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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 I_A phosphatidylinositol (PI) 3-kinases and increased intracellular concentrations of the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). 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 PIP_3, thereby acting as a negative regulator of insulin signaling [9]. The demonstration that the lethality associated with loss of dPTEN in *Drosophila* is rescued by a mutant form of dPKB with impaired affinity for PIP_3 indicates that PKB is a key effector of this pathway [10]. Genetic and biochemical studies have identified two critical targets of PKB, namely forkhead transcription factors of the FOXO subfamily and the Tuberous Sclerosis Complex 2 (TSC2) tumor suppressor protein.

In *C. elegans*, the only FOXO transcription factor is encoded by *daf-16*. Loss-of-function mutations in *daf-16* completely suppress the dauer-constitutive and longevity phenotypes associated with reduced function of insulin-signaling components. On the basis of knowledge about DAF signaling in *C. elegans*, forkhead transcription factors belonging to the FOXO subfamily have been identified as direct targets of insulin/IGF signaling in mammals [11-13]. The mammalian DAF-16 homologs comprise the proteins FOXO1 (FKHR), FOXO3a (FKHRL1) and FOXO4 (AFX). Their phosphorylation by the insulin-activated kinases PKB and serum- and glucocorticoid-regulated protein kinase (SGK) creates binding sites for 14-3-3 proteins, and this leads to inactivation of FOXO proteins via cytoplasmic sequestration [12,14]. The result of this process is an insulin-induced transcriptional repression of FOXO target genes, which are involved in the response to DNA damage [15] and oxidative stress [16,17], apoptosis [12,18], cell-cycle control [19-21] and metabolism [22]. In addition to their transcriptional activation capabilities, FOXO proteins 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

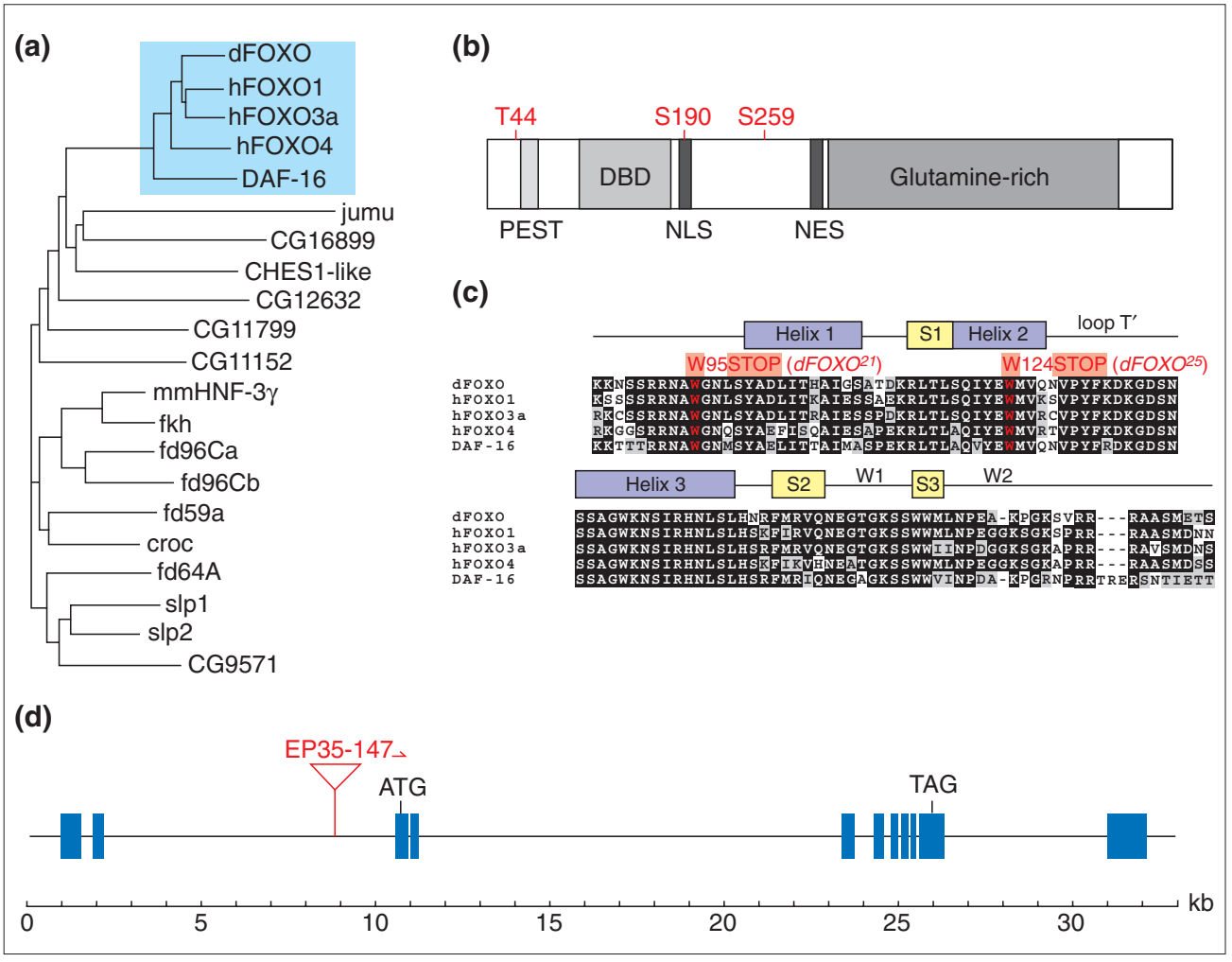


Figure 1
dFOXO is the only *Drosophila* FOXO/DAF-16 homolog. A TBLASTN search of the *Drosophila* genome for known and predicted genes encoding forkhead transcription factors retrieved 16 genes. **(a)** A phylogenetic tree calculated from a multiple sequence alignment of the forkhead domains of these 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-l6 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-l6.

