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Article

dDOR Is an EcR Coactivator that Forms a Feed-Forward Loop Connecting Insulin and Ecdysone Signaling

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Summary

Background: Mammalian *DOR* was discovered as a gene whose expression is misregulated in muscle of Zucker diabetic rats. Because no *DOR* loss-of-function mammalian models are available, we analyze here the in vivo function of *DOR* by studying flies mutant for *Drosophila DOR* (*dDOR*).

Results: We show that dDOR is a novel coactivator of ecdysone receptor (EcR) that is needed during metamorphosis. dDOR binds EcR and is required for maximal EcR transcriptional activity. In the absence of dDOR, flies display a number of ecdysone loss-of-function phenotypes such as impaired spiracle eversion, impaired salivary gland degradation, and pupal lethality. Furthermore, *dDOR* knockout flies are lean. We find that *dDOR* expression is inhibited by insulin signaling via FOXO.

Conclusion: This work uncovers dDOR as a novel EcR coactivator. It also establishes a mutual antagonistic relationship between ecdysone and insulin signaling in the fly fat body. Furthermore, because ecdysone signaling inhibits insulin signaling in the fat body, this also uncovers a feed-forward mechanism whereby ecdysone potentiates its own signaling via dDOR.

Introduction

Thyroid hormone receptor (TR) is an important regulator of development and metabolism in animals [1, 2]. TR is a type II nuclear hormone receptor (NR). It resides in the nucleus and binds DNA regardless of ligand binding, and it heterodimerizes with retinoid X receptor (RXR) [2]. In the absence of ligand, TR is complexed with corepressors to inhibit transcription, whereas in the presence of ligand, it binds coactivators and activates transcription [2, 3]. One recently discovered TR coactivator is DOR [4]. *DOR* was first identified as a gene that is downregulated in muscle of diabetic rats [4]. DOR was then shown to have two functions. It acts as a coactivator of thyroid hormone receptor $TR_{\alpha}1$, binding $TR_{\alpha}1$ and impacting its transcriptional activity [4]. Furthermore, DOR has a second life outside the nucleus, as a regulator of autophagy [5, 6]. Together, these data implicate DOR as a regulator of NR function and of metabolism. However, no DOR mutant animals have yet been reported, and the in vivo function of DOR remains to be studied.

We study here the *Drosophila* homolog of *DOR*. *Drosophila* has 18 nuclear receptors, including ecdysone receptor (EcR). EcR shares many commonalities with type II NRs, in that it heterodimerizes with the fly RXR homolog USP, binds DNA constitutively, complexes with either coactivators or corepressors depending on its state of ligand binding, and can form a functional complex with mammalian RXR [1, 7–10]. The EcR/USP complex senses and responds to the hormone 20-hydroxyecdysone (20E) to regulate developmental timing and metabolism. 20E triggers all developmental transitions, such as the molts from one larval stage to the next, and many events occurring during metamorphosis. These include termination of larval feeding, apoptosis, and elimination of larval salivary glands and larval fat body, as well as many morphological changes in tissues that will give rise to the adult fly [1, 7, 8]. Several EcR corepressors and coactivators have been identified and characterized, including Alien [11], SMRTER [12], bonus [13], Trithorax-related gene (TRR) [14], Taiman [15], and rigor mortis [16]. However the coactivator(s) of EcR required for proper pupal development and metamorphosis remain to be described.

Interestingly, crosstalk has recently come to light between ecdysone signaling and insulin signaling, which regulates the growth and metabolism of animals [17]. Ecdysone regulates insulin signaling and vice versa [18–20]. In particular, in the fat body of the fly, ecdysone signaling inhibits PI3K activity and thereby insulin signaling, suggesting an antagonistic relationship between these two hormonal signaling pathways [18, 21–24]. The molecular mechanisms underlying these regulatory events, however, are not fully understood.

Results

dDOR Encodes the *Drosophila* Homolog of Mammalian DOR

In order to study the function of DOR in an in vivo animal model, we searched the *Drosophila* genome for homologs of human *DOR* (*hDOR*). A BLAST search through all predicted *Drosophila* proteins with the sequence of hDOR yielded CG11347 as the top hit, which we rename *Drosophila* DOR (dDOR) (Figure S1, available online).

