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The Drosophila Forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling

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Abstract

Background: Forkhead transcription factors belonging to the FOXO subfamily are negatively regulated by protein kinase B (PKB) in response to signaling by insulin and insulin-like growth factor in Caenorhabditis elegans and mammals. In Drosophila, the insulin-signaling pathway regulates the size of cells, organs, and the entire body in response to nutrient availability, by controlling both cell size and cell number. In this study, we present a genetic characterization of dFOXO, the only Drosophila FOXO ortholog.

Results: Ectopic expression of dFOXO and human FOXO3a induced organ-size reduction and cell death in a manner dependent on phosphoinositide (PI) 3-kinase and nutrient levels. Surprisingly, flies homozygous for dFOXO null alleles are viable and of normal size. They are, however, more sensitive to oxidative stress. Furthermore, dFOXO function is required for growth inhibition associated with reduced insulin signaling. Loss of dFOXO suppresses the reduction in cell number but not the cell-size reduction elicited by mutations in the insulin-signaling pathway. By microarray analysis and subsequent genetic validation, we have identified d4E-BP, which encodes a translation inhibitor, as a relevant dFOXO target gene.

Conclusion: Our results show that dFOXO is a crucial mediator of insulin signaling in Drosophila, mediating the reduction in cell number in insulin-signaling mutants. We propose that in response to cellular stresses, such as nutrient deprivation or increased levels of reactive oxygen species, dFOXO is activated and inhibits growth through the action of target genes such as d4E-BP.
Background

Receptors for insulin and insulin-like growth factors (IGFs) are central regulators of energy metabolism and organismal growth in vertebrates and invertebrates. In mammals, the insulin receptor regulates glucose homeostasis and embryonic growth [1], whereas the insulin-like growth factor 1 receptor (IGF1-R) regulates embryonic and postembryonic growth [2] and longevity [3]. In Caenorhabditis elegans, DAF-2 - the homolog of the mammalian insulin/IGF receptor - controls organismal growth in response to poor nutrient conditions indirectly by controlling formation of the long-lived, stress-resistant dauer stage during larval development, and lifespan in the adult [4]. In Drosophila, the insulin/IGF receptor homolog DInr controls organismal growth directly by regulating cell size and cell number [5]. Furthermore, reduced insulin signaling causes female sterility and independently increases lifespan [6,7]. The striking conservation of insulin receptor function is also reflected in the conservation of the intracellular signaling cascade. Binding of insulin-like peptides to their receptor tyrosine kinases leads to the activation of class IA phosphatidylinositol (PI) 3-kinases and increased intracellular concentrations of the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3). This results in recruitment to the membrane, and activation, of the protein kinases phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (PKB/AKT), both of which contain pleckstrin homology (PH) domains and which in turn modulate the activity of downstream effector proteins [8]. The lipid phosphatase PTEN (phosphatase and tensin homolog on chromosome 10) catalyzes the 3-dephosphorylation of PIP3, thereby acting as a negative regulator of insulin signaling [9]. The demonstration that the lethality associated with loss of dPTEN in C. elegans, forkhead transcription factors belonging to the FOXO subfamily have recently been shown to induce cell-cycle arrest by repressing transcription of genes encoding D-type cyclins [23,24]. FOXO transcription factors mediate insulin resistance in diabetic mice [25], and have been proposed to be tumor suppressors, as several chromosomal translocations disrupting FOXO genes are found in cancers [26,27], and overexpressed FOXO proteins can inhibit tumor growth [23].

TSC2, the second target of PKB, forms a complex with TSC1 and acts as a negative regulator of growth in Drosophila, and as a tumor suppressor in mammals. Overexpressed activated PKB phosphorylates TSC2 and thereby disrupts the TSC1/2 complex in Drosophila and in mammalian cells [28,29]. In Drosophila, the TSC1/2 complex functions by negatively regulating two kinases, dTOR (homolog of the mammalian target of rapamycin) [30] and dS6K (homolog of the mammalian ribosomal protein S6 kinase) [31]. Recent genetic and biochemical evidence indicates that TSC1/2 regulates S6K activity by acting as a GTPase-activating protein (GAP) for the small GTPase Rheb [32-35]. Interestingly, flies lacking dS6K function are reduced in size because of a reduction in cell size but not in cell number [36]. The growth control pathways regulating cell size and cell number therefore bifurcate either at dPKB or between dPKB and dS6K.

In this study, we describe the identification of dFOXO, the single FOXO ortholog in Drosophila. Although dFOXO function is not essential for development and organismal growth control under normal culture conditions, it mediates the reduction in cell number associated with reduced insulin signaling. Our results show that dFOXO regulates expression of d4E-BP, which mediates part of the cell-number reduction in dPKB mutants. We propose that dFOXO upregulates d4E-BP transcription under conditions of low insulin signaling. Furthermore, our observations suggest that dFOXO is required for resistance against oxidative stress in adult flies.

