Notch1 maintains dormancy of olfactory horizontal basal cells, a reserve neural stem cell

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The remarkable capacity of the adult olfactory epithelium (OE) to regenerate fully both neurosensory and nonneuronal cell types after severe epithelial injury depends on life-long persistence of two stem cell populations: the horizontal basal cells (HBCs), which are quiescent and held in reserve, and mitotically active globose basal cells. It has recently been demonstrated that down-regulation of the ΔN form of the transcription factor p63 is both necessary and sufficient to release HBCs from dormancy. However, the mechanisms by which p63 is down-regulated after acute OE injury remain unknown. To identify the cellular source of potential signaling mechanisms, we assessed HBC activation after neuron-only and sustentacular cell death. We found that ablation of sustentacular cells is sufficient for HBC activation to multipotency. By expression analysis, next-generation sequencing, and immunohistochemical examination, down-regulation of Notch pathway signaling is coincident with HBC activation. Therefore, using HBC-specific conditional knockout of Notch receptors and overexpression of N1ICD, we show that Notch signaling maintains p63 levels and HBC dormancy, in contrast to its suppression of p63 expression in other tissues. Additionally, Notch1, but not Notch2, is required to maintain HBC dormancy after selective neuronal degeneration. Taken together, our data indicate that the activation of HBCs observed after tissue injury or sustentacular cell ablation is caused by the reduction/elimination of Notch signaling on HBCs; elimination of Jagged1 expressed by sustentacular cells may be the ligand responsible.

Notch | olfactory epithelium | reserve stem cell | trp63

The neurogenic and regenerative capacity of the adult olfactory epithelium (OE) in both rodents and humans is unmatched elsewhere in the nervous system (1–3). Two stem cell populations maintain life-long neurogenesis in the adult rodent OE: the globose basal cells (GBCs) and horizontal basal cells (HBCs). The GBC population is a heterogeneous mix of both label-retaining and nonlabel-retaining progenitor cells that is further subdivided on the basis of transcription factor expression (4–7). In contrast, HBCs are a reserve stem cell population and are molecularly and morphologically homogeneous and similar to basal cells of other epithelia (8). HBCs emerge perinatally, form a complete monolayer adherent to the basal lamina by approximately postnatal day 14, and rarely contribute to tissue maintenance in the uninjured OE (9, 10). However, as a consequence of severe tissue injury and the wholesale loss of both neurons and sustentacular (Sus) cells, HBCs lose their attachment to the basal lamina, proliferate, transition into GBCs, and give rise to all types of cellular constituents of the OE during its regeneration, a constellation of responses that we term "activation" (9–11). In contrast, existing data suggest that selective neuronal loss in response to ablation of the olfactory bulb does not result in HBC activation (9), although another laboratory has observed an enhanced HBC contribution to the epithelium after bulb ablation (12). The effect of the targeted death of Sus cells has not been investigated.

It has recently been demonstrated that the transcription factor p63, a member of the p53 family of transcription factors, is both necessary and sufficient to maintain HBC dormancy in the adult OE (10, 11). *p63* has two transcription start sites (TSS) subserving alternate N-terminal isoforms: full-length TAp63 and truncated ΔNp63, which has a shorter transactivation domain. In addition, alternative splicing generates five potential C-terminal domains: α, β, γ, δ, ε (13). ΔNp63α is the dominant form in the OE by far (14). ΔNp63α expression typifies the basal cells of several epithelia, including the epidermis, prostate, mammary glands, vagina, and thymus (15). In skin, *p63* is required for both the establishment (16) and maintenance (17) of the stem cell populations. Additionally, shRNA knockdown and overexpression studies in other tissues have revealed that ΔNp63 has a multitude of transcriptional targets, including genes involved in cell adhesion, cell cycle control, and cross-talk with various signaling pathways. For example, ΔNp63 has been shown to regulate expression of basal cell markers (K5/14) (18) and cell adhesion in mammary epithelial cells and keratinocytes (19), while also participating in the response to a variety of signaling pathways, including Notch, Wnt, Bmp, and FGF (20). From this finding, it is evident that ΔNp63 likely serves as a nexus onto which many signals converge to regulate the behavior of the basal cells. However, neither the nature of the cellular injury required to activate HBCs nor the upstream molecular signaling pathways governing ΔNp63α down-regulation following tissue injury that leads to HBC activation has been elucidated.

A multitude of studies have demonstrated the Notch signaling pathway can serve as an integral cell–cell signaling pathway for embryogenesis, tissue homeostasis, and stem cell dynamics

Significance

Self-renewing tissues require both facultative and injury-activated reserve stem cells to maintain integrity. Horizontal basal cells (HBCs), dormant reserve stem cells of the olfactory epithelium, are roused when tissue damage leads to the suppression of the transcription factor ΔNp63, and regenerate all epithelial cell types, including sensory neurons. We show that the targeted death of the sustentacular cells, but not of neurons, leads to activation. Signaling via Notch1 receptors, possibly driven by Jagged1 on sustentacular cells, holds HBCs dormant by maintaining *p63* expression; Notch 2 does not regulate *p63* here. In contrast, *p63* is suppressed by Notch signaling in skin and other tissues. Understanding p63 regulation in olfactory epithelium may inform efforts to alleviate the age-related decline in olfactory function.

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through its ability to regulate cellular proliferation, differentiation, and apoptosis (21–25). The Notch signaling pathway in mammals—consisting of ligands Jagged1, Jagged2, Delta-like1–4 (Jag1–2, Dll1–4), receptors Notch1–4, and cofactors RBPJ and Mastermind-like that bind to the cleaved intracellular domain (NICD) of the receptors in the signal-receiving cell—has a multitude of effects, including the regulation of canonical target genes, such as the Hes family of genes (26). The Notch signaling pathway is highly active in quiescent neural stems cells of the subgranular zone and subventricular zone of the adult CNS, and it has been demonstrated that canonical Notch-ON, RBPJ-dependent signaling maintains the undifferentiated and quiescent state of neural stems cells in vivo (27–29). More recently, it has been shown that Dll1 resides in proximity to the quiescent neural stem cells (NSCs) of the subventricular zone in adult mice, and conditional knockout of Dll1 in cells adjacent to the NSC population reduces the number of quiescent NSCs with an accompanying increase in activated NSCs and transit-amplifying cells (30).

Cross-talk between Notch and p63 has been well characterized in some tissues. For example, in the basal cells of the epidermis, Notch signaling antagonizes p63 by inhibiting *p63* expression, whereas p63 inhibits expression of Notch receptors and effector genes (31). Similarly, Notch2 knockout in the mammalian lens increases *p63* transcript levels (32). Contrary to these reports of Notch-p63 antagonism, however, Notch and p63 have also been noted to positively regulate each other on rare occasion, suggesting that the consequences of Notch-p63 cross-talk are cell-type–specific. In NIH 3T3 cells, knockout of *p63* inhibits Notch-mediated transcription of *Jagged1* (33). In the other direction, p63 can feedback to activate Notch pathway gene expression in some tissues (34–36). Thus, the relationship between Notch and p63 tends to be antagonistic, but is not absolutely so. Accordingly, the Notch-p63 relationship in HBCs of the OE warrants investigation as a formidable exemplar of the regulation of reserve stem cells and their activation after injury.

Results

Systematic Cell-Specific Ablation and HBC Activation. It has been well established that wholesale loss of both neurons and Sus cells after severe OE injury caused, for example, by inhalation of MeBr gas, evokes the activation of HBCs, which contribute to regeneration of the epithelium (9, 10). Whether activation of HBCs requires damage to both cell populations has yet to be determined. One study that traced HBC lineage after injury reported that HBCs remain dormant after the selective loss of mature neurons that follows olfactory bulbectomy (OBX) (9). However, the same kind of injury produced a different result in animals bearing a leaky RU486-responsive CrePR transgene driven by a Krt5 promoter (12). In the latter paradigm, some HBCs were apparently activated to multipotency following OBX. However, it is important to note that the *Krt5.CrePR* transgene did not require RU486 injection for activity and, as a consequence, is functional from a very early age (postnatal day 3, if not earlier), when there is considerable flux between GBC and HBC populations (37). In light of these inconsistent findings, we undertook the reexamination of whether HBC activation was dependent on neuron-only injury and to investigate the role of Sus cell-only loss.

