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Department of Molecular Biology

Creation of Drosophila melanogaster mutants for multiple sirtuin genes (Sirt2, Sirt6 and Sirt7)

Bachelor Thesis

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Biological Chemistry

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Annotation

The aim of this thesis was to create a fruit fly that is a mutant for multiple sirtuin genes, Sirt2,Sirt6 and Sirt7, by meiotic recombination and Crispr/Cas 9 technology. This fly would be used in order to achieve the creation of a fly mutant for all sirtuin genes, whose phenotype would be characterized by testing it under stress conditions.

Affirmation

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Paraskevi Tziortzouda

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1. INTRODUCTION

1.1 DROSOPHILA MELANOGASTER: A MAJOR MODEL ORGANISM

1.1.1 Drosophila Genetics

The systematic use of the fruit fly Drosophila melanogaster for research in the laboratories of Biology and Genetics started after 1910, when T.H. Morgan, the father of modern experimental genetics, and his students, tested the chromosome theory of inheritance on the fruit fly and found out the linkage between the gene for eye color and the X sex chromosome.

A study of the National Human Genome Research Institute in 2000 has shown that approximately 75% of human disease genes have a match in Drosophila genome, correlating to one out of two fly proteins being homologous to mammalian proteins. The size of Drosophila genome (1.75×10^8 base pairs in haploid state, roughly 13.600 genes) is about 5% the size of the human genome (3×10^9 base pairs, roughly 21.000 genes) packed in only four pairs of chromosomes, designated as 1-4, with the low number of chromosomes being the main reason for simplification of genetics in the fruit fly. The X chromosome is chromosome 1 and chromosomes 2,3 and 4 are autosomes (A). Even though females are XX and males are XY, the Y chromosome does not affect sex determination, because the sex is determined by the X:A ratio, with XXY zygotes with X:A ration of 1 being female flies and XO zygotes with a ratio of 0.5 being non-fertile males. The only role of the Y chromosome is to make males fertile and this is why it is not assigned a number. In Fig.1, the acrocentric X chromosome, the metacentric chromosomes 2 and 3 and the small acrocentric chromosome 4 are observed. In every chromosome, there is heterochromatin present close to the centromere, but the Y chromosome is almost exclusively covered by heterochromatin. Approximately 21% of the 170.000 kb DNA of Drosophila, is the so called “satellite DNA”, which is located on the heterochromatic regions of the chromosomes. With another 9% of the DNA being transposable elements and 3% being repeated genes encoding for sRNA, rRNA and histone proteins, only 67% of the genome is “euchromatic”.

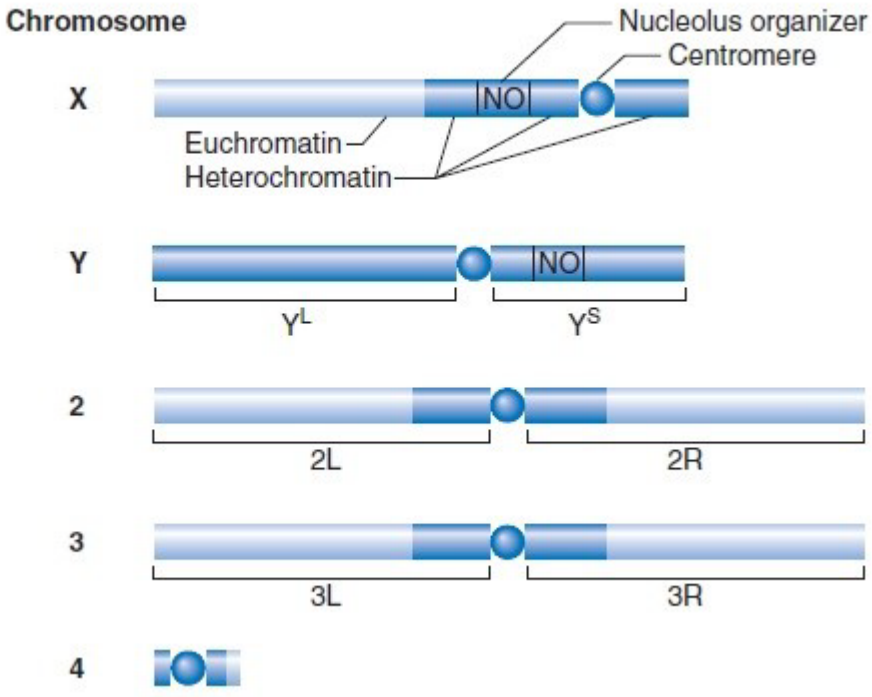

Fig.1: The chromosomes of Drosophila melanogaster. Source: [8] Reference D, Genetic portrait of the fruit fly. (http://biologii.net/files/ngu/Reference_D.pdf)

1.1.2 Meiotic Recombination in Drosophila and the power of Balancer Chromosomes

In Drosophila, crossing-over is absent in males, since they are the heterogametic sex. This is beneficial for the researcher, as we know that if a gene on a chromosome is inherited through the male parent, it will surely be present in the offspring without being recombined away on the other chromosome. In females, each main chromosome arm undergoes several crossovers during meiosis, with no crossing-over taking place in the 4th chromosome or in the heterochromatic regions of all chromosomes. Meiotic recombination is a driver of genetic diversity, which allows recombination of mutations onto the same chromosome and simulation of several disease models, but sometimes it could be a threat. In order to avoid unwanted recombination, balancer chromosomes can be used, which typically contain a dominant marker that can be followed through the crosses, and a lethal recessive allele that does not allow the survival of homozygotes. Balancer chromosomes can also be associated with some physical marker (e.g. eye color or hair length). They are

important for stock maintenance, because they do not allow meiotic recombination between homologous chromosomes and hence they prevent the appearance of genotypes that differ from the parental generation.

1.1.3 The life cycle of the fruit fly

Drosophila melanogaster has a high capacity for reproduction. Normally, a fertilized female stores sperm from the male in its receptaculum seminis and later lays and fertilizes hundreds of eggs. After ~21 hours at 25℃, the embryo is obtained (Fig.2). Later, the 1st instar larva needs two days to molt into the 2nd and 3rd instar larva, which then keeps on feeding on the bottom of the vial for one more day, during the so called “foraging stage”, and eventually leaves the food and starts wandering (wandering stage) before it pupariates. While in the pupal stage, histolysis and metamorphosis occur, meaning that the pupal larval organs degenerate and are re-structured into the adult fly organs (Fig.3). Finally, in a total time frame of 10 days, the adult fly emerges. Under 25℃, virgin females can be collected within 8 hours, the time needed for sexual maturation of the males. In the case of 18℃, all the times mentioned should be doubled.

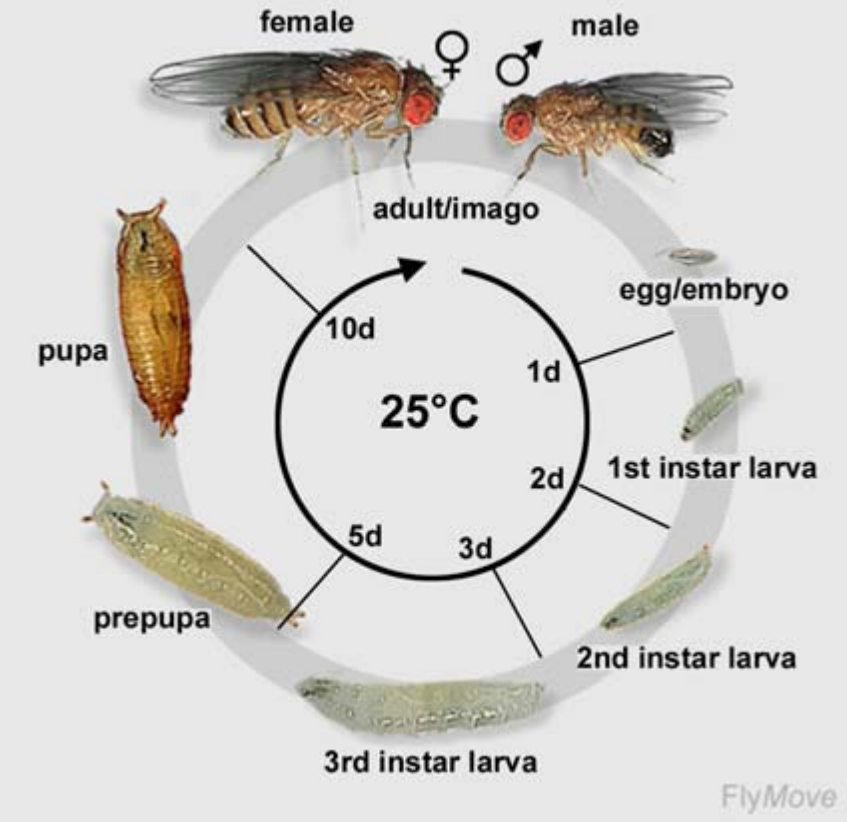

Fig.2: The life cycle of Drosophila melanogaster. Source: [1] A. Prokop - A rough guide to *Drosophila* mating schemes.

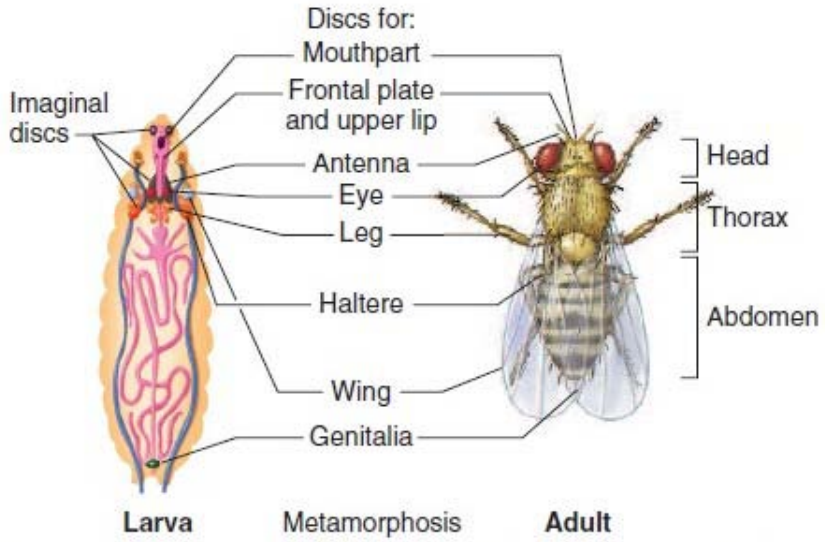

Fig.3: The metamorphosis of larva to the adult fly. Source: [8] Reference D, Genetic portrait of the fruit fly.

Later, the distinguishing between males and females is based on certain physical appearance criteria (Fig.4). First of all, a characteristic that only males exhibit is sex combs on the front legs. Secondly, females are larger than males, and have distinct black dorsal stripes, whereas in males these stripes are fused, resulting in a black spot. Finally, the anal plates of males are more complex and darker and do not exhibit the pin-like extension that females' anal plates have.

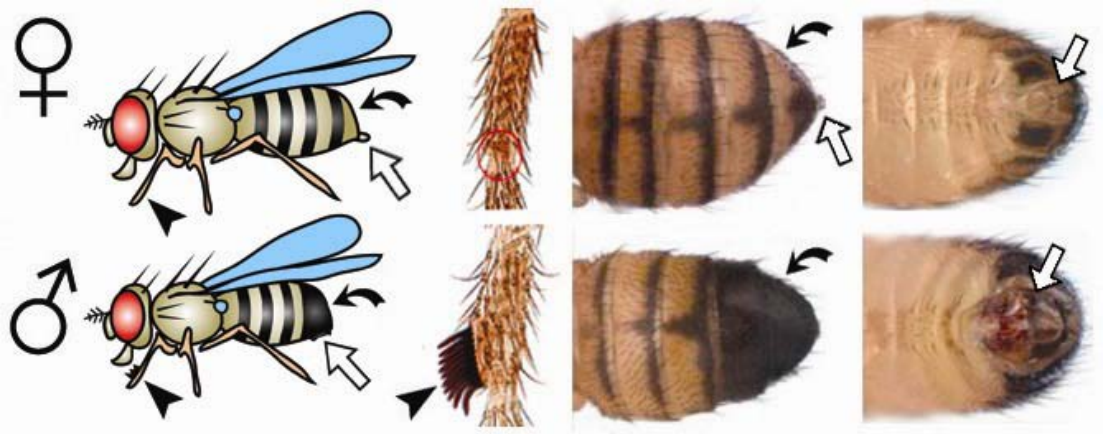

Fig.4 The anatomical differences between males and females. Source: [1] A. Prokop - A rough guide to *Drosophila* mating schemes.

