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Creation of *Drosophila melanogaster* mutant for SIRT2 and SIRT7 genes

Bachelor Thesis

Laboratory of developmental biology

DEPARTMENT OF MOLECULAR BIOLOGY

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Annotation: The aim of the thesis was to create a fly that is a mutant for two of the sirtuin genes, SIRT2 and SIRT7. This was achieved either by recombining existing SIRT2 and SIRT7 mutations on the same chromosome or by using the Crisper technology to introduce de novo mutation in SIRT7 on the background of SIRT2 mutant. The fly genotypes were verified by sequencing. The flies served as an important middle step towards the creation of flies with all five sirtuin genes mutated.

Affirmation: I hereby declare that I have worked on the submitted bachelor thesis independently. All additional sources are listed in the bibliography section. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

Acknowlegdements: First I want to thank my supervisor Alena Krejčí, who accepted me in the lab and helped me patiently during my practical and theoretical work. I would also like to thank to the whole team in the lab for their never-ending optimism and creation of a great collective. Many thanks go to my friends and classmates who made the whole studies a bit easier. Finally, I want to thank my family, especially my mom who was always there for me to give me another perspective and valuable advices all along the way.

CONTENTS

1. INTRODUCTION

1.1. *Drosophila* as model organism

One of the most well-known and oldest model organisms being studied for more than a century is the fruit fly, *Drosophila melanogaster*[1]**.** It has been used for basic research of cell and developmental biology, as well as for certain aspects of cancer research. It has an important role in a broad range of scientific areas like neurosciences and genetics, in which it also serves as a model for human diseases[2–4].

About 75% of human disease genes have a match in the genome of fruit flies according to a study of the National Human Genome Research Institute from 2000[5]. The genome of the fruit fly (1.75 x 108 base pairs in haploid state, approximately 13.600 genes) is packed in four pairs of chromosomes, designated 1-4, whereas number 1 is the X chromosome and 2-4 are the autosomes[6]. This low number of chromosomes leads to the simplification of genetics and allow us using the fruit fly as a perfect model for human diseases and genetic modifications.

One pair of sex chromosomes also known as heterosomes (X/X females or X/Y males) and three pairs of autosomes are found in *Drosophila Melanogaster*. The sex is determined by the X:A ratio, which means that the sex is determined by the ratio of the number of X chromosomes to the number of copies of each autosome. XO zygotes with an X:A ratio of 0.5 develop to be morphologically normal but sterile males while XXY zygotes with an X:A ratio of 1 develop as normal females. Since the Y chromosome doesn't play any role in sex determination it is only required to confer male fertility[6].

It has been identified that the X chromosome is acrocentric and large, whereas chromosomes 2 and 3 are metacentric and large. An exception is the chromosome 4, which is a tiny acrocentric "dot" chromosome with only 2% the size of the main autosomes[6]**.**

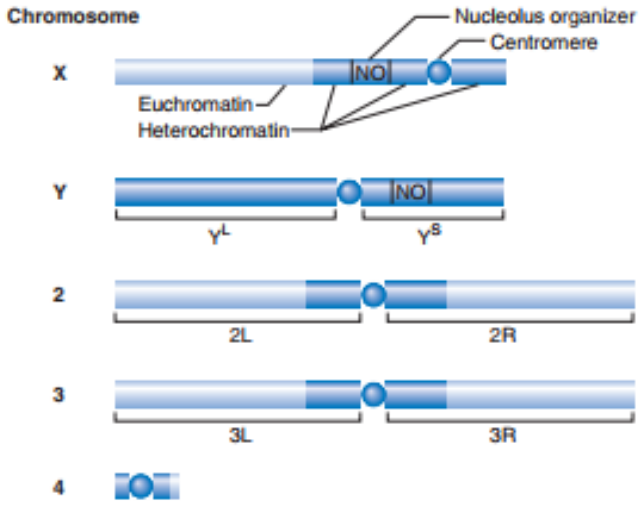


Fig.1: Structure of *Drosophila* chromosomes prior to S phase DNA replication[6].

There are many reasons for choosing the fruit fly *Drosophila melanogaster* for genetic studies. Among them it is the small size of the genome, its fast reproduction cycle and low maintenance cost. The vast mutant collections available in fly banks and collections of RNAi and other genetic tools make the fly ideally suited for basic research to study function of genes on cellular level.

The flies can be kept in glass vials closed with cotton wool and their food consist of corn, flour, yeast, agar and glucose. However, the knowledge about the fruit fly is enormous and its methods for manipulation of the *Drosophila melanogaster* are well-established.

1.2. Life cycle of *Drosophila melanogaster*

The fruit fly completes its life cycle from fertilization to the emergence of an adult fly within 10 days at a temperature of 25°C. The fertilization of more than hundreds of eggs is possible due to special sperm storage organs (the spermatheca and seminal receptacle) in the female fly. It can produce about 3000 progenies and a single male can sire well more than 10,000 offspring. Not even 24 hours are needed that the embryo completes its development[6].

The fruit fly undergoes four stages for complete development which can be seen in Fig.2:

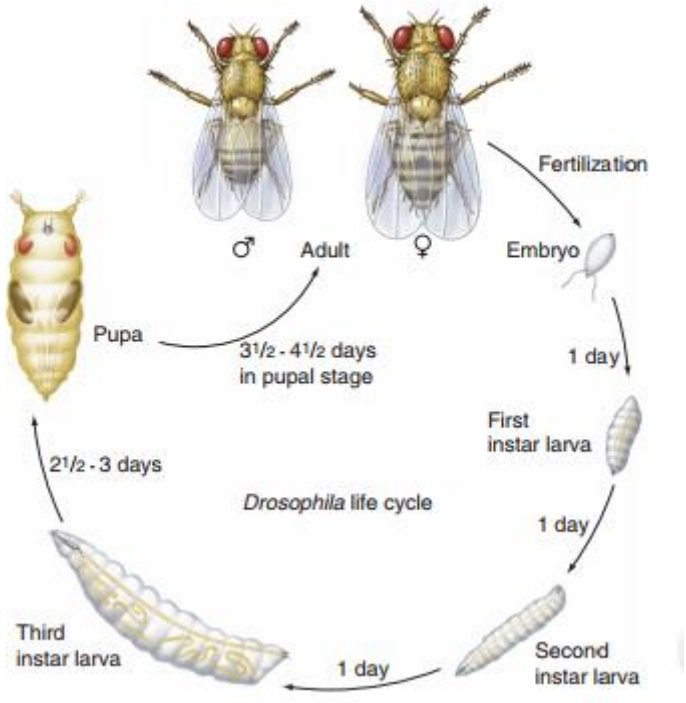


Fig.2: The life cycle of the *Drosophila melanogaster*

Hatching: Transition from an embryo to a first instar larva.

Molts: Transitions between larval instars.

Pupariation: Conversion of a third instar larva to a pupa.

Eclosion: Emergence of the adult fly from the pupal case.[6]

With the hatching of the first stage (first instar) larva the embryogenesis ends. To produce second and third instar larvae, the wormlike first instar larva dramatically increases in body size by molting 24 and 48 hours after hatching. The third instar larva completes its growth, crawl out of the food and pupate about three days after the second molt. Metamorphosis occurs inside the protective pupal case which leads to dramatic reorganization of the body plan of the fruit fly which takes about four days. During these four days adult structures are produced by disintegration of larval tissues and replacements through differentiation and proliferation of cells. Imaginal discs develop from small groups of cells set aside the early embryo and are responsible for the creation of structures specific to adults like legs, genitalia, wings, eyes …. During the pupal and larval stages, the imaginal discs go through extensive growth. The adult abdominal epidermis is formed by adult-forming cells which are clustered in nests within each abdominal segment. The last step is the expansion of the wings, pigmentation and hardening of the exoskeleton after metamorphosis. After they emerge, virgin fruit flies can be collected

within 8 hours because the sexual maturation of the males happens within this period. Often the flies are kept in media of 18°C, in this case the times mentioned are doubled[6].

The distinguishing of flies is based on the physiological appearance of the fruit fly. Females display separated dark stripes at the posterior tip of their abdomen, which are merged in males and the size of female flies is slightly larger than that of the male ones. In the males, the anal plates are darker and more complex whereas in the females display a pin-like extension. Another slightly difference are the sex combs on the first pair of legs in the male fly. A secure indicator if a fly is a virgin is the greenish spot on the abdomen of the female fly which can be obtained during a very short period of after eclosion[7].

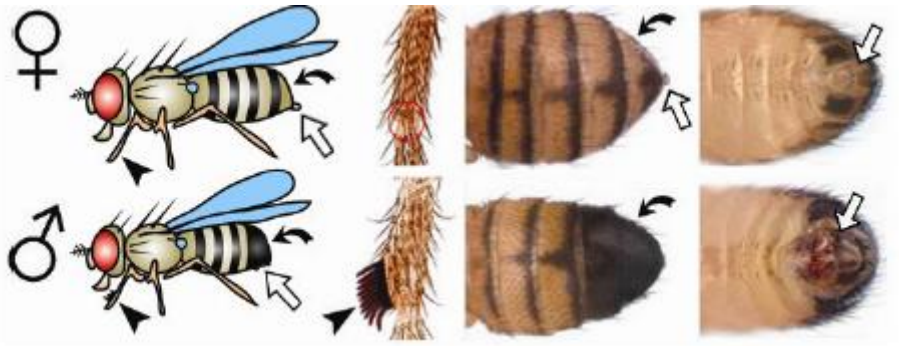


Fig.3: Criteria for the selection of the right fly[7]

1.3. Genetic recombination and the use of balancer chromosomes

Genetic recombination, also known as crossing over, is the exchange of genetic material either between different regions of the same chromosome or multiple chromosomes. The sequence identity is required between the homologous regions of DNA that line up in arrangement for exchange[8]. Genetic recombination allows the creation of offspring with combinations of traits that differ from those traits found in either parent. Frequent recombination occurs in meiosis when gametes are formed and it is very rare during mitosis (unless genetic tricks are used by the researchers). Moreover, in *Drosophila* meiotic recombination occurs only in females and is absent in males. In our case we recombined existing SIRT2 and SIRT7 mutations coming from two different parental flies on the same chromosome of the progeny of these two flies. Recombination is a highly complex process, where two homologues DNA strands are aligned, precisely broken into each strand, exchanged, and sealed again[8]. In every main chromosome arm only one or two crossovers take place per meiosis[6]. Neither in the centromeric heterochromatin nor in the fourth chromosome crossing-over occurs[6].

