Notch2 regulates BMP signaling and epithelial morphogenesis in the ciliary body of the mouse eye

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The ciliary body (CB) of the mammalian eye is responsible for secreting aqueous humor to maintain intraocular pressure, which is elevated in the eyes of glaucoma patients. It contains a folded two-layered epithelial structure comprising the nonpigmented inner ciliary epithelium (ICE), the pigmented outer ciliary epithelium (OCE), and the underlying stroma. Although the CB has an important function in the eye, its morphogenesis remains poorly studied. In this study, we show that conditional inactivation of the Jagged 1 (Jag1)-Notch2 signaling pathway in the developing CB abolishes its morphogenesis. Notch2 is expressed in the OCE of the CB, whereas Jag1 is expressed in the ICE. Conditional inactivation of *Jag1* in the ICE or *Notch2* in the OCE disrupts CB morphogenesis, but neither affects the specification of the CB region. Notch2 signaling in the OCE is required for promoting cell proliferation and maintaining bone morphogenetic protein (BMP) signaling, both of which have been suggested to be important for CB morphogenesis. Although Notch and BMP signaling pathways are known to cross-talk via the interaction between their downstream transcriptional factors, this study suggests that Notch2 maintains BMP signaling in the OCE possibly by repressing expression of secreted BMP inhibitors. Based on our findings, we propose that Jag1-Notch2 signaling controls CB morphogenesis at least in part by regulating cell proliferation and BMP signaling.

The mammalian eye is composed of the anterior segment, the posterior retina and the vitreous. The anterior segment consists of cornea, lens, and ciliary body (CB), whereas the posterior retina contains six types of retinal neurons and Müller glial cells, which are organized into three distinct cell layers. The light passes through the cornea and is then focused by the lens and detected by photoreceptors in the retina. Both the aqueous humor anteriorly and the vitreous humor posteriorly function together to sustain intraocular pressure (IOP) in the eye and thereby maintain its shape. Pressure regulation is particularly important because abnormally high IOP is a major risk factor for glaucoma (1). In the eye, the CB is responsible for producing aqueous humor (2). Although high IOP is often attributed to the blockage of the drainage system for the vitreous, known as the trabecular meshwork, abnormal CB function might also contribute to high IOP formation because of excess aqueous humor production. Finally, contraction of the muscles in the CB controls lens accommodation for near versus far vision. Despite its important biological functions and potential medical significance, the formation and development of the CB remain poorly studied.

The CB contains two layers of apically adhered epithelial sheets, the pigmented outer ciliary epithelium (OCE) and the nonpigmented inner ciliary epithelium (ICE), and the underlying stroma (2). It forms at the periphery of the developing optic cup and first segregates from the retina and then from the iris. One previous study suggested that blood vessel capillaries underneath the CB are believed to control the formation of folds (2). In addition, increased cell number and cell volume in the CB are associated with fold formation (3–5). Moreover, normal IOP is also required for fold formation in the CB (5). Finally, neural crest cell–derived stromal cells underneath the CB also contribute to CB morphogenesis (6, 7). These findings indicate that complex cellular interactions are critical for CB formation and morphogenesis.

Genetic studies in mice have identified a number of signaling pathways and factors that are important for CB formation and morphogenesis. FGF signaling is important for segregation from the retina, whereas Wnt signaling is critical for segregation from the iris (8–12). In mice, the CB begins its morphogenesis at around birth (P0), and continues the process during the first week to form three to four folds (13, 14) (Fig. 1 *A*–*D*). Through conditional knockout (CKO), *Dicer1*, which encodes the key enzyme in the microRNA pathway, has recently been shown to be required for CB fate specification (15). The bone morphogenetic protein (BMP) signaling pathway and transcription factors orthodenticle homeobox 1 (Otx1) and paired box 6 (Pax6) are critical for CB morphogenesis (16–20). BMP4 and BMP7 are expressed in both ICE and OCE of the CB, and disruption of BMP signaling leads to severe CB morphogenesis defects (10, 17, 21, 22). Although BMP signaling, Otx1, and Pax6 have been shown to be important for CB morphogenesis, how they are integrated in the CB to control the coordinated cellular events important for CB morphogenesis still remain poorly understood.

In mammals, four Notch receptors (Notch 1–4) and five ligands [Delta-like 1, 3, and 4 as well as Jagged (Jag) 1 and 2] play important roles in both developmental processes and disease pathogenesis (23). Upon engagement between the Notch receptor and its ligand, the Notch intracellular domain (NICD) is released from the transmembrane domain via a series of proteolytic cleavages and then translocates into the nucleus where it binds a recombination signal binding protein for immunoglobulin kappa J region (RBPJK) to activate transcription of its target genes, including the hairy and enhancer of split (*Hes*) and hairy/enhancer-of-split related with YRPW motif (*Hey*) gene families (23). In development, Notch signaling controls cell fate determination, tissue boundary formation, proliferation, and other cellular processes (24). Additionally, defective Notch signaling is associated with various cancers, including leukemia and breast and colon, and also with heritable genetic diseases such as Alagille syndrome and Hajdu-Cheney syndrome (25, 26). Notch and BMP pathways synergistically control gene expression via regulation of expression or physical interaction of two pathway components (27). In this study, we have revealed a unique cooperation between the two pathways in the regulation of CB morphogenesis.

