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Enhancer Interaction Networks as a Means for Singular Olfactory Receptor Expression

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SUMMARY

The transcriptional activation of one out of ~2800 olfactory receptor (OR) alleles is a poorly understood process. Here, we identify a plethora of putative OR enhancers and study their in vivo activity in olfactory neurons. Distinguished by an unusual epigenetic signature, candidate OR enhancers are characterized by extensive interchromosomal interactions associated with OR transcription and share a similar pattern of transcription factor footprints. In particular, we establish the role of the transcription factor Bptf as a facilitator of both enhancer interactions and OR transcription. Our observations agree with the model whereby OR transcription occurs in the context of multiple interacting enhancers. Disruption of these interchromosomal interactions results in weak and multigenic OR expression, suggesting that the rare coincidence of numerous enhancers over a stochastically chosen OR may account for the singularity and robustness in OR transcription.

INTRODUCTION

The olfactory system has the ability to detect and distinguish among an astounding number of olfactory stimuli (Bushdid et al., 2014). This vast receptive field is afforded by the large repertoire of olfactory receptors (OR), which, in most mammals, are encoded by more than a thousand genes located in numerous genomic clusters throughout the genome (Buck and Axel, 1991; Sullivan et al., 1996; Zhang et al., 2004). ORs are expressed in olfactory sensory neurons (OSNs) in a monogenic, monoallelic, and seemingly stochastic fashion (Chess et al., 1994) in such a way that each neuron expresses only one out of the ~2,800 available alleles. For each OSN, the identity of the expressed OR determines the spectrum of chemicals that

it responds to and its connectivity to the brain (Wang et al., 1998). The dual role of ORs in odor detection and axon guidance makes the singularity of their expression critical for olfactory perception; were multiple ORs coexpressed in each OSN, the topographic map of OSN projections to the olfactory bulb would be perturbed, likely resulting in reduced olfactory sensitivity and resolution.

The continuous transcription of a single OR is maintained by an OR-elicited feedback signal that stabilizes the expression of the chosen OR and prevents the activation of additional ones (Ferreira et al., 2014; Lewcock and Reed, 2004; Serizawa et al., 2003; Shykind et al., 2004). In mammals, this feedback uses components of the unfolded protein response (UPR) to detect the newly translated OR in the endoplasmic reticulum and to induce transient translation of transcription factor Atf5 (Dalton et al., 2013). Atf5 orchestrates, among others, the expression of Adcy3, the major adenylyl cyclase in the OSNs that is necessary for stable OR transcription and OSN differentiation. Adcy3 expression makes OR choice permanent by signaling for the downregulation of Lsd1, a lysine demethylase with dual coactivator and corepressor activities that regulate OR expression (Lyons et al., 2013). This feedback system is only possible because OR silencing, via the hallmarks of constitutive heterochromatin, occurs early, during OSN differentiation, and before the onset of OR transcription (Magklara et al., 2011). This epigenetic silencing is reinforced by the nuclear convergence of OR loci into a few, OR-specific heterochromatic foci (Clowney et al., 2012). Active OR alleles escape these foci, supporting a role of the spatial compartmentalization between active and inactive OR alleles to the singular OR expression (Armelin-Correa et al., 2014). Indeed, disrupting the nuclear architecture of OSNs violates the "one receptor per neuron" rule, causing coexpression of multiple OR alleles per neuron, albeit at reduced levels (Clowney et al., 2012).

These observations provide the molecular underpinnings of the feedback signal and emphasize the importance of gene silencing in OR gene regulation; however, they do not explain how a single OR allele is selected for transcriptional activation
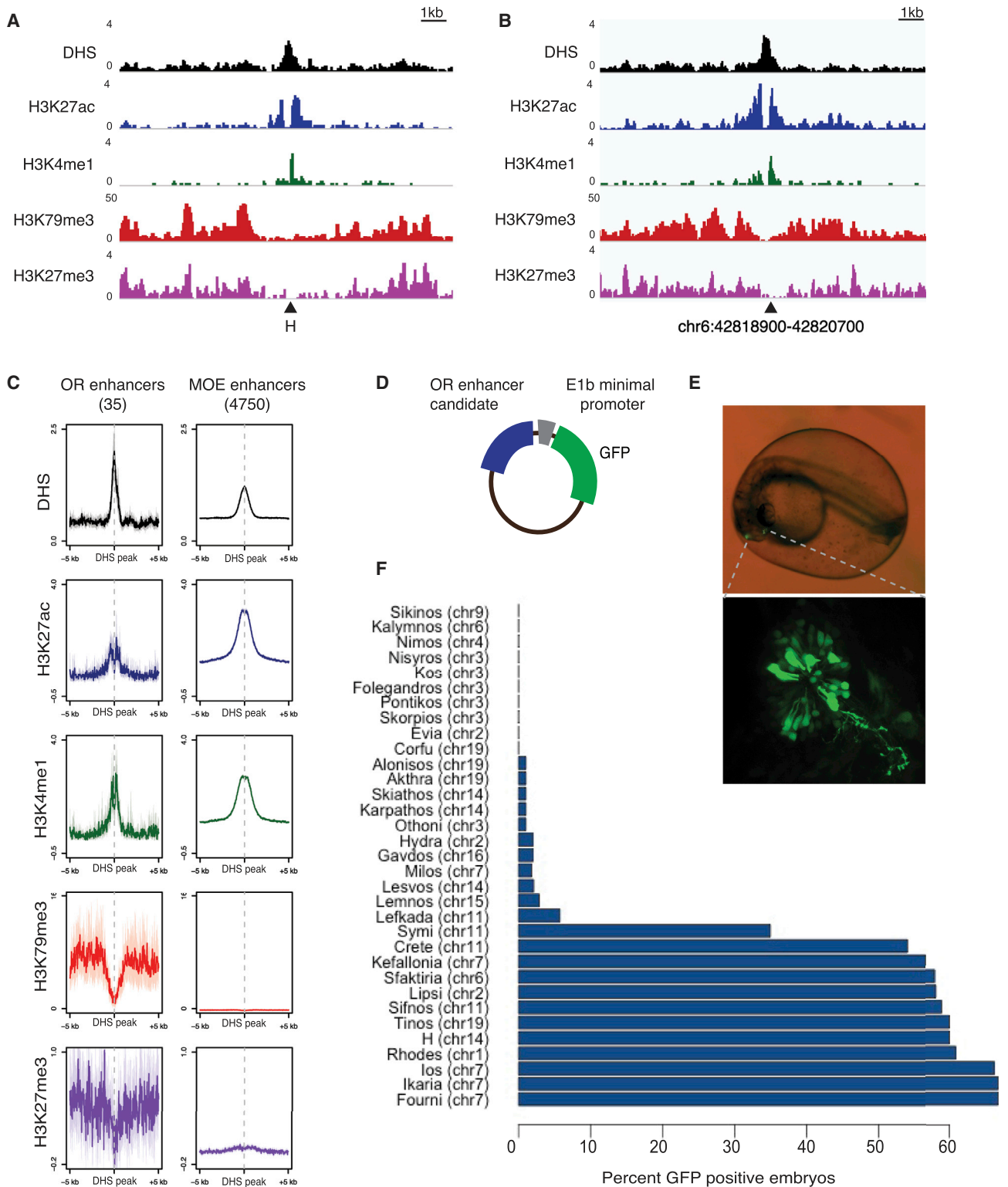
Figure 1. An Epigenetic Signature for Putative OR Enhancers

(A) Sequencing tracks for DHS-seq and ChIP-seq. Each row displays the number of reads for each track. Triangle indicates H enhancer.

(B) DHS-seq and ChIP-seq tracks over potential OR enhancer Sfaktiria (coordinates are mm9).

(C) Aggregate plots of ChIP-seq and DHS-seq reads over all potential OR enhancers (left) and all potential MOE enhancers (right). y axis is RPKM, and error is bootstrapped 95% confidence intervals. x axis is centered at DHS peaks.

(legend continued on next page)

at the beginning of this process. We previously hypothesized that an intergenic OR enhancer, H, could provide this singularity because it frequently associates with transcriptionally active OR alleles from the same or different chromosomes (Lomvardas et al., 2006). However, deletion of the H enhancer affects only the expression of three linked and proximal ORs (Khan et al., 2011). Redundancy for the function of H as a *trans* enhancer, provided by additional H-like elements, could explain why the physical association with H appears to be genetically superfluous for the transcription of most ORs (Williams et al., 2010). This model, which predicts an intricate network of genomic interactions (Bargmann, 2006), ascribes two distinct roles to each intergenic OR enhancer: a critical function as a *cis* regulatory element, which may open up the local chromatin architecture, orchestrating the first step of OR choice, and a redundant function as a *trans* enhancer which, together with other enhancers, facilitates high rates of OR transcription.

