INVESTIGATION OF THE ROLE OF *IROC* IN ASYMMETRIC CELL DIVISION

by

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"Knowledge should mean a full grasp of knowledge:
Knowledge means to know yourself, heart and soul.
If you have failed to understand yourself,
Then all of your reading has missed its call."

Yunus Emre

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Finally, I thank my ready to publish book, and I thank my future books. Even thinking about them gave me joy and strength to carry on.

To all, may Aten shine brightly upon your day.

ABSTRACT

INVESTIGATION OF THE ROLE OF *IROC* IN ASYMMETRIC CELL DIVISION

Drosophila melanogaster olfactory system has a high range of neural diversity. During pupal development, Olfactory Receptor Neurons (ORNs) are diversified within the sensory organ lineage emerges from a Sensory Organ Precursor (SOP). ORN diversification is the initial phase increases the neural diversity in olfactory system. Various cell fate determinants act on precursor cells of ORNs in a combinational code and enable ORNs to acquire different cell fates. Cell fate determinants are distributed throughout the sensory organ lineage by asymmetric cell division and they can be used as cell type specific markers.

By using these markers, we aimed to elucidate the function of our gene family of interest, *IroC (Iroquois complex)* in asymmetric cell division during olfactory system development. The *Iroquois Complex* (*IroC*) consists of three different genes in *Drosophila*: *araucan (ara), caupolican (caup),* and *mirror (mirr)* that are located on the third chromosome. *IroC* is shown to be expressed in antenna and maxillary palp, two olfactory organs of *Drosophila*.

I aimed to understand the expression profile of *IroC* and confirm the previously determined expression pattern using Gal4 lines. To achieve this aim, I conducted experiments by using different constructs to get a better idea about the expression pattern of iro proteins. To study function of iro proteins, I conducted clonal analysis experiments with using different cell-type specific markers in *IroC* triple mutant and *caup* single mutant backgrounds. Analysis showed that in the absence of iro proteins, Notch protein cannot be functioned and Notch downstream genes are negatively affected. Thus, iro proteins act as cell fate determinants.

ÖZET

IROC GENLERİNİN ASİMETRİK HÜCRE BÖLÜNMESİ ÜZERİNDEKİ ROLLERİNİN ARAŞTIRILMASI

Drosophila melanogaster koku sistemi geniş bir nöral çeşitliliğe sahiptir. Koku reseptör nöronları (ORN), duyu organı prekürsörlerinden (SOP) kökenlenen duyu organ soyağacı içinde, pupa gelişimi sırasında farklılaşırlar. ORN farklılaşması, koku sistemindeki nöral çeşitliliği arttıran ilk fazdır. Çeşitli hücre kaderi belirleyiciler, ORN prekürsörleri üzerine kombinasyon halinde etki ederler ve ORNlerin farklı hücre kaderleri kazanmasını sağlarlar. Hücre kaderi belirleyiciler, duyu organ soyağacı boyunca asimetrik hücre bölünmesi ile dağıtılırlar ve hücre tipi-spesifik markör olarak kullanılabilirler.

Biz de bu markörları kullanarak, ilgilendiğimiz gen ailesi olan *IroC*'nin (Iroquois kompleksi) koku sistemi gelişimi sırasında asimetrik hücre bölünmesi üzerine etkisini araştırmayı hedefledik. *IroC, Drosophila'*da üçüncü kromozom üzerinde bulunan üç genden oluşur; *araucan (ara), caupolican (caup),* and *mirror (mirr). IroC*'nin *Drosophila* koku organları olan anten ve maxiller palpta ifade edildiği gösterilmiştir.

IroC gen ifadesi profilini anlamayı ve Gal4 sinek hatları kullanılarak daha önceden belirlenmiş gen ifadesi profilini teyit etmeyi amaçladım. Bu amacı gerçekleştirmek için ve iro proteinlerinin ifade profilleri hakkında daha iyi bir fikre sahip olmak için farklı yapılar kullanarak deneyler yaptım. Sonrasında, iro proteinlerinin fonksiyonunu çalışmak için farklı hücre tipi-spesifik markörler kullanarak klon analizi deneyleri gerçekleştirdim. Analiz sonucunda Notch proteininin iro proteinleri yokken çalışamayacağı ve hedef genlerin de negative yönde etkilendiği görüldü. Yani, iro proteinlerinin hücre kaderi belirleyici olarak çalıştıkları görüldü.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

bp	Base pair
g	Gram
kb	Kilobase
ml	Milliliter
M	Molar
µl	Microliter
µm	Micrometer

LIST OF ACRONYMS/ABBREVIATIONS

aa	Amino acid
Acj6	Abnormal chemosensory jump 6
AL	Antennal lobe
APF	After puparium formation
ara	Araucan
BD	Binding domain
caup	Caupolican
CNS	Central nervous system
DE	Dorsal eye
DI	Delta
DNA	Deoxyribonucleic acid
E(spl)	Enhancer of split
fng	Fringe
FRT	FLP recombination target
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
Ham	Hamlet
IroC	Iroquois complex
Lz	Lozenge
Mam	Mastermind
Min	Minute
Mirr	Mirror
nt	Nucleotide
OR	Olfactory receptor
ORN	Olfactory receptor neuron
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
RNA	Ribonucleic acid
RNA-Seq	RNA Sequencing
pros	Prospero
RT	Room temperature
sens	Senseless
Ser	Serrate
slou	Slouch
Su(H)	Suppressor of hairless
SOP	Sensory organ precursor
spdo	Sanpodo
T	Trichoid
UAS	Upstream activating sequence
Vg	Vestigial

1. INTRODUCTION

Contributions of the olfactory sense to survival of life has been undervalued for a long time (Sarafoleanu *et al.,* 2009) . Olfaction is the oldest sense in terms of evolution and is used to guide organisms to distinguish food, to identify enemies and to socially connect with allies. In fact, a fragrance made of sweet-smelling lotus flower and citrus, named as “a scent of the gods” strengthened the reign of pharaoh Hatshepsut, one of the most powerful women in human history, who ruled over 3400 years ago in a male-dominated land (Wise and Odor, 2009).

From survival instincts to behavioral and social skills, having a more sophisticated olfactory system allowed organisms to interact with their environment in a better way. This interaction is achieved by a diverse range of olfactory receptors. The power of odorant recognition is directly proportional to an increase in the diversity of olfactory receptors. The *Drosophila melanogaster* olfactory system combines these properties and thus has become a great model to study olfaction.

1.1. Olfaction

A proper sense of smell relies on two functional systems: a peripheral system for odorant recognition and a control system for odorant discrimination. Investigation of these two systems is the baseline to acknowledge how odorous information is gathered, transmitted and processed. Mechanisms underlying this phenomenon has been studied in vertebrates as well as insects. The *Drosophila* olfactory system is anatomically similar to its vertebrate counterpart and offers a powerful system to study with.

In olfactory systems, an Olfactory Receptor Neuron (ORN) is responsible for gathering odorous information from the environment. Odorants are recognized by Olfactory Receptors (ORs) located in the dendrites. In *Drosophila*, each ORN expresses a specific OR from a large

repertoire, which act as an identity card for ORNs. OSNs project their axons to the first relay station in the brain; the olfactory bulb in vertebrates and the antennal lobe (AL) in *Drosophila*. Axons reaching the first synaptic zones of the central nervous system meet their secondary neurons, namely projection neurons in *Drosophila*, and form synapses with their dendrites. Then, odorous information is further transmitted to higher brain regions including the mushroom body and the lateral horn (Semaniuk, 2015).

As a simpler replica of its vertebrate counterpart, the *Drosophila* olfactory system is more manageable. For instance, around 2 million ORNs and around 1000 ORs are present in mice, while *Drosophila* is estimated to have around 1300 ORNs and 62 well identified ORs. Additionally, a fly brain contains approximately 100,000 neurons, as considered to be quite convenient (Semaniuk, 2015).

1.2. Olfactory System Organization of *Drosophila melanogaster*

Drosophila olfactory organs; antenna and maxillary palp are covered with hair-shaped structures called sensilla (Figure 1.1.). These are sensory hairs that house ORNs. Nearly 410 sensilla cover the antenna while the maxillary palp contains around 60. According to their functions and morphological differences, sensilla are divided into 3 classes: basiconic, trichoid and coeloconic sensilla. A basiconic sensillum is morphologically club-shaped and it houses 2 or 4 ORNs. A trichoid sensillum is morphologically longer than the other types of sensilla and it houses 1, 2 or 3 ORNs (T1, T2 and T3, respectively). A coeloconic sensillum is morphologically peg-shaped and it can house 2 or 3 ORNs. All types of sensilla are distributed in stereotyped areas of the olfactory organ surfaces (Figure 1.2.). The antenna is covered with all types of sensilla while the maxillary palp only contains 60 basiconic sensilla housing 120 ORNs. Here, each sensillum contains 2 ORNs and there are 3 different sensillum subtypes: pb1, pb2, pb3 (Schweisguth, 2015).

The *Drosophila* olfactory pathway starts in the sensilla. ORNs are excited by odorant molecules. Each OR recognizes different odorous compounds and response differently in

electrophysiological studies (Guo and Kim, 2007). After odorant binding, the information is carried to the AL. ORNs interact with projection neurons in specific synaptic zones called glomeruli. ORNs expressing the same OR project their axons to the same glomerulus. Projection neurons carry odorous information to higher brain regions; the mushroom body and lateral horn. The mushroom body is reported to be important for learning and memory. Thus, the sense of smell also contributes to these mental processes (Laissue and Vosshall, 2008).

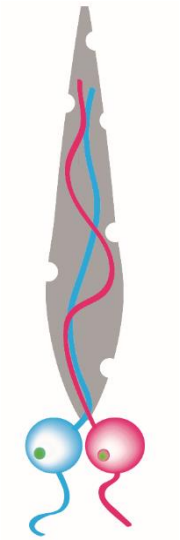

Figure 1.1. Schematic representation of a single sensillum housing two ORNs. Odorant recognition is achieved by olfactory organs covered with sensilla. A sensillum can house ORNs in different numbers and characteristics.

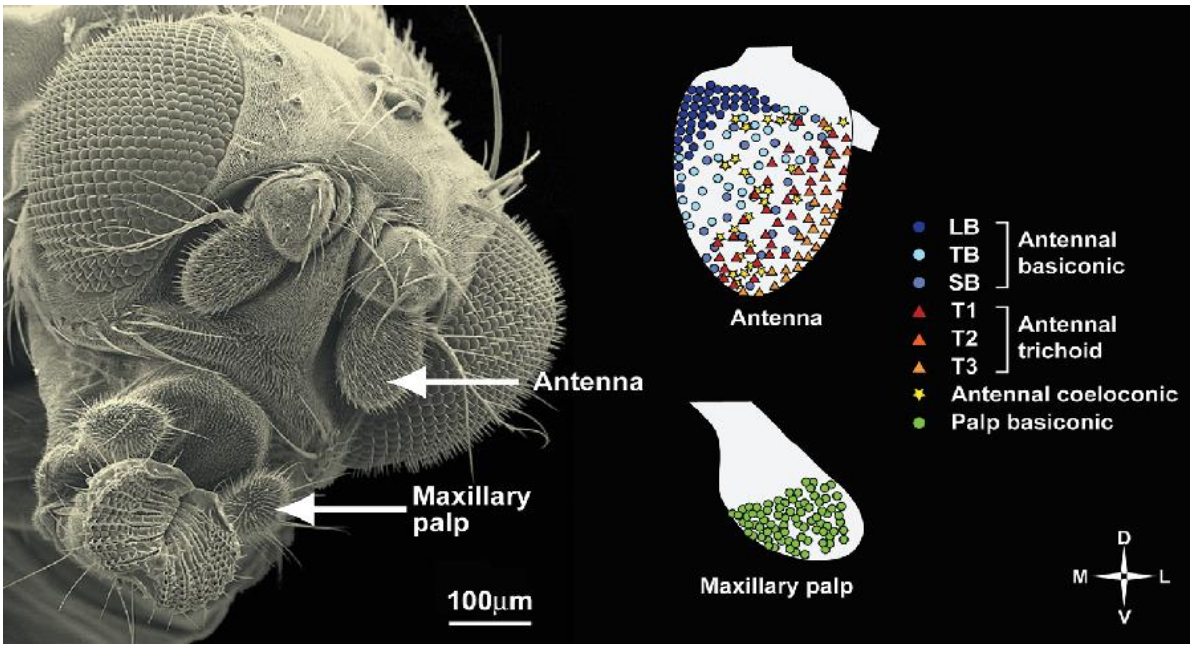

Figure 1.2. Organization of the *Drosophila* olfactory system. Antenna and maxillary palp (arrows) are covered with specific sensilla types. According to their functions and morphological differences, sensilla are divided into 3 classes: basiconic, trichoid and coeloconic sensilla (taken from Laissue and Vosshall, 2008)

Antenna and maxillary palps develop from the antennal disc of a larval eye-imaginal disc. The antennal disc is specified by homeodomain proteins Homothorax, Extradenticle and Distalless (Haynie and Bryant, 1986). Then cells in the antennal disc are prepatterned by the combination of different genes; *engrailed, wingless, decapentaplegic* and *hedgehog* (Cohen and di Nardo, 1993). These genes act in a gene regulatory network and separate the cells in the antennal disc according to their differentiation potential.

In the next step sensory organ precursors (SOPs) are selected out of these epidermal cells. SOP selection occurs via differential expression of the transcription factors *atonal*, *amos* and *lozenge* (Jhaveri *et al.*, 2000). At this step, *lozenge* activates *amos* expression and together they define SOPs, which will give rise to basiconic and trichoid sensilla fates. *atonal* expression is needed to form coeloconic sensilla precursors. On the other hand, the transcription factor

senseless (*sens*) acts in a regulatory network to regulate Notch signaling in SOPs. The role of *sens* in SOP selection will be further discussed in this chapter.

After SOP selection, the neurogenesis stage of olfactory system development starts. SOPs undergo a series of asymmetric cell divisions to generate all cells in the olfactory system. In other words, cells of the olfactory system are derived from SOPs in a defined order during development and are presented in a sensory organ lineage.

1.3. Asymmetric Cell Division and *Drosophila* Sensory Organ Lineage

Asymmetric cell division is an evolutionary conserved and a multistep process that relies on the asymmetric distribution of cell fate determinants during cell division. A polarized mother cell gives rise to two developmentally differentiated daughter cells. Cell fate determinants are distributed unequally throughout the mitotic cell. At the cytokinesis stage of cell division, cell fate determinants are segregated along the mitotic spindle. Finally, daughter cells inherit distinct levels of cell fate determinants and gain different cell identities. (Schweisguth, 2015) (Figure 1.3.).

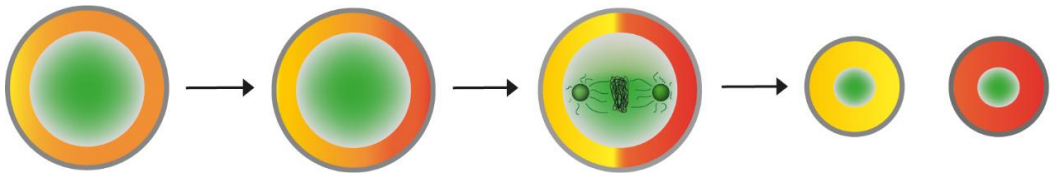

Figure 1.3. Asymmetric cell division is a multistep process. Cell fate determinants (yellow and red) are distributed unequally during cell division. Daughter cells inherit distinct levels of cell fate determinants and gain different cell identities.

A single SOP gives rise to several precursor cells and from these, differentiated cells are generated in a consecutive manner. SOP and precursor cells divide asymmetrically to produce cells with different characteristics. The most distinguishable character is whether cells have an active Notch signal or not. In fact, cells within a sensory organ lineage are named by whether

they have active Notch signaling (Notch ON) or not (Notch OFF) (Das *et al.*, 2010) (Figure 1.4.).

SOP daughter cells pIIa and pIIb acquire two different characters, the pIIa cell gains a Notch ON profile while the pIIb expresses a Notch antagonist, Numb and gains a Notch OFF profile (Endo *et al.*, 2007). Through the sensory organ lineage, several asymmetric cell divisions occur and Notch/Numb are distributed by the same process at every division. However, Notch/Numb activity is not the only determining factor in acquisition of terminal characteristics of sensory organ cells. Downstream effects of these genes shape progenitor cell identities. For example, the transcription factor *cut* is specifically expressed in supporting cell progenitors to generate supporting cells (Figure 1.4.). Genes such as *cut* that determine cellular identity work in a combinational manner and produce cells with various differentiation profiles, thus directly affecting the terminal identity of cells within a sensory organ lineage. These genes are called cell fate determinants.

1.3.1. Cell Fate Determinants of *Drosophila* Sensory Organ Lineage

A single SOP gives rise to a specific type of sensillum lineage in *Drosophila* olfactory organs. At the end of the sensory organ lineage, four supporting cells and four neuronal cells are produced. If a sensillum normally contains less than four neurons, cells in the neuronal class undergo apoptosis. Finally, ORNs in the same sensillum and four supporting cells are derived from the same SOP.

In the sensory organ lineage, “pII” refers to SOP daughters, and “a” refers to active Notch signaling while “b” refers to inactive Notch signaling. pIIa gives rise to the non-neuronal cells, which contains 4 cells known as shaft and socket cells. On the other hand, pIIb gives rise to 4 cells of neuronal class, which include the ORNs and ORN-associated sheath cells. The fourth cell within this class survives only when it originates in the coeloconic sensilla lineage.

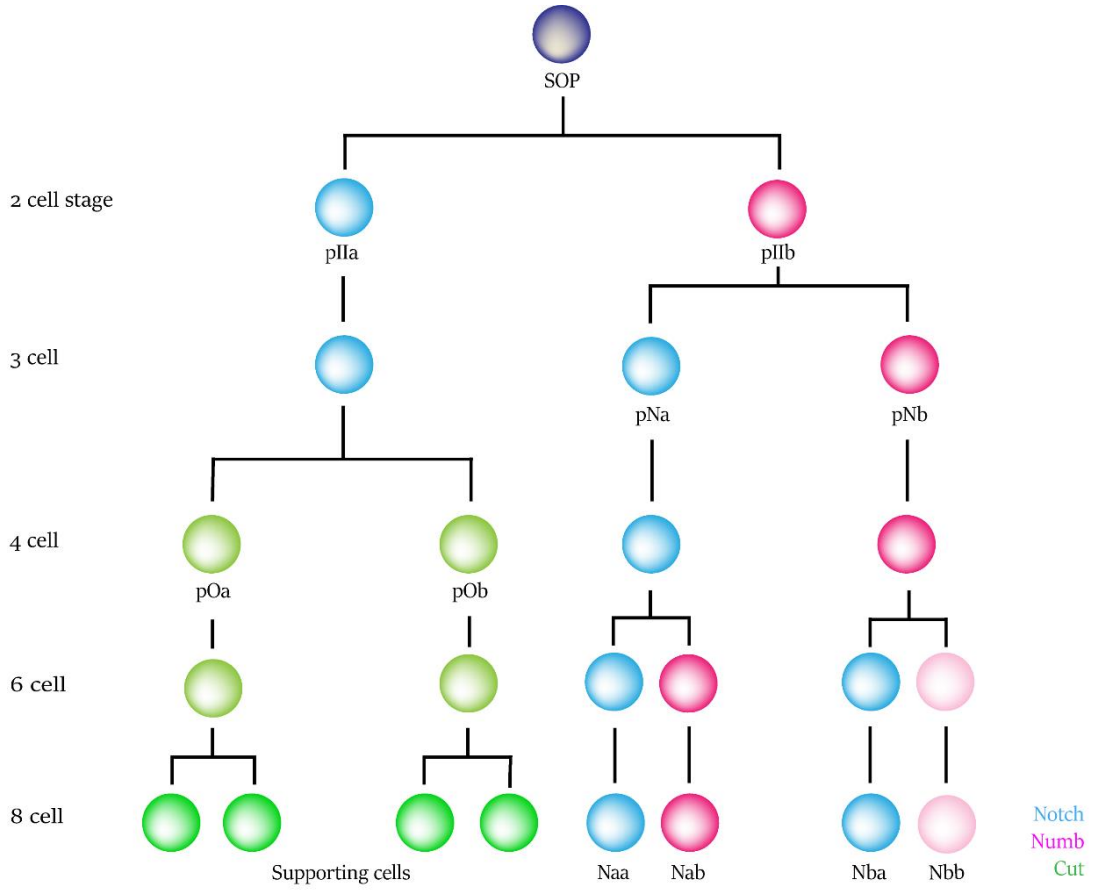

Figure 1.4. Sensory organ lineage. SOP and precursor cells divide asymmetrically to produce cells with different characteristics. Asymmetric cell division occurs at the end of 2-cell, 3-cell and 6-cell stages. Cells are recognized by whether they have an active Notch signal (blue) or not (pink). Also, the transcription factor Cut (green) allows recognition of supporting cells.

A sensory organ lineage is classified by cell stages with increasing number of cells through cell divisions. Many transcription factors that are differentially expressed in these cells have been identified and can be used to trace the identity of cells (Figure 1.5.). pIIa is Notch ON while pIIb is Notch OFF, thus, only pIIb expresses Numb at the 2-cell stage. On the other hand, transcription factors *senseless* (*sens*) and *prospero* (*pros*) are expressed in pIIb. pIIb asymmetrically divides; pNa gains a Notch ON profile while pNb inherits the Numb protein and expresses *sens* and *pros* at the 3-cell stage. Also, at this stage, Notch signaling activates the

transcription factor *cut* in pIIa. Cell divisions are not synchronized in the *Drosophila* sensory organ lineage, pIIa divides later than pIIb. pIIa gives rise to pOa and pOb daughter cells. At the 4-cell stage, pOa and pNa are Notch active while pOb and pNb are Numb active. Sens is expressed in pNa and pNb as it is expressed in all cells in the neuronal lineage. Through the 6-cell stage, pOb starts to express *pros*, while pNa and pNb are dividing. Then, differentiation of the neuronal class terminates. pNa daughter Naa specifically expresses the receptor protein Seven-up. After division of pOa and pOb, finally, the generation of 4 supporting and 4 neuronal cells from a sensory organ precursor is completed (Chai *et al.*, 2019; Endo *et al.*, 2007; Lai and Orgogozo, 2004; Schweisguth, 2015). Additionally, it is reported that elav is slightly expressed in the neuronal class of the sensory organ lineage (not shown in Figure 1.5.) (Sen *et al.*, 2003).

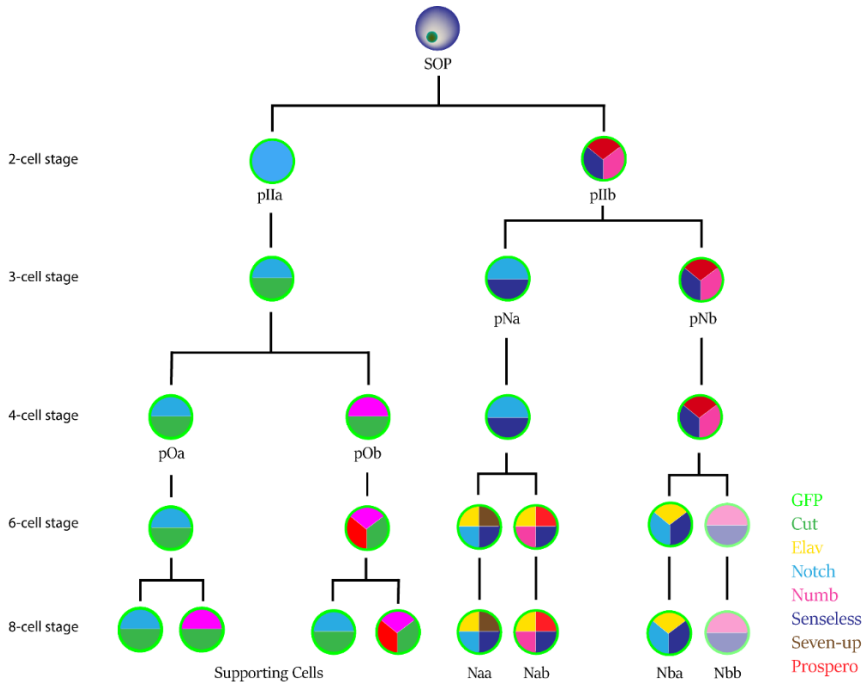

Figure 1.5. Cell fates in *Drosophila* sensory organ lineage is shaped via combinational expression of cell fate determinants. Cells within the lineage can be distinguished by the expression profile of these determinants. Colors indicate the expression profiles of different markers: Notch ON (pale blue) and Notch OFF (Numb-positive, magenta) cells, Sens-positive neuronal cells (dark blue), Cut-positive supporting cells (dark green), Prospero-positive cells (red), Seven-up (brown) and elav-expressing (yellow) cells.

1.3.1.1. Notch/Numb. Before SOP division, Notch contributes to SOP selection within a regulatory network including sens. During SOP division, Notch mediates cell fate acquisition of pIIa and pIIb. It is reported that loss of Notch leads to a pIIa to pIIb-like cell transformation (Figure 1.6.), while an activated Notch signal results in a pIIb to pIIa-like transformation. Delta (DI) and Serrate (Ser) ligand activity is required for proper function of Notch in pIIa (Benton e*t al.*, 2006). Importantly, DI and Ser locate on the membrane of pIIb thus the two daughter cells affect each other.

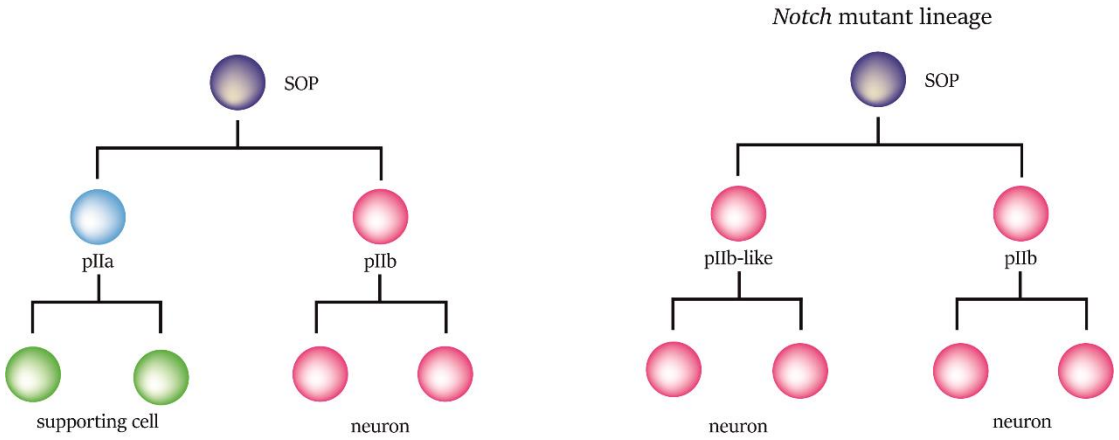

Figure 1.6. Loss of Notch leads to a pIIa to pIIb-like cell transformation. In a Notch loss-of-function background pIIa loses its characteristics and becomes a pIIb-like cell. Subsequently, more neurons are generated in such a sensory lineage.

On the other hand, Numb localizes at the basal-anterior cortex of SOPs and asymmetrically segregates to the pIIb cell. Thus, loss of Numb leads to a pIIa-cell like transformation of the pIIb cell (Figure 1.7.). These data confirm that Notch and Numb act as cell fate determinants in the sensory organ lineage (Ayer and Carlson, 1992).

Aside the fact that the inhibitory activity of Numb towards Notch is controversial, the main hypothesis is that Numb promotes disruption of Notch receptor trafficking by removal of the Notch receptor from the membrane into endosomes (Benton *et al.*, 2006). It is known that Numb regulates the endosomal sorting of Notch-Sanpodo (Spdo) complexes. In SOPs, Spdo

regulates Notch endocytosis, it interacts with Numb and it is considered as the main factor responsible for Notch/Numb antagonism. Numb inhibits the recycling of Spdo, and as a result, it inhibits the activity of Notch. Additionally, loss of Spdo activity leads to a pIIa-to-pIIb transformation (Lai and Orgogozo, 2004).

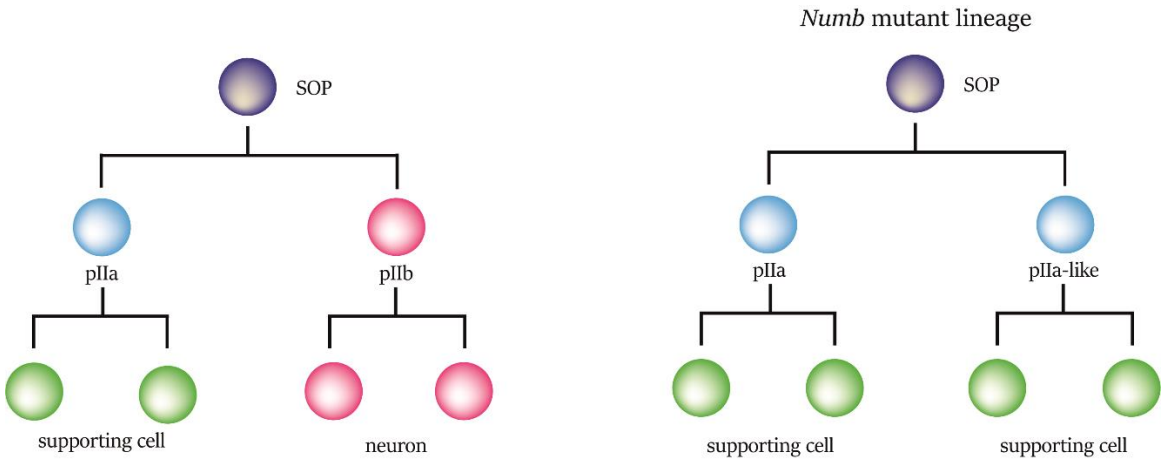

Figure 1.7. Loss of Numb leads to a pIIb to pIIa-like cell transformation. pIIb loses its characteristics and behavea as a pIIa cell in a loss of Numb background. As a result, more supporting cells are generated at the end of cell divisions.

