dFOXO-independent effects of reduced insulin-like signaling in *Drosophila*

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Summary

The insulin/insulin-like growth factor-like signaling (IIS) pathway in metazoans has evolutionarily conserved roles in growth control, metabolic homeostasis, stress responses, reproduction, and lifespan. Genetic manipulations that reduce IIS in the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the mouse have been shown not only to produce substantial increases in lifespan but also to ameliorate several age-related diseases. In *C. elegans*, the multitude of phenotypes produced by the reduction in IIS are all suppressed in the absence of the worm FOXO transcription factor, DAF-16, suggesting that they are all under common regulation. It is not yet clear in other animal models whether the activity of FOXOs mediate all of the physiological effects of reduced IIS, especially increased lifespan. We have addressed this issue by examining the effects of reduced IIS in the absence of dFOXO in *Drosophila*, using a newly generated null allele of *dfoxo*. We found that the removal of dFOXO almost completely blocks IIS-dependent lifespan extension. However, unlike in *C. elegans*, removal of dFOXO does not suppress the body size, fecundity, or oxidative stress resistance phenotypes of IIS-compromised flies. In contrast, IIS-dependent xenobiotic resistance is fully dependent on dFOXO activity. Our results therefore suggest that there is evolutionary divergence in the downstream mechanisms that mediate the effects of IIS. They also imply that in *Drosophila*, additional factors act alongside dFOXO to produce IIS-dependent responses in body size, fecundity, and oxidative stress resistance and that these phenotypes are not causal in IIS-mediated extension of lifespan.

Key words: *Drosophila*; aging; FOXO; insulin signaling.

Introduction

The insulin/insulin-like growth factor (IGF)-like signaling (IIS) pathway of metazoans regulates such diverse processes as growth, developmental timing, body size, metabolism, stress responses, reproduction, and lifespan (Kenyon, 2005; Giannakou & Partridge, 2007). Genetic manipulations that inhibit IIS in the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the mouse not only increase lifespan but also delay the onset of age-related pathology and disease (Tatar et al., 2003; Kenyon, 2005; Bonkowski et al., 2006; Cohen et al., 2006; Wessells & Bodmer, 2007; Selman et al., 2008; Wessells et al., 2009). Direct downstream targets of IIS in worms, flies, and mammals are the FOXO (forkhead bOX-containing protein, subfamily O) proteins, a highly conserved family of transcription factors. Phosphorylation of FOXOs by the insulin-activated protein kinases PKB/AKT and SGK leads to their sequestration within the cytoplasm and, as a result, transcriptional inactivation of target gene expression (van der Horst & Bruning, 2007; Partridge & Bruning, 2008). Several direct FOXO target genes have been identified that function during cell cycle control, metabolism, apoptosis, and the regulation of cellular stress responses (Greer & Brunet, 2005, 2008; Partridge & Bruning, 2008; Salih & Brunet, 2008). Hence, the activation of FOXOs and their target genes has been under intense study to identify the transcriptional changes associated with IIS-dependent lifespan extension.

Lifespan extensions induced by decreasing the activity of the insulin/IGF1-like receptor, DAF-2, or downstream components of the IIS pathway in *C. elegans* are completely dependent upon the activity of the worm FOXO transcription factor, DAF-16 (Kenyon et al., 1993). Thus, mutation of *daf-16* or reductions in its expression by RNAi can completely abrogate the lifespan extension observed in mutants for *daf-2*, the worm insulin/IGF receptor orthologue, or *age-1*, the worm phosphatidylinositol 3-kinase orthologue (Kenyon et al., 1993). In other model organisms, FOXOs clearly play important roles during lifespan determination: overexpression of dFOXO protein in the adult fat body increases lifespan in *Drosophila* (Tatar et al., 2003; Giannakou et al., 2004; Hwangbo et al., 2004), while heterozygous knockouts for the insulin receptor substrates, IRS1 or IRS2, are long lived and show increased activity of FOXO1 target genes in murine models (Taguchi et al., 2007; Selman et al., 2008). Furthermore, genetic variation in the Foxo3a gene is associated with longevity in several different human populations (Kuningas et al., 2007; Willcox et al., 2008; Flachsbart et al., 2009). However, it has yet to be shown in these other animal models whether the effects of reduced IIS on lifespan are directly dependent on FOXO activity.

In addition to lifespan extension, decreasing IIS in the worm produces a number of other phenotypic responses that are all dependent upon DAF-16, suggesting that they may all be regulated by a common mechanism. For example, *daf-2*-dependent reproductive delay and oxidative stress resistance are completely suppressed by knockdown of *daf-16* expression by RNAi (Larsen, 1993; Honda & Honda, 1999; Dillin et al., 2002). Also, DAF-16 mediates both stress resistance and reduced adult fecundity in *age-1* mutants (Larsen, 1993; Tissenbaum & Ruvkun, 1998; Honda & Honda, 1999). dFOXO-dependent effects on IIS-mediated growth control and germline stem cell (GSC) proliferation have been reported in *Drosophila* (Junger et al., 2003; Puig et al., 2003; Hsu et al., 2008). Nevertheless, in other animals, it remains unclear whether FOXOs mediate all of the phenotypic effects of reduced IIS and thus whether this feature of the signaling pathway has been conserved through evolution.

In this study, we have examined whether several IIS-dependent phenotypes, including increased lifespan, reduced fecundity, increased stress resistance, developmental delay, and growth inhibition, are...
dependent on dFOXO activity in Drosophila. We combined a newly generated null allele of dfoxo with several models of reduced IIS in Drosophila including ubiquitous expression of a kinase-dead, dominant negative version of the Drosophila insulin receptor both during development and specifically restricted to adulthood, late ablation of the dillp-producing median neurosecretory cells (MNCs) and adult-specific ubiquitous expression of a dominant negative form of PI3 kinase, all of which show phenotypes typical of reduced IIS. We found that mutation of dfoxo almost completely blocked lifespan extension in these IIS-compromised flies. However, removal of dfoxo failed to rescue IIS-mediated developmental delay, small body size, reduced egg laying, and resistance to paraquat. In contrast, increased resistance to the xenobiotic toxin, dichlorodiphenyltrichloroethane (DDT), was completely dependent on dFOXO activity. Our results show that, unlike in C. elegans, where all phenotypic traits produced by reduced IIS are DAF-16 dependent, additional factors besides dFOXO have evolved in Drosophila to mediate the full IIS response.

Results

Generation and characterization of a new dfoxo null allele

Several loss-of-function mutants for the Drosophila dfoxo transcription factor have already been described. These include dfoxo$^{27}$ and dfoxo$^{25}$, both of which contain chemically induced nucleotide transversions within the dfoxo coding region, resulting in premature stop codons within the proposed DNA-binding domain of the protein (Junger et al., 2003) and dfoxo$^{94}$, which contains a P-element insertion within the first intron of the dfoxo locus (Weber et al., 2005; Fig. 1A). Heteroallelic combinations of these mutants produce viable adults but no detectable protein by western blot analysis and are therefore considered to function as genetic nulls (Junger et al., 2003; Giannakou et al., 2008; Min et al., 2008). We have performed chromatin immunoprecipitation (ChIP) experiments on chromatin extracts prepared from dfoxo$^{94}$/dfoxo$^{25}$ transheterozygous flies using a specific dFOXO antibody, the epitope for which would still be present within any translated mutant protein (Fig. 1A). dFOXO DNA binding was assessed using a promoter region of the Drosophila SH2B-encoding gene, Lnk, which we have previously demonstrated to be bound by dFOXO (Slack et al., 2010). Surprisingly, quantitative PCR (qPCR) after dFOXO ChIP from dfoxo$^{94}$/dfoxo$^{25}$ samples showed enrichment of the Lnk promoter fragment relative to a control genomic region, similar to wild-type controls (Fig. 1B). Thus, despite the apparent absence of dFOXO protein in dfoxo$^{94}$/dfoxo$^{25}$ mutants (Fig. 1C), there still appears to be residual DNA-binding activity in these flies. This allelic combination may therefore function more as a dominant negative rather than as a true null. Interestingly, dominant effects of the dfoxo$^{27}$ allele have been observed in other studies (Nielsen et al., 2008).

