Reduced insulin signalling in adulthood protects soma and germline under mutation accumulation

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Abstract

Dominant theory maintains that organisms age due to resource allocation trade-offs between the immortal germline and the disposable soma. Strikingly, adulthood-only downregulation of insulin signalling, an evolutionarily conserved pathway regulating resource allocation between reproduction and soma, increases lifespan and offspring fitness without fecundity cost in the nematode, *Caenorhabditis elegans*. Nevertheless, theory suggests that reduced germline maintenance can be a hidden cost of lifespan extension. We ran a mutation accumulation (MA) experiment and downregulated insulin signalling in half of the 400 MA lines by silencing *daf-2* gene expression using RNA interference (RNAi) across 40 generations. Adulthood-only *daf-2* RNAi reduced extinction of MA lines both under UV-induced and spontaneous mutation accumulation. Fitness of the surviving UV-induced MA lines was higher under *daf-2* RNAi. Our results suggest that reduced insulin signalling protects the soma and the germline and imply that suboptimal gene expression in adulthood is a major driver of organismal ageing.

Main

Ageing, the physiological deterioration of an organism leading to increased probability of death and decreased reproduction with advancing adult age, is taxonomically ubiquitous but remains incompletely understood[1-4]. While there is broad agreement that ageing evolves because natural selection gradients on traits decline after reproductive maturity[5-9], the proximate causes of late-life deterioration are less clear[1,2,10,11]. The “disposable soma” theory of ageing suggests that ageing evolves as a result of competitive resource allocation between the germline and the soma[12-14]. It follows that increased investment in somatic maintenance leading to longer lifespan trades-off with traits associated with reproduction, such as the number or size of progeny[15,16]. Despite considerable support for trade-offs between somatic maintenance and reproduction, growing empirical work questions the universality of such resource allocation trade-offs in the evolution of ageing[2,11,17,18]. Several studies have shown that experimentally increased lifespan, often via the downregulation of genes in nutrient-sensing signalling pathways in adulthood, is not detrimental to reproduction measured as offspring number or offspring fitness[2,18,19,20,21]. These results are in line with the hypothesis that a gradual decline in selection gradients with advancing age after reproductive maturity results in suboptimal gene expression in adulthood contributing to somatic deterioration[2,22-27].

However, it has been proposed that reduced germline maintenance can be a hidden cost of increased lifespan[18]. Germline maintenance, the repair and surveillance of genomic and proteomic integrity in germline stem cells and gametes, is energetically expensive[18,28] and germline signalling plays a key role in resource allocation to somatic maintenance[18,28,29,30]. Germline ablation results in increased somatic maintenance and lifespan in *Drosophila melanogaster* fruitflies[31] and

Caenorhabditis elegans nematodes[32-36], although lifespan extension in *C. elegans* requires an intact somatic gonad[32]. It is important to note that the requirement of the intact somatic gonad for lifespan extension in germline-less worms does not negate the possibility of a resource allocation trade-off as sometimes implied, but only shows that germline signalling is required to mediate the effect[18,31,33]. Similarly, recent work in *Danio rerio* zebrafish suggests that germline ablation increases somatic maintenance under stress[37]. Furthermore, nutritional stress in *C. elegans* results in germline reduction and increased lifespan[38]. When soma-to-germline communication is disrupted, the number of germ cells is unaffected and lifespan extension is abolished[38]. Taken together, these results suggest that germline maintenance is costly and can trade-off with somatic maintenance and lifespan[18,28]. Therefore, the "expensive germline" hypothesis predicts that increased investment into somatic maintenance reduces resources available for germline maintenance, leading to an increased germline mutation rate and reduced offspring quality[18].

We tested this prediction by manipulating insulin/IGF-1 signalling (IIS), an evolutionarily conserved pathway that regulates the physiological response of organisms to their environment[3]. IIS mechanistically links nutrient intake with development, growth, reproduction and lifespan across diverse taxa[3,30,39]. Reduced IIS, via genetic and environmental interventions, consistently extends lifespan[30]. Here, we combined a mutation accumulation (MA) approach[40-43] with reduced IIS in adulthood via *daf-2* RNA interference in *C. elegans* to test the "expensive germline" hypothesis, by assessing extinction rate, life history traits and fitness of MA lines under spontaneous and induced mutation accumulation.

Results and Discussion

Parental *daf-2* RNAi in adulthood extends lifespan and improves offspring fitness under UV-induced stress. Adulthood-only *daf-2* RNAi in N2 wildtype *C. elegans* nematodes, significantly extended parental lifespan relative to empty vector (e.v.) controls under benign conditions (no irradiation) and under ultraviolet-C ("UV") irradiation-induced stress (Cox proportional hazards mixed effects model, coxme, with matricides censored, RNAi: z=10.530, df=2, p<0.001; UV: z=0.070, df=3, p=0.940; RNAi x UV: z= 1.500, df=4, p=0.130; matricides classed as dead: RNAi: z=10.400, df=2, p<0.001; UV: z=0.250, df=3, p=0.800; RNAi x UV: z= 1.310, df=4, p=0.190; Fig. 1a).

There was no cost to parental reproduction, neither under benign conditions nor when parents were UV-irradiated (Fig. S1). Reproduction was estimated as age-specific offspring production (fecundity, Generalised Poisson, RNAi x UV x Age: z = -2.546, p=0.0109), as individual fitness (lambda, generalised linear model, GLM, RNAi: t=-0.065, df=1, p=0.948; UV: t=-3.328, df=1, p=0.0104; RNAi x UV: t= 0.159, df=1, p=0.874) and as total lifetime reproduction (LRS) (GLM, RNAi: t=-1.887, df=1, p=0.0606; UV: t=-3.665, df=1, p<0.001; RNAi x UV: t= 0.066, df=1, p=0.948).

However, *daf-2* RNAi significantly increased offspring fitness both under benign conditions and when parents were UV-irradiated (lambda, GLM: RNAi: t=-2.305, df=1, p=0.0222; UV: t=-1.625, df=1, p=0.1059; RNAi x UV: t=- 0.772, df=1, p=0.441; Fig. 1b). Offspring of UV-irradiated parents did not have significantly lower fitness, even though irradiation reduced fitness and total reproduction of their parents, suggesting effects on offspring may have been buffered to some extent, perhaps via germline repair mechanisms[18,37,44].

