Transcriptional feedback control of insulin receptor by dFOXO/FOXO1

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The insulin signaling pathway, which is conserved in evolution from flies to humans, evolved to allow a fast response to changes in nutrient availability while keeping glucose concentration constant in serum. Here we show that, both in *Drosophila* and mammals, insulin receptor (InR) represses its own synthesis by a feedback mechanism directed by the transcription factor dFOXO/FOXO1. In *Drosophila*, dFOXO is responsible for activating transcription of dInR, and nutritional conditions can modulate this effect. Starvation up-regulates mRNA of dInR in wild-type but not dFOXO-deficient flies. Importantly, FOXO1 acts in mammalian cells like its *Drosophila* counterpart, up-regulating the InR mRNA level upon fasting. Mammalian cells up-regulate the InR mRNA in the absence of serum, conditions that induce the dephosphorylation and activation of FOXO1. Interestingly, insulin is able to reverse this effect. Therefore, dFOXO/FOXO1 acts as an insulin sensor to activate insulin signaling, allowing a fast response to the hormone after each meal. Our results reveal a key feedback control mechanism for dFOXO/FOXO1 in regulating metabolism and insulin signaling.

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Metazoan metabolism adapted throughout evolution to deal with changes in nutrient availability. Fasting and feeding states cycle regularly during the life of an organism, so mechanisms have been developed to allow cells to regulate metabolism by responding rapidly to changes in nutrients. The insulin signaling pathway, which is conserved in evolution from flies to humans, constitutes an example of a key regulatory mechanism that evolved to allow these organisms to adapt to changes in nutrient availability while keeping glucose concentration constant in serum. After a meal, when glucose levels are high, insulin levels rise, triggering a vast array of metabolic responses that activate glucose storage. Conversely, during periods of fasting, when glucose is scarce, insulin levels drop and a different set of responses are activated, finally leading to an increase in liver glucose production, allowing its utilization by the different cells in the organism.

Insulin exerts its effects by binding to the insulin receptor (InR), which activates a cascade of events that, among others, results in the activation of Akt kinase. Akt in turn influences gene expression through the forkhead-related FOXO family of transcription factors FOXO1, FOXO3a, and FOXO4 (Burgering and Kops 2002) by phosphorylating these proteins at three conserved Ser/Thr residues. This leads to retention of FOXO transcription factors in the cytoplasm, thereby down-regulating RNA synthesis of specific target genes that affect cell cycle progression (Alvarez et al. 2001) and apoptosis (Brunet et al. 1999). Recent evidence suggests that FOXO1 is also a key element in insulin-mediated regulation of metabolism. Several groups have shown that FOXO1 is inhibited by insulin through PI3K/Akt and also through PI3K-dependent kinases different from Akt (Biggs et al. 1999; Guo et al. 1999; Nakae et al. 1999, 2001; Rena et al. 1999; Tang et al. 1999). Interestingly, FOXO1 regulates expression of several genes involved in glucose metabolism, including insulin-like growth factor-binding protein-1 (Durham et al. 1999), phosphoenolpyruvate carboxy kinase (Hall et al. 2000), and glucose-6-phosphatase (Schmoll et al. 2000). In addition, FOXO1 has also been implicated in myoblast (Hribal et al. 2003) and adipocyte differentiation (Nakae et al. 2003) and pancreatic β cell growth (Kitamura et al. 2002; Nakae et al. 2002), cells that are all pivotal in the regulation of glucose metabolism.

We previously cloned the *Drosophila* equivalent of FOXO1 and showed that dFOXO transcriptionally activates novel downstream as well as upstream targets of the insulin signaling cascade (Puig et al. 2003). For example, dFOXO activates transcription of the translational inhibitor initiation factor 4E-binding protein (d4EBP), partly explaining the growth inhibition elicited by dFOXO (Puig et al. 2003). Interestingly, we also found that dFOXO activates transcription of dInR itself, which provided the first evidence for a transcriptional feedback mechanism in the InR pathway. This feedback mecha-

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nism would, in principle, allow the cells to regulate growth and metabolism by responding rapidly to changes in nutrients (Puig et al. 2003). We reasoned that in flies, when nutrients are abundant, elevated levels of *Drosophila* insulin-like peptides (DILPs, the *Drosophila* equivalent of human insulin) are secreted to activate the dInR pathway, down-regulating dInR transcription by dFOXO inactivation. However, under situations in which nutrients are limiting, DILPs would be secreted at a reduced rate and the dInR pathway would be inactivated, freeing dFOXO to activate its target genes. Since dFOXO would be active when nutrients are limited, dInR becomes up-regulated by dFOXO under these conditions. This situation permits the cells to accumulate higher levels of dInR in the membrane, thus establishing a sensitized and primed state to signal when triggered by changes in DILP levels. In this way, when nutrient conditions change, the cells would be able to respond rapidly by turning on the mechanisms that stimulate growth, including shutting down dFOXO via dAkt phosphorylation. Therefore, with this feedback mechanism, regulation of dInR transcription by dFOXO could allow exquisitely fine-tuned and balanced insulin signaling.

Our initial experiments that revealed this potential feedback control were performed by overexpression of dFOXO in *Drosophila* S2 cells. In the current study, we investigate this putative mechanism under more physiological conditions. We activated endogenous dFOXO by nutrient deprivation in S2 cells as well as starvation in flies, and studied whether the dInR would be up-regulated in these conditions. In addition, since the insulin signaling pathway is well conserved between flies and mammals, we also analyzed the potential up-regulation of mammalian InR by FOXO1. We depleted muscle or liver cells of nutrients and growth factors to determine whether the InR mRNA would be up-regulated under these conditions. In addition, we directly measured binding of FOXO1 to the InR promoter in vivo and in vitro. Finally, we assessed whether FOXO1 up-regulation of InR produced an increased sensitivity of the InR pathway. Our results reveal that the FOXO1 transcription factor plays a key role in a feedback control mechanism that regulates metabolism and insulin signaling both in flies and mammals.

Results

dFOXO up-regulates InR transcription in S2 cells

We previously showed that in *Drosophila* S2 cells, overexpression of dFOXO activates transcription of the dInR promoter (Puig et al. 2003). We hypothesized that dFOXO would regulate insulin signaling by a transcriptional feedback mechanism in order to respond efficiently to changes in insulin levels, which parallels changes in nutrient levels (Deeney et al. 2000; Ikeya et al. 2002). We reasoned that when nutrients are limiting, insulin levels should decrease and signaling of the PI3K/Akt kinase cascade would be abrogated. Under these conditions, dFOXO would be in a dephosphorylated, nuclear-localized, and active state. Consequently, transcription of dInR would be up-regulated, and the receptor would accumulate in the cell membrane, primed to signal when triggered by changes in insulin levels. In this way, when nutrient conditions change, the cells would be highly sensitized and be able to respond rapidly by turning on the mechanisms that stimulate metabolism (Puig et al. 2003).

