Cell-Nonautonomous Effects of dFOXO/DAF-16 in Aging

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SUMMARY

Drosophila melanogaster and Caenorhabditis elegans each carry a single representative of the Forkhead box O (FoxO) family of transcription factors, dFOXO and DAF-16, respectively. Both are required for lifespan extension by reduced insulin/igf signaling, and their activation in key tissues can extend lifespan. Aging of these tissues may limit lifespan. Alternatively, FoxOs may promote longevity cell non-autonomously by signaling to themselves (FoxO to FoxO) or other factors (FoxO to other) in distal tissues. Here, we show that activation of dFOXO and DAF-16 in the gut/fat body does not require dfoxo/daf-16 elsewhere to extend lifespan. Rather, in Drosophila, activation of dFOXO in the gut/fat body or in neuroendocrine cells acts on other organs to promote healthy aging by signaling to other, as-yet-unidentified factors. Whereas FoxO-to-FoxO signaling appears to be required for metabolic homeostasis, our results pinpoint FoxO-to-other signaling as an important mechanism through which localized FoxO activity ameliorates aging.

INTRODUCTION

Forkhead box O (FoxO) transcription factors (TFs) are involved in a plethora of cellular processes to regulate whole-organism physiology and are major determinants of animal lifespan (Partridge and Brüning, 2008; Salih and Brunet, 2008). Activation of FoxO-family TFs mediates the lifespan-extending effects of dampered insulin/insulin-like growth factor-like signaling (IIS) in both worms and flies (Kenyon et al., 1993; Slack et al., 2011; Yamamoto and Tatar, 2011). This evolutionary conservation appears to extend to humans, because certain genetic variants of Foxo3A are robustly associated with human longevity (Flachsbart et al., 2009; Kuningas et al., 2007; Wilcox et al., 2008). Indeed, Forkhead-like TFs can even extend lifespan in a single-celled eukaryote, budding yeast (Postnikoff et al., 2012).

In Drosophila melanogaster, tissue-restricted activation of Drosophila foxo (dfoxo) is sufficient to extend lifespan (Demontis and Perrimon, 2010; Giannakou et al., 2004; Hwangbo et al., 2004). Such an increase in dfoxo activity confined to key tissues could promote whole-organism survival in two mutually compatible ways: cell autonomously and cell nonautonomously. The lifespan of the animal could be limited by pathology in a particular organ, so that cell-autonomous action of dfoxo in that organ alone could promote longevity (Rera et al., 2013). In addition, healthy aging may involve the coordinated action of multiple organ systems, with dfoxo in one organ altering whole-organism physiology through systemic changes (Demontis and Perrimon, 2010; Hwangbo et al., 2004; Rera et al., 2013). For example, adult-onset induction of dfoxo in the midgut and abdominal fat body (equivalent to mammalian liver and adipose) activates the transcription of Drosophila insulin-like peptide (dilp) 6 in the fat body, whereas in muscle dfoxo represses the activin ligand dowdle, and these endocrine signals have a distal effect on the median neurosecretory cells (mNSCs) in the brain, resulting in lowered DILP2 peptide in circulation (Bai et al., 2012, 2013). Importantly, upregulation of dilp6 is required for the beneficial effect of dfoxo on lifespan (Bai et al., 2012). However, whether this requires dfoxo in tissues other than the ones producing the DILP6 signal remains unexamined.

The single Caenorhabditis elegans FoxO ortholog, DAF-16, can act both cell autonomously and cell nonautonomously to regulate gene expression (Libina et al., 2003; Murphy et al., 2007; Qi et al., 2012; Zhang et al., 2013). DAF-16 activity in one tissue can induce DAF-16 activity in another in a process of tissue entrainment mediated by altered expression of an insulin-like peptide (Murphy et al., 2007), which is highly reminiscent of the situation in the fly. For this reason, it has been widely believed that the fruit fly’s dfoxo acts from specific cells to activate dFOXO in the whole animal in an instance of dfoxo-to-dfoxo signaling (Bai et al., 2012, 2013; Demontis and Perrimon, 2010; Hwangbo et al., 2004). However, the relevance of this tissue entrainment for Drosophila lifespan has not been experimentally tested. Indeed, there is a growing awareness that FoxOs in one tissue can also signal to other factors elsewhere, i.e., FoxO-to-other signaling. In the worm, DAF-16 activity in one tissue can elicit daf-16-independent responses in the receiving tissues (Qi et al., 2012; Zhang et al., 2013). The existence and relevance of dfoxo-to-other inter-tissue signaling is unexplored in Drosophila.

Here, we establish the relevance to aging of the cell-nonautonomous effects of dfoxo, differentiating between dfoxo-to-dfoxo
and dfoxo-to-other signaling in adult Drosophila. We find that

\[ \text{dfoxo-to-dfoxo signaling does not affect aging and confirm that} \]

the same is true of the worm daf-16. On the other hand, dfoxo in the gut and fat body can promote health of the neuro-muscular system, possibly via transcriptional regulation of a

secreted neuropeptide-like molecule, and dfoxo in mNSCs can extend lifespan. Both effects are independent of dfoxo’s presence in other tissues, demonstrating the relevance of
dfoxo-to-other signaling in Drosophila aging. At the same time, dfoxo-to-dfoxo signaling is required for the metabolic effects of localized dfoxo induction, showing that distinct physiological effects of tissue-restricted dfoxo activation are mediated by different signaling routes.

RESULTS

**dfoxo-to-dfoxo Signaling in Drosophila Is Dispensable for Extension of Lifespan by Gut/Fat Body or mNSC dfoxo**

To examine whether activation of dFOXO in other tissues contributes to the lifespan-extending effects of induction of dfoxo in the adult gut and fat body, we generated strains where the tissue-restricted induction of dfoxo could be triggered by the RU486 inducer in either an otherwise wild-type or a dfoxo-null background (S106>dfoxo or dfoxoΔ/Δ S106>dfoxo). We used females, where the effects of dfoxo activation on aging are clearly observed (Giannakou et al., 2004). Because the lifespan effects of ectopic dfoxo expression can be conditional on the nutritional status of the animal (Bai et al., 2012; Min et al., 2008), we used a food with the optimal amount of dietary yeast (10% weight/volume) for lifespan under our laboratory conditions (Bass et al., 2007) and where expression of dfoxo targeted to adult gut and fat body robustly extends lifespan (Giannakou et al., 2008). Importantly, on this food, lifespan is maximized so that the effects of dfoxo can be studied as additional to the beneficial effects of the diet.