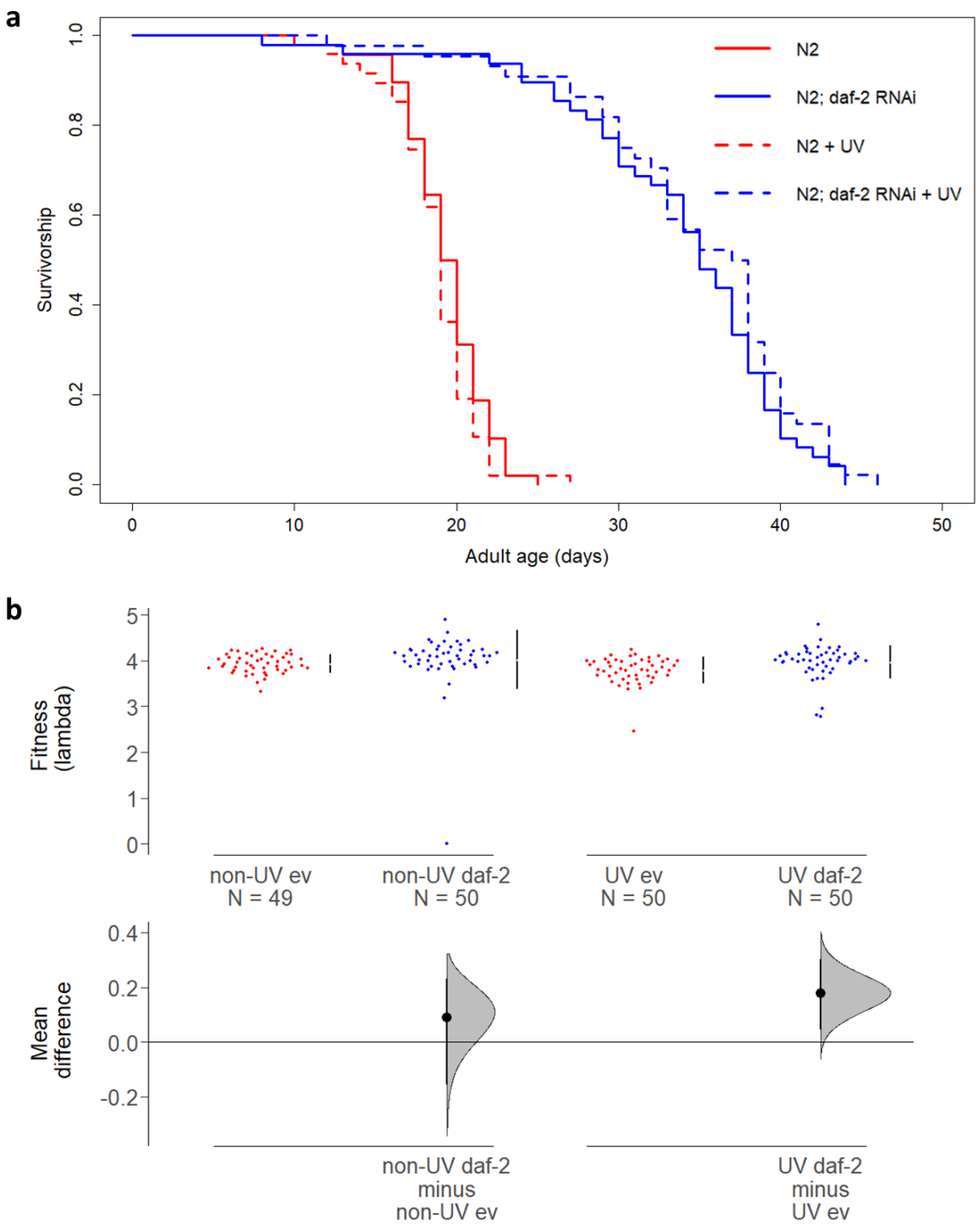

Fig. 1| Adulthood *daf-2* RNAi in UV-irradiated and non-irradiated parents increases parental lifespan and offspring fitness. a, Parental survival, n=50 per treatment. **b,** Offspring fitness. UV irradiation status and adulthood RNAi treatment (either *daf-2* RNAi, 'daf-2', or empty vector control, 'ev') of parents is indicated. Offspring were maintained on empty vector and were not irradiated. Mean (effect

size) and 95% confidence intervals shown were derived using non-parametric bootstrap resampling in 'dabestr' R package[90].

The impact of parental *daf-2* RNAi and UV treatment on offspring age-specific reproduction varied with offspring age as did the number of offspring with zero fecundity (zero-inflated generalised Poisson, ZIGP, RNAi x age: $z=4.864$, $p<0.001$; UV x age: $z=3.678$, $p<0.001$; RNAi x UV: $z=-0.175$, $p=0.861$; ZI varied with age: $z=-0.227$, $p=0.00656$ and age^2: $z=3.301$, $p<0.001$; Fig. S2). There was no effect of parental treatment (neither *daf-2* RNAi, UV irradiation nor their interaction) on offspring total lifetime reproduction (GLM, RNAi: $t=-1.141$, $df=1$, $p=0.255$; UV: $t=-1.626$, $df=1$, $p=0.106$; RNAi x UV: $t= -0.053$, $df=1$, $p=0.958$; Fig. S2).

Our findings show that under adulthood-only *daf-2* RNAi, parents increase investment into somatic maintenance resulting in increased lifespan with no cost to themselves or their offspring under benign conditions validating previous work in *C. elegans* [19,20]. Importantly, we reveal that the absence of a longevity-fecundity trade-off in parents persisted under stressful conditions, when organisms have to invest into repairing UV-induced damage.

Furthermore, we show that *daf-2* knockdown in adult parents primarily influenced the timing of reproduction in their offspring. Specifically, it caused a shift to increased early life reproduction, improving offspring individual fitness, rather than an increase in total reproduction. Whilst an earlier study found increased total reproduction in the first generation of offspring from *C. elegans* parents treated with *daf-2* RNAi, across N2 wild-type and two other genetic backgrounds, this was also accompanied by increased offspring fitness[20], in agreement with our results here.

Under UV-induced mutagenesis, the costs of germline maintenance would be expected to become more apparent[28,37,45]. However we show that adulthood-only *daf-2* RNAi improves fitness of F1 offspring even when parents are under stress.

Multigenerational *daf-2* RNAi in adulthood protects against extinction. To test the effects of *daf-2* RNAi in adulthood for germline maintenance, we conducted a mutation accumulation (MA) experiment for 40 generations in a total of 400 N2 wild-type *C. elegans* MA lines. In 200 of the MA lines, we reduced IIS via *daf-2* RNAi in adult worms in every generation, whereas the other 200 lines served as controls. Mutations were either allowed to accumulate spontaneously or were induced each generation via UV-C irradiation (46 J/m^2) (i.e. n=100 MA lines per treatment). We aimed to determine whether an increased investment in somatic maintenance under *daf-2* RNAi would trade off with reduced germline maintenance leading to fitness costs and, consequently, faster MA line extinction.

We found that *daf-2* RNAi reduced extinction under both UV-induced and spontaneous MA across 40 generations (Cox proportional hazards regression analysis, coxph, RNAi: z=2.159, df=2, p=0.031; UV: z=11.469, df=2, p<0.001; RNAi x UV: z= -0.537, df=4, p=0.591; Fig. 2a). Extinction results did not differ significantly between two independent experimental blocks (coxph, z=-0.942, df=3, p=0.346; Fig. S3). The major causes of MA line extinction (Fig. 2b) were infertility (failure to lay eggs), or sterility (the production of eggs that did not hatch), indicative of the underlying germline damage. Infertility and sterility were likely linked with observed reproductive abnormalities such as a deformed vulva, abnormal external growth close to the vulva (possible tumour) and cavities in the reproductive tract in place of

embryos or oocytes (Fig. 2c). It is known that vulva-less *C. elegans* mutants are unable to lay eggs and can die from internal hatching[46,47].

The reduction in extinction observed in our MA lines with *daf-2* RNAi shows the benefits of increased investment into germline maintenance that became more pronounced across multiple generations of UV-induced and spontaneous MA.

