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 homeostasis, 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 over-expression 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-bounds 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 (dfoxoN/A) flies that completely lacked the dFOXO protein (Slack et al., 2011). None of the peaks identified in the wild type were present in the dfoxoN/A (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 WWACA (Biggs et al., 2001) were enriched, such as WWWRTAASA/WAA and WNTTATAAACWNNR (Table 1), indicating that these are a good match to the motif recognised
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 forkhead-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 highly enriched in our ChIP-recovered sequences by Clover (Table I), indicating that ADF1, a Drosophila Myb-
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 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 ($P=2 \times 10^{-14}$), 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

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**Table 1** Representative enriched motifs identified by Clover

| Motif TF | All sequences bound by dFOXO Raw score | Raw score | $P^*$
|----------|--------------------------------------|-----------|---------|
| VCGCGAGCGCCCTGCGCGCG | ADF1 | 665 | $<10^{-3}$ |
| WWWWRTAASAWAA | BRCZ4 | 659 | $<10^{-3}$ |
| WNTATAAACAWNNR | XFD2 | 222 | $<10^{-3}$ |
| dFOXO-bound and gene(s) downregulated in dfoxo | Raw score | $P^*$
| NNNGCCACAGRKGCSSNN | CTCF | 117 | $<10^{-3}$ |
| TRTAAACAANWN | FOXO3A | 104 | 0.003 |
| dFOXO-bound and gene(s) upregulated in dfoxo | Raw score | $P^*$
| WWWWRTAASAWAA | BRCZ4 | 75.2 | $<10^{-3}$ |
| TRTAAACAANWN | FOXO3A | 56.7 | $<10^{-3}$ |
| dFOXO-bound and gene(s) downregulated in dfoxo | Raw score | $P^*$
| NNNGCCACAGRKGCSSNN | CTCF | 271 | $<10^{-3}$ |
| TRTAAACAANWN | FOXO3A | 208 | $<10^{-3}$ |
| dFOXO-bound and gene(s) upregulated in dfoxo | Raw score | $P^*$
| NWAACACAN | FOXO1 | 63.5 | $<10^{-3}$ |
| TRTAAACAANWN | FOXO3A | 57.8 | 0.002 |

For the comprehensive lists please refer to Supplementary information.

*Relative to wild type.

Relative to daGAL4 > UAS-dlnRDNb.

*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.

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For the comprehensive lists please refer to Supplementary information.
up/downregulated in dfoxoΔ/Δ 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 dfoxoN/A 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 dip3, a Drosophila insulin-like peptidase gene (Brogiole et al., 2001) (Figure 3B), imply that dfoxoN/A 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 dfoxoN/A 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).

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dFOXO binding is enriched within the genes upregulated upon 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 (dFOXOp) 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 daughterlessGAL4 driver (daGAL4). Importantly, daGAL4 > UAS-dInRDN 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 genomic locations are unaltered but binding is increased upon stress or IIS reduction

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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-dInRDN 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-dInRDN flies were again enriched for forkhead-recognition motifs relative to all dFOXO-bound sequences (Table I), indicating
that transcriptional activity correlates with a high density of binding motifs.

The set of direct dFOXO targets in daGAL4 > UAS-dlnRDN 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-dlnRDN 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-dlnRDN 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-dlnRDN 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-dlnRDN flies to those that were changed in the opposite direction upon removal of dfoxo in the background of daGAL4 > UAS-dlnRDN (Figure 7A). Interestingly, we found a highly significant overlap between genes repressed in daGAL4 > UAS-dlnRDN and those activated upon removal of dfoxo in that genetic context ($P < 10^{-15}$), while the overlap between genes activated in daGAL4 > UAS-dlnRDN 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/A/A and dfoxo/A/A daGAL4 > UAS-dlnRDN 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-dlnRDN 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-dlnRDN flies. On the other hand, none of the predominantly metabolic functions downregulated in daGAL4 > UAS-dlnRDN 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-dlnRDN 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-dlnRDN flies, and this TF may in turn be required for gene repression in daGAL4 > UAS-dlnRDN flies. Rigorous demonstration of GATAd as a mediator of dFOXO actions awaits further study.

Interestingly, a substantial number of genes changed in daGAL4 > UAS-dlnRDN 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 dlnRDN in a dfoxo/A/A background. We compared the transcriptome response to the induction of dlnRDN in a dfoxo/A/A background to the response observed in the presence of dfoxo. We found that 176 genes upregulated in daGAL4 > UAS-dlnRDN were still upregulated in dfoxo/A/A daGAL4 > UAS-dlnRDN, while 29 genes downregulated in daGAL4 > UAS-dlnRDN 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 dlnRDN 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 5** Enrichment of dFOXO-bound genes within IIS transcriptional response. Overlaps between the genes regulated in whole daGAL4 > UAS-dlnRDN 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.
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...
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 two animals. Out of the 121 genes present in the overlap, 44 were disregulated upon dfoxo deletion in the wild-type or daGAL4->UAS-dlnRDN 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.
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 (Telemen 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...
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', 14-3-3c, and JNKs (slpr and TAK1), among others. PP2A, 14-3-3c 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 superoxide dismutase (Curtis et al., 2008), 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<sup>N/A</sup> 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 Towner, 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 homeostasis (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 homeostasis.

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 GATA<sub>d</sub> mRNA levels in both the wild-type and IIS-compromised flies, and this in effect may constitute an IIS feed-forward loop, since GATA<sub>d</sub> 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 TFs (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 day-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 (Wolfurin et al., 2003). Indeed, Foxa2 is directly inactivated by Akt via phosphorylation of a single site that is conserved in the fly Fkh (Wolfurin 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-dfoxo DS (Wu et al., 2005) and dfoxo NS (Slack et al., 2011) were backcrossed at least six times into Dahomey background carrying w1118 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 SVA 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 dfoxo DS 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 52 cells based on a published method (Andrulis et al., 2000; Puig et al., 2003). In all, 10 ml of 5 × 10⁵ cells/ml were incubated in Schneider’s medium without serum for 2 h at 25°C with 0.5% formaldehyde which was added to 0.1% for 10 min, then washed 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 0.1 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 DS.
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 (Frohlich 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 McElwée 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 define lists of differentially expressed genes. For all other worm data sets, selected gene lists were already provided. The define lists of differentially expressed genes. For all other worm data sets, selected gene lists were already provided. The define lists of differentially expressed genes.

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).

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