

# Evolution of immune chemoreceptors into sensors of the outside world

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Edited by Thomas C. Kaufman, Indiana University, Bloomington, IN, and approved June 2, 2017 (received for review March 10, 2017)

**Changes in gene expression patterns represent an essential source of evolutionary innovation. A striking case of neofunctionalization is the acquisition of neuronal specificity by immune formyl peptide receptors (Fprs). In mammals, Fprs are expressed by immune cells, where they detect pathogenic and inflammatory chemical cues. In rodents, these receptors are also expressed by sensory neurons of the vomeronasal organ, an olfactory structure mediating innate avoidance behaviors. Here we show that two gene shuffling events led to two independent acquisitions of neuronal specificity by Fprs. The first event targeted the promoter of a V1R receptor gene. This was followed some 30 million years later by a second genomic accident targeting the promoter of a V2R gene. Finally, we show that expression of a vomeronasal Fpr can reverse back to the immune system under inflammatory conditions via the production of an intergenic transcript linking neuronal and immune Fpr genes. Thus, three hijackings of regulatory elements are sufficient to explain all aspects of the complex expression patterns acquired by a receptor family that switched from sensing pathogens inside the organism to sensing the outside world through the nose.**

olfaction | vomeronasal organ | olfactory receptor | gene evolution | neofunctionalization

Genetic variability, the substrate for selective forces during evolution, relies on genetic accidents. These accidents can trigger a variety of changes, of innovations ranging from the alteration of protein identity to the modulation of transcriptional activity. In mammals, genes expressed in the olfactory system (more precisely, those coding for olfactory chemosensors) evolve very quickly, because unusual selective pressures act on this system (1–3). Thus, studying the dynamics of olfactory chemoreceptor diversity offers a unique opportunity to identify the molecular events at the origin of the genetic novelties that underlie evolutionary processes.

To extract information from an unknown outside world, mammals use a wide variety of olfactory chemosensory receptors. These receptors, most of which are G protein-coupled receptors, are expressed by neurons localized in the main olfactory neuroepithelium or in the vomeronasal organ (VNO). Based on their amino acid sequences, they are classified into various families, including the odorant receptor (Or), trace amine receptor (Taar), type A membrane-spanning four-domain protein (Ms4a), vomeronasal type 1 (V1r) and type 2 (V2r) receptor, and formyl peptide receptor (Fpr) families (1).

The olfactory sensory toolbox used by mammals is remarkable because of the extraordinary size diversity of the gene repertoires encoding it. For example, mice, humans, and elephants dispose of 1,200, 450, and 2,000 different Or genes, respectively. Similarly, the mouse and the rat genomes each contain more than 200 V1r genes, whereas the human, snake, and fish genomes harbor fewer than 10 Vrs. Moreover, the toolbox also differs from one species to another, even closely related ones, in the identity of the members pertaining to a given sensor family. This interspecies chemosensor repertoire variability reaches an extreme with the

olfactory Fpr family. Indeed, Fpr olfactory expression appears to have been acquired by rodents and to be restricted to this clade, despite its expression in the immune system of all mammals. Thus, mice, rats, and gerbils express Fprs in a punctate and monogenic pattern in vomeronasal sensory neurons (4, 5), which is the singular rule characterizing the transcription of olfactory chemoreceptor genes (6).

The mouse genome contains seven Fpr genes that are expressed by immune cells, by olfactory neurons, or by both cell types. *Fpr1* and *Fpr-rs2* are transcribed by monocytes/macrophages, *Fpr-rs1* is transcribed by immune cells and vomeronasal sensory neurons, and *Fpr-rs3*, *-rs4*, *-rs6*, and *-rs7* are transcribed only by sensory neurons (4, 5). Both immune and vomeronasal Fprs recognize ligands that are linked to pathogens or to pathogenic states (4, 7, 8); what makes them different is apparently the world that they probe.

