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Absence of effects of Sir2 over-expression on lifespan in *C. elegans* and *Drosophila*

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Abstract

Over-expression of sirtuins (NAD^+-dependent protein deacetylases) has been reported to increase lifespan in budding yeast, *Caenorhabditis elegans* and *Drosophila melanogaster*[1-3]. Studies of gene effects on ageing are vulnerable to confounding effects of genetic background[4]. We re-examined the reported effects of sirtuin over-expression on ageing and found that standardisation of genetic background and use of appropriate controls abolished the apparent effects in both *C. elegans* and *Drosophila*. In *C. elegans*, outcrossing of a line with high level *sir-2.1* over-expression[1] abrogated the longevity increase, but not *sir-2.1* over-expression. Instead, longevity co-segregated with a second-site mutation affecting sensory neurons. Outcrossing of a line with low copy number *sir-2.1* over-expression[2] also abrogated longevity. A *Drosophila* strain with ubiquitous over-expression of *dSir2* using the UAS-GAL4 system was long-lived relative to wild-type controls, as previously reported[3], but not relative to the appropriate transgenic controls, and nor was a new line

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with stronger over-expression of *dSir2*. These findings underscore the importance of controlling for genetic background and the mutagenic effects of transgene insertions in studies of genetic effects on lifespan. The life extending effect of dietary restriction (DR) on ageing in *Drosophila* has also been reported to be *dSir2* dependent[3]. We found that DR increased fly lifespan independently of *dSir2*. Our findings do not rule out a role for sirtuins in determination of metazoan lifespan, but they do cast doubt on the robustness of the previously reported effects on lifespan in *C. elegans* and *Drosophila*.

Keywords

Ageing; *C. elegans*; *Drosophila*; sirtuin; genetic background

The role of sirtuins in ageing was discovered in budding yeast (*Saccharomyces cerevisiae*), where over-expression of *SIR2* increases replicative lifespan[5]. It was then reported that elevated sirtuin levels increase lifespan in the nematode *C. elegans*[1,2,6] and the fruitfly *Drosophila*[3], suggesting an evolutionarily ancient role of sirtuins in longevity assurance[7]. Dietary restriction (DR), reduced food intake short of starvation, extends lifespan in organisms ranging from yeast to mammals[8], and initial studies suggested that DR increases lifespan by activating sirtuins in yeast[9], *C. elegans*[10] and *Drosophila*[3]. Pharmacological activation of sirtuins has thus been widely promulgated as a potential means to mimic DR and slow ageing in humans[11]. However, several aspects of the role of sirtuins in ageing have proved controversial[12]. Subsequent studies have suggested that sirtuins do not mediate DR effects on ageing, at least in budding yeast and *C. elegans*[13,14]. The plant-derived polyphenol resveratrol and other compounds have been reported to activate sirtuins and extend lifespan[15,16]. More recent findings have challenged both effects[17-20]. We therefore re-examined the effects of sirtuin over-expression on lifespan in *C. elegans* and *Drosophila*. In particular, we wished to exclude the possibility that the increased longevity observed in strains with sirtuin gene over-expression are caused by differences in genetic background, or by the mutagenic effects of transgene insertion, which frequently confound studies of the genetics of ageing[4].

We first examined a high copy number *sir-2.1* transgenic *C. elegans* strain (LG100) carrying the integrated transgene array *geIn3 [sir-2.1 rol-6(su1006)]* (Ref. 1). As expected, this strain was long lived (Fig. 1a; Table S1). However, outcrossing (x5) of *geIn3* to wild type (N2) abrogated the increase in longevity (Fig. 1a, Table S1) without affecting SIR-2.1 protein levels (Fig. 1b). This loss of longevity upon outcrossing was verified by an independent research team (Table S2).

