

An Epigenetic Trap Stabilizes Singular Olfactory Receptor Expression

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SUMMARY

The molecular mechanisms regulating olfactory receptor (OR) expression in the mammalian nose are not yet understood. Here, we identify the transient expression of histone demethylase LSD1 and the OR-dependent expression of adenylyl cyclase 3 (*Adcy3*) as requirements for initiation and stabilization of OR expression. As a transcriptional coactivator, LSD1 is necessary for desilencing and initiating OR transcription, but as a transcriptional corepressor, it is incompatible with maintenance of OR expression, and its downregulation is imperative for stable OR choice. *Adcy3*, a sensor of OR expression and a transmitter of an OR-elicited feedback, mediates the downregulation of LSD1 and promotes the differentiation of olfactory sensory neurons (OSNs). This novel, three-node signaling cascade locks the epigenetic state of the chosen OR, stabilizes its singular expression, and prevents the transcriptional activation of additional OR alleles for the life of the neuron.

INTRODUCTION

Olfactory receptors (ORs) are G protein-coupled receptors that detect odors and regulate the projection of olfactory sensory neurons (OSNs) to the brain (Buck and Axel, 1991). In the mouse, ORs are encoded by ~1,400 genes (Young et al., 2002) organized in gene clusters found on most chromosomes (Sullivan et al., 1996; Zhang and Firestein, 2002). OR genes have promoter elements that share similar regulatory sequences (Clowney et al., 2011; Michaloski et al., 2006) and are expressed in the main olfactory epithelium (MOE) in a monogenic and monoallelic fashion (Chess et al., 1994). An unusual, MOE-specific epigenetic signature of OR loci, characterized by enrichment for H3K9me3 and H4K20me3, likely governs what is a seemingly stochastic OR expression pattern (Magklara et al., 2011). This epigenetic silencing is reinforced by the aggregation of silenced OR genes in a few heterochromatic foci that preserve the expression of only one OR allele in each OSN (Clowney et al.,

2012). The active OR allele in each OSN escapes from the OR aggregates and relocates to a euchromatic territory where it frequently interacts with a distant OR enhancer, the H enhancer (Clowney et al., 2012; Lomvardas et al., 2006). The singularity of OR expression is essential for olfactory perception because ORs are localized in the OSN dendrites and axons (Barnea et al., 2004) and participate in both odorant detection and axon targeting (Wang et al., 1998). Neurons expressing the same OR converge their axons in distinct glomeruli of the olfactory bulb (Mombaerts et al., 1996), by a process that relies on the identity of the OR protein and its basal activity levels (Mori and Sakano, 2011). Thus, maintaining the stable and singular expression of the same OR throughout the life of the neuron is necessary for the integrity of the topographic map in the olfactory bulb, such that coherent OR expression may be required for proper odor decoding in the brain.

Although the molecular mechanisms that stabilize OR expression are not known, it is established that the expression of transgenic ORs elicits a negative feedback that prevents the expression of endogenous OR genes (Lewcock and Reed, 2004; Nguyen et al., 2007; Serizawa et al., 2003). Moreover, lineage-tracing experiments monitoring the expression of OR alleles from their endogenous loci showed that OR expression elicits a positive feedback signal that stabilizes its own choice and prevents OR gene switching in most OSNs (Shykind et al., 2004). Together, these observations raise questions regarding possible mechanisms that could stabilize the transcription of one OR allele, while simultaneously preventing the expression of all the other OR genes. A simple model that could account for the existence of an OR-elicited feedback signal emerged from the discovery that epigenetic silencing of ORs occurs prior to detectable OR transcription, and that OR choice coincides with a switch from the repressive histone H3K9 methylation to the activating H3K4 methylation (Magklara et al., 2011). Based on these observations, singular OR expression could become permanent if the choice of an intact OR allele suppresses H3K9 and H3K4 demethylases, so that one active OR allele and ~1,000 silent OR genes preserve their distinct epigenetic states for the life of the neuron.

Demethylation of H3K9me3 and H3K4me3 are both stepwise processes that require removal of one methyl group first, creating H3K9me2 and H3K4me2, respectively, which are then further demethylated by different enzymes. LSD1 (lysine-specific

demethylase 1, *Kdm1a*), an amine oxidase, is the only protein with the enzymatic ability to catalyze lysine demethylation reactions for both intermediates, H3K9me2 and H3K4me2, and therefore act as transcriptional coactivator or corepressor, respectively (Metzger et al., 2005; Shi et al., 2004). The exact mode of action of LSD1 is influenced by the context of the transcription factor that recruits it to a specific locus and the nature of the local histone modifications. For example, LSD1 recruitment by androgen receptor to specific loci results in H3K9me2 demethylation and transcriptional activation, whereas LSD1 demethylates H3K4me2 and represses transcription as a component of the CoREST complex (Metzger et al., 2005; Shi et al., 2005; Wang et al., 2007; Wissmann et al., 2007).

Here, we show that LSD1, which is transiently expressed during OSN differentiation, is involved in both OR gene activation and post-choice gene switching and, thus, plays a dual role in OR regulation. Genetic ablation of LSD1 activity prior to OR choice results in widespread loss of OR expression and failure of the OSNs to mature and to project axons to the brain. Deletion of LSD1 immediately after OR activation has no detectable consequences in OR expression and OSN targeting, suggesting that LSD1 activity is needed only during the initial derepression of the selected OR. OR expression induces the subsequent expression of adenylyl cyclase 3 (*Adcy3*), which promotes OSN maturation and LSD1 downregulation. Lineage-tracing experiments reveal increased OR gene switching in *Adcy3* knockout (KO) mice, suggesting a requirement for timely LSD1 downregulation for the stabilization of OR expression. Ectopic expression of transgenic LSD1 in mature OSNs also perturbs the stability of OR choice, suggesting that *Adcy3* stabilizes OR transcription by downregulating LSD1. Thus, our data connect OR choice with the terminal differentiation of olfactory neurons and uncover the molecular underpinnings of a feedback loop that preserves the epigenetic state of active and silent ORs for the life of the neuron.

RESULTS

Dynamic LSD1 Expression Is Required for Initiation but Not Maintenance of OR Expression

We hypothesized that initiation of OR transcription requires H3K9 demethylation, whereas stabilization of singular OR transcription requires suppression of H3K9 and H3K4 demethylases involved in OR regulation. Because LSD1 has both H3K9 and H3K4 demethylase activities, we asked whether its expression pattern is compatible with such a role in OR regulation. RNA-sequencing (RNA-seq) analysis (Magklara et al., 2011) from mature OSNs (mOSNs, olfactory marker protein [OMP]-positive) and progenitor/immature neuronal populations (Neurogenin-1 [Ngn1]-positive) shows that LSD1 is expressed in the Ngn1-positive cells but reduced by 3.6-fold during differentiation to the OMP-positive stage (Figure 1A). Immunofluorescence (IF) reveals high levels of LSD1 protein in the nuclei of Ngn1-positive cells and their immediate progeny and significant reduction of LSD1 in more apical, mOSN populations of the adult MOE (Figure 1B). Two-color RNA fluorescence in situ hybridization (FISH) experiments demonstrate the mutually exclusive expression patterns of LSD1 and OMP, verifying the transcriptional downregulation of LSD1 in OR-expressing mOSNs (Figure 1C).

The dynamic pattern of LSD1 expression, together with recent microarray analyses showing that injury-induced neurogenesis in the MOE coincides with LSD1 upregulation (Krolewski et al., 2013), supports a role for this protein in OR regulation.

To functionally test the role of LSD1 in initiation and maintenance of OR gene expression, we used a conditional LSD1 KO (Wang et al., 2007) that we deleted at three distinct developmental time points: prior, during, and after OR gene activation. This was accomplished by using *Foxg1-Cre* (Hébert and McConnell, 2000), *MOR28-IRES-Cre* (Shykind et al., 2004), and *OMP-IRES-Cre* (Eggen et al., 2004), respectively (Figure 1D). LSD1 deletion before OR expression results in widespread loss of OR expression, based on both ISH with a pool of OR RNA probes and IF with OR antibodies as well as a general targeting deficit of the OSN axons (Figure 1E and Figures S1A and S1B available online). This analysis was performed in embryonic day (E) 18.5 MOE sections due to perinatal lethality. In contrast to the early LSD1 KO, IF for MOR28 shows that LSD1 deletion immediately after MOR28 activation has no measurable effects on OR expression or OSN targeting (Figures 1F–1H and S1C). Similarly, RNA ISH and IF as above show that LSD1 deletion in mOSNs has no detectable effects on OR expression (Figures 1G and S1D). These data suggest that LSD1 activity is necessary for OR desilencing and initiation of OR transcription but dispensable for OSN function following OR choice, at least within the kinetic restrictions imposed by available genetic strategies.