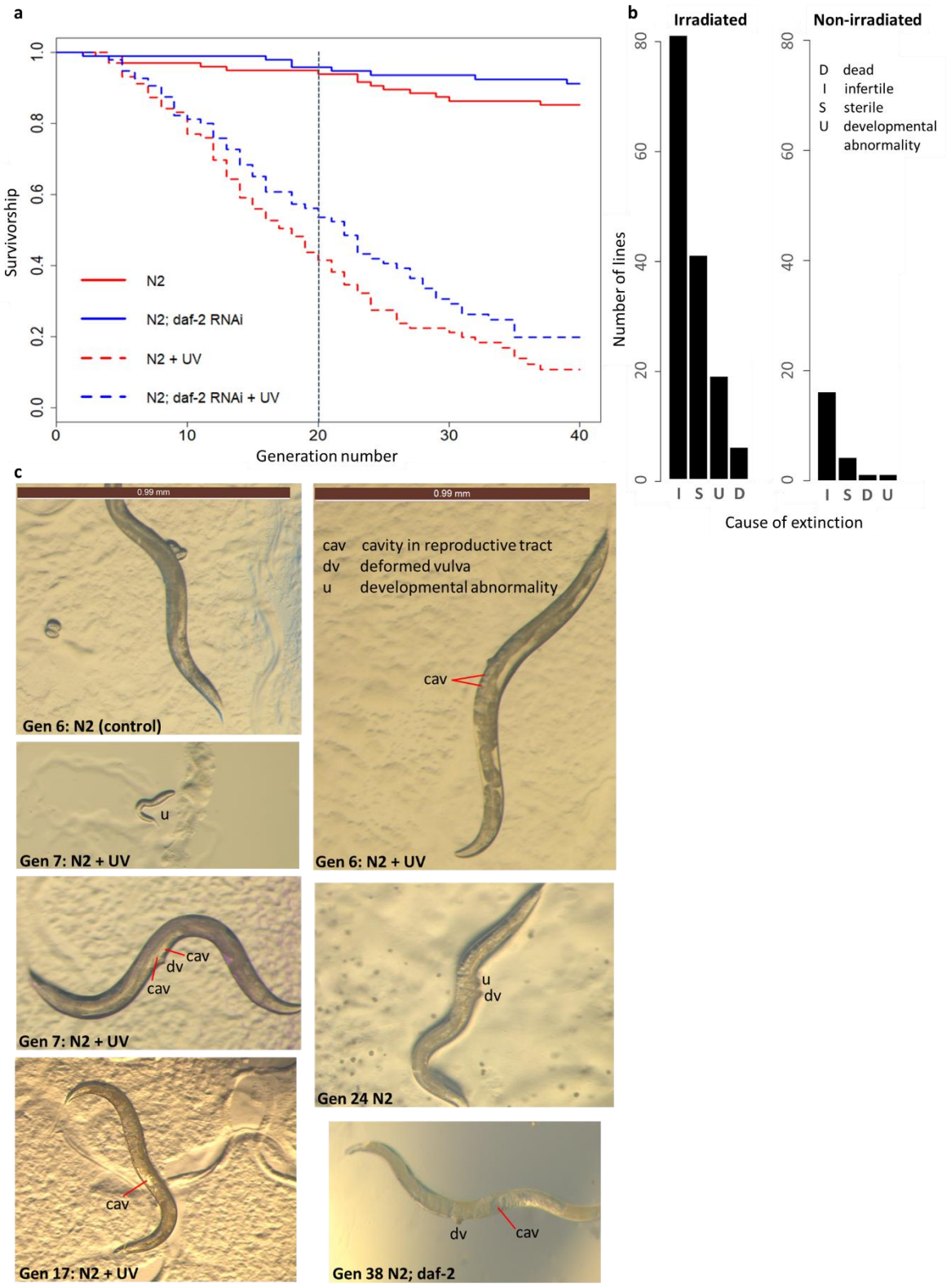

Fig. 2| Reduced adulthood IIS, via *daf-2* RNAi, protects against N2 wild-type extinction under mutation accumulation. a, Transgenerational survival in N2 under spontaneous and UV-induced mutation accumulation. Vertical dotted line at

generation 20 indicates timing of life history assay. Sample size of 100 lines per RNAi strain by irradiation treatment combination. **b,** Causes of extinction of N2 wild-type MA lines indicate germline damage. **c,** Representative images of germline damage. Brown scale bar of 0.99 mm for all images.

Multigenerational *daf-2* RNAi increased fitness of surviving MA lines. We next tested for the effects of *daf-2* RNAi on the life history traits of surviving MA lines. We were interested in how IIS influences potentially detrimental effects of MA that are not sufficiently severe to cause line extinction. We assayed age-specific reproduction, egg size, male production and adult heat shock resistance in grand-offspring from the spontaneous and UV-induced MA lines on *daf-2* RNAi versus control treatments, at generation 20 of MA, following two generations of rearing under common garden conditions (no irradiation, on empty vector control) to attenuate direct effects of irradiation and RNAi. Generation 20 was a point at which there was a pronounced benefit of *daf-2* RNAi for protection against extinction in the UV-induced MA lines, but no clear difference in extinction trajectories for the spontaneous (non-irradiated) MA lines (Fig. 2a).

Adulthood-only *daf-2* RNAi across 20 generations of mutation accumulation significantly increased individual fitness in the surviving irradiated MA lines, but there was no effect of *daf-2* RNAi on the fitness of non-irradiated lines (GLM, irradiated, RNAi: t=-2.804, p=0.006; non-irradiated, RNAi: t=0.647, p=0.518; all data, RNAi x UV: t=-2.886, p=0.004; Fig. 3a), in agreement with the results on line extinction at generation 20 (Fig. 2a).

The increased fitness of irradiated *daf-2* RNAi MA lines was driven by their improved early life fecundity (Day 1 and Day 2 offspring production), relative to irradiated controls (ZIGP, RNAi x age: z=-1.981, p=0.0475; RNAi x age^2: z=3.334, p<0.001; Fig. 3b), an effect absent in the non-irradiated lines (ZIGP, non-UV, RNAi: z=0.05, p=0.963; all data: RNAi x UV x age: z=-2.50, p=0.0125; RNAi x UV x age^2: z=3.58, p<0.001; ZI intercept: z=-18.27, p<0.001). There was no effect on total reproduction (GLM, UV: t=-0.811, p=0.419; non-UV: t=0.875, p=0.383; UV x RNAi: t=-1.138, p=0.256; Fig. S4).

We suggest that the fitness benefits of *daf*-2 RNAi for irradiated MA lines were most likely due to genetic differences between the treatments, as the benefits persisted after two generations of common garden rearing and therefore were not due to the direct exposure of offspring to RNAi (neither as adults nor as eggs). This conclusion is reinforced by the finding that parental *daf*-2 RNAi effects on offspring fitness do not persist beyond F1 (see below, Fig. 5). Such genetic differences have likely arisen from the lower rate of MA in *daf-2* RNAi lines leading to lower fitness costs. Variation in fitness was considerably greater across irradiated than across non-irradiated MA lines (Fig. 3a), suggesting that irradiation-induced *de novo* mutations generated greater genetic and phenotypic variation compared to spontaneous MA. The spontaneous mutation rate in N2 wild type *C. elegans* is estimated as one *de novo* mutation per individual per generation, under standard conditions[48,49]. The close association between extinction trajectories during the first 20 generations of MA (Fig. 2a) and the fitness of the surviving MA lines (Fig. 3a) suggests that a threshold of accumulating deleterious mutations needs to be crossed before the effect size is sufficiently severe to result in extinction.