Although *Notch1* and *Notch2* are expressed in the developing vertebrate eye (28, 29), only Notch1 has been shown to prevent premature differentiation of retinal progenitors and control

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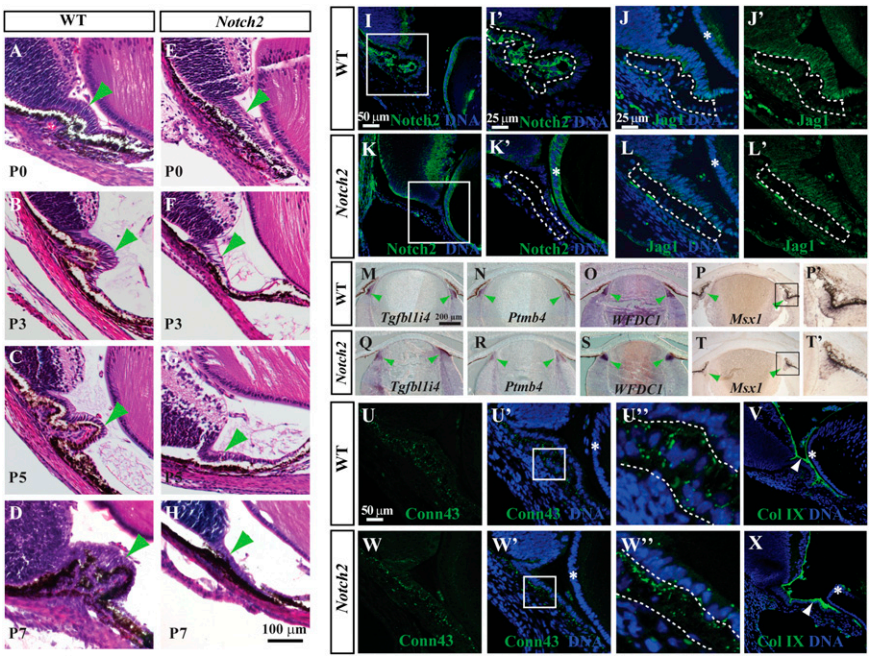

Fig. 1. Notch2 is required for CB morphogenesis. (*A–D*) The CB undergoes gradual morphogenesis at P0 (*A*), P3 (*B*), P5 (*C*), and P7 (*D*). (*E–H*) The CB fails to undergo any morphogenesis in the *Notch2 CKO* mutant at P0 (*E*), P3 (*F*), P5 (*G*), and P7 (*H*). Both Notch2 (*I*) and Jag1 (*J*) proteins are expressed in the developing P3 WT CB. Notch2 is expressed in the OCE, whereas Jag1 is expressed in the ICE. Notch2 (*K*) and Jag1 (*L*) protein expression in the *Notch2 CKO* mutant CB. Notch2 is efficiently deleted in the OCE, whereas Jag1 is reduced on the apical side of the ICE. *Tgfbl1i4* (*M* and *Q*), *Ptmb4* (*N* and *R*), *WFDC1* (*O* and *S*), and *Msx1* (*P* and *T*) mRNAs are expressed in the control E17.5 CB (*M–P*) and in the *Notch2 CKO* mutant CB (*Q–T*). Green arrows indicate the newly specified CB-iris region. *P′* and *T′* represent highlighted areas in *P* and *T* in a higher magnification. Conn43 and Collagen IX are expressed normally in the *Notch CKO* mutant CB (*U* and *V*) as in the control CB (*W* and *X*). *Lens. (Scale bars: *A–H*, 100 μm; *I* and *K*, 50 μm; *I′*, *J′*, *K′–L′*, and *U–X*, 25 μm; *M–T*, 200 μm.)

retinal progenitor proliferation and retinal lineage specification in the developing retina (30, 31). In this study, we use a retinal pigmented epithelium (RPE)-specific Cre line, tyrosinase related protein 1 (*Trp1*)-*Cre*, to conditionally inactivate the function of *Notch2* and show that it is required in the OCE to control CB morphogenesis. Furthermore, we show that Notch2 signaling regulates OCE cell proliferation and BMP signaling.

