The University of South Bohemia in České Budějovice
Faculty of Science

Sirtuins as regulators of apoptosis-induced proliferation in the eye of *Drosophila melanogaster*

Bachelor's thesis

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Annotation:

The effect of Sirtuins on the activity of mTOR, a regulator of apoptosis induced proliferation, was studied in the eye of *Drosophila melanogaster*. The eye sizes of wildtype flies and mutants with knockdown of a Sirtuin, respiratory Complex I or both were compared by quantifying the amount of red eye pigment with colorimetry.

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

Place Seitenstetten, Austria **Date** 30.3.2023

Student's signature Philipp Hebesberger

Acknowlegdements: My supervisor Alena Bruce Krejčí has my deep gratitude for supporting me over the years despite the setbacks and guiding me through the process of understanding the topic and writing this thesis. Likewise, my co-supervisor Valentina Magnin, who taught me how to conduct my experiments, stayed patient with me despite the difficulties and I thank her for that. From many different students I met during my time as a student I received so much help and kindness, even if none of them knew me well, which means the world to me. And if it weren't for my parent's support and their faith in me, all my effort would have been impossible.

Contents

1. Introduction

1.1 Functions of the mitochondrion

Mitochondria are organelles with two membranes, which are found in all eukaryotes, as they generate most of the cell's energy in the form of adenosine triphosphate (ATP) [1]. To do this they require acetyl coenzyme A (CoA), that enters the so-called Krebs-cycle, where it reacts with oxaloacetate to citrate, that is subsequently oxidized back to oxaloacetate. Acetyl-CoA is obtained from the catabolism of sugars (glycolysis), triglycerides and branched chain amino acids (BCAAs) [2,3]. Hexoses are broken down to two pyruvate molecules each and shuttled into the mitochondrial matrix, where they react with CoA to acetyl-CoA under decarboxylation. Triglycerides are hydrolysed into fatty acids and glycerol, the latter of which enters the glycolysis pathway as dihydroxyacetone phosphate (DHAP) [4,5]. Fatty acids react to fatty acid-CoA, are then transported into the mitochondria by bonding to carnitine and undergo β-oxidation to acetyl-CoA. BCAAs are transported into the mitochondria and enter the Krebs-cycle as acetyl CoA [3]. The electrons released during this process reduce nicotinamide adenine dinucleotide (NAD^+) to NADH and flavine adenine dinucleotide (FAD) to $FADH_2$ [2].

The energy stored within these electrons is then harvested by the electron transport chain (ETC) located in the inner mitochondrial membrane (Fig.1). It consists of four protein complexes: NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome bc_1 (complex III), cytochrome c oxidase (complex IV) and ATP-synthase (complex V). The NADH generated during the Krebs cycle is oxidized to NAD^+ by complex I and the electrons are transferred to ubiquinone, reducing it to ubiquinol. Analogously, complex II oxidizes $FADH_2$ to FAD, followed by ubiquinone reduction. Ubiquinol is then oxidized by complex III and the electrons are transferred to the iron atom of cytochrome C. It is oxidized by complex IV, which uses the electrons to catalyse the reaction between O_2 and H^+-ions to water. The electrons are transported within the four complexes between redox centres, which is an exergonic process. Complexes I, III and IV use the released energy to transfer H^+-ions from the matrix to the intermembrane space, establishing an electrochemical gradient. The resulting potential energy is harvested by complex V, which allows H^+-ions to travel back into the matrix. The released energy is used to catalyse the reaction between adenosine diphosphate (ADP) and phosphate to ATP [2].

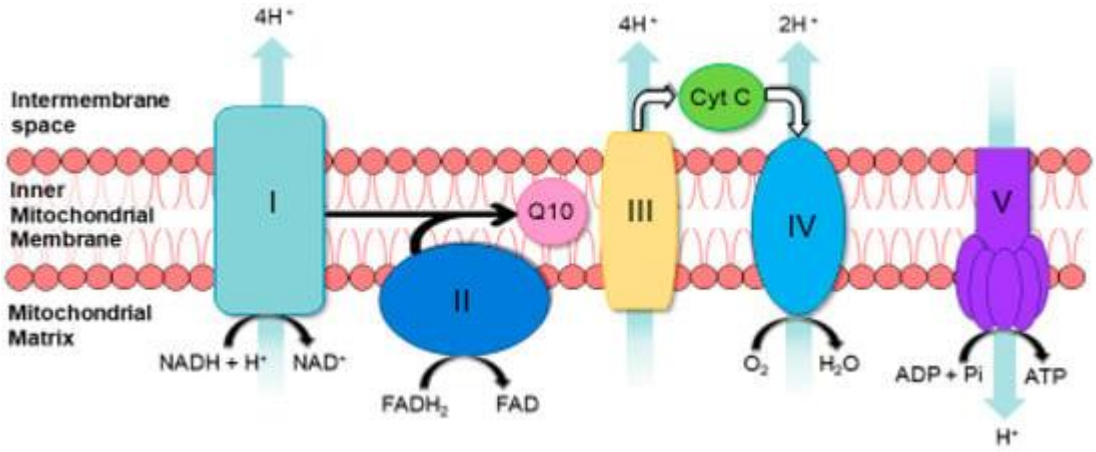

Fig. 1 The Electron Transport Chain [6].

1.2 Complex I deficiency

Isolated deficiency of Complex I of the respiratory chain is the most common cause of mitochondrial disease in children and adults [7,8]. The disorder is heterogenous; variations occur in age of onset and presentation of symptoms, such as Leigh syndrome (LS; severe neurological disorder), fatal infantile lactic acidosis (FILA), Leber's hereditary optic neuropathy (LHON) and hypertrophic cardiomyopathy (HCM). Complex I consists of a peripheral arm protruding into the matrix for electron transport and an arm embedded into the inner mitochondrial membrane, enabling proton translocation. It consists of seven mitochondrial-encoded NADH-dehydrogenase (ND) subunits and at least 38 nuclear-encoded subunits. Generally, it is very difficult to determine which mutations lead to certain phenotypes due to the extreme heterogeneity of these diseases, but both nuclear and mitochondrial defects have been identified in patients with such disorders. Nuclear mutations are the major cause of Complex I deficiency [9].

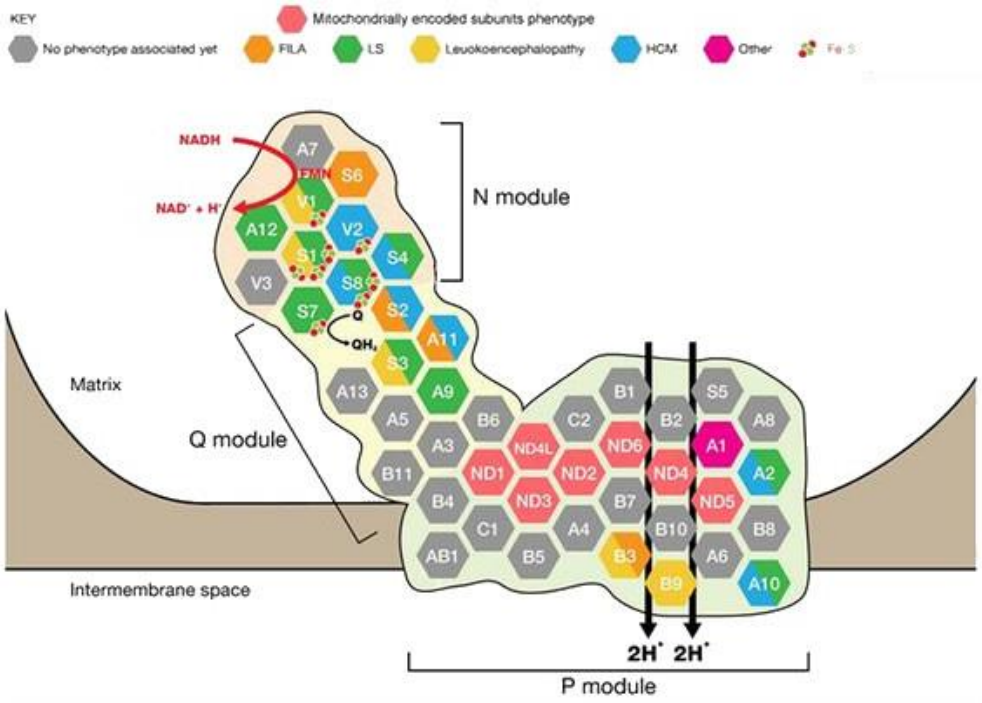

Fig. 2 The arrangement of Complex I subunits. They are colour-coded according to the associated clinical phenotype(s) and gene locations. The N-module oxidizes NADH, the Q-module reduces ubiquinone and the P-module enables proton translocation [9].

