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Title of project:

"Obtainment and characterization of null Hexim mutants in Drosophila Melanogaster"

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I would also like to thank my greek professors, who support me and accept my project.

ABBREVIATIONS

- 7SK snRNA: 7SK small nuclear RNA
- CTD: Carboxyl Terminal Domain
- Cdk9: Cyclin Dependent Kinase 9
- P-TEFb : Positive Transcription Elongation Factor b
- HEXIM: Hexamethylene Bisacetamide- Inducible protein
- hm7SK snRNA: Human 7SK small nuclear RNA
- Bin3: Bicoid Interacting protein 3
- GFP: Green Fluorescent protein
- LARP7: La-related Protein 7

Abstract

Last years it is increasingly shown the importance of non-coding RNAs in essential activities of eukaryotic cells. The 7SK snRNA is a non-coding RNA, which even if it was known for more than 30 years, just recently it proved its importance in the regulation of transcriptional elongation by RNA polymerase II in mammals. 7SK RNA is responsible for inhibition of P-TEFb. However, 7SK snRNA is not sufficient to inhibit P-TEFb alone . Thay means another protein is necessary for the above regulation, which is Hexim protein. The aim of this project was to investigate the importance of Hexim protein in development by using Drosophila Melanogaster as a model. This aim achieved by obtaining of null mutants with deletion in Hexim, which is a factor that takes part in the above regulation. Experimental method was mutagenesis by P element. The obtained results indicated that Hexim is really important not only for the development of the organism, but first of all for its viability.

Introduction

1. Eukaryotic Transcription

In Eukaryotes the genetic material (DNA) is primarily localized to the nucleus, where is separated from the cytoplasm by the nuclear membrane. DNA is transcribed into different RNAs (m-RNA, r-RNA, t-RNA, sn-RNA) from different RNA Polymerases. Specifically:
- RNA Polymerase I : synthesizes r-RNAs
- RNA Polymerase II: synthesizes m-RNAs
- RNA Polymerase III: synthesizes t-RNAs, small RNAs (r-RNA 5S), snRNAs (p.ex. 7SK)

Eukaryotic transcription takes place in three steps: initiation, elongation and termination. Therefore, when termination is completed successfully, maturation of RNAs starts, before they will be released into the cytoplasm.

2. 7SK snRNA in vertebrates

2.1 Characteristics of 7SK snRNA

Human 7SK RNA is an abundant non-coding 331nt snRNA[1] (approximately $2*10^5$ per cell) , which is transcribed from a single human gene on chromosome 6 by RNA Polymerase III [3]. 7SK RNA was considered as a highly conserved vertebrate innovation. It has been found that there are numerous repeated truncated 7SK pseudogenes, which are dispersed in vertebrate genomes[4].

The hm7SK snRNA ribonucleoprotein particle (RNP) is found in cell extracts as a 12S RNP complex composed of RNA with other proteins [5]. The hm7SK snRNA specifically interacts with up to eight different proteins including CDK9 and Cyclin T that make up the P-TEFb complex[6] together with some others that have been recently discovered (Bin3, hnRNPs and LARP7)

Recent studies have revealed the presence of 7SK snRNA in other mammals, birds, reptiles, amphibians [4], echinoderms, as well as in *Drosophila Melanogaster*.

2.2 Putative structure of 7SK snRNA

Similar to spliceosomal U6 gene, the hm7SK gene belongs to the class III genes that are transcribed by RNA Polymerase III. Class III genes posses a promoter located exclusively upstream of the transcription initiation site [7]. The 7SK promoter contains three common elements including a proximal sequence element, a TATA box-like element and a distal enhancer element. The putative secondary structure of the 7SK snRNA displays several hairpin loops involved in protein interactions.

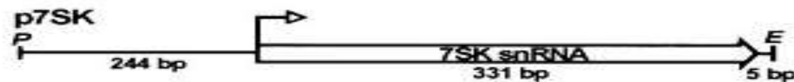


Figure1: The schematic structure of the 7SK gene

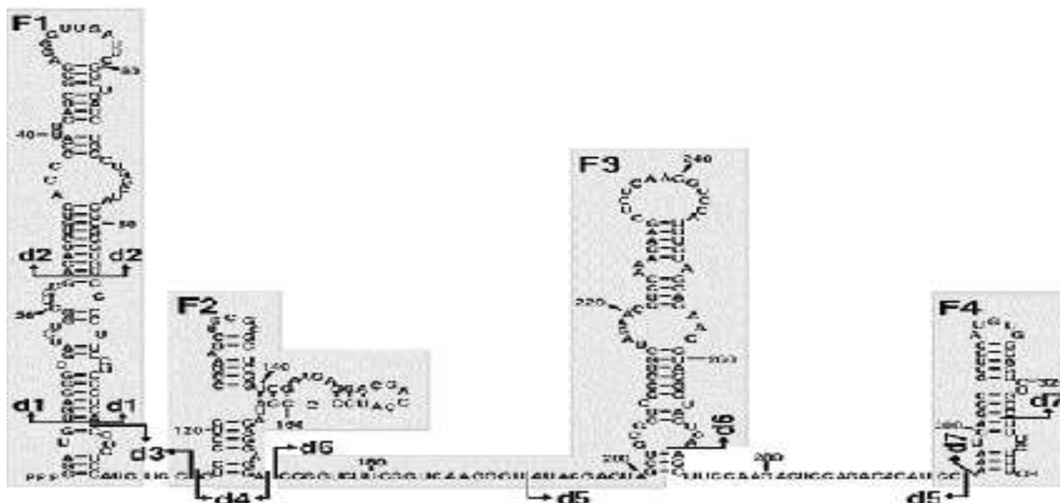


Figure 2: The putative secondary structure of the hm7SK snRNA with hairpin loops involved in protein interaction. The structure is quite well-conserved across animal phyla despite the extreme divergence at sequence level.

2.3 Function of 7SK snRNA

7SK snRNA was discovered in 1976 by Zieve and Penman, but its role was unknown for a lot of years. Thanks to Bensaude and Zhou groups (2001) now it is well characterized and it is known that its human form acts in the inhibition of transcription elongation [3].

It is transcribed by RNA polymerase III (RNA PIII) and is located in the nucleus. Together with associated cellular proteins, 7SK snRNA regulates the activity of the positive transcription elongation factor b (P-TEFb). In humans, this regulation is accomplished by the recruitment of P-TEFb by the 7SK snRNA-binding proteins, hexamethylene bisacetamide (HMBA)-induced

mRNA 1 or 2 (HEXIM1 or HEXIM2), which inhibit the kinase activity of P-TEFb [8] 7SK snRNA should be viewed as the RNA scaffold on which an elaborate P-TEFb regulatory machine is assembled and the reversible association of P-TEFb with 7SK snRNP is an important regulatory mechanism for eukaryotic gene expression. [8]

3. P-TEFb

The positive transcription elongation factor P-TEFb is a pivotal regulator of gene expression in higher cells. [9] Specifically, it plays a key role in RNA Polymerase II elongation control. [10]

There are two distinct P-TEFb complexes in vivo which differ in size, composition and activity. The small P-TEFb complex has a strong kinase activity (active form) and is composed of CDK9 and one of four C-type cyclin regulatory subunits termed Cyclin T1, Cyclin T2a, Cyclin T2b and Cyclin K. [11,12] The active form is free of Hexim and 7SK, and interacts with a variety of cellular factors, including NF-κB , c-Myc, MyoD and Brd4, in order to regulate gene transcription.[10] In contrast, the large P-TEFb complex (inactive form) has a very weak kinase activity of CDK9 (at least 15-fold weaker than the active form). Last complex is that one contains 7SK snRNA and Hexim proteins. [6,9,10,13,14]