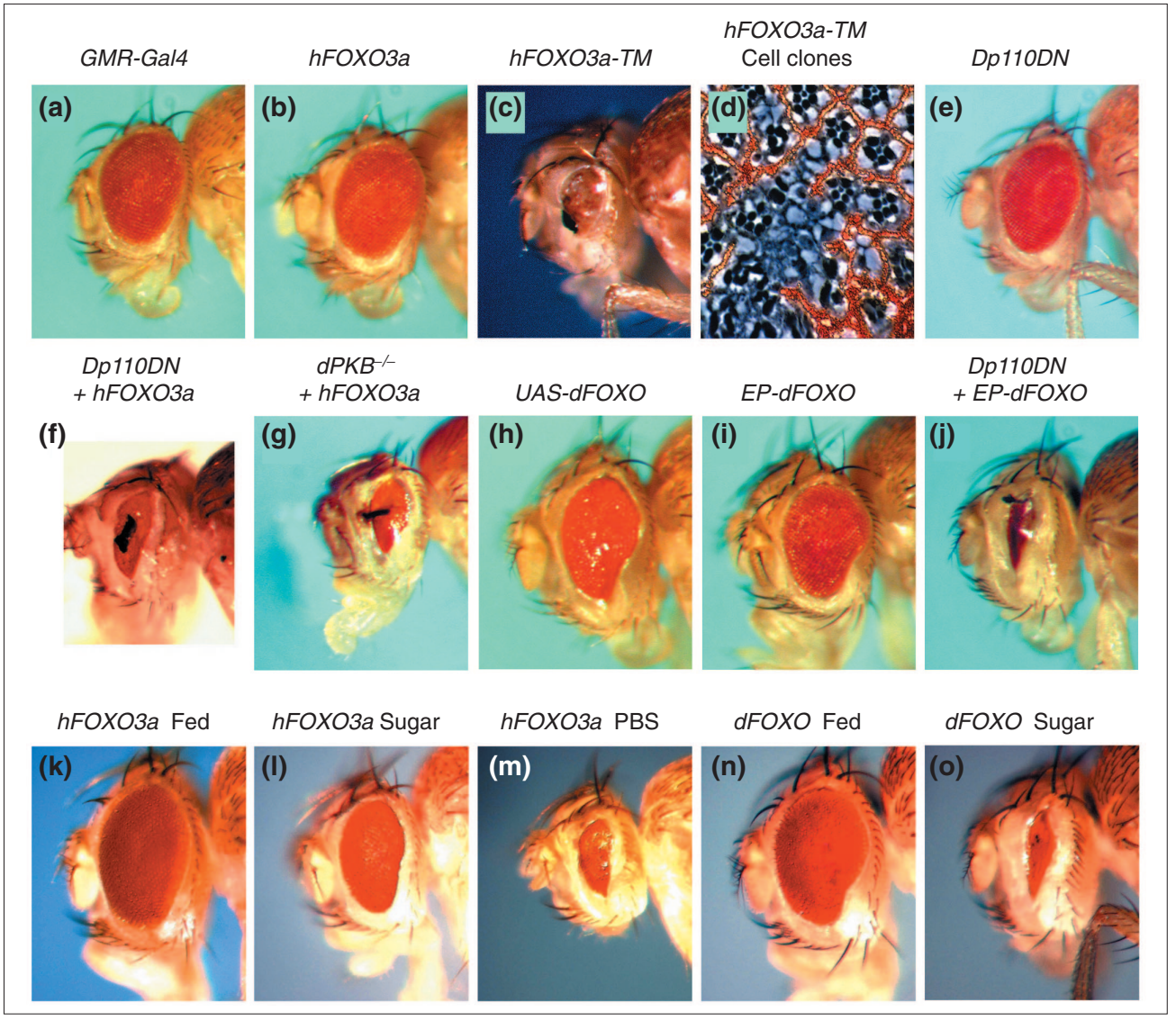


Figure 2
Targeted *hFOXO3a* and *dFOXO* expression in the developing *Drosophila* eye induces organ-size reduction and cell death, and the phenotypes are sensitive to insulin signaling and nutrient levels. **(a)** *GMR-Gal4*-expressing control fly. **(b)** No discernible phenotype results from *hFOXO3a* 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 mimicks 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/y 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*^{3}*/dPKB*^{1}; (h) *y w; UAS-dFOXO/GMR-Gal4*; (i) *y w; GMR-Gal4/+; EP-dFOXO/+*; (j) *y w; GMR-Gal4 UAS-Dp110DN/+; EP-dFOXO/+*; (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 *hF0X03a* 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 2j) 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 F_1 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 *($dFOXO^{21}$)* and Figure 3d *($dFOXO^{25}$)*. In $dFOXO^{21}$ and $dFOXO^{25}$, 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 homozygous-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 $dFOXO^{21}$ and $dFOXO^{25}$ 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 figure on the next page)*
Null *dFOXO* mutants are viable, have no overgrowth phenotype and are hypersensitive to oxidative stress. **(a)** *Dp110DN* expressing control fly. **(b)** *EP*-driven coexpression of *dFOXO* elicits a necrotic eye phenotype. **(c,d)** EMS-induced mutations in *dFOXO* lead to a reversion of the overexpression phenotype. **(e,f)** Selective removal of dFOXO from the head (right) does not lead to an organ-size alteration compared to a control fly (left). **(g)** w^--marked dFOXO-deficient photoreceptor cells are the same size as wild-type cells. **(h)** In contrast to *dPTEN*, *dFOXO* null mutants have no organismal growth phenotype. For each genotype, the left bar indicates the body weight of females and the right bar the weight of males. Values are shown ± standard deviation (SD). **(i)** *dFOXO* mutants are hypersensitive to oxidative stress. The graph shows a survival curve of male adult flies on PBS/sucrose gel containing 5% hydrogen peroxide. The observed hypersensitivity is more pronounced in males, but is also observed in females (not shown). The increased resistance of homozygous *EP-dFOXO* flies might be caused by low basal *dFOXO* overexpression from the *EP* element, which occurs due to leakiness of *UAS* enhancers in the absence of Gal4. Control flies placed on PBS/sucrose without oxidant survived during the time window shown. Genotypes are: (a) *y w; GMR-Gal4 UAS-Dp110DN/+*; (b) *y w; GMR-Gal4 UAS-Dp110DN/+; EP-dFOXO/+*; (c) *y w; GMR-Gal4 UAS-Dp110DN/+; EP-dFOXO^{21}/+*; (d) *y w; GMR-Gal4 UAS-Dp110DN/+; EP-dFOXO^{25}/+*; (e,f) *y w ey-flp/y w; FRT82/FRT82 cl3R3 w$^+$* (left); *y w ey-flp/y w; FRT82 EP-dFOXO^{21}/FRT82 cl3R3 w$^+$* (right); (g) *y w hs-flp/y w; FRT82 EP-dFOXO^{21}/FRT82 w$^+$*.

