CHARACTERIZATION OF A NOVEL R7-SPECIFIC GENE IN THE DROSOPHILA VISUAL SYSTEM

by

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ABSTRACT

CHARACTERIZATION OF A NOVEL R7-SPECIFIC GENE IN THE DROSOPHILA VISUAL SYSTEM

The proper functioning of the nervous system depends on the correct specification of many different cell types and the establishment of proper connections between neurons during development. The visual system of *Drosophila* represents a good model to study these mechanisms. The *Drosophila* eye consists of 800 small eye-like structures called ommatidia each containing eight photoreceptors (PRs). Photoreceptors are grouped into two; outer and inner, and each expresses sensory receptor molecule called Rhodopsin (Rh). In an attempt to identify novel genes playing a role in photoreceptor specification, the enhancer trap line for *CG7985* showing an R7-specific expression was selected. The aim of this thesis was to characterize this gene functionally to understand its role in visual system development. *CG7985* was shown to encode a hexosaminidase enzyme that is conserved in evolution. Hexosaminidases are involved in the hydrolysis of GM2 gangliosides. The human homolog of *CG7985*, *HexDC*, has not been investigated extensively, while other well-studied homologs are represented by HexA and HexB, which give rise to diseases like Tay - Sachs and Sandhoff that are characterized by symptoms that include blindness, loss of motor function, paralysis and psychosis. As *CG7985* has not been studied previously, we generated tools necessary for its functional analysis. Using Flp/FRT recombination a mutant has been generated. In addition, a transgenic line carrying a targeting construct for homologous recombination was generated. A UAS-line for ever-expression analyses was generated. To identify the endogenous expression of *CG7985* a genomic BAC construct encoding a fusion protein CG7985::GFP has been engineered and transgenic flies carrying this construct have been analyzed. Initial analyses of the mutant did not reveal any phenotypes affecting Rh choice in the R7 cell. Western blot analysis of R7-specific and glycosylated proteins did not reveal any changes in the glycosylation pattern of these proteins. More studies are needed to elucidate the function of CG7985 protein.

ÖZET

FOTOALMAÇ-7 HÜCRESİNE SPESİFİK ÖZGÜN GENİN SİRKE SİNEĞİ GÖRME SİSTEMİNDE KARAKTERİZASYONU

Sinir sistemi gibi karmaşık sistemlerin sorunsuz çalışmaları, yoğun bir hücre özelleşmesi ve nöronların sorunsuz bağlanması ile mümkündür. Sirke sineği sinir sisteminin takibi basit olduğundan, oldukça uygun bir model teşkil eder. Sirke sineği gözü yaklaşık 800 adet basit göz benzeri yapıdan oluşmaktadır. Bu yapılardan her biri 8 fotoalmaç hücresi ihtiva eder. Fotoalmaç hücreleri dış ve iç fotoalmaç olarak iki gruba ayrılırlar. Her bir fotoalmaç, rodopsin reseptörünün ifadesini sağlar. Fotoalmaç özelleşmesinde yeni genler bulmak için yapılan taramada *CG7985* genine rastlanmış ve oluşturduğu R7 ye özel ifade örtüsünden dolayı detaylı analiz için seçilmiştir. Bu tezin hedefi *CG7985* geninin işlev analizi yapılarak görme sistemi içerisindeki rolünü keşfetmektir. *CG7985* geninin bir hexosaminidase enzimini kodladığı ve evrimde korunduğu gösterilmiştir. Heksozaminidazlar GM2 gangliositlerin yani, beta-1-4-bağlı N-acetylheksozaminlerinin hidrolizinde görev alırlar. *CG7985*in insan homoloğu, *HexDC* geni üzerinde işlev yönünden herhangi bir çalışma bulunmamaktadır. Fakat, aynı enzim ailesinin diğer elemanları HexA ve HexB iyi çalışılmıştır. Bu genlerin Tay-Sachs ve Sandhoff sendromları gibi hastalıklarda etkin oldukları gösterilmiştir. *CG7985* geni ile daha önce çalışılmadığı için, işlevsel analiz için gerekli teknik ve aletler tasarlanıp elde edildi. Flp/FRT sistemini kullanarak bir mutant oluşturuldu, bunun yanı sıra homolog rekombinasyon için gerekli yapı hazırlandı. UAS–tabanlı yüksek ifade sağlayan bir sinek elde edildi. İlk olarak CG7985 anlatım profili analiz edildi. İçsel ifadeyi yakalayabilmek için, BAC mühendisliği kullanılarak CG7985 proteini GFP etiketli üreten bir sinek elde edildi ve analiz edilip sistemin çalıştığı gösterildi. Oluşturulan mutantların ilk analizleri henüz bir fenotiple sonuçlanmadı. Western analizleri de CG7985 enziminin hedef proteinini gösteremedi. CG7985 enziminin R7 hücre biyolojisindeki rolünü göstermek için daha fazla analiz yapılması gerekmekedir.

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LIST OF ACRONYMS / ABBREVIATIONS

BAC	Bacterial Artificial Chromosome
bp	Base Pairs
cDNA	Complementary DNA
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
FLP	Flippase
FRT	Flip Recombinase Targets
GFP	Green Fluorescent Protein
eGFP	Enhanced Green Fluorescent Protein
HRP	Horse Radish Peroxidase
PBS	Phosphate Saline Buffer
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
pH	Power of Hydrogen
PR	Photoreceptor
RNA	Ribonucleic Acid
RNAi	RNA Interference
RT-PCR	Real Time PCR
UAS	Upstream Activating Sequence
HexDC	Hexosaminidase domain containing
GH	Glycoside Hydrolase
GPCR	G-Protein Coupled Receptor
Rh3	Rhodopsin 3
N	Notch
Rh4	Rhodopsin 4
EGFR	Epidermal Growth Factor Receptor
lGMR	Long Glass Multiple Reporter
pros	Prospero
UV	Ultraviolet

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1. INTRODUCTION

1.1. Visual System

Perception of the surrounding world and its proper representation in the brain depends on the five senses. The visual system belongs to the central nervous system that enables organisms to perceive and process light and visual details. The eye, the outer-most member, gathers light information and projects it onto the brain through nerves to produce functional information about the environment

In order to study the development of the visual system, the *Drosophila* eye is used as a model. *Drosophila melanogaster* has a relatively simple nervous system compared to humans. The size of the fly nervous system and the number of total neuron and glia cells is several orders of magnitudes lower than that of human's. Additionally, the number and complexity of genes is also lower, allowing an easier dissection of gene function. Although the physiology differs significantly in many cases, most of the genes and molecular mechanisms important for the proper functioning of the nervous system are conserved.

1.2. The *Drosophila melanogaster* Eye as Model

The human eye is a camera-type eye. The retina contains two types of PRs namely the cones which are necessary for color perception and rods which are necessary for dim light vision. *Drosophila* have compound eyes that consist of ~800 ommatidia, which are the basic eye units. *Drosophila* also has two types of PRs namely the inner PRs (named according to their position) resemble the cones and are necessary for color perception. The outer PRs resemble the vertebrate rods and are necessary for dim light detection. Humans have different cone cells that can sense blue, green and red light while *Drosophila* has adapted differently and has PRs that can sense blue, green, and UV-light. In both of these organisms the spatial distribution of different types of PRs is stochastic and the mechanism of development of PRs resembles each other (Cook *et al.*, 2001).

1.3. Drosophila Ommatidia

The eye of *Drosophila melanogaster* is a widely used model organ, especially for studying mechanisms of tissue specification and cell fate determination (Sprecher *et al.*, 2008). It is composed of almost 800 independent small eye-like subunits, called ‘‘ommatidia’’. (Ready *et al.*, 1976). Each ommatidium is composed of 8 PRs (Charlton-Perkins *et al.*, 2010). Six of these are motion detecting PRs (R1–R6) located on the perimeter of each ommatidium and are called outer PRs and two of them are smaller, color detecting PRs (R7 & R8) in the middle, called inner PRs (Kirschfeld *et al.*, 1977). Outer PRs express the photopigment Rh1, whereas inner PRs express either of two photopigments (Figure 1.1a). Three major subtypes can be differentiated: pale, yellow and dorsal rim area (DRA) (Figure 1.1b).

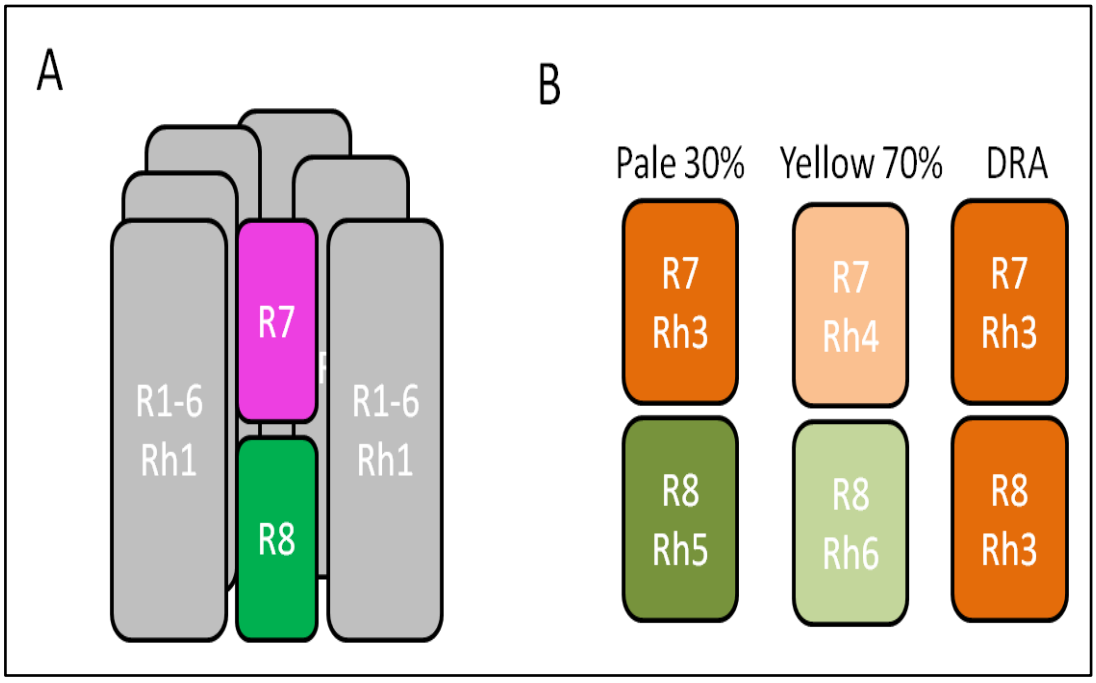

Figure 1.1. Representation of photoreceptors (A) in adult *Drosophila* ommatidium, (B) the different subtypes are determined by the expression of different types of rhodopsins.

Ommatidia develop from the eye-antennal imaginal disc (Figure 1.2) and are clusters of undifferentiated cells, which proliferate during larval stages and differentiate

during late larval and the pupal stage. From this particular disc, the cells are sequentially committed to become PRs or support cells.

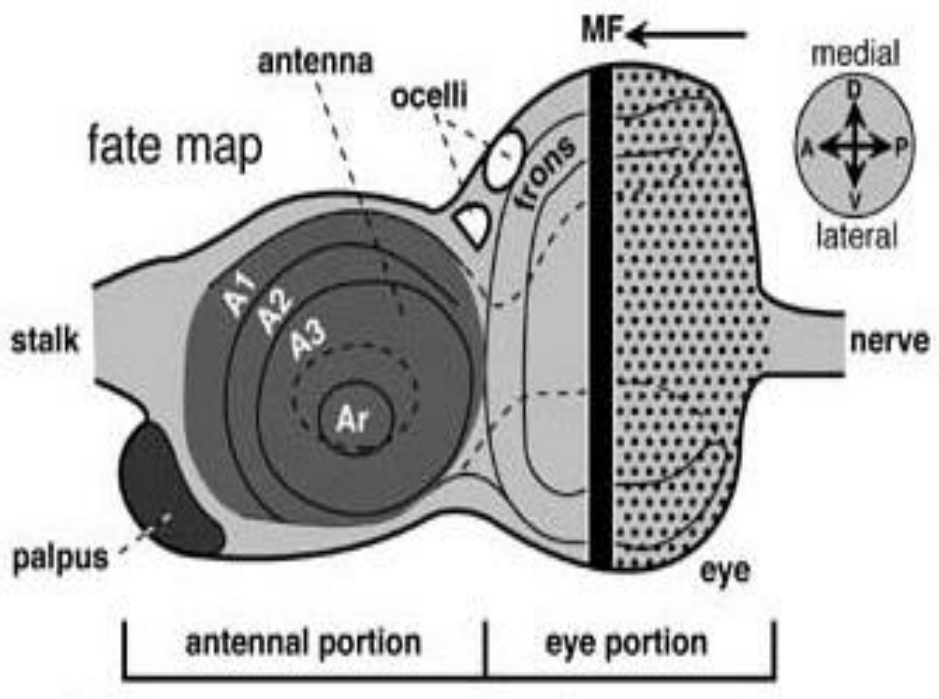

Figure 1.2. *Drosophila* eye-antennal imaginal disc by Lewis I. Held, Jr, 2005

Sequential differentiation starts from the posterior part of the disc through the movement of the morphogenetic furrow (MF) and terminates at the anterior. The MF is an imaginary linear wave that passes through the cells from posterior to anterior and in its wake crystallizes clusters of cells which give rise to the PRs of the eye by recruiting precursor cells (Kirschfeld *et al.,* 1977). The cells themselves do not migrate, however they change physically; their shape and size, biologically; their cell-cycle state and gene expression choices.

Numerous signaling pathways and hundreds of downstream genes are active within this MF so that cell specification will occur in a determined and timely fashion. PRs respond to different light intensities and wavelengths because of expression of different Rhodopsin (Rh) genes, which encode photosensitive G protein–coupled receptors (GPCRs) (Terakita *et al.,* 2005).

1.4. Specification and Differentiation of Photoreceptors

Specification of PRs in *Drosophila* depends on strong directed signaling and represents a stepwise process. Formation of the eye primordium initiates with the activity of "master regulators", which are the *Drosophila* homologs of the vertebrate *Pax6* gene namely *eyeless* (*ey*) and *twin of eyeless* (*toy*). They are called master regulators since they can create ectopic eye tissues. They start a signaling cascade that activates *sine oculis* (*so*), *eyes absent (eya*), and *dachshund* (*dac*), which are important genes necessary for eye tissue formation (Figure 1.1) (Morante *et al.*, 2007).

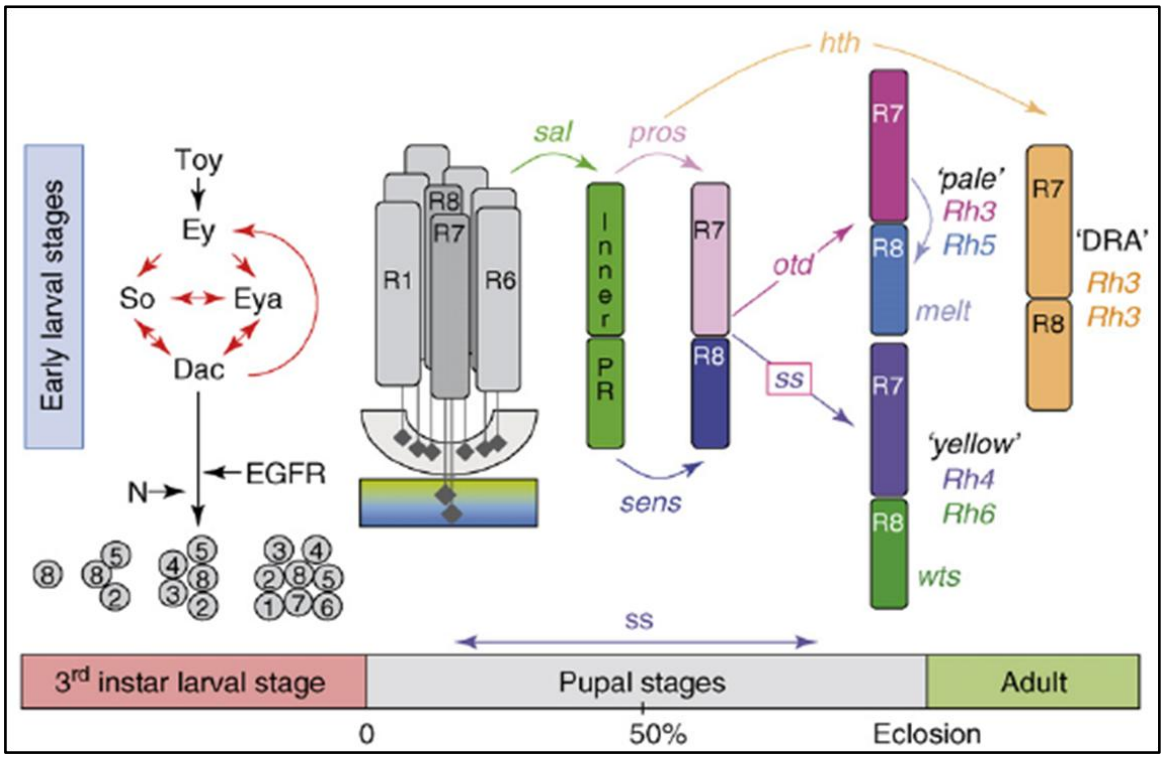

Figure 1.1. Signals involved in the specification and differentiation of PRs from larval through pupal up to the adult stage, it requires a strong and precise signaling so that each step can be carried out without errors (Morante *et al.*, 2007).

After the eye tissue formation starts, Notch (N) and epidermal growth factor (EGF) signaling pathways are activated and PRs start to form out of the epithelial cell cluster. First one to form is R8, then comes R2-R5, R3-R4, R1-R6, and then R7 (Figure 1.2) (Greenwood *et al.*, 1999). Cone cells and pigment cells join together to form the

ommatidial structure. The last PR specified, the R7 has a special importance since this study will mainly focus on the function of a R7-specific gene, *CG7985*. First of all, Sevenless (Sev) is expressed in R7 cells, which binds to Bride of Sev (Boss) that is expressed on R8 cells prior to Sev expression. This step both represses non-neuronal fate in R7 cells and activates Ras pathway. Key transcription factor in R7 specification is Prospero (Pros), whose absence turns all R7 cells into R8 cells.

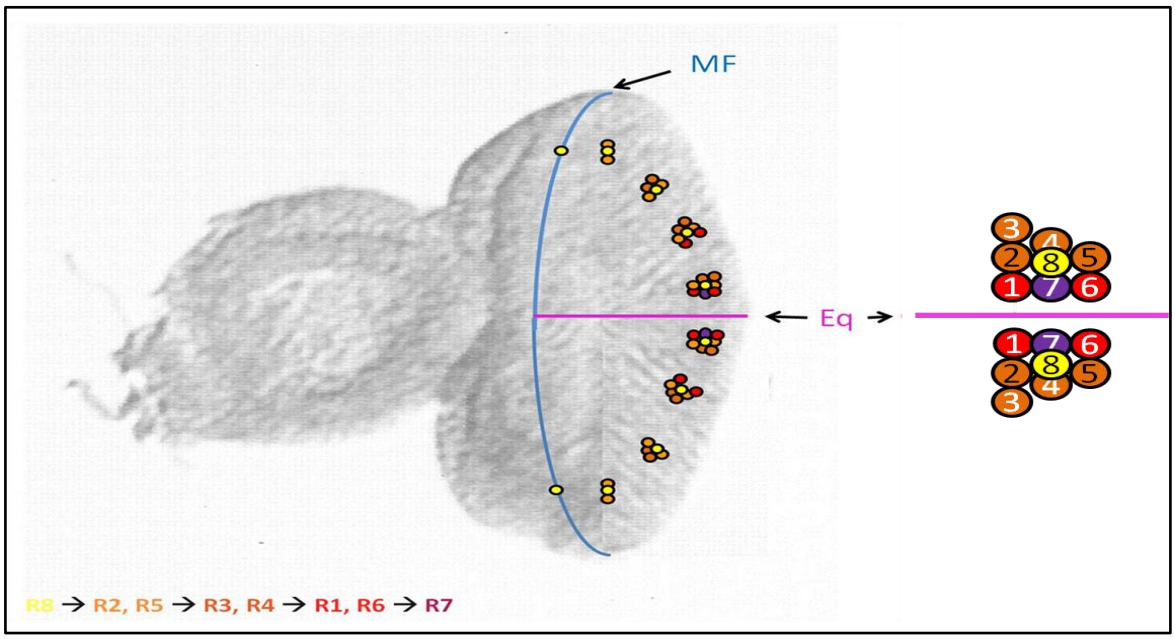

Figure 1.2. Specification of PRs during the larval stage. PRs are formed in the order, R8 being the first and R7 being the last. PRs rotate to create the final formation. PRs have mirror image rotation at the other side of the equator.

PR differentiation continues in the pupal stage, where PRs decide to follow different fates. The first decision is to become either outer or inner PRs. As summarized (Figure 1.1), the *spalt* (*sal*) gene is the main transcription factor that causes R7 and R8 to become inner PRs and in this way distinguishes them from outer PRs. In case of a mutation in the *spalt* gene, R7 and R8 acquire the outer PRs fate (Mollereau *et al.*, 2001). The inner PRs have one more step to go: subtype specification. Another transcription factor *prospero* (*pros*) causes R7 to express either *rh3* or *rh4* by inhibiting R8 specific rhodopsin promoters. This has been shown by mutating *prospero* and observing that all R7 express

R8 specific rhodopsins (Cook *et al.,* 2003). Yet, this result does not suggest that R8 cells do not need any further transcription factors for Rhodopsin choices. A well known transcription factor *senseless* (*sens*) is necessary for R8 cells to acquire their proper fate, which is to express Rh5 or Rh6, the R8-specific Rhodopsins (Xie *et al.*, 2008).

After PRs are specified and R7-R8 are differentiated, subtype specification mechanism initiates. There are 2 major and 1 minor subtypes in the fly retina; pale, yellow and dorsal rim area (DRA) (Figure 1.1). DRA is the minor subtype and consists of R7 and R8 both expressing rh3. Main transcription factor for DRA specification is *homothorax* (*hth*) (Wernet *et al.,* 2003). The main subtypes are stochastically distributed throughout the retina with a constant ratio of 30% pale and 70% yellow. Pale/Yellow subtype specification mechanism is not fully understood but key regulator *spineless* (*ss*) and *orthodenticle* (*otd*) are shown to be required for pale/yellow subtype specification (Wernet *et al*., 2006).

1.5. Glycosylation and Deglycosylation of Proteins

One of the most abundant types of post translational modifications of proteins is the glycosylation. Glycans, which are the molecules introduced during glycosylation have many different structures, purely depending on the monosaccharide used. Although there are structural differences between different organisms in terms of glycans used, the mechanism of glycosylation remains identical. Glycosides (molecules with sugar group attached) and glycosyltransferases (the enzyme that transfers monosaccharide to target molecule, e.g. protein) generate additional complexity so that different cells express different proteins using limited protein and glycan reservoirs. Different glycan attachments help same protein act different e.g. act on different receptors or different response on the same receptor. (Easton *et al.*, 2011)

Analysis of protein regulation system showed that deficiency in glycosylation and deglycosylation disrupt function and/or efficiency of protein activity (Acar *et al.*, 2007).

1.6. Glycoside Hydrolase

Glycoside hydrolases are enzymes that catalyze the hydrolysis of the glycosidic linkage to release sugar mono/polymers. They are a broad family with functions ranging

from degradation of biomass to pathogenesis mechanisms. Their importance for gene activity is related with the functional catalytic machinery composed of glycosyltransferases and depends on synthesis and breakage of glycosidic bonds. For example; the enzyme beta-galactosidase (LacZ), is involved in regulation of expression of the lac operon in *E. coli* (Monad *et al.*, 1962).

In higher organisms, glycoside hydrolases are located in the endoplasmic reticulum and Golgi apparatus to act in the processing of N-linked glycoproteins (King *et al.*, 1996). Mass spectrometry studies have enlightened the structure of N-linked glycans that are functionally attached to protein substrates during *Drosophila* embryonic development (Figure 1.3) (Aoki *et al.*, 2007).

