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## **Notch2 is required for maintaining sustentacular cell function in the adult mouse main olfactory epithelium**

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### **Abstract**

*Notch* receptors are expressed in neurons and glia in the adult nervous system, but why this expression persists is not well-understood. Here we examine the role of the *Notch* pathway in the postnatal mouse main olfactory system, and show evidence consistent with a model where *Notch2* is required for maintaining sustentacular cell function. In the absence of *Notch2*, the laminar nature of these glial-like cells is disrupted. *Hes1*, *Hey1*, and *Six1*, which are downstream effectors of the *Notch* pathway, are down-regulated, and cytochrome *P450* and *Glutathione S-transferase (GST)* expression by sustentacular cells is reduced. Functional levels of GST activity are also reduced. These disruptions are associated with increased olfactory sensory neuron degeneration. Surprisingly, expression of *Notch3* is also down-regulated. This suggests the existence of a feedback loop where expression of *Notch3* is initially independent of *Notch2*, but requires *Notch2* for maintained expression. While the *Notch* pathway has previously been shown to be important for promoting gliogenesis during development, this is the first demonstration that the persistent expression of *Notch* receptors is required for maintaining glial function in adult.

### **Keywords**

*Notch*; glia; olfactory; sustentacular; neurodegeneration; *Hes1*; *Hey1*; *Six1*; *Notch2*; *GST*

### **Introduction**

The *Notch* pathway is involved in a wide array of cell fate decisions during development (Louvi and Artavanis-Tsakonas, 2006). However, *Notch* receptors are also expressed in the adult in astrocytes, Müller glia, olfactory ensheathing glia, and Bergmann glia (Carson et al., 2006; Furukawa et al., 2000; Givogri et al., 2006; Higuchi et al., 1995; Irvin et al., 2001; Tanaka et al., 1999). Although some of these glia are likely to represent progenitor populations (Gaiano and Fishell, 2002), why *Notch* receptors continue to be maintained within apparently committed, mature glia is not known.

One hypothesis would be that *Notch* receptor expression is required to prevent a change in cell fate. Experiments in fly (Fortini et al., 1993) and in frog (Coffman et al., 1993) show that transient expression of *Notch* only temporarily alters cell fate. In postmitotic retinal neurons,

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misexpression of *Notch1* can cause a subset of these neurons to become Müller glia (Jadhav et al., 2006). Thus, expression of *Notch* receptors even in differentiated cell-types can alter cell fate, suggesting that maintained expression of *Notch* receptors may be required to permanently establish fate, at least in some cell-types. On the other hand, activation of the *Notch* pathway in neural crest stem cells initiates an irreversible switch from neurogenesis to gliogenesis (Morrison et al., 2000). Similarly, in the CNS, brief induction of *Notch1* or *Notch3* signaling can irreversibly induce astrocyte differentiation (Tanigaki et al., 2001). These experiments suggest that transient activation of the *Notch* pathway is sufficient to promote gliogenesis.

An alternative hypothesis to explain the persistent expression of *Notch* receptors in adult would be that the *Notch* pathway acts to maintain function in adult glia. *Notch1* and *Notch2* are expressed in adult neurons (Sestan et al., 1999). Although studies on adult neuronal *Notch* function are rare, these studies indicate that the *Notch* pathway is employed in the adult in a manner distinct from its role during development. In adult hippocampal neurons, for example, reduced *Notch1* expression impairs long-term potentiation (Wang et al., 2004). *Notch* receptors in the cortex are thought to influence neurite outgrowth (Sestan et al., 1999). Thus, the *Notch* pathway acts in the adult to influence and maintain neuronal function. But why *Notch* receptors are expressed in adult glia has yet to be examined.

Here we have examined the function of *Notch2* in the postnatal mouse main olfactory epithelium. The nasal epithelium is a pseudostratified epithelium containing four major cell-types separated into distinct layers (Farbman, 1992). Sustentacular cells are located apically, with olfactory sensory neurons (OSNs) basal to these cells. Deep to the OSNs lie the progenitor cells of the epithelium, which can be divided into globose and horizontal cell-types. Although olfaction is mediated by receptors expressed by OSNs, the OSNs themselves are supported by sustentacular cells, which perform a number of glial-like functions (Nomura et al., 2004; Vogalis et al., 2005; Weiler and Farbman, 1998). For example, sustentacular cells express high levels of cytochrome *P450* isoforms (Gu et al., 1998) as well as *Glutathione S-transferases* (*GSTs*; (Whitby-Logan et al., 2004)). Expression of these enzymes is consistent with sustentacular cells acting in a neuroprotective manner (Ling et al., 2004).

We show that *Notch2* and *Notch3* expression persists in postnatal and adult animals within sustentacular cells. We use a conditional mutant of *Notch2* to show that the canonical *Notch* pathway is important for maintaining sustentacular cell function. In the absence of *Notch2*, the laminar organization of the epithelium is disrupted. Expression of the transcription factors *Hes1*, *Hey1*, and *Six1*, which are downstream effectors in the *Notch* pathway, are down-regulated in sustentacular cells. Moreover, expression of cytochrome *P450* isoforms and *GST* enzymes in the epithelia are reduced. Functional levels of GST activity are also reduced. These changes in the sustentacular layer are accompanied by neurodegeneration of OSNs, consistent with the interpretation that the glial-like function of sustentacular cells has been affected. This work therefore provides evidence that, in addition to its role during development in promoting gliogenesis, the *Notch* pathway is required for maintaining the function of differentiated glia in the adult. Finally, we show that *Notch3* expression in *Notch2* mutants is down-regulated, suggesting that maintenance of *Notch3* expression also requires *Notch2*.

## Materials and methods

### Mice

All animal protocols were approved by Cornell's IACUC. *Notch2* mutant mice were generated as previously described (McCright et al., 2006) and are maintained in a mixed 129Sv/C57BL/6 background. *Notch2<sup>lox/lox</sup>* (*N2<sup>lox/lox</sup>*) animals were crossed to *Foxg1*-Cre mice (Hebert and McConnell, 2000), also maintained in a mixed 129Sv/C57BL/6 background, to generate F1 *N2<sup>lox/+</sup>;Foxg1<sup>Cre/+</sup>* animals. These mice were crossed to *N2<sup>lox/lox</sup>* animals to generate the

mutant  $N2^{flox/flox};Foxg1^{Cre/+}$  animals and controls ( $N2^{flox/+};Foxg1^{Cre/+}$  and  $N2^{flox/flox}$ ). Within-litter, sex matched comparisons were performed for all studies. The day a vaginal plug was observed was termed day 0.5.

### ***In situ* hybridization**

P0, 2.5 week-old, and adult (8-19 weeks) mice were euthanized and decapitated. Heads were embedded in OCT (TissueTek; Sakura-FineTek) and fresh-frozen in liquid nitrogen cooled isopentane. 10-20 $\mu$ m thick cryosections were collected and processed for single and double-label *in situ* hybridization as previously described (Williams et al., 2007). Probes used were cloned by PCR or purchased from clonesets. Probes corresponded to the following regions for each gene: *Notch1* (nt 7819-9029), *Notch2* (nt 594-1653), *Notch3* (nt 519-1369 and 7038-7943), *Hes1* (nt 234-1379), *Hes5* (nt 15-1271), *Scg10* (BMAP clone 30G10), *Mash1* (nt 664-1810), *OMP* (IMAGE clone IRAKp961I03127Q), *Hey1* (nt 781-1288), *Six1* (NIA 7.4k clone H4070G06). Sections were hybridized at 62-70°C.

### **TUNEL**

10 $\mu$ m sections were fixed and rinsed with PBS before incubating in pre-cooled ethanol:glacial acetic acid (2:1) for 5 min. at -20°C. After rinsing with PBS, endogenous peroxidase activity was quenched by incubating in 3% hydrogen peroxide for 10 min. at room temperature. After further PBS washes, slides were incubated with equilibration buffer (Chemicon) for 10 minutes and then incubated with terminal transferase (New England Biolabs (NEB)) in incubation buffer (1x CoCl<sub>2</sub> (NEB), 1x restriction buffer 4 (NEB), 0.5 nM biotin-dUTP (Roche)) for 3.5 hrs at 37°C. Reactions were stopped with stop buffer (Chemicon), rinsed with PBS, incubated with streptavidin-HRP (Zymed) and reacted using the AEC staining protocol (Zymed).

### ***Mash1* and TUNEL Morphometry**

Sections from matched littermates were serially photographed. Number of positive signals/mm were obtained by drawing a line between positive signals, counting the number of positive spots within this range, and obtaining an average. The spots that were connected was arbitrarily chosen, but selected so as not to have extreme distances between spots. To compensate for the arbitrary nature of the selection, between 5-26 mm (*Mash1*) and 3-18 mm (TUNEL) of linear distance were counted and averaged per animal. Cells were binned into basal, neuronal, and apical layers based upon their relative location within the epithelium. Cells were only defined as apical if they were clearly adjacent to the lumen. Cells were only defined as basal if they were within 2-3 cell diameters of the lamina propria.

### **RNA isolation**

Whole epithelia were dissected out and flash-frozen in liquid nitrogen. Total RNA was purified using Trizol reagent as per manufacturer's instructions (Invitrogen).

### **RT-PCR**

5 $\mu$ g of total RNA was primed with oligo-dT and reverse transcribed using Superscript III (Invitrogen). The product was precipitated and 1/100<sup>th</sup> of the reaction was used per PCR reaction. A control RNA sample containing no reverse transcriptase was performed for all three RNA samples. PCR primers for *Notch2* were identical to those described previously (Carson et al., 2006). We found that the *Notch3* sequences described in this paper overlapped with a *Notch* pseudogene. We designed new primers for *Notch3* with the following sequences: AAGGTGGAAGTGCATAGACAAG and ATCTTG TAGGCAGTCCCGAGTAT to produce a product of 506 bp.

## Northern Blot

10µg of total RNA from P0, 2.5 week, and 3 month old adult epithelia was electrophoresed through an agarose gel containing 2% formaldehyde in Hepes buffer. The RNA was transferred to a nylon-backed membrane and the filter hybridized in Church buffer at 65°C (Church and Gilbert, 1984). After washing, membranes were exposed to Biomax MS film (Kodak).

## Immunohistochemistry

Epithelia from adult matched *Notch2* mutants and wild-type controls were fixed in Bouin's fixative (LabChem) overnight at room temperature and washed in 70% ethanol. Samples were embedded in wax and 5µm sections were deparaffinized and processed as described previously (Carson et al., 2006) except samples were microwaved in citric acid. NOTCH3 antibody (Santa Cruz Biotechnology #sc-5593) at a dilution of 1:50 was applied overnight at 4°C, and bound antibody was detected using a FITC goat anti-rabbit secondary (Vector Labs) and an Alexa488 anti-FITC tertiary antibody (Molecular Probes). Sections were imaged on a Leica DMRE upright microscope fitted with bandpass filters.

## Histology

Samples were embedded in wax and processed for histology by the Cornell Diagnostic Laboratory as described (Luna, 1992; Luna et al., 1968; Preece, 1972).

## Scanning electron microscopy

Samples were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate (pH 7.4) at 4°C for at least 24 hours. Samples were then treated in 30% potassium hydroxide as described (Nomura et al., 2004), washed in 0.1M sodium cacodylate, fixed overnight in 1% osmium tetroxide (0.1M sodium cacodylate; Electron Microscopy Sciences), washed, and dehydrated through an ethanol gradient containing 70% ethanol with 2% uranyl acetate. Samples were dried in a critical point dryer (Bal-Tec), cracked, and sputter-coated in Au/Pd for 2 min. (Bal-Tec). Samples were viewed on a Hitachi S4500 SEM at an accelerating voltage of 10kV.

## DAPI

Cryostat sections were fixed in buffered 4% paraformaldehyde, stained (15 min. at 1 µg/ml in 1x PBS; Roche), washed, and mounted in 90% glycerol containing 0.5% npropyl gallate and 20 mM Tris pH 8.0.

## Quantitative RT-PCR

qPCR was performed using Universal ProbeLibrary (UPL; Roche) reagents, and appropriate primers were designed using the Roche Universal ProbeLibrary Assay Design website (<https://www.roche-applied-science.com/sis/rtPCR/upl/index.jsp>). Each RNA sample was tested in triplicate and the data normalized using rodent GAPDH (Applied Biosystems) as an endogenous control.