Mice subjected to unilateral OBX were killed either 1 or 4 wk after injury. Successful OBX injury was verified by the disappearance of olfactory marker protein (OMP)-expressing olfactory neurons 1 wk after OBX (Fig. S1). The genetically labeled HBCs in the *K5CreER^{T2};fl(stop)TdTomato* animal remained dormant and both TdT^{+} and CK14^{+}, despite near complete absence of mature neurons at time points 1 wk or more postinjury (Fig. S1 *C* and *D*), compared with the uninjured control side (Fig. S1 *A* and *B*).

The results from the OBX lineage-trace experiment are consistent with the previous demonstration that neuronal loss alone is insufficient to activate HBCs to multipotency (9). Thus, we tested whether targeted loss of Sus cells activated HBCs. We used a multigenic approach to induce specific ablation of Sus cells concurrent with HBC lineage tracing. Mice carrying a *Cyp2g1-rtTA* driver, which is Sus cell- and Bowman's duct/gland cell-specific (38), a *TetO-diphtheria toxin A* (*DTA*) transgene, and the *K5CreER^{T2}* and *fl(stop)TdTomato* alleles were used. In quadrigenic mice with this genotype, the presence of doxycycline causes the death of Sus and duct/gland cells in the OE (Fig. 1). For a negative control, we substituted a *TetO-GFP* element for the *TetO-DTA* transgene to label the cells in which exposure to doxycycline led to activation of the *TetO*-containing constructs.

Tamoxifen was administered to quadrigenic mice at 6 wk of age to induce the heritable expression of TdTomato by HBCs. Two weeks after tamoxifen induction, we started animals on 2 g/kg doxycycline chow ad libitum for 1 mo and then collected the tissue (Fig. 1).

In the *K5CreER^{T2};fl(stop)TdTomato;Cyp2g1-rtTA;TetO-GFP* control mice, doxycycline administration led to GFP-labeling of a substantial population of Sus cells, with persistent HBC dormancy under these conditions (Fig. 1*C*). In the *K5CreER^{T2};fl(stop)TdTomato;Cyp2g1-rtTA;TetO-DTA* mice, in contrast, doxycycline treatment gave rise to TdTomato^{+} neurons and Sus cells (Fig. 1 *D* and *E*). The latter group of transgenic animals produced threefold more clusters of non-HBCs per tissue section and 13-fold more non-HBC cells per tissue section than the controls (Fig. 1*F*). Non-Sus cells are also dying within the epithelium of the DTA-expressing mice. However, there is no evidence for accelerated death of GBCs and olfactory sensory neurons (OSNs) in the DTA group compared with the GFP-expressing control mice after 1 mo of doxycycline chow, as counts of Caspase3^{+}, non-Sus cells are not significantly different between the two groups at this time (Fig. 1*F*). That the death of OSNs is equivalent between the two groups suggests that HBC activation reflects the accelerated death of Sus cells by itself; the overwhelming predominance of HBC-derived Sus cells and duct/gland cells in the DTA mice also supports that interpretation.

Postlesion RNA-Seq Analysis Reveals the Notch Pathway Is a Candidate Pathway. The foregoing results suggest that the death of Sus and duct/gland cells boosts HBCs from dormancy, resulting in the generation of both neurons and Sus cells from within the HBC lineage. Preliminary microarray data comparing HBCs harvested from uninjured control mice with HBCs isolated 48 h after MeBr exposure suggested that the Notch signaling pathway is differentially regulated after injury. In an additional effort to identify candidate cell-to-cell signaling pathways for further investigation, we undertook an in-depth transcriptomic analysis comparing HBCs from uninjured OE vs. HBCs isolated 18 h after MeBr exposure, when levels of p63 protein are at a minimum (10). The cells were FACS-isolated on the basis of TdTomato expression in tamoxifen-treated *K5CreER^{T2};fl(stop)TdTomato* mice. RNA-seq analysis was carried out, as described in *Materials and Methods*.

Quality control of the samples by hierarchical clustering, analysis of *p63* levels, and t-SNE (t-distributed stochastic neighbor embedding) dimension reduction indicated that one of the MeBr-exposed samples did not lesion, and was eliminated from further analysis (Fig. S2*A*). Additional quality-control plots demonstrated no need for further normalization nor for a method of filtering out genes below the detection threshold (Fig. S2*B*). A subsequent volcano plot of these data showed that both *p63* and *Hes1* were significantly down-regulated following lesion (Fig. S2*C*), and the latter change suggested a reduction in Notch signaling. Pathway analysis demonstrated that other members of the Notch signaling pathway are significantly down-regulated 18 h following MeBr lesion (Fig. 2), confirmed previous microarray data, and further supported Notch as a candidate pathway involved in postinjury HBC activation.

Given the potential involvement of Notch signaling in regulating p63 expression, we performed promoter analysis in silico on a total of 1.35 kb of genomic sequence of the *p63* gene,

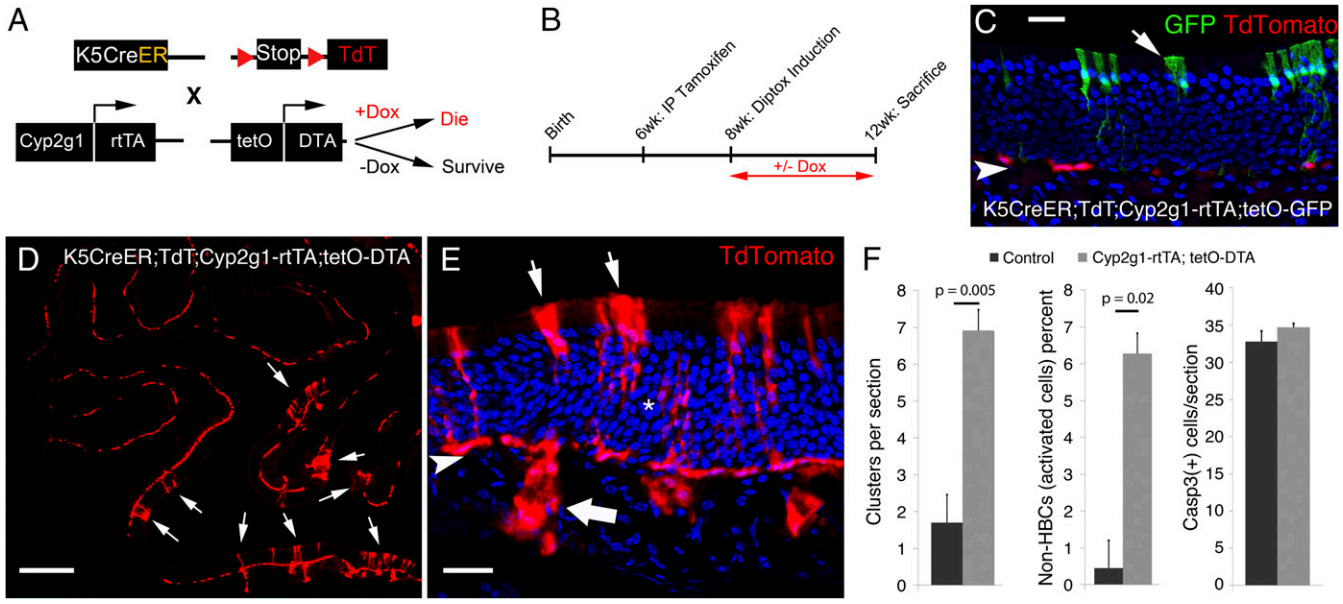

Fig. 1. HBCs activate in response to selective Sus cell depletion. (*A*) Quadrigenic genotype used for tracing HBC lineage following Sus cell ablation. Cyp2g1 is selectively expressed in the Sus cells and Bowman's duct/gland cells. (*B*) Experimental timeline. (*C*) In reporter animals, in which tetO-GFP is substituted for tetO-DTA, administration of doxycycline drives GFP expression in Sus cells (arrow) without concomitant activation of TdTomato-labeled HBCs, which remain dormant at the basal lamina. (*D*) Low-magnification view of clusters of TdTomato$^+$ cells (arrows) derived from activated HBCs following Sus cell depletion. (*E*) Confocal micrograph of cell clusters derived from activated HBCs following Sus cell ablation. The HBC-derived cells illustrated here include Sus cells (thin arrows), cells of Bowman's gland (thick arrows), and a rare neuron (asterisk), as well as the monolayer of HBCs found immediately superficial to the basal lamina. (*F*) Quantification of cell clusters (*Left*), TdTomato$^+$/non-HBCs derived from HBCs (*Center*), and the number of Caspase3$^+$ GBCs and OSNs in the vicinity of dead and replacement Sus cells (*Right*). Note that activation is pronounced when Sus cells die, despite comparable and low levels of neuronal death in the control vs. Sus cell ablated OE. Arrowheads demarcate the basal lamina. (Scale bars: 30 μm in *D* and *F*; 300 μm in *E*.)

