**Lysine-specific demethylase-1 (LSD1) depletion disrupts
monogenic and monoallelic odorant receptor (OR) expression
in an olfactory neuronal cell line**

Rutesh Vyas, Diane Meredith, and Robert P. Lane^{*}

Robert P. Lane

Abstract

Function of the mammalian olfactory system depends on specialized olfactory sensory neurons (OSNs) that each express only one allele (“monoallelic”) of one odorant receptor (OR) gene (“monogenic”). The lysine-specific demethylase-1 (LSD1) protein removes activating H3K4 or silencing H3K9 methylation marks in a variety of developmental contexts, and is thought to be important for proper OR regulation. Most of the focus in the field has been on a potential “activating” function for LSD1; e.g., in the demethylation of H3K9 associated with the expressed OR allele. Here we show that depletion of LSD1 in an immortalized olfactory-placode-derived cell line (OP6) results in multigenic and multiallelic OR transcription per cell, while not seemingly disrupting the ability of these cells to activate new OR genes during clonal expansion. These results are consistent with LSD1 having a role in silencing additional OR alleles, as opposed to being required for the activation of OR alleles, within the OP6 cellular context.

Keywords:

Olfactory receptor, Lysine specific demethylase-1 (LSD1/KDM1A), Allelic exclusion, Mutually exclusive expression, Monogenic OR expression, Olfactory placode (OP6) cell line, Chromatin, Epigenetics, H3K4 methylation, H3K9 methylation

Introduction

The proper function of the mouse olfactory system depends on the development of specialized sensory neurons that each respond to a narrow range of odorant chemistry. This specialization is accomplished by the expression of one allele (“monoallelic”) of one odorant receptor (OR) gene (“monogenic”) from a repertoire of ~1400 OR genes encoded in the mouse genome ([Buck and Axel, 1991](#); [Reviewed in McClintock, 2010](#); [Rodriguez, 2013](#)). A very active area of research and the focus of this study concerns the mechanism whereby each olfactory sensory neuron (OSN) transcribes OR genes/alleles in a mutually exclusive manner.

Recent evidence points to epigenetic mechanisms contributing to mutually exclusive OR transcription ([Reviewed in Monahan and Lomvardas, 2015](#)). Three compelling lines of evidence are summarized here. First, identical OR transgene cassettes incorporated into the same mouse genome are expressed in a mutually exclusive manner ([Serizawa et al., 2000](#); [Serizawa et al., 2003](#)), indicating that this phenomenon must be governed at a level beyond the DNA sequence per se. Second, disruption of epigenetic

states, including nuclear chromocenter structure ([Clowney et al., 2012](#)) or deletion of proteins that establish heterochromatic marks ([Lyons et al., 2014](#)), results in multiple OR genes transcribing per cell. And third, two heterochromatic histone marks, H3K9me3 and H4K20me3, are deposited at all OR gene loci prior to choice, and are subsequently removed exclusively on the selected OR gene allele, and a histone mark associated with activation, H3K4me3 is exclusively acquired on the selected OR gene allele ([Magklara et al., 2011](#)), suggesting that OR activation involves selective de-repression. The simplest model to account for these observations is one in which OR genes are initially sequestered to a silencing chromatin compartment, with one allele only being liberated from this heterochromatin so that it can access transcriptional machinery.

A prediction of this model is that there exists a de-repressive protein complex to which competing OR gene loci have restricted access. The lysine-specific demethylase-1 (LSD1) is a plausible candidate constituent of such a putative complex. LSD1 is a versatile protein utilized in a broad range of activating and repressing functions in various developmental contexts ([Cai et al., 2014](#); [Foster et al., 2010](#); [Laurent et al., 2015](#); [Maes et al., 2015](#); [Mosammaparast and Shi, 2010](#); [Ray et al., 2014](#); [Rusconi et al., 2015](#); [Shin et al., 2015](#); [Su et al., 2009](#); [Toffolo et al., 2014](#); [Tsai et al., 2008](#); [Wang et al., 2007](#)). The protein functions in both the H3K4 and H3K9 histone demethylation pathways, the latter important for gene de-repression; the H3K9 demethylation observed exclusively at the selected OR gene locus ([Magklara et al., 2011](#)) suggests a possible role for LSD1 in OR activation. LSD1 is expressed in the early cells of the OSN lineage, but it is down-regulated later in the lineage after OR choice has occurred ([Kilinc et al., 2016](#); [Krolewski et al., 2013](#)), and the deletion of mouse LSD1 in vivo results in a dramatic decrease in OR

expression within the mature olfactory epithelium ([Lyons et al., 2013](#)). Finally, we recently reported that mouse LSD1 protein is compartmentalized in the nucleus during a narrow developmental window within the earliest post-mitotic cells of the OSN lineage when OR selections are thought to be occurring; these LSD1 compartments appear to interact with one or a small number of OR loci at a time, consistent with possibly playing a role in mutually exclusive selection ([Kilinc et al., 2016](#)). Together, these findings provide momentum for this study, where we directly investigate the consequence of LSD1 depletion in OR regulation within an immortalized olfactory-placode derived cell line (OP6).

The OP6 cell line is derived from an immortalized post-progenitor receptor neuron from E10 mouse olfactory placode ([Illing et al., 2002](#)). The onset of OR expression is thought to occur by E9.5 ([Rodriguez-Gil et al., 2010](#)), or before the embryonic state from which OP6 cells were derived (E10). Individual OP6 cells in culture appear to express OR genes monoallelically and monogenically ([Kilinc et al., 2014](#); [Pathak et al., 2009](#)). However, full OSN maturation including up-regulation and stabilization of the selected OR gene, presumably via the unfolded protein response (UPR)-mediated feedback loop ([Dalton et al., 2013](#)), is not realized in OP6 cultures. Therefore, we suggest that OP6 cells might represent a stage between “OR choice” and “OR commitment”, and thus an opportunity to distinguish between these two regulatory events not easily separable in vivo. Consistent with this perspective, we observe that OR expression levels in OP6 cells are much lower than the levels found in more mature OSNs of the olfactory epithelium, thus probably below a threshold required to trigger the UPR feedback pathway; perhaps consequently, we also observe frequent OR switching

during OP6 cell growth indicating a lack of post-feedback commitment ([Pathak et al., 2009](#)). Moreover, OP6 cells do not express the olfactory marker protein, OMP, which is up-regulated in mature OSNs after OR commitment; moreover, OP6 cells express both GAP43 and LSD1 ([Kilinc et al., 2016](#); [Pathak et al., 2009](#)), which are down-regulated in mature OSNs after OR commitment ([Dalton et al., 2013](#); [Lyons et al., 2013](#)). The latter observation is noteworthy, since it has been previously argued that down-regulation of LSD1 might be necessary to stabilize OR selection by preventing additional OR activation events ([Lyons et al., 2013](#)). Thus, one of the motivations of our study was to investigate whether OR choice indeed becomes stabilized when LSD1 is artificially down-regulated as normally occurs post-feedback in vivo.