The *dDOR* locus is predicted to encode six different transcripts, giving rise to three different polypeptides (Figure 1A) [25]. The *-RA*, *-RB*, *-RD*, and *-RE* isoforms encode a 387 amino acid protein hereafter referred to as DOR_{long}, whereas the *-RC* isoform encodes a shorter protein, of 273 amino acids, which we refer to as DOR_{short} (Figures 1A and 1C). The *-RF* isoform encodes an even shorter protein similar to DOR_{short} but lacking 44 amino acids at the N terminus. While performing RT-PCR with oligonucleotides specific for the long isoform, we noticed the presence of two differently sized PCR products. Sequencing revealed that one of the products corresponded to the predicted "long" isoform. The second product corresponded to an unannotated isoform consisting of the "long" isoform plus a 90 bp extension of the third exon, resulting

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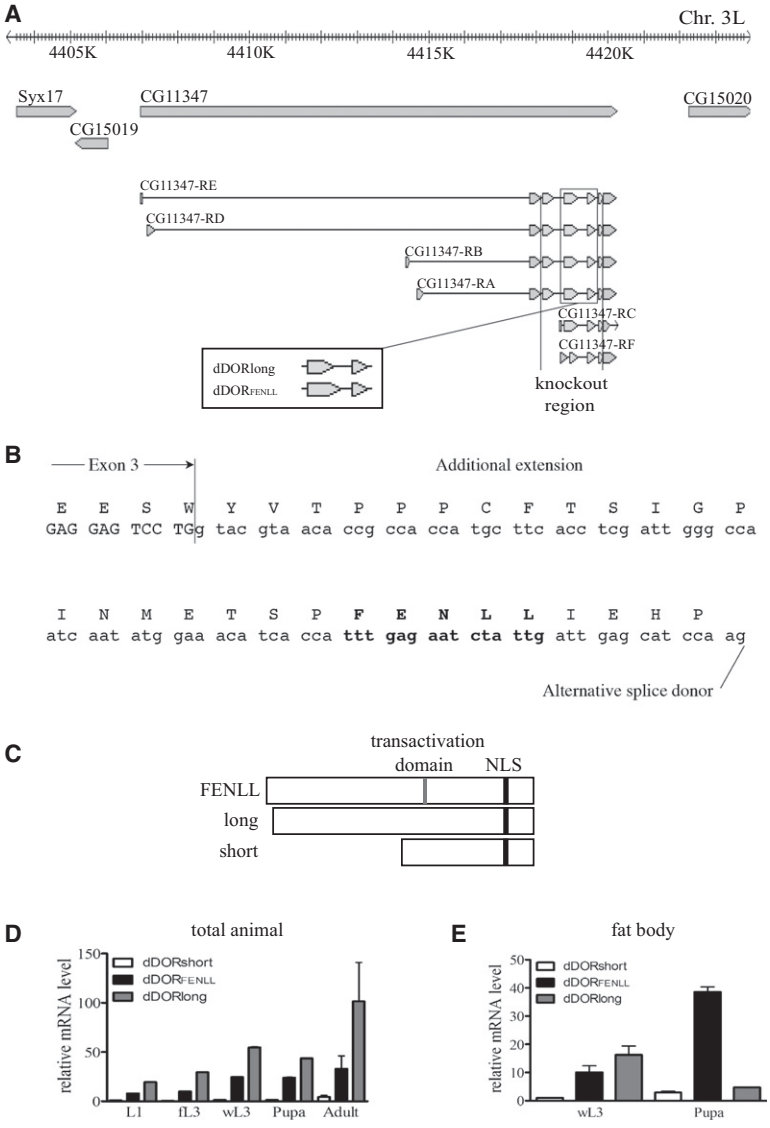

Figure 1. *dDOR* Gene Structure and Splice Isoforms

(A) Overview of the *dDOR* (*CG11347*) genomic region. *dDOR* codes for six splice variants (*-RA* to *-RF*) plus an additional, previously unannotated splice variant that we term $dDOR_{FENLL}$, containing an extended third exon. The knockout region, as indicated, encompasses the entire open reading frame of all isoforms.

(B) Additional nucleotide and amino acid sequence present in the splice variant with an extended third exon ($dDOR_{FENLL}$ isoform). This splice form encodes a protein containing a motif (in bold) similar to the LXXLL motif found in nuclear receptor cofactors.

(C) Schematic representation of the three dDOR protein isoforms: FENLL, long (RA, RB, RD, and RE), and short (RC). NLS denotes nuclear localization signal.