1.3.1.2. Senseless. *sens* encodes the zinc finger transcription factor Senseless that enhances pro-neural gene expression in SOPs thus, directly contributes to SOP selection. Pro-neural genes encode basic helix-loop-helix transcription factors and are key regulators of neuronal differentiation. Pro-neural genes activate the transcription of *sens* and *Enhancer of split E(spl)* complex genes, which takes part in Notch signaling (Benton *et al.*, 2006). Then, Sens protein can act directly on pro-neural genes to take part in this autoregulatory loop or inhibit transcription of *E(spl)* genes (Figure 1.8.). It is known that the expression of *E(spl)* genes is subsequently reduced in the SOPs to permit their specification (Ayer and Carlson, 1992). In this situation, proneural gene expression is further upregulated and the Delta signal is sent to the neighboring cells that do not express Sens. Here, neighboring cells start to ectopically express E(spl) proteins and cause E(spl) accumulation, gaining a Notch ON profile.

In summary, Delta expression is upregulated in Sens expressing cells (Tichy *et al.,* 2008) while E(spl) expression is upregulated in cells that do not express or express very low levels of Sens. Sens is therefore an essential component of the pro-neural Notch signaling pathway by enhancing the reduction of Notch signaling in SOPs (Benton *et al.,* 2006) sens is expressed in pIIb cell where it suppresses E(spl) activity and contributes to an impaired Notch signaling profile.

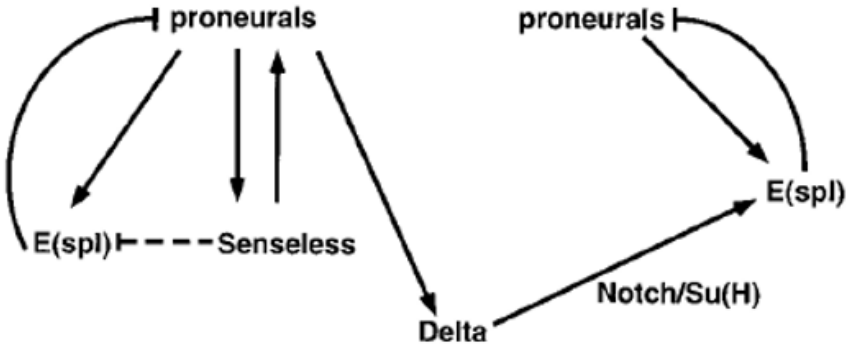

Figure 1.8. Sens protein action model on Notch pathway. *E(spl)* complex genes and *sens* are activated by pro-neural genes. Sens either acts on pro-neural genes directly or inhibits transcription of *E(spl)* complex genes. In the second scenario, pro-neural gene expression is further upregulated and Delta signal is sent to the neighboring cells that do not express Sens (adapted from Nolo *et al.,* 2000).

1.3.1.3. Cut. *cut* encodes homeobox transcription factor Cut that functions as a critical bimodal switch between different cell fates in the sensory organ lineage. In *Drosophila,* Cut accumulates in all external sensory cells (supporting cells) and their precursor cells. Similar to other cell fate determinants, loss of Cut function or mis-expression of Cut protein causes alterations in cell fate (Benton *et al.,* 2006). It is known that Cut is a direct Notch target and its activity depends on Notch signaling (Ayer and Carlson, 1992).

In the *Drosophila* sensory organ lineage, Notch is localized to pIIa and its daughter cells, thus enabling Cut expression. After several cell divisions, cut-positive supporting cells are

generated. Although Notch is also expressed in some of the cells of the neuronal lineage, the presence of Sens activity in these cells does not allow Cut to function since Sens and Cut are reported to have an antagonistic relationship between them. It has been previously observed that Sens overexpression causes a dramatic downregulation of Cut protein levels (Tichy *et al.*, 2008).

1.3.1.4. Prospero. *pros* encodes the homeodomain transcription factor Prospero (Pros) that act as an intrinsic signal for the specification of cell fates within the sensory organ lineage. Pros is asymmetrically localized to intermediate precursor cells in the Central Nervous System, but not asymmetrically localized in cells at any stage of the sensory organ lineage. Here, Pros is expressed first in the nucleus and then generally found in the cytosol of pIIb cells (Münch and Galizia, 2016). Pros controls pIIb identity with Numb protein (Reddy and Rodrigues, 1999). As previously mentioned, Numb antagonizes Notch and Pros is expressed in cells where Notch protein is depleted. It is known that Loss of Notch function or mis-expression of Numb protein results in the ectopic expression of Pros in pIIa cells (Manning and Doe, 1999).

1.4. Acquisition of Terminal ORN Cell Fates

At the end of the sensory organ lineage divisions, ORNs in different numbers and with different terminal cell fates are generated. The last step of ORN differentiation is OR gene choice. ORNs express G-protein coupled receptor-like (GPCR-like) ORs that have seven transmembrane domains (Benton *et al.*, 2006). OR gene choice is regulated by the effect of different transcription factor proteins. Acj6 is a POU domain transcription factor and it is expressed in both of the olfactory organs in *Drosophila* to regulate OR expression and define ORN identity (Ayer and Carlson, 1992). On the other hand, another POU domain transcription factor Pdm3 also regulates for selecting OR type. Additionally, Pdm3 is important for axonal targeting (Tichy *et al.*, 2008). After OR gene selection is completed, different ORs are expressed in ORNs. In addition to specifying the odorant response profile ORs determine the projection patterns of ORNs to the central brain. ORs are present in a complex with a co-receptor, Or83b (Orco), which is needed for a functioning OR.

1.5. Iroquois Gene Complex

The *Iroquois Complex* (*IroC*) consists of three different genes in *Drosophila*: *araucan (ara)*, *caupolican (caup)*, and *mirror (mirr)* that are located on the third chromosome. *IroC* genes are transcription factors and their proteins share a highly conserved 63 amino acid (aa) homeodomain. Contrary to 60 aa homeodomains, iro proteins have 3 aa loop extension that include iro proteins to TALE class homeodomain proteins. In addition to a homeodomain, iro proteins have an iro box, which represents an EGF-like protein-protein interaction domain (Figure 1.9A). This domain is similar to the Notch receptor interaction domain (Gömez-Skarmeta *et al.*, 1996). While mirr can function as a homodimer, ara and caup can form hetero- and homo- dimers (Bilioni *et al.*, 2005; Carrasco-Rando *et al.*, 2011).

The genes *ara* and *caup* are thought to be redundant since their expression pattern is similar and they are closely located on the genome and share common cis-regulatory regions (Gömez-Skarmeta *et al.*, 1996). However, *mirr* is more divergent and encodes a protein that has distinct functions in different tissues. It is suggested that *IroC* homologs are present in all multi-cellular organisms (Cavodeassi *et al.*, 2001; Bilioni *et al.*, 2005). Human and mouse homologs are shown in Figure 1.9B.

IroC genes have distinct roles during development. Iro proteins contribute to the patterning of the dorsal mesoderm along the anterior-posterior axis which is important for proper development. Iro proteins define the dorsal compartments of the eye and head by being expressed in the larval eye imaginal disc (Irvine, 1999). On the other hand, in the wing imaginal disc Iro proteins define the dorsal compartment of the thorax (Gömez-Skarmeta *et al.*, 1996). Iro proteins act as cell fate determinants in the *Drosophila* muscle system (Carrasco-Rando *et al.*, 2011), and they also regulate heart development by affecting precursor cells (Mirzoyan and Pandur, 2013).

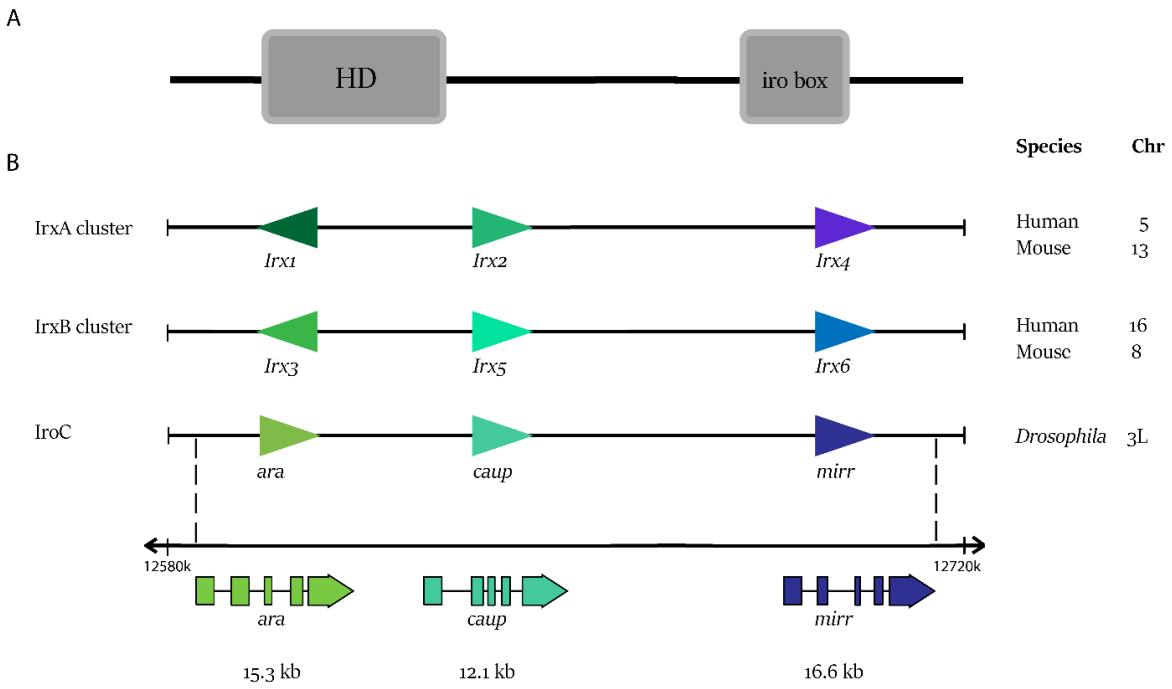

Figure 1.9. The transcription factors of the *Iroquois Complex* (IroC) are encoded by three different genes in *Drosophila*: *araucan (ara)*, *caupolican (caup)*, and *mirror (mirr)*. (A) All iro proteins have two conserved domains: a homeodomain of the TALE class (HD) and the iro box. (B) Genomic physical map of *Drosophila melanogaster Iro* genes. Vertebrate paralogous genes are labeled with similar colors (Adapted from Cavodeassi *et al.*, 2001 and Gomez-Skarmeta *et al.*, 2002).

1.5.1. An Investigation on the Known Relationship of *IroC* and Notch Signaling

In particular, we hypothesize that iro proteins act as cell-fate determinants. As previously mentioned, the Notch signaling pathway is crucial for cell fate acquisition in the *Drosophila* sensory organ lineage. Thus, I would like to review the known relationship between Notch and *IroC* to have a clear view on the possible effects of *IroC* on differentiation occurring in the sensory organ lineage.

There is a lot of evidence that suggests a complicated relationship between *IroC* and the Notch pathway. Overall, it has been suggested that Notch acts downstream of *IroC*.

Fringe (*fng*) is a glucosyltransferase that increases the affinity of Notch to its ligand Delta. The relationship of *IroC* and *fng* in the larval eye-imaginal disc can be taken as an example. *IroC* is known to suppress *fng*, especially, mirr has been shown to directly bind to fng through the Iro Binding Domain (IBD) and to act as a transcriptional repressor. When the IBD on fng is mutated, *mirr* repression onto *fng* is lost. Besides the fact that there are no identified IBDs, ara and caup also can regulate fng expression. Mis-expression of *caup* disturbs *fng* activity and leads to inadequate eye development. Iro proteins are expressed in the dorsal part of the eye-imaginal disc while fng expression is restricted to the ventral side. Additionally, elimination of fng or blocking Notch has no effects on IroC (Froldi *et al.,* 2015). Thus, taking these data together, it can be concluded that the Fng/Notch pathway acts downstream of the *IroC* genes.

Additionally, the effects of loss of IroC on progenitor cell specification is reported in hearth and muscle progenitor cells. Because of the fact that *IroC* regulates Notch activity, loss of IroC leads to an imbalance of progenitor cell specification and results in an abnormal number of heart cells in *Drosophila* (Monastirioti *et al.,* 2009). Also, it is presented that *ara* and *caup* are cell identity genes in muscles and iroC proteins are sufficient for acquisition of specific cell types in the *Drosophila* muscle system. iro proteins act as transcriptional repressors of *slouch* (*slou*) and *vestigial* (*vg***)** genes in a combinational manner (Froldi *et al.,* 2015). Slou represses the expression of the transcription factor *Ladybird* and results in the gain of Notch-activity. On the other hand, iro proteins repress the activation of vestigial, which is normally activated by Suppressor of Hairless Su(H) that is a part of Notch signaling.

RNASeq analyses performed in our lab on antenna and maxillary palp showed that in loss of IroC background, some of the transcription factors important for Notch signaling were differentially expressed (Taşkıran, 2018). In *IroC*-deficient tissues, *nerfin-1* expression was significantly increased (8-fold). Under normal circumstances, Nerfin-1 represses Notch activity (Monastirioti *et al.,* 2009). It could be suggested that iro proteins repress nerfin-1, thus prevent repression of Notch in the sensory organ lineage in wild-type background. Under these terms; when iro proteins are missing, Notch signal is expected to be depleted in olfactory organs. On the other hand, the transcription factor *hey* is significantly downregulated in *IroC* mutant background. Hey is a direct Notch target and is activated in the same way as *E(Spl)* genes

(Monastirioti *et al.,* 2009). This downregulation again supports the idea that the Notch signal is lost in olfactory organs when IroC is not present. In addition, in the RNASeq data Numb appeared to be overexpressed. Taken all these data together, it seems clear that Notch signaling is disrupted in loss of IroC.

IroC and Notch signaling have a complex relationship in different tissues. Notch acts downstream of *IroC* as shown in various loss of IroC cases. *IroC* affects Notch signaling but loss of Notch has no impact on IroC.

1.6. Genetic Tools for *Drosophila*

The fruit fly *Drosophila melanogaster* provides many sophisticated tools for gene manipulation. Here, two of them have been used extensively in this study; the Gal4/UAS binary system (Brand and Perrimon, 1996) and the Mosaic Analysis with a Repressible Cell Marker (MARCM) (Li and Luo, 1999). The Gal4/UAS system is a binary expression system that makes use of a cell-specific promoter and a promotor sequence. A cell-specific promoter drives the expression of Gal4. This protein is a transcription factor normally expressed in yeast. The Gal4 protein binds to the promoter sequence called Upstream Activating Sequence (UAS) and drives expression of a transgene. Thus, the transgene will only be expressed in cells defined by the cell-specific promoter driving Gal4 (Duffy, 2000)

In order to visualize single cells in a developing neuronal tissue and investigate the loss of function effect of a gene on them, clonal cells that are homozygous for the mutant version of the gene of interest should be generated. The MARCM system is used for this purpose. Homozygous mutant cells are derived from heterozygous precursors via mitotic recombination. This enables one to investigate the function of the gene only in a subset of cells. Mitotic recombination is induced via a heat shock inducible Flippase enzyme. Another yeast protein, GAL80 is used together with the GAL4-UAS binary system in MARCM. The GAL80 protein inhibits the activity of GAL4. At the end of the FRT-mediated mitotic recombination, GAL80 is removed from one of the daughter cells, thus a cell-specific promoter drives the expression of Gal4 only in this daughter cell. A chromosome arm that is *in trans* to the chromosome arm

containing the GAL80 transgene, contains a mutation and at the end of this cell division, labeled daughter cell become homozygous for the mutant background of interest. For a MARCM experiment following alleles are needed: Mutation of the gene of interest recombined on a suitable FRT site in one allele of a fly line, and a hs-flp, a tub-Gal4, a tub-Gal80 and UAS-GFP constructs on one allele of another fly line (Figure 1.10.). The second fly line is called as MARCM ready line.

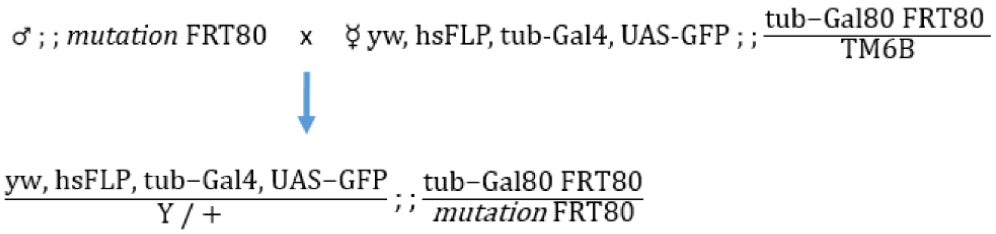

Figure 1.10. A typical MARCM cross. A mutation recombined on a suitable FRT site in one allele of a fly line and hs-flp, tub-Gal4, tub-Gal80 and UAS-GFP transgenes on different alleles of another fly line are crossed to generate the MARCM line that can be analyzed for mutant effects.

2. AIM OF THE STUDY

It is known that iro proteins regulate cell identities of precursor cells in various systems in *Drosophila*, thus, directly contribute to the acquisition of terminal cell fates. In this study, our main aim was to elucidate the possible contribution of *IroC* genes to cell fate determination in the olfactory system.

Iro proteins are expressed in developing antennae where cell fate determination takes place. Asymmetric cell division plays the most important role in cell fate determination by regulating the distribution of cell fate determinants to newborn precursor cells. Thus, this study aimed to reveal the role of *IroC* in asymmetric cell division by clonal analysis.

Furthermore, I aimed to analyze the *IroC* expression pattern by using different constructs to control and confirm *IroC* expression in the olfactory system.

3. MATERIALS AND METHODS

3.1. Biological Material

Drosophila melanogaster lines were stored in air-permeable transparent tubes including fly food at 25°C and 70% humidity with a 12h:12h light and dark cycle. Commercially available fly food (Genesee Scientific Nutri-FlyTM Bloomington Formulas) was used for maintenance. *Drosophila melanogaster* lines used in this study are listed in Table 3.1.

Table 3.1. Fly Lines used in this study

Name of Line	Chromosome No.	Description
Gal4 Drivers		
tubP-Gal4	I	Expresses Gal4 under the control of the promoter of *tubulin*
IroC-Gal4	III	Expresses Gal4 under the control of *IroC*
Mirr (DE)-Gal4	III	Gal4 expression in the dorsal part of the eye and adult antenna
LexA Drivers		
GMR33C10-LexA	II	Expresses lexA under the control of DNA sequences in *mirr*
GMR80B11-LexA	II	Expresses lexA under the control of DNA sequences in *caup*
GMR34C07-LexA	II	Expresses lexA under the control of DNA sequences near *mirr*
GMR34B11-LexA	II	Expresses lexA under the control of DNA sequences in *mirr*

Table 3.1. Fly Lines used in this study (cont.).

Name of Line	Chromosome No.	Description
LexA Drivers		
GMR33E14-LexA	II	Expresses lexA under the control of DNA sequences near *mirr*
GMR34D12-LexA	II	Expresses lexA under the control of DNA sequences in *mirr*
UAS and LexAop constructs		
UAS-mCD8::GFP	I, II	Encodes GFP fused cell membrane bound protein under the control of UAS
UAS-GFP.nls	I	Encodes GFP with nuclear localization signal under the control of UAS
LexAop-mCD8::GFP	I	Encodes GFP fused cell membrane bound protein under the control of LexAop
Chromosomal Deficiency Lines		
$iroC^{DFM3}$	III	Chromosomal deficiency spanning *araucan*, *caupolican* and the promoter of *mirror*
caup	II	Chromosomal deficiency spanning *caupolican*
minute	III	Chromosomal deficiency spanning *minute*
General Stocks		
Canton S		Standard laboratory wild-type Drosophila melanogaster
W^{1118}	I	Presents white eye phenotype due to the mutant allele of eye pigment gene

Table 3.1. Fly Lines used in this study (cont.).

Name of Line	Chromosome No.	Description
General Stocks		
yw; QB	I, II, III	Fly line that consists of flies carrying eye marker w, body marker y and balancer chromosomes Sp / CyO ; TM2 / TM6B
hs-FLP	I	Expresses FLP-recombinase (Flippase) under the control of the promoter of h*sp70* (heat-shock promoter)
FRT80B	III	Allow FLP-mediated site-specific recombination on the chromosome arm 3L
tubP-Gal80 FRT80B	III	Expresses Gal80 under the control of the promoter of *tubulin* and localized on FRT80B

3.2. Chemicals and Supplies

3.2.1. Chemical Supplies

Chemical supplies used in this study are listed in Table 3.2.

Table 3.2. Chemical supplies used in this study

Chemical	Producing Company
Paraformaldehyde	Sigma-Aldrich, USA (P6148)
Triton X-100	AppliChem, USA (A4975)
$MgCl_2$	Riedel-de Haen, Germany (13152)
Ethidium Bromide Solution	Sigma Life Sciences, USA (E1510)

Table 3.2. Chemical supplies used in this study (cont.).

Chemical	Producing Company
100 bp Marker	NEB, USA
Bovine Serum Albumin (BSA)	Sigma-Aldrich, USA (A9647)
Horse Serum	Thermo Fisher (26050070)

3.2.2. Solutions

Solutions used in this study are listed in Table 3.3.

Table 3.3. Solutions used in this study

Solution	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% Triton X-100
PBX3	PBS (1x) 0.3% Triton X-100
TAE Buffer (1x)	40 mM Tris-Cl 1 mM EDTA 0.1% Acetic acid

Table 3.3. Solutions used in this study (cont.).

Solution	Ingredient
Squish Buffer	10 mM Tris, pH 8.0 1 mM EDTA 25 mM NaCl
Proteinase K	20 mg/mL

3.2.3. Oligonucleotides

Oligonucleotides were synthesized commercially at Macrogen (South Korea). Dissolved oligonucleotides were stored at -20°C as 100mM stock. PCRs were performed using diluted stock solutions at 10 uM oligonucleotides. Oligonucleotides used in this study are listed in Table 3.4.

Table 3.4. Oligonucleotides used in this study

Name	Sequence (5’ - 3’)
Forward_screen_caup	TTTTCCCTTTGGCATCTTTG
Reverse_screen_caup	ATGTGGAGAGACCCTTGTGG

3.2.4. Antibodies

Antibodies used in this study are listed in Table 3.5.

Table 3.5. Antibodies used in this study

Name	Antigen	Species	Dilution	Supplier
Primary Antibodies				
GFP	GFP	Rabbit	1:500	Invitrogen
Elav	Elav	Rat	1:250	Hybridoma
Cut	Cut	Mouse	1:50	Hybridoma
Cmnb-1	Numb	Mouse	1:500	Hybridoma
Notch Intracellular Domain	Notch Intracellular Domain	Mouse	1:100	Hybridoma
Svp	Seven up	Mouse	1:5	Hybridoma
Pros	Prospero	Mouse	1:50	Hybridoma
Sens	Senseless	Guinea pig	1:1000	Bellen Lab
nc82	Bruchpilot	Mouse	1:50	Hybridoma
Secondary Antibodies				
Alexa 405	Rabbit	Goat	1:800	Invitrogen
Alexa 405	Mouse	Goat	1:800	Invitrogen
Alexa 488	Rabbit	Goat	1:800	Invitrogen
Alexa 555	Mouse	Goat	1:800	Invitrogen
Alexa 555	Rat	Goat	1:800	Invitrogen
Alexa 555	Guinea pig	Goat	1:800	Invitrogen
Alexa 637	Rat	Goat	1:800	Invitrogen
Alexa 647	Rat	Goat	1:800	Invitrogen
Alexa 647	Mouse	Goat	1:800	Invitrogen

3.2.5. Embedding Media

As embedding media, Vectashield embedding medium (Vector Laboratories, Inc.), Fluoromount-G embedding medium (Southern Biotech) or 80% glycerol were used. Embedded tissues were kept in the dark at 4°C until visualization.

3.2.6. Disposable Labware

Disposable labware used in this study are listed in Table 3.6.

Table 3.6. Disposable labware used in this study

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

3.2.7. Equipment

Equipment used in this study are listed in Table 3.7.

Table 3.7. Equipment used in this study

Equipment	Manifacturer
Autoclave	Astell Scientific Ltd., UK
Centrifuges	Eppendorf, Germany (Centrifuge 5424, 5417R)
Confocal Microscopes	Leica Microsystems, USA (TCS SP5) Leica Microsystems, USA (TCS SP8 Inverted)
Electrophoresis Equipment	Bio-Rad Labs, USA
Fluorescence Stereomicroscope	Leica Microsystems, USA (MZ16FA)
Freezers	Arçelik, Turkey
Gel Documentation System	Bio-Rad Labs, USA (Gel Doc XR)
Incubators	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)

3.3. Molecular Biology Techniques

3.3.1. Genomic DNA Extraction

In order to perform PCR, template DNAs from each fly line of interest were extracted. In this protocol, a single fly from each line was frozen in a PCR tube and squeezed in 50 µl

squish buffer and 0.5 µl Proteinase K (20mg/mL). The samples were incubated at 37°C for 30 minutes and 95°C for 2 minutes. The extracted DNA was directly used as the template in Polymerase chain reactions (PCR).

3.3.2. PCR

PCRs were performed in a total volume of 25 µl containing 1 µl genomic DNA as a template, 0.125 µl One Taq polymerase, 1x One Taq Standart Buffer, 10 mM dNTP, 5 pmol of forward primer, 5 pmol of reverse primers. The reaction conditions were 2’ 95°C, followed by (30’’ 95°C, 30’’ 54°C, 30’’ 68°C) x 30 cycles, and 10’ 68°C. PCR products were run on a 2% agarose gel.

3.3.3. Agarose Gel Electrophoresis

2% agarose gel was prepared with 1x TAE buffer and 30 ng/ml ethidium bromide solution. Loading dye was added to samples and concentration of dye was finalized to 1x. 100 bp Marker was used as size control. The gel was run at 120 V for 30-40 min, and visualized under UV (Gel-DOC, Bio-Rad, USA).

3.4. Histological Techniques

3.4.1. Immunohistochemistry

3.4.1.1. Dissection and Immunohistochemistry of Third Instar Eye Imaginal Disc. Third instar larvae were selected and eye imaginal discs were dissected using forceps together with larval brains and mount hooks. Tissues were placed on a 3-well cavity dish containing PBS on ice and fixed with 200 µl of the fixation solution (4% paraformaldehyde in 0.1% PBST (PBS with 0.1% Triton X-100)), incubated for 20 minutes at room temperature (RT). Then eye imaginal discs were washed 3x15 minutes each in 0.3% PBST and blocked in 200 µl PAXD for 2 hours at RT. Then, the tissue was incubated with primary antibodies diluted in 200 µl PAXD for 24 hr at 4°C. After incubation, eye imaginal discs were washed 3x15 minutes each in 0.3% PBST and

incubated with secondary antibodies diluted in 200 µl PAXD for 24 hr at 4°C. Then, eye imaginal discs were washed again 3x15 minutes each in 0.3% PBST, mounted and visualized under a Leica TCS SP5 confocal microscope.

3.4.1.2. Dissection and Immunohistochemistry of Adult Antennae. Flies were anesthetized on CO_2 pad and third segment of the antennae was cut using forceps. Antennae were placed on a 3-well cavity dish containing PBS on ice and fixed with 90 µl of the fixation solution and incubated for 40 minutes at RT. Then, antennae were washed in 0.4% PBST (PBS with 0.4% Triton X-100), 3x 10 minutes each and blocked in 90 µl of 5% normal horse serum in 0.1% PBST for 20 minutes at RT. After removing the blocking solution, antennae were incubated with primary antibodies diluted in 0.1% PBST containing 5% horse serum for 48 hr at 4°C in a moistened sealed box. Then antennae were washed in 0.4% PBST, 6x 10 minutes each and incubated with secondary antibodies diluted in 5% normal horse serum in 0.1% PBST for 48 hr at 4°C. Antennae were washed again in 0.4% PBST, 6x 10 minutes each. The PBST solution was removed and antennae were cleared in 40% glycerol for 1 minute and then 80% glycerol for 1 minute. Then, the antennae were mounted and visualized under a Leica TCS SP5 confocal microscope.

3.4.1.3. Dissection and Immunohistochemistry of Adult Brain. Flies were anesthetized on ice and the head was cut from the edges of the retina using forceps. Brains were placed in a 3-well cavity dish containing PBS on ice and then placed into 1.5 ml test tubes. Tissues were fixed in 500 µl of 4% PFA and incubated for 25 minutes at RT. Then, brains were washed in 0.5% PBST (PBS with 0.5% Triton X-100), 9x10 minutes in each and blocked in 500 µl of PAXD for 1 hr at RT. After removing the blocking solution, tissues were incubated with primary antibodies diluted in PAXD for 48 hr at 4°C in a moistened sealed box. Then brains were washed in 0.5% PBST, 9x10 minutes each and incubated with secondary antibodies diluted in PAXD for 48 hr at 4°C. Then antennae were washed again in 0.5% PBST, 9x10 minutes each. PBST was removed and tissues were cleared in Vectashield embedding medium for 30 min to increase the visualization quality. Then, the brains were mounted and visualized under Leica TCS SP5 confocal microscope.

3.4.1.4. Dissection and Immunohistochemistry of Developing Antennae. Pupae were selected at a specific time point according to their development and pupal cases were removed using forceps. The soft bodies of pupae were placed on a 3-well cavity dish containing PBS on ice without harming the integrity of the pupae and fixed with 200 µl of 4% PFA in 0.1% PBST and incubated for 1 hr at 4°C. Then, pupae were carefully placed in a test tube containing 70% sterile ethanol and incubated for 24 hr at 4°C. After removing the ethanol, pupal bodies were dissected on a dissection pad by removing the head capsules to expose the heads. Developing antennae were dissected carefully while they were still attached to the membrane. Then developing antennae were incubated in 200 µl of 4% BSA in 0.1% PBST for 1 hr at 4°C, washed 3x15 minutes each in 0.3% PBST and incubated with primary antibodies diluted in 4% BSA in 0.1% PBST for 48 hr at 4°C. After incubation, developing antennae were washed 3x15 minutes each in 0.3% PBST and incubated with secondary antibodies diluted in 4% BSA in 0.1% PBST for 48 hr at 4°C. Then, developing antennae were washed again 3x15 minutes each in 0.3% PBST, mounted and visualized under a Leica TCS SP5 confocal microscope.

