ROLE OF IROC IN THE FLY OLFACTORY SYSTEM

by

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ABSTRACT

ROLE OF IROC IN THE FLY OLFACTORY SYSTEM

Sensory neurons, such as the visual and olfactory neurons, tend to express only one sensory receptor per neuron in order to prevent sensory overlap and then send their axons to particular target regions in the brain to form specific connections. The molecular mechanisms underlying either of these processes are largely unknown. The olfactory system represents an extreme example as only one receptor from a very large repertoire of receptor genes (up to 1200 in mouse) is selected for expression. The olfactory system of *Drosophila* represents an ideal system to study these mechanisms as it is quantitatively reduced and very well described. We have observed expression of a transcription factor family, *IroC*, in the maxillary palps, one of the two olfactory organs in the fly, and especially in the subset of olfactory receptor neurons, which coexpresses two olfactory receptor genes, *OR33c* and *OR85e*. *IroC* has previously been shown to regulate the coexpression of two rhodopsin genes in the fly retina and to contribute to retinal axon guidance. We thus aimed to identify the putative role of this family of transcription factors in the fly olfactory system by performing classical loss-of-function and gain-of-function experiments. Our results attribute three functions to *IroC* in the fly olfactory system. (i) Iro proteins repress the expression of *OR33c* and *OR85e* genes. (ii) Iro proteins may have a role in the specification of pb2B neurons from progenitor cells. (iii) Iro proteins contribute to the proper targeting of olfactory receptor neurons.

ÖZET

IROC'NİN SİNEK KOKU ALMA SİSTEMİNDEKİ ROLÜ

Görme ve koku alma nöronları gibi duyu nöronları duyusal örtüşmeyi engellemek amacıyla nöron başına sadece bir duyu reseptörü sentezleme eğilimindedirler ve beyindeki belirli bölgelere aksonlarını göndererek özel bağlantılar oluşturmaktadırlar. Bahsi geçen her iki sürecin de altında yatan moleküler mekanizmalar büyük ölçüde bilinmemektedir. Koku alma sistemi sadece tek bir reseptör geninin çok geniş bir repertuardan (farede 1200 civarında) seçilip ifade edilmesi itibariyle uç bir örnek teşkil etmektedir. *Drosophila*'nın koku alma sistemi, sayıca az hücre ve reseptörden oluşması ve çok iyi tanımlanmış olması itibariyle bu mekanizmaları çalışmak için ideal bir sistem örneğidir. *Drosophila*'nın iki koku alma organından biri olan maxillary palp'ta, özellikle de iki koku reseptör geninin, *OR33c* ve *OR85e*'nin, birlikte sentezlendiği bir alt grup koku alma nöronlarında, *IroC* transkripsiyon faktör ailesinin ifadesini gözlemledik. Daha önce *IroC*'nin iki rodopsin geninin sinek retinasında birlikte sentezlenmesini düzenlediği ve retina hücrelerinin aksonlarının bağlantısına katkıda bulunduğu gösterilmiştir. Bu bilgiler ışığında klasik işlev kaybettirme ve işlev kazandırma deneyleri yaparak bu gen ailesinin sinek koku alma sistemindeki olası rolünü tespit etmeyi amaçladık. Sonuçlarımız *IroC*'ye sinek koku alma sisteminde üç işlev atfetmektedir. (i) Iro proteinleri, *OR33c* ve *OR85e* genlerinin ifadesini baskılamaktadır. (ii) Iro proteinlerinin, pb2B nöronlarının progenitör hücrelerden özelleşmesinde rolü olabilir. (iii) Iro proteinleri koku alma nöronlarının beyinde doğru bağlantıları oluşturmasına katkıda bulunmaktadır.

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LIST OF ACRONYMS/ABBREVIATIONS

Acj6	Abnormal Chemosensory Jump 6
AL	Antennal Lobe
ara	Araucan
BAC	Bacterial Artificial Chromosome
bp	Base Pairs
bHLH	Basic Helix-Loop-Helix
caup	Caupolican
cDNA	Complementary DNA
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
Dscam	Down Syndrome Cell Adhesion Molecule
FRT	Flip Recombinase Targets
GFP	Green Fluorescent Protein
GPCR	G-Protein Coupled Receptor
GR	Gustatory Receptor
IR	Ionotropic Receptor
Iro	Iroquois
IroC	Iroquois Complex
LN	Local Interneuron
MB	Mushroom Body
mirr	Mirror
N	Notch
OBP	Odor Binding Protein
OR	Olfactory Receptor
ORN	Olfactory Receptor Neuron
pb	Palp Basiconic
PBP	Pheromone Binding Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pdm3	POU Domain Motif 3

PFA	Paraformaldehyde
pH	Power of Hydrogen
PN	Projection Neuron
PR	Photoreceptor
RFP	Red Fluorescent Protein
Rh3	Rhodopsin3
Rh4	Rhodopsin4
RNA	Ribonucleic Acid
RNAi	RNA Interference
RT-PCR	Real Time PCR
SEM	Standard Error of the Mean
SOP	Sense Organ Precursor
UAS	Upstream Activating Sequence

1. INTRODUCTION

Because we, as human beings, are so visually oriented, it is rather difficult for us to appreciate the vital importance of chemical sensation. In fact the ability to detect, interpret and respond to chemical stimuli is the primary skill for most of the animal species. This ability, indeed, modulates our feeding, mating and social behaviors and also processes like learning and memory. This is why the understanding of olfactory organization, one such chemosensory system, was awarded with a Nobel Prize in Physiology or Medicine to Dr. Linda Buck and Dr. Richard Axel in 2004.

1.1. Olfaction: Common Principles across Phyla

Olfaction, the sense of smell, is probably the most ancient of sensory modalities in animals (Strausfeld and Hildebrand, 1999). As in the case of other senses like hearing and seeing, olfactory organization with respect to its neural organization is similar in remotely related species (Strausfeld and Hildebrand, 1999). It has been suggested that common/identical selective pressures resulted in the convergent evolution to solve common/identical physicochemical constraints (Ache and Young, 2005).

The cells responsible for odor detection are termed olfactory receptor neurons (ORNs) and inhabit appendages what we call nose. These cells are bipolar neurons in which dendrites terminate under the epidermis in an array of filamentous processes, whereas axons extend into the olfactory processing centers in the central nervous system (CNS) where they synapse to secondary projection neurons (Hildebrand and Shepherd, 1997; Fuss and Ray, 2009). Secondary projection neurons in turn transmit the signal to higher brain centers (Hildebrand and Shepherd, 1997) (Figure 1.1).

Dendrites are not in direct contact with the environment, but instead they project into a fluid-filled compartment -the lymph-, which contacts the environment directly (Ache and Young, 2005). Odor molecules (airborne, volatile odorants for terrestrial animals or odorants in solution for aquatic animals) are solved in this fluid-filled compartment before they bind to the responsive receptors found on the dendritic surface of ORNs (with the aid

of OBP or PBP) (Ache and Young, 2005). Binding of an odorant molecule to its receptor depolarizes the cell. Depolarized axons then transmit the signal to projection neurons, which further relay the signal to higher brain centers (Hildebrand and Shepherd, 1997).

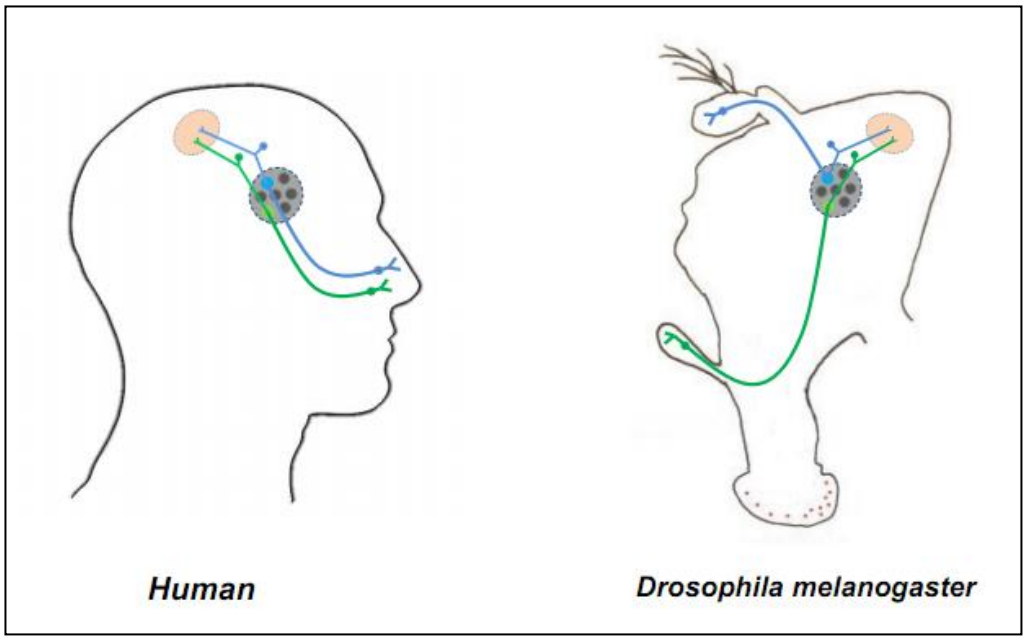

Figure 1.1. Olfaction: common principles. Bipolar ORNs housed in peripheral olfactory organs recognize the odor molecules. Binding of an odor molecule to its receptor depolarizes the ORN. Depolarized neurons transmit the signal to the olfactory processing centers in the CNS and synapse to secondary projection neurons which in turn relay the signal to higher brain centers.

In addition to these striking morphological similarities across species, mechanisms underlying olfactory discrimination are fundamentally similar. For most animals to detect and respond differentially to an odor is of vital importance. Despite other minor factors there are two main events that determine the way of deciphering odors: receptor choice and specific targeting of axons. First of all, there is a general phenomenon called “one neuron-one receptor” rule. According to this phenomenon, each olfactory neuron tends to express only one olfactory receptor (OR) from a large repertoire of receptors (Mazzoni *et al.*, 2004; Hallem and Carlson, 2004). Secondly, the neurons, which express the same olfactory receptor project their axons to the same units in the olfactory processing centers (Hallem and Carlson, 2004; Fuss and Ray, 2009) (Figure 1.2).

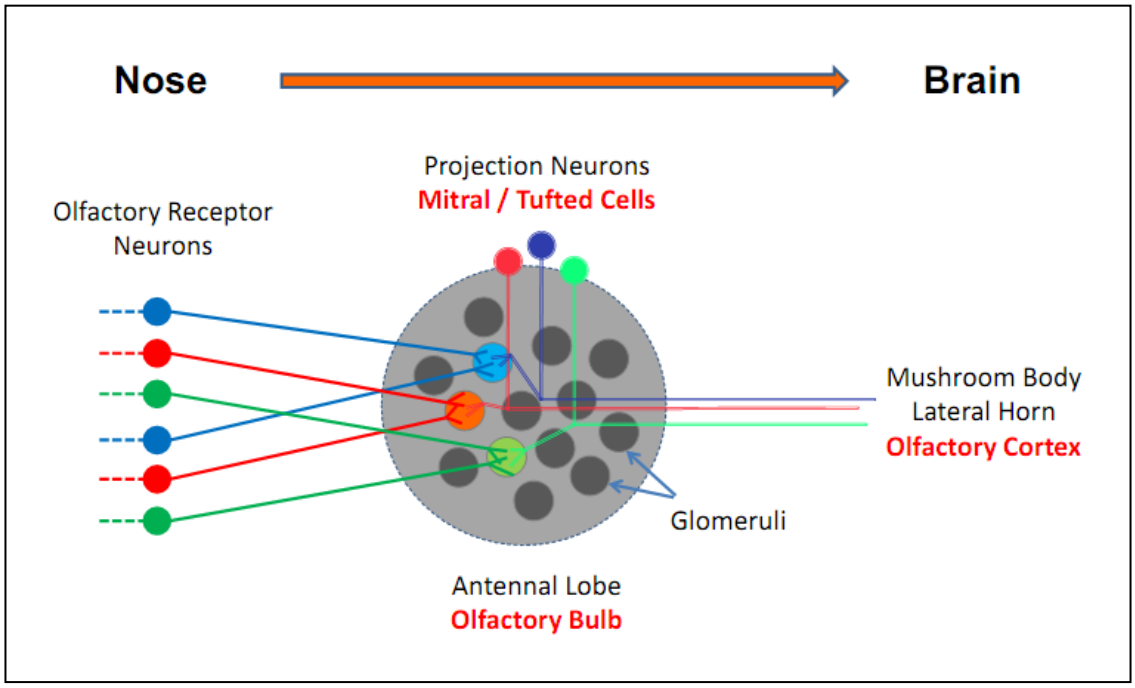

Figure 1.2. Schematic representation of the *Drosophila* olfactory system, with mammalian counterparts labeled in red. Axons of ORNs that express the same receptor converge on structures in the antennal lobe called glomeruli; hence they create a spatial odor map. This spatial map is mostly preserved by the corresponding projection neurons that synapse with the ORNs in higher brain centers (adapted from Jefferis *et al.*, 2001).

It does not matter whether these similar solutions to odor detection and recognition either evolved early and were retained in evolution or evolved convergently in animals. Either way, the similarities in olfactory organization make the model organisms valuable in the investigation of the sense of smell (Ache and Young, 2005). The fruit fly *Drosophila melanogaster* has a relatively simple olfactory system (Vosshall and Stocker, 2007). The availability of genetic and molecular approaches, plus the ease of *in vivo* measurement, either physiological or behavioral, make *Drosophila* a perfect model to study the olfactory system (Carlson, 1996).

1.2. Organization of the *Drosophila melanogaster* Adult Olfactory System

There are two pairs of olfactory organs in adult *Drosophila*, the - third segment of - antennae and the maxillary palp (Figure 1.3A). Each antenna, the main olfactory organ,

contains about 1200 ORNs, whereas each maxillary palp contains about 120 ORNs. The surface of both organs is covered with more than 450 hair-like-structures called sensilla and ORNs together with non-neuronal support cells are housed in these sensilla (Shanbhag *et al.* 1999; Hallem and Carlson, 2004) (Figure 1.3B). Support cells protect ORNs from the enviroment, secrete sensillum lymph and keep each sensillum electrically insulated from its neighbor (Vosshall and Stocker, 2007). There are three morphological types of sensilla, basiconic, coeloconic and trichoid, that differ in number, size and morphology (Shanbhag *et al.* 1999; Vosshall and Stocker, 2007). The antenna includes all of these three morphologically different types of sensilla, concentrated in distinct regions [large basiconic sensilla clustered at the medial-proximal side of the antenna, trichoid sensilla clustered at the lateral-distal edge of the antenna and small basiconic and coeloconic sensilla are interspersed in the middle region of the antenna (Shanbhag *et al.* 1999)], whereas the maxillary palp contains only basiconic sensilla (Vosshall and Stocker, 2007). Usually two ORNs are housed in each sensillum, but this number can change between 1 and 4. The sensilla in the maxillary palp have always two ORNs (Hallem and Carlson, 2004).

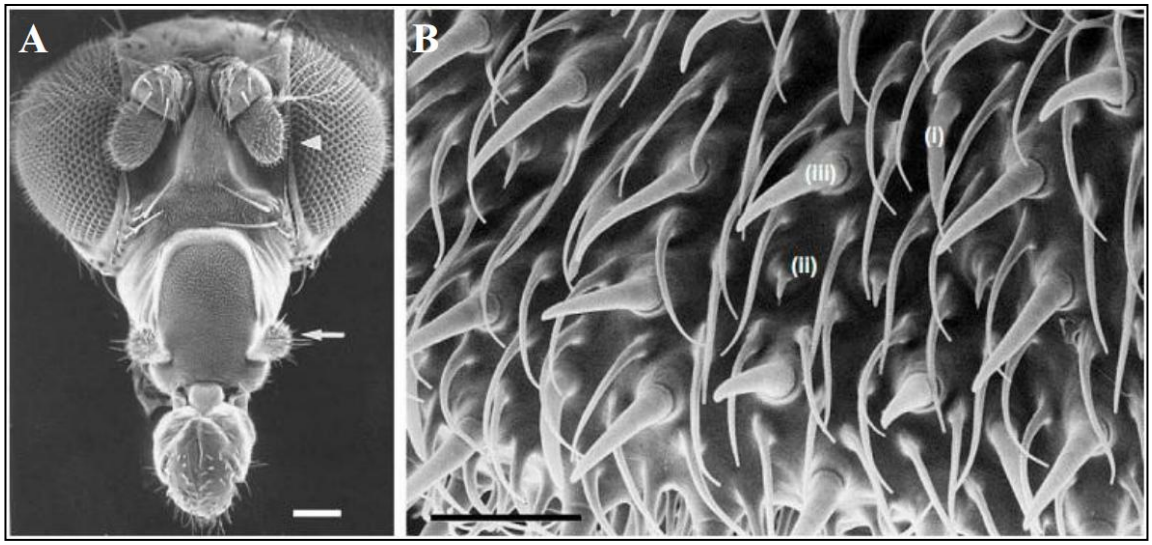

Figure 1.3. The *Drosophila* olfactory organs. (A) The adult head with antennae (arrow head) and maxillary palps (arrow). Scale bar=100 µm. (B) Three morphological types of olfactory sensilla on the antennal surface: (i) basiconic, (ii) coeloconic and (iii) trichoid. Scale bar=10 µm (taken from Hallem and Carlson, 2004).

The olfactory sensilla of both organs derive from antennal imaginal discs and two basic helix-loop-helix (bHLH) transcription factors, Atonal and Amos, are required for initial specification of undifferentiated cells in the antennal disc (Brochtrup and Hummel, 2010). Atonal determines the fate of coeloconic sensilla in the antenna and basiconic sensilla in the maxillary palp (Gupta and Rodrigues, 1997; Jhaveri *et al.*, 2000a), whereas Amos determines the fate of both basiconic and trichoid sensilla in the antenna (Gupta *et al.*, 1998; Goulding *et al.*, 2000; zur Lage *et al.*, 2003). Together with Amos, the relative amount of another transcription factor, Lozenge, distinguishes basiconic from trichoid sensilla (Gupta *et al.*, 1998; Goulding *et al.*, 2000; zur Lage *et al.*, 2003).

Different models have been proposed for the development of ORNs (Simpson, 1990; Campos-Ortega, 1993; Ray and Rodrigues, 1995; Sen *et al.*, 2003; Lai and Orgogozo, 2004; Endo *et al.*, 2007). Recent findings suggest that ORNs together with support cells of a sensillum develop from a common sensory organ precursor (SOP) (Figure 1.4). Initially, together with the expression of proneural genes, all cells in the antennal disc are undifferentiated and competent to become a SOP. Upon lateral inhibition via Notch signaling, accumulation of proneural proteins occurs, which results in the specification of a SOP. Subsequent divisions of SOPs, together with varying levels of Notch activity, produce all different components of the sensillum (Endo *et al.*, 2007).

ORNs send their axons to the antennal lobe (AL) which is functionally similar to the olfactory bulb in vertebrates (Figure 1.2). The AL is composed of spherical units called glomeruli. In the ALs ORNs synapse to the second order neurons called projection neurons (PNs), which in turn transmit the signal to higher brain centers, like mushroom body (MB) and lateral horn. Other than these two types of neurons, ORNs and PNs, the glomeruli also contain the processes of local interneurons (LNs), which generally branch and innervate multiple glomeruli. By providing lateral connections, LNs are responsible for information transfer between the glomeruli.

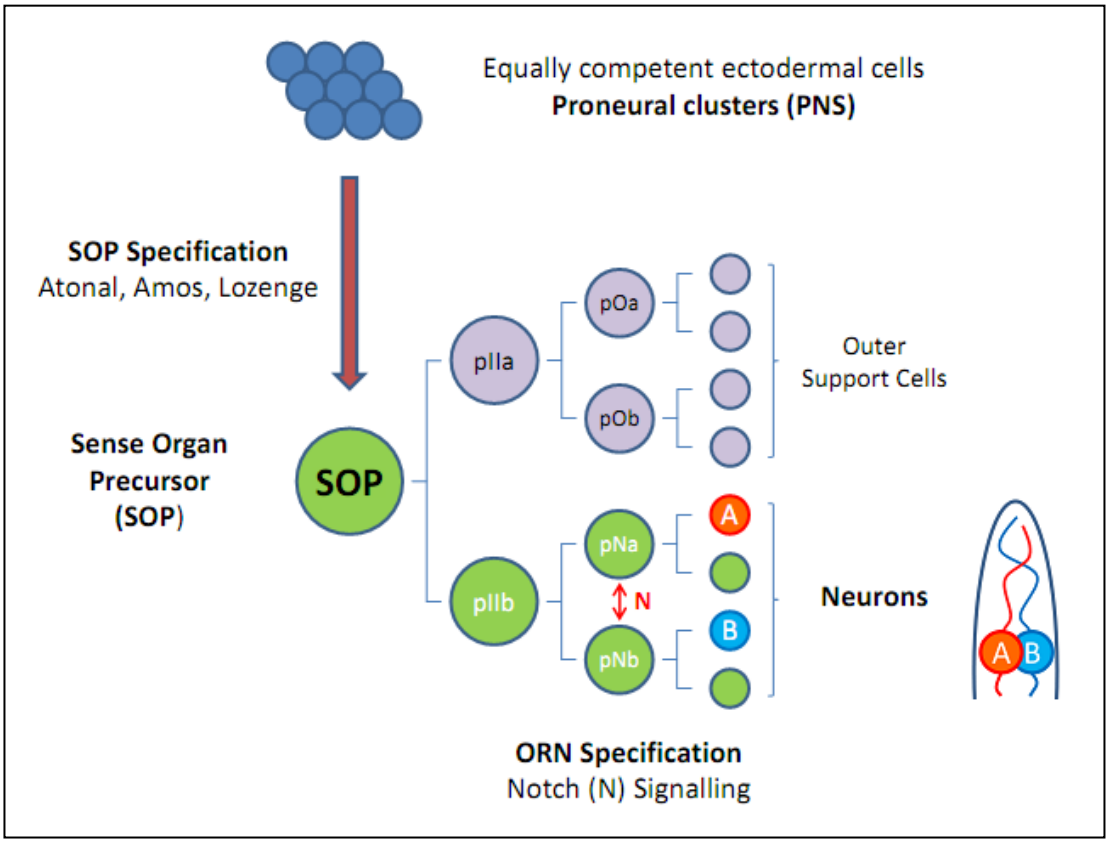

Figure 1.4. Development of ORNs in *Drosophila*. Accumulation of proneural proteins upon Notch signaling results in the specification of SOPs. Subsequent divisions of SOPs, together with varying levels of Notch activity, produce all different components of the sensillum.

1.3. Olfactory Receptor Genes

The olfactory organs have been known for a long time; however, the molecular mechanism by which odorz evoke responses were largely unknown. At the end of the 90s several groups identified some candidate olfactory receptor genes based on homology searches to vertebrate counterparts (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999). Studies have shown that these newly identified candidate olfactory receptors are selectively expressed in ORNs and are the prime sites where odor recognition occurs (Clyne *et al.*, 1999; Vosshall *et al.*, 1999; Elmore *et al.*, 2003). Initially the number of ORs was 57 (Vosshall *et al.*, 2000). Further analysis enlarged the number of ORs to 62 which are expressed from a total of 60 OR genes through alternative splicing (Robertson *et al.*,

2003; Robertson, 2009) (Table 1.1). ORs are named according to their cytogenetic position (Drosophila Odorant Receptor Nomenclature Committee, 2000).

OR genes encode receptor proteins with seven trans-membrane domains and although once thought to be similar to the known G-protein-coupled receptors (GPCRs) (which actually directed the initial fly OR search throughout the genome), they share no sequence similarity with those GPCRs (Vosshall *et al.*, 1999; Benton *et al.*, 2006; Withstrand *et al.*, 2006) and apparently they have inverted membrane topology relative to that of GPCRs (Benton *et al.*, 2006; Lundin *et al.*, 2007) (Figure 1.5). Studies suggest that fly ORs have evolved independently of chemoreceptors found in other animals and they represent a novel family of membrane proteins (Vosshall *et al.*, 1999; Benton *et al.*, 2006). Recent studies have indicated, although with different outcomes, that insect ORs together with noncanonical, widely expressed OR83b function as odor-gated ion channels (Sato *et al.*, 2008; Wicher *et al.*, 2008), whereas vertebrate ORs function as metabotropic GPCRs (Kaupp, 2010).

The fly OR family genes are evenly distributed across the genome. The lack of large clusters of OR genes (only a few instances) and the low overall amino acid homology (20%) suggests that the gene family has a very ancient origin (Robertson *et al.*, 2003).

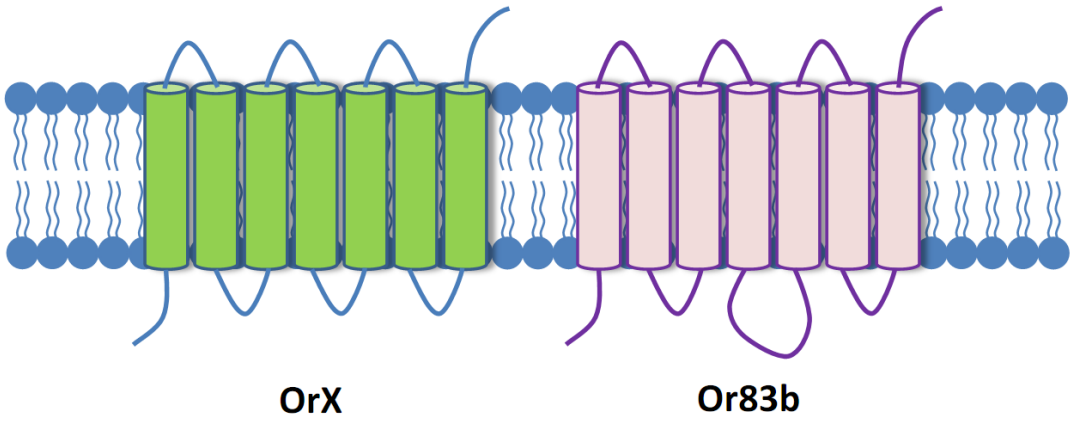

Figure 1.5. Membrane topology of ORs. Drosophila ORs and OR83b have a novel membrane topology with N-termini inside the cytoplasm. OR-OR83b receptor complex functions as a non-selective cation channel responsible for olfactory transduction.

Table 1.1. Distribution of *Drosophila* ORs among olfactory organs. A comprehensive list of ORs expressed in each olfactory organ, subdivided by functional class and sensillum. Data compiled are from Clyne *et al.* (1999), Gao and Chess (1999), Vosshall *et al.* (1999), Vosshall *et al.* (2000), Suh *et al.* (2004), Couto *et al.* (2005), Fishilevich *et al.* (2005), Fishilevich and Vosshall (2005), Goldman *et al.* (2005), Kreher *et al.* (2005), Yao *et al.* (2005), Benton *et al.* (2006), Hallem and Carlson (2006), Jones *et al.* (2007), Kwon *et al.* (2007), Vosshall and Stocker (2007) and Benton *et al.* (2009). Some ORNs express gustatory receptors (GRs) or ionotropic receptors (IRs) alone or in combinations.

Antenna						**Maxillary Palp**	**Larva**	
Basiconic Sensilla			**Trichoid Sensilla**	**Coeloconic Sensilla**		**Basiconic Sensilla**	**Dorsal Organ**	
ORX / OR83b		GR21a / GR63a	ORX / OR83b	ORX / OR83b	IRs	ORX / OR83b	ORX / OR83b	
OR7a	OR59b	GR63a	OR2a	OR35a	IR8a	OR33c	OR1a	OR45b
OR9a	OR67a		OR19a		IR21a	OR42a	OR2a	OR47a
OR10a	OR67b		OR19b		IR25a	OR46aA	OR7a	OR49a
GR10a	OR67c		OR23a		IR31a	OR59c	OR13a	OR59a
OR13a	OR69aA		OR43a		IR40a	OR71a	OR22c	OR63a
OR22a	OR69aB		OR47b		IR64a	OR85d	OR24a	OR67b
OR22b	OR82a		OR65a		IR75a	OR85e	OR30a	OR74a
OR33a	OR83c		OR65b		IR75b		OR33a	OR82a
OR33b	OR85a		OR65c		IR75c		OR33b	OR83a
OR42b	OR85b		OR67d		IR75d		OR35a	OR85c
OR43b	OR85f		OR88a		IR76a		OR42a	OR94a
OR47a	OR92a				IR76b		OR42b	OR94b
OR49a	OR98a				IR84a		OR45a	
OR49b	OR98b				IR92a			
OR56a					IR93a			

1.4. Olfactory Receptor Gene Choice

Mechanisms underlying the specific expression of OR genes are not well understood. Various models that use either stochastic or deterministic mechanisms have been proposed (Fuss and Ray, 2009). In mammals it has been proposed that expression of one OR inhibits the expression of others (Serizawa *et al.*, 2003; Lewcock and Reed, 2004; Shykind *et al.*, 2004). Contrary to the vertebrate model, there is no negative-feedback in OR gene expression which was thought to be the main regulator of the "one neuron-one receptor" rule. Early expression of an ectopic OR did neither result in the absence nor in the wrong selection of ORs (Dobritsa *et al.*, 2003; Ray *et al.*, 2007). Endo *et al.* (2007), suggest that selection of ORs is linked to the identity of the cell subclass, meaning OR selection occurs through a lineage-related mechanism through asymmetric division. These initial findings are further supported using mutants for the components of the Notch signaling pathway (Ray *et al.*, 2007). In case of *mastermind* mutants (low Notch activity), both of the ORNs residing in the same sensillum express only one of the two OR genes that are normally expressed in the sensillum; however, in case of *numb* mutants (high Notch activity), both of the ORNs express the other OR gene of the pair. Plus, early over-expression of *mastermind* across the maxillary palp results in the ectopic expression of one of the two OR genes in both neurons of the sensillum (Ray *et al.*, 2007). These results suggest that an asymmetric division process which results in low- and high-Notch sibling fates could trigger downstream events that eventually lead to the correct expression of the appropriate OR gene (Ray *et al.*, 2007).

Examination of transgenic fly lines constructed with OR upstream regions of variable length suggested that relatively short regions of upstream DNA are necessary and sufficient to drive appropriate expression (Vosshall *et al.*, 2000; Gao *et al.*, 2000; Dobritsa *et al.*, 2000; Couto *et al.*, 2005; Fishilevich and Vosshall, 2005; Goldman *et al.*, 2005). These initial observations were further supported by the finding that short regions, like 500 bps upstream of an OR start codon, are necessary and sufficient to drive the appropriate expression pattern (Ray *et al.*, 2007). These observations suggested that cis-elements regulate the expression of OR gene expression. A careful analysis of cis-elements in the maxillary palp showed that regulatory elements play a role in a combinatorial manner. Identified motifs, such as Dyad-1 and Oligo-1, not only promote expression of OR genes in

the maxillary palp, but also repress their expression in the antenna (Ray *et al.*, 2007). Thus by acting together these two cis-motifs ensure the correct expression of maxillary palp OR genes.

Some transcription factors, such as Acj6 (Abnormal chemosensory jump 6), Pdm3 (POU domain motif 3), Lozenge and Scalloped, have been shown to play a role in OR gene expression (Ray *et al.*, 2007, 2008; Tichy *et al.*, 2008). Careful analysis of mutant lines for both *acj6* and *pdm3* showed that these transcription factors are required for the expression of a specific subset of OR genes, but not for others (Ray *et al.*, 2007, 2008; Tichy *et al.*, 2008). For example, examination of *acj6* and *pdm3* mutant lines resulted in three ORN populations, one depending on both of these transcription factors, one depending on Acj6 alone and one that does not need either Acj6 or Pdm3 for OR expression (Tichy *et al.*, 2008). These studies suggested that OR gene choice relies on a combinatorial code of transcription factors. Regulation of OR gene expression is further enriched by different splice forms of the same gene. Bai *et al.* (2009) showed that splice forms of acj6 can either activate or repress OR gene expression. Rescue experiments with only one splice form of *acj6* gene in *acj6* mutant background also suggested that some ORNs may require more than one splice form in a combinatorial fashion to express the appropriate OR gene (Bai and Carlson, 2010).

Figure 1.6 summarizes the known mechanisms of OR gene expression.

1.5. Projection of Olfactory Receptor Neurons

Before the onset of OR gene expression ORNs send their axons to the AL, where PN dendrites have already established a coarse positional map: a proto-AL structure (Jefferis *et al.*, 2001, 2004). Class-specific assembly of ORNs and PN dendrites, together with the isolation of protoglomerular units by glial membranes, eventually maturate the glomeruli and hence the AL (Jhaveri *et al.*, 2000b; Jefferis *et al.*, 2004).

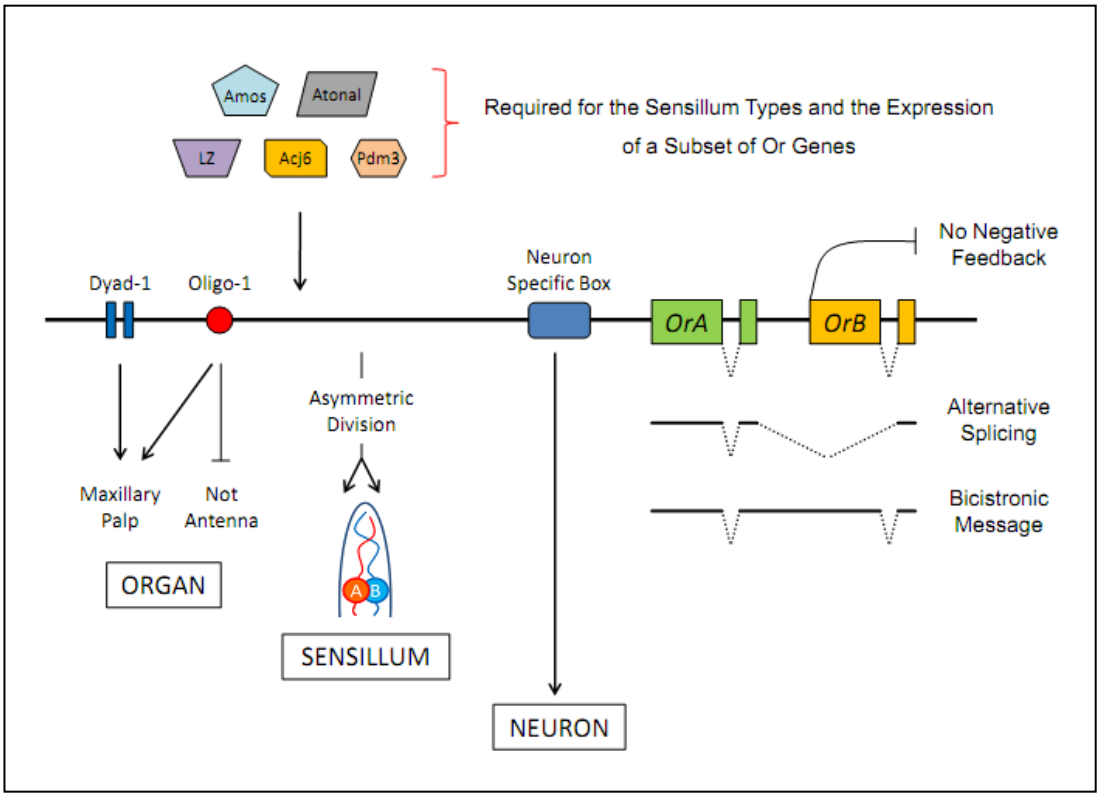

Figure 1.6. Summary of known mechanisms for receptor gene choice: a deterministic model. Transcription factors which are also responsible for the specification of sensillum subtype are expressed in overlapping subsets of ORN classes and a combinatorial code of those transcription factors may have a role in the regulation of OR gene expression. Positive and negative regulatory elements found on the upstream region (eg. Dyad-1 and Oligo1) are responsible for OR gene expression at the organ level. Additionally, elements found in the upstream region of OR genes may dictate which OR is expressed in each ORN class. Lz, Lozenge; Acj6, Abnormal chemosensory jump 6; Pdm3, POU domain motif 3 (adapted from Ray *et al.*, 2007).

The newly born ORNs in the periphery send their axons to the central nervous system and make specific connections in a stepwise fashion via responding to short- and long-range cues that can either attract or repel growth cones (Yu and Bargmann, 2001; Hummel *et al.*, 2003; Rodrigues and Hummel, 2008). For the initial protoglomerulus formation cell-adhesion molecules, such as N-Cadherin, play a role (Hummel and Zipursky, 2004). The glomerulus- specific segregation of ORNs in the AL depends on both local neuron-neuron (axon-axon) (Lattemann *et al.*, 2007; Hummel *et al.*, 2003; Sweeney

et al., 2007; Jhaveri *et al.*, 2004) and neuron-glia interactions (Yao *et al.*, 2007; Sakurai *et al.*, 2009). For correct targeting of some specific ORN classes another cell surface protein, Dscam, is required (Hummel *et al.*, 2003). Studies governing the downstream effectors of Dscam -Dock and Pak- have further confirmed this initial finding (Ang *et al.*, 2003). Another study suggests a combinatorial code of Robo receptors, which act to position sensory terminals within the antennal lobe through short-range interactions (Jhaveri *et al.*, 2004). The distribution of ORN axons into distinct glomeruli and proper targeting of late-arriving ORN axons from the maxillary palp are achieved through the repulsive interaction between Semaphorin-1a and its receptor Plexin A found on ORN axons (Sweeney *et al.*, 2008).