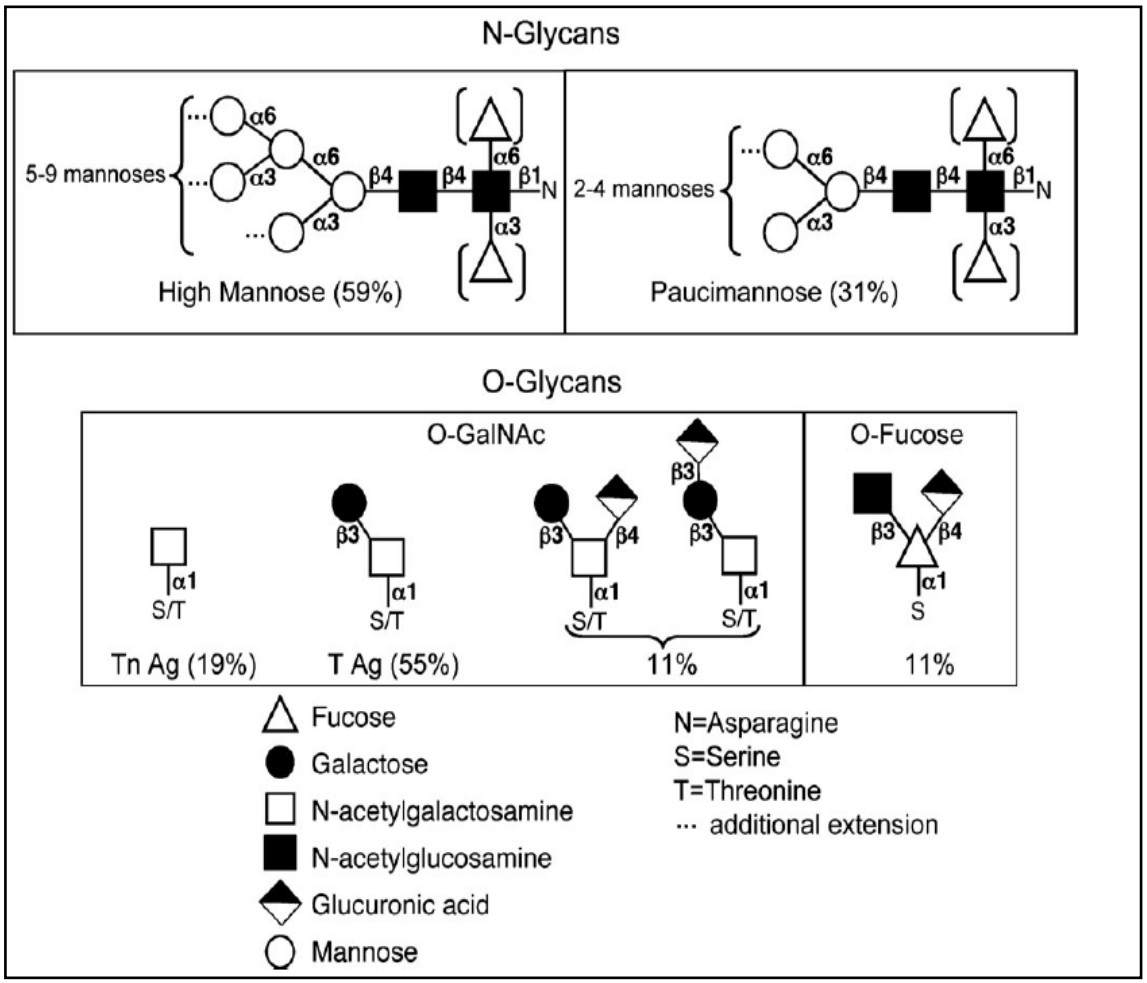

Figure 1.3. Glycans of *Drosophila melanogaster*
(ten Hagen *et al.*, 2009)

Glycoside hydrolases were shown to directly modify receptor molecules namely rhodopsins (Cao *et al.,* 2011). They also have been shown to modify and promote monoubiquitination of histones, presumably for transcriptional activation (Fujiki *et al.,* 2011).

The N-glycan structures of *Drosophila melanogaster* differ considerably from mammals (Gagneux *et al.,* 1999). But, *Drosophila* N-glycans are more closely related to mammalian N-glycans than to other studied organism (Cipollo *et al.,* 2005; Hanneman *et al.,* 2006). What makes these enzymes so crucial is that the N-glycan structure profile of *Drosophila* that changes as it develops. This can be considered as key evidence for a highly regulated glycosylation machinery that has roles for certain glycan structures during the course of developmental (Aoki *et al.,* 2008).

1.7. Glycosylation and Deglycosylation of Rhodopsins

N-linked glycosylation is important for functional folding, assembly into proper structure, oligomerization with other molecules (Kornfeld *et al.,* 1985; Helenius *et al.,* 1994). Studies have shown the importance of functional N-glycosylation sites, such that when the sites are lost, protein secretion levels are reduced significantly (Fiedler *et al.,* 1995). Proteins are distributed through the ER and during transportation to the cell surface the oligosaccharide side chains of glycoproteins are exchanged with new sugar residues. This process creates the glycosylation status of membrane proteins which makes an important factor for the specificity and efficiency of protein (Varki *et al.,* 1993; Dwek *et al.,* 1996).

It has been shown that N-glycosylation of the Rhodopsin, in PR neurons, is critical for its distribution from the ER and for its proper targeting to light sensory organelles (Hargrave *et al.,* 1977). It has also been shown that when Rhodopsin glycosylation is blocked pharmacologically, newly synthesized Rhodopsin is unable to maturate (Fliesler *et al.,* 1984). Importantly, mutations at the sites of N-glycosylation in Rhodopsin have been identified in patients of autosomal dominant *retinitis pigmentosa* (Dryja *et al.,* 1995) a retinal degeneration disorder that causes visual defects including night blindness and tunnel vision. Rh1, the most abundant and major Rhodopsin in *Drosophila*, undergoes N-

glycosylation during its biosynthesis (Brown *et al.,* 1994). Mutation of residue Asn20, the single site of N-glycosylation in Rh1, retains Rh1 in the secretory pathway and drastically reduces the level of mature Rh1 (Webel *et al.,* 2000). Interestingly, the oligosaccharide chain is either extensively trimmed or completely removed in mature Rh1 (Huber *et al.,* 1990), indicating that Rh1 undergoes deglycosylation during its transport from ER to the light sensory organelle rhabdomere (Satoh *et al.,* 1997). However, the enzymes mediating this process remain unknown.

1.8. UAS/Gal4 System

The UAS/Gal4 system is used for spatiotemporally controlled expression of genes in the fly, one of the greatest tools that make *Drosophila* such a good model organism. *Saccharomyces cerevisiae*, the donor organism of the Gal4 transcription factor protein gene, *Gal4* encodes a protein that specifically binds a short section of the promoter region, UAS (Upstream Activation Sequence) to activate transcription of the flanking gene (Figure 1.4). For an enhancer trap screen a P element carrying the Gal4 gene (P[Gal4]) is randomly inserted into the genome. If the P element enters downstream of an enhancer, the target gene expression will reflect the pattern of enhancer activity (Brand *et al.,* 1993; Wixon *et al.,* 2000). The Gal4 carrying line is named ‘driver line’. The UAS/Gal4 system is used for overexpression of a target gene or downregulation via RNA interference.

The UAS/Gal4 binary system can be repressed by Gal80 which is an inhibitor of Gal4 directly (Lee *et al.,* 1999). In certain strains Gal80 is linked to a thermosensible (ts) promoter that is only active at low temperatures (Matsumoto *et al.,* 1978). UAS-tagged gene expressed together with Gal4 and $Gal80^{ts}$ allows target gene expression at permissive temperature (29ºC), but not at restrictive temperature (18ºC), providing strict control. With these techniques the gene of interest can be expressed in the desired tissue or cells, in the desired time interval.

In addition to UAS/Gal4 binary system, there are LexA: VP16-LexAOperon and QF-QUAS binary systems. These systems are useful when there is a need for second or third binary system in order to observe effects of different genes in the same cell.

Combinations of different binary systems remove the problem of different promoters or enhancers interfering with different reporter genes (Potter *et al.*, 2010).

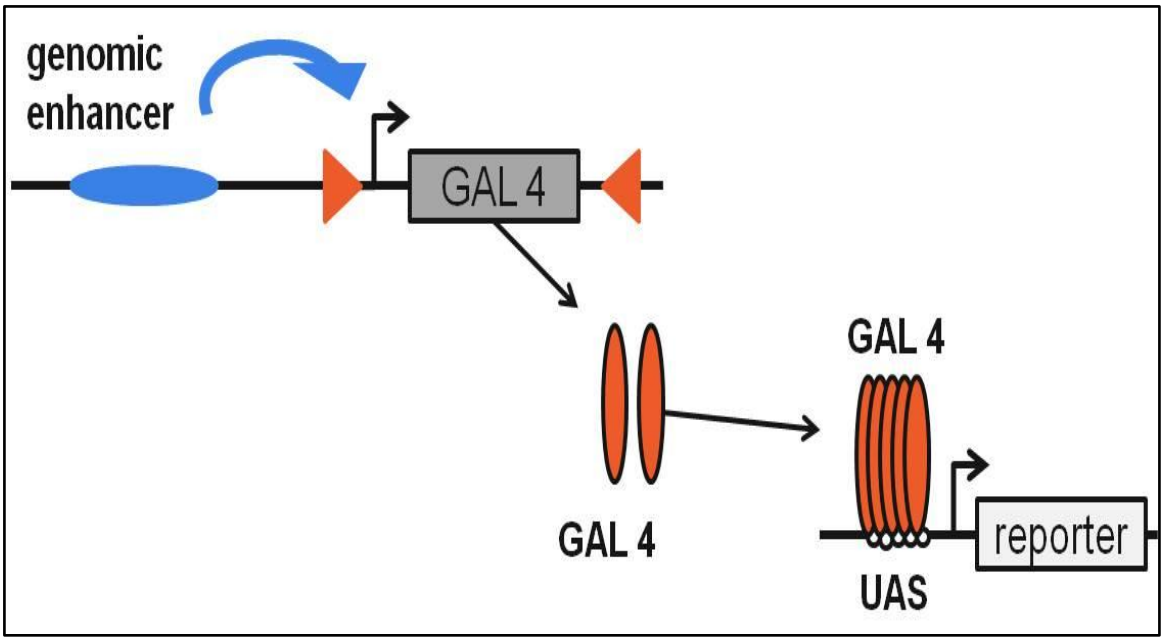

Figure 1.4. UAS/GAL4 binary system. Gal4 protein is expressed under the control of a tissue-specific enhancer/promoter. Gal4 binds to UAS sequence and leads to tissue specific expression of the reporter.

1.9. FLP/FRT System

The FLP/FRT system is another tool adapted to the fly from yeast (McGuire *et al.*, 2004). This system represents a site directed recombination method and makes use of flippase recombinase enzyme (FLP) and flippase recognition target (FRT). Once the enzyme is active it binds to the FRT and creates an FRT-mediated cleavage, where a physical crossover happens (Figure 1.5). The system is analogous to Cre-Lox recombination.

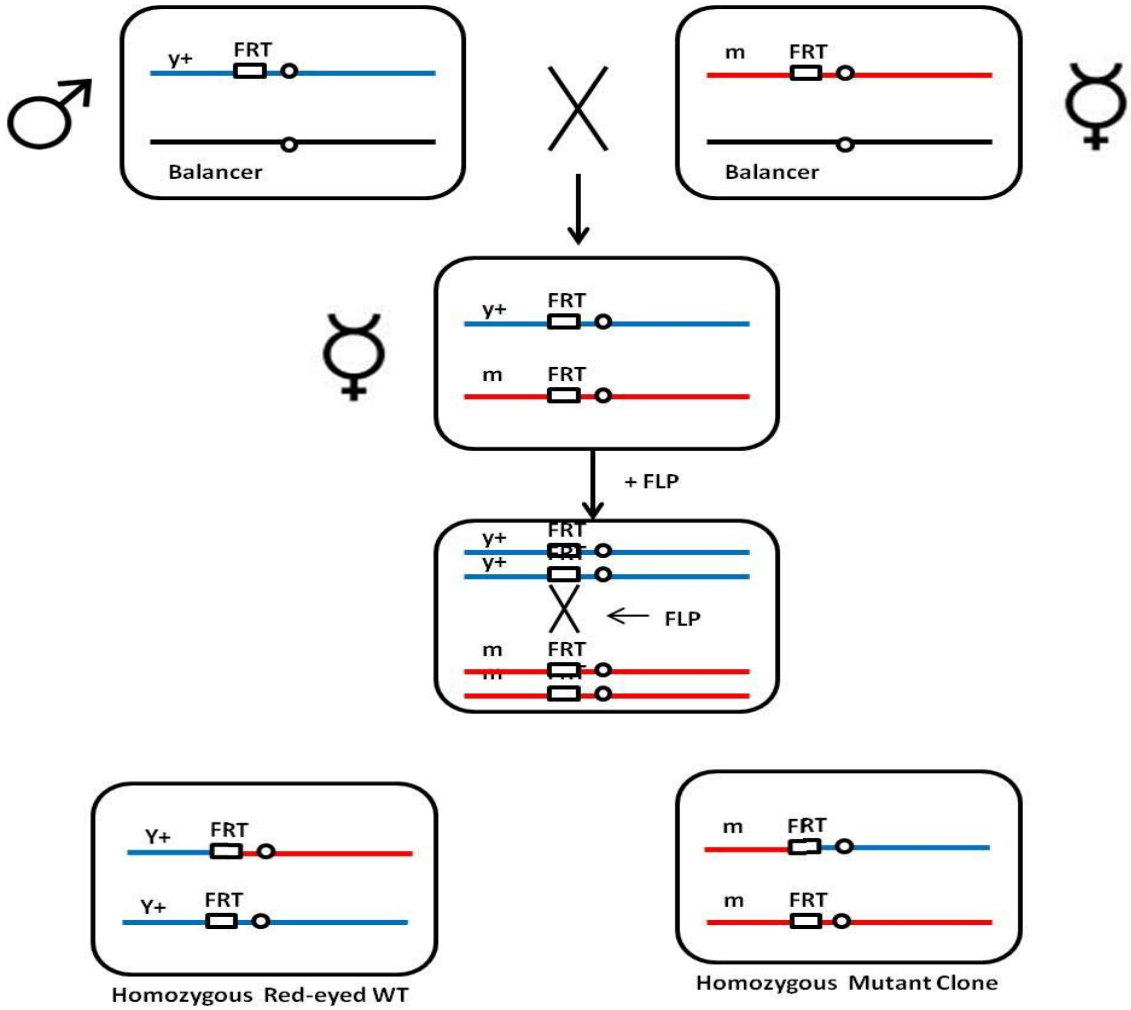

Figure 1.5. Generation of homozygous mutant clones by FLP/FRT. Males with y^+ (black body) the gene at the position of m (mutant target gene) are crossed with females, carrying the mutant gene; offsprings will create mosaic tissues, depending on the source of FLP.

The FLP/FRT system can be manipulated to create chimeric tissues, recombining chromosomes in only a subset of cells, generating genetic mosaics. Using this technique, it is possible to study homozygous lethal mutations in heterozygous animals, thus allowing the study of genes causing lethality is avoided. Since FLP is a recombinase targeting FRT sites it can be overall induced via heat shock or in specific tissues using the UAS/Gal4/Gal80^{ts} system. The daughter cells will have either two wild type copies of the gene, or two mutated copies, in an otherwise heterozygous animal (Theodosiou *et al.*, 1998).

2. PURPOSE

The nervous system is the most complex structure in our body and its proper functioning relies on the correct specification of cells and the establishment of proper connections during development. We study the fly visual system, which represents an excellent model system because of its reduced complexity as compared to vertebrate nervous systems and the well-studied specification and differentiation mechanisms including axonal targeting to the brain.

Here, we identify an R7 cell specific gene, *CG7985*, which encodes a glycosyl hydrolase and aim to investigate its function in visual system development. As this gene has not been investigated previously one of our major aims is to generate all the necessary tools to study its function. Our specific aims can be summarized as; (i) developmental analysis of *CG7985* expression, using the enhancer-trap line, (ii) generation of tools to study endogenous gene expression: GFP-tagged fusion protein expression analysis in transgenic flies (and antibody generation), (iii) generation of mutants for functional analysis: site-specific excision by Flp/FRT and homologous recombination using IMAGO, (iv) generation of a transgenic line for overexpression analysis, and (v) functional analysis of mutants.

3. MATERIALS AND METHODS

3.1. Biological Material

Flies used in this study, if not explained otherwise, were raised at 25 ºC with a 12h day -12h night cycle and 80% humidity in a temperature and humidity controlled chamber. They were fed commercially available fly food (Bloomington formula, Flystuff) was used and prepared fresh twice a week.

Fly lines, used in this study, are listed in Table 3.1.

Table 3.1. Fly lines used in this study.

Lines	Chr. No.	Description
CyO	II	Balancer allele
TM2	III	Balancer allele
TM3	III	Balancer allele
TM6	III	Balancer allele
ey-FLP	II	Expresses Flippase under the control of *eyeless* promoter
GMR-Hid	III	Expresses apoptotic hid gene under the control of glass multiple reporter
UAS-CG7985	II	UAS fused to CDS of *CG7985* gene
UAS-CG7985-RNAi	II	UAS fused to *CG7985* dsRNA
Elav-Gal4	II	Expresses Gal4 under the control of *elav* promoter
AC887-Gal4	III	Enhancer trap line, inserted into the first intron of *CG7985* gene
lGMR-Gal4	II	Expresses Gal4 under the control of *long glass multiple reporter* promoter
UAS-nGFP	I	UAS fused to GFP cDNA
FRT82B	III	FRT site in III chromosome that allows FLP-mediated recombination
w^{1118}		Loss of function, *white* gene
QB		Balancer flies (yw; Sp/ CyO ; TM2 / TM6B)

3.2. Chemicals and Supplies

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

3.2.1. Enzymes

Restriction enzymes and buffers were from Biolabs, Go Taq polymerase from Promega, T4 ligase and buffers were from Roche and Biolabs.

3.2.2. Chemical Supplies

Table 3.2. Chemicals used in this study.

Chemical	**Manufacturer**
1 kb Marker	NEB, USA (N3232L)
Bovine Serum Albumin (BSA)	Sigma-Aldrich, USA (A9647)
Ethidium Bromide solution	Sigma Life Sciences, USA (E1510)
Formamide	Sigma-Aldrich, USA (A9037)
Marker X	Roche, USA (11498037001)
$MgCl_2$	Riedel-de Haen, Germany (13152)
NaCl	Sigma-Aldrich, USA (S7653)
Paraformaldehyde	Sigma-Aldrich, USA (P6148)
Phenol	Chloroform : Isoamyl : Sigma-Aldrich, USA (P2069)
Agarose	Biomax (104514PR)
Triton X-100	AppliChem, USA (A4975)
Tris	Sigma-Aldrich, USA (T6066)
Trizol	MRC, USA (TR118)
Tween 20	Roche, USA (11332465001)
Xgal	Sigma-Aldrich, USA (B42529905)
Acrylamide	Sigma-Aldrich, USA (A3574)
Ampicillin	Sigma-Aldrich, USA (A9518)
DTT	Fisher, USA (A/P470/46)
Glycerol	Sigma-Aldrich, USA (G5516)
LB Agar	Sigma Life Sciences, USA (SL08394)
Kanamycin	Sigma-Aldrich, USA (K4000)
LB Broth EZMix^{TM} Powder	Sigma-Aldrich, USA (L7658)
Milk Powder	Cell Signaling, USA (9999S)

3.2.3. Buffers and Solutions

Solutions, if necessary, are autoclaved for 20` at 121 bars. Buffers and solutions used in this study are summarized in Table 3.3.

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

Name	Content
BNT	PBS (1X) 0,3% Triton X-100 1% BSA 250 mM NaCl
Elution Buffer	10 mM Tris-Cl, pH 8,5
Embryo Lysis Buffer	50 mM NaCl 50 mM Tris-Cl, pH 7,5 10% Glycerol 320 mM Sucrose 1% Triton-X 100 0,5% NP-40 0,5% Na-DOC 1X PIC
Laemmli	50 mM Tris-Cl, pH 7,5 2% SDS 10% Glycerol 0,1% Bromophenol blue 100 mM DTT
LB Agar	5 g/l NaCl 10 g/l Tryptone 5 g/l Yeast extract 14 g/l Agar
LB Broth	5 g/l NaCl 10 g/l Tryptone 5 g/l Yeast extract
Loading Buffer	50% Glycerol 0,0005% Bromophenol Blue
Lysis Buffer	200 mM NaOH 1% SDS (w/v)
Paraformaldehyde (16%, pH 7, 4)	8g Paraformaldehyde 500 mL distilled water
PBS (1X)	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4
PBST	PBS (1x) 0.1% Tween-20
PBSTX	PBS (1x) 0.05% Triton X-100
PBX3	PBS (1x) 0.3% Triton X-100
Resuspension Buffer	50 mM Tris-Cl, pH 8.0 10 mM EDTA 100 µg/ml RNase A

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

Name	Content
TAE (1X)	40 mM Tris-Cl 1 mM EDTA 0.1% Acetic acid
TBS-T (pH 8,3)	2 mM Trisma Base 15 mM NaCl 0, 1% Tween-20
Transfer Buffer (pH 8,3)	25 mM Tris base 190 mM glycine 20% ethanol
Trizol (exact content confidential)	Phenol Guanidine isothiocyanate Red dye Proprietary components

3.2.4. Oligonucleotide Primers

Lyophilized primers were dissolved with double distilled sterile water to obtain a stock concentration of 100 pmol/µl. Stocks were stored at -20ºC. Stock primers were diluted by double distilled sterile water to obtain 10 pmol/µl working concentration.

Table 3.4. Primers used in this study.

Primer Name	Primer Sequence	T_m ºC
FRT_strategy_R_	GAGAATGGGCAGGGATTATGACGC	66,9
FRT_strategy_F_	CGTATTTGTATTAGCGGCTCCCCGGC	71,1
CG7985_newmidoligo_R	TCGAGCCGCGGACCGGTGACGTCGTTAACGCTAGCC	83,6
CG7985_newmidoligo_F	CTAGGGCTAGCGTTAACGACGTCACCGGTCCGCGGC	83,6
Upstream_FRT_F	TGCGCTCGCGAAAGAGAGTAGAA	64,6
Downstream_FRT_F	GACTGGGAGCGGCAAACGCC	66,6
FRT_common_R_1	ATCACGTAAGTAGAACATGAAATAACAATATAAA	64,6
FRT_common_R_2	GCATTAAGCGCNGCGGGTGTG	65,3
Actin79b_rtpcr_cont_F	ATGTATCCAGGTATCGCTGAC	59,5
Actin79b_rtpcr_cont_R	TGCTTGGAGATCCACATCTG	58,4
CG7985_CDS_pet30_F_NcoI_	CCATGGATATGCACTCAAATCTTTGGATA	66,2

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

Primer Name	Primer Sequence	T_m °C
CG7985_CDS_pet30_R_BamHI	GGATCCTCATTGTTGCTCCTGCTGATG	69,9
CG7985_CDS_pet30_mid_F	GAGTTCTTCAGCCGCTGGCCGCGC	73,8
CG7985_RT_PCR_F	GCTTTCTCAAGCAACTGCTGCC	64,2
CG7985_RT_PCR_R	CTCGAAGCCCGAGAGTTTGAGG	65,8
CG7985_upstream_2	GTGAGCCTGTGTGAAGTGCGGT	65,8
CG7985_upstream_3	GGAGCAGACAGTGATGCTCTTGGC	68,5
CG7985_downstream_5	CCTGCCTATTAGCACCTGCTC	63,2
CG7985_downstream_4	CGGCCTTAGTCTAGCCTACAAGTTC	67,4
CG7985_downstream_3	ACAACTACCGGACTGGGAGCGG	67,9
CG7985_downstream_2	GAAGATGAGGTCGTGAGGCCG	65,3
CG7985_upstream_5	CCGCTGCGAGCTGAGAAACGCAC	69,9
CG7985_upstream_4	GAAGCCCAAGGGCTGAGTACCT	65,8
CG7985_upstream_KpnI_F	GGTACCGTGCGCTGGGTAAGGTG	69,9
CG7985_upstream_AvrII_R	CCTAGGCAATCGCGAGCAGTTGCA	68,5
CG7985_downstream_NheI_F	GCTAGCAAACCTCTTATTGCCGTCTTC	68,2
CG7985_downstream_SacII_R	CCGCGGTTCGGCCTAAGTTCAACAAATG	71,8
hexdc_pet30_NcoI_F	CCATGGATAGCGTCTCGTTCATCTGGAC	71,8
hexdc_pet30_BamHI_R	GGATCCTCATTGTTGCTCCTGCTGATG	69,9
hexdc_pFastBac_NcoI_F	CCATGGCAAAATGAGCGTCTCGTTCATCTGGAC	75,4
hexdc_pFastBac_NheI_R	CGATCGTCATTGTTGCTCCTGCTGATG	69,9
hexdc_R	GGATCCTCATTGTTGCTCCTGCTGATG	69,9
hexdc_F	CCATGCATAGCGTCTCGTTCATCTGGAC	71,8
cg7985_galk_F	GCCTCTGCCCTTGAAAGTGCAGGAGGACATGGGACTGGTGA CGCATCAGCAGGAGCAACAACCTGTTGACAATTAATCATCG GCA	91
cg7985_galk_R	ATGGCATTATATTTCAGGTATACTTAAGGTAACTGCCAACAG GGCACTCACCTGGCGCCTCTCAGCACTGTCCTGCTCCTT	88,9
CG7985_F	CGTCTCCAAGGGCTACTTCA	60,5
CG7985_R	TCAGGATCAAGAGCCGAGTC	60,5

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

Primer Name	Primer Sequence	T_m °C
eGFP-overlap_R	CCAGTGAAAAGTTCTTCTCCTTTACTCATGCCGCTGCCTTGTTGCTCCTGCTGATGCGT	85,9
eGFP-overlap_F	GATTACACATGGCATGGATGAACTATACAAAGGCAGCGGCTGAGAGGCGCCAGGTGAGT	86,7
CG7985_eGFP_Seq_R1	AGAGGCAGTGGTCTCTGTG	59,5
CG7985_eGFP_Seq_R2	TGTGTCCAAGAATGTTTCCATCT	59,2
CG7985_eGFP_Seq_F1	CCGAAGGTTATGTCCAGGAA	58,4
CG7985_eGFP_Seq_F2	CCTCGGGCCAAATATGAGT	57,5
CG7985_eGFP_Final_Seq_F	TGGTGAGAGCTTGTTGGTGC	60,5

3.2.5. Antibodies

Antibodies used in this study are summarized in Table 3.5.