We therefore generated a new deletion mutant of dfoxo by imprecise excision of a P-element positioned upstream of the first noncoding exon of the dfoxo gene. This deletion (dfoxo$^{94}$) spans over 20 kb of the dfoxo locus, removing part of the predicted promoter region as well as several

![Fig. 1 Molecular characterization of the dfoxo$^{94}$ deletion.](image)
coding exons. Homozygotes for the deletion were adult viable, and neither dFOXO protein expression nor DNA-binding activity was detected in these flies (Fig. 1B,C). However, the deletion removes the sequence encoding the epitope site for the dFOXO antibody, and so we could not exclude the possibility that some mutant protein is produced. We therefore examined the expression of dfoxo mRNA by RT-PCR using primers that anneal outside of the deleted region and found that homozygous mutants were completely devoid of dfoxo transcript expression (Fig. 1D). Consequently, this deletion appears to represent a true null allele of dfoxo.

dfoxo<sup>394</sup> homozygotes were delayed in egg–adult development time and were also smaller in size than their controls, with significant reductions in both body weight and wing area (Fig. 2A,B). No obvious effects on developmental time or body weight have been previously reported for other dfoxo mutants, with only a small decrease in wing size described for dfoxo<sup>27/27</sup> dfoxo<sup>25/25</sup> transheterozygotes (Junger et al., 2003). Nevertheless, delayed egg–adult development and reduced body size were also observed in transheterozygous dfoxo<sup>27</sup>/dfoxo<sup>394</sup> flies as well as in hemizygous Df(3R)ED5624/dfoxo<sup>394</sup> flies using a deficiency that removes the entire dfoxo locus (Supplementary Fig. S1), confirming that the observed effects on developmental time and body size in dfoxo<sup>394</sup> homozygotes are specific to the dfoxo<sup>394</sup> genetic lesion.

The small body size of dfoxo<sup>394</sup> homozygotes suggested that dFOXO activity could potentially regulate cell growth or proliferation. To examine this, we generated clones of dfoxo<sup>394</sup> mutant cells in an otherwise heterozygous animal by mitotic recombination. Both clone size and dfoxo<sup>394</sup> mutant cell size were normal (Fig. 2C), as has been observed with other dfoxo alleles (Junger et al., 2003), suggesting that dFOXO does not act cell-autonomously to restrict cell proliferation or growth. Hence, the effects of the dfoxo<sup>394</sup> mutation on growth must occur via nonautonomous mechanisms. Similar to other dfoxo allelic combinations, homozygous dfoxo<sup>394</sup> females were shorter lived than their controls (Fig. 2B) and also laid fewer eggs (Fig. 2C).

The dfoxo<sup>394</sup> deletion was then combined with several mutations or genetic manipulations that reduce IIS in Drosophila including a dlp2-3,5 triple mutant (Gronke et al., 2010), median neurosecretory cell (mNSC) ablation (Broughton et al., 2005), chico<sup>1</sup> mutants (Bohni et al., 1999), and Lnk<sup>205S</sup> mutants (Slack et al., 2010). All resulted in preadult lethality when combined with homozygosity for dfoxo<sup>394</sup>. Interestingly, the lethality of chico<sup>1</sup>; dfoxo<sup>394</sup> homozygotes was rescued by the expression of a UAS-dfoxo transgene within the MNCs, suggesting that a dFOXO-dependent transcriptional response specifically within the MNCs is both necessary and sufficient for the viability of chico<sup>1</sup> homozygotes.

Viable flies were obtained when the dfoxo<sup>394</sup> mutant was combined with either overexpression of a dominant negative form of the Drosophila insulin receptor (UAS-InRDN) under the control of the ubiquitous and constitutive daughterless-GAL4 driver (daGAL) or late ablation of the mNSCs by the expression of UAS-reaper (UAS-rpr) under the control of the InsP3-GAL4 driver (InsP3GAL). In addition, we examined adult-onset ubiquitous expression of UAS-InRDN as well as adult-onset ubiquitous expression of a catalytically inactive, dominant negative form of PI3 kinase (UAS-Dp110DN) using the inducible daughterless-GeneSwitch (daGS) driver. daGS only drives transgene expression in the presence of the RU486 steroid.

![Fig. 2 Phenotypic analysis of dfoxo<sup>394</sup> homozygous mutant flies.](image-url)
drug. Treatment with RU486 had no effect on the lifespan, fecundity, or stress resistance of daGS/+ flies themselves (Supplementary Fig. S2).

**IIS-mediated longevity requires dFOXO activity**

In the presence of dfoxo, daGAL > UAS-InRDN females lived significantly longer than controls, with a 10–15% increase in median lifespan and 6–10% increase in maximum lifespan (Fig. 3A). In a dfoxo– background, all groups were shorter lived compared with their wild-type counterparts, yet daGAL > UAS-InRDN dfoxo– flies showed an age-related increase in survival compared with both genetic controls (daGAL/+ dfoxo– and UAS-InRDN/+ dfoxo–) (Fig. 3A). When all flies were considered in the analysis, daGAL > UAS-InRDN dfoxo– flies were significantly longer lived than both genetic controls (P < 0.001; log-rank test). However, no significant differences in survival were apparent between groups during the first 50 days of the experiment (P = 0.55; log-rank test), whereas survival at ages beyond 50 days was increased in daGAL > UAS-InRDN dfoxo– flies compared with both controls (P < 0.0001; log-rank test) (Fig. 3A). Hence, maximum lifespan, calculated as the median age of the oldest 10% of the population to die, was significantly increased by approximately 8%.
(P < 0.0001; log-rank) for daGS > UAS-InRDN dfoxo−/− flies (66 days, n = 14) relative to both genetic controls (54 days, n = 11 for daGS/+ dfoxo−/− flies and 59 days, n = 16 for UAS-InRDN dfoxo−/− flies). This apparent age-related difference in survival was observed in a second, independent experiment, suggesting that ubiquitous and constitutive expression of UAS-InRDN can increase survival later in life and hence extend maximum lifespan even in the absence of dFOXO activity.

InsP3GAL > UAS- rpr females also lived significantly longer than both genetic controls (InsP3GAL/+ and UAS-rpr+/+) in the presence of dfoxo−/− with approximately 30% increase in median and approximately 20% in maximum lifespan (Fig. 3B). Again, all groups were shorter lived in a dfoxo−/− background, but in contrast to daGS > UAS-InRDN flies, InsP3GAL > UAS-rpr were not significantly longer lived than their genetic controls in a dfoxo−/− background (Fig. 3B).

In both daGS > UAS-InRDN and daGS > UAS-Dp110DN flies, induction of transgene expression by RU486 increased lifespan in both genotypes, with median lifespan increased by 24% and 7%, respectively, and maximum lifespan by 5% for both genotypes compared with uninduced flies of the same genotypes (Fig. 3C,D). No extension of lifespan was observed in either daGS > UAS-InRDN and daGS > UAS-Dp110DN flies in a dfoxo−/− background upon RU486-induced transgene expression compared with uninduced controls (Fig. 3C,D).

Taken together, these data show that loss of dFOXO activity is sufficient to almost completely inhibit the longevity of IIS mutants in Drosophila. By contrast, treatment of dfoxo−/− females with the TOR kinase inhibitor, rapamycin, significantly increased median lifespan by approximately 10%, as was observed in rapamycin-treated wDahomey control females (Supplementary Fig. S3). Furthermore, dfoxo−/− females also showed an increase in lifespan under dietary restriction (Supplementary Figs. S4 and Table S1). Thus, loss of dFOXO activity specifically abrogates the increase in lifespan from reduced IIS.

