Supporting Information

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SI Materials and Methods

Immunohistochemistry and Microscopy. For BrdU labeling, pups were injected i.p. with BrdU at the dosage of 0.1 mg/g body weight 2 h before the eyes were enucleated. A drop of tissue-marking dye (Triangle Biomedical Sciences) or Histogel (Thermo Scientific) was dripped onto the dorsal side of the eyes upon enucleating. Eyes were fixed in 4% formaldehyde at 4 °C overnight and then processed through standard tissue processing techniques. For detecting the phosphorylated form of SMAD proteins 1, 5, and 8 (Smad1/5/8), a phosphatase inhibitor mixture (Calbiochem) was added during tissue fixation. The sectioned eye tissues on the slides were treated with heat in 1× citrate buffer for antigen retrieval, blocked with Powerblock (Biogenex), and incubated with primary antibodies overnight at 4 °C. Primary antibodies used in this study include: pSMAD1/5/8 (Cell Signaling, #9511); Notch2 (Developmental Studies Hybridoma Bank or DSHB, C651.6DbHN); Jagged (Jag)1 (Santa Cruz Biotechnology, H-66); BrdU (Megabase Research Products, #BP40250); GFP (Invitrogen, A10262); Connexin43 (Cell Signaling, #3512); orthodenticle homeobox 1 (Otx1) (DSHB, Otx-5F5); paired box 6 (Pax6) (Zymed, #426600); and Collagen IX (DHSB, D1-9). All secondary antibodies conjugated with fluorescent chromes (Alexa 488 and Alexa 568) were purchased from Invitrogen. After immunostaining, tissue sections were counterstained with DAPI (Invitrogen) and mounted with Vectashield Mounting Media (Vector Laboratories). Apoptosis was detected using the ApopTag Fluorescein In-Situ Apoptosis Detection Kit (Chemicon). For histological analysis of *Jag1* mutant mice, eyes were fixed by immersion in 2.5% glutaraldehyde and 2% paraformaldehyde at 4 °C for 24 h and processed as previously described (1). Only sections going through the middle of the eye and containing optic nerve were examined.

Western Blotting. The outer ciliary epithelium (OCE) layer of the control and *Notch2 CKO* mutant P3 ciliary bodies (CBs) was dissected, and then ground in SDS sample buffer. The protein lysates were subjected to SDS/PAGE electrophoresis. The primary antibodies used for Western blotting were: bone morphogenetic protein (BMP) receptors Bmpr1a, Bmpr1b, Bmpr2, Smad1, Smad5, Smad8 (Santa Cruz); pSMAD1/5/8 (Cell Signaling); and β-actin (Abcam). The secondary HRP-conjugated antibodies were purchased from Promega, and Western Lightning Plus-ECL reagent (PerkinElmer) was used for signal development.

Lentivirus Production, Intraocular Injection, and Cell Culture. cDNAs for full-length coding sequence of (Chordin-like 1) *Chrdl1* and (Neuroblastoma 1) *Nbl1* were cloned by PCR and cloned into the vector pIRES2-enhanced green fluorescent protein (EGFP) (Addgene). Coding sequences of *Chrdl1* and *Nbl1* together with IRES-EGFP was subcloned into the pSicoR lentivirus vector (Addgene). High-titer lentiviruses were produced by cotransfecting the construct and packaging plasmids psPAX2 and pMD2.G (Addgene) into 293T cells and were then injected into the CB region of around birth (P0) CD1 pups. Eyeballs were collected at P3 for further analysis. For the in vitro assays, after lentiviruses had been added to cultured 293T cells for 4 h, culture medium was replaced by fresh medium supplemented with either DMEM only or DMEM containing 2 ng/mL, 5 ng/mL, and 10 ng/mL recombinant human BMP4 (PeproTech). Cells were harvested 12 h later for Western blotting analysis.

1. Harder JM, Libby RT (2011) BBC3 (PUMA) regulates developmental apoptosis but not axonal injury induced death in the retina. *Mol Neurodegener* 6:50.

