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Structural and Functional Analysis of RIC8B Variants Regulating Olfactory Receptor Responses

嗅覚受容体応答を調節する RIC8B バリエーションの構造・機能解析

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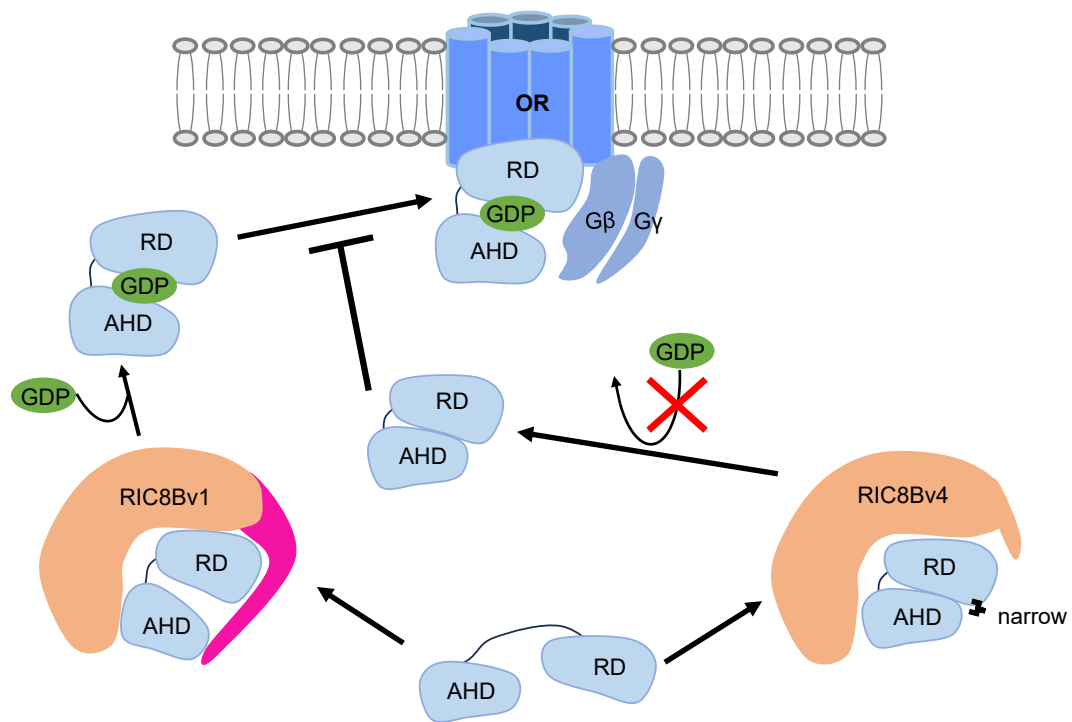
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Abstract

Resistance to inhibitors of cholinesterase 8B (RIC8B) functions as a chaperone and guanine nucleotide exchange factor (GEF) for G α s/olf. In this study, I focused on RIC8B variant 1 (v1), which is abundantly expressed at the mRNA level, and variant 4 (v4), which lacks the C-terminal cradle Loop Helix (CLH) domain. Together with three closely related variants (v2, v3, and v10), I evaluated a total of five variants for their chaperone activity. HEK293T cells were co-expressed with olfactory receptors (ORs; OR51E1, OR51E2, OR1A1), and cAMP production in response to odorants was used as an indicator. As a result, only v4 consistently and significantly suppressed the cAMP responses. Complex structure prediction using AlphaFold3 revealed that v1 forms multiple hydrogen bonds with G α s through its CLH domain, whereas v4, lacking CLH, failed to establish these interactions. These findings suggest that v4 may be unable to stably adopt the proper binding conformation with G α s, resulting in reduced chaperone activity. Consequently, improperly folded G α s may exert a dominant-negative-like effect, thereby suppressing OR responses.

Keywords: olfactory receptors, RIC8B, cAMP, chaperone, G protein



Graphical abstract caption

RIC8Bv1, containing the CLH domain (magenta), functions as a chaperone, enabling properly folded Gas to associate with ORs and transmit signals. In contrast, RIC8Bv4 lacks the CLH domain, causing the AHD and RD of Gas to approach each other, thereby preventing GDP (green) binding and suppressing cAMP production.

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General Introduction

Olfaction as a Fundamental Chemosensory System

Olfaction is a fundamental sensory modality that enables organisms to detect volatile chemical compounds in the external environment and to rapidly evaluate information that is beneficial or harmful for survival. Through this capacity, olfaction plays indispensable roles in essential biological behaviors such as feeding, avoidance of danger, and reproduction. Despite its central importance, however, the molecular mechanisms underlying olfactory perception remained elusive for much of the twentieth century, particularly when compared with other sensory systems.

Until the latter half of the twentieth century, studies of olfaction relied primarily on electrophysiological and behavioral approaches. Although it was well established that odorant stimulation is converted into electrical signals within the olfactory epithelium, the molecular identity of the receptors responsible for odor detection—the molecules that directly receive odorants—remained unknown (Ottoson, 1956; Getchell, 1973; Firestein, 2001). In contrast to vision, in which rhodopsin had been identified, or taste, in which taste receptors had been characterized, the olfactory reception mechanism was regarded as a “black box.” Consequently, the question of how organisms discriminate an enormous diversity of odorants persisted as one of the long-standing unresolved problems in neuroscience (Baylor, 1996; Firestein, 2001; Buck, 2004).

Discovery of Olfactory Receptors and the GPCR Paradigm

A decisive breakthrough in olfactory research occurred in 1991, when Buck and Axel cloned genes from a rat olfactory epithelium–derived cDNA library and demonstrated that olfactory receptors (ORs) constitute a vast multigene family of seven-transmembrane G protein–coupled receptors (GPCRs) (Buck and Axel, 1991). This discovery fundamentally transformed the field of olfaction. By identifying a large and diverse set of GPCR genes expressed specifically in the olfactory epithelium, they established that olfactory perception is not mediated by a single receptor, but rather by a molecular recognition system based on numerous receptors acting in concert.

This conceptual shift firmly positioned olfaction within the framework of GPCR-mediated chemosensation. In recognition of this contribution, Buck and Axel were awarded the Nobel Prize in Physiology or Medicine in 2004 (Buck, 2004; Axel, 2004). Their work provided the molecular foundation for understanding how chemical information from the environment is translated into neural signals.

Diversity and Evolution of the Olfactory Receptor Gene Family

OR genes form one of the largest gene families in mammals. In humans, approximately 400 functional OR genes coexist with a large number of pseudogenes, whereas in mice, more than 1,000

OR genes have been reported, highlighting substantial interspecies differences (Zhang and Firestein, 2002). This remarkable diversity is thought to reflect differences in ecological niches and survival strategies, and ORs have therefore attracted attention as indicators of evolutionary adaptation (Niimura and Nei, 2005; Nei et al., 2008).

Thus, the OR gene family represents not merely a collection of sensory receptors, but a molecular repertoire that has been shaped by strong evolutionary pressure to enable organisms to acquire and interpret environmental chemical information efficiently.

Organizational Principles of Olfactory Signal Processing

In the late 1990s, fundamental principles governing OR gene expression and neural wiring were elucidated. Olfactory sensory neurons (OSNs) were shown to obey the “one neuron–one receptor” rule, whereby each neuron expresses only a single OR gene in a mutually exclusive manner (Chess et al., 1994). Furthermore, OSNs expressing the same OR were found to project their axons to the same glomerulus in the olfactory bulb (Mombaerts et al., 1996). These findings established the organizational principle of olfactory information processing described as “one neuron–one receptor–one glomerulus” (Mombaerts, 2006).

This architecture demonstrates that olfactory information is represented in the central nervous system as a spatial map, in which the chemical features of odorants are translated into distinct patterns of neural activity (Feinstein and Mombaerts, 2004).

Molecular Basis of Olfactory Signal Transduction

ORs function as class A GPCRs that activate heterotrimeric G proteins upon odorant binding. In the olfactory system, G α olf serves as the principal G α subunit and stimulates adenylyl cyclase III (ACIII), resulting in an increase in intracellular cyclic AMP (cAMP) levels (Jones and Reed, 1989; Bakalyar and Reed, 1990; Buck and Axel, 1991). The elevation of cAMP opens cyclic nucleotide-gated (CNG) channels, leading to Ca²⁺ influx and subsequent depolarization of olfactory sensory neurons (Nakamura and Gold, 1987; Firestein et al., 1991).

This cAMP-dependent signaling cascade is now established as the molecular basis of olfactory reception and serves as a canonical example of GPCR-mediated sensory transduction (Firestein, 2001).

Combinatorial Coding and Functions Beyond Olfaction

Subsequent studies demonstrated that odor identity is encoded not by individual receptors but by combinatorial patterns of activation across multiple ORs, a concept known as combinatorial coding (Malnic et al., 1999). This framework provides a theoretical explanation for how a limited number of receptors can discriminate an immense variety of odorants.

More recently, ORs have been detected in numerous non-olfactory tissues, where they function as chemical sensors involved in diverse physiological processes. These findings have prompted a redefinition of ORs as broadly acting chemosensory receptors whose roles extend beyond classical olfaction (Spehr and Munger, 2009; Maßberg and Hatt, 2018).

Challenges in Functional Analysis of Olfactory Receptors

Despite forming the largest GPCR family, ORs have historically lagged behind other GPCRs in terms of functional characterization (Buck, 2000; Firestein, 2001). Comprehensive functional analysis requires systematic identification of ligand–receptor pairs, yet studies using native olfactory sensory neurons are constrained by technical and experimental limitations (Malnic et al., 1999). As a result, heterologous expression systems have been widely employed.

However, ORs are characterized by high hydrophobicity and structural instability, making their efficient trafficking to the cell surface extremely difficult in heterologous systems such as HEK293 cells (Lu et al., 2003; McClintock and Sammeta, 2003). Overcoming these limitations has therefore been a central challenge in OR research.

RIC8 Family Proteins as Key Regulators of Gas/olf Maturation

The functional expression of ORs in heterologous systems became feasible in the 2000s with the identification of accessory proteins and signal peptides (Krautwurst et al., 1998; Saito et al., 2004). OR signaling proceeds through Gas/olf (Jones and Reed, 1989; Belluscio et al., 1998), and proper folding and maturation of Gα proteins are indispensable for effective signal transduction.

Accordingly, the maturation and quality-control mechanisms governing Gα proteins represent critical regulatory checkpoints in OR signaling (Tall et al., 2003; Von Dannecker et al., 2006).

Among the factors involved in Gas/olf maturation, the Resistance to inhibitors of cholinesterase 8 (RIC8) family has emerged as a central regulator. Originally identified in *Caenorhabditis elegans* as an intracellular signaling regulator (Miller et al., 1996; Miller et al., 1999), RIC8 exists in mammals as two isoforms, RIC8A and RIC8B (Tall et al., 2003). RIC8A primarily acts on Gαi/o/q in the nervous system and during development, whereas RIC8B is expressed in multiple tissues, including the olfactory system, and selectively targets Gas/olf (Von Dannecker et al., 2006; Kerr et al., 2008). Beyond their role as guanine nucleotide exchange factors (GEFs), RIC8 proteins function as molecular chaperones that regulate the folding and stabilization of nascent Gα subunits (Chan et al., 2013; Papasergi et al., 2015; Papasergi-Scott et al., 2023).

Structural Prediction and the Significance of RIC8B Splice Variants

Recent advances in structural biology have substantially deepened our understanding of the three-dimensional structure of RIC8B and its interaction with Gas/olf (Chan et al., 2013; Papasergi et al.,

2015; Papasergi-Scott et al., 2023). Nevertheless, human RIC8B is known to exist in multiple splice variants (Von Dannecker et al., 2006; Kerr et al., 2008), the functional significance of which remains incompletely understood. In particular, systematic comparisons between variants highly expressed in neural tissues and closely related variants lacking key structural features have been largely absent. The possibility that alternative splicing–induced structural diversity qualitatively regulates signal transduction represents an important and largely unexplored issue in GPCR research (Markovic and Challiss, 2009; Einstein and Jordan, 2014).

Concurrently, protein structure analysis has been transformed by advances in artificial intelligence–based prediction methods. The introduction of AlphaFold2 revolutionized single-protein structure prediction (Jumper et al., 2021), and the subsequent development of AlphaFold3 (AF3) in 2024 extended these capabilities to macromolecular complexes involving protein–protein interactions, nucleic acids, and small-molecule ligands (Abramson et al., 2024). These computational approaches complement experimental techniques by enabling analysis of transient interactions and flexible structural regions that are otherwise difficult to characterize. For dynamic molecular systems such as G proteins and their accessory factors, integrating structure prediction with functional analysis has become increasingly essential for elucidating signal transduction mechanisms.

Aim of This Doctoral Thesis

In this doctoral thesis, I aim to elucidate how structural features of RIC8B splice variants, as revealed by AF3-based structure prediction, contribute to the regulation of odorant-induced olfactory receptor responses mediated by G α s/olf. By integrating functional assays with computational structural analysis, this work seeks to deepen our understanding of olfactory signal transduction and to provide comprehensive insights into the roles of accessory factors in GPCR signaling.

