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Department of molecular biology and genetics

Characterisation of *Drosophila melanogaster* with mutations in all genes of the Sirtuin family

Master thesis

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Experimental biology: molecular and cell biology and genetics

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Annotation

The aim of my study was to create a *Drosophila* line lacking the expression of all Sirtuin genes, check its developmental phenotype and characterise its response in stress conditions. The flies had bigger weight than controls, they had decreased fertility and fecundity and they developed more slowly. They showed a trend towards increased resistance to chill coma, but they did not show a significant difference in starvation or oxidative stress assay. Its effect on lifespan is being investigated.

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1 INTRODUCTION

1.1 Sirtuins and function

Sirtuins are a group of NAD+ dependent deacetylase proteins responsible for many metabolic and physiological pathways in the organism of archaea, bacteria and eukaryotes. They were first discovered in *Saccharomyces cerevisiae* in 1979 in connection with the regulation of gene silencing and lately also in connection with the regulation of lifespan and, importantly, in connection with regulation of metabolism (1) (2). Sirtuins in various organisms are homologs of the yeast Sir2 gene (Silent-mating-type information regulation 2) that in yeast silences transcription in three loci: HM mating-type locus, telomeres and ribosomal DNA. In cell ageing process the complex of Sir2-Sir3-Sir4 translocates from telomeres and HM locus to rDNA that leads to slowing down of accumulation of extrachromosomal rDNA (ERCs) that would otherwise accelerate replicative ageing in yeasts. After the addition of an extra copy of SIR2 into the yeast genome, the accumulation of ERCs was inhibited, which results in inhibition of replicative ageing (3).

In 2000, the enzymatic function of Sir2 was discovered (4). It functions as a deacetylase removing acetylated residues from many cellular proteins, causing activation or inhibition of their function. Likewise, the ADP-ribosyl group can be added by Sirtuins (ADP-ribosyl transferase enzymatic reaction), or other posttranslational modification of proteins can be deacylated (5). All these enzymatic activities of Sirtuins are NAD+ dependent (4).

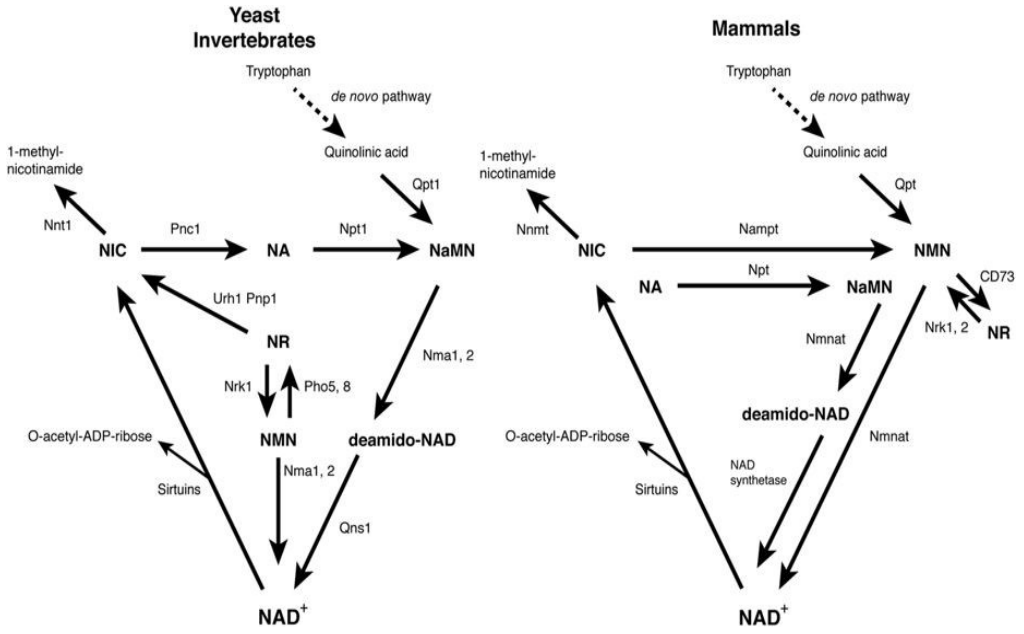

Figure 1: Comparison of biosynthesis of NAD+ in yeast/invertebrates and mammals *(6)*.

Figure 1 shows the biosynthetic pathways for NAD^+ synthesis. (6). In yeast and invertebrates, the NAD^+ is made from precursors such as nicotinamide (NIC), nicotinic acid (NA) and nicotinamide riboside (NR). The *de novo* pathway is another option, in which nicotinic acid mononucleotide (NaMN) is created from tryptophan through phosphoribosyl transferase Qpt, and then deamido-NAD is formed through adenyl transferase Nma (1,2). Lately, due to NAD synthetase activity, the NAD+ is created. The NAD biosynthetic pathways are slightly different in mammals, although they use similar NAD precursors. In yeast and invertebrates, the *de novo* pathway is dominant in the formation of NAD+. On the contrary, the nicotinic amide pathway is more utilised in mammals (6) (7). Both in yeast, invertebrates or mammals, Sirtuins use NAD+ during their enzymatic steps to create nicotinamide and ADP ribose (6) (7). The more NAD+ there is, the higher is the activity of Sirtuins. (6) The level of NAD+ can be changed due to nutrients and stress (8). Furthermore, the change in NAD+/NADH ratio can affect transcription and genome stability. In the yeast experiment, the increase of Sirtuins activity and Pnc1 was observed during caloric restriction (glucose reduction in food from 2 % to 0,5 %), leading to life extension (8) Increasing nicotinic amidase (D-NAAM) expression and hence Sir2-activation caused life extension in *Drosophila* (9). In lower eukaryotes, the caloric restriction also positively impact lifespan (10). In mammals, the

enzyme Nampt affects the activity of Sirtuins, and its overactivation also results in life extension (11). The model how activation of Sirtuins by caloric restriction, stress or exercise can lead to prevention of aging and to life extension is showed in figure 2.

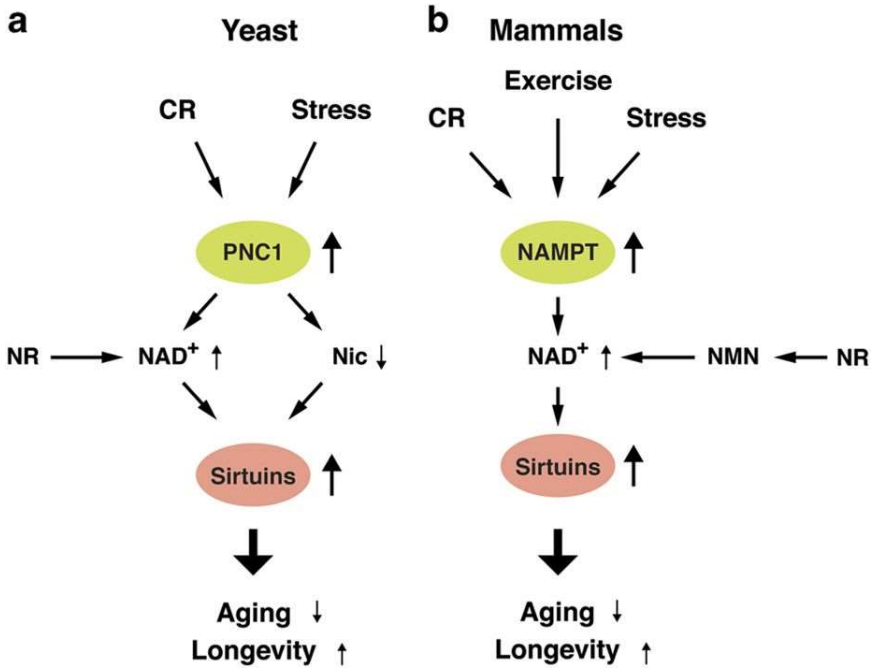

Figure 2: **a.** Effect of stress and caloric restriction (CR) on life longevity in yeasts, **b.** Effect of CR, stress and exercise on life longevity in mammals *(6)*.

The orthologs of the Sir2 gene in mammals comprise a group of seven proteins (SIRT1-SIRT7) with deacylase or mono-ADP ribosyl transferases activities (12). Sirtuins belong to class III histone deacetylases (HDAC), and they can remove acetylate groups from histones as well as from many other nonhistone protein substrates (13). The nonhistone deacetylation is responsible for many different functions related to Sirtuin activity and it closely connects Sirtuins with regulation of various cellular processes, such as protection against oxidative damage generally connected with ageing, obesity, diabetes II. type, cardiovascular diseases, cancer, dementia, arthritis or osteoporosis (14), (15). The scheme explaining the connection between decrease of Sirtuin activity and aging is shown in Fig.2.

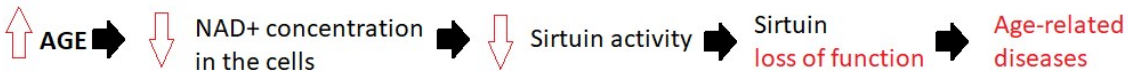

Figure 3: Demonstration of the relationship between ageing, the concentration of NAD+ and Sirtuin activity. (own creation, undertook from Paraskevi, bachelor thesis)

Sirtuins are located in the nucleus, nucleolus, cytosol or mitochondria. Every gene of this group has an enzymatic function and its preferable substrates, but in case of an impaired function of one gene, the substitution by another gene from this group can be utilised to some extent. Table 1 shows the overview of the function and localisation of Sirtuins and their targets (15).

Table 1: Overview of function, localisation and target in SIRT1-7 *(16)*.

Sirtuin and localization	**Enzymatic activity**	**Targets and substrates**			**Function**	**Tissue expression**	**Ageing and age-related diseases**	
		Modification	Activation	Inhibition			Increase/ involvement in CR	Decrease
SIRT1 nuclear/cytosolic	Deacetylase	H1, H3, H4, (H1K26, H1K9, H3K9, H3K56, H3K14, H4K16) α tubulin, p53- (stabilization)	Suv39h1, LKB1, AMPK, NBS1, XPA, Mn-SOD, WRN, Ku70 FOXO, PGC-1α	NFκB, p300, p66shc, mTOR	DNA repair, glucose metabolism, differentiation, neuroprotection, insulin secretion, vascular protection	Brain, adipose tissue, heart, kidney, liver, retina, skeletal muscle, vessels, uterus	Cell survival, longevity, physical activity/increase in CR	Cellular senescence, oxidative stress, inflammation, neurodegeneration, cardiovascular diseases, adiposity, insulin resistance, liver steatosis
SIRT2 cytosolic/nuclear	Deacetylase	α tubulin, H4K16	FOXO	NFκB, p53	Cell-cycle control (transition from G2 to M phase), adipose tissue development and functionality	Adipose tissue, brain, heart, kidney, liver, skeletal muscle, vessels	Longevity/increase in CR	Oxidative stress, neurodegeneration

Sirtuin and localization	Enzymatic activity	Targets and substrates			Function	Tissue expression	Ageing and age-related diseases	
		Modification	Activation	Inhibition			Increase/ involvement in CR	Decrease
SIRT3 mitochondrial/nuclear/cytosolic	Deacetylase	H3, H4 (H3K9, H4K16)	FOXO, Ku70, Mn-SOD, catalase, IDH2	p53, HIF1α	Regulation of mitochondrial metabolism, ATP production	Adipose tissue, brain, heart, kidney, liver, oocytes, skeletal muscle, vessels	Longevity, metabolic health, glucose homeostasis/increase in CR	Oxidative stress, neurodegeneration, cardiac hypertrophy, adiposity, liver steatosis
SIRT4 mitochondrial	ADP-ribosyl-transferase			GDH, AMPK	Insulin secretion, regulation of mitochondrial metabolism, DNA repair	Brain, heart, kidney, liver, vessels, pancreatic β-cells		Fatty acid oxidation
SIRT5 mitochondrial/cytosolic/nuclear	Deacetylase demalonylase desuccinylase		SOD1		Urea cycle	Brain, heart, kidney, liver, vessels, thymus, testis, skeletal muscle	Increase in CR	Oxidative stress, fatty acid oxidation

Sirtuin and localization	Enzymatic activity	Targets and substrates			Function	Tissue expression	Ageing and age-related diseases	
		Modification	Activation	Inhibition			Increase/ involvement in CR	Decrease
SIRT6 nuclear (associated with chromatin)	Deacetylase, ADP-ribosyl-transferase	H2B, H3 (H2BK12, H3K9, H3K56), WRN (stabilization)	FOXO, PARP1, CtIP	NFκB, IGF-1	DNA repair, telomere protection, genome stability, cholesterol homeostasis, regulation of glycolysis and gluconeogenesis	Brain, heart, kidney, liver, vessels, retina, skeletal muscle, thymus, testis, ovary	Longevity, glucose homeostasis/increase in CR	Cardiac hypertrophy, adiposity, liver steatosis, inflammation, insulin resistance
SIRT7 nucleolar/nuclear	Deacetylase	H2A, H2B, H3 (H3K18)	FOXO	RNA poly-merase I	Regulation of rRNA transcription, cell cycle regulation, cardioprotection	Heart, vessels, liver, brain, skeletal muscle, peripheral blood cells, spleen, testis	Increase in CR	Cardiac hypertrophy

1.1.1 SIRT1

At this moment, SIRT1 is the most studied gene of the Sirtuin group, having an essential role in cell metabolic pathways and immune response of an organism, affecting general metabolic health and aging. SIRT1 activity can be stimulated by different ways, including phosphorylation by a protein AMPK kinase that is activated by adenosine-5'-monophosphate (AMP). SIRT1 has a considerable effect on the immune response that can be controlled directly or indirectly through the metabolic pathway. SIRT1 can deacetylate various transcription factors involved in inflammatory immune response such as NF-κB (nuclear factor kappa-light-chain enhancer of activated B cells) and HIF1α (hypoxia-inducible transcription factor-1 alpha subunit) (17) (15) and Fig.4.

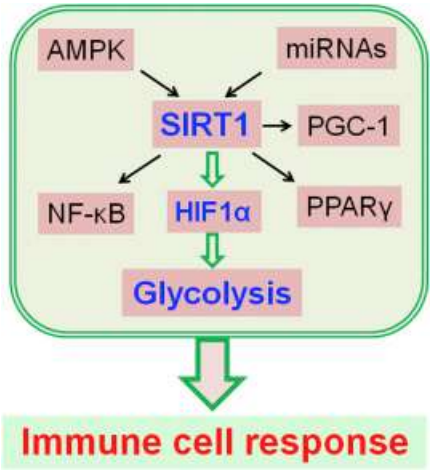

Figure 4: Direct and indirect way of controlling immune response by SIRT1 *(17)*.

As mentioned before in the text, Sirtuin activity can be stimulated by light starvation of organism where the positive effect on ageing can be observed. The observed effects of SIRT1 on life extension are explained by SIRT1 dependent histone deacetylation (18), deacetylation of nuclear receptors and transcriptional coactivators PGC-1α (19), FOXO proteins (20) or other transcriptional factors (table 1). SIRT1 activated by caloric restriction can stimulate PGC-1α leading to the expression of gluconeogenic genes. This process lowers the repressive effect PGC-1α to glycolytic genes resulting in life extension. PGC-1α is a principal regulator of mitochondrial genes biogenesis that cooperates with transcriptional factors essential for mitochondrial gene expression. Nevertheless, the mutual interaction between SIRT1 and PGC-1α can only occur during metabolic stress, such as caloric

restriction. Metabolic stress induces the increase in AMP/ATP ration that activates AMPK that in turn stimulates SIRT1 activity. AMPK also phosphorylates and activates PGC-1α. The pathway AMPK/SIRT1/PGC-1α is frequently used by hormones (for example leptin, adiponectin) to mediate increase in mitochondrial metabolism (21). The increase in mitochondrial respiration is associated with lipolysis (22). Moreover, SIRT1 can inhibit the biosynthesis of lipids. Another protein that is activated by SIRT1 during metabolic deprivation, such as caloric restriction, is PPARgamma, also responsible for the increase in fatty acid oxidation induced by SIRT1.

SIRT1 takes an important part also in the formation of heterochromatin that contributes to higher genome integrity in stress response. SIRT1 can cause deacetylation of histones such as H4K16, H3K9 or H1K26, leading to formation of facultative heterochromatin (15).