Results

***Notch2* Is Required in the OCE to Control CB Morphogenesis in the Mouse Eye.** *Notch2* is strongly expressed in the pigmented epithelium of the developing eye, including the CB region, but its role in eye development has not been investigated (28, 29). To investigate the role of *Notch2* in the regulation of RPE development in the developing eye, we used a floxed allele of *Notch2*, *Notch2*$^{fx/fx}$ (32), and an RPE-specific Cre line, *Trp1-Cre* (the *Cre* gene under the control of the *Trp1* promoter) (33), to conditionally inactivate *Notch2* function in the developing pigmented epithelium. Surprisingly, RPE-specific *Notch2 CKO* mutant adult eyes show no other discernible phenotype except lack of CB morphogenesis in comparison with the control (Fig. S1). The *Trp1-Cre* line exhibits some degree of RPE degeneration in the eye as recently reported (34), but its CB region is normal. The mutant CB phenotype is very consistent on the ventral side of the eye, but is more variable on the dorsal side ranging from no morphogenesis to normal morphogenesis (Fig. S1). This is likely caused by the uniform expression of *Trp1-Cre* in the CB on the ventral side of the developing eye and the highly mosaic expression on the dorsal side (Fig. S2). Consequently, our analysis of the *Notch2* mutant CB phenotype in this study is focused on the ventral side. These results indicate that *Notch2* is required for driving CB morphogenesis.

As reported previously (12, 35), the CB and iris regions are specified before birth, and the CB morphogenesis takes place within the first week after birth. The first fold in the CB region forms within the first 3 d (Fig. 1 *A* and *B*). The second fold forms from the third day to the fifth day (Fig. 1*C*), and the folding process finishes by the seventh day (Fig. 1*D*). In contrast, there are no detectable CB folds from P0 to P7 in the *Notch2 CKO* mouse eyes in comparison with the corresponding control (Fig. 1 *E–H*). Based on the persistence of the mutant phenotype in the adult (Fig. S1), we conclude that *Notch2* plays a necessary role in CB morphogenesis.

Notch2 Signaling Is Dispensable for the Specification of the CB Fate. Consistent with its mRNA expression patterns, Notch2 protein is expressed in the OCE of the CB at P3, and Jag1 protein is expressed only in the ICE (Fig. 1 *I* and *J*). In contrast, Notch2 protein is not detectable in the OCE of the *Notch2 CKO* mutant CB (Fig. 1*K*). Although Jag1 protein remains detectable in the ICE of the *Notch2 CKO* mutant CB, its expression is drastically reduced and is no longer restricted to the apical side, suggesting that the absence of Notch2 leads to down-regulation, degradation, and/or mislocalization of Jag1 (Fig. 1 *L* and *L′*). These expression results have further confirmed the efficient deletion of Notch2 in the OCE on the ventral side and further suggest that Jag1 might be the ligand for activating Notch2 in the CB.

One potential explanation for the failure of the morphogenesis of the *Notch2 CKO* mutant CB is that the CB cell fate fails to be specified in the mutant because Notch signaling often regulates cell fate specification (24). The WT control CB has been previously shown to express WAP four-disulfide core domain 1 (*WFDC1*), thymosin beta 4 (*Ptmb4*), transforming growth factor beta-1-induced transcript 4 (*Tgfbli4*), and Msh homeobox 1 (*Msx1*) (Fig. 1 *M–P′*) (36, 37). Our mRNA in situ results indicate that the *Notch2 CKO* mutant CB still expresses *WFDC1*, *Ptmb4*, *Tgfb1i4*, and *Msx1* at normal levels (Fig. 1 *Q–T′*). In addition, we also examined Connexin43 and Collagen IX protein expression for CB secretion function in the control and *Notch2 CKO* mutant CBs. Connexin43 forms gap junctions between ICE and OCE and controls aqueous humor production (38), whereas Collagen IX is secreted by the CB and accumulates on the surface of the CB and the retina (39). Both the control and *Notch2 CKO* mutant CBs exhibit similar levels of Connexin43 (Conn43) expression in the OCE and secreted Collagen IX protein on the surface of the CB (Fig. 1 *U–X*). These results suggest that Notch2 is dispensable for the specification of the CB region and its protein secretion.

Jag1 Is Required in the ICE to Control CB Morphogenesis. The expression patterns in the control and *Notch2 CKO* mutant CBs suggest that Jag1 might be the Notch2 ligand (Fig. 1 *I–L*). To further test this idea, we conditionally inactivated *Jag1* in the ICE using a sine oculis-related homeobox 3 (*Six3*)-*Cre* line and a *Jag1* floxed allele, *Jag1*$^{fx/fx}$. *Six3-Cre* can mediate gene deletion in the ICE (40), whereas *Jag1*$^{fx/fx}$ is a conditional

allele for allowing efficient deletion of *Jag1* (41). Similarly, the conditional inactivation of *Jag1* in the CB leads to a reduction or complete elimination of the folds at P3 and adults in comparison with the corresponding control CBs, indicating that Jag1 is required for CB morphogenesis (Fig. 2 *A–D*). *Otx1* and *Msx1* are markers for the developing CB-iris region, whereas *Hes5* is a marker for the neural retina and is normally excluded from the CB-iris region (36, 37). Consistent with the findings from the *Notch2 CKO* mutant, mRNA in situ hybridization results show that *Otx1*, *Hes5*, and *Msx1* exhibit similar expression patterns in both control and *Jag1 CKO* mutant CBs, indicating that the ciliary margin is correctly specified (Fig. 2 *E–J*). Taken together, our results suggest that Jag1 serves as the Notch2 ligand to control CB morphogenesis.

