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Generation of Odorant Receptor-QF2 Knock-In Drivers for Improved Analysis of Olfactory Circuits in *Drosophila*

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ABSTRACT

Drosophila melanogaster has provided numerous insights into the olfactory system, primarily relying on a series of transgenic *Gal4* drivers. The combined use of *Gal4/UAS* and a second binary expression system, such as the *QF/QUAS* system, provides the opportunity to manipulate the two distinct cell populations, thereby accelerating the elucidation of the olfactory neural mechanisms. However, resources apart from the *Gal4/UAS* system have been poorly developed. In this study, we generated a series of odorant receptor (*Or*)-*QF2* knock-in driver (*Or-QF2^{KI}*) lines for 23 Ors using the CRISPR/Cas9 knock-in method. In these lines, the QF2 protein is cotranslated with each Or product. The expression pattern of the *Or-QF2^{KI}* drivers mostly corresponded to that of the *Or-Gal4* drivers. In addition, the *Or42a-QF2^{KI}* driver identified the additional expression pattern of Or42a, which is consistent with the data of single-nucleus RNA sequencing and is attributed to the *Or-QF2^{KI}* drivers' ability to reflect the endogenous expression of the *Or* genes. Thus, these *Or-QF2^{KI}* drivers can be used as valuable genetic tools for olfactory research in *Drosophila*.

1 | Introduction

Olfaction plays a vital role in detecting the surrounding environment to enhance an individual's survival chance. Wandering albatrosses rely on olfaction to forage for the patchily distributed prey over the large open ocean (Nevitt et al. 2008). Rabbits, rodents, deer, etc., exhibit avoidance behavior toward the urine of predators, such as the wolf, through the olfactory detection of pyrazine analogs contained in the urine (Osada et al. 2013, 2015). Odorants are detected by olfactory receptors (ORs) expressed by olfactory receptor neurons (ORNs) located in the olfactory organ (Buck and Axel 1991; Zhao et al. 1998). In mammals, ORs form the superfamily of G-protein-coupled receptors; for instance, a mouse has approximately 1000 OR genes, and a human has approximately 400 OR genes (Godfrey et al. 2004; Malnic et al. 2004). Furthermore, each OR is activated by multiple ligands rather than a single ligand, which allows animals to detect tens of thousands of odorants (Malnic et al. 1999).

The fruit fly, *Drosophila melanogaster*, is a powerful model animal that has been used to reveal various biological phenomena, including the olfactory system. It detects odorants by ORNs housed in the sensory hair covering its antenna and maxillary palp. Similar to vertebrates, the majority of ORNs express just one OR gene from 126 OR genes containing mainly two types: 60 Odorant receptors (Ors) and 66 Ionotropic receptors (Irs) (Benton et al. 2009; Croset et al. 2010; Gomez-Diaz et al. 2018; Robertson et al. 2003; Su et al. 2009). Furthermore, ORNs that express the same receptor project their axons to a specific glomerulus in the antennal lobe, which is the primary center of the insect olfactory system (Couto et al. 2005; Fishilevich and Vosshall 2005; Silbering et al. 2011). In the glomerulus, ORN axons form synapses with the dendrites of the projection neurons (PNs), which are the second-order olfactory neurons, and the olfactory information is transferred to the higher brain region (Vosshall and Stocker 2007). For instance, the Or56a$^+$ ORNs exclusively respond to the odorant geosmin, which is produced by harmful microbes, and their axons project to the specific glomerulus called DA2. The activation of Or56a$^+$ ORNs by geosmin induces the activation of PNs, which form synapses with Or56a$^+$ ORN axons at DA2 and result in the elicitation of innate avoidance behavior from harmful microbes (Stensmyr et al. 2012). Thus, elucidating the olfactory system is essential for understanding animal behavior elicited by odorants.

The vast majority of recent *Drosophila* research has employed the *Gal4/UAS* system, in which the Gal4 activator expressed under the promoter of interest binds to the upstream activation sequence (UAS) and induces the expression of the gene of interest under the *UAS* (Brand and Perrimon 1993). The combination of numerous *Gal4* driver and *UAS* effector lines allows for the manipulation of the expression of any genes in any tissues or cell populations. Furthermore, secondary binary expression systems, such as the *QF/QUAS* (Potter and Luo 2011; Riabinina et al. 2015) and *LexA/LexAop* (Lai and Lee 2006) systems, can work in parallel with the *Gal4/UAS* system, which enables the manipulation of two distinct cell populations, thereby promoting the elucidation of neural circuits (Cachero et al. 2020; Okumura et al. 2016; Qian et al. 2018; Task et al. 2022; Xu et al. 2024). The study of *Drosophila* olfaction has primarily relied on the *Gal4/UAS* system. For all *Or* and *Ir* genes, *Gal4* lines that carry the promoter of each *Or* or *Ir* gene have been established (Figure 1A; Couto et al. 2005; Fishilevich and Vosshall 2005; Sánchez-Alcañiz et al. 2018; Silbering et al. 2011). However, the number of driver lines for *Or* or *Ir* in other binary expression systems remains limited (Table 1). The generation of secondary *Or-* or *Ir*-driver lines that work in parallel with the *Gal4/UAS* system would facilitate a better understanding of the olfactory system through which the activation of ORNs is associated with animal behavior.