LG100 exhibited a neuronal dye-filling (Dyf) defect[22] that did not segregate with the transgene upon outcrossing (Fig. S2A). Dyf mutants often exhibit extended lifespan[23]. To determine whether the longevity of LG100 might be attributable to a *dyf* mutation, we derived from it three Dyf, non-Rol lines (lacking *geIn3*) and three non-Dyf, Rol lines (carrying *geIn3*). Dyf, non-Rol lines were long-lived and showed wild-type SIR-2.1 protein levels (Fig. 1c,d, Table S3). Non-Dyf, Rol lines showed elevated SIR-2.1 protein levels but had wild-type lifespans. Dyf mutant longevity appeared to be partially *daf-16* dependent (Fig. S2B), as seen previously for other Dyf mutants[23]. The co-segregation of longevity with this *dyf* mutation but not *geIn3* was previously noted by another research team (S.S. Lee, Cornell University, personal communication). Furthermore, knock-down of *sir-2.1* expression in LG100 using RNA-mediated interference did not suppress longevity, despite lowering SIR-2.1 protein to wild-type levels (Fig. 1e,f; Table S4). Taken together, these results imply that the longevity of LG100 is attributable to an unidentified *dyf* mutation (or

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possibly another mutation closely-linked to the *dyf* locus), and that high level over-expression of *sir-2.1* is not sufficient to increase lifespan in these strains.

A low copy number transgenic strain (NL3909) over-expressing *sir-2.1* (Ref. 7) is also long-lived[2]. We confirmed the increased lifespan of NL3909 (*pkIs1642 [sir-2.1 unc-119] unc-119(ed3)*) relative to the control strain NL3908 (*pkIs1641 [unc-119] unc-119(ed3)*) (Fig. 1g, Table S5). We also observed an apparent elevation of SIR-2.1 protein in NL3909 relative to NL3908 (Fig. 1h). Outcrossing (x6) of NL3909 once again abrogated longevity (Fig. 1g, Table S5) without affecting SIR-2.1 protein levels (Fig. 1h, Fig. S1c). This effect of outcrossing was independently verified (Table S6). Thus, the longevity of NL3909 also appears to be attributable to genetic background effects rather than to *pkIs1642*.

The duplication *mDp4* includes the *sir-2.1* locus, and the *mDp4*–containing strain DR1786 is long lived[1]. We too found that DR1786 is long-lived, and also shows elevated *sir-2.1* expression. However, longevity was not suppressed by *sir-2.1* RNAi (Fig. S3, Table S7) suggesting causation by factors other than *sir-2.1*, either on *mDp4* or elsewhere in the genome.

In *Drosophila* over-expression of *dSir2* reportedly increases lifespan relative to wild-type controls[3]. Over-expression was achieved using the GAL4, UAS binary system[24], with the largest increases in lifespan produced by combination of EP-UAS-*dSir2* (*dSir2*^{EP2300}) with a ubiquitously-expressed tubulin-GAL4 driver. We outcrossed these two transgenes (x6) into the control white Dahomey (w^{Dah}) background. Assayed on a medium similar to that used in the original study, EP-UAS-*dSir2*/tubulin-GAL4 flies were longer lived than wild-type controls, as previously reported[3] (Fig. 2a). However, they were not longer lived than the tubulin-GAL4/+ control flies (Fig. 2a). This implies that life extension is due to transgene-linked genetic effects other than over-expression of *dSir2*. Lifespan was assayed on a range of food media (see Methods for details) to test for nutrient-dependence of any effect. However, in no case were EP-UAS-*dSir2*/tubulin-GAL4 flies longer lived than one or both transgenic controls (Fig. S4).

Lack of an observable effect on lifespan could reflect the relatively modest increase in *dSir2* expression in EP-UAS-*dSir2*/tubulin-GAL4 flies, both in terms of levels of mRNA (Fig. S5) and protein (+35% relative to wild type, Fig. S6). We therefore created lines with a higher level of over-expression of *dSir2* (UAS-*dSir2*-Myc9/tubulin-GAL4). Here *dSir2* mRNA and protein levels were robustly increased relative to wild type (+318% relative to wild-type protein levels; Fig. S5, S6). We examined recombinant protein raised in *E. coli* to check that the presence of the Myc tag did not interfere with *dSir2* histone deacetylase (HDAC) activity, as measured by deacetylation of the fluorophore-containing p53 (Fluor de Lys) or native acetylated histone H4 substrates, and it did not (Fig. S7). We also found that *dSir2* HDAC activity was unaffected by addition of resveratrol in either assay (Fig. S7). We saw no increase in lifespan in UAS-*dSir2*-Myc/tubulin-GAL4 flies relative to tubulin-GAL4/+ controls, on food medium similar to that used in the original study (Fig. 2b) or relative to either control on a range of other media (Fig. S4b,c,f). An independent research team also saw no increase in lifespan in tubulin-GAL4/UAS-*dSir2*-Myc9 flies (Fig. S8). These results suggest that the previously observed longevity of EP-UAS-*dSir2*/tubulin-GAL4 flies was not attributable to elevated expression of *dSir2*, and that stronger, ubiquitous over-expression of *dSir2* also does not extend fly lifespan.