Notch2 Signaling Regulates Cell Proliferation in the CB. Differential proliferation between ICE and OCE of the CB during the first postnatal week has been proposed as important for CB morphogenesis (5, 13). To assess cell proliferation rates in both ICE and OCE of the control and *Notch2 CKO* mutant CBs, we performed comprehensive BrdU labeling experiments on different developmental stages ranging from E17.5 to P7. At E17.5, the cells in both ICE and OCE of the control CB proliferate at rapid and comparable rates (Fig. 3*A* and Fig. S3*A*). However, during P0–P5, cells in the ICE of the control CB proliferate two to three times slower than those at E17.5, whereas cells in the OCE continuously proliferate at rates comparable to or slightly lower than those at E17.5 (Fig. 3 *C*, *E*, and *G* and Fig. S3*A*). At P7, when the CB morphogenesis ends, cells in both CB layers proliferate at comparable but slower rates than earlier stages (Fig. 3 *I* and Fig. S3*A*). The peak proliferation differences between ICE and OCE takes place during the P3–P5 period, in which the most dramatic changes in folding and morphogenesis occur (13) (Fig. S3*A*). These results suggest that different proliferation rates between ICE and OCE of the CB could potentially generate the driving force during CB fold generation by producing more cells in the OCE.

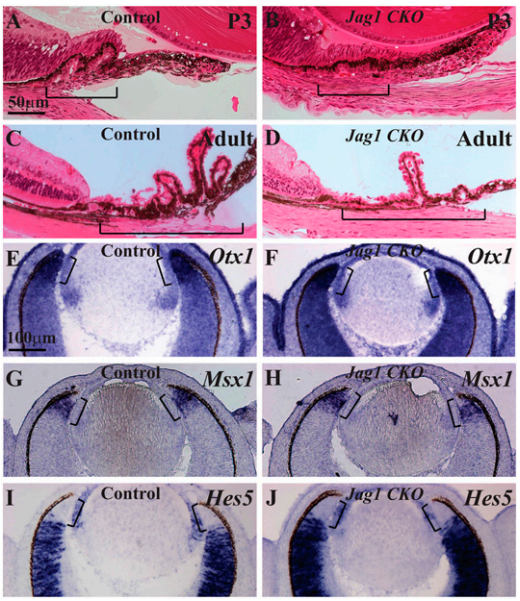

Fig. 2. Jag1 likely serves as the ligand for Notch2 in the developing CB. The folded CB (bracket) is detected in the P3 control (*A*), but is largely absent in the P3 *Six3;Jag1*$^{fx/fx}$ mutant (*B*). The folded CB (bracket) is detected in the adult control (*C*) but most of CB folds are lost in the adult *Six3;Jag1*$^{fx/fx}$ mutant (*D*). *Otx1* (*E* and *F*), *Hes5* (*G* and *H*), and *Msx1* (*I* and *J*) mRNAs show similar expression patterns in the E15.5 control and *Six3;Jag1*$^{fx/fx}$ mutant eyes. (Scale bar: *A–D*, 50 μm; *E–J*, 100 μm.)

Interestingly, cells in the ICE and OCE of the control and *Notch2 CKO* mutant E17.5 CBs proliferate at similar rates, indicating that Notch2 is likely dispensable for cell proliferation in the embryonic CB (Fig. 3 *A*, *B*, and *K*; Fig. S3*B*). However, cells in the OCE of the *Notch2 CKO* mutant CB proliferate significantly and drastically slower than their counterparts in the control CB during P0–P7 (Fig. 3 *C–K*). Furthermore, the proliferation rates for cells in the ICE of the *Notch2 CKO* mutant CB do not change significantly in comparison with those for the cells in the control ICE (Fig. S3*C*). Consequently, the proliferation difference between ICE and OCE of the WT CB is lost in the *Notch2 CKO* mutant CB (Fig. S3*B*). Similarly, in the P3 *Jag1 CKO* mutant CB, cells in the ICE proliferate at comparable rates to those in the P3 control CB, but cells in the OCE proliferate at significantly lower rates than those in the control CB (Fig. 3 *L–N*). To ensure that the reduction in cell number was due solely to decreased proliferation, we also performed the TUNEL assay to assess any potential changes in apoptosis. Our results show that there is no increase in apoptosis in both ICE and OCE of the *Notch2 CKO* mutant CB in comparison with those in the control (Fig. S4). Taken together, our findings argue strongly that Notch2 maintains the rapid proliferation in the OCE, which might contribute to CB morphogenesis.