Introduction

Olfactory receptors (ORs) are seven-transmembrane G protein–coupled receptors (GPCRs) expressed on the cilia of human olfactory sensory neurons (OSNs), where they play a central role in odorant recognition (Lankford et al., 2020). The human genome contains 388 OR genes, which account for approximately half of all functional GPCRs (Niimura and Nei, 2003; 2005). In OSNs, a single OR species is expressed per cell in a mutually exclusive manner (Pourmorady et al., 2023), and each OR can be activated by multiple odorant molecules. Thus, odor discrimination is thought to be achieved through a combinatorial pattern recognition based on the activation of multiple ORs (Malnic et al., 1999).

When odorants bind to ORs, heterotrimeric G proteins composed of G_{α} /olf, G_{β} , and G_{γ} are activated. GDP-bound G_{α} /olf is converted to its GTP-bound form, which stimulates adenylyl cyclase (AC), thereby inducing cAMP production. The resulting cAMP opens cyclic nucleotide–gated (CNG) ion channels, leading to Na^+ and Ca^{2+} influx and subsequent membrane depolarization (Buck, 2004). Accordingly, the degree of OR activation is frequently quantified by measuring cAMP production or the associated cellular responses (Nakamura and Gold, 1987) (**Figure 1**).

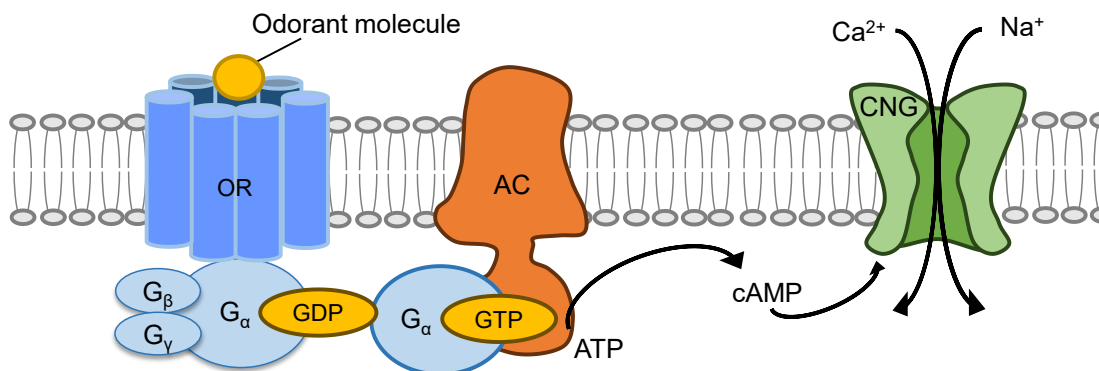


Figure 1. Schematic representation of G_{α} -cAMP signaling mediated by olfactory receptors.

Olfactory receptors (ORs) are seven-transmembrane G protein–coupled receptors (GPCRs) that detect odorant molecules. Upon odorant binding, heterotrimeric G proteins are activated, leading to the dissociation of the G_{α} subunit. Activated G_{α} stimulates adenylyl cyclase (AC), resulting in increased intracellular cyclic AMP (cAMP) production. Elevated cAMP levels open cyclic nucleotide–gated (CNG) channels, allowing cation influx and ultimately causing membrane depolarization of olfactory sensory neurons.

ORs are generally classified into class I, which respond to hydrophilic odorants, and class II, which respond to hydrophobic odorants. Among class I ORs, OR51E1 and OR51E2 are highly homologous (approximately 60%) and are responsive to small molecules such as fatty acids (Billesbølle et al.,

2023). Notably, these receptors have been reported to be ectopically expressed in non-neuronal tissues such as prostate cancer, suggesting possible functions beyond olfaction (Pronin and Slepak, 2021).

Heterologous expression systems, such as those using HEK293T cells, have been widely employed to study odorant responses of human ORs (hORs). However, hORs alone are inefficiently trafficked to the cell surface, and only about 30% of all hORs exhibit detectable responses (Zhuang and Matsunami, 2007). To overcome this limitation, several strategies have been developed, including the addition of secretion signal peptides (Rho tag, Lucy tag) to the N-terminus of hORs (Saito et al., 2004; Shepard et al., 2013), co-expression with OR accessory proteins (ORAPs) such as the OR chaperone RTP1S (Wu et al., 2012), the OR-specific G protein *G_{as}/olf* (Jones et al., 1989), and the *G_α* chaperone Resistance to inhibitors of cholinesterase 8B (RIC8B) (Dannecker et al., 2005; 2006). RIC8 was originally identified as an intracellular signaling factor through the analysis of an aldicarb-resistant mutant in *Caenorhabditis elegans* (Miller et al., 1996; Miller et al., 1999). In mammals, two isoforms, RIC8A and RIC8B, have been characterized. RIC8A is expressed in the nervous system, embryonic tissues, and cortex, where it specifically acts on *G_{ai/o/q}* and is essential for neuronal development, cell polarity, and asymmetric division (Ruisu et al., 2013). In contrast, RIC8B is expressed in the olfactory bulb, heart, lung, and brain, where it acts selectively on *G_{as}/olf* and is required for the development and function of the olfactory system. Both isoforms exhibit guanine nucleotide exchange factor (GEF) activity (Chan et al., 2011; Hinrichs et al., 2012) as well as chaperone activity for *G_α* proteins (Chan et al., 2013; Papasergi et al., 2015; Papasergi-Scott et al., 2023).

Structurally, RIC8B adopts a crescent-shaped superhelical structure composed of five Armadillo (ARM) repeats and four HEAT (Huntingtin, elongation factor 3, PR65/A, TOR) repeats, with a cradle Loop Helix (CLH) domain and a flexible C-terminal tail (PDB: 8EL7, 8EL8) (Papasergi-Scott et al., 2023) (**Figure 2**). The CLH interacts with the $\alpha 5$ helix of *G_{as}/olf* and is considered critical for functional complex formation. In mice, deletion of exon 9, which encodes the CLH, greatly reduces the interaction between RIC8B and *G_{as}/olf* (Dannecker et al., 2005; Papasergi-Scott et al., 2023). Multiple splice variants of human RIC8B also exist (GTEx (Genotype-Tissue Expression; The GTEx Consortium, 2020)), yet functional differences remain poorly understood, particularly between RIC8B variant 1 (RIC8Bv1), which is highly expressed in neural tissues, and RIC8B variant 4 (RIC8Bv4), which lacks the CLH domain (**Figure 3**). In this study, I investigated the effects of these variants on odorant-induced responses of ORs.

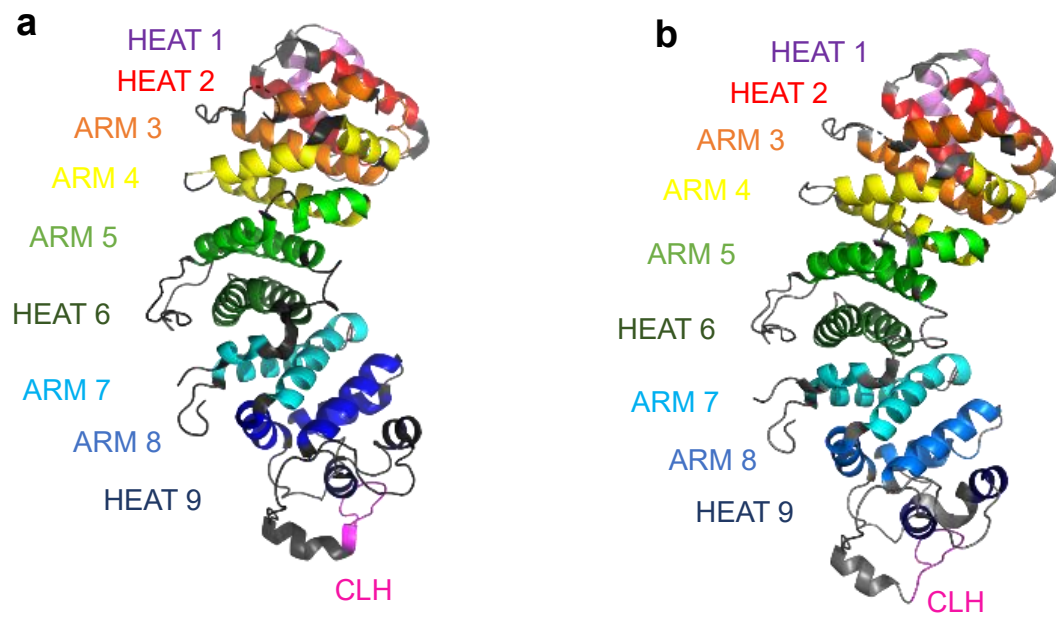


Figure 2. Structure and splice variants of RIC8B.

(a) Domain-colored representation of RIC8B (PDB ID: 8EL7). (b) Domain-colored representation of RIC8B (PDB ID: 8EL8). RIC8B forms a crescent-shaped superhelical structure composed of five ARM repeats and four HEAT repeats, with a C-terminal CLH domain and a flexible tail region.

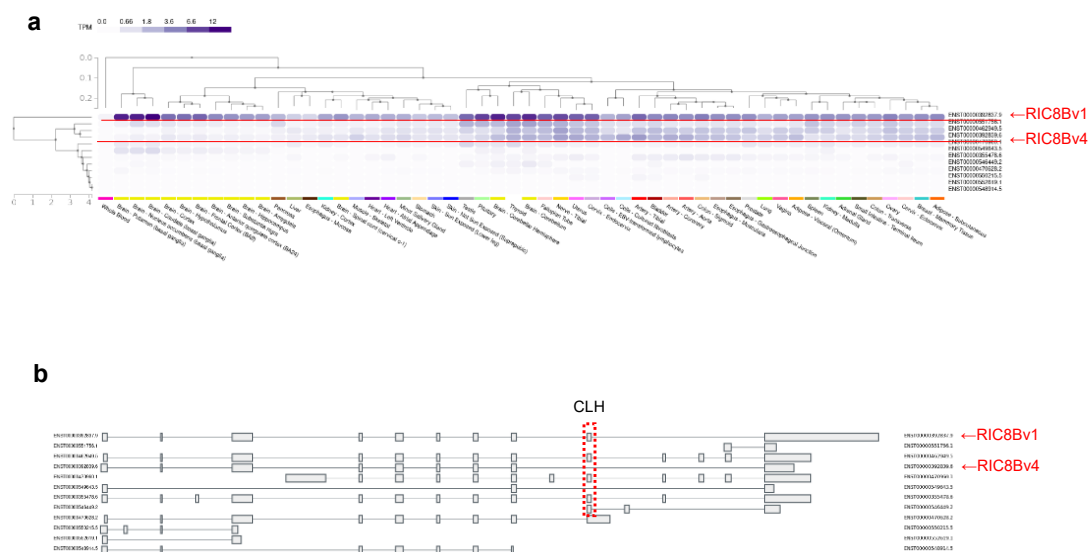


Figure 3. Expression of RIC8B splice variants in human tissues.

Multiple splice variants of human RIC8B are expressed across diverse tissues, as shown by GTEx (Genotype-Tissue Expression; The GTEx Consortium, 2020).

(a) Tissue-wide expression levels of RIC8B splice variants. Higher expression levels are indicated by darker purple shading.

(b) Exon structures of expressed RIC8B splice variants, arranged from top to bottom in order of decreasing expression abundance.

Notably, the functional differences between RIC8B variant 1 (RIC8Bv1), which is highly expressed in neural tissues, and RIC8B variant 4 (RIC8Bv4), which lacks the cradle Loop Helix (CLH) domain, remain largely unexplored.

Materials and Methods

Cell culture

Human embryonic kidney–derived HEK293T cells were obtained from RIKEN BRC (Tsukuba, Ibaraki, Japan) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂ (Fujita et al., 2019). Cells were routinely passaged every 2–3 days, and experiments were performed using cells with a low passage number. To improve transfection efficiency, cells were split at a 1:2 ratio on the day prior to transfection and used for experiments on the following day.

Plasmids

Expression plasmids for OR51E1 (NCBI Accession No.: NM_152430.4), OR51E2 (NM_030774.3), and OR1A1 (NM_014565.3) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Synthetic DNA fragments encoding each OR with tandem Lucy and Rho tags fused to the N-terminus were cloned into the pcDNA5 vector under the control of the CMV promoter.

Synthetic DNA constructs encoding five splice variants of RIC8B (RIC8Bv1, NM_001330145.2; v2, NM_001330146.2; v3, NM_001330147.2; v4, NM_018157.4; v10, NM_001351367.2) were obtained from GeneScript (Piscataway, NJ, USA) (**Figure 4**) and inserted into the pcDNA3.1(+) vector downstream of the CMV promoter. RIC8Bv1 del-Loop is a deletion mutant generated by removing residues N484–E505 from RIC8Bv1. RIC8Bv1 del-Helix is a deletion mutant lacking residues E506–S523 of RIC8Bv1. Expression plasmids for RIC8Bv1 del-Loop, RIC8Bv1 del-Helix, and RIC8Bv1 R75E were constructed by GenScript based on the RIC8Bv1 expression plasmid. The expression plasmid for RIC8Bv4 R75E was generated using the RIC8Bv4 expression plasmid as a template by the QuikChange site-directed mutagenesis method. The Lucy-Rho-OR51E1-GFP and Lucy-Rho-OR1A1-GFP expression plasmids were generated by inserting the GFP gene (PV365804.1) at the C-terminus of the ORs, based on the Lucy-Rho-OR51E1 and Lucy-Rho-OR1A1 expression plasmids, respectively. The pGloSensor-22F plasmid (Cat# E2301; Promega, Madison, WI, USA) was used as a cAMP biosensor, and a GFP expression plasmid was prepared as described previously (Fujita et al., 2021). The empty vector (pBApo-CMV Neo, Cat# 3240) was obtained from Takara Bio (Kusatsu, Shiga, Japan).

