Genome-wide dFOXO targets and topology of the transcriptomic response to stress and insulin signalling

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FoxO transcription factors, inhibited by insulin/insulin-like growth factor signalling (IIS), are crucial players in numerous organismal processes including lifespan. Using genomic tools, we uncover over 700 direct dFOXO targets in adult female *Drosophila*. dFOXO is directly required for transcription of several IIS components and interacting pathways, such as *TOR*, in the wild-type fly. The genomic locations occupied by dFOXO in adults are different from those observed in larvae or cultured cells. These locations remain unchanged upon activation by stresses or reduced IIS, but the binding is increased and additional targets activated upon genetic reduction in IIS. We identify the part of the IIS transcriptional response directly controlled by dFOXO and the indirect effects and show that parts of the transcriptional response to IIS reduction do not require *dfoxo*. Promoter analyses revealed GATA and other forkhead factors as candidate mediators of the indirect and *dfoxo*-independent effects. We demonstrate genome-wide evolutionary conservation of dFOXO targets between the fly and the worm *Caenorhabditis elegans*, enriched for a second tier of regulators including the *dHR96/daf-12* nuclear hormone receptor.
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Introduction

The insulin/insulin-like growth factor (IGF) signalling (IIS) pathway, conserved throughout the animal kingdom, affects a variety of traits, including growth and development, metabolic homoeostasis, stress resistance, fecundity and adult lifespan (for review see Russell and Kahn, 2007; Piper *et al*, 2008). Forkhead Box-O (FoxO; note that we use FoxO to refer to all the members of the group) transcription factors (TFs) are regulated by IIS. Stimulation of IIS activates the Akt kinase, which in turn inactivates Foxo3A through phosphorylation resulting in nuclear exclusion (Brunet *et al*, 1999). Conversely, inactivation of IIS results in activation of FoxOs. FoxOs are also controlled by other signalling pathways, and have complex and important roles during animal development and adulthood. They are involved in metabolism, stress protection, cellular differentiation, cell-cycle arrest and apoptosis (for review see Greer and Brunet, 2008; Partridge and Bruning, 2008; Salih and Brunet, 2008). Recently, FoxOs have been shown to act as lineage-restricted tumour suppressors and to

be important in stem cell maintenance in mice (Paik *et al*, 2007, 2009; Tothova *et al*, 2007).

Reduced IIS activity extends lifespan in distantly related model organisms such as the nematode worm *Caenorhabditis elegans*, the mouse *Mus musculus* and the fruit fly *Drosophila melanogaster*, at the same time delaying or diminishing age-associated functional decline (Kenyon *et al*, 1993; Clancy *et al*, 2001; Tatar *et al*, 2001; Wessells *et al*, 2004; Martin and Grotewiel, 2006; Selman *et al*, 2008). The molecular basis of this lifespan extension is currently under intense investigation. Work on *C. elegans* has established the critical role of FoxOs in lifespan. The single worm FoxO orthologue (*daf-16*) is essential for prolonged lifespan and other traits upon reduction in IIS (Kenyon *et al*, 1993), indicating that transcriptional reprogramming effected by DAF-16 is the basis of this enhanced longevity. Indeed, *daf-16* is crucial for the transcriptional response to reduced IIS (Murphy *et al*, 2003). However, the requirement for *dfoxo* in the transcriptional or lifespan response to reduced IIS in *Drosophila* or other organisms has not been defined.

FoxOs have a role in lifespan beyond the IIS pathway: they are also required for lifespan extension achieved by manipulations of the Jun N-terminal kinase (JNK) pathway in flies (Wang *et al*, 2005) and of the Ste20-like kinase (MST) and AMP-activated protein kinase in worms (Lehtinen *et al*, 2006; Greer *et al*, 2007), and also for some forms of dietary restriction in the worm (Greer *et al*, 2007; Honjoh *et al*, 2009; Zhang *et al*, 2009). Furthermore, adult-onset and tissue-restricted overexpression of the single *Drosophila* FoxO orthologue (*dfoxo*) is sufficient to enhance longevity in the fly (Giannakou *et al*, 2004; Hwangbo *et al*, 2004). Further emphasising the pivotal and evolutionarily conserved role that FoxOs have in lifespan, genetic variation in the *Foxo3A* gene in humans is strongly associated with longevity (Kuningas *et al*, 2007; Willcox *et al*, 2008; Flachsbart *et al*, 2009). Thus, FoxOs are emerging as potentially important targets for intervention into ageing and ageing-related diseases of humans.

A crucial part of understanding the functioning of TFs, such as dFOXO, is determining their *in vivo* genome-wide binding locations and the specific transcriptional programmes they orchestrate from these locations. In the case of FoxOs, such information is only emerging. A number of genes are bound by DAF-16 in the worm, but <100 transcriptionally regulated direct targets are known (Oh *et al*, 2006; Schuster *et al*, 2010). In *Drosophila*, genome-wide dFOXO targets have been only examined in larvae during starvation (Teleman *et al*, 2008) and these may have only limited relevance to adult-specific traits such as ageing.

In this study, we use genomic approaches to discover >700 direct dFOXO targets in the adult female fly. We show that the dFOXO genomic binding locations do not change during stress or downregulation of IIS, but that different target genes are regulated in wild-type and IIS mutant flies. We define the part of the IIS response that requires the action of dFOXO directly as well the indirect effects. Surprisingly, we uncover a substantial portion of the IIS response that does not require *dfoxo*. In parallel to this study and corroborating our findings, Slack *et al* (2011) have shown that *dfoxo* is only required for a subset of physiological changes brought on by reduced IIS in the fly, unlike the situation in *C. elegans* where all known phenotypic outputs of reduced IIS require *daf-16*. Despite this difference in the architecture of the IIS response between the worm and the fly, we find conservation of FoxO-dependent transcriptional effects, and a significant genome-wide conservation of genes bound by dFOXO and DAF-16.

Results

dFOXO binds ∼1400 genomic locations in the adult female fly that are distinct from those bound in larvae or cultured cells

dfoxo has an important role in adult fly physiology, as evidenced by a substantial reduction in lifespan upon removal of *dfoxo* function (Giannakou *et al*, 2008; Min *et al*, 2008; Slack *et al*, 2011), a reduction that is also observed in loss-of-function mutants for the worm orthologue *daf-16* (Larsen *et al*, 1995; Garigan *et al*, 2002). This prompted us to capture a snapshot of genomic locations bound by dFOXO in adult flies kept under normal conditions. We prepared chromatin from 7-day-old

females and pulled-down dFOXO-associated DNA with an affinity-purified anti-dFOXO antibody (Giannakou *et al*, 2007). As a control, we performed a mock immunoprecipitation (IP) using the pre-immune serum. By hybridisation of the pulled-down DNA to genome-wide tiling arrays and determination of binding peaks (see Materials and methods), we identified 1423 dFOXO-bound genomic regions, averaging 908 bp in length. The sites bound by dFOXO tended to cluster together in a non-random manner: 78% of the peaks were within 10 kb of another, whereas one peak per 99 kb would be expected by chance. An example of the peaks identified is given in Figure 1A. The locations of the bound regions, as well as all other lists mentioned in the paper are given as Supplementary information. The binding was reproducible, as demonstrated by high concordance of the three biological replicates (Supplementary Figures 1 and 2; Supplementary Figure 2 shows Parson correlations of all ChIP-chip experiments performed). To validate the array data, we tested for enrichment of the bound regions by qPCR. Eight out of eight dFOXO-bound and three out of three non-bound regions were verified by qPCR (Figure 1B), indicating high reliability of the data set. To further establish the specificity of the antibody used, we performed ChIP-chip on *dfoxoΔ/dfoxoΔ* (*dfoxo*$^{Δ/Δ}$) flies that completely lacked the dFOXO protein (Slack *et al*, 2011). None of the peaks identified in the wild type were present in the *dfoxo*$^{Δ/Δ}$ (for an example see Figure 1A), confirming that these genomic regions were specifically bound by dFOXO.

The sites bound by dFOXO in the adult fly were distinct from those previously described as occupied in larvae (Figure 2A) (Teleman *et al*, 2008), and the overlap was slightly less than expected by chance (overlap of nine peaks expected by chance, three observed, $P=0.02$). This revealed that dFOXO binding may be influenced by developmental stage and/or tissue composition of the animal. The sites bound were also distinct from those previously observed in cell culture. For example, ectopically expressed dFOXO was bound to the promoter of the *Drosophila* insulin receptor (*dInR*) gene in cultured cells (Puig *et al*, 2003; Puig and Tjian, 2005), whereas we found it bound to the coding region of the gene in adult females. To confirm that this difference was not due to different antibodies or different ChIP protocols used in our and previous studies, we examined the binding of endogenous dFOXO to DNA in S2 cells after 2 h serum starvation. We found that dFOXO was bound to the P1 promoter of the *dInR* gene in S2 cells, while it bound the coding region of the same gene in adult females (Figure 2B and C). Since the same antibody and the same IP conditions were used, this difference reflects a true difference in dFOXO binding in S2 cells and adults. Hence, the sites of dFOXO binding are dependent on cell type. Note that the binding within coding/transcribed regions was a general feature of dFOXO binding in adult female flies (Supplementary Figure 3).