Genetic overexpression of protein deacetylase Sir2 increases longevity in a variety of lower organisms (23) (24) (3) although the effect in vertebrates is less clear. In experiment (25) the authors compared *wt* mice and mice with triple overexpression of SIRT1 under its endogenous regulatory elements and found lower levels of DNA damage, decreased expression of the ageing-associated gene $p16^{INK4A}$, a better general health and fewer spontaneous carcinomas and sarcomas. The effect of Sirt1 was however evident in mice model susceptible to liver cancer where Sirt1 activation significantly decreased the cancer occurrence and improved the liver metabolic health. These results provided direct proof of the anti-ageing activity of Sirt1 in mammals and of its tumour suppression activity in ageing- and metabolic syndrome-associated cancer. Sirt was also shown in mice to prevent glucose intolerance and osteoporosis (17)

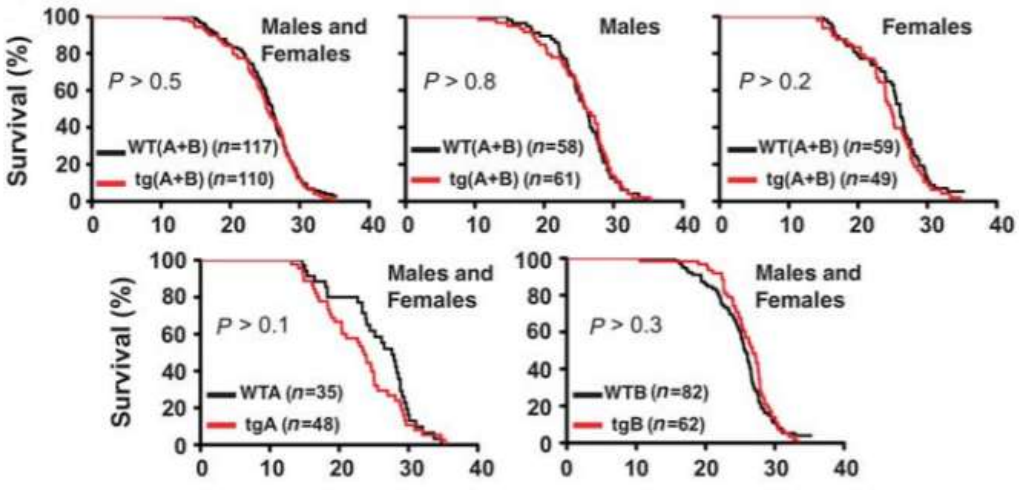

Figure 5: Comparison of lifespan in *wt* mice and mice with SIRT1 overexpression in the liver (tg). The lifespan of *wt* and tg was not statistically different (P>0,05) *(17)*.

In *D. melanogaster*, SIRT1 (or dSir2) is located in II. chromosome. It has one annotated transcript and translates into one polypeptide. For long time there was a controversy whether Sirt1 activation affects lifespan in *Drosophila,* but recent experiments with Sirt1 mild overexpression in carefully controlled genetic background proved convincingly that low activation of Sirt1 promotes lifespan extension (while strong Sirt1 overexpression has the opposite effect) (26) (27). In fact, it was enough to activate Sirt1 only in the fat body to achieve lifespan extension (12) (15) (26).

1.1.2 SIRT2

SIRT2 is a deacetylase situated in cell cytosol. Enzymatic function of SIRT2 is primarily focused on transcriptional factor FOXO1 and FOXO3. Through deacetylation of histones H4 and H3, SIRT2 is crucial in the creation of condensed chromatin and transition to/from the mitotic phase of the cell cycle. Thus, SIRT2 acts as a control point important in cancer cell proliferation.and it is considered as a tumour suppressor (28). Tumour suppressor function of SIRT2 was confirmed for example by the experiment (29), in which SIRT2 deficient mice developed hepatocellular and mammary gland cancer. Furthermore, lower level of SIRT2 were observed in human cancer diseases, correlating with hyperacetylation of SIRT2 target H3K53 (30).

SIRT2 gene expression is highest in the brain, especially in myelin-producing cells – oligodendrocytes. Since the SIRT2 deacetylation of FOXO3 helps neural stem cells to manage ageing, there is a hypothetic impact of SIRT2 to cell ageing of these cells. SIRT2 can also deacetylate PAR-3, the main regulator of cell polarity. Therefore, there is a decrease in the activity of signaling part of protein kinase C during the process myelinization, that can affect peripheral neuropathy in diabetes (31). SIRT2 also has a potential role in the treatment of sclerosis, which is characterised by the progressive loss of myelin (32). SIRT2 can also deacetylate RelA subunit NF-κB contributing to the stabilisation of gluconeogenesis. Thus, SIRT2 can affect cell metabolism and ageing (15).

1.1.3 SIRT3

SIRT3 is the main mitochondrial deacetylases. Nonetheless it can also be found in cell cytosol. It contributes to mitochondrial DNA repair and mitochondrial integrity protection. Moreover, SIRT3 can prevent apoptotic cell death during oxidative stress by deacetylation of the glycosylase OGG1 (33).

By its enzymatic function SIRT3 controls activity of many metabolic enzymes, such as acetyl CoA synthetase (AceCS2), therefore is SIRT3 crucial for metabolism regulation (34). The interaction of SIRT3 and AceCS3 affects the cell cycle progression, apoptosis and cell growth (35). SIRT3 increase the level of cellular ATP by fatty acids decomposition. It is also involved in protection against the adverse impact of reactive oxygen species (ROS) that are increasing with age and damage cellular structures (36). SIRT3 deletion caused obesity, a higher level of glucose and hyperlipidemia in transgene mice (35). Other study proved defects in β-oxidation in mice mutated for SIRT3 (37).

FOXO3a, a member of the transcriptional factors group located in mitochondria, is firmly connected to SIRT3 and life extension (38). SIRT3 and FOXO3a both play a part in cascade pathway in mitochondrial signalisation. This is crucial for age regulation through ROS. SIRT3 can detoxicate ROS and subsequently act as tumour suppressors. However, SIRT3 can also act as an oncogenic factor, for example in breast or lung cancer (35).

Since the participation of SIRT3 in ATP level regulation, its impact on metabolic changes during light starvation was studied. Increased level of SIRT3 in hepatic mitochondria was observed during starvation (39). It is well known that caloric restriction also reduces

DNA oxidative damage (40) and the rate of DNA oxidative damage is significantly lower in *wt* mice than in SIRT3 mutant mice, probably by regulation of the glutathione ROS defense system and by regulation of NADPH (41). These effects dare dependent on Sirt3 deacetylation and activation of isocitrate dehydrogenase (Idh2) (42), (43), Figure 6.

Figure 6: The effect of SIRT3 on ageing (summarized from (40), (35)).

1.1.4 SIRT4

SIRT4 is another mitochondrial Sirtuin with wide-ranging activity on insulin signaling, lipid metabolism, TCA cycle, pyruvate metabolism and amino acid oxidation (44) (45) (46) (47) (48). Main enzymatic function of SIRT4 is mono-ADP-ribosyl transferase activity (44). Sirt4 is probably a negative regulator of gene transcription in fatty acids oxidation (49).

Whether the Sirt4 also has some impact on lifespan or metabolism function was focused study, where the flies lacking Sirt4 were compared to flies with overexpressed Sirt4 (50). As expected, flies overexpressing Sirt4 showed a life extension, and on the contrary, Sirt4 knockout flies lived shorter time. Moreover, when the starvation assay was performed in the same study, flies lacking Sirt4 were more sensitive in comparison to controls and Sirt4 overexpressed flies survived longer. Furthermore, Sirt4 knockout flies exhibited impaired ability to use energy stores during fasting. These results confirmed a crucial role of Sirt4 in metabolic changes and in lifespan (50). During the calorie restriction, SIRT4 inhibits gluconeogenesis, oppositely as SIRT3, thereby Sirt4 inhibits insulin secretion (50).

1.1.5 SIRT5

Last Sirtuin gene located in mitochondria is SIRT5, and its function is deacetylation but more importantly demalonyation and desuccinylation (51). One of the targets of SIRT5, CPS1 (carbamoyl phosphate synthetase 1), is involved in the urea cycle (52). During fasting, amino acids are used as energy source, and the levels of NAD+ in liver mitochondria are

increased. This activates SIRT5 deacetylating CPS1 and adapting the organism to the increase in amino acids catabolism. CPS1 is known for detoxification of excess ammonia and in its deficiency leads to hyperammonemia, mental retardation and death (53). Thus, there is a suggestion that SIRT5 is crucial during ammonia detoxification and disposal by activating CPS1 (52) (54). These findings support the role of SIRT5 in adaptation during metabolic stress and highlight SIRT5 as another possible target for treatment of metabolic disorders (52).

1.1.6 SIRT6

Sirt6 is another nuclear Sirtuin. It is able to poly-ADP-ribosylate PARP-1 that leads to an increase of PARP-1 activity and more efficient DNA repair (15). Deletion of SIRT6 can result in transcriptional changes in hundreds of genes. Similarly to SIRT1, SIRT6 can decrease the activity of NF-κB, via its interaction with the RelA subunits of NF-κB, and this way it has an impact on gene expression of many genes, including genes contributing to cell ageing and ageing of the organism in general (55) (56).

Mice with SIRT6 deletion were born weak and had various metabolic defects. After reaching the age of 2 or 3 weeks, the mice showed age-related abnormalities (57). The life-prolonging effect of SIRT6 was confirmed in male mice with SIRT6 overexpression in brain, but it was not apparent in females (58) and Fig.7.

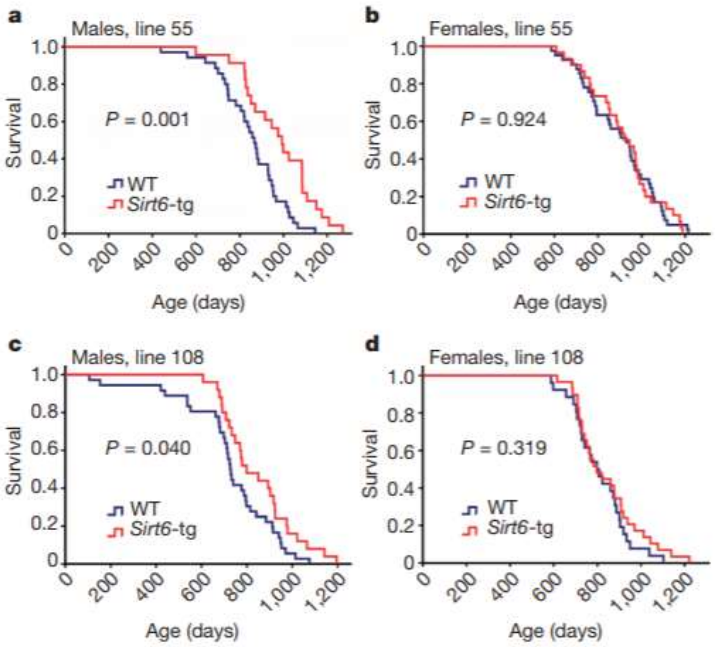

Figure 7: Comparison of lifespan during high fat diet in *wt* mice and mice with SIRT6 overexpression in brain (SIRT6-tg). Statistical analysis proved a difference in both male strains (strain 55 P=0,001, line 1018 P=0,04). There was no significant difference in female mice (strain 55 P=0,924, strain 108 P=0,319). *(58)*

As mentioned before, caloric restriction has a positive impact on ageing in connection to the SIRT1 gene. Light starvation induced not only SIRT1 activity but also SIRT6 levels and in mice. Moreover, SIRT6 activity positively affects glucose homeostasis and genome stability in general. SIRT6 is, therefore, one of the Sirtuins associated with lifespan extension after mild overactivation (59).

1.1.7 SIRT7

The last member of Sirtuin family is SIRT7 that is present in nucleus. During mitosis SIRT7 associates with condensed heterochromatin (60) (61). The enzymatic function of SIRT7 is deacetylation, with histone H3K18 being a its typical substrate (62). Moreover, SIRT7 is associated with active ribosomal RNA genes, and it binds to histones and RNA polymerase I, in order to stimulate transcription (60). This role in transcriptional activation contrasts with the silencing function of other Sirtuins (63).

When the SIRT7 deletion was induced in mice, the inflammatory cardiomyopathy, cardiac hypertrophy, fibrosis, hyperacetylation of p53, increased apoptosis and reduced resistance to oxidative stress was developed. Besides, the transgene mice showed shorter lifespan (figure 8) (64). SIRT7 might also work as an oncogene since the SIRT7 knockout delays tumorigenicity (62).

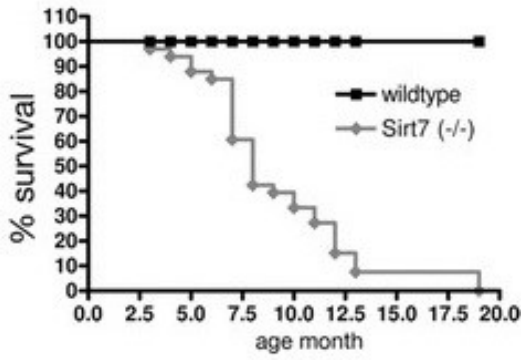

Figure 8: Percentage of survival during the lifespan assay. Mean lifespan was decreased in Sirt7 lacking mice in comparison to *wt* mice *(64).*

Developmental defects of Sirtuin knockout mice:

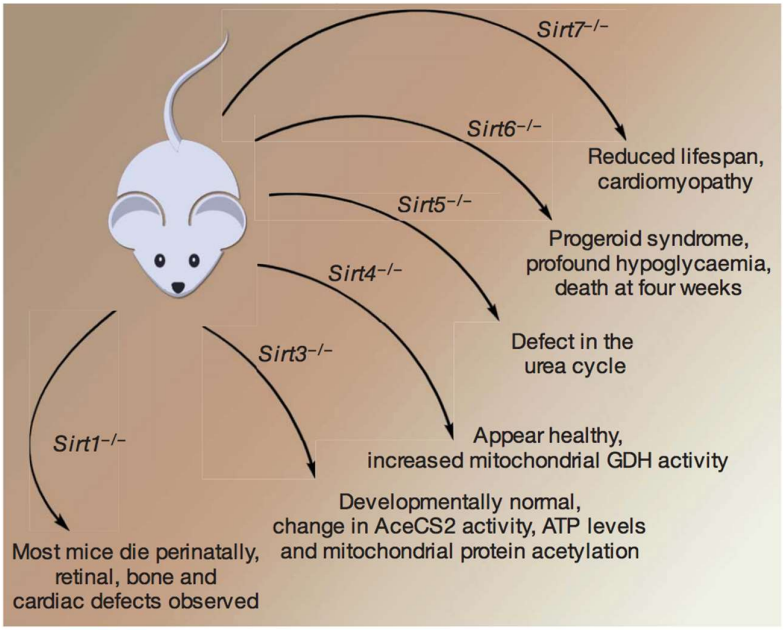

Figure 9: Mouse knockout models as tools for exploring Sirtuin function. The phenotypes of Sirtuin KO mice include a reduction in median lifespan, ranging from a usual survival of days

(SIRT1) to weeks (SIRT6) or months (SIRT7). In contrast, although biochemical phenotypes have been reported, Sirt3$^{-/-}$ and Sirt4$^{-/-}$ mice appear outwardly normal. Initial reports suggest that Sirt5$^{-/-}$ mice also exhibit no obvious phenotype. Sirt1$^{-/-}$ implies lethality only in inbred genetic background *(65)*.

Most of the Sirtuin knockout mice develop normally, but they display various metabolic and physiological defects during their life. The phenotype of Sirt1 knockout mice depends on the genetic background, and it is lethal only in some of the inbred mice strains (66). Whole-body deletion of SIRT1 leads to elevated prenatal death in inbred mice (67) (68). The few pups born displayed marked cardiac and neurological problems, leading to death very early in the postnatal period (67) (68). In order to bypass this situation, SIRT1 deletion was performed in outbred mice. These mice were viable, but smaller, and displayed a marked metabolic inefficiency (69) (66).

1.2 *D. Melanogaster* and Sirtuins

Sirtuins are highly conserved proteins located in various organisms from yeast to human. From the phylogenetic aspect, Sirtuins are divided into several classes (I-IV and U) according to their variations in a region close to the catalytic center of the protein (70). This is probably the reason why Sirtuins differ in their localisation, function and target substrates (71) (61).

D. melanogaster, used in this thesis as a model organism, has conserved five genes of the Sirtuin group, i.e. SIRT1, SIRT2, SIRT4, SIRT6 and SIRT7. Until now, only SIRT1, 2 and 4 were studied in *Drosophila*.

1.2.1 SIRT1

There was used GAL4 drivers for overexpression of SIRT1 in a ubiquitous manner in the experiment. This study showed a positive effect on *Drosophila* lifespan (figure 10) (72). Moreover, another study supports the statement about SIRT1 and two positive effects on lifespan in *Drosophila* (73).

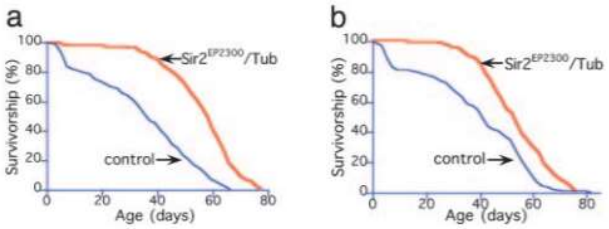

Figure 10: The survivorship of flies with SIRT1 overexpression induced by Tub-GAL4 driver (red) compared with controls (blue). The increased was 29 % for females and 18 % for males *(72)*. The increase of lifespan mediated by Sirt1 is only apparent on food that contains relatively high amount of yeast (high caloric food) where Sirt1 protects against the high caloric diet induced shortening of lifespan (Benerjee, 2012).