We found no detectable expression of dFOXO protein or of dfoxo transcript in the dfoxoΔ/Δ S106>dfoxo females in the absence of the inducer (Figures 1A and 1B). Feeding RU486 for 5 days resulted in equivalent increases in dfoxo transcript in S106>dfoxo and dfoxoΔ/Δ S106>dfoxo females (Figure 1B; see Table 1 for detailed statistical analysis). The S106 driver has been thoroughly characterized and, in the female fly, only drives expression in the gut and fat body (Poirier et al., 2008). To ensure the flies are experiencing the same nutritional conditions, we examined their feeding behavior with the proboscis-extension assay (Wong et al., 2009) and found no significant differences (Figure S1A).

We examined the effect on lifespan resulting from the presence of the inducer in the S106>dfoxo and dfoxoΔ/Δ S106>dfoxo lines in two sequential, independent, experimental trials (Figure 1C), recording deaths of over 1,000 flies in total. The presence of RU486 from day 2 of adulthood extended the median lifespan of S106>dfoxo females on average by 6% (log-rank test p < 0.05 for each trial; Figure 1C). The magnitude of the effect was less than previously reported (Giannakou et al., 2004) but is consistent with more recent work in our laboratory (Giannakou et al., 2008) and with six other independent trials performed in the course of the last 4 years (2008–2012, average median extension 5%; Figure S1B). The lifespan of dfoxo-null flies was also extended by a similar percentage (average 10%, log-rank test p < 0.05 for each trial; Figure 1C). Thus, the presence of dfoxo in the rest of the body is not required for the lifespan-extending effects of its induction in the gut/fat body.

Flies lacking dfoxo have short lifespans (Figure 1C), possibly due to developmental effects of the mutation (Slack et al., 2011), complicating the direct comparison between effects of dfoxo-null and wild-type flies to RU486, we combined the two experimental trials and analyzed the survival data using a mixed-effects Cox proportional hazards (CPH) model (Table 1). Both RU486 (30% reduction in risk of death, $p = 2 \times 10^{-3}$) and the presence of genomic dfoxo ($p < 10^{-15}$) had a significant effect on lifespan, but their interaction did not ($p = 0.95$). The absence of a significant interaction confirms that the effect of RU486 did not differ between the lines and hence that the presence of dfoxo elsewhere in the body does not affect the extension of lifespan by induction of dfoxo in the gut and fat body. Thus, tissue enrichment through dfoxo-to-dfoxo signaling is not required for longevity.

This result indicated that either dfoxo acts cell autonomously to extend lifespan or that dfoxo in one tissue activates dfoxo-independent longevity-assurance mechanisms in other tissues. The latter would occur through dFOXO-to-other signaling, as has been observed for DAF-16 (Qi et al., 2012; Zhang et al., 2013). To further test for dfoxo-to-other signaling, we manipulated the levels of dfoxo in cells whose prominent function is in adult endocrine signaling. mNSCs in the adult brain play an important role in aging by producing DILP2, DILP3, and DILP5 (Broughton et al., 2005) and possibly other endocrine signals. Expressing dfoxo specifically in the mNSC, using a dlap2-GAL4 driver, extended the lifespan of female flies in both wild-type and dfoxo nulls ($p < 0.01$ to either control in both backgrounds; Figure 1D; Table 1). CPH analysis found significant effects of the genomic dfoxo ($p < 10^{-13}$) and its induction in dlap2GAL4>dfoxo flies ($50\%$ reduction in risk of death, $p = 2.2 \times 10^{-3}$) on lifespan but no evidence for a significant interaction between them ($p = 0.33$; Table 1). This confirmed that the effect on lifespan is independent of dfoxo in tissues other than the mNSCs.

This longevity phenotype must represent a gain of function in the mNSC, because the ablation of mNSCs, representing a loss of function in these cells, requires dfoxo to extend lifespan (Slack et al., 2011). Indeed, we observed no significant changes in the mRNA levels of dlap2, dlap3, and dlap5 upon induction of dfoxo in mNSCs (Figures S1C and S1D). Furthermore, we found no changes in the mRNA levels of any dlap detectable in whole adults (dlap2 through to dlap7), including dlap6 (Figure S1D), or their binding partner and regulator, Imp-L2 (Alc et al., 2011b) (Figure S1E), upon activation of dfoxo in the mNSCs, confirming that dlap2GAL4>dfoxo flies are not experiencing any alterations in systemic IIS activity. Because the principal role of these cells is
in endocrine signaling, the physiological effects of \textit{dfoxo} activation in the mNSCs are most likely to be mediated by \textit{dfoxo}-to-other signaling.

**Gut/Fat Body \textit{dfoxo} Acts at a Distance Independently of \textit{dfoxo} in Target Tissues**

To further investigate the role of \textit{dfoxo}-to-other signaling in fly physiology, we examined the beneficial effects of gut/fat body induction of \textit{dfoxo} on the neuromuscular system, an organ system distal to the site of \textit{dfoxo} activation in our model. The ability of flies to climb a vertical surface is a suitable physiological measure of the performance of this organ system and is susceptible to aging (Cook-Wiens and Grotewiel, 2002). We scored the number of low, medium, and high climbers in three cohorts of \textit{S\textsubscript{1106}>dfoxo} or \textit{dfoxo\textsubscript{D}/D} \textit{S\textsubscript{1106}>dfoxo} genotype in the presence or absence of RU486 over \textasciitilde10 weeks.

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**Figure 1. \textit{dfoxo-to-dfoxo} Signaling Is Not Required for the Antiaging Effects of Increased dFOXO Activity in the Gut/Fat Body or mNSC**

(A) Western blots of dFOXO and the tubulin loading control on whole-fly protein extracts from \textit{S\textsubscript{1106}>dfoxo} or \textit{dfoxo\textsubscript{D}/D} \textit{S\textsubscript{1106}>dfoxo} female flies in the absence of the inducer.