Additionally, for the selection of virgin females, another criterion of virginity is a dark green spot in the female's abdomen (Fig.5). Concerning the maintenance of fly stocks, the flies are kept in glass vials closed with cotton wool, and their food consists of corn flour, yeast, agar and glucose (Fig6).

Fig.5 Black spot in the abdomen, the indication of the fly's virginity. Source: *Drosophila Melanogaster* and Mendelian Genetics, by Pete Geiger, University of Arizona. (The Berg Lab, University of Washington, Drosophila oogenesis).

Fig.6: Flies in the laboratory. Source: Genetics, Key Stage: KS4/5, School of Life Sciences, Warwick, School of Life Sciences, Gibbet Hill Campus, The University of Warwick, Coventry, CV4 7AL.

1.1.4 Advantages of using Drosophila as a model organism

Drosophila is such a successful model due to its numerous advantages, with one of them being its simply organized genome. Moreover, the adult fly emerges within 10 days, so experiments can progress fast and keeping the flies in the laboratory is cheap and easy, so many independent stocks can be maintained. Also, a variety of methods can be used to manipulate the genes of the flies or to create clones and the small size and low complexity of the fly's organs, give the possibility to easily observe tissues and cells. Finally, maybe one of the most crucial advantages of the

fruit fly, is the tremendous amount of knowledge and resources existing, after a century of its usage in the laboratory.

1.2 OVERVIEW OF THE SIRTUIN PROTEINS

1.2.1 Functions of sirtuins

Over the years, many researchers have focused on sirtuins, and nowadays there is an unquestionable link between sirtuins and longevity, since sirtuin activity protects from age related diseases. (Sirtuins: Guardians of mammalian healthspan by William Giblin Mary E. Skinner and David B. Lombard)

Sirtuins are proteins involved in biological pathways of archaea, bacteria and eukaryotes. They are the homologs of the yeast Sir2 gene, with Sir2 meaning Silent-mating-type information regulation 2, which regulates gene silencing in yeast. Not all sirtuins, but some of them have the important function of protein deacetylase, while others are ADP-ribosylases, acylases or lipoamidases. There are seven members of the sirtuin family in mammals and they are all NAD^+ -dependent for their enzymatic activity.

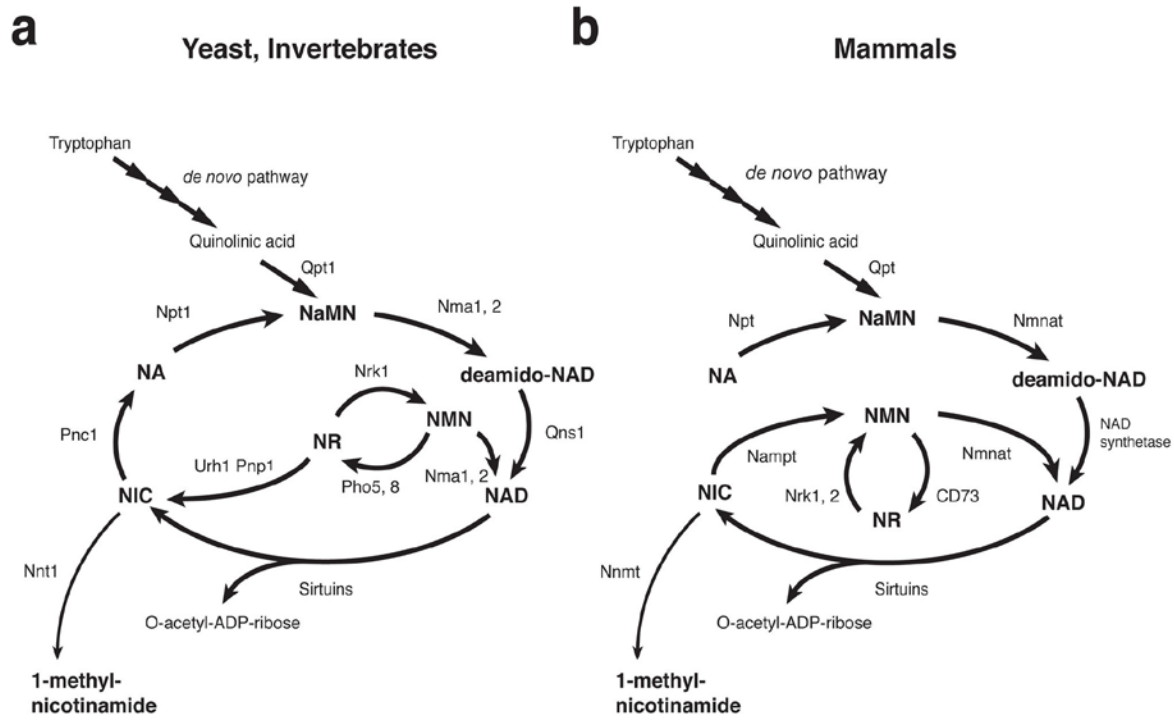

Fig.7: Biosynthesis of NAD^+ in a) yeast and invertebrates and b) mammals. (Source: [13] NAD^+ and Sirtuins in Aging and Disease by Shin-ichiro Imai and Leonard Guarente.)

In Fig.7, the participation of sirtuins on breaking NAD^+ into nicotinamide and O-Acetyl-ADP-ribose is shown in yeast, invertebrates and mammals. NAD^+ can be synthesized by the contribution of three molecules, which are Tryptophan (Trp), nicotinamide (NIC) and nicotinic acid (NA). In this process, nicotinamide riboside (NR) can be converted to nicotinamide mononucleotide (NMN) via NR kinases (Nrk) 1 or 2, which increases the levels of NAD^+ in the cell, meaning that supplementing the NAD^+ precursors (NMN and NR), produces more NAD^+ and causes reactivation of Sirtuins. Apart from the function of Sirtuins as nicotinamide adenine dinucleotide (NAD^+)_dependent deacetylases, they can also function as mono-ADP-ribosyltransferases, deacylases and lipoamidases. In general, Sirtuins regulate cellular homeostasis and they have been associated to genomic instability and metabolic alterations, typical characteristics of cancer, but also to aging and neurodegenerative diseases, metabolic dysfunction, inflammation and even cardiovascular disease.

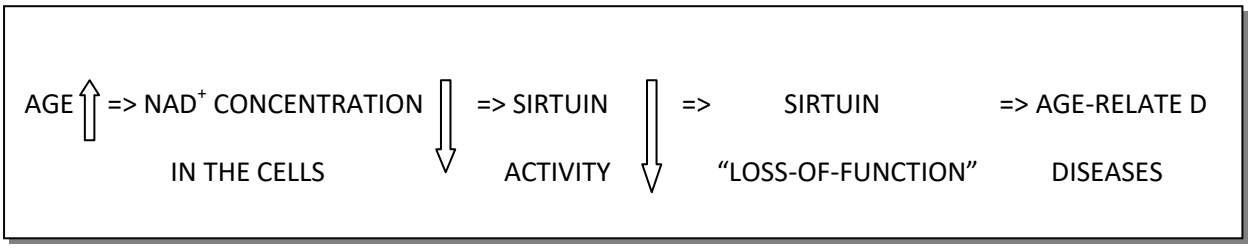

Fig.8: the dependence of sirtuin activity on age, leading to age associated diseases.

1.2.2 Localization and Structure

Mammals have seven sirtuins (SIRT1-SIRT7) whereas in Drosophila there are five members of the sirtuin family present. These can be localized in three different regions of the cell, the nucleus, the cytoplasm or the mitochondria. All sirtuin proteins/enzymes contain a core region of catalytic activity composed of approximately 275 amino acids that code for the NAD^+ binding and zinc binding domain, but they differ in their N- and C- termini.

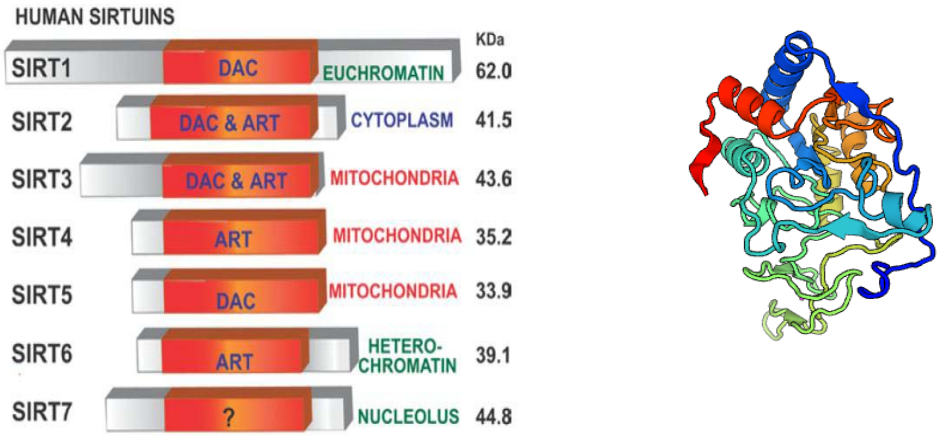

Fig.9: The human sirtuins are enzymatically mainly NAD^+ dependent protein deacetylases (DAC) or ADP-ribosyl transferases (ART) and they have different localization. (Source: [9] Sirtuins in mammals: insights into their biological function by Shaday MICHAN and David SINCLAIR).

On the right the typical NAD binding Rossmann fold is shown. (Source: PDB Protein Data Bank)

1.2.3 Specific functions of sirtuins

SIRT1, mainly localized in the nucleus, is involved in many cellular processes like lipid metabolism, deacetylation of substrates in glucose metabolism to increase gluconeogenesis and hinder glycolysis, responding to DNA Double Strand Breaks (DSBs) and promoting DNA repair, thus resulting in accelerated tumorigenesis when missing. It is the most studied enzyme from all the sirtuin family due to its role in lifespan extension [14], [15]. SIRT7, localized in the nucleolus and nucleus, is a histone deacetylase. It also participates in lipid metabolism, but interestingly in the case of cancer, SIRT7 overexpression seems to protect cancer cells, suggesting a potential oncogenic role of SIRT7 [15]. SIRT3, localized in mitochondria, is a mitochondrial deacetylase regulating production of Reactive Oxygen Species (ROS) and enzymes of metabolism, so the loss of SIRT3 leads to high levels of ROS inside the cell [14], [15]. SIRT4, also located in the energy factories of the cell, is associated with glutaminolysis of the TCA cycle and ammonia production, a by-product of glutaminolysis (Fig.10) [15]. SIRT5, located in the mitochondria, participates in the urea cycle and detoxification of ammonia. It also exhibits NAD^+-dependent desuccinylase and demalonylase activity [15].

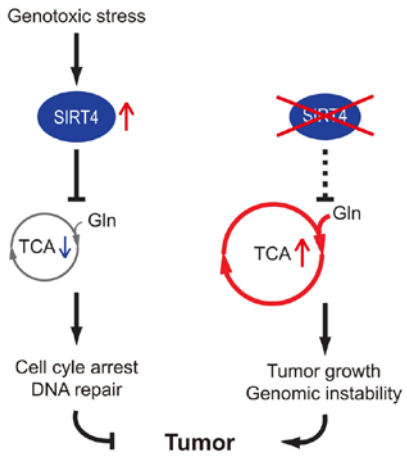

Fig.10: The role of SIRT4 in TCA cycle.
Source: [15] Sirtuins in Cancer: a Balancing Act between Genome Stability and Metabolism Seung Min Jeong1,2,*,and Marcia C. Haigis3,*.

SIRT2, mainly localized in the cytoplasm, acts as a regulator of the cell cycle [15]. For example, it affects acetylation of H4K16 during mitosis and has an effect on the compaction of chromatin, resulting in a prolonged cycle when over-expressed and cycle arrest when down-expressed. When SIRT2 function is completely lost, defects

of mitosis appear, eventually leading to cell death. So, SIRT2 is an important Sirtuin for genome stability, since it participates in the control of the cell cycle and in the replication of DNA. Apart from the crucial role of SIRT2 in genomic integrity, its critical function is obvious in metabolic homeostasis as well, during modulation of glucose and lipid metabolism, with SIRT2 deacetylating the enzyme Phosphoenolpyruvate Carboxykinase (PEPCK), thus promoting gluconeogenesis. Additionally, when SIRT2 is transcriptionally repressed, it may result in development of obesity.