Loss of dFOXO activity fails to rescue IIS-mediated fecundity defects

IIS plays a complex role during oogenesis in Drosophila: autonomous IIS within the germline directly controls germline cyst development, vitellogenesis, and the rate of GSC divisions (LaFever & Drummond-Barbosa, 2005; Hsu et al., 2008; Hsu & Drummond-Barbosa, 2009), while IIS via dFOXO−/−, and its genetic backgrounds (Fig. 4A). Similar results were observed for InsP3GAL > UAS-rpr females: egg laying was reduced compared with both InsP3GAL/+ and UAS-rpr/+ controls in both wild-type and dfoxo−/− backgrounds (Fig. 4B). Ubiquitous adult-specific induction of UAS-InRDN or UAS-Dp110DN expression also decreased female fecundity and, again, this decrease in female egg laying was still observed in a dfoxo−/− background (Fig. 4C,D). Taken together, these data show that removal of dfoxo−/− is not sufficient to rescue the reduced fecundity of IIS-compromised females. Furthermore, in a dfoxo−/− mutant background, InsP3GAL > UAS-rpr females laid significantly fewer eggs than their dfoxo−/− genetic controls (P < 0.05, t-test) and both daGS > InRDN dfoxo−/− and daGS > Dp110DN dfoxo−/− females treated with RU486 laid significantly fewer eggs than their uninduced controls (P < 0.001, ANOVA), suggesting that loss of dFOXO activity and reduced IIS in these flies acted additively to reduce egg laying.

Mutation of dfoxo can reverse the reduction in GSC proliferation caused by the loss of chico function, demonstrating that dFOXO is required in the GSCs for at least some of the effects of lowered IIS on reproduction (Hsu et al., 2008). To further examine the effects of dFOXO activity more specifically within the germline, we generated flies overexpressing a dFOXO transgene in the GSCs, using the maternal GAL4 driver, mata-GAL4. Despite significant overexpression of dFOXO protein in the ovaries of these females, they were still fertile with no overall gross morphological defects in ovarian structure (Fig. 4E). However, egg laying by these females was reduced by approximately 30% (Fig. 4E), demonstrating that increased dFOXO activity specifically within the germline is sufficient to reduce egg production.

In daGS > UAS-InRDN females, the dominant negative insulin receptor is expressed in all somatic cells but not in the germline. Therefore, the reduction in egg laying in these females must be mediated via indirect

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**Fig. 3** Effects of dfoxo removal on the survivorship of IIS-compromised flies. (A) Survival curves of female flies overexpressing a dominant negative version of the insulin receptor (daGS > UAS-InRDN) and their genetic controls (daGS/+ and UAS-InRDN/+ ) in both wild-type and dfoxo−/− backgrounds (representative of two independent experiments). In a wild-type background: for daGS > UAS-InRDN median survival = 71 days, maximum survival = 82 days, n = 93; for daGS/+ median survival = 61 days, maximum survival = 77 days, n = 114; for UAS-InRDN/+ median survival = 64 days, maximum survival = 75, n = 115. The survival of daGS > UAS-InRDN flies was significantly different from each of the controls (P < 0.0001; log-rank test). No significant difference in survival was detected between the two controls (P = 0.6, log-rank test). In a dfoxo−/− background: for daGS > UAS-InRDN median survival = 53 days, maximum survival = 64 days, n = 114; for daGS/+ median survival = 49 days, maximum survival = 59 days, n = 109; for UAS-InRDN/+ median survival = 49 days, maximum survival = 57 days, n = 106. The survival of daGS > UAS-InRDN dfoxo−/− flies is significantly different from each of the controls (P < 0.001; log-rank test). No significant difference in survival was detected between the two controls (P = 0.5, log-rank test). (B) Survival curves of female flies with late ablation of the median neurosecretory cells (InsP3GAL > UAS-rpr) and their genetic controls (InsP3GAL/+ and UAS-rpr+/+) in both wild-type and dfoxo−/− backgrounds. In a wild-type background: for InsP3GAL > UAS-rpr median survival = 82 days, maximum survival = 100 days, n = 99; for InsP3/+ median survival = 60 days, maximum survival = 82 days, n = 94; for UAS-rpr/+ median survival = 62 days, maximum survival = 82 days, n = 93. The survival of InsP3GAL > UAS-rpr flies was significantly different from each of the controls (P < 10−11, log-rank test). No significant difference in survival was detected between the two controls (P = 0.8, log-rank test). In a dfoxo−/− background: for InsP3GAL > UAS-rpr median survival = 44 days, maximum survival = 58 days, n = 94; for InsP3/+ median survival = 41 days, maximum survival = 56 days, n = 108; for UAS-rpr/+ median survival = 44 days, maximum survival = 59 days, n = 95. The survival of InsP3GAL > UAS-rpr flies was not significantly different from either of the controls (P > 0.2, log-rank test). (C) Survival curves of female daGS > UAS-InRDN flies induced to ubiquitously express the dominant negative insulin receptor by feeding RU486-containing food from day 3 of adulthood (wild-type +RU486: median survival = 83 days, maximum survival = 95 days, n = 94; dfoxo−/− +RU486: median survival = 46 days, maximum survival = 70 days, n = 81) compared with uninduced controls (wild-type –RU486: median survival = 67 days, maximum survival = 90 days, n = 97; dfoxo−/− –RU486: median survival = 44 days, maximum survival = 65 days, n = 79). The survival of wild-type daGS > UAS-InRDN +RU486 was significantly different from the –RU486 control (P < 10−6, log-rank test). The survival of dfoxo−/− daGS > UAS-InRDN +RU486 was not significantly different from the –RU486 control (P = 0.2, log-rank test). (D) Survival curves of female daGS > UAS-dp110DN flies induced to ubiquitously overexpress a dominant negative form of Dp110 by feeding RU486-containing food from day 3 of adulthood (wild-type +RU486: median survival = 80 days, maximum survival = 97 days, n = 91; dfoxo−/− +RU486: median survival = 51 days, maximum survival = 65 days, n = 95) compared with uninduced controls (wild-type –RU486: median survival = 75 days, maximum survival = 92 days, n = 103; dfoxo−/− –RU486: median survival = 54 days, maximum survival = 65 days, n = 97). The survival of wild-type daGS > UAS-dp110DN +RU486 was significantly different from the –RU486 control (P < 0.001, log-rank test). The survival of dfoxo−/− daGS > UAS-dp110DN +RU486 was not significantly different from the –RU486 control (P = 0.4, log-rank test). Representative of two independent experiments.
Fig. 4 Effects of dfoxo removal on the female fecundity. (A–D) Average number of eggs laid per 7-day-old female or after 7 days of RU486 treatment. Data are presented as the mean number of eggs laid per female over a 24-h period ± SEM. Eggs were counted from ten separate vials, and each vial contained ten females. (A) Females with constitutive and ubiquitous expression of the dominant negative insulin receptor. In both wild-type and dfoxo− backgrounds, daGAL > UAS-InRDN females laid significantly fewer eggs than both daGAL+/− and UAS-InRDN+/− controls (**P < 0.05, t-test). There was no significant difference between daGAL > UAS-InRDN and daGAL > UAS-InRDN dfoxo− flies. (B) Females with late ablation of the median neurosecretory cells. In both wild-type and dfoxo− backgrounds, InsP3GAL > UAS-rpr laid significantly fewer eggs than both InsP3GAL+/− and UAS-rpr+/− controls (**P < 0.05, t-test). (C) Females induced to ubiquitously overexpress the dominant negative insulin receptor by feeding RU486-containing food from day 3 of adulthood. In both wild-type and dfoxo− backgrounds, daGS > UAS-InRDN females induced with RU486 (+RU486) laid significantly fewer eggs uninduced controls (−RU486) (**P < 0.05, t-test). (D) Females induced to ubiquitously overexpress dominant negative Dp110 by feeding RU486-containing food from day 3 of adulthood. In both wild-type and dfoxo− backgrounds, daGS > UAS-Dp110DN females induced with RU486 (+RU486) laid significantly fewer eggs uninduced controls (−RU486) (**P < 0.05, t-test). (E) dFOXO protein expression in the germline. Western blot analysis of dFOXO protein expression in ovaries overexpressing two independent dFOXO transgenes (dfoxo-p8 and dfoxo-p13) within the germline under the control of the mataGAL4 driver. Blots were probed with anti-tubulin as a control for protein loading. Ovaries from these females overexpressing dFOXO protein in the germline look structurally wild-type, but egg production is significantly reduced. Eggs were collected from 7-day-old females over a 24-h period and counted. Data are presented as the mean number of eggs laid per female over these 24-h periods ± SEM (**P < 0.05, ANOVA). (F) Quantitative RT-PCR analysis of yolkless mRNA expression in female flies of the indicated genotypes normalized to actin5C. Data are presented as means ± SEM (n = 5). yolkless expression is upregulated in daGAL > UAS-InRDN females compared with controls (**P < 0.05, t-test) in both wild-type and dfoxo− backgrounds.
responses of the germline to somatic signals. IIS can indirectly affect egg production through the regulation of yolk protein uptake into the oocyte during vitellogenesis (Richard et al., 2005). It is therefore possible that dFOXO transcriptional activity is required for the expression of yolk protein transcripts themselves. However, we found no significant difference in the expression of yolk protein 2 (YP2) in daGAL > UAS-InRDN females, in either a wild-type or dfxoo- background, indicating that YP2 expression is unresponsive to both IIS itself and dFOXO activity. In contrast, transcription of the yolk protein receptor, yolkless, whose expression is normally restricted to the oocyte, was significantly increased in daGAL4 > UAS-InRDN females compared with controls (Fig. 4F). However, this increase in yolkless expression was still present in dfxoo- mutants (Fig. 4F) and so occurs independently of dFOXO-mediated transcriptional regulation.

