CHARACTERIZATION OF LYSINE-SPECIFIC DEMETHYLASE 1 AS A POTENTIAL OLFACTORY RECEPTOR REGULATOR IN THE DEVELOPING MOUSE OLFACTORY EPITHELIUM

By

Alyssa L. Savarino

Faculty Advisor: Dr. Robert P. Lane

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Abstract

Mouse olfactory sensory neurons (OSNs) express a variety of >1000 odorant receptor (OR) genes, and each OSN expresses only one OR from this large repertoire. The expressed OR lacks H3K9 tri-methylation (me3) and contains H3K4me3, while the opposite is true for the remaining silenced genes. A candidate demethylase, lysine-specific demethylase 1 (LSD1) has recently been shown to be linked to OR gene regulation, acting on either H3K4 or H3K9 methylation marks. We have shown that LSD1 protein exhibits variable organization in the nuclei of developing OSNs, consolidating into a single compartment at the edges of chromocenters in vivo; singular compartmentalization of the protein occurs within the nuclei of early post-mitotic p27Kip1-positive cells in the mouse olfactory epithelium (MOE). Using an immortalized cell line derived from the olfactory placode, we found that LSD1 also forms the singular compartment in vitro, consolidating in G1 phase. LSD1 consolidation occurs sequentially, exhibiting a poly-punctate phenotype prior to the formation single focus, observed both in vitro and in vivo. Based on DNA-immunoFISH studies, we found that LSD1 compartments associate with a small subset of OR genes at a high frequency. We show an invariable co-compartmentalization of LSD1 with CoREST by immunofluorescence, a complex which has been characterized in chromatin-modifying roles in both activation (removal of H3K9 methylation) or repression (removal of H3K4 methylation) depending on its binding partners; the association of the two proteins in
complex was also confirmed by co-immunoprecipitation. We hypothesize that this singularity may mediate monogenic OR expression. Further in vitro examination of DNA damage marks characteristic of LSD1 activity and histone marks that influence its substrate specificity provide insight on the function of this singularity. All together, our data suggest that the LSD1-CoREST complex forms a singular compartment during a narrow developmental time window concurrent with the timing of OR choice in maturing neurons in vivo.
Introduction

The olfactory system presents an interesting problem in gene regulation where specialized neurons in the nose detect odorants and convey the information to the brain to produce the sensation of smell. There is an enormous repertoire of olfactory receptor genes in the genome, yet a single gene expressed per olfactory sensory neuron. Thus a pressing question in the field of olfaction is: how does each sensory neuron express a single gene while keeping 99% of the genes off? This study addresses the singular expression pattern by investigating a candidate demethylase, LSD1, which may play a role in selection of a single olfactory receptor allele for transcriptional activation. This protein interestingly aggregates into a singular nuclear compartment, a singularity that may be key to the underlying epigenetic mechanism in the olfactory epithelium.

1.1 The cellular and molecular basics of olfaction

The odorant binding cascade causes olfactory perception

Olfactory perception involves the interaction of odorant ligands and olfactory receptors on the surface of olfactory sensory neurons. Mature olfactory sensory neurons (mOSNs) reside on the exterior of the olfactory epithelium lining the nasal turbinates and septum. These mOSNs are bipolar neurons that contain the olfactory receptors on ciliated dendrites with the other end of the unmyelinated axon extending to its final location in the olfactory bulb (Mombaerts 1999).
Olfactory receptor (OR) genes comprise the largest gene family in vertebrate genome (Buck and Axel 1991, Levy, Bakalyar et al. 1991). They are a type of G-protein coupled receptor (GPCR), which are seven-transmembrane proteins that activate an intracellular G-protein upon binding of odorant ligand. Once activated, the olfactory-specific G protein (denoted Golf) triggers an internal signaling cascade beginning with adenylate cyclase to convert ATP into cyclic AMP (cAMP) (Figure 1). The production of cAMP leads to the opening of cyclic-nucleotide-gated ion channels, consequently generating the action potential (Jones and Reed 1989, Kaneko 2001). Sensory information is communicated to the olfactory bulb by depolarization of the OSN triggered by odorant ligand binding to the olfactory receptor.
Figure 1. The olfactory transduction cascade. When an odorant molecular binds to the olfactory receptor (OR), it triggers G-protein signaling and activation of adenylate cyclase 3 (AC3). Activation of AC3 leads to the production of cyclic AMP (cAMP), which binds to the cyclic nucleotide-gate (CNG) channel that allows for the influx of sodium and calcium cations to depolarize the cell membrane. Interestingly, OSNs are characterized by high levels of cAMP in the soma to regulate the phosphorylation of crucial transcription factors for growth of OSNs (Zou, Chesler et al. 2009).
The mouse olfactory epithelium (MOE) cell lineage

The olfactory sensory epithelium consists of three major cell populations: basal cells that give rise to the neurons, the olfactory sensory neurons (OSNs) themselves, and sustentacular cells that provide support to the epithelium. The cell types will be discussed from most basal to apical, with the basal cells sitting above the lamina propria (Figure 2). First, the olfactory neuroepithelium contains a population of peripheral adult neuroglial progenitor stem cells that reside above the basal lamina of the tissue (Graziadei and Graziadei 1979); these multipotent basal cells are divided into two types, horizontal basal cells (HBCs) and globose basal cells (GBCs). The HBCs sit directly above the lamina propria and are the more specialized of the two progenitor cells, proliferating at a low rate (Carter, MacDonald et al. 2004). The globose basal cells (GBCs) are the small, round basal cells that proliferate rapidly giving rise to the rest of the epithelial cell types (Schwob 2002).

The second broad cell category is the olfactory sensory neuron (OSN). Within the interior of the epithelium, there is a subgroup of immature OSNs arising from the GBCs that represent a developmental progression towards the terminally differentiated mature OSNs (Schwob 2002). The post-mitotic immature OSNs have selected an olfactory receptor gene for expression (Fan and Ngai 2001), however they do not yet have extended cilia, characteristic of the mature neurons. The mature OSNs (mOSNs) are bipolar cells with dendrites that extend cilia to the surface of the OE and an unbranched, unmyelinated axon
extending into a glomerulus of the olfactory bulb. A glomerulus is a spherical structure within the olfactory bulb that is the first site of synaptic processing of the odorant information. The mOSNs are the most peripheral subtype of neurons and express olfactory marker protein (OMP) once terminally differentiated (Monti-Graziadei, Margolis et al. 1977).

The final cell type is the sustentacular (Sus) cell, which provides structural support to the epithelium. Sus cells are non-neuronal support cells coated with microvilli, as opposed to cilia on mature neurons. Sus cells are abundant with endoplasmic reticulum suggesting that they perform a role in detoxification of the OE and are capable of phagocytizing dead neurons (Schwob 2002).
Figure 2. Cell populations of the mouse olfactory epithelium. The horizontal basal cells (HBCs) and globose basal cells (GBCs) are the self-renewing stem cells of the population. GBCs are multipotent (GBC_{mpp}) and mitotically active to generate the other cell types of the epithelium. The transit amplifying (GBC_{ta}) and immediate neuronal precursor basal cells (GBC_{inp}) are in the process of differentiation to a neuronal status. The sustentacular cells (Sus) are the support cells of the epithelium. The mature OSNs (mOSNs) are the only cells that express OMP (Schwob 2002).
Axonal wiring of OSNs to the olfactory bulb

Studies of the spatial patterning of olfactory receptor expression in the MOE have shown that cells expressing the same olfactory receptor are restricted to four zones (Ressler, Sullivan et al. 1993, Vassar, Ngai et al. 1993). These zones are designated at the earliest stages of development, in the rodent embryo, before OR expression is detectable and prior to the formation of the olfactory bulb (Mombaerts 1999). Within a zone, neurons expressing different ORs are not segregated, instead appearing randomly organized (Iwema, Fang et al. 2004). However there exists an underlying organization of the axonal projections to the olfactory bulb (Figure 3). OSNs expressing a given olfactory receptor (OR) project their axons to converge upon one medial and one lateral glomerulus within each olfactory bulb (Mori, Mataga et al. 1992, Katoh, Koshimoto et al. 1993). When the OSNs are ablated, the epithelium does not precisely repopulate the previous ablated OSN types, meaning that not exactly the same OR expression is recapitulated (Gogos, Osborne et al. 2000). Experiments that substitute the coding sequence of an OR into another OR sequence revealed a change in axonal targeting to the olfactory bulb (Mombaerts, Wang et al. 1996).

This suggests a role for the odorant receptor itself in the instruction of axonal guidance to distinct glomeruli. It has been proposed that there exists a set of molecules that guide the axons to the distinct glomeruli to develop this glomerular map of the olfactory system (Serizawa, Miyamichi et al. 2006). The
OR proteins function to detect these signal molecules and form the appropriate axonal bundles, or glomeruli, in response. The signal molecules were identified as OR-derived cAMP signals, regulating the anterior-posterior topography of the axonal projections (Imai, Suzuki et al. 2006, Imai and Sakano 2007). Thus, the proper wiring of OSNs is governed by the particular OR that is expressed in each individual neuron (Serizawa, Miyamichi et al. 2003), which is determined during development of the epithelium. It is clear that the choice of a single OR per neuron is the genetic basis for the OR-instructed projection of axons, but the genetic mechanism behind monogenic OR expression has yet to be elucidated.
Figure 3. OSN axonal convergence to the olfactory bulb. Schematic illustrating the convergence of axons from olfactory sensory neurons expressing the same odorant receptor to the same glomerulus in the olfactory bulb. The olfactory receptors, depicted in blue/red/green/purple, are located on the surface of the olfactory epithelium in the turbinates of the nose wiring back to the appropriate glomeruli in the olfactory bulb of the brain (Dulac and Torello 2003).
1.2 **Olfactory receptor gene regulation**

*Mutually exclusive OR gene expression*

The olfactory receptor (OR) gene family, one of the largest known families of genes in the vertebrate genome, is comprised of more genes than even the immunoglobulin gene family (Glusman, Yanai et al. 2001). These genes exist in large clusters throughout the genome (Reed, Bakalyar et al. 1992, Ben-Arie, Lancet et al. 1994). ORs are encoded by intron-less coding regions and are highly expressed in each individual OSN (Mombaerts 1999). It has been estimated that approximately 1000 copies of the OR mRNA exist in a single neuron (Mombaerts 1999). Remarkably, only a single OR gene is expressed in each individual neuron. Moreover, it has also been demonstrated that the expression of OR genes is monoallelic; only one of the two alleles corresponding to an OR gene is expressed in any given OSN (Chess, Simon et al. 1994). As the transcription of a single OR gene determines the response specificity to an odorant stimulus and the axonal targeting to a glomerulus, the monogenic and monoallelic transcription of an OR gene is at the core of the olfactory circuit.

The monoallelic expression of ORs was demonstrated when it was observed that the two alleles encoding a certain OR replicate asynchronously (Chess, Simon et al. 1994), which is characteristic of allelic inactivation of one of the two alleles for a gene. Later mutually exclusive OR expression confirmed that when copies of identical OR transgenes were introduced, which should theoretically bind exactly the same transcription factors, only a single transgene
was expressed (Serizawa, Ishii et al. 2000). Additionally, expression of either the transgenic or endogenous allele led to separate axonal wiring to glomeruli (Ishii, Serizawa et al. 2001). Mutually exclusive expression of the transgenes experiment also verified that the mechanism behind monoallelic expression did not solely involve a cis-regulatory sequence since the same upstream sequences were present in each transgene, yet only one was expressed per cell.

“Minigenes” were shown to be functional units of expression (~9 kb) paralleling the characteristics of actively expressed endogenous OR genes (Vassalli, Rothman et al. 2002). Such transgene-expressing OSNs are phenotypically identical to mature OSNs, exhibiting OSN-specific expression that was punctate (present in non-adjacent cells), located in the appropriate MOE zone, and monoallelic. The smallest transgene (minigene) studied that was able to recapitulate the endogenous OR expression required an upstream region for expression; this indicated a role for proximal control regions in addition to the results that suggested a role for long-ranging enhancers acting in trans.

A distant OR enhancer was identified, called the H-region, that was found to exclusively interact with the transcriptionally active OR locus in trans (Lomvardas, Barnea et al. 2006). This predicated the development of a stochastic model for OR genes to compete for a single complex acting in trans that worked with the H-region, or a similar enhancer yet to be identified, to select an OR. When the H-region was knocked out, there was no resultant loss in global OR gene transcription indicating that there must be other unidentified
OR enhancers at play (Fuss, Celik et al. 2007, Nishizumi, Kumasaka et al. 2007). Another OR enhancer, the P-element, has been identified as a distant element that participates in OR transcription (Bozza, Vassalli et al. 2009). Many other candidate OR enhancers have recently been identified (Markenscoff-Papadimitriou, Allen et al. 2014) and may interact with a complex in trans. Importantly, enhancers like the H- and P-elements only regulate the likelihood that a particular OR gene be selected; these enhancers elements do not affect overall OR transcript levels (Khan, Vaes et al. 2011).

A transcription factor for olfactory receptors: Lhx2

In search of the mechanism that liberates a single OR to be expressed, the field has focused on singular OR choice at the transcriptional level. In parallel to examination of cis-regulatory elements, the field has evaluated trans-factors that bind OR promoters. One such trans-factor that has been identified is Lhx2, expressed in the olfactory stem cell progenitors and neurons (Kolterud, Alenius et al. 2004, Rodriguez 2013). Homeodomain sites were identified in the promoter and upstream regions of many OR genes. Lhx2 is a LIM-homeobox transcription factor that binds to the homeodomain site of the promoter for OR M71 (Hirota and Mombaerts 2004). Lhx2 mRNA is highly abundant in OSNs and found to be the most common binding partner of OR promoters among eight candidate transcription factors (Hirota and Mombaerts 2004, McClintock 2010).
Lhx2-knockout mice die \textit{in utero} due to its essential role in normal embryonic brain (forebrain, eyes, and olfactory system) and liver development (Lee, Brenner et al. 2011). Inactivation of Lhx2 in OSNs led to a cell autonomous defect in OSN axons not innervating their targets in the olfactory bulb (Berghard, Hagglund et al. 2012). Since the OR gene choice and subsequent production of OR protein is critical in the wiring of axons to the appropriate glomeruli (Serizawa, Miyamichi et al. 2003), it seems that Lhx2 is probably part of the OR selection process as its absence causes a defect in axonal projections. In Lhx2-deficient mice, the morphology of the olfactory epithelium looks normal, yet there is no expression of OMP suggesting that there are no mature OSNs (Hirota and Mombaerts 2004). Thus without Lhx2, immature neurons cannot terminally differentiate, perhaps because an OR has not been selected for expression.

\textit{The spatial organization of OR genes due to epigenetic marks}

The monoallelic nature of OR expression along with the fact that OR promoters share common regulatory elements suggest that the mechanism regulating OR expression is more complex than simple regulation by transcription factors that turn on or off the expression of a gene by binding to a \textit{cis} regulatory sequence. An “epigenetic” mechanism, where a modification independent of DNA sequence influences gene expression, may allow one allele of a gene to be consistently expressed while the (perhaps identical) allele on the homologous chromosome is consistently repressed, as well as other non-homologous alleles in the genome.
OR genes are subject to the kinds of chromatin marks that can mediate such epigenetic mechanisms: the transcribed OR allele contains an H3K4me3 mark, a signature associated with transcriptionally active genes (Magklara, Yen et al. 2011). The non-transcribed OR genes exhibit H4K20me3 and H3K9me3 marks (Magklara, Yen et al. 2011). It was shown that these heterochromatic marks appear before OR choice, meaning the initial selection of an OR. This suggests a general model where OR regulation commences with a widespread early developmental silencing of all OR genes (i.e. before any OR genes are expressed) as a basis for monogenic OR expression (model discussed in section 1.3; see Figure 4).

