GENERATION OF CRISPR/CAS-MEDIATED MUTANTS OF *IROC* GENES AND PROTEIN TAGGING FOR ENDOGENOUS EXPRESSION ANALYSIS IN *DROSOPHILA*

by

Ecem Çayıroğlu

B.S., Genetics and Bioengineering, Yeditepe University, 2015

Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science

Graduate Program in Molecular Biology and Genetics

Boğaziçi University

2019

For those who cause happiness wherever they go…

ACKNOWLEDGEMENTS

This study was supported by the funds of TUBITAK project (215Z369) carried out at Bogazici University, Department of Molecular Biology and Genetics from April 2016 to April 2018.

First of all, I would like to thank my supervisor, Assoc. Prof. Dr. Arzu Çelik for being always supportive and challenging at the same time. She trusted me with great responsibilities during this study and helped me to gain both knowledge and self-confidence in the lab. I am also thankful to Assoc. Prof. Dr. İbrahim Yaman and Assoc. Prof. Dr. Batu Erman for evaluating this thesis by devoting their valuable time.

I also would like to thank my labmates Duran Emre Kanacı, İbrahim İhsan Taşkıran, Bilal Başdağ, Emir Erkol, Gökhan Özturan, Serhat Beyaz, Onur Deniz, Ece Sönmez, Metin Özdemir, Yiğit Kocagöz, Selif Eski, Uğurcan Sakızlı and Mehmet Can Demirler for their infinite supports throughout this thesis.

İbrahim Taşkıran and Emre Kanacı deserve more than a thank you. Besides being my IroC partners, they are also great friends and it was always a pleasure to learn with them. Also, a huge hug goes to my best friends Atakan Kesim, Selvin Selvi, Deniz Yaşar Öztaş, Melis Bardakcı, Cansu Yıldırım and Osman Emre Dai for always being there for me whenever I needed.

Finally, I am grateful to my dear father Ayhan Çayıroğlu, mother Semra Çayıroğlu, brother Bora Çayıroğlu, grandmother Şükran Bozkurt and the rest of my family for their great patience and endless love. Without all my friends and family, I could not have survived.

ABSTRACT

GENERATION OF CRISPR/CAS-MEDIATED MUTANTS OF *IROC* GENES AND PROTEIN TAGGING FOR ENDOGENOUS EXPRESSION ANALYSIS IN *DROSOPHILA*

In the olfactory system, correct olfactory receptor neuron (ORN) development depends on proper olfactory receptor (OR) gene expression and proper projection of axons to higher brain centers. Thus, during development, ORNs go through two important decisions: selecting one OR gene from a large repertoire of OR genes and project their axons to a particular location in the brain. The availability of genetic tools and its relative simplicity make *Drosophila* an important model organism to uncover the molecular basis of the olfactory system development and function. Our studies focus on the function of *IroC (Iroquois complex)*, a transcription factor family, in ORN specification. The Iro family includes three genes called *araucan (ara), caupolican (caup)* and *mirror (mirr)*, which are conserved as clusters in all multi-cellular organisms. Previous analysis of enhancer trap lines showed that *IroC* is expressed in two olfactory organs, antennae and maxillary palp and using a triple mutant of *IroC*, RNA Sequencing analysis was performed on olfactory organs and iroC target genes were characterized.

In the framework of this study, to investigate *IroC* genes individually we wanted to generate novel tools, single knock-outs and individually-tagged proteins of iroC using CRISPR/Cas9 technology. To identify and compare target gene repertoires, I have generated single *IroC* mutants which will be used for RNASeq analysis in future studies. Also, I have generated individual fluorescently-tagged iro proteins to study their endogenous expression patterns. Overall, the generated tools will help to study iroC function in general and help to clarify the role of these proteins in olfactory system development.

ÖZET

DROSOPHILA'DA IROC GENLERİNİN CRISPR/CAS YÖNTEMİ KULLANILARAK MUTANTLARININ VE ENDOJEN İFADELERİNİN ANALİZİ İÇİN ETİKETLENMİŞ PROTEİNLERİNİN OLUŞTURUMLASI

Koku sisteminde, doğru koku reseptör nöronu (ORN) gelişimi, uygun koku reseptör (OR) gen ifadesine ve aksonların daha yüksek beyin merkezlerine uygun şekilde projeksiyonuna bağlıdır. Bu nedenle, gelişim sırasında, ORN'ler iki önemli karar alır: büyük bir OR gen repertuarından bir OR genini seçmek ve aksonlarını beyinde belirli bir yere iletmek. Genetik aletlerin mevcudiyeti ve nispi sadeliği *Drosophila*'yı koku sisteminin moleküler temelini ortaya çıkarmak için önemli bir model organizma haline getirmektedir. Çalışmalarımız, bir transkripsiyon faktör ailesi olan *IroC*'nin (Iroquois kompleksi) ORN spesifikasyonundaki fonksiyonu üzerine odaklanmaktadır. Iro ailesi, tüm çok hücreli organizmalarda kümeler halinde korunmuştur ve *araucan (ara), caupolican (caup)* ve *mirror (mirr)* adı verilen üç gen içerir. Transkripsiyon hızlandırıcı tuzak hatlarının önceki analizi, *IroC*'nin iki koku organında, antenlerde ve maksiller palpta ifade edildiğini göstermiştir. üçlü mutant sinekler kullanılarak RNA sekanslama analizi yapılmış ve koku sistemindeki iroC hedef genlerini karakterize edilmiştir.

Bu çalışma çerçevesinde *IroC* genlerini ayrı ayrı araştırmak için CRISPR/Cas9 teknolojisini kullanarak yeni sinek hatları üretildi; IroC genleri için tekli mutantlar ve iroC'nin etiketli proteinleri. Tekli *IroC* mutantları, hedef genlerin ne kadarının çakıştığını belirlemek için, gelecekteki çalışmalarda RNASeq analizi yapılması amacıyla tarafımdan üretildi. Ayrıca, endojen ifade şablonlarını incelemek için floresan etiketli tekli iro proteinleri tarafımdan üretildi. Genel olarak, üretilen araçlar iroC fonksiyonunu incelemeye ve bu proteinlerin koku alma sistemindeki rolünü netleştirmeye yardımcı olacaktır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

bp	Base pair
g	Gram
kb	Kilobase
L	Liter
ml	Milliliter
mm	Millimeter
M	Molar
ng	Nanogram
nm	Nanometer
rcf (g)	Relative centrifugal force (gravity)
rpm	Revolutions per minute
v	Volume
w	Weight
µg	Microgram
µm	Micrometer
µl	Microliter

LIST OF ACRONYMS/ABBREVIATIONS

Acj6	Abnormal chemosensory jump 6
AD	Activating domain
AL	Antennal lobe
BD	Binding domain
cDNA	Complementary DNA
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
FRT	FLP recombination target
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
Gr	Gustatory receptor
gRNA	Guide RNA
HDR	Homology directed repair
Ir	Ionotropic receptor
IroC	Iroquois complex
Lz	Lozenge
NHEJ	Non-homologous end-joining
nt	Nucleotide
NTC	No template control
OR	Olfactory receptor
ORN	Olfactory receptor neuron
PAM	Protospacer adjacent motif
pb	Palp basiconic
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
Pdm3	POU domain motif 3
PFA	Paraformaldehyde
pH	Power of hydrogen
PN	Projection neuron
Q-PCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNA-Seq	RNA Sequencing
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SOP	Sensory organ precursor
TF	Transcription factor
UAS	Upstream activating sequence

1. INTRODUCTION

The fruit fly, *Drosophila melanogaster*, has an advanced olfactory sensory system that allows hundreds of different odorants to be recognized and distinguished. For the animal, assessment of these odorants is requisite to define appropriate food sources and egg - laying sites. With novel tools, the molecular basis of this discriminatory power has begun to come out. Many Drosophila odorant receptors, transcription factors, elements were discovered. However, the transmission and interpretation of olfactory signals by the brain, stereotypic neuron-receptor expression still remain as an unsolved issue. The availability of genetic tools and a complete sequence of genomes render *Drosophila* an important model organism to uncover the molecular basis of olfactory system.

1.1. Olfaction

Our knowledge about the eye, how we process light and which properties of light are measured by the eye, led to the production of cameras and displays. We don't have the same knowledge about olfaction which is a key sensory modality to control many aspects of behavior. The relationship between the molecular features of a stimulus and the sensory response is still unknown (Hildebrand, 1995; Saberi and Seyed-allaei, 2016).

Initial odor recognition in flies is mediated by olfactory receptor neurons (ORNs), specialized bipolar neurons, which house olfactory receptors (ORs). Dendrites gather the environmental information with ORs and a single axon from each neuron transmits this signal to the central nervous system. There, synapses are formed with projection neurons (PNs), which then transmit the signals to higher brain centers such as the mushroom body and lateral horn (John G. Hildebrand and Shepherd, 1997).

Across species, olfactory system morphologies and underlying mechanisms are fundamentally similar. Detection and response to an odor is highly important for most animals. In general, ORNs display two main differentiation properties in the peripheral sensory epithelium: choosing single OR gene expression out of a large repertoire of OR genes and specific axonal targeting. Each ORN that expresses the same OR targets the same glomerulus in the olfactory processing center, the antennal lobe (Fuss and Ray, 2009; Hallem and Carlson, 2004). In addition to this, there is a general phenomenon called the "one neuron-one receptor" rule that states that each ORN tends to express only a single OR (Fuss and Ray, 2009; Hallem and Carlson, 2004; John G. Hildebrand and Shepherd, 1997). However, there are also some contrasting findings that show that a sensory neuron can express more than one receptor at the same time (Goldman *et al*., 2005; Mazzoni *et al*., 2008). The underlying reason for this co-expression are unknown, although in case of the photoreceptors it has been shown that the iroC genes are responsible for the co-expression of Rh3 and Rh4.

The similarity of olfactory systems in different animals makes it suitable to study the system in model organisms, such as *Drosophila*. For instance, while in mice there are ~2 million ORNs and ~1000 ORs, the *Drosophila melanogaster* olfactory system has a reduced numerical complexity. There are ~1300 ORNs and 62 ORs have been identified. Thus, the availability of a large repertoire of genetic tools and molecular and behavioral approaches make *Drosophila* a perfect model to study (Adams *et al*., 2000; Carlson, 1996; Rubin *et al*., 2000).

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

In adult *Drosophila* there are two main olfactory organs, the antennae and the maxillary palps. Both of these appendages are covered with sensilla, a hair-like structure. In the maxillary palp each sensillum houses 2 ORNs, thus a total of ~120 ORNs. However, antennae have a more complex structure and each sensillum houses 1-4 ORNs in total ~1200 ORNs. In terms of morphology, sensilla can be divided into three groups: trichoids, ceoloconics, and basiconics. In addition, they can be subdivided based upon their different

ORN combinations (Brochtrup and Hummel, 2011; Rodrigues and Hummel, 2008; Vosshall *et al*., 1999) (Figure 1.1.). Dendrites of each ORN are exposed to the environment to gather odor information and their axons project to glomeruli, spherical structures that are found in the antennal lobe. In these class-specific glomeruli, axonal terminals of each ORN synapse with PNs dendrites. Then PNs transmit the signal to higher brain centers. Local interneurons (LNs) are also found in glomeruli, which provide lateral connections between multiple glomeruli (Bhalerao *et al*., 2003; Fishilevich and Vosshall, 2005; Vosshall *et al*., 2000).

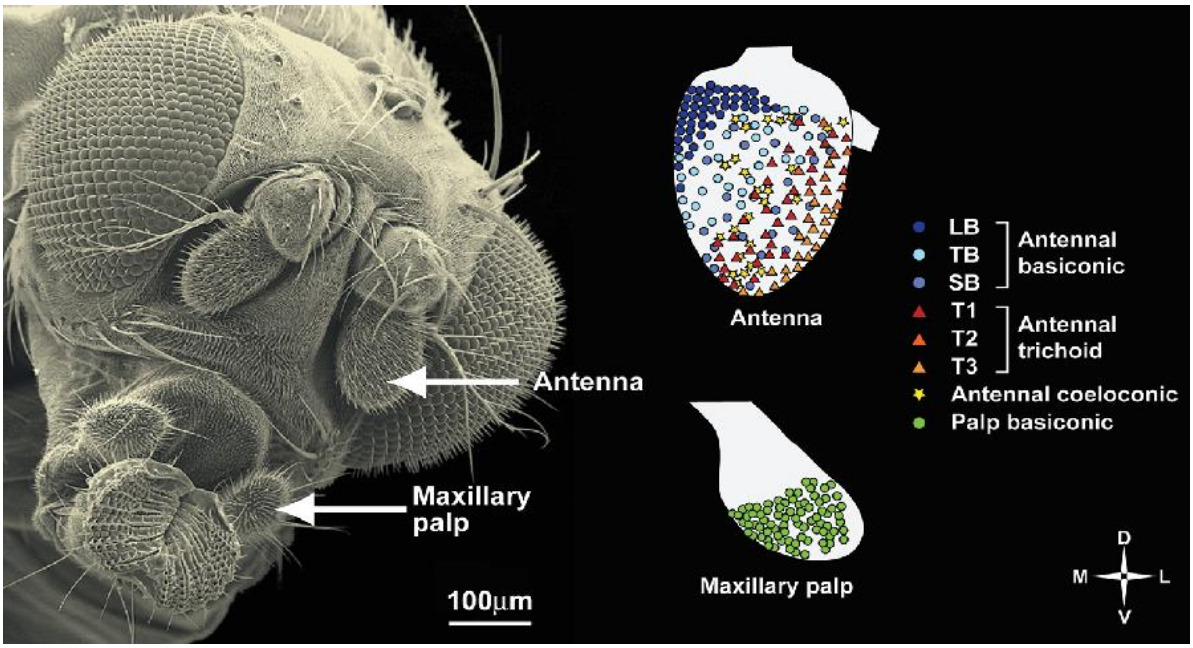

Figure 1.1.Organization of the *Drosophila* olfactory system. At left, arrows show the maxillary palp and the antenna, two major olfactory organs in adult *Drosophila* head. At right, schematic view of sensilla types and locations were shown on both organs (Laissue and Vosshall, 2008).

The olfactory organs arise from the eye-antennal imaginal disc found in *Drosophila* larvae. During pupal metamorphosis these discs give rise to adult structures. The expression of homeotic genes during embryonic development specifies the identity of these discs and antennal fate is mainly controlled by *Homothorax* (Morata, 2001). For the proper antennal

development and morphological identity three important factors are expressed in larval- and pupal- antennal discs; *amos*, *atonal* and *lozenge (lz).* At the beginning of third instar larval stage, *lz*, a member of AML- 1/Runt transcription factor family, controls both basiconic and trichoid sensilla fates. *lz* is a positive regulator of *amos*, a bHLH transcription factor, which is also required for basiconic and trichoid sensilla formation (Goulding *et al*., 2000; B P Gupta and Rodrigues, 1997; Bhagwati P Gupta *et al*., 1998). Around the initiation of puparium formation *amos* is expressed right after *lz* and low-level expression of *lz* with *amos* results in trichoid fate, high level of expression of *lz* with *amos* results in basiconic fate. On the other hand, formation of coeloconic sensilla requires *atonal*, another bHLH transcription factor, which is expressed in early pupal development (Goulding *et al*., 2000). Subsequent to this initial fate determination, Notch signaling initiates ORN diversification upon lateral inhibition. Unlike morphological divisions of sensilla, subtype diversifications require additional factors such as *Rotund (Rn), Dachshund (Dac)* and *Engrailed (En)* which specify sensillar subtype identity (Li *et al*., 2013) (Figure 1.2.).

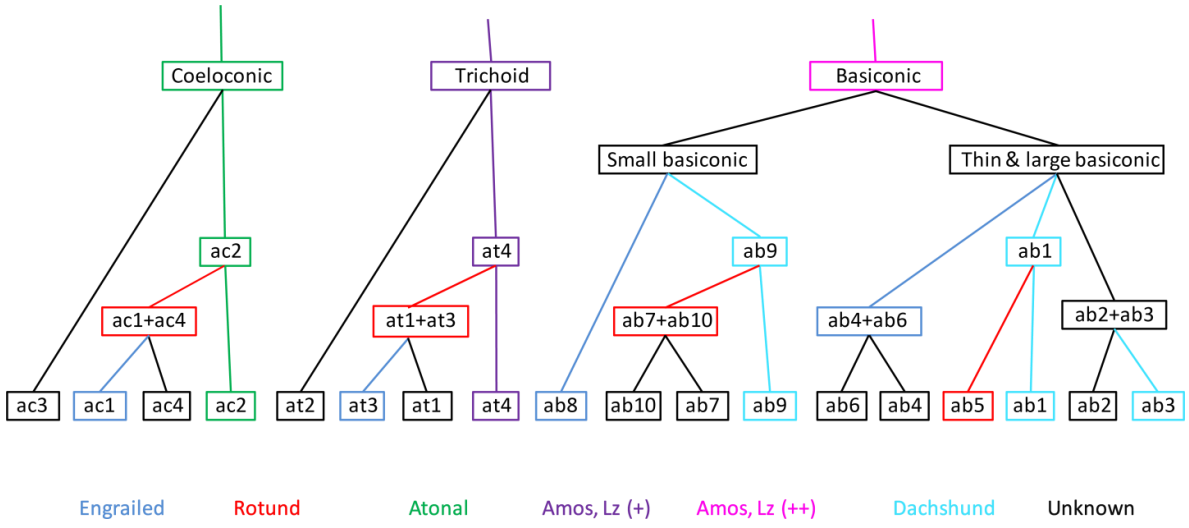

Figure 1.2. SOP decision tree based upon combinatorial expression of transcription factors. Expression of *Atonal*, *Amos* and *Lozenge* define sensillar morphological classes (green, purple and pink, respectively). *Engrailed*, *Rotund* and *Dachshund* are expressed later and define sensillar subtypes (dark blue, red and light blue, respectively) (adapted from Barish and Volkan, 2015).

Last steps of development of ORNs are axon guidance to antennal lobe and the selection of OR gene expression. To form synapses with proper PN partners, ORNs must navigate themselves in the antennal lobe (Dhanisha Jhaveri *et al*., 2000). Dscam and Robo receptors are known axon guidance molecules that are necessary for this navigation. Both of them use Dreadlocks (Dock), SH2/SH3 adaptor protein, and the serine/threonine kinase Pak. With this insight, it is suggested that Dscam and Robo receptor actions are coordinated and both of them are involved in proper glomerular targeting. There are three Robo receptors and around 38,000 alternative splice isoforms of Dscam in *Drosophila*. All Robo receptors and all isoforms of Dscam are known to be involved in axon guidance, yet not exactly known how (Hummel *et al*., 2003; D. Jhaveri *et al*., 2004). In addition, Notch signaling and transcription factors *Acj6* and *Pdm3* play a critical role in control of axonal targeting (Endo *et al*., 2007; Komiyama *et al*., 2004).

1.3. Olfactory Receptor Genes

Odorant receptors which are encoded by OR genes have seven transmembrane domains. In vertebrates they belong to the G-protein coupled receptor (GPCR) family which make them metabotropic receptors (Benton *et al*., 2006). Nevertheless, it is a topic of debate for insects. The topology of ORs differs from vertebrates and they are thought to be ionotropic receptors, which can also use metabotropic signaling (Sato *et al*., 2008; Wicher *et al*., 2008). In addition, unlike vertebrates, most insect ORs are found together with Orco (Or83b), a widely expressed co-receptor and function in the presence of it. The list of *Drosophila* ORs and the glomerulus they target can be seen in Table 1.1.

Many ORN properties, such as onset and termination dynamics, spontaneous firing rate, and signaling mode are determined by the odorant receptor. Moreover, for different chemical stimuli, each receptor has a different response spectrum that has been determined by electrophysiological studies (Guo and Kim, 2007).

Table 1.1. Overview of the *Drosophila* olfactory receptor classes (adapted from Martin *et al.*, 2013).

SENSILLUM CLASS	NEURON	RECEPTOR	CORECEPTOR	GLOMERULUS
BASICONIC (ANTENNA)	ab1A	Or42b	Orco	DM1
	ab1B	Or92a	Orco	VA2
	ab1C	Gr21a, Gr63a		V
	ab1D	Or10a, Gr10a	Orco	DL1
	ab2A	Or59b	Orco	DM4
	ab2B	Or33b, Or85a	Orco	DM5
	ab2B	Or33b	Orco	DM5
	ab2B	Or85a	Orco	DM5
	ab3A	Or22a, Or22b	Orco	DM2
	ab3B	Or85b	Orco	VM5d
	ab4A	Or7a	Orco	DL5
	ab4B	Or33a, Or56a	Orco	DA2
	ab4B	Or33a	Orco	DA2
	ab4B	Or56a	Orco	DA2
	ab5A	Or82a	Orco	VA6
	ab5B	Or33b, Or47a	Orco	DM3
	ab5B	Or33b	Orco	DM3
	ab5B	Or47a	Orco	DM3
	ab6A	Or13a	Orco	DC2
	ab6B	Or49b	Orco	VA5
	ab7A	Or98a	Orco	VM5v
	ab7B	Or67c	Orco	VC4
	ab8A	Or43b	Orco	VM2
	ab8B	Or9a	Orco	VM3
	ab9	Or67b	Orco	VA3
	ab9	Or69aA, Or69aB	Orco	D
	ab10A	Or67a	Orco	DM6
	ab10B	Or49a, Or85f	Orco	DL4
	ab10B	Or49a	Orco	DL4
TRICHODEA (ANTENNA)	at1A	Or67d	Orco	DA1
	at2A	Or83c	Orco	DC3
	at2B	Or23a	Orco	DA3
	at3A	Or19a, Or19b	Orco	DC1
	at3	Or2a	Orco	DA4m
	at3	Or43a	Orco	DA4l
	at4A	Or47b	Orco	VA1v
	at4B	Or65a, b, c	Orco	DL3
	at4C	Or88a	Orco	VA1d
COELOCONICA (ANTENNA)	ac1	Ir31a	Ir8a	VL2p
	ac1	Ir75d	Ir25a	VL1
	ac1	Ir92a	Ir25a, Ir76b	VM1
	ac2	Ir75a	Ir8a	DP1l
	ac2	Ir75d	Ir25a	VL1
	ac2	Ir41a	Ir25a, Ir76b	VC5
	ac3A	Ir75a, Ir75b, Ir75c	Ir8a	DL2d/v
	ac3B	Or35a	Orco, Ir76b	VC3
	ac3B	Or35a	Orco, Ir76b	VC3
	ac4	Ir84a	Ir8a	VL2a
	ac4	Ir75d	Ir25a	VL1
	ac4	Ir76a	Ir25a, Ir76b	VM4
BASICONICA (PALP)	pb1A	Or42a	Orco	VM7d
	pb1B	Or71a	Orco	VC2
	pb2A	Or33c, Or85e	Orco	VC1
	pb2B	Or46a	Orco	VA7l
	pb3A	Or59c	Orco	VM7v
	pb3B	Or85d	Orco	VA4

1.4. Olfactory Receptor Gene Choice

The last step for ORN development is receptor gene choice. Selection and expression of olfactory receptors is regulated by expression of several transcription factors. One of the first transcription factors that was shown to play a role in olfactory gene choice is Acj6. Acj6 is a POU domain transcription factor, which regulates OR expression directly and is expressed both in maxillary palps and antennae. It defines ORN identity in both organs by regulating OR choice (Ayer and Carlson, 1992; Bai and Carlson, 2010; Clyne *et al*., 1999). Another POU domain transcription factor, Pdm3, is also necessary for the generation of ORN identity. It is also important for axonal targeting (Tichy *et al*., 2008). Unlike mammalian ORNs, *Drosophila* ORNs use a combinatorial code of transcription factors for OR expression. For instance, in case of Or42a, Pdm3 and Acj6 work in combination to regulate its expression (Tichy *et al*., 2008). More recently, six novel transcription factors (zf30c, sim, xbp1, fer1, E93, and onecut) have been identified that work in combination with Acj6 to express ORs in the antenna (Jafari *et al*., 2012).