To identify elements that may provide redundancy for H as a *trans* enhancer, we performed a genome-wide search for intergenic OR enhancers. DNase I hypersensitivity (DHS)-sequencing (DHS-seq) and chromatin immunoprecipitation sequencing (ChIP-seq) experiments uncovered 35 predicted enhancer elements linked to OR gene clusters. Reporter assays in zebrafish embryos, followed by transgenic and knockout experiments in the mouse, support the function of at least 12 elements as OSN-specific enhancers that likely regulate OR transcription. Importantly, circularized chromosome conformation capture sequencing (4C-seq) analyses of sorted OSNs, combined with two- and three-color DNA fluorescent in situ hybridization (FISH) experiments, demonstrate the convergence of multiple enhancers over the chosen OR. Hi-C analysis with chromatin from the whole main olfactory epithelium (MOE), along with DNA FISH experiments, revealed extensive inter- and intrachromosomal interactions between most of these elements in olfactory neurons. Finally, two independent genetic manipulations that disrupt robust OR expression, ectopic expression of Lbr in mature OSNs and conditional deletion of Bptf, a transcription factor predicted to bind to these enhancers by in vivo footprinting experiments, result in significant decrease of *trans* enhancer interactions accompanying the significant reduction of OR transcription. Our experiments, which reveal the regulatory landscape of OR enhancers, propose a model of singular OR choice that depends upon the convergence of multiple enhancers in a three-dimensional nucleoprotein complex that regulates robust OR expression.

RESULTS

To examine the epigenetic state of the H enhancer and to use this information for the identification of novel OR enhancers, we performed DHS-seq (Thurman et al., 2012) and ChIP-seq for H3K4me1 and H3K27ac (Table S1 available online). DHS and enhancer mark enrichment is observed at enhancers of OSN-transcribed genes, such as the protocadherin alpha enhancers (Ribich et al., 2006) (Figure S1A). The H enhancer sequence is also enriched for H3K4me1 and H3K27ac and has a well-defined DHS peak (Figure 1A), suggesting that H is transcriptionally engaged in more cells than the small fraction of neurons that express the proximal OR genes on which we do not detect activating histone marks in whole MOE preparations. The second known OR enhancer, the P element (Khan et al., 2011), also has the same features as H.

To identify additional elements that share the chromatin pattern of the H enhancer, we performed a computational search for intergenic ChIP-seq and DHS-seq peaks using SICER (Zang et al., 2009). To remove enhancers that might generally specify neuronal cell types, we filtered out regions that overlapped with H3K4me1 and H3K27ac peaks from cerebellum ChIP-seq experiments that we performed in parallel (Figure S1B). However, even upon this filtering, there are 4,750 intergenic sequences that have these characteristics in a MOE-specific fashion. To reduce this number, we restricted the search to only intergenic regions residing within OR gene clusters (and 100 Kb upstream and downstream of these clusters). This strategy reduced the number of positive hits to 35, with an average distance of ∼∼35 Kb from the nearest OR (Figure S1B and Table S2).

We also searched for other epigenetic marks that may be used to distinguish OR enhancers from the rest of the MOE-specific regulatory elements. Two histone modifications associated with repression, H3K79me3 and H3K27me3 (Barski et al., 2007; Ernst et al., 2011), have a unique distribution at the H locus—they are missing from the actual enhancer sequence but are enriched in the flanking sequences (Figure 1A). Visual inspection of the remaining OR enhancer candidates shows that this pattern is shared among 23 of the 35 potential enhancer elements (example in Figure 1B). Aggregate plots comparing ChIP-seq and DHS-seq reads on OR proximal elements and predicted MOE enhancers located outside OR clusters highlight the specificity of this epigenetic signature (Figure 1C). Indeed, although 65% of predicted OR enhancers overlap with regions of H3K79me3 enrichment, only 2.6% (126/4,750) of all the predicted MOE enhancers show this overlap (Figure S1B). Interestingly, in *Drosophila* embryos, H3K79me3 enrichment is found on developmentally regulated enhancers (Bonn et al., 2012).

Functional Analysis of Predicted OR Enhancers

We sought a functional assay that is appropriate for a high-throughput in vivo enhancer screen. We performed transient reporter assays in zebrafish embryos and scored for MOE-specific reporter expression as previously described (Booker et al., 2013). Although the predicted OR enhancer sequences are not conserved to zebrafish, the H element supports reporter expression in zebrafish OSNs (Nishizumi et al., 2007), suggesting that this assay is appropriate for the functional identification of OR

(D) Schematic depicting the E1b-tol2 expression construct.

(E) Zebrafish embryo injected with Sfaktiria-GFP at 24 hr postfertilization (hpf). Olfactory epithelium is indicated with dotted lines. Bottom, OSN cell bodies and axons expressing GFP.

(F) Results of zebrafish enhancer screen. Percent of injected zebrafish embryos with GFP-positive olfactory neurons at 48 hpf for each OR enhancer candidate. See also Figure S1 and Tables S1 and S2.

enhancers. Inspired by a previous description of enhancers as "islands" composing a "regulatory archipelago" (Montavon et al., 2011), we named the potential OR enhancers after Greek islands, a nomenclature that will be followed throughout the manuscript. We cloned the DHS peaks of 32 islands into a Tol2 retrotransposon-based reporter vector with a minimal promoter and GFP (Figure 1D and Table S3B) (Li et al., 2010). Each construct was injected into one-cell stage zebrafish oocytes, and GFP expression was monitored during embryogenesis at 24 and 48 hr postfertilization. Specific GFP expression was observed in OSNs of the olfactory epithelium for 12 of the sequences screened (Figures 1E, 1F, S1C, and S1D). Empty vector controls do not support GFP expression in zebrafish OSNs.

To identify potential differences between active and inactive enhancers, we summarized the levels of histone modifications around DHS peaks using principal component analysis. The first principle component (PC1) of each modification robustly represents its overall level at each enhancer locus. Ordering candidate OR enhancers by H3K79me3 PC1 grouped together enhancers that were active in zebrafish OSNs, as well as the H and P elements (Figure S1E). This set of enhancers had high levels of flanking H3K79me3. Ordering by H3K4me1 PC1 shows that enhancers that were active in zebrafish OSNs had relatively lower levels of H3K4me1 (Figure S1F). Interestingly, OR genes proximal to these 11 elements are more highly expressed than the average OR by RNA-seq of mouse OSNs (Figure S2A).

To further validate the zebrafish reporter assay, we generated transgenic β-galactosidase (β-gal) reporter lines using a reporter vector driven by the hsp68 minimal promoter (Kothary et al., 1988). We tested three zebrafish-positive elements, Sfaktiria, Lipsi, and Kefallonia (Table S2), referred to as Sfaktiria-lacZ, Lipsi-lacZ, and Kefallonia-lacZ in the rest of the manuscript. Whole-mount x-gal staining of these transgenic mice shows widespread reporter expression specifically in the MOE similar to the H-lacZ transgenic, which we generated as positive control (Figures 2A–2D and S2B). Immunofluorescence (IF) for β-gal in the MOE of the Sfaktiria-lacZ mouse also shows widespread expression (Figure 2E). In the olfactory bulb, β-gal-positive axons target multiple glomeruli and express the glutamate transporter Vglut2 (Figures 2F and 2G), indicating that this enhancer drives expression in mature OSNs. In contrast, there is no β-gal IF signal in Neurogenin-1 positive neurons (Figure S2C), which suggests that enhancer activity is synchronous to OR expression. Similar results were obtained from Lipsi-lacZ and Kefallonia-lacZ transgenics (data not shown). In contrast, β-gal is coexpressed with olfactory receptor Olfr1507 in Sfaktiria-lacZ transgenics crossed to Olfr1507iresGFP knockin mice (Barnea et al., 2004) (Figure 2H).

These transgenic reporter assays demonstrate an OSN-specific enhancer activity for the candidate OR enhancers. To test the requirement of these elements in OR expression, we deleted one of them, Lipsi, which is located on chromosome 2 between Ofr362 and Olfr364. Our targeting strategy deleted, by homologous recombination, 1,000 bp of conserved sequence corresponding to the DHS peak at this location (Figure 2I). qRT-PCR analysis on RNA prepared from wild-type and Lipsi KO littermates shows marked reduction in expression of the eight genomically linked ORs that reside within this genomic cluster on

chromosome 2, whereas ORs from a distant genomic cluster in the same or different chromosomes are unaffected (Figure 2J). RNA in situ hybridization (ISH) analysis in MOE sections from Lipsi KO and wild-type littermates confirms that expression of ORs from this genomic cluster is abolished (Figures 2K and S2D).