To determine the extent of the transcriptional effects on OR expression, we performed RNA-seq analysis with complementary DNA (cDNA) libraries prepared from the MOEs of control and LSD1 KO mice at E18.5. This approach also shows significant reduction of OR transcription, both regarding the total number of OR messenger RNA (mRNA) reads and the number of ORs that can be detected in the LSD1 KO mice (Figure 2A). In control mice, we detect 662 ORs, and in LSD1 KO mice, we only detect 212 ORs with ~4-fold fewer reads in those ORs that are expressed in LSD1 KO MOEs. This analysis also revealed that transcription factors known or suspected to activate OR transcription, such as *Emx2* and *Lhx2* (Hirota and Mombaerts, 2004; McIntyre et al., 2008), are still expressed in the LSD1 KO mice (Figure 2B), supporting a direct role of LSD1 in OR regulation. Developmental markers of progenitor cells, such as *Neurod1*, are not affected by LSD1 deletion (Figure 2C). Importantly, developmental markers that are postmitotic and synchronous to OR expression, such as *GAP43* and *NCAM1* or *Stmn1*, *Dpysl5*, *Marcksl1*, and *Ablim1* (Iwema and Schwob, 2003; Krolewski et al., 2013; Nickell et al., 2012), are also only moderately affected by LSD1 deletion, based on our RNA-seq and ISH experiments (Figures 2C and S2, respectively). This suggests that the loss of LSD1 activity and not some downstream developmental deficit is the cause of OR downregulation in the LSD1 KO MOE. In contrast, mOSN markers are markedly downregulated in the LSD1 KO MOEs (Figures 2F and 2G). The loss of the mOSN layer in the LSD1 KO likely explains the thinner expression pattern of *GAP43* and *NCAM1* and the ~2-fold reduction observed by RNA-seq (Figures 2C and S2), given that at this developmental stage, there is some overlap of these immature markers with mOSN markers.

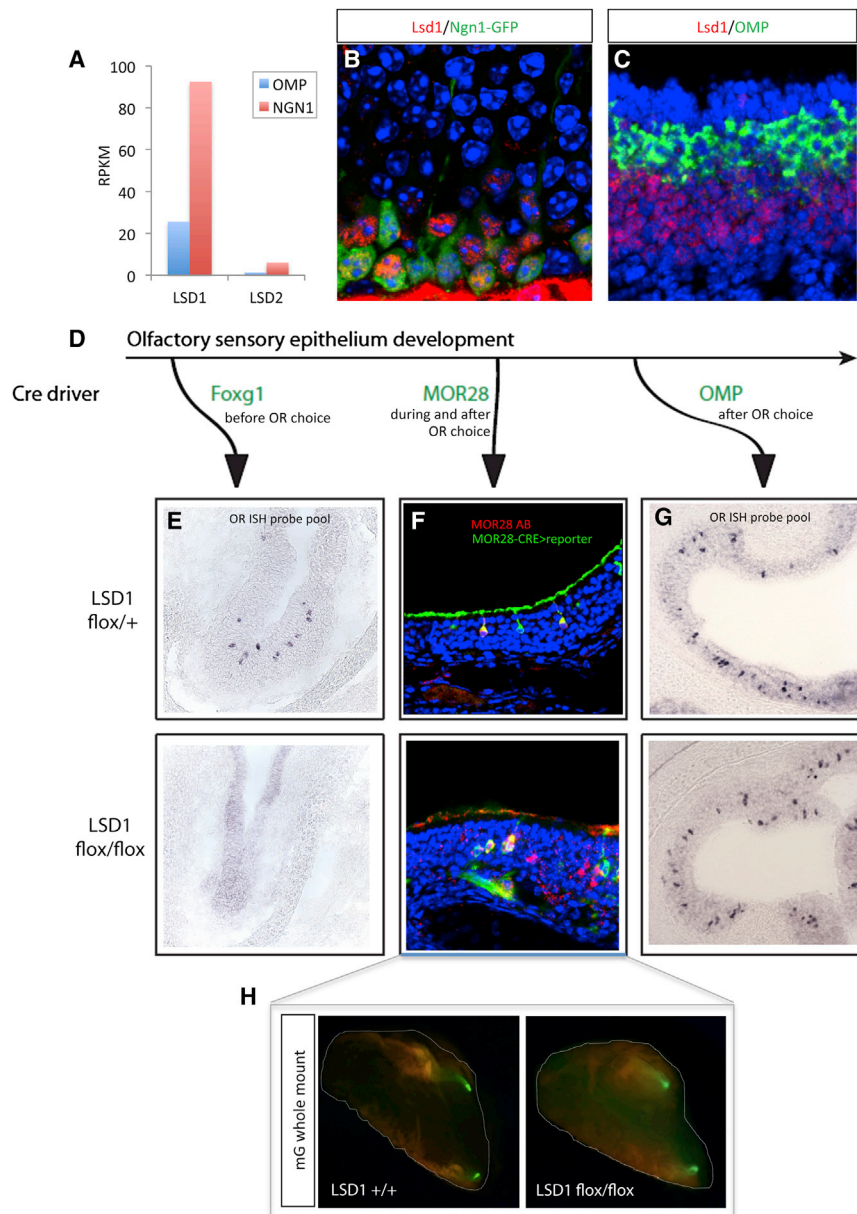


Figure 1. Transient LSD1 Expression Is Required for OR Expression

(A) mRNA-seq reads per million mapped per thousand base pairs of exon model (RPKM) for LSD1 and LSD2 in the mature versus immature/globose basal cells (Ngn1⁺). (B) LSD1 immunofluorescence (IF, red) in the Ngn1-GFP⁺ MOE at P30. (C) LSD1 and OMP two-color RNA ISH at P5. DAPI nuclear stain is shown in blue. (D) Removal of LSD1 over developmental time with three different MOE-specific Cre recombinase mouse lines. (E) OR ISH probe pool for 8 class II OR genes in *Foxg1-Cre; LSD1flox/+* and *Foxg1-Cre; flox/flox* (class I OR ISH is shown in Figure S1). (F) MOR28-IRES-Cre-mediated Cre reporter (green) in MOE with MOR28 immunofluorescence (red); coexpressing cells are stably expressing MOR28 in the absence of LSD1. (G) Class II OR ISH in *OMP-IRES-Cre; LSD1 flox/+* and *flox/flox* MOEs at P1. (H) Olfactory bulbs of *MOR28-IRES-Cre; LSD1^{+/+}* and *MOR28-IRES-Cre; LSD1flox/flox* animals at P30 with a two-color membrane-bound Cre-reporter: mT before Cre; mG after Cre (mT/mG; Muzumdar et al., 2007). See also Figure S1.

preserve the expression of transgenic MOR28 in mOSNs. These three alleles were put into the *Foxg1-Cre; LSD1 KO* background (Figure 3B). Embryos were collected at E18.5 and subjected to whole-mount X-gal staining (Figure S3). Many LacZ-positive neurons are detected in the MOEs of these mice, and the X-gal-stained cells have dendrites that reach the lumen of the olfactory epithelium (Figures 3B and S3). Strikingly, IF shows that ectopic expression of MOR28 restores *Adcy3* immunoreactivity in the *LSD1 KO* mice (Figure 3B), showing that OR expression controls *Adcy3* expression.