P-TEFb is required for the transition from abortive elongation into productive elongation of most class II genes. [15] One of the major targets of the kinase activity of P-TEFb is the carboxyl-terminal domain (CTD) of the largest subunit of RNA Polymerase II, and this phosphorylation of the CTD by P-TEFb occurs during transcription elongation. [10]

4. The 7SK-HEXIM- P•TEFb complex

In the current model of elongation regulation by the 7SK-Hexim complex, P-TEFb is maintained in a functional equilibrium by dynamic associations with its positive and negative regulators. In the nucleus, a major fraction of P-TEFb is sequestered by the 7SK-Hexim complex where the kinase activity of P-TEFb is inhibited. P-TEFb, therefore, is unable to phosphorylate the RNA Polymerase II and is channeled into an abortive elongation mode. Under certain stress or signals, the rapid disruption of the 7SK-HEXIM- P•TEFb complex results in the release of P-TEFb and the formation of Brd4- P•TEFb complex (active form). This conversion results in the increased recruitment of P•TEFb by Brd4 to transcriptional templates, and the stimulation of productive elongation. However, also under a certain signal, P•TEFb can be shifted to the inactive 7SK-Hexim complex. Thus, the dynamic associations of P•TEFb with its positive and negative regulators are kept under tight cellular control in response to the transcriptional demand of the cell. (Figure 3)

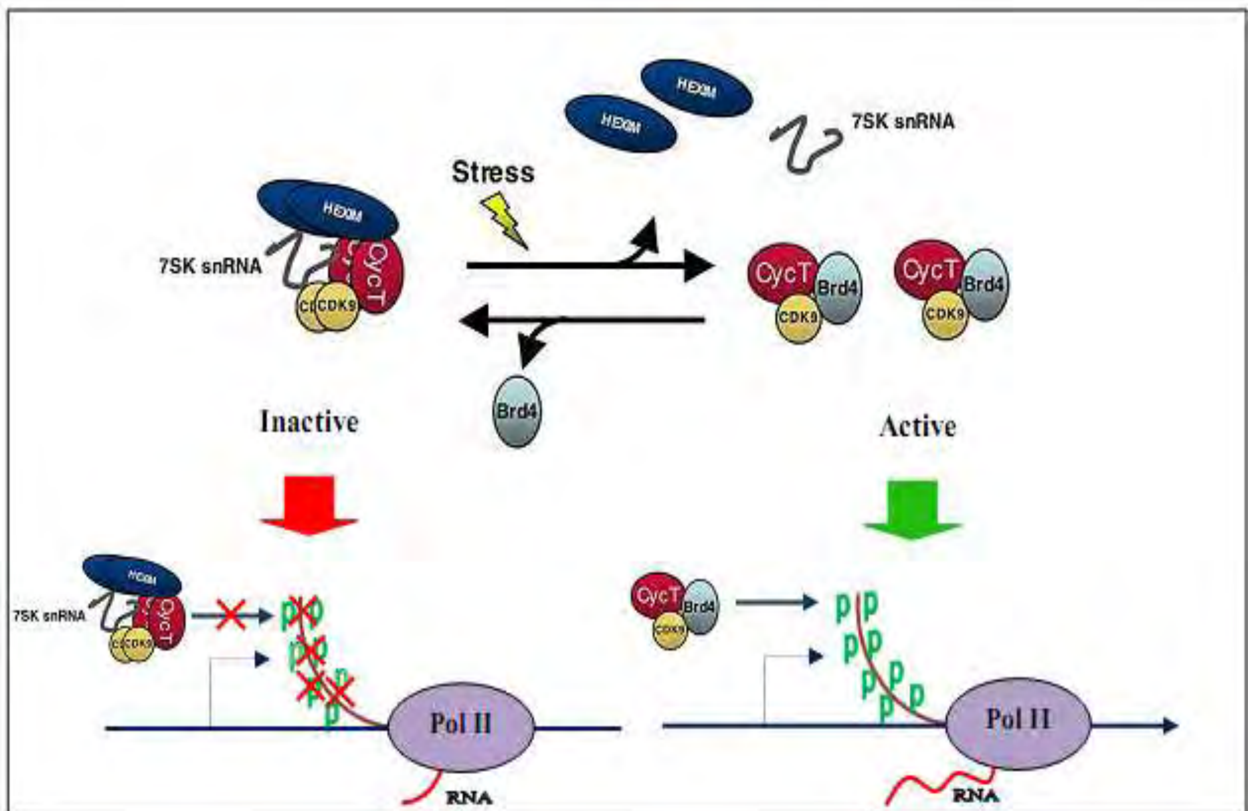


Figure 3: Elongation regulation by the 7SK – HEXIM complex in vivo. In the left side P-TEFb acts as negative regulator and in the right side as positive regulator. Regulations depend on triggering signals and the response of the cell under a certain condition.

5.Two Hexim isoforms

Hexim 1

Hexim 1 is a highly conserved protein containing an RNA binding domain, a nuclear localization signal and many other highly conserved regions with unknown function [12]. Hexim 1, consisting of 359 amino acids (80 kDa) and its role is to regulate the elongation of transcription by RNA Polymerase II, by associating with P-TEFb. Specifically, Hexim 1 has been identified as the third protein component of the 7SK- P-TEFb snRNP formed in vivo, potently and specifically inhibits the kinase and transcriptional activities of P-TEFb in a 7SK-dependent manner[16] , by binding in one of the four Cyclins in P-TEFb. Hexim 1 by itself assembles into dimers in vivo[11], and remains as dimers after binding to 7SKsnRNA. Each dimer can bind to only one through their RNA binding motifs and to two P-TEFb complexes [17]. (Figure 4)

Hexim 1 can be found in both cytoplasm and nucleus. Hexim 1 m-RNA and protein levels markedly increase in murine leukemia cells undergoing terminal differentiation and during differentiation of neuroblastoma cells. Futhermore, the ectopic expression of Hexim 1 causes growth inhibition and

promotes neuronal differentiation [18]. In addition, it has been suggested that most of Hexim 1 could be associated with a variety of RNAs, and even form a distinct complex with glucocorticoid receptor (GR) without involving the 7SK snRNA, CDK9, nor Cyclin T1[19]. However, the 7SK snRNA is probably the major Hexim1 ligand [20] Recently, Hexim 1 has been reported as a protein accumumlating in heart tissue during early embryogenesis in mouse and also as a growth inhibitor that is down-regulated in breast cancer.[12]

Hexim 2

Hexim 2 is similar with Hexim 1 (paralogue genes), but its difference is that it is less abundant and shorter than Hexim 1 by 73 amino acids at the N-terminal domain [21]. The human Hexim 2 gene is localized less than 10kb downstream of the Hexim 1 gene, on chromosome 17 .[12]

Hexim 2 regualtes P-TEFb activity, like Hexim1, throught its association with 7SK snRNA. Thus, when Hexim 1 is knocked down, Hexim 2 is able to fuctionally compensate for the loss of Hexim 1 [22] to inactive the P-TEFb complex resulting in the abortive elongation. Sequence analysis reveals that Hexim proteins are highly conserved throughout vertebrates [12].