Multiple Enhancers Interact in *trans* with a Transcriptionally Active OR

Our data thus far provide a comprehensive epigenetic and genetic characterization of intergenic DNA elements that may act as OR enhancers. To examine whether these elements associate with active OR genes in *trans*, like the H enhancer (Lomvardas et al., 2006), we performed 4C on an isolated population of OSNs expressing Olfr1507. We chose Olfr1507 for this experiment because its expression depends on the genomically linked H enhancer. Thus, it provides an ideal gene locus for testing the hypothesis that a *cis* enhancer may act in concert with *trans* enhancers. 4C was performed on fluorescence-activated cell (FAC)-sorted neurons from Olfr1507iresGFP knockin mice, and libraries were amplified with inverse PCR primers anchored at the *Olfr1507* promoter as previously described (Clowney et al., 2012). 4C libraries from GFP+ and GFP− cells were analyzed by qPCR to quantitate the relative enrichment of various DNA loci. Several of the newly identified sequences are enriched in the library corresponding to GFP+ cells at levels approaching the enrichment levels of H (Figure 3A). Enrichment is significantly reduced in GFP− cells, which suggests that these associations are restricted to cells that transcribe *Olfr1507*. Two-color DNA FISH analysis (Figure 3B) verified that the *Olfr1507* locus frequently colocalizes in *trans* with the three most highly enriched elements, Lipsi (chr2), Sfaktiria (chr6), and Crete (chr11), in OSNs immunolabeled with an anti-Olfr1507 antibody (~63% of Olfr1507+ OSNs, n = 124 for each pair). Similar results were obtained by FISH in Olfr1507iresGFP MOEs using anti-GFP immunolabeling (Figure S3A). Three-color DNA FISH (Figure 3C) revealed that Olfr1507 colocalizes with both Lipsi and Crete in 16% of Olfr1507+ OSNs, a highly significant increase over the frequency of colocalization of the three loci in Olfr1507− OSNs (0.2%, n = 406, p = 2E-10, chi-square test) (Figure 3D). Thus, as the network of enhancer interactions becomes more complex, it remains associated with OR transcription.

To explore the long-range interactions of *Olfr1507* in an unbiased fashion, we generated 4C-seq libraries generated from GFP+ and GFP− cells from Olfr1507iresGFP mice (Figure S3B). In agreement with our qPCR analysis, 4C-seq revealed multiple contacts between *Olfr1507* and 15 candidate OR enhancers, nine of which are functional in zebrafish OSNs (Figures S3C–S3E and Table S5). Interactions with OR enhancers were significantly (p < 0.01, Wilcoxon t test) stronger in Olfr1507+ OSNs compared to the negative population (Figure 3E). The network of interchromosomal interactions between Olfr1507 and predicted OR enhancers is depicted in Figure 3F.

Extensive *cis* and *trans* Interactions between Potential OR Enhancers in OSNs

To examine the extent of putative enhancer interactions in OSNs, we performed Hi-C analysis in the whole MOE. To specifically interrogate the interactions of OR enhancer candidates, we
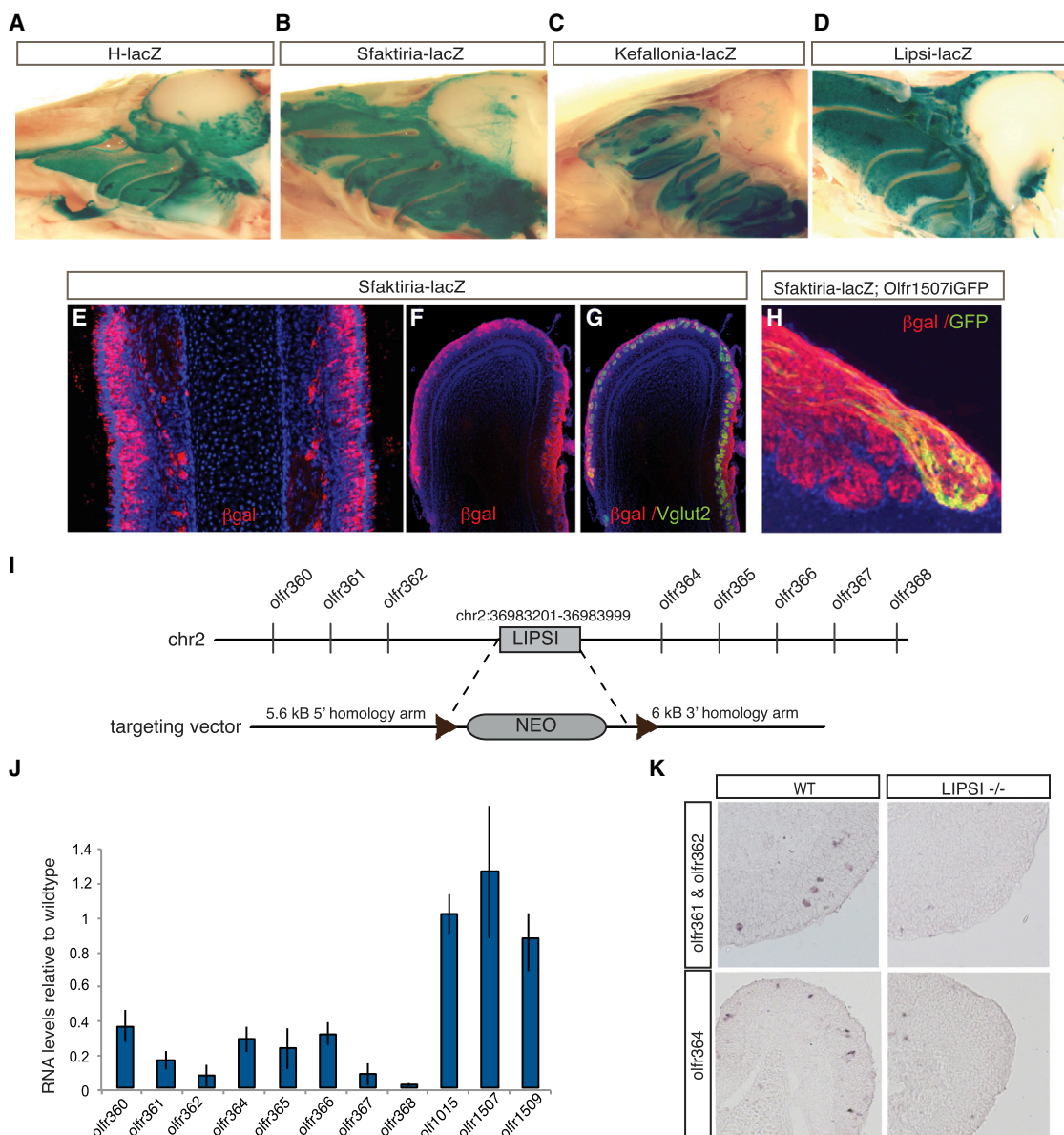
Figure 2. Genetic Verification of Enhancer Function in Mouse OSNs

(A–D) Whole-mount x-gal staining of MOE from H-lacZ, Sfaktiria-lacZ, Kefallonia-lacZ, and Lipsi-lacZ enhancer transgenic mice, respectively.

(E) IF for β-gal (red) in Sfaktiria-lacZ olfactory epithelium. DAPI nuclear stain (blue).

(F) IF for β-gal (red) in Sfaktiria-lacZ olfactory bulb.

(G) IF for β-gal (red) and Vglut2 (green) in Sfaktiria-lacZ olfactory bulb.

(H) IF for β-gal (red) and GFP (green) in Sfaktiria-lacZ; Olfr1507iresGFP olfactory bulb. DAPI nuclear stain (blue).

(I) Targeted deletion of Lipsi allele on chromosome 2. 998 bp were replaced with a floxed neo cassette via homologous recombination. Coordinates are mm9.

(J) RT-qPCR from Lipsi KO and Lipsi WT adult MOE. RT-qPCR levels for each primer set were normalized to OMP, and the results are shown as fold difference of Lipsi KO over Lipsi WT. Error bars represent SEM over duplicate experiments.

(K) RNA ISH for *Olfr361* and *Olfr362* pooled probes and *Olfr364* in Lipsi KO and WT MOE at P1.

