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Chromatin-based regulation of olfactory receptor genes

by

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DISSERTATION

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I dedicate this work to my parents, Nancy Artz and Frank Lyons.

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Abstract

There are roughly 2600 olfactory receptor (OR) genes in the diploid mouse genome. Each olfactory sensory neuron expresses only a single OR in a monogenic and monoallelic fashion. The molecular mechanisms regulating receptor expression in the mammalian nose are poorly understood. We have identified 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 co-activator, LSD1 is necessary for de-silencing and initiating OR transcription, but as a transcriptional co-repressor, it is incompatible with maintenance of OR expression and its downregulation is imperative for stable OR choice. Adcy3, 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.

We have gone on to show that ER stress and specifically PERK kinase likely detects the OR protein and thus serves as a key intermediary in the pathway leading to LSD1 downregulation and OR choice stabilization. The activation of PERK via the UPR triggers the translation of nuclear ATF5, which is required for the upregulation of Adcy3. Thus LSD1 is needed to activate one OR from a repressed chromatin environment which initiates the cascade of molecular events at the ER that lead to its eventual downregulation. This dynamic regulation of LSD1 via ATF5 is central to the generation coherent olfactory input to the brain.

We have evidence suggesting that epigenetic silencing, which is mediated by histone H3 lysine 9 methyltransferases G9a and G9a-like protein (GLP), is essential for both the stochastic and the monogenic nature of OR expression. Conditional deletion of both G9a and GLP results in transcriptional domination by a few ORs, loss of monogenic expression, and significant downregulation of the rest of the family members. Therefore, heterochromatin at OR genes creates an epigenetic platform for generating transcriptional and cellular diversity.

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Chapter 1: an evolutionary perspective on our sense of smell

Olfaction facilitated the rise of mammals

The sense of smell is perhaps our most primal. Among terrestrial mammals, the ability to detect odorants is tantamount to survival. The significant anatomical investment in generating olfactory input that is seen throughout nearly all terrestrial vertebrates attests to this fact. The mammalian forebrain is largely composed of the neuroanatomical region called the olfactory bulb that is directly enervated by the olfactory sensory neuron, the cell type which directly interacts with odorants in the nasal cavity. Although vision and audition are arguably the most important senses for humans today, and perhaps the most well understood, it is olfaction which has allowed our early mammalian ancestors to take advantage of new ecological niches and to rise to form the crown group that mammals are today (Luo 2007).

The initial radiation of mammals during the early Mesozoic Era about 200 million years ago correlates with an increase in olfactory sensitivity (as measured by forebrain volume, where the olfactory bulbs occupy a defined anterior region). Solid evidence of such a correlation comes from the fossil record, where forebrain volume correlates well with the increase in olfactory turbinate elaboration and likely an increase in olfactory sensory neuron diversity (Rowe et al. 2011). Regions of the brain involved in tactile sensation likely also increased during this very early period in the evolution of mammals from their reptilian ancestry, as mammals gained a large amount of sensory input from the newly evolved

mammalian integument (e.g. whiskers and fur) that required neural interpretation (Rowe et al. 2011).

Thus olfaction is a at the very core of our origins: in facilitating the early exploration of novel niches unavailable to diurnal reptiles, the reliance on olfaction helped to change the shape of the mammalian central nervous system and reallocate resources to the brain, allowing a major transition in lifestyle from an ectothermic ancestry.

Hardware for smelling: the olfactory receptor

Olfactory neurons deploy rhodopsin-type (class A) G-protein coupled receptors (GPCRs) to detect odorous ligands (Buck and Axel 1991; Nei et al. 2008). However, it is unclear whether these GPCRs arose explicitly to detect photons. Considering the biochemical requirements for detecting light, such as the use of a retinal or retinal-like cofactor, it seems as likely that the very first "rhodopsin-type" GPCRs were in fact olfactory receptors which do not have obligate cofactor requirements for their function. The emergence of a protein whose 7-pass transmembrane topology could be later co-opted for the detection of light energy with the addition of a cofactor is one parsimonious explanation for the evolutionary relationships we see today that exist between ORs and opsins.

Whether the ability to detect light or odorants emerged first, olfaction is a sensory modality that arose in the most recent common ancestor of all metazoa over a billion years ago (Perez 2003). The olfactory system has been undergoing profound changes in response to what

appears to be a very limited selective pressure on receptor stasis, but rather a massive pressure for receptor variation. Especially for tetrapods, olfactory receptors are both very numerous and highly varied in sequence composition across genera (Freitag et al. 1998; Ji et al. 2009; Niimura 2012; Steiger et al. 2009). This observation supports the hypothesis that the evolutionary advantage is given to those animals with the ability to expand their olfactory perception to the broadest possible extent.

Olfactory receptor pathway conservation in vertebrates

The sense of smell is mediated by the OR binding one of its numerous cognate ligands that occur in the environment. These receptors are present throughout all vertebrates analyzed to date, as are the well-conserved downstream signaling components called G-proteins. GPCR binding to a cognate ligand induces conformational changes to the receptor which initiates the dissociation of the G-protein heterotrimer which is docked on the cytoplasmic face of the unbound OR protein (Schild and Restrepo 1998). Indeed this basic signal relay module is, in its simplest form, conserved throughout all of eukarya.

As a unique biochemical pathway, olfaction arises from the ability of G-protein alpha to bind adenylyl cyclase 3 (Adcy3) and trigger its activation (Bakalyar and Reed 1990; Pace et al. 1985). Adcy3, which is the "olfactory cyclase," catalyzes the generation of cyclic adenosine monophosphate (cAMP) from the free ATP in the neuron, such that both Adcy3 and G-

alpha(olf) are absolutely required for the sense of smell (Belluscio et al. 1998; Wong et al. 2000). This critical transition from a signal outside to a signal inside of the cell constitutes the beginnings of a well-conserved molecular cascade that potentiates the ability of the neuron to send electrical signals. The conversion of what is an extracellular ligand-binding event to an increase in the intracellular concentration of a small molecule involved in myriad signaling processes, namely cAMP, represents the core physiochemical transformation that engenders our sense of smell and divulges its basal origins.

Distribution of vertebrate-type olfactory receptors across chordates

The earliest diverged true chordates appear to have GPCRs that closely approximate OR charateristics, namely a cytoplasmic domain between the 3th and 4th transmembrane domains with the tripeptide motif Aspartate-Arginine-Tyrosine (D-R-Y) which directly interacts with the G-protein complex (Breer 2003; Krishnan et al. 2013). However, organisms such as the tunicate *Ciona intestinalis* do not possess a well-developed central nervous system and do not have a peripheral olfactory organ (Mazet and Shimeld 2005). This suggests that the OR genes and the membrane-localized signaling network that is initiated by the GPCR-to-G-protein interaction was an genetic module coopted in order to subserve the needs of the central nervous system (CNS) as it became increasingly complex in the last common ancestor of *Ciona* and the branch of chordates that led to vertebrates.

The observation that OR expression occurs in vertebrate gametes such as mouse sperm (Vanderhaeghen et al. 1997) suggests a simple explanation for the high conservation of this and other OR protein motifs—the OR-type GPCRs were used to facilitate sperm-egg recognition

before the emergence of chordates. The signaling pathway for the two events are essentially identical and GPCRs have a well-known role in chemoattraction in single-celled organisms generally. The link between chemoattraction and odorant detection (Spehr et al. 2004) is a seductive hypothesis and would help to explain the high conservation of this protein type across disparate taxa. Perhaps further bolstering this hypothesis, discussed at length in the following, is that OR protein is needed to guide the OSN axon into the forebrain from the periphery.

Chapter 2: Anatomical and gene regulatory hallmarks of the olfactory system in the mouse *Mus musculus*

Olfactory receptors are expressed monoallelically and monogenically

The olfactory receptor (OR) superfamily was discovered in the early 1990s by Linda Buck and Richard Axel (Buck and Axel 1991). Soon thereafter it was realized that OR genes are expressed at very high levels in the olfactory sensory neuron (OSN), and their expression pattern was essentially indeterministic, aside from very broad zones of expression with the MOE (see Figures 1 and 2). This expression pattern suggested that OR genes may be stochastically activated within the MOE which led to speculation about whether they, like most genes, are expressed from both paternal and maternal chromosomes (i.e. biallelically) (see Figure 1 for an example of OR expression). From the genome sequence of the mouse, we know that there are approximately 1300 olfactory receptors, meaning there are 2600 total OR alleles from which to choose (Zhang and Firestein 2009).

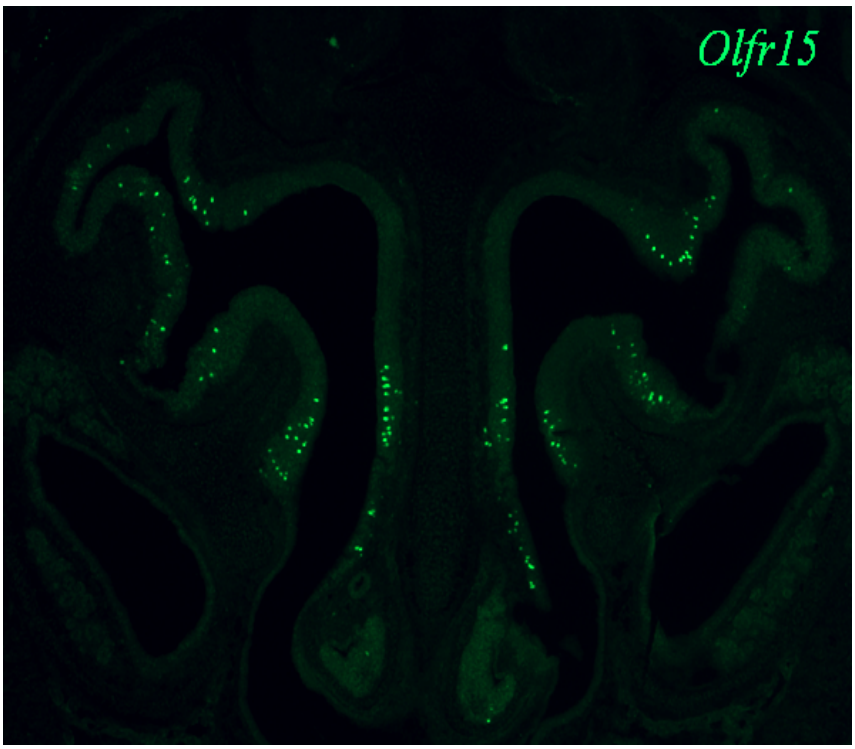

Figure 1: an example of stochastic expression pattern of olfactory receptor gene Olfr15.

Cryosection of an E18.5 MOE, which stains light green (background reactivity), while supporting tissue and surround area of cranium is darker. Very bright spots are individual neurons expressing *Olfr15*. The stochastic choice occurs within a domain, which is discernable here as a swath of the MOE where signal is present, and this will be discussed later in this chapter.

In order to assess whether mouse OR genes are expressed monoallelically, two lines of work have been carried out with similar results. With a genetically hybrid F1 mouse (M. spretus X M. musculus), derived from a cross of Mus musculus and a distantly related member of the genus, Mus spretus, it was possible to detect with RNA hybridization the presence of only one of two parental OR alleles in a given single neuron, and never alleles from both in a single

cell (Chess et al. 1994). This differential parental allele detection is possible due to the genetic variation, such as SNPs, of genes in distantly related species. This remarkable finding in conjunction with the unique, indeterminate expression pattern strongly suggested that the OR genes were controlled by a gene regulatory network that was different from the typical combinatorial logic that permit the transcriptional activation of genes irrespective of allele. More recent work from the Shykind and Mombaerts labs has shown that differentially labeled OR genes are indeed expressed in a mutually exclusive manner, such that for instance, P2-IRES-GFP from one parent and P2-IRES-tau::lacZ from the other will not be present in the same neuron under normal circumstances (Feinstein and Mombaerts 2004; Shykind 2005; Strotmann et al. 2000). The neuroanatomical significance of this monoallelic expression will be touched upon later in further detail.

Monoallelic gene expression in other systems: within mammals

The niche of a given organism is not static. Organisms must respond to an ever-changing environment and this means they must evolve mechanisms to recognize a diversity of unknowns. The chemicals in the air and water are some such unknowns, and are the pertinent drivers of selection when considering olfaction. The olfactory system of mammals and perhaps all vertebrates has evolved to express only one or a few of all possible OR alleles at a high level from the birth of the neuron (Sato et al. 2007). In the case of the mouse, this generates 2600 discrete cell types, each of which there are many thousands filling the sensory epithelium of the nose, the main olfactory epithelium (MOE).

There are a number of other systems in which a large diversity of cell types is generated to serve the end goal of cellular diversification. In none of these other systems are the genes responsible for conferring cellular identity strewn throughout the genome as with olfaction. Rather, in these other systems, the locus or loci responsible for the generation of this diversity are localized to a discrete, singular region of the genome. Nonetheless there are interesting similarities between these other monoallelic systems which are of some interest when considering how the olfactory receptor regulatory network evolved.

X chromosome inactivation

Within the mouse there are at least 5 other monoallelic expression modalities, the first of which is perhaps the most obvious: X-chromosome inactivation in females. This phenomenon is a response to the need of mammalian cells to express genes at a particular level across both sexes (Lyon 1974). Wild-type males have only one X chromosome, while females have 2. All other things being equal, the transcription from X chromosomal genes would be twice in females what it is in males. This increased gene dosage is deleterious, and has been avoided by the evolution of X-inactivation whereby one of the two X chromosomes in all cells of the female will undergo a random silencing event which will then propagate clonally through the daughter cells of that progenitor. The timing of X-inactivation is variable and thus many of the tissues in the mature adult mouse are composed of a collection of variegated cells, rather than all of the cells arising from a single cell or single clone which inactivated a particular X. X-inactivation relies on the formation of heterochromatin that is nucleated by a long non-coding RNA called Xist (Marahrens et al. 1997; Panning et al. 1997). (Interestingly, there are OR

genes on the X chromosome, which means that X chromosome OR expression is secondarily monoallelic, the monoallelicism being pre-established well before OR expression in stem cell progenitors of the MOE.) The other mammalian monoallelic modalities in the mouse are less general than X-inactivation and like OR expression, subserve the purpose of some organ or cell population.

Vomeronasal Receptors

The first monoallelic expression system I will discuss is the one most like OR gene expression—namely, the vomeronasal receptors (Dulac and Axel 1995). These receptors are not the focus of the work herein but are inextricably linked to the olfactory capabilities of the mouse via a peripheral olfactory organ called the vomeronasal organ. The neurons in this organ, which is located just dorsal to the palette in the mouse rostrum, project axons to a structure closely apposed to the olfactory bulb called the accessory olfactory bulb. These receptor genes are composed of two distinct classes: V1Rs, which are apically located and detect volatiles, and V2Rs, which are basal to V1R-neurons, and detect peptides. Both V1- and V2Rs are deployed in a monoallelic fashion, although V2Rs are usually coexpressed with one other VR protein in a combinatorial way, such that family A, B, or D receptors will be expressed in neurons also expressing a family C receptor (Ishii and Mombaerts 2011).

There are other chemosensory receptor types called Trace-Amine Associated Receptors (TAARs) occurring on chromosome 10 (Liberles and Buck 2006); all 15 TAARs are present in a single gene cluster. Both the VR- and TAAR-positive neurons in the olfactory system express receptors that are stochastically chosen, based on their expression pattern and our existing

genetic knowledge. The last olfactory class of receptor that is known is the Formyl-peptide receptors (FPRs) which are deployed in the VNO and were very recently discovered to have a role in olfaction (Liberles et al. 2009; Rivière et al. 2009).

Clustered Protocadherins

Similar to non-OR olfactory receptors, the clustered Protocadherin (Pcdh) genes are present in singular singular genomic regions. Protocadherins are also transmembrane proteins, like the ORs, VRs, and TAARs, but are only single-pass, rather than seven-pass. These proteins are involved in self-recognition such that a given cell will stochastically express one of many potential splice isoforms of Pcdh based on which of the promoters is chosen (Chen & Maniatis, 2013). There are 3 major classes of Pcdh within the single chromosome 18 cluster: alpha, beta, and gamma. The arrangement of these genes is unique: the variable extracellular domain is encoded by the 5'-most exon and the membrane-spanning and intracellular domains are encoded by constitutive regions coded for by a common set of 3 exons in the 3' end of the gene. The stochastic process occurs at the level of promoter choice. For example, the choice of the 5'-most alpha exon will cause the transcription all other alpha exons, but these exons are then spliced out from the mature mRNA which would consist of the 5'-most variable exon and the 3 constant exons of that particular cluster (Tasic et al., 2002).

This is in stark contrast to the ORs where only promoter choice is required for stochastic expression to occur. Because OR gene coding sequences are almost exclusively contained within a single exon, we are others (Gentles & Karlin, 1999) hypothesize that, relative to other mechanisms, alternative splicing is of minimal importance in OR and other GPCR regulation.

B and T cell receptors

Unlike the chemosensory receptors discussed above, the immune system possesses the remarkable capacity to permanently recombine somatic cell DNA in unpredictable ways in order to produce tremendous genotypic variation. This variation allows for the production hundreds of millions of different antibodies and receptors.

Both B and T cell receptors are generated by a complex process of DNA recombination followed by the repression of this recombination machinery to set the allelic choice in place (Schatz & Swanson, 2011). The repression of this recombination machinery occurs to allow a single allele to be expressed for the life of the immune system cell. Like olfactory receptors and the clustered Protocadherins, as well as the other gene families I briefly touched upon, the B and T cell receptors are transmembrane receptors that are involved in the detection of compounds in the extracellular milieu.

For the OSN, the detection of odorants is the key, while for B cells, the key is binding to antigen and for T cells it is binding to MHC-presented antigen. For B cells, productive transcript can be generated only following the DNA recombination between 2 loci termed V and J. This recombination brings the promoter of the resulting transcriptional unit into a region that can be acted upon by the relevant enhancer (Matheson & Corcoran, 2012). As a segment is generated by so-called VDJ recombination, the transmembrane immunoglobulin is processed and the recombinase is shut off via a negative feedback signal. The targeting of one of the two alleles for recombination occurs via a process involving specific histone lysine methylation

(Daniel & Nussenzweig, 2012), which will be discussed in detail in the coming section. The exact mechanism of maternal versus paternal allele choice is not understood.

Monoallelic gene expression in other systems: antigenic variation in Plasmodium

The ability of our immune system to detect pathogens with high efficiency suggests that because these invaders continue to persist, they must possess a remarkable host defense evasion program (Deitsch et al. 2009; Magklara & Lomvardas, 2013). The causative agent of the tropical disease malaria is a bloodborne pathogen (*Plasmodium falciparum*) which infects erythrocytes and causes them to be stabilized rather than get cleared by the spleen, as would happen to a typically infected red blood cell. *P. falciparum* achieves this evasion by expressing 1 of about 60 transmembrane proteins called PfEMP1 at random and switching from one to another allele. Each individual pathogen has been shown to have a unique repertoire of these genes, such that somatic mutation almost certainly is at play. The expression of the chosen var gene in a given parasite is temporary and is likely mediated by the interplay of chromatin dynamics and nuclear structure (Voss et al., 2006).

Mouse main olfactory epithelium development as it relates to OSN differentiation

The MOE begins as the nasal pit, a divot in the nascent mouse face. This placode which has begun to invaginate into a bowl-shaped structure is of non-neuronal ectodermal origin. This morphogenetic process is set in motion shortly after gastrulation, around embryonic day 10 (E9), during neurulation (Treolar et al. 2010). The very first olfactory sensory neurons appear histologically about 4 days after this, but olfactory receptor expression has begun in earnest well before this stage, around E11.5, for a limited number of OR genes (Rodriguez-Gil et al., 2010). There are very few cells in the anlage, and even fewer neurons, and thus the function of OR expression at this pre-neuronal stage is unlikely to serve the purpose of detecting odorants.

The initial placode specification is achieved via apparently redundant function of Pax6, Sox2, and Oct1. The Pax6 mutant mouse lacks well-developed eyes but also lacks nasal pits. Morphogenesis proceeds as a signaling loop induced by FGF8 and BMP4, with Shh playing an antagonistic role. As mentioned before, the OSN acquires a unique developmental fate via the OR it chooses to express. The OSN emerges from a pool of transient progenitor cells that express neurogenic basic helix-loop-helix transcription factors, such as Neurogenin-1 and MASH1.

The mature neurons of the MOE are replaced as they age. Thus in addition to the transiently amplifying stem cell population described above (i.e. MASH1+ and Ngn1+), the MOE is host to a basal cell population termed the horizontal basal cell (HBC) which is the resident quiescent stem cell, able to completely repopulate the MOE with the diverse cell types to which it is host (Jang et al. 2013). Only under extreme conditions are HBCs caused to proliferate (Leung et al. 2007). They appear to be a true adult stem cell population that is in that they are

absent in the embryonic MOE. The HBC population is histologically distinct both by morphology and the expression of a number of unique cell surface proteins such as Keratin-5 and -14. These cells appear sometime around birth (Schwob, 2005).

Thus the MOE is highly organized in a basal-apical fashion such that the more potent, stem cells of the tissue are located basally while the mature OSNs are near the apical surface. This apparent organization belies the reality that the MOE is actually pseudostratified, and all cells of the organ are in active contact with both the basal and apical surface. The most apical cells, the glia-like sustentacular cells comprise the final cell type relevant to the discussion here. These cells serve a largely mysterious role in the MOE but do cytologically appear to serve a protective role for the OSNs soma, which reside immediately basal to them. The sustentacular cells are both able to be generated by the resident basal stem cells of the MOE as well as undergo clonal expansion on their own. It is a layer of sustentacular cells through which the OSNs project their ciliary dendrites to the nasal cavity, and thus it may be surmised that the sustentacular cells play an indispensible role in the ability of the OSN to receive ligands from the environment. Nonetheless, aside from suggestion that they are resident de-toxifying cells (Miyawaki et al.1996) and due to a lack of genetic evidence, their role in the overall function of the MOE remains largely a mystery. The OSN thus resides between stem cells basally and sustentacular cells apically. They are a post-mitotic cell type sandwiched between two mitotically active layers.

Olfactory sensory neuron targeting of the olfactory bulb

The OSN projects an unmyelinated, unbranched axon to the lamina propria immediately subtending the region of the MOE where it resides. Here, axonal bundles form via a process termed fasciculation to facilitate targeting of the posteriorly located olfactory bulb, the region of the forebrain dedicated as a first relay station for incoming odorant information. The coalescence of the OSN axons to form these axonal fibers is temporary, for as the fibers cross the region separating the periphery from the CNS, the axons separate to find their respective targets in this anterior region of the brain (Mombaerts et al., 1996). It is here where the second important role for olfactory receptor genes becomes apparent.

Each olfactory receptor-expressing cell will target a neuropilar structure in a which only like neurons are allowed to target, termed glomeruli (singular: glomerulus). The OSN expressing ORx, for instance, will target the region of the olfactory bulb that receives axons from OSNs expressing that OR only, while those OSNs expressing ORy will target to a different region. (This topological neuronal sorting is the subject of extensive research currently and is well outside the purview of my dissertation work, although I will return to this issue of OSN targeting in subsequent sections, but only superficially.) In the above example (also see Figure 2), ORx is a gene, not an allele, such that both maternal and paternal copies of the OR gene expressed will drive targeting to a common glomerulus. Thus OR expression permits the detection of an odorous ligand in the nasal cavity at the dendrite, as well as facilitates the relationship the OSN will have with the CNS (Mombaerts et al., 1996) for its approximately 3 month lifetime (Hinds et al. 1984). This is in stark contrast to the means by which invertebrates such as *Drosophila melanogaster* achieves olfactory sensory neuron targeting of higher neural

processing centers, as those neurons can target in the absence of a functional receptor (Dobritsa et al. 2003).

