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dSir2 and longevity in *Drosophila*

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Abstract

The silent information regulator 2 (Sir2 or Sirtuin) family of proteins is highly conserved and has been implicated in the extension of longevity for several species. Mammalian Sirtuins have been shown to affect various aspects of physiology including metabolism, the stress response, cell survival, replicative senescence, inflammation, the circadian rhythm, neurodegeneration, and even cancer. Evidence in *Drosophila* implicates Sir2 in at least some of the beneficial effects of caloric restriction (CR). CR delays age-related pathology and extends life span in a wide variety of species. Here we will review the evidence linking *Drosophila* Sir2 (dSir2) to longevity regulation and the pathway associated with CR in *Drosophila*, as well as the effects of the Sir2 activator resveratrol and potential interactions between dSir2 and p53.

Keywords

Sir2; Drosophila melanogaster; calorie restriction; aging

INTRODUCTION

Silent information regulator 2 (Sir2) proteins, or Sirtuins, are members of a highly conserved family of proteins that act as either nicotinamide adenine dinucleotide (NAD⁺)-dependent protein deacetylases or mono-ADP-ribosyltransferases [1,2]. The founding member of this family is yeast Sir2, but Sirtuins are found in a variety of species ranging from bacteria to humans. Yeast Sir2 regulates silencing of the mating type loci, homologous recombination at the rDNA loci [3], and silencing of subtelomeric regions. Aging in yeast has been measured by counting the number of times a mother cell can divide and produce a daughter cell, usually called replicative life span. The role of Sir2 in yeast longevity was discovered when it was found that having an extra copy of the *sir2* gene extended replicative life span while *sir2* mutants have a shorter life span [4]. Since that time Sirtuins in multicellular organisms have been linked to multiple physiological processes including metabolism, stress responses, cell survival, replicative senescence, inflammation, circadian rhythm, neurodegeneration, cancer, and others [1,2].

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There are seven members of the Sirtuin family in mammals, SIRT1 to SIRT7. While they all have highly conserved catalytic and NAD⁺ binding domains, sequences vary at their two termini [5]. Furthermore, they differ with regard to intracellular location and deacetylation targets, and consequently are involved in different physiological processes [2]. There are five members of the Sir2 family in *Drosophila melanogaster*. Based on sequence similarity, dSir2 is the *Drosophila* homologue of yeast Sir2 and human SIRT1. *Drosophila* has been instrumental in confirming the role of Sir2 in regulating the longevity of model organisms.

dSir2 enzymatic activity and cofactors

Sir2 as a NAD⁺ dependent deacetylase targets histone as well as non-histone targets. Sirtuins catalyze deacetylation of the substrates and release nicotinamide (NAM) and O-acetyl-ADP-ribose. NAM is a strong non-competitive Sir2 inhibitor [2]. In yeast, worms and flies NAD⁺ is regenerated in a 4-step process, starting with the enzyme pyrazinamidase/nicotinamidase 1 (PNC1). Overexpression of *PNC1* in yeast increases longevity by decreasing the levels of nicotinamide, thereby decreasing direct inhibition of Sir2, as well as by increasing the NAD⁺/NADH ratio [6]. Yeast PNC1 levels and activity are induced in response to CR and mild stress, and PNC1 is required for yeast longevity extension caused by CR [6]. Knockdown of worm *pnc1* decreases survivorship, however, overexpression of worm *pnc1* increases oxidative stress resistance but not longevity in a Sir2-dependent manner [7]. It has been recently reported that the *Drosophila* homologue of PNC1, D-NAAM (*Drosophila* nicotinamide amidase), has a similar beneficial effect on fly longevity [8]. Overexpression of D-NAAM results in up to a 30% extension of the mean life span and 20% extension of maximal life span. Overexpression D-NAAM in a *dsir2* mutant background did not extend fly life span, suggesting that the longevity effect of D-NAAM requires the presence of Sir2 [8]. The levels of D-NAAM are affected by oxidative stress but CR, heat shock, and target of rapamycin (Tor) inhibitors do not affect the levels of D-NAAM in S2-cells [8], in contrast to the findings in yeast. However, in human neuroblastoma cells resistance to oxidative stress, induced by NOC-9, is increased by overexpression of D-NAAM. Furthermore, this resistance is Sir2-dependent. Although these data imply an important role of Sir2 in mediating the beneficial effects of D-NAAM, there is still a possibility that other targets may be involved. For instance, it was found that the nicotinamide effect on yeast longevity is not solely mediated by sirtuins [9].