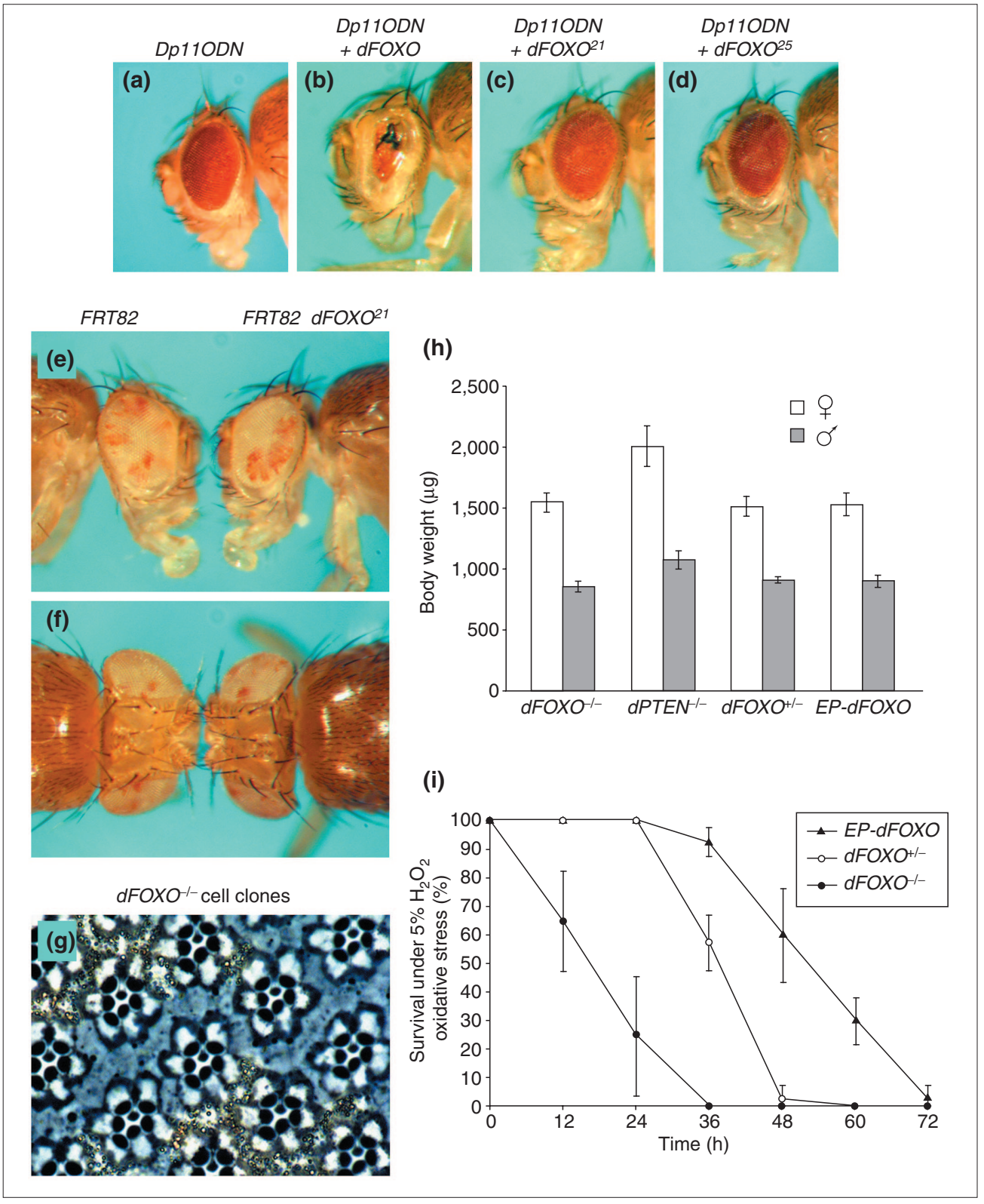


Figure 3 *(see legend on the previous page)*

The growth-deficient phenotypes of *DInr*, *chico*, *Dp110* and *dPKB* mutants are significantly suppressed by loss of dFOXO

We performed genetic epistasis experiments to examine whether the growth phenotypes of DInr-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 *DInr*, *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 5l). 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 $dPTEN^{-/-}$ bighead 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 *DInr* [52]. It may be that in a *dPTEN*-mutant background dFOXO activates DInr expression in a negative-feedback loop. In this model, concomitant loss of dFOXO would alleviate the *dPTEN* overgrowth phenotype by lowering DInr 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)TAAACAA [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