Balancer chromosomes can be used to avoid unwanted recombination. They typically contain dominant marker which can be followed through the whole process of crossing and a lethal recessive allele that suppresses the formation of homozygotes. They do not allow meiotic recombination between homologous chromosomes which is important for the stock maintenance. Via this technique genotypes that differ from the parental generation are not formed in a balanced stock.

1.4. CRISPR/CAS9 and its history

Precise and targeted changes to the genome of living cells is a goal for every biomedical researcher. One of the ways to achieve an efficient and reliable result is a tool based on a bacterial CRISPR-associated protein-9 nuclease (CAS9) from Streptococcus pyogenes[9]. CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. They are segments of prokaryotic DNA that contain short and repetitive sequences. They were firstly discovered in E. coli in the 1980s but their function was unknown until Barrangou and his colleagues confirmed it 2007 by using a genome fragment of an infectious virus and adding it into the CRISPR locus of a S. thermophilus[10]. The result showed that the S. thermophilus showed resistance against a bacteriophage[10]. Out of free types of CRISPR mechanisms, type II is the most studied one[9]. In this type, the foreign DNA of the plasmid or virus is incorporated into the middle of the locus, CRISPR locus (short repeats, 20bp), by cutting the DNA into small fragments[9]. Next step is the transcription of the loci, where afterwards the transcripts are processed to generate small so-called crRNA (CRISPR RNA)[11]. The purpose of the crRNA is to guide the Cas9 effector endonuclease to specific place on the DNA via its connection to tracrRNA, to allow Cas9 to cut the foreign DNA due its sequence homology with tracrRNA[11]:

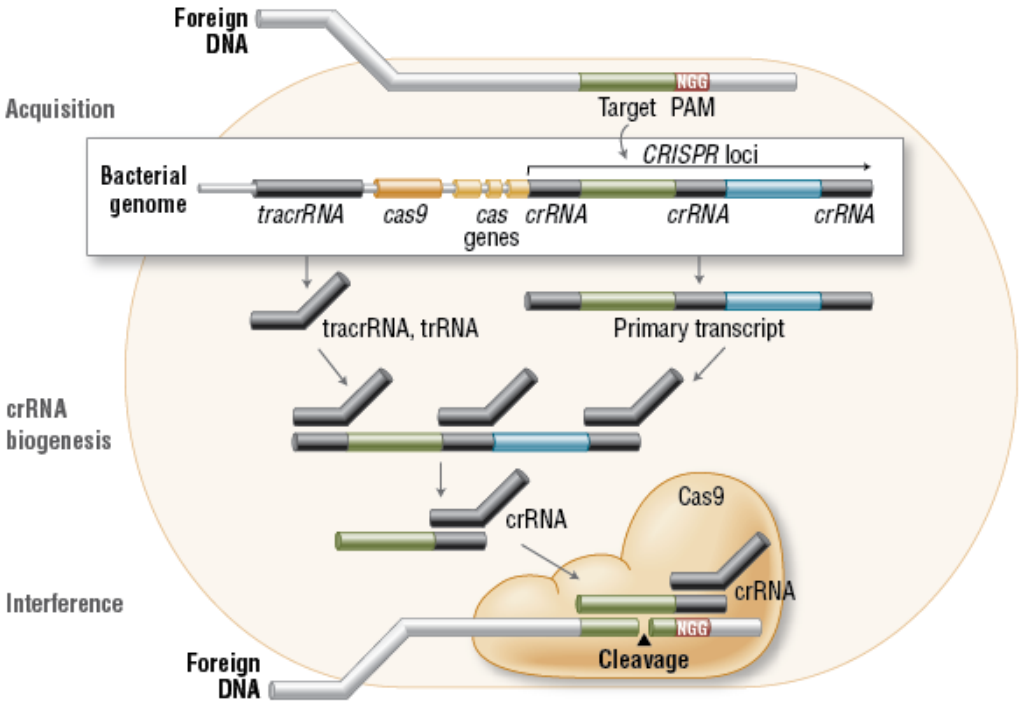


Fig.4: The incorporation of invading DNA into the bacterial genome at the CRISPR loci can be seen in the acquisition phase. Transcription of the CRISPR loci and the creation of crRNA are the next steps during the biogenesis. During interference a complex of Cas9 endonuclease, crRNA and separate tracrRNA is formed. The invading DNA which contains a 20-nucleotide crRNA complementary sequence next to the PAM sequence can be cleaved off via tracrRNA[9].

A huge role in CRISPR mechanism especially type II CRISPR systems is the Cas protein Cas9 which has been shown through knockdown and rescue experiments to be a key player[9]. It requires only one Cas protein (Cas9) for gene silencing which makes it in comparison to other CRISPR systems unique[12]. Cas9 in type II systems is responsible for destroying the target DNA and participates in the processing of crRNAs[11,12]. This can only be achieved by presence of two nuclease domains, the RuvC-like nuclease domain that resides at the amino terminus and the HNH-like nuclease domain located in the mid-region of the protein[13].

For the cleavage and recognition of the site-specific DNA, Cas9 must be complexed with a separate trans-activating crRNA which can be tracrRNA or trRNA and a crRNA[11]. The trans-activating crRNA (tracrRNA or trRNA) must be partially complementary to the crRNA[11]. In the presence of RNase III and Cas9 the tracrRNA is needed for crRna maturation which is encoding multiple pre-crRNAs from a primary transcript[12].

The two nuclease domains cut both DNA strands during the destruction of the target DNA, creating double-stranded breaks at sites which are defined by a 20-nucleotide target sequence

within the crRNA transcript[11,14]. With the help of the RuvC domain the noncomplementary strand is cleaved off whereas the HNH domain cleaves the complementary strand[9].

Another important thing which makes this system complete is a short-conserved sequence, (2-5 nts) known as protospacer-associated motif (PAM) which follows immediately 3´-of the crRNA complementary sequence[15]. Without a PAM sequence, even a fully complementary sequence is ignored by CAS9-RNA[16].

In 2012 the lab of Doudna and Charpentier developed a new tool in molecular biology by the simplification of the type II CRISPR nuclease using a two-component system by combining trRNA and crRNA into a synthetic so called single guide RNA[11].

Three different modifications of the Cas9 nuclease have been introduced (see Fig.5)[9]:

The first one, the wild-type Cas9, cleaves site-specifically double-stranded DNA[9]. This process allows the activation of the double strand break (DSB) repair machinery, which repairs double strand breaks by the cellular Non-Homologous End Joining (NHEJ) pathway[17]. The result is the disruption of the targeted locus by insertions and/or deletions which occur[17]. It was discovered that double strand breaks can be also repaired by the homology-directed repair (HDR) pathway, if a donor template with homology to the targeted locus is added, replacement mutations are able to be performed[17,18].

Another form of Cas9 is the mutated Cas9D10, which was developed by Cong and his colleagues for more precision, minimizing the off-target effects[19]. The mutated Cas9D10 shows only nickase activity; cleaving only one DNA strand without activating NHEJ[9]. Two gRNA/Cas9 complexes need to meet at a specific place in the genome to introduce the double stranded DNA break and a subsequent mutation/insertion via the HDR repair [11,19,20].

The third form of Cas9 is a nuclease -deficient Cas9 (dCas9) in which mutations in the RuvC and HNH domain were performed to inactivate cleavage activity but still enable DNA binding[11,21,22]. This technique does not introduce any DNA mutations. However, it can be used for very specific, targeted gene silencing or activation and as a visualization tool by combining it with different effector domains[21,23–27].

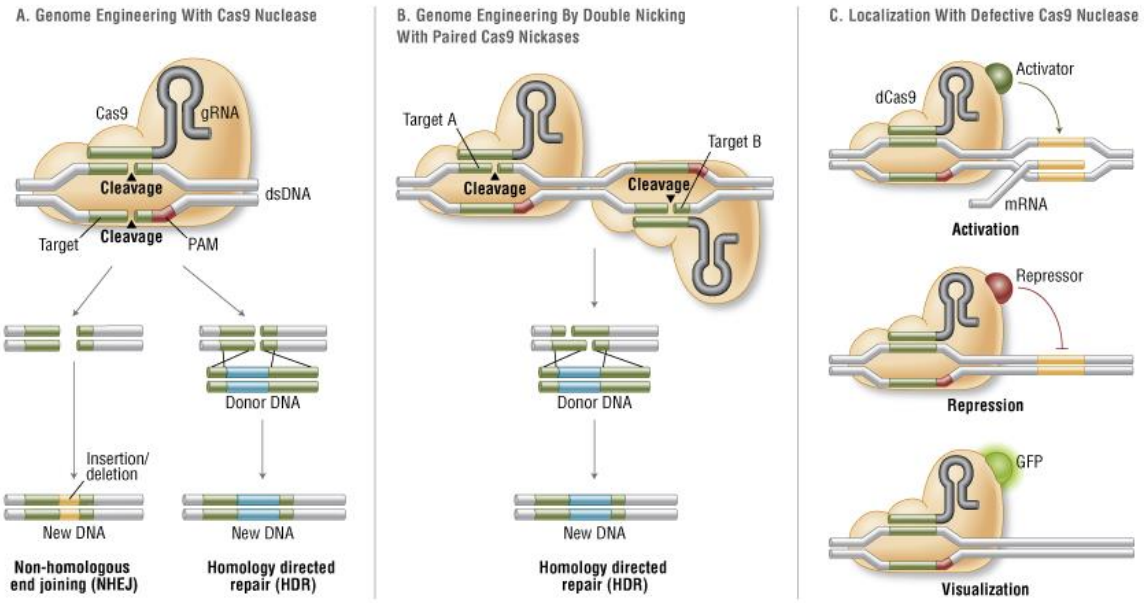


Fig.5: CRISPR/Cas 9 applications.

A) Using wild type Cas9 enzyme, non-homologous end joining (NHEJ) is carried out in the absence of a homologous repair template or the insertion of knock-ins is enabled by adding a homologous repair DNA template and using homology directed repair pathway (HDR).

B) Using the Cas9D10 mutant of Cas9, a staggered double-stranded break can be created using two single guide RNAs. Homology directed repair can then occur, leading to insertion of heterogenic DNA and hence mutation.

C) Using a nuclease -deficient Cas9 (dCas9), repressors, fluorescent proteins or transcriptional activators can be directed to specific places in the genome[9].

1.4.1. Advantages and Disadvantages of Crisper method

Crispr/Cas9 is a very simple technique with much better efficiencies in comparison to TALENs and ZFNs[28]. The only disadvantage it has is the so called off-target effect[29,30].

These mutations appear often at sites which show a difference of few nucleotides in comparison to the original sequence that are neighbored to a PAM sequence[29,30].