Table 3.5. Antibodies used in this study.

Name	Antigen	Species	Dilution	Source
Primary Antibody				
Anti-Elav	Elav	Rat	1:50	DSHB
Anti-Elav	Elav	Mouse	1:50	DSHB
Anti-GFP	GFP	Rabbit	1:1000	Invitrogen
Anti-GFP	GFP	Mouse	1:1000	Promega
Anti-Prospero	Prospero	Mouse	1:5	DSHB
Anti-NC82	Bruchpilot	Mouse	1:100	DSHB
Anti-Rh3	2B1	Mouse	1:250	Steve Britt
Anti-Rh4	11E6	Mouse	1:250	Steve Britt
Anti-Notch int.	C17.9C6	Mouse	1:250	DSHB
Anti-HexDC	HexDC	Mouse	1:250	ABCAM

Table 3.5. Antibodies used in this study (cont.).

Name	**Antigen**	**Species**	**Dilution**	**Source**
Primary Antibody				
Anti-His	6x His	Mouse	1:1000	CST
Secondary Antibody				
Alexa 488	Mouse IgG	Donkey	1:800	Invitrogen
Alexa 488	Rabbit IgG	Goat	1:800	Invitrogen
Alexa 488	Rat IgG	Donkey	1:800	Invitrogen
Alexa 555	Mouse IgG	Goat	1:800	Invitrogen
Alexa 647	Rat IgG	Donkey	1:800	Invitrogen
HRP linked	Mouse IgG	Horse	1:1000	ABCAM

3.2.6. Embedding Media

Vectashield Embedding Medium (Vector Laboratories, Inc) was used as embedding medium for samples stained with fluorescently labeled secondary antibodies.

3.2.7. Disposable Labware

Table 3.6. Disposable labware used in this study.

Labware	**Manufacturer**
96 well plate sealers	Bio-Rad, USA
96 well plates PCR / Culture	Bio-Rad, USA
Culture tubes, 14 ml	Greiner Bio-One, Belgium
Filter tips	Greiner Bio-One, Belgium
Microscope cover glass	Fisher Scientific, UK
Microscope slides	Fisher Scientific, UK
Nylon membrane (0.2 μm)	Sartorius Stedim Biotech, France
PCR tubes (200 μl)	Bio-Rad, USA
Petri dish	Greiner Bio-One, Belgium

3.2.8. Equipment

Table 3.7. Equipment used in this study.

Equipment	**Manufacturer**
Autoclave	Astell Scientific Ltd., UK
Centrifuges	Eppendorf, Germany (Centrifuge 5424, 5417R)
Cold Room	Birikim Elektrik Soğutma, Turkey
Confocal Microscope	Leica Microsystems, USA (TCS SP5)
Electronic Balances	Sartorius, Germany (TE412)
Electrophoresis Equipment	Bio-Rad Labs, USA (ReadySub-Cell GT Cells)
Environmental Test Chamber	Sanyo, Japan (MLR 351H)
Fluorescence Stereomicroscope	Leica Microsystems, USA (MZ16FA)
Freezers	Arçelik, Turkey Thermo Electron Corp., USA (Thermo Forma 723)
Gel Documentation System	Bio-Rad Labs, USA (Gel Doc XR)
Heating Block	Fisher Scientific, France (Dry-bath incubator)
Heating magnetic stirrer	IKA, China (RCT Basic)
Incubator	Weiss Gallenkamp, UK (Incubator Plus Series)
Inverted Microscope	Zeiss, USA (Axio Observer, Z1)
Laboratory Bottles	Isolab, Germany
Micropipettes	Eppendorf, Germany
Microwave oven	Vestel, Turkey
pH meter	WTW, Germany (Ph330i)
Real Time PCR machine	Thermo Scientific Piko Real 96
Rotamax	Heidolph
Stereo Microscope	Olympus, USA (SZ61)
Thermal Cycler	Bio-Rad Labs, USA (C1000 Thermal Cycler)
Vortex Mixer	Scientific Industries, USA (Vortex Genie2)
Water Bath	Grant Instruments, UK (JB Aqua 12)

3.3. Molecular Biological Techniques

3.3.1. Isolation of Genomic DNA

Embryo lysis buffer was prepared freshly using 200 ug/ml Proteinase K. Single whole fly was placed into a 2,0 ml tube and smashed with a pipette tip containing 50 µl of lysis buffer, which is released slowly while smashing. Mixture was incubated at RT for 20 min. Proteinase K was heat-inactivated at 95^{o}C for 2 min. After a brief spin down, supernatant was transferred into a fresh 1, 5ml test tube.

3.3.2. Isolation of BAC and Plasmid DNA

To obtain plasmid DNA the QIAprep Spin Miniprep Kit (QIAGEN) and/or Roche High Pure Miniprep kit (Roche) were used according to the manufacturer's protocol. Briefly, overnight cultured bacterial cells (5ml) were centrifugated in a 12 ml culture tube at 4400 rpm for 5 min. The supernatant (LB) was removed and the pellet (bacterial cells) was resuspended in 250 µl of resuspension buffer. After that 250 µl of lysis buffer was added and mixed thoroughly by inverting the tube. Then, 350 µl of neutralization buffer was added and mixed thoroughly by inverting the tube. The mixture observed to be covered with whitish staff and was centrifuged for 5 min at 13000 rpm in a table top microcentrifuge. The supernatant was applied to the spin columns of the kits and centrifuged for 30 sec at 11000 rpm. The flow-through was discarded and the spin column was washed by adding 500 µl of wash buffer and centrifuged for 30 sec at 11000 rpm. The flow-through was discarded and the spin column was placed in a 1.5 ml test tube, up to 50 µl of elution buffer was added and centrifuged for 2 min at maximum speed (14600 rpm).

For BAC isolation and for large scale plasmid DNA isolation, QIAGEN midiprep kit and / or Roche High Pure midiprep kit was used according to the manufacturer's protocol. Overnight cultured bacteria (25 ml) were collected in a 50 ml test tube by centrifuging at 5500 rpm for 30 min at 4ºC. The supernatant (LB) was discarded and the pellet was resuspended in 4 ml of resuspension buffer by vortexing. After that 4 ml of lysis buffer was added, mixed thoroughly by inverting the tube and incubated at RT for 5 min. Then, 4 ml of ice-cold neutralization buffer was added mixed thoroughly by inverting the tube and incubated on ice

for 15 min. To precipitate the pellet the mixture was centrifuged for 45 min at 5500 rpm at 4ºC. The supernatant re-centrifuged in a new 50 ml falcon tube for 30 min at 5500 rpm at 4ºC. QIAGEN-100 column was equilibrated by addition of 4 ml of equilibration buffer. The supernatant was applied to the column after the equilibration buffer flows through. The column was washed 2 times with 10 ml of washing buffer. The DNA was eluted with 5 ml of eluting buffer into a new 15 ml falcon test tube. DNA was precipitated by addition of 3.5 ml of ice-cold isopropanol and centrifuged for 30 min at 6000 rpm at 4ºC. The supernatant was removed carefully; keeping the pellet in place, 2 ml of 70% EtOH was added to wash off the isopropanol and centrifuged for 15 min at 6000 rpm at 4ºC. The supernatant was removed and the pellet was air-dried. At last, the pellet was dissolved in 50 µl of EB or distilled water, according to the follow up experiment and transferred into a new 1.5 ml test tube.

3.3.3. Isolation of Total RNA

In order to obtain RNA from fly stocks, approximately 20 flies were decapitated and the heads were placed into a 2 ml test tube containing 800 µl of Trizol reagent under the hood. Tissues were homogenized with a small size homogenizer. The mixture was incubated at RT for 5 min to allow nucleoprotein complexes to dissolve. 160 µl of chloroform was added, mixed and incubated at RT for 15 min. The solution was centrifuged at 12000 rpm for 15 min at 4ºC. The aqueous phase was transferred into a new 1,5 ml test tube, without disturbing other layers and 400 µl of isopropanol was added. The solution was incubated at RT for 10 min and centrifuged at 10000 rpm for 10 min at 10ºC. The supernatant was discarded and the pellet was washed with 800 µl 75% EtOH and centrifugation at 7500 rpm for 5 min. The EtOH was discarded and the pellet was air-dried. Finally, the pellet was dissolved in 20 µl of DEPC-treated distilled water.

3.3.4. Transformation of Plasmid DNA

$CaCl_2$ competent bacteria (*E. coli TOP10, BL21, Rosetta2, JM109, and DH5α)* were thawed on ice. Plasmid DNA or the ligation product was added to the thawed bacteria and incubated for 30 min on ice. The mixture was heat-shocked at 42ºC for 90 sec. and

incubated on ice for 2 min. Depending on the antibiotics, bacteria were either directly spread on plates or first grown in 1 ml LB at 37ºC for 1 hour shaking, then 100 µl of the mixture was spread on an LB agar plate that contains the appropriate antibiotic and incubated overnight at 37ºC.

3.3.5. Restriction Digestion of DNA

Digestion of DNA was performed using restriction enzymes according to the manufacturer's suggestions, usually for 1µg of DNA is restricted with 1U of restriction enzyme in 50µl solution. Restriction enzymes were heat inactivated whenever possible and treated as required for the follow up experiments, usually purified either by agarose gel extraction or by PCR purification.

3.3.6. Dephosphorylation

Whenever vector DNA is linearized using single restriction enzyme digestion or by two enzymes that produce compatible ends, to prevent religation of vector DNA during ligation reactions, the 5' phosphate of linearized vector DNA was removed using Calf Intestinal Phophatase (CIP, NEB) according to the manufacturer's protocol. The enzyme was heat inactivated at 65ºC for 10 min and vector DNA was extracted from agarose gel to remove CIP.

3.3.7. Reverse Transcription and cDNA synthesis

Reverse Transcription and cDNA synthesis was done using Invitrogen, SuperScript First-Strand kit according to the manufacturer's protocol. Briefly, approximately 1 µg of RNA was isolated from fly heads was incubated with 1 µl of oligo dT primers in a total volume of 5 µl for 5 min at 70ºC to remove secondary structures. Then the mixture was incubated on ice for 5 min. The reaction mix was prepared by adding 6.5 µl of distilled water, 2 µl of reverse transcriptase buffer, 4 µl of 2 mM $MgCl_2$, 1 µl of 25 mM dNTP, 0.5 µl of 1U RNase inhibitor and 1 µl 1U of reverse transcriptase. The reaction mixture and the sample mixture were combined and incubated for 5 min at 25ºC, then at 42ºC for 1 hour.

Finally, the mixture was incubated at 70ºC for 15 min to heat-inactivate the reverse transcriptase.

3.3.8. Ligation

Ligations were performed using T4 DNA Ligase (NEB or Roche) overnight at 16ºC in 10 µl volume, according to the manufacturer's protocol.

3.3.9. Real Time Quantitative PCR

RT-qPCR reaction was carried out using iQ^{TM} SYBR Green Supermix. 1µl of freshly prepared cDNA was placed into the SYBR Green Supermix with primers that are specific to the cDNA and was run on a Thermo Scientific Piko Real 96 real time PCR machine that can detect light signal which is emitted by formation of dsDNA. The data was analyzed using Microsoft Excel.

3.3.10. Colony PCR

In order to screen for positive colonies after a ligation colony PCR was used. Colonies were picked with toothpicks or sterile tips and were transferred into the PCR tubes containing 11, 9 µl distilled water, and streaked on a bacterial plate, so that the colony can later be amplified once they are verified. PCR tubes were heated up to 95ºC for 15 min to burst the cells open. After that the master mix of PCR solution was prepared by mixing 1 U home-made Taq polymerase, 1X Taq polymerase buffer, 1,2 mM $MgCl_2$, 0,2 mM dNTP and 0,5 µM of primers and distributed on to the burst colonies in PCR tubes. The PCR was carried out according to the standard protocols. The PCR products were run on a 1% agarose gel.

3.3.11. BAC Recombineering

BAC recombineering requires combination of 3 unique techniques together. (i) BACs generated in P[acman] library, (ii) SW102/SW105 *E. coli* strains that are capable of recombining, (iii) efficient selection method, GalK.

A BAC clone carrying the gene of interest and its flanking regions was ordered from the P[acman] library and was electro-transformed into SW102 strain. SW102 cells containing the BAC clone were cultured at 32ºC overnight and diluted 1:50 the next day. After OD reached 0, 6 the recombineering genes were activated by 42ºC heat induction for 15 min. Bacteria were harvested and made electro competent (Warming *et al.,* 2005). After transformation with desired PCR product, cells were recovered for 3h at 32ºC.

3.3.12. Agarose Gel Electrophoresis

Unless otherwise stated, 1% agarose gel (w/v) was prepared with 1x TAE buffer and 30ng/ml ethidium bromide solution. Samples were prepared by mixing with loading dye to a final concentration of 1x and loaded on the agarose gel. 100bp or 1 kb Marker was used as size marker. The gel was run at 100V for less than an hour and visualized under UV (Bio-Rad, USA).

3.3.13. Gel Extraction of DNA

After DNA samples were run on an agarose gel, the DNA fragment of interest was cut from the agarose gel with a clean scalpel and placed into a 2 ml test tube. Roche High Pure Gel Extraction Kit (Roche) was used according to the manufacturer's protocol. Briefly, binding buffer was added to the agarose gel slice 3 times the weight of the slice and incubated for 20 min at 65ºC on a shaking hot plate. 300 µl of isopropanol was added to the sample to precipitate the DNA. The sample was applied to the spin column of the kit and centrifuged for 1 min at 11000 rpm. The flow-through was discarded and the column was washed with 500 µl of washing buffer twice and centrifuged for 30 sec at 11000 rpm.

Finally, the DNA was eluted using 50 µl of elution buffer and centrifuged for 2 min at maximum speed (14600 rpm). The samples are stored at -20ºC.

3.3.14. Sequencing Analysis

Purified DNA samples were directly subjected to sequencing. Samples were sequenced at Macrogen Inc. (Korea) and DNA sequences were analyzed by using Vector NTI (Invitrogen) or ApE (a plasmid editor) by M. Wayne Davis softwares.

3.4. Generation of Transgenic Flies

Generated constructs were amplified in bacteria and harvested with Qiagen™ midi kit and sent to Genetivision Inc, USA for injection.

3.4.1. Generation of an UAS-misexpression construct:

An EST clone containing the full-length coding sequence of *CG7985* gene was ordered from Berkeley Drosophila Genome Project (BDGP) (Rubin *et al.,* 2000) and amplified with HF DNA Polymerase Advantage™ using primers "CG7985_CDS_F_XhoI and CG7985_CDS_R_XbaI" and cloned into pGEMT-Easy vector system. Ligation products were transformed into *E.coli* and spreaded on plates. Candidate colonies were confirmed with colony PCR using universal primers (M13F and M13R). 6 colonies were sequenced to check for possible mutation and one mutation free clone was chosen. Both pGEMT-Easy with CDS and pUAST were cut with XhoI and XbaI. After confirming ligation midi kit was used for a large-scale DNA preparation and the final construct was sent to Genetivision, Inc to for injection.

3.5. Biochemical Methods

3.5.1. IPTG induction

IPTG inducible cells were inoculated in 5ml LB + appropriate antibiotics and grown at 37°C overnight. The overnight culture was diluted in fresh LB + appropriate antibiotics 1:50 total volume of 50 ml and grown at 37ºC for approximately 4 hours (until OD_{600} = 0, 6 – 0, 8). 1 ml of the culture was used as uninduced control. 1µl of 1M IPTG was added per each ml of culture to the rest of the culture. Both induced and uninduced cultures were grown at 37°C for 3 hr. Cultures were then centrifuged and pellets collected and stored at -80ºC.

3.5.2. Protein Extraction

Approximately 20 adult fly heads were collected and homogenized in 100 µl of embryo lysis buffer. The mixture was incubated on ice for 15 min allowing structures to dissolve. The mixture was then centrifuged for 10 min at 4ºC, 10000 rpm. The supernatant was transferred into a new 1, 5 ml test tube and 3x volume Laemli's buffer, 1x volume 2mM DTT was added and the samples and boiled for 5 min.

3.5.3. SDS-PAGE

SDS-PAGE gels are prepared with a resolving gel prepared and polymerized first and a stacking gel was prepared and polymerized on top of the resolving gel. Bio-Rad gel preparation system was used.

Table 3.8. SDS-PAGE ingredients for each gel.

Ingredients	Resolving gel (5ml)	Stacking gel (3ml)
ddH_2O	1,7 ml	2,175 ml
Tris-Cl (pH 8,8)	1,875 ml	-
Tris-Cl (pH 6,8)	-	375 µl
Acrylamide-Bisacrylamide 29:1	1,35 ml	397,5 µl
SDS (20%)	25 µl	15 µl
APS (10%)	50 µl	30 µl
TEMED	3 µl	7,5 µl

3.5.4. Western Blot

Polyvinylidene difluoride (PVDF) membranes were activated in methanol for 30 sec and washed in transfer buffer. SDS-PAGE gel, was cut to match the membrane size and placed into the transfer chamber. Depending on the expected protein size, transfer is achieved under a 200mA current for 2 hours. After blotting, the efficiency of the transfer the membrane was verified using Panacea's Red staining and washed by TBS-T for 3 times 10 min each. The membrane was blocked in 5% non-fat milk (in TBS-T) for 1 hour. Primary antibodies were used according to the manufacturer's protocol and prepared in 2% non-fat milk (in TBS-T) and incubated overnight. The next day, the membrane was washed with TBS-T, 3 times for 10 min. The secondary antibody, HRP coupled anti-mouse IgG was prepared 1:1000 in 5% non-fat milk (in TBS-T) and incubated with the membrane for 2 hours at RT. Membrane was washed with TBS-T 3 times for 10 min each. HRP revealing kit 20x LumiGLo was diluted in 700 TBS-T and membranes were incubated with this solution for 3 min.

3.6. Histological Techniques

3.6.1. Immunohistochemistry

All of the immunohistochemistry experiments except western blots were carried out as stated; Tissues were dissected in ice-cold PBS on dissecting plate under the microscope; dissected tissues were transferred into the staining dish containing 0,3% PBSX, on ice. In less than 30 min, PBSX was removed and replaced by 150 µl of 4% PFA (paraformaldehyde) for fixation and incubated on 100 rpm shaker for 20 min at RT. PFA was then removed and tissues were washed with 150 µl 0,3% PBX at 100 rpm shaker for three times, 10 min each, at RT. The supernatant was removed and tissues were blocked with 150 µl BNT for one hour on a shaker at RT. Primary antibody solution was prepared in BNT as shown in Table 3.4. BNT was removed and 150 µl of primary antibody solution was added on top of the tissues and incubated on 100 rpm shaker for overnight at 4° C. Next day primary antibody was removed and tissues were washed with 150 µl 0,3% PBX on 100 rpm shaker for 10 min at RT, three times. The appropriate secondary antibodies

were prepared as 1:800 dilutions in BNT and 150 µl of secondary antibody solution was added on top of the tissues and incubated on 100 rpm shaker at RT for 2 hours. Tissues were washed with 0, 3 % PBX, 3 times, mounted and visualized by confocal microscopy.

3.6.2. Antibody Staining of Larval Eye Imaginal Discs

The protocol was carried out as explained in immunohistochemistry section.

3.6.3. Antibody Staining of Whole Mount Adult Eye

Adult fly heads were collected, the proboscis was removed and then the eye was separated from residual brain and cuticle particles. Lamina parts were removed to enhance the antibody staining efficiency. Antibody stainings were carried out as explained in immunohistochemistry section.

3.6.4. Antibody staining of Adult Brains

The brains were collected in 2% PFA (in PBS, with 0.05% Tween-20) on ice and the brains were fixed at RT for 90 min. rest is carried out as explained in Section 3.6.1.

3.7. Bioinformatics

3.7.1. Phylogenetic Analysis

NCBI Protein Blast was the selected database of analysis. All homologs were chosen form reference sequence database among proteins experimentally shown to exist. Alignment of the respective amino acid sequences of the proteins was performed ClustalW server of EMBL-EBI. Mega5 was the denoted program for phylogenetic analysis using the

Kimura2 algorithm parameter with Neighbor-Joining method and/or Maximum Likelihood method based on the Tamura-Nei algorithm.

Additionally, Bootstrap test of phylogeny with repeats of 100, the initial phylogeny was constructed (Tamura *et al.*, 1993).

3.8. Experiments for Functional Knock-down of *CG7985*

3.8.1. Knockdown by RNAi

Vienna Drosophila Research Center has a database of publicly available UAS-RNAi lines for almost every single gene. UAS-RNAi lines for the *CG7985* gene were ordered and crossed to the driver lGMR-Gal4, ey-Gal4 in order to knock down the *CG7985* gene specifically in the eye.

UAS-Dicer was added to the background to enhance RNAi efficiency.

3.8.2. Crosses

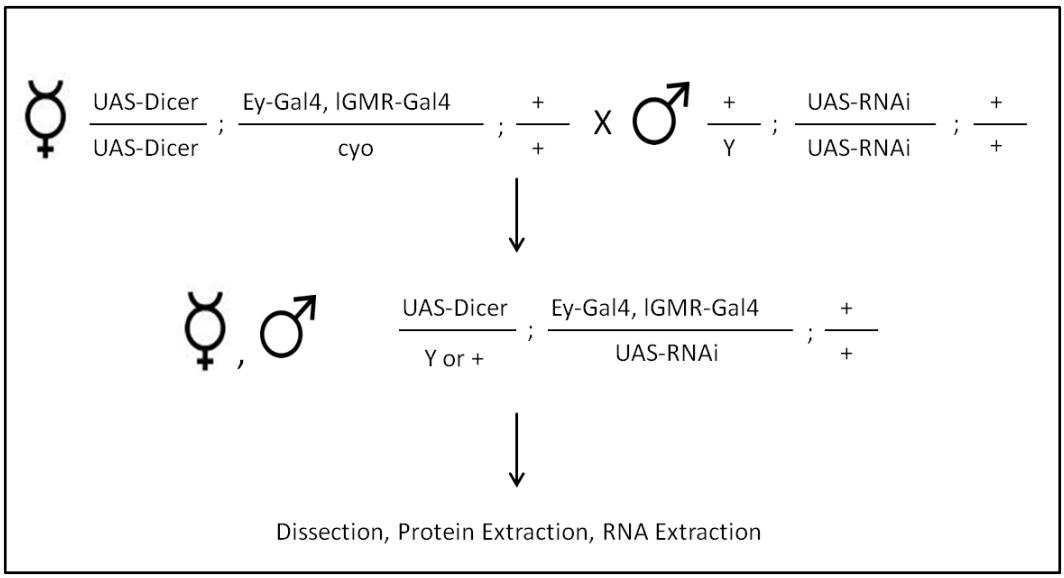

Figure 3.1 Crossing scheme for down regulation of *CG7985* gene using RNA interference F1 flies were selected against CyO to prevent recombination.

3.8.3. Analysis

Whole mount adult eyes were stained against Rh5/Rh6 to check for any change in Rhodopsin ratio. Mounted samples were visualized under the confocal microscope. RNA was extracted from adult head and used for quantitative real time PCR to check the efficiency of down regulation at the mRNA level.

Driver flies were used as control in quantitative real time PCR. Protein extracted from adult heads was used for Western blot analysis using anti-Rh3, anti-Rh4, and anti-Notch intracellular domain antibodies.

3.9. Experiments for Misexpression of *CG7985*

3.9.1. Generation of an misexpression construct for *CG7985*

Flies carrying UAS-CG7985 construct were created as explained in Generation of Transgenic Flies section. In order to misexpress *CG7985* gene specifically in photoreceptor cell, lGMR-Gal4 was used as a driver.