See also Figure S2 and Tables S2 and S3.

performed a modified Capture-C protocol (Hughes et al., 2014) (see Extended Experimental Procedures). Our analysis revealed that 32/35 elements associate in high frequency with other enhancers from this repertoire. These interactions appear to be highly specific; there is a significant (p < 0.01, Wilcoxon t test)

20-fold enrichment for reads that span two different potential OR enhancers compared to reads that span an OR enhancer and one of the other MOE enhancers (Figure 4A). Within the observed repertoire, there are "promiscuous" enhancers that form frequent interactions with many other elements (Evia and
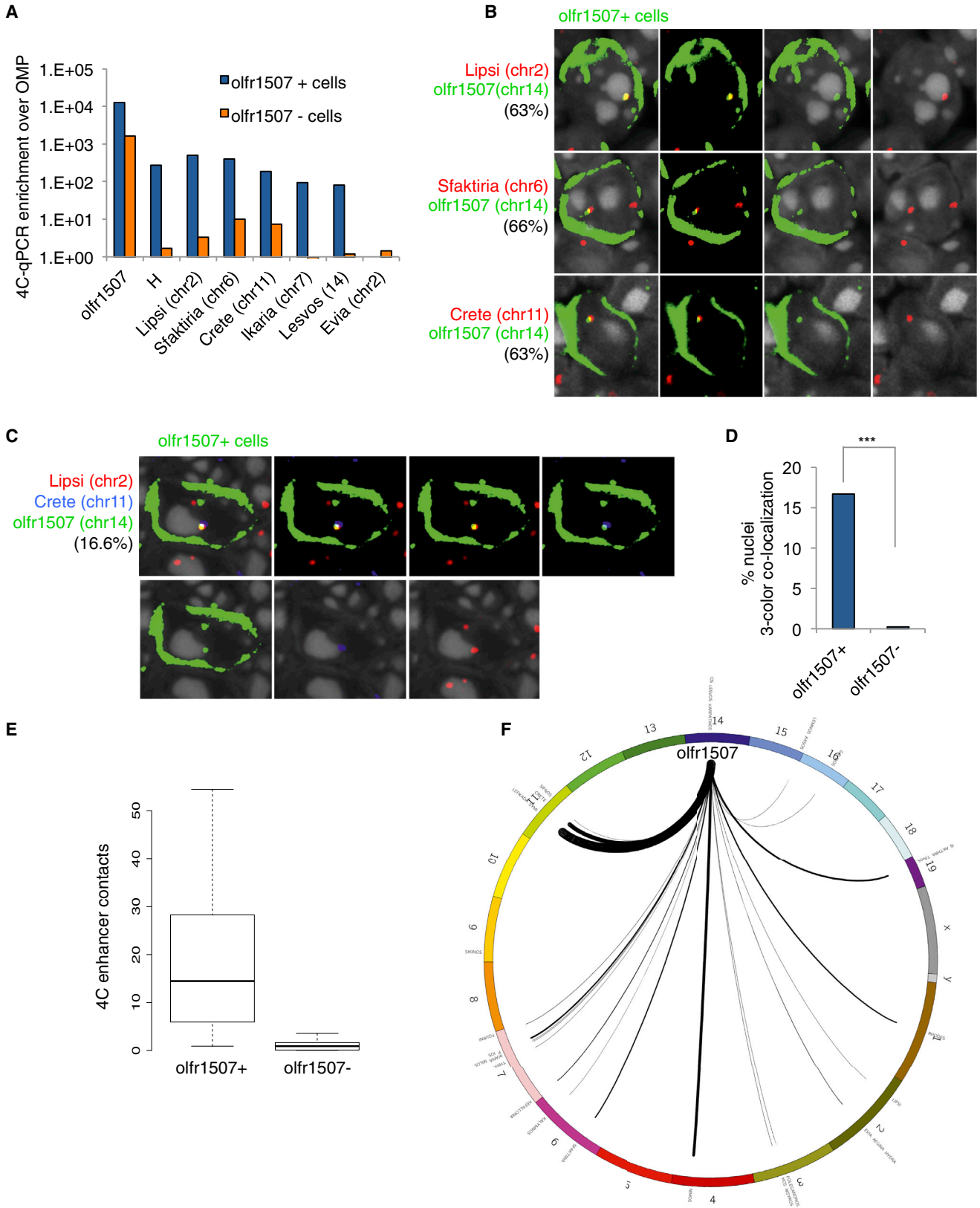
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Gavdos), whereas others form fewer interactions (Nimos and Lemnos). A contact matrix depicting the pairwise frequencies of these interactions organized by hierarchical clustering reveals the existence of four clusters of potential enhancers exhibiting similar frequencies of interactions (Figure 4B). Enhancers located on chromosomes 2, 3, 7, and 16 make the most frequent contacts with each other and with enhancers from other chromosomes (Figure 4C).

To independently verify the Hi-C data, we performed two-color DNA FISH analysis on sections of the MOE (Figures 4D, 4E, and S4). DNA sequences that interact infrequently by Hi-C, like Nimos, exhibit a low frequency of colocalization with other OR enhancers both in OSNs and in sustentacular cells, a non-neuronal cell type of the MOE that we used as an internal control (Figure 4D). In contrast, increased Hi-C interactions generally correspond to frequent colocalizations by DNA FISH that are restricted to OSNs. On average, enhancer-enhancer colocalizations are ~6 times more prevalent in OSNs than in sustentacular cells (n = 3,264 nuclei; p = 10^{-44}, chi-square test). As in the Hi-C experiment, enhancer-enhancer interactions are specific to OR enhancers, as colocalizations with predicted MOE enhancers are not significantly increased in OSNs compared to sustentacular cells (Figure 4D). It is worth noting that sequences engaged in interchromosomal contacts include those that were not functional in the zebrafish reporter assay, suggesting that they may have functional regulatory roles in the mouse.

The Regulatory Landscape of OR Enhancers

Because enhancer-bound transcription factors mediate long-range genomic interactions (Nolis et al., 2009), we searched for proteins that bind on candidate OR enhancers in order to obtain mechanistic insight into the formation of these intricate interchromosomal associations. For an unbiased search of regulatory sequences, we sought to isolate DNase I protected sequences on the candidate enhancers. In vivo footprinting by DHS-seq was recently used to identify, in a high-throughput fashion, regulatory sequences and factors that control cell-type-specific differentiation (Neph et al., 2012a, 2012b). To increase the depth of our DHS-seq reads on the 35 putative enhancers, we employed a similar sequence capture strategy as done for Hi-C, using oligos tiling the OR enhancer candidates (Figure 5A) and modifying a recently published enrichment strategy (Stergachis et al., 2013).

After enrichment, DHS-seq read coverage of the OR enhancers increased ~10,000-fold (Figure 5A). Mapping DNase I cleavage sites across the H enhancer revealed multiple sequences that are protected from DNase I digestion (Figure S5A) and that can be computationally identified as footprints (Neph et al., 2012b). We performed a motif search of footprinted sequences in the 12 enhancers with confirmed zebrafish activity using MEME (Bailey and Elkan, 1994). This analysis revealed four protected sequence motifs, which match predicted binding sites for Atf5, Evx2/Lhx2, Olf/Ebf, and Hdx (Figures 5B and S5B). Interestingly, three of these factors, Atf5, Olf/Ebf, and Lhx2, have reported roles in OR gene regulation (Dalton et al., 2013; Hirota and Mombaerts, 2004; Rothman et al., 2005). ChIP experiments for Lhx2, for which we were able to obtain a ChIP-quality antibody, revealed enrichment on OR enhancers containing Lhx2 motifs (Figure S5C, see Extended Experimental Procedures), providing independent verification of the in vivo occupancy data.

Next, we expanded our search to the complete repertoire of enhancers identified by epigenetic analysis. Across the 35 potential enhancers, 1,040 footprints were identified with significant footprint occupancy scores (FOS < 0.5) (Table S4A). Eleven TF motifs from the TRANSFAC database had median footprint scores of less than one, indicating DNase I protection (Figure 5C). Homeodomain TF motifs comprise the majority of the footprinted motifs. Four TF motifs (Nobox, Foxj2, Cdx, and C/EBPgamma) have p values < 0.05 and exhibit protection of the consensus sequence across all OR enhancers (Figure 5D). Among consensus sequences with reduced FOS, the predicted binding motif for transcription factor Bptf (bromo and PHD-finger transcription factor) (Jordan-Sciutto et al., 1999) appeared as a promising candidate protein that may be involved in the formation or stabilization of long-range genomic interactions. As the histone binding component of the NURF chromatin remodeling complex, Bptf binds acetylated and methylated histone tails from different histones through the bromo and PHD-finger domains, respectively (Ruthenburg et al., 2011), which could potentially mediate the bridging of chromatin fibers from different chromosomes. Bptf has previously been shown to facilitate expression of Hox genes via chromatin remodeling at cis-regulatory sequences (Wysocka et al., 2006) but has yet to be implicated in OR expression.

To examine the functional significance of Bptf binding on OR-proximal enhancers, we conditionally deleted Bptf in the MOE by

Figure 3. Multiple Enhancers Interact in *trans* with a Chosen OR

(A) 4C from Olfr1507iresGFP FAC-sorted cells, library constructed by inverse PCR off *Olfr1507* promoter. qPCR enrichment of candidate OR enhancers relative to OMP in GFP$^+$ and GFP$^-$ cells. Error bars display SEM between triplicates.

(B) IF staining for Olfr1507 (green) and two-color DNA FISH for Olfr1507 DNA (green) and Lipsi, Sfaktiria, and Crete DNA (red). DAPI is nuclear stain (gray). Percent of Olfr1507-positive cells containing colocalized red and green probes is indicated for each pair.

(C) IF staining for Olfr1507 (green) and DNA FISH for Olfr1507 (green), Lipsi (red), and Crete (blue) DNA. DAPI is nuclear stain (gray). Percent of Olfr1507$^+$cells containing three-color colocalization is indicated.