ORs Induce *Adcy3* Expression

This developmental arrest invites the hypothesis that OR expression might be a prerequisite for OSN maturation. Consistent with this, an “empty” OSN that does not express OR protein, like olfactory receptor neurons generated in *Drosophila* (Hallem and Carlson, 2006), has yet to be described in the mouse, where neurons with detectable pseudogene OR expression are immature and OMP negative (Shykind et al., 2004). Thus, we sought to rescue the *LSD1 KO* phenotype by ectopic OR expression (Figure 3A). We crossed a tetO-MOR28-IRES-LacZ transgene (Clowney et al., 2012) to two tTA drivers: *Gγ8-tTA* (Nguyen et al., 2007), which is not affected by the *LSD1* deletion since its expression initiates in immature OSNs (Ryba and Tirindelli, 1995), and *OMP-IRES-tTA* (Yu et al., 2004), which might

The fact that *Adcy3* constitutes a faithful marker for OR expression, in addition to being a marker of OSN maturation, allowed us to also test whether the levels of *LSD1* affect the kinetics of OR choice and OSN maturation. We detect a significant reduction in the number of *Adcy3*-positive cells in heterozygote *LSD1 KO* mice compared to wild-type (WT) littermates (Figure 3C). This suggests that the levels of *LSD1* are not saturating during OR choice, which may result in a slow and inefficient process of OR activation contributing to the singularity of OR choice.

Adcy3 Promotes OR Choice Stabilization and OSN Differentiation via *LSD1* Downregulation

The intriguing observation that *Adcy3* expression is mutually exclusive with *LSD1* expression and depends upon OR

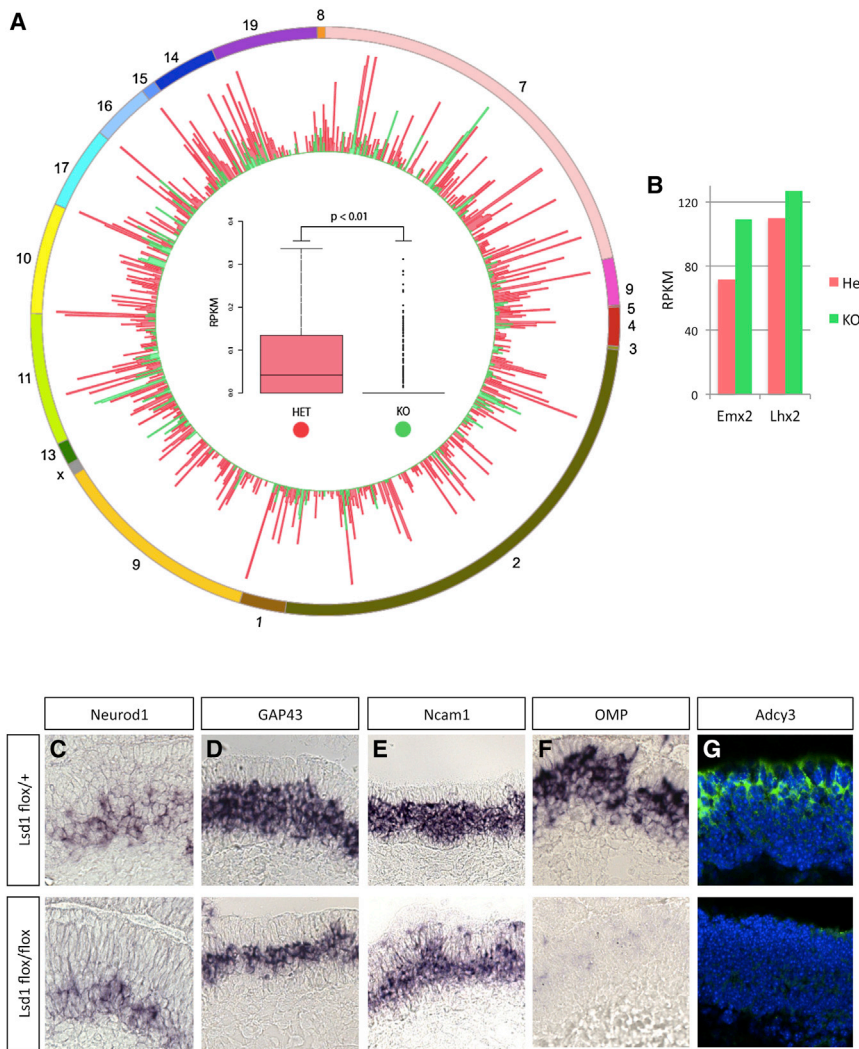


Figure 2. Early Deletion of LSD1 with Foxg1-Cre Causes Massive Reduction in OR Gene Expression and Developmental Arrest at a Differentiation Stage Synchronous to the Onset of OR Transcription

(A) mRNA-seq RPKM for each Refseq OR in mouse genome from E18.5 MOE sample. Each spoke of a given color is the expression value for that OR in the MOE of that genotype (red: Foxg1-Cre; Lsd1flox/+; green: Foxg1-Cre; Lsd1flox/flox). External chromosome ring reflects number of OR genes, not actual length of chromosome. Summary boxplot is shown within Circos plot; p value < 0.001, unpaired Student's t test.

(B) RPKM values of the two known transcriptional activators of OR genes in the LSD1 heterozygote and KO.

(C–F) Chromogenic ISH for developmental markers in LSD1 heterozygote (top panels) and KO (bottom panels), respectively: Neurod1, GAP43, NCAM1, and OMP.

(G) IF for Adcy3 at same embryonic stage; DAPI nuclear stain is shown in blue.

See also Figure S2.

residing below the sustentacular cells (Figure 4C). Similarly, IF for β -galactosidase, which is expressed instead of Adcy3 in this KO strain, shows that transcription of the Adcy3 locus is also restricted to the most apical OSN layer, providing further evidence for a stabilizing positive-feedback loop, whereby stable Adcy3 expression is contingent on OR and Adcy3 proteins (Figures 4D and S4C). Notably, the Adcy3 KO MOEs have a thin mOSN layer at this age, suggesting that eventually a small proportion of neurons settle to a stable and robust

expression prompted us to test whether this protein plays a role in the downregulation of LSD1 and the stabilization of OR choice. Adcy3 is the main adenylyl cyclase in OSNs, and previous reports have shown that Adcy3 KO OSNs have severe targeting defects (Chesler et al., 2007; Col et al., 2007; Zou et al., 2007). A role of Adcy3 in stabilization of OR expression could account for these targeting deficits, together with activity-dependent processes that regulate axon guidance. Given that gene switching requires the repression of the previously chosen OR and the desilencing of a new OR allele, both of which could be accomplished in part by the dual enzymatic activities of LSD1, we examined whether Adcy3 deletion affects LSD1 expression.

In agreement with an instructive role for Adcy3 in LSD1 gene regulation, we find that Adcy3 deletion causes a dramatic extension of LSD1 immunoreactivity toward the mOSN layers at postnatal day 21 (P21) (Figures 4A and S4A). Similarly, GAP43 expression is also apically expanded, showing that Adcy3 deletion delays the terminal differentiation of these neurons (Figures 4B and S4B). RNA ISH for OMP shows that the mOSN layer is reduced and restricted only to the most apical OSN layer,

choice of a single OR, as previously reported (Zou et al., 2007), possibly via low-level, paralogous adenylyl cyclase activity. This is also evident by the OR expression pattern because at P21, OR IF shows robust OR expression in the thin LSD1-negative apical layer of the Adcy3 KO MOE (Figure 4E).