Many threads of evidence refer to close relationship between Sirtuins and caloric restriction (CR). According to the study (72), the level of Sir2 mRNA was increased in CR *Drosophila*. However, no additive effect on life extension was observed in Sir2 overexpressed *Drosophila*. Another study focused on the mobility of flies during CR showed a lower activity of Sir2 mutant flies on a low-calorie diet in comparison to a high-calorie diet (74). Hence, there is a suggestion that the increase in activity requires SIRT1 (74). Probably, the lower activity of Sir2 mutant is caused by inefficient metabolism and possible damage of mitochondrial function (75). As in mammals, the tumour suppressor gene p53 is a target of Sir2 in *Drosophila*. Many studies suggest a crucial role of p53 in the ageing process in mammals, likewise in *Drosophila* (76) (77). To sum up, p53 interacts with Sir2 and probably with another component of this pathway and might be related to genotoxic stress (75).

On the contrary, there is a study proving no impact of Sir2 to lifespan in *Drosophila* (26). This study aimed to exclude the possible effect of different genetic background or mutagenic effects of transgene insertion which can confound the results of studies in ageing (78). For overexpression GAL4, UAS binary system (EP-UAS-*dSir2* with a ubiquitously-expressed tubulin-GAL4 driver), as in original study (72). After outcrossing mentioned transgenes, the white control Dahomey background was created. The assay was performed according to the original study (72). Flies lived longer than *wt* controls. However, the results show the same life longevity in both flies dSir2útub-GAL4 and dSir2/+ (figure 11). This indicates that overexpression of Sir2 probably does not affect lifespan in *Drosophila*. The life extension in the original study (72) was possibly caused by the transgene-linked genetic effect. Besides, this study disproved the suggestion of resveratrol effect on lifespan (79), because there was

no life extension observed in tested flies (26). Moreover, the relativity of caloric restriction, life extension and Sir2 was tested too. All the tested genotypes acted normally. Therefore, Sir2 is not probably required for the life extension caused by caloric restriction (26).

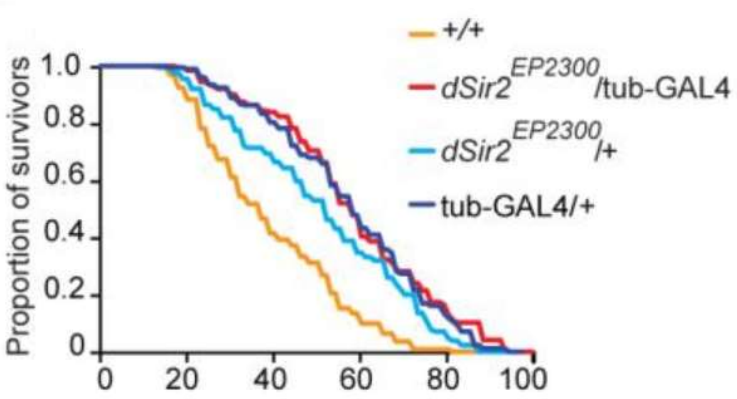

Figure 11: Lifespan of wt flies, Sir2/tub-GAL4 and Sir2/+. Lifespan in flies over-expressing dSir2EP2300 driven via tubulin-GAL4 (tub-GAL4) is longer than wild type, but not than the tubulin-GAL4 /+ genetic control. Median lifespans: +/+ = 39 days, dSir2EP2300/tubulin-GAL4 = 59 days, dSir2EP2300/+ = 53 days, tubulin-GAL4 /+ = 60 days *(26)*.

Fat body specific knockdown of SIRT1 affects insulin signaling and metabolic homeostasis; SIRT1 is a key component that links dietary inputs with organismal physiology and survival (80). A recent study showed that SIRT1 not only plays an important role during aging, but it also regulates lipid metabolism and obesity. Endurance exercise improved climbing capacity, cardiac contraction, and *Sir*t1 expression, it reduced body and heart triacylglycerol levels, heart fibrillation and mortality of flies kept on high fat diet and also in aging flies (81). Physical activity of flies lacking Sirt1 is lower on low calorie food compared to high caloric food (82). Similarly, increasing Sirt1 activity by feeding flies resveratrol, a CR mimetic, increased spontaneous physical activity of flies on high caloric food. In *Drosophila*, spontaneous physical activity therefore closely mimics life span in its dependence on Sirt1 (82). Another work showed that loss of SIRT1 leads to age-progressive metabolic disease with symptoms similar to those of type 2 diabetes (83). This study also provided a new genetic model of insulin resistance in *Drosophila* and established HNF4 (hepatocyte nuclear factor) as a critical downstream target in the Sir2 signaling pathway (83).

SIRT1 is functionally closely connected to the FOXO transcription factor present in mammals (20) and in *Drosophila* (84). Therefore, there was a hypothesis that FOXO mediates the rescue of mitochondrial integrity and function induced by SIRT1 (85). The results showed that the role of SIRT1 and FOXO are to suppress mitochondrial enlargement in dopaminergic neurons during neuronal degeneration in Parkinson disease (85). Moreover, SIRT1 is involved in mediating apoptotic cell death in Drosophila through activation of JNK and FOXO pathways (84). Overexpression of SIRT1 induced cell death by caspase-dependent apoptosis in the eye (84).

The ability of SIRT1 mutations to enhance PcG (Polycomb) mutant phenotypes and to perturb PRE-mediated silencing indicates that SIRT1 plays a role in Polycomb silencing (86). However, *Drosophila* SIRT1 mutants are viable under standard laboratory conditions, and they do not exhibit obvious PcG phenotypes (86). This could be due to functional redundancy between Sirtuins (12).

1.2.2 SIRT2

Complex V is a fifth mitochondrial respiratory subunit complex that stimulates ATP biosynthesis (87). Impairment of mitochondrial respiration is strongly associated with aging and pathology in neurodegenerative disorders, cardiovascular diseases, diabetes, and cancer (87). *Drosophila* SIRT2 is an important regulator of mitochondrial function as it deacetylates ATP synthase β, the catalytic subunit of respiratory complex V, and this way it makes the complex more active (88). This study also suggests that ceramide increase affects NAD+ level and Sirtuin activity. Ceramide, a central intermediate in sphingolipid metabolism, mediates many stress responses, and recent literature highlights that perturbations in ceramide levels can affect glucose and fat metabolism (88). Moreover, the neuroprotective function of SIRT2 was examined in models of Huntington's disease (HD) in *Drosophila* (89). SIRT2 was inhibited in genetic and pharmacological manner, and both inhibitors achieved significant neuroprotection in HD flies (89).

1.2.3 SIRT4

Drosophila SIRT4 was also shown to impact lifespan (50). A transgenic fly overexpressing *Drosophila* SIRT4 in the fat body were long-lived compared with genetically matched controls. These findings demonstrate that increasing SIRT4 expression can extend the lifespan in the fly and that this effect may be primarily mediated through SIRT4 actions in

the fat body (50). Furthermore, this study also provides evidence that flies lacking SIRT4 were sensitive to starvation and died much sooner than the genetically matched wild-type cohort (50). Conversely, the authors found that flies overexpressing SIRT4 were resistant to starvation and able to survive longer than genetically matched uninduced controls in the absence of food (50). These results suggest that *Drosophila* SIRT4 is responsive to nutritional inputs and that it is important for mediating metabolic changes associated with fasting (50). The results of this study also suggest that the impaired ability of the SIRT4 knockout flies to resist starving is due to its problems to use up available energy stores, as the SIRT4 knockout flies displays lower decay of total TAGs (triglycerides, primary form of energy storage in the fly), glycogen, trehalose and glucose in comparison to the starving control (50). There is a recent study indicating that Sir4 mutant is short lived independently on the type of diet (90).

2 AIMS OF STUDY

In mammals, there are seven members of the Sirtuin family, where deletion of SIRT1 is lethal, and deletion of SIRT6 and SIRT7 lead to developmental and phenotypic defects. As noted before, *Drosophila melanogaster* has five members of the Sirtuin family. However, none of the genes is lethal when knocked-out. This probably implies functional alternation of an individual gene in a certain way. In mammals, nor fruit flies, there was not created an organism missing all Sirtuin genes.

The aim of my thesis was

1. To create a *Drosophila* line lacking all five Sirtuin genes, by combination of strains mutant for individual Sirtuins available in our lab.

2. To test the impact of combined mutations of all five Sirtuin genes on the *Drosophila* development, on the lifespan of adult flies on high and low yeast diet, on weight of adult flies and on their resistance to stress conditions in following assays:
    - Chill coma recovery assay
    - Starvation assay
    - Oxidative stress resistance assay

3 MATERIALS AND METHODS

3.1 Strains of used *Drosophila melanogaster*

Before setting up any experiments, we had to think about which control we should use as to compare with our mutant. Individual strains of *Drosophila* can differ in their phenotypes due to different genetic background. In the ideal case, we should clean the Sirtuin penta mutant ($Sirt^{-/-}$) into the selected wide type genetic background (for example by crossing to w^{1118} strain for at least six generations) and only then perform our experiments. In our case, however, this is almost impossible, because none of the Sirtuin mutants display an obvious phenotype and the $Sirt1^{2A711}$, $Sirt6^{d2}$ and $Sirt7^{d10}$ mutations have no selection marker either (they do not bear w+ allele). Consequently, the screening of flies during the cleaning process would be tough and expensive. Therefore, we decided to compare our $Sirt^{-/-}$ mutant with several controls at once, including w^{1118}, yw and Oregon R strains. We hoped to identify a robust phenotype of $Sirt^{-/-}$ mutant that would differ from all the controls.

The CG18446 ^{MI02952} served as a positive / negative technical control because it was already tested in our lab in most of the assays I performed (and therefore I knew which result to expect if the assay was performed correctly).

Used strains:

- Sirt1 ^{2A711} – SIRT1 coding region deleted, created by *Xie and Golic, 2005*
- Sirt2 $^{5B-2-35\ -}$ SIRT2 coding region deleted, created by *Xie and Golic, 2005*
- Sirt4 $^{white+1}$- SIRT4 coding region deleted, created by *Xie and Golic, 2005*
- Sirt6 ^{d2} - 2 bp deletion close to the start of ORF, created by Crisper/Cas9
- Sirt7 ^{d10} - 10 bp deletion close to the start of ORF, created by Crisper/Cas9
- $Sirt2^{5B-2-35,}$ $Sirt6^{d2}$, $Sirt7^{d10}$ – triple mutant for SIRT2, SIRT6 and SIRT7 gene created by Paraskevi Tziortzouda (91)
- Balancer – Sp/Cyo; Dr/TM3
- Sirt4 $^{white+1}$; $Sirt1^{2A711}$; TM3/TM6
- $Sirt^{-/-}$ - fly mutant for all 5 genes of the Sirtuin family
- CG18446 ^{MI02952} - transposon insertion close to the start of ORF in CG18446 gene
- w^{1118} (control)
- OregonR (OR, control)

- yw (control)

3.2 Creation of *D. melanogaster* strain mutant for all genes of Sirtuin family

The *Drosophila* SIRT4 gene is located on the first chromosome, SIRT1 on the second chromosome and SIRT2, SIRT6 and SIRT7 on the third chromosome. The very first step towards the creation of *Drosophila* line mutant for all five Sirtuins was to create a strain with the mutant alleles for $Sirt2^{5B\text{-}2\text{-}35}$, $Sirt6^{d2}$ and $Sirt7^{d10}$ on one chromosome by meiotic recombination. Bachelor student Paraskevi Tziortzouda and Aljosa Smajlic made the meiotic recombination between $Sirt2^{5B\text{-}2\text{-}35}$ and $Sirt^{d2}$ and subsequently, de novo mutation of $Sirt7^{d10}$ was introduced using Crisp/Cas9 method (91). My thesis aimed to combine the mutations on I., II., III. chromosome and to create a *Drosophila* mutant for all five Sirtuins.

At first, the $Sirt2^{5B\text{-}2\text{-}35}$, $Sirt6^{d2}$ and $Sirt7^{d10}$ mutant was crossed with a double balancer line (table 2) to collect males with curly wings and shortened hair on the scutum (+/Cyo; $Sirt2^{5B\text{-}2\text{-}35}$, $Sirt6^{d2}$ and $Sirt7^{d10}$ / TM3). These males were then crossed with the females from the Sirt4 $^{white+1}$; $Sirt1^{2A711}$; TM3/TM6 stock that we already had in the lab. The presence of both TM3 and TM6 balancers gives the fly a black colour of the body and homozygotes for TM3 or TM6 are not viable. In the next generation, the males with curly wings and either TM3 or TM6 balancers (Sirt4 $^{white+1}$/y; $Sirt1^{2A711}$ /Cyo; $Sirt2^{5B\text{-}2\text{-}35}$, $Sirt6^{d2}$, $Sirt7^{d10}$ /TM3(6)) were crossed again with female Sirt4 $^{white+1}$; $Sirt1^{2A711}$; TM3/TM6. Final stock was established by selecting either both TM3 male and TM3 female or TM6 male and TM6 female, to create stable line with Sirt4 $^{white+1}$; $Sirt1^{2A711}$; $Sirt2^{5B\text{-}2\text{-}35}$, $Sirt6^{d2}$ and $Sirt7^{d10}$/TM3(6) genotype (see the crossing scheme in figure 12). In the next generation we selected flies with no balancers and established a homozygous line $Sirt4^{white+1}$; $Sirt1^{2A711}$; $Sirt2^{5B\text{-}2\text{-}35}$, $Sirt6^{d2}$, $Sirt7^{d10}$. In this theses, we describe it by its shorter name as " $Sirt^{-/-}$ ".

Table 2

BALANCER	PHENOTYPE
Sb (stubble)	Shortened hair on the thorax, II. chromosome
Dr	Reduced eyes, III. chromosome
Cyo	Curly of oster, II. chromosome, curly wings
TM3	Shortened hair on the scutum, III. chromosome
TM6	Shortened hair on the side of thorax, III. chromosome

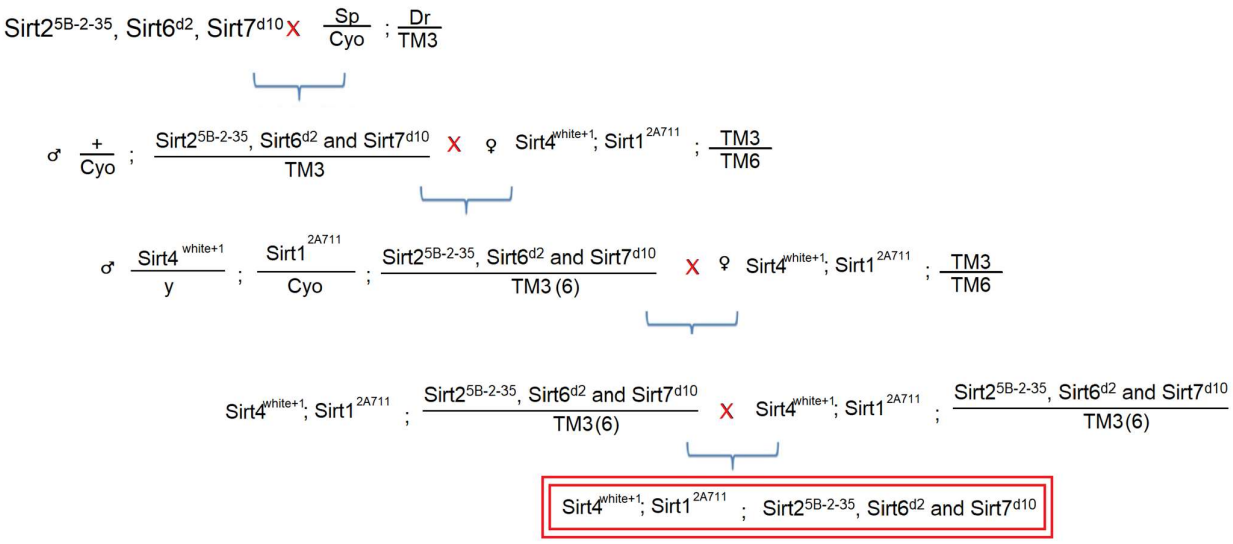

Figure 12: Crossing scheme to make the *Drosophila* line with all five Sirtuin genes mutated (Sirt4 $^{white+1}$; Sirt1^{2A711}; Sirt2$^{5B-2-35}$, Sirt6^{d2} and Sirt7^{d10}). We describe the homozygous stock as " Sirt$^{-/-}$ " in this thesis.

3.3 Staging of flies for all experiments

To avoid the influence of nutrient availability and stress during development or the effect of the age of the adults used for subsequent experiments, we bred all tested strains in strictly regulated conditions. We tried to keep the same number of developing larvae in the flasks, the same diet and and we used the same age of adults for all our experiments. We used 4-5 days old adult flies for all subsequent assays.

All flies were bred at 25 °C on food of the following composition (Table 3):

Table 3: The composition of food we used to breed all our flies.

INGREDIENTS	AMOUNT
water	900 ml
corn flour	80 g
sugar	75 g
instant yeasts	15 g
agarose	4 g
10 % Nipagin	20 ml

For the optimization of the number of larvae in each flask (to achieve similar larvae density for all the lines we used) we used the following approach: First we set cages containing approximately 250 adult females and 250 males, and we allowed the flies to lay eggs to juice Petri dishes (Table 4), and we let them adjust to the cage conditions for 3 days.