***Drosophila* Sir2: expression, localization, and role as a transcriptional modulator**

Drosophila Sir2 (dSir2) is expressed widely in the early embryo but becomes restricted to the nervous system by late embryogenesis [10], showing both a nuclear and cytoplasmic localization that shifts over the course of embryogenesis [10,11]. After embryogenesis expression of dSir2 protein has been determined for whole bodies, with low levels in larvae and moderate levels in pupae and adults [11,12]. When adults were examined using immunolocalization, dSir2 protein was present in the nuclei of neurons and in the nuclei and cytoplasm of fat body cells [13].

dSir2 localizes to numerous euchromatic and heterochromatic sites on salivary gland chromosomes [10,11]. dSir2 chromatin binding sites have been mapped in KC cells (of embryonic origin) using an *in situ* methylation assay [14]. This also shows an association of dSir2 with multiple euchromatic and heterochromatic sites. dSir2 mutations have a modest effect on position-effect variegation, a form of heterochromatin-mediated silencing [10,15]. There is evidence for genetic interactions between dSir2 and transcription factors such as Foxo [16], Hairy [11] and p53 [17], and evidence of direct physical interactions with Hairy and p53 [11,17]. Overexpression of dSir2 in S2 cells (of embryonic origin) changes the expression of several hundred euchromatic genes (approximately 100 genes are up-regulated and 215 genes are down-regulated) [12]. Mammalian SIRT1 interacts with 34 known

deacetylation targets and six binding partners [18], so additional dSir2 targets may be identified in the future.

Flies with homozygous dSir2 null mutations are fully viable, fertile, and develop at normal rates [10,15]. Homozygous null mutants have reduced life span compared to controls [15], whereas null flies that are transheterozygotes for two different null mutations have only slightly reduced life spans [10]. No effect on life span was observed in flies heterozygous for a null mutation in dSir2 [19]. When dSir2 expression was reduced using RNAi transgenes, ubiquitous inhibition of dSir2 mediated by the actin 5C Gal4-driver resulted in severely reduced viability while inhibition only in the nervous system gave normal viability [20]. Life span was examined under the latter condition and found to be reduced. Discrepancies between the results with RNAi and null mutations might be explained by pleiotropic effects of the RNAi transgenes.

Effects of dSir2 on *Drosophila* longevity

Overexpression of Sir2 extends longevity in yeast and worms [4,21]. Similarly, flies that overexpress dSir2 live longer compared to genetically-matched controls [13,17]. Using three different UAS-lines (*dSir2^{EP2300}*, *dSir2^{EP2384}* and *dSir2^{EY03602}*) and five different GAL-4 drivers, it was found that overexpression of dSir2 increases fly longevity in both male and female flies but the magnitude of the longevity effects depend on the timing, place and levels of dSir2 overexpression. The biggest increase in mean life span, 57%, was observed when tubulin-GAL4 was used to drive ubiquitous overexpression of dSir2 using the *dSir2^{EP2300}* line. Under these conditions dSir2 expression was 4-fold increased compared to controls. The mean increase in longevity for ubiquitous overexpression, averaged across all three UAS lines, was 29% for females and 18% for males [13]. No effect on longevity was observed when the weak armadillo-GAL4 driver was used. Life span can still be increased in male and female flies when dSir2 is expressed pan-neurally throughout development. When there is pan-neural expression only during adult life, median and maximal life span were extended in females but only maximal life span was extended in males. Another study has confirmed life span extension when dSir2 expression is induced in the *dSir2^{EP2300}* line [19]. A report examining developmental defects caused by Sir2 overexpression in the eye imaginal disc showed overexpression of both *dSir2* and the adjoining gene *dnaJ-H* when the *dSir2^{EP2300}* line was crossed to the *gmr-GAL4* driver [16]. However, when the same transgenic line, *dSir2^{EP2300}*, was crossed to an inducible pan-neural driver and dSir2 was overexpressed only in adults there was life span extension but no overexpression of *dnaJ-H* [17]. Two concentrations of inducer were used, causing 3-fold and 5-fold increases in dSir2 expression, and greater life span extension was obtained when there was higher overexpression. At both concentrations of inducer there was barely any change in *dnaJ-H* expression compared to controls, indicating life span extension was solely due to dSir2 [17]. It is likely that the other transgenic lines used in [13] also induce dSir2 but not *dnaJ-H*, as their transgene insertion sites are more distant from the *dnaJ-H* transcription start site. The effects of dSir2 null mutations on CR-mediated longevity extension provide independent confirmation for a role of dSir2 in life span regulation (see next section).