We used RNAi to knockdown LSD1 expression to undetectable levels in a high percentage (~90%) of OP6 cells in culture. We find that LSD1 depletion does not appear to interfere with the ability of OP6 cells to switch from one OR gene to another during culturing, a result not predicted if LSD1 was required for de novo OR activation events. This result also seems consistent with results obtained in LSD1 knockout mice, where OR genes are able to express in this context as well, albeit at reduced levels ([Lyons et al., 2013](#)). Thus, neither study supports a model in which LSD1 activity is necessary for initial OR selection. Surprisingly however, we observe that LSD1 depletion in OP6 cells disrupts both monoallelic and monogenic OR transcription, suggesting that LSD1 is normally required to prevent multiple OR activation events per cell. This result suggests that LSD1 plays a role in suppressing competing OR genes/alleles.

Methods

Mouse OP6 cell preparation and immunofluorescence

The OP6 cell line was cultured at 33°C in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (Gibco), as described previously ([Illing et al., 2002](#)). In some studies, we generated clonal colonies of various sizes from single OP6 cells. For subsequent immunofluorescence and FISH analyses, cells were seeded on 22cm² coverslips coated with 0.1% gelatin (Sigma) in a 6 well plate at about 50% confluency and expanded for one day to near confluency. Briefly, cells were fixed with 3% paraformaldehyde in PBS for 10 minutes, permeabilized in 0.5% Triton-X (Sigma) for 10 minutes, and blocked in 4xSSC, 0.20% Tween, 4.0% BSA for 20 minutes at 37°C. The primary and secondary antibody incubations were performed at 37°C for 45 minutes in a humidified chamber. The primary antibodies used in this study were rabbit anti-Lsd1 (Abcam, ab129195, 1:100), mouse anti-CoREST (Millipore, MABN486, 1:250), rabbit anti-LBR (Abcam, 122919, 1:50), and mouse anti-methylcytosine (Millipore, MABE345, 1:200). The secondary antibodies used in this study were donkey anti-mouse-Cy3 (Jackson ImmunoResearch, 715-165-150, 1:100), donkey anti-rabbit Alexa 488 (Jackson ImmunoResearch, 711-545-152, 1:100), and goat anti-rabbit-Cy3 (Millipore, AP132C, 1:800). Images were acquired using a Deltavision RT imaging system (Applied Precision) adapted to an Olympus (IX71) microscope equipped with XYZ motorized stage. Each image was sectioned with 0.5 µm intervals to ensure complete coverage of the nucleus. Images were processed using Softworx (Applied Precision).

LSD1 knockdown by RNAi

We adopted an RNAi protocol with slight modifications described at (http://www.broadinstitute.org/rnai/public/resources/protocols_2012). Briefly, lentiviral constructs containing mouse LSD1 shRNA (RMM3981-201787935) and scramble (non-targeted) shRNA (RHS6848, GE-Dharmacon Life Science) were transiently transfected along with packaging plasmids pCMV-dR8.2 dvpr (Plasmid#8455, Addgene) and pCMV-VSV-G (Plasmid#8454, Addgene) ([Sarbassov et al., 2005](#)) into HEK-293T cells (ATCC) using standard calcium phosphate reagents according to manufacturer protocols (Invitrogen). Virus-containing supernatants were collected at 36 and 60 hours after transfection and concentrated by ultracentrifugation. Approximately 10^5 host OP6 cells were subjected to spin-infection at RT for 90 minutes in the presence of 8 μ g/ml polybrene ([Stewart et al., 2003](#)). Lentivirus-transduced OP6 cells (LSD1-knockdown and scramble controls) were selected using puromycin and clonally passaged.

cDNA analyses

Total RNA was extracted from transduced OP6 cells using RNeasy Mini kit (Qiagen), and cDNA generated using superscript reverse transcriptase per manufacturer's protocols (Invitrogen). In some studies, we produced cDNA from single cells using the SMARTer PCR cDNA synthesis kit (Clontech). All cDNA experiments included negative control samples in which reverse transcriptase was omitted ("no-RT controls"). Quantitative PCR (qPCR) was conducted on an ABI 7300 real-time PCR machine (Applied Biosystems). qPCR primer sequences used in this study are available upon request. For colony studies, we seeded individual OP6 cells (confirmed by microscopy) and grew ~200-cell clones. cDNA produced from these colonies were subjected to degenerate OR PCR using the following primers ([Clowney et al., 2012](#); [Michaloski et al., 2006](#)):

ATGGCITAYGAYMGITAYGTIGCIATHTG (135R),
RTTICKIARISWRTAIAATRAAIGGRTT (P8), *GCITAYGAYCGITAYGTIGCIATITG* (P26),
and *ACIACIGAIAGRTGIGAISCRAIGT* (P27). Restriction digests were conducted on
degenerate PCR products using *MseI*, *MluCI*, or *HinfI* (New England Biolabs).

Degenerate PCR products were cloned into pCR-TOPO4.0 (Invitrogen), and several
clones per sample were selected for plasmid preparation and sequencing.

Random sampling simulations to estimate OR complexity

We simulated random sampling from 200-cell colonies across various levels of OR
complexity to identify a complexity level in which the average simulation yields the
observed OR redundancy in that random sample. To exemplify, we obtained 41 distinct
OR genes from a random sample of 60 sequenced templates from the Scr-12 colony; this
degree of redundancy (41 distinct ORs, 19 redundant ORs in a random sample size of 60
sequences) is the average outcome in a simulation where the overall complexity in the
product is 73 OR genes. The results of the simulations for each colony are shown in
Table 1. In addition, we tested the null hypothesis that OR representation is not
significantly different between KD and Scr colonies by simulating random sampling from
a pair of colonies and estimating the expected extent of OR-gene overlap from the two
samples assuming an equal representation (73 distinct genes all equally likely to be
sampled). To exemplify, a random sampling of 41 ORs from Scr-12 and 19 ORs from
KD1 produces an average of 10.2 overlapping ORs between the two samples in the
simulation; the observed 9 overlapping ORs between these two samples is not
significantly different than the simulation ($p \approx 0.35$ to observe 9 or fewer overlaps). The
results of these simulations for pairs of colonies are shown in Table 2.

Western blot analyses

Total protein was extracted from $\sim 10^6$ OP6 cells using 1X RIPA buffer (Cell-Signaling) containing 1X protease inhibitor cocktail (Roche). Protein levels were measured using the Pierce BCA Protein assay kit (Thermo Scientific). Western blot experiments were conducted on 8-10% SDS-PAGE gels transferred to polyvinylidene membranes (Thermo Scientific), blocked in 5% BSA in TBST, followed by overnight incubation with primary antibodies (see below). Following washes, membranes were incubated for 1 hour using anti-rabbit AP (Abcam), washed, and antigen visualized using NBT/BCIP reagent (Roche). Blots were imaged using G-Box F3 (Syngene) and densitometry analyses conducted using ImageJ software (NIH). The following primary antibodies were used for Western blots: anti-LSD1 (ab129195), anti-Beta-actin (ab8227), and anti-acetylated histone H4 (06-866, Millipore). For the global histone modification analysis, we followed the protocol described in ([Rumbaugh and Miller, 2011](#)).