3.5. Experiments for Functional Analysis

3.5.1. Loss of Heterozygosity and MARCM Analysis

Offspring of MARCM crosses were raised at 18°C. Third instar larvae were picked and heat-shocked in a water bath at 37° for 30 minutes. After heat shock, the larvae were transferred back to 18°C for synchronization of development until white pupae emerge. Then the temperature was switched to 29°C for the rest of development. Pupae at a specific developmental timepoint were dissected.

4. RESULTS

4.1. *IroC* Expression Pattern Library

Iro proteins are widely expressed in both Central and Peripheral Nervous System in *Drosophila melanogaster* (Bilioni *et al.,* 2005; Carrasco-Rando *et al.,* 2011; Cavodeassi *et al.,* 2001; Mazzoni *et al.,* 2008a; Mirzoyan and Pandur, 2013) . In this study, I first conducted experiments in order to understand the expression profile of *IroC* and confirm the previously determined expression pattern using Gal4 lines. The main aim of these experiments was to get a better idea about the expression pattern of individual iro proteins.

Several fly lines were used: Dorsal-Eye Gal4 (DE-Gal4) and IroC-Gal4 lines, and a set of transgenic fly lines (LexA driver lines).

DE-Gal4 line was developed by replacing the $mirr^{DE}$ P[lacZ] enhancer trap element with a P[Gal4] element (Morrison and Halder, 2010) and this line is thought to represent *mirr* expression pattern. IroC-Gal4 line was developed by replacing the iro^{rF209} P[lacZ] enhancer trap element with a P[Gal4] element (Mazzoni *et al.,* 2008b) and it is thought to represent the *ara* and *caup* expression pattern (Figure 4.1.) (Gomez-Skarmeta *et al.,* 1996; Ikmi *et al.,* 2008).

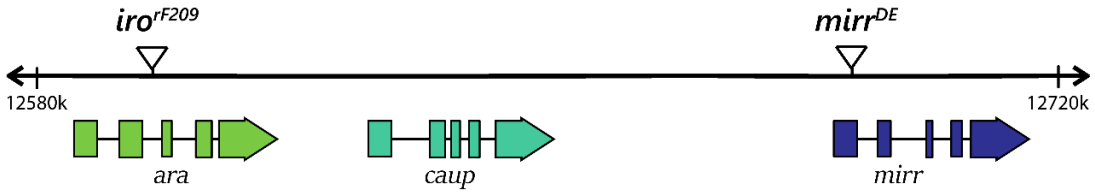

Figure 4.1. Physical map of the *IroC* locus. *ara* and *caup* are closely located to each other and *mirr* is located approximately 4.5 kb downstream of *caup*. P-element insertion sites for iro^{rF209} and $mirr^{DE}$ that are replaced by Gal4 are represented as triangles.

Analysis of DE-Gal4, UAS-GFP line showed that *mirr* is expressed at the mid-pupal stage in developing antenna and in a subset of neurons in the adult antenna as shown by co-staining with the neuronal marker Elav (Figure 4.2.A, A'). The neuron-specific antibody Elav is not only expressed in mature ORNs but also in precursors of ORNs (Sen *et al.*, 2003). Analysis of the IroC-Gal4 line crossed with UAS-mGFP showed that *ara* and *caup* are expressed in the developing antenna (Figure 4.2.B), but to a much lower extent than in the adult (Figure 4.2.B'). Thus, analysis of the expression patterns of DE-Gal4 and IroC-Gal4 indicates that *ara* and *caup* expression gets lost in the adult stage but *mirr* expression is still present.

The developing olfactory system contains distinct types of precursor cells that give rise to neurons of the olfactory organs and the expression of iroC during development of the olfactory organs indicates that iroC might take part in the specification of neuronal cell fates during olfactory system development. It is still unclear however in which sensory organ lineages *IroC* is expressed in. One way to determine the subset of ORNs that express iroC would be to co-stain with individual ORs. OR promoter Gal4 lines have been previously generated, however cannot be used in conjunction with the iroC-Gal4 line. Thus, alternative approaches are needed. One way could be to look at the projection pattern of the iroC-Gal4 line as labeled neurons would project their axons to the antennal lobe and label glomeruli. The location of labeled glomeruli could then be used to determine the subset in which iroC is expressed. Brains of iroC-Gal4 and mirr-Gal4 lines were stained against GFP and co-stained with bruchpilot a general synaptic marker (Fig. 4.3.). While expression in the antennal lobe was observed, the expression was not restricted to the antennal lobe and thus could not be used to deduct the OR subsets

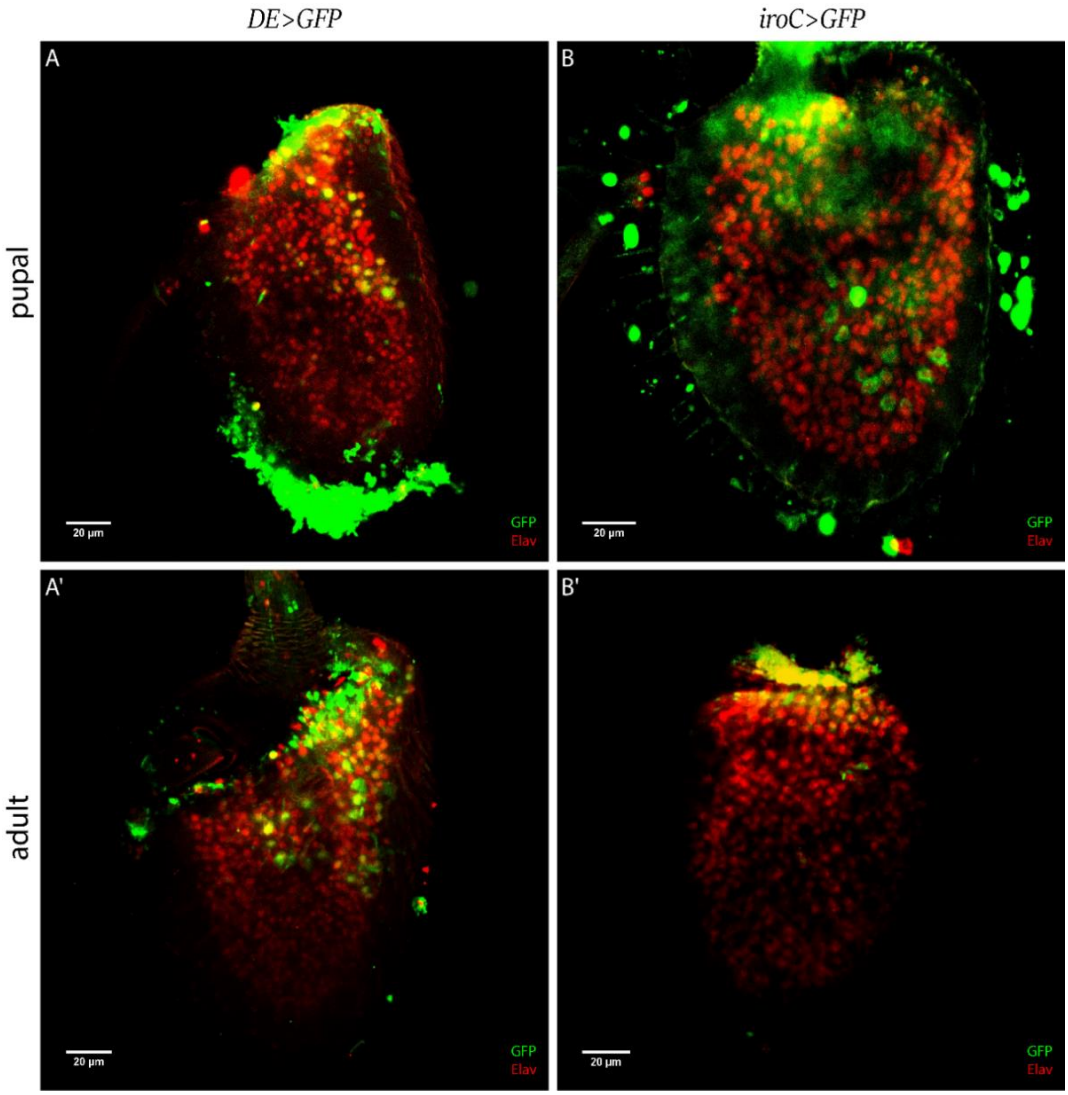

Figure 4.2. *IroC* is expressed in antenna. Pupal antenna stained using antibodies against GFP (green) and neuronal marker elav (red). *mirr* is expressed in the developing antenna (A) as well as *ara* and *caup* (B). Adult antenna stained using antibodies against GFP (green) and elav (red). *mirr* is expressed in adult antenna.

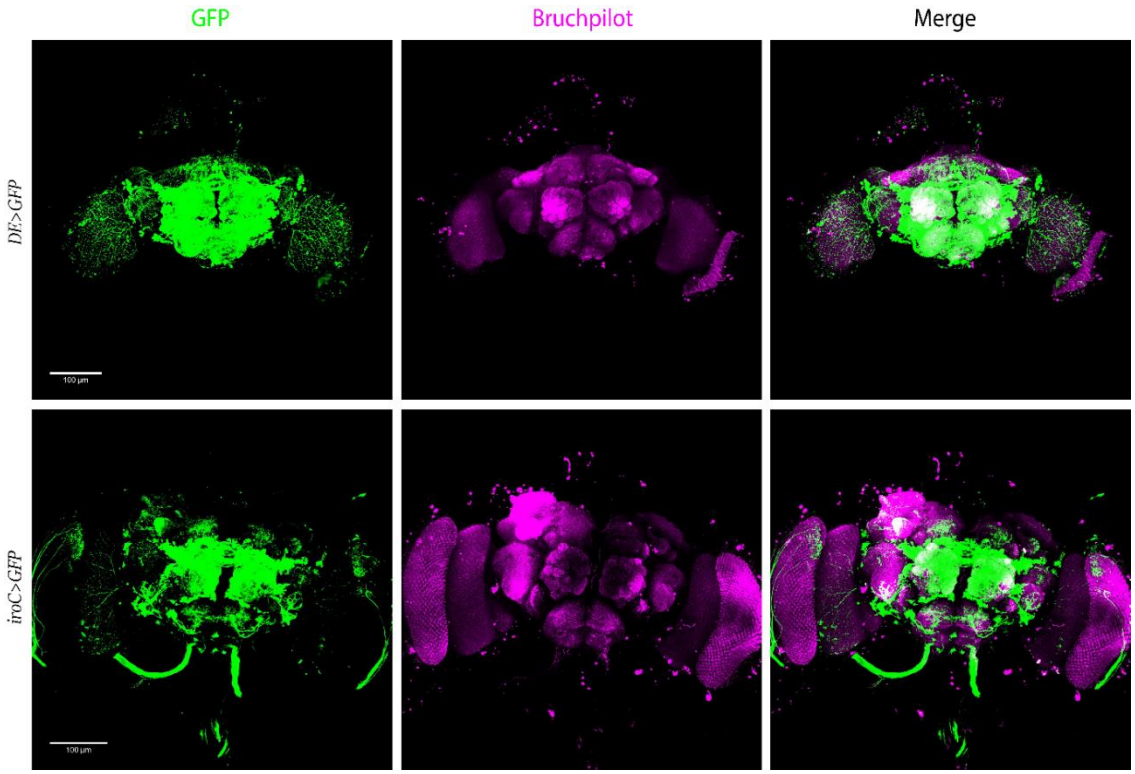

Figure 4.3. *IroC* is expressed in the adult brain. Adult brain stained using antibodies against GFP (green) and Bruchpilot (magenta). *mirr, ara* and *caup* are expressed in different subsets of neurons. Bruchpilot stains presynaptic active zones and acts as a background in brain staining.

In an attempt to overcome this problem we made use of driver lines generated by FlyLight, an initiative to generate a library of driver lines by cloning approximately 3 kb fragments of the whole *Drosophila* genome into Gal4 or lexA vectors (Pfeiffer *et al.*, 2008). The lexA lines were preferred so that they could be combined with the OR-Gal4 lines in co-localization studies. The lexA-lexAop system is another heterologous system that works similar to the Gal4-UAS system where the lexA transcription factor binds selectively to lexAop sites when present. This library contains six fragments from the iroC genomic locus (shown in Figure 4.4.) and were used in this study. Except for line 54974 that contains a fragment of the *caup* gene all other lines were derived from the *mirr* gene.

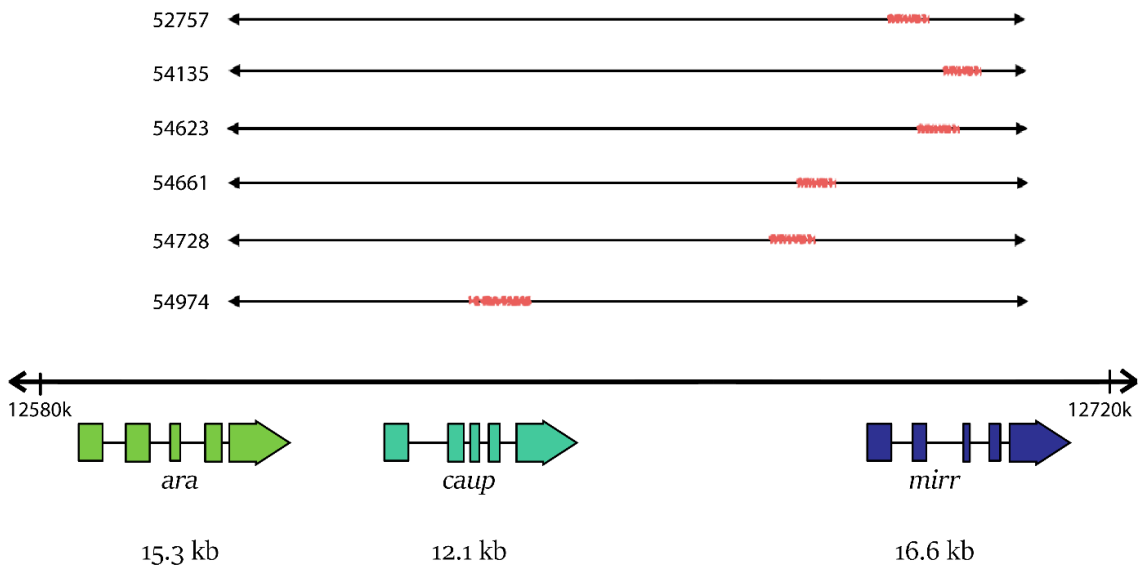

Figure 4.4. Representation of the genomic locations of *IroC* fragments in the *IroC* locus used to generate transgenic fly lines in FlyLight project.

Analysis of transgenic LexA line 52757 showed no expression in the antennal lobe. In accordance with this observation, no expression was observed in antenna or the maxillary palp (Figure 4.5.). Similarly, no expression in olfactory organs and glomeruli was observed for the other transgenic lines, although expression in optic lobes and other parts of the brain was present. Overall, my analysis showed that these fragments generally drive LexA expression in areas that correspond to iroC expression domains, however, none of these lines showed expression within glomeruli or the peripheral organs and thus could not be used in further analyses to determine OR subsets (Figure 4.5.)

An alternative approach taken in our lab was the direct tagging of IroC proteins with GFP using CRISPR/Cas technology. We hope that analysis of those lines will give us more insight into the subset-specific expression pattern of each of the iroC genes. It will also allow us to evaluate the expression pattern observed with the iroC-Gal4 and DE-Gal4 lines and determine how much they represent the endogenous expression or the iroC genes.

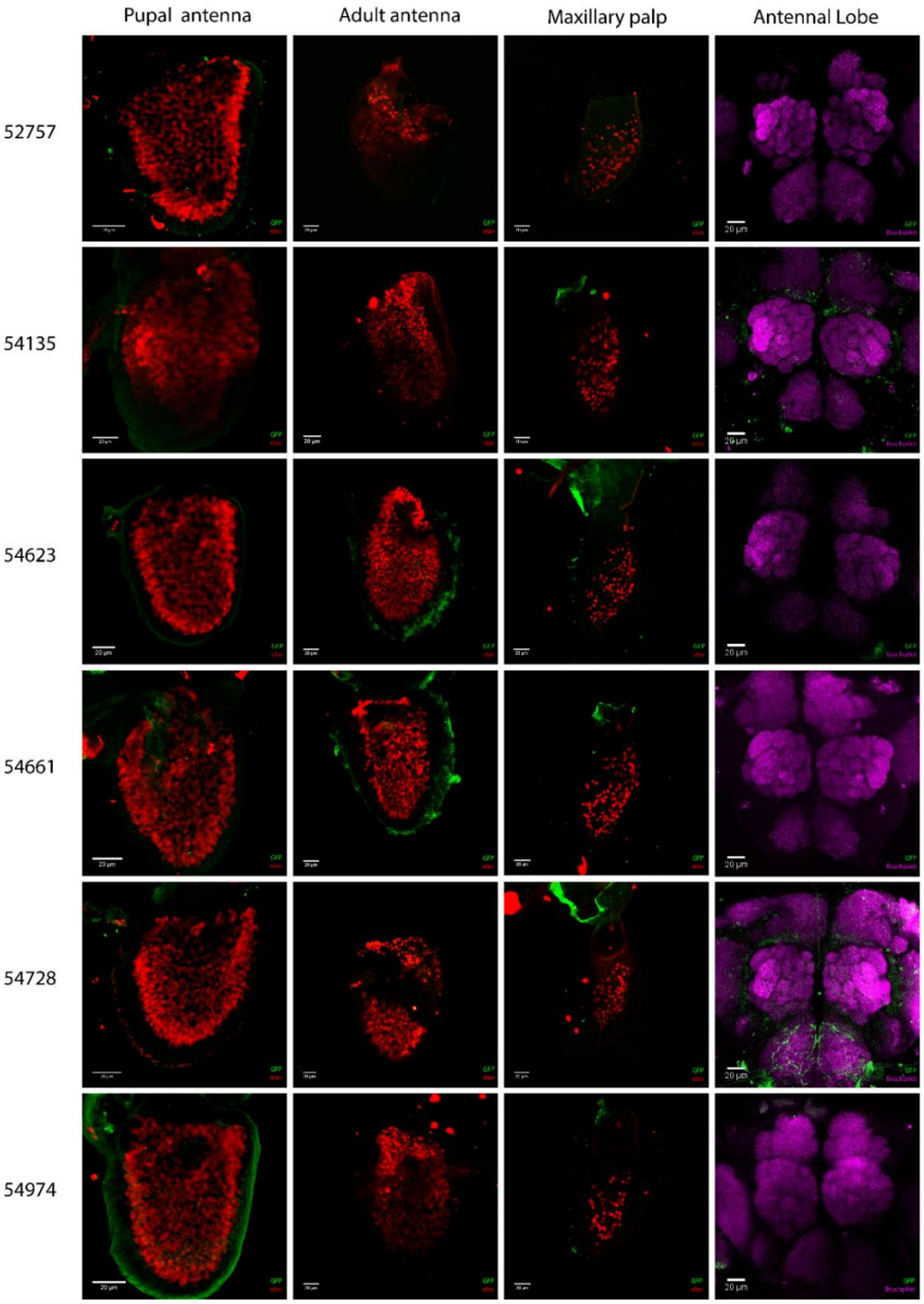

Figure 4.5. Analysis of transgenic LexA lines. Adult brain stained using antibodies against GFP (green) and Bruchpilot (magenta). Olfactory organs stained using antibodies against GFP (green) and Elav (red). Expression pattern in antennal lobe was not observed in none of the analyzed transgenic LexA lines.

4.2. Analysis of iroC Mutants for their Role in Asymmetric Cell Division

In order to elucidate the involvement of *IroC* in asymmetric cell division in the sensory organ lineage two different iroC mutations were used and the fate of the cells was determined by immunohistochemical staining using specific markers.

4.2.1. iroC Mutants

In our laboratory, several iroC mutant fly lines were obtained in order to study the function of *IroC* genes (Figure 4.6.). iro^{DFM3} is a triple mutant in which *ara* and *caup* sequences and the regulatory region of *mirr* is deleted. $mirr^{E48}$ is a single mutant in which *mirr* sequences are deleted, while *ara* and *caup* are not disrupted. *Caup* is a single mutant that has been generated in our laboratory (Çayıroğlu, 2019).

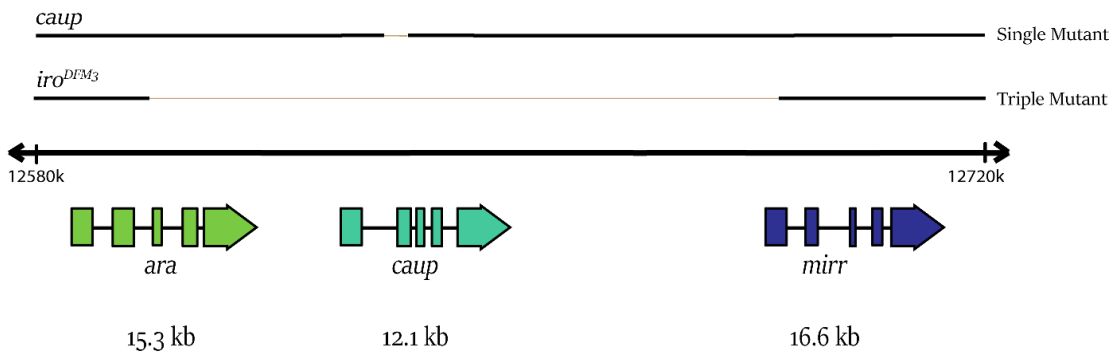

Figure 4.6. Physical Map of the IroC Locus and representation of iroC mutants

IroC mutations are homozygous lethal, except for the *caup* mutant. Nevertheless, to be able to perform MARCM analysis, induce the mutation at a specific time, and label the progeny the *caup* mutation had to be recombined on an FRT chromosome.

4.2.2. Recombination of *caup* mutant onto FRT80 Site

MARCM is a technique that enables one to investigate the function of a gene in a specific subset of cells. In the developing antenna for instance, sensory organ precursors derive from SOPs. It was necessary to restrict the number of cells within multiple sensory organ lineages in order to analyze single precursor cells within a lineage as it is not possible to track single cells within a crowded epidermal field. The MARCM technique makes this possible by labeling only some of the subsets of cells with GFP. Other subset of cells in the developing organ are not labeled and thus are excluded from the analysis. The second benefit of the MARCM technique is that only the subsets of cells that are homozygous for the mutation of interest are labeled with GFP. These cells are called MARCM clones and are homozygous for the mutation of interest, while other cells in the developing organ are heterozygous for the mutation of interest. Analyzing homozygous mutant cells is the most reliable approach to study the effects of a relevant gene.

Homozygous mutant cells are derived from heterozygous precursors via mitotic recombination which is mediated by FLP/FRT. Mitotic recombination is induced via a heat shock-inducible flippase enzyme (FLP) that recognizes a pair of flippase recombinase target (FRT) sequences on *in trans* chromosome arms and causes recombination between these two FRT sites. In MARCM, one FRT sequence flanks a repressible cell marker while the other FRT sequence flanks a genomic region of interest (Blair, 2003). Mitotic recombination results in three different cell types: daughter cells homozygous for the mutation of interest that are labeled with a GFP reporter, daughter cells homozygous for a repressible cell marker that are not labeled with a reporter protein, and heterozygous cells, that are also not labeled.

To use the *caup* mutation in a MARCM experiment it was recombined onto a FRT80 chromosome that is located on the third chromosome. The FRT80 site was selected because it is located on the same chromosomal arm on the third chromosome as *caup*. As a first step, the recombination frequency was calculated. Recombination frequency is generally calculated by using charts which statistically compare genomic position of selected regions (in centiMorgans) and cytological positions of these selected regions that are thought to recombine during meiosis

(Singh *et al.,* 2005). The cytological positions of *caup* and *FRT80* were used to determine the recombination distance in centiMorgan (cM). The difference between recombination distances is equal to the recombination frequency. Here, recombination rate between genomic position of *caup* and FRT80 site was calculated as 9%. In other words, 9% of the offspring would be recombinants between *caup* and *FRT80*. Thus, to obtain at least one recombinant 50 crosses were set.

Table 4.1. Recombination frequency. Using recombination distance, it is possible to estimate recombination frequency.

	Sequence Coordinates	**Cytological Position**	**Recombination Distance** (in centi Morgan)	**Recombination Frequency** (distance in centi Morgan)
caup	3L: 12607930-12736869	69D1	38	47-38=9
FRT80	3L: 12607930-12736869	80	47	

The *caup* mutant line was crossed to fly line carrying the *FRT80* site and offspring (F1) carrying both alleles were selected. Since *Drosophila* males segregate their chromosomes by a mechanism that completely lacks crossing overs during meiosis, recombination occurs only in females (Hughes *et al.,* 2018). Thus, virgin F1 females were collected and crossed to a wild-type fly line. The flies were put into vials containing approximately 10 ml of fly food and 100 µl of 25 mg/ml neomycin (G418) per vial (Figure 4.7.) to select for the *FRT80* transgene that carries a neomycin resistance gene. Flies that survive on neomycin-supplemented fly food are assumed to carry the *FRT80* site (F2). Flies that did not recombine with *FRT80* and carried only the *caup* mutation did not survive. Wild-type flies were used as negative control and iro^{DFM3} mutant containing a verified *FRT80* site was used as a positive control to test the effectivity of the neomycin treatment. As expected, neomycin was lethal to wild-type offspring but not to iro^{DFM3} mutant offspring. Male flies that survived neomycin selection (F2) were crossed one by one to a wild-type fly line. The resulting fly lines include flies that carry only the *FRT80* site or

flies that are recombinants between *caup* and *FRT80*. To distinguish between these possibilities genomic PCR analysis on offspring of single crosses (F3) was performed to test for the presence of the *caup* mutation.

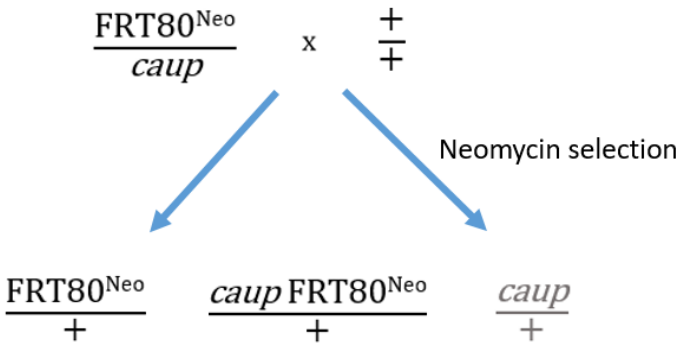

Figure 4.7. Neomycin selection. Virgin flies which contain *caup* mutation and *FRT80* alleles crossed to wild-type fly line and offspring were grown on Neomycin containing medium. As *FRT80* transgene contains a *neomycin* resistance gene, flies that survive on neomycin-containing medium were carrying *FRT80* site which include flies carrying recombined version of *caup* onto *FRT80* site (*caup FRT80*) and flies carrying only *FRT80* site. Flies carrying only *caup* mutation die due to neomycin toxicity.

The $caup^{17}$ mutant has a 106 base pair deletion in the coding region of the *caup* gene and thus a functional protein cannot be produced (Çayıroğlu, 2019). After neomycin selection, 41 single crosses were set for $caup^{17}$. 20 crosses produced viable progeny (F3) and were used for genomic DNA extraction and subsequent PCR analysis in order to validate the recombination event. PCR screening was performed using primers flanking the mutation as described in Çayıroğlu, 2019. Genomic DNA of w^{1118} was used as a negative control and genomic DNA of $caup^{17}$ homozygous mutant was used as a positive controls (Figure 4.14.). PCR results showed a wild-type band in all lines and a mutant band in fly lines 17.7, 17.13,17.17 (Figure 4.8.A), 17.24, 17.25, 17.29 and 17.32 (Figure 4.8.B). Thus, the recombination event was verified in 7 out of 20 lines, which indicates a recombination frequency of 35%, which is much higher than expected.

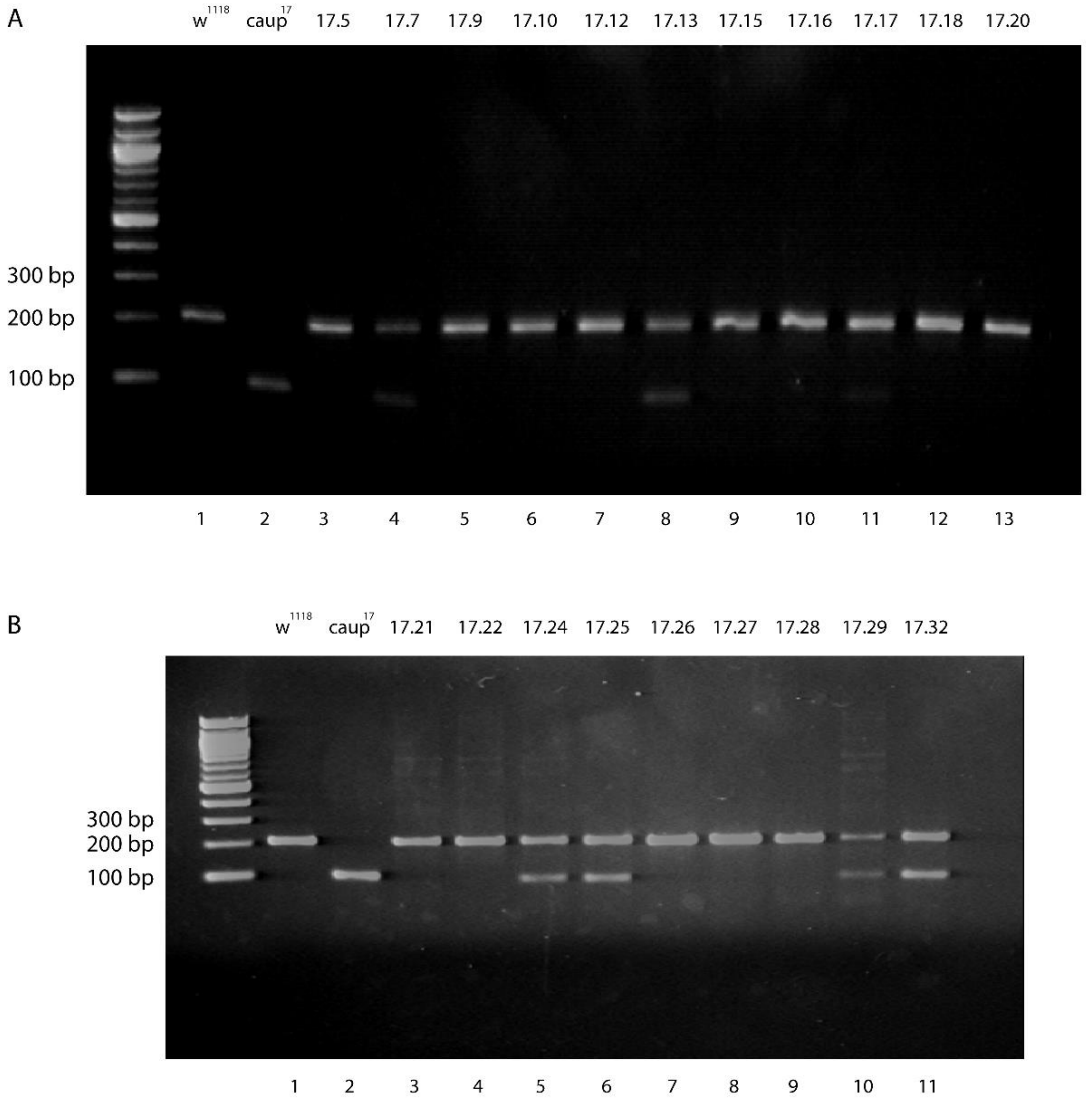

Figure 4.8. PCR screening to validate *caup*^{17} recombination onto FRT80. There are two bands in lane 4, 8 and 11; represents 17.7, 17.13 and 17.17 fly lines (A). There are two bands in lane 5, 6, 10 and 11; represents 17.24, 17.25, 17.29 and 17.32 fly lines (B) Heavy band on the gel is for wild-type allele and band size is ~207 bp long. Lighter band is for mutant allele and band size is ~100 bp long because of the deletion that created by CRISPR-Cas9 (Çayıroğlu, 2019). In lane 2 (A, B) and only lighter band is present.