Since Complex I oxidizes NADH, cells with Complex I deficiency have low amounts of NAD^+ and high amounts of NADH [10,11]. The excessive NADH stalls oxidations which produce this compound, including steps of the Krebs-cycle, glycolysis, β-oxidation and glutamate deamination. This contributes to the decreased rate of oxidative phosphorylation observed in patients with Complex I [2,12].

Complex I serves also as the major site of ROS production in the cell [13]. ROS are known to cause cellular damage and are thought to contribute to aging and cancer development. Conversely, they also play an important role as signalling molecules and contribute to the health and longevity of the organism [14]. Complex I deficiencies and inhibitors such as Rotenone, which inhibits the electron transfer of Complex I to ubiquinone, lead to an increased production of ROS, causing apoptosis [15,16]. In theory, the same effect should be caused by a mutation leading to a loss of ubiquinone reduction by Complex I. Indeed, a mutation of NDUFS2, a Complex I subunit directly involved in this process, leads to decreased glycolysis, ATP-production, membrane integrity and increased ROS levels inducing apoptosis [17].

The reduced level of ATP means the cell has to slow down its rate of protein synthesis, a very energy consuming process, hence why low ATP inhibits cell growth and division. This is mediated by AMP-dependent kinase (AMPK), which senses the low ATP/AMP ratio and deactivates the mammalian Target of Rapamycin (mTOR) [18].

1.3 mTOR

mTOR is a primarily cytosolic serine/threonine kinase located on the surface of lysosomes, which mediates anterograde (nucleus to mitochondria) and retrograde (mitochondria to nucleus) communication. Its most well-known targets are p70-6SK, an activator of translation which is activated by mTOR and 4E-BP1, an inhibitor of translation, which is deactivated by mTOR in order to enhance protein synthesis. These pathways are activated when the cell has the nutrients necessary for large-scale protein translation, cell growth and division [19]. Despite mTOR's ability to promote anabolism and inhibit autophagy, upregulation of mTOR can also lead to apoptosis; these effects are situational. Upregulation of mTOR has been observed in Type 2 diabetes, obesity, cancer, epithelial wound healing and respiratory dysfunctions. Thus, disabling Complexes I, III and IV leads to activation of mTOR, resulting in downstream activation of Notch, JNK and JAK-STAT pathways, which can lead to apoptosis induced proliferation (AIP) of nearby cells [20]. The Notch pathway has many roles in intercellular signalling. It is implicated in various developmental and physiological processes, for example neurogenesis and regulating binary cell-fate decisions in stem cell progeny [21]. The JAK-STAT pathway influences the immune system, cell growth regulation and early embryonic development [22]. JNKs are kinases, which are activated in response to stress signals, such as cytokines, ROS or UV-light [23], are responsible for the high ROS-production after knockdown of Complex I and are pro-apoptotic signal molecules [24]. All negative phenotypes associated with Complex I deficiency are rescued by mTOR-RNAi, suggesting that mTOR overactivation triggers apoptosis [20].

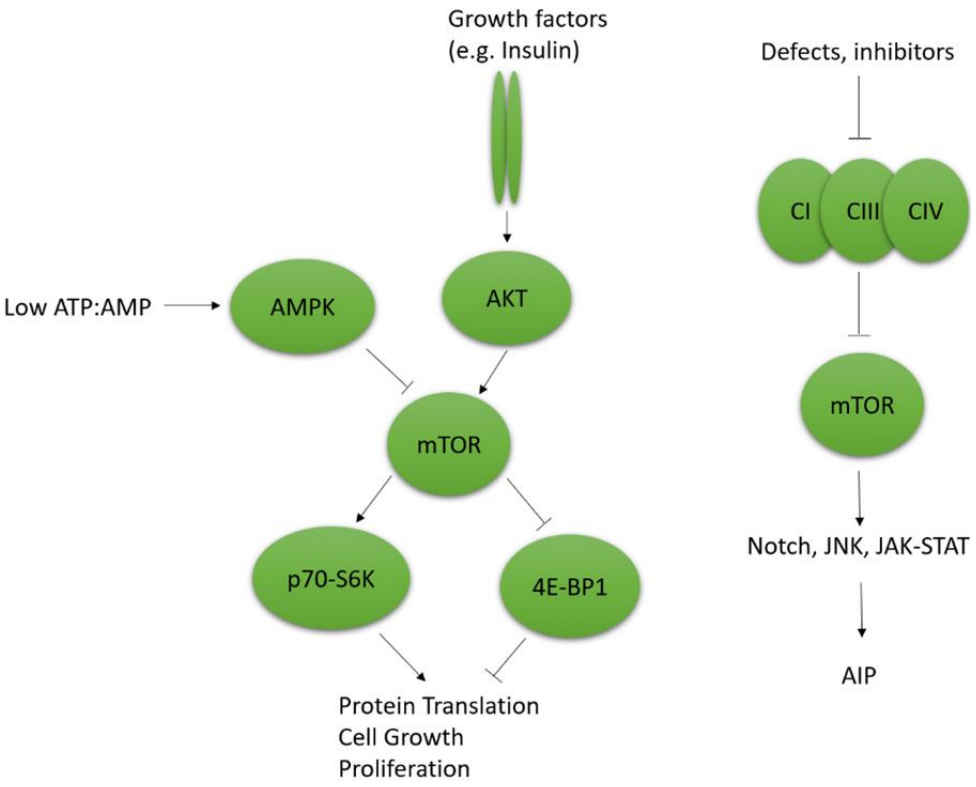

Fig. 3 A brief overview of mTORs involvement in regulating homeostasis [19,20].

This interaction offers a potential way for treating Complex I disorders, and several studies showed positive effects of rapamycin-inhibition of mTOR on mitochondrial diseases in mice [25,26]. This highlights the importance of understanding how mTOR's activity is influenced, and to this effect the Silent Information Regulator Proteins (Sirtuins) will be discussed below. As NAD^+-dependent deacetylases, they sense the decreased NAD^+/NADH-ratio caused by Complex I dysfunction, placing them in the role of nutrient sensors, which regulate many important functions related to the lifespan and health of the cell [27].

1.4 Sirtuins

The first discovered Sirtuin protein Sir2 was described in 1979 in yeast and is involved in gene silencing [28]. Since then, orthologs have been found in many organisms, from bacteria to humans [29]. In the latter seven different homologues have been found, called Sirt1-7. Other than being NAD^+-dependent protein deacetylases, some have additional activities such as ADP-dependent ribosyltransfer. The Sirtuins have different subcellular localizations; these are summarized in Table 1.

Table 1 Localization of the mammalian Sirtuins [30,31,32,33].