In this study, we generated 23 *Or-T2A-QF2* knock-in lines (*Or-QF2^{KI}*), in which QF2 is cotranslated with the Or protein, thus enabling the manipulation or labeling of ORNs simultaneously with the *Gal4/UAS* or *LexA/Lexop* system. Most *Or-QF2^{KI}* exhibited the same expression pattern compared with the transgenic *Or-Gal4* drivers, with some exceptions. *Or42a-QF2^{KI}* revealed that *Or42a* is expressed in the ORNs projecting to the VL2p glomerulus in addition to the known ORNs projecting to the VM7 glomerulus, which is consistent with the Fly Cell Atlas, single-nucleus RNA sequencing dataset (Li et al. 2022). Together, these *Or-QF2^{KI}* lines provide new insight into the olfactory system through their combined use with other binary expression systems.

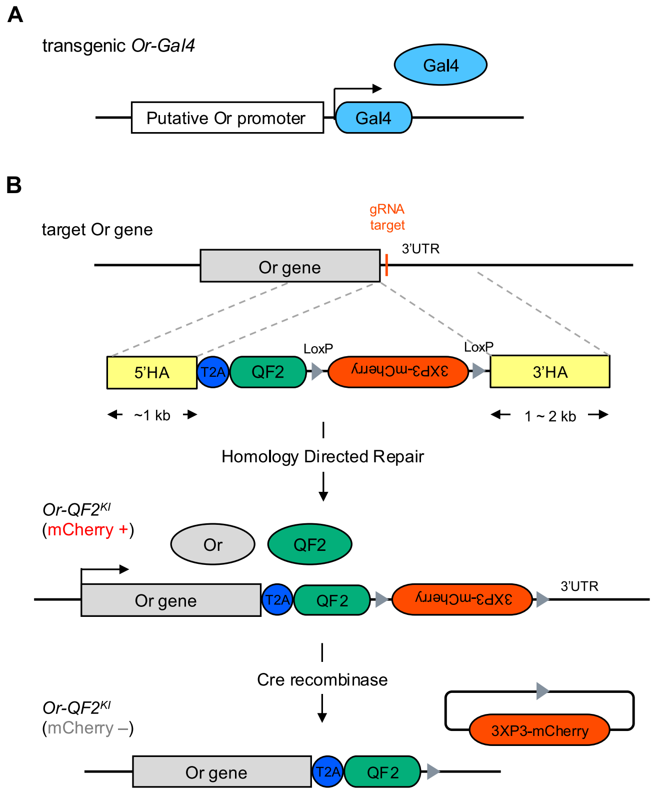

FIGURE 1 | Establishment of *Or-QF2^{KI}* lines. (A) Schematic of transgenic *Or-Gal4* strain. Gal4 transcription factor is expressed under the putative promoter of *Or.* (B) Strategy for the establishment of the *Or-QF2^{KI}* lines. One gRNA induces a double-strand break in the 3′ UTR, and a knock-in construct was inserted (top). In the construct, *T2A-QF2* and *3XP3-mCherry* (eye marker) were included between the 5′ homology arm (5HA) and the 3′ homology arm (3HA) of the target Or. Following the target Or expression, the knock-in line induces the expression of *QF2 (middle). 3XP3-mCherry* is excised by Cre recombinase (bottom).

2 | Results

2.1 | Generation of Odorant Receptor-T2A-QF2 Knock-In Lines

We generated *Or-QF2^{KI}* lines according to the method described by Task et al. (2022). In this method, the T2A-QF2 cassette and 3xP3-mCherry selection marker are inserted before the stop codon of the Ors mediated by the CRISPR/Cas9 system (Figure 1B). T2A induces ribosomal skipping, which leads to the translation of two proteins, Or and QF2. As a result, the expression of QF2 reflects the endogenous expression of *Or* genes. *D. melanogaster* uses a different set of Ors between the larval and adult stages (Couto et al. 2005; Fishilevich et al. 2005; Kreher et al. 2005). We established 23 *Or-QF2^{KI}* lines targeted to the odorant receptors, including Or13a, Or22b, Or33a, Or42a, Or43b, Or49a, Or49b, Or56a, Or59c, Or65a, Or67b, Or69a, Or71a, Or83c, Or85a, Or85b, Or85f, and Or98P, which are only expressed in adults or both adults and larvae, and Or22c, Or24a,

TABLE 1 | Olfactory receptor drivers using a second binary expression system.