The spatial organization of the OR genes within the nucleus appears to be different between expressed and non-expressed loci (Armelin-Correa, Gutiyma et al. 2014) due to the documented epigenetic states of the loci. Active alleles exist in the euchromatic (mildly compact chromatin that is actively transcribed) domains while silent OR genes reside in the heterochromatic (densely packed chromatin preventing transcription) regions. The non-transcribed OR genes are also more centrally located within the nuclei, contrary to the common aggregation of silenced genes to the nuclear periphery. Relating this to the localization of homologous OR alleles, it was determined that the two different alleles exist separately, one in the constitutively heterochromatic chromocenter and one in the surrounding facultative chromatin (Armelin-Correa, Gutiyma et al. 2014).
The three dimensional nuclear organization of the wildtype nuclei demonstrates that mature olfactory neurons are unique in exhibiting a nuclear architecture with a single large mass of constitutive heterochromatin termed the chromocenter (Clowney, LeGros et al. 2012). Constitutive heterochromatin is composed of major satellite and repetitive DNA sequences enriched for H3K9 tri-methylation and H4K20 tri-methylation, remaining compacted throughout the cell cycle to maintain silencing (Campos and Reinberg 2009, Almouzni and Probst 2011). Areas of facultative heterochromatin are localized around the central chromocenter and nuclear periphery (Ruault, Dubarry et al. 2008). Facultative heterochromatin is a form of reversible heterochromatin enriched for H3K27 methylation, subject to developmentally regulated decompaction (Trojer and Reinberg 2007, Ruault, Dubarry et al. 2008). The chromatin compaction within heterochromatic compartments prevents its association with RNA polymerase factories, and thus the transcription of these sequences (Markaki, Gunkel et al. 2010).

The organization of the chromatin in this way (besides the obvious epigenetic marks) is likely due to the lamin B receptor (LBR) protein which is critical to OR regulation (Clowney, LeGros et al. 2012). In most cell types other than those of the olfactory epithelium, LBR exhibits a peripheral organization in the nucleus to maintain heterochromatic organization tethered to the nuclear lamina (Makatsori, Kouroumli et al. 2004, Hirano, Hizume et al. 2012, Kind, Pagie et al. 2013). LBR is developmentally regulated in the MOE involving its down-
regulation throughout OSN differentiation (Clowney, LeGros et al. 2012). Loss of LBR as differentiation progresses is accompanied by the internalization of heterochromatin from the nuclear periphery to the aforementioned internal nuclear chromocenters. Studies of LBR loss-of-function mutations leads to OR aggregation in basal and Sus cells, thus disrupting the later interchromosomal aggregation of OR genes in mOSNs. When LBR is engineered to be expressed in mOSNs, there is a resultant disruption in the aggregation of OR foci (Clowney, LeGros et al. 2012).

1.3  

**A model for OR “choice”**

*Proposed model for mutually exclusive OR transcription: “silence all, de-repress one”*

The consensus model for the regulation of OR genes in a monoallelic fashion involves the initial silencing of all OR gene loci followed by a de-repression event on a single OR locus. Yet this model does not distinguish cause-effect relationships (i.e. it is unclear how the initial silencing effect is produced or when in the process the de-repression event is occurring). This model aligns with the epigenetic marks observed on active and inactive OR gene loci as all inactive alleles contain H3K9me3 while the singular active allele is marked with H3K4me3. The foundation of this model is built by three important observations. First, as mentioned, the repressive tri-methylation of histone-3 lysine-9 is widespread early in the OSN lineage (basal cells and immature
neurons) before OR selection (Magklara, Yen et al. 2011). Such repressive marks are not observed on the single transcribed OR allele later in development.

Second, the deletion of the candidate protein lysine-specific demethylase 1 (LSD1; to be discussed at length in Section 1.5), which may be involved in the removal of H3K9 methylation, causes global impairment in OR expression (Lyons, Allen et al. 2013). Third, all non-expressed (inactive) OR genes reside in the heterochromatic chromocenters in mature OSNs, thus maintaining the widespread silenced state and prevents OR genes from interacting with active RNA polymerase II (Clowney, LeGros et al. 2012).

The proposed series of events is still a working model as only one of the mechanisms behind these assumptions is known. It was unclear how the H3K9me3 marks are initially distributed to all OR loci, which is the proposed mark producing the ground state of silencing before OR selection. This silencing methylation mark makes the OR genes inaccessible to polymerase factories as they are confined to heterochromatic chromocenters. Recently histone methyltransferases, G9a and GLP, both responsible for heterochromatic silencing, were shown to be vital for stochastic and monogenic expression of ORs (Lyons, Magklara et al. 2014). This study reaffirmed that H3K9 methylation marks on these OR loci lead to their localization to constitutive heterochromatin, an epigenetic platform to regulate mutually exclusive OR gene choice. Based on the known spatial organization of active versus inactive alleles (Armelin-Correa, Gutiymama et al. 2014), it is unclear how the eligible (chosen) OR gene loci are
sequestered from within chromocenters, and most puzzling is how a single OR locus is selected for expression. Along the same lines, if an unproductive OR choice is made, it is unclear how a neuron is able to switch to an alternative functional OR choice (Serizawa, Miyamichi et al. 2003, Lewcock and Reed 2004).

To address the question of sequestration of ORs from the chromocenters, the most parsimonious explanation would be a random demethylation event on the H3K9 residues of a single OR locus. This would provide a mechanism for a single OR to exit a chromocenter. A candidate H3K9 demethylase, lysine-specific demethylase 1 (LSD1) was identified and knocked out in mice; however this only led to a reduction in OR expression as a small subset of ORs was still expressed (Lyons, Allen et al. 2013). Assuming this model, either there are other demethylases in the cell that would explain the LSD1 knockout result, or removal of H3K9 methylation is not the required criteria for initial selection of an OR locus.

If H3K9 demethylation is not the rate limiting step in initial OR selection, it is plausible that a different chromatin modifier is functioning up- or downstream of LSD1 activity. Another hypothesis could be that demethylation is not the event responsible for choosing an OR; rather a single OR may access an RNA polymerase II factory earlier in development so that it is not recruited to the chromocenters at all. These two contrasting hypotheses differ primarily in the developmental window when OR choice is made; either an initial silencing event is followed by recruitment of a single OR locus from the chromocenters, or
an early event selects an OR locus and its later sequestration and transcription locks in the OR choice. When the histone methyltransferases G9a and GLP were knocked out in vivo, there was a subsequent loss of singular OR expression (Lyons, Magklara et al. 2014), suggesting that H3K9 methylation is required at some point in regulation of ORs, either to select a single OR, or to maintain singular OR choice.
Figure 4. Model of the epigenetic mechanism governing olfactory receptor expression: “silence all, de-repress one” (Magklara, Yen et al. 2011). Initially in the olfactory epithelial stem cells (HBCs and GBCs), there is a ground state of H3K9me2 on OR loci. Upon developmental progression to an immature OSN, it acquires the heterochromatic silencing marks, H3K9me3 and H3K20me3. As a mature OSN, the single OR allele chosen for expression acquires the transcriptionally active histone modification, H3K4me3, while the remaining OR genes maintain the heterochromatic silencing.
Feedback loops may maintain gene expression subsequent to OR choice

Each OR allele (as opposed to each gene) should be considered a single target, thus making the question of monogenic and monoallelic choice the same. OR choice is a required intermediate step in the developmental progression towards a mature OSN. The selection of a functional OR gene and subsequent production of OR protein is required for the maintenance of the specific OR choice (Serizawa, Miyamichi et al. 2003). Without functional OR protein, the neuron will not mature to a fully differentiated OSN.

The current consensus in the field involves a model in which OR choice and stabilization require a ground state of silencing, a singular escape from the silenced state, and an OR activity-mediated feedback loop to lock in proper OR choice (addressed in depth later in Figure 9) (Lewcock and Reed 2004, Dalton, Lyons et al. 2013). Two pathways can occur once a neuron makes a choice. If a functional OR had been selected, its singular transcription is maintained. However if an OR pseudogene is initially chosen, the neuron will re-enter the process for OR selection to choose a functional OR gene (Lewcock and Reed 2004). This is considered an OR-mediated feedback loop as OR choice is stabilized for transcription while the OR quality check takes place. Although not previously mentioned, OR pseudogenes are quite abundant in mammalian genomes (in some species, pseudogenes make up greater than half the OR repertoire) (Glusman, Yanai et al. 2001) and their potential selection could lead to suboptimal sensory processing, affirming the need for a developmental
checkpoint. The process of changing a selection from an OR pseudogene to a functional OR is referred to as “switching” (Shykind, Rohani et al. 2004).

It is not clear whether the feedback loop is positive or negative. In the first case where a functional OR is chosen, it may function as a negative feedback signal to prevent further OR choice and to maintain monogenic expression. The second scenario, where an OR pseudogene is chosen, might result in a positive feedback loop. It is worth noting that OR choice could be limited to subsets of several genes (functional and pseudogenes), maybe due to the presence of a de-repressor (as the genes are all initially silenced with H3K9me3 marks) or a transcriptional factory that permits only a single OR for expression (Dalton, Lyons et al. 2013).

Based on the current knowledge in the field of olfaction, from the topographical zones of the epithelium and axonal convergence in the olfactory bulb to the identification of monogenic and monoallelic OR expression, there seem to be three critical steps that result in monogenic (and monoallelic) OR expression. First, during embryonic development, the spatial zones of the olfactory epithelium are designated to specify the subset of olfactory receptors that will be expressed in that region (the developmental process of MOE “zoning” is unknown). Next, selection of a single OR among the designated subset occurs in what appears to be a stochastic manner. OSN maturation culminates in the commitment to, or locking in of, an OR choice mediated by a feedback mechanism.
1.4  
A model system for studying OR switching and choice

The basal progenitor cells (HBCs and GBCs) that produce the mOSNs first appear in the embryonic olfactory placode (OP), a structure that appears at embryonic day E10. In order to make cell lines that reproduce the same developmental cell lineages of the olfactory epithelium, the Roskams lab (University of British Columbia, Vancouver) generated clonal lines from the E10 stage of the mouse olfactory placode. Sixty individual OP cells were isolated and infected with a retrovirus containing the large-T antigen, which causes the cells to restart the cell cycle. The OP6 and OP27 cell lines were chosen for further studies of the OE based on initial RT-PCR screenings, which revealed that both lines express a combination of early neuronal markers. These initial studies confirmed that the OP6 and OP27 cell lines mimic cells from early stages of the MOE lineage before any differentiation has occurred (Figure 5). OP6 cells specifically resemble the developmental stage of an immature OSN (OSNi) while OP27 cells exhibit the less mature characteristics of immediate neuronal precursors (GBCinp). Perhaps most important to our work is that both OP6 and OP27 cells express endogenous olfactory receptors. It is also critical to note that these lines are each clonal derivatives from a single ancestral progenitor cell from the OP, based on Southern blot analysis which showed that genomic DNA from each cell line hybridized to a single band as opposed to multiple bands indicative of clonality (Illing, Boolay et al. 2002).
To generate cells that are similar to mature OSNs in vitro, retinoic acid is used to stimulate neuronal differentiation of these cell lines (Illing, Boolay et al. 2002). Treatment with retinoic acid along with incubation of the cells at a non-permissive temperature to inactivate the large-T antigen effectively stimulates cells to exit the cell cycle and begin the process of differentiation. Visual changes in cell morphology accompany exit from the cell cycle; these include a change from a triangular appearance to a bipolar neuronal phenotype, of an elongated cell body and two distinct processes. Immunodetection assays can also reveal markers specific for undifferentiated versus differentiated OP cells. G<sub>olf</sub> is expressed at the permissive and non-permissive temperatures, suggesting that these immortalized cells have the ability to function in chemosensory signaling. Adenylate cyclase III (Adcy3) is present at both temperatures, however Adcy3 is expressed within the cell body in undifferentiated cells and localizes to the extended axons upon differentiation. OMP, normally found in the cytosol of mature OSNs, is absent from both cell lines at the permissive temperature but present upon differentiation (Illing, Boolay et al. 2002).

In this study, we work with the OP6 cell line to study the underlying factors influencing OR gene regulation in vivo. OP6 cells have shown to be constantly making OR selections; presumably the founder OP6 cell had selected an OR gene without locking in its choice, explaining why we observe regular OR gene switching. Because the cells can be differentiated, we can also study the stage when an olfactory receptor has been selected and is actively expressed.
Previous studies in our lab indicated that stable OR gene choice is not a property of the cell line as gene switching was observed during cell culturing (Pathak, Johnson et al. 2009). Single cell analyses confirmed that OP6 cells demonstrate monogenic OR transcription in both undifferentiated and differentiated states comparable to what is observed in vivo (Pathak, Johnson et al. 2009, Kilinc, Meredith et al. 2014).

Yet we previously observed that there are some major differences between the cell line and mature OSNs. Like mOSNs, OR loci are enriched within the heterochromatic chromocenters, but they are also found at the nuclear periphery and within interchromatin compartments in OP6 cells (Kilinc, Meredith et al. 2014). Unlike the spatial organization of the active allele in a euchromatic region and the inactive allele in the chromocenter in mOSNs, both alleles localize outside of chromocenters in OP6 cells.

