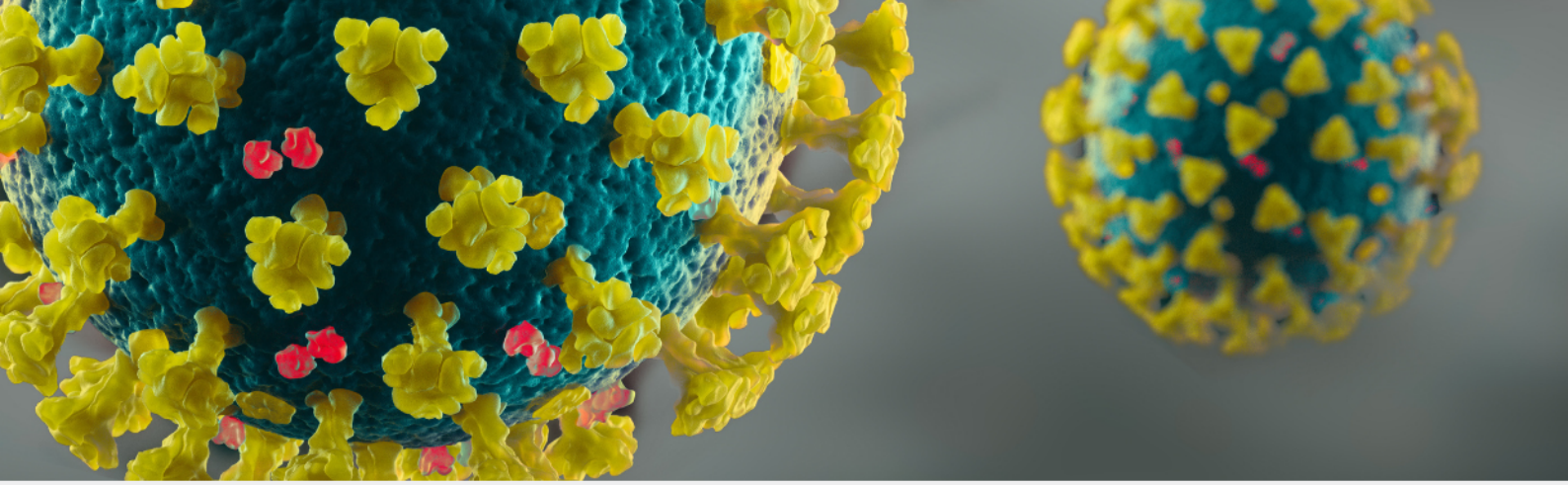


Vaccine Discovery and Development: Lessons from COVID-19

Free eBook

Emerging infectious diseases (EIDs) can evolve into a global healthcare crisis or pandemic. Scientists have previously required years to develop vaccines or therapeutics. The use of high throughput technology can greatly broaden the insights collected during discovery, augment efficiency and safety of handling EIDs, and shorten timelines.

Download this publication for an overview of many lessons learned in virology, immunology, and vaccine research during COVID-19 vaccine development.

Download here

SARTORIUS

CURRENT PROTOCOLS
A Wiley Brand

Received: 5 February 2021 | Revised: 9 April 2021 | Accepted: 15 April 2021

DOI: 10.1096/fj.202100224R

RESEARCH ARTICLE

THE FASEB JOURNAL

An evolutionary conserved olfactory receptor for foodborne and semiochemical alkylpyrazines

Patrick Marcinek[1,2] | **Franziska Haag**[1] | **Christiane Geithe**[1,3] | **Dietmar Krautwurst**[1]

[1]Leibniz-Institute for Food Systems Biology at the Technical University of Munich, Freising, Germany

[2]Hamilton Germany GmbH, Gräfelfing, Germany

[3]Brandenburg University of Technology Cottbus – Senftenberg, Senftenberg, Germany

Correspondence
Dietmar Krautwurst, Leibniz-Institute for Food Systems Biology at the Technical University of Munich, Lise-Meitner-Str. 34, 85354 Freising, Germany.
Email: d.krautwurst.leibniz-lsb@tum.de

Abstract

Molecular recognition is a fundamental principle in biological systems. The olfactory detection of both food and predators via ecological relevant odorant cues are abilities of eminent evolutionary significance for many species. Pyrazines are such volatile cues, some of which act as both human-centered key food odorants (KFOs) and semiochemicals. A pyrazine-selective odorant receptor has been elusive. Here we screened 2,3,5-trimethylpyrazine, a KFO and semiochemical, and 2,5-dihydro -2,4,5-trimethylthiazoline, an innate fear-associated non-KFO, against 616 human odorant receptor variants, in a cell-based luminescence assay. OR5K1 emerged as sole responding receptor. Tested against a comprehensive collection of 178 KFOs, we newly identified 18 pyrazines and (2*R*/2*S*)-4-methoxy-2,5-dimethylfuran-3(2H)-one as agonists. Notably, OR5K1 orthologs in mouse and domesticated species displayed a human-like, potency-ranked activation pattern of pyrazines, suggesting a domestication-led co-evolution of OR5K1 and its orthologs. In summary, OR5K1 is a specialized olfactory receptor across mammals for the detection of pyrazine-based key food odors and semiochemicals.

KEYWORDS

chemical ecology, chemosensory evolution, odorant receptor, olfaction

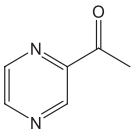
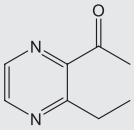
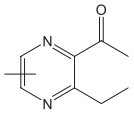
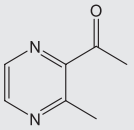
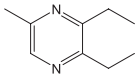
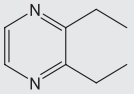
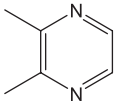
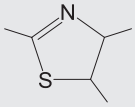
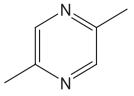
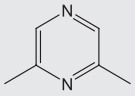
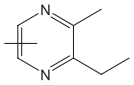
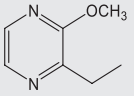
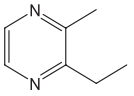
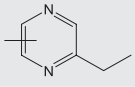
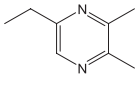
1 | INTRODUCTION

Pyrazines are a ubiquitous group of compounds widely found in nature.[1,2] Pyrazines are mainly known as aroma-enhancing products of food browning, formed via the Maillard reaction[3] during the heating of food. Notably, some pyrazines have lowest odor thresholds in the pg/L range, and occur in foods at concentrations above their odor thresholds, thereby critically determining the aroma of the food as "key food odorants" (KFOs; Table 1).[4,5] Pyrazines also form under physiological conditions,[6] and display a certain permanence in the environment.[1] Thus, many pyrazines have been reported as semiochemicals.[7-11] "Semiochemicals" are mostly, but not exclusively, volatile compounds that allow the transfer of

Abbreviations: cAMP, cyclic adenosine monophosphate; Gαolf, olfactory type G-protein α subunit; Gγ13, G protein γ subunit 13; KFO, key food odorant; OR, odorant receptor.

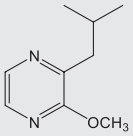
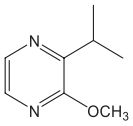
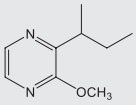
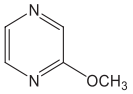
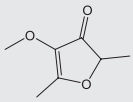
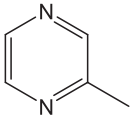
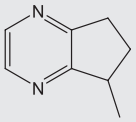
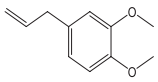
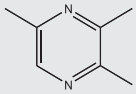
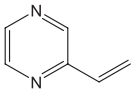
Patrick Marcinek and Franziska Haag contributed equally to this work.

TABLE 1 Alphabetical list of compounds utilized in the present study

Compound number	Structure	Compound name	Compound acronym	CAS	
1		2-Acetylpyrazine	2-AP	22047-25-2	K[a]/-
2		2-Acetyl-3-ethylpyrazine	2-A-3-EP	32974-92-8	-/-
3		2-Acetyl-3,5(6)-dimethylpyrazine, mixture of isomers	2-A-3,5(6)-DMP	54300-08-2	-/-
4		2-Acetyl-3-methylpyrazine	2-A-3-MP	23787-80-6	-/-
5		2,3-Diethyl-5-methylpyrazine	2,3-DE-5-P	18138-04-0	K/S[b]
6		2,3-Diethylpyrazine	2,3-DEP	15707-24-1	-/-
7		2,3-Dimethylpyrazine	2,3-DMP	5910-89-4	-/-
8		2,5-Dihydro-2,4,5-trimethylthiazoline	TMT	4145-93-1	-/S
9		2,5-Dimethylpyrazine	2,5-DMP	123-32-0	-/S
10		2,6-Dimethylpyrazine	2,6-DMP	108-50-9	-/S
11		2-Ethyl-3,5(6)-dimethylpyrazine, mixture of isomers	2-E-3,5(6)-DMP	27043-05-6	K/S
12		2-Ethyl-3-methoxypyrazine	2-E-3-MOP	25680-58-4	-/-
13		2-Ethyl-3-methylpyrazine	2-E-3-MP	15707-23-0	-/-
14		2-Ethyl-5(6)-methylpyrazine, mixture of isomers	2-E-5(6)-MP	13360-64-0	-/S
15		5-Ethyl-2,3-dimethylpyrazine	5-E-2,3-DMP	15707-34-3	-/-

(Continues)

TABLE 1 (Continued)

Compound number	Structure	Compound name	Compound acronym	CAS	
16		2-Isobutyl-3-methoxypyrazine	2-IB-3-MOP	24683-00-9	K/S
17		2-Isopropyl-3-methoxypyrazine	2-IP-3-MOP	25773-40-4	K/S
18		2-(Butan-2-yl)-3-methoxypyrazine	2-B-2-3-MOP	24168-70-5	K/S
19		2-Methoxypyrazine	2-MOP	3149-28-8	-/-
20		(2R/2S)-4-Methoxy-2,5-dimethylfuran-3(2H)-one, sum of isomers (Methoxyfuraneol)	MF	4077-47-8	K/-
21		2-Methylpyrazine	2-MP	109-08-0	-/-
22		5H-5-Methyl-6,7-dihydrocyclopenta-[b] pyrazine	5-M-6,7-DPP	23747-48-0	-/-
23		Methyl eugenol	ME	93-15-2	K/S
24		2,3,5-Trimethylpyrazine	2,3,5-TMP	14667-55-1	K/S
25		2-Vinylpyrazine	2-VP	4177-16-6	-/-

Abbreviation: CAS, CAS number.