This occurs because of the ability of the protein CAS9 to recognize up to 5 base mismatches within single base difference in the PAM sequence or the protospacer region[29,30]. Whole-genome sequencing is required to detect the presence of off-target mutations[29,30]. The more complex genome the more of a chance that the off-target mutation will occur. As *Drosophila*

has 10x smaller genome than human the off-target effect is much less of a problem. Using the Cas9D10 system can greatly reduce the off-target effect.

1.5. The role of the SIRT family in mammals

Sirtuins are proteins that connects metabolism with longevity in worms, flies and yeast and that can be found also in mammals[31]. There are seven orthologues of the yeast NAD-dependent deacetylase SIR2 in mammals, called SIRT1-7[31]. SIR2 stands for 'silent information regulator' and it is involved in many biochemical processes such as DNA repair, chromatin silencing and chromosome fidelity during meiosis[32]. It has been shown that deleting SIR2 shortens lifespan in yeast and that an extra copy of the SIR2 gene in yeast extends the replicative lifespan[33]. Toxic extrachromosomal rDNA circles (ERCs) in yeast can be suppressed by SIR2 leading to a longer lifespan[34]. Due to researches in Escherichia coli a homolog of SIR2 (CobB)was shown to act as a catalysator in phosphoribosyl transferase reaction in cobalamin biosynthesis, indicating that the SIR2 possesses NAD-dependent ADP-ribosyltransferase activity on top of its deacetylase activity[31,35,36]. Sirtuins are involved in NAD-dependent histone deacetylation where they cleave NAD+ in each reaction, linking their activity with metabolism[37–39]. During this process, new metabolites 2' and 3'-O-acetyl-ADP-ribose are generated which have an important role in regulating other cellular processes[40–43].

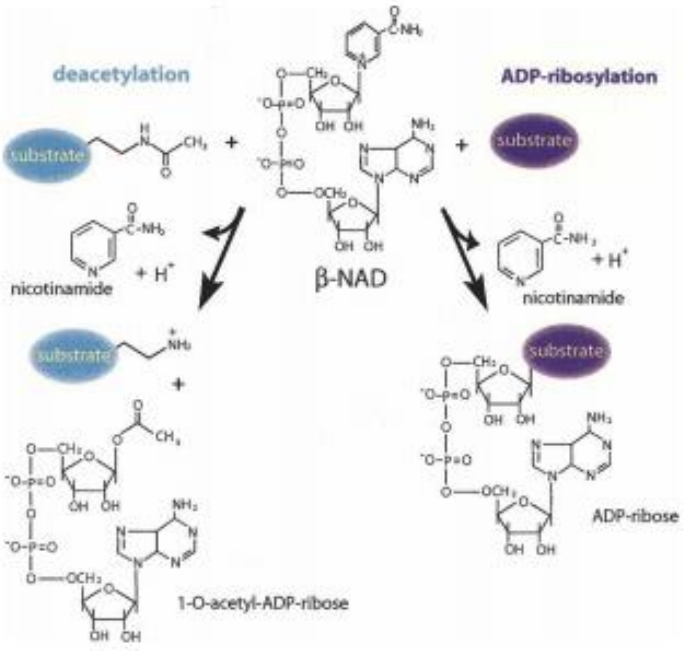


Fig.6: The use of NAD+ by sirtuins.

Sirtuin use NAD+ to perform ADP-ribosylation or deacetylation of their substrates. Nicotinamide is released by both of these reactions. During the deacetylation reaction, another metabolite of NAD+, the 1-O-acetyl-ADP-ribose, is formed[31].

SIRT1-7 have a high conserved NAD-binding sirtuin core domain[44]. However, they differ from each other in a broad range of parameters, starting from the localization (Tab.1) to their substrates and specific cellular functions (Tab.2 and 3)[45,46]. They also differ in their catalytic properties; for example SIRT4-7 exhibit weak deacetylase activity in vitro whereas only SIRT1-3 possess efficient deacetylase activity in vitro[47]. Recent experiments showed that some of the SIRTs which possess weak deacetylase activity show more efficient activity for the removal of other acyl lysine modifications[47]. Tab. 3 summarizes the role of the human sirtuins.

Tab.1: Localization of different mammalian sirtuins[45].

SIRTUIN	**LOCATION**
SIRT1	Nucleus, Cytoplasm
SIRT2	Cytoplasm, Nucleus (transiently)
SIRT3	Mitochondria
SIRT4	Mitochondria
SIRT5	Mitochondria, Cytoplasm, Nucleus
SIRT6	Nucleus
SIRT7	Nucleolus

Tab.2: Sirtuin functions in yeast[46]

	Activity	**Target**	**Cellular and molecular function**
Yeast			
SIR2	Deacetylation	H4K16, H3K56, H3K4	Heterochromatin, gene silencing
Hst1	Deacetylation	H3K4	Transcription repression
Hst2	Deacetylation	H4K16	Telomere and nucleolar silencing
Hst3	Deacetylation	H3K56	DNA repair and replication
Hst4	Nucleus	H3K56	DNA repair and replication

In Drosophila melanogaster only five sirtuin genes exist, while dSIR2 (Sirt1) is the closest homologue to the yeast SIR2[46]. SIRT1 plays a role in histone deacetylation activity, protein deacetylation activity and transcription factor binding, it has been also obtained that SIRT1 has positive effect on Notch activation during wing development and in the context of sensory organ precursor specification[48–53]. In SIRT2,4,6 the main function is histone deacetylation activity, whereas the molecular function of SIRT7 remains unknown[50].

Mutations of the sirtuin genes SIRT1, SIRT2 and SIRT4 were performed and showed that none of them was lethal on its own and showed no obvious developmental phenotype. The introduction of mutations in SIRT6 and SIRT7 genes made in Krejčí lab lead also to viable flies with no phenotypes.

Tab.3: Sirtuin functions of mammals [45,46]

Mammal	Activity	Target	Cellular and molecular function
SIRT1	Deacetylation	H1K26, H4K16, p53, PGClα, NF-κB, FOXO1, FOXO3, FOXO4, Notch, HIFlα, 14-3-3, PI3K, DNMT1, TORC1, HSF1, Ku70	Insulin signaling, tumorigenesis, apoptosis, transcription silencing, mitochondria regulation cell proliferation and survival, tissue regeneration, differentiation, stress response
SIRT2	Deacetylation Demyristolation	H4K16, Tubulin, PAR-3, FOXO1, FOXO3, CDH1, CDC20, PGClα	Brain aging, adipocyte differentiation, genome integrity, oxidative catabolism, Mitosis, nerve myelination and regeneration,
SIRT3	Deacetylation Decrotonylation	LCAD, IDH2, GDH, ACS2, SOD2, HMGCS, OTC	TCA cycle, oxidative phosphorylation, oxidative stress, Fatty acid oxidation, Tumor suppression, Regulation of mitochondrial activity
SIRT4	ADP-ribosylation Deacetylation Lipoamidation	GDH, IDE, ANT2, ANT3, MCD, PDH	Fatty acid oxidation, TCA cycle Glucose metabolism Amino acid catabolism Tumor suppression
SIRT5	Deacetylation Desuccinylation Demalonylation Deglutarylation	CPS1 UOX	Urea cycle Amino acid metabolism Fatty acid metabolism
SIRT6	Deacetylation ADP-ribosylation Deacetylation Deacylation	H3K9, H3K56, PARP1, HIF1α, TNFα, GCN5	Telomere silencing, Genome stability
SIRT7	Deacetylation	RNA polymerase	rDNA transcription

In humans, a common catalytic core of around 250 amino-acids is found in the SIRT proteins. The N-and C-terminal regions differ in length. These regions seem to possess the ability of acquiring different conformational states. The binding of multiple partners and the different functions in cells may be explained upon the differences in primary structure of sirtuins as well as in differences in their post-translational modifications[54].

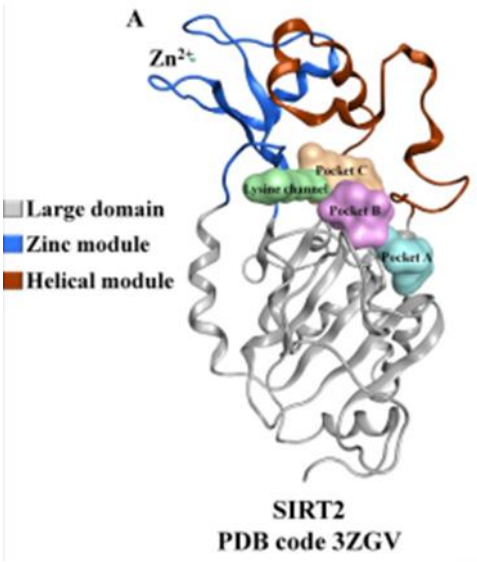


Fig.7: Three-dimensional structure of Sirtuins; SIRT2 as an example for the visualization[54].

Fig.8: The structure of the seven human SIRTs. Showing N-terminal domain in blue, C-terminal domain in green and catalytic domain in brown[54]:

The most studied sirtuin is SIRT1 that is also the closest homologue of the yeast SIR2 protein[46]. It is mainly localized to the nucleus and it plays a key role in H4K16 and H1K26 deacetylation and many other non-histone targets[55]. It has been extensively studied regarding its role in lifespan extension[56]. Reduced apoptosis is linked with the deacetylation of p53 by SIRT1 upon oxidative stress and DNA damage[57,58].It regulates mitochondria activities and biogenesis as well[59]. The acetylation state of the transcription factors FOXO which are as well regulated by SIRT1 are suggested to participate in regulation of metabolism and stress response by directing them to specific targets[60]. Activation of PGC1α is mediated by SIRT1 through deacetylation[59]. SIRT1 can be shuttled out from the nucleus to the cytoplasm by inhibiting the insulin signal pathway[46]. New functions of SIRT1 like delaying replicative senescence in primary fibroblasts, neuroprotection against diverse neurodegenerative diseases, stem cell differentiation, liver function promotion and cell fate determination have been nowadays established[61–67].