Moreover, Hexim1 and Hexim2 were found to form stable homo and hetero-oligomers [23] possessing different physiological functions. Until now, there is no clear answer for the remaining question about the existence of two Hexim genes in the cells. It is supposed that these genes might allow more diverse control of P-TEFb and the utilization between Hexim 1 and Hexim 2 can be differently regulated in vivo [12]. This point of view suggests that the expression levels of these two Hexims should be different in various tissues and cells [11].

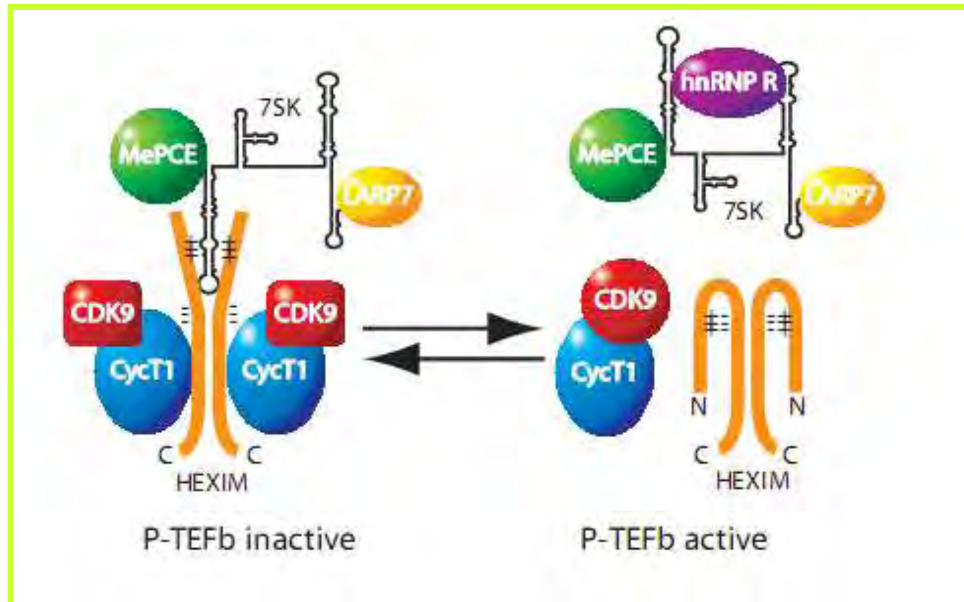


Figure 4: Interacting partners of 7SK snRNA. Regardless of the inactive or active form of P-TEFb, MePCE and LARP7 stably bind to 7SK snRNA while CDK9 and Cyclin T dissociate from this complex and Hexim to switch on active form.

6. P element

A **P element** is a transposon that is present specifically in the fruit fly Drosophila melanogaster and is used widely for mutagenesis and the creation of genetically modified flies used for genetic research.
The P element is a class II transposon, which means that its movement within the genome is made possible by a transposase. The complete element is 2907 bp and is autonomous because it encodes a functional transposase; non-autonomous P elements which lack a functional transposase gene due to mutation also exist. Non-autonomous P elements can still move within the genome if there are autonomous elements to produce transposase. The P element can be identified by its terminal 31-bp inverted repeats, and the 8 bp direct repeat produced by its movement into and out of the DNA sequence. A typical P-strain fly has 30-50 copies of the P element in its genome. However many of these copies contain internal deletions meaning that they do not encode the transposase. They therefore rely on other P elements to produce transposases in order for them to move.
The P element has found wide use in *Drosophila* research as a mutagen. The mutagenesis system typically uses an autonomous but immobile element, and a mobile nonautonomous element.Naturally-occurring P elements contain:

- Coding sequence for the enzyme transposase
- Recognition sequences for transposase action

Transposase is an enzyme that regulates and catalyzes the excision of a P element from the host DNA, cutting at two recognition sites, and then reinserts

randomly. It is the random insertion that may interfere with existing genes, or carry an additional gene, that can be used for genetic research.
To use this as a useful and controllable genetic tool, the two parts of the P element must be separated to prevent uncontrolled transposition.

7. Objectives of the Project

Until now, almost all of the studies about 7SK snRNA/HEXIM complex have been carried out in cells cultures or in vitro. It is very difficult to assess globally and properly about the role of 7SK snRNA/Hexim complex in living organisms. With this project we propose an alternative model in Drosophila Melanogaster that not only overcomes the impediments of study on human model but also makes a chance to understand and assess to developmental role and function of 7SK snRNA/Hexim complex during development of a whole living organism in vivo.
The first aim of this project is to investigate the importance of Hexim protein for development by obtaining null mutants with deletion in Hexim gene. Specifically, that is coming possible by mutagenesis for imprecisely excision of P element.
And the second aim is to ensure our hypothesis that when deletion of Hexim is homozygous, is responsible for lethality. In that case, GFP analysis allow us to follow Drosophila in its life cycle and to understand in which step it deceases.