Consistent with a delay in the stabilization of OR expression, RNA-seq analysis of WT and Adcy3 KO MOEs at P21 shows that overall OR mRNA levels are moderately reduced in a statistically significant manner (Figure 4F, left panel). Because Adcy3 is expressed after OR choice and in an OR-dependent manner, these effects likely do not reflect a developmental delay in the initiation of OR expression but, rather, post-OR choice instability. Indeed, our RNA-seq analysis shows that overall expression of pseudogene ORs is not decreased but rather slightly increased, both at absolute and relative levels (Figure 4F, right panel and Figure S4D, respectively), supporting the notion that LSD1⁺ OSNs continue to search ORs, even after the choice of an intact OR. Because OR pseudogenes can be chosen at the same frequency as intact ORs (Shykind et al., 2004), but their expression is less stable, a general deficit in stabilization of

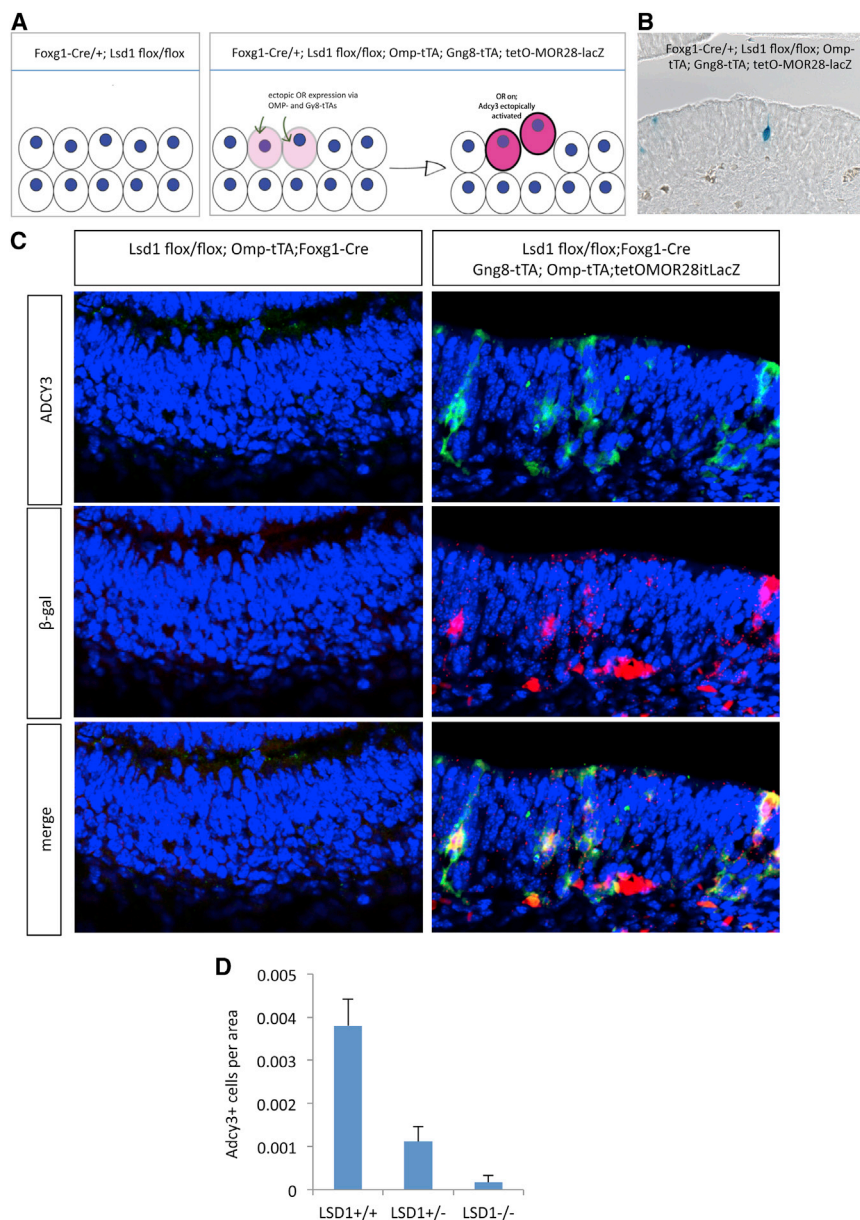


Figure 3. Ectopic Expression of Transgenic MOR28 in the LSD1 KO MOE Can Rescue the Loss of Adcy3 Expression

(A) Model summary of findings from mis-expression study. Using two tTA drivers, one active in the immature neuron (Gng8-tTA), and one active in the mature neuron (OMPitTA), it is possible to express high levels of MOR28 in the LSD1 KO MOE in a sporadic fashion. We find that OR expression is followed by the onset of Adcy3 protein expression.

(B) X-gal staining in sections of LSD1 KO MOE shows infrequent transgenic MOR28 expression under the control of two tTA drivers. Whole-mount image is shown in Figure S3.

(C) Foxg1-Cre; tetO-MOR28-lacZ MOE at E18.5 with either Lsd1 flox/flox; OMPitTA (left panels) or Lsd1 flox/flox; Gng8-tTA; OMPitTA (right panels). Adcy3 IF (green); Beta-galactosidase IF (red); and merge.

(D) LSD1 dosage positively correlates with Adcy3 immunoreactivity. Adcy3⁺ cells in E18.5 MOEs were quantified per unit area in ImageJ. y axis units are Adcy3⁺ cells per micron of MOE area considered. Barplot shows mean of two quantified regions of MOE from one experiment. Error bars are mean \pm standard error of the mean (SEM).

this age. At this age, the majority of the OSNs are immature, yet we detect OR-expressing OSNs in Adcy3 KO and an apical expansion of LSD1 expression, which is less pronounced than in the older mice (Figures 4G, S4E, and S4F). Lineage tracing, however, shows that Adcy3 KO mice have an \sim 2-fold increase (Student's t test; $p = 0.007$) of single-positive (Cre⁺ or GFP⁺) over the number of double-positive neurons (Figures 4H and 4I), supporting the frequent switching phenotype suggested by the increase of pseudogene expression.

The rapid OR switching phenotype observed in Adcy3 KO pups suggests that the ectopic LSD1 expression in the Adcy3 KO is the cause of post-choice OR downregulation, and that sustained LSD1 expression is incompatible with OR transcription. To directly test the post-choice effects of LSD1 expression, we generated transgenic tetO-LSD1, which we crossed to OMPitTA mice to drive expression of LSD1 in mOSNs (Figures 5A and 5B). OSNs of these mice retain high levels of LSD1 even after OR choice, causing significant downregulation of OR expression by IF (Figure 5B and quantification in Figure S5A) and RNA ISH (Figure 5C). Feeding these mice doxycycline for 3 weeks shuts off tTA-driven LSD1 expression in mOSNs and restores robust OR expression (Figures 5B and S5A).

We also brought the P2-IRES-taulacZ reporter (Mombaerts et al., 1996) into the LSD1-overexpressing background. Consistent with the aforementioned decrease in OR expression, there

intact OR expression would favor the representation of pseudogenized ORs.

To directly test the role of Adcy3 in the stability of OR expression, we performed lineage-tracing experiments (Shykind et al., 2004) in control and Adcy3 KO mice by crossing MOR28-IRES-Cre mice to a Cre-inducible GFP reporter (Muzumdar et al., 2007). If intact ORs switch in the absence of Adcy3, then a fraction of GFP-positive neurons should stop expressing the MOR28-IRES-Cre allele that recombined and activated the reporter, generating GFP⁺/Cre⁻ neurons. Moreover, if switching is rapid, then a fraction of Cre-expressing neurons should be GFP negative because Cre-mediated recombination takes 6–24 hr (Hayashi and McMahon, 2002; Nakamura et al., 2006). We performed this analysis at P2 because the Adcy3 KO mice fail to thrive, and mice with all four alleles did not survive beyond