[a]Categorizing compounds as KFO (K) according to Dunkel et al,[4] plus 2-E-3,5(6)-DMP.

[b]categorizing compounds as semiochemical (S) according to Table S3.

chemical cues between individuals of the same and/or different species, most often eliciting a standardized behavior.[12-15] Pyrazines function both intraspecies, for example, as pheromones sensu stricto,[16-18] as well as interspecies, for example, as allomones (to the benefit of the "sender")[7,8] and kairomones (to the benefit of the "receiver").[9,10,19] Despite pyrazines' eminent role as information carriers for the chemical senses of humans and other animals, the molecular chemoreceptive mechanisms of their chemosensory perception remained largely unknown, so far.

Odorant receptors (OR),[20] the predominant type of receptor with which we perceive volatiles, represent the largest gene family within the human genome, with approximately 400 protein-coding genes.[21,22] These receptors have evolved to best detect ecologically relevant groups of volatiles, such as KFOs or semiochemicals.[4,23] However, there is a considerable overlap between the latter two groups of compounds, and for example, body odors.[24,25] Such a diverse function has been known for certain alkylpyrazines, which are hedonic food quality indicators, while simultaneously acting as volatile cues with impact

on both intra- and interspecies communication on a very basal level.[9,10,26] A pronounced aspect of said communication is represented by fear-associated avoidance, either within the same species, where territory demarcations are involved,[27] or in-between species, where prey species try to avoid their predators.[10] Such volatile-induced avoidance behavior appears to be evolutionary hard-coded into species, to a degree where, even after a predator has not been present in a given territory for generations, the innate response within the prey species remains.[28,29]

Despite the importance of pyrazines function in olfactory communication, a highly specific and sensitive olfactory receptor for this class of chemicals still remains unknown. The innate fear-inducing semiochemical 2,5-dihydro-2,4,5-trimethylthiazoline (Table 1, TMT, "fox odor")[30,31] has been shown to act as an aversive odor, detected via both the olfactory system[32,33] and a nociceptive mechanism, for example, via the ligand-gated ion channel Trpa1 in mice.[34] As with pyrazines, a TMT-specific human OR has not been identified, so far. However, a TMT-specific OR was found in mice (Olfr1019).[33]

Here we set out to identify the best receptor for 2,3,5-trimethylpyrazine (2,3,5-TMP, Table 1), which qualifies as a KFO[4] and a fear-inducing odorant,[10,11] and for the innate fear-inducing semiochemical TMT.[30,31] Following a sensitivity-improved, dual screening strategy, we first screened 2,3,5-TMP and TMT against our in-house OR library of 616 human allelic variant IL-6-HaloTag-ORs,[35] using the GloSensor technology.[36,37] Vice versa, we then screened the sole identified receptor versus a comprehensive collection of known KFOs.[4] Finally, we compared pyrazine and TMT responses between ORs from orthologous genes across 9 species.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Dulbecco´s MEM (#F0435), FBS superior (#S0615), L-glutamine (#K0282), penicillin (10 000 U/mL)/streptomycin (10 000 U/mL) (#A2212), trypsin/EDTA solution (#L2143) (formerly Biochrom, Berlin, Germany, now Merck KGaA), calcium chloride dehydrate (#22322.295), D-glucose (#101174Y), dimethyl sulfoxide (DMSO) (#83673.230), HEPES (#441476L), potassium chloride (#26764.230), sodium hydroxide (#28244.295) (VWR International GmbH, Darmstadt, Germany), sodium chloride (#1064041000, Merck KGaA, Darmstadt, Germany), and D-luciferin (beetle) monosodium salt (#E464X, Promega, Madison, USA), 2,5-dihydro-2,4,5-trimethylthiazoline (CAS# 4145-93-1) (#AB494350, abcr GmbH, Karlsruhe, Germany).

Pyrazines and other odorants are listed in Table S1. Compounds of particular interest for our experiments are listed alongside their assigned compound numbers, CAS numbers, structures, acronyms, and designation as KFO and/or semiochemical in Table 1. Compounds utilized in the KFO screen were as previously published[38,39] (Table S2).

2.2 | Molecular cloning of human ORs and mammalian orthologs

Orthologs were identified using OrthoDB v10.1.[40] The protein-coding regions of human, mouse, and bovine ORs were amplified from genomic DNA by polymerase chain reaction (PCR) using gene-specific primers (Table S3) in a touchdown protocol: 1x (98°C, 3 minutes), 10x[(98°C, 30 seconds), (60°C to 50°C, 30 seconds, 1°C decrement), (72°C, 1 minute)], 25x[(98°C, 30 seconds), (50°C, 30 seconds), (72°C, 1 minute)], 1x (72°C, 7 minutes). Amplicons were either MfeI/NotI-digested (#R0589S/#R0189S, New England BioLabs, Ipswich, USA) or EcoRI/NotI-digested (#R6017/#R6435, Promega, Madison, USA), ligated into expression plasmid IL6-HaloTag-pFN210A[35] using T4-DNA ligase (#M1804, Promega, Madison, USA), and verified by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany).

2.3 | PCR-based site-directed mutagenesis for the construction of ptOR5K1 and ppOR5K1

The *Pan troglodytes* homolog ptOR5K1 (LOC470870) was generated from human OR5K1 by PCR-based, site-directed mutagenesis, using overlapping primers (Table S3) in a two-step touchdown PCR, and sub-cloned as described above. The *Pan paniscus* OR5K1 homolog ppOR5K1 (LOC100974684) was generated from the *Pan troglodytes* homolog ptOR5K1 by PCR-based, site-directed mutagenesis, and subcloned as described above.

2.4 | Gene synthesis for clOR5K1, oaOR5K1, pcOR5K1, vvOR5K1

The predicted protein-coding regions of the OR5K1 equivalents of *Canis lupus familiaris* (clOR5K1, LOC100856070), *Ovis aries* (oaOR5K1, LOC101105868), *Puma concolor* (pcOR5K1, LOC112850120), *Vulpes vulpes* (vvOR5K1, LOC112913751) were synthesized and cloned into expression plasmid IL6-HaloTag-pFN210A[35] by BioCat GmbH (Heidelberg, Germany).

2.5 | Sequencing and phylogenetic trees

All sub-cloned OR-coding amplicons were identified as being identical to their respective NCBI-reference sequences by Sanger sequencing (Eurofins Genomics,

Ebersberg, Germany) using vector-internal primers (Table S3).

The evolutionary history of OR5K1 orthologs was inferred by using the Maximum-Likelihood method and a Jones-Taylor-Thornton (JTT) matrix-based model.[41] The bootstrap consensus tree inferred from 100 replicates[42] was taken to represent the evolutionary history of the taxa analyzed. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA X.[43] A pairwise comparison table for OR5K1 and its investigated orthologs was constructed via amino acid alignment and analysis in CLC Main Workbench 20 (Qiagen, Hilden, Germany).

2.6 | Chemical clustering tree

A hierarchical physicochemical clustering tree of compounds was constructed utilizing the clustering toolbox from ChemMine Tools.[44] The resulting tree was modified utilizing Mega X,[43] to fit with the evolutionary trees in appearance.

2.7 | Cell culture and transient DNA transfection

We used HEK-293 cells,[45] a human embryonic kidney cell-line, authenticated in 2021 (Eurofins genomics, Ebersberg, Germany), as a test cell system for the functional expression of ORs, as described previously.[37] In short, in 96-well plates, 12,000 cells/well were transfected as follows: 100 ng/well of the respective OR construct and 50 ng/well of each the chaperone RTP1S,[46] the G-protein subunit $G\alpha_{olf}$,[47,48] olfactory G-protein subunit Gγ13,[49] and of pGloSensor-22F, coding for a genetically engineered, cAMP-dependent luciferase[36] (Promega, Madison, USA) using 0.75 µL/well ViaFect (#E4981, Promega, Madison, USA. As "mock"-control, we transfected empty pFN210A vector without OR coding region.

2.8 | Luminescence assay

Concentration/response relations were measured 42 hours post-transfection as described previously.[35,37] The automated screen of an OR cDNA expression library,[50] comprising 616 cDNAs coding for 391 human OR types (NCBI reference sequences) and 225 of their most frequent variants, against 100 µM 2,3,5-TMP was carried out as previously shown,[50] in HEK-293 cells, utilizing a Fluent Automation Workstation base for liquid-handling (Tecan, Männedorf, Switzerland), including transfection, with an integrated Spark multimode microplate reader (Tecan, Männedorf, Switzerland) for luminescence read-out. We utilized the previously published[51] combination of OR1A1 and 30 µM (-)-carvone as positive control for each plate. We used an empty pFN210A vector without OR coding region as "mock" transfection control, as described above.

2.9 | Key food odorant screen

The key food odorant screen with 177 (out of ca. 230) known KFOs[4,52] plus 2-*E*-3,5(6)-DMP was carried out according to Geithe et al (2017),[38] and according to the transfection protocol described above. Utilized compounds are listed in Table S4.