Other factors than these general cell-adhesion molecules, that enable each ORN axon to bind specifically are not known. Some transcription factors which also affect OR gene expression, such as Acj6 and Pdm3, also play a role in the precise axon targeting of a subset of ORNs (Komiyama *et al.*, 2004; Tichy *et al.*, 2008). This suggests that regulation of the sensory and synaptic identity of ORNs is based on common transcription factors.

1.6. Maxillary Palp - A Model Olfactory Organ

The second olfactory organ, the maxillary palp, is well studied and serves as a perfect and relatively simple model organ to study olfaction (Singh and Nayak, 1985). de Bruyne *et al.* (1999) provided an extensive description of the maxillary palp. It contains about 60 sensilla of a single category, sensilla basiconica, each of which houses two neurons of two particular classes combined according to a pairing rule, giving a total number of about 120 ORNs (Stocker, 1994; de Bruyne *et al.*, 1999, Goldman *et al.*, 2005). Extracellular single-unit physiological recordings reveal that the ORNs fall into six functional classes, thereby yielding three sensillum subtypes (Figure 1.7A) (de Bruyne *et al.*, 1999; Goldman *et al.*, 2005). These three sensillum subtypes have been termed as palp basiconic 1 (pb1), pb2 and pb3. Each of these functional classes of ORNs exhibits distinct odorant sensitivity. The neurons in each sensillum (subtype) are named as A and B cells. The neuron that gives a firing response with greater amplitude is referred to as the A cell and the other, with smaller spikes, as the B cell. Hence, six types of ORNs with

distinguishable response profiles are termed as pb1A, pb1B, pb2A, pb2B, pb3A and pb3B (Figure 1.7B) (de Bruyne *et al.*, 1999; Goldman *et al.*, 2005).

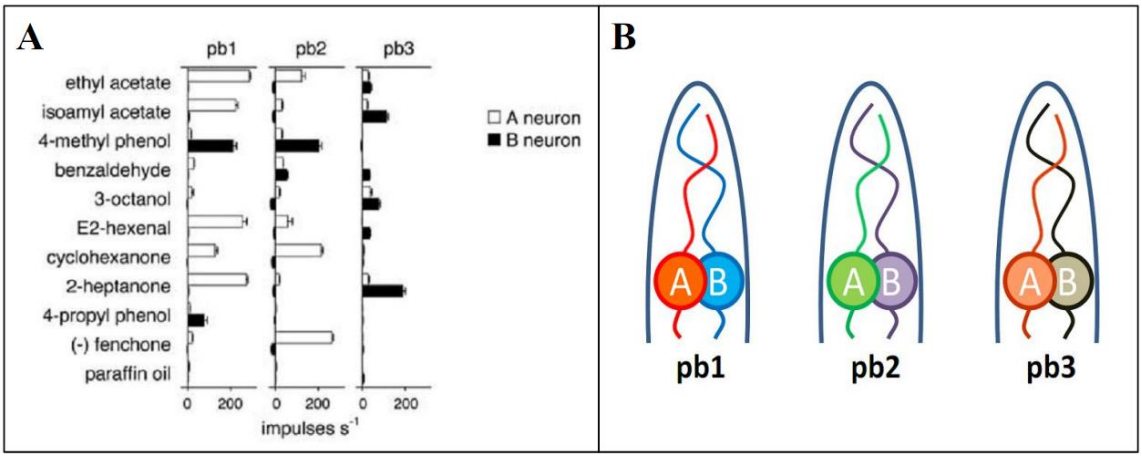

Figure 1.7. ORN classes in the maxillary palp. (A) Odorant response profiles of the three types of sensilla of the maxillary palp in wildtype flies. Error bars show SEM (taken from Goldman *et al.*, 2005). (B) Schematic representation of the three types of sensilla of the maxillary palp.

The three sensillum types have a mixed, partially overlapping, but not random, distribution across the surface of the maxillary palp. They are not in mutually exclusive zones and the precise positions of individual sensilla are not fixed. Random recordings confirm that the numbers of sensilla of each type were approximately equal: 36 ± 2, 31 ± 3, and 33 ± 4% for pb1, pb2, and pb3, respectively (de Bruyne *et al.*, 1999).

Figure 1.8. summarizes the time course of elements of Drosophila olfactory pathway.

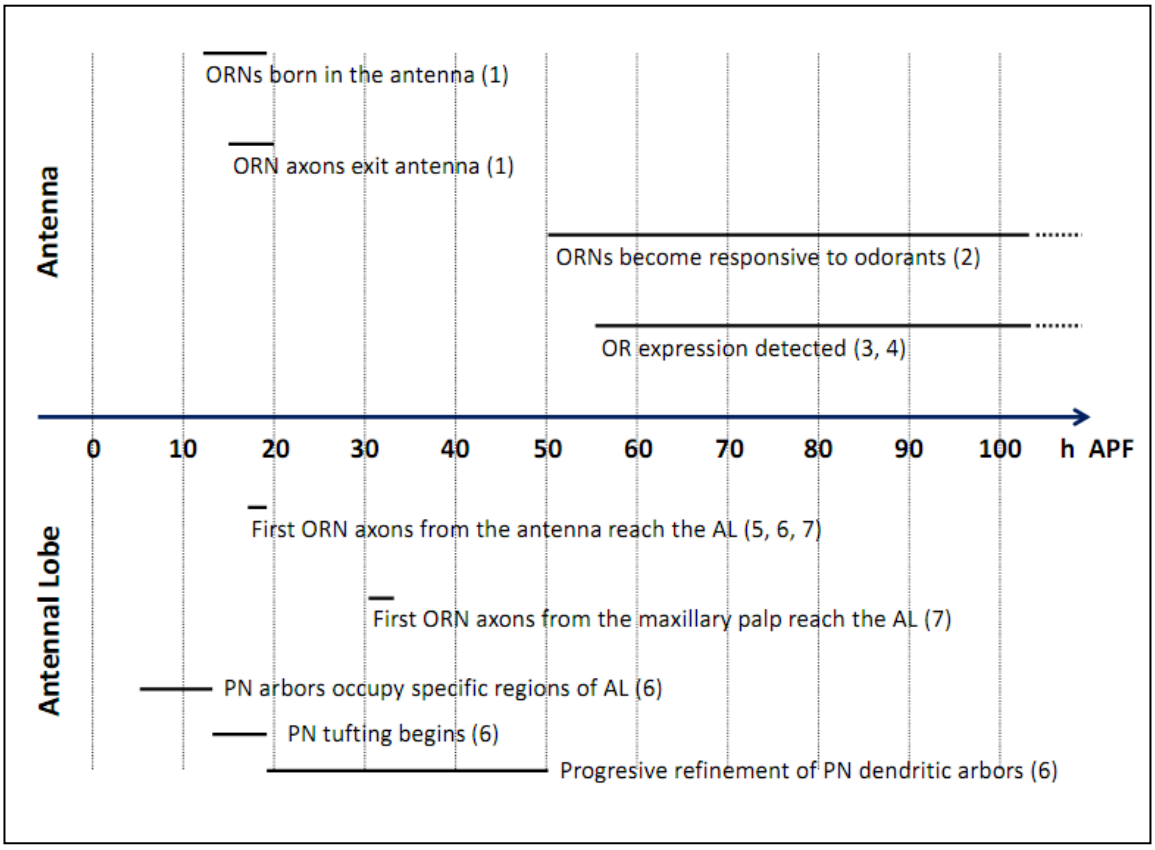

Figure 1.8. Developmental chart of *Drosophila* olfactory pathway. AL, antennal lobe; OR, olfactory receptor; ORN, olfactory receptor neuron; PN, projection neuron. Data are collected from (1) Ray and Rodrigues (1995), (2) Dubin and Harris (1997), (3) Clyne *et al.* (1999), (4) Larsson *et al.* (2004), (5) Jhaveri *et al.* (2000b), (6) Jefferis *et al.* (2004) and (7) Sweeney *et al.* (2007). Adapted from Oland *et al.* (2008).

1.7. Coexpression of Two ORs in One Neuron

Goldman *et al.* (2005) accomplished the construction of a receptor to neuron map for all classes of ORNs in the maxillary palp and this is the first study that shows a complete map for an (insect or vertebrate) olfactory organ. Based on RT-PCR experiments and *in situ* hybridization assays, Goldman *et al.* (2005) showed that 7 OR genes are expressed in the maxillary palp: *OR33c*, *OR42a*, *OR46a*, *OR59c*, *OR71a*, *OR85d* and *OR85e*. Because there are 6 ORN classes, the expression of 7 OR genes suggests that at least one ORN class expresses more than one OR gene. By conducting electrophysiological recordings, using genetic tools and performing double *in situ* hybridizations, a complete receptor to neuron

map was constructed and it was shown that *OR33c* and *OR85e* co-localize in the same ORNs, the pb2A neurons (Figure 1.9A). ORNs expressing the same odorant receptor converge upon the same glomerulus in the antennal lobe of *Drosophila*. Again, using genetic tools the corresponding glomeruli were labeled separately and hence, the coexpression of *OR33c* and *OR85e* in the same ORNs was independently confirmed (Figure 1.9B).

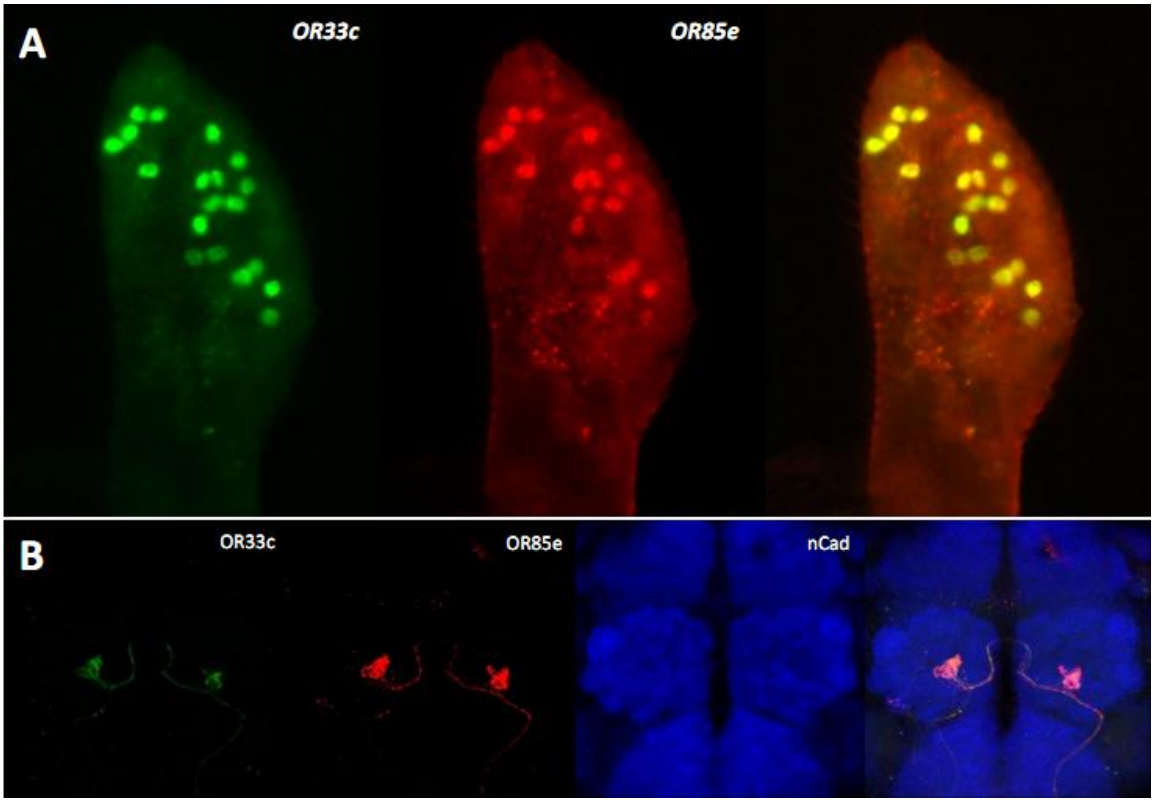

Figure 1.9. Coexpression of *OR33c* and *OR85e*. (A) Double *in situ* hybridization assay shows that *OR33*c (green) and *OR85e* (red) localize to the same ORNs. (B) ORNs coexpressing *OR33c* and *OR85e* project to the VC1 glomerulus in the antennal lobe. PrOR33c-mCD8::GFP ORNs are labeled with anti-GFP antibody (green), OR85e-Gal4 > UAS-mRFP ORNs are labeled with anti-DsRed antibody (red), and the glomeruli are stained with anti-nCad antibody (blue).

In an attempt to question the functionality of both receptors Goldman *et al.* (2005) used an *in vivo* expression system (Dobritsa *et al.*, 2003; Hallem *et al.*, 2004). They ectopically expressed *OR33c* and *OR85e* in a particular mutant antennal ORN class, ab3A, which shows no response due to a deletion that removes the endogenous expression of

OR22a and *OR22b* genes, and found that both of the genes, *OR33c* and *OR85e*, encode functional receptors (Figure 1.10).

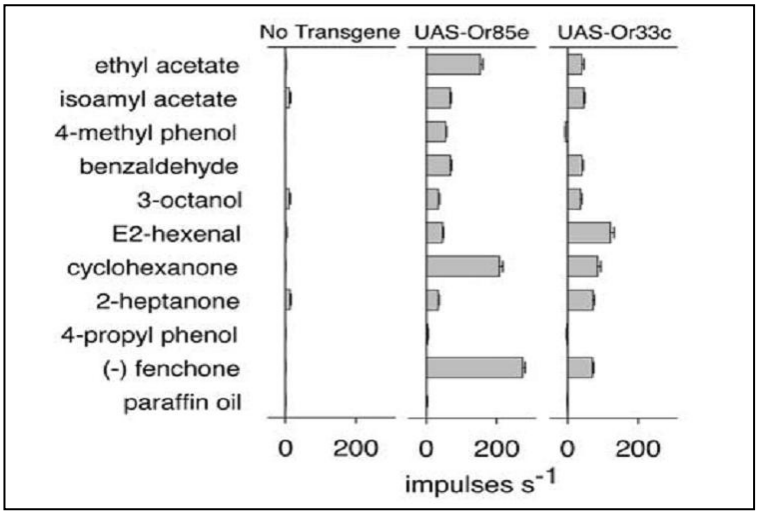

Figure 1.10. Both *OR85e* and *OR33c* genes encode functional odorant receptors. Odorant response profiles of ab3A neurons from *ab3A*$^{\Delta}$ flies: with no OR transgene (left), with ectopic *OR85e* expression (middle), and with ectopic *OR33c* expression (right). Error bars show SEM (taken from Goldman *et al.*, 2005).

The receptor to neuron map of the maxillary palp is illustrated in Figure 1.11 showing the expressed ORs and their corresponding sensillum class (Goldman *et al.*, 2005; Ray *et al.*, 2007).

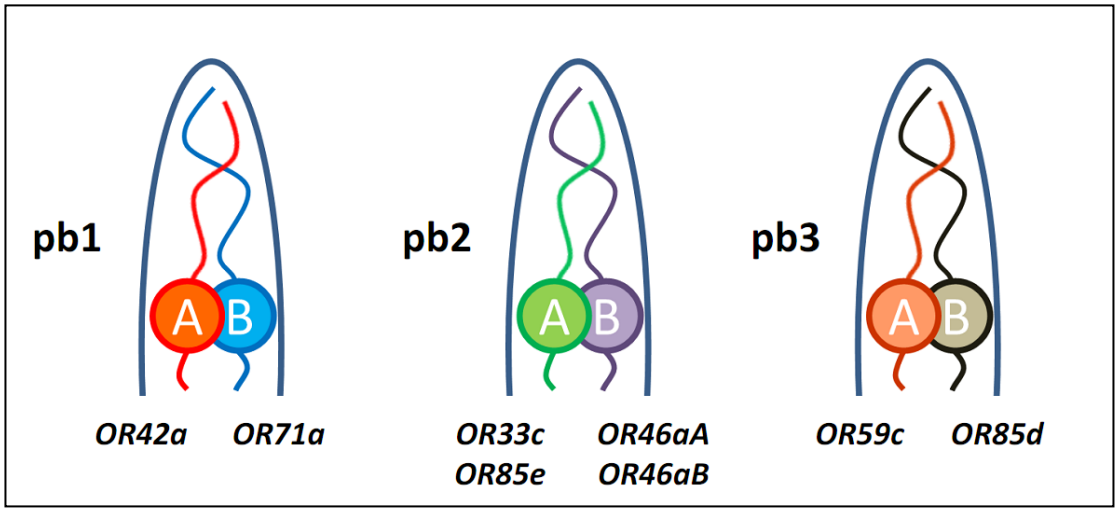

Figure 1.11. Receptor to neuron map of the maxillary palp in wildtype flies.

OR33c and *OR85e* genes map to different chromosomes, hence the coexpression of these two genes is not a coincidental by-product of a common chromosomal location. They are not products of a recent duplication event; in fact, according to bioinformatic analysis they only share 16% amino acid homology. Also by confirming their coexpression with double *in situ* hybridizations in *Drosophila pseudoobscura*, which diverged from *Drosophila melanogaster* about 46 million years ago, they showed that this coexpression is evolutionary conserved (Goldman *et al.*, 2005).

Reported instances of OR coexpression together with the coexpression of *OR33c* and *OR85e* are listed in Table 1.2. Studies revealed that at least 20% of ORN subtypes express two (or three) canonical receptors (Couto *et al.*, 2005; Fishilevich and Vosshall, 2005; Goldman *et al.*, 2005; Vosshall and Stocker, 2007; Ray *et al.*, 2007).

Table 1.2. Reported instances of OR coexpression in *Drosophila*.

Organ	Receptor 1	Receptor 2	Receptor 3
Antenna	OR10a	GR10a	
Antenna	OR19a	OR19b	
Antenna	OR22a	OR22b	
Antenna	OR33a	OR56a	
Antenna / Larval Dorsal Organ	OR33b	OR47a	
Antenna	OR33b	OR85a	
Antenna	OR49a	OR85f	
Antenna	OR65a	OR65b	OR65c
Antenna	OR69aA	OR69aB	
Antenna	OR85b	OR98b	
Maxillary Palp	OR33c	OR85e	
Maxillary Palp	OR46aA	OR46aB	
Larval Dorsal Organ	OR94a	OR94b	

1.8. Iroquois Complex

The Iroquois (Iro) family of genes were discovered in *Drosophila* in a mutagenesis screen aimed to identify possible actors that affect the patterning of sensory organs, essentially bristles (Dambly-Chaudiere and Leyns, 1992; Leyns *et al.*, 1996). In a mutant line (*iro*^{1}) of the family all lateral bristles of the notum of the fly were missing, but the large and small bristles of the central notum remain (Figure 1.12). This phenotype

resembled the hairstyle of the Iroquois American Indians and hence it is at the origin of the name of the mutation (Leyns *et al.*, 1996; Cavodeassi *et al.*, 2001).

Figure 1.12. Lack of lateral bristles on the notum *iro*^{1} flies. (A) Lateral view of wild-type flies. (B) Lateral view of *iro*^{1} flies. All lateral bristles, macro- and micro-chaetes, on the notum are missing (arrow) (taken from Leyns *et al.*, 1996).

The Iro family of transcription factors consists of three genes in *Drosophila*: *araucan (ara)*, *caupolican (caup)* and *mirror (mirr)* (Gomez-Skarmeta *et al.*, 1996; McNeill *et al.*, 1997) (Figure 1.13A). Together they form the gene cluster called *Iroquois Complex (IroC)*. *ara* and *caup* genes are closely related, share common cis-regulatory regions and although having some intrinsic distinct roles they are mostly genetically redundant. However, *mirr* encodes a more divergent protein, is expressed mostly in different tissues and is not redundant to *ara* and *caup* (Gomez-Skarmeta *et al.*, 1996; McNeill *et al.*, 1997).

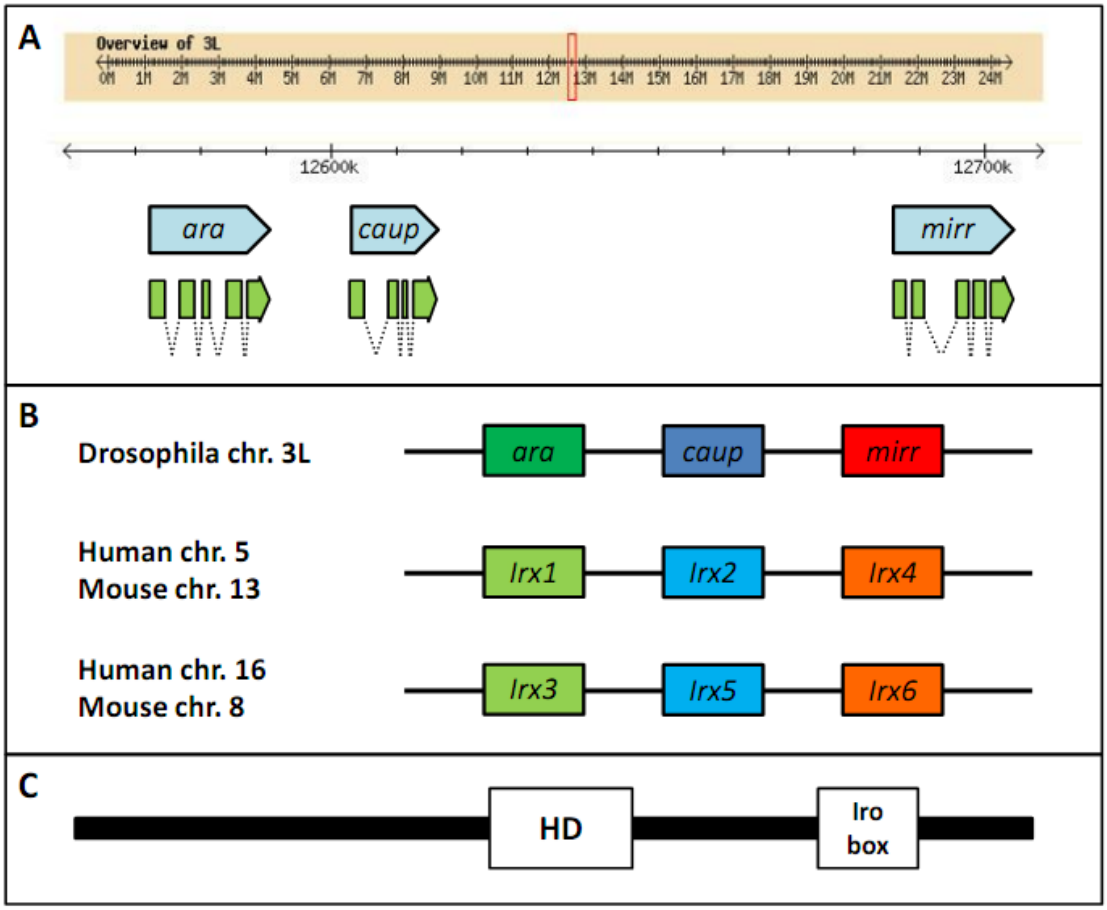

Figure 1.13. Genomic organization of *IroC* genes and structure of the proteins. (A) Detailed physical map of *Drosophila melanogaster IroC*. Transcription units of the three *Drosophila Iro* genes are shown in green. (B) *Iro* genes are chromosomally arranged in groups of three genes. Vertebrate paralogous genes are labeled with similar colors. (C) All Iro proteins have the same structure with two strongly conserved domains: a homeodomain of the TALE class (HD) and the Iro box (adapted from Cavodeassi *et al.*, 2001 and Gomez-Skarmeta and Modolell, 2002).

Molecular analysis of *IroC* genes allowed further identification of homologs in other organisms and studies suggest that the complex is found in all multi-cellular organisms. (Cavodeassi *et al.*, 2001; Bilioni *et al.*, 2005) (Figure 1.13B). All Iro proteins share a highly conserved homeodomain (HD), but there is little sequence similarity outside the HD (Billioni *et al.*, 2005) (Figure 1.13C). Classic Hox transcription factors have a characteristic 60-aa HD; however Iro proteins have 63-aa HD with a 3-aa loop extension (TALE), which places the Iro family into the TALE family of transcription factors (Bilioni *et al.*, 2005). Outside the HD all Iro proteins have a 9-aa region of homology called Iro

box, a motif resembling the central part of the EGF repeats of the Notch receptor protein (Gomez-Skarmeta *et al.*, 1996; Cavodeassi *et al.*, 2001; Bilioni *et al.*, 2005). Additionally Billioni *et al.* (2005) showed that Iro proteins can form homo- and hetero-dimers, and each of them can bind to the "ACAnnTGT" motif.

Iro proteins have been shown to be essential for diverse processes, such as specification of ventricular identity of the heart (Bao *et al.*, 1999), differentiation of the neural plate (Bellefroid *et al.*, 1998; Gomez-Skarmeta *et al.*, 1998), formation of the organizer during gastrulation (Kudoh and Dawid, 2001), organization of planar cell polarity in the eye (McNeill *et al.*, 1997; Cavodeassi *et al.*, 1999; Yang *et al.*, 1999), compartmentalization of sensory organs (Gomez-Skarmeta *et al.*, 1996; Diez del Corral *et al.*, 1999), axonal path-finding in the CNS (Jin *et al.*, 2003; Sato *et al.*, 2006), and regulation of receptor expression (Mazzoni *et al.*, 2008).

1.9. Gal4-UAS Binary System

What makes the fruit fly *Drosophila melanogaster* a powerful model organism is the availability of many genetic tools. The most prominent tool that is widely used in studies concerning *Drosophila* is the Gal4-UAS binary system (Duffy, 2002). This system enables targeted gene expression of any cloned gene in a tissue- or cell-specific manner. The Gal4 gene, identified in the yeast *Saccharomyces cerevisiae*, encodes for a protein of 881 amino acid length. The Gal4 protein has both DNA binding and transcriptional activation functions. It binds specifically a DNA sequence called Upstream Activating Sequence (UAS) through its DNA binding domain (BD) and activates the downstream gene through its activating domain (AD). Fisher *et al.* (1988) demonstrated that the Gal4 protein is able to induce a reporter gene under the control of an UAS sequence in *Drosophila* and expression of this protein has no deleterious effect on flies. These initial findings have directed the studies of Brand and Perrimon and lead them to publish the landmark article describing the Gal4-UAS binary system for targeted gene expression (Brand and Perrimon, 1993).

In this bipartite system, the expression of the gene of interest – the targeted gene - is controlled by the presence of an UAS sequence and in the absence of Gal4 protein there is

no expression of the targeted gene. Hence, if these transgenic elements are kept in separate transgenic lines, they need to be crossed in order to observe the expression of the targeted gene in the offspring. In the upstream region of the Gal4 gene either a tissue specific promoter or a tissue specific enhancer (plus minimal promoter) is cloned. This Gal4, termed "driver", then expresses a particular pattern that resembles the functional activity of the cloned upstream region. Upon expression and binding of the Gal4 protein to the UAS sequence, the targeted gene is activated and starts to be expressed (Figure 1.14A). A detailed description of the system and its various applications are described in the review paper of Duffy (2002).

The Gal4-UAS binary system is a repressible system (Lee and Luo, 1999). The Gal80 protein binds to the AD domain of Gal4 and inhibits its activator function. Upon expression of Gal80 within Gal4 and UAS containing flies, the system is repressed (Figure 1.14B). This beautiful feature is applied in many approaches like lineage tracing and clonal analysis (Lee and Luo, 1999 and 2001).

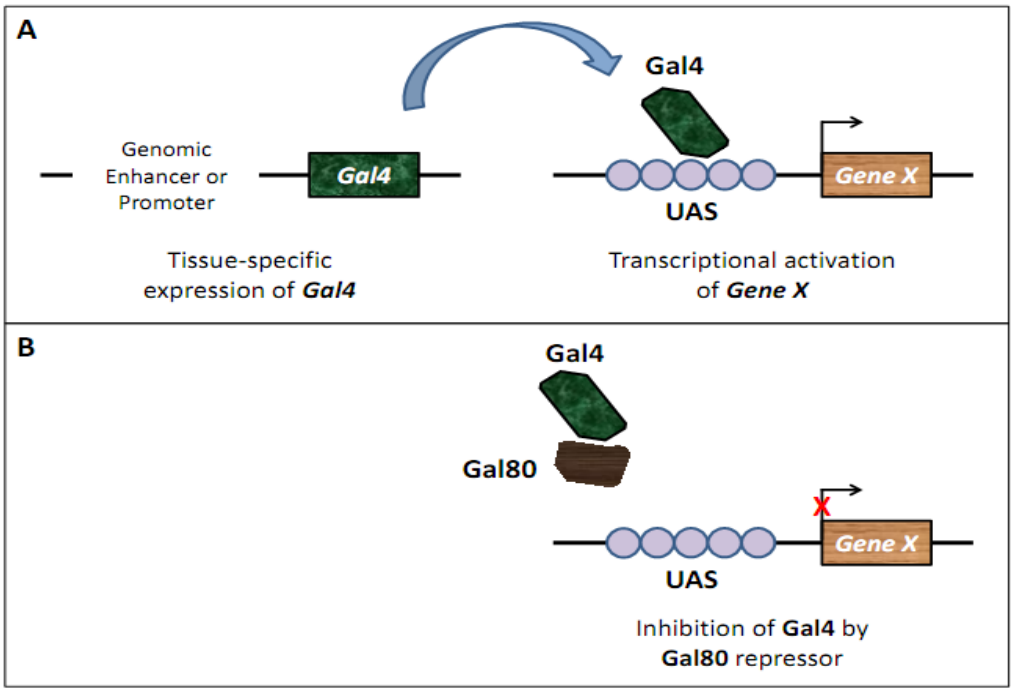

Figure 1.14. Gal4-UAS binary system. (A) Upon expression and binding of the Gal4 protein to the UAS sequence, the transcription of the target gene found downstream of the UAS sequence starts. (B) The Gal80 protein binds to the Gal4 protein and inhibits its activity.

Other than the Gal4-UAS system two additional binary systems which are used often have been developed: LexA:VP16-LexAOperon and QF-QUAS (Q system) binary systems (Lai and Lee, 2006; Potter *et al.*, 2010). These systems rely on the same logic as the Gal4-UAS system and usage of two binary system along with the Gal4-UAS system, which do not cross-react with each other enabled fly investigators to mark and effect different populations of cells in the same organism (Lai and Lee, 2006; Potter *et al.*, 2010).

2. PURPOSE

To prevent sensory overlap, ORNs tend to express only one receptor per neuron from a large repertoire of genes and send their axons to particular regions in the brain to form specific connections. The mechanisms underlying either of these two processes are poorly understood.

The selection and expression of a single OR from a large repertoire of genes is a daunting task and the mechanisms that enable this selection are still not clear. While in general every sensory neuron expresses only one receptor exceptions to this rule have been reported. While the functional significance of the coexpression of two sensory receptors is not known understanding the mechanism that allows the coexpression of two receptors might lead to an understanding of how the mechanism is repressed in general to allow the expression of only one receptor per sensory neuron.

In order to get insight into this selection mechanism the main purpose of this study is to identify the putative role of a transcription factor family, *IroC*, in the regulation of odorant receptor gene choice in the fly olfactory system. The expression of *IroC* in a subset of ORNs which coexpress two OR genes in the maxillary palp, namely *OR33c* and *OR85e*, has been observed. Based on an earlier study on the role of *IroC* in the regulation of rhodopsin genes (Mazzoni *et al.*, 2008), it was hypothesized that *IroC* genes might have a similar function in the olfactory system and are responsible for the induction of at least one of the coexpressed OR genes. In order to test this hypothesis I aimed to characterize the expression of *IroC* in detail, generate the necessary tools to do functional experiments in the maxillary palp and then perform classical loss-of-function and gain-of-function experiments to analyze the role of *IroC* in these varying backgrounds.

In a second aim the role of *IroC* in axonal targeting of ORNs to the antennal lobe was investigated, based on a previous study that showed involvement of *IroC* in proper targeting of photoreceptor cells (Sato *et al.*, 2006). Axonal targeting was analyzed by downregulating *IroC* in ORNs and visualizing their targeting patterns.

3. MATERIALS AND METHODS

3.1. Biological Material

Unless otherwise stated flies were raised at 25 ± 1°C with a 12:12 day:night cycle and 70-80% humidity in temperature and humidity-controlled incubators. Commercially available fly food (Applied Scientific, USA) was used (113 g fly food: 700 ml water) and prepared freshly every week. Fly strains used in the experiments were listed and described in Table 3.1.

Table 3.1. Fly strains used in this study.

Name of Line	Chr. No.	Description
GAL4 drivers		
act(FRT)CD2(FRT)-Gal4	III	Expresses Gal4 under the control of *actin* promoter upon elimination of CD2 casette by FLP mediated recombination
Appl-Gal4	I	Expresses Gal4 in post-mitotic neurons under the control of *appl*
Elav-Gal4	I, II, III	Expresses Gal4 in post-mitotic neurons under the control of *Elav*
IroC-Gal4	III	Expresses Gal4 under the control of *IroC*
nSyb-Gal4	III	Expresses Gal4 in post-mitotic neurons under the control of *synaptobrevin*
OR83b-Gal4	I	Expresses Gal4 under the control of the promoter of *OR83b*
OR85e-Gal4	III	Expresses Gal4 under the control of the promoter of OR85e
pb-Gal4	II	Expresses Gal4 under the control of the promoter of *proboscipedia*
SG18.1-Gal4	II	Expresses Gal4 in most of the ORNs
tub-Gal4	II	Expresses Gal4 under the control of the promoter of *tubulin*
UAS constructs		
UAS-ara	II, III	UAS fused to *araucan* cDNA
UAS-ara RNAi *(VDRC 49079)*	III	UAS fused to *araucan* dsRNA
UAS-ara RNAi *(VDRC 101903)*	II	UAS fused to *araucan* dsRNA
UAS-caup	II	UAS fused to *caupolican* cDNA
UAS-caup RNAi *(VDRC 2931)*	II	UAS fused to *caupolican* dsRNA
UAS-caup RNAi *(VDRC 105705)*	II	UAS fused to *caupolican* dsRNA
UAS-Dicer2	II	UAS fused to Dicer2 cDNA
UAS-mCD8::GFP	I, II, III	UAS fused to membrane targeted GFP cDNA

Table 3.1. Fly strains used in this study (continued).

Name of Line	Chr. No.	Description
UAS constructs		
UAS-mirr	I	UAS fused to *mirror* cDNA
UAS-mRFP	III	UAS fused to RFP with a nuclear localization sequence
UAS-myrRFP	II, III	UAS fused to cell membrane RFP
UAS-nGFP	I	UAS fused to nuclear GFP cDNA
Chromosomal Deficiency Lines		
iro^{DFM3}	III	Chromosomal deficiency spanning *araucan*, *caupolican* and the promoter of *mirror*
Other Lines		
ey-FLP	I	Expresses FLP-recombinase (Flippase) under the control of the *eyeless* promoter
FRT80	III	Allow FLP-mediated site specific recombination on the chromosome arm 3L
$Gal80^{ts}$	III	Expresses Gal80 in a temperature dependent manner
hs-FLP	I	Expresses FLP-recombinase (Flippase) under the control of a heat-shock promoter (*hsp70*)
PrOR33c-mCD8::GFP	III	Expresses cell surface GFP under the control of OR33c promoter
PrOR42a-mCD8::GFP	II, III	Expresses cell surface GFP under the control of OR42a promoter
PrOR46a-mCD8::GFP	III	Expresses cell surface GFP under the control of OR46a promoter
PrOR47a-syt::GFP	III	Expresses pre-synaptic GFP under the control of OR47a promoter
PrOR67d-mCD8::GFP	II	Expresses cell surface GFP under the control of OR67d promoter
PrOR83b-Gal80	II	Expresses Gal80 under the control of OR83b promoter
PrOR85e-mCD8::GFP	II	Expresses cell surface GFP under the control of OR85e promoter
tub-Gal80	III	Expresses Gal80 under the control of tubulin promoter
yw67; QB		Flies carrying balancer chromosomes Sp / Cyo ; TM2 / TM6B

3.2. Chemicals and Supplies

Unless otherwise stated all chemicals used in this study were from Sigma, Roche, Fisher Scientific or Molecular Probes.

3.2.1. Enzymes

Restriction enzymes and buffers were used from New England Biolabs or Fermentas. GoTaq polymerase and polymerase buffers were used from Promega. T4 DNA ligase and T4 ligase buffer were used from New England Biolabs.

3.2.2. Chemical Supplies

1 kb Marker	:	NEB, USA (N3232L)
Bovine Serum Albumin (BSA)	:	Sigma-Aldrich, USA (A9647)
CHAPS	:	Roche, USA (810118)
Denhardts (50x)	:	Sigma-Aldrich, USA (D2532)
Ethidium Bromide solution	:	Sigma Life Sciences, USA (E1510)
Formamide	:	Sigma-Aldrich, USA (A9037)
Glutaraldehyde	:	Sigma-Aldrich, USA (G5882)
Heparin	:	Sigma-Aldrich, USA (H4784)
Herring sperm DNA	:	Promega,USA (D181B)
Marker X	:	Roche, USA (11498037001)
$MgCl_2$	:	Riedel-de Haen, Germany (13152)
NaCl	:	Sigma-Aldrich, USA (S7653)
Paraformaldehyde	:	Sigma-Aldrich, USA (P6148)
Phenol:Chloroform:Isoamyl alcohol	:	Sigma-Aldrich, USA (P2069)
SeaKem LE agarose	:	Biomax (104514PR)
SSC	:	Sigma-Aldrich, USA (C8532)
Triton X-100	:	AppliChem, USA (A4975)
Tris	:	Sigma-Aldrich, USA (T6066)
Trizol	:	MRC, USA (TR118)
Tween 20	:	Roche, USA (11332465001)
Xgal	:	Sigma-Aldrich, USA (B42529905)

3.2.3. Buffers and Solutions

Table 3.2 summarizes the buffers and solutions used in this study.