MATERIALS AND METHODS

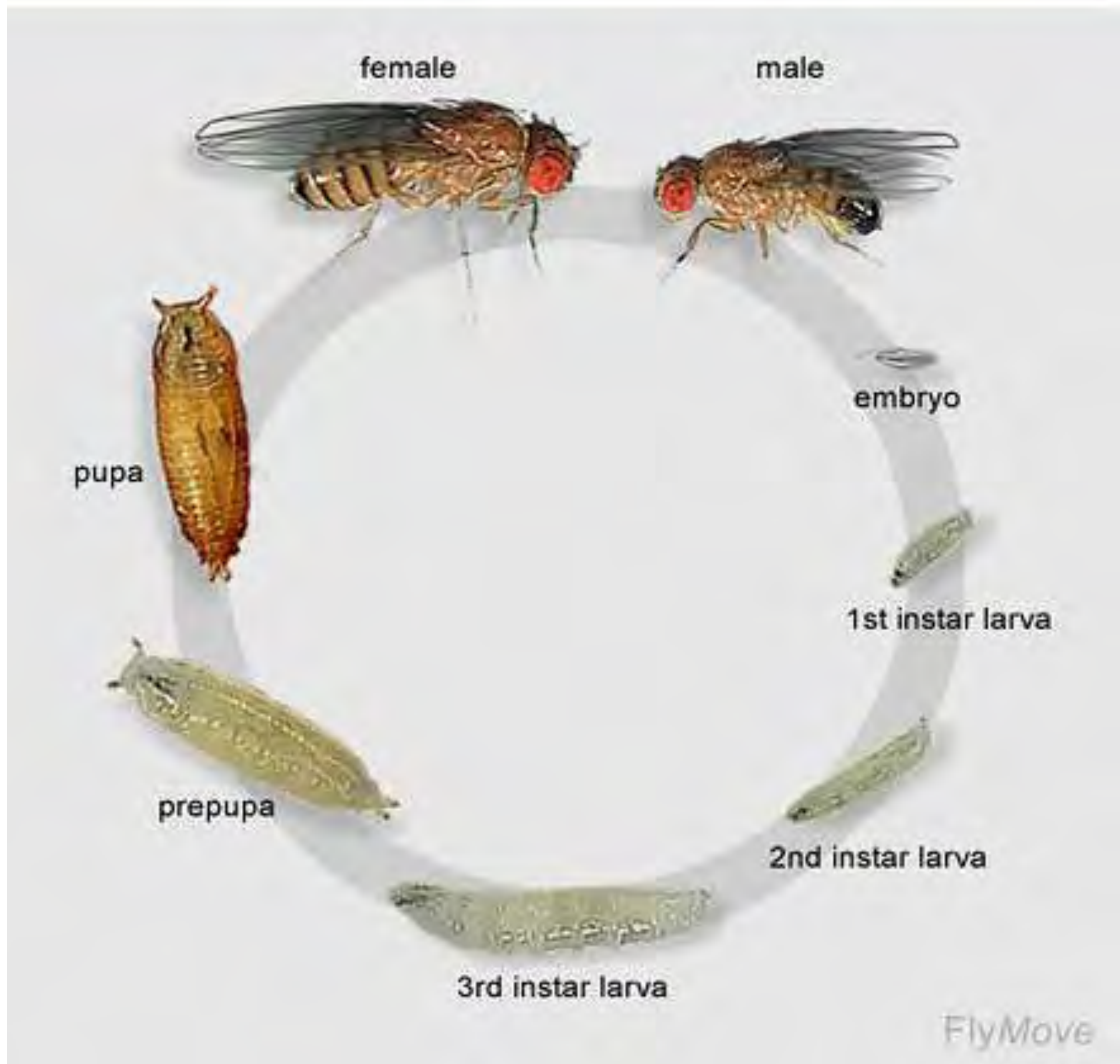
1.MATERIALS

1.1 Model animal: Drosophila Melanogaster

Drosophila Melanogaster is a two-winged insect that belongs to the Diptera and is commonly known as the fruit fly. It is one of the most widely used and genetically best-known of all eukaryotic organisms in Biology. Thanks to many advantages it has, Drosophila is a useful tool in genetics and developmental biology:
-It is small and easy to grow in the laboratory
-It has a short generation time (10 days at room temperature- 25 C)
-It has a high fecundity (one egg every 30 minutes with sufficient food)
-It has only four pairs of chromosomes: three autosomes and one sex chromosome
-Males do not show meiotic recombination, facilitating genetic studies
-The mature larvae show giant chromosomes in the salivary glands called polytene chromosomes
-Its complete genome is sequenced
-You can anesthetize them easily and manipulated individuals with very unsophisticated equipment
-Drosophila are sexually dimorphic (males and females are different), making it is quite easy to differentiate the sexes
-Virgin females are easily isolated because they are physically distinctive from mature adults

Life cycle of Drosophila melanogaster

D. melanogaster exhibits complete metamorphism, meaning the life cycle includes an egg, larval (worm-like) form, pupa and finally emergence (eclosure) as a flying adult. This is the same as the well-known metamorphosis of butterflies and many other insects. The larval stage has three instars, or molts.
Life cycle by day
Day 0: Female lays eggs
Day 1: Eggs hatch
Day 2: First instar (one day in length)
Day 3: Second instar (one day in length)
Day 5: Third and final instar (two days in length)
Day 7: Larvae begin roaming stage. Pupariation (pupal formation) occurs 120 hours after egg laying
Day 11-12: Eclosion (adults emerge from the pupa case). Females become sexually mature 8-10 hours after eclosion.



In our experiments Drosophila is sustained at 25C on a growth medium of the following composition:

-1kg maize
-1kg yeast
-155g agar
-6L distilled water
-600ml of Neplagine -1L pure alcohol 100% with 100gr of methylhydroxy-4-benzoate).
Neplagine prevents the growth of bacteria.

1.2 Balancer Chromosomes

Drosophila has one more advantage that makes her a really great organism model and that is the use of Balancer Chromosomes. Recessive lethal "balancer chromosomes" carrying visible genetic markers can be used to keep stocks of lethal alleles in a heterozygous state without recombination due to multiple inversions in the balancer. These markers allow to following the deletion of Hexim (in our case), as they are located in the same Chromosome and they are easily identifiable either with the naked eye or under a microscope.
Many balancers exist for the X, 2 and 3 chromosomes, but they are not necessary for chromosome 4 because there is no exchange on that chromosome.
For this experiment, the balancers which are used are : Tm3 and Tm6.
Specifically, both of them are in the third chromosome and they carry on a marker:

-Tm3: Serrate (Ser), that expressed as wings notched

-Tm6: SbTb → Sb: stubble (hairs are shorter and thicker than wild type)
Tb: tubby (small body- good marker for larval and pupal stages)

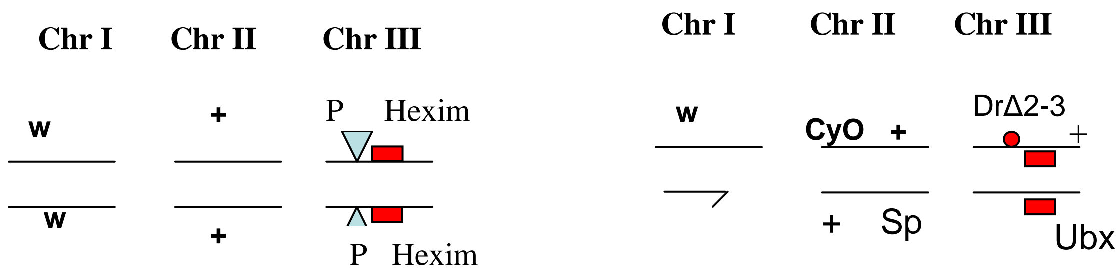
2. METHODS

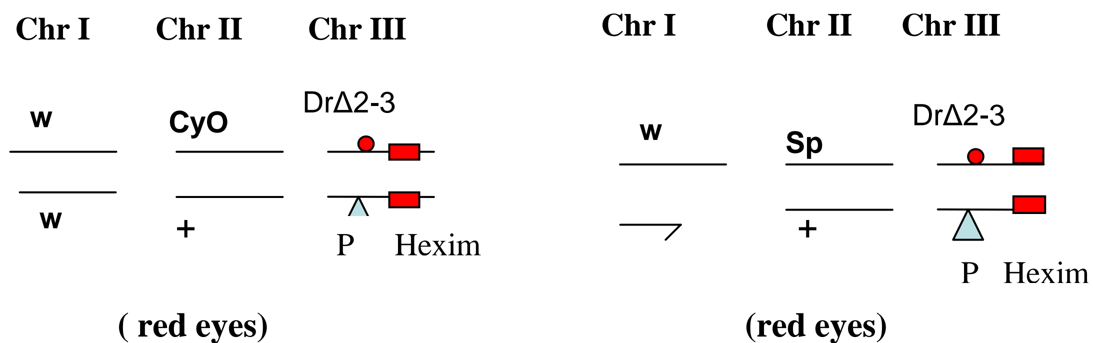
2.1 Crossings- Mutagenesis

Mutagenesis took place in four steps of crossings: *(protocol 1-Annexes)*

1st cross: In this step P element (transposon) and transposase (Dr Δ2-3) are not in the same fly. After those crossings, it is expected for having offspring flies that carry on both of them. Specifically:

Female: w/w; +/+ ; PW+ / PW+ X Male: w/Y ; CyO/ Sp; DrΔ2-3/ Tm6 Ubx
(red eyes) (white eyes)