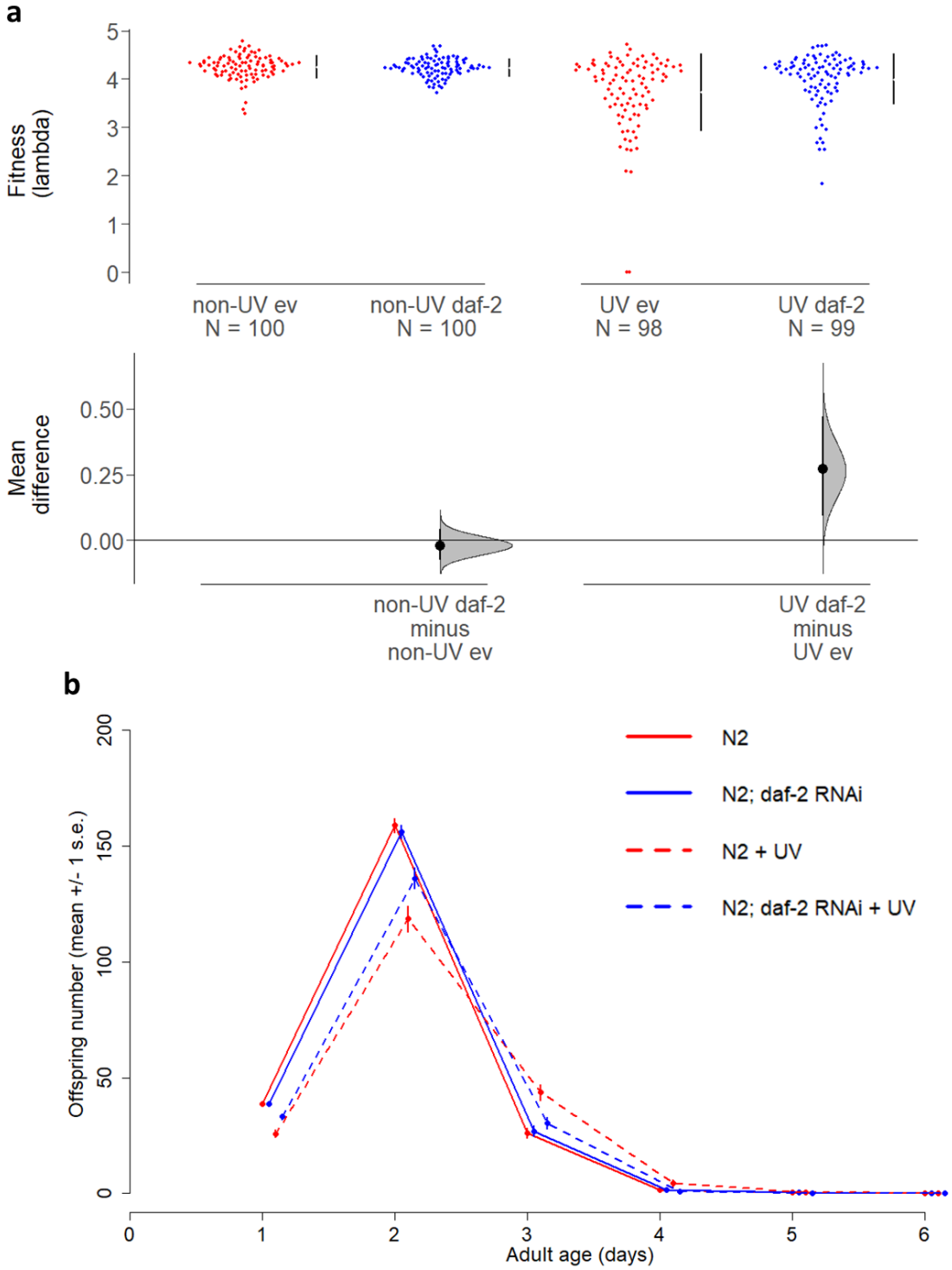

Fig. 3| The effect of *daf-2* RNAi on fitness and age-specific reproduction after 20 generations of MA. a, Individual fitness (lambda) of the grand-offspring of N2 wild-type MA lines at generation 20. Fitness was assayed in a standard common garden environment, on the empty vector control and no irradiation; following two

generations of rearing under standard conditions, from MA generation 20. Mean and 95% confidence intervals shown. **b,** Age-specific reproduction in the grand-offspring of N2 MA lines at generation 20.

To determine if increased offspring fitness in the surviving *daf-2* RNAi-treated MA lines, was associated with greater parental resource allocation into their eggs, we measured egg size, as a proxy for parental investment (n=75 eggs measured, one per individual taken from the Generation 20 fitness assay). Previous work has shown that reduced IIS, either via dietary restriction or via *daf-2* RNAi, increases mean egg size[20,50].

We found that grandparental *daf-2* RNAi resulted in grand-offspring (F2) that laid smaller eggs if their grand-parents from the MA lines had been irradiated, but there was no significant effect on the size of eggs laid by F2 offspring descended from non-irradiated grandparents treated with *daf-2* RNAi following 20 generations of MA (GLM, UV lines, RNAi: t=2.362, df=1, p= 0.0195; non-UV lines, RNAi: t=-1.246, df=1, p=0.215; all data, RNAi x UV: t=2.642, df=1, p=0.00869; RNAi x UV x Block: t=-0.774, df=1, p=0.440; Fig. 4). This is contrary to the increase in F1 egg size under reduced parental IIS found in previous work under benign conditions[20,50].

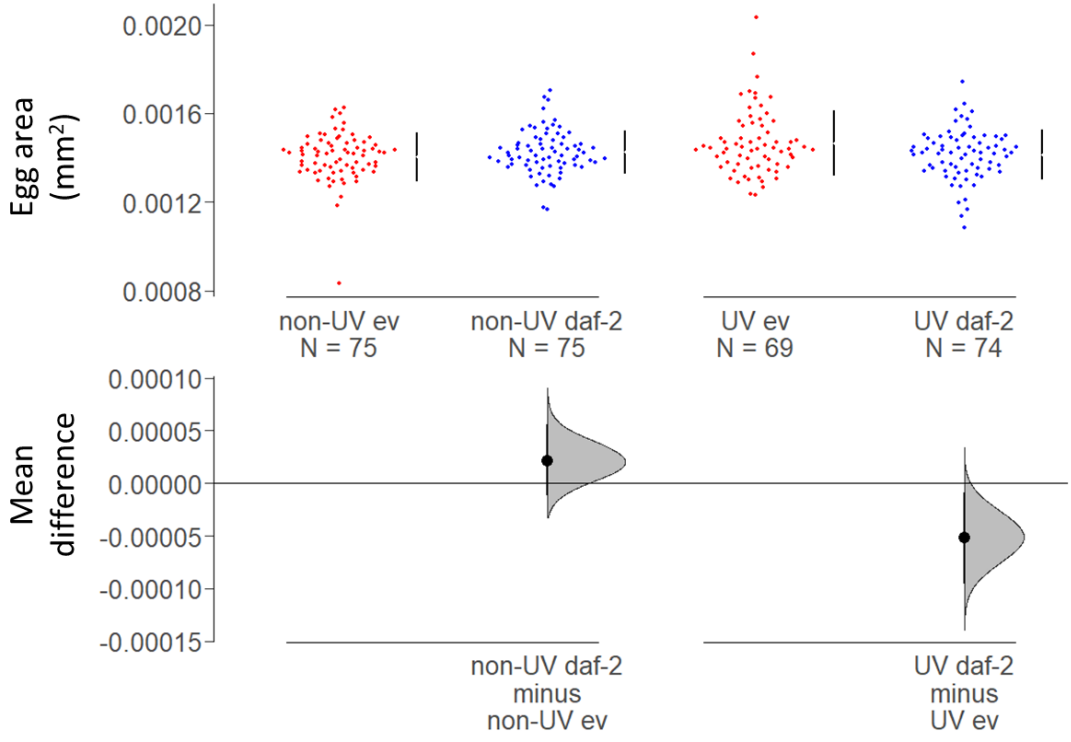

Fig. 4| The effect of *daf-2* RNAi on egg size after 20 generations of spontaneous or UV-induced MA. Egg size (area, mm^2) was reduced in the grand-offspring of UV-induced MA lines on *daf-2* RNAi ('daf-2') relative to those from UV-irradiated MA lines on empty vector control ('ev'). However, this difference in egg size was absent from the spontaneous ('non-UV') MA lines, following 20 generations of MA. Grand-offspring were all non-irradiated and kept on ev. Mean and 95% confidence intervals are shown.

Our results indicate that even though F2 offspring from irradiated *daf-2* RNAi MA lines laid smaller eggs, this did not seem to impact negatively on the quality (fitness) of offspring they produced. It is possible that a smaller egg size could be a phenotypically plastic response to UV radiation, perhaps to improve stress resistance to irradiation. Alternatively, it could be the result of a trade-off between

improved investment into germline genetic quality and egg size. At present, it is unclear why grandparental *daf*-2 RNAi resulted in smaller eggs in F2.

Exposure to certain environmental stresses (e.g. high temperature[51], starvation[52], increased mutation rate[53]), increases male production and outcrossing in *C. elegans*. However, we found no increase in the proportion of males produced by the grand-offspring in irradiated MA lines. Only one male developed from the 293 eggs assayed.