2.10 | Data analysis of the cAMP-luminescence measurements

The raw luminescence data obtained from the GloMax Discover microplate reader/Spark multimode microplate reader were analyzed in the case of concentration/response assays by averaging each data point of basal levels and data points after odorant application. For a given luminescence signal, the respective averaged basal level was subtracted, and the now corrected data set was normalized to the maximum amplitude within a data set. The data set for the mock control was subtracted, and effective concentration at 50% of maximum (EC_{50}) values and curves were derived from fitting the function[53]

$$f(x) = \left(\frac{(\min - \max)}{\left(1 + \left(\frac{x}{EC_{50}}\right)^{\text{Hillslope}}\right)} \right) + \max$$

to the data by nonlinear regression (SigmaPlot 14.0, Systat Software). All data are presented as mean ± SD of at least 3 independent transfection experiments performed in triplicates. Significances were calculated by paired two-tailed Student's *t* test.

2.11 | EC_{50}/human odor threshold correlation analysis

Compounds for which a measurable EC_{50} in human OR5K1 could be established are listed alongside their published human odor thresholds in water (µg/kg) in Table S6. In case

of more than one published threshold, the lowest one was chosen for further analysis.

Correlation analysis was carried out using the R package ggplot2[54] and Spearman's correlation.

3 | RESULTS

We have chosen 2,3,5-TMP for our search to identify cognate human ORs, because of its double function as an alkylpyrazine KFO,[4,55] and as a semiochemical identified in predator feces/urine[9,56] (Tables S2, S4, S5).

3.1 | OR5K1 of all human ORs solely responded to 2,3,5-TMP, and to "fox odor" TMT, across mammalian orders

OR5K1 emerged as the sole responder in an automated screen of 2,3,5-TMP (24) against an OR-library of 616 OR-gene variants (Figure 1A). In order to test the veracity of the signal, we established concentration/response-relationships of 2,3,5-TMP with OR5K1 and its most commonly found haplotype OR5K1-F_{62}L (minor allele frequency = 0.05, Figure 1A, insert). EC_{50} values were 139.04 ± 7.08 μM and 376.04 ± 44.81 μM for OR5K1 and OR5K1-F_{62}L, respectively. To test whether OR5K1 is the sole responder among paralog members of the human OR5K phylogenetic clade, we screened a variety of compounds similar to 2,3,5-TMP (ie, pyrazines) with a concentration of 300 μM against OR5K2, OR5K3, and OR5K4 (Figure S1). However, neither of the paralogs performed within measurable parameters.

We then asked whether the activity of OR5K1 was pyrazine-specific. In order to investigate this, we attempted to test the receptor against "fox odor" TMT, a semiochemical that triggered innate fear reactions in mice,[30,31] and is structurally similar to 2,3,5-TMP. Screening TMT against our OR-library, revealed OR5K1 as the sole responder (Figure 1B). As in the screen against 2,3,5-TMP, the human paralog OR5K2-4 did not respond to TMT. We then established TMT-specific concentration/response activities of OR5K1 and its orthologs from apes (*Pan paniscus* [ppOR5K1], *Pan troglodytes* [ptOR5K1]), as well as from other mammalian species (*Bos taurus* [btOR5K1], *Canis lupus familiaris* [clOR5K1], *Mus musculus* [Olfr173], *Ovis aries* [oaOR5K1], *Puma concolor* [pcOR5K1], and *Vulpes vulpes* [vvOR5K1]) (Figure 1B, insert). The human receptor OR5K1 remained the strongest responder among the simian receptors. Among the prey species, the receptors btOR5K1 and oaOR5K1 derived from ungulates are the better responders, with regard to their EC_{50} values, as compared to the mouse ortholog Olfr173. Among the predators, the *Vulpes vulpes* ortholog vvOR5K1 demonstrated the strongest activity.

3.2 | OR5K1 is highly selective for alkyl pyrazines out of 178 KFOs tested

We then characterized the food-centered agonist space of human OR5K1 by testing against 177 key food odorants (KFOs, listed in Table S2)[4] at 300 μM each (Figure 1C). We also decided to include 2-E-3,5(6)-DMP, as a good number of publications have proposed this mix of isomers in part or its entirety as a key odorant in various foods since Dunkel et al (2014) was published.[57-59] This extended the number of compounds measured in this KFO screen to 178. Beyond 2,3,5-TMP, the majority of activating compounds were pyrazines as well: 2,3-DE-5-P, 2-IP-3-MOP, 2-IB-3-MOP, and 2-B-2-3-MOP. Further activating compounds were methoxyfuraneol (MF) and the previously published methyl eugenol (ME)[60] (Figure 1C). We established concentration-response-relationships and determined EC_{50} values for most KFO hits, except for compounds 2-AP and 2-B-2-3-MOP, which did not go into saturation. Similarly, ME tested on both ape orthologs failed to produce signals that could be fitted to obtain an EC_{50} (Table S6).

3.3 | Mouse homologs of the human OR5K subfamily diversified in function as compared to their human orthologs

The phylogenetic relationships of mouse orthologs with the human OR5K subfamily, according to OrthoDB,[40] are depicted in Figure 2A (excluding the Olfr173 paralog Olfr172 for its lack of response in a pre-screen not shown here). Godfrey et al (2004) reported that 54% of shared subfamilies have more members in mouse than in human, and hypothesized that larger sizes of the subfamilies may result in a greater sensitivity of detection or in the ability to discriminate between closely related odorants.[61] Here, we put this to the test for human OR5K1 and its mouse subfamily homologs, by establishing concentration-response relations for the KFO 2-E-3,5(6)-DMP, which had the second-lowest EC_{50} in OR5K1 (21.18 ± 2.06 μM) and the lowest EC_{50} in the mouse ortholog Olfr173 (37.85 ± 4.35 μM) (Table S6), as well as for the fear-inducing semiochemical TMT (OR5K1: 102.15 ± 1.05 μM; Olfr173: 651.36 ± 123.46 μM). Human OR5K1 and its mouse ortholog Olfr137 were on average fivefold and 17-fold more sensitive for the KFO 2-E-3,5(6)-DMP over TMT, respectively. In sharp contrast, the homolog Olfr175 responded fivefold more sensitively to the semiochemical TMT (53.61 ± 3.96 μM) as compared to the KFO 2-E-3,5(6)-DMP (265.17 ± 16.34 μM) (Figure 2B, Table S6).

We obtained similar results for Olfr1019, a mouse OR unrelated to the OR5K subfamily, which has been reported previously to respond to TMT[33] (Figure 2B). The EC_{50} values of