The vomeronasal sensory neuroepithelium is pseudostratified and composed of two functionally different types of neurons, organized into a basal layer and an apical layer. *Fpr-rs1* is transcribed by basal vomeronasal neurons, whereas *Fpr-rs3*, *-rs4*, *-rs6*, and *-rs7* are expressed by those located in the apical part of the neuroepithelium (4, 5). The axonal projections of like vomeronasal sensory neurons (i.e., those expressing the same chemoreceptor gene) reach the accessory olfactory bulb in the brain, where they

## Significance

**Immune formyl peptide receptors (Fprs) evolved in rodents from expression in immune cells to be transcribed in olfactory sensory neurons. Explaining the initial neuronal acquisition, we found that an Fpr coding exon landed in front of a vomeronasal receptor promoter, hijacking its expression pattern. This type of gene shuffling occurred twice in the mouse lineage, many million years apart, leading to the exclusive expression of Fprs in the two main populations of vomeronasal sensory neurons. Finally, we demonstrate that the immune expression of one of the mouse vomeronasal Fprs can be restored via the production of an intergenic transcript. Thus, we provide the complete history of genomic events that led to a model case of evolutionary neofunctionalization in a mammal.**

Author contributions: Q.D., J.T., A.C., and I.R. designed research; Q.D., J.T., L.R., and G.L. performed research; M.R. contributed new reagents/analytic tools; Q.D., J.T., L.R., A.C., and I.R. analyzed data; and A.C. and I.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. [MF175578–MF175624](https://doi.org/10.1073/pnas.1704009114)).

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1704009114/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1704009114/-DCSupplemental).

coalesce and form multiple structures called glomeruli. This is true for V1rs (9, 10), V2rs (11), and Fprs (12). Vomeronasal sensory neurons located in the apical and basal zones do recognize different types of chemicals, mainly kairomones (semiochemicals that mediate interspecific interactions). Activation of these neuronal populations often leads to innate and stereotyped behaviors (13–15).

In this study, we show that two independent exon shufflings associated with the production of an intergenic splice variant explain how, starting from an immune specificity, Fprs acquired their complex expression patterns in neurons during evolution.

## Results and Discussion

**The Different Identities of Olfactory and Immune Fprs.** Patterns of Fpr expression are complex. In the mouse, *Fpr1* and *Fpr-rs2* are transcribed by monocytes/macrophages, *Fpr-rs1* is transcribed by immune cells and basal vomeronasal sensory neurons, and *Fpr-rs3*, *-rs4*, *-rs6*, and *-rs7* transcripts are found only in apical vomeronasal sensory neurons (4, 5, 16) (Fig. 1A). To study the evolutionary history of the Fpr repertoires, we performed homology searches across genome assemblies of 32 mammalian species, from which we retrieved 101 Fpr coding sequences. In addition, we harvested tissue samples from 15 Eumuroidea species and identified 39 new Fpr sequences with a degenerate PCR approach. The resulting Fpr gene phylogeny demonstrates the massive diversification of the repertoires (Fig. 1A). Supporting the peculiar dynamics of the Fpr family, a synteny analysis of the Fpr cluster between the rat and the mouse shows high rates of gene birth and death (Fig. S1A and B).

To determine the time at which Fprs first acquired neuronal expression, we tested multiple species for vomeronasal and immune Fpr expression. In most Eumuroidea species tested (a group representing 23% of mammalian species living today), we found at least one Fpr gene transcribed in vomeronasal sensory neurons, and none outside of this rodent family (Fig. 1A and D and Fig. S2). Analysis of the tree thus suggests that the split between immune and apical vomeronasal Fprs occurred at the root of the Eumuroidea group, and that *Fpr-rs3* (which is present across the Eumuroidea lineage, whereas the *Fpr-rs4*, *-rs7*, and *-rs6* orthologs are found only in the Murinae group), corresponds to the first vomeronasal Fpr (Fig. 1A).

Neofunctionalization of vomeronasal Fprs is associated with the development of specific agonist properties (4, 7, 8) and signatures of positive selection (5). To investigate protein characteristics that could have been acquired by vomeronasal Fprs, we analyzed the sequence of FPR-rs3 in 17 rodent species. We identified 10 amino acids that were fixed in FPR-rs3 of all tested species and that were always absent from immune Fprs (Fig. 1B and Fig. S3). The position of one of these differentially conserved amino acids (His106) was previously identified by modeling approaches as likely having a role in ligand recognition by Fprs (17). To evaluate the selective pressures acting on the immune and the vomeronasal Fprs, we then calculated pairwise  $K_a/K_s$  ratios [the rate of nonsynonymous substitutions ( $K_a$ ) over the rate of synonymous substitutions ( $K_s$ )] for *Fpr-rs3*, *Fpr-rs2*, and *Fpr1* of the different rodent species. The ratios show that both immune and vomeronasal Fprs are under purifying selection. They also indicate lower values when comparing *Fpr1* and *Fpr-rs2* sequences than when comparing *Fpr1* and *Fpr-rs3* sequences (Fig. 1C).