The role of sirtuins in the extension of lifespan by DR in yeast and *C. elegans* is controversial, with multiple groups reporting that sirtuins are not required for life span extension from DR in both organisms[8]. In *Drosophila*, it was reported that DR does not increase lifespan in *dSir2* deletion mutant flies[3]. We tested this too, using the deletion alleles

dSir2$^{4.5}$ (tested previously[3]) and *dSir2*^{17}. We first outcrossed these alleles (Fig. S9a) into the Canton S wild type (see Methods), used in the previous DR study[3]. We then checked the effect of each allele on *dSir2* gene expression. *dSir2*^{17} abrogated *dSir2* mRNA, implying that this is a null allele. By contrast, *dSir2*$^{4.5}$, which contains a relatively small deletion at the 5′ end of the gene, did not reduce *dSir2* mRNA levels (Fig. S9b,c).

To reassess the role of *dSir2* in DR in *Drosophila*, we compared lifespans of wild-type (Canton S), *dSir2*$^{4.5}$ and *dSir2*^{17} homozygotes. All genotypes responded similarly and normally to DR in trials conducted by two independent research teams (Fig. 2c, Fig. S10), hence the effect of DR on lifespan did not require *dSir2*.

In this study, we were unable to verify the effect of sirtuin over-expression on lifespan in either *C. elegans* or *Drosophila*. Increased lifespan was seen in two *C. elegans* lines with elevated *sir-2.1* expression derived from independent studies, as previously reported, but in each case this was abrogated by outcrossing. *sir-2.1* over-expression does exert effects on traits other than lifespan. For example, *geIn3* is neuroprotective in a worm model of neuron dysfunction in Huntington's disease[25] and, importantly, this effect is not attributable to the *dyf* mutation (Fig. S11). Moreover, both NL3909 and its outcrossed derivative are thermotolerant (M. Somogyvári, C. Sőti, unpublished data). In *Drosophila*, lines over-expressing *dSir2* were longer-lived than wild type controls, as previously reported, but they were not longer lived than lines containing the appropriate transgenic controls. That all transgenic lines were longer lived than the Dahomey wild type into which they had been outcrossed could reflect heterosis in the vicinity of the transgene inserts, or a mutagenic effect of the GAL4 insert.

Lifespan was not increased by over-expression either of *sir-2.1* from its own promoter in *C. elegans,* or *dSir2* ubiquitously from a heterologous promoter in *Drosophila*. Our findings call into question the robustness of earlier reports of a role of sirtuins in longevity-assurance based upon over-expression in *C. elegans* and *Drosophila*, and also on the role of *dSir2* in the response to DR in *Drosophila*. However, sirtuins can affect lifespan in animals under certain conditions: *C. elegans daf-2(e1370)* mutants are hypersensitive to genetic effects on lifespan[26], and here deletion of *sir-2.1* reproducibly increases lifespan[6] (Fig. S12).

Our finding that resveratrol does not activate HDAC activity of *dSir2* using a native histone H4 peptide is consistent with earlier findings with yeast *SIR2* and mammalian SirT1 (Ref. 17,18). Resveratrol increased *Drosophila* lifespan in one study[27] but not another[21]. In principle, this could reflect sensitivity of resveratrol effects to subtle differences in culture conditions. If this were the case, our findings would imply that such effects are not attributable to direct activation of *dSir2* by resveratrol.

METHODS SUMMARY

Nematode strains and maintenance

Nematodes were maintained on nematode growth medium (NGM) agar at 20°C, with *Escherichia coli* OP50 bacteria as a food source. Nematode strains used included: wild type (N2), GA707 *wuEx166 [rol-6(su1006)]* (*rol-6* control), LG100 *geIn3 [sir-2.1 rol-6(su1006)] dyf-?(wu250)*, NL3909 *pkIs1642 [sir-2.1 unc-119] unc-119(ed3)*, and the control strain NL3908 *pkIs1641 [unc-119] unc-119(ed3)*.