Gene	Left primer	Right primer	UPL#
<i>Cyp1a2</i>	gactgactcccacaactctgc	gaagccatctgtaccactg	19
<i>Cyp2a5</i>	accaaggacaccaagtcttg	agagcccagcataggaaaca	52
<i>Cyp2g1</i>	tgatgccacatttcagtcct	ccttgaccgaagtacacagt	21
<i>GST mu1</i>	gcagctcatcatgctctgtta	ttttctcagggatggtctca	106
<i>GST mu2</i>	agttggccatggtttgctac	agcttcatctctcaggagagac	106

## GST assay

Dissected epithelia were snap frozen in liquid nitrogen before being homogenized in a solution of 10% glycerol, 1 mM EDTA, 0.1M DTT, 1 mM PMSF (Sigma), and 1 mM PTU (Sigma) using a TissueLyser (Qiagen). Homogenates were centrifuged for 20 min. at 10,000g and the supernatant quantified with a BCA protein assay (Pierce). GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB; Sigma), a generic substrate, was determined spectrophotometrically as previously described (Habig and Jakoby, 1981; Whitby-Logan et al., 2004) using 10 $\mu$ M assay and GSH (Sigma) at 2.5 mM. Change in absorbance at 340 nm was recorded at 30 second intervals for at least 5 minutes. Each reaction was performed in duplicate or triplicate. Statistical comparison of mutant and control slopes was performed using the JMP statistical package (SAS Institute).

## Results

### Expression of *Notch* receptors in the postnatal main olfactory epithelium

We examined the expression of *Notch1-4* using *in situ* hybridization. Although *Notch* receptors have been previously examined for expression in the olfactory system, the various studies have not been entirely consistent with one another. *Notch1* is found in the basal epithelium by Orita et al. (Orita et al., 2006), Mitsiadis et al. (Mitsiadis et al., 2001), Doi et al. (Doi et al., 2004), and Lindsell et al. (Lindsell et al., 1996), but not by Carson et al. (Carson et al., 2006). Doi et al. do not observe any *Notch2* expression at all within the epithelium, whereas Carson et al., Mitsiadis et al., and Lindsell et al. show *Notch2* expression within the sustentacular layer. Finally, *Notch3* is not detected in the sustentacular layer by Carson et al., but is by Mitsiadis et al. However, Doi et al. detected *Notch3* in the basal OE at postnatal stages.

Given the variation in described expression patterns for the *Notch* receptors, we reexamined the expression patterns for the four receptors during postnatal stages using *in situ* hybridization. We find *Notch1* is expressed from P0 to adult in the basal epithelium, consistent with Orita et al. but not Carson et al. Clusters of *Notch1* expressing cells are observed at all stages (Fig. 1A-C), although this expression is significantly reduced by adulthood (defined as 8 weeks and older). Interestingly, expression of *Notch1* appears to be restricted to the dorsal recess (data not shown). In contrast, we found that *Notch2* is apically expressed within the sustentacular layer at all stages (Fig. 1D-F), consistent with Carson et al. but not Doi et al. We found that *Notch3* expression is present in the sustentacular layer from P0 through adulthood (Figs 1G-I). Both *Notch2* and *Notch3* are also expressed in the lamina propria (data not shown; (Carson et al., 2006)). *Notch4* expression is only found within the lamina and not within the epithelium itself (data not shown).

These data suggested that *Notch2* and *Notch3* are co-expressed within sustentacular cells. To more closely examine this expression, we performed a series of double-label *in situ* hybridization experiments on adult epithelia. To first confirm that expression does occur within sustentacular cells, we used a known marker of sustentacular cells, *Carbonyl reductase 2* (*Cbr2*) (Yu et al., 2005). Expression of *Notch2* clearly colocalizes with *Cbr2* (Fig. 2A-C). To show that expression does not occur within the neuronal layer, we used *Olfactory marker protein* (*OMP*) ((Margolis, 1982), Fig. 2D-F). Finally, we asked whether or not *Notch2* and *Notch3* are co-expressed among sustentacular cells, and found significant overlapping expression (Fig. 2G-I). These results show that *Notch2* and *Notch3* are expressed within sustentacular cells. Neuronal expression was not detected using these approaches.

We were surprised at the observed expression of *Notch3*, as prior studies using immunohistochemistry had not detected *Notch3* in the sustentacular layer (Carson et al., 2006; Doi et al., 2004), and RT-PCR experiments suggested the transcript was not present in

adult (Carson et al., 2006). We therefore performed additional tests to validate our observed *Notch3* expression pattern. We cloned two non-overlapping regions of *Notch3* and used each for *in situ* hybridization (see Materials and methods). *Notch3* was detected with both probes in the sustentacular layer (data not shown). We also performed immunohistochemistry and detected expression of NOTCH3 protein in adult animals in sustentacular cells (Fig. 2J). No expression was detected in the sustentacular layer in the absence of primary antibody (Fig. 2K). Finally, Northern blot analysis and RT-PCR with whole epithelial RNA isolated from all three postnatal stages clearly detected *Notch3* RNA (Fig. 2L). Collectively, these experiments show that *Notch3* is expressed postnatally in the epithelium by sustentacular cells.

### Conditional elimination of *Notch2* with *Foxg1-cre*

The continued expression of *Notch2* and *Notch3* within the sustentacular layer during postnatal stages led us to further investigate the role of *Notch* receptor expression in the adult. Sustentacular cells make up 15-25% of all olfactory epithelial cells (Farbman, 1992). Based on similarities in function with glia, these cells are thought to act as support cells for OSNs, and have been termed “glial-like” (Weiler and Farbman, 1998). What is the purpose of the maintained expression of *Notch2* and *Notch3* in postnatal sustentacular cells? We used a conditional null mutant of *Notch2* (McCright et al., 2006) and a null mutant of *Notch3* (Krebs et al., 2003) to examine the effects of removing these *Notch* receptors on sustentacular cells. We used the *Foxg1-Cre* line (Hebert and McConnell, 2000) to selectively inactivate *Notch2* in the epithelium and telencephalon. Although it has previously been shown that *Foxg1*<sup>Cre/+</sup> animals have haploinsufficient phenotypes within the CNS (Shen et al., 2006), no qualitative differences were observed among the two control populations (*N2*<sup>flox/+</sup>; *Foxg1*<sup>Cre/+</sup> and *N2*<sup>flox/flox</sup>; data not shown).

To show that *Foxg1-Cre* is expressed within the epithelium, we crossed these mice with a *Rosa26-LacZ* reporter line (Soriano, 1999). Expression of  $\beta$ -galactosidase is detected throughout the epithelium (Fig. 3A). To confirm the efficacy of the deletion, we performed *in situ* hybridization using a probe corresponding to the exon deleted in the conditional *Notch2* mutant (Fig. 3B,C). No signal was observed in mutant animals (Fig. 3C) as compared against controls (Fig. 3B). These experiments show that *Notch2* has been effectively deleted from the sustentacular layer during development.

Unexpectedly, we found that mutant mice were significantly smaller than their heterozygous littermates (Fig. 3D). Although there was no apparent weight difference at P0, at later stages mutant animals weighed 27% (2.5 weeks) and 47% (adult) less than controls. In preliminary studies, we had found that *Foxg1* is expressed within Rathke's pouch at embryonic day 10 (E10). Similarly, *Notch2* is also expressed in the pouch at E10 (data not shown). As activated *Notch2* has been shown to affect pituitary differentiation (Raetzman et al., 2006), one interpretation is that *Notch2* deletion in the pituitary leads to the reduced size of the mutant animals. Consistent with this, *Notch2* mutant pituitaries are significantly smaller in size than that of wild-type littermates (data not shown). Although mutant animals were born in approximately Mendelian ratios (21%), nearly two-thirds did not survive to adulthood. While the mortality rate was high, we did not find any evidence of non-specific effects upon the olfactory system, as described below.

### Absence of *Notch2* disrupts epithelial organization

We initially used histological stains to assess the overall structure of the epithelium in mutants. In sagittal sections of adult *Notch2* mutants, clear alterations in hematoxylin/eosin and Bielschowsky staining were observed (Fig. 4). Hematoxylin/eosin staining revealed that the relatively uniform spacing of sustentacular nuclei seen in control animals was disrupted in regions in the mutant (compare Fig. 4A,B). The morphology of the nuclei was also altered,

and they appeared smaller and more irregularly shaped. Pyknotic nuclei could be seen as well in the epithelium (Fig. 4B). Bielschowsky staining was used to reveal axonal and dendritic processes of OSNs. Significantly fewer dendritic processes were observed in the mutant, leading to gaps and a reduction in the number of dendritic tufts at the apical surface (Fig. 4C,D).

The histological analysis suggested that the laminar nature of the epithelium had been disrupted. Using scanning electron microscopy, we were able to confirm this disorganization. In wild-type animals, OSNs are organized into relatively ordered arrays (Fig. 4E). However, in three different mutants, regions of the epithelium can be found where OSNs appear disorganized (Fig. 4F), consistent with what was observed with the hematoxylin/eosin and Bielschowsky analysis.

We next examined olfactory bulbs of mutant mice for defects. Because of the observed difference in size between mutant and control animals and the potential effect of *Notch2* on the pituitary, we based our study on data from mice bearing a knockout mutation in *IGF1* (Pichel et al., 2003). Hypophysectomized animals show highly reduced levels of *IGF1* (Murphy et al., 1987), and *IGF1* mutants weigh less than controls at embryonic day 18.5 (E18.5). In addition, the mitral layer is disorganized and disrupted, and 70% of mitral neurons are missing in these *IGF1* mutants. In contrast, no alterations in the laminar structure of the mitral layer were observed in *Notch2* mutant bulbs (Fig. 4G,H).

The histological and SEM analysis showed that the epithelium appeared disorganized. However, we noted that this disorganization was not generally observed throughout the epithelium. While some areas were significantly disrupted, other areas appeared relatively normal. The variability in phenotype led us to examine the overall structure in a coronal plane of the epithelium. Strikingly, in several mutants, significant differences in the thickness of the epithelium could be observed as compared with controls (Fig. 5). DAPI staining showed that some regions of the epithelium were dramatically thinner than others (Fig. 5B,D; compare with Fig. 5A,C). These differences are highlighted in cartoon form in Fig. 5E,F. This variability in epithelial thickness was not present in all mutants. However, all of the phenotypes described below were present in varying degrees within all *Notch2* mutant animals.

### Neurodegeneration in *Notch2* mutants within the epithelium

The DAPI stained images (Fig. 5) clearly showed significant degeneration had occurred in some mutants. However, it was unclear whether other areas of the epithelium that were apparently unchanged in thickness and other mutants that did not show dramatic alterations in epithelial thickness would also show the presence of degeneration. Moreover, it was unclear whether both neurons and sustentacular cells were being affected. We therefore used TUNEL analysis to examine and quantify the levels of apoptosis in adult *Notch2* mutants and to define the laminar location of this expression (see Materials and methods; 3-8mm of linear distance counted per adult). We defined apical TUNEL-positive cells as those adjacent to the lumen, and basal TUNEL-positive cells as being within 2-3 cell diameters of the basal lamina. Most of the observed TUNEL signal appeared to be within the neuronal layer (Fig. 6B; compare with 6A), although occasional cells were detected in the sustentacular and basal layer. Quantification of TUNEL signal (n=3 pairs; see Materials and methods) showed no significant difference in the number of TUNEL-positive cells per millimeter in the basal layer of adults (Fig. 6D). Significant differences did exist between mutant and control in the apical layer, although the overall number of positive cells was small (0.3 cells/mm vs 0/mm). The great majority of the increase in TUNEL-expressing cells were located within the neuronal layer.

Interestingly, we found that some regions of the mutant epithelium showed dramatically higher TUNEL expression (Fig. 6C). We note that the thickness of the epithelium was not a strict predictor of the degree of observed TUNEL expression. In areas of the epithelium that were

extremely thin (e.g. Fig. 5D), little or no TUNEL expression was detected, presumably because relatively few cells remained. Areas that did have extremely high TUNEL expression were not included in our quantitation as it was impossible to identify individually stained cells. As a result, our analysis underestimates the level of cell death in mutants.