dSir2 and Caloric restriction

Caloric restriction (CR) without malnutrition delays age-related pathologies and extends survivorship in a variety of species. CR also extends survivorship in flies, but the magnitude of the effect varies with genetic background. CR has multiple effects on fly physiology, such as decreased weight and fecundity, increased physical activity and oxidative stress resistance, and delayed lipid oxidative damage [22,23,24]. [Please see Tatar review on the effects of CR in this issue, 25]

It has been proposed that Sirtuins mediate the beneficial effects of CR in yeast and worms [21,26]. However, the role of Sir2 in CR has proven more complex in these model systems. In yeast, the link between Sir2 and CR varies depending on the experimental conditions and genetic background [27]. In nematodes SIR2 overexpression extends life span, but one report indicates that longevity extension mediated by CR requires Sir2 [28] while other reports indicate that it does not [29,30].

In *Drosophila* several lines of evidence suggest that dSir2 mediates life span extension by CR. First, CR flies have increased mRNA levels of dSir2; second, longevity increases mediated by dSir2 overexpression are not additive to increases mediated by CR; third, *dsir2* mutant flies do not have CR-mediated longevity changes; and fourth, *dsir2* mutant flies do not exhibit increased mobility on CR [13,24,31]. Mobility has been a particularly useful assay for CR-mediated physiological changes. CR increases the spontaneous physical activity of male and female flies in a dSir2-dependent fashion, and *dsir2* mutant flies are less active on low calorie food compared to the *dsir2* mutant flies on high calorie food. Similar to the latter finding, *sirt1*^{-/-} mutant mice on CR do not show the increased activity usually observed in mice on CR, as measured by walking, jumping, and distance traveled [32,33]. The lower activity of *dsir2* mutant flies could result from changes similar to what has been observed in *sirt1*-null mice, such as inefficient metabolism and changes in mitochondrial function.

dSir2 is part of a pathway mediating the longevity effect of CR

Decreased levels of Rpd3 extend the longevity of flies but do not affect the fertility of females [31]. A similar effect is obtained when adult flies are fed a histone deacetylase inhibitor [34]. Like Sir2, Rpd3 is a histone deacetylase, and like Sir2, it has a role in silencing both euchromatin and heterochromatin [35]. Fly longevity is not affected when heterochromatin silencing is increased or decreased independently of Sir2 or Rpd3 [35]. Therefore, it is most likely that the effect of Sir2 and Rpd3 on fly longevity is mediated by their effects on euchromatin.

Several lines of evidence link Rpd3 to CR-mediated life span extension and Sir2 [13,31]. 1) Rpd3-mediated life span extension is not additive with CR-mediated extension. 2) Sir2 transcription is increased by CR, and a similar increase is obtained in *rp3* mutant flies relative to controls. 3) The life span extensions obtained with CR or *rp3* mutations are suppressed by Sir2 mutations. Independent confirmation of life span extension by *rp3* mutations was recently published [19]. This same group found that reduced levels of either Rpd3 or Sir2 decreased neurodegeneration in a *Drosophila* model of Huntington's disease, and that when both were decreased at the same time there was added protection from neurodegeneration. Decreasing the levels of other histone deacetylases had no effect. This group failed to see a significant effect of *rp3* mutations on Sir2 message levels, but results may vary depending on genetic background and the presence of balancer chromosomes [19].

The findings from *Drosophila* point to at least two transcriptional regulators that mediate the effects of CR on longevity, Rpd3 and Sir2. It has been proposed that Sir2 is a direct metabolic sensor, potentially transducing the redox status of yeast and mammalian cells into transcriptional changes [36]. However, the *Drosophila* data place Sir2 in a sequential pathway leading from CR to Rpd3 to Sir2. Deacetylation targets of dSir2 include yet another transcription factor, p53 (see section below on p53), histones, and perhaps other proteins yet to be identified. The FOXO transcription factor has been shown to be a deacetylation target of SIRT1 in mammalian cells [18], and *Drosophila* FOXO has been linked to longevity regulation mediated by the *Drosophila* insulin signaling pathway [see review by L. Partridge in this issue, 37]. The metabolic sensors upstream from Rpd3 have not been definitively

characterized, but are likely to include olfaction [38]. The Tor signaling pathway has been also implicated as a mediator of CR and is covered in another review in this issue [37].