Gene	Method	Stock #	References
Or7a	LexA::p65 replaced with the endogenous gene	BDSC605638	Zhang et al. (2024)
	nls-LexA::p65 expressed under the Or7a promoter	—	Gugel et al. (2023)
Or13a	T2A-QF2 inserted before the stop codon	DGGR119657	This study
Or22a	LexA::p65 replaced with the endogenous gene	BDSC605639	Zhang et al. (2024)
	nls-LexA::p65 expressed under the Or22a promoter	BDSC80543	Eliason et al. (2018)
Or22b	T2A-QF2 inserted before the stop codon	DGGR119658	This study
Or33a	T2A-QF2 inserted before the stop codon	DGGR119661	This study
Or42a	nls-LexA::p65 expressed under the Or42a promoter	—	Zocchi et al. (2022)
	T2A-QF2 inserted before the stop codon	DGGR119662	This study
Or43b	T2A-QF2 inserted before the stop codon	DGGR119663	This study
Or47a	LexA::p65 replaced with the endogenous gene	BDSC605641	Zhang et al. (2024)
Or47b	LexA expressed under the Or47b promoter	—	Hueston et al. (2016)
Or49a	T2A-QF2 inserted before the stop codon	DGGR119664	This study
Or49b	QF expressed under the Or49b promoter	—	Macpherson et al. (2015)
	T2A-QF2 inserted before the stop codon	DGGR119665	This study
Or56a	LexA::p65 replaced with the endogenous gene	BDSC605642	Zhang et al. (2024)
	T2A-QF2 inserted before the stop codon	DGGR119666	This study
Or59b	LexA::p65 replaced with the endogenous gene	BDSC605643	Zhang et al. (2024)
Or59c	T2A-QF2 inserted before the stop codon	DGGR119667	This study
Or65a	LexA::p65 replaced with the endogenous gene	BDSC605644	Zhang et al. (2024)
	T2A-QF2 inserted before the stop codon	DGGR119669	This study
Or67b	T2A-QF2 inserted before the stop codon	DGGR119670	This study
Or69a	T2A-QF2 inserted before the stop codon	DGGR119671	This study
Or71a	T2A-QF2 inserted before the stop codon	DGGR119672	This study
Or82a	LexA::p65 replaced with the endogenous gene	BDSC605646	Zhang et al. (2024)
	LexA expressed under the Or82a promoter	BDSC80588	Eliason et al. (2018)
	nls-LexA::GAD expressed under the Or82a promoter	—	Kidd et al. (2015)
Orco	T2A-QF2 inserted before the stop codon	BDSC92400	Task et al. (2022)
Or83c	T2A-QF2 inserted before the stop codon	DGGR119674	This study
Or85a	LexA::p65 replaced with the endogenous gene	BDSC605647	Zhang et al. (2024)
	T2A-QF2 inserted before the stop codon	DGGR119675	This study
Or85b	LexA::p65 replaced with the endogenous gene	BDSC605648	Zhang et al. (2024)
	T2A-QF2 inserted before the stop codon	DGGR119676	This study
Or85c	LexA::p65 replaced with the endogenous gene	BDSC605648	Zhang et al. (2024)
Or85f	T2A-QF2 inserted before the stop codon	DGGR119677	This study
Or88a	LexA::p65 replaced with the endogenous gene	BDSC605649	Zhang et al. (2024)
Or98P	T2A-QF2 inserted before the stop codon	DGGR119679	This study
Ir8a	T2A-QF2 inserted before the stop codon	BDSC92398	Task et al. (2022)

(Continues)

TABLE 1 | (Continued)

Gene	Method	Stock #	References
Ir21a	QF2w expressed under the Ir21a promoter	—	Castaneda et al. (2024)
Ir25a	T2A-QF2 inserted before the stop codon	BDSC92392	Task et al. (2022)
Ir40a	LexA:VP16 expressed under the Ir40a promoter	—	Silbering et al. (2015)
Ir52a	LexA::VP16 fused to IR52a 5′ flanking region	BDSC60692	Koh et al. (2014)
Ir56d	LexA:VP16 expressed under the Ir40a promoter	BDSC81253	Sánchez-Alcañiz et al. (2018)
Ir76b	T2A-QF2 inserted before the stop codon	BDSC92396	Task et al. (2022)
Ir84a	lexA::VP16 expressed under the Ir84a promoter	BDSC41751	Grosjean et al. (2011)
Ir93a	LexA expressed under the Ir93a promoter	—	Frank et al. (2015)
Ir94a	LexA expressed under the Ir94a promoter	—	Frank et al. (2015)
Ir94e	nls-LexA::P65 replaced with the endogenous gene	—	McDowell et al. (2022)
Larval specific receptor			
Or22c	T2A-QF2 inserted before the stop codon	DGGR119659	This study
Or24a	T2A-QF2 inserted before the stop codon	DGGR119660	This study
Or63a	T2A-QF2 inserted before the stop codon	DGGR119668	This study
Or83a	T2A-QF2 inserted before the stop codon	DGGR119673	This study
Or94b	T2A-QF2 inserted before the stop codon	DGGR119678	This study

Note: The list of *QF2* or *LexA* drivers under the control of the expression of the *Or* or *Ir* genes. How to express a driver is described in method column. BDSC: the stock number at Bloomington *Drosophila* stock center. DGGR: the stock number at Kyoto *Drosophila* stock center.

Or63a, Or83a, and Or94b, which are expressed only in larvae (Table 1).