Reduced IIS increases the activity of heat shock factor 1 which mediates lifespan extension[54,55]. Heat shock responses are conserved across diverse taxa and positively associated with lifespan (as reviewed by[56,57]). To test the stress resistance of post-reproductive (Day 7) adults, we assayed survival and locomotion under acute heat shock for 1 hour and 45 minutes at 37°C (following[58]). We found no effect of MA line treatment (neither UV irradiation nor *daf-2* RNAi) on the survival of untreated F2 offspring following heat shock. Only 4% of the 371 individuals were dead by 24 hours after heat shock. However F2 offspring from non-irradiated *daf-2* RNAi MA lines recovered normal locomotion faster following heat shock than F2 from non-irradiated MA control lines, but this benefit was not seen in descendants from irradiated MA lines (Binomial GLM, 3h post-heat shock, RNAi: z=-2.274, df=1, p=0.0229; UV: z=-2.078, df=1, p=0.0377; RNAi x UV: z=2.304, df=1, p= 0.0212; 24h post-heat shock, UV: z=-0.206, df=1, p=0.837; RNAi: z=-0.045, df=1, p=0.964; RNAi x UV: z=1.347, df=1, p=0.178; Fig. S5). This is in line with previous work, which found survival after heat shock to more than double with age, from the first to the fourth day of adulthood in *C. elegans*[58].

Parental *daf-2* RNAi does not affect offspring fitness transgenerationally.

Transgenerational epigenetic inheritance of RNAi can occur in *C. elegans* via transmission of small interfering RNAs[59,60]. This transgenerational inheritance of RNAi can last for several generations[60-62] and requires the germline argonaut protein, HRDE-1, that is absent in *hrde-1* (heritable RNAi defective- 1) mutants[63]. To determine whether there was direct transgenerational transfer of *daf-2* RNAi via the germline and thus for how many generations the effects of parental *daf-2* RNAi could persist, we assayed the fitness effects of three generations of offspring (F1, F2 and F3) from *daf-2* RNAi treated versus control parents. Two genetic backgrounds were used: *C. elegans* N2 wild-types, and *hrde-1* mutants that did not transfer RNAi transgenerationally.

Parental effects of *daf-2* RNAi on the fitness of descendants, were absent after the first generation of offspring in the N2 wild-types and absent across all generations of offspring in the *hrde-1* mutants (GLM, F1, RNAi x genotype: t=-2.160, df=1, p=0.0329; F2, RNAi x genotype: t=0.372, df=1, p=0.711; RNAi: t=1.090, df=1, p=0.278; genotype: t=7.044, df=1, p<0.001; F3, RNAi x genotype: t=- 0.973, df=1, p=0.332; RNAi: t=0.359, df=1, p=0.721; genotype: t=5.302, df=1, p<0.001; all data: RNAi x generation: F=4.450, df=2, p=0.0124; Fig. 5). Furthermore, there was no effect of *daf-2* RNAi on age-specific reproduction after the first generation of offspring, for either N2 or *hrde-1* backgrounds (Fig. S6; Table S1). There was also no *daf-2* RNAi effect on total reproduction for F2 and F3 offspring generations (Fig. S7; Table S1).

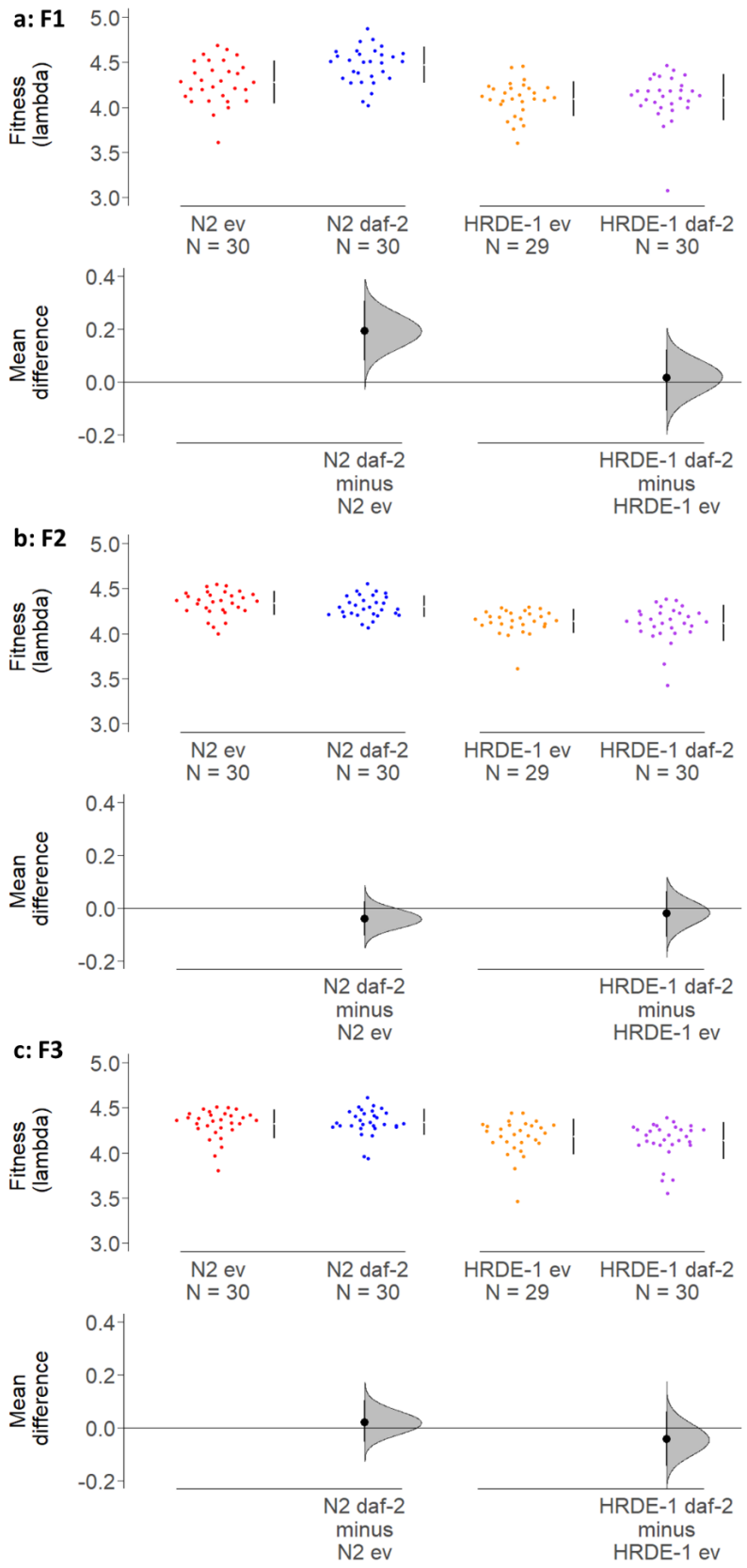

Fig. 5| The effects of *daf-2* RNAi on offspring fitness do not persist beyond the first offspring generation. Fitness of the: **a,** first (F1); **b,** second (F2) and **c,** third

(F3) generation of offspring from parents treated with *daf-2* RNAi ('daf-2') or an empty vector control ('ev'), in N2 wild-type and RNAi inheritance deficient *hrde-1* mutant backgrounds. All offspring generations were untreated (kept on ev).

The absence of fitness benefits in the second and third generations of offspring from *daf*-2 RNAi parents strongly suggests that the life-history differences between the *daf-2* RNAi and control irradiated MA lines at generation 20 in the common garden experiment were due to genetic differences and not the direct effects of RNAi. These results also imply a lack of transgenerational inheritance of *daf-2* RNAi beyond the first generation of offspring. Interestingly, the heritable RNAi deficiency 1 (*hrde-1*) gene was required for the fitness benefits of parental *daf-2* RNAi in irradiated offspring, as these offspring fitness benefits were absent in the *hrde-1* mutant background. The *hrde-1* mutants had overall lower fitness and total reproduction than N2 wild-type.