(D) mRNA expression levels of the three *dDOR* isoforms in the whole animal during development, measured by quantitative RT-PCR normalized to rp49.

(E) mRNA expression levels of the three *dDOR* isoforms in fat bodies of wandering third-instar larvae and pupae, measured by quantitative RT-PCR normalized to rp49.

Error bars represent standard error of the mean (SEM).

from use of an alternate splice donor (Figures 1A and 1B). As a result, 30 amino acids are inserted in the middle of the $dDOR_{long}$ protein. Contained in these 30 amino acids is the sequence FENLL (Figure 1B in bold), which is similar to the LXXLL nuclear-receptor-interacting motif found in nuclear receptor coactivators [3, 26]. This FENLL sequence aligns to the transactivation domain motif of human DOR (LEDLL) when the two proteins are aligned to each other (Figure S1 in bold). We refer to this isoform as $dDOR_{FENLL}$. The domain of $dDOR_{FENLL}$ surrounding the FENLL sequence has 75% identity and 85% homology to human DOR (Figure S1). The three isoforms of *dDOR* that we study in this manuscript are summarized schematically in Figure 1C.

In order to measure the relative abundance of the three isoforms in vivo, we performed quantitative RT-PCR with isoform-specific primers on RNA extracted from animals of various stages of development (Figure 1D). The most abundant isoform is the long one, followed by the FENLL isoform (roughly half the level of the long isoform), whereas the short isoform is expressed at comparatively low levels (Figure 1D). This relative expression of the three isoforms is also observed in fat body of wandering third-instar larvae whereas in fat body of early pupae the FENLL isoform strongly predominates (Figure 1E). Indeed, the FENLL isoform is highly enriched in fat body of early pupae when compared to the rest of the body (***t test < 0.0001, Figure S1B).

dDOR Knockout Flies Display Defects during Metamorphosis

In order to study dDOR function in vivo, we generated *dDOR* knockout flies by targeted homologous recombination, replacing the entire open reading frame of all the splice variants with the *mini-white* gene (Figure 1A) [27]. We backcrossed female flies heterozygous for the *dDOR* knockout to w^{1118} flies for four generations in order to obtain two fly stocks with similar genetic backgrounds, differing only by the presence or absence of the *dDOR* mutation. The resulting stocks will be referred to as *dDOR* knockout flies and control flies, respectively, and were used for all experiments described here. dDOR knockout flies had no detectable expression of *dDOR* mRNA as measured by quantitative RT-PCR (Figure 2A) or of dDOR protein (Figure 2B).

dDOR knockout larvae are viable, have no obvious defects, and pupate at almost the same time as control larvae (Figure 2C). A number of defects, however, become apparent during metamorphosis. The viability of *dDOR* knockouts drops significantly during metamorphosis, so that only 59% of animals eclose as adults, compared to 91% of controls (*t test = 0.02, Figure 2D). This viability defect, as well as other defects mentioned here, is rescued by expression of *dDOR* from a UAS transgene, showing that it is specific for *dDOR* loss of function. This will be discussed in more detail below. *dDOR* knockout animals also display defects in major ecdysone-triggered biological responses. *dDOR* knockouts have impaired anterior spiracle eversion, with 35% of *dDOR* knockout pupae displaying this phenotype (n = 53), compared to just 2% of control pupae (n = 51) (Figure 2E). This defect is not due to the mild delay in pupation of *dDOR* mutants, because the 35% of animals that do not evert their spiracles at pupation still display the same phenotype 48 hr after pupation and eventually die. Furthermore, destruction of larval