(B) \textit{dfoxo} transcript levels (relative to Act and with \textit{S\textsubscript{1106}>dfoxo} -RU486 set to 1) in \textit{S\textsubscript{1106}>dfoxo} or \textit{dfoxo\textsubscript{D}/D} \textit{S\textsubscript{1106}>dfoxo} female flies fed or not RU486.

(C) Survival of \textit{S\textsubscript{1106}>dfoxo} or \textit{dfoxo\textsubscript{D}/D} \textit{S\textsubscript{1106}>dfoxo} female flies in presence or absence of RU486 determined in two experimental trials (top and bottom panel).

(D) Survival of \textit{dilp2-GAL4>dfoxo} female flies, or the two genetic controls (\textit{dilp2-GAL4} or \textit{UAS-dfoxo} alone), in wild-type (WT) or \textit{dfoxo\textsubscript{D}/D} backgrounds.

(E) The proportion of high climbers (top panel) or combined medium and high climbers (bottom panel) in three cohorts (combined) of \textit{S\textsubscript{1106}>dfoxo} or \textit{dfoxo\textsubscript{D}/D} \textit{S\textsubscript{1106}>dfoxo} female flies in the presence or absence of RU486.

Note the same color code is used in (B), (C), and (E) and is given in (C). See Table 1 for statistical analysis of data in (B)–(E). Where used, box plots indicate median, first and third quartile, data range, and outliers. See also Figure S1.
Induction of \textit{dfoxo} expression in the gut and fat body enhanced the climbing ability of female flies throughout their lifespan, observed as an increase in the proportion of high, or combined medium and high, climbers (Figure 1E). This enhancement could be seen in both the wild-type and \textit{dfoxo}-null backgrounds, revealing that it is independent of \textit{dfoxo} in other tissues. Indeed, statistical analysis (mixed-effects ordinal logistic model, Table 1) confirmed that the effect of RU486 (\(p = 1.8 \times 10^{-3}\)) and \textit{dfoxo} (\(p = 5.4 \times 10^{-3}\)) were both significant but that their interaction was not (\(p = 0.12\)). Hence, local action of \textit{dfoxo} in the gut and fat body has a beneficial effect on the performance of a distal organ system. This could occur through systemic effects of healthy gut and fat body or through specific signaling events. In the latter case, its independence from \textit{dfoxo} in the distal cells is again consistent with \textit{dfoxo}-to-other signaling.

**Gut/Fat Body \textit{dfoxo} Regulates Expression of \textit{Nplp4}**

In order to trigger \textit{dfoxo}-to-other signaling, the gut/fat body \textit{dfoxo} may regulate the expression of a secreted factor other than \textit{dilp6}. To identify such a factor, we determined the whole-fly, genome-wide, transcriptional changes induced by RU486 in the \(S_{1}106\text{-}\textit{dfoxo}\) flies (Table S1; Figure 2A). We found that, besides the documented changes in \textit{dilp6} (Bai et al., 2012), induction of the gut/fat body \textit{dfoxo} altered the mRNA levels of another gene encoding a signal peptide targeting its protein product for secretion, \textit{neuropeptide-like precursor 4} (\textit{Nplp4}). The mature product of this gene is a YSY peptide of previously unknown function (Nässel and Winther, 2010). Quantitative PCR confirmed that activation of \textit{dfoxo} led to repression of this gene (Figure 2B). Hence, \textit{Nplp4} is a candidate for a secreted factor mediating \textit{dfoxo}-to-other signaling. Interestingly, this gene was repressed in both heads and bodies of \(S_{1}106\text{-}\textit{dfoxo}\) females fed RU486 (\(p = 0.048\) for RU486, \(p = 0.19\) for body part:RU486 interaction; Figure 2B; Table 1), whereas, as expected, the induction of \textit{dfoxo} itself was confined to the body, (\(p = 0.053\) for body part:RU486 interaction; Figure 2C; Table 1), indicating \textit{Nplp4} responds to \textit{dFOXO} both locally and distally.

**Importance of \textit{dfoxo}-to-\textit{dfoxo} Signaling to \textit{Drosophila} Metabolism**

Although \textit{dfoxo}-to-\textit{dfoxo} signaling is not required for lifespan extension, it may be required for other physiological effects in response to the activation of \textit{dFOXO} in gut and fat body. To query the existence of these other physiological effects, we examined whether there are transcriptional changes in response to RU486 in \(S_{1}106\text{-}\textit{dfoxo}\) flies that do not occur in \textit{dfoxo}\(\Delta\)/\(Δ\) \(S_{1}106\text{-}\textit{dfoxo}\) females. We reasoned that the genes and processes that respond to RU486 in \(S_{1}106\text{-}\textit{dfoxo}\) flies but fail to do so in the \textit{dfoxo}\(\Delta\)/\(Δ\) \(S_{1}106\text{-}\textit{dfoxo}\) females may be regulated through \textit{dfoxo}-to-\textit{dfoxo} signaling.

Among the genes regulated by RU486 in \(S_{1}106\text{-}\textit{dfoxo}\) females, we identified all those for which the RU486-induced transcriptional change was altered by mutation of \textit{dfoxo} by finding the genes whose transcript levels show a significant interaction between the presence of genomic \textit{dfoxo} and its induction by RU486 in the relevant linear model (Figure 2A; Table S1). The magnitude of fold-change for these genes was reduced on average in \textit{dfoxo}\(\Delta\)/\(Δ\) \(S_{1}106\text{-}\textit{dfoxo}\) compared to \(S_{1}106\text{-}\textit{dfoxo}\) females (Figure 2A), indicating they require the presence of genomic \textit{dfoxo} for correct expression. We confirmed the significance of this effect using a linear model (\(p = 1 \times 10^{-7}\);Figure 2A and the associated caption). Note that \textit{Nplp4} was equally repressed in \textit{dfoxo}\(\Delta\)/\(Δ\) \(S_{1}106\text{-}\textit{dfoxo}\) and \(S_{1}106\text{-}\textit{dfoxo}\) females (Table S1).