dFOXO is not required for IIS-mediated oxidative stress resistance

Genetic interventions that inhibit IIS often result in enhanced resistance to various stresses including oxidative stress (Clancy et al., 2001; Broughton et al., 2005). We therefore examined the effects of paraquat, an intracellular ROS generator, on the survival of daGAL4 > UAS-InRDN, InsP3GAL-L > UAS-rpr and daGS > Dp110DN flies. In a wild-type background, daGAL > UAS-InRDN, InsP3GAL > UAS-rpr and daGS > Dp110DN flies all survived for significantly longer on food supplemented with 20 mM paraquat compared with their respective controls (Fig. 5A, B, C). Interestingly, we still observed a small but significant and proportionally similar increase in the survival of daGAL4 > UAS-InRDN dfxoo- and InsP3GAL-L > UAS-rpr dfxoo- and daGS > Dp110DN dfxoo- flies over their
Fig. 6 Functions for dFOXO during IIS-mediated dichlorodiphenyltrichloroethane (DDT) resistance. (A) Survival curves in response to DDT of female flies overexpressing a dominant negative version of the insulin receptor (daGAL > UAS-InRDN) and their genetic controls (daGAL/+ and UAS-InRDN/+ in both wild-type and dfoxo− backgrounds. In a wild-type background: for daGAL > UAS-InRDN median survival = 4.9 days, maximum survival = 4.9 days, n = 76; for daGAL/+ median survival = 2.4 days, maximum survival = 3.5 days, n = 97; for UAS-InRDN/+ median survival = 2.4 days, maximum survival = 3.5, n = 100. The survival of daGAL > UAS-InRDN flies was significantly different from each of the controls (P < 10−24; log-rank test). In a dfoxo− background: for daGAL > UAS-InRDN median survival = 1.8 days, maximum survival = 2.4 days, n = 55; for daGAL/+ median survival = 1.8 days, maximum survival = 2.4 days, n = 89; for UAS-InRDN/+ median survival = 1.8 days, maximum survival = 2.1 days, n = 65. The survival of daGAL > UAS-InRDN dfoxo− flies was not significantly different from each of the controls (P > 0.1; log-rank test). (B) Survival curves in response to DDT of female flies with late ablation of the median neurosecretory cells (InsP3GAL > UAS-rpr) and their genetic controls (InsP3GAL/+ and UAS-rpr/+ in both wild-type and dfoxo− backgrounds. In a wild-type background: for InsP3GAL > UAS-rpr median survival = 3.6 days, maximum survival = 4.5 days, n = 92; for InsP3GAL/+ median survival = 2.6 days, maximum survival = 3.1 days, n = 83; for UAS-rpr/+ median survival = 2.6 days, maximum survival = 2.6 days, n = 94. In a dfoxo− background: for InsP3GAL > UAS-rpr median survival = 1.9 days, maximum survival = 2.6 days, n = 90; for InsP3GAL/+ median survival = 1.6 days, maximum survival = 2.6 days, n = 90; for UAS-rpr/+ median survival = 1.6 days, maximum survival = 2.3 days, n = 94. The survival of InsP3GAL > UAS-rpr dfoxo− flies was not significantly different from each of the controls (P > 0.08; log-rank test). (C) Survival curves in response to DDT of female flies induced to overexpress dominant negative Dp110 (daGS > UAS-Dp110DN) using RU486 (+RU486) and their uninduced controls (−RU486) in both wild-type and dfoxo− backgrounds. In a wild-type background (top panel): for +RU486 median survival = 3.3 days, maximum survival = 4.0 days, n = 88; for −RU486 median survival = 2.9 days, maximum survival = 4.0 days, n = 99. The survival of +RU486 flies was significantly different from the uninduced controls (P < 0.001; log-rank test). In a dfoxo− background (bottom panel): for +RU486 median survival = 2.3 days, maximum survival = 2.9 days, n = 76; for −RU486 median survival = 2.3 days, maximum survival = 2.9 days, n = 85. The survival of +RU486 flies was not significantly different from the uninduced controls (P = 0.6; log-rank test).
respective controls (Fig. 5A,BC), showing that in the absence of dFOXO activity, reductions in IIS can still increase resistance to paraquat treatment.

IIS-dependent xenobiotic metabolism is dFOXO-dependent

In both worms and flies, long-lived IIS mutants show increased expression of genes involved in xenobiotic metabolism (McElwee et al., 2007), and IIS mutants in Drosophila show increased survival in the presence of the xenobiotic toxin, DDT (Gronke et al., 2010). In a wild-type background, daGAL4 > UAS-InRDN, InsP3GAL > UAS-rpr and daGS > Dpi110DN flies survived for longer in the presence of DDT compared with their controls (Fig. 6A,B,C). In a dfoxo− background, all experimental and control groups showed increased sensitivity to DDT and unlike with paraquat treatment, daGAL4 > UAS-InRDN dfoxo−, InsP3GAL > UAS-rpr