Across species, Fpr genes are consistently grouped in the genome and neighbor a cluster of vomeronasal receptor genes (Fig. 1D and Fig. S1C). In non-Eumuroidea species, a split between Fprs on one side and the Vr gene cluster composed of V1rs but lacking V2rs on the other side is observed, with no intermingling (Fig. 1D and Fig. S1C). In contrast, in the Eumuroidea group (18), multiple Fprs are also embedded inside the Vr cluster that contains both V1rs and V2rs. It is these Fprs, located outside of the ancestral immune Fpr cluster, that are expressed in VSNS

(Fig. 1A and D). Thus, the physical proximity of Fprs with Vrs (and therefore with their regulatory elements) is correlated both with the arrival of V2rs in the Fpr/Vr cluster and with the acquisition of neuronal specificity, suggesting a causative link between Fpr/Vr intermingling and vomeronasal expression.

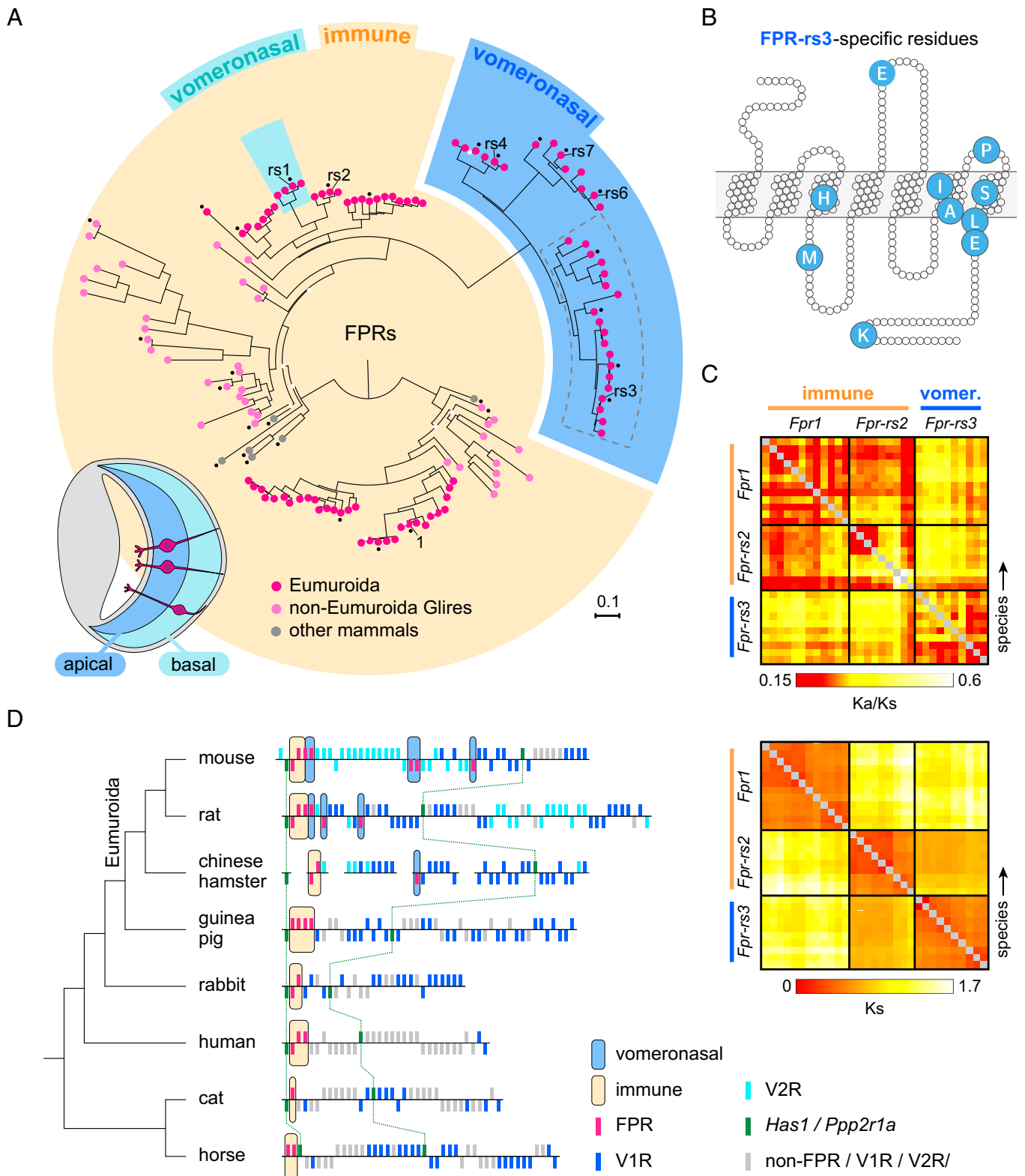
### Fpr Promoters Driving Apical Vomeronasal Neuron Expression Were Initially V1r Gene Promoters.