Artificial OR promoter reporter constructs are sufficient for mirroring OR expression. Therefore, this endogenous promoter locus was studied for the encoded information and two motifs that are necessary for regulating olfactory organ specific OR gene expression were identified. The Dyad1 motif is required to induce OR expression in the maxillary palp and Oligo1 motif is required to prevent the same ORs to be expressed in the antenna (Fuss and Ray, 2009). Upstream regions of OR genes also include binding sites for the transcription factors that are mentioned above (Miller and Carlson, 2010). A summary of genes and motifs involved in OR gene choice can be seen in Figure 1.3.

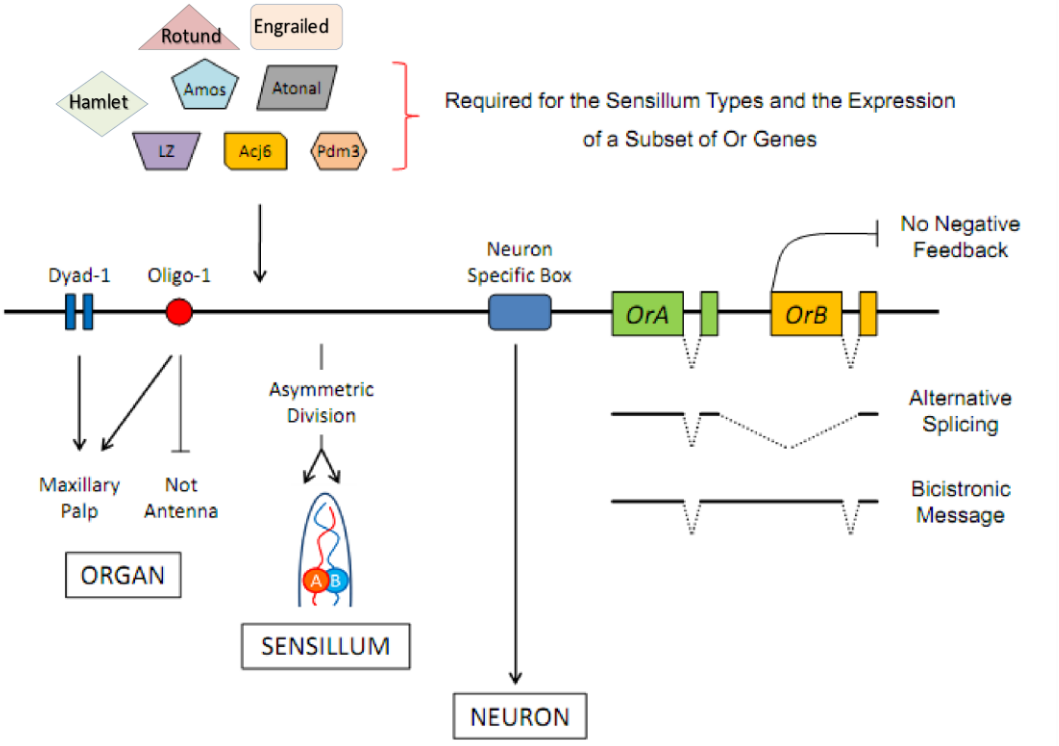

Figure 1.3. Schematic view of known receptor gene expression mechanisms (adapted from Ray *et al.*, 2007)

1.5. Iroquois Complex

In *Drosophila*, the *Iroquois complex (IroC)* consists of three genes; *araucan (ara), caupolican (caup)* and *mirror (mirr)*. These genes encode for highly conserved TALE homeodomain proteins. Unlike classic hox genes, TALE class genes have a homeodomain of 3 amino acid loop extension. Other than the highly conserved homeodomain, all iro proteins have an iro box, an EGF-like motif. This motif is a protein-protein interaction domain and is similar to the Notch receptor interaction domain (Gómez-Skarmeta *et al.*, 1996; McNeill *et al.*, 1997).

All three genes have a conserved homeodomain and an iro box. However, according to alignment results of these proteins, *ara* and *caup* are more similar and share same cis-regulatory elements. Furthermore, expression patterns of *ara* and *caup* are similar and redundant to each other in different tissues (Gómez-Skarmeta *et al.*, 1996). Mirr is a more divergent protein and has more distinct functions. Homologs of *IroC* are found in all multi-cellular organisms. In vertebrates, two *IroC* paralogs are conserved as clusters, and consist of three homologs of *ara*, *caup* and *mirr* in each cluster (Florencia Cavodeassi *et al.*, 2001) (Figure 1.4.).

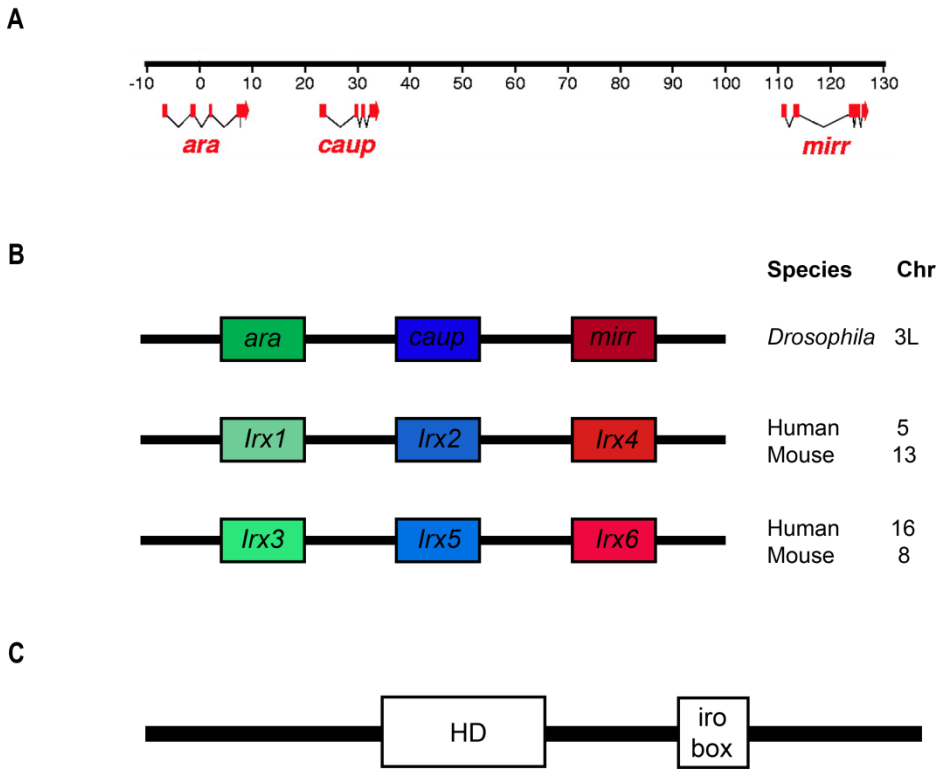

Figure 1.4. Genomic organization and protein structure of *IroC* members. A) Detailed physical map of *IroC* genes shown with red. B) Three members of *IroC* are clustered together and vertebrate paralogous genes are shown with similar colors. C) Protein structure of IroC. All Iro proteins have a highly conserved homeodomain of the TALE class (HD) and an iro box (adapted from Cavodeassi, Modolell and Gómez-Skarmeta, 2001).

Iro proteins can form homo- and hetero- dimers and there are identified binding sites for them. Mirr functions in a homodimer form and binds to the binding site AAAACACGTGTTAA. In addition, it was shown that the ACANNTGT (Bilioni *et al*., 2005) sequence is enough for for minimal recognition by Mirr. Ara and Caup can form hetero- and homo- dimers and their binding site has been determined to be the sequence ACAN(2–8)TGT (Carrasco-Rando *et al*., 2011). Later, Andreu *et al.*, (2012) proposed another binding site for Mirr, ACACGTGT.

1.6. Known IroC Expression Pattern

During the development of *Drosophila*, Iro genes play key roles. The early pattern of expression of the *IroC* factors suggests that they contribute to the patterning of the dorsal mesoderm along the anterior-posterior axis to ensure the normal development of its derivatives. Moreover, only *mirr* is shown to be expressed in the follicle cells and regulates follicle cell organization in the ovaries (Andreu *et al*., 2012; F Cavodeassi *et al*., 2000; Diez del Corral *et al*., 1999).

At the beginning of the second larval instar, their expression in the eye precursor (the eye/antenna imaginary disc) defines the dorsal compartments of the eye and head (F Cavodeassi *et al*., 2000). At the same time, the extent of the notum (dorsal mesothorax) territory is defined by *IroC* expression in the mesothorax and wing precursor (the wing imaginal disc) (Gömez-Skarmeta *et al*., 1996; Morata, 2001). In addition, Iro genes are involved in the territorial growth of imaginal eye and wing discs by creating boundaries in the confrontation between Iro-expressive and non-expressive cells (F Cavodeassi *et al*., 1999). Created DE-Gal4 line thought to give the expression pattern of mirr, confirmed previous findings. Analysis of DE-Gal4 line showed dorsal expression pattern in third instar eye disc, dorsal expression pattern in second instar eye disc and in wing disc and haltere disc expression is found in nota, pleura and pouch regions. Moreover, in stage 11 embryo expression was detected segmentally repeating pattern in the mesoderm (Morrison and Halder, 2010).

Iro proteins are also involved in cell fate specification and pattern formation. In combination with other transcription factors they regulate the expression of proneural genes in proneural clusters (F Cavodeassi *et al*., 2001). Furthermore, *ara* and *caup* are known to be expressed in muscle cells and to specify the lateral transverse muscle fate by controlling the expression of the *slouch* gene (Carrasco-Rando *et al*., 2011). The regulation of planar polarity in the eye and follicle cell organization in the ovaries depends on the regulation of *fng* by *mirr*. By regulating pipe, the dorsal area of the follicular epithelium of the egg chamber is defined, which is necessary to establish the embryonic axes correctly (Andreu *et al*., 2012). Finally, *IroC* members are expressed in a subpopulation of Even-skipped expressing pericardial cells and seven pairs of heart-associated cells which affect heart development in *Drosophila* (Mirzoyan and Pandur, 2013). A summary of expression domains can be seen in Figure 1.5.

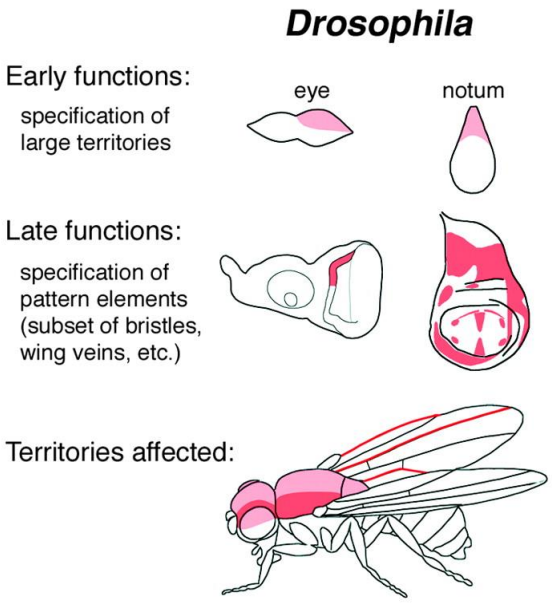

Figure 1.5. Early and late functions of *IroC* in *Drosophila* imaginal discs. *IroC* members are expressed in the dorsal regions of second instar eye and wing discs and in third instar larvae, more resolved pattern is observed. *IroC* absence leads lack of sensory bristles, wing veins and probably abnormal transformation of neurons (adapted from Cavodeassi, Modolell and Gómez-Skarmeta, 2001).

1.7. CRISPR/Cas9 System

Our ability to modify endogenous genomic sequences has been revolutionized by the development of genome engineering technologies. Previously, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were used for genome engineering (Gaj, 2014). Nowadays, these methods are replaced by CRISPR/Cas, which was initially discovered as a bacterial adaptive immunity mechanism. Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated proteins (Cas) are part of a bacterial defense mechanism guided by RNA against the invader (Rath *et al*., 2015). Because of its more efficient double stranded break response and cheapness (Song *et al*., 2016), it is now regarded as an irreplaceable genome engineering tool.

In this system, the Cas endonuclease protein and a guide RNA (gRNA) form a complex and localize to the intended part of DNA. The target DNA (the protospacer) should contain a protospacer adjacent motif (PAM), required for Cas protein, and also should be complementary to the gRNA (Doudna and Charpentier, 2014; Hsu *et al*., 2014). The gRNA includes two parts. First 20 nucleotides of the gRNA act as a guide to target the Cas protein to the target sequence. The second part has a secondary structure for direct binding to Cas endonuclease. After this complex formation, the Cas protein recognizes the PAM (NGG) sequence, cleaves the DNA 3 nucleotides upstream of the PAM region and creates a double-stranded break (DSB) (Huai *et al*., 2017).

To maintain genomic stability, such breaches must be repaired. As a response, organisms have developed a repair mechanism to mend the instability caused by the DSB. The two most widely studied DSB responses are non-homologous end-joining (NHEJ) and homology-directed repair (HDR). The NHEJ pathway repairs DNA by the addition or deletion of random nucleotides (Zhu *et al*., 2015). On the other hand, the HDR pathway uses a donor DNA homologous to the DNA near the DSB site and repairs it by homologous recombination (Bassett and Liu, 2014) (Figure 1.6.).

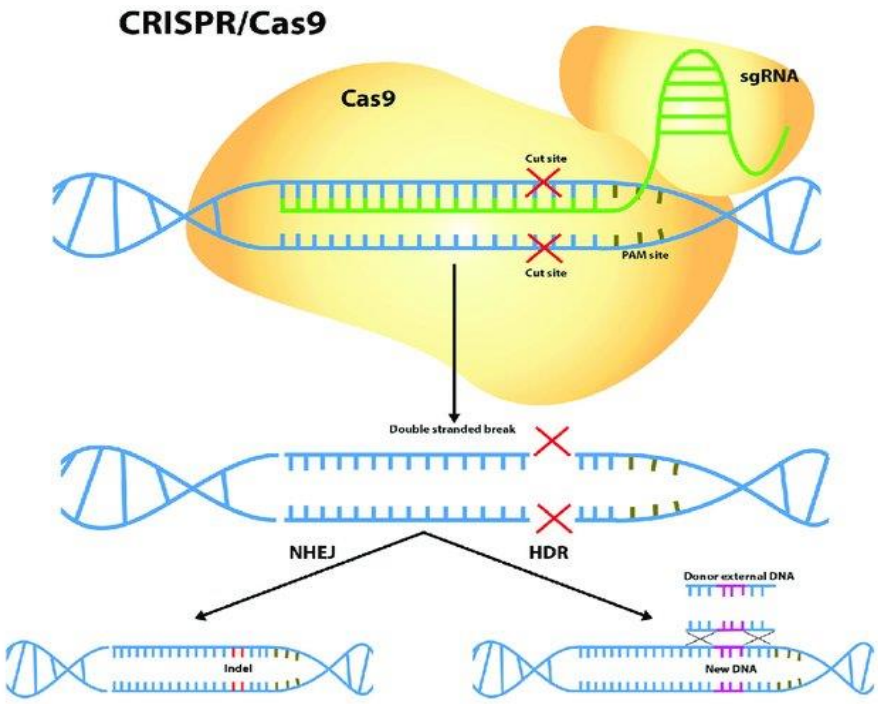

Figure 1.6. Schematic view of the CRISPR/Cas9 system. 20 nucleotide gRNA recognizes the target DNA region followed by PAM sequence. As a result of DSB, either NHEJ or HDR repair pathways are activated (Cribbs and Perera, 2017).

2. AIM OF THE STUDY

It has been shown that Iro proteins are essential for various processes, however, lack of tools for iroC was limiting more detailed analysis. Our main purpose in this study was to fill this tool gap by generation of single knock-outs and fluorescently-tagged iro proteins mediated by CRISPR/Cas9.

Our further studies using these generated lines will focus on iroC role in olfactory system development. Individual knock-out lines were generated to perform transcriptome-wide analysis to find differentially expressed olfactory receptor genes and transcription factors that are regulated by iro proteins. To identify endogenous expression pattern of iroC, fluorescently tagged iro proteins were generated, and for mirr, expression profile was determined.

Our second aim was to verify the differential expression of transcription factors identified by RNA Sequencing of *IroC* triple mutant flies (İbrahim İhsan Taşkıran, 2018). The data were confirmed using QPCR.

3. MATERIALS AND METHODS

3.1. Biological Material

Drosophila melanogaster lines were stored at 25ºC and 70% humidity in air-permeable disposable vials including fly food. Fly food was commercially available (Genesee Scientific Nutri-Fly™ Bloomington Formula) and prepared according to company's user guide. All flies were reared in fly incubators maintaining a 12h light and 12h dark cycle and once a month they were transferred to new vials with freshly prepared fly food. Fly lines used in this study are listed in Table 3.1.

Table 3.1. Fly lines used during this study.

Transgene	Inserted Chr. No.	Description
nos-Cas9	1	Cas9 ecpressionunder the control of *nanos* promoter. Expression of Cas9 only happens in germline cells.
w^{1118}	1	Mutant allele of eye pigment gene causes white eye phenotype.
yw ; QB		Flies carrying balancer chromosomes Sp / CyO ; TM2 / TM6B
yw ;; TM3/TM6B		Flies carrying balancers for third chromosome TM3 / TM6B
U6:1-gRNA(a1) *U6:3-gRNA*(a2)	2	Expresses gRNA under the control of RNA promotor U6:1 and U6:3. Expressed gRNAs specific to target sequences (a1 and a2) on *ara* gene.

Table 3.1. Fly lines used during this study (cont.).

Transgene	**Inserted Chr. No.**	**Description**
U6:1-gRNA(c1) *U6:3-gRNA*(c2)	2	Expresses gRNA under the control of RNA promotor U6:1 and U6:3. Expressed gRNAs specific to target sequences (c1 and c2) on *caup* gene.
U6:1-gRNA(m1) *U6:3-gRNA*(m2)	2	Expresses gRNA under the control of RNA promotor U6:1 and U6:3. Expressed gRNAs specific to target sequences (m1 and m2) on *mirr* gene.
CL	3	Putative cell lethal mutation
GMR-hid	3	Recombined construct of pro-apoptotic gene hid under the control of GMR promoter
FRT80B	3	Allow FLP-mediated site-specific recombination on the chromosome arm 3L
iro^{DFM3}	3	Chromosomal deficiency spanning *araucan*, *caupolican* and the promoter of *mirror*

3.2. Chemicals and Supplies

All commercially available chemicals and supplies used in this study are listed under the corresponding titles.

3.2.1. Chemical Supplies

Table 3.2. Chemicals used in this study.

Chemical	Producing Company
1 kb Marker	NEB, USA (N3232L)
100 bp Marker	NEB, USA
Ethidium Bromide Solution	Sigma Life Sciences, USA (E1510)
$MgCl_2$	Riedel-de Haen, Germany (13152)
NaCl	Sigma-Aldrich, USA (S7653)
Tris	Sigma-Aldrich, USA (T6066)
EDTA	Sigma-Aldrich, USA (59417C)
DEPC	
DMSO	
Isopropanol	
SeaKem LE Agarose	Biomax (104514PR)
Phenol:Chloroform:Isoamyl alcohol	Sigma-Aldrich, USA (P2069)
Triton X-100	AppliChem, USA (A4975)
Trizol	Invitrogen (15596-026)
Tween-20	Roche, USA (11332465001)

3.2.2. Buffers and Solutions

Table 3.3. Contents of buffers and solutions used in this study.

Solution / Mixture	Ingredient
Formaldehyde Solution (4%)	160 g/l PFA, pH 7.4
PAXD	50 g BSA 3 g Sodium Dexoycholate 0.3% Triton X-100 In PBS
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
EB (Elution Buffer)	10 mM Tris-Cl, pH 8.5
Loading Buffer (10x)	50% Glycerol 0,0005% Bromophenol Blue
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	Confidential / Commercial

Table 3.3. Contents of buffers and solutions used in this study (cont.).

Solution / Mixture	Ingredient
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)
TAE Buffer (1x)	40 mM Tris-Cl 1 mM EDTA 0.1% Acetic acid
Squish Buffer	10 mM Tris, pH 8.0 1 mM EDTA 25 mM NaCl

3.2.3. Oligonucleotide Primers

Oligonucleotides were synthesized commercially at Macrogen (South Korea). Lyophilized oligonucleotides were dissolved in dH_2O to obtain a final concentration of 100 µM. Dissolved primers were stored at -20ºC as stock. For double gRNA PCR, 1:50 diluted (2 µM) oligonucleotides were used. All other PCRs were performed with 1:10 diluted (10 µM) oligonucleotides. Oligonucleotides used in this study are listed in Table 3.4.