The overall increase in neurodegeneration seen in adult mutants led us to ask when this phenotype first occurred. We examined TUNEL expression in P0 and 2.5 week-old animals (n=3 pairs for each timepoint; 6-18mm/P0 animal, and 7-15mm/2.5 week animal). Quantitation showed no difference in the total number of TUNEL-positive cells per millimeter between mutant and control (Fig. 6E). No difference was observed in the apical-basal distribution of TUNEL-positive cells at either stage as well (data not shown). Collectively, these results show that neurodegeneration does not occur during early postnatal life, but does increase as the animal ages. To control for potential non-specific apoptosis that may occur as a result of altered *Notch2* function in the pituitary, we examined mutant adult bulbs for evidence of increased TUNEL expression. No differences were observed between mutants and controls (data not shown), suggesting that widespread apoptosis throughout the nervous system is not present.

### Deletion of *Notch2* does not alter sustentacular cell fate in the adult

One possible reason for the persistent expression of *Notch2* in postnatal sustentacular cells may be to prevent alterations in sustentacular cell fate. It has previously been shown that *Hes1* is important for repressing neuronal fate in sustentacular cells (Cau et al., 2000). In the absence of *Hes1*, increased numbers of OSNs are produced. It was hypothesized that *Notch* receptor expression is required for mediating the effects of *Hes1* on sustentacular cells during development (Cau et al., 2000). If *Notch* reprises this role in the adult olfactory system, the loss of *Notch2* may lead to a change in cell fate among sustentacular cells. We therefore examined the epithelia of mutant animals at P0 (n=18), 2.5 weeks (n=8) and adult (8-19 weeks; n=13) for alterations in cell fate using a variety of neuronal markers.

We initially screened adult epithelia for *Scg10*, a marker of early neuronal development (Pellier-Monnin et al., 2001), and *Olfactory marker protein (OMP)*, a marker of mature OSNs (Danciger et al., 1989). In adult animals, the location of *Scg10* expression is similar in controls (Fig. 7A) and mutants (Fig. 7B). The expression of *Scg10*, however, is more variable in the mutant. In some areas, expression of *Scg10* is significantly increased relative to control (Fig. 7B left panel vs. Fig. 7A), while in others, *Scg10* expression is patchy, with areas of weak or absent expression (Fig. 7B right panel vs Fig. 7A). No expression of *Scg10* was observed apically in the sustentacular layer.

Like *Scg10*, *OMP* is expressed in mutant epithelia (Fig. 7C,D). However, in adult mutants, the apical surface of *OMP* expression was often irregular (Fig. 7D). This expression correlates with the disorganized, laminar structure observed with the histological stains (Fig. 4B). Occasionally, *OMP* expression was seen to extend into the apical layer (Fig. 7D). We interpret this to indicate that OSNs are being displaced into the apical layer due to disruption of the sustentacular layer (Fig. 4B, F).

We also examined expression of several genes that are markers of sustentacular fate. *Cytokeratin8 (Krt8)* is a structural protein expressed by sustentacular cells (Suzuki and Takeda, 1991). *Krt8* expression appeared relatively unaffected in mutants (Fig. 7 E,F). Similarly, we looked at expression of *O-MACS*, a member of the *CoA-synthetase* family that is expressed exclusively in the dorsal epithelium in a zonal manner (Oka et al., 2003). *O-MACS* is expressed by sustentacular, neuronal, and basal cells. Its expression was unaffected in the mutant, demonstrating that zonal identity for these dorsal-most sustentacular cells was not affected by the absence of *Notch2* (Fig. 7G,H). Finally, we looked at expression of *Carbonyl reductase2 (Cbr2)*, an enzyme produced by sustentacular cells that is thought to be important for clearance



of odorants (Yu et al., 2005). Expression of *Cbr2* in general appeared similar between mutant and control, although the level of expression seemed somewhat reduced in the mutant (data not shown). Collectively, these experiments show that no dramatic change in cell fate markers have occurred in either the sustentacular layer or in the neuronal layer of *Notch2* mutants. However, disruption of the normal domains of *OMP* expression and *Scg10* were visible. Although most regions of the epithelia showed variation in the laminar expression of *OMP* (Fig. 7D), in some areas of mutant epithelium, *OMP* expression was dramatically affected (Fig. 8A,B). The epithelium in these regions was highly variable in thickness, and *OMP* expression was strongly reduced and sometimes absent. We also examined mutant olfactory bulbs using a variety of probes, including *Neurotensin*, *IGF1*, and *Glutamate receptor* (subunit 1). No differences were seen between mutants and controls (data not shown).

### Increase in progenitor cell number in *Notch2* mutants

We next examined expression of *Mash1*, a marker of olfactory neuronal progenitors (Guillemot et al., 1993). During development, *Mash1* labels cells that are migrating from the apical layer to the basal epithelium where they will give rise to progenitor populations (Cau et al., 2002). At P0, there are large numbers of *Mash1*-expressing cells in the neuronal and basal layer in control animals (Fig. 9A). A small number of positive cells can also be detected in the apical layer, as previously described (Cau et al., 2002). By 2.5 weeks, there are fewer *Mash1*-positive cells in the neuronal layer and an increasing number of basally located cells (Fig. 9C). In the adult, the great majority of *Mash1*-positive cells are basally located (Fig. 9E). As previously described (Manglapus et al., 2004), *Mash1* expression in the dorsal recess tended to be lighter than those in the more ventral and lateral epithelium in adults. In P0 and 2.5 week-old mutant animals, the overall pattern of *Mash1*-expressing cells was similar to that of controls (Fig. 9B,D). However, in the adult mutant, increased numbers of *Mash1* cells were observed in the neuronal and apical layer (Fig. 9F). As with *OMP* expression, in some regions of mutant epithelia, a dramatic increase in *Mash1* expressing cells was detected (Fig. 9G,H).

We quantified the distribution of *Mash1* in P0 (n=3 pairs; 8-15mm/animal), 2.5 week (n=3 pairs; 5-26mm/animal), and adult (n=3 pairs; 7-14mm/animal) mutants and controls (Fig. 9I). No difference was observed in the total number of *Mash1*-expressing cells per millimeter at P0. Although no significant difference was observed in 2.5 week-old animals, there were areas of epithelia in the mutant which possessed increased numbers of *Mash1*-positive cells (data not shown). However, these regions constituted a relatively small proportion of the epithelium, and the overall quantitation showed no significant differences.

In adult mutants, there were significantly greater numbers of *Mash1*-positive cells per mm as compared with control (Fig. 9I). We quantified the apical-basal distribution of these cells in a manner analogous to that for the TUNEL analysis. Approximately equal numbers of cells are found in the basal layer between control and mutant (Fig. 9J). However, in both the neuronal and apical layers, significantly higher numbers of *Mash1*-positive cells were observed in the mutant. We note that we did not include any areas in this quantitation where there were excessively large numbers of *Mash1*-positive cells (e.g. Fig. 9G,H), as it was impossible to quantify the number of cells within these regions. Thus, as with the TUNEL analysis, this quantification represents an underestimate of the number of *Mash1*-positive cells and their distribution in *Notch2* mutants. While the small but significant increase in apically-detected *Mash1* expression is consistent with a cell fate change among sustentacular cells, only a small number of such cells are detected (Fig. 9J; 3.0 cells/mm vs 0.1 cells/mm). Moreover, the distribution of *Mash1* cells in highly affected regions is reminiscent of what was observed in olfactory epithelia that have been chemically ablated (Manglapus et al., 2004). In these epithelia, both neurons and sustentacular cells have been ablated, and *Mash1* cells can be

observed in the apical layer of the regenerating epithelia. Over time, this expression disappears, and *Mash1* is again primarily found in the basal epithelium.

### Deletion of *Notch2* alters expression of downstream *Notch* pathway effectors

Although *Notch2* expression is widespread in the sustentacular layer, we were unable to identify widespread changes in cell fate among sustentacular cells in *Notch2* mutants. We next considered that *Notch2* may instead be required for maintained sustentacular function. It had previously been suggested, based upon the continued expression of *Hes1* in the adult, that *Hes1* may be required for maintaining the function of sustentacular cells (Manglapus et al., 2004). We therefore examined whether or not *Hes1* and *Hey1*, which are both *in vitro* downstream targets of *Notch2* (Maier and Gessler, 2000; Shimizu et al., 2002), are affected in mutants.

*Hes1* is expressed in the sustentacular layer during postnatal stages in *Notch2* mutants and in controls (Fig. 10A-E). At P0, *Hes1* expression could be detected in the apical epithelium of *Notch2* mutants. Although this expression appeared somewhat lighter than control, no significant differences were found (Fig. 10A,B). At 2.5 weeks, however, gaps in *Hes1* expression could be detected apically (Fig. 10C), which increased in frequency in the adult (Fig. 10D). This can be contrasted with the relatively higher control expression of *Hes1* in adults (Fig. 10E) and at 2.5 weeks (data not shown). Thus, although *Hes1* was detected at all stages in *Notch2* mutants, progressively fewer cells expressed *Hes1* in the apical layer as the animal aged. While the reduced and intermittent apical *Hes1* expression was the predominant phenotype, in some areas of adult mutant epithelia, *Hes1* expression was dramatically increased (Fig. 8C,D). The epithelium was also reduced in thickness within these areas. The distribution of *Hes1* was also markedly higher throughout the apical-basal extent. This distribution is reminiscent of what was observed in epithelia that have been ablated and are undergoing regeneration (Manglapus et al., 2004).

An examination of *Hey1* expression in P0, 2.5 week and in adult *Notch2* mutants showed that, like *Hes1*, gaps and reduced levels of *Hey1* are observed in the sustentacular layer in the adult (compare Fig. 10I with 10J). Thus, expression of both *Hes1* and *Hey1* are missing in a large subset of sustentacular cells in *Notch2* mutants. Also like *Hes1*, this is often accompanied by increased numbers of *Hey1*-positive cells basally. However, *Hes1* is only beginning to be affected in 2.5 week-old mutants and is more strongly affected in adults, while *Hey1* is dramatically reduced at all stages (compare Fig. 10G with 10F). This suggests that although both *Hes1* and *Hey1* are downstream targets of the *Notch* receptors, there are differences in activation of these two transcription factors by the various receptors, as previously suggested (Shimizu et al., 2002).

*Six1* has been shown in zebrafish to be affected by mutants in the *Notch* pathway (Bricaud and Collazo, 2006). We found that *Six1* is expressed in the sustentacular layer in control animals at all ages (Fig. 10K,O). It is also strongly expressed basally in P0 controls, but this basal expression becomes weaker by 2.5 weeks, and is much reduced in expression by adulthood. In mutants, the pattern of *Six1* distribution in P0 and 2.5 week-old appears minimally disrupted (Fig. 10L,M). No significant difference in expression between mutant and control could be detected, although expression appeared to be somewhat weaker in 2.5 week-old mutants. In contrast, in adult mutants, the uniform expression of *Six1* is interrupted apically in mutants by domains that express little or no *Six1* (Fig. 10N). The alterations in *Hes1*, *Hey1*, and *Six1* show that transcriptional regulation is variably disrupted in the absence of *Notch2* in an age-dependent manner. *Hey1* is disrupted apically at all postnatal stages, *Hes1* expression only appears altered beginning at 2.5 weeks, while *Six1* is most strongly affected in adults.

### **Notch3 expression is absent in Notch2 adults**

Both *Notch2* and *Notch3* are expressed in sustentacular cells (Fig. 1, 2). Both are known to activate *Hes1* and *Hey1* *in vitro* (Maier and Gessler, 2000; Shimizu et al., 2002) and are thought to serve similar functions *in vivo* (Louvi and Artavanis-Tsakonas, 2006). We were therefore surprised at the fact that there was reduced *Hes1* and *Hey1* in *Notch2* mutants, as we expected that *Notch3* might compensate for the lack of *Notch2*. Interestingly, we found that *Notch3* expression was strongly affected. In P0 mutants, *Notch3* was still detectable in the sustentacular layer, although expression was reduced relative to control (compare Fig. 10Q with 10P). Occasional gaps in *Notch3* expression could also be seen at P0. We found *Notch3* could also be detected in E14 mutants (data not shown). But in both 2.5 week-old animals (Fig. 10R) and in adult mutants (Fig. 10S), *Notch3* expression was essentially absent. Unlike the expression of *Hes1*, *Hey1*, and *Six1*, which are still present in the adult at a low level, *Notch3* expression was undetectable by *in situ* hybridization. This surprising result suggests that *Notch3* expression initially does not require *Notch2*. However, as development proceeds, maintenance of *Notch3* directly or indirectly requires *Notch2*.