**Fig. 7** Effects of dFOXO removal on IIS-mediated developmental delay and growth inhibition. (A) Egg-to-adult development time is delayed in females overexpressing the dominant negative insulin receptor (daGAL > UAS-InRDN) irrespective of the presence or absence of dfoxo. Only the eclosion period of the adult flies is shown. Data are shown as percentage of flies eclosing. (n = 118 for daGAL > UAS-InRDN, n = 110 for daGAL > UAS-InRDN dfoxo−; n = 120 for daGAL+/+, n = 102 for daGAL/− dfoxo−; n = 107 for UAS-InRDN/+, n = 82 for UAS-InRDN/+ dfoxo−). (B) Body weights and wing areas as indicators of adult fly body size in females overexpressing the dominant negative insulin receptor (daGAL > UAS-InRDN). Data are presented as means ± SEM (n = 10 for each measurement). daGAL > UAS-InRDN females in both wild-type and dfoxo− backgrounds are significantly reduced in body size compared with control flies (daGAL+/+ and UAS-InRDN+/+) (**P < 0.05, t-test). (C) Body weights and wing areas as indicators of adult fly body size in females with late ablation of the median neurosecretory cells (InsP3GAL > UAS-rpr). Data are presented as means ± SEM (n = 10 for each measurement). InsP3GAL > UAS-rpr females in both wild-type and dfoxo− backgrounds are significantly reduced in body size compared with control flies (InsP3GAL+/+ and UAS-rpr+/+) (**P < 0.05, t-test). (D) Removal of dFOXO rescued the growth inhibition effects of overexpressing the dominant negative insulin receptor in a tissue-restricted manner. UAS-InRDN was specifically expressed in the developing eye using eyGAL. This resulted in a smaller eye compared with control (middle panel compared with left panel). This tissue-restricted growth inhibition was fully rescued in homozygous dfoxoΔ170 (dfoxo−) mutant flies (right panel).
dfoxo− and daGS > Dp110DN dfoxo− flies did not show any increased resistance to DDT over their respective controls (Fig. 6A,B,C). Thus, removal of dFOXO activity not only increased sensitivity to DDT treatment but completely abrogated the increased resistance to DDT of IIS-compromised flies.

dFOXO-independent effects on developmental delay and growth

daGAL > UAS-InRDN females were delayed in egg–adult development time by over 24 h compared with both the daGAL+/+ and UAS-InRDN+/+ controls (Fig. 7A). They also showed a significant reduction in both body weight and wing size (Fig. 7B). Removal of dfoxo did not rescue either the developmental delay or reduced body size of daGAL > UAS-InRDN females (Fig. 7A,B). InsP3GAL > UAS-rpr females were not delayed in their development time but were significantly smaller than both InsP3+/+ and Uas-rpr/+ controls (Fig. 7C). Again, InsP2GAL > UAS-rpr females were still significantly smaller than controls in a dfoxo− mutant background (Fig. 7C). Thus, global removal of dfoxo is not sufficient to rescue the small body size of IIS mutant flies. Interestingly, when we restricted the expression of the dominant negative insulin receptor to the developing eye using eyGAL4 (which produces a smaller eye under wild-type conditions), we observed a complete rescue of growth inhibition in the dfoxo− mutant background (Fig. 7D). It is therefore possible that dFOXO activity is required for the production of systemic growth factors that are required for proper organismal growth.

Candidates for systemic growth factors regulated by dFOXO are the Drosophila insulin-like peptides or dilps. Three of these peptides (dilp-2, dilp-3, and dilp-5) are expressed in the MNCs of the Drosophila brain. Ablation of the MNCs or genetic deletion of all three MNC-expressed dilps produces small flies owing to systemic effects on IIS-mediated growth (Ikeya et al., 2002; Broughton et al., 2005; Gronke et al., 2010). Furthermore, expression of dilp3 has been shown to be dependent on dFOXO activity (Broughton et al., 2008). In dfoxo− mutant backgrounds, the expression of all three MNC-expressed dilps was significantly reduced compared with control flies (Fig. 8A).

Effects of dfoxo deletion on dFOXO target gene expression

The translational regulator 4E-BP (encoded by Thor) has been well documented as a direct target of dFOXO (Junger et al., 2003; Puig et al., 2003). 4E-BP expression is upregulated when dFOXO is activated either in response to low IIS or upon exposure to stressors such as paraquat. We therefore examined the effects of the dfoxo− mutation on 4E-BP expression under various conditions in which dFOXO activity would normally be induced. Thus, 4E-BP expression was upregulated in both daGAL > UAS-InRDN flies and wild-type flies exposed to 20 mM paraquat (Fig. 8B,C). Surprisingly, 4E-BP expression was also increased to a comparable level in dfoxo− homozygous mutants themselves. However, in dfoxo− homozygous genotypes, no further increases in 4E-BP expression were observed in daGAL > UAS-InRDN flies or upon exposure to paraquat (Fig. 8B,C).

Discussion

As a result of the pleiotropic effects of IIS on animal physiology, extension of lifespan by reduced IIS is often accompanied by other phenotypic responses, including reduced or delayed reproduction, growth inhibition, increased stress resistance, and metabolic dysregulation. In C. elegans, all of the phenotypic outcomes of reduced IIS are under a common regulatory mechanism, because they are all dependent on the transcriptional activity of the FOXO transcription factor, DAF-16 (Kenyon et al., 1993; Dillin et al., 2002). In Drosophila and mammals, many of the same physiological traits are affected by reduced IIS, but a requirement for FOXO transcriptional activity in mediating all of the phenotypic responses to reduced IIS, especially lifespan extension, in these other animal models is less well understood. In this study, we have combined a novel deletion...
mutant of dfoxo that is devoid of dfoxo mRNA expression with several models of reduced IIS in Drosophila to investigate the consequences of dfoxo removal on lifespan, fecundity, development, growth, and stress resistance.

In C. elegans, IIS-mediated lifespan extension is entirely dependent on the activity of DAF-16, and genetic manipulations that reduce IIS cannot extend the lifespan of daf-16 mutant or RNAi-treated worms (Kenyon et al., 1993; Dillin et al., 2002). We have observed similar results in Drosophila in that mutation of dfoxo completely blocked the lifespan extension associated with late ablation of the MNCs as well as adult-specific expression of either a dominant negative form of the Drosophila insulin receptor or a dominant negative form of Dp110. However, we did observe an age-specific increase in survival late in life in dfoxo mutants with ubiquitous expression of the dominant negative insulin receptor during development. In these flies, no differences in survival were observed until after the flies were aged 50 days, after which experimental flies consistently out-lived controls, resulting in a significant extension of their maximum lifespan. While these effects on survival were reproduced in independent replicate experiments, they were not observed with any other IIS manipulation, suggesting that developmental expression of the dominant negative insulin receptor may produce effects that are not necessarily linked to reduced IIS. It is, however, intriguing to note that at least one genetic manipulation that reduces IIS in Drosophila can still increase lifespan in the absence of dFOXO activity. In contrast to its effects in IIS-mediated lifespan extension, dfoxo mutants still showed increased survival in response to treatment with rapamycin, a specific TOR kinase inhibitor, and in response to dietary restriction. Thus, the inhibition of lifespan extension by the removal of dfoxo appears to be specific to the downregulation of IIS.

In worms, DAF-16 is also required to mediate the reduction in brood size associated with the genetic perturbation of daf-2 or age-1 expression (Tissenbaum & Ruvkun, 1998). However, we have found that in Drosophila, removal of dfoxo activity fails to rescue the reduction in egg laying associated with reduced IIS and consequently the reduced fecundity of IIS-compromised females does not appear to be dFOXO-dependent. In fact, low IIS and removal of dFOXO activity actually had additive effects, causing further reductions in egg laying than reduced IIS alone. The nature of the genetic manipulations used in our study to reduce IIS exclude direct reductions within the germline itself, and so the observed effects on female fecundity must be mediated by disruption of somatic signals to the germline. Our data would suggest that these somatic signals act independently of dFOXO. In support of this, we observed dFOXO-independent effects on the expression of the vitellogenic gene, yolKB, with reduced somatic IIS. Our study also highlights important differences between worms and flies in the timing requirements for IIS during reproduction. In C. elegans, daf-2 RNAi initiated at egg hatching caused a delay in reproduction, whereas daf-2 RNAi during adulthood had no effect (Dillin et al., 2002). Our data have shown that reductions to IIS specifically during adulthood by expression of the dominant negative insulin receptor or dominant negative Dp110 are sufficient to reduce female fecundity.