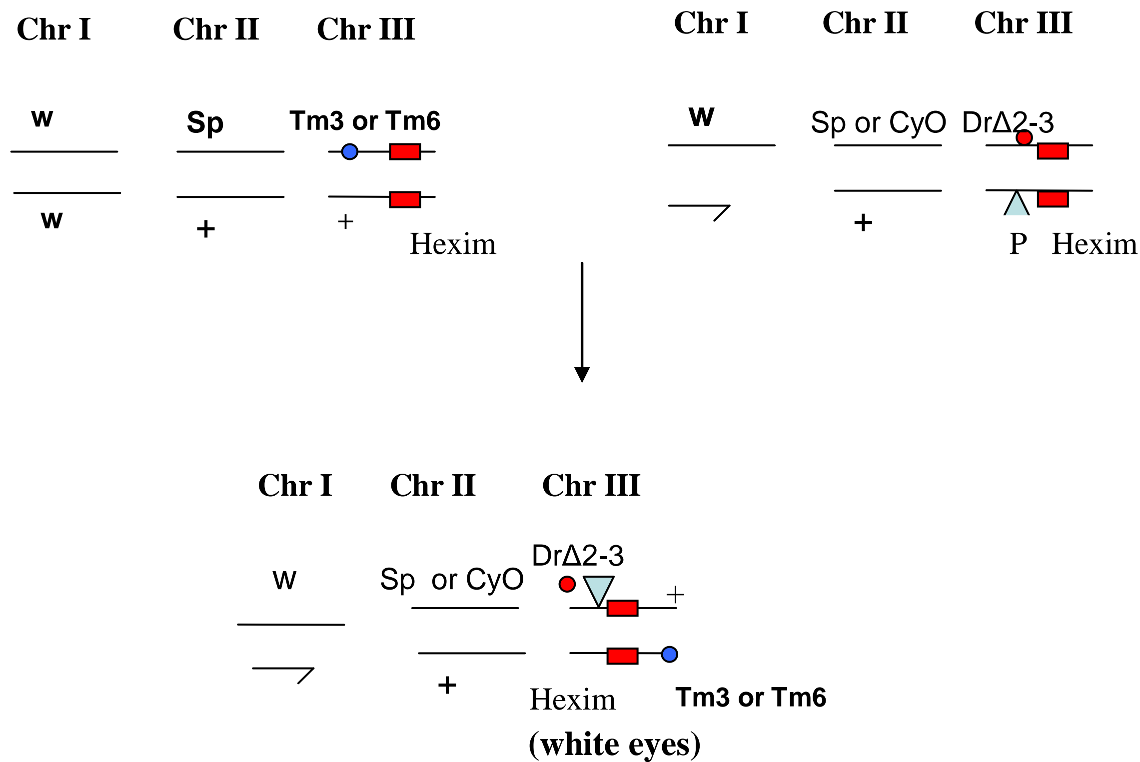


(red eyes) **(red eyes)**

In new generation there are four different phenotypical males and females, but we are interested only in males with above genotypes.

2nd Cross: Crossing of virgin females that carry on a balancer with the males of first cross(offspring), which have in the third chromosome P element and transposase.

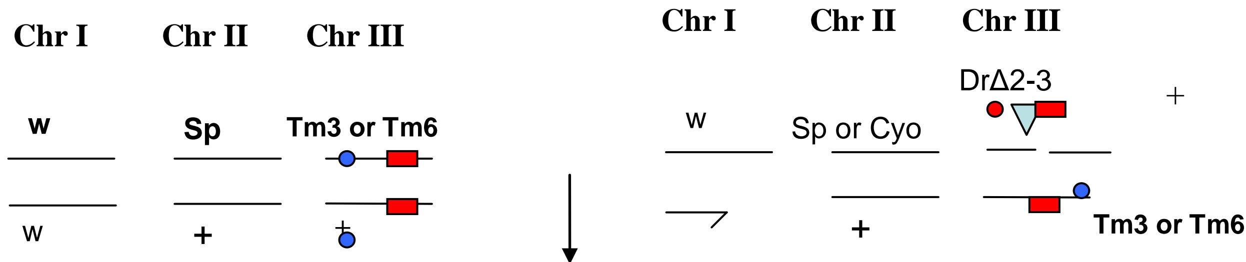
Female: w/w; +/+ ; Tm3 Ser/Tm6 SbTb X Male: w/Y ; CyO or Sp/ +; DrΔ2-3/PW⁺
(white eyes) (red eyes)

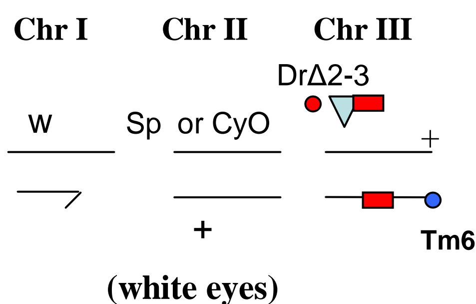


We are interested only in males with above genotypes.

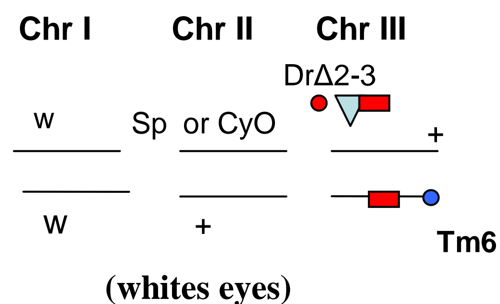
3rd cross: Crossing virgin females with same genotype like in the second cross with males from the second cross (offspring)

Female: w/w; +/+; Tm3 Ser/Tm6 SbTb X Male: w/Y; CyO or Sp/ +; PΔW⁺/Tm3 or Tm6
(white eyes) (white eyes)





(white eyes)



(whites eyes)

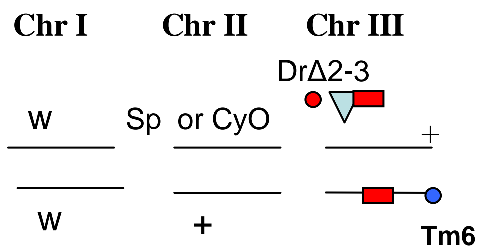
We are interested only in males and females with above characteristics, even if we can recognise and other phenotypes under the microscope. Throw away different phenotypes.

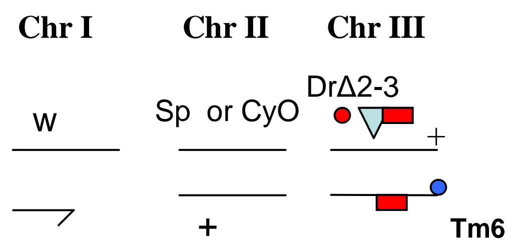
4th cross: Crossing of the descendants from cross 3. This crossing will allow the creation of stock where included only flies with desirable deletion if the experiment is successful.

Female: w/w; CyO or Sp/ +; PΔW+/Tm3 or Tm6 SbTb
(white eyes)

X

Male: w/Y; CyO or Sp/ +; PΔW+/Tm3 or Tm6
(white eyes)





DrΔ2-3	Transposase
P element	Transposon located in Chr III
Tm3	Balancer in chromosome III
Tm6	Balancer in chromosome III
Cyo	Wings curled upward instead of flat
Ubx	Haltere larger and rounder than normal

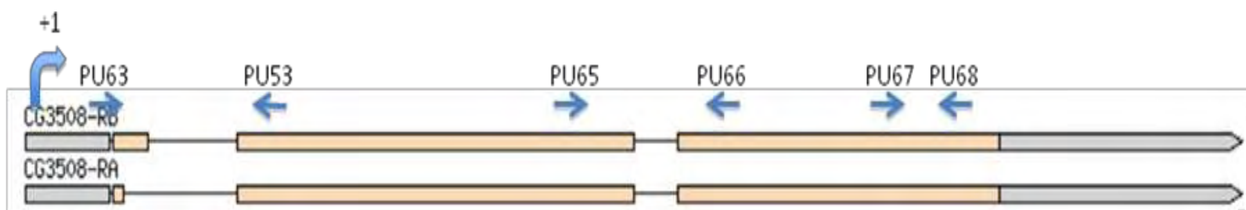
*In every step we keep the crossings in 25 °C.