Notch2 Maintains BMP Signaling in the CB. Although *Otx1* and *Pax6* have been shown to be important for CB morphogenesis (18, 20), their protein expression in the P0 *Notch2* mutant OCE remains unchanged in comparison with the P0 control OCE, suggesting that Notch2 signaling must regulate other pathways to control CB morphogenesis (Fig. S5). Because BMP4 haploinsufficiency and Noggin overexpression cause a similar CB morphogenesis defect to that of the *Notch2 CKO* mutant (16, 17), we then determined if Notch2 signaling affects BMP signaling in the developing CB by examining the expression of the phosphorylated form of SMAD proteins 1, 5, and 8 (pSMAD1/5/8). Secreted BMP proteins can bind to receptor complexes composed of at least one of type I receptors (BMPR1a and BMPR1b) and a type II receptor (BMPR2), leading to SMAD1/5/8 phosphorylation (42). In developing retinal progenitors, OCE cells, and differentiating lens fiber cells, BMP signaling also results in pSMAD1/5/8 production (17, 43, 44). In the control P3 CB, cells in the OCE, but not those in the ICE, show strong pSMAD1/5/8 expression, indicating that the BMP pathway is active in the OCE (Fig. 4 *A* and *A′*). However, in the *Notch2 CKO* mutant CB, pSMAD1/5/8 expression diminishes in the OCE (Fig. 4 *B* and *B′*). pSMAD1/5/8 expression remains normal in the lens, suggesting that BMP signaling reduction is restricted to the CB region (Fig. 4 *A* and *B*). These results show that Notch2 is required for maintaining active BMP signaling in the OCE.

One of the potential ways for Notch2 to regulate BMP signaling is to control the expression of BMP pathway components. To further investigate the level at which Notch2 might regulate BMP signaling, we examined the expression of BMPR1a, BMPR1b, BMPR2, SMAD1, SMAD5, SMAD8, and pSMAD1/5/8 using Western blotting on the control and *Notch2 CKO* mutant P3 OCE cells. All BMP receptors and unphosphorylated SMAD1/5/8 proteins show comparable protein expression levels between the control and the *Notch2 CKO* mutant CBs (Fig. 4 *C* and *D* and Fig. S6*A*). As expected, pSMAD1/5/8 proteins exhibit a significant reduction in *Notch2 CKO* mutants, further confirming our immunohistochemistry results (Fig. 4 *C* and *D*). These results suggest that Notch2 regulates BMP signal transduction in the CB likely by regulating the activity, but not the expression, of BMP pathway components.

Gene Expression Changes in the *Notch2 CKO* Mutant OCE Might Help Explain Defective BMP Signaling and Cell Proliferation. To gain a more global view of how Notch2 might regulate cell proliferation and BMP signaling during CB morphogenesis, we performed microarray-based gene profiling to examine the gene expression differences between *Notch2 CKO* mutant and control OCE cells

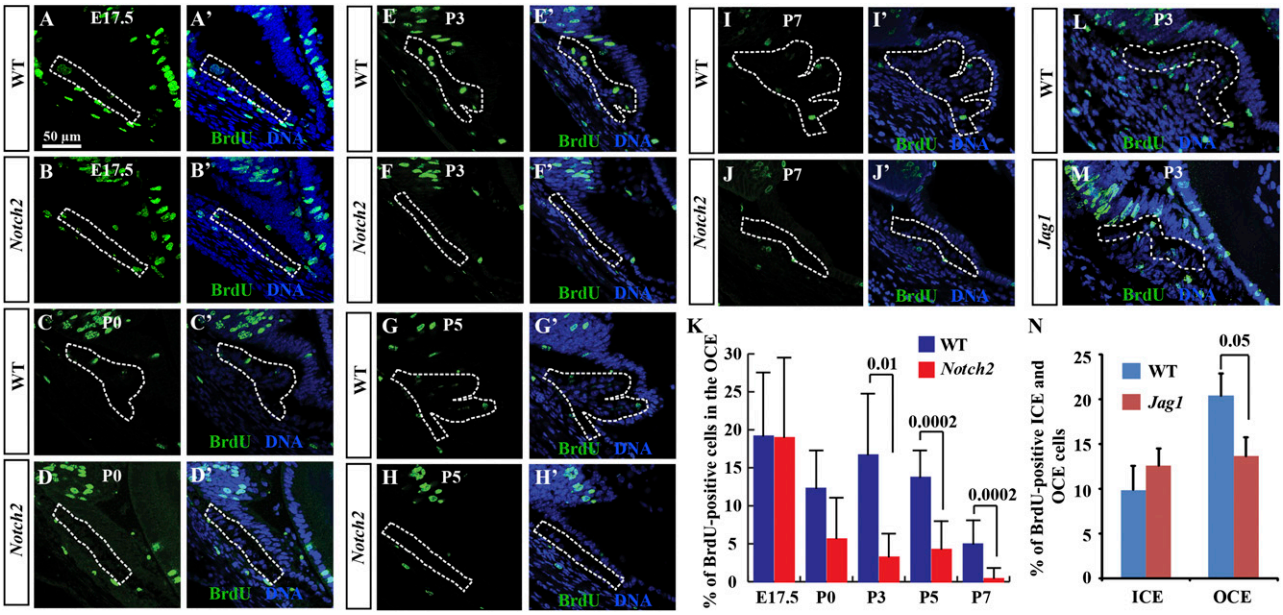

Fig. 3. Notch2 is required for cell proliferation in the CB. (*A–J*) Confocal images show BrdU-labeled cells in outer (highlighted by broken lines) and inner layers of control (*A*, *C*, *E*, *G*, and *I*) and *Notch2 CKO* mutant (*B*, *D*, *F*, *H*, and *J*) CBs during E17.5-P7. (*K*) Percentages of BrdU-positive cells in the outer layer of the control and *Notch2 CKO* mutant CBs change with age ($n = 14$ 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). (*L–N*) *Jag1* mutant P3 OCE cells (highlighted by broken lines in *M*) exhibit significantly lower percentages of BrdU-positive cells than control OCE cells (highlighted by broken lines in *L*). *N* shows quantitative results ($n = 8$ for WT; $n = 16$ for *Jag1*). (Scale bar: 50 μm.)