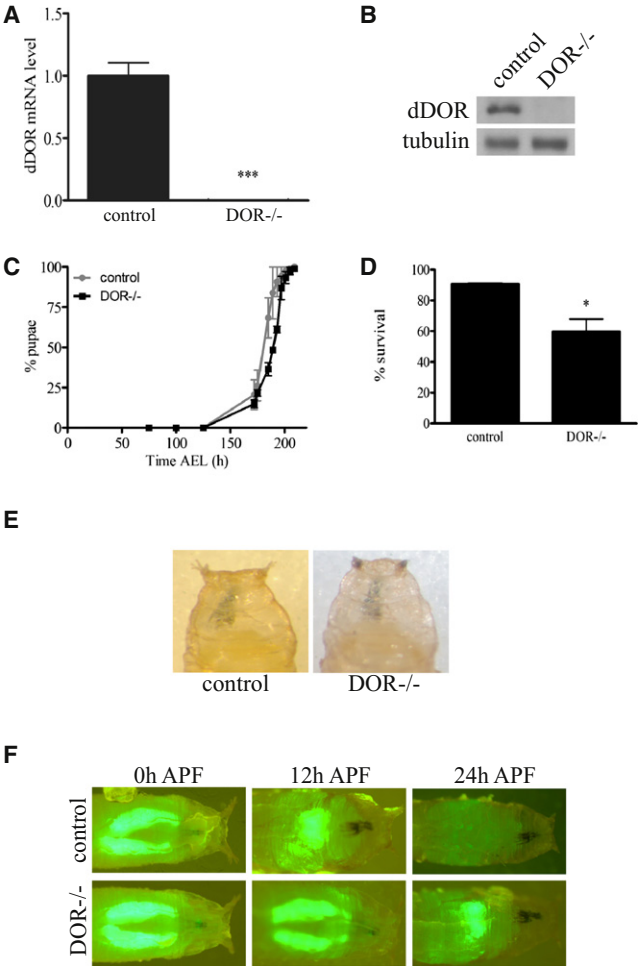

Figure 2. *dDOR* Knockout Flies Have Reduced Viability, Pupation Defects, and Impaired Ecdysone Signaling

(A and B) *dDOR* knockout animals have no detectable dDOR mRNA or protein.

(A) mRNA levels of all *dDOR* splice isoforms in wandering third-instar larvae measured by quantitative RT-PCR, normalized to rp49. Error bars represent SEM. *** t test = 0.001.

(B) Protein extracts from control and *dDOR* knockout wandering third-instar larvae probed with anti-dDOR and anti-tubulin antibody.

(C) *dDOR* knockout animals are not strongly delayed in development. Pupation curves show the percentage of pupated animals as a function of time after egg laying (AEL) for control animals (gray curve, n = 82) and *dDOR* knockout animals (black curve, n = 78). Error bars represent SEM.

(D) *dDOR* knockout flies have reduced viability. The percentage of live adults relative to collected first-instar larvae for animals of indicated genotypes is shown. Error bars represent SEM. * t test = 0.02.

(E) Representative images of defective anterior spiracle eversion, observed in 35% of *dDOR* knockout pupae.

(F) Larval salivary gland death during metamorphosis, observed via salivary-gland-specific GFP expression in control and *dDOR* knockout pupae, 12 hr and 24 hr after puparium formation (APF). Sixty-six percent of *dDOR* mutants display delayed degradation of salivary glands, as observed by residual GFP 24 hr APF.

salivary glands is impaired in *dDOR* knockouts. Using flies expressing GFP in the larval salivary glands (*esg-GAL4, UAS-GFP*), we followed the fate of the salivary glands in vivo during metamorphosis. Whereas larval salivary glands were completely removed in wild-type animals by 24 hr after pupation, 66% of *dDOR* knockouts still had visible GFP at this time (Figure 2F).

dDOR Knockouts Have Impaired Ecdysone Signaling

Human DOR functions as a transcriptional coactivator of the TRα1 thyroid hormone nuclear receptor, which shares some properties with the *Drosophila* ecdysone receptor. Furthermore, ecdysone plays a fundamental role during metamorphosis, regulating for instance spiracle eversion and salivary gland degradation. Therefore, we asked whether *dDOR* mutants have defects in ecdysone signaling. At the end of larval third instar, a pulse of ecdysone induces larvae to wander out of their food and to begin pupating [1]. This correlates with induction of several direct transcriptional targets of the ecdysone receptor including *Eip75B* and the *broad complex* (*BR-C*) [1]. We performed a time-course analysis of gene expression in carefully staged animals, from 24 hr prior to pupation to 12 hr after pupation, measuring mRNA levels of *Eip75B isoform A* (*E75A*) and *BR-C* by quantitative RT-PCR (Figure S2). As expected from previous studies [28], both *BR-C* and *E75A* expression increased dramatically in control animals during the "wandering" stage, 0–6 hr prior to pupation (Figures S2A and 2B, black lines). In contrast, induction of both genes was severely blunted in *dDOR* knockouts (Figures S2A and 2B, gray lines). A similar defect in induction of EcR target gene expression could be observed when measuring all *Eip75B* isoforms combined (*E75*) (Figure S2C) or another direct EcR target gene, *E74* (Figure S2D). In contrast, two genes that are not direct "early response" genes of ecdysone signaling, β*FTZ-F1* and *DHR3*, were less affected (Figures S2E and S2F). To quantify the induction of *E75* and *BR-C* in response to the ecdysone pulse that causes larvae to wander out of the food, we focused on two time points: "feeding" animals 24 hr prior to pupation (before the ecdysone pulse) versus "wandering" animals 0–6 hr prior to pupation (after the ecdysone pulse). In control animals, expression of *E75* and *BR-C* was upregulated roughly 20-fold in wandering animals compared to feeding animals (Figure 3A). In contrast, in *dDOR* knockout animals, induction of both EcR targets was significantly impaired (**t test < 0.001, Figure 3A).