That said, if the OP6 cells are truly mimicking the OR gene regulation events as in immature OSNs, it seems that OR sequestration from within chromocenters may function in a later silencing event once the single OR locus is chosen for expression. We must also note another key difference between OP6 cells and the OSN lineage in vivo, which is that the lamin B receptor (LBR) expression in undifferentiated OP6 cells is non-peripheral unlike in vivo (Kilinc, Meredith et al. 2014). Upon differentiation though, OP6 cells lose LBR expression just like the down-regulation of LBR observed in vivo in mature OSNs (Clowney, LeGros et al. 2012, Kilinc, Meredith et al. 2014). In the past, we
speculated that the internalization of LBR in cycling OP6 cells might be an intermediate step to drive the aggregation of heterochromatin to the nuclear interior. But undifferentiated OP6 cells contain many dispersed chromocenters (Kilinc, Meredith et al. 2014), so the presence of LBR in the interior may not be enough to stimulate chromocenter aggregation in this intermediate stage. This OP cell line continues to be an excellent model system for studying OR gene regulation because we are observing cells that are constantly making de novo OR choices as they continuously proceed through the cell cycle.
Figure 5. OP6 immortalized cell line. OP6 cells represent immature olfactory receptor neurons from the olfactory sensory neuron lineage. Developing OSNs go through a series of developmental stages from basal cell (HBCs and GBCs) to immediate neuronal precursor (GBC_{inp}), immature OSN (OSN_{i}), and finally mature OSN (OSN_{m}). The stage of OP6 depicted in this diagram indicates its stage at the permissive temperature. When exposed to the non-permissive temperature, the large T-antigen is turned off, and the cells become mature OSNs (Illing, Boolay et al. 2002).
1.5 Lysine-specific demethylase 1

A candidate demethylase: LSD1

Considering the idea that a de-repression of gene expression may be a critical aspect of the mechanism that promotes monogenic expression of ORs, a candidate demethylase was identified. Lysine-specific demethylase 1 (KDM1A, AOF2, BHC110, or simply LSD1), the first known lysine-specific histone demethylase, is a flavin-dependent amine oxidase functioning as an H3K4 and H3K9 demethylase, active in the removal of di- and mono-methylation marks on these histone lysine residues (Finley and Copeland 2014). As previously mentioned, H3K4me3 and H3K9me3 mark active and inactive OR loci, respectively. LSD1 has additionally been shown to act on non-histone residues in a study of the methylated form of Lys 370 on the p53 transcription factor, a tumor suppressor, as well as being recruited by p53 in order to repress transcription of a tumor marker during liver development (Cloos, Christensen et al. 2008, Tsai, Nguyen et al. 2008). LSD1 was even shown to act in conjunction with DNA methylase DNMT1/3L, which recognizes histone H3 tails that are unmethylated at H3K4. This role is interesting because it raises the possibility that LSD1-dependent demethylation of H3K4 residues may be crucial during de novo DNA methylation processes. This would suggest that LSD1 plays a role in the formation and spreading of heterochromatin, consistent with its function as a co-repressor (Forneris, Binda et al. 2008).
LSD1 contains a central SWIRM domain (named for the proteins present, Swi3, Rsc8, and Moira), a motif that provides a scaffold for protein-protein interactions, as well as a C-terminal amine oxidase domain including the active site for its demethylation activity (Figure 6) (Metzger, Wissmann et al. 2005). This protein is unique from other histone demethylases as it produces reactive oxygen species upon demethylating its substrate (Hou and Yu 2010). Both hydrogen peroxide and formaldehyde are byproducts of LSD1 activity (Figure 7) (Anand and Marmorstein 2007). In cells, hydrogen peroxide selectively oxidizes guanine nucleosides to 8-oxoguanosines (8-oxoG). This DNA damage mark, in turn, has been shown to recruit the DNA repair protein, oxoguanine glycosylase 1 (OGG1), to bind the 8-oxoG specifically at LSD1-regulated gene promoters (Perillo, Ombra et al. 2008, Amente, Bertoni et al. 2010). OGG1 produces nicks on the DNA strands adjacent to the 8-oxoG damage, as part of the repair process. The consequent DNA nicks provide a means of looping enhancers to the upstream promoter or downstream polyadenylation sites due to increased DNA flexibility (Abbondanza, De Rosa et al. 2011, Ombra, Di Santi et al. 2013).
Figure 6. LSD1 domains. Analysis of the full length LSD1 sequence based on the ELM database, which contains a collection of linear motifs with corresponding sequences of known function. In the first line, the LSD1 domains are identified including the mentioned SWIRM and Tower domain, as well as the amine oxidase catalytic domain (AOD). The GlobPlot indicates the intrinsic protein disorder (brown represents disordered regions, yellow depicts the low-complexity regions, and green indicates the globular domains). The third line represents the annotated linear motifs that are the likely functional sites of the protein (Forneris, Battaglioli et al. 2009).

Figure 7. LSD1 demethylation reaction. Chemical reaction mechanism of LSD1 removing a methyl group from a dimethylated lysine. The oxidation reaction can proceed from a dimethylated residue to an unmethylated residue (Shi and Whetstine 2007).
LSD1 is the key enzymatic component of a number of complexes with opposing co-activator and co-repressor functions. These complexes are recruited to target genes to dictate gene expression programs throughout development and mammalian organogenesis (Wang, Scully et al. 2007). LSD1 was initially characterized as a repressor for target genes through its H3K4 demethylation ability (Shi, Lan et al. 2004). It has shown to be required for plasma cell differentiation in association with the repressor Blimp-1, HDAC1/2, and methyltransferase G9a. The complex silences the mature B-cell expression cascade and thus allows for plasma differentiation (Su, Ying et al. 2009). LSD1 is required for the proliferation of neural stem cells as it is co-recruited with HDAC5 to bind to TLX promoters (Sun, Alzayady et al. 2010). TLX is a nuclear receptor whose target genes must be repressed via H3K4 demethylation to maintain cell proliferation. Drug inhibition and knockdown of LSD1 confirmed its essential role as these experiments lead to a reduction in neural stem cell proliferation (Sun, Alzayady et al. 2010).

In line with its association with histone deacetylases, LSD1 associates with the nucleosome remodeling and deacetylase (NuRD) complex to regulate the TGFβ1 signaling pathway prevalent in several types of cancers (Wang, Zhang et al. 2009). In the context of breast cancer cells, the LSD1-NuRD complex has a range of abilities as a chromatin remodeler with ATPase activity along with deacetylation and demethylation (Wang, Zhang et al. 2009). In embryonic stem cells, the LSD1-NuRD complex neutralizes enhancers that maintain the ESC state
to allow for differentiation into mature cell types. ESCs lacking LSD1
demethylase activity do not differentiate fully (Whyte, Bilodeau et al. 2012).

LSD1 is a well-known component of the enzymatic core of the REST
repressor complex, associated with REST, CoREST, BHC80, and BRAF35. This
complex is pertinent as it suppresses neuronal gene expression in non-neuronal
cells through the demethylation of H3K4 (Mosammaparast and Shi 2010).
Silencing is maintained through the recruitment of other chromatin modifying
machinery like H3K9 methyltransferase, G9a. However, a perhaps more
interesting and less characterized role of LSD1 is its H3K9 demethylase activity.
H3K9 methylation is associated with the condensation of constitutive
heterochromatin and thus suppression of gene expression (Nakayama, Rice et al.
2001). By demethylating H3K9 residues, LSD1 can act as a transcriptional
activator of target genes (Shin, Ming et al. 2015).

LSD1 has specifically been shown to perform as a transcriptional
activator in the context of androgen receptor transcription where it
demethylates repressive histone marks on the H3K9 residues, therefore causing
the de-repression of androgen receptor target genes. When LSD1 is knocked
down in this context, there is a halt in androgen transcriptional activation and
decrease in cell proliferation (Metzger, Wissmann et al. 2005). During this AR-
dependent gene activation, LSD1 works in coordination with the trimethyl
demethylase, Jumonji C domain-containing protein, JMJD2C, to fully demethylate
H3K9 residues (Figure 8) (Wissmann, Yin et al. 2007).
Figure 8. LSD1-bound complex in androgen receptor transcription. Schematic of LSD1 and member of Jumonji C domain containing protein, JMJD2C, interaction with the androgen receptor proteins, where LSD1 functions as an H3K9 demethylase. This activates the transcription of androgen responsive genes (Kooistra and Helin 2012).
LSD1 in vitro: cell cycle studies

LSD1 regulation is cell-cycle dependent based on in vitro studies of embryonic stem cells and immortalized cell lines (Nair, Ge et al. 2012). LSD1 is recruited to the chromatin of interphase nuclei (G1/S/G2 phases) and excluded from the chromatin during M phase, perhaps to allow gene transcription. In an embryonic stem cell study, LSD1 seemed to function as a co-repressor during G1/S/G2 phases by demethylating H3K4me1,2 and the loss of LSD1 from the chromatin during M phase correlates with an up-regulation of LSD1 target genes (Nair, Ge et al. 2012). This mechanism suggests that transient interaction with LSD1 throughout the cell cycle can lead to short-term gene expression changes.

Another study found that LSD1 is indirectly required for proper chromosome segregation and centrosome duplication during mitosis. LSD1 locally demethylates repressive H3K9me1 histone marks on promoters of BUBR1 and MAD2 which play an active role in chromosome segregation (Lv, Bu et al. 2010).

A cell cycle-dependent regulation of the Epstein-Barr virus latency period identified LSD1 in a different complex, associating specifically and exclusively with the retinoblastoma protein, Rb. The LSD1-Rb complex binds to the C promoter (Cp) of the virus during S phase to create a decrease in H3K4 methylation and thus a decrease in Cp transcription (Chau, Deng et al. 2008). From these studies, it is apparent LSD1-induced demethylation is dynamically regulated throughout the cell cycle.
Patterns of LSD1 expression in vivo

LSD1-mediated histone demethylation has been studied in a variety of model organisms, all of which suggest that LSD1 is dynamically regulated during development in vivo (Shi, Matson et al. 2005). Like many demethylases, it demonstrates restricted patterns of embryonic and adult expression. For example, the mammalian LSD1 is highly expressed in mouse testes and other germline tissues consistent with lower levels of H3K4 di-methylation in these areas. In the fly, mutations of the LSD1 homolog result in sex-specific embryonic lethality for males, leaving the surviving female offspring sterile (Lan, Nottke et al. 2008).

In zebrafish, it has been shown that embryos treated with an LSD1 inhibitor result in a 1.6- to 1.8-fold decrease in olfactory receptor-positive cells, which suggests that H3K9 demethylation (potentially performed by LSD1) is connected to OR gene activation (Ferreira, Wilson et al. 2014). Consistent with this idea, Lyons et al. found that LSD1 is necessary for de-repressing and thus initiating OR gene transcription in a mouse model system (Lyons, Allen et al. 2013). They show that the down-regulation of LSD1 is concurrent with differentiation to a mature OSN and its down-regulation is essential for stabilizing OR choice (i.e. preventing another OR from being selected). Once LSD1 is at lower levels in the epithelium, the cells cease to make additional OR choices (Lyons, Allen et al. 2013).
Going back to the previously mentioned feedback loops defining OR choice and expression, Dalton et al. have recently suggested a detailed model for the OR-mediated feedback loop, which incorporates a role for LSD1 in the selection process (Figure 9) (Dalton, Lyons et al. 2013). In this model, LSD1 transcriptionally activates an OR (via H3K9 demethylation) and OR protein functions as a feedback signal activating the unfolded protein response (UPR) in the endoplasmic reticulum (ER). The UPR is a quality control pathway for protein production in the ER, adjusting the environment when unfolded proteins are detected to increase protein-folding capacity; the increase in folding decreases the ER load in the cell (Ron and Walter 2007). In response to the detection of unfolded protein, the UPR begins with Perk (an ER kinase)-mediated phosphorylation of the translation initiation factor, eif2a in order to inhibit translation. Under the conditions of inhibited translation, the activating transcription factor (ATF4) induces transcriptional changes to rid the ER of misfolded proteins. ATF5 is a MOE-specific paralog of ATF4 (Harding, Zhang et al. 2000) whose translation triggers the transcription of adenylate cyclase 3 (Adcy3) which subsequently drives the repression of LSD1, effectively eliminating the initial stress (i.e. the initial production of OR protein).

Upon LSD1 down-regulation, the OSN matures. Within this feedback loop scenario, LSD1 may function as a transcriptional co-activator in the immature neurons of the OE, responsible for the singular activation of an OR. In the more mature cells that have committed to an OR choice, Adcy3-mediated repression of
LSD1 may be the mechanism allowing for stable choice to be persistently locked in, meaning that LSD1 can no longer remove active marks on the expressed gene or H3K9 methylation on the silenced ORs (Dalton, Lyons et al. 2013).
Figure 9. Model of the OR-mediated feedback system (Rodriguez 2013), as proposed by Dalton et al. 2013. Here, LSD1-dependent demethylation of an OR allele leads to the production of an OR protein activating the unfolded protein response in the endoplasmic reticulum. This triggers a cascade of events that ultimately leads to the down-regulation of LSD1 observed in mature OSNs, which are no longer involved in the OR selection process.
**CoREST associates with LSD1**

LSD1 can accommodate either H3K9me1/2 or H3K4me1/2 in its active site, acting as either a transcriptional co-activator or co-repressor, respectively. The action of LSD1 is context-dependent, specifically influenced by the transcription factors recruited to the gene locus and local histone modifications. As stated, LSD1 has been extensively studied in the context of the androgen receptor, which recruits LSD1 to loci for its co-activator function, leading to demethylation of H3K9me2 and subsequent gene transcription (Shi, Lan et al. 2004, Metzger, Wissmann et al. 2005).

The multi-protein BHC complex, or BRAF-HDAC complex, has been identified as a key regulator in development by repressing neuronal-specific genes in non-neural cell types. This complex contains histone deacetylase activity (HDAC1/2) and histone demethylase activity via LSD1. It was first shown to lead to an increase in H3K4 demethylation when compared to mutated LSD1. Another component of this complex is the RE1-silencing transcription factor (REST), which recruits the co-repressor for element 1-silencing transcription factor, CoREST (Rcor1). CoREST is a fundamental protein that facilitates the binding of the complex, specifically LSD1, to nucleosomes and protects LSD1 from proteasomal degradation (Lee, Wynder et al. 2005, Shi, Matson et al. 2005). Studies of hematopoietic cell commitment and differentiation have identified LSD1 and CoREST in complex with Gfi-1/1b, zinc finger repressors that maintain stem cell competence. Repression of Gfi-1/1b by
the LSD1-CoREST complex requires a co-repressor role to eliminate the H3K4 methylation on Gfi1/1b in order to abolish stem cell characteristics and stimulate differentiation (Saleque, Kim et al. 2007). In the germline, particularly during spermatogenesis, LSD1 and CoREST simultaneously occupy histone genes with SFMBT1 (Scm [Sex comb on midleg] with four MBT [malignant brain tumor] domain 1) throughout the cell cycle. The co-assembly of these three proteins correlates with a loss of RNA polymerase II at the histone gene promoters (Zhang, Bonasio et al. 2013).

Studies of the LSD1-CoREST complex structure have identified an extended helical region of LSD1, named the “Tower” domain, which interacts with CoREST. The C-terminal SANT domain of CoREST is responsible for directly binding the target DNA (Nair, Ge et al. 2012). Further structural data suggested that the LSD1-CoREST complex acts an “ergonomic clamp” by detaching the histone H3 tail from the nucleosomal DNA to ensure its availability to sit in the LSD1 active site (Pilotto, Speranzini et al. 2015). When CoREST is depleted both in vivo (in human embryonic kidney cells) and in vitro, the result is a de-repression of REST-target genes and an increase in H3K4 methylation (Lee, Wynder et al. 2005). In the study of CoREST/LSD1-mediated demethylation, hyperacetylated nucleosomes were found to be less prone to demethylation, suggesting that the complex may be targeting hypoacetylated nucleosomes as preferred substrates (Shi, Matson et al. 2005). Because LSD1 is associated with HDAC1/2 in this complex, the first step in the chromatin modifying program
could be elimination of the acetyl groups from the K4 residues by the HDACs followed by H3K4 demethylation by LSD1 and subsequent recruitment of other chromatin remodelers that mark the gene loci for transcriptional repression (Forneris, Binda et al. 2006).