To determine whether or not the converse is true, and if *Notch3* also influences *Notch2* expression, we examined *Notch3* mutants (Krebs et al., 2003) for phenotypes in the adult olfactory epithelium. We found that expression of *Notch2* was unaffected in *Notch3* mutants (data not shown), suggesting that *Notch3* is not required for either the initiation or maintenance of *Notch2* expression. We also found no histological or molecular alterations with any of the markers used to analyze *Notch2* mutants (data not shown). Unfortunately, efforts to generate the triple mutant (*N2<sup>flox/flox</sup>; Foxg1<sup>Cre/+</sup>; N3<sup>-/-</sup>*) have been unsuccessful so far. Together, these results suggest that *Notch2* can compensate for *Notch3*, but that *Notch3* can only partially compensate for *Notch2*.

### **Impairment of sustentacular cell function**

One of the hypothesized roles of sustentacular cells is that they function in a neuroprotective role towards OSNs (Whitby-Logan et al., 2004). This is based in large part on the fact that sustentacular cells express high levels of enzymes known to participate in metabolic modification of toxicants. These include cytochrome *P450* isoforms (Ling et al., 2004) and *Glutathione S-transferases* (Weech et al., 2003; Whitby-Logan et al., 2004). Indeed, the epithelium can display higher levels of *P450* activity than the liver (Hadley and Dahl, 1983). Moreover, some cytochrome *P450* forms are either expressed most highly in the epithelium or are uniquely expressed in the epithelium (Ling et al., 2004). Where expression of these enzyme isoforms has been examined, the majority are expressed primarily or exclusively in sustentacular cells and/or in Bowman's glands. There are three major *P450* isoforms – *Cyp1a2*, *Cyp2a5*, and *Cyp2g1* - which collectively constitute >35% of the total *P450* in the epithelium (Gu et al., 1998). At least a dozen other isoforms are also expressed (Ling et al., 2004). Similarly, of the various *GST* isoforms, *mu1* and *mu2* are most highly expressed (Ben-Arie et al., 1993).

We hypothesized that impairment of sustentacular cell function may ultimately lead to neuronal degeneration. Toxicology experiments have shown that exposure to some nasal inhalants can lead to cell death in select regions of the epithelium, which is attributable to airflow-driven deposition in these areas (Harkema et al., 2006). Notably, these areas include the dorsal medial meatus and proximal regions of the lateral and middle medial meatus, areas which are strongly affected in several of our mutants (Fig. 5). Thus, the variability of our phenotype may be dependent, at least in part, upon the ability of sustentacular cells to respond to environmental inhalants and the frequency to which these cells are exposed to airflow bearing these inhalants.

We therefore analyzed by quantitative RT-PCR the level of RNA expression of several major *P450* and *GST* isoforms in the epithelium (Fig. 11A). This analysis showed decreases of 40-50% in four of five genes assayed. These results indicate that expression of these metabolically important modification enzymes are down-regulated, consistent with a reduction in the ability of sustentacular cells to detoxify and/or modify inhalants and other toxins. Interestingly, *Cyp2a5* appeared to be modestly up-regulated in two samples but strongly down-regulated in a third. The first two samples were from animals that were 4 weeks of age while the third sample is from one that is 9 weeks old. As we have shown previously, there appears to be a progressive increase in neurodegeneration from 2.5 weeks to adulthood (8 weeks or older). As such, RNA levels of *Cyp2a5* may be minimally affected at 1 month, but are eventually down-regulated as the animal ages. We also performed a functional analysis to determine if the level of GST activity in mutant epithelia correlates with the decrease in *GST mu1* and *mu2* RNA. Graphing of GST activity (n=3) clearly revealed lower levels in mutants as compared with controls (Fig. 11B). The ratio of the two slopes show that the mutant pairs possessed 80-87% of control levels of GST activity (Fig. 11C), confirming GST levels are impaired in *Notch2* mutants.

## Discussion

Here we show evidence consistent with a model where *Notch2* acts to maintain sustentacular function in the adult main olfactory epithelium. In the absence of *Notch2*, a progressive reduction of *Hes1*, *Hey1*, and *Six1* expression occurs as postnatal development proceeds. Further, we show that sustentacular cell function is impaired, as assessed by RNA expression of *P450* and *GST* isoforms and functional assays of GST activity. These alterations in sustentacular cell morphology, gene expression, and function are associated with neurodegeneration among OSNs. Finally, we show that *Notch2* is required to maintain, but not initiate, *Notch3* expression. *Notch3*, however, is not required for *Notch2* initiation or maintenance of expression.

### ***Notch2* and *Notch3* are co-expressed in sustentacular cells**

We have demonstrated that *Notch2* and *Notch3* are co-expressed within the sustentacular layer of the epithelium. There is conflicting evidence regarding the expression of *Notch3* in the olfactory system. Our results are inconsistent with other studies that show no expression of *Notch3* in sustentacular cells (Carson et al., 2006; Doi et al., 2004). We use *in situ* hybridization with two non-overlapping probes, double-label *in situ* hybridization, immunohistochemistry, Northern blot analysis, and RT-PCR to show *Notch3* RNA and protein are present in the epithelium and are expressed by sustentacular cells. We cannot explain why our results vary from these other studies. It is possible that the antibody used for immunohistochemistry by Carson et al. and Doi et al. may not be the same as that used in these studies (see Materials and methods).

Sustentacular cells possess many similarities with glial cells. Like glial cells, sustentacular cells express high levels of cytochrome *P450* isoforms (Chen et al., 1992) and *Glutathione S-transferases* (Krishna et al., 1994), and participate in detoxification. Like Müller glia, sustentacular cells span the epithelium and encapsulate OSN cell bodies, forming columns of cells within the epithelia (Nomura et al., 2004). Similar to glia, sustentacular cells electrically isolate OSNs, and ensure that their dendrites do not make direct contact with one another (Getchell et al., 1984). Collectively, these functions indicate that sustentacular cells support and maintain OSN function. However, they also express markers more commonly found on keratinocytes, including cytokeratins (Suzuki and Takeda, 1991). These cells have therefore been termed “glial-like” instead of glia (Weiler and Farbman, 1998). Despite the inferred function of sustentacular cells in supporting OSNs, this has not been formally demonstrated.

Part of this is due to a lack of any genetic model or chemical ablation paradigm that selectively removes sustentacular cells while leaving OSNs intact.

### ***Notch2* mutants do not possess widespread alterations in sustentacular cell fate**

We initially hypothesized that the persistence of *Notch2* expression in adult may be required to prevent alterations in cell fate by sustentacular cells. Although transient expression of the *Notch* pathway is sufficient to initiate an irreversible switch from gliogenesis to neurogenesis in some systems (Morrison et al., 2000; Tanigaki et al., 2001), in others, transient expression only temporarily alters cell fate (Dorsky et al., 1997; Fortini et al., 1993). As *Notch* receptor function had not been previously examined in the olfactory system, it was unclear which paradigm would apply to sustentacular cell development. We found alterations in *Hes1*, *Hey1*, and *Six1* expression in *Notch2* mutants. All three transcription factors have been shown to repress neuronal cell fate during development. Loss of *Hes1* in the epithelium leads to increased numbers of OSNs (Cau et al., 2000). Misexpression of *Hey1* promotes astrocyte formation by repressing *Mash1* and *Math3* (Sakamoto et al., 2003). Finally, *Six1* acts in zebrafish to repress neuronal differentiation (Bricaud and Collazo, 2006). Thus, all three genes affected in *Notch2* mutants have been shown to inhibit neuronal cell fate during development.

But despite the known effects of *Hes1*, *Hey1*, and *Six1* on cell fate during development, our results suggest that once the fate of sustentacular cells has been determined, this process is not reversible, consistent with other studies (Morrison et al., 2000; Tanigaki et al., 2001). If significant alteration in cell fate had occurred among sustentacular cells, we would expect to find a large increase in neuronal marker expression in the apical epithelium. However, markers of neuronal fate, such as *Scg10* and *OMP*, were not widely expressed in mutants in the sustentacular layer. Moreover, markers of sustentacular fate, including *Krt8* and *Cbr2*, are still expressed in apparently normal expression patterns. This argues against a wholesale change in cell fate by sustentacular cells. However, we cannot exclude the possibility that other markers not tested here may produce results more consistent with a change in cell fate.

One other possible interpretation for the continued postnatal expression of *Notch2* in the sustentacular layer may be to maintain progenitor populations within the apical epithelium. This would be analogous to its proposed role in the cortex to inhibit differentiation among progenitor cells (Ever and Gaiano, 2005). However, there appears to be little evidence that sustentacular cells harbor a population of progenitor cells in the adult. First, proliferation occurs primarily in the basal layer in adults (Smart, 1971; Weiler and Farbman, 1998). Second, transplantation experiments have not demonstrated that sustentacular cells are able to function as stem cells (Chen et al., 2004). Finally, in cases where chemical ablation has been used to destroy sustentacular and neuronal cells within the epithelium, sustentacular cells are replaced by dividing basal cells (Manglapus et al., 2004). Thus, results from mitotic profile analyses, transplantation studies, and ablation experiments show adult sustentacular cells are likely to represent a population of mature, differentiated cells.

### ***Notch2* is required for maintaining sustentacular function**

Our experiments are most consistent with the interpretation that *Notch2* is required to maintain sustentacular cell function. We demonstrate reduced RNA levels for major *P450* and *GST* isoforms known to be expressed by sustentacular cells, as well as show an overall reduction in *GST* activity in mutants. Consistent with a loss of the normal, supporting function of sustentacular cells, these alterations are associated with neurodegeneration among OSNs. One possible interpretation for the variability in our phenotype may be the relative exposure within the epithelium to airflow patterns during inhalation. Toxicology experiments have shown that inhaled compounds, including that from soiled cage bedding (Mery et al., 1994), can have differential effects on various regions of the epithelium due to airflow-driven deposition.

Although we found that nearly all areas of the epithelium possessed higher TUNEL and Mash1 expression than controls, some areas were often more highly affected, consistent with these prior studies.

How does *Notch2* act to maintain sustentacular cell function once cell fate has been established? The ability of the *Notch* pathway to influence a wide variety of different developmental decisions is thought to be context dependent (Gaiano and Fishell, 2002). Although *Hes1* and *Hey1* are canonical downstream effectors of the *Notch* receptors, a handful of other genes have been identified that are also directly activated by *Notch*. *Brain lipid binding protein (BLBP)*, for example, has been shown to be a target of the *Notch* pathway during radial glia formation (Anthony et al., 2005). Although *Hes5* is also activated during the genesis of radial glia, *BLBP* expression occurs after *Hes5*. The sequential nature of *Hes5* and *BLBP* expression suggests that the panoply of genes that are activated by the *Notch* pathway can change over time. Thus, early in radial glial development, *Hes* genes would be expressed in response to *Notch* signaling, presumably to repress *Mash1* and inhibit neural differentiation. After this process, glial transcription targets, such as *BLBP*, are then expressed, leading to activation of a glial differentiation pathway.

We propose that a similar model holds in the olfactory epithelium. During development, activation of *Hes1* by *Notch* receptors is important to inhibit neuronal differentiation by sustentacular cell precursors. This is supported by the observation that in *Hes1* mutants excess numbers of OSNs are produced (Cau et al., 2000). However, once this cell-fate decision has been established, The *Notch* pathway then acts to promote glial differentiation. This is likely to involve *Hes1* and *Hey1*, which act with or upon other genes, such as *Six1*, ultimately leading to expression of cell-type specific markers such as *Krt8* and other genes necessary for sustentacular function (e.g. *GSTs*). During postnatal stages, maintenance of sustentacular function requires continued expression of *Notch2*. In combination with *Notch3*, *Hes1*, *Hey1*, and *Six1* continue to be expressed within the postnatal and adult animal.