Chromatin immunoprecipitation (ChIP).

Native chromatin was prepared from $\sim 10^7$ OP6 cells as described previously ([Brand et al., 2008](#)). Briefly, nuclei were extracted, digested with micrococcal nuclease (Thermo Scientific) and purified using micro-spin hydroxyapatite chromatography columns (The Nest Group) to yield mono-nucleosomes. Purified chromatin was incubated with pre-mixed Protein-A Dynabeads (Invitrogen) plus either anti-H3K4me3 (04-745, Millipore), anti-H3K4me2 (07-030, Millipore), anti-H3K9me3 (ab8898, Abcam) or anti-H3K9me2 (ab1220, Abcam). Immunoprecipitated DNA was isolated by phenol-chloroform extraction and ethanol precipitation. Enrichment relative to input fractions was determined using qPCR; primer sequences for various control and OR genes used in

various ChIP experiments are available upon request. All enrichment levels were noise-subtracted using a negative control presumed to not contain any histones with the modification in question, and normalized using a positive control presumed to not vary between samples in the histone modification in question.

RNA FISH

Gene-specific probe templates were generated by PCR (primer sequences available upon request) and cloned into pCRII-TOPO vector (Invitrogen) containing flanking SP6 and T7 promoters to enable in vitro production of sense and antisense probes. All cloned products and their orientations within the vector were verified by sequencing. Following linearization, in vitro transcription was carried out by either SP6 or T7 (Roche) RNA polymerases using a digoxigenin (DIG) RNA labeling mix (Sigma; 11277073910) or biotin RNA labeling mix (Sigma; 11685597910). RNA FISH was performed as described previously ([Kilinc et al., 2014](#); [Schaeren-Wiemers and Gerfin-Moser, 1993](#)). Briefly, cells were permeabilized in CSK buffer with 2 mM vanadyl ribonucleoside complex (NEB) and 0.5% Triton-X100 (Sigma), then fixed in 4% paraformaldehyde, followed by dehydration in a 70%-80%-95%-100% ethanol series prior to incubation in 50% formamide/2X-SSC. Approximately 50-100 ng of probe was added to the hybridization buffer (50% formamide, 2X-SSC, 10% dextran sulfate, 5 µg Cot1-DNA, 500 µg/ml salmon sperm DNA) and incubated overnight at 37°C in a humidified chamber. Following washes (maximum stringency = 50% formamide, 0.5X-SSC at 37°C), samples were blocked for subsequent antibody incubations (4% BSA, 4X-SSC, 0.2% Tween-20). DIG signals were visualized using sheep anti-DIG FITC (11207741910, Roche) and donkey anti-sheep FITC (sc-2476, Santa Cruz Biotech)

antibodies. For RNA FISH experiments conducted with biotinylated probes, we amplified signals by including a third antibody incubation as follows: primary incubation using avidin-rhodamine (A-2012, Vector Laboratories), secondary incubation using biotinylated anti-avidin (ab7235, Abcam), and a third incubation using avidin-rhodamine (A-2012, Vector Laboratories). For two-color RNA FISH experiments, we combined primary antibodies and secondary antibodies in respective incubations.

Results

We have utilized a cell line derived from E10 mouse olfactory placode, the OP6 cell line, to investigate the molecular mechanisms underlying mutually exclusive OR transcription. The founder cell to the OP6 line has been staged as a post-mitotic immature neuronal cell ([Illing et al., 2002](#)). We have previously shown that OP6 cells express OR genes in a mutually exclusive manner (both monallelically and monogenically), although at much lower transcript level per cell than is the case in mature olfactory sensory neurons (OSNs) ([Kilinc et al., 2014](#)). We have also shown that OP6 cells “switch” their OR choice during culturing ([Pathak et al., 2009](#)). Together, the reduced expression level and the lack of stability in OR choice suggest that OP6 cells represent an intermediate state between OR selection and commitment.

OR commitment is thought to involve a feedback system designed to prevent the activation of a second gene once an initial selection has been successfully realized, and presumably to amplify the expression level of the selected OR so the neuron has robust odorant-binding function. The selected OR protein generates this feedback via activating

the unfolded protein response (UPR) pathway, with one downstream consequence being the down-regulation of the LSD1 protein ([Dalton et al., 2013](#)), which might prevent the cell making an additional OR selection ([Lyons et al., 2013](#)). In the absence of OR protein and this feedback process, the cell will “switch” to another OR ([Lewcock and Reed, 2004](#); [Serizawa et al., 2003](#); [Shykind et al., 2004](#)). The fact that OP6 cells “switch”, as well as express LSD1 at pre-feedback levels, is further indication that OP6 cells represent an intermediate state between OR selection and commitment.

LSD1 knockdown in OP6 cells. A simple model has been proposed that links the down-regulation of LSD1 to the commitment of the neuron to its initial OR choice, in which OR selection is dependent on LSD1-mediated H3K9 demethylation activity ([Lyons et al., 2013](#); [Tan et al., 2013](#); [Tian et al., 2016](#)). Thus, this model predicts that the down-regulation of LSD1 would be sufficient to prevent additional OR activation events. A problem with this model is that LSD1 knockout mice appear to make OR selections, albeit at reduced expression levels ([Lyons et al., 2013](#)), suggesting that LSD1-mediated H3K9-demethylation might instead be important with OR enhancement or some other downstream function, as opposed to being required for initial OR selection per se.

To further investigate a role for LSD1 in OR selection and commitment pathways, we used RNAi to deplete LSD1 from OP6 cell cultures. Following selection of transfected cells, resulting OP6 populations exhibit stable ~90% knockdown of LSD1 mRNA (through Day 21), as compared to control OP6 populations that were transfected with a “scrambled” cassette that should not target any known gene in the mouse genome (Fig. 1A). We also analyzed LSD1 protein immunofluorescence levels and Western blot

levels in knockdown (KD) versus scrambled control (Scr) OP6 cell populations, and observe robust depletion of LSD1 in KD populations as compared to Scr populations (Fig. 1B). The immunofluorescence experiments indicate that the majority of KD cells (51/59) exhibit no detectable LSD1 protein, whereas a small subset of these cells (8/59) exhibit approximately the same level of LSD1 immunofluorescence as the control populations (Fig. 1B). This observation seems to indicate that the residual ~10% LSD1 mRNA measured in KD populations (~90% knockdown level, Fig. 1A) is probably due to a bimodal population (“all or nothing”) as opposed to a homogeneous partial knockdown. We also observe several other interesting phenotypes in the LSD1-depleted cells, including a significant decrease in global 5-methyl-cytosine DNA methylation (Supplemental Figure 1), a significant increase in global histone H4 acetylation (Supplementary Figure 1), a significant down-regulation of the LSD1-corepressor protein, CoREST (Supplementary Figure 2), and a significant up-regulation of the matrix-associated lamin-B-receptor (Supplementary Figure 3). None of these preliminary observations will be discussed further in this paper (Supplementary Figures are located at the bottom of this manuscript).