2.2 Characterization of mutants by PCR

There are four samples that are coming from mutagenesis, where mutants are homozygous viable for a deletion. It is supposed that this deletion is not in Hexim, because there is the hypothesis when the mutant is homozygous for Hexim deletion is lethal. To verify this hypothesis, the samples are checked by PCR for presence or absence of Hexim gene.

The PCR will compare the presence or absence, but also the level of expression among the samples: wild type flies(Canton S), flies 20799(mutants with overexpression of Hexim) and homozygous viable samples. Hexim gene is detected at 300bp.

PCR allows the amplification of specific DNA sequences and dramatically increase the amount of them. It is necessary to be known the sequence of regions which delimit the DNA in order to determine the sequence of DNA primers. The number of PCR cycles realized by DNA polymerase is generally between 25 and 40. Every cycle of PCR consists of three different phases in three different temperatures. (Protocol 2-Annexes)



In the picture are depicted the limits of primers on Hexim gene. Specifically:

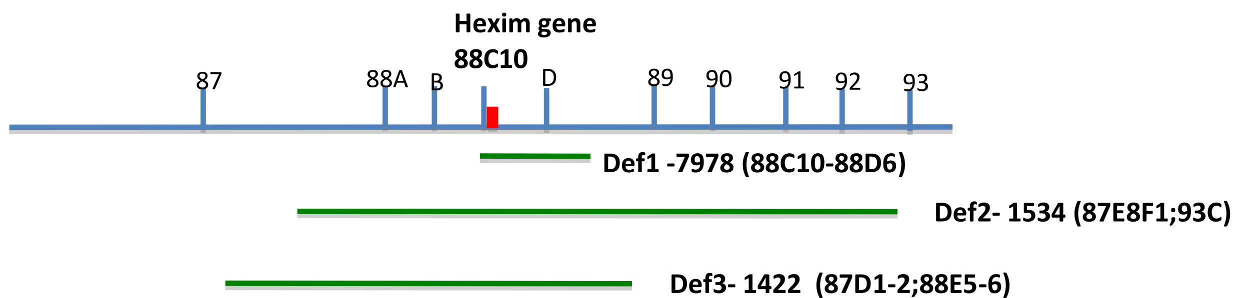
- primers PU63 and PU53 recognising a part in the beginning of the gene
- primers PU65 and PU66 recognising a part late in the gene
- primers PU67 and PU68 are the positive control of PCR

Electrophoresis in agarose gel 1,2%

For verifying and visualizing the size and the quantity of PCR's products, it has been used analysis by electrophoresis in agarose gel and they are compared with a score of size (Smart Ladder SF). The detection of DNA on this kind of gel is possible when it is exposed in UV radiation, after reaction with BET (ethidium promide), which is intercalating in the DNA strains.

2.3 Deficiency (Deletion) of Hexim

Crossing flies that have deficiences of Hexim with the mutants of mutagenesis in order to see if the mutants of mutagenesis have a deletion in Hexim. Hypothesis is that the loss of Hexim is homozygous lethal. However, deficiencies do not have a deletion only to Hexim, but also in other genes around Hexim. That is why, it is necessary to cross also deficiencies together, in order to ensure that they have the same deletion.

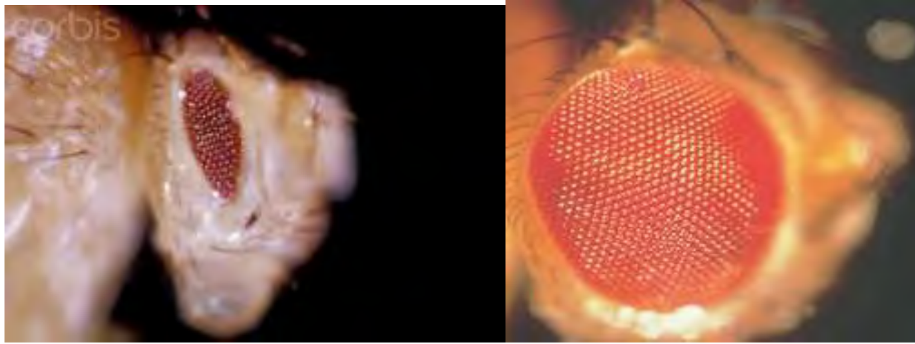


In the picture is depicted the location of Hexim and the size of deciciences.

2.4 Characterization of lethality step by using GFP

The female mutants of mutagenesis that carry on a deletion in Hexim gene are crossed with males that carry on a balancer in X Chromosome (FM7) and two balancers in Chromosome III (Tm6 and Tm3).

- FM7 balancer carries the dominant marker Bar, which is recognized as oval shape of Drosophila's eye (eye narrower than usual)



Left: Bar eye **Right: normal eye**

- Tm6 balancer carries on the marker B^{tb} , which is recognised by two long bristles.
- Tm3 balancer carries on the GFP.

Crossings:

1st step:

Female: w/w; +/+ ; ΔHexim/ Tm6 SbTb

X

Male: FM7,GFP ; +/+;Tm6 B^{tb}/Tm3 GFP

After doing the above crosses appeared many phenotypes, but we are interested only in:

- females with genotype **FM7,GFP/+; ΔHexim/Tm3-GFP**
- and males with genotype: +/+; **ΔHexim/Tm3-GFP**

2nd step:

Female: FM7,GFP/+; ΔHexim/Tm3-GFP X **Male: +/+; ΔHexim/Tm3-GFP**

Crossing of descendants from first cross obtaining mutants that are:

- homozygous for deletion of Hexim (it is supposed to be lethal)
- heterozygous for deletion of Hexim and carry on Tm3 balancer with GFP marker
- homozygous for balancer Tm3-GFP (lethal)

3rd step:

Under specific microscope observing every developmental stage of Drosophila in order to find out until which stage homozygous Drosophila for deletion of Hexim will stay alive. This fly do not fluorescent under the suitable microscope.

RESULTS AND DISCUSSION

1. Mutagenesis

In order to study what is the impact of the Hexim's gene deletion in Drosophila, it is necessary to obtain mutants that are homozygous for this deletion. The hypothesis is when Hexim deletion is homozygous , the phenotype is lethal, because Hexim takes part in a very important complex for transcriptional elongation. Specifically, the obtainment of the null mutants in Hexim deletion needs four steps of crossings:

1 step: female fly has the transposon (P element) and male the transposase (DrΔ2-3) . Transposon needs to be in the same chromosome with transposase in the same fly in order to be activated and jump. This goal has been successed in this cross.

2step: Collection of males from first cross that carry on transposon and transposase and cross them with virgin females, which have a balancer chromosome (Tm3 or Tm6) in Chromosome III. We expect that transposase will activate transposon and it will jump with Hexim gene or at least with a part of it. P element is located before the beginning of Hexim. We verified that P element jumped with Hexim, when white eyes appeared to descendants. However, P element is possible to jump precisely that means without Hexim gene. In that case, there will not be any change in eye colour.

3step: Collection of males that carry on the transposon, the transposase and one of the chromosome balancers (either Tm3 or Tm6). Cross of these males with virgin females as in the second step.