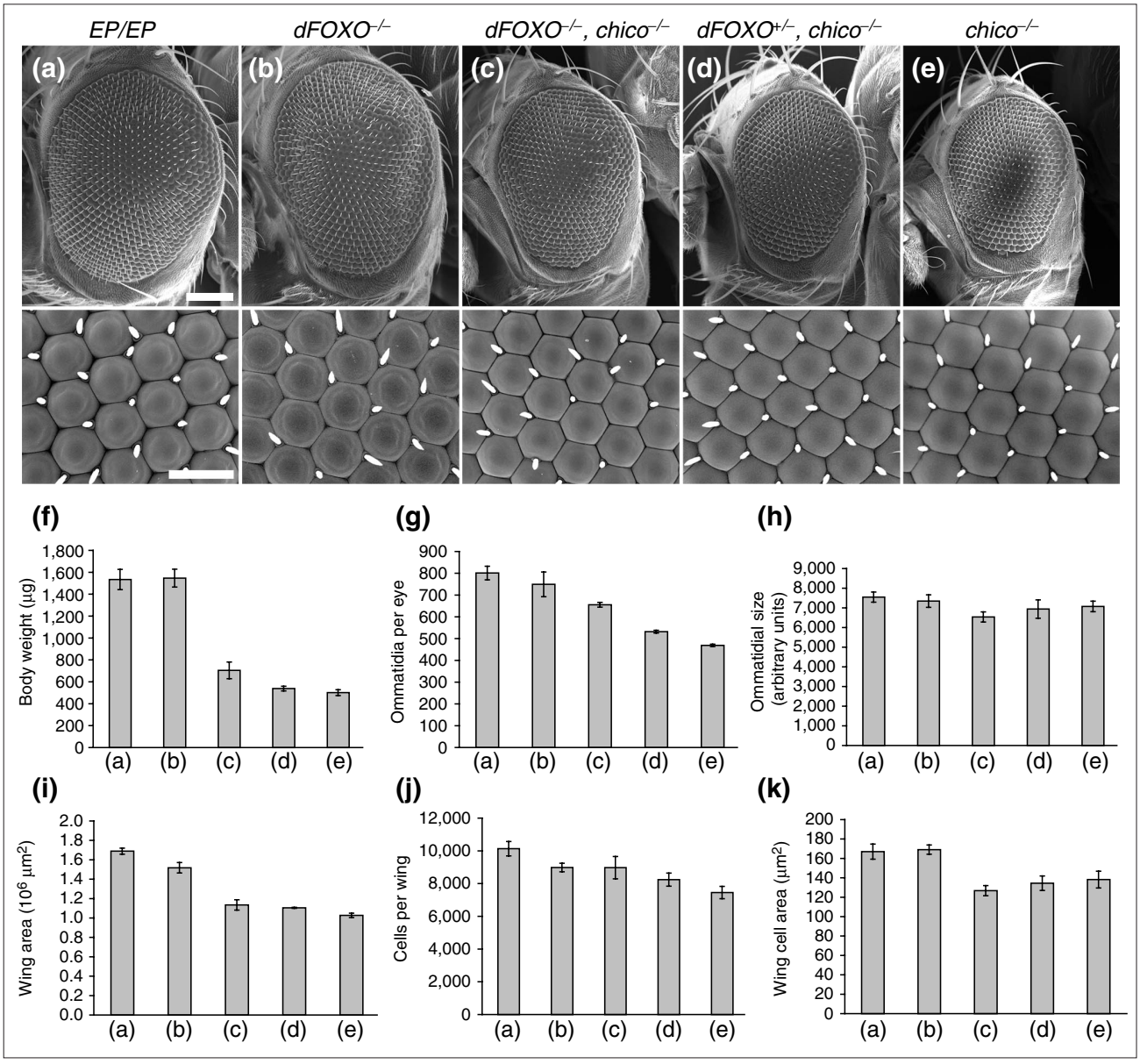


Figure 4

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-dFOXO*^{21}*/EP-dFOXO*^{25}; (c) *y w; chico*^{1}*/chico*^{2}*; EP-dFOXO*^{21}/+; (d) *y w; chico*^{1}*/chico*^{2}*; EP-dFOXO*^{21}*/ EP-dFOXO*^{25}; (e) *y w; chico*^{1}*/chico*^{2}.

[57]. The *d4E-BP* gene encodes a translational repressor and was initially identified as the immune-compromised *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 *d4E-BP* locus. The d4E-BP protein is negatively

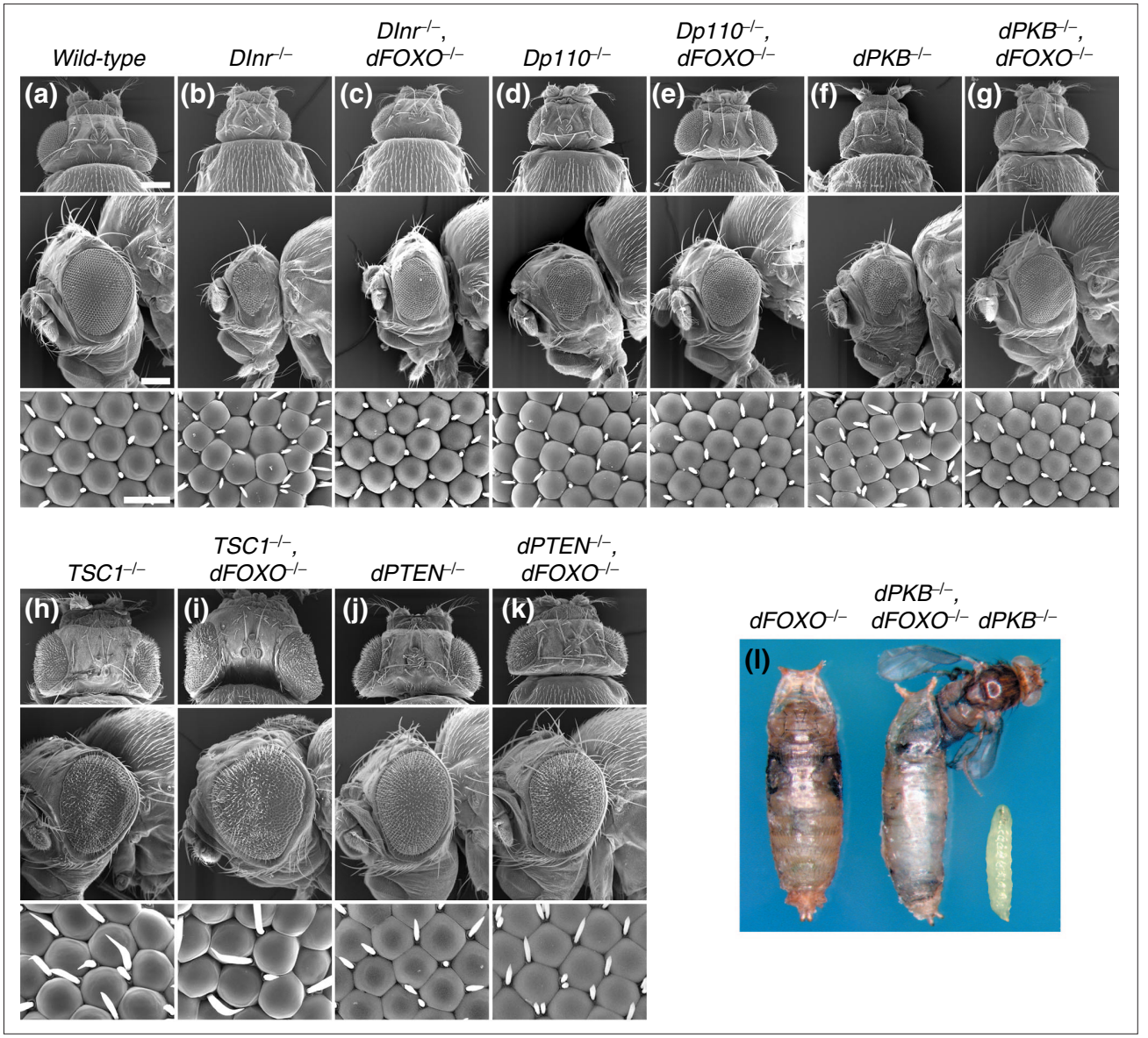
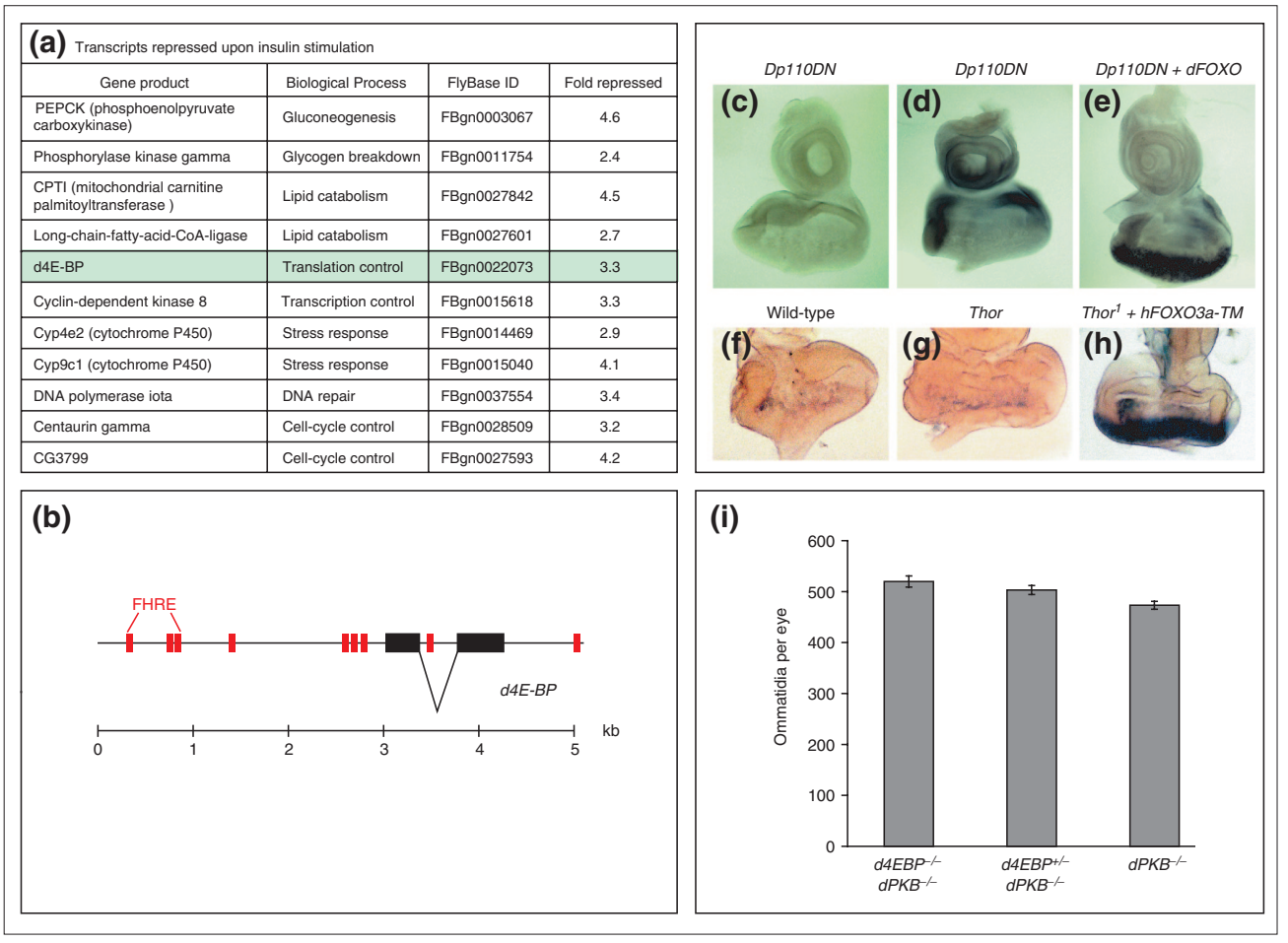