Table 4: The composition of juice plates used for collecting Drosophila eggs.

INGREDIENTS	AMOUNT
water	1000 ml
100 % apple juice	300 ml
agarose	30 g
10 % Nipagin	25 ml

The yeast paste (~ 1 tsp) was added on the top of the medium to attract the females to lay eggs. Each 16 – 24 hours of egg laying the Petri dishes were changed for three days in order to let the flies adjust to the cage conditions. The collection of egg for experimental flies took place the fourth day. The plates were washed by phosphate buffer (PBS), eggs were carefully brushed away by cotton wool brush and then poured into the 15ml tubes. After the egg settled at the bottom of the tube, 32 µl of eggs were transferred to the fresh flask (table 3). In the case of CG18446 ^{MI02952} the amount had to be increased to 100 µl and in case of $Sirt^{-/-}$ to 200 µl of eggs in order to hatch similar number of larvae and collect similar number of adult flies (empirically titrated). Flasks were kept at 25 °C incubator. The process of egg collection was initiated one day earlier with $Sirt^{-/-}$ because it took longer for the adults to emerge. After 9 - 10 days after transferring the eggs into the flasks (one day after emerging of adults) newly emerged flies were removed and the flies emerged after that were allowed to mate for 48 hours. Two days old flies were separated according to sex under light CO_2 anesthesia. We were careful not to exceed the maximal time of anesthesia over 4 minutes to avoid the impact of CO_2 on the experiments. After the separation by sexes, the flies were stored in glass vials (20 flies/vial). Next steps differed according to specific assay used and the procedures are described later in the text.

January 2019

Wk	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
1		1	2	3	4	5	6
2	7	8	9	10 Adjusting to the cage	11 Adjusting to the cage	12 Adjusting to the cage	13 Egg collection Sirt-/-
3	14 Egg collection controls	15	16	17	18	19	20
4	21	22 Removal of emerge flies Sirt-/-	23 Removal of emerge flies controls	24 Mate	25 CO_2	26	27
5	28	29	30	31	1		

Figure 13: An example for the time scheme that we used for the staging process of flies for our experiments.

3.4 Characterisation of Sirt$^{-/-}$ flies in stress assays

3.4.1 Chill coma recovery assay

The male or female flies were transferred to vials with *Drosophila* food (table 3) that were stored in 25 °C incubator in a horizontal position to prevent death of the flies by sticking to the medium. The flies were allowed to rest for 48 hours to fully recovery from anesthesia. Subsequently, the vials were inserted to a box with ice until all the flies fell into the chill coma. Then the individual flies were carefully distributed to pre-cooled 96-wells plate by tweezers. The plate was closed, covered with aluminum foil and buried into a box of ice that was stored in a 6 °C cold room for 16 hours (92). After this time, we took the flies out of the ice, spread them on a filter paper on room temperature and waited until they start to show signs of recovery from the chill coma. A fly standing on its feet was counted as fully recovered and the time of recovery was noted. If the fly did not recover by 90 minutes, it was considered as dead. Experiment was performed in 4 biological replicates.

3.4.2 Oxidative stress resistance assay

After separation of sexes the flies were transferred to vials containing the following diet (Table 5):

Table 5: Composition of diet for oxidative stress assay.

INGREDIENTS	AMOUNT
water	1000 ml
sugar	13 g
agarose	10 g
catalase inhibitor (3 – amino – 1, 2, 4 – triazol)	0,4204 g

At first, the catalase inhibitor 3 – amino – 1, 2, 4 – triazole (Sigma) was in 100 ml of distilled water and subsequently added into the 900 ml of medium cooled to the 40 °C to prevent quality damage of chemical. Vials with flies were stored in a horizontal position in 25 °C incubator for 60 hours. After this time, the flies were flipped to vials containing a medium with 3 – AMINO – 1, 2, 4 – TRIAZOL AND 0,5 % H_2O_2, to induce the oxidative stress (Table 6). The H_2O_2 was added into the medium in the same manner as adding the catalase inhibitor.

Table 6: Food medium used in oxidative stress assay.

INGREDIENTS	AMOUNT
water	1000 ml
sugar	13 g
agarose	10 g
30 % H_2O_2	16,67 ml
3-amino-1,2,4-triazol	0,42 g

Subsequently, the vials with flies were stored at 25 °C incubator, and every 2 hours the fly mortality was recorded. In every recording, the position of vials was randomly changed to avoid uneven heat effect of the incubator. Experiment was performed in 3 biological replicates.

3.4.3 Starvation assay

After separation of sexes, the flies were transferred to vials containing 1 % agarose (bacteriological agar, Amresco) for 6 hours to starve the flies and but to prevent the stress possibly caused by thirst. Subsequently, the flies were flipped to vials with the usual food (table 3) for 2 hours to achieve the make sure all the flies eat well before the starvation assay. After this, flies were again transferred to vials with 1 % agarose and stored in 25 °C incubator in a horizontal position. The fly mortality was recorded every 4 hours. The position of vials was randomly changed during this time to avoid the uneven heat effect in the incubator. Every second day, the flies were flipped to vials with fresh 1 % agarose to prevent deaths caused by thirst. Experiment was performed in 4 biological replicates.

3.4.4 Assessing the flies' lifespan

After separation of sexes the flies were transferred to vials with two types of diet – high yeast (50 %) and low yeast diet (2,5 %) as you can see in Table 7 and 8:

Table 7: High-yeast medium for lifespan assay.

INGREDIENTS	AMOUNT
Water	1000 ml
Corn flour	86 g
sugar	50 g
Instant yeasts	50 g
agarose	6 g
10 % Nipagin	20 ml

Table 8: Low-yeast medium for lifespan assay.

INGREDIENTS	AMOUNT
water	1000 ml
Corn flour	86 g
sugar	50 g
Instant yeasts	2,5 g
agarose	6 g
10 % Nipagin	20 ml

Vials with flies were stored in 25 °C incubator in a horizontal position. The flies were flipped to fresh medium every 2-3 days. Counting of the dead flies was performed every flipping. In every observation, the position of vials was randomly changed to prevent uneven heat effect of the incubator. Experiment was performed in 2 biological replicates.

3.4.5 Assessing the flies' dry body weight

The flies were distributed according to their sexes under light anesthesia and stored in Eppendorf tubes, which were three times preweight individually for determination of their weight without flies. The tubes were opened, and the neck of the tubes were secured with a parafilm perforated by the needle in order to allow the water to escape during the drying process. Samples were dried in a lyophilizer, and their weight was measured three times again, and average value was calculated from the three measurements. The weight differences of tubes after lyophilisation and empty tubes determined the dry weight of the flies. We weight 50-100 flies of each genotype, and we calculated an average dry weight of one fly. Experiment was performed in 3 biological replicates.

3.4.6 Assessing the flies' fecundity and fertility

The flies were separated according to their sexes under light anesthesia. Thirty females and fifteen males were selected and transferred to a small cage for each strain. Flies were allowed to rest and lay eggs for two days in 25 °C incubator. Subsequently, the Petri dishes with fly food (table 4) were changed every 4 hours (8:00, 12:00, 16:00 and 20:00) and the eggs were counted. After 24 hours the larvae in the first instar were counted again in 4-hour interval.

3.5 Verification of genotypes

Before the experiments started, we decided to verify the genotype of the $Sirt^{-/-}$ and CG18446 ^{MI02952} strains. The verification was repeated several time during the cause of the experiments. We used real-time PCR and sequencing to verify the presence of the desired mutations.

3.5.1 Extraction of genomic DNA (Fly DNA miniprep)

1. 20 - 50 flies carefully squish in 250 µl H buffer (tube 1,6 ml)
2. add 15 µl of proteinase K (10 mg/ml) + 25 µl 10 % SDS
3. put samples in heat block 50 °C for 4 hours or overnight
4. cool down to room temperature and add 250 µl DEPC, 2x extract DNA in phenol: chloroform: isoamyl alcohol (25:24:1)
    i. add 500 µl P:CH: IAA to the sample, mix with overturning
    ii. centrifuge 13 500 rpm/ 4‘/4 °C
    iii. Discard the supernatant (upper part) with DNA and go to the point i. moreover, repeat
5. Extract DNA in chloroform: isoamyl alcohol (24:1)
    i. add 450 µl CH: IAA to the sample, mix with overturning
    ii. Centrifuge 13 500 rpm/4‘/4 °C
6. Transfer the supernatant to new tube and add NaAc (1/10 volume of supernatant = ca 15 µl) and 100 % EtOH (2x bigger volume than supernatant), lastly add ethanol, precipitate DNA in -20 °C, overnight
7. Centrifuge 13 500 rpm/4‘/4 °C
8. Pour off the alcohol, centrifuge for a few seconds and carefully pour off the rest, pellet = DNA, wash with 500 µl 70 % EtOH, centrifuge 13 500 rpm/4‘/4 °C, pour off

the alcohol, centrifuge for a few seconds, discard the rest carefully, let it dry, add 50 µl of DEPC and melt the pellet, incubation in 68 °C for 5 minutes

Table 9: Composition of H buffer

COMPOSITION	VOLUME
160 mM saccharose	1,095 g
100 mM TRIS-CR (ph=8)	2 ml
80 mM EDTA	3,2 ml
Distilled water	14,8 ml

Table 10: The recipe for 10 % SDS solution

COMPOSITION	VOLUME
electrophoresis-grade SDS	10 g
Distilled water	90 ml

The procedure for H buffer: mix and heat to 68 % for the melting, set the pH to 7,2, add the volume to 100 ml, sterilise with filtration

3.5.2 Verification of Sirtuin genes mutations

First, the extraction of DNA was performed (chapter 3.4.3) and either normal PCR or the real-time quantitative PCR (rt-qPCR) was performed with the following primers:

- **Sirt1**$^{2A\text{-}7\text{-}11}$

  SIRT1 sequence location: 2L:13,165,564-13,169,551 [+] (www.flybase.org)

Primers inside the ORF of SIRT1 gene:

Sir2_ORFreals_OK: GGA TGC ATG TGA TCT GAA CG (2L:13,168,358-13,168,378)

Sir2_ORFreala_OK: TAA TGC AGG CGG TTC TTT TC (2L:13,168,481-13,168,501)

The $Sir2^{2A-7-11}$ allele is a deletion constructed using ends-out gene targeting method (93). No PCR product should be amplified from $Sir2^{2A-7-11}$ flies as they lack the whole coding region of SIRT1.

- **$Sirt2^{5B-2-35}$**

SIRT2 sequence location**:** 3R:20,328,465-20,330,915 [+] (www.flybase.org)

CG5085-C1 (Fwd): GCC CCA GGC TAG TCT AAA TAG (3R:20,328,667-20,328,687)

CG5085-C2 (Rev): GAA AGA AAG CTC GCG CTA TTA G (3R:20,330,028-20,330,049)

The $Sirt2^{5B-2-35}$ is a deletion constructed in a similar manner as $Sir2^{2A-7-11}$, by ends-in gene targeting method (93). The primers are located at the edges of the $Sirt2^{5B-2-35}$ deletion; therefore, the products in mutant should be approximately 200 bp, whereas in *wild type* it should be 1382 bp.

- **Sirt4** $^{white+1}$

SIRT4 sequence location: X:5,664,628-5,665,940 [-] (www.flybase.org)

Sirt4 ORF s (Rev): GGG AGA TAACAAAACAATGCGTGTGG (X:5,665,858-5,665,880)

Sirt4 ORF a (Fwd): GGG AGA AAAGTCCTCCAGCCGTTTG (X:5,665,746-5,665,764)

The Sirt4$^{white+1}$ is a deletion constructed using ends-out gene targeting method. The mini-white marker was not removed by Cre-mediated recombination (93). No PCR product should be amplified from Sirt4$^{white+1}$ flies as they lack the whole coding region of SIRT4.

- **Sirt6^{d2}**

SIRT6 sequence location: 3R:10,255,349-10,261,849 [-] (www.flybase.org)

SIRT6 CRISPR ver s (Fwd): ACG TTG CAG GGA TTT TTG AC (3R:10,261,249-10,261,268)

SIRT6 CRISPR a (Rev): TTT GTA GCG TTA CGG ATA CGG (3R:10,261,856-10,261,878)

The primers surround the deletion made in SIRT6 gene coding region. The size of the products will be very similar for Sirt6^{d2} mutated allele as well as for wild type because the Sirt6^{d2} mutation in the open reading frame comprises only 2 bp. We, therefore, sequenced the PCR product in order to confirm the presence of the mutation.

- **Sirt 7 ^{d10}**

SIRT7 sequence location: 3R:29,055,927-29,059,204 [+] (www.flybase.org)

SIRT7 CRISPR ver s (Fwd): GGA AGC GAG TCA TTC CTA CG (3R:29,055,871-29,055,890)

SIRT7 CRISPR ver a (Rev): GCT TCT TGG TCG TCT TCA CC (3R: 29,056,606-29,056,625)

The primers surround the deletion made in SIRT7 gene coding region. The size of the products will be very similar for Sirt7^{d10} mutated allele as well as for wild type

because the Sirt7^{d10} mutation in the open reading frame comprises only 10 bp. We, therefore, sequenced the PCR product in order to confirm the presence of the mutation.

3.5.3 Verification of the CG18446 ^{MI02952} allele

- CG18446 ^{MI02952} sequence location 2R: 9,871,470.9,873,646 [+] (www.flybase.org)

MiMIC REAL a: 5' CATGTTGGGTGAGGTGCTC 3'

MiMIC REAL s: 5' GCTGGTCAAGGAGATTCTGG 3'

The CG18446^{MI02952} allele is a transposon insertion close to the transcription start site of the gene. The primers surround the inserted transposon MI02952. If the transposon is present the product will not be created in rtqPCR reaction (product of ca 2000 bp) (www.flybase.org). The product from wild type CG18446 gene should be 2115 bp long.

3.5.4 Quantitative real-time PCR

The standard curve was made in each run with each primer set, and it was based on 10-fold dilution of genomic DNA from wild type flies.

1. Dilution of genomic DNA10x, 100x, 1 000x, 10 000x – take 27 µl distilled water and 3 µl of DNA, resuspend and transfer 3 µl from this tube to the new one with pre-prepared 27 µl distilled water (for 100x diluted standard), repeat for 1 000x and 10 000x diluted standard.
2. Pipet all samples in duplicates (studied gene and normalisation gene CG16941)
3. Make a qPCR master mix, containing the following composition multiplied by number of samples:

Table 11 : Composition of rt-qPCR reaction per one sample

COMPOSITION	VOLUME
Primer anti-sense	0,3 µl
Primer sense	0,3 µl
SYBR green	5 µl
Distilled H2O	3,9 µl
Sample DNA	0,5 µl

4. Vortex and centrifuge for 1 minute, distribute 9,5ul into each well of the 96 well PCR plate
5. Add 0,5 µl DNA
6. Cover with plastic foil
7. Centrifuge 830 rcf/1 minute insert into the PCR machine (CFX96 Touch™ Real-Time PCR Detection System, Bio Rad)
8. Run the following rtPCR protocol

Table 12 : Real time PCR protocol

	Temperature	Time	Notes
1. Initial denaturation	95 °C	3 min	
2. Denaturation	94 °C	15 s	
3. Annealing, extension, reading the fluorescence	57 °C	30 s	
	72 °C	30 s	To step 2, repeat 40x
	57 °C	5 s	
	95 °C	5 s	

4 RESULTS

4.1 Creation of Sirt$^{-/-}$ line

The crosses for the creation of Sirt$^{-/-}$ were performed according to the scheme in Materials and methods, and we successfully made the homozygous Sirt$^{-/-}$ line. The flies not only survived in the homozygous state, but they were fully viable with no obvious developmental defects.

4.2 Verification of Sirt-/- genotype

In order to verify the genotype of the Sirt$^{-/-}$ line, we performed a series of real time quantitative PCR for verification of Sirt1$^{2A-7-11}$, Sirt2$^{5B-2-35}$ and Sirt4$^{white+1}$ and we sequenced the coding region of Sirt6^{d2} and Sirt7^{d10}. DNA from 50 flies was extracted, and quantitative real-time PCR was run with primers described in the Method section. The verification was performed several times during the cause of the experiments.

Verification of the Sirt1$^{2A-7-11}$ mutation:

Using a primer pair that amplifies a region inside the ORF of SIRT1 gene (see material and methods) no product will be created in rtqPCR reaction (as the whole coding region is deleted in Sirt1^{2A711}). To exclude the possibility that the lack of the SIRT1 PCR product was only due to a poor quality of genomic DNA we also amplified another gene (CG16941) that is present in two copies in the wild type diploid genome. We then calculated the ratio between the amounts of SIRT1 and CG16941 products. There were three possible outcomes of this calculation:

- ➔ homozygotes Sirt1^{2A711}/ Sirt1^{2A711} did not create any product with Sir2_ORFreal_OK primers, whereas products with CG16941 primers were amplified. The ratio was close to 0.
- ➔ heterozygote Sirt1^{2A711}/+ created twice less product with Sirt1^{2A711} Sir2_ORFreal_OK primers (from only one copy of genomic DNA) than with CG16941 primers (from both copies of genomic DNA), ratio 1:2
- ➔ +/+ created equal amounts of product with Sirt1^{2A711} Sir2_ORFreal_OK primers and with CG16941 primers, ratio 1:1

Real time PCR results from one of the Sirt1^{2A711} verification are shown in Table 13.