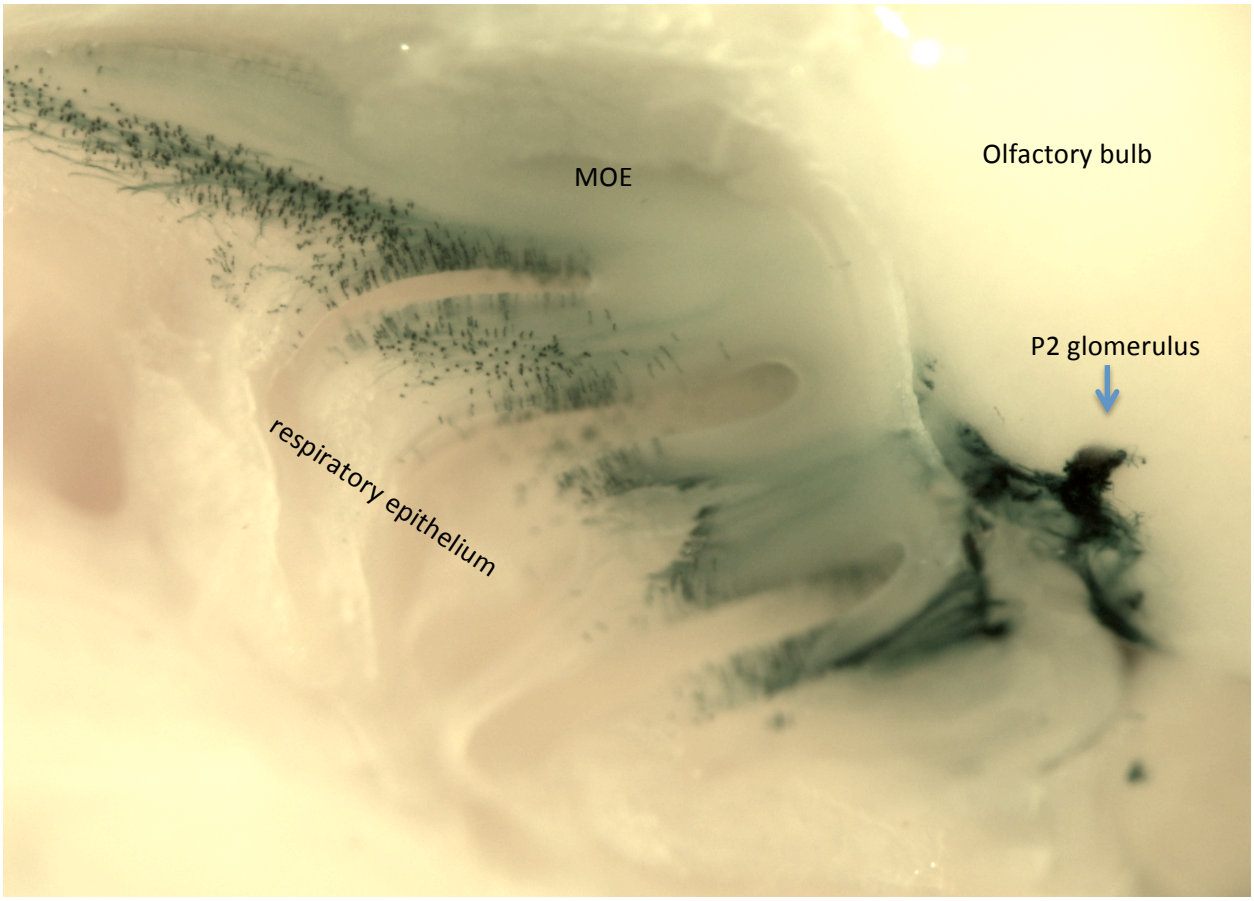

Figure 2: OSNs are instructed to target a particular glomerulus by what OR they express.

Whole mount X-gal stained MOE from P2-IRES-tau::lacZ/+ mouse at 4 weeks after birth. The very intense staining to the right of the MOE is the region of the forebrain where OSN axons innervate the glomerulus, a region populated with mitral cell dendrites corresponding to that particular OR class.

The olfactory bulb consists of a collection of inhibitory and excitatory neurons. The neurons to which OSNs directly synapse are called mitral cells, and the activity of the excitation from OSN to mitral cells is modulated by adjacent, inhibitory cells termed granule cells. Each

mitral cell receives input to its dendrite via only a single glomerulus, and there are roughly 30 mitral cells enervating a single glomerulus. Nearby tufted cells can also serve excitatory roles in the modulation of OSN signals (Mori, Nagao, & Yoshihara, 1999).

OR gene regulation thus must be tightly regulated in order to allow for the singular expression of the protein that is both conferring upon the neuron a specific ligand sensitivity as well as a unique CNS targeting capacity. What allows for a particular OR gene to be the one and only one selected in a given OSN? How could the cell facilitate the choosing of one in nearly three thousand alleles, and then how is this gene expression choice locked in place for the life of the neuron so that the region of the brain collecting information from this neuron will have "reliable" information about that which is in the periphery? It is a molecular puzzle that must be put together first in the nucleus where the genes encoding the OR proteins reside. I will briefly remark on the relevant workings of transcription and the control of gene expression as it bears on eukaryotic gene families such as the olfactory receptors.

Control of transcription at the level of chromatin

The most basic of eukaryotes to the most "complex" such as mammals contain their genomes on a proteinaceous spool, with the DNA thread winding around. This spool acts as a means by which to control and package the DNA and all the important information that it contains. DNA is an acidic molecule, carrying a negative charge at neutral pH, while the spooling proteins termed histones are basic. It is this fundamental chemical attraction that drives the energetically favorable compaction of DNA in the nucleus of the cell. The canonical histone

proteins are maintained in an octamer, with 2 of each of the following subunits: H2A, H2B, H3, and H4 (Murray 1965). Each histone is relatively small and encoded by multiple identical loci in the mouse genome. They are extremely well-conserved proteins, such that the difference in amino acid sequence for histone H3 between the pea plant *Pisum* and the cow *Bos* is less than 4 percent. The very earliest eukaryotes had these proteins and they have been heavily selected for through evolutionary time to remain the same, just as the basic make-up of DNA has remained nearly identical across disparate taxa (Reeck et al. 1978).

The DNA wraps around the histone octamer in such a way that 147 base pairs of double stranded nucleic acid are protected when the native DNA is exposed to a nuclease that cleaves the DNA backbone. X-ray crystallographic data have supported this notion and have taken it a step further to illustrate that there is a "ramp" onto which the DNA winds as it is bound to its histone spool (Luger et al. 1997). Thus the DNA and histone are in extremely close apposition.

The histone proteins, in conjunction with the DNA, constitute the fundamental unit upon which transcriptional machinery must exert its forces. These two things together, DNA and histones, are termed chromatin, and it is the chromatin environment which is what is altered by the cell to facilitate or slow the process of gene expression. There are many thousands of potential sites at which control could be exercised at a given locus when one is considering only the histone:DNA unit. The level to which eukarya have increased the sophistication of transcription is variable across clades, but all have the commonality of histone or some basic DNA-spooling proteins containing the DNA genome. Additionally all known

eukarya require a large complex of proteins to make transcription possible, and these complexes exist in 2 main categories.

The first requirement is the RNA polymerase itself, which is exclusively RNA polymerase II in mammals. This enzyme is the catalytic component of the transcription machinery and it has no fewer than 7 main subunits that must be in place in order for transcription to be possible, namely TFIIA, -B, -D, -E, -F, -G, and –H. The second category is composed of a very large complex that is in fact larger than the RNA pol II complex, termed Mediator (Kim, Björklund, Li, Sayre, & Kornberg, 1994). Mediator consists of 17 subunits at its most basic and is an absolute requirement for eukaryotic transcription.

Both the RNA pol II and Mediator complexes must interact with the chromatin environment at genes. The specific sequence elements that are responsible for recruiting the intiation complex (X. Liu, Bushnell, & Kornberg, 2013) (a subclass of the RNA pol II machinery, TFIID, along with TAF proteins such as TBP) must be able to gain access to the DNA substrate in order to facilitate transcription. Decreasing the ability of RNA pol II subunits to gain access to the chromatin around particular genes is one way to prevent the expression of a given gene (Felsenfeld, 1996; Hathaway et al., 2012). Conversely, making a gene more accessible to the soluble transcription factors that set gene expression in motion is a means by which the cell can activate transcription (Lomvardas & Thanos, 2002).

The process of opening or closing chromatin for the transcription machinery to enter and engage the DNA is a tightly controlled one. Chromatin of genes that are typically expressed in all cell types (so-called housekeeping genes) is readily accessible to enzymatic digestion by

exogenous DNAses under native conditions. Chromatin of genes that are highly regulated in time and space are generally less accessible (Trojer & Reinberg, 2007), with a spectrum of accessibility for genes falling between these opposite regulatory poles. The OR genes represent an extreme class of genes that are very highly regulated, as I will now discuss.

Genomics and epigenetic features of the olfactory receptor genes in MOE

The olfactory receptor genes are found on all but 3 somatic chromosomes, and they are also on only the X chromosome but not the Y (specifically, there are no functional or pseudogene ORs found on chromosomes 5, 12, or 18) (Zhang & Firestein, 2009). There are between 50 and 75 clusters of OR genes, depending on how one defines "cluster," but suffice it to say that the OR genes are nearly all found in gene clusters throughout the genome such that a given OR will nearly certainly have a neighboring gene that is also an OR. There are about a dozen OR genes that are "alone," surrounded by non-OR genes, but these are the exception to the rule, as >99% of OR genes are clustered. The regions in which ORs reside are riddled with repetitive elements, especially non-LTR retrotransposons, nearly all of which are transcriptional inactive in the mouse today (Kambere & Lane, 2009). This pattern has led to the suggestion that OR genes have gone through numerous rounds of expansion by inadvertent transposition facilitated by reawakened transposable elements, a mechanism which has led to a vastly different raw numbers of OR genes across relatively closely related taxa. For instance, domesticated cows (*Bos taurus*) have nearly 2000 functional OR genes, while humans have about 500 (Nei et al., 2008). The frog *Xenopus tropicalis* has about 800 functional ORs and 800

functional pseudogene ORs, and nearly all of these ORs stem from a frog-specific radiation of the class II ORs, meaning these ORs are all more closely related to each other than to other OR genes in other vertebrate taxa (Ji et al. 2009). Additionally, OR genes are found in AT-rich isochores of the genome, regions of genome which are cytologically separable with Giesma staining from typical gene-rich regions and tend to be enriched for repetitive elements.

In addition to having unique isochoric and repeat-associated genetic features, the OR genes are decorated with a heterochromatin-like epigenetic composition (Magklara et al., 2011). (The term epigenetic here refers to the non-canonical DNA information contained in chromatin.) As mentioned above, the chromatin includes the DNA along with the very tightly associated proteins, the histones. Histone proteins are comprised of helix-turn-helix domains which interact with each other at the core octamer. The amino tail of histones resides outside of the region circumscribed by the spooling of DNA and are largely unstructured. The external, amino tail of histone polypeptides, as well as the core alpha-helical regions are targets of a vast array of chromatin-modifying enzymes. As mentioned above, a main means by which transcription can be controlled is by changing the accessibility of the DNA to which histones are bound. Critically, the recruitment of certain activating or silencing factors can be induced by specific post-translational modifications (PTMs) generated on the histone itself (Kouzarides, 2007).

The histone then can be viewed as an actively involved player in the transcriptional regulatory process. Movement of the histone octamer from the region of DNA to which it is bound is energy intensive and alternative mechanisms of regulating transcription have evolved.

The direct modification of histones is catalyzed by a large number of enzymes, all of which have a similar goal: to modify or maintain the transcriptional state of a particular region of the genome. These PTMs of the histone proteins are often reversible and consist of methylation, acetylation, ubiquitination, sumoylation, and ADP-ribosylation to name a few.

At the OR genes, the histone modification pattern is unique among genes in the mammalian genome. In the OSN, the degree to which histone H3 is modified at lysine 9 (K9) by methylation is pronounced. Generally, this particular lysine residue resides in the larger amino terminal tail context of NH3-ARTKQTARKST and is often found to be trimethylated only at pericentromeric repeat elements, such as the major satellite repeats (Fodor, Shukeir, Reuter, & Jenuwein, 2010). Intriguingly, the OSN carries an OR-specific enrichment for this trimethyl mark that is roughly equivalent to that seen at these pericentromeric repeats (Magklara et al. 2011). In non-OSN tissue such as liver there is far less H3K9me3 at OR genes, while the pericentromeric enrichment remains relatively constant. The only other gene family found to have a specific enrichment for this K9me3 modification is the KRAB-ZNF family of transcription factors. However, unlike ORs, these genes are enriched for this mark throughout the organism, irrespective of cell type. H3K9me3 at the pericentromeric repeat elements suggests a transcriptionally repressive role for this mark, as often repetitive regions of the genome are host to potentially mobile genetic elements such as retrotransposons which via transcription and subsequent cDNA synthesis are “copied and pasted” across the genome. The presence of these transposable elements at the pericentromeric regions as well as within the OR gene clusters unites these parts of the genome in a sense—both these regions must be

transcriptionally silenced, in the former case to keep transposition to a minimum, and in the latter, to ensure that only one OR gene is activated in a single OSN.

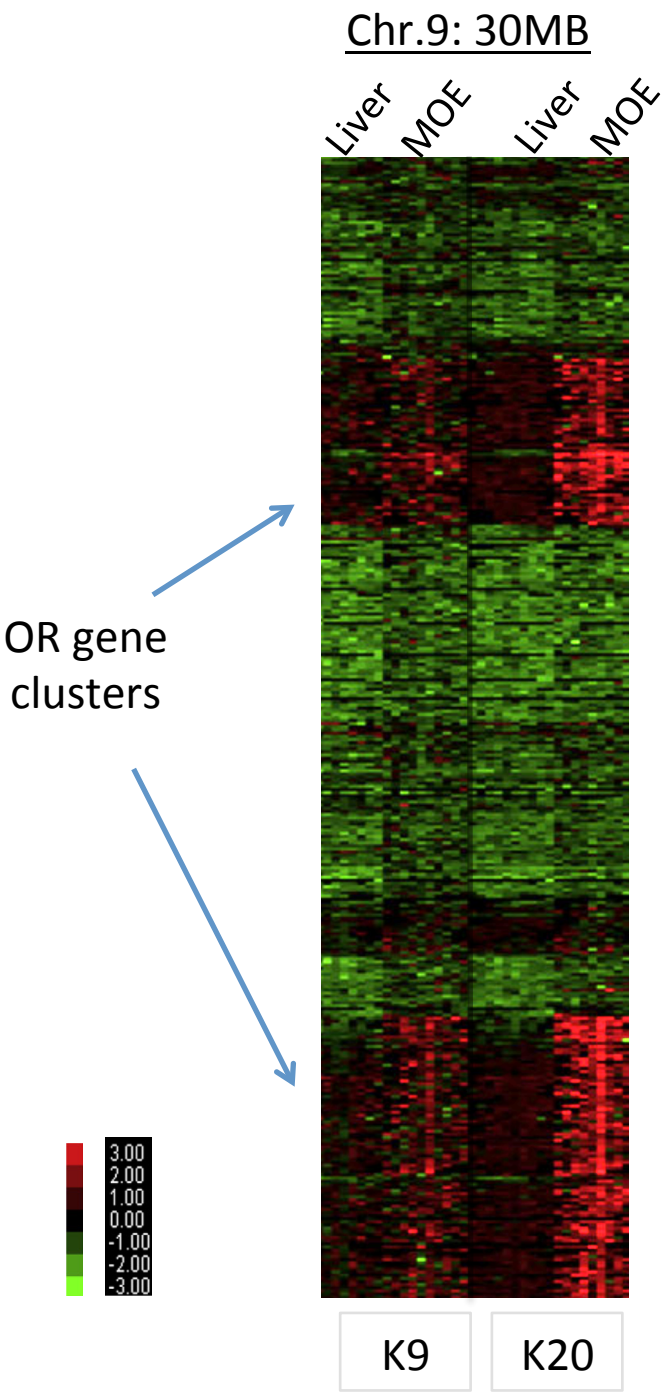

Figure 3: Enrichment for 2 important transcriptionally-silencing histone modifications specifically at the OR loci in the MOE.

H3K9 (left) and H4K20 (right). ChIP-chip experiment illustrating lack of enrichment (green) at genes immediately neighboring OR gene clusters on a representative interval of Chromosome 9 from the mouse. From Magklara et al. (2011).

Thus the OR genes are transcriptionally silenced with a PTM that causes the recruitment of repressor complexes to the host chromatin environment, and this happens in a cell-specific fashion, unlike the way in which pericentromeres are silenced. The PTM is the same in both cases however. This similarity at the level of PTM suggests that a mechanism arose convergently to facilitate both the silencing of OR genes in the OSN and the much more ancient requirement of silencing the pericentromeric repeat elements. As the number of OR genes expanded and their sequence diversity increased, the singularity became far more important. Interestingly the spread of the OR genes was likely driven in large part by the activity of transposable elements, and it is the cellular recognition of these repeat elements that allows them and flanking sequence to be transcriptionally repressed. Being the tinkerer that nature is, the means by which the OR genes spread throughout the genome also provided for a mechanism to maintain their tight epigenetic regulation.

OR gene silencing: specific mechanism and timing

The repressive chromatin environment which is unique to OR genes in the OSN arises just prior to the final cell divisions which generate the post-mitotic cell population of the MOE. That is to say, in the immediate progenitor cells of the MOE (Neurogenin-1 positive, as

discussed above), the OR genes have already gained the K9me3 that they carry for the life of the OSN. The OR gene, however, is known to become transcriptionally active only after the final cell division of its lineage. Thus a potential quandary presents itself: if the OSN must repress all ORs but the one OR it is express for its whole life, but all of the choices are already transcriptionally silenced, how can the OSN express an OR at all?

Histone H3K9 methylation recruits factors that have transcriptionally repressive qualities. One key factor that is recruited by this mark is Cbx1 (HP1-beta) which binds to K9me3 via its chromodomain. Interestingly, this protein, which is roughly the mass of a histone, contains a region to bind itself as well, termed the "chromoshadow" domain (Canzio et al., 2011). HP1 is thus able to polymerize across a transcriptionally repressed region of the genome, in a sense "locking it away" from potential activators. Cbx1 appears to be present on the OR genes prior to the final division based on immunofluorescence (IF), but the density of its signal increases following the OSN's maturation and OR expression (Clowney et al., 2011). An additional level of regulation occurs at the level of HP1 binding by a class of proteins called Lamin-binding receptor (LBR). The LBR proteins engage both HP1 and the nuclear lamina, a structural component of the inner nuclear membrane. LBR is found to be expressed transiently in the OSN lineage such that immediately upon entering into a post-mitotic state, the immature OSN downregulates LBR, thereby releasing HP1 and associated chromatin from the peripheral location at the nuclear membrane. This release correlates to the increased compaction of nuclear DNA and is important for the normal regulation of OR gene expression, such that preventing this release will cause the OR genes to be expressed from multiple loci in a single OSN and at decreased overall levels.

As mentioned above the amino acid sequence context of the amino-terminal proximal part of histone H3 contains 2 lysine residues. The first lysine, at position 4 (H3K4), is typically methylated at histones over genes being transcribed or those genes which are "destined" to be transcribed. Critically, the methylation of K4 is thought to be mutually exclusive to that of K9 (Shi et al., 2011), such that proteins which are known activators of transcription, which will typically bind to the K4me3 residue and acetylate K9 cannot bind K4 when K9 is in its trimethyl state. Thus the "histone code" at these two residues suggests that genes in post-mitotic cell that are marked with meK9 are stuck in transcriptionally silent mode (Wang et al., 2001) unless one of 2 things happens: either the histone bearing the repressive mark is somehow removed from the nucleosome particle and replaced with an unmodified histone, or the histone tail is covalently modified in a way that returns it to its "ground state" or its unmodified form. Due to the ATP requirement for histone displacement, it may seem reasonable to suspect that the covalent modification of histone tails via a demethylase is the mechanism by which cells typically return histone tails that are K9me back to K9 unmethylated. The return to "ground state" for the histone tail would allow for activating K4 methyl to be added, and in turn, K9 could be acetylated, further accelerating the transcriptional activation program and preventing the silencing activity of chromodomain-containing proteins such as Cbx1.

Based on lineage tracing experiments conducted with an OR-Cre knock-in mouse in which endogenous Olfr1507 was replaced with Olfr1507-IRES-Cre, it is believed that OR expression follows the last cell division (Shykind et al., 2004). The Cre-mediated recombination of a reporter transgene is seen largely in separate OSNs that do not appear to have emerged from a single cell and expanded clonally. Furthermore, careful in situ hybridization (ISH) with

markers for post-mitotic neurons (such as GAP43) have revealed that the OR expression almost always comes after the onset of such markers (Iwema & Schwob, 2003)(a feature which can be assessed due to the pseudostratified nature of the MOE, as mentioned previously). Unpublished experiments I have carried out lend credence to this notion: Cre+ neurons from this knock-in allele are never found to be positive for DNA synthesis or mitosis-associated antibodies by IF.

Given this timing of OR onset, the repressive K9me should be removed from the activated OR allele and be replaced with K4me. This is indeed what was found to be the case at the Olfr17 locus ("P2"), which similar to the Olfr1507-IRES-Cre, was genetically tagged with a knock-in strategy to allow the creation of a wild-type OR gene product but with a GFP reporter rather than a Cre. GFP+ neurons were sorted from heterozygote P2-GFP/+ MOE and K4me3 ChIP showed that only the GFP-tagged allele was enriched for this activating histone PTM. Meanwhile, the wild-type allele was positive only for the repressive K9me3 (Magklara et al. 2011). Thus it was concluded that the OR allele that is activated undergoes an "epigenetic switch" from repressed to activated, allowing for the high levels of OR transcription to take place in a stochastic fashion on the allele which is activated and thereby de-repressed.

This model provided a framework to address the long-standing questions: how does the OSN choose only one OR allele? And how is the first OR transcribed typically the only OR allele activated in that particular neuron to provide for the one-OR, one neuron rule? We address these questions in the work below, which was previously published in July 2013.

Chapter 3: A role for histone demethylase LSD1 in OR gene regulation

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 co-activator, LSD1 is necessary for de-silencing and initiating OR transcription, but as a transcriptional co-repressor, 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 ~1400 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 ~1000 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 LSD1 activity is needed only during the initial de-repression 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 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-seq analysis (Magklara et al., 2011) from mature OSNs (mOSNs, Olfactory Marker Protein (OMP)-

positive) and progenitor/immature neuronal populations (Neurogenin1 (Ngn1)-positive) shows that LSD1 is expressed in the Neurogenin-1 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 Neurogenin-1 positive cells and their immediate progeny, and significant reduction of LSD1 in more apical, mOSN population of the adult MOE (Figure 1B). Two-color RNA 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), support 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) which we deleted at three distinct developmental time points: prior, during, and after OR gene activation, using Foxg1-Cre (Hebert and McConnell, 2000), MOR28-IRES-Cre (Shykind et al., 2004) and OMP-IRES-Cre (Eggan 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 supplemental Figure S1A, B). This analysis was performed in E18.5 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 (Figure 1F-H and S1C). Similarly, RNA ISH and IF as above show that LSD1 deletion in mOSNs has no detectable effects on OR expression (Figure 1G and S1D). These data suggest

that LSD1 activity is necessary for OR de-silencing 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 using cDNA libraries prepared from the MOE 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 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 (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 post-mitotic 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 RNAseq and ISH experiments (Figure 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 (Figure 2F,G). The loss of the mOSN layer in the LSD1 KO likely explains the thinner expression pattern of GAP43 and NCAM1 and the ~1-fold reduction observed by

RNAseq (Figure 2C and S2), since at this developmental stage there is some overlap of these immature markers with mOSN markers.

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 ORNs 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: G**γ**8-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 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 MOE of these mice and the X-gal stained cells have dendrites that reach the lumen of the olfactory epithelium (Figure 3B and supplemental Figure 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.

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 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 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. Since gene switching requires the repression of the previously chosen OR and the de-silencing 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 towards the mOSN layers at PND21 (Post-natal day 21) (Figure 4A and S4A). Similarly, GAP43 expression is also apically expanded, showing that Adcy3 deletion delays the terminal differentiation of these neurons (Figure 4B, S4B). RNA ISH for OMP shows that the mOSN layer is reduced and restricted only to the most apical OSN layer, 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 (Figure 4D, 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 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, since at PND21 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 wild type and Adcy3 KO MOEs at PND21 shows that overall OR mRNA levels are moderately reduced in a statistical 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 RNAseq 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 supplemental Figure S4D, respectively), supporting the notion that $LSD1^{+}$ OSNs continue to search ORs, even after the choice of an intact OR. Since 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 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 hours (Hayashi and McMahon, 2002, Nakamura et al., 2006). We performed this analysis at PND2 because the Adcy3 KO mice fail to thrive and mice with all four alleles did not survive with any reliability beyond this age based on our observations. 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 (Figure 4G and S4E, F). Lineage tracing, however, shows that Adcy3 KO mice have a ~2 fold increase (Student's T-test; P=0.007) of single positive (Cre^+ or GFP^+) over the number of double positive neurons (Figure 4H,I), 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 (Figure 5A,B). 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 supplemental 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 (Figure 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 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 technically impossible to detect the binding of LSD1 on an active OR by ChIP. This technical hurdle would be bypassed if we could detect a molecular "apparition" for the presence of LSD1 at the active OR allele. Such a mark could be generated by hydrogen peroxide, which, in addition to formaldehyde, is a localized chemical byproduct of LSD1-mediated lysine de-methylation (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 a ~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-qPCR analysis with DNA prepared from E18.5 MOEs from wild type, 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 very low fraction of cells.

To test whether *8*-oxodG enrichment stems from transcriptionally active OR alleles, we FAC-sorted GFP^{+} neurons expressing olfactory receptor P2 from P2-IRES-GFP knock-in mice. Using DIP-qPCR analysis we quantified the relative enrichment of *8*-oxodG on the active and inactive OR alleles. We detect a ~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). Since 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 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 knock-in 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 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 wild type 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 4 quartiles of transcription levels and we plotted *8*-oxodG levels for each quartile. The mean *8*-oxodG RPKMs are essentially identical between the 4 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).