4.2.3. Clonal Analysis

One of the recombinants generated in 4.2.2. was selected for use in MARCM clonal analysis experiments. In the meantime, the MARCM ready line containing all the other necessary alleles for a successful MARCM experiment such as hs-flp, tub-Gal4, tub-Gal80 and UAS-GFP was ordered and tested. Additionally, the MARCM ready line contains the *Minute* (*Min*) mutation on the third chromosome. Dominant *Min* mutation is reported to slow the cell division rate by altering ribosome functions (Blair, 2003). Since *Min* is found recombined to *tub-Gal80 FRT80*, it is not segregated to clonal cells and does not affect their division rate while other cells become smaller. The cross shown in Figure 4.9 was set in excess and offspring carrying all above-mentioned alleles were selected and subjected to heat-shock treatment.

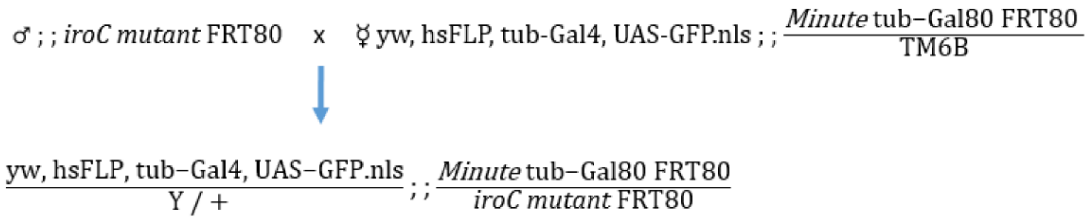

Figure 4.9. A crossing scheme for conducting MARCM experiment using iroC mutants. *IroC* genes are located on the third chromosome, thus, FRT80 site located on the third chromosome is needed for this study.

The MARCM workflow shown in Figure 4.10 was used. Flippase expression was induced by a heat-shock treatment at the larval stage where SOP specification has not started yet, in order to generate clones that involve all cells in a sensory organ lineage. This ensures that clonal cells are derived from a single SOP, and are not derived from different cells in the sensory organ lineage. Thus, it was possible to investigate the role of *IroC* at every step of the sensory organ lineage.

After heat shock, larvae were raised at 18°C to synchronize their development and for allowing more effective silencing of the Gal4 protein by Gal80 in dividing SOPs. Once

puparium formation started, animals were transferred to 29°C incubation to advance pupal growth and to increase the efficiency of Gal4 protein in homozygous clone cells (del Valle Rodríguez *et al.*, 2012). This procedure enhanced the GFP signal of the homozygous clone cells. At 18-21 hours After Puparium Formation (APF), pupae were dissected and immunostained with antibodies against relevant proteins that are expressed at specific stages of the sensory organ lineage. Alternatively, pupae were fixed and stored at 4°C for future experiments.

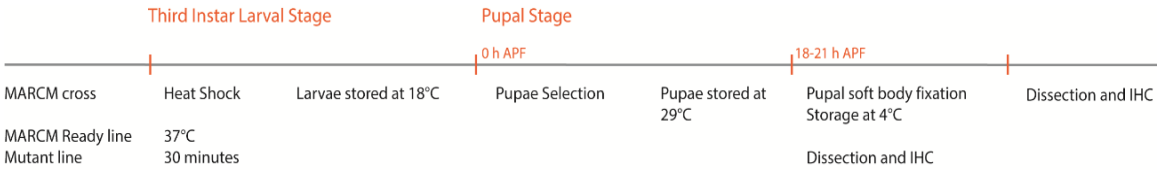

Figure 4.10. MARCM workflow. Larvae were heat-shocked and incubated at 18°C. Pupae were selected according to their phenotypes and incubated in 29°C. After around 21 hours, developing antennae were directly dissected or pupal soft body was fixed and stored at 4°C for future experiments.

4.2.3.1. Clonal Analysis Using Cell Type-Specific Markers. The *Drosophila melanogaster* peripheral sensory organ lineage starts to form by division of a stem cell, SOP. A single SOP gives rise to a specific type of sensillum lineage. At the end of such a sensory organ lineage, 4 supporting cells and 4 neuronal cells are produced. In a sensory organ lineage, Notch-mediated asymmetric cell divisions occur successively by differential distribution of the Numb protein.

Cell fate acquisition by cell fate determinants in sensory organ lineage is achieved by a "combinational code" in which cell fate determinants are differentially expressed and every cell has a specific character in the lineage. Thus, cell fate determinants can be used as "cell type-specific markers" while conducting clonal analysis experiments to understand the fate of each cell.

4.2.3.2. Notch/Numb. Notch/Numb are the first cell fate determinants affecting cell identities in the sensory organ lineage (Nolo *et al.,* 2000). Notch receptor trafficking is disrupted by Numb; Numb inhibits the recycling of Spdo, and thus results in inactive Notch.

Clonal analysis of iro^{DFM3} triple mutants and *caup* single mutants are summarized in the following figures.

Immunohistochemical analysis of MARCM clones in the iro^{DFM3} mutant background shows that in 21 different 2-cell clones analyzed none of the SOP daughter cells express Notch. Also, 4 different 2-cell clones analyzed in the *caup* mutant background shows that Notch is not expressed in SOP daughter cells. Unlike the wild-type clones the pIIa cell becomes inactive for Notch. At this point it is unclear which of the cells in the MARCM clone are originally pIIa or pIIb, thus, they were not marked in the summary diagram (Figure 4.11. and Figure A.1.- A.3.). However, it can be concluded that the pIIa cell is transformed to a pIIb-like cell.

Analysis of 12 different 3-cell clones in iro^{DFM3} mutant background and 8 different 3-cell clones in *caup* mutant background using Notch Intracellular Domain (NID) and GFP antibodies showed that none of the clonal cells expressed NID. This is not due to the fact that the antibody is not working as other cells in the tissue were stained for Notch (Figure 4.12. and Figure A.4.). Thus, lack of iroC leads to the transformation of these cells to a Notch-OFF phenotype, where pIIa is transformed to a pIIb-like cell. Neither pIIb cells nor its progeny expresses Notch. These data are consistent with the data obtained with 2-cell clones.

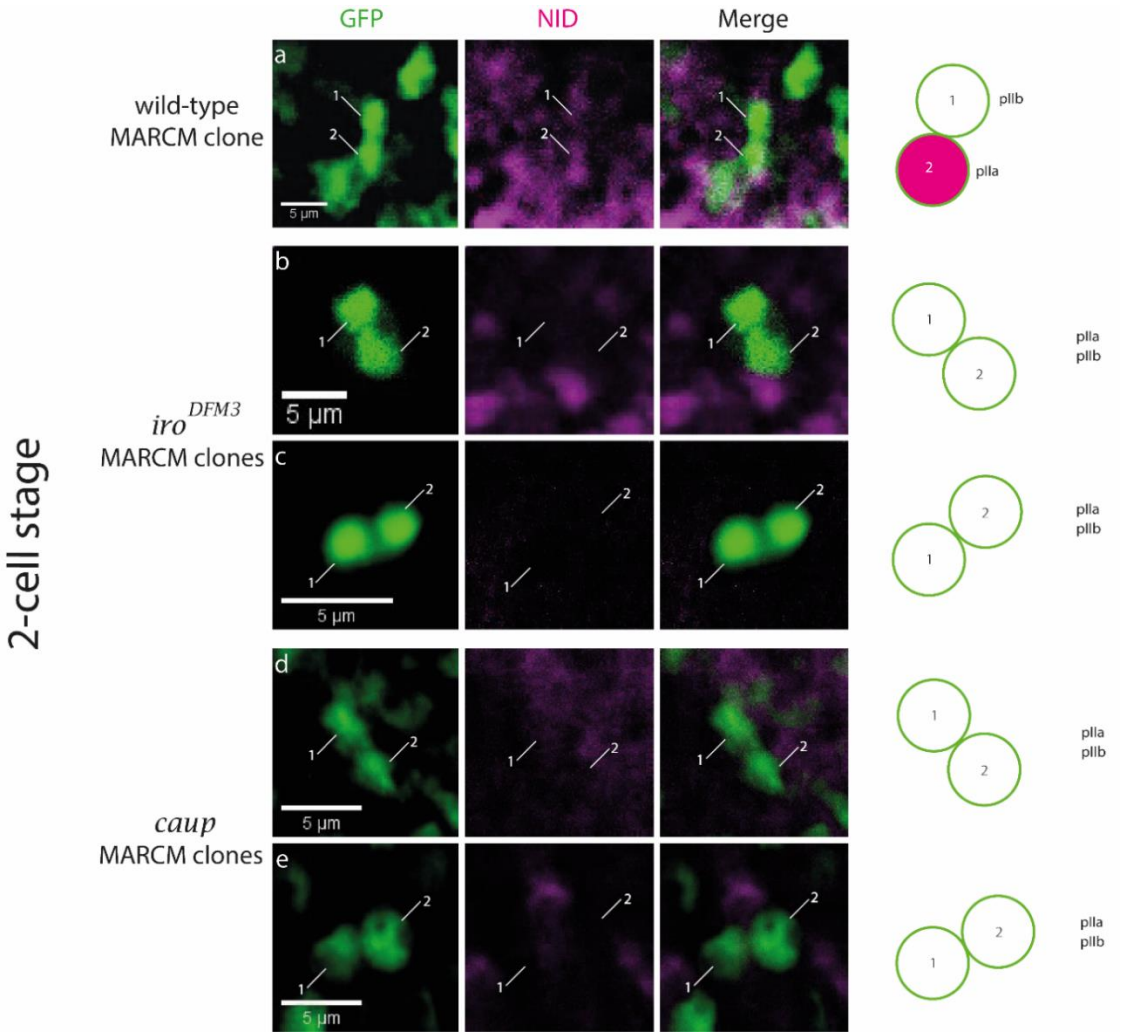

Figure 4.11. 2-cell MARCM clones in wild-type background, *iro*^{DFM3} and *caup* mutant backgrounds. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Notch Intracellular Domain (magenta). Notch is expressed in one of the SOP daughters, pIIa in wild-type clones (a). None of the SOP daughter cells express Notch in *iro*^{DFM3} clones (b, c) and *caup* mutant clones (d, e). Each cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

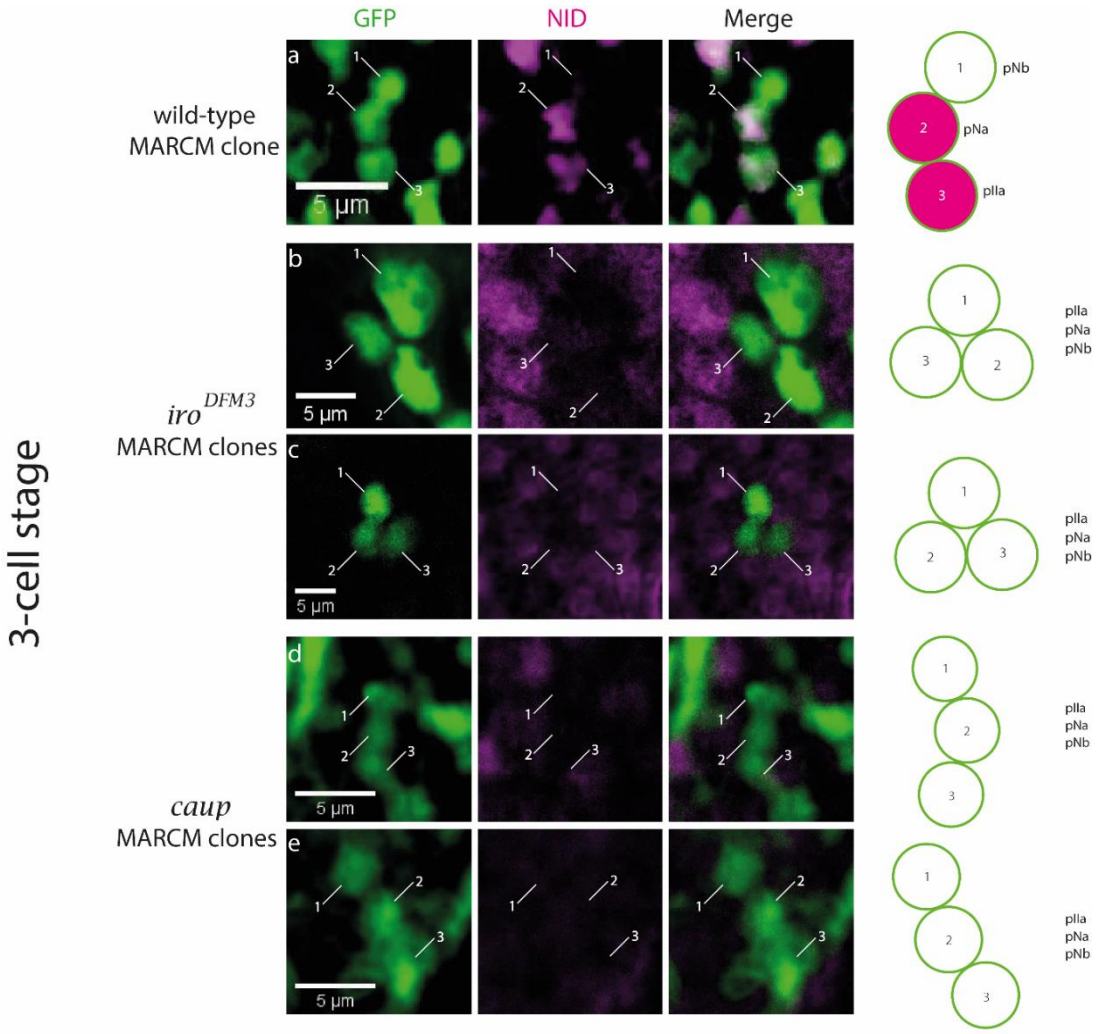

Figure 4.12. 3-cell MARCM clones in wild-type background, *iro*^{DFM3} and *caup* mutant backgrounds. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Notch Intracellular Domain (magenta). Notch is expressed in pIIa and pNa in wild-type clones (a). None of the cells express Notch in *iro*^{DFM3} clones (b, c) and *caup* clones (d, e). Each individual cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

At the 4-cell stage, Notch is expressed in pOa and pNa in wild-type clones. In 8 different 4-cell *iro*^{DFM3} MARCM clones and 4 4-cell *caup* MARCM clones, none of the clonal cells express Notch suggesting that the cell lineage continues to branch without Notch activity (Figure 4.13 and Figure A.5). Notch was not activated during SOP specification in the lack of iroC and cells of the sensory organ lineage differentiate without Notch expression. On the other hand, *caup* phenotype is the same as the phenotype gathered from *iro*^{DFM3} MARCM clones suggesting that the absence of iroC proteins affects Notch similarly.

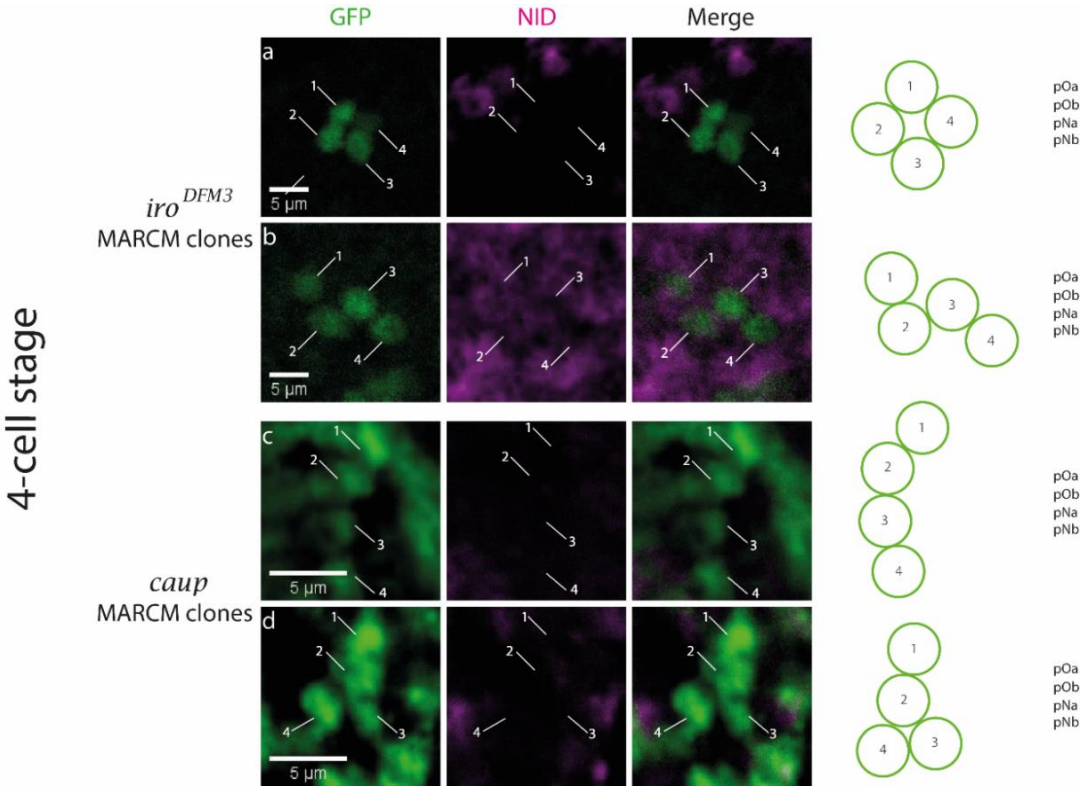

Figure 4.13. 4-cell MARCM clones in *iro*^{DFM3} and *caup* mutant backgrounds. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Notch Intracellular Domain (magenta). Notch is not expressed in *iro*^{DFM3} clones (a, b) and *caup* clones (c, d). Each individual cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

Notch is expressed in pOa, Naa, Nba at the both 5-cell stage and 6-cell stage in wild-type background. Additionally, it starts to be expressed in pOb cell at the 7-cell stage. However, single MARCM clone analysis per each of these cell stages showed that none of the cells express Notch in *iro*^{DFM3} mutant background (Figure 4.14.)

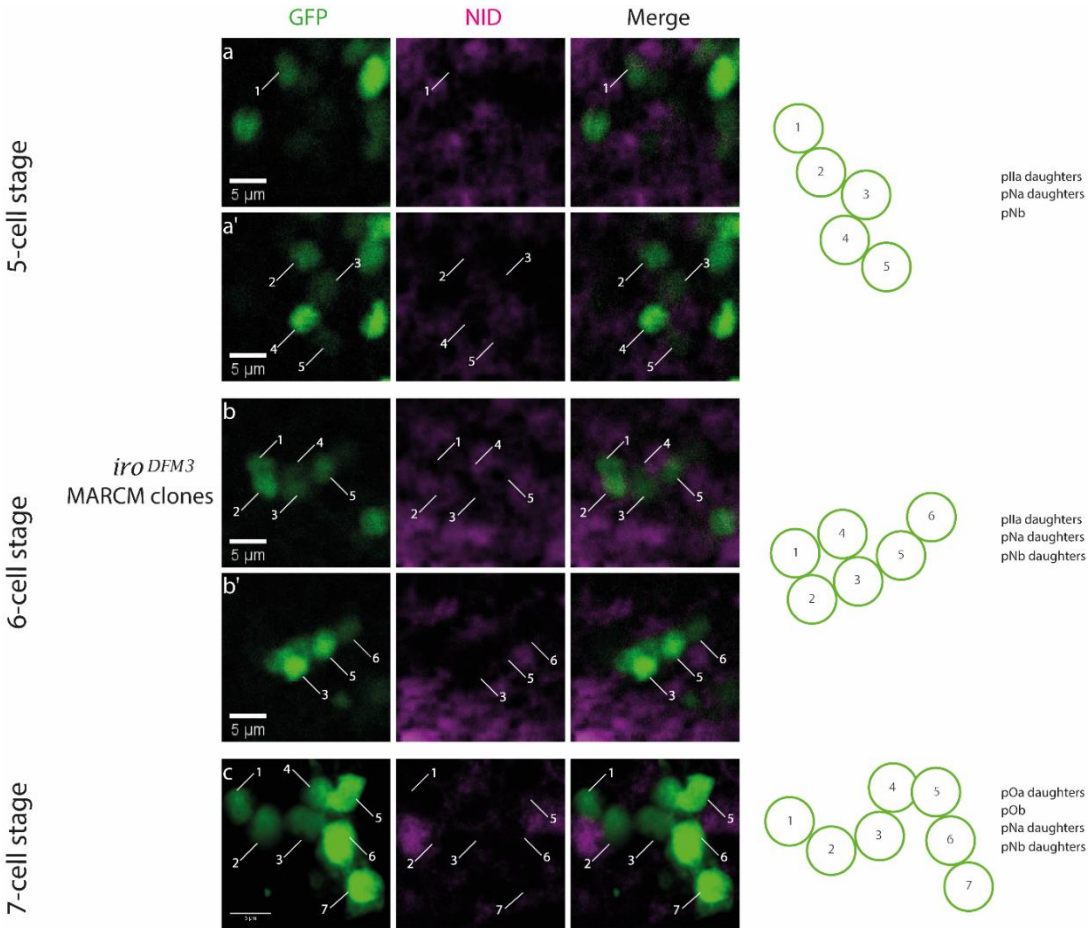

Figure 4.14. 5-cell, 6-cell and 7-cell MARCM clones in *iro*^{DFM3} mutant background. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Notch Intracellular Domain (magenta). Notch is not expressed at the 5-cell stage (a, a'), 6-cell stage (b, b') and at the 7-cell stage (c). Each cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

Clonal analysis using NID is summarized in Figure 4.15. Notch is widely expressed in sensory organ lineage to specify precursors of supporting cells and neuronal cells (Figure 4.15A). However, my data clearly show that Notch expression is lost in lack of iroC (Figure 4.15B). This suggests that terminal cell fates are changed in *IroC* mutant clones and that iro proteins act as cell fate determinants.

Clonal analysis results for wild-type, $iroC^{DFM3}$ and *caup* are summarized in Table 4.2., and show that the ratio of Notch-positive cells is actually zero.

Table 4.2. Ratio of cell type-specific marker Notch in wild-type and mutant background.

Cell Stages (wild type)	**Number of cells expressing marker**	**Ratio**
2	1	0,50
3	2	0,66
4	2	0,50

Cell Stages ($IroC^{DFM3}$)	**Number of Replicates**	**Number of observed cells**	**Number of cells expressing marker**	**Ratio**
2	21	42	0	**0**
3	12	36	0	**0**
4	8	32	0	**0**
Cell Stages (*caup*)	**Number of Replicates**	**Number of observed cells**	**Number of cells expressing marker**	**Ratio**
2	4	8	0	**0**
3	8	24	0	**0**
4	4	16	0	**0**

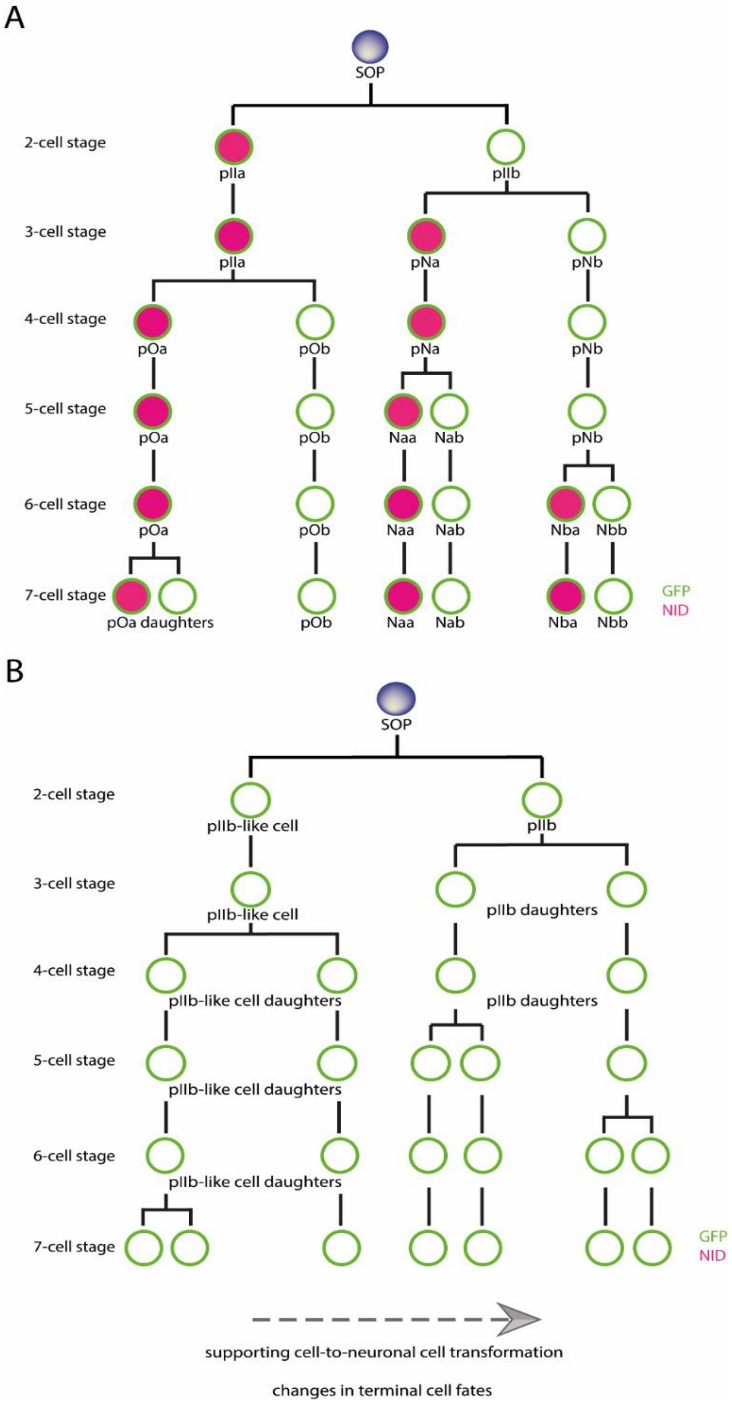

Figure 4.15. Representation of Notch expression pattern in the sensory organ lineagein wild-type and iroC mutants. Notch contributes to cell fate determination of cells within sensory organ lineage (A). In the absence of iroC, Notch expression is lost leading to alterations in precursor cell fates and terminal cell fates (B).

Immunohistochemical analysis of MARCM clones was also performed for the Notch antagonist Numb. Numb is inherited by pIIb cell at the 2-cell stage in wild-type background. However, analysis of 3 different 2-cell MARCM clones shows that in the *iro*^{DFM3} mutant background both of the SOP daughters express Numb (Figure 4.16.). Again, these data suggest that the pIIa cell is transformed to a pIIb-like cell.

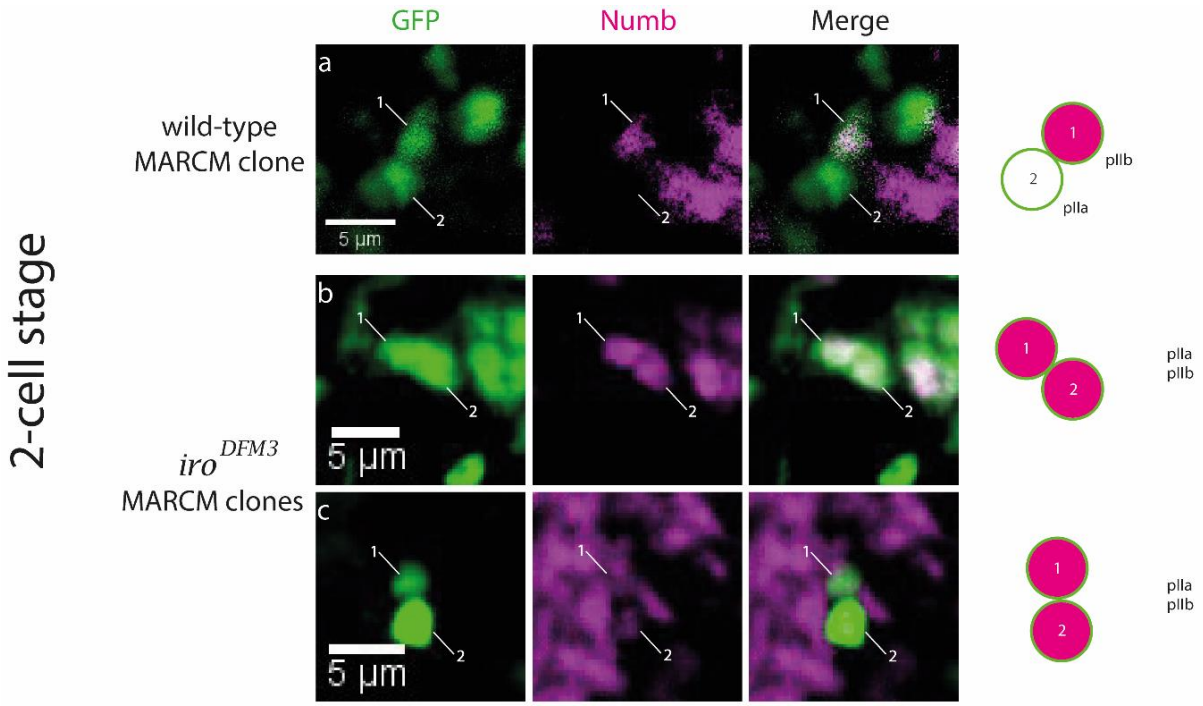

Figure 4.16. 2-cell MARCM clones in wild-type and *iro*^{DFM3} mutant background. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Numb (magenta). SOP daughter pIIb inherits Numb in wild type background (a). Both of the SOP daughters express Numb in the absence of iroC (b, c). Each individual cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

Further analysis of Numb in larger clones (3 different 4-cell MARCM clones) showed that Numb is expressed in both of the branches of the sensory organ lineage in the iroC mutant background, although it is normally expressed only in the branch that gives rise to ORNs (Figure 4.17.).