Figure 1. LSD1 mRNA and protein knockdown in OP6 cells. **A. Left Panel:** Average LSD1 cDNA levels as measured by qPCR in knockdown (white bars) relative to scrambled control (black bars) OP6 cell populations normalized to GAPDH. The average LSD1 knockdown level relative to negative controls for four independent clones at Day 4 post-transfection was 6.6%, for 2 independent clones at Day 11 post-transfection was 6.8%, and for three independent clones at Day 21 post-transfection was 7.6%. **Right Panel:** Relative LSD1 cDNA levels as measured by qPCR in four individual knockdown cells (white bars) relative to the average of two scrambled control cells (black bar) normalized to GAPDH. **B. Left Panels:** LSD1 immunofluorescence (green) within a typical knockdown (KD) versus scramble control (Scr) cell nucleus. **Upper Right Panel:** Average LSD1 immunofluorescence in 60 scramble control cells (black bar) versus 59 knockdown cells (white bar). Standard deviations are indicated. The high standard deviation in the KD sample is due to a bimodal distribution, with 51 of these cells (86%) exhibiting no immunofluorescence and 8 of these cells (14%) exhibiting close to control levels of LSD1 immunofluorescence (right two bars in histogram). **Lower Right Panel:** Western blots from scramble control and knockdown populations (10 μ g, 15 μ g, 20 μ g total protein loaded; beta-actin control shown) with histogram relative quantification of band intensities (scrambled control black bars, knockdown white bars). Cell nuclei are labeled with DAPI.

LSD1 depletion does not prevent OR switching during OP6 clonal expansion. Our specific interest was to investigate the impact of LSD1-depletion on OR gene regulation. A prediction of a model in which LSD1-mediated demethylation is required for de novo

OR activations during OP6 cell culturing (i.e., “switching”), is that no new ORs would get selected during expansion of an OP6 cell clone. We tested this prediction by growing ~200-cell clones from isolated single OP6 cells. We conducted RT-PCR on LSD1-depleted and control clones using degenerate PCR primers designed against conserved OR sequences. The specific degenerate PCR primers utilized have been shown previously to amplify a large fraction of OR genes from a complex template mixture ([Clowney et al., 2012](#); [Michaloski et al., 2006](#)). We then digested these degenerate PCR products using a variety of frequent-cutting (4-base) restriction enzymes, where the complexity of the digested fragments will be proportional to the complexity of the original PCR product. For example, we observe only two bands when digesting a gene-specific OR PCR product (whose fragment sizes exactly add up to the size of the original PCR product), whereas we observe hundreds of bands (whose fragments sizes add up to much more than the size of the original PCR product) when digesting a degenerate RT-PCR product from a large OP6 culture expressing numerous OR genes (Fig. 2). When applying this assay on LSD1-depleted and control cell clones, we observe no obvious reduction in the complexity of KD samples, as might be predicted if these clones were unable to switch and activate new ORs during colony expansion (Fig. 2, Table 1). We also sequenced several OR templates generated from LSD1 knockdown colonies, as well as from scrambled control colonies to confirm that multiple OR genes are expressed in all colonies (Table 1). Consistent with the OR heterogeneity observed in restriction digest patterns shown in Figure 2, we identified multiple OR genes present in degenerate PCR templates derived from KD samples (15, 19, and 24 distinct OR genes from the KD7, KD1, and KD6 colonies, respectively; Table 1). Assuming that our sequenced sample is

unbiased and representative (see Methods), we used the degree of redundancy observed at various depths of sequencing of these products to arrive at an approximation of the total OR complexity represented in the Scr and KD degenerate PCR products (Table 1). While these estimates are likely quite error-prone, given that the sample is probably not random due to gene-specific bias in the amplicon (see Methods), we note that these estimates (~76 and ~65 OR genes, respectively) are similar to previous estimates of the overall OR complexity within a typical OP6 culture as measured by cDNA microarrays (~80 OR genes; not shown). Together, our results suggest that LSD1 depletion does not dramatically impair the ability to select new OR genes during colony expansion: KD clonal colonies appear to exhibit a comparably rich array of OR genes. While “OR switching” per se is evident in KD populations (OR complexity is >1 in KD clones), we remain cautious about possible subtle differences in switching tendencies that might arise as a consequence of LSD1 absence.

Figure 2. Colony switching assays indicate comparable levels of OR complexity in knockdown versus control colonies. *Upper Panel:* Degenerate OR RT-PCR products (using P135-P8 degenerate primers) from wild-type OP6 whole populations (WP), two scrambled control clonal colonies (Scr, 200 cell colonies each), and two LSD1-knockdown clonal colonies (KD, 200 cell colonies each) are shown undigested (u, 531-bp product) and digested with *MseI* restriction enzyme (M). A single OR gene product (OR31, 320 bp) is digested with *MseI* to control for complete digestion and false-positive complexity. *Lower Panel:* Additional degenerate OR RT-PCR products from two knockdown (KD) and two scramble control (Scr) colonies each digested with three restriction enzymes (Ml=*MluCI*, Ms=*MseI*, H=*HinfI*).

LSD1 depletion does not seem to alter OR representation in OP6 cell populations. We

were next curious to know whether LSD1 depletion impacted the representation of OR genes commonly and reliably represented in OP6 cultures. We used RNA FISH to determine the percentage of cells in LSD1 knockdown and control cultures for four specific OR genes (Olfr 58, Olfr 70, Olfr860, and Olfr1414) that are commonly represented in OP6 populations, as well as with several BAC-sized RNA FISH probes. Anecdotally, we were unable to identify any gains or losses in OR representation in

LSD1-depleted populations using this non-exhaustive method. We also analyzed the OR sequences obtained from degenerate OR RT-PCR from LSD1-depleted and control populations. While the sampling is again non-exhaustive, we do observe numerous common ORs represented in both the KD and Scr degenerate PCR products. We simulated a random sampling from each of the clones to predict an expected degree of OR overlap, assuming both KD and Scr populations contained the same 76 OR genes (estimated complexity from Table 1), and found that the observed OR overlap was within statistical probability for all pairwise overlaps (except the Scr12-KD6 overlap, which was more divergent than expected; Table 2). That is, the observed overlap in OR representation between Scr and KD sequence samples is not significantly less than the null hypothesis might predict (i.e., if there was no change in OR representation). Therefore, the sampling methods utilized thus far (degenerate RT-PCR and RNA FISH) seem to indicate that LSD1 depletion does not dramatically influence OR representation in OP6 cell populations. A more rigorous analysis of OR representation/switching tendencies in KD versus Scr OP6 cultures would depend on an exhaustive RNA-seq approach.