SIRT6, mainly localized in the nucleus of the cell, like SIRT1 and SIRT7 [14], [15]. SIRT6 has two of the main functions of sirtuins, which means that it acts as an ADP-ribosyltransferase and as a NAD^+ -dependent deacetylase, leading to premature aging when missing, because of telomere malfunctions. The unquestionable role of SIRT6 in genomic stability and in the DNA Damage Response (DDR) that the cells have developed to avoid "genomic imbalance", is confirmed by SIRT6 KO mice being very sensitive to DNA damage [15]. DNA repair is also a part of the protein's activity, as it is being promoted by SIRT6. Moreover, concerning cell metabolism, SIRT6 acts as a modulator of glucose metabolism by actively participating in gluconeogenesis (SIRT6 inhibits gluconeogenesis in the liver by suppressing the expression of gluconeogenic genes and inactivating the transcription factor PGC-1α, avoiding the danger of diabetes) and resulting in increased levels of glycolysis when its function is lost. Being a tumor suppressor is also added in the list of the protein's functions, after recent studies proving that SIRT6 KO mice exhibit enhanced carcinogenesis. In addition to these roles, SIRT6 also affects fat metabolism, with SIRT6 overexpression protecting against accumulation of fat. Finally, SIRT6 seems to protect mammals even from heart disease, like cardiac hypertrophy (CH) and recent studies have even suggested an anti-inflammatory role of SIRT6.

In Drosophila melanogaster, researchers have studied mutations of the sirtuin genes Sirt1, Sirt2 and Sirt4, but none of them was shown to be lethal on its own. Mutations for Sirt6 and Sirt7 genes were created by members of Krejci lab, but they turned out to be also viable with no obvious phenotypes. The next logical step is to combine more than one mutations together creating double, triple, quadruple and penta mutants, and test their lethality or other phenotypes. The goal of my thesis fitted into this scheme.

Sirtuin	SIRT1	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
Class	I.	I.	I.	II.	III.	IV.	IV.
NAD^+ K_m value	94-96 μM	83 μM	880 μM	35 μM	980 μM	26 μM	N.A.
Localisation	Nucleus Cytoplasm	Nucleus Cytoplasm	Mitochondria	Mitochondria	Mitochondria (Cytoplasm, Nucleus)	Nucleus	Nucleolus
Molecular function	Deacetylation (S) Decrotonylation	Deacetylation (S) Decrotonylation	Deacetylation (S) Decrotonylation ADP ribosilation	Deacetylation (W) ADP ribosilation Lipoamidation	Deacetylation (W) Demalonylation Desuccinylation Deglutyrylation	Deacetylation (W) ADP ribosilation Demyrystoylation	Deacetylation
Null phenotype	Developmental defects, lethal in some backgrounds	Normal development	Normal development	Normal development	Normal development	Premature ageing	Smaller size, short lifespan, heart defects
Biological function	Known substrates						
Histones	H1, H3, H4	H3, H4	H3, H4	N.A.	N.A.	H3	H3
Chromatin remodeling	p300, HDAC1, MOF, TIP60, Suv39h1	p300					
DNA repair	Ku70, XPA, NBS1, PARP1		Ku70		CtIP, PARP1, DNA pol-β		
Stress response	p53, FOXO1, FOXO3, FOXO4, NF-κB, HIF1A, HIF2α, c-FOS, C-JUN, E2F1	p53, FOXO1, FOXO3A, NF-κB, c MYC,	p53, FOXO3A, SOD2			HIF1A, NF-κB	p53
Metabolism	ACS1, PGC-1α, CXR, FXR, SREBP-1c, LKB1, TORC1, TORC2, eNOS	PEPCK1	LCAD, HMGCS2, IDH2, GDH, SOD2, OXPHOS Complexes (I, II, III, V)	GDH, PDH, MCD	CPS1, SDH, PDH, Glutaminase, Urate oxidase,		
Others	NICD, RIP1, Cortactin, RAR-β, TAU	α-Tubulin, CDH1, CDC20	Cyclophylin D				RNA pol I

Table 1:Overview of basic information about mammalian sirtuins and some of their substrates. Underlining in molecular function refers to efficiency of the reaction type: double underlining - high efficiency, single underlining - average efficiency, no underlining – low efficiency (adapted from Haigis & Sinclair 2010; Nakagawa & Guarente 2011; Martínez-Redondo & Vaquero 2013; Cantó et al. 2015; Chen et al. 2015; Pougovkina & de Boer 2016; Yang & Sauve 2016, Horvath, 2017).

Name of the sirtuin (SIRT)	Functions	Subcellular localization
SIRT1	Cellular longevity, tumor promoter, tumor suppressor, inflammation, oxidative stress, glucose homeostasis, cell adhesion, cell metabolism	Nucleus
SIRT2	Mitotic check point, tumor promoter, tumor suppressor	Cytoplasm/nucleus
SIRT3	Tumor suppressor, tumor promoter, mitochondrial oxidation, stress responsive deacetylase	Mitochondria
SIRT4	Glutamine catabolism, TCA cycle, ADP-ribosylation	Mitochondria
SIRT5	Glycolysis, cancer metabolism, fatty acid oxidation, lysine succinylation, malonylation, glutarylation	Mitochondria
SIRT6	Telomere and genome stability, DNA repair, inflammation, glucose homeostasis	Nucleus
SIRT7	Ribosomal production	Nucleus (nucleoli)

Table 2: Certain functions of sirtuins. Source: [18] Sankarathi Balaiya,1,2 Khaled K. Abu-Amero,1,3 Altaf A. Kondkar,3 and Kakarla V. Chalam1, Sirtuins Expression and Their Role in Retinal Diseases, Oxidative Medicine and Cellular Longevity

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1.3 CRISPR / CAS9 TECHNOLOGY

1.3.1 Principle of the Technique

CRISPR is an acronym for “Clustered regularly interspaced short palindromic repeats”. CRISPR are segments of prokaryotic DNA that contain short and repetitive sequences. Since they are palindromes, the nucleotide sequence must be the same in both 5’->3’ and 3’->5’ direction. Additionally, each palindromic repeat is followed by the so called “spacer DNA”, which are small segments of DNA that resulted from exposure to alien DNA in the past (e.g. exposure of the bacterium to a phage or a plasmid). The third “component” of this system is the Cas (CRISPR-associated system) genes, that can be observed along the side of the CRISPR sequences. The last “component” of the system is the Photospacer Adjacent Motif (PAM) DNA sequence, a 2-6 bp sequence that follows the sequence marked for cutting by Cas9 Nuclease. It is necessary for the correct targeting of the DNA sequence by Cas9. The PAM sequence is not a component of the bacterial genome, but rather a residue of virus or plasmid past invasion. The role of the CRISPR/Cas9 system is to offer immunity to foreign genetic material (e.g. phage DNA trying to invade a bacterium), so it is used as a defense system in bacteria. Out of all the bacterial genomes that have been sequenced up to date, 40% of bacteria contain CRISPRs, whereas in archaea this number goes up to 90%.

For the purposes of genomic engineering and manipulation of genetic material by removal of present genes and addition of new ones, the CRISPR RNA-Guided Cas9 Nuclease system can be used, where Cas9 nuclease is being delivered into the cell to specific DNA sequences by the short synthetic guide-RNA. There, it can bring about position-specific DSBs in the eukaryotic genome. As a result, the CRISPR RNA/Cas9 system can be successfully used in Drosophila melanogaster, or other species, for purposes of genome engineering and modifications in the germline that will be passed on to the offspring.

In the CRISPR type II system from Streptococcus pyogenes, which is a four component system, two small RNA molecules are included, the CRISPR RNA (crRNA), that has a complementary sequence to the foreign DNA, and the trans-

activating CRISPR RNA (tracr RNA). Both of them interact with the Cas9, in order to direct the sequence specific cut of the DNA. After researchers realized the great potential of this technique, it has been further modified into an easier two-component system, by the fusion of the two RNA molecules into a single “guide RNA”, that can lead the Cas9 to the correct position for the cleavage.

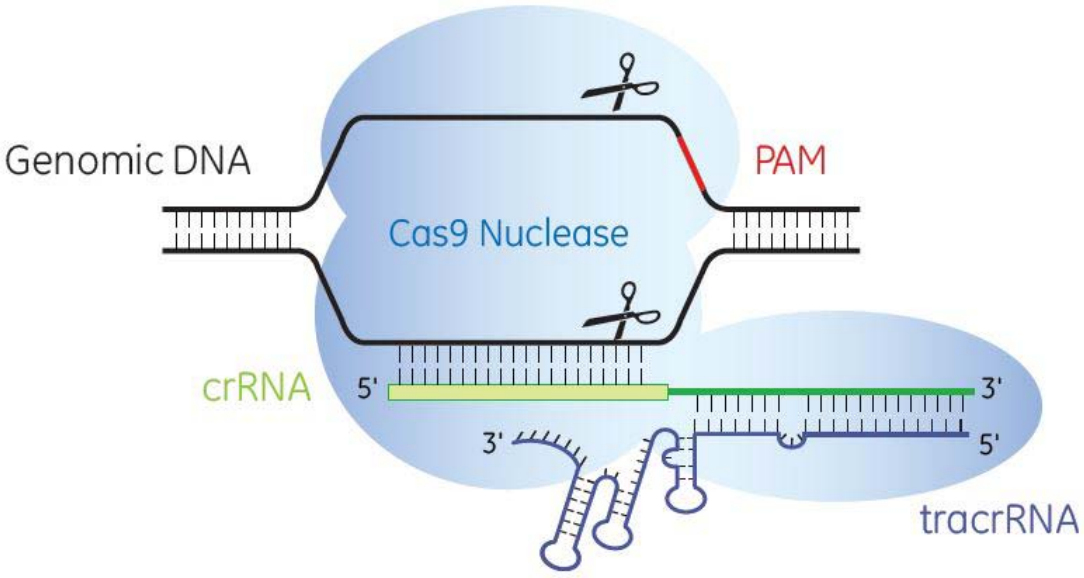


Fig.11: Basic principle of the CRISPR/Cas9 system. The components of the system are shown as a simple scheme, explaining the components as described above. (Source: CRISPR Guide RNA, GE Healthcare Dharmacon Inc.)