CoREST is a SANT-domain-containing protein that serves as a chromatin modifying complex's bridge between HDACs and LSD1. After identifying its role in the BHC complex, CoREST was found to be a component of another complex containing the carboxy-terminal binding protein (CtBP) and LSD1, which functions as a co-repressor complex, demethylating H3K4 residues, specifically in the developing pituitary gland (Wang, Scully et al. 2007). However the CtBP complex with LSD1 and CoREST was shown to remove repressive H3K9 methylation marks in gastrointestinal endocrine cells (Ray, Li et al. 2014). In the endocrine context, the two associated proteins bind together to promoters containing bound NeuroD1, a transcription factor known as an activator of insulin gene transcription, at actively transcribed genes. When LSD1 was suppressed by treatment with pargyline, an inhibitor of LSD1, there was a resultant increase in H3K9me2 levels at the secretin gene promoter (secretin being a peptide hormone endogenous to gastrointestinal tract and critical for digestion) confirming that in this complex, LSD1 is responsible for the removal of the repressive H3K9 methylation marks (Ray, Li et al. 2014).

LSD1 activity on H3K9 residues, functioning as a co-activator, is its less well-characterized role. Other signal-dependent DNA-binding transcription
factors and nuclear receptors have been shown to be substrates of LSD1 activity on H3K9 methyl groups in a mechanism that prevents constitutive gene activation. One example is a role for LSD1 in estradiol-dependent gene activation (Garcia-Bassets, Kwon et al. 2007). By associating with the proline glutamic and leucine-rich protein 1 (PELP1) and estrogen receptor-α (ERα), the substrate specificity of LSD1 effectively switches from its default H3K4me1/2 to H3K9me1/2. Data shows that PELP1 functions as a reader of the histone H3 methylation marks in this PELP1-LSD1-ERα complex to selectively choose H3K9 methylated gene loci (Nair, Nair et al. 2010). Another critical piece of data in estradiol-dependent gene activation is that the demethylation of H3K9 by LSD1 leads to oxidative-driven DNA looping of promoter and enhancer sites. In the absence of H3K9 demethylation (i.e. LSD1 inhibition), no oxidative species are produced to create DNA nicks consequently abolishing DNA looping and subsequent transcription of target E2-responsive genes (Abbondanza, De Rosa et al. 2011). Also in the context of nuclear receptor gene activation, LSD1 has been characterized as a mediator linking interacting gene loci to interchromatin granules, which are dynamic nuclear structures typically associated with active transcription sites or as storage for splicing factors (Hu, Kwon et al. 2008).
Additional histone marks influence LSD1-CoREST complex activity: phosphorylated $H3T6$ and $H3T11$

In the androgen receptor model, androgen receptor-induced expression leads to interactions with LSD1 that dictate a change in its substrate specificity from H3K4 dimethyl groups to H3K9 dimethyl groups. The change in substrate specificity also signifies a change in function from a silencing to activating role in gene regulation (Culhane and Cole 2007). So in addition to context-dependent binding partners, two histone modifications have been identified that correlate with LSD1 acting as an H3K9 demethylase.

Phosphorylation of histone H3 at threonine 11 ($H3T11\text{ph}$) was shown to correlate with increased androgen receptor transcription. That is, the protein-kinase-C-related kinase (PRK1) specifically phosphorylates H3T11 and this event stimulates the recruitment of JMJD2C to demethylate the tri-methyl group on histone H3 lysine residues (Metzger, Yin et al. 2008). JMJD2C functions prior to LSD1 to produce a mono- or di-methyl lysine substrate for LSD1. PRK1 has therefore been dubbed the gatekeeper protein to the androgen receptor gene activation cascade.

Next, phosphorylation of histone H3 at threonine 6 ($H3T6\text{ph}$) by protein kinase C beta I (PKCβI) was demonstrated as a key event that switches LSD1 substrate specificity to H3K9me2 by preventing H3K4 demethylation (Metzger, Imhof et al. 2010). In vitro experiments showed that histone H3 peptides that were methylated at lysine 4 and phosphorylated at threonine 6 were no longer
available to the LSD1 active site. Thus H3T6ph acts as a block to LSD1 H3K4 demethylation, perhaps insulating certain gene promoters from being silenced. The kinase, PKCβ, associates with the AR and LSD1 directly on the target gene promoters after androgen-induced gene expression to phosphorylate H3T6 and maintain the expression of the target gene (Metzger, Imhof et al. 2010).

LSD1 inhibition experiments using a known inhibitor, pargyline, showed that at androgen receptor-negative, LSD1-positive sites there were increased levels of H3K9me1 (Cai, He et al. 2014). Among the sites tested were REST sites where LSD1 typically performs as a co-repressor. This result suggests a potential broader role of LSD1 co-activator activity since its H3K9 demethylation activity may not be exclusive to AR-stimulated genes. These two studies have shown that LSD1 can co-activate transcription factors like the androgen receptor for a small set of genes that are marked with H3T11 and H3T6 phosphorylation. The data suggests that the combination of these marks is enough to switch the substrate specificity of LSD1 from H3K4 to H3K9 mono- and di-methyl groups. However, although its substrate specificity has changed, it still retains the ability to demethylate H3K4me1/2. It has been speculated that the maintenance of this H3K4 demethylase role may be part of a negative feedback loop to suppress gene expression in the absence of the initial triggering signal, for example, absence of the androgen stimulating the expression of the androgen receptor. Analysis of these histone marks in relation to LSD1 activity have not been studied in other contexts, yet such histone modifications may be critical for the
change in the LSD1 active site. It would be interesting to see whether phosphorylation marks influence LSD1 activity in relation to OR expression or provide a scaffold for LSD1 exclusivity in monogenic expression.

1.6 **Questions addressed in this study**

Preliminary studies on the OP6 cell line revealed several nuclear phenotypes of LSD1, one of which presents a singular compartment of LSD1 in the nucleus. The primary aims of this study include characterization of the nuclear phenotypes of LSD1 as regulated through the cell cycle and throughout developmental regulation of the mouse olfactory epithelium. Understanding the singularity aspect of LSD1 distribution in OP6 cells may provide insight on the monogenic and monoallelic nature of olfactory receptor expression. We hypothesize that this singularity may provide the trigger for selecting a single OR allele for expression in an OSN. We are asking whether compartmentalized LSD1 is interacting with olfactory receptor genes, and if so we want to determine the functional activity of the compartment. Is this singularity actively demethylating H3K9 residues on OR loci? Is it a transcriptionally active compartment feeding a single OR locus into an RNA polymerase II factory? By studying LSD1 organization *in vitro* and *in vivo*, we are trying to link the compartment to monogenic OR expression, examining novel binding partners (e.g. CoREST), a transcription factor (Lhx2) tightly linked to OR transcriptional activation, and
further histone modifications that may provide insight on LSD1’s substrate specificity in this nuclear compartment.
Methods

2.1 Maintenance of immortalized cell lines

The OP6 and OP27 cell lines, generously provided by Jane Roskam's lab, as well as GD25 fibroblast cell line were used for the in vitro studies. OP6 and OP27 cells were grown in tissue culture flasks and enzymatically passaged with 0.25% trypsin/ethylenediaminetetraacetic acid solution (Gibco) when they reached 90-100% confluency in the dish. OP6/OP27 medium was composed of DMEM (Life Technologies) and fetal bovine serum (Gibco, final concentration 10% by volume). Cycling cells were incubated at 33°C and 5% carbon dioxide.

OP6/OP27 cells were differentiated into olfactory sensory neurons by turning off the large-T-antigen at 39°C for 4-15 days after growing the cells to confluency in regular OP6/OP27 medium. Differentiation medium was composed of DMEM/F12 (Life Technologies), fetal bovine serum (Gibco, final concentration 2% by volume), N-2 Supplement (Gibco), ascorbic acid prepared in water (Sigma, final concentration 100μM), and retinoic acid prepared in dimethyl sulfoxide (Sigma, final concentration 10μM).

GD25 fibroblasts were used as a control cell line separate from the OSN lineage. GD25 cells are also adherent and thus passaged with 0.25% trypsin/EDTA when they reached 90-100% confluency in the dish. GD25 medium was composed of DME with L-glutamine (Gibco), fetal bovine serum (Gibco, final concentration 10% by volume), and penicillin/streptomycin
solution (Gibco, final concentration 1% by volume). The GD25 cells were incubated at 37°C and 5% carbon dioxide.

For subsequent immunocytochemistry and FISH analysis, cells were seeded on 22cm² coverslips coated with 0.1% gelatin (Sigma) in a 6 well plate at about 50% confluency and expanded for an additional day for near 100% confluency.

2.2 **Blocking the cell cycle for time course studies**

For cell cycle analyses, confluent OP6 cells were incubated in 500μM L-mimosine for G1/S block or 100μM nocodazole for G2/M block, both diluted in the appropriate growth media, for 16 hours and released into regular media at specific time points prior to fixation and subsequent immunofluorescence experiments.

2.3 **Immunocytochemistry**

Cells were fixed with 3% paraformaldehyde for 10 minutes and permeablinized in 0.5% Triton X-100 (Sigma) in PBS for 10 minutes. Cells were then blocked in 1% bovine serum albumin (BSA) in PBS for 20 minutes at 37°C. Primary and secondary antibodies were diluted in the 1% BSA blocking solution. Primary antibody dilutions were applied to the cells on a slide and incubated at 37°C for one hour in a humidifying chamber. After 3 washes in 1xPBS with agitation, the cells incubated in appropriate secondary antibody dilutions at 37°C for one hour
in a humidifying chamber. Cell nuclei were stained with DAPI (1mg/ml diluted 1:1000 in PBS) and cells were mounted to slides using Vectashield (Vector Labs). See Tables 1-2 for the primary and secondary antibodies used.

2.4 Production of in situ DNA probes: single OR BACs and pan-OR

BAC DNA (1.5μg) was nick-translated using the Roche Nick Translation Kit with DIG-11-dUTP or Biotin-16-dUTP. See Table 3 for the list of BAC probes and the encompassed genes per probe. The nick translation reactions incubated at 16°C for 2-4 hours, verified to be a smear between 100-800 base pairs by gel electrophoresis, and stopped by addition of 0.5M EDTA. Nick translated DNA was extracted from the reactions by ethanol precipitation. Before proceeding with the probe preparation for DNA FISH, the nick translated DNA product efficiency was confirmed using a dot blot.

Dot blot verification required the spotting of 10-fold serial dilutions of the nick-translated DNA (1:10, 1:100, and 1:1000) on a nitrocellulose membrane. Once dried, the membrane incubated in 1x blocking solution for 30 minutes at room temperature with agitation. The membrane then incubated in the primary antibody, either anti-DIG-AP or anti-Biotin-AP diluted 1:5000 in 1x blocking solution for 30 minutes at room temperature with agitation. After two washes in 1x washing buffer, each for 15 minutes, the membrane was developed in 1x detection buffer supplemented with NBT/BCIP. Adequate DIG or biotin incorporation into the probes was determined by the appearance of all three
dilutions on the blot. A DIG wash and block buffer kit (Roche, Cat. No. 11585762001) was used to produce the solutions used in the dot blot procedure.

Approximately 100ng nick translated probe was mixed with 5μg Cot1-DNA (Invitrogen; 1mg/mL) and 11μg salmon sperm DNA (Sigma; 11mg/mL) per DNA FISH hybridization reaction; for DNA-immunoFISH, 200ng nick translated probe was mixed with 10μg Cot1-DNA and 22μg salmon sperm DNA. After addition of 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol, the probes were stored at -80°C for one hour. The probes were then pelleted at 4°C, dissolved in 100% formamide (Sigma), and incubated at 37°C for proper dissolution. Just prior to the application to cells (see section 2.6 DNA-immunoFISH protocol), both 50% dextran sulfate and 20xSSC were added to the probes which were then denatured for 10 minutes at 85°C and then competed at 37°C for 30 minutes with vigorous agitation.

For some experiments, a “pan OR” DNA FISH probe was produced by PCR with degenerate primers designed against well conserved OR sequences. Three different degenerate PCR assays were performed on genomic DNA template:

135 (5’ ATGGCITAYGAYMGITAYGTIGCIATHTG3’)/P8 (5’ RTTICKIARISWRTAIATRAAIGGRTT3’)

P26 (5’ GCIYTAYGAYGTIGCIATITG3’)/P27 (5’ ACIACIGAIAGRTGIGAISCRCAIGT3’)

5B (5’ CCCATGTAYTTTBTYCTCDSYAAYYTRTC3’)/P8 (5’ RTTICKIARISWRTAIATRAAIGGRTT3’)

50
Like the nick-translated BAC probes, DIG-11-dUTP or Biotin-16-dUTP was incorporated, by PCR amplification in this context. The degenerate products were digested with MluCI and pooled together. The pan-OR probe was initially tested using Southern blot analysis with both OR-containing and non-OR-containing DNA. The OR-containing DNA consisted of the following BAC probes: RP23-54M12, RP23-172N22, RP23-275I18, RP24-65B23. The non-OR-containing DNA consisted of the following BACs: RP23-358O6, RP24-149A5, RP23-105L18, RP23-155O16. The Southern blots showed hybridization signals for OR-containing fragments for each of the OR BACs and no hybridization for the non-OR BAC fragments.

2.5 DNA FISH to confirm pan-OR probe

Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and permeabilized with 0.5% Triton-X for 10 minutes at room temperature with agitation. Following permeabilization, the cells were dehydrated in an 80%, 95%, and 100% ethanol series followed by incubation in 50% formamide/2x saline-sodium citrate (SSC) buffer (pH 7.4) for 20 minutes at room temperature. Next, cells were denatured at 85°C for 30 minutes and then hybridized with heat-denatured probes overnight at 37°C.

Following hybridization, the cells were washed three times with 50% formamide/2xSSC for 5 minutes and blocked in 4% BSA/4xSSC/0.2% Tween-20 for 20 minutes at 37°C in a humidifying chamber. Fluorescent anti-DIG or anti-
avidin primary antibody incubations in 1% BSA/4xSSC/0.2% Tween-20 were performed for 45 minutes at 37°C, again in a humidifying chamber. Following three washes in 4xSSC/0.2% Tween-20, the cells incubated in secondary antibodies diluted in 1% BSA/4xSSC/0.2% Tween-20 for 45 minutes at 37°C in a humidifying chamber. If biotin labeled, a third antibody incubation was required with fluorescent anti-avidin in 1% BSA/4xSSC/0.2% Tween-20 for 45 minutes at 37°C in a humidifying chamber. In between all antibody incubations, cells were washed 3 times with 4xSSC/0.2% Tween-20 at 37°C. Cell nuclei were lastly stained in DAPI (1mg/ml diluted 1:1000 in PBS) and mounted using Vectashield (Vector Labs).

2.6 DNA immuno-fluorescent in situ hybridization (FISH)

A modified DNA FISH protocol was performed on cells first stained by immunocytochemistry (i.e. stain cells for proteins first and then proceed to DNA probe hybridization). Following this initial staining, cells were post-fixed for one hour in 3% paraformaldehyde at room temperature, and then permeabilized in 0.7% Triton X-100 for 20 minutes at room temperature with agitation. Cells were then dehydrated in an 80%, 95%, 100% ethanol series followed by incubation in 50% formamide/2x saline-sodium citrate (SSC) buffer (pH 7.4) for 20 minutes at room temperature. Next, cells were denatured at 85°C for 45 minutes and then hybridized with heat-denatured probes overnight at 37°C.
Following hybridization, the cells incubated in primary and secondary antibodies (tertiary antibody if biotin-labeled) according to the same procedure as mentioned in the DNA FISH protocol (see Section 2.5).