4 step: In this step, by crossing the descendants of the third cross that carry on a deletion in Hexim, we create stock of them. Tm3 balancer is less credible than Tm6, that is why in this step we prefer mutants carrying Tm6 and we reject mutants with Tm3 balancer.
We suppose that our mutagenesis is successful because we have mutants with white eyes (deletion), that means P element jumped with a part of a gene. But still we cannot be sure if the deletion is in our candidate gene or in another close to P element.

Candidates	Number of samples
Initial candidates	131
Deletion of the gene	50
Homozygous lethal	2

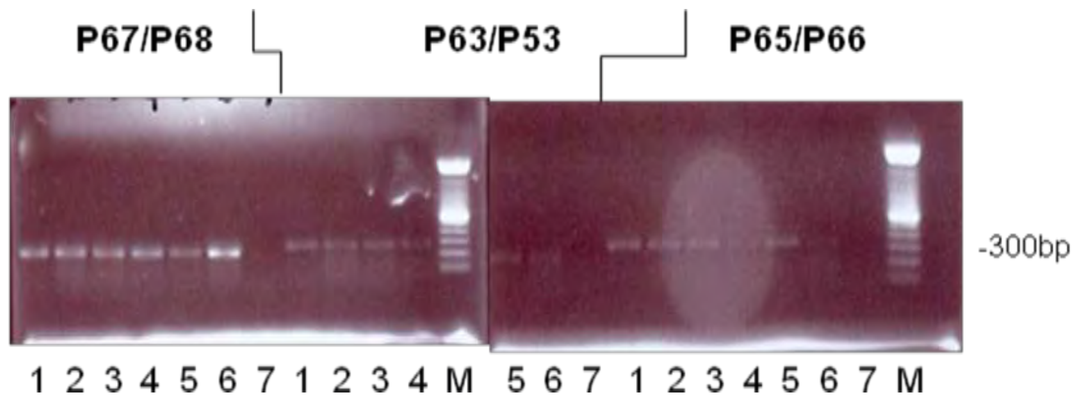
Table1: The number of candidates in every step of mutagenesis

Figure 5: Above Drosophila with deletion, down wild type Drosophila.

2. Characterization of mutants by PCR

We would like to verify our hypothesis that the homozygous deletion of Hexim is lethal. But, we observed in our mutant that some of them have a deletion (white eyes), nevertheless, this deletion is “homozygous viable”. That is coming in contrast with our hypothesis. By using PCR we checked if these mutants have deletion of Hexim or not.

The results we obtained are:



Picture 6: There is detection of Hexim gene in every mutant apart from control sample that does not contain DNA.

1,2,3,4: Homozygous viable mutants
5: Canton S. – wild type fly
6: 20799 fly- fly that has over-expression of Hexim gene
7: Control- no sample

So, after obtaining the above result we made sure that there is no deletion in Hexim and mutants are "homozygous viable" for other gene deletion. That allows us to continue our hypothesis about "homozygous lethal" phenotype.

3. Deficiency (Deletion) of Hexim

In order to check if the deletion in homozygous lethal phenotypes of mutagenesis is in Hexim, we cross these mutants with deficiencies, which include deletion of Hexim. Deficiencies is a kind of measure for the area of deletion. A cross between the mutant of mutagenesis and the deficiency is supposed to give lethal phenotype. However, it is necessary to cross deficiencies together in order to be sure that they have deletion in same area and in our case to have deletion in Hexim (lethal phenotype).

Deficiencies	Observed phenotype	
	Candidate 1	Candidate 2
1	Not lethal	Not lethal
2	Lethal	Lethal
3	Lethal	Lethal

Table 2: Observed results of deficiencies

We conclude that Deficiency 1 does not have the same deletion with our candidates, but Deficiencies 2 and 3 appeared lethal phenotypes probably means there is Hexim deletion. However, to ensure this hypothesis firstly we cross these two deficiencies together and expecting for observing lethal phenotype. The result of this cross was lethal phenotype, so we purpose both of them have Hexim deletion.
About Deficiency 1, we purpose that it has deletions in other regions but not in region of our candidate gene. So, the problem in this case is that Deficiency includes Hexim gene. After using PCR method by Patricia Uguen(supervisor of the stage), she realized that our hypothesis is right.

However, still we cannot be absolutely sure that the deletion in common among our candidates, deficiency 2 and deficiency 3 is the right deletion. It is possible that P element has jumped with a region of another gene that is in common with both deficiencies and it is observed lethal babies. If it can be true this hypothesis, we have a wrong deletion.

4. Characterization of lethality step by using GFP

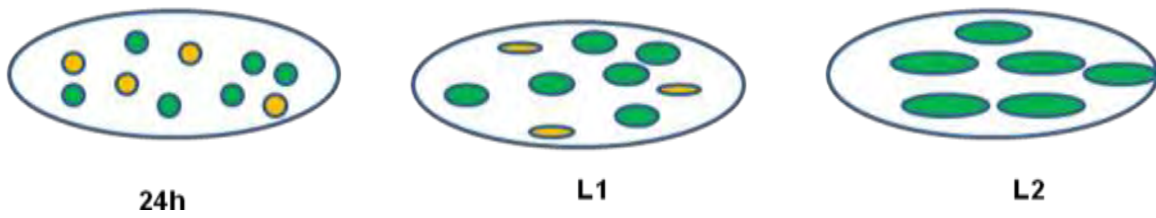
With this method we obtain mutants that can fluorescent under specific microscope. But, these are not the mutants that are homozygous for the deletion, because GFP is not in the same allele with the Hexim deletion. Observing the mutants in every developmental stage we could see some mutants fluorescent and some others not. But this observation was until first instar larva (L1). In candidates we were interested in were these do not fluorescent, because that means were homozygous for the deletion and they did not carry on the allele with GFP marker. Specifically:

Stage	Mutants-GFP	Mutants-homozygous lethal
Embryogenesis	present	present
First instar larva (L1)	present	present
Second instar larva (L2)	present	absence
Third instar larva (L3)	present	absence

Table 3: Presence and Absence of mutants in every developmental stage

According to above results we conclude that the mutant, which is homozygous for the Hexim deletion can survive until L1 stage but not more. We purposed that

mutant can stay alive until L1 thanks to mother's contribution, but when this contribution will be over mutant will die.



Picture 7: It is depicted the stages until "homozygous lethal" phenotype disappeared.

CONCLUSION AND PERSPECTIVES

This project had two aims firstly to investigate the importance of Hexim gene in viability and secondly to examine the stage of life that Drosophila can reach without production of Hexim protein. Both of these aims have been successed, as we managed obtaining mutants with deletion of Hexim in both alleles. It is difficult to obtain these mutants, because you cannot be sure for the imprecise jump of P element. Also, needs a lot of time (3 months) and many crossings.
Futhermore, we observed that Hexim is really important gene for Drosophila's viability and development, as its absence causes death. That probably means that deletion of Hexim gene provokes modifications in the regulation of transcription. Consequently, this impact on transcription affects in some other genes that are important for life and death is coming. However, Drosophila manages to be alive at embryogenesis and first larva stage (L1) thanks to mother's contribution, but after that stage it dies.