2.2 | Expected Expression of the *Or-QF2^KI^* Driver

To examine QF2 expression in the ORNs, the *Or-QF2^{KI}* lines were crossed with the *10xQUAS-6xGFP* reporter line (Figure 2). Previous studies identified the projecting pattern of ORN axons to the glomerulus using transgenic *Or-Gal4* drivers, which express *Gal4* under the putative promoter fragment of *Or* genes (Couto et al. 2005; Fishilevich et al. 2005; Fishilevich and Vosshall 2005; Kreher et al. 2005). All of the established *Or-QF2^{KI}* drivers labeled the expected glomerulus corresponding to the pattern that the *Or-Gal4* drivers labeled (Figure 2). Previously, the *Or59c-Gal4* driver labeled two glomeruli (1 and VM7) (Couto et al. 2005). In the *Or59c-QF2^{KI}* driver, a single glomerulus, VM7, was labeled, which resembles the case of Or67d, where *Gal4* knock-in at the endogenous *Or67d* gene locus labeled a single glomerulus, while the transgenic *Or67d-Gal4* driver using the putative promoter of Or67d labeled two glomeruli (Couto et al. 2005; Fishilevich and Vosshall 2005; Kurtovic et al. 2007). Some *Or-QF2^{KI}* drivers (Or42a, Or49b, Or56a, Or69a, and Or85f) exhibited an expanded expression pattern rather than the expected glomerulus (Figure 3). A previous study mentioned that the selection marker downstream of the driver sequence partially causes this additional labeling (Zhang et al. 2024). In our case, the elimination of the *3xP3-mCherry* selection marker via Cre recombinase abolished or reduced the additional signal in *Or49b-*, *Or56a-*, *Or69a-*, and *Or85f-QF2^{KI}* (Figure 3A), further suggesting that the selection marker would interfere with the specific expression of Ors.

2.3 | Identification of the Novel Expression Pattern of Or42a

Surprisingly, the *Or42a-QF2^{KI}* driver strongly labeled the additional glomerulus even after the selection marker was removed (Figure 3B). The *Or42a-QF2^{KI}* driver unexpectedly labeled the VL2p glomerulus in addition to the VM7 glomerulus labeled by the transgenic *Or42a-Gal4* driver, which uses the putative promoter region of the *Or42a* gene to express *Gal4* (Figure 4A; Couto et al. 2005; Fishilevich and Vosshall 2005). Previously, a certain transgenic *Or42a-Gal4* driver used by Chou et al. (2010) labeled the glomerulus VL2p and V as well as VM7, but it was considered an ectopic expression of Gal4. To validate the expression of Or42a in the ORNs projecting to the VL2p glomerulus, we analyzed the Fly Cell Atlas (FCA), a single-nucleus RNA sequencing dataset derived from dissected olfactory organs, such as the antenna and maxillary palp, at the age of 5 days (Li et al. 2022). The VL2p glomerulus is innervated by axons of the antennal ORNs expressing Ir31a (Silbering et al. 2011). We found that some ORNs express both Or42a and Ir31a in the antennal data of FCA, even though Or42a is known to be expressed in the maxillary palp ORNs rather than the antennal ORNs (Figure 4B; Couto et al. 2005; Fishilevich and Vosshall 2005). Consistently, the green fluorescent protein (GFP) signal derived from *Or42a-QF2^{KI}* was detected in the maxillary palp as well as in the antenna (Figure 5A). These