To test whether *dDOR* mutant tissue has an impaired ability to sense and respond to ecdysone hormone, we explanted fat bodies from control and *dDOR* knockout animals, treated them with exogenously supplied ecdysone (1 μM 20-hydroxyecdysone, 20E) for 4 hr, and then measured induction of *E75* and *BR-C* by quantitative RT-PCR. Expression of both *E75* and *BR-C* was strongly induced in fat body explants from control animals upon ecdysone treatment (Figure 3B, white bars). In comparison, induction of *E75* and *BR-C* was impaired in fat body explants from *dDOR* knockouts (*t test = 0.01; **t test = 0.003; Figure 3B, black bars), indicating that intracellular ecdysone signaling is defective in *dDOR* mutant tissue. We also asked whether dDOR is required for effective ecdysone signaling in Kc167 cells, which are known to be ecdysone responsive [29, 30]. When Kc cells were treated with 20E (1 μM) for 12 hr, expression of both *E75* and *BR-C* was strongly induced (Figure 3C, white bars). In comparison, induction of *E75* and *BR-C* was significantly blunted in Kc cells in which *dDOR* expression had been knocked down by dsRNA treatment (t test = 0.001 and 0.01, respectively, Figure 3C, black bars). To test whether the defect in *E75* and *BR-C* induction is due specifically to reduced function of the ecdysone receptor complex, we made use of a luciferase reporter containing tandem repeats of the ecdysone receptor response element (EcRE) [30]. Kc cells transfected with the EcRE reporter had markedly increased luciferase activity upon treatment with 20E