Results
dFOXO is the only Drosophila homolog of FOXO and DAF-16

The Drosophila genome contains a single homolog of the DAF-16/FOXO family of transcription factors. This notion is
supported by the phylogenetic tree diagram calculated from the multiple sequence alignment (Figure 1a). The dFOXO gene is more closely related to the mammalian FOXO subfamily and daf-16 than any other Drosophila forkhead gene. The amino-acid sequences of the predicted 613 amino-acid dFOXO protein and hFOXO3a are 27% identical over the full protein length, and 82% identical within the forkhead DNA-binding domain. Furthermore, dFOXO is the only Drosophila forkhead gene encoding a putative protein containing conserved PKB phosphorylation sites [37]. The orientation of the three PKB consensus sites relative to the forkhead domain (Figure 1b) is conserved among the mammalian FOXO

![Figure 1](http://jbiol.com/content/2/3/20)

**Figure 1**

dFOXO is the only Drosophila FOXO/DAF-16 homolog. A TBLASTN search of the Drosophila genome for known and predicted genes encoding forkhead transcription factors retrieved 16 genes. (a) A phylogenetic tree calculated from a multiple sequence alignment of the forkhead domains of these 16 proteins and of the human FOXO proteins FOXO1 (FKHR), FOXO3a (FKHRL1) and FOXO4 (AFX), the C. elegans DAF-16 and mouse Foxa3 (HNF-3γ; protein names on the figure are from GenBank). The similarity of dFOXO to FOXO proteins is highlighted in blue. (b) dFOXO has three PKB phosphorylation sites in the same orientation as those of mammalian FOXO proteins. The sites are indicated above the protein; PEST (destruction), nuclear localization (NLS), nuclear export (NES) and DNA-binding sequences are also shown. (c) A multiple amino-acid sequence alignment of the dFOXO, human FOXO and DAF-16 forkhead domains illustrates the high degree of sequence conservation especially within the DNA-binding domain. The secondary structure is indicated above the alignment. Similar and identical amino-acid residues are shaded in gray and black, respectively. The region encoding helix 3 of the forkhead domain, which is the DNA-recognition helix contacting the major groove of the DNA double helix, is identical in the five proteins. Given the high structural similarity between the DNA-binding domains of FOXO4 (AFX) and HNF-3γ [86], it is likely that FOXO proteins contact insulin response elements through helix 3. Two EMS-induced point mutations described in this study are shown in red. (d) The dFOXO gene spans a genomic region of 31 kilobases (kb) and contains 11 exons (blue bars). The EP35-147 transposable element is inserted in the second intron upstream of the open reading frame, allowing GAL4-induced expression of endogenous dFOXO.
proteins, DAF-16 and dFOXO. Figure 1c shows the high degree of sequence conservation between dFOXO and FOXO/DAF-16 proteins within the DNA-binding domain. Taken together, these observations strongly suggest that dFOXO is the only Drosophila homolog of the mammalian FOXO transcription factors and C. elegans DAF-16.

Figure 2
Targeted hFOXO3a and dFOXO expression in the developing Drosophila eye induces organ-size reduction and cell death, and the phenotypes are sensitive to insulin signaling and nutrient levels. (a) GMR-Gal4-expressing control fly. (b) No discernible phenotype results from hFOXO3a expression. (c) Expression of hFOXO3a-TM in the eye disc leads to pupal lethality; escapers at 18°C show a necrotic phenotype and severely disrupted cell specification. (d) Expression in w-marked clones of cells induces a similar phenotype at 25°C. (e) Dp110DN expression slightly reduces eye size, and (f) co-expression of wild-type hFOXO3a partially mimics the hFOXO3a-TM escaper phenotype. (g) The same enhancement of hFOXO3a activity was observed in a dPKB−/− background. (h,i) Expression of transgenic or endogenous dFOXO results in a small-eye phenotype, which is also dramatically enhanced by (j) Dp110DN. (k-o) hFOXO3a and dFOXO phenotypes are progressively exacerbated by protein deprivation ('sugar') and complete starvation ('PBS'). Flies like the one shown in (m) die within one day, and complete starvation of dFOXO-expressing flies resulted in pupal lethality (not shown). Genotypes are: (a) y w; GMR-Gal4/+; (b) y w; GMR-Gal4/+; UAS-hFOXO3a/+; (c) y w; GMR-Gal4/+; UAS-hFOXO3a-TM/+; (d) y w hs-flp w; GMR > FRT- w+ STOP - FRT > Gal-4/+; UAS-hFOXO3a-TM/+; (e) y w; GMR-Gal4 UAS-Dp110DN/++; (f) y w; GMR-Gal4 UAS-Dp110DN/++; UAS-hFOXO3a+/+; (g) y w; UAS-hFOXO3a/GMR-Gal4; dPKB−/−; (h) y w; UAS-dFOXO/GMR-Gal4; (i) y w; GMR-Gal4/++; EP-dFOXO/+; (j) y w; GMR-Gal4 UAS-Dp110DN/++; EP-Dp110DN/+; (k-m) y w; GMR-Gal4/++; UAS-hFOXO3a+/+; (n,o) y w; GMR-Gal4/++; EP-dFOXO+/+
Overexpressed dFOXO is responsive to insulin signaling and nutrient levels, inducing organ-size reduction and cell death

To assess whether dFOXO has a key function in insulin signaling like that of DAF-16 in *C. elegans*, we tested whether overexpression of wild-type or mutant forms of hFOXO3a and dFOXO could antagonize insulin signaling. Elimination of the three PKB consensus phosphorylation sites in mammalian FOXO3a prevents its phosphorylation, subsequent binding to 14-3-3 proteins, and sequestration in the cytoplasm [12]. This leads to constitutive nuclear localization of the mutant FOXO3a and transcriptional activation of its target genes. Assuming that blocking the PKB signal would have the same activating effect on dFOXO, we overexpressed wild-type and triple PKB-phosphorylation-mutant variants of both dFOXO and human FOXO3a. Furthermore, we identified an *EP* transposable element insertion in the second *dFOXO* intron, which permits the GAL4-induced overexpression of endogenous *dFOXO* (Figure 1d). We used the GMR-Gal4 construct to drive UAS-dependent expression in postmitotic cells in the eye imaginal disc [38]. While expression of wild-type hFOXO3a in the developing eye did not result in a visible phenotype (Figure 2b), hFOXO3a-TM expression caused pupal lethality. Few escaper flies eclosed and displayed a strong necrotic eye phenotype (Figure 2c). A block of cell differentiation and necrosis was also observed when hFOXO3a-TM was expressed in cell clones in the developing eye (Figure 2d).