Sirtuin	Location
Sirt1	Nucleus, Cytosol
Sirt2	Cytosol
Sirt3	Mitochondria
Sirt4	Mitochondria
Sirt5	Mitochondria, Cytosol, Peroxisomes, Nucleus
Sirt6	Nucleus
Sirt7	Nucleolus

Sirt1, involved in heterochromatic gene silencing as a histone deacetylase [32], seems to be directly regulating ROS levels by deacetylating the Forkhead Box transcription factors of class O (FoxO), which decrease ROS production. There is evidence that Sirt1 can promote and prevent apoptosis by interacting with p53, a known tumor suppressor, depending on the ROS-concentration [30]. Although primarily located in the nucleus [30], Sirt1 can be shuttled into the cytosol, which can explain its ability to interact with TSC2 leading to TOR downregulation [34]. This is also achieved by Sirt1's interaction with AMPK: Either it is upregulated leading to higher NAD^+ levels and activating Sirt1, or Sirt1 can activate AMPK via deacetylation. Sirt6 is associated with chromatin and promotes DNA-repair, genomic stability, and downregulates genes involved in glucose uptake, glycolysis, ribosome and protein synthesis [30]. Interestingly, mTOR was found to inhibit Sirt6 in the nucleus of brown adipose tissue [35], which underscores the complexity of mTOR pathways. Sirt7 is associated with RNA-polymerase I and upregulates rRNA synthesis [36], and it also seems to be involved in regulating oxidative stress, possibly by interacting with Sirt1 [30]. mTOR was found to localize in the nucleoli, allowing it to interact with Sirt7, which is thought to enable synthesis of RNA-polymerases I, II and III. The interaction between Sirt7 and mTOR does not seem to involve deacetylation [36].

Sirt2 plays an important role for cell division as a tubulin deacetylase and activating cholesterol biosynthesis [37]. Sirt2 downregulates mTOR in kidney cells but inhibits primary cilia formation in retinal epithelial cells by activation of mTOR, suggesting a tissue dependent function [38,39]. Under fasting conditions, it promotes gluconeogenesis by stabilizing phosphoenolpyruvate carboxykinase. It also reduces ROS levels in several ways; examples

are activation of FoxO, NRF2 which may be mediated by AKT, and glucose-6-phosphate dehydrogenase to boost NADPH levels [30].

Sirt3 deacetylates FoxO to activate antioxidants [30] and activates isocitrate dehydrogenase 2 (IDH2) to boost NADPH and reduce GSH in response to caloric restriction (CR) [40]. Sirt3 upregulates AMPK in mice kidneys, thus inhibiting mTOR, promoting mitochondrial autophagy and protecting against sepsis-induced kidney-dysfunction [41]. It also increases pyruvate dehydrogenase activity, which reduces glycolytic rate, and induces fatty acid oxidation (FAO). Sirt3 may contribute to increased gluconeogenesis from amino acids by boosting glutamate dehydrogenase (GDH) activity by deacetylation. Sirt4, on the other hand, downregulates GDH via ADP-ribosylation, PDH with its lipoamidase activity and suppresses FAO, thereby downregulating mitochondrial metabolism [30]. Sirt4 also upregulates hepatocyte mTOR activity in a fed state by AMPK inhibition; this interaction is even present during nutrient deprivation [42]. Sirt5 assists Sirt4 in downregulating PDH, but it promotes FAO, activates IDH2 and superoxide dismutase to reduce ROS levels. Under oxidative stress it also inhibits pyruvate kinase, thus diverting glucose away from the mitochondria into the pentose phosphate pathway, leading to more NADPH production [30].

1.5 *D. melanogaster* and Sirtuins

While the location, protein targets and modifications catalysed by the Sirtuins may differ between them, they are all directly involved in maintaining the health and longevity of the cell in many different and context-dependent ways. To understand how these proteins interact with mTOR during Complex I deficiency, experiments were conducted in *Drosophila melanogaster* mutants. It is important to consider that it is only distantly related to mammals, which may lead to differences in the activities of the Sirtuins.

D. melanogaster rose to prominence as a model organism for genetics and biomedical sciences by Thomas Hunt Morgan, who used it to prove the chromosomal theory of inheritance over 100 years ago. Since then, a wide variety of tools and techniques have been discovered to manipulate the *Drosophila* genome, which, combined with its low cost and short generation time, makes *D. melanogaster* very useful for human disease modelling [43]. Various disorders can be created in specific tissues rather than the entire organism to increase the viability of the mutant. The Gal4/UAS system is one of the most important tools for tissue specific expression of transgenic elements. The principle is that Gal4 is expressed downstream of a gene only

active in the tissue of choice; Gal4 then binds to the UAS-promoter upstream of the transgene. It is a useful tool for knocking down target proteins with RNAi [44].

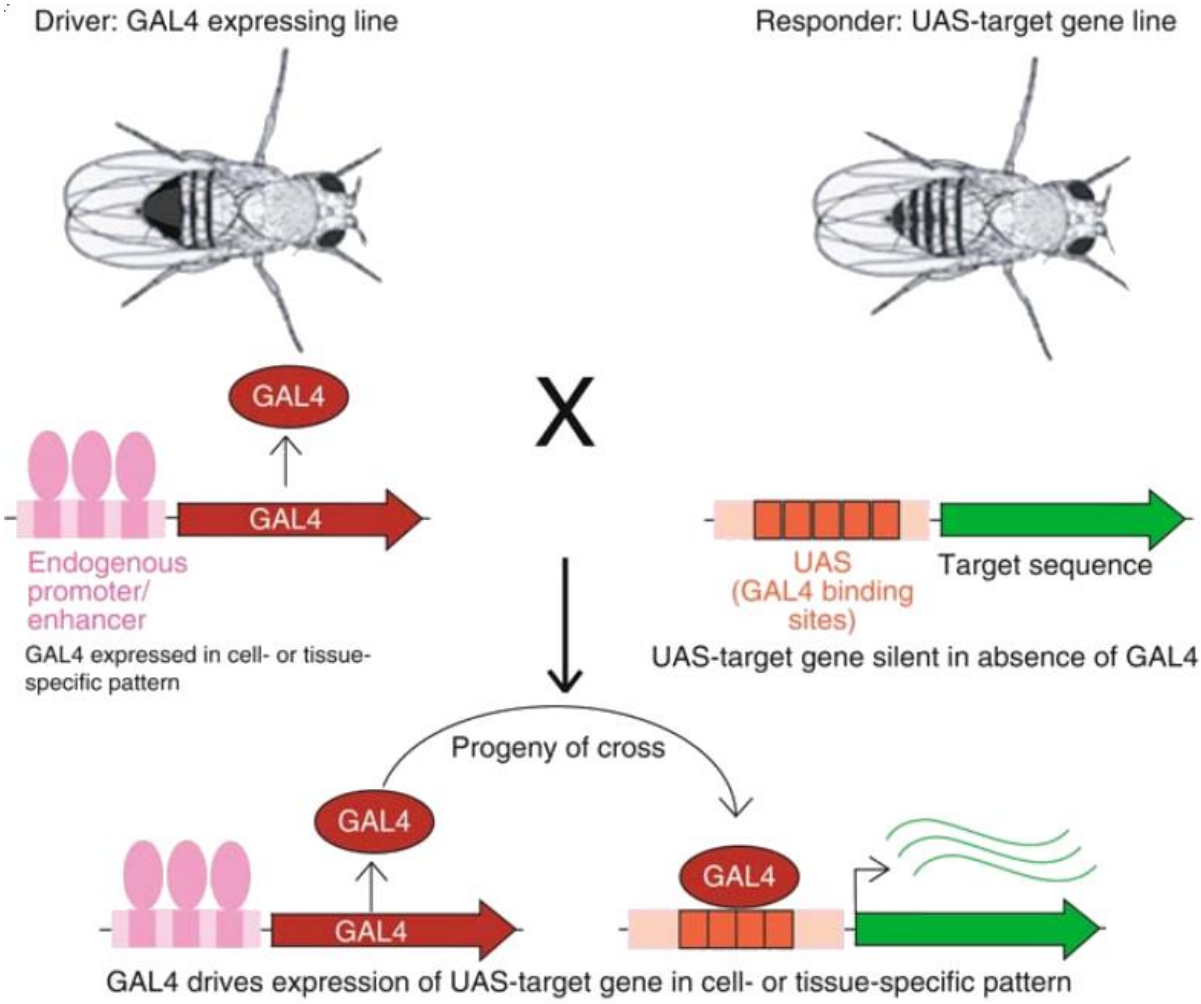

Fig. 4 The Gal4/UAS-system [44].