An alternative interpretation to our results is that *Notch2* may also be expressed in OSNs at levels below our limit of detection, and that loss of *Notch2* within neurons leads to neurodegeneration. However, others have also shown *Notch2* is apically expressed at various developmental and postnatal stages (Carson et al., 2006; Lindsell et al., 1996; Mitsiadis et al., 2001). Moreover, we examined expression of *Notch2* as early as E10. At all epithelial stages where the laminar structure of the epithelium could be distinguished, *Notch2* was expressed only by sustentacular cells (data now shown). Nevertheless, mutations in *Notch* receptors have previously been shown to be directly associated with neuronal apoptosis (Mason et al., 2006). Thus, we cannot formally rule out that the absence of low levels of *Notch2* within OSNs forms the basis of our phenotype.

Another interpretation of our results would be that loss of *Notch2* during developmental stages may somehow affect neuronal development, leading to increased apoptotic susceptibility at postnatal stages. However, we examined embryonic *Notch2* E14 mutants (n=3) for various neuronal and sustentacular markers and found no overt phenotypes (data not shown). Thus, it is unlikely that our results can be explained by an early, embryonic effect that would influence postnatal survival of OSNs or sustentacular function.

### ***Notch2* affects *Notch3* expression in the epithelium**

An unexpected result from these studies is that there exists communication among *Notch* receptors within the sustentacular layer. Initiation of *Notch3* appears to be independent of *Notch2*, as there is clear expression of *Notch3* in sustentacular cells at P0 (Fig. 10) and in E14 mutants (data not shown). But in 2.5 week-old postnatal *Notch2* mutants and in adult, *Notch3* expression essentially is undetectable. The converse was not true, as *Notch2* expression

was unaffected in *Notch3* mutants. This suggests that *Notch3* expression is directly or indirectly dependent upon *Notch2*, but *Notch2* expression is independent of *Notch3*. How or why *Notch3* is affected by *Notch2* is unknown. While we have no mechanistic explanation for the loss of *Notch3* in *Notch2* adults, one possibility is that the reduced levels of *Hey1* and *Hes1* may lead to a feedback loop that results in the elimination of *Notch3* expression in the mutant. Classic studies in *Drosophila* have shown that expression of *Delta* by presumptive neuroblasts is regulated indirectly by upregulation of *Enhancer of split* expression caused by *Notch* activation. This in turn leads to a positive feedback loop where *Delta* expression is down-regulated as *Notch* activation increases (Heitzler et al., 1996). In the cochlea of mammalian *Hes1* mutants, *Notch1* expression is reduced, suggesting *Notch1* expression is dependent upon the *Hes* genes to some extent for expression (Zine and de Ribaupierre, 2002). If a similar model holds in the sustentacular layer, we would predict that the presence of *Notch3* at P0 and at E14 is able to partially activate *Hey1* and *Hes1*. But the overall reduction in *Hey1* and *Hes1* activation would ultimately lead to lower levels of *Notch3*. Over time, this feedback loop would reduce or eliminate *Notch3*. In the absence of both *Notch2* and *Notch3*, *Hes1* and *Hey1* expression would be down-regulated, affecting *Six1* and other as yet unidentified genes, ultimately impacting sustentacular function. Genetic proof that *Notch2* and *Notch3* interact would require the generation of triple mutants of *Notch2*, *Foxg1-Cre*, and *Notch3*, but these efforts have so far proven unsuccessful.

Prior studies have shown that the various *Notch* receptors may indirectly interact with one another by differential binding or activation of downstream *Notch* effectors. *Notch3* has been shown *in vitro* to antagonize *Notch1* activity (Beatus et al., 1999), while *Notch2* has been shown to antagonize *Notch1* and *Notch3* activity (Shimizu et al., 2002). On the other hand, chimeric fusions of *Notch1* and *Notch2* suggest functional redundancy between these receptors (Kraman and McCright, 2005). It has similarly been argued in *Notch3* mutants that the unaltered expression of *Notch1* and *Notch2* suggests functional redundancy among the *Notch* receptors at E13.5 (Kitamoto et al., 2005). However, *Notch3/Notch1* double mutants show no synergistic effects, suggesting a lack of functional overlap prior to E9.5 (Krebs et al., 2003). These various conflicting results suggest that *Notch* receptors may function either in a similar or antagonistic manner depending upon the specific context in which the receptors are expressed. In none of these studies, however, has it been suggested that the receptors directly or indirectly regulate the expression of one another. Outside of the nervous system, transfection of the *Notch2* intracellular domain into C2C12 myoblasts decreases *Notch3* expression, while transfection of *Notch2* siRNA increases *Notch3* levels (Ono et al., 2007). Thus, manipulation of *Notch2* has been shown to affect *Notch3* in a non-neuronal system.

### Non-specific neurodegeneration is not observed in *Notch2* mutants

We have described alterations in *Hes1*, *Hey1*, *Six1*, and *Notch3* expression in *Notch2* mutants, as well as increased neurodegeneration of OSNs. *Notch2* is also known to be important for pituitary development (Raetzman et al., 2006). Given the reduced size and weight of *Notch2* mutants, our phenotypes may be caused indirectly by non-specific effects associated with pituitary defects. We found that *Foxg1* is expressed in the pituitary (data not shown), and therefore it is likely that *Notch2* pituitary function has been impaired in these mutants. We are able to functionally separate some of our phenotypes from any potential effects caused by pituitary impairment. At P0, we have observed alterations in *Hey1* and *Notch3* expression. However, there is no apparent difference in weight between mutant and controls at this stage. Thus, it is unlikely that pituitary impairment would be the primary reason for the change in *Hey1* and *Notch3* expression. Although *Hes1* and *Six1* are relatively unaffected at P0, they are affected in older mutants. We observed clear weight differences at 2.5 weeks and in adult between mutants and controls, and therefore are unable to directly show that *Hes1* and *Six1* are down-regulated in response to absence of *Notch2* in the epithelium. However, given the

extensive evidence that *Hes1* is a downstream target of the *Notch* receptors, and that *Six1* is affected by mutations in the *Notch* pathway, it seems reasonable to assume that these effects are also due to loss of *Notch2*.

We have also shown increased neurodegeneration in adult mutant epithelia. This phenotype cannot be directly separated from any potential indirect effects associated with pituitary impairment. Indeed, *IGF1* has been shown to be important for neuronal survival (Russo et al., 2005). However, when we examined olfactory bulbs for increased apoptosis by TUNEL, we found no difference between mutant and control (data not shown). Thus, there appears to be no widespread, non-specific increase in apoptosis in *Notch2* mutants. We have also compared our mutant phenotype with those of others affecting pituitary development. *IGF1* mutants are reduced in weight relative to control at E18.5 (Pichel et al., 2003). In our *Notch2* mutants, no weight differences were observed at P0. *IGF1* mutants also show disruptions in bulbar architecture and variably reduced bulbs that are smaller in size due in part to reduced numbers of mitral neurons (Pichel et al., 2003). As disrupting bulbar structure can cause apoptosis of OSNs (Costanzo and Graziadei, 1983), we examined *Notch2* mutant bulbs at P0, 2.5 weeks, and adults using histological stains and by *in situ* hybridization. We did not observe any alteration of the mitral layer at any stage, as described for *IGF1* mutants, nor did the size of the bulb appear to be affected. No difference in gene expression were identified in mutant bulbs as compared with controls. As such, several distinctions can be made between our *Notch2* mutants and those affecting *IGF1*. Given the presumed supporting role that sustentacular cells play in maintaining OSN function, it seems reasonable to assume that loss of *Notch2* in sustentacular cells ultimately would affect OSN survival. Nevertheless, we cannot completely exclude the possibility that impairment of other hormonal aspects of pituitary function may lead to increased neurodegeneration in the epithelium.

### Feedback between the sustentacular and basal layers

In the adult *Notch2* mutant, the absence of both *Notch2* and *Notch3* is associated with down-regulation of *Hes1* in the apical layer. Concomitant with this, we noticed an increase in *Hes1* expression basally. As mentioned previously, this is consistent with chemical ablation studies showing the generation of *Hes1*-expressing cells basally following ablation (Manglapus et al., 2004). These cells will presumably migrate apically and continue differentiating into sustentacular cells. Similarly, *Hey1* and *Six1* expression are also upregulated basally in *Notch2* mutant adults.

As both *Hes1* and *Hey1* are expressed apically during development, we have assumed that they are activated in response to expression of *Notch2* and *Notch3*. However, in adult *Notch2* mutants and in chemical ablation paradigms, it is unclear how these basally-derived replacement sustentacular cells are able to initiate expression of *Hes1* and *Hey1*. One possibility is that *Notch1*, which we have shown is expressed in a subset of basal cells postnatally, mediates this expression. Upregulation of progenitor cell division by *Notch1* in response to injury can lead to increased numbers of glial cells being produced (Givogri et al., 2006). Alternatively, we note that *Hes1* has been shown during early epithelial development to be expressed in a *Mash1*-independent manner (Cau et al., 2000). Only after E10 does *Hes1* appear to require *Mash1* for expression. Perhaps this basal *Hes1* expression follows a similar mechanism, and does not require *Notch* for its initial expression.

We observed an increase in *Hes1*, *Hey1*, and *Six1* expression basally despite the fact that the apical layer was still present in *Notch2* mutants. Although alterations in the structure of the sustentacular layer have occurred, *O-MACS*, *Krt8*, and *Cbr2* are still expressed by cells in the apical layer. Thus, it is intriguing to speculate why basal cells are triggered to produce these presumed replacement sustentacular cells if at least some markers of sustentacular cells are still present. One possibility is that a feedback mechanism associated either with



neurodegeneration of OSNs or with disruption in sustentacular function is detected by basal cells. *GDF11* (Wu et al., 2003) and *Npy* (Hansel et al., 2001) provide feedback to basal cells to modulate neurogenesis. Perhaps a similar mechanism regulates production of sustentacular cells by basal cells.

### The *Notch* pathway and neurodegeneration

Sustentacular cells have long been called support cells for OSNs because of their many glial-like properties. Our observed degeneration phenotype also has parallels with the role of astrocytes in neurodegeneration. In amyotrophic lateral sclerosis (ALS), Huntington's Disease, and Alzheimer's Disease (AD), alterations in astrocyte function strongly affects the survival and likelihood of injury to neurons (Maragakis and Rothstein, 2006). As a result, the astrocytes may still be present but the supported neurons will die. In keeping with these findings, sustentacular cell death appears to be minimal in *Notch2* mutants despite the increase in OSN degeneration.

In addition, *Notch1* has been associated with Alzheimer's disease (Berezovska et al., 1998). While a great deal of attention has focused on the possible role of *Notch1* expression in neurons as contributing towards the progression of this disease (Sestan et al., 1999), it is tempting to speculate that one explanation for the loss of nasal sensitivity that often precedes diagnosis of Alzheimer's disease may be due to loss of *Notch2* expression in sustentacular cells leading secondarily to OSN degeneration.

### Conclusions

The *Notch* pathway plays multiple, pleiotropic roles during development, and its effect is dependent upon the specific temporal and cellular context. *Notch* receptor expression in progenitor cells is thought to maintain these cells in an undifferentiated state, while at later stages the *Notch* pathway is involved in delineating neuronal versus glial fates. Still later, *Notch* receptors may act to promote astrocyte fate while inhibiting oligodendrocyte fate. In the adult, *Notch* receptors may act to regulate neurite outgrowth. Thus, while the immediate downstream effectors of the *Notch* pathway may remain the same, the functional output of the genes that are activated are likely to vary based upon the specific cell-type and timing of differentiation.

Here we have provided evidence to support the model that *Notch2* acts to maintain sustentacular function in the epithelium. Although the *Notch* receptors are expressed in the adult nervous system, most efforts have centered around understanding *Notch* receptor function in neurons (Presente et al., 2001; Presente et al., 2004; Wang et al., 2004). We propose that persistent *Notch* receptor expression in at least some glial populations is important for maintaining their functional role in supporting neuronal survival.