Assuming that the lack of a phenotype observed upon UAS-hFOXO3a expression is due to hFOXO3a inactivation by endogenous DInr signaling in the eye disc, we performed the same experiment in a background of reduced insulin signaling. Indeed, in the presence of a dominant-negative (DN) form of Dp110 (encoding the PI 3-kinase catalytic subunit) [39], hFOXO3a expression induced a necrotic phenotype similar to the one observed with the hyperactive phosphorylation mutant (Figure 2f). To confirm that hFOXO3a is responsive to *Drosophila* insulin signaling and rule out artificial coexpression effects, we expressed hFOXO3a in flies mutant for either *dPKB* (Figure 2g) or Dp110 (not shown), and observed similar phenotypes to those seen upon coexpression of *Dp110DN*. *Drosophila* FOXO has qualitatively similar, but stronger effects. Expressing the wild-type form of *dFOXO* causes a weak eye-size reduction and disruption of the ommatidial pattern even in a wild-type background (Figure 2h,i), and the phenotype is strongly affected by Dp110DN as well (Figure 2j). The UAS-dFOXO-TM transgene appears to cause lethality even in the absence of a Gal4 driver, as we did not obtain viable transgenic lines with this construct. Furthermore, we examined the effects of nutrient deprivation on FOXO-expressing tissues. If nutrient availability is limited, FOXO should be more active in response to lowered insulin signaling. Indeed, we observed that the overexpression phenotypes of both hFOXO3a and dFOXO are enhanced under conditions of starvation. *Drosophila* larvae that are starved until 70 h after egg laying (AEL) die within a few days. But if the onset of nutrient deprivation occurs after they have surpassed the metabolic ’70 h change’ [40,41], they survive and develop into small adult flies. We therefore subjected larvae expressing hFOXO3a or dFOXO (under GMR control) to either protein starvation (sugar as the only energy source) or complete starvation, starting 80-90 h AEL, and analyzed the effect on the adult’s eyes. Both phenotypes (Figure 2k,n) were progressively exacerbated by protein starvation (Figure 2l,o) and complete starvation (Figure 2m), the latter condition being accompanied by early adult or larval lethality, in the case of hFOXO3a or dFOXO, respectively. The resulting phenotypes are due to the FOXO transgenes, as wild-type control flies that have been starved during development display only a body-size reduction while maintaining normal proportions and normal eye structure.

The *dFOXO* overexpression phenotype (Figure 2i,j) does not appear to be caused by the activation of any of the known cell-death pathways. Expression of the caspase inhibitors *p35* or *DIAP1*, or of *p21*, an inhibitor of p53-induced apoptosis [42], and loss of *eiger*, which encodes the *Drosophila* homolog of tumor necrosis factor (TNF) [43], did not suppress the eye phenotype (data not shown). In agreement with our results, it was observed in a parallel study that the GMR-*dFOXO* overexpression phenotype is insensitive to caspase inhibitors, and is not accompanied by increased acridine-orange-detectable apoptosis in the imaginal disc [44]. It therefore remains unclear whether high levels of nuclear *dFOXO* induce a specific caspase-independent cell-death program or whether nuclear accumulation of overexpressed *dFOXO* leads to secondary necrosis in a rather nonspecific fashion. Furthermore, the necrotic eye phenotype does not reflect the phenotype observed following a complete block in insulin signaling. Loss-of-function mutations in insulin-signaling components reduce cell size and cell number but do not increase cell death in larval tissues [45,46]. In summary, our overexpression experiments are consistent with a model in which, under normal conditions, excess FOXO transcription factor is phosphorylated by dPKB and kept inactive in the cytoplasm. Under conditions of reduced insulin-signaling activity or nutrient deprivation, dFOXO or hFOXO3a protein translocates to the nucleus and induces growth arrest and necrosis.

**dFOXO loss-of-function mutants are viable, have no overgrowth phenotype and are hypersensitive to oxidative stress**

Although the overexpression experiments described above did not reveal the physiological function of dFOXO, they
provided the entry point for isolation of loss-of-function mutations. We made use of the EP35-147 element, which permits the generation of the necrotic eye phenotype (Figure 2) by driving expression of endogenous dFOXO in the presence of Dp110DN. We mutagenized homozygous EP males, mated them to homozygous GMR-Gal4 UAS-Dp110DN females and then screened the F1 generation for reversion of the strong gain-of-function phenotype and its associated semilethality. Several loss-of-function alleles of dFOXO were isolated and molecularly characterized. Two such revertants are shown in Figure 3c (dFOXO21) and Figure 3d (dFOXO25). In dFOXO21 and dFOXO25, the codons for W95 and W124 within the forkhead domain are mutated to stop codons, respectively (Figure 1c), so they are assumed to be null alleles of dFOXO. We performed the subsequent phenotypic and epistasis analyses with these two lines.