Figure 5
Growth-deficient phenotypes of *DInr*, *Dp110* and *dPKB* mutants are suppressed by loss of dFOXO. **(a)** Control fly. **(b)** Selective removal of DInr from the head leads to a pinhead phenotype, which is partially suppressed by the loss of dFOXO **(c)**. The same suppression is observed in *Dp110*-, and *dPKB*-pinheads **(d-g)**. The *TSC1*$^{-/-}$ bighead phenotype **(h)** is enhanced by mutations in *dFOXO* **(i)**, but the *dPTEN*$^{-/-}$ bighead **(j)** is slightly suppressed **(k)**. **(l)** Living without PKB. In contrast to the larval lethality of *dPKB* null mutants, *dPKB-dFOXO* double mutants develop into small pharate adults, most of which fail to eclose. Bar sizes are 200 μm (low magnification) and 20 μm (high magnification). Genotypes are: (a) *y w ey-flp/y w; FRT82/FRT82 cl3R3 w*$^{+}$; (b) *y w ey-flp/y w; FRT82 DInr*^{304}*/FRT82 cl3R3 w*$^{+}$; (c) *y w ey-flp/y w; FRT82 DInr*^{304} *EP-dFOXO*^{25}*/FRT82 cl3R3 w*$^{+}$; (d) *y w ey-flp/y w; FRT82 Dp110*^{5W3}*/FRT82 cl3R3 w*$^{+}$; (e) *y w ey-flp/y w; FRT82 Dp110*^{5W3} *EP-dFOXO*^{25}*/FRT82 cl3R3 w*$^{+}$; (f) *y w ey-flp/y w; FRT82 dPKB*^{1}*/FRT82 cl3R3 w*$^{+}$; (g) *y w ey-flp/y w; FRT82 dPKB*^{1} *EP-dFOXO*^{25}*/FRT82 cl3R3 w*$^{+}$; (h) *y w ey-flp/y w; FRT82 dTSC1*^{Q87X}*/FRT82 cl3R3 w*$^{+}$; (i) *y w ey-flp/y w; FRT82 dTSC1*^{Q87X} *EP-dFOXO*^{25}*/FRT82 cl3R3 w*$^{+}$; (j) *y w ey-flp/y w; FRT40 dPTEN*$^{117-4}$*/FRT40 cl2L3 w*$^{+}$; (k) *y w ey-flp/y w; FRT40 dPTEN*$^{117-4}$*/FRT40 cl2L3 w*$^{+}$; *FRT82 EP-dFOXO*^{25}*/FRT82 cl3R3 w*$^{+}$; (l) *y w;; EP-dFOXO*^{21}*/EPdFOXO*^{25} (left), *y w;; dPKB*^{1} *EP-dFOXO*^{21}*/dPKB*^{1} *EP-dFOXO*^{25} (middle), *dPKB*^{1}*/dPKB*^{1} (right).



(a) Transcripts repressed upon insulin stimulation

Gene product	Biological Process	FlyBase ID	Fold repressed
PEPCK (phosphoenolpyruvate carboxykinase)	Gluconeogenesis	FBgn0003067	4.6
Phosphorylase kinase gamma	Glycogen breakdown	FBgn0011754	2.4
CPTI (mitochondrial carnitine palmitoyltransferase)	Lipid catabolism	FBgn0027842	4.5
Long-chain-fatty-acid-CoA-ligase	Lipid catabolism	FBgn0027601	2.7
d4E-BP	Translation control	FBgn0022073	3.3
Cyclin-dependent kinase 8	Transcription control	FBgn0015618	3.3
Cyp4e2 (cytochrome P450)	Stress response	FBgn0014469	2.9
Cyp9c1 (cytochrome P450)	Stress response	FBgn0015040	4.1
DNA polymerase iota	DNA repair	FBgn0037554	3.4
Centaurin gamma	Cell-cycle control	FBgn0028509	3.2
CG3799	Cell-cycle control	FBgn0027593	4.2