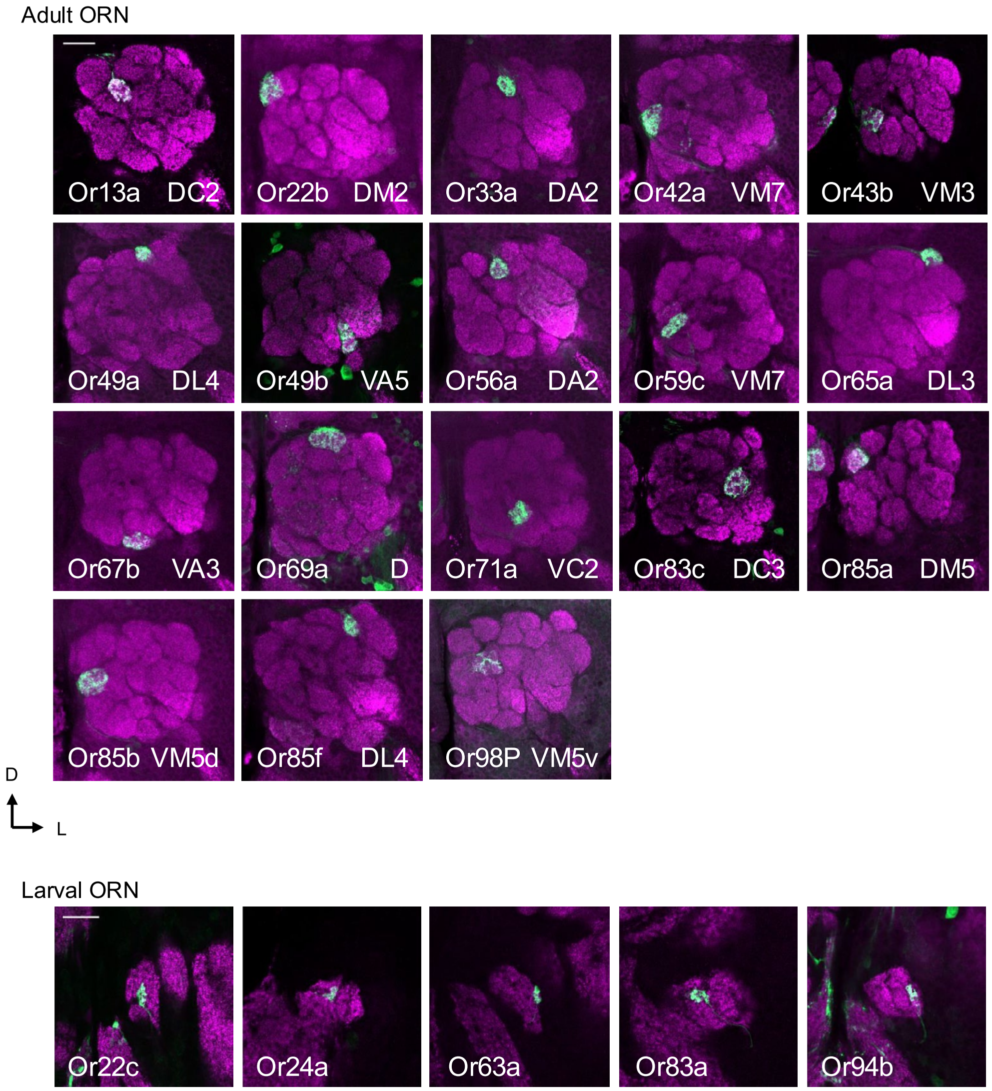

FIGURE 2 | Expression of the *Or-QF2*^{KI} drivers in ORNs. The *Or-QF2*^{KI} lines were crossed with *10xQUAS-6xGFP*. The images shown are a single section of the antennal lobe. GFP was stained with an anti-GFP antibody (green), and the presynaptic marker was stained with an nc82 antibody (magenta) to visualize the glomeruli. Scale bar = 20 μm.

data indicate that the antennal ORNs expressing Or42a project their axons to the VL2p glomerulus.

Drosophila Or forms a ligand-gated ion channel with a co-receptor, Orco, which is broadly expressed in ORNs (Benton et al. 2006; Sato et al. 2008; Wicher et al. 2008). Although the antennal ORNs expressing Or42a project their axons to the VL2p glomerulus, the ORNs expressing Orco do not project to VL2p, which was reported using the *Orco-QF2*^{KI} driver (Task et al. 2022). To examine the possibility that the antennal ORNs expressing Or42a do not express Orco, we performed the staining with an anti-Orco antibody and analysis of the FCA data. Most of the maxillary palp Or42a$^+$ ORNs expressed Orco both in the staining experiment and the maxillary palp FCA data (Figure 5B,C). However, most of the antennal ORNs expressing Or42a did not express Orco, neither in the staining experiment nor in the antennal FCA data (Figure 5D,E). This suggests that the antennal ORNs expressing Or42a do not express Orco.

3 | Discussion

The comprehensive establishment of olfactory receptor Gal4 drivers has primarily been dedicated to the development of the study of *Drosophila* olfaction (Couto et al. 2005; Fishilevich and Vosshall 2005; Sánchez-Alcañiz et al. 2018), whereas other binary expression systems, such as the *QF/QUAS* or *LexA/LexAop* systems, to manipulate ORNs have only been partially generated (Table 1; Lai and Lee 2006; Potter et al. 2010; Zhang et al. 2024). In this study, we generated 23 *Or-QF2*^{KI} knock-in drivers, which reflect the endogenous expression of Ors using the CRISPR/Cas9 knock-in technique. Most *Or-QF2*^{KI} drivers labeled the corresponding glomerulus identified by the *Or-Gal4* driver, thereby supporting the reliability of this developed tool. The combination of the *Or-QF2*^{KI} driver and the other Gal4 or LexA driver allows the simultaneous manipulation of multi-type cells. For instance, one can monitor the neural activity of the higher-order olfactory neurons, such as the mushroom body neurons expressing a calcium sensor by a *Gal4* or *LexA* driver,