consisting of 1.1 kb upstream of the *ΔNp63* TSS and 250 bp of the TSS, the location of which was based on published reports (39, 40). FIMO (find individual motif occurances) scanning using the generally acknowledged consensus binding motif for RBPJ (41, 42) revealed not only scattered binding sites far upstream, but also two distinct clusters, one located 250 bp upstream from the TSS and a smaller one directly at the TSS, supporting direct RBPJ regulation of the Δ*N*-p63 locus. In addition, analysis using a longer consensus binding motif that enriches for sites of coincident RBPJ/NICD binding (42) predicted two such sites in the more upstream area, consistent with published reports suggesting that in general, NICD/RBPJ binding occurs further away from the TSS, whereas NICD-independent RBPJ binding is more enriched closer to the TSS (Fig. S2*D*).

Components of the Canonical Notch Signaling Pathway Are Present in the Uninjured Adult OE. Given the RNA-seq demonstration that Notch receptors and pathway genes are transcribed by HBCs and differentially regulated following tissue injury, we wanted to confirm expression of the corresponding proteins in the OE. To that end, we stained tissue sections of adult OE with antibodies targeting Notch ligands, receptors, targets, and cofactors to establish the distribution of the Notch signaling components in the adult OE. Antibodies against the canonical Notch cofactor RBPJ stained all cells of the OE, with non-HBCs labeling with the most intensity (Fig. 3*A*). Hes1, the canonical downstream target of NICD/RBPJ Notch signaling, labeled all HBCs to a variable extent, which were identified by staining for the HBC marker CK14 (Fig. 3, thin arrows). Additionally, colabeling of CK18 and Hes1 demonstrated that all Sus and duct (Fig. 3, thick arrows) cells are Hes1$^+$, with the Sus cells labeling with the highest intensity (Fig. 3*B*).

The RNA-seq data indicate that HBCs transcribe Notch1 and Notch2 but not Notch3 and Notch4 (Fig. 2). Immunostaining with Notch1 antibody confirmed the presence of Notch1 protein in not only HBCs, but also ducts (Fig. 3*C*, thick arrow), as well as a small GBC population found just apical to the HBCs (Fig. 3*C*, double arrows). Notch2, by comparison with Notch1, is expressed by the HBCs and Sus cells (Fig. 3*D*). Interestingly, GBCs, whether Notch1$^+$ or Notch1$^-$, do not label with Notch2.

Canonical Notch ligands of the Jagged and Delta family are also expressed in the OE (Fig. 2). Jag1 exclusively labels Sus cells and does not label the HBCs (arrows, Fig. 3*E*). The distribution of Dll1 was mapped using Dll1-LacZ reporter mice, as antibody labeling was unsuccessful. In this case, colabeling of β-gal with the HBC marker CK14 was exclusive and extensive (Fig. 3*F*). We were unable to detect positive staining for Notch3, Notch4, or Jag2 in the OE.

The Notch Signaling Pathway in HBCs Responds to Acute MeBr Injury. The differential regulation of Notch signaling components in response to injury that was observed with RNA-seq was confirmed by qPCR and by immunohistochemistry (IHC). Naris-plugged mice were exposed to MeBr gas and then killed for tissue harvest 18 h later (10). The naris-plugged side served as an internal control, because it is largely spared the effect of the gas. By IHC, staining for the Notch1 receptor was initially more pronounced in HBCs after lesion (which retain much reduced but detectable expression of p63) (Fig. 4 *A* and *A1*), whereas Notch2 expression did not change significantly (Fig. 4 *B* and *B1*). However, labeling for Hes1, the downstream effector of canonical Notch signaling, was decreased after injury compared with the uninjured side of the OE (Fig. 4 *C* and *C1*). On the other hand, the canonical coactivator, RBPJ, was increased after injury compared with the control side and insufficient to maintain Hes1 levels in the absence of active Notch signaling (Fig. 4 *D* and *D1*).

qPCR analysis revealed a complex pattern of gene expression as a function of time during the acute postinjury period. The analysis of multiple Notch pathway components at 18 h after

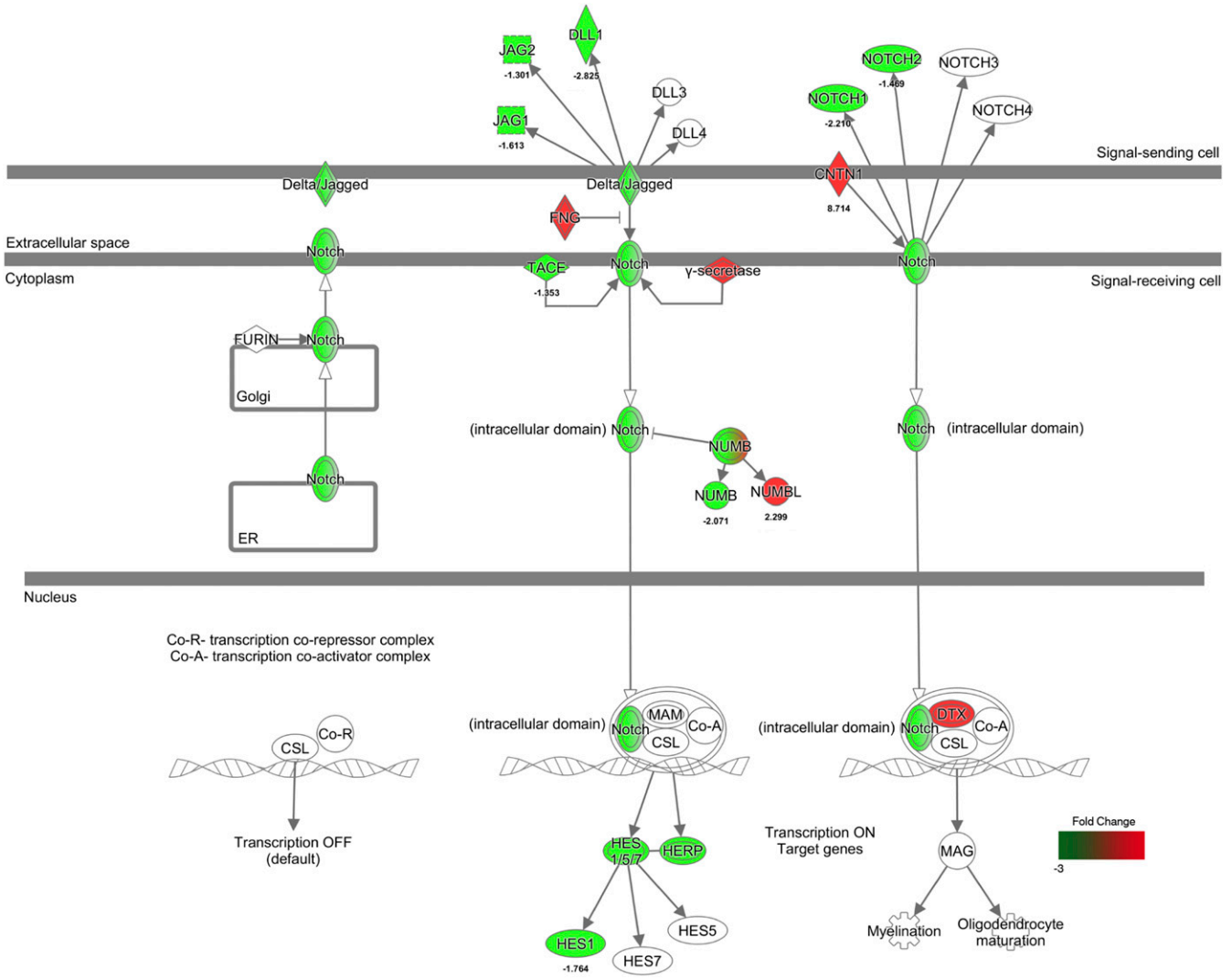

Fig. 2. Transcriptomic analysis of HBCs reveals that the Notch pathway is differentially regulated during activation from dormancy. Ingenuity Pathway Analysis diagram showing that a large number of canonical Notch signaling members are significantly down-regulated 18-h postinjury.

lesion parallels the RNA-seq observation described earlier and indicates that mRNA levels are significantly reduced by comparison with uninjured controls (Fig. 4*E*). The decline in mRNA levels was tracked a multiple time points—0 (i.e., at the end of the exposure), 12, 18, and 24 h post-MeBr—for selected HBC-specific components: *Notch1*, *Notch2*, *Hes1*, and *p63*. *Notch1* levels at the end of exposure period are nearly 16-fold increased, but then fall 2-fold to a nadir at 18 h, which anticipates the decline in *Hes1* and *p63* mRNAs. *Notch2* mRNA levels display a more subdued response to injury (Fig. 4*F*). The enhanced immunoreactivity for Notch1 protein in HBCs at 18 h postinjury may reflect that initial increase in *Notch1* mRNA.