Table 3.4. Oligonucleotides used in this study.

Name	Sequence (5' - 3')
Fwd_ara_pCFD4_gRNA	TATATAGGAAAGATATCCGGGTGAACTTCGGGACGG TGGTTGGAGCAGGGTTTTAGAGCTAGAAATAGCAAG
Rev_ara_pCFD4_gRNA	ATTTTAACTTGCTATTTCTAGCTCTAAAACGTGGCCA GATCCGCTGGCCCGACGTTAAATTGAAAATAGGTC
Fwd_caup_pCFD4_gRNA	TATATAGGAAAGATATCCGGGTGAACTTCGGGACCG GCAGTCAGGCCGGGTTTTAGAGCTAGAAATAGCAAG
Rev_caup_pCFD4_gRNA	ATTTTAACTTGCTATTTCTAGCTCTAAAACCCATGGC CGCTCTGGCTACCGACGTTAAATTGAAAATAGGTC
Fwd_mirr_pCFD4_gRNA	TATATAGGAAAGATATCCGGGTGAACTTCGGGAGGC GTGTTGGGCGACGGTTTTAGAGCTAGAAATAGCAAG
Rev_mirr_pCFD4_gRNA	ATTTTAACTTGCTATTTCTAGCTCTAAAACCCCTCGA CGCTGCCCACGCCGACGTTAAATTGAAAATAGGTC
Fwd_screen_ara_Nhej	GAGGTCAGGATTGTCAGGGT
Rev_screen_ara_Nhej	CTCACAGCATGATCCACCAC
Fwd_screen_caup_Nhej	TTTTCCCTTTGGCATCTTTG
Rev_screen_caup_Nhej	ATGTGGAGAGACCCTTGTGG
Fwd_screen_mirr_Nhej	TGTTCATAATCAACTTGGACAGC
Rev_screen_mirr_Nhej	GTAGATGGTGCGACCCGTAT
Fwd_HDR_ara_gRNA	TGCA GTTCATCGGGTAACCGCTGG
Rev_HDR_ara_gRNA	AAAC CCAGCGGTTACCCGATGAAC
Fwd_HDR_caup_gRNA	TGCA GAGTAATGCCTATCTAACGG
Rev_HDR_caup_gRNA	AAAC CCGTTAGATAGGCATTACTC
Fwd_HDR_mirr_gRNA	TGCA GTAGAGTTACGATGCACCGA
Rev_HDR_mirr_gRNA	AAAC TCGGTGCATCGTAACTCTAC
Fwd_ara_LHA	TCGCATGCTCCCGGCCGCGGAAGTGGTAGTGGTGGT TAC
Rev_ara_LHA	GTTGGTGAACTTCTTGAAGAG
Fwd_ara_RHA	CATTATGGGATCGTGCAAGAATCGGACGGATTTGTG TAATAT

Table 3.4. Oligonucleotides used in this study (cont.).

Name	Sequence (5’ - 3’)
Rev_ara_RHA	CAGGCGGCCGCGAATTCAGGTTATTGTAGTAGCACTATGGAAA
Fwd_caup_LHA	TCGCATGCTCCCGGCCGCGTGGACAGTAGTGGTGACCAG
Rev_caup_LHA	TACTAGTGACGCCCATGTGCC
Fwd_caup_RHA	CGGTTAGTGGAGAGCAGTTAG
Rev_caup_RHA	CAGGCGGCCGCGAATTCATCGAACCAGTTTGCCGAACG
Fwd_mirr_LHA	TCGCATGCTCCCGGCCGCCGTGCCGCCTCGTTTGCTACT
Rev_mirr_LHA	CGCACTGAACCGACGTATGTG
Fwd_mirr_RHA	CTGTTTAGTAATGGTGCGTATATCAATTTG
Rev_mirr_RHA	CAGGCGGCCGCGAATTCATGTCGCTGATGTTGCTGTTGC
Fwd_mirr_LHA_new	GGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTCGTGCCGCCTCGTTTGCTAC
Rev_mirr_LHA_new	TAAAGCAAATCTTAGAGTTACGATGCACCGATCCACTCGCGCACTGAACCGACGTATGTG
Fwd_mirr_RHA_new	CAGCACAGTGCACAGTAATGAAACCGGTATCTGTTTAGTAATGGTGCGTATATCAATTTG
Rev_mirr_RHA_new	AACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTGTCGCTGATGTTGCTGTTG
Rev pgemt	AATCGAATTCCCGCGGCCGCCAT
Rev pgemt ncoi	GCGGCCGGGAGCATGCGACGT
Fwd_mirr_screen_HDR	GGTCACACAGGCGGTAGATAC
Rev_mirr_screen_HDR	CTGCATGTTGTATCATCGGTCG

Table 3.4. Oligonucleotides used in this study (cont.).

Name	Sequence (5’ - 3’)
Fwd_mirr_screen_HDR_GFP	CGTTATCCCGACCACATGAAGC
Rev_mirr_screen_HDR_GFP	GCTTCATGTGGTCGGGATAACG
Gapdh1-qPCR-F	GACTCGACTCACGGTCGTTT
Gapdh1-qPCR-R	CACCACATACTCGGCTCCAG
Pdm3-qPCR-F	TCCATCGTCCAGAAGCGAAC
Pdm3-qPCR-R	TCCAGATCAATGCCGTCCAC
Acj6-qPCR-F	GATTCGAGAGCCTGACCCTG
Acj6-qPCR-R	TTCTTTTCTTTTCGCCCGCC
Or49b-qPCR-F	TCACCTTCGACGTTCCACTG
Or49b-qPCR-R	TGGTCCTTGCTCGTTCATCC
Or85e-qPCR-F	TTCTGCACTGCTCACAGGAG
Or85e-qPCR-R	GACTGAGGAGTTCGCCACAA
Gr21a-qPCR-F	GATCCGCTGGAACTGGACAA
Gr21a-qPCR-R	ACAACAATGGTCGTCTGGCA

3.2.4. Antibodies

Table 3.5. Antibody list used in this study

Name	Antigen	Species	Dilution	Source
Primary Antibodies				
Elav	Elav	Rat	1:20	Hybridoma
GFP	GFP	Rabbit	1:500	Invitrogen
Secondary Antibodies				
Alexa 488	Rabbit	Goat	1:800	Invitrogen
Alexa 637	Rat	Goat	1:800	Invitrogen

3.2.5. Embedding Media

Vectashield embedding medium (Vector Laboratories, Inc.) was used in immunohistochemistry experiments. For further visualization, embedded tissues were kept at 4ºC.

3.2.6. Disposable Labware

Table 3.6. Disposable labware used in this study

Material	**Manufacturer**
Micropipette Tips	Greiner Bio-One, Belgium
Microscope cover glass	Fisher Scientific, UK
Microscope slides	Fisher Scientific, UK
PCR tubes (200 µl)	Bio-Rad, USA
Material	Manufacturer
Pipette Tips (10 - 200 - 1000 µl)	VWR, USA
Plastic Pasteur pipettes	TPP Techno Plastic Products AG, Switzerland
Syringe (1cc)	Becton, Dickinson and Company, USA
Test Tubes, (0.5 - 1 - 1,5 - 2 ml)	Citotest Labware Manufacturing, China
Test Tubes, (15 - 50) ml	Becton, Dickinson and Company, USA
Culture tubes (14 ml)	Greiner Bio-One, Belgium
Filter Tips	Greiner Bio-One, Belgium
Petri Dishes, 60 x 15 mm	TPP Techno Plastic Products AG, Switzerland

3.2.7. Equipment

Table 3.7. Equipment used in this study

Equipment	Manufacturer
Autoclave	Astell Scientific Ltd., UK
Centrifuges	Eppendorf, Germany (Centrifuge 5424, 5417R)
Cold Room	Birikim Elektrik Soğutma
Electrophoresis Equipment	Bio-Rad Labs, USA
Freezers	Arçelik, Turkey
Gel Documentation System	Bio-Rad Labs, USA (Gel Doc XR)
Heating Block	Fisher Scientific, France
Heating Magnetic Stirrer	IKA, China (RCT Basic)
Incubator	Weiss Gallenkamp, USA (Incubator Plus Series)
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)
Vortes Mixer	Scientific Industries, USA (Vortex Genie2)
Microscope cover glass	Fisher Scientific, UK
Microscope slides	Fisher Scientific, UK
Confocal Microscope	Leica Microsystems, USA (TCS SP5)

3.2.8. Enzymes

Restriction enzymes and buffers were used from New England Biolabs or Promega. OneTaq, Q5 polymerases and polymerase buffers, PNK, T4 DNA ligase and T4 ligase buffer were used from New England Biolabs.

3.3. Molecular Biological Techniques

3.3.1. Isolation of plasmid DNA

3.3.1.1. Small Scale Plasmid DNA Isolation. For miniprep Thermo-Scientific GeneJET Plasmid Miniprep Kit was used. Single colony from a plate was chosen to inoculate in 2-4ml LB media and incubated for 16 hours at 37ºC while shaking at 250rpm. Bacterial cultures were harvested in 2ml tubes at 6800 x g in a microcentrifuge for 2min at room temperature and the medium was discarded. After this, all steps were performed at room temperature and centrifugation steps were carried out in a microcentrifuge with max rpm (≥ 12000 x g). Pellets were resuspended in 250µl of Resuspension Solution that includes RNase-A. Then 250µl of Lysis Solution was added. Tubes were inverted 4-6 times and incubated for 5 min. 350µl of Neutralization Solution was added to the tubes and inverted 4-6 times immediately and centrifuged for 5 min. To bind the DNA, supernatants containing the plasmid DNA were transferred to GeneJET spin columns and centrifuged for 1 min. The flow-through was discarded and 500 µl of Wash Solution was added to the columns and centrifuged for 1 min. The washing step was performed twice. Then the columns were placed into a clean 1.5 ml tube and 50 µl Elution Buffer was added carefully to the center of the column membrane. After incubation for 2 min and centrifugation for 2 min, the flow-through containing the plasmid was collected and stored at -20°C for further usage.

3.3.1.2. Medium Scale Plasmid DNA Isolation. QIAGEN Plasmid Plus Midi Kit was used for medium scale plasmid DNA isolation. Cultured bacteria in 50ml LB were harvested by centrifugation at 6000 x g for 15min at 4°C. The supernatant was removed and the pellet was resuspended in 4 ml Buffer P1 containing RNase-A. 4ml Buffer P2 was added and the sample was inverted 6-7 times at room temperature for 5 min. 4ml pre-chilled Buffer P3 was added, mixed thoroughly immediately by vigorously inverting 4–6 times. Sample was centrifuged at ≥20,000 x g for 30 min at 4°C while equilibrating a QIAGEN-tip with 4ml Buffer QBT by gravity flow. The supernatant was taken carefully and applied to the equilibrated QIAGEN-tip. After all supernatant entered the resin by gravity flow, 10ml Buffer QC was added and allowed to move through the QIAGEN-tip by gravity flow for two times. The DNA was eluted from the resin using 5ml Buffer QF into a 15ml falcon tube and precipitated by adding 3.5ml isopropanol, mixed carefully and centrifuged at ≥15,000 x g for 30 min at 4°C. The supernatant was carefully decanted. The pellet was washed with 2ml 70% ethanol at room temperature and centrifuged at ≥15,000 x g for 10 min. The ethanol was decanted and the pellet was left to air-dry for 5-10 min. DNA was eluted with an appropriate volume (≥100 µl) of Elution Buffer or double distilled water.

3.3.2. Isolation of RNA

From adult flies, 140 antennae and 140 maxillary palp were dissected on CO_2 fly pads and directly collected in 300µl Trizol reagent. Collected tissues were mechanically homogenized using hand held homogenizer and incubated at RT for 5 min. 60 µl chloroform was added to sample tube then shaken vigorously by hand for 15 seconds and incubated at RT for 3 min. Sample was centrifuged at 12000 x g at 4ºC for 15 min. The aqueous phase was transferred into new tube. Little amount of RNA could be isolated from these tissues due to their smallness. To visualize the RNA pellet, 10 µg of RNase free glycogen was added to the tube. 200 µl isopropanol was added to the tube and incubated at RT for 10 min. Sample was centrifuged at 12000 x g at 4ºC for 10 min. Supernatant was discarded and pellet was washed with 400 µl 75% EtOH. After centrifugation at 7500 x g at 4ºC for 5 min, EtOH was discarded

and the pellet was left to air-dry for 5 minutes. Air-dried pellet was dissolved in 30 µl of DEPC-treated dH_2O and stored in -80°C freezer.

3.3.3. cDNA Synthesis

cDNA synthesis kit (Invitrogen) was used according to the manufacturer's suggestions to synthesize cDNA from isolated RNA. 500ng isolated RNA, 1 µl 10mM dNTP mix and 1 µl 500 µg/ml oligo(dT) primer in a total volume of 10 µl was incubated at 65ºC for 5 min then chilled on ice at least 1 min. In the mean-time a reaction mix was prepared with 2 µl 10x reverse transcriptase buffer, 4 µl 25mM $MgCl_2$, 2 µl 0.1M DTT, and 1 µl 40U RNaseOUT. Prepared reaction mix and sample mix were mixed gently and incubated at 42ºC for 2 min. Then, 1 µl of reverse transcriptase was added and the sample was incubated at 42ºC for 50 min, at 70ºC for 15 min and chilled on ice. Finally, 1µl RNaseH was added, incubated at 37ºC for 20 min and stored at -20ºC.

3.3.4. Genomic DNA Extraction from A Single Fly

A single fly of desired genotype was placed in a 200 µl PCR tube and frozen at -20ºC until ready to process. For each tube 50 µl Squish Buffer with freshly added 0.5 µl Proteinase K (20mg/mL) was prepared. This prepared buffer was pipetted into a yellow tip. With the same tip, the fly was mechanically homogenized then buffer was ejected. Sample was mixed slowly using pipette, then incubated at 37ºC for 30 min and 95ºC for 2 min in a thermocycler. Sample centrifuged for 7 min at max rpm in microcentrifuge, supernatant was collected in a clean tube. Extracted DNA was stored at +4ºC or -20ºC for later use.

3.3.5. Restriction Digestion of DNA

DNA digestion was performed according to the manufacturer's suggestions. For analytical digestion 2.5 µl (final concentration 1X) of appropriate buffer, 10U restriction enzyme, 500ng DNA sample were used in a 25ul total volume. Mixture was incubated at 37ºC for at least an hour.

3.3.6. Transformation

Competent bacteria (*Escherichia coli TOP10 or DH5α)* were prepared by chemical treatment for chemical transformation and stored at -80ºC. 50 µl competent bacteria were thawed on ice. 10-100ng DNA was added slowly to thawed bacteria and incubated on ice for 30 min. Heat-shock at 42ºC was applied to bacteria for 45sec and put back on ice for 5 min. 750µl LB medium was added next to a Bunsen Burner, mixed gently and incubated at 37ºC for 1 h while shaking at 250rpm. 100 µl of the mixture was spread on an LB agar plate containing the appropriate antibiotic and incubated overnight at 37ºC.

3.3.7. Amplification of DNA by PCR

3.3.7.1. Conventional PCR. For mutant fly screening home-made Taq polymerase was used. Each reaction was performed in 25 µl volume which contains 1xTaq buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTP, 10 µM forward primer, 10 µM reverse primer ~100 ng of template genomic DNA, and 5 U of Taq polymerase. The reaction conditions were 3 min at 95ºC, (30sec at 95ºC, 30sec at 50-60ºC, 1min at 68ºC) x 30 cycles, 10min at 68ºC. PCR products were run on 1% agarose gel. If products were shorter than 500bp, 2-4% agarose gel was used.

3.3.7.2. Colony PCR. Colony PCR was used to screen a large number of bacterial colonies. A master mix was prepared in a total volume of 25 µl as described in 3.3.7.1. Single colonies were picked from the plate using a pipette tip and resuspended in the prepared PCR master mix. PCR was performed using appropriate reaction conditions and PCR products were run on 1% agarose gel.

3.3.7.3. Reverse Transcription Polymerase Chain Reaction (RT-PCR). To verify the differential expression of genes identified in the RNA-Sequencing analysis (İbrahim İhsan Taşkıran, 2018) RT-PCR was performed. cDNA obtained from antennae and maxillary palps of triple mutants was used as template. 2.5 µl Taq polymerase buffer (10x), 2 µl $MgCl_2$ (25mM), 1 µl cDNA, 0.5 µl forward primer (10 pmol/µl), 0.5 µl reverse primer (10 pmol/µl), 0.5 µl dNTP (10mM), 0.125 µl (5U) Taq polymerase, and 17.875 µl dH_2O was mixed in a

total volume of 25 µl. The reaction conditions were 3 min at 95ºC, (30sec at 95ºC, 30sec at 56ºC, 30sec at 68ºC) x 30 cycles, 10min at 68ºC. PCR products were run on 2% agarose gel.

3.3.7.4. Quantitative Polymerase Chain Reaction (Q-PCR). Differentially expressed genes found by RNA-Sequencing (İbrahim İhsan Taşkıran, 2018) analysis were confirmed using Q-PCR. Obtained cDNAs from mutant and control flies were used as templates. 2 µl cDNA (1:10 diluted), 0.25 µl forward primer (10 µM), 0.25 µl reverse primer (10 µM), 5 µl Master Mix containing SYBR Green, and 2.5 µl dH_2O were mixed together in a final volume of 10 µl. The reaction conditions were 10 min at 95ºC, (15sec at 95°C, 45sec at 57°C) for 30 cycles in SCAN mode. Melting curve was started from 60°C and increased 1°C/sec until 95ºC. For each gene, 3 biological replicates and for each, 3 technical replicates and no template control (NTC) were prepared. Fold change was calculated using ΔΔ Ct values.

3.3.8. Agarose Gel Electrophoresis

1% (w/v) agarose gel was prepared with 1x TAE buffer unless stated otherwise. Agarose was dissolved in 1x TAE in a microwave, and after cooling down 30ng/mL EtBr was added to the mixture in the hood and poured to an appropriate tray with a comb. After solidification, the comb was removed and the tray was placed into a tank. Samples were loaded to wells with loading dye that had a final concentration of 1x. Additionally 1kb or 100bp ladders were loaded to the first well as size markers. The gel was run at 90-120V for 40-60 min and visualized under a transilluminator (Bio-Rad, USA).

3.3.9. Gel Extraction of DNA

DNA fragments were cut out from the agarose gel with a clean scalpel on a UV light box and transferred to a 1.5ml tube. For gel purification High Pure PCR Product Purification Kit (Roche) was used according to the manufacturer's suggestions. 300 µl Binding Buffer was added to tube for each 100 mg of gel. The tube was incubated at 56ºC for 10 min with periodical vortexing. 150 µl isopropanol was added for each 100mg gel slice and vortexed thoroughly. Samples were transferred to filter columns assembled on appropriate tubes and centrifuged for 1 min at max speed in a microcentrifuge. The flow-through was discarded and

500 µl Wash Buffer was added. The samples were centrifuged for 1 min at max speed and the flow-through was discarded. A second wash step was performed with 200 µl of Wash Buffer. After centrifugation the filter tube was transferred to a clean 1.5ml tube and 30 µl Elution Buffer was added to the center of the filter. The tube was incubated for 10 min at room temperature and centrifuged at max speed for 1 min. To increase the yield, the elution step could be performed twice. Purified DNA fragment was stored at -20ºC until further use.

3.3.10. Gibson Assembly

NEB Gibson Assembly Kit was used according to manufacturers' suggestions. For 1-2 fragment cloning, a total of 0.02-0.5 pmol insert and backbone fragments; for 4-6 fragment cloning, a total of 0.2–1.0 pmol were recommended by NEB. 10 µl 2x Gibson Assembly Master Mix were added to DNA fragments and the total volume was adjusted to 20 µl with dH_2O. The samples were incubated at 50ºC for at least an hour. The assembled fragments were stored at -20ºC for subsequent transformation.

3.3.11. Ligation

0.020 pmol Vector DNA, 0.060 pmol insert DNA, 1x T4 Ligation Buffer (NEB), and 1 U of T4 DNA Ligase (NEB) were mixed in a microcentrifuge tube in a total volume of 15 µl. Ligation was performed at 23ºC for 2 h and transformed into *E. coli*.

3.3.12. Sequencing Analysis

DNA samples were sequenced in order to verify the correctness of the constructs. All samples were sequenced at Macrogen Inc. (Korea). Sequences of DNA were analyzed by using MUSCLE (EMBL-EBI) alingment software and ExPASy (SIB) *in silico* translation software.

3.4. Generation of NHEJ Mediated *IroC* Knockouts by CRISPR/Cas9

3.4.1. Selection of gRNA Targets

In order to select appropriate gRNAs an online design tool, CRISPRscan algorithm, was used. A high score for suggested gRNA sequences indicates the presence of less off-target sites and a higher binding efficiency. For the generation of individual mutants of *IroC*, the largest exons closest to the transcription start site, were targeted with two selected gRNAs.

3.4.2. gRNA Cloning to pCFD4 Vector

72bp long primers containing gRNA and homology sequences of the pCFD4 vector were designed. For each gene (*ara, caup, mirr*) PCR was performed, using pCFD4 as a template, and a 600bp insert was obtained. On the other hand, 4µg pCFD4 (6.4 kb) was linearized with the *Bbs*I restriction enzyme to create the backbone for cloning. Inserts and backbone were run on a 1% agarose gel and the desired fragments were excised from the gel. After purification, insert and backbone fragments were assembled using the Gibson Assembly kit (NEB). Gibson Assembly cloning was performed for each gene separately combining the insert and backbone. Then 5 µl of each mixture was transformed into competent cells. Several colonies were selected and plasmids were isolated. Analytical digestion with the restriction enzymes *Sac*II and *Xba*I was performed and positive colonies were sent for sequencing for further verification.

3.4.3. Embryo Injection

Verified constructs were purified in medium scale as described in 3.3.1.2. and sent to Rainbow Transgenic Flies (USA, Inc.) for embryo injection. The pCFD4 vector carries a *vermillion* marker that would change the eye color in a *vermillion* mutant background. Therefore, a phiC31 expressing, *vermillion* mutant fly line was chosen for injection: *y,v; attP40* (Bloomington stock 25709). G0 flies were crossed with a balancer line, *y,v*

;Gla Bc/CyO, and screened for transformant flies. Transformant flies were balanced and stocks were established.

