic DNA was digested with BamHI. R esults for the inner and 5'outer probe depicted in (G) are shown. Similar results were observed with the 3' probe. (I and J) PCR genotyping for the different alleles generated after crossing with a germline Flp line (I) or a germline Cre line (J). (K) Western blot analysis of WT and $p130Cas^{\Delta/\Delta}$ 11.5 dpc embryos. Although a truncated form of p130Cas is still present in $p130Cas^{\Delta/\Delta}$ mice, total p130Cas expression levels are almost completely abolished. In $p130Cas^{\Delta/\Delta}$ mutants, the levels of phospho-p130Cas are greatly reduced compared to WT. These results, in combination with our observation that $p130Cas^{\Delta/\Delta}$ mice die at the same developmental stage as the null mutants, suggest that the $p130Cas^{\Delta}$ allele behaves as a null (or a very strong hypomorphic) *p130Cas* allele. Protein size for each band is expressed in kDa and presented on the left side. Yellow arrowheads mark the ILM. Scale bar, 50 μm.

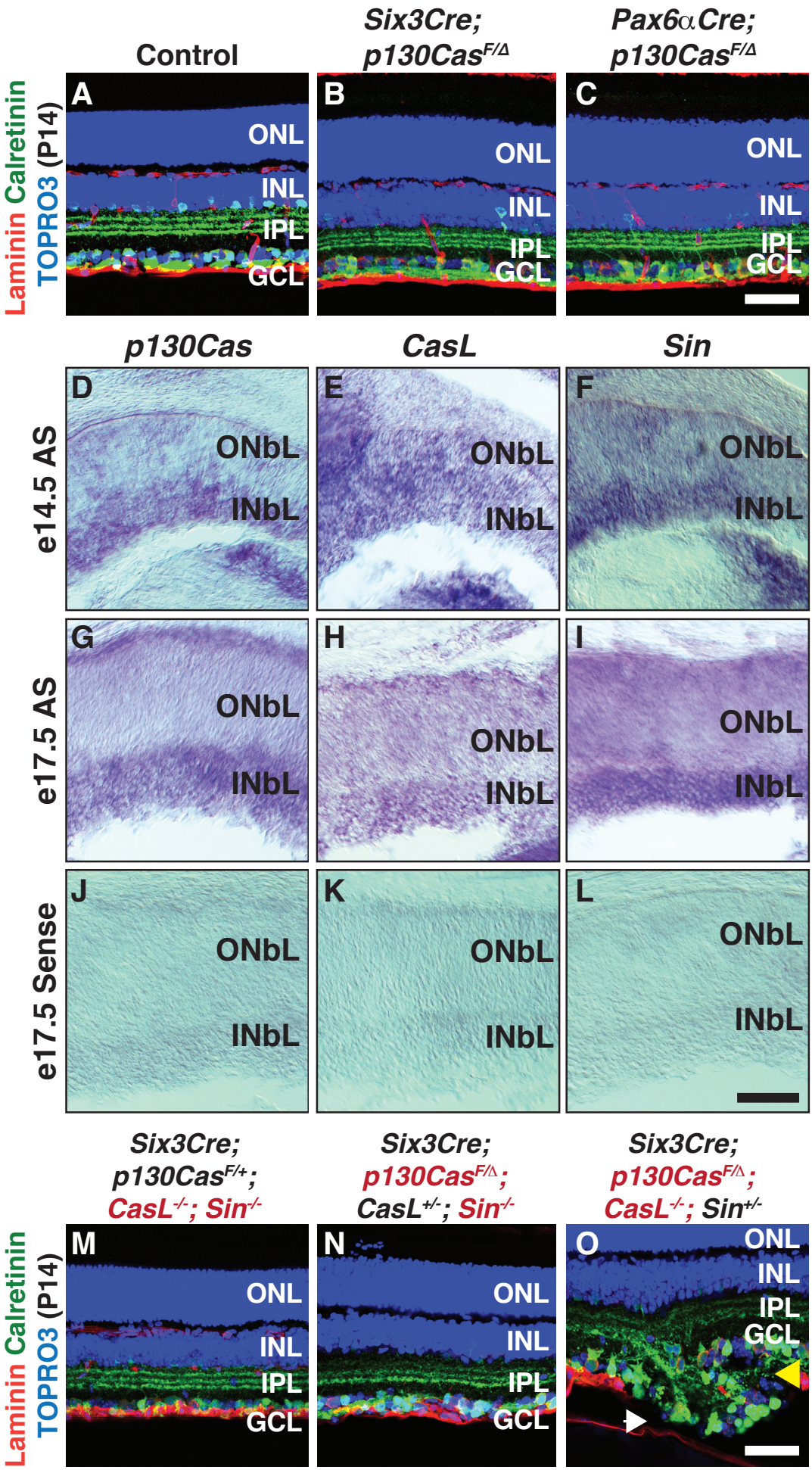
Control
Six3Cre; p130Cas F/Δ
Pax6αCre; p130Cas F/Δ
Laminin Calretinin TOPRO3 (P14)
A
ONL
INL
IPL
GCL
B
ONL
INL
IPL
GCL
C
ONL
INL
IPL
GCL
p130Cas
CasL
Sin
e14.5 AS
D
ONbL
INbL
E
ONbL
INbL
F
ONbL
INbL
e17.5 AS
G
ONbL
INbL
H
ONbL
INbL
I
ONbL
INbL
e17.5 Sense
J
ONbL
INbL
K
ONbL
INbL
L
ONbL
INbL
Six3Cre; p130Cas F/+; CasL -/-; Sin -/-
Six3Cre; p130Cas F/Δ; CasL +/-; Sin -/-
Six3Cre; p130Cas F/Δ; CasL -/-; Sin +/-
Laminin Calretinin TOPRO3 (P14)
M
ONL
INL
IPL
GCL
N
ONL
INL
IPL
GCL
O
ONL
INL
IPL
GCL