### Acknowledgments

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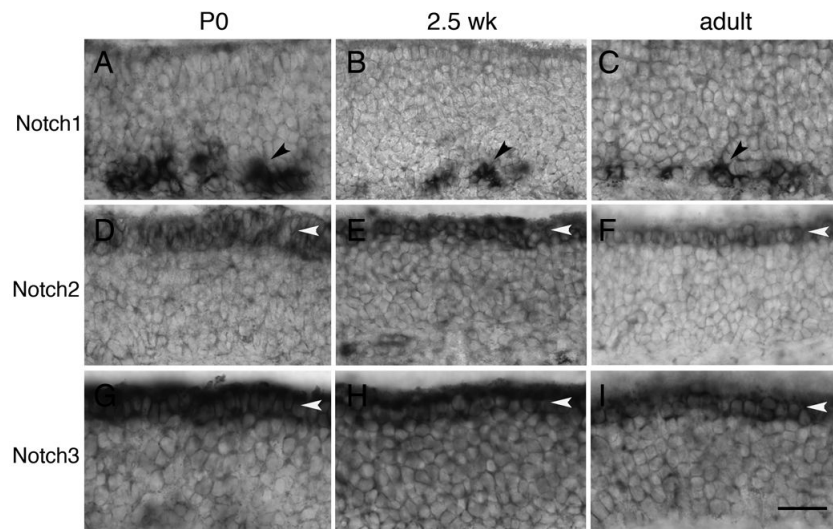
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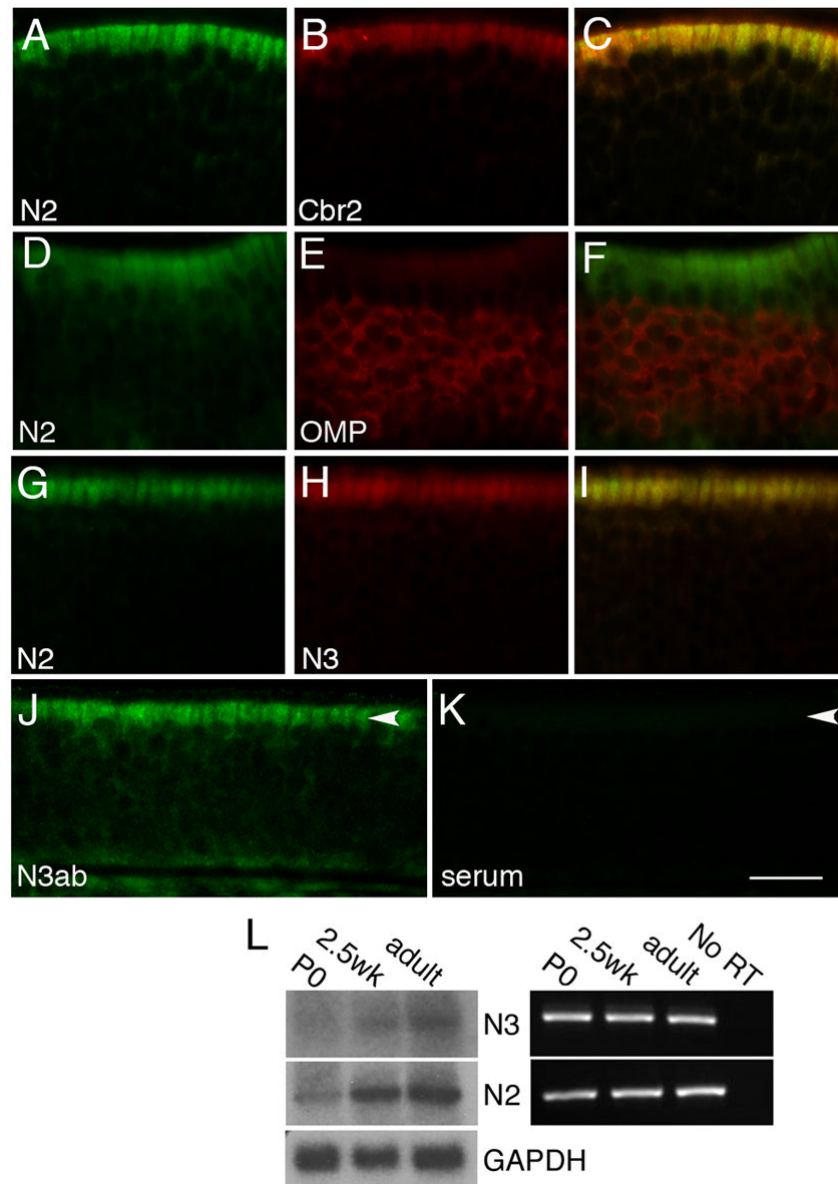
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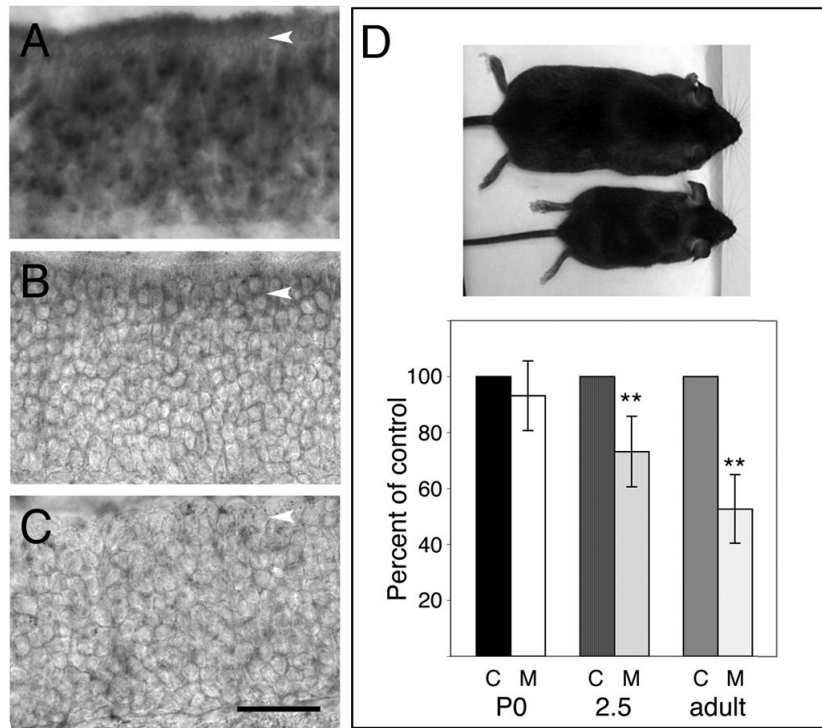
**Figure 1. Expression of *Notch* receptors during postnatal stages**

A-C) *Notch1* is expressed in the basal epithelium (black arrowheads) by a subset of cells located primarily in the dorsal recess. This expression is present at P0, 2.5 weeks, and in adult (8-19 weeks). D-F) *Notch2* is expressed by sustentacular cells (white arrowheads) at P0, 2.5 weeks, and adult. Expression is also detected in the lamina propria (data not shown). G-I) *Notch3* is expressed by sustentacular cells (white arrowheads) at P0, 2.5 weeks, and adult. Expression is also detected in the lamina propria (data not shown). Scale bar=40 $\mu$ m.



### Figure 2. *Notch* receptor expression in sustentacular cells

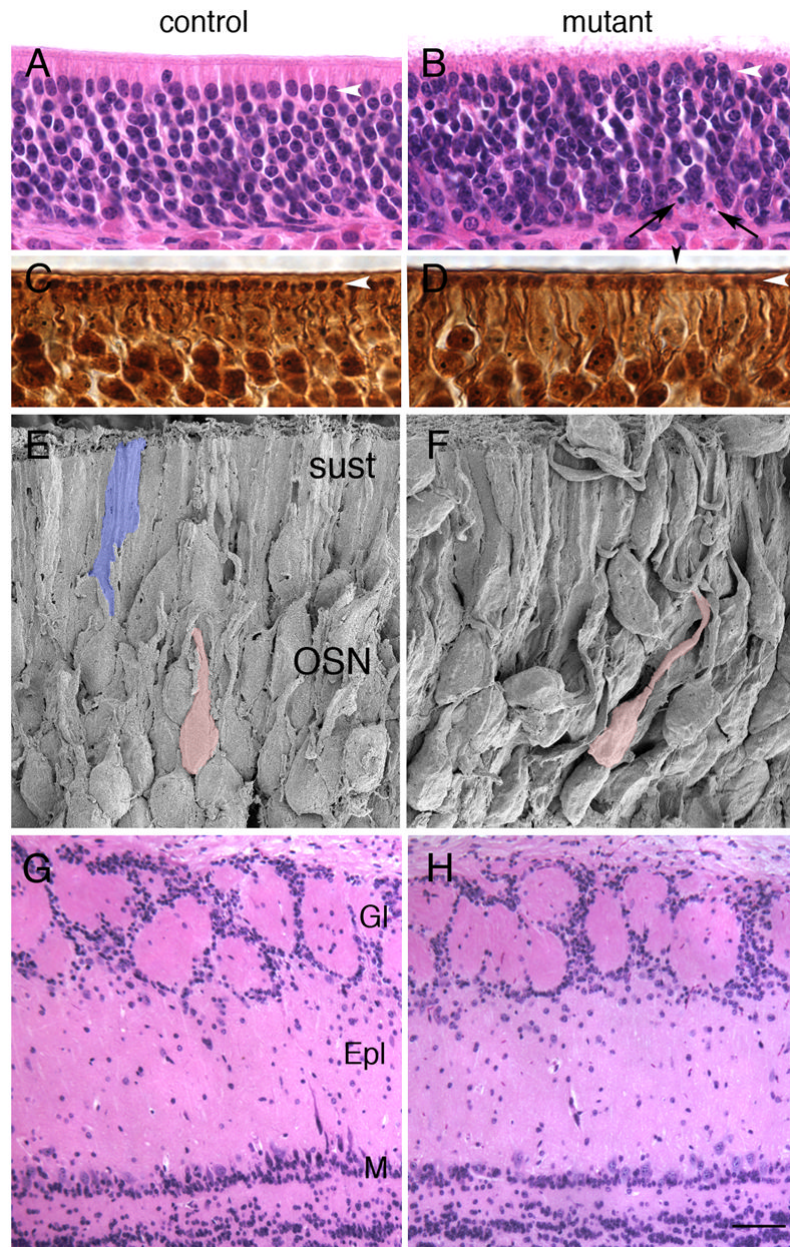
A-C) Double-label *in situ* hybridization in adult epithelia of *Notch2* (A) and *Cbr2* (B), an enzyme expressed by sustentacular cells, show that both are localized to the sustentacular layer (C). D-F) *Notch2* (D) and *OMP* (E), a marker for mature OSNs, are not co-expressed in the neuronal layer (F). G-I) *Notch2* (G) and *Notch3* (H) are both expressed in the sustentacular layer (I). J) Immunohistochemical detection of NOTCH3 protein in adult animals in sustentacular cells (arrowhead). K) No signal is detected in sustentacular cells (arrowhead) in the absence of anti-NOTCH3 antibody. Scale bar=25 $\mu$ m for A-I, 17 $\mu$ m for J-K. L) RNA isolated from whole epithelia was used for Northern blot and RTPCR. *Notch2* and *Notch3* message can be detected at P0, 2.5 weeks, and adult stages. *GAPDH* was used as a loading control for the Northern blot.



**Figure 3. *Notch2* is effectively deleted in mutant mice and mutant animals are smaller than control siblings**

A) *Foxg1-cre* animals (Hebert and McConnell, 2000) were crossed to the *Rosa26-LacZ* reporter strain (Soriano, 1999). Sections from P0 progeny were assayed for β-galactosidase activity and counterstained with eosin. β-galactosidase expression was detected in the sustentacular layer (arrowhead) and throughout the apical-basal extent of the epithelium. B) Exon 3 (260 bp; deleted in *N2<sup>lox/lox</sup>; Foxg1-cre* animals (McCright et al., 2006)) was used as probe to detect expression of *Notch2* in control and mutant animals. Message could be detected within sustentacular cells of adult control animals (arrowhead). C) No *Notch2* message could be detected with the exon 3 probe in *N2<sup>lox/lox</sup>; Foxg1-cre* animals (arrowhead). D) *Notch2* mutant animals were smaller than their control siblings (top mouse-control adult; bottom mouse-mutant adult). Animals were weighed at P0, 2.5 weeks, and adult (8-19 weeks) stages to determine when this weight difference became apparent. At P0, the average weight of controls (c-control, 1.6±0.1g) did not differ significantly from mutants (m-mutant, 1.8±0.2g; n=4 pairs; p=0.3, Student's *t*-test). However, by 2.5 weeks, mutants were on average 27% less in weight than control siblings (n=8 pairs; control: 8.1±1.4g; mutant: 5.8±1.0g; p=0.003). Although adult animals were weighed at different ages (8-19 weeks), control siblings were always compared with mutant siblings. On average, mutants weighed 47% less than controls (n=13 pairs; control: 24.7g±4.3; mutant: 12.8g±3.0; p=2.1×10<sup>-8</sup>, Student's *t*-test). Scale bar=40μm.