3.9.2. Crosses

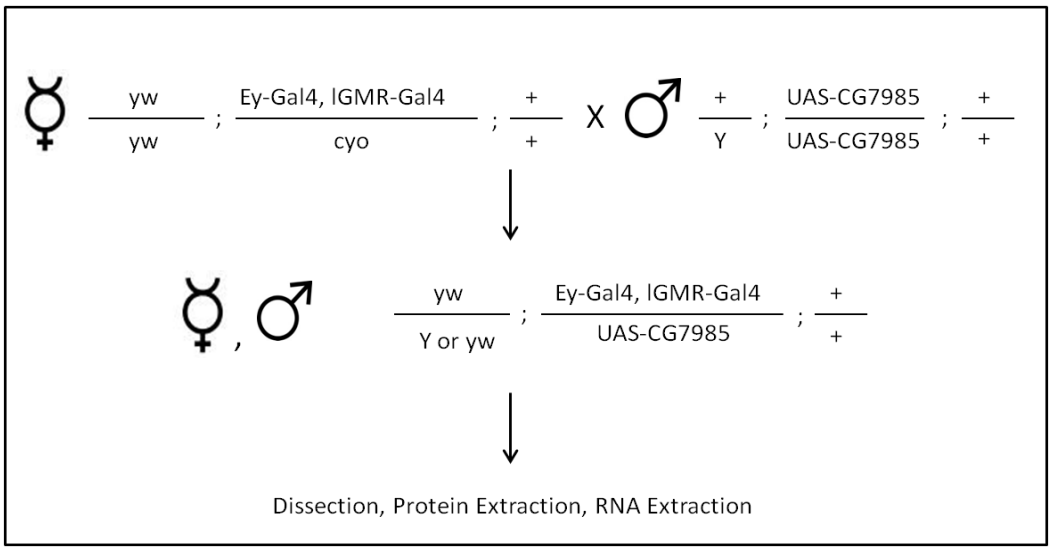

Figure 3.2 Crossing scheme for misexpression of *CG7985* gene in photoreceptor cells F1 flies were selected against CyO to prevent recombination.

3.9.3. Analysis

RNA was extracted from adult heads and used for quantitative real time PCR to check the efficiency of down regulation at the mRNA level. RNA extracted from driver flies were used as control in quantitative real time PCR. Proteins extracted from adult heads were used for western blot analysis using anti-Rh3, anti-Rh4, and anti-Notch intracellular domain antibodies.

4. RESULTS

The *CG7985* gene referred as Dmel\CG7985 was identified in an enhancer-trap screen (Arzu Çelik, unpublished). It was selected for further analysis because of its interesting expression pattern in R7 cells It is predicted to be a protein-coding gene from *Drosophila melanogaster* and has an annotated 80kDa polypeptide. CG7985 protein was suggested to have cation binding activity, hydrolase activity, hydrolyzing O-glycosyl compounds. It was also suggested that CG7985 protein is involved in carbohydrate metabolism having sequence homology to glycoside hydrolase family 20 members' reference sequence data. In this study, the aim was generate tools necessary to study the function of *CG7985*.

4.1. Bioinformatic Analyses of *CG7985*

Bioinformatic analysis using DNA sequences did not reveal any direct homologues of *CG7985*. This is thought to be due o the high divergence of insect gene sequences during evolution. Thus, alignments were carried out using its protein sequence. The protein sequence of *CG7985* was taken from Flybase, a public database for *Drosophila melanogaster*. Candidate protein sequences were found using NCBI Blast and collected for alignments. ClustalW aligned proteins were used to drive the phylogenetic tree. Many different algorithms were used for fine tuning of the branches but basically, bootstrap method was the major evaluation method.

4.1.1. *CG7985* is Homologous to Human HexDC

The results of the phylogenetic analyses showed that *CG7985* is the homolog of human HexDC, hexosaminidase domain containing protein (Figure 4.1). The homology was 71% on the amino acid level. Databases were analyzed for HexDC and identified two reports for *HexDC*. The first study identifies *HexDC*, shows that it has hexosaminidase

activity and suggests that it has the activity to hydrolyze terminal glycans (Gutternigg *et al.,* 2009).

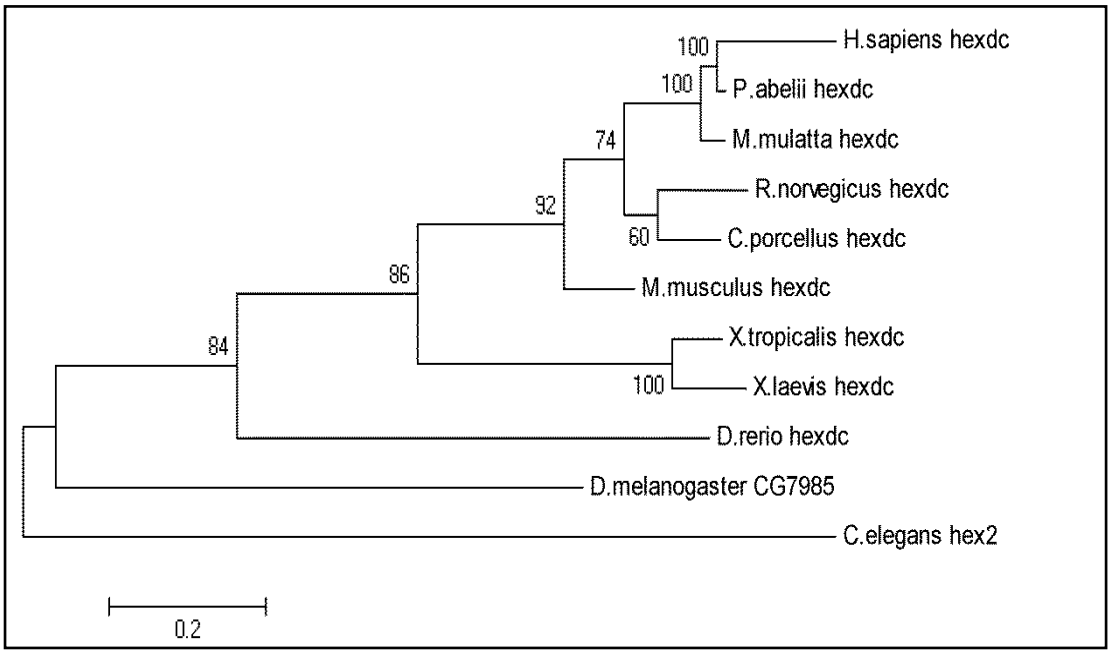

Figure 4.1. Bootstrap algorithm-driven homology tree for *CG7985.* The tree shows that *CG7985* is conserved from *C. elegans* to *H. sapiens*.

TMpred analysis of CG7985 protein sequence suggests that there is a strong possibility that amino acids between positions 14 and 35 represent transmembrane helices. These helices have a score of 2542 on a scale where over 500 are considered significant. When *Drosophila* CG7985 was compared to Human HexDC (Figure 4.2) it was observed that the human version was 122aa shorter than *Drosophila* version. The difference between the two proteins is that Human HexDC lacks a C terminal domain, which has the TM domain in *Drosophila* homolog. The number of aa residues was observed to be equal in the enzyme domain (480aa for both proteins) and the homology reached 70% similarity in the enzyme domain. This aligned domain completely covers the active enzyme domain of human HexDC.

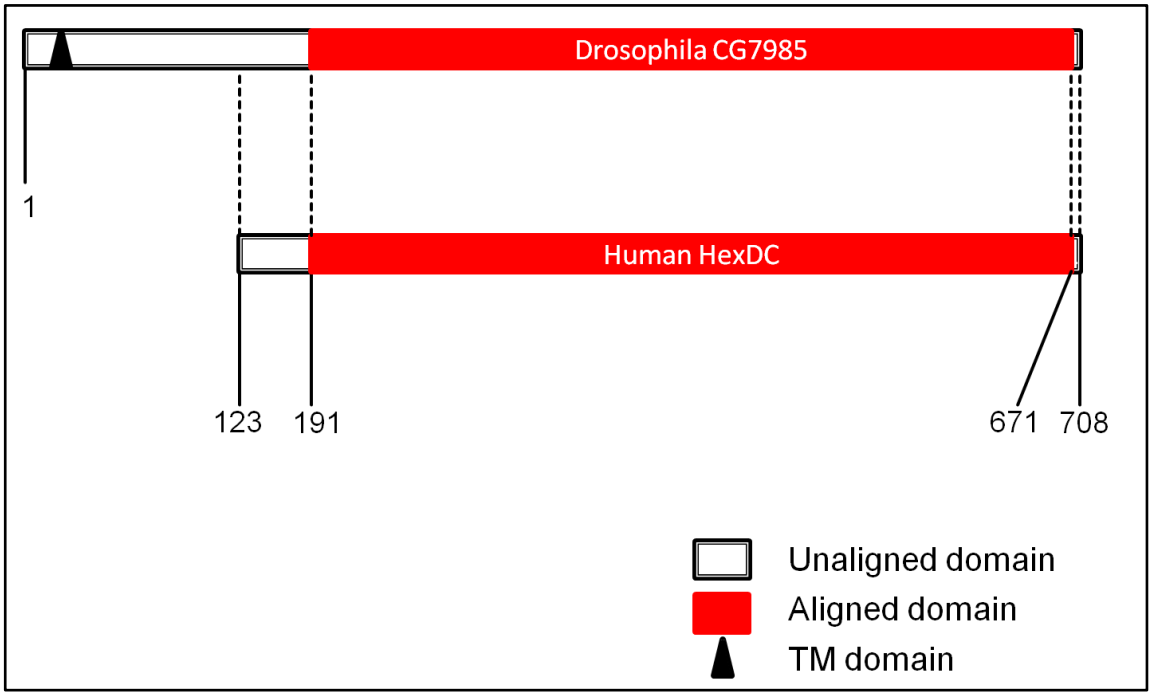

Figure 4.2. Schematic representation of alignment between human HexDC and *Drosophila* CG7986. Human HexDC lacks 122aa from the C terminal compared to CG7985. Aligned domain is also the active enzyme domain as HexDc.

4.2. Expression of *CG7985* drived by an Enhancer Trap Insertion

AC887, the enhancer trap insertion (PiggyBac [Gal4]) which was shown to be inserted into the first intron of *CG7985* gene was generated by Arzu Çelik. This line was localized and initial expression analyses were performed by a former master student (Arzu Öztürk, 2010).

AC887 enhancer trap line was crossed to GFP reporter lines with different properties and the resulting patterns were analyzed. *CG7985* expression in pupal eye discs was stained for GFP which is the reporter for the enhancer trap, *elav* which is the marker for all photoreceptors, and *prospero* which is the marker for R7. GFP, elav double staining was seen to give a specific pattern for GFP as it was expressed in a single photoreceptor. GFP, pros staining was shown to overlap (Arzu Öztürk, 2010) meaning that *CG7985* gene is expressed in R7.

The pattern of the enhancer trap line expression in pupal eye discs (Figure 4.3a) was also single photoreceptor specific as it was in larval eye imaginal discs. It was observed that there are a few ommatidia in which there is no GFP expression and there are a few ommatidia in which there were 2 cells express GFP. This might indicate an early subtype specification, but needs further analysis.

In adult brain stainings (Figure 4.3b) UAS-CD8:: GFP was used as a reporter in order to visualize cell membranes. This allows to trace the whole cell surface including axonal projections. We observed that AC887 was expressed in medulla neurons, projection neurons in the antennal lobe and in the mushroom body.

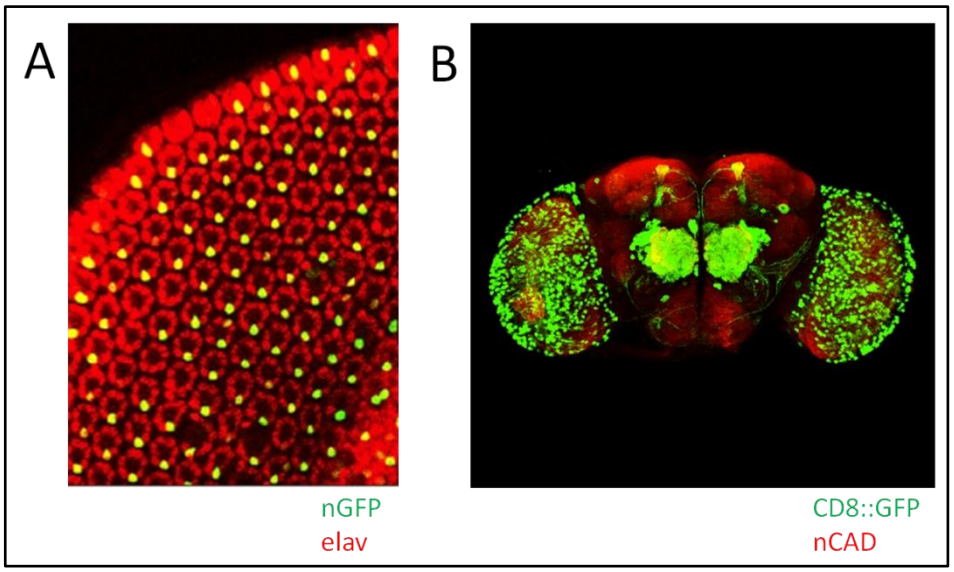

Figure 4.3. AC887 is expressed in R7 photoreceptors, medulla neurons, projection neurons, antennal lobe, mushroom body (A) AC887 > nGFP in pupal discs; elav as marker (Ece Terzioğlu Kara) (B) AC887> CD8: GFP in adult brain; nCAD amarker (Mustafa Talay).

4.3. Generation of an eGFP-Tagged CG7985 BAC Transgenic Line

While the enhancer-trap line is inserted into the first intron of *CG7985* it is not clear if the observed expression really reflects endogenous gene expression. In order to visualize endogenous protein localization a transgenic *Drosophila* line, that expresses

CG7985::eGFP C-terminal fusion protein under endogenous regulatory control using BAC transgenesis.

4.3.1. Generation of an eGFP-Tagged CG7985 BAC-Transgenic Construct

BAC transgenesis involves the generation of a transgenic construct using recombination in bacteria. For this purpose three different tools were combined, (i) a BAC from the P[acman] library containing sufficient upstream and downstream sequences of the gene of interest (Venken *et al.*, 2006), (ii) SW102/SW105 *E.Coli* strain, which allows for homologous recombination, (iii) efficient galK selection system. For the initial step of BAC recombineering, we referred to the P[acman] database of *Drosophila* genome fragments, which are cloned into a ready-to-inject P-element vector. Once the recombination steps were achieved, the BAC was directly injected into fly embryos, where it integrates into the genome. The BAC clone CH322-82G07 containing upstream and downstream regions of CG7985 and having the size of 22kb (Figure 4.4) was ordered.

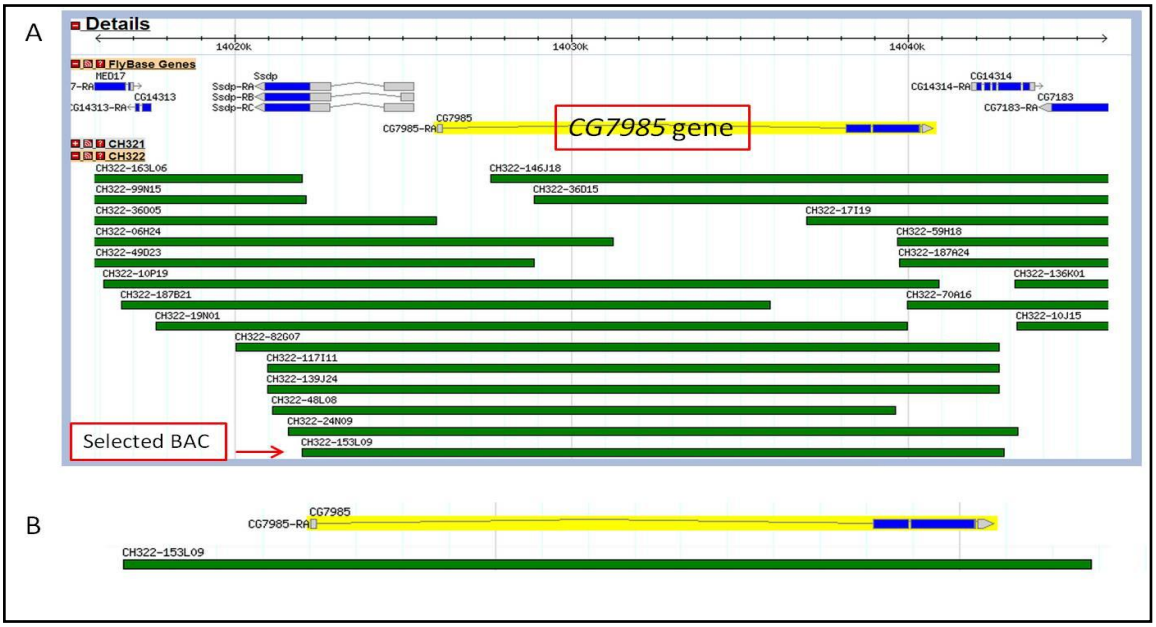

Figure 4.4. P[acman] library of *Drosophila* genome fragments showing the region of *CG7985*. (A) All fragments for *CG7985* region and selected BAC for recombineering. (B) Schematic summary for comparing *CG7985* gene to the selected BAC.

BAC DNA was prepared and digested (Figure 4.5) for analytic analysis using EcoRI enzyme, to verify the correctness of the received BAC. Expected band sizes 3,2 kb, 4kb 7kb and 11 kb were observed thus validating the BAC clone.

The verified BAC clone was transformed into bacteria of the SW102 strain, as explained in section 3.3.11, and positive colonies were selected for recombination. Two PCR products containing the homology arms and the reporter DNA were produced; (i) *LH_galK_RH* was generated using primer pair CG7985_galK_F and CG7985_galK_R and (ii) *LH_eGFP_RH* using primer pair eGFP-overlap_F and eGFP-overlap_R.

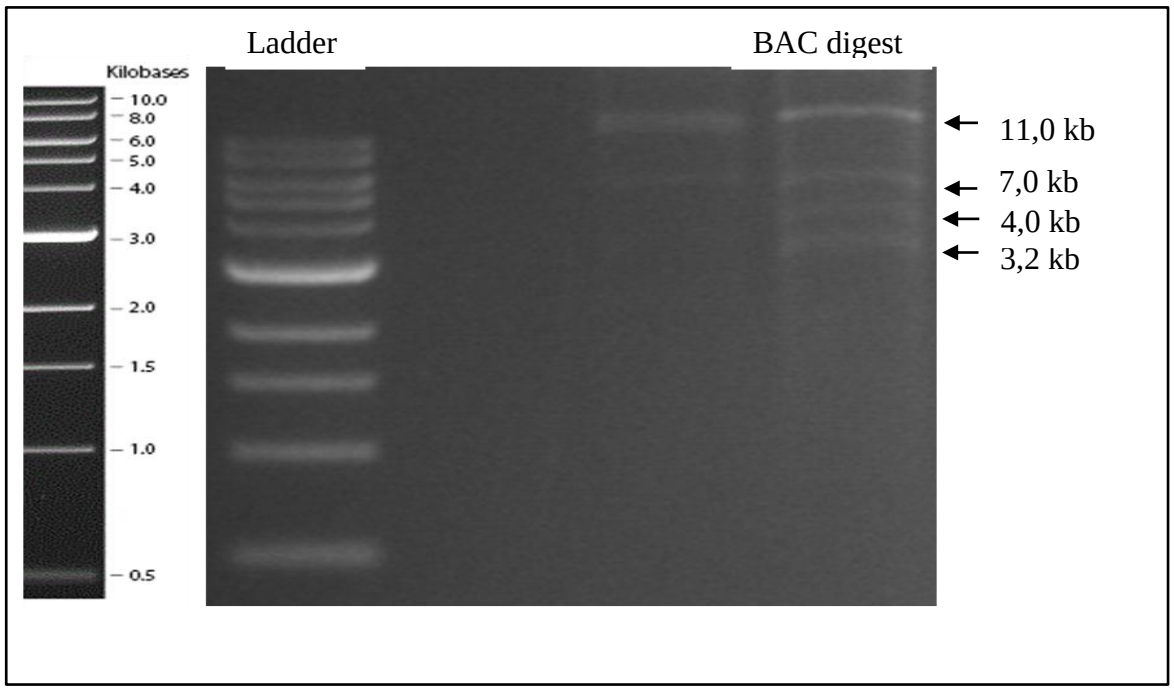

Figure 4.5. Analytic digest of ordered BAC to verify. Expected band sizes of the BAC digest were; 3,2 kb, 4kb, 7kb, and 11 kb and all were observed.

Detailed recombination steps were carried out according to (Warming *et al.,* 2005). Primers were designed in order to amplify GalK and GFP sequences from appropriate templates and right and left homology arms were attached to the primer sequences. PCR amplifications were performed using high fidelity polymerase to reduce possible mutations

that could interfere with proper recombination. Template DNAs were DpnI digested and PCR products were isolated (Figure 4.6) from agarose gels.

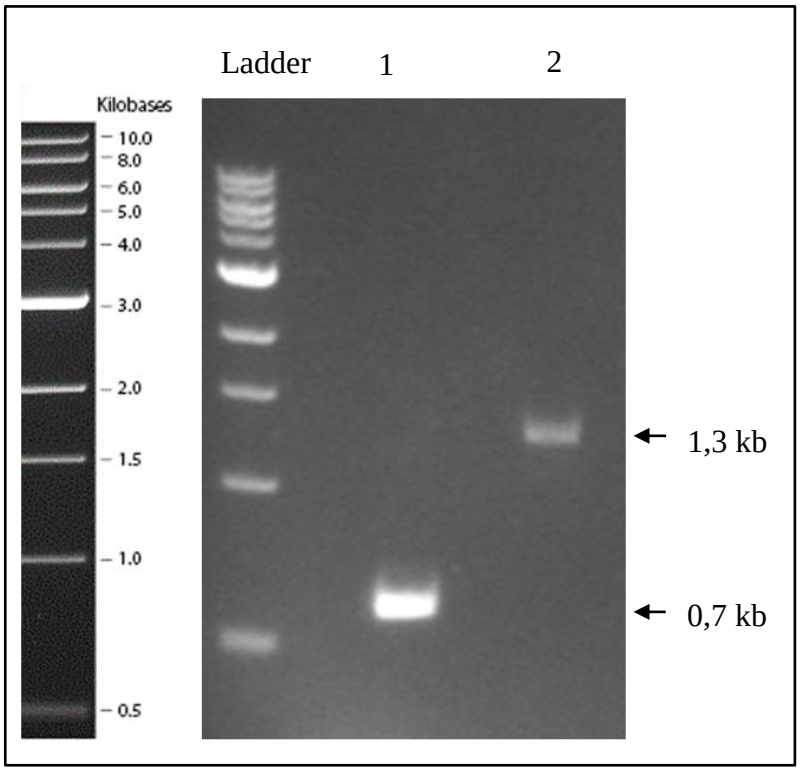

Figure 4.6. Agarose gel results of PCR products for BAC recombineering. Lane 1: eGFP with homology sequences with expected size of 0,7kb; lane 2 :galK sequence containing homology arms with expected size of 1,3kb.

The first step in BAC recombination involves the introduction of GalK into the endogenous BAC locus. Thus, the *LH_galK_RH* PCR product was transformed into a recombination proficient bacterial strain containing our BAC clone. Successful introduction of the GalK sequence into the BAC DNA by recombination can be screened simply by plating the bacteria on GalK selection plates. Only GalK-bearing colonies can survive as these can use the GalK source present in the plates. In a second selection step, surviving colonies were spread on MacConkey agar plates on which GalK bearing colonies give reddish color. Double-positive verified colonies were selected and grown for the second step of recombination. In the second step *LH_eGFP_RH* PCR product was used. The aim was to replace GalK with eGFP sequence and screen using GalK counter plates.

After recombination, colonies were recovered and plated on GalK counter selection plates (containing deoxygalactose), on which GalK bearing colonies cannot survive. Surviving colonies were isolated and grown. Midi kit protocol was used to isolate the modified BAC clone and the sequence was verified both for lack of mutations and insertion position. The BAC recombineering strategy is summarized in Figure 4.7.