Figure S3

Figure S3, related to Figure 3. Cas Adaptor Proteins Act Redundantly During Retina Development

(A-C) Histological assessment of Control (A), *Six3Cre; p130Cas$^{f/\Delta}$* (B) and *Pax6αCre; p130Cas$^{f/\Delta}$* (C) retina sections using antibodies against laminin (red), calretinin (green), and TOPRO3 (blue). *Six3Cre; p130Cas$^{f/\Delta}$* (B) and *Pax6αCre; p130Cas$^{f/\Delta}$* (C) retinas are stratified, show normal lamination, and are indistinguishable from Control (A). (D-L) *In situ* hybridization of e14.5 (D-F) and e17.5 (G-L) WT retinas with antisense (D-I, AS) and sense control (J-L) probes for *p130Cas* (D, G and J), *CasL* (E, H and K) and *Sin* (F, I and L). The three distinct *Cas* genes are expressed in an overlapping fashion and are mainly enriched in the INbL. (D-I). (M-O) Immunostaining of *Six3Cre;p130Cas$^{f/+}$;CasL$^{-/-}$;Sin$^{-/-}$* (M), *Six3Cre;p130Cas$^{f/\Delta}$;CasL$^{+/-}$;Sin$^{-/-}$* (N) and *Six3Cre;p130Cas$^{f/\Delta}$;CasL$^{-/-}$;Sin$^{+/-}$* (O) P14 retinas using antibodies against laminin (red) and calretinin (green), and the nuclear counterstain TOPRO3 (blue). *Six3Cre;p130Cas$^{f/+}$;CasL$^{-/-}$;Sin$^{-/-}$* (M) and *Six3Cre;p130Cas$^{f/\Delta}$;CasL$^{+/-}$;Sin$^{-/-}$* (N) retinas show normal lamination and stratification, but *Six3Cre;p130Cas$^{f/\Delta}$;CasL$^{-/-}$;Sin$^{+/-}$* (O) retinas fully recapitulate the *Six3Cre;TcKO (Six3Cre;p130Cas$^{f/\Delta}$;CasL$^{-/-}$;Sin$^{-/-}$)* phenotypes. INbL: inner neuroblastic layer; ONbL: outer neuroblastic layer. Yellow arrowheads mark the ILM; white arrows mark the ectopic aggregates. Scale bar, 50 μm for A-C and M-P, and 100 μm for D-L.

Control
Six3Cre;TcKO
CTB-488 CTB-546 (P14)
CTB-546 T3 (Adult)
SCN
SC
LGN
SC + MTN
LGN
A
A'
B
B'
C
C'
D
D'
E
E'
P0
P14
P3
Lam Calr T3
PECAM T3
GS T3
βGal T3
Six3Cre;Rosa26LZ/+
F
G
H
I
J
K
L
M
N
N'
ONL
INL
IPL
GCL

Figure S4

Figure S4, related to Figure 4. RGC Axonal Central Projections are Formed in the Absence of Cas Adaptor Protein Function, and Histological Analysis of Müller Glia and ILM Disruption