Table 1. Degenerate-OR PCR product complexity in knockdown versus control colonies. The number of sequenced OR templates from degenerate RT-PCR products for two scrambled control colonies (Scr-11, Scr-12) and three LSD1 knockdown colonies (KD-7, KD-1, KD-6) is shown, as well as the number of distinct OR genes identified in the sequenced sample. The degree of redundancy observed in the sequencing sample was used to estimate the total OR complexity in the degenerate OR PCR product (right column; see Methods). Similarly, the total number of distinct ORs identified across both scrambled control (Scr-total) and all three knockdown (KD-total) was used to estimate the total complexity in these respective colonies.

Clone	Sequenced	Distinct ORs	Complexity
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Scr-11	13	11	35
Scr-12	60	41	73
Scr (total)	73	47	76
KD-7	17	15	63
KD-1	26	19	38
KD-6	40	24	35
KD (total)	83	47	65

Table 2. Degenerate-OR PCR product overlap between knockdown and control colonies. The number of distinct ORs observed in various scramble control (Scr) and knockdown (KD) clones, and the expected (e) number of overlapping OR genes in the pairwise comparison assuming a simulated random selection from a total complexity of 76 ORs estimated in degenerate RT-PCR products (see Table 1). The observed number of overlapping OR genes (o) is not significantly different than predicted by the hypothesis (*p*-values from simulation shown, see Methods), with the exception of the Scr12-KD6 pair, where there are significantly fewer overlaps observed than predicted (*p*=0.04).

Pair	ORs	e	o	<i>p</i>
Scr11-Scr12	11,41	5.9	5	0.42
KD1-KD6	19,24	6.1	5	0.43
KD1-KD7	19,15	3.7	4	0.68
KD6-KD7	24,15	4.8	5	0.70
Scr11-KD1	11,19	2.8	2	0.45
Scr11-KD6	11,24	3.5	2	0.26
Scr11-KD7	11,15	2.2	2	0.60
Scr12-KD1	41,19	10.2	9	0.35
Scr12-KD6	41,24	13.0	9	0.04
Scr12-KD7	41,15	8.1	8	0.57

LSD1 depletion disrupts monoallelic and monogenic OR expression in OP6 cells. In our RNA FISH experiments conducted on the LSD1-depleted OP6 populations at passage-8 (35 days post-infection), we were surprised to observe that all four OR genes exhibited an approximately 2-fold higher percentage of positive cells in knockdown cultures (Fig. 3). Even more surprisingly, we observed several of the positive cells for a given probe exhibited two positive signals per nucleus. In numerous RNA FISH experiments over the previous several years, we have never before observed a multi-spot signal in wild-type OP6 cells. Therefore, LSD1 depletion in OP6 cells appears to disrupt allelic exclusion: while 100% of OP6 cells exhibit one-spot RNA FISH signals consistent with monoallelic expression, an average >10% of the positive cells for a given OR probe

in knockdown populations exhibit two-spot RNA FISH signals consistent with a modest incidence of biallelic expression. We note that sense control probes were cleanly negative in all experiments (not shown).

FISH data using a single gene probe, demonstrating a disruption of allelic exclusion, led us to wonder if LSD1-depleted cells might also express multiple genes per cell. Such an outcome would predict a systematic increase in the percentage of positive cells for each representative OR; for example, a systematic ~2-fold increase in positive cells per OR tested in knockdown versus control populations (as evident in Fig. 3) would be consistent with each cell now expressing an average of two ORs per cell, assuming the overall OR representation in the population is unchanged. To seek evidence for multigenic OR expression in LSD1-depleted OP6 cells, we conducted RNA FISH experiments using pooled OR probes (Fig. 3). We make three observations from these experiments. First, we observe that RNA FISH experiments conducted with pools of probes suffer from a substantial increase in the false-negative rate as compared to experiments conducted with single probes. We conclude this because the percentage of positive cells in the pool of three probes is less than the sum of percentages observed with each of the probes when tested individually. In one pooled experiment, we estimate that the additional false-negative rate due to pooling is ~38% (Fig. 3B, top histogram); in a second pooled experiment, we estimate that the additional false-negative rate due to pooling is ~56% (Fig. 3B, bottom histogram). Second, we observe an even higher percentage of multi-spot nuclei than is observed in experiments conducted with individual probes. That is, in the first pooled experiment, we observe 3.5% multi-spot nuclei in the pool, whereas the incidence of multi-spot nuclei in the three individual

experiments would only predict a total of 2% incidence in the pool (Fig. 3A); similarly, in the second pooled experiment, we observe 1.7% multi-spot nuclei in the pool, whereas the incidence of multi-spot nuclei in the three individual experiments would only predict a total of 1% incidence in the pool (Fig. 3B). While perhaps within variation expected by chance alone, this increase in multi-spot incidence in the pool as compared to the sum of the parts suggests some incidence of multigenic OR expression above and beyond the incidence of multiallelic OR expression. And third, we observe an incidence of some nuclei with three or more RNA FISH signals when pooling probes that was not observed in any of the experiments conducted with individual probes. This observation also suggests some incidence of multigenic OR expression above and beyond the incidence of multi-allelic OR expression.

Figure 3. RNA FISH with individual and pooled OR probes. A. Representative RNA FISH signals (green) in scrambled control (Scr; solid bars) and knockdown (KD; open bars) cell nuclei for three OR

antisense probes (58, 70, 860), as well as a pool of all three probes. Rare multi-spot RNA FISH signals are observed only in knockdown populations, including some cells in the pooled probe experiment that exhibit more than two spots. Sense control probes were all negative (not shown). **B.** Histograms showing the percentage of cell nuclei (n =number of cell nuclei scored for each probe) exhibiting a positive RNA FISH signal for each individual probe, as well as the pool, in two independent experiments. Hatched bars indicate the percentage of these positive cells that exhibit a multi-spot signal. Cell nuclei are labeled with DAPI.