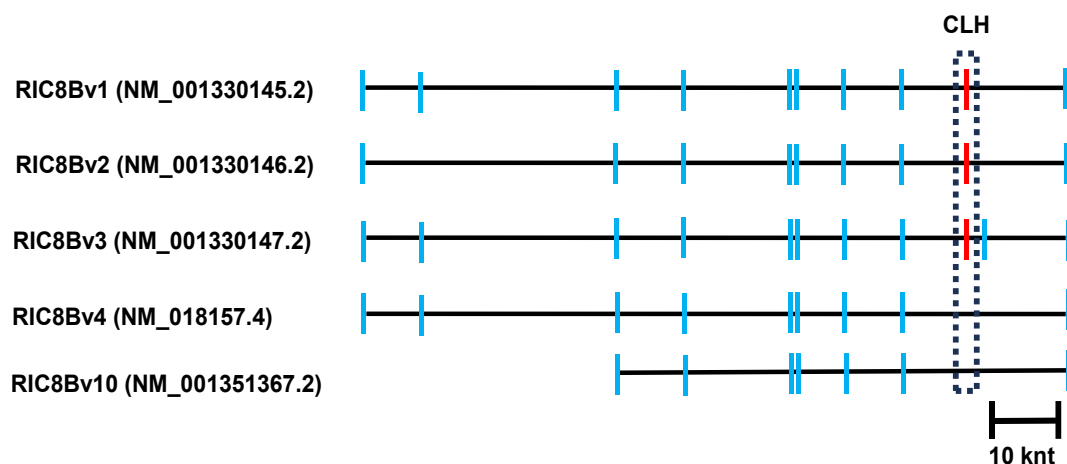


Figure 4. RIC8B splice variants used in this study.

Exon structures of the RIC8B splice variants analyzed in this study. The cradle Loop Helix (CLH) domain is indicated by a red line enclosed in a dashed box. Relative to RIC8B variant 1 (RIC8Bv1), RIC8B variant 4 (RIC8Bv4) lacks the exon encoding the CLH domain, RIC8B variant 2 (RIC8Bv2) lacks an N-terminal exon, RIC8B variant 3 (RIC8Bv3) contains an additional C-terminal exon, and RIC8B variant 10 (RIC8Bv10) lacks two N-terminal exons relative to RIC8Bv4.

Reagents

Isovaleric acid (Cat# M0182; TCI Chemicals, Tokyo, Japan), propionic acid (Cat# 194-03012; Fujifilm-Wako, Osaka, Japan), and R-(–)-carvone (Cat# 035-10921; Fujifilm-Wako) were dissolved in HBSS(+) buffer (phenol red-free, Cat# 084-08965; Fujifilm-Wako) and diluted as required. 3-Isobutyl-1-methylxanthine (IBMX, Cat# I5879; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (Cat# 046-21981; Fujifilm-Wako) and further diluted with HBSS(+) buffer prior to use.

cAMP assay

Intracellular cAMP levels were measured using the GloSensor cAMP assay system (Promega). This system employs a genetically modified firefly luciferase whose luminescence intensity changes in response to cAMP binding, enabling real-time monitoring of intracellular cAMP levels. In the present study, the GloSensor 22F plasmid was used (**Figure 5**).

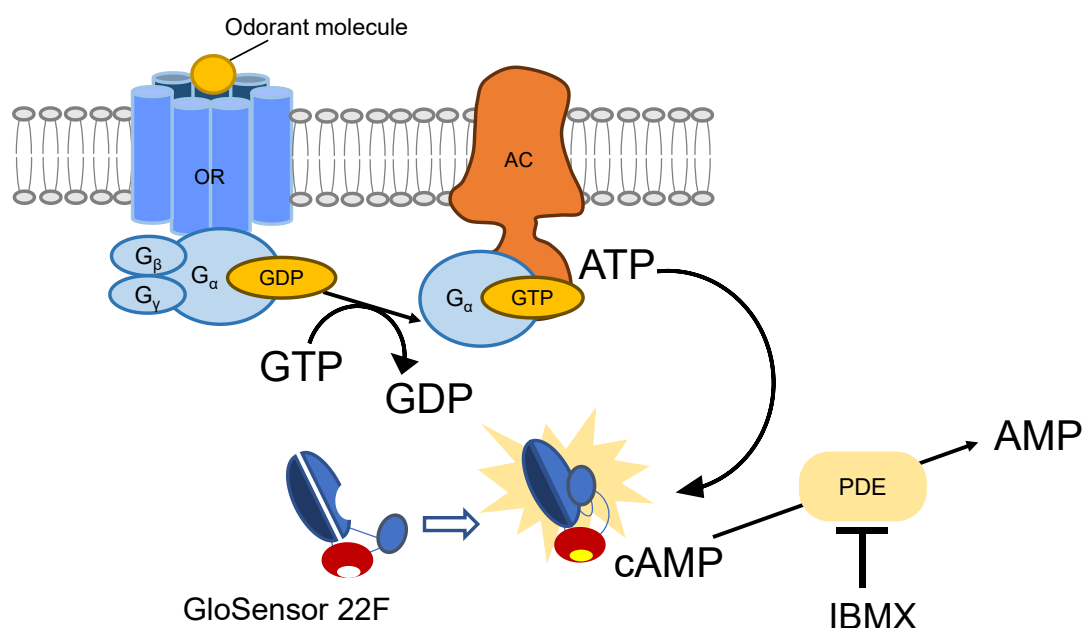


Figure 5. Principle of the GloSensor cAMP assay system.

Stimulation of olfactory receptors (ORs) by odorant molecules activates heterotrimeric G proteins through GDP–GTP exchange on the G_α subunit. Activated G_α stimulates adenylyl cyclase (AC), resulting in increased intracellular cyclic AMP (cAMP) production. The generated cAMP binds to GloSensor 22F, a genetically modified firefly luciferase containing a cAMP-binding domain, and induces a conformational change that enhances luminescence. In this assay, intracellular cAMP levels were quantitatively evaluated based on changes in luminescence intensity.

Poly-L-lysine-coated 96-well white plates were prepared by adding 50 μ L of poly-L-lysine (Cat# 236105; Thermo Fisher Scientific) to each well of 96-well white plates (Cat# VBH-SPL01; Cosmo Bio) and incubating for 1 h at room temperature, followed by washing with 200 μ L of phosphate-buffered saline (PBS). HEK293T cells ($\sim 4 \times 10^5$ cells/mL) were seeded at 50 μ L per well onto the coated plates.

For transfection, plasmid DNA mixtures containing GloSensor (50 ng), GFP (100 ng), Lucy–Rho–OR51E1 or Lucy–Rho–OR1A1, and RIC8B or empty vector (150 ng), for a total of 300 ng per well,

were diluted in Opti-MEM (Cat# 31985062; Thermo Fisher Scientific) and transfected using PolyMagNeo (Cat# PG60200; OZ Biosciences, Marseille, France). For experiments using OR51E2, plasmid DNA mixtures containing GloSensor (50 ng), GFP (75 ng), Lucy-Rho-OR51E2 (200 ng), and RIC8B or empty vector (150 ng), for a total of 400 ng per well, were prepared and transfected in the same manner. After medium replacement, cells were incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO₂, and GFP fluorescence was measured using a Synergy 2 plate reader (BioTek, Winooski, VT, USA).

Cells were then incubated for 2 h at room temperature in 75 µL of HBSS(+) containing GloSensor cAMP reagent (2% GloSensor reagent; Cat# E1290; Promega). Subsequently, 25 µL of HBSS(+) containing ligands and IBMX (final concentration, 0.5 mM) was added using an eight-channel pipette, and luminescence was measured immediately at 2-min intervals.

Normalized relative luminescence units (RLU) were calculated as follows:

1. GFP fluorescence intensity was measured for each well.
2. A correction factor (Co) was calculated as: $Co = (\text{fluorescence intensity of OR-expressing cells}) / (\text{fluorescence intensity of non-OR-expressing cells})$.
3. RLU_raw was calculated as: $(\text{maximum luminescence in ligand-stimulated OR-expressing cells}) / (\text{maximum luminescence in ligand-unstimulated non-OR-expressing cells})$.
4. Normalized RLU was obtained by dividing RLU_raw by Co.

All experiments were performed three times using completely independent wells, and the results are presented as mean $\pm$ standard deviation (SD). To account for day-to-day variability in absolute relative luminescence unit (RLU) values, comparative analyses were conducted using data obtained under identical experimental conditions on the same day (**Figure 6**).

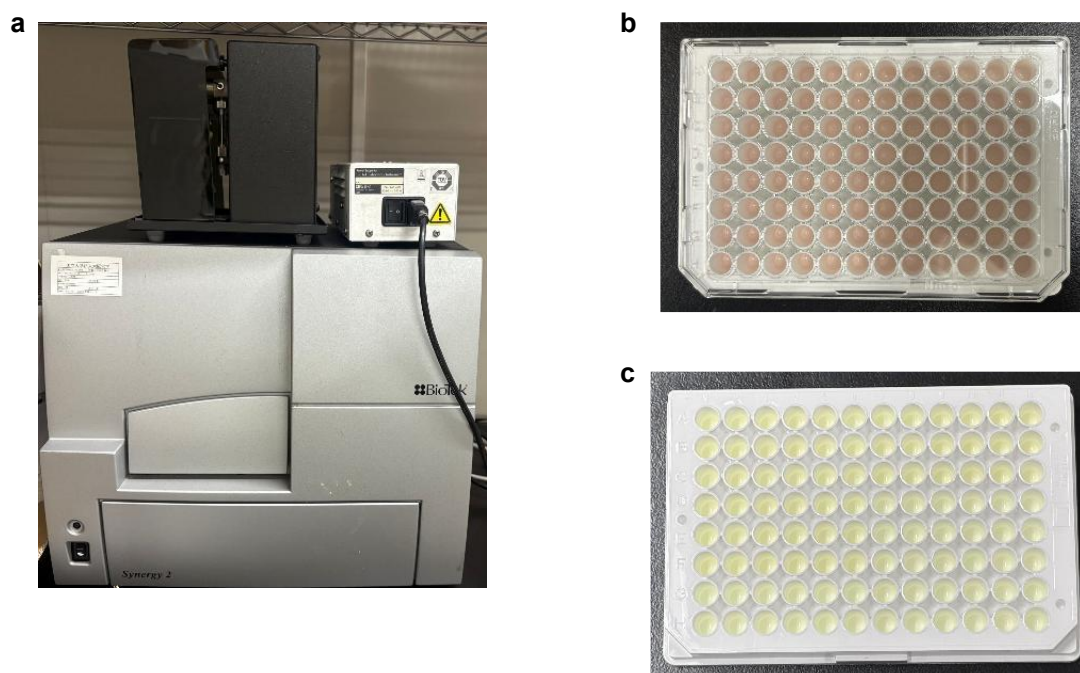


Figure 6. Experimental setup and cell plates used for luminescence measurements.

- (a) Synergy 2 plate reader used for luminescence detection (BioTek, Winooski, VT, USA).
- (b) HEK293T cells seeded in poly-L-lysine-coated 96-well white plates (poly-L-lysine, Cat# 236105; Thermo Fisher Scientific; plates, Cat# VBH-SPL01; Cosmo Bio).
- (c) Representative image of a cell plate immediately prior to measurement, following incubation with 2% GloSensor reagent in HBSS(+).

In vitro assessment of OR expression following co-expression of RIC8B splice variants

HEK293T cells ($\sim 4 \times 10^5$ cells/mL) were seeded at 50 μ L/well in poly-L-lysine-coated 96-well white plates. Plasmid DNA mixtures containing Lucy-Rho-OR-GFP (200 ng), RIC8B (100 ng) or empty vector, for a total of 300 ng/well, were dissolved in Opti-MEM and transfected using PolyMagNeo. After medium replacement, cells were incubated for 24 h at 37 °C in a humidified 5% CO₂ atmosphere, and GFP fluorescence was measured using a Synergy 2 plate reader.

Structure prediction using AlphaFold3

The RIC8B–Gas complex was predicted using the AlphaFold server (AlphaFold3 model; Google DeepMind & Isomorphic Labs) (Abramson et al., 2024). Per-residue confidence was assessed using the predicted Local Distance Difference Test score (pLDDT; 0–100), with values ≥ 70 considered

high confidence. The accuracy of overall folding was evaluated by the predicted TM-score (pTM; 0–1), and the accuracy of inter-subunit relative positioning by the inter-chain predicted TM-score (ipTM; 0–1), with higher values indicating higher confidence. Structural alignment with the known structure (PDB: 8EL7) was performed using PyMOL v2.5.3 (Schrödinger, LLC; RRID:SCR_000305), and structural similarity was quantified by root-mean-square deviation (RMSD).