To identify the genomic events that led to the acquisition of vomeronasal specificity by Fpr genes, we analyzed the genomic elements that potentially control their expression. We initially focused our attention on the *Fpr-rs3* gene, because it is conserved among Eumuroidea and thus can be used for interspecies comparisons (Fig. 1A). We divided the sequence of *Fpr-rs3* into three regions: the *Fpr-rs3* promoter, the *Fpr-rs3* first and noncoding exon, and the *Fpr-rs3* coding sequence. Homologies with these sequences were searched in the mouse, rat, rabbit, guinea pig, horse, and cat genomes. High sequence homologies (up to 90% identity) were found between the *Fpr-rs3* promoter and all promoters of Fpr genes transcribed in the apical layer of the VNO (Fig. 2A). No homology was found with the promoters of *Fpr-rs1* (expressed in basal vomeronasal neurons) and with the two immune Fpr genes. Interestingly, we found high sequence homologies (59–70%) of the promoter and the first noncoding exon of *Fpr-rs3* with V1r genes of the rabbit, guinea pig, horse, and cat (Fig. 2A), whereas only vomeronasal Fpr sequences were matched in the mouse. This suggests that the gene shuffling event had led to the hijacking of a V1r regulatory sequence, with the functional elimination of its coding sequence (CDS) as a side effect. This was confirmed by the identification of V1r CDS remnants between the first noncoding exons of vomeronasal Fpr genes and their CDSs, which we identified in the mouse and rat genomes (Fig. 2A–D and Fig. S4A and B). A V1r phylogeny found that these remnants correspond to a V1r family (which we term V1rx) that disappeared in Eumuroidea following the exon shuffling (Fig. S4C). Taken together, these findings indicate that an exon shuffling event involving the last and coding exon of an immune Fpr gene (a gene more closely related to *Fpr-rs2* than to *Fpr1* based on phylogenetic analyses) targeted a V1r gene in a rodent living between 31 and 71 Mya (19). This provided a novelty to the rodent chemosensory toolbox, an innovation that has been expanded and maintained until today.

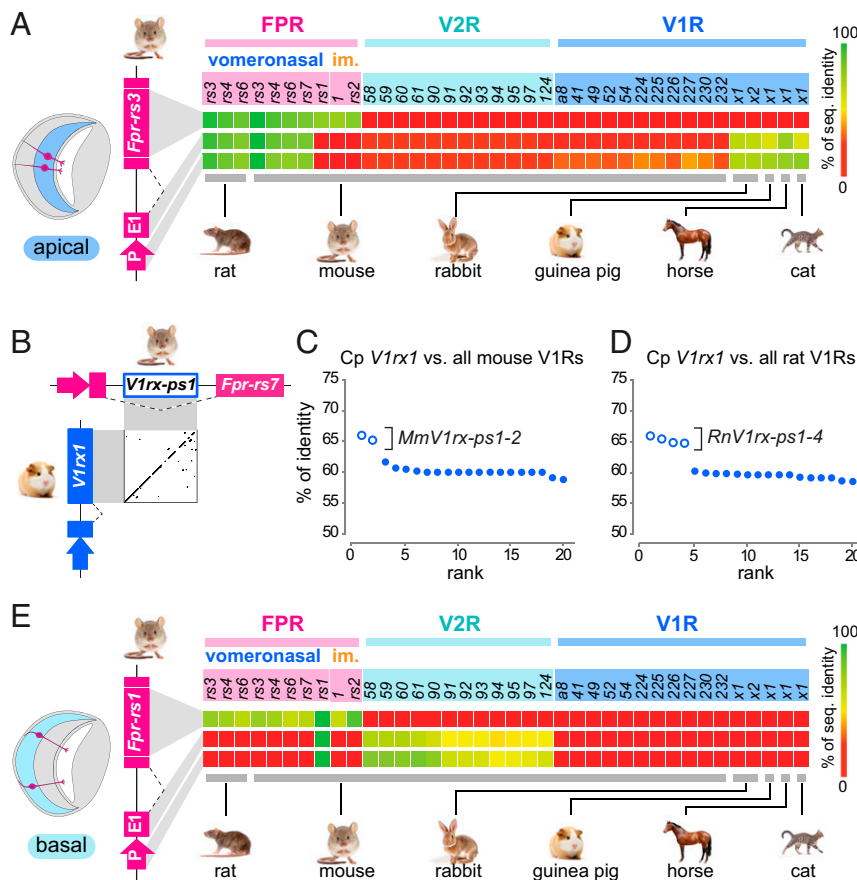
### The Fpr Promoter Driving Basal Vomeronasal Neuron Expression Was Initially a V2r Gene Promoter.