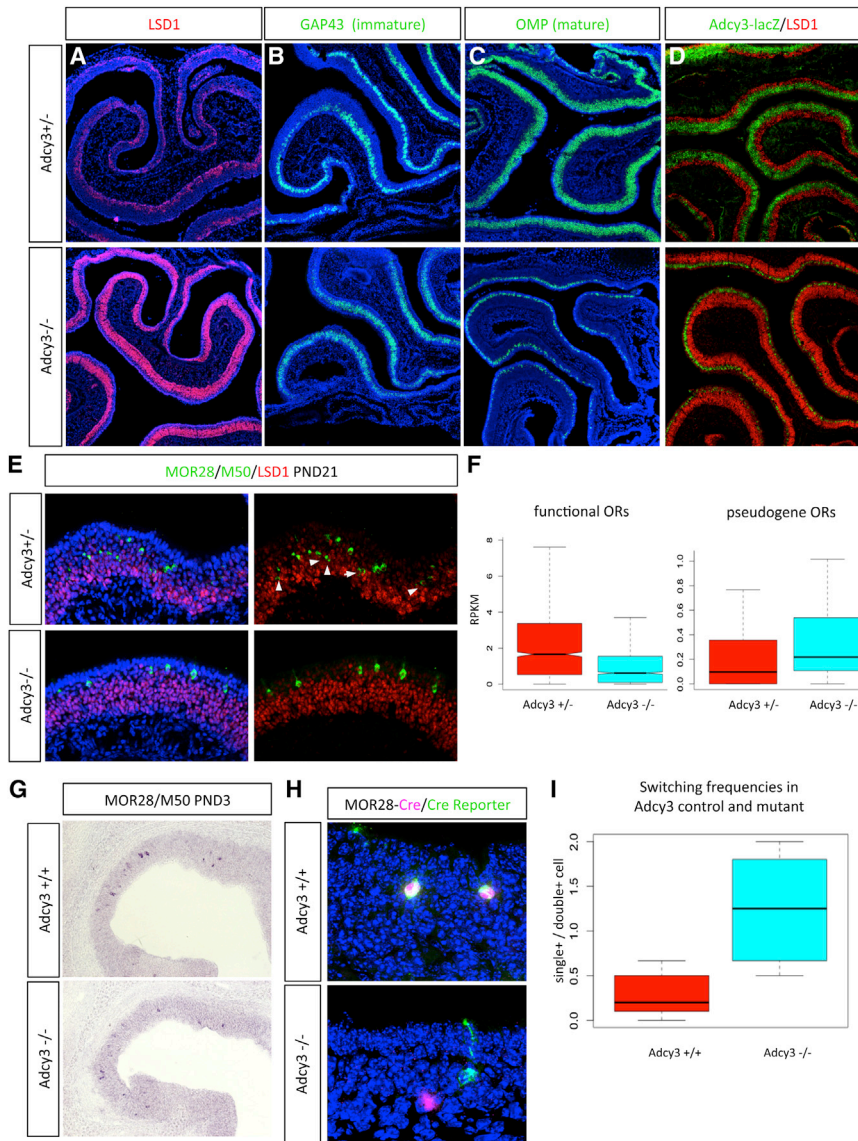


Figure 4. Adcy3 Removal Triggers Upregulation of LSD1 Protein Levels and Increase in OR Gene Switching

(A) P21 sections with IF for LSD1 in *Adcy3*^{+/-} (top) and ^{-/-} (bottom), respectively. See Figure S4 for quantification.

(B and C) Fluorescent RNA ISH for immature (GAP43) and mature (OMP) neurons in *Adcy3*^{+/-} (top) and ^{-/-} (bottom), respectively.

(D) Beta-gal (green) and LSD1 (red) IF in *Adcy3*^{+/-} (top) and ^{-/-} (bottom), respectively. A lacZ reporter is knocked into the *Adcy3* locus. See the Extended Experimental Procedures for details.

(E) P21 sections from *Adcy3*^{+/-} (top) and ^{-/-} (bottom), stained with OR (green, MOR28 and M50) and LSD1 (red) antibodies. ORs are detected in a more basal layer in *Adcy3*^{+/-} but not in *Adcy3*^{-/-} (arrowheads).

(F) Boxplot of RNA-seq RPKM values for all detected Refseq OR genes (left, *n* = 1072; *p* value < 0.01; unpaired Student's *t* test) and OR pseudogenes (right, *n* = 48, not significant) in corresponding genotype from P21 MOE. RPKMs of unexpressed intact and pseudogene ORs were excluded. See also Figure S4.

(G) P3 RNA ISH for M50 and MOR28 as in (E). IF for ORs and LSD1 is shown in Figure S4.

(H) P2 MOE from MOR28-IRES-Cre; Cre-reporter mice in *Adcy3* WT or KO background. Cre IF (magenta) and mT/mG reporter (green) are shown.

(I) Quantification of experiment in (H). Single- (CRE- or GFP-positive) and double-positive cells from ten sections of P2 WT and *Adcy3* KO mice were counted and plotted as a ratio of single- to double-positive cells. Single animal, ten sections quantified. *P* value = 0.007, Student's unpaired *t* test.

was a dramatic reduction of this OR reporter gene (Figure 5D). Moreover, olfactory-bulb targeting becomes perturbed in the LSD1-overexpressing MOE, with regional targeting, by and large, unaffected but the total number of targeted glomeruli increasing from 1–2 in the control to roughly a dozen in the OMPitTA; tetO-LSD1 (Figure 5E and data not shown), further supporting that LSD1-overexpressing OSNs are unable to settle on a single OR and switch frequently to other OR alleles from the same zone.

LSD1 Triggers Guanine Oxidation of the Active OR DNA

The likely transient interaction of LSD1 with a chosen OR makes it technically impossible to detect by chromatin immunoprecipitation (ChIP) the binding of LSD1 on an active OR. This technical hurdle would be bypassed if we could detect a molecular “signature” for the presence of LSD1 at the active OR allele. Such a mark could be generated by hydrogen peroxide, which, in addi-

tion to formaldehyde, is a localized chemical byproduct of LSD1-mediated lysine demethylation (Anand and Marmorstein, 2007). Importantly, other histone demethylases do not involve the generation of reactive oxygen species like FAD-dependent LSD1 (Hou and Yu, 2010). Hydrogen peroxide tends to selectively oxidize guanosine to 8-oxoguanosine (8-oxodG), thus, we reasoned that the extensive demethylation of an OR locus would generate enough hydrogen peroxide to locally modify guanosines of the chosen allele, as has been implied by the recruitment of OGG1, a DNA repair protein that binds to 8-oxodG at LSD1-regulated promoters (Perillo et al., 2008).

To detect 8-oxodG on a genomic locus, we developed a DNA immunoprecipitation (DIP) assay with an antibody specific for this modified base. We performed preliminary titration experiments with a synthetic DNA template derived from the Cre sequence. This analysis showed that a commercially available antibody could immunoprecipitate this PCR-synthesized Cre DNA with an ~10-fold higher efficiency than its unmodified counterpart (Figure S6A; see Experimental Procedures). Thus, a DIP-based strategy is sensitive and specific enough for the detection of this modified base on an active OR allele. DIP-quantitative