Results

Effects of RIC8B variants on OR responses

HEK293T cells were co-transfected with Lucy-Rho-tagged OR51E1 (class I OR) or OR1A1 (class II OR) together with one of five RIC8B splice variants (v1, v2, v3, v4, or v10). Odorant-induced cAMP production was measured using the GloSensor cAMP assay, with isovaleric acid used to activate OR51E1 and R-(–)-carvone used to activate OR1A1. Odorant concentrations were set at sub-EC₅₀, EC₅₀, and near-saturating levels, and cAMP responses were compared across conditions. Real-time changes in relative luminescence units (RLU) are shown in **Figure 7**. For quantitative

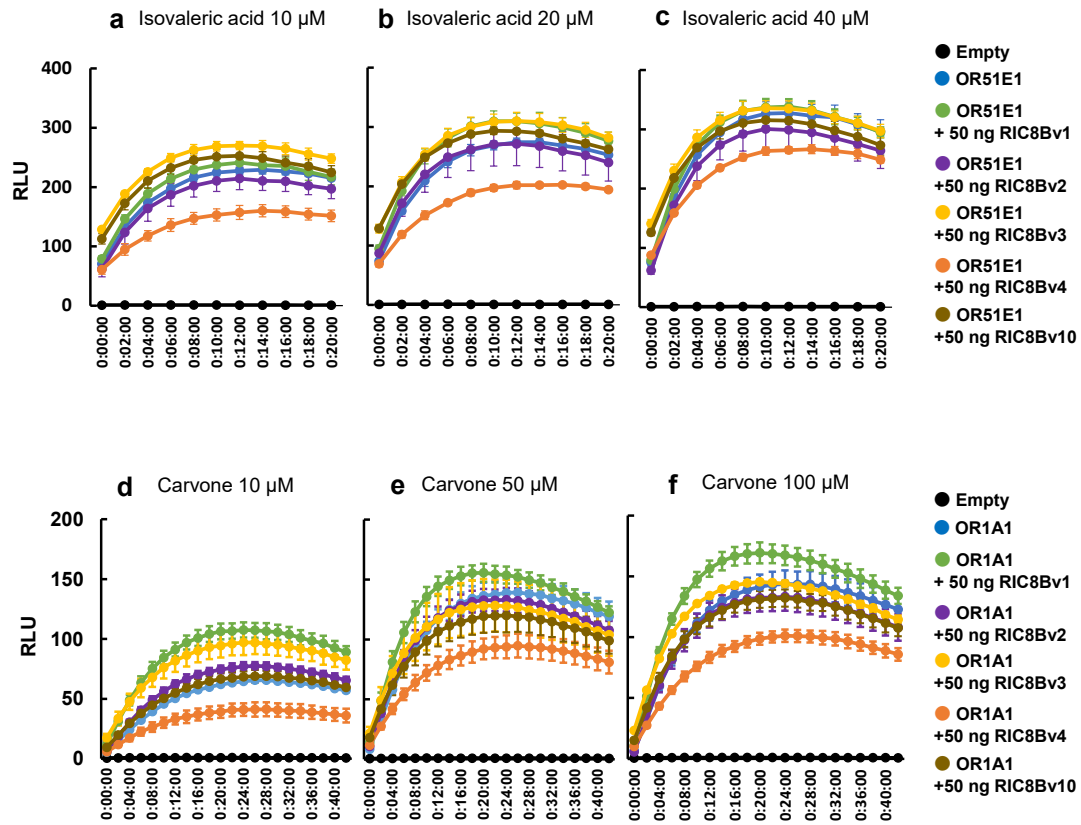


Figure 7. Real-time changes in relative luminescence units (RLU).

The vertical axis represents relative luminescence units (RLU), and the horizontal axis represents time (min). Odorant concentrations were set at sub-EC₅₀, EC₅₀, and near-saturating levels. Blue, OR alone; green, co-expression with RIC8Bv1; purple, co-expression with RIC8Bv2; yellow, co-expression with RIC8Bv3; orange, co-expression with RIC8Bv4; brown, co-expression with RIC8Bv10. Panels a–c show responses of OR51E1, and panels d–f show responses of OR1A1. Data are presented as geometric means ($n = 3$), with error bars indicating standard deviation. The maximum response values from each trace are plotted in **Figure 8**.

comparison, the maximum response values obtained for each odorant stimulation were extracted and plotted as shown in **Figure 8**. RIC8Bv1, v2, v3, and v10 showed effects similar to, or slightly enhancing, those observed with OR alone (**panels a–c, e–h, j**). In contrast, RIC8Bv4 consistently and significantly suppressed odorant-induced cAMP responses in both ORs (**panels d, i**). These results indicate that RIC8Bv4 specifically suppresses cAMP responses mediated by *G α s*, regardless of the type of OR.

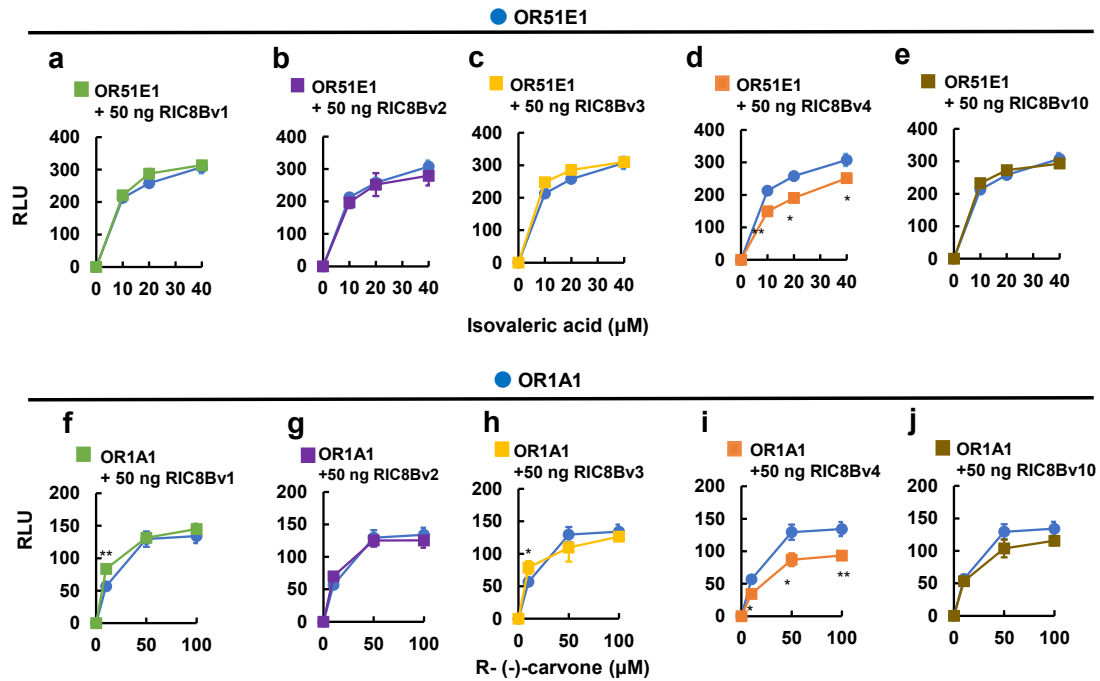


Figure 8. Odorant responses of ORs co-expressed with five RIC8B splice variants.

The vertical axis indicates relative luminescence units (RLU), and the horizontal axis indicates odorant concentration. Isovaleric acid was used as the odorant for OR51E1, and R-(–)-carvone was used for OR1A1. Odorant concentrations were set at sub-EC₅₀, EC₅₀, and near-saturating levels. Blue, OR alone; green, co-expression with RIC8Bv1; purple, co-expression with RIC8Bv2; yellow, co-expression with RIC8Bv3; orange, co-expression with RIC8Bv4; brown, co-expression with RIC8Bv10. Panels a–e show responses of OR51E1, and panels f–j show responses of OR1A1. Data are presented as geometric means (n = 3), with error bars indicating standard deviation. Data shown side by side were obtained from experiments performed on the same day. Statistical analysis was performed using Dunnett’s test. *P ≤ 0.01; **P ≤ 0.001.

Specific suppression of OR responses by RIC8Bv4

To examine whether the suppressive effect of RIC8Bv4 was dose-dependent, I repeated the GloSensor cAMP assay by varying the amounts of RIC8Bv1, v4 or v10 plasmid DNA (25, 50, and

75 ng/well) (**Figure 9**). In cells expressing OR51E1 with RIC8Bv1, odorant concentration–dependent cAMP production in response to isovaleric acid was confirmed, but no significant effect of v1 co-expression was observed (**panel a**). In contrast, co-expression of RIC8Bv4 clearly suppressed cAMP production in a dose-dependent manner (**panel b**). Specifically, upon stimulation with 40 μ M isovaleric acid, cAMP production decreased to 82.1%, 76.8%, and 46.6% of control levels with 25, 50, and 75 ng/well of RIC8Bv4 plasmid, respectively. RIC8Bv10 showed no significant effect at 25 or 50 ng/well; however, at 75 ng/well, it exhibited a weaker inhibitory effect than RIC8Bv4 only for OR51E1 (**panel c**). Similar analyses were performed with OR51E2 (class I OR) and OR1A1. For OR51E2, which is activated by propionic acid, measurements were conducted at 500 and 1000 μ M, as higher concentrations were technically difficult to assess. RIC8Bv1 showed little effect at any expression level, whereas RIC8Bv4 consistently suppressed cAMP responses (**panels d-f**). A comparable suppressive effect of RIC8Bv4 was also reproduced in OR1A1 responses to R-(–)-carvone (**panels g-i**). Collectively, these findings indicate that suppression of OR activation by RIC8Bv4 is observed across multiple ORs and likely arises from structural features unique to this variant.

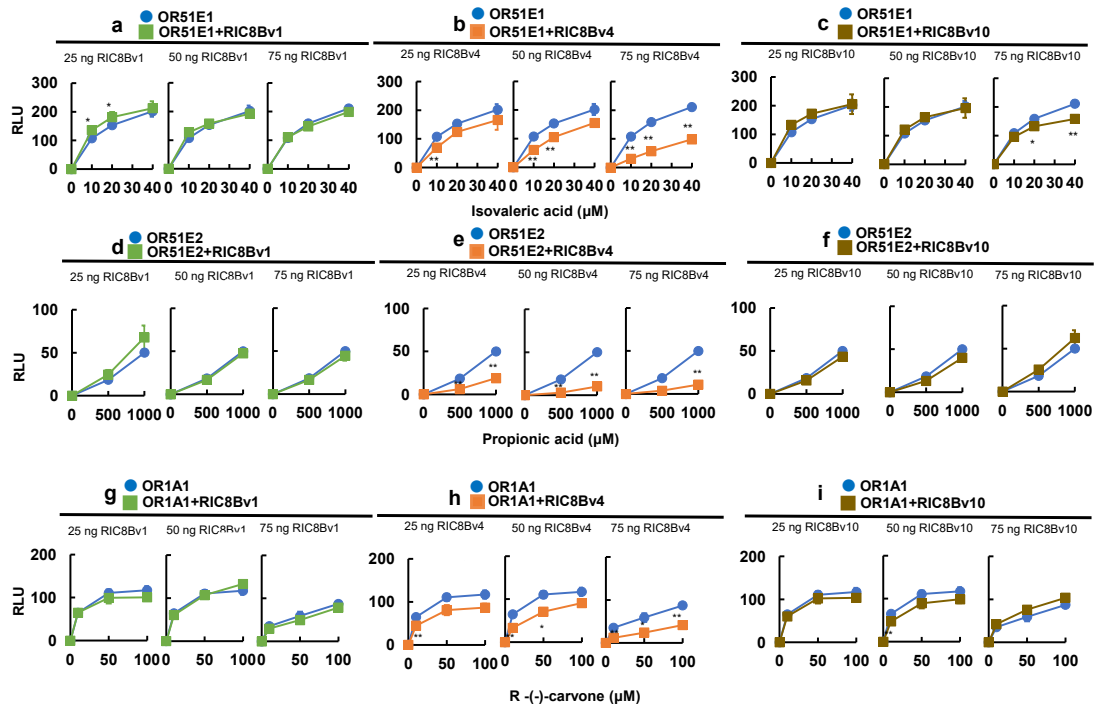


Figure 9. Dose-dependent effects of RIC8B splice variants on odorant-induced OR responses.

The vertical axis indicates relative luminescence units (RLU), and the horizontal axis indicates odorant concentration. Isovaleric acid was used for OR51E1, propionic acid for OR51E2, and R-(-)-carvone for OR1A1. Odorant concentrations were set at sub-EC₅₀, EC₅₀, and near-saturating levels. For OR51E2, measurements were performed at 500 μM and 1000 μM propionic acid due to technical limitations at higher concentrations. Blue, OR alone; green, co-expression with RIC8Bv1; orange, co-expression with RIC8Bv4; brown, co-expression with RIC8Bv10. Panels a–c show responses of OR51E1, panels d–f show responses of OR51E2, and panels g–i show responses of OR1A1. Data shown side by side were obtained from experiments performed on the same day. Data are presented as geometric means (n = 3), with error bars indicating standard deviation. Statistical analysis was performed using Dunnett's test. *P ≤ 0.01; **P ≤ 0.001.

Changes in OR expression levels by co-expression of RIC8B variants

HEK293T cells expressing Lucy-Rho-OR51E1-GFP or Lucy-Rho-OR1A1-GFP were co-transfected with one of five RIC8B splice variants (v1, v2, v3, v4, or v10) (**Figure 10**). In OR51E1-expressing cells, co-expression with RIC8Bv2, v3, v4, v10 increased OR expression by 45.4-58.0% (**panel a**). In OR1A1-expressing cells, co-expression with RIC8Bv1, v2, v4, v10 increased OR expression by 34.7-58.0% (**panel b**). The increase in expression levels of OR51E1 and OR1A1 upon co-expression with RIC8B splice variants suggests that RIC8B, which functions as a chaperone for Gas, indirectly

modulates OR expression levels. However, at least for OR51E1 and OR1A1, the suppression of OR responses by RIC8Bv4 is likely attributable to factors other than changes in OR expression levels, given that an increase in OR protein expression was observed.