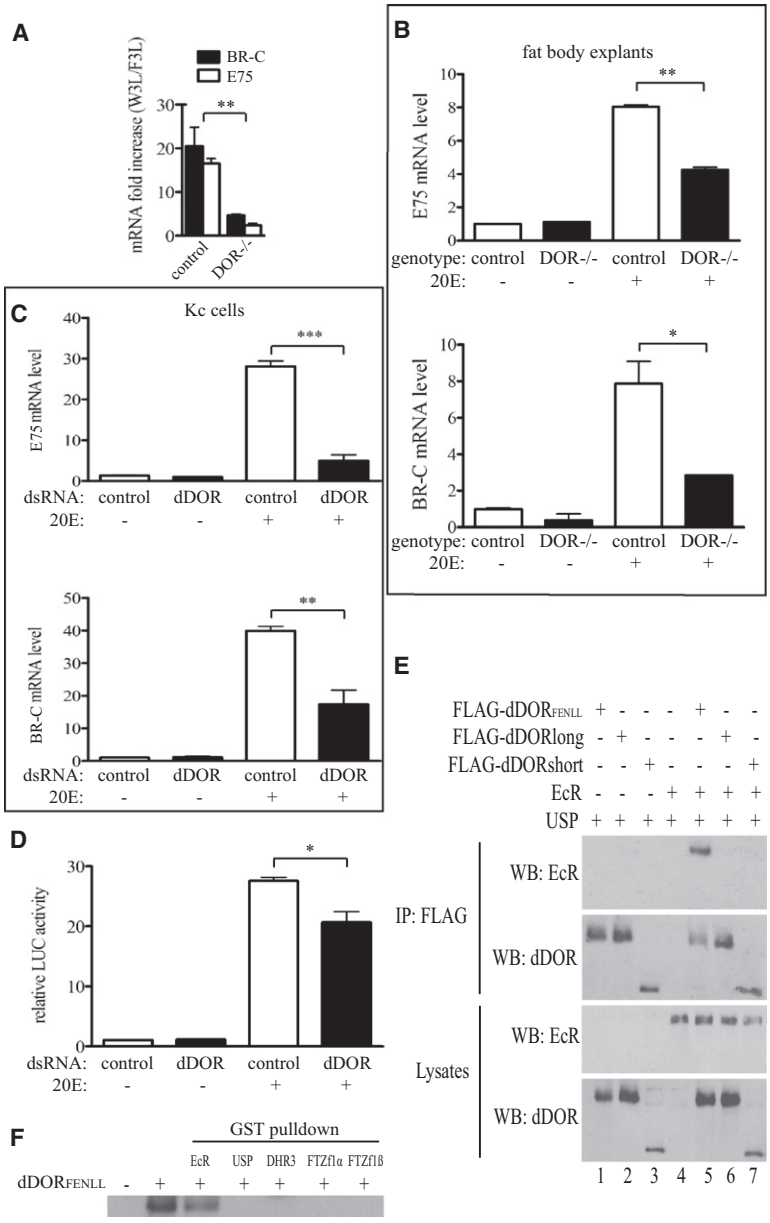

Figure 3. dDOR Binds Physically to EcR and Potentiates EcR Transcriptional Activation

(A) *dDOR* knockout animals have impaired ecdysone signaling. Fold induction in expression of two direct target genes of ecdysone receptor, *broad* ("*BR-C*," black bars) and *Eip75B* (all isoforms, "*E75*," white bars). Induction is measured in wandering third-instar larvae (0–6 hr prior to pupation) compared to feeding third-instar larvae (24 hr prior to pupation), by quantitative RT-PCR relative to *rp49*, for animals of indicated genotypes. Error bars represent SEM. **t test < 0.001.

(B) *dDOR* mutant tissue is impaired in its response to exogenously applied ecdysone. Fat body explants from control and *dDOR* knockout wandering L3 larvae were cultured in Grace's insect medium in the presence or absence of 20-hydroxyecdysone (20E) (1 μM for 4 hr), and expression of the EcR target genes *E75* (top panel) and *BR-C* (bottom panel) was measured by quantitative RT-PCR relative to rp49. Error bars represent SEM. *t test = 0.01; **t test = 0.003.

(C) Knockdown of *dDOR* in Kc167 cells causes an impaired transcriptional response to ecdysone. Kc cells treated with control dsRNA or dsRNA against *dDOR* were cultured in the presence or absence of 20E (1 μM, 12 hr), and expression of the EcR target genes *E75* (top panel) and *BR-C* (bottom panel) was measured by quantitative RT-PCR relative to rp49. Treatment of Kc cells with dsRNA against *dDOR* reduced *dDOR* expression at the RNA level by 58%, measured by quantitative RT-PCR (not shown). Error bars represent SEM. **t test = 0.01; *** t test = 0.001.

(D) Knockdown of *dDOR* in Kc167 cells impairs induction of an ecdysone-receptor-responsive reporter. Kc167 cells, transfected with an EcRE-dependent firefly luciferase reporter, together with a constitutive renilla luciferase construct for normalization, were stimulated with 20E (10 μM) for 12 hr. Relative firefly/renilla luciferase activity is indicated. Error bars represent SEM. *t test = 0.02.

(E) EcR interacts physically with the FENLL isoform of dDOR, but not the short or long isoforms of dDOR lacking the LXXLL-like motif. S2 cells were transfected with constructs for expression of EcR (RB isoform), USP, and various isoforms of dDOR, as indicated, and were treated with 10 μM 20E for 12 hr. Immunoprecipitation of the various dDOR isoforms was performed with anti-FLAG antibody. Lysates and IPs were probed with anti-EcR and anti-dDOR antibodies.

(F) $dDOR_{FENLL}$ interacts specifically with EcR. GST-tagged *Drosophila* nuclear receptors (EcR, USP, DHR3, FTZf1α, and FTZf1β) were expressed in bacteria and purified with Gluthathione-S-sepharose beads. Beads containing equal amounts of the various recombinant nuclear receptors were incubated with lysates of S2 cells expressing $dDOR_{FENLL}$. S2 lysates and GST pull-downs were probed with anti-dDOR antibody.