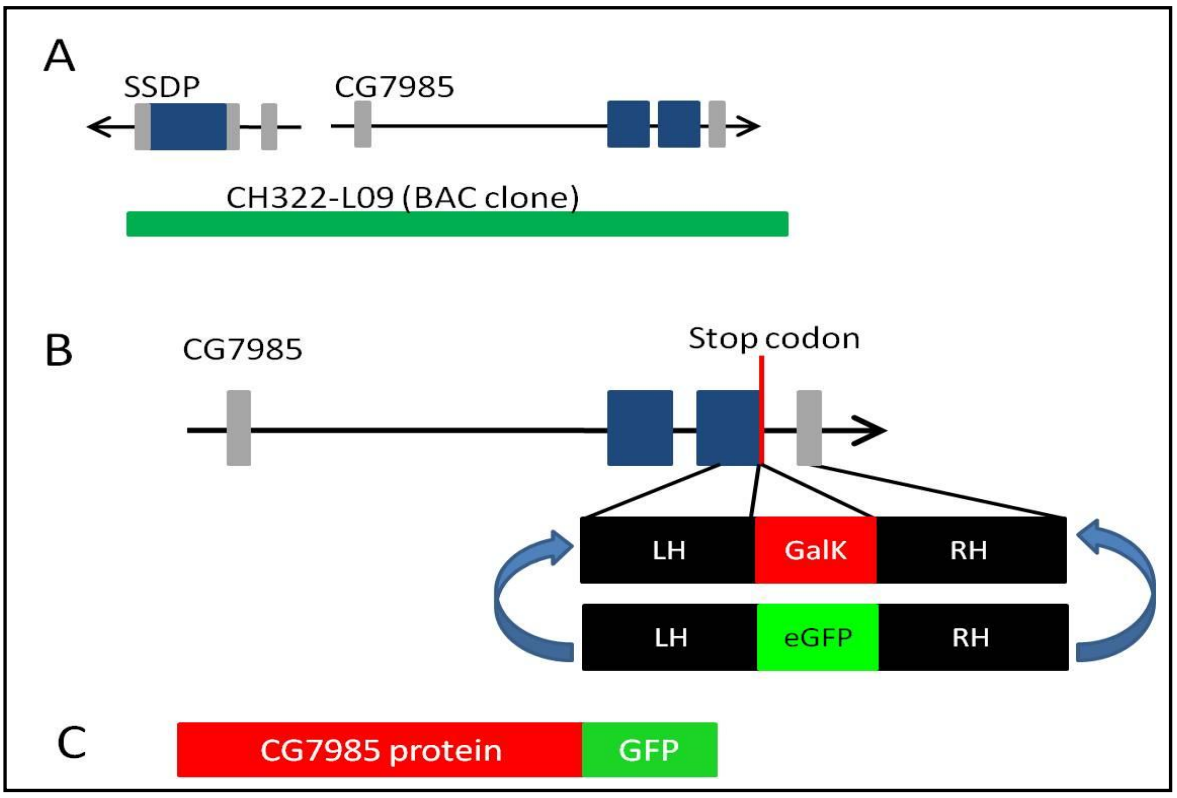

Figure 4.7. Summary of BAC recombineering strategy. (A) Ordered BAC comparison to the genomic region of *CG7985*. (B) Position of modification and summary of PCR products, first GalK inserted later replaced with eGFP. (C) Expected result, fusion protein.

4.3.2. Generation and Analysis of eGFP-Tagged *CG7985* Transgenic Line

The generated construct was prepared in large-scale and sent to injection after confirmation the correctness of the sequence. Injections were done into the landing site attB 40 and a transgenic line was generated. The expression of the fusion protein was visualized in third instar larval eye imaginal discs of eGFP fused CG7985 expressing line

(Figure 4.8) stained with anti GFP antibody. The expression pattern was weakly observable in a pattern that appeared to be specific to a single photoreceptor. Due to a problem with the confocal microscope, which was unavailable for a long time better analyses could not be performed in the framework of this thesis.

There are no available antibodies against CG7985, thus the direct visualization of endogenous CG7985 protein could not be determined. Thus, the validity of the CG7985::eGFP BAC transgenic expression will be verified later.

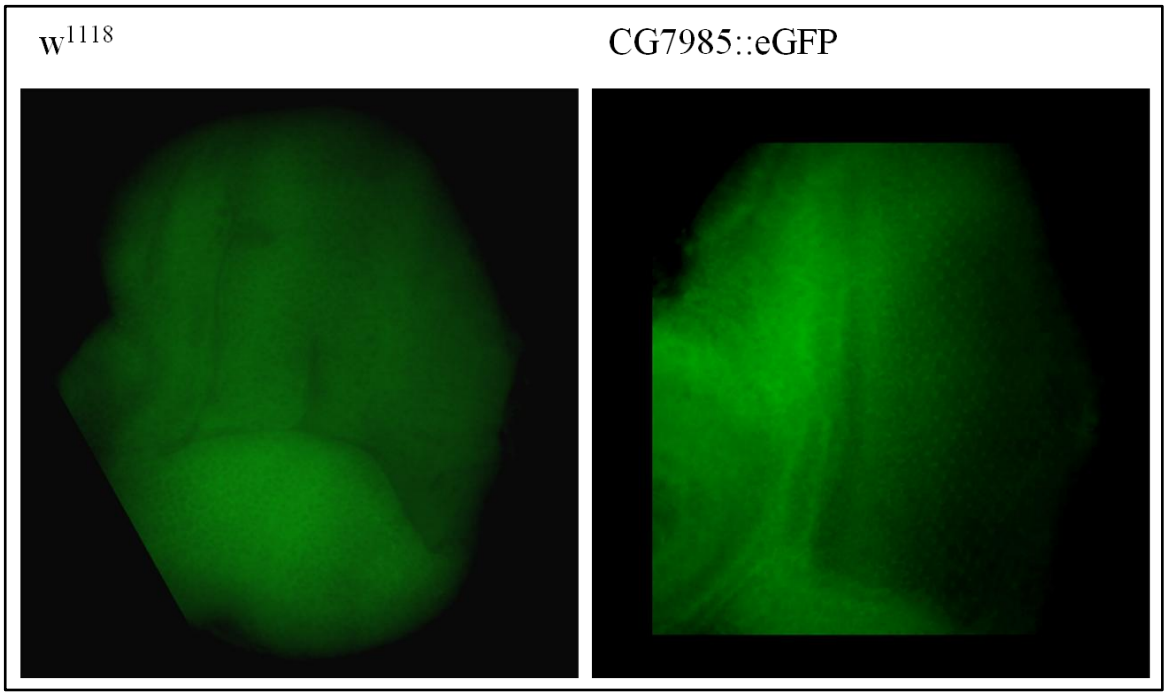

Figure 4.8. Anti-GFP antibody staining of wild type and CG7985 eGFP third instar imaginal discs. Notice the dot-like structure, that is not present in w^{1118} line.

4.4. Antibody Generation for CG7985 Protein

The ability to visualize endogenous gene expression and protein localization are important tools for studying gene function. To generate a tool to independently visualize *CG7985* and confirm its R7-specific expression we set out to generate a CG7985-specific antibody.

Antibody generation (Figure 4.9) requires 4 steps; (i) cloning of CDS into an expression vector, (ii) expression of a tagged fusion protein, (iii) purification of protein using affinity purification by making use of the tag, (iv) injection into host animals, for antibody production.

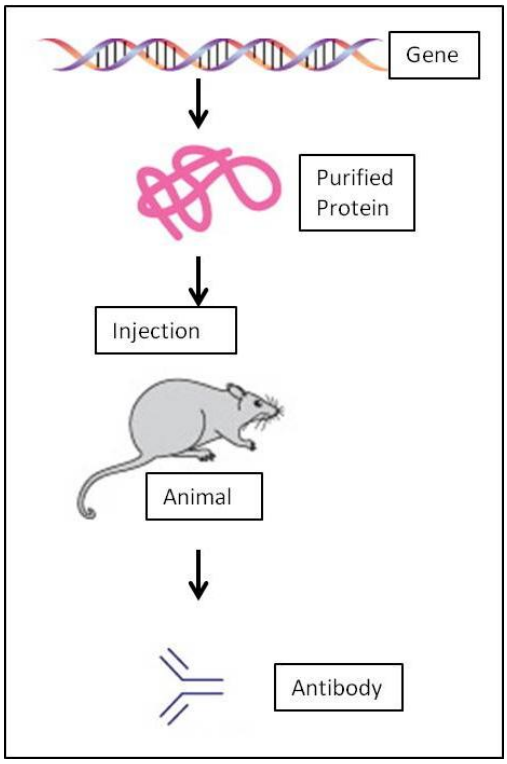

Figure 4.9. Summary of antibody generation steps. First, target CDS is cloned into an expression vector and the protein is expressed. Second, the protein is purified, and finally the protein is injected into the host animal, expecting to cause antibody production.

Initially, CDS of *CG7985* was amplified using primers pairs of CG7985_CDS_pet30_F_NcoI and CG7985_CDS_pet30_R_BamHI (Figure 4.10) and high fidelity enzymes. The PCR product was cloned into pGEMT-Easy vector and sequenced. Using NcoI and BamHI to cut both vector and insert, CDS in pET30 the construct was created. The generated construct was transformed into Rosetta2^{TM}, which is a protein expression strain of *E.Coli*. Protein production was induced by IPTG as explained in section 3.5.1. The bacteria were harvested and protein lysate was collected.

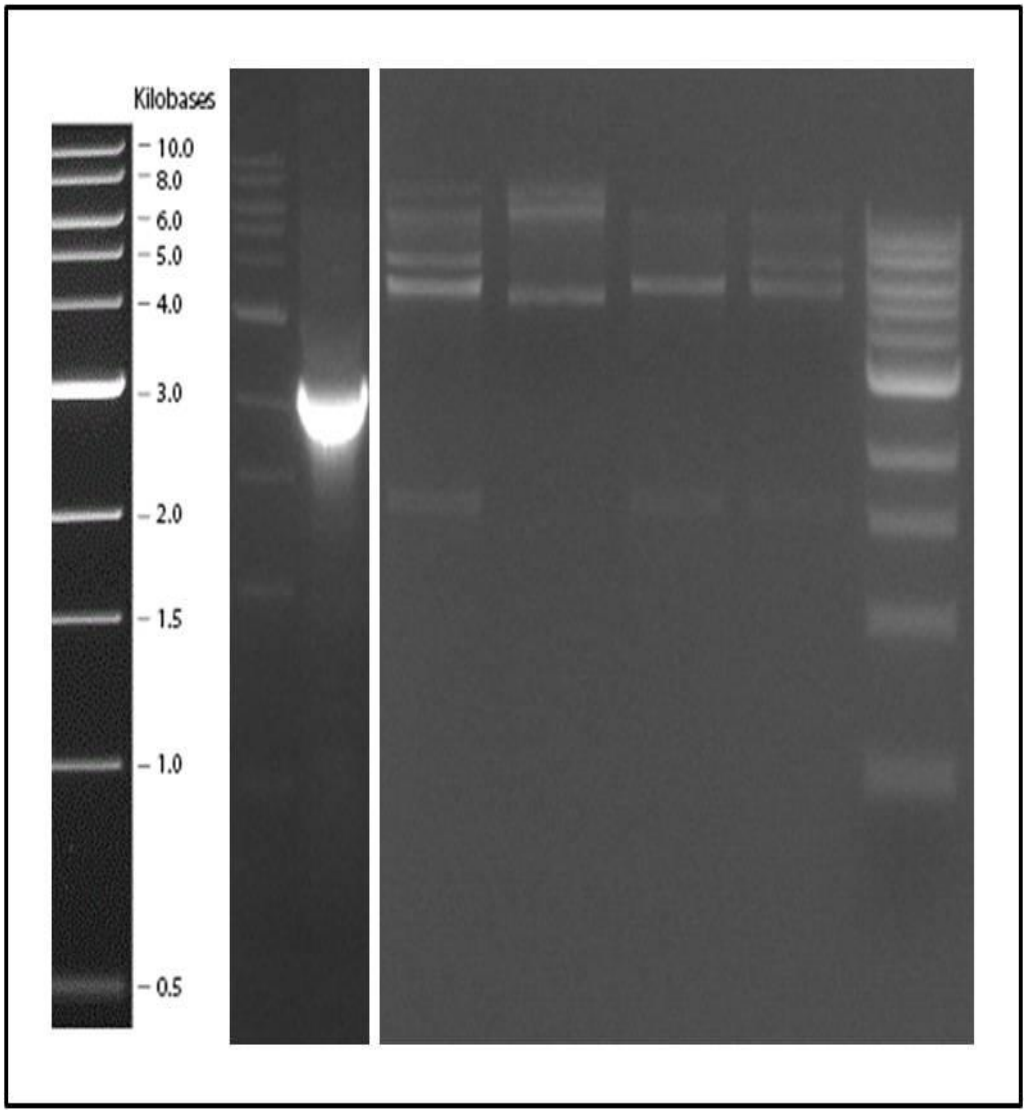

Figure 4.10. Gel photos of cloning of CDS into pET30. Left, ladder; middle, cloning of CDS (expected size 2211bp); right, analytic digest of CDS in pET30 with SmaI.

CG7985 was successfully cloned and that it could be induced to produce tagged CG7985 protein (Figure 4.11). However we were not able to isolate this his-tagged protein from the bacterial lysate. Although Rosetta2 strain was useful in expressing trans-proteins, the amino acid residues of CG7985 appeared problematic.

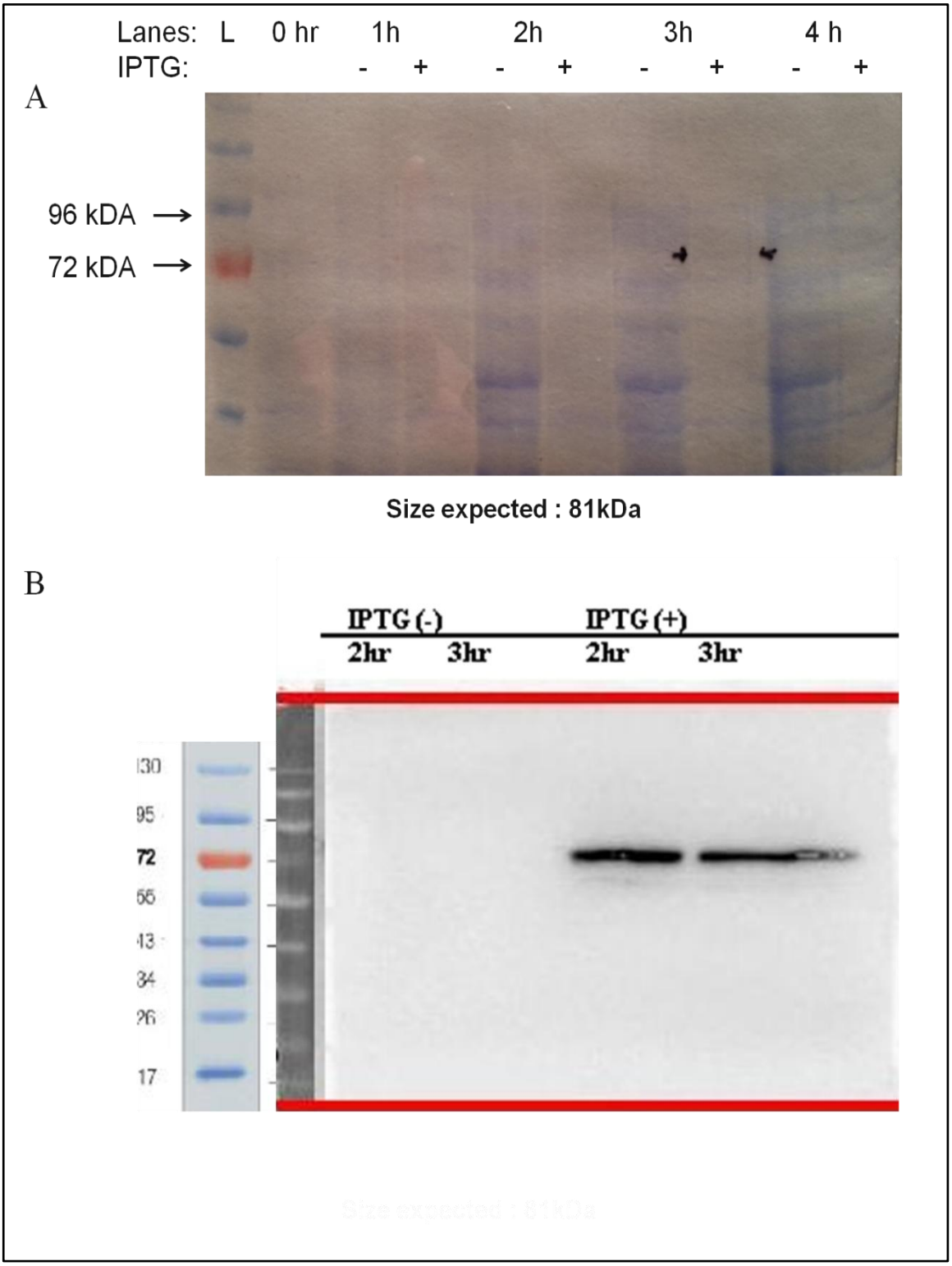

Figure 4.11. A) Commassie staining of IPTG induced (+) and IPTG (-) Rosetta 3 strains. There is a weak band at 80kDa at IPTG (+) and not in IPTG (-). B) Western results for anti-his staining of protein extracted from Rosetta 2 strains with CDS in pET30 construct.

We ordered a commercially available antibody against human HexDC, which is generated by an epitope shared also in CG7985 but we could not observe any cross reaction. Stainings on HeLa cells (Figure 4.12.) revealed that human HexDC protein is expressed strongly in the nucleus.

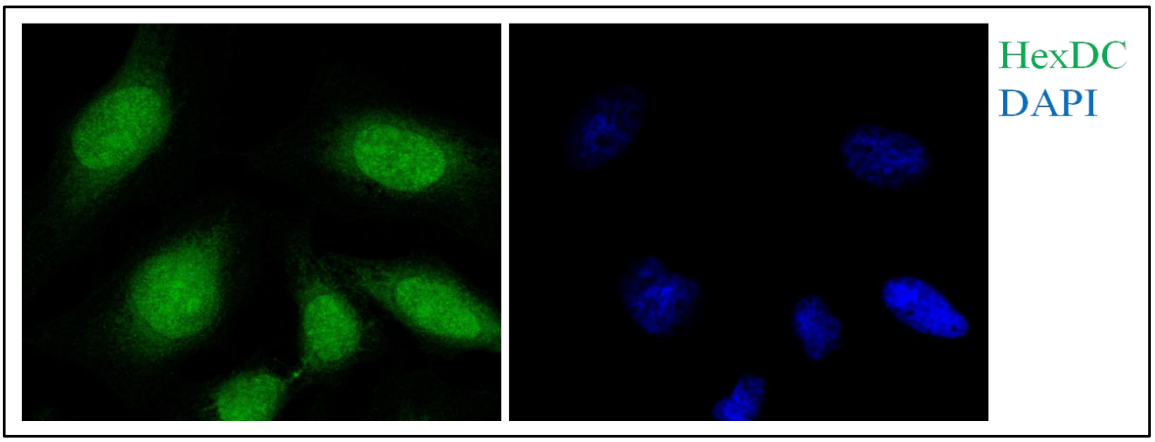

Figure 4.12. Immunohistochemical staining of HeLa cells with anti human HexDC antibody. Cells were co-stained with nuclear stain DAPI. HexDC was visualized with Alexa-488. DAPI is shown in blue. Human HexDC is localized highly in the nucleus.

4.5. Generation of Tools for Functional Analysis of *CG7985*

Studies of gene function rely heavily on the availability of mutants and their analysis. As previously described *CG7985* has not been investigated in *Drosophila* at all, thus the generation of mutants was essential. The classic way of generation of mutants is by P-element excision. In some cases two P-elements flanking the gene of interest can be used to delete the region between them. A more specific way would be the use of site-specific recombinases that act on particular target sites that flank the region of interest. The most elegant way is the use of targeting constructs to replace the gene of interest with a marker gene by homologous recombination. This strategy also allows the addition of target sites that can later be used to insert other genes, eg. GFP, lacZ, or the human homolog of *CG7985*. Here, we chose to follow two strategies: first; we aimed to generate a targeting construct for homologous recombination, second; we employed already available PiggyBac elements containing FRT sites for site-specific recombination.

4.5.1. Generation of a *CG7985* Mutant using FLP/FRT Site-Specific Recombination

The aim of this strategy is the generation of a mutant using of FLP/FRT technology. This technique relies on site-specific recombination in which the flippase (FLP) recombinase acts on its recombination targets, FRT sites (short asymmetric sequences that the enzyme recognizes and recombines directly) to create breakpoints in the DNA (Senecoff *et al.,* 1988). The DNA rearranges according to the positions of the FRT sites. Thus, it can be used for the (i) inversion of genes, (ii) duplication of genes, or (iii) knock-out of genes (Golic *et al.,* 1996). In order to create a mutant by using the FLP/FRT knock out strategy, it is necessary to have two FRT sites arranged in the same orientation, which are located upstream and downstream of the genomic region to be removed. (Golic *et al.,* 1996). In order to create a mutant by using the FLP/FRT knock out strategy, it is necessary to have two FRT sites arranged in the same orientation, which are located upstream and downstream of the genomic region to be removed.

We screened fly databases for available transgenic elements that are inserted within or surrounding *CG7985* and ordered two transgenic fly lines (141891 and 141583) from the DGRC database with the necessary properties. These lines were carrying PiggyBac elements containing FRT sites which are in the same orientation. The first one was located upstream of *CG7985* and the second one was located downstream of *CG7985*. In order to induce the recombination event these two elements should be present together in one fly, so that FLP can act on both sites and delete the sequences in between by inducing a recombination event. For this purpose these two lines were crossed together (Figure 4.13) then to a fly carrying a heat-shock (hs) flp source. Males that carry both FRT sites and the hsflp source were heat-shocked for 1h at 37°C within the first day of adulthood. It was expected to observe recombination between the FRT sites, thus generating 2 deficient alleles; (Figure 4.14) a duplication and a deletion.

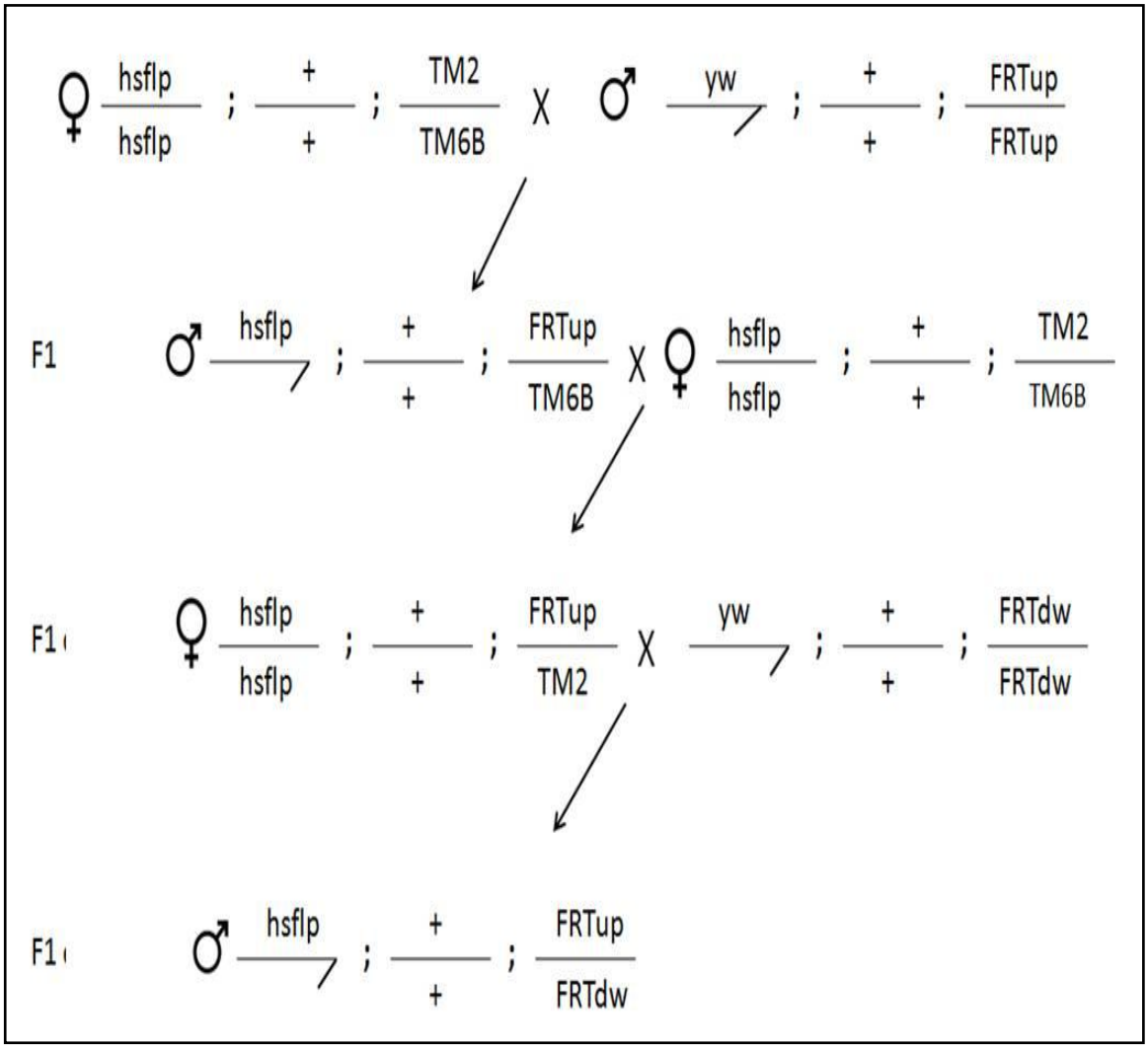

Figure 4.13. Crossing scheme of the FLP/FRT main stock. Males obtained were heat shocked to activate *hsflp* and crossed to balancer virgins to generate stable stocks carrying the putative mutation and lacking *hsflp*. Every stock generated was screened by PCR.