Figure 6

dFOXO regulates transcription of the *d4E-BP* gene. **(a)** A selection of microarray-identified genes that are transcriptionally downregulated after 2 h of insulin stimulation in Kc167 cells and contain forkhead response elements (FHREs) in their genomic upstream or intronic sequences. **(b)** FHREs (red) at the *d4E-BP* locus; black boxes are exons. **(c,d)** Overexpression of *Dp110DN* alone does not induce transcription of *d4E-BP* in imaginal discs, but **(e)** coexpression of *dFOXO* strongly upregulates the gene. **(f-h)** Expression of human *FOXO3a-TM* induces expression of the *d4E-BP* enhancer trap line *Thor*^{1}. **(i)** *d4E-BP* and *dPKB* interact genetically. The *Thor*^{1} mutation increases the ommatidial number in *dPKB*-mutants by 9% without affecting cell size. Values are shown ± SD. Genotypes are: (c) *y w; GMR-Gal4 UAS-Dp110DN/+*; (d) *y w; GMR-Gal4 UAS-Dp110DN/+*; (e) *y w; GMR-Gal4 UAS-Dp110DN/+; EP-dFOXO/+*; (f) *y w*; (g) *y w; Thor*^{1}*/+*; (h) *y w; Thor*^{1}*/GMR-Gal4; UAS-hFOXO3a-TM/+*; (i) from right to left: *y w;; dPKB*^{3}*/dPKB*^{1}, *y w; Thor*^{1}*/+; dPKB*^{3}*/dPKB*^{1}, *y w; Thor*^{1}*/Thor*^{1}*; dPKB*^{3}*/dPKB*^{1}.

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 *Thor*^{1} 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*^{1} mutation slightly but significantly suppressed the reduced cell-number phenotype in a dose-dependent manner. The *Thor*^{1} 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 suppresses 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 dTSC2, resulting in increased activation of dS6K [29]. Under normal culture conditions this regulation does not seem critical, however, loss of dPKB function does not lower dS6K activity in larval extracts [54]. Second, under physiological conditions, dPDK1 regulates dPKB as well as dS6K [63]. Third, 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

d4E-BP, which encodes an inhibitor of translation initiation. When conditions improve, the insulin and TOR signaling pathways can stimulate translation by disrupting the 4E-BP/eIF4E complex via phosphorylation of 4E-BP, and in parallel by repressing FOXO-dependent 4E-BP expression. Fifth, under even more severe starvation or stress conditions, full activation of dFOXO upregulates expression of the insulin receptor itself, thus rendering the cell hypersensitive to low insulin levels (see [52]). These multiple positive and negative interactions ensure a continuous fine adjustment of the growth rate to changing environmental conditions.

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 [37].

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

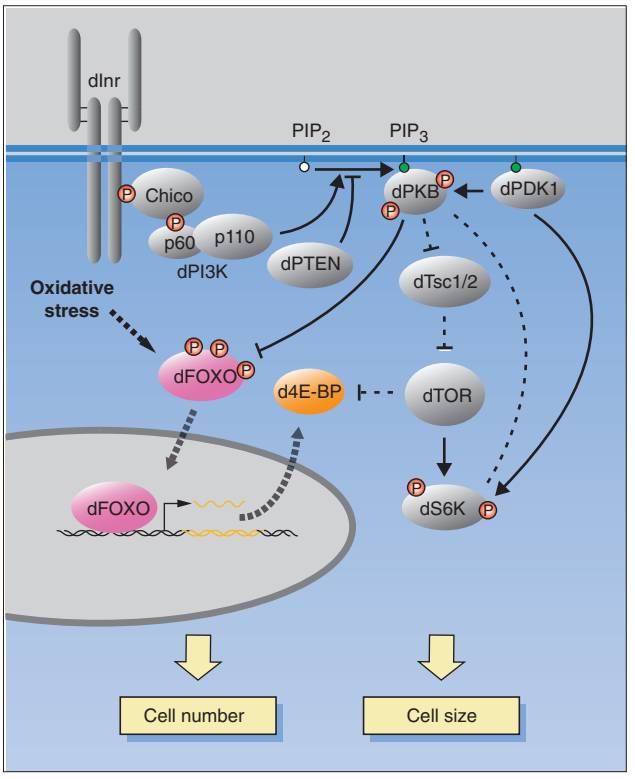


Figure 7
dFOXO may be an integrator of cellular stress. We propose a model in which dFOXO senses different forms of cellular stress (that is, nutrient deprivation or reactive oxygen species) and induces cellular responses, such as proliferation arrest, in part by repressing translation via upregulation of *d4E-BP*. The various signaling proteins shown in the figure are discussed in the text.

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 *Eco*RI-*Asp*718 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 *Bgl*II-*Xba*I fragments. Fragments were excised from the pECE clones via complete digestion with *Xba*I followed by partial *Bgl*II 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 F_1 progeny were screened for suppression of semilethality and the eye phenotype shown in Figure 3b. F_1 revertants were retested for transmission of the reversion to F_2 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 *Thor*^{1} 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^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 $1x10^6$ 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™ 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™ 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 μM cadmium. For the starvation test, flies were transferred from normal food to empty vials closed with a wet foam stopper. For oxidative-stress challenge, flies were starved in empty vials for 6 h and then transferred to vials containing a gel of phosphate-buffered saline (PBS), 10% sucrose, 0.8% low-melt agarose and the respective oxidative agent (either 5% H_2O_2 or 20 mM paraquat). The oxidant was added to the solution after cooling to 40°C. A control population of flies was placed in vials containing the PBS-sucrose gel without oxidant. 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 transfering 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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References

1. Takahashi Y, Kadowaki H, Momomura K, Fukushima Y, Orban T, Okai T, Taketani Y, Akanuma Y, Yazaki Y, Kadowaki T: **A homozygous kinase-defective mutation in the insulin receptor gene in a patient with leprechaunism.** *Diabetologia* 1997, **40:**412-420.
2. Baker J, Liu JP, Robertson EJ, Efstratiadis A: **Role of insulin-like growth factors in embryonic and postnatal growth.** *Cell* 1993, **75:**73-82.
3. Holzenberger M, Dupont J, Ducos B, Leneuve P, Geloen A, Even PC, Cervera P, Le Bouc Y: **IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice.** *Nature* 2003, **421:**182-187.
4. Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G: ***daf*-2, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*.** *Science* 1997, **277:**942-946.
5. Brogiolo W, Stocker H, Ikeya T, Rintelen F, Fernandez R, Hafen E: **An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control.** *Curr Biol* 2001, **11:**213-221.
6. Tatar M, Kopelman A, Epstein D, Tu MP, Yin CM, Garofalo RS: **A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function.** *Science* 2001, **292:**107-110.
7. Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E, Leevers SJ, Partridge L: **Extension of life-span by loss of CHICO, a *Drosophila* insulin receptor substrate protein.** *Science* 2001, **292:**104-106.
8. Cantley LC: **The phosphoinositide 3-kinase pathway.** *Science* 2002, **296:**1655-1657.
9. Maehama T, Dixon JE: **The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate.** *J Biol Chem* 1998, **273:**13375-13378.
10. Stocker H, Andjelkovic M, Oldham S, Laffargue M, Wymann MP, Hemmings BA, Hafen E: **Living with lethal PIP3 levels: viability of flies lacking PTEN restored by a PH domain mutation in Akt/PKB.** *Science* 2002, **295:**2088-2091.