2.7 Mouse olfactory epithelium sections

FVBN (Neurog1-eGFP) and B6/129S mice were used for LSD1/neurogenin/P27 and LSD1/CoREST immunohistochemistry experiments, respectively. Mice were anesthetized using a triple cocktail of ketamine, xylazine, and acepromazine to keep the heart pumping followed by intracardiac flush with phosphate buffered saline (PBS; pH 7.2) and perfusion with 1% paraformaldehyde-lysine-sodium periodate (for the Ngn-GFP animals) or 4% paraformaldehyde. After gross dissection and extraction of the olfactory epithelium, tissue was immersed in fixative under a vacuum for 2-3 hours and subsequently decalcified in saturated ethylenediaminetetraacetic acid (EDTA) overnight. Tissue was then cryoprotected overnight with 30% sucrose and stored in OCT, frozen via liquid nitrogen before cryosectioning at 10µm.

2.8 Immunohistochemistry and tyramide signal amplification

Mouse sections were treated with 3% hydrogen peroxide in methanol for 5 minutes, boiled in 0.01M citric acid buffer (pH 6.0) using a commercial food steamer, and incubated in normal donkey block (NDB; 10% serum + 5% non-fat dry milk + 4% BSA +0.1% Triton X-100) for 15 minutes at room temperature.
The sections were then incubated with the primary antibody diluted in blocking solution (rabbit anti-LSD1, chicken anti-GFP, mouse anti-P27, and mouse anti-CoREST) for 1 hour at room temperature. Visualization of the anti-LSD1 staining was performed using tyramide signal amplification (TSA) with FITC. To perform TSA, sections incubated in biotin anti-rabbit antibody diluted in NDB post-primary antibody, for 1 hour at room temperature. The sections were then blocked in Tris-NaCl blocking buffer (TNB) for 15 minutes followed by SA-HRP antibody incubation diluted in TNB for 1 hour. The last step is incubation in FITC-tyramide diluted 1:100 along with BSA (1:100) in amplification diluent for 9 minutes at room temperature.

Following TSA, the sections incubated in secondary antibodies conjugated to fluorescein cyanine-3 and Alexa-647 for one hour at room temperature. Between each antibody incubation step, sections were washed three times in PBS. See Tables 4-5 for antibodies used for immunohistochemistry. Nuclei were stained with DAPI for 10 minutes and the sections were sealed with n-propyl gallate.

2.9 Fluorescence microscopy and phenotype analysis

Microscopy and image processing was performed using a Deltavision RT imaging system (Applied Precision) adapted to an Olympus (1X71) microscope, courtesy of Amy MacQueen. Each image was sectioned with 0.5-micron interval slices to cover the entire nucleus.
The degree of LSD1 protein consolidation was determined using Softworx software (Applied Precision). The LSD1 phenotypes scored as punctate (i.e. mono and poly compartments) exhibit twice the LSD1 immunofluorescence intensity compared to three other randomly chosen areas in the nucleus. The mono-punctate cells exhibited only one area in the nucleus that fit these criteria, while the poly-punctate cells exhibited 2-4 highly intense areas per nucleus. The same criteria were applied for analysis of the CoREST protein consolidation upon finding the identical phenotype for CoREST.

2.10 Co-immunoprecipitation (Co-IP)

Co-IP assays were performed using a Nuclear Complex Co-IP Kit (Active Motif, Cat No. 54001) and Protein G Agarose Columns (Active Motif, Cat. No 53039). OP6 cells were grown to 100 percent confluency. Nuclear extraction was performed by washing and collecting of cells with PBS/phosphatase inhibitors, pelleting the cells, and resuspending in 1xHypotonic Buffer. Detergent was added to the extracts and the nuclear fraction was pelleted, allowing for discard of the cytoplasmic fraction. The nuclear pellet was resuspended in complete digestion buffer (100mM PMSF + Protease Inhibitor Cocktail + Digestion Buffer, Active Motif), an enzymatic shearing cocktail was added, and the suspension was incubated for 10 minutes at 37°C. To stop the reaction, 0.5M EDTA was added and the suspension was centrifuged. The supernatant was saved for the Co-IP.
Immunoprecipitation was performed with 5 µg of antibody and 400 µg of nuclear extract. The IP buffer was made using the kit’s 5x low stringency IP buffer supplemented with final concentrations of 150mM NaCl and 1% detergent. After incubation at 4°C overnight, the antibody-extract mixture incubated in a Protein G-Agarose column for 1 hour at 4°C and washed extensively with IP wash buffer supplemented with bovine serum albumin (5xLow IP Buffer + Protease Inhibitor Cocktail + 1M DTT + water, Active Motif). The IP was eluted using 2x Reducing Buffer (130mM Tris pH 6.8, 4% SDS, 0.02% bromophenol blue, and 100mM DTT) and 5µl pure glycerol was added to each elution.

2.11 Western blotting

The Co-IP samples were boiled at 95-100°C for 5 minutes and loaded onto a 10% SDS-PAGE Gel with Precision Plus Protein Kaleidoscope Standards (BioRad). Western blotting was performed using PVDF membrane pre-wetted in methanol. After transfer to PVDF, the membrane incubated in 1xTBST/5% BSA (Tris-buffered saline/0.1% Tween-20/5% bovine serum albumin) for 30 minutes at room temperature and incubated in primary antibody diluted in 1xTBST/5% BSA at 4°C overnight on a rotator. The membrane was washed with 1xTBST and incubated in secondary antibody diluted in 1xTBST/5% BSA at room temperature for 1 hour on a rotator. Following secondary antibody incubation, the membrane was washed with 1xTBST and developed with NBT/BCIP in 1x
alkaline phosphatase buffer. See Table 6 for antibodies used for Co-IP and Western blotting.
Table 1. Primary antibodies used for *in vitro* experiments.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Cat. No.</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Chicken anti-α tubulin</td>
<td>Abcam</td>
<td>ab89984</td>
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<tr>
<td>Mouse anti-8-oxoguanine</td>
<td>Abcam</td>
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<td>Mouse anti-CoREST</td>
<td>Millipore</td>
<td>MABN486</td>
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<td>Mouse anti-LSD1</td>
<td>Millipore</td>
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<td>Mouse anti-γ tubulin</td>
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<td>Rabbit anti-OGG1</td>
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Table 2. Fluorescent (secondary and tertiary) antibodies used for *in vitro* experiments.

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<td>A-2012</td>
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<td>Roche</td>
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<td>1:100</td>
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<td>715-165-150</td>
<td>1:100</td>
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<td>Donkey anti-rabbit Alexa 488</td>
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<td>711-545-152</td>
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<td>Donkey anti-sheep FITC</td>
<td>Santa Cruz Biotechnology</td>
<td>Sc-2476</td>
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<td>Goat anti-chicken Alexa 488</td>
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<td>Life Technologies</td>
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Table 3. BAC clones obtained from BACPAC Resource Center for DNA FISH experiments.

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<th>BAC Clone</th>
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<tr>
<td>RP23-155016</td>
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<td>RP23-220F2</td>
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<td>RP23-289G7</td>
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<tr>
<td>RP23-133O2</td>
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<td>RP24-149O10</td>
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<tr>
<td>RP23-21E22</td>
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<tr>
<td>RP23-6D17</td>
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<td>RP23-172N22</td>
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Table 4. Primary antibodies used for *in vivo* experiments.

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<tr>
<th>Antibody</th>
<th>Company</th>
<th>Cat. No.</th>
<th>Dilution</th>
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</thead>
<tbody>
<tr>
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<td>Chicken anti-GFP</td>
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<td>1:400</td>
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<tr>
<td>Mouse anti-CoREST</td>
<td>Millipore</td>
<td>MABN486</td>
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<td>1:25</td>
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<td>Jackson Immuno Research</td>
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Table 5. Fluorescent (secondary and tertiary) antibodies used for *in vivo* experiments.

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<th>Antibody</th>
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<th>Cat. No.</th>
<th>Dilution</th>
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<td>Donkey anti-mouse-647</td>
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<tr>
<td>Donkey anti-mouse-cy3</td>
<td>Jackson Immuno Research</td>
<td>715-165-150</td>
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Table 6. Antibodies used for Co-IPs and Western blots.

<table>
<thead>
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<th>Antibody</th>
<th>Company</th>
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<td>Goat anti-mouse-AP</td>
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<td>Mouse anti-CoREST</td>
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<td>Mouse anti-rabbit-AP</td>
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<td>Rabbit anti-LSD1</td>
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<td>ab129195</td>
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</table>
Results

3.1  *LSD1 exhibits three nuclear phenotypes* in vitro

The first pertinent question for studying LSD1 in the context of OR regulation was to find out whether the demethylase was expressed in the OP6 cell line, and if so, determine if it displayed a unique nuclear phenotype.

Immunocytochemistry was performed on cycling OP6 cells, staining for LSD1 using a monoclonal antibody. We found that LSD1 exhibits three varying nuclear phenotypes that we have characterized as diffuse, poly-punctate, and mono-punctate (Figure 10). The diffuse phenotype designation describes cells which have LSD1 broadly distributed throughout the nucleus with no compartmentalization of protein. The diffuse cells largely predominate the cycling cell populations at a frequency of 74%. The poly-punctate designation refers to cells that have multiple discrete compartments of LSD1. The poly-punctate phenotype occurred in 15% of cycling OP6 cells. The mono-punctate phenotype exhibited a single nuclear compartment of LSD1 throughout all of the z-stacks of a nucleus. This singularity is present in 11% of cycling cell populations. Nuclear compartments were quantified (by Softworx software) to have at least double the fluorescence intensity in the region of interest as compared to the baseline fluorescent levels of the nucleus.
Figure 10. The three nuclear phenotypes of LSD1 in OP6 cycling cells: (A) diffuse, (B) poly-punctate, and (C) mono-punctate. Cells were stained for LSD1 (red) and DNA (DAPI, blue). The images shown are projections of all the z-stacks comprising each nucleus.
3.2 Cell cycle regulation of LSD1

Upon discovering that LSD1 presented a unique singularity in a significant percentage of OP6 cycling cells, we wanted to determine if the nuclear organization and compartmentalization of the protein were dynamically regulated as a function of the cell cycle. To address this question, we blocked the cells at various stages of the cell cycle. Treatment with the drug L-mimosine stalled cells in G1/S phase; nocodazole treatment blocked cells in G2/M phase. By examining 100 cell nuclei in triplicate for both blocking conditions plus cycling cells, we found a higher incidence of singular compartmentalization of LSD1 at the G1/S boundary while poly-punctate LSD1 reached a maximum in G2/M phase (Figure 11). During G1/S, the LSD1 phenotypic frequencies were as follows: ~89% diffuse, ~6% poly-punctate, and ~5% mono-punctate (Figure 11). For G2/M blocked cells, the frequencies for diffuse, poly-punctate, and mono-punctate LSD1 were 58%, 39%, and 3%, respectively (Figure 11). To complete the full characterization of the nuclear organization of LSD1 in vitro, OP6 cells were differentiated at the non-permissive temperature, effectively turning off the large T-antigen, with the addition of differentiation-specific supplements (i.e. retinoic acid). LSD1 consolidation was not observed in differentiated cell populations.

Presuming cell cycle-dependent consolidation, the results of the cell cycle block experiments identified the need to determine the exact brief time window when the singularity arises. Since the frequency of the mono-punctate
phenotype nearly dropped out at the G2/M boundary, we hypothesized that the compartmentalization was probably occurring during M phase or G1. To address this hypothesis, we designed a time course experiment initially synchronizing the cells at the G2/M boundary. After incubating for sixteen hours in nocodazole, the cells were permitted to cycle together with a release from the drug for fourteen hours. We stained cells for LSD1 at a series of twelve separate time points post-G2/M release to monitor the appearance of LSD1 compartmentalization, scoring for the combined incidence of both the poly-punctate and mono-punctate phenotypes. At the same time, cells were scored for incidence of telophase by α-tubulin staining which is well characterized as a marker of distinct phases throughout the cell cycle (Figure 12A-B).

We found that LSD1 is excluded from chromatin during mitosis, which was previously described in other cell types (Nair, Ge et al. 2012) and a common feature of many transcription factors (Kadauke and Blobel 2013). As OP6 cells progress through mitosis and enter telophase, LSD1 begins to return to the nucleus. LSD1 consolidated into discrete compartments predominantly in early G1, based on the α-tubulin being asymmetrically distributed around the daughter cell nucleus and the presence of condensed chromatin. The data observed for both the incidence of LSD1 consolidation and cells in telophase throughout the time course appeared bimodal (Figure 12C). Two reasons could explain the apparent noise in this data. Either there was a lack of complete synchronization with nocodazole treatment, or there was a lag-time for most of
the cells to start cycling again. There are two distinct peaks in LSD1 compartmentalization at +3 hours and +8 hours that are immediately following the telophase peaks at +2 hours and +5 hours after G2/M release. After the first shoulder of the second peak in compartmentalization (+8 hours), 7.3% of the cells were poly-punctate and 6.6% were mono-punctate. At the later shoulder of the peak (+10 hours), 5.2% exhibited the poly-punctate phenotype while 6.8% were mono-punctate. This suggested that the poly-punctate phenotype precedes compartmentalization of LSD1 into a single focus, occurring after cells complete mitosis in early G1.

The possibility that the cells were not adequately synchronized led us to design a more rigorous time course study. We introduced an additional technique to produce a uniformly synchronized cell population after treating the cells with nocodazole. Mitotic shake-off was performed after nocodazole treatment on the basis that mitotic cells are less firmly attached to the culture dish (i.e. gelatin-coated coverslips) (Fox, Read et al. 1987). This method successfully produced a more synchronized population with less noise, as we did not observe bimodality in either cell synchronization or LSD1 compartmentalization peaks (Seda Kilinc, personal communication, unpublished). To determine the level of synchrony we used a different scoring method, identifying the number of cells in cytokinesis, as cells in telophase should be mostly eliminated due to the mitotic shake-off. In the cleaner data set (not shown), there is a rise in the mono-punctate LSD1 phenotype in G1 phase.
The decline in the peak after ~10 hours post-G2/M release is probably due to the fact that the cells are exiting G1 and proceeding through the cell cycle (Seda Kilinc, personal communication, unpublished).

We also performed an alternative time-course experiment to examine whether turning off the large-T antigen (i.e. differentiation) has an effect on LSD1 compartmentalization; note that our previous observation was an absence in LSD1 consolidation in differentiated cell populations. This experiment was designed to directly test the hypothesis that LSD1 consolidation depends on progression through the cell cycle. The prediction of this hypothesis would be that OP6 cells that are not progressing through M-phase, due to differentiation, would not exhibit LSD1 compartments. As shown above, we did not find any incidence of LSD1 consolidation in differentiated OP6 cells as all differentiated OP6 cells exhibit diffuse distribution of LSD1. We wanted to determine if this result was due to OP6 developmental progression or loss of the cell cycle. First, we determined the percentage of OP6 cells exhibiting the combined poly- and mono-punctate phenotypes under cycling conditions. This was followed by deactivation of the large T-antigen at the non-permissive temperature for three days, reactivation of the large T-antigen for two days, and a second deactivation of the large T-antigen for two days. All of these conditions were performed in the absence of differentiation factors like retinoic acid. Deactivation of the large T-antigen begins the process of differentiation, which developmentally
corresponds to cells exhibiting a loss of LSD1 in vivo (noting that we observe a diffuse pattern of LSD1 expression in differentiated OP6 cells).