Once males were heat shocked, it was expected to have a recombination event between the two FRT sites. Each male was crossed with a balancer female to generate stable stocks carrying the desired mutation and lacking *hsflp*. Stocks were PCR screened with primers designed specifically to recognize the knock-out (Figure 4.14) allele. A total of 170 stocks were screened (Figure 4.15A) and Stock Nr. 168 was the only one giving any PCR product. Four PCR bands of 1,2 kb, 0,75 kb, 0,6 kb and 0,4 kb were amplified including the expected band size of 1,2 kb, which is the total sequence of an FRT region used in the case of this study. Sequence results (Figure 4.15B) confirmed the deletion.

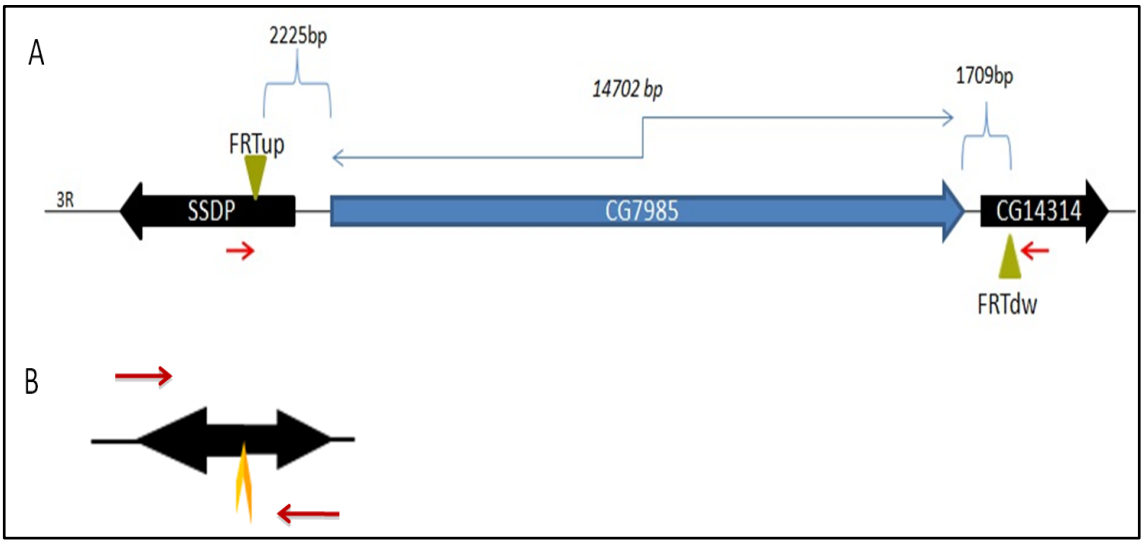

Figure 4.14 FLP/FRT deletion strategy. (A) Relative postions of FRT sites. (B) Expected mutant allele. Red arrows indicate the location of primers designed for PCR screen.

The mutant stock number 168 was amplified and we observed that it is homozygous lethal. While it is possible that the lethality is due to the deficiency of *CG7985* is also plausible that it is due to the deficiency caused by the deletion of the upstream and downstream genes.

As it can be seen in Figure 4.13A the FRT elements used for generating the deletion are located in the *SSDP* gene located upstream and the *CG14314* gene located downstream of *CG7985*. *SSDP* and *CG14314* appear to encode housekeeping genes. In order to rescue the lethality and possible phenotypes caused by the deletion of these two genes we ordered two BAC clones (Figure 4.16) that cover the *SSDP* and *CG14314* genomic regions .As the BACs were chosen from the PACMAN library, they contain attachment sites for targeted insertion. For both we chose insertion sites on the second chromosome. In addition a rescue construct for *CG7985* was ordered and send for injection as well. These will be brought into the mutant background to rescue the expression of these genes and thus to have a clean and probably a homozygous viable mutant that can be analyzed.

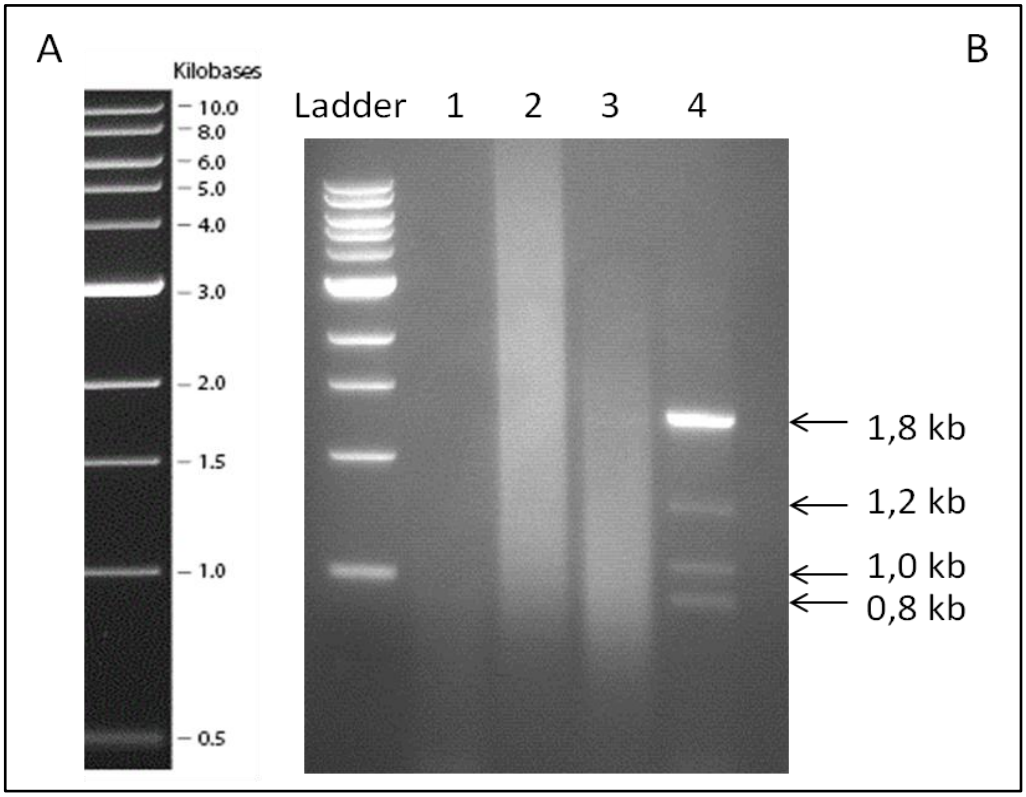

Figure 4.15. FLP/FRT knock out screen results. (A) A representative gel of the PCR screen, only Stock Nr. 168 (lane 4) gave PCR products, which were isolated and sent to sequencing.

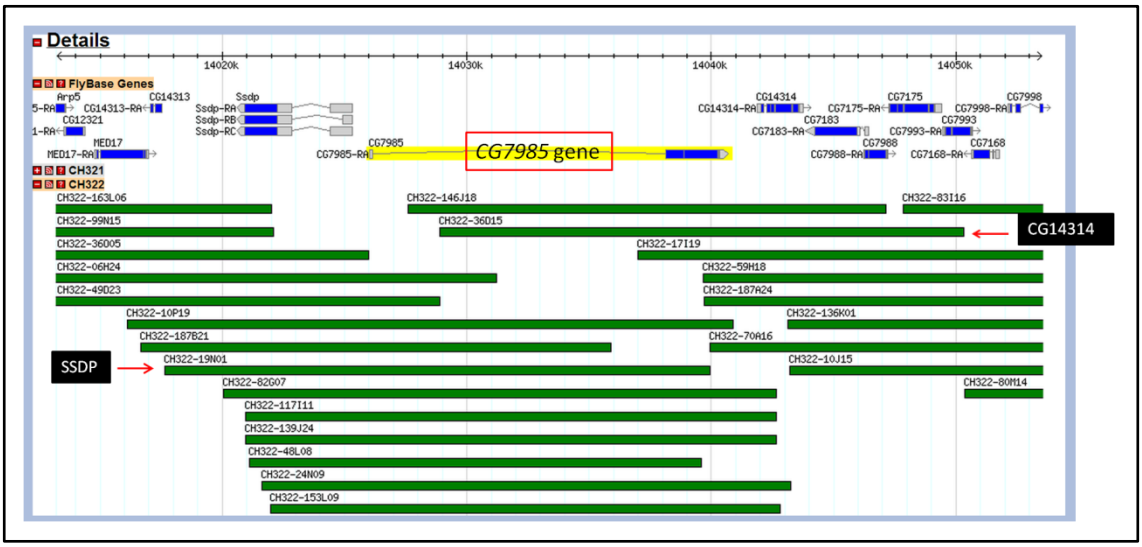

Figure 4.16. Rescue BAC constructs that were selected for *SSDP* and *CG14314*. Their relative genome coverage can be seen. These clones have been ordered and injected to flies to generate transgenic lines.

Since it was not expected that the analysis of heterozygous flies would show the mutant phenotype, we resourced to a technique that relies again on the use of the FLP/FRT system to generate homozygous mutant tissue in an otherwise mutant background. For this purpose the mutant allele has to be recombined to a relative FRT source, in our case, FRT82B. The flies that were used to create the mutant already carry the necessary FRT82B source. To verify that it is functional a test was done using GMR-hid (Figure 4.17). The GMR-hid transgene kills photoreceptors because of the eye-specific expression of the cell death gene *hid* (Stowers *et al.*, 1999), thus flies carrying this transgene have no eye tissue. The *CG7985* mutation present on a putative FRT82B chromosome was crossed to the FRT82B *GMR-hid* containing chromosome. After induction of mitotic recombination in only the eye tissue using eyeless-flippase (ey-flp), which is only possible if both alleles carry a functional FRT82B site, the eye tissue will be rescued by loss of the GMR-hid. The resulting eye tissue will thus be homozygous mutant, while the rest of the fly will be heterozygous mutant. Our analysis showed that we could rescue the eye tissue and thus that our mutant tissue is located on a functional FRT82B allele. Thus, this method can be used to study mutant phenotypes in the eye.

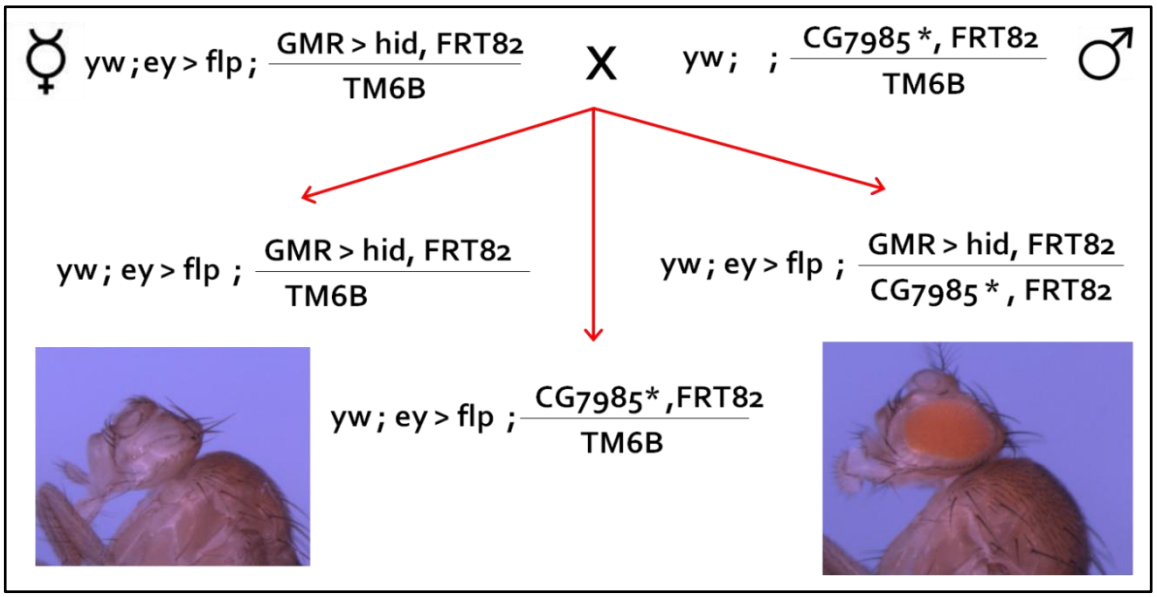

Figure 4.17. Verification of the presence of a FRT82B site in mutant background. The GMR>hid construct kills eye tissue (left photo) even in heterozygous state. When another allele bearing FRT82B is present in the background, it can rescue the phenotype.

4.5.2. Generation of a *CG7985* Knock out Line using homologous recombination IMAGO Method

In order to create targeted knock-outs the homologous recombination strategy was employed. For this purpose a targeting construct carrying upstream and downstream regions of the target gene as well as a marker (e.g. *white*) need to be constructed. The construct preparations have many steps that need to be treated with extreme precision and caution. Single mistake in one step causes whole construct to be wasted. Many methods were used to make sure construct was correctly prepared; sequencing analytic digest, run on gel and size difference.

The targeting construct is then injected to *Drosophila* embryos to create a transgenic line. The construct is later mobilized to allow the homology arms to make Holliday Junctions and the target gene to be replaced by the marker to create a knock-out.

We have chosen to use one of the novel techniques to create knock-outs by ends-out recombination. The IMAGO technique, integrase-mediated approach for gene knock-out (Choi *et al.,* 2009). In this method, in addition to ends out-homologous recombination by replacing the target gene with the *white* marker, the targeting construct integrates *attP* sites to both sides of *white*, making it a replaceable reporter gene.

Any *attB* sequence-carrying construct can replace *white* and create a different transgenic line, all based on one single recombination.

To generate an IMAGO (Figure 4.17) targeting construct a genomic BAC clone (BACR27G04) carrying the *CG7985* gene region was identified and ordered. The BAC clone was prepared and digested to verify its identity.

Only then it was used as a template to amplify the 5’ and 3’ homology arms of the construct. The upstream and downstream regions (3,7kb and 3,4kb respectively) were amplified (Figure 4.19a) using primer pairs; CG7985_upstream_F, CG7985_upstream_R and CG7985_downstream_F, CG7985_downstream_R, respectively.

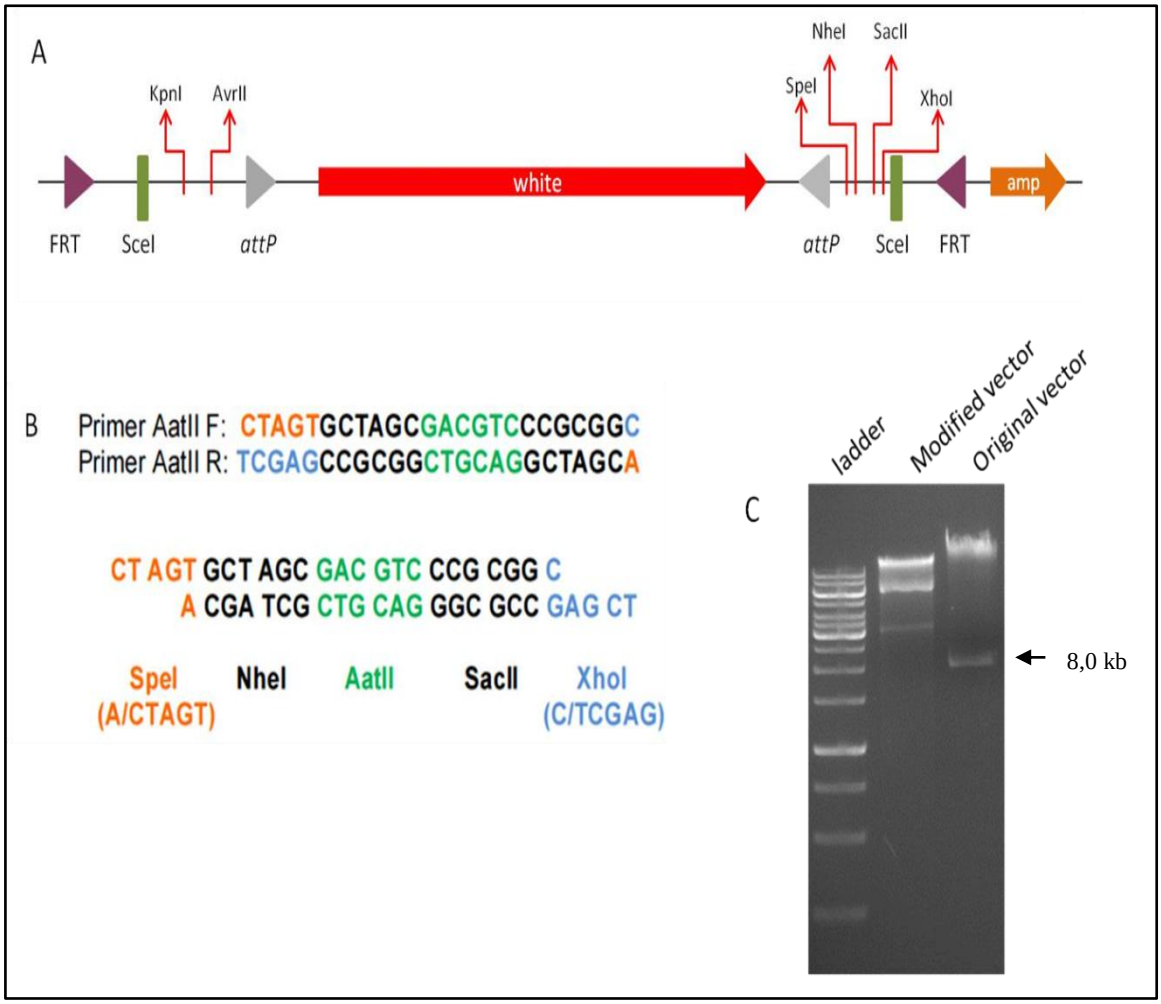

Figure 4.18. Modified IMAGO construct. (A) IMAGO vector map, (B) Modification on the downstream enzyme domain, (C) Analytic digest to verify the modification using NheI. The original vector was not cut (no NheI site) and modified vector gave a 8kb band.

PCR products (Figure 4.19a,b) were cloned into the pGEMT-Easy vector and sequenced. The restriction sites KpnI and AvrII were used for the upstream homology arm and NheI and SacII were used for the downstream arm and ligated to create the up_imago_dw construct.

The IMAGO vector was modified to fit our cloning strategy. Downstream enzyme domain was cut with SpeI and XhoI and specific primer dimer (Figure 4.18b) was ligated to add NheI and SacII enzyme domains.

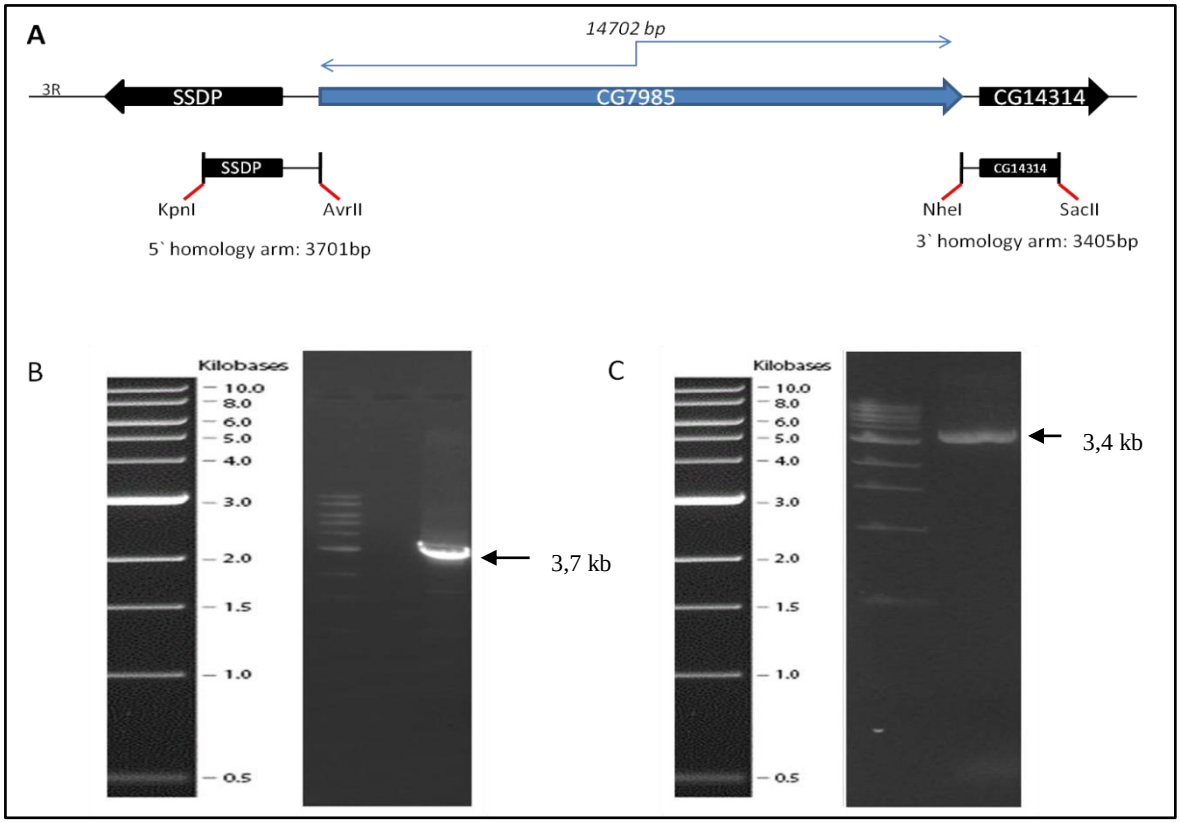

Figure 4.19. Cloning of IMAGO homology arms. (A) *CG7985* gene region used in generating IMAGO constructs, (B) Cloning of upstream and (C) downstream arms as the scheme suggests.

The generated IMAGO targeting construct was sent to injection into white (-) flies and transgenic flies (red eye color) were obtained. Crossing schemes and heat shock protocols were carried out as suggested in the IMAGO paper by Choi *et al.*, 2009. Basically, the IMAGO flies were balanced to identify the insertion on the second chromosome (*CG7985* is located on the 3rd). Then, IMAGO flies were crosses to hsflp, *hsSceI* source flies and the main stock *yw; IMAGO/CyO; hsflp, hsSceI* was generated. Virgins were collected from the main stock and heat shocked at 37°C for 1h on the first day after eclosion and later crossed to balancer flies. When the second chromosome was fully balanced (*sp/CyO*) the red eye color was used to screen, since the red eye color source is the IMAGO construct on the second chromosome. 400 virgins were heat-shocked and over 1000 progeny were screened. Unfortunately, no candidate could be identified for further selection. Thus, the screen needs to be continued by increasing the numbers of flies.

4.5.3. Generation of UAS-CG7985 Line for Gain of Function Analysis

UAS-CG7985 overexpression line was generated by amplifying the coding sequence of CG7985 and ligating into pUAST vector. (See section 3.4.1 and Figure 4.20). The construct was sent to be injected into flies and transgenic flies were successfully generated.