A role for dFOXO within the germline itself during oogenesis, however, cannot be excluded. Previous studies have shown that the effects of low IIS on GSC proliferation can be reverted by a reduction in dFOXO activity, suggesting that at least some aspects of oogenesis are regulated by dFOXO (Hsu et al., 2008), and we have shown here that overexpression of dFOXO alone specifically within the germline is sufficient to reduce egg laying. Hence, dFOXO-dependent and dFOXO-independent processes may mediate the full effects of reduced IIS on oogenesis. The proliferation of the GSCs in response to diet has been shown to be regulated via both dFOXO-dependent and dFOXO-independent processes (Hsu et al., 2008). Moreover, the Ras-binding domain of Drosophila P3K is required for maximal P3K activity during egg laying (Orme et al., 2006). Therefore, signaling via both dFOXO and Ras/Mapk may together mediate the full IIS response during oogenesis. Removal of dFOXO alone would hence be insufficient to rescue IIS-mediated defects in egg laying.

In C. elegans, DAF-16 regulates the expression of several oxidative stress responsive genes such as sod3, mit-1, ctl-1, and ctl-2 (Honda & Honda, 1999; Murphy et al., 2003). Furthermore, oxidative stress resistance in daf-2 mutant worms is entirely dependent on the presence of DAF-16 (Honda & Honda, 1999). In contrast, we have found that all of the models of reduced IIS tested in this study showed increased resistance to the intracellular ROS generator, paraquat, even in the absence of dFOXO. dFOXO-independent effects may therefore contribute, at least in part, to the increased survival of IIS-compromised flies in response to paraquat treatment. Thus, IIS-dependent oxidative stress resistance can be uncoupled from IIS-dependent lifespan extension based on their requirements for dFOXO, suggesting that they are mediated via nonoverlapping mechanisms. Hence, oxidative stress resistance is not causal in IIS-mediated lifespan extension. By comparison, increased resistance to the xenobiotic toxin, DDT, was completely abolished in dfoxo mutants, suggesting that it is entirely dependent upon dFOXO activity. Furthermore, dfoxo mutants were more sensitive to DDT treatment than wild-type controls, indicating that dFOXO activity is required for survival in the presence of DDT. Our findings that IIS-dependent DDT resistance and lifespan extension require dFOXO suggests that enhanced xenobiotic metabolism may contribute to longevity in long-lived IIS mutant flies. Interestingly, transcriptome analyses of IIS mutants from worms, flies, and mammals have shown that the regulation of cellular detoxification is an evolutionarily conserved function of long-lived IIS mutants in all three model organisms (McElwee et al., 2007).

Perhaps our most surprising observation was that in combination with several IIS mutants, removal of dFOXO caused developmental lethality, for example, chico; foxo double mutants were lethal at preupal stages. Furthermore, we were able to rescue the lethality of chico; foxo double mutants by the expression of dFOXO within the MNCs. Thus, a dFOXO-dependent transcriptional response specifically within the MNCs is required for the viability of chico mutants. The MNCs express the Drosophila insulin-like peptides dilp-2, dilp-3, and dilp-5, and we have shown that dFOXO is required for the basal expression of all three MNC-expressed dilps because their expression is reduced in dfoxo mutant heads. This raises the possibility that dFOXO activity itself may regulate systemic IIS, supported by our observations that removal of dFOXO activity has nonautonomous effects on growth. Nonautonomous inhibition of both somatic and GSC divisions has also been reported for other dfoxo mutants (Junger et al., 2003; Hsu et al., 2008).

In C. elegans, the group of genes identified as direct targets of DAF-16 and that are differentially expressed in response to IIS are enriched for IIS pathway genes, suggesting that when IIS is low, DAF-16 increases insulin sensitivity by upregulating the expression of IIS pathway genes (Schuster et al., 2010). It is probable that a similar feedback mechanism operates in Drosophila, mediated by dFOXO transcriptional regulation. In a previous study, we identified the dilps among several Drosophila IIS pathway genes that show increased expression in an IIS mutant, suggestive of such transcriptional feedback (Slack et al., 2010).

Several dFOXO target genes have been well characterized in Drosophila, including the translational regulator 4E-BP. We have demonstrated that 4E-BP expression is upregulated in response to both reduced IIS and paraquat exposure in a dFOXO-dependent manner. Interestingly, 4E-BP expression was also increased upon removal of dFOXO itself. It is possible...
that dFOXO functions to restrict basal 4E-BP expression under normal conditions. Alternatively, the effects on 4E-BP expression upon removal of dFOXO may be indirectly mediated via dFOXO-dependent effects on the activity of other transcription factors. 4E-BP has recently been shown to be a potential target for the Drosophila FoxA transcription factor, fork-head (FKH), in response to low TOR signaling (Bulow et al., 2010). 4E-BP expression is suppressed by the loss of FKH activity and elevated upon FKH overexpression (Bulow et al., 2010). dFOXO and FKH share the conserved forkhead DNA-binding domain, and so it is possible that they compete for binding at the same target genes. The increase in 4E-BP expression observed in dFOXO mutants may therefore occur as a result of FKH-mediated transcriptional regulation. However, increased expression of other potential FKH target genes such as cubitus and CG6770 have not been observed in dfoxo mutants (N. Alic, C. Slack and L. Partridge, unpublished data).

Taken together, our data have shown that unlike in C. elegans, where all of the phenotypic effects of reduced IIS are dependent on DAF-16 activity, in Drosophila, several IIS-dependent phenotypes appear to be regulated, at least in part, through dFOXO-independent mechanisms. These results therefore have important implications when analyzing the requirements for IIS in particular phenotypic traits. In worms, the standard protocol would be to remove DAF-16 activity and look for abrogation of the response or phenotype. In flies, and possibly higher organisms, such experiments may prove misleading. For example, dfoxo mutants display a normal response to DR, but overexpression of the dominant negative insulin receptor or removal of clps-2, -3 and -5 almost completely blocks the DR response (Grandison et al., 2009; Broughton et al., 2010; Gronke et al., 2010). In conclusion, our data suggest that there is evolutionary divergence in the downstream effectors of IIS, and so in higher organisms, additional factors may act in concert with FOXOs to mediate the full response to reduced IIS.

### Experimental procedures

#### Fly stocks and maintenance

The dfoxo allele was generated by conventional imprecise excision using P[GT1]foxo flies that carry an P[GT1] element transposon in the 5'upstream region of the dfoxo gene, approximately 130 nucleotides upstream of the dfoxo transcriptional start site (Dionne et al., 2006). The 5' and 3' breakpoints of the dfoxo deletion were mapped to the genomic sequence by PCR and sequencing. UASp-dFOXO transgenic flies for germline expression of dFOXO were generated using standard procedures. The P[GT1]foxo flies were crossed for at least 6 generations into the control background. Flies were reared and housed as for lifespan experiments until 7 days of age, and then flies were starved for 5 h on 1% agar before being transferred to fly food containing either 20 mM paraquat or 0.03% (w/v) DDT.

#### Growth analysis

Body weights of 7-day-old flies (n = 10 for each genotype) were measured using a precision balance. Wing areas were measured as previously described (Bohni et al., 1999). For clonal analysis of growth, clones of dfoxo mutant cells were induced in the larval wing disks at 24–48 h after egg deposition by heat-shocking larvae of the genotype y, w, hs-flp/w; FRT82, ubi-GFP/FRT82, dfoxo for 1 h at 37°C. Larval wing disks were dissected out, fixed in 4% formaldehyde for 20 min at room temperature, and mounted in Vectashield mounting medium containing DAPI.

#### Western blots

Western blots were carried out on protein extracts made from whole flies using a TCA-based extraction protocol. Equal amounts of protein as quantified using the Bio-Rad protein assay reagent were loaded onto SDS–PAGE gels and blotted according to standard protocols. Blots were probed with either anti-dFOXO antibody (Giannakou et al., 2007) at a dilution of 1:5000 or anti-tubulin antibody (Sigma, Gillingham, UK) at 1:2500 dilution. Secondary antibodies conjugated to HRP (AbCam, Cambridge, UK) were used, and the signals were detected by chemiluminescence using the Enhanced ECL kit (GE, Amersham, UK).