Table 3.2. Contents of buffers and solutions used in this study.

Buffer/Solution	Content
Detection Buffer	100 mM Tris-Cl, pH 8.0 100 mM NaCl 10 mM $MgCl_2$
DIG (TN) Buffer	100 mM Tris-Cl, pH 7.5 150 mM NaCl

Table 3.2. Contents of buffers and solutions used in this study (continued).

Buffer/Solution	Content
EB (Elution Buffer)	10 mM Tris-Cl, pH 8.5
Hybridization Buffer	50% Formamide 5x SSC 5x Denhardts 0.25 mg/ml yeast tRNA 0.5 mg/ml herring sperm DNA 0.05 mg/ml heparin 2.5 mM EDTA 0.25% CHAPS 0.1% Tween-20
LB Agar	5 g/l NaCl 10 g/l Tryptone 5 g/l Yeast extract 14 g/l Agar
LB Broth	5 g/l NaCl 10 g/l Tryptone 5 g/l Yeast extract
Loading Buffer (10x)	50% Glycerol 0,0005% Bromophenol Blue
N3	Confidential / Commercial
P1 (Resuspension Buffer)	50 mM Tris-Cl, pH 8.0 10 mM EDTA 100 µg/ml RNase A
P2 (Lysis Buffer)	200 mM NaOH 1% SDS (w/v)
P3 (Neutralization Buffer)	3.0 M Potassium Accetate , pH 5.5
PB (Binding Buffer)	Confidential / Commercial
PBS (1x)	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4
PBST	PBS (1x) 0.1% Tween-20
PBSTX	PBS (1x) 0.05% Triton X-100
PBX3	PBS (1x) 0.3% Triton X-100
PBX3 + 1% BSA	PBS (1x) 1% BSA 0.3% Triton X-100
PE	Confidential / Commercial
QBT (Equilibration Buffer)	750 mM NaCl 50 mM MOPS, pH 7.0 15% Isopropanol (v/v) 0.15% Triton X-100 (v/v)
QC (Wash Buffer)	1 M NaCl 50 mM MOPS, pH 7.0 15% Isopropanol (v/v)
QF (Elution Buffer)	1.25 M NaCl 50 mM Tris-Cl, pH 8.5 15% Isopropanol (v/v)

Table 3.2. Contents of buffers and solutions used in this study (continued).

Buffer/Solution	Content
TAE Buffer (1x)	40 mM Tris-Cl 1 mM EDTA 0.1% Acetic acid
TE (1x)	10 mM Tris-Cl, pH 8.0 1 mM EDTA
TNB	100 mM Tris-Cl, pH 7.5 150 mM NaCl 0.5% NEN Blocking Reagent (Roche)
TNT	100 mM Tris-Cl, pH 7.5 150 mM NaCl 0.05% Tween-20
Xgal Staining Solution	7.2 mM NaH_2PO_4, pH 7.2 2.8 mM Na_2HPO_4 150 mM NaCl 3 mM $K_3[Fe(CN)_6]$ 3 mM $K[Fe(CN)_6]$ 0.1% Tween 20 and 0.1% X-Gal

3.2.4. Oligonucleotide Primers

PrOR33c and PrOR85e primers were designed to amplify 1000 bp upstream of *OR33c* and *OR85e* genes, respectively and ordered from Iontek (Turkey). Primers were diluted with double distilled sterile water to obtain a final concentration of 100 pmol/ µl. Diluted primers were kept at -20°C. Table 3.3 summarizes the primers used in this study.

Table 3.3. Primers used in this study.

Primer Name	Primer Sequence (5'-3')	T_m (^{o}C)
actin79b-RTPCR-F	TGCTTGGAGATCCACATCTG	57
actin79b-RTPCR-R	ATGTATCCAGGTATCGCTGAC	57
ara-RTPCR-F	GTTTGGCCAAAGATGAGACC	57
ara-RTPCR-R	CCCATGAAATGGCAACATACC	57
caup-RTPCR-F	CAGAAGCTTTCGATCCCGGC	61
caup-RTPCR-R	GTTGGTGGTGTTGCATTTGCT	57
GFP-R	AACTTGTGGCCGTTTACGTCGC	64
M13F	TGTAAAACGACGGCCAGT	53
M13R	GGAAACAGCTATGACCATG	55
mCherry-CoPCR-R	CCGTCCTCGAAGTTCATCAC	59
PrOR33c-F	CCTAATGCACATTTCCCAGCAAG	60
PrOR33c-R	GTCGGGTGTCACGGAAATCG	61

Table 3.3. Primers used in this study (continued).

Primer Name	Primer Sequence (5'-3')	T_m (^oC)
PrOR85e-F	AAATGGCTTGACGGCAGAGC	59
PrOR85e-R	GATTTCGGCTGCCTAATCGAGCTG	64
PrOR33c-CoPCR	GGCTAACCAACCAGGCATTT	57
PrOR85e-CoPCR	GACGCACATTGCGAGGGATT	59
T3	AATTAACCCTCACTAAAGGG	54
T7	AATACGACTCACTATAGGG	53

3.2.5. Antibodies

Antibodies used in this study are listed in Table 3.4.

Table 3.4. Antibodies used in this study.

Name	Antigen	Species	Dilution	Supplier
Primary Antibodies				
β-galactosidase	β-galactosidase	mouse	1:500	Promega
Elav	Elav	rat	1:50	Hybridoma Bank
GFP	GFP	rabbit	1:1000	Invitrogen
GFP	GFP	mouse	1:1000	Promega
nCad	N-Cadherin	rat	1:20	Hybridoma Bank
DsRed	RFP	rabbit	1:1000	Clontech
DIG-AP	Digoxigenin	sheep	1:500	Roche
FLU-POD	Fluorescein	sheep	1:100	Roche
Secondary Antibodies				
Alexa 488	mouse	donkey	1:800	Invitrogen
Alexa 488	rabbit	goat	1:800	Invitrogen
Alexa 488	rat	donkey	1:800	Invitrogen
Alexa 555	mouse	goat	1:800	Invitrogen
Alexa 647	rat	donkey	1:800	Invitrogen
Cy3	rabbit	goat	1:800	Jackson Laboratory
Cy3	rat	donkey	1:800	Jackson Laboratory
Cy5	mouse	goat	1:800	Jackson Laboratory
Cy5	rabbit	donkey	1:800	Jackson Laboratory
Cy5	rat	donkey	1:800	Jackson Laboratory

3.2.6. Embedding Media

Vectashield Embedding Medium (Vector Laboratories, Inc) or 50% glycerol were used as embedding medium for samples that have been stained with fluorescent substrates and dyes in the course of this study

3.2.7. Disposable Labware

Culture tubes, 14 ml	:	Greiner Bio-One, Belgium
Filter tips	:	Greiner Bio-One, Belgium
Microscope cover glass	:	Fisher Scientific, UK
Microscope slides	:	Fisher Scientific, UK
Nylon membrane (0.2 µm)	:	Sartorius Stedim Biotech, France
PCR tubes (200 µl)	:	Bio-Rad, USA
Petri dish	:	Greiner Bio-One, Belgium
Pipette tips	:	VWR, USA
Syringe (1 cc)	:	Becton, Dickinson and Company, USA
Test tubes, 0.5 ml	:	Citotest Labware Manufacturing, China
Test tubes, 1.5 ml	:	Citotest Labware Manufacturing, China
Test tubes, 2 ml	:	Citotest Labware Manufacturing, China
Test tubes, 15 ml	:	Becton, Dickinson and Company, USA
Test tubes, 50 ml	:	Becton, Dickinson and Company, USA

3.2.8. Equipment

Autoclave	:	Astell Scientific Ltd., UK
Centrifuges	:	Eppendorf, Germany (Centrifuge 5424, 5417R)
Cold Room	:	Birikim Elektrik Soğutma, Turkey
Confocal Microscope	:	Leica Microsystems, USA (TCS SP5)
Cryostat	:	Leica Microsystems, USA (CM3050S)

Electrophoresis Equipment	:	Bio-Rad Labs, USA (ReadySub-Cell GT Cells)
Environmental Test Chamber	:	Sanyo, Japan (MLR 351H)
Fluorescence Stereomicroscope	:	Leica Microsystems, USA (MZ16FA)
Freezers	:	Arçelik, Turkey Thermo Electron Corp., USA (Thermo Forma 723)
Gel Documentation System	:	Bio-Rad Labs, USA (Gel Doc XR)
Heating Block	:	Fisher Scientific, France (Dry-bath incubator)
Heating magnetic stirrer	:	IKA, China (RCT Basic)
Incubator	:	Weiss Gallenkamp, UK (Incubator Plus Series)
Inverted Microscope	:	Zeiss, USA (Axio Observer, Z1)
Laboratory Bottles	:	Isolab, Germany
Micropipettes	:	Eppendorf, Germany
Microwave oven	:	Vestel, Turkey
pH meter	:	WTW, Germany (Ph330i)
Refrigerators	:	Arçelik, Turkey
Stereo Microscope	:	Olympus, USA (SZ61)
Thermal Cycler	:	Bio-Rad Labs, USA (C1000 Thermal Cycler)
Vortex Mixer	:	Scientific Industries, USA (Vortex Genie2)
Water Bath	:	Grant Instruments, UK (JB Aqua 12)

3.3. Molecular Biological Techniques

3.3.1. Isolation of DNA and RNA

3.3.1.1. Small Scale Plasmid DNA Isolation (Miniprep). To obtain plasmid DNA the QIAprep Spin Miniprep Kit (QIAGEN) was used according to the manufacturer's suggestions. Briefly, cultured bacteria were precipitated in a 2 ml test tube by centrifuging at 14000 rpm for 1 min. The supernatant was removed and the pellet was resuspended in 250 µl of Buffer P1. 250 µl of Buffer P2 was added and mixed thoroughly by inverting the tube. 350 µl of Buffer N3 was added and mixed thoroughly by inverting the tube several times. The mixture was centrifuged for 10 min at 13000 rpm in a table-top microcentrifuge. The supernatant was applied to a commercially available spin column (QIAGEN) and centrifuged for 30 s at 11000 rpm. The flow-through was discarded. The spin column was washed by adding 750 µl of Buffer PE and centrifuged for 30 s at 11000 rpm. The flow-through was discarded and the column was centrifuged for an additional 30 s to remove the remaining was buffer. The spin column was placed in a new 1.5 ml test tube, 50 µl of pre-warmed Buffer EB was added and centrifuged for 1 min at maximum speed (14600 rpm).

3.3.1.2. Large Scale Plasmid DNA Isolation (Midiprep). To obtain plasmid DNA in larger sclae the QIAGEN Plasmid Midi Kit (QIAGEN) was used according to the manufacturer's suggestions. Cultured bacteria were precipitated in a 50 ml test tube by centrifuging at 5500 rpm for 25 min at 4°C. The supernatant was removed and the pellet was resuspended in 4 ml of Buffer P1. 4 ml of Buffer P2 was added, mixed thoroughly by in inverting the tube several times and incubated at room temperature for 5 min. 4 ml of ice-cold Buffer P3 was added, mixed well and placed on ice for 15 min. The mixture was centrifuged for 30 min at 5500 rpm at 4°C. The supernatant was poured into a 50 ml falcon test tube and centrifuged again for 30 min at 5500 rpm at 4°C. Meanwhile the commercially available column (QIAGEN-100) was equilibrated by adding 4 ml of Buffer QBT. The supernatant was applied to the column. After flow through the column was washed 2 times with 10 ml of Buffer QC. The DNA was eluted with 5 ml of QF into a new 15 ml falcon test tube. 3.5 ml of ice-cold isopropanol was added, mixed well and centrifuged for 30 min at 6000 rpm at 4°C. The supernatant was removed carefully, 2 ml of 70% EtOH was added and

centrifuged for 15 min at 6000 rpm at 4°C. The supernatant was removed as much as possible and the pellet was air-dried. The pellet was then resuspended in 50 µl of EB and transferred into a new 1.5 ml test tube.

3.3.1.3. Isolation of BAC (Bacterial Artificial Chromosome). Cultured bacteria were precipitated in a 2 ml test tube by centrifuging at 14000 rpm for 1 min. The supernatant was removed and the pellet was resuspended in 300 µl of Buffer P1. 300 µl of Buffer P2 was added, mixed thoroughly by inverting the tube several times and incubated for 5 min at room temperature. 300 µl of Buffer P3 was added, mixed thoroughly by inverting the tube several times and placed on ice for 5 min. The mixture was centrifuged for 10 min at 13000 rpm in a table-top microcentrifuge at 4°C. The supernatant was transferred to a new 1.5 ml test tube, 800 µl of ice-cold isoproponal was added and mixed by inverting the tube several times. The mixture was centrifuged for 15 min at maximum speed (14600 rpm) at 4°C. The supernatant was removed carefully, 500 µl of 70% EtOH was added and centrifuged for 5 min at maximum speed at 4°C. The supernatant was removed as much as possible and the pellet was air-dried. The pellet was then resuspended in 50 µl of Buffer EB or TE (1x).

3.3.1.4. Isolation of Total RNA. 10 fly heads were collected and put into a 2 ml test tube containing 800 µl of Trizol reagent under the hood and were homogenized with a tissue homogenizer. To allow nucleoprotein complexes to dissolve the mixture was left at room temperature for 5 min. 160 µl chloroform was added and mixed well by shaking. The mixture was first incubated at room temperature for 15 min and then centrifuged at 12000 rpm for 15 min at 4°C. The aqueous phase was transferred into a new 1.5 ml test tube and 400 µl of isopropanol was added. The mixture was incubated at room temperature for 10 min and centrifuged at 10000 rpm for 10 min at 10°C. The supernatant was discarded and washed by adding 800 µl 75% EtOH and centrifuging at 7500 g for 5 min. The EtOH was discarded and the pellet was left to air-dry for 3-5 min. The pellet than was dissolved in 20 µl of DEPC-treated dH_2O.

3.3.2. Restriction Digestion of DNA

Digestion of DNA was performed using the stated restriction enzymes according to the manufacturer’s suggestions. The used enzyme was heat inactivated and the reaction was purified before further use.

3.3.3. Dephosphorylation

In order to prevent the religation of vector DNA during ligation reactions the 5' phosphate of linearized vector DNA was removed using Antarctic Phophatase (NEB) according to the manufacturer's suggestions. The enzyme was heat inactivated at 65°C for 10 min and the reaction was purified before further use.

3.3.4. Filling of 5' Overhangs

To fill 5' overhangs (recessed 3' termini) DNA Polymerase I, Klenow (NEB), was used according to the manufacturer's suggestions.

3.3.5. Ligation

Ligations were performed using T4 DNA Ligase (New England Biolabs) overnight at 16°C according to the manufacturer's suggestions.

3.3.6. Transformation

50 µl of competent bacteria (*Escherichia coli TOP10)* prepared by chemical treatment and stored at -80°C was thawed on ice. The thawed bacteria were then added to the ligation product (or plasmid DNA for retransformation) in a 1.5 ml test tube and incubated on ice for 30 min. The mixture was then heat-shocked at 42°C for 1 min and 30 sec. The tube was put back on ice at least for 5 min. 1 ml LB was added and the mixture was incubated at 37°C for 1 h while shaking. 100-200 µl of the mixture then was plated on an LB agar plate that contains the appropriate antibiotic and incubated overnight at 37°C.

3.3.7. Reverse Transcription and cDNA Synthesis

To synthesize cDNA from obtained RNA a cDNA synthesis kit (Invitrogen, SuperScript First-Strand) was used according to the manufacturer's suggestions. Briefly, 1 µg of RNA was incubated with 1 µl of oligo dT primer in a total volume of 5 µl for 5 min at 70°C. The mixture was then incubated on ice for at least 5 min. Meanwhile the reaction mix was prepared by mixing 6.5 µl of dH_2O, 2 µl of reverse transcriptase buffer (10x), 4 µl of $MgCl_2$, 1 µl dNTP, 0.5 µl RNase inhibitor and 1 µl of reverse transcriptase. The reaction mixture was added to the sample mixture and incubated for 5 min at 25°C. The reaction was incubated further at 42°C for at least 1 h. Finally the mixture was incubated at 70°C for 15 min to heat-inactivate the reverse transcriptase.

3.3.8. Polymerase Chain Reaction (PCR)

3.3.8.1. Conventional PCR. The promoter regions of *OR33c* and *OR85e* gene were amplified by polymerase chain reaction from the commercially available BACs, BACR37P12 and BACR01F13, that span the genomic locus of *OR33c* and *OR85e* genes, respectively. Reactions were performed in a total volume of 30 µl containing 0.5 µl isolated BAC DNA, 1.5U Advantage polymerase, 3 µl $MgCl_2$ containing Advantage polymerase buffer (10x), 0.1 mM dNTP, 0.5 µM of each primer (PrOR33c_F and PrOR33c_R; PrOR85e_F and PrOR85e_R). The reaction conditions were 5' 94°C, (30'' 94°C, 40'' 59°C, 1' 72°C) x 24 cycles, 10' 72°C. PCR products were run on a 1% agarose gel.

3.3.8.2. Colony PCR. To find a positive colony the colonies on the plate were screened by colony PCR. Using a pipette tip a colony was transferred from the plate and was resuspended in a PCR tube containing 21.6 µl dH_2O. The pipette tip was then streaked on a fresh plate to allow for the growth of the bacteria. The tube then was heated up to 95°C for 15 min to break the cells. Meanwhile a master mix was prepared by mixing 0.3 µl home-made Taq polymerase, 3 µl Taq polymerase buffer (10x), 1.5 mM $MgCl_2$, 0.1 mM dNTP and 0.5 µM of each primer of interest for each sample colony. After the breakdown of the cells, PCR was performed by adding the prepared master mix to the plasmid containing

water and following the appropriate reaction conditions. The PCR products were run on a 1% agarose gel.

3.3.8.3. Semi-Quantitative RT-PCR. To determine the relative abundance of expressed *IroC* genes between control and the RNAi flies semi-quantitative RT-PCR was performed. The synthesized cDNAs were used as template and primers were designed as to bridge exon-exon junctions in order to control for genomic DNA contamination. As an internal control the *actin79b* gene was used. Reactions were performed in a total volume of 30 µl containing 0.3 µl of synthesized cDNA, 1.5U GoTaq polymerase, 6 µl GoTaq Buffer (5x), 1.5 mM $MgCl_2$, 0.1 mM dNTP, 0.5 µM of each primer (actin79b-RTPCR-F and actin79b-RTPCR-R; ara-RTPCR-F and ara-RTPCR-R; caup-RTPCR-F and caup-RTPCR-R) and 18.3 µl distilled water. The standard PCR program comprised a cycle of 5' at 94°C, followed by 30'' at 94°C, 40'' at 56°C and 30'' at 72°C for a number of cycles to be determined and finally 10' at 72°C. The optimal number of cycles where amplification was in the exponential range was determined according to the internal control gene, *actin79b*. PCR products were run and compared on the same 1% agarose gel (Figure A.1).

3.3.9. Phenol-Chloroform Extraction

Samples were adjusted to 300 µl by adding H_2O and phenol-chloroform extracted using an equal volume of phenol-chloroform-isoamylalcohol (25:24:1, v/v). The mixture was vortexed and centrifuged for 10 min at maximum speed (14600 rpm) at 4°C. The aqueous phase was transferred to a new tube and extracted again using 300 µl phenol-chloroform-isoamylalcohol and centrifuging for 10 min at maximum speed at 4°C. The aqueous phase then transferred to a new tube and extracted using 300 µl choloroform and centrifuging for 2 min at maximum speed at 4°C. The aqueous phase was then ethanol precipitated.

3.3.10. Ethanol Precipitation

DNA was precipitated using 0.3 M sodium acetate (NaAc) and three volumes of ice-cold absolute ethanol. RNA was precipitated using 0.4 M lithium chloride (LiCl) and 2.5 volumes of ice-cold absolute ethanol. Precipitation was allowed at -80°C for 30 min. The

mixture was then centrifuged at maximum speed (14600 rpm) at 4°C for 30 min in case of DNA and 15 min in case of RNA. The pellet was washed with 70% ethanol, air-dried and resuspended in the appropriate buffer or DEPC-treated dH_2O.

3.3.11. Agarose Gel Electrophoresis

1% agarose gel (w/v) was prepared with 1x TAE buffer and 30 ng/ml ethidium bromide solution. Samples were prepared by addition of loading dye so that the concentration of loading dye was finalized to 1x and loaded on the agarose gel. Additionally Marker X or a 1 kb Marker was used as size marker. The gel was run at 120V for 40 min and visualized under a transilluminator (Bio-Rad, USA).

3.3.12. Gel Extraction of DNA

The DNA fragment was excised from the agarose gel with a clean scalpel. To obtain DNA fragments of the desired molecular weight QIAquick Gel Extraction Kit (QIAGEN) was used according to the manufacturer's suggestions. Briefly, the gel was placed in a 2 ml test tube, 1000 µl of Buffer QG was added and incubated for 20 min at 50°C with periodical vortexing. 350 µl of isopropanol was added to the sample and mixed. The sample was then applied to the QIAquick spin column and centrifuged for 30 sec at 11000 rpm. The flow-through was discarded and the column was washed with 500 µl of Buffer QG to remove residual agar and centrifuged for 30 sec at 11000 rpm. The bound DNA was washed by addition of 750 µl Buffer PE and centrifuging for 30 sec at 11000 rpm. To elute DNA, 30 µl of Buffer EB was added and centrifuged for 1 min at maximum speed (14600 rpm).

3.3.13. Sequencing Analysis

Purified DNA samples were directly subjected to sequencing. Samples were sequenced at Macrogen Inc. (Korea) and DNA sequences were analyzed by using Vector NTI (Invitrogen) software.

3.3.14. Generation of Transgenic Flies

For the generation of PrOR33c-mCherry and PrOR85e-TLNΔCherry constructs an attB site containing vector (pJR8) was modified. In order to be able to use the XbaI restriction site downstream of the GFP sequence for cloning, the pJR8 vector was first retransformed into a DAM$^-$ and DCM$^-$ *Epicurian SCS110* bacterial strain. The multiple cloning site and the GFP sequence were replaced with the multiple cloning site and the YFP sequence of pACSF_eYFP vector using the XbaI sites to create the pJR8_eYFP vector. The correct plasmid was selected by performing colony PCR using the T3 and GFP-R primers. The YFP sequence of pJR8_eYFP vector was either replaced with the mCherry sequence obtained from pRSETB_mCherry to generate pJR8_mCherry vector or replaced with TLNΔCherry sequence obtained from pUAST_TLNΔCherry (Nicolai *et al.*, 2010) to generate the pJR8_TLNΔCherry vector.

The mCherry sequence was obtained from pRSETB_mCherry vector by EcoRI digestion, blunting and partial digestion with NcoI. The YFP sequence of pJR8_eYFP vector was excised by NotI digestion, blunted and partially digested with NcoI. Then, the mCherry sequence was ligated to the remaining pJR8_eYFP vector backbone to generate the pJR8_mCherry vector. The correct plasmid was selected by performing colony PCR using the T3 and mCherry-CoPCR-R primers.

The TLNΔCherry sequence was obtained from pUAST_TLNΔCherry using the EcoRI and XbaI sites. The YFP sequence of the pJR8_eYFP vector was excised out by EcoRI digestion and XbaI partial digestion. The TLNΔCherry sequence was then ligated to the remaining pJR8_YFP vector backbone to generate the pJR8_TLNΔCherry vector. The correct plasmid was selected by performing colony PCR using the T3 and mCherry-CoPCR-R primers.

Promoters obtained by PCR from the commercially available BACs were cloned into the pGEMT-Easy vector to generate PrOR33c_ pGEMT and PrOR85e_pGEMT vectors. The cloned fragments were sequenced for correctness before proceeding to the next cloning step. To generate the PrOR33c-mCherry vector, the PrOR33c sequence was excised out from the pGEMT_PrOR33c vector using EcoRI sites and ligated to the

pJR8_mCherry vector, which was linearized by EcoRI digestion and dephosphorylated. To generate PrOR85e-TLNΔCherry vector, the PrOR85e sequence was excised out from the pGEMT_PrOR85e vector using EcoRI sites and ligated to the pJR8_TLNΔCherry vector, which was linearized by EcoRI digestion and dephosphorylated. The direction of the cloning was tested through colony PCR using orientation-specific primers: PrOR33c-CoPCR and SV40-R or PrOR85e-CoPCR and SV40-R.

The generated vectors, PrOR33c-mCherry and PrOR85e-TLNΔCherry, were purified by Midiprep and sent to the company Genetivision Inc (USA) for site-directed transformation of flies using the available phiC31 sites, VK37(2L)22A3 and VK20(3R)99F8, respectively (Venken *et al.*, 2006).

Vector maps of all of the indicated vectors are given in the Appendix B.

3.3.15. Labeling of RNA Using Digoxigenin or Fluorescein by *in vitro* Transcription

RNA probes labeled with digoxigenin- or fluorescein-labeled UTP (DIG- or FLU-UTP) were generated by in vitro transcription according to the manufacturer's instructions. Before beginning the transcription reaction, the template DNA was generated using vector specific primers and the DNA was purified by phenol-chloroform extraction and ethanol precipitation. The labeling reaction was performed in a total volume of 20 µl and about 1 µg DNA was used as template. Transcription buffer (Roche) and DIG-labeling mix (or FLU-labeling mix) (Roche) were added to a final concentration of 1x. 50 U of RNA polymerase (T3, T7 or Sp6; Roche) and 20 U of RNAse inhibitor (Roche) were also added. The reaction was incubated 2-3 hours and terminated by addition of 2 µl of EDTA (0.2 M, pH 8.0). The RNA transcript was ethanol precipitated, resuspended in 50 µl DEPC treated dH_2O and analyzed for size using agarose gel electrophoresis.

3.4. Histological Techniques

3.4.1. *In Situ* Hybridization

In situ hybridization was performed as described in Goldman *et al.* (2005). Briefly, whole proboscises with maxillary palps attached were dissected in PBS (1x) and collected in 4% paraformaldehyde (PFA) in PBS with 0.05% Tween-20 on ice. Fixation was continued at room temperature for 30 min. Samples were washed 5 times for 5 min in PBST at room temperature and incubated in hybridization buffer for 1 hr at 65°C for pre-hybridization. The tissues were then hybridized with digoxigenin- and fluorescein-labeled RNA probes at 55°C overnight (Probes were diluted 1/100 in hybridization buffer, denatured at 95°C for 5 min and chilled on ice for 5 min). On the second day, the samples were washed 4 times over 4 hr in hybridization buffer, 3 times for 20 min in 0.2x SSC at 55°C and 4 times for 5 min in PBST at room temperature. The tissues were then equilibrated in DIG Buffer for 10 min and blocked in TNB for 1 h at room temperature. The tissues were then incubated with 1/500 diluted anti-DIG-AP (Roche) and 1/100 diluted anti-FLU-POD (Roche) in TNB at 4°C overnight. On the third day, the tissues were washed 3 times for 15 min in TNT. For the visualization of FLU-labeled probes, Alexa-488 or Cy5 conjugated Tyramide (Perkin Elmer) was applied (1/100 diluted in 1x Plus amplification diluent) for 15 min and washed 3 times for 15 min TNT. To visualize the DIG-labeled probes, the tissues were first equilibrated by washing 2 times for 10 min in detection buffer and then incubated in freshly prepared HNPP/Fast Red TR (Roche) substrate containing detection buffer (10 µl Fast Red TR, 10 µl HNPP and 1 ml detection buffer were mixed and filtrated with 0.2 µm nylon membrane) for 30 min at room temperature followed by 3 washes for 15 min with TNT. Maxillary palps were then dissected and mounted in Vectashield (Vector Laboratories, Inc) and visualized using a Zeiss Observer Z1 inverted fluorescent microscope or a Leica TCS SP5 confocal microscope.

For triple stainings (double *in situ* hybridization and antibody staining), the double *in situ* hybridization protocol was followed in general. During the detection step, the primary antibody against GFP (in a dilution of 1/50) was applied to the samples along with anti-DIG-AP and anti-FLU-POD in TNB at the end of the second day. The secondary antibody

diluted in TNB was applied to the samples for 3 hr at room temperature after the *in situ* hybridization steps were finished. Finally, the samples were washed 3 times for 15 min in TNT and mounted in Vectashield (Vector Laboratories, Inc).

3.4.2. Immunohistochemistry

Tissues were dissected in PBS (1x) and collected in 4% PFA/PBS with 0.05% Tween-20 on ice. Fixation was continued at room temperature for 30 min. The samples were then washed three times for 15 min in PBX3 and blocked for 1 hr in PBX3 + 1% BSA. The tissues were then incubated with primary antibodies diluted in PBX3 + 1% BSA overnight at 4°C. On the second day, the samples were washed three times for 15 min in PBX3 and incubated with secondary antibodies diluted in PBX3 + 1% BSA for 3 hr at room temperature. The samples were then washed three times for 15 min in PBX3, mounted in Vectashield (Vector Laboratories, Inc) and visualized using a Zeiss Observer Z1 inverted fluorescent microscope, a Leica MZ16FA fluorescence stereomicroscope or a Leica TCS SP5 confocal microscope. Primary and secondary antibodies and their corresponding dilution ratios are listed in Table 3.3.

In the case of brain dissection and stainings the same procedure was performed with minor modifications. The brains were collected in 2% PFA/PBS with 0.05% Tween-20 on ice and fixation was continued at room temperature for 1 h and 30 min.

3.4.3. Xgal Staining

Detection of β-galactosidase was performed by Xgal staining as described in Emmons *et al.* (2007). Tissues were collected in 0.5% glutaraldehyde/PBSTX on ice and fixed for additional 15 min at room temperature. Tissues were washed 2 times for 5 min in PBSTX and incubated in freshly prepared 0.1% Xgal in Xgal staining solution for 30 min (at 37°C) to overnight (at room temperature). Tissues were then washed in dH_2O, mounted on slides and visualized using a light microscope.

3.5. Experiments For Functional Analysis

Functional analysis of *IroC* genes was performed in two major ways: loss-of-function and gain-of-function experiments.

3.5.1. Loss-of-Function Experiments: RNA Interference

The Vienna Drosophila Research Center (VDRC) has generated publicly available UAS-RNAi lines for every gene in the *Drosophila* genome. Fly RNAi lines for the members of the *IroC* family, *araucan* and *caupolican*, were ordered (two lines for each) from the VDRC. Flies were balanced using the double balancer *yw*^{67}*; Sp/CyO ; TM2/TM6B* for subsequent crosses.

UAS-RNAi lines were crossed to ElaV-Gal4 or IroC-Gal4 as shown in Figure 3.1, Figure 3.2 and Figure 3.3 and analyzed by *in situ* hybridization or immunohistochemistry against GFP, respectively.

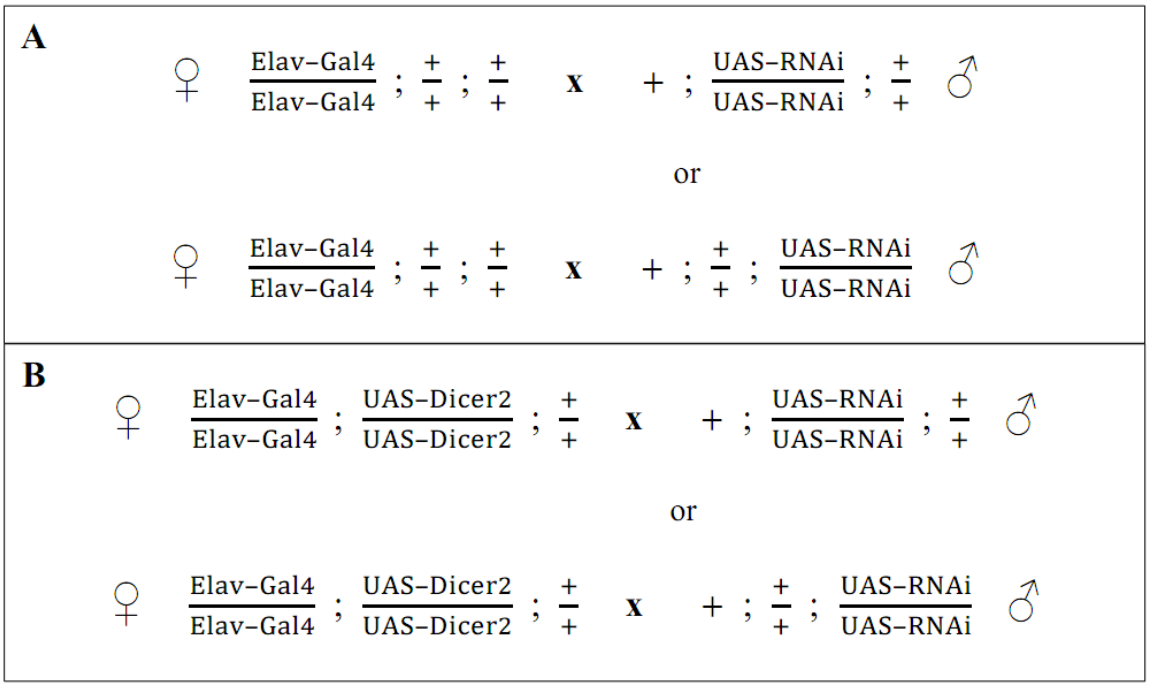

Figure 3.1. RNAi crosses using the Elav-Gal4 driver.

A

♀ $\frac{+}{+}$; $\frac{+}{+}$; $\frac{\text{iroC-Gal4, PrOR33c-mCD8::GFP}}{\text{TM6B}}$ **x** + ; $\frac{\text{UAS-RNAi}}{\text{UAS-RNAi}}$; $\frac{+}{+}$ ♂

or

♀ $\frac{+}{+}$; $\frac{+}{+}$; $\frac{\text{iroC-Gal4, PrOR33c-mCD8::GFP}}{\text{TM6B}}$ **x** + ; $\frac{+}{+}$; $\frac{\text{UAS-RNAi}}{\text{UAS-RNAi}}$ ♂

B

♀ $\frac{+}{+}$; $\frac{\text{PrOR85e-mCD8::GFP}}{\text{cyo}}$; $\frac{\text{iroC-Gal4}}{\text{TM6B}}$ **x** + ; $\frac{\text{UAS-RNAi}}{\text{UAS-RNAi}}$; $\frac{+}{+}$ ♂

or

♀ $\frac{+}{+}$; $\frac{\text{PrOR85e-mCD8::GFP}}{\text{cyo}}$; $\frac{\text{iroC-Gal4}}{\text{TM6B}}$ **x** + ; $\frac{+}{+}$; $\frac{\text{UAS-RNAi}}{\text{UAS-RNAi}}$ ♂

Figure 3.2. RNAi crosses using the IroC-Gal4 driver.

A

♀ $\frac{\text{Elav-Gal4}}{\text{Elav-Gal4}}$; $\frac{\text{PrORXX-mCD8::GFP}}{\text{PrORXX-mCD8::GFP}}$; $\frac{+}{+}$ **x** + ; $\frac{\text{UAS-RNAi}}{\text{UAS-RNAi}}$; $\frac{+}{+}$ ♂

or

♀ $\frac{\text{Elav-Gal4}}{\text{Elav-Gal4}}$; $\frac{\text{PrORXX-mCD8::GFP}}{\text{PrORXX-mCD8::GFP}}$; $\frac{+}{+}$ **x** + ; $\frac{+}{+}$; $\frac{\text{UAS-RNAi}}{\text{UAS-RNAi}}$ ♂

B

♀ $\frac{\text{Elav-Gal4}}{\text{Elav-Gal4}}$; $\frac{+}{+}$; $\frac{\text{PrORXX-mCD8::GFP}}{\text{PrORXX-mCD8::GFP}}$ **x** + ; $\frac{\text{UAS-RNAi}}{\text{UAS-RNAi}}$; $\frac{+}{+}$ ♂

or

♀ $\frac{\text{Elav-Gal4}}{\text{Elav-Gal4}}$; $\frac{+}{+}$; $\frac{\text{PrORXX-mCD8::GFP}}{\text{PrORXX-mCD8::GFP}}$ **x** + ; $\frac{+}{+}$; $\frac{\text{UAS-RNAi}}{\text{UAS-RNAi}}$ ♂

Figure 3.3. RNAi crosses for targeting experiments using the Elav-Gal4 driver.

3.5.2. Loss-of-Function Experiments: Clonal Analysis

IroC deficiency, iro^{DFM3}, is homozygous lethal, hence cell clones homozygous for *IroC* deficiency were generated in a heterozygous cell by Mosaic Analysis with a Repressible Cell Marker (MARCM) (Lee and Luo, 1999). iro^{DFM3} and tubGal80 were recombined with FRT80B in different flies. These flies were crossed and for the recombination event to happen both FLP recombinase under the control of *eyeless* promoter and FLP recombinase under the control of heat-shock promoter were used (Figure 3.4). For inducing the flies with heat-shock, flies at different developmental stages during larva were subjected to 37°C for 1 hr. F1 flies were genotyped and dissected. The expression of olfactory receptor genes, *OR33c* and *OR85e*, in *IroC* deficient clones was analyzed by double *in situ* hybridization followed by immunohistochemistry.