(D) Quantification of (C). Percent Olfr1507$^+$and Olfr1507$^-$OSNs containing three-color colocalizations.

(E) 4C-seq from Olfr1507iresGFP-positive and -negative cells, library constructed by inverse PCR from Olfr1507 promoter. Average number of contacts for OR enhancers are plotted. y axis is RPKM that span two different putative enhancers at an expected ligation site.

(F) Circos plot (Krzywinski et al., 2009) of OR enhancer chromosomal locations and 4C-seq contacts with the Olfr1507 promoter in Olfr1507$^+$ cells. Lines are weighted by frequency of interaction (RPKM that span two different putative enhancers at an expected ligation site). See Table S5 for enhancer names, locations, and interaction frequencies.

See also Figure S3.
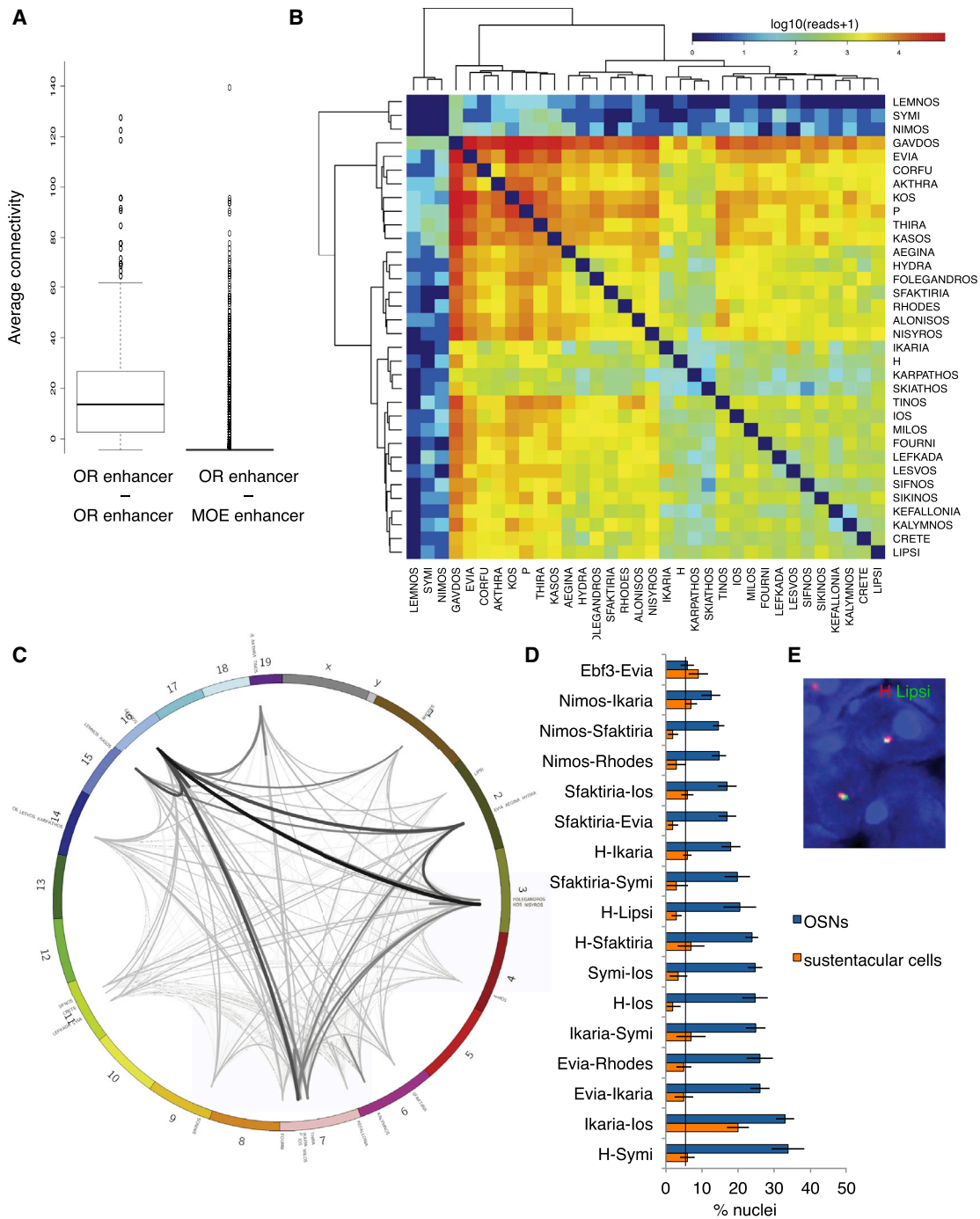
Figure 4. An Intricate Network of Enhancer Interactions in Mouse OSNs

(A) Average Hi-C connectivity of OR enhancers with other OR enhancers compared to average connectivity of OR enhancers to MOE enhancers. y axis is reads spanning two different genomic regions normalized to the total number of reads.

(B) Contact matrix depicting interaction frequency between candidate OR enhancers (red, highest interaction frequency; blue, lowest interaction frequency). Normalized read counts spanning two enhancer regions were divided into 20 bins, with 5 bins representing each color shade (color key provided for quantitation). Interactions between enhancers are hierarchically clustered.

(C) Circos plot of OR enhancer chromosomal locations and Hi-C contacts. Lines are weighted according to frequency of enhancer-enhancer interactions.

(D) Results of DNA FISH screen. x axis is percent nuclei containing two-color colocalization between OR enhancer candidates, and y axis is enhancer candidate pairs tested. OSN and sustentacular cell nuclei indicated. Vertical line is baseline OSN colocalization frequency. Ios and Ikaria were not significantly reduced in

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crossing a floxed Bptf allele (Landry et al., 2011) to a Foxg1-Cre driver that is expressed before the onset of OR expression (Hébert and McConnell, 2000). We refer to the Foxg1-cre, Bptf flox/flox mouse as the Bptf KO. These mice die perinatally, thus restricting our analysis to E18.5 embryos. IF for Olfr1507 in sections from Bptf KO and control MOEs showed complete loss of expression of this OR in the Bptf KO (Figure 6A). RNA ISH using a complex probe detecting several hundred ORs (see Extended Experimental Procedures) also showed a dramatic reduction in OR expression (Figures 6B and 6G). *GAP43* and *Ncam1*, markers of immature OSNs that are synchronous to OR expression (Lyons et al., 2013), are still expressed in Bptf KO MOEs, suggesting that the loss of OR expression is not caused by loss of the OSN lineage (Figures 6C and 6D). Finally, a general deficit in OR expression is corroborated by the loss of mature OSN markers, such as OMP, Adcy3, and Vglut2, in the Bptf KO MOE (Figures 6E and 6F).

To test whether Bptf participates in the establishment or maintenance of associations between potential OR enhancers, we performed two-color DNA FISH in sections of control and Bptf KO MOEs. This analysis revealed a significant decrease in the frequency of interactions between H-Lipsi and H-Sfaktiria in Bptf KO OSNs (Figure 6H; p = 0.0005 and p = 0.002, respectively, chi-square test; n = 356 nuclei). Importantly, the overall chromatin architecture of Bptf KO OSNs remains intact, and the aggregation of OR foci is not impaired (Figure 6I), suggesting a rather specific role of this protein in enhancer interactions and OR gene activation.

OR Gene Aggregation Facilitates Enhancer Interactions in *trans*

An important question emerging from our analyses is whether frequent enhancer interactions are a consequence of OR gene aggregation in OSN nuclei. The fact that we detect enhancer colocalization with *Olfr1507* more frequently in Olfr1507^{+} cells suggests that these associations would preferentially occur outside the repressive OR foci, which is where the transcriptionally active ORs also reside (Clowney et al., 2012). Two-color DNA FISH shows that 60% of H or Lipsi alleles colocalize with a complex DNA FISH probe that recognizes most OR loci (panOR probe) (Figure 7A). However, three-color DNA FISH shows that H and Lipsi colocalization occurs preferentially outside the OR foci ($p < 10^{-12}$; n = 1,530; chi-square test) within euchromatic nuclear territories (Figures 7A and 7B). Thus, enhancer interactions in *trans* may be favored by—but are not a simple product of—the convergence of linked ORs, a result that is consistent with the observation that Bptf deletion impairs enhancer interactions, but not OR aggregation.

It is possible that the differentiation-dependent aggregation of OR loci instead facilitates enhancer interactions by bringing the elements into close proximity. To test this, we analyzed the frequency of interchromosomal associations between potential OR enhancers in OSNs that express Lamin b receptor (Lbr).