Notch Signaling Up-Regulates p63 and Fosters HBC Dormancy in the Uninjured OE. That the decline in mRNA levels of *Notch1* and downstream components of the signaling pathway, such as *Hes1* mRNA, anticipated the nadir of *p63* gene expression suggests that Notch signaling maintains *p63* levels in this tissue. We tested directly whether Notch signaling exhibits a positive upstream regulation of *p63* transcription, first by eradicating Notch1 via conditional knockout and, second, by enhancing Notch signaling via overexpression of the constitutively active Notch1 intracellular domain (N1ICD). Furthermore, we assayed whether elimination of Notch signaling causes a decreased threshold for HBC activation in the uninjured OE as a consequence of p63 down-regulation.

We analyzed transcript levels by qPCR in FACS-purified HBCs from either *Notch1*-conditional knockout or constitutive *N1ICD*-expressing mice in which the *K5CreER*^{T2} driver was used to target the gene mutation to HBCs specifically, while simultaneously expressing a TdTomato reporter for lineage tracing and cell sorting. Cells were harvested by FACS 2 wk after tamoxifen administration. Compared with wild-type TdTomato^{+} HBCs, constitutive *N1ICD* overexpression resulted in a nearly fourfold increase in *Notch1*, as well as a threefold increase in *Hes1* mRNA, as expected, and also a threefold increase in *p63* expression (Fig. 5*A*). Conversely, following conditional knockout of *Notch1* in HBCs, *Notch1* mRNA trended downward, *Hes1* held at normal levels, but *p63* was significantly decreased (Fig. 5*A*). It is likely that the changes in gene expression are attenuated by incomplete recombination at the *Notch1* locus compared with the *ROSA26-fl(stop)TdTomato* locus (see below for the demonstration that Notch1 is retained in some HBCs in Tam-treated homozygous knockout animals).

Although levels of *p63* mRNA respond to Notch signaling modulation, we sought to determine whether there were functional consequences of altered expression. Specifically, we assayed the

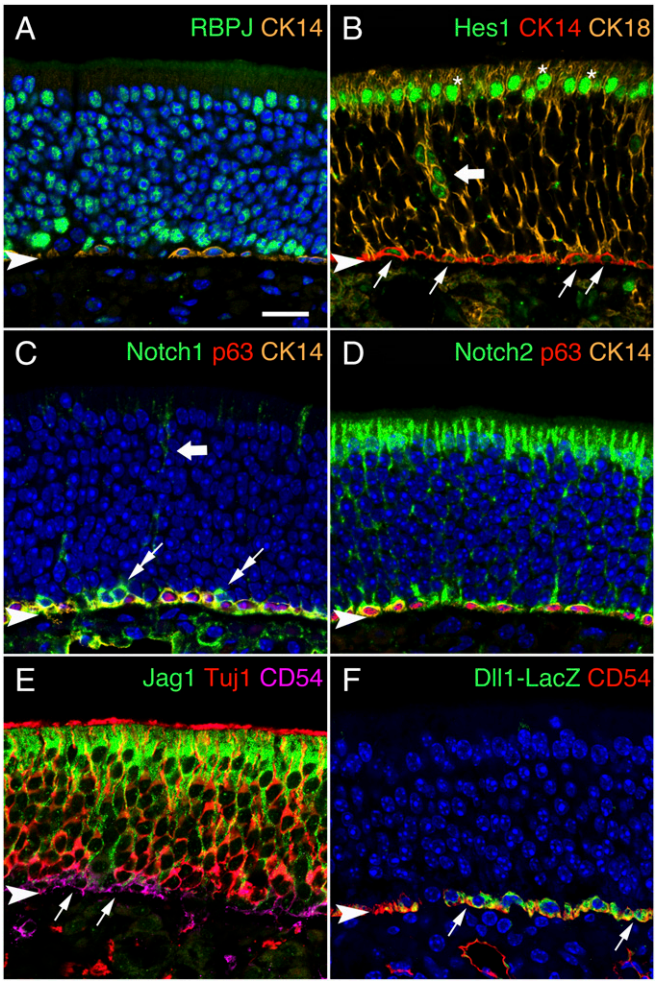

Fig. 3. Notch signaling components are prominent in HBCs in the adult mouse OE. (*A*) RBPJ, the canonical Notch transcriptional coactivator, is present in all cells of the OE, including HBCs (colabeled with CK14). (*B*) Hes1 is found in basal HBCs (colabeled with CK14, arrows), apical Sus cells (colabeled with CK18, asterisks), and duct cells (colabeled with CK18, thick arrow). (*C*) Notch1 is present in HBCs ($p63^+$), a subset of GBCs (double thin arrows), and duct cells (thick white arrow); Sus cells may be faintly labeled as well. (*D*) Notch2 is present in apical Sus cells and basal HBCs ($p63^+$). (*E*) Jag1 is exclusively found in Sus cells, as it does not colabel with Tuj1 or CD54. Note the close association Jag1 and the surface of HBCs (arrows). (*F*) Dll1 expression by LacZ reporter is found exclusively in basal HBCs (arrows). Arrowheads demarcate the basal lamina. (Scale bar, 20 μm, applies to all panels.)

consequences of *Notch1* conditional knockout on the threshold for HBC activation in the uninjured OE. To that end, *K5CreER^{T2};fl(stop)TdTomato;Notch1$^{fl/fl}$* mice were perfused 3 mo after tamoxifen treatment. Compared with both wild-type *K5CreER^{T2};fl(stop)TdTomato* animals and animals with deletion of the RBPJ DNA binding domain, animals with HBC-specific deletion of *Notch1* demonstrated increased spontaneous activation of HBCs in the uninjured OE (Fig. 5 *C* and *D*). Because efficient recombination at all floxed alleles required maximization of the tamoxifen dose, clonal analysis of HBC activation was not possible. Nonetheless, we quantified the number of epithelial patches in which $TdTomato^+$ non-HBCs form a contiguous group as clusters. The number of clusters of labeled non-HBCs increased threefold with *Notch1* deletion. We also counted the number of $TdTomato^+/CK14^-$ cells (i.e., those cells that are descended from HBCs but have become another type of cell) (Fig. 5 *C* and *D*). In this case as well, non-HBCs also increased sixfold with Notch1 deletion. Despite the high-dose tamoxifen, not all $TdTomato^+$ HBCs lack immunodetectable Notch1 (thin white arrow in Fig. 5*D*), which indicates that full recombination was still elusive. Because many HBCs do lack detectable Notch1 labeling (white-on-black arrows, Fig. 5*D*), the loss of Notch1 apparently biases toward, but does not ensure, spontaneous activation, which may explain the variability in activation of HBCs observed between biological replicates.

Given enhanced activation of HBCs in the absence of Notch1, we also sought to determine whether mutating *RBPJ*, the cofactor with which the intracellular domain of all Notch1–4 receptors bind to accomplish downstream signaling, would cause a more pronounced down-regulation of *p63* transcription and greater activation. To that end, we carried out conditional recombination in the HBCs of *RBPJ$^{fl(ex6\text{-}7)/fl(ex6\text{-}7)}$* mice using the *K5CreER^{T2}* driver and a *TdTomato* reporter, which has the effect of excising the DNA-binding domain. We found that *Hes1* mRNA levels increased somewhat in HBCs (3-fold) as did *p63* message (2.4-fold) (Fig. 5*B*), which is opposite to the effect of *Notch1* deletion. However, it is well established that the RBPJ protein binds upstream of the *Hes1* promoter in the absence of Notch signaling to inhibit *Hes1* transcription (43–45). Moreover, the manner by which the interaction of RBPJ with NICD relieves that inhibition is tissue-specific (46). For example, in a breast cancer cell line, RBPJ deficiency results in a Notch-like gene-expression signature, such that the canonical target *Hey* is up-regulated as a result of de-repression (47), which effect resembles the outcome observed in HBCs as well.