Examination of the Gene Ontology categories enriched in this group of genes revealed “proteolysis” as overrepresented (\(p = 3.1 \times 10^{-7}\); Table S1), hinting that protein metabolism may be regulated through a \textit{dfoxo}-to-\textit{dfoxo} signal. Pursuing this lead, we found that RU486 feeding triggered a small (12%) but significant reduction in total protein content of \(S_{1}106\text{-}\textit{dfoxo}\) females and that this effect was blocked by deletion of \textit{dfoxo} (Figure 2D; \(p = 3.1 \times 10^{-3}\) for RU486 by genotype interaction; Table 1). Similar significant changes were not observed in total triglyceride, total trehalose, or total glycogen content (Figure S2A). However, due to assay variability, we cannot discount possible subtle changes in these metabolites. On the other hand, total body mass followed closely the protein content (Figure 2E; Table 1).

Surprisingly, both deletion of \textit{dfoxo} and its induction in the gut and fat body reduced total protein content and fly weight. The two manipulations may act in different ways. The small size of \textit{dfoxo} nulls is due to the developmental effects of the mutation (Slack et al., 2011) and, together with their reduced fecundity (Slack et al., 2011) (Figure S2B), could explain the lowered body weight and protein content. On the other hand, \(S_{1}106\text{-}\textit{dfoxo}\) was induced in adulthood and had no effect on fecundity in either wild-type or \textit{dfoxo}-null females (Figure S2B), but it had an effect on body weight and protein content. Hence, the latter two metabolic phenotypes of \textit{dFOXO} activation in gut/fat body may depend on \textit{dfoxo}-to-\textit{dfoxo} signaling. However, because \textit{dfoxo} was absent in all tissues throughout development, we cannot exclude the possibility that the inability of \textit{dfoxo} nulls to respond to RU486, for these traits, is due to developmentally altered gut/fat body function. Note that the expression pattern of the proteolysis genes, which initially led us to this phenotype, cannot mechanistically explain the loss of protein in \(S_{1}106\text{-}\textit{dfoxo}\) females upon RU486 feeding, because these genes are repressed in this condition (Figure S2C), but may rather be part of a homeostatic mechanism. Nevertheless, the results strongly indicate the effects on lifespan and metabolism of tissue-restricted activation of \textit{dFOXO} can be separated by their requirement for \textit{dfoxo}-to-other or \textit{dfoxo}-to-\textit{dfoxo} signaling.

**daf-16-to-daf-16 Signaling in \textit{C. elegans} Is Dispensable for Extension of Lifespan by Gut daf-16**

The worm intestine serves a functionally similar role to the gut and fat body in \textit{Drosophila}. It is an important \textit{daf-16} signaling center, and increased \textit{DAF-16} activity in this organ activates \textit{DAF-16} elsewhere (Libina et al., 2003; Murphy et al., 2007). However, in the context of reduced IIS resulting from mutation in \textit{daf-2}, \textit{daf-16} presence solely in the intestine is sufficient substantially, but not completely, to restore the \textit{daf-2} mutant longevity in otherwise \textit{daf-16}-deficient worms (Libina et al., 2003). This indicates that the observable \textit{daf-16}-to-\textit{daf-16} signaling is not essential for lifespan extension. However, the
### Table 1. Statistical Analysis

| Relevant Figure | Model and Description | Random Effect | Coefficient | Estimate | SE   | p Value | Description |
|-----------------|-----------------------|---------------|-------------|----------|------|---------|-------------|
| **Figure 1B**   | mixed-effects linear  | n = 7–8 batch | intercept   | 0.81     | 0.15 | <10^-4 | dfoxo       |
|                 |                       |               | dfoxo       | 0.571    | 7.1 x 10^-2 |      |
|                 |                       |               | RU486       | 0.311    | 7.1 x 10^-2 | 2.0 x 10^-4 |
|                 |                       |               | dfoxo:RU486 | 6.6 x 10^-2 | 7.1 x 10^-2 | 0.36 |
| **Figure 1C**   | MECPH                 | 1,050 deaths (1,078 total) experimental trial | dfoxo | -2.1 | 0.11 | <10^-15 |
|                 |                       |               | RU486       | -0.34    | 9.1 x 10^-2 | 2 x 10^-4 |
|                 |                       |               | dfoxo:RU486 | 8.5 x 10^-3 | 0.13 | 0.95 |
| **Figure 1D**   | CPH                   | 533 deaths (545 total) NA | dfoxo | -3.26 | 0.22 | <10^-15 |
|                 |                       |               | UAS-dfoxo   | -0.23    | 0.15 | 0.13 |
|                 |                       |               | dlap2GAL4>dfoxo | -0.65 | 0.15 | 2.2 x 10^-5 |
|                 |                       |               | dfoxo:UAS-dfoxo | 0.28 | 0.21 | 0.19 |
|                 |                       |               | dfoxo:dlap2GAL4>dfoxo | 0.21 | 0.22 | 0.33 |
| **Figure 1E**   | mixed-effects ordinal logistic | total observations = 2,179 biological repeat (vial) time | dfoxo | -7.1 x 10^-2 | 7.8 x 10^-3 | <10^-15 |
|                 |                       |               | RU486       | 0.81     | 0.26 | 1.8 x 10^-3 |
|                 |                       |               | time:dfoxo  | -5.4 x 10^-3 | 9.8 x 10^-3 | 0.58 |
|                 |                       |               | time:RU486  | 2.9 x 10^-3 | 0.01 | 0.78 |
|                 |                       |               | dfoxo:RU486 | -0.59    | 0.38 | 0.12 |
|                 |                       |               | time:dfoxo:RU486 | 8.1 x 10^-3 | 1.3 x 10^-2 | 0.54 |
| **Figure 2B**   | linear                | n = 5 NA      | intercept   | 3.4      | 0.25 | <10^-4 |
|                 |                       |               | head        | 2.6      | 0.25 | <10^-4 |
|                 |                       |               | RU486       | -0.54    | 0.25 | 4.8 x 10^-2 |
|                 |                       |               | head:RU486  | -0.34    | 0.25 | 0.19 |
| **Figure 2C**   | linear                | n = 5 NA      | intercept   | 2.8      | 0.25 | <10^-4 |
|                 |                       |               | head        | 1.2      | 0.25 | <10^-4 |
|                 |                       |               | RU486       | 0.38     | 0.25 | 1.8 x 10^-2 |
|                 |                       |               | head:RU486  | -0.28    | 0.25 | 5.4 x 10^-2 |
| **Figure 2D**   | mixed-effects linear  | n = 8–10 batch | intercept   | 0.12     | 1.4 x 10^-2 | 6.8 x 10^-2 |
|                 |                       |               | dfoxo       | 1.7 x 10^-2 | 1.8 x 10^-3 | <10^-4 |
|                 |                       |               | RU486       | -5.5 x 10^-3 | 1.8 x 10^-3 | 4 x 10^-3 |
|                 |                       |               | dfoxo:RU486 | -5.7 x 10^-3 | 1.8 x 10^-3 | 3.1 x 10^-3 |
| **Figure 2E**   | mixed-effects linear  | n = 27–30 batch | intercept   | 1.6      | 0.03 | <10^-4 |
|                 |                       |               | dfoxo       | 9.6 x 10^-2 | 1.3 x 10^-2 | <10^-4 |
|                 |                       |               | RU486       | -3.8 x 10^-2 | 1.3 x 10^-2 | 3.3 x 10^-3 |
|                 |                       |               | dfoxo:RU486 | -2.6 x 10^-2 | 1.3 x 10^-2 | 0.04 |
| **Figures 3A and S3A** | MECPH | 944 deaths (1,128 total) experimental trial | daf-16 | -2.5 | 0.11 | <10^-15 |
|                 |                       |               | intestinal daf-16 | -1.3 | 0.10 | <10^-15 |
|                 |                       |               | daf-16:intestinal daf-16 | 1.1 | 0.14 | 2 x 10^-15 |