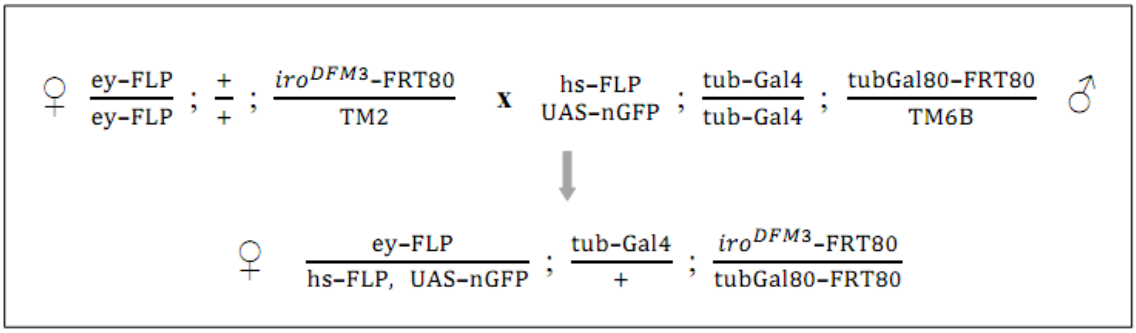

Figure 3.4. MARCM strategy.

3.5.3. Gain-of-Function Experiments

For tissue specific gain of function studies *IroC* genes, *ara*, *caup* and *mirr*, either alone or in combination with each other were misexpressed. Three strategies were applied. In the first strategy cell clones that ubiquitously express all *IroC* genes, along with RFP with a nuclear localization sequence, were created by FLP-out technique. act(FRT)CD2(FRT)-Gal4 flies were crossed to UAS-*ara*, UAS-*caup*, UAS-*mirr* flies. FLP expression is induced by heat-shock for 8 min at 0 ± 6 h APF (Figure 3.5). In the second strategy all *IroC* genes were misexpressed using the Elav-Gal4 driver, but the misexpression was inhibited until 42 ± 6 h APF by using temperature sensitive Gal80

protein (Figure 3.6). In the third strategy *IroC* genes either alone or in combination with each other were misexpressed early using the SG18.1-Gal4 line or late using the OR83b-Gal4 line (Figure 3.7 and Figure 3.8). The expression of olfactory receptor genes was analyzed by performing double *in situ* hybridization. In cases where cell clones were generated by using the FLP-out technique additionally immunohistochemistry for RFP was carried out.

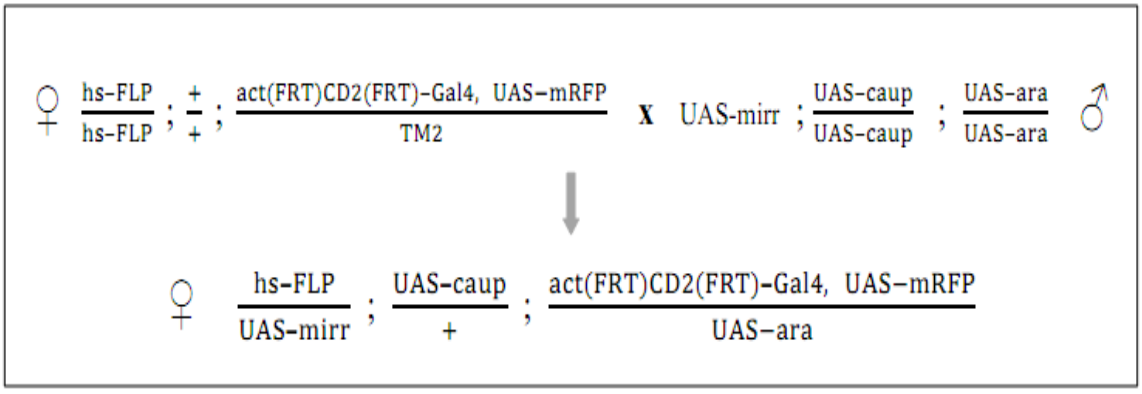

Figure 3.5. Generation of cell clones by the FLP-out technique. Cell clones induced by heat-shock ubiquitously express *IroC* genes and a RFP marker which is used to label these cells.

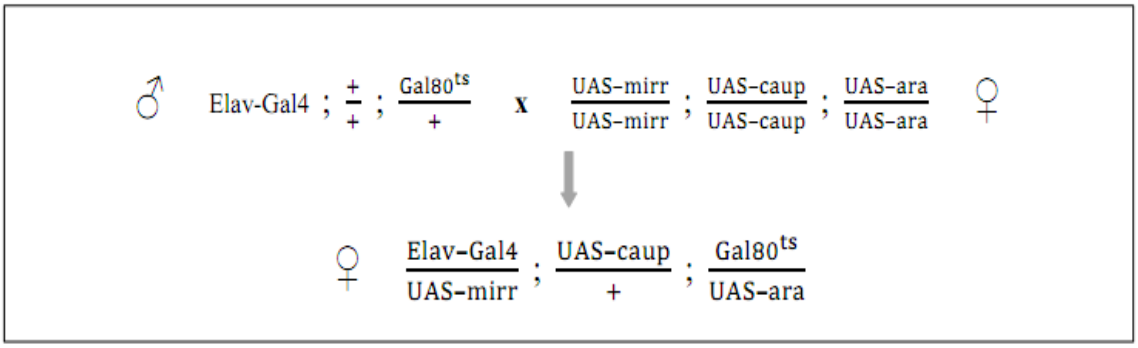

Figure 3.6. Misexpression of *IroC* genes using the Elav-Gal4 driver under the control of temperature-sensitive Gal80 protein. Pupa were synchronized and aged for 42 ± 6 h at 18°C, transferred to 29°C to misexpress the *IroC* genes with the Elav-Gal4 and stained at adult stage.

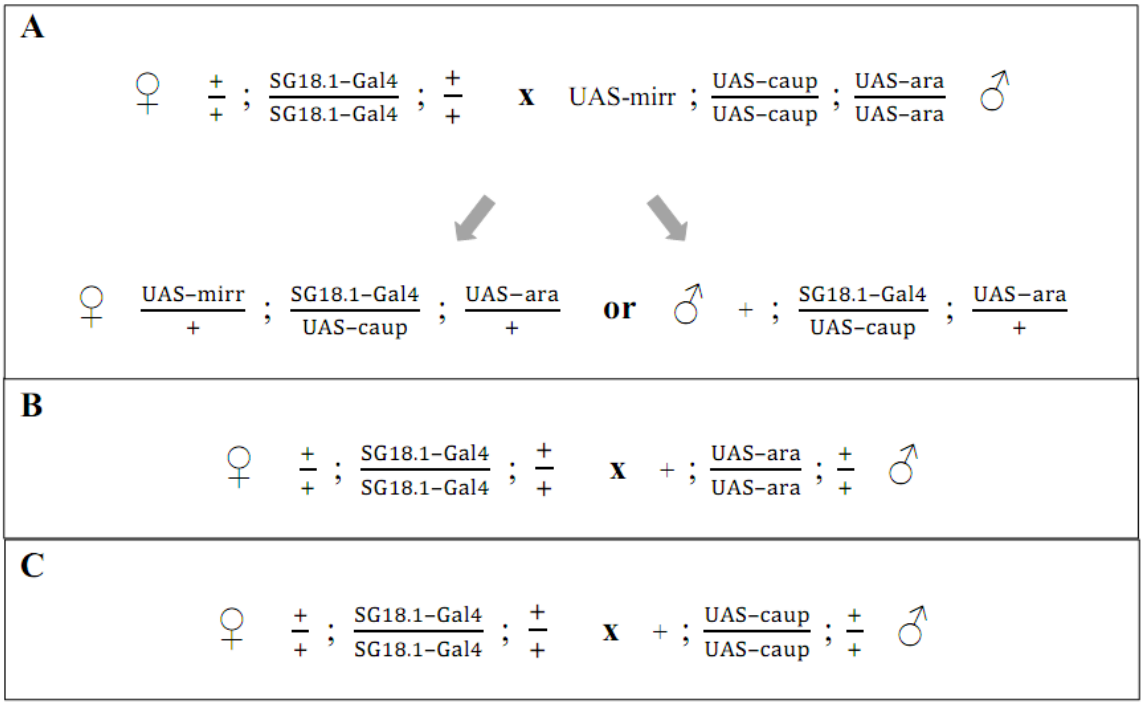

Figure 3.7. Early misexpression of *IroC* genes using the SG18.1-Gal4 driver. (A) Misexpression of all *ara* and *caup* genes together or all *IroC* genes together. (B) Misexpression of *ara* gene alone. (C) Misexpression of *caup* gene alone.

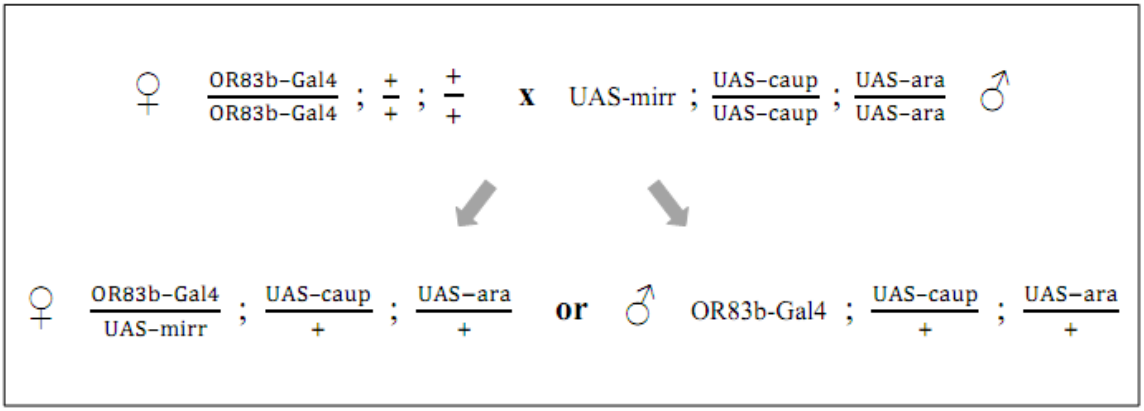

Figure 3.8. Late misexpression of *IroC* genes by using the OR83b-Gal4.

4. RESULTS

4.1. *IroC* Is Expressed in a Subset of Olfactory Receptor Neurons in the Maxillary Palp

IroC genes are known to control the development of various organs (Cavodeassi *et al.*, 2001; Bilioni *et al.*, 2005). As many peripheral appendages share common transcription factors during development (Campos-Ortega, 1993; Lai and Orgogozo, 2004), the initial aim of this study was to analyze the expression pattern of *IroC* in the olfactory system. To analyze the expression pattern of *IroC* an IroC-Gal4 line was used, which is a P[Gal4] insertion in the *IroC* locus and was prepared by the replacement of an earlier identified P[lacZ] enhancer trap element (ara^{rF209}) with P[Gal4] element. The insertion is believed to reflect the expression pattern of *ara* and *caup* genes (Figure 4.1) (Gomez-Skarmeta *et al.*, 1996; Sepp and Auld, 1999; Mazzoni *et al.*, 2008; Ikmi *et al.*, 2008).

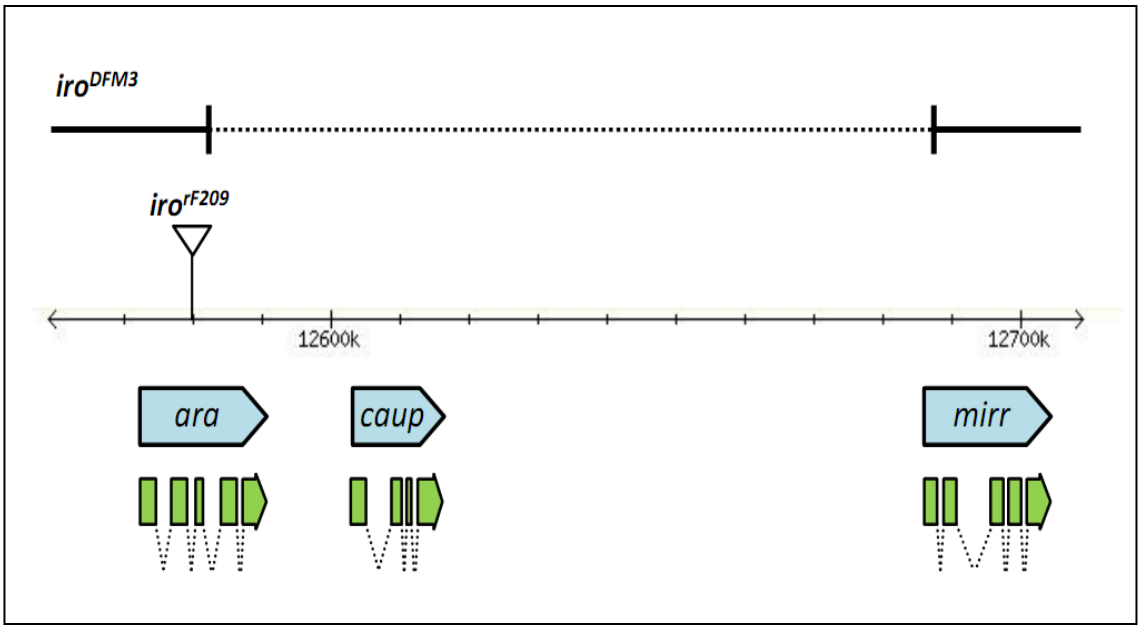

Figure 4.1. Physical map of the *IroC* locus. The triangle represents the insertion of P[lacZ] (iro^{rF209}), which was replaced with a P[Gal4] element by targeted transposition to create the IroC-Gal4 reporter line.

Analysis of *IroC* expression in the olfactory system at the adult stage showed that the IroC-Gal4 reporter is expressed in the maxillary palps but not in the antenna (Figure 4.2A). There are about 120 ORNs in the maxillary palp. A closer view of the IroC-Gal4 reporter expressing cells in the maxillary palp showed that it is not expressed in all, but in a subset of ORNs (Figure 4.2B).

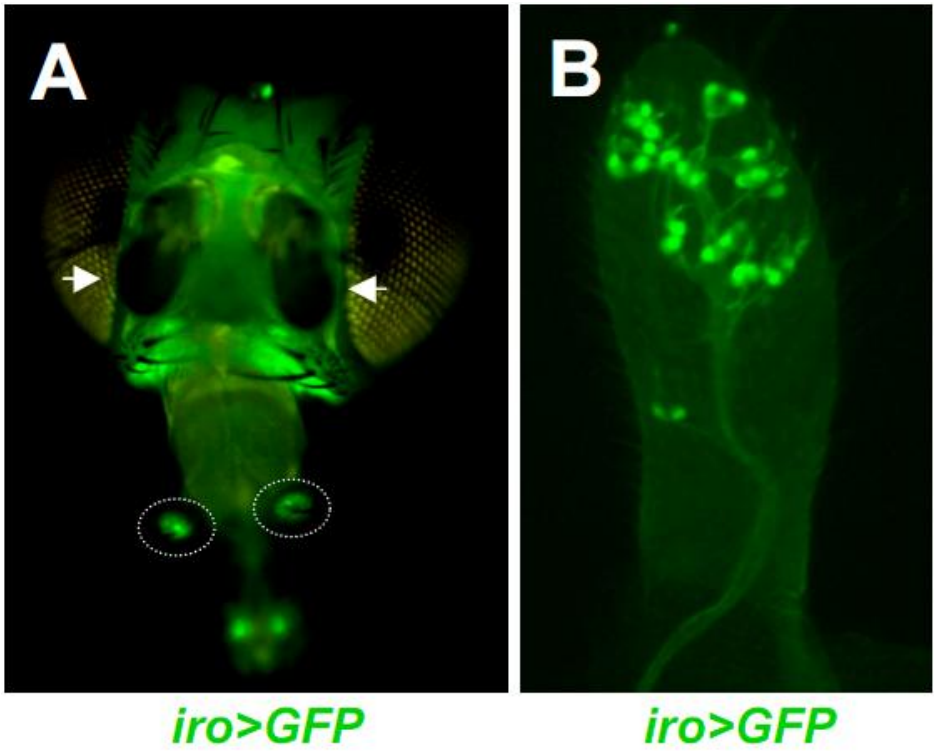

Figure 4.2. *IroC* expression in the olfactory system. (A) IroC-Gal4 reporter is expressed in the maxillary palps (circled) but not in the antenna (arrow). (B) A closer view of *IroC* expression in the maxillary palp. IroC-Gal4 reporter is expressed in a subset of ORNs.

There are six functional ORN classes paired in three palp basiconic sensillum subtypes (Figure 1.11). To investigate if the subset of IroC-Gal4 expressing cells corresponds to a functional subset of maxillary palp neurons, extracellular single-unit recordings have been carried out specifically on the IroC-Gal4 expressing cells (Dobritsa *et al.*, 2003; Goldman *et al.*, 2005). The odor-response profile of *IroC*-positive cells was compared to previously identified profiles of ORN subtypes (de Bruyne *et al.*, 1999; Goldman *et al.*, 2005). This analysis showed that the odor-response profile of *IroC*-expressing cells resembles the response profile of pb2 cells (Figure 4.3). This resemblance clearly demonstrates that *IroC* is specifically expressed in pb2 cells.

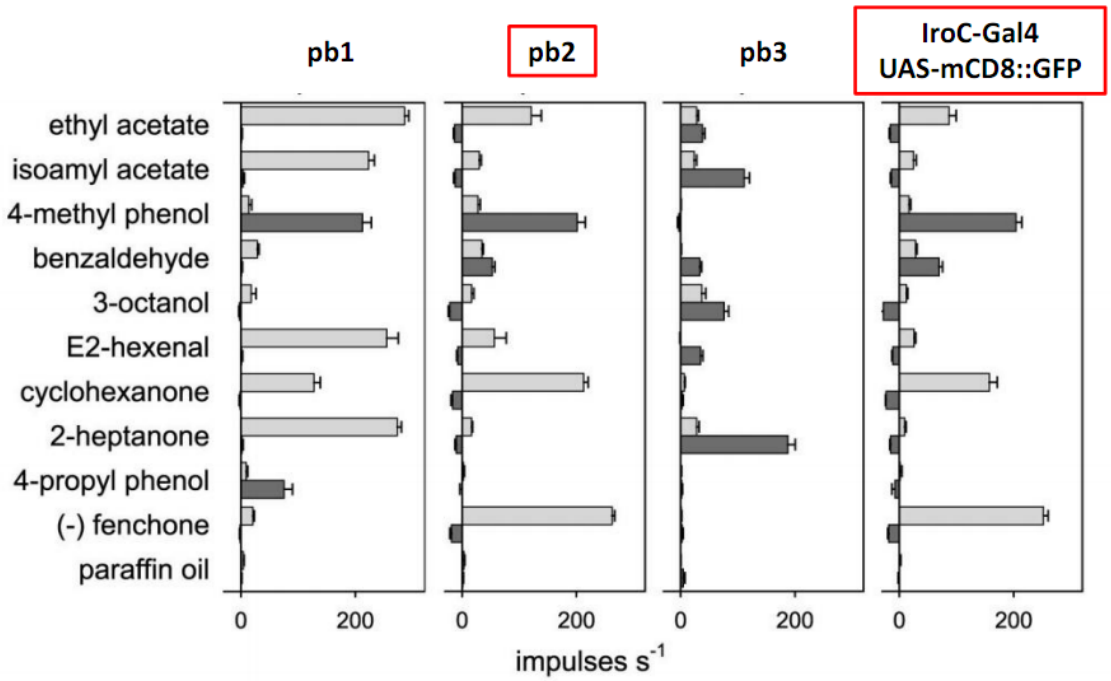

Figure 4.3. Odor response profile of *IroC* expressing cells. Single-unit recordings have been performed specifically on *IroC* expressing cells, which are labeled with membrane tagged GFP. The response profile of *IroC* positive cells resembles the response profile of pb2 cells.

Since extracellular recordings are carried out through the lymph, which contains dendrites of both neurons, the method does not distinguish the origin of the odor-response. To identify which pb2 cell expresses *IroC*, double *in situ* hybridization followed by antibody staining against GFP was carried out (Figure 4.4A). These experiments suggest that *IroC* is expressed in both of the pb2 cells: pb2A and pb2B. This initial finding was further confirmed by using genetic tools, eg. the *IroC* reporter was shown to colocalize with GFP expression under the control of *OR33c* gene promoter in pb2A cells. In addition, *IroC* reporter expression was also observed in the neighboring pb2B cells (Figure 4.4B).

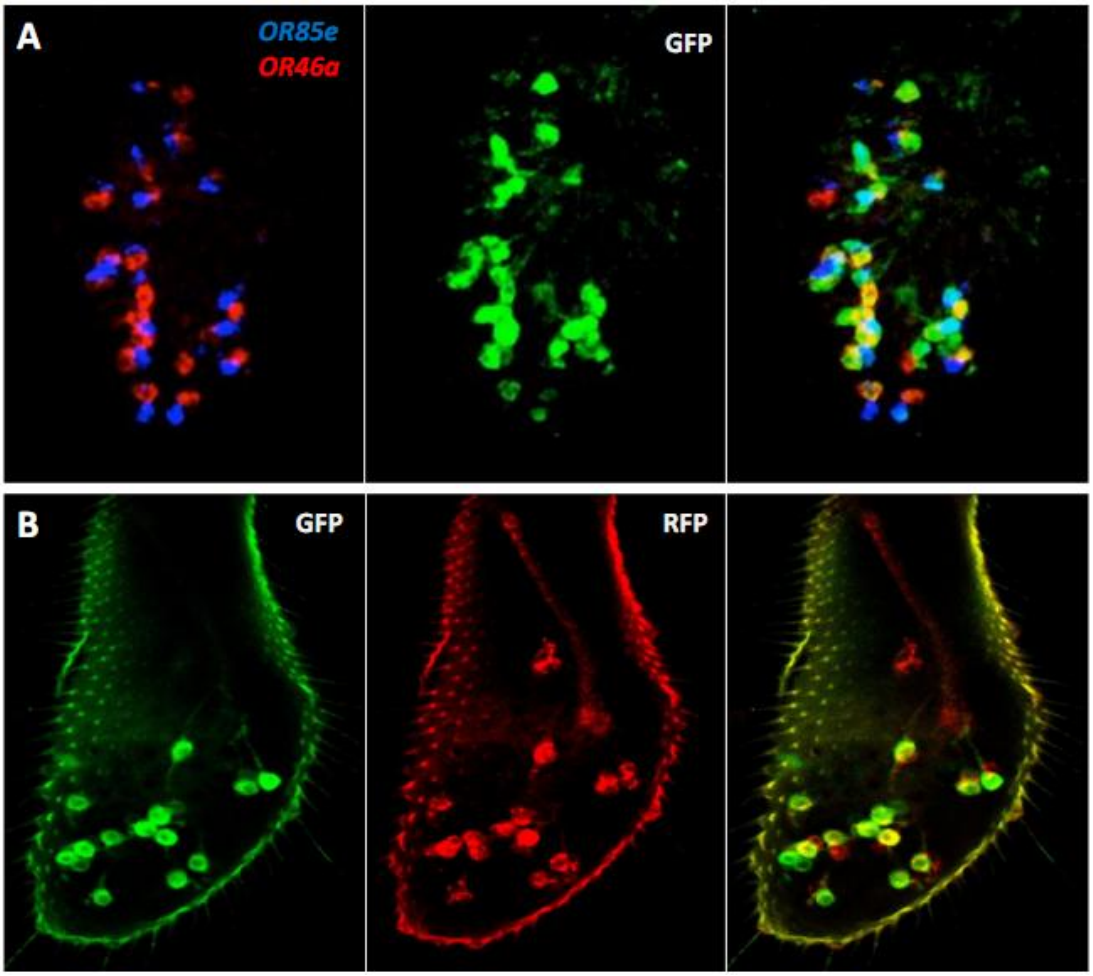

Figure 4.4. *IroC* expression localizes to pb2 cells of the maxillary palp. (A) Double *in situ* hybridization was carried out to highlight pb2 cells by using probes for *OR85e* (blue, pb2A cells) and *OR46a* (red, pb2B cells) genes. *IroC* reporter was detected by using an antibody against GFP (green). (B) GFP expression under the control of *OR33c* gene promoter and RFP expression driven by IroC-Gal4 colocalized in the pb2A cells. Note that there is also RFP expression detected in the neighboring pb2B cells.

These observations were further confirmed by looking at the targeting of *IroC* reporter expressing cells. *IroC* expressing cells send their axons to VC1 and VA7l glomeruli, which are the targeting glomeruli of pb2A and pb2B ORNs, respectively (Figure 4.5). The additional labeled glomeruli are not due to the innervations of those glomeruli by *IroC* expressing ORNs, but due to the innervations of those glomeruli by *IroC* expressing projection neurons and local neurons. This observation was further confirmed by specifically blocking *IroC* reporter expression in ORNs with Gal80 expression under the control of an *OR83b* gene promoter. *OR83b* is expressed in most of

the antennal ORNs and in all of the maxillary palp ORNs (Benton *et al.*, 2006). These data show that the *IroC* reporter is expressed in the pb2 sensillum subset of the maxillary palp but not in the antenna.

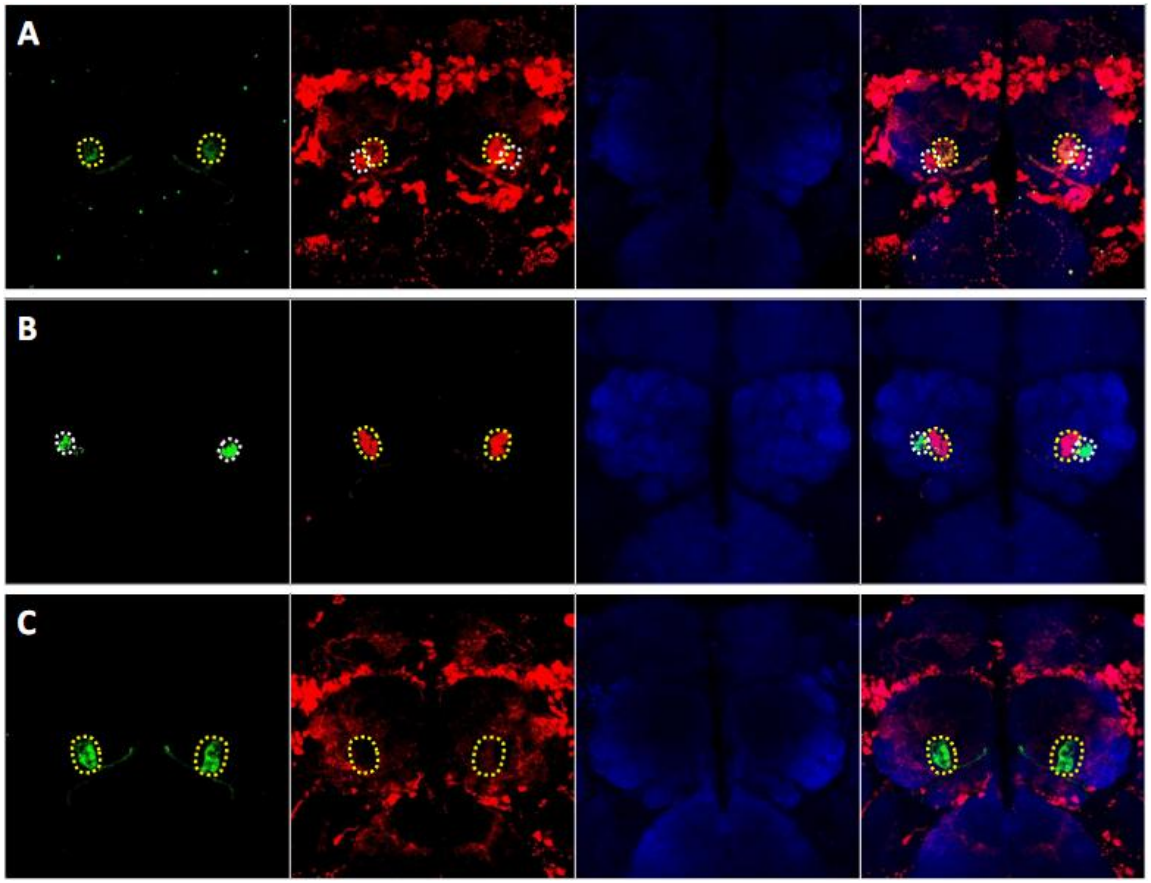

Figure 4.5. *IroC* expression localizes to the glomeruli of pb2 cells. (A) Membrane-tagged RFP expression driven by IroC-Gal4 reporter (red) colocalizes with GFP expression under the control of *OR33c* gene promoter (green) in VC1 glomeruli, the target glomeruli of pb2A cells. Note that there is also RFP expression in the neighboring VA7l glomeruli, the target glomeruli of pb2B cells. (B) Synaptotagmin::GFP (syt::GFP) is expressed under the control of *OR46a* gene promoter and membrane tagged RFP expression is driven by OR85e-Gal4 reporter. (C) There is no RFP expression driven by IroC-Gal4 reporter in either VC1 (yellow dashed lines) or VA7l glomeruli (not shown). RFP expression driven by IroC-Gal4 reporter is specifically inhibited in ORNs by Gal80 protein expressed under the control of *OR83b* gene promoter. In all pictures white dashed circles indicate the VA7l glomeruli and yellow dashed circles indicate the VC1 glomeruli.

Gene regulation of sensory receptors in sensory neurons happens mainly through transcriptional regulation. In a recent study *IroC* has been implicated in the regulation of rhodopsin genes in the fly retina (Mazzoni *et al.*, 2008). There are two ommatidial subtypes in the *Drosophila* eye: yellow and pale subtypes (Morante *et al.*, 2007). These mutually exclusive subtypes are distinguished by distinct rhodopsins that are expressed in inner photoreceptors (R7 and R8). Each photoreceptor cell expresses one rhodopsin gene in accordance with the "one neuron-one receptor" rule. The pale subtype contains *Rh3* in R7 and *Rh5* in R8, whereas the yellow subtype contains *Rh4* in R7 and *Rh6* in R8 (Chou *et al.*, 1999). Mazzoni *et al.* (2008) identified a novel class of ommatidia in the dorsal edge of the eye, which coexpress *Rh3* and *Rh4* in R7 cells and found that dorsally expressed *IroC* is both necessary and sufficient for the coexpression of *Rh3* and *Rh4* rhodopsins. This finding and the observation that *IroC* is expressed in a subset of ORNs that coexpresses two OR genes let us to hypothesize that *IroC* might be involved in the coexpression of OR genes *OR33c* and *OR85e* in pb2A neurons (see Figure 1.9). In order to test this hypothesis, classical loss-of-function (LOF) and gain-of-function (GOF) experiments were carried out.

4.2. *IroC* Binding Sites in the Upstream of Maxillary Palp OR Genes

Bilioni *et al.* (2005) identified the "ACAnnTGT" as the minimal Mirr binding site and showed that other Iro proteins can recognize the same sequence although with different affinity. As the palindromic nature of the binding site suggests, Iro proteins can bind to the site as both homo- and hetero-dimers. The upstream regions of OR genes expressed in the maxillary palps were analyzed for putative *IroC* binding sites. It turned out that some of the OR genes, genes that are expressed in the pb2 and pb3 classes, have varying numbers of *IroC* binding site in their upstream region (Figure 4.6). Since this binding site has a high occurrence throughout the genome (data not shown), functional analysis of these identified sites in the upstream region of OR genes, needs to be performed. Nevertheless, the presence of this binding site(s) in the upstream region of OR genes initiated investigations of the potential role of *IroC* genes as regulators of OR gene expression in the maxillary palp.

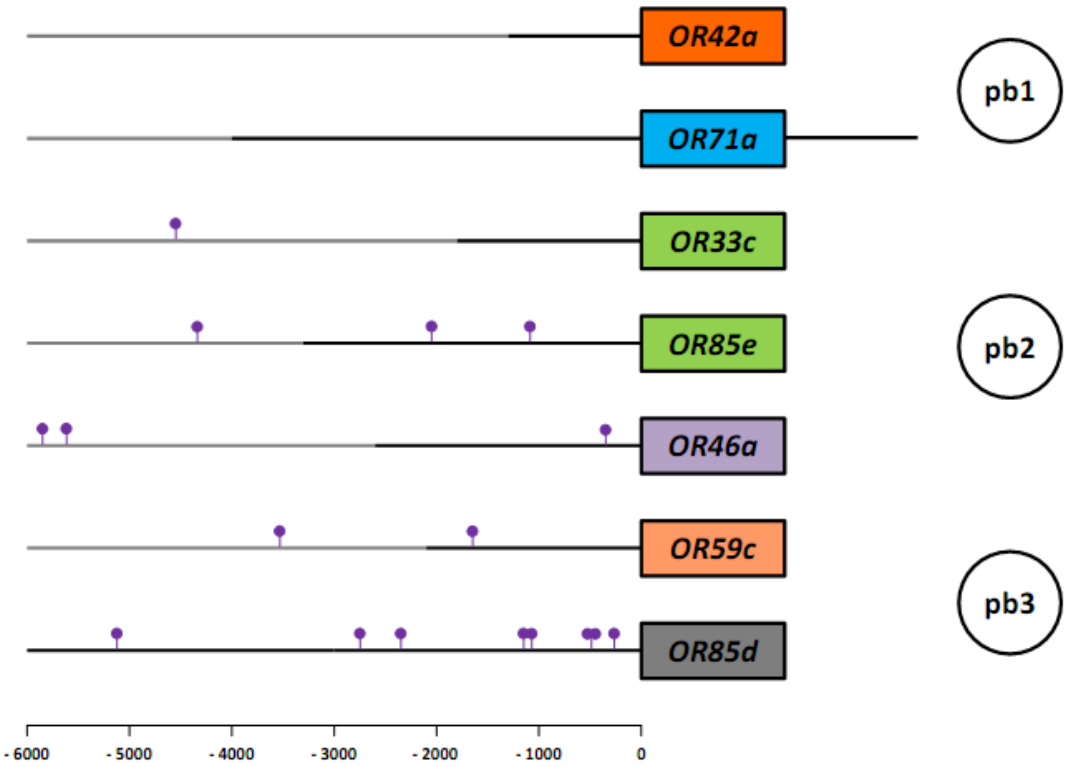

Figure 4.6. Identified *IroC* binding sites on the upstream regions of OR genes expressed in the maxillary palps. Black lines indicate the identified promoter regions that drive faithful expression of GFP in Couto *et al.* (2005). In case of *OR71a* downstream region, which is needed for correct expression, is also included (Goldman *et al.*, 2005).

4.3. Setting Up the System

4.3.1. Characterization of Gal4 Lines for Use in LOF and GOF Experiments

To perform LOF and GOF experiments in a tissue-specific and time-dependent manner tools that will allow for this manipulation are necessary. As the maxillary palp has not been studied extensively so far tools to perform the necessary experiments had to identified and generated. Initially several Gal4 lines that have been previously described in the literature to show expression in the maxillary palp were obtained and have been characterized in more detail in this study. Table 4.1 summarizes the Gal4 lines used in this study.

Table 4.1. Gal4 lines used during this study.

Name of the Gal4 line	Reference
Elav-Gal4	Jefferis *et al.*, 2004
Appl-Gal4	Torroja *et al.*, 1999
nSyb-Gal4	Verstreken *et al.*, 2009
tub-Gal4	Lee and Luo, 1999
ey-Gal4	Hummel *et al.*, 2003; Bonini *et al.*, 1997
pb-Gal4	Benassayag *et al.*, 2003
OR83b-Gal4	Larsson *et al.*, 2004; Benton *et al.*, 2006
SG18.1-Gal4	Shyamala and Chopra, 1999; Jhaveri and Rodrigues, 2002

Elav-Gal4 (Figure 4.7A), Appl-Gal4 (Figure 4.7B) and nSyb-Gal4 are all post mitotic pan-neural drivers. They are not specific to ORNs but since their expression onset is early they are widely used. Tub-Gal4 is expressed in all cells at any developmental stage (Lee and Luo, 1999). The ey-Gal4 line has been prepared by cloning a fragment which reports *eyeless* staining in eye progenitor cells into the Gal4 vector (Bonini *et al.*, 1997). It is expressed in the eye-antennal imaginal disc and additionally in the developing antennae and maxillary palps. The pb-Gal4 line has been prepared by using the upstream region of the *proboscipedia* gene, which is a homeotic gene and required for the development of adult mouthparts including maxillary palps (Bennasayag *et al.*, 2003) (Figure 4.7C). The OR83b-Gal4 line is prepared by using the promoter region of the *OR83b* gene, which is expressed in most of the ORNs in the antenna and in all ORNs in the maxillary palp. This Gal4 line is specific to ORNs; however, since the expression onset is late, it is mostly used in late rescue experiments (Larsson *et al.*, 2004) (Figure 4.7D). The SG18.1-Gal4 line has been identified in an enhancer trap screen. It is expressed in the primary organs of the adult olfactory system and their presumptive areas in the imaginal discs and widely used in research covering ORN axon targeting (Shyamala and Chopra, 1999; Jhaveri and Rodrigues, 2002) (Figure 4.7E).