SIRT2 deacetylates tubulin and regulates skeletal muscle differentiation[60]. It can be found in the cytoplasm or nucleus of mammals[60]. According to latest researches it accumulates in neurons in the central nervous system in aging brains[68]. It has been found that its microtubule deacetylase activity is involved in the pathology of neurodegenerative and brain aging diseases[68]. It appears important for nerve myelination and regeneration by deacetylating Par-3 in the peripheral nervous system[69]. Par-3 is a critical regulator of myelin assembly in Schwann cells and cell polarity[69]. Through deacetylating PCG1α and FOXO1 it regulates metabolism in adipocytes[60,70]. It has been shown that FOXO3 is targeted for degradation and polyubiquitination when deacetylation occurs by SIRT1 or SIRT2[71]. Also reduced levels of SIRT2 were obtained in hepatocellular carcinoma and tissues from human breast cancers where the SIRT2 plays a role in regulating the anaphase-promoting complex (APC) activity in these tissues by deacetylating CDC20 and CDH1 to antagonize tumorigenesis and maintain genome integrity[46,72]. In humans it fulfills diverse funcitons[73]. It is important for cell differentiation, cell proliferation and plays a role as transcription factors that are involved in synaptic plasticity as well as in transcriptional repression of genes which are encoding for DNA binding proteins, in the nucleus[74]. In the cytoplasm, it has been obtained that it is involved in the stabilization of microtubules and deacetylates proteins of the cytoskeleton, one of these is the α-tubulin[75]. Further, some of the SIRT2 inhibitors play a role in reducing α-synuclein toxicity, tau phosphorylation, brain cholesterol levels in cell cultures and increase of the brain derived neurotrophic factor (BDNF) in the brain of mice[73,76–79].

Three of the sirtuins, SIRT3, SIRT4 and SIRT5 are localized mainly to the mitochondria[46]. SIRT3 targets diverse enzymes which play crucial roles in maintaining metabolic homeostasis as wells as enzymes required for generating AceCS2 and acetyl-coA[80]. SIRT3 is the major mitochondrial deacetylase which is as well involved in the fatty acid oxidation pathway where it deacetylates the long chain acyl CoA dehydrogenase (LCAD)[46]. It has been shown that glutamate dehydrogenase (GDH) and isocitrate dehydrogenase (IDH2) are targets of SIRT3 indicating the role of SIRT3 in the TCA cycle[46]. In oxidative phosphorylation it is involved in deacetylation of components in each step of electron transport[81]. SIRT3 is also involved in protection against oxidative stress due to deacetylation and activation of SOD2 (mitochondrial oxidant)[80].New studies showed that SIRT4 negatively regulates fatty acid oxidation in muscle and liver cells, showing an opposite effect of SIRT3[60]. It is also known that SIRT4 is mainly an ADP-ribosylase targeting glutamate dehydrogenase (GDH)[60]. Succinyl or malonyl groups are removed in a very similar manner to deacetylation by targeting the carbonyl phosphate synthetase (CPS1) by SIRT5[60].

The function of SIRT6 is the deacetylation of H3K9 and H3K56 histone and it is localized in the nucleus, more functions can be obtained from Tab.3 [46]. According to new studies SIRT6 is an ADP-ribosylase for PARP1 and supports DNA repairs by stimulating the PARP1 function[82].

SIRT7 is found in the nucleolus and it plays a role in the activation of RNA polymerase I transcription[60]. Diverse pathologies like hepatic steatosis, cardiac hypertrophy and deafness were linked to the deficiency of SIRT7[83]. It positively controls ribosome production, via direct interaction with the PolI machinery, at coding regions and promoters of ribosomal genes[83]. According to new studies SIRT7 may have the ability to act as an oncogene, thanks the ability of transcriptional regulation of a certain set of genes through direct interaction with the EKL4 transcription factor[83]. Via histone H3K18 deacetylation, SIRT7 negatively regulates the transcription of genes outside of the rDNA repeats[83]. Although it's catalytic activity and targets remain in some cases unknown plenty interaction partners have been identified like RNApoII and rDNA transcription factor UBF[46].

2. AIMS OF THE THESIS

We know that there are five types of the sirtuin genes present in the fruit fly Drosophila melanogaster and none of them is lethal on its own when mutated nor shows any physiological phenotypes, which could be due to a functional redundancy amongst the sirtuin genes or the fact that they are predominantly needed during stress conditions. This is based on the research of the team of Alena Krejčí, where mutations in genes SIRT1, SIRT2, SIRT4, SIRT6 and SIRT7 were studied. The phenotypic consequences of a combination of more than one sirtuin mutation has so far not been investigated in any organism. Therefore, the Krejčí lab pursued the idea of the creation of a "super fly" that has all the five sirtuins genes mutated. The goal was to create such a fly and then check its lethality and characterize its phenotype, amongst other by various stress tests like temperature, oxidative and nutritional stress. If the "super fly" is lethal or has an interesting phenotype, we could then identify the combination of sirtuins genes that is responsible for it. However, before our lab could start with this project, a fly that would bear SIRT2, SIRT6 and SIRT7 mutations on the same chromosome was needed, since all these three sirtuins are located on the third chromosome.

The aim of my thesis was to create a fly that is a mutant for two sirtuin genes, SIRT2 and SIRT7, that would then serve as a platform for the creation of the triple SIRT2,6,7 mutant fly and, ultimately, for the creation of the 'super fly' with all five sirtuin genes mutated.

The creation of the double mutant fly for SIRT2 and SIRT7 genes was achieved by using two different approaches:

1. Meiotic recombination of existing SIRT2 and SIRT7 mutations on the third chromosome.

2. Crispr/Cas9 technology to introduce *de novo* mutation in SIRT7 on the background of SIRT2 mutant.

3. METHODS

Plenty of methods have been applied for the success of my project, starting from the fruit fly breeding and genetics till the usage of several molecular biology tools, one of them the latest one; CRISPR/Cas9 system.

3.1. The fruit flies maintenance

The fruit flies were kept in glass vials closed with cotton wool and their food consisted of corn, flour, yeast, agar and glucose. Dry pieces of yeast were put on the top of the food mixture.

Tab.4: Ingredients

Ingredients:	
corn flour	160g
yeast	30g
agar	9g
glucose	150g
water	1,9l

Incubators with a media of 25°C and 18°C were used for the storage of the fruit flies. Dependent on the media the virgins were collected during a certain period of time; at 25°C media the fruit flies were collected within 8 hours whereas for 18°C media the collection time was within 18 hours. The appearance of the fly was also important for choosing the virgin flies, a dark green spot on their abdomen as well as their pale appearance helped to choose correctly. It was important to supply the flies with fresh food, water and clean vials to keep the healthy and viable.

Different strains of the fruit fly *Drosophila Melanogaster* were used for my project which can be seen in Tab 5.

Tab.5: Strains of fruit flies I used during my project, showing the chromosomal location of genes and transgenic elements

	Chromosome	Stock
SIRT2	III	
SIRT7	III	
SIRT7 gRNA	II	
Nos-Cas9/cyo	II	
TM3/TM6	III	balancer stock for III
If/cyo; TM3/TM6	II, III	double balancer stock
Sp/cyo; TM3/TM6	II, III	double balancer stock

3.2. Verification of genotypes

Before starting the experiment, we needed to verify the genotypes of the SIRT2 and SIRT7 mutant flies, to ensure that the flies were indeed carrying these mutations. The presence of SIRT2 and SIRT7 mutations needed to be verified also in the potential double mutant flies I created during my project.

3.3. The verification of the SIRT2 mutations

After the extraction of DNA from a single fly polymerase chain reaction (PCR) was used to verify the SIRT2 mutation. The DNA fragments were visualized by electrophoresis to obtain if the occurrence of the mutation on the agarose gel is present or not. The primers were designed in such a manner that they span the SIRT2 gene; if mutation is present the resulting PCR product is short (0.2kb) while in wildtype allele the product will be 1.4 kb. Dependent on the size of the PCR product on an agarose gel we were able to decide if the flies contain the SIRT2 mutation.

The DNA fragment size is measured in base pairs (kb)

Following sequence of primers for SIRT2 verification were used:

Tab.6: primers for verification of SIRT2 mutation

CG5085-C1:	**CG5085-C2:**
GCCCCAGGCTAGTCTAAATAG	GAAAGAAAGCTCGCGCTATTAG

The DNA isolation, polymerase chain reactions (PCRs) and electrophoreses were performed according to two protocols:

3.3.1. Protocol I

Extraction of DNA from a single fly for PCR analysis

Firstly, a male fly is taken out of the stock and transferred into 50µl squashing buffer (SB) containing 0.5µl of proteinase K (20mg/ml stock).

Then the male fly is mashed until the solution becomes cloudy

Incubation for 30 min at 37°C in the heat block

Incubation for 2 min at 95°C in the heat block

Centrifugation at 14000 rpm for 1 min

Take out 0.5µl of extracted DNA for 20µl PCR reaction

PCR

Each reaction is carried out in in 20µl using OneTaq DNA polymerase from NEB. A master stock, for every sample, is prepared by mixing the substances in the following order:

Tab7: The composition of PCR reaction used to amplify genomic DNA from flies

Substances	Amount
DEPC H2O	14.5µl
OneTaq standard reaction buffer	4µl
dNTP	0.4µl (10mM)
5'primer	0.4µl (10µM)
4'primer	0.4µl (10µM)
Taq Polymerase	0.2µl

During mixing, all the solutions have to be kept on ice. It is important to place the Taq Polymerase in a cooling block during this process. 0.5µl of extracted DNA are mixed then with the prepared 19.5µl of the master mix in a thin walled PCR tube. Afterwards the mixture is centrifuged and placed in the PCR cycler with a block temperature of 94°C. Specific programs are chosen for different screenings.

Thermocycling conditions for a routine PCR

Tab.8: Order of steps for thermocycling conditions for a routine PCR with OneTaq polymerase from NEB.

Step	Temperature	Time
Initial Denaturation	94°C	30 seconds
30 cycles	94°C 55°C 68°C	20 seconds 30 seconds 40 seconds
Final Extension	68°C	5 minutes
Hold	4-10°C	

Electrophoresis

1µl of 6x loading buffer is used which is mixed with 5µl of the centrifuged PCR product. The solution is run in 1.5% agarose gel (Tab. 9).

Tab.9: Composition of agarose gel

Substance	Amount
agarose	1.5g
TBE buffer (0.5x)	100ml
EtBr	3µl

A DNA ladder is run along the side of the extracted DNA for the determination of the DNA fragments size. We use 1kb plus DNA ladder from NEB. Fig.9 shows the sizes of all the fragments of this ladder.

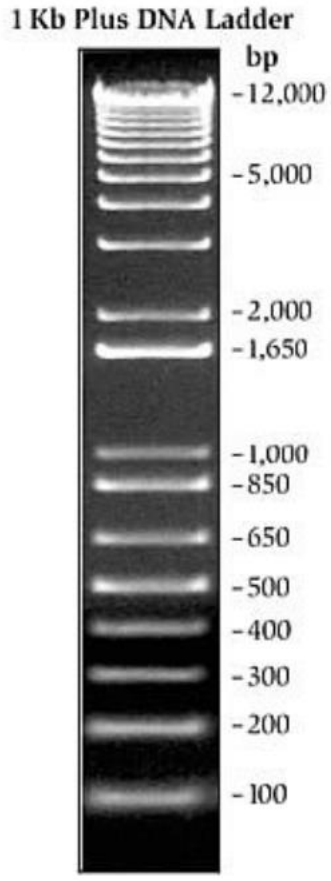


Fig.9: The 1kb plus DNA ladder from NEB.