A key aspect of this hypothetical feedback control was the notion that dInR transcriptional activation by dFOXO would be modulated by insulin availability. To test this directly in a system that did not involve overexpression of proteins, *Drosophila* S2 cells were grown in M3 complete medium or fasted by incubation in Hank's balanced salt solution (HBSS) for 6 h. After HBSS incubation, a significant proportion of endogenous dFOXO becomes unphosphorylated and active (Fig. 1A, lane 2). Under these conditions, dFOXO effectively activates transcription of dInR, as measured by quantitative RT–PCR (qPCR) (Fig. 1B, lane 2). The levels of EF1α mRNA used as a control for specificity remained unchanged (Fig. 1B, lanes 3,4). We next determined whether dFOXO is actually bound to the dInR promoter in vivo

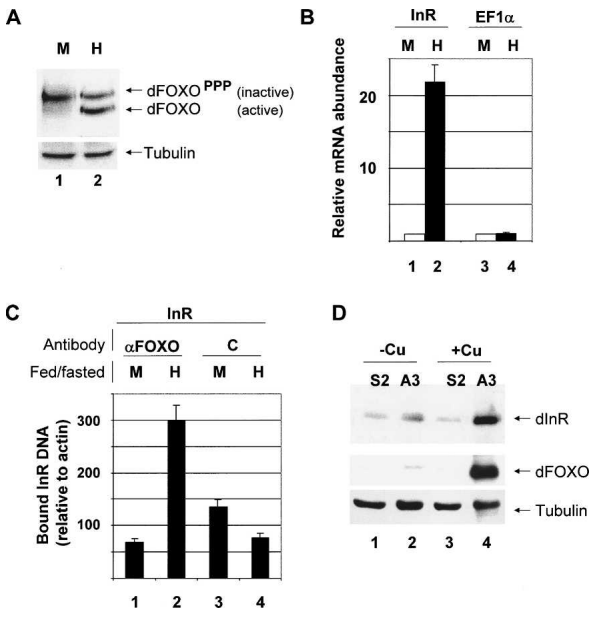

Figure 1. dFOXO activates InR mRNA transcription in *Drosophila* S2 cells upon starvation. (*A*) The Western blot shows that dFOXO gets dephosphorylated upon starvation. S2 cells were incubated for 6 h in HBSS (H) or in complete medium (M), and dFOXO was detected with specific antibodies. (*B*) qPCR shows that S2 cells incubated in HBSS specifically up-regulate dInR mRNA levels. (*C*) dFOXO binds to the dInR promoter in S2 cells upon starvation. ChIP was performed in S2 cells incubated in HBSS (H) or complete medium (M) and measured by qPCR. (*D*) dFOXO activity up-regulates InR protein levels in S2 cells. Control wild-type S2 cells (lanes *1*,*3*) or S2 dFOXOA3 cells (lanes *2*,*4*) were grown in the absence (lanes *1*,*2*) or presence (lanes *3*,*4*) of 600 μM $CuSO_4$ for 24 h to induce dFOXOA3 expression. dInR, dFOXO, and tubulin levels were analyzed by Western blot analysis with specific antibodies.

by using chromatin immunoprecipitation (ChIP). S2 cells were grown for 8 h in complete medium or in HBSS. Cells were cross-linked with formaldehyde, extracts were made, and chromatin was immunoprecipitated with antibodies raised against dFOXO. Figure 1C shows that dFOXO is specifically bound to the dInR promoter upon fasting (Fig. 1C, lane 2) but not to a U6 promoter used as control (data not shown). To test whether an increase in dInR mRNA levels produced by dFOXO leads to an increase in dInR protein levels, we performed Western blot analysis in S2 cells. S2 dFOXOA3 cells, which contain a constitutively active version of dFOXO regulated by a methalotionein promoter (Puig et al. 2003), were grown with $CuSO_4$ (to activate dFOXOA3 expression) or without it for 24 h. As a control, wild-type cells were grown in the same conditions. Subsequently, whole cell extracts were analyzed by Western blot using antibodies against dInR. Cells overexpressing dFOXOA3 display a three- to fivefold increase in the 170-kDa form of dInR (Marin-Hincapie and Garofalo 1995) compared with control S2 cells (Fig. 1D, cf. lanes 3 and 4). Taken together, these results indicate that dFOXO activates dInR gene expression under physiological conditions when nutrients and growth factors are absent.

Flies up-regulate dInR through dFOXO upon starvation

To test if a similar response also occurs in the whole organism, we designed a starvation experiment in flies. Two-day-old wild-type or dFOXO-deficient (Junger et al. 2003) flies were grown for 4 d in complete medium (containing yeast) or in medium containing only agarose, phosphate buffered saline (PBS), and 10% sucrose. After 4 d, dInR mRNA was analyzed by qPCR. Wild-type flies responded to starvation by up-regulating dInR mRNA more than twofold (Fig. 2A, cf. lanes 3 and 4). In contrast, flies lacking dFOXO showed no significant differences in the levels of dInR mRNA (Fig. 2A, lanes 1,2). As expected, control mRNAs such as actin (Fig. 2B) or EF1α (data not shown) were unaffected. These results suggest

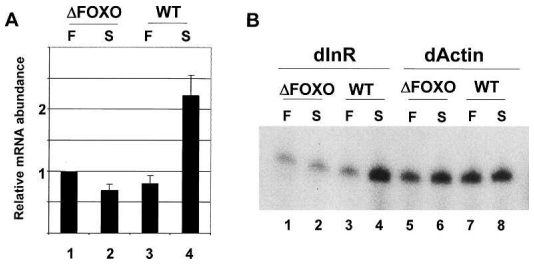

Figure 2. dFOXO up-regulates dInR mRNA in wild-type but not in *dfoxo* mutant flies upon starvation. Flies were starved for 4 d and mRNAs quantitated by qPCR. (*A*) dInR mRNA levels from flies starved (S) or fed (F) were quantitated by qPCR. Actin mRNA was used to normalize. (*B*) dInR and actin mRNAs were quantitated by RT–PCR using [α-^{32}P]dCTP and a PhosphorImager. dInR mRNA is up-regulated (2.7-fold) only in wild-type flies upon starvation.

that dFOXO is a key transcriptional regulator that responds to nutrient conditions by activating expression of dInR. An increase in the concentration of dInR in the plasma membrane would prime target cells, thus keeping them in a "ready to fire" state. Consistent with this scenario, dFOXO mutant flies are more sensitive to low nutrients, and as a consequence they are short-lived under starvation conditions, suggesting that dFOXO may be important for metabolic homeostasis (Junger et al. 2003; Kramer et al. 2003). These observations suggest that dFOXO may be a key transcriptional regulator that mediates nutrient response and metabolic balance by modulating dInR transcription.

The sensitivity of the InR signaling pathway is increased by dFOXO activity

We next tested whether as a consequence of the dFOXO-dependent increase in InR protein, the InR pathway is more sensitive in response to insulin changes. We used a kinase assay to detect InR autophosphorylation, which directly correlates with activation of the pathway in response to insulin (Kahn and White 1988). Extracts from S2 dFOXOA3 cells (grown for 24 h with $CuSO_4$ to activate dFOXOA3 expression) were prepared, and dInR protein was immunoprecipitated with specific antibodies. Then dInR autophosphorylation was determined by an in vitro kinase assay. As control, extracts from S2 wild-type cells grown in the same conditions were used. As shown in Figure 3A, lane 4, a polypeptide of 170 kDa corresponding to dInR shows increased phosphorylation in the extracts coming from cells that overexpress dFOXOA3. This band is precipitated by dInR-specific antibodies (Fig. 3A, lane 4) and it appears only when precipitated dInR is preincubated with 100 nM insulin before ^{32}P-ATP is added in the kinase assay (data not shown), confirming that it represents dInR autophosphorylation. Therefore, these results indicate that dInR sensitivity as measured by dInR autophosphorylation is increased by dFOXOA3 expression.