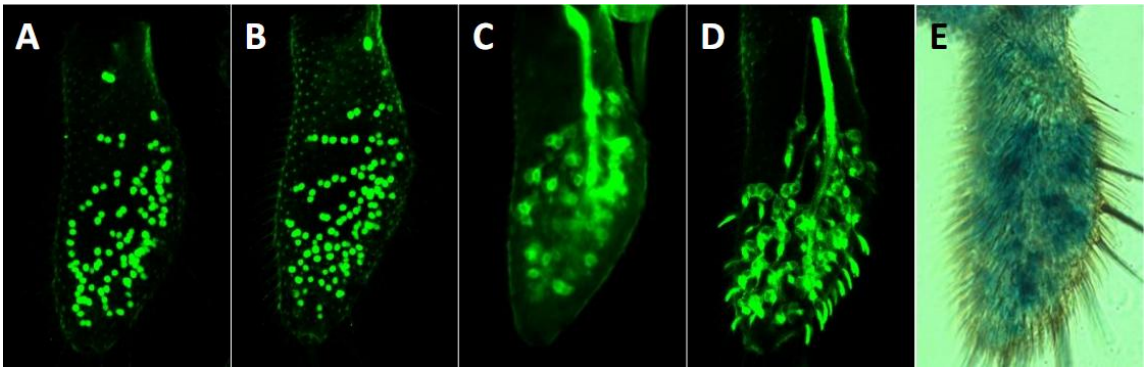

Figure 4.7. Characterization of Gal4 lines in the maxillary palp. (A) Elav-Gal4 and (B) Appl-Gal4 drive the expression of GFP with a nuclear localization signal. (C) SG18.1-Gal4 and (D) OR83b-Gal4 drive the expression of membrane tagged GFP. GFP is stained by using an antibody against GFP. (E) pb-Gal4 drives the expression of lacZ, which was detected by Xgal staining.

4.3.2. An Alternative Detection System: Generation of Transgenic Reporter Lines

During this study the effect of LOF and GOF of *IroC* was measured by changes in the expression of OR genes as detected by *in situ* hybridization. While *in situ* hybridization experiments give a direct visual readout of changes in expression it has also drawbacks, as it is very tedious and a long process prone to variation. To simplify the experimental setup the possibility of using direct promoter fusion lines of the receptors of interest, which will give the same visual readout of changes was explored. Transgenic fly lines that express GFP under the control of promoters of various OR genes have been generated (Couto *et al.*, 2005). While GFP tagged versions of *OR33c* and *OR85e* were available red fluorescent protein tagged versions of these promoters were not available. However, as detection of changes in the coexpression of these two OR genes was one of the main aims, differently tagged versions of these promoters were necessary. For this purpose mCherry versions of two ORs were generated by targeted insertion of promoter constructs into the fly genome: PrOR33c-mCherry and PrOR85e-TLNΔCherry. Analysis of the transgenic lines showed that the generated PrOR33c-mCherry line shows no expression of Cherry at all (Figure 4.8A). While PrOR85e-TLNΔCherry showed some expression in the maxillary palps, the expression was very weak even after antibody staining (Figure 4.8B). Additionally, the Cherry expression under the control of *OR85e* gene promoter did not colocalize with the

GFP expression driven by the promoter of *OR33c* in some cases (Figure 4.8C). As a result, no faithful promoter fusion constructs were generated that could replace and/or complement the *in situ* hybridization experiments. Thus, all LOF and GOF experiments were performed by detecting the expression of the OR genes by fluorescence double *in situ* hybridization.

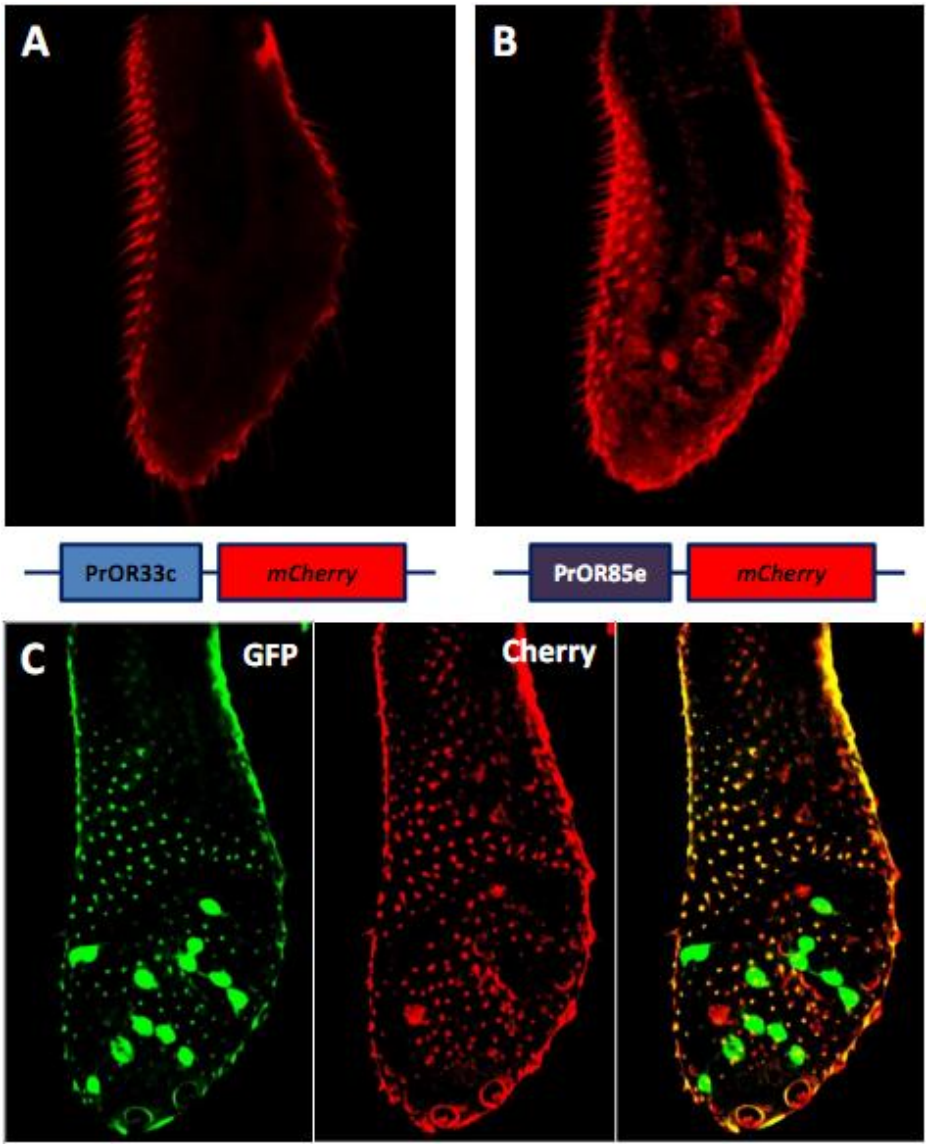

Figure 4.8. Expression analysis of Cherry protein in transgenic fly lines generated by targeted insertion of promoter fusion constructs into the fly genome. (A) PrOR33c-mCherry line. No Cherry expression under the control of *OR33c* gene promoter was observed. (B) PrOR85e-TLNΔCherry line. Cherry expression was visualized using anti-DsRed antibody. (C) GFP expression under the control of *OR33c* gene promoter does not colocalize with the Cherry expression driven by the *OR85e* gene promoter.

4.4. Lack of *IroC* Does Not Result in the Loss of *OR33c* and *OR85e* Expression

In order to test the hypothesis that iroC controls the co-expression of two OR genes first classical loss-of-function experiments were carried out. Two strategies were used: downregulation by RNA interference and analysis of *IroC* deficient flies. For the downregulation of *IroC* genes by RNA interference publicly available UAS-RNAi lines for *IroC* genes were used. Two UAS-RNAi lines for *ara* and *caup* genes, respectively, are publicly available. These UAS-RNAi lines were crossed to the pan-neural driver Elav-Gal4 at three different temperatures and fluorescent double *in situ* hybridization for OR genes *OR33c* and *OR85e* were carried out on maxillary palps (Table 4.2). While crosses are carried out normally at 25°C, raising the temperature to 29°C raises the activity of the driver and lowering the temperature to 18°C lowers the activity of the driver. Thus, lowering the temperature is known to help to overcome possible lethality problems.

Table 4.2. Downregulation of *IroC* genes. There are 2 UAS-RNAi lines for each *ara* and *caup* genes. These lines were crossed to the pan-neural driver Elav-Gal4 at different temperatures. F1 flies of checked crosses were dissected and the expression of olfactory receptor genes was analyzed by double *in situ* hybridization.

X		**Elav-Gal4**			**Elav-Gal4; UAS-Dicer2**		
		18°C	**25 °C**	**29 °C**	**18 °C**	**25 °C**	**29 °C**
UAS	**ara^{RNAi} VDRC 49079**		✓	✓		✓	✓
	ara^{RNAi} VDRC 101903	✓	lethal	lethal		lethal	lethal
	caup^{RNAi} VDRC 2931		✓	✓		✓	✓
	caup^{RNAi} VDRC 105705		✓	✓		✓	✓

Downregulation of *IroC* genes *ara* and *caup*, did not result in any change in the coexpression of *OR33c* and *OR85e* (Figure 4.9A). To enhance the RNA interference effect a UAS-Dicer2 transgene has been included, as this was reported to enhance the activity of RNAi in neurons (B. Dickson, personal communication). However, even under these conditions no change in the coexpression of OR genes have been observed (Figure 4.9B).

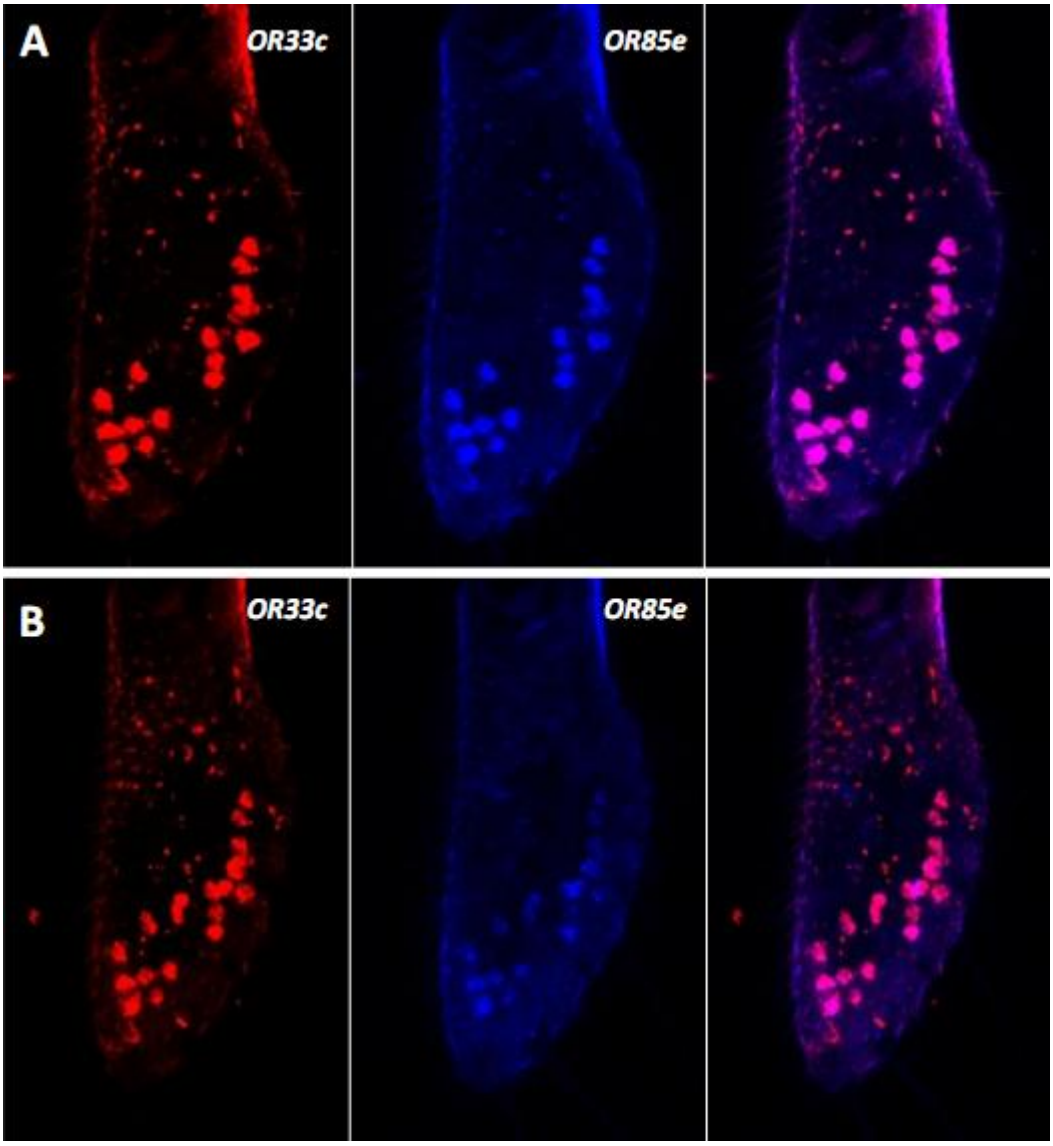

Figure 4.9. Downregulation of *ara* and *caup* did not result in any change in the coexpression pattern of *OR33c* and *OR85e*. (A) Elav-Gal4 drives UAS-ara$^{RNAi\ 49079}$ at 29°C. (B) Elav-Gal4 drives both UAS-ara$^{RNAi\ 49079}$ and UAS-Dicer2 at 29°C.

During the RNA interference experiments a change in the number of cells that coexpress *OR33c* and *OR85e* was noticed. The cells were counted and an increase in one of the RNAi crosses was noticed, which is thought to be the strongest RNAi line. Figure 4.10 summarizes the number of cells that were detected in each of the RNAi crosses by *in situ* hybridization. The number of cells counted in the control maxillary palps is in accordance with previous findings (Ray *et al.*, 2007). These results indicate that lack of *IroC* may result in an increase of the pb2A cells. Thus, rather than affecting the expression of OR genes *IroC* might regulate the number of cells in the pb2 subset.

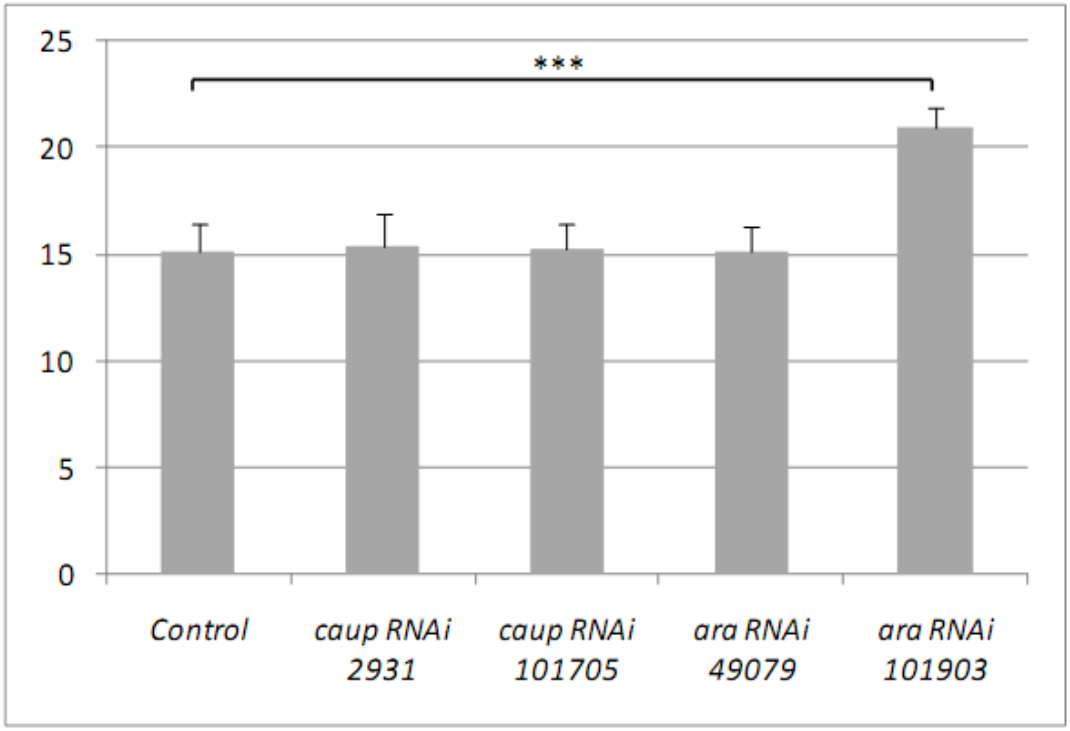

Figure 4.10. Increase in the number of *OR33c* and *OR85e* expressing cells. UAS-RNAi lines were crossed to Elav-Gal4. n= 12, p*** < 0,001, error bars indicate SEM.

In order to confirm the RNA interference results another experiment was performed, in which downregulation of *IroC* was achieved using the IroC-Gal4 driver and *OR33c* and *OR85e* reporter lines were used as read-out for the pb2A cell. While this experiment does not give information about whether *IroC* plays a role in the coexpression of *OR33c* and *OR85e* it allows the determination of the number of cells that are GFP-positive and thus represent the pb2A subset. These results were then compared to the numbers obtained by *in situ* hybridization. Stainings showed that control animals have about 15 pb2A cells per maxillary palp; however, downregulation of *ara*, but not *caup*, resulted in an increase in the number of pb2A cells. Figure 4.11 summarizes the number of counted cells that are positive for GFP. These results confirm our previous findings with the Elav-Gal4 driver, indicating that *IroC* genes, especially *ara*, restrict the number of pb2A cells.

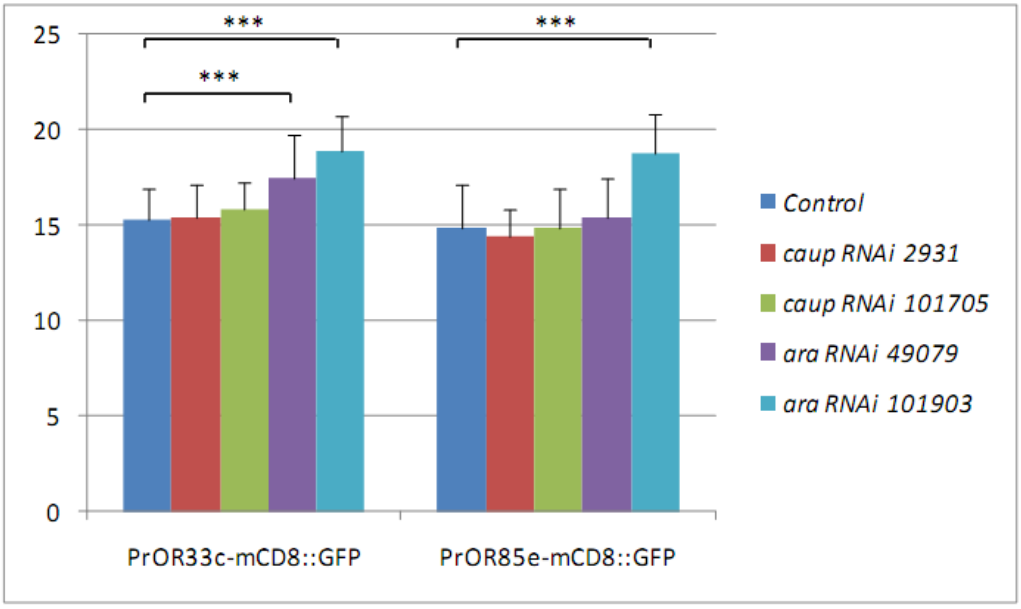

Figure 4.11. Number of PrOR33c-GFP and PrOR85e-GFP positive cells after downregulation by RNAi of *ara* and *caup* by IroC-Gal4. n= 20, p*** < 0.001, error bars indicate SEM.

The observed increase in the number of cells that express *OR33c* and *OR85e* may be due to either a general increase in the number of pb2 ORNs or abnormal segregation of a regulatory protein which may result in the misexpression of OR genes, *OR33c* and *OR85e*, that are normally expressed in the pb2A cells, in the pb2B cells. To test these possibilities *in situ* hybridization against 3 probes was performed; *OR33c* and *OR85e* to label the pb2A subset and *OR46a* to label the pb2B subset. As a result of these experiments a parallel increase in the number of *OR46a* expressing pb2B cells was obeserved (data not shown), which suggests that *IroC* downregulation either results in a general increase in the number of ORNs or in an increase in the number of pb2 cells. Additionally in two instances RNAi-based *IroC* downregulation resulted in the misexpression of *OR33c* and/or *OR85e* in the *OR46a* expressing pb2B cell (Figure 4.12) (*OR33c* and *OR85e* were labeled with the same label and detected with the same detection method; thus, the staining does not distinguish these two probes in this experiment). It has been reported that *IroC* genes modulate Notch signaling (Irvine, 1999; Bilioni *et al.*, 2005), which plays a role in specifying ORN identity and eventually in OR expression (Endo *et al.*, 2007; Ray *et al.*, 2007). The observed misexpression of OR genes suggests that IroC may play a role in the asymmetric cell division of A and B cells of pb2 sensillum that derive from a common progenitor.

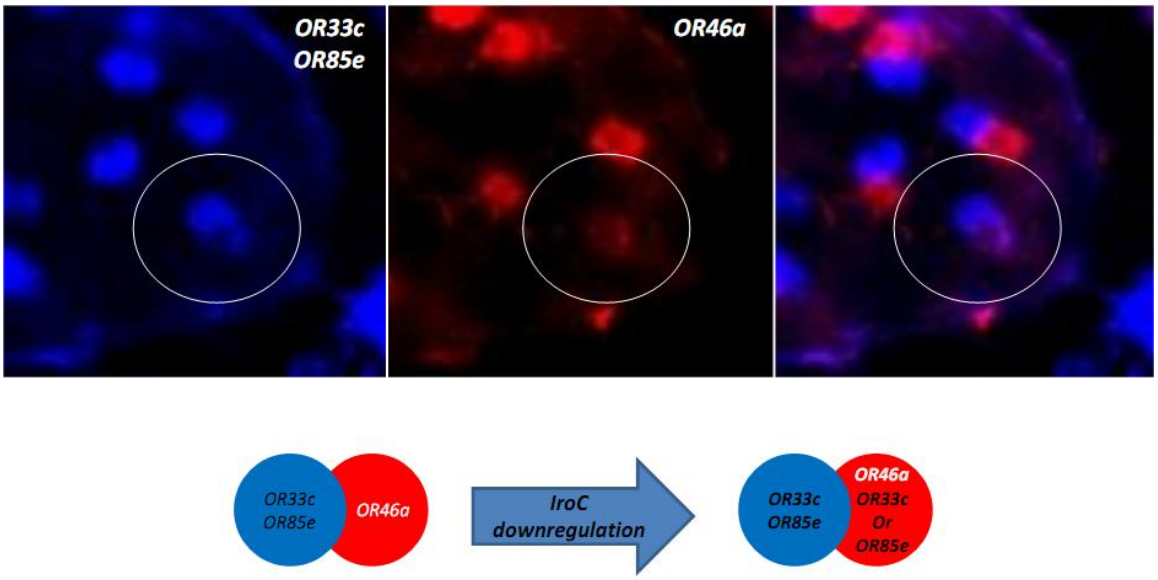

Figure 4.12. Downregulation of *IroC* results in the misexpression of *OR33c* and/or *OR85e* in the pb2B cell. Downregulation of *ara* by RNAi using the Elav-Gal4 resulted in the misexpression of *OR33c* and/or *OR85e* (blue) in the *OR46a* expressing pb2B cell (red).

A more reliable way to investigate the role of a gene is to look at null mutants. *IroC* deficient lines (null mutant flies) exist and the deficiency (iro^{DFM3}) cover the *ara*, *caup* genes and delete most of the regulatory sequences of *mirr* (see Figure 4.1) (Gomez-Skarmeta *et al.*, 1996; Diez del Corral *et al.*, 1999; Mazzoni *et al.*, 2008). However, these lines are homozygous lethal. The lethality problem can be circumvented by generating mutant clones of cells in the maxillary palp in an otherwise heterozygous animal. Thus, clones of *IroC* mutant cells were generated by using the Mosaic Analysis with a Repressible Cell Marker (MARCM) technique (Lee and Luo, 1999) (Figure 4.13). This method adds the possibility to positively mark the generated mutant clones. Thus, to do this analysis a mutant iro^{DFM3} allele that is recombined on an FRT chromosome and a tub-Gal80 transgene that is recombined on another FRT chromosome was used. When these alleles are present in the same fly together with a UAS-GFP transgene no GFP expression is observed because Gal80 inhibits Gal4. Upon expression of a FLP recombinase that is under the control of a desired promoter (in this case *eyeless*) mitotic recombination between FRT sites is triggered. One of the resulting daughter cells will be homozygous for iro^{DFM3}, and the other cell will be homozygous for *tub-Gal80*. Due to the loss of the repressor (Gal80) in homozygous iro^{DFM3} daughter cells, repression of Gal4 is relieved and GFP can now be expressed. Thus, using this technique, homozygous mutant cells can be

generated in a heterozygous mutant background and identified by their GFP expression. Initial experiments showed that ey-FLP does not create too many clones in the maxillary palps (Sweeney *et al.*, 2007; unpublished observation). Therefore, to generate more cell clones deficient for *IroC* the amount of FLP recombinase was increased by using a heat-inducible promoter. For inducing the flies with heat-shock, flies at different developmental stages during larval stages were subjected to 37°C for 1 hr. After optimizing the heat-shock protocol this background was used to analyze the effect of the *IroC* deficiency on the coexpression of *OR33c* and *OR85e* by double *in situ* hybridization followed by antibody staining against GFP. In accordance with the RNA interference results, *IroC* deficient cells continued to express *OR33c* and *OR85e* (Figure 4.14). Thus, in contrast to the hypothesis that *IroC* regulates coexpression of ORs in pb2A cells, these results suggest that it does not seem to have an effect on the expression of ORs.

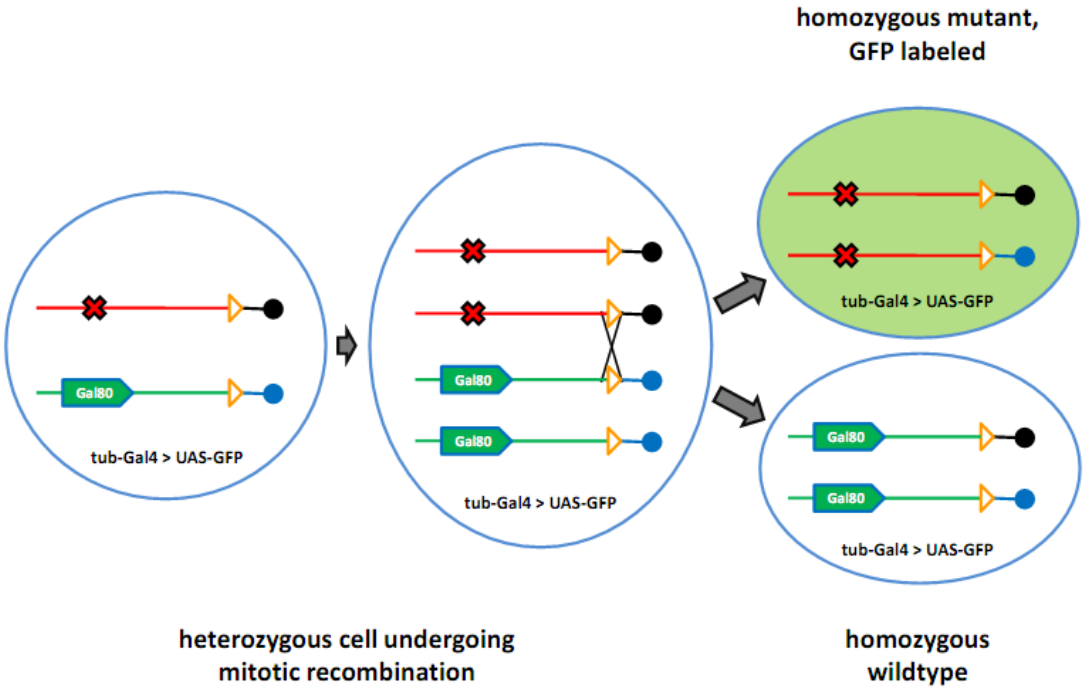

Figure 4.13. MARCM system. After site-specific recombination the heterozygous mother cell give rise to two daughter cells: one of which is homozygous wildtype and have two copies of Gal80 repressor and the other cell is homozygous mutant and labeled with GFP due to the loss of Gal80 repressor. Red filled X marks the *IroC* deficiency (*iro*^{DFM3} mutant allele), blue and black filled circles represent the centromeres, white filled triangles show FRT sites (FRT80) and black X stands for FLP-mediated recombination.

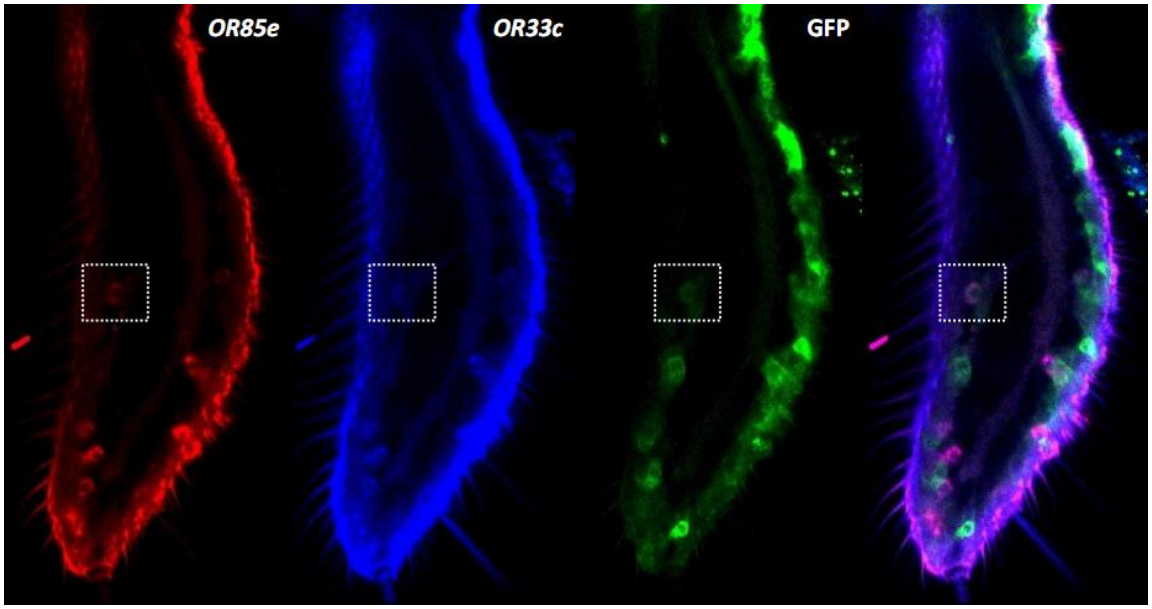

Figure 4.14. Coexpression of *OR33c* and *OR85e* persists in *IroC* deficient clones marked positively with GFP. FLP expression induces recombination between FRT sites and hence creates cells homozygous mutant for *IroC*. Due to the loss of the repressor Gal80 these cells are labeled with GFP. Double *in situ* hybridization followed by antibody staining against GFP shows that *IroC* deficient cells coexpress *OR33c* (blue) and *OR85e* (red).

4.5. Misexpression of *IroC* Results in the Repression of OR Genes

Along with the loss-of-function experiments, a series of misexpression experiments were carried out to study whether *IroC* genes are sufficient to induce OR coexpression or at least expression of one of the coexpressed genes across the maxillary palp. For the misexpression experiments various Gal4 driver lines were used (Table 4.3). Most of the used driver lines are not solely specific to ORNs but are either expressed in all neurons or are general drivers expressed in all cells; hence early overexpression of *IroC* resulted in lethality at various developmental stages. Even decreasing the temperature to 18°C to lower the Gal4 activity resulted in lethality.

Table 4.3. Misexpression of *IroC* genes with various Gal4 drivers at different temperatures.

			UAS			
			ara	*caup*	*ara caup*	*ara caup mirr*
Gal4	Elav	18°C	lethal	lethal	lethal	lethal
		25°C	lethal	lethal	lethal	lethal
	Appl	18°C	lethal	lethal	lethal	lethal
		25°C	lethal	lethal	lethal	lethal
	nSyb	18°C	lethal	lethal	lethal	lethal
		25°C	lethal	lethal	lethal	lethal
	tub	18°C	lethal	lethal	lethal	lethal
		25°C	lethal	lethal	lethal	lethal
	ey	18°C	lethal	lethal	lethal	lethal
		25°C	lethal	lethal	lethal	lethal
	pb	18°C	lethal	lethal	lethal	lethal
		25°C	lethal	lethal	lethal	lethal
	OR83b	25°C	✓	✓	✓	✓
	SG18.1	25°C	✓	✓	✓	✓

In order to overcome the lethality problem and to misexpress *IroC* early (earlier than OR expression onset) during development different strategies were applied. In the first strategy clones of cells that ubiquitously express *IroC* genes by using a Gal4 line under the control of the *actin* (*Act5C*) promoter were generated. However, in this experiment the actin promoter is separated from Gal4 and can only be activated upon elimination of a CD2 cassette that is present between the promoter and the Gal4 sequence flanked by two FRT sites. The FRT sites can be recombined by heat shock induced FLP recombination (Pavlopoulos *et al.*, 2001) (Figure 4.15). This recombination will result in the FLP-out of

the *CD2* cassette juxtaposing the *actin* promoter to Gal4 and hence driving UAS-*IroC* gene expression at a low uniform level in all cells of the clone along with an UAS-*mRFP* with a nuclear localization sequence to label the cell clones.

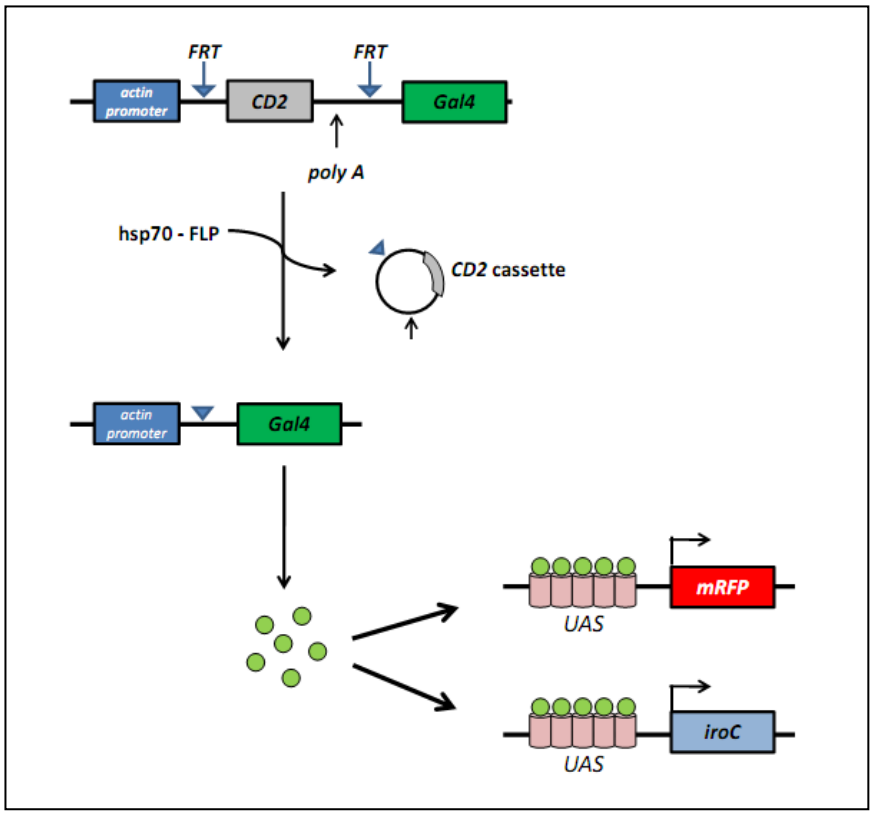

Figure 4.15. FLP-out technique. Upon heat-shock, FLP recombinase is induced. FLP recombines the two FRT sites flanking the *CD2* cassette and removes it. Elimination of the *CD2* cassette juxtaposes the *actin* promoter to Gal4 and hence drives the expression of the target genes, in this case *IroC*, along with RFP to label the cells.

Clones were generated by FLP expression induced by heat-shock for 8 min at 0 ± 6 h APF and analyzed by *in situ* hybridization against 3 probes, *OR33c* and *OR85e* to label the pb2A subset and *OR46a* to label the pb2B subset. As there was no possibility to do four-color imaging, two of the probes (*OR33c/OR85e*) were labeled with the same label and detected with the same detection method. Thus, the staining does not distinguish these two probes in this experiment. The second label was used for *OR46a* and the third channel was used to detect the RFP-labeled *IroC* positive cells. Analysis of more than 30 clones showed that early expression of *IroC* never correlates with the expression of OR genes of the pb2 subset (Figure 4.16).

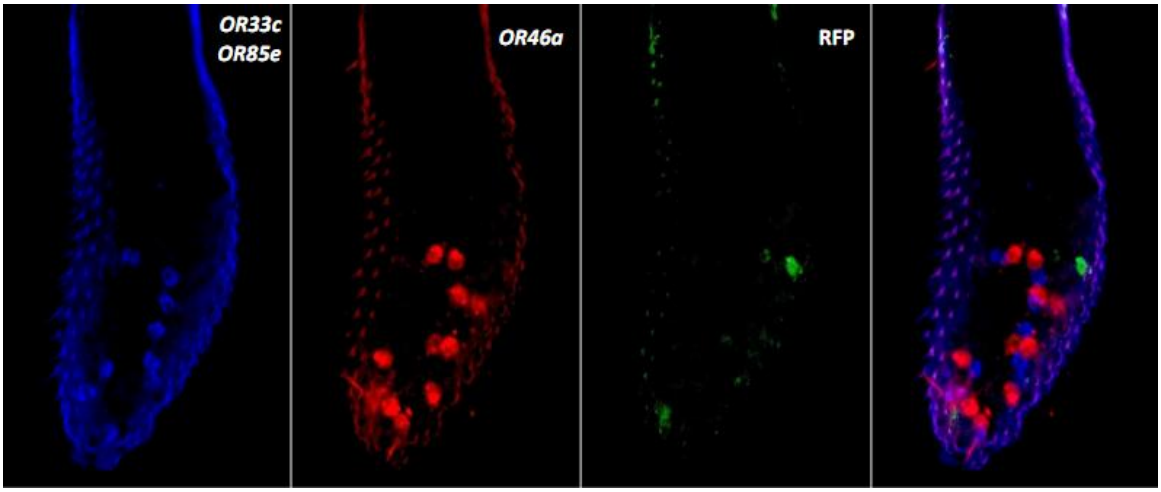

Figure 4.16. *IroC* positive cell clones do not express *OR33c*, *OR85e* or *OR46a*. *IroC* positive induced clone labeled with RFP (green) does not colocalize either with the *OR33c* and *OR85e* coexpressing pb2A cells (blue) or *OR46a* expressing pb2B cells (red).