3.4. Verification of SIRT7 mutations

The verification of the SIRT7 mutant is performed in a different manner than the verification of SIRT2 mutation. The SIRT7 mutation was introduced into the genomic DNA by CRISPR that causes a deletion of a few base pairs in the open reading frame of the genes, causing a frame shift during translation. As only few base pairs are missing in the mutant in comparison to wild type allele, the presence of SIRT7 mutations could not be tested simply by PCR. Instead, we used T7 endonuclease digest of a PCR product.

A T7 endonuclease can be used to identify small mutations in DNA isolated from organisms heterozygous for particular gene of interest. It recognizes structural defects caused by DNA heteroduplexes and cleaves it at the site of this mismatch. We used PCR to amplify region from genomic DNA of heterozygous files presumably bearing the mutation in SIRT7 and digested it with T7 endonuclease. When SIRT7 mutation was present we saw two extra small DNA bands on the agarose gel after electrophoresis (principle of the method can be seen in Fig.10)[9].

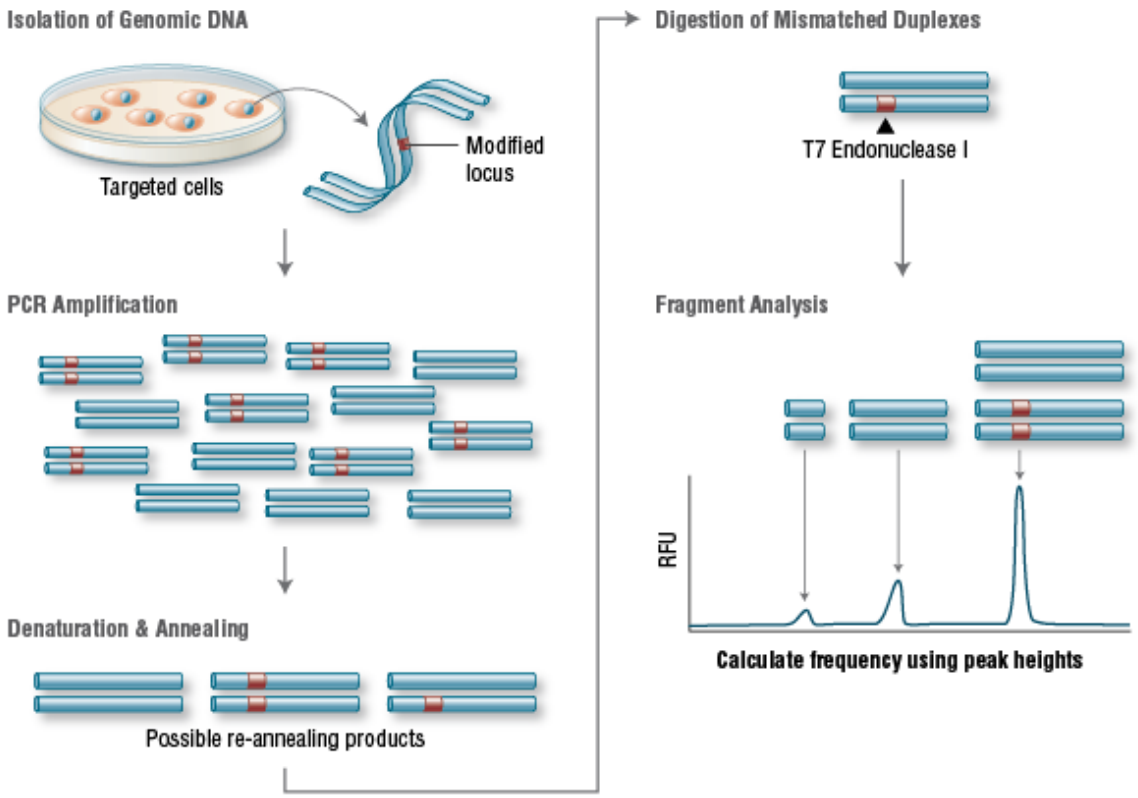


Fig.10: The principle of DNA mutation detection by T7 endonuclease. PCR with a specific primer pair is used to amplify genomic DNA spanning the presumptive mutation, using heterozygous flies. Different DNA structures occure after denaturing and re-annealing of the PCR product. A mismatch in DNA duplexes is cleaved off by T7 endonuclease. The DNA can then be separated by electrophoreses[9]

T7 endonuclease recognizes and cleaves off non-perfectly matched DNA along the double strand at the position where a mismatch or a loop is created. Therefore, we used to test heterozygous flies. The region around the site of SIRT7 mutation is amplified by PCR using flies with TM3 balancer.

The resulting PCR product mixture, if the SIRT7 mutation occurs on the non-balancer chromosome, would contain wild type and mutated products of PCR. After random hybridization, the wild type and the mutated strands would form a heteroduplex that would allow the T7 endonuclease to recognize and precisely cleave it off.

The digested PCR product was run on the agarose gel via electrophoresis leading to one band of the size of 650 bp if the mutation is absent or two smaller bands of 400 and 250 bp when digestion occurred. As a positive control, we used a fly strain that was previously sequenced for the presence of SIRT7 mutations.

Afterwards the DNA is isolated from a homozygous fly, amplified by PCR and sent for DNA sequencing to obtain the exact sequence of the mutation present.

3.4.1. Protocol II

DNA Isolation and PCR analysis (as described above)

Following sequence of primers are used:

Tab.10: Primers for PCR amplification of SIRT7 and SIRT6 region from genomic DNA containing a presumptive SIRT7 mutation.

SIRT6 CRISPR ver s:	**SIRT7 CRISPR ver s:**
ACGTTGCAGGGATTTTTGAC	GGAAGCGAGTCATTCCTACG
SIRT6 CRISPR ver a:	**SIRT7 CRISPR ver a:**
TTTGTAGCGTTACGGATACGG	GCTTCTTGGTCGTCTTCACC

Digestion of purified PCR product by T7 endonuclease

In a 1.5ml Eppendorf tube the PCR product is transferred, mixed with 60µl DEPC water (H2O) and 8µl of 3M Sodium Acetate (CH3COONa). The mixture is vortexed for a short time.

250µl of 100% ethanol, which serve as a molecular biology grade, are added and mixed by inverting the mixture couple of times. After mixing it is stored at -20°C overnight or -80°C for some hours.

Centrifugation for 20 min at 20000rpm at 4°C.

The ethanol is carefully poured away.

Remaining DNA pellet is treated with approximately 0.5ml of 70% ethanol and mixed by inverting the mixture couple of times.

Centrifugation for 10 min at 20000 rpm at 4°C.

The ethanol is carefully poured away. The remaining ethanol is centrifuged and removed by a yellow tip allowing the pellet to be attached to the side of the tube.

The DNA pellet is dried at room temperature by opening the lid of the Eppendorf.

16µl DEPC water are added to the tube and 1µl is used to measure concentration and purity.

The working solution of T7 endonuclease is prepared from the stock solution, with concentration of 2 units per µl:

Tab.11: Working solution of T7 endonuclease to obtain concentration of 2u/µl.

Substance	**Amount**
DEPC H2O	4.4µl
T7 endonuclease	1.1µl (10 units/µl)

During preparation, all the solutions have to be kept on ice. Fast adding to the reaction mixture is recommended. The following reaction is prepared:

Tab.12: Solution for the T7 endonuclease reaction

Substance	Amount
DEPC H2O	12.5µl
PCR product	5µl
Buffer 2	2µl
T7 endonuclease working solution	0.5µl

The digestion is carried on by incubation at 37°C for 2 hours.

Electrophoresis

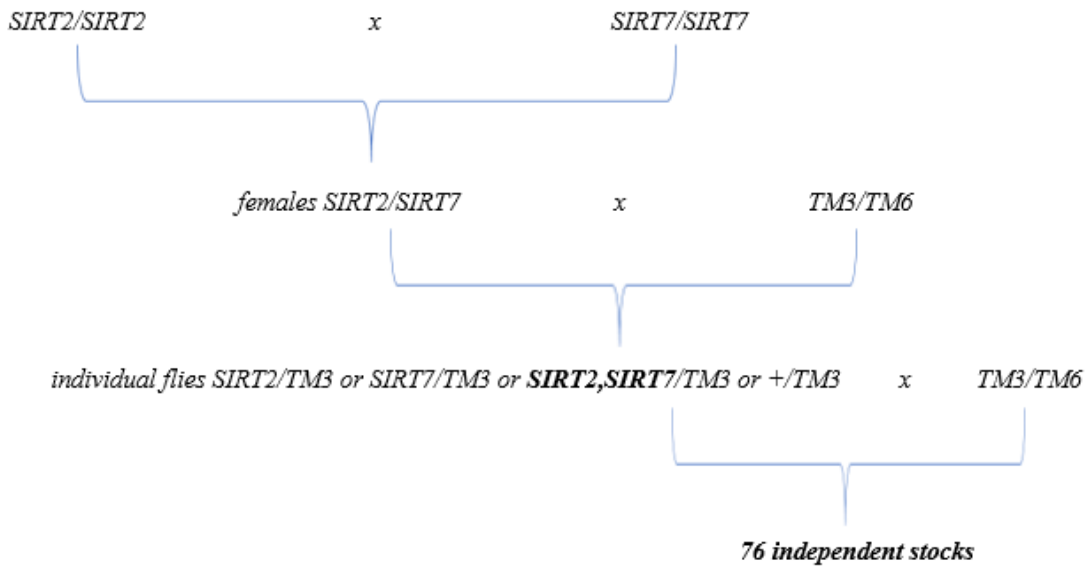
4µl of loading dye are mixed with the DNA and stored on ice. 10µl are run on a 2% gel.

3.5. Crossing procedure

Creating double mutant for SIRT2 and SIRT7 by meiotic recombination

Thanks to the locations of SIRT2 gene at the position 92E3-92E3 and SIRT7 gene at the position 98F6-98Fg at the III chromosome, the creation of a double mutant by meiotic recombination was expected to happen with low recombination frequency.