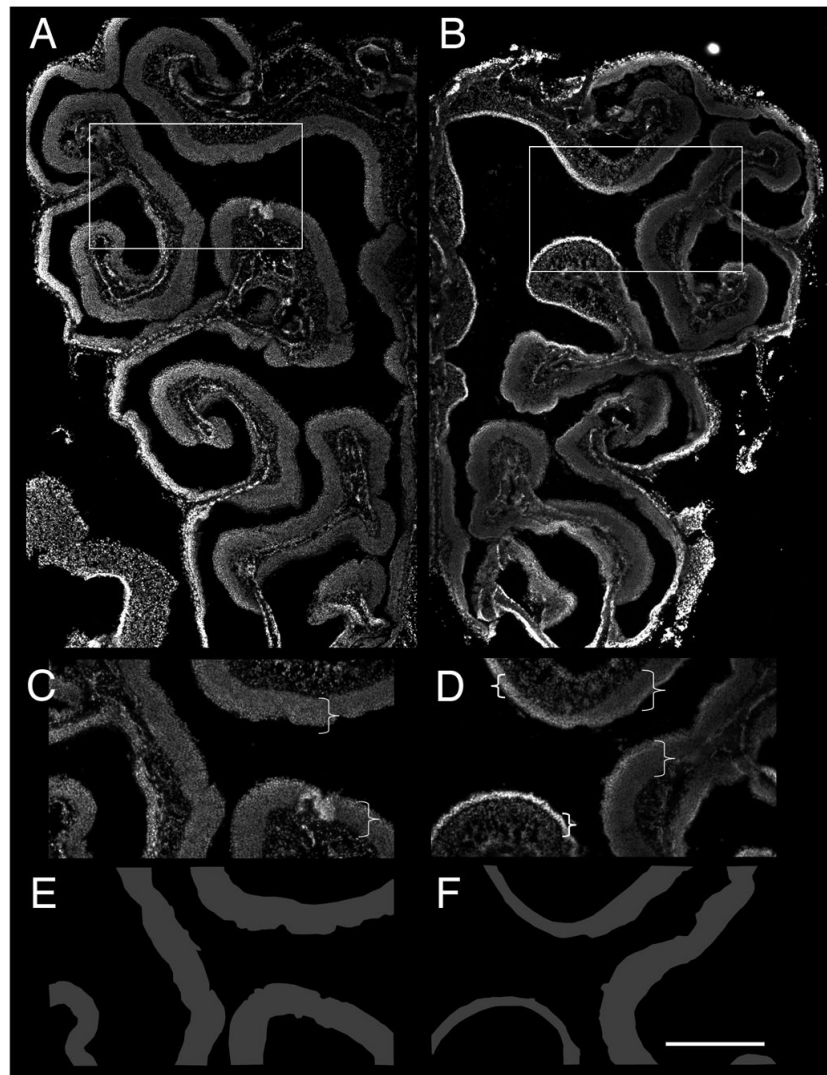




**Figure 4. Disruption of epithelial structure in adult *Notch2* mutants**

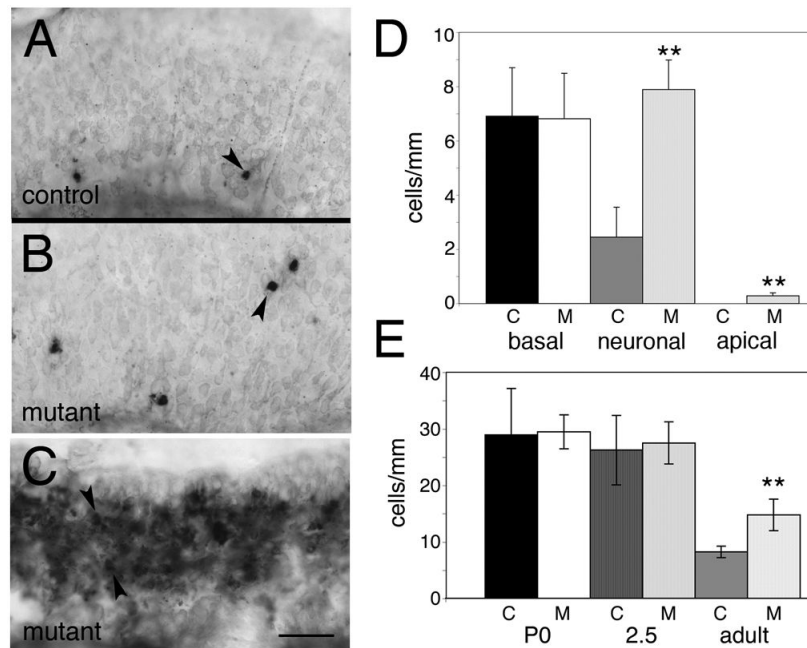
White arrowheads indicate sustentacular layer in A-D. A,B) Hematoxylin and eosin staining of control (A) and mutant (B) adult animals. Disruption of the organization of the sustentacular layer is apparent in mutants as compared with controls (white arrowhead). Pyknotic nuclei (black arrows) could be seen in mutant sections. C,D) Bielschowsky staining revealed gaps (black arrowhead) in the relatively uniform distribution of dendritic tufts (white arrowhead) in mutants (D) as compared against controls (C). E,F) Scanning electron micrograph from control (E) and mutant (F) adult animals show increased disorganization within the neuronal layer. Compare dendritic path of two neurons pseudocolored pink. Typical sustentacular cell pseudocolored blue (E). G,H) Hematoxylin and eosin staining of control (G) and mutant (H) adult olfactory bulbs. No differences in laminar structure were observed. Gl: glomerular layer,

Epl: external plexiform layer, M: mitral layer. Scale bar=20 $\mu$ m for A-B, 10 $\mu$ m for C-D, 5 $\mu$ m for E-F, 62.5 $\mu$ m for G-H.



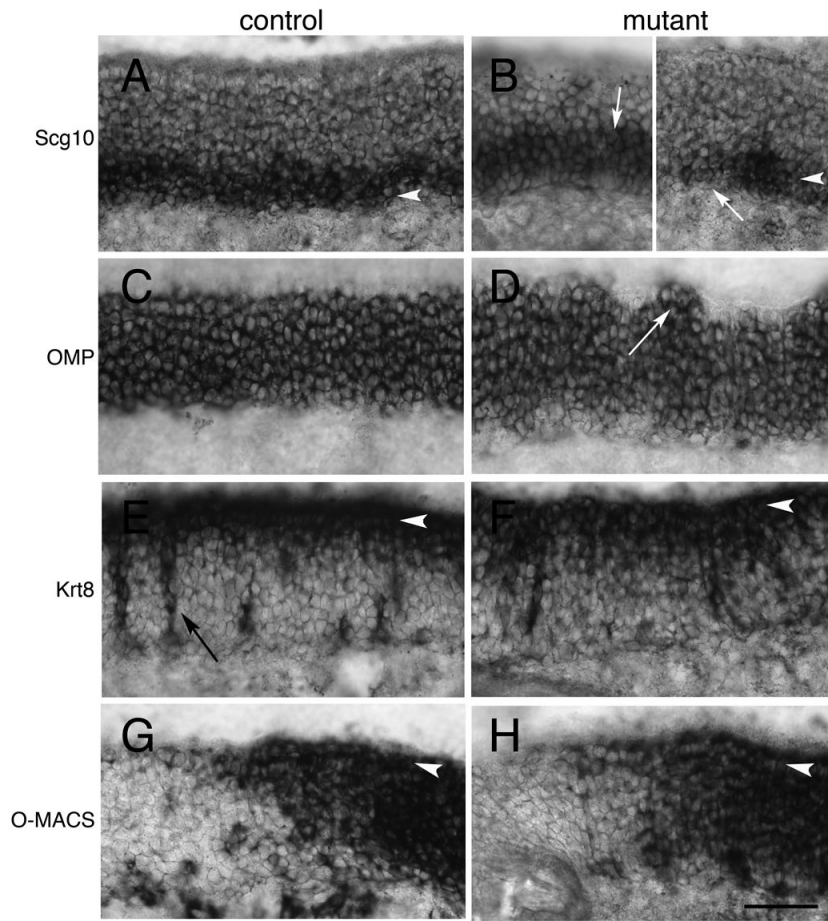
**Figure 5. Variability in epithelial degeneration in *Notch2* mutants**

A-B) Low power images of DAPI stained coronal sections from control (A) and mutant (B) epithelium. Boxed areas are represented in (C) and (D). Brackets highlight regions of epithelia (C,D). E,F) Tracing of epithelia of in (C) and (D) to illustrate variability in mutant epithelium as compared with control. Scale bar=500 $\mu$ m for A-B, 300 $\mu$ m for C-D.



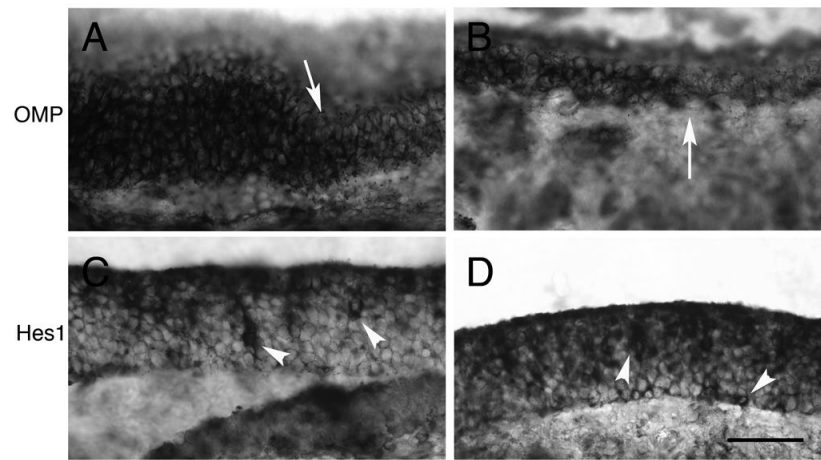
### Figure 6. Increased apoptosis in *Notch2* mutants

A) TUNEL staining was used to assay levels of apoptotic cell death in control animals. Apoptotic cells were sparsely distributed in adult animals, and were generally detected in the basal and neuronal layers (arrowhead). B) TUNEL staining in *Notch2* mutants appeared more widespread, with many areas outside the basal layer showing increased expression relative to control (arrowhead). C) TUNEL staining in some regions of *Notch2* mutant epithelia was dramatically elevated (e.g. arrowheads). D,E) Quantitation of TUNEL expression. “C” indicates control and “M” indicates mutant. D) The distribution of TUNEL positive cells in control and mutant adults were binned by location into basal, neuronal, and apical layers. No significant differences were observed in the basal layer (control:  $6.9 \pm 1.8$  cells/mm; mutant:  $6.8 \pm 1.7$  cells/mm;  $p=0.7$ ; Student's *t*-test). However, in the neuronal layer (control:  $2.4 \pm 1.1$  cells/mm; mutant:  $7.9 \pm 1.1$  cells/mm;  $p=0.005$ ) and in the apical layer (control: 0 cells/mm; mutant:  $0.3 \pm 0.1$  cells/mm;  $p=0.01$ ) significant differences were observed. The bulk of the TUNEL-positive cells in the mutant were present in the neuronal layer. E) Quantification of total TUNEL cells per millimeter throughout the apical-basal extent of the epithelium was performed in P0 ( $n=3$  pairs: 6-18mm/animal), 2.5 week-old ( $n=3$  pairs: 7-15mm/animal), and adult ( $n=3$  pairs: 3-8mm/animal) control and mutant animals. No significant differences were detected at P0 (control:  $29.0 \pm 8.2$  cells/mm; mutant:  $29.5 \pm 3.0$  cells/mm;  $p=0.9$ ), or at 2.5 weeks (control:  $26.3 \pm 6.2$  cells/mm; mutant:  $27.5 \pm 3.7$  cells/mm;  $p=0.8$ ). However, in adult animals, there were significant differences between control and mutant (control:  $8.2 \pm 1.0$  cells/mm; mutant:  $14.8 \pm 2.8$  cells/mm;  $p=0.02$ ; asterisks indicate significant differences relative to control). Quantitation of data in (D) and (E) excluded all areas similar to those shown in (C), as it was impossible to determine the number of positive cells per millimeter in such regions. Scale bar=25 $\mu$ m.