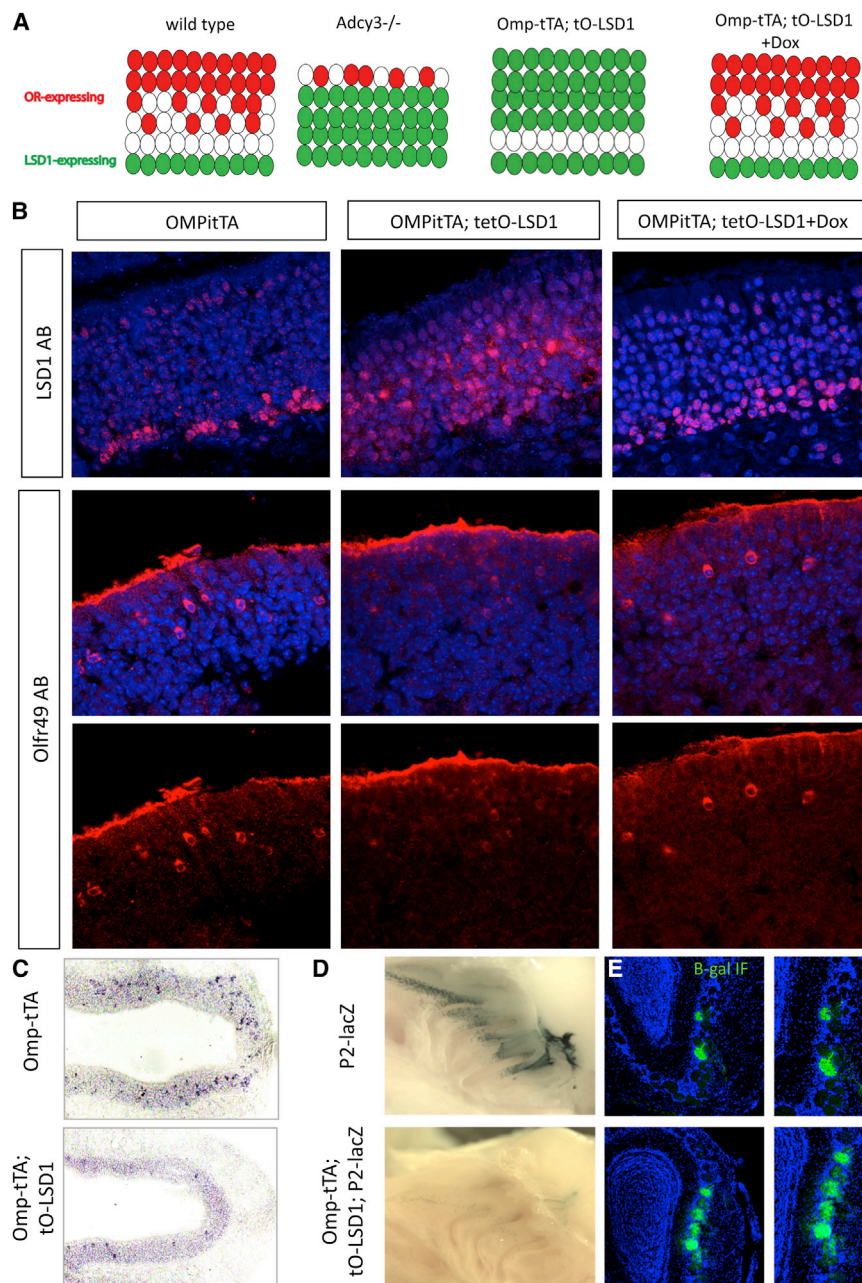


Figure 5. Ectopic Expression of Transgenic LSD1 in the Mature Neuron Layer Causes Reversible Destabilization of OR Expression

(A) Model summarizing results in adult MOE regarding the expression pattern of ORs and LSD1 under different genetic manipulations: OR-expressing OSNs are prevalent in LSD1-negative layer regardless of genotype. Weakly OR-expressing OSNs are present in OMPitTA; tetO-LSD1 mice before dox, but robust expression returns following dox and the reduction of LSD1 misexpression.

(B) Adult OMP-tTA; tetO-LSD1 mice were raised until 3 weeks and either placed on doxycycline for 3 weeks to shut off tTA activation or maintained on dox-free food. Control littermate mice (OMPitTA only) were also placed on doxycycline for 3 weeks. Six-week-old MOEs were harvested, and IF was performed for LSD1 (red top panel) or Olf49 (C6) (red two bottom panels with or without DAPI). See also Figure S5.

(C) Misexpressing LSD1 in the MOE with OMPitTA reduced OR expression in the MOE. Chromogenic ISH OR pool (15 OR probes total) in OMPitTA (left) and OMPitTA; tetO-LSD1 (right).

(D) P2-lacZ (top left) and P2-lacZ; OMPitTA; tetO-LSD1 (bottom left) MOE and bulb following whole-mount X-gal staining in P25 mice. Olfactory bulb sections (right) from the same genotype are shown with beta-galactosidase IF (green). Despite the low levels of beta-gal at the cell bodies due to switching, the protein appears stable at the axons (Clowney et al., 2012), which allows the visualization of additional glomeruli.

quantified the relative enrichment of 8-oxodG on the active and inactive OR alleles. We detect an ~ 3 -fold higher enrichment of 8-oxodG on the P2 allele, compared to the enrichment of this base on the inactive OR genes tested (Figure 6B). Because the majority of the sorted P2 neurons are mature, their transcription was initiated days or weeks before, and thus they have long ago downregulated LSD1. The enrichment levels we obtained imply that 8-oxodG

PCR (qPCR) analysis with DNA prepared from E18.5 MOEs from WT, heterozygote, and homozygote LSD1 KO mice shows LSD1-dependent 8-oxodG enrichment on two OR loci tested, P2 and MOR28 (Figure 6A). The enrichment levels for 8-oxodG are low in this experiment and comparable with the enrichment of a control locus, likely because we performed DIP in whole MOE populations in which the two OR alleles are expressed in a very low fraction of cells.

To test whether 8-oxodG enrichment stems from transcriptionally active OR alleles, we fluorescence-activated cell-sorted (FACS) GFP⁺ neurons expressing olfactory receptor P2 from P2-IRES-GFP knockin mice. Using DIP-qPCR analysis, we

is stable on OR DNA, due to a probably inefficient DNA repair process stemming from the low expression levels of OGG1 and NEIL1 (Klungland and Bjelland, 2007), as shown by our RNA-seq analysis (data not shown). To test this, we used a Cre-OR knockin line whereby Cre replaces the coding sequence of MOR28. MOR28-delete-Cre-expressing OSNs treat this allele like a pseudogene OR and switch from it in order to express a functional OR, often the functional MOR28 allele (Shykind et al., 2004). We crossed this delete-Cre line to the membrane-GFP (mT/mG) Cre reporter (Muzumdar et al., 2007) and isolated the GFP-positive neurons with FACS. Although transcription from the deleted MOR28 allele has ceased in most GFP-positive

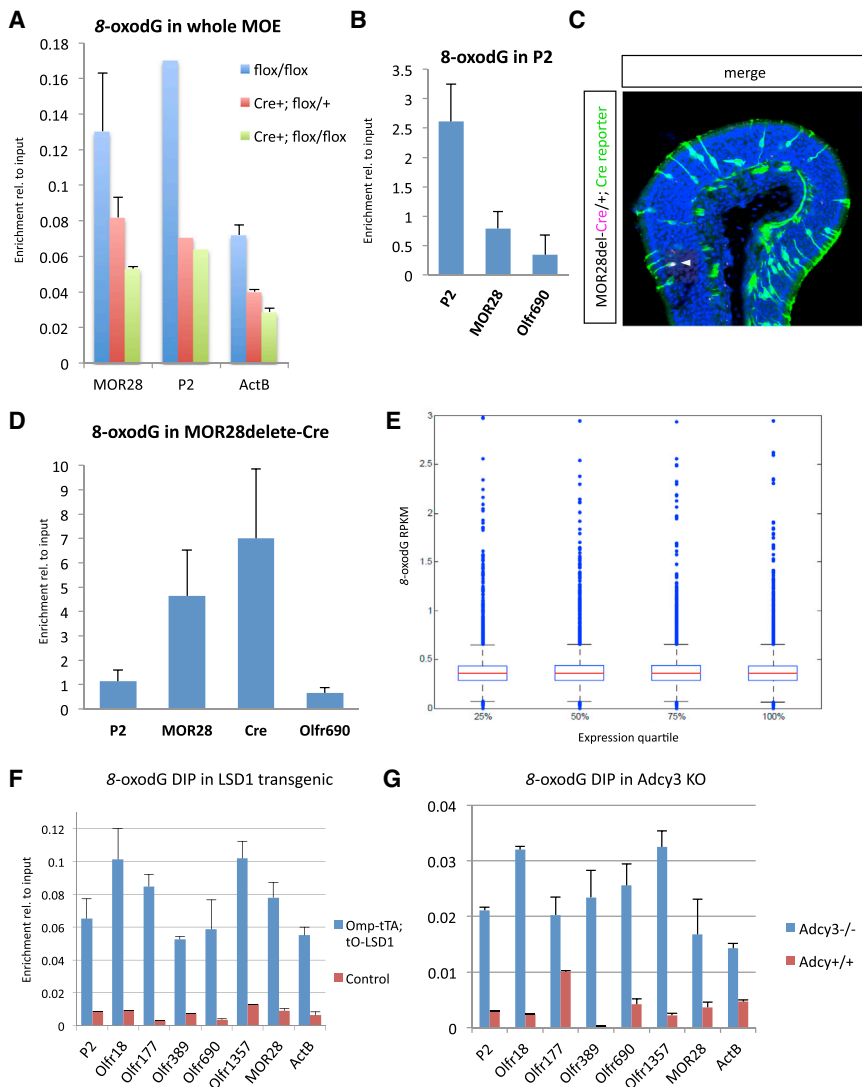


Figure 6. LSD1 Generates Stable 8-oxodG at Active OR Genes

(A) 8-oxodG DIP was performed on sonicated genomic DNA (gDNA) from LSD1 WT, heterozygote, and KO MOEs at E18.5.

(B) 8-oxodG-DIP-qPCRs from gDNA of P2-GFP-sorted cells from P30 mice; values shown are mean of qPCR performed in duplicate from one representative experiment. Error bars represent the SEM.