The crosses were performed by following procedure:



First, of all, we crossed SIRTSIRT2/SIRT2 with SIRT7/SIRT7

As recombination only occurs in females, around 6-8 SIRT2/SIRT7 virgin females were collected and crossed to 4 TM3/TM6 males for balancing

110 individual virgin female or male flies over TM3 or TM6 were collected from the previous cross and crossed again with TM3/TM6 female virgin or male flies.

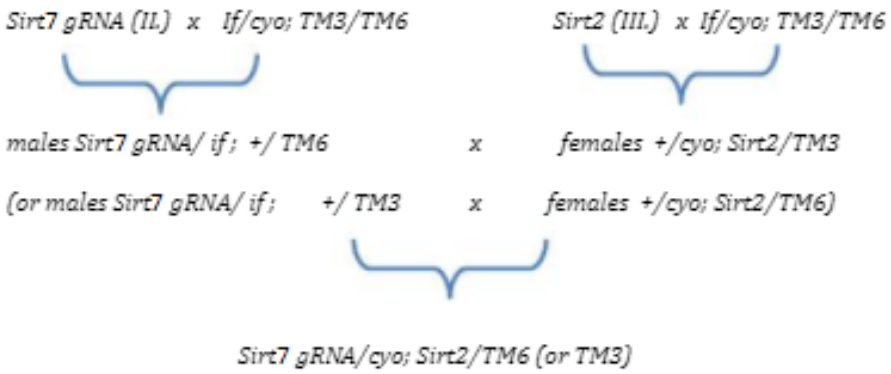
76 independent stocks were created by collecting and crossing brothers and sisters for each vial. They either contained the SIRT2 allele, SIRT7 allele, the recombinant of SIRT2+SIRT7 on the same chromosome or the reciprocal wild type allele, always over a balancer chromosome. However, we could not simply distinguish the genotypes from each other. Therefore, PCR was used to screen these 76 stocks for the detection of the SIRT2 and then SIRT7 mutation.

First, stocks were screened fir the presence of SIRT2 mutation and then only the SIRT2 positive flies were taken for the T7 endonuclease analysis to detect the presence of the SIRT7 mutation.

Creating double mutant for SIRT2 and SIRT7 by CRISPR/Cas9 technology

In order to create the double mutant for SIRT2 and SIRT7 by Crispr/Cas9 system we first needed to make a fly carrying SIRT7gRNA and SIRT2 mutation. By crossing it to a fly expressing Cas9 in the germline, it allowed us to create a double mutant through CRISPR induced mutagenesis of SIRT7 on the SIRT2 mutant background.

The following scheme shows the crosses we set to obtain the SIRT7 gRNA; SIRT2 flies.



Afterwards the above described stock was made homozygous and crossed to a fly expressing Cas9 nuclease under the control of germline specific promoter from the nanos gene (nos):

SIRT7gRNA; SIRT2 x nos-Cas9/cyo

Flies of the SIRT7gRNA/nos-Cas9; SIRT2/+ genotype were collected. In these flies, the SIRT7 gRNA and Cas9 meet in order to introduce SIRT7 mutation.

Around 30 individual flies were crossed with a TM3/TM6 stock to establish independent lines.

PCR was used for verification of the presence of SIRT2 mutation and the presence of SIRT7 mutation was tested by T7 endonuclease according to protocol II.

The sample was sent for sequencing if both mutations occurred.

4. RESULTS

4.1. Optimization of the T7 endonuclease digest

To ensure proper and accurate function of the T7 endonuclease, the optimization of the enzymatic reaction had to be performed, since many factors can affect the function and quality of an enzyme. Incubation time was chosen to be 37°C and the enzyme and DNA concentration was optimized in the reaction mixture. This was performed in cooperation with my colleague Paraskevi Tziortzouda (who worked on the creation of SIRT2, SIRT6 double mutant fly).

Firstly, the genomic DNA was isolated from a SIRT6 and SIRT7 mutated homozygous males. The DNAs were then mixed in a 1:1 ratio together leading to heterozygosity in SIRT6 and SIRT7 alleles. We mixed the DNA of SIRT6 and SIRT7 homozygous flies but we could easily use genomic DNA form a heterozygous SIRT6 or SIRT7 males as well. PCR was performed with SIRT6 primers as described above and digested by various concentrations of T7 endonuclease, with different amounts of DNA. Electrophoreses was used to run 10µl of the digest on a 2% agarose gel.

The appearance of additional smaller bands in the gel was the result if the digestion occurred. If no mutation was present, only one band would appear on the gel.

The results from the optimization of the T7 endonuclease can be observed from Fig.11. By mistake, DNA ladder was mixed with all the samples leading to extra bands in lanes 3-6 (A-D); these are not products of a digest but represent the DNA ladder fragments. Nevertheless, the results of the experiments can still be interpreted.

As apparent from Fig.11, both concentrations of T7 endonuclease with both the concentrations of DNA lead to DNA digest. Based on these optimization reactions we decided to use these conditions for the T7 digest for verification of all our potential SIRT2, SIRT7 mutants:

A: 12.5 DEPC water, 5ul PCR product, 2ul buffer 2, 0.5µl T7 endonuclease working stock (1u).

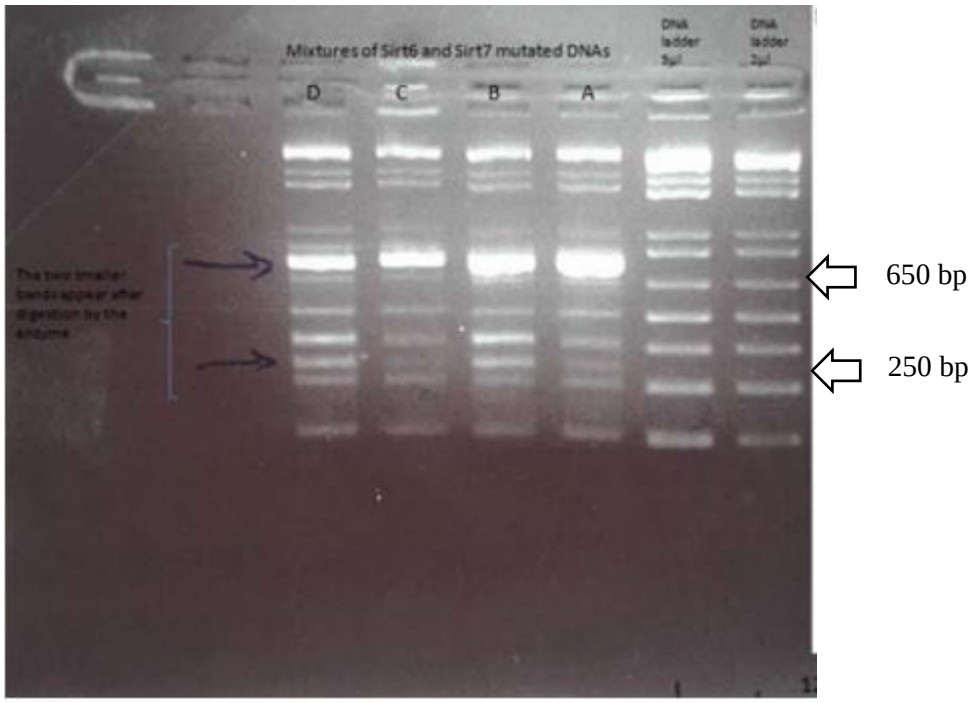


Fig.11: The result of the optimization reactions for T7 digest of PCR product amplified from region spanning the presumptive SIRT6 deletion. We tested different amounts of enzyme and PCR product to obtain the optimal condition for T7 digest. The first arrow indicates the undigested product, the second arrow represents the first of the two fragments of the digested product having 250bp. The second digested fragment overlaps with the 400bp ladder band. By mistake, DNA ladder was mixed with all the samples leading to extra bands in lanes 3-6 (A-D). These are not products of a digest but represent the DNA ladder fragments.

Different amounts of T7 enzyme were used as well as 2 different amounts of DNA. A: 12.5 DEPC water, 5ul PCR product, 2ul buffer 2, 0.5µl T7 endonuclease working stock (1u). B: 11.5µl DEPC water, 5µl PCR product, 2µl buffer 2, 1.5µl T7 endonuclease working stock (3u). C: 15µl DEPC water, 2.5µl PCR product, 2µl buffer 2, 0.5µl T7 endonuclease working stock (1u). D: 14µl DEPC water, 2.5µl PCR product, 2µl buffer 2, 1.5µl T7 endonuclease working stock (3u). Incubation of 2 hours was performed. The last two lanes represent the 1kb pus DNA ladder, 5µl and 2µl.

4.2. Creation of double mutant for SIRT2 and, SIRT7 created by meiotic recombination

As mentioned above, two approaches were used to combine the mutation for the sirtuin genes SIRT2 and SIRT7 on the same chromosome; meiotic recombination of existing SIRT2 and SIRT7 mutants and CRISPR mediated mutagenesis of SIRT7 on the background of existing SIRT2 mutation.

Firstly, 76 independent lines were established by recombining existing SIRT2 and SIRT7 mutations on the same chromosome, as described in the Methods. Since there is no phenotypic marker for SIRT2 and SIRT7 mutations we screened those independent lines by PCR for the

presence of SIRT2 mutation in their genomic DNA. The result of this screening is shown in gels on picture 12 A-D (some lines were tested more than once). We obtained 29 positive results which were afterwards tested for the presence of SIRT7 mutation by T7 digestion.