To more rigorously distinguish between multiallelic and multigenic OR expression in LSD1-depleted OP6 cells, we conducted two-color RNA FISH experiments with the Olfr288, Olfr860, and Olfr58 probes (Fig. 4). As observed in the previous one-color experiments reported in Figure 3, there is an approximate doubling of positive cells in KD versus Scr populations. The average percentage of positive cells in the three experiments conducted with individual probes (Olfr288 only, Olfr860 only, and Olfr58 only) increased from 2.2% (Scr) to 4.7% (KD), and the average percentage of positive cells in the two experiments conducted with pairs of probes (Olfr288+Olfr860 and Olfr288+Olfr58) increased from 3.7% (Scr) to 8.0% (KD) (Fig. 4B). As reported in Figure 3, we also observed an incidence of multi-allelic expression in only KD samples for these single-probe experiments (3 of a total of 21 positive cells exhibited a two-spot signal) (Fig. 4). In the two-color experiments conducted with pooled pairs of OR probes, we observed examples of multiallelic OR expression (e.g., top left panel in Fig. 4A) and multigenic OR expression (top right panel in Fig. 4A). We also observed an example of a 3-spot signal (in 24 total positive cells) that shows both multiallelic and multigenic OR expression in a single cell (lower left panel in Fig. 4A). Overall, the incidence of multiallelic cells was equal to the incidence of multigenic cells in these experiments (~1.7% incidence each; Fig. 4B), suggesting that the probability of selecting the other allele is approximately the same as the probability of selecting any other random gene. Of the 7 total cells exhibiting multi-colored RNA FISH signals (i.e., with at least one red and

one green spot), we find one example of a yellow spot that would suggest a shared RNA polymerase (shown in the lower right panel in Fig. 4A); the other 6 cells are exemplified by the image shown in the upper right panel in Figure 4A, where the red and green spot are clearly separated in space suggesting transcription from a distinct RNA polymerase location in the nucleus. This finding seems to dismiss the notion of a dedicated OR-specific RNA polymerase factory for which ORs might compete as part of a mechanism to ensure mutual exclusivity, at least during this initial selection (pre-feedback) phase. Of note, one gene pair used in this experiment (Olfr860 and Olfr58) are ~70kb apart within the same OR cluster on chromosome 9, whereas the other gene pair (Olfr860 and Olfr288) are from different OR clusters on chromosome 9 and 15, respectively. We observed a roughly equal tendency for multigenic expression in the two experiments (~22% of positive cells co-express the gene pair from the same chromosomal cluster; ~20% of positive cells co-express the two genes from two different chromosomal clusters). The one observed yellow spot is from the OR pair from the same chromosomal locus (Olfr860 and Olfr58; lower right panel in Fig. 4A), so this co-localized transcriptional

Figure 4. Two-color RNA FISH on LSD1-depleted cells using pairs of OR probes. **A.** Representative two-color RNA FISH images conducted with a pair of OR probes (Olf860=red, Olf58=green). *Upper left:* an example of a multiallelic cell showing two positive RNA FISH signals from the same probe/color. *Upper right:* an example of a multigenic cell showing two positive RNA FISH signals, one from each OR probe/color. *Lower left:* an example of a cell with three positive RNA FISH signals, with both Olf860 alleles expressing (red) along with the Olf58 gene (green). *Lower right:* an example of a cell with Olf860 (red) and Olf58 (green) expressing from the same physical location in the nucleus, evident by a yellow spot (inset panels: same cell shown in the individual red and green channels). **B.** Histogram summarizing results from three experiments conducted with each of the three OR probes individually (left) and the two experiments conducted with pooled pairs of OR probes (right). The incidences of monoallelic/monogenic OR expression (open portions of bars), multiallelic OR expression (gray portions of bars), and multigenic OR expression (black portion of bars) are shown for LSD1-depleted cells (KD) and scrambled controls (SCR). All sense probe controls were negative (not shown). Cell nuclei are labeled with DAPI.

event may very well arise from cis co-activation as opposed to two different chromosomes looping into the same RNA polymerase factory. At this point, we do not have evidence with our very limited sampling to suggest gene proximity bias for OR co-expression in LSD1 depleted OP6 cells.

H3K4 and H3K9 methylation states at OR loci in LSD1 depleted cells. We conducted chromatin immunoprecipitation (ChIP) experiments using antibodies against H3K4me2, H3K4me3, H3K9me2, and H3K9me3, in order to investigate the impact of LSD1 depletion for these marks at various OR promoter regions (Fig. 5). The H3K4me2/me3 levels at OR loci were predictably near background levels as established by the zfp560 negative control; the maximum enrichment level observed was for Olfr944, where the noise-adjusted H3K4me3 signal strength was ~7% that observed for GAPDH in KD populations (Fig. 5A, lower panel). Therefore, our general conclusion from H3K4 ChIP is that most OR loci continue to exhibit a paucity of this mark, as would be predicted if H3K4 is only rarely acquired at OR loci (e.g., most of the templates for a particular OR gene are not transcribed in the population at a given point in time).

To more rigorously investigate the long-term impact of LSD1 depletion at OR loci, we compared H3K4me2 levels in late- versus early-passage LSD1-depleted cell populations. We observe a systematic increase in H3K4me2 levels at higher passage numbers in LSD1-depleted cultures (Fig. 5C). The H3K4me2 enrichment levels even after prolonged absence of LSD1 for several generations remain very near background levels. Since we are measuring values close to zero in all cases, we presume that even small amounts of experimental error/variation might confound interpretation. Nevertheless, we observe a highly reproducible trend in which all 21 OR promoters tested (across four independent ChIPs for two independent chromatin preps) exhibit a small, but statistically significant elevation in this mark (pairwise T-test, p-value $\sim 10^{-5}$). We make two arguments that this systematic trend is probably not an experimental artifact (e.g., due to systematic differences in the chromatin preparations or ChIP

efficiencies). First, as noted, this trend is reproducible across independent preparations, and so it seems unlikely that a systematic bias within the experiment would be reproduced in multiple attempts. Second, we observe the opposite directionality (i.e., marginally higher H3K4me2 levels in the early- versus late-passage) in three of the four experimental replicas for the positive control GAPDH gene assumed to maintain consistently high, LSD1-independent levels of H3K4me2. We also observe the opposite trend for several developmental gene promoters (MyoD, Gap43, G-olf, Ngn1, and AC3) tested in one of the experiments (not shown); these gene promoters, like OR promoters, exhibit low levels of H3K4me2, but probably are not regulated by LSD1 and therefore might be expected to retain constant H3K4me2 levels between KD and Scr populations. Together, our data suggest that LSD1 depletion has a gradual and subtle impact on H3K4 methylation in a systematic way across all tested OR loci. Importantly, the gradual yet systematic increase in H3K4 methylation at OR loci is predicted if LSD1 normally functions to ensure these active histone marks are only rarely acquired at OR gene promoters.

As anticipated, the H3K9 methylation levels at OR gene promoters are uniformly high and approximate or exceed those observed at the zfp560 positive control (Fig. 5B). These data therefore recapitulate previous reports indicating that the H3K9 methylation is strongly associated with OR promoters ([Magklara et al., 2011](#)), presumably as part of a mechanism to ensure the silencing of most ORs per cell. We do not observe a significant or systematic difference in H3K9me3 at OR loci in LSD1-depleted cells (Fig. 5B, lower panel). In contrast, we do observe a slight but apparently systematic increase in H3K9me2 levels (Fig. 5B, top panel; paired T-test $p < 0.02$), albeit across a small

experimental sample. An accumulation of H3K9me2 at activated OR loci might be predicted if the first LSD1-independent step in the H3K9me3 demethylation pathway at a selected OR allele is transpiring normally (H3K9me3 to H3K9me2) but the second LSD1-dependent step in this pathway (H3K9me2 to H3K9me1/0) is perturbed.