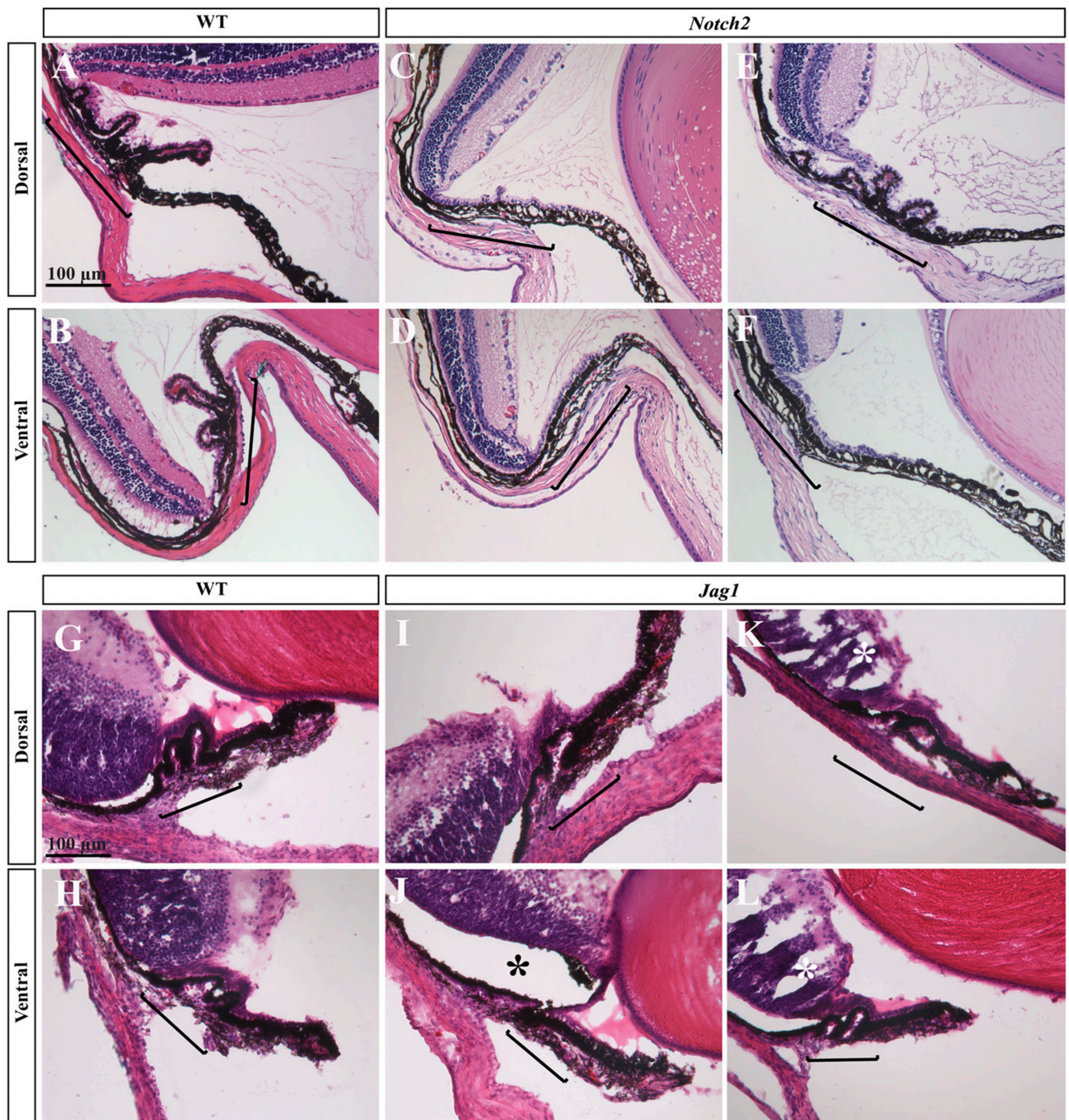

Fig. S1. The morphogenesis defect persists in the adult *Notch2* conditional knockout (*CKO*) mutant CB. (*A*–*F*) H&E staining of paraffin sections, whereas *G*–*L* represent H&E staining on cryosections. The bracket in *A*–*L* indicates the CB region. (*A* and *B*) The control CB on the dorsal and ventral of the adult control eye. (*C*–*F*) The morphogenesis is completely disrupted on the ventral side (*D* and *F*), but more variable on the dorsal side (*C* and *E*) of the adult *Notch2 CKO* eye. (*G* and *H*) The control CB on the dorsal and ventral of the P3 control eye. (*I*–*L*) The CB morphogenesis is completely or partly disrupted on both dorsal (*I* and *K*) and ventral sides (*J* and *L*) of the P3 *Jag1 CKO* eye. *Artifact caused by cryosectioning. (Scale bar: 100 μm.)