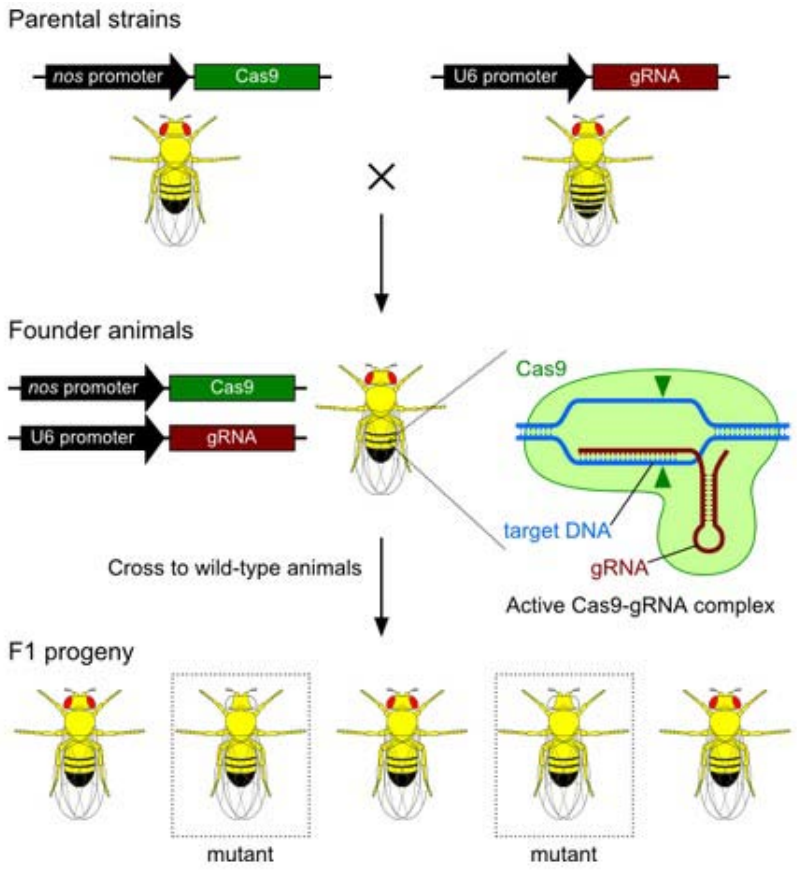


Fig.12: The use of Crispr/Cas9 system for the introduction of mutations in Drosophila melanogaster. Similar approach was used in the project, to obtain the Sitr2,Sirt6 and Sirt2,Sirt6,Sirt7 mutants. (Source: [6] Kondo S[1], Ueda R., Highly improved gene targeting by germline-specific Cas9 expression in Drosophila. Genetics. 2013 Nov;195(3):715-21. doi: 10.1534/genetics.113.156737. Epub 2013 Sep 3)

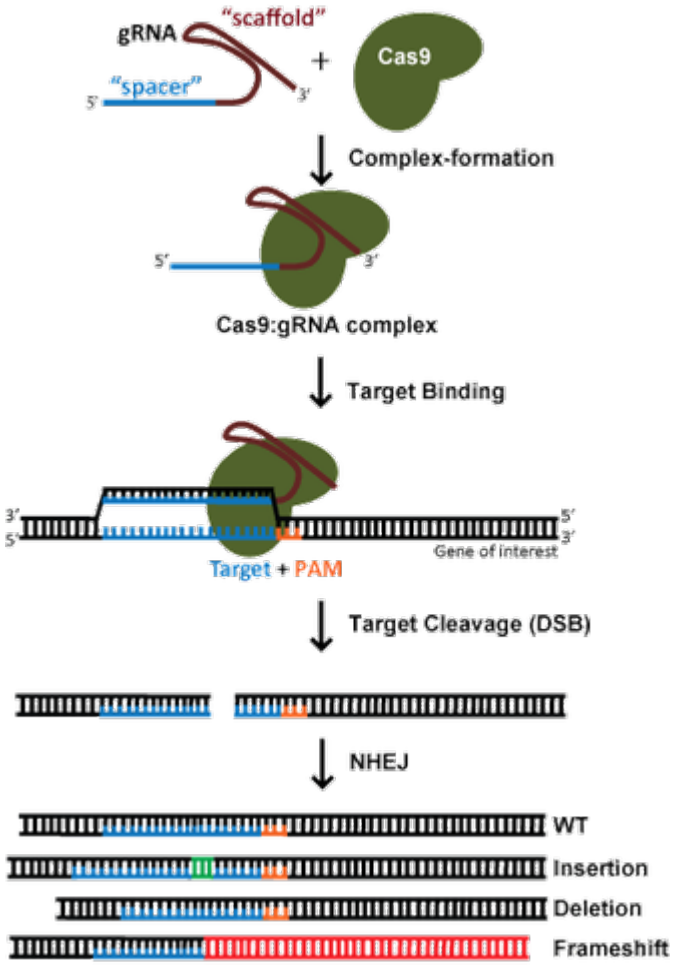


Fig.13: General description of genome engineering by Crispr/Cas9 technology. The Cas9- gRNA complex is formed and spots the gene of interest, locating itself next to the PAM sequence and starting editing the genome. The DSBs are then repaired by NHEJ (non-homologous end joining) and the possible outcomes are: wild-type (WT, meaning no mutation created), insertion of new piece of DNA, deletion of a part of the gene and frameshift (insertions or deletions [indels] of a number of DNA monomers which is not divisible by three, leading to a whole new set of codon triplets). (Source: CRISPR/Cas9 Guide, addgene, The non-profit plasmid repository.)

Crispr / Cas9 system can be used in many different ways in genome modification experiments. An example of use of Crispr / Cas9 in Drosophila, is the important role that it plays in activation of transcription [2]. Scientists have proven that transcription is inhibited when the gRNA binds downstream of the transcription starting site. This is promising for using the Crispr / Cas9 system in overexpression studies, in a simple and easily applicable way. So, Crispr is not used only for making deletions from the genome, but it is much more than that. It can be used for targeted

mutagenesis or insertion of a certain DNA piece into specific regions in the genome (e.g. tagging an endogenous protein with GFP).

1.3.2 A little bit of History

CRISPRs were first discovered in Escherichia coli back in 1987, when Y. Ishino accidentally cloned a part of a CRISPR together with the gene of his interest and saw that it was composed of unusual interrupted clustered repeats, but at that time the function of these sequences was not known yet. In 1993, researchers that were studying Mycobacterium Tuberculosis also reported these repeated sequences, but it was not until 2001 when F. Mojica together with R. Jansen described the function of these sequences and proposed the name CRISPR. Prior to that, F. Mojica had identified the palindromic repeats in 20 different species of microorganisms.

The discovery of Cas9 and PAM originated from A. Bolotin and his studies on the bacterium Streptococcus Thermophilus. He observed the cas genes and that one of them was encoding the large nuclease Cas9. He also noticed that all spacers have the so called PAM sequence at their end, playing the role of the target component. The proof that CRISPR system can have DNA as its target molecule and not only RNA came from L. Marraffini and E. Sontheimer, with Moineau coming in 2010 to confirm and add that DSBs are created three nucleotides away from PAM sequence and only one protein (Cas9) is needed. Later, in 2011, E. Charpentier showed that tracrRNA together with crRNA, guide the Cas9 to the target sequence. By June 2012, it was known that these two types of RNA can be fused and give the single guide RNA. Finally, the ability of CRISPR system to manipulate eukaryotic genomes was demonstrated by F.Zhang in 2013.

1.3.3 Advantages and Disadvantages of the Method

The Crispr/Cas9 technology is a relatively new method as it has been used for only a few years in the field of Biology. However, conclusions on the advantages and disadvantages of this genetic engineering method can already be drawn. First of all, Crispr/Cas9 is a very simple technique. Its simplicity is accompanied by its high

efficiency and precision. For example, in mice, the use of embryonic stem cells can be avoided, since the Crispr technique can be applied directly to the mouse embryo. In Drosophila, the guide RNA can very easily guide the Cas9 to the target DNA and introduce deletion on the desired position by simply crossing the appropriate fly stocks. Nowadays, with Bioinformatics as a powerful tool in hand, researchers can readily synthesize guide RNAs with the most efficient sequences. This means that the success of the technique is almost guaranteed, and a very high percentage of the progeny is expected to carry the desired deletion. On the other hand, the efficiency is much lower when Crispr is used to insert a certain sequence into specific part of the genome, like when tagging an endogenous protein. Nevertheless, the efficiency is still higher than the older techniques like homologous recombination. Probably the most apparent disadvantage of the Crispr system is the possibility of introducing mutations at regions other than the primarily designed target of interest, so called off-target effect. This is a problem with more complex genomes, but concerning Drosophila, the off-target effects are minimal.

2. AIMS OF THE THESIS

In Drosophila, there are five types of the sirtuin genes present and none of them, when mutated, is lethal on its own and none of them shows any obvious developmental or physiological phenotypes (published data for Sirt1, Sirt2 and Sirt4 and unpublished results from our lab for Sirt6 and Sirt7). This is contrary to mammals where Sirt1 is lethal in majority of genetic background and Sirt6 and Sirt7 display developmental defects or phenotypes apparent shortly after birth. The lack of phenotypes in sirtuin mutants may be an indication of functional redundancy between the five sirtuins. In this case, combining more than one sirtuin mutated gene in one animal could lead to a more severe phenotype. This has so far not been explored, not in mammals or in Drosophila. The ultimate goal in Krejci laboratory is to create a "superfly" with all of the five sirtuins deleted and test its lethality or characterize its phenotype under stress conditions)e.g. food stress, temperature stress, oxidative stress). If this "superfly" turns out to be lethal or have interesting phenotype, it will be desirable to create combinations of sitruin mutations to identify which of the combinations are lethal, or if, for example, any single sirtuin member is able o substitute for the function of the other remaining members.

Three if the five Drosophila sirtuins are located on the III. chromosome (Sirt2, Sirt6 and Sirt7). Therefore, before being able to make the "superfly" or various combination of sirtuin mutants, there was the need to create a fly that would bear Sirt2, Sirt6 and Sirt7 mutations on the same chromosome.

The main aim of my thesis was to create a fly that is a mutant for two sirtuin genes, Sirt2 and Sirt6. This was achieved by using two different approaches:

1. Meiotic recombination of existing Sirt2 and Sirt6 mutations on the same chromosome, and

2. Crispr/Cas9 technology in order to introduce de novo mutation in Sirt6 on the background of Sirt2 mutant.

Next goal was to create a fly that is a mutant for the three sirtuin genes on the III. chromosome, Sirt2, Sirt6 and Sirt7. This was be achieved by using the Crispr/Cas9

technology in the same way, to introduce de novo mutation in Sirt7 on the background of the already obtained Sirt2,Sirt6 mutant.

3. METHODS

3.1 FLIES

All the flies were kept in glass vials with cotton wool on a food with corn flour, yeast, glucose and agar as ingredients (corn flour 160g, yeast 30g, glucose 150g, agar 9g and water 1.9L) with some extra dry pieces of yeast on the top of the food mixture. Flies were kept under ambient conditions or at 25℃ incubators. Fly virgins were collected every <8 hours during the day if left at room temperature (or 25℃) and after <18 hours when left at 18℃. Extra confirmation for virgin females was their pale appearance and a dark green spot in their abdomen, which is their last food as larvae. The flies were always kept in clean vials and fresh food, in order to keep them healthy and viable. Water was applied regularly to not let the food dry out.

These are the strains of Drosophila that I used (numbers in brackets indicate the chromosome where the gene is located or the transgenic element inserted):

- Sirt2 mutant (III.)
- Sirt6 mutant (II.)
- TM3/TM6 (double balancer stock)
- If/cyo ; TM3/TM6 (double balancer stock)
- Sp/cyo; TM3/TM6hu (double balancer stock)
- Sirt6 gRNA (II.)
- nos-Cas9/cyo
- Sirt7 gRNA (II.)

3.2 VERIFICATION OF GENOTYPES

Before starting the experiments, the flies' genotypes had to be verified to ensure that the Sirt2 mutant and the Sirt6 mutant flies needed were indeed carrying these mutations. Similarly, the final recombinant flies were to be verified as well.

Verification of the Sirt2 mutations:

For the verification of Sirt2 mutant, polymerase chain reaction (PCR) was used after extraction of DNA from a single fly of a stock. Then, electrophoresis was performed to view the DNA fragments on the agarose gel and conclude if the mutation is present or not. The primers were designed so as they span the Sirt2 gene. In the wild type (WT) fly, the DNA fragment's size should be 1.4kb, whereas if a Sirt2 mutation was present, the DNA fragment should have a size of 0.2kb.

The sequence of the primers was the following:

Sirt2 verification primers

CG5085-C1:
GCCCCAGGCTAGTCTAAATAG
CG5085-C2:
GAAAGAAAGCTCGCGCTATTAG

The following protocol (Protocol 1) was used for all the DNA isolations, PCRs and Electrophoreses:

PCR and Electrophoresis:

a. Extraction of DNA from a single fly for PCR analysis:

1. One male fly is taken and put in 50ul of SB (squashing buffer) containing 0.5ul of proteinase K (20mg/ml stock).

2. The fly is mashed until the solution becomes cloudy.

3. Incubation for 30min at 37℃ (in the heat block).

4. Incubation for 2min at 95℃ (in the heat block).

5. 1min spin at 14000 rpm, 0.5ul of the extracted DNA are taken for the 20ul PCR reaction.

b. PCR using OneTaq DNA Polymerase (NEB):

(one reaction contains 20ul)

The master stock is created by mixing the following in one mix for all samples tested:

- 14.5ul DEPC H_2O
- 4ul buffer (OneTaq standard reaction buffer)
- 0.4ul dNTP (10mM each)
- 0.4ul of the 5' primer (10μM)
- 0.4ul of the 3' primer (10μM)
- 0.2ul of Taq Polymerase

The master stock is always made by mixing the components in this order. Everything is kept on ice all the time and polymerase on a cool block. 19.5ul of the master mix are mixed with 0.5ul of extracted DNA in a thin walled PCR tube. The mixture is spinned briefly and placed in the PCR cycler with heated lid and block temperature of 94°C. (Specific programs are chosen for different screenings).

c. Electrophoresis:

5ul of the PCR product are mixed with 1ul of 6x loading buffer and run on a 1.5% agarose gel (1.5g agarose, 100ml TBE buffer 0.5x, 3ul EtBr). Along the side of the extracted DNA, a DNA ladder is also run for the determination of the DNA fragments' size.