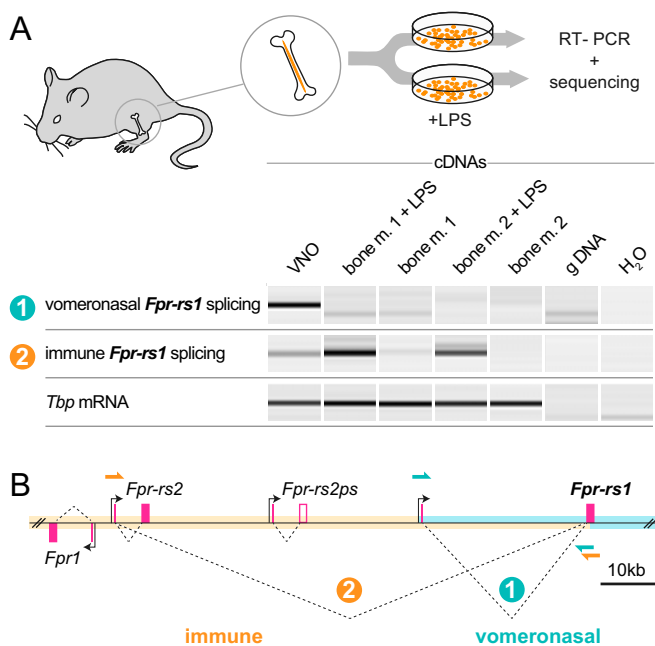
In the mouse VNO, *Fpr-rs1* is transcribed exclusively in basal vomeronasal neurons (4, 5). Because V2rs are expressed in the basal part of the vomeronasal epithelium, unlike V1rs, we wondered whether a similar type of genomic accident—that is, an exon shuffling but this time involving a V2r gene—could be at the origin of this novel Fpr transcription pattern. We found significant sequence homologies (up to 75%) between the mouse *Fpr-rs1* promoter or its first noncoding exon and mouse V2r promoters (Fig. 2E). We observed a complete lack of homology between the *Fpr-rs1* CDS and the V2R CDSs and an 81.5% identity with the one of the immune *Fpr-rs2* (Fig. 2E). This Fpr expression in the basal vomeronasal zone likely represents a recent acquisition that postdates the split between rats and mice, given that we found no sequence homologies between Fpr promoters and V2r promoters in the rat, hamster, or guinea pig. Moreover, we did not find *Fpr-rs1* in *Apodemus sylvaticus*, suggesting that the acquisition is restricted to the *Mus* genus.

The acquisition of Fpr expression by both apical and basal vomeronasal sensory neurons is not just more of the same, because although these two types of sensory neurons mediate the perception of pheromones and kairomones, they are not equivalent. They each project to well-defined and distinct parts of the accessory olfactory bulb (9, 10), a segregation maintained at the level of the projections of the corresponding secondary neurons