Table 13: The real time PCR results from one of the verifications of Sirt1^{2A711} genotype. Two bottles of Sirt $^{-/-}$ were tested: old one where we spotted white eyed flies and presumed contamination of the stock (Sirt $^{-/-}$ old), and a new bottle with non-contaminated Sirt $^{-/-}$ (Sirt $^{-/-}$ new). The correct stock should have the CG18446 ^{MI02952}:CG16941 ratio close to 0. The yw and w^{1118} were also included in the real time verification, and their ratio to CG16941 should be close to 1.

Sample	SIRT1 primers	Average	CG16941 primers	Average	SIRT1/CG16941
Sirt1^{2A711}	0,00000	0,00002	0,05315	0,04802	0,00047883
Sirt1^{2A711}	0,00005		0,04290		
yw	0,09488	0,07637	0,07753	0,07540	1,012822391
yw	0,05786		0,07327		
w	0,07556	0,07628	0,06816	0,06816	1,119193177
w	0,07700		0,00000		
Sirt $^{-/-}$ new	0,00000	0,00002	0,07370	0,07193	0,000336703
Sirt $^{-/-}$ new	0,00005		0,07016		
Sirt $^{-/-}$ old	0,08381	0,07893	0,11086	0,10156	0,777167317
Sirt $^{-/-}$ old	0,07405		0,09227		

Verification of Sirt4$^{white+1}$ mutation:

Using a primer pair that amplifies a region inside the ORF of SIRT4 gene (see material and methods) no product will be created in rtqPCR reaction (because the coding region of SIRT4 is deleted in Sirt4$^{white+1}$ mutation). To exclude the possibility that the lack of the SIRT4 PCR product was only due to a poor quality of genomic DNA we also amplified another gene (CG16941) that is present in two copies in the wild type diploid genome. We then calculated the ratio between the amounts of Sirt4$^{white+1}$ and CG16941 products. There were three possible outcomes of this calculation:

- ➔ homozygotes Sirt4$^{white+1}$/ Sirt4$^{white+1}$ did not create any product with Sirt4$^{white+1}$ SIRT4 ORF primers, whereas products with CG16941 primers did not be amplified. The ratio was close to 0.
- ➔ heterozygote Sirt4$^{white+1}$/+ created twice less product with Sirt4$^{white+1}$ SIRT4 ORF primers (from only one copy of genomic DNA) than with CG16941 primers (from both copies of genomic DNA), ratio 1:2
- ➔ +/+ created equal amounts of product with Sirt4$^{white+1}$ SIRT4 ORF primers and with CG16941 primers, ratio 1:1

Table 14: The real time PCR results from one of the verifications of Sirt4$^{white+1}$ genotype. The correct stock should have the Sirt$^{-/-}$:CG16941 ratio close to 0. As a positive control CG18446^{MI02952} was used.

Sample	SIRT4 primers	Average	CG16941 primers	Average	Ratio
Sirt$^{-/-}$	0,00008	7,66E-05	0,09785	0,101784	0,000753
Sirt$^{-/-}$	0,00008		0,10572		
CG18446^{MI02952}	0,00285	0,002756	0,00240	0,002383	1,156507
CG18446^{MI02952}	0,00266		0,00236		

Verification of Sirt2$^{5B-2-35}$ mutation:

Using the C1, C2 primers that surround the region deleted in Sirt2$^{5B-2-35}$ mutant (see Materials and methods) a product will of ca 200 bp will be amplified. In wild type situation (like in w1118 strain) the product will be 1382 bp. We run normal PCR reaction and visualized the PCR product on an electrophoretic gel.

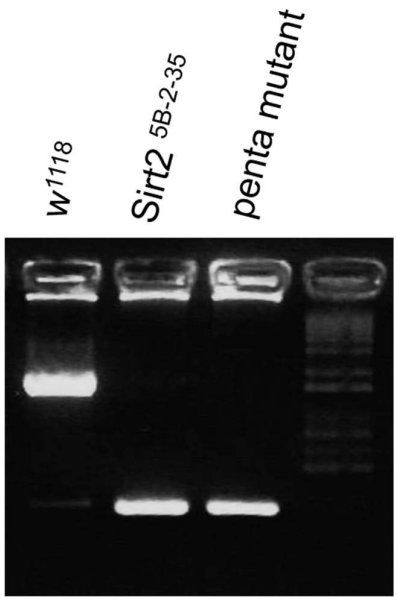

Figure 14: PCR product from Sirt2$^{5B-2-35}$ and w^{1118} strain using C1 and C2 primers. The small product amplified from Sirt2$^{5B-2-35}$ flies indicates that they really bear the Sirt2$^{5B-2-35}$ mutation.

Verification of the Sirt6 ^{d2} and Sirt7 ^{d10} mutations:

Mutations of Sirt6^{d2} and Sirt7^{d10} were created by Crispr/Cas9 system and consist of deletion of a small number of nucleotides close to the open reading frame of the genes, causing a frameshift during translation. Consequently, there should be no functional protein product. The region around the deletion was amplified by PCR and sequenced. The sequencing results are shown in figure 15 and 16.

```
1   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ATGAGCTGCAACTACGCGGATGGATTGTCAGCCTACGACAACAAGGGAATTTTGGGAGCAC  61
                                                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1   AATAGACGCTTTTGAAGTAGATCAAACTTTTATCAAACCATGAGCTGCAACTACGCGGATGGATTGTCAGCCTACGACAACAAGGGAATTTTGGGAGCAC  100

62  CAGAGAGTTTCGACAGCGATGAGGTTGTGGCCGAAAAGTGCCAGGAATTGGCTGAATTGATCAAGAAATCGGGACACGTTGTCCTCCACACGGGAGCTGG  161
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
101 CAGAGAGTTTCGACAGCGATGAGGTTGTGGCC----------AGGAATTGGCTGAATTGATCAAGAAATCGGGACACGTTGTCCTCCACACGGGAGCTGG  190

162 GATCAGTACGTCTGCAGGAATTCCGGATTTCCGCGGACCCAAGGGCGTTTGGACCCTGGAGGAGAAGGGCGAGAAGCCGGACTTCAATGTTTCCTTCGAT  261
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
191 GATCAGTACGTCTGCAGGAATTCCGGATTTCCGCGGACCCAAGGGCGTTTGGACCCTGGAGGAGAAGGGCGAGAAGCCGGACTTCAATGTTTCCTTCGAT  290
```

Figure 15: Sequencing of Sirt6^{d2} flies. Upper strand shows wild type SIRT6 sequence, lower strand shows sequencing result from Sirt6^{d2} flies.

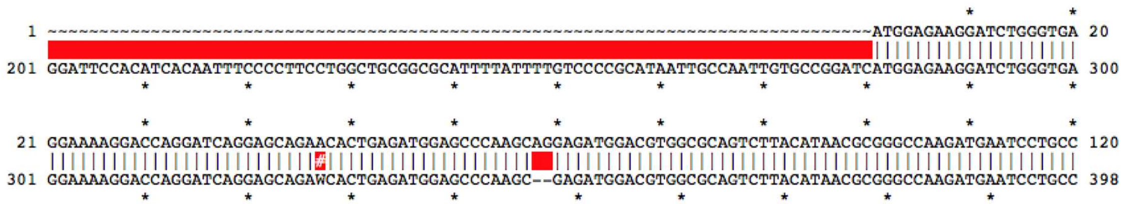

Figure 16: Sequencing of Sirt7^{d2} flies. Upper strand shows wild type SIRT7 sequence, lower strand shows sequencing result from Sirt7d^{10} flies.

Verification of the CG18446^{MI02952} mutation:

We used primers surrounding the location of transposon MI02952 insertion into gene CG18446 (primers MiMIC REAL). If there the transposon is present the product will not be created in rtqPCR reaction (product of ca 2000 bp). To exclude the possibility that the lack of the CG18446 ^{MI02952} PCR product was only due to a poor quality of genomic DNA we also amplified another gene (CG16941) that must be present in two copies in the wild type diploid genome. We then calculated the ratio between the amounts of CG18446 ^{MI02952} and CG16941 products. There were three possible outcomes of this calculation:

- ➔ homozygotes CG181446^{MI02952}/ CG181446^{MI02952} did not create any product with CG18446 MIMIC REAL primers, whereas products with CG16941 primers did not be amplified. The ratio was close to 0.
- ➔ heterozygote CG18446 ^{MI02952}/+ created twice less product with CG18446^{MI02952} MIMIC REAL primers (from only one copy of genomic DNA) than with CG16941 primers (from both copies of genomic DNA), ratio 1:2
- ➔ +/+ created equal amounts of product with CG18446^{MI02952} MIMIC REAL primers and with CG16941 primers, ratio 1:1

Table 15 shows a typical result from the real time PCR verification of CG18446^{MI02952} allele.

Table 15 The real time PCR results from one of the verifications of CG18446 MI02952 genotype. The correct stock should have the CG18446 MI02952:CG16941 ratio close to 0. yw strain was used as a positive control.

Sample	CG18446 MI02952	Average	CG16941	Průměr	Ratio
CG18446 MI02952	0,00033	0,00078	0,20385	0,21391	0,00364647
CG18446 MI02952	0,00123		0,22397		

Testing Sirt$^{-/-}$ resistance to stress

As mentioned before at the beginning of this section, the fly lacking all Sirtuin gene was fully viable. As Sirtuins are involved in whole range of metabolic regulations and also in ROS generation, we decided to test if the Sirt$^{-/-}$ flies become susceptible to various types of stress conditions. We tested the flies in three types of stress: cold stress, starvation and oxidative stress.

4.3 Cold stress tolerance

It is known that in temperatures close to 0 °C the chill coma is induced in *Drosophila*, as it loses its ability to maintain resting muscle potential (94) (95). After the temperature increase, most of the flies can recover from the coma without any obvious damage. However, the chill coma and subsequent recovery cause immense energetic stress to the organism and the recovery requires sufficient supplies of glycogen and fatty acids from fat body to the peripheral tissues. These supplies must be mobilised on time, and they need to be allowed to enter to the peripheral tissues. We wanted to test whether the Sirtuins could play a role in this process.

Test of chill coma recovery assay was repeated four times. We used 4-5 days old flies in every repetition, and the flies were kept each time under the same conditions (see Material and methods). The 96-wells plates with flies were buried in the box of ice in the same depth to maintain similar conditions for all repeats of the experiment. 120 adult males from every strain were tested in each replicate of the experiment. Due to more complicated metabolism of females (caused by laying eggs) only the males were tested in this assay.

As showed in Figure 17, Sirt$^{-/-}$ flies recovered earlier than two from three controls. For statistical analysis, the unpaired t-test was utilised showing significant differences between

OR and Sirt$^{-/-}$ or w and Sirt$^{-/-}$. Difference between Sirt$^{-/-}$ and yw was not significant, as was the difference between w^{1118} and CG18446 ^{MI02952} flies.

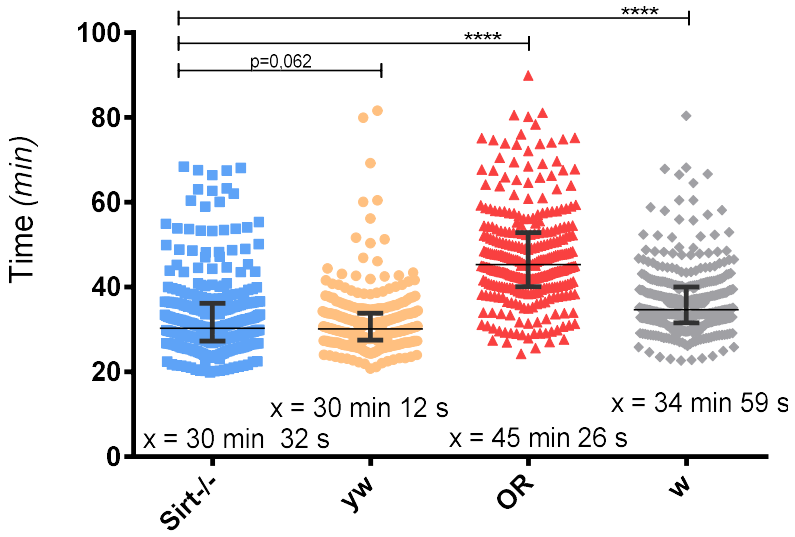

Figure 17: Recovery time of males after chill coma. Results combined from all 4 replicates of the experiment and compared using unpaired t-test (**** p<0,0001). *X* stands for the median of recovery time of each strain.

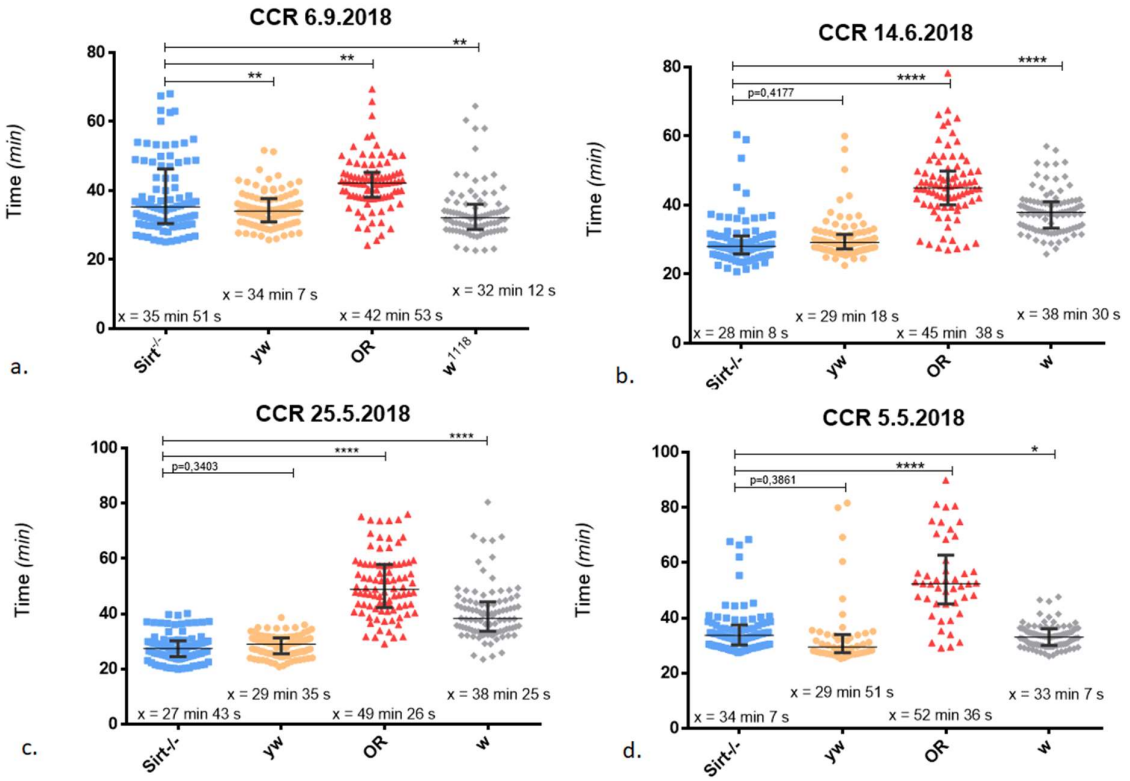

Figure 18: Values from 4 biological replicates of chill coma recovery (CCR) assay. **a.** significant difference between Sirt$^{-/-}$ and other controls (p >0,01), **b**. significant difference between Sirt$^{-/-}$ and OR and w^{1118} (p >0,0001), difference between Sirt$^{-/-}$ and yw was not significant (p = 0,4177), **c.** significant difference between Sirt$^{-/-}$ and OR and w^{1118} (p >0,0001), difference between Sirt$^{-/-}$ and yw was not significant (p= 0,3403), **d.** significant difference between Sirt$^{-/-}$ and OR and w^{1118} (p >0,0001), difference between Sirt$^{-/-}$ and yw was not significant (P = 0,3861). *X* stands for the median of recovery time.

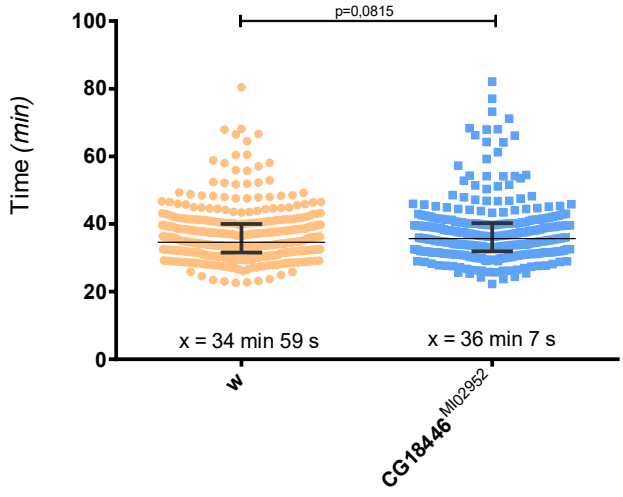

Figure 19: Chill coma recovery assay in the negative control CG18446^{MI02952}. Values from all 4 biological replicates, compared using unpaired t-test (**** p <0,0001). *X* stands for the median of recovery time.