at P0. The gene profiling results indicate that 339 and 229 genes are up-regulated and down-regulated twofold or more in the *Notch2 CKO* mutant CB at P0 than in the control CB, respectively. Among the known Notch target genes, the expression of *Hey1* significantly decreases in the *Notch2 CKO* mutant CB (Table S1 and Fig. S7). For BMP signaling, our gene profiling results have shown that at least four BMP ligands are expressed in the CB layer at high levels, including *Bmp2*, *4*, *5*, and *7* (Table S2). Among them, *Bmp2* and *Bmp4* are down-regulated and up-regulated 1.6-fold and 2.7-fold in the *Notch2 CKO* mutant CB, respectively, and *Bmp5* and *Bmp7* remain unchanged (Table S1). Consistent with their protein expression, the genes encoding BMP receptors and BMP-specific SMAD show no dramatic expression changes in the *Notch2 CKO* mutant CB (Table S2). Consistent with the pSMAD1/5/8 expression results, *inhibitor of DNA binding 2*, a known BMP target gene, exhibits a significant decrease in expression in the *Notch2 CKO* mutant CB, indicating that BMP signaling activity decreases in the *Notch2 CKO* mutant CB (Table S2 and Fig. S7). These gene expression results support the idea that Notch2 controls BMP signaling not by regulating the expression of BMP receptors and SMADs.

The gene profiling results also provide insight into how Notch2 controls cell proliferation in the OCE of the CB. In the *Notch2 CKO* mutant CB, the expression of only one cell-cycle inhibitor, cyclin-dependent kinase inhibitor 3, declines significantly, but the expression of 12 important positive cell-cycle regulators, including cyclin A2 and cyclin B2, are significantly decreased (Table S1 and Fig. S7). Cell division cycle 20 homolog (*Cdc20*) and pituitary tumor-transforming gene 1 (*Ptg1*) have recently been shown to positively regulate cell-cycle progression in human cells (45). Therefore, the expression changes for cell-cycle regulators might explain why cell proliferation decreases in the *Notch2 CKO* mutant CB.

Notch2 Represses the Expression of two BMP Signaling Inhibitor Genes, *Chrdl1* and *Nbl1*, in the OCE of the CB. Inactivation of *Notch2* function in the OCE results in reduced BMP signaling activity not only in the OCE but also in the underlying stromal cells, suggesting that Notch2 can regulate BMP signaling in both cell-autonomous and non-cell autonomous manners (Fig. 4 *A* and *B*). One of the possibilities is that Notch signaling normally represses the expression of a gene(s) encoding a secreted BMP inhibitor in the OCE. Based on the microarray results, *Chrdl1* (Chordin-like 1) and *Nbl1* (Neuroblastoma 1) appear to be up-regulated in the *Notch2* mutant OCE, but different probes in microarray chips yield inconsistent results. We have used quantitative RT (qRT)-PCRs to confirm that *Chrdl1* and *Nbl1* are up-regulated in the *Notch2* mutant OCE 2.1-fold and 1.7-fold, respectively (Fig. 4*E*). We have further shown that they indeed repress BMP signaling activities in human 293T cells when overexpressed, which is consistent with published results (46, 47) (Fig. S6 *B–H*). To directly test if *Chrdl1* and *Nbl1* are capable of inhibiting BMP signaling in the developing CB, we injected lentiviruses carrying *CMV-Chrdl1-IRES-gfp* [the *CMV* promoter controlling the expression of *Chrdl1* and *gfp* genes linked by internal ribosome entry site (IRES)] and *CMV-Nbl1-IRES-gfp* into the CBs of the P0 eyes. Following *Chrdl1* overexpression, pSMAD1/5/8 expression is severely reduced in both the OCE and the underlying mesenchymal cells of the P3 CBs and CB fold formation is also disrupted, indicating that *Chrdl1* overexpression is capable of repressing BMP signaling and disrupting CB morphogenesis (Fig. 4 *F* and *G*). Although *Nbl1* overexpression can also decrease pSMAD1/5/8 expression, it is less effective in repressing BMP signaling and disrupting CB morphogenesis than *Chrdl1* in the CB, which is consistent with the results in cultured human 293T cells (Fig. S6 *B–H*). Therefore, we propose that Notch2 controls BMP signaling possibly by repressing *Chrdl1* and *Nbl1* expression in the OCE.