Finally, we measured the enrichment levels of *8*-oxodG in Adcy3 KO and OMPitTA; tetO-LSD1 MOE, which have abnormally high levels of LSD1 protein. We find significant increases of *8*-oxodG levels in both the Adcy3 KO and the LSD1 overexpressing mice (Figure 6F,G). Notably, in wild type adults, the baseline *8*-oxodG levels are lower than in embryos, which is probably explained by the significantly higher proportion of LSD1-expressing cells in embryonic than 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 becomes ectopically demethylated. A 10-20 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 wild type 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 wild type 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 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 stabilizes 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 makes the subsequent downregulation of LSD1 imperative for the stabilization of OR choice.

These observations pose a significant question: why LSD1 activates OR transcription before an OR is chosen but represses 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 possible that the downregulation of the chosen OR is not a direct consequence of H3K4 demethylation by LSD1, but indirect effect of the fact that an additional OR has been activated. Finally, as 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 of 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 vs feed-forward 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, de-silence 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 de-silenced 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 feed-forward 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 feed-forward

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 co-activator and co-repressor activites, as an initiator of this cascade, provides the additional advantage of auto-correction, 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 previous studies failed to implicate OR activity in the feedback signal. Deletion of various components of OR signaling, such as G_{olf}**(α)** 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**(α)** 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**(α)**-independent mechanisms by which an OR activates Adcy3 signaling, or that LSD1 dowregulation 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, since 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. Since 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, since they are post-mitotic 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 inter-species polymorphisms observed in this gene family, since *8*-oxodG frequently pairs with adenosine rather than cytosine during DNA replication (Grollman and Moriya, 1993). Thus, LSD1 mediated derepression in the germ line could explain both the drift towards 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 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 seldomly used, affording individualized adaptation to an extremely plastic system (Santoro and Dulac, 2012).

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Figures and Figure Legends

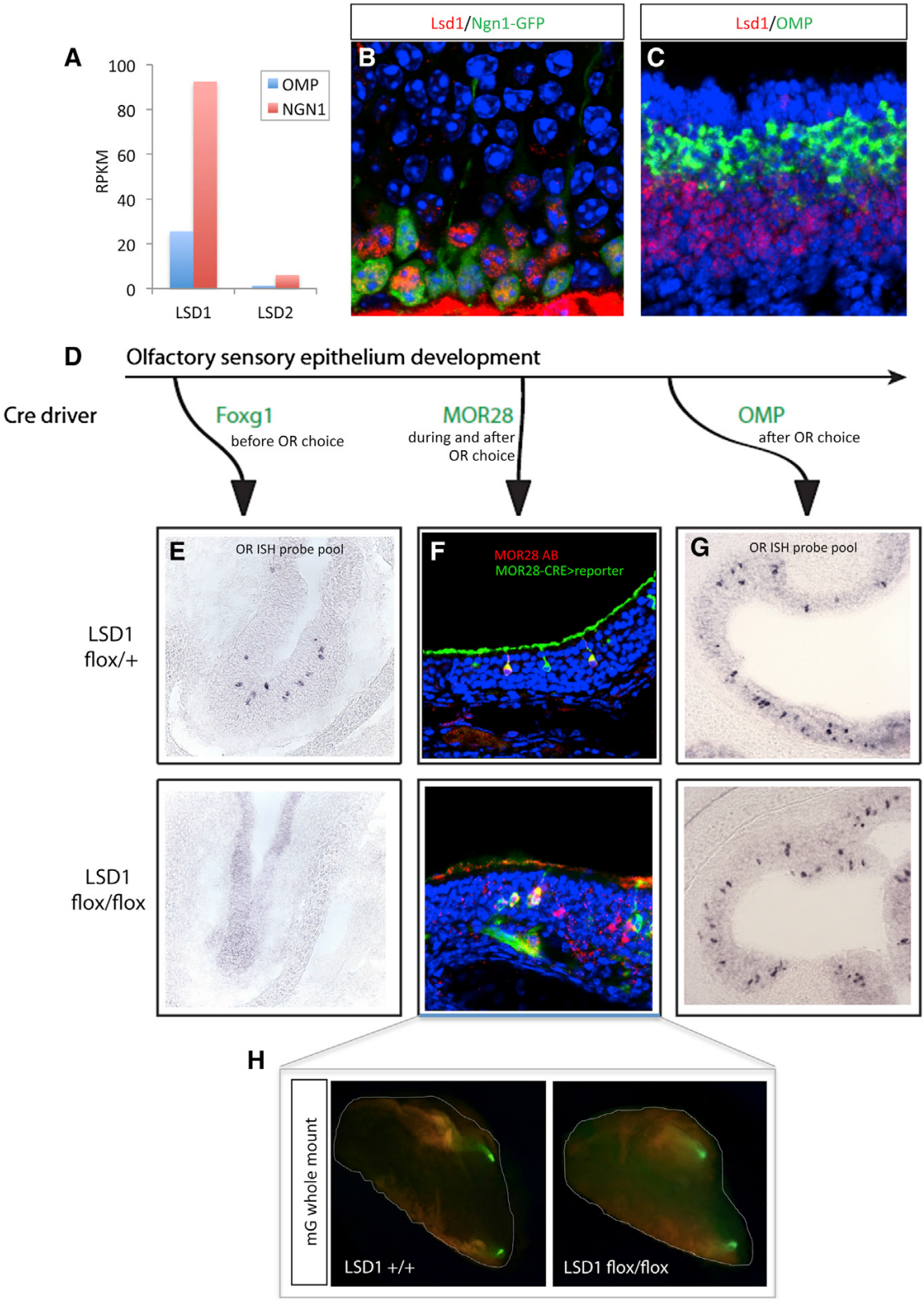

Figure 1*: Transient LSD1 expression is required for OR expression*

(A) mRNA-seq reads per million mapped per thousand basepairs 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 PND30.

(C) LSD1 and OMP 2-color RNA in situ hybridization (ISH) at PND5. DAPI nuclear stain is shown in blue.

(D) Removal of LSD1 over developmental time with 3 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 MOE at PND1.

(H) Olfactory bulbs of MOR28-IRES-Cre; LSD1+/+ and MOR28-IRES-Cre; LSD1flox/flox animals at PND30 with a 2-color membrane-bound Cre-reporter: mT before Cre; mG after Cre (mT/mG; Muzumdar et al. 2007). See also Figure S1.

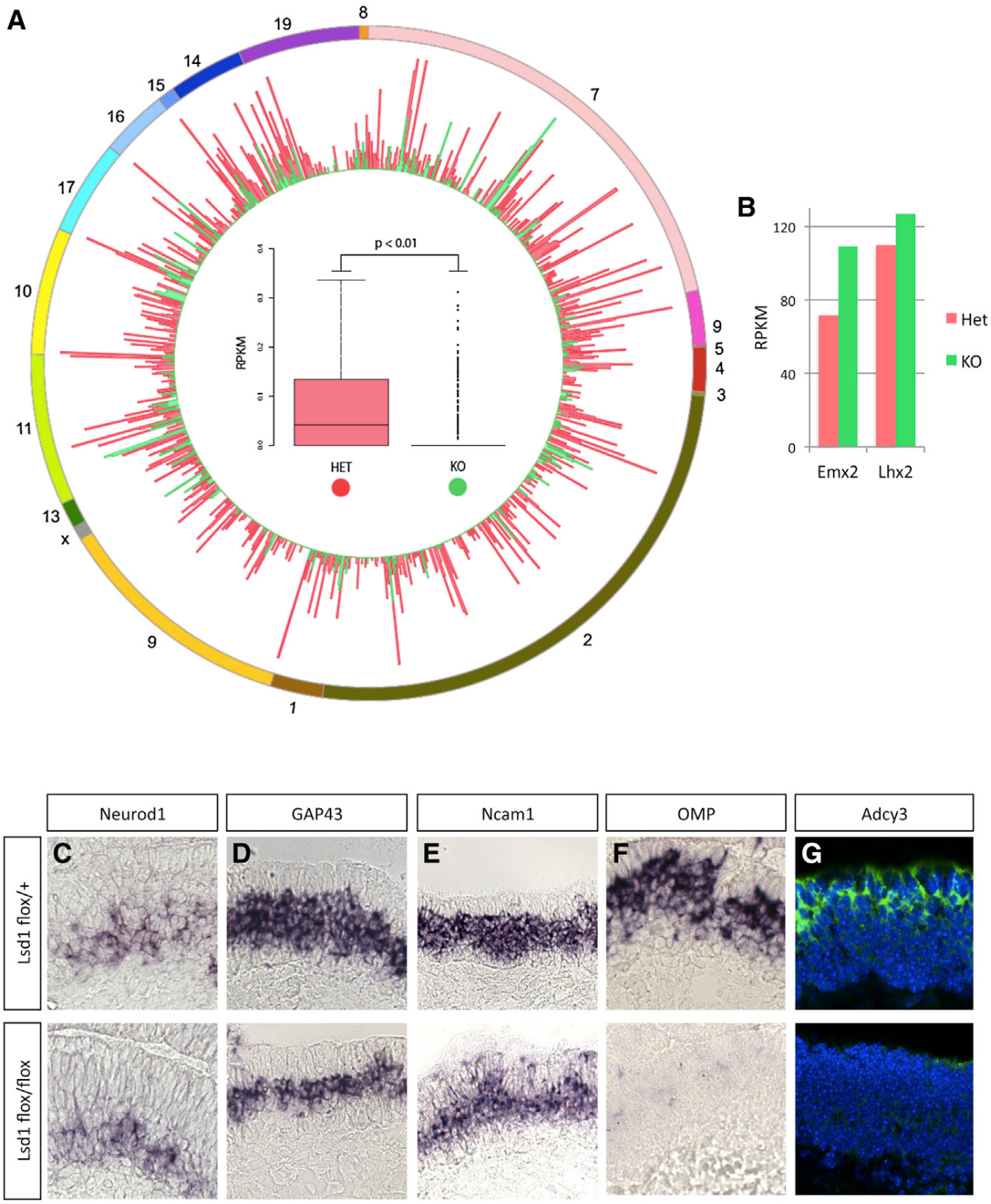
A
8
19
14
15
16
7
17
10
9
5
4
3
11
13
x
9
2
1
p < 0.01
RPKM
HET
KO
B
120
80
40
0
RPKM
Het
KO
Emx2
Lhx2
Neurod1
GAP43
Ncam1
OMP
Adcy3
Lsd1 flox/+
Lsd1 flox/flox
C
D
E
F
G

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 value for that OR in MOE of that genotype (red: Foxg1-Cre; LSD1flox/+; green: Foxg1-Cre; Lsd1flox/flox). External doughnut represents relative chromosomal location of each OR gene. Summary boxplot is shown within Circos plot; student's paired t-test used for significance testing.

(B) RPKM values of the 2 known transcriptional activators of OR genes in the LSD1 heterozygote and knockout.

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

(G) IF for Adcy3 at same embryonic stage, DAPI nuclear stain is shown in blue. See also Figure S2.

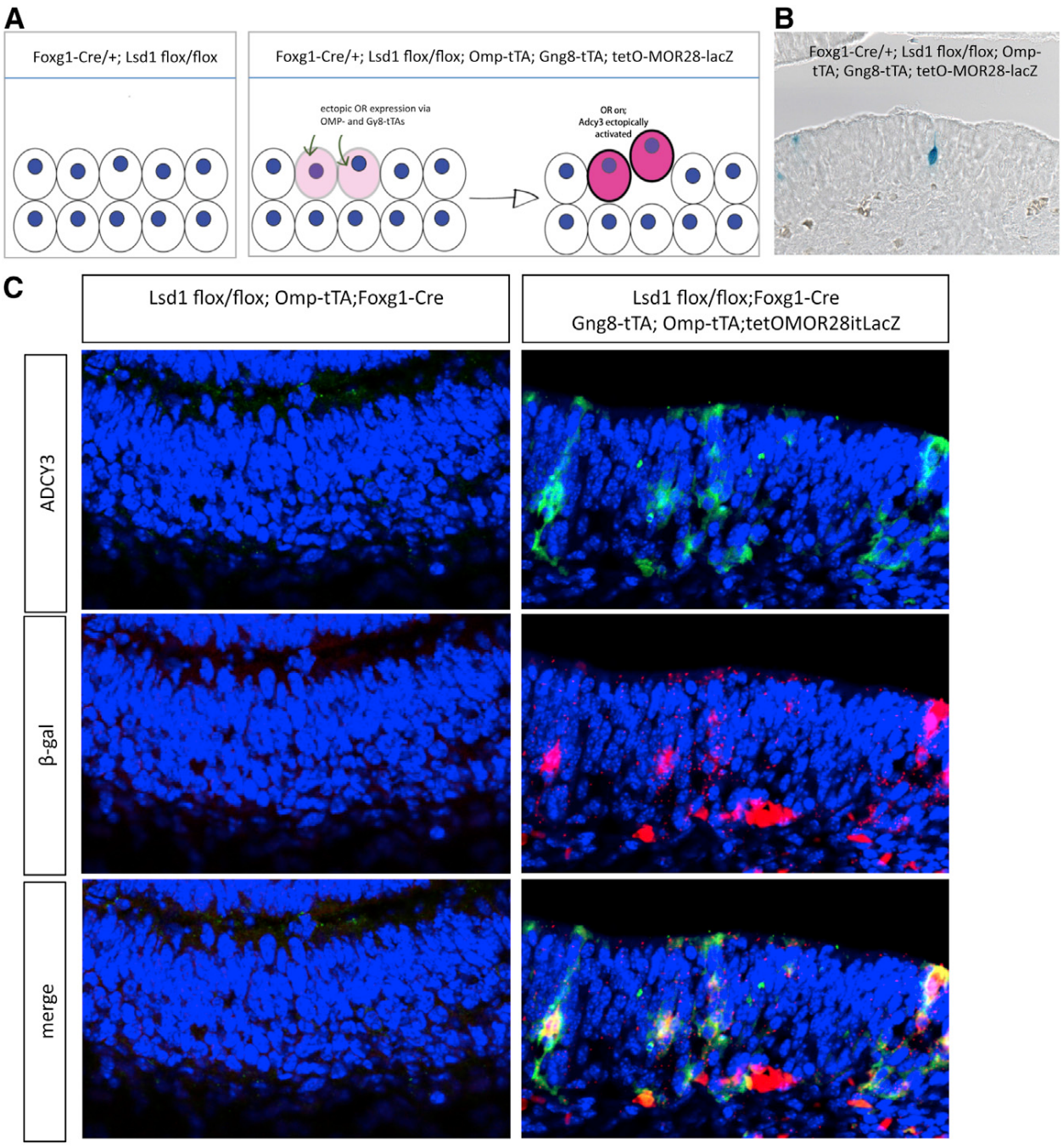
A
Foxg1-Cre/+; Lsd1 flox/flox
Foxg1-Cre/+; Lsd1 flox/flox; Omp-tTA; Gng8-tTA; tetO-MOR28-lacZ
ectopic OR expression via OMP- and Gy8-tTAs
OR on: Adcy3 ectopically activated
B
Foxg1-Cre/+; Lsd1 flox/flox; Omp-tTA; Gng8-tTA; tetO-MOR28-lacZ
C
Lsd1 flox/flox; Omp-tTA;Foxg1-Cre
Lsd1 flox/flox;Foxg1-Cre
Gng8-tTA; Omp-tTA;tetOMOR28itLacZ
ADCY3
β-gal
merge
D
Adcy3+ cells per area
0.005
0.004
0.003
0.002
0.001
0
LSD1+/+
LSD1+/-
LSD1-/-

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 misexpression study. Using two tTA drivers, one active in the immature neuron (G**γ**8-tTa), and one active in the mature neuron (OMPitTA), it is possible to express high levels 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) Xgal 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/+; OMPitTA (left panels) or Lsd1 flox/flox; G**γ**8-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 MOE were quantified per unit area in ImageJ. Y axis units are Adcy3+ cells per micron of MOE area considered. Error bars show standard error of 2 quantified regions of MOE from one experiment.

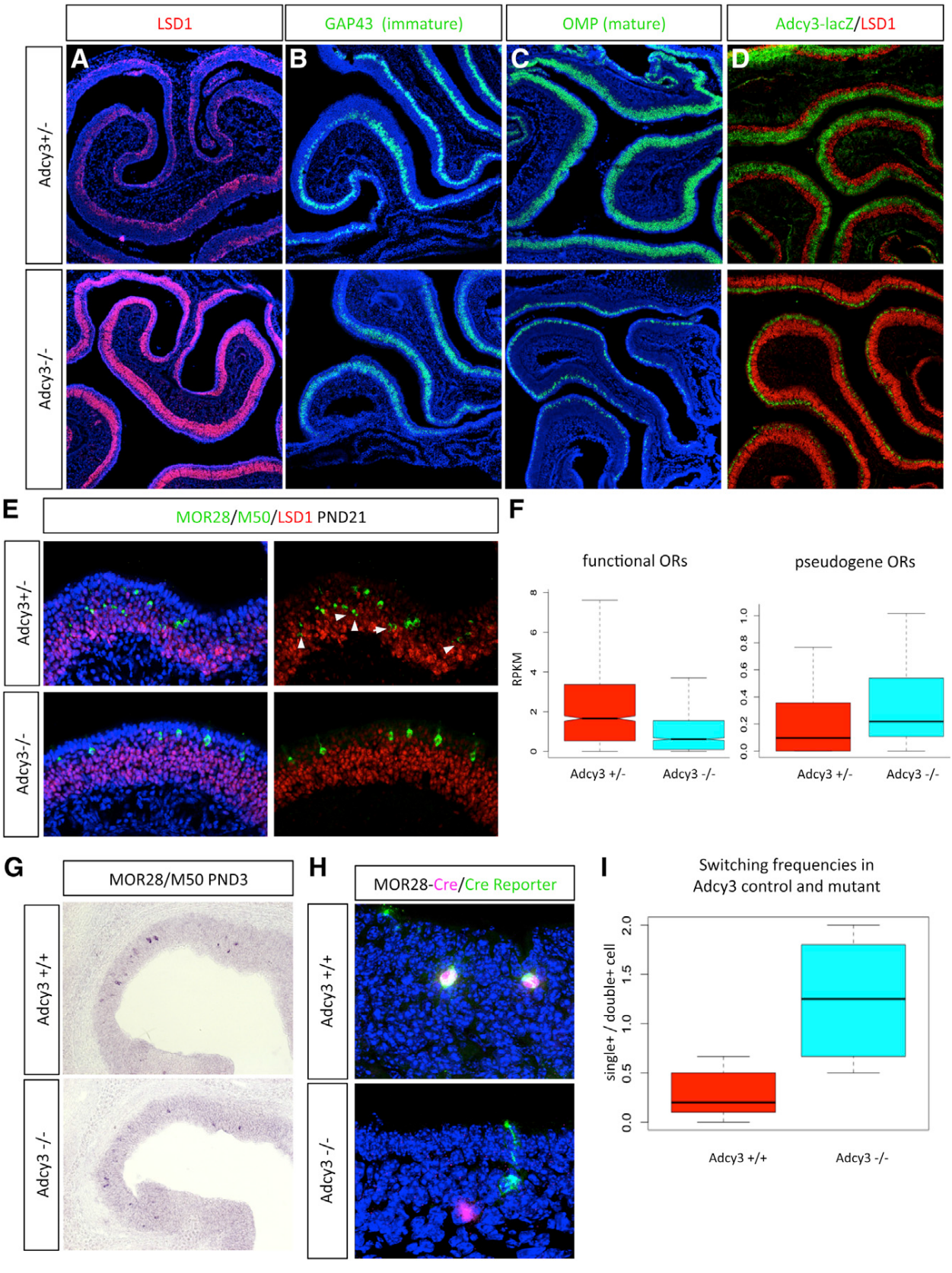
LSD1
GAP43 (immature)
OMP (mature)
Adcy3-lacZ/LSD1
A
B
C
D
Adcy3+/-
Adcy3-/-
E
MOR28/M50/LSD1 PND21
Adcy3+/-
Adcy3-/-
F
functional ORs
pseudogene ORs
RPKM
Adcy3 +/-
Adcy3 -/-
Adcy3 +/-
Adcy3 -/-
G
MOR28/M50 PND3
Adcy3 +/+
Adcy3 -/-
H
MOR28-Cre/Cre Reporter
Adcy3 +/+
Adcy3 -/-
I
Switching frequencies in
Adcy3 control and mutant
single+ / double+ cell
Adcy3 +/+
Adcy3 -/-

Figure 4: Adcy3 removal triggers upregulation of LSD1 protein levels and increase in OR gene switching.

(A) PND21 sections with IF for LSD1 in Adcy3+/- (top) and -/- (bottom), respectively. See Figure S4 for quantification. (B,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 Adcy3 locus. See supplemental experimental procedures for details.

(E) PND21 sections from Adcy3+/- (top) and -/- (bottom), stained with OR (green, MOR28 and M50) and LSD1 (red) antibodies.

(F) RNA-seq RPKM values for all expressed Refseq ORs (n=1072) and OR pseudogenes (n=48) in corresponding genotype from PND21 MOE. RPKMs of unexpressed intact and pseudogene ORs were excluded. See also Fig S4. (G) PND3 RNA ISH for M50 and MOR28 as in (E). IF for ORs and LSD1 shown in supplemental Figure 4 (H) PND2 MOE from MOR28-IRES-Cre; Cre-reporter mice in Adcy3 wildtype or knockout background. Cre IF (magenta) and mT/mG reporter (green).

(I) Quantification of experiment in (H). Single (CRE or GFP positive) and double positive cells from 10 sections of PND2 wild type and Adcy3 KO mice were counted and plotted as ratio of

single to double positive. SE represents variation across sections, single animal, 10 sections quantified. P value=0.007, calculated with Student's unpaired T-test.

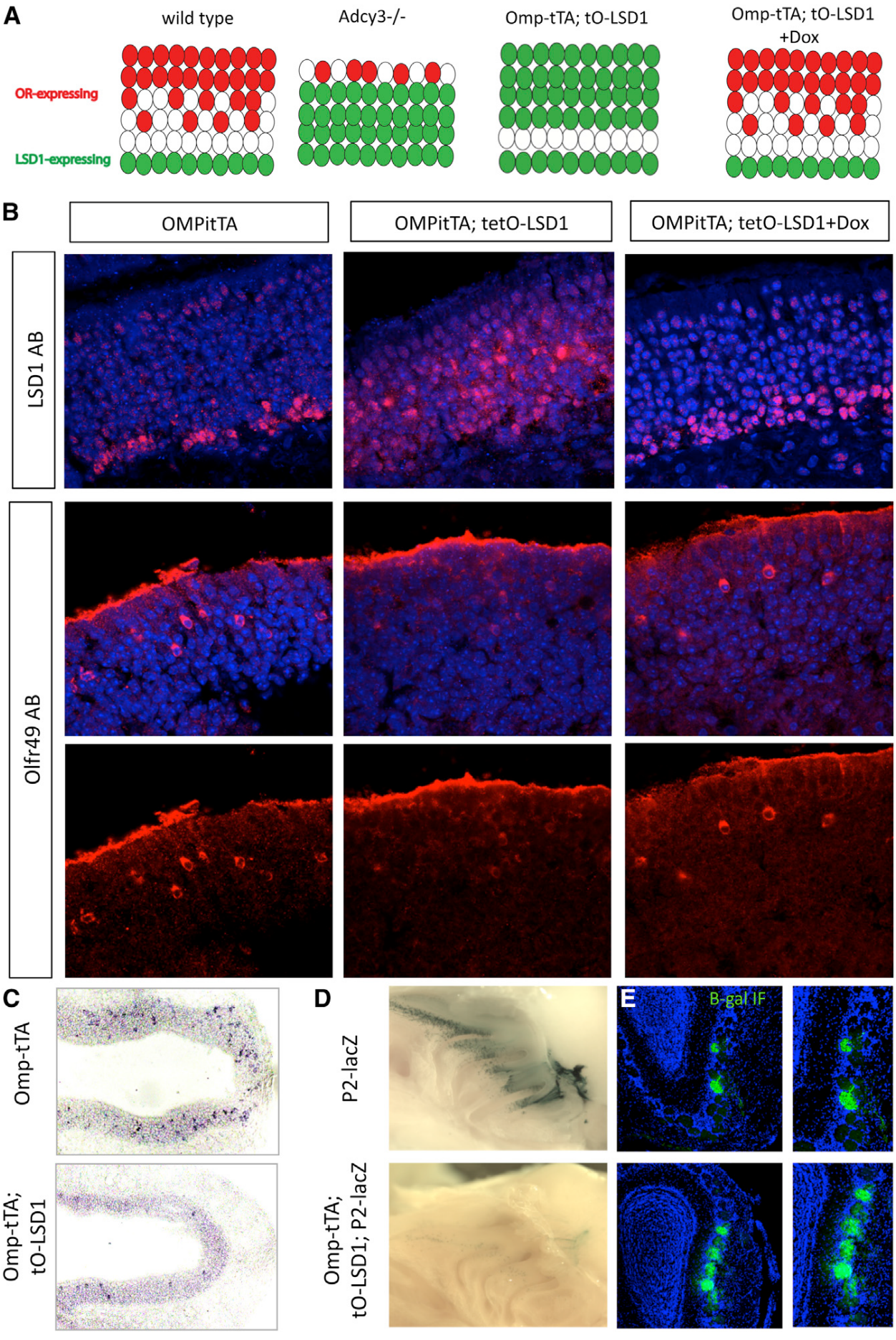
A
wild type
Adcy3-/-
Omp-tTA; tO-LSD1
Omp-tTA; tO-LSD1 +Dox
OR-expressing
LSD1-expressing
B
OMPitTA
OMPitTA; tetO-LSD1
OMPitTA; tetO-LSD1+Dox
LSD1 AB
Olfr49 AB
C
Omp-tTA
Omp-tTA; tO-LSD1
D
P2-lacZ
Omp-tTA; tO-LSD1; P2-lacZ
E
B-gal IF

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. 6 week old MOE were harvested and IF was performed for LSD1 (red top panel) or Olfr49 (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 PND25 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, 2013), which allows the visualization of additional glomeruli.