We previously showed that ectopic Lbr expression in OSNs disrupts the aggregation of OR genes and the interaction of the H enhancer with OR genes in *trans*, but not in *cis* (Clowney et al., 2012). 4C-qPCR analysis of control and Lbr[+] FAC-sorted OSNs revealed significant reduction in the frequency of *trans* interactions between potential OR enhancers upon Lbr expression (Figure 7C), supporting a role for the unusual nuclear architecture of OSNs, and possibly for the aggregation of OR loci, in the frequent association of predicted OR enhancers from different chromosomes. Importantly, because OR transcription is significantly reduced in Lbr[+] OSNs (Clowney et al., 2012), these data provide an independent genetic manipulation whereby the specific disruption of *trans* enhancer interactions reduces OR transcription rates. Thus, the same process that contributes to the effective silencing of OR transcription, the aggregation of OR genes in heterochromatic foci, may also facilitate the activation of a single allele by increasing the probability of enhancer-enhancer interactions.

DISCUSSION

Our experiments revealed 35 intergenic OR-linked sequences that share common epigenetic properties with the H element, the prototypical OR enhancer. In addition to common enhancer features, these sequences are characterized by high flanking levels of H3K79me3. Currently, it is not clear whether these epigenetic marks coexist on the same enhancer alleles or whether they reflect different states of these elements in the total cellular population. In any case, our reporter screen showed that 12 of the 32 elements that were tested regulate OSN-specific expression in zebrafish OSNs and revealed a positive association between flanking H3K79me3 enrichment and enhancer activity. We demonstrated the activity of three of these elements (Lipsi, Sfaktiria, and Kefallonia) as OSN enhancers in the mouse, and we showed that Lipsi is necessary for the expression of proximal OR genes. Because zebrafish reporter assays for mammalian enhancers generate false negatives (Ariza-Cosano et al., 2012; Booker et al., 2013; McGaughey et al., 2008), it is likely that many of the DNA elements that did not activate transcription in zebrafish OSNs are mammalian or mouse-specific OR enhancers, especially in light of their extensive interactions with verified enhancers. It is worth noting that our screen is far from being saturated because enhancers that are transcriptionally engaged in a smaller cell population would not meet our computational thresholds, and enhancers with a different epigenetic signature would be ignored.

Our experiments revealed an unusual network of genomic interactions occurring predominantly between putative OR-associated enhancers from different chromosomes. Interactions identified by Hi-C were verified by extensive DNA FISH experiments, which demonstrated that sequences from different chromosomes colocalize in up to 35% of OSN nuclei. 4C-seq from FAC-sorted OSNs and three-color DNA FISH experiments

sustentacular cells, probably due to the genomic linkage of these DNA elements, which both reside on chromosome 7 at 9 MB distance. Error bars are SEM from multiple sections of the MOE.

(E) Representative DNA FISH for H (red) and Lipsi (green) in OSN nucleus. The blue nuclear stain is DAPI.

See also Figure S4.
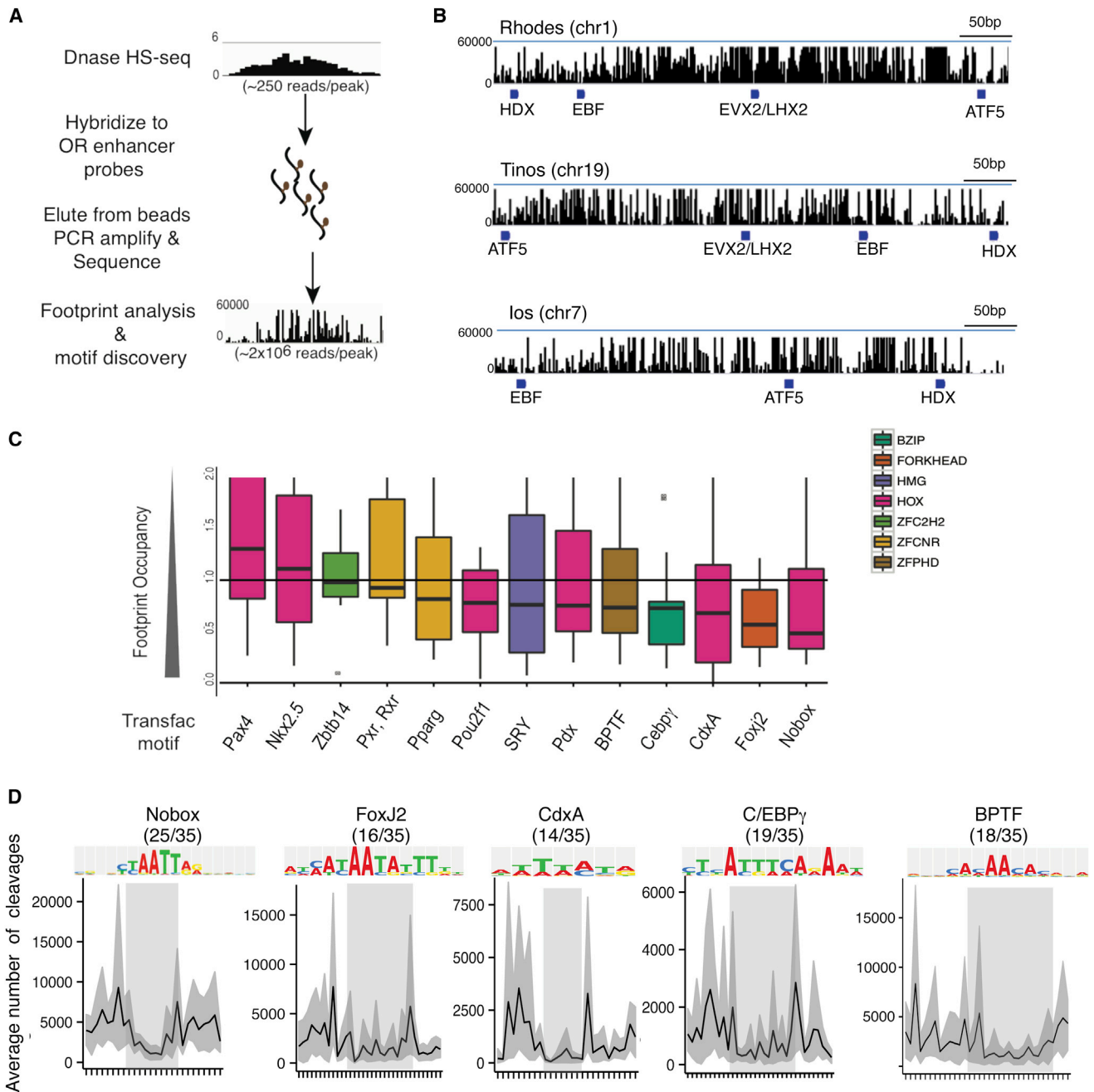
Figure 5. Shared Transcription Factor Footprints on OR Enhancers

(A) Schematic of sequence-capture-based method of DNase I library construction.

(B) DNase I cleavages mapped over OR enhancer candidates Rhodes, Tinos, and Ios. Blue bars indicate footprints containing ATF5, HDX, EVX2/LHX2, and EBF motifs.

(C) Transcription factor (TF) motifs from the TRANSFAC database that are enriched on OR enhancer candidates and average footprint score for each TF motif. Low FOS scores indicate greater footprint occupancy. TF motifs are color coded according to TF family.

(D) Average DNase I cleavages for TRANSFAC motifs on 35 candidate OR enhancers. The x axis is centered at the consensus sequence (shaded box). Fraction of OR enhancers containing each TF motif is indicated in parentheses. Error is bootstrapped 95% confidence intervals.

See also Figure S5 and Table S4 for footprint and motif locations.

suggest that more than one enhancer colocalizes with an OR gene in the neurons that transcribe that OR. Previous work showed that H interacts in *cis* and in *trans* with the transcription-