3.4.4. Generation of NHEJ-mediated *ara, caup and mirr* mutants

gRNA expressing transgenic fly lines generated as described in 3.4.3. were crossed to nos-Cas9 transgenic flies that express germline-specific Cas9. Using a germline-specific Cas9 allows for the occurrence of mutations in the germ cells of F1 flies and increases the probability of these mutations to be inherited. Therefore, each F2 is expected to have a unique mutation in the third chromosome where iroC genes are located. Each F2 fly was crossed individually with a w;;TM3/TM6B balanced line. At least 100 single crosses were established for each gene. Putative mutations were screened with PCR using primers Fwd_ara_screen_nhej and Rev_ara_screen_nhej for ara, Fwd_caup_screen_nhej and Rev_caup_screen_nhej for caup, Fwd_mirr_screen_nhej and Rev_mirr_screen_nhej for mirr as described in Table 3.4. and sent for sequencing for further analysis.

3.5. Generation of HDR-Mediated Fluorescently Tagged IroC Proteins by CRISPR/Cas9

HDR-mediated gene editing requires the generation of two vectors: one including a gRNA and one for donor DNA that includes GFP.

3.5.1. Selection of gRNA Targets

An online designing tool, flyCRISPR Target Finder algorithm was used for gRNA selection. For *ara* and *caup* the C terminus was chosen for tagging and single gRNAs were selected. For the *mirr* gene the N terminus was targeted due to the presence of different mRNA variants for *mirr* and the wish for tagging both of the variants. These selected gRNAs were within 100bp distance from the target sites. gRNAs without any off-target were chosen.

3.5.2. Single gRNA Cloning to pCFD5 Vector

Selected gRNAs were ordered as desalted oligos with 4 base addition to their 5' end. For the top oligo TGCA and for the bottom oligo AAAC sequences were added. These overhangs are necessary for the correct ligation. The bottom oligo gRNA sequence was chosen to be reverse complementary to the top oligo. Oligos were resuspended in dH_2O to a concentration of 100 µM. To anneal and phosphorylate these oligos 1 µl top oligo (100 µM stock), 1 µl bottom oligo (100 µM stock), 1 µl 10x T4 Ligation Buffer (NEB), 6.5 µl ddH_2O and 0.5 µl (5U) T4 Polynucleotide Kinase (NEB) were mixed into a 200 µl PCR tube. The mixture was incubated in a thermocycler at 37°C for 30 min, 95°C for 5 min and ramped down to 20°C at 5°C/min. 4µg pCFD5 was digested with *Bbs*I restriction enzyme and the linearized band was isolated from the agarose gel. Ligation was performed using X µl *Bbs*I-digested pCFD5 (60 ng), 1 µl annealed oligos diluted 1:200 in ddH_2O, 1x T4 Ligation Buffer (NEB), 1 µl (10U) T4 DNA Ligase (NEB) and adjusted with ddH_2O to a total volume of 15 µl. The reaction was incubated at 23°C for at least one hour. 2 µl of the ligation reaction were transformed into 50 µl competent cells and plates were incubated at 37°C overnight. Plasmids from positive colonies were isolated and sequenced for further verification.

3.5.3. Donor Template DNA Preparation

Homology arms were selected 1kb upstream and downstream of the gRNA target sites. Commercially synthesized G-blocks including GFP were intended to be knocked-in with the help of homology arms. pGEM-T Easy plasmid was selected as backbone and digested with *Nco*I and *Spe*I restriction enzymes. First upstream and downstream homology arms were amplified by PCR separately for each gene. Upstream homology arm forward primers had 30 overlapping sequences with the digested pGEM-T Easy backbone. Upstream homology arm reverse primers and downstream forward primers had 30 bp overlap with the G-blocks. All downstream reverse primers had 30 bp overlapping sequence with the digested pGEM-T Easy vector. Once all fragments were ready they were joined by with Overlap Extension PCR and Recombination (OEPR) method (Liu *et al.*, 2017). Upstream homology arms were used as forward primers. 5µl from this reaction was directly transformed into competent cells. After

analytical digestion with *Nco*I, plasmids that had correct band sizes were sequenced for verification. Verified plasmids were purified using medium scale preparation as described in 3.3.1.2. before injection into fly embryos.

3.5.4. Embryo Injection

Injection was performed at GenetiVision (USA, Inc.). gRNA-pCFD5 and donor template DNA constructs were co-injected to 240 nos-Cas9 transgenic fly embryos. G0 flies were crossed to yw; sp/CyO; TM/TM6B balanced line. Flies in the F1 generation are putative carriers of the GFP knock-in through their target sites. Therefore, flies from the F1 generation were crossed individually to the balanced line.

3.5.5. Screening of Fluorescently Tagged iroC

IroC is known to be expressed in the dorsal part of the eye. Thus, each F2 line analyzed under a fluorescent stereomicroscope for GFP expression. Knock-in for *mirr* was also verified with PCR using primers Fwd_mirr_screen_HDR and Rev_mirr_screen_HDR_GFP, Fwd_mirr_screen_HDR_GFP and Rev_mirr_screen_HDR.

3.6. Histological Techniques

3.6.1. Immunohistochemistry

Eye-antennal imaginal disc, wing disc, leg disc and brain from larvae were dissected in 1x PBS on dissection pad. Dissected samples were collected in a tube that contained 1x PBS on ice then fixed in 4% PFA for 30 minutes at RT. Then, samples washed 3 times with PBX3 for 15 min at RT and incubated overnight with primary antibodies diluted in PAXD at 4ºC. Samples were washed 3 times with PBX3 for 15 min at RT and incubated with secondary antibody at RT for 2 hours. Samples were washed 3 times with PBX3 for 15 minutes. Final dissection was performed if it was necessary and mounted on a slide using Vectashield. Mounted samples were observed under confocal microscope. All washing and incubation steps were performed on a shaker.

4. RESULTS

4.1. Available Fly Lines to Study *IroC*

IroC is a transcription factor family that includes *ara*, *caup* and *mirr* genes and they involved in the development of various organs (Bilioni *et al*., 2005; F Cavodeassi *et al*., 2001). To study their roles, triple null mutant of iroC, iro^{DFM3}, line was used generally. In iro^{DFM3}, *ara* and *caup* and the regulatory region of *mirr* are deleted. To determine expression pattern of iroC, *iroC*-Gal4 and DE-Gal4 lines had been used. IroC-Gal4 was obtained by the replacement of P[lacZ] enhancer trap element (iro^{rF209}) with P[Gal4] element (Mazzoni *et al*., 2008), which is believed to give the expression pattern of *ara* and *caup* (Figure 4.1) (Gomez-Skarmeta *et al*., 1996; Ikmi *et al*., 2008). DE-Gal4 is believed to reflect mirr expression pattern that was obtained by replacement of P[lacZ] enhancer trap element ($mirr^{DE}$) with P[Gal4] element (Morrison and Halder, 2010) (Figure 4.1.).

In our lab *iroC* is shown to be expressed in olfactory organs and using iro^{DFM3} line RNA Sequencing was performed and differentially expressed genes were identified (Mustafa Talay, 2011; İbrahim İhsan Taşkıran, 2018). However, antibodies against ara, caup and mirror do not work in the olfactory system and *in situ* hybridization did not work probably due to their low-level expression. In addition, there are no commercially available individual mutant flies and RNAi does not lead to 100% downregulation. Therefore, in this study using CRISPR/Cas9, I aimed to generate individual knock-outs of iroC genes and generate fluorescently-tagged iro proteins to identify their endogenous expression patterns.

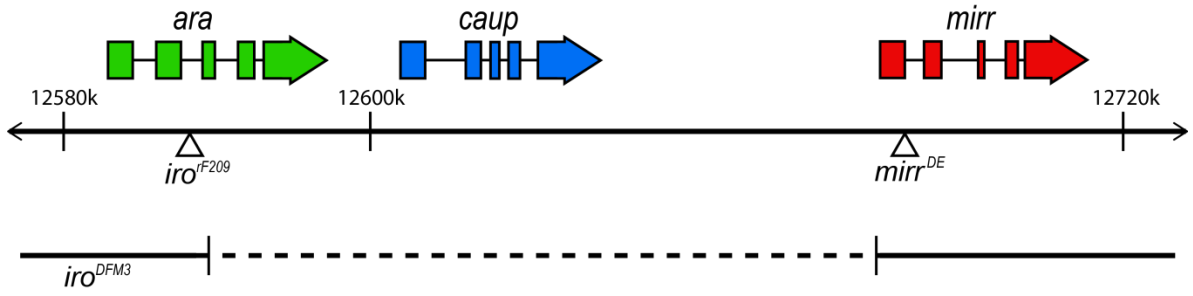

Figure 4.1. Representation of the *IroC* Locus. In terms of genomic location, *ara* and *caup* are closer and *mirr* is more divergent. Triangles indicate P-element insertion sites for iro^{rF209} and $mirr^{DE}$ that are replaced by Gal4. iro^{DFM3} is shown in the bottom. Dashed line indicates deletion of *ara*, *caup* and regulatory region of *mirr*.

4.2. Generation of *ara, caup* and *mirr* mutants by CRISPR/Cas

Individual functions of iroC members could be determined using the generated mutants. Also, we would like to compare individual mutant RNA-seq results with the results from the triple mutant. To accomplish this, our plan was to induce NHEJ repair pathway in flies and create random mutations in DNA. For this reason, gRNAs were chosen, cloned into gRNA expression vector and integrated into the fly genome. Only gRNA expressing flies were generated to prevent any further experimental problems that would cause fly loss. Generated flies were crossed with nos-Cas9 flies. Offsprings of the gRNA and Cas9 expressing flies were putative mutants for our gene of interests.

4.2.1. Preparation of gRNA Constructs

In order to be able to study the function of individual iroC genes we aimed to generate individual mutants for each gene in the iro complex using CRISPR/Cas. The strategy was to create frameshift mutations in the coding regions and deplete the function of iroC proteins. *ara*, *caup* and *mirr* have 5 exons, however, first exons of iroC genes have very short sequences (51bp, 51bp and 42bp respectively). Thus, they are not very suitable to select

gRNA target sites. Therefore, their second exons were analyzed using the CRISPRscan gRNA selecting tool (https://crisprscan.org/) and two gRNAs were chosen for each gene (Table 4.1; Figure 4.2). The selected sequences were followed by a PAM sequence (NGG), which is necessary for gRNA recognition. For each gene the selected gRNAs were separated by 25bp for *ara*, 65bp for *caup* and 22bp for *mirr* and they do not have any predicted off-target (Table 4.1.). Using two gRNA, the aim was to generate larger deletions and to increase the chance of inducing a frameshift mutation.

Table 4.1. Selected gRNAs for each gene, their locus in the genome and off-targets.

Gene of Interest	**Target Locus**	**Selected gRNA**	**Off-targets**
ara	3L:12585948-12585971 (+)	GGGACGGTGGTTGGAGCAGG	None
	3L:12585993-12586016 (+)	GGGCCAGCGGATCTGGCCAC	None
caup	3L:12616902-12616925 (-)	GGGACCGGCAGTCAGGCCGG	None
	3L:12616820-12616843 (+)	GGTAGCCAGAGCGGCCATGG	None
mirr	3L:12696524-12696547 (-)	GGGAGGCGTGTTGGGCGACG	None
	3L:12696565-12696588 (-)	GGCGTGGGCAGCGTCGAGGG	None

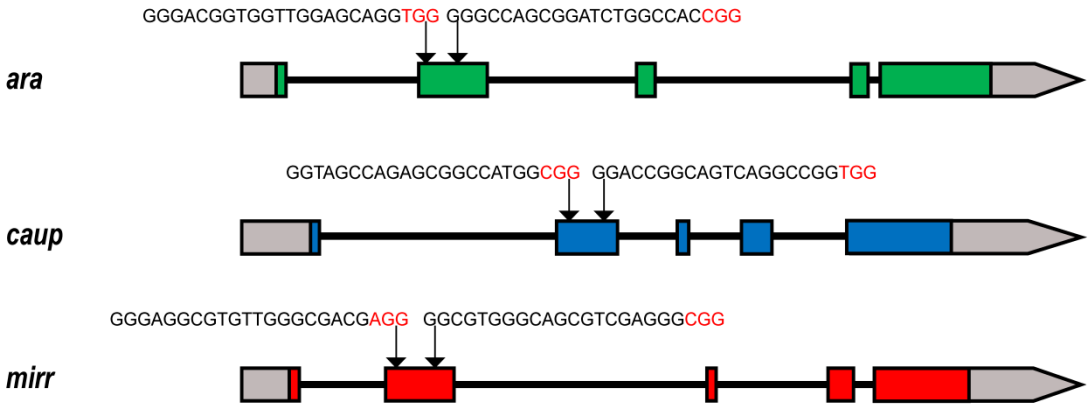

Figure 4.2. Schematic drawing indicating gene structures and *ara*, *caup* and *mirr* gRNA targeting sites. For *ara* green boxes, for *caup* blue boxes and for *mirr* red boxes correspond to exons and grey boxes correspond to UTRs. gRNA target regions are shown by arrows and red sequences indicate the PAM sites.

gRNAs were cloned into the pCFD4 vector. pCFD4 is a tandem expression vector, which means that it has two gRNA cores and two U6 promoters that enable expression of two different gRNAs at the same time (Figure 4.3A). Primers were chosen as shown in Figure 4.3B. The forward primer includes the first gRNA and the reverse primer includes the second gRNA. In addition, each primer has homology sequences that allow restriction enzyme-free cloning.

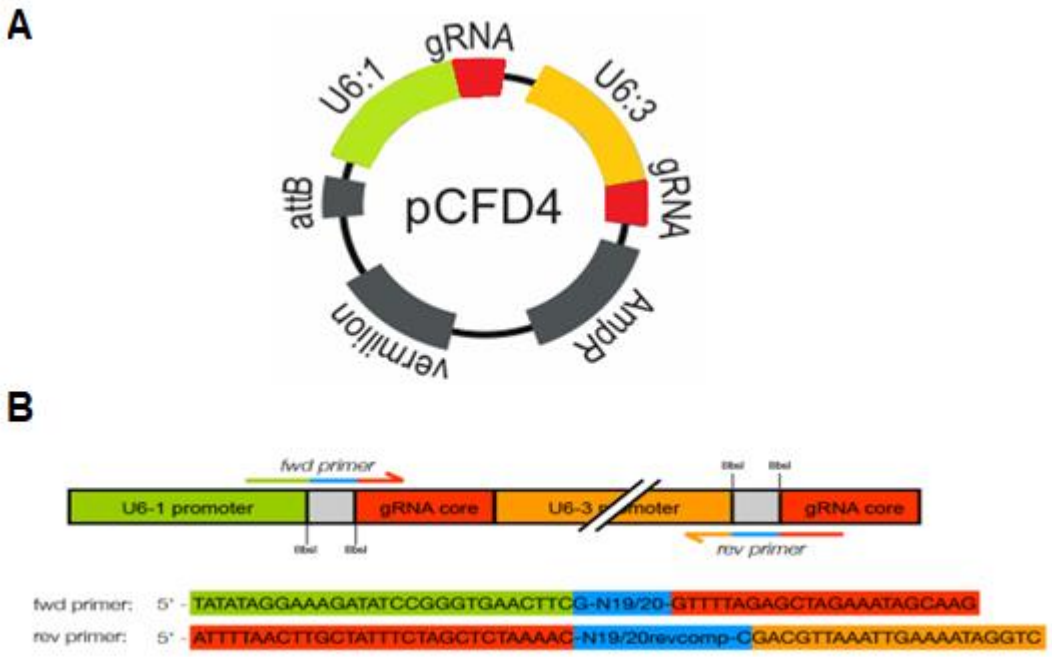

Figure 4.3. Schematic view of ligation independent pCFD4 vector with two U6 promoter. A) U6 promoters are shown in green and orange while the two gRNA cores are shown in red. B) Green, red and orange-colored sequences indicate the homology regions found in primers. Blue sequences correspond to selected gRNAs.

A 600bp insert was amplified using pCFD4 as a template using the primers including the gRNAs (Figure 4.4A). In parallel, 6.4kb pCFD4 vector was prepared by *Bbs*I digestion (Figure 4.4B). The inserts and vector was gel-purified and the isolated fragments were assembled using Gibson assembly. One, 11 and 10 colonies were selected for *ara*, *caup* and *mirr*, respectively, and were verified by analytical double digestion with *Xba*I and *Sac*II. The

expected fragment of 2.5kb and 5.5kb was obtained for *ara*, *caup* and *mirr*, respectively (Figure 4.4C,D,E). The positive colonies were verified by sequencing.

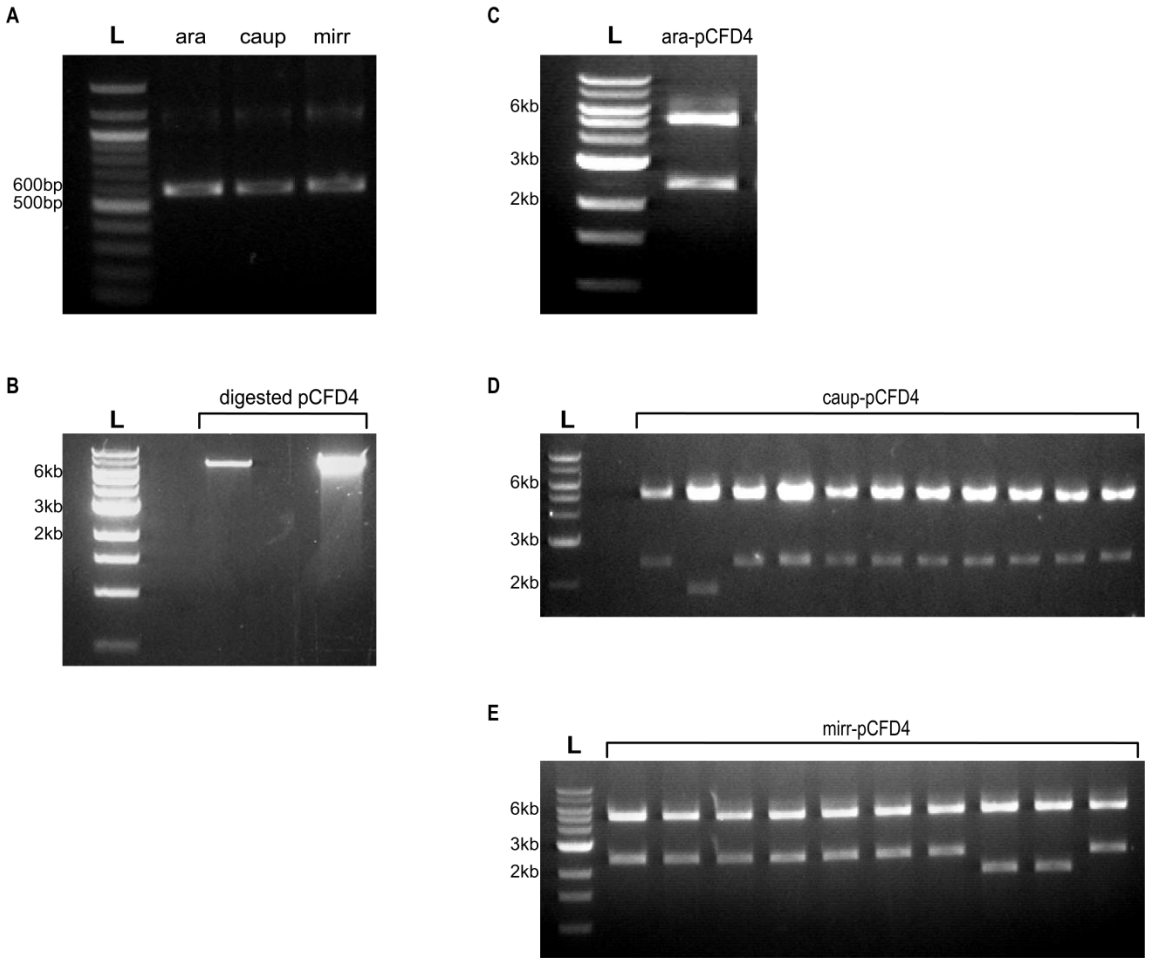

Figure 4.4. Preparation and cloning of insert and backbone using Gibson assembly. (A,B) PCR products of gRNA inserts for *ara, caup* and *mirr* were 600bp as expected and digested pCFD4 was obtained as expected 6.4kb band. (C,D,E) After analytical digestion expected band sizes 2.5kb and 5.5kb were observed, positive colonies were observed for *ara, caup* and *mirr* respectively.

4.2.2. Generation and Screening of Mutant Lines

The successfully generated gRNA constructs were prepared in large scale for embryo injection. The pCFD4 plasmid contains *vermillon* as a selectable marker. Flies mutant for vermillion have bright red eyes. The injection of a plasmid carrying the *vermillion* gene would rescue the mutant phenotype and turn their eyes dark red and allow for selection of recombinant flies (Figure 4.5). Thus, flies with a *vermillion* mutant background were chosen for injection. In addition, the pCFD4 plasmid also contains an attachment site, which allows for its targeted insertion into the fly genome that has attachment sites engineered on different chromosomes (Port *et al*., 2014). The target genes *ara*, *caup* and *mirr* are located on the third chromosome. To allow for the selection of the mutants and the crossing out of the gRNA plasmid and the Cas9 source that is present on the X chromosome, the plasmids were targeted to the second chromosome for insertion. In particular, we chose the fly line yv;attP40 (Bloomington 25709).