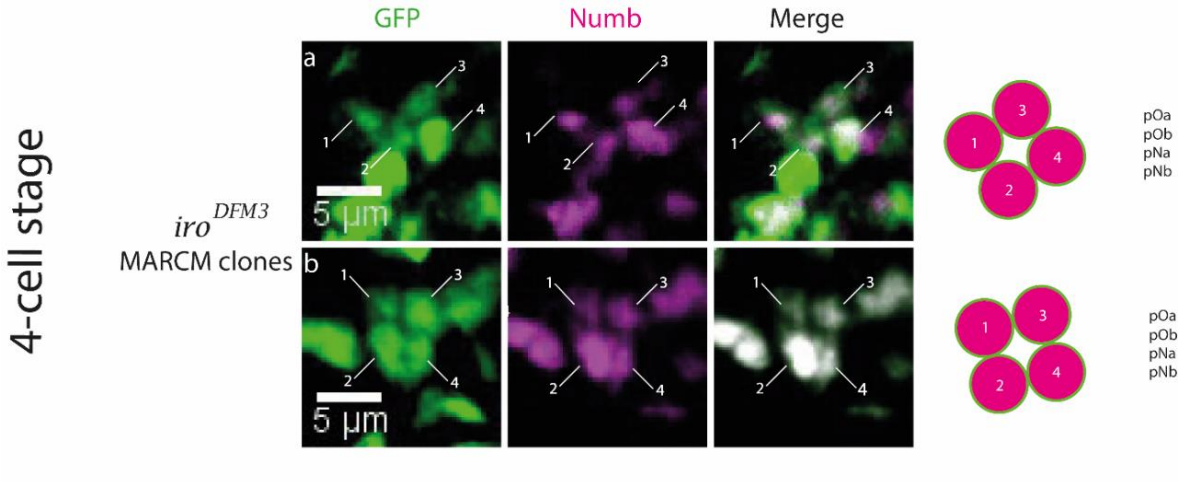

Figure 4.17. 4-cell MARCM clones in *iro*^{DFM3} mutant background. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Numb (magenta). All of the cells at the 4-cell stage express Numb in *iro*^{DFM3} mutant background (a, b). Each individual cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

Clonal analysis results for Numb are summarized in Figure 4.18. Numb is known to be expressed in pIIb at the 2-cell stage and pOb and pNb at the 4-cell stage (Figure 4.18A). However, all of the cells express Numb suggesting changes in cell fates (Figure 4.18B).

Table 4.3. summarizes MARCM analysis data and clearly shows the increase in Numb expression in the *iro*^{DFM3} mutant background.

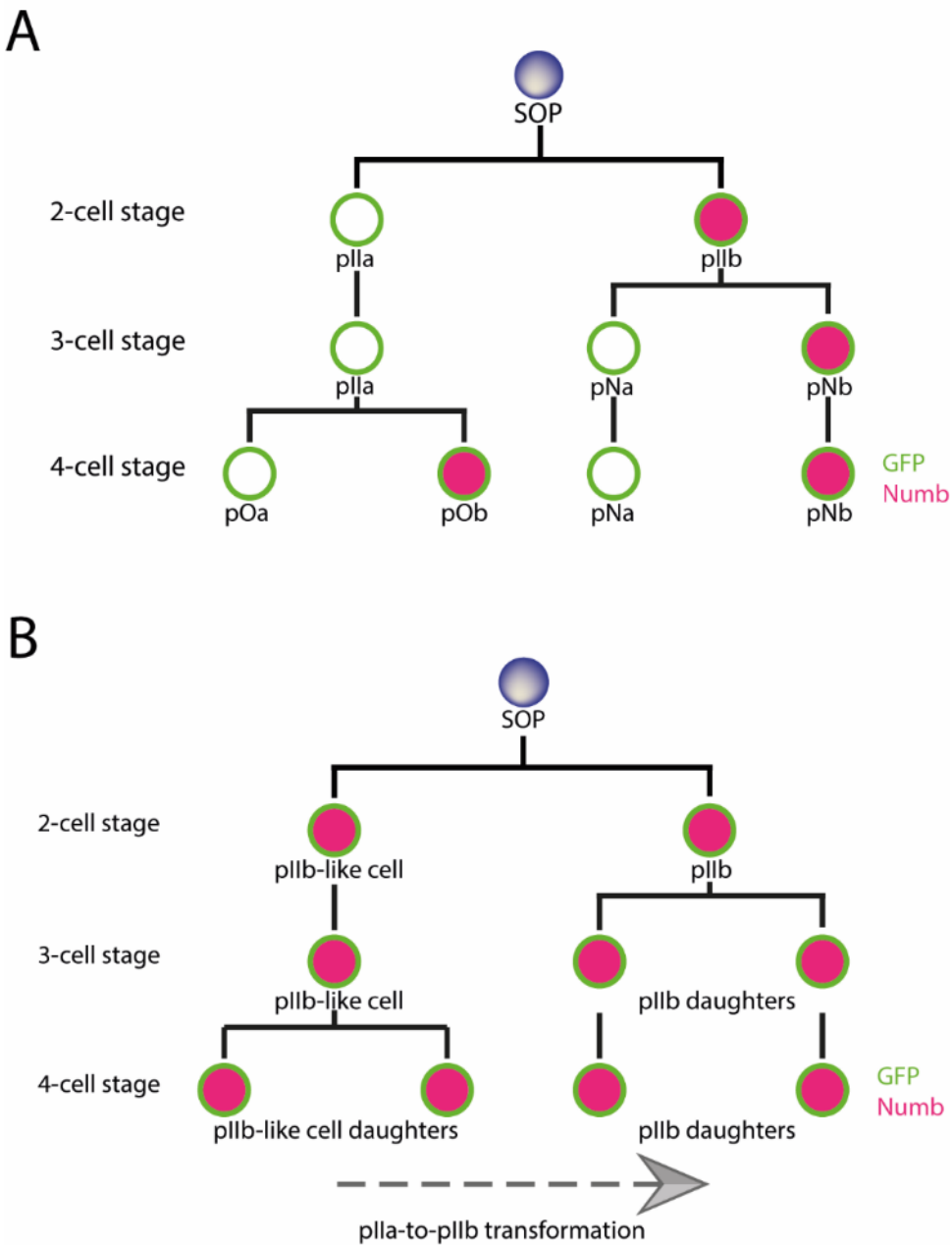

Figure 4.18. Representation of Numb expression pattern in the sensory organ lineage. (A) Numb is inherited by pIIb at the 2-cell stage and expressed in pOb and pNb at the 4-cell stage. All of the cells at the 2- and 4-cell stage express Numb in *iro*^{DFM3} mutant background

Table 4.3. Ratio of cell type specific marker Numb in wild-type and mutant background.

Cell Stages (wild type)	**Number of cells expressing marker**	**Ratio**
2	1	0,50
4	2	0,50

Cell Stages (*IroC*^{DFM3})	**Number of Replicates**	**Number of observed cells**	**Number of cells expressing marker**	**Ratio**
2	3	6	6	**1**
4	3	12	12	**1**

4.2.3.3. Senseless. Sens is expressed in the pIIb cell where it suppresses E(spl) activity and contributes to impaired Notch signaling profile. Although it was assumed that *sens* is also expressed in pIIa (Endo *et al.*, 2007), later it was reported that *sens* is very slightly expressed in some of the pIIa cells but expression is lost in the same cells (Chai *et al.*, 2019). It is lost because sens is a repressor of cut and these transcription factors cannot function in the same cell. At the 3-cell stage, pIIa starts to express cut when sens expression is lost.

Sens is only expressed in pIIb cell in wild-type clones at the 2-cell stage. Immunohistochemical analysis of MARCM clones in the *iro*^{DFM3} mutant background showed that in 11 2-cell clones analyzed both of the SOP daughter cells express sens. Similarly, 5 2-cell clones in the *caup* mutant background showed that *sens* is expressed in both of the SOP daughter cells (Figure 4.19.). On the other hand, 6 *iro*^{DFM3} 3-cell clones showed that sens is expressed not only in the pIIb cell, but also in the pIIa daughters (Figure 4.20.). These phenotypes are consistent with the previously analyzed MARCM clones; the pIIa cell is transformed to a pIIb-like cell in the absence of iroC.

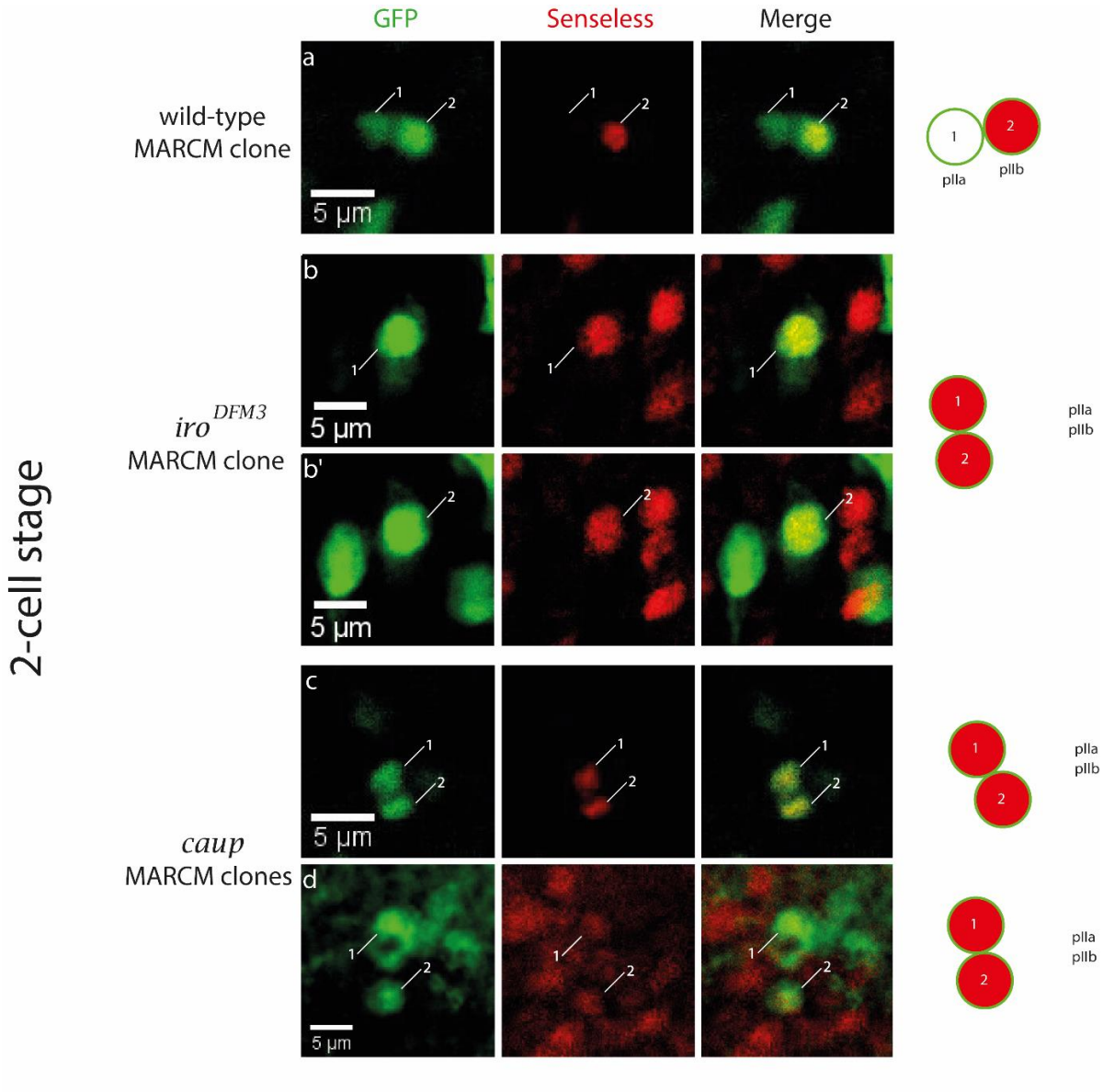

Figure 4.19. 2-cell MARCM clones in wild-type background, *iro*^{DFM3} and *caup* mutant backgrounds. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Sens (red). Unlike the wild-type phenotype (a), both of the SOP daughters express *sens* in *iro*^{DFM3} and *caup* mutant backgrounds (b-d). Each individual cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

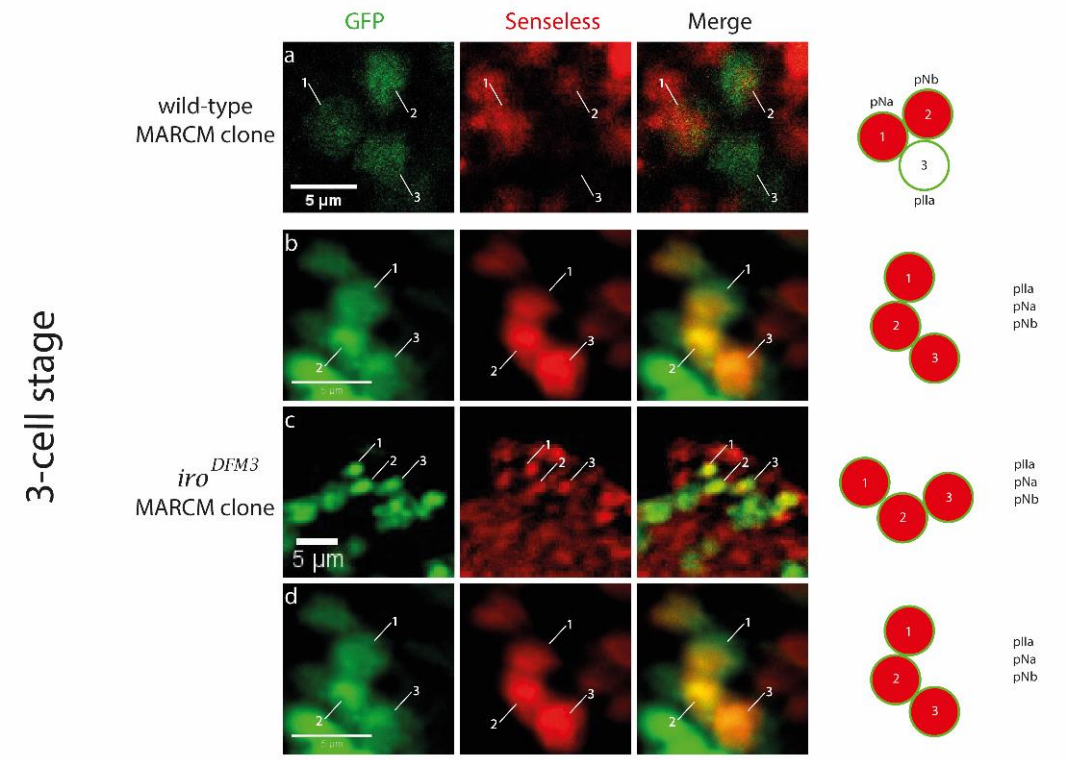

Figure 4.20. 3-cell MARCM clones in wild-type and *iro*^{DFM3} mutant backgrounds. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Sens (red). *sens* is only expressed in pIIb cell in wild-type clones (a). In *iro*^{DFM3} mutant background, sens is expressed not only in the pIIb cell but also in the pIIa daughters suggesting that the pIIa cell is transformed to a pIIb-like cell in the absence of iroC (b, c). Scale bar = 5 µm

Alterations in the cell fates are clearly observed at the 4-cell stage when iroC is deficient (Figure 4.21.). In wild-type clones, pIIb daughters pNa and pNb express sens while pOa and pOb do not. pOa and pOb normally give rise to the supporting cells of the sensory organ lineage. However, their *sens* expression profile in the absence of iroC supports cell fate transformation from supporting to neuronal cell fates. 5 and 2 different 4-cell clones were analyzed for *iro*^{DFM3} and *caup* mutants, respectively.

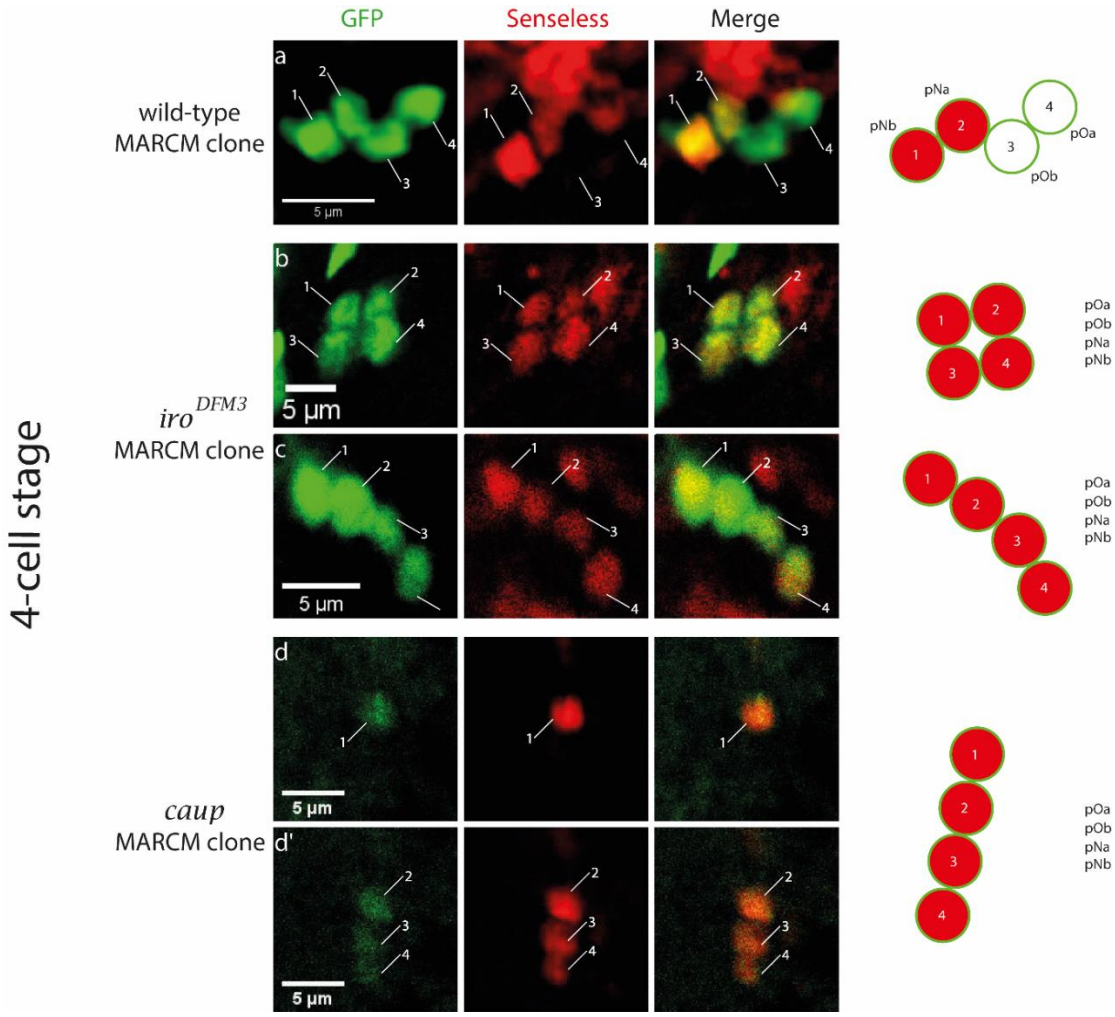

Figure 4.21. 4-cell MARCM clones in wild-type background, *iro*^{DFM3} and *caup* mutant backgrounds. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Sens (red). *sens* is only expressed in the cells give rise to supporting cells (a). In lack of iroC, sens is expressed in all of the cells at the 4-cell stage (b-d'). Each individual cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

Clonal analysis results for Sens are summarized in Figure 4.22. Sens is expressed in the branch of sensory organ lineage which generates ORNs (Figure 4.22A). My analysis suggested that in the absence of iroC, all of the cells express sens. Thus, these results support the observation that cell fates were altered starting from SOP daughters (Figure 4.22B).

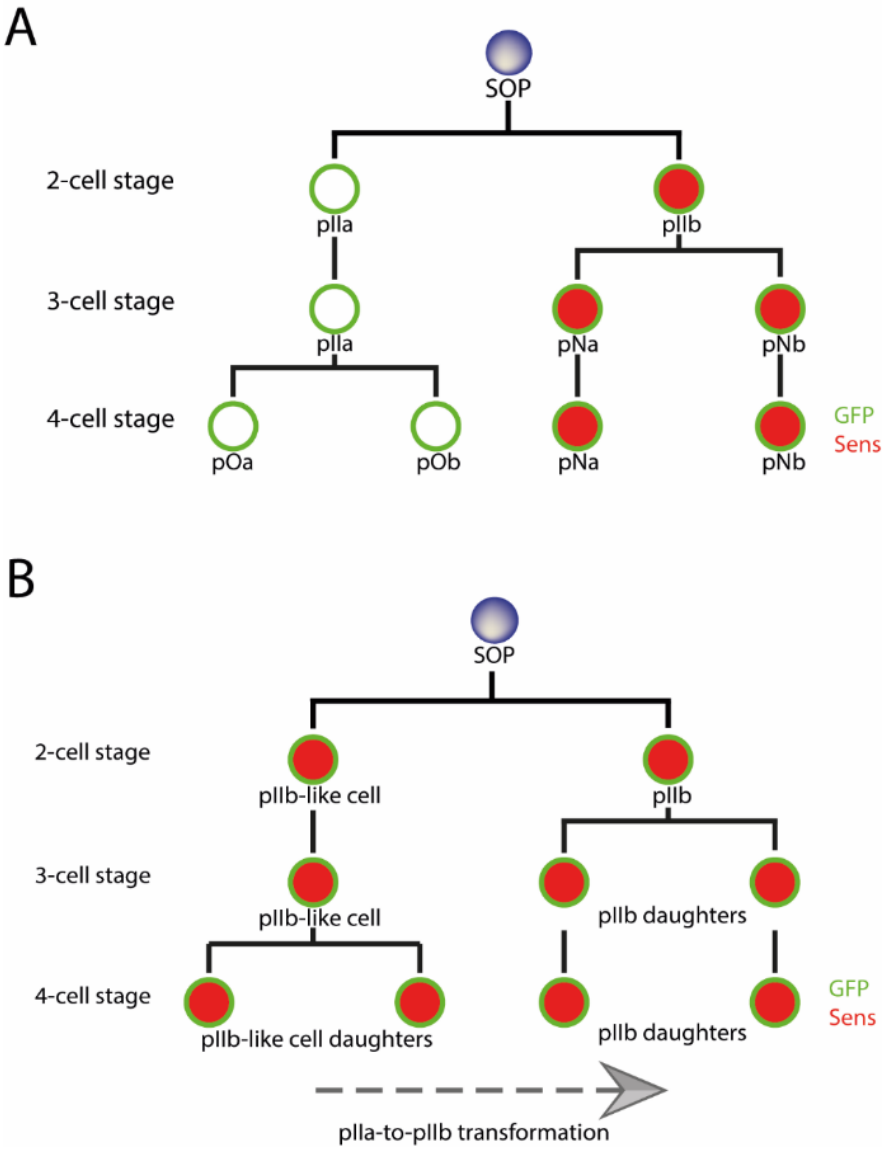

Figure 4.22. Representation of Sens expression pattern in the sensory organ lineage. *sens* expression is seen only in pIIb cell and the branch derives from pIIb (A). In lack of iroC, sens is expressed in all of the cells of 2-, 3- and 4-cell stages in the sensory organ lineage (B).

Clonal analysis data for sens are presented in Table 4.4.

Table 4.4. Ratio of cell type specific marker sens in wild-type and mutant background.

Cell Stages (wild type)	**Number of cells expressing marker**	**Ratio**
2	1	0,50
3	2	0,66
4	2	0,50

Cell Stages (*IroC*^{DFM3})	**Number of Replicates**	**Number of observed cells**	**Number of cells expressing marker**	**Ratio**
2	11	22	22	**1**
3	6	18	18	**1**
4	5	20	20	**1**
Cell Stages (*caup*)	**Number of Replicates**	**Number of observed cells**	**Number of cells expressing marker**	**Ratio**
2	5	10	10	**1**
4	2	8	8	**1**

4.2.3.4. Cut. The transcription factor cut is expressed in supporting cells and their precursor cells. Cut is a direct Notch target and its activity depends on Notch signaling (Reddy and Rodrigues, 1999). Additionally, it is known that the transcription factor sens inhibits *cut* expression.

Cut expression is first observed at the 3-cell stage where it is activated by Notch in pIIa. Also; *cut* is distinctively expressed in pIIa daughters pOa and pOb in wild-type background. Unlike the wild-type expression profile, cut expression is not observed in 3- and 4-cell stages when iroC is not present (Figure 4.23., Figure 4.24., Figure A.6.- Figure A.11.). This phenotype is caused by Sens misexpression and Notch depletion in the lack of iroC (29 and 19 replicates for 3- and 4- cell stages were analyzed, respectively).

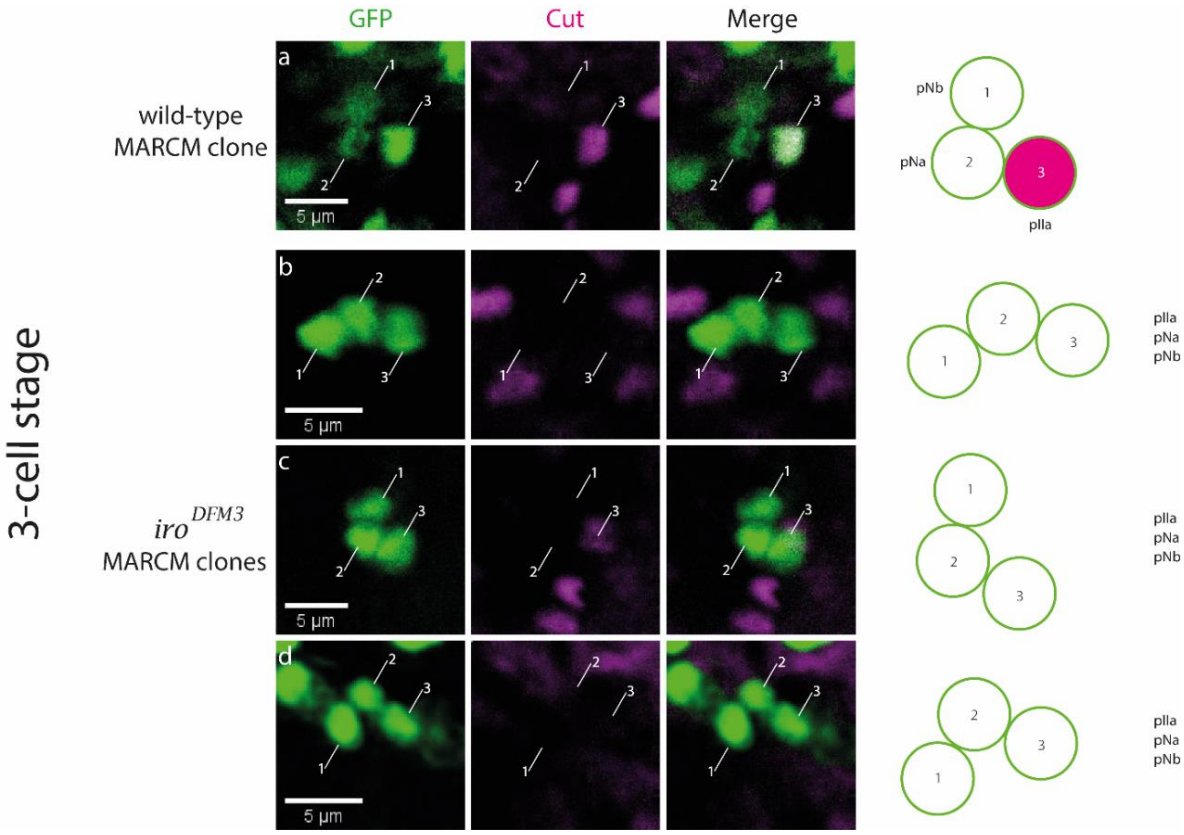

Figure 4.23. 3-cell MARCM clones in wild-type and *iro*^{DFM3} mutant backgrounds. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Cut (magenta). In sensory organ lineage, cut is firstly expressed in pIIa cell (a). None of the cells express cut in lack of iroC (b-d). Each cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

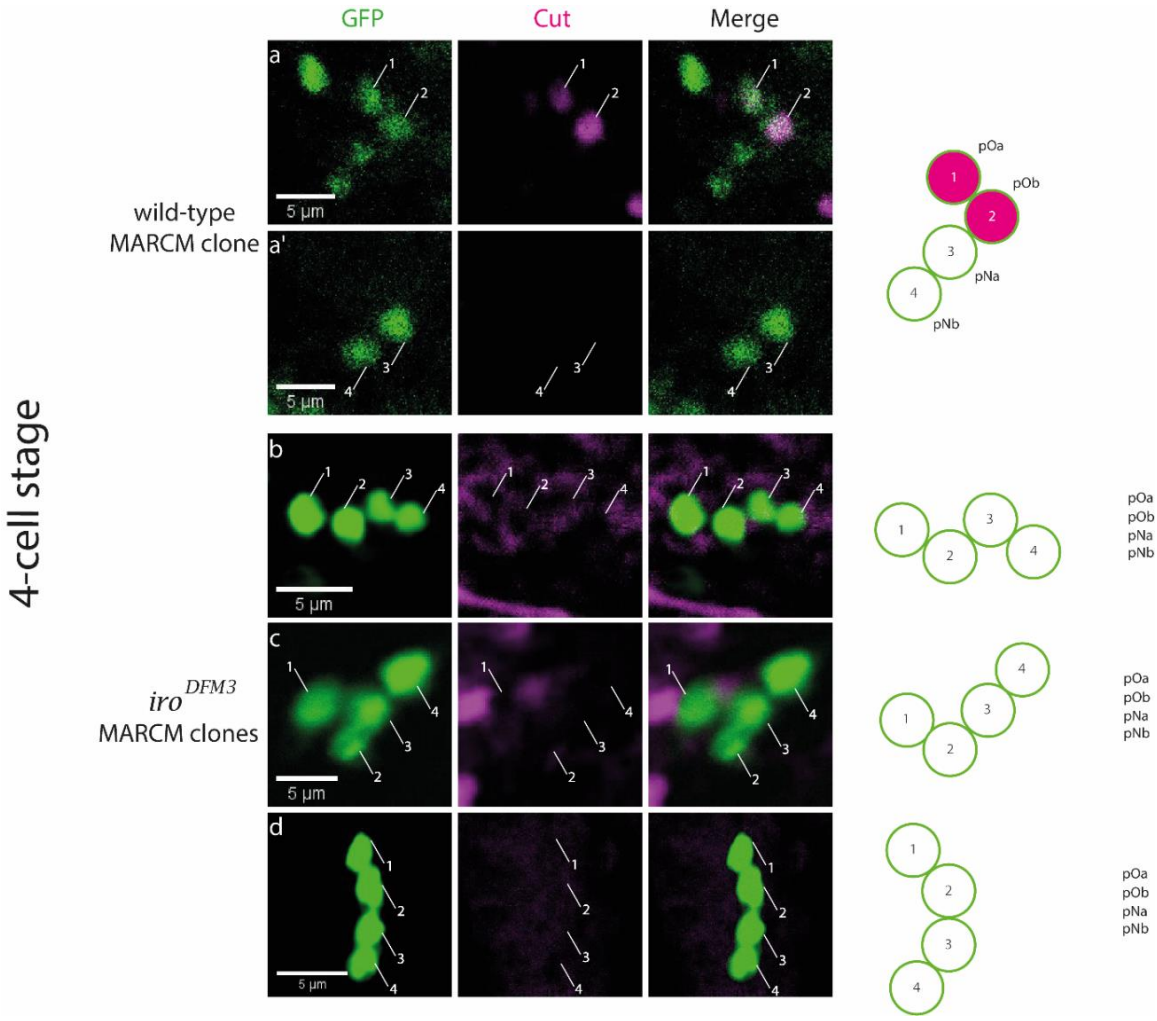

Figure 4.24. 4-cell MARCM clones in wild-type and *iro*^{DFM3} mutant backgrounds. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Cut (magenta). Cut is expressed in pOa and pOb cells in 4-cell clone (a-a') in wild-type background. None of the cells express cut in *iro*^{DFM3} mutant background. Each individual cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

Analysis of 8 5-cell clones showed that cut is not expressed in any of the cells. This phenotype also confirms that Notch cannot be activated in the later stages of sensory organ lineage. On the other hand, sens misexpression keeps cut silenced in the absence of iroC. Thus,

precursor cells of the supporting cells cannot be specified by the transcription factor cut (Figure 4.25, Figure A.12.).