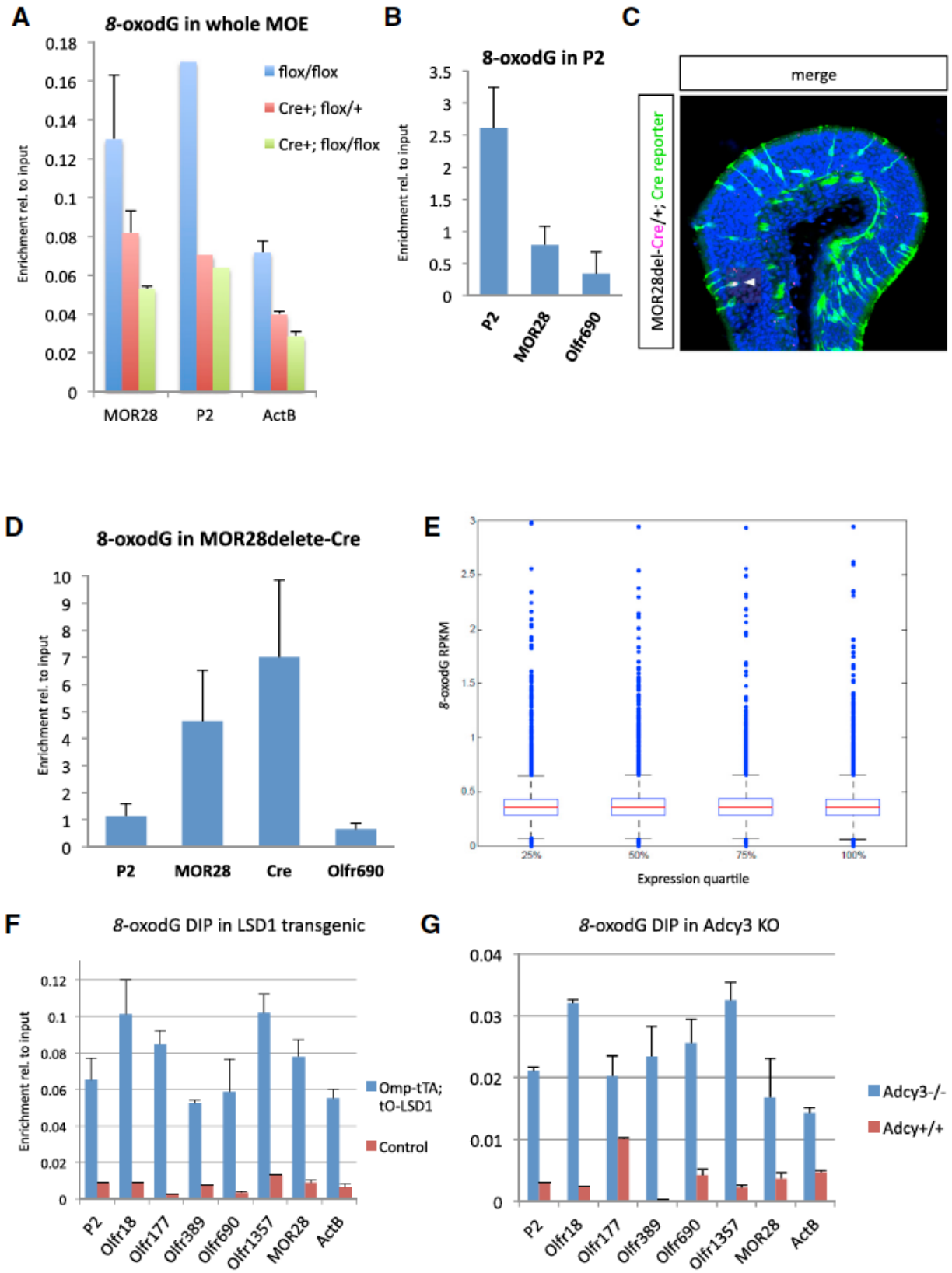
A
8-oxodG in whole MOE
Enrichment rel. to input
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
flox/flox
Cre+; flox/+
Cre+; flox/flox
MOR28
P2
ActB
B
8-oxodG in P2
Enrichment rel. to input
3.5
3
2.5
2
1.5
1
0.5
0
P2
MOR28
Olfr690
C
merge
MOR28del-Cre/+; Cre reporter
D
8-oxodG in MOR28delete-Cre
Enrichment rel. to input
10
9
8
7
6
5
4
3
2
1
0
P2
MOR28
Cre
Olfr690
E
8-oxodG RPKM
3
2.5
2
1.5
1
0.5
0
25%
50%
75%
100%
Expression quartile
F
8-oxodG DIP in LSD1 transgenic
Enrichment rel. to input
0.12
0.1
0.08
0.06
0.04
0.02
0
Omp-tTA; tO-LSD1
Control
P2
Olfr18
Olfr177
Olfr389
Olfr690
Olfr1357
MOR28
ActB
G
8-oxodG DIP in Adcy3 KO
0.04
0.03
0.02
0.01
0
Adcy3-/-
Adcy+/+
P2
Olfr18
Olfr177
Olfr389
Olfr690
Olfr1357
MOR28
ActB

Figure 6*: LSD1 generates stable 8-oxodG at active OR genes.*

(A) 8-oxodG DIP was performed on sonicated genomic DNA (gDNA) from LSD1 wildtype, heterozygote, and knockout MOE at E18.5.

(B) 8oxodG-DIP-qPCRs from gDNA of P2-GFP sorted cells from PND30 mice.

(C) PND30 MOE of MOR28-del-Cre; Cre-reporter mouse, with Cre IF (magenta) 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) 8oxodG-DIP-qPCRs from Cre-reporter-positive neuron gDNA at PND30, as shown in (C).

(E) DIP-seq analysis of an E18.5 wild-type 8-oxodG library. Expression quartiles from the RPKM values generated from Fig. 1 mRNA-seq. y-axis is 8-oxodG RPKM. Boxplots show mean 8-oxodG RPKM for each expression quartile demarcated by horizontal red bar.

(F, G) 8oxodG-DIP-qPCRs from gDNA from whole MOE of LSD1 overexpressing mice and Adcy3 knockout mice, respectively. Error bars are standard error from 2 PCR replicates from one representative experiment. See also Figure S6 for control experiments.

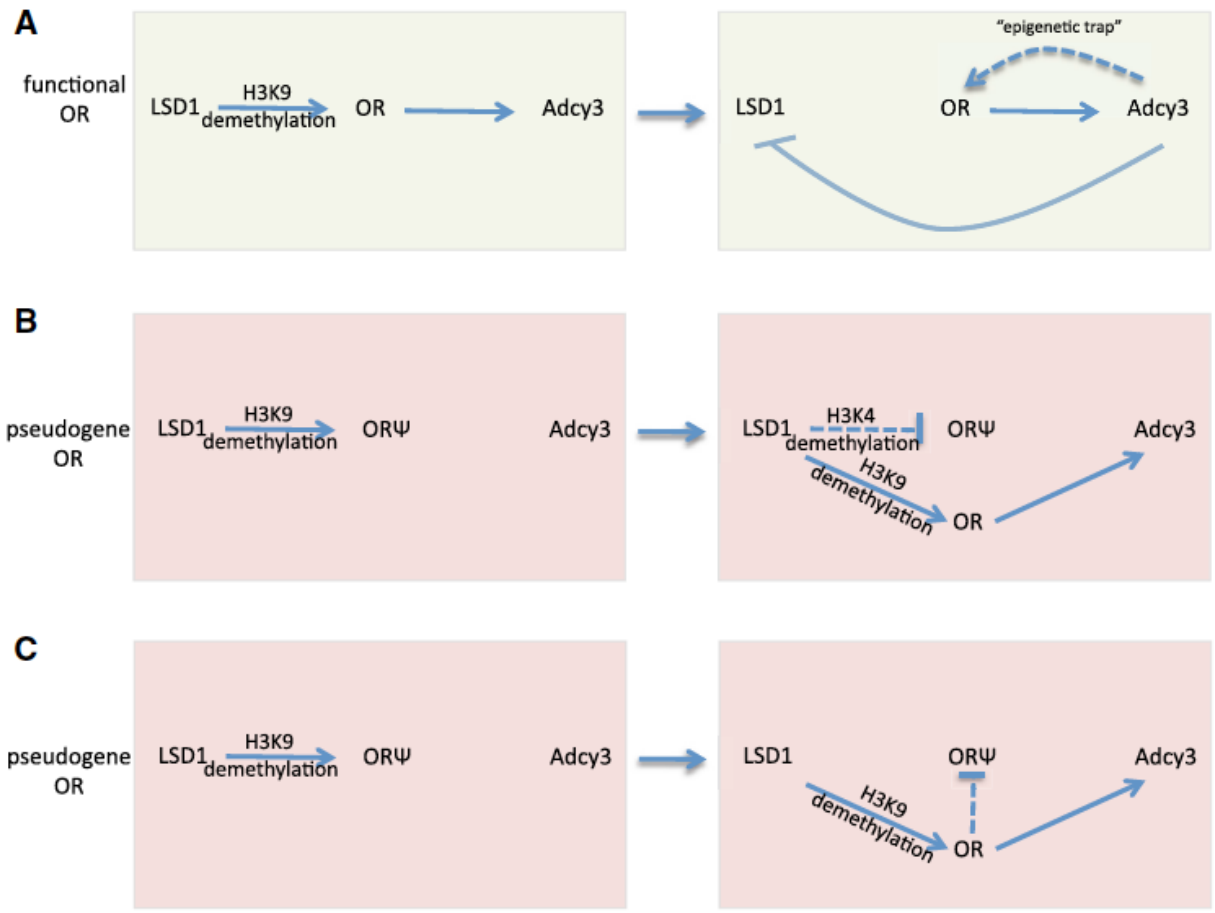

***Figure 7**: A three node signaling cascade combined with a feedback signal that generates epigenetic memory.*

(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 silence directly the previously chosen OR, but causes its repression by activating an additional OR allele.

Experimental Procedures

Mice and strains used

All mice were housed in standard conditions with a 12-hour 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 Supplemental 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 Supplemental Experimental Procedures .

Confocal images were collected with the Zeiss LSM 700 and brightfield images were collected on the Zeiss Axioskop 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 minutes 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 NaAOc 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 IIx or HiSeq2000 (Illumina). Sequencing libraries were prepared with standard methods (Magklara et al. 2011) but in the case of the mRNA, the ScriptSeq kit (Epicentre) was used. Detailed information can be found in Supplemental Experimental Procedures.

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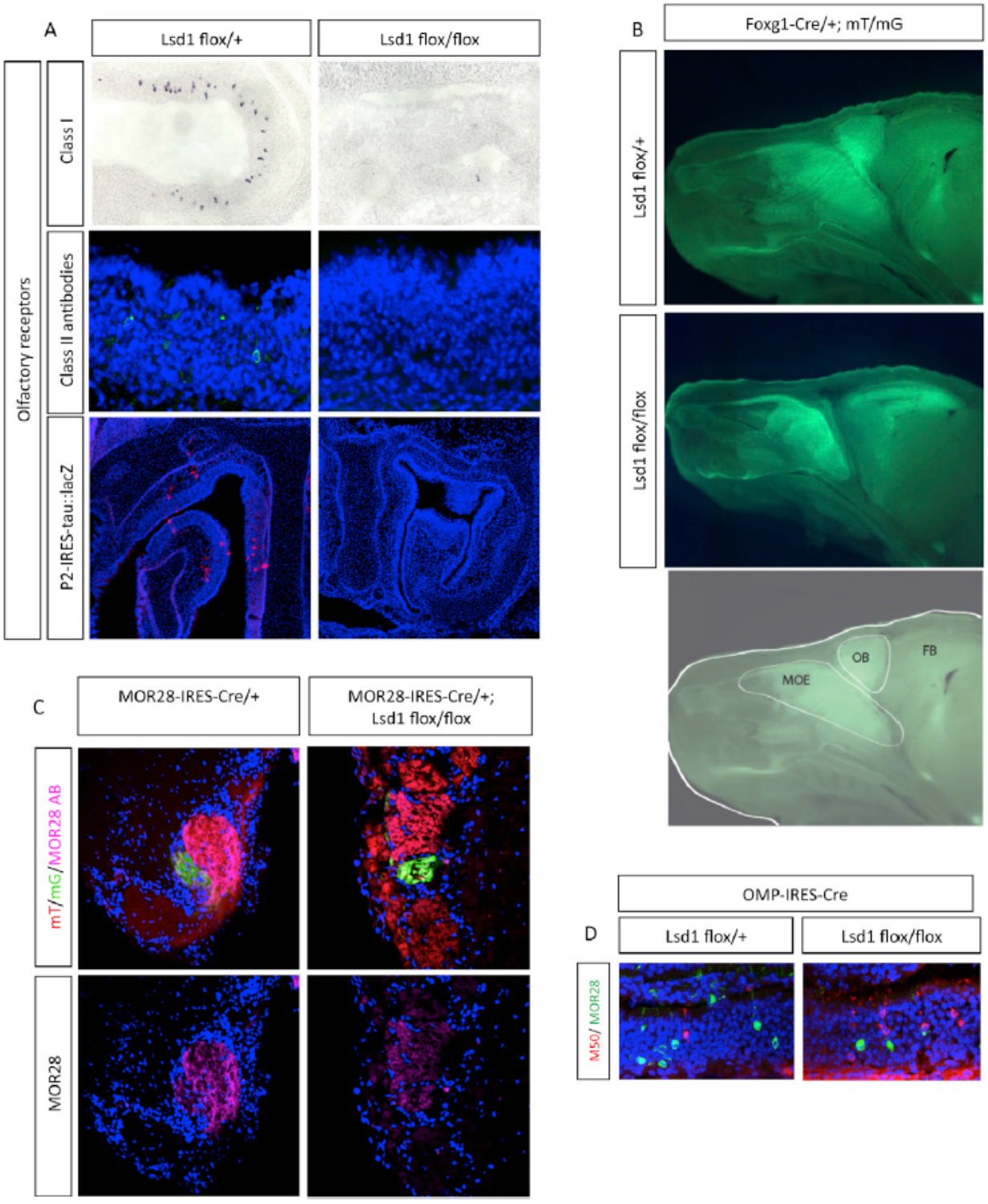
Supplemental Figures and Figure Legends



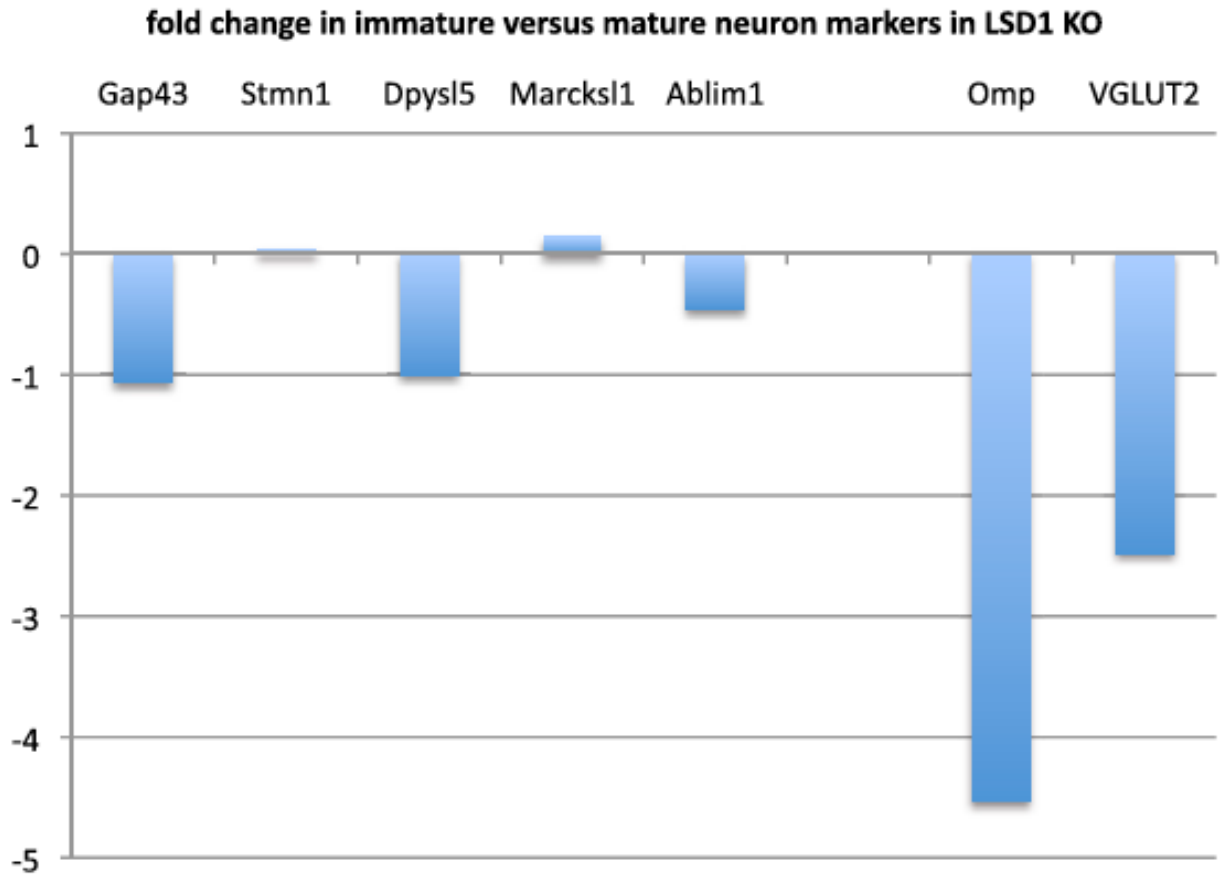
Supplemental Figure 1 *(relevant to Figure 1): Transient LSD1 expression is required for OR expression.*

(A) OR ISH probe pool for 7 class I OR genes (top), IF with a pool of antibodies for ORs MOR28, M50, M71 and C6 (middle) and IF for bgal for the detection of P2-IRES-tauLacZ expression (bottom) in Foxg1-cre; LSD1flox/+ (left) and Foxg1;cre;flox/flox (right).

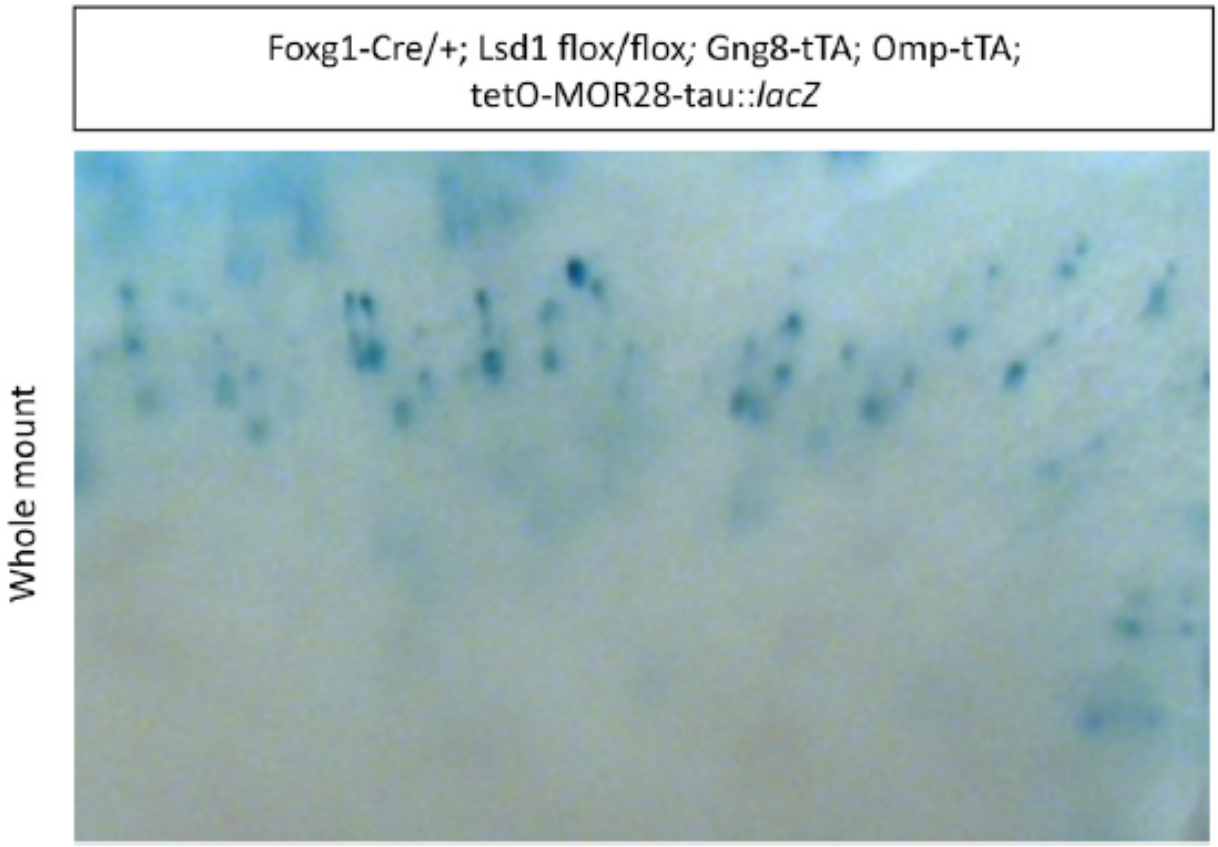
(B) Whole mount GFP visualization of OSN projections in Foxg1-cre; LSD1flox/+ (top) and Foxg1;cre;flox/flox (middle) mice with a CRE inducible GFP reporter. The bottom panel highlights the position of the main olfactory epithelium (MOE) and olfactory bulb (OB).

(C) Sections of the olfactory bulbs corresponding to the whole mount images shown in Figure 1H. MOR28 is detected by IF (magenta) and is present in the GFP positive glomeruli (green) regardless of whether the projecting OSNs have a functional copy of LSD1 (left) or not (right).

(D) IF for M50 (red) and MOR28 (green) in OMP-IRES-Cre (left) or OMP-IRES-Cre;LSD1flox/flox mice.

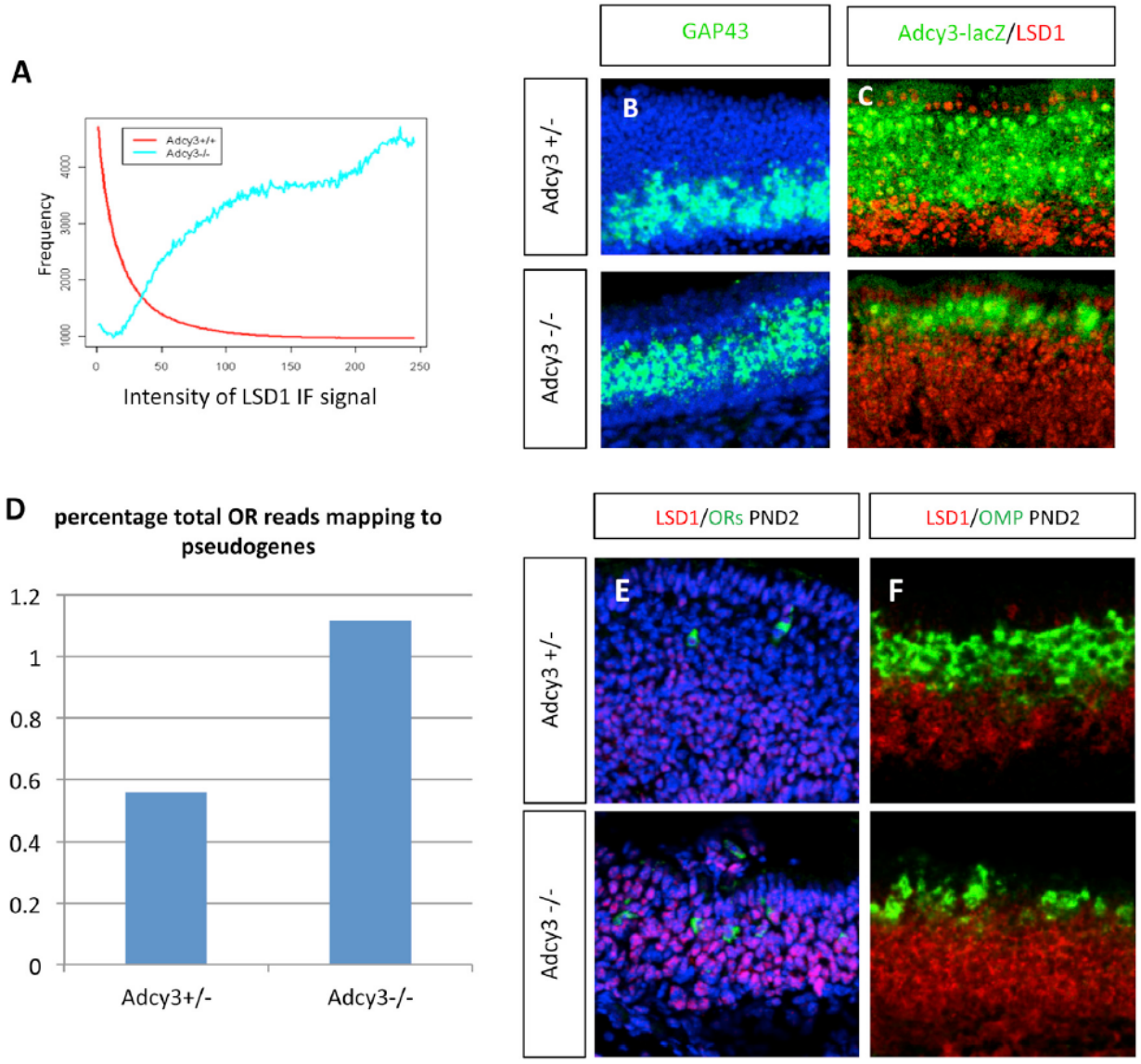


Supplemental Figure 2 *(relevant to Figure 2): RNA-seq log2-fold changes are shown for immature (left) and mature (right) neuron marker genes.*



Supplemental Figure 3 (relevant to Figure 3): Ectopic MOR28 expression in LSD1 KO MOEs.