To gain an insight into the DNA sequence recognised by dFOXO in adult females, we looked for statistical over-representation of known binding motifs in the DNA recovered from ChIP using Clover analysis (Frith *et al*, 2004). Several forkhead-like motifs containing the core FoxO-recognition sequence WWAACA (Biggs *et al*, 2001) were enriched, such as WWWRTAAASAWAA and WNTATAAACAWNNR (Table I), indicating that these are a good match to the motif recognised
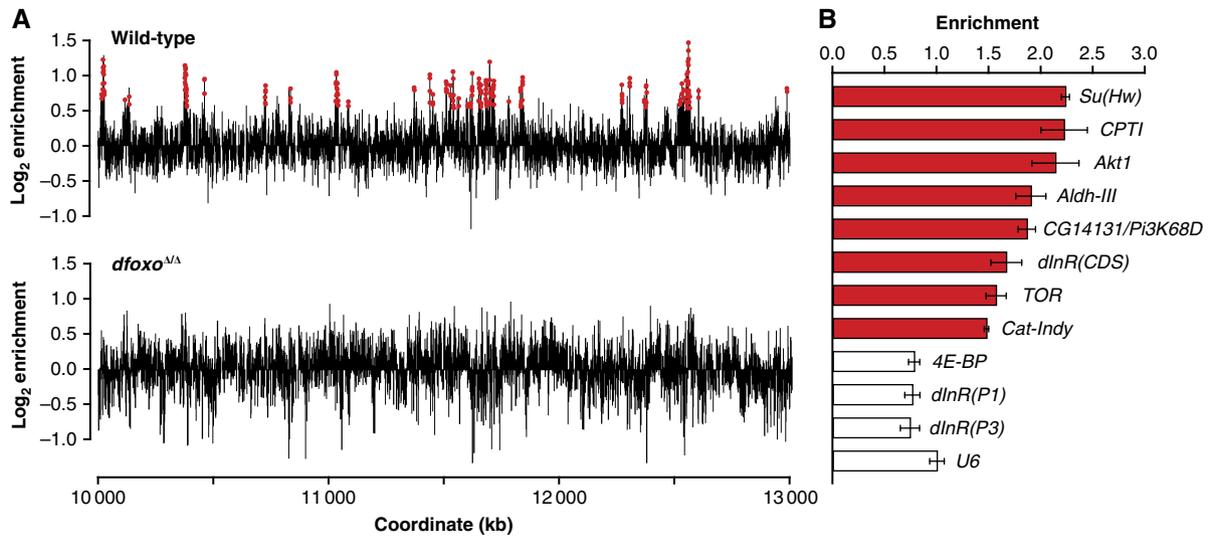
Figure 1 Genome-wide dFOXO binding in whole adult flies. (**A**) ChIP-chip assays were carried out on 7-day-old females using anti-dFOXO antibody. ChIP-chip traces, showing the enrichment (log$_2$-transformed) of the dFOXO-immunoprecipitated DNA over total chromatin, are averages of three biological repeats after subtraction of the mock (pre-immune serum) control and are shown over a 3-Mbp region of chromosome 2R in wild-type flies (top) or *dfoxo$^{\Delta/\Delta}$* flies (bottom). Red dots denote the peaks identified in the ChIP-chip signal. Note that no peaks were identified in this region in the *dfoxo$^{\Delta/\Delta}$* flies. (**B**) qPCR was used to confirm the enrichment observed in dFOXO ChIP-chip in the three biological repeats of the wild-type chromatin. Relative enrichment was calculated as proportion of chromatin recovered in the IP for a single region divided by average recovered for all regions for that chromatin, with *U6* enrichment set to one. The data are presented as means with standard errors. Red indicates regions that were expected to be enriched, white indicates those that were not. Significant difference was detected by ANOVA ($P < 10^{-4}$, $n = 3$), and *t*-test revealed that the regions indicated in red were significantly different from the others ($P < 0.05$).
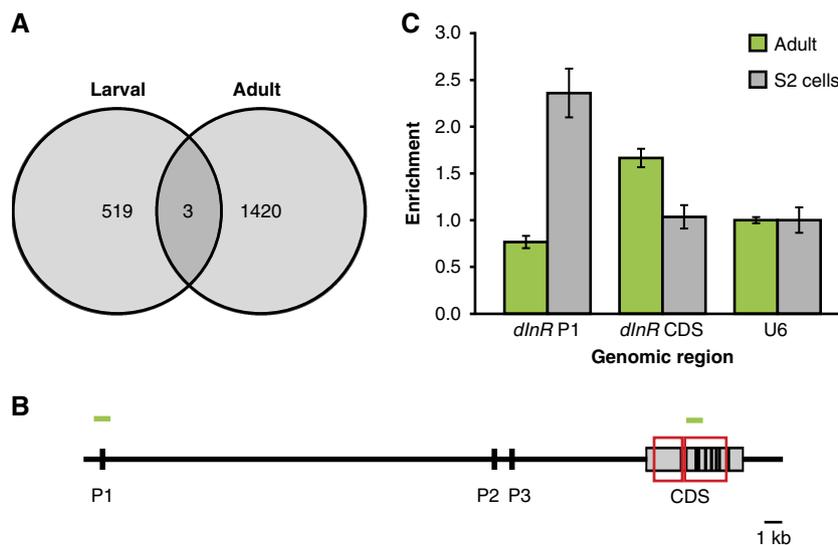

Figure 2 dFOXO-binding sites in adults are distinct from those in larvae or S2 cells. (**A**) Overlap between the genomic sites bound by dFOXO in larvae and adults. The data for larvae were generated by Teleman *et al* (2008). The observed overlap was slightly smaller than expected by chance ($P = 0.02$). Expected overlap of nine peaks was determined from simulation of 10^3 random peak sets, of identical size, length and chromosomal distribution. (**B**) A schematic of the *dInR* locus is given with grey boxes representing exons, black marks the P1, P2 and P3 promoters (Casas-Tinto *et al*, 2007), red boxes the sites bound by dFOXO in adult flies (observed in ChIP-chip data) and green bars the location of amplicons (left—P1, right—coding region) used for ChIP-qPCR shown in (C). (**C**) dFOXO binding within the *dInR* locus in adults and S2 cells. The qPCR results show relative enrichment of the P1 promoter and the coding region (CDS) of *dInR*, or the *U6* control, in three biological repeats of adult chromatin, or three IPs from a single chromatin sample from 2 h serum-starved S2 cells. The data are presented as in Figure 1B. ANOVA detected significant differences in enrichment ($n = 3$, $P < 10^{-4}$), with P1 promoter being enriched in S2 cells and the coding region in adults (*t*-test, $P < 0.05$).

by dFOXO. We attempted to generate *de novo* the dFOXO motif present in the genomic DNA bound by dFOXO using MEME analysis. Unfortunately, MEME failed to identify a forkead-like motif but isolated variants of a CTGCTG sequence (Supplementary Table 1). This sequence is similar to the motif bound by ADF1 (England *et al*, 1990), the motif that was also identified as highly enriched in our ChIP-recovered sequences by Clover (Table I), indicating that ADF1, a *Drosophila* Myb-

Table I Representative enriched motifs identified by Clover

Motif	TF	Raw score	P^*
	All sequences bound by dFOXO		
VCGCYGCMGYCGCTGMCNGCG	ADF1	665	$< 10^{-3}$
WWWRTAAASAWAA	BRCZ4	659	$< 10^{-3}$
WNTATAAACAWNNR	XFD2	222	$< 10^{-3}$

Motif	TF	Raw score	P^{**}
	dFOXO-bound and gene(s) downregulated in $dfoxo^{\Delta/\Delta}$ [a]		
NNNGCCASCAGRKGGCRSNN	CTCF	117	$< 10^{-3}$
TRTAAACAANWN	FOXO3A	104	0.003

Motif	TF	Raw score	P^{**}
	dFOXO-bound and gene(s) upregulated in $dfoxo^{\Delta/\Delta}$ [a]		
WWWRTAAASAWAA	BRCZ4	75.2	$< 10^{-3}$
TRTAAACAANWN	FOXO3A	56.7	$< 10^{-3}$

Motif	TF	Raw score	P^{**}
	dFOXO-bound and gene(s) downregulated in $dfoxo^{\Delta/\Delta}$ $daGAL4 > UAS\text{-}dInR^{DN}$ [b]		
NNNGCCASCAGRKGGCRSNN	CTCF	271	$< 10^{-3}$
TRTAAACAANWN	FOXO3A	208	$< 10^{-3}$

Motif	TF	Raw score	P^{**}
	dFOXO-bound and gene(s) upregulated in $dfoxo^{\Delta/\Delta}$ $daGAL4 > UAS\text{-}dInR^{DN}$ [b]		
NWAAACAAN	FOXO1	63.5	$< 10^{-3}$
TRTAAACAANWN	FOXO3A	57.8	0.002

Motif	TF	Raw score	P^{***}
	Promoters of genes downregulated in $daGAL4 > UAS\text{-}dInR^{DN}$ indirectly dependent on $dfoxo$		
NNWGATAASA	GATA2	80	$< 10^{-3}$
MNAGATAANR	GATA1	39.9	$< 10^{-3}$

Motif	TF	Raw score	P^{***}
	Promoters of genes upregulated in $daGAL4 > UAS\text{-}dInR^{DN}$ independent of $dfoxo$		
NNNWAAAYAAAYANNNNN	FOXJ2	115	0.004
WWWRTAAASAWAA	BRCZ4	110	$< 10^{-3}$

Motif	TF	Raw score	P^{***}
	Promoters of genes downregulated in $daGAL4 > UAS\text{-}dInR^{DN}$ independent of $dfoxo$		
NNWGATAASA	GATA2	8.11	$< 10^{-3}$
NCWGATAACA	GATA1	4.77	0.003

For the comprehensive lists please refer to Supplementary information.
[a]Relative to wild type.
[b]Relative to $daGAL4 > UAS\text{-}dInR^{DN}$.
*Relative to whole chromosome 2L.
**Relative to all sequences bound by dFOXO.
***Relative to all promoter sequences of the genes present on the expression arrays.

like transcriptional activator (Cutler *et al*, 1998), may share genomic sites with dFOXO.