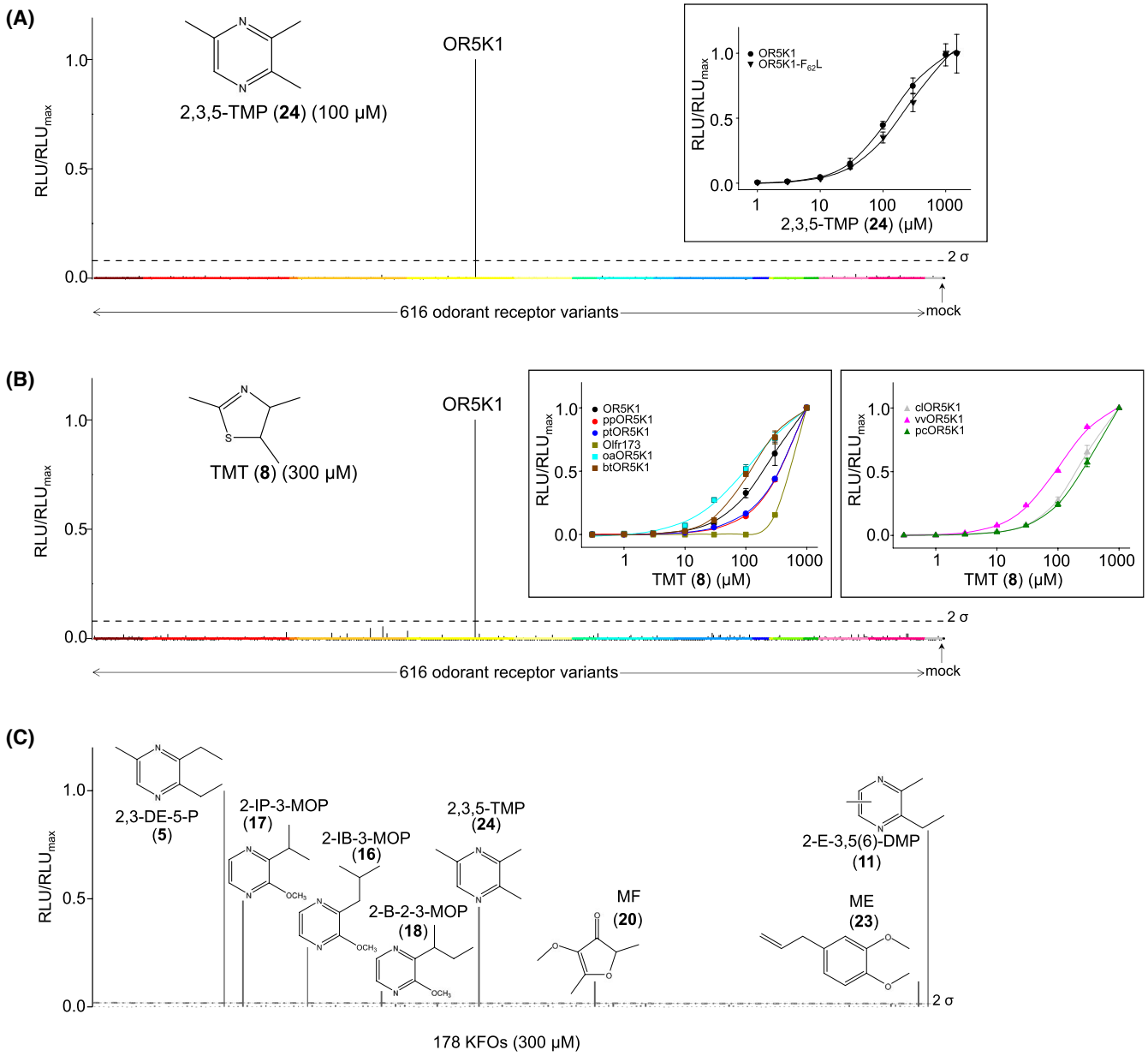


FIGURE 1 OR5K1 soley responds to 2,3,5-trimethylpyrazine and TMT. OR5K1 is the sole responder in an automated screen of 616 OR variants against 100 µM 2,3,5-TMP (A), and TMT at 300 µM (B). Data were normalized to the maximum response (OR5K1). OR families are color-coded and sorted in ascending numerical order. Dashed line indicates 2σ-threshold as signifier of activation. The negative control was a mock plasmid. Insert (A): Both the OR5K1 reference and its most common variant, OR5K1-F_{62}L, presented measurable concentration/response-relationships with. Inserts (B): Concentration/response-relationship of various OR5K1 orthologs versus. Values are normalized to the highest signal of each respective receptor's maximum concentration/response-relation (RLU/RLU_{max}). Data are presented as mean ± SD (n = 3). ● = apes, ■ = prey species, ▲ = predators, bt = Bos *taurus*, cl = Canis *lupus familiaris*, oa = Ovis *aries*, pt = Pan *troglodytes*, pp = Pan *paniscus*, pc = Puma *concolor*, vv = Vulpes *vulpes*. C, Alkylpyrazines are the best KFO agonists of OR5K1. 177 key food odorants (listed in Table S2) according to Dunkel et al (2014)[4] plus 2-E-3,5(6)-DMP were screened at 300 µM against OR5K1. Data are means of triplicate determinations, and were normalized to the maximum response of 2,3-DE-5-P. RLU = relative luminescence units. Bold numbers identify compounds according to Table 1

TMT and 2-E-3,5(6)-DMP on Olfr1019 differed significantly (n = 3, P <.05, two-sided t test), with 51.7 µM ± 31.0 µM and 457.6 µM ± 32.5 µM, respectively. Olfr1019, much like Olfr175, responded about 10-fold more sensitively to TMT than to the pyrazine compound 2-E-3,5(6)-DMP, and both Olfr1019 and Olfr175 responded to TMT, with comparable EC_{50} values of 51.7 µM ± 31.0 µM and 53.61 ± 3.96, respectively. However, TMT activated Olfr175 with a

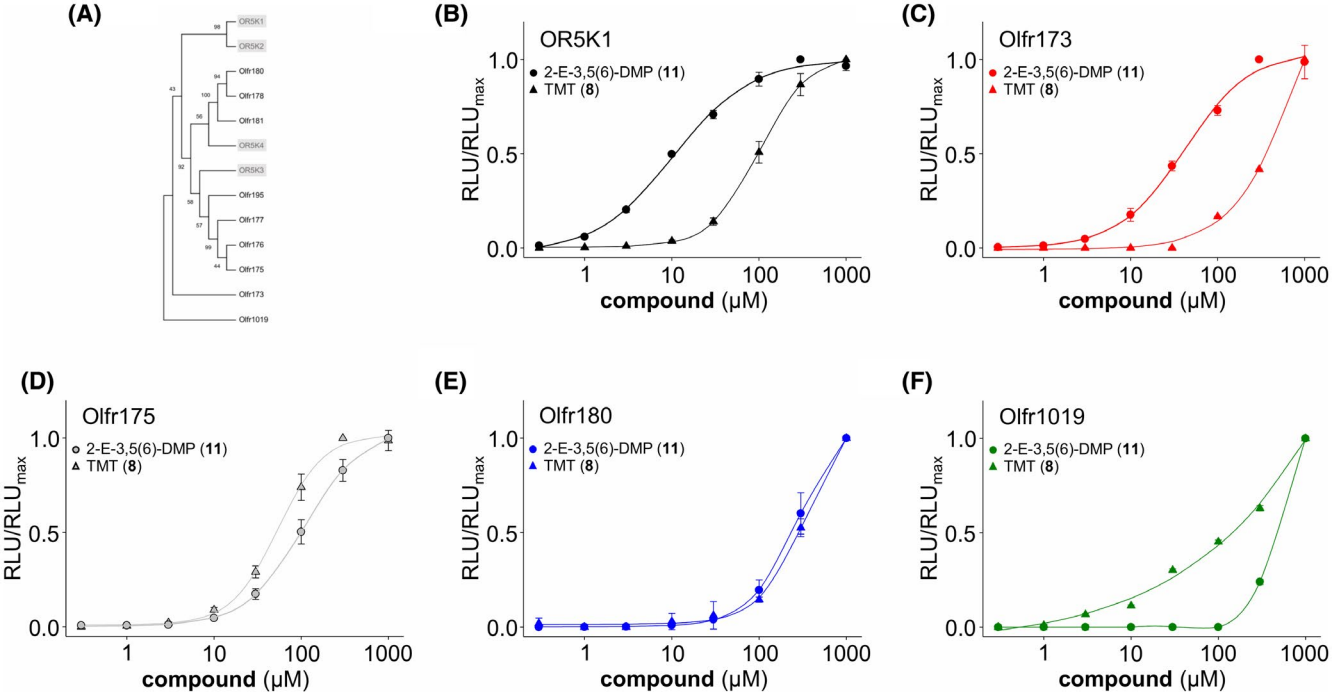


FIGURE 2 Mouse orthologs of the OR5K subfamily have diversified functions in response to 2-Ethyl-3,5(6)-dimethylpyrazine and TMT. A, Maximum-Likelihood-Tree inferring the evolutionary relationships of the OR5K-clade and its mouse orthologs plus Olfr1019. Numbers next to branches indicate percentage of replicate trees in which the associated taxa clustered together in a bootstrap test with 100 replicates. Grey boxes indicate human receptors. B-F, Concentration/response curves of OR5K1 (B), mouse OR5K-clade orthologs Olfr173 (C), Olfr175 (D), Olfr180 (E) and Olfr1019 (F), activated by both TMT and 2-E-3,5(6)-DMP. ▲ indicates receptor interaction with TMT, while ● indicates interaction with 2-E-3,5(6)-DMP. Data are normalized to the highest signal of each respective receptor's maximum response (RLU/RLU_{max}), and are presented as mean ± SD (n = 3). RLU, relative luminscence units

significantly (P <.05 by two-sided t test) better efficacy at maximum activating concentration (1 mM) than Olfr1019 (Figure S2A). Here, it should be noted that neither TMT nor 2-E-3,5(6)-DMP activated in our OR library screens the human homolog of Olfr1019, OR5AR1, sufficiently high enough to be considered a "hit" (for TMT, see Figure 1B, the screen with 2-E-3,5(6)-DMP is not shown).

Testing TMT against all human and mouse members of the OR5K-subfamily, interestingly, and unlike with pyrazines, of all tested mouse receptors Olfr175 responded best (Figures 2B and S2A, Table S6). Notably, the OR5K4 relative, Olfr180, had comparable EC_{50} values for both TMT and 2-E-3,5(6)-DMP (Table S6), underlining the differential odorant selectivities in OR5K subfamily-related mouse ORs.

We further screened OR5K-subfamily mouse ORs against both 2-E-3,5(6)-DMP and 5-E-2,3-DMP, the latter of which has not yet been described as a KFO, but was identified as innate fear-associated by Osada et al (2017).[11] For both 2-E-3,5(6)-DMP and 5-E-2,3-DMP, Olfr173 (OR5K1/OR5K2 clade) emerged as the strongest responder, followed by Olfr175 (OR5K3 clade), Olfr180 (OR5K4 clade), and Olfr178 (OR5K4 clade) (Figure S2B, Table S6). Our results demonstrate that each OR5K clade has at least one mouse ortholog responding to TMT and/or to pyrazines. In sharp contrast, none of the human OR5K1 paralogs (OR5K2, OR5K3, OR5K4) tested here, responded neither to TMT (Figures 1A,B and S2A), nor to any of the pyrazines tested (Figure S1). Our findings support the observation that orthologous rather than paralogous ORs correspond in their function.[33,60,62]

3.4 | OR5K1 and orthologs are most potently activated by odorants with a known KFO/semiochemical double function

We then contrasted compounds against cognate receptors in a heat map encoding the EC_{50} values of the respective compound/receptor combinations (Table S6) in a clustered fashion (Figure 3). We sorted the compounds according to their physico-chemical relationships with each other and identified them as key food odorants (according to Dunkel et al (2014)[4]), or semiochemicals (according to Table S4), specifying whether they were found to be present in predator urine or feces, as we assume these to be a major source for fear-inducing volatiles among mammals (as opposed to, for example, insect semiochemicals). For the receptors, we clustered them according to their reference amino acid