Because FOXO transcription factors have been proposed to be the primary effectors of insulin signaling, on the basis of epistasis of daf-16 over daf-2 in C. elegans, it seemed reasonable to expect an overgrowth phenotype in dFOXO−/− flies as is observed in dPTEN loss-of-function mutants. To our surprise, dFOXO loss-of-function mutants are homozygously viable and display no obvious phenotype under normal culturing conditions (Figure 3h). Thus, dFOXO is not essential for development. Only close inspection of the dFOXO mutants revealed that their wing size is significantly reduced (Figure 4i). But cellular and organismal growth are unaffected by dFOXO mutations. To assess whether dFOXO-mutant tissue grows to a different size than wild-type tissue, we recombined the dFOXO21 and dFOXO25 alleles onto the FRT82 chromosome and induced genetic mosaic flies with the ey-Flp/FRT system [47]. When the eye and head capsule were composed almost exclusively of dFOXO−/− tissue (w-marked in Figure 3e,f, on the right), no head-size difference was observed compared to the control fly with a head homozygous for the FRT82 chromosome without the dFOXO mutation (Figure 3e,f, left). This is consistent with experience from extensive genetic screens for recessive growth mutations carried out in our lab. An ey-Flp-screen on the right arm of chromosome 3 did not reveal any mutations in dFOXO based on an altered head-size phenotype (H.S. and E.H., unpublished observations).

We next asked whether cell size, like organ size, was not affected by the loss of dFOXO. For this purpose, we used a heat shock-inducible Flp construct to generate clones of homozygous dFOXO−/− photoreceptor cells and wild-type cells within one adult eye (Figure 3g). The cells lacking dFOXO are marked by the absence of pigment granules. Consistent with the absence of a 'bighead' phenotype, dFOXO−/− cells and wild-type cells have the same size. Similarly, no significant difference in the body weight of mutant and control flies was observed (Figure 3h). In contrast, flies with a viable heteroallelic combination of dPTEN loss-of-function alleles are significantly bigger than wild-type flies [48]. Taken together, these results argue that with the exception of the slight wing-size reduction, dFOXO is not required to control cellular, tissue, or organismal growth in a wild-type background.

A critical role has been reported for mammalian and C. elegans FOXO proteins in resistance against various cellular stresses, in particular oxidative stress [16,17,49], DNA damage [15] and cytokine withdrawal [50]. We tested the stress resistance of adult dFOXO mutant flies by measuring survival time following different challenges. Among starvation on water, oxidative-stress challenge, bacterial infection, heat shock, and heavy-metal stress, the only condition for which hypersensitivity was observed is oxidative stress. When placed on hydrogen-peroxide-containing food, dFOXO mutant flies display a significantly reduced survival time compared to control flies (Figure 3i). A very similar effect is elicited by paraquat feeding. These observations are consistent with the paraquat hypersensitivity of daf-16 mutants in C. elegans [51], suggesting that a role for FOXO proteins in protecting against oxidative stress is conserved across species.
Figure 3 (see legend on the previous page)
The growth-deficient phenotypes of Dlnr, chico, Dp110 and dPKB mutants are significantly suppressed by loss of dFOXO

We performed genetic epistasis experiments to examine whether the growth phenotypes of Dlnr-signaling mutants are dependent on dFOXO function. For this purpose, we either generated double-mutant flies or investigated the double-mutant effect only in the head using the ey-Flp/FRT system. In contrast to the absence of a growth phenotype in single dFOXO mutant flies, lack of dFOXO significantly suppresses the growth-deficient phenotype observed in flies mutant for the insulin receptor substrate (IRS) homolog chico (Figure 4). Flies mutant for chico are smaller because they have fewer and smaller cells [45]. Loss of one dFOXO copy dominantly suppresses the cell-number reduction in chico mutant flies without affecting cell size. The suppression is more pronounced when both copies of dFOXO are removed in a chico mutant background. In this situation, the chico small body-size phenotype is partially suppressed. Homozygous chico-dFOXO double-mutant flies have more, and even slightly smaller, cells than homozygous chico single mutants. It seems that removal of dFOXO accelerates the cell cycle at the expense of cell size in a chico background.

We next asked whether dFOXO interacts with other components of the Drosophila insulin-signaling pathway. The ey-Flp/FRT system was used to generate heterozygous insulin-signaling mutant flies with heads homozygous for each mutation. Removal of Dlnr, Dp110 or dPKB leads to a characteristic 'pinhead' phenotype, which is substantially suppressed by the presence of a dFOXO loss-of-function allele on the same FRT chromosome as the insulin-signaling mutation. In all three cases, we observed a partial rather than a complete rescue of the tissue growth repression, consistent with the finding that dFOXO mutations affect only the cell-number aspect of the chico phenotype. Surprisingly, loss of dFOXO dramatically delays lethality in dPKB mutants. Complete loss of dPKB leads to larval lethality in the early third instar, but homozygous dPKB-dFOXO double mutants are able to develop into pharate adults of reduced size, most of which fail to eclose (Figure 5i). The lethality associated with the complete loss of dPKB is therefore largely due to hyperactivation of dFOXO.