An additional assay was performed in S2 cells to confirm this apparent insulin sensitivity. dFOXOA3 S2 cells grown in full medium in the presence of $CuSO_4$ for 24 h (to express dFOXOA3) were incubated with increasing concentrations of insulin for 6 min. dInR autophosphorylation was analyzed by Western blot with a commercial antibody that recognizes InR only when it is phosphorylated. Although this antibody was originally raised against phosphorylated mammalian InR, it cross-reacts with phosphorylated dInR (Fig. 3B). As control, wild-type S2 cells were treated under the same conditions. As expected, upon exposure to increased concentrations of insulin, phosphorylation of dInR in cells that overexpress dFOXOA3 increases dramatically (Fig. 3B, lanes 1–6). Interestingly, this response is blunted in wild-type S2 cells (Fig. 3B, lanes 7–12), indicating that up-regulation of dFOXO sensitizes the dInR pathway to changes in insulin concentration. Taken together, these results suggest that dFOXO activation leads to an in-

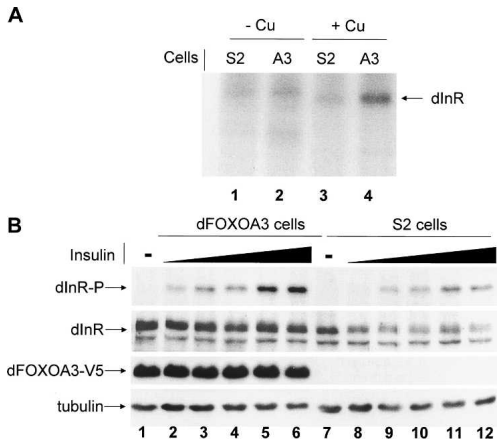

Figure 3. Autophosphorylation of InR is increased by dFOXO activity. (*A*) Control wild-type S2 cells (lanes *1*,*3*) or S2 dFOXOA3 cells (lanes *2*,*4*) were grown in the absence (lanes *1*,*2*) or presence (lanes *3*,*4*) of 600 µM $CuSO_4$ for 24 h to induce dFOXOA3 expression. Extracts were made and dInR was precipitated with specific antibodies bound to protein A-coupled Sepharose beads. Beads were washed, a kinase assay was performed in the beads, and the proteins bound to the beads were analyzed by SDS-PAGE. A 170-kDa phosphorylated band corresponding to dInR is present only in samples coming from cells overexpressing dFOXOA3. (*B*) S2 dFOXOA3 cells (lanes *1–6*) or control S2 cells (lanes *7–12*) were grown for 24 h in the presence of $CuSO_4$ to induce dFOXOA3 expression. Subsequently, insulin was added in increasing concentrations (0, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M). dInR autophosphorylation was analyzed by Western blot with a specific antibody.

crease in InR protein levels, which allows for an enhanced insulin sensitivity in *Drosophila*. These findings confirm the role of dFOXO in regulating dInR expression and sensitizing the dInR signaling pathway.

FOXO1 activates InR promoter in mammalian cells

Although it has been well established that the FOXO signaling pathway is highly conserved between flies and mammals, it was not known whether FOXO proteins directly regulate the mammalian InR gene. We therefore searched for FOXO recognition elements (FREs) present in the human InR promoter. Interestingly, InR has a consensus FRE (Furuyama et al. 2000) upstream from the InR transcription start sites, and this FRE is conserved in mouse, rat, and human (Fig. 4A). To test whether FOXO1, the mammalian homolog of dFOXO most abundant in hepatocytes (Nakae et al. 1999), can, indeed, activate transcription from this promoter, we transfected human 293 cells with a luciferase gene driven by the human InR promoter (Araki et al. 1987, 1989; Lee et al. 1992). Cotransfection of a constitutively active version of FOXO1 (FOXO1A3, in which all three Akt phosphorylation sites are mutated to Ala) led to a robust increase in luciferase activity (Fig. 4B). We shortened the human InR promoter to a set of minimal sequence elements (–486 to –205 upstream of the ATG initiation codon) that retained the ability to respond to FOXO1; this minimal promoter contains the conserved FRE (–469 to –461). Subsequently, we introduced mutations in the FRE and measured FOXO1 activity (Fig. 4C). Mutating the putative FRE severely reduced FOXO1 dependent activation (Fig. 4C, cf. lanes 2 and 4). As a control, we used a different transcription factor, c-Jun, which activates its own promoter (Angel et al. 1988; data not shown) but does not activate the InR promoter (Fig. 4C, lane 3). Our findings suggest that FOXO1 likely binds specifically to the upstream FRE of the InR promoter to activate transcription of this cognate promoter.

FOXO1 specifically binds InR promoter

In order to determine whether FOXO1 can specifically and directly bind the FRE upstream of the InR promoter, we performed band-shift assays by using recombinant human FOXO1 expressed in bacteria and a DNA probe derived from the InR promoter fragment containing the FRE. As shown in Figure 4D, recombinant FOXO1 binds efficiently to the DNA fragment containing the FRE from the InR (Fig. 4D, lane 2) but does not bind to a DNA fragment in which the FRE has been mutated (Fig. 4D, lane 4), indicating that FOXO1 can bind the InR promoter through the cognate FRE. Interestingly, ChIP experiments showed that FOXO1 can also bind the InR promoter in vivo (see below). These results confirm that human FOXO1 functions much like its *Drosophila* counterpart by activating InR in a feedback loop, thus auto-regulating a key member of the insulin signaling pathway.

FOXO1 activates transcription of InR upon insulin depletion

Two of the major insulin-sensing cell types in mammals are muscle and liver. We therefore investigated whether mammalian FOXO1 would autoregulate transcription of InR in an insulin-dependent manner in these two tissue types. C2C12 (mouse muscle) and Hepa 1–6 (mouse liver) cells were incubated in complete medium or in HBSS for 8 h, and InR mRNA was analyzed by qPCR. As expected, nutrient and growth factor deprivation up-regulated InR mRNA (Fig. 5A; data not shown). Under these fasting conditions, FOXO1 remains unphosphorylated and active (Fig. 5B, cf. lanes 1 and 2). To assess the phosphorylation state of FOXO1, we used an antibody that specifically detects the phosphorylated form of FOXO1 (Fig. 5C, lane 2). As expected, phosphatase treatment of the cell extracts abolished this specific antibody signal (Fig. 5C, lane 1). These experiments indicate that depleting C2C12 cells of nutrients, hormones, and growth factors up-regulates InR through a FOXO1-dependent mechanism. The same results were observed with Hepa 1–6 cells (data not shown).

HBSS is a balanced salt solution containing only essential ions required for normal metabolism and a buff-

