Absence of effects of Sir2 overexpression on lifespan in C. elegans and Drosophila

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Overexpression of sirtuins (NAD+-dependent protein deacetylases) has been reported to increase lifespan in budding yeast (Saccharomyces cerevisiae), Caenorhabditis elegans and Drosophila melanogaster1–3. Studies of the effects of genes on ageing are vulnerable to confounding effects of genetic background14. Here we re-examined the reported effects of sirtuin overexpression on ageing and found that standardization of genetic background and the 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 overexpression15 abrogated the longevity increase, but did not abrogate sir-2.1 overexpression. Instead, longevity co-segregated with a second-site mutation affecting sensory neurons. Outcrossing of a line with low-copy-number sir-2.1 overexpression16 also abrogated longevity. A Drosophila strain with ubiquitous overexpression of dsir2 using the UAS-GAL4 system was long-lived relative to wild-type controls, as previously reported17, but was not long-lived relative to the appropriate transgenic controls, and nor was a new line with stronger overexpression of dsir2. These findings underscore the importance of controlling for genetic background and for the mutagenic effects of transgene insertions in studies of genetic effects on lifespan. The life-extending effect of dietary restriction on ageing in Drosophila has also been reported to be dsir2-dependent18. We found that dietary restriction 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 of sirtuins on lifespan in C. elegans and Drosophila.

The role of sirtuins in ageing was discovered in budding yeast, where overexpression of SIR2 increases replicative lifespan19. It was then reported that elevated sirtuin levels increase lifespan in the nematode C. elegans20,21 and the fruitfly Drosophila22, indicating an evolutionarily ancient role of sirtuins in longevity assurance23. Dietary restriction (reduced food intake short of starvation) extends lifespan in organisms ranging from yeast to mammals24, and initial studies indicated that dietary restriction increases lifespan by activating sirtuins in yeast25, C. elegans26 and Drosophila22. Pharmacological activation of sirtuins has therefore been widely promulgated as a potential means to mimic dietary restriction and slow ageing in humans27. However, several aspects of the role of sirtuins in ageing have proved controversial28. Subsequent studies have indicated that sirtuins do not mediate the effects of dietary restriction on ageing, at least in budding yeast and C. elegans29,30. The plant-derived polyphenol resveratrol and other compounds have been reported to activate sirtuins and extend lifespan31,32, but more recent findings have challenged both effects33–36.

We therefore re-examined the effects of sirtuin overexpression on lifespan in C. elegans and Drosophila. In particular, we wished to exclude the possibility that the increased longevity observed in strains with overexpression of sirtuin genes is caused by differences in genetic background, or by the mutagenic effects of transgene insertion, which frequently confound studies of the genetics of ageing37.

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 sla1006] (ref. 1). As expected, this strain was long-lived (Fig. 1a and Supplementary Table 1). However, outcrossing (∼X5) of geIn3 to wild type (N2) abrogated the increase in longevity (Fig. 1a and Supplementary Table 1) without affecting SIR-2.1 protein levels (Fig. 1b). This loss of longevity upon outcrossing was verified by an independent research team (Supplementary Table 2).

LG100 showed a neuronal d-filling (Dyf) defect38 that did not segregate with the transgene upon outcrossing (Supplementary Fig. 2a). Dyf mutants often show extended lifespan39. To determine whether the longevity of LG100 might be attributable to a dyf mutation, we derived from this strain 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 Sir2-2.1 protein levels (Fig. 1c, d and Supplementary Table 3). Non-Dyf, Rol lines showed elevated Sir2-2.1 protein levels but had wild-type lifespans. Dyf mutant longevity seemed to be partially dependent on daf-16 (Supplementary Fig. 2b), as seen previously for other Dyf mutants40. The co-segregation of longevity with this dyf mutation, but not with geIn3, was previously noted by another research team (S. S. Lee, personal communication). Furthermore, knockdown 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 and Supplementary Table 4). Taken together, these results indicate that the longevity of LG100 is attributable to an unidentified dyf mutation (or possibly another mutation closely linked to the dyf locus), and that high-level overexpression of Sir-2.1 is not sufficient to increase lifespan in these strains.

A low-copy-number transgenic strain (NL3909) overexpressing sir-2.1 (ref. 7) is also long-lived41. We confirmed the increased lifespan of NL3909 (pKls1642 [sir-2.1 unc-119] unc-119(ed3)) relative to the control strain NL3908 (pKls1641 [unc-119] unc-119(ed3)) (Fig. 1g and Supplementary Table 5). We also observed an apparent increase in SIR-2.1 protein levels in NL3909 relative to NL3908 (Fig. 1h). Outcrossing (∼X6) of NL3909 once again abrogated longevity (Fig. 1g and Supplementary Table 5) without affecting SIR-2.1 protein levels (Fig. 1h and Supplementary Fig. 1c). This effect of outcrossing was independently verified (Supplementary Table 6). Thus, the longevity of NL3909 also seems to be attributable to effects of genetic background rather than to pKls1642.