dFOXO directly regulates 356 genes in the wild-type adult female

In all, 1755 unique genes were no further than 1 kb away from a dFOXO-bound site, defining a large set of potential dFOXO target genes. To identify which of these genes are direct dFOXO targets in the wild type, we identified the genes that require *dfoxo* for their normal expression in the adult female under standard conditions. A large portion of the transcriptome (2036 genes) was altered upon *dfoxo* removal, confirming the importance of this TF to adult physiology. Furthermore, there was a significant overlap ($P = 10^{-10}$) between the genes whose expression changed in $dfoxo^{\Delta/\Delta}$ and the set of putative dFOXO targets obtained from ChIP-chip, revealing a total of 356 direct dFOXO targets in the adult female (Figure 3A). The enrichment was specific to the subset of genes that were downregulated upon deletion of *dfoxo* ($P = 2 \times 10^{-14}$), indicating that dFOXO tends to act as an activator of transcription, while also directly repressing some genes. The DNA sequences bound by dFOXO and associated with the 356 direct targets that were
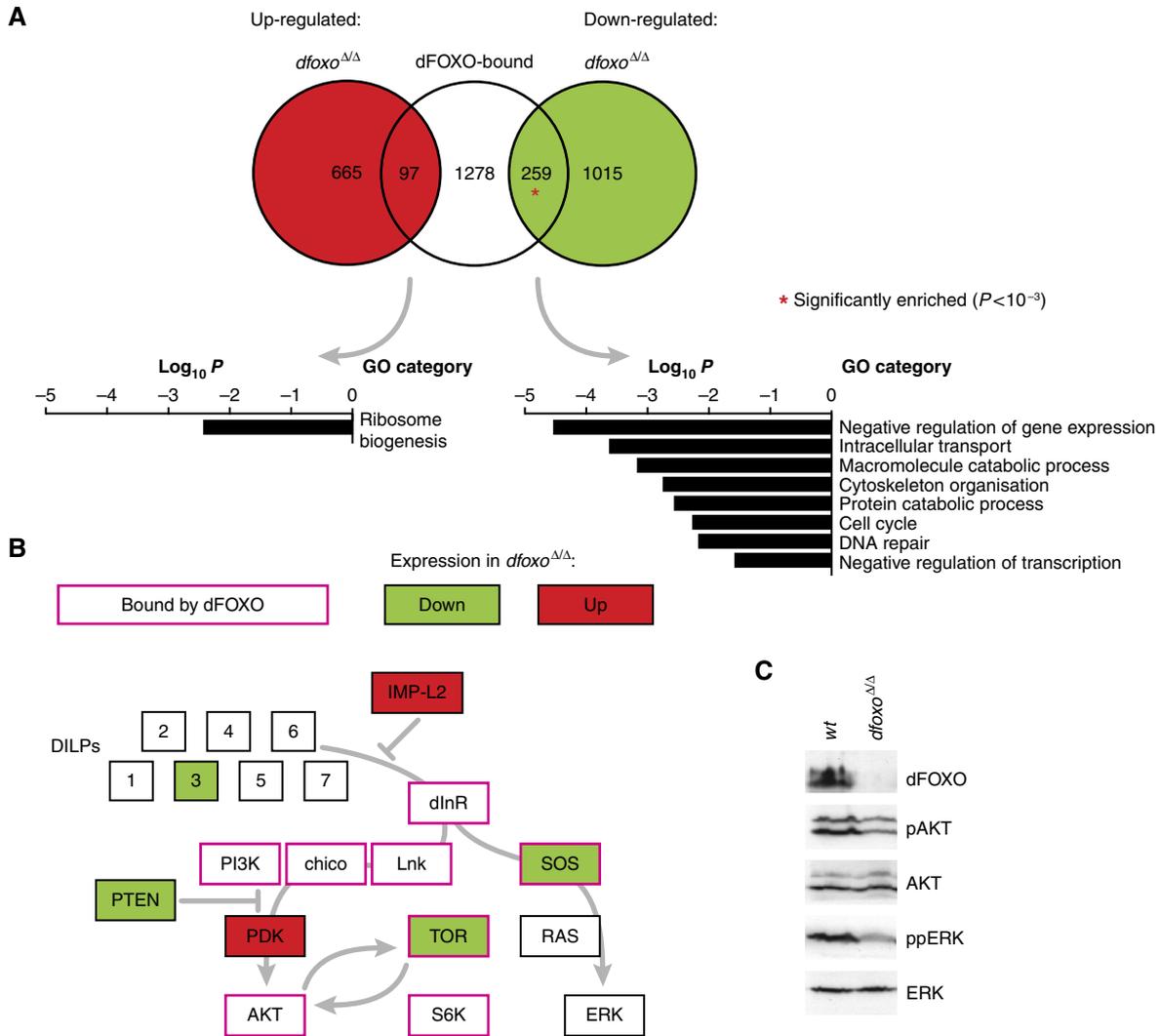
Figure 3 Direct dFOXO targets in wild-type adult flies. (**A**) Overlap between the genes that neighbour a dFOXO-bound site and those with transcript levels altered in *dfoxo*$^{\Delta/\Delta}$ flies relative to wild type. For consistency with later experiments, both *dfoxo*$^{\Delta/\Delta}$ and wild-type flies also carried the *daGAL4* driver. The probability of overlap was calculated based on hypergeometric distribution and an overlap significantly larger than expected by chance ($P < 10^{-3}$) is indicated with a red asterisk. Note that only the dFOXO-bound genes that were present on the expression arrays were taken into account. Representative biological functions enriched within the overlaps are shown. (**B**) dFOXO binding and regulation of IIS components. dFOXO binding and altered transcript levels in *dfoxo*$^{\Delta/\Delta}$ flies were mapped onto a schematic of IIS. Note that PI3K denotes the p110 subunit. (**C**) The levels of Serine 505-phosphorylated AKT (pAKT) and the dually phosphorylated ERK (ppERK) were measured in wild-type and *dfoxo*$^{\Delta/\Delta}$ females, as well as the levels of total AKT, ERK and dFOXO. *dfoxo*$^{\Delta/\Delta}$ females had 70% ($\pm 10\%$) of the wild-type pAKT/AKT ratio, and 40% ($\pm 3\%$) of wild-type ppERK/ERK. In both cases, the difference to wild type was significant ($P < 0.05$, $n = 3$, *t*-test).

up/downregulated in *dfoxo*$^{\Delta/\Delta}$ flies were further enriched for forkhead-like motifs relative to all dFOXO-bound sites (Table I). Thus, a high density of binding motifs correlates with observable transcriptional control.

Functional analysis of direct dFOXO targets (Figure 3A) revealed dFOXO to be an activator of genes involved in cell cycle, DNA repair, cytoskeletal organisation, intracellular transport and protein catabolism. dFOXO also directly repressed certain ribosome biogenesis genes. Interestingly, a significant number of genes involved in repression of gene expression, particularly at the level of transcription, were downregulated in the absence of *dfoxo*, including the insulator proteins *su(Hw)*, *CTCF* (Bushey *et al*, 2008) and a member of a polycomb group protein complex—*dSfmbt* (Muller and

Verrijzer, 2009), revealing that dFOXO might be important for establishment, demarcation and maintenance of repressive chromatin states. Interestingly, CTCF-recognised DNA motifs were enriched in the sequences bound by dFOXO and associated with loss of transcription in *dfoxo* nulls (Table I), indicating that CTCF may be important at sites of dFOXO-driven transcriptional activation.

Furthermore, we found that dFOXO directly regulated the expression of several important sequence-specific TFs, including *Bigmax*, *Mio* and *dHR96*, thus uncovering a substantial second tier of regulators. *Bigmax* and *Mio* are a pair of basic helix-loop-helix leucine zipper TFs that are the fly orthologues of the MondoA and Mlx TFs involved in regulating metabolism in mammals (Sans *et al*, 2006), while *dHR96* encodes a nuclear

hormone receptor regulating xenobiotic resistance in flies (King-Jones *et al*, 2006). Metabolic and detoxification genes were not significantly represented within the direct dFOXO targets, even though FoxOs have been implicated in the control of metabolic and detoxification processes (McElwee *et al*, 2003, 2007; Murphy *et al*, 2003; Matsumoto *et al*, 2007). In the fly, substantial control of these processes may be mediated via secondary effectors, such as *Bigmax/Mio*, which are directly repressed by dFOXO, and *dHR96*, which is directly activated by dFOXO.

Feedback regulation of *dInR* by dFOXO through transcriptional upregulation has been previously demonstrated in experiments with cultured *Drosophila* cells (Puig *et al*, 2003). This feedback onto the IIS pathway may be more extensive than previously thought, because dFOXO was also bound to the insulin-receptor substrates *chico* and *Lnk*, the *Akt* kinase and the *Sos* adaptor protein genes, as well as to components of the IIS-interacting TOR signalling pathway, *S6K* and *TOR* itself (Figure 3B). Importantly, dFOXO was directly required for the maintenance of *TOR* and *Sos* transcription in the adult female, since these genes were both bound by dFOXO and their mRNA decreased in the *dfoxo*$^{\Delta/\Delta}$ mutant. Interestingly, the transcriptional changes to IIS pathway components, including the upregulation of *Imp-L2*, an IGF-binding protein homologue and a negative regulator of IIS (Honegger *et al*, 2008; Alic *et al*, 2011), and a downregulation of *dilp3*, a *Drosophila* insulin-like peptide gene (Brogiolo *et al*, 2001) (Figure 3B), imply that *dfoxo*$^{\Delta/\Delta}$ flies may behave as mutants with reduced IIS activity with respect to the components upstream and/or parallel to dFOXO itself. At the same time, the observed changes in PTEN and PDK could partially compensate for this loss of IIS (Figure 3B).

To further investigate the effect of the direct regulation by dFOXO of *TOR* and *Sos* transcription, we determined the consequences of loss of *dfoxo* on the relevant signalling pathways. AKT is phosphorylated on S505 by the TOR kinase as part of the TOR complex 2 (Sarbassov *et al*, 2005), while SOS activity results in phosphorylation and activation of the ERK kinase (Biggs *et al*, 1994). In *dfoxo*$^{\Delta/\Delta}$ flies, levels of both S505-phosphorylated AKT and phosphorylated ERK were significantly reduced (Figure 3C), demonstrating that dFOXO-mediated regulation of signalling components has an effect on downstream signalling events. Note that a reduction in AKT S505 phosphorylation in a *dfoxo* mutant has also been observed by others (Shen and Tower, 2010).

dFOXO genomic locations are unaltered but binding is increased upon stress or IIS reduction

dfoxo is thought to be an important regulator of stress responses, with a well-documented role in resistance to oxidative stress and starvation (Junger *et al*, 2003; Puig and Tjian, 2005; Zheng *et al*, 2007; Teleman *et al*, 2008; Villa-Cuesta *et al*, 2010). These two assaults may pose different demands on fly physiology, and it might thus be expected that dFOXO would change its binding locations to regulate different groups of genes during these two different stresses.

We determined conditions of paraquat (a superoxide generator) or starvation exposure that activated dFOXO, by

examining its phosphorylation status. In cell extracts, AKT-phosphorylated, inactive dFOXO is retarded on SDS–PAGE (Puig *et al*, 2003). Two bands were also present in extracts from 7-day-old female flies, and the proportion of the slower migrating, phosphorylated dFOXO (dFOXO$_{ppp}$) was increased in flies injected with recombinant human insulin compared with mock-injected or uninjected controls (Figure 4A), consistent with AKT phosphorylation. The phosphorylation of the top band was confirmed with calf intestinal phosphatase (CIP) treatment. Treatment of flies with 20 mM paraquat in food (18 h) or starvation (48 h) resulted in an increase in the proportion of unphosphorylated dFOXO (Figure 4B), indicating its activation.