ally active OR allele (Lomvardas et al., 2006). Because the inactive *Olfr1507* allele is epigenetically similar to other silent ORs in Olfr1507+ OSNs (Magklara et al., 2011) and likely spatially
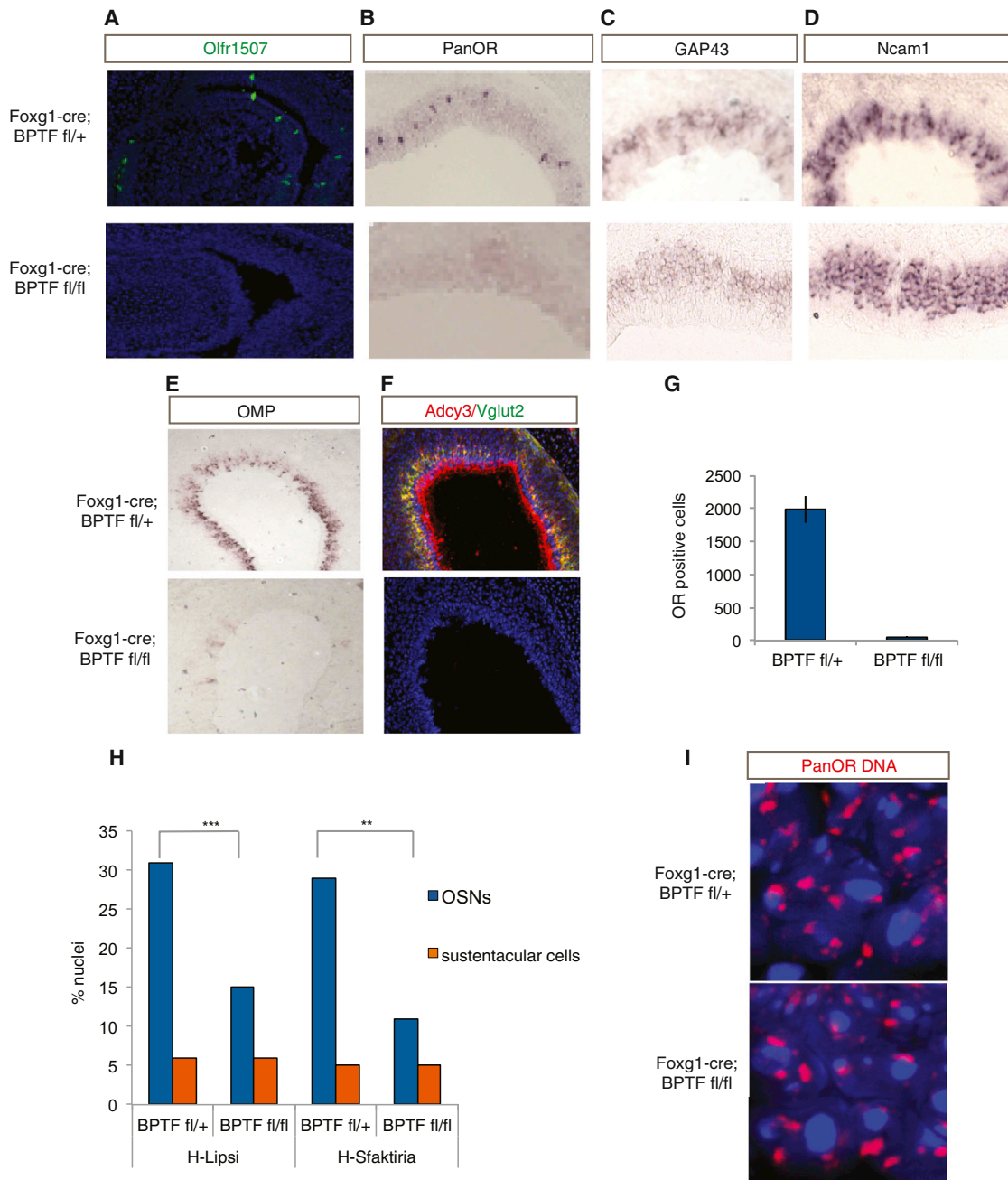
Figure 6. Bptf Is Required for OR Expression and Enhancer Interactions

(A) Olfr1507 IF (green) in Foxg1-Cre; Bptf flox/+ (heterozygote) and Foxg1-Cre; Bptf flox/flox (KO) MOE at e18.5. DAPI is the nuclear stain (blue).

(B) PanOR RNA ISH in Bptf heterozygote and KO.

(C–E) ISH for developmental markers in Bptf het (top) and KO (bottom).

(F) Adcy3 IF (red) and Vglut2 IF (green) in Bptf het (top) and KO (bottom). DAPI (blue) is nuclear stain.

(G) Quantification of OR ISH experiment (B) in Bptf het and KO. Error bars represent the variance over duplicate experiments.

(H) Quantification of DNA FISH colocalizations between H and Sfaktiria BAC probes and between H and Lipsi BAC probes in Bptf het and KO. The y axis is percent OSN and sustentacular cell nuclei containing colocalized probes.

(I) DNA FISH with complex pan olfactory receptor (PanOR) probe (red) in Bptf het and KO OSNs. DAPI is nuclear stain (blue).

indistinguishable from them (Armelin-Correa et al., 2014), the parsimonious assumption is that multiple OR enhancers also coalesce over the transcriptionally active OR allele.

A question emerging from our observations regards the functional significance of the convergence of multiple enhancer elements over the chosen OR. Recent data revealed that many
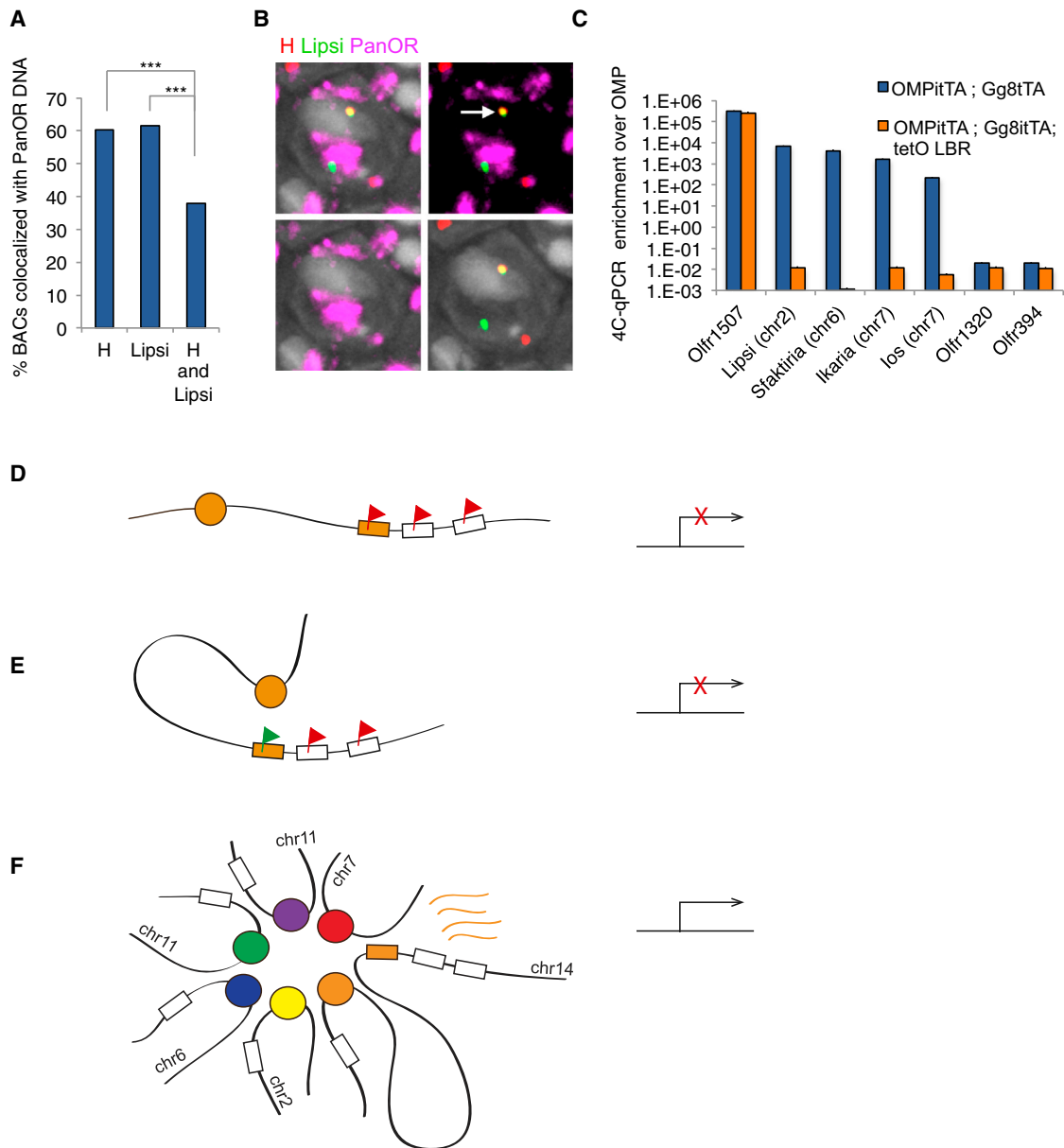
Figure 7. OR Gene Aggregation Promotes Enhancer Interactions

(A) Percent of OSN nuclei in which individual BAC probes for H and Lipsi are colocalized with PanOR probe (two-color DNA FISH) and in which colocalized H and Lipsi BAC probes are colocalized with PanOR probe (three-color DNA FISH).

(B) Three-color DNA FISH with PanOR probe (magenta), H BAC (red), and Lipsi BAC (green). DAPI is nuclear stain (gray). Arrow indicates colocalized enhancer probes located outside of OR foci.

(C) 4C-qPCR analysis in Lbr-overexpressing mice (orange bars) and control mice (blue bars). 4C library is generated by inverse PCR from the H enhancer. Enrichment of candidate OR enhancers and OR promoter sequences on different chromosomes are normalized to control gene OMP. Error bars are SEM from duplicates.