Upon initial deactivation, there is a complete lack of LSD1 compartmentalization. After reactivation of the large T-antigen, there is a very small return of the LSD1 consolidation. Upon the second deactivation, the LSD1 returned to its diffuse state in the nuclei (Figure 12D). The loss of LSD1 consolidation with the deactivation of the large T-antigen demonstrates that this phenotype is likely cell cycle-dependent. Although the cells were not terminally differentiating, the loss of LSD1 in this state is consistent with published in vivo data where LSD1 is not expressed in cells that are differentiating to mOSNs (Krolewski, Packard et al. 2013). When the large T-antigen was reactivated, it effectively rescued the LSD1 phenotype, albeit at low levels, so this further indicated that LSD1 consolidation correlates with developmental stage. Keeping the grand “silence all, depress one” model in mind, the consolidation of LSD1 seems to be cell cycle-dependent in OP6 cells (as well as dependent on an undifferentiated state) which are constantly making and switching OR gene choice.
Figure 11. Histogram showing the phenotypic distribution of LSD1 throughout the cell cycle as well as in differentiated cells as determined by immunocytochemistry. An analysis of 100 cell nuclei (n = 3) was performed to calculate the standard deviations, denoted by the error bars.
Figure 12. LSD1 consolidation characterized by time course studies. (A) LSD1 (red) is absent from the nucleus during metaphase (top) and anaphase (bottom), based on α-tubulin staining (green) of mitotic spindle organization throughout the cell cycle. (B) LSD1 consolidation into the poly-punctate phenotype is observed in early G1 phase (top) with asymmetric α-tubulin and condensed DNA staining (DAPI; blue), followed by the mono-punctate phenotype later in G1 phase (bottom) with a more symmetrical distribution of α-tubulin around the nucleus and less condensed DNA. Scale bars represent 3 μm. (C) Plot of the combined incidence of poly- and mono-punctate LSD1 (red line) overlays the histogram representing the number of cells in telophase (gray). X-axis reflects the number of hours post-G2/M release. The error bars represent one standard deviation (200 scored cells per experiment, n=2). (D) Plot of the combined incidence of poly- and mono-punctate LSD1 consolidation under initial (cycling), deactivation, reactivation, and deactivation of the large T-antigen. Error bars reflect one standard deviation (n=3). All data in this figure was provided by Seda Kilinc.
3.3 *The LSD1 singularity is present in vivo*

To explore whether this singularity might be biologically relevant during development, we performed immunohistochemistry (IHC) on tissue from the mouse olfactory epithelium. We first observed that the LSD1 singularity was present in the epithelium, as well as the poly-punctate phenotype, both in distinct phases along the OSN lineage. However, to stage the developmental window when the compartmentalization is occurring, we needed to stain for developmental markers along with LSD1. Neurogenin-1 (Ngn1) is a transcription factor that regulates neuronal differentiation and is present in the late globose basal cells (Ma, Sommer et al. 1997, Cau, Casarosa et al. 2002, Kam, Raja et al. 2014). We proceeded with IHC on Ngn1-GFP mice. Due to steam treatment of the tissue for proper antigen-retrieval, the inherent GFP fluorescence is eliminated; so, we used anti-eGFP antibodies to detect the Ngn1 in the late GBCs. P27^Kip1 (P27) is a cyclin-dependent kinase inhibitor that prevents cell cycle progression into G1 (Guo, Packard et al. 2010), and is thus a marker of post-mitotic cells.

Three-color Ngn1-P27-LSD1 IHC on MOE tissue revealed a partial overlap of double-positive Ngn1/P27 cells in the basal region of the epithelium (Figure 13A). This Ngn1+/P27+ population is most likely representative of the most nascent cells of the OSN lineage that have recently exited the cell cycle with persisting GFP expression from recently expressed Ngn1. Interestingly, P27 is only expressed in cells adjacent to the basal cell layers (and sustentacular cells),
but does not maintain expression in all post-mitotic cells; this finding was consistent with previous P27 staining in the MOE (Guo, Packard et al. 2010). The majority of the MOE is negative for P27 expression, despite the mature layers being post-mitotic. From this pattern, we deduced that P27 must only be required during early OSN differentiation (i.e. in the earliest post-mitotic cells of the lineage).

LSD1 is expressed in the innermost layers (approximately the bottom quarter) of the epithelium, those that are, Ngn1-positive, Ngn1/P27 double positive, and P27-positive (Figure 13B). LSD1 expression is down-regulated in the mature OSNs as described previously (Krolewski, Packard et al. 2013), which are Ngn1/P27 double-negative. In the Ngn1-positive and Ngn1/P27 double-positive cells, LSD1 is primarily organized in the poly-punctate phenotype analogous to our observations in OP6 cells. However in the P27-positive layer that have just recently down-regulated Ngn1, LSD1 strikingly exhibits the mono-punctate phenotype in 59% of the cells, with the remaining exhibiting the diffuse phenotype (Figure 13). The P27-positive only cells represent the earliest post-mitotic cells that are beginning the process of OSN differentiation, presumably when OR gene selection is occurring, meaning that LSD1 compartmentalization into a single foci correlates with the timing of OR selection. This aligns with our hypothesis that LSD1 may be providing the singular rare event to de-repress a single OR for expression.
Another important observation was made from studying LSD1 compartmentalization in the MOE. In mono-punctate cells, the LSD1 compartment is always localized to the periphery of a chromocenter (n=34 cells, example in Figure 14A). For the poly-punctate cells, LSD1 compartments are localized at a chromocenter 65% of the time (n = 375 compartments in 111 cells). Each poly-punctate cell nevertheless had an average of 2.2 LSD1 compartments at the edge of a chromocenter and an average of 1.2 compartments not confined to a chromocenter. This observation is pertinent to our study as eligible olfactory receptor genes are localized in the facultative heterochromatin at the periphery of chromocenters (Armelin-Correa, Gutiyyama et al. 2014).

By examining the incidence of the two compartmentalized phenotypes developmentally, we observed a pattern of poly-punctate LSD1 preceding mono-punctate LSD1 in the MOE (Figure 14B). LSD1 consolidates transiently and progressively (poly- to mono-punctate compartmentalization) as the cells mature from the more basal layers of the MOE to the beginning of neuronal specialization when an OR must be selected. With this in mind, the study progressed to ask whether this compartment is interacting directly with olfactory receptor genes.
Figure 13. LSD1 exhibits poly- and mono-punctate phenotypes in vivo. (A) MOE section oriented with the most basal layers at the bottom. Expression of P27$^{\text{Kip1}}$ (P27; green) is confined to the earliest post-mitotic cells of the OSN lineage and sustentacular cells (labeled “s”). Late GBCs express Neurogenin-1-eGFP (Ngn; red). Circles around individual nuclei of the immature cells are identifying the cells that are Ngn1-positive, P27-positive, or double positive for both markers. Scale bar represents 10μm. (B) Same MOE section shown in A, showing DNA (DAPI; blue) and LSD1 (green). LSD1 is expressed from the basal layer to the P27-positive cells, in the bottom quarter of the MOE shown below the dotted line. (C) Zoomed-in view of the same MOE section as the above panels. Boxes are drawn around a Ngn-P27 double-positive cell (yellow) that exhibits poly-punctate (P) LSD1, and a P27-positive cell (green) exhibiting mono-punctate (M) LSD1. Scale bar represents 5μm.
Figure 14. LSD1 singularity localizes to the edges of chromocenters and consolidation is developmentally regulated in vivo. (A) LSD1 singularity (green) in a P27-positive cell is consolidated on the edge of a nuclear chromocenter (red). Scale bar represents 3μm. (B) Histogram depicting the average frequencies of poly-punctate (light gray) and mono-punctate (dark gray) LSD1 in the MOE cell populations as staged by Neurogenin-1 and P27\textsuperscript{Kip1} expression. The mature OSNs were characterized as cells in the outermost layers of the epithelium with a single consolidated chromocenter. Error bars represent one standard deviation (n=2).
3.4 *The LSD1 singularity associates with olfactory receptor genes*

Next we asked whether the LSD1 compartments interacted with OR gene loci, keeping in mind that the singularity could play a role in regulation of monogenic and monoallelic OR expression. To address this question, we performed DNA-immunoFISH, staining for LSD1 and OR loci *in vitro* using OP6 cells. FISH (fluorescent *in situ* hybridization) probes were produced from BACs (bacterial artificial chromosomes) containing portions of OR gene clusters. There was a low incidence of co-localization of ORs (examples shown in Figure 15A) and the singular LSD1 compartment in experiments using a single BAC probe, effectively a single OR gene cluster. Specifically, for twenty-two FISH experiments, each with a different individual OR-containing BAC probe, 30-50 OP6 cell nuclei exhibiting mono-punctate LSD1 were analyzed (per probe). Because we were only seeing 3 or less co-localizations per probe, this was not an adequate sample size. Assuming that LSD1 may be part of the OR gene selection process, we did not expect to see many co-localizations for individual probes since a population of cells was likely to make a variety of OR gene choices.

From these experiments, it was estimated that the average frequency of co-localization was ~1% per probe. When multiple BAC probes were pooled for the same DNA-immunoFISH experiment, we observed a single OR signal within the LSD1 compartment, but at about the same frequency as the individual BAC probe experiments. Additional larger pooled DNA FISH experiments have been conducted that exhibit a frequency of co-localization that is proportional to the
pool size (Seda Kilinc, unpublished personal communication), consistent with an interpretation that OR cluster interactions with the LSD1 compartment are rare and possibly exclusive (i.e. one OR cluster per LSD1 compartment).

In efforts to observe more OR clusters at once, we produced a “pan-OR” DNA FISH probe by degenerate PCR against conserved regions of OR genes using mouse genomic DNA as the template. This technique was performed by other groups for which degenerate PCR was shown to identify 50-70% of the mouse OR templates (Dulac and Axel 1995, Malnic, Hirono et al. 1999, Clowney, LeGros et al. 2012). To confirm that the pan-OR probe hybridized specifically to OR genes, we performed two proof-of-concept experiments. A southern blot was performed using high stringency conditions with 24 OR-containing BACs and 9 non-OR-containing BACs (negative control). When probed with the pan-OR degenerate product, no hybridization was observed on the non-OR BACs, and 61% hybridization to ORs in the OR-containing BACs (consistent with the 50-70% success rate mentioned above for degenerate PCR). A false-positive rate of less than 1% was determined as there were 5-false positive bands from a total of ~2700 possible non-OR bands. Second, a DNA FISH experiment was performed, labeling the pan-OR probe in one color and a pool of five OR-containing BAC probes in a second color. We observed 77% co-localization of BAC and pan-OR signals. The pan-OR probe exhibits an average of 110 +/- 30 spots in each OP6 nucleus, presumably representing clusters of about 4 OR genes per spot (Figure 15B).
Upon confirming the high-specificity of the pan-OR probe, DNA-immunoFISH was performed on the OP6 cells using the pan-OR FISH probe and staining for LSD1. The overwhelming majority (>99%) of the pan-OR signals are not associated with the LSD1 compartment in a nucleus. Nevertheless, a majority (~70%) of the singular LSD1 compartments (mono-punctate phenotype) contained a single pan-OR signal (Figure 15C). This suggests that LSD1 compartments are interacting with a small number (or perhaps only one) of OR loci in an exclusive way. DNA-immunoFISH was also performed in vivo using the pan-OR probe as well as pooled BAC probes, confirming that the LSD1 mono-compartment interacts with only a small subset of OR genes at a time (Seda Kilinc, personal communication, unpublished).
Figure 15. The LSD1 compartment co-localizes with single OR gene clusters in vitro. (A) DNA FISH probes made from individual OR-containing BACs (378K9 and 359J17) as well as a pool of five BACs (green) in DNA-immunoFISH experiment staining for LSD1 (red). (B) DNA-immunoFISH with pan-OR probe (green) and individual BAC probe (red) to confirm pan-OR detects OR clusters. (C) DNA-immunoFISH with pan-OR probe (green) co-localizing with a monopunctate LSD1 compartment (red). All scale bars represent 3μm. These DNA-immunoFISH experiments were performed by Seda Kilinc.
3.5 Identification of OSN lineage-specific binding partners of LSD1

With data showing that compartmentalized LSD1 is interacting with single OR clusters \textit{in vitro} and \textit{in vivo}, we next wanted to identify putative binding partners of LSD1 in its compartmentalized form. Based on the extensive literature characterizing the LSD1-CoREST complex, we tested whether this form of consolidated LSD1 is associated with CoREST in OP6 cells. Two-color immunocytochemistry staining for LSD1 and CoREST showed that CoREST exhibits the same three nuclear phenotypes as LSD1 \textit{in vitro:} poly-punctate, mono-punctate, and diffuse. In fact, we found that the LSD1 and CoREST compartments for both the poly-punctate and mono-punctate cells co-localize 100% of the time (Figure 16A). This suggests that LSD1 and CoREST form a complex together in their consolidated states.

We also investigated possible LSD1-CoREST in MOE tissue sections. However, unlike the stark result we observed with 100% co-consolidation in OP6 cells, CoREST did not show any obvious compartmentalization in the mouse olfactory epithelium (Figure 16B). Nevertheless, we did observe that CoREST is co-expressed with LSD1 in the bottom quarter of the MOE composed of the basal and immature cell types. We speculate that CoREST still consolidates with LSD1 \textit{in vivo}, however, a higher level of CoREST expression in these cells might result in non-consolidated protein as well. It is also possible that CoREST-LSD1 complexes are disrupted as a consequence of differing fixation conditions required for \textit{in vitro versus in vivo}. 
Immunofluorescence of both OP6 cells and the MOE suggested a co-consolidation and expression similar cell populations, respectively, so we proceeded to perform co-immunoprecipitation (Co-IP) experiments to confirm the association of LSD1 and CoREST in a complex. Co-IP of OP6 cell nuclear extracts revealed that LSD1 and CoREST are in complex in OP cell nuclei (Figure 16C). We pulled down LSD1 first with a monoclonal antibody against LSD1 and subsequently performed a Western blot with this fraction staining for CoREST; this was performed vice versa as well (CoREST pull-down with subsequent LSD1 staining), both yielding the same conclusion that CoREST and LSD1 are interacting in these cells. It is important to note that this result only confirms a general interaction between LSD1 and CoREST as we were not able to specifically isolate the consolidated forms of LSD1, although the striking co-consolidation of both proteins makes this a likely occurrence.