ChIP-chip performed on paraquat-treated or starvation-exposed flies revealed that the substantial majority of binding locations remained the same as those in the untreated controls (Supplementary Table 2; Supplementary Figure 2), and visual inspection of the remaining sites indicated that they were actually present in untreated controls but below the peak-calling threshold. While there appeared to be essentially no change in the location of dFOXO, the ChIP-chip data indicated a general increase in the intensity of the dFOXO-bound peaks. Comparison of non-normalised array replicate data showed that the peak height (the ratio of the height of peak probes to background probes) was significantly higher in the treated samples than in the untreated controls (Figure 4C). This general trend was confirmed for four target regions by qPCR (Figure 4D). Hence, upon stress, more dFOXO localises to the same sites already occupied in the absence of stress.

We also determined whether dFOXO binds to different target sites when it is activated by a reduction in IIS, by performing ChIP-chip on flies with dampened IIS through ubiquitous expression of a dominant-negative form of *dInR* using the *daugtherlessGAL4* driver (*daGAL4*). Importantly, *daGAL4 > UAS-dInR*^{DN} flies have an extended lifespan (Ikeya *et al*, 2009). This genetic intervention also resulted in increased binding to pre-existing sites on a genome-wide scale (Figure 4E), and this was confirmed for four specific regions by qPCR (Figure 4F). Thus, upon activation, dFOXO increases its occupancy on pre-existing sites.

dFOXO binding is enriched within the genes upregulated upon IIS reduction

To further examine the relationship between the dFOXO regulon and IIS, we looked at the proportion of dFOXO-bound genes among the genes that are regulated by IIS (Figure 5). As a canonical model of IIS reduction, we generated and compared the expression profiles of *daGAL4 > UAS-dInR*^{DN} flies to their controls, the same genotypes that were used for ChIP-chip above. dFOXO-bound genes were enriched within the genes upregulated in the whole *daGAL4 > UAS-dInR*^{DN} flies (178 genes, $P = 3 \times 10^{-11}$), confirming that dFOXO functions as an important transcriptional activator within the IIS response. Interestingly, out of the 198 genes bound by dFOXO and regulated in *daGAL4 > UAS-dInR*^{DN} flies, only 38 had mRNA levels detectably altered in *dfoxo*$^{\Delta/\Delta}$. This overlap was significant ($P < 10^{-15}$ with respect to all genes on expression arrays) but was not complete. Hence, dFOXO may regulate
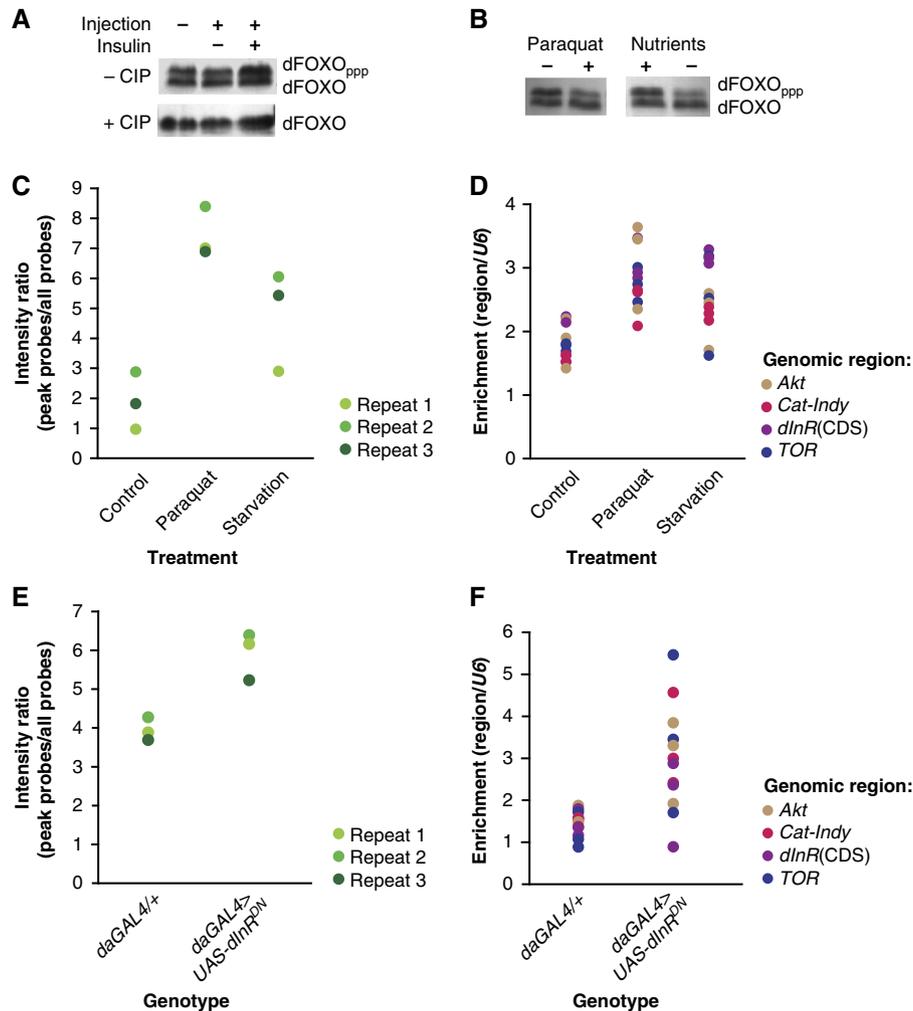
Figure 4 dFOXO binding under stress conditions or on downregulation of IIS. (**A**) Phosphorylation of dFOXO upon insulin injection. In all, 7-day-old females were injected with recombinant human insulin, mock-injected or not injected, and frozen after 5 min. The proteins were extracted, some treated with CIP and separated by SDS–PAGE. The phosphorylated (dFOXOppp) and unphosphorylated dFOXO is indicated. (**B**) dFOXO phosphorylation after 18 h of 20 mM paraquat administration or after 48 h of starvation. (**C**) Increased genome-wide enrichment of dFOXO-bound regions upon stress. Three biological repeats of the ChIP-chip assay were performed with anti-dFOXO antibody on flies treated with paraquat, starved or untreated controls. The intensity ratios of the peak probes (bound by dFOXO) to all probes, each taken at 0.75 quantile, is shown and was significantly greater for all treatment replicates (Wilcox rank sum test, $n=3$, $P=0.024$). (**D**) Increased region-specific enrichment of dFOXO-bound regions upon stress. The IPs were repeated on the same chromatin samples and the enrichment relative to *U6* of *Akt*, *dInR*, *TOR* and the region between the *Cat* and *Indy* genes was determined by qPCR. The effect of treatment was found to be significant (two-way ANOVA, $n=3$, effect of treatment $P<10^{-4}$, effect of genomic region $P=0.02$, no significant interaction of the two main effects). The same genome-wide (**E**) or region-specific (**F**) analysis was performed on $daGAL4 > UAS\text{-}dInR^{DN}$ flies or the driver alone control ($daGAL4$). This resulted in significant increase in the enrichment of dFOXO-bound regions, both on genome-wide scale (Wilcox rank sum test, $n=3$, $P=0.05$) and to the four target regions examined (two-way ANOVA on log-transformed data, $n=3$, effect of treatment $P=7 \times 10^{-4}$, no significant effect of genomic region).

different genes under different conditions even though its binding locations stay the same.

dFOXO is directly required for correct expression of 645 genes upon IIS reduction

While dFOXO locations on the genome were unaltered upon reduction in IIS, it was possible that different dFOXO-bound genes required *dfoxo* for correct expression in an IIS mutant and in the wild-type fly. To determine direct targets of dFOXO in $daGAL4 > UAS\text{-}dInR^{DN}$ flies, we compared the mRNA expression profiles of these flies in the presence or absence of *dfoxo*. A very large set of genes (3520) was misregulated upon deletion of *dfoxo* in this genetic context. There was a significant overlap between this set of genes and dFOXO-bound genes ($P<10^{-15}$), revealing 645 direct targets of dFOXO (Figure 6A). Again this overlap was specifically significant for genes downregulated upon *dfoxo* deletion ($P<10^{-15}$), confirming that dFOXO predominantly acts as a transcriptional activator within the IIS response. The dFOXO-bound sequences from which it was active in $daGAL4 > UAS\text{-}dInR^{DN}$ flies were again enriched for forkhead-recognition motifs relative to all dFOXO-bound sequences (Table I), indicating

Upregulated:

daGAL4>UAS-dInR^{DN}
VS *daGAL4*   dFOXO-bound

Downregulated:

daGAL4>UAS-dInR^{DN}
VS *daGAL4*

690   179   1438   19   390
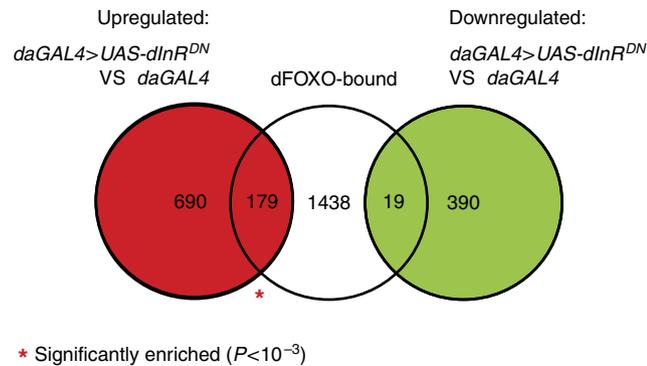
* Significantly enriched ($P<10^{-3}$)

Figure 5 Enrichment of dFOXO-bound genes within IIS transcriptional response. Overlaps between the genes regulated in whole *daGAL4>UAS-dInR^{DN}* flies relative to driver only controls (*daGAL4*) and genes bound by dFOXO. A red asterisk denotes an overlap significantly larger than expected by chance ($P<10^{-3}$), as computed from a hypergeometric distribution.

that transcriptional activity correlates with a high density of binding motifs.