Nematode lifespan measurements

These were performed as described[28], at 20°C. To prevent progeny production, 5-fluoro-2′-deoxyuridine (FUdR) was added to seeded plates, to a final concentration of 10, 40 or 50 μM. Before testing effects of RNA-mediated interference (RNAi) on lifespan, worms were

kept for 2 generations on the RNAi bacteria. Statistical significance of effects on lifespan was estimated using the log rank test, performed using JMP, Version 7 (SAS Institute).

Fly stocks and maintenance

tubulin-GAL4 and *dSir2*^{EP2300} were obtained from the Bloomington Stock Center. *dSir2-Myc2* and *dSir2-Myc9* lines were generated by germ-line transformation into strain w^{04}. *dSir2*$^{4.5}$/SM6B, *dSir2*^{17}/Cyo and Canton S were gifts from S. Pletcher, J. Rine and S. Helfand. All lines were outcrossed at least 6 times into the relevant controls. Experiments were performed at 25°C on a 12h : 12 h light-dark cycle at constant humidity.

Fly lifespan assays

Flies were bred at standard density, allowed to mate for 48 hours after emerging then sorted into 10 females per vial. Vials were changed every 48 hours, and deaths per vial scored until all flies were dead. Over-expression studies n=200. *dSir2* mutant studies n=100. For statistical methodology, see above.

dSir2 deacetylation assays

We used both the SirT1 Fluorimetric Drug Discovery Kit (Enzo Life Sciences) and an HPLC-based acetyl-histone H4 deacetylation assay[29]. *dSir2* and *dSir2*-Myc were cloned into pET SUMO (Invitrogen) and purified on HisPur cobalt spin columns (Thermo Scientific).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A

METHODS

C. elegans

Nematode strains and maintenance—*C. elegans* were cultured under standard monoxenic conditions[31,32]. Strains used included N2 (wild type), GA707 *wuEx166 [rol-6(su1006)]*, HT1593 *unc-119(ed3)*, LG100 *geIn3* [*sir-2.1 rol-6(su1006)*] *dyf-?(wu250)*, NL3908 *pkIs1641* [*unc-119*] *unc-119(ed3),* and NL3909 *pkIs1642* [*sir-2.1 unc-119*] *unc-119(ed3).*

Outcrossing of strains—LG100 was outcrossed with N2, and the Rol trait used to detect the presence of *gIn3*. NL3908 and NL3909 were outcrossed using HT1593 *unc-119(ed3)*. Rescue of Unc was used to detect the presence of the transgene array.

Isolation of Dyf, non-Rol and non-Dyf, Rol lines—LG100 was crossed with N2, and lines established from individual F2 animals exhibiting Dyf, non-Rol or non-Dyf, Rol phenotypes. The Dyf phenotype was identified by staining with the dye DiI, and looking for absence of dye uptake into the amphid and phasmid neurons. Non-Dyf, Rol F2 animals that were heterozygous for the *geIn3* transgene array (the *rol-6* marker is dominant) were identified by the presence of non-Rol animals in the F3, and excluded.

RNA-mediated interference (RNAi)—Animals were fed *E. coli* containing the HT115 vector either with or without a portion of the *sir-2.1* gene cloned into it. The *sir-2.1* feeding strain was obtained from the Ahringer RNAi library[33]. Worms were maintained on RNAi feeding strains for two generations prior to lifespan measurements. One day before starting measurements, 5-fluoro-2′-deoxyuridine (FUDR) was applied to seeded plates to 10 μM to prevent progeny production.

Analysis of SIR-2.1 protein levels—Protein was prepared from synchronous nematode cultures (L4 larvae and young adults) raised on *E. coli* OP50 or RNAi bacteria for two generations. Western blots were performed with anti-actin monoclonal antibodies (Santa-Cruz Biotechnology), anti-SIR-2.1 polyclonal antibody (kindly provided by A. Gartner[34]). For all assays, 3-5 replicate worm cultures were used.