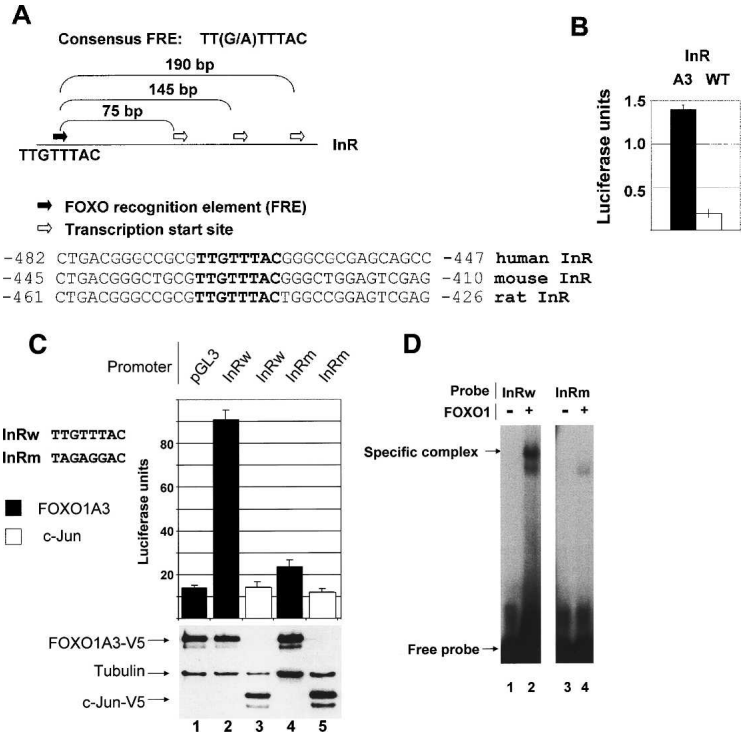

Figure 4. Mammalian FOXO1 activates the InR promoter. (*A*) The InR promoter contains a putative FRE upstream from the transcription start sites. The FRE (in bold) in the InR is conserved in human, rat, and mouse. (+1) The A in the ATG initiation codon. (*B*) Transfection assays show that human InR can be activated by FOXO1A3 in 293 cells in the presence of insulin (200 nM). (A3) Triple Ala mutant FOXO1; (WT) wild-type FOXO1. (*C*) The InR promoter containing mutated versions of the putative FRE no longer responds to FOXO1 activation. (InRw) Wild-type InR promoter (lanes *2*,*3*); (InRm) mutant InR promoter (lanes *4*,*5*). (Lane *1*) Empty vector was used as control. (Black bars) A plasmid driving the expression of FOXO1A3-V5 was cotransfected; (white bars) a plasmid driving the expression of c-Jun-V5 was cotransfected. (*D*, lanes *1*,*2*) Band shift performed with recombinant human FOXO1 purified from *E. coli* and a DNA probe containing the putative FRE from the InR promoter. In lanes *3* and *4*, a DNA probe containing the same mutation used in *C* was used.

ering system to maintain the physiological pH. In contrast, C2C12 culture medium (DME) is a rich broth containing salts, amino acids, vitamins, glucose, and serum, which includes numerous hormones and growth factors. We therefore wanted to determine which component present in the culture medium is necessary and sufficient to inhibit FOXO1 activity. In particular, we wanted to know whether insulin alone would be sufficient to achieve this effect. First, we measured FOXO1 inhibition by analyzing its phosphorylation state, which directly reflects its ability to activate InR promoter, both in *Drosophila* and mammalian cells (see Figs. 1, 5). We designed an experiment in which C2C12 cells were incubated for 8 h in serum-free culture media lacking one of the components present in the complete medium (amino acids, glucose, vitamins) with or without insulin. As shown in Figure 6A, depleting cells of each of these essential components (amino acids, glucose, or vitamins) had no measurable effect on FOXO1 phosphorylation. FOXO1 phosphorylation levels were the same in complete serum-free medium; in serum-free medium lacking either amino acids, glucose, or vitamins; or in a salt solution equivalent to HBSS (formulated by removing amino acids, glucose, and vitamins from the serum-free complete medium) (Fig. 6A, cf. lanes 1, 3, 5, 7, and 9). Interestingly, addition of insulin to 200 nM was enough to achieve full FOXO1 inhibition, independently of the presence of the other components (amino acids, glucose, or vitamins) (Fig. 6A, cf. lanes 2, 4, 6, 8, and 10). Thus, FOXO1 phosphorylation and inactivation are driven largely by insulin, and no other medium component appears to be required for triggering this effect. Next, we tested different insulin concentrations to determine if there was any difference between serum-free medium and HBSS on FOXO1 inhibition. C2C12 cells were incubated for 8 h with either HBSS or serum-free medium. Insulin was then added at different concentrations. There was no significant difference observed on FOXO1 phosphorylation when cells were incubated in serum-free medium or HBSS for all insulin concentrations tested, 25 nM to 1 μM (Fig. 6B). These experiments suggest that FOXO1 is regulated by a direct feedback loop in which insulin activates InR, and this signaling pathway, in turn, down-regulates FOXO1 activity, at least at the concentrations of insulin we have tested.

It has been reported that FOXO1 is inactivated by phosphorylation by the PI3K/Akt pathway, but it can also be inactivated by kinases other than Akt (Nakae et al. 1999). We therefore wanted to determine whether inactivation of FOXO1 by insulin occurred at least partly through the Akt kinase. We transfected human 293 cells with a luciferase gene driven by the human InR promoter together with FOXO1 to activate InR promoter transcription. Cotransfection of Akt1 reduced luciferase activity by almost 50% (Fig. 6C, lane 1), while cotransfection of a kinase-inactive version of Akt1 (AktKD) had no effect (Fig. 6C, lane 4). The result obtained with Akt1 is reversed by the PI3K-specific inhibitor LY294002 (Fig. 6C, lane 3). These results confirm that inactivation of FOXO1 by insulin is, at least in part, regulated by Akt. Interestingly, Akt1 did not inhibit FOXO1 activity completely, even when cotransfected at equal molar ratios with FOXO1 (Fig. 6C, lane 2), suggesting that other kinases also play a role in the regulation of FOXO1.

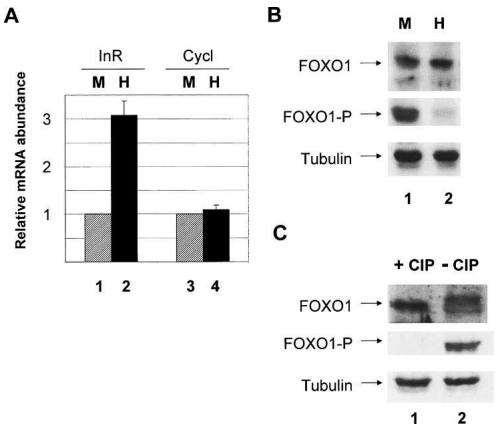

Figure 5. FOXO1 up-regulates InR transcription upon fasting. (*A*) Mouse C2C12 cells were fasted for 8 h in HBSS (H) or kept in complete medium (M). Total RNAs were extracted, and mRNAs for InR and cyclophilin were quantitated by qPCR. Actin mRNA was used to normalize. (*B*) Western blot of FOXO1 shows that in fasted C2C12 cells, FOXO1 is dephosphorylated (H) when compared with FOXO1 from fed cells (M). (*C*, lane *1*) Western blot of cell extracts treated with calf intestinal phosphatase (+CIP), and detected with a phospho-FOXO1-specific antibody, show that FOXO1 is dephosphorylated, indicating that the phospho-specific antibody recognizes FOXO1 only when it is phosphorylated.

FOXO1 binds InR promoter in vivo

We next tested whether FOXO1 binds the InR promoter in vivo in an insulin-dependent fashion. ChIP was performed on C2C12 cells that had been incubated in serum-free medium plus or minus insulin for 8 h. As shown in Figure 7A, lane 3, an InR promoter fragment was specifically coprecipitated by anti-FOXO1 antibodies from cells incubated in serum-free medium minus insulin as assayed by qPCR. In contrast, the level of promoter DNA coprecipitated from cells incubated in serum-free medium plus insulin is similar to control background levels (Fig. 7A, lane 4). In the same experiment, we measured FOXO1 phosphorylation (Fig. 7B) and InR mRNA up-regulation (Fig. 7C). Both parameters correlate well with InR promoter binding by FOXO1, further confirming that insulin is sufficient to abolish FOXO1 binding to InR promoter by keeping it in a phosphorylated and inactive state. The same results were observed with Hepa 1–6 cells (data not shown). These results reveal that FOXO1 is efficiently bound to the InR promoter in muscle and liver cells in vivo only in the absence of insulin.