(C) P30 MOE of MOR28-del-Cre; Cre-reporter mouse, with Cre IF (magenta, arrowhead) and mT/mG Cre-reporter (green are cells that have expressed Cre to levels sufficient to recombine reporter locus). DAPI nuclear stain is blue.

(D) 8-oxodG-DIP-qPCRs from Cre-reporter-positive neuron gDNA at P30, as shown in (C). Values shown are mean of qPCR performed in duplicate from one representative experiment. Error bars represent the SEM.

(E) DIP-seq analysis of an E18.5 WT 8-oxodG library. Expression quartiles from the RPKM values generated from Figure 1 mRNA-seq. Boxplots show mean 8-oxodG RPKM for each expression quartile, where mean is demarcated by red bar.

(F and G) 8-oxodG-DIP-qPCRs from gDNA from whole MOE of LSD1-overexpressing mice and Adcy3 KO mice, respectively. Relative enrichment is \pm SEM as above. See also Figure S6 for control experiments.

neurons (Figure 6C), we detect significant enrichment for 8-oxodG on the Cre locus in the GFP-positive cells (Figure 6D). Interestingly, we also detect enrichment for this modified base on the WT MOR28 allele, which is explained by the high frequency by which these OSNs switch to this allele.

To test the possibility that 8-oxodG is a reflection of unprotected DNA due to transcription and not a direct consequence of LSD1-dependent demethylation, we performed Illumina sequencing in DIP from the whole MOE. DIP-seq analysis shows that the enrichment for 8-oxodG does not correlate with levels of transcription. We sorted the mouse genes into four quartiles of transcription levels, and we plotted 8-oxodG levels for each quartile. The mean 8-oxodG reads per kilobase per million mapped reads (RPKM) are essentially identical between the four expression quartiles, suggesting that the enrichment of this base on the chosen OR allele is not a byproduct of the unusual transcription rates of OR alleles, but rather it is indicative of LSD1's proximity to that OR locus (Figure 6E).

expressing cells in embryonic than in adult MOEs. Interestingly, under these overexpression conditions, there is an expected loss of specificity at the LSD1-dependent DNA oxidation. Moreover, these results also show that in the OMPitTA; tetO-LSD1 MOE, only a small fraction of OR genes become ectopically demethylated. A 10- to 20-fold increase of 8-oxodG levels in DIPs from mixed MOE populations suggests that each OR allele is H3K9 free in 1%–2% of the cells instead of 0.1% that is calculated in WT MOEs. In agreement with the notion that ORs remain epigenetically silenced in >95% of the cells, and that in each OSN, the vast majority of OR genes remain heterochromatinized, ChIP-qPCR for H3K9me3 shows similar enrichment levels between WT and LSD1-overexpressing MOEs (Figure S6C).

DISCUSSION

Our understanding of OR regulation changed by the realization that OR expression elicits a feedback signal that prevents the

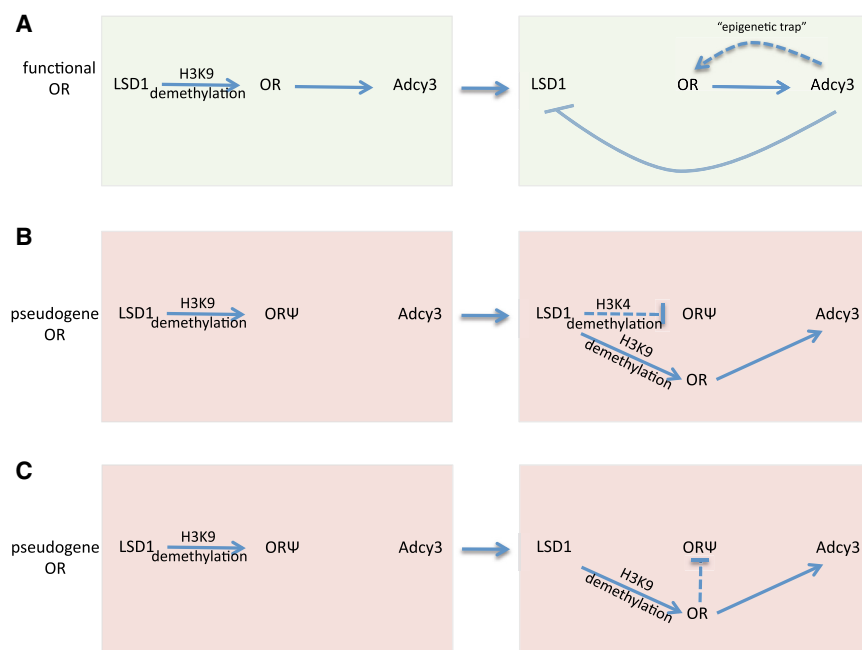


Figure 7. A Three-Node Signaling Cascade Combined with a Feedback Signal that Generates Epigenetic Memory

(A) Stabilization of OR expression is achieved by an Adcy3-dependent “trap” such that the functional chosen OR cannot be turned off once LSD1 is downregulated by its induction of Adcy3. This trap is caused by removing LSD1 from the signaling circuit, which allows stable transcription to ensue (represented by dashed line that reflects the indirect OR stabilization by Adcy3).

(B) Pseudogene ORs (OR Ψ) are unable to activate Adcy3, and thus OSNs that have chosen these ORs maintain the ability to re-choose and use LSD1 to transcriptionally silence the OR Ψ .

(C) Alternatively, LSD1 may not directly silence the previously chosen OR but instead cause its repression by activating an additional OR allele.

possible that the downregulation of the chosen OR is not a direct consequence of H3K4 demethylation by LSD1 but an indirect effect of the fact that an additional OR has been activated. Finally, as

expression of additional ORs and/or stabilizes the expression of the chosen one (Lewcock and Reed, 2004; Nguyen et al., 2007; Serizawa et al., 2003; Shykind et al., 2004). However, neither the mechanism of OR gene activation nor the pathway that stabilizes this activation were previously understood. We recently showed that ORs undergo heterochromatic silencing in the MOE, at a stage prior to OR expression, and that OR choice coincides with an epigenetic switch from H3K9me3 to H3K4me3 at the chosen allele (Magklara et al., 2011). The data presented here demonstrate that the epigenetic signature of active and silent ORs affords the deployment of a feedback mechanism that prevents the activation of additional ORs, while at the same time stabilizing the expression of the chosen allele. This epigenetic switch, combined with the dual function of LSD1 as H3K9 and H3K4 demethylase, not only renders the chosen OR immune to the feedback signal but also makes the subsequent downregulation of LSD1 imperative for the stabilization of OR choice.

These observations pose a significant question: why does LSD1 activate OR transcription before an OR is chosen but repress OR transcriptions after OR choice? Before OR choice, ORs are marked only by H3K9 methylation, thus LSD1 can only demethylate H3K9 in an OR locus and activate transcription. However, after OR choice, the chosen OR switches from H3K9 to H3K4 methylation (Magklara et al., 2011). Therefore, at this stage, if LSD1 is still present and recruited to the chosen OR, it can only demethylate H3K4, resulting in the repression of this OR. Thus, the same molecule before OR choice is by default an activator but after choice is a repressor for an already activated OR and a potential activator for the remaining silenced OR genes. Therefore, local epigenetic context determines the exact action of LSD1 and makes the chosen OR susceptible to LSD1-mediated repression. Alternatively, if the OSN cannot support OR transcription from two different loci simultaneously, it is

in every genetic manipulation, indirect effects from either the deletion or the overexpression of LSD1 could contribute to the observed phenotypes.

It is worth emphasizing that the genetic manipulations presented here affected only the stability of OR choice and not the singularity of expression, unlike when we disrupted nuclear OR aggregation by ectopic LBR expression (Clowney et al., 2012). The fact that ORs aggregate in large nuclear foci makes the majority of ORs inaccessible to LSD1, explaining why most ORs remain heterochromatic after LSD1 overexpression. Thus, the mechanism that affords the selection of only one out of thousands of OR alleles is different than the mechanism that makes this selection permanent.