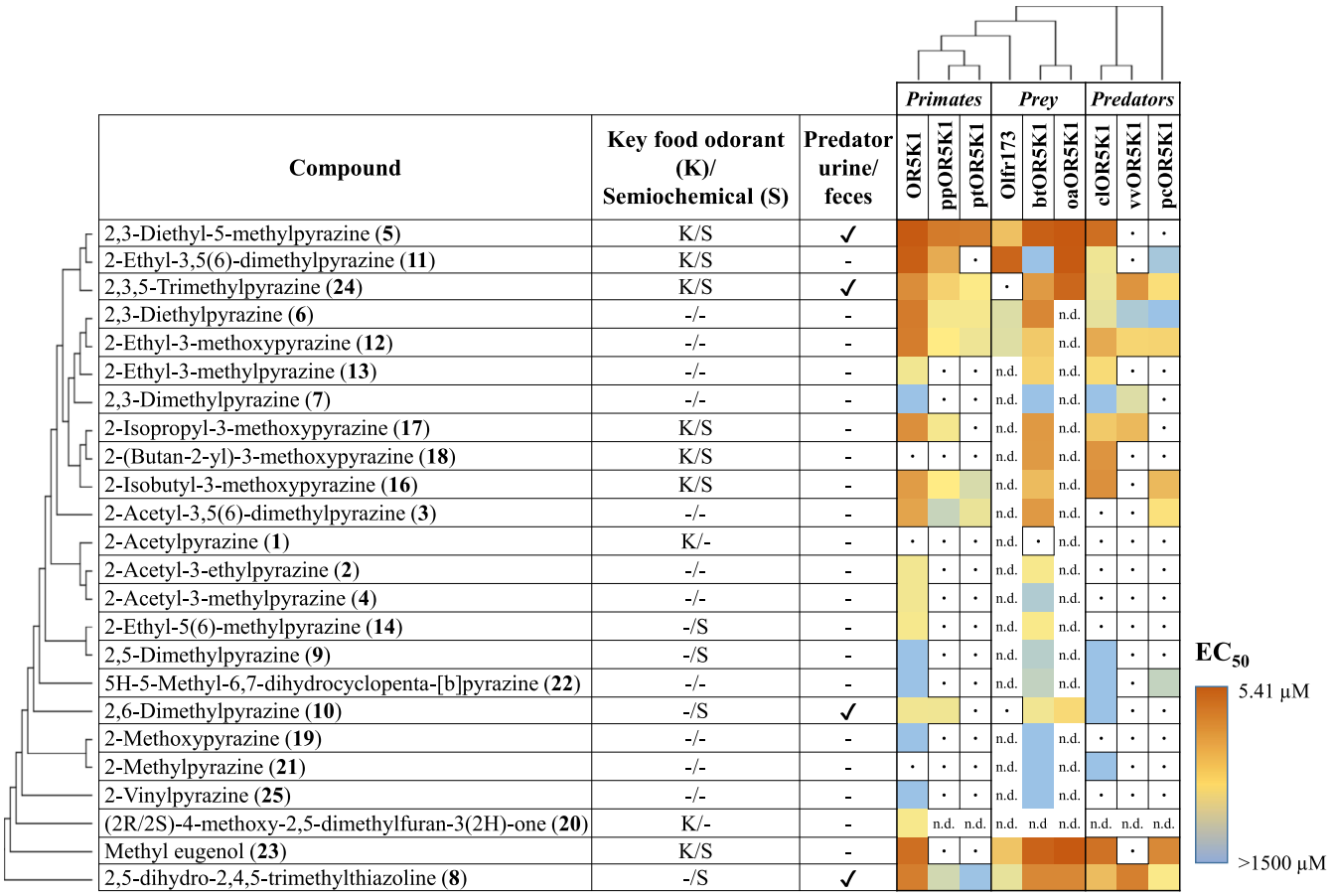


FIGURE 3 Compound and evolutionary clustering reveal evolutionary conserved agonist/receptor relationships for OR5K1-orthologs. Clustering of compounds by their physico-chemical properties was done using the clustering toolbox from ChemMine Tools.[44] Key food odorants were identified according to Dunkel et al (2014), with addition of 2-ethyl-3,5(6)-dimethylpyrazine (2-E-3,5(6)-DMP). Studies that identified compounds as semiochemicals are referenced in Table S4. The evolutionary history of OR5K1, ppOR5K1 (*Pan paniscus*), ptOR5K1 (*Pan troglodytes*), Olfr173, btOR5K1 (*Bos taurus*), oaOR5K1 (*Ovis aries*), clOR5K1 (*Canis lupus familiaris*), vvOR5K1 (*Vulpes vulpes*), and pcOR5K1 (*Puma concolor*) was inferred from applying the Maximum-Likelihood method and the Jones-Taylor-Thornton (JTT) matrix-based model.[41] The inferred evolutionary relationships and shared sequence identities of the receptors investigated are given in Figure S3. Color scale is based on EC_{50} values ranging from 5.41 µM (lowest measured) to ≥1500 µM. The corresponding numerical values of the heat map are given in Table S6. ● indicates measurable concentration-dependent responses that failed to fit to an EC_{50}. n.d., not determined. Data represent means of n = 3. Bold numbers in parentheses identify compounds according to Table 1

sequences, inferring evolutionary relationships. Additionally, we classified the ORs as belonging to "Primates," "Prey," and "Predators" (Figure 3).

2,3-DE-5-P, 2-E-3,5(6)-DMP, and 2,3,5-TMP elicited the strongest receptor responses particularly across the primates and prey categories (Figure 3). Notably, all three compounds are both KFOs and semiochemicals, of which 2,3-DE-5-P and 2,3,5-TMP have been found in predator urine. In contrast, the semiochemical TMT performed well across all receptors tested, whereas it activated the non-human ape receptors less potently. Interestingly, across categories, the receptors from human, cow, and dog displayed a largely similar EC_{50}-ranking pattern for the odorants investigated (Figure 3).

3.5 | Bioassay-based EC_{50} values of OR5K1 agonists correlate with their respective odor-thresholds

Since OR5K1 resulted as the sole human alkyl pyrazine receptor, we then asked, whether our in-vitro determined EC_{50} values somehow correlate with human odor thresholds, which we identified by a literature research (Table S5). For mixtures of isomers, we used the lower threshold. The concentration-response relation of 2-MOP did not go into saturation, and thus prevented a logistic fit function-derived EC_{50} (Figure S4). Therefore, we had to assume a least EC_{50} value for 2-MOP according to its concentration of maximum effect (1500 µM). There is a significant positive correlation

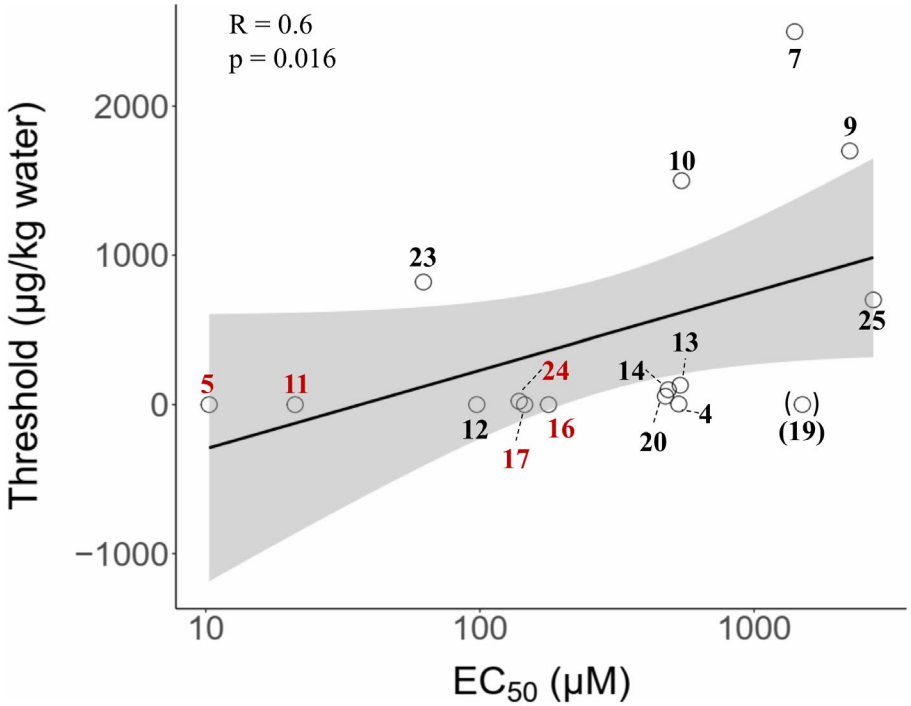


FIGURE 4 Pyrazines' in-vitro EC_{50} values on OR5K1 correlate with human odor thresholds. Odor thresholds and corresponding EC_{50} values can be found in Table S5. Thresholds are detection thresholds, determined orthonasally in water. Bold numbers identify compounds according to Table 1. Pyrazines identified as both KFO and semiochemical are demarcated with red numbers. A non-saturating concentration-response relation of 2-MOP prevented a logistic fit function-derived EC_{50} (Figure S4). Therefore, we assumed a least EC_{50} value for 2-MOP according to its concentration of maximum effect (1500 µM). R, Spearman's correlation coefficient. A 95%-confidence interval is shaded in grey

(Spearman's correlation coefficient = .60; $P < .05$) of in-vitro determined EC_{50} values and odor threshold concentrations in humans (Figure 4). Particularly on the lower end, EC_{50} values below 300 µM correlate with low odor thresholds, with the sole exception being the non-pyrazine ME. For example, the compound cluster 2,3-DE-5-P, 2,3-DMP, and 2,3,5-TMP exhibiting lowest EC_{50} values (Figure 3), is also found at the lower odor threshold concentration end of the correlation plot (Figure 4).