Important to keep in mind when maintaining flies carrying a mutation, transgene or other genetic alterations is that these can be lost due to homologous recombination. The discovery of balancer chromosomes was therefore of great importance, as these carry inversions which prevent crossovers during meiosis. Recessive lethal or sterile mutations ensure that flies homozygous for the balancer will not contaminate the stock [45].

Despite these advantages, the model has its limitations due to the rather distant evolutionary relationship between *D. melanogaster* and homo sapiens, though homologues can have a very high similarity. This makes it possible to study disorders which are caused by a single gene or the function of a highly conserved pathway, but it is often more difficult to look at more complex processes [46]. The study of Sirtuins in *Drosophila* highlights this, as only five Sirtuins exist in the fruit fly: dSirt1, 2, 4, 6 and 7 [47]. The most striking difference is seen in the mitochondria: *D. melanogaster* only has one (dSirt4), while mammals have three

mitochondrial Sirtuins. dSirt4 promotes FAO, glycolysis, trehalose hydrolysis and BCAA catabolism, and is thus particularly important during periods of starvation. Unlike in mammals, dSirt4 is an agonist of mitochondrial metabolism and is upregulated during CR, opposite to its mammalian counterpart. As the only Sirtuin in *D. melanogaster* its tasks overlap with all three mammalian Sirtuins, seeming closest to Sirt3 in its function [48]. dSirt1, 2 and 6 seem to have the same or similar functions in *D. melanogaster* as compared to mammals, and dSirt7's catalytic domain is highly conserved in a wide variety of species [49,50,51,52].

2 Aim of the thesis

Ample research exists on the effects of the Sirtuins on lifespan and caloric restriction that have been tested in various biological systems including *D. melanogaster* [27]. There is limited evidence from mammals that Sirtuins may interact with mTOR complexes, including direct [35,36] and indirect interactions [34,38,39,41,42]. However, unlike for mammals, it is less clear how the Sirtuin family interacts with mTOR and Complex I in the fruit fly.

Using the model of Complex I deficiency characterized in the lab [20] the aim of my thesis was to test the genetic interactions between ND-49 (NDUFS2 in mammals) deficiency and Sirtuins in the *Drosophila* eye tissue. As the size of the *Drosophila* eye is smaller in ND-49 mutant due to apoptosis [20], it can be easily modified by genetic interactions. Specifically, I was using Sirtuin-RNAi lines and the UAS/Gal4 system to knock out Sirtuins in the eye tissue and quantify the size of the eye with colorimetry. The aim of my thesis was to test if the Sirtuin genes genetically interact with complex I deficiency in the context of the *Drosophila* eye tissue.

3 Methods

3.1 Husbandry

The strains of *D. melanogaster* (see table 1) were provided by Dr. Alena Krejci and Valentina Magnin, Department of Moleular Biology and Genetics, University of South Bohemia. The GFP and Sirtuin-RNAi flies were bought from the Bloomington *Drosophila* Stock Centre (https://bdsc.indiana.edu) and VDRC stock center (https://stockcenter.vdrc.at). The ND49-RNAi;Ey-Gal4 stock was created by Valentina Magnin. All stocks were maintained on standard food, that is agar food enriched with a cornmeal, glucose and yeast (agar 9 g, glucose 150 g, cornmeal 160 g, yeast 30 g, methylparaben 50 mL, water 1900 mL) having additional grains of dry yeast on the top of the food. The food dries out when only few flies live in a vial, and liquifies when the population of larvae is large, that was counteracted by adding water and paper tissue respectively. The flies were kept in incubators at 25 °C or 18 °C. The flies spent 3-21 days in their vials before being transferred to a new vial ("flipped").

3.2 Crossing

Female virgins were collected from the GFP-RNAi and Sirtuin-RNAi-lines once on Monday afternoon and twice per day from Tuesdays to Fridays. This could be done after the stock had been flipped at least once, which leaves vials containing offspring. Females remain virgins 8h after eclosure at 25°C and 16h at 18°C and were collected within these time frames at the respective storage temperature. They were kept in separate vials and used after at least one week to make sure they did not store any sperm.

Eight of the Sirtuin-RNAi or control GFP-RNAi virgins were crossed with UAS - ND49-RNAi/CyO; Ey-Gal4/TM6B males or the control UAS - GFP-RNAi/CyO; Ey-Gal4/TM6B males; CO_2 was used to put them to sleep first to facilitate the process. The offspring was collected 9 days after emergence and stored at -20°C. The II. balancer chromosomes I used had the CyO gene, that leads to curly wings. This was used to identify progeny lacking ND49-RNAi and were thus discarded.

Table 2 The fruit fly lines used for the experiments. The chromosome number is shown in brackets. The balancer chromosomes are CurlyO (CyO, curly wings) and TM6B (tubby body).

Function	RNAi-line (chromosome)	Genotype
GFP-RNAi	BL9331 (II)	w[1118]; P{w[+mC]=UAS-GFP.dsRNA.R}143
dSirt1-RNAi	BL31636 (III)	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01423}attP2
	BL32481 (III)	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00484}attP2
dSirt2-RNAi	BL31613 (III)	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01397}attP2
	KK102741 (II)	P{KK102741}VIE-260B
dSirt4-RNAi	BL31638 (III)	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01426}attP2/TM3, Ser[1]
	BL33984 (III)	BL33984 (III) y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00944}attP2
	BL36588 (III)	BL36588 (III) y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.GL00548}attP2
	KK108211 (III)	P{KK108211}VIE-260B
dSirt6-RNAi	BL31399 (III)	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01583}attP2
	BL34530 (III)	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01009}attP2
	BL36801 (II)	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.GL01008}attP40
dSirt7-RNAi	BL31093 (III)	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01558}attP2
	BL32483 (III)	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00486}attP2/TM3, Sb[1]
GFP-RNAi in eyes		UAS - GFP-RNAi/CyO; Ey-Gal4/TM6B
ND-49-RNAi in eyes		UAS - ND49-RNAi/CyO; Ey-Gal4/TM6B

3.3 Colorimetry

Five batches of crosses were conducted to lower the workload per experiment. For each assay, GFP-RNAi and several Sirtuin-RNAi virgins were crossed both with ND49-RNAi;EyGal4 males or GFP-RNAi;EyGal4 males to collect the heads of their progeny with tweezers, which were put on ice in Eppendorf tubes. 10 heads were needed for each tube, and since the experiments were conducted in triplets, at least 30 flies were needed from each cross. Afterwards, 200 µL of 30% ethanol at pH=2 was transferred into each tube and their contents were homogenized with a microcentrifuge pestle. The tubes were then covered with aluminium foil and stored at 25°C for 63 hours; this prevented the eye pigment from bleaching out while it dissolved in the solution. Lastly, the solutions were transferred onto a 96-well plate and placed into a Sunrise Microplate reader by Tecan, which measured the absorption at 480 nm [53].