(D–F) A model for essential and redundant functions of an OR enhancer in *cis* and in *trans* respectively. Schematic representations of different states in the OSN nucleus (left) and corresponding transcriptional outputs (right). An OR gene (orange box) located proximal to an enhancer (orange circle) is repressed by H3K9me3 (red flag, D). The *cis*-proximal enhancer may facilitate derepression of the OR chromatin landscape (green flag, E) but is not sufficient for OR transcription. Multiple *trans*-interacting enhancers (colored circles) aggregate around the transcribed *cis*-proximal OR (orange box, F).

developmentally regulated genes require numerous enhancers for their proper expression. Experiments in the developing mouse embryo have shown that multiple enhancer sequences act in a coordinated fashion to activate Hox genes in various tissues, and similar observations have been made for the activation of protocadherin gene clusters (Andrey and Duboule, 2014;

Delpretti et al., 2013; Guo et al., 2012; Montavon et al., 2011; Noordermeer et al., 2014). Thus, enhancers may act in an additive or even synergistic fashion to increase transcription rates. Given that ORs likely represent the most abundant protein-coding mRNAs in OSNs, it is possible that the convergence of multiple enhancers contributes to the robustness of OR transcription. Coordinated action between multiple loci in *trans* has been also described for the activation of the human IFNβ gene, which is also expressed in a stochastic but robust fashion upon virus induction (Apostolou and Thanos, 2008). Finally, genetic experiments suggest that a DNA sequence may act as a *trans* enhancer during the stochastic photoreceptor gene choice in *Drosophila* ommatidia (Johnston and Desplan, 2014). Thus, while the Hox genes, with restrictive spatiotemporal expression requirements, use an "archipelago" of *cis* regulatory elements to achieve precise expression patterns, systems that tolerate or even seek stochasticity may utilize *trans* interactions to obtain robust but low probability transcriptional outputs.

Currently, there is no direct evidence of a role of these interactions in OR transcription. Previous work showed that deletion of H does not affect expression of more than three proximal OR alleles, despite the physical association of this enhancer with multiple other ORs. Similarly, deletion of P or Lipsi appears to affect the expression of linked OR alleles only, a result also observed in H,P double-KO mice (Khan et al., 2011). However, with at least 15 enhancer elements interacting frequently with the *Olfr1507* promoter in Olfr1507+ OSNs, it is expected that deletion of a single *trans* enhancer does not elicit transcriptional consequences. In contrast, if each of these elements is required for a critical step in the transcriptional activation of *cis*-linked OR genes, such as orchestrating the desilencing of the linked ORs, then each individual deletion may be sufficient for a detectable transcriptional effect in *cis* (Figures 7D–7F), explaining why these enhancers are required in *cis* but are redundant in *trans*.

To test this model of *trans* enhancement, we perturbed the ability of these elements to interact with each other in *trans*. Two independent experiments, deletion of Bptf and expression of Lbr in OSNs, resulted in significant downregulation of OR transcription, to the extent that ORs are not detectable by RNA in situ hybridization (ISH) or IF in the mutant OSNs. Although in both cases we cannot directly attribute the downregulation of OR transcription to the reduced frequency of *trans* interactions, these results are consistent with our model. Recent experiments showed that an increase in the number of homeodomain and O/E sites on the promoters of transgenic ORs increases the frequency by which they are transcribed (Vassalli et al., 2011). Because the only way to increase the local concentration of binding sites for these transcription factors near an OR promoter is to recruit sites from other genomic regions, this result is consistent with the hypothesis that each enhancer on its own is required, but not sufficient, for the expression of proximal ORs.

There are alternative interpretations of our data that do not invoke coordinated action between distant OR enhancers. For example, enhancer convergence may reflect the existence of specialized nuclear bodies or factories with high affinity for these enhancers and for the protein complex that supports OR transcription. Such a nuclear body is described in the regulation of VSG genes in trypanosome (Navarro and Gull, 2001). Moreover,

sequestering these putative enhancers into distinct nuclear territories may primarily prevent them from interacting with their proximal ORs, essentially "decommissioning" a large number of elements in each OSN. Thus, enhancer convergence may serve two functions: to eliminate the possibility of simultaneous choice of multiple ORs and to ensure robust expression of the single active OR.

In summary, our data are consistent with a model in which the robust transcription of an OR requires an enhancer in *cis* and numerous enhancers in *trans*. High levels of OR expression may be necessary for activation of the Perk pathway via ER stress, and it is likely that only ORs expressed above a certain threshold can elicit this feedback. This prediction is consistent with the observation that transgenic ORs expressed at low levels from heterologous promoters can be coexpressed with endogenous ORs (Zhou and Belluscio, 2012). Because the vetting mechanism that stabilizes OR choice may screen for both the quality and quantity of OR protein, ORs transcribed at suboptimal levels will be turned off by sustained Lsd1 expression. If the number of enhancers associating with an OR promoter indeed determines expression levels, then stable OR expression will occur only once a sufficient number of enhancer elements associate with an OR promoter. Simple modeling of the observed experimental frequencies of pairwise enhancer interactions predicts that the colocalization of 16 different enhancers from a repertoire of 35 will occur only once in each OSN nucleus (see Extended Experimental Procedures). Thus, depending on the actual number of enhancers needed to achieve feedback-eliciting levels of OR transcription, the limited, or even unique, generation of a nucleoprotein complex with sufficient number of enhancers may provide the elusive singularity of OR choice.

It seems counterintuitive that a sensory system critical for survival and reproduction would rely on a molecular mechanism as inefficient and probabilistic as the interchromosomal convergence of a large number of enhancer elements. However, unlike most developmental systems that are built upon tight spatiotemporal regulation, the peripheral olfactory system may be able to tolerate such a variable and often nonproductive process because an efficient feedback mechanism is in place to ensure that terminal OSN differentiation occurs only upon OR choice. This mechanism is compatible with the rapid evolution of the OR gene family, which is characterized by significant copy number variations among closely related species and significant polymorphisms within species in accordance with the essential function of this gene family in adaptation. It remains to be seen if other fast-evolving gene families involved in the perception of—and the protection from—the constantly changing external environment (Clowney et al., 2011) may be employing similar radical mechanisms for stochastic and mutual exclusive gene expression.

EXPERIMENTAL PROCEDURES

Mouse Strains

Mice were treated in compliance with the rules and regulations of IACUC. The Lipsi enhancer knockout mouse was generated by homologous recombination in embryonic stem cells (ESCs) (see Extended Experimental Procedures). Other mouse strains used are described in the Extended Experimental Procedures.

ChIP-Seq and DHS-Seq

Nuclei from the olfactory epithelium of 6- to 8-week-old wild-type mice were isolated, and native chromatin ChIPs were performed as described (Magklara et al., 2011). For DHS-seq, nuclei from the olfactory epithelium were isolated and digested briefly with DNase I (Ambion). For Illumina sequencing, 100–500 bp fractions were gel extracted and prepared. See Extended Experimental Procedures for more detailed protocols.

ChIP-Seq and DHS-Seq Analysis

Sequencing reads were mapped to the mouse genome using Bowtie2 (Langmead and Salzberg, 2012). SICER (Zang et al., 2009) was run on reads that mapped to OR clusters or the whole genome to identify OR and MOE potential enhancers, respectively. OR clusters were defined as genomic regions containing one or more OR genes extending to the nearest non-OR Refseq gene. Peaks located within 5 kB of a Refseq gene or an OR promoter (Clowney et al., 2011) were subtracted. DHS peaks that intersected H3K4me1 or H3K27ac peaks were selected, and DHS peaks that intersected with cerebellum H3K4me1 or H3K27ac peaks were subtracted.

Transgenic Zebrafish and Mouse Assays

Enhancer candidate sequences were amplified from mouse genomic DNA using primers targeting DHS peaks (Table S3B). PCR products were cloned into the E1b-GFP-tol2 vector (Li et al., 2010) containing a minimal promoter followed by GFP and injected using standard protocols into at least 75 one-cell-stage zebrafish oocytes per construct as described in Smith et al. (2013). GFP expression was observed at 24 and 48 hpf. Embryos containing at least one GFP-positive OSN were counted. Embryos with GFP expression in other tissues were excluded. For transgene reporter mice, the same PCR products were cloned into a vector containing the Hsp68 minimal promoter followed by the lacZ reporter gene (Kothary et al., 1988).

DHS-Seq Sequence Capture

DHS-seq library was further amplified for 10 PCR cycles, and 1 μg was hybridized to Seqcap EZ choice probes (NImblegen, Roche) designed to target and tile OR enhancer regions (Table S3A). See also Extended Experimental Procedures.

ChIP qPCR

Crosslinked ChIPs were performed on chromatin isolated from MOE of 3-week-old wild-type mice using anti-Lhx2 (kind gift from Mark Roberson). See Extended Experimental Procedures for detailed protocols.

ACCESSION NUMBERS

Raw sequencing data can be accessed at Gene Expression Omnibus (GEO) GSE55174 and GSE52464.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.09.033.

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