Once identifying CoREST as a key component of the LSD1 complex, we set out to identify other components of the complex. Since Lhx2 was shown to be a critical transcription factor for OR genes (Hirota and Mombaerts 2004, Kolterud, Alenius et al. 2004, Lyons, Allen et al. 2013) and might therefore be a component of this putative activating complex, we performed two-color immunocytochemistry on OP6 cells, staining for LSD1 and Lhx2. We observe that mono-punctate LSD1 compartments commonly (in ~50% of the LSD1 compartments) contain a co-localized Lhx2 signal (Figure 17). Unfortunately, we were not able to optimize Lhx2 staining in the tissue to confirm this finding.
*in vivo.* As with CoREST, co-immunoprecipitation was performed by pulling down LSD1 from OP6 cell nuclear extract and staining for Lhx2 on a Western blot. Lhx2 did not co-immunoprecipitate with LSD1 suggesting that the proteins do not interact, or that the LSD1 compartment is interacting with such a small fraction of Lhx2 that is present in the nucleus that the specific LSD1-interacting portion is undetectable. To confirm this interaction, we will need to perform co-immunoprecipitation experiments followed by protein mass spectrometry, a more sensitive technique that will also identify other potential co-factors in the LSD1-CoREST complex.
Figure 16. LSD1 associates with CoREST. (A) LSD1 (green) and CoREST (red) co-consolidate in the mono- and poly-punctate LSD1 compartments in OP6 cells, shown in the top and bottom panels respectively. Condensed DNA is visualized with DAPI (blue). Scale bars represent 3μm. (B) CoREST (red) and LSD1 (green) both express in the immature cell layers of the MOE. Yellow spots indicate LSD1 consolidation co-localizing with CoREST. Scale bar represents 10μm. (C) Western blots of Co-IP experiments showing the presence of LSD1 (~100 kD) when using anti-LSD1 antibody (left two lanes) and CoREST (~58 kD) when using anti-CoREST antibody (right two lanes). Nuclear extract control (e) and LSD1-immunoprecipitated fraction (IP).
Figure 17. LSD1 associates with OR-specific transcription factor, Lhx2. (A) LSD1 (red) and Lhx2 (green) in a mono-punctate LSD1 compartment. Condensed DNA is visualized with DAPI (blue). Scale bar represents 3μm. (B) Histogram showing frequency of LSD1-Lhx2 interactions in vitro. The interactions were scored as either co-localized or touching (Lhx2 on periphery of LSD1 compartment), and the association bar indicates the combination of co-localized and touching interactions. An average of 30 OP6 nuclei were scored exhibiting the mono-punctate LSD1 phenotype (n =2). Error bars reflect one standard deviation.
3.6 Determining the enzymatic activity of the LSD1 singularity

The logical next series of questions involved identifying the function of the LSD1-CoREST co-consolidated complex. All of the following experiments addressing function were performed solely in vitro.

a. Is the compartment a “transcriptional activation” focus?

If the LSD1 compartment were an expression compartment, the prediction would be that the LSD1 compartment would co-localize with RNA polymerase II (Pol II) in the nucleus. We performed two-color immunocytochemistry staining for LSD1 and Pol II in OP6 cells. There are many Pol II factories in the nucleus, as observed by hundreds of Pol II foci, but the LSD1 mono-punctate compartment was never observed to co-localize with a Pol II compartment (Figure 18A). The LSD1 compartment does not appear to be an expression compartment based on immunofluorescence, however it is possible that we are not able to visualize a small Pol II molecule within the compartment. To further address whether LSD1 is involved in OR gene transcription, RNA FISH using OR RNA probes could determine whether OR RNA is overlapping with the LSD1 compartment. We note that in vivo, LSD1 is down regulated in mature OSNs (Figure 13B), despite ongoing OR transcription, so we already presumed that this compartment was not necessary for maintaining OR transcription. This suggests that the LSD1-CoREST complex is probably acting upstream of transcriptional activation, perhaps as part of the OR gene selection process (i.e. the “de-repress one” step of
the OR regulation model), removing H3K9 methylation on the chosen OR gene and prior to transferring the locus to a Pol II factory. It is also formally possible that LSD1 compartments act downstream of OR gene transcription through a feedback loop to prevent the selection of additional OR genes (i.e. demethylation of H3K4 activating marks). The active site of LSD1 can accommodate H3K4me3 and H3K9me3 in different contexts; so once OR choice is established, the LSD1 compartment sitting on the edge of a chromocenter could be demethylating H3K4 residues on from previously “eligible” subset of OR loci. In this way, LSD1 could be functioning downstream to maintain monogenic expression of ORs.

b. Is the compartment a “hub” of histone demethylation activity?

As a byproduct of demethylation, LSD1 produces hydrogen peroxide causing 8-oxoguanosine (8-oxoG) damage on the adjacent DNA (Perillo, Ombra et al. 2008, Hou and Yu 2010). This damage mark can serve as a proxy for LSD1 demethylation in immunofluorescence experiments. We asked whether LSD1 compartments are actively demethylating histone residues and performed immunocytochemistry on OP6 cells, staining for LSD1 and 8-oxoG damage. 8-oxoG staining revealed hundreds of foci within the nucleus as well as in the cytoplasm. This was expected, as 8-oxoG is a common DNA damage mark, not solely specific to LSD1 activity; 8-oxoG damage is also present in mitochondrial DNA, explaining the cytoplasmic foci. Enrichment of 8-oxoG foci within and around LSD1 mono-punctate compartments was observed 20% of the time
(Figure 18B). It is also important to note that the 8oxoG foci found within the LSD1 compartments were substantially smaller than the compartment itself, suggesting that only a portion of the complex is actively demethylating at any given time; thus, demethylation within the compartment may be a rare event. Therefore, the LSD1-CoREST compartment does not appear to be a hub of robust demethylation activity; rather, its demethylase activity is limited temporally and/or spatially within the compartment. This result aligns with the hypothesis that the de-repression of a single OR is a selective and rare event, perhaps as part of a mechanism that ensures only one OR is demethylated per nucleus.

We can imagine two caveats to the observation of the observed low level of demethylation within compartments. First, upon LSD1 demethylation of a substrate, the DNA could be rapidly evicted from the compartment, which could explain the low incidence of DNA damage that we observe within the complex. Alternatively, the damaged DNA (due to demethylase activity) could be immediately repaired causing us to under-estimate of the compartment’s demethylase activity. Although the “eviction” hypothesis is difficult to prove, we do note that there is no enrichment whatsoever of 8oxoG-marked DNA in the region surrounding the LSD1 compartment, as might be predicted if the compartment was a hub of activity and eviction was very efficient. To test the question about efficient DNA repair as an explanation for a low incidence of 8oxoG DNA, we investigated the oxoguanine glycosylase (OGG1) repair protein,
which is recruited to specifically repair 8-oxoG damage (Perillo, Ombra et al. 2008, Amente, Bertoni et al. 2010).

We stained OP6 cells simultaneously for LSD1 and OGG1. OGG1 foci, like 8oxoG damage marks, are highly abundant in OP6 cells. We did commonly observe OGG1 signals within LSD1 compartments (~90%); however, we note again that these foci were located within a very small portion of the compartmentalized space, typically with only one tiny signal at most per compartment (Figure 18C). This result suggested that 8-oxoG repair might be occurring quickly, yet even with this interpretation, we presume that only a very small portion of the entire LSD1 compartment is active at any given moment. From these data, we therefore tentatively conclude that demethylation is probably a rare event within a given compartment of consolidated LSD1. A future study of the H3K9 methylation states of the OR gene within the LSD1 compartment will shed more light on the relative activity of the compartment. For instance if the OR genes contain H3K9 tri-methylation, it would suggest that the rate limiting step of the compartment’s demethylase activity is dependent on the binding of an additional co-factor, or some other trigger for LSD1 demethylation. If no level of methylation is found on these residues, it would support the idea that this form of LSD1 is highly active and demethylating the lysine residues upon entering the compartment.
Figure 18. Characterizing the activity of the singular LSD1 compartment. (A) LSD1 compartment (green) never co-localizes with RNA polymerase II (red); DNA (DAPI, blue). Scale bar represents 3μm. Pol II experiment performed by Seda Kilinc. (B) 8-oxoG (green) is observed in the LSD1 compartment (red) ~20% of the time. 8-oxoG vicinity analysis scored 30 mono-punctate nuclei (n =1). Scale bar represents 3μm. (C) OGG1 (green) co-localizes with LSD1 (red) compartment >90% of the time. An average of 30 mono-punctate nuclei were scored (n=2). Scale bar represents 5μm.
3.7  The effect of histone phosphorylation on LSD1 substrate specificity

LSD1 has been shown to change its substrate specificity from H3K4me3 to H3K9me3 in the context of androgen receptor transcriptional activation (Metzger, Imhof et al. 2010, Cai, He et al. 2014). The phosphorylation of the residues H3T6 and H3T11 correlate with its change in specificity. To gain clues for the identification of the substrate specificity of compartmentalized LSD1 in our study, we investigated these two phosphorylated residues. Initial two-color immunocytochemistry experiments were performed on OP6 cells, with the combinations of CoREST-H3T6ph and CoREST-H3T11ph. Approximately 65% of mono-punctate LSD1-CoREST compartments co-localize with H3T6ph and H3T11ph signals. Poly-punctate compartments co-localize with H3T6ph at an incidence of 58% and H3T11ph 44% of the time (Figure 20A-C). CoREST was used as a proxy for LSD1 in these experiments since it was shown previously to have 100% co-consolidation with LSD1, and there was antibody species-incompatibility between the LSD1 and phosphorylated histone antibodies.

After identifying that these phosphorylation marks are commonly within the compartment, we asked whether they were present on the OR clusters observed within the LSD1-CoREST complex. We focused on the more well characterized mark in the literature, H3T6ph (Cai, He et al. 2014). Three-color DNA-immunoFISH was performed staining for CoREST, H3T6ph, and individual OR-containing BAC probes. For six different OR cluster-containing BAC probes, an average of twenty-five OP6 cell nuclei exhibiting the LSD1 mono-punctate
compartment were analyzed. As in previous experiment with single BAC probes, we found a low incidence of co-localization of OR signals and the LSD1 compartment. However, H3T6ph was never found to be co-localizing with the OR cluster signal within the LSD1 singular compartments (Figure 20D). This result could be interpreted in several ways. Either this phosphorylation mark is not applicable to LSD1 substrate specificity in the context of OR gene activation, or if it does apply, the substrate specificity of LSD1 may be its default H3K4me3. It is possible that the chromatin is decondensed inside the active site of LSD1 and we are not visualizing H3T6ph on the BAC signal, in which case the phosphorylation may still be functionally relevant. We can also imagine that the phosphorylation and subsequent demethylation of an OR locus within the compartment is a transient event and we are not catching it with immunofluorescence. Chromatin immunoprecipitation (ChIP) is a more sensitive technique that may better address this question in the future; that is, the chromatin collected from the LSD1 immunoprecipitation would be isolated to identify the phosphorylation state of the ORs associated with LSD1.
Figure 19. Histone phosphorylation marks are associated with the mono-punctate LSD1 (COREST) compartment, but not OR genes. (A) Mono-punctate CoREST compartment (red) associates with H3T6ph (green). (B) Mono-punctate CoREST compartment (red) associates with H3T11ph (green). (C) Histogram showing percentage of LSD1 compartment association with each phosphorylated histone mark. An average of 23 cells were scored for each phenotype (n=3). Error bars reflect one standard deviation. (D) Mono-punctate nucleus representative image from a DNA-immunoFISH experiment with CoREST (left, red), single OR-containing BAC (RP24-65B23) probe (middle, white), and H3T6ph (green, right). Middle inset: BAC signal (white) co-localized with LSD1 compartment (red). Right inset: BAC signal (red) and H3T6ph (green), noting that no H3T6ph is present on the BAC signal. Scale bars represent 5μm.
This study has shown that LSD1 presents a compelling singularity both *in vitro* and *in vivo* that fits the “silence all, de-repress one” model for monogenic OR transcription. The compartmentalization of this demethylase is developmentally regulated, occurring in the same developmental window as initial OR selection (i.e. the most recently post-mitotic cells of the OSN lineage). The LSD1 singular compartment co-localizes with a small number of OR genes at a time, consistent with the possible selection of a single OR gene for expression. One consistent binding partner of this singularity has been identified, CoREST, an adaptor that facilitates LSD1 binding to nucleosomes. It does not appear to be a high-throughput, general expression compartment or a highly active demethylase complex, rather a rare, perhaps selective demethylation compartment waiting for a trigger (e.g. binding of an additional co-factor) to remove di- and mono-methyl marks on its lysine residue substrate. Interestingly, the LSD1-CoREST singular compartment transiently associates with OR-specific transcription factor, Lhx2, which further argues that this complex is relevant to OR transcriptional regulation. Future studies of the LSD1 singular nuclear compartment will address additional binding partners and the functionality of the complex.
Discussion

4.1  *LSD1 presents a compelling singularity* in vitro and in vivo

Previous studies in different model systems have identified LSD1 as necessary for olfactory receptor gene activation (Lyons, Allen et al. 2013, Ferreira, Wilson et al. 2014). LSD1 is down regulated upon OSN differentiation in the mouse olfactory epithelium, a developmental time when OR choice has been locked in for the mature neuron (Krolewski, Packard et al. 2013, Lyons, Allen et al. 2013). Although there has been a growing notion that LSD1 could be the demethylase responsible for demethylating the actively expressed OR gene, the nuclear organization of LSD1 within the OSN lineage has never before been addressed. This study describes the observation of a compelling nuclear consolidation of LSD1 into a discrete compartment.

The presence of a singularity in biological systems is unique and quite rare. One notable singularity is the RNA polymerase I body found in the bloodstream form of the African trypanosome (Navarro and Gull 2001). Like the choice of a single OR allele per OSN, the trypanosome expresses a single allele for the variant surface glycoprotein that will compose the parasite’s antigenic coat (Horn 2014). A transcriptionally active extranucleolar body of RNA Pol I exhibits singular compartmentalization in the bloodstream form of the parasite, responsible for the monoallelic expression pattern (Navarro and Gull 2001). The presence of a singular LSD1 compartment emulates the discrete Pol I body.
Without considering the specific monogenic and monoallelic transcription of ORs, there are several general reasons why LSD1 protein might consolidate in this manner. Consolidation of the protein could facilitate strictly controlled demethylation of histone lysine residues (H3K4 or H3K9), promoting selectivity while preventing widespread access to the compartment. In a tightly controlled demethylation process, most of the consolidated protein would have to be inactive, or providing little demethylation activity in order to maintain its selective functionality. Compartmentalization of the protein could also provide a scaffold for multiple other protein co-factors to bind. A multi-protein complex might determine the substrate specificity and identify gene targets more efficiently than the LSD1 protein alone. For instance, other proteins within the complex may provide more efficient scanning abilities to identify gene targets or may bind gene targets with higher affinity. If involved in a chromatin regulatory pathway, the hypothesized complex could function to monitor the chromatin states of target genes throughout development or cell differentiation. In this scenario, a highly efficient multi-protein consolidated complex would be providing constant demethylation to target gene loci. To address these possibilities, a future focus on co-immunoprecipitation of LSD1 and bound co-factors will narrow down the substrate specificity of the complex and thus the developmental function.
4.2 The developmental timing of LSD1 consolidation correlates with the timing of OR selection in vivo

We chose to initially investigate the nuclear organization of LSD1 in OSN-specific populations based on the theory that LSD1 is a key player in mutually exclusive OR transcription. Our *in vivo* immunohistochemistry results have replicated the expression patterns found previously in the MOE (Krolewski, Packard et al. 2013, Lyons, Allen et al. 2013) with LSD1 abundant in the more basal cell types (particularly the GBCs) and a general down regulation as the tissue matures to the outer OSN layers where LSD1 is absent. It seems that there is a developmental progression of LSD1 compartmentalization from a poly-punctate phenotype in the Neurogenin 1-P27-double positive cells to a mono-punctate consolidation in P27-single positive cells. The double positive cells are earlier in the OSN lineage with Neurogenin 1 expression lingering upon exiting the cell cycle from their former stem-like progenitor state. Yet, we have shown that the consolidation of LSD1 into a single focus occurs in the P27-positive cells, the most recent post-mitotic cells in the lineage that do not have lingering stem-like characteristics (i.e. Neurogenin 1 is no longer expressed). The establishment of singular OR choice during this developmental time triggers the beginning of differentiation to an OSN (Fan and Ngai 2001). The compartmentalization of LSD1 into a single compartment therefore correlates with the timing of presumed OR choice *in vivo*.
One hypothesis for the incremental consolidation of LSD1 could be that the multiple compartments of LSD1 are recognizing an eligible subset of OR loci within the progenitor cells in a particular zone of the epithelium. Maybe another co-factor is required to maintain the stability of the complex positioned at a single OR locus, which may be what we visualize as the mono-punctate compartment in the P27-positive cells. To test that hypothesis, one could perform FACS on the MOE to separate the Neurogenin-P27 double positive cells from the P27-positive cells, followed by co-immunoprecipitation of LSD1 and protein mass spectrometry. If different proteins are associated with LSD1 in these different cell types, or even the presence of a single crucial factor, it would suggest that the singular compartment is stabilized by a different set of factors to theoretically maintain its selectivity for a single OR locus.