***hrde-1* is required for *daf-2* RNAi to confer germline protection under MA.** *hrde-1* encodes an Argonaut protein that plays a key role in nuclear RNAi, RNAi inheritance and promoting germline immortality[64]. To determine if functional *hrde-1* was necessary for the protective effects of *daf-2* RNAi under spontaneous and UV-induced MA, we ran 400 MA lines in parallel to the N2 MA experiment, using the *C. elegans hrde-1* mutant background and reduced IIS, via *daf-2* RNAi, in half of the MA lines. The heritable RNAi deficiency (*hrde-1* mutant) resulted in the rapid extinction of irradiated MA lines and the loss of the protective effects of *daf-2* RNAi under UV-induced MA, across 25 generations (RNAi: $z=-4.016$, $df=2$, $p<0.001$; UV: $z=12.370$, $df=2$, $p<0.001$; RNAi x UV: $z=-1.758$, $df=3$, $p=0.079$; Fig. 6). In fact,

irradiated *daf-2* RNAi lines went extinct faster than controls, in the *hrde-1* mutant background. The major cause of extinction in irradiated *hrde-1* mutant MA lines was developmental arrest, followed by infertility (Fig. S8). This strongly suggests that functional RNAi is required for *daf-2* RNAi protection against extinction, and for normal development and reproduction under UV-induced MA.

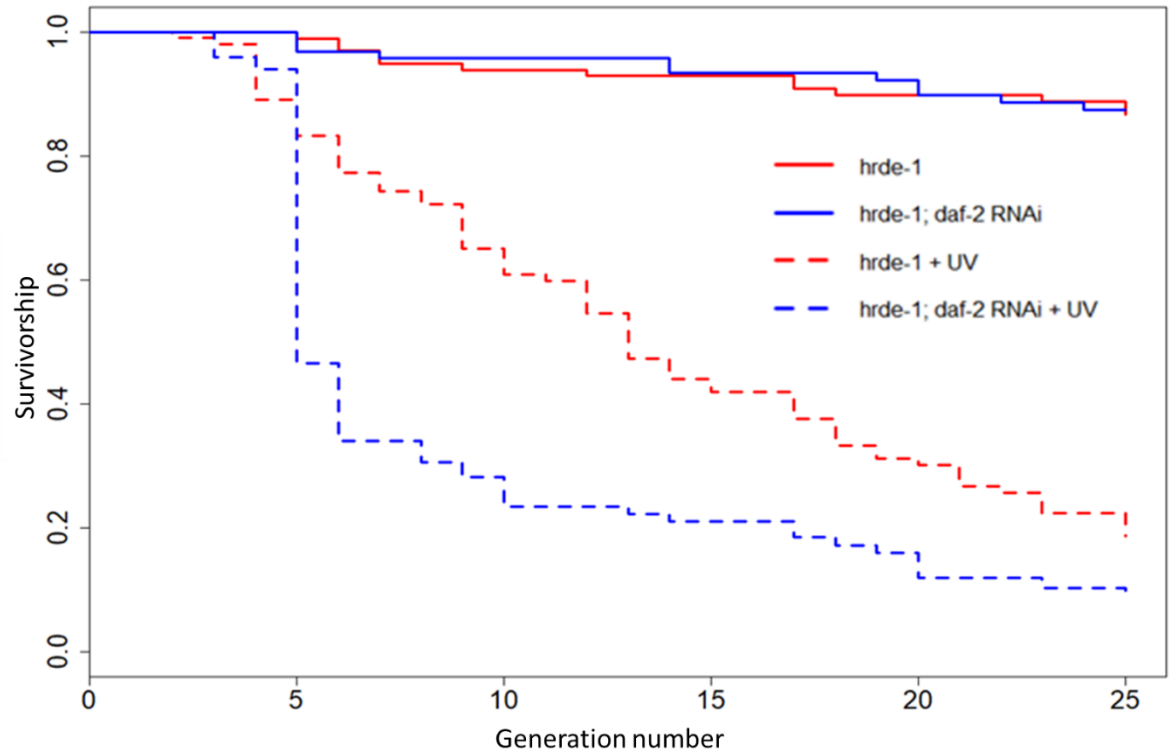

Fig. 6| The effect of *daf-2* RNAi on *hrde-1* mutant multigenerational survival under mutation accumulation. Sample size of 100 MA lines for each RNAi by UV irradiation treatment combination.

We show the important role of *hrde-1* in the *daf-2* RNAi-mediated protection of irradiated offspring under mutation. Our results suggest that the interaction between UV-induced germline damage and a deficiency in transgenerational inheritance of RNAi reverses the protective benefits of *daf-2* RNAi. This supports previous work suggesting that *hrde-1* mutants suffer from progressive sterility under high

temperatures implying their increased sensitivity to environmental stresses driven in part by defects in gametogenesis[63].

Conclusion

We found that reduced insulin signalling in adulthood, via *daf-2* RNAi, protects against extinction under both UV-induced and spontaneous mutation accumulation in *C. elegans*. Furthermore, the fitness of the surviving UV-irradiated MA lines was higher under *daf-2* RNAi. Most extinctions occurred because of infertility, egg hatching failure and developmental abnormalities suggesting that mutation accumulation directly contributed to the observed differences between the RNAi treatments. Germline protection under *daf-2* RNAi requires nuclear argonaut *hrde-1* because fitness of *hrde-1*; *daf-2* RNAi worms was reduced both in one-generation and in multi-generation experiments. This is in line with previous work suggesting that *hrde-1* is required for transgenerational inheritance and germline immortality[63,64]. We set out to test whether adulthood-only *daf-2* RNAi, known to extend lifespan without an obvious cost to parental fecundity[19,20], trades-off with germline maintenance, resulting in the accumulation of germline mutations and detrimental fitness effects across multiple generations. Positive effects on F1 offspring fitness observed in previous work[20] are likely driven by parental effects because we showed here that they disappear after one generation. However, we found that multigenerational downregulation of *daf-2* via adulthood-only RNAi has positive effects on germline maintenance and protects mutation accumulation lines from extinction. This was particularly so when germline mutation rate was increased by

low-level UV radiation. Our results therefore suggest that wild-type level of *daf*-2 expression is sub-optimally high in adulthood.

Antagonistic pleiotropy theory of ageing (AP) maintains that genes that increase fitness in early life at the expense of fitness late in life can be overall beneficial for fitness and go to fixation[6]. In line with AP, downregulation of *daf-2* expression during development reduces fitness[19], but what is the physiological basis of the putative trade-off? Our findings that adulthood-only downregulation of *daf-2* expression protects both the germline and the soma under benign conditions and under UV-induced stress, argue against the idea that resource allocation underlies this trade-off. However, the results are in line with the hypothesis that selection optimises gene expression for development and early-life reproduction but fails to optimise gene expression later in life[2,10,22-24]. This could be either because selection on insulin signalling in adulthood is too weak in *C. elegans*[65] or because of constraints that prevent the evolution of age-specific gene expression[66,67]. Future studies should focus on investigating the relative importance of trade-offs and constraints in the evolution of ageing across taxa. Our findings support the idea that insulin signalling is directly linked with the repair and maintenance of the germline and the soma, and highlight reduced insulin signalling as an important target for potentially cost-free extension of healthy lifespan.

Methods

We conducted four experiments to our test our main hypotheses about the effects of reduced insulin/IGF-1 signalling (IIS) in adulthood, via adult-only *daf-2* RNAi, on the soma and the germline:

1. Inter-generational effects of reduced IIS in parents under UV-induced stress, on parental and offspring fitness and reproduction.

2. Effects of reduced IIS on 40 generations of spontaneous and UV-induced mutation accumulation, in N2 wild-type and RNAi inheritance deficient (*hrde-1*) mutant backgrounds.

3. Life history and fitness effects of 20 generations of spontaneous and UV-induced mutation accumulation, on the surviving MA lines.

4. Transgenerational effects of *daf-2* RNAi on offspring fitness in N2 and *hrde-1* mutant backgrounds.

Nematode stocks and culture. The nematode (roundworm) *Caenorhabditis elegans* N2 wild-type (Bristol) and heritable RNAi deficiency 1 (*hrde-1*) mutant strains were defrosted from stocks acquired from Caenorhabditis Genetics Center (University of Minnesota, USA, funded by NIH Office of Research Infrastructure Programs, P40 OD010440) and from the lab of Prof. Eric Miska (Gurdon Institute, University of Cambridge, UK), respectively, and stored at -80°C until use. All experimental lines were kept at 20°C, 60% relative humidity and in darkness, consistent with standard *C. elegans* rearing protocol[68]. *C. elegans* is a valuable model system due to its short life cycle, ease of genetic manipulation and its normal reproductive state as self-fertilising hermaphrodites. Males were excluded from our experiments and occur at a very low frequency of approximately 0.3% under benign lab conditions and in nature[69,70].