For the verification of the Sirt6 mutant we could not use the PCR method since these flies are not constructed by deleting the coding region but by introducing a mutation by the transcription start site (by Crispr) that causes a frame shift I translation. Instead individual heterozygous flies were tested by using the enzyme T7 endonuclease, that digests dsDNA in positions of mismatch along the double strand where there is a loop. We first amplified by PCR the region around the site of Sirt6 mutation, using flies with TM3 balancer. If the non-balancer mutant chromosome contained irt6 mutation, the resulting PCR product mixture would contain both the wild type and mutated PCR product. When they hybridize together in random fashion there will be significant amount of dsDNA products that contain one strand of wild

type and one strand of mutated Sirt6 region (mismatch). These will be recognized by T7 endonuclease and cleaved. Afterwards, the digested PCR product was run on electrophoresis and if the mutation was absent, only one band was obvious (the original one, non digested), whereas if the Sirt6 mutation was present, two smaller bands appeared, as digestion had occurred. As positive control we used Sirt6 mutant that has been sequenced before and it was certain that the mutation was present. After finding a mutation "candidate" fly, its DNA was isolated again but this time from a homozygous fly, the same PCR region was amplified and sent for sequencing. This way we knew the exact sequence of the mutation present.

Verification of Sirt6 or Sirt7 mutations:

The following protocol (Protocol 2) was followed for every verification of Sirt6 mutations:

a.DNA Isolation and PCR (as described above) followed by DNA sequencing:

PCR performed using the Sirt6 primers:

Sirt6 crispr ver s: ACGTTGCAGGGATTTTTGAC

Sirt6 crispr ver a: TTTGTAGCGTTACGGATACGG

Sirt7 crispr ver s: GGAAGCGAGTCATTCCTACG

Sirt7 crispr ver a: GCTTCTTGGTCGTCTTCACC

b. Digestion of purified PCR product by T7 endonuclease:

1. The PCR product is transferred to 1.5 ml a eppendorf tube, 60ul DEPC water are added and 8 ul 3M Sodium Acetate. The mixture is mixed by short vortex.
2. 250ul 100% ethanol (molecular biology grade) are added, and by inverting a couple of times the liquids are mixed. The mixture is stored at -20℃ overnight or -80℃ for some hours.
3. Centrifugation for 20 minutes at 20000rpm at 4℃.
4. The ethanol is carefully poured away, while the DNA pellet should remain attached to the side of the tube.
5. cca 0.5ml of 70% ethanol are added ad the liquids are mixed by inverting the tube.
6. Spinning for 10 minutes at 20000rpm at 4℃.

7. The ethanol s carefully poured away and the pellet should remain attached to the side of the tube.

8. A short spin follows for a few seconds and then the rest of ethanol is taken away by a yellow tip.

9. The pellet is left to dry at room temperature for a few minutes until all ethanol evaporates.

10. 16ul DEPC water is added in the tube and 1ulis used to measure concentration and purity.

11. The working dilution of T7 endonuclease is prepared from the stock solution, with concentration of 2 units per ul:

4.4 ul DEPC water

1.1 ul endonuclease (10units/ul)

All the work is performed on ice and very fast, so that the stock is not sitting on ice for too long before adding to the reaction mixture.

12. The following reaction is prepared:

* 12.5 ul DEPC water

* 5ul PCR product

* 2ul buffer 2

* 0.5ul T7 endonuclease working solution (1u)

The reaction is incubated at 37℃ for 2 hours, so that the digestion takes place.

c. Electrophoresis:

Loading dye (4ul) is added, mixed with the DNA and stored on ice. 10ul are run on a **2%** gel.

Similar approach was used for verification of Sirt7 mutants. These have been created the same way as Sirt6 mutants (by Crispr) and therefore contained only short deletion that could not b verified by conventional PCR electrophoresis.

3.3 CROSSING SCHEMES

3.3.1 Creating double mutant for Sirt2+Sirt6 by meiotic recombination

The location of Sirt2 gene on the III. chromosome is at position 92E3-92E3 and the position of Sirt6 is 86A2-86A3. Therefore, there was an option to create the double mutant by meiotic recombination, although with not very high expected recombination frequency. The following procedure was followed and these crosses were performed:

1. Sirt2/Sirt2 x Sirt6/Sirt6

2. Around 5-6 Sirt2/Sirt6 virgin females were collected and crossed to 4 TM3/TM6 males to balance.

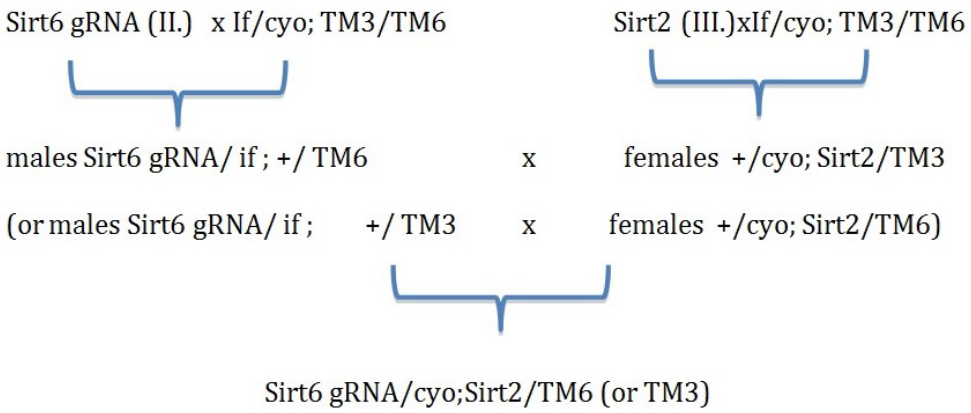
3. From this cross, 100 individual virgin females or males over TM3 or TM6 are collected and crossed to TM3/TM6 males or virgin females.

4. Brothers and sisters were collected and crossed , to establish 100 independent stocks. They should either contain Sirt2 allele (over the balancer), Sirt6 allele, the recombinant of Sirt2+Sirt6 on the same chromosome, or the reciprocal wild type allele.

5.Finally, these 100 stocks were screened by PCR for the detection of the Sirt2 and Sirt6 mutations. First, the presence of Sirt2 was tested and only the Sirt2 positive flies were taken for T7 endonuclease analysis to detect the presence of Sirt6 mutation.

3.3.2 Double mutant for Sirt2+Sirt6 created by Crispr/Cas9 technology

For the creation of the double mutant through Crispr induced mutagenesis on the Sirt2 mutant background the fly carrying Sirt6gRNA and Sirt2 mutation had to be created first (Protocol 3):



Then, the following cross was performed:

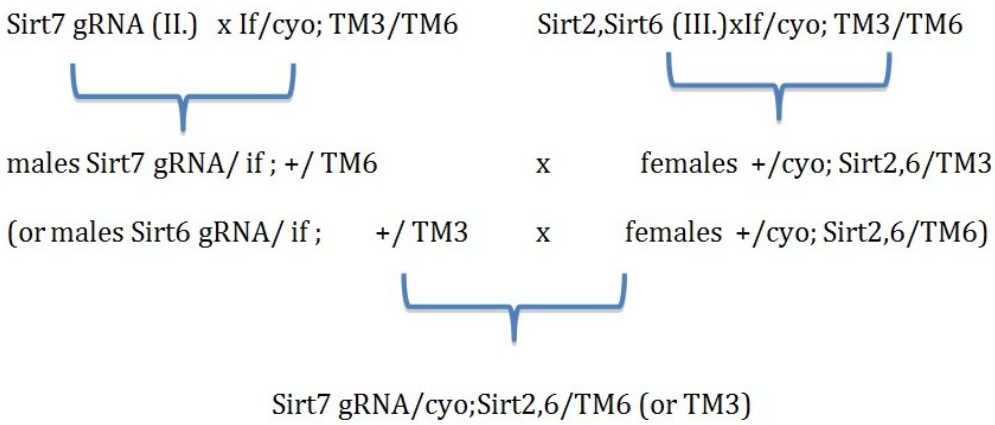
Sirt6gRNA; Sirt2 (homozygous for both chromosomes) x nos-Cas9/cyo

Flies of the **Sirt6gRNA / nos-Cas9; Sirt2/+** were collected. Then, 20-30 individual flies were crossed to TM3/TM6 stock, and independent lines were established.

Finally, the Sirt6 gene was amplified by PCR and tested by T7 endonuclease according to protocol 2. If the mutation was present, the sample was sent for sequencing for further confirmation.

3.3.3 Triple mutant by Crispr/Cas9 technology

For the creation of the triple mutant by Crispr/Cas9 method, the following crosses were performed (Protocol 4):



Then, the following cross is performed:

Sirt7gRNA ; Sirt2,Sirt6 (homozygous for both chromosomes) x nos-Cas9/cyo

Flies of the following phenotype were collected:

Sirt7gRNA / nos-Cas9 ; Sirt2,Sirt6/ +

20-30 individual flies were crossed to TM3/TM6 stock and independent lines were established.

Finally, the presence of Sirt2 mutation was tested by PCR and the presence of Sirt6 and Sirt7 mutations were tested by digestion with T7 endonuclease.

4. RESULTS

4.1 OPTIMIZATION OF THE T7 ENDONUCLEASE DIGEST

Before using the T7 endonuclease for detecting the Sirt6 mutation, optimization of the enzyme had to be performed, to ensure its proper and accurate function, since many factors can easily affect the quality and function of an enzyme. We decided to optimize the concentration of the enzyme in the reaction mixture and the time of incubation at 37°C.

First of all, genomic DNA was isolated from a Sirt6 mutated homozygous male, and a Sirt7 mutated homozygous male (that had normal wild type allele for Sirt6). Then, the DNAs were mixed together in a 1:1 ratio, so as the Sirt6 allele is heterozygous (we could have simply used heterozygous Sirt6 male but mixing the Sirt7 and Sirt6 homozygous was also an option as we had the DNA ready from previous experiments). PCR was conducted with primers Sirt6 (described above) and digested by various concentrations of T7 endonuclease, for different time point of incubation. 10μl of the digest was run on a 2% agarose gel electrophoresis.

In Fig.14, the results from the optimization of the T7 endonuclease can be observed. A digested product would result in the appearance of two additional smaller bands in the gel picture. On the other hand, if no mutation was present, only the original band would appear in the gel. This difference is shown in Fig.14 and Fig.15 below.