Only one SIRT7 positive result was obtained from the 29 lines screened, indicating that the flies from stock 52 are double mutants, see Fig.16. Afterwards genomic DNA was isolated from homozygous flies and PCR with SIRT7 primers was performed and sent for sequencing to verify if mutation occurred and to check that it is not in-frame mutation. Fig.14 shows the alignment of the SIRT7 wild type genomic DNA and the sequencing results from line 52, confirming a presence of 5 bp mutation in SIRT7 gene close to the open reading frame:

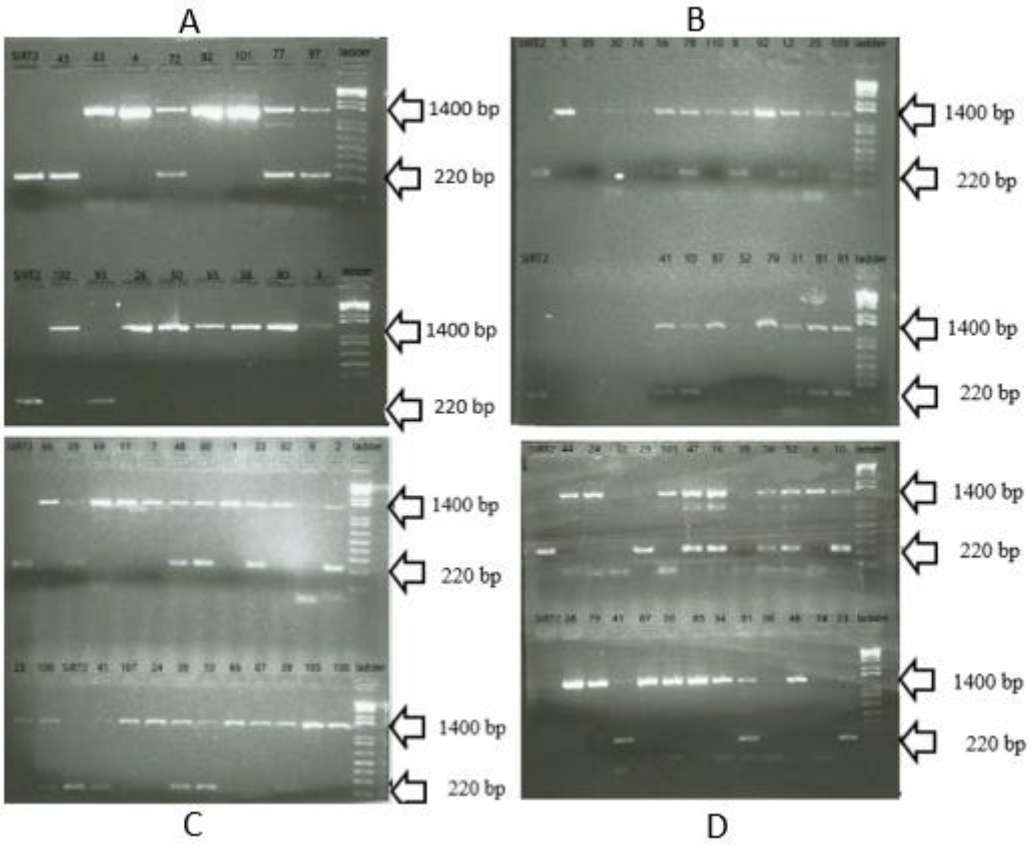


Fig.12: Result of screening by PCR for the presence of SIRT2 mutation in potential SIRT2,SIRT7 mutant flies created by meiotic recombination. If SIRT2 mutation is present, we detect a band at around 220bp, whereas wildtype allele amplifies a band of 1400 bp. Positive results in stock #93, #97, #77, #72 and #43 (gel A), #41, #10, #31, #81, #91, #109, #25, #12, #8, #78, #56 and #30 (gel B), #109, #41, #10, #39, #2, #33, #80, #48(gel C) and #51, #23, #10, #52, #76, #16, #47, #29(gel D).

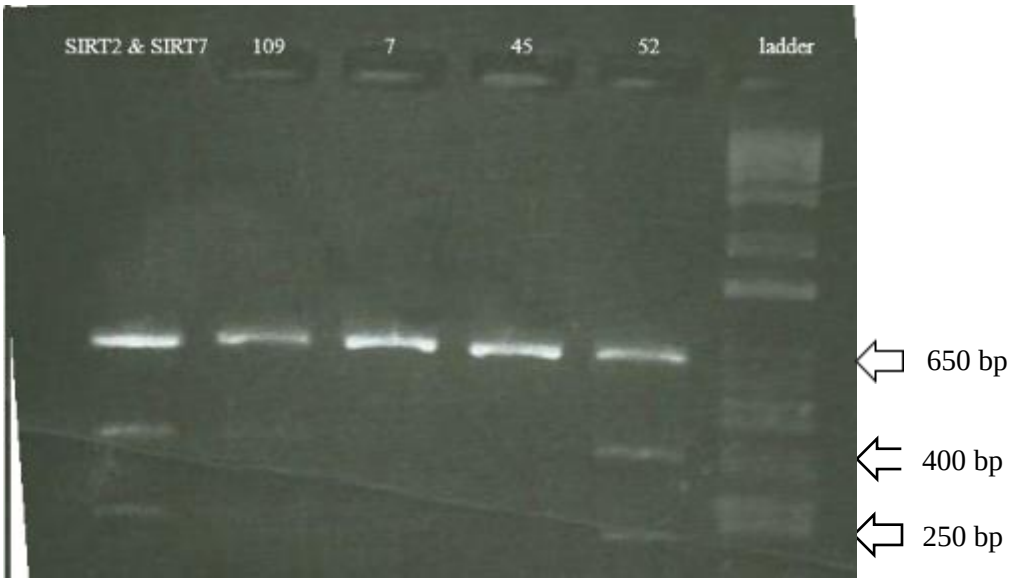


Fig.13: The verification of SIRT7 mutation by T7 endonuclease digest in potential SIRT2, SIRT7 flies created by meiotic recombination. The fly #52 is a double mutant for the two sirtuin genes SIRT2 and SIRT7. Same fragments (around 400bp and 250bp) are obtained in the #52 DNA as in the positive control (mixture of SIRT2 and SIRT7 mutated DNA), indicating the mutation. The other PCR products from fly lines 7 and 45 show only one fragment of 650bp, indicating no mutation. Line 109 may also be positive for SIRT7 mutation but we have not tested it further.

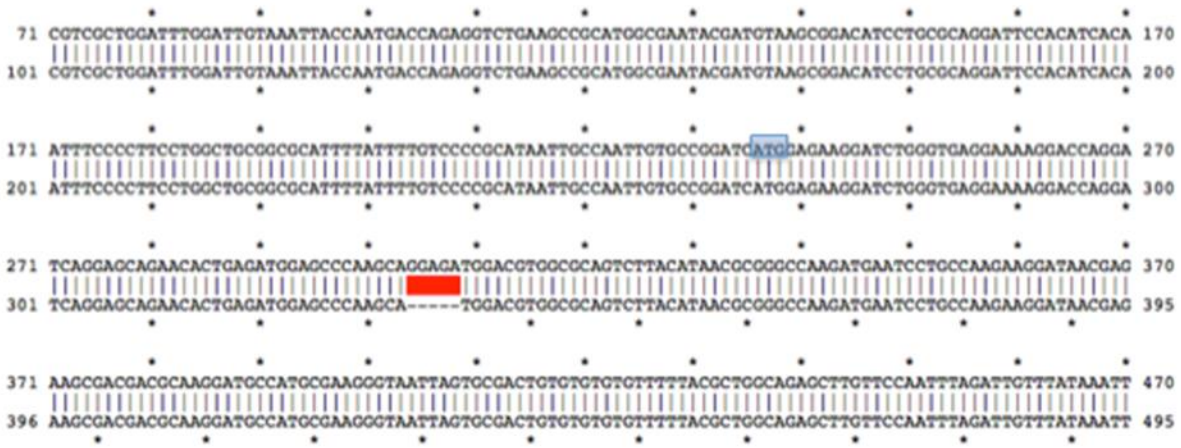


Fig.14: The alignment of SIRT7 wild type genomic sequence (upper line) with the result of the sequencing reaction from fly strain 52 (bottom line). The 5-nucleotide deletion in the open reading frame of SIRT7 gene in fly strain 52 is indicated in red, start od translation in blue.

4.3. Creation of the double mutant for SIRT2 and SIRT7 by the CRISPR/Cas9 technology

Here we introduced the SIRT7 mutation *de novo* by CRISPR/Cas9 technology into *Drosophila melanogaster* with the SIRT2 mutation already present.

30 independent lines were created (see Methods) and we needed to test them for the presence of SIRT2 and SIRT7 mutations. The genomic DNA was isolated from 16 heterozygous fly lines. Due to the crossing scheme (see Methods) our expectation for the detection of SIRT2 mutations was around 50% which we nicely confirmed (7 positive lines from 16 screened, see Fig.15). The presence of SIRT7 was again verified by T7 endonuclease digestion but we selected only these 7 lines where we confirmed the presence of SIRT2 mutation. This way we identified 3 fly lines bearing mutations for SIRT2 and SIRT7 (Fig.16). We sequenced the SIRT7 gene from line 17 to confirm the mutation and see if it is not in-frame. The alignment can be seen in Fig.17 and indicates that we introduced 1 bp deletion close to the open reading frame of SIRT7 gene.

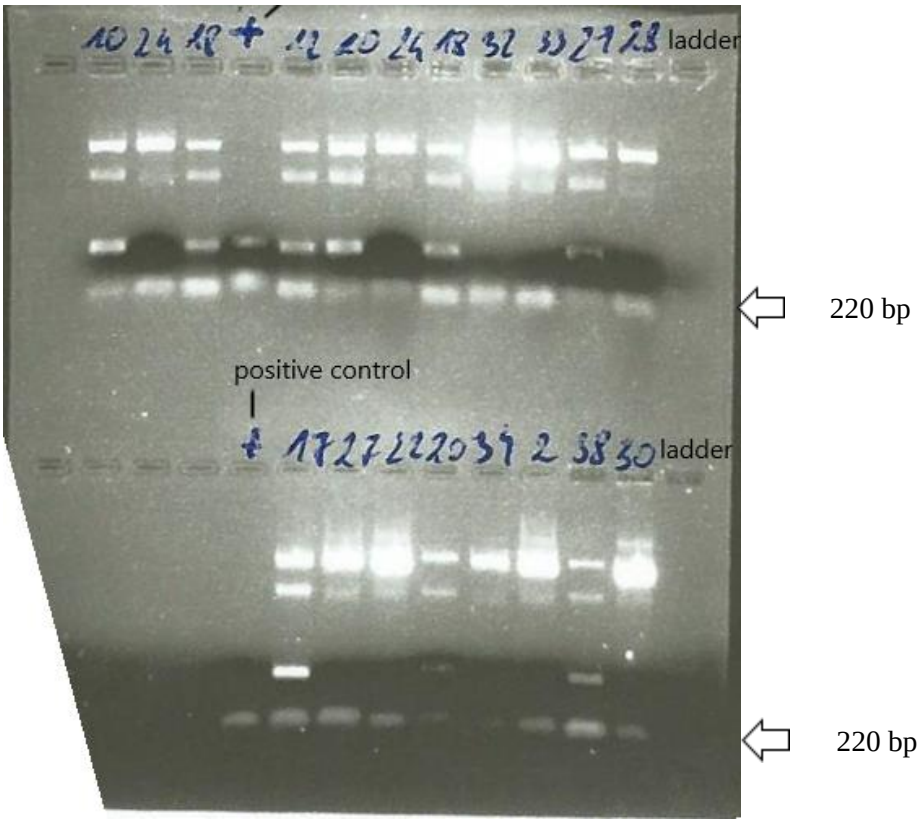


Fig.15: Results of the verification of the presence of SIRT2 mutations in different stocks potentially bearing the SIRT2 and SIRT7 mutations, created by Crispr/Cas9 methodology. The appearance of a band of 220 bp indicates that SIRT2 mutation is present in the stocks #10, #18, #12, #20, #29, #38, #17. The positive control (+) indicates a PCR product from SIRT2 homozygous line.