4 | DISCUSSION

ORs have evolved to best detect agonists out of ecologically relevant groups of volatiles, such as KFOs or semiochemicals.[4,23] To what degree these two agonist spaces overlap is, however, largely unknown. Here, certain pyrazines, in particular alkylpyrazines, are role models, because of their relative stability,[1] and their frequent double function as KFOs[4,63] and as semiochemicals.[9-11] In our study, OR5K1 emerged as the sole human OR to detect both. Indeed, we newly identified 18 pyrazines and the non-pyrazine compound MF as agonists for OR5K1, for which the sole previously identified KFO agonist was ME,[60] a phenylpropene that can be found in essential oils, for example, from basil leaves.[4] The function of human OR5K1 as an alkylpyrazine-selective OR at the intersection of KFOs and semiochemicals appeared to be evolutionary conserved at the level of apes (*Pan paniscus* and *Pan troglodytes*). We investigated the the OR5K1 orthologs from these two species, since they underwent the same reduction in OR gene functionality observed for many apes,[64,65] including human. Indeed, here, we validated the most potent agonist of human OR5K1, 2,3-DE-5-P, also as best alkylpyrazine agonist in both OR5K1 orthologs, ppOR5K1 and ptOR5K1. In contrast, ME, which already had an at least sixfold higher EC_{50} value than 2,3-DE-5-P for human OR5K1, elicited concentration-dependent responses in both ape orthologs that were shifted to higher concentrations by several orders of magnitude, without measurable EC_{50} values. Therefore, ME must no longer be considered as lead compound for human OR5K1.

Mice are both synanthropes (ie, profiteers of human civilization) and classic prey animals. We thus reasoned that recognizing both food- and fear-associated aspects of alkyl pyrazines should be beneficial to mice, suggesting at least one pyrazine-responding mouse OR5K1 homolog. Godfrey et al (2004) suggested that larger sizes of mouse OR subfamilies may result in an increased ability to discriminate between closely related odorants.[61] While solely human OR5K1, but none of its paralogs, responded to any of the pyrazines or

TMT tested in the present study, indeed, each OR5K-clade harbors one pyrazine-responsive mouse homolog, with different best receptors for example, KFO/semiochemical 2,3-DMP (Olfr173) and non-KFO/semiochemical TMT (Olfr175), suggesting that during the diversification of the OR5K subfamily in mice different receptors evolved to selectively detect different, yet structurally related alkylpyrazines. This is corroborated by previous studies reporting that phylogenetically related mouse ORs had overlapping odorant specificities.[66,67]

Another compound that was found in wolf urine and initially has been considered to be fear-inducing is 2,6-DMP.[9] In further tests by Osada et al (2011), this particular compound, however, failed to significantly induce fear reactions—its status as a semiochemical, thus, remains unclear.[11] In our hands, 2,6-DMP had the highest measurable EC_{50} for human OR5K1, and no measurable EC_{50} for its mouse orthologs, suggesting that this compound has little physiological relevance as a semiochemical via the OR5K subfamily.

TMT has been reported previously to induce innate fear in mice.[30,31] Kobayakawa et al (2007) genetically inactivated the dorsal zone of the mouse olfactory bulb in order to create TMT-non-responsive individuals.[68] By this approach, the transcript levels of two OR5K1 homologs were notably reduced: Olfr175 (designated the alternative name Olfr174 in that article) and Olfr180,[68] corroborating our results. In the present study, out of the OR5K1 homologs from nine species investigated, the receptors from mouse (Olfr175) and fox emerged as the most sensitive receptors for TMT, closely followed by the receptors from cow and sheep. In contrast to mouse, however, OR5K1 emerged as the sole human OR responding to TMT, albeit with a comparable sensitivity as mouse Olfr175. Our results suggest that human OR5K1 has evolved to preferably detect KFOs, with, however, a mostly conserved function as semiochemicals. Pyrazines as KFOs derive from the roasting of food, an unnatural process. They are, therefore, not generally associated to food in the natural world, but may well be associated with food in the human ecological niche. Therefore, some KFO pyrazines may be attractive, at least for some domesticated species, while pyrazines in a semiochemical context, for example, predator-prey relationships, may be largely aversive. Therefore, the ecological and sensory context of the organism receiving the odor likely determines the final interpretation of the information received, towards either attraction or aversion.[69,70] Whether the definition of food odors may be extrapolated to non-human species will require systematic de-orphaning of non-human ORs with KFOs and behavioural studies.

Saito et al (2017) previously published TMT and its structural homolog 4-methyl-thiazoline as agonists for another mouse receptor, Olfr1019,[33] which is not related to the OR5K subfamily of mouse ORs, displaying only about 44% amino acid identity to Olfr175. However, in the present study, Olfr1019 responded best to TMT, with comparable EC_{50} values as Olfr175, but with lower efficacy. The flattened concentration-response relation may be due to the presence of different structural forms of TMT. Since TMT is a mixture of isomers, the individual molecules may differentially activate their cognate receptor (see 38,51). Altogether, we have now identified four mouse ORs that redundantly responded to the predator odor TMT, with different sensitivities (Olfr1019 = Olfr175 $\gg$ Olfr180 > Olfr173) and efficacies (Olfr175 $\gg$ Olfr1019 > Olfr180 > Olfr173). Our data show that the chemoreception of fear-associated alkylpyrazines in general, and of predator odor TMT in particular, is more complex in mice than in humans, at least on a molecular level. This suggests a phylogenetic clade-independent, evolutionary valuable redundancy, enabling the detection of a predator odor over a wide concentration range, which may be the molecular basis of an olfactory intensity coding for such ecologically relevant odorant cues.[61] A similar observation has been reported previously for (Z)-5-tetradecen-1-ol, which, via mouse Olfr288, elicited attraction behavior at low concentrations, and, via an extended OR activity pattern, activated an aversion behavior at higher concentrations.[71]

It has been questioned whether a single molecule, such as TMT, or rather a combination of volatiles from predator urine, for example, pyrazines, is an adequate stimulus to sufficiently trigger avoidance behavior in prey animals under natural conditions.[72,73] Indeed, a mixture of pyrazines from wolf urine was more efficient to trigger freezing behavior in mice than the single components tested alone.[9] Moreover, pyrazines and TMT are not the sole inducers of innate fear.[74] Recent studies have demonstrated the importance of multi-receptor activation in steering olfaction-induced behavior in mice.[71,75] In particular, Saraiva et al (2016)[75] demonstrated the importance to consider not just ORs, but also other volatile-associated receptors in this context, as in their hands the attractive TAAR5-ligand trimethylamine managed to ablate the aversive effect of TMT in mice. Recently, Dewan et al (2018)[76] proposed both a functional redundancy across ORs and TAARs, as well as odor detection thresholds (though not behavioral thresholds) being set by the receptor most sensitive to a given compound. Future studies regarding fear-associated semiochemicals will have to take further combinatorial approaches, akin to those just described, and much like with food aroma recombinates.[77,78]

ORs play an eminent role in the speciation process.[79-81] Thus, an evolutionary clustering of OR sequences separated well between the mammalian orders "primates", "prey", and "predators".[82] A domestication-led co-evolution[83-85] may be the reason for the overall similar clustering and EC_{50}-ranking response patterns for pyrazines and semiochemicals of OR5K1-orthologs, at least in human, cow, sheep, and dog.[86-89] For sheep and goats, for example, evidence for a transspecific genomic signature and phenotype of

domestication has been described previously.[88] Thus, domestication may be causative for a similar OR5K1 phenotype. For instance, the OR5K1 ortholog of *Canis lupus familiaris*, clOR5K1, demonstrated an increased, human-like diversity of pyrazine agonists, as compared to both predator orthologs, pcOR5K1 and vvOR5K1.[87,90] Interestingly, the receptor of, for example, *Bos taurus*, which shares 87% sequence identity with human OR5K1, has an agonist spectrum that is largely similar to that of the human receptor, whereas the two chimp orthologs, with 98%-99% sequence identity to human OR5K1 deviate significantly, with a largely reduced pyrazine agonist spectrum. Man et al (2008) proposed that sequence-based homology comparisons of a generalized odorant binding pocket of ORs,[91] rather than comparing an overall sequence homology, may allow to infer the similarities and differences in OR function.[92] However, the amino acids constituting a proposed generalized odorant binding pocket of ORs[91] are identical in human and chimp.[39] We had previously described a similar disparity of detection of heated onion KFO 3-mercapto-2-methylpentan-1-ol by OR2M3 orthologs from humans and other apes, which may reflect an adaptation to modern human's nutritional behavior toward onions in particular, and/or the heating of food across cultures in general.[39] While apes are not a typical prey for, eg, wolves and foxes, sheep and their offspring are. Our results show that the sheep ortholog of human OR5K1 was the most sensitive receptor for pyrazines found in predator urine or feces, and may, thus, reflect an adaptation to sheep's life as prey.

Based on previously published OR agonist information and their analysis of OR transcript levels in the olfactory epithelium, Saraiva et al (2019) suggested that highly abundant mouse and human OR subtypes detect ecologically relevant odorants, such as semiochemicals or KFOs.[23] Indeed, OR5K1 or homologs Olfr173 and Olfr175 range among the 5.7% most abundant human ORs[23,93] or 25% most abundant mouse ORs,[23] respectively. Moreover, the in-vitro determined EC_{50} values of pyrazines in this study overall correlated with their published human odor thresholds.

A cautionary note: odor thresholds, however, may vary by orders of magnitude, for example, 2-IP-3-MOP, with 0.004 μg/kg[94] versus 0.0004 μg/kg.[95] Moreover, we may have missed other pyrazine-responsive ORs or responsive genetic variants, (**i**) since our receptor screening experiments did include the 225 most frequent, but not all genetic human OR variants, and (**ii**) some receptors of our OR library may not work with the assay used in our study. Beyond ORs, other receptors have been reported to detect, for example, TMT, for example, bitter taste receptor Tas2r143,[96] or chemesthetic receptor Trpa1.[34]

In summary, here, we identified OR5K1 as a human KFO and semiochemical alkylpyrazine-selective receptor, with 18 pyrazines, as well as non-pyrazines TMT and MF as new agonists. OR5K1 is evolutionary conserved across mammalian orders, with an extended and functionally diversified OR5K subfamily in mouse.