(Figure 3D, white bars). This induction was significantly impaired when cells were treated with dsRNA against *dDOR* (*t test = 0.02, Figure 3D, black bars), indicating that depletion of dDOR blunts the ability of EcR to activate transcription via EcR response elements.

The FENLL Isoform of dDOR, but Not the Other Isoforms of dDOR, Binds EcR

The data presented above indicate that dDOR is required for the ecdysone receptor complex to function properly. Because dDOR has a nuclear hormone coactivator-like motif, we tested whether dDOR binds physically to EcR. We expressed FLAG-tagged versions of the three dDOR isoforms (FENLL, long, and short) together with EcR and its partner USP in S2 cells and precipitated dDOR with anti-FLAG antibody. Although we could not detect EcR in the immunoprecipitates of $dDOR_{short}$ or $dDOR_{long}$ (Figure 3E, lanes 6 and 7), we could readily detect EcR in the immunoprecipitate of the FENLL form of dDOR (Figure 3E, lane 5). The fact that EcR coimmunoprecipitates only with $dDOR_{FENLL}$ but not the other two isoforms of dDOR highlights the specificity of this interaction. Particularly striking is the comparison between the $dDOR_{FENLL}$ and $dDOR_{long}$ immunoprecipitates, because the two proteins differ by only 30 amino acids containing the FENLL motif. Specific mutation of the amino acids FENLL to AENAA reduced binding of the FENLL isoform to EcR by 59% (Figure S2H).

In order to test the specificity of binding of dDOR to EcR, we performed a GST-pull-down assay with a panel of recombinantly expressed *Drosophila* nuclear receptors (EcR, USP, DHR3, FTZf1α, and FTZf1β) (Figure 3F). Whereas beads containing GST-EcR readily coimmunoprecipitated dDOR from lysates of S2 cells expressing dDOR-FENLL, beads containing equal amounts of the other nuclear receptors did not (Figure 3F and Figure S2I).

We previously reported that human DOR has a dual function, being involved both in transcriptional coactivation in the nucleus and in regulation of autophagy in the cytoplasm by binding to the human autophagy protein Atg8/GATE16 [5]. Therefore, we tested whether dDOR can bind the *Drosophila* homologs of Atg8, Atg8a, and Atg8b. We expressed the three dDOR isoforms (FENLL, long, and short) together with HA-tagged Atg8a and Atg8b in S2 cells and immunoprecipitated the Atg proteins via their HA tags. Both the FENLL and long isoforms of dDOR bound the Atg proteins, but the short isoform of dDOR did not (Figure S3). This indicates that the FENLL-containing domain of dDOR is not required for binding to the Atgs, whereas it is required for binding to EcR. This also suggests that the long isoform of DOR, which binds the Atg proteins but not EcR, might be dedicated to regulating autophagy.

Ecdysone Receptor Binding by dDOR Is Required for Proper Ecdysone Signaling and Animal Viability

The differential ability of $dDOR_{FENLL}$ and $dDOR_{long}$ to bind to the ecdysone receptor allowed us to test which defects of the *dDOR* knockout animals occur as a consequence of defective ecdysone signaling. We generated animals homozygous for the *dDOR* knockout mutation in which either the $dDOR_{FENLL}$ or the $dDOR_{long}$ isoforms were ubiquitously expressed by using a *UAS* transgene together with the *tubulin-GAL4* driver. This essentially generates animals in which *dDOR* expression is replaced with expression of only one specific *dDOR* splice isoform. As negative controls, we also generated flies homozygous for the *dDOR* knockout mutation carrying either of the *UAS* constructs alone (which by themselves do not express) or only the *Tubulin-GAL4* driver. Unlike *dDOR* knockouts, *dDOR* homozygous knockout animals expressing $dDOR_{FENLL}$ were able to properly upregulate expression of the EcR target genes *E75* and *BR-C* during the wandering third-instar stage (***t test < 0.0001, Figure 4A, columns 13 and 14). In contrast, the $dDOR_{long}$ isoform was unable to rescue induction of these EcR target genes (Figure 4A, columns 11 and 12). This is in agreement with our coimmunoprecipitation results showing that the FENLL isoform binds EcR whereas the long isoform does not (Figure 3E), and it indicates that binding of dDOR to EcR is required for proper ecdysone signaling in *Drosophila*. Expression of $dDOR_{FENLL}$ also rescued the viability defects of *dDOR* knockouts (*t test = 0.03, Figure 4B, column 7) whereas $dDOR_{long}$ did not (Figure 4B, column 6), demonstrating that the mortality of *dDOR* knockouts during metamorphosis is due to impaired ecdysone signaling. We noted that in explanted fat bodies from *dDOR* knockouts, induction of EcR target genes in response to exogenously applied 20E was blunted, but not completely abrogated (Figure 3B). This indicates that although dDOR plays an important role in potentiating the transcriptional activity of the EcR complex, EcR is still able to weakly induce target gene expression in the absence of dDOR. This raised the possibility that feeding 20E to *dDOR* knockouts might compensate for the blunted activity of EcR in these flies, thereby rescuing their lethality. Indeed, feeding 20E to *dDOR* knockout animals was able to significantly rescue their viability defect (***t test = 0.0003, Figure 4C).