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question remains whether daf-16-to-daf-16 signaling contributes to the longevity promoted by DAF-16 alone.

To investigate this, we tested for a role of daf-16-to-daf-16 signaling in longevity induced by daf-16 overexpression in the intestine. Increasing DAF-16 activity alone, within otherwise wild-type worms, has two opposing effects on lifespan. DAF-16 stimulates germline hyperplasia in a cell-nonautonomous manner (Qi et al., 2012). This shortens the animal's lifespan and masks the second, prolongevity effect of DAF-16 (Qi et al., 2012). Indeed, semiquantitative overexpression of daf-16 extends lifespan only if the germline cell proliferation is blocked, e.g., by administration of 5-fluoro-2'-deoxyuridine (FUdR) (Qi et al., 2012).

Similar to Qi and coworkers, we used FUdR to reveal the lifespan extension caused by overexpression of daf-16 from the intestinal-specific ges-1 promoter in otherwise wild-type worms and asked whether this effect required the presence of daf-16 in other tissues (Figure 3A). We found that intestinal activation of DAF-16 can extend lifespan of both wild-type and daf-16-deficient (daf-16(mu86)) worms fed HTT15 bacteria (log-rank p < 0.05 in three out of five and three out of three assays, respectively; Figures 3A and S3A). We confirmed these findings using a second, independently derived transgene (Figure S3B).

Upon further examination, we found that the ability of the intestinal daf-16 to extend wild-type lifespan was conditional on the food source and had a small but opposite effect when worms were fed on OP50 bacteria (Figure S3C). This is similar to the effect of gut/fat body expression of dfoxo on Drosophila lifespan, which can depend on available nutrition (Bai et al., 2012; Min et al., 2008). Importantly, however, the ability of the intestinal daf-16 to extend lifespan in the absence of daf-16 elsewhere was observed under all conditions, including the worms fed OP50 (Figures 3 and S3A–S3C). MECPH analysis of the combined data obtained with one of the transgenes on HTT15 bacteria (Figures 3 and S3A) confirmed that both the effects of daf-16 presence in the whole worm and its intestinal induction were significant (p < 10⁻⁴ for both) and revealed a significant interaction of the two main effects (p = 2 × 10⁻⁸; Table 1). Thus, intestinal daf-16 extended the lifespan of the mutant more than that of the wild-type worms (Figure 3), confirming that, as in the fly (Figure 1C) and during IIS dampening in the worm (Libina et al., 2003), tissue entrainment through daf-16-to-daf-16 signaling is not required for lifespan extension and, indeed, could even have the opposite effect.

Prompted by the findings in the fly (Figure 2D), we also examined if the induction of intestinal daf-16 in worms had an effect on their total protein content. We found that the intestinal daf-16 reduced whole-worm protein content (p < 10⁻⁴; Figure 3B; Table 1) on HTT15 bacteria. In contrast to the fly, we found no evidence that this reduction is prevented by the absence of daf-16 in other tissues, and, in fact, found that mutation of daf-16 increases the overall protein content (Figure 3B; Table 1). We obtained similar results with the second transgene (Figure S3D). Hence, this phenotype is mediated either cell autonomously by daf-16 in the intestine or through daf-16-to-other signaling. Thus, whereas the physiological effect appears conserved between the fly and worm, the way it is mediated differs.

It is also of note that, similar to the lifespan effect of intestinal daf-16 in an otherwise wild-type worm, we found this modulation of protein content conditional on the bacterial food source and neither the transgenes nor mutation of daf-16 had any significant effect when worms were fed OP50 bacteria (data not shown). For both lifespan and protein content, the alteration of phenotype between OP50 and HTT15 is reminiscent of the lifespan effects of certain sensory mutants in C. elegans (Maier et al., 2010) and suggests that intestinal DAF-16 plays a role in food perception.

DISCUSSION

In the fly, tissue-restricted dFOXO triggers endocrine factors to cause a drop in overall, systemic, IIS activity (Bai et al., 2012, 2013; Demontis and Perrimon, 2010; Hwangbo et al., 2004). Because insulin signals repress the activity of FoxOs (Brunet et al., 1999), this will result in body-wide activation of dFOXO (tissue entrainment), including further activation of dFOXO in the specific tissue (positive feedback). Our results show that the tissue entrainment is not required for the beneficial effects of dfoxo on lifespan or on healthspan. The regulation of systemic IIS by local dfoxo can still be relevant to lifespan as part of a positive feedback loop. For example, the upregulation of dilp6 by dFOXO in the fat body triggers a reduction in global IIS activity, and this, in turn, could be affecting lifespan by fine-tuning the activity of dFOXO in the fat body itself.