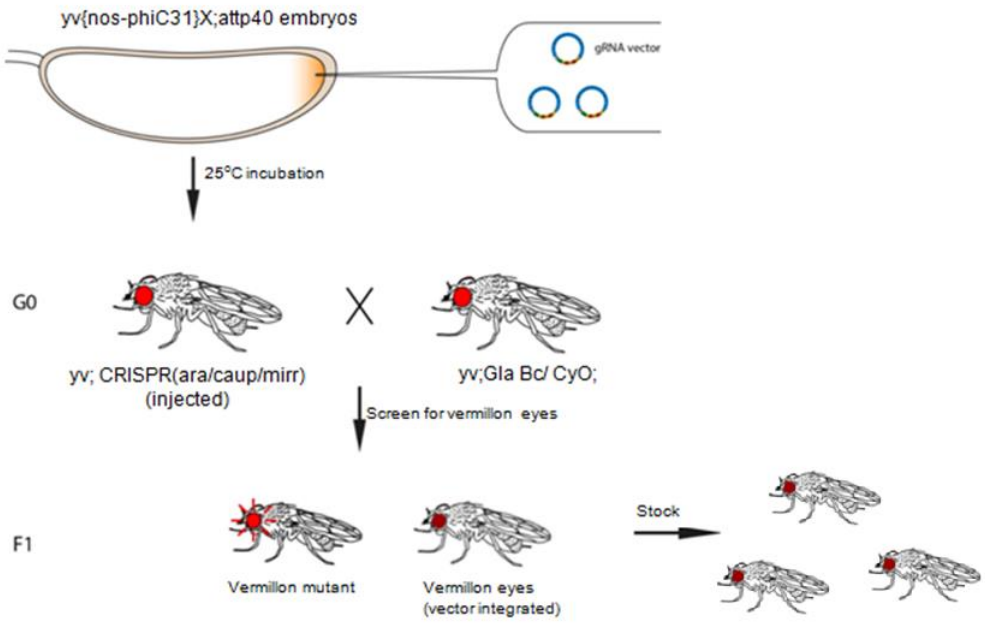

Figure 4.5. Schematic view of embryo injection and recombinant fly selection. *Vermillion* mutant embryos were chosen for gRNA-pCFD4 construct injection. Injected flies were crossed with *vermillion* mutant flies for recombinant fly selection by looking at the eye phenotype.

240 embryos were injected for each construct. Table 4.2. summarizes the number of obtained larvae after injection and how many transformant lines were obtained.

Table 4.2. Numbers of hatched larvae, eclosed male and female flies, and generated transformant lines are shown.

Sample Name	**Larvae**	**Male**	**Female**	**Cross**	**Sterile**	**Transformant**
ara-gRNA-pCFD4	110	41	38	51	13	8
caup-gRNA-pCFD4	135	44	42	53	19	7
mirr-gRNA-pCFD4	170	42	45	51	15	7

To generate the individual mutants one of the transformant lines was selected for each gene. Transformant flies carrying the gRNA-pCFD4 constructs were crossed with *nos*-Cas9 flies. *nos* (*nanos*) is a germline-specific promoter that will allow the expression of Cas9 only in germ cells increasing the probability of germline transformation (Port *et al*., 2014). Flies carrying both constructs were crossed to balancer lines to remove the *nos*-Cas9 and gRNA carrying alleles. Since every fly generated from this cross was potentially a mutant, each one had to be tested for indel mutations. To screen for mutations, primers flanking the gRNA target site were designed and used in single fly genomic PCR reactions (Figure 4.6).

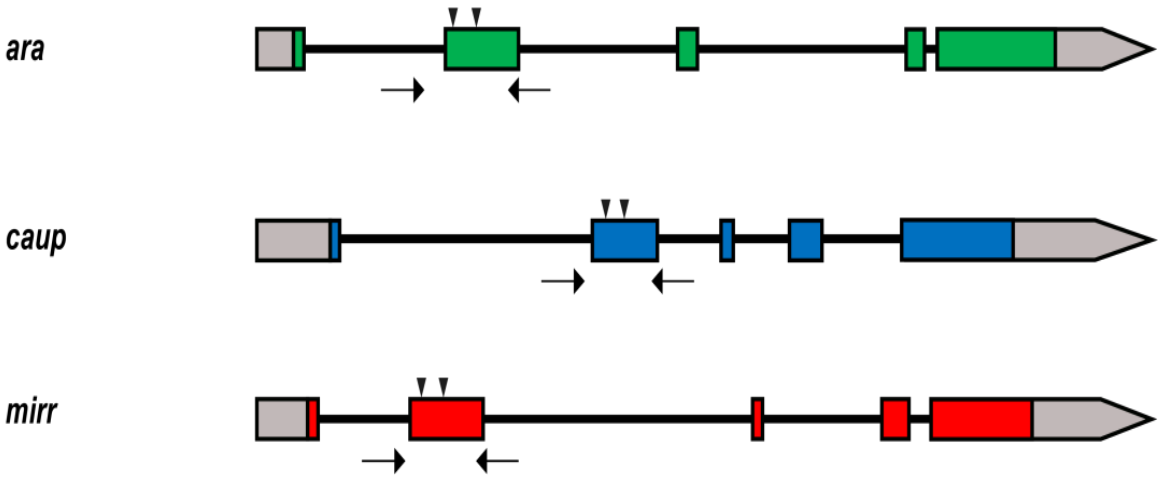

Figure 4.6. Mutant screening primers. Arrowheads show the gRNA target sites and arrows show the primers that flank the gRNA target sites.

For *ara* 300 single crosses, for *caup* 100 single crosses and for *mirr* 300 single crosses were screened. Because two gRNAs were used, large deletions that could be observed on an agarose gel were expected. In wild type flies expected band sizes for *ara*, *caup* and *mirr* were 245bp, 207bp and 225bp, respectively. Genomic DNA was extracted from heterozygous flies, thus, two bands were expected on the gel one for the wild type and one for the mutant allele. 4% agarose gels were prepared to separate the expected small bands more efficiently. Only one mutant could be obtained for *ara*, line 64 (Figure 4.7A). 5 mutants were obtained for *caup* line 7, 10, 14, 17 and 24 (Figure 4.7B). Unfortunately, not a single mutant was found for *mirr* out of 300 single crosses (Figure 4.7C).

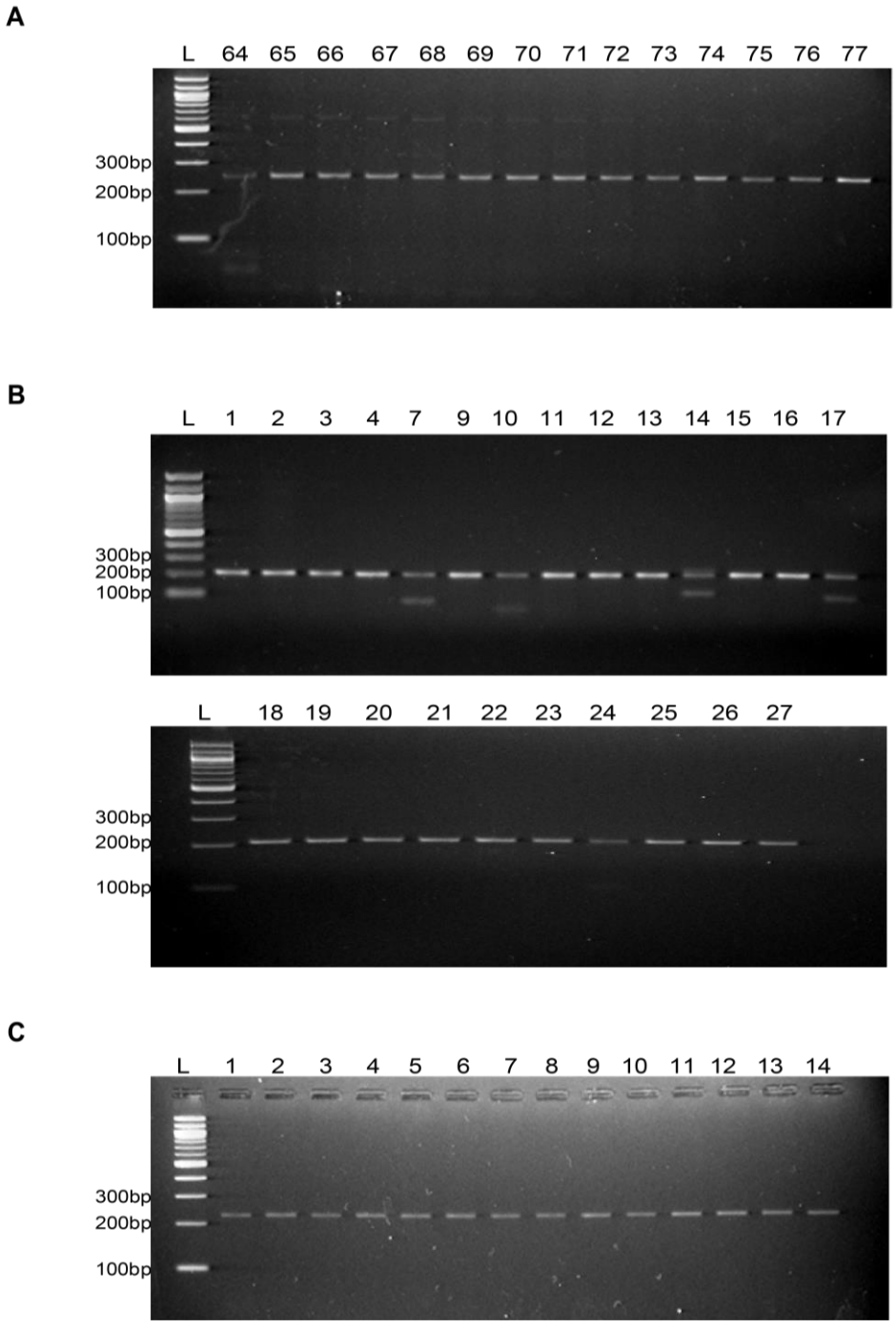

Figure 4.7. Screening for the mutant flies by PCR. A) 300 cross for *ara* was screened, one mutant (64) was found. B) Mutant bands were observed for heterozygous flies that are found in vials 7, 10, 14, 17 and 24. C) For *mirr*, no mutant bands could be identified by PCR.

4.2.3. Characterization of Mutant Lines

Sequence alignments were performed by using the Multiple Alignment tool MUSCLE and *in silico* translation of proteins were performed using the online translation tool ExPASy. A total of 300 flies were screened and 1 mutant was found for *ara*. As a result of sequencing, a 54 bp deletion was observed in ara^{64} on the DNA level (Figure 4.8A). However, this deletion does not cause a frameshift at the protein level (Figure 4.8B). The length of the new protein formed was shortened to 691aa only effecting the first exon and not any domains of protein. Unfortunately, the presence of the protein could not be confirmed by Western blot analysis, since we do not have an antibody against ara.

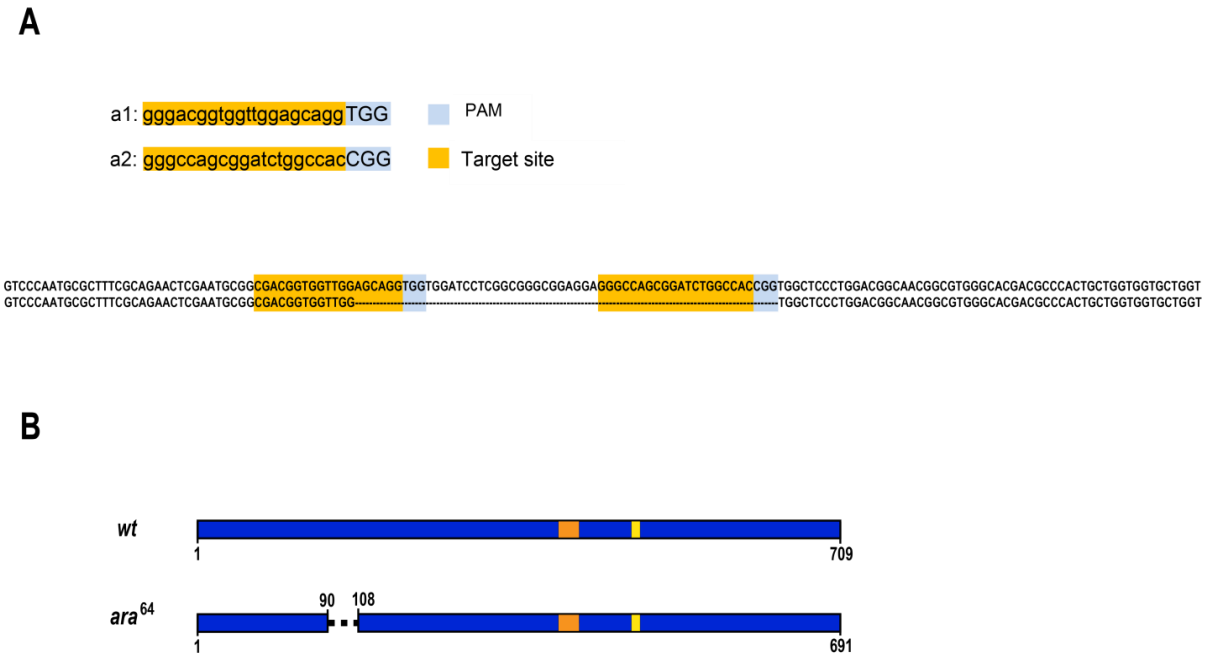

Figure 4.8. Representation of CRISPR / Cas9-generated deletions in the *ara* gene. A) Investigation of gRNA target sequences and sequencing of mutations. The 20-base gRNA sequences for a1 and a2 correspond to the orange-labeled sequences next to the blue-labeled NGG (PAM). The top sequence represents wild type and the bottom sequence represents the mutant ara^{64} line. B) Representation of the deletions at the protein level.

The 5 fly lines detected for *caup* were also sequenced and further characterized. The sequencing results showed 126 bases deletion in *caup*^{7}, 156 bases in *caup*^{10}, 90 bases in *caup*^{14}, 106 bases in *caup*^{17} and *caup*^{24} (Figure 4.9A). Both of the gRNAs for *caup* worked efficiently. At the protein level, these deletions do not cause a frameshift for *caup*^{10}. For *caup*^{14}, the 55th and 56th amino acids differ with the wild type DNA sequence, but other than that, there is no frameshift. *Caup*^{7}, *caup*^{17} and *caup*^{24} mutations cause frameshift mutations and lead to early stop codons. The predicted final size of the proteins is 116 and 143 amino acids, respectively, for *caup*^{7} and *caup*^{17} (Figure 4.9B).

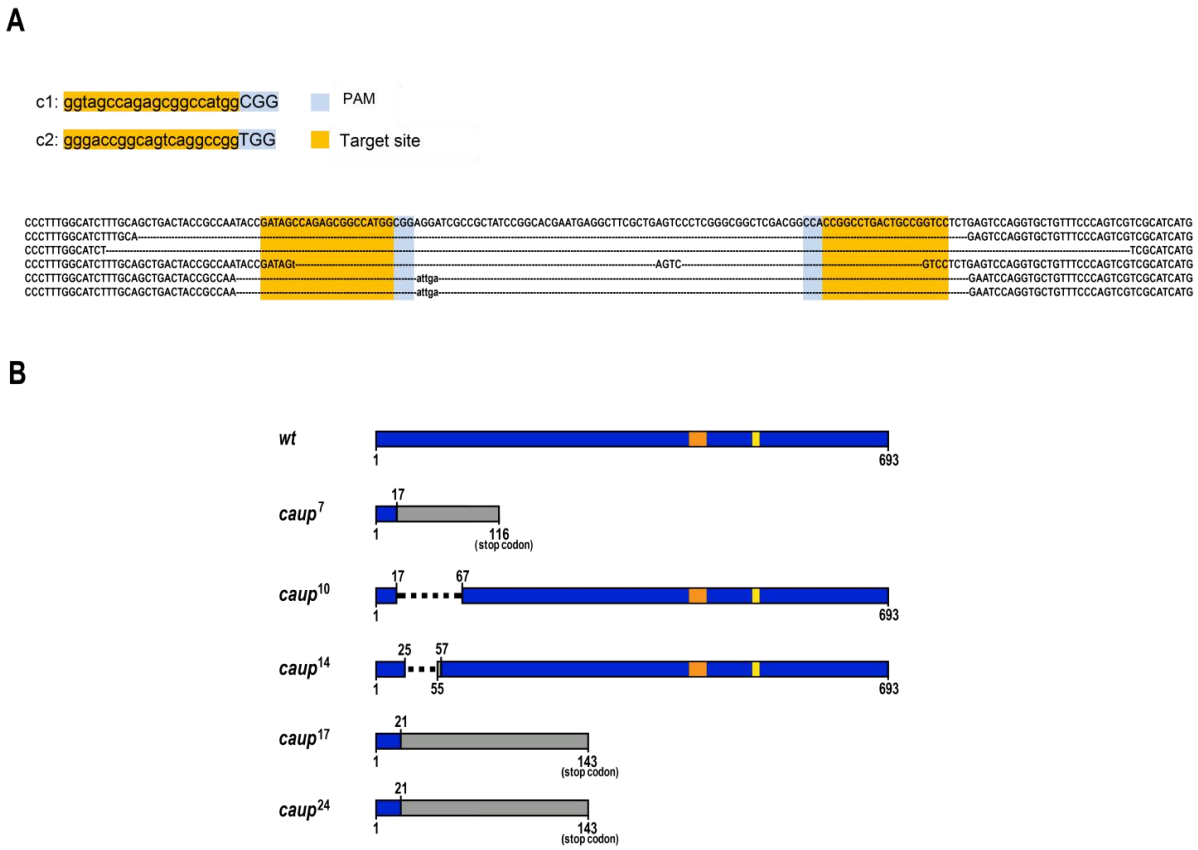

Figure 4.9. Representation of CRISPR / Cas9-generated deletions in the *caup* gene. A) The sequence at the top of represents the wild type sequence and subsequent sequences represent the mutant fly sequences in following order *caup*^{7}, *caup*^{10}, *caup*^{14}, *caup*^{17}, and *caup*^{24}. B) Representation of the deletions in the protein level. Wild type caup encodes a 693 amino acid protein shown in navy blue. Early stop codons were generated in *caup*^{7}, 17 and 24.

While performing these experiments, we expanded our tool search from literature and found out there is a mutant fly line for *mirr* (*mirr*^{e48}) which is not commercially available. Later, found line was kindly shared with our laboratory.

In our lab, RNA-seq analysis was performed by İbrahim İhsan Taşkıran, 2018 using iroC triple mutant line (*IroC*^{DFM3}). This line lacks *ara* and *caup* and the regulatory region of *mirr*. The aim was to create single mutants and perform RNA-seq with those lines. Using generated mutant *caup* lines and obtained *mirr* mutant individual analysis could be performed.

4.3. Generation of Fluorescently Tagged iroC proteins by CRISPR/Cas

To analyze the expression pattern of *iroC*, *iroC*-Gal4 line had been used in our lab. It was obtained by the replacement of P[lacZ] enhancer trap element (*iro*^{rF209}) with P[Gal4] element (Mazzoni *et al*., 2008), which is believed to give the expression pattern of *ara* and *caup*. Since, we do not have the antibodies for *IroC* members, we cannot confirm if this line shows the endogenous expression pattern. To solve this issue, we aimed to generate fusion protein and see the endogenous pattern of *IroC* members separately.

For the addition of fluorescent protein C terminus was chosen for *ara* and *caup* because N terminus was well preserved through the evolutionary process. Since there were three mRNA variants and all of them had a different end of C terminus, a fluorescent protein was added to the N terminus in case of *mirr* (Figure 4.10.). The gRNAs were selected and are shown in Figure 4.11A.

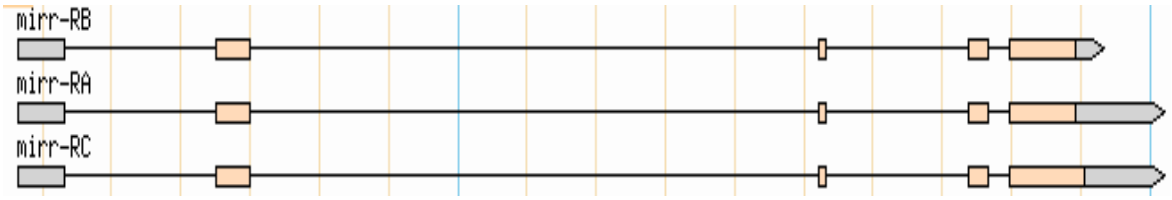

Figure 4.10. *mirr* transcript variants. Their C terminus and 3'UTR sequences are different for each transcript while rest of the sequences are same (Grey boxes indicate UTR and pink boxes indicate exons).

To clone the gRNAs the more advanced and efficient vector pCFD5 was selected. This vector has a U6:3 promoter and a tRNA-based system (Port and Bullock, 2016). gRNAs are followed by tRNAs and freed by endogenous RNases. This system allows the use of more than one gRNA with the same promoter as compared with pCFD4, which uses two different promoters for the two gRNAs cloned. To introduce the GFP, a donor DNA template that has ~1kb homology arms flanking cutting site is necessary.

The ara template contains a 978bp homology arm upstream of the cut site followed by a linker sequence (GGSx4), 3xHA, GFP (711bp) with a stop codon and a 953bp downstream homology arm. The caup template contains 1050bp homology arm upstream of the cut site followed by a linker sequence (GGSx4), 3xV5, GFP (711bp) with a stop codon and a 1011bp downstream homology arm. The mirr template contains a 986bp homology arm upstream of the cut site followed by 3xFLAG with a start codon, GFP (711bp), a linker sequence (GGSx4) and a 969bp downstream homology arm (Figure 4.11B).