11. Burgering BM, Kops GJ: **Cell cycle and death control: long live Forkheads.** *Trends Biochem Sci* 2002, **27:**352-360.
12. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME: **Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor.** *Cell* 1999, **96:**857-868.
13. Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, Burgering BM: **Direct control of the Forkhead transcription factor AFX by protein kinase B.** *Nature* 1999, **398:**630-634.
14. Brunet A, Park J, Tran H, Hu LS, Hemmings BA, Greenberg ME: **Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a).** *Mol Cell Biol* 2001, **21:**952-965.
15. Tran H, Brunet A, Grenier JM, Datta SR, Fornace AJ, Jr., DiStefano PS, Chiang LW, Greenberg ME: **DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein.** *Science* 2002, **296:**530-534.
16. Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM: **Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress.** *Nature* 2002, **419:**316-321.
17. Furukawa-Hibi Y, Yoshida-Araki K, Ohta T, Ikeda K, Motoyama N: **FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress.** *J Biol Chem* 2002, **277:**26729-26732.
18. Dijkers PF, Medema RH, Lammers JW, Koenderman L, Coffer PJ: **Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1.** *Curr Biol* 2000, **10:**1201-1204.
19. Dijkers PF, Medema RH, Pals C, Banerji L, Thomas NS, Lam EW, Burgering BM, Raaijmakers JA, Lammers JW, Koenderman L, *et al.*: **Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1).** *Mol Cell Biol* 2000, **20:**9138-9148.
20. Alvarez B, Martinez AC, Burgering BM, Carrera AC: **Forkhead transcription factors contribute to execution of the mitotic programme in mammals.** *Nature* 2001, **413:**744-747.
21. Medema RH, Kops GJ, Bos JL, Burgering BM: **AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1.** *Nature* 2000, **404:**782-787.
22. Barthel A, Schmoll D, Kruger KD, Bahrenberg G, Walther R, Roth RA, Joost HG: **Differential regulation of endogenous glucose-6-phosphatase and phosphoenolpyruvate carboxykinase gene expression by the forkhead transcription factor FKHR in H4IIE-hepatoma cells.** *Biochem Biophys Res Commun* 2001, **285:**897-902.
23. Ramaswamy S, Nakamura N, Sansal I, Bergeron L, Sellers WR: **A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR.** *Cancer Cell* 2002, **2:**81-91.
24. Schmidt M, de Mattos SF, van der Horst A, Klompmaker R, Kops GJ, Lam EW, Burgering BM, Medema RH: **Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D.** *Mol Cell Biol* 2002, **22:**7842-7852.
25. Nakae J, Biggs WH, 3rd, Kitamura T, Cavenee WK, Wright CV, Arden KC, Accili D: **Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1.** *Nat Genet* 2002, **32:**245-253.
26. Borkhardt A, Repp R, Haas OA, Leis T, Harbott J, Kreuder J, Hammermann J, Henn T, Lampert F: **Cloning and characterization of AFX, the gene that fuses to MLL in acute leukemias with a t(X;11)(q13;q23).** *Oncogene* 1997, **14:**195-202.
27. Sublett JE, Jeon IS, Shapiro DN: **The alveolar rhabdomyosarcoma PAX3/FKHR fusion protein is a transcriptional activator.** *Oncogene* 1995, **11:**545-552.
28. Inoki K, Li Y, Zhu T, Wu J, Guan KL: **TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling.** *Nat Cell Biol* 2002, **4:**648-657.
29. Potter CJ, Pedraza LG, Xu T: **Akt regulates growth by directly phosphorylating Tsc2.** *Nat Cell Biol* 2002, **4:**658-665.
30. Gao X, Zhang Y, Arrazola P, Hino O, Kobayashi T, Yeung RS, Ru B, Pan D: **Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling.** *Nat Cell Biol* 2002, **4:**699-704.
31. Radimerski T, Montagne J, Hemmings-Mieszczak M, Thomas G: **Lethality of *Drosophila* lacking TSC tumor suppressor function rescued by reducing dS6K signaling.** *Genes Dev* 2002, **16:**2627-2632.
32. Stocker H, Radimerski T, Schindelholz B, Wittwer F, Belawat P, Daram P, Breuer S, Thomas G, Hafen E: **Rheb is an essential regulator of S6K in controlling cell growth in *Drosophila*.** *Nat Cell Biol* 2003, **5:**559-566.
33. Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D: **Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins.** *Nat Cell Biol* 2003, **5:**578-581.
34. Saucedo LJ, Gao X, Chiarelli DA, Li L, Pan D, Edgar BA: **Rheb promotes cell growth as a component of the insulin/TOR signalling network.** *Nat Cell Biol* 2003, **5:**566-571.
35. Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, Kozma SC, Hafen E, Bos JL, Thomas G: **Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2.** *Mol Cell* 2003, **11:**1457-1466.
36. Montagne J, Stewart MJ, Stocker H, Hafen E, Kozma SC, Thomas G: ***Drosophila* S6 kinase: a regulator of cell size.** *Science* 1999, **285:**2126-2129.
37. Alessi DR, Caudwell FB, Andjelkovic M, Hemmings BA, Cohen P: **Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase.** *FEBS Lett* 1996, **399:**333-338.
38. Hay BA, Wassarman DA, Rubin GM: ***Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death.** *Cell* 1995, **83:**1253-1262.
39. Leevers SJ, Weinkove D, MacDougall LK, Hafen E, Waterfield MD: **The *Drosophila* phosphoinositide 3-kinase Dp110 promotes cell growth.** *EMBO J* 1996, **15:**6584-6594.
40. Beadle G, Tatum E, Clancy C: **Food level in relation to rate of development and eye pigmentation in *Drosophila melanogaster*.** *Biol Bull* 1938, **75:**447-462.
41. Zinke I, Schutz CS, Katzenberger JD, Bauer M, Pankratz MJ: **Nutrient control of gene expression in *Drosophila*: microarray analysis of starvation and sugar-dependent response.** *EMBO J* 2002, **21:**6162-6173.
42. Ollmann M, Young LM, Di Como CJ, Karim F, Belvin M, Robertson S, Whittaker K, Demsky M, Fisher WW, Buchman A, *et al.*: ***Drosophila* p53 is a structural and functional homolog of the tumor suppressor p53.** *Cell* 2000, **101:**91-101.
43. Igaki T, Kanda H, Yamamoto-Goto Y, Kanuka H, Kuranaga E, Aigaki T, Miura M: **Eiger, a TNF superfamily ligand that triggers the *Drosophila* JNK pathway.** *EMBO J* 2002, **21:**3009-3018.
44. Kramer JM, Davidge JT, Lockyer JM, Staveley BE: **Expression of Drosophila FOXO regulates growth and can phenocopy starvation.** *BMC Dev Biol* 2003, **3:**5.
45. Bohni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andruss BF, Beckingham K, Hafen E: **Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4.** *Cell* 1999, **97:**865-875.
46. Weinkove D, Neufeld TP, Twardzik T, Waterfield MD, Leevers SJ: **Regulation of imaginal disc cell size, cell number and organ size by *Drosophila* class I(A) phosphoinositide 3-kinase and its adaptor.** *Curr Biol* 1999, **9:**1019-1029.
47. Newsome TP, Asling B, Dickson BJ: **Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics.** *Development* 2000, **127:**851-860.
48. Oldham S, Stocker H, Laffargue M, Wittwer F, Wymann M, Hafen E: **The *Drosophila* insulin/IGF receptor controls growth and size by modulating PtdInsP(3) levels.** *Development* 2002, **129:**4103-4109.
49. Honda Y, Honda S: **The *daf-2* gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*.** *FASEB J* 1999, **13:**1385-1393.
50. Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L, Coffer PJ: **FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity.** *J Cell Biol* 2002, **156:**531-542.