Our ChIP experiments demonstrated that insulin signaling results in the loss of FOXO1 bound to the InR promoter. It had previously been shown that phosphorylated FOXO transcription factors are retained in the cytoplasm (Brunet et al. 1999; Brownawell et al. 2001), thus preventing their activity in the nucleus. However, there is some evidence suggesting that FOXO1 could be regulated by phosphorylation in ways other than merely cytoplasmic retention (Zhang et al. 2002; Tsai et al. 2003). Interestingly, Ser 256 in FOXO1 is located in the DNA-binding domain, and its phosphorylation by Akt could potentially lead to altered binding of FOXO1 to InR promoter. In order to investigate this possibility, we compared the binding of phosphorylated and unphosphorylated FOXO1 to the InR promoter in vitro. Purified recombinant wild-type FOXO1 or mutant FOXO1A3 expressed in bacteria was first incubated with recombinant Akt kinase and ATP to carry out in vitro phosphorylation. Figure 7D shows that only FOXO1 is phosphorylated in Ser 256 by the Akt kinase as detected with phospho-specific antibodies (Fig. 7D, lane 1), while FOXO1A3 remains unphosphorylated at that residue (Fig. 7D, lane 2). Subsequently we used both proteins in band-shift experiments with a DNA probe containing the InR promoter including the FRE (same as in Fig. 4D).

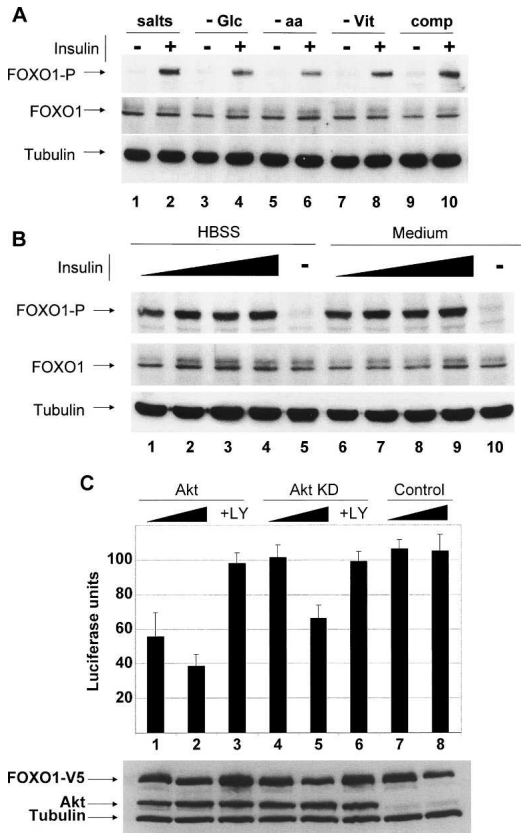

Figure 6. Insulin is sufficient to inactivate FOXO1. (*A*) C2C12 cells were incubated in serum-free complete medium (comp), in medium without glucose (–Glc), without amino acids (–aa), without vitamins (–Vit), or in medium without amino acids, glucose, and vitamins (salts) for 8 h. Insulin (200 mM) was added every 2 h (even lanes). (*B*) C2C12 cells were incubated for 8 h in HBSS or serum-free complete medium, and insulin was added every 2 h at 25 nM (lanes *1*,*6*), 100 nM (lanes *2*,*7*), 300 nM (lanes *3*,*8*), or 1 µM (lanes *4*,*9*). In lanes *5* and *10*, no insulin was added. (Lanes *5*,*10*) The appearance of a slower migrating species does not correlate with FOXO1 phosphorylation. (*C*) 293 cells were cotransfected with a luciferase reporter gene driven by the InR promoter, and plasmids expressing FOXO1 (all lanes), Akt (lanes *1–3*), Akt KD (lanes *4–6*), or pcDNA3.1 as control (lanes *7*,*8*). In lanes *3* and *6*, LY294002 at 20 µM was added 6 h before analyzing luciferase activity.

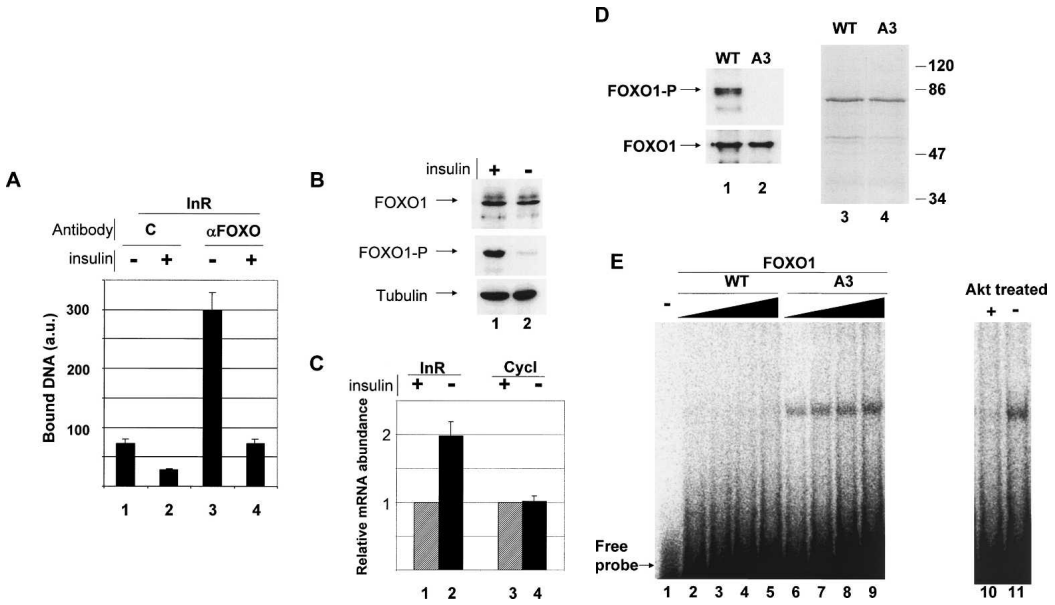

Figure 7. FOXO1 binds and activates InR promoter in the absence of insulin. (*A*) ChIP was performed in C2C12 cells incubated in serum-free medium with (1 μM) or without insulin. Antibodies against FOXO1 specifically precipitate InR promoter only in the absence of insulin. Actin was used to normalize the qPCR between samples, and each value was independently normalized to its input. (*B*) FOXO1 is dephosphorylated in the absence of insulin. (*C*) mRNA for InR is up-regulated in the absence of insulin. (*D*) FOXO1 (lane *1*) and FOXO1A3 (lane *2*) were phosphorylated in vitro by Akt kinase and detected by Western blot with anti-phospho-FOXO1-specific antibodies (*upper* panel) or FOXO1-specific antibodies (*lower* panel). (Lanes *3*,*4*) Coomassie gel of both proteins. (*E*) Phosphorylation by Akt prevents binding of FOXO1 to the InR promoter probe. Band shift performed with recombinant human FOXO1 (shown in *D*) and a DNA probe containing the putative FRE from the InR promoter. (Lane *1*) No protein. (Lanes *2–5*) FOXO1. (Lanes *6–9*) FOXO1A3. (Lanes *10*,*11*) Band shift was performed with FOXO1 after (+) or before (–) in vitro kinase reaction.

As shown in Figure 7E, lanes 6–9, the mutant FOXO1A3 that cannot be phosphorylated by Akt at Ser 256 binds efficiently to the InR promoter. In contrast, wild-type FOXO1, which is phosphorylated by Akt kinase at Ser 256 (Fig. 7D), showed little or no binding to the InR promoter (Fig. 7E, lanes 2–5). This is not due to some nonspecific loss of binding activity during protein purification because this same protein binds efficiently to the InR probe when it is not phosphorylated, prior to incubation with Akt (Fig. 7E, lanes 10,11). These results suggest that phosphorylation of FOXO1 at Ser 256 can affect its binding activity to the InR promoter, which in part could explain the loss of FOXO1 bound to this promoter observed in vivo upon insulin signaling.