**Fig. 1.** The different identities of olfactory and immune Fprs. **(A)** Phylogenetic relationships between Fprs pertaining to 32 different mammalian species (16 Eumuroida, 12 non-Eumuroida glires, and 4 other mammals). The tree was inferred from a protein alignment with a maximum likelihood approach. The scale bar corresponds, for each branch, to amino acid substitutions per site. Branches with low bootstrap support (<30%) are indicated by a white disk. Black dots indicate species for which Fpr tissue specificity (vomeronasal or immune) was evaluated by qPCR, RNA-seq, or immunohistochemistry. On the bottom left part of the tree, the schematic represents a coronal section through a VNO with its basal and apical neuroepithelium. The dotted area encompasses all FPR-rs3 orthologs. **(B)** Conserved amino acid residues present in FPR-rs3 of all tested species and never observed in FPR-rs2 or FPR1. Colored residues are conserved at 100% in FPR-rs3 of all tested rodent species ( $n = 17$  species, 18 alleles) (Fig. S3). **(C)** Pairwise  $K_a/K_s$  ratios and  $K_s$  rates of immune and vomeronasal Fprs from various rodent species (the row order follows the order in Fig. S3). **(D)** Intermingling between Fpr and Vr genes is associated with vomeronasal Fpr expression. As landmarks of the syntenic, the *Has1* and *Ppp2r1a* nonfactory genes are shown in green. Genomic coordinates are indicated in Table S6. Genes (colored rectangles) are arranged according to their transcriptional orientation.





**Fig. 3.** Reacquisition of immune specificity by *Fpr-rs1*. (A, Upper) Schematic representing the experimental design. (A, Lower) RT-PCR (primer locations indicated in B) showing two *Fpr-rs1* transcript variants, one driven by the *Fpr-rs1* promoter and the other driven by the immune *Fpr-rs2* promoter. The abundance of this latter intergenic splice variant increases in bone marrow cells after LPS treatment. (B) Schematic corresponding to the vomeronasal (1) and immune (2) *Fpr-rs1* transcripts.

neurons expressing this mutant allele lose their ability to coalesce and to form glomeruli in the accessory olfactory bulb; however, when this V1r coding sequence is swapped with the one of an Or (Ors share no sequence homologies with V1rs), vomeronasal axons regain the ability to form glomeruli (9). Thus, a few simple and specific hijackings of regulatory elements were sufficient to provide an entire mammalian clade with a completely novel sensory toolbox to extract information from the outside world.

## Methods

**Identification of Chemosensory Receptor Gene Repertoires.** The whole mouse gene repertoires encoding V1rs, V2rs, and Fprs were retrieved from the mouse genome assembly GRCm38/mm10 using the Ensembl Biomart interface and filtering for InterPro signatures IPR004072 (vomeronasal receptor type 1), IPR004073 (vomeronasal receptor type 2), and IPR000826 (formyl peptide receptor-related) (25). The corresponding protein sequences were used to identify each species repertoire (Table S1) with tblastn searches (e-value < 1e-20). ORFs with fewer than seven TMHMM-predicted (26) transmembrane domains were discarded. For each repertoire, a neighbor-joining tree was built using closely related protein families as outgroups (T2rs for V1rs, T1rs and *Casr* for V2rs, and *GPR32* for Fprs). Genes branching with the outgroup were filtered out. For *Fpr* gene identification in nonsequenced rodent species, rodent tissue samples were taken from our own collection (*Mesocricetus auratus*, *Phodopus roborovskii*, *Acomys dimidiatus*, *Meriones unguiculatus*, and *Phodopus sungorus*) and from the Natural History Museum of Geneva (*Rattus exulans*, *Rattus rattus*, *Niviventer langbianis*, *Microtus regalis*, *Microtus arvalis*, *Apodemus alpicola*, *Apodemus flavicollis*, *A. sylvaticus*, and *Micromys minutus*). DNAs were extracted, and homologs of mouse *Fpr1*, *Fpr-rs2*, and *Fpr-rs3* were amplified by PCR with degenerate primers located on ortholog-specific conserved sequence segments (Table S2). All sequences are available in Dataset S1, and those absent from genome databases were submitted to GenBank (accession nos. MF175578–MF175624).

**Gene Phylogenies.** Multiple sequence alignments were performed with the MAFFT aligner program using the G-INS-i setting (27). Because V2r subfamilies have nonhomologous exons, we kept the alignable part of the proteins and trimmed their alignment before the PxxC[ST]xxC motif

(located upstream of the first transmembrane domain) and after the end of the last transmembrane domain. For all alignments, columns containing >90% gaps were discarded from the phylogenetic analysis. Protein duplicates corresponding to silent polymorphic variants were discarded as well. ProtTest 3 (28) was then used to determine the best-fitting model of evolution to build the trees. Maximum likelihood phylogenies were calculated using PhyML 3 (29). Branch supports were assessed using the nonparametric Shimodaira–Hasegawa-like approximated likelihood ratio test and, for the *Fpr* phylogeny, also by 1,000 bootstrap replicates. Subsequent phylogenies were used to establish the *Fpr*, V1r, and V2r gene synteny in the *Fpr/Vr* gene cluster between the mouse and the rat. We adapted our species tree to the phylogeny published by Fabre et al. (30).