3.4 Photography

Eye pictures of females from each cross were taken with an Olympus SZX12 fluorescence microscope, using flies that had been stored at -20°C in Eppendorf tubes. These were observed under the microscope on petri dishes and pictures of the whole fly were taken once the lighting was adjusted. The magnification and distance from the lens were kept constant to make the comparison between pictures meaningful. Later, the pictures were cropped in Paint 3D to only show the fly heads.

3.5 Statistics

To evaluate the data, double-tailed, homoscedastic t-tests were conducted to measure levels of significance between the two GFP-RNAi-crosses, and between the GFP-ND49-RNAi and each Sirtuin-ND-49-RNAi cross. The levels of significance are indicated by stars, where ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$ and ns = no significance. They were calculated for the comparison of the two GFP-crosses and the comparison between the GFP-ND-49-RNAi and each Sirtuin-ND-49-RNAi cross.

4 Results

4.1 All Sirtuins upregulate mTOR in the context of Complex I deficiency

The data, including error bars, is presented in five graphs (Fig. 5-9), one for each type of Sirtuin protein, even though five colorimetry assays were conducted on different days with different crosses, the five result for each control GFP cross were combined into one due to their similarity in absolute values of absorbance. Likewise, the results for the RNAi-lines, that were tested in biological duplicates, were also merged for the same reason.

The results presented in the graphs indicate that a rescue of the eye size was achieved with most dSirt-RNAis in combination with ND-49-RNAi, except for BL31093 (dSirt7).