In contrast to the enhanced activation of HBCs following conditional knockout of Notch1, the excision of the DNA-binding domain of *RBPJ* did not result in any increase in the appearance of HBC-derived neurons, Sus cells, or other non-HBCs relative to wild-type control mice (Fig. 5*C*). Furthermore, the elimination of the DNA-binding domain of *RBPJ* still permits transcription of the canonical Notch target *Hes1* because IHC staining with anti-Hes1 strongly labels the nuclei of HBCs that lack a detectable RBPJ DNA-binding domain (assessed by staining with a domain-specific antibody) (Fig. S3). These data are congruent with the qPCR studies demonstrating that *Hes1* and *p63* transcription are increased in HBCs in which RBPJ has been knocked out (Fig. 5*B*). Furthermore, the increase in *Hes1* with RBPJ knockout suggests that Notch signaling in HBCs is not maximal in the context of the uninjured OE, which fits with the response of HBCs to OBX (Fig. 6).

Notch1, Not Notch2, Maintains HBC Dormancy After OBX. Although OBX and the consequent initial and ongoing loss of mature OSNs cause no or very infrequent HBC activation (Fig. S1) (9, 12), we assayed how OBX alters Notch signaling in HBCs and whether Notch1 knockout interacts with neuronal injury to markedly enhance HBC activation. To that end, we determined the level of Notch pathway mRNAs by qPCR in HBCs from uninjured OE vs. OE harvested 7-d post-OBX (Fig. S4). In stark contrast to the eventual decline in the mRNA of Notch pathway components as a consequence of the wholesale loss of Sus cells and neurons following MeBr exposure, we observed a marked increase in Notch-related genes in OBX mice (Fig. S4). Similarly, upon IHC assessment, we found that the staining for Hes1, p63, and Notch1 was increased in HBCs on the OBX side compared with the unoperated side, which also indicates enhanced Notch signaling when neuronal degeneration is maximal (Fig. 6).

The marked mRNA and protein increases seen after the death of OSNs by qPCR and IHC, respectively, prompted us to determine whether Notch1 receptor signaling played a functional role in maintaining HBC dormancy in the setting of massive retrograde neuronal degeneration. In the first test, tamoxifen treatment of *K5CreER^{T2};fl(stop)TdTomato;Notch1$^{fl/fl}$* mice preceded unilateral OBX by 2 wk followed by an additional 2 wk survival following surgery (Fig. 7*A*). On the ablated side, we observed thousands

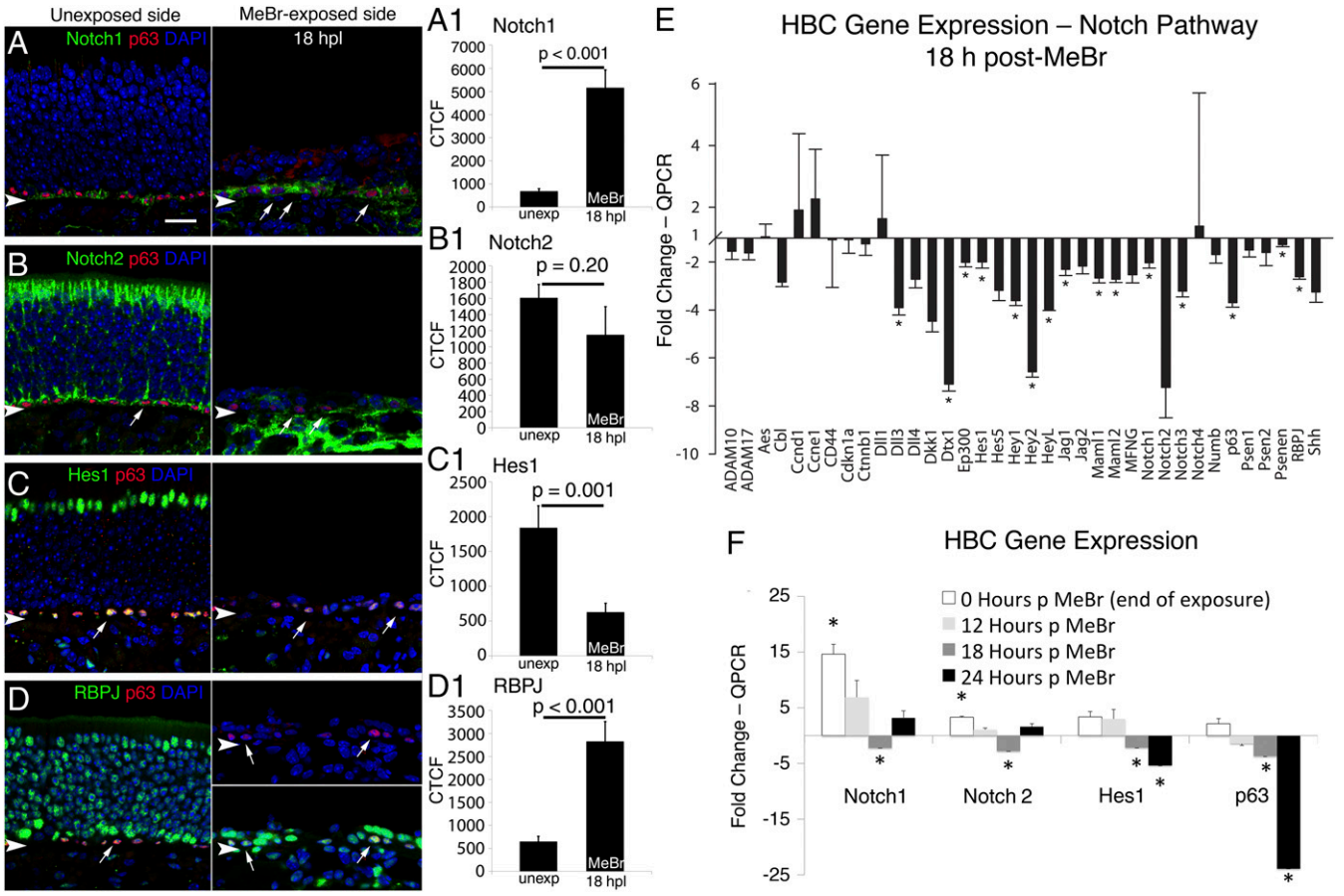

Fig. 4. Notch signaling components and targets change following MeBr injury in wild-type mice. (*A*) Notch1, (*B*) Notch2, (*C*) Hes1, and (*D*) RBPJ IHC labeling 18 h after MeBr gas injury by comparing the naris-plugged and unexposed side (*Left*) vs. the exposed (*Right*) side of the nose. Confocal micrographs are taken from matching areas of the OE on the two sides of the same tissue section. In *A*, HBCs exhibit enhanced staining for Notch1 on the lesioned side (arrows). In *B* and *C*, the decline in the expression level of p63 in HBCs on the lesioned side is apparent as well as the reduction in Notch2 (*B*) and Hes1 (*C*) staining (arrows). In *D*, by way of contrast, RBPJ staining is enhanced in HBCs on the injured side (arrows). (*A1–D1*) Corrected total cell fluorescence (CTCF) measurements of Notch1, Notch2, Hes1, and RBPJ IHC labeling on unexposed and MeBr-exposed. (*E*) Relative fold-change in expression of Notch pathway mRNAs in FACS-purified HBCs as determined by qRT-PCR analysis 18 h after MeBr injury. As a consequence of injury, the large majority of pathway components have declined; asterisks indicate significant differences in gene expression corrected for false discovery. Error bars represent SEM. (*F*) Time course of mRNA changes by qRT-PCR analysis of Notch receptor, Hes1, and p63 gene expression in HBCs at 0, 12, 18, and 24 h following MeBr injury; $*P < 0.05$. As noted previously (10), the decline in p63 protein levels precedes the maximal decline in *p63* gene expression. Conversely, Notch1 protein expression lags the decline in mRNA levels. See Table S2 for detailed statistical information. Arrowheads demarcate the basal lamina. (Scale bar in *A*, 20 μm, applies to *B–D* as well.)

of TdTomato$^+$ neurons, Sus cells, and non-HBC basal cells vs. a handful in the wild-type and heterozygote mice (compare Fig. 7 *B* and *C*). The statistical comparison of conditional heterozygote vs. knockout mice confirms this, as the difference in the number of activation-derived non-HBCs between OBX and spared sides of the conditional knockout mice is significant (Fig. 7*D*). In contrast, there is no significant difference between injured and unoperated sides in the heterozygotes (Fig. 7*D*). As before, the large dose of tamoxifen required for efficient recombination precludes clonal analysis. For this analysis, the unoperated side was used as an internal control; the interval between OBX and analysis was relatively short in comparison with the survivals required to see spontaneous activation in the uninjured OE, which explains the lack of activation on the unoperated side (Fig. 5).