The set of direct dFOXO targets in *daGAL4>UAS-dInR^{DN}* flies contained the majority of the direct targets observed in the wild type (298 out of 357; Figure 6B) but was larger. This increase in the number of direct targets may have resulted from an increase in dFOXO binding leading to more sites passing a threshold of bound dFOXO required for transcriptional regulation. Alternatively, the regulation of dFOXO function in *daGAL4>UAS-dInR^{DN}* flies might have occurred through processes independent of dFOXO binding, such as activation of cofactors. This increased number of direct dFOXO targets was also reflected in an increased feedback to IIS, including the TOR signalling pathway. For example, the *chico* and *S6 kinase* genes were revealed as direct targets of dFOXO in *daGAL4>UAS-dInR^{DN}* flies (Figure 6C). Functions enriched within these direct dFOXO targets included cell cycle, catabolism, intracellular transport, cytoskeleton organisation, sexual reproduction and tRNA metabolism (Figure 6A). Interestingly, genes involved in protein phosphorylation as well as regulation of transcription were also over-represented, revealing again a potentially important second tier of regulators.

dFOXO is required for only a part of the transcriptional response to reduced IIS

The direct targets of dFOXO in *daGAL4>UAS-dInR^{DN}* flies identified above could require dFOXO for activation or repression in response to a reduction in IIS, or may require dFOXO for sustained basal level transcription during IIS reduction. To discern between these two possibilities, and uncover genes that require *dfoxo* for active transcriptional regulation upon reduction in IIS, we compared the genes whose transcripts were altered in *daGAL4>UAS-dInR^{DN}* flies to those that were changed in the opposite direction upon removal of *dfoxo* in the background of *daGAL4>UAS-dInR^{DN}* (Figure 7A). Interestingly, we found a highly significant overlap between genes repressed in *daGAL4>UAS-dInR^{DN}* and those activated upon removal of *dfoxo* in that genetic

context ($P<10^{-15}$), while the overlap between genes activated in *daGAL4>UAS-dInR^{DN}* and repressed upon *dfoxo* deletion was marginally significant ($P=0.01$). Hence, *dfoxo* is mainly required for repression of genes during IIS reduction. On the other hand, the comparison of dFOXO ChIP-chip data with expression in *dfoxo^{Δ/Δ}* and *dfoxo^{Δ/Δ} daGAL4>UAS-dInR^{DN}* flies (Figures 3 and 6) showed that dFOXO tends to act as an activator of transcription. Mapping the ChIP-chip data onto the expression data overlaps (Figure 7A) revealed that the genes requiring *dfoxo* for activation during IIS reduction were enriched for direct dFOXO targets, while those requiring *dfoxo* for repression tended to be indirect targets.

The functional categories enriched within the set of genes that require *dfoxo* for upregulation in *daGAL4>UAS-dInR^{DN}* flies included RNA processing, signal transduction, transcription and cytoskeleton organisation (Figure 7B). Genes involved in RNA processing were directly upregulated by dFOXO in *daGAL4>UAS-dInR^{DN}* flies. On the other hand, none of the predominantly metabolic functions downregulated in *daGAL4>UAS-dInR^{DN}* flies in a *dfoxo*-dependent manner were directly regulated by dFOXO.

To uncover a potential mechanism whereby dFOXO indirectly regulates gene repression in an IIS mutant, we searched for TF-binding motifs over-represented in the promoters of genes that require *dfoxo* for repression in *daGAL4>UAS-dInR^{DN}* flies, but are not directly bound by dFOXO. Clover analysis identified several GATA-like motifs (Table I). Indeed, dFOXO directly activates transcription of *GATAd* in both the wild-type and *daGAL4>UAS-dInR^{DN}* flies, and this TF may in turn be required for gene repression in *daGAL4>UAS-dInR^{DN}* flies. Rigorous demonstration of GATAd as a mediator of dFOXO actions awaits further study.

Interestingly, a substantial number of genes changed in *daGAL4>UAS-dInR^{DN}* flies were not altered upon deletion of *dfoxo* (Figure 8). Hence, *dfoxo* appears to be required only for a part of the IIS response in flies. To confirm this surprising finding, we looked at what happens when we induce *dInR^{DN}* in a *dfoxo^{Δ/Δ}* background. We compared the transcriptome response to the induction of *dInR^{DN}* in a *dfoxo^{Δ/Δ}* background to the response observed in the presence of *dfoxo*. We found that 176 genes upregulated in *daGAL4>UAS-dInR^{DN}* were still upregulated in *dfoxo^{Δ/Δ} daGAL4>UAS-dInR^{DN}*, while 29 genes downregulated in *daGAL4>UAS-dInR^{DN}* were still downregulated in the absence of *dfoxo*. This directly demonstrates that a substantial portion of the IIS response, at least 16% of the detectable changes, is independent of *dfoxo* in adult *Drosophila*.

To identify the TFs that may mediate this *dfoxo*-independent aspect of the IIS response, we looked at over-representation of known TF-binding motifs in the promoters of the 176 genes upregulated and the 29 downregulated upon induction of *dInR^{DN}* irrespective of the absence of *dfoxo*. Numerous forkhead-like motifs were associated with the upregulated genes (Table I), indicating that another forkhead factor mediates the *dfoxo*-independent transcriptional activation in an IIS mutant. The promoters of the downregulated genes were enriched for GATA-like motifs, indicating a GATA factor, but probably not *GATAd*, is required for gene repression in an IIS mutant, but further studies will be needed to directly demonstrate this mechanism.
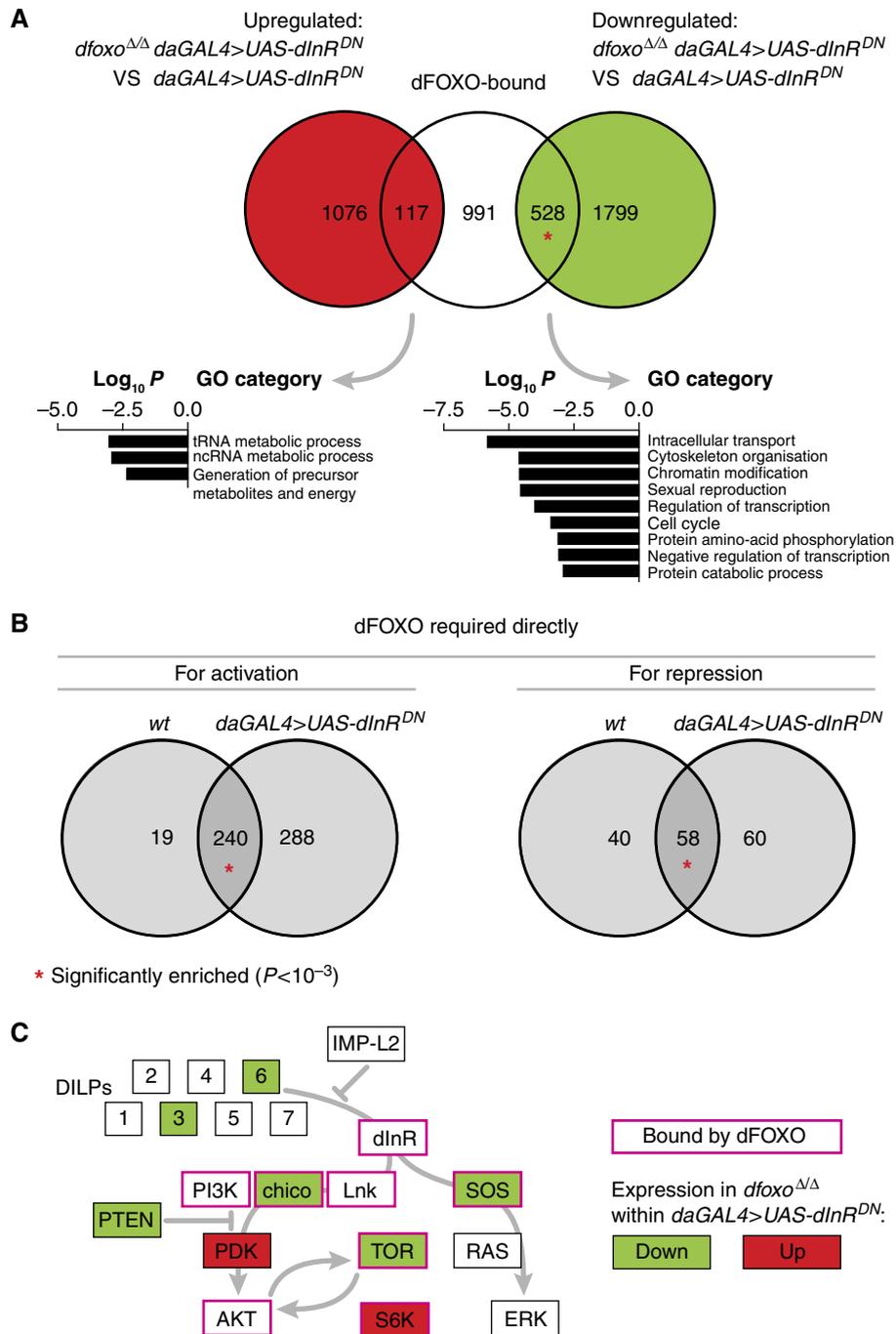
Figure 6 Direct dFOXO targets in an IIS mutant. (**A**) Overlap between the genes that neighbour a dFOXO-bound site and those with transcript levels altered in *dfoxo*$^{\Delta/\Delta}$ *daGAL4 > UAS-dInR*^{DN} flies relative to *daGAL4 > UAS-dInR*^{DN}. Representative biological functions enriched within the overlaps are shown. (**B**) Comparison of direct dFOXO targets in wild-type and in *daGAL4 > UAS-dInR*^{DN} flies. The Venn diagram on the left compares genes that directly require dFOXO for activation of transcription in the two genetic contexts (i.e. genes bound by dFOXO and downregulated upon deletion of *dfoxo*), while the one on the right compares genes that directly require dFOXO for repression in the two genetic contexts (i.e. genes bound by dFOXO and upregulated upon deletion of *dfoxo*). In both (A, B), the probability of overlap was calculated based on hypergeometric distribution and an overlap significantly larger than expected by chance ($P < 10^{-3}$) is indicated with a red asterisk. Note that only the dFOXO-bound genes that were present on the expression arrays were taken into account. (**C**) dFOXO binding and regulation of IIS components. dFOXO binding and altered transcript levels in *dfoxo*$^{\Delta/\Delta}$ *daGAL4 > UAS-dInR*^{DN} flies were mapped onto a schematic of IIS. Note that PI3K denotes the p110 subunit.