We also observed that dFOXO interacts with the tumor suppressors dTSC1 and dPTEN. Tissue-specific removal of either gene from the head leads to a bighead phenotype (Figure 5h,j). The dTSC1+/− bighead phenotype is enhanced by loss of dFOXO (Figure 5i). This observation is consistent with the recently reported negative feedback loop between dS6K and dPKB. Mutant dTSC1 larvae have elevated levels of dS6K activity, which in turn downregulates dPKB activity [31]. This reduction in dPKB activity probably leads to enhanced activation of dFOXO, which in turn partially mitigates the overgrowth phenotype by slowing down proliferation. The dTSC1 phenotype can therefore be enhanced by loss of the inhibitory function of dFOXO. Unexpectedly, the dPTEN+/− bighhead phenotype was slightly suppressed by dFOXO mutations (Figure 5k). From the current model, it would be expected that in a dPTEN mutant dPKB activity is high and dFOXO is to a large extent inactive in the cytoplasm. Thus, removal of dFOXO function should have no effect on the dPTEN phenotype. At present, we can only speculate about possible explanations for this observation. In a parallel study, it has been shown that dFOXO can induce transcription of Dlnr [52]. It may be that in a dPTEN-mutant background dFOXO activates Dlnr expression in a negative-feedback loop. In this model, concomitant loss of dFOXO would alleviate the dPTEN overgrowth phenotype by lowering Dlnr levels. Another possible explanation is that dFOXO has additional functions when localized to the cytoplasm or during its nuclear export, such as interacting with other proteins. Loss of dFOXO might affect the function of interaction partners that have a role in dPTEN signaling.

In summary, our epistasis analysis provides strong genetic evidence that dFOXO is required to mediate the organismal growth arrest that is elicited in insulin-signaling mutants.

dFOXO upregulates transcription of the d4E-BP gene

We have shown previously that Drosophila embryonic Kc167 cells respond to insulin stimulation with upregulated activities of dPKB and dS6K [53,54]. We performed mRNA profiling experiments using the Affymetrix GeneChip system to measure on a genome-wide scale the transcriptional changes induced by insulin in these cells. On the basis of the currently held model that FOXO transcription factors are transcriptional activators that are negatively regulated by insulin, we expected potential dFOXO target genes to be repressed in Kc167 cells upon insulin stimulation. Figure 6a shows a selection of dFOXO target gene candidates that are transcriptionally downregulated by a factor of two or more upon insulin stimulation and whose promoter regions contain one or more conserved forkhead-response elements (FHREs) with the consensus sequence (G/A)JTAAAACAA [55]. Three of these candidate gene products are each involved in one of two biological processes known to be negatively regulated by insulin, namely gluconeogenesis (PEPCK) and lipid catabolism (CPTI and long-chain-fatty-acid-CoA-ligase). The remaining candidates are involved in stress responses (cytochrome P450 enzymes), DNA repair (DNA polymerase iota), transcription and translation control (d4E-BP and CDK8), and cell-cycle control (centaurin gamma and CG3799). Several of the insulin-repressed genes have been reported to be transcriptionally induced in
Drosophila larvae under conditions of complete starvation (d4E-BP and PEPCK) or sugar-only diet (CPTI and long-chain-fatty-acid-CoA-ligase) [41,56]. We chose d4E-BP for further investigation, because it has previously been reported to be insulin-regulated at the level of protein phosphorylation, but not at the level of gene expression.

Figure 4
Loss of dFOXO suppresses the cell-number reduction in chico mutants. (a–e) Partial rescue of the chico phenotype by mutations in dFOXO. Bar sizes are 100 µm (low magnification) and 20 µm (high magnification). Each graph displays the variation of a single parameter between the five genotypes shown in (a–e): (f) body weight, (g) cell number in the eye, (h) cell size in the eye, (i) wing area, (j) cell number in the wing, and (k) cell size in the wing. (f) dFOXO−/− partially suppresses the low-body-weight phenotype of chico−/−. The suppression is less pronounced in the wing (i), because dFOXO−/− null mutants have significantly smaller wings than control flies, although their body weight is the same. In a chico−/− background, loss of dFOXO leads to increased cell numbers in the eye (g) and in the wing (j) compared to the chico single mutant. Although organ and tissue size is increased, cell size significantly decreases in the chico-dFOXO double mutant both in the eye (h) and in the wing (k). It seems that loss of dFOXO in a chico−/− background leads to increased proliferation rates. All values are shown ± SD. Genotypes are: (a) y w; EP-dFOXO/EP-dFOXO; (b) y w; EP-dFOXO21/EP-dFOXO25; (c) y w; chico1/chico2; EP-dFOXO21/EP-dFOXO25; (d) y w; chico1/chico2; EP-dFOXO21/EP-dFOXO25; (e) y w; chico1/chico2.
The \textit{d4E-BP} gene encodes a translational repressor and was initially identified as the immune-compromised \textit{Thor} mutant in a genetic screen for genes involved in the innate immune response to bacterial infection [58,59]. Figure 6b shows the presence of several FHREs in the genomic region around the \textit{d4E-BP} locus. The \textit{d4E-BP} protein is negatively
regulated by insulin through LY294002- and rapamycin-sensitive phosphorylation [57], suggesting involvement of the Dp110 and dTOR signaling pathways. Phosphorylation of d4E-BP leads to the dissociation of d4E-BP from its binding partner, the translation initiation factor deIF4E, which then participates in the formation of a functional initiation complex. Positive transcriptional regulation of d4E-BP by dFOXO, which corresponds to negative transcriptional regulation by insulin, would be a complementary mechanism of regulation.