Removal of the olfactory bulb might cause systemic effects (secondary to bleeding, inflammation, and so forth) that act in concert with the loss to neurons to incite the activation of HBCs in the absence of Notch1. Accordingly, we sought to isolate the consequences of accelerated neuronal loss from the immediate effects of OBX by treating with tamoxifen after the initial response to ablation is past (Fig. 7*E*) (48). In this case, tamoxifen administration to *Notch1$^{fl/fl}$* mice 10 d after OBX, followed by killing 7 d after tamoxifen treatment, also demonstrated enhanced HBC activation. As before, TdTomato$^+$ non-HBCs were numerous (Fig. 7 *F–I*). We observed cells situated apical to the HBC layer that were classified as probable GBCs because they did not label with either PGP9.5 (a neuronal marker) or CK14 (Fig. 7*G*, *Inset*). In some areas, neurons bearing dendritic processes are labeled (arrows, Fig. 7 *G* and *H*). In addition, HBCs were dividing at a higher rate than normal as a consequence of Notch1 knockout and subsequent OBX, as demonstrated by Ki67 staining (Fig. 7*I*). Although it is usual to observe far less than one dividing HBC per millimeter length of OE in tissue from OBX wild-type mice (9, 49), we observed three dividing HBCs (identified by Ki67/TdTomato/p63 immunostaining) in a cluster adjacent to one another (arrows, Fig. 7*I*). We have never observed this phenomenon in uninjured tissue. Counts of clusters (which were possible in this experiment because the extent of recombination was less in these animals) and of activation-derived non-HBCs again demonstrated statistically significant differences between the lesioned and spared sides of conditional knockout animals (Fig. 7*J*).

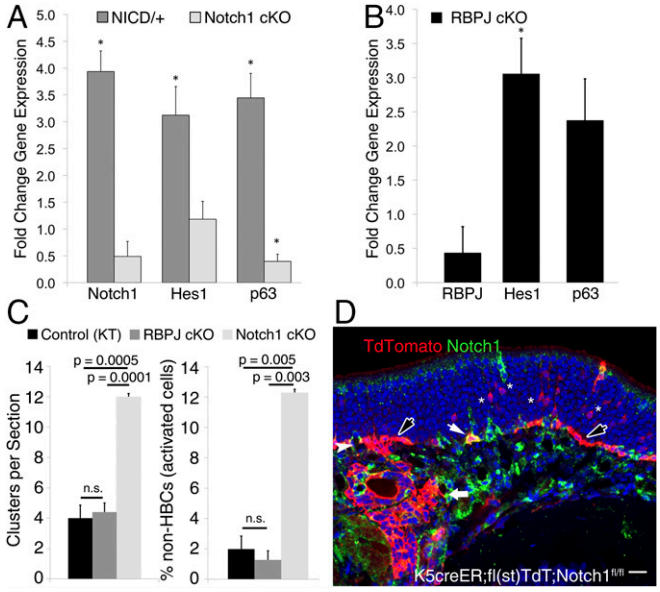

Fig. 5. Notch signaling modulates p63 expression and decreases the threshold for HBC activation in the uninjured OE. (*A*) qRT-PCR of Notch1, Hes1, and p63 in FACS-purified HBCs overexpressing N1ICD/+ and Notch1 cKO. (*B*) qRT-PCR of RBPJ cKO HBCs for RBPJ, Hes1, and p63; all fold-changes are normalized to wild-type (equal to 1). (*C*) Quantitation of HBC activation in Notch-modulated HBCs by counting numbers of clusters containing HBC-derived non-HBC epithelial cells and the percentage of HBC-derived non-HBCs. (*D*) Representative example of HBC activation in the setting of Notch1 cKO. Not all TdTomato$^+$ HBCs have undergone excision of Notch1 (thin white arrow), but most have (white on black arrows). HBC-derived neurons are marked by asterisks; n.s., not significant. A very large aberrant nest of HBC-derived cells has invaded the lamina propria and lack Notch1 labeling (thick arrow). See Table S2 for detailed statistical information. (Scale bar, 10 μm.)

Given that HBCs express both Notch1 and Notch2 receptors (Fig. 2 *C* and *D*), we also investigated the extent to which signaling via Notch2 might also influence HBC activation by itself. Accordingly, we assayed HBC activation following OBX in *Notch2$^{fl/fl}$* mice. Perhaps unexpectedly, in Notch2 conditional knockout mice, all of the TdTomato$^+$ cells on the ablated and unoperated sides of the Notch2 conditional knockout mice remained HBCs, as in the *K5CreER^{T2};fl(stop)TdTomato* control animals (Fig. S5). Thus, Notch2-knockout HBCs did not activate to multipotency as a consequence of OBX in contrast to the effect of Notch1 conditional knockout.

Discussion

The results presented here demonstrate that targeted killing of Sus cells of the OE is sufficient to shift HBCs from dormancy to active proliferation and multipotency, whereas the abrupt, massive loss of OSN is not. Furthermore, Notch1 signaling apparently and positively regulates p63 expression in HBCs, the master regulator of HBC dormancy, whose decline is both necessary and sufficient for HBC activation. Neuronal death does become capable of overcoming dormancy when both *Notch1* alleles are excised. The expression of Jag1 by Sus cells and of Notch1 by HBCs may constitute the signaling dyad responsible for the effect that the Sus cells exert on p63 and HBCs. In contrast, Notch2, although expressed by HBCs, is apparently irrelevant to the regulation of p63 either by itself or in combination with Notch1. Additionally, the loss of functional RBPJ does not produce a pan-Notch knockout effect on p63, as others have suggested; rather, in HBCs, RBPJ on its own seems to play an inhibitory role in the transcription of Notch target genes because conditional excision of its DNA-binding domain appears to relieve repression of the canonical target Hes1.

Sus Cell Ablation, but Not OSN Ablation, Results in Increased HBC Activation. HBC activation is observed following ablation of Sus cells that is relatively minor in extent, judging by the limited numbers of Sus cells that become labeled in response to doxycycline using the same rtTA-expressing Cyp2G1 transgenic driver and a Tet-responsive GFP construct. Whether the ongoing, low-level loss of OSNs is also necessary cannot be ruled out, although the loss of Sus cells at the level achieved here was not associated with an increase in apoptotic, Caspase3$^+$ OSNs. The degree of activation is particularly striking, given the persistence of dormancy in the face of constant piecemeal loss of OSNs via normal turnover in the uninjured OE and of the wholesale death of mature OSNs observed with OBX. The response to Sus cell ablation strongly suggests that tissue integrity, as denoted by Sus cell status, provides a critical signal to HBCs, instructing them to maintain or escape their dormant state. In contrast, the lack of activation in response to the death of neurons—whether constant (in uninjured OE), accelerated (observed as a chronic