121 dFOXO-bound genes are also bound by DAF-16 in the worm

Several physiological roles of FoxOs, as well as of IIS, are conserved across distantly related animals. However, examination of transcriptional changes in worm, fly and mouse IIS mutants failed to identify any significant co-regulation of orthologous genes in the three organisms (McElwee *et al*, 2007). We realised that the regulatory architecture of the transcriptional response in the worm and
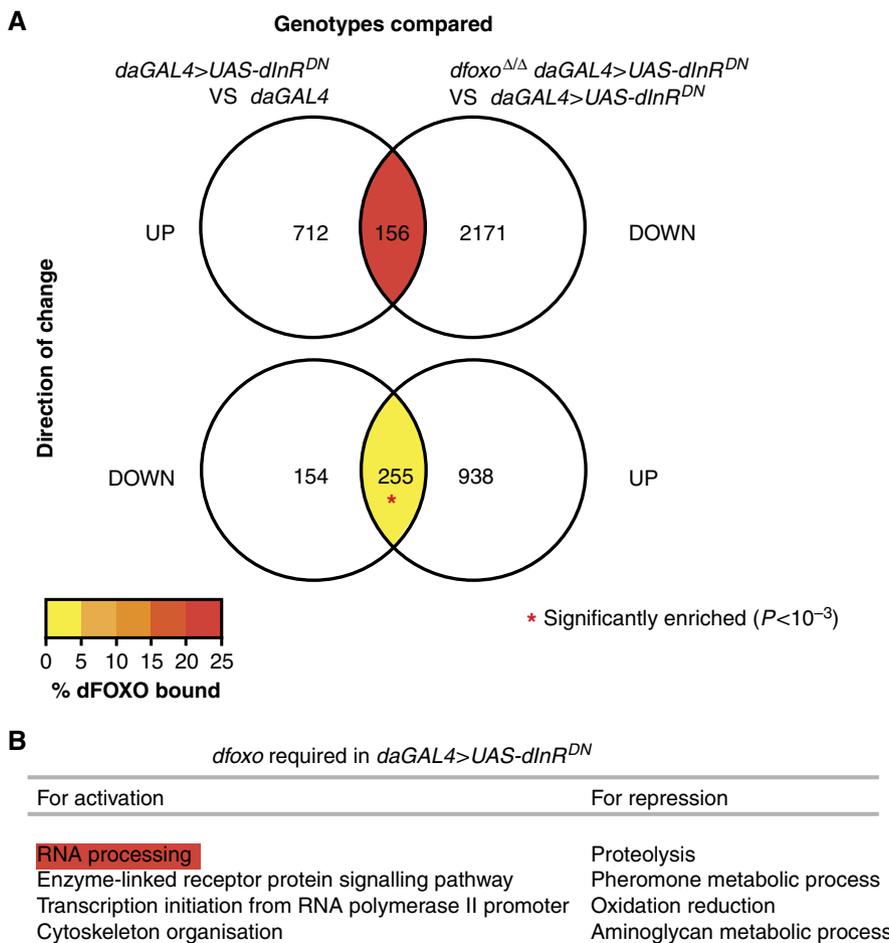
A

Genotypes compared

daGAL4>UAS-dInR^{DN}
VS *daGAL4*

dfoxo$^{\Delta/\Delta}$ daGAL4>UAS-dInR^{DN}
VS *daGAL4>UAS-dInR^{DN}*

Direction of change

UP   712   156   2171   DOWN

DOWN   154   255   938   UP
*

0 5 10 15 20 25
% dFOXO bound

* Significantly enriched ($P<10^{-3}$)

B

dfoxo required in *daGAL4>UAS-dInR^{DN}*

For activation	For repression
RNA processing	Proteolysis
Enzyme-linked receptor protein signalling pathway	Pheromone metabolic process
Transcription initiation from RNA polymerase II promoter	Oxidation reduction
Cytoskeleton organisation	Aminoglycan metabolic process

Figure 7 *dfoxo*-dependent part of the transcriptional response to altered IIS. (**A**) Sets of genes that are transcriptionally upregulated in response to over-expression of *dInR^{DN}* (*daGAL4>UAS-dInR^{DN}* vs *daGAL4*; left) or downregulated upon deletion of *dfoxo* in a fly over-expressing *dInR^{DN}* (*dfoxo$^{\Delta/\Delta}$ daGAL4>UAS-dInR^{DN}* vs *daGAL4>UAS-dInR^{DN}*; right) were compared in the Venn diagram above. The overlap is composed of the genes that require *dfoxo* for upregulation in *daGAL4>UAS-dInR^{DN}* flies. The Venn diagram below shows the reciprocal situation, and the overlap is composed of the genes that require *dfoxo* for downregulation in *daGAL4>UAS-dInR^{DN}* flies. The probability of overlap was calculated based on hypergeometric distribution and an overlap significantly larger than expected by chance ($P<10^{-3}$) is indicated with a red asterisk. The proportion of the genes in the overlaps that are direct dFOXO targets (bound by dFOXO) is indicated with colour. (**B**) Non-redundant functional categories that are enriched ($P<0.05$) in the genes within the overlaps in (A). Those that are enriched within the direct dFOXO targets within the same overlaps are highlighted in red. Note that 'proteolysis' included predominantly extracellular proteases.

the fly is different since in the fly the IIS response only partially requires *dfoxo*. This prompted us to re-examine the conservation of the transcriptional response between the worm and the fly.

Transcriptional response to IIS changes in the worm has been examined from the perspective of *daf-2/daf-16* epistasis. Since we have now performed the equivalent *dInR/dfoxo* epistasis experiments for the fly, we compared our data set to the ones already published for the worm (McElwee *et al*, 2003, 2007; Murphy *et al*, 2003), making sure that equivalent gene sets were being compared. We identified significant conservation of the genes that require *dfoxo* for downregulation between the worm and the fly. The comparisons that produced statistically significant overlaps are shown in Figure 9A and B. Hence, there is actually evolutionary conservation of *dfoxo*-dependent aspects of the IIS transcriptional response.

Even though the set of genes requiring *dfoxo* for repression in the fly is comprised predominantly of indirect dFOXO targets, the evolutionary conservation within this set strongly suggested that there would be an underlying conservation at the level of direct dFOXO targets. Hence, we examined if evolutionary conservation could be observed at the level of dFOXO binding between the worm and the fly. We compared the set of genes that are bound by dFOXO in the adult fly with the composite set of DAF-16-bound genes identified by ChIP-cloning (Oh *et al*, 2006) or Dam-ID (Schuster *et al*, 2010). Strikingly, we found that there was a significant tendency for orthologous genes to be bound by dFOXO in the fly and DAF-16 in the worm (Figure 9C; $P<10^{-15}$), indicating significant conservation of dFOXO-bound genes between the two animals. Out of the 121 genes present in the overlap, 44 were disregulated upon *dfoxo* deletion in the wild-type or *daGAL4>UAS-dInR^{DN}* flies. The overlap was significantly enriched for genes acting in signal transduction (Figure 9D), including *Sos*, *Akt* and *PP2A-B'*. Hence, direct regulation of signalling components is an evolutionarily conserved role of FoxOs.
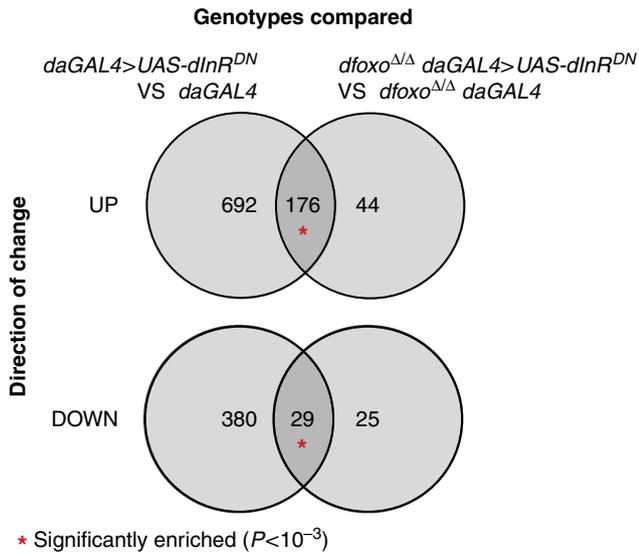
Genotypes compared

Figure 8 *dfoxo*-independent part of the transcriptional response to altered IIS. Sets of genes that are transcriptionally upregulated in response to over-expression of $dInR^{DN}$ in the wild-type fly ($daGAL4 > UAS\text{-}dInR^{DN}$ vs $daGAL4$; left) or upregulated upon over-expression of $dInR^{DN}$ in an *dfoxo* null fly ($dfoxo^{\Delta/\Delta}$ $daGAL4 > UAS\text{-}dInR^{DN}$ vs $dfoxo^{\Delta/\Delta}$ $daGAL4$; right) were compared in the Venn diagram above. The overlap is composed of the genes that do not require *dfoxo* for upregulation in $daGAL4 > UAS\text{-}dInR^{DN}$ flies. The Venn diagram below shows the reciprocal situation, and the overlap is comprised of genes that do not require *dfoxo* for downregulation in $daGAL4 > UAS\text{-}dInR^{DN}$ flies. The probability of overlap was calculated based on hypergeometric distribution and an overlap significantly larger than expected by chance ($P < 10^{-3}$) is indicated with a red asterisk.

This overlap not only included conservation of signalling feedback loops, but also of control of other signalling pathways (e.g. *CaMKII*) and extended to several TFs. For example, FoxOs may link steroid hormone signalling to IIS in both flies and worms through regulation of the *dHR96/daf-12* TF.