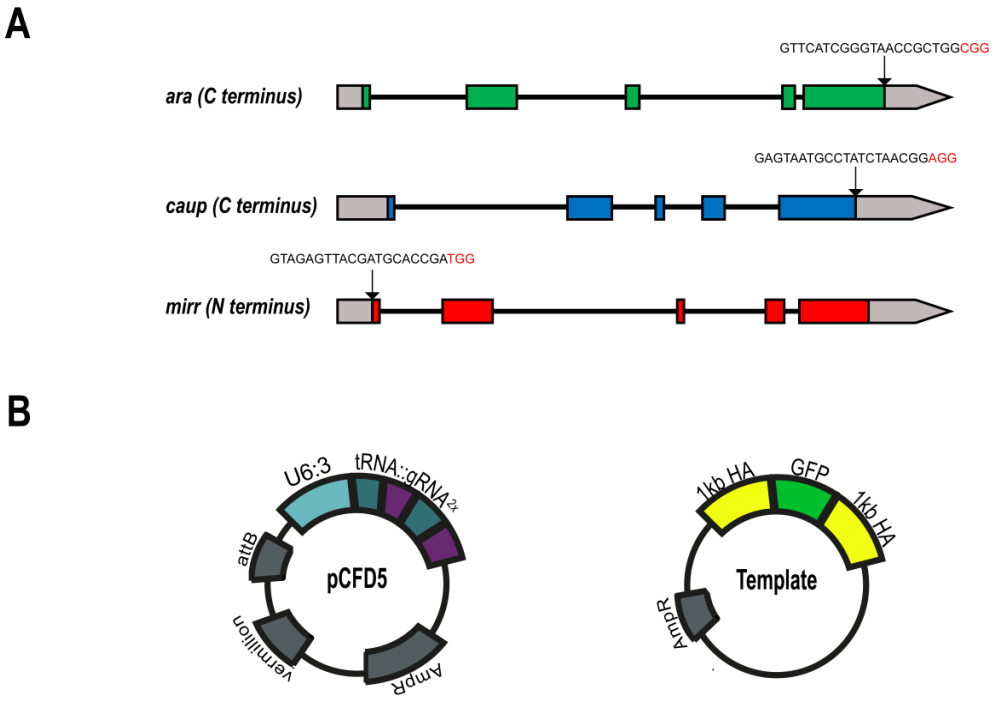

Figure 4.11. Addition of fluorescent protein to iroC genes by CRISPR / Cas9. A) gRNA target sequences were shown for each *IroC* gene. The *ara*, *caup* and *mirr* exon regions were green, blue and red respectively. It is desired to add the fluorescent protein to the C terminus for the ara and caup, the N terminus for the mirr. B) Selected gRNAs were cloned into plasmid pCFD5. The template DNA was obtained by 1kb homology arms and GFP.

In order to be able to compare the localization of the different iroC genes with each other in a pairwise manner (caup-ara, ara-mirr and caup-mirr) in the same tissue, in addition to GFP different tags were added for the three proteins (3xHA+GFP for ara, 3xV5+GFP for caup, 3xFLAG+GFP for mirr). A linker sequence was also designed to prevent that GFP affects iroC protein localization. Linker, GFP and small tag sequences were ordered as G-blocks (Figure 4.12). G-blocks are double stranded, sequence verified synthetic DNA fragments. In addition, altered PAM sites were included to ordered G-blocks to prevent binding of gRNA to template.

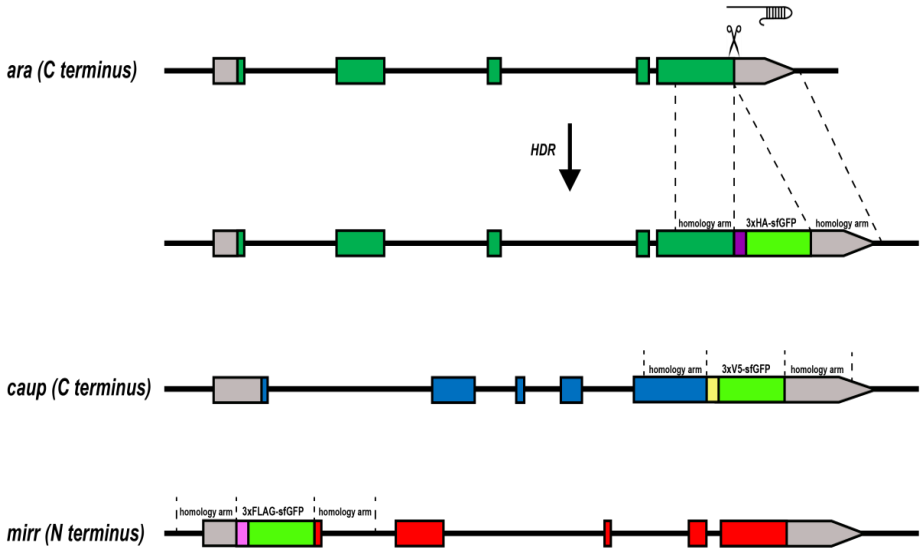

Figure 4.12. Schematic drawing of *IroC* genes after addition of G-blocks by CRISPR/Cas9. In addition to GFP, *ara*, *caup* and *mirr* were also tagged with 3xHA (purple box), 3xV5 (yellow box) and 3xFLAG (pink box), respectively. Dashed lines indicate selected 1kb homology arms.

4.3.1. Preparation of gRNA Constructs

gRNAs were chosen in an area covering 100 bp upstream and 100 bp downstream of the target site (C terminus for ara and caup, N terminus for mirr). The 20bp gRNAs were selected using flyCRISPR Target Finder tool. Chosen gRNAs did not have any predicted off-target (Table 4.3).

Table 4.3. Selected gRNAs for each gene, their locus in genome and off-targets are shown.

Gene of Interest	Target Locus	Selected gRNA	Off-targets
ara	3L:12595501-12595523 (-)	GTTCATCGGGTAACCGCTGG	None
caup	3L:12620659-12620681 (+)	GAGTAATGCCTATCTAACGG	None
mirr	3L:12694246-12694268 (-)	GTAGAGTTACGATGCACCGA	None

The top primer comprises the gRNA sequence and the bottom primer comprises the complementary gRNA sequence. Top and bottom primers were phosphorylated and annealed, then ligated with pCFD5, which was linearized with *Bbs*I restriction enzyme (Figure 4.13A). Due to the short length of the 20bp insert, the success of the ligation could not be assessed on a conventional agarose gel (Figure 4.13B). DNA from selected colonies was isolated and sequenced. As a result, gRNAs were successfully cloned into pCFD5 plasmid for all three genes (Figure 4.13C).

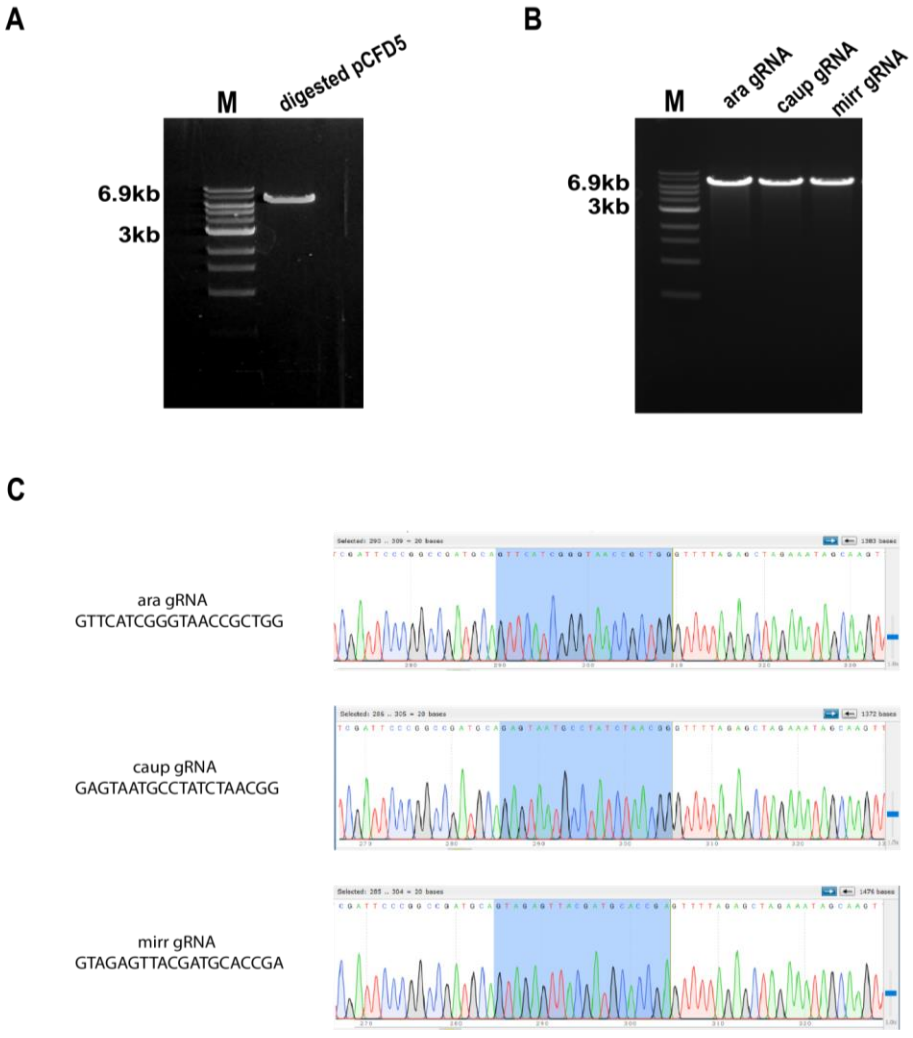

Figure 4.13. Cloning of selected gRNAs for the generation of transgenic lines. (A) pCFD5 was linearized with the *Bbs*I restriction enzyme and displayed the expected 6.9 kb band. (B) The gRNAs were ordered as 20 bp top and bottom primers, annealed and ligated to linearized pCFD5. (C) Sequencing result for all three genes, verifying the successful cloning of gRNAs.

4.3.2. Preparation of Donor DNA Template Constructs

In order to generate the template for HDR 4 different fragments needed to be assembled: the vector backbone, the right homology arm (downstream), the left homology arm (upstream) and the GFP (+ individual tags).

To generate the vector backbone the pGEM-T Easy plasmid was used and digested with *Nco*I and *Spe*I enzymes and 3kb expected band was isolated (Figure 4.14A). To be able to use Gibson Assembly for cloning, primers to amplify the homology arm were designed to include 21bp overlapping sequences with the previous and the next fragment to be joined. For all three genes, using these primers with the overlapping sequences, approximately 1kb right and left homology arms were amplified by PCR. Obtained fragments, backbone, right and left homology arms and G-blocks were combined using the Gibson Assembly method (Figure 4.14B). Very few colonies could be obtained and none of the isolated plasmids had the expected band size 6kb (Figure 4.14C). The procedure was repeated for all three genes, but no positive result was obtained.

To increase Gibson Assembly efficiency, for the Mirr donor plasmid, the overlapping base size was increased to 30 bases, primers were re-ordered and the homology arms were amplified by PCR again approximately 1kb long. Prepared 4 fragments were assembled with Gibson Assembly, but again the expected results could not be obtained (Figure 4.14D,E). Later, overlapping sequence for Mirr template was increased to 60bp. Same procedure repeated. However, again, successful cloning could not be obtained with Gibson (data not shown).

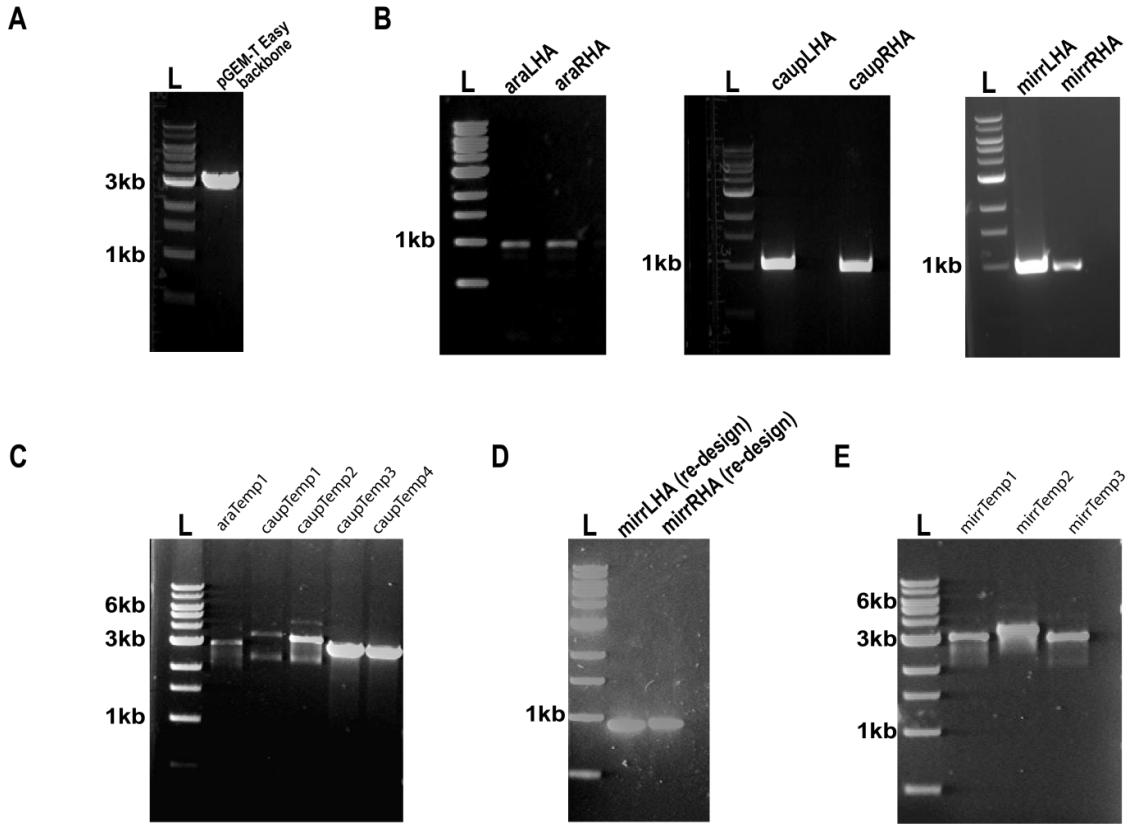

Figure 4.14. Preparation of Donor DNA Plasmid. A) pGEM-T Easy plasmid was digested and 3kb band was isolated. (B) For all three genes, approximately 1kb right and left homology arms were amplified by PCR. (C) Cloning failed with Gibson Assembly. (D) *mirr* primers were re-designed and homology arms were amplified again. (E) Expected bands could not be obtained by Gibson Assembly.

Later, recently published OEPR (Overlap Extension PCR and Recombination *in vivo*) cloning strategy was chosen instead of Gibson Assembly. The same fragments that were used in Gibson Assembly could be employed. In OEPR, all fragments were amplified separately using PCR just like the preparation of Gibson fragments. Therefore, previously amplified left and right homology arms for iroC genes, G-blocks and the linearized pGEM-T Easy backbone were used. Since, all of the formed products have homology sequences to another fragment overlap extension could occur in PCR. All fragments put in a PCR tube, using first fragment as forward primer and others as template. As reverse primer, simple 20bp oligo which covers

both 5’end of the first fragment and 3’end of the backbone was designed and ordered. In my case, I had 3 insert fragments and a backbone. Using homology sequences, all fragments come together in PCR and a linear product was formed. Last step was to transformation of this linear product to *E. coli*. *E. coli* have homologous recombination enzymes which makes our linear product circular plasmids, in my case, donor DNA templates. Analytical digestion with the restriction enzyme *Nco*I resulted in the expected donor template size of 6kb for *caup* and *mirr* (Figure 4.15). Sequencing selected plasmids verified the successful cloning.

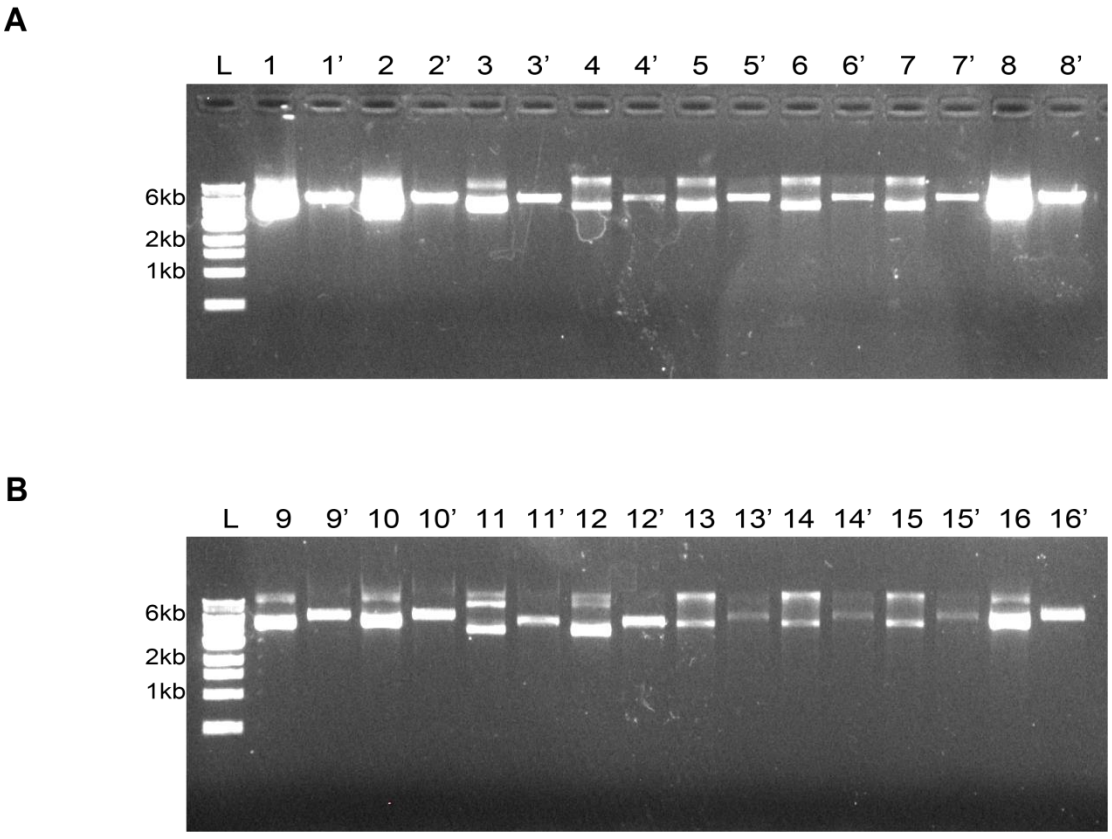

Figure 4.15. Analytical digestion of *caup* and *mir*r donor DNA templates. Numbers indicate undigested plasmids, numbers with apostrophe represent the corresponding digested plasmids. A) All isolated plasmids gave the expected band sizes for *caup* donor DNA. B) 9, 10 and 16 numbered plasmids gave the expected band sizes for *mirr* donor DNA.

4.3.3. Generation and Screening of Tagged Fly Lines

Generated gRNA and donor DNA constructs were isolated in medium scale for embryo injection. gRNA and donor DNA constructs were co-injected to Nos-Cas9 embryos. For mirr, 240 embryos were injected, crossed with ywQB. From their offspring 350 single crosses were put again with ywQB. caup and ara constructs were also recently injected (Figure 4.16.).

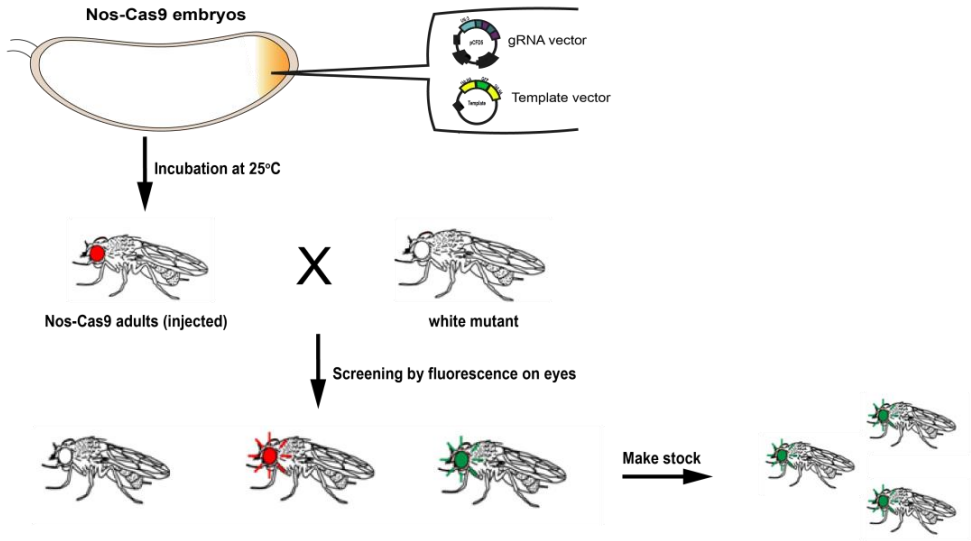

Figure 4.16. Schematic view of embryo injection and recombinant fly selection. Nos-Cas9 embryos were co-injected with pCFD5-gRNA and donor DNA template constructs. Injected flies were crossed with balancer flies and Nos-Cas9 allele was removed. After single crosses, transgenic flies were selected.

Since *IroC* is known to be expressed in the dorsal part of the eye green fluorescence was expected to be observed in the dorsal part of the eyes of the transgenic flies. However, because iroC expressed in low levels, we could not observe any GFP signal under fluorescence microscope directly and thus could not be used for screening of positive integration. To confirm the successful HDR, insert flanking primers were designed and PCR was performed to verify correct tagging (Figure 4.17.). PCR results for *mirr* showed that 2, 8, 34 and 35 numbered flies had the correct insertion (Figure 4.18.).

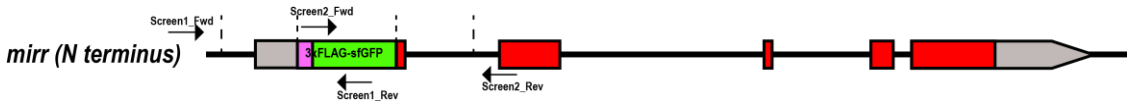

Figure 4.17. Correct HDR screening primers for *mirr*. Dashed lines in introns respresents whole construct including homology arms. Two screenings (Screen1, Screen2) were designed, both have one primer from GFP and one from outside the whole construct.