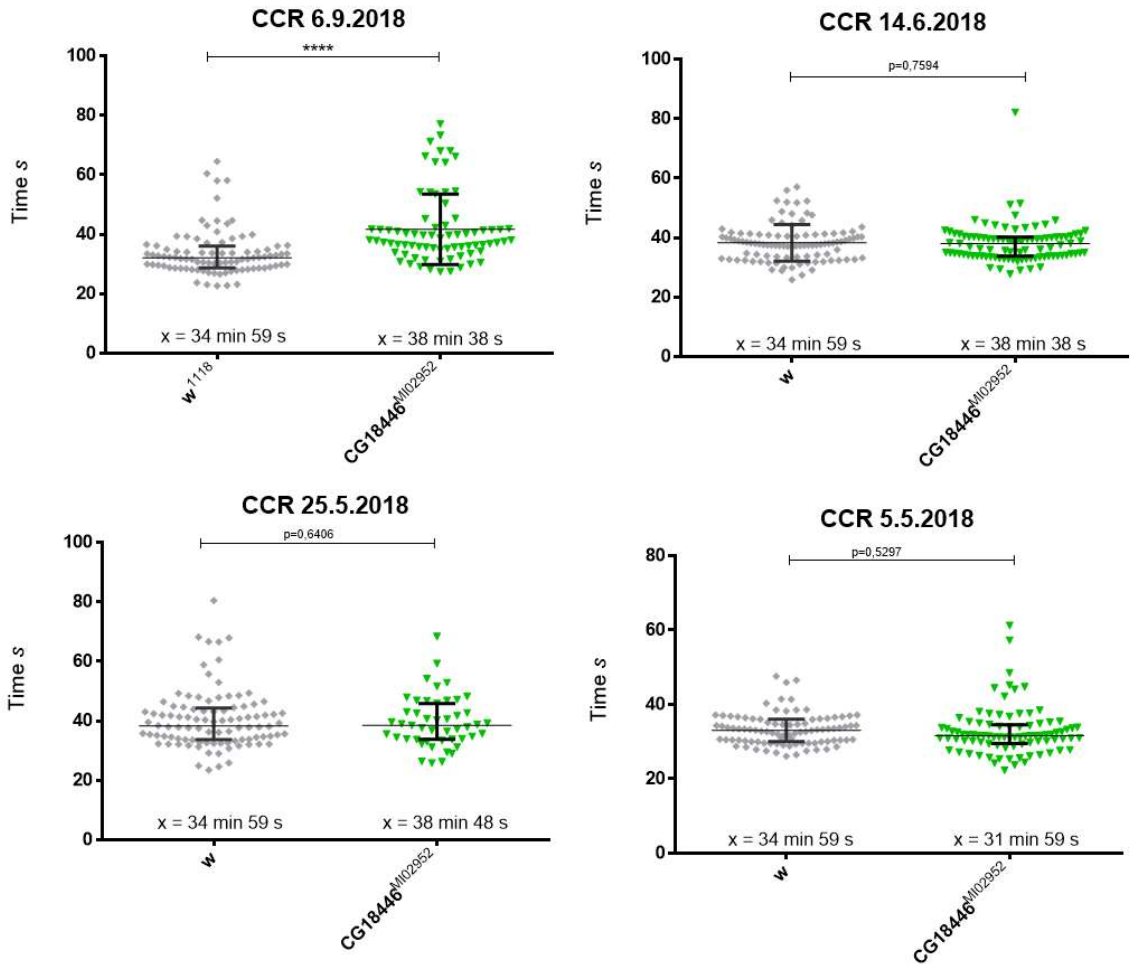

Figure 20: Graphs of 4 biological replicates of chill coma recovery (CCR) assay. Comparison between negative control $CG18466^{MI02952}$ and w^{1118}. Only significant difference occurred in the first graph. *X* stands for the median of recovery time.

4.4 Oxidative stress

Due to the close relationship between Sirtuins and regulation of ROS mentioned previously in the text, we decided to test the oxidative stress resistance of $Sirt^{-/-}$ flies. Oxidative stress was induced by peroxide (0,5 % H_2O_2) added into the food after its cooling to 40 °C. First, the inhibitor of catalase 3 – AMINO – 1, 2, 4 – TRIAZOLE was added into to diet to maximize of H_2O_2 effect (96). Otherwise, the flies' naturally expressed catalase would decompose the hydrogen peroxide to water and molecular oxygen, thereby largely neutralised the effects of H_2O_2.

Test of resistance against oxidative stress was repeated three times. There were 4-5 days old flies in every repetition, and the flies were kept under the same conditions (see Material and methods). 120 adult males from every strain of flies were chosen for experiment.

The hypothesis we had presumed a worse resistance to oxidative stress in $Sirt^{-/-}$ mutants, but it was not confirmed since $Sirt^{-/-}$ did not differ from control (Fig. 21) and the median values were analysed using unpaired t test (no significance, $p > 0,05$). The $CG18446^{MI02952}$ control was less resistant to oxidative stress, as expected (Fig. 21). However the significance was not proven ($p > 0,05$, median values).

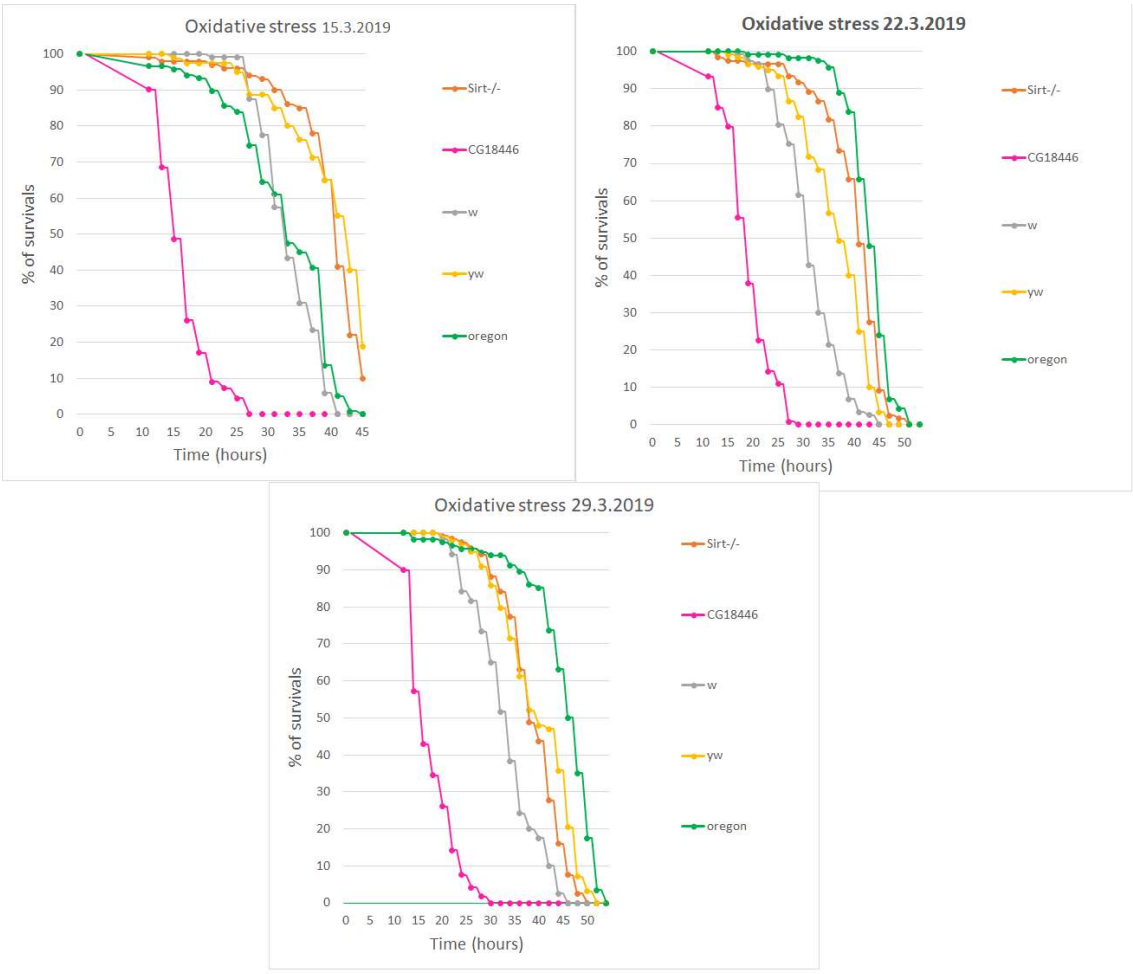

Figure 21: The percentage of surviving males of individual fly strains during oxidative stress. Three biological replicates.

4.5 Starvation assay

During starvation, the organism switches its metabolism in order to utilize the energy stores. It includes the use of fatty acids oxidation, leading to higher levels of acetyl coenzyme A and subsequent formation of ketone bodies that can result in a decrease of ratio NAD+/NADH (97). As to NAD+/NADH ratio closely influences Sirtuin function, the next test chosen for the characterisation of our mutant flies was the test of resistance to starvation (98).

Test of resistance in starvation was repeated four times. There were 4-5 days old flies in every repetition, and the flies were kept under the same conditions (see Material and methods). 120 adult males and 120 females from every strain of flies were chosen for each biological replicate of the experiment.

We expected disrupted metabolism in $Sirt^{-/-}$ mutants and thereby shorter time of survival during starvation in comparison to control strains. However, this hypothesis was not confirmed, since the results prove no significant differences (unpaired t test from median values, $p > 0,05$). In the $CG18446^{MI02952}$ control, the time of survival was shorter as expected. However, the significant difference was displayed only in males (unpaired t test from median values, $CG18446^{MI02952}$ vs w^{1118} p=*, $p < 0,01$).

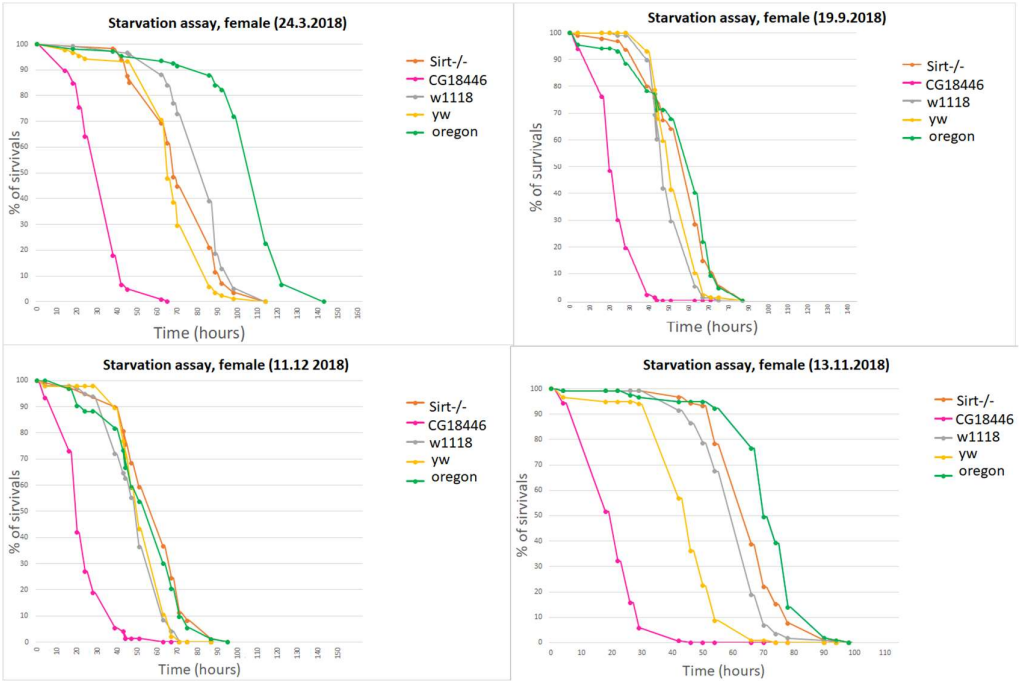

Figure 22: Percentage of survivals of female flies under starvation. Graphs from 4 biological replicates. Negative control $CG18446^{MI02952}$ survived the shortest time in all experiments as

expected.

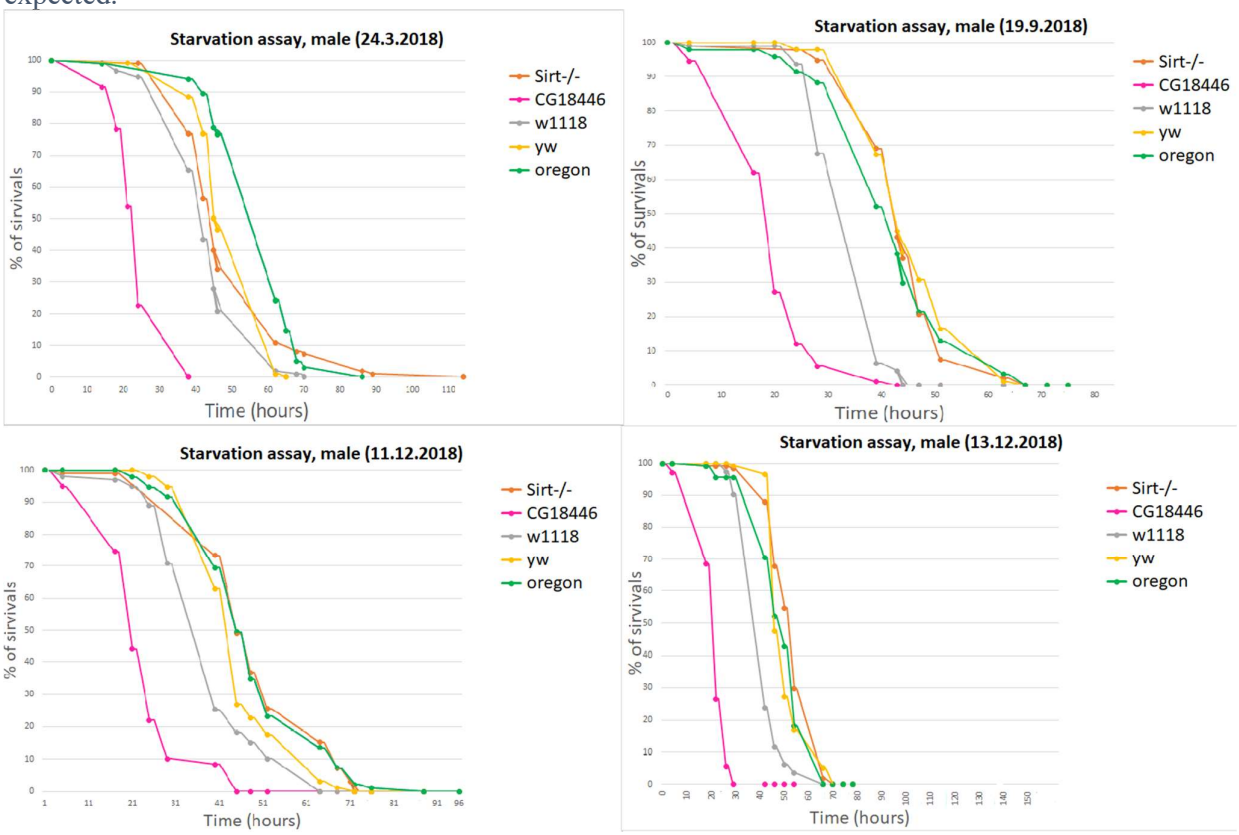

Figure 23: Percentage of survivals of male flies under starvation. Graphs from all 4 biological replicates. Negative control CG18446^{MI02952} survived the shortest time in all experiments as expected.

4.6 Lifespan

A 100 male individuals of each strain were used for testing lifespan. After distribution under light anesthesia flies were transferred into the vials with fresh food either with high or low amount of yeast (see Methods) and stored in 25 °C incubator. The position of vials was randomised and changed with every counting od dead flies.

We expect shorter lifespan of Sirt$^{-/-}$ and CG18446 ^{MI02952} in comparison to control strains. This experiment runs in duplicates, and the results will be discussed in defense of the master's thesis. At this moment, the control CG18446 ^{MI02952} control appear to have shorter lifespan than the rest of the strains (except OR). Sirt$^{-/-}$ shows shorter life span on both types of diet. Current state of the results are shown in the Figure 24.