ACKNOWLEDGMENTS

We thank Tim Frey, Sandra Hoffmann, and Julia Bauer for expert technical assistance. FH has been supported partially by a collaboration project with Altria Client Services Inc, Richmond, VA, USA. CG has been supported partially by a collaboration project with MARS Inc, McLean, VA, USA.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

D. Krautwurst and P. Marcinek designed the study. P. Marcinek, F. Haag, and C. Geithe conceptualized and performed screening and concentration-response experiments, and analyzed data. P. Marcinek performed phylogenetic and cluster analysis. P. Marcinek wrote the first draft. F. Haag and D. Krautwurst contributed substantially to revisions, and D. Krautwurst wrote the manuscript.

DATA AVAILABILITY STATEMENT

Data underlying the results have been deposited in Mendeley (https://doi.org/10.17632/7436r2hmjy.1).

ORCID

Patrick Marcinek https://orcid.org/0000-0003-4003-9308
Franziska Haag https://orcid.org/0000-0003-4210-0475
Christiane Geithe https://orcid.org/0000-0002-7302-8851
Dietmar Krautwurst https://orcid.org/0000-0002-3350-8682

REFERENCES

1. Muller R, Rappert S. Pyrazines: occurrence, formation and biodegradation. *Appl Microbiol Biotechnol*. 2010;85:1315-1320.
2. Kosuge T, Kamiya H. Discovery of a pyrazine in a natural product: tetramethylpyrazine from cultures of a strain of Bacillus subtilis. *Nature*. 1962;193:776.
3. Hellwig M, Henle T. Baking, ageing, diabetes: a short history of the Maillard reaction. *Angew Chem Int Ed Engl*. 2014;53:10316-10329.
4. Dunkel A, Steinhaus M, Kotthoff M, et al. Nature's chemical signatures in human olfaction: a foodborne perspective for future biotechnology. *Angew Chem Int Ed Engl*. 2014;53:7124-7143.
5. Wagner R, Czerny M, Bielohradsky J, Grosch W. Structure-odour-activity relationships of alkylpyrazines. *Z Lebensm Unters Forsch A*. 1999;208:308-316.
6. Henning C, Glomb MA. Pathways of the Maillard reaction under physiological conditions. *Glycoconj J*. 2016;33:499-512.
7. Bohman B, Phillips RD, Menz MH, et al. Discovery of pyrazines as pollinator sex pheromones and orchid semiochemicals: implications for the evolution of sexual deception. *New Phytol*. 2014;203:939-952.

8. Silva-Junior EA, Ruzzini AC, Paludo CR, et al. Pyrazines from bacteria and ants: convergent chemistry within an ecological niche. *Sci Rep*. 2018;8:2595.
9. Osada K, Kurihara K, Izumi H, Kashiwayanagi M. Pyrazine analogues are active components of wolf urine that induce avoidance and freezing behaviours in mice. *PLoS ONE*. 2013;8:e61753.
10. Osada K, Miyazono S, Kashiwayanagi M. The scent of wolves: pyrazine analogs induce avoidance and vigilance behaviors in prey. *Front Neurosci*. 2015;9:363.
11. Osada K, Miyazono S, Kashiwayanagi M. Structure-activity relationships of alkylpyrazine analogs and fear-associated behaviors in mice. *J Chem Ecol*. 2017;43:263-272.
12. Regnier FE. Semiochemical—structure and function. *Biol Reprod*. 1971;4:309-326.
13. Sbarbati A, Osculati F. Allelochemical communication in vertebrates: kairomones, allomones and synomones. *Cells Tissues Organs*. 2006;183:206-219.
14. Charpentier MJ, Boulet M, Drea CM. Smelling right: the scent of male lemurs advertises genetic quality and relatedness. *Mol Ecol*. 2008;17:3225-3233.
15. Nielsen BL, Rampin O, Meunier N, Bombail V. Behavioral responses to odors from other species: introducing a complementary model of allelochemics involving vertebrates. *Front Neurosci*. 2015;9:226.
16. Law JH, Regnier FE. Pheromones. *Annu Rev Biochem*. 1971;40:533-548.
17. Wyatt TD. Pheromones. *Curr Biol*. 2017;27:R739-R743.
18. Li YY, Lu YY, Lu M, Wei HY, Chen L. HPLC separation of 2-Ethyl-5(6)-methylpyrazine and its electroantennogram and alarm activities on fire ants (*Solenopsis invicta* Buren). *Molecules*. 2018;23:1661.
19. Pickett JA, Barasa S, Birkett MA. Vertebrate pheromones and other semiochemicals: the potential for accommodating complexity in signalling by volatile compounds for vertebrate management. *Biochem Soc Trans*. 2014;42:846-850.
20. Buck L, Axel R. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell*. 1991;65:175-187.
21. Olender T, Waszak SM, Viavant M, et al. Personal receptor repertoires: olfaction as a model. *BMC Genom*. 2012;13:414.
22. Niimura Y. Evolutionary dynamics of olfactory receptor genes in chordates: interaction between environments and genomic contents. *Hum Genomics*. 2009;4:107-118.
23. Saraiva LR, Riveros-McKay F, Mezzavilla M, et al. A transcriptomic atlas of mammalian olfactory mucosae reveals an evolutionary influence on food odor detection in humans. *Sci Adv*. 2019;5:eaax0396.
24. Verhulst NO, Mbadi PA, Kiss GB, et al. Improvement of a synthetic lure for Anopheles gambiae using compounds produced by human skin microbiota. *Malar J*. 2011;10:28.
25. Krautwurst D, Kotthoff M. A hit map-based statistical method to predict best ligands for orphan olfactory receptors: natural key odorants versus "lock picks". *Methods Mol Biol*. 2013;1003:85-97.
26. Laska M, Persson O, Hernandez Salazar LT. Olfactory sensitivity for alkylpyrazines-a comparative study in CD-1 mice and spider monkeys. *J Exp Zool A Ecol Genet Physiol*. 2009;311:278-288.
27. Llaneza L, Garcia EJ, Lopez-Bao JV. Intensity of territorial marking predicts wolf reproduction: implications for wolf monitoring. *PLoS ONE*. 2014;9:e93015.
28. Chamaille-Jammes S, Malcuit H, Le Saout S, Martin JL. Innate threat-sensitive foraging: black-tailed deer remain more fearful of wolf than of the less dangerous black bear even after 100 years of wolf absence. *Oecologia*. 2014;174:1151-1158.
29. Sahlen E, Noell S, DePerno CS, Kindberg J, Spong G, Cromsigt JP. Phantoms of the forest: legacy risk effects of a regionally extinct large carnivore. *Ecol Evol*. 2016;6:791-799.
30. Rosen JB, Asok A, Chakraborty T. The smell of fear: innate threat of 2,5-dihydro-2,4,5-trimethylthiazoline, a single molecule component of a predator odor. *Front Neurosci*. 2015;9:292.
31. Rosen JB, Pagani JH, Rolla KL, Davis C. Analysis of behavioral constraints and the neuroanatomy of fear to the predator odor trimethylthiazoline: a model for animal phobias. *Neurosci Biobehav Rev*. 2008;32:1267-1276.
32. Ayers LW, Asok A, Heyward FD, Rosen JB. Freezing to the predator odor 2,4,5 dihydro 2,5 trimethylthiazoline (TMT) is disrupted by olfactory bulb removal but not trigeminal deafferentation. *Behav Brain Res*. 2013;253:54-59.
33. Saito H, Nishizumi H, Suzuki S, et al. Immobility responses are induced by photoactivation of single glomerular species responsive to fox odour TMT. *Nat Commun*. 2017;8:16011.
34. Wang Y, Cao L, Lee C-Y, et al. Large-scale forward genetics screening identifies Trpa1 as a chemosensor for predator odor-evoked innate fear behaviors. *Nat Commun*. 2018;9:2041.
35. Noe F, Geithe C, Fiedler J, Krautwurst D. A bi-functional IL-6-HaloTag((R)) as a tool to measure the cell-surface expression of recombinant odorant receptors and to facilitate their activity quantification. *J Biol Methods*. 2017;4:e82.
36. Binkowski B, Fan F, Wood K. Engineered luciferases for molecular sensing in living cells. *Curr Opin Biotechnol*. 2009;20:14-18.
37. Geithe C, Andersen G, Malki A, Krautwurst D. A butter aroma recombinate activates human class-I odorant receptors. *J Agric Food Chem*. 2015;63:9410-9420.
38. Geithe C, Noe F, Kreissl J, Krautwurst D. The broadly tuned odorant receptor OR1A1 is highly selective for 3-Methyl-2,4-nonanedione, a key food odorant in aged wines, tea, and other foods. *Chem Senses*. 2017;42:181-193.
39. Noe F, Polster J, Geithe C, Kotthoff M, Schieberle P, Krautwurst D. OR2M3: a highly specific and narrowly tuned human odorant receptor for the sensitive detection of onion key food odorant 3-mercapto-2-methylpentan-1-ol. *Chem Senses*. 2017;42:195-210.
40. Kriventseva EV, Kuznetsov D, Tegenfeldt F, et al. OrthoDB v10: sampling the diversity of animal, plant, fungal, protist, bacterial and viral genomes for evolutionary and functional annotations of orthologs. *Nucleic Acids Res*. 2019;47:D807-D811.
41. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. *Comput Appl Biosci*. 1992;8:275-282.
42. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*. 1985;39:783-791.
43. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol*. 2018;35:1547-1549.
44. Backman TW, Cao Y, Girke T. ChemMine tools: an online service for analyzing and clustering small molecules. *Nucleic Acids Res*. 2011;39:W486-W491.
45. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol*. 1977;36:59-74.