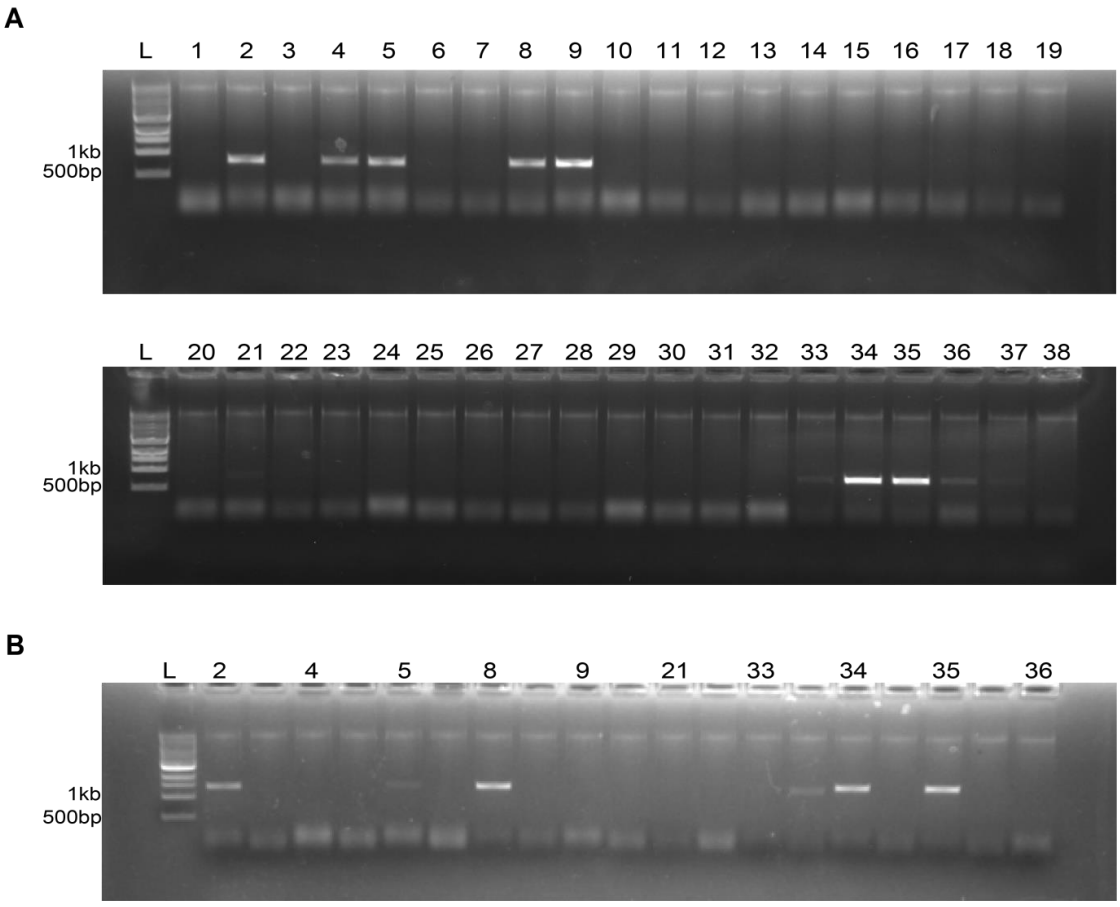

Figure 4.18. Screening for the transgenic flies by PCR for *mirr*. A) Screen1 was performed for 50 single cross. 2, 4, 5, 8, 9, 34, 35 numbered wells gave the expected band size 785bp. 21,33 and 36 numbered wells also had less intense bands with the expected size. B) Screen2 was performed to confirm other half of the construct. 2, 8, 34 and 35 numbered wells gave the expected band size 1342bp.

4.3.4. Analysis of 3xFLAG-GFP Fused Mirr Expression Patterns

4 fly lines were confirmed to have the correctly fused forms. Previously, DE-Gal4 line was generated which is believed to reflect *mirr* expression (Morrison and Halder, 2010). We wanted to compare its expression pattern to our fused protein in 3rd instar larval eye-disc, brain wing disc and leg disc. Immunohistochemical analysis using Elav (neuronal marker) and GFP antibodies, showed the specific expression of *mirr* can be shown with tagged mirr in a more specific manner (Figure 4.19.). GFP activity is restricted to dorsal part of eye-antennal imaginal disc, however, not active in neurons. Also in antennal disc, GFP is active in the ventral side. Presumptive nota, hinge and pleura regions in wing disc showed GFP activity. Leg disc showed a little signal and in larval brain there is GFP activity in ventral nerve cord and in medial lobe, but not in optic lobe (Figure 4.19.).

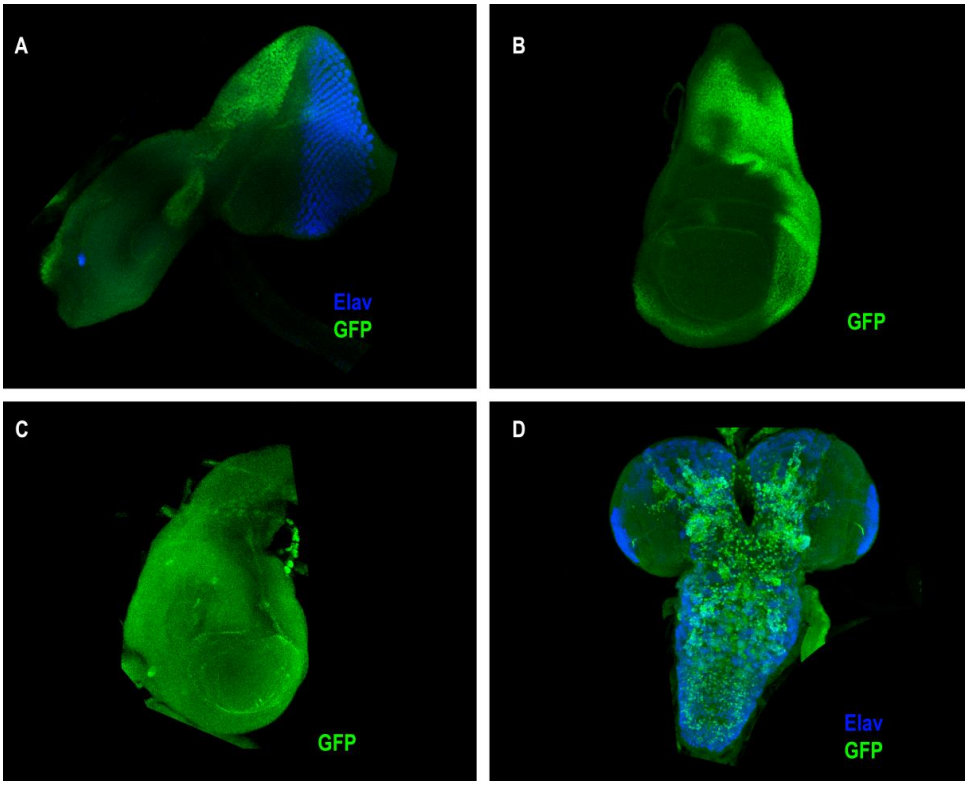

Figure 4.19. Tagged mirr expression pattern assayed by Elav and GFP antibodies in 3rd instar larvae. A) Eye-antennal imaginal disc displayed dorsally restricted GFP activity. B) Wing disc displayed GFP activity in presumptive nota, hinge and pleura regions. C) Leg disc showed a little signal. D) In larval brain mirr seems to be expressed in medial lobe and ventral nerve cord.

Using generated fusion proteins would give us more specific results in case of their expression pattern during development. Generation of fusion proteins for ara and caup are still in process. Donor DNA template and gRNAs were injected to 240 Nos-Cas9 embryos just like mirr. Injected flies will be crossed with ywQB and from their offspring 350 single crosses will be put again with ywQB. After obtaining putatively transgenic flies, to confirm the successful HDR, PCR will be performed using insert flanking primers to verify correct tagging.

4.4. Validation of RNA Sequencing Results by QPCR

In our lab İbrahim İhsan Taşkıran, 2018 was performed RNA-Seq analysis using triple mutant of *IroC* (Iro^{DFM3}). Iro^{DFM3} mutant lacks *ara, caup* and the regulatory regions of *mirr*. To have more reliable results, he double-checked RNA-Seq results with QPCR. However, he only checked OR genes, but not any transcription factor nor non-effected genes as control. Also, he did not show downregulated antennal iontropic receptor (Ir) genes that are expressed in coeloconic sensilla and antennal gustatory receptors (Gr) that are expressed in antennal basiconic sensilla in his study. Differentially expressed transcription factors, olfactory, iontropic and gustatory receptors are shown in Table 4.4.

Table 4.4. Differentially expressed transcription factors, olfactory, iontropic and gustatory receptors. (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

Gene Group	Gene Name	Mean Count	Fold Change (Log2)	P Value
Transcription Factors	Pdm3	2908.72	0.47	**
	Acj6	1578.32	0.47	**
Iontropic Receptors	Ir60a	147.68	1.11	****
	Ir25a	5992.05	0.54	**
	Ir76b	5139.94	0.42	*
	Ir92a	427.01	0.43	*

Table 4.4. Differentially expressed transcription factors, olfactory, iontropic and gustatory receptors. (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001) (cont.)

Gene Group	Gene Name	Mean Count	Fold Change (Log2)	P Value
Gustatory Receptors	Gr21a	944.52	0.51	*
	Gr63a	1733.32	0.48	*
Olfactory Receptors	Or49b	370.08	-0.37	Not significant
	Or85e	576.93	0.27	Not significant

In this study, I performed Q-PCR analysis to validate RNA-Seq results of differentially expressed *acj6, pdm3, Gr21a* genes. To normalize the results *Gapdh1* gene was used as control. As non-affected gene, *Or85e* was chosen as another control, since its expression level did not change in *iroC* mutant background. *Or49b* also had a special aim, it is differentially expressed in a P value of 0.07 according to RNA-Seq results. This P value seems insignificant, but, RNA-Seq analysis did not performed with homozygous tissue. Tissue was mosaic for iroC mutant and wild type. Therefore, even though 0.07 looks bigger than 0.05 (min significant value) we wanted to analyze if the Q-PCR would show an increase in mRNA level.

The cDNA was isolated from maxillary palp and antenna of control and clonal samples and used as template. Primer-BLAST web browser (Ye *et al.*, 2012) was used to design specific primers to ensure they span exon-axon junctions to avoid DNA contamination. All primers were selected to form smaller PCR products than 250bps. 3 biological replicates and for each, 3 technical replicates were prepared. In replicates contained 2 no-template control reactions. Obtained results from Q-PCR were consistent with RNA-Seq results. The fold changes in Q-PCR for *acj6, pdm3, Gr21a, Or49b* and *Or85e* were 0.27, 0.6, 0.46, 1.92, and 1.07 respectively. *Or49b* seems to be upregulated as expected. However, not all biological replicates gave an accurate result for *Or49b* which again seems to be insignificant upregulation (Figure 4.20.).

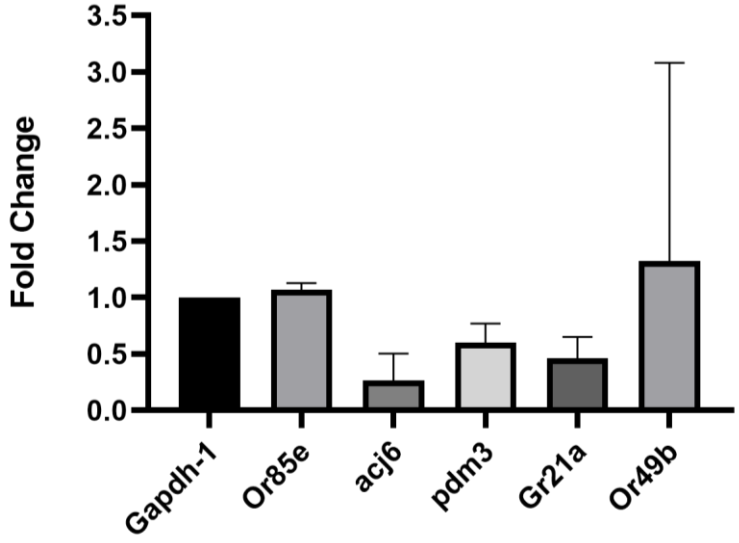

Figure 4.20. Q-PCR analysis result. *Gapdh1* gene was used as a control. According to Q-PCR results, *acj6*, *pdm3* and *Gr21a* were downregulated, *Or49b* was upregulated and *Or85e* did not change which is consistent with RNA-Seq results.

5. DISCUSSION

The fundamental knowledge acquired about the structure and function of the eye, how we process light and which properties of light are measured by the eye, led to the production of cameras and displays. The level of knowledge we have about olfaction which is a key sensory modality to control many aspects of behavior is not that advanced yet. In this sensory modality the relationship between the molecular features of a stimulus and the sensory response is still unknown.

The fruit fly, *Drosophila melanogaster*, has an advanced olfactory sensory system that allows hundreds of different odorants to be recognized and distinguished. For the animal, assessment of these odorants is requisite to define appropriate food sources and egg - laying sites.

With the advent of novel fly lines, the molecular basis of this discriminatory power has begun to be uncovered. Many *Drosophila* odorant receptors, transcription factors, DNA motifs were discovered. However, the transmission and interpretation of olfactory signals by the brain and the stereotypic neuron-receptor expression still remain unsolved. The availability of genetic tools and a complete sequence of its genome render *Drosophila* an important model organism to uncover the molecular basis of the olfactory system.

In the framework of this thesis my main aim was to generate novel tools for each member of the *IroC*. *IroC* is a transcription family that consists of three genes; *ara*, *caup* and *mirr* were previously shown to be expressed in the olfactory organs (Mustafa Talay, 2011). *IroC* is known to be involved in the regulation of rhodopsin gene expression, fate determination and proper appendage development. Therefore, we hypothesized that *IroC* could have a similar role in olfactory system. Using a commercially available fly line, a triple

mutant of *IroC*, transcriptome-wide analysis of its target genes was performed and significant downregulation of Or genes and related transcription factors were identified (İbrahim İhsan Taşkıran, 2018). However, the lack of tools limited a more detailed analysis into the role of each gene in the complex. Here, with the generation of knock-outs and tagged proteins of iroC, we aimed to contribute to further analysis of the role of these transcription factors.

Firstly, knock-outs of individual *IroC* genes were generated via CRISPR/Cas9 technique. Generated individual mutant flies are now used for lineage tracing and transcriptome-wide analysis.

Next step was to generate tagged individual proteins of iroC via CRISPR/Cas9. This allowed the analysis of the individual expression patterns of these proteins as available antibodies and *in situ* hybridization did not work in the peripheral olfactory organs.

5.1. Generation of Individual Knock-outs of iroc Using CRISPR/Cas9

Since there are no commercially available individual mutant fly lines, previously RNA interference analysis was used for loss of function experiments. Unfortunately, 100% downregulation of *IroC* couldn't be achieved by this method (Mustafa Talay, 2011). Thus, we chose to generate single mutants using CRISPR/Cas9.

The main aim was to create a frameshift mutation that would lead to an early stop codon and prevent proper protein synthesis. To achieve this, the second exon of *ara*, *caup* and *mirr* was chosen as target site and specific gRNAs were designed using the CRISPRScan algorithm tool. The DSB in the DNA at the gRNA target site is expected to induce the NHEJ repair pathway, whose activation leads to the creation of random indel mutations in the genome at this site. We hoped that some of these indel mutations would cause the desired frameshift mutations.

Two 20nt gRNAs were chosen for each gene to generate larger deletions and cloned into a gRNA expression vector that has two U6 promoters. The *IroC* gene family is located on the 3rd chromosome of *Drosophila*. Therefore, to avoid any disruption, the generated constructs were integrated by site-directed recombination to attB sites located on the 2nd chromosome. The generated transgenic flies carry the gRNA constructs and are able to express them, but do not express the Cas9 enzyme which is required for DSB. The gRNA-carrying flies were crossed to flies expressing Cas9 under the control of the nanos promoter. *nos* is a germline-specific promoter and thus restricts Cas9 expression to germ line cells. This restriction decreases lethalty by preventing any somatic mutation and increases the probability of germline transmission. Putatively, from this point, all the offspring of flies that were expressing both Cas9 and gRNA, should have unique indel mutations. For screening, for each gene 100 single crosses were set and primers that flank the gRNA sites were designed. Screening on genomic DNA extracted from each of the generated lines was performed by PCR due to lack of any visible marker. In addition, the idea was that deletions caused by the use of two gRNAs could be easily observed on gel. After screening for *caup*, 5 mutants out of 100 crosses were identified. Unfortunately, for *ara* and *mirr* no mutations were found. Therefore, I increased the cross number from 100 to 400 for *ara* and *mirr*. Only one mutant could be obtained for *ara*, however, despite screening these many lines, no mutant for *mirr* was found. Selected gRNAs had the score of 79 out of 100 due to CRISPRScan gRNA prediction tool. This outcome showed that the selected gRNAs for *ara* and in particular for *mirr* were not as efficient as expected. gRNA efficiency relies on many things such as secondary structure, hairpin formation, chromatin structure, low and high GC content of gRNA, as well as the downstream and upstream sequence of PAM. Moreover, it has been shown that gRNAs targeting non-transcribed strands were more effective than targeting of transcribed strands and gRNAs targeting ribosomal genes were more likely to be depleted (Doench *et al*., 2014; Wang *et al*., 2014). So, selection algorithms should be improved further to increase efficiency.

The generated mutants were further analyzed by sequencing. In the *ara* mutant ara^{64} a 54-nucleotide deletion was detected, which corresponds to 18 amino acids. Unfortunately, on the protein level, this deletion does not lead to any frameshift or stop codon and thus could not

be used for functional analysis. On the other hand, 3 out of 5 mutants of *caup* had frameshift mutations that lead to early stop codons. In case of $caup^{7}$ on the protein level the frameshift begins after 17 amino acids and continues with 99 irrelevant amino acids after which a stop codon occurs resulting in a 116 amino acid protein. $caup^{17}$ and $caup^{24}$ start with the initial 21 amino acids and a frameshift causes a translation stop, leading to a protein of 143 amino acids. The wild-type caup protein is composed of 693 amino acids. The described 3 mutants lack 4 proper exon formation out of 5 and lack DNA-binding domain, homeodomain and iro box. Thus, are considered full mutants and could be used for further analysis. On the other hand, the other two *caup* knock-outs; $caup^{10}$ lacks 50 amino acids and $caup^{14}$ lacks 30 amino acids without causing any frameshift.

While performing these experiments, we were in contact with other labs interested in iroC and found that there is a mutant fly line for *mirr* ($mirr^{e48}$). This line was kindly shared with our laboratory by Sonsoles Campuzano. However, so far there is still no single mutant for ara.

5.2. Generation of Fluorescently Tagged iro Proteins

In the literature, the *IroC* expression pattern has been followed by using the IroC-Gal4 driver, which is believed to reflect the expression pattern of *ara* and *caup* (Ikmi *et al*., 2008; Mazzoni *et al*., 2008). Moreover, there are reported antibodies for *caup* (also recognizes *ara*) (Diez del Corral *et al*., 1999) and *mirr* (CH *et al*., 1999), which are not commercially available and in general not available any more. Another Gal4 line, DE-Gal4, was generated later and is believed to reflect *mirr* expression only (Morrison and Halder, 2010). In imaginal discs *in situ* assays have been performed to look at iroC expression.

In the olfactory system we have previously used the Gal4 lines to investigate iroC expression (Talay, 2011; Taşkıran, 2018). However, it is not 100% clear if these Gal4 lines reflect the endogenous expression of *iroC* genes. To clarify the endogenous *IroC* expression

pattern in general and in the olfactory system in particular we aimed to generate endogenously tagged iroC proteins that could be used for expression analysis.

As mentioned before, transcription factors work in a combinatorial manner in the olfactory system. Iro proteins are known to form homo- and hetero-dimers. Individual tagging of ara, caup and mirr would tell us if these proteins are all expressed together and play a role in the olfactory system or only some of them. Tagging of Iro proteins was performed using the CRISPR/Cas9 system by inducing the HDR pathway.

The aim was to tag each protein with GFP for direct visualization and to add a smaller epitope to be able to compare the localization of iro proteins to each other in double labeling experiments. *IroC* is a highly conserved transcription family, thus, generation of fusion proteins should be studied carefully to prevent any disruption of their function. I compared homologous sequences to each other, their conserved regions, mRNA variants and the protein homologies. *ara* and *caup* have a high sequence homology and are very high conserved in their N terminus. Consequently, the C terminus was chosen for tagging ara and caup. Mirr also has conserved regions in the N terminus. Nevertheless, the N terminus was chosen for tagging because of the presence of alternatively spliced mRNAs varying in the C terminus.

The induction of a HDR pathway requires two specific constructs: a gRNA plasmid to target a specific sequence and a donor DNA template that will be used during repair and introduce the tag. A gRNA was chosen from an area between 100 base upstream and downstream of the tagging site using the flyCRISPR Target Finder tool. The selected 20 nt gRNAs were then cloned under a U6 promoter in a gRNA expression vector for each gene. The donor DNA template consisted of 3 fragments that needed to be cloned; a 1kb upstream homology arm, a G-block (consisting of GFP, a small epitope, and a linker sequence), and 1kb downstream homolog arm. As small epitopes, we chose 3xHA, 3xV5 and 3xFLAG for *ara, caup* and *mirr*, respectively. The linker sequence was used to prevent misfolding of the protein and disruption of protein function. The necessary mutation of the PAM sites for these donor DNA templates (to prevent destruction of the DNA template by targeting by Cas9) were

included into the G-blocks. Therefore, I did not need to perform any site-directed mutagenesis to obtain the templates.

gRNA and template constructs were co-injected into nos-Cas9 embryos directly. G0 flies were crossed with ywQB line. F1 lines represent the putatively tagged flies. 350 single crosses were set for the *mirr* line. The initial screening was performed under a fluorescent microscope since we know *IroC* expressed in the dorsal part of the eye. Unfortunately, the GFP signals in adult eye were too low to be detected. Thus, around 100 flies were screened by genomic PCR using primers flanking the target site and 5 positive fly lines were identified. These lines were used for expression analysis in imaginal discs and larval brain for which the expression pattern has been described in the literature. The pattern appeared similar to already published patterns but future analysis is necessary to cross these lines to DE-Gal4 and compare the pattern in more detail. These lines will also be used to assess the expression in the olfactory system.

The lines for ara and caup took longer to be generated and are currently in the crossing phase. Once the transgenic lines are generated the expression patterns will be compared to iroC-Gal4.