Neuroprotection assays—To test for sirtuin protection from expanded polyglutamines (polyQs), we crossed GA919 (*geIn3* dissociated from *dyf-?(wu250)*) to strains carrying integrated polyQ arrays. These polyQ strains co-express the first 57 amino acids of human huntingtin (htt) with either 19 or 128 Glns fused to CFP, expressed from the *mec-3* promoter, and YFP expressed from the *mec-7* promoter in touch receptor neurons[25]. Response to touch at the tail was tested as described[25]. Three trials were performed and 150-200 animals/genotype tested.

Lifespan analysis—Lifespans of synchronized population cohorts were measured as previously described[28]. FUDR was applied to the plates, to 10, 40 or 50 μM (see Supplemental Tables). Lifespan experiments were performed at 20°C. A small proportion of animals were censored, usually due to uterine rupture, which mainly occurred at mid-adulthood (~day 9-11).

Statistical analysis—Statistical significance of effects on lifespan were estimated using the log rank test, performed using JMP, Version 7 (SAS Institute).

Drosophila

Fly stocks and maintenance—tubulin-GAL4 and *dSir2*^{EP2300} lines were obtained from the Bloomington Stock Center. *dSir2-Myc2* and *dSir2-Myc9* lines were generated by germ-line transformation. These were outcrossed into white Dahomey (w^{Dah}). *dSir2*$^{4.5}$/SM6B (Ref. 35), *dSir2*^{17}/Cyo (Ref. 36), kindly provided by S. Pletcher and J. Rine, were outcrossed into Canton S. All lines were out-crossed at least 6 times. The presence of the deletion was detected by PCR using the following primers: 149F (5′-AGATATGACATAAGGCAGTGGC-3′), 1427R (5′-TCCCGTTAGCACAATGATCTTC-3′); 3909R (5′-GAAGGCGGTAGCAATGG TGACAA-3′). Flies were maintained at 25°C on a 12h : 12 h light-dark cycle at constant humidity.

Myc-tagged *dSir2*—The Myc tag was added to RE27621 from Riken using standard techniques and cloned into pUASP. The construct was microinjected into w^{04} and the transformant lines *dSir2-Myc2* and *dSir2-Myc9* recovered. Primers: Sir5'R2 (5′-

CAAGAATTCCAACGAGAATTTTACACAGGTCGTGTG-3′), Sir3'Xba (5′-ATC GAGTCTAGACACTGCTGCTAACTGTCCTGGAGG-3′) MYC3'Xba (5′-GAGCT ATCTAGAGGATCCGAGGAGCAGAAGCTGATC-3′).

Lifespan assays—Flies were bred at standard density, allowed to mate for 48 hours after emerging (once mated) then sorted into 10 females per vial (UCL) or 35 per vial on 15% SYA (University of Michigan). Vials were changed every 48 hours, and deaths per vial scored until all flies were dead. Numbers of flies used in lifespan assays: over-expression studies, n, ~200 (UCL) or ~350 (U. Michigan). DR studies, n=100. For the over-expression studies, the fly food recipes were as follows. SYA (100 g yeast, 50 g sugar, 15 g agar, 30 ml nipagin and, in most trials, 3 ml propionic acid per litre food), ASG (20 g yeast, 85 g sugar, 10 g agar, 60 g maize, per litre food), ASG[1] (31 g yeast, 124 g sugar, 9 g agar, 53 g cornmeal, 25 ml nipagin per litre food), 15% SYA (150 g yeast, 150 g sugar, 21 g agar, 15 ml tegosept). For the DR trials the food dilutions used were as follows. 15 g agar, 30 ml nipagin, 3 ml propionic acid, with yeast and sugar both altered to final concentrations of 10 g, 50 g, 100 g, 150 g, 200 g per litre food. All food was prepared as previously described[21].

Genetic crosses—tubulin-GAL4/TM3 males were crossed to *dSir2*^{EP2300}, *dSir2-Myc2* or *dSir2-Myc9* virgin females, and *dSir2*^{EP2300}/+; tubulin-GAL4/+, *dSir2-Myc2*/+; tubulin-GAL4/+ or *dSir2-Myc9*/+; tubulin-GAL4/+ females were selected from the progeny. For the controls, tubulin-GAL4/TM3 males or *dSir2*^{EP2300}, *dSir2-Myc2* or *dSir2-Myc9* virgin females were crossed to w^{Dah} and *dSir2*^{EP2300}/+, tubulin-GAL4/+, *dSir2-Myc2*/+ or *dSir2-Myc9*/+ females were selected from the progeny.