More interestingly, an *IroC*-misexpressing pb2A cell clone, which does not express either *OR33c* or *OR85e* was observed (Figure 4.17). Additionally, OR expression in the neighboring pb2B cell, *OR46a*, persisted. The lack of *OR33c* and *OR85e* expression in a pb2A cell which misexpresses *IroC* suggests that *IroC* represses the expression of *OR33c* and *OR85e*.

In a parallel experiment the temperature-sensitive *Gal80* (*Gal80*^{ts}) gene has been employed. Misexpression of *IroC* genes in all neurons using the pan-neural Elav-Gal4 driver results in lethality at larval stages. To overcome this lethality problem ubiquitously expressed *Gal80*^{ts} was used. At the permissive temperature (18°C), the GAL80 protein is active and represses the misexpression of *IroC* genes by inhibiting the GAL4 protein expressed under the control of the Elav promoter. At the restrictive temperature (29°C), GAL80 is inactivated, allowing the Gal4 protein to turn on the misexpression of *IroC* genes in all neurons (Figure 4.18).

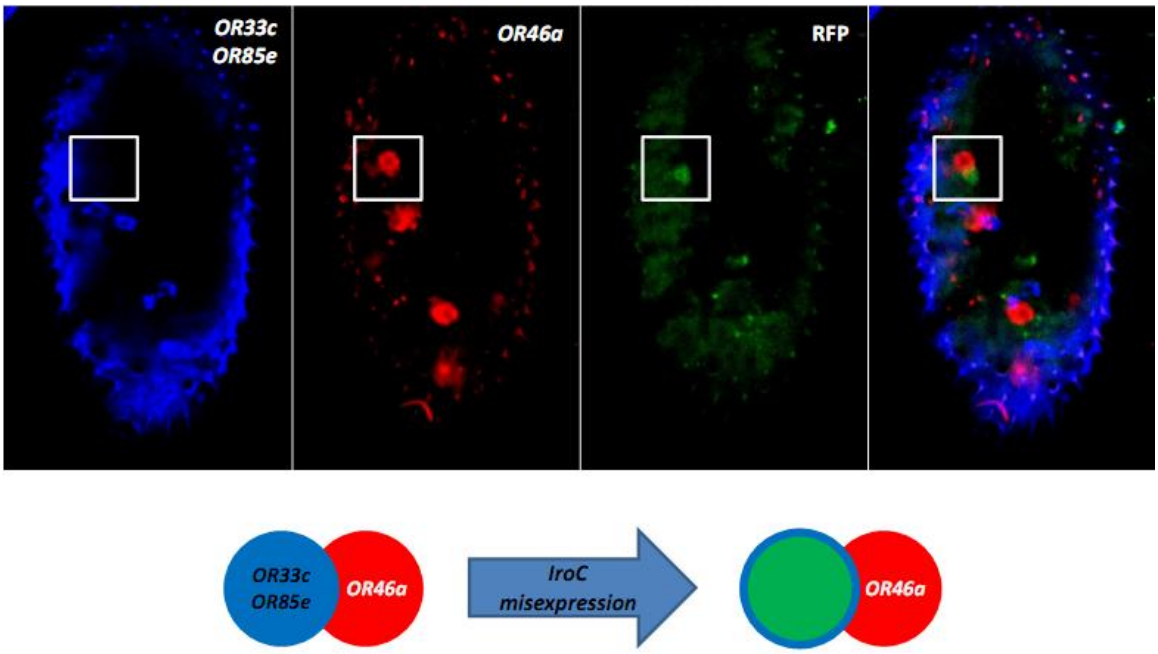

Figure 4.17. *IroC*-misexpressing pb2A cell clone does not express *OR33c* or *OR85e*. *IroC* positive induced pb2A cell clone labeled with RFP (green) does not express either *OR33c* or *OR85e (blue)*. *OR46a* expression in the neighboring pb2B cell persists (red).

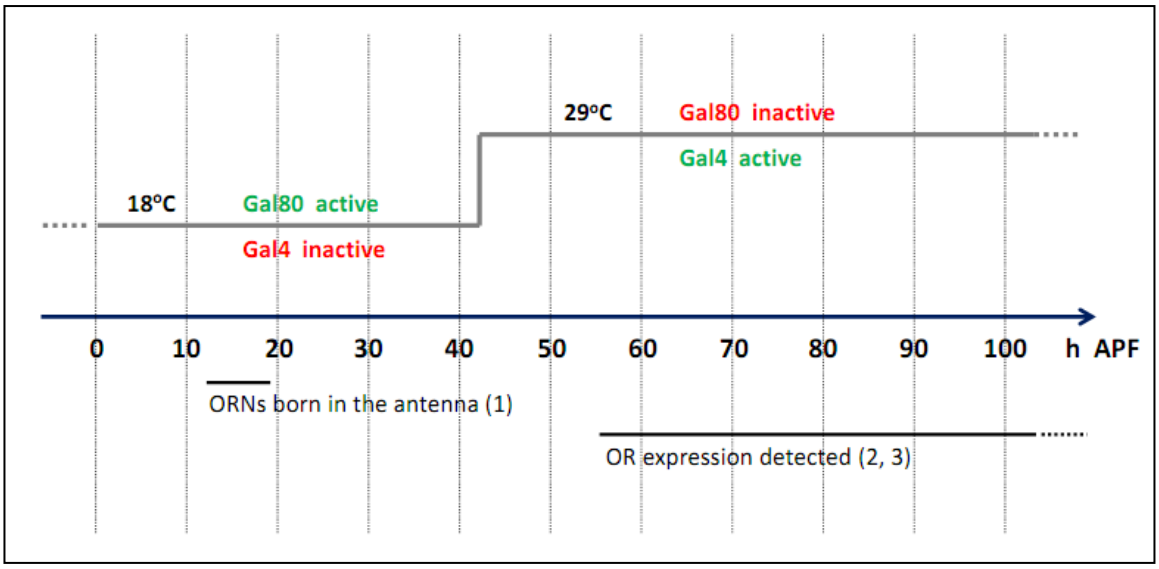

Figure 4.18. Schematic representation of the *Gal80*^{ts} system. Pupae were synchronized and aged for 42 ± 6 h at 18°C, transferred to 29°C to misexpress the *IroC* genes with pan-neural driver Elav-Gal4 and stained at adult stage. (1) Ray and Rodrigues, 1995, (2) Dubin and Harris, 1997, and (3) Clyne *et al.*, 1999.

In contrast to the hypothesis that *IroC* induces the expression of ORs in the pb2 neurons early misexpression of *IroC* genes using this method resulted in the repression of *OR85e* (Figure 4.19). Control flies of the same background that were not shifted to the restrictive temperature showed normal expression of *OR85e*, about 17 pb2A cells per maxillary palp (Figure 4.19A); however, flies transferred from 18°C to 29°C during development showed almost no expression of *OR85e* (Figure 4.19B). Cells were quantified and show a significant decrease ($p < 0.001$) in *OR85e* cells (Figure 4.20). These results indicate that *IroC* might be doing the opposite and rather repressing - at least - *OR85e*.

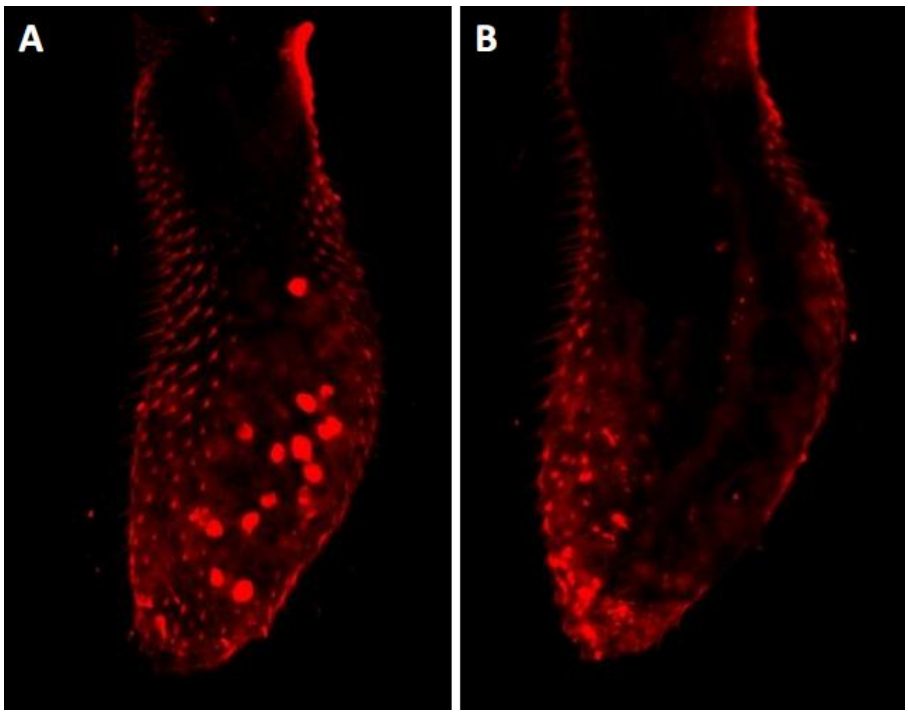

Figure 4.19. Misexpression of *IroC* genes results in the absence of *OR85e* gene expression. (A) *In situ* hybridization showing control flies left at 18°C with normal pattern of *OR85e* expression. (B) Flies transferred from 18°C to 29°C during development (42 ± 6 h APF) induce *IroC* expression and show no *OR85e* expression.

In order to investigate the time frame when *IroC* is required, *IroC* genes were expressed alone or in combination before the onset of OR gene expression. For this purpose the SG18.1-Gal4 driver was used. This reporter is expressed in most of the ORNs in the antenna (not shown) and in all of the ORNs found in the maxillary palp (see Figure 4.7C) (Shyamala and Chopra, 1999; Jhaveri and Rodrigues, 2002). While the exact onset of this Gal4 line is not known exactly, it is known to be expressed in the presumptive areas

from which olfactory organs will develop and been widely used in ORN axon targeting experiments (Yao *et al.*, 2007), suggesting that the Gal4 line is expressed prior to OR expression.

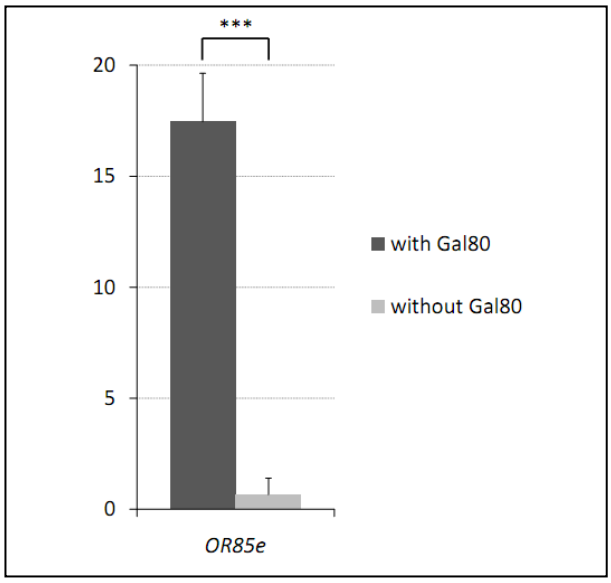

Figure 4.20. Reduction in the number of *OR85e* expressing cells upon early *IroC* expression. Elav-Gal4 drives the expression of *IroC* genes: *UAS-ara*, *UAS-caup* and *UAS-mirr*. n= 20, p*** < 0.001, error bars indicate SEM.

Contrary to the suggested hypothesis misexpression of *IroC* using the SG18.1-Gal4 driver line did not result in an increase of cells that express either *OR33c* or *OR85e* or both. Misexpression of *IroC* rather resulted in a dramatic decrease of this subset of cells (Figure 4.21). This repression was observed in all experiments in which either *ara* alone, *caup* alone, *ara* and *caup* together or *ara*, *caup* and *mirr* together were misexpressed. Thus, these results suggest that *IroC* either plays a role in the specification of pb2A cells or represses the expression of *OR33c* and *OR85e*. In order to investigate further which of these possibilities is true additional experiments were undertaken.

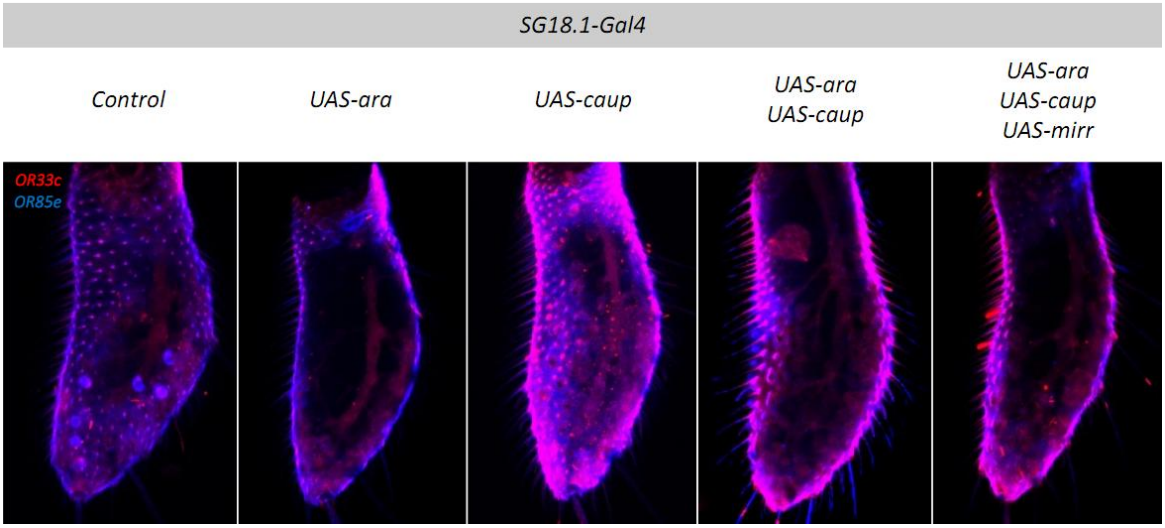

Figure 4.21. Misexpression of *IroC* genes using SG18.1-Gal4 represses the expression of *OR33c* (red) and *OR85e* (blue). Double *in situ* hybridization shows that misexpression of *IroC* genes either alone or together using the SG18.1-Gal4 driver results in the repression of both OR genes expressed in the pb2A cells.

Earlier studies show that Notch signaling plays a role in specifying ORN identity and eventually in OR expression (Endo *et al.*, 2007; Ray *et al.*, 2007). Activation of Notch in both of the cells found in a sensillum (Notch-ON) results in the expression of one OR in each of these cells. On the other hand, inhibition of Notch in both of these cells (Notch-OFF) results in the expression of other OR genes in each of these cells. It has been reported as well that *IroC* genes repress the expression of *fringe (fng)*, which is a modulator of Notch signaling (Irvine, 1999; Bilioni *et al.*, 2005). It was hypothesized that the decrease in the number of cells that express *OR33c* and/or *OR85e* could be a result of a cell fate change. Thus, the fate of the OR gene that is expressed in the neighboring pb2B cells, *OR46a*, was investigated. Contrary to the repression of *OR33c* and *OR85e* misexpression of *IroC* genes does not seem to affect the expression of *OR46a* in most of the maxillary palps analyzed (Figure 4.22). Only in the case of misexpression of *ara*, *caup* and *mirr* together some repression was observed. Cell numbers were counted and showed that the decrease in cell number is significant in most cases ($p < 0.001$) (Figure 4.23). These results suggest that *IroC* genes, *ara* and *caup*, on their own, have no role in the expression of *OR46a*, whereas *mirr* might have a role in the repression of *OR46a*. Unfortunately, it was not possible to test the overexpression of *mirror* alone as this line died and was not available for analysis.

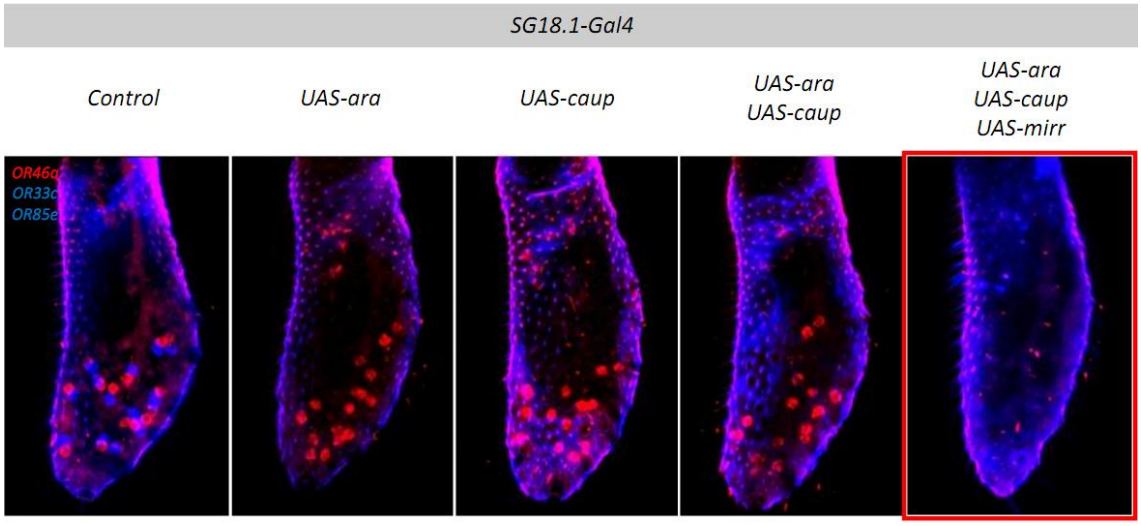

Figure 4.22. *IroC* genes play different roles in the expression of *OR46a*. Double *in situ* hybridization with three probes using two detection methods (*OR33c* and *OR85e*, blue; *OR46a*, red) shows that misexpression of *ara* and *caup* alone or together do not affect the expression of *OR46a*, whereas additional misexpression of *mirr* results in the repression of *OR46a* (n= 20).

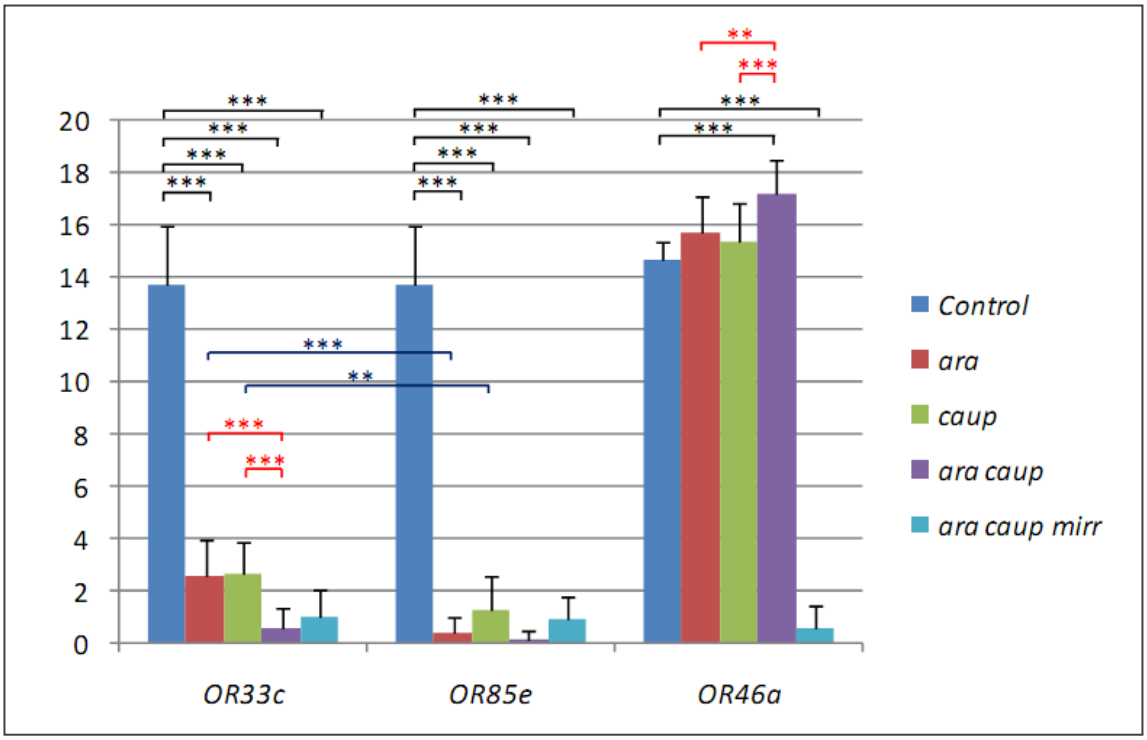

Figure 4.23. Quantification of cells after misexpression of *IroC* genes in the maxillary palp. SG18.1-Gal4 drives the expression of *IroC* genes: *UAS-ara*, *UAS-caup* and *UAS-mirr*. n= 20, p** < 0.005, p*** < 0.001, error bars indicate SEM.

To further investigate the time dependence of this repression late misexpression experiments with *ara* and *caup* genes by using the OR83b-Gal4 driver was performed. This experiment resulted in a decrease of cells that express *OR33c* and *OR85e*, but the number of cells that express *OR46a* did not change significantly (Figure 4.24). Although the decrease was still significant late misexpression of all *IroC* genes together rescued the phenotype observed by the mixexpression of *ara* and *caup* to some degree.

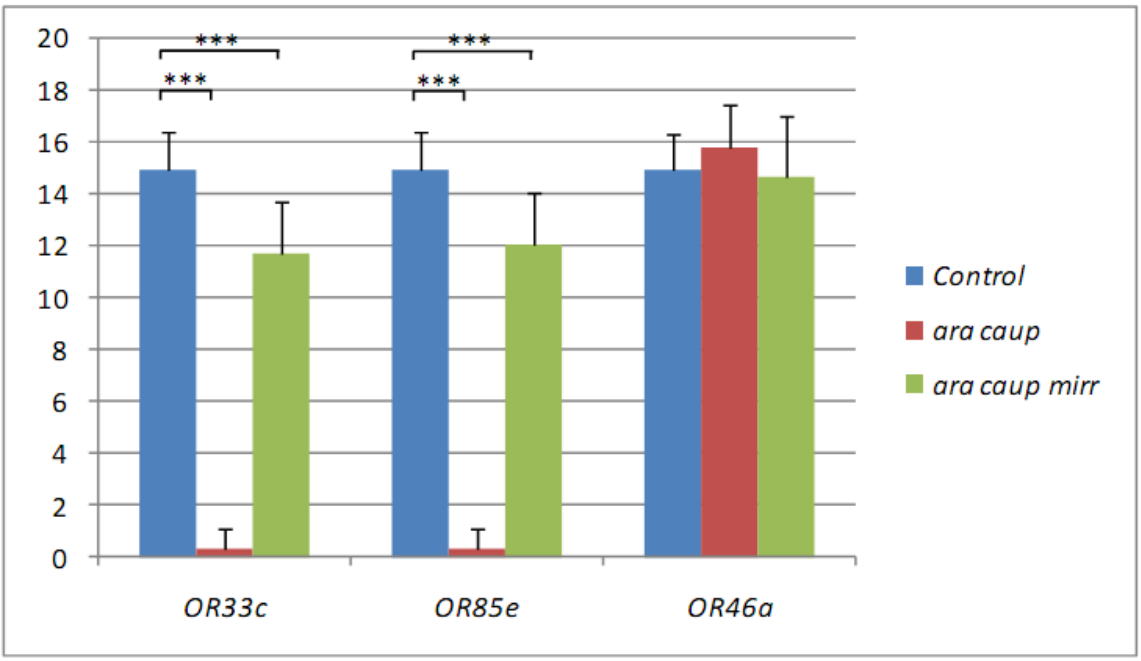

Figure 4.24. Summary of cells after misexpression of *IroC* genes using the OR83b-Gal4 driver. OR83b-Gal4 drives the expression of *iroC* genes: *UAS-ara*, *UAS-caup* and *UAS-mirr*. n= 25, p*** < 0,001, error bars indicate SEM.

4.6. *IroC* May Have a Role in ORN Axonal Targeting

In has been shown that in several cases transcription factors that are involved in the regulation of sensory receptors are also important for the targeting of sensory receptor neurons (eg. acj6) (Komiyama *et al.*, 2004). In a study analyzing retinal projections Sato *et al.* (2006) showed that interaction between Dwnt4 and its receptor Dfrizzled2 contributes to retinal axon guidance, and suggests that dorsally expressed *IroC* attenuates the competence of Dfrizzled2 to respond to Dwnt4.

Since *IroC* is expressed in ORNs, the possibility that it might contribute to ORN targeting in the antennal lobe was investigated as well. Using the Elav-Gal4 driver *caup* and *ara* genes were downregulated by RNAi and targeting of several OR-expressing neurons were analyzed by visualizing the neurons with GFP fusion proteins. In particular the projections of three ORN classes from the maxillary palp (pb2A, pb2B and pb1A) and two ORN classes from the antenna (at1 and ab5) were investigated. Three criteria were used to classify targeting defects: (i) position of the glomerulus of interest, (ii) ectopic glomerulus formation and (iii) presence or absence of the commissure. This analysis showed that downregulation of *caup* by RNAi with Elav-Gal4 has no effect on axon guidance of the examined ORN classes (Figure 4.25). However, downregulation of *ara* gene by RNAi resulted in defects in axonal targeting of some of the analyzed ORN classes in addition to a general defect of antennal lobe organization. These results suggest that *IroC* may have a role in the correct targeting of some ORN classes. If this is done in a cell-autonomous or non-cell autonomous fashion (as *IroC* genes are also expressed in the projection neurons) needs to be investigated.

In order to look at targeting of OR expressing neurons after downregulation of *IroC* genes by RNAi using the IroC-Gal4 driver was used. In agreement with the phenotypes observed using the Elav-Gal4 driver, downregulation of *IroC* genes using the IroC-Gal4 driver, defects of ORN axon targeting were observed (Figure 4.26). The observed phenotypes were not as severe as those observed when using the Elav-Gal4 driver. In contrast to the previous experiment with Elav-Gal4, in this experiment defects upon downregulation of the *caup* gene as well were observed. Thus, these results confirm earlier findings and suggest that *IroC* genes may have a role in ORN targeting.

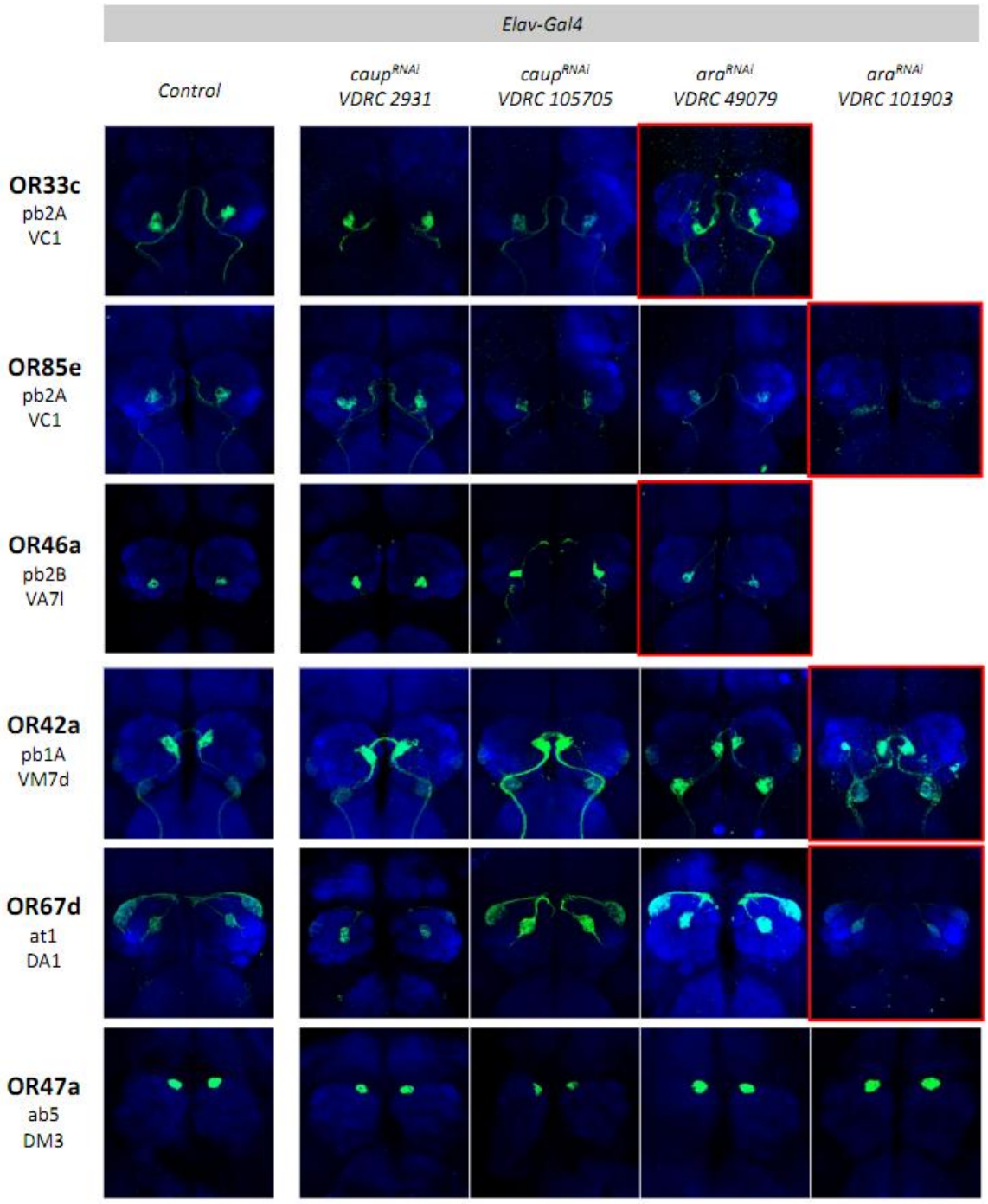

Figure 4.25. Downregulation of *IroC* genes using the Elav-Gal4 results in defects of ORN axon targeting. UAS-RNAi lines are crossed to the Elav-Gal4 driver. Red margins mark the instances where defects in ORN axon targeting were oserved.

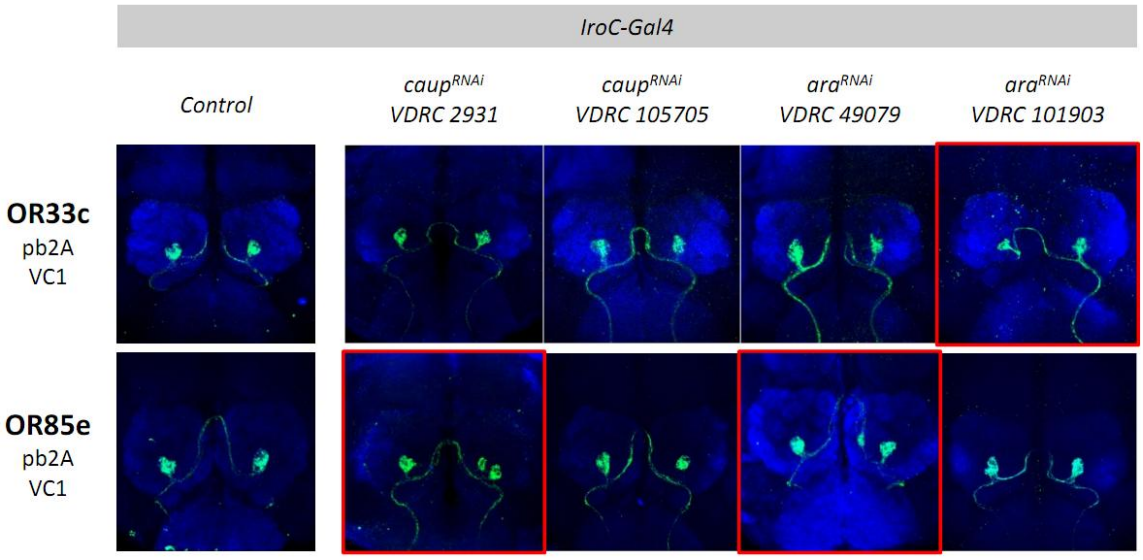

Figure 4.26. Downregulation of *IroC* genes using the IroC-Gal4 results in defects of ORN axon targeting. UAS-RNAi lines are crossed to the iroC-Gal4 driver. Red margins mark the instances where defects in ORN axon targeting were observed.

4.7. *IroC* Expression in the Olfactory Organs during Development

GOF and LOF phenotypes (repression of OR expression and defects in ORN axon targeting) suggest that the expression onset of *IroC* genes should be early. However, initial analyses showed that in the adult *IroC* is expressed in a subset of maxillary palp ORNs but not in the antenna. To look at the onset of *IroC* gene expression during development of the olfactory organs, the iroC-Gal4 reporter line was analyzed during pupal stages. It turned out to be very difficult to localize the maxillary palp and antenna during early stages of pupal development. Pupae were dissected starting at 24 h APF (After Puparium Formation); however, maxillary palps could only be identified as early as 48 h APF (Figure 4.27). Analyses showed that *IroC* is expressed in the maxillary palp between 48-96 h APF. Interestingly, expression of *IroC* was also observed in a subset of neurons in the antenna starting at 24 h APF. *IroC* expression in the brain and antennal lobe was observed starting at 24 h.

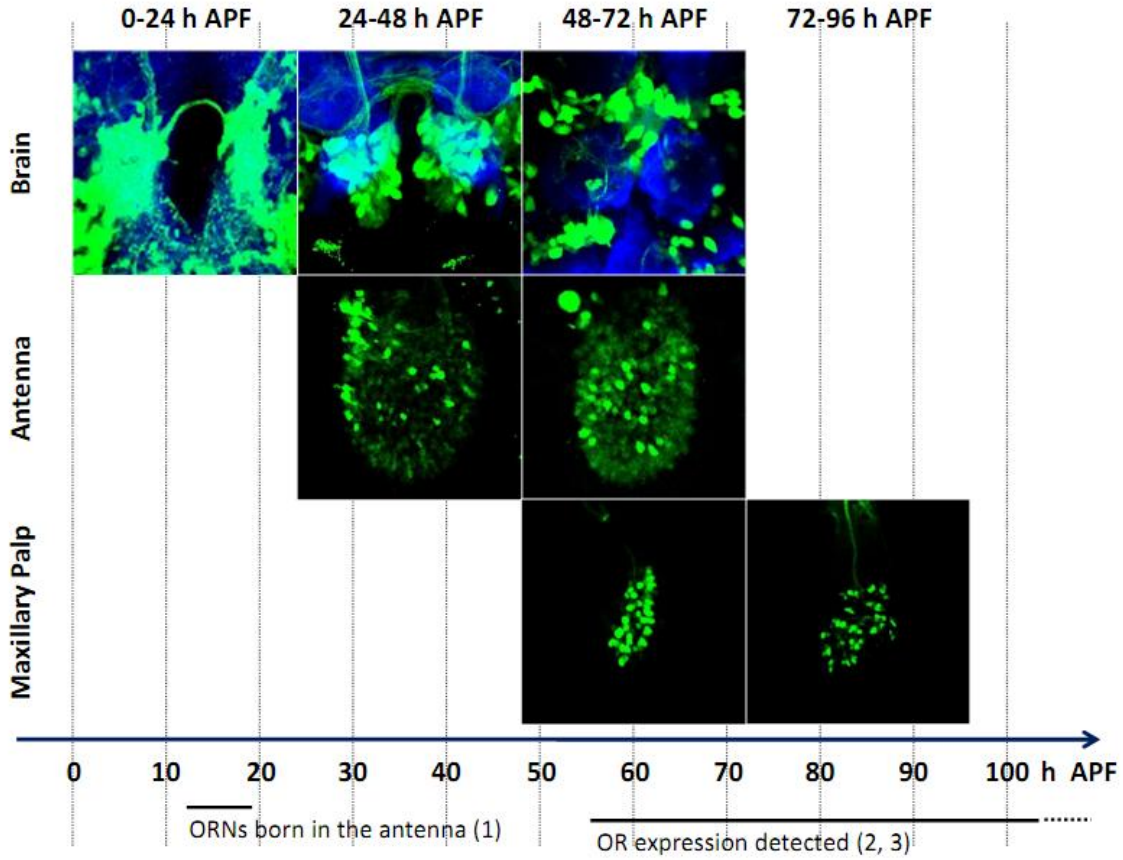

Figure 4.27. Analysis of *IroC* expression during pupal stages. Pupae were synchronized and the IroC-Gal4 reporter was analyzed in the maxillary palps, in the antenna and in the antennal lobe through the expression of GFP.