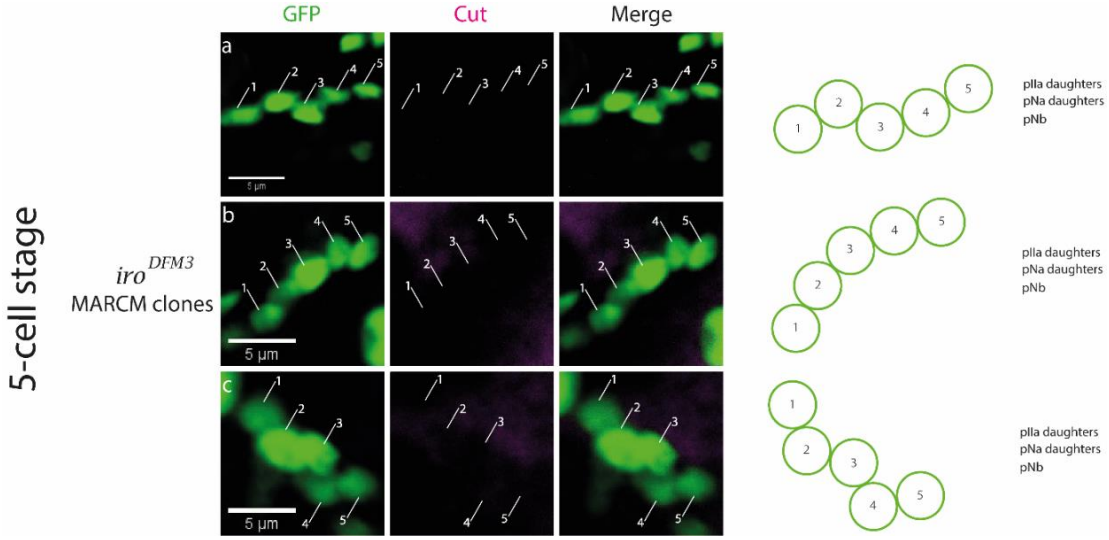

Figure 4.25. 5-cell MARCM clones in *iro*^{DFM3} mutant background. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Cut (magenta). Cut is not expressed in any of the cells in *iro*^{DFM3} mutant background. Each individual cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

Analysis of single clones of 6-cell and 8-cell stages suggests that cut is not expressed during terminal cell fate acquisition in the sensory organ lineage (Figure 4.26.). It is possible that more ORNs are produced in the absence of iroC. Further analysis is required to clarify this possibility. Clonal analysis results are summarized in Figure 4.27.

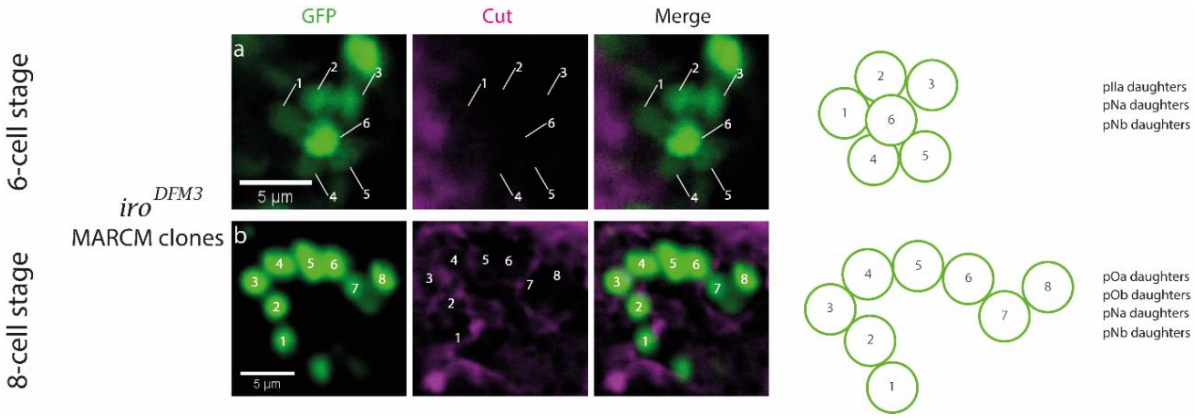

Figure 4.26. 6-cell and 8-cell MARCM clones in *iro*^{DFM3} mutant background. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker Cut (magenta). None of the cells express cut suggesting that supporting cells were failed to be produced. Each individual cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

Table 4.5. summarizes the decrease in cut expression in *iro*^{DFM3} mutant background.

Table 4.5. Ratio of cell type specific marker cut in wild-type and mutant backgrounds.

Cell Stages (wild type)	**Number of cells expressing marker**	**Ratio**
2	0	0
3	1	0,33
4	2	0,50

Cell Stages (*IroC*^{DFM3})	**Number of Replicates**	**Number of observed cells**	**Number of cells expressing marker**	**Ratio**
2	0	0	0	0
3	29	87	0	**0**
4	19	76	0	**0**
5	8	40	0	**0**

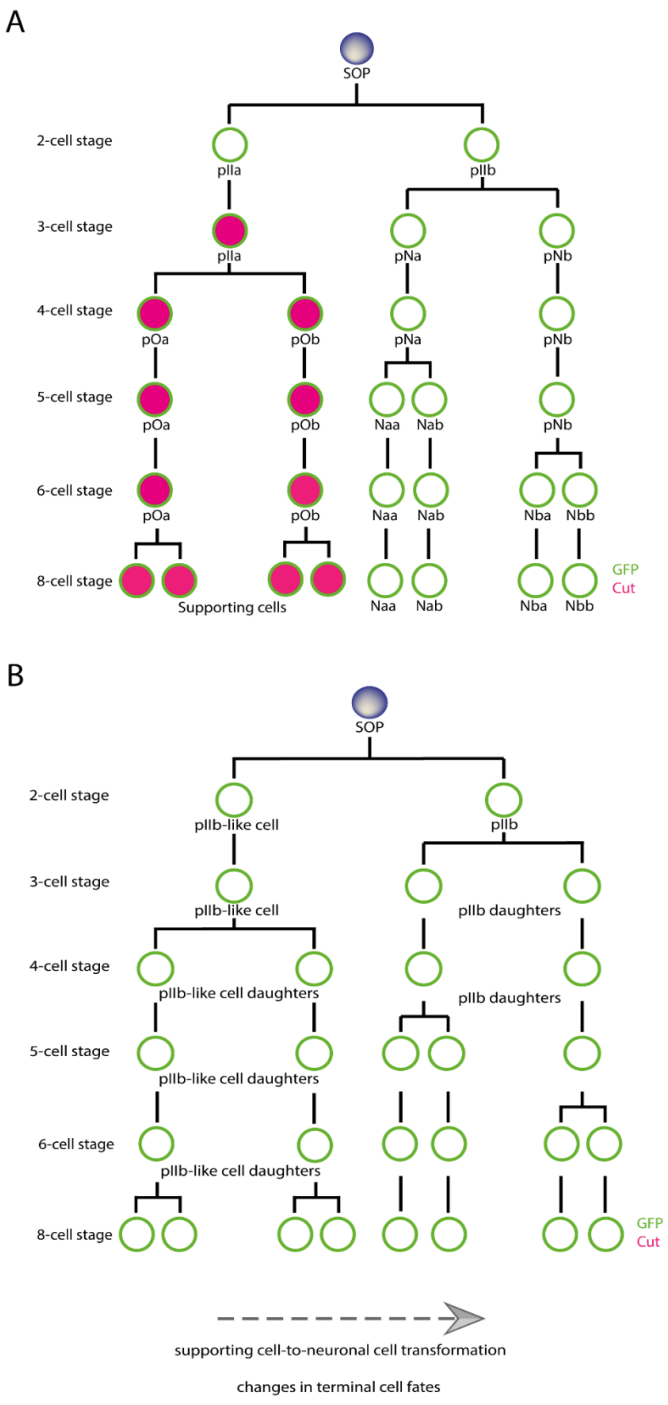

Figure 4.27. Representation of cut expression pattern in the sensory organ lineage. cut is expressed in supporting cell branch of sensory organ lineage in wild-type background (A). In lack of iroC, none of the cells express cut thus supporting cell to neuronal cell transformation occurs (B).

4.2.3.5. Prospero. Expression or the transcription factor pros is Numb-dependent. Numb antagonizes Notch and thus pros is expressed in the cells which do not express Notch. Also, *nerfin-1* has been shown to be downstream of *pros*. Nerfin-1 is overexpressed in iro^{DFM3} mutant background, thus, *pros* expression is expected to be seen out of its normal range in the absence of iroC. However, Notch/Numb are not the only factors that regulate pros activity.

Pros is expressed in one of the SOP daughters, pIIb, which has a Notch OFF profile in wild-type background. At the 3-cell stage, the pIIb daughter pNb cell acquires a Notch OFF profile and expresses pros. Additionally, this expression profile continues to show itself at the 4-cell stage in wild-type background. In the absence of iroC, an additional cell expresses pros at the 3-cell stage (4 replicates of 3-cell clones were analyzed). At the 4-cell stage, pros is expressed in an additional cell (2 replicates of 4-cell clones were analyzed) (Figure 4.28.- Figure 4.29.).

Pros expression varies in iro^{DFM3} mutant background. At the later cell stages, *pros* expression is more diverse. It is expressed in 4 cells in one 5-cell clone, while it is expressed in 2 in another. At the 7 and 8-cell stages, pros is expressed in 4 cells, yet it looks like these cells are different from each other. Although, the data gathered for pros clonal analysis are consistent in itself, it is not possible to define the character of pros-positive cells in the iro^{DFM3} mutant background (Figure 4.30.). Increased expression of pros was expected in the conditions which Numb misexpression and Notch depletion were observed in the absence of iroC proteins. However, pros expression is not increased as much as Numb expression. The responsible for this phenotype can be the asymmetric cell division. Notch/Numb are asymmetrically segregated to newborn cells and their distribution is always predefined and restricted to a specific zone of the cell in sensory organ lineage. On the other hand, pros is not asymmetrically segregated; it is first found in the nucleus and then found in the cytosol of pros-positive cells. Additionally, there must be other factors that affect pros expression pattern because data showed that is not in a binary relationship with Notch/Numb.

Table 4.6. summarizes increase in pros expression in *iro*^{DFM3} mutant background and Figure 4.31. summarizes overall pros phenotype in wild-type and *iro*^{DFM3} mutant backgrounds.

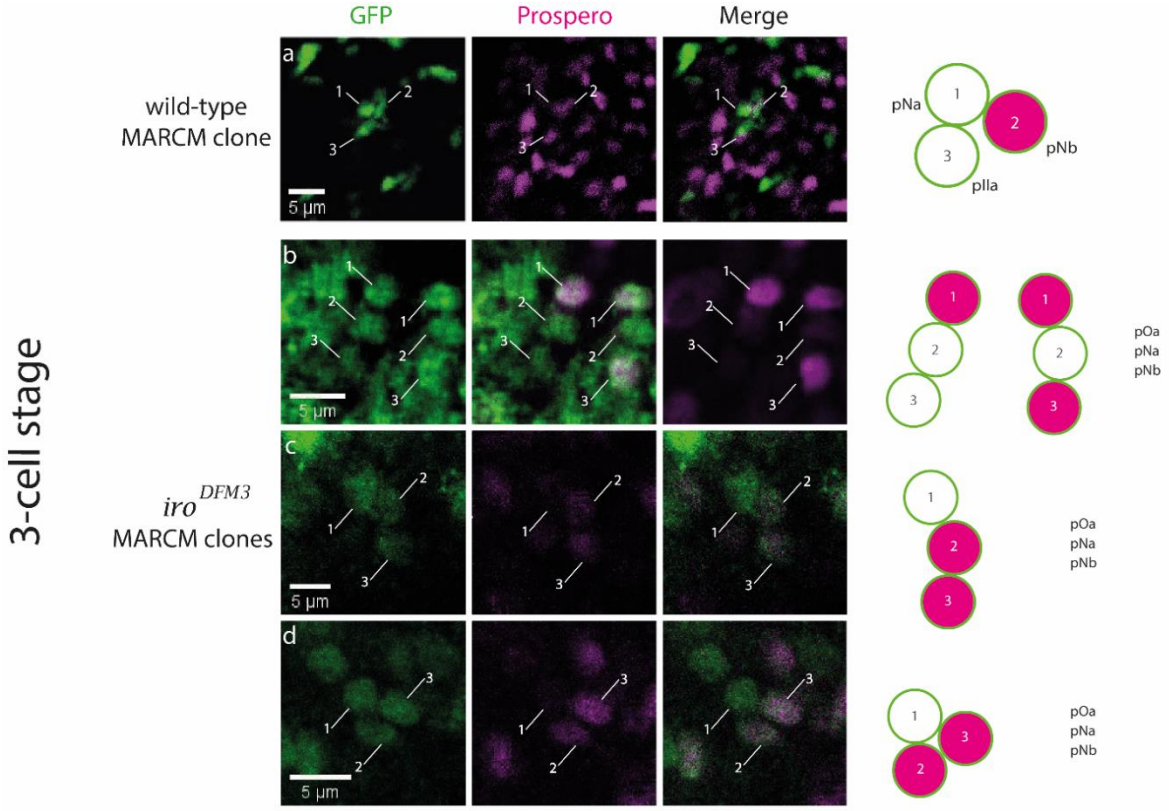

Figure 4.28. 3-cell MARCM clones in *iro*^{DFM3} mutant background. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker pros (magenta). Only pNb cell express pros in wild-type clones (a) while one more cell express pros in the absence of iroC (b-d). Each individual cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

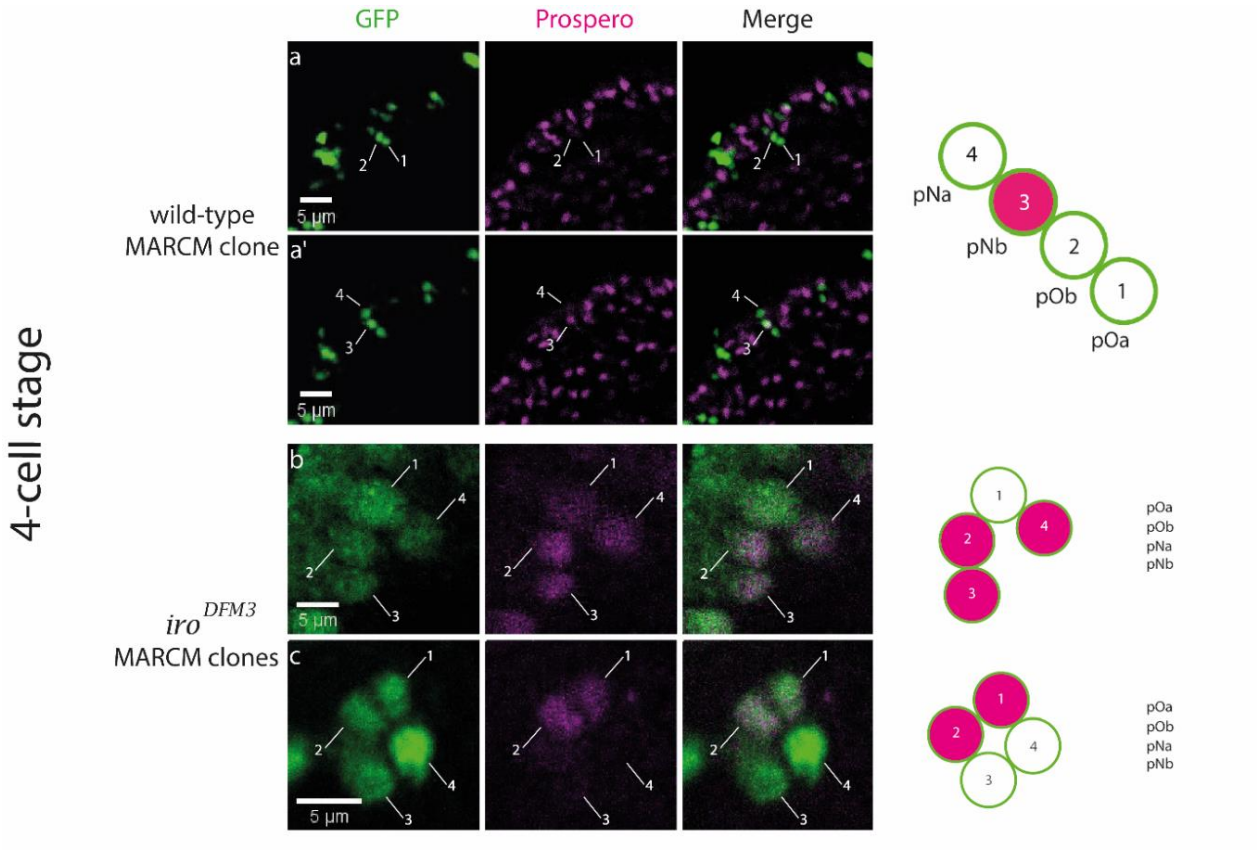

Figure 4.29. 4-cell MARCM clones in *iro*^{DFM3} mutant background. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker pros (magenta). Only pNb cell express pros in wild-type clones (a, a'). At the 4-cell stage, where pNb cell is still present, pros is expressed additionally in one or more cells (b, c). Each individual cell in the MARCM clone is numbered and represented by the same number in the summary diagram. Scale bar = 5 µm

Table 4.6. Ratio of cell type specific marker pros in wild-type and mutant background.

Cell Stages (wild type)	Number of cells expressing marker	Ratio
2	1	0,50
3	1	0,33
4	1	0,25

Cell Stages ($IroC^{DFM3}$)	Number of Replicates	Number of observed cells	Number of cells expressing marker	Ratio
3	4	12	7	**0,58**
4	2	8	5	**0,62**
5	2	10	6	**0,60**

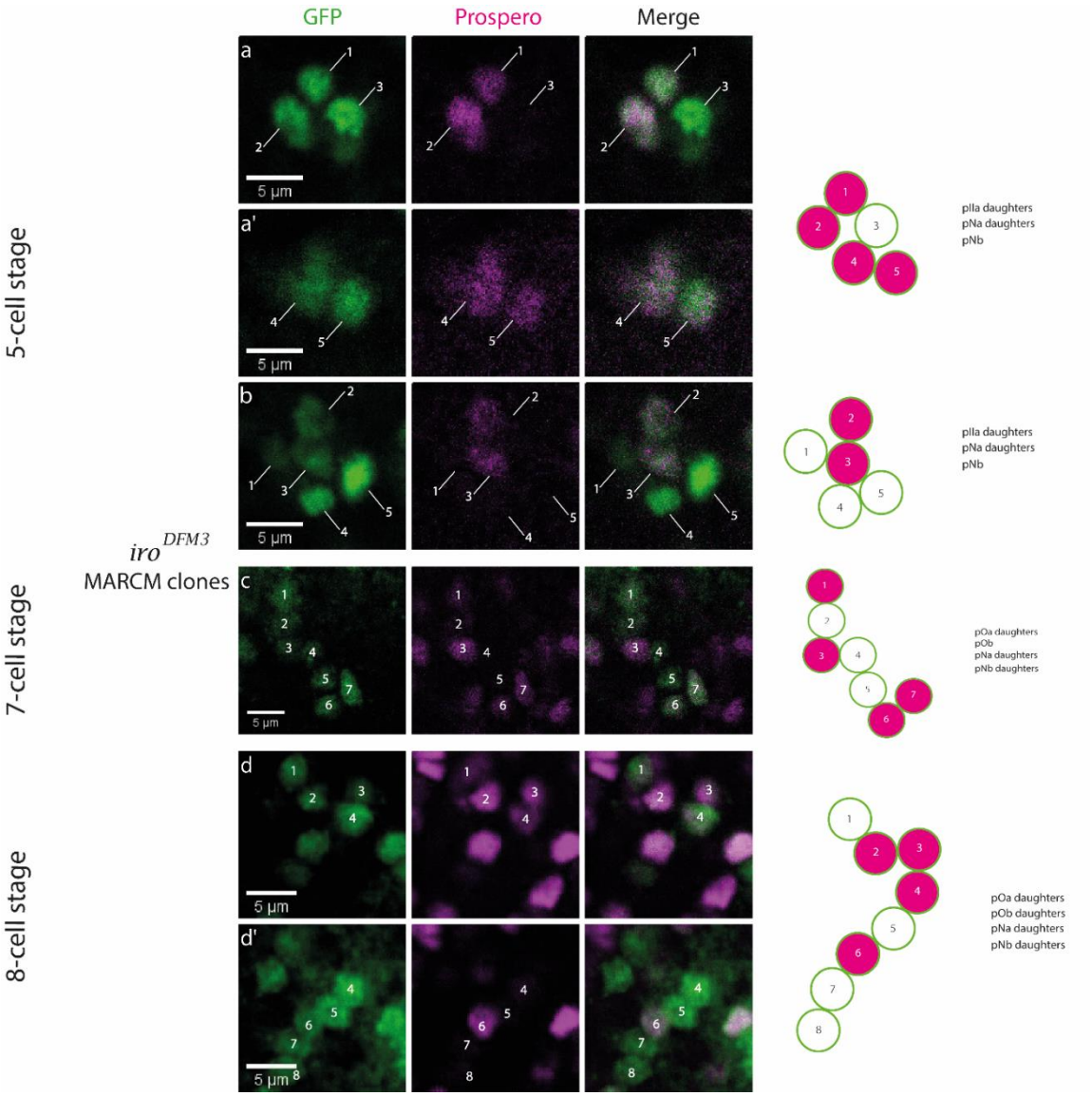

Figure 4.30. 5-, 7- and 8-cell MARCM clones in *iro*^{DFM3} mutant background. Developing antenna immunostained using antibodies against GFP (green) and cell type-specific marker pros (magenta). Through the later cell stages, *pros* expression is diverse. It is expressed in 4 cells in one 5-cell clone (a, a'), and it is expressed in 2 cells in another (b). At the 7 and 8-cell stages (c-d'), it is expressed in 4 cells which look like different from each other. Scale bar = 5 µm

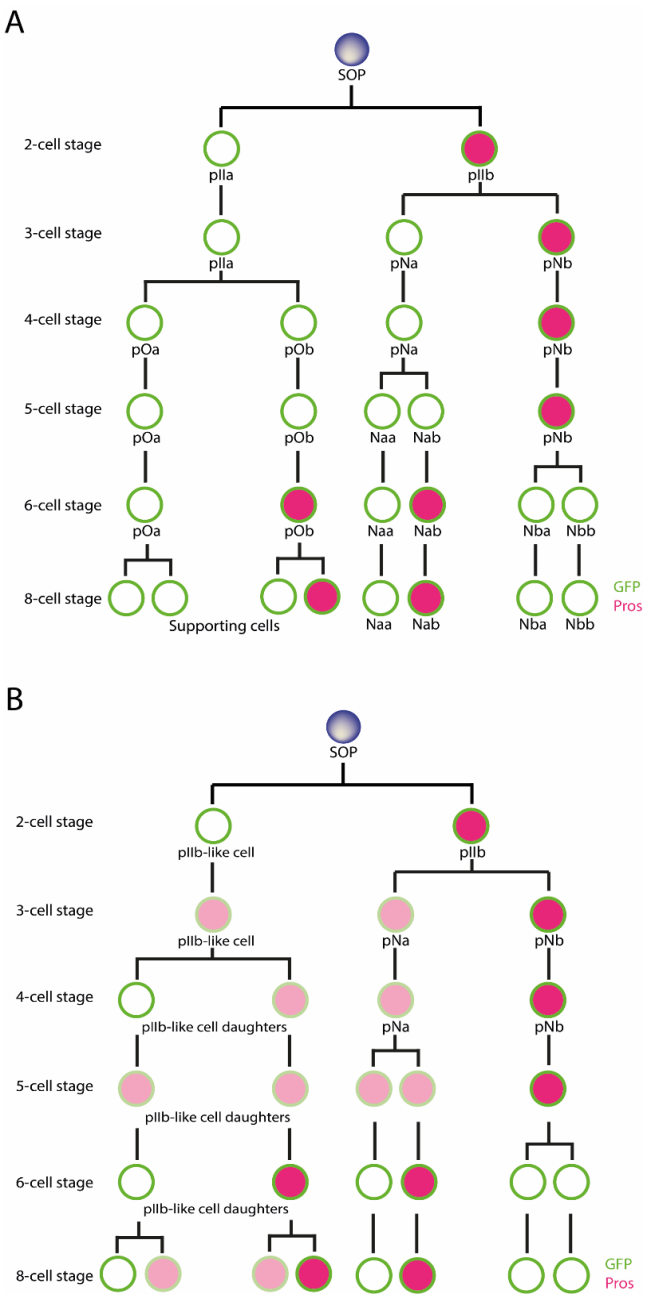

Figure 4.31. Representation of pros expression pattern in the sensory organ lineage. pros is expressed in pIIb and its daughter pNb cell. It is also expressed in one of the supporting cells (pOb daughter) in wild-type background (A). In lack of iroC, pros expression varies. It is expressed in 4 cells in one 5-cell clone, while it is expressed in 2 in another. At the 7 and 8-cell stages, pros is expressed in 4 cells. There must be other factors that affect pros expression pattern because data shows that pros is not in a binary relationship with Notch/Numb (B).

5. DISCUSSION

Olfaction is the oldest sense in terms of evolution and contributes to survival of life in terms of food detection, identification of enemies and allies, and mating. A sophisticated olfactory system allows organisms to interact with the environment in a better way. This is achieved by a diverse range of olfactory receptors. The power of odorant recognition is directly proportional to an increase in different types of olfactory receptors.

The peripheral system for odorant recognition and control system for odorant discrimination are the main elements of a functioning olfactory system. In *Drosophila*, odorant recognition is achieved by antenna and maxillary palp. Odorant discrimination on the other hand, is done in the brain; in the antennal lobe and mushroom body. Using this pathway, *Drosophila* olfactory system can recognize and distinguish hundreds of different odorants, thanks to great diversity in ORs and various cell types of olfactory system.

In the framework of this thesis my main task was to investigate the contribution of *IroC* to cell diversity of *Drosophila* olfactory system. Diversity is initially achieved by asymmetric cell division. Asymmetric cell division is an evolutionary conserved and a multistep process that relies on the asymmetric distribution of cell fate determinants during cell division and causes two daughter cells to acquire distinct cell fates. During development of the *Drosophila* olfactory system, Notch signaling-mediated asymmetric cell division takes place. A SOP gives rise to precursor cells and through the pupal stage, terminal fates of olfactory system cells are defined by contribution of different transcription factors. These genes are called cell fate determinants. We mainly asked whether *IroC* transcription factors are one of these cell fate determinants or not in the olfactory system.