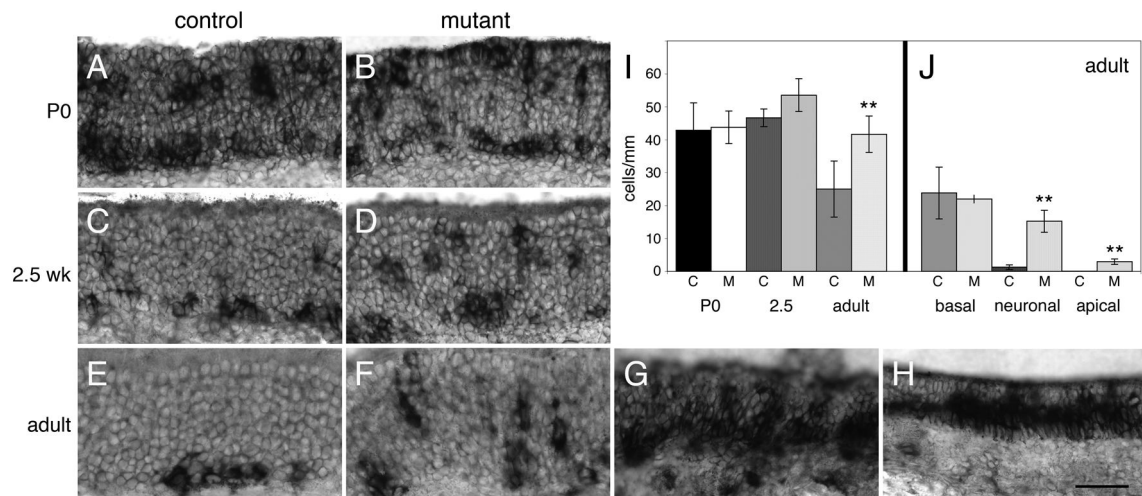
**Figure 7. Cell fate is unaffected in *Notch2* mutant adults**

>A) *Scg10* expression in adult control animals is basally located in developing immature OSNs (arrowhead). B) Expression of *Scg10* can be detected in *Notch2* mutants (arrowhead), but this expression is uneven. Some areas have significantly elevated expression (B-left panel; white arrow), while others have patchy or no *Scg10* expression (B-right panel; white arrow). C) *OMP* expression in control animals is detected in mature OSNs within the neuronal layer. D) *OMP* expression in mutant animals is similar to that in control. However, the apical surface of *OMP* expression is less laminar in appearance. In several areas, *OMP* expression appears to extend into the apical layer (white arrow). E) Control expression of *Cytokeratin8* (*Krt8*) is detected in the sustentacular layer (arrowhead) and in Bowman's glands (black arrow). F) *Krt8* is detected in the sustentacular layer of mutant animals (arrowhead). G) *O-MACS* expression in control animals is detected in the dorsal-most zone of the epithelium (arrowhead shows sustentacular layer). H) *O-MACS* is also detected in a similar pattern in *Notch2* mutants in the sustentacular layer (arrowhead). Scale bar=50 $\mu$ m.



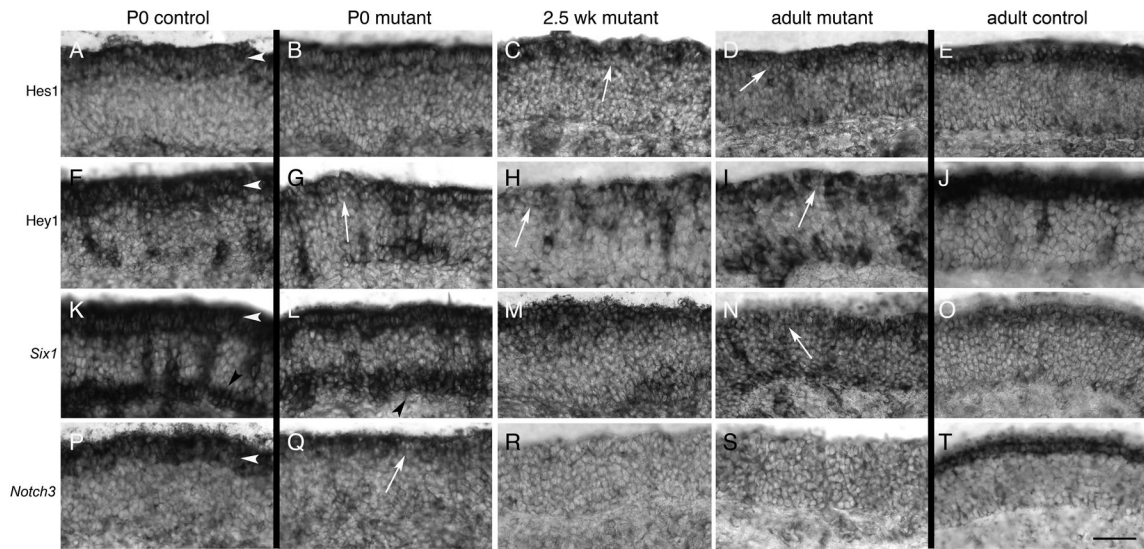
**Figure 8. Extreme examples of degenerating epithelia in *Notch2* mutants**

All images are taken from *Notch2* adult mutants. A,B) *OMP* expression is dramatically affected in some areas of epithelia (areas to right of arrows). C,D) *Hes1* expression is strongly affected in some areas of mutant epithelia, with an increase in the number of *Hes1*-positive cells in the basal and neuronal layers (arrowheads). Scale bar=50 $\mu$ m.



**Figure 9. *Mash1* expression is elevated in adult mutants**

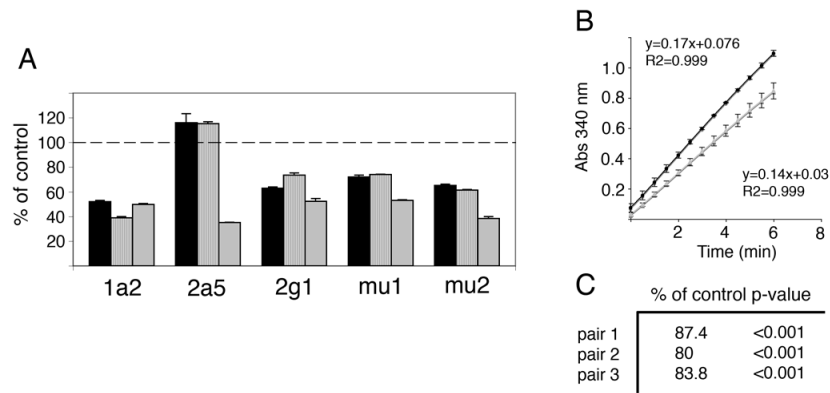
A,B) *Mash1* expression in P0 control (A) and mutant (B) animals are similar. C,D) *Mash1* expression in 2.5 week-old control (C) and mutant (D) are also similar. E,F) Multiple regions can be found in adult mutant animals that appear to have increased numbers of *Mash1*-expressing cells (F) as compared against control (E). G,H) In some extreme instances, very high numbers of *Mash1*-expressing cells can be found in regions of mutant epithelia (compare with (E)). I) Quantitation of the total number of *Mash1*-expressing cells per millimeter (c-control; m-mutant) showed no significant differences at P0 (8-15mm counted/animal; control:  $42.9 \pm 8.3$  cells/mm; mutant:  $43.7 \pm 5.0$  cells/mm;  $p=0.9$ , Student's *t*-test). No differences were found at 2.5 weeks (5-26mm counted/animal; control:  $46.6 \pm 2.7$  cells/mm; mutant:  $53.5 \pm 10.3$  cells/mm;  $p=0.3$ ). However, significant differences were found in adult (7-14mm counted/animal; control:  $25 \pm 8.5$  cells/mm; mutant:  $41.7 \pm 5.5$  cells/mm;  $p=0.05$ ; asterisks indicate significant differences relative to control). J) We quantified the distribution of *Mash1*-expressing cells in adults in the basal, neuronal, and apical layers of the epithelium. No significant differences were found in the basal layer (control:  $23.8 \pm 7.9$  cells/mm; mutant:  $22.0 \pm 1.2$  cells/mm;  $p=0.7$ ). However, in the neuronal (control:  $1.3 \pm 0.7$  cells/mm; mutant:  $15.2 \pm 3.3$  cells/mm;  $p=0.004$ ) and apical layers (control:  $0.1 \pm 0.1$  cells/mm; mutant:  $3.0 \pm 0.8$  cells/mm;  $p=0.008$ ), significant differences were identified between mutant and control. Quantification does not include areas of epithelia with extremely high levels of *Mash1* expression (e.g. G,H). Scale bar=50 $\mu$ m.



**Figure 10. Down-regulation of *Hes1*, *Hey1*, *Six1*, and *Notch3* in the absence of *Notch2***

In all panels, arrowheads point to the position of the sustentacular layer. For comparison, P0 and adult controls for each probe are shown. A,E) Expression of *Hes1* at P0 (A) and adult (E) control animals. B-D) No significant differences in *Hes1* expression could be detected at P0 in mutants (B). Subtle differences were observed at 2.5 weeks, as there was an apparent decrease in the number of *Hes1*-expressing cells in the apical epithelium (C; arrow). However, a clear reduction in *Hes1* expression could be seen in adult mutants (D: white arrow shows large gap in *Hes1* expression). *Hes1* positive cells can also be detected scattered in the neuronal and basal layers. F,J) *Hey1* is evenly expressed in sustentacular cells at P0 (F) and in adult (J) controls. *Hey1* is also expressed in an unknown population of cells distributed deep to the sustentacular layer. G-I) *Hey1* expression is strongly affected at P0 (G), 2.5 weeks (H), and adult (I) in *Notch2* mutants. Significant disruption of apical *Hey1* expression is observed at all stages, with gaps in the normal, uniform apical expression (arrows). Increased expression is also seen in basal cells in mutants relative to controls (I). K,O) Expression of *Six1* in P0 (K) and adult (O) control animals. *Six1* is expressed in P0 animals strongly in the sustentacular and basal layers, and in cells distributed in the neuronal layer. Only a small number of basally-located cells express *Six1* in control adults (O). L-N) *Six1* expression in P0 (L) and 2.5 week (M) mutant animals appears minimally or subtly affected. However, in adult (N) mutant animals, gaps in *Six1* expression could be seen apically (arrow). P,T) Expression of *Notch3* in P0 (P) and adult (T) control animals. Q-S) Expression of *Notch3* in P0 mutant (Q) animals is clearly detectable in the sustentacular layer, but this expression is more variable than control, and is also reduced in expression (arrow). Weak or no expression of *Notch3* is observed in 2.5 week-old (R) or adult (S) *Notch2* mutants. Scale bar=50 $\mu$ m.





**Figure 11. Quantitation of *P450* and *GST* expression and determination of *GST* activity**  
 A) Quantitative RT-PCR of *P450* and *GST* isoform expression levels in three mutant epithelia as compared with control. Reduction of *P450* isoforms *Cyp1a2* and *Cyp2g1* and *GST* isoforms *mu1* and *mu2* RNA are seen in all three mutant epithelia relative to control. Expression of *Cyp2a5* is somewhat increased in two mutants, but strongly decreased in the third mutant. This may be a result of the ages of the mutant samples. The first two epithelia are obtained from 1 month old animals, while the third is from an animal 9 weeks old. B) Example graph of *GST* activity from one paired mutant and control assay. Black line (circles) represents control epithelial extract and gray line (triangles) represents mutant epithelial extract. Graph indicates increasing accumulation of GS-DNB conjugate over time, as determined by absorbance at 340nm. Note reduced slope of mutant relative to control. C) Percent of control *GST* activity from three separate mutant-control epithelial pairs.