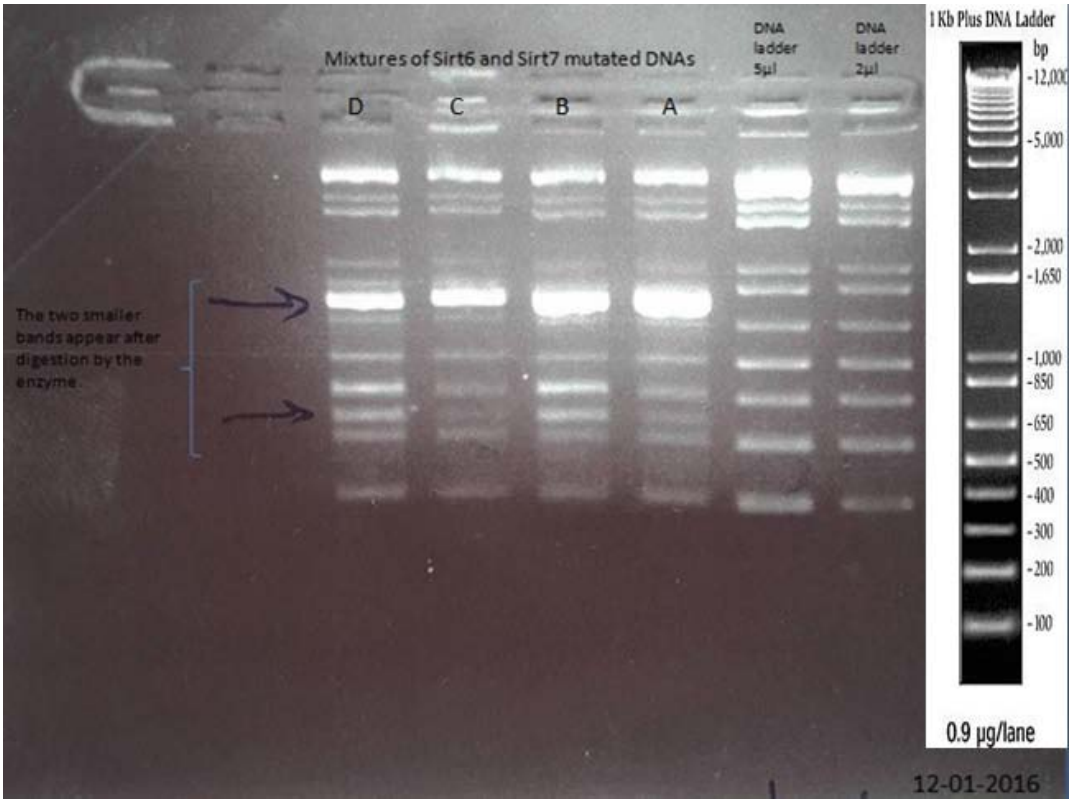


Fig.14 Results of the T7 endonuclease digest of PCR product amplified from region spanning the presumptive Sirt6 deletion. Here we tested the optimal conditions for T7 digest (amount of PCR product and amount of enzyme). When the mutation is present, two smaller bands should appear, which is the case in the picture above. The first arrow indicates the undigested product, the second arrow represents the first of the two fragments of the digested product (250bp). The second digested fragment overlaps with the 400bp ladder band. Apart from using 2 different amounts of DNA ladder (5µl and 2µl), different amounts of T7 enzyme were also used. A: 12.5 ul DEPC water, 5ul PCR product, 2ul buffer 2, 0.5 ul T7 endonuclease working stock (1u). B: 11.5 ul DEPC water, 5 ul PCR product, 2 ul buffer 2, 1.5 ul T7 endonuclease working stock (3u). C: 15 ul DEPC water, 2.5 ul PCR product, 2 ul buffer 2, 0.5 ul T7 endonuclease working stock (1u). D: 14 ul DEPC water, 2.5 ul PCR product, 2 ul buffer 2, 1.5 ul T7 endonuclease working stock (3u). The same time of incubation was used for all 4 reactions (2 hours). By mistake, DNA ladder was mixed with all the samples so the other bands in lanes 3-6 (A-D) are not products of a digest but represent the DNA ladder fragments.

 Fig.15: The comparison between T7 digested and undigested PCR product of the Sirt6 gene. The most left run shows a digested PCR product, indicating the presence of the mutation, whereas the other two runs are undigested, meaning that the mutation is not present.

4.2 THE DOUBLE MUTANT FOR Sirt2,Sirt6 CREATED BY RECOMBINATION

In order to create the fly that is a mutant for two of the sitruin genes, Sirt2 and Sirt6, we decided to test two approaches – meiotic recombination of existing Sirt2 and Sirt6 mutants and Crispr mediated mutagenesis (as described in the Aims and Methods). When recombining the existing Sirt2 and Sirt6 mutations on the same chromosome we established 86 independent lines but we could not easily tell if they contain the Sirt2 and Sirt6 mutations alone or in combination, as there is not phenotypic marker for the Sirt2 or Sirt6 mutations.

We first screened the 86 lines by PCR for the presence of Sirt2 mutation in their genomic DNA. Out of these we selected 46 positive lines and tested for the presence of Sirt6 mutation by T7 digestion (protocol 2). As it is shown in Fig.16, one double mutant (#56) was found already during the first round of screening.

Genomic DNA from homozygous male from stock 56 was isolated, PCR with primers Sirt6 was performed and it was sent for sequencing to verify the presence of Sirt6 mutation. The sequencing reaction confirmed that Sirt6 mutation was really present (11 nucleotides missing close to the ATG start of the Sirt6 gene translation).

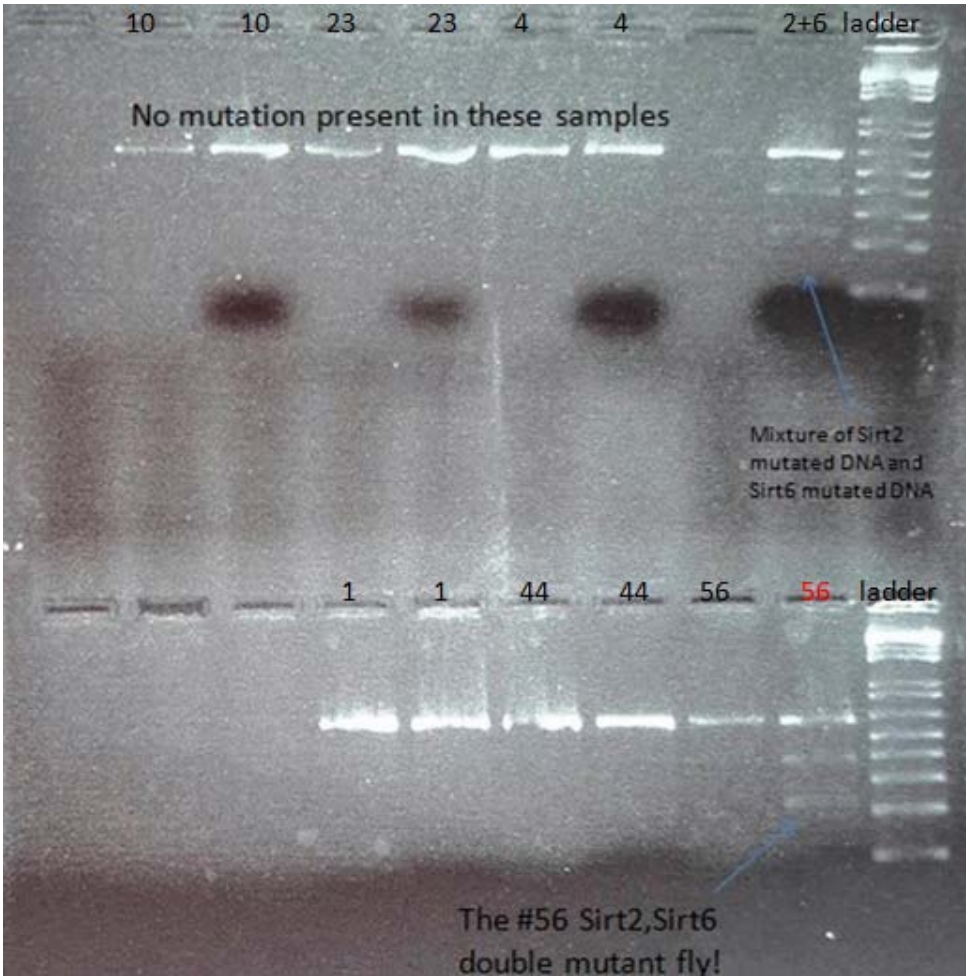


Fig.16: The double mutant fly #56 for the two sirtuin genes Sirt2 and Sirt6, by meiotic recombination method. Only the #56 DNA shows the same fragments as the mixture of Sirt2 and Sirt6 mutated DNA. The size of the undigested product is around 650bp, whereas the size of the 2 digested fragments are around 400bp and 250bp respectively. All the other PCR products showed only one big fragment (original) and no smaller fragments, indicating that the Sirt2+Sirt6 mutation is not present. Each number is present twice, as the undigested product was always loaded next to the 2h digested product.

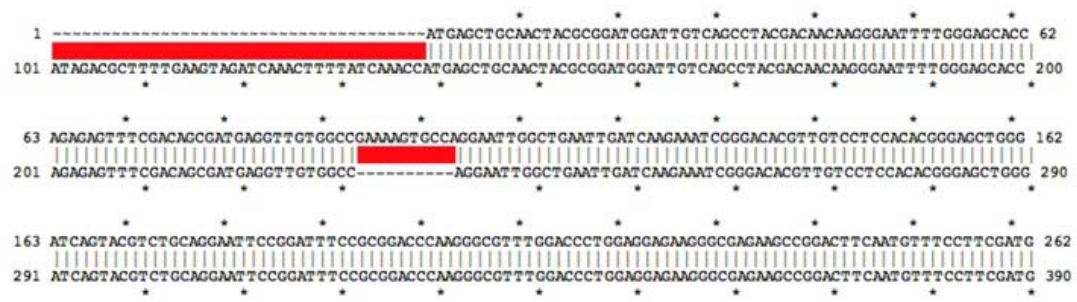


Fig.17: The alignment of the sequence for Sirt6 gene from homozygous fly from the #56 stock with the wild type Sirt6 gene sequence. The upper DNA strand represents genomic DNA from wild type and the lower DNA strand represents the results of the sequencing reaction from genomic DNA from stock #56. The 11 nucleotides missing in the open frame of Sirt6 gene in #56 stock are apparent, resulting in a frameshift and production of an unfunctional protein.

Nevertheless, we performed another round of screening and found several more Sirt2,Sirt6 double recombinants (Fig.18). Results presented below indicate the discovery of five more Sirt2,Sirt6 double mutants from meiotic recombination. One out of the five stocks was sent for sequencing and was further confirmed to be a double mutant fly, so overall two confirmed independent stocks were finally available for further experiments (#56 and #10).

```
CLUSTAL format alignment by MAFFT (v7.310)

16_1096_001_Sir GMCKG-ATCTCGATGGACTCATTTAGCCGTACAAAATATAAACAAATTAATACCAAAAAG
16_1096_002_Sir GMASRCATCTCGATGG-MTCATTTAGCCGTACAAAATATAAACAAATTAATACCAAAAAG
                *      **********  ****************************************

16_1096_001_Sir ACATAATAGACGCTTTTGAAGTAGATCAAACTTTTATCAAACCATGAGCTGCAACTACGC
16_1096_002_Sir ACATAATAGACGCTTTTGAAGTAGATCAAACTTTTATCAAACCATGAGCTGCAACTACGC
                ************************************************************

16_1096_001_Sir GGATGGATTGTCAGCCTACGACAACAAGGGAATTTTGGGAGCACCAGAGAGTTTCGACAG
16_1096_002_Sir GGATGGATTGTCAGCCTACGACAACAAGGGAATTTTGGGAGCACCAGAGAGTTTCGACAG
                ************************************************************

16_1096_001_Sir CGATGAGGTTGTGGCCA----------GGAATTGGCTGAATTGATCAAGAAATCGGGACA
16_1096_002_Sir CGATGAGGTTGTGGCCRARAAKTGCCWGGAATTGKCTGAATTGATCAACAAATCGGGACC
                ****************          ******* ************* **********

16_1096_001_Sir CGTTGTCCTCCACACGGGAGCTGGGATCAGTACGTCTGCAGGAATTCCGGATTTCCGCGG
16_1096_002_Sir CGTTGTCCTCCACACGGCAGCTGGGATCCRTACGTTCGCGAGAATTCCGGATTTCCGCGS
                ********************  **********  *****..**..*****************

16_1096_001_Sir ACCCAAGGGCGTTTGGACCCTGGAGGAGAAGGGCGAGAAGCCGGACTTCAATGTTTCCTT
16_1096_002_Sir ACCCGAACSCGTTTGGACCCTGGAGAARAASGGCGATAAGCCGGACTTCTCTGTTTCCTT
                ****.*.  ****************.* ** ***** ************  *********

16_1096_001_Sir CGATGAAGCCAGACCAACTAAAACCCACATGGCTATCATAGCCCTGATTGAAAGTGGCTA
16_1096_002_Sir CGATGAACCCAGACCAACTAGRACCCACATGGCTATCATAGCCCTGATTGARAGTGGCTA
                ******* ************. ****************************** ********

16_1096_001_Sir TGTGCAGTACGTTATCTCACAGAATATTGATGGTCTCCACTTGAAATCCGGACTGGATCG
16_1096_002_Sir TGTGCAGTACGTAATCTCGCAGAATATTGATGGTCTCCACTTGAGATCCGGACTGGATCG
                ************ *****.**************************.***************

16_1096_001_Sir GAAGTATCTTTCCGAATTGCACGGCAACATTTACATCGAACAGTGTAAGAAATGCAGACG
16_1096_002_Sir TAAGAATCTTTCCGAATTGCACGGMTMCATTTACATCRRACAKTGWRASAGATGCAKACG
                 *** ******************    **********  *** **  * *.***** ***

16_1096_001_Sir GCAATTTGTGAGCCCATCTGCCGTGGAAACAGTGGGTCAAAAACCCGGGGAAMSKTAA
16_1096_002_Sir GCAATTTGTGASCCCATCTGCCGTGGACACAGWGGYYRAMAAAGCYTG-CCAMSKAAA
                *********** *************** **** **   * *** *  *   *    **
```

Fig.18: The alignment of the sequence for Sirt6 gene from homozygous fly from the #10 stock with the wild type Sirt6 gene sequence. The upper DNA strand represents the results of the sequencing from the stock #10 and the lower DNA strand represents genomic DNA from wild type. The 10 nucleotides inserted in the open frame of Sirt6 gene in #10 stock are apparent, resulting in a frameshift and production of an unfunctional protein.