The *Iroquois Complex* (*IroC*) consists of three different genes in *Drosophila*: *araucan (ara), caupolican (caup),* and *mirror (mirr).* Iro proteins share a highly conserved 63 amino acid (aa) homeodomain and an iro box, an EGF-like protein-protein interaction domain. This domain

is similar to the Notch receptor interaction domain (Münch and Galizia, 2016). Iro proteins act as cell fate determinants in the *Drosophila* muscle system (Carrasco-Rando *et al.,* 2011) and they also regulate heart development by affecting precursor cells (Bao *et al.,* 1999).

Taking this information into account, *IroC* is thought to be important for the generation of cellular diversity in the sensory organ lineage of *Drosophila*. The first aim was to identify the subset of ORs in which IroC is expressed. The second aim was to investigate the functions of IroC in generation of cellular diversity. For this purpose, two mutants, IroC triple mutant $IroC^{DFM3}$ and the single mutant $caup^{17}$, have been chosen and used for clonal analysis. The fate of the cells was followed using different cell-type specific markers.

5.1. *IroC* Expression Pattern

It is known that *IroC* is expressed in a subset of ORNs in the adult olfactory system. In maxillary palp, both of the neurons in pb2 sensillum subtype express *IroC* (Talay, 2011). In the antenna, it is unclear in which ORN *IroC* is expressed.

Analysis of DE-Gal4, UAS-GFP line presented that *mirr* is expressed at the mid-pupal stage in developing antenna where precursors of ORNs and newly generated ORNs are present. Analysis of adult antenna using the same fly line presented that *mirr* is also expressed in a subset of neurons in the adult antenna. On the other hand, analysis of IroC-Gal4 line crossed with UAS-mGFP presented that *ara* and *caup* are expressed at the mid-pupal stage in developing antenna but not in the adult antenna. Thus, data indicates that *mirr*, *ara* and *caup* are expressed in precursor cells of developing antenna and in subset of ORNs at the pupal stage; *ara* and *caup* expression gets lost in the adult stage but *mirr* expression is still present in the adult stage. Although it is unclear in which subset of cells *IroC* is expressed in sensory organ lineage, we hypothesize that *IroC* is involved in specification of neuronal cell fates during olfactory system development.

Additionally, analysis of DE-Gal4, UAS-GFP line and IroC-Gal4 line crossed with UAS-mGFP showed that *IroC* expression pattern corresponds to basiconic sensilla on the antenna. It is known that transcription factor amos acts in the antennal disc to define SOPs which

give rise to basiconic sensilla subtype (Jhaveri *et al.,* 2000). It can be hypothesized that *IroC* is expressed in ORNs housing basiconic sensilla, thus, iro proteins would take roles during SOP selection along with amos at the antennal disc. RNASeq analyses performed in our lab on antenna and maxillary palp supports this hypothesis (Taşkıran, 2018). It is shown that eleven OR genes were differentially expressed in the absence of IroC. In fact, these OR genes are known to be expressed in the ORNs housing basiconic sensilla (Münch and Galizia, 2016), except *OR47b* gene. *OR47b* is expressed in ORNs housed by antennal trichoid sensilla. *OR47b* was downregulated in IroC mutant background because of the fact that there are 3 identified ara/caup binding sites on *OR47b* (Taşkıran, 2018).

In Central Nervous System, Iro proteins are expressed in both central brain and optic lobes. *IroC* expression has also been observed in the antennal lobe where glomeruli are located. It is expected since there are *IroC* expressing ORNs in both antennae and maxillary palp. Yet it was not possible to define subset of ORNs that express *IroC* by analyzing the glomeruli since other cells including projection neurons also express *IroC*.

To solve this problem and reveal the type of IroC-positive OR subsets, we made use of a number of lexA lines generated by cloning of 3 kb fragments from the iroC locus, in the hope that drivers with less complex expression patterns of iroC could be identified and used in conjunction with OR-Gal4 drivers.

These lines were LexA driver lines and they were developed by site-specific recombination in the FlyLight project (Jenett *et al.*, 2012). Analysis of six transgenic fly lines that represent the expression pattern of sequences near or inside from *mirr* and inside of *caup* gene showed that these fragments are expressed in adult brain but not expressed in antenna or maxillary palp or glomeruli in the antennal lobe. In total, relevant analysis showed that these fragments generally drive LexA expression in optic lobes and other parts of the brain, which are part of the general iroC expression pattern, but do not seem to contain the enhancers that drive expression in the olfactory system and thus could not be used towards determination of iroC-positive OR subsets.

We hope that analysis of direct fusion proteins of each of the iroC with GFP generated by CRISPR/Cas will be useful in achieving this goal.

5.2. Analysis of iroC Mutants for their Role in Asymmetric Cell Division

MARCM technique is a suitable technique to restrict the number of sensory organ lineage cells for single cell analysis as it is not possible to track single cells within a crowded epidermal field. MARCM technique makes this possible via labeling only some of cells with GFP. At the same time, GFP-marked cells are homozygous for the mutant of interest. Obtaining homozygous mutant cells is the most reliable approach to study the function of a relevant gene.

Clonal analysis of $iroC^{DFM3}$ and $caup^{17}$ mutants was performed using the MARCM technique. For this purpose, it was necessary to recombine the $caup^{17}$ mutant onto a suitable FRT chromosome; since *IroC* is located on the third chromosome, *FRT80* site located on the same arm of the chromosome as iroC was used. $caup^{17}$ was successfully recombined onto *FRT80* and the $caup^{17}$ mutant became suitable to conduct MARCM experiments.

$iroC^{DFM3}$ and $caup^{17}$ mutant were analyzed by examining the expression profile of several cell fate determinants. Cell fate determinants are generally transcription factors that define characteristics of precursor cells and contribute to the acquisition of terminal fates of ORNs and supporting cells. They are asymmetrically distributed throughout the sensory organ lineage.

After SOP specification, SOP undergoes Notch signaling-mediated asymmetric cell division. Thus, experiments including control of Notch signaling were performed. It is shown that in both single mutant and triple mutant of IroC, Notch signal was lost.

The Notch signaling pathway is the main cell fate determinant in *Drosophila* sensory organ lineage. In order to clarify the possible causes of Notch phenotype in IroC mutant background, I would like to review the known relationship between *IroC* and Notch signaling

and to discuss Notch regulation by *IroC*. Additionally, how Notch is regulated specifically in *Drosophila* sensory organ lineage should be discussed here.

Previous studies suggested that *IroC* and Notch signaling pathway has a complicated relationship in different tissues, yet, it has become clear that Notch acts downstream of *IroC* in these tissues.

Notch regulation by IroC activity in larval eye imaginal disc can be an example here. *Fringe* (*fng*) is a glucosyltransferase that increases the affinity of Notch to its ligand Delta. mirr has been shown to directly bind to fng through the Iro Binding Domain (IBD) and to act as a transcriptional repressor. On the other hand, it is shown that mis-expression of *caup* disturbs *fng* activity and leads to inadequate eye development. It shows that caup alone can act as a cell fate determinant. As a result of the repression of fng by Iro proteins, *fng* expression is restricted to the ventral side of the eye-imaginal disc while *IroC* is expressed in the dorsal part (Bilioni *et al.,* 1999). Thus, Notch pathway is regulated indirectly by protein-protein interaction and Fng/Notch pathway acts downstream of *IroC* in the larval eye-imaginal disc.

Relationship between *IroC* and Notch signaling is also shown in *Drosophila* heart and muscle tissues. In the heart, loss of IroC leads to an imbalance of progenitor cell specification and results in an abnormal number of heart cells (Bao *et al.,* 1999). On the other hand, *ara* and *caup* are shown to be cell identity genes and IroC proteins are sufficient for acquisition of specific cell types in the *Drosophila* muscle system. Here, Iro proteins act as transcriptional repressors of *slouch* (*slou*) and *vestigial* (*vg*) genes in a combinational manner (Carrasco-Rando *et al.,* 2011). Slou represses the expression of the transcription factor *Ladybird* and results in the gain of Notch-activity. On the other hand, iro proteins repress the activation of vestigial, which is normally activated by Su(H) that is a part of Notch signaling.

Additionally, RNASeq analyses performed in our lab also showed that in IroC mutant background, some of the transcription factors important for Notch signaling were differentially expressed (Taşkıran, 2018). Nerfin-1 normally represses Notch activity (Xu *et al.*, 2017). In *IroC*-deficient tissues, *nerfin-1* expression is reported to be 8-fold increased. It could be suggested that iro proteins repress nerfin-1, thus prevent repression of Notch in the sensory

organ lineage in wild-type background. On the other hand, the transcription factor *hey* is significantly downregulated in *IroC* mutant background. Hey is revealed to be a direct Notch target and it is activated in the same way as *E(Spl)* genes (Monastirioti *et al.*, 2009). In addition, in the RNASeq data Numb appeared to be overexpressed.

Taken all these data together, it seems clear that Notch signaling is impaired in loss of IroC in olfactory system. Therefore, it would be beneficial to discuss Notch regulation in the olfactory system, here. Notch regulation by cell safe determinants takes place at the different stages of the developing olfactory system, from SOP specification to terminal cell fate determination.

Before SOP division, Notch signaling pathway is regulated within a regulatory network. This regulatory network includes the zinc finger transcription factor Sens and directly contributes to SOP selection. Sens enhances pro-neural gene expression in SOPs. Pro-neural genes encode basic helix-loop-helix transcription factors and are key regulators of neuronal differentiation. Pro-neural genes activate the transcription of *sens* and *E(spl)* complex genes (Benton *et al.*, 2006). Then, Sens protein can act directly on pro-neural genes to take part in this autoregulatory loop or inhibit transcription of *E(spl)* genes. When transcription of *E(spl)* genes is inhibited, pro-neural gene expression is further upregulated and the Delta signal is sent to the neighboring cells that do not express Sens. Here, neighboring cells start to ectopically express E(spl) proteins and cause E(spl) accumulation, gaining an active Notch signaling profile (Benton *et al.*, 2006). Thus, Sens is an essential component of the pro-neural Notch signaling pathway by enhancing the reduction of Notch signaling in SOPs.

After SOP selection, precursor cells are generated through Notch signaling-mediated asymmetric cell divisions. At the 3-cell stage, pIIa cell that will produce supporting cells of the sensory organ start to express homeobox transcription factor Cut. It is known that Cut is a direct Notch target and its activity depends on Notch signaling (Ayer and Carlson, 1992; Blochlinger *et al.*, 1990). Also, *sens* and *cut* antagonizes each other since Sens inhibits Notch signaling. It is shown that Sens overexpression causes a dramatic downregulation of Cut protein levels (Nolo *et al.*, 2000)**.**

On the other hand, another cell fate determinant Numb antagonizes Notch. Numb promotes disruption of Notch receptor trafficking and leads to removal of the Notch receptor from the membrane into endosomes by inhibiting Spdo. Spdo regulates Notch endocytosis, it interacts with Numb and it is considered as the main factor responsible for Notch/Numb antagonism. Numb inhibits the recycling of Spdo, and as a result, it inhibits the activity of Notch (Fortini and Bilder, 2009; Kandachar and Roegiers, 2012).

Additionally, transcription factor Prospero defines the pIIb cell fate that is sufficient for the acquisition of neural cell fates. Pros activity is Numb-dependent (Reddy and Rodrigues, 1999). Numb antagonizes Notch and thus pros is expressed in the cells which do not express Notch. Also, *nerfin-1* has been shown to be downstream of *pros*. Nerfin-1 is overexpressed in iro^{DFM3} mutant background, thus, *pros* expression is expected to be seen out of its normal range in the absence of iroC. However, Notch/Numb are not the only factors that regulate pros activity.

Finally, there are other cell fate determinants which contributes to the Notch signaling and to cell fate acquisition in *Drosophila* sensory organ lineage. Pro-neural gene *mastermind* (*mam*) encodes nuclear protein Mam which acts downstream of Notch signaling. Mam acts as a coactivator of Su(H) protein and it is shown that Mam interacts with Su(H) in the presence of Notch intracellular domain. Thus, its activity depends on active Notch signaling. It is shown that loss of Mam leads to pIIa to pIIb-like cell transformation and loss of precursors of neural branch (Endo *et al.*, 2007). Also, ORN terminal cell fates and ORN projection patterns were changed in *mam* mutant background (Endo *et al.*, 2007). Additionally, *Hamlet* (*Ham*) transcription factor is expressed only in the pNa-derived ORN identities in the sensory organ lineage. Ham interacts with E(spl) loci and it is shown that Ham alters the accessibility for Su(H) binding at the enhancer, in other words, Ham controls chromatin-modification events at specific Notch targets. Because of this, Ham activity is capable of erasing Notch activity in ORNs, specifically in Naa neuron (Endo *et al.*, 2012). It is shown that in lack of Ham, Naa cell fate is cannot be acquired; ORN cell type and projection pattern was changed (Endo *et al.*, 2012). These data support the idea that differential Notch activity in ORN precursors regulates the specification of both odorant receptor expression and axonal targeting.

In this study, it is shown that Notch signaling is altered in IroC mutant backgrounds. Notch is not expressed in SOP daughters pIIa and pIIb, suggesting Notch impairment in SOP. In SOP, sens regulates Notch signaling via inhibiting transcription of *E(spl)* genes during SOP selection. Without *IroC*, sens is kept expressed and inhibits Notch signaling. Since sens protein structure is not identified, it is not known that whether sens has an Iro Binding Domain or not. Even so, it can be suggested that *sens* acts downstream of *IroC*. Analysis of sensory organ lineage in sens mutant background can reveal the role of sens in this network which *IroC* and *sens* act to select SOPs and regulate Notch signaling. Since Notch is not present in the beginning of the lineage, it cannot be asymmetrically distributed to newborn cells. The presence of sens in the whole sensory organ lineage is consistent with these data.

Clonal analysis using an antibody against Numb protein supported this observation as well. Since Notch is inhibited in pIIa, Numb becomes expressed in both of the daughter cells of SOP. My data suggest that it is also expressed throughout the sensory organ lineage. Analysis of cut activity, which is Notch-dependent, showed that cut expression is completely lost in the sensory organ lineage, which again is in line with previous observations. On the other hand, analysis of the distribution of pros was not as clear. It is not asymmetrically distributed. Normally, pros is expressed in Notch-inactive cells. In IroC mutant background, the number of pros-expressing cells is increased at different cell stages. Presumably, these cells were Numb-positive, since Notch is not expressed and Numb becomes expressed throughout the sensory organ lineage.

Overall, I propose that *IroC* genes are cell fate determinants in *Drosophila* sensory organ lineage. Data presented here show that in lack of IroC, from SOP selection to terminal cell fate determination, Notch becomes inactive via a regulatory network between Notch and sens. It is also shown that caup protein is important for cell fate determination in the sensory organ lineage. It is thought that *ara* and *caup* are redundant and *ara* could be compensating for the lack of *caup* in antennal development, but this has not been observed. Our data supports the previous observations discussed above that iro proteins can act separately on cell fate determination. Nevertheless, the expression levels of *ara* and *mirr* should be determined in the *caup* mutant. Analysis of the *mirr* mutant alone would be also interesting to determine if it has a role in this

process at all, as all the phenotypes observed with the triple mutant were recapitulated with the *caup* mutant alone. To further investigate this, rescue experiments using UAS-caup and UAS-ara can be set. For example, if introducing UAS-caup into *iroC*^{DFM3} mutant background rescues the phenotype, that means only *caup* is responsible for alterations in cell fates in sensory organ lineage. If not, *ara* and *mirr* can also be responsible. Similarly, if introducing UAS-ara into the same background rescues the phenotype, *ara* can be identified as a single cell fate determinant.

Additionally, it is possible to tract projection patterns of ORNs in different *IroC* mutant backgrounds by conducting MARCM experiments. This approach would reveal the alterations in the terminal cell fates of ORNs by analyzing antennal lobe since ORNs project their axons to a defined glomerulus. Here in this study, it is shown that terminal cell fates were shifted and more neurons were produced in lack of IroC but it is still not possible to name them. After *IroC* expressing ORNs are revealed using fly lines containing GFP-tagged versions of iro proteins in the future, MARCM experiments can be conducted specifically to reveal the possible increase in the number of these ORNs.

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APPENDIX A: MARCM CLONES IN IROC MUTANT BACKGROND

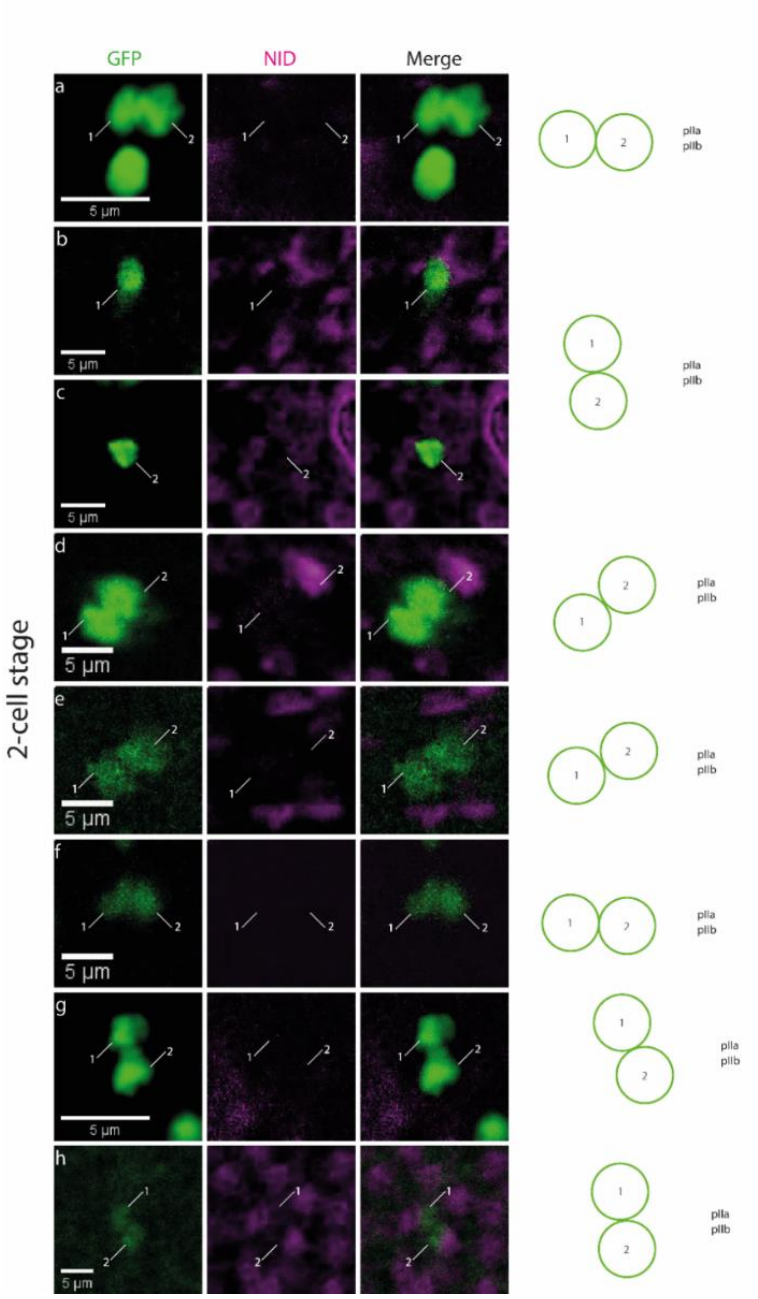

Figure A.1. Analysis of 2-cell MARCM clones using NID marker in *IroC*^{DFM3} mutant background.

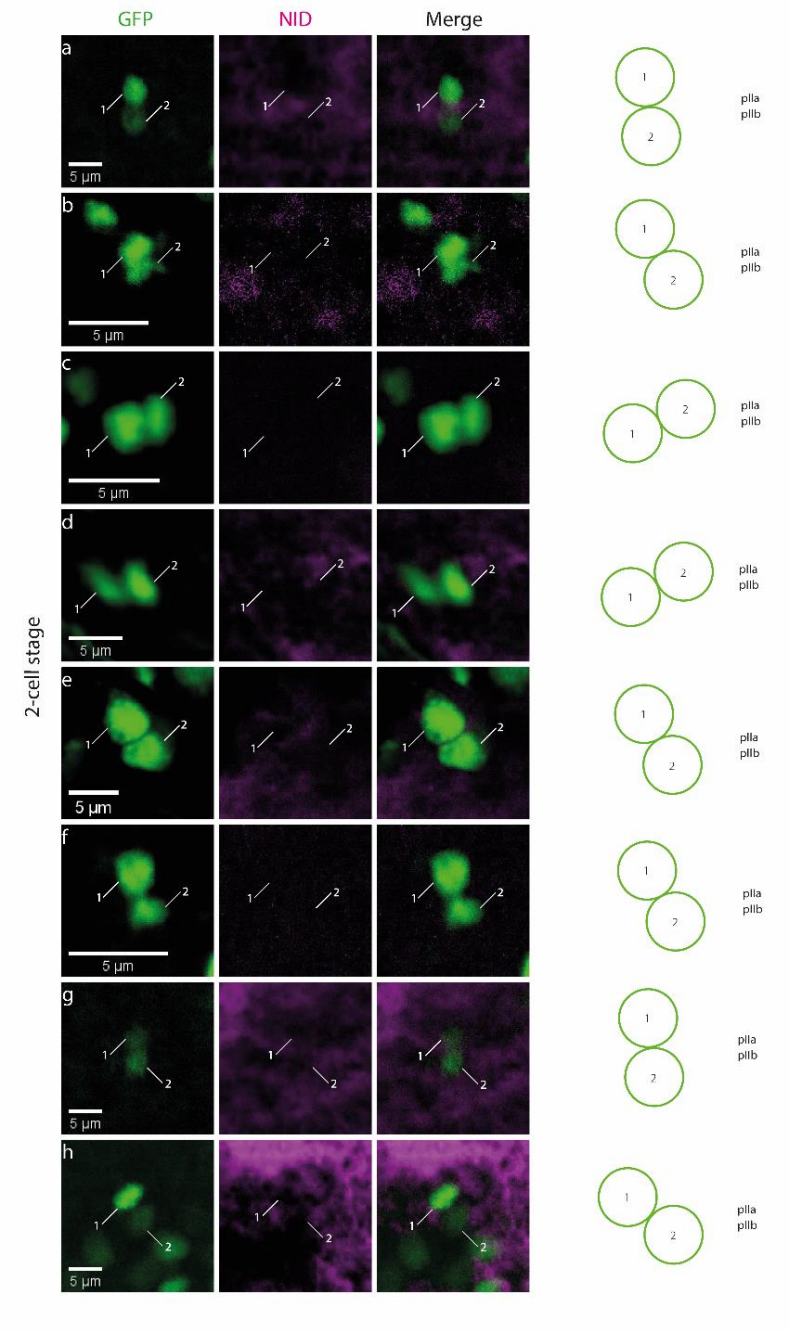

Figure A.2. Analysis of 2-cell MARCM clones using NID marker in *IroC*^{DFM3} mutant background.

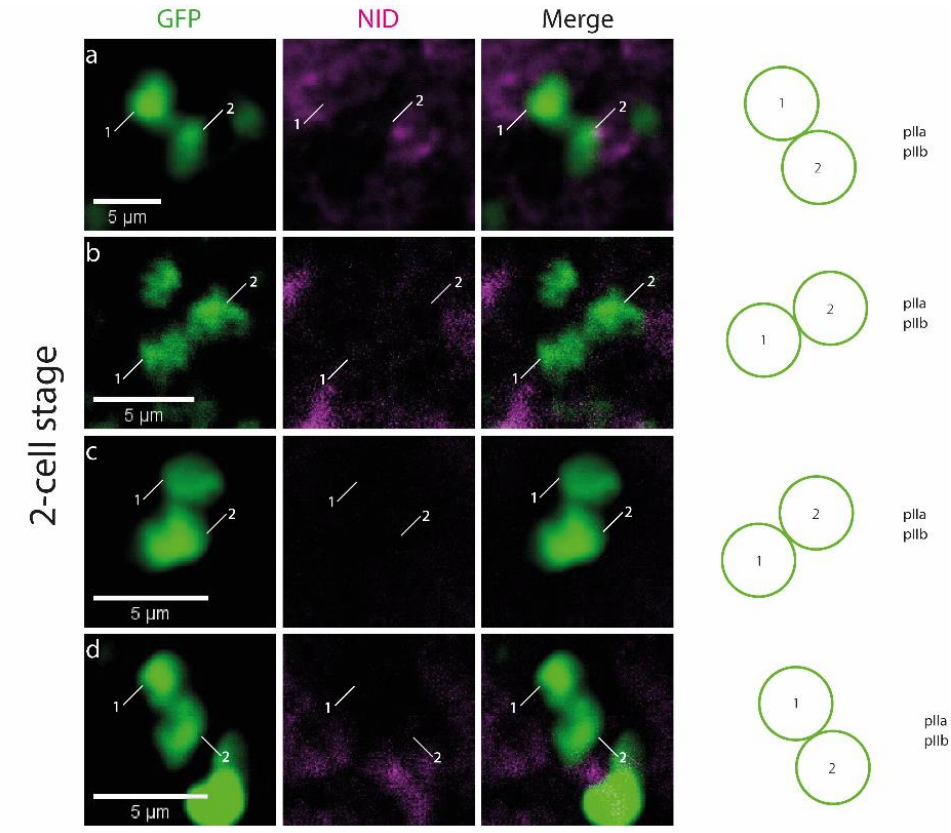

Figure A.3. Analysis of 2-cell MARCM clones using NID marker in *IroC*^{DFM3} mutant background.

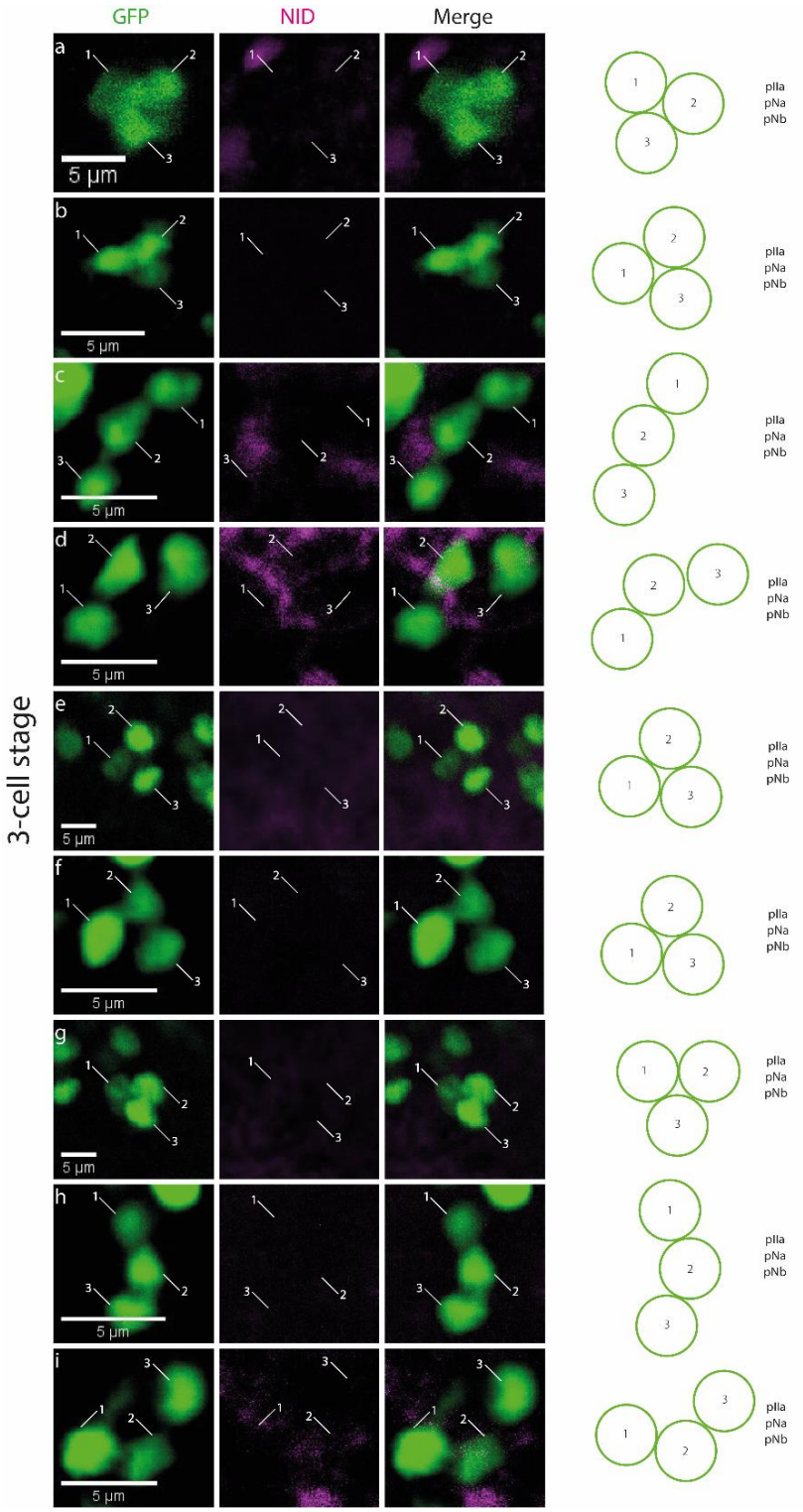

Figure A.4. analysis of 3-cell MARCM clones using NID marker in *IroC*^{DFM3} mutant background.