Table 1. Continued

| Relevant Figure | Model and Description | Random Effect | Coefficient⁵ | Estimate⁵ | SE | p Value |
|-----------------|-----------------------|---------------|--------------|-----------|----|---------|
| Figure 3B       | linear                |               | intercept    | 0.52      | 7.9 × 10⁻³ | <10⁻⁴ |
|                 | n = 8–16              | NA            | daf-16       | -2.3 × 10⁻² | 7.9 × 10⁻³ | <10⁻⁴ |
|                 |                       |               | intestinal daf-16 | -3.7 × 10⁻² | 7.9 × 10⁻³ | 6.2 × 10⁻³ |
|                 |                       |               | daf-16:intestinal daf-16 | -9.3 × 10⁻³ | 7.9 × 10⁻³ | 0.24 |

⁴In all models, the effect of presence of dfoxo (daf-16) or its induction (overexpression) is examined; dilp2-GAL4 was used as reference for dfoxo; “body” was used as reference for body versus head comparisons; “:“ indicates interaction term.

⁵For mixed-effect linear models and linear models, the coefficient estimates either have no units because they are derived from the dfoxo/Act or Nplp4/Act transcript ratios (for Figures 1B, 2B, and 2C) or are given in mg (for Figures 2D and 2E) or μg (Figure 3B). For MECPH and CPH models, the coefficient estimate is the natural log of the hazard ratio, where a negative value indicates a beneficial effect on survival. For mixed-effect ordinal logistic, these are log-transformed odds of climbing high, where a negative value indicates a reduction in climbing ability.
Under certain experimental conditions, the lifespan effects of ectopic dfoxo expression can be conditional on the nutrients available to the animal (Bai et al., 2012; Min et al., 2008). Hence, tissue entrainment may also have conditional relevance. In addition, our results indicate that dfoxo-to-dfoxo signaling is required for the metabolic effects of localized dfoxo induction, namely a drop in protein content and fly weight, and further examination may reveal roles for dfoxo-to-dfoxo signals in yet other aspects of physiology.

Both DAF-16 in C. elegans and dFOXO in Drosophila can extend lifespan from the gut/fat body without being present in other tissues. The gut and/or fat body may represent the organs most vulnerable to aging, so that DAF-16/dFOXO directly prevents the otherwise lethal age-related pathologies in these organs. This, in turn, could have indirect benefits for other organs. Indeed, there is some evidence that the health of the Drosophila gut limits lifespan (Rera et al., 2013). Furthermore, DAF-16/dFOXO could regulate key metabolic genes in these tissues, such as lipases, fatty acid catabolic genes, and others, effecting a shift in energy utilization toward prolonged health and survival. However, dFOXO activity in other tissues, such as the muscle (Demontis and Perrimon, 2010) or the mNSC (Figure 1D), can also extend lifespan. Although it is conceivable that multiple tissues independently and simultaneously limit lifespan, in at least some of these interventions, the relevant effects must be cell nonautonomous.

DAF-16 in one tissue is known to trigger DAF-16-independent responses in other tissues (Qi et al., 2012; Zhang et al., 2013). In one case, this is mediated by induction of a transcriptional mediator, mdt-15, and is required in part for the beneficial effects of the intestinal activation of DAF-16 by daf-2(−) on whole-organism aging (Zhang et al., 2013). Our results indicate that dFOXO
may also initiate dfoxo-independent processes in the receiving tissues that counteract whole-organism aging. This is the most likely mechanism whereby its activity in the Drosophila mNSC can extend lifespan, and a similar mechanism may underlie the health benefits observed when it is induced in the gut and fat body. The search for the factors that mediate this effect of dFOXO at a distance is now of interest, and we identified Nplp4 as a candidate. The evolutionary persistence of this FoxO-to-other signaling between the fly and the worm strongly suggests that its relevance may extend to mammals.

**EXPERIMENTAL PROCEDURES**

**Fly Husbandry and Experiments**

All transgenes and the dfoxo mutant were backcrossed at least six times into the wild-type outbred Dahomey population carrying the w1118 mutation and cured of Wolbachia infection and frequently outcrossed back into the wild-type population. The Dahomey stock was collected in 1970 in Dahomey (now Benin) and has been kept in population cages maintaining its lifespan and fecundity at levels similar to freshly caught stocks. The lines were maintained, and all experiments performed, at 25°C with 60% humidity and 12 hr:12 hr light:dark cycle on sugar-yeast-agar (1SYA) food (Bass et al., 2007). Experimental flies developed at standardized densities and once-mated females were sorted on day 2 of adulthood onto food containing 200 μM RU486 (Sigma) or control food as required (15 per vial for climbing assays, five for feeding, and ten for all others). Flies were harvested on day 7 for weight and metabolite measurements and protein and RNA analysis. Sample preparation and hybridizations to Droso Affymetrix arrays were performed and data analyzed with LIMMA, essentially as described elsewhere (Alic et al., 2011a). For further details, see Supplemental Experimental Procedures. Gene lists are given in Table S1.

**Worm Husbandry and Experiments**

Worms were maintained at 20°C unless otherwise indicated. Prior to experiments, animals were maintained at the permissive temperature and grown for at least one generation in the presence of food to assure full viability. Lifespan assays were performed on HT115 bacteria carrying empty pL4440 vector, or OP50 bacteria, in the presence of 10 μM FUDR. Worms were placed on these plates at the L4 stage and scored as dead or alive every 2–3 days. For further details, see Supplemental Experimental Procedures.

**Statistical Analysis**

Analyses were performed in JMP (SAS) or R. Further details are given in Table 1 and Supplemental Experimental Procedures. To determine difference in slopes of the regression lines between the two gene sets in Figure 2A, the linear model was fitted with RU486-induced response in dfoxo D/D S1106>dfoxo as the dependent variable and the response in S1106>dfoxo (continuous) and gene set (categorical) as explanatory variables, testing for the significance of the interaction term.