(A-D') Coronal sections showing RGC axonal projections in the main retinorecipient brain areas of Control (A-D) and *Six3Cre; TcKO* (A'-D') adult mice injected with Alexa-488 cholera toxin (CTB-488, green) in the right eye and Alexa-546 cholera toxin B (CTB-546, red) in the left eye. The RGC projections in *Six3Cre; TcKO* adult mice (A'-D') are indistinguishable from projections in Control animals (A-D). (E and E') Injection sites of CTB-Alexa546 in the LGN for RGC retrograde labeling. (F-M) Immunohistological analyses of Control (F, H, J, L) and *Six3Cre; TcKO* (G, I, K, M) retinas. (F and G) Immunostaining of Control (F) and *Six3Cre; TcKO* (G) P0 retinas with anti-Calretinin (Calr, Green) and anti-laminin (Lam, Red) antibodies, and the nuclear stain TOPRO3 (T3, blue). The laminin-rich ILM is only disrupted at sites where aggregates form (white arrows). (H-K) Immunohistochemistry of Control (H, J) and *Six3Cre; TcKO* (I and K) P14 retinas with an antibody against the vascular marker PECAM reveals exuberant vascularization of ectopic GCL aggregates (white arrow). (L and M) Immunostaining with anti-glutamine synthetase (GS) shows mildly disrupted Müller glia endfeet organization in *Six3Cre; TcKO* (M) animals (yellow arrowhead), as compared to control (L). (N and N') Immunohistochemistry of *Six3Cre; Rosa26*$^{LZ/+}$ P3 retinas with anti-βGal antibody (green) and TOPRO3 (T3, blue) reveals widespread Cre activity in the retina, with the exception of the neuroepithelial cells that form the ILM (red arrowheads). SCN, suprachiasmatic nucleus; SC, superior colliculus; LGN, lateral geniculate nucleus; MTN, medial terminal nucleus. Scale bars, 250 μm for A-E', 200 μm for F-I, 100 μm for N, and 50 μm for I-L and N'.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals

The day of vaginal plug observation was designated as embryonic day 0.5 (e0.5) and the day of birth in this study was designated as postnatal day 0 (P0). Generation of the *β1-integrin*$^{f/f}$ (*Itgb1*$^{f/f}$), *Six3Cre*, *Pax6αCre*, *CasL*$^{-/-}$ and *Sin*$^{-/-}$ mouse lines has been described previously (Donlin et al., 2005; Furuta et al., 2000; Marquardt et al., 2001; Raghavan et al., 2000; Seo et al., 2005). Control animals were *Itgb1*$^{f/+}$ for conditional integrin mutants or *CasL*$^{-/-}$*; Sin*$^{-/-}$*; P130Cas*$^{+/f}$ for the *Six3Cre;TcKO Cas* and *Pax6αCre*; *TcKO Cas* mutants.

Generation of p130Cas conditional allele

The mouse line carrying a targeted allele for the *Bcar1* (*p130Cas*) locus was generated using the vector PL253, modified by Bac recombineering (Liu et al., 2003). The *p130Cas*$^{neo/+}$ allele was engineered to carry two loxP sites (target recognition sequences for Cre recombinase) flanking Exon 2 of the *p130Cas* gene (Figure S2), and an FRT-flanked *neomycin* cassette. Germline transmission of the *p130Cas*^{neo} allele was verified by Southern Blot and a PCR genotyping strategy (Figure S2). Deletion of coding exon 2 to generate the *p130Cas*$^{\Delta}$ allele was induced by crossing recombinant *p130Cas*$^{neo/+}$ mice with *EIIa-cre* mice, which carry the *Cre*-transgene under the control of the adenovirus *EIIa* promoter (Lakso et al., 1996). In order to eliminate the deleterious effects of the *neomycin* gene, *p130Cas*$^{neo/+}$ animals were crossed to deleter mice carrying ACT-FlpE,

which expresses FlpE recombinase under the direction of the human ACTB promoter (Rodriguez et al., 2000). FlpE mediates germline excision of the *neomycin* gene and leaves a single FRT in the genomic DNA, as well as two *loxP* sites flanking exon 2 ($p130Cas^{f/+}$). All breedings at the chimera stage were done into the C57/Bl6J mouse strain and were then backcrossed into the 129/SvJ background. PCR genotyping of $p130Cas^{\Delta/f}$ mice was done using the following primers: Loxp5BcarF: 5'-caagttctaggatagccaagg-3'; Loxp5BcarR: 5'-tcatctactaggctgccaatg-3' and BcarDeltaR: 5'-ccacaggctttatgttcacatc-3'.