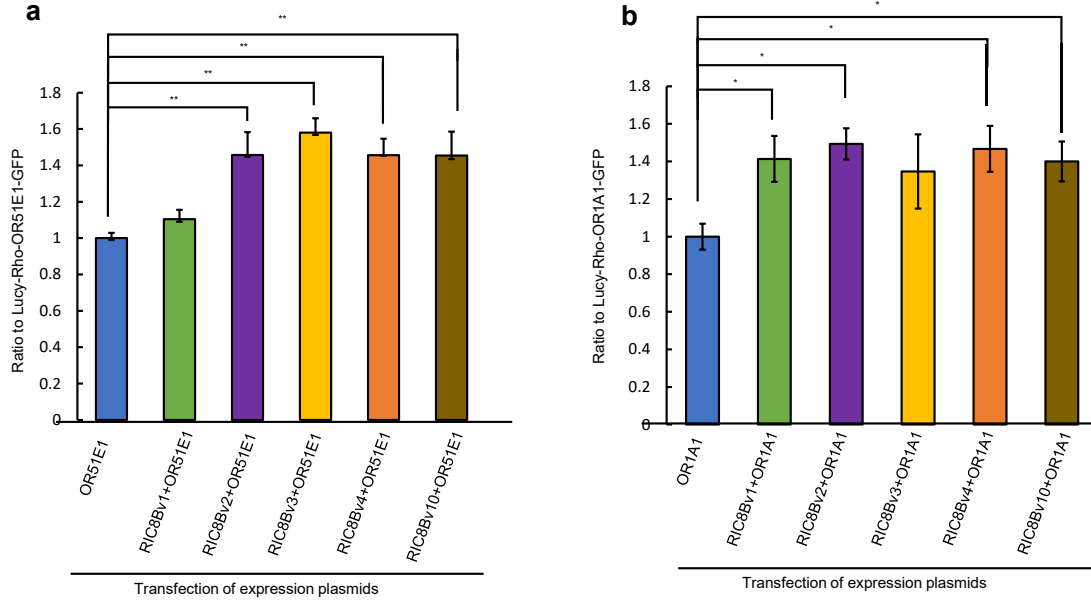


Figure 10. Effects of RIC8B splice variants co-expression on OR51E1 and OR1A1 protein expression in HEK293T cells.

Cells were transfected with a total of 300 ng plasmid DNA/well (200 ng OR plasmid, 100 ng RIC8B plasmid). Fluorescence intensity is presented as a relative value, with the fluorescence of Lucy-Rho-OR-GFP alone set to 1. Data are presented as geometric means ($n = 3$), with error bars indicating standard deviation. (a) Relative fluorescence intensity of Lucy-Rho-OR51E1-GFP. (b) Relative fluorescence intensity of Lucy-Rho-OR1A1-GFP. Statistical analysis was performed using Dunnett's test. * $P \leq 0.01$, ** $P \leq 0.001$.

Structural prediction of RIC8B splice variant–Gas complexes

RIC8B is known to act as a chaperone promoting proper folding of Gas/olf and enhancement of GPCR signaling depends on direct binding between them (Dannecker et al., 2005). To assess whether the unique suppressive effect of RIC8Bv4 arises from a distinct binding mode, I predicted the structures of complexes between Gas and each RIC8B variant (v1, v2, v3, v4, and v10) using AlphaFold3 (AF3) (**Figure 11**). The AF3-predicted RIC8Bv1–Gas complex was superimposed on the known cryo-EM structure (PDB ID: 8EL7), and structural validity was evaluated by RMSD and pTM/ipTM scores. The results (RMSD: 1.296 Å; pTM: 0.71; ipTM: 0.78; **panel a**) were favorable, particularly in the N-terminal binding pocket of RIC8B interacting with the C-terminus of Gas. Similar results were obtained for v2 (RMSD: 1.346 Å, pTM: 0.70, ipTM: 0.78; **panel b**), v3

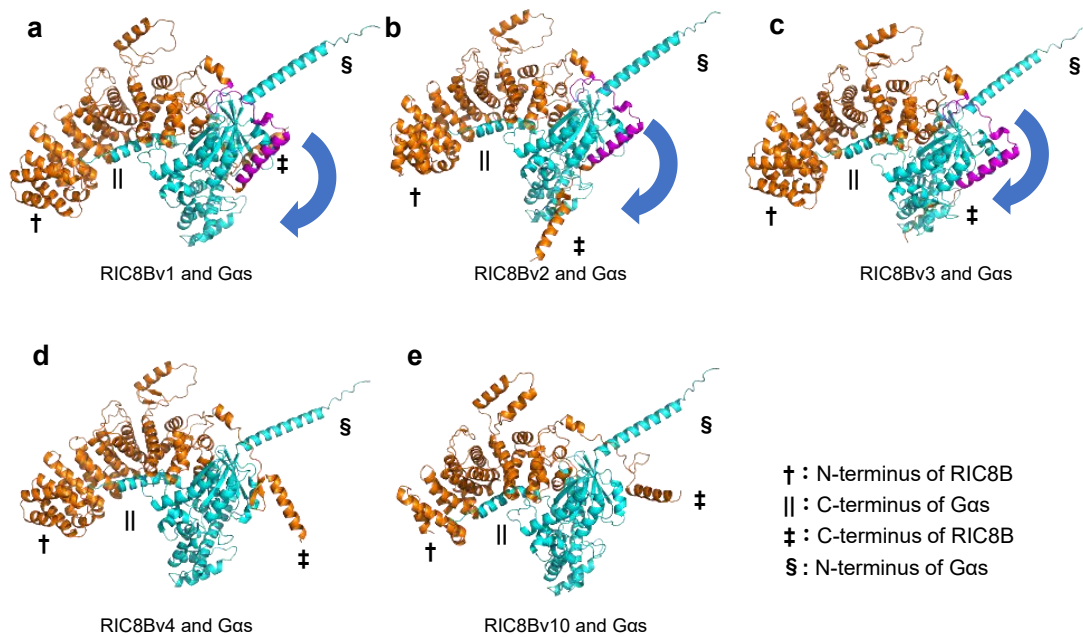


Figure 11. Predicted structural models of RIC8B splice variants in complex with Gas.

RIC8B is shown in orange, Gas in cyan and CLH in magenta. The “embracing” conformation is shown with blue arrows. †: N-terminal region of RIC8B; ||: C-terminal region of Gas; ‡: C-terminal region of RIC8B; §: N-terminal region of Gas. (a) RIC8Bv1–Gas, (b) RIC8Bv2–Gas, (c) RIC8Bv3–Gas, (d) RIC8Bv4–Gas and (e) RIC8Bv10–Gas.

(RMSD: 1.318 Å, pTM: 0.77, ipTM: 0.85; **panel c**), and v4 (RMSD: 1.319 Å, pTM: 0.72, ipTM: 0.78; **panel d**). In contrast, v10, lacking both CLH and a complete N-terminal binding pocket, showed reduced accuracy despite an RMSD of 1.191 Å, with lower pTM/ipTM scores (0.67/0.69), suggesting poorer folding and inter-subunit positioning (**panel e**).

Region-wise confidence evaluation by AF3 pLDDT (**Figure 12**) indicated that the N-terminal regions were consistently rated as “high to very high” across all variants, whereas confidence for the C-terminal CLH ranged from “low to high” in v1–v3 and from “low to very low” in v4 and v10. Overall, while AF3 predictions alone are insufficient to establish the functional importance of the CLH sequence, the models for variants retaining the CLH (v1, v2, and v3) were compatible with an “embracing” conformation around Gas (**panels a–c**). In contrast, such a conformation was not apparent in the models for v4 and v10 (**panels d and e**).

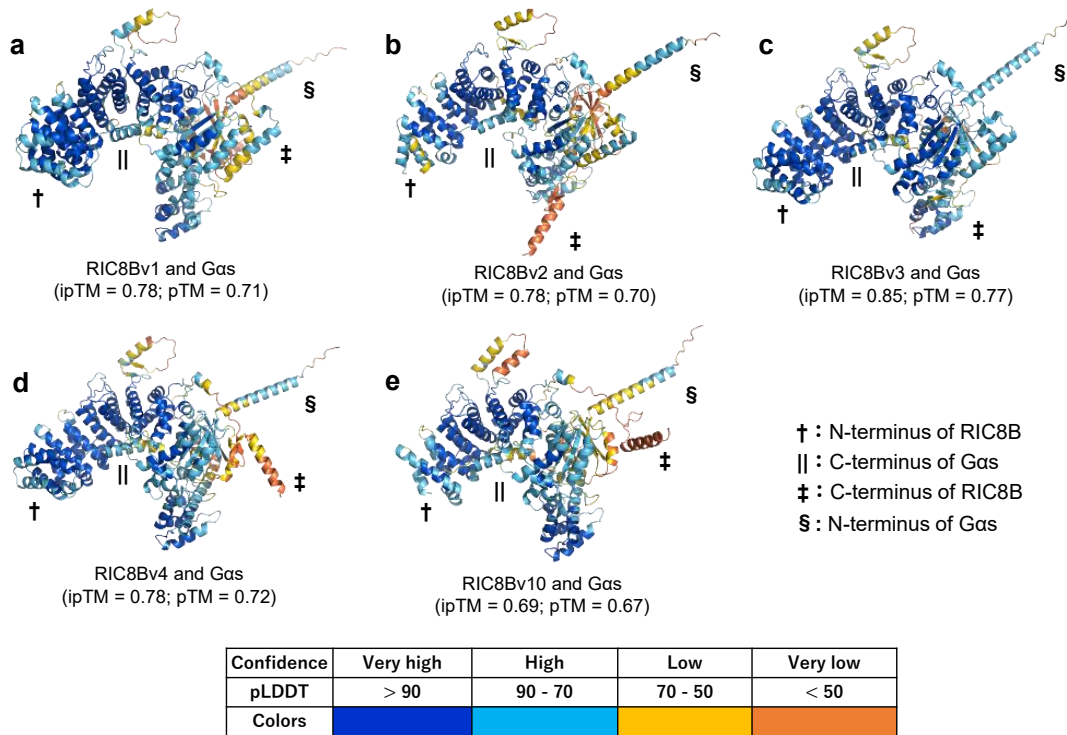


Figure 12. Predicted confidence of RIC8B splice variant–Gas complexes by pLDDT.

(a) RIC8Bv1–Gas, (b) RIC8Bv2–Gas, (c) RIC8Bv3–Gas, (d) RIC8Bv4–Gas and (e) RIC8Bv10–Gas. Structures are colored according to pLDDT: >90, blue; 90–70, cyan; 70–50, yellow; and <50, orange. Predicted accuracy scores: RIC8Bv1 ipTM = 0.80, pTM = 0.71; RIC8Bv2 ipTM = 0.74, pTM = 0.70; RIC8Bv3 ipTM = 0.81, pTM = 0.76; RIC8Bv4 ipTM = 0.76, pTM = 0.72; and RIC8Bv10 ipTM = 0.64, pTM = 0.64. Lower accuracy was observed in the C-terminal region of RIC8B, which was unresolved in the cryo-EM structure (PDB ID: 8EL7).

Comparison of binding modes between RIC8B splice variants and Gas

Based on AF3 models, hydrogen bond interactions were compared among variants. For v1, six hydrogen bonds (average 2.78 ± 0.08 Å) were predicted between the N-terminal binding pocket and the C-terminus of Gas, in addition to six hydrogen bonds mediated by the CLH domain, suggesting dual anchoring and a stable “embracing” conformation (**Figure 13 panels a, d, e**). For v4, seven hydrogen bonds (2.83 ± 0.31 Å) were predicted in the N-terminal pocket–Gas interface, but CLH-derived bonds were absent (**panels b, f**). For v10, only three hydrogen bonds (2.63 ± 0.25 Å) were predicted at the N-terminal pocket, and CLH-mediated interactions were also absent; the loss of residue R75, present in v1/v4, likely contributed to this instability (**panels c, g**). Taken together, these results suggest that both the N-terminal binding pocket and the CLH domain of RIC8B are

indispensable for proper folding and functional expression of Gas. Although RIC8Bv4 can bind Gas, the absence of CLH results in an unstable binding mode, insufficient chaperone activity, and potential sequestration of Gas precursors, leading to misfolded proteins that show dominant-negative-like effect on GPCR signaling pathways (**Figure 14**). This interpretation is consistent with previous yeast two-hybrid assays (Dannecker et al., 2005). In contrast, the even weaker binding capacity of RIC8Bv10 likely prevents effective sequestration of Gas precursors, thereby failing to inhibit the generation of functional Gas.