5.3. Loss of *IroC* Genes Results in the Decrease of Transcription Factors and OR Gene Expression

As mentioned in the beginning, we would like to perform RNA-Seq analysis with individual mutant lines. The first one was performed by İbrahim İhsan Taşkıran with a triple mutant of *IroC* (Taşkıran, 2018). After RNA-Seq analysis he confirmed the downregulation of OR genes with Q-PCR. However, he did not analyze any of the transcription factors or non-effected genes by Q-PCR. According to P values obtained from the RNA-Seq data, transcription factors *acj6* and *pdm3* and GR gene *Gr21a* were significantly downregulated, OR gene *Or49b* was upregulated and there was no significant change in *Or85e* expression in

the *iroC* mutant background. Q-PCR related to these genes showed correlated results with RNA-Seq.

pdm3 is a POU domain transcription factor which is downregulated in iroC mutant background. *pdm3* is known to be involved in OR gene regulation, mostly the regulation of *Or42a* expression in the maxillary palp, as well as proper axon targeting (Tichy *et al*., 2008). Thus, if iroC is regulating *pdm3* and pdm3 is regulating *Or42a,* we would expect a downregulation in *Or42a* expression. However, in our RNA-Seq results *Or42a* expression does not change. The reason could be a change in the number of cells that express *pdm3* but not *pdm3* itself or that even small amount of *pdm3* expression is enough for regulation of *Or42a* expression. *acj6* is another POU domain transcription factor that is involved ir OR gene regulation and proper axon targeting (Bai *et al*., 2009). It was previously shown that Acj6 regulates *Or42a, Or33c*, *Or85e, Or46a*, and *Or59c* expression in the maxillary palp and *Or42b*, *Or92a, Or85a,* and *Or85b* expression in the antenna. However, only *Or33c* and *Or85a* are downregulated in the *iroC* mutant background, which indicates that iroC is probably an upstream effector of *acj6* like in the *pdm3* case.

Or49b is expressed in the ab6B neuron and seems to be upregulated in the *iroC* mutant background. However, according to RNA-Seq results, this upregulation is not significant with a P value of 0.07. It is important to point out that the tissue that was used for RNA-Seq analysis was not homozygous mutant for iroC, but was mosaic. Therefore, we wanted to check if the Q-PCR would show a significant increase in mRNA level. However, not all biological replicates gave an accurate result for *Or49b* and one can interpret that as an insignificant upregulation. Gr21a and Gr63a are expressed together in ab1C neuron and appeared to be significantly dowregulated in the *iroC* mutant background. These gustatory odorant receptors function in carbon dioxide detection and avoidance behavior. *Or85e* is co-expressed in the pb2A neuron with *Or33c* and its expression level does not change, while *Or33c* appears to be significantly downregulated. It seems iroC is involved in the Or gene regulation in cells where Or genes are co-expressed, which is in line with İbrahim İhsan Taşkıran data.

In addition, a detailed analysis of RNA-Seq data showed that *Ir92a, Ir60a, Ir25a, Ir76b*, antennal iontropic receptors, are also significantly downregulated. *Ir92a* is expressed in ac1 and *Ir25a, Ir76b* function as co-receptors in coeloconic sensilla such as Orco. *Ir60a* is a novel receptor only known to be expressed in coeloconic sensilla, since its expression is gone in *atonal* mutant background (Croset *et al*., 2010; Menuz *et al*., 2014). Since almost all known co-receptors are downregulated there could be two interpretations: 1- *IroC* directly regulates Or gene expression or 2- mis-specification of cells leads to neuron loss, which appears as downregulation. Further analysis should be performed to clarify this issue and in order to differentiate the expression changes of genes, *in situ* hybridization experiments need to be performed.

In summary, to see the whole picture clearer, more experiments need to be done. Now, we know that *iroC* genes have a role in olfactory gene regulation. Using individual mutant lines, this study could be extended with transcriptome-wide analysis. During development, the expression pattern of iroC genes can now be tracked by using generated tagged versions of iro proteins. These constructs will allow us to differentiate the expression of these three genes. Like previously reported transcription factors, Iro proteins may be a part of the combinatorial network which should be elucidated in the future.

REFERENCES

Adams, M. D., S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne, P. G. Amanatides, … J. C. Venter, 2000, "The Genome Sequence of Drosophila Melanogaster.", *Science*, Vol. 287, No. 5461, pp. 2185–95.

Andreu, M. J., E. Gonzalez-Perez, L. Ajuria, S. Gonzalez-Crespo, S. Campuzano, and G. Jimenez, 2012, "Mirror Represses Pipe Expression in Follicle Cells to Initiate Dorsoventral Axis Formation in Drosophila", *Development*, Vol. 139, No. 6, pp. 1110–1114.

Ayer, R. K., and J. Carlson, 1992, "Olfactory Physiology in TheDrosophila Antenna and Maxillary Palp:Acj6 Distinguishes Two Classes of Odorant Pathways", *Journal of Neurobiology*, Vol. 23, No. 8, pp. 965–982.

Bai, L., and J. R. Carlson, 2010, "Distinct Functions of Acj6 Splice Forms in Odor Receptor Gene Choice", *Journal of Neuroscience*, Vol. 30, No. 14, pp. 5028–5036.

Bai, L., A. L. Goldman, and J. R. Carlson, 2009, "Positive and Negative Regulation of Odor Receptor Gene Choice in Drosophila by Acj6", *Journal of Neuroscience*, Vol. 29, No. 41, pp. 12940–12947.

Barish, S., and P. C. Volkan, 2015, "Mechanisms of Olfactory Receptor Neuron Specification in Drosophila.", *Wiley interdisciplinary reviews. Developmental biology*, Vol. 4, No. 6, pp. 609–21.

Bassett, A. R., and J. Liu, 2014, "CRISPR/Cas9 and Genome Editing in Drosophila", *Journal of Genetics and Genomics*, Vol. 41, No. 1, pp. 7–19.

Benton, R., S. Sachse, S. W. Michnick, and L. B. Vosshall, 2006, "Atypical Membrane Topology and Heteromeric Function of Drosophila Odorant Receptors In Vivo", *PLoS Biology*, Vol. 4, No. 2, pp. e20.

Bhalerao, S., A. Sen, R. Stocker, and V. Rodrigues, 2003, "Olfactory Neurons Expressing Identified Receptor Genes Project to Subsets of Glomeruli within the Antennal Lobe of Drosophila Melanogaster", *Journal of Neurobiology*, Vol. 54, No. 4, pp. 577–592.

Bilioni, A., G. Craig, C. Hill, and H. Mcneill, 2005, "Iroquois Transcription Factors Recognize a Unique Motif to Mediate Transcriptional Repression in Vivo", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 102, No. 41, pp. 14671–14676.

Brochtrup, A., and T. Hummel, 2011, "Olfactory Map Formation in the Drosophila Brain: Genetic Specificity and Neuronal Variability", *Current Opinion in Neurobiology*, Vol. 21, No. 1, pp. 85–92.

Carlson, J. R., 1996, "Olfaction in Drosophila: From Odor to Behavior.", *Trends in genetics : TIG*, Vol. 12, No. 5, pp. 175–80.

Carrasco-Rando, M., A. S. Tutor, S. Prieto-Sánchez, E. González-Pérez, N. Barrios, A. Letizia, P. Martín, S. Campuzano, and M. Ruiz-Gómez, 2011, "Drosophila Araucan and Caupolican Integrate Intrinsic and Signalling Inputs for the Acquisition by Muscle Progenitors of the Lateral Transverse Fate", *PLoS Genetics*, Vol. 7, No. 7,.

Cavodeassi, F., R. Diez Del Corral, S. Campuzano, and M. Domínguez, 1999, "Compartments and Organising Boundaries in the Drosophila Eye: The Role of the Homeodomain Iroquois Proteins.", *Development*, Vol. 126, No. 22, pp. 4933–4942.

Cavodeassi, F., J. Modolell, and S. Campuzano, 2000, "The Iroquois Homeobox Genes Function as Dorsal Selectors in the Drosophila Head", *Development*, Vol. 127, No. 9, pp. 1921–1929.

Cavodeassi, F., J. Modolell, and J. L. Gómez-skarmeta, 2001, "The Iroquois Family of Genes : From Body Building to Neural Patterning", *Development*, Vol. 2855, pp. 2847–2855.

CH, Y., S. MA, H. McNeill, C. H. Yang, and M. a Simon, 1999, "Mirror Controls Planar Polarity and Equator Formation Through Repression of Fringe Expression and Through Control of Cell Affinities.", *Development*, Vol. 126, No. 24, pp. 5857–5866.

Clyne, P. J., S. J. Certel, M. de Bruyne, L. Zaslavsky, W. A. Johnson, and J. R. Carlson, 1999, "The Odor Specificities of a Subset of Olfactory Receptor Neurons Are Governed by Acj6, a POU-Domain Transcription Factor.", *Neuron*, Vol. 22, No. 2, pp. 339–47.

Cribbs, A. P., and S. M. W. Perera, 2017, "Science and Bioethics of CRISPR-Cas9 Gene Editing: An Analysis Towards Separating Facts and Fiction.", *The Yale journal of biology and medicine*, Vol. 90, No. 4, pp. 625–634.

Croset, V., R. Rytz, S. F. Cummins, A. Budd, D. Brawand, H. Kaessmann, T. J. Gibson, and R. Benton, 2010, "Ancient Protostome Origin of Chemosensory Ionotropic Glutamate Receptors and the Evolution of Insect Taste and Olfaction", *PLoS Genetics*, Vol. 6, No. 8, pp. e1001064.

Diez del Corral, R., P. Aroca, J. L. G mez-Skarmeta, F. Cavodeassi, and J. Modolell, 1999, "The Iroquois Homeodomain Proteins Are Required to Specify Body Wall Identity in Drosophila.", *Genes & development*, Vol. 13, No. 13, pp. 1754–61.

Doench, J. G., E. Hartenian, D. B. Graham, Z. Tothova, M. Hegde, I. Smith, and D. E. Root, 2014, "Rational Design of Highly Active SgRNAs for CRISPR-Cas9-Mediated Gene Inactivation.", *Nature biotechnology*, Vol. 32, No. 12, pp. 1262–7.

Doudna, J. A., and E. Charpentier, 2014, "The New Frontier of Genome Engineering with CRISPR-Cas9", *Science*, Vol. 346, No. 6213, pp. 1258096–1258096.

Endo, K., T. Aoki, Y. Yoda, K. Kimura, and C. Hama, 2007, "Notch Signal Organizes the Drosophila Olfactory Circuitry by Diversifying the Sensory Neuronal Lineages", *Nature Neuroscience*, Vol. 10, No. 2, pp. 153–160.

Fishilevich, E., and L. B. Vosshall, 2005, "Genetic and Functional Subdivision of the Drosophila Antennal Lobe", *Current Biology*, Vol. 15, No. 17, pp. 1548–1553.

Fuss, S. H., and A. Ray, 2009, "Mechanisms of Odorant Receptor Gene Choice in Drosophila and Vertebrates", *Molecular and Cellular Neuroscience*, Vol. 41, No. 2, pp. 101–112.

Gaj, T., 2014, "ZFN, TALEN and CRISPR/Cas Based Methods for Genome Engineering", *Trends in Biotechnology*, Vol. 31, No. 7, pp. 397–405.

Goldman, A. L., W. Van der Goes van Naters, D. Lessing, C. G. Warr, and J. R. Carlson, 2005, "Coexpression of Two Functional Odor Receptors in One Neuron", *Neuron*, Vol. 45, No. 5, pp. 661–666.

Gömez-Skarmeta, J. L., R. D. Del Corral, E. De La Calle-Mustienes, D. Ferrés-Marcó, and J. Modolell, 1996, "Araucan and Caupolican, Two Members of the Novel Iroquois Complex, Encode Homeoproteins That Control Proneural and Vein-Forming Genes", *Cell*, Vol. 85, No. 1, pp. 95–105.

Gomez-Skarmeta, J. L., R. Diez del Corral, E. de la Calle-Mustienes, D. Ferré-Marcó, and J. Modolell, 1996, "Araucan and Caupolican, Two Members of the Novel Iroquois Complex, Encode Homeoproteins That Control Proneural and Vein-Forming Genes.", *Cell*, Vol. 85, No. 1, pp. 95–105.

Goulding, S. E., P. zur Lage, and A. P. Jarman, 2000, "Amos, a Proneural Gene for Drosophila Olfactory Sense Organs That Is Regulated by Lozenge.", *Neuron*, Vol. 25, No. 1, pp. 69–78.

Guo, S., and J. Kim, 2007, "Molecular Evolution of Drosophila Odorant Receptor Genes", *Molecular Biology and Evolution*, Vol. 24, No. 5, pp. 1198–1207.

Gupta, B. P., G. V Flores, U. Banerjee, and V. Rodrigues, 1998, "Patterning an Epidermal Field:DrosophilaLozenge, a Member of the AML-1/Runt Family of Transcription Factors, Specifies Olfactory Sense Organ Type in a Dose-Dependent Manner", *Developmental Biology*, Vol. 203, No. 2, pp. 400–411.

Gupta, B. P., and V. Rodrigues, 1997, "Atonal Is a Proneural Gene for a Subset of Olfactory Sense Organs in Drosophila.", *Genes to Cells*, Vol. 2, No. 3, pp. 225–33.

Hallem, E. A., and J. R. Carlson, 2004, "The Odor Coding System of Drosophila", *Trends in Genetics*, Vol. 20, No. 9, pp. 453–459.

Hildebrand, J. G., 1995, "Analysis of Chemical Signals by Nervous Systems.", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 92, No. 1, pp. 67–74.

Hildebrand, J. G., and G. M. Shepherd, 1997, "Mechanisms of Olfactory Discrimination:Converging Evidence for Common Principles Across Phyla", *Annual Review of Neuroscience*, Vol. 20, No. 1, pp. 595–631.

Hsu, P. D., E. S. Lander, and F. Zhang, 2014, "Development and Applications of CRISPR-Cas9 for Genome Engineering.", *Cell*, Vol. 157, No. 6, pp. 1262–78.

Huai, C., G. Li, R. Yao, Y. Zhang, M. Cao, L. Kong, … Q. Huang, 2017, "Structural Insights into DNA Cleavage Activation of CRISPR-Cas9 System", *Nature Communications*, Vol. 8, No. 1, pp. 1–9.

Hummel, T., M. L. Vasconcelos, J. C. Clemens, Y. Fishilevich, L. B. Vosshall, and S. L. Zipursky, 2003, "Axonal Targeting of Olfactory Receptor Neurons in Drosophila Is Controlled by Dscam.", *Neuron*, Vol. 37, No. 2, pp. 221–31.

Ikmi, A., S. Netter, and D. Coen, 2008, "Prepatterning the Drosophila Notum: The Three Genes of the Iroquois Complex Play Intrinsically Distinct Roles", *Developmental Biology*, Vol. 317, No. 2, pp. 634–648.

Jafari, S., L. Alkhori, A. Schleiffer, A. Brochtrup, T. Hummel, and M. Alenius, 2012, "Combinatorial Activation and Repression by Seven Transcription Factors Specify Drosophila Odorant Receptor Expression", *PLoS Biology*, Vol. 10, No. 3, pp. e1001280.

Jhaveri, D., S. Saharan, A. Sen, and V. Rodrigues, 2004, "Positioning Sensory Terminals in the Olfactory Lobe of Drosophila by Robo Signaling", *Development*, Vol. 131, No. 9, pp. 1903–1912.

Jhaveri, D., A. Sen, and V. Rodrigues, 2000, "Mechanisms Underlying Olfactory Neuronal Connectivity in Drosophila—The Atonal Lineage Organizes the Periphery While Sensory Neurons and Glia Pattern the Olfactory Lobe", *Developmental Biology*, Vol. 226, No. 1, pp. 73–87.

Komiyama, T., J. R. Carlson, and L. Luo, 2004, "Olfactory Receptor Neuron Axon Targeting: Intrinsic Transcriptional Control and Hierarchical Interactions", *Nature Neuroscience*, Vol. 7, No. 8, pp. 819–825.

Laissue, P. P., and L. B. Vosshall, 2008, "The Olfactory Sensory Map in Drosophila.", *Advances in experimental medicine and biology*, Vol. 628, , pp. 102–14.

Li, Q., T. S. Ha, S. Okuwa, Y. Wang, Q. Wang, and P. C. Volkan, 2013, "Combinatorial Rules of Precursor Specification Underlying Olfactory Neuron Diversity", *Current Biology*, Vol. 23, No. 24, pp. 2481–2490.

Liu, C.-J., H. Jiang, L. Wu, L.-Y. Zhu, E. Meng, and D.-Y. Zhang, 2017, "OEPR Cloning: An Efficient and Seamless Cloning Strategy for Large- and Multi-Fragments.", *Scientific reports*, Vol. 7, , pp. 44648.

Martin, F., T. Boto, C. Gomez-Diaz, E. Alcorta, and W. O. Library, 2013, "Elements of Olfactory Reception in Adult Drosophila Melanogaster", *The Anatomical Record*, Vol. 296, No. 9, pp. 1477-1488.

Mazzoni, E. O., A. Celik, M. F. Wernet, D. Vasiliauskas, R. J. Johnston, T. A. Cook, F. Pichaud, and C. Desplan, 2008, "Iroquois Complex Genes Induce Co-Expression of Rhodopsins in Drosophila", *PLoS Biology*, Vol. 6, No. 4, pp. e97.

McNeill, H., C. H. Yang, M. Brodsky, J. Ungos, and M. A. Simon, 1997, "Mirror Encodes a Novel PBX-Class Homeoprotein That Functions in the Definition of the Dorsal-Ventral Border in the Drosophila Eye.", *Genes & Development*, Vol. 11, No. 8, pp. 1073–82.

Menuz, K., N. K. Larter, J. Park, and J. R. Carlson, 2014, "An RNA-Seq Screen of the Drosophila Antenna Identifies a Transporter Necessary for Ammonia Detection", *PLoS Genetics*, Vol. 10, No. 11, pp. e1004810.

Miller, C. J., and J. R. Carlson, 2010, "Regulation of Odor Receptor Genes in Trichoid Sensilla of the Drosophila Antenna", *Genetics*, Vol. 186, No. 1, pp. 79–95.

Mirzoyan, Z., and P. Pandur, 2013, "The Iroquois Complex Is Required in the Dorsal Mesoderm to Ensure Normal Heart Development in Drosophila", *PLoS ONE*, Vol. 8, No. 9, pp. 1–15.

Morata, G., 2001, "How Drosophila Appendages Develop", *Nature Reviews Molecular Cell Biology*, Vol. 2, No. 2, pp. 89–97.

Morrison, C. M., and G. Halder, 2010, "Characterization of a Dorsal-Eye Gal4 Line in Drosophila.", *Genesis*, Vol. 48, No. 1, pp. 3–7.

Port, F., H.-M. Chen, T. Lee, and S. L. Bullock, 2014, "Optimized CRISPR/Cas Tools for Efficient Germline and Somatic Genome Engineering in Drosophila", *Proceedings of the National Academy of Sciences*, Vol. 111, No. 29, pp. E2967–E2976.

Rath, D., L. Amlinger, A. Rath, and M. Lundgren, 2015, "The CRISPR-Cas Immune System: Biology, Mechanisms and Applications", *Biochimie*, Vol. 117, , pp. 119–128.

Ray, A., W. van der G. van Naters, T. Shiraiwa, and J. R. Carlson, 2007, "Mechanisms of Odor Receptor Gene Choice in Drosophila.", *Neuron*, Vol. 53, No. 3, pp. 353–69.

Rodrigues, V., and T. Hummel, 2008, Development of the Drosophila Olfactory System, In *Brain Development in Drosophila melanogaster*, Vol. 628, pp. 82–101.

Rubin, G. M., M. D. Yandell, J. R. Wortman, G. L. Gabor Miklos, C. R. Nelson, I. K. Hariharan, … S. Lewis, 2000, "Comparative Genomics of the Eukaryotes.", *Science* , Vol. 287, No. 5461, pp. 2204–15.

Saberi, M., and H. Seyed-allaei, 2016, "Odorant Receptors of Drosophila Are Sensitive to the Molecular Volume of Odorants", *Scientific Reports*, Vol. 6, No. 1, pp. 25103.

Sato, K., M. Pellegrino, T. Nakagawa, T. Nakagawa, L. B. Vosshall, and K. Touhara, 2008, "Insect Olfactory Receptors Are Heteromeric Ligand-Gated Ion Channels", *Nature*, Vol. 452, No. 7190, pp. 1002–1006.

Song, G., M. Jia, K. Chen, X. Kong, B. Khattak, and L. Mao, 2016, "CRISPR/Cas9: A Powerful Tool for Crop Genome Editing", *Crop Journal*, Vol. 4, No. 2, pp. 75–82.

Talay, M., 2011, "Role of *IroC* in the Fly Olfactory System", M.Sc. Thesis, Boğaziçi University.

Taşkıran, İ. İ., 2018, "Transcriptome-Wide Analysis of Iroc Target Genes in the *Drosophila* Olfactory System", M.Sc. Thesis, Boğaziçi University.

Tichy, A. L., A. Ray, and J. R. Carlson, 2008, "A New Drosophila POU Gene, Pdm3, Acts in Odor Receptor Expression and Axon Targeting of Olfactory Neurons", *Journal of Neuroscience*, Vol. 28, No. 28, pp. 7121–7129.

Vosshall, L. B., H. Amrein, P. S. Morozov, A. Rzhetsky, and R. Axel, 1999, "A Spatial Map of Olfactory Receptor Expression in the Drosophila Antenna.", *Cell*, Vol. 96, No. 5, pp. 725–36.

Vosshall, L. B., A. M. Wong, and R. Axel, 2000, "An Olfactory Sensory Map in the Fly Brain.", *Cell*, Vol. 102, No. 2, pp. 147–59.

Wang, T., J. J. Wei, D. M. Sabatini, and E. S. Lander, 2014, "Genetic Screens in Human Cells Using the CRISPR-Cas9 System.", *Science*, Vol. 343, No. 6166, pp. 80–4.

Wicher, D., R. Schäfer, R. Bauernfeind, M. C. Stensmyr, R. Heller, S. H. Heinemann, and B. S. Hansson, 2008, "Drosophila Odorant Receptors Are Both Ligand-Gated and Cyclic-Nucleotide-Activated Cation Channels", *Nature*, Vol. 452, No. 7190, pp. 1007–1011.

Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, and T. L. Madden, 2012, "Primer-BLAST: A Tool to Design Target-Specific Primers for Polymerase Chain Reaction", *BMC bioinformatics*, Vol. 13, No. 1, pp. 134.

Zhu, L., H. Mon, J. Xu, J. M. Lee, and T. Kusakabe, 2015, "CRISPR / Cas9-Mediated Knockout of Factors in Non-Homologous End Joining Pathway Enhances Gene Targeting in Silkworm Cells", *Nature*, No. November, pp. 1–13.