51. Yanase S, Yasuda K, Ishii N: **Adaptive responses to oxidative damage in three mutants of *Caenorhabditis elegans (age-1, mev-1* and *daf-16)* that affect life span.** *Mech Ageing Dev* 2002, **123:**1579-87.
52. Puig O, Marr MT, Ruhf ML, Tjian R: **Control of cell number by *Drosophila* FOXO: downstream and feedback regulation of the insulin receptor pathway.** *Genes Dev,* in press.
53. Radimerski T, Mini T, Schneider U, Wettenhall RE, Thomas G, Jeno P: **Identification of insulin-induced sites of ribosomal protein S6 phosphorylation in *Drosophila melanogaster*.** *Biochemistry* 2000, **39:**5766-5774.
54. Radimerski T, Montagne J, Rintelen F, Stocker H, van der Kaay J, Downes CP, Hafen E, Thomas G: **dS6K-regulated cell growth is dPKB/dPI(3)K-independent, but requires dPDK1.** *Nat Cell Biol* 2002, **4:**251-255.
55. Furuyama T, Nakazawa T, Nakano I, Mori N: **Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues.** *Biochem J* 2000, **349:**629-634.
56. Zinke I, Kirchner C, Chao LC, Tetzlaff MT, Pankratz MJ: **Suppression of food intake and growth by amino acids in *Drosophila*: the role of *pumpless*, a fat body expressed gene with homology to vertebrate glycine cleavage system.** *Development* 1999, **126:**5275-5284.
57. Miron M, Verdu J, Lachance PE, Birnbaum MJ, Lasko PF, Sonenberg N: **The translational inhibitor 4E-BP is an effector of PI(3)K/Akt signalling and cell growth in *Drosophila*.** *Nat Cell Biol* 2001, **3:**596-601.
58. Bernal A, Kimbrell DA: ***Drosophila* Thor participates in host immune defense and connects a translational regulator with innate immunity.** *Proc Natl Acad Sci USA* 2000, **97:**6019-6024.
59. Rodriguez A, Zhou Z, Tang ML, Meller S, Chen J, Bellen H, Kimbrell DA: **Identification of immune system and response genes, and novel mutations causing melanotic tumor formation in *Drosophila* melanogaster.** *Genetics* 1996, **143:**929-940.
60. Paradis S, Ruvkun G: ***Caenorhabditis elegans* Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor.** *Genes Dev* 1998, **12:**2488-2498.
61. Potter CJ, Huang H, Xu T: ***Drosophila* Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size.** *Cell* 2001, **105:**357-368.
62. Gao X, Pan D: **TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth.** *Genes Dev* 2001, **15:**1383-1392.
63. Rintelen F, Stocker H, Thomas G, Hafen E: **PDK1 regulates growth through Akt and S6K in *Drosophila*.** *Proc Natl Acad Sci USA* 2001, **98:**15020-15025.
64. Apfeld J, Kenyon C: **Cell nonautonomy of *C. elegans daf-2* function in the regulation of diapause and life span.** *Cell* 1998, **95:**199-210.
65. Tatar M, Yin C: **Slow aging during insect reproductive diapause: why butterflies, grasshoppers and flies are like worms.** *Exp Gerontol* 2001, **36:**723-738.
66. Partridge L, Gems D: **Mechanisms of ageing: public or private?** *Nat Rev Genet* 2002, **3:**165-175.
67. Shimada H, Furuno H, Hirai K, Koyama J, Ariyama J, Simamura E: **Paraquat detoxicative system in the mouse liver postmitochondrial fraction.** *Arch Biochem Biophys* 2002, **402:**149-157.
68. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, *et al.*: **The genome sequence of *Drosophila melanogaster*.** *Science* 2000, **287:**2185-2195.
69. Rubin GM, Hong L, Brokstein P, Evans-Holm M, Frise E, Stapleton M, Harvey DA: **A *Drosophila* complementary DNA resource.** *Science* 2000, **287:**2222-2224.
70. **FlyBase** [http://flybase.org]
71. **Stratagene** [http://www.stratagene.com]
72. Brand AH, Perrimon N: **Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.** *Development* 1993, **118:**401-415.
73. Basler K, Christen B, Hafen E: **Ligand-independent activation of the sevenless receptor tyrosine kinase changes the fate of cells in the developing *Drosophila* eye.** *Cell* 1991, **64:**1069-1081.
74. Ashburner M: *Drosophila.* Cold Spring Harbor Laboratory Press: Cold Spring Harbor; 1989.
75. **Gene Codes Corporation** [http://www.genecodes.com]
76. **NIH Image** [http://rsb.info.nih.gov/nih-image]
77. Xu T, Rubin GM: **Analysis of genetic mosaics in developing and adult *Drosophila* tissues.** *Development* 1993, **117:**1223-1237.
78. Lehmann R, Tautz D: ***In situ* hybridization to RNA.** *Methods Cell Biol* 1994, **44:**575-598.
79. O'Neill JW, Bier E: **Double-label *in situ* hybridization using biotin and digoxigenin-tagged RNA probes.** *Biotechniques* 1994, **17:**870-875.
80. **Promega** [http://www.promega.com]
81. **Roche** [http://www.roche-applied-science.com]
82. **Invitrogen life technologies** [http://www.invitrogen.com]
83. **Affymetrix** [http://www.affymetrix.com]
84. **Qiagen** [http://www.qiagen.com]
85. Lemaitre B, Reichhart JM, Hoffmann JA: ***Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms.** *Proc Natl Acad Sci USA* 1997, **94:**14614-14619.
86. Weigelt J, Climent I, Dahlman-Wright K, Wikstrom M: **Solution structure of the DNA binding domain of the human forkhead transcription factor AFX (FOXO4).** *Biochemistry* 2001, **40:**5861-5869.