We then investigated whether overexpression of endogenous dFOXO could induce transcriptional upregulation of the d4E-BP gene. On the basis of our overexpression results, we chose the Dp110DN-dFOXO coexpression to efficiently activate dFOXO. Eye imaginal discs from Dp110DN-expressing third instar larvae display a low level of basal d4E-BP transcription throughout the disc, which is not induced by the driver construct alone (Figure 6d). Coexpression of dFOXO elicited a dramatic upregulation of d4E-BP transcription posterior to the morphogenetic furrow (Figure 6e). Consistent with this observation, we were able to induce expression of the d4E-BP enhancer trap line Thor1 with human FOXO3a-TM (Figure 6f-h). It remained unclear, however, whether regulation of d4E-BP expression by dFOXO is of physiological relevance.
It has been previously reported that overexpression of d4E-BP partially suppresses the dPKB overexpression phenotype [57], but as ectopic expression experiments have to be interpreted with some caution, we assessed whether loss of d4E-BP function suppresses the cell-number reduction in insulin-signaling mutants as does loss of dFOXO function. We generated double-mutant flies for dPKB and d4E-BP and observed that the Thor
 mutation slightly but significantly suppressed the reduced cell-number phenotype in a dose-dependent manner. The Thor
 mutation itself had no effect on ommatidial number compared to wild-type flies (data not shown), so we can rule out additive effects of d4E-BP and dPKB. These observations strongly argue that under conditions of reduced insulin-signaling activity the dFOXO-dependent reduction in cell number is in part mediated by the transcriptional upregulation of its target d4E-BP. Microarray studies in both mammalian [23] and Drosophila [52] cells imply that FOXO transcription factors exert their physiological functions by modulating expression of large sets of target genes.

**Discussion**

Forkhead transcription factors of the FOXO subfamily mediate insulin-regulated gene expression in C. elegans and mammals. In this study, we provide genetic evidence that the *Drosophila* FOXO/DAF-16 homolog dFOXO is an important downstream effector of *Drosophila* insulin signaling and a regulator of stress resistance.

**dFOXO is a critical target of dPKB but mediates only part of its function**

Genetic studies in *C. elegans* and *Drosophila* have led to two models regarding the output of the insulin pathway. First, the complete epistasis of *daf-16* over the insulin pathway mutants *daf-2*, *age-1*, *akt-1* and *akt-2* suggests that the primary function of PKB is to inactivate FOXO transcription factors [60]. Second, it has been proposed that the TSC tumor suppressor complex is the major target of PKB [61,62] in the regulation of cell growth in *Drosophila*. Our analysis of *Drosophila* FOXO indicates that it is indeed a critical PKB target, but that it mediates only one aspect of PKB function. Several lines of evidence support this model. Firstly, the effects of ectopic overexpression of dFOXO and hFOXO3a in the developing *Drosophila* eye are altered by Dp110 and dPKB signaling as well as by nutrient levels. Under conditions of lowered insulin signaling, the phenotypes resulting from expression of dFOXO and hFOXO3a were dramatically enhanced. This situation was mimicked by expressing a dPKB-insensitive phosphorylation mutant, suggesting that endogenous dPKB signaling is required to mitigate the effects of ectopically expressed dFOXO and hFOXO3a. Secondly, the physiological relevance of dFOXO in dPKB signaling is most vividly demonstrated by our observation that the larval lethality associated with the complete loss of dPKB is rescued by dFOXO mutations to the extent that some flies develop to pharate adults. The lethality associated with loss of dPKB function is therefore to a large extent due to the hyperactivation of dFOXO. Thirdly, loss of dFOXO function suppresses the effects of insulin-signaling mutations only partially; dFOXO mediated a reduction in cell number but not in cell size in response to reduced insulin signaling.

**dFOXO controls the reduction in cell number in body-size mutants**

Genetic analysis of the control of body size in *Drosophila* has revealed two classes of mutations. Flies carrying mutations in *chico* or viable allelic combinations of *DInr*, *Dp110*, and dPKB are reduced in body size by up to 50% owing to a reduction in both cell size and cell number. Conversely, flies mutant for dS6K exhibit a more moderate reduction in body size, caused almost exclusively by a reduction in cell size [36]. This suggests that the pathways controlling cell number and cell size bifurcate at or below dPKB. Although dFOXO single mutants have no obvious size phenotype, loss of dFOXO substantially reduces the cell-number reduction observed in insulin-signaling mutants. It appears that dFOXO mediates the repression of proliferation in flies mutant for *DInr*, *chico*, *Dp110*, and dPKB without being required for the reduction in cell size. *Chico-dFOXO* double mutant flies even have slightly smaller cells than *chico* mutants, suggesting that removal of dFOXO permits cell-cycle acceleration under conditions of impaired insulin signaling. The pathway controlling body size in response to insulin therefore bifurcates at the level of dPKB: dPKB controls cell number by inhibiting dFOXO function and dPKB controls cell size, at least under some conditions, by regulating S6K activity by phosphorylation of dTSC2 [29].