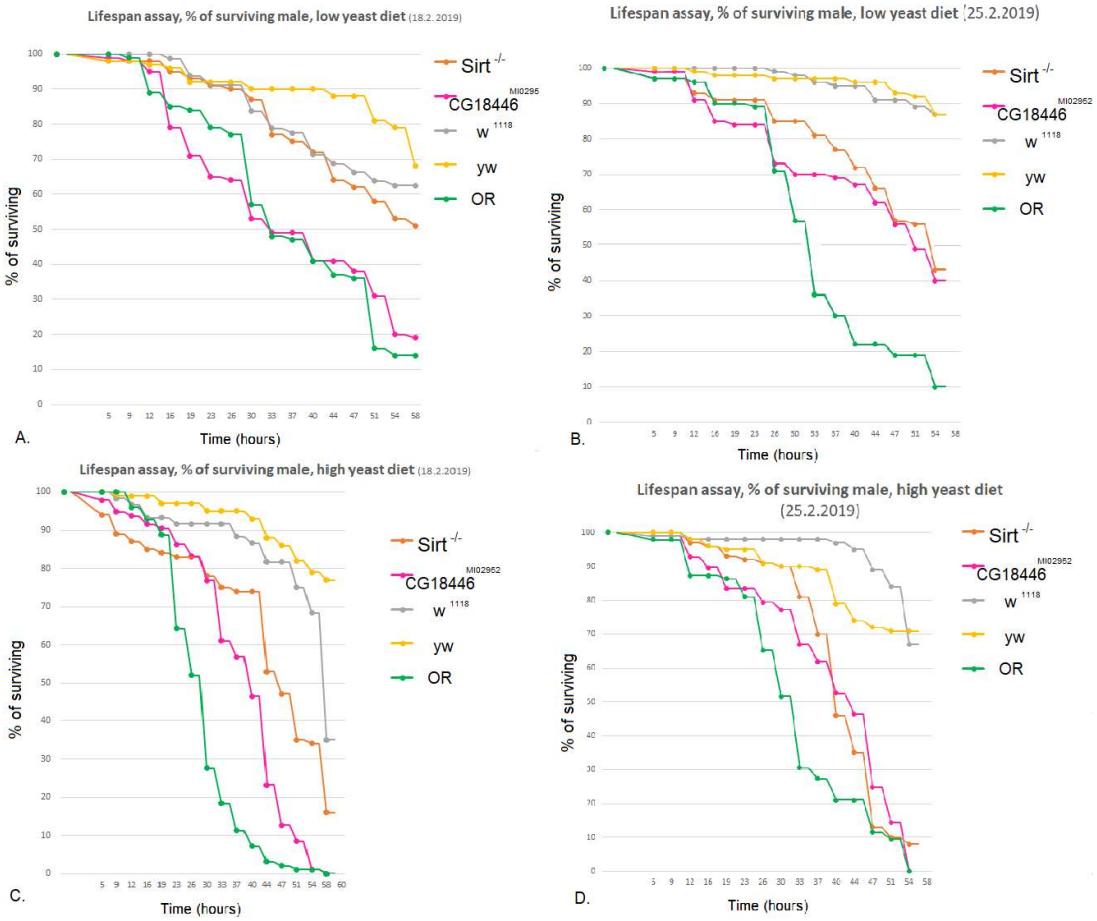

Figure 24: Summary of the ongoing lifespan experiments. Percentage of surviving males on two types of diet (low yeast and high yeast), graphs from 2 biological replicates (current results). Sirt$^{-/-}$ implies shorter life span on high yeast diet. Final results will be available during the defense of the thesis.

4.7 Weighting

We noticed immediately that the Sirt$^{-/-}$ flies were bigger than any of the control flies. We, therefore, decided to assess their weight.

Two hundred individuals of the same sex, strain and age that were bred in the same larvae density (see chapter Methods and materials) were inserted in a tube, lyophilized and the dry weight of per fly calculated. The weighting was repeated in three biological replicates

We expected a higher weight of Sirt$^{-/-}$ in comparison to control strains as the Sirt$^{-/-}$ mutants seemed to be bigger. This presumption was confirmed ($p < 0{,}0001$). On the other

hand, the CG18446^{MI02952} control flies had lower weight in comparison to w^{1118} ($p < 0,0001$). Unpaired t-test was used for statistical analysis.

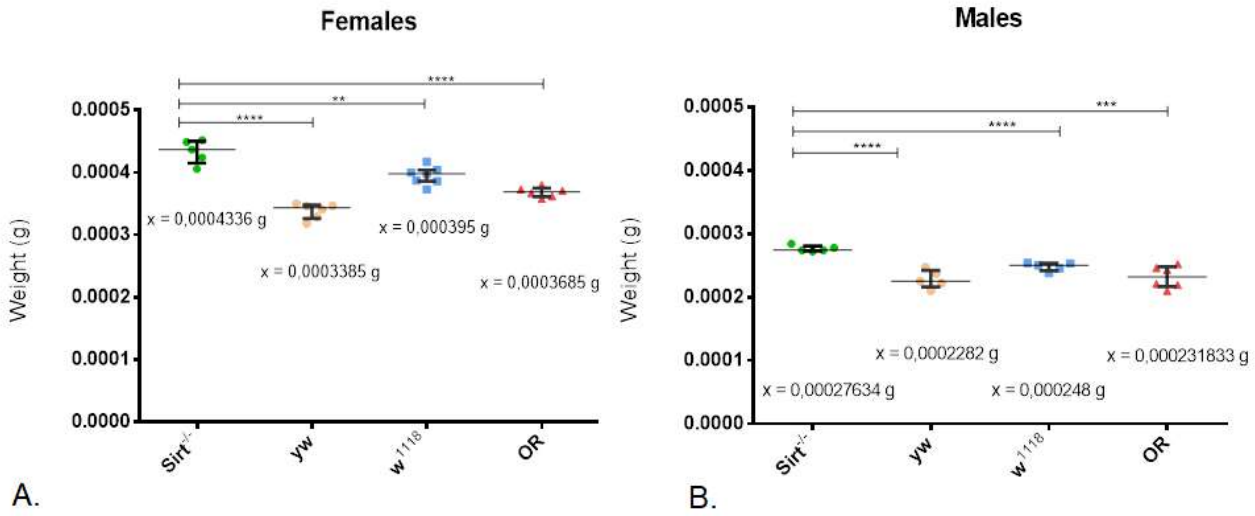

Figure 25: **A.** Average dry weight per female fly of indicated genotype. Values from 3 biological replicates. The significant difference were observed (**** $p < 0,0001$, ** $p< 0,01$). x stands for average value. **B.** Average dry weight per male fly of indicated genotype. Values from 3 biological replicates. The significant difference were observed (**** $p < 0,0001$, *** $p< 0,001$). x stands for average value.

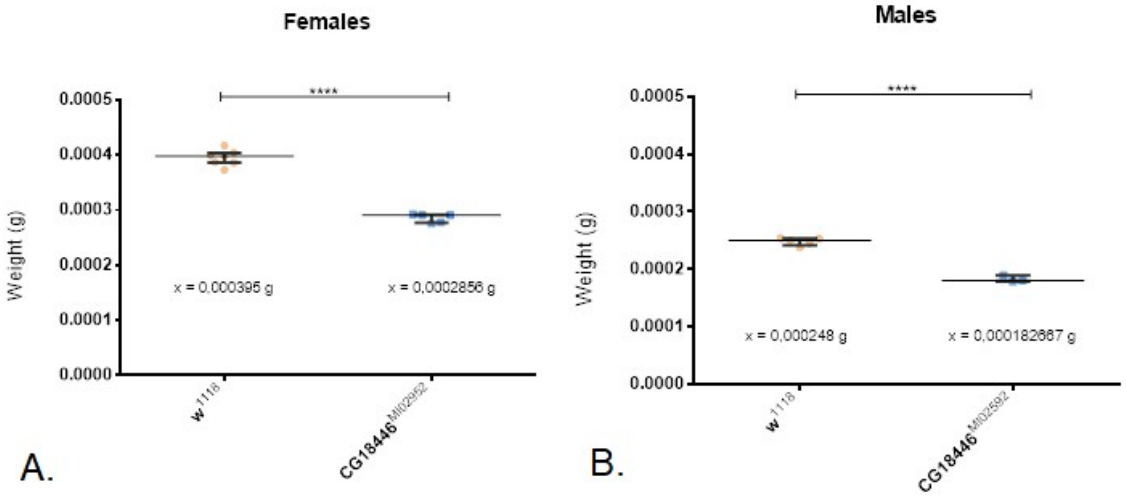

Figure 26: **A.** Average dry weight per female fly of indicated genotype. Values from 3 biological replicates. The significant difference were observed (**** $p < 0,0001$). x stands for average value. **B.** Average dry weight per female fly of indicated genotype. Values from

3 biological replicates. The significant difference were observed (**** p < 0,0001). x stands for average value.

4.8 Fecundity and fertility

During the optimization of the staging process of the flies, we observed a lower number of eggs laid by $Sirt^{-/-}$ in comparison to control strains. When the equal number (32 µl) of eggs was transferred from juice plates to flasks, the flasks with $Sirt^{-/-}$ had lower number of hatched larvae and fewer adult flies. For those reasons we decided to count the number of eggs (fecundity) and the number of hatched larvae (fertility) in $Sirt^{-/-}$ and other control strains.

Thirty females and fifteen males of every strain were distributed to small cages with agar juice plates. After two days of adjusting period, the plates were changed four times in 24h, the number of laid eggs was recorded. The following day, the hatched larvae were counted on the same plates.

We expected worse results in $Sirt^{-/-}$ in comparison to control strain. This presumption was confirmed (Figure 27), except comparison with yw strain (p=0,1193). The percentage of laid eggs was statistically analyzed using unpaired t test (all values p <0,05). The $CG18446^{MI02952}$ control no difference in fecundity in comparison to w^{1118}. However, the number of hatched larvae (fertility) was significantly lower for both $Sirt1^{-/-}$ and for $CG18446^{MI02952}$ (Fig. 28,29), reaching only 10 % of hatching success for $Sirt1^{-/-}$ flies.

These results suggests that $Sirt1^{-/-}$ flies have not only problems to lay eggs but, importantly, that the eggs have problem to develop into a larva. In future experiments it would be interesting to test whether the unhatched eggs are simply not fertilized or whether the development of the embryo arrests at certain stage of development. Nevertheless, from the results of our stress assays it is obvious that despite problems with the $Sirt^{-/-}$ fertility, those eggs that eventually develop give rise to adults that are not severely affected.

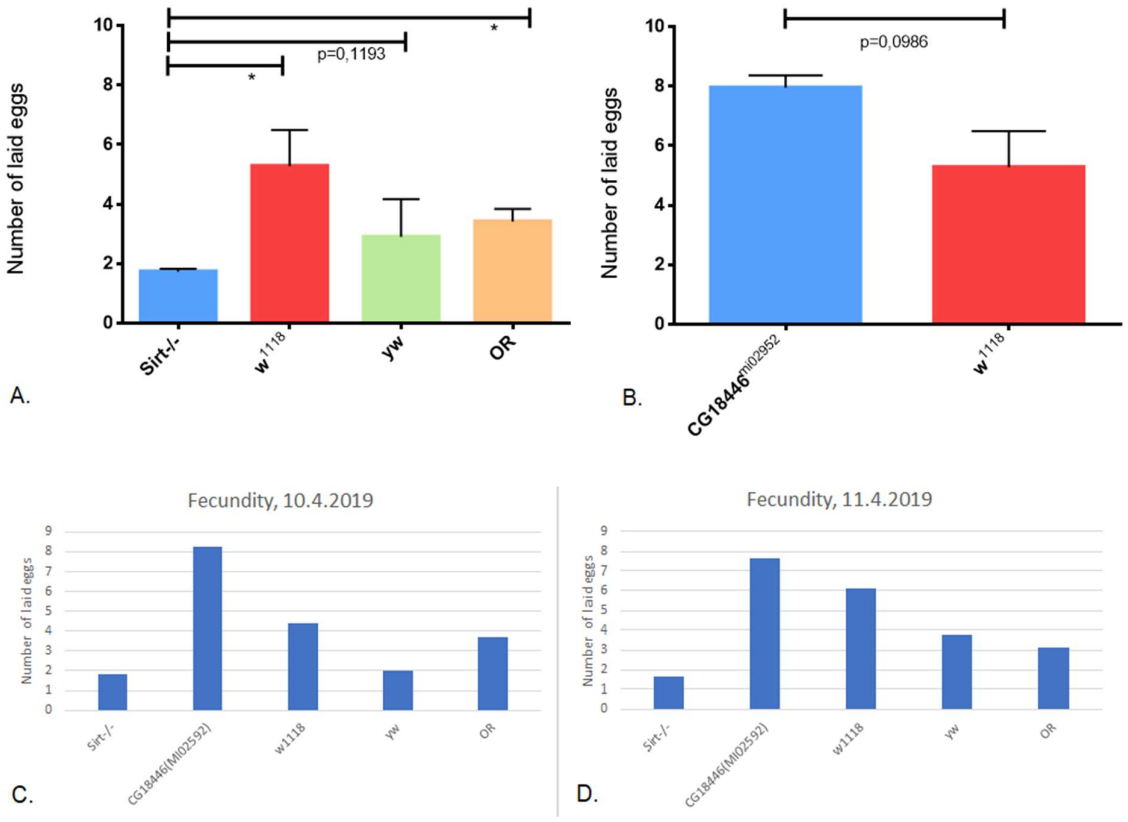

Figure 27: Number of eggs laid in 24 hours by 1 female in the cage. Graphs represent averages from 2 biological replicates. **A**. Significant differences was displayed between $Sirt^{-/-}$ and w^{1118} and OR (*, $p < 0,05$), no difference in comparison to yw strain, **B**. No significant difference monitored between $CG18446^{MI02952}$ and w^{1118} ($p > 0,05$). **C** + **D.** Data from each experiment.

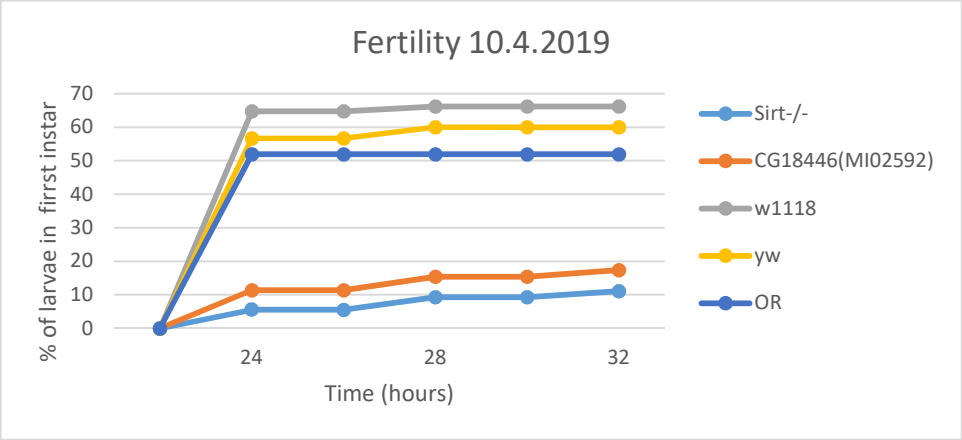

Figure 28: Percentage of hatched larvae; $Sirt^{-/-}$ 11,1 %, $CG18446^{MI02952}$ 17,4 %, OR 51,9 %, yw 60 %, w^{1118} 66,2 %.

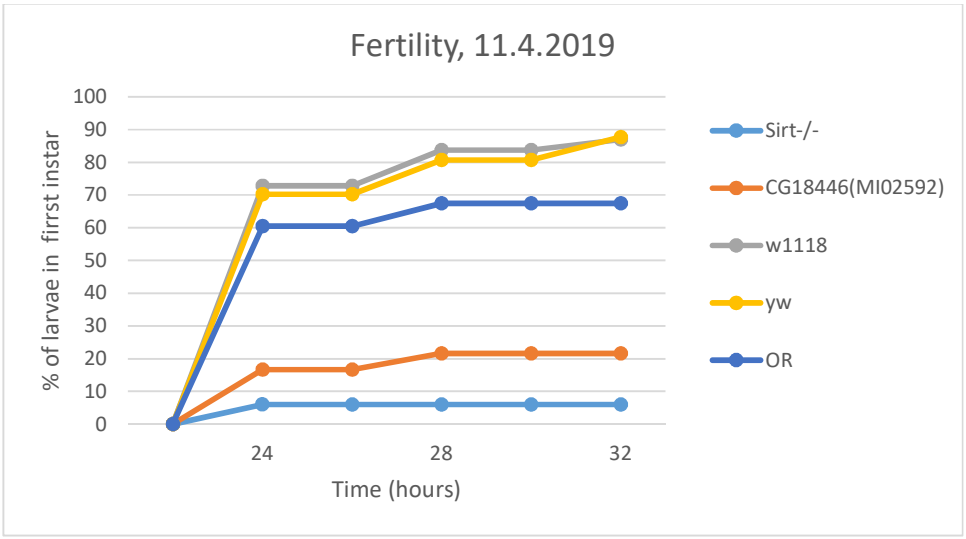

Figure 29: Percentage of hatched larvae; $Sirt^{-/-}$ 6 %, $CG18446^{MI02952}$ 21,6 %, OR 67,4 %, yw 87,7 %, w^{1118} 87 %.