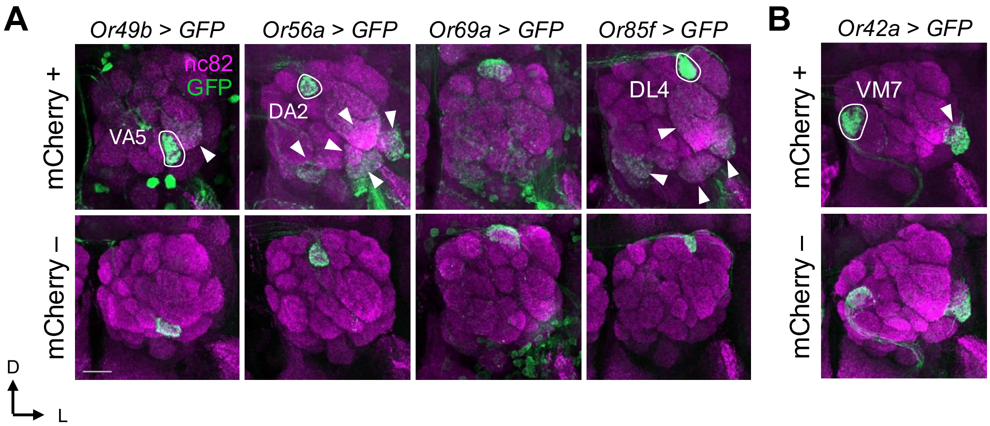

FIGURE 3 | Some *Or-T2A-QF2*^{KI} drivers weakly labeled the additional glomerulus. *Or49b-*, *Or56a-*, *Or69a-*, *Or85f-QF2*^{KI} (A), and *Or42a-QF2*^{KI} (B) were crossed with *10xQUAS-6xGFP* lines before (top) and after (bottom) the elimination of the 3XP3-mCherry selection marker using Cre recombination. The image shown is the full z-stack of the adult antennal lobe. GFP was stained with an anti-GFP antibody (green), and the presynaptic marker was stained with an nc82 (magenta) antibody to visualize the glomeruli. The expected glomerulus is outlined. Arrowheads indicate the additional glomerulus labeled by the *Or-QF2*^{KI} driver. Scale bar = 20 μm.

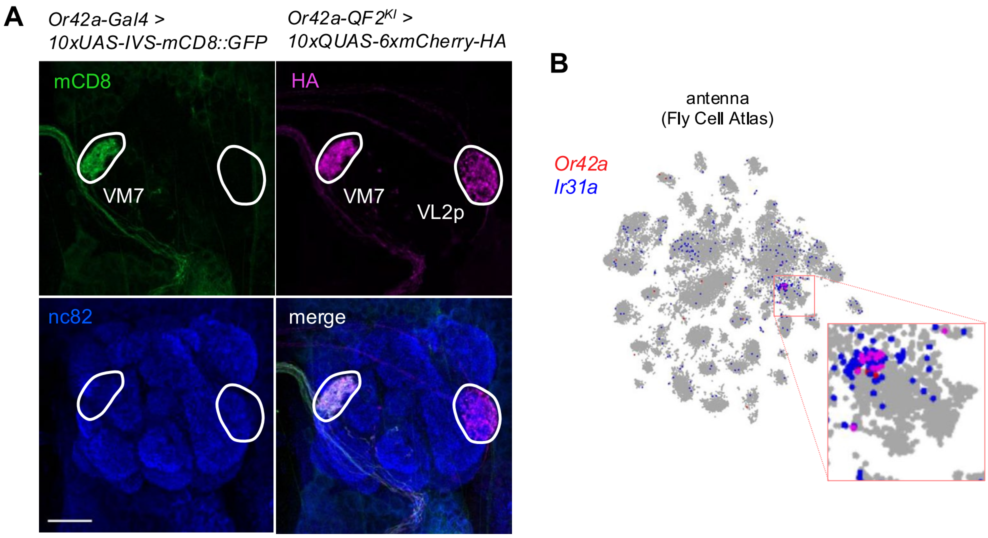

FIGURE 4 | *Or42a-QF2*^{KI} driver reveals a novel expression pattern. (A) Or42a ORNs were labeled by the *Or42a-Gal4* (Fishilevich and Vosshall 2005) and *Or42a-QF2*^{KI} drivers. The image shown is the full z-stack of the adult antennal lobe. mCD8::GFP (green) was expressed under the Gal4 driver, and 6xmCherry-HA (magenta) was expressed under the *QF2*^{KI} driver. The presynaptic marker was stained with a nc82 antibody (blue) to visualize the glomeruli. Scale bar = 20 μm. (B) *t*-Distributed Stochastic Neighbor Embedding (*t*-SNE) plot of the antenna (10× stringent dataset) in the Fly Cell Atlas (Li et al. 2022). Or42a^{+} cells: red; Ir31a^{+} cells: blue; and Or42a^{+} Ir31a^{+} cells: magenta.

while manipulating the activity of specific ORNs by the *Or-QF2*^{KI} driver. In addition, the molecular mechanism underlying the synaptic assembly of ORN-PN pairs can be analyzed by the combinatorial use of the *Or-QF2*^{KI} driver, which labels ORNs, and the *PN-Gal4* driver, which allows genetic manipulation in PNs. Thus, *Or-QF2*^{KI} strains lead to a better understanding of the function and development of olfactory neural circuits.

We found that the *Or42a-QF2*^{KI} driver labeled the Or42a^{+} ORNs, projects their axons to the VL2p glomerulus as well as to the VM7 glomerulus. Based on the FCA analysis, there are two types of Or42a^{+} ORNs: one is in the maxillary palp and the other is in the antenna. The antennal Or42a^{+} ORNs also express Ir31a and send axons to the VL2p glomerulus. Intriguingly, immunostaining using an anti-Orco antibody confirmed that the antennal Or42a^{+} ORNs are unlikely to express Orco, which is thought to be essential for olfactory function. These observations raise several possibilities: (1) Or42a solely forms the homotetramer. *Machilis hrabei*, the basal insect, has a small number of olfactory receptor genes, which do not contain Orco (Brand et al. 2018). Without a co-receptor, MhOR5 forms the homotetramer (del Mármol et al. 2021). Another recent report suggests that *Drosophila* Or42b also forms a homotetramer without Orco (Lee et al. 2025). These indicate the possibility that Or42a solely forms the homotetramer without Orco. (2) Or42a forms a complex with other partners, such as the Ir co-receptor, rather than with Orco. To validate these possibilities, further analysis is required, for instance, extracellular electrophysiological recordings to measure the odor-evoked activity and coimmunoprecipitation to identify the partner of Or42a. Furthermore, the application of the T2A-QF2 knock-in strategy to other ORs