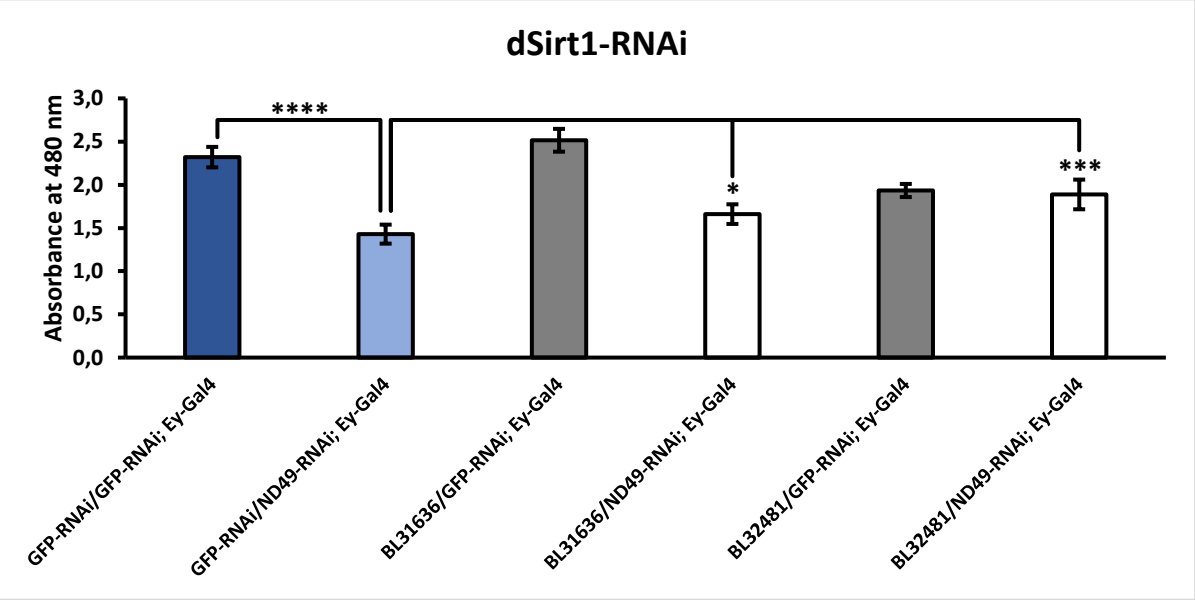

Fig. 5 Absorbance measurements of the dSirt1-RNAi-crosses

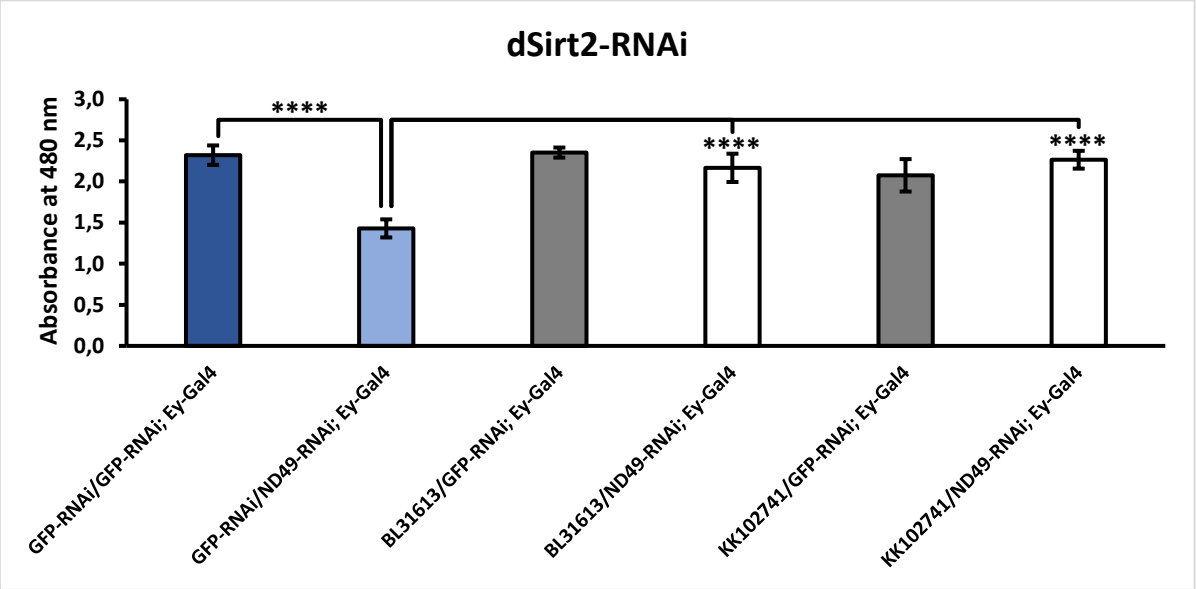

Fig. 6 Absorbance measurements of the dSirt2-RNAi-crosses

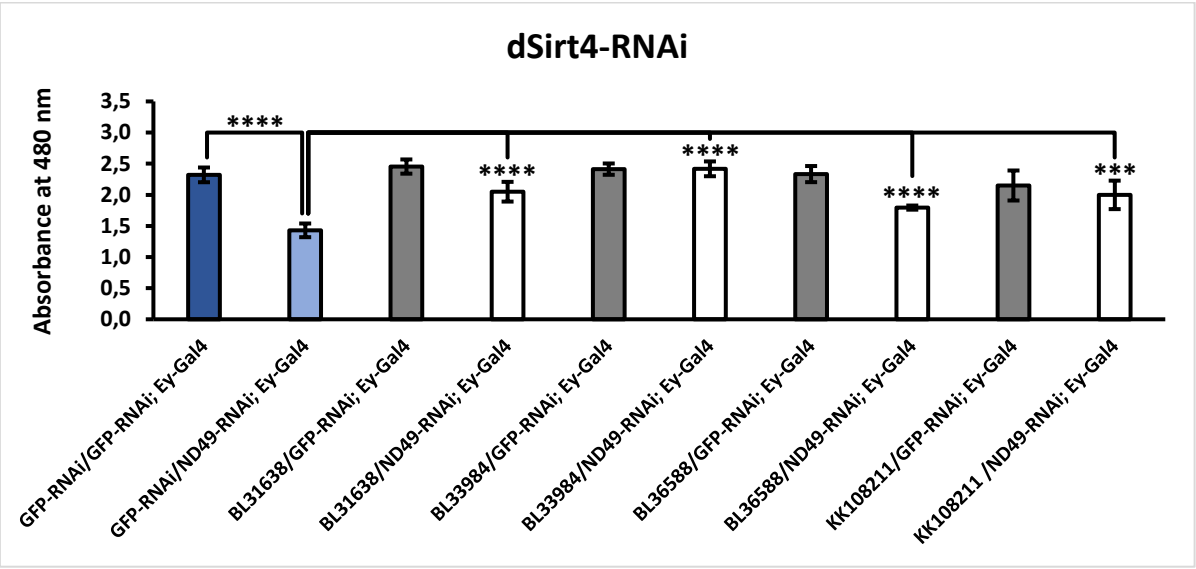

Fig. 7 Absorbance measurements of the dSirt4-RNAi-crosses

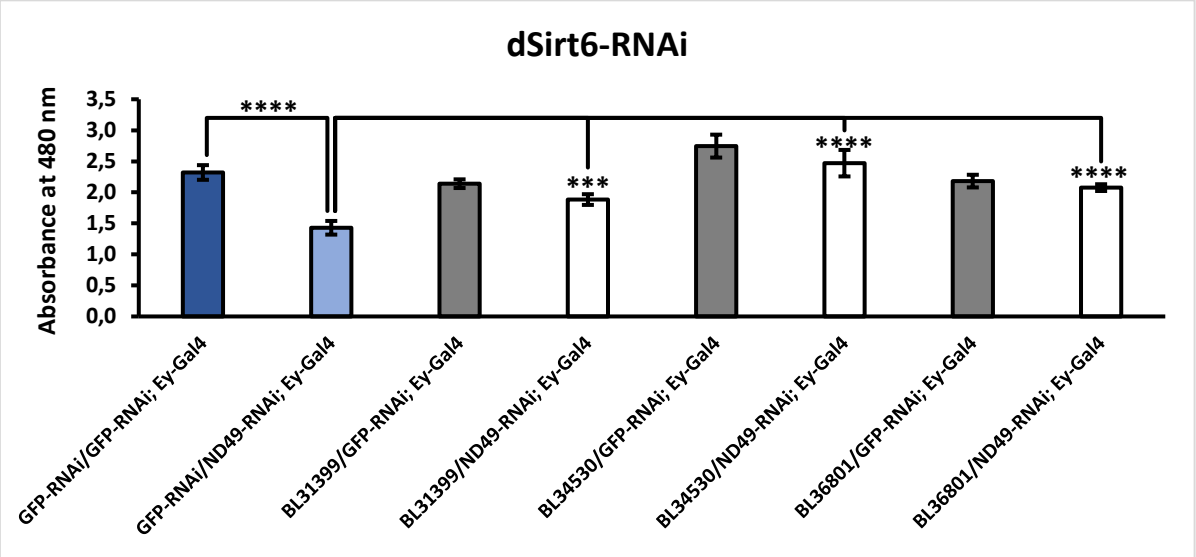

Fig. 8 Absorbance measurements of the dSirt6-RNAi-crosses

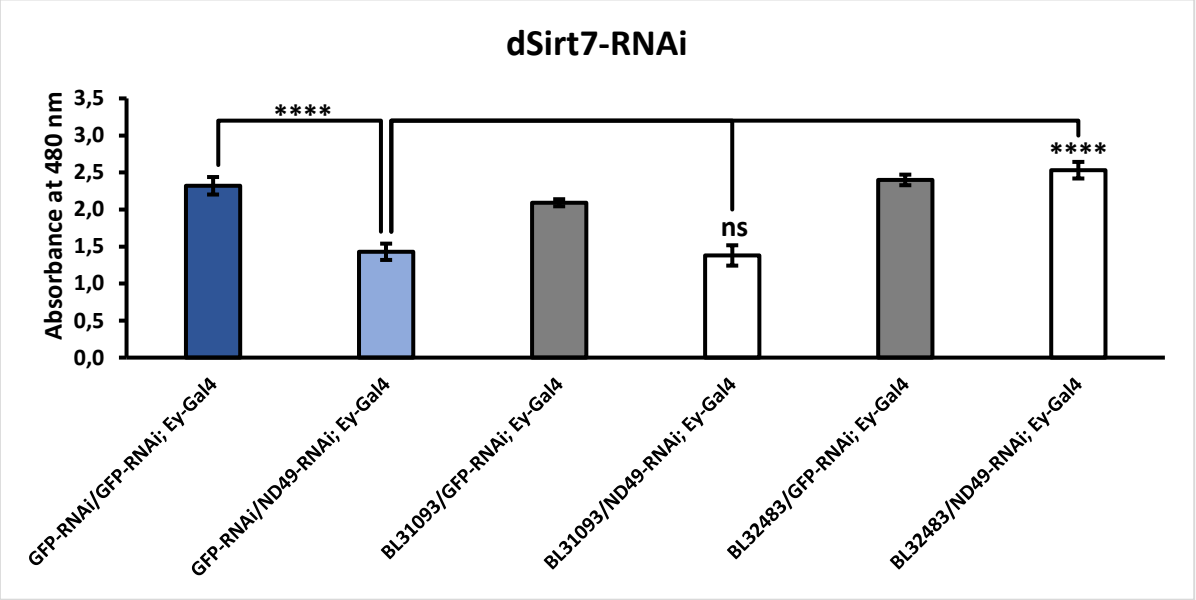

Fig. 9 Absorbance measurements of the dSirt7-RNAi-crosses

4.2 Eye Pictures

The pictures in this section serve as an additional means of comparison between the differences in eye sizes of the individual crosses. Therefore, the representative pictures are to reflect the results shown in Fig. 5-9.