InR protein levels are up-regulated by FOXO1

We have shown that FOXO1 binds and activates transcription from the InR promoter in the absence of insulin. Next, we wanted to know whether the increase in mRNA levels produced by FOXO1 leads to a parallel increase in InR protein levels. Western blot analysis was performed with C2C12 cells that were incubated for 12 h in serum-free medium with or without 100 nM insulin. Subsequently, InR protein was quantitated by Western blotting with specific antibodies. InR protein levels increase in parallel with its mRNA levels when cells are grown without insulin (Fig. 8A, lane 2). Under these conditions, FOXO1 is dephosphorylated and active (Fig. 8A, lane 2). Thus, like the *Drosophila* system, an increase in InR mRNA levels directed by FOXO1 leads to a parallel increase in InR protein levels, confirming a key role for FOXO1 in the regulation of InR expression during fasting.

Activation of InR by FOXO1 sensitizes the mammalian signaling pathway

We have shown that FOXO1 activates transcription of the InR promoter, which increases the levels of InR protein present in the cells. Next we wanted to test if, as in the *Drosophila* system, increased levels of InR lead to an enhanced sensitivity of the pathway. As before, we used an in vitro kinase assay to detect InR autophosphorylation upon treatment with insulin. C2C12 cells were incubated for 12 h in serum-free medium plus or minus 100 nM insulin. Extracts were obtained, and InR was partially purified by precipitation using wheat germ agglutinin (WGA) coupled to Sepharose beads. Subsequently, InR was eluted from the beads, and a kinase assay was performed to determine InR autophosphorylation. A phosphorylated polypeptide of 95 kDa corresponding to mouse InR was observed only in extracts obtained from cells grown in the absence of insulin (Fig. 8B, lane 4). This polypeptide species is phosphorylated only when samples are preincubated with insulin before adding ^{32}P-ATP (Fig. 8B, cf. lanes 2 and 4), confirming that it represents InR autophosphorylation. No such la-

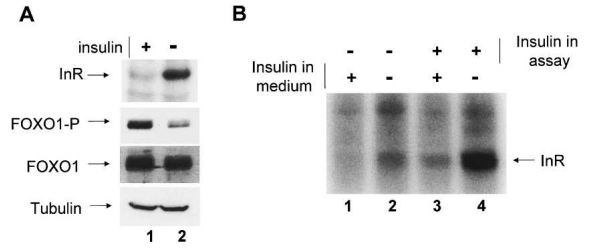

Figure 8. InR pathway is sensitized by FOXO1. (*A*) FOXO1 activity up-regulates InR protein levels in C2C12 cells. Cells were grown in serum-free medium with (lane *1*) or without (lane *2*) 100 nM insulin. InR, FOXO, and tubulin levels were analyzed by Western blot analysis with specific antibodies. (*B*) Autophosphorylation of InR is increased by FOXO1 activity. C2C12 cells were grown in serum-free medium plus (lanes *1*,*3*) or minus (lanes *2*,*4*) 100 nM insulin. Extracts were made, and InR was precipitated with WGA-coupled Sepharose beads. InR was eluted from the beads, and samples were preincubated (lanes *3*,*4*) or not (lanes *1*,*2*) with 100 nM insulin before a kinase assay was performed. Proteins were analyzed by SDS-PAGE. A 95-kDa band corresponding to phosphorylated InR is present only in samples coming from cells grown in the absence of insulin.

beled polypeptide is obtained from cells that had been grown in serum-free medium in the presence of insulin (Fig. 8B, lane 3), conditions that inhibit FOXO1. These results indicate that the InR pathway is more "activated" as a consequence of FOXO1-directed stimulation of InR protein levels. Thus, our results suggest that, as in the *Drosophila* case, FOXO1 activation also produces an increase in the InR protein levels that results in an enhanced insulin receptor activity in mammalian cells. These findings confirm the central role of dFOXO/FOXO1 in regulating InR expression, which leads to enhanced sensitivity of the InR signaling pathway both in flies and mammals.

Discussion

FOXO1 regulates InR transcription

It is well known that the expression and activity of the InR can be regulated by a wide variety of factors and that changes in the numbers of receptor molecules plays a pivotal role in several physiologic and pathologic states (Kahn 1985). The lowered sensitivity of cells to insulin and the hyperinsulinemia observed in obesity and type II diabetes mellitus is often accompanied by a reduced number of insulin receptors (Czech 1985). Insulin is thought to down-regulate its own receptor by a variety of mechanisms that can influence its synthesis as well as degradation (Knutson et al. 1982, 1985; Mamula et al. 1990). Interestingly, it has been shown that the number of InR molecules correlates with nutritional conditions both in tissue culture cells and in animals. Thus, levels of InR in growing HepG2 cells are relatively low, and they increase substantially if cells are starved (Hatada et al. 1989). In addition, states of chronic hyperinsulinemia produce a reduction in the number of InR present in the plasma membrane (Gavin et al. 1974; Bar et al. 1976). InR mRNA levels also change in animals depending on fasting–feeding conditions. For example, rats fed a high-fat diet display a decreased number of InR molecules in liver plasma membranes (Sun et al. 1977), and InR mRNA levels in rat skeletal muscle or liver increase after fasting, returning to normal levels after insulin treatment or refeeding (Knott et al. 1992; Tozzo and Desbuquois 1992). Interestingly, tissues other than muscle or liver might have similar regulation. For example, mRNA and protein levels of rat intestinal InR increase up to 230% in fasting conditions, and these effects are fully reversed by refeeding (Ziegler et al. 1995). Similar observations have been made in other organisms (Bisbis et al. 1994; Dupont et al. 1998). These effects indicate a nutritional influence on the abundance of the InR. Importantly, insulin levels in serum change in parallel to nutrient availability, both in flies and mammals (Deeney et al. 2000; Ikeya et al. 2002). Thus, when nutrients are high—that is, after a meal—insulin levels increase, while they decrease upon fasting. In *Drosophila* it has been shown that the InR/PI3K pathway coordinates cellular metabolism with nutritional conditions (Britton et al. 2002). Inhibiting this pathway phenocopies the cellular and organismal effects of starvation, while activating it bypasses the nutritional requirements for cell growth. The InR/PI3K pathway regulates the activity of FOXO1 in mammals (Nakae et al. 1999; Rena et al. 1999; Tang et al. 1999), *Caenorhabditis elegans* (Henderson and Johnson 2001), and *Drosophila* (Junger et al. 2003; Kramer et al. 2003; Puig et al. 2003), so nutrient activation of the PI3K pathway results in inactivation of FOXO1 by phosphorylation. However, despite this accumulated base of information, the molecular mechanism linking FOXO1 and InR expression had not been revealed.

Here we show that mammalian FOXO1 and its *Drosophila* counterpart dFOXO directly regulate insulin-signaling response to nutritional conditions through a feedback mechanism that involves activation of transcription from the InR promoter. Incubating C2C12 cells with a balanced salt solution or with serum-free medium up-regulated insulin receptor mRNA. Under these conditions, FOXO1 becomes dephosphorylated and actively binds to the InR promoter. When insulin was added to the medium, InR mRNA was down-regulated, even in the absence of serum, vitamins, amino acids, and glucose. Concomitantly, phosphorylation of FOXO1 increased and binding to InR promoter decreased. These results indicate that FOXO1 regulates InR transcription through a direct feedback mechanism that senses insulin levels in serum, which is, in turn, a reflection of nutrient load. It is important to note that, at this point, we cannot rule out that the increased InR protein levels we see caused by FOXO1 could be due to other mechanisms in addition to increased transcription from the InR promoter (i.e., affecting mRNA stability, or protein translation).