Resveratrol – a potential activator of Sir2 and mimetic of CR

Genetic studies showing that overexpression of Sir2 extends longevity raised a related question, could induction of Sir2 enzymatic activity extend longevity as well? A screen for Sir2-activating agents identified several polyphenolic compounds, the most potent being resveratrol [39]. Studies *in vivo* showed that addition of resveratrol to food extends the life span of yeast, worms, fruit flies, and fish [39–45]. Resveratrol has no additive effect on the longevity of wild type flies subjected to CR, consistent with it being a CR mimetic. *dsir2* mutant flies and *sir2.1* mutant worms fed resveratrol do not live longer on a high calorie diet compared to controls [40], providing solid evidence linking Sir2 and resveratrol *in vivo*. However, the longevity effect of resveratrol in fruit flies and worms was not observed in some studies [46,47]. Addition of resveratrol to the food of *ad libitum*-fed mice causes many changes similar to those observed in CR mice or mice fed every other day, a CR-like regimen [48]. For instance, gene expression profiles are similar to those found in CR mice [49].

Resveratrol confers multiple physiological benefits to mice on a high calorie diet, changing systemic metabolism, increasing mitochondrial activity and biogenesis, and increasing physical activity [50,51,52]. In flies resveratrol increases the low spontaneous physical activity associated with a high calorie diet [24], similar to the effect in mice. Administration of resveratrol to mice at 12 months of age decreases age-associated pathologies such as inflammation, proteinuria, and cataracts but does not lead to an extension of maximum life span [48].

Several issues may prove important to the interpretation of resveratrol's effects: the dose response properties of resveratrol, the effects of genetic background, and the effects of resveratrol on non-Sir2 targets *in vivo*. Titrations of the response may vary depending on the assay and the source of resveratrol. In our laboratory the survivorship of female flies is increased by resveratrol starting at 5 and peaking at 200 μmol . The survivorship of male flies is increased starting at 5 and peaking at 100 μmol . In mice high doses of resveratrol increase rotaroid performance and endurance running but decrease total physical activity [50]. In yeast low doses of resveratrol can increase yeast replicative life span by 70% but higher doses cause only minor effects [39]. Genetic background effects are detected with resveratrol in flies, where the wild type *CS* and control *yw* strains both have increased physical activity on a high calorie diet with high resveratrol (200 μmol) but on a CR diet high resveratrol decreases the activity of *CS* flies with no effect on the activity of *yw* flies [24]. *In vitro* assays of Sir2 deacetylase activity using fluorescent substrates have come under scrutiny with regards to resveratrol-mediated activation [39,46,53] and a recent study failed to show activation with non-fluorescent substrates [54]. It has recently been shown that cyclooxygenases and AMP-stimulated protein kinase are activated by resveratrol independently of Sir2 [55,56,57]. Given the many proven beneficial effects of resveratrol in vertebrate and invertebrate systems, the mechanism of resveratrol's effects on Sir2 and non-Sir2 targets promises to be an active field of study. An excellent review of the physiological effects of resveratrol and its use as a CR mimetic has been recently published [52].

dSir and p53

The tumor suppressor gene p53 regulates repair of DNA damage, the cell cycle, apoptosis, and compensatory proliferation. Mammalian p53 was one of the first identified targets of Sir2 [58,59]. Deacetylation of p53 by Sir2 inhibits the transcriptional activity of p53 and p53-mediated apoptosis. Several studies indicate a role of p53 in mammalian aging.

Hyperactivation of p53 in mice prevents tumor formation but decreases survivorship [60]. Mice with hyperactivated p53 show signs of a premature aging phenotype marked by osteoporosis, decreased stress resistance and organ atrophy [61]. In contrast, mice that overexpress both p53 and Arf (a p53 binding protein) show delayed aging and a 16% increase in median life span [62]. Longevity extensions have also been obtained with p53 in *Drosophila*, but the effects are dependent on the level, time and place of overexpression and frequently display a marked sexual dimorphism. Overexpression of wild type p53 throughout development is lethal at high levels but lower level expression in larvae leads to median life span extension in both sexes [64]. When wild type p53 is only expressed in adults, moderate changes in median life spans occur, with an increase in males and decrease in females when ubiquitously expressed but with a decrease in males and an increase in females when pan-neurally expressed [65]. Flies lacking p53 are sick with shortened life spans [63]. However, pan-neural expression of a dominant negative form of p53 (DN-p53) during development and adulthood causes 32% and 58% median life span increases in males and females respectively [63]. Pan-neural expression of DN-p53 only in adults also leads to increased life span in females, though less than that obtained in the preceding experiment, and no increase in males [17]. When DN-p53 is expressed outside of the nervous system (muscle or fat bodies) during adult life there is a decrease in longevity [63]. The results with DN-p53 are dependent on the presence of wild type p53, as the aforementioned life span effects are not seen in a p53 mutant genetic background [66].