Discussion

Using ChIP-chip we have defined >1400 genomic locations occupied by dFOXO in the adult fly. Interestingly, we find these locations to be distinct from those observed by others in larvae (Teleman *et al*, 2008) and in cell culture (Puig *et al*, 2003). It is possible that the differences between our adult data and the published larval data stem from differences in protocols (e.g. the antibody used) or even experimental design (e.g. sex of the flies used). Importantly, however, we show that the observed differences between S2 cells and adults, in the case of the promoter (P1) and the coding region of the *Drosophila InR*, represent true biological differences. It is not surprising that dFOXO would occupy different locations during development and in the adult fly. A similar observation has been made for a number of transcriptional events, and even the *dInR* gene alone is transcribed from three promoters under tight spatio-temporal control (Casas-Tinto *et al*, 2007). Furthermore, some differences will stem from cell- and tissue-specificity of dFOXO action. Indeed, FoxO factors are known to elicit tissue-specific transcriptional changes in the mouse (Paik *et al*, 2007; Tothova *et al*, 2007), and the same tissue-restricted action by dFOXO
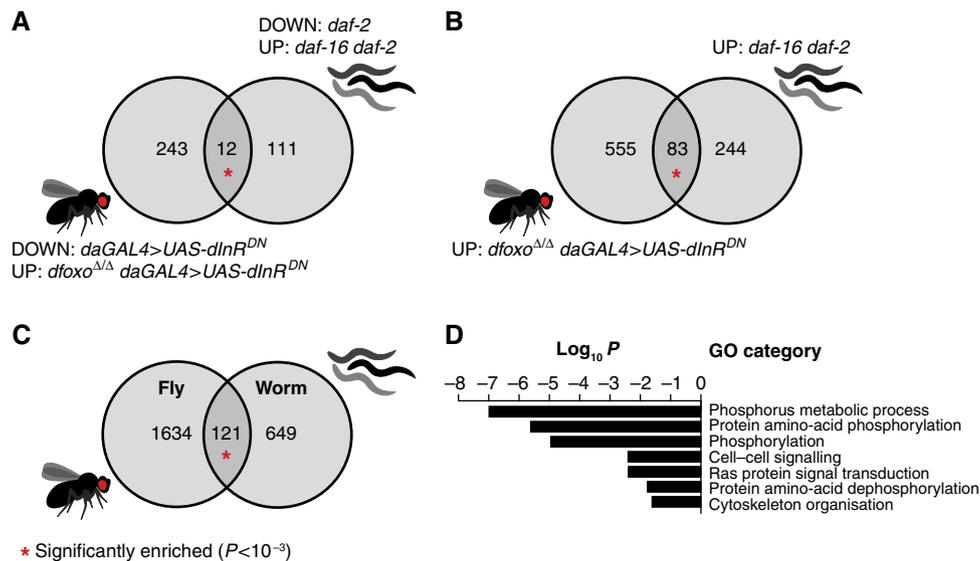

Figure 9 dFOXO-bound genes are conserved between the fly and the worm. (**A**) Overlap between the fly orthologues of the genes that are downregulated by reduced function of *daf-2* and upregulated by reduced function of *daf-16* in the worm (Murphy *et al*, 2003), on the one hand, and those downregulated in $daGAL4 > UAS\text{-}dInR^{DN}$ and upregulated in $dfoxo^{\Delta/\Delta}$ $daGAL4 > UAS\text{-}dInR^{DN}$ in the fly, on the other (hypergeometric distribution, $P = 7 \times 10^{-6}$). (**B**) Overlap between the fly orthologues of the genes that are upregulated by reduced function of *daf-16* in a *daf-2* background in the worm (McElwee *et al*, 2007), on the one hand, and upregulated in $dfoxo^{\Delta/\Delta}$ $daGAL4 > UAS\text{-}dInR^{DN}$ in the fly, on the other (hypergeometric distribution, $P < 10^{-15}$). (**C**) Overlap between the genes bound by dFOXO in the fly and the fly orthologues of the genes bound by DAF-16 in the worm (Oh *et al*, 2006; Schuster *et al*, 2010). (**D**) Representative functional categories enriched within the overlap shown in (C).

on the transcription of the *myc* gene has been observed in *Drosophila* larvae (Teleman *et al*, 2008). By binding to different locations in a spatially and temporally determined manner, dFOXO would be able to orchestrate different responses to suit its function in different life stages and tissues. Interestingly, we find a substantial portion of dFOXO bound in transcribed regions. In yeast, forkhead factors regulate Pol II elongation (Morillon *et al*, 2003), and dFOXO may perform a similar function.

We observe dFOXO bound to a number of genes encoding IIS signalling components. Furthermore, *dfoxo* may also exert feedback onto other pathways that regulate it: dFOXO was bound near the genes encoding *PP2A-B′*, *14-3-3ε* and JNKKKs (*slpr* and *TAK1*), among others. PP2A, *14-3-3ε* and JNK have all been shown to regulate FoxO activity (Wang *et al*, 2005; Nielsen *et al*, 2008; Yan *et al*, 2008). A number of these dFOXO-activated genes is also activated on over-expression of super-oxide dismutase (Curtis *et al*, 2007), suggesting that dFOXO, like its mammalian counterparts (Nemoto and Finkel, 2002; Dansen *et al*, 2009), may be redox regulated. Interestingly, as is evident from Figures 3B and 6C, we detect binding to only the intracellular components of IIS such as *chico*, *Lnk* and *Akt*, while the genes with altered expression level in *dfoxo*$^{\Delta/\Delta}$ include extracellular cell-to-cell signalling molecules, such as those encoded by *dilp3*, *dilp6* and *Imp-L2*. The latter genes have a more localised expression pattern, for example *dilp3* is expressed in only ∼14 cells in the whole adult fly (Broughton *et al*, 2005). It is possible that genes such as *dilp3* are also bound and directly regulated by dFOXO but that we did not observe this in the whole fly ChIP-chip due to a very small number of cells in which this binding occurs.

4E-BP (a.k.a. *Thor*) has been shown to be bound by dFOXO in larvae (Teleman *et al*, 2008) and cell culture (Puig *et al*, 2003), and its regulation has been reported as consistent with dFOXO acting as a direct activator of its expression (Junger *et al*, 2003; Puig *et al*, 2003). On the other hand, we do not observe dFOXO binding in the vicinity of this gene in adults (see Figure 1B), and the *4E-BP* transcript is actually elevated in a *dfoxo* null. It is possible that dFOXO is required for direct activation of this gene in only a limited number of cells/tissues in the adult, thus escaping detection by ChIP-chip on whole animals. Furthermore, the role of dFOXO in *4E-BP* regulation may be sexually dimorphic, as has recently been indicated (Shen and Tower, 2010). Alternatively, *4E-BP* might be a target of a different forkhead factor in the adult female fly. Indeed, *Forkhead* (*Fkh*, the fly FoxA orthologue) is able to activate transcription of *4E-BP* in larvae (Bulow *et al*, 2010). Since *dfoxo* nulls have reduced levels of *TOR*, and TOR is an inhibitor of Fkh activity (Bulow *et al*, 2010), it is likely that Fkh is activated in *dfoxo* nulls leading to increased levels of the *4E-BP* transcript. It remains to be established whether Fkh might indeed be directly binding to the *4E-BP* locus in adult flies.

From the 1400 dFOXO-bound locations, using transcriptional profiling of *dfoxo* null flies under normal conditions or with reduced IIS, we define >700 direct transcriptional targets of dFOXO in the adult. Several functions associated with these genes have been linked with FoxO biology previously, such as cell cycle (Medema *et al*, 2000), DNA repair (Tran *et al*, 2002), cytoskeleton organisation (Kamei *et al*, 2004), negative regulation of gene expression such as translation (Puig *et al*,

2003; Teleman *et al*, 2008) and regulation of protein catabolism (Stitt *et al*, 2004). dFOXO is known to be involved in the repression of protein synthetic machinery via *myc* in larvae (Teleman *et al*, 2008) but our study also revealed a significant regulation of ribosome biogenesis genes effected directly by dFOXO in the adult female. We also identified other, previously unknown functions, such as control of negative regulators of transcription and chromatin modifiers, hinting at the importance of dFOXO in establishment and maintenance of repressive chromatin states. Yet other functions were completely unexpected. For example, dFOXO appears as a positive regulator of sexual reproduction, including oogenesis, in an IIS mutant (see Figure 6C). This surprising finding is backed up by phenotypic epistasis analysis that shows removal of *dfoxo* to exacerbate the fecundity defect of several IIS mutants (Slack *et al*, 2011). Hence, dFOXO actually positively regulates some aspects of IIS. Indeed, one of the most surprising findings of our study is that dFOXO is directly required for expression of several components of IIS and interacting pathways, including *TOR* and *Sos*, in the wild-type fly, with consequences for the downstream signalling events. Importantly, this is not just simple feedback in response to alteration in the levels of insulin/IGF-like signal, but rather dFOXO is active in the normal adult and its activity promotes signalling through the IIS pathway. This observation can also explain why *dfoxo* deletion is lethal in combination with certain IIS mutants (Slack *et al*, 2011), since the combined reduction in IIS will be too great for the flies to survive. This potentiation of IIS by FoxOs could also explain why mice with reduced IIS through mutation of IRS1 have mild insulin resistance but preserved old-age glucose homoeostasis (Selman *et al*, 2008). In this case, the mild insulin resistance would be the primary effect of the mutation of IRS1, while the resulting activation of FoxOs would be responsible for sustained IIS in old age and thus for the observed preservation of glucose homoeostasis.

dFOXO directly regulates an extensive second tier of regulators; throughout this study we have repeatedly encountered different transcriptional and post-transcriptional regulators as predominant dFOXO targets. This aspect of dFOXO biology is also conserved in the worm (Schuster *et al*, 2010). Indeed, some of the potential secondary effectors are directly conserved between the worm and the fly, such as the nuclear hormone receptor *dHR96/daf-12*, highlighting their importance. Our study also illustrates the role this second tier of regulators may play. dFOXO is directly required for the maintenance of *GATAd* mRNA levels in both the wild-type and IIS-compromised flies, and this in effect may constitute an IIS feed-forward loop, since GATAd in turn may be an important transcriptional repressor in response to reduced IIS. Hopefully, subsequent studies will demonstrate the existence of such a feed-forward loop.