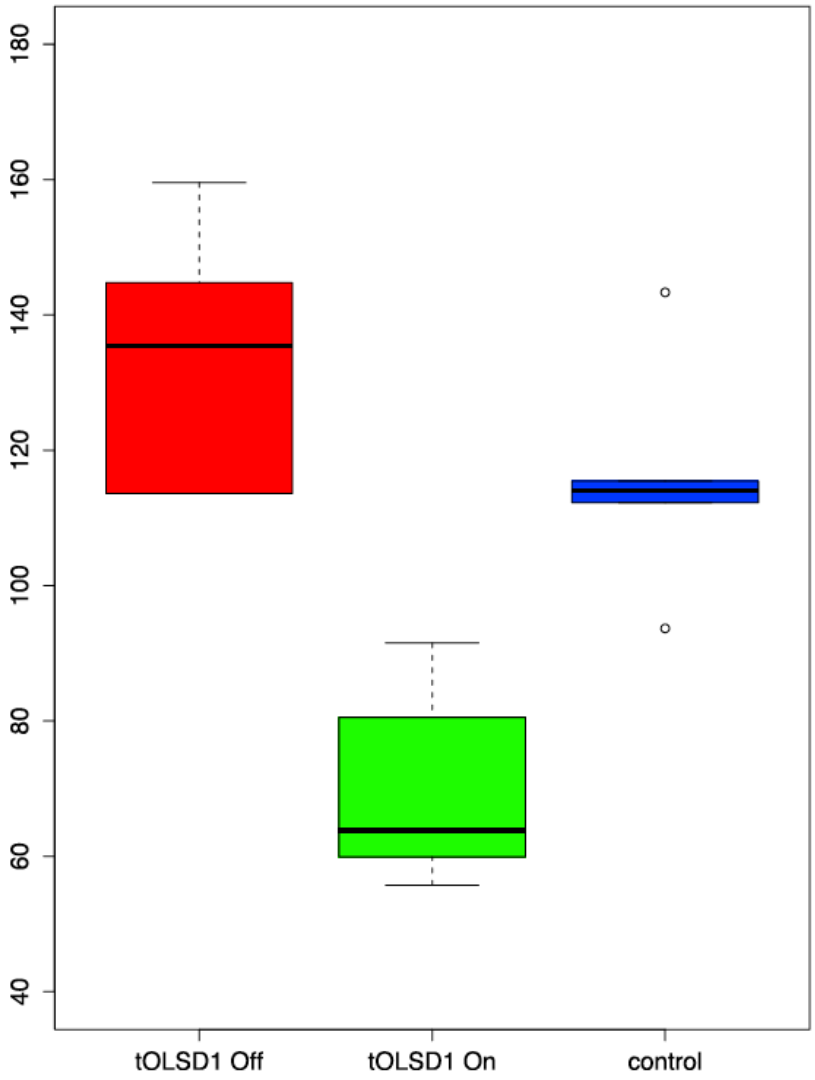
Figure shows whole mount view of X-gal staining of the “rescue” mice described in Figure 3.



Supplemental Figure 4 (relevant to Figure 4): LSD1 IF in Adcy3 control and KO.

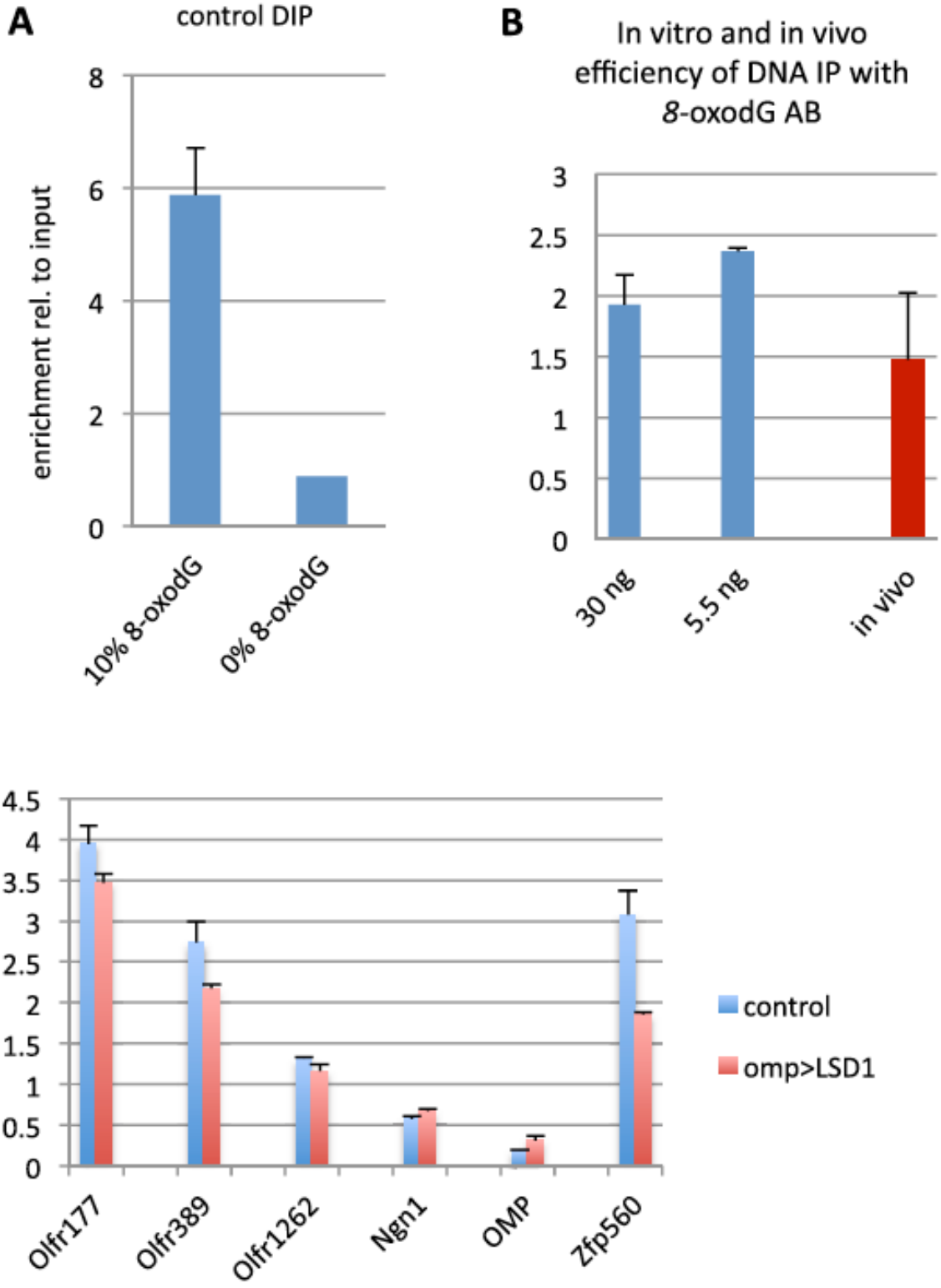
(A) Quantification of LSD1 IF signal in MOE sections from wild type and Adcy3 KO littermates. (B,C) Same images as shown in Figure 4B and 4D with Adcy3+/- (top) and -/-(bottom), enlarged to illustrate extent of GAP43 expansion relative to total depth of MOE (left panels) and the mutually-exclusive nature of Adcy3-lacZ (green) expression compared to LSD1 (red) (D) The proportion of OR reads that mapped to pseudogenes relative to total functional OR reads in control and knockout. Total and pseudogene mapped reads totals were calculated by summing

their respective RPKMs. (E) PND2 sections from Adcy3+/- (top) and -/- (bottom), stained with OR (green, MOR28 and M50) and LSD1 (red) antibodies. (F) PND2 sections from Adcy3+/- and -/- (top and bottom, respectively) probed with antisense RNA probes for OMP (green) and LSD1 (red).



Supplemental Figure 5 (relevant to Figure 5): Quantification of Olfr49 IF signal.

Counting of sections of control mice (blue bar) or OMPitTA;tetOLSD1 mice that have been fed dox (tetOLSD1 “off”, red bar) or not (tetOLSD1 “on”, green bar). Cells with IF signal much lower than in the wild type sections were included in the counts.


A
control DIP
enrichment rel. to input
8
6
4
2
0
10% 8-oxodG
0% 8-oxodG
B
In vitro and in vivo efficiency of DNA IP with 8-oxodG AB
3
2.5
2
1.5
1
0.5
0
30 ng
5.5 ng
in vivo
C
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Olfr177
Olfr389
Olfr1262
Ngn1
OMP
Zfp560
control
omp>LSD1

Supplemental Figure 6 *(relevant to Figure 6): 8-oxodG DIP of oxodized PCR product is immunoprecipitated at levels similar to in vivo 8-oxodG DIP.*

(A) qPCR illustrating that 8-oxodG-enriched Cre in vitro synthesized fragment is immunoprecipitated greater than 6-fold above control DIP. (B) Addition of 8-oxodG enriched Cre PCR fragment to sonicated genomic DNA at approximate physiological concentration (1 femtogram PCR fragment per nanogram of sonicated genomic DNA) allows efficient immunoprecipitation of this oxidized fragment in the nanogram range that is used for DIP. Enrichment for *in vitro*-generated *8*-oxodG DIP (black bars) are on the order of that observed in a separate, *in vivo* experiment (red bar). Error bars ± SEM; qPCR performed in duplicate.

(C) H3K9me3 ChIP-qPCR analysis from wild type and LSD1 overexpressing littermates. Error bars ± SEM; qPCR performed in duplicate.

Supplemental material and methods

Mouse strains

Mouse strains used are as follows: Foxg1-Cre (Hebert and McConnell 2000); Gng8-tTA (Nguygen et al. 2007); OMP-IRES-GFP; OMPitTA (Yu et al. 2004); tetO-MOR28-IRES-tau::*lacZ* (Clowney et al.,2012); P2-IRES-tau::lacZ (Mombaerts et al. 1996) Lsd1 conditional knockout (Wang et al. 2007), Adcy3 null (Deltagen, Adcy3^{tm1Dgen}), MOR28-IRES-Cre (Shykind et al. 2004); MOR28-delete-Cre (Shykind et al. 2004), OMP-IRES-Cre (Eggan et al. 2004); mT/mG Cre reporter (Muzumdar et al. 2007) and tetO-LSD1 (synthesized full length mouse LSD1 was inserted into a modified pTRE2 vector (Invitrogen), linearized, and injected into at the Gladstone Institute Transgenic Mouse Core). The neo-resistance cassette has been removed from all the knock-in and knockout mice, especially MOR28-IRES-Cre, to assure that we are studying switching and not "twitching". Removal of the new cassette did not change the switching frequencies previously published.

Riboprobes, antibodies and ISH protocol

All RNA probes were generated using *in vitro* transcription with T7 or Sp6 RNA polymerase to synthesize digoxigenin-labeled UTP (Roche). Probes used for the 2 classes of olfactory receptors are as follows: *Olfr571*, *-676, -677, -690, -552, -556* (Class I) *and 1277, -6, -124, -713, -715, -1310, -1356, -16, -802, -2, -1507, -693* (Class II) using previously described sequences (Hirota *et al,.*2007; Fuss *et al.,* 2007). Antibody and in situ hybridization (ISH) was carried out on embryonic day 18.5 unless otherwise noted. Embryonic and perinatal tissue was frozen in OCT and sectioned at 16 microns with pre-fixation. Adult MOE were fixed for 2 hours prior to OCT embedding and sectioning. Antibody staining was carried out following standard procedures, with antibodies used as follows: Adenylyl Cyclase 3 (Santa Cruz); Lsd1 (Abcam); Cre (Novagen);

Beta-galactosidase (Abcam). Both Adcy3 and LSD1 antibodies were tested in the Adcy3 and LSD1 KO MOEs and produced no IF signal (data not shown). OR antibodies were designed by G.B (Barnea *et al.* 2004): MOR28 (*Olfr1507*), C6 (*Olfr49*), M71 (*Olfr151*), M50 (*Olfr6*). ISH as well as Immunofluorescence/ISH was carried out as described in Clowney et al (2012).

RNAseq

Read counts for these sequencing runs used for the data shown here are as follows (40-bp reads on Genome Analyzer II): LSD1 mutant cDNA: 56,229,900; LSD1 control cDNA: 51,290,921; LSD1 control *8*-oxodG: 18,125,232; LSD1 mutant *8*-oxodG: 14,110,756; (100-bp reads on Hi-Seq 2000): Adcy3 heterozygote: 33,264,169; Adcy3-/- knockout: 35,843,348. An in-house Python script was used to generate RPKM (reads per kilobase per million mapped reads) values for olfactory receptor genes for both mRNA-seq and DIP-seq libraries for LSD1 and 8-oxodG datasets, as in Magklara et al. (2011). Adcy3 expression values were generated using the Cufflinks suite (Trapnell et al. 2010). Regarding Figure 6, for mRNA-seq, only reads falling on the olfactory receptor exons were counted; for DIP-seq, reads falling on the entire annotated gene body were counted. The radial visualization of olfactory receptor expression in Figure 2 was generated using Circos (Krzywinski et al. 2009), and the boxplots in Figures 2 and 6 were created in MATLAB. Boxplots and statistical calculations in Figure 4 and 5 were made using R.

Chapter 4: a link between OR expression and Adcy3-mediated LSD1 repression

OR induces Adcy3: the outstanding gap in understanding

From our work on LSD1, it was clear that this histone demethylase is required for normal levels of OR transcription and it was this high level of OR transcription and its subsequent translation that likely induced Adcy3. The major remaining problem with relation to the pathway described in Figure 7 of Chapter 2 was the absence of a tangible regulatory link between the OR and Adcy3. This induction from OR to Adcy3 is required to shutoff LSD1 and stabilize OR choice, as shown in Chapter 2. One potential link is the cytosolic region of the OSN, where both OR and Adcy3 transcript converged. The unpublished observation that Adcy3 transcript is present, albeit it at reduced levels, in the LSD1 knockout MOE suggested that Adcy3 translation regulation could be positively regulated by OR translation. One hypothesis was that in the absence of OR transcription, Adcy3 transcript "waits" for a cue to be translated from the newly generated OR protein.

Alternatively, the Adcy3 transcript seen in the LSD1 KO could be insufficient to trigger downregulation of LSD1 even if it is translated, as it is about 3-fold reduced from control levels by RPKM. Instead, highly-transcribed OR genes could induce the upregulation of an intermediary which is able to relay an upregulatory signal to either the Adcy3 gene promoter or an Adcy3 transcriptional activator. During and after the publication of the above LSD1 work, we explored these possibilities and identified a bZIP transcription factor, Atf5 (Hansen et al., 2002), which is transiently translationally upregulated in response to OR gene expression (Dalton, Lyons, & Lomvardas, 2013) and appeared to be an excellent candidate for the "OR protein

monitor" which could bridge the gap in signaling between OR protein and Adcy3 activation. I will now discuss the evidence in support of this claim and address issues with the overall model, from both Chapter 2 and this chapter, to conclude.

Atf5 mRNA possesses 4 open reading frames from which translation can occur and its mRNA is highly transcribed throughout the MOE. Its main mode of regulation is translational based on current data. The first 2 ORFs, which are out of frame with the downstream 2 ORFs, serve unknown purposes. The downstream 2 ORFs are in-frame with eachother and encode the transcription factor long form (Atf5l) and short form (Atf5s), respectively. The translation of the long form or short form thus generates a bZIP transcription factor that can translocate to the nucleus and effect transcriptional change (Watatani et al., 2008). In its essence, both of these forms serve to alter the global transcriptional profile for a brief period of time during a period of cellular duress.

The 2 upstream ORFs of Atf5 mRNA will be productively engaged during physiological, low-stress conditions in the cell. However, the downstream 2 ORFs which encode Atf5 transcription factor will be translated only if the ribosome is able to access their start codon. The primary means by which this is accomplished is via ribosome scanning, a process which occurs in response to a shortage of methionine used to encode the start amino acid. Typically, start methionine is provided to the ribosome via an initiation factor termed Eif2alpha. A well-studied cellular stress pathway that blocks global translation is Eif2alpha phosphorlaytion by ER stress sensors of IRE1-type kinases (Ron & Walter, 2007). These ER-spanning proteins possess chaperone-like domains that detect unfolded protein in the ER lumen and phosphorylate

targets in the cytoplasm in response. PERK is one such kinase that is well expressed in the MOE, and it is known to be required for normal translation of the Atf5l and/or Atf5s.

In Dalton et al. (2013) we show that global KO of Atf5 prevents the stabilization of OR choice due to the lack of downregulation of LSD1, apparently due to the broad loss of Adcy3 protein. Intriguingly, in the absence of LSD1, ectopic OR expression is sufficient to stimulate translation of nuclear Atf5 (Figure 1), while removal of either PERK or introduction of Eif2alpha phospho-mutant is sufficient to prevent Adcy3 expression, and thus prevent LSD1 downregulation. Thus the transient passage of the immature OSN through a state of ER stress is needed to ensure the stable choice of an OR gene, insofar as it allows the upregulation of a transcription factor, Atf5, to trigger the protein synthesis of Adcy3. Further, in the absence of LSD1, global stress induced with tunicamycin (an N-glycosylation inhibitor; White & Speake, 1979) triggers the translation of Atf5 and remarkably generates some neurons that express Adcy3 at levels high enough to detect by IF.

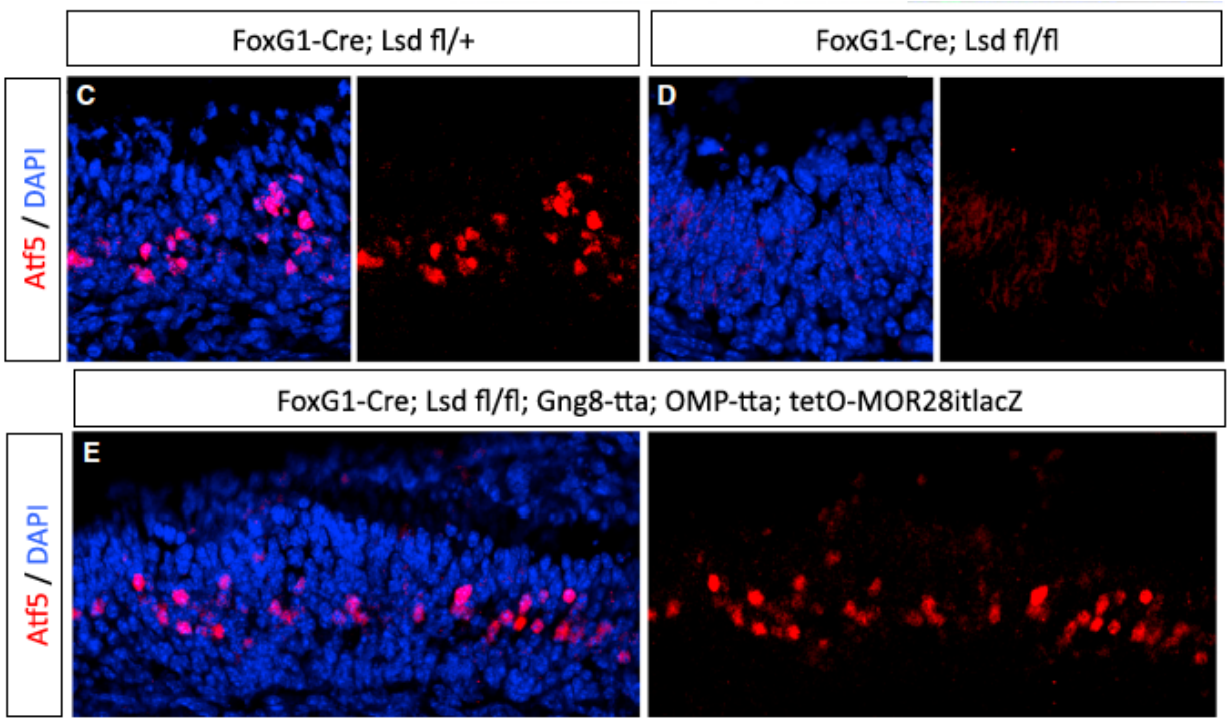


Figure 1: Atf5 translation is induced by OR expression.

(C) OR expression occurs normally in the LSD1 heterozygote mouse at E18.5, however (D) does not occur in the LSD1 knockout. (E) Misexpression of OR in the LSD1 knockout rescues Atf5 translation.

Some major questions that remain regarding OR feedback

How does Adcy3 signal to LSD1 in order to shut LSD1 off? Is it primarily at the transcription level, since the transcript levels fall from the immature to mature OSN? Or is the signal mediated first at the post-transcriptional level, or is there a protein degradation component? There are innumerable possibilities regarding how an increase in Adcy3 and the

beginning of a functional mature OSN could lead to the downregulation of LSD1. There are numerous predicted PKA phosphorylation sites on LSD1 (unpublished data). It is plausible to imagine that phosphorylation can trigger the degradation of LSD1 following the beginnings of basal Adcy3 activity. Alternatively the activity of the OR encoded by the chosen allele may differentially signal to Adcy3 and this might also help to explain the observed differences in OR gene switching among different OR genes (e.g. M71-Cre-reporter+ OSNs rarely if ever switch, while in our hands MOR28-Cre-reporter+ OSNs are seen to be expressing non-MOR28 alleles about 30% of the time). In this model, the kinetics with which LSD1 is degraded defines the number of ORs chosen. For those ORs that are less active (Reisert, 2010) the likelihood of being disengaged by the "OR choice machinery" would be thus increased.

Chapter 5: the removal of repressive histone lysine methylation at OR genes—the making of a clean slate

The preceding has illustrated a clear role for the requirement for LSD1 in the transcriptional activation of OR genes, and the work has linked LSD1 to the maturation of the OSN via its induction of a stabilizing feedback signal centered around Adcy3 and Atf5. We show that LSD1 likely acts transiently on the histone substrate at the OR locus which is "to-be-chosen" for activation and is forced off by its own action, a self-generated "trap" into which it must walk by virtue of its chemical affinities for methylated histones and cofactors.

In this chapter, I begin to define the role of the OR repression in the exquisite transcriptional control of the olfactory system. The surprising outcome of this set of studies is that although the transcriptional repression of OR genes is needed to maintain normal OR expression diversity, it is not needed to ensure OR gene expression. When histone K9 methylation is greatly reduced, our expectation was that many ORs would be expressed per cell. What we find instead is counterintuitive but fascinating: OR choice is heavily biased toward the selection of about a dozen OR genes scattered throughout the genome.

What allows for the stochastic process of OR choice?

There is an inherent randomness to the choice of the OR within a given zone that is mediated by an unknown mechanism. Due to requirement for a very large number of unique OSNs that endow the nose with exquisite olfactory sensitivity and discriminatory power, the molecular

nature of this “randomness” generator is of central importance to the animal's olfactory capabilities. Additionally, animal nervous systems generally deploy stochastic gene transcription to permit self- from non-self identification yet the basic molecular mechanisms of these gene regulatory processes remain elusive (Lomvardas Magklara 2013). One such mechanism is evinced by the clustered protocadherins, as discussed in chapter I.

G9a and its homolog GLP are expressed in main olfactory epithelium

The genome of the adult olfactory sensory neuron (OSN) is enriched for 2 known repressive histone modifications across the ~50 OR gene clusters termed trimethyl (me3) H3K9 and me3H4K20 (Magklara et al. 2011). To delineate the contribution of these histone modifications on the expression of OR genes, we used conditional knockout mouse lines crossed to a Cre that is active in the olfactory placode, ensuring gene ablation throughout the entire MOE, as discussed in Chapter II.