In summary, we observe an apparent systematic accumulation of H3K4me2 (and possibly H3K9me2) in LSD1-depleted cell populations, consistent with the interpretation that LSD1 normally functions to remove these marks at OR gene promoters. These increases are modest on a gene-by-gene basis across the cell population; in addition, we observe no obvious trend with H3K9me3, a mark previously shown to be associated with OR silencing. We emphasize that these ChIP experiments were conducted on whole populations, whereas we anticipate that the regulation of H3K9 (and H3K4) methylation is likely occurring at a given OR locus within a very small fraction (<5%) of the population; i.e., in only those rare cells where a given OR gene has been activated. Therefore, even if the number of expressing cells for a given OR gene has doubled (e.g., from ~1% to ~2% of the population), the anticipated decrease in H3K9 methylation from ~99% to ~98% of templates in the population (or the anticipated increase in H3K4 methylation from ~1% to ~2% of templates in the population) is likely to be within the margin of experimental error and noise parameters for this methodology. A more informative investigation of LSD1's role in regulating H3K4 and H3K9 methylation at OR loci would depend on a single-cell ChIP methodology and/or application on more

homogeneously expressing cell populations.

Figure 5. Chromatin immunoprecipitation (ChIP) to measure changes in H3K4 and H3K9 levels at OR loci in LSD1-depleted OP6 cells. A. and B. Enrichment levels in all chromatin ChIP experiments are relative to the input fraction, noise-subtracted using a negative control locus presumed to lack the mark, and normalized to a positive control locus to correct for experimental variation (see text). **A.** Relative H3K4me2 (top) and H3K4me3 (bottom) enrichment levels in LSD1-knockdown OP6 cell populations (open bars) and scrambled control OP6 cell populations (solid bars) for various odorant receptor promoter regions (“Olf” numbers indicated) using Zfp560 for noise subtraction (negative control) and GAPDH for normalization (positive control). Only Olf944 and Olf995 seem to exhibit above-threshold signal for these histone marks. **B.** Relative H3K9me2 (top) and H3K9me3 (bottom) enrichment levels in LSD1-knockdown OP6 cell populations (open bars) and scrambled control OP6 cell populations (solid bars) for various odorant receptor promoter regions (“Olf” numbers indicated) using GAPDH for noise subtraction (negative control) and Zfp560 for normalization (positive control). Two independent H3K9me3 experiments were conducted due to higher noise levels (experiment#1: 350, 944, 995, 385, 1497; experiment#2: 350, 944, 995, 389, 860, 58 as noted above histograms). **C.** Relative H3K4me2 enrichment levels between early passages (solid bars) and late passages (open bars) of LSD1-depleted OP6 cell populations at 21 OR gene promoters. All levels are normalized to input fraction to correct for PCR bias and noise-subtracted using a satellite DNA negative control to establish background thresholds. Standard deviation bars are shown for data collected from two independent chromatin preparations used to produce four independent ChIP experiments (except for *Olf389*, *Olf177*, and *Olf39* where only a single experiment is shown for each).

Discussion

We have depleted the LSD1 protein in a cell line clonally derived from olfactory placode founders that were previously staged as immature neurons in the developing olfactory lineage ([Illing et al., 2002](#)). The “immature neurons” of the olfactory lineage remain a subject of active research and characterization. In ([Hanchate et al., 2015](#)), these cell populations were subdivided into “early” and “late” immature stages based on developmental gene profiles and the probability of multiple transcribed ORs per cell, with the latter subpopulation being enriched for cells expressing a dominant OR (like OP6 cells). Both subpopulations exhibit OR expression levels that are significantly lower than in mature OSN populations (like OP6 cells). We previously characterized the OP6 cell line as a likely intermediate stage along the OR activation pathway, having already selected one and only one OR gene and allele to be transcribed per cell at premature levels of transcript, yet uncommitted to this choice, likely due to the non-execution of the feedback inhibition process ([Pathak et al., 2009](#)) resulting in persistence of the LSD1 protein ([Kilinc et al., 2016](#)) and frequent switching of OR genes during culturing ([Pathak et al., 2009](#)). Until the olfactory sensory neuronal lineage is more precisely described, we are reluctant to map the OP6 founder cell as to an exact developmental stage within the OE. Moreover, we note that much of the characterization of the olfactory neuronal lineage, and especially attributes of OR gene regulation within this lineage, stem from studies involving late embryonic, post-natal or adult mice, whereas little is known about OSN staging, OR expression profiles, and mechanistic details of OR regulation within an

early embryonic developmental context, including the nascent E10 olfactory placode. Nevertheless, we propose that the OP6 cell line was derived from a developmental stage when OR genes are first expressing at low levels, yet incapable of the UPR-mediated feedback loop associated with OSN maturation, as well as robust OR expression and commitment.

We have worked with the OP6 cell line for more than a decade, and have never observed multigenic or multiallelic OR expression using a variety of methods. Herein we show that depletion of the LSD1 protein in OP6 cells disrupts both monoallelic and monogenic OR expression in this cell line. This result is consistent with LSD1 functioning in a “repressive” pathway otherwise important for maintaining the silencing of competing OR alleles/genes, which contrasts previous published studies that focus on possible “de-repressive” functions for LSD1.

We speculate on two possible “repressive” mechanisms in the context of maintaining the silencing of competing OR alleles/genes. First, LSD1 might be important not just for H3K9 regulation at OR loci, but also H3K4 regulation prior to the UPR-mediated OR commitment and further differentiation into a mature OSN. Several papers in the past year have reported single-cell transcriptome data within the OSN lineage, finding that multiple OR genes per cell are detected in less mature subpopulations ([Hanchate et al., 2015](#); [Tan et al., 2015](#)). This observation raises the possibility of an iterative process, whereby an initial small subset of ORs might transcribe at low levels, but competitors are eventually silenced as the process ensues ([Abdus-Saboor et al., 2016](#); [Tan et al., 2013](#); [Tian and Ma, 2008](#)). LSD1 might be critical in such an iterative

silencing process, e.g., by removing H3K4 methylation at competing loci ([as discussed in Tian et al., 2016](#)).