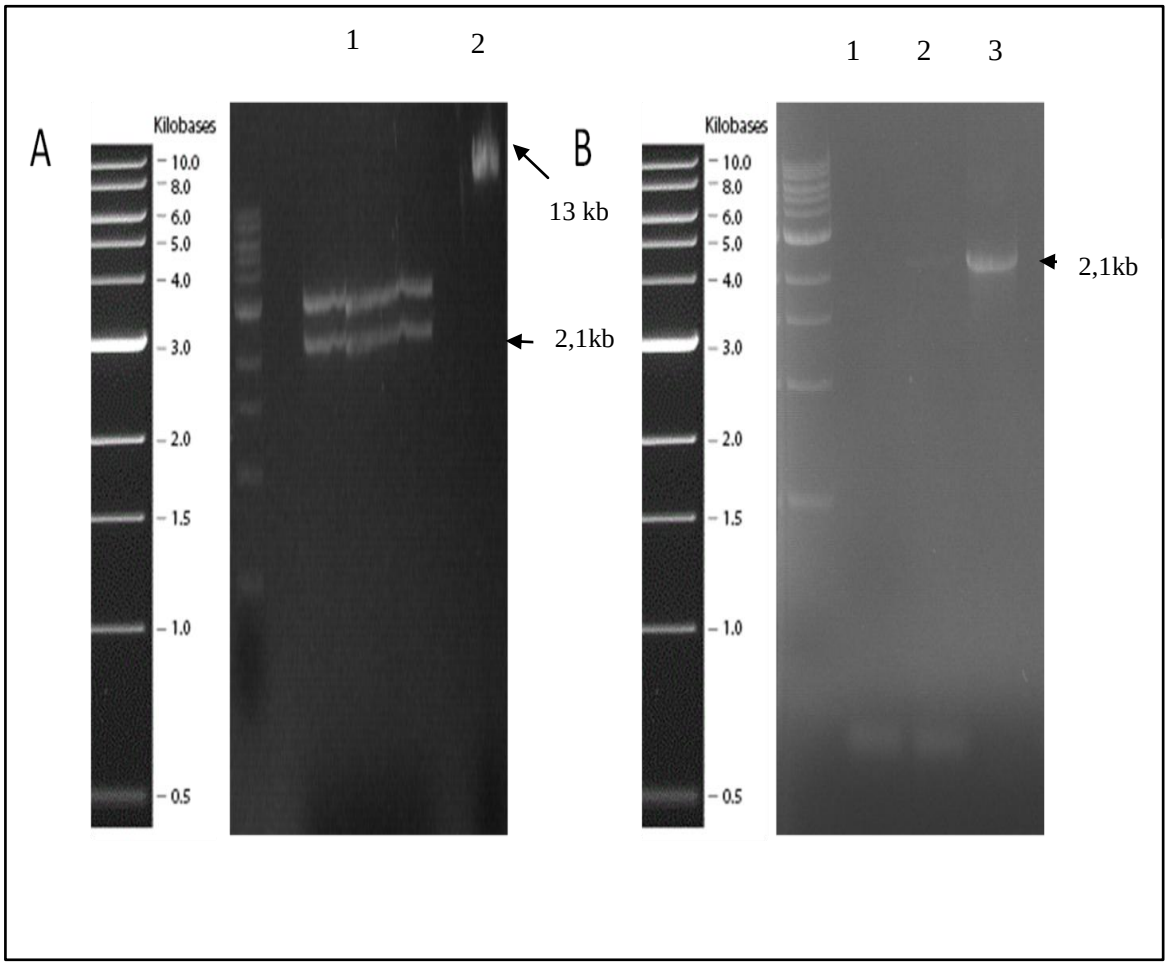

Figure 4.20. Gel photos of UAS-CG7985 cloning. (A) Both pUAST and CDS of CG7985 in pGEMT-easy constructs were linearized with EcoRI, expected sizes 2,1 kb and 13 kb were observed, (B) Directional colony PCR, only clone 3 was positive.

4.5.4. Verification of Functionality of Misexpression and Downregulation Lines

In order to get an idea how well up-and down-regulation of expression of *CG7985* works quantitative RT-PCR was used. Q-RT PCR allows quantitation of mRNA levels and the comparison of increase and decrease of mRNA by direct comparison.

4.5.5. QRT-PCR Results for CG7985-RNAi and UAS-CG7985 Lines

In order to verify that the UAS-line that was generated in he framework of this study and the UAS-RNAi lines obtained from the VDRC stock center are working and to determine their efficiency we made use of quantitative RT-PCR. The lines were crossed to a strong Gal4 driver and flies carrying the UAS line and the driver were dissected to collect the fly heads, the RNA was isolated and reverse-transcribed into cDNA. The cDNA was then used as a template to perform a qRT-PCR.

The primer pair used here was; CG7985_RTprimer_F and CG7985_RTprimer_R. Fly heads of each line were collected and RNA was isolated, and then reverse-transcribed into cDNAs. 1µl of each cDNA sample was used in QRT-PCR reaction. cDNAs generated from RNA extracted from driver lines and the results were normalized to these controls. As expression drivers, lGMR-Gal4 and UAS-Dicer / lGMR-Gal4 lines were used (see table 3.1) and actin levels were used as normalization control.

The results showed that (Figure 4.21), wild type flies as well as the driver lines only have no significant effect on *CG7985* mRNA level. RNAi line, driven by lGMR-Gal4 and enhanced by UAS-Dicer decreases *CG7985* mRNA level by 68%. UAS-CG7985 was observed to increases *CG7985* mRNA level by 244%.

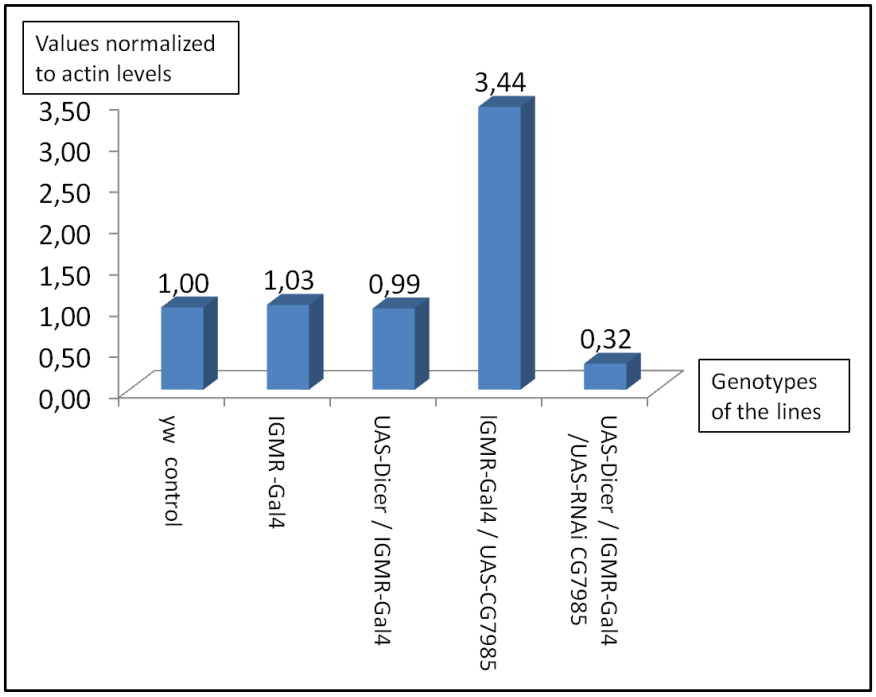

Figure 4.21. QRT-PCR results for overexpression and downregulation lines. As controls, wild type and driver lines only were used and did not change, *CG7985* mRNA level. RNAi line decreases 68% and UAS line increases %244 mRNA level of *CG7985*.

4.6. Biochemical and Immunohistochemical Analysis of *CG7985* Function

4.6.1. Cell-Type Specific Analysis of *CG7985* FLP/FRT Knockout Line

Homozygous mutant eyes were generated for functional analysis. Several markers were tested important for R7 cell specification were tested by antibody staining of larval eye imaginal discs. The first marker used was *Cut*, which stains four cone cells surrounding the PRs. *pros* is a transcription factor that specifically marks R7 cells and also stains cone cells weakly. Both wild type and *CG7985* homozygous knock out mutant, generated by FRT flip out, larval eye imaginal discs were stained for *cut, pros* and *elav.*

Stainings for *cut* (Figure 4.23) was expected to give 4 cone cells around the main PR structure, which indirectly shows R7 differentiation as cone cells develop downstream of R7. Comparing wild type to homozygous mutant, there was no significant difference, neither in structure nor in position.

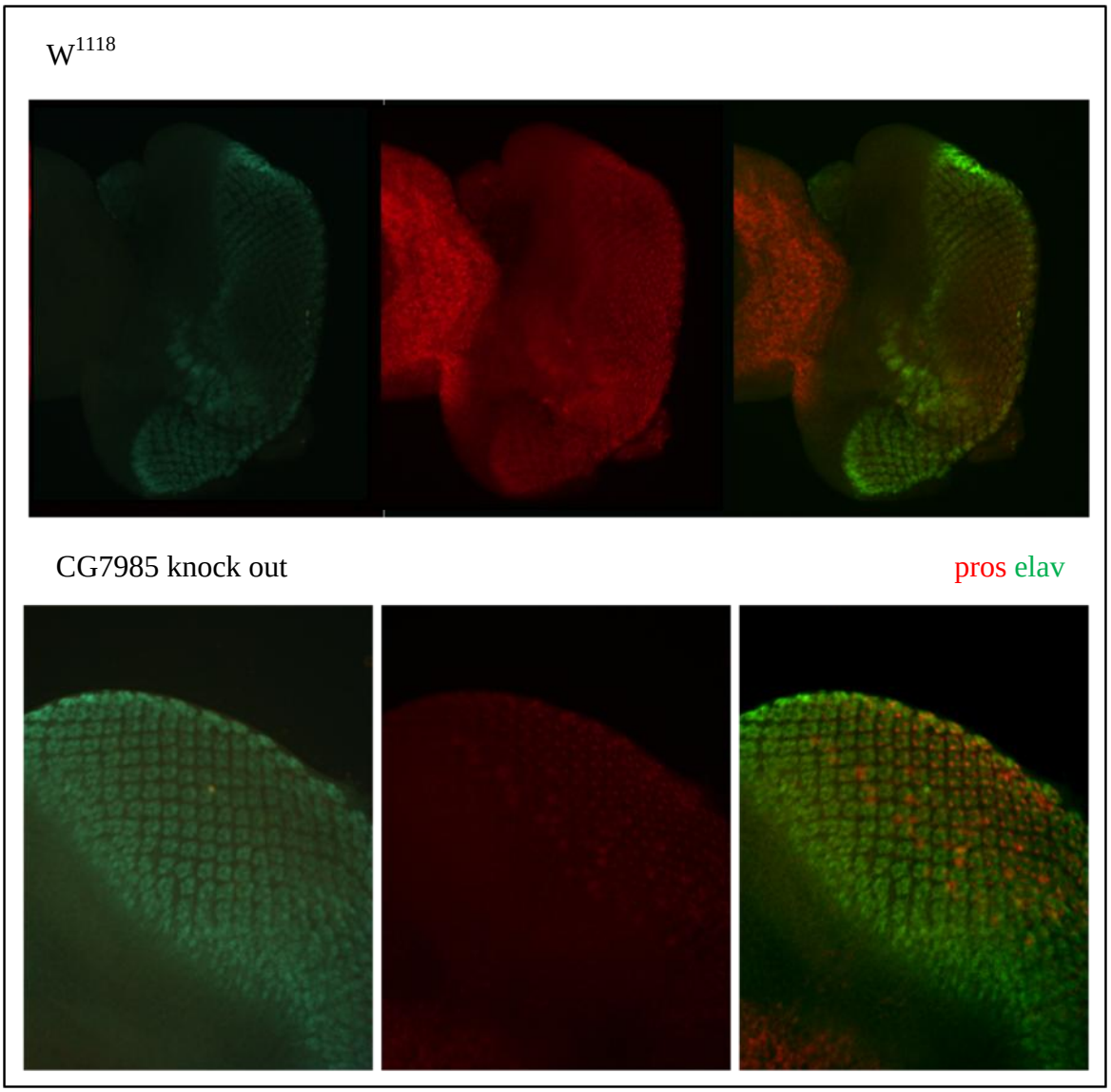

Figure 4.22. Antibody staining of larval eye imaginal discs for *pros* and *elav*. Top part shows wild type discs, and bottom part shows *CG7985* homozygous knock out mutant generated by FRT flip out. *pros* and *elav* staining show no significant difference.

Stainings for *pros* (Figure 4.23) was expected to stain R7 cells strongly and cone cells around the main PR structure, weakly. Comparing wild type to homozygous mutant imaginal discs, no significant difference, in either structure or in position was observed.

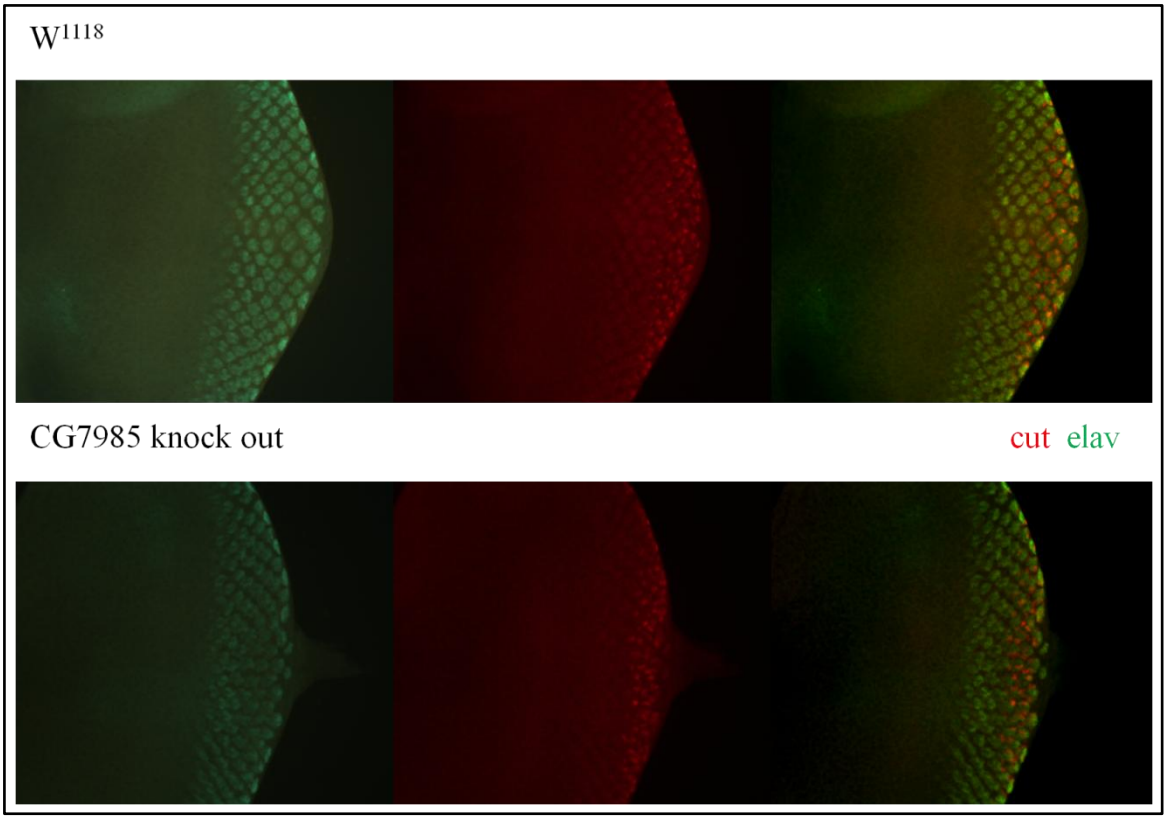

Figure 4.23. Antibody staining of larval eye imaginal discs for *cut* and *elav*. Top part shows wild type discs, and bottom part shows *CG7985* homozygous knock out mutant generated by FRT flip out. *cut* and *elav* staining show no significant difference.

4.6.2. Analysis of CG7985 Protein Target by Western Blotting

CG7985 encodes a putative glycosyl hydrolase. CG7985 was cloned into expression vectors to test for its activity in biochemical glycosylation/deglycosylation assays. Unfortunately, we were not able to get proper expression of the protein to test in biochemical assays. We thus decided to test its function *in vivo* by trying candidate targets.

As CG7985 is expressed in R7 cells we asked if CG7985 might be deglycosylating proteins that are important in photoreceptor specification and differentiation that are known to be glycosylated. We chose Notch as well as Rh3, Rh4 as candidates as it was previously described that they are glycosylated and glycosylation is important for their function.

Proteins were extracted from wild-type and mutant heads and probed with Rh3, Rh4 and Notch antibodies. The expectation was to see a shift in the running pattern of not properly de-glycosylated proteins.

Not deglycosylated proteins would be heavier and run slower in the gel as compared to proteins extracted from wild-type flies. We used primary antibodies against Rh3 and Rh4 and against the intracellular and extracellular domains of Notch.

It is the extracellular domain of Notch that is glycosylated, however the antibody did not work in our hands and no difference for the intracellular domain was observed, as expected.

Firstly, we used CG7985-RNAi line, *CG7985* heterozygous knock out mutant and wild type as control, then after generating *CG7985* homozygous knock out eye mutants we repeated the experiment.

Since C7985 was hypothesized to deglycosylate the predicted targets, we expected to see an increase in size of the target proteins in the mutants, since glycans will not be removed and will stay attached to the proteins.

Although Rh and Notch were the main targets no differences in the running pattern of these proteins were observed (Figure 4.24). Rh3 mature protein has the size of 39kDA. In mutants probed with Rh3 No change in the running pattern was visible.

The wild type mature Rh4 protein has the size of 39kDa, but here as well no shift in size was observed in mutants.

The same is true for notch intracellular domain, wild type mature protein has the size of 79kDa, and mutant animal Notch intr. protein has the same size showing that CG7985 has no effect on Rh3, Rh4 and on Notch intr. proteins.

Testing for the informative Notch extracellular domain was not successful because the antibody in our hands did not work in Western blots. Thus, Notch is still a strong candidate and needs to be evaluated.

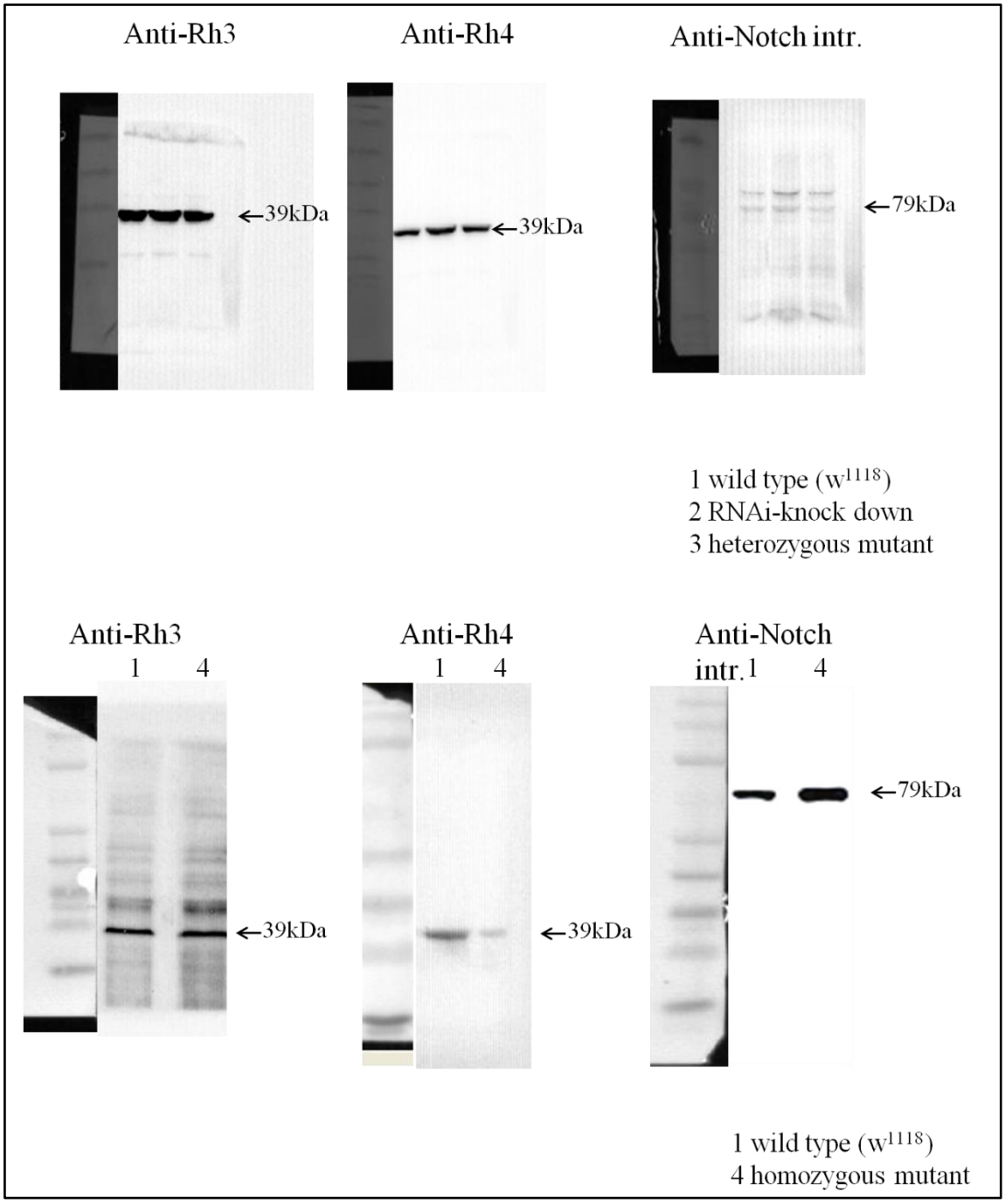

Figure 4.24. Western blot analysis of Rh3, Rh4, and Notch intr. domain. Wild type, RNAi knock down, heterozygous and homozygous mutant lines were tested for Rh3, Rh4, and Notch intr. domain. None was observed to give any significant size difference.

5. DISCUSSION

The fundamental problem of a developing nervous system is the generation of many different neuronal subtypes. The visual system represents a very good model to study the generation of neuronal diversity.

In *Drosophila* color vision is achieved by the differential expression of Rhodopsin molecules with different sensitivities in R7 and R8 photoreceptors. Color ommatidia fall into two classes, pale and yellow that is defined by the expression of different sets of rhodopsin molecules in the inner PR cells R7 and R8. Pale and yellow ommatidia are distributed randomly in the fly retina with a ratio of 30:70 and results from a stochastic decision in R7 cells, which is then communicated to the underlying R8 cell. While the genetic pathways regulating the initial steps of the differentiation of inner PRs have been understood in some detail later steps of PR differentiation are widely unknown.

In an attempt to identify novel genes involved in photoreceptor differentiation an enhancer-trap screen has been performed and a particular focus has been on genes expressed in R7 and R8 cells. One of the genes identified in this enhancer-trap screen and showing R7-specific gene expression is CG7985. Bioinformatic analysis showed that CG7985 is a conserved protein with 70% homology to vertebrate HexDC, which encodes a glycosyl hydrolase. Interestingly, our analysis also indicated that there is a TM domain in CG7985 between amino acids 14 and 35, which does not exist in HexDC. This pointed towards the possibility of CG7985 being anchored to the cell membrane. In line with this finding, a recent study by Pásztói *et al.,* 2012 suggests that there are anchoring proteins of HexDC that attach it to the cell membrane. However, it is unclear which part of HexDC is important for this membrane attachment as the TM domain of CG7985 does not exist in HexDC.

Hexosaminidases from the same family as HexDC have been studied extensively. For example HexA together with an activator protein catalyzes the degradation of G_{M2} gangliosides as well as other molecules that contain terminal N-acetyl hexosamines (Knapp *et al.,* 1996). HexA is a heterodimer composed of an alpha and a beta subunit. While the alpha subunit is encoded by HexA, the beta subunit is encoded by HexB. Gene

mutations in the alpha subunit (HexA gene) are the cause for Tay-Sachs disease; here, G_{M2} gangliosides accumulate in nerve cells and cause premature cell death (Mark *et al.,* 2003). Mutations in the beta subunit (HexB gene) cause Sandhoff disease here cell death is induced by glycolipid globoside accumulation in the nerve cells (Sandhoff *et al.,* 1968).

Infants with Tay–Sachs usually die after two years of age but before the age of six, as disease takes time to accumulate, from aspiration and pneumonia. The disease, Tay–Sachs, causes vision loss and cerebral degeneration. There is no cure or an available treatment, yet.

It turns out that all of these diseases are caused by a simple fact: glycoside hydrolases are necessary to remove glycans from proteins once the proteins are no more needed in cells. GH removes glycans and lysosomal enzymes target those proteins. Once GH is mutated and cannot remove glycans, lysosomes fail to degrade those proteins and they start to aggregate (Helenius *et al.,* 1994). Main targets are G_{M2} gangliosides and other molecules containing terminal N-acetyl hexosamines.

Glycosylation of proteins is important for the functional folding, assembly into proper structure, and oligomerization with other molecules (Kornfeld *et al.,* 1985; Helenius *et al.,* 1994). Previous studies have shown the importance of functional glycosylation sites; their loss reduces protein secretion significantly (Fiedler and Simons 1995). Proteins are distributed from the ER and during transport to the cell surface the oligosaccharide side chains of glycoproteins are exchanged with new sugar residues. Considering there are many proteins circulating in specializing cells, glycosylation is a new level of regulation of proteins, thus the signaling within or even to the outside of cell.

Thus, *Drosophila* HexDC appeared as a good candidate for detailed analysis. Since this gene has not been studied in flies at all, we generated a set of tools to be able to do functional analysis.

We took two approaches to generate a mutant for *CG7985.* In the first approach we made use of two existing genetic elements in order to delete the region in between. As both of these elements contained FRT sites we made use of a hsflp source to induce recombination between these two sites. The 170 generated fly lines were screened by PCR for the desired deletion and we obtained one line, which results in a frequency of 1/170 of this event. This line appeared to be homozygous lethal. The lethality is thought to result

from the partial deletion of the upstream (*Sequence-specific single-stranded DNA-binding protein, SSDP*) and downstream (*CG14314*) genes. Thus, initially only heterozygous flies were analyzed. In parallel, we generated rescue constructs, genomic clones contained in BACs, of the upstream and downstream genes to see if lethality can be rescued. These experiments are ongoing, but as they involve the generation of transgenic fly lines and then the crossing of all the transgenes together into the mutant background this part could not be accomplished in the framework of this thesis. As an alternative we generated mutant whole eye clones using ey-flp and GMR-hid and used these flies in our functional analysis studies.