H3K9 is mono- and dimethylated by protein complexes containing G9a (EHMT2, KMT1C) and its homolog GLP (EHMT1, KMT1D). Dimethylated lysine residues are substrates for trimethylation by a different family of histone methyltransferases, SetDB1 and SetDB2, thus we reasoned that achieving a repressed transcriptional state would require G9a and/or GLP, at least in gene-rich, euchromatic regions where these molecules have been described as being active (Shinkai & Tachibana, 2011). Additionally, the adult stem cells of the MOE, the HBCs, possess an enrichment for H3K9me2 at the OR genes (Magklara et al. 2011), suggesting that developmentally, dimethylation is established before trimethylation. (Although initial studies of recombinant G9a found that it can contribute to all three methylation states at lysine 9; see (Patnaik et al., 2004)) Both of these genes are expressed in embryo and adult in a dynamic

pattern whereby the progenitor cells of the MOE express high levels while the mature OSN downregulates these genes (Figure 1A and -B). Foxg1-Cre; G9a flox/+ and G9a flox/flox (control and cKO henceforth) MOEs were assayed for mature markers at the day prior to parturition, due to perinatal lethality in these cKO mice. Olfactory Marker Protein (OMP) and VGLUT2 are both only slightly reduced in the G9a cKO (Figure 1C-E) suggesting that there was no dramatic alteration in olfactory receptor expression in this mutant. However, olfactory receptor immunofluorescence (IF) revealed there was an overt reduction in the relative number of neurons expressing MOR28 and M50 while there was no discernable change in M71+ OSN abundance (data not shown).

OR genes are predominantly downregulated in the G9a cKO, but a few are upregulated

We next sought to define what caused loss of MOR28+ OSNs and reduction in M50+ OSNs in the E18.5 G9a cKO MOE. We generated mRNA-seq libraries from control and G9a cKO MOE at this age and found a significant decrease in OR expression on a global scale (unpaired t-test, p-value=0.0079). Approximately a third of the 980 ORs detected in the control MOE were completely undetected in the cKO (Figure 3B) and of the ~300 genes that are 2-fold or more downregulated in the cKO, ~50% are OR genes, and of these genes fewer than 10 have a control RPKM >5 (data not shown). This indicates removal of G9a has a relatively subtle overall effect on the MOE transcriptome generally. However, a handful of OR genes are dramatically upregulated in the G9a cKO including Olfr231 and -877, suggesting that the G9a cKO does not have a deficit in OR expression but rather the cKO OSN is unable to randomly select an OR and undergoes some form of a biased selection process. This selection process generates high levels of a single OR from only ~13 clusters on 9 chromosomes despite the presence of hundreds of ORs to choose from on these chromosomes (data not shown). Most notably,

Olfr231 is expressed at roughly 5-fold its normal level based on RNA-seq (Figures 2 and 4), a finding supported by our fluorescent RNA in situ hybridization (ISH, Figures 2 and 4).

The relative abundance of the most highly expressed functional OR in the control MOE (Olfr15) is shunted by 2 OR genes that are very poorly represented in the control MOE, Olfr231 and -877 (Figure 2A). ChIP-qPCR indicate a paucity of repressive chromatin modification at both of these loci which suggests a role for histone-based repression in promoting balanced, random OR choice. Despite the marked increased prevalence of OSNs expressing these 2 ORs, there were very few neurons at E18.5 in which Olfr231 was coexpressed with Olfr877 (Figure 2C), supporting the notion that there is a self-reinforcing feedback for the chosen OR (Shykind et al. 2004) and suggesting that this feedback does not require nor entail histone repression *per se*. Furthermore, we assayed neighboring OR genes in the Olfr231 cluster to determine if monogenic OR expression was affected by the G9a cKO reduction of repressive H3K9 and H4K20 methyl marks (Figure 4). Surprisingly, OR genes immediately flanking Olfr231 are not affected by the loss of G9a. OR gene clusters are known to reside in heterochromatic foci in the OSN nucleus. We assayed the impact of G9a cKO on nuclear architecture by H3K9 me3 and me2 IF. Both suggest there is little overt nuclear phenotype in the G9a cKO, which is in agreement with findings of Yokochi et al. (2009) in their study of mouse ES cells, although we do observe a pronounced aggregation of me2H3K9 signal in the G9a cKO (Figure 5A).

Histone demethylase LSD1 plays a key role in OR expression and subsequent OSN maturation via upregulation of second messenger system components. Due to the loss of OR gene repression at the chromatin level, the role for LSD1 may accordingly be reduced in the G9a cKO. LSD1 deposits 8-oxoguanosine near sites of its enzymatic activity, with relatively high stability in the OSN (see Chapter 3). In agreement with the notion that LSD1 may have an attenuated role in the G9a cKO MOE, the levels of 8-oxoguanosine are nearly identical in control and cKO at diverse loci including 2 highly upregulated ORs that would likely possess

enrichment for 8-oxoguanosine were there a requirement for LSD1 enzymatic activity (Figure 3D).

GLP acts in concert with G9a to ensure random OR expression

Although the reduction in me3 H3K9 levels is pronounced in the G9a cKO MOE, we sought to rule out the possibility that GLP, or G9a-like-protein, is important in MOE development. There is limited biochemical evidence suggesting GLP performs monomethylation slightly more efficiently than G9a and G9a and GLP often form heterodimers in ES cells, although homodimers have been observed as well (Tachibana et al., 2005). To conditionally remove both G9a and GLP, we performed a similar genetic cross as described above with Foxg1-Cre but with both G9a and GLP flox mice in order to generate a double conditional knockout MOE (Tachibana et al. 2005; Schaefer et al., 2009). Remarkably, at E18.5 the levels of Olfr231 are increased well beyond those seen in the cKO of G9a (Figure 6). The number of Olfr877+ neurons is decreased while Olfr231+ neurons increase in prevalence, suggesting that G9a and GLP cooperate to repress OR genes. Interestingly, the zonality of Olfr231 appears to become expanded in the dcKO, as Olfr231 is observed in a large number of vomeronasal organ (VNO) neurons (Figure 6C and Figure 7).

A default OR choice mechanism

Together these data suggest H3K9 methylation acts as a substrate of OR choice, providing the OSN an opportunity to select an OR before a default expression program is set in motion. This "default OR" is expressed in a larger number of OSNs ectopically in the G9a cKO and ~3-fold that number of OSNs lacking both G9a and GLP. We speculate that this OR does not behave as a typical OR early in the life of the animal in that it is not repressed as highly as

most OR genes (see Figure 2). Other more highly repressed ORs are selected via the LSD1-AC3 signaling circuit that ensures monogenic expression and prevents the activation of other ORs. We propose that there exists a parallel, default circuit in OSNs that have become unable to select from the repressed OR genes. The presence of such a parallel OR expression circuit is suggested by the finding that Olfr231 does not have a well-restricted zone, suggesting it is competent to be expressed broadly in the MOE unlike most other ORs which are tightly restricted (Ressler et al. 1993; Vassar et al. 1993). Furthermore, the OR gene cluster in which Olfr231 resides carries little H3K9 repression early on, unlike most OR gene clusters we have assayed whereby repression appears to be relatively stable throughout the life of the MOE. This means paradoxically that Olfr231 is less repressed around birth, and in the absence of the appropriate repressive substrate for the canonical OR choice pathway to act, Olfr231 is transcribed at high levels, inducing stabilization of Adcy3 expression, which serves as the common denominator in the two OR choice circuits. The increased Adcy3 expression essentially finalizes OR choice by repressing LSD1, the histone demethylase required for activation of repressed, canonical OR gene members.

Knockout of G9a in mature olfactory sensory neuron does not cause OR deregulation

Although repressive H3K9 and H4K20 appear severely reduced in the G9a cKO, the expression pattern of G9a suggests that it may have a role in the mature OSN as well earlier in the neuronal lineage. We used an OMP-IRES-Cre mouse line (as described in Chapter 3) to ablate G9a exclusively in the mature OSNs and performed ISH and IF for ORs (Figure 5B). No overt phenotype was observed in the OMP-G9a cKO, unlike for the Foxg1-Cre G9a cKO. MOR28+ neurons were as numerous in the OMP-G9a cKO as in the control MOE. Furthermore, a pool of OR ISH probes illustrate that there is no reduction in the abundance of OR genes that are highly repressed "canonical" ORs, nor is there an increase in Olfr231 or Olfr877 by chromogenic ISH. No obvious loss of mature neurons was observed in the OMP-G9a cKO by

VGLUT2 IF (data not shown) or OMP ISH (Figure 5B). Thus G9a likely has a transient role in the maintenance of a balanced OR choice in the developing MOE as suggested by IF and RNA-seq data (shown in Figure 2).

H4K20 methylation is dispensable for canonical, random OR choice

Previous work has shown that the OR genes are highly repressed with trimethylation at H4K20 in addition to H3K9 (Magklara et al. 2011). Unlike K9 repression, however, K20 methylation is believed to be more stable, as there is no known me3K20 demethylase (Kouzarides 2007; note: PHF8 can demethylate monomethyl but not di- or trimethylK20 (W. Liu et al., 2010; Qi et al., 2010)). The SET-domain containing proteins responsible for adding these histone H4 marks are termed SUV4-20H1 and -H2 (Schotta et al., 2004). SUV4-20H1 null mice die perinatally while SUV420-H2 null mice can thrive and are fertile. The SUV4-20H1 conditional knockout allele was used in conjunction with the -H2 knockout to cross to Foxg1-Cre in order to generate SUV4-20H1/H2 dKO (H1/2 dKO). We sought to generate these mice to answer 2 questions: first, what is the order of operations in the repression of OR genes and, second, what effect will removing only K20-specific methyltransferases have on the stable expression of the OR?

Removal of both SUV4-20H1 and -H2 allowed us to probe these questions. We found that the double knockout eliminated H4K20me3 at OR genes but did not effect the levels of H3K9me3 at OR genes (Figure 8C-D). Similar to the G9a cKO, there was no gross morphological defect in the MOE and there were normal amounts of mature neurons at E18.5. However, these H1/2 dKO oftentimes survived into their second month of life (n=6), although they were unable to thrive and often had difficulties acquiring enough food even when isolated from their littermates. OR expression by qPCR at PND30 illustrate no overt changes and normal levels of mature neuron marker expression. Neither Olfr231 nor 877 is upregulated in the H1/2 dKO (Figure 8B). Together these data suggest H4K20 methylation is a redundant layer of

transcriptional repression that perhaps aids in the large-scale organization of the OR genes in topological foci within the nucleus of the mature OSN (Clowney et al. 2012) and that its role is related to the fine tuning of OR transcript levels to complement the apparently more important role that K9 methylation is playing.

Conclusions from the methyltransferase studies

In Chapter 4, I have described remarkably specific effects caused by deleting histone methyltransferases in the MOE. While it is surprising that the OSN is essentially normal in the absence of H4K20 methylation, it is perhaps more remarkable that there is what appears to be a default or alternative OR expression program that can serve as a backup operation in the absence of available repressed ORs. These findings suggest that the mammalian nose is evolved to the idea that the need for fine olfactory acuity is second in importance to some degree of olfaction. Exquisite sensitivity has evolved in the olfactory system of mammals as result of massive receptor family expansion and diversification and a feedback mechanisms to engage a single OR allele (Shykind et al. 2004, Serizawa et al. 2003, Lewcock et al. 2004), but transgenic mice that express only a single OR in all of their OSNs are fertile and can thrive and reproduce despite that their olfactory acuity suffers (Fleischmann et al., 2008). The highly ordered synaptic inputs of OSN targets in the brain are not reflective of an absolute "odor map," as this topological information is discarded and personalized in the piriform cortex and other olfactory cortices. This complex, non-linear relationship of the brain to the OSN and the ORs they express may explain why a default OR expression system exists in the mouse: the maturation of a cell type that is required for the very basic first steps of post-birth survival rely on a limited number of genes such as LSD1, which has been shown to possess a mutagenic potential via its 8-oxoguanosine generation (Lyons et al. 2013; Perillo et al., 2008). Such a system might fail occasionally in nature but its role is too important to not safeguard with a form of “backup” insurance against total system failure.

We have shown evidence for a requirement of H3K9 but not H4K20 trimethylation in the balanced OR choice process and we uncovered the surface of a novel default OR expression pathway that is in stark contrast to the standard mode of OR choice involving the recently described LSD1-based mechanism that ensures representation of many hundreds of receptors. It is tempting to speculate that the G9a cKO OR phenotype is a reflection of atavism with the former OR choice mechanism having been completely bypassed in all but a few olfactory neurons to accommodate for an olfactory "arms race" which occurred following large genome duplications and other genomic-scale events early in tetrapod history (Nei et al. 2009). Central to the ability of the animal to survive is its olfactory acuity, and this selection pressure may have led to the fixation of OR alleles in ancestral mammals populations that actively recruited heterochromatin nucleating factors such as G9a and GLP so as to provide a substrate for stochastic OR choice.

Figures for Chapter 5

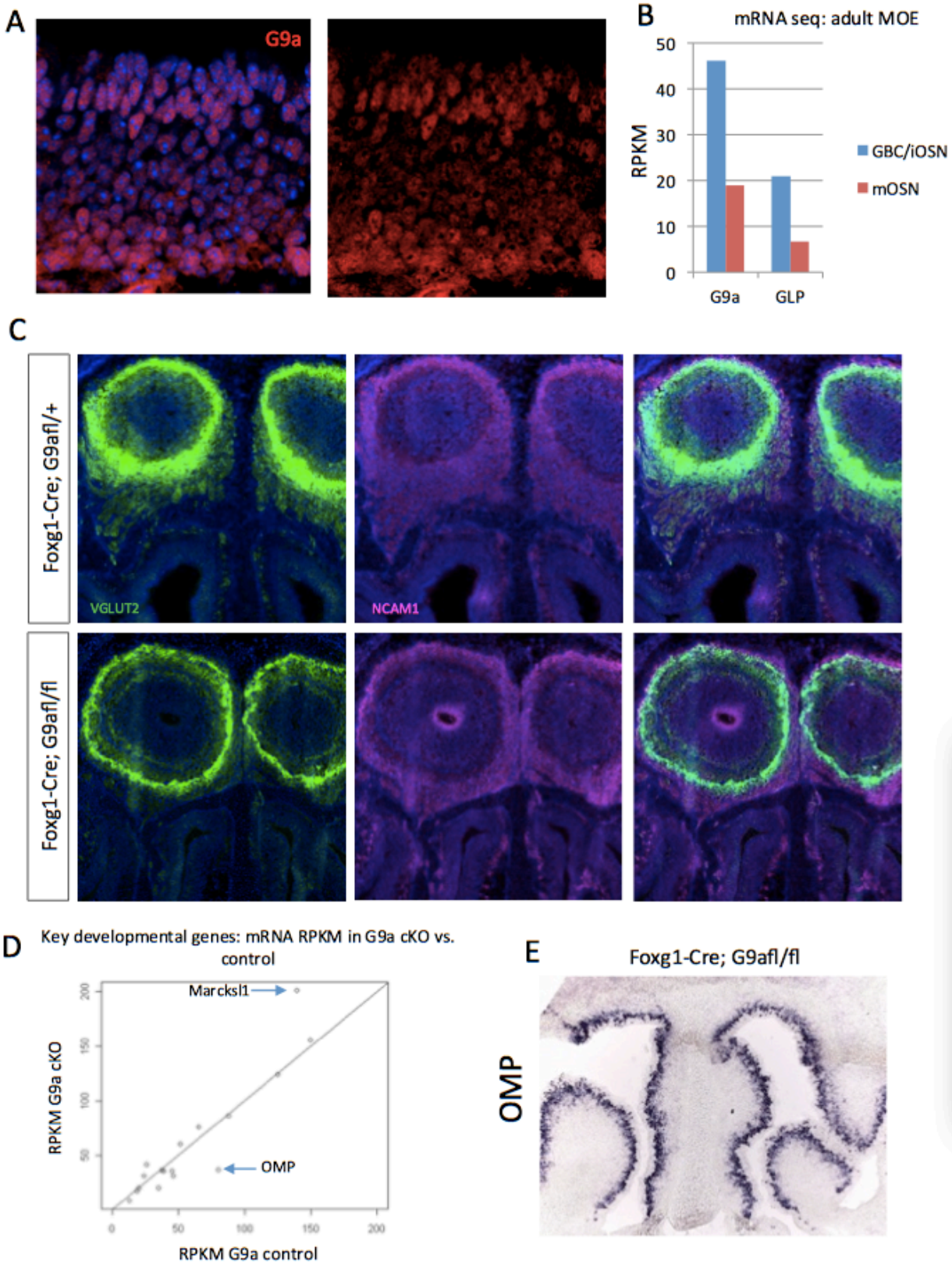


Figure 1: G9a knockout mice do not display overt developmental defects.

A. G9a immunofluorescence (red) in the E18.5 MOE with DAPI nuclear stain (blue). Note the reciprocal staining pattern whereby G9a occupies the regions of the nucleus that are less enriched for DAPI.

B. G9a and GLP RPKM values from RNA-seq in adult OMP-GFP (mature OSN) and Ngn1-GFP (globose basal and immature neuron) cells as reported in Magklara et al. (2011). Note the reduction in transcript abundance in the mature OSN (mOSN) relative to the immature neuron (iOSN).

C. G9a control and G9a cKO E18.5 MOE and olfactory bulb cryosections were stained with NCAM1 (magenta, immature plus mature OSN) and VGLUT2 (green, mature OSN) marker antibodies. The olfactory bulbs in both control (top panels) and cKO (bottom panels) are phenotypically similar at the level of innervation by OSNs.

D. Scatterplot displaying the RPKM values for the following developmentally important genes, from least to most highly expressed in control MOE: Slc17a6, Ebf4, Emx2, Dpysl5, Stmn4, Ebf2, Ablim1, Lhx2, Ebf3, Ebf1, Lsd1, Stmn3, Omp, Ncam1, Stmn2, Marcksl1, Gap43. Note OMP and Marcksl1 labeled as the only 2 heavily affected genes.

E. OMP ISH at E18.5 in the G9a cKO. Despite relatively severe decrease in RNA-seq RPKM, OMP is highly expressed in the cKO based on ISH.

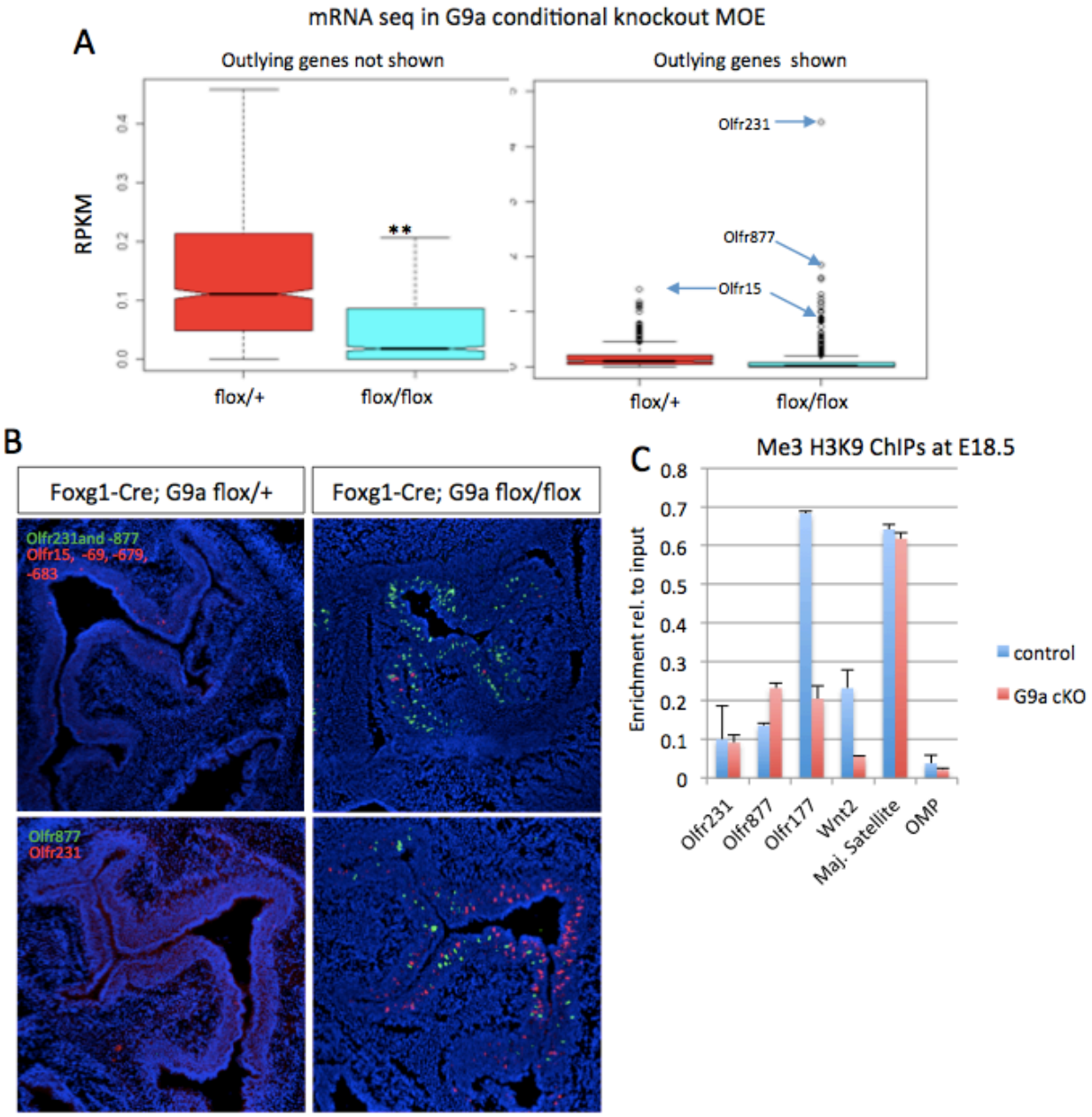

A
mRNA seq in G9a conditional knockout MOE
Outlying genes not shown
Outlying genes shown
RPKM
**
Olfr231
Olfr877
Olfr15
flox/+
flox/flox
flox/+
flox/flox
B
Foxg1-Cre; G9a flox/+
Foxg1-Cre; G9a flox/flox
Olfr231and -877
Olfr15, -69, -679, -683
Olfr877
Olfr231
C
Me3 H3K9 ChIPs at E18.5
Enrichment rel. to input
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Olfr231
Olfr877
Olfr177
Wnt2
Maj. Satellite
OMP
control
G9a cKO

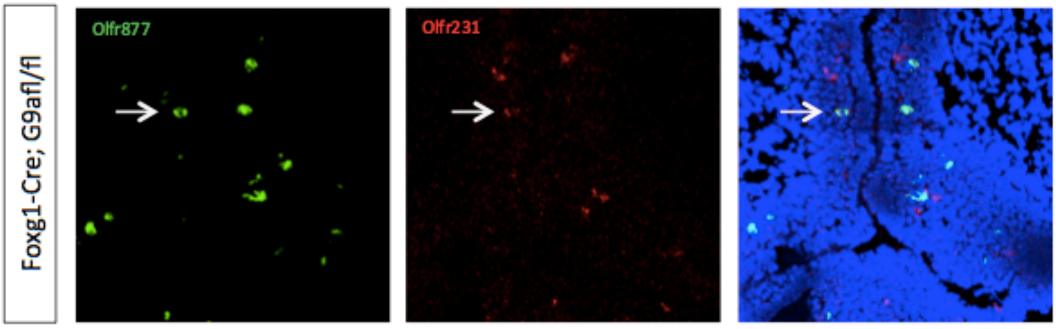
Figure 2: Massive increase in a subset of OR genes in the G9a knockout mouse MOE.

A. RNA-seq RPKM for all Refseq OR genes detected in either G9a control or G9a cKO MOE at E18.5. Plotted without (left panel) and with outliers (right panel) to emphasize the significant decrease of OR diversity in the MOE following loss of G9a with a relative increase in a small number of OR transcripts. P-value<0.001; Welch's T-test.

B. MOE cryosections with 2-color RNA FISH at E18.5 illustrating the increase in the G9a cKO of Olfr231 and -877 (green) versus 4 other OR genes (red; compare top panels, knockout on right). Lower panels show Olfr231 (red) and Olfr877 (green). Note vast increase in the number of the OSNs expressing these ORs. DAPI nuclear dye in blue.

C. Chromatin immunoprecipitation for me3 histone H3K9 (top) and me3 histone H4K20 (bottom) in the E18.5 MOE. Note the paucity of me3K9 at Olfr231 and Olfr877 loci in the control MOE (analogous to the enrichment at OMP, a highly expressed gene). Also note the loss of me3K20 in G9a cKO. Olfr177 serves as a representative "repressed" OR gene locus. Error bars are SEM, PCR performed in duplicate.