The signaling systems controlling cell size and cell number are tightly interconnected. Genetic and biochemical analyses have revealed five different links between the dTSC-dTOR-dS6K pathway and the DInr-dPKB-dFOXO pathway. First, under conditions of unnaturally high insulin-signaling activity (that is, following the oncogenic activation of dPKB) dPKB phosphorylates and inactivates dS6K itself downregulates dPKB activity in a negative feedback loop [31]. Fourth, under severe starvation conditions, nuclear dFOXO presumably activates target genes that reduce cell proliferation. One of these target genes is
control of the insulin pathway in aging, and possibly diapause, is also under the process in a manner similar to dauer formation in worms. Dauer formation (regardless of length or temperature) and arrest development or the aging response to diverse environmental cues (nutrients, day length or temperature) and arrest development or the aging process in a manner similar to dauer formation in worms [65]. Ageing, and possibly diapause, is also under the control of the insulin pathway in Drosophila [65,66]. It has recently been shown that heterozygous IGF-1R mutant mice also exhibit a prolonged lifespan [3]. It therefore appears that the function of the insulin pathway, its components, and possibly at least some of its targets, have been conserved throughout evolution.

**Evolutionary conservation of insulin signaling and FOXO function**

Genetic dissection of signaling by insulin and its target DAF-16 has been pioneered in C. elegans and has helped to unravel the role of this pathway in dauer formation and longevity. Our analysis shows that the same pathway with the homologous nuclear targets operates in flies in the control of cell growth and proliferation, processes that do not involve insulin signaling in worms. Dauer formation and possibly longevity affect the entire organism and do not depend on cell-autonomous functions of the insulin signaling pathway [64]. The cell-growth phenotype in Drosophila, however, depends on the cell-autonomous functioning of the insulin-signaling cascade [45]. Insects enter diapause in response to diverse environmental cues (nutrients, day length or temperature) and arrest development or the aging process in a manner similar to dauer formation in worms [65]. Ageing, and possibly diapause, is also under the control of the insulin pathway in Drosophila [65,66]. It has recently been shown that heterozygous IGF-1R mutant mice also exhibit a prolonged lifespan [3]. It therefore appears that the function of the insulin pathway, its components, and possibly at least some of its targets, have been conserved throughout evolution.

**dFOXO may integrate different forms of cellular stress**

The longevity phenotype of IGF-1R-deficient mice is associated with enhanced resistance to oxidative stress [3]. It is likely that this phenomenon is due to hyperactivation of FOXO proteins, as several studies have shown that FOXO transcription factors play a role in the oxidative-stress response in mammalian cells [16,17] as well as in C. elegans [49]. Our observation that dFOXO mutant flies are hypersensitive to oxidative stress confirms that, in addition to their role in insulin signaling, the role of FOXO proteins in protecting against cellular stress is highly conserved. The mechanism by which dFOXO confers oxidative-stress resistance is not yet known. In our microarray experiment, we identified several genes encoding cytochrome P450 enzymes as dFOXO target gene candidates (Figure 6a). As it has been shown that cytochrome P450 enzymes reduce the toxic effects of paraquat in mice [67], they might partially mediate the protective effect of dFOXO. Furthermore, it remains to be established whether the regulation of dFOXO by insulin is required for dFOXO’s protective properties. It is tempting to speculate that distinct stress-induced signaling pathways activate dFOXO under conditions of cellular stress, in addition to the negative input from the insulin cascade, as several stress-induced phosphorylation sites are conserved between hFOXO3a and dFOXO (A Brunet and ME Greenberg, personal communication). This view is supported by our observation that overexpression of a FOXO variant that cannot be inactivated by PKB elicits cell death, a phenotype not observed in larval tissues lacking insulin-signaling components [45]. This result argues that dFOXO induces cellular responses that are independent of insulin.

The emerging model postulates that positive and negative inputs converge on FOXO proteins in response to different environmental conditions, making them central and important integrators controlling cellular (cell-cycle progression) and organismal adaptations (dauer formation, diapause and longevity; see Figure 7). Elucidating the positive inputs that converge on FOXO, by mutating conserved phosphorylation sites in the single Drosophila homolog of this class, should help us to better understand dFOXO’s integrator function.

**Materials and methods**

**Identification of dFOXO**

We searched the Drosophila genome [68] using a TBLASTN algorithm for sequences with homology to the DNA-binding domain of human FOXO3a (amino acids 157-251). The resultant matches were further assessed for the presence of consensus PKB phosphorylation sites R-X-R-X-X-S/T [57]. We used a genomic DNA stretch flanking the only identified region fulfilling these criteria to search a collection of Drosophila expressed sequence tags [69], which eventually identified two clones (LD05569 and LD18492) containing identical full-length cDNA sequences of 3.7 kb length. The dFOXO gene is annotated in FlyBase [70] (FBgn0038197) under the name foxo.

**Generation of plasmids and transgenic flies**

The cDNA clone LD05569 contains the full-length dFOXO cDNA within the pBS-SK(+) vector (Stratagene [71]). To generate a triple PKB phosphorylation mutant of dFOXO, we used PCR-based site-directed mutagenesis (QuickChange, Stratagene) to introduce the three point mutations T44A, S190A and S259A. Primer sequences are available upon
request. The mutated sequence was confirmed by double-stranded DNA sequencing. To generate UAS constructs, the cDNA inserts from both wild-type dFOXO and triple-mutant dFOXO were subcloned from pBS-SK(+) into the pUAST transformation vector [72] as EcoRI-Asp718 fragments. The corresponding UAS constructs containing the cDNA encoding wild-type and triple-mutant hFOXO3a [12] were generated by subcloning the inserts from pECE-HA-hFOXO3a and pECE-HA-hFOXO3a-TM (generous gifts of Anne Brunet) into pUAST as BglII-XbaI fragments. Fragments were excised from the pECE clones via complete digestion with XbaI followed by partial BglII digestion. All sequences were confirmed by double-stranded DNA sequencing. The four resultant UAS constructs are referred to as UAS-dFOXO, UAS-dFOXO-TM, UAS-hFOXO3a and UAS-hFOXO3a-TM.