#### Quantitative RT-PCR

Total RNA was extracted from ten whole adult flies or 20 adult heads per genotype using standard Trizol (Invitrogen, Paisley, UK) protocols. cDNA was prepared using oligo(dT) primer and Superscript II reverse transcriptase according to the manufacturer’s protocol (Invitrogen). Quantitative RT-PCR was performed using the PRISM 7000 sequence detection system and Fast SYBR® Green PCR Master Mix (ABI, Warrington, UK). Relative quantities of transcripts were determined using the relative standard curve method and normalized to actin5C. Four or five independent RNA extractions were used for each genotype. Primer sequences are available upon request.

#### Chromatin immunoprecipitation

Chromatin immunoprecipitations were carried out essentially as described (Slack et al., 2010). For quantitative PCR, a suitable dilution of total chromatin and IP was used for the quantification using the PRISM 7000 sequence detection system and Fast SYBR® Green PCR Master Mix (ABI). For ChiP analysis, relative amounts of the target DNA
recovered after ChIP compared with total chromatin were determined using two or three independent biological replicates. The relative proportion of DNA binding was calculated by dividing the proportion of DNA binding in the ChIP for a single region by the average recovered for all regions for that chromatin to normalize for plate–plate differences.

Immunohistochemistry and confocal microscopy

Immunohistochemical analysis of dFOXO protein overexpression in whole mount ovaries of 7-day-old females was performed using the anti-dFOXO antibody at a dilution of 1:250 followed by AlexaFluor 488 labeled anti-rabbit secondary antibody (Invitrogen). Nuclei were visualized using DAPI. Images were acquired using a Zeiss LSM 700 confocal microscope and zen (Zeiss, Welwyn Garden City, UK) software.

Statistical analyses

Statistical analyses were performed using sas (version 7) software (SAS Institute Inc., Cary, NC, USA). Lifespan data were subjected to survival analysis (Log-rank tests). Maximum lifespans were calculated as the median of the last surviving 10% of the population. Other data were tested for normality using the Shapiro–Wilk W test on studentized residuals (Sokal & Rohlf, 1998). One-way analyses of variance (ANOVA) were performed normality using the Shapiro–Wilk W test on studentized residuals (Sokal & Rohlf, 1998). One-way analyses of variance (ANOVA) were performed, and planned comparisons of means were made using Tukey–Kramer HSD (Honestly Significant Difference) or Student’s t-test.

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Author contributions

CS participated in the design and coordination of the study, carried out most of the experiments, and drafted the manuscript; MEG participated in the design of the study; AF and MG participated in the experimental work; LP conceived the study, participated in its design and coordination, and drafted the manuscript.

References

Bass TM, Grandison RC, Wong R, Martinez P, Partridge L, Piper MD (2007) Optimization of dietary restriction protocols in Drosophila. J. Gerontol. A Biol. Sci. Med. Sci. 62, 1071–1081.

Bohni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andrus BF, Beckingham K, Hafen E (1999) Autonomaous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS-1. Cell 97, 865–875.

Bonkowski MS, Parmenter RW, Rocha JS, Masternak MM, Panici JA, Bartke A (2006) Long-lived growth hormone receptor knockout mice show a delay in age-related changes of body composition and bone characteristics. J. Gerontol. A Biol. Sci. Med. Sci. 61, 562–567.

Broughton SJ, Slack C, Alic N, Metaxakis A, Bass TM, Drieye Y, Partridge L (2010) DILP-producing median neurosecretory cells in the Drosophila brain mediate the response of lifespan to nutrition. Aging Cell 9, 335–346.

Buch S, Melcher C, Bauer M, Katzenberger J, Pankrath MJ (2008) Opposing effects of dietary protein and sugar regulate a transcriptional target of Drosophila insulin-like peptide signaling. Cell Metab. 7, 321–332.

Bulow MH, Aebihold R, Pankrath MJ, Junger MA (2010) The Drosophila FoxA ortholog fork head regulates growth and gene expression downstream of target of rapamycin. PLoS ONE 5, e15171.

Clancy DJ, Gems D, Hershman LG, Oldham S, Stocker H, Hafen E, Levers SJ, Partridge L (2001) Extension of lifespan by loss of CHICO, a Drosophila insulin receptor substrate protein. Science 292, 104–106.

Cohen E, Bieschke J, Perciavalle RM, Kelly JW, Dillin A (2006) Activating mutations protect against age-onset proteotoxicity. Science 313, 1604–1610.

Dillin A, Crawford DK, Kenyon C (2002) Timing requirements for insulin/IGF-1 signaling in C. elegans. Science 298, 830–834.

Dionne MS, Pham LN, Shirasu-Hiza M, Schneider DS (2006) Akt and FOXO dysregulation contribute to infection-induced wasting in Drosophila. Curr. Biol. 16, 1977–1985.

Flachsbart F, Caliebe A, Kleindorp R, Blanche H, von Eller-Eberstein H, Nikolaus S, Schreiber S, Nebel A (2009) Association of FOXO3A variation with human longevity confirmed in German centenarians. Proc. Natl Acad. Sci. USA 106, 2700–2705.

Giannakou ME, Partridge L (2007) Role of insulin-like signalling in Drosophila lifespan. Trends Biochem. Sci. 32, 180–188.

Giannakou ME, Goss M, Junger MA, Hafen E, Levers SJ, Partridge L (2004) Long-lived Drosophila with overexpressed dFOXO in adult fat body. Science 305, 361.

Giannakou ME, Goss M, Jacobson J, Virti G, Levers SJ, Partridge L (2007) Dynamics of the action of dFOXO on adult mortality in Drosophila. Aging Cell 6, 429–438.

Giannakou ME, Goss M, Partridge L (2008) Role of dFOXO in lifespan extension by dietary restriction in Drosophila melanogaster: not required, but its activity modulates the response. Aging Cell 7, 187–198.

Grandison RC, Piper MD, Partridge L (2009) Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. Nature 462, 1061–1064.

Greer EL, Brunet A (2005) FOXO transcription factors at the interface between longevity and tumour suppression. Oncogene 24, 7410–7425.

Greer EL, Brunet A (2008) FOXO transcription factors in ageing and cancer. Acta Physiol. (Oxf) 192, 19–28.

Gronke S, Clarke DF, Broughton S, Andrews TD, Partridge L (2010) Molecular evolution and functional characterization of Drosophila insulin-like peptides. PLoS Genet. 6, e1000857.

Honda Y, Honda S (1999) The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. FASEB J. 13, 1385–1393.

van der Horst A, Burgering BM (2007) Stressing the role of FoxO proteins in lifespan and disease. Nat. Rev. Mol. Cell Biol. 8, 440–450.

Hsu HJ, Drummond-Barbosa D (2009) Insulin levels control female germline stem cell maintenance via the niche in Drosophila. Proc. Natl Acad. Sci. USA 106, 1117–1211.

Hsu HJ, LaFever L, Drummond-Barbosa D (2008) Diet controls normal and tumour germline stem cells via insulin-dependent and -independent mechanisms in Drosophila. Dev. Biol. 313, 700–712.

Hwangbo DS, Gershman B, Tu MP, Palmer M, Tatar M (2004) Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature 429, 562–566.

Ikeya T, Galic M, Belawat P, Naiz K, Hafen E (2002) Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. Curr. Biol. 12, 1293–1300.

Junger MA, Rintelen F, Stocker H, Wasserman JD, Vegh M, Radimerski T, Greenberg ME, Hafen E (2003) The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signalling. J. Biol. 2, 20.

Kenyon C (2005) The plasticity of aging: insights from long-lived mutants. Cell 120, 449–460.

Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A. elegans mutant that lives twice as long as wild type. Nature 366, 461–464.