The duplication mDp4 includes the sir-2.1 locus, and the mDp4-containing strain DR1786 is long-lived41. We found that DR1786 is

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Indeed long-lived, and also shows elevated sir-2.1 expression. However, longevity was not suppressed by sir-2.1 RNA interference (RNAi) (Supplementary Fig. 3 and Supplementary Table 7) indicating causation by factors other than sir-2.1, either on mDp4 or elsewhere in the genome.

In Drosophila, overexpression of dSir2 reportedly increases lifespan relative to wild-type controls. Overexpression was achieved using the GAL4-UAS binary system, with the largest increases in lifespan being produced by the combination of EP-UAS–dSir2 (dSir2EP2300) with a ubiquitously expressed tubulin–GAL4 driver. We outcrossed these two transgenes (×6) into the control white Dahomey (wDah) background. When 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 (Fig. 2a). However, they did not live longer than the tubulin–GAL4/+ control flies (Fig. 2a). This implies that lifespan extension is due to transgene-linked genetic effects other than the overexpression 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 (Supplementary Fig. 4).

The lack of an observable effect on lifespan could reflect the relatively modest increase in dSir2 expression in EP-UAS–dSir2/tubulin–GAL4 flies.
flies, both in terms of messenger RNA levels (Supplementary Fig. 5) and protein levels (increased by 35% relative to wild type; Supplementary Fig. 6). We therefore created lines with a higher level of overexpression of dSir2 (UAS–dSir2–Myc9/tubulin–GAL4). Here, dSir2 mRNA and protein levels were robustly relative to wild type (an increase of 318% relative to wild-type protein levels; Supplementary Figs 5 and 6). We examined recombinant protein raised in Escherichia coli to check that the presence of the Myc tag did not interfere with dSir2 histone deacetylase activity, as measured by deacetylation of the fluorophore-containing p53 substrate (Fluor de Lys) or of native acetylated histone H4 substrates, and it did not (Supplementary Fig. 7). We also found that dSir2 histone deacetylase activity was unaffected by addition of resveratrol in either assay (Supplementary Fig. 7). We saw no increase in lifespan in UAS–dSir2–Myc9/tubulin–GAL4 flies relative to tubulin–GAL4/+ controls, either on a food medium similar to that used in the original study (Fig. 2b), or relative to either control on a range of other media (Supplementary Fig. 4b, c, f). An independent research team also saw no increase in lifespan in UAS–dSir2–Myc9/tubulin–GAL4 flies (Supplementary Fig. 8). These results indicate that the previously observed longevity of EP-UAS–dSir2/tubulin–GAL4 flies was not attributable to elevated expression of dSir2, and that stronger, ubiquitous overexpression of dSir2 also does not extend fly lifespan.

The role of sirtuins in the extension of lifespan by dietary restriction in yeast and C. elegans is controversial, with several groups reporting that sirtuins are not required for lifespan extension via dietary restriction in both organisms4. In Drosophila, it was reported that sirtuin restriction does not increase lifespan in dSir2 deletion-mutant flies2. We tested this too, using the deletion alleles dSir24,5 and dSir217. We first outcrossed these alleles (Supplementary Fig. 9a) into the Canton S wild type (see Methods), which was used in the previous dietary-restriction study4. We then checked the effect of each allele on dSir2 gene expression. The allele dSir217 abrogated dSir2 mRNA, indicating that this is a null allele. By contrast, dSir24,5, which contains a relatively small deletion at the 5′ end of the gene, did not reduce dSir2 mRNA levels (Supplementary Fig. 9b, c).

To reassess the role of dSir2 in dietary restriction in Drosophila, we compared lifespans of wild-type (Canton S), dSir24,5 and dSir217 homozygotes. All genotypes responded similarly and normally to dietary restriction in trials conducted by two independent research teams (Fig. 2c and Supplementary Fig. 10), hence the effect of dietary restriction on lifespan did not require dSir2.

In this study, we were unable to verify the effect of sirtuin overexpression 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 overexpression. Overexpression of sir-2.1 does exert effects on traits other than lifespan. For example, gln3 is neuroprotective in a worm model of neuron dysfunction in Huntington’s disease24 and, notably, this effect is not attributable to the dyf mutation (Supplementary Fig. 11). Moreover, both NL3909 and its outcrossed derivative are thermotolerant (M. Somogyva, unpublished data). In Drosophila, lines overexpressing dSir2 were longer-lived than wild-type controls, as previously reported, but they were not longer-lived than lines containing the appropriate transgenic controls. The fact 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 either by overexpression of sir-2.1 from its own promoter in C. elegans, or by ubiquitous overexpression of dSir2 from a heterologous promoter in Drosophila. Our findings call into question the robustness of earlier reports of a role for sirtuins in longevity assurance on the basis of overexpression in C. elegans and Drosophila, and also the role of dSir2 in the response to dietary restriction in Drosophila. However, sirtuins can affect lifespan in animals under certain conditions: C. elegans daf-2(e1370) mutants are hypersensitive to genetic effects on lifespan25, and in these mutants, deletion of sir-2.1 reproducibly increases lifespan (Supplementary Fig. 12).