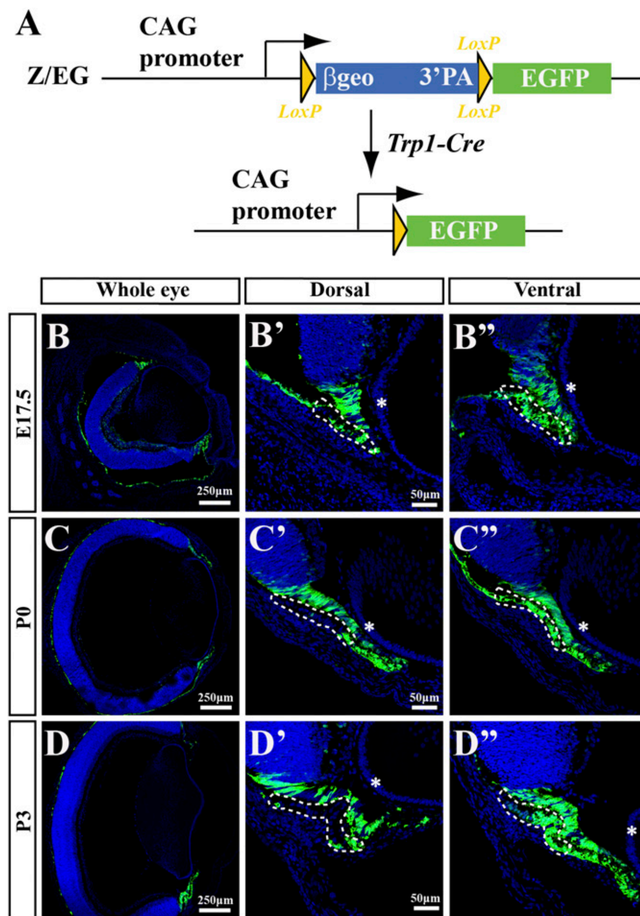

Fig. S2. Tyrosinase related protein 1 (*Trp1*)-*Cre* efficiently catalyzes LoxP-mediated recombination in the OCE of the ventral but not dorsal CB. (*A*) *Trp1-Cre* removes the LoxP-flanked the *β-galactosidase* and *neomycin* fusion gene (*βneo*) in *Z/EG* to activate GFP expression. (*B–D*) The CBs on the dorsal side of E17.5 (*B*), P0 (*C*), and P3 (*D*) *Trp1-Cre; Z/EG* eyes show highly mosaic GFP expression in the OCE (broken lines) on the dorsal side (*B′*, *C′*, and *D′*) and uniform GFP expression in the OCE (broken lines) on the ventral side (*B″*, *C″*, and *D″*). (Scale bars: *B–D*, 250 μm; *B′*, *B″*, *C′*, *C″*, *D′*, *D″*, 50 μm.)