46. Saito H, Kubota M, Roberts RW, Chi Q, Matsunami H. RTP family members induce functional expression of mammalian odorant receptors. *Cell*. 2004;119:679-691.
47. Shirokova E, Schmiedeberg K, Bedner P, et al. Identification of specific ligands for orphan olfactory receptors. G protein-dependent agonism and antagonism of odorants. *J Biol Chem*. 2005;280:11807-11815.
48. Jones DT, Reed RR. Golf: an olfactory neuron specific-G protein involved in odorant signal transduction. *Science*. 1989;244:790-795.
49. Li F, Ponissery-Saidu S, Yee KK, et al. Heterotrimeric G protein subunit gamma13 is critical to olfaction. *J Neurosci*. 2013;33:7975-7984.
50. Noe F, Frey T, Fiedler J, Geithe C, Nowak B, Krautwurst D. IL-6-HaloTag((R)) enables live-cell plasma membrane staining, flow cytometry, functional expression, and de-orphaning of recombinant odorant receptors. *J Biol Methods*. 2017;4:e81.
51. Geithe C, Protze J, Kreuchwig F, Krause G, Krautwurst D. Structural determinants of a conserved enantiomer-selective carvone binding pocket in the human odorant receptor OR1A1. *Cell Mol Life Sci*. 2017;74:4209-4229.
52. Kreissl J, Mall V, Steinhaus P, Steinhaus M. Leibniz-LSB@TUM Odorant Database, Version 1.0.; 2019.
53. DeLean A, Munson PJ, Rodbard D. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol*. 1978;235:E97-E102.
54. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag; 2016.
55. Buttery RG, Orts WJ, Takeoka GR, Nam Y. Volatile flavor components of rice cakes. *J Agric Food Chem*. 1999;47:4353-4356.
56. Guan D, Lu YY, Liao XL, Wang L, Chen L. Electroantennogram and behavioral responses of the imported fire ant, *Solenopsis invicta* Buren, to an alarm pheromone component and its analogues. *J Agric Food Chem*. 2014;62:11924-11932.
57. Seyfried C, Granvogl M. Characterization of the key aroma compounds in two commercial dark chocolates with high cocoa contents by means of the sensomics approach. *J Agric Food Chem*. 2019;67:5827-5837.
58. Wagner J, Schieberle P, Granvogl M. Characterization of the key aroma compounds in heat-processed licorice (succus liquiritiae) by means of molecular sensory science. *J Agric Food Chem*. 2017;65:132-138.
59. Wagner J, Granvogl M, Schieberle P. Characterization of the key aroma compounds in raw licorice (*Glycyrrhiza glabra* L.) by means of molecular sensory science. *J Agric Food Chem*. 2016;64:8388-8396.
60. Adipietro KA, Mainland JD, Matsunami H. Functional evolution of mammalian odorant receptors. *PLoS Genet*. 2012;8:e1002821.
61. Godfrey PA, Malnic B, Buck LB. The mouse olfactory receptor gene family. *Proc Natl Acad Sci U S A*. 2004;101:2156-2161.
62. Jiang Y, Matsunami H. Mammalian odorant receptors: functional evolution and variation. *Curr Opin Neurobiol*. 2015;34:54-60.
63. Wagner R, Czerny M, Bielohradsky J, Grosch W. Structure odour-activity relationships of alkylpyrazines. *Z Lebensm Unters Forsch A*. 1999;208:308-316.
64. Rouquier S, Blancher A, Giorgi D. The olfactory receptor gene repertoire in primates and mouse: evidence for reduction of the functional fraction in primates. *Proc Natl Acad Sci U S A*. 2000;97:2870-2874.
65. Matsui A, Go Y, Niimura Y. Degeneration of olfactory receptor gene repertories in primates: no direct link to full trichromatic vision. *Mol Biol Evol*. 2010;27:1192-1200.
66. Li J, Haddad R, Santos V, Bavan S, Luetje CW. Receptive range analysis of a mouse odorant receptor subfamily. *J Neurochem*. 2015;134(1):47-55.
67. Bautze V, Bär R, Fissler B, et al. Mammalian-specific OR37 receptors are differentially activated by distinct odorous fatty aldehydes. *Chem Senses*. 2012;37:479-493.
68. Kobayakawa KO, Kobayakawa R, Matsumoto H, et al. Innate versus learned odour processing in the mouse olfactory bulb. *Nature*. 2007;450:503-508.
69. Anson JR, Dickman CR. Behavioral responses of native prey to disparate predators: naiveté and predator recognition. *Oecologia*. 2013;171:367-377.
70. Lindström L, Rowe C, Guilford T. Pyrazine odour makes visually conspicuous prey aversive. *Proc Biol Sci*. 2001;268:159-162.
71. Horio N, Murata K, Yoshikawa K, Yoshihara Y, Touhara K. Contribution of individual olfactory receptors to odor-induced attractive or aversive behavior in mice. *Nat Commun*. 2019;10:209.
72. Apfelbach R, Parsons MH, Soini HA, Novotny MV. Are single odorous components of a predator sufficient to elicit defensive behaviors in prey species? *Front Neurosci*. 2015;9:263.
73. Rampin O, Jerôme N, Saint-Albin A, et al. Where is the TMT? GC-MS analyses of fox feces and behavioral responses of rats to fear-inducing odors. *Chem Senses*. 2018;43:105-115.
74. Sievert T, Laska M. Behavioral responses of CD-1 mice to six predator odor components. *Chem Senses*. 2016;41:399-406.
75. Saraiva LR, Kondoh K, Ye X, Yoon KH, Hernandez M, Buck LB. Combinatorial effects of odorants on mouse behavior. *Proc Natl Acad Sci U S A*. 2016;113:E3300-E3306.
76. Dewan A, Cichy A, Zhang J, et al. Single olfactory receptors set odor detection thresholds. *Nat Commun*. 2018;9:2887.
77. Grosch W. Evaluation of the key odorants of foods by dilution experiments, aroma models and omission. *Chem Senses*. 2001;26:533-545.
78. Schieberle P, Hofmann T. Mapping the combinatorial code of food flavors by means of molecular sensory science approach. In: Jelen H, ed. *Food Flavors—Chemical & Functional Properties of Food Components*. Boca Raton: Taylor & Francis Group; 2011:413-438.
79. Gardiner A, Barker D, Butlin RK, Jordan WC, Ritchie MG. Drosophila chemoreceptor gene evolution: selection, specialization and genome size. *Mol Ecol*. 2008;17:1648-1657.
80. Schweizer RM, Robinson J, Harrigan R, et al. Targeted capture and resequencing of 1040 genes reveal environmentally driven functional variation in grey wolves. *Mol Ecol*. 2016;25:357-379.
81. Paudel Y, Madsen O, Megens HJ, et al. Copy number variation in the speciation of pigs: a possible prominent role for olfactory receptors. *BMC Genom*. 2015;16:330.
82. Springer MS, Stanhope MJ, Madsen O, de Jong WW. Molecules consolidate the placental mammal tree. *Trends Ecol Evol*. 2004;19:430-438.
83. Larson G, Fuller DQ. The evolution of animal domestication. *Annu Rev Ecol Evol Syst*. 2014;45:115-136.
84. Sullivan AP, Bird DW, Perry GH. Human behaviour as a long-term ecological driver of non-human evolution. *Nat Ecol Evol*. 2017;1:65.
85. Hunter P. The genetics of domestication: research into the domestication of livestock and companion animals sheds light both on their "evolution" and human history. *EMBO Rep*. 2018;19:201-205.

86. Inlamea OF, Soares P, Ikuta CY, et al. Evolutionary analysis of *Mycobacterium bovis* genotypes across Africa suggests co-evolution with livestock and humans. *PLoS Negl Trop Dis*. 2020;14:e0008081.
87. Schleidt WM, Shalter MD. Co-evolution of humans and canids. *Evol Cogn*. 2003;9:57-72.
88. Alberto FJ, Boyer F, Orozco-terWengel P, et al. Convergent genomic signatures of domestication in sheep and goats. *Nat Commun*. 2018;9:813.
89. Zhang K, Lenstra JA, Zhang S, Liu W, Liu J. Evolution and domestication of the Bovini species. *Anim Genet*. 2020;51:637-657.
90. Son S, Oh JD, Lee SH, Shin D, Kim Y. Comparative genomics of canine *Lactobacillus reuteri* reveals adaptation to a shared environment with humans. *Genes Genomics*. 2020;42:1107-1116.
91. Man O, Gilad Y, Lancet D. Prediction of the odorant binding site of olfactory receptor proteins by human-mouse comparisons. *Protein Sci*. 2004;13:240-254.
92. Man O, Willhite DC, Crasto CJ, Shepherd GM, Gilad Y. A framework for exploring functional variability in olfactory receptor genes. *PLoS ONE*. 2007;2:e682.
93. Verbeurgt C, Wilkin F, Tarabichi M, Gregoire F, Dumont JE, Chatelain P. Profiling of olfactory receptor gene expression in whole human olfactory mucosa. *PLoS ONE*. 2014;9:e96333.
94. Masanetz C, Grosch W. Key odorants of parsley leaves (*Petroselinum crispum* [Mill.] Nym. ssp. *crispum*) by Odour–activity values. *Flavour Frag J*. 1998;13:115-124.
95. Neta ER, Miracle RE, Sanders TH, Drake MA. Characterization of alkylmethoxypyrazines contributing to earthy/bell pepper flavor in farmstead cheddar cheese. *J Food Sci*. 2008;73:C632-C638.
96. Moine F, Brechbuhl J, Nenniger Tosato M, Beaumann M, Broillet MC. Alarm pheromone and kairomone detection via bitter taste receptors in the mouse Grueneberg ganglion. *BMC Biol*. 2018;16:12.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Marcinek P, Haag F, Geithe C, Krautwurst D. An evolutionary conserved olfactory receptor for foodborne and semiochemical alkylpyrazines. *The FASEB Journal*. 2021;35:e21638. https://doi.org/10.1096/fj.202100224R