Our finding that resveratrol does not activate the histone deacetylase activity of dSir2 using a native histone H4 peptide is consistent with earlier findings using yeast Sir2 and mammalian SirT1 (refs 17, 18). Resveratrol increased Drosophila lifespan in one study26 but not in another27. In principle, this could reflect sensitivity of resveratrol effects to subtle differences in culture conditions. If this were the case, our findings would indicate 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 agar at 20 °C, with E. coli OP50 bacteria as a food source. Nematode strains used included wild type (N2), GA707 wuEx166 [rol-6(su1006)] (rol-6 control), LG100 geln3 [sir-2.1 rol-6(su1006)] Dyf(wu250), NL3909 pkl1642 [sir-2.1 unc-119] unc-119(ed3) and the control strain NL3908 pkl1641 [unc-119] unc-119(ed3).

Nematode lifespan measurements. These were performed as previously described, 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 the effects of RNAi on lifespan, worms were kept for two generations on the RNAi bacteria. The statistical significance of effects on lifespan was estimated using the log-rank test, performed using JMP, Version 7 (SAS Institute).

Drosophila stocks and maintenance. Tubulin–GAL4 and dSir22,29 lines were obtained from the Bloomington Stock Center. The dSir2–Myc2 and dSir2–My9 lines were generated by germline transformation into strain w1118. The dSir212,29/M66b, dSir227/Cyo and Canton S lines were gifts from S. Fletcher, J. Rine and S. Helfand. All lines were outcrossed at least six times into the relevant controls. Experiments were performed at 25 °C on a 12 h:12 h light:dark cycle at constant humidity.

Drosophila lifespan assays. Flies were bred at standard density, allowed to mate for 48 h after emerging, then sorted into ten females per vial. Vials were changed every 48 h, and deaths per vial were scored until all flies were dead. In overexpression studies, n = 200. In dSir2-mutant studies, n = 100. For statistical methodology, see earlier.

dSir2 deacetylation assays. We used both the SirT1 Fluorimetric Drug Discovery Kit (Enzo Life Sciences) and an HPLC-based acetyl-histone-H4 deacetylation assay30. dSir2 and dSir2–Myc were cloned into pET SUMO (Invitrogen) and proteins were purified on HisPur cobalt spin columns (Thermo Scientific).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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**METHODS**

Nematode strains and maintenance. *Caenorhabditis elegans* were cultured under standard monoxenic conditions. Strains used included N2 (wild type), GA707 (N2 with unc-119(ed3)), HT1593 unc-119(ed3), LG100 gen3 [sir-2.1 rol-6 (su1006)], dyf-7(wu250), NL3908 pki1641 [unc-119] unc-119(ed3) and NL3909 pki1642 [sir-2.1 unc-119] unc-119(ed3).

Outcrossing of nematode strains. LG100 was outcrossed with N2 and the Rol trait was used to detect the presence of grn3. NL3908 and NL3909 were outcrossed using HT1593 unc-119(ed3). Rescue of Unc (uncoordinated movement) 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 were selected from individual F$_1$ animals with Dyf, non-Rol or non-Dyf, Rol phenotypes. The Dyf phenotype was identified by staining with the dye 1,1'-dicyclohexyl-3,3',5'-tetramethyldifluoroacetocyanine (Dil) and looking for absence of dye uptake into the amphid and phasmid neurons. Non-Dyf, Rol F$_2$ animals that were heterozygous for the gen3 transgene array (the rol-6 marker is dominant) were identified by the presence of non-Rol animals in the F$_3$ and were excluded.

**RNAi in C. elegans.** 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 Arrhinger RNAi library. Worms were maintained on RNAi feeding strains for two generations before lifespan measurements. One day before starting measurements, FUDR was applied to seeded plates at 10 μM to prevent progeny production.

Analysis of SIR-2.1 protein levels in *C. elegans.* Protein was prepared from expanded polyglutamines (polyQs), crossed GA919 (gen3 dissociated from dyf-7(wu250)) to strains carrying polyQ arrays. These polyQ strains co-express the first 57 amino acids of human huntingtin with either 119 or 128 Glu residues fused to cyan fluorescent protein and expressed from the mec-3 promoter, and YFP expressed from the mec-7 promoter in touch-receptor neurons. The response to touch at the tail was tested as previously described.

Three trials were performed and 150–200 animals were tested per genotype.

Lifespan analysis in *C. elegans.* Lifespans of synchronized population cohorts were measured as previously described. For all assays, 3–5 replicate worm cultures were used.

Neuroprotection assays in *C. elegans.* To test siruin-mediated protection from expanded polyglutamines (polyQs), we crossed GA919 (gen3 dissociated from dyf-7(wu250)) to strains carrying polyQ arrays. These polyQ strains co-express the first 57 amino acids of human huntingtin with either 119 or 128 Glu residues fused to cyan fluorescent protein and expressed from the mec-3 promoter, and YFP expressed from the mec-7 promoter in touch-receptor neurons. The response to touch at the tail was tested as previously described.

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