Fig. S3. The cells in inner ciliary epithelium (ICE) and OCE layers proliferate at different rates during CB morphogenesis. (*A* and *B*) The cells in the OCE (O) proliferate at faster rates than those in the ICE (I) based on the percentages of BrdU-positive cells during CB morphogenesis (P0–P5), but not before (E17.5) and after (P7) the morphogenesis. The proliferation difference between the two layers is almost completely abolished in the *Notch2 CKO* mutant eye during CB morphogenesis (P0–P5). (*C*) The cell proliferation rates in the ICE of the *Notch2 CKO* mutant CB do not decrease significantly in comparison with those for the control CB (n = 14 for E17.5 WT; n = 3 for E17.5 *Notch2*; n = 11 for P0 WT; n = 12 for P0 *Notch2*; n = 12 for the rest).

Fig. S4. *Notch2* deletion does not affect CB cell survival. (*A* and *C*) In the E17.5 *Notch2 CKO* mutant CB (*C* and *C′*), there is no increase in TUNEL-positive cells in comparison with the control E17.5 CB (*A* and *A′*) in the OCE (broken lines). (*B* and *D*) In the P3 *Notch2 CKO* mutant CB (*D* and *D′*), there is no increase in TUNEL-positive cells in comparison with the control P3 CB (*B* and *B′*) in the OCE (broken lines).

Fig. S5. Otx1 and Pax6 protein expression remains unchanged in the *Notch2* mutant OCE. (*A* and *B*) Immunostaining results show that Otx1 and Pax6 proteins exhibit similar expression levels in both the P0 control (*A*–*A″*) and *Notch2* mutant (*B*–*B″*) OCEs (broken lines). (Scale bar: 50 μm.)

Fig. S6. *Chrdl1* and *Nbl1* can repress BMP signaling in cultured 293T cells. (*A*) Western blots show that BMP1a, BMPR1b, and BMPR2 protein levels remain unchanged in *Notch2* mutant OCE cells in comparison with control OCE cells. (*B* and *C*) Overexpression of *Chrdl1* and *Nbl1* but not *GFP* can significantly reduce pSMAD1/5/8 expression in 293 T cells when treated with BMP4. *B* exhibits the Western blot, whereas *C* shows quantitative results of three independent replicates. $*P < 0.05$. (*D*–*G*) Following lentivirus infection, high levels of *Chrdl1* and *Nbl1* mRNAs can be detected by RT-PCR (*D*) and GFP expression (*E*–*G*). *E* represents the GFP only control. (*H* and *H″*) *Nbl1*-overexpression in GFP-positive CB cells (*H′*) decreases, but does not eliminate, pSMAD1/5/8 expression (*H″*), and does not disrupt CB morphogenesis (*H*).

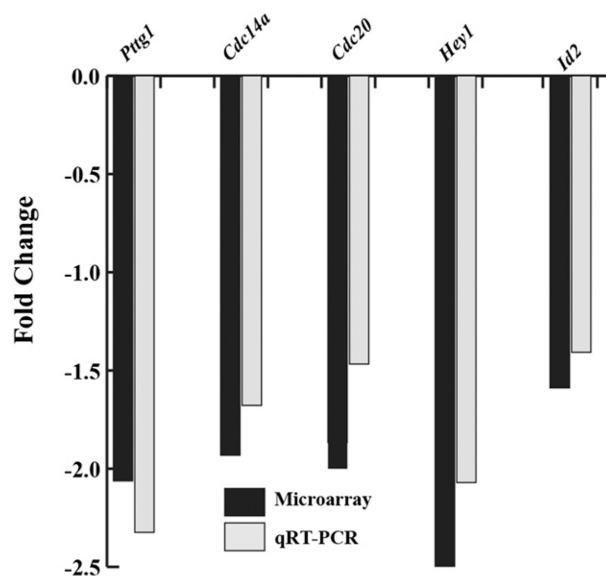

Fig. S7. The Notch target gene hairy/enhancer-of-split related with YRPW motif 1 (*Hey1*) and cell-cycle regulators are down-regulated in the *Notch2 CKO* mutant CB. Quantitative (q)RT-PCR results confirm the microarray finding that pituitary tumor-transforming gene 1 (*Pttg1*), cell division cycle (*Cdc*)*14a*, *Cdc20*, *Hey1*, and inhibitor of DNA binding 2 (*Id2*) are down-regulated in the *Notch2 CKO* mutant CB.