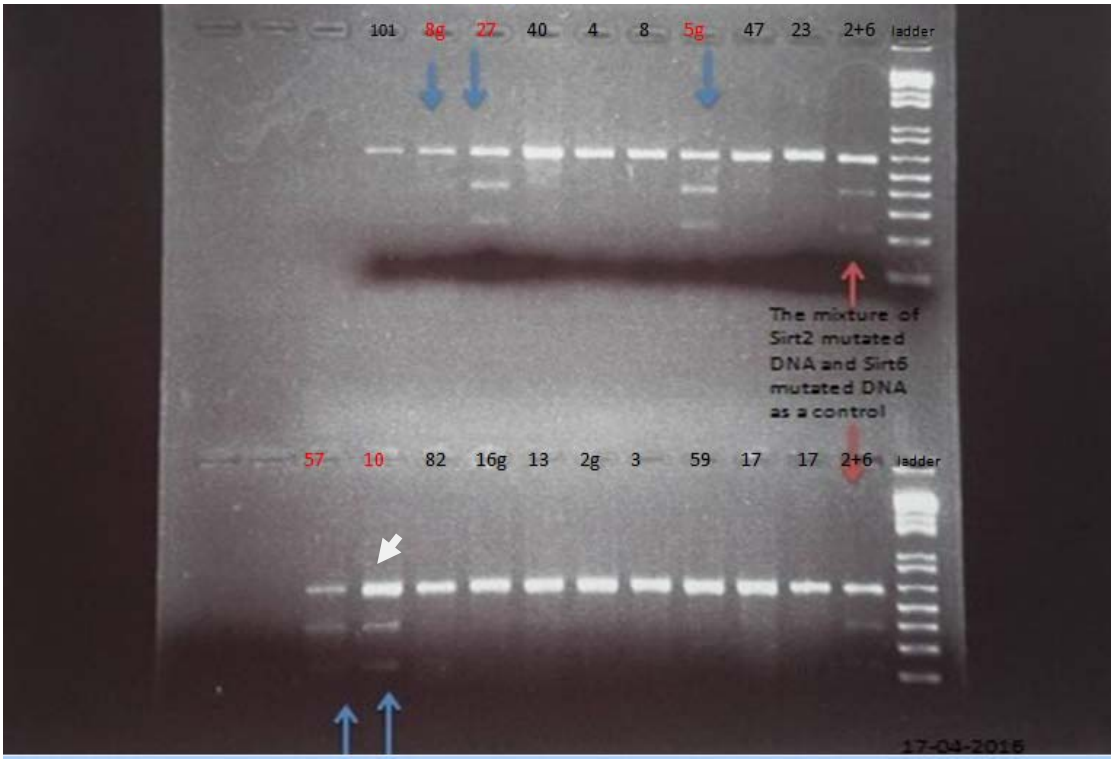


Fig.19: Five more potential Sirt2,Sirt6 double mutants discovered by using the recombination method (#57, #10, #8g, #27 and #5g), one confirmed by sequencing (white arrow #10). In this gel, the screening for Sirt6 mutation is presented, in which we included only stocks that were tested before and were positive for the Sirt2 mutation.

4.3 THE DOUBLE MUTANT FOR Sirt2,Sirt6 CREATED BY THE CRISPR/Cas9 TECHNOLOGY

A mutated fly for the Sirt2 and Sirt6 genes was also created by using a second method, the so called Crispr/Cas9 technology. In this case, we introduced the Sirt6 mutation *de novo* by Crispr system into a fly that already had Sirt2 mutation present (as described in Aims and Methods).

We crossed flies as described in Methods and established 30 independent lines. We then isolated genomic DNA from 9 heterozygous flies and tested them for the presence of Sirt2 mutation. Out of the 9 tested lines 6 had the Sirt2 mutation present, according to our expectations (1/2 of flies should have the Sirt2 mutation present based on the crossing scheme). We also tested the flies for the presence of Sirt6 mutation by T7 digest. Out of these 9 lines tested again, 7 showed positive signal for Sirt6 mutation after T7 digest. In total, 5 flies were double mutated for Sirt2 and Sirt6 genes. This correlates to about 56 % efficiency of the Crispr mediated mutagenesis, that is an excellent result.

Moreover, each of these Sirt2,Sirt6 positive lines represent an independent mutant for Sirt6 gene (bearing probably different size of deletion in Sirt6 gene). In order to verify the nature of the Sirt6 mutation we needed to sequence the lines. Genomic DNA from homozygous male from stocks 1, 2, 4 and 5 was isolated, PCRs with primers Sirt6 were performed and sent for sequencing (protocol 1). We needed to verify the presence of Sirt6 mutation but also to check whether this mutation really resulted in a frame shift (as missing 3 nucleotides would only result in a missing amino acid and no frameshift). The sequencing reaction confirmed that we deleted 1 nucleotide from the Sirt6 gene in line 2, 2 nucleotides in line 4 and 10 nucleotides in line 5, hence creating a frameshift and an unfunctional protein.

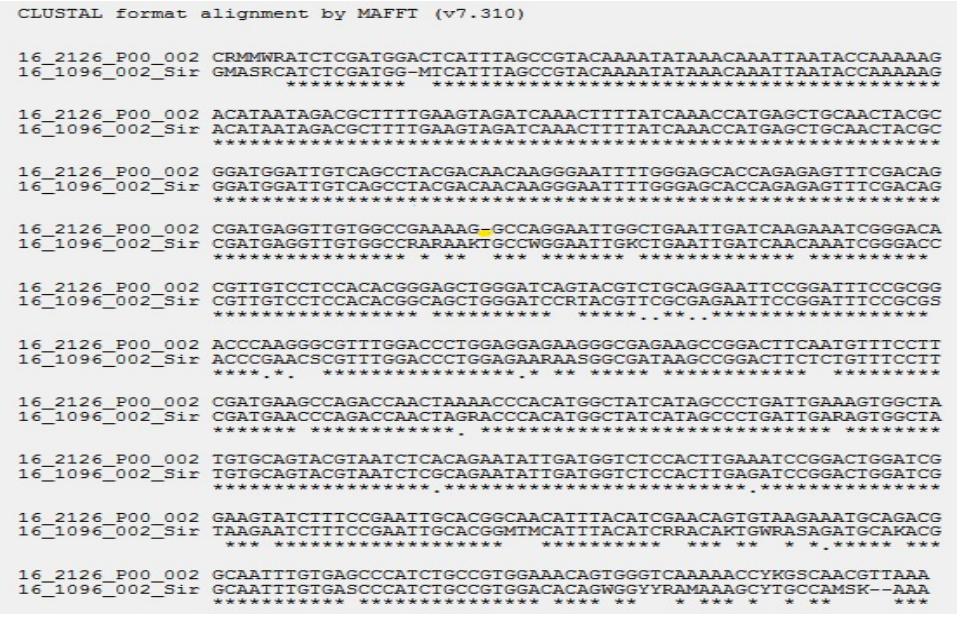


Fig.20: The alignment of the sequence for Sirt6 gene from homozygous fly from the #2 stock with the wild type Sirt6 gene sequence. The upper DNA strand represents the results of the

sequencing from the stock #2 and the lower DNA strand represents genomic DNA from wild type. The 1 nucleotide inserted in the open frame of Sirt6 gene in #2 stock is apparent, resulting in a frameshift and production of an unfunctional protein.

```
CLUSTAL format alignment by MAFFT (v7.310)

16_2126_P00_003 AAMATRAATCTCGATGGACTCATTTAGCCGTACAAAATATAAACAAATTAATACCAAAAA
16_1096_002_Sir -GMASRCATCTCGATGG-MTCATTTAGCCGTACAAAATATAAACAAATTAATACCAAAAA
                 . *   **********  ****************************************

16_2126_P00_003 GACATAATAGACGCTTTTGAAGTAGATCAAACTTTTATCAAACCATGAGCTGCAACTACG
16_1096_002_Sir GACATAATAGACGCTTTTGAAGTAGATCAAACTTTTATCAAACCATGAGCTGCAACTACG
                ************************************************************

16_2126_P00_003 CGGATGGATTGTCAGCCTACGACAACAAGGGAATTTTGGGAGCACCAGAGAGTTTCGACA
16_1096_002_Sir CGGATGGATTGTCAGCCTACGACAACAAGGGAATTTTGGGAGCACCAGAGAGTTTCGACA
                ************************************************************

16_2126_P00_003 GCGATGAGGTTGTGGCCGAAATTGTGGCCAGGAATTGGCTGAATTGATCAAGAAATCGGG
16_1096_002_Sir GCGATGAGGTTGTGGCCRARA--AKTGCCWGGAATTGKCTGAATTGATCAACAAATCGGG
                ****************** * *   .  *** ******* ************* ********

16_2126_P00_003 ACACGTTGTCCTCCACACGGGAGCTGGGATCAGTACGTCTGCAGGAATTCCGGATTTCCG
16_1096_002_Sir ACCCGTTGTCCTCCACACGGCAGCTGGGATCCRTACGTTCGCGAGAATTCCGGATTTCCG
                ** ****************** **********  ******..**..****************

16_2126_P00_003 CGGACCCAAGGGCGTTTGGACCCTGGAGGAGAAGGGCGAGAAGCCGGACTTCAATGTTTC
16_1096_002_Sir CGSACCCGAACSCGTTTGGACCCTGGAGAARAASGGCGATAAGCCGGACTTCTCTGTTTC
                ** ****.*.  ****************.* ** ***** ************  ******

16_2126_P00_003 CTTCGATGAAGCCAGACCAACTAAAACCCACATGGCTATCATAGCCCTGATTGAAAGTGG
16_1096_002_Sir CTTCGATGAACCCAGACCAACTAGRACCCACATGGCTATCATAGCCCTGATTGARAGTGG
                ********** ***********. ****************************** *****

16_2126_P00_003 CTATGTGCAGTACGTAATCTCACAGAATATTGATGGTCTCCACTTGAAATCCGGACTGGA
16_1096_002_Sir CTATGTGCAGTACGTAATCTCGCAGAATATTGATGGTCTCCACTTGAGATCCGGACTGGA
                **********************.*************************.************

16_2126_P00_003 TCGGAAGTATCTTTCCGAATTGCACGGCAACATTTACATCGAACAGTGTAAGAAATGCAG
16_1096_002_Sir TCGTAAGAATCTTTCCGAATTGCACGGMTMCATTTACATCRRACAKTGWRASAGATGCAK
                *** *** ******************   **********  *** **  * *.*****

16_2126_P00_003 ACGGCAATTTGTGAGCCCATCTGCCGTGGAAACAGTGGGTCAAAACCTKKSSSMMMCKTA
16_1096_002_Sir ACGGCAATTTGTGASCCCATCTGCCGTGGACACAGWGGYYRAMAAAGCYTGCCAMS---K
                ************** *************** **** **   * **  .
```

Fig.21: The alignment of the sequence for Sirt6 gene from homozygous fly from the #4 stock with the wild type Sirt6 gene sequence. The upper DNA strand represents the results of the sequencing from the stock #4 and the lower DNA strand represents genomic DNA from wild type. The 2 nucleotides missing in the open frame of Sirt6 gene in #4 stock are apparent, resulting in a frameshift and production of an unfunctional protein.