In *Drosophila* a similar mechanism occurs. Incubation of S2 cells with complete medium kept dFOXO phosphorylated and inactive, while incubation in HBSS dephosphorylated dFOXO. dInR mRNA was up-regulated only when dFOXO was dephosphorylated and active. In

addition, wild-type flies starved for 4 d up-regulated dInR, and this effect requires an intact *dfoxo* gene. Our studies indicate that in *Drosophila*, the PI3K/Akt pathway also senses insulin levels and regulates binding of dFOXO to the dInR promoter accordingly. These results underscore the importance of the InR/PI3K/Akt pathway in sensing nutrients and insulin, a function that has been conserved during evolution. They also highlight the role of FOXO1 as a sensor for insulin levels, promoting accumulation of InR in the absence of insulin, thereby allowing a fast response to the hormone after each meal. Under conditions in which insulin levels are chronically elevated, for example, in obese animals or patients, down-regulation of InR transcription would occur and insulin sensitivity would be impaired. These results establish the FOXO1 transcription factor as a key player in a feedback control mechanism that regulates metabolism and insulin signaling.

FOXO1 regulates multiple components of the InR pathway

Our results show that in conditions in which insulin levels are low, mammalian FOXO1 activates InR. Interestingly, we have observed that FOXO1 also activates the insulin receptor substrate-2 (IRS-2) promoter under fasting conditions, and, as it occurs with InR, insulin is sufficient to reverse this effect (our unpublished results). FOXO1 binds IRS-2 promoter in vitro and in vivo and activates IRS-2 transcription when muscle or liver cells are fasted. In addition, we have observed that FOXO1 activates IRS-2 promoter in luciferase assays, and this activation depends on the presence of a consensus FRE present in the IRS-2 promoter, because mutating this FRE abolishes FOXO1-dependent activation. Thus, we have observed that FOXO1 regulation of IRS-2 is parallel to InR regulation. While our experiments with the IRS-2 promoter were being performed, Ide et al. (2004) reported that SREBPs compete with FOXO transcription factors for binding to the IRS-2 promoter in liver; while SREBPs inhibit IRS-2 production, FOXO1 was found to activate IRS-2 transcription. They also found that fasting promoted binding of FOXO1 to the FRE of the IRS-2 promoter. Therefore, these findings strongly support our conclusions that FOXO1 regulates insulin signaling through a feedback mechanism that impinges on the insulin receptor and at least one of its substrates, IRS-2 (Fig. 9). After a meal, high levels of insulin peptide hormone activate its cognate receptor, which leads to repression of InR and IRS-2 transcription, resulting in subsequent dampening of the pathway by reducing the number of receptors on the cell surface and by limiting its ability to signal downstream through IRS-2. Conversely, fasting causes reduced levels of InR signaling, which in turn activates FOXO1, leading to increased transcription of InR and IRS-2. Once this transcription mechanism is activated, feedback regulation and phosphorylation of FOXO1 via the insulin signaling cascade automodulates InR expression. Insulin sensitivity could, therefore, be significantly affected by FOXO1 regulation. Regulation of insulin sensitivity by a feedback loop through FOXO1 would allow the cells to keep an exquisite metabolic balance between feeding and fasting states, permitting a faster response of the tissues to insulin changes. This feedback mechanism could well be disrupted in pathological states with abnormally increased insulin levels as is found in the case of insulin-resistant diabetes.

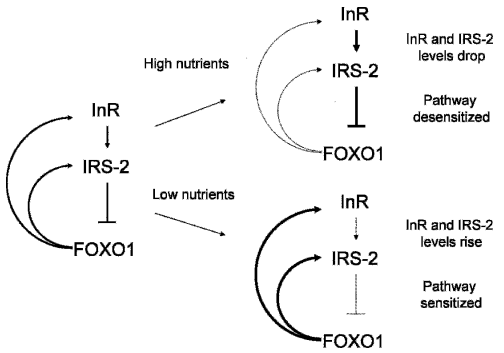

Figure 9. Proposed mechanism by which FOXO1 regulates the sensitivity of the InR signaling pathway through a feedback mechanism that involves up-regulation of InR and IRS-2 upon fasting. When nutrients are high, the InR pathway turns down FOXO1 activity and the levels of InR and IRS-2 drop, dampening the signaling through the InR pathway. When nutrient conditions change, FOXO1 is active and it sensitizes the pathway by activating the transcription of InR and IRS-2.

Materials and methods

Constructs, Drosophila *strains, antibodies, and cell types*

The human InR promoter was a gift from E. Araki (Kumamoto University, Kumamoto, Japan) and S. Tsai (Baylor College of Medicine, Houston, TX) (Araki et al. 1987; Lee et al. 1992). Mutated versions of this construct were produced by PCR. Plasmids expressing FOXO1 were gifts from F. Barr (University of Pennsylvania Medical Center, Philadelphia, PA) and T. Unterman (Veterans Affairs Chicago Health Care System, Chicago, IL) (Rena et al. 1999; Tang et al. 1999). FOXO1 was subcloned in the pcDNA-V5 expression vector (Invitrogen), which expresses FOXO1 tagged with the V5 epitope. c-Jun-V5 and c-Jun promoter were kind gifts from K. Geles (University of California at Berkeley, Berkeley, CA). Plasmids expressing Akt and a kinase dead mutant form of Akt (AktKD) were a kind gift of D. Stokoe (University of California at San Francisco, San Francisco, CA) and T. Asano (University of Tokyo, Tokyo, Japan). All constructs were checked by sequencing. The flies used in these study were compound heterozygous for $dFOXO^{21}$ and $dFOXO^{25}$ alleles (Junger et al. 2003). As control line, EP, a strain with a clean excision of the P element from EP35–147 (parental for $dFOXO^{21}$ and $dFOXO^{25}$) was used. EP, $dFOXO^{21}$, and $dFOXO^{25}$ were gifts from M. Juenger and E. Hafen (Universitat Zurich, Zurich, Switzerland). 293 (ATCC CRL-1573), C2C12 (ATCC CRL-1772), and Hepa 1–6 (ATCC CRL-1830) were obtained from the American Tissue Culture Collection. Polyclonal antibodies against dFOXO (used at 1:1000 dilution) were described before (Puig et al. 2003); antibodies against FOXO1 (used at 1:1000 dilution) were raised in rabbits against a GST-FOXO1 fusion protein containing amino acids 271–655 of human FOXO1; antibodies against dInR were raised in rabbits by using a GST fusion protein of a dInR peptide as described (Fernandez at al 1995); antibodies against FOXO1-P Ser 256 (used at 1:1000

dilution) and mInR-P Tyr 1146 (which also detects dInR-P; used at 1:1000) were obtained from Cell Signaling; and antibodies against mInR (used at 1:1000) were obtained from Santa Cruz Biotechnology. Monoclonal antibodies against tubulin (clone DM1A; Sigma) and anti-V5 (Invitrogen) were used at 1:5000 and 1:10,000 dilutions, respectively. Insulin (Roche) was dissolved in water, and its concentration was determined by absorbance at 278 nm.

Cell culture, transfection, and extract preparation

Drosophila S2 cells were grown as described (Puig et al. 2003). Human 293 cells and mouse C2C12 and Hepa 1–6 cells were grown in DME medium (Sigma) with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin at 37°C. S2 cells were transfected as described (Puig et al. 2003). 293 cells were transfected by the calcium-phosphate method with 500 ng of expression vector and 100 ng of reporter per well on a 24-well plate. For Akt cotransfection, 100 or 500 ng of Akt, AktKD, or pcDNA3.1 was added. Luciferase was measured using the dual luciferase reporter (Promega). Empty vector pGL3Basic was used as a reporter negative control. Protein concentration (measured by a Bradford assay; Bio-Rad) was used to normalize for loading differences between samples. Each experiment was repeated at least three times. Errors represent standard deviation of three measurements. Extracts for Western blots were obtained by lysing the cells directly in SDS-PAGE loading buffer. To detect FOXO1-P or dInR-P, phosphatase inhibitors (Na_3VO_4 at 5 mM and NaF at 50 mM) were added to blocking and antibody-binding buffers, as recommended (Sharma and Carew 2002).