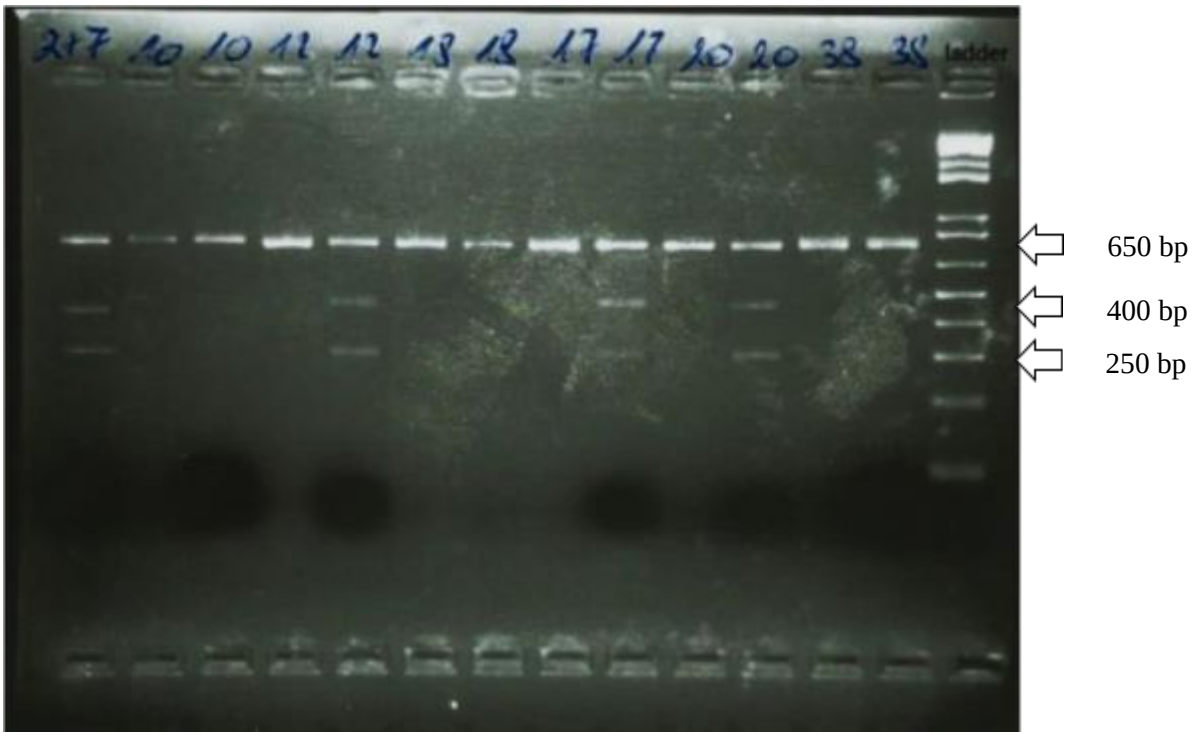


Fig.16: Testing the presence of SIRT7 mutation in the potential double SIRT2, SIRT7 flies created by CRISPR/Cas9 technology. SIRT7 mutation appears to be present in the stocks #12, #20, #17. The size of the undigested product is around 650 bp, whereas the size of the 2 digested fragments are around 250bp and 400bp. The undigested product was always loaded next to the digested product, therefore sample numbers appear twice on the picture. First lane in the gel represents a positive control.

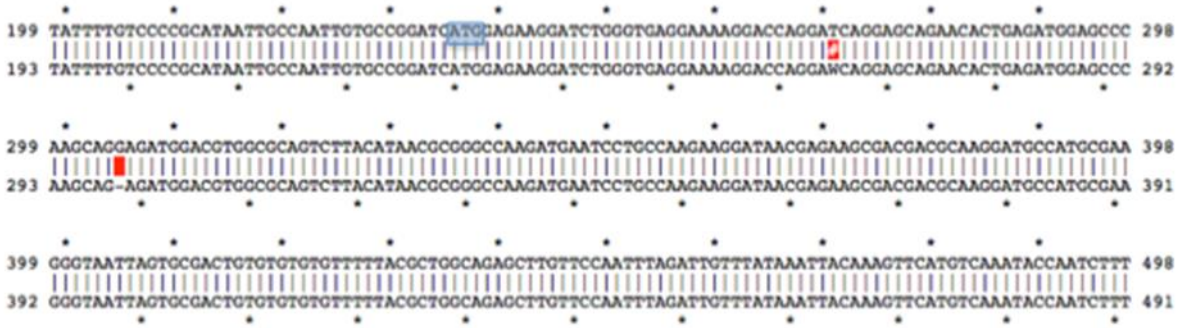


Fig.17: The alignment of SIRT7 wild type genomic sequence (upper line) with the result of the sequencing reaction from fly strain 17 (bottom line). The 1 nucleotide deletion in the open reading frame of SIRT7 gene in fly strain 17 is indicated in red, start od translation in blue. Red square with a hash represent an inaccuracy in the sequencing reaction but manual checking confirmed the right sequence (base T).

5. DISCUSSION

5.1. Comparing the efficiencies between meiotic recombination and CRISPR method

In my thesis, I successfully employed two different methods for the creation of a Drosophila melanogaster mutant bearing SIRT2 and SIRT7 mutation: meiotic recombination of the two existing mutations on the same chromosome and CRISPR/Cas9 technology to introduce de novo SIRT7 mutation on SIRT2 mutant background. This result leaded to several independent stocks which can be used for further genetic experiments. Also, the success of these two different methods allows us to compare them in terms of their efficiency. With the meiotic recombination, out of 76 independent fly stocks only 1 fly was a double mutant for SIRT2 and SIRT7 showing an efficiency of 1,32%. By using CRISPR/Cas9 a much better result was obtained, 18,75%, where 3 out of 16 fly stocks showed double mutations. The success of meiotic recombination depends on the distance of the two genomic elements of interest on the chromosome; the further apart they are the bigger recombination rates[8]. In our case SIRT2 and SIRT7 genes lie relatively close to each other so the recombination rate was extremely low. In other cases, however, the recombination rates could be much better. The Crisp/Cas9 approach guarantees a success but it requires the creation of gRNA flies or injection of the gRNA into the embryos of the Cas9 flies, which is time consuming in the first place and requires good injections skills. We were lucky to have the gRNA flies already available in the lab, otherwise the whole approach would require much more time. Therefore, meiotic recombination could sometime provide faster results.

5.2. The double mutant for SIRT2 and SIRT7 lacks any developmental phenotype

We were curious to see if the double mutant fly will show any phenotype, indicative of a problem during development. Although the individual knock-outs of sirtuins have been studied in mammals[46], nobody tested the phenotype of animal's mutant for more than one sirtuins. To our surprise, the SIRT2, SIRT7 flies looked completely normal, with no obvious developmental defects in any part of the body. This could mean that either there is a functional redundancy between the sirtuins and the SIRT2 and SIRT7 enzymatic activities are taken over by any of the other three sirtuin members still present in the fly. SIRT2 and SIRT7 are present in the nucleus and cytoplasm[45] so maybe SIRT1 takes over for them since it is also found in the nucleus/cytoplasm. Alternatively, we did not see the developmental phenotype in the double SIRT2, SIRT7 mutant because they affect the fly's physiology and/or response under stress conditions. We planned to perform various stress tests (like starvations stress, chill coma

recovery stress, oxidative stress) but this was not possible in the time frame of my bachelor thesis.

5.3. The advantages and caveats of genome engineering by CRISPR/Cas9

Four different approaches have been used so far as tools for the effective genome editing : transcription activator-like effector nucleases (TALENs), Zinc-finger nucleases (ZFNs) based on eukaryotic transcription factors, meganucleases from microbial mobile genetic elements and the CRISPR/Cas9 system[19,84–87]. It was shown that the RNA-guided DNA endonuclease Cas9 from the type II bacterial adaptive immune system CRISPR has the highest efficiency and the lowest customization costs[84,88]. In comparison with the two sequence-directed nucleases (TALENs) and (ZFNs) which, in pairs, can generate targeted double stranded breaks (DSBs), the custom modifications for the CRISPR/Cas9 system are much easier [88]. The system consists of a common nuclease, Cas9, which is directed to the targeted loci through simple base pairing by a small RNA molecule[88]. Another benefit is the ease of the technique, where introduction of mutations via injection of different gRNAs can be established, without the need of a transgenic fly with stable gRNA insertion.

One of the problems connected with the CRISPR-introduced mutations is the frequency of in-frame mutations, resulting in a protein missing several amino acids instead of a nonsense protein. Although a mutation occured, in this case it may not affect the protein function and produce the expected mutant phenotype.

Another serious problem is so called off target mutation/cleavage effect[9,29,88,89]. Cas9 generates double sited breaks (DSBs) with its two nuclease domains, this leads to a cleavage specificity of only 22 nucleotides[88]. However, it also tolerates mismatches at the 5' end of the targeting sequence and therefore, it could pair with more than one place in the genome. This would lead to the introduction of a undesired mutation, since it would target another region than we aimed for[9,29,30,88–95]. By adding two extra guanin (G) nucleotides to the 5' end or by establishing a truncated gRNA which truncates within the crRNA-derived sequence, some improvements can be performed to reduce the off-target mutation/cleavage[94,96]. Another way is by using leveraging computation for the creation of webbased tools to ease the identification of potential CRISPR target sites, for example ZiFiT Targeter[97,98]. A smart approach was introduced by Cong and his colleagues[19] where the researches mutated Cas9 so as it has only a nickase activity and therefore creates just a ssDNA breaks, not DSBs. Two Cas9/gRNA are then needed to meet at the target of interest and only if two ssDNA cuts are introduced the DSB happens and a mutation can be created. The target specificity then doubles and off-target effect is minimal

since ssDNA break can be easily repaired without introducing a mutation, if it happens on its own.

To detect an off-target mutation is virtually impossible since we could not know where it happened and it would require sequencing of the whole genome. However, we can 'clean' individual chromosomes by crossing and recombining for at least six generations with a wild type strain. In this project, it would not make really a sense, since our flies do not have a phenotype or a dominant marker that we would need during this approach. We would need to sequence all the flies which would be a very time consuming and expensive process.

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