Since *daf-16* is strictly required for all phenotypic outputs of reduced IIS in the worm (Kenyon *et al*, 1993; Gems *et al*, 1998), and also appears strictly required for the transcriptional response to reduced IIS (Murphy *et al*, 2003), it was very surprising to find that dFOXO was only required for part of the transcriptional response to reduced IIS in the fly. On the other hand, this is in accordance with phenotypic epistasis experiments in the fly where lifespan extension and xenobiotic resistance are dependent on *dfoxo*, while lowered fecundity

and body size, delayed development and resistance to paraquat are not (Slack *et al*, 2011). This implies that phenotypes such as fecundity are negatively regulated via other factors in the fly. Our study indicates that GATA factors are the most likely candidates for mediating transcriptional repression in response to reduced IIS. Studies in the worm have also revealed the presence of a GATA-recognition sequence in the promoters of IIS-regulated genes (Murphy *et al*, 2003; Budovskaya *et al*, 2008). Furthermore, at least one of the 14 worm GATA TF (*elt-3*) is regulated by IIS, and reduced function in any of the three GATA TFs (*elt-3*, *egr-1*, *egl-27*) blocks the lifespan extension by a *daf-2* mutant (Budovskaya *et al*, 2008). The role of GATA factors in lifespan in other organisms awaits examination. At the same time, our study reveals the potential involvement of other forkhead factors, besides dFOXO, in the transcriptional activation response to IIS reduction. Fkh is the prime suspect, since it is regulated by TOR signalling in the fly (Bulow *et al*, 2010), and Foxa2 is involved in the IIS response in mammals (Wolfrum *et al*, 2003). Indeed, Foxa2 is directly inactivated by Akt via phosphorylation of a single site that is conserved in the fly Fkh (Wolfrum *et al*, 2003). While our study provides hints, further work will be needed to determine the identity of other TFs involved in the fly IIS response.

Our study reveals that the transcriptional response to IIS in the fly is clearly more complex than that in the worm. The parallel genetic study performed by Slack *et al* (2011) shows that the genes directly regulated by dFOXO must still effect the lifespan extension by reduction in IIS. Importantly, we have now identified these genes. Their characterisation is the next step towards understanding the physiological and molecular changes that can extend animal lifespan, keeping in mind that it is now crucial to determine the architecture of the mammalian response to reduced IIS.

Materials and methods

Fly handling

For experiments on wild-type flies, the Dahomey stock (Clancy *et al*, 2001) was used. *daugterlessGAL4* (Bloomington Stock Center), *UAS-dInR^{DN}* (Wu *et al*, 2005) and *dfoxo^{Δ94}* (Slack *et al*, 2011) were backcrossed at least six times into Dahomey background carrying *w^{1118}* mutation (Giannakou *et al*, 2004), and which was *Wolbachia* positive. All experiments were performed at 25°C, 12-h light/dark cycle and controlled humidity. Flies were reared at standard density on SYA food (5% sucrose, 10% yeast, 1.5% agar) and females were sorted on day 3 of adulthood. For chromatin preparation, flies were kept at 200 females per bottle, 10 per vial for all other experiments. For starvation, flies were kept on 1% agar for 48 h, and for paraquat treatment for 18 h on food containing 1% agar, 5% sucrose, 20 mM paraquat, starting on day 5, and immediately frozen in liquid nitrogen. In all other cases, the flies were frozen on day 7. For insulin injections, 20 7-day-old females were gassed with carbon dioxide, injected with 50 nl of PBS with 0.1 μg/ml blue food dye (FD&C Blue Dye no. 1) with or without insulin (10 IU/ml, Actrapid, Novo Nordisk), allowed to wake up for 5 min and frozen.

Chromatin preparation, IP, array hybridisation and qPCR

Biological triplicates were done for all fly chromatin preparations. For each experiment, all the batching was done so that the treatments to be compared were carried out in parallel. The ChIP protocol as described by Kuras and Struhl (1999) was adapted for adult *Drosophila*. In all, 1000 females were crushed to a fine powder under liquid nitrogen and re-suspended in 6 ml of PBS supplemented with Protease Inhibitors Cocktail (10 μl/ml; Sigma). The flies over-expressing *dInR^{DN}* were smaller than their controls so that they were re-suspended in 4 ml PBS to maintain the fly weight/buffer volume ratio. Cross-linking was performed with 0.5% formaldehyde for 10 min and quenched with addition of 1.5 ml of 2.5 M glycine. The cross-linked chromatin was recovered by centrifugation and washed twice with FA/SDS buffer (50 mM Hepes-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Na deoxycholate, 0.1% SDS, 1% Triton X-100 and 1 mM PMSF) re-suspended in the same and incubated for 1 h at 4°C. Chromatin was again recovered by centrifugation and sheared to an average size of 400 bp by sonication, giving on average 6 ml of chromatin in FA/SDS. For IPs, 1 μl of affinity-purified anti-dFOXO antibody (Giannakou *et al*, 2007), or 1 μl of the corresponding pre-immune serum (mock control) were bound to Protein-G Dynabeads (Invitrogen) and incubated for 2 h at room temperature with 450 μl of chromatin. Beads were washed once with FA/SDS, three times with FA/SDS containing 500 mM NaCl, once with TE and once with 10 mM Tris–HCl pH 8, 250 mM LiCl, 1 mM EDTA, 1% NP40 and 0.5% Na deoxycholate. DNA was recovered, treated with protease, de-cross-linked, treated with RNase and purified with Qiagen PCR purification kit (Qiagen, UK).

For array hybridisation, the entire IP after volume reduction, or 50 ng of total chromatin DNA, were amplified two times (Whole Genome Amplification kit, Sigma) as per the manufacturer's instructions. The material from the IP was hybridised against the input material. The labelling and hybridisations were carried out by Nimblegen Systems, using custom *Drosophila* whole-genome tiling arrays with probes spaced approximately every 300 bp, as described (Choksi *et al*, 2006).

Chromatin was prepared from S2 cells based on a published method (Andrulis *et al*, 2000; Puig *et al*, 2003). In all, 10 ml of 5×10^6 cells/ml were incubated in Schneider's medium without serum for 2 h at 25°C after which formaldehyde was added to 0.1% and quenched 3 min later with 0.5 ml of 2.5 M glycine for 3 min. The cells were collected by centrifugation and taken up in 2 ml of FA/SDS supplemented with PMSF. The chromatin was washed, sheared by sonication and the IPs performed as for whole flies above.

For qPCR, a suitable dilution of total chromatin and IP was used for quantification with primer pairs indicated, using Power SYBR Green PCR Master Mix (ABI) on ABI Prism 7000. Unless otherwise noted, the value reported is the percentage of the total chromatin recovered in the IP for the target sequence divided by the same for the U6 control. The primers used are given in Supplementary information.

Peak identification and analysis

ChIP-chip data were normalised using the LIMMA package (Smyth and Speed, 2003) in Bioconductor (Gentleman *et al*, 2004), applying loess normalisation within each array and quantile normalisation between arrays. Replicate information was pooled by taking the median probe value for each set of arrays and was smoothed along each chromosome using a running median within a window of three probes. Experimental signal was adjusted by mock control (pre-immune serum) data by direct subtraction of median probe intensity values. Peaks were called using the Ringo package (Toedling *et al*, 2007) in R, using a y0 threshold of 0.97 and a distance cutoff of 600 bp. Peaks were padded with 1000 bp upstream and downstream of the outermost peak probe position and genes were considered associated with the peak where any part of a gene taken from the FlyBase release 4.3 gene set (Drysdale and Crosby, 2005) overlapped with this region. When required, the observed peak set was compared with simulations of 1000 random peak sets, of identical size, length and chromosomal distribution.

RNA isolation, expression array hybridisation and analysis

RNA was extracted using Trizol (Invitrogen) from four biological repeats of 10 females of the following genotypes: *daGAL4*, *dfoxo^{Δ/Δ}*

daGAL4, daGAL4 > UAS-dInR^{DN}, dfoxo^{Δ/Δ} daGAL4 > UAS-dInR^{DN}. All the batching was done so that the treatments to be compared were carried out in parallel. RNA was purified with RNeasy columns (Qiagen) and its quality and concentration were determined using an Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA). The RNA was further processed into cRNA using standard Affymetrix protocols and hybridised to the Affymetrix Drosophila Genome 2.0 Genechip. The data were summarised and normalised using RMA (Bolstad *et al*, 2003; Irizarry *et al*, 2003a, b) and quantile normalisation as implemented in the LIMMA package. Differential expression between different samples was assessed using linear models and the empirical Bayes moderated *t*-statistic implemented in LIMMA. Highly differentially expressed genes were selected by applying an adjusted *P*-value <0.005 cutoff.

DNA motif identification, EASE analysis and comparison to *C. elegans* data sets

Identification of known DNA motifs with a statistical over-representation was done using the Clover program (Frith *et al*, 2004) and the TransFac database (Matys *et al*, 2003) for input motifs. *De novo* identification of motifs from peak sequences was conducted with MEME (Bailey *et al*, 2006) on regions 500 bp padded from the most intense probe in the peak and repeat-masked.

Gene function over-representation analysis within gene sets was conducted using EASE in DAVID v6.7 online (Dennis *et al*, 2003; Huang da *et al*, 2009).

For comparison with *C. elegans* data sets, the two colour array data from McElwee *et al* (2003) were retrieved from the Stanford Microarray Database (Hubble *et al*, 2009) and processed using LIMMA in order to define lists of differentially expressed genes. For all other worm data sets, selected gene lists were already provided. The *C. elegans* data sets were mapped to fly genes using orthology relationships that were retrieved from TreeFam (Li *et al*, 2006; Ruan *et al*, 2008) and InParanoid (O'Brien *et al*, 2005; Berglund *et al*, 2008) using the FlyMine resource (Lyne *et al*, 2007).

Western blots

The proteins were obtained by TCA extraction and separated on 8% SDS–PAGE and western blots performed as previously described (Giannakou *et al*, 2007). Anti-phospho-AKT, phospho-ERK, total AKT and total ERK were obtained from Cell Signaling. Where reported, the blots were quantified as described (Alic *et al*, 2011).

Statistical analysis

Analyses were performed in R, Excel or JMP. Where required, the data were log-transformed to fit a normal distribution. The details of tests used are given in figure captions.

Note

Array data are available from ArrayExpress under accession numbers E-TABM-751 (ChIP-chip data) and E-TABM-757 (expression data).

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

Author contributions: NA, MEG, CS, HMC and LP designed the experiments. NA, MEG, CS and MPH performed the experiments. TDA, IP, ES and JT designed the data analysis. TDA, IP, NA and ES performed the analysis. NA, HMC and LP wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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