Table S1. Expression of many cell-cycle regulators is down-regulated in the *Notch2 CKO* mutant CB

Gene symbol	Gene name	Fold change*	*P* value	Note
Hey1	Hairy/enhancer-of-split related with YRPW motif-like	−2.5	0.004	Known Notch target gene
				Regulation of cell-cycle progression
Cdkn3	Cyclin-dependent kinase inhibitor 3	−2.1	0.04	Negative
Cdc20	Cell division cycle 20 homolog	−2.0	0.006	Positive
Cks2	CDC28 protein kinase regulatory subunit 2	−2.1	0.03	Positive
Cdc25c	Cell division cycle 25 homolog C	−2.1	0.01	Positive
Cdca3	Cell division cycle associated 3	−2.1	0.007	Positive
Cdca2	Cell division cycle associated 2	−2.1	0.006	Positive
Ttk	Ttk protein kinase	−2.2	0.01	positive
Ccna2	Cyclin A2	−2.2	0.02	Positive
Ccnb1	Cyclin B1	−2.2	0.01	Positive
Pttg1	Pituitary tumor-transforming gene 1	−2.3	0.02	positive
Cdca8	Cell division cycle–associated 8	−2.4	0.02	Positive
Plk1	Polo-like kinase 1	−2.5	0.01	Positive
Cdca5	Cell division cycle–associated 5	−2.5	0.009	Positive

*A negative number means down-regulation in gene expression.

Table S2. Expression levels of BMPs and their downstream components in the OCE between control and *Notch2* CKO mutant CBs

Gene symbol	Gene name	WT (mean ± STD)	*Notch2 CKO* (mean ± STD)	Fold change*	*P* value
BMP2	Bone morphogenetic protein 2	1,541 ± 74	981 ± 79	−1.6	0.01
BMP4	Bone morphogenetic protein 4	1,504 ± 300	4,105 ± 710	+2.7	0.09
BMP5	Bone morphogenetic protein 5	1,702 ± 277	2,151 ± 28	+1.2	0.2
BMP6	Bone morphogenetic protein 6	221 ± 28	320 ± 9	+1.5	0.02
BMP7	Bone morphogenetic protein 7	2,645 ± 225	2,639 ± 645	1.0	0.99
BMP8a	Bone morphogenetic protein 8a	36 ± 3	34 ± 3	−1.1	0.5
BMP8b	Bone morphogenetic protein 8b	129 ± 6	112 ± 7	−1.1	0.1
BMP10	Bone morphogenetic protein 10	137 ± 6	122 ± 14	−1.1	0.4
BMP15	Bone morphogenetic protein 15	58 ± 8	59 ± 7	1.0	0.9
BMPR1a	Bone morphogenetic protein receptor 1A	2,813 ± 172	2,864 ± 85	1.0	0.7
BMPR1b	Bone morphogenetic protein receptor 1B	208 ± 41	203 ± 34	1.0	0.9
BMPR2	Bone morphogenetic protein receptor II	8,826 ± 443	8,364 ± 579	1.0	0.5
SMAD1	MAD homolog 1	7,228 ± 333	6,971 ± 287	1.0	0.04
SMAD4	MAD homolog 4	10,661 ± 556	11,254 ± 607	+1.1	0.4
SMAD5	MAD homolog 5	240 ± 9	209 ± 15	−1.1	0.2
SMAD8	SMAD8 protein	106 ± 3	110 ± 3	1.0	0.3
ID2	Inhibitor of DNA binding 2	5,861 ± 170	3,652 ± 489	−1.6	0.08

Symbols + and – indicate an increase and a decrease in gene expression, respectively.
*For fold change, 1.0 means no change in gene expression.