The early expression of *IroC* in the antenna was surprising, as it does not persist in the adult. This earlier expression of *IroC* in the antenna suggests that it may have an earlier role during the development of the antenna. The second surprising observation is the changing number of cells that express *IroC* in the maxillary palp during pupal development. The number of *IroC*-positive cells is initially very high and gradually decreases as the fly develops (Figure 4.27). The expression of *IroC* in the maxillary palp and antenna of newly eclosed (0-24 h) and 20 days old flies was analyzed. A decrease in cell number was also observed in the adult as the flies age when compared to cell numbers just after eclosion (Figure 4.28A). In contrast to earlier findings in this study a few GFP-positive cells were observed in the antenna in newly eclosed and aged flies (Figure 4.28B). A quantification of cells expressing *IroC* in the maxillary palp is summarized in Figure 4.29.

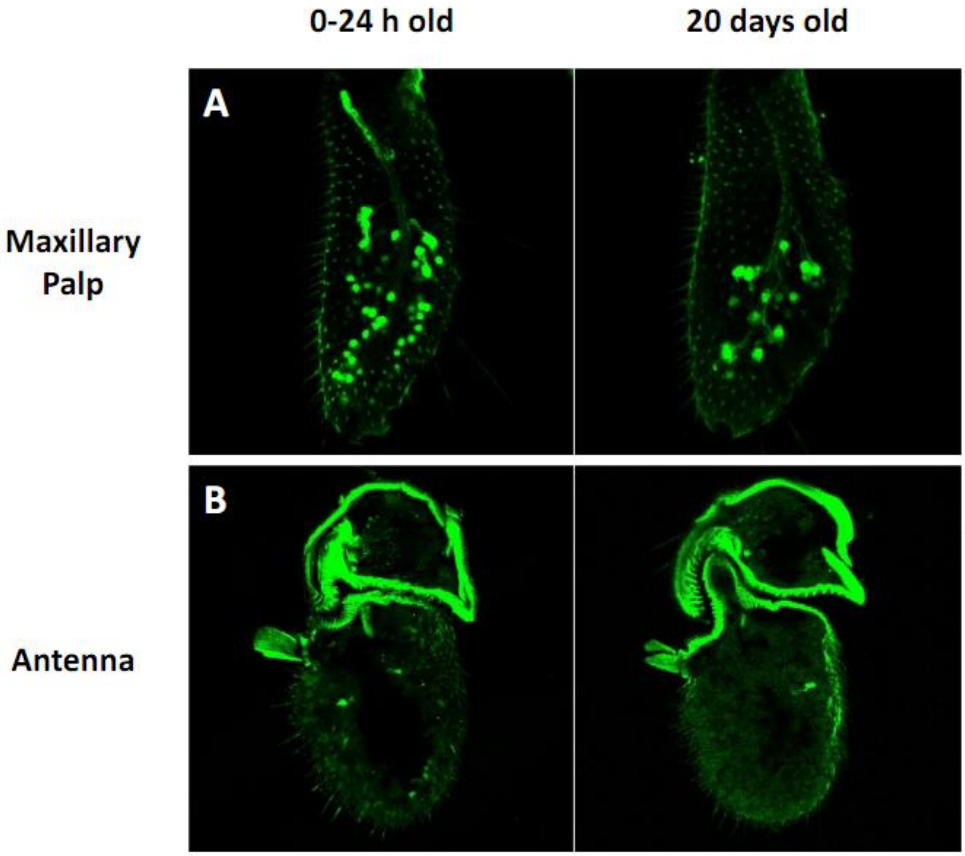

Figure 4.28. Analysis of *IroC* expression in olfactory organs in newly eclosed and aged adult flies. *IroC* is expressed both in the maxillary palp (A) and in the antenna (B) in young and old flies.

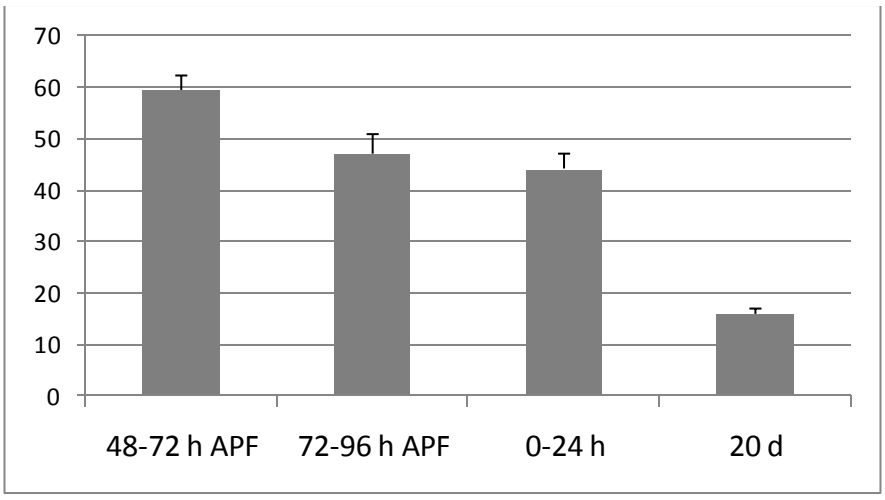

Figure 4.29. Summary of cell numbers of IroC-positive cells at different time points. IroC-Gal4 drives the expression of UAS-GFP. n=8, error bars indicate SEM.

In order to find out what type of cells expressed *IroC* early and lost its expression in the adult cell lineage analysis using the FLP-out technique was performed (Weigmann and Cohen, 1999) (Figure 4.30). In this method IroC-Gal4 drives the expression of UAS-FLP. The expressed FLP eliminates a *stop* cassette that is flanked by two FRT sites located between an *actin* promoter and a *β-galactosidase* reporter gene. The FLP-out of the *stop* cassette juxtaposes the *actin* promoter to *β-galactosidase* and eventually results in the expression of *β-galactosidase* labeling all cells that at some point of their life expressed *IroC*, even if the cell does not express IroC-Gal4 any more.

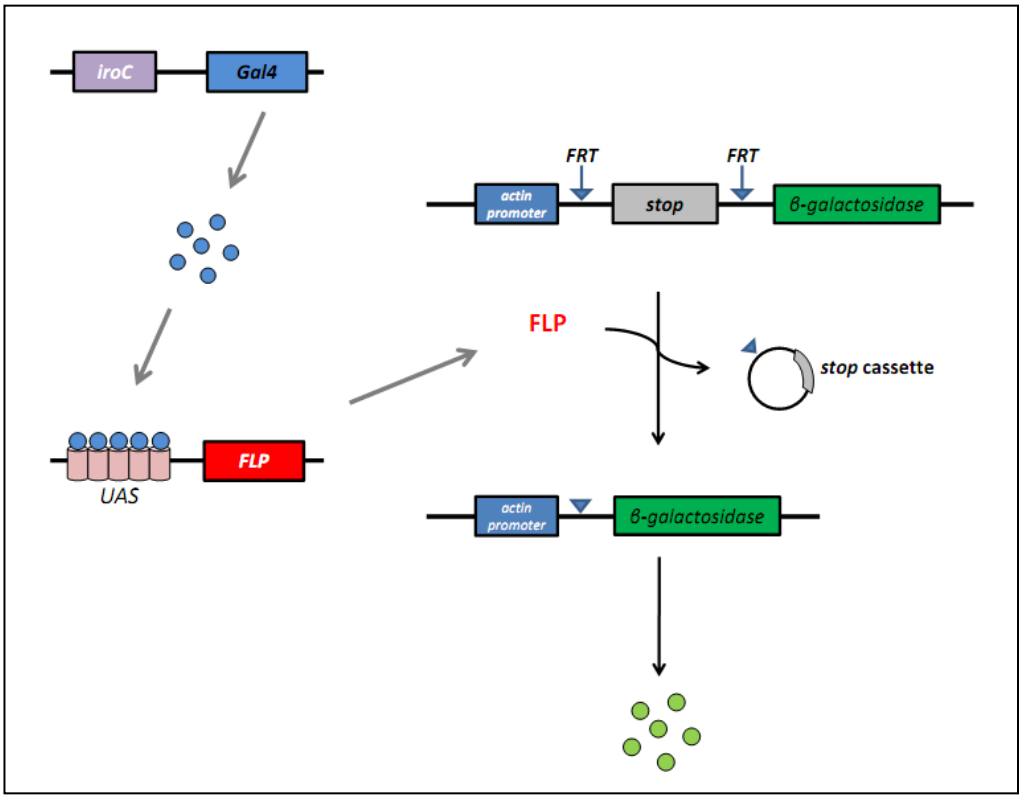

Figure 4.30. Cell lineage analysis using FLP-out technique. IroC-Gal4 drives the expression of UAS-FLP. FLP recombines two FRT sites flanking the *stop* cassette and removes it. Elimination of the *stop* cassette juxtaposes the *actin* promoter to *β-galactosidase* and hence drives the expression of the *β-galactosidase*, which is used to label the cells.

Figure 4.31 shows the high number of cells, both in the maxillary palp and the antenna, that once expressed *IroC* during their development. The observed high number of cells in the maxillary palp confirms previous findings obtained during this study and suggests that *IroC* may have an additional earlier role in the development of ORNs in the maxillary palp. The observed dramatic increase in the number of ORNs found in the antenna positive for *β-galactosidase* expression suggests that *IroC* may also have a role in the development of some ORNs found in the antenna.

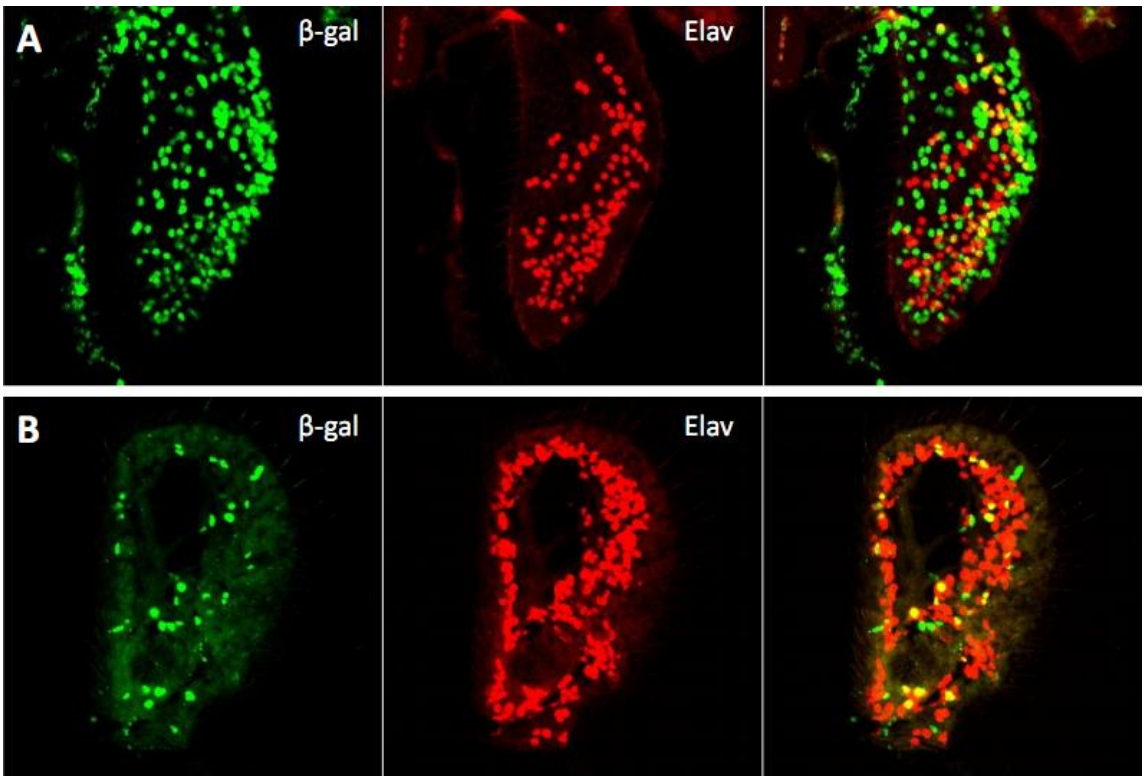

Figure 4.31. Cell lineage analysis of IroC expression in the maxillary palp (A) and in the antenna (B). β-galactosidase (green) labels all cells ever expressing *IroC*, even if the cell does not express it anymore; Elav (red) labels all neurons.

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5. DISCUSSION

Humans mostly rely on the visual system for probing the environment, while in animals the sense of smell is still more important. Olfaction is the sensory modality that shapes most of our behaviors and modulates processes such as learning and memory. In fact the chemosensory nature of olfaction, to detect and respond to chemical signals, which is present even in the simplest of the life forms, makes it probably the oldest modality and it is the primary skill for most of the animals. These simple facts constitute the basic need for research trying to understand the mechanisms which govern the perception of odors.

The olfactory system is one of the most complex sensory systems as the sense of smell relies on a large repertoire of olfactory receptors (1200 in mice, 400 in humans, 100 in fish) (Ache and Young, 2005). On the other hand only one of these receptors is expressed in each olfactory receptor neuron and thus represents a major gene regulatory problem on how to express only one and silence all the other receptors. Elucidating how receptor gene choice works in this complex system will broaden our understanding of gene regulatory mechanisms in general.

Olfactory receptor neurons target specific regions in the antennal lobe where they connect to target neurons in regions called olfactory glomeruli. While there is some understanding how this happens in the fly, the mechanism is not fully understood. In many cases transcription factors that regulate OR gene expression are also involved in axonal targeting of ORNs.

In the framework of this thesis the role of a transcription factor family, namely *IroC*, in the olfactory system of *Drosophila*, which are expressed in a subset of cells that coexpress two olfactory receptors was investigated. Finding out how the exclusive expression of receptors is alleviated will give some insight on how receptor gene choice is regulated normally. In addition the possible involvement of *IroC* in the targeting of ORNs was investigated.

5.1. Problems with the Experimental Set-Up

The mechanisms which govern OR gene regulation and specific targeting of ORN axons are poorly understood. Many transcription factors, cell adhesion molecules, sequence motifs have been identified; however, the picture is still not complete. Here, the role of a transcription factor family, *IroC*, was investigated in receptor gene choice and axonal targeting of ORNs in the olfactory system of the fly.

IroC expression was initially observed in the maxillary palp and in particular in the ORN subtype where coexpression of two OR genes, *OR33c* and *OR85e*, occurs. A recent study concerning the regulation of *rhodopsin* genes in the retina identified a novel class of ommatidia in the dorsal edge of the eye, which coexpress *rhodopsin Rh3* and *Rh4* (Mazzoni et al., 2008). This ommatidial subclass was shown to express *IroC* and the authors showed that *IroC* expression is both necessary and sufficient for the coexpression of *Rh3* and *Rh4* rhodopsins. This finding and the observation of *IroC* expression in pb2 cells along with the presence of *IroC* binding sites in the upstream regions of the OR genes in the maxillary palp constitute the main aim of this thesis which is to investigate the role of *IroC* genes in the olfactory system of the fly.

To test the role of *IroC* classical loss-of-function and gain-of-function experiments were performed. For this purpose, several Gal4 driver lines that show expression in the olfactory organs, especially in the maxillary palps were characterized (Table 4.1). As an alternative way to detect OR gene expression promoter fusion constructs that drive GFP expression of various OR genes were generated. While it was previously reported that short upstream regions are enough to drive faithful expression of OR genes (Ray *et al.*, 2007) the lines generated in the framework of this study did not recapitulate the endogenous expression of OR genes.

The reasons for the weak or lack of expression might be various. Fishilevich *et al.* (2005) and Couto *et al.* (2005) used various lengths of upstream regions (up to 8000 bp) to create either direct GFP fusion lines or Gal4 lines. Ray *et al.* (2007) made a series of deletions in the promoter regions of some OR genes expressed in the maxillary palp, *OR85e* and *OR46a*, and using these regions they created Gal4 lines. Examination of these

Gal4 lines showed that short upstream regions such as 500 bp are sufficient to drive the faithful expression in the maxillary palp. Although in this study 1000 bp upstream region was used instead of 500 bp as suggested by Ray *et al.* (2005) to generate the transgenic lines, only weak expression in the case of PrOR85e-TLNΔCherry was observed. While the cloned upstream region seems to contain most of the required regions for the expression of the reporter, the expression of the reporter seems not strong enough. In the case of the previously published studies Gal4 lines were generated, which are crossed to UAS-reporter lines leading to the amplification of the signal to several orders of magnitude. The reason for the observed instances where Cherry does not colocalize with the GFP expression under the control of *OR33c* gene promoter indicates the lack of sequences necessary for the restriction of OR gene expression to its ORN class. In the case of PrOR33c-mCherry, the cloned 1000 bp upstream region seems not to contain the necessary regulatory regions. Ray *et al.* did not examine the upstream region of *OR33c* to find the minimal promoter. Our observations suggest that 500 bp of OR upstream region might not be sufficient to drive the faithful expression of this particular OR gene. These lines should be generated by using longer upstream regions to observe a stronger expression or by adding a general enhancer. P-element based transformations occur randomly (Rubin and Spradling, 1982; Ryder and Russell, 2003), but since these transgenic lines were created by targeted site-specific transformation using the phiC31 system (Venken *et al.*, 2006), the possibility of genomic variation on the observed phenotypes was observed.

5.2. Loss of *IroC* Genes Results in an Increase in the Number of pb2A Cells

In the framework of this study loss-of-function experiments were perfomed by using the RNA interference method. The pan-neural driver Elav-Gal4 was used to downregulate *IroC* genes by crossing it to UAS-RNAi lines and the maxillary palps of the progeny were analyzed by double *in situ* hybridization for *OR33c* and *OR85e*. Analyses showed that coexpression of the two OR genes persisted in this background suggesting that *IroC* has no role in their coexpression. Additionally, to enhance the level of RNA interference, a UAS-Dicer2 has been included, as this has been suggested to enhance the activity of RNA interference in neurons (B. Dickson, personal communication). However, even under these conditions, no change in the coexpression of *OR33c* and *OR85e* has been observed.

RNAi interference does not lead to 100% downregulation of *IroC* thus, a deficiency for *IroC* was analyzed as well. The *iroC*^{DFM3} allele has been reported as a null mutant allele, a deficiency of *ara*, *caup* and the promoter region of *mirr* (Gomez-Skarmeta *et al.*, 1996; Diez del Corral *et al.*, 1999; Mazzoni *et al.*, 2008). Since *iroC*^{DFM3} is homozygous lethal, *IroC* deficient cell clones were generated by performing MARCM. Double *in situ* hybridization for *OR33c* and *OR85e* followed by antibody staining to label the *IroC* deficient cell clones was carried out. Initially a total of more than 300 *IroC* deficient cell clones were generated in different experiments but none of them was positive for either *OR33c* or *OR85e*. This high number of mutually exclusive staining suggested that maybe the pb2A cell does not exist at all in an *IroC* deficient background. However, after repeating the experiment several times a few *IroC* deficient cell clones, which do express both of the coexpressed OR genes, were observed.

During RNA interference experiments a change in the number of cells coexpressing *OR33c* and *OR85e* was observed. The cells were counted and found that downregulation of the *ara* gene results in a significant increase in the number of cells that express *OR33c* and *OR85e*. These results were further confirmed by additional experiments in which IroC-Gal4 was used to downregulate *IroC* genes specifically in *IroC* positive cells and previously generated GFP reporter lines that show expression under the promoters of *OR33c* or *OR85e* genes were used. GFP-positive cells in different RNAi crosses were counted and the number of GFP-positive cells were found to increase upon downregulation of *ara*. These findings suggest that *IroC* genes, at least *ara*, repress the expression of *OR33c* and *OR85e* in a cell-autonomous manner.

If the increase in the number of cells is assumed to be a direct result of de-repression, than late downregulation of the *ara* and *caup* genes by using the OR83b-Gal4, should give a similar phenotype. One might perform these experiments and see whether late downregulation will de-repress the expression of *OR33c* and *OR85e*. Additionally, one might also question the expression level of the OR gene that is expressed in the neighboring ORNs, *OR46a*. Moreover generating two-cell clones and performing *in situ* hybridization for *OR33c* or *OR85e* and *OR46a* on those cells might give an idea about the reasons for the observed increase in the number of pb2A cells.

5.3. *IroC* Genes Repress OR Gene Expression

To further investigate the function of *IroC*, gain-of-function experiments were performed by misexpressing *IroC* genes in the maxillary palp. Since *IroC* genes play many roles during development early misexpression of *IroC* genes using pan-neural postmitotic drivers resulted in lethality. To overcome this lethality problem several approaches were used. In the first approach clones that express *IroC* genes ubiquitously by using the FLP-out technique were generated. While many clones were generated, none of these clones was either *OR33c* or *OR85e* positive. In the second approach the pan-neural driver Elav-Gal4 was used to misexpress all of the *IroC* genes at a later time point. To achieve this, the misexpression of *IroC* was inhibited until 42 ± 6 h APF using temperature sensitive *Gal80* expression to overcome the lethality problem. Misexpressing of *IroC* genes at a late time point but prior to OR gene expression onset resulted in an almost complete repression of *OR85e*. These finding suggested that *IroC* may have a role in the repression of *OR85e*. In a third experiment two Gal4 lines were used to misexpress *IroC* genes, SG18.1-Gal4 and OR83b-Gal4, the expression of which are more restricted and specific to ORNs. Early misexpression of *IroC* genes, either alone or in combinations with each other, using the SG18.1-Gal4 resulted in a decrease in the number of cells that express *OR33c* and *OR85e*. This suggests that Iro proteins may act as a repressor of OR gene expression.

These results are in accordance with published data related to the activity of Iro proteins. Earlier studies showed that Iro proteins can recognize and bind to the same sequence found on the DNA, but with different affinities (Bilioni *et al.*, 2005). Additionally, Ara and Caup are redundant to each other and have overlapping activities. Results obtained in this study suggest that both Ara and Caup proteins have repressor activity, however with different efficiency on the *OR33c* and *OR85e* genes: e.g. repression of *OR85e* is more severe. Upstream regions of *OR33c* and *OR85e* were analysed and a varying number of *IroC* binding sites were found. The more severe repression observed with the *OR85e* gene may be explained by the additional *IroC* binding sites found upstream of the transcription start site. Misexpression of *ara* and *caup* together resulted in a more severe repression for both of the genes suggesting a combinatorial action of these proteins. It is known that Iro proteins bind to the identified *IroC* binding site as homo- and hetero-dimers, so combinatorial expression of these proteins may be the reason for the

observed additional repression. Even if these results are not statistically significant yet, they also suggest that additional misexpression of *mirr* along with *ara* and *caup* de-represses the observed phenotype slightly.

Iro proteins are known to be modulators of the Notch signaling pathway (Irvine, 1999; Bilioni *et al.*, 2005). Earlier studies show that Notch signaling plays a role in ORN identity and eventually in OR expression (Endo *et al.*, 2007; Ray *et al.*, 2007). Activation of Notch in both of the cells found in a sensillum (Notch-ON) results in the expression of one OR in both of the cells, whereas inhibition of Notch in both of the cells (Notch-OFF) results in the expression of other OR genes in both of the cells. The observed decrease in the number of cells that express *OR33c* and *OR85e* could be a result of a similar mechanism. To investigate this possibility the expression of the OR gene in the neighboring cells, *OR46a* was analysed. Examination of the *OR46a* gene in the same experimental conditions showed that Ara and Caup proteins do not repress the expression of *OR46a*; however, even if it is not statistically significant misexpression of Ara and Caup alone did result in an increase in the number of cells. Additionally, misexpression of both Ara and Caup proteins together resulted in a statistically significant increase in the number of *OR46a* expressing cells. This increase may be the result of specification of more progenitor cells as pb2B cells, as well as, the result of an abnormal segregation of a protein, which in turn leads to the misexpresion of *OR46a* in the neighboring pb2A cells. Since the ORs expressed in the pb2A cells are repressed, not a single case where *OR46a* is expressed along with *OR33c* and *OR85e* in pb2A cells could be observed, but nevertheless this result does not exclude this possibility.

More interestingly misexpression of Mirr along with Ara and Caup resulted in the repression of *OR46a*. While misexpression of Mirr alone could not be performed these results suggest that Mirr represses the expression of *OR46a*. It has been reported that Ara and Caup have more overlapping expression and similar/redundant activities, but the expression pattern of Mirr is more diverse and it is not redundant to the other two Iro proteins, therefore the observed decrease in the number of *OR46a* positive cells may be due to the distinct activity of the Mirr protein. Additionally, the presence of *IroC* binding sites in the upstream region of *OR46a* supports this suggestion.

Late misexpression of *ara* and *caup* together using the OR83b-Gal4 line resulted in the repression of *OR33*c and *OR85e*, but did not change the expression of *OR46a*. These findings are in accordance with the misexpression results using the SG18.1-Gal4 line. Since OR83b-Gal4 is a late driver, it could have no effect on the specification of ORNs or asymmetric division of progenitor cells. Therefore, the observed decrease in the number of cells positive for either *OR33c* and/or *OR85e* genes suggests that this decrease is a direct result of OR gene repression.

Late expression of *mirr* along with *ara* and *caup* together using the OR83b-Gal4 also did not result in a change of *OR46a* expression, but it resulted in a decrease in the number of cells expressing *OR33c* and *OR85e*; however, it was not as severe as the decrease in the misexpression of Ara and Caup proteins together. These results suggest that either Mirr rescued the phenotype to some degree or Mirr inhibited the activity of Ara and Caup by forming hetero-dimers with them. Mirr may also have a higher binding affinity and its binding to the *IroC* binding sites may mask the sites from the repression effect of Ara and Caup.

Early misexpression of *mirr* along with *ara* and *caup* genes resulted in a decrease of *OR46a* expressing cells; however, late misexpression did not change the number of cells positive for *OR46a* expression significantly. If it is assumed that the decrease in the number of cells positive for *OR46a* gene expression is simply an issue of repression, then the different results obtained by different Gal4 lines suggest that SG18.1-Gal4 is stronger than OR83b-Gal4. But if it is assumed that these Gal4 lines have similar strengths than these results suggest that the observed decrease is a direct outcome of not repression but an outcome of ORN class identity change.

The simplest interpretation of these findings is that Iro proteins act as transcriptional repressors. This finding is in agreement with previous findings, since in the literature it has been stated that Iro proteins mostly function as transcriptional repressors (for review Bilioni *et al.*, 2008). However, Matsumoto *et al.* (2004) showed that phosphorylation can convert the vertebrate Iro protein, Irx2, from a transcriptional repressor to an activator. Therefore, misexpression of *IroC* genes may result in the novel expression of other OR genes, which are normally expressed in the antenna or larval dorsal organ, but may also

result in an increase in the number of cells that express the other OR genes that are expressed in the maxillary palp but were not examined during this study. An earlier study trying to identify the possible role of Abnormal chemosensory jump 6 (Acj6) protein showed that a transcription factor can act both as a repressor and activator in the regulation of OR genes (Bai *et al.*, 2009). This finding supports the idea presented here and emphasizes the need to carry out electrophysiological recordings both to confirm our findings and test whether there is additional expression of other OR genes that could be identified from the response profiles of the pb2 neurons.

Finally, if it is assumed that Iro proteins act as OR gene repressors, one might also generate *IroC* deficient cell clones that are labeled with GFP as in the MARCM experimental setup, perform single unit cell recordings specifically on those GFP expressing cells and see whether the loss of *IroC* makes the expression of other OR genes possible by comparing the obtained odor response profile with earlier identified profiles.

5.4. *IroC* Contributes to the Proper Targeting of ORNs

In a relatively recent study Sato *et al.* (2006) show that interaction between Dwnt4 and its receptor Dfrizzled2 contributes to retinal axon guidance, and suggest that dorsally expressed *IroC* attenuates the competence of Dfrizzled2 to respond to Dwnt4. Based on this finding, *IroC* function was investigated in the context of targeting of ORNs in the antennal lobe. Using the Elav-Gal4 driver *caup* and *ara* genes were downregulated by the RNA interference method and targeting of several GFP-expressing ORNs were analyzed: three ORN classes from the maxillary palp (pb2A, pb2B and pb1A) and two ORN classes from the antenna (at1 and ab5). Antibody stainings indicate that downregulation of *IroC* genes, in particularly the *ara* gene, resulted in defects in the targeting of some ORNs. These results suggest that indeed *IroC* genes may have a role in ORN axon targeting. Since Elav-Gal4 is a pan-neural driver, it downregulates *IroC* genes not only in the ORNs but also in all neurons including projection neurons (PNs). Prior to the arrival of ORN axons to the antennal lobe, processes of PNs generate a proto-antennal lobe structure. Downregulation of *IroC* genes in PNs as well may be the primary reason for the observed phenotypes. However, downregulation of *ara* and *caup* genes by using the IroC-Gal4

reporter line also resulted in defects in the targeting of examined pb2A ORNs, although not as severe as the phenotypes observed using the Elav-Gal4 driver. These results suggest that *IroC* may have a role in the precise targeting of ORNs both from the antenna and the maxillary palps.

To test this observation conclusively MARCM should be performed. By inducing cell clones that show marker expression under the control of various OR gene promoters, one can examine the axon of one ORN that is deficient for *IroC* (Figure 5.1).

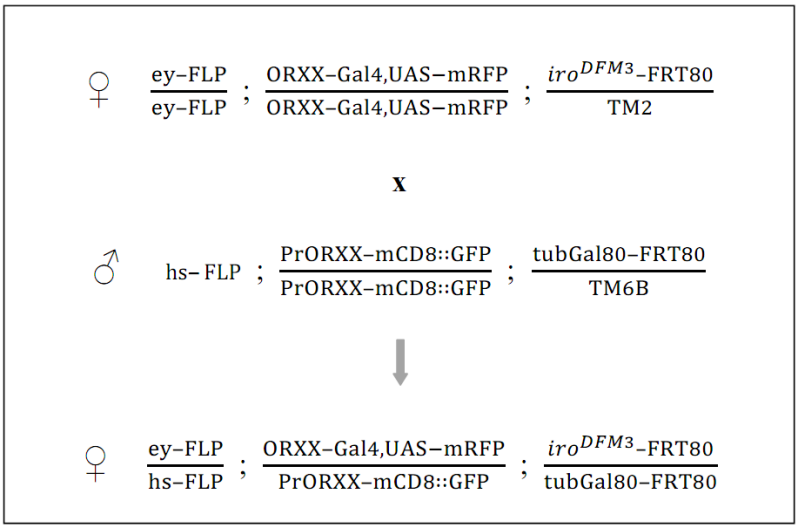

Figure 5.1. A proposed MACRM analysis for axonal targeting.

5.5. *IroC* May Have a More General Role in the Olfactory System

Analysis of *IroC* expression during pupal stages showed that the number of cells positive for *IroC* expression decreases during development. This decrease persisted at the adult stage. Additionally, expression of *IroC* was observed in the antenna. Furthermore, a careful analysis of the antenna for *IroC* expression in the adult using cryostat sectioning showed a few *IroC*-positive cells. This finding was surprising as no *IroC* expression was observed at the beginning of this study in the antenna. This might however be due to the thickness of the cuticle of the antenna that hinders the observation of GFP across the cuticle and the low number of *IroC* positive cells.

These observations initiated cell lineage analyses using the FLP-FRT system. The results indicated the presence of far more cells that express *IroC* in either organ than were initially observed. This finding suggests that *IroC* may have a more general role involving other ORN classes and their corresponding OR genes in both of the olfactory organs. While previously only the upstream regions of maxillary palp ORs were analysed for *IroC* binding sites this analysis should be extended to includethe whole OR repertoire.

In particular the upstream regions of 7 OR genes expressed in the maxillary palp were analysed and in 5 of them varying number of *IroC* binding sites were identified. Additionally, identification of this binding site in two of three sensillum subtypes further suggests that *IroC* expression may affect OR expression in a subset dependent manner and in a combinatorial fashion with other identified transcription factors that play role in OR gene regulation.

Earlier studies showed that another transcription factor, *pannier (pnr)*, and *IroC* are expressed in a complementary and mutually exclusive manner in thoracic epithelium (Simpson, 1996). One should also look at the expression pattern of *pnr* in the maxillary palp, and if it were, investigate its role in the olfactory system and analyze its interaction with *IroC*.

In summary, our findings complement each other and suggest the following: Iro proteins have a role in the specification of an ORN class as observed for pb2B cells probably by modulating Notch signaling, they repress the expression of OR genes, *OR33c* and *OR85e* and contribute to the proper targeting of some ORN classes (Figure 5.2).

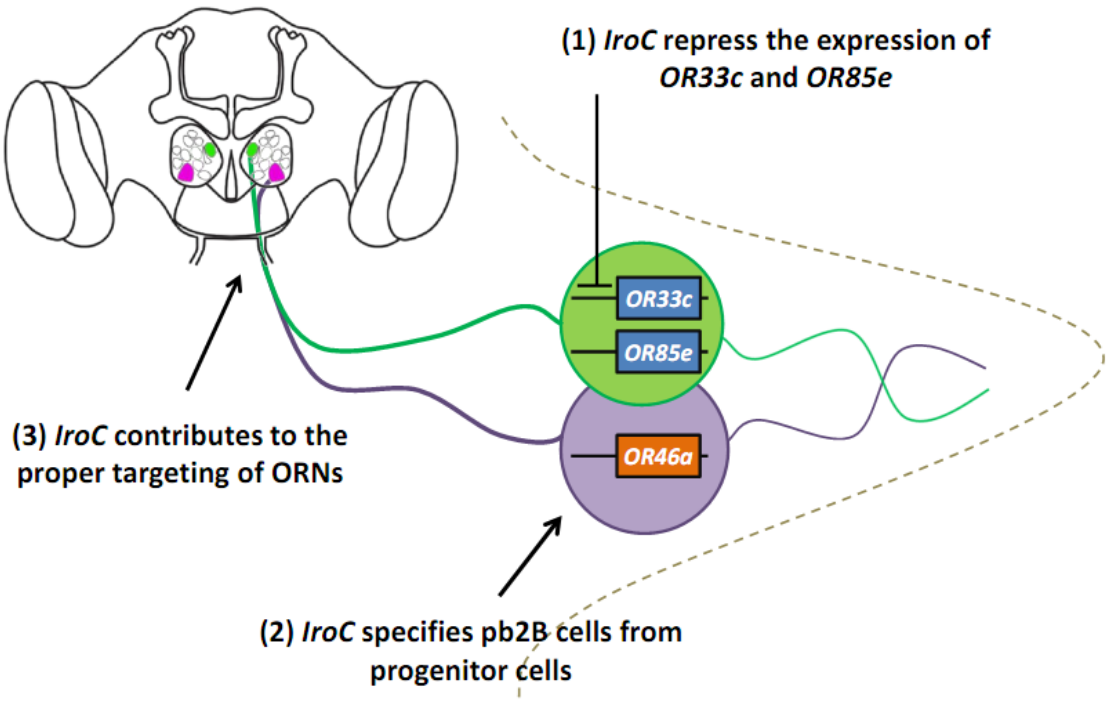

Figure 5.2. Characterized roles of *IroC* in the fly olfactory system. (1) *IroC* repress the expression of OR genes, *OR33c* and *OR85e*. (2) *IroC* specifies pb2B cells from progenitor cells. (3) *IroC* contributes to the proper targeting of some ORN classes.

How do the observations obtained in this study fit to the restricted expression pattern of *IroC* in pb2 neurons in the adult maxillary palp? If *IroC* is responsible for the repression of *OR33c* and *OR85e* genes how can it be only expressed in cells that express those receptors?

During this study the expression of *IroC* is detected by using the IroC-Gal4 reporter. This reporter is believed to show the expression pattern of *ara* and *caup* (Ikmi *et al.*, 2008; Mazzoni *et al.*, 2008) and the observed expression pattern of this reporter line in the adult eye and in the eye-antenna imaginal disc support this idea (Mazzoni *et al.*, 2008; data not shown). However, since no antibodies against each of the Iro proteins are available and *in situ* hybridization for these factors did not work probably due to low expression levels, it could not be confirmed whether the observed expression reflects truly the endogenous expression pattern of *IroC*. Thus, it cannot be excluded with absolute certainty that the expression of these genes in the maxillary palp is more dynamic than initially thought.

How direct is the involvement of *IroC* in the regulation of OR genes? Are there other explanations that would fit the observations made throughout this study? The transcription factors that regulate the expression of *IroC* genes are not fully identified. A regulatory protein found upstream of *IroC* might regulate the transcription of another factor as well, which in turn might drive the expression of *OR33c* and/or *OR85e* genes (Figure 5.3). Additionally phosphorylation of Iro proteins might inhibit their repressor activity (Matsumoto *et al.*, 2004), thus permitting the expression of OR genes.

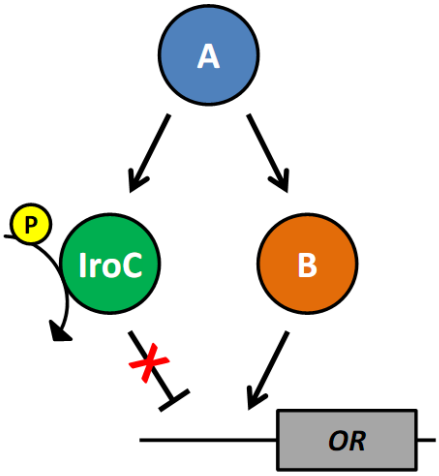

Figure 5.3. A possible mechanism regulating the expression of OR genes. Factor A drives the expression of both IroC and an unknown factor B, which in turn drives the expression of OR genes. Phosporylation of IroC inhibits its repressor activity, thus enables the expression of OR genes.