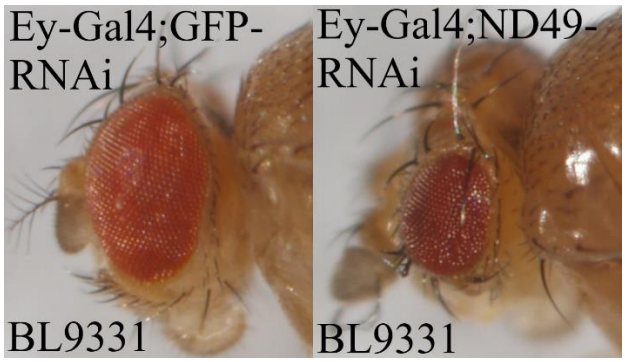

Fig. 10 GFP-RNAi crosses

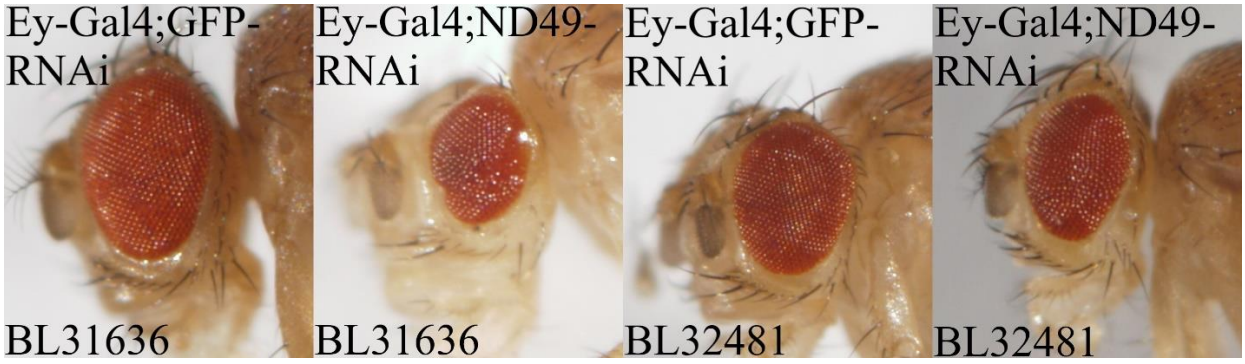

Fig. 11 dSirt1-RNAi crosses

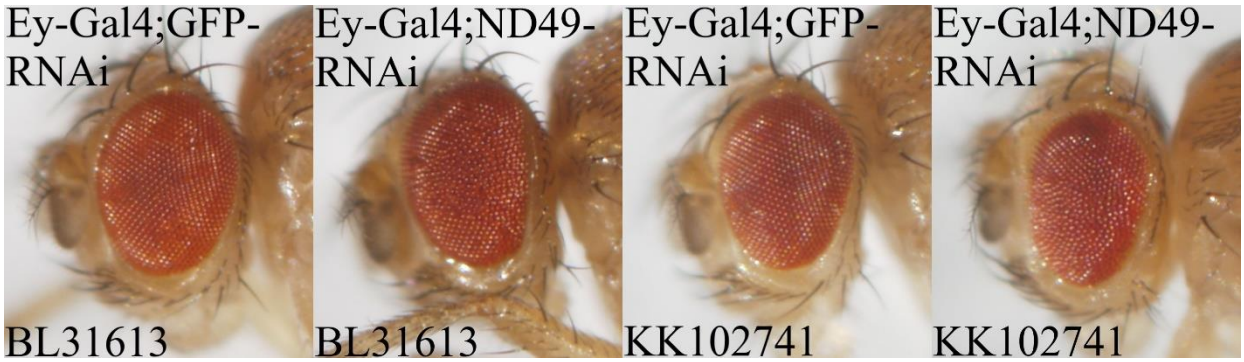

Fig. 12 dSirt2-RNAi crosses

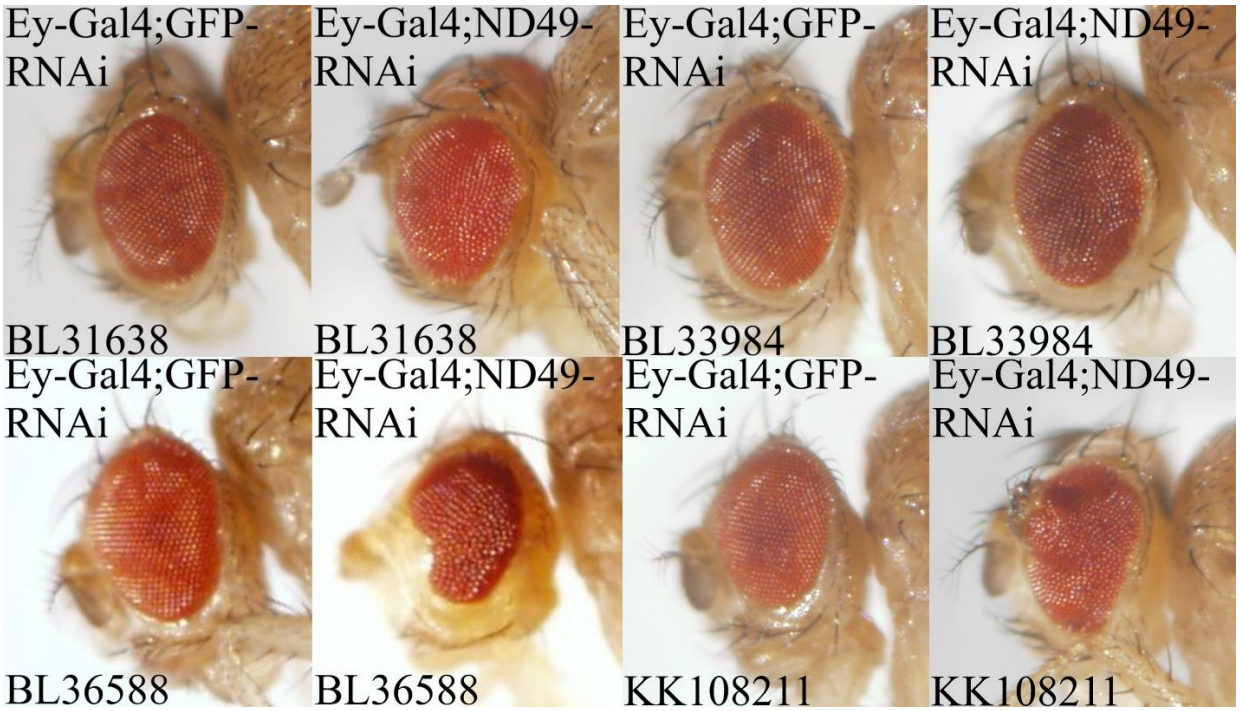

Fig. 13 dSirt4-RNAi crosses

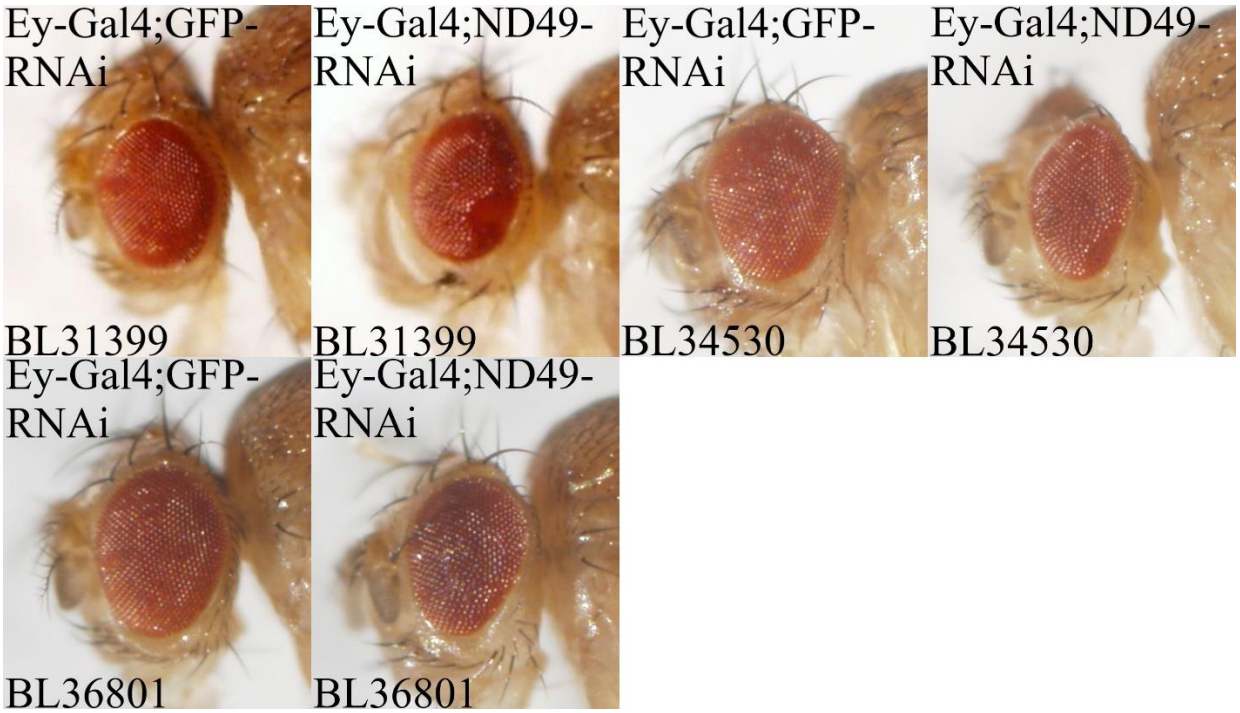

Fig. 14 dSirt6-RNAi crosses

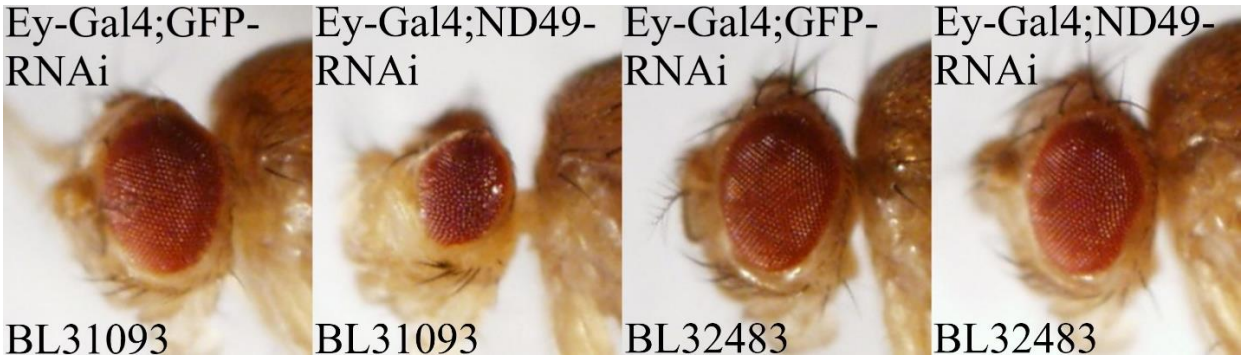

Fig. 15 dSirt7-RNAi crosses

5 Discussion

The results of my experiments showed that the expression of each of the *Drosophila* Sirtuin-RNAis in combination with ND-49-RNAi resulted in eye sizes significantly larger than the ND-49-RNAi alone. In other words, the apoptosis-induced proliferation (AIP) caused by the ND-49-RNAi phenotype in the eye could be rescued by the simultaneous expression of Sirtuin-RNAi. As a smaller eye size indicates more apoptosis and a more active mTOR pathway [20], my results suggest that during Complex-1 deficiency any of the Sirtuins in the fruit fly eye activates mTOR activity. This is a surprising result as during Complex-I deficiency the NAD+/NADH ratio decreases and hence Sirtuins should be less active, as they use NAD+ for their enzymatic activities. We could speculate that it is a non-enzymatic function of Sirtuins that becomes more important in low NAD+/NADH conditions and blocks mTOR activity (Fig. 16). This could be because of direct physical interactions between mTOR and Sirtuins or indirectly via Sirtuins affecting a function of another protein involved in the mTOR pathway.

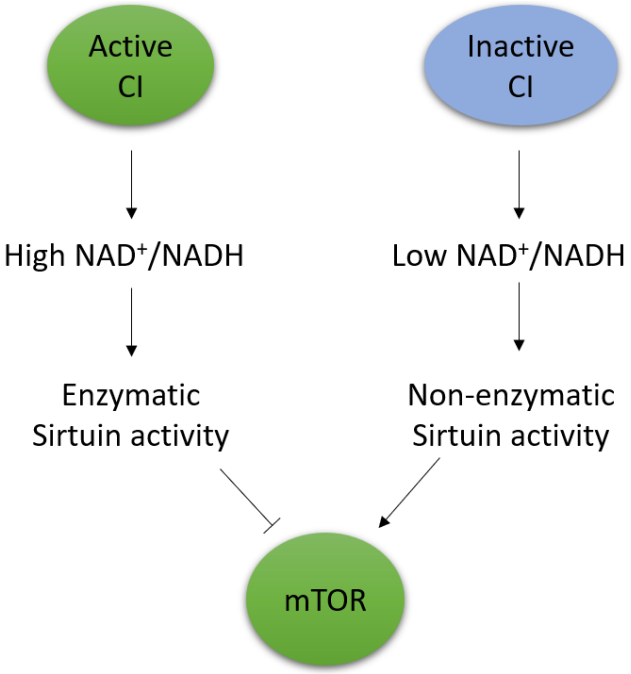

Fig. 16 Proposed model for the Sirtuin interaction with mTOR at varying NAD^+/NADH-ratios.

As my experiments look only at genetic interactions, we cannot distinguish on a cellular level whether it is the apoptosis or proliferation of the eye tissue that is affected by Sirtuin-RNAi. More proliferation and bigger eye tissue could be achieved via Sirtuin mediated stimulation of proliferation but also by increasing apoptosis of the tissue that would stimulate apoptosis-induced proliferation. Immunostaining experiments would be needed to address this question.

It would also be interesting to look on the activity of other pathways that have been described in the context of ND-49-RNAi downregulation, such as the JNK, JAK/STAT and Notch pathways [20,24]. We could use genetically encoded reporters to detect the activity of these pathway after Sirtuin downregulation by RNAi.