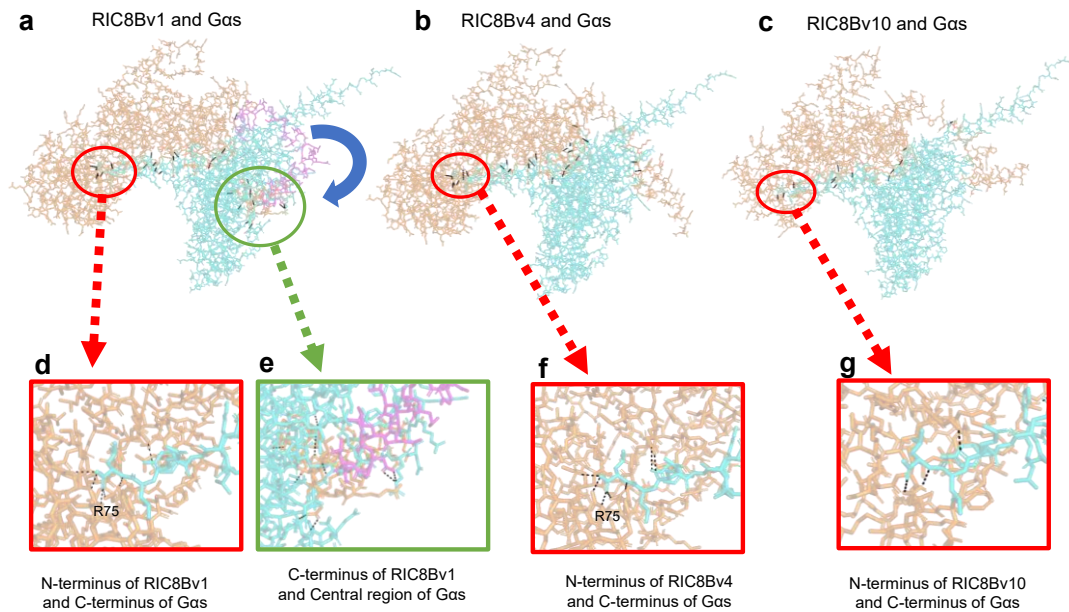


Figure 13. Predicted hydrogen bonds models of RIC8B splice variants with Gas.

Cyan: Gas; orange: RIC8B; magenta: CLH; and dashed black lines: hydrogen bonds.

(a) RIC8Bv1–Gas, (b) RIC8Bv4–Gas, and (c) RIC8Bv10–Gas, showing overall structures.

(d) Enlarged view of panel a (red circle), showing the interaction between the N-terminal region of RIC8Bv1 and the C-terminal region of Gas.

(e) Enlarged view of panel a (green circle), showing CLH-mediated interactions of RIC8Bv1.

(f) Enlarged view of panel b (red circle), showing the interaction between the N-terminal region of RIC8Bv4 and the C-terminal region of Gas.

(g) Enlarged view of panel c (red circle), showing the interaction between the N-terminal region of RIC8Bv10 and the C-terminal region of Gas.

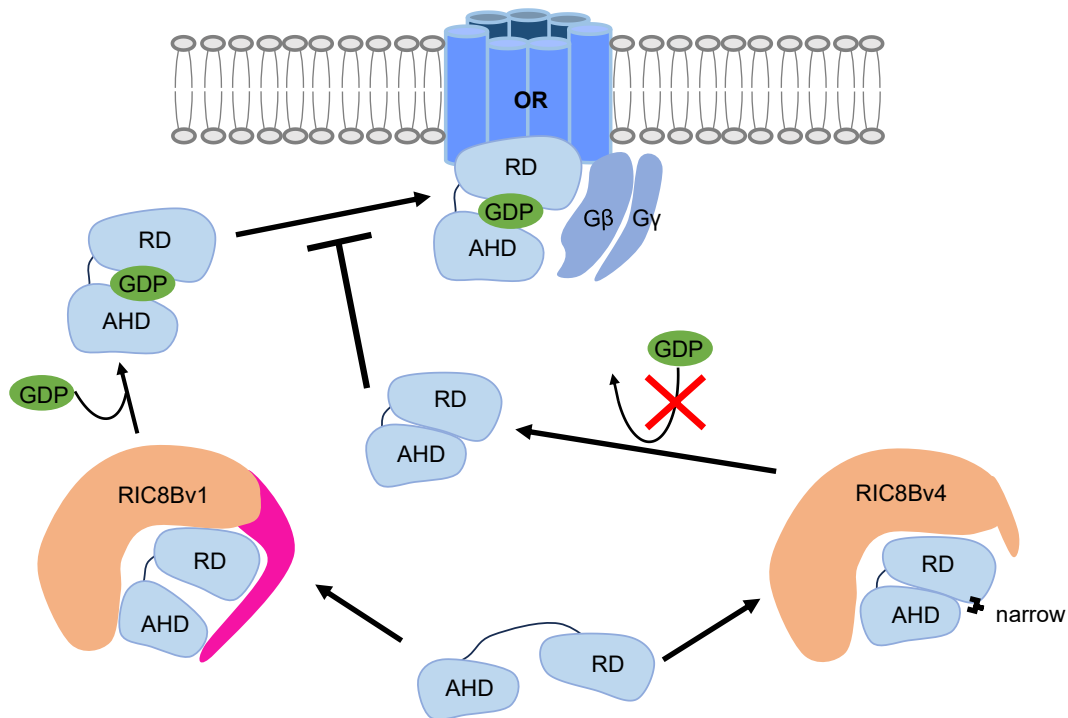


Figure 14. Mode of function of RIC8Bv1 and v4

RIC8Bv1, containing the CLH domain (magenta), functions as a chaperone, enabling properly folded Gas to associate with ORs and transmit signals. In contrast, RIC8Bv4 lacks the CLH domain, causing the AHD and RD of Gas to approach each other, thereby preventing GDP (green) binding and thereby suppressing cAMP production.

Effects of the RIC8B cradle Loop and cradle Helix on odorant-induced OR responses

The CLH of RIC8Bv1 was subdivided into two regions—the cradle Loop (N484–E505) and the cradle Helix (E506–S523)—and deletion mutants lacking each region were generated to examine the contributions of these elements to odorant-induced OR responses. HEK293T cells were co-transfected with OR51E1, OR51E2, or OR1A1 together with RIC8Bv1, RIC8Bv1 del-Loop, RIC8Bv1 del-Helix, or RIC8Bv4, and odorant-induced responses were evaluated using a cAMP assay (**Figure 15**).

Co-expression of RIC8Bv1 or RIC8Bv1 del-Helix resulted in odorant responses comparable to those observed with OR expression alone (**panels a, c, e, g, i, and k**). In contrast, RIC8Bv1 del-Loop exhibited an inhibitory effect on the odorant responses of OR51E1 and OR51E2, although this effect was weaker than that observed with RIC8Bv4 (**panels b, d, f, and h**). A very weak inhibitory effect was also observed for the odorant response of OR1A1 (**panels j and l**). These results suggest that the cradle Loop region of RIC8B plays an important role in its chaperone activity toward Gas.

To further investigate the structural basis underlying these functional differences, the structures of the deletion mutants were predicted using AlphaFold3 (AF3) (**Figure 16**). Neither RIC8Bv1 del-Loop nor RIC8Bv1 del-Helix was predicted to adopt the characteristic “embracing” conformation observed for RIC8Bv1 in complex with Gas (**panels c and d**). Notably, RIC8Bv1 del-Loop was predicted to undergo substantial structural rearrangement relative to RIC8Bv1 (**panel c**). These observations suggest that deletion of the cradle Loop disrupts the structural integrity required for effective chaperone function, thereby impairing the ability of RIC8Bv1 to support proper folding and maturation of Gas.

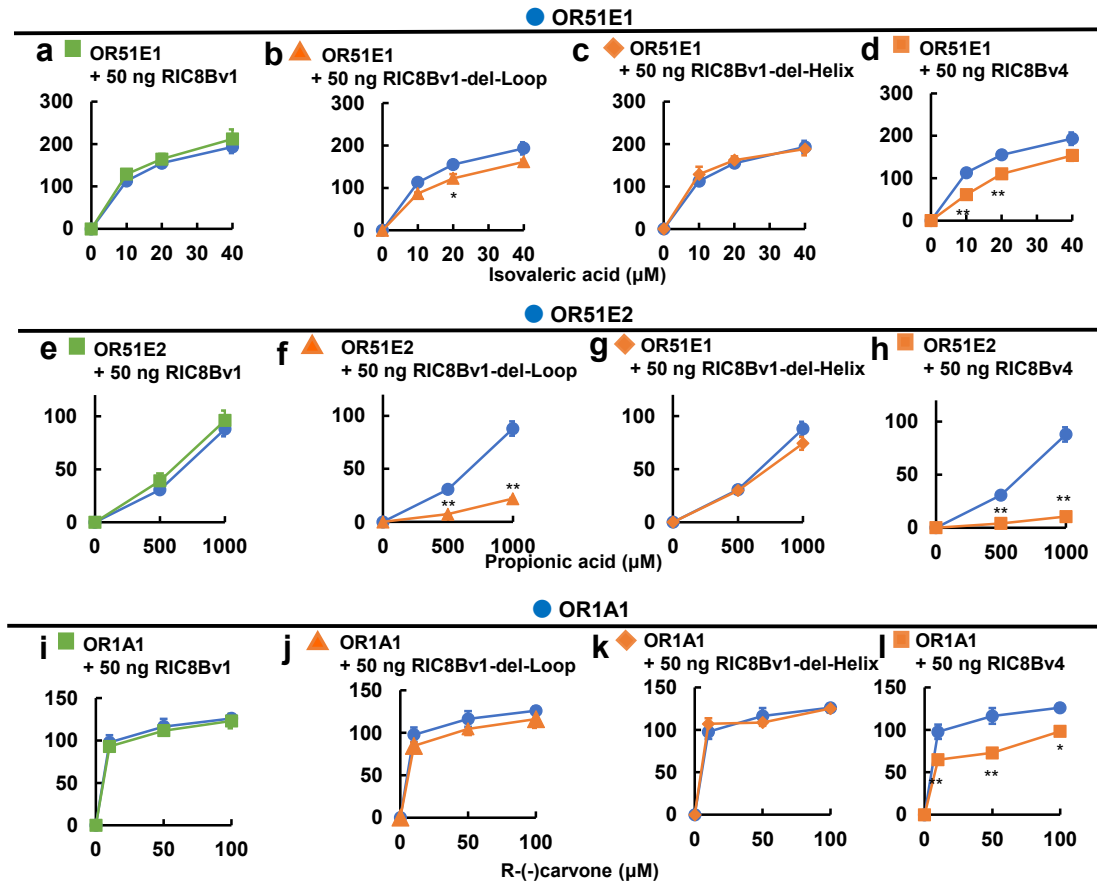


Figure 15. Effects of CLH subdomain deletions in RIC8Bv1 on odorant-induced OR responses.

The vertical axis indicates relative luminescence units (RLU), and the horizontal axis indicates odorant concentration. Isovaleric acid was used for OR51E1, propionic acid for OR51E2, and R-(-)-carvone for OR1A1. Odorant concentrations were set at sub-EC₅₀, EC₅₀, and near-saturating levels. For OR51E2, measurements were performed at 500 μ M and 1000 μ M propionic acid due to technical limitations at higher concentrations. Blue, OR alone; green squares, co-expression with RIC8Bv1; orange triangles, co-expression with RIC8Bv1 del-Loop; orange diamonds, co-expression with RIC8Bv1 del-Helix; orange squares, co-expression with RIC8Bv4. Panels a–d show responses of OR51E1, panels e–h show responses of OR51E2, and panels i–l show responses of OR1A1. Data shown side by side were obtained from experiments performed on the same day. Data are presented as geometric means ($n = 3$), with error bars indicating standard deviation. Statistical analysis was performed using Dunnett's test. * $P \leq 0.01$; ** $P \leq 0.001$.

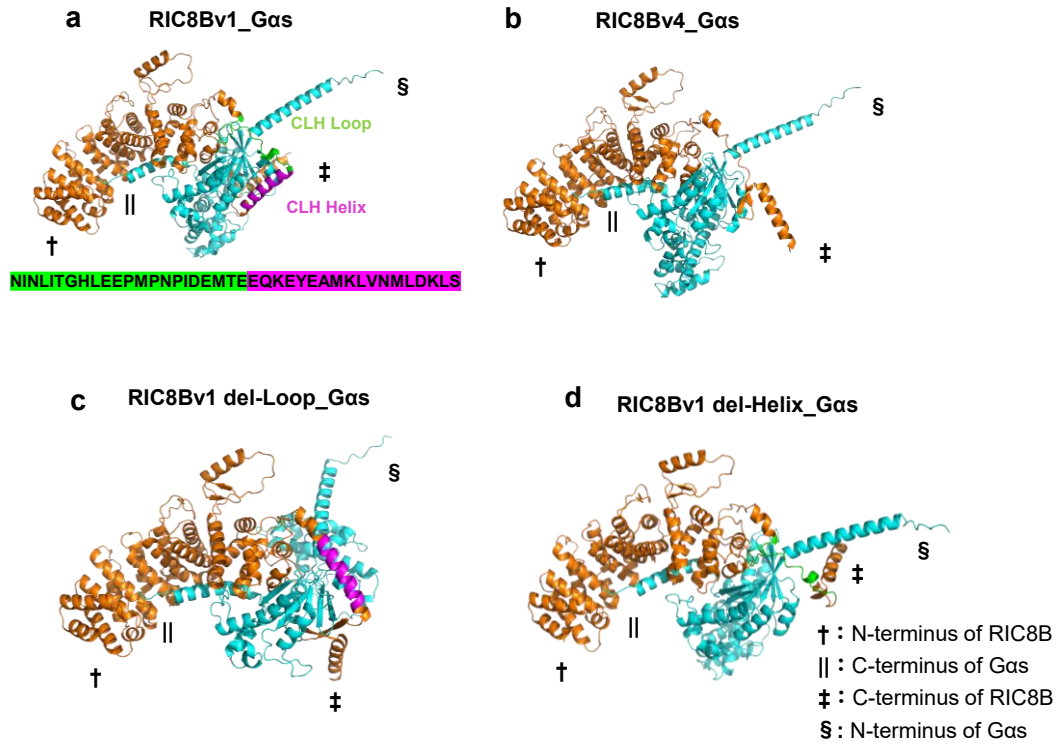


Figure 16. Predicted structural models of RIC8Bdel-Loop and RIC8Bv1 del-Helix in complex with Gas.