**ACCESSION NUMBERS**

The ArrayExpress accession number for the array data reported in this paper is E-MTAB-1232.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.01.015.

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Supplemental Information

Cell-Nonautonomous Effects of dFOXO/DAF-16 in Aging

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David Gems, and Linda Partridge
Figure S1
Figure S1

A Feeding events as proportion of total observed events (fly occurrences) per vial for the $S_{106}>dfoxo$ and $dfoxo\Delta/\Delta$ $S_{106}>dfoxo$ females (15 and 9 vials per food condition, respectively) on food with or without RU486. The data were analysed with a Generalised Linear Model with binomial distribution and an overdispersion parameter. There was no significant effect of genotype (p=0.4), presence of RU486 (p=0.08) or their interaction (p=0.4). B Box plot representing the median lifespan extension (as % of control) caused by RU486 feeding in $S_{106}>dfoxo$ females in six independent trials. Log-rank test revealed that RU486 had a significant effect in each case (p<0.05). C mRNA levels of dilp2, dilp3 and dilp5 in heads of female flies of the indicated genotype. Data were analysed with a Linear Model and no significant effect of transcript or genotype, or any interaction, was found (p>0.05, n=4-5). D mRNA levels of dilp2 through to dilp7 in whole female flies of the indicated genotype, with the level in dilp2GAL4 control set to 1 and n=6. Data were analysed with a Linear Model and no significant effect of genotype was found (p=0.2), while there was a significant effect of the transcript (p<10^{-4}) and a significant interaction of the two (p=2x10^{-4}), but in no case was dilp2GAL4>dfoxo significantly different to both of the controls by t-test (p>0.05). Note the levels of dilp4 in the UAS-dfoxo control are different to dilp4 levels in the two other genotypes, and this is most likely due to the effect of the driver alone on dilp4 levels. dilp1 transcript is undetectable in whole adult females. E mRNA levels of Imp-L2 in whole female flies of the indicated genotype, with the level in dilp2GAL4 control set to 1 and n=6. ANOVA revealed no significant differences (p=0.3).
Figure S2
**Figure S2**

**A** Trehalose, TAG and glycogen were quantified per fly for the four conditions shown (n=8-15 per condition). Data were analysed per compound with a Mixed Effect Linear Model with genotype and RU486 as main effects, their interaction, and experimental batch as random effect. Only the effect of genotype for glycogen was borderline significant (p=0.050), all others were not. **B** Eggs laid per female per 24h for the same four conditions over the first ~4 weeks of life determined in two experimental trials (two panels, means ± SEM). The data were analysed with Mixed Effect Linear Model with time, genotype and RU486 as main effects, their interactions, and experimental trial as random effect. The effects of genotype (genomic dfoxo), time and their interaction were all significant (p<10^-4) but RU486 or any of its interactions were not (p>0.05). The same colour code is used in A and B. **C** Heatmap of the expression levels detected by microarray analysis for proteolysis genes that show a different response to RU486 in $S_{106}>dfoxo$ and $dfoxo\Delta/\Delta S_{106}>dfoxo$ females. Each column represents a separate array with the expression levels scaled to the average of the $S_{106}>dfoxo$ -RU486 condition.
Figure S3
**Figure S3**

A Further experimental trials looking at the effect of gut over-expression of *daf-16* (*muEx211[ges-1p::GFP::daf-16]*) in wild-type and *daf-16* deficient worms on HT115 bacteria. Each panel is a separate experimental trial. B The effect of an independently derived transgene (*muEx227[ges-1p::GFP::daf-16]*) on lifespan in wild-type and *daf-16* deficient worms on HT115 bacteria. Log-rank test detected significant differences in both wild-type and *daf-16* deficient worms (*p*<10^{-4}). C Two experimental trials looking at the effect of gut over-expression of *daf-16* (*muEx211[ges-1p::GFP::daf-16]*) in wild-type and *daf-16* deficient worms on OP50 bacteria. Log-rank test detected significant differences in both wild-type and *daf-16* deficient worms (*p*<0.01 and *p*<10^{-4}, respectively) in both trials. MECPH model revealed significant interaction between genomic *daf-16* and its intestinal induction (*p*<10^{-15}). D Protein content of worms with *daf-16* induced in the intestine (*muEx227[ges-1p::GFP::daf-16]*) in otherwise *daf-16(+) or *daf-16(mu86)* worms. The data were analysed with a Linear model and the effect of transgene found significant (*p*=0.012), the effect of *daf-16* mutation borderline significant (*p*=0.069) and no significant interaction (*p*=0.86, n=8-16). The colour code is the same in all panels and is given in A.
Table S1 Supplemental Data

Separate Excel File containing:

List of genes differentially expressed (10% FRD) in S106>dfoxo females with RU486 treatment, with indication of the ones that show significant interaction between genotype (S106>dfoxo or dfoxo null S106>dfoxo) and RU486 (10% FDR).

Biological process categories over-represented in genes with significant interaction term in list above.
Extended Experimental Procedures

**Fly husbandry, lifespan, feeding and climbing assays**

S1106 (Giannakou et al., 2004; Poirier et al., 2008), UAS-dfoxo (Giannakou et al., 2004), dilp2-GAL4 (Ikeya et al., 2002) and dfoxoΔ94 (Slack et al., 2011) were backcrossed at least 6 times into the wild-type outbred Dahomey population carrying the w1118 mutation and cured of Wolbachia infection, and frequently outcrossed back into the wild-type population. The Dahomey stock was collected in 1970 in Dahomey (now Benin) and has been kept in population cages maintaining its lifespan and fecundity at levels similar to freshly caught stocks. Combinations of transgenes/mutants were created using standard fly genetic techniques while avoiding population bottlenecks. dfoxoΔ94 allele was tracked with PCR (Slack et al., 2011). The lines were maintained, and all experiments performed, at 25°C with 60% humidity and 12h:12h light:dark cycle, on sugar-yeast-agar (1SYA) food (Bass et al., 2007). Experimental flies developed at standardised densities and once-mated females were sorted on day two of adulthood onto food containing 200 µM RU486 (Sigma) or control food as required (15 per vial for climbing assays, 5 for feeding, 10 for all others). Note that the food from the exact same cook was used for the - and + RU486. Lifespan measurements were performed essentially as described (Giannakou et al., 2004). Climbing ability was scored once a week. Flies were placed in a plastic pipette (15 flies per pipette, 3 pipettes per condition), tapped to the bottom and allowed to climb for 45 seconds. Their position was then scored as low (climbing below 2 cm), medium (above 2 and below 20) and high (above 20 cm). This scoring was repeated 3 times for each pipette and averaged to the nearest fly. Feeding
observations were performed essentially as described (Wong et al., 2009) for one hour on days 7 and 8 by two observers, blinded to the experimental conditions, with flies feeding (proboscis extended to the food) or not scored multiple times for each vial. The observations were summed per vial before analysis. Flies were harvested on day seven for weight and metabolite measurement, protein and RNA analysis, by freezing in liquid N₂.