This paradigm predicts that the loss of LSD1 might result in multiple expressed OR loci per cell due to the accumulation of H3K4 methylation marks at more than one locus per cell. To seek evidence in support of this hypothesis, we conducted ChIP at OR loci, where the ~doubling in the incidence of OR activations in KD populations (e.g., from ~1% to ~2%) might correlate with a corresponding ~doubling in H3K4 methylation at expressed OR loci. The impact on H3K4 methylation in KD populations appears to be more subtle than this hypothesis might predict. At early passages, most ORs tested exhibit H3K4 methylation near the background noise levels; nevertheless, we cannot dismiss the possibility of a systematic doubling of H3K4 methylation at OR loci, since doubling the incidence might still be at or near detection thresholds (e.g., from ~1% of templates to ~2% of templates for a given OR within a heterogeneous cell population). At later passages, we observe a striking systematic increase in H3K4 methylation across a broad panel of ORs tested. This observation affirms that LSD1 would seem to play a role in maintaining lower levels of H3K4 methylation at OR promoters at the pre-commitment stage. However, we do not observe a systematic increase in OR expression levels at later passages as compared to early passages (not shown), as would be predicted if further accumulation of this histone mark was alone sufficient to drive more frequent OR expression.

An alternative hypothesis attributes the perturbation of singular OR expression per cell in LSD1-depleted populations as an indirect consequence of interactions with other regulatory factors as opposed to a direct consequence of H3K4 elevation. For

example, a critical activation factor might normally be sequestered within an LSD1 protein complex, thus limiting its access to competing OR promoters; the depletion of LSD1 in turn might lead to liberation of this putative activator so that it can interact with additional OR promoters (i.e., more than one OR). The LSD1 compartments described previously ([Kilinc et al., 2016](#)) might represent a scaffold for such an activator to prevent multiple/widespread OR associations. Unlike the H3K4-mediated activation hypothesis described above, this hypothesis does not predict a cumulative effect on OR expression within an LSD1-depleted environment, since once the LSD1 complex is disrupted, presumably the same access to a putative activator would be granted to competing ORs irrespective of how long the culture has grown in the absence of LSD1.

The two above hypotheses are not mutually exclusive - for example, acquisition of H3K4 methylation at multiple OR genes per cell might increase the probability of multiple activations per cell, and in addition, a putative activator normally sequestered by/complexed with LSD1 might also permit only a limited number of activation events per cell. A hallmark of both models is the notion of a limiting factor enabling OR activation (i.e., either H3K4 methylation being limiting and/or a putative activator protein being limiting). Thus, both models might predict that even when the system is perturbed, there might be an upper limit on the number of OR genes that can be activated per cell and/or the total OR RNA output that can be generated when more than one OR gene is active per cell. Such a consideration would account for why both LBR overexpression and G9a/GLP double-knockout mice that perturb monogenic OR expression result in a finite number of ORs expressed per cell ([Clowney et al., 2012](#); [Lyons et al., 2014](#)), as

well as a compensatory reduction in OR expression levels per cell when multiple ORs are activate ([Clowney et al., 2012](#); [Hanchate et al., 2015](#)).

An important observation in this study is the fact that LSD1 depletion did not perturb de novo OR activation events that occur during “switching” (Fig. 2). The LSD1 knockout mouse ([Lyons et al., 2013](#)) similarly suggests that LSD1 is dispensable for de novo OR activations, since these mice nevertheless express a broad set of OR genes, albeit at reduced levels. If LSD1-mediated removal of H3K9 marks is not required for OR activation per se, it could mean that this chromatin modification might occur downstream of selection (as opposed to a pre-requisite for selection). For example, perhaps LSD1-mediated H3K9 demethylation at the selected OR is important to ensure robust expression levels once an OR is chosen, thus explaining why LSD1 knockout mice exhibit lower OR expression levels, but not abolished OR expression ([Lyons et al., 2013](#)), and/or to ensure that this chosen OR escapes recruitment to nuclear chromocenters (where the other ORs are sequestered, possibly as a consequence of retaining this mark). It is also important to note that LSD1 targets the H3K9me2 mark, not the H3K9me3 mark, so it remains possible that the previous demethylation step from H3K9me3 to H3K9me2 (by an as yet uncharacterized protein) might be a prerequisite to OR activation, even if the subsequent LSD1-mediated demethylation step to follow is unnecessary at this stage.

We attempt to reconcile a model that is consistent with all the published data, including observations made in the OP6 cell line. Rather than proposing a single, rate-limiting step for OR selection (e.g., K9 demethylation), we instead propose a model where both K9 and K4 methylation states are contributing to initial OR selection.

Proteins such as G9a ([Lyons et al., 2014](#)), along with LSD1 ([Ooi and Wood, 2007](#); [Roopra et al., 2004, herein](#)), might collaborate to ensure that K9 methylation (added/maintained by G9a) dominates across OR loci and acquisition of K4 methylation is rare (aided by LSD1-mediated removal). Perturbation of either enzyme (G9a, ([Lyons et al., 2014](#)); LSD1, herein) might shift the equilibrium state such that K9 is less predominant (and K4 is less rare), thereby increasing the probability of multiple OR activations per cell. The disruption to monogenic OR expression observed here is reminiscent to the G9a knockout mouse – neither results in a dramatic perturbation of K9/K4 methylation states and both seem to exhibit a modest multigenic phenotype – suggestive of a more subtle role for both proteins in maintaining an appropriate balance of the two marks. Such a model might also predict stochastic imbalances arising in vivo, leading to rare and/or transient multi-OR cells during the initial selection stages, an observation that has recently been made by two independent studies ([Hanchate et al., 2015](#); [Tan et al., 2015](#)). Importantly, we suggest that initial choice might be more of a dynamic process in which management of K9 and K4 marks leads to one (or a small number) of preliminary candidates, that will subsequently be refined, amplified, and stabilized during the feedback-mediated commitment process. There are certainly other models that could account for all current observations, but a key aspect of the described model is a shift away from regarding LSD1 as the essential and primary activating factor, as well as H3K9 demethylation as the direct causative molecular event in the establishment of monogenic OR expression, while raising new perspective about LSD1's role in the process of excluding competitor OR loci.

Finally, our results might inspire two interesting questions within the field of epigenetic transcriptional regulation that warrant further study. First, the LSD1 requirement for maintaining OR monoallelic expression is perhaps surprising, given it would be reasonable to assume that allelic regulation is established in a non-lineage-specific manner in the early embryo, as it seems to be for other gene loci exhibiting allelic exclusion ([Chess, 2012, 2013](#); [Eckersley-Maslin and Spector, 2014](#); [Ensminger and Chess, 2004](#); [Savova et al., 2013](#)). We note that in our two-color RNA FISH experiments, we observed approximately the same incidence of multiallelic and multigenic OR expression (Fig. 4B). This finding seems to support a hypothesis that the second OR allele in a given cell is treated equivalently with respect to selection as any other OR gene locus. Second, the simultaneous duality of LSD1 as possibly both a positive ([Lyons et al., 2013](#)) and negative (herein) regulator of OR transcription (i.e., within the same cellular and developmental context), has not yet been established for other LSD1 regulatory targets, which broadens our perspective on the versatility of this important regulatory factor. This latter consideration might be especially critical in the context of OR gene switching, where presumably both activation and deactivation must be precisely coupled.

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