**Transcription Start Site Identification.** To determine *Mus musculus Fpr-rs1* and *Fpr-rs3* transcription start sites (TSSs), their first noncoding exons were identified by assembling RNA sequencing (RNA-seq) reads from whole VNOs. The assembled transcripts were then blasted against the mouse genome (GRCm38/mm10) to define their coordinates.

**Promoters, First Exons, and CDS Comparisons.** Sequences upstream from TSSs and first exons from mouse *Fpr-rs1* and *Fpr-rs3* were used as blastn queries in genomes from different species to retrieve sequences that share similarities. These sequences constituted a dataset to which first exon sequences and sequences upstream of previously identified V1r and V2r TSSs (31) were added. Promoters, first exons, and CDSs were aligned with MAFFT using the E-INS-i setting. They were trimmed manually and curated with G-blocks (32) before the identity matrix analyses. Promoter and first exon sequence identity matrices were built with nucleotide sequences, whereas the CDS matrix identity was built with amino acid sequences. Genomic coordinates of these sequences are provided in Table S3.

**Gene Expression Analyses.** Before tissue extraction, animals were flushed of blood under anesthesia. Bone marrow was taken from femur, tibia, and humerus. mRNA samples were DNase-treated, and cDNAs were synthesized with random hexamers and poly-T oligonucleotides (PrimeScript RT Reagent Kit; TaKaRa). Room temperature controls were performed for all cDNAs. Quantitative PCR (qPCR) reactions were performed in triplicate in 384-well plates. SDS 2.2.1 software was used to analyze data, with a detection threshold at  $\Delta Rn = 0.3$ . An in-house spreadsheet was used to analyze and convert Ct values to relative values. Outliers with  $\Delta Ct > 0.5$  relative to the median within a given technical triplicates were discarded. The geNorm algorithm was used to analyze the variance of the normalization genes and select them. Normalization genes and V1r- and *Fpr*-specific primers are listed in Table S4. For the species for which mRNA samples were disposed of, we blasted *Fpr* sequences on publicly available bone marrow RNA-seq reads (rabbit, SRX110712; cat, SRX1610312; horse, SRX752839, SRX752840, SRX752841, SRX752842, and SRX752843). For RNA-seq, RNAs were ribodepleted, and 100-bp reads were sequenced with the Illumina HiSeq 2500 system.

**Amino Acid Conservation.** To identify FPR-rs3-specific sites, we used an alignment including all identified FPR1, FPR-rs2, and FPR-rs3 sequences from Eumuroidea species. FPR-rs1 sequences from *Mus* species were discarded because they are supposed to be expressed in both the immune and the vomeronasal system. FPR-rs3-specific sites were identified as being 100% conserved among all FPR-rs3 and absent in FPR1 or FPR-rs2 at homologous positions. For  $K_a/K_s$  estimations, the alignment of codons was inferred from the protein alignment. We then selected a subset of sequences separated with a minimum evolutionary distance ( $K_s \geq 0.1$ ). Positions with gaps were removed, and estimated  $K_a/K_s$  ratios were computed for all pairs using the pairwise mode of codeml in the PAML 4.9c package (33) (runmode = -2, CodonFreq = 0, fix\_kappa = 1).

***Fpr-rs1* Expression.** Bone marrow was flushed from mouse femurs and tibias. Bone marrow cells were resuspended at  $4 \times 10^6$  cells/mL, plated at  $1 \times 10^7$  cells per 60-mm dish, and left for 2 h at 37 °C. PBS or PBS + 150  $\mu$ g/mL of LPS (*Salmonella enteritidis*) was added to the cultures, which were then maintained at for 8 h at 37 °C. mRNAs were extracted and reverse-transcribed. PCR conditions are specified in Table S5. All PCR amplicons were sequenced.

**ACKNOWLEDGMENTS.** We thank Benoît von der Weid, Francisco Resende, Chenda Kan, and Véronique Jungo for expert technical help, and thank the Milinkovitch laboratory for *Acomys dimidiatus* genomic DNA. This work was supported by the Claraz Foundation, and the Swiss National Science Foundation Grants 31003A\_170114 (to I.R.), CR3313\_143723 (to I.R. and A.C.), and 31003A\_172878 (to A.C.).

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