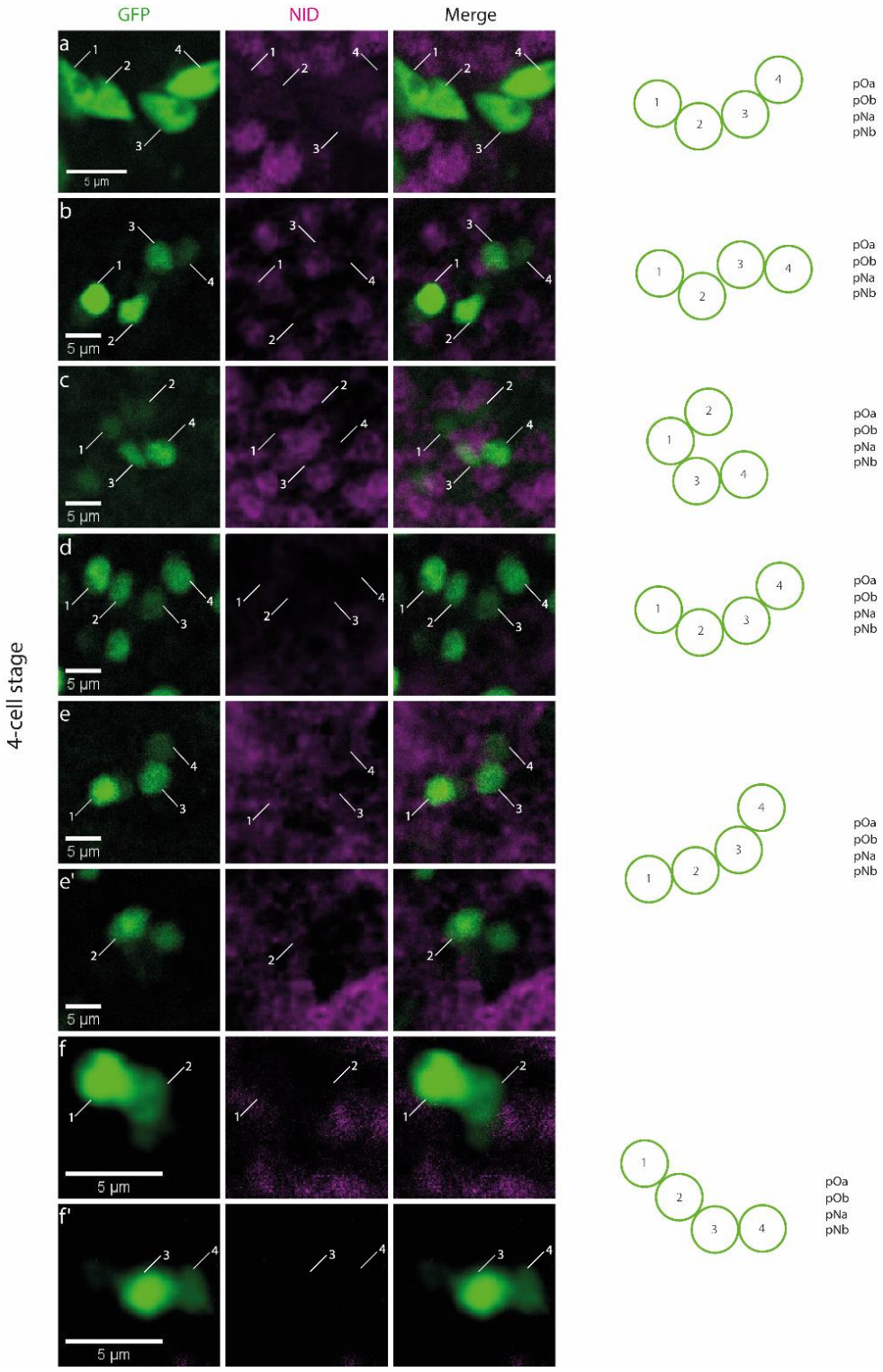

Figure A.5. Analysis of 4-cell MARCM clones using NID marker in *IroC*^{DFM3} mutant background.

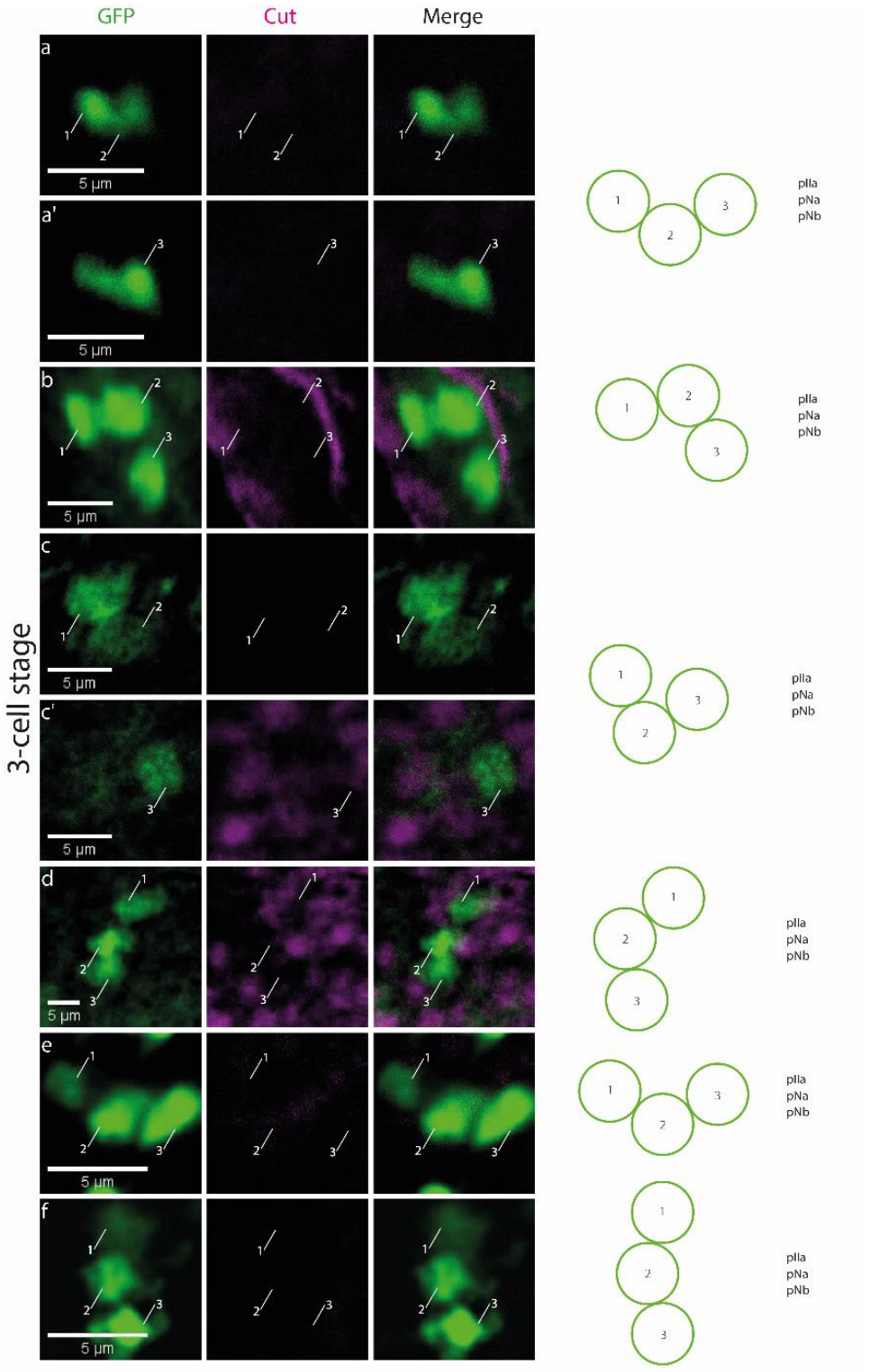

Figure A.6. Analysis of 3-cell MARCM clones using Cut marker in *IroC*^{DFM3} mutant background.

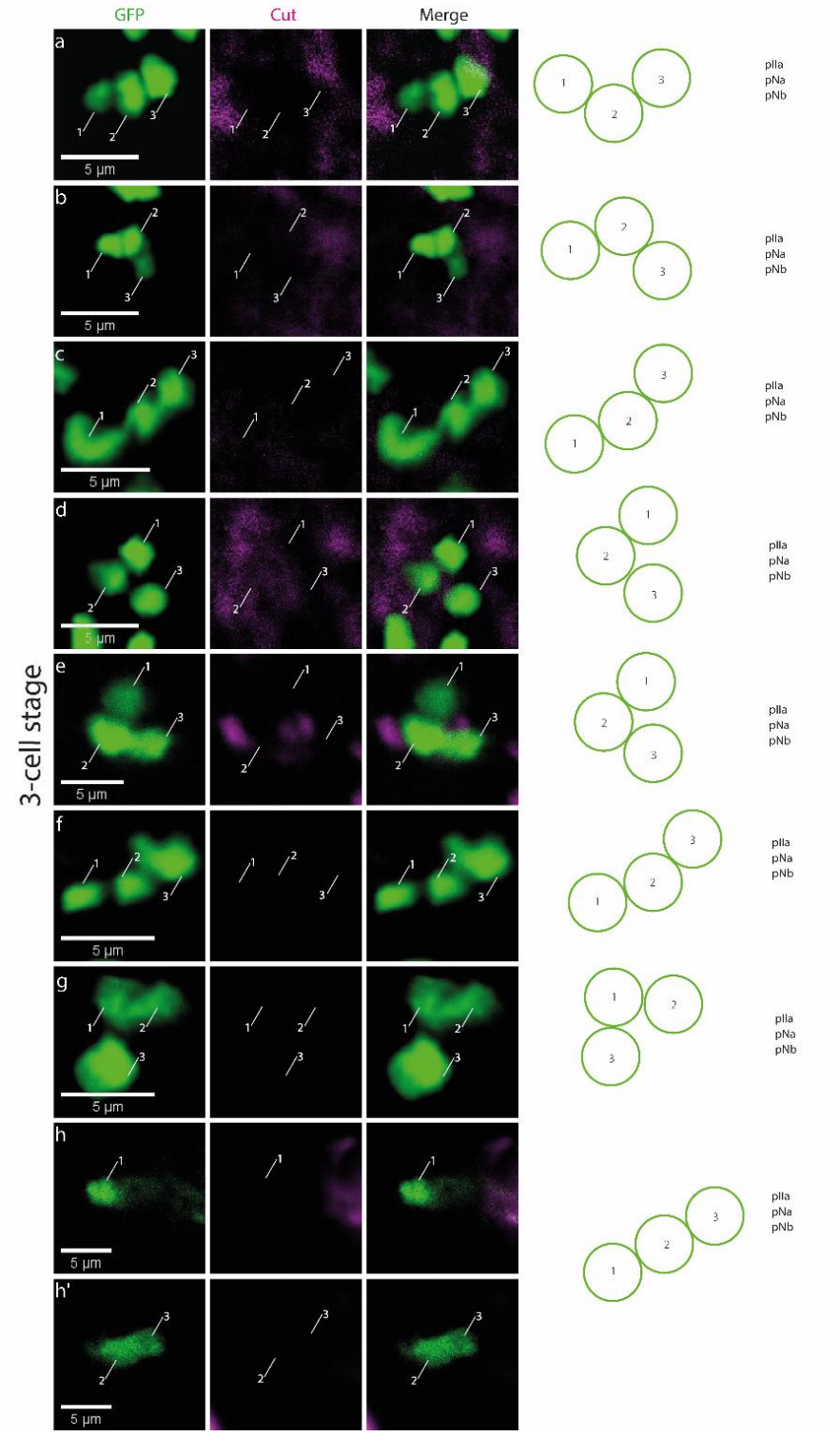

Figure A.7. Analysis of 3-cell MARCM clones using Cut marker in *IroC*^{DFM3} mutant background.

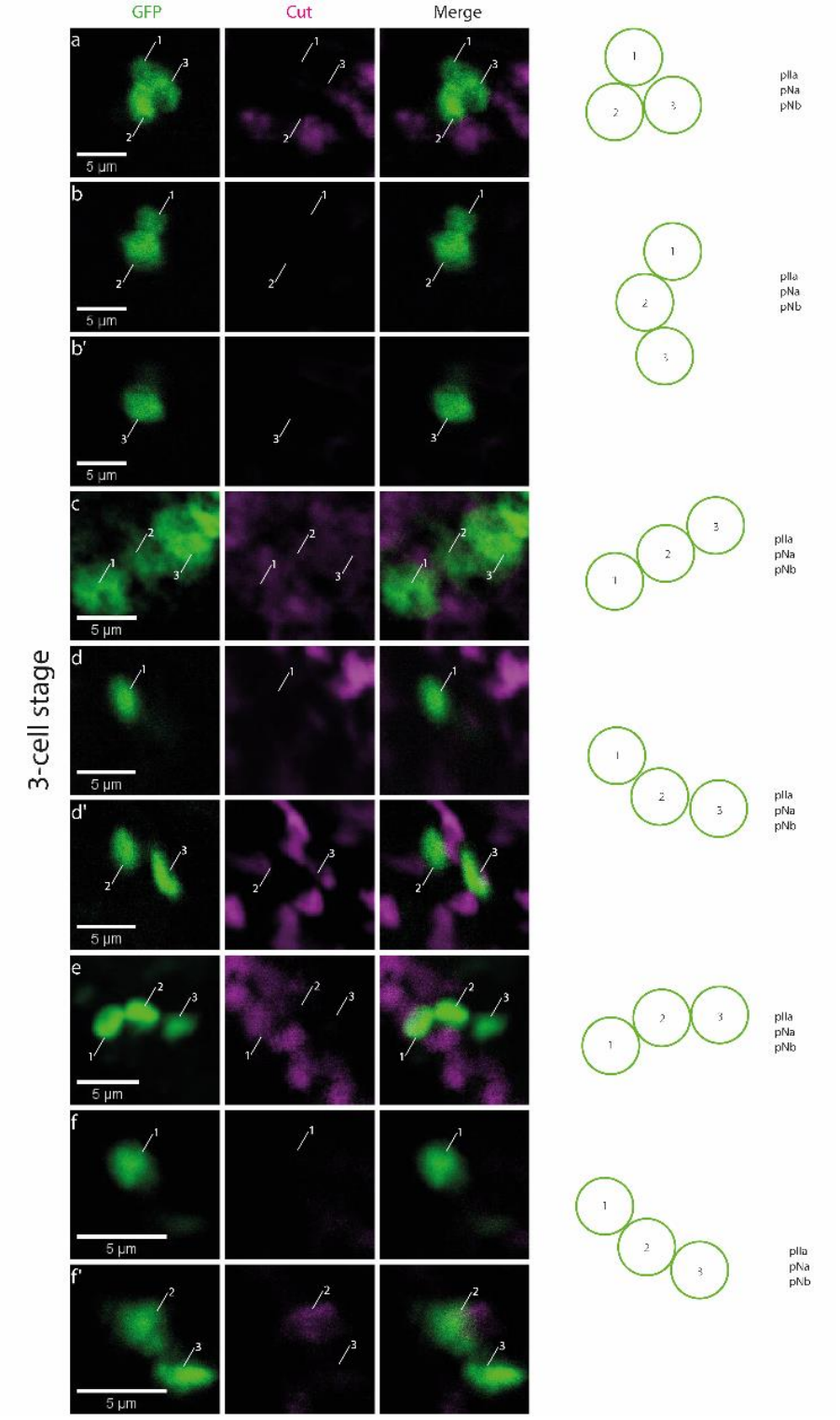

Figure A.8. Analysis of 3-cell MARCM clones using Cut marker in *IroC*^{DFM3} mutant background.

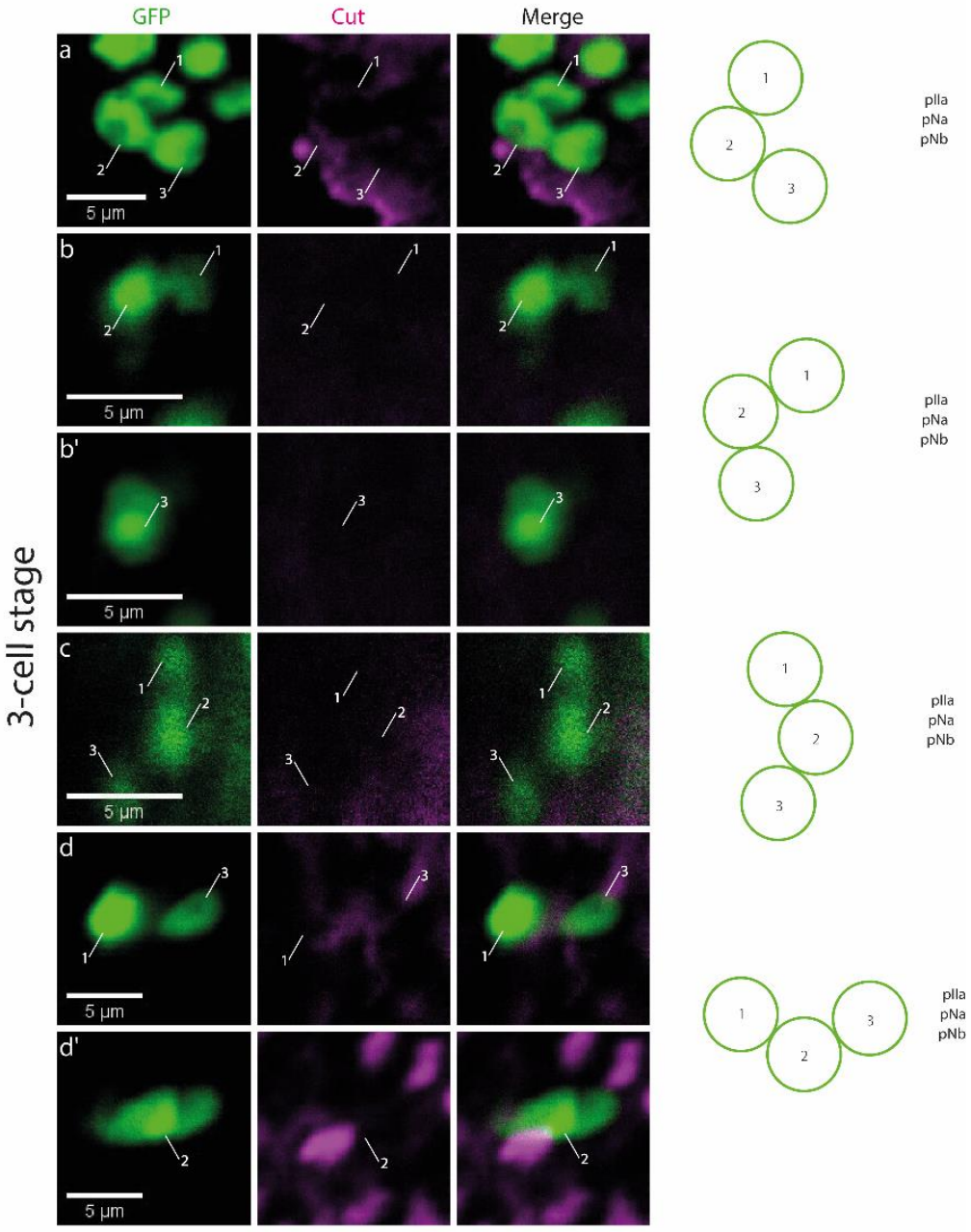

Figure A.9. Analysis of 3-cell MARCM clones using Cut marker in *IroC*^{DFM3} mutant background.

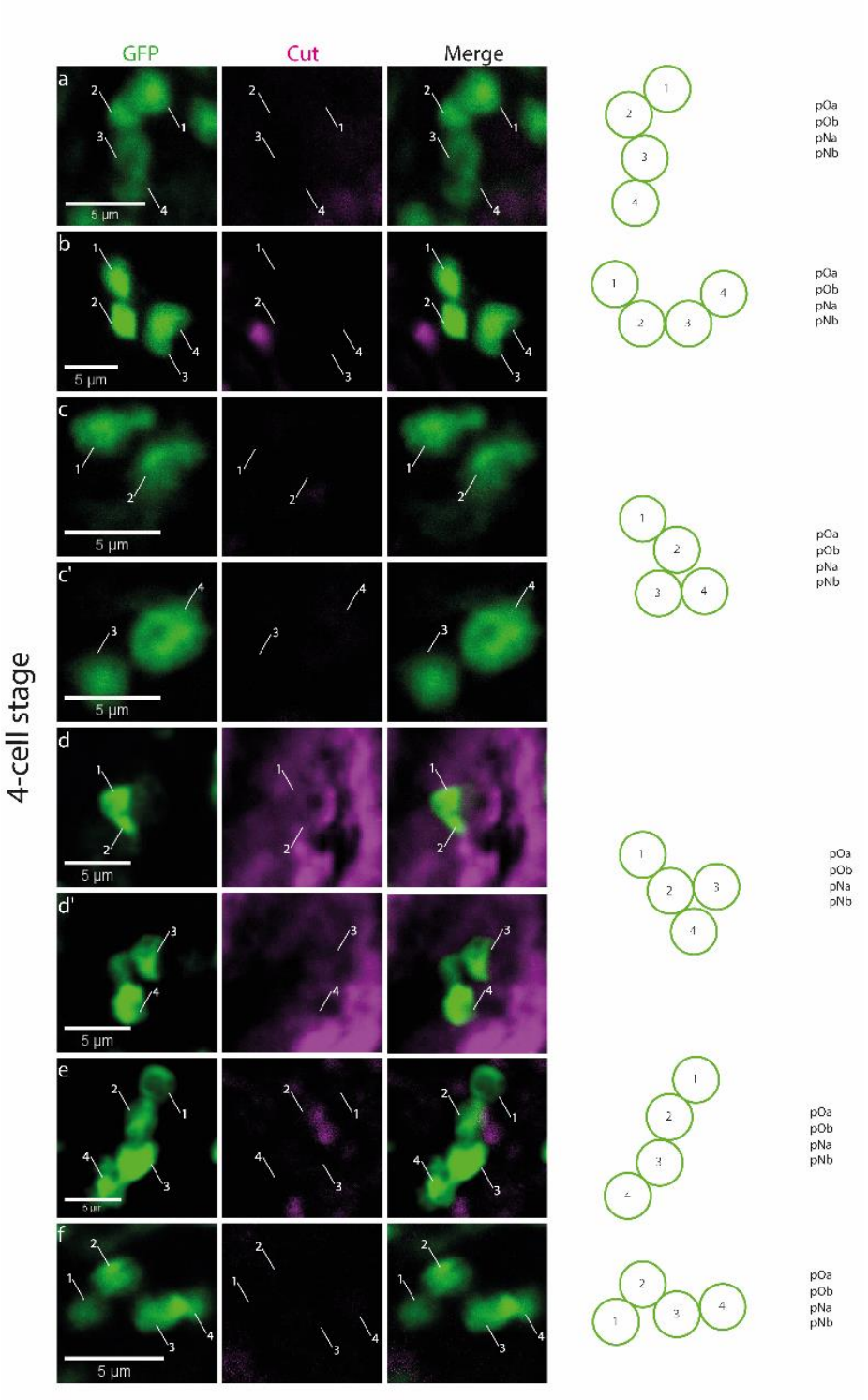

Figure A.10. Analysis of 4-cell MARCM clones using Cut marker in *IroC*^{DFM3} mutant background.

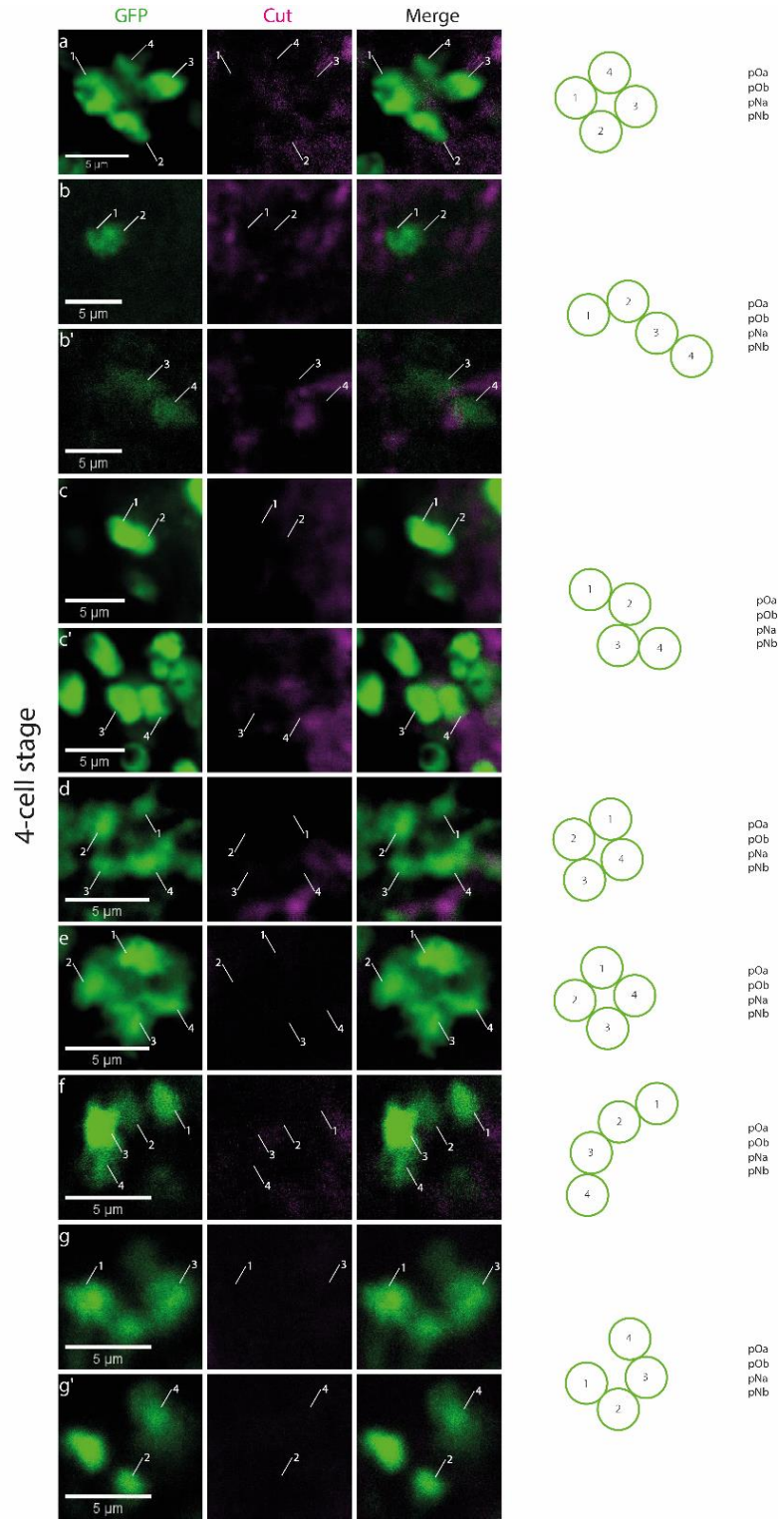

Figure A.11. Analysis of 4-cell MARCM clones using Cut marker in *IroC*^{DFM3} mutant background.

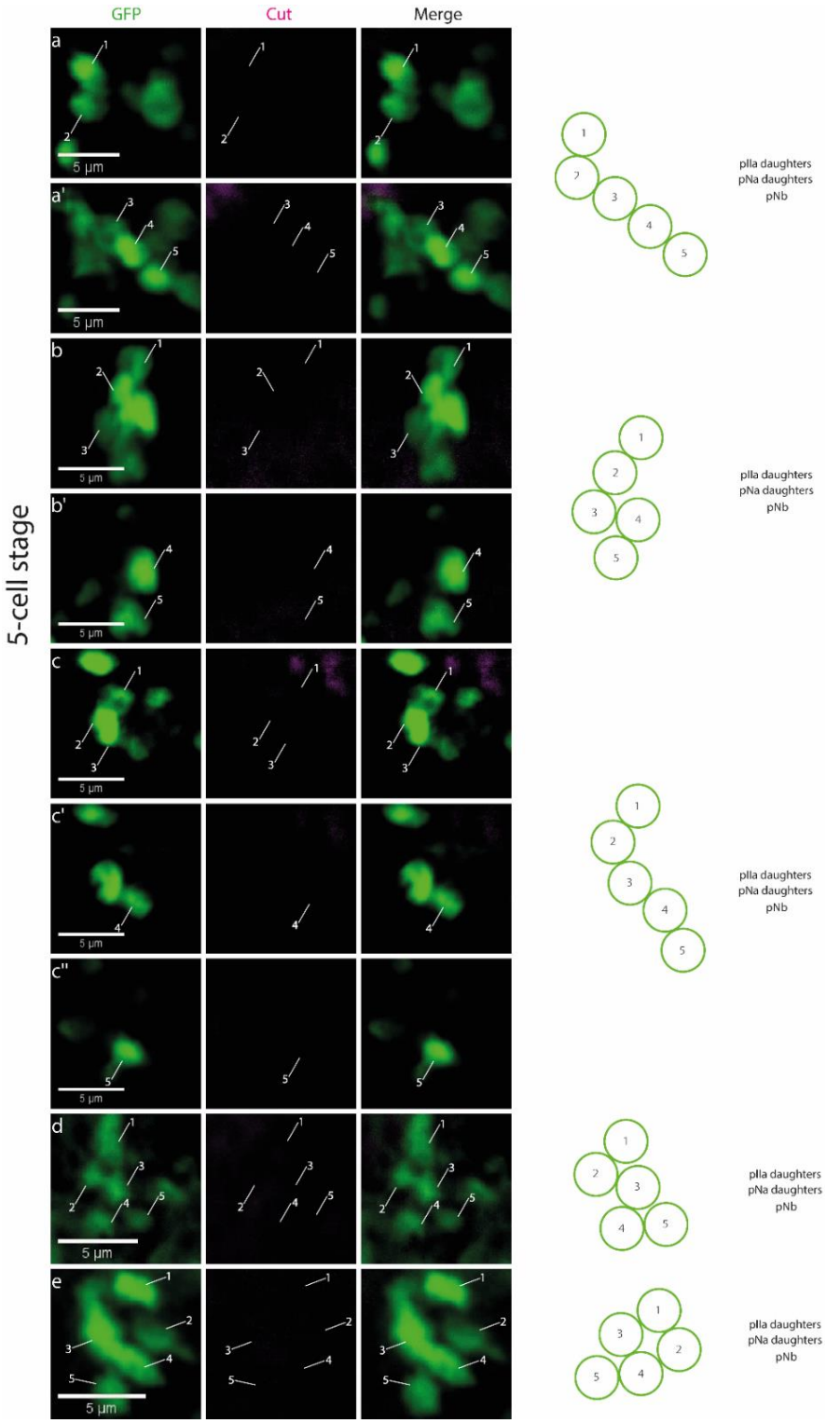

Figure A.12. Analysis of 5-cell MARCM clones using Cut marker in *IroC*^{DFM3} mutant background.