Another explanation could be that the presence of another transcription factor may inhibit the activity of Iro proteins. It has been suggested that transcription factors are expressed in overlapping subsets of ORNs and they act in a combinatorial fashion to regulate OR gene expression (Ray *et al.*, 2007). In this respect Iro proteins may be part of this combinatorial network and the interactions between these proteins needs to be elucidated in the future.

APPENDIX A: SEMI-QUANTITATIVE RT-PCR

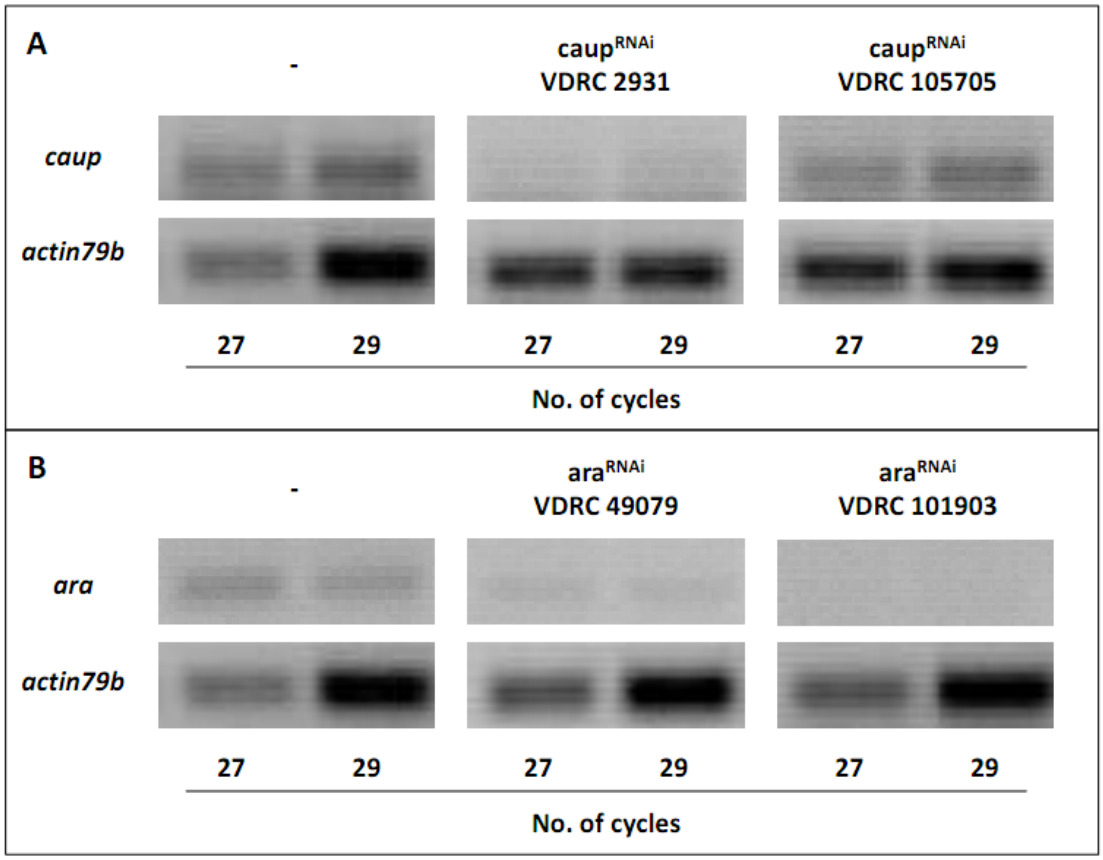

Figure A.1. Semi-quantitative RT-PCR. Publicly available UAS-RNAi lines were crossed to the pan-neural driver Elav-Gal4. F1 generation was collected and mRNA levels of *IroC* genes were detected using RT-PCR. Crossing of *caup*^{RNAi} VDRC 2931 line did result in a decrease of caup mRNAs; however, crossing of caupRNAi VDRC 105705 line did not change the caup mRNA amount significantly. Crossing of both *ara*^{RNAi} lines (VDRC 49079 and 101903) resulted in a decrease of *ara* mRNAs (the latter one is more efficient).

APPENDIX B: VECTOR MAPS

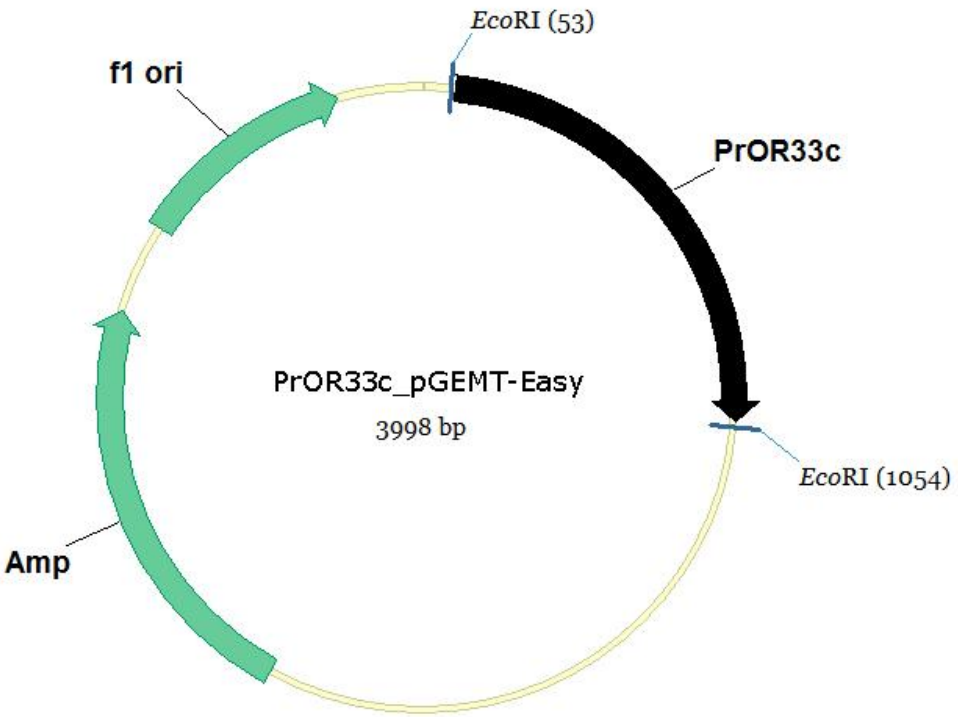

Figure B.1. Vector map of PrOR33c_pGEMT-Easy.

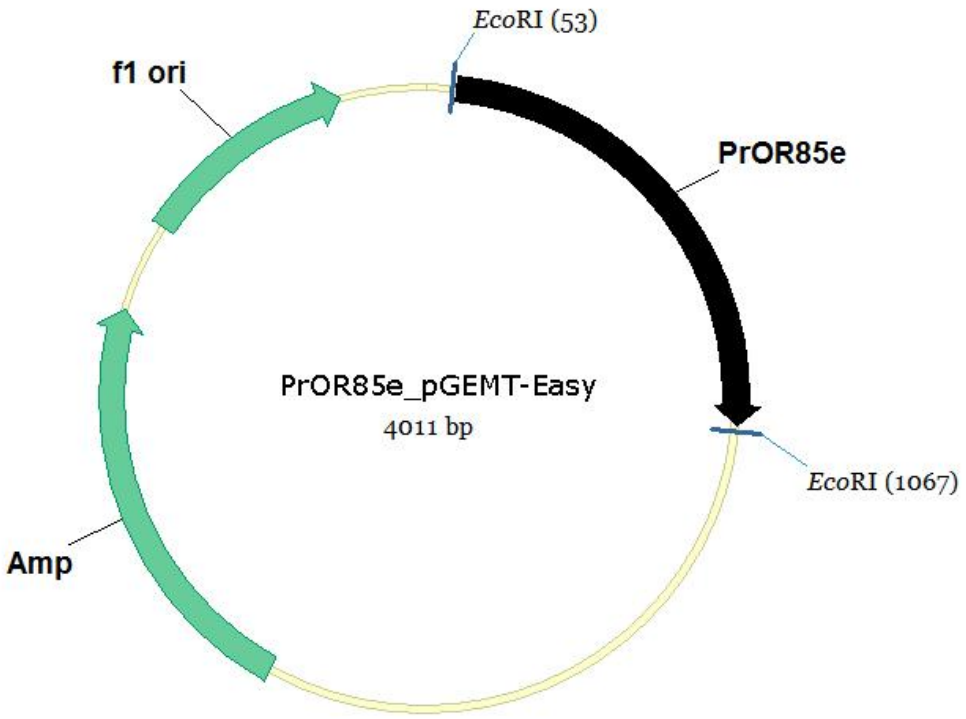

Figure B.2. Vector map of PrOR85e_pGEMT-Easy.

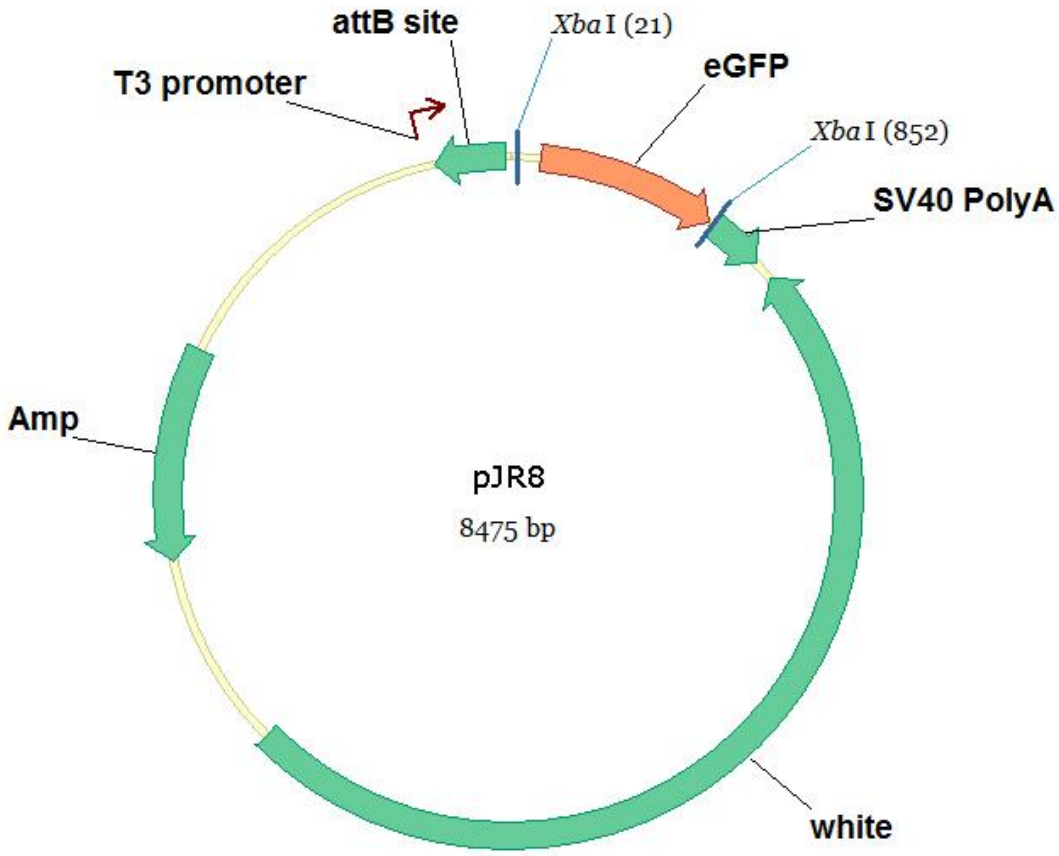

Figure B.3. Vector map of pJR8.

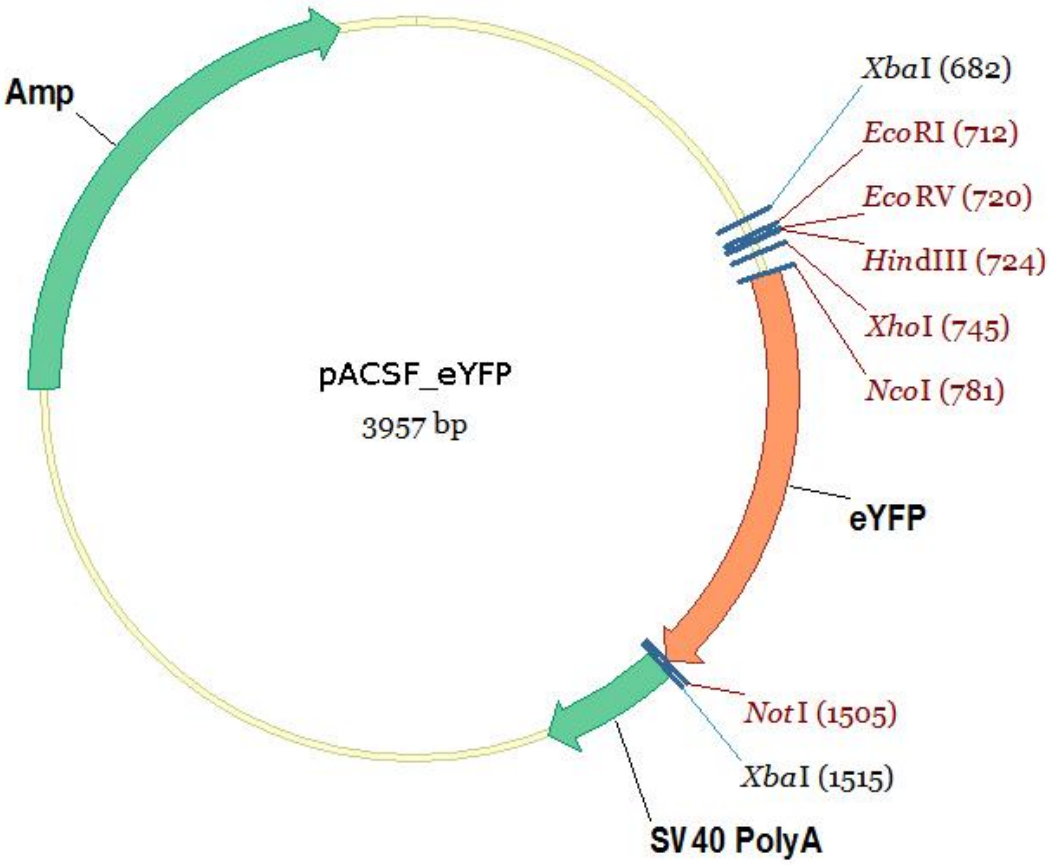

Figure B.4. Vector map of pACSF_eYFP.

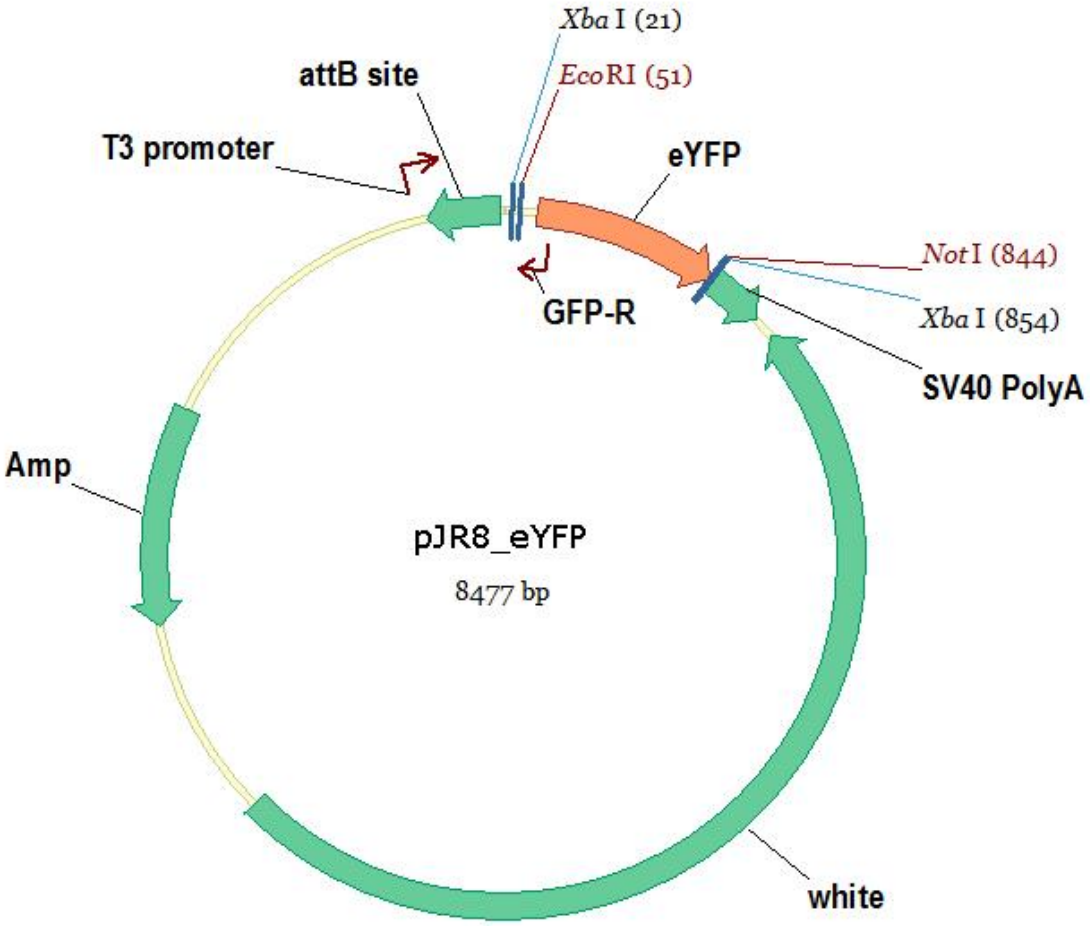

Figure B.5. Vector map of pJR8_eYFP.

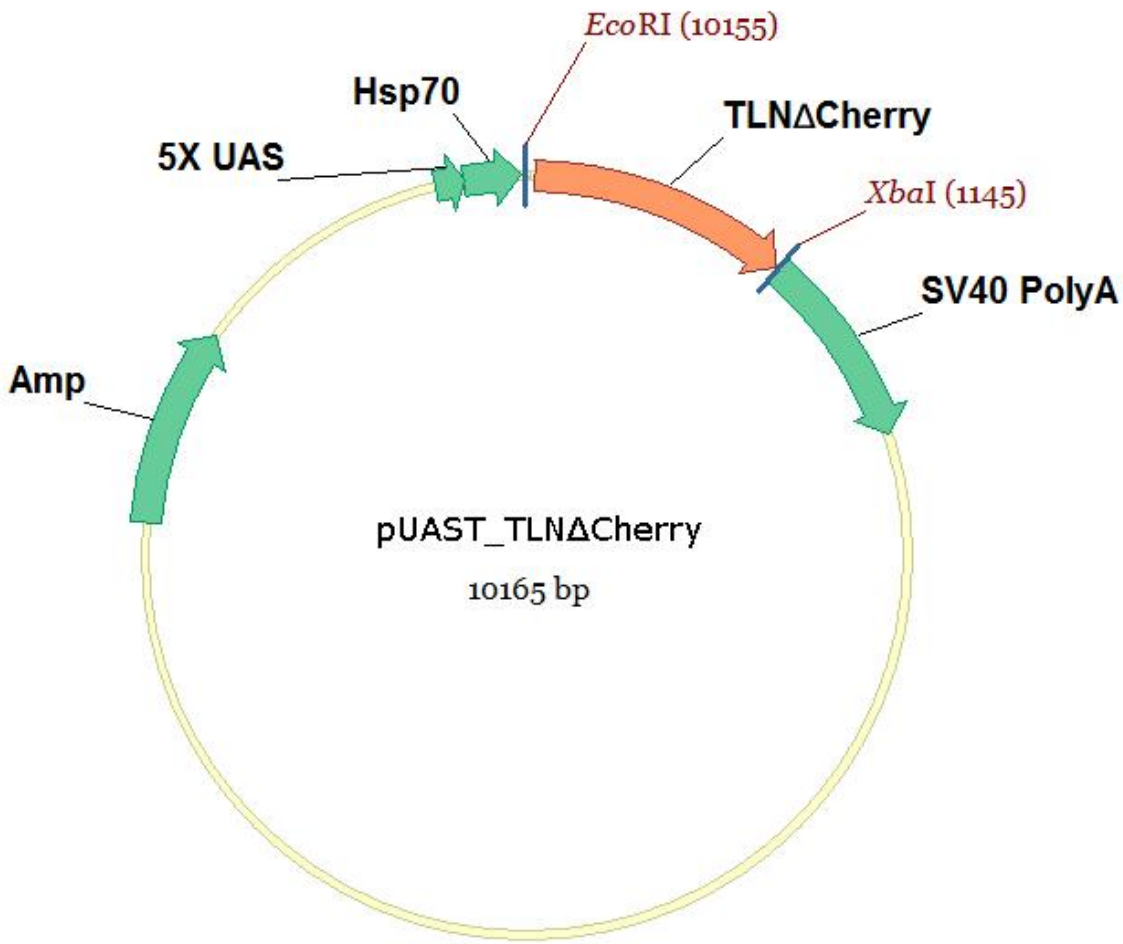

Figure B.6. Vector map of pUAST_TLNΔCherry.

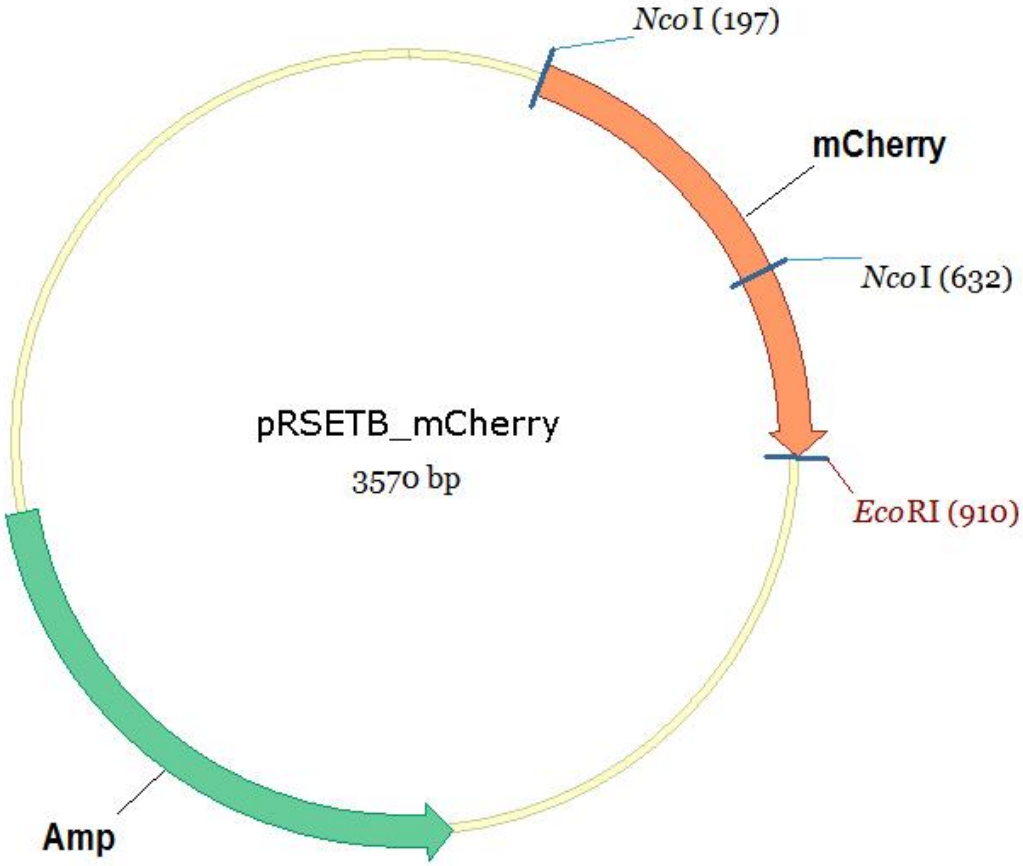

Figure B.7. Vector map of pRSETB_mCherry.

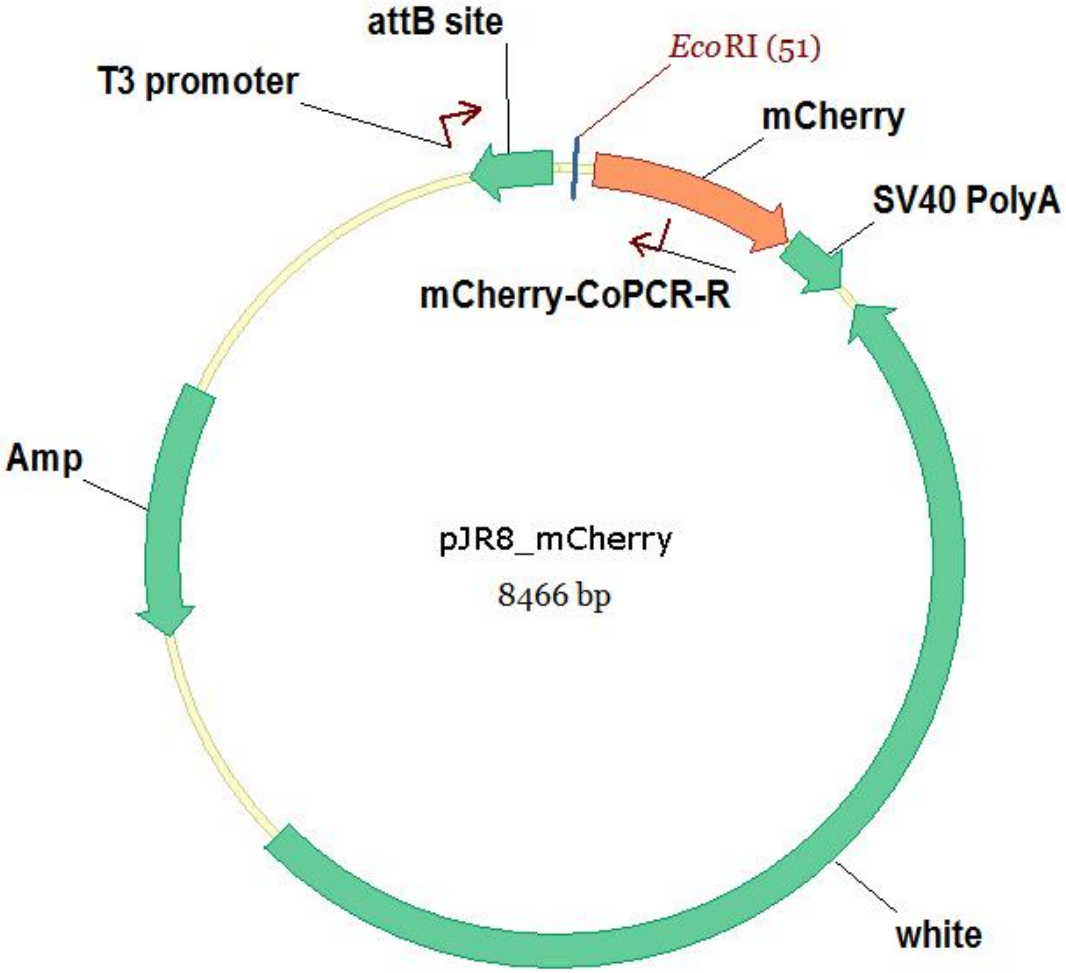

Figure B.8. Vector map of pJR8_mCherry.

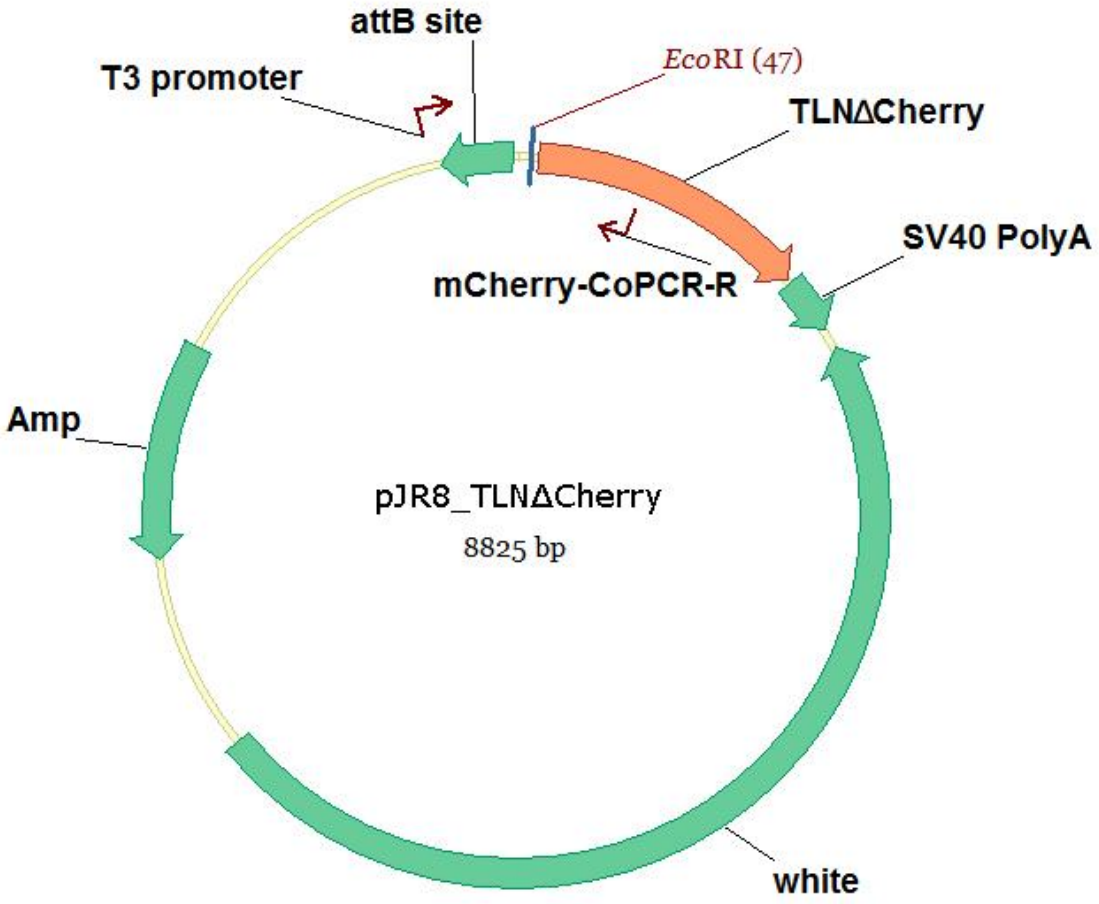

Figure B.9. Vector map of pJR8_TLNΔCherry.

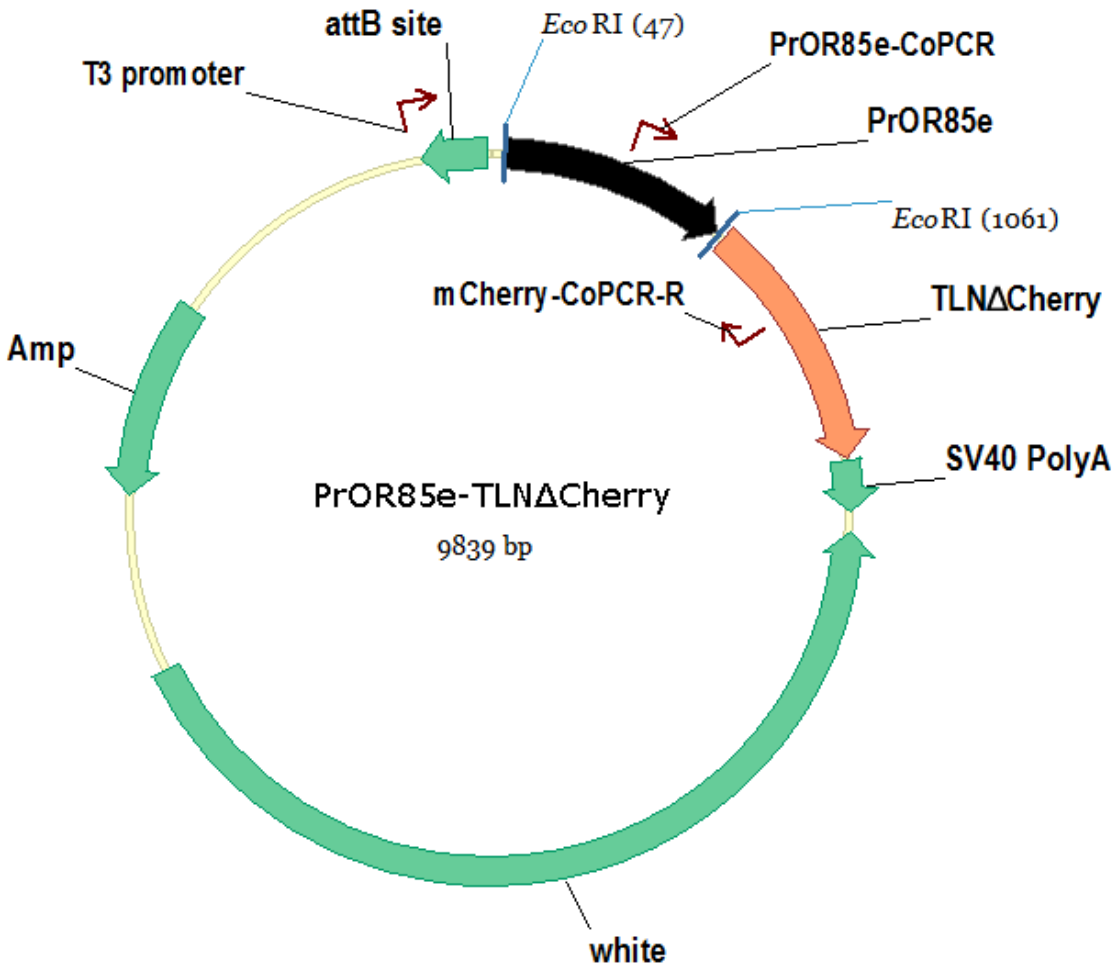

Figure B.10. Vector map of PrOR85e-TLNΔCherry.

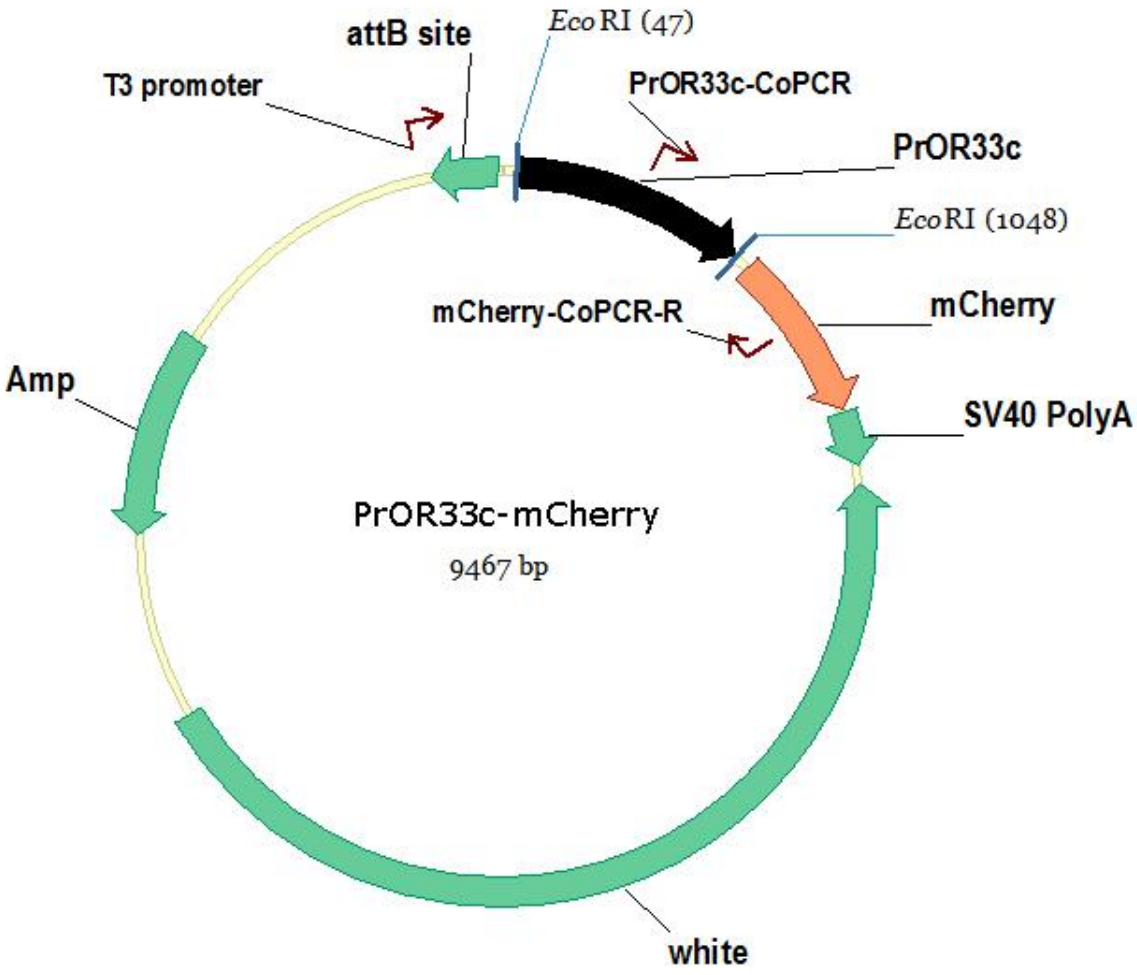

Figure B.11. Vector map of PrOR33c-mCherry.

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