Discussion

Although BMP signaling, Pax6, and Otx1 have recently been shown to be required for CB morphogenesis (17, 18, 20), it remains unclear how they work together to control CB morphogenesis at the molecular and cellular levels. In this study, we show that Notch2 signaling in the OCE drives CB morphogenesis at least in part by maintaining BMP signaling and promoting cell proliferation (Fig. 4*H*). Defective Notch2 signaling in the OCE decreases cell proliferation and BMP signaling. Mechanistically,

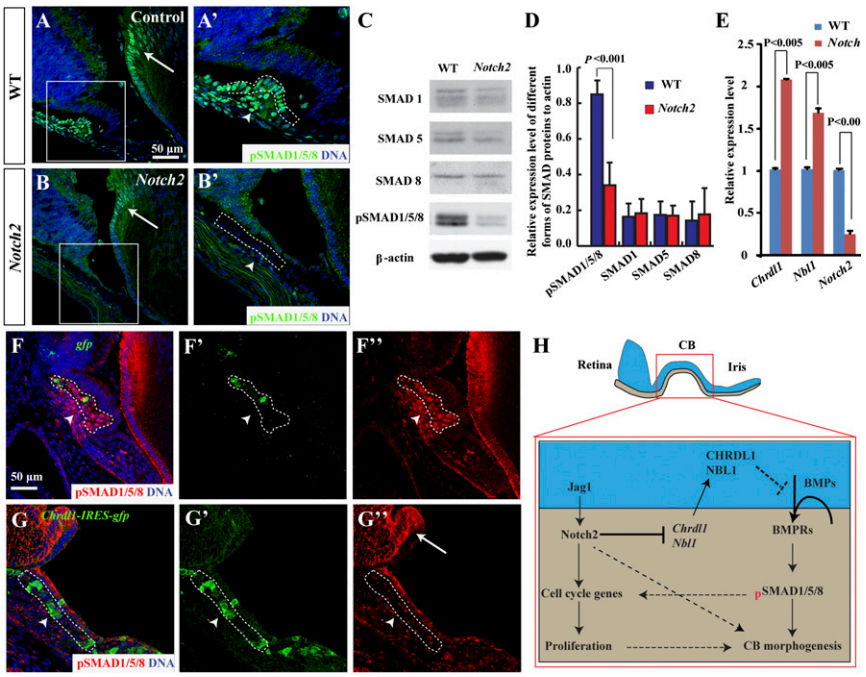

Fig. 4. Notch2 controls BMP signaling in the OCE of the CB possibly by repressing *Chrdl1* and *Nbl1* expression. (*A*–*B*′) The P3 control CB shows strong pSMAD1/5/8 expression in OCE (broken lines) and stromal cells (arrowhead), which diminishes in the P3 *Notch2* mutant CB (*A*′ and *B*′ show the boxed areas in *A* and *B*, respectively). Arrows indicate the lens. (*C*) Western blots show similar expression levels of BMP transducers in P3 control and *Notch2* mutant OCE cells. (*D*) Quantitative results (three biological replicates normalizing to β-actin) show that the *Notch2* mutant CB expresses significantly lower pSMAD1/5/8 than the control CB. (*E*) qRT-PCR results show that *Chrdl1* and *Nbl1* mRNA levels are significantly lower in the P3 *Notch2* mutant OCE than in the P3 control (WT: $n = 4$; *Notch2*: $n = 5$). (*F* and *G*) P3 CB carrying GFP-positive *Chrdl1*-overexpressing OCE cells (*G*; the arrow indicates the misfolded ICE resulting from detachment from the OCE) shows diminished pSMAD1/5/8 expression in both the OCE (broken lines) and the stromal cells (arrowhead) in comparison with the P3 injection control CB containing GFP-positive OCE cells (*F*). (*H*) A working model for Jag1-Notch2 signaling in the regulation of CB morphogenesis. (Scale bars: *A*, *B*, *F*, and *G*, 50 μm.)

Notch2 signaling controls BMP signaling at least in part by repressing the expression of *Chrdl1* and *Nbl1*, encoding two secreted BMP inhibitors. It controls cell proliferation by directly or indirectly (via BMP signaling) regulating the expression of cell cycle–related genes. In contrast with the previous findings that Notch and BMP signaling cooperate with each other by targeting their downstream transcription factors to the promoters of common target genes (48, 49), this study has revealed a unique strategy for cross-talk between Notch and BMP pathways. BMP signaling and cell proliferation have been proposed to regulate CB morphogenesis (5, 13, 14, 16, 17). Although Notch signaling has been demonstrated to play a critical role in cell fate specification (24), both *Jag1* and *Notch2* are dispensable for the specification of the CB fate based on the expression of multiple CB markers. Therefore, we propose that Notch2 signaling regulates cell proliferation and BMP signaling in the CB and consequently CB morphogenesis (Fig. 4*H*). However, our study does not rule out the possibility that Notch2 signaling also controls CB morphogenesis independent of BMP signaling (Fig. 4*H*).