To generate transgenic Drosophila lines, P-element-mediated germline transformation was carried out as described previously [73]. Several independent transformant lines were recovered for each construct with the exception of UAS-dFOXO-TM, for which we did not obtain a viable transformant line.

**EMS reversion mutagenesis**

To generate dFOXO loss-of-function mutants, homozygous y w; EP35-147 males were mutagenized with 27 mM ethyl methanesulfonate (EMS) according to standard procedures [74]. Mutagenized males were mated to homozygous y w; GMR-Gal4 UAS-Dp110DN virgins. Roughly 60,000 F1 progeny were screened for suppression of semilethality and the eye phenotype shown in Figure 3b. F1 revertants were retested for transmission of the reversion to F2 and positive candidate lines were then balanced over TM3 Sb Ser. To characterize the mutations, the dFOXO open reading frame from each individual mutagenized chromosome was amplified by RT-PCR and sequenced. The cDNA derived from the unmutagenized EP35-147 chromosome was used as a reference sample to identify mutations. Promising mutations were verified by double peak analysis of PCR fragments amplified from genomic DNA using the Sequencher program (Gene Codes Corporation [75]).

**Drosophila strains**

The EP-35-147 line was kindly provided by Konrad Basler, the GMR-Gal4 driver was a gift from M. Freeman. The GMR-Gal4, UAS-Dp110DN line was obtained from Sally Leevers, the eiger mutants from Masayuki Miura, and the Thor1 line from Paul Lasko.

**Phenotype analyses**

All phenotypes were analyzed in females raised at 25°C unless indicated otherwise. Body weight, cell size and cell number were determined as described previously [5]. The body weight experiment was performed in duplicate, and male and female flies were measured separately (n = 12 for each gender and genotype; the highest and lowest values were excluded from the analysis). Flies were reared under identical, non-crowding conditions and were of identical age (2 d) at the time of the experiment. The sizes of ommatidia and rhabdomeres were quantified with the program NIH Image 1.61. [76].

**Clonal analysis**

To induce loss-of-function clones, we used the FLP/FRT and ey-FLP systems to generate mosaic flies by mitotic recombination [47,77]. Overexpression clones were generated as described [63].

**In situ hybridizations**

**In situ** hybridizations to eye imaginal discs was performed as described [78,79]. The d4E-BP cDNA was PCR-amplified with Pfu polymerase from Promega [80] from total double-stranded cDNA derived from adult y w flies and cloned.
into the pCAP\textsuperscript{S} vector (PCR blunt-end cloning kit from Roche [81]). Insert orientation was determined by sequencing. Vector-specific PCR primers flanking the multiple cloning site (MCS) and containing either T7 or SP6 RNA polymerase promoters were used to synthesize double-stranded DNA templates for the labeling in vitro transcription reaction. The sense probe was transcribed with T7 and the antisense probe with SP6 RNA polymerase.

**Cell culture**

*Drosophila* embryonic Kc167 cells were maintained as described elsewhere [53]. Briefly, cells were grown at 25°C in Schneider’s *Drosophila* medium (Gibco/Invitrogen [82]) supplemented with 10% heat-inactivated fetal calf serum, FCS. Cells were split and diluted to a density of 1x10\textsuperscript{6} per ml twice a week. For the microarray experiment, cells were grown into the stationary phase for 7 d and then stimulated with 100 nM bovine insulin for 2 h.

**Microarray experiment**

The microarray experiment was performed at the Functional Genomics Center Zürich (FGCZ) using the Affymetrix GeneChip\textsuperscript{TM} system [83]. Total RNA was extracted from untreated control cells and insulin-treated cells 2 h after stimulation using the RNeasy Mini kit (Qiagen [84]) according to the manufacturer’s instructions. From each cell population, three independent samples were taken, processed in parallel and hybridized to three separate microarrays. Synthesis of cDNA and labeled cRNA, array hybridization and scanning were performed according to the standard Affymetrix protocols. The .chp files for the individual scanned microarrays were imported into the Affymetrix Data Mining Tool\textsuperscript{TM} software for data analysis.

**Stress treatments**

Stress-resistance experiments were performed with 3-day-old adult flies, and males and females were assayed separately. For bacterial infection experiments, adult flies were pricked with a thin needle which had been dipped in a concentrated bacterial culture [85]. Bacterial strains tested were the Gram-negative *Erwinia carotovora carotovora* and the Gram-positive *Micrococcus luteus*. Heat shock was performed by continuous exposure to 37°C. Resistance to heavy metals during development was assayed by rearing flies on food containing either 2.5 mM copper, 6 mM zinc or 200 \mu M cadmium. For the starvation test, flies were transferred from normal food to empty vials containing either 2.5 mM copper, 6 mM zinc or 200 \mu M cadmium. Dead flies were counted every 12 h (n = 80 for each gender and genotype). The hydrogen peroxide and paraquat experiments were each done in triplicate. Larval starvation was performed by rearing larvae on normal fly food until 80 h after egg deposition, then floating them in 30% glycerol, washing with water and transferring batches of 30-40 larvae to vials containing a gel of either PBS, 20% sucrose and 0.8% agarose (sugar condition) or PBS-agarose only (complete starvation).

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