Metabolite and weight measurements

Whole fly trehalose was determined as described (Alic et al., 2011b). For triacylglycerol (TAG) and protein measurements, a single female was homogenised in 400 µl of 0.05% Tween-20 and TAG measured with Infinity TAG Reagent (Thermo Scientific), protein with BCA Protein Assay kit (Pierce) against a suitable standard. For glycogen measurements, a single female was homogenised into 100 µl of PBS 0.1% TritonX-100, and the glucose released by incubation with amyloglucosidase in the samples and glycogen standards determined with Infinity Glucose Reagent (Thermo Scientific). Flies were weighted singly or in pairs, and the two highest/lowest measurements for each condition discarded.

Western blots, qPCR and microarray analysis

Proteins were extracted and western blots performed as described (Alic et al., 2011b). RNA extractions and qPCR were performed essentially as described (Alic et al., 2011a). dfoxo primers used were: GTTGGCCCCAGGAGACTC and GATGAGATCCGCATAGGATAG; Actin5C: CACACCAAATCTTACAAATGTGTGA and AATCCGGCCTTGCACATG; Nplp4: CCGGACCATGCATTGCAATG and GCGCATATCCGTAATCCGT. dfoxo mRNA was deemed detectable when the qPCR signal was above that
observed in the absence of reverse transcriptase. mRNA levels of *dilp2* through to 7 and *Imp-L2* were quantified as described (Alic et al., 2011b; Broughton et al., 2005; Gronke et al., 2010). Sample preparation and hybridisations to Dros2 Affymetrix arrays were performed as described (Alic et al., 2011a). The microarray data were analysed in R. They were summarised and normalised using RMA, differential expression was assessed using Linear Models and the empirical Bayes moderated t-statistic implemented in LIMMA (Bolstad et al., 2003; Irizarry et al., 2003a; Irizarry et al., 2003b) and FDR was controlled using the described procedure (Benjamini and Hochberg, 1995). Genes differentially expressed on RU486 feeding in S;106>dfoxo females were determined (at FDR=10%), and amongst these genes the ones with significant interaction between genotype and RU486 (FDR=10%) were determined by a Linear Model (full factorial design) fitted to all the data (the four conditions). Gene Ontology Enrichment was determined with David EASE (Dennis et al., 2003). Gene lists are given in Supplemental Data. Raw microarray data are available from ArrayExpress under accession code: E-MTAB-1232.

**Worm husbandry, lifespan and protein assays**

Worms were maintained at 20°C unless otherwise indicated. The following strains were used: N2s derived from the Caenorhabditis Genetics Center male N2 line, XA2954 daf-16(mu86) derived from CF1038 daf-16(mu86) (Lin et al., 2001), GA1064 muEx211[ges-1p::GFP::daf-16], GA1065 daf-16(mu86) muEx211[ges-1p::GFP::daf-16], GA1067 muEx227[ges-1p::GFP::daf-16], GA1066 daf-16(mu86) muEx227[ges-1p::GFP::daf-16]. The latter four strains were created by crossing CF1514 daf-16(mu86); daf-
2(e1370) muEx211[ges-1p::GFP::daf-16] or CF1595 daf-16(mu86); daf-2(e1370) muEx227[ges-1p::GFP::daf-16] (Libina et al., 2003) with the N2 strain and isolating wild type and daf-16 mutant worms carrying the extrachromosomal array using three-primer PCR: F: CGGTGACCATCTAGAGTCACA; In: CCAATAGCTGGAGAAACACGA and; R: CTAGGAGGAAAAGCCATTTGT (Love et al., 2010), without further outcrossing. Prior to experiments animals were maintained at the permissive temperature and grown for at least one generation in the presence of food to assure full viability. Lifespan assays were performed on HT115 bacteria carrying empty pL4440 vector, or OP50 bacteria, in the presence of 10μM FUdR. Worms were placed on these plates at the L4 stage and scored as dead or alive every 2-3 days. For protein measurements, 20 L4s were picked from OP50 maintenance plates and allowed to mature for 24h on HTT15 bacteria carrying empty pL4440 vector, picked into 100 μl of 0.05% Tween-20, the bacteria removed by centrifugation and the worms lysed with glass beads in a Ribolyser. Protein concentration was determined against a BSA standard as for the fly. 10-20 repeats were performed for each sample and 5% lowest and 5% highest measurements removed from each group.

**Statistical Analysis**

Mixed Effect Linear Models, Linear Models, Generalised Linear Models, post-hoc tests and Log-rank comparisons were performed in JMP (SAS), all other analysis in R. MECPH analysis was performed using the coxme package (Therneau, T., 2012, http://CRAN.R-project.org/package=coxme), CPH using survival (Therneau, T., 2013, http://CRAN.R-project.org/package=survival), Mixed Effects Ordinal Logistic analysis using
**ordinal** (Christensen, R. H. B., 2012 http://www.cran.r-project.org/package=ordinal). Further details are given in Table 1. To determine difference in slopes of the regression lines between the two gene sets in Figure 2A, Linear Model was fitted with RU486-induced response in \textit{dfoxo}Δ/Δ \textit{S106>dfoxo} as the dependant variable and the response in \textit{S106>dfoxo} (continuous) and gene set (categorical) as explanatory variables, testing for the significance of the interaction term.
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