Kleiber M, Magi R, Westendorp RG, Slagboom PE, Remm M, van Heemst D (2007) Haplotypes in the human Foxo1a and Foxo3a genes; impact on disease and mortality at old age. Eur. J. Hum. Genet. 15, 294–301.

LaFever L, Drummond-Barbosa D (2005) Direct control of germline stem cell division and cyst growth by neural insulin in Drosophila. Science 309, 1071–1073.
Larsen PL (1993) Aging and resistance to oxidative damage in Caenorhabditis elegans. Proc. Natl Acad. Sci. USA 90, 8905–8909.

McElwae JJ, Schuster E, Blanc E, Piper MD, Thomas JH, Patel DS, Selman C, Wthers DJ, Thornton JM, Partridge L, Gems D (2007) Evolutionary conservation of regulated longevity assurance mechanisms. Genome Biol. 8, R132.

Min KI, Yamamoto R, Buc H, Pankraz M, Tatar M (2008) Drosophila lifespan control by dietary restriction independent of insulin-like signaling. Aging Cell 7, 199–206.

Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Arrhringer J, Li H, Kenyon C (2003) Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature 422, 277–283.

Nielsen MD, Luo X, Bietas B, Syverson K, Jasper H (2008) 14-3-3 Epsilon antagonizes FoxO to control growth, apoptosis and longevity in Drosophila. Aging Cell 7, 688–699.

Orme MR, Altrubiae S, Bradley GL, Walker CD, Leevs SJ (2006) Input from Ras is required for maximal PI3K signalling in Drosophila. Nat. Cell. Biol. 8, 1298–1302.

Partridge L, Bruning JC (2008) Forkhead transcription factors and ageing. Oncogene 27, 2351–2363.

Puig O, Marr MT, Ruhf ML, Tjian R (2003) Control of cell number by FOXO: downstream and feedback regulation of the insulin receptor pathway. Genes Dev. 17, 2006–2020.

Richard DS, Rybczynski R, Wilson TG, Wang Y, Wayne ML, Zhou Y, Partridge L, Harshman LG (2005) Insulin signaling is necessary for vitellogenesis in Drosophila melanogaster independent of the roles of juvenile hormone and ec dys teroids: female sterility of the chsc1 insulin signaling mutation is autonomous to the ovary. J. Insect Physiol. 51, 455–464.

Saith DA, Brunet A (2008) FOXO transcription factors in the maintenance of cellular homeostasis during aging. Curr. Opin. Cell Biol. 20, 126–136.

Schuster E, McElwae JJ, Tullet JM, Doonan R, Matthijssen F, Reece-Hoyes JS, Hope IA, Vanfleteren JR, Thornton JM, Gems D (2010) DamiD in C. elegans reveals longevity-associated targets of DAF-16/FoxO. Mol. Syst. Biol. 6, 399.

Selman C, Lingham S, Choudhry AI, Battenham RL, Clare M, Clements M, Ramadani F, Okkenhaug K, Schuster E, Blanc E, Piper MD, Al-Qassab H, Speakman JR, Carmignac D, Robison IC, Thornton JM, Gems D, Partridge L, Withers DJ (2008) Evidence for lifespan extension and delayed age-related biomarkers in Drosophila embryo transcriptome null mice. FASEB J. 22, 807–818.

Slack C, Wrez C, Wieser D, Aich N, Foley A, Stocker H, Withers DJ, Thornton JM, Hafen E, Partridge L (2010) Regulation of lifespan, metabolism, and stress responses by the Drosophila SH2B protein, Lnk. PLoS Genet. 6, 1000881.

Sokol RR, Rohlf FJ (1998) Bimetry. New York, WH Freeman.

Taguchi A, Wartschow LM, White MF (2007) Brain IIS2 signaling coordinates life span and nutrient homeostasis. Science 317, 369–372.

Tatar M, Bartke A, Antebi A (2003) The endocrine regulation of aging by insulin-like signals. Science 299, 1346–1351.

Tissenbaum HA, Ruvkun G (1998) Biometry. New York, WH Freeman.

Tricoire H, Battisti V, Trannoy S, Lasbleiz C, Pret AM, Monnier V (2009) The ste-FoxO and insulin-like signaling, C. Slack

Tricoire H, Battisti V, Trannoy S, Lasbleiz C, Pret AM, Monnier V (2009) The ste-FoxO and insulin-like signaling, C. Slack

Wesells RJ, Bodmer R (2007) Age-related cardiac deterioration: insights from Drosophila. Front. Biosci. 12, 39–48.

Wesells RJ, Fitzgerald E, Piazza N, Ocorr K, Morley S, Davies C, Lim HY, Elmen L, Hayes M, Oldham S, Bodmer R (2009) dI4eBP acts downstream of both dTOR and dFoxo to modulate cardiac functional aging in Drosophila. Aging Cell 8, 542–552.

Willcox B, Donlon TA, He Q, Chen R, Grove JS, Yano K, Masaki KH, Willcox DC, Rodriguez B, Curb JD (2008) FOXO3A genotype is strongly associated with human longevity. Proc. Natl Acad. Sci. USA 105, 13987–13992.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 (A) Survival curves of female daGS/+ flies +RU486 and −RU486. Experiments were started with 100 flies per condition. No significant difference in survival was detected between treatments by Log-rank test. (B) Average number of eggs laid per female after 7 days of RU486 treatment (+RU486) or untreated (−RU486). Data are presented as the mean number of eggs laid per female over a 24-h period ± SEM. Eggs were counted from ten separate vials and each vial contained ten females. No significant difference was detected between treatments by ANOVA. (C) Survival of daGS/+ flies in the presence of 20 mM paraquat after 7 days of RU486 treatment (+RU486) or untreated (−RU486). Experiments were started with 100 flies per treatment. No significant difference was detected between treatments by Log-rank test. (D) Survival of daGS/+ flies in the presence of DDT after 7 days of RU486 treatment (+RU486) or untreated (−RU486). Experiments were started with 100 flies per treatment. No significant difference was detected between treatments by Log-rank test.

Fig. S2 (A) Egg to adult development time in males and females of the indicated genotypes. Only the eclosion period of the adult flies is shown. Data are presented as means ± SEM. (B) Body weights and wing areas as indicators of adult fly body size in females of the indicated genotypes. Data are presented as means ± SEM (n = 10 for each measurement). The reduced body size of daGS homozygotes is also observed in transheterozygotes of daGS+/− and hemizygous daGS+/Δ-fl(3R)ED5624. (Males: wDahomey, n = 114; daGS+/+, n = 94; daGS+/Δ-fl(3R)ED5624, n = 106; daGS+/Δ-fl(3R)ED5624, n = 98; daGS+/Δ-fl(3R)ED5624, n = 112; daGS+/Δ-fl(3R)ED5624, n = 84. Females: wDahomey, n = 107; daGS+/+, n = 106; daGS+/Δ-fl(3R)ED5624, n = 116; daGS+/Δ-fl(3R)ED5624, n = 114; daGS+/Δ-fl(3R)ED5624, n = 118; Δ-fl(3R)ED5624, n = 93; daGS+/Δ-fl(3R)ED5624, n = 99). (B) Body weights and wing areas as indicators of adult fly body size in females of the indicated genotypes. Data are presented as means ± SEM (n = 10 for each measurement). The reduced body size of daGS homozygotes is also observed in transheterozygotes of daGS+/− and hemizygous Δ-fl(3R)ED5624/daGS+/Δ-fl(3R)ED5624 (***P < 0.05, t-test).

Fig. S3 Effects of rapamycin treatment on the survivorship of daGS+/Δ-fl(3R)ED5624 mutants.

Fig. S4 Lifespan and fecundity responses of daGS+/Δ-fl(3R)ED5624 mutant females to dietary restriction.

Table S1 Median lifespans and statistical analysis of daGS+/Δ-fl(3R)ED5624 mutants and control flies across a food dilution series.

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