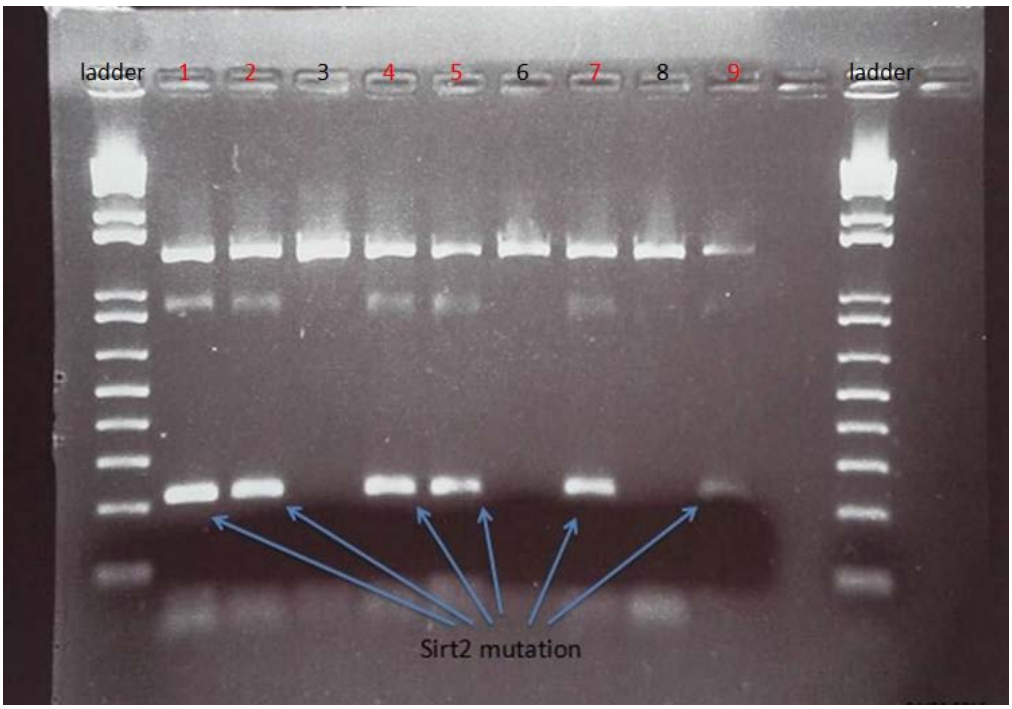


Fig.22: The 9 fly stocks screened for Sirt2 mutation. The Sirt2 mutation appears to be present in numbers 1,2,4,5,7 and 9, meaning 6/9 fly genomes, as mentioned earlier. The Sirt2 mutation was present in the flies from the beginning of the Crispr/Cas9 crossing method, and here it is checked in order to see in which fly stocks it was present in the end.

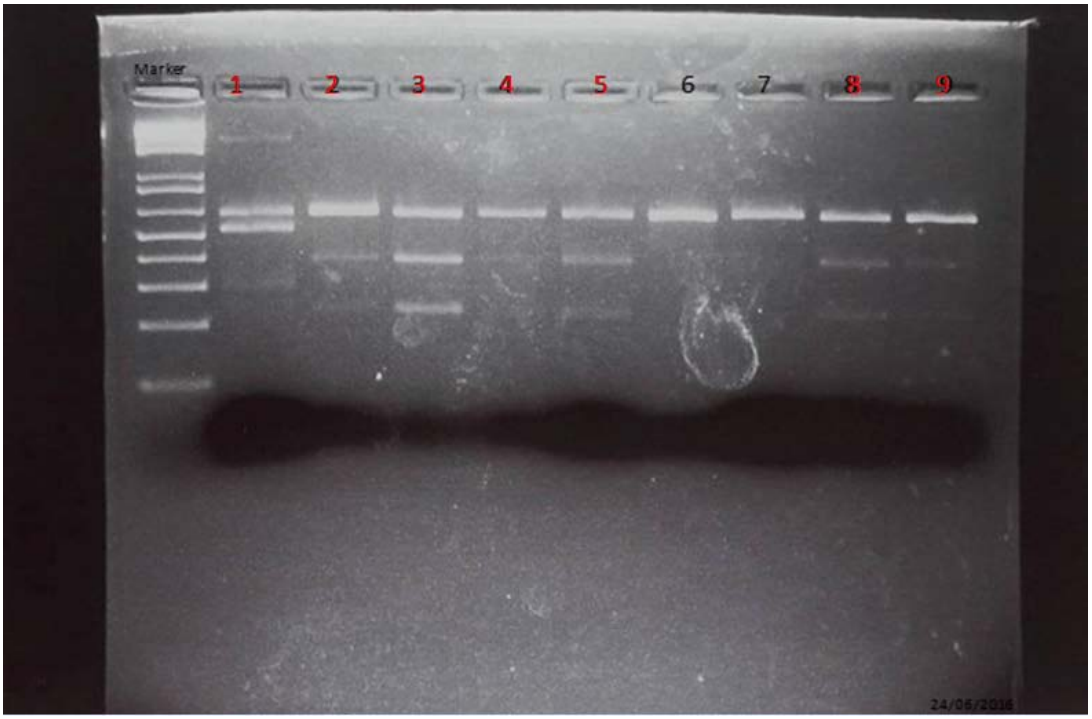


Fig.23: The Sirt6 mutation created by Crispr/Cas9 technology, screened in the PCR products of fly stocks 1-9, with only #6 and #7 not showing smaller fragments. So, as shown in this gel, 7 out of 9 fly genomes carry the Sirt6 mutation. After analyzing the 2 gel pictures together (Fig.22 and Fig.23), we could see that in total 5 fly lines had the double mutation (#1, #2, #4, #5 and #9). This indicates the high efficiency of the Crispr /Cas9 system, as 7/9 flies screened had the Sirt6 mutation.

Taking together, the Crispr/ Cas9 method appears to be much more efficient than the meiotic recombination method, which supports and verifies the fact that Crispr / Cas9 technology is said to bring a new era in biology, wit this high potential, efficiency and reliability.

4.4 A STEP CLOSER TO THE SUPERFLY: THE TRIPLE MUTANT FLY

After the successful creation of the double mutant fly for Sirt2 and Sirt6 genes, the project continued with starting the creation of a triple mutant fly for the genes Sirt2, Sirt6 and Sirt7. For the purpose of this experiment, we used the double mutant fly #56, which was obtained by recombining Sirt2 and Sirt6 mutations on the same chromosome and introduced Sirt7 mutation de novo by Crispr system. After several crosses were performed, the experiment reached the point indicated in the picture below by the red arrow. Later, after the end of the academic year in České Budějovice, it was continued by my supervisor Alena Krejci. These flies were crossed to the nos-Cas9 and independent lines were screened for the presence of Sirt7 mutation. The triple recombinant was found and then combined with the Sirt4 mutation on the X. chromosome and Sirt1 mutation in the II. chromosome, in order to finally create the "superfly".

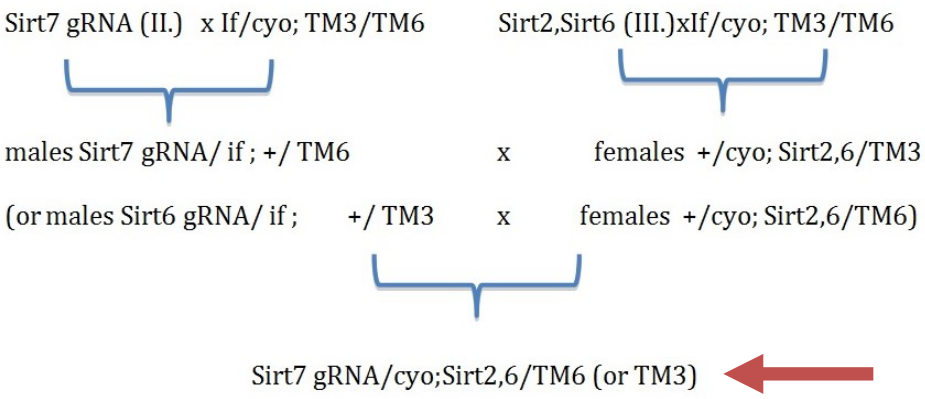


Fig.24: The crossing scheme for the creation of the Sirt2,Sirt6,Sirt7 triple mutant. The point indicated by the red arrow, is the point the project had reached by the end of the academic year.

5. DISCUSSION

The fly that is a mutant for two of the sirtuin genes, Sirt2 and Sirt6, was created. Two different methods were used for this purpose, the meiotic recombination of the two existing mutations on the same chromosome, and the Crispr/Cas9 technology. As a result, several independent stocks of flies were created, that can be used for further genetic or behavior observation experiments. By comparing the efficiency of the two approaches it was apparent that meiotic recombination is much less efficient than Crispr mediated mutagenesis (56% versus 5.8%).

5.1 GENETIC ENGINEERING BY CRISPR/Cas9

The Crispr/Cas9 method was used for the acquisition of the double mutant fly, but is also the strategy for the obtainment of the Sirt2,Sirt6,Sirt7 triple mutant fly. Crispr/Cas9 is a powerful tool for genome editing and has brought a lot of excitement into the biological and medical research field. This can be understood, as this method has several tremendous advantages over other older techniques. First of all, the guide RNA that leads the way for the Cas9 nuclease, can be easily designed with no unaffordable costs. It also has very high specificity, because it does not depend on DNA/protein recognition, so it can simply target any desired sequence. Moreover, a variation of the technique allows the injection of different gRNAs, which lead to the introduction of mutations in many genes simultaneously. Finally, the traditional meiotic recombination technique can be avoided, since the Crispr/Cas9 can create mutations in a much faster and efficient way.

Nevertheless, no technique comes without drawbacks and Crispr is also characterized by some of them. The most important risk the method entails is the so called “off-site effects”. This means that the mutation could be introduced to other loci than the target position. This could happen because of high similarity of these loci to the real target, leading to other than the desired mutations. This is difficult to spot unless the whole genome would be sequenced, but that would be expensive and therefore not realistic. An additional problem of the method is the fact that it could introduce in-frame mutation, so not all mutations created could cause a frame-shift.

This would result in a protein missing one or certain amino acids, which may have no severe impact on the function of the protein.

The off-target mutations could be removed from the fly's genome in several ways. In general, the chromosome could be "cleaned" by allowing the flies to cross and recombine with a wild type chromosome for at least six generations or at least to exchange the other two chromosomes where the Crispr mediated mutation is not present. In the case of this project, white-eye flies were used, so no phenotypic marker could be exploited and the "cleaning" would be difficult, as we would need to sequence all flies from each generation for the verification of Sirt6 mutation. This would be a very time and money consuming process. There would be an advantage if instead of simply introducing deletion in Sirt6 gene we would insert a dominant marker at the same time (like white gene or GFP tag). However, the process of Crispr mediated sequence insertion is much less efficient than simple mutation, so we decided to go for the easier option.

5.2 WHAT COMES NEXT..?

Triple mutant flies for other than Sirt2, Sirt6 and Sirt7 genes have already been created and the stock appeared to be viable with no obvious developmental effects or health problems. This may be explained by assuming that the sirtuin genes have a kind of functional redundancy. Maybe Sirt1 or Sirt4 substitutes the loss of function of Sirt2, Srt6 and Sirt7. Additionally, flies are always kept under ambient light and temperature conditions, with sufficient food and water supply. This implies that the flies do not have to "use" the Sirtuin proteins so much, as no metabolic or other stress occurs. If stress conditions are applied (e.g. lack of food, temperature stress, etc.), the flies may need to express more of these metabolism regulating proteins. So, in this case, if the sirtuin genes are mutated, the proper expression of the sirtuin proteins will not be possible and the significance of the mutation for the fly's organism may finally be obvious and a phenotype may be characterized. If this holds true, it will conclude to the important role of sirtuin genes as "stress defense" genes.

After the obtainment of the triple mutant fly, the next step will be the creation of the penta mutant for Sirt1, Sirt2, Sirt4, Sirt6, Sirt7 and Sirt4 genes, the "superfly"

as we call it. At that point, there should finally exist an obvious phenotype or, most probably, result in early developmental death. If this is not true, then we would expect a big response under stress conditions, as it would be difficult to imagine that the fly would carry five genes in the genome of which none would be needed.

By observing how the mutations of the sirtuin genes affect Drosophila melanogaster, we may gain insight into the function of each sirtuin gene. Such a finding would constitute a critical and meaningful achievement, revealing “molecular secrets” and providing information on more important functions of the sirtuins, the regulators of genome stability and metabolism.

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