We have also observed a comparable result in OP6 cells in which LSD1 compartmentalization is cell cycle-dependent. That is, LSD1 is completely absent from cells during mitosis. There was an observed increase in LSD1 consolidation into the single compartment in G1 phase based on time course studies. This corresponds to the P27-positive cells in the MOE, as P27\textsuperscript{kip1} is a cyclin-dependent kinase inhibitor that effectively stops cells from re-entering the cell cycle, thus halting the cell cycle progression at G1 phase. A poly-punctate phenotype is observed in the OP6 cells, with its highest incidence at the G2/M boundary. This is also consistent with our findings \textit{in vivo} as the poly-punctate consolidation pattern is only observed in cells that are cycling, the
progenitor cells of the populations (the globose basal cells of the MOE expressing Neurogenin 1). When we induced OP6 cell differentiation by turning off the large T-antigen, we saw a subsequent lack of LSD1 consolidation, paralleling the loss of LSD1 expression in the differentiating cell types of the MOE to the mature OSNs. All together, LSD1 singular consolidation seems to be dependent on recent exit from the cell cycle and down regulated upon the expression of differentiation-specific factors.

4.3 The LSD1 singular compartment interacts with a small subset of OR genes at a time

The LSD1 singular compartment localizes to the edges of heterochromatic chromocenters in vivo. Nuclear chromocenters are structures composed of the most compact chromatin, as a way of silencing genes and repetitive satellite sequences, and are characterized by the presence of H3K9 tri-methylation (Guenatri, Bailly et al. 2004, Almouzni and Probst 2011). Inactive OR genes were previously shown to be enriched around the periphery of nuclear chromocenters in mature OSNs (Clowney, LeGros et al. 2012, Armelin-Correa, Gutiymama et al. 2014, Kilinc, Meredith et al. 2014). We have also shown that the LSD1 compartment interacts (i.e. co-localizes) with OR clusters in (probably) a mutually exclusive manner based on DNA-immunoFISH experiments using pooled OR-containing BAC probes and the pan-OR probe. Further our in vivo DNA-immunoFISH studies show that the LSD1 singular compartment co-
localizes with a single foci of OR-containing BAC probes at once, with a higher incidence of LSD1-OR interactions upon addition of more BACs in pooled probe experiments.

Although FISH does not have the resolution to see individual genes, we do observe single OR clusters (encompassing anywhere from 1-14 OR genes) with its corresponding allelic cluster separate from the compartment, as seen in individual OR-containing BAC probe DNA-immunoFISH experiments. This interaction suggests the LSD1 is involved in the mechanism facilitating monogenic OR transcription. The specific localization of LSD1 to the periphery of chromocenters in vivo may suggest that the compartment functions to deliver previously active genes to the chromocenters upon H3K4 demethylation, or it may function to license a single OR gene to exit the chromocenter upon H3K9 demethylation to be expressed. We cannot rule out that LSD1 may be functioning downstream of the transcription process as a way to prevent transcription of other OR genes once an OR is expressed. However the latter explanation is more favorable as it would indicate that LSD1 plays a role in initial OR choice and is more consistent with our findings in vivo since an OR is not previously expressed in the progenitor cells or immature neurons. Given that we find a very small sub-region of the compartment in vitro to contain 8-oxoG, OGG1, or Lhx2 loci (albeit the combination were not examined at the same time), it seems that a small portion of the compartment contains the active site for demethylation. This is consistent with the notion that the multiple ORs in a
cluster within the broad LSD1 compartment are not all being demethylated, rather a single locus containing one of the mentioned marks above.

A role for the LSD1 singular compartment in initial OR gene selection is consistent with the recently proposed OR-mediated feedback model (Figure 9) (Dalton, Lyons et al. 2013). The consolidation of LSD1 may provide the scaffold for a singular OR demethylation event upstream of transcription. This may effectively license the single OR gene to be transcribed and the presence of OR protein would thus trigger the unfolded protein response (UPR). The UPR pathway would then lead to the production of adenylyl cyclase III to inhibit LSD1 expression (and therefore prevent consolidation into a singular compartment) in differentiated OSNs which have made their characteristic OR gene selection. Our data is consistent with this reasoning, as we do not observe LSD1 expression in the more mature layers of the mouse olfactory epithelium where cells have committed to an OR choice.

4.4 Identification of the functionality of the LSD1 compartment

To determine the function of the LSD1 compartment, we investigated a combination of OR regulators, DNA damage marks, and histone modifications that could indicate LSD1’s activity *in vitro*. First, we found that LSD1 always co-consolidates with CoREST, an adaptor protein that facilitates the complex binding to nucleosomes (Lee, Wynder et al. 2005, Shi, Matson et al. 2005). Identification of CoREST as a co-factor however did not narrow down the
particular activity of LSD1, as this complex has been shown to demethylate H3K9 and H3K4 substrates in different contexts (Metzger, Wissmann et al. 2005, Saleque, Kim et al. 2007, Wang, Scully et al. 2007, Zhang, Bonasio et al. 2013, Ray, Li et al. 2014). We then found that a potential OR regulator, the transcription factor Lhx2 (Hirota and Mombaerts 2004, Kolterud, Alenius et al. 2004), is present within ~half of the LSD1 compartments at any given time. Along with the DNA-immunoFISH data that demonstrated the presence of single OR clusters in the LSD1 compartment, the presence of Lhx2 further provides evidence that LSD1 consolidation may be important to OR regulation in developing OSNs. A future three-color DNA-immunoFISH experiment will distinguish whether Lhx2 is located on the one OR clusters inside the LSD1 compartment, which would link the transcription factor directly to the ORs found inside the LSD1-CoREST complex.

We showed that consolidated LSD1 never co-localizes with RNA polymerase II factory, however it is possible that we are not able to visualize a single Pol II molecule using immunofluorescence. An additional RNA FISH experiment could substantiate the conclusion that this is not an OR expression compartment, as we would not expect to see OR RNA within the LSD1 compartment. By studying the DNA damage mark, 8-oxoG, indicative of LSD1 demethylation activity and shown to be on active OR loci in vivo (Perillo, Ombra et al. 2008, Amente, Bertoni et al. 2010, Lyons, Allen et al. 2013), we determined that the compartment contains some demethylase activity, at least 20% of the
time. If the 8-oxoG marks provide an accurate measure of LSD1-mediated demethylation and are not immediately repaired or ejected from the compartment, the majority of the consolidated LSD1 compartment may be a consolidation of mostly inactive protein.

However, we considered the possibility that the demethylase activity could be masked by the immediate downstream repair by OGG1 in vitro (Perillo, Ombra et al. 2008, Amente, Bertoni et al. 2010). OGG1 is abundant in OP6 cells and is found within greater than 90% of LSD1 compartments. Yet despite the generally high incidence of an OGG1 focus within the compartment, there is not an enrichment of OGG1 foci around the compartment nor is OGG1 covering the entire compartment. This repair protein, like 8-oxoG damage, is only found within a small portion of the much larger LSD1-CoREST compartment. Thus it does not look like the consolidated complex is a hub of high-throughput demethylation. Again, this result is consistent with “silence all, de-repress one” model for OR gene regulation (Figure 4) (Magklara, Yen et al. 2011) as demethylation of a single OR may be a rare and selective event. The LSD1 compartment may be awaiting the binding of an additional partner to trigger its demethylase activity, perhaps JMJD2C that removes the tri-methyl group on H3K9 residues.

Recent studies showed enrichment of 8-oxoG damage at the active OR locus in vivo, which may act as a beacon to recruit enhancers for commitment to a single OR choice (Lyons, Allen et al. 2013). Based on microarray data from
another group, OGG1 does not seem to be present in the MOE at detectable levels (unpublished, personal communication with James Schwob), which may explain why we observe a lack of OR choice commitment (i.e. switching) in the OP6 cell line; that is, OGG1 may be repairing the damage preventing a final commitment to an OR gene in OP6 cells. As another method of selectivity, this damage could be facilitating the formation of chromatin loops. Looping would be an effective way to provide greater selectivity for LSD1 demethylation of a single OR locus.

In light of the recent list identifying more OR enhancers, or locus control regions (LCRs) (Markenscoff-Papadimitriou, Allen et al. 2014), it seems that these regions are also essential to the selectivity mechanism for OR selection. Since we visualize an OR cluster (per BAC signal) within the LSD1 compartment, LCRs may also be functioning inside the compartment to restrict a single locus to the small region of the complex that is actively demethylating.

Lastly we considered two different phosphorylation marks that were shown to influence a change in LSD1 substrate specificity from H3K4 to H3K9 methylation marks (Metzger, Wissmann et al. 2005, Metzger, Yin et al. 2008, Metzger, Imhof et al. 2010, Cai, He et al. 2014). We rationalized that if these marks were present on the on the OR cluster within the LSD1 compartment, that it would fit with the hypothesis that the LSD1 compartment is demethylating H3K9 marks on ORs as a way to license a single OR for expression. Curiously, we found H3T6ph and H3T11ph marks within the LSD1 compartments around 50% of the time, but the marks were not observed on the OR-BAC probe signals.
within the compartment. Since these phosphorylation marks are present within the compartment, at least part of the complex could be demethylating H3K9 residues. We can imagine two possibilities to explain this observation. Either the LSD1 compartment is demethylating H3K9 residues on a non-OR locus, or if it is demethylating an OR, the locus may be decompacted and we simply are not visualizing the decompaction. Based on the DNA-immunoFISH results, it does not appear that the OR clusters within the LSD1 compartment contain H3T6ph, however future ChIP studies of the OR sequences interacting with LSD1 will better address this question.

4.5 Future studies of the LSD1 compartment

To clarify the role of the singular LSD1 compartment, future studies will focus on the identification of other factors in complex with LSD1 and CoREST. This will include chromatin immunoprecipitation to identify the OR DNA associated with LSD1; perhaps decompacted OR DNA within the compartment will contain H3T6 phosphorylation, indicative of H3K9 demethylation activity. Co-immunoprecipitation experiments followed with protein mass spectrometry will help to identify other co-factors to LSD1. Co-IP will not be able to isolate solely consolidated LSD1 compartments, so candidate proteins would need to subsequently be examined using immunofluorescence to test whether they are specifically components of the LSD1-CoREST complex. The most informative experiment, eluded to earlier, would be to collect nuclear fractions from P27-
positive cells (Neurogenin 1-negative) in the mouse olfactory epithelium, which would seemingly be enriched for LSD1 singular compartments, and perform LSD1 Co-IP and mass spectrometry.

It is also unclear when in the process of OR selection that consolidated LSD1 may be functioning. Based on its localization on the periphery of chromocenters where inactive ORs are typically held in a silenced states, an LSD1 compartment could be a first step in OR selection by demethylating H3K9 on an OR loci for subsequent delivery to an RNA polymerase factory. Perhaps Co-IP studies will identify chaperone proteins that bring genes to transcriptional factories. Otherwise, the LSD1 compartment could be delivering OR genes to the chromocenters via H3K4 demethylation as a method of silencing. If LSD1 compartmentalization is instead a generic process rather than one specific to OR selection, the LSD1-CoREST complex may be demethylating a broader range of heterochromatic gene loci which happens to include OR loci. It is possible to imagine that consolidation of the demethylase is serving a general epigenetic function to provide selectivity during the cell cycle or cell differentiation. To distinguish whether it is demethylating the OR locus within the compartment, three color DNA-immunoFISH with LSD1, 8-oxoG, and OR probes would identify whether active demethylation is occurring at an OR locus. To address whether it is specific to ORs, a variety of non-OR-containing BAC probes would need to be investigated to see the incidence of co-localization; if the compartment is specific
to OR regulation, we would not expect to see other BAC probes within the compartment.

Lastly, the limited demethylase activity within the compartments, per the 8-oxoG and OGG1 studies *in vitro*, brings up the question of what the rate-limiting step for consolidated LSD1 demethylation could be; this is likely related to the rate limiting step in the selection of an OR gene for activation based on the “silence all, de-repress one” model. It will be important to address the H3K9 methylation states of the OR clusters within the singular LSD1 compartment using both immunofluorescence and ChIP. If the compartment were holding ORs with H3K9 tri-methylation, it would suggest the need for the binding of a cofactor that triggers LSD1-demethylation activity (e.g., JMJ2C removes the tri-methyl group first enabling LSD1 to remove di- and mono-methyl groups). In this scenario, the rate-limiting step in selection would be the first step that removes the tri-methylation mark. If on the other hand, the status of OR loci within the compartment are di-methylated, it would suggest that the LSD1 step itself is rate limiting. Finally, if the status of OR loci are un-methylated, it would suggest that demethylation occurs rapidly, but transfer out of the compartment is rate limiting (e.g., awaiting some kind of chaperone).

Identification of the LSD1 singular nuclear compartment is consistent with the “silence all, de-repress one” consensus model in the field of OR gene regulation. This study lays the groundwork for identifying a singular event, potentially pivotal in the establishment of a single olfactory receptor gene
choice. The singular LSD1-CoREST compartment consolidates during the same developmental timing as OR gene choice and interacts with single OR gene clusters. There are many exciting avenues for future studies, including identification of binding partners that may trigger its demethylase activity. These will help to elucidate the function of LSD1 on its associated OR genes, which may be crucial to the development of monogenic and monoallelic olfactory receptor gene expression.
References


Ma, Q., L. Sommer, P. Cserjesi and D. J. Anderson (1997). "Mash1 and neurogenin1 expression patterns define complementary domains of neuroepithelium in the developing CNS and are correlated with regions expressing notch ligands." J Neurosci 17(10): 3644-3652.


Pathak, N., P. Johnson, M. Getman and R. P. Lane (2009). "Odorant receptor (OR) gene choice is biased and non-clonal in two olfactory placode cell lines, and OR RNA is nuclear prior to differentiation of these lines." J Neurochem 108(2): 486-497.