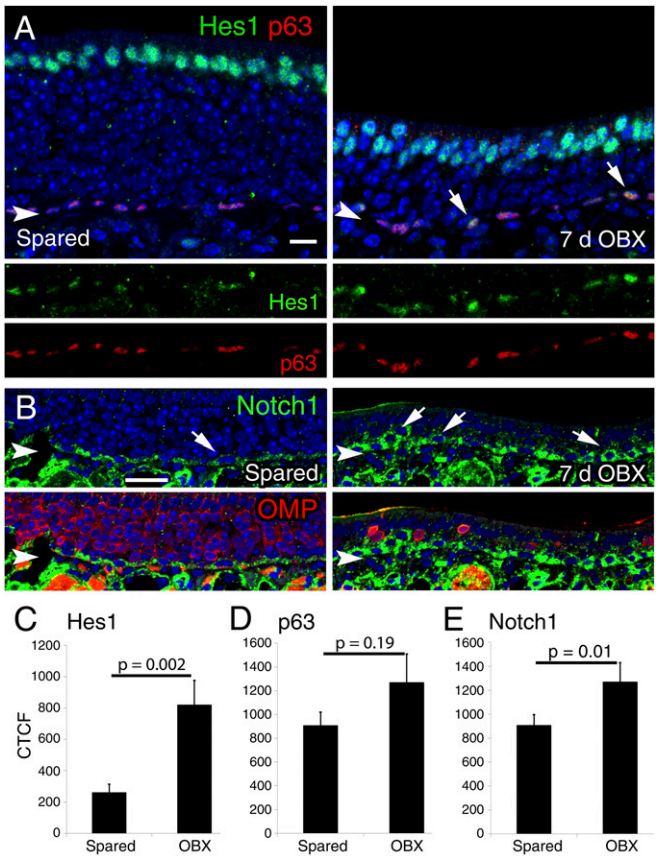

Fig. 6. Notch signaling contributes to HBC quiescence in the setting of neuronal injury. (*A*) IHC of Hes1 and p63 following unilateral OBX. Note the relative increase of Hes1 in the nuclei of HBCs on the OBX side compared with the spared side (arrows). *Lower* panels illustrate channels separately for clarity. (*B*) Notch1 IHC on the septum of a unilateral OBX animal. Note the relative increase of Notch1 labeling on the OBX side compared with the spared side (arrows). Decreased OMP on the OBX side demonstrates the completeness of the lesion. Arrowheads demarcate the basal lamina. (Scale bars: 10 μm in *A*; 20 μm in *B*.) (*C*–*E*) CTCF quantification of Hes1, p63, and Notch1 IHC labeling on spared and OBX sides. See Table S2 for detailed statistical information.

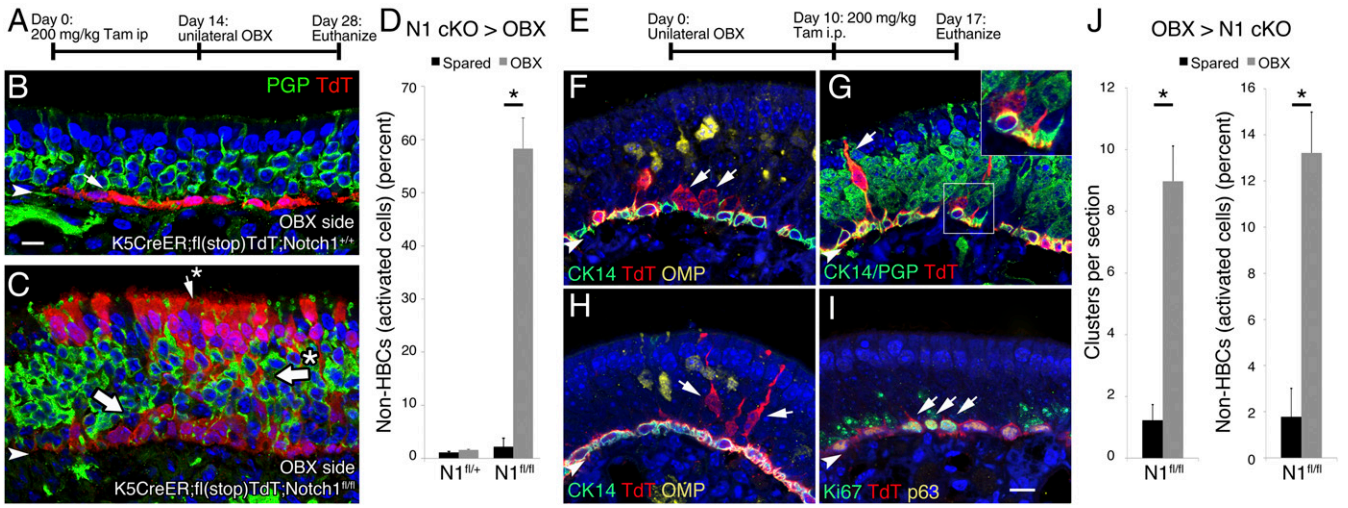

Fig. 7. Notch1 contributes to HBC quiescence in the setting of ongoing accelerated neurogenesis. (*A*) Experimental timeline for assessing the effect of Notch1 gene excision in advance of the unilateral ablation of the olfactory bulb (OBX). Six-week-old transgenic *K5-CreER^{T2};N1$^{fl/fl}$;fl(stop)TdTomato* mice were used. (*B* and *C*) OBX side. (*B*) HBCs do not activate in the post-OBX OE of Notch1$^{+/+}$ mice 7 d after the procedure, as all TdTomato$^+$ cells remain HBCs (arrow). (*C*) HBCs do activate in the post-OBX OE of Notch1$^{fl/fl}$ mice, giving rise to GBCs (thick arrow), OSNs (thick arrow/asterisk), and Sus cells (arrow/asterisk). [Scale bar in *B* (also for *C*), 10 μm.] (*D*) Counts of TdTomato$^+$ cells in the OE that are not HBCs as a measure of HBC-derived progeny demonstrate enhanced activation with OBX after Notch1 cKO. *$P < 0.05$. (*E*) Experimental timeline assessing the effect of Notch1 gene excision subsequent to unilateral OBX. (*F–I*) OBX side. (*F*) HBCs have lost CK14 expression and migrated apically (arrows). (*G* and *H*) HBC-derived CK14$^-$/PGP$^-$ GBCs (*Inset* in *G*) and PGP$^+$ and OMP$^-$ immature OSNs (*G* and *H*, respectively) (arrows) are evident within a week after Notch1 deletion. (*I*) Ki67$^+$ GBCs are numerous immediately superficial to the layer of TdT$^+$ HBCs, in keeping with the acceleration of neurogenesis. Moreover, Ki67$^+$ HBCs are evident, indicating markedly heightened proliferation (arrows). [Scale bar in *I* (also for *F–H*), 10 μm; *G*, 2×.] (*J*) Counts of TdTomato$^+$ clusters (*Left*) and cells in the OE (*Right*) that are not HBCs as a measure of HBC-derived progeny demonstrate enhanced activation when OBX is followed by conditional knockout of Notch. *$P < 0.05$. See Table S2 for detailed statistical information. Arrowheads demarcate the basal lamina.

consequence of OBX), or massive (seen acutely following OBX)—has the effect of maintaining the HBC reserve.

The lack of response to neuronal death that we observe closely matches a previous report demonstrating that OBX does not result in HBC activation (9). However, the current findings stand in opposition to observations by another laboratory using a different driver line in which HBCs did contribute to the OE of uninjured mice and to an enhanced degree following OBX (12). Two confounding factors might explain the difference in results. First, the latter driver line, although expressing a mutated progesterone receptor fused with Cre recombinase, was active in neonatal animals in the absence of the RU486 ligand, at which time HBCs might function more broadly as progenitors (12). Second, OBX in mice, when done too aggressively, can kill other epithelial cell types in addition to the OSNs because the vascular supply to the OE is compromised. Both of these features may be sufficient to account for the discrepancy.

Notch Signaling in HBCs Responds to both Direct Epithelial Injury (MeBr Exposure) and Neuron-Specific Depletion (OBX) but in Opposite Directions. Microarray and RNA-seq analyses of HBCs with and without injury demonstrated that Notch signaling was down-regulated after MeBr-triggered epithelial injury and subsequent delamination of neurons and Sus cells, which concomitantly eliminates the Sus cell-expressed Notch ligand Jag1. Down-regulation of Notch signaling in HBCs is not unexpected in the absence of a *trans* ligand normally expressed in the Sus cells. Nonetheless, injury to other tissues often has context-specific effects on the Notch pathway. In the lung, Notch signaling activity is up-regulated in tissue harvested from an acute lung injury mouse model (50). However, in the CNS, Hes1, a Notch signaling downstream target, is down-regulated following traumatic brain injury in association with enhanced hippocampal neurogenesis (51).