Defrosted *C. elegans* strains were reared through two generations prior to set-up, on NGM agar supplemented with the fungicide nystatin and antibiotics streptomycin and ampicillin, to prevent infection (each at 100 µg/ml, as standard recipes, e.g.[71]) and

seeded with the antibiotic-resistant *Escherichia coli* bacterial strain OP50-1 (pUC4K, from J. Ewbank at the Centre d'Immunologie de Marseille-Luminy, France). We bleached eggs from the grandparents of experimental individuals, to standardise parental age and remove any infection or temperature effects from defrosting, prior to experiments.

Reducing IIS via *daf-2* RNAi feeding in adulthood. To downregulate adulthood expression of the insulin-like sensing signalling receptor homolog, *daf-2*, we fed late-L4 larvae with *Escherichia coli* bacteria expressing *daf-2* double-stranded RNA (dsRNA), that decreases mRNA levels of the complementary transcribed *daf-2* systemically[59,72]. The *daf-2* gene is upstream of and inhibits the action of master regulator daf-16/FOXO. RNase-III deficient, IPTG-inducible HT115 *E. coli* bacteria with an empty plasmid vector (L4440) was used as the control (as[19,20,72]). As RNAi treatment was started from the late-L4 stage, immediately prior to the onset of adult sexual maturity, all individuals developed on empty vector (e.v.) control *E. coli* prior to this stage. Normal development, which requires functional *daf-2*, was therefore unaffected[19,30]. RNAi clones were acquired from the Vidal feeding library (Source BioScience, created by M. Vidal lab, Harvard Medical School, USA) and tested for efficacy, prior to delivery.

During all experiments, worms were kept on 35mm NGM agar plates (supplemented with 1mM IPTG and 50µg/mL of antibiotic ampicillin, to inhibit the growth of bacteria other than our antibiotic resistant *E. coli*) and seeded with 0.1mL of the e.v. control or *daf-2* RNAi bacteria, 24 to 48 hours before use, for *ad libitum* bacterial growth. Bacterial cultures were prepared prior to the experiments, by growing in LB supplemented with 50µg/mL ampicillin (as[73]).

Ultraviolet wavelength C (UV-C) irradiation. To induce mutations we UV-irradiated Day 2 adults, with a calibrated ultraviolet-C (UV-C) radiation dose of 46J/m^2 (wavelength 254nm), via 20 second exposure to the UV-C radiation emitted from the lamp of a Thermo Scientific Heraguard ECO Safety Cabinet (calibration details in Supplementary Methods). This dose is in the range of previous UV-C irradiation doses for *C. elegans* adults or eggs[74-77]. Our pilot work showed that this dose reduced the fecundity of Day 2 adults laying at 20°C by 61% compared with un-exposed sham controls (data not shown). Non-irradiated control worms received a sham-irradiation, by being positioned in identical orientation under the UV-C lamp for 20 seconds, while it was switched off.

UV irradiation was timed at exactly 24 hours (+/- 30 minutes) after the RNAi treatment began at the onset of adulthood and prior to peak reproduction, to allow time for reduced IIS in individuals on *daf-2* RNAi[72,78]. This timing allowed us to induce germline mutagenesis, as adult somatic tissue in *C. elegans* is post-mitotic and very resistant to irradiation, whereas germline tissue (eggs, developing oocytes and germline stem cells) is still actively dividing (undergoing meiosis and mitosis) and so is more sensitive to UV irradiation[35]. As spermatogenesis is completed during the late-L4 stage[79], it would not have been directly affected by irradiation.

Nematodes were transferred to new seeding immediately after UV irradiation, in case the seeding or plate was affected by UV irradiation, and worms were placed just outside the seeding on the new plate, to minimise contamination with mutated bacteria (as[80,81]). UV-irradiated worms were allowed 8 hours (+/- 30 minutes) recovery, to provide sufficient time for expulsion of irradiated embryos, before starting egg laying[76,82]. Eggs laid were therefore most likely irradiated either as oocytes, or as germline stem cells[79,82].

Inter-generational effects of UV irradiation and reduced IIS in parents on parental and offspring fitness and reproduction. Wild-type N2 parents were either UV-irradiated or not as Day 2 adults and maintained on *daf-2* RNAi or an empty vector control for the whole of adulthood, in fully factorial design. Offspring were all non-irradiated and maintained on empty vector throughout life. We assayed the daily offspring production from unmated, singly-held hermaphrodite parents, for their entire reproductive period (first six days of adulthood) and also from the first generation of their offspring, by daily transfers to fresh plates. To assess parental survival, daily mortality checks were made, with death being defined as no observed movement after gentle prodding. Worms were grouped as ten worms per plate after the six-day reproductive period, for logistical reasons and these groups were maintained as independent non-mixing units, for daily transfers across lifetime so that plate identity could be included as a random effect in analysis.

Mutation accumulation (MA) lines. To determine the effects of reduced adulthood IIS, via *daf-2* RNAi, on spontaneous and UV-induced mutation accumulation, we established 800 MA lines, across two genetic backgrounds. The eight experimental treatments were the full-factorial combinations of genotype (N2 or *hrde-1* mutant), RNAi treatment (empty vector or *daf-2* RNAi) and irradiation (UV or sham), with 100 MA lines per treatment. We ran each of the eight treatments in parallel, divided into two time-staggered independent blocks of 50 lines per treatment, for logistical reasons and to capture any between-block variation.

Each MA line was propagated as a single individual hermaphrodite per generation, to create successive genetic bottlenecks, allowing *de novo* deleterious mutations to accumulate in the relative absence of selection[40-42], if they had no effect on developmental viability, and in the absence of mating. MA is common approach used

in several species, to study the evolutionary genetics of *de novo* mutation rates[43]. In wild type *C. elegans*, the spontaneous mutation rate in MA lines is positively correlated with the rate of fitness decay[83].

Each generation, we allowed mid-day 2 to mid-day 3 adults to lay eggs onto empty vector plates, from which we picked a single late-L4 larva per line to form the next generation of MA. The late-L4 stage is easily identifiable in *C. elegans* (vulva cells are visible in the vulva lumen and prior to vulval protrusion at sexual maturity), which allowed repeatable and consistent age-controlled set-up of each generation. The duration of the egg lay period was optimised, to account for differences in developmental timing between treatments. Age of parental egg lay was alternated between mid-Day 2 and mid-Day 3 every second generation, to limit selection on parental age at reproduction and so the offspring forming each new MA generation came from parents during their period of peak reproduction.

We recorded the generation at which extinction occurred and the cause of extinction (death, failure to produce viable eggs or failure to reach sexually mature adult). Individuals that were lost, underwent matricide (internal hatching that killed the parent) prior to the Day 2 egg lay, died from the expulsion of internal tissue, became infected or desiccated on the plate wall were censored. We photographed a sample of Day 1 adult worms with visible developmental or reproductive abnormalities under light microscopy (camera specifications in egg size measurement section below), including worms with stunted growth, apparent aberrant external growth (tumour), deformed vulva, or abnormal cavities in the reproductive tract in place of oocytes or embryos.

Effect of 20 generations of spontaneous and UV-induced mutation accumulation on fitness and life history of surviving MA lines. To test for life history differences between the N2 wild-type MA lines after 20 generations of MA, we assayed age-specific reproduction, egg size, male production and adult heat shock resistance, in all remaining UV-irradiated N2 MA lines and an equally-sized random sample of the non-irradiated N2 MA lines. In total we assayed 100 grand-offspring from each of the four N2 MA treatments, equally from the two independent experimental blocks. Prior to the life history assay, individuals taken from MA lines at generation 20 were reared for two subsequent generations under common garden conditions (no irradiation, on empty vector control), to reduce direct parental effects from exposure to RNAi and UV, and hence look for genetic differences between MA treatments. The life history assay was conducted in the common garden environment.