In a second approach that is genetically more accurate we aimed to generate a mutant by homologous recombination using an integrase-mediated approach. For this purpose a targeting construct has been generated and injected into fly embryos to generate a transgenic line. Later this transgene has been mobilized by heat-shock methods in order to replace the endogenous locus with the white gene. We have generated the transgenic flies which carry the target construct that will remove *CG7985* and replace it with *white.* So far, 400 lines have been screened; however no recombinants have been obtained so far. Since homologous recombination happens randomly in cells it is not very common and thus it will be necessary to repeat the experiment by increasing the numbers of flies to screen.

Initial analysis was done by staining homozygous larval imaginal discs for *cut, pros* and *elav* to test the correct specification of PRs. No changes in the staining paterns were observed suggesting that *cut, pros* and *elav* patterning were not disrupted by CG7985 enzyme.

We have generated a transgenic overexpression line by cloning CDS of *CG7985* into a pUAST vector. We also ordered a knock down line from a public stock centers, *CG7985* RNAi. We tested these tools for their effect on *CG7985* mRNA level. QRT-PCR was done to each tool using lGMR-Gal4 or ey-Gal4 as drivers and we showed that the RNAi line decreases *CG7985* mRNA level by 68% and UAS-CG7985 line increases *CG7985* mRNA level by 244%.

Since CG7985 is homologous to HexDC, we hypothesized that it will hydrolize glycans from proteins specifically in R7 cells, which may have a role in differentiation and

/ or specification of the R7 cell. As possible targets we chose to investigate Rh3, Rh4 and Notch, because they were previously shown to be glycosylated (Stanley *et al.,* 2007) and they are specifically expressed in R7 cells. We have tested this hypothesis on Western blots using specific antibodies for Rh3, Rh4 and Notch intracellular domain in different mutant backgrounds. We tested the wild type, RNAi line, heterozygous and homozygous lines. We were unable to visualize Notch extracellular domain, although that's the part of Notch that is glycosylated. In none of the cases the running pattern of the three proteins analyzed appeared to be changed. None of them was retarded in the gel. This strongly suggests that CG7985 does not hydrolyze these three proteins. As Rh3, Rh4 and Notch don't appear to be targets of CG7985, it will be necessary to take a more unbiased approach to find the actual targets, which should involve some proteomics approaches.

We have also checked Rhodopsin choice ratio, corresponding to the pale to yellow ommatidium subtype ratio, which is 30 %:70 %. Experiments were carried out using RNAi knock down and Rh5 to Rh6 ration were examined. Compared to wild type there were no significant difference in Rh ratios, but since about a third of *CG7985* mRNA was still present in the knock down line, and the corresponding protein levels are unknown, it will be tested using homozygous *CG7985* knock out animals to verify one way or the other.

There are no available tools to visualize CG7985 protein directly, so we took several approaches to visualize endogenous CG7985 expression. We ordered a commercially available human HexDC antibody, making sure that it shares a common antigen with CG7985. However, this antibody did not work on either Western blots against fly protein or immunohistochemical stainings in imaginal discs. In parallel, human HexDC antibody was tested in immunohistochemical stainings on HeLa cells. The stainings showed that human HexDC is strongly localized to the nucleus and weakly to the cytosol.

In order to be able to visualize CG7985 protein, an attempt to generate an antibody was made. CG7985 full-length protein was expressed, however, the protein could not be isolated. Western blots showed that the protein expression level was too low for isolation. This could be attributable to a common phenomenon called rare codon problem, each amino acid can be coded by more than one codon, however tRNA levels for each codon differs, adding another level of protein regulation. According to the literature 7 out of 8 rare codons can be expressed using protein expression in *E. coli* strains. It was observed that CG7985 protein sequence contains many of the last 8^{th} codon (bacteria have 0, 3% as

compared to CG7985 has 13%), which might account for the observed low expression levels.

An alternative way for generating a tool for protein visualization is to tag CG7985 protein, using a relatively new method, BAC recombineering mainly because we can manipulate huge BAC DNAs without the need for digestion and ligation. This method is a combination of 3 novel techniques. It depends on an *E. coli* strain that has the capacity to do ends-out recombination, SW102. A BAC containing the genomic region of CG7985 from the P[acman] library was modified to tag CG7985 with eGFP in a two-step process. In the initial recombination the BAC is modified with a galK cassette. The galK gene is used to monitor the success of the recombination event. In a second recombination step the galK gene is replaced by eGFP. BAC transgenic flies were obtained after injection of the construct into embryos and analyzed by antibody stainings against GFP. The expression of the construct was tested by Western blot analysis against GFP to visualize the transgene and confirmed its successful expression. The expression in imaginal discs appears to be specific to a single cell per ommatidium. However, a more detailed analysis is necessary to fully understand the expression profile.

Following injection of embryos a transgenic line was expressing eGFP tagged CG7985 was obtained and examined. CG7985::eGFP flies were observed to have relatively weak but still recognizable expression. When we checked the expression pattern with an epifluorescence microscope it looked like a single PR specific expression, which follows the MF. It was observed to look more like nucleic stainings, tiny dots with no leakage to surroundings. It is necessary to further analyze the pattern and stainings with confocal microscopy using marker protein stainings.

These tools, though it takes quite some time to analyze it in details, it will be useful to understand the role of *CG7985* in *Drosophila* eye development. We strongly believe that this enzyme has a target protein which has a role in differentiation or specification of R7, maybe even signaling of R8 specification. Once the function of this gene is identified and understood well, the knock out fly lines may be used to model the HexDC deficient systems. Diseases related to malfunctioning of HexA and HexB were observed to be strong phenotypes, resulting in deaths and HexDC presence in autoimmune disease was shown (Pásztói M *et al.*, 2012). The function of HexDC is yet unknown, making disease studies harder. The future experiments on *Drosophila CG7985* will enlighten us about the function

of the gene and probably will be able to corelate it with the human homolog, hoping to provide a model for the treatment of any related diseases.

APPENDIX A : VECTORS

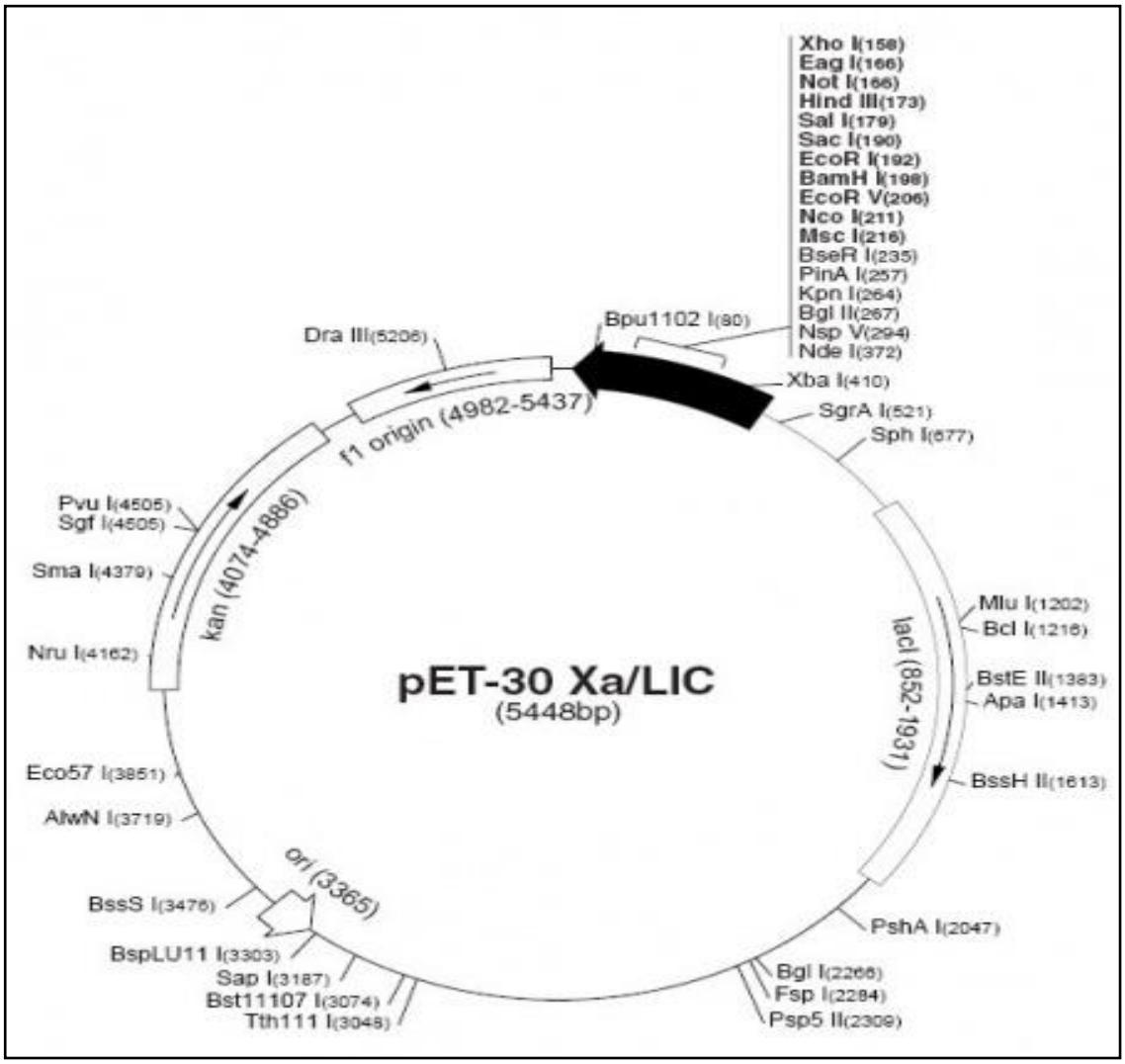

Figure A.1. Vector map of pET-30 Xa/LIC.

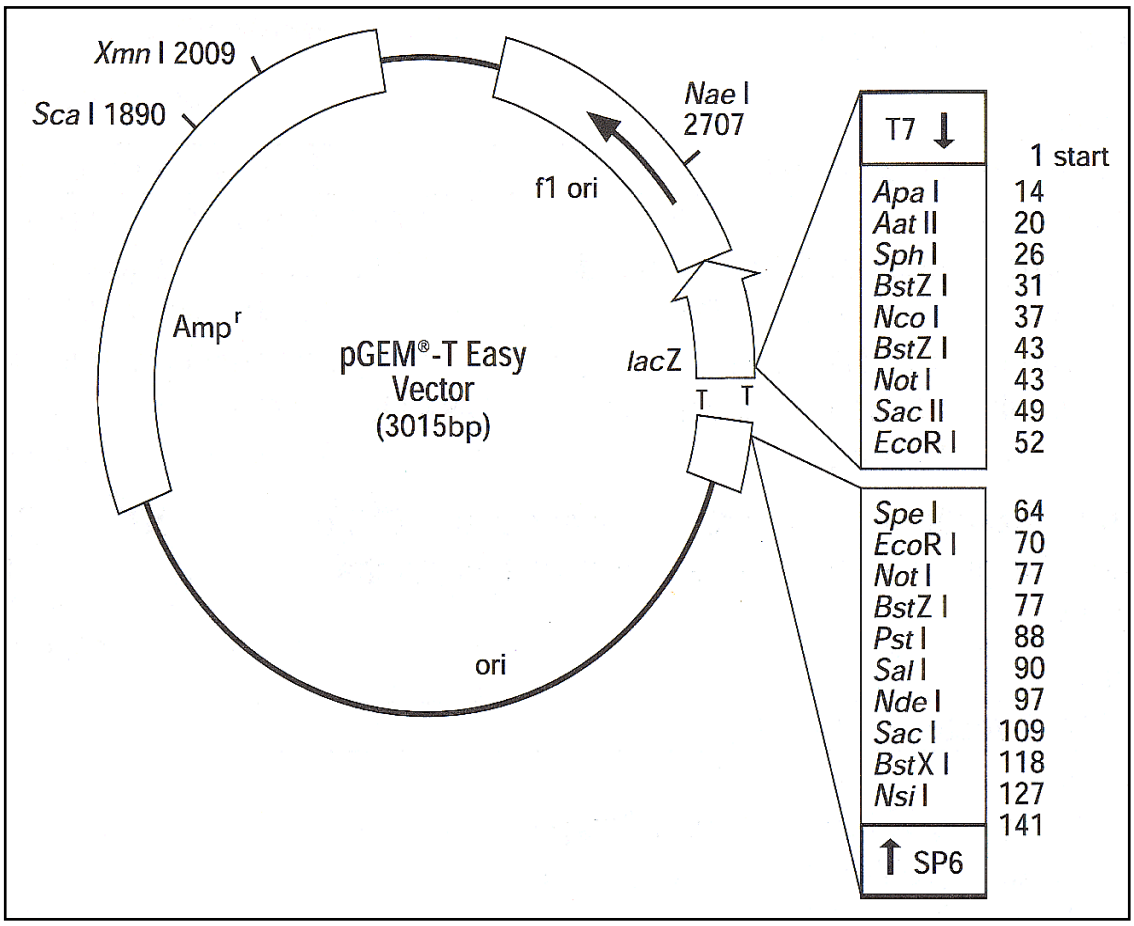

Figure A.2. Vector map of pGEM-T Easy.

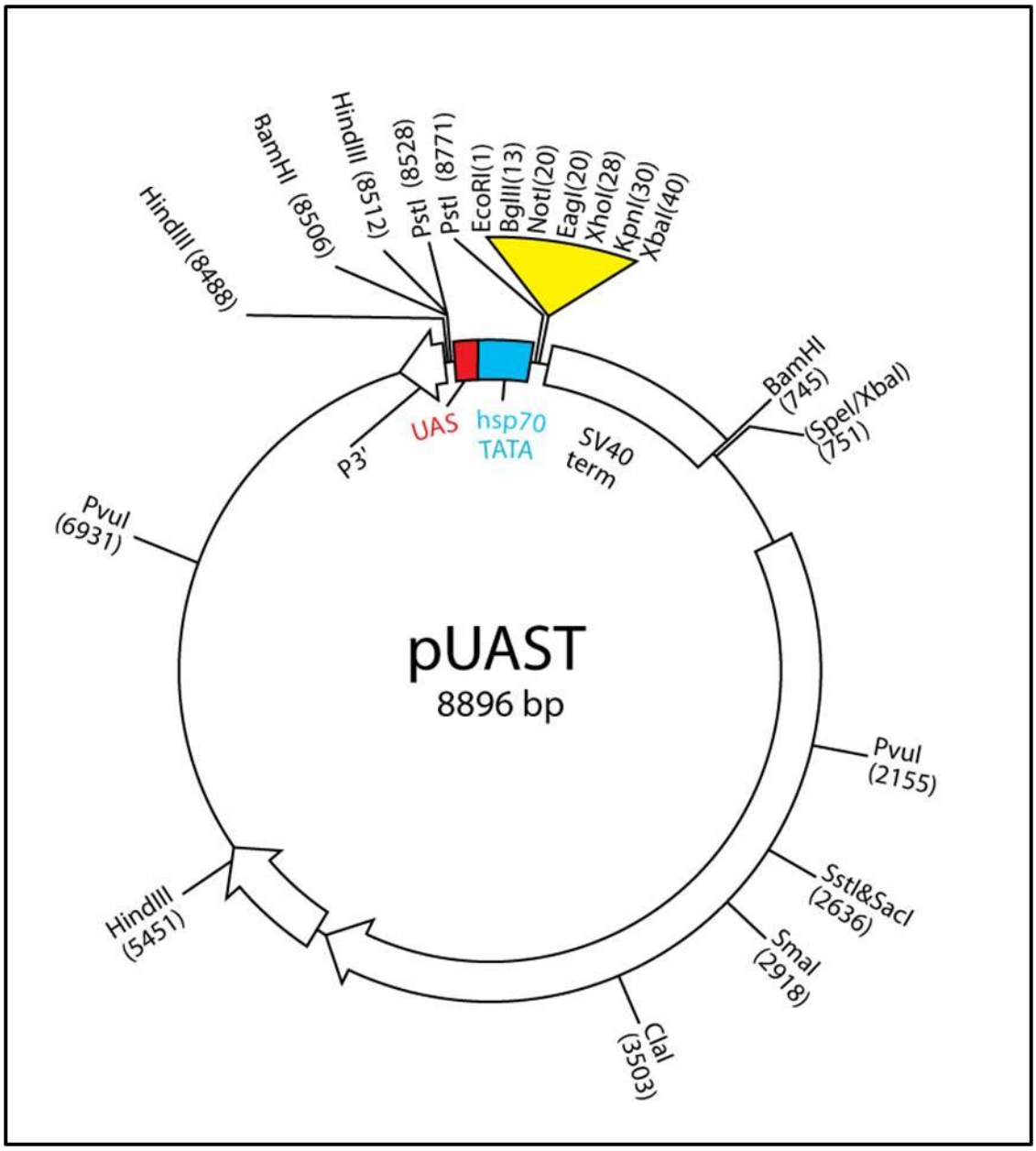

Figure A.3. Vector map of pUAST.

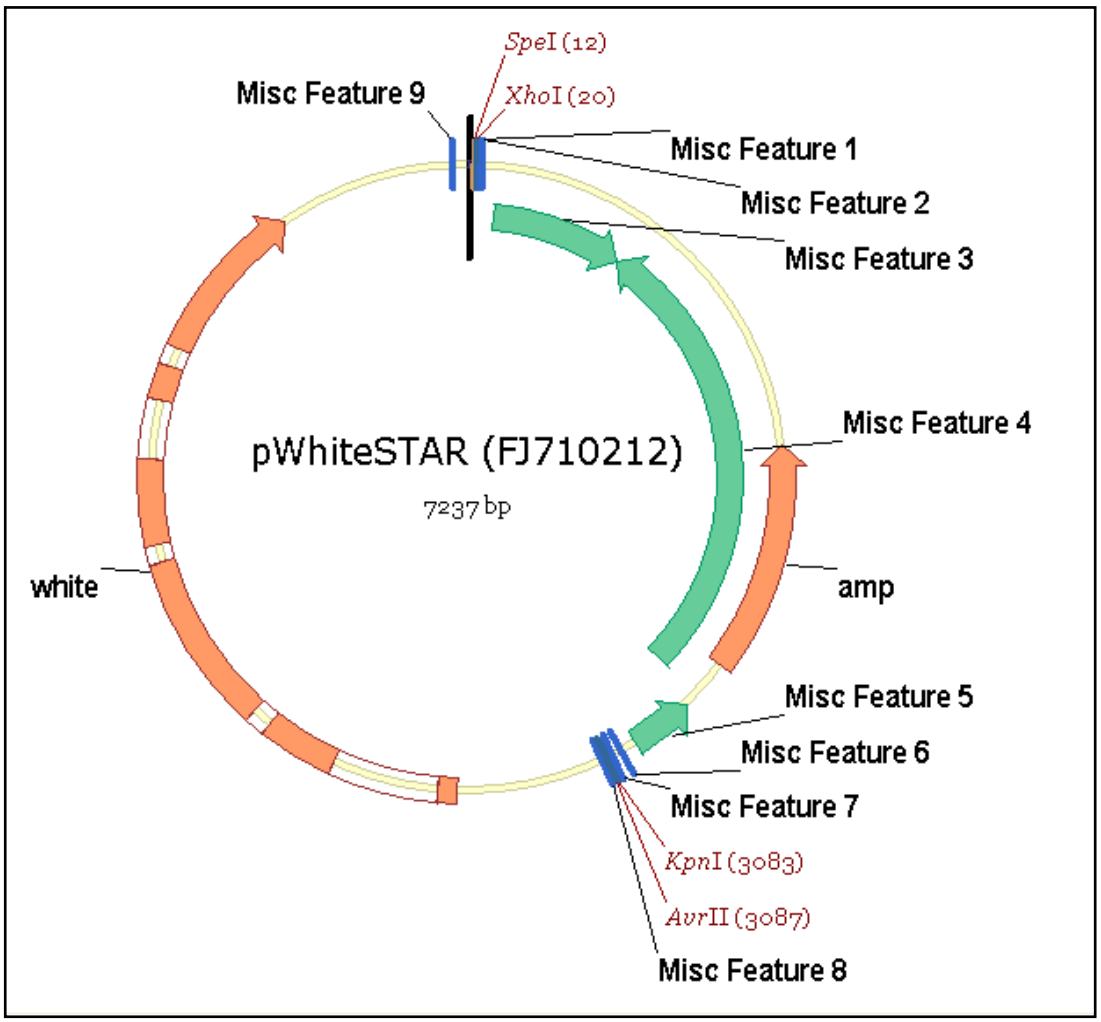

Figure A. 4. Vector map of pWhiteSTAR, the IMAGO cnstruct.

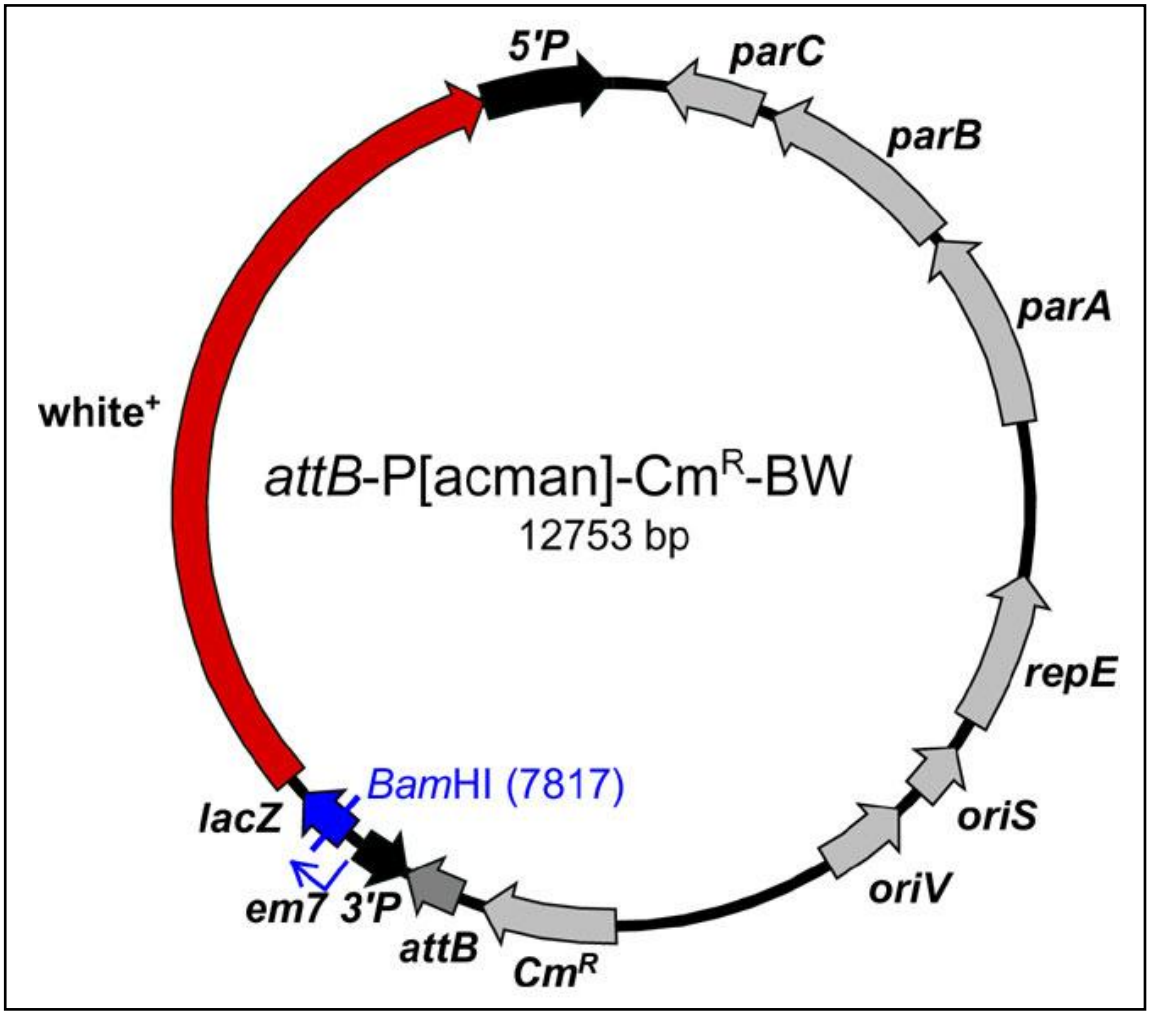

Figure A.5. Vector map of P[acman] backbone plasmid.

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