5 DISCUSSION

So far, neither in mammals nor in *Drosophila* anybody has created an organism lacking all Sirtuin genes. The aim of our laboratory was to create such a fly and to characterize its phenotype. After a demanding crossing procedure, the penta mutant fly ($Sirt^{-/-}$) was successfully created. We expected that $Sirt^{-/-}$ will not be viable. To our surprise, however, the fly was not only fully viable even with no obvious developmental defects (although with decreased fecundity and fertility and with increased body weight). The arising question is, why does the Drosophila have all five sirtuin genes if it is not essential for its life? Furthermore, Sirtuin genes are conserved across many organism, from *S. cerevisiae* (1) to humans (99) (12). If Sirtuins genes were redundant, they would probably vanish during evolutionary development. These are only more reasons to think that flies lacking all Sirtuin genes should either have problems during development or it should affect their responses under stress conditions. The results of my thesis, however, show that this is not the case and the phenotype of the sirtuin penta mutant ($Sirt^{-/-}$) fly is only mild, in fact, milder than the phenotype of the individual sirtuin mutants alone.

5.1.1 Knockout of Sirtuins results in heavier *Drosophila* adults

The first phenotypical characteristics that we have observed was the bigger size of $Sirt^{-/-}$ flies in comparison to controls (w^{1118}, yw, OR). Therefore, we decided to weight the mutant flies, and our presumption was confirmed (Fig 25). The bigger weight could be due to $Sirt^{-/-}$ flies being obese or because of overactivation of pathways involved in the regulation of body size, such as the mTOR or insulin pathway.

There is a lot of evidence connecting sirtuins to obesity (80) (100) and fatty acids metabolism (101). Drosophila SIRT1 (dSir2) plays a pivotal role in the activation of PGC-1 and FOXO function via their NAD-dependent deacetylation, indicating that SIRT1 and NAD+ levels are associated with glucose metabolism (102) (103) (104). Total glucose and triglyceride levels of SIRT1 were increased in mutant flies ($Sir2^{2A711}$) and decreased in flies with SIRT1 overexpression, clearly showing that SIRT1 regulates fat metabolism (Fig. 30 and (80)). However, no significant difference in the weight of $Sir2^{2A711}$ mutant flies was observed in this study (80). A recent study has confirmed that SIRT1 acts in the fat body to maintain insulin sensitivity and it regulates the expression of metabolic gene regulators such as *dHNF4*

(hepatocyte nuclear factor 4 in Drosophila) (83). This study also showed that SIRT1 mutants develops age-progressive symptoms of diabetes leading to obesity (83). We have not measured directly the amount of lipids or other metabolites in the $Sirt^{-/-}$ flies, but we could speculate that the phenotype of our $Sirt^{-/-}$ flies could possibly be explained simply by the effect of the $Sir2^{2A711}$ mutation.

Controversly however, the mutant for SIRT7, was reported to have the opposite effect on fat metabolism, as SIRT7 mutant flies had a lower level of TAGs than control strains (105) (106). The overall bigger size of the $Sirt^{-/-}$ flies could therefore be explained also by other mechanisms than by affecting the amount of fats or glucose metabolites in the fly, for example by the effect of sirtuins on mTOR or insulin signaling that are the master regulators of cell and organismal size (107) (108) (109).

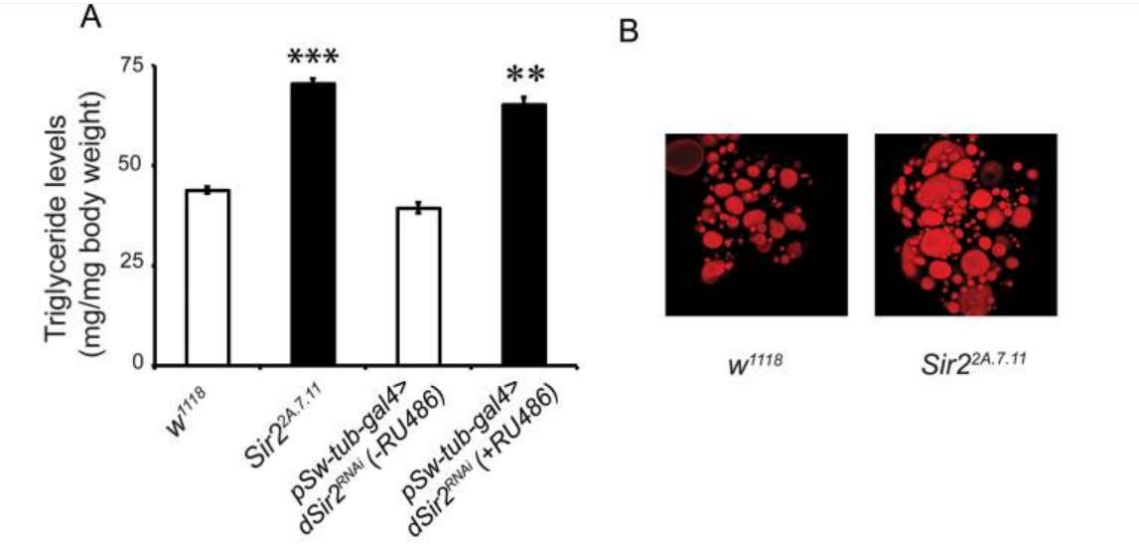

Figure 30: A. Levels of TAGs in SIRT1 mutant ($Sir2^{2A711}$), in whole-body induced Sirt1-RNAi (pSw-tub-gal4>$dSir2^{RNAi}$ (+RU486)) and in control conditions. B. The deregulated fat metabolism and increased triacylglycerol (TAG) levels were corroborated by oil red staining of fat bodies of SIRT1 mutant flies *(80)*.

5.1.2 $Sirt^{-/-}$ flies indicates poor fecundity, fertility and delayed development of adults

Another phenotype of $Sirt^{-/-}$ flies that we noticed immediately was their defects in fecundity and fertility, thus lower number of laid eggs and subsequently lower number of larvae. The larval development lasted one day longer than that of any other control strains we

used. Moreover, it took a longer time for the Sirt$^{-/-}$ flies to emerge in comparison to controls. We speculate that this might have associations with the increased body weight, as a result of poor ability to release energy reserves like triglyceride (lipid) (110) (111) and glycogen (glucose) (112) from the stores in order to support the larval development. Drosophila stores release chemical energy in the form of glycogen and lipids. Although glycogen accumulates also in muscles (113), the main storage organ for both carbohydrates and lipids is the fat body (114). Above mentioned study (80) showed that besides the defects in fat body in SIRT1 mutant, there is no effect on the muscle tissue. In addition, the association between fertility and SIRT4 was explored in recent study (50). Flies lacking SIRT4 in fat body displayed decreased fertility, with 33% fewer eggs produced than wild-type controls on both high- and low-calorie content food (50). We may speculate that the balance between storing and releasing energy in Sirt$^{-/-}$ flies is shifted towards energy storage, and this could be the reason for increased body size but also slower development and poor fecundity and fertility. The poor egg hatching could simply be explained by problems with egg fertilization, either due to improper development of sperms or the eggs. As SIRT6 and SIRT7 are expressed most abundantly in the ovary, their mutations could be responsible for the fertility problems of Sirt$^{-/-}$ flies.

5.1.3 Sirt$^{-/-}$ do not show changes in the resistance to starvation

In connection to poor energy management of individual Sirtuin mutants and presumably also of Sirt$^{-/-}$ flies, we expected premature deaths of Sirt$^{-/-}$ mutants in starvation assay. Nevertheless, Sirt$^{-/-}$ flies survived approximately as long as other control strains in our experiments. In mammals, SIRT1 mediated transcription of genes involved in metabolic homeostasis during starvation (115) (19) (116). In support of this observation, another study showed that SIRT1 activity increases in response to starvation and its absence decreases starvation resistance in Drosophila (80). Further, Drosophila SIRT1 is a critical factor in fat mobilization from the fat body during starvation. Flies mutant for SIRT1 in the fat body failed to mobilize energy reserves that negatively affected their starvation surviving (80).The long-lived *Methuselah* mutants have increased starvation resistance coinciding with increase in their fat stores (117). Also, there was a study selecting *Drosophila* strains based on their increased starvation resistance that correlated with increased fat storage (118) (119). From these reasons, we expected lower survival of Sirt$^{-/-}$ during starvation. On the other hand, however, there is also evidence of starvation resistant fly lines with reduced body fat stores

(120). These findings indicate that increase in body fat may or may not affect the starvation survival.

Further, other sirtuins can affect the *Drosophila* metabolism during starvation. Flies with downregulation of SIRT4 in a fat body exhibit impaired ability to use energy stores during fasting, similarly to SIRT1 mutant (50). Sensitivity to starvation could be caused by differences in total energy stores; rates of synthesis, mobilization, or catabolism of energy stores; or a combination of these factors. Drosophila SIRT4 mutant flies show moderately higher level of TAGs. In this study (50), the maintenance of energy resources (TAGs, glycogen, trehalose, glucose) during fasting were measured (Fig 31). Drosophila SIRT4 knockout flies starved more rapidly and consistently maintained higher levels of energy storage metabolites during fasting, indicating that they may suffer from an inability to properly catabolize energy reserves during periods of fasting (50). However, despite the fact that $Sirt^{-/-}$ flies do not have SIRT1 and SIRT4 they do not show significant changes in the resistance to starvation. This indicates that the $Sirt^{-/-}$ flies do not simply phenocopy the sum of phenotypes from the individual Sirtuin mutants.

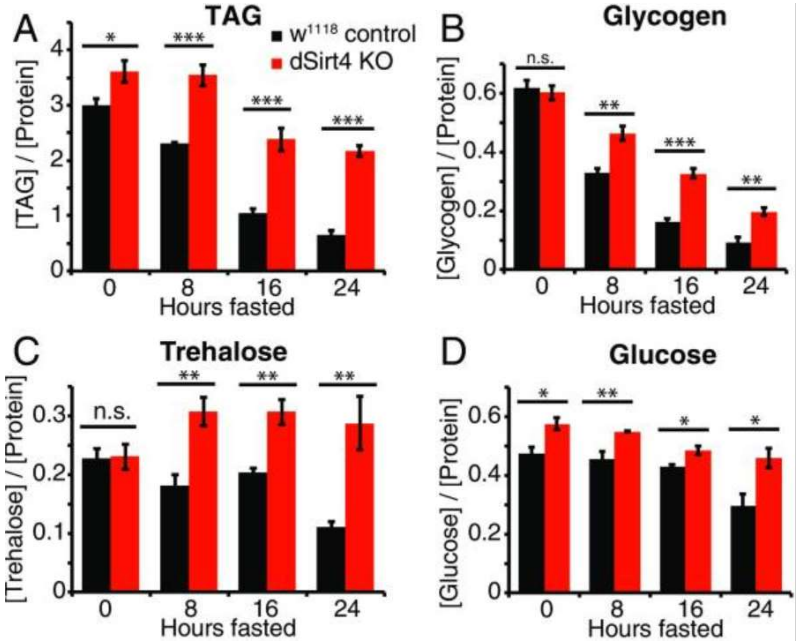

Figure 31 : Levels of energy storage metabolites in Drosophila SIRT4 (dSIRT4) fat body specific knockout (KO) flies during fasting. (A) TAG levels in control (black) and dSirt4 KO (red) male flies, (B) levels of glycogen, (C) levels of trehalose, (D) levels of glucose *(50).*

5.1.4 Sirt$^{-/-}$ flies can cope well with H_2O_2-induced oxidative stress

Due to our initial observations (weight, fertility, fecundity, development), we anticipated that Sirt$^{-/-}$ flies may have some metabolic defects, and therefore it may also produce more ROS, as it is the case of mutants for SIRT1 or SIRT2. Besides that, there is an evidence that obesity and higher level of ROS (reactive oxygen species) could have a negative effect on performance in stress assays (121) (122) (123). On the other hands, longevity models of Drosophila (TOR (124), IIS (125), Methuselah mutants (126)), that are also obese, have increased resistance to paraquat, a commonly used to evoke oxidative stress. These findings speculates on a potential antioxidant role of the lipid droplets in these long-lived obese flies (127). The function of sirtuins is associated with protection against oxidative stress and decrease of ROS levels. Moreover, the oxidative stress damages of DNA can result in acetylation of p53 and thereby, induce cellular senescence. As mentioned before in the text, p53 is also in close relationship with sirtuins. Another study showed that oxidative stress induced by H_2O_2, as in our experiment, leads to depletion of NAD^+ resources and to decrease in sirtuin activity (128). This is next reason, why we expected mutant flies Sirt$^{-/-}$ to have lower resistance under the oxidative stress. Nonetheless, no differences were observed in comparison to controls.

5.1.5 Sirt$^{-/-}$ flies recovered from chill coma slightly earlier

Only little is known about the cold stress tolerance and Sirtuins in *Drosophila*. However, the expression of mammalian SIRT3 is increased when exposed to the cold (37) (129). The closest homologue of the mammalian SIRT3 is Drosophila SIRT2 according to the BLAST (Basic Local Alignment Search Tool) searches. Nevertheless, there is no evidence about the connection of Drosophila SIRT2 with cold stress. In our cold stress tolerance assay we expected that Sirt$^{-/-}$ flies might have higher amount of circulating glucose (as SIRT1 mutant alone), and therefore they could recover from chill coma earlier than control strains (although the release of the energy from the fat body might be negatively affected, as we discuss in the above sections). Interestingly, Sirt$^{-/-}$ mutants really recovered from chill coma slightly earlier but only when compared to two of the three control strains. The results of this assay, therefore, cannot be unequivocally interpreted.

5.1.6 Sirt$^{-/-}$ implies to be short-lived on high yeast diet

In *Drosophila*, overexpression of SIRT1 extended the lifespan on high caloric (high yeast) diet (72) (130) although earlier studies without properly controlled genetic background showed shortened lifespan (131). The overexpression of *Drosophila* SIRT1 in the pan-neuronal cells or fat body extended the lifespan, but the SIRT1 induction in motoneuron or muscles had no effect on the lifespan (72) (130). These findings imply that the life extension is tissue-specific. Furthermore, the overexpression of Drosophila SIRT4 in the whole body or in the fat body results in lifespan extension and increase the resistance to starvation (50). Male, but not female, transgenic mice overexpressing *SIRT6* have a significantly longer lifespan than wild-type mice, but the role of SIRT6 in *Drosophila* has not yet been investigated (58).

Moreover, several selection experiments revealed the existence of a genetically based variation in the fat metabolism and lifespan, suggesting that the fat storage and longevity could be controlled by analogous principle (132) (133) (118) (119).

According to these studies, we expect Sirt$^{-/-}$ flies to have shortened lifespan in comparison to control strains. Current results are showing a trend towards life shortening on both types of diet. The final results will be available during the defense of the thesis.

5.1.7 What comes next?

One of the possible caveats connected with Sirt$^{-/-}$ flies is he fact that we can not strictly control the genetic background. Many studies has been doubted due to different genetic background that affected the phenotype of the gene under investigation (72) (26) (134) (135). The stock was created by a complicated crossing scheme, and therefore its genetic background is not easily defined. This could be rectified in our case by cleaning the Sirt$^{-/-}$ into the selected wild type genetic background, such as crossing it to w1118 strain for at least six generations. However, this would be very difficult since none of the Sirtuins mutants display an obvious phenotype and the Sirt1^{2A711}, Sirt2$^{5B-2-35}$, Sirt6^{d2} and Sirt7^{d10} mutations have no selection marker either (they do not bear w+ allele or similar marker). From this reason, we decided to compare our penta mutant (Sirt$^{-/-}$) to several controls at once. It would certainly be useful to try to clean the genetic background of Sirt$^{-/-}$ in some way in the future and see if

some minor phenotypic differences become more obvious in comparison to a genetically matched control.

Further, we can not exclude the possibility that the small deletions in $SIRT6^{d2}$ and $SIRT7^{d10}$ created by Crispr/Cas9 may not fully prevent reinitiating of translation later in the transcript. It is, therefore, possible that the protein product of SIRT6 and SIRT7 is still created, although in a limited manner. As mentioned before, the sirtuins have overlapping function (CIT). Thus, SIRT6 and SIRT7 could alternate some function of other knockout sirtuins. The SIRT6 and SIRT7 genes in *Drosophila* were not explored yet. However, mutants made in our lab did not display any defects (unpublished data). To test whether a protein product is created from the $SIRT6^{d2}$ and $SIRT7^{d10}$ transcripts we would need antibodies against Drosophila SIRT6 and SIRT7 gene that are however not available. Without performing a western blot with SIRT6 and SIRT7 specific antibodies, we can not be sure whether the $SIRT6^{d2}$ and $SIRT7^{d10}$ alleles are loss of function.

An important line of future research of $Sirt^{-/-}$ strain would be to measure its metabolic parameters, such as the levels of TAGs, DAGs, glycogen, trehalose or glucose. This approach could again be difficult to properly interpret without having the $Sirt^{-/-}$ in a clean genetic background. Nevertheless, it would be interesting to see if there are at all any metabolic changes in $Sirt^{-/-}$ flies or whether the metabolic changes occur but do not have any significant impact on the $Sirt^{-/-}$ resistance in stress assays.

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