In stark contrast to the effect of direct epithelial lesion by MeBr inhalation, Notch signaling is enhanced in HBCs after OBX. At present, the mechanism underlying the Notch-ON state after OBX is unclear. However, the HBCs do undergo a number of changes as a consequence of neuronal degeneration, which may reflect the close physical association between them and bundled olfactory axons exiting the epithelium (8).

Notch1 Contributes to HBC Dormancy in the OE. Altered Notch1 signaling influences the maintenance of HBCs as dormant reserve stem cells. Enhanced Notch activity has the consequence of increasing the expression of *p63* in HBCs, a change that would oppose their activation given the necessity and sufficiency of eliminating p63 in shifting HBCs from dormancy. In contrast, in the absence of Notch1, reserve HBCs exhibit a tendency toward spontaneous activation in the uninjured OE that is markedly enhanced following OBX. The alterations in *p63* expression in response to manipulations of Notch pathway activity in the HBCs are opposite to the effect of Notch on p63 in other tissues. For example, in keratinocytes, Notch1 blocks p63 expression and promotes differentiation. Conversely, p63 antagonizes Notch1 and prevents differentiation (31, 34, 52, 53). Similarly, in mammary epithelial cells, Notch signaling reduces levels of ΔNp63 and mimics ΔNp63 depletion (35). Finally, in the trachea, Notch3 knockout results in an increased number of K5-expressing basal cells (54), and Notch signaling is required for differentiation of basal cells (55), both of which imply no effect on—or inhibition of—p63 expression by Notch. It is true that transduction of fibroblasts with NICD increases p63 expression (33), but as far as we can determine this is the only instance other than olfactory HBCs where this effect has been observed.

Given the apparent regulation of p63 levels by Notch1, the nature of the role of the canonical downstream Notch effector and repressive cofactor RBPJ in that regulation is unclear. RBPJ can play either an instructive or a permissive role in canonical Notch signaling when bound to N1ICD (46). In a permissive role, N1ICD binding to RBPJ removes RBPJ from DNA and alleviates its repression of gene expression. In an instructive role, the

N1ICD/RBPJ dimer becomes incorporated into or recruits a transcriptional complex to induce target gene transcription. In HBCs, the increase in expression of Notch1-targets *p63* and *Hes1* demonstrates that RBPJ function in HBCs is permissive with respect to these genes because mutation relieves repression, whereas an instructive role posits that N1ICD/RBPJ presence is required for gene transcription. Certainly, the in silico analysis of *p63* upstream of the TSS presented in Fig. S2 suggests that RBPJ binds to the promoter and may directly regulate *p63* expression. However, in the absence of ChIP data providing evidence that RBPJ directly binds to the p63 promoter or within the p63 loci, the notion that p63 is a direct target of RBPJ in HBCs can only be suggested at this time. Alternate indirect pathways by which Notch signaling could alter p63 transcription have been demonstrated in other tissues. For example, Notch and Wnt are known to have an antagonistic relationship (56), and it is possible that Notch1 or RBPJ deletion alters Wnt signaling, which in turns alters p63 expression. Importantly, we provide evidence that functional RBPJ is not required for transcription of the canonical Notch target Hes1 or of p63 in HBCs of the adult OE.

Whereas deletion of Notch1 enhances activation of HBCs to a degree in the absence of injury and to a greater extent following OBX, there are plenty of Notch1$^{-/-}$ HBCs even at long survivals after injury. Thus, it is evident that the Notch pathway does not serve as the master regulator of p63 in the same way that p63 serves as the master regulator of HBC dormancy. In contrast, Notch signaling has been characterized as the master regulator of p63 in other tissues, such as the skin (31). In the case of the OE, p63 levels in quiescent HBCs are presumably set in response to multiple different niche-derived cues, the integration of which determines whether levels of p63 decline to a level consonant with HBC activation. In this formulation, Notch1 deletion alters the rheostat that sets p63 levels and the probability of HBC activation, such that a tissue perturbation that does not normally elicit activation (e.g., OBX) is better able to shift the HBCs out of dormancy.

Notch2 Is Not Required for Maintenance of HBC Quiescence. Although it is evident that Notch2 protein is present in HBCs by IHC and RNA-seq analysis, Notch1 and Notch2 do not play redundant roles in maintaining HBC quiescence in the setting of neuronal injury. Excision of *Notch2*, in contrast to the enhanced rate of activation observed with *Notch1* knockout, seems to have little or no effect on HBCs. Although the morphology of *Notch2*-deleted HBCs is altered somewhat after OBX, they remain locked in dormancy, whether *Notch2* is knocked out before or after OBX. It is not uncommon for Notch1 and Notch 2 to play different, even countervailing, roles in tissue. For example, the consequences of *Notch1* knockout are more severe during gestation than those of *Notch2* (57, 58). In specific terms, Notch2 but not Notch1 is responsible for inhibiting endochondral bone formation during limb development (59). Similarly, Notch2 has been shown to play a key role in the establishment and survival of the Sus cell population of the OE (60), but Notch1 does not. With respect to disease processes, it is known that Notch1 and Notch2 play opposite roles in oncogenesis and have been used as opposing cancer prognostication factors (61–63). Additionally, Notch1 and Notch2 play different roles in diabetic nephropathy (64). Nonetheless, the interaction between the two receptors can be synergistic: for example, in gut (65) and in immune cells (66). Thus, tissue context looks to be determining their individual roles.

Conclusions

The data presented herein indicate that Sus cell injury is a key cellular event that leads to activation of HBCs. Furthermore, signaling via Notch1 plays a significant role in maintaining the expression of p63 in the context of low-level and accelerated neuronal turnover, and therefore ensuring HBC dormancy; the enhanced p63 signaling in the context of dying neurons has the likely effect of preserving, protecting, and defending the HBC reserve stem cell population. Surprisingly, the maintenance of p63 by the Notch1 pathway is opposite to its role in most, if not all, other epithelial tissues. In the OE, the elimination of the Notch ligand Jagged1 by the destruction of Sus cells may, in turn, be part of the mechanism by which tissue injury causes a decline in p63 levels and consequently HBC activation. Of course, other injury-associated cues—whether signals that accelerate the degradation of p63 and suppress its expression or ones that fail to maintain p63 levels, which role Notch1 seems to fill—are likely to contribute to the response of HBCs to tissue damage. Additional work will be required to elucidate the other molecules and pathways that control HBC activation from dormancy and its reestablishment as part of healing. Nonetheless, our findings also have significant implications for the aging of the OE and olfactory dysfunction in the elderly. Despite the remarkable capacity for lifelong neurogenesis in the OE, we have previously demonstrated in both humans and mice that the aged OE has areas of aneuronal tissue, where the active GBC population has been exhausted and neurogenesis has ceased (67–69). However, the HBCs in this setting remain dormant and fail to regenerate the functional neuronal tissue, perhaps because Sus cells remain intact. The current data, which demonstrate that Notch1 maintains HBC quiescence in the setting of massive, near-complete absence of neurons following OBX, suggest that the Notch signaling pathway could serve as a potential target for therapy in the aged neuroepithelium.

Materials and Methods

Mice. All animals were housed in a heat- and humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care-accredited vivarium operating under a 12:12-h light:dark cycle, and animals were maintained on an ad libitum rodent chow and water. The Committee for the Humane Use of Animals at Tufts University School of Medicine, where the animals were housed and the experiments conducted, approved all protocols using vertebrate animals. See *SI Materials and Methods* for origins of wild-type and transgenic animals.

Surgical Procedures and IHC. OBX was performed as previously described (70). MeBr lesions were performed as previously described (71). IHC and cell dissociations were performed as previously described (5, 72). For a full description of the experimental procedures and staining conditions used in this study, please see *SI Materials and Methods*.

RNA-Seq, Bioinformatic, and Statistical Analysis. HBCs were isolated from both uninjured and 18 h post-MeBr lesioned *K5-CreERT2;fl(stop)TdTomato* transgenic mouse lines after Tam induction of labeling. RNA were subjected to deep sequencing using the NuGEN Ovation kit on an Illumina HiSEq. 2500 at 100 M reads per sample. Samples Nml3 and 18HPL3 were discarded after quality control and clustering. Promoter analysis was done using FIMO scanning of published promoters and consensus motifs for RBPJ binding. Additional details can be found in *SI Materials and Methods*. The primary antibody dilutions, the details of their working conditions, and the methods for their detection are listed in Table S1. Detailed information of statistical data can be found in Table S2.

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