Mammalian p53 directly interacts with SIRT1, and data indicate a similar interaction between *Drosophila* p53 and Sir2 [17]. When flies overexpress tagged wild type p53, immunoprecipitation using the p53 tag also precipitates Sir2. Recombinant dSir2 deacetylates p53 substrates *in vitro*. Additionally, when wild type p53 is cotransfected into S2 cells with a p53-responsible luciferase reporter construct, luciferase transcription is inhibited by resveratrol.

CR does not further increase the life span of flies overexpressing DN-p53. If dSir2 and DN-p53 are overexpressed at the same time, life span extension is no greater than Sir2 overexpression by itself [17]. Similarly, life span extension observed in cep-1 mutant worms, the worm homologue of p53, was not-additive to CR or Sir2.1 overexpression [67]. Ubiquitous overexpression of wild type p53 in flies that lack Sir2 does not change life span, consistent with an overlap between the longevity effects mediated by p53 and Sir2 [68]. However, pan-neural expression of wild type p53 in flies lacking Sir2 led to similar changes in life span compared to flies containing Sir2 (decreases in males and increases in females) [68]. DN-p53 has not been overexpressed in flies lacking Sir2.

There is evidence for interactions between p53 and the Tor and insulin signaling pathways [66,68]. [Please see reviews on insulin and Tor signaling pathway in this issue, 37]. The evidence so far suggests that p53 functions as a regulatory link in multiple signaling pathways analogous to its role in mammalian cells, where it is a common link in diverse pathways related to DNA damage, apoptosis, and cell proliferation. In summary, *Drosophila* p53 interacts with dSir2 and components of other signaling pathways, perhaps integrating inputs related to genotoxic stress.

Sir2 is part of a larger pathway and network of interactions

Genetic manipulation of Rpd3, Sir2 and p53 can extend life span in *Drosophila*, and there is clear evidence that these three genes are part of the CR pathway for longevity regulation [13,17,31]. CR is hypothesized to reduce Rpd3 levels, which in turn increases dSir2 levels, and one of the targets of dSir2 is p53. Rpd3 and dSir2 have other deacetylation targets, and it is likely that CR influences other effectors besides Rpd3. Further support for this hypothesis has recently come from gene expression profile studies performed in the Helfand

laboratory [69]. They sought to identify genes whose activity in whole female flies is affected by three life-extending conditions: CR, increased dSir2 and decreased p53 activity. Several hundred gene activity changes are shared by CR and dSir2 induction but far fewer are shared between CR and p53 down regulation, consistent with dSir2 being a major effector of CR and p53 being one of several effectors for dSir2. This study also shows the power of the *Drosophila* system for discovering mechanisms of longevity regulation. One gene that was upregulated among all three life-extending conditions, *takeout*, was subsequently shown to be upregulated under additional life extending conditions (mutations decreasing the levels of *Indy*, *Rpd3*, the insulin receptor substrate *chico*, and *Methuselah*). The role of this gene in life span extension was then directly corroborated by overexpression in adult neurons, pericerebral fat bodies and abdominal fat bodies. The mechanism of the *takeout* effects on longevity is not clear at this time and it will be the subject of future research.

Conclusions

Sirtuins are a highly conserved family of proteins that have been implicated in regulating diverse aspects of physiology including metabolism, stress response, cell survival, replicative senescence, inflammation, circadian rhythm, neurodegeneration, cancer, etc in variety of species [1,2]. A remarkable amount of work supports the key role of Sir2 in longevity regulation, CR, and disease processes, while also highlighting the complexity of the interaction between Sir2/SIRT1 and its numerous targets. *Drosophila* has been useful in revealing the role of dSir2 in aging and longevity. For instance, flies have provided compelling evidence that dSir2 mediates at least some of the beneficial effects of CR. Research on the functions of Sir2/SIRT1 has increasingly focused on its role in diseases and whether it can be a therapeutic target, resulting in the identification of several drugs for potential therapeutic use. Perhaps the most exciting work on SIRT1 in mammals concerns the prevention or amelioration of metabolic and neurological conditions that affect the elderly. *Drosophila* can contribute to many aspects of this research due to the availability of genetic and molecular tools, as shown by recent work in flies highlighting the role of Sir2 in neurodegeneration and Huntington disease. These studies show how flies can model pathologies associated with aging, and flies could potentially be integrated into the testing of new drugs. While aging is a complex process, an intervention based on evolutionarily conserved processes might be within our reach. Genetic manipulations in flies are providing the framework for these future benefits.

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