My results are in line with mammalian studies on Sirt1, 6 and 7 which all showed interactions between these proteins and mTOR [34,36,54]. The role of Sirt2 in mTOR regulation is less straightforward, as it has been shown to be important for cell proliferation and cilia formation in retinal epithelial cells, while downregulating mTOR in kidney cells [38,39]. The latter effect was reproduced in the *Drosophila* eye. The Sirt4 effect in the fruit fly is significantly different than in mammals, serving as an antagonist of mitochondrial metabolism [48], that matches with the observed AIP in Sirt4 and Complex I deficient flies. The two RNAi-strains that only showed very little or no rescue phenotype (Sirt1-BL31636 and Sirt7-BL31093) likely had low efficiency of the RNAi construct.

Previous studies highlighted the importance of caloric restriction (CR) for metabolic regulation and described its influence on Sirtuin activity [27]. Based on nutrient availability and other factors such as cellular damage these proteins and other nutrient sensors "decide" cell fate by being able to initiate apoptosis [20]. Sirt2 was also shown to have opposite effects on mTOR activity in different tissues. For these reasons the dSirt-mTOR interactions should be studied at different caloric intakes and in other tissues to see how well the results produced by my study can be generalized. Moreover, it would be interesting to measure the NAD+/NADH ratio in our genotypes. It is presumed, and the data in our lab using a NADH sensitive reporter also indicate, that the NAD+/NADH ratio is decreased after ND-49-RNAi, but we do not know if this is still true when adding Sirtuin-RNAi. The cell could raise its cytosolic NAD+-levels by decreasing mitochondrial metabolism and switching to lactate fermentation. This seems plausible, because all Sirtuins in *D. melanogaster* promote mitochondrial metabolism and their deactivation should be tied to a higher nutrient influx, which was confirmed for Sirt6 [30]. Accordingly, if the lactate fermentation pathway is activated, a high-caloric diet could aid the recovery of the eye (by recycling the NAD+) while a low-calorie diet would exacerbate the symptoms of Complex I deficiency.

The binding affinity between Sirtuins and mTOR complex could be affected as well by means such as allosteric regulation of mTOR resulting from mitochondrial stress. However, the primary localization of mTOR to the surface of lysosomes and various localizations of Sirtuins (see Table 1) makes this scenario less likely. Nevertheless, such mechanisms could explain

how it can promote cell growth, proliferation and apoptosis under varying conditions. Last but not least, since ROS production increases upon inhibition of Complexes I, III and IV [13,55], they may play an important role in affecting mTORs downstream targets, either directly or indirectly, and Sirtuins may help to regulate the redox homeostasis under these conditions.

6 References

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