RIC8B is shown in orange, Gas in cyan, cradle Loop in green and cradle Helix in magenta.

†: N-terminal region of RIC8B; ||: C-terminal region of Gas; ‡: C-terminal region of RIC8B; §: N-terminal region of Gas. (a) RIC8Bv1–Gas, (b) RIC8Bv4–Gas, (c) RIC8Bv del-Loop–Gas and (d) RIC8Bv del-Helix–Gas.

Effects of R75 residue in RIC8Bv1 and RIC8Bv4 on odorant-induced OR responses

Structural analyses of RIC8Bv1 by cryo-EM (Papaserghi-Scott et al., 2023) and AF3-based structure prediction (**Figure 13, panels d and f**) suggest that residue R75 in RIC8Bv1 and RIC8Bv4 is involved in hydrogen bonding with the C-terminal region of Gas. Based on these observations, I generated the RIC8Bv1 R75E mutant, which has been reported to exhibit reduced chaperone activity (Papaserghi-Scott et al., 2023), as well as the corresponding RIC8Bv4 R75E mutant. HEK293T cells were co-transfected with OR51E1 or OR1A1 together with RIC8Bv1, RIC8Bv4, RIC8Bv1 R75E, or RIC8Bv4 R75E, and odorant-induced responses were evaluated using a cAMP assay (**Figure 17**). RIC8Bv1 R75E did not affect odorant-induced OR responses, similar to wild-type RIC8Bv1 (**panels a, c, e, and g**). In contrast, the inhibitory effect of RIC8Bv4 on odorant-induced OR responses was abolished in the RIC8Bv4 R75E mutant (**panels b, d, f, and h**). Previous studies reported that RIC8Bv1 R75E lacks chaperone activity (Papaserghi-Scott et al., 2023); however, HEK293T cells

used in this study express endogenous RIC8Bv1 (Diamant et al., 2024; unpublished data). Therefore, I consider that the presence of endogenous RIC8Bv1 may have masked differences between RIC8Bv1 and RIC8Bv1 R75E in the present assay. Future experiments using RIC8B knockdown or knockout systems will be required to unambiguously isolate the variant-specific contributions of exogenously expressed RIC8B mutants and to rigorously assess their intrinsic chaperone activities in the absence of endogenous RIC8B. Taken together, these results suggest that disruption of R75-mediated hydrogen bonding compromises the interaction of RIC8Bv4 with Gas, thereby abolishing its dominant-negative-like effect on odorant-induced OR responses (**Figure 14**).

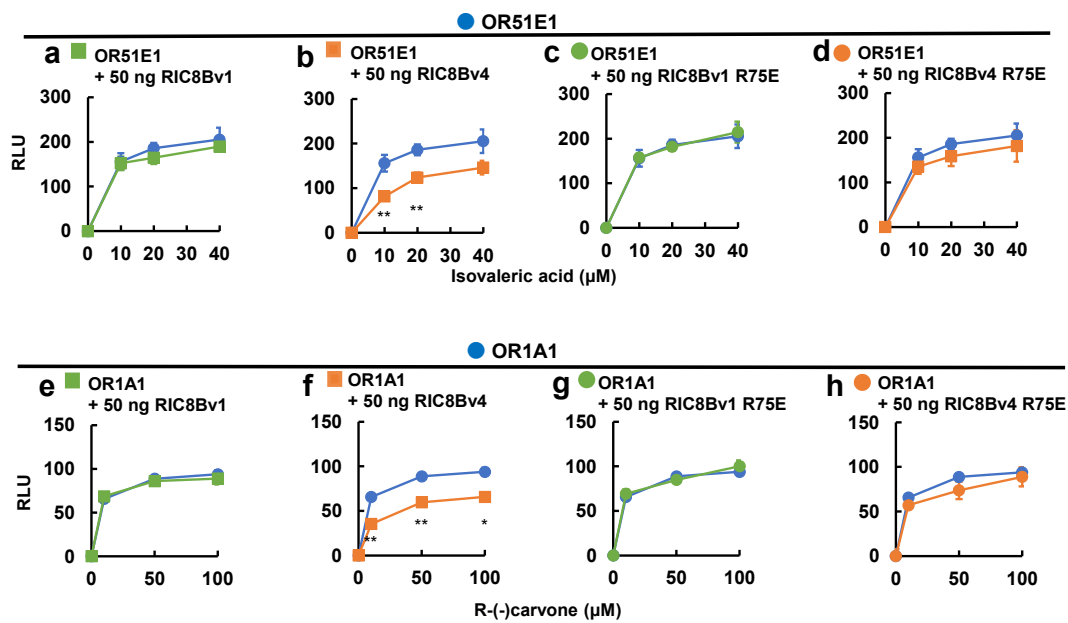


Figure 17. Effects of R75 mutations in RIC8B variants on odorant-induced OR responses.

The vertical axis indicates relative luminescence units (RLU), and the horizontal axis indicates odorant concentration. Isovaleric acid was used for OR51E1, and R-(-)-carvone was used for OR1A1. Odorant concentrations were set at sub-EC₅₀, EC₅₀, and near-saturating levels. Blue, OR alone; green squares, co-expression with RIC8Bv1; orange squares, co-expression with RIC8Bv4; green circles, co-expression with RIC8Bv1 R75E; orange circles, co-expression with RIC8Bv4 R75E. Panels a–d show responses of OR51E1, and panels e–h show responses of OR1A1. Data shown side by side were obtained from experiments performed on the same day. Data are presented as geometric means (n = 3), with error bars indicating standard deviation. Statistical analysis was performed using Dunnett’s test. *P ≤ 0.01; **P ≤ 0.001.

Discussion

Alternative splicing of RIC8B as a qualitative regulator of Gas signaling

Achieving functional expression of human ORs in heterologous systems such as HEK293T cells remains challenging. Prior strategies have included appending N-terminal Rho or Lucy tags (Saito et al., 2004; Shepard et al., 2013) and co-expressing OR accessory proteins (e.g., the RTP family, Gas/olf, and RIC8B) (Zhuang and Matsunami, 2007). In the present study, I successfully detected OR responses in HEK293T cells by employing Lucy–Rho–tagged OR51E1, OR51E2 and OR1A1. Consistent with Dannecker et al. (2005), RIC8B indirectly facilitates functional OR expression by stabilizing Gas/olf, while also acting as both a chaperone and a GEF for Gas/olf (Hinrichs et al., 2012). In my experiments, co-expression of RIC8B variants tended to increase OR protein levels (**Figure 10**), suggesting that correctly folded Gas, assisted by RIC8B variants, forms complexes with ORs to promote intracellular trafficking and reduce ER-associated degradation. These findings align with reports that RIC8B co-expression improves OR surface and functional expression (Saito et al., 2004; Zhuang and Matsunami, 2007). Taken together, my results extend the current view of RIC8B function. Specifically, my findings suggest that alternative splicing of RIC8B does not merely fine tune chaperone efficiency but may constitute a regulatory mechanism that qualitatively switches Gas signaling competence.

Structural basis for the dominant-negative-like effect of RIC8Bv4

A key finding of this work is that RIC8Bv4, which lacks the CLH domain, significantly suppresses odorant-evoked responses of both class I ORs (OR51E1, OR51E2) and class II OR (OR1A1) (**Figures 7-9**), suggesting a broad inhibitory effect on cAMP signaling across multiple ORs. Structural predictions using AF3 suggest that RIC8Bv1 engages Gas via a dual-anchoring “embracing” mode that involves both the N-terminal binding pocket (particularly residue R75) and the CLH domain, especially cradle Loop. This mode of interaction is likely to stabilize the RIC8Bv1-Gas complex and support chaperone activity (**Figures 11–13, 15**).

In contrast, although RIC8Bv4 is predicted to retain the ability to interact with Gas via the N-terminal binding pocket, the absence of CLH may result in a less stable interaction and insufficient chaperone activity. Under such conditions, RIC8Bv4 may sequester Gas precursor forms, thereby interfering with proper folding and potentially leading to the accumulation of non-functional Gas species that may show dominant-negative-like effect on GPCR pathways. Consistent with this idea, RIC8Bv10 appears to interact with Gas even more weakly than RIC8Bv4, which may render precursor sequestration inefficient and thus unlikely to substantially inhibit the production of functional Gas. Furthermore, analysis using the RIC8Bv4 R75E mutant, in which the residue R75—previously shown to be essential for the chaperone activity of RIC8Bv1 (Papasergi-Scott et al.,

2023)—was substituted with glutamate, demonstrated that the suppressive, dominant-negative-like effect of RIC8Bv4 on odorant-induced OR responses also requires R75-mediated interaction with *Gas* (**Figure 17**).

Geometric analysis of the GDP-binding site within the RIC8B–*Gas* complex further supports this model. AF3 suggests that residues E50, S51, G52, K53, S54, T55, L198, R199, N292, K293, D295, and A366 of *Gas* can participate in hydrogen bonding with GDP (**Figure 18 panels a, b**).

Comparing *Gas* folded in the presence of RIC8Bv1 versus RIC8Bv4, the position of A366 differed markedly: the A366–G52 distance was 6.1 Å with v1 and 5.1 Å with v4, and the C352–L198 distance was 7.4 Å with v1 and 6.4 Å with v4 (**Figure 18 panel c**). Thus, in the v4 context, the α -helical domain (AHD) and Ras-like domain (RD) are predicted to approach one another, narrowing the GDP-binding pocket and thereby potentially favoring misfolded conditions (**Figure 14**). These inferences are consistent with yeast two-hybrid data showing variant-dependent differences in RIC8B–*Gas* interactions (Dannecker et al., 2005). Recent work further posits that the C-terminal region immediately following the CLH of RIC8Bv1 transiently prevents premature AHD–RD association in *Gas*, maintaining an intermediate competent for correct folding and subsequent GDP binding (Papasergi-Scott et al., 2023).

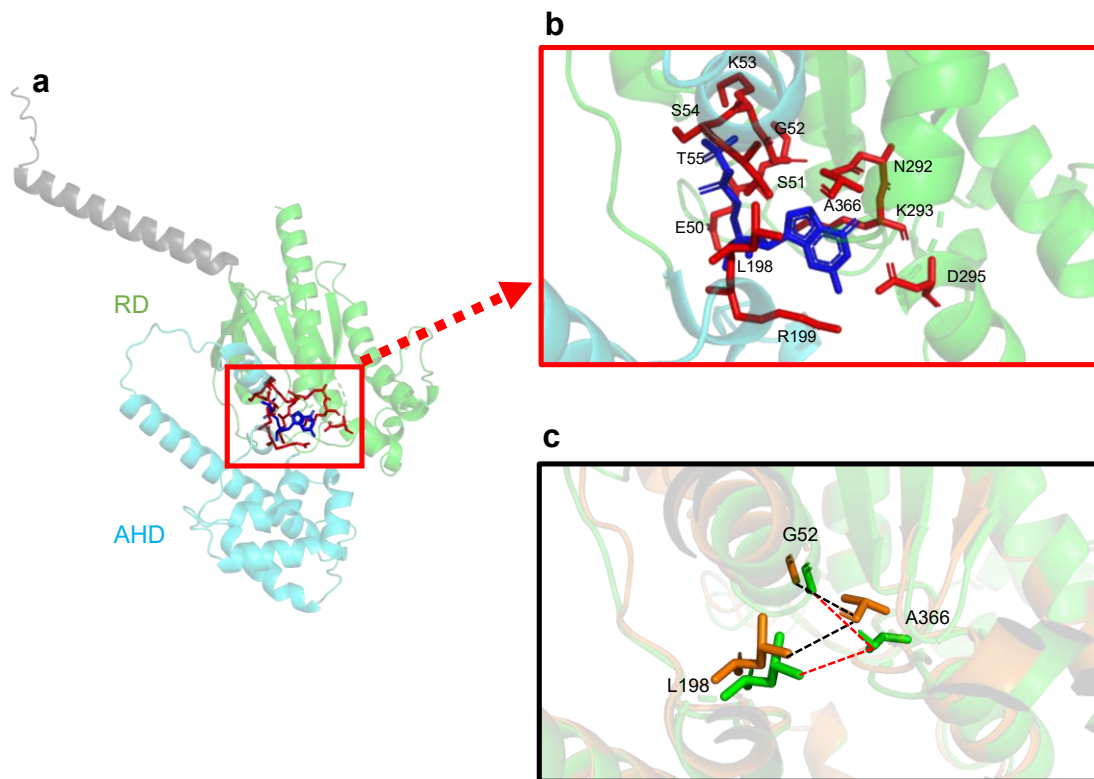


Figure 18. Predicted GDP-bound Gas structure.