Individual age-synchronised, late-L4 hermaphrodites were set up on separate e.v. plates, labelled with a unique identifier, to blind experimenters to treatment identity, and thus avoid bias. The two experimenters conducted the life history assay in two simultaneous time blocks and each block had an identical and equal representation of individual worms from all four treatments (n=50 per treatment per block).

Age-specific reproduction, fitness and total lifetime reproduction assays. For all experiments, we assayed age-specific offspring production (fecundity) over the first 6 days of adult life (the reproductive window for *C. elegans* hermaphrodites), by transferring to fresh plates every 24h, to acquire daily reproduction counts. Plates were scored for viable adult offspring 2.5 days later. Individual fitness (lambda) was calculated- a measure weighted by early reproduction and analogous to the intrinsic rate of population growth[15,20,84,85]. The first 4 days of adult reproduction were used to

calculate fitness and to analyse age-specific reproduction, as day 5 and 6 reproduction was zero for almost all individuals. Parental fitness and age-specific reproduction were analysed for day 2 to 4 inclusive, for the first intergenerational experiment, as UV-irradiation was administered at the start of day 2 of adulthood. Total lifetime reproduction (lifetime reproductive success) was calculated for each individual as the sum of age-specific offspring counts across the first 6 days of adulthood.

Egg size measurement. To determine if reduced IIS influenced parental resource allocation into eggs, under MA, we measured the area of a single egg (in mm^2) produced by 75 individuals per treatment in the generation 20 age-specific reproduction assay (half of Block 1 and all of Block 2 individuals), at peak reproduction (Day 2). Egg size is commonly used as a proxy for parental investment[20,50]. We photographed eggs, within 2 hours of lay, under 12x magnification light microscopy (Leica M165C with Lumenera Infinity 2-7C digital microscope camera). Only eggs laid at the normal gastrula (approximately 30-cell) stage of development were photographed, as egg shape and size can vary after 9 hours of ex-utero development. Egg size (area in mm^2 enclosed within a free-drawn ellipse around the egg perimeter) was calculated from photos using *Image J Fiji program* v.1.51[86], whilst blind to treatment identity. To minimise experimenter error, each measurement was taken twice and an average taken, and the same experimenter measured all eggs. Egg measurement was completed across three measurement days, using identical ImageJ settings and scale calibration. Treatments and blocks were stratified across measurement days and randomly dispersed within each day, to avoid bias. Egg area measurements were strongly repeatable between replicate measurements on the same egg (Spearman's rank

correlation coefficient, rho= 0.899), there was no temporal autocorrelation between measurement days (Kruskal-Wallis rank sum test, χ^2=299, df=314, p=0.721) and only weak temporal autocorrelation on the first measurement day (Spearman's rank, rho=0.398).

Male production. We determined the percentage of eggs that developed into adult males (n= 75 eggs assayed/treatment), one egg per individual Day 2 worm, as above, to determine if the stress induced by irradiation or mutation accumulation would increase male production above standard N2 wild-type levels, as seen previously for heat stress and starvation in *C. elegans*[51,52].

Adult heat shock resistance. To test the stress resistance of post-reproductive (Day 7) adults, we assayed survival following acute heat shock of 1 hour and 45 minutes at 37°C (following[58]), in the same individuals used for the Generation 20 age-specific reproduction assay. At 3 and 24 hours post-heat shock we recorded any individuals that had died and categorised locomotion as normal movement (crawling spontaneously or when gently prodded), uncoordinated movement of head and tail with no forward or backward trajectory, or just head movements; following[87,88]. Previous work confirmed that from 12 hours after heat shock is a reliable time point to score long-term survival in *C. elegans* adults[58]. All worms were heat-shocked simultaneously, grouped by treatment, five worms per plate and the positions of plates in the oven were stratified by treatment, to control for any positional differences in heat exposure.

Transgenerational effects of *daf-2* RNAi on offspring fitness in N2 and *hrde-1* mutant backgrounds. To determine whether *daf-2* RNAi was transferred transgenerationally via the germline, we assayed, in a separate experiment, whether

offspring fitness benefits persisted across three generations of offspring (F1, F2 and F3) from Day 2 parents treated with *daf-2* RNAi or empty vector control throughout adulthood, in N2 wild-type and *hrde-1* mutant backgrounds. Offspring of each generation were maintained on empty vector (n=30 individuals per treatment and per genotype) and assayed for daily reproduction (fecundity). Offspring generations were produced from day 2 parents and set-up as late-L4 larvae across all treatments.

Statistical analyses. All analyses were conducted in R version 4.0.0[89]. Fitness and total lifetime reproduction data were plotted using the R package 'dabestr' (data analysis using bootstrap-coupled estimation[90]). The 'dabest' Cummings estimation plots display all datapoints from each treatment as a swarm-plot, with the mean ± standard deviation of treatment as a gapped line adjacent to the points. The mean difference (effect size) and its 95% confidence interval (CI) is estimated for pairwise comparisons of treatments via non-parametric bootstrap resampling (n=5000) and displayed below the main plots. When the 95% CI vertical bar does not cross the x-axis (mean difference equal to zero), there is a significant difference between the pairwise treatment comparison.

Parental survival in the intergenerational (first) experiment was analysed using a Cox proportional hazards mixed effects model[91] ('coxme'), fitting RNAi, UV and their interaction as fixed effects and plate as a random effect. Separate analyses were conducted with matricides either classed as deaths or as censors. The age at death response variable contained a coding variable to distinguish deaths from censored individuals (due to accidental losses). Extinction (survival trajectories) under mutation accumulation was analysed using Cox proportional hazards regression analysis ('coxph' function in 'survival' package). A maximal model was fitted using a three-way RNAi x UV x block interaction and step-wise model simplification

conducted[92]. Block was included as a fixed effect to test for repeatability between blocks.

Age-specific reproduction was analysed using generalised linear mixed effects models to account for temporal pseudoreplication of repeated measures on the same individuals across lifetime, with the template model builder package ('glmmTMB' in R[93,94]). Models fitted with Poisson, Zero-Inflated Poisson, Generalised Poisson and Zero-Inflated Generalised Poisson error structure were compared using AIC values of model fit ('AICtab' function in 'bbmle' package), following[94], to account for under- or over-dispersion and for zero-inflation, when it was found to occur in simulated residuals generated with the 'DHARMa' package[95]. Age and a quadratic form of age (age^2) were fitted as fixed effects in both the conditional and zero-inflation model formula, and significance assessed in each case. The age^2 term controlled for the curved (non-linear) trajectory of reproduction across age[96]. UV irradiation, RNAi treatment and their three-way interaction with age or age^2 were fitted as fixed effects, and experimental block, observer and a unique plate identifier as random effects. Genotype was substituted with UV as a fixed effect to assess the transgenerational effects of *daf-2* RNAi on N2 versus *hrde-1* mutants. Models that did not converge were not included in model comparison, and the converging model with best AIC fit was presented (as[94]).

Fitness (lambda) was calculated using the 'lambda' function in the 'popbio' package. Fitness, total reproduction and egg size data were analysed using a generalised linear model (GLM) with Gaussian error structure ('lm' function in 'stats' package). Locomotion was coded as a binary response variable (normal or abnormal movement, as defied above) and analysed using a GLM with binomial error structure.

Data availability. The data will be made freely available on Figshare upon publication.

Code availability. The code for analyses will be made freely available on GitHub upon publication.

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Author contributions

E.D., A.A.M. and H.C. conceived and designed the study, with T.C. and S.I. providing additional advice. E.D., H.C. and K.S. performed the experiments and collected the data, which E.D. and K.S. analysed. E.D. and A.A.M. wrote the manuscript, with comments from all authors.

Competing interests

The authors declare no competing interests.