QRT-PCR—RNA was extracted from ten 10 day old females using standard techniques and transcribed into cDNA. 4 biological replicates were run per genotype, each in triplicate. Samples were normalised to either Actin5C or RP49. Primers: Sir2-4 5′-GCTCTCCACCGTTGTCTGAGGGCC-3′ (Ref. 3), Sir2-5 5′-GGCGGCAGCTGTGCTGCGATGAG-3′ (Ref. 3), Actin5CF 5′-CACACCAAATCTTACAAAATGTGTGA-3′, ActinCR 5′-AATCCGGCCTTGCACATG-3′, RP49F 5′-ATGACCATCCGCCCAGCATCAGG-3′, RP49R 5′-ATCTCGCCGCAGTAAACG-3′.

Analysis of *dSir2* protein levels—Protein was extracted from 30 females aged 7 days. Western blots were performed using antibodies c-myc 9E10 (Santa Cruz Biotechnology), p2E2 (Developmental Studies Hybridoma Bank), and Tubulin (Sigma).

dSir2* deacetylation assays—dSir2* (RE27621) and *dSir2*-Myc were cloned into pET SUMO (Invitrogen) and purified on HisPur cobalt spin columns (Thermo Scientific). For the *Fluor de Lys* assay, using the SirT1 Fluorimetric Drug Discovery Kit (Enzo Life Sciences), results presented are the mean ± S.E.M. of 3 biological replicates. Within each biological replicate samples were run in triplicate. Final concentrations: Resveratrol and suramin 0.2 mM, NAD^+ 0.1 mM. Deacetylation of native acetyl histone H4 peptide was monitored by HPLC. Deacetylation of histone H4 N-terminal peptide (SGRGKGGKGLGKGGA(acetyl-K)RHRC) (Biomatik) was carried out using 500 μM NAD^+, 100 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM DTT and 0.05% Triton X-100 and monitored by HPLC (Agilent 1100) with an ACE C8-300 150×3.0mm column. The elution profiles were analyzed using Chemstation for LC 3D software.

Statistical analyses—Survivorships and the response to DR were compared using the log-rank test and analyses were performed using JMP, Version 7 (SAS Institute).

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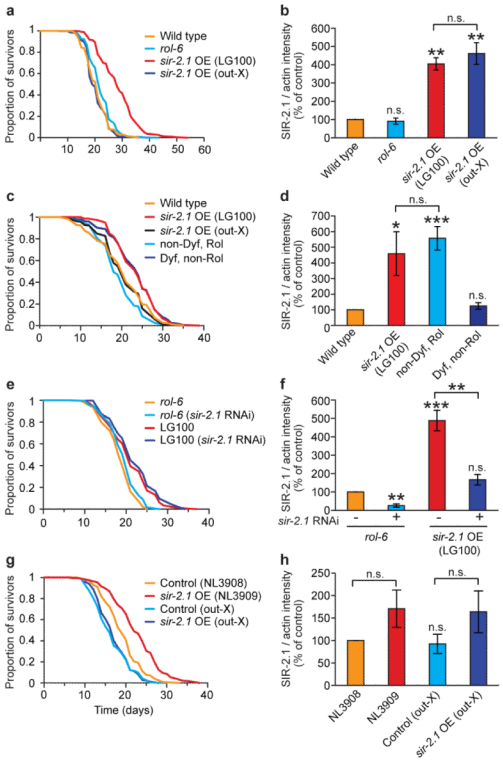