The predicted structure was generated with AF3 and colored using PyMOL.

(a) Overall structure of Gas, with the α -helical domain (AHD) in cyan, the Ras-like domain (RD) in green, GDP in blue, and GDP-interacting residues in red.

(b) GDP-binding pocket.

(c) Comparison of Gas folded with RIC8Bv1 (green) versus RIC8Bv4 (orange). GDP-binding residues are shown as sticks. The positions of G52, L198 and A366 differ between v1- and v4-folded Gas.

Context-dependent expression and functional implications of RIC8B splice variants

GTEx analysis indicates that RIC8Bv1 (ENST00000392837.8) is highly expressed (0.1–11.8 TPM) across more than 50 tissues and cell types, while RIC8Bv4 (ENST00000392839.6) is also appreciably expressed (0.1–2.6 TPM) in many contexts. Although v1 predominates in most tissues, primary cultured fibroblasts and EBV-immortalized lymphocytes exhibit comparable levels of v1 and v4 that exceed those of other isoforms. Accordingly, in tissues or cells where RIC8Bv4 is relatively enriched, it may function as a suppressive modulator of GPCR signaling mediated by Gas. This study has several limitations. First, it relies on in vitro experiments using HEK293T cells,

which may not fully recapitulate the behavior of RIC8B variants in the native olfactory system. Second, while AF3-based structural predictions are highly informative, they remain computational and require validation by experimental structural biology. Third, it remains unresolved whether the suppressive effect of RIC8Bv4 is attributable solely to G α s misfolding; contributions from additional interaction partners cannot be excluded.

Despite these limitations, my integrated approach—systematically analyzing how RIC8B variants influence OR signaling and combining structural prediction with functional assays—highlights a previously unappreciated inhibitory mechanism. I believe this framework provides a valuable basis for future studies aimed at dissecting variant-specific chaperone functions and their broader implications for G α s-coupled GPCR signaling.

Physiological relevance and potential pathological implications of RIC8Bv4

Finally, I consider the physiological implications of my findings regarding RIC8Bv4. Olfactory receptors are known to be expressed not only in the olfactory epithelium but also in a variety of non-olfactory tissues. In prostate cancer cells, OR51E2 and OR51E1 are referred to as the prostate-specific G-protein-coupled receptor (PSGR) and PSGR2, respectively, and signaling from these receptors has been shown to suppress cell proliferation through the MAPK (Mitogen-Activated Protein Kinase) cascade (Neuhaus et al., 2009; Maßberg et al., 2016). Furthermore, analysis of UCSC Xena datasets (Goldman et al., 2020) comparing the relative mRNA expression levels of RIC8Bv1, v4, v8, and v11 between normal prostate tissue and prostate cancer samples revealed that only RIC8Bv4 exhibited an increased expression proportion in cancer cells (**Figure 19**). Taken together, these observations suggest that, in prostate cancer, increased expression of RIC8Bv4 may suppress ligand-induced signaling of PSGR and PSGR2, thereby attenuating their reported anti-proliferative effects and potentially promoting cancer cell proliferation.

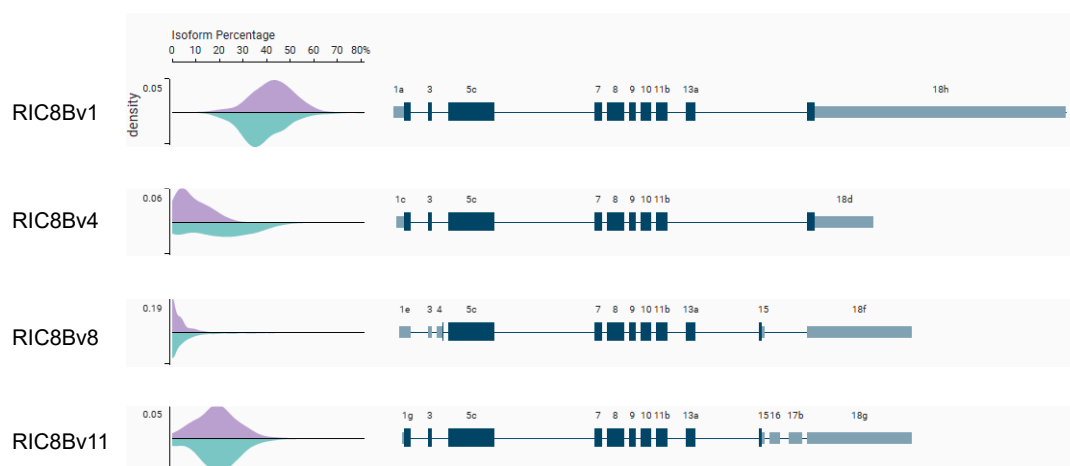


Figure 19. Changes in the expression of RIC8B splice variants in normal prostate and prostate cancer tissues.

Data were obtained from UCSC Xena. Isoform percentages are shown for RIC8B splice variants that are close to the full-length sequence. Normal prostate samples (GTEx prostate) are shown in purple, and prostate cancer samples (TCGA Prostate Adenocarcinoma) are shown in cyan. The exon–intron organization of human RIC8B splice variants is also shown, with exons indicated by blue boxes and introns by thin lines.

Comprehensive Discussion

Aim and Overall Framework of This Study

In this doctoral thesis, I focused on odorant-induced olfactory receptor (OR) responses mediated by *G α s* and investigated the roles of RIC8B splice variants, which function as central regulatory factors in this signaling pathway. By integrating functional analyses with structure prediction, this study demonstrates that RIC8B splice variants act as molecular switches that qualitatively regulate the signaling competence of *G α s*. Although ORs transmit odor information through *G α s*-dependent cAMP signaling, it has been recognized that the efficiency of this response is influenced not only by the intrinsic properties of the receptors themselves but also by intracellular regulatory factors. By specifically focusing on alternative splicing of RIC8B, this study examined the possibility that previously unrecognized regulatory mechanisms modulate OR signaling.

Functional comparison of the major RIC8B splice variants, RIC8Bv1 and the CLH-deficient RIC8Bv4, revealed that these variants exert distinct effects on the functional maturation and signaling capacity of *G α s*. In this chapter, the findings obtained throughout this thesis are integrated, and their conceptual significance is discussed.

Regulatory Principles Revealed by Integrating Structural Information and Functional Analysis

A defining feature of this study is the integrative use of functional assays together with structure prediction. Recent advances in computational structural biology, particularly the development of AlphaFold3 (AF3), have made it feasible to formulate structural hypotheses for protein complexes and folding intermediates that have been difficult to characterize experimentally. Against this background, the present work provides a structural interpretation of the dynamic process of chaperone-dependent *G α s* maturation.

AF3-based structural predictions played a central role in generating hypotheses regarding the interaction modes between RIC8B variants and *G α s*. Notably, RIC8Bv1 was predicted to adopt an “embracing” interaction mode involving the cradle Loop Helix (CLH) domain, whereas this stabilizing interaction was absent in RIC8Bv4. This structural distinction provides a rational framework for explaining the functional differences between these variants. Furthermore, while both RIC8Bv1 and RIC8Bv4 retain the ability to capture *G α s* through residue R75, RIC8Bv10 lacks this residue, offering an explanation for its reduced ability to engage *G α s*.

Taken together, these findings suggest that R75 in RIC8B intervenes in the folding trajectory of *G α s* to support its maturation into a functional conformation, whereas CLH-deficient variants such as RIC8Bv4 may arrest this process at an incomplete stage. Regulation at the level of protein folding represents a layer of control that has been largely overlooked in classical GPCR signaling studies. In

this regard, the present work highlights the importance of protein maturation as a determinant of signaling competence.

Why RIC8Bv10 does not act as a dominant-negative despite lacking CLH

Although RIC8Bv10, like RIC8Bv4, lacks the C-terminal cradle Loop Helix (CLH) domain, it did not exhibit a robust dominant-negative effect on odorant-induced OR responses in this study.

Structural predictions provide a plausible explanation for this difference. In addition to the absence of CLH, RIC8Bv10 lacks two N-terminal exons relative to RIC8Bv4, resulting in an incomplete N-terminal binding pocket that is critical for stable interaction with Gas. Notably, RIC8Bv10 also lacks residue R75, which is conserved in RIC8Bv1 and RIC8Bv4 and was shown to be essential for effective engagement with the C-terminal region of Gas.

AF3-based models predict that, as a consequence of these combined deficiencies, RIC8Bv10 interacts only weakly and transiently with Gas. Such interactions are likely insufficient to sequester Gas folding intermediates or to interfere with productive chaperone-mediated maturation. In contrast, RIC8Bv4 retains an intact N-terminal binding pocket and R75-mediated contacts while lacking CLH, a combination that may permit stable yet non-productive association with Gas, thereby promoting misfolding and dominant-negative inhibition. These observations indicate that dominant-negative activity requires not merely the absence of CLH, but a specific structural balance in which partial Gas binding is preserved while chaperone-mediated stabilization is compromised, as supported by the predicted interaction modes shown in **Figures 11 and 13**.

Extension from OR Signaling to General GPCR Signaling

RIC8B is known to function as a chaperone for Gas/olf, suggesting that its regulatory influence may extend beyond ORs to other Gas-coupled GPCR signaling pathways (Chan et al., 2013; Papasergi et al., 2015; Papasergi-Scott et al., 2023). Representative examples of Gas-coupled receptors include the β 2-adrenergic receptor and the dopamine D1 receptor, in which the stability and activation efficiency of Gas are key determinants of signaling strength.

In this study, co-expression of the CLH-deficient variant RIC8Bv4 significantly suppressed odorant-induced OR responses. This suppression could not be fully explained by reduced OR protein levels alone, suggesting that the Gas-mediated signaling process itself was compromised. Specifically, RIC8Bv4 may fail to adequately support correct folding of Gas, resulting in reduced cAMP production upon receptor stimulation.

If this mechanism is correct, the inhibitory effect of RIC8Bv4 may not be restricted to ORs but could also affect other Gas-dependent GPCR systems. However, because ORs exhibit intrinsically low surface expression efficiency and rely heavily on OR accessory proteins including RIC8B for functional expression (Dannecker et al., 2006), the impact of RIC8B variants is likely to be more

pronounced in OR systems than in other GPCRs. Clarifying the extent to which the CLH domain of RIC8B universally contributes to G α s-coupled GPCR signaling will require comparative analyses using receptors beyond the olfactory system.

GPCR Signaling Mediated by G α Subunits Other Than G α s/olf

RIC8A shares high sequence and structural similarity with RIC8B and is thought to operate through a conserved molecular mechanism within the RIC8 family (Papaserghi-Scott et al., 2023).

Functionally, RIC8A primarily exhibits chaperone and GEF activities toward G α i/o/q family members (Ruisu et al., 2013; Srivastava et al., 2019), whereas RIC8B selectively targets G α s/olf. Despite this difference in substrate specificity, both proteins possess a conserved CLH structure at the C-terminus that is believed to support proper folding of G α subunits.

In light of the inhibitory effects observed for the CLH-deficient RIC8B variant, it is conceivable that analogous phenomena may occur for RIC8A. Specifically, CLH-deficient variants of RIC8A may be unable to sufficiently support the functional maturation of G α i/o/q, leading to reduced downstream signaling in corresponding GPCR pathways. Such effects are more likely to reflect impaired G α folding or activation efficiency rather than changes in receptor expression or membrane localization. These considerations suggest that RIC8A and RIC8B may regulate GPCR signaling strength and stability through a shared CLH-dependent chaperone mechanism, despite targeting different G α subfamilies.

Limitations and Methodological Considerations

In this study, three ORs that have been widely used in previous functional analyses and that yield reproducible odorant responses in heterologous systems were selected for investigation. This strategy minimized variability arising from experimental instability and enabled quantitative assessment of the effects of RIC8B variants on OR signaling.

Nevertheless, ORs generally exhibit low surface expression efficiency in heterologous systems, and only a subset of receptors displays robust functional responses. As a result, many ORs with extremely weak responsiveness could not be analyzed, and inhibitory effects similar to those observed for RIC8Bv4 may be difficult to detect due to technical limitations. Furthermore, for OR51E2, maximal responses could not be achieved even at ligand concentrations up to 1000 μ M, and higher concentrations were precluded by cytotoxicity and pH-related nonspecific effects.

Accordingly, it is difficult to directly extrapolate the observed inhibitory effects of RIC8Bv4 to all ORs. However, the consistent suppression observed across multiple representative ORs—including class I receptors (OR51E1 and OR51E2) and a class II receptor (OR1A1)—strongly suggests that the regulatory mechanism identified in this study reflects a fundamental aspect of OR signaling rather than an isolated phenomenon. Future studies employing olfactory epithelium-derived cells,

organoids, or improved expression systems may enable broader validation of these findings.

Future Perspectives and Conceptual Significance

Taken together, this study proposes a novel molecular mechanism by which alternative splicing of RIC8B qualitatively regulates G α s signaling competence. This concept extends beyond the traditional view of GPCR signaling as a process governed solely by receptor activation and suggests that signaling capacity may already be determined at the stage of protein maturation.

Integration of in vivo analyses and high-resolution structural studies will be essential for further elucidating the physiological relevance and generality of this regulatory mechanism. The conceptual framework proposed here not only deepens our understanding of GPCR signaling but also highlights alternative splicing of accessory factors as a potential target for modulating signal transduction pathways.

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