A

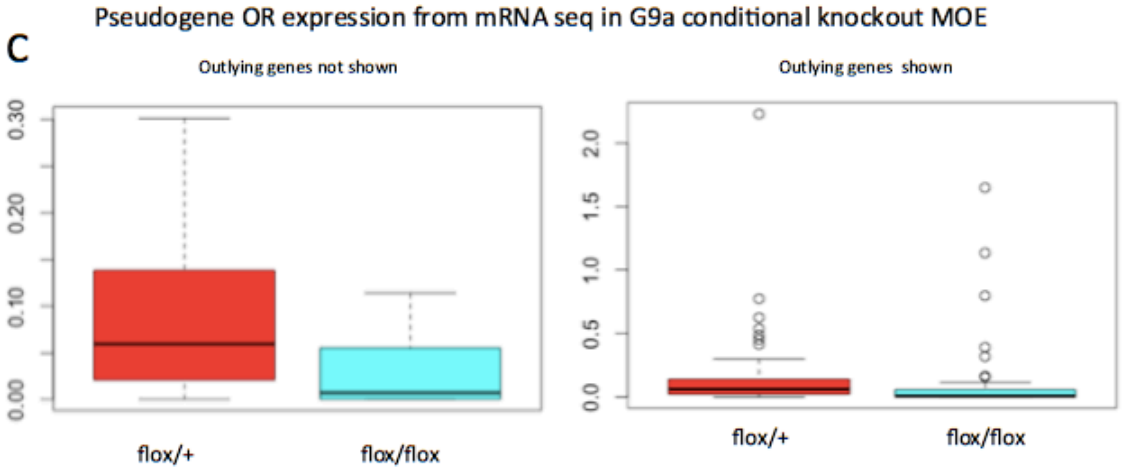


B

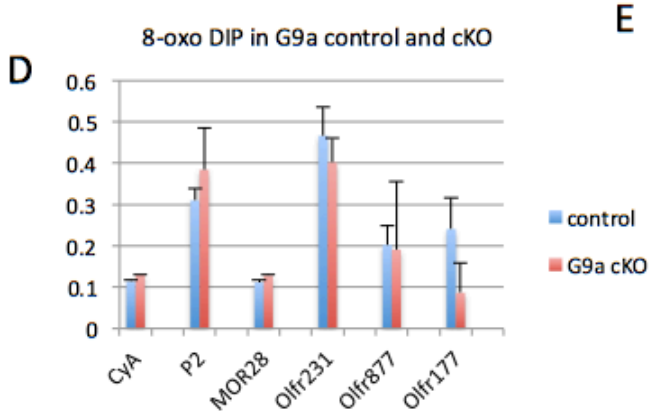
number of OR types detected in G9a control and cKO

	functional	pseudogenes
Foxg1-Cre; G9a flox/+	942	61
Foxg1-Cre; G9a flox/flox	607	36

C



D



E

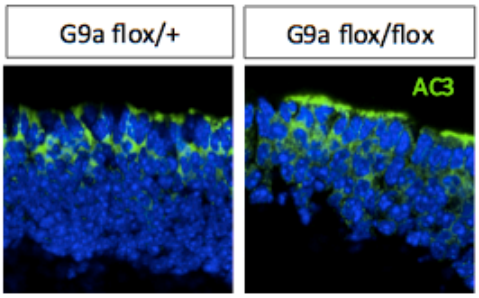


Figure 3: The G9a mutant mouse coexpress ORs at low frequency and does not appear to need LSD1 in OR activation.

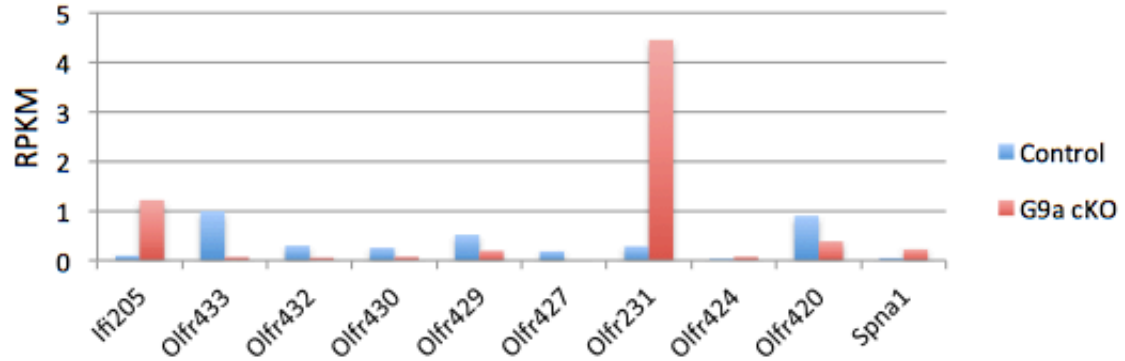
A. MOE cryosections with 2-color RNA FISH at E18.5. Olfr231 (red) and Olfr877 (green) are rarely found in the same OSN despite being massively upregulated in the absence of G9a.

B. Table showing the number of ORs detected, divided by class (functional versus putatively pseudogenized, based on mm9 Mus musculus genome assembly).

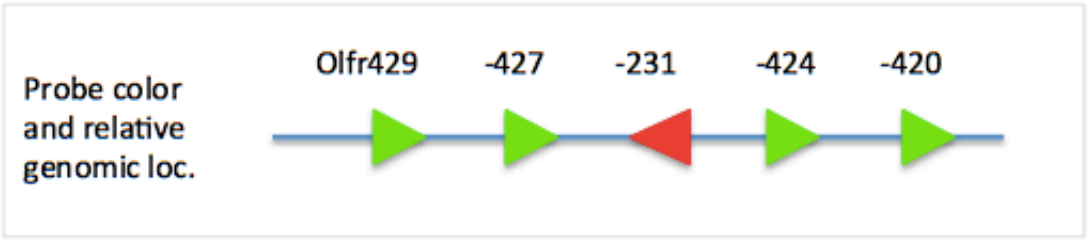
C. RNA-seq RPKM for all Pseudogene ORs detected in either G9a control or G9a cKO MOE at E18.5. Plotted without (left panel) and with outliers (right panel) to contrast with the differences identified in Figure 1A.

D. 8-oxoguanosine DNA immunoprecipitation to detect regions of LSD1 activity in the MOE. Note absence of significant differences for loci assayed. PCR performed in duplicate, error bars are +/- SEM.

A


Olfr231 gene cluster expression levels at E18.5
RPKM
5
4
3
2
1
0
Ifi205
Olfr433
Olfr432
Olfr430
Olfr429
Olfr427
Olfr231
Olfr424
Olfr420
Spna1
Control
G9a cKO

B


Probe color
and relative
genomic loc.
Olfr429
-427
-231
-424
-420

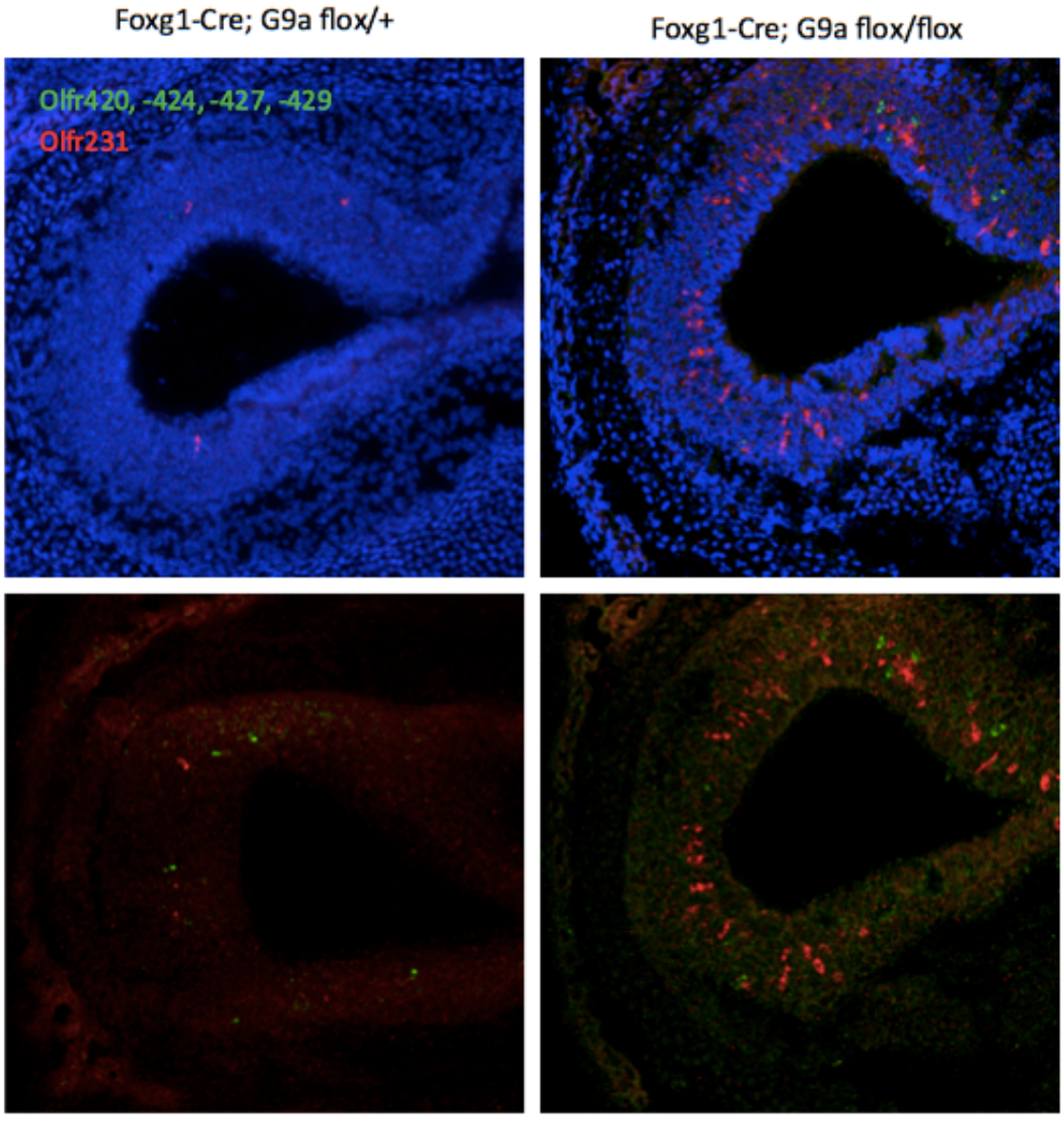
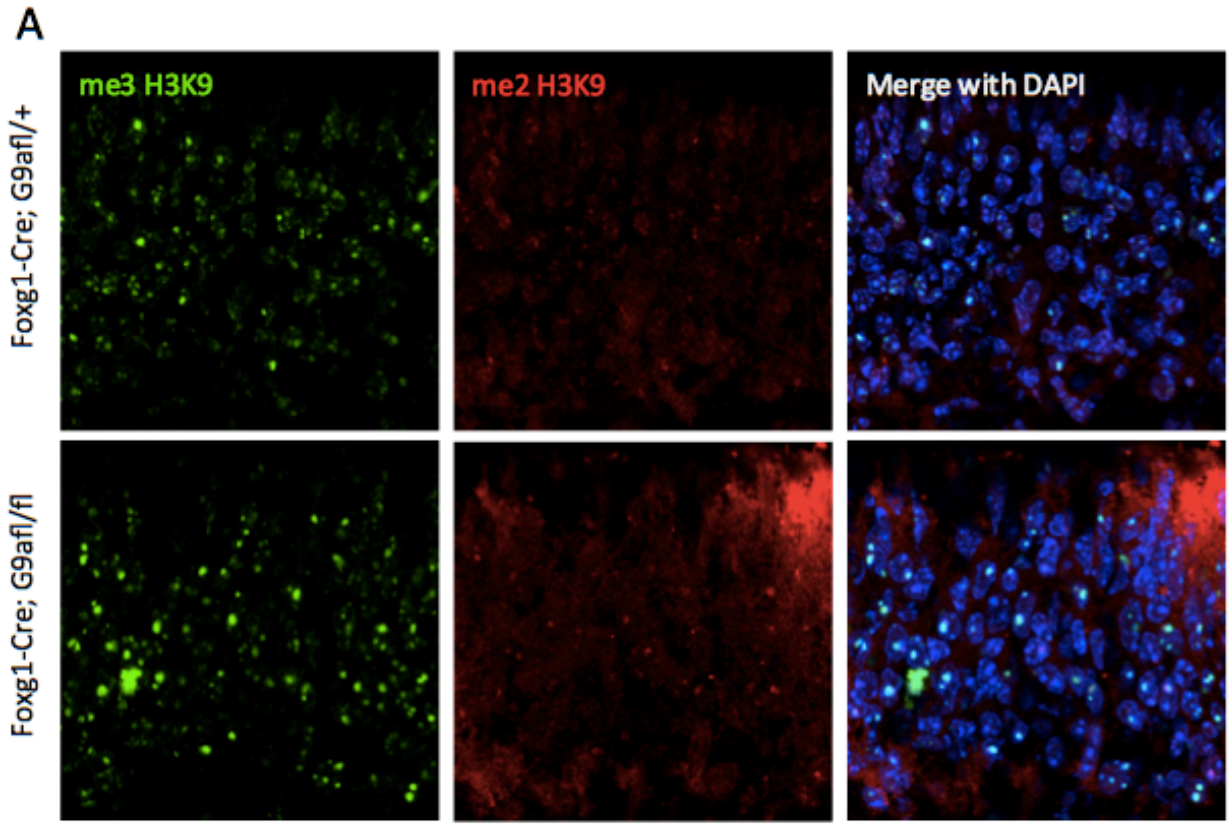

Foxg1-Cre; G9a flox/+
Foxg1-Cre; G9a flox/flox
Olfr420, -424, -427, -429
Olfr231

Figure 4. Olfr231 neighboring genes are not affected by the removal of G9a.

A. RNA-seq RPKM barplot for the Olfr231 cluster of 8 OR genes with 2 nearest neighboring genes, Spna1 and Ifi205, illustrating how G9a deletion affects Olfr231 disproportionately.

B. MOE cryosections with 2-color RNA FISH at E18.5 for Olfr231 (red) and 4 immediately neighboring OR genes (green; Olfr424, -427, -420, and -429). ISH signal shows a clear increase in Olfr231+ neurons while neighboring genes are not overrepresented in the G9a cKO (left panel) relative to the control (right panel).


A
me3 H3K9
me2 H3K9
Merge with DAPI
Foxg1-Cre; G9afl/+
Foxg1-Cre; G9afl/fl

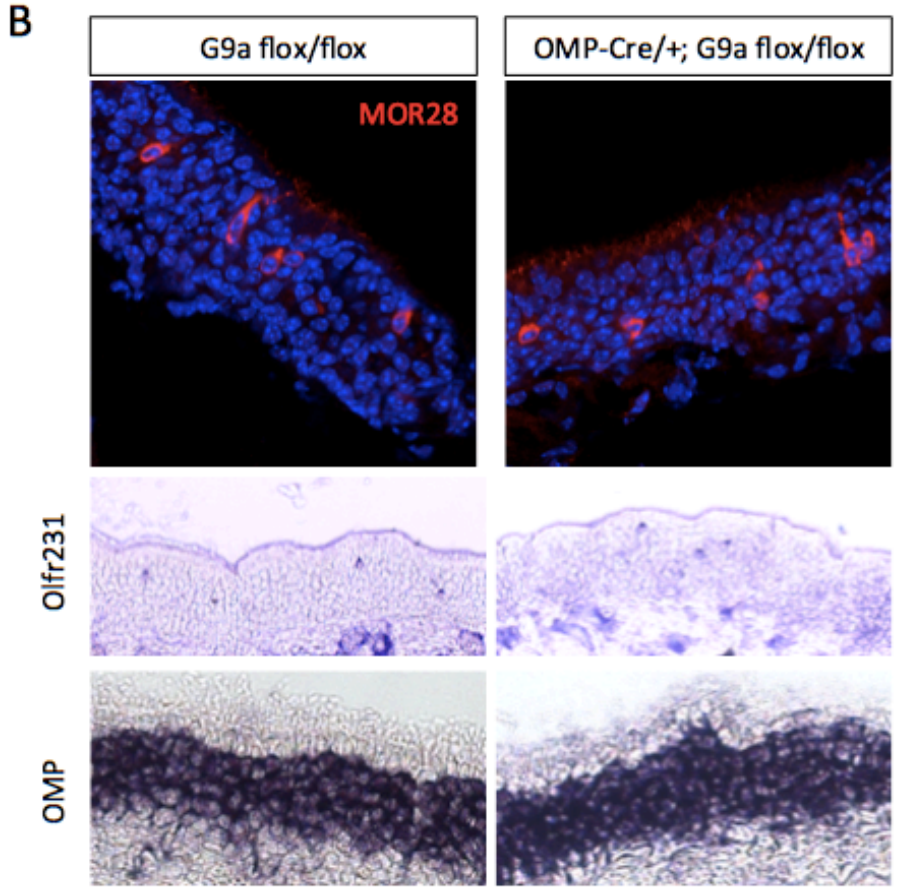
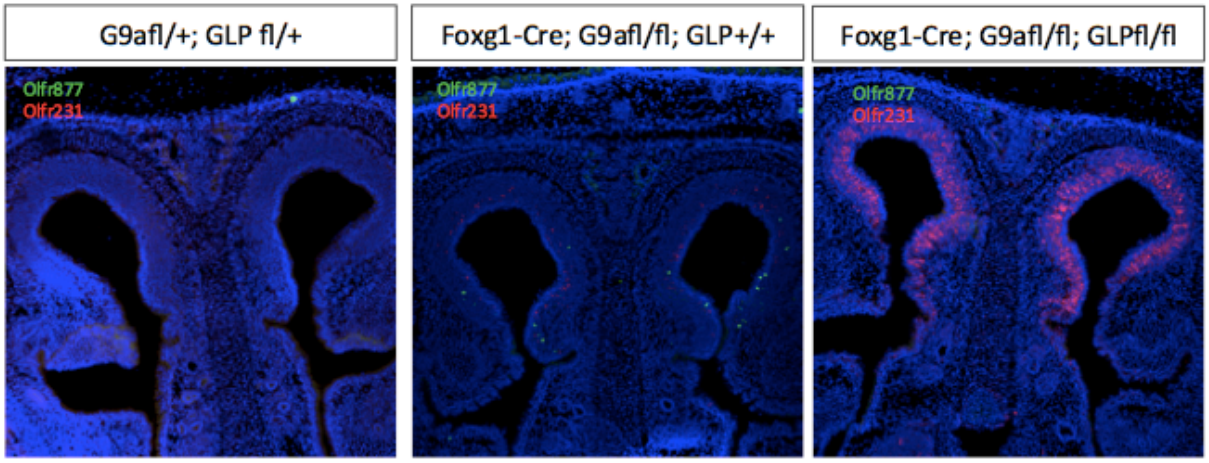
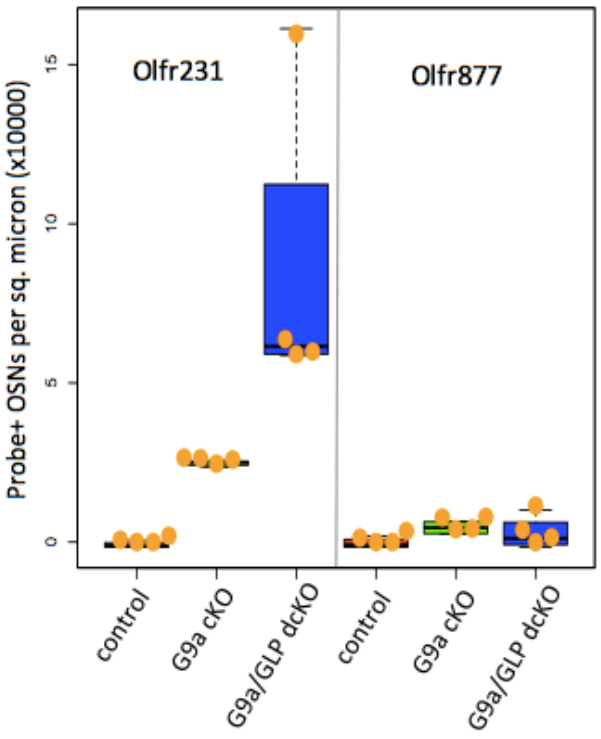

B
G9a flox/flox
OMP-Cre/+; G9a flox/flox
MOR28
Olfr231
OMP

Figure 5. Post-mitotic removal of G9a does not lead to a discernable phenotype.

A. Immunofluorescence for modified H3K9 residues associated with gene repression and deposited by G9a. Me3 H3K9 (green) and Me2 (red) in the control E18.5 MOE. DAPI, blue.

B. OMP-Cre mediated deletion of G9a (right panels) does not alter the relative abundance of MOR28+ OSNs unlike the Foxg1-Cre mediated deletion. Olfr231 ISH and OMP ISH both are normal in the absence of G9a in the mature OSN as well. Animals are PND30.


G9afl/+; GLP fl/+
Foxg1-Cre; G9afl/fl; GLP+/+
Foxg1-Cre; G9afl/fl; GLPfl/fl
Olfr877
Olfr231


Olfr231
Olfr877
Probe+ OSNs per sq. micron (x10000)
0
5
10
15
control
G9a cKO
G9a/GLP dcKO
control
G9a cKO
G9a/GLP dcKO

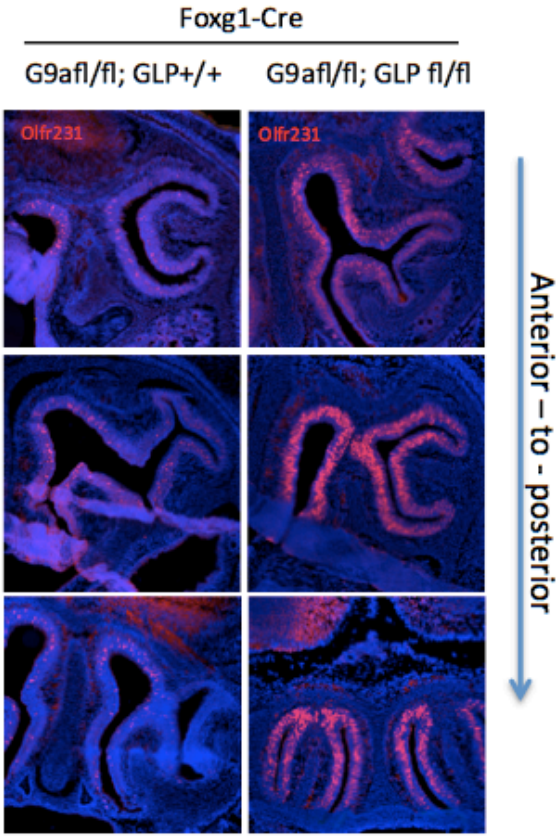

Foxg1-Cre
G9afl/fl; GLP+/+
G9afl/fl; GLP fl/fl
Olfr231
Olfr231
Anterior – to - posterior

Figure 6. GLP contributes to the stochastic choice of ORs.

A. G9a/GLP double knockout MOE at E18.5 with 2-color RNA FISH for Olfr231 (red) and Olfr877 (green). Leftmost panel is Cre-negative. Center panel is G9a cKO only, while right panel is the double cKO. Note the increase in Olfr231 and the concomitant decrease in Olfr877.

B. Quantification of the number of Olfr231+ or Olfr877+ OSNs in the three genotypes from (A). Note large increase in Olfr231 prevalence in the cdKO. OR+ OSN were counted per section for 4 different sections.

C. Olfr231 is expanded into all MOE zones in the absence of both methyltransferases. Topmost panels show more anterior sections, while lower panels represent more posterior sections, illustrating three regions from anterior-to-posterior order. Olfr231 mRNA is shown in red.

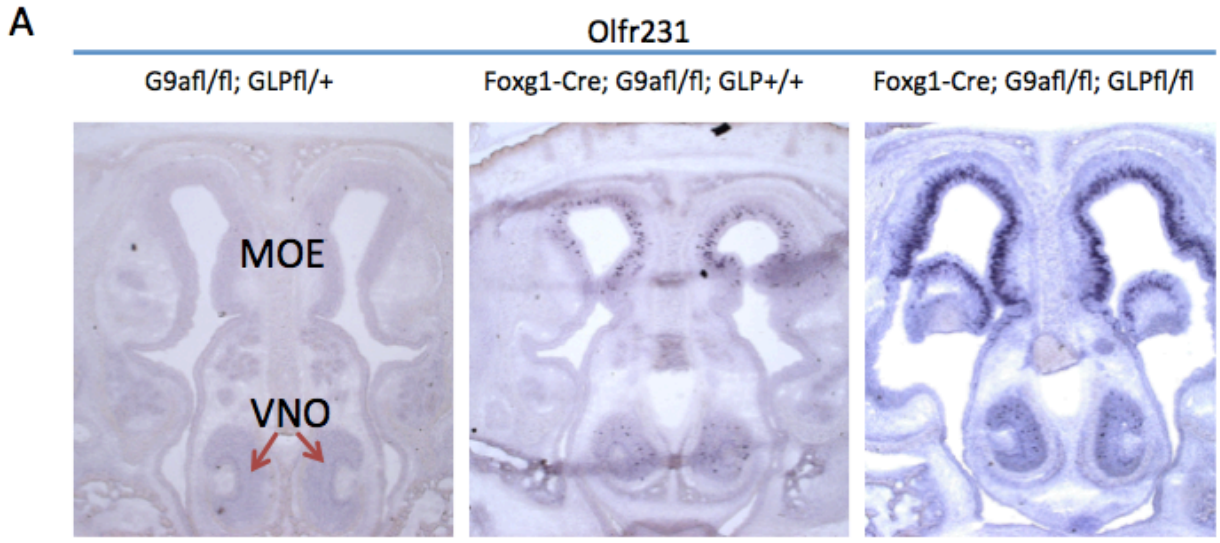


Figure 7. VNO-specific misexpression of Olfr231 in the G9a/GLP double knockout.