Figure 1. *C. elegans*: Longevity of LG100 and NL3909 is not attributable to *sir-2.1* over-expression

a, **b**. Outcrossing of LG100 removes life extension without affecting SIR-2.1 protein levels. Data in **b** derived from Western blots (mean of three trials each using an independent protein preparation). A representative Western blot is shown in Fig S1a. qRT-PCR showed that *sir-2.1* mRNA is also elevated in both strains (data not shown). **c**, LG100-derived Dyf, non-Rol segregant lines are long-lived while non-Dyf, Rol lines are not. **d**. Non-Dyf Rol segregant lines have elevated SIR-2.1 levels, while Dyf, non-Rol lines do not. **e**, **f**. *sir-2.1* RNAi does not suppress LG100 longevity, but reduces SIR-2 protein levels. **g**, **h**. Outcrossing of NL3909 removes life extension without affecting SIR-2.1 protein levels. See Tables S1, S3, S4 and S5 for lifespan statistics for **a**, **c**, **e** and **g**, respectively. Error bars, S.E.M.. *0.01< P < 0.05; ** 0.001 < P < 0.01; *** P < 0.001, n.s., not significant; Student's t test (two tailed). One remaining possibility is that the outcrossed *sir-2.1* strains both contain second site mutations that suppress longevity effects. However, *daf-2* RNAi strongly induced longevity in both (data not shown), arguing against the presence of a general suppressor of longevity in each case.

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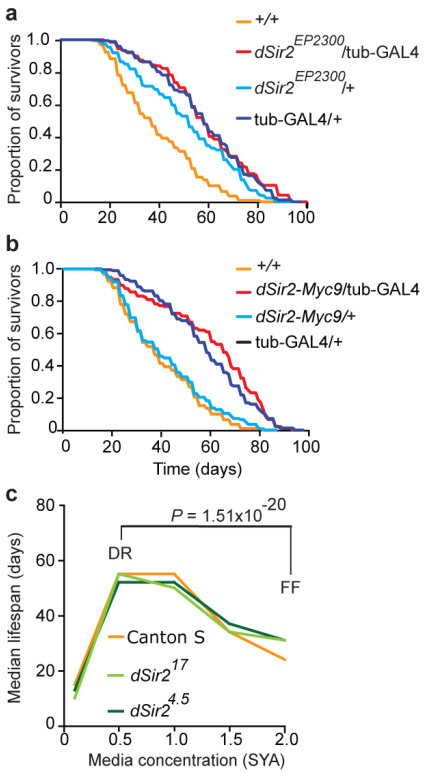

Figure 2. *Drosophila*: Absence of effects of *dSir2* on lifespan

All lines were outcrossed into w^{Dah} (+/+) **a**, Lifespan in flies over-expressing $dSir2^{EP2300}$ driven via tubulin-GAL4 (tub-GAL4) is longer than wild type, but not than the tubulin-GAL4 /+ genetic control. Median lifespans: +/+ = 39 days, $dSir2^{EP2300}$/tubulin-GAL4 = 59 days, $dSir2^{EP2300}$/+ = 53 days, tubulin-GAL4 /+ = 60 days. $dSir2^{EP2300}$/tubulin-GAL4 *vs.* $dSir2^{EP2300}$/+, $P = 0.0006$; $dSir2^{EP2300}$/tubulin-GAL4 *vs.* tubulin-GAL4 /+, $P = 0.9295$; $dSir2^{EP2300}$/tubulin-GAL4 *vs.* +/+, $P < 0.0001$. **b**, Lifespan in flies over-expressing *dSir2-Myc9* is longer than in wild type, but not than in the tubulin-GAL4 control. Median lifespans: +/+ = 39 days, *dSir2-Myc9*/tubulin-GAL4 = 67, *dSir2-Myc9*/+ = 41 days, tubulin-GAL4/+ = 60 days. *dSir2-Myc9*/tubulin-GAL4 *vs. dSir2-Myc9*/+, $P = 0.0001$; *dSir2-Myc9*/tubulin-GAL4 *vs.* tubulin-GAL4/+, $P = 0.1354$; *dSir2-Myc9*/tubulin-GAL4 *vs.* +/+, $P < 0.0001$. Compared using log rank test, n=200. **c**, Effect of dietary restriction on *Drosophila* lifespan is not *dSir2* dependent. Flies were assayed over five concentrations of SYA media and data are presented as the median lifespan on each food concentration. All lines were outcrossed into Canton S (+/+). *P* values confirm that all flies respond normally to DR when median lifespans are compared for DR *vs.* fully-fed (FF) conditions[30].