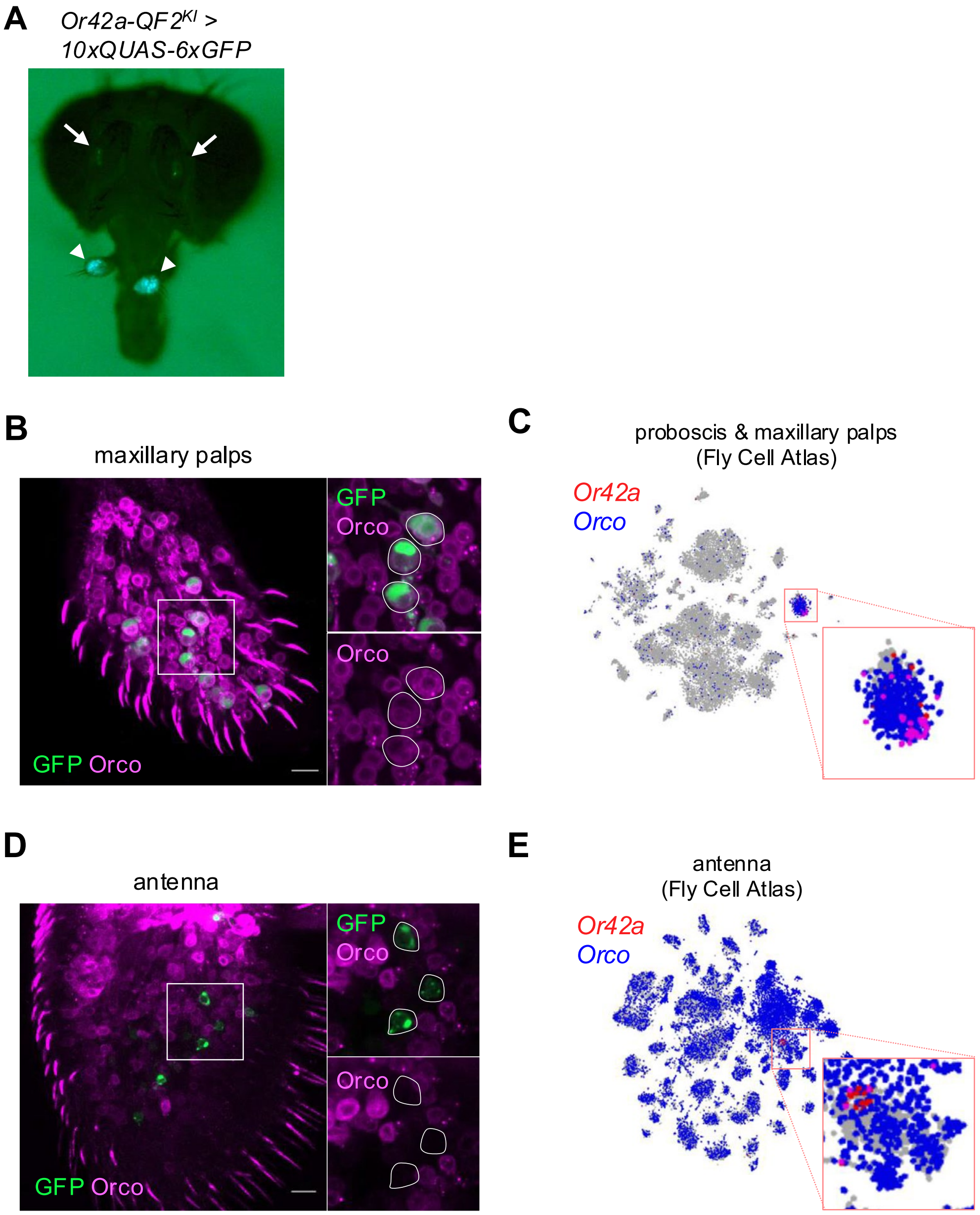

FIGURE 5 | Antennal ORNs expressing *Or42a* did not express Orco. (A) The antenna and maxillary palp were labeled by the *Or42a-QF2^{KI}* driver. The GFP signal was derived from the *Or42a-QF2^{KI}* driver in the adult fly head. The arrowhead indicates the maxillary palp, and the arrow indicates the antenna. (B) Whole-mount staining of the maxillary palp with anti-Orco antibody (magenta) in *Or42a-QF2^{KI}>10xQUAS-6xGFP* (green). The right figures show an enlargement of the boxed area. (C) *t*-SNE plot of the proboscis and maxillary palp (10× stringent dataset) in the FCA (Li et al. 2022). Or42a^{+} cells: Red; Orco^{+} cells: blue; and Or42a^{+} Orco^{+} cells: magenta. (D) Whole-mount staining of the antenna with anti-Orco antibody (magenta) in *Or42a-QF2^{KI}>10xQUAS-6xGFP* (green). The right figures show an enlargement of the boxed area. (E) The *t*-SNE plot of the antenna (10× stringent dataset) in the FCA (Li et al. 2022). Or42a^{+} cells: red; Orco^{+} cells: blue; and Or42a^{+} Orco^{+} cells: magenta. Scale bar = 10 µm.

would contribute toward revealing novel insights into the molecular mechanisms of ORs.