G9a/GLP double knockout MOE at E18.5 with ISH for Olfr231 to highlight the increase in VNO-specific expression of this OR. Leftmost panel is Cre-negative. Center panel is G9a cKO only, right panel is the double cKO.

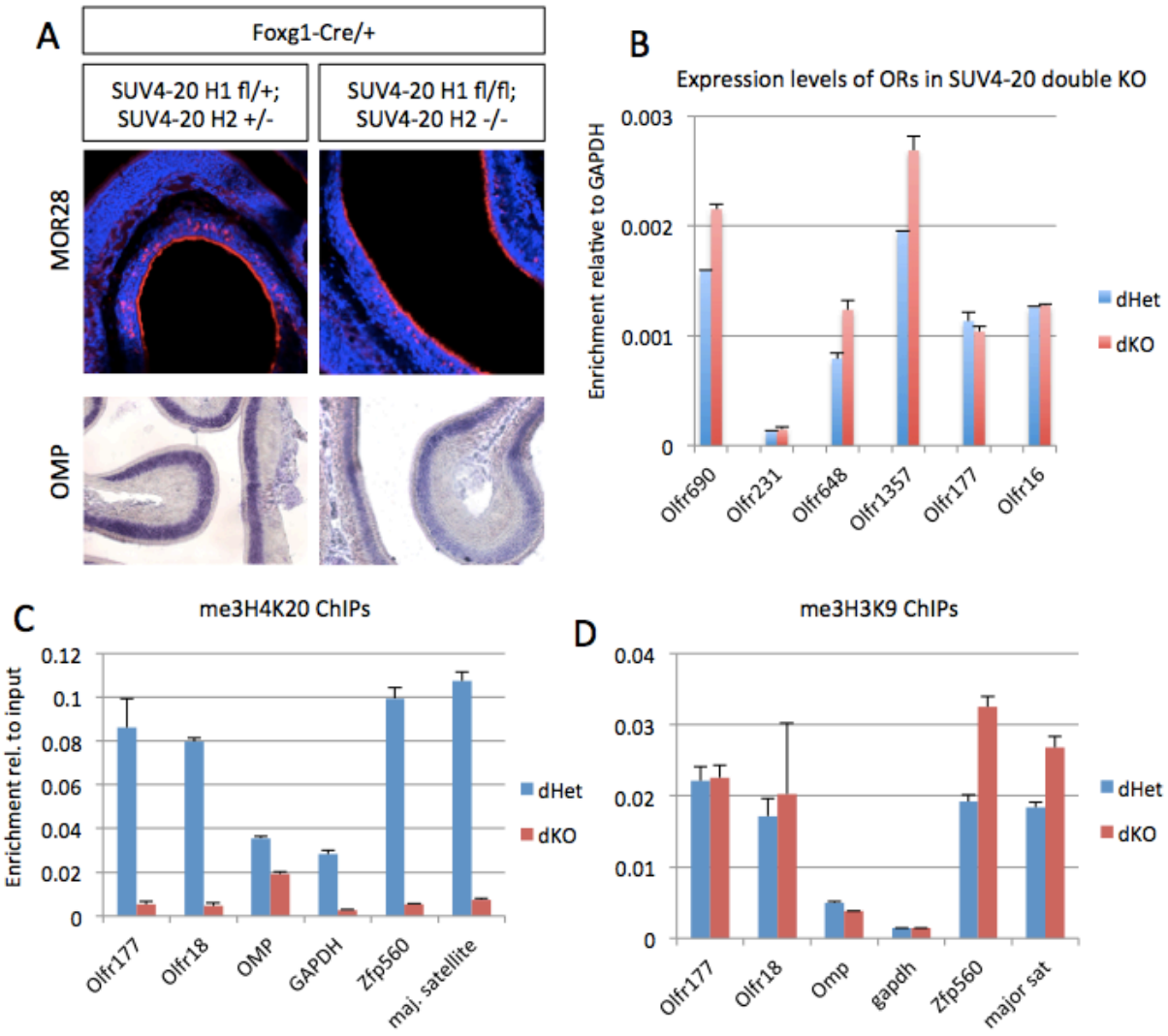


Figure 8. H4K20 methylation is dispensible for the random choice mechanism.

A. MOR28 immunofluorescence in PND30 SUV4-20 H1 flox/+; -H2 +/- (left panels) and double knockout (right panels) MOEs. Distribution and frequency of MOR28+ OSNs is normal in these mutant animals. OMP ISH (bottom panels) shows no differences between the two genotypes.

B. RT-qPCR on cDNA generated from PND30 control (double heterozygote, dHet) and double knockout (dKO) MOEs normalized to GAPDH. No significant differences were found. Note the very low expression level of Olfr231 in the adult MOE. Error bars are SEM, PCR performed in duplicate.

C. Chromatin immunoprecipitation for me3 histone H4K20 in the PND30 MOE. Error bars are SEM, PCR performed in duplicate.

D. Chromatin immunoprecipitation for me3 histone H3K9 in the PND30 MOE. Note lack of reduction for this mark. Compare to Figure 1. Error bars are SEM, PCR performed in duplicate.

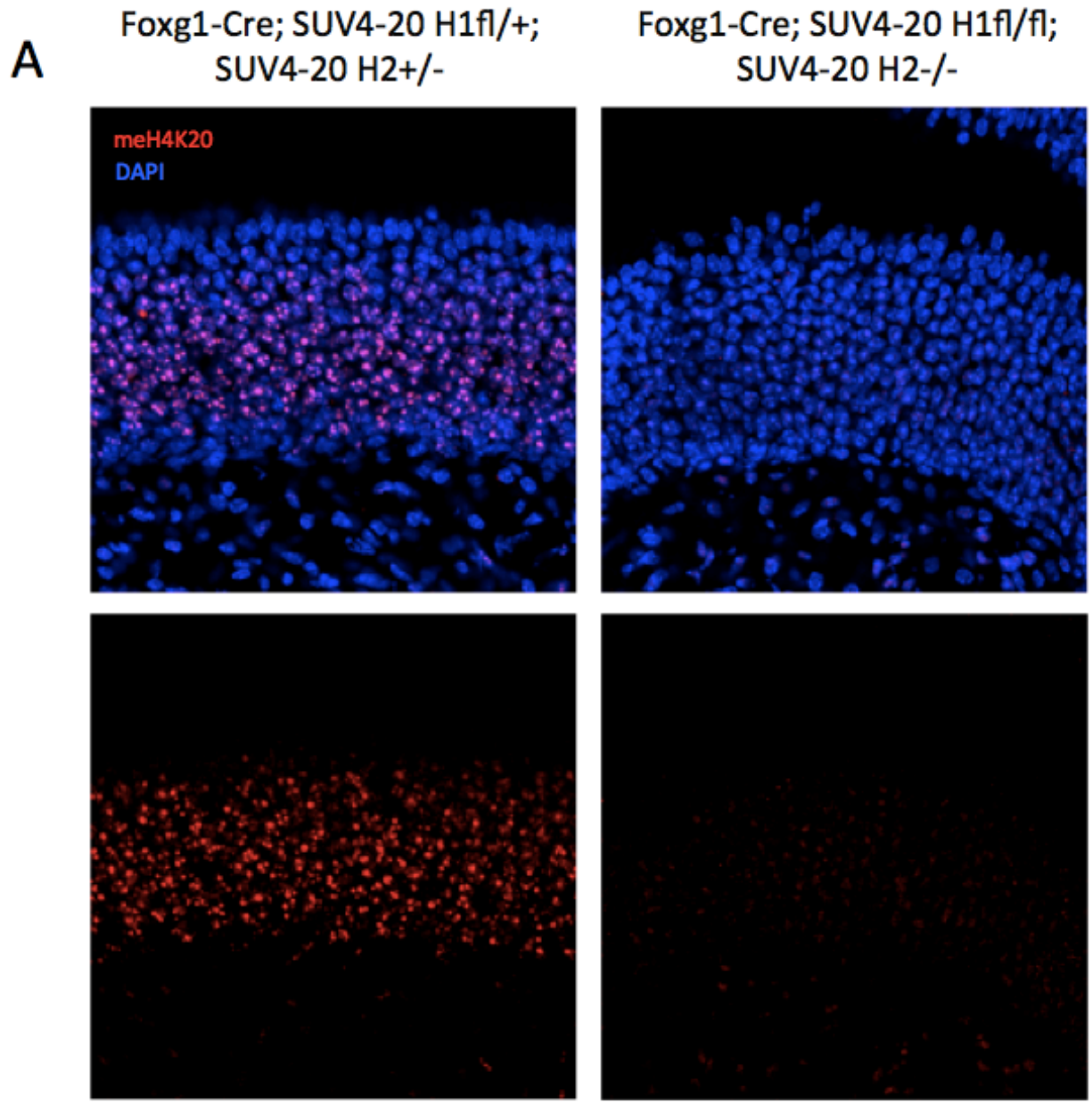


Figure 9. SUV4-20 enzymes are responsible for the vast majority of K20 trimethylation in the MOE.

A. Immunofluorescence for Me3H4K20 (red) in PND30 SUV4-20 H1 flox/+; -H2 +/- (left panels) and double knockout (right panels) MOEs. DAPI, blue. Marked decrease in Me3K20 is observed with some residual signal lingering in the DAPI focus of some OSNs, perhaps remaining from prior to the Cre activity.

Experimental procedures for Chapter 4

Mice and strains used

All mice were housed in standard conditions with a 12-hour 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. Mouse strains used are as follows: Foxg1-Cre (Hébert & McConnell, 2000), OMP-IRES-Cre (Eggan et al., 2004), G9a flox and GLP flox (Tachibana et al., 2005), SUV4-20H1 flox and SUV4-20H2 knockout (Schotta et al., 2004).

Chromatin and DNA Immunoprecipitation

Performed as described in Magklara et al. (2011).

DNA Deep Sequencing

Sequencing was performed on the Illumina HiSeq 2000. cDNA libraries for both G9a cKO and control MOE were prepared with the ScriptSeq kit V2 (Epicentre), with total mappable reads as follows: G9a control: 69,620,496 and G9a cKO: 73,023,480.

In situ hybridization and immunofluorescence

Performed as described in Lyons et al. (2013). For 2 color ISH, both probes were detected using TSA signal amplification (Perkin Elmer) with a 30% hydrogen peroxide wash between development steps. Olfr231 and Olfr877 RNA probes were generated from a plasmid containing the entire ORF of these genes. Me3H3K9 antibody (abcam), Me2H3K9 (abcam), and Me3H4K20 (abcam) were all used at a 1:1000 dilution. G9a/Ehmt2 antibody (R&D Diagnostics) was used at 1:500. VGLUT2 antibody (Millipore) was used at 1:3000.

Chapter 6: The making of a stochastic system—evidence and speculation

Of all of the features that make olfaction a unique sensory modality, the genomics of the olfactory receptor gene family is perhaps the most striking. Together with the "one receptor per neuron" rule, we see a system that relies largely on chance to generate an exquisitely sensitive peripheral sense organ charged with detecting vast ranges of innumerable chemical moieties. The complexity of the neural outcomes elicited by the act of smelling is mirrored by what appears to be the daunting task of regulating tens of hundreds of olfactory receptor alleles, yet as I have begun to show with the work in the preceding chapters, this potential regulatory complexity can be distilled into a few key parameters which allow the OR genes and perhaps other monoallelic systems to behave in the stochastic way that they do.

I. Clustered genes – prehistoric signatures of a transposon-mediated gene family expansion

OR genes are nearly exclusively found in cluster, as mentioned in Chapter 1. These clusters are critical determinants in how ORs are regulated and tell us that these gene duplications were most likely a result of transposition events (Kambere & Lane, 2009). The clusters, of which there are roughly 50 in the haploid mouse genome, typically contain one or more enhancers (Eirene Markenscoff-Papdimitriou, unpublished data), which is able to control or influence expression of many OR genes. Thus the clusters assist in centralizing transcriptional power to a

single seat, the locus-specific enhancer, which can then provide a local singularity to which the receptor gene promoter will randomly interact with via chromosomal bending locally.

II. Facultatively heterochromatinized genes

Furthermore, the clustered layout allows for OR genes to be the subject of repression via pre-existing mechanisms of heterochromatin spreading, such as self-polymerization observed for Cbx1. If the OR genes each had their own unique enhancer and resided in a typical euchromatic region, control of its expression in a monogenic and monoallelic fashion could not depend on heterochromatin spreading without invoking the need for some insulators of this repressive spread. The complexity of shutting down ORs in the precursor cells of the OSN lineage would be enormous, time-consuming, and energy intensive.

The mammalian genome is well-equipped to repress newly transposed DNA, via DNA methylation and repressive histone modification. The fact that ORs are always nested in a region rich with non-LTR retrotransposons suggests that the cell allowed for its natural response to the invasion to take place but then turned the system around in its favor to keep non-chosen OR gene transcription to a minimum. Given that G9a and GLP are key mediators of this repression, it is interesting to note that they are typically not involved in true pericentromeric heterochromatin maintenance; this is instead fulfilled by members of the SU-VAR family of SET-domain-containing proteins, most importantly SUV39H1 and –H2. The facultative heterochromatin we observe at the quasi-heterochromatic regions containing OR genes are accessed and repressed by G9a and GLP, making them a unique domain of the

genome. Interestingly, the G9a protein itself contains a "reader" motif termed an ankyrin repeat which allows G9a protein to bind its own K9me (Collins & Cheng, 2010). It is not clear how G9a is targeted to OR genes to begin with, but it could be simply via K9me recognition following cell division. The larger question relates to how the ORs are repressed to the trimethyl state when G9a and GLP are only capable of generating dimethyl lysine. Nonetheless, the clustering of OR genes no doubt assists in the efficacy of a protein like G9a in the efficient spreading of its repressive methyl-mark, and this clustering is thus not simply an artifact or little importance. Rather the clusters allow G9a to rapidly generate the template from which stochastic transcriptional activation can proceed, as directed by enhancer/promoter interactions that likely involve the concentration of histone modifying enzymes such as LSD1.

III. Information-poor promoters

The usefulness of gene clustering is also seen by the fact that the genes behave as meristic elements with roughly equivalent affinities for activating transcription factors. The OR gene promoters are notoriously information-poor (Clowney et al., 2011; Michaloski et al., 2011) and possess homeodomain and O/E motifs that are common to nearly all OR gene promoters, thus providing little differentiability. The removal of Lhx2 from the mouse MOE reveals for instance that this factor is required for the activation of all class II OR genes, while revealing nothing about the way in which a single OR gene is chosen or stabilized from the heterochromatin environment (Hirota & Mombaerts, 2004).

The repetitive nature of OR genes with similar promoters in a cluster means that a given OR will have a similar likelihood of being activated if they were all exposed to same limiting concentration of activator. This model seems plausible for the expression of OR genes at their origin, such that a non-deterministic mechanism could be fulfilled over a small number of highly unique OR genes, such as are seen in zebrafish, especially given that zebrafish seem to tolerate multiple OR gene expression in a given OSN (Sato et al. 2007). However in an OSN where nearly 3000 alleles are competing for a single transcription factor to drive activation, the attractiveness of this model declines. A more reasonable hypothesis is that this transcription factor is made available to the region where the heterochromatin has been cleared away by LSD1 and other histone modifiers. The equality of the promoters in this setting suggests that relatively large fluctuations in critical transcription factors will be tolerated and so control at this locus can be relaxed. This would make sense because, in the case of Lhx2, it is needed across the genome in the CNS to diversify cell types and tuning it down in OSN lineage would provide a regulatory impediment. The singularity of monoallelic expression is instead more robust when heterochromatin is recruited and the promoters are very similar, allowing for a single time frame during which LSD1 is active and access to the OR promoter is possible.

A hypothesis on the role of 8-oxoguanosine in OR choice

A more controversial idea is that this localization of LSD1 to a focal part of nucleus by virtue of its interaction with the enhancer permits a truly stochastic process to occur: LSD1-mediated

histone demethylation triggers the small-scale evolution of hydrogen peroxide and this peroxide triggers the conversion of guanosine to 8-oxoguanosine essentially at random. Another reason to house the ORs in clusters would be to facilitate the truly random base oxidation caused by LSD1, which would then trigger the recruitment of an as-yet-to-be-determined factor which could drive the opening of the chromatin to allow robust transcriptional activation. Although there is some bias for oxidation of guanosine in a particular nucleotide context, the oxidation would be theoretically far more random than the interactions of large protein demethylase complexes with chromatin. Given that ORs are centralized in the immature neuron into a small number of foci, this could also explain the choice of ORs that are very distant from enhancers or for ORs that apparently lack enhancers altogether. A characteristic that would have clear selective advantage in nature is anything that would increase the ability of the organism to detect subtle differences in the enviroment, and so it is not a far stretch to imagine that such a potentially destructive system would have arose to permit monoallelic expression of the OR gene family.

The fact that LSD1 activity is tightly linked with the expression of Adcy3, a protein that is expressed only after the OR is recognized as functional, supports the notion that the cell has evolved means to detect damage to OR genes that could render it non-functional. By tying this ER quality control checkpoint into the feedback, LSD1 mediated activation can be vetted by ensuring that the transcript is encoding a functional OR from a DNA template that is not permanently mutagenized from the 8-oxoguanosine, just as the feedback detects pseudogenes. Similarly, mRNA damage caused by this peroxide could be overlooked if the OR CDS maintains its integrity in the message, meaning that small, insignificant damage would be tolerated, while

protein altering changes would likely not be permitted, especially if they affected major aspects of OR topology or secondary modification such as N-glycosylation. In other words, 8-oxoguanosine-driven RNA modifications on the OR transcript would likely be tolerated because they would by default be randomly located along the molecule. DNA oxidation on the other hand would generate a modified template, driving the incorporation of adenosine rNTPs complementary to the oxidized DNA guanosine for the life of the neuron or as long as the base modification remains.

A dual role for olfaction in speciation

Regardless of the role of 8-oxoguanosine mutagenesis on OR diversity, the extreme diversity of OR genes across relative narrow phylogenetic constraints, for instance within the Rodents, suggests there is a mechanism to ensure that OR genes are allowed to "explore" mutational space more than average proteins. Additionally, it appears that even very "useful" ORs (that is ORs that detect odorants which are most likely to be important in the fitness of the organism such as noxious compounds) are summarily pseudogenized (Gilad, Man, Pääbo, & Lancet, 2003).

However, the OR genes are the ultimate case of genetic redundancy: the binding affinity for a particular organic compound for a given receptor is likely only slightly higher than the next best receptor. This redundancy is thus critical for the ability of the OR genes to come and go in their "boom and bust" cycle of expansion and contraction at the whim of transposon mobilization. This exploratory phenomenon has been rendered possible by the combination of

transposition along with the negative feedback mechanism in place in the OSN. ORs that are more highly relied upon by other organs such as the kidney and sperm are often the most highly conserved through phylogenetic space (De la Cruz et al., 2009). For the typical OR gene which is deployed in the MOE only, the only selective forces which is of relevance is whether the protein can trigger the feedback. The level of redundancy is so great that the selective advantage conferred by the gain of a new receptor is likely minimal except in extraordinary circumstances. However, due to the nature of OR selection and the likely enzymatic kinetics involved in altering the chromatin landscape to access the OR-to-be-activated, it is advantageous to have a very large number of OR genes so as to maximize the likelihood of an efficient initial selection process. The ORs are located very broadly throughout the mouse genome, on all chromosomes except for 3 autosomes and the Y chromosome. In this pattern of genome population by the ORs, what comes to mind is the notion of "the selfish gene." The OR gene itself is the unit on which selection is acting, and its deployment in the OSN guarantees at least that a new OR gene will not be selected against.

In the extraordinary circumstances to which I refer to above, it seems highly probable that the novel OR genes could be providing in circumstances of population separation a relatively rapid speciation mechanism. There are the obvious potential mechanisms that would drive speciation, such as the ability of newly pseudogenized OR genes to block productive reintegration of separated populations. A perhaps more radical yet far more realistic possibility, is that the expansion and contraction of the OR genes which happens, to our knowledge, at random during the passage of time, drives the genomes of separated populations apart. In contrast to the standard model of population divergence, whereby the

genes that are needed for male/female compatibility are lost, I posit that the expansion and contraction of the OR subgenome by transposition or otherwise, is playing a key role in reproductive isolation by virtue of the altered chromatin territories and a resulting chromosomal incompatibility during meiosis. As an example, the OR gene divergence across human ethnicities, which are all highly related relative to natural occuring mammalian populations across the world, are very divergent above background. The high frequency of copy number variants in OR genes compared to non-OR genes in the human population (Young et al., 2008) for instance supports a role for OR gene diversity in generating genomic diversity that hypothetically could lead to reproductive isolation if given many thousands of generations of separation.

The OR gene family thus provided one of many places to begin mammalian evolution in the early-to-mid Mesozoic Era, helping to diversify the OSN population and drive the expansion of the forebrain. How this exactly was facilitated in the mammalian and not other lineages remains unclear, as some amphibians possess over 1000 OR genes (Ji et al. 2009). The general trend however is that the very massive OR expansion occurred in the mammalian lineage, and these early OR subgenomes have devolved and re-evolved since then. The plasticity in the OR subgenome is tolerated in part because of the OSN feedback, as well as by a mysterious force that may be driving populations apart and thus helping to force stochastic speciation events during relatively short periods of genetic isolation. This unknown mechanism may be the reactivation of quiescent transposons associated with OR genes or perhaps it may involve replicative errors (Arlt, Wilson, & Glover, 2012), but the generation of many thousands of

mammalian species in the Mesozoic may have been assisted in large part to the unique properties of OR genes.

Concluding remarks

Olfaction is a remarkable peripheral sense. In contrast to the more familiar modality of vision, in which coherent properties of a finite spectrum of electromagnetism are detected by fewer than half a dozen GPCRs, olfaction is charged with perceiving the unimaginable complexity of the small molecules at a broad range of different concentrations (Can Güven & Laska, 2012). There are differential neuronal responses to compounds of identical structure that differ to the slightest possible degree. For instance, compounds synthesized from deuterium rather than the lighter hydrogen can be differentiated between (however slight the perceived difference (Franco et al. 2011; Hara, 1977). Enantiomers of the same compound elicit distinctly different response profiles (Brookes, Horsfield, & Stoneham, 2009). The number of organic compounds that could be detected is so vast that it is essentially unknowable (Touhara & Vosshall, 2009).

Olfaction thus must convey to the brain a collection of information about what properties a molecule possesses. This property or set of properties is conveyed to a subset of glomeruli, where the stereotypical neuronal activity which represents a given odor across members of a given species. This pattern of glomerular activation, which could be thought of as the first "impression" or image of the smell, is then passed to the next set of neurons in olfactory cortex. The pattern is nearly completely lost here; only very vague partitioning occurs. The brain thus takes advantage of associative nature of recollection to link a unique set

of neurons in the cortex to a stereotyped pattern in the olfactory bulb. Thus each individual's perception of an odor is almost certainly unique to that individual. The interpretation of the world is made without any conscious effort, but then the activity of higher brain centers redefines what is provided into a unique signature and attempts to link the incoming information to memories of other sensory experiences. This explains how a mouse with only a single OR in the vast majority of its MOE can detect a variety of odors (Fleischmann et al. 2008): the sense is purely associative in the cortical centers.

Central to this reinterpretation by the brain of the odorant world is the reliably robust odorant-induced glomeruli activity. The colors of the world are the same due the physical properties of light, while the constant in olfaction is a staggering array of chemical forms. Thus in the face of extreme molecular diversity, where there are few if any constants, it is critical to provide a singular identity to the olfactory sensory neuron. Were the OSN to change affinities midlife, the resulting neuronal response to odor X would potentially shift to the profile of more akin to that of odor Y. This switching is incompatible with survival, as the smell of roses is pleasant and non-toxic, while the smell of cadaverine is noxious. The identities provided by the OSN to the outside world must remain constant. Furthermore, the singularity of expression is a necessity because the alleles are often different, such that maternal allele that would detect odor X may be complementary to paternal allele that does not detect odor X as well or at the same affinity. In extreme cases of divergence between two alleles, the targeting of the bulb is different between OSNs expressing the different alleles. Thus switching between alleles would also be detrimental to survival. The stable choice of a single OR gene is the critical determinant of our interpretation of the outside odorant world and establishes a set of neural constants in

the olfactory bulb that the brain can then interpret in a variety of ways. The stability of information supplied by the olfactory system has evolved to rely on a chromatin-based system that has integrated an ER stress module to ensure the robustness of OR choice. The olfactory receptor stabilization pathway thus brings the transcriptional response to transposition into collaboration with another system designed to detect foreign proteins, the UPR. Allowing and investing in this high degree of evolutionary tinkering (Jacob, 1977) is not surprising given the likely critical importance of this modality to evolutionary success.

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