In the future, this project could continue by investigating more details about Hexim's function and specifically, which part of Hexim is deleted or in other words deletion of which part is responsible for death. For example, using the method "sequencing" we could have some information about the exact region of deletion in Hexim, but also we can discover if there is deletion and in other genes.
Another thing, that we could do in order to ensure the importance of Hexim gene for viability is to cross the "homozygous lethal" mutants with wild type flies. In this case, every descendant will have an allele with Hexim gene and it supposed to be viable. (rescue of mutants)

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ANNEXES

Protocol 1: Mutagenesis

Cross 1: female vierge W/W; +/+; PW^+/PW^+ (red eyes)
X male W/Y; CyO/Sp; DrΔ2-3/Tm6 Ubx (white eyes)

Prepare 20 tubes, where every one will contain one female with 4-5 males. Remove parents in new tube.

male / female	w; CyO; DrΔ2-3 ou (1/4) Y; CyO; DrΔ2-3	w; CyO; Tm6 Ubx ou (1/4) Y; CyO; Tm6 Ubx	w; Sp; DrΔ2-3 ou (1/4) Y; Sp; DrΔ2-3	w; Sp; Tm6 Ubx ou (1/4) Y; Sp; Tm6 Ubx
w; +; PW^+ 1	w; CyO; DrΔ2-3 / w; + ; PW^+ (Fem, Curly, Dr, YO) ou w; CyO; DrΔ2-3 / Y; + ; PW^+ (male, Curly, Dr, YO)	w; CyO; Tm6 Ubx / w; + ; PW^+ (Fem, Curly, Ubx, YO) ou w; CyO; Tm6 Ubx / Y; + ; PW^+ (male, Curly, Ubx, YO)	w; Sp ; DrΔ2-3 / w; + ; PW^+ (Fem, Sp, Dr, YO) ou w; Sp ; DrΔ2-3 / Y; + ; PW^+ (male, Sp, Dr, YO)	w; Sp ; Tm6 Ubx / w; + ; PW^+ (Fem, Sp, Ubx, YO) ou w; Sp ; Tm6 Ubx / Y; + ; PW^+ (male, Sp, Ubx, YO)

We are interested in: male, Curly (ou Sp), Dr, YO

Keep the males that you collect at 18°C , don't use them immediately for second cross.

Cross 2: 1 male w/Y; CyO ou Sp/+; DrΔ2-3/PW^+ (red eyes)
X female w/w; +/+; Tm3 Ser/Tm6 SbTb (white eyes)

Put one only male in a tube with 4-5 females. Prepare more than 50 tubes.

male / female	w; CyOouSp; DrΔ2-3 ou (1/2) Y; CyOouSp; DrΔ2-3	w; CyOouSp; PW^+ ou (1/2) Y; CyOouSp; PW^+	w; CyOouSp; $PΔW^+$ ou (?) Y; CyOouSp; $PΔW^+$
w; +; Tm3 Ser (1/2)	w; CyOouSp; DrΔ2-3 / w; + ; Tm3 Ser (Fem, Curly ou Sp, Dr, Ser, RE) ou w; CyOouSp; DrΔ2-3 / Y; + ; Tm3 Ser (male, Curly ou Sp, Dr, Ser, RE)	w; CyOouSp; PW^+ / w; + ; Tm3 Ser (Fem, Curly ou Sp, Ser, RE) ou w; CyOouSp; PW^+ / Y; + ; Tm3 Ser (male, Curly ou Sp, Ser, RE)	w; CyOouSp; $PΔW^+$ / w; + ; Tm3 Ser (Fem, Curly ou Sp, Ser, WE) ou w; CyOouSp; $PΔW^+$ / Y; + ; Tm3 Ser (male, Curly ou Sp, Ser, WE)
w; +; Tm6 SbTb (1/2)	w; CyOouSp; DrΔ2-3 / w; + ; Tm6 SbTb (Fem, Curly ou Sp, Dr, SbTb, RE) ou w; CyOouSp; DrΔ2-3 / Y; + ; Tm6 SbTb (male, Curly ou Sp, Dr, SbTb,RE)	w; CyOouSp; PW^+ / w; + ; Tm6 SbTb (Fem, Curly ou Sp, SbTb, RE) ou w; CyOouSp; PW^+ / Y; + ; Tm6 SbTb (male, Curly ou Sp, SbTb, RE)	w; CyOouSp; $PΔW^+$ / w; + ; Tm6 SbTb (Fem, Curly ou Sp, SbTb, WE) ou w; CyOouSp; $PΔW^+$ / Y; + ; Tm6 SbTb (male, Curly ou Sp, SbTb, WE)

We are interested in : males Curly ou Sp, Ser ou SbTb, white eyes

Cross 3: 1 male w/Y; CyO ou Sp/+; PΔW[+]/Tm3 ouTm6 (white eyes)
X femelle w/w; +/+; Tm3 Ser/Tm6 SbTb (white eyes)

Put one only male in a tube with 4-5 females. Prepare more than 50 tubes.

male / femelle	w; CyOouSp; PΔW+ ou (1/2) Y; CyOouSp; PΔW+	w; CyOouSp; Tm3 ouTm6 ou (1/2) Y; CyOouSp; Tm3 ouTm6
w; +; Tm3 Ser (1/2)	w; CyOouSp; PΔW+ w; + ; Tm3 Ser (Fem, Curly ou Sp, Ser, WE) Ou w; CyOouSp; PΔW+ Y; + ; Tm3 Ser (male, Curly ou Sp, Ser, WE)	w; CyOouSp; Tm3 ouTm6 w; + ; Tm3 Ser (Fem, Curly ou Sp, Ser, SbTb ou lethal, WE) ou w; CyOouSp; Tm3 ouTm6 Y; + ; Tm3 Ser (male, Curly ou Sp, Ser, SbTb ou lethal, WE)
w; +; Tm6 SbTb (1/2)	w; CyOouSp; PΔW+ w; + ; Tm6 SbTb (Fem, Curly ou Sp, SbTb, WE) Ou w; CyOouSp; PΔW+ Y; + ; Tm6 SbTb (male, Curly ou Sp, SbTb, WE)	w; CyOouSp; Tm3 ouTm6 w; + ; Tm6 SbTb (Fem, Curly ou Sp, Ser, SbTb ou lethal, WE) ou w; CyOouSp; Tm3 ouTm6 Y; + ; Tm6 SbTb (male, Curly ou Sp, Ser, SbTb ou lethal, WE)

We are interested in : male et female Curly ou Sp, SbTb, white eyes. We could collect also the mutants that have deletion of Hexim and as balancer Tm3, but Tm3 balancer is less good than Tm6.

Cross 4: Cross together the brothers and sisters from the third cross. In this way, you will have a stock with desirable mutants.

Protocol 2: PCR

Test 50ng of genomic DNA from each sample: four “homozygous viable”, one wild type, one mutant with overexpression of Hexim, control.

Synthesis of DNA template:

For final volume of 25µL:

- Distilled water H_2O : 17,7 µL
- Tp 10X TaqAM : 2,5 µL
- $MgCl_2$ (25mM): 1.5 µL
- dNTPs (2,5 Mm/each) : 1 µL
- Taq AM: 0,3 µL
- Genomic DNA (100ng/ µL) : 0,5 µL
- Primer 1 (10 µM) : 0,75 µL
- Primer 2 (10 µM) : 0,75 µL

For each pair of oligos you mix all the elements together except for genomic DNA.

Set up the PCR program:

-94 C 5min

-94 C 30sec
-58 C 30 sec } repeated 30 cycles
-72C 30 sec

-72 C 7 min

Migrate 10 µL of each PCR sample + 2 µL of loading buffer on gel agarose 1.2% with wider wells. Also, use 6 µL of ladder of size (Smart Ladder SF) for electrophoresis.

Set up electrophoresis program: 100V for 25min.