Band-shift analysis and ChIP

Band-shift analysis was performed as described (Puig et al. 2003) using recombinant FOXO1 expressed in *Escherichia coli* and oligonucleotides containing the putative FRE for human InR. The oligonucleotide sequences were InR w, TGACGGGCCGC GTTGTTTACGGGCGCGAGCAG; InR m, TGACGGGCCGC GT**A**G**AGG**ACGGGCGCGAGCAG. Double-stranded DNA probes for each construct were obtained by denaturing and reannealing equal molar amounts of each oligonucleotide (+ and – strands). ^{32}P labeling was performed after annealing. ChIP was performed as described (Puig et al. 2003) by cross-linking S2 cells with 0.1% formaldehyde and mouse cells with 1% formaldehyde. For immunoprecipitation, anti-dFOXO (Puig et al. 2003) or FOXO (generated against a GST-FOXO4 fusion protein containing amino acids 1–204 of human FOXO4) specific antibodies were used. qPCR, used to quantitate bound DNA in the pellets, was performed with SybrGreen PCR Mix (QIAGEN or Bio-Rad) on an MJ Research Opticon-2 system. The U6 snRNA promoter was used as an internal control with *Drosophila* samples, and the actin gene was used with mouse samples. All values represented are independently normalized to their respective inputs.

In vitro kinase reactions were performed with human recombinant FOXO1 and FOXO1A3. Purification of both proteins was performed with Ni-NTA agarose beads (QIAGEN) in 50 mM phosphate buffer (pH 7.4), 500 mM NaCl, 1% Triton X-100, 10% glycerol, 20 mM β-mercaptoethanol, protease inhibitors (Roche), and 20 mM imidazol. Beads were washed in the same buffer with 50 mM imidazol, and proteins were eluted in the same buffer with 250 mM imidazol. Subsequently, proteins were purified with a Superdex 75 gel filtration column (Pharmacia) in 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 20 mM β-mercaptoethanol. One-milliliter fractions were collected and analyzed by electrophoresis on SDS-PAGE. Fractions containing each pure (>95%) protein (FOXO1 or FOXO1A3) were pooled, and proteins were concentrated with an Amicon 10 kDa concentrator (Millipore) following the manufacturer's instructions. Equal amounts of FOXO1 and FOXO1A3 as determined by Bradford assay (Bio-Rad) were kinased in vitro by using recombinant Akt kinase (New England Biolabs) and 400 µm of ATP for 30 min at 30°C. Proteins were used for band-shift experiments immediately after the kinase reaction.

Expression analysis and starvation experiments

Hepa 1–6 cells were grown in complete medium (DME at pH 7.2; Sigma). C2C12 cells were grown to confluence in serum-containing medium and then differentiated in DME medium containing 2% horse serum for 2 d. Cells were incubated with either Hank's balanced salt solution (HBSS; without phenol red and glucose at pH 7.2; Sigma) or complete medium for 8 h. The starvation experiment of Figure 5A was done by incubating cells for 8 h in solutions containing DME, DME without amino acids (–aa), DME without glucose (–Glc), DME without vitamins (–Vit), or DME without amino acids, glucose, and vitamins (salts). The pH for all solutions was adjusted at 7.2. Insulin (1 µM to 25 nM) was added every 2 h when necessary. In all experiments in which insulin was added, bovine serum albumin (RIA grade; Sigma) was added at a final concentration of 0.125% to avoid insulin precipitation, in both plus and minus insulin samples. Cells were pelleted, and total RNA was obtained with trizol (Invitrogen). cDNA was synthesized with random hexamers and Superscript II (Invitrogen) or the iScript kit (Bio-Rad). qPCR was performed with the SybrGreen PCR Mix (QIAGEN or Bio-Rad) on an MJ Research Opticon-2 system. Actin was used as an internal control to normalize differences in loading for all *Drosophila* and mouse samples. All figures represent relative values of starved versus fed samples for each mRNA analyzed. Errors are the standard deviation of three measurements. All experiments have been performed at least two times. Primer sequences for each gene are available upon request. To starve flies, 2-d-old male flies were kept for 4 d on a diet with 10% sucrose and 2% yeast paste (control), or only with 10% sucrose (starved), dissolved in PBS plus 0.8% low melting agarose. Subsequently, flies were ground in trizol, and total RNA was extracted. RT–PCR in Figure 2B was performed as described above except that [α-^{32}P]dCTP was incorporated in the amplification mix, and samples were run in a denaturing 6% PAGE gel.

Autophosphorylation analysis of InR

S2 cells containing an inducible version of dFOXOA3 have been described before (Puig et al. 2003). A3 and S2 control cells were incubated for 24 h with 600 µM $CuSO_4$ to induce the expression of dFOXOA3. Equal numbers of cells were incubated in lysis buffer (50 mM HEPES at pH 7.6, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 100 mM NaF, 10 mM Na orthovanadate, protease inhibitors [Roche, 1 tablet every 50 mL of buffer]) for 1 h at 4°C with gentle shaking and then sonicated two times for 30 sec each with a Branson sonifier (450 model at output 1). After centrifugation to remove cell debris, protein concentration of the extracts was determined with a Bradford assay (Bio-Rad). Equal amounts of total protein were used for immunoprecipitation with protein A-coupled Sepharose beads (Pharmacia) that had been preincubated with polyclonal anti-dInR antiserum or with a control serum. Extracts (1 mL) were rotated for 2 h at 4°C, washed six times with washing buffer (20 mM HEPES at pH 7.6, 0.1% Triton X-100, 150 mM NaCl), and the beads were directly used in kinase assays.

For mInR purification C2C12, cells were grown in 9-cm plates

in DME plus 0.125% BSA with (fed) or without (fasted) 100 nM insulin for 12 h. Cells were scraped from the plate, pelleted, and resuspended in lysis buffer (same as above) and incubated with gentle shaking at 4°C for 1 h. Cells were sonicated as above, and cell debris was separated by centrifugation. Supernatants (1 mL) were added to Sepharose beads coupled with WGA (Vector Laboratories) and rotated at 4°C for 2 h. Beads were washed as above, and mInR was eluted from the beads by incubating them for 2 h at 4°C in wash buffer with 0.6 M N-acetyl-glucosamine (Sigma). mInR partially purified this way was directly used for kinase assays.

Sepharose beads containing dInR or partially purified mInR in WGA beads were directly used in kinase assays. InR was preincubated for 1 h at room temperature with or without 100 nM insulin in wash buffer in a final volume of 60 µL. Then, the kinase reaction was initiated by addition of 20 µL containing $MgCl_2$ (10 mM final conc), $MnCl_2$ (8 mM final conc), ATP (50 µM final conc), and 38 µCi of ^{32}P-ATP. After 10 min at room temperature, the reaction was stopped by addition of 40 µL of 3× SDS loading buffer containing DTT and by boiling for 5 min. Proteins were separated in 8% SDS-PAGE gels. Gels were dried and analyzed with a PhosphorImager.

InR autophosphorylation in vivo

Drosophila S2 or dFOXOA3 cells were incubated with 600 µM $CuSO_4$ to induce dFOXOA3 expression. Equal numbers of S2 or A3 cells were incubated with different concentrations of insulin (10^{-10} to 10^{-6}) for 6 min at 37°C in a final volume of 1 mL. Cells were rapidly pelleted, and the reaction was stopped by resuspending the cells in 100 µL of 1× SDS loading buffer and boiling for 5 min. Proteins were separated in 8% SDS-PAGE gels, and dInR autophosphorylation was analyzed by Western with the InR-P phospho-specific antibody (Tyr 1146) from Cell Signaling.

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