Although Notch2 signaling regulates cell proliferation and BMP signaling in the CB, it remains unclear whether it controls CB morphogenesis through regulation of cell proliferation, BMP signaling, or both. Consistent with the previous report (13), we also show that the proliferation rate in the OCE is significantly higher than that in the ICE, which results in an increase of cell volume and perhaps cell shape changes resulting from cell crowding in the OCE. The changes in cell volume and shape in the developing CB have been proposed to regulate CB morphogenesis (3–5, 13, 14). Interestingly, such differential proliferation between ICE and OCE of the *Notch2 CKO* mutant CB is abolished. Mechanistically, we show that Notch2 is required to directly or indirectly control the expression of cell-cycle regulators in the OCE, including Cyclin A and B as well as CDC25. These results suggest that the extra cells produced in the OCE resulting from differential proliferation between two CB layers might provide a driving force behind CB morphogenesis. However, it remains formally possible that differential proliferation between two CB layers is secondary to CB morphogenesis for compensating the surface difference on the two layers caused by morphogenesis. BMP signaling has been shown to be essential for CB morphogenesis (17) and is also known to be linked to cell proliferation in a variety of cell types, including cancer cells (42). Therefore, Notch2 could also regulate cell proliferation through modulating BMP signaling. This idea can be directly tested in the future by examining cell proliferation in the CB-specific inactivation of BMP receptors or SMADs.

The cross-talk between BMP and Notch signaling pathways has been shown to exist in different cellular contexts. In endothelial cells, the activin A receptor-like 1 (Alk1)/BMPR-mediated BMP signaling pathway and the delta-like 4 (Dll4)-activated Notch signaling pathway work together to transcriptionally activate the expression of *Hey1* and *Hey2* genes (48, 49). In addition, Notch and BMP signaling pathways can block myogenic differentiation of C2C12 cells by regulating the expression of Hey1 through a direct interaction between Smad1 and NICD (50). In the zebrafish pineal gland, BMP signaling is a competence factor for Notch signaling to efficiently activate its target gene expression (51), whereas in the regulation of the initial formation of the olfactory nerve BMP signaling negatively affects Notch signaling to achieve the balance between the two pathways (52). In these two cases, it remains unclear how the two pathways are integrated. In this study, we have shown that Notch signaling controls BMP signaling activity in the developing CB possibly by repressing *Chrdl1* and *Nbl1* expression in the OCE. In addition, we show that *Chrdl1* overexpression in the OCE can inhibit BMP signaling in both the OCE and the underlying mesenchymal cells of the developing CB, which is similar to *Notch2* inactivation specifically in the OCE. Although *Nbl1* is also capable of repressing BMP signaling in the CB, it is less effective than *Chrdl1* and its overexpression is not sufficient to disrupt CB morphogenesis. The important unanswered questions remain: whether *Chrdl1* and *Nbl1* are also expressed in the ICE to contribute to BMP regulation, how Notch2 signaling represses *Chrdl1* and *Nbl1* expression in the OCE at the molecular level, and whether OCE-specific inactivation of *Chrdl1*, *Nbl1*, or both sufficiently restore BMP signaling and morphogenesis in the *Notch2* mutant CB. In summary, we have identified the Jag1-Notch signaling pathway as a key signaling pathway to control CB morphogenesis at least in part by regulating BMP signaling and cell proliferation.

Materials and Methods

Mouse Strains. *Trp1-Cre*, *Six3-Cre*, *Notch2*$^{fx/fx}$, and *Jag1*$^{fx/fx}$ strains were previously described (32, 33, 40, 41, 53). The *Z/EG* reporter mice were crossed

into *Trp1-Cre; Notch2*$^{fx/fx}$ to generate GFP mosaics (54). All experiments were performed under the authorization of the Institutional Animal Care and Use Committees at either the Stowers Institute for Medical Research or the University of Rochester.

mRNA in Situ Hybridization. *Ptmb14* and *Tgfb1li4* probes were made from PCR products on the mouse brain RNAs. The following primers were used to clone the PCR products into pGEM-T easy vector (Promega): *Tgfb1li4*-GACTTCAGCAGCTAGATTCC, GAACAGACCGAAGAGATGTGCT and *Ptmb14*-CACCTCATTTGCATAGAAGC, TACATCCTGCAGGACATC, whereas the probes for *WFDC1*, Otx1, Hes5, and Msx1 were made from cDNA clones. Digoxigenin-labeled RNA probes were synthesized according to the manufacturer's instructions (Roche). Embryos were fixed in 4% (vol/vol) paraformaldehyde in PBS buffer at 4 °C for 2–12 h, then cryoprotected in 30% sucrose in PBS and followed by rapid freezing in tissue freezing medium (TBS, Inc.). Embryos were sectioned at a thickness of 20 μm, and the sections were processed for in situ hybridization as documented previously (28).

Microarray Analysis. The outer, pigmented epithelial layer of the control and *Notch2 CKO* mutant CBs was dissected from P0 pups. Total RNA was extracted with TRIzol reagent (Invitrogen) and was subjected to one round of amplification. All experiments were done with three biological replicates. All analysis was done in R.

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