4 | Experimental Procedures

4.1 | *Drosophila* Stocks and Husbandry

The following strains were obtained from the Bloomington *Drosophila* Stock Center: *10xQUAS-6xGFP* (#52263, #52264), Crey+ 1B (#766, #851), *10xQUAS-6xmCherry-HA* (#52270), *10xUAS-IVS-mCD8::GFP* (#BL32186), and *Or42a-Gal4* (#9969). All flies were maintained at 25°C under standard laboratory conditions.

4.2 | Generation of the *Or-QF2^{KI}* Knock-In Lines

Donor plasmids for the *Or-QF2^{KI}* knock-in lines were constructed using the *pHACK-QF2* plasmid (Addgene #80274). The plasmid was digested with *Mlu*I for the 5′ homology arm and *Spe*I for the 3′ homology arm. The homology arms were amplified from the genomic DNA of the *Canton-S* strain and were assembled into the digested plasmid using the NEBuilder

HiFi DNA Assembly Master Mix (New England Biolabs, #E2621L). gRNAs were designed to target downstream from the stop codon and as close as possible to the stop codon using flyCRISPR (https://flycrispr.org). The annealed oligos for the gRNAs were cloned into a *Bbs*I-digested *U6b-sgRNA-short* plasmid (a kind gift from N. Perrimon) using Ligation high Ver. 2 (TOYOBO, #LGK-201). The donor and gRNA plasmids were injected into *nos-Cas9* flies (NIG-FLY #CAS-0011, #CAS-0012). The primers for the homology arms and oligos for the gRNAs are presented in Table S1. The flies exhibiting the mCherry signal in their eyes were screened. To ensure the proper knock-in, all *Or-QF2^{KI}* lines were required to fulfill three criteria: genomic PCR, sequencing of the region around the border between the exon and T2A-QF2 cassette, and the activity of the *Or-QF2^{KI}* driver in the ORNs by crossing with the *10xQUAS-6xGFP* reporter. These *Or-QF2^{KI}* strains are available at Kyoto *Drosophila* Stock Center.

4.3 | Removal of 3xP3-mCherry

To remove the 3XP3-mCherry cassette, the eye selection marker, *Or-QF2^{KI}* lines were crossed with Crey+ 1B flies, in which Cre recombinase is expressed in both the germline and somatic tissue (Siegal and Hartl 1996).

4.4 | Immunostaining

The brains (1–5 days old adults and wandering larvae), antennae (3 days old adults), and maxillary palps (3 days old adults) were dissected in 1× phosphate-buffered saline with 0.3% Triton X-100 (0.3% PBST) and fixed in 4% paraformaldehyde (PFA) in 0.3% PBST for 20–30 min at room temperature (RT). The fixed samples were rinsed three times with 0.3% PBST for 20 min each at RT. The samples were blocked with 5% normal goat serum in 0.3% PBST for 1 h at RT and incubated with primary antibody diluted with 0.3% PBST overnight at 4°C. The samples were rinsed three times with 0.3% PBST for 20 min each at RT and incubated with the secondary antibody diluted with 0.3% PBST overnight at 4°C. After washing three times with 0.3% PBST for 20 min each at RT, the samples were mounted in SlowFade Diamond (Thermo Fisher, S36972). The primary antibodies used were nc82 (DSHB, 1:40), rabbit anti-GFP (Invitrogen, A6455, 1:1000), rat anti-HA (3F10) (Roche, 11,867,423,001, 1:500), rat anti-mCD8 (Invitrogen, MCD0830, 1:100), and rabbit anti-Orco (gifted by Leslie Vosshall, 1:100). The secondary antibodies used were goat anti-rabbit Alexa 488 (Invitrogen, A11034, 1:2000), goat anti-mouse Alexa 555 (Invitrogen, A32723, 1:125), goat anti-rat Alexa 568 (Invitrogen, A11036, 1:500), goat anti-rabbit Alexa 568 (Invitrogen, A11077, 1:500), and goat anti-mouse Alexa 633 (Invitrogen, A21052, 1:500). Images were obtained using a laser scanning confocal microscope (Zeiss LSM900).

4.5 | Expression Analysis via the FCA

The expression of olfactory receptor genes in the antenna and maxillary palp (Figures 4B and 5C,E) was analyzed using the single-nucleus RNA-seq data of the proboscis and maxillary palp (10× stringent dataset) and the antenna (10× stringent dataset) from the FCA. HVG t-SNE plots were visualized using SCope (https://scope.aertslab.org/#/FlyCellAtlas/*/welcome; Davie et al. 2018). To color the cells expressing each gene at lower levels, the upper threshold in the scale tool was lowered.

Author Contributions

Y.U. and T.C. conceived the project. Y.U. and R.S. performed the experiments, except for the injections. K.M. and K.S. performed the injections to generate the knock-in lines. T.C. and M.O. supervised the project. The manuscript was written by Y.U. and T.C. with input from all authors.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.