Feedback Signal versus Feedforward Loop

Our data, together with the established requirement of intact OR protein for the generation of the feedback signal, lead to the following regulatory model: LSD1, in complex with an as-yet-unidentified H3K9me3 demethylase, desilences a previously heterochromatinized OR allele, allowing H3K4 trimethylation and transcriptional activation. If this allele encodes an intact OR, then it will induce Adcy3 expression, LSD1 downregulation, and OSN maturation, generating an “epigenetic” trap that will preserve OR expression, cellular identity, and targeting specificity, as long as the underlying transcription factor milieu remains unaltered (Figure 7A). In contrast, if the initially chosen allele is a pseudogene and does not produce OR protein, then this particular choice cannot induce Adcy3 expression, and LSD1 will not be turned off. With LSD1 activity still present, an additional OR can become desilenced via H3K9 demethylation, but also the previously chosen OR allele can become demethylated at H3K4 and turned off. Thus, failure to terminate LSD1 activity results in OR gene switching, and this process will continue until an intact OR is expressed (Figure 7B).

Different versions of feedforward developmental loops in transcription factor regulation were recently described in the differentiation of *Drosophila* photoreceptor neurons (Johnston et al., 2011) and in various examples of mammalian differentiation (Neph et al., 2012). In these systems, as in a plethora of cases where a network of interactions has been mapped (Alon, 2007), establishment of cellular identity did not require downregulation of the activator that initiates a specific differentiation program, as is the case with LSD1 here. A major difference in OR regulation is the existence of an extraordinary number of similar promoters and a strict requirement for singularity in OR expression, which likely makes a feedforward circuit ineffective. Instead, the three-node signaling cascade described here, which locks the epigenetic states of the chosen allele and of the silent ORs, can assure both singularity and robustness. Using LSD1, which has coactivator and corepressor activities, as an initiator of this cascade provides the additional advantage of autocorrection, through the post-choice repression of pseudogenes.

Adcy3 Has Multiple Functions in the MOE

The finding that Adcy3, which requires the LSD1-dependent OR gene activation to be expressed, induces rapid LSD1 downregulation makes this protein both a *sensor* for a productive OR choice and a *transmitter* of the feedback signal that stabilizes its own expression and the expression of the chosen OR, while promoting OSN differentiation. It is intriguing that Adcy3 induces LSD1 downregulation because the role of OR activity in the feedback signal is unclear. Although odorant-induced activity is required for singular OR expression in the septal organ (Tian and Ma, 2008), deletion of various components of OR signaling, such as $G_{\text{olf}}(\alpha)$ and Cnga2, do not affect OR expression (Belluscio et al., 1998; Brunet et al., 1996), unlike in *Drosophila* photoreceptor neurons (Vasiliauskas et al., 2011). Furthermore, mutation of the DRY motif, which prevents OR interaction with $G(\alpha)$ proteins, has no impact on the singularity of OR expression (Imai et al., 2006). Although the effects of the DRY mutation in the stability of OR expression were not addressed, it is possible that there are additional, $G(\alpha)$ -independent mechanisms by which an OR activates Adcy3 signaling, or that LSD1 downregulation requires only OR-dependent Adcy3 expression and not OR-dependent Adcy3 activation. In either scenario, only low levels of cAMP, generated by basal Adcy3 activity, might be sufficient to induce LSD1 downregulation and OSN maturation, as in the Adcy3 KO mice, some OSNs eventually mature and turn off LSD1. Low expression of other adenylyl cyclases may eventually generate enough cAMP to elicit a feedback signal in the Adcy3 KO OSNs. In any case, Adcy3 occupies a critical checkpoint role in the development of the peripheral olfactory system: it regulates the stability of OR choice, the targeting of OSN axons, and the longevity of olfactory neurons (Santoro and Dulac, 2012).

A Transcriptional Regulator with Mutagenic Potential

The detection of 8-oxodG on the chosen OR allele is suggestive of extensive LSD1-mediated demethylation activity in close proximity to the chosen OR allele. Given that ORs are embedded in continuous blocks of methylated H3K9 (Magklara

et al., 2011), demethylation of this lysine residue during OR activation is a plausible explanation for the local production of hydrogen peroxide and the accumulation of 8-oxodG at the chosen OR. Guanosine oxidation, by default, would not have consequences in the genomic stability of OSNs, as they are postmitotic and relatively short-lived. However, were an LSD1-mediated demethylation responsible for OR activation during spermatogenesis (Fukuda et al., 2004), this could provide a mechanistic explanation for the high AT-rich content of OR genes and the extreme intra- and interspecies polymorphisms observed in this gene family, as 8-oxodG frequently pairs with adenosine rather than cytosine during DNA replication (Grollman and Moriya, 1993). Thus, LSD1-mediated derepression in the germline could explain both the drift toward high AT content (Glusman et al., 2001) and the evolutionary plasticity of olfactory receptor genes (Clowney et al., 2011; Niimura and Nei, 2007). Moreover, the observation that deletion of Adcy3 results in substantial increase of DNA oxidation in the OSN nuclei invites speculation regarding the role of neuronal activity pathways in protecting central nervous system (CNS) neurons from DNA oxidation and its deleterious long-term effects.

In summary, ORs provide an unusual example in biology, whereby a transmembrane receptor protein specialized in odorant detection functions also as a molecular organizer of the sensory neuron. The finding that Adcy3 expression and OSN differentiation depend upon OR expression suggests that there are no temporal restrictions or developmental windows for OR choice; an immature OSN will remain as such until it chooses a functional OR, allowing a slow, inefficient, and stochastic process for the choice of only one out of thousands of available alleles. The pleiotropic function of ORs in odor detection, OSN maturation, axonal wiring, and OSN longevity makes the peripheral olfactory system “self-organizing” and centered solely around the identity of the OR, which may have facilitated the rapid expansion of this gene family during tetrapod evolution (Niimura and Nei, 2007). To accommodate adaptation in novel and variable ecological niches, olfaction has remained extremely plastic, both at the level of the genomic integrity of the chemoreceptors and at the transmission and interpretation of odorant information in piriform cortex (Choi et al., 2011). For a sensory system that lacks “labeled lines” and where polymorphisms appear constantly, ascribing such a central developmental role to the receptor protein itself prevents pseudogene ORs from compromising the sensitivity and discriminatory power of the olfactory system (Shykind et al., 2004). The initial screening for OR quality is further fortified by a secondary, activity-dependent screen that gradually eliminates OSNs that are seldom used, affording individualized adaptation to an extremely plastic system (Santoro and Dulac, 2012).

EXPERIMENTAL PROCEDURES

Mice and Strains Used

All mice were housed in standard conditions with a 12 hr light/dark cycle and access to food and water ad libitum and in accordance with the University of California IACUC guidelines. All strains were maintained on a mixed genetic background. Detailed information on the various mouse strains used is provided in the [Extended Experimental Procedures](#).

In Situ Hybridization and Immunofluorescence

IF and ISH was performed as previously described (Clowney et al., 2012). Information on the riboprobes and antibodies used can be found in the [Extended Experimental Procedures](#). Confocal images were collected with the Zeiss LSM 700, and brightfield images were collected on the Zeiss Axio-skop Plus. All image processing was carried out with ImageJ (NIH).

Chromatin Immunoprecipitation

ChIP was performed as described in Magklara et al. (2011).

DNA Preparation and Immunoprecipitation

Genomic DNA was purified either following FACS or total MOE dissection using DNeasy genomic DNA isolation kit (QIAGEN). Purified genomic DNA was sonicated in PBS with 0.5% Tween-20 to a peak around 400 bp fragments using the Bioruptor (Diagenode). For sorted cells, fragmentation of DNA was assumed to be complete following 15 to 30 min of sonication using medium-to high-power output with samples in ice water. 8-oxodG monoclonal antibody (Trevingen) was incubated with DNA rotating overnight at 4°C. Immunoprecipitation and washes were carried out in PBS-Tween 0.05%, and DNA elution buffer consisted of 0.1M NaOAc and 1% SDS in TE (pH 8).

DNA Deep Sequencing

Oligonucleotide reads were generated for Lsd1 and Adcy3 mutant and control mRNA libraries as well as 8-oxodG libraries using the Genome Analyzer Ix or HiSeq2000 (Illumina). Sequencing libraries were prepared with standard methods (Magklara et al., 2011), but in the case of the mRNA, the ScriptSeq kit (Epicenter) was used. Detailed information can be found in the [Extended Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.06.039>.

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