Immunohistochemistry

Eyes were fixed and processed as previously described (Matsuoka et al., 2011). Primary antibodies used in this study include: mouse anti-CD29 (β1-integrin, BD Biosciences, 1:200), rabbit anti-p130Cas C terminal (Santa Cruz, 1:200), rabbit anti-p130Cas PY165 (Cell Signaling Technology, 1:100), rabbit anti-laminin (Sigma, 1:1000), rabbit anti-GFP (Lifescience Technologies, 1:500), chicken anti-GFP (AVES, 1:1000), rabbit anti-Calbindin (Swant, 1:2000), goat anti-Calretinin (Swant, 1:2000), goat anti-ChAT (Millipore, 1:100), mouse anti-Brn3a (Millipore, 1:20), mouse anti-Brn3b (gift from Dr. Jeremy Nathans, 1:200), mouse anti-protein kinase C α (Millipore, 1:500), Guinea pig anti-vGlutI (Millipore, 1:500), rabbit anti-vGAT (Synaptic Systems, 1:500), Rat anti-heparan sulfate proteoglycan (Perlecan) (Millipore, 1:500), rat anti-PECAM (BD biosciences, 1:500), Mouse anti-glutamine synthetase (GS) (Millipore, 1:500).

In situ hybridization

In situ hybridization was performed on fresh frozen retina sections (20 μm thickness) using digoxigenin-labeled cRNA probes as previously described (Giger et al., 2000). cDNA templates for the cRNA probes were generated using the following primers: *CasL* F:5- GGG TCT AGA ACC GCG GTG GAC AAA GTA GAG C-3, R:5- GGG GAA TTC AGA GGG CGT CGA TGG CGT TGA G -3; *p130Cas* F:5- GGG TCT AGA ATC TAC CAA GTT CCT CCA TCT CTG-3, R: 5- GGG GAA TTC ACA CCA TCG TCA ACT ACA CTC CC-3; *Sin* F: 5- GGG TCT AGA CGT GGC AGA GGT CTA TGA TGT G-3, R: 5- GGG GAA TTC GCT GGA TCA TTG GCT ACC TCC C-3. PCR products were then cloned using the Strataclone Blunt Cloning Kit (Stratagene).

Wholemount retinal staining

Wholemount retina ICC was performed as previously described (Matsuoka et al., 2011), with a few modifications. Briefly, enucleated eyes were fixed in 4% paraformaldehyde (PFA) for 1 hour at 4°C. The eyecups were dissected out and incubated for 4-5 days at room temperature with primary antibodies in PBS containing 10% donkey serum, 0.5% Triton X-100 and 20% dimethyl sulphoxide (DMSO). Retinas were washed with PBS+0.5% Triton X-100 5 times for 1 hour at room temperature, and then incubated with secondary antibodies in PBS+0.5% Triton X-100 overnight at 4°C. Retinas were washed in PBS+0.5% Triton X-100 5 times for 1 hour at room temperature and then flat

mounted. Confocal images were taken using a Zeiss Axioskop2 Mot Plus, LSM 5 Pa confocal microscope.

Cholera toxin subunit B (CTB) injection

Bilateral CTB injection was performed as previously described (Matsuoka et al., 2011). Briefly, the animals were anesthetized using isoflurane, and then injected with 2 μL CTB-Alexa-555 or CTB-Alexa-488 (Lifescience Techologies, 1mg/mL), bilaterally into the vitreous of each eye. For retrograde labeling, 0.3 μL CTB-Alexa-555 were injected bilaterally into the LGN using a stereotactic frame. Two days after the procedure, the animals were perfused with 4% PFA; and the brains were dissected out and further sectioned using Leica VT1000 S Fully Automatic Vibrating Blade Microtome. The eyes were dissected out, post-fixed in 4% PFA, cryoprotected and sectioned using a cryostat.

***In utero* retina electroporation**

In utero retina electroporation was performed as previously described with a few modifications (Garcia-Frigola et al., 2007; Petros et al., 2009). Briefly, e13.5 *p130Cas*$^{F/F}$*; CasL*$^{-/-}$*; Sin*$^{-/-}$ embryo retinas were injected intravitreally with pCAGGS-Cre-IRES-EGFP plasmid (4 μg/μL) and electroporated with five 40 volts, 50 ms square pulses at a frequency of 60 Hz. As a result, EGFP is expressed in the same cells that express Cre recombinase. The embryos were allowed to develop until e18.5 before being sacrificed and further processed.

SUPPLEMENTAL REFERENCES

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