dDOR-Mediated Regulation of Ecdysone Signaling Is Required for Proper Animal Metabolism

Expression of *DOR* in rats was reported to be aberrant upon development of diabetes [4]. Either this occurs as

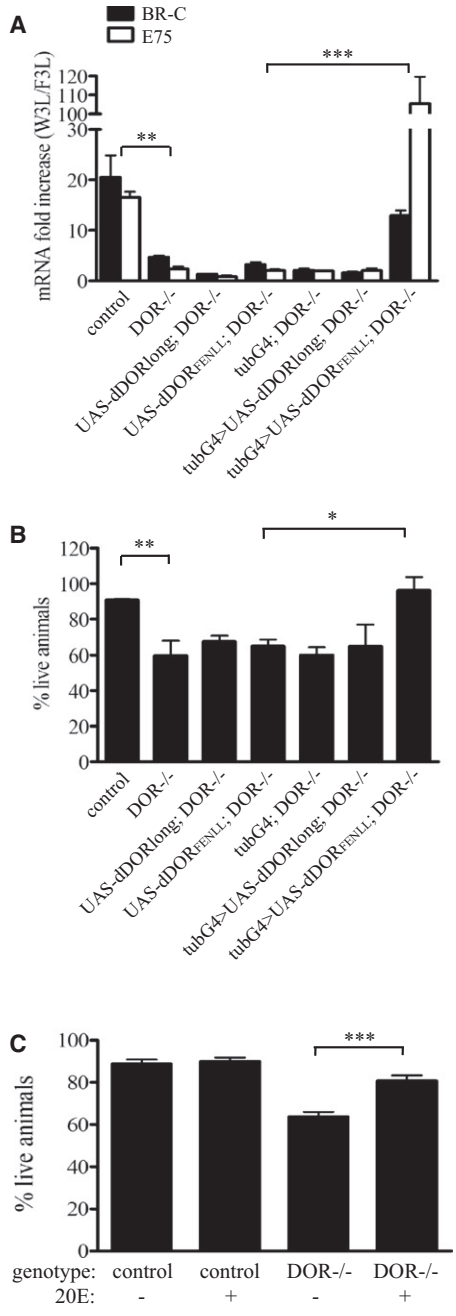

Figure 4. Rescue of *dDOR* Mutant Phenotypes by Isoform-Specific Expression of *dDOR*

(A) Impaired ecdysone signaling in *dDOR* knockout animals is rescued specifically by the $dDOR_{FENLL}$ isoform. Fold induction in expression of two direct target genes of ecdysone receptor, broad ("*BR-C*," black bars) and Eip75B ("*E75*," white bars). Induction is measured in wandering third-instar larvae (0–6 hr prior to pupation) compared to feeding third-instar larvae (24 hr prior to pupation), by quantitative RT-PCR relative to *rp49*, for animals of indicated genotypes. Error bars represent SEM. **t test < 0.001; *** t test < 0.0001.

(B) Reduced viability of *dDOR* knockout flies is rescued specifically by the $dDOR_{FENLL}$ isoform. The percentage of live adults relative to collected first-instar larvae for animals of indicated genotypes is shown. Error bars represent SEM. *t test = 0.03; **t test = 0.02.

(C) Ecdysone feeding rescues the viability of *dDOR* knockout animals. The percentage of live adults relative to collected first-instar larvae for control and *dDOR* knockout animals either fed 0.5 mg/ml 20E or not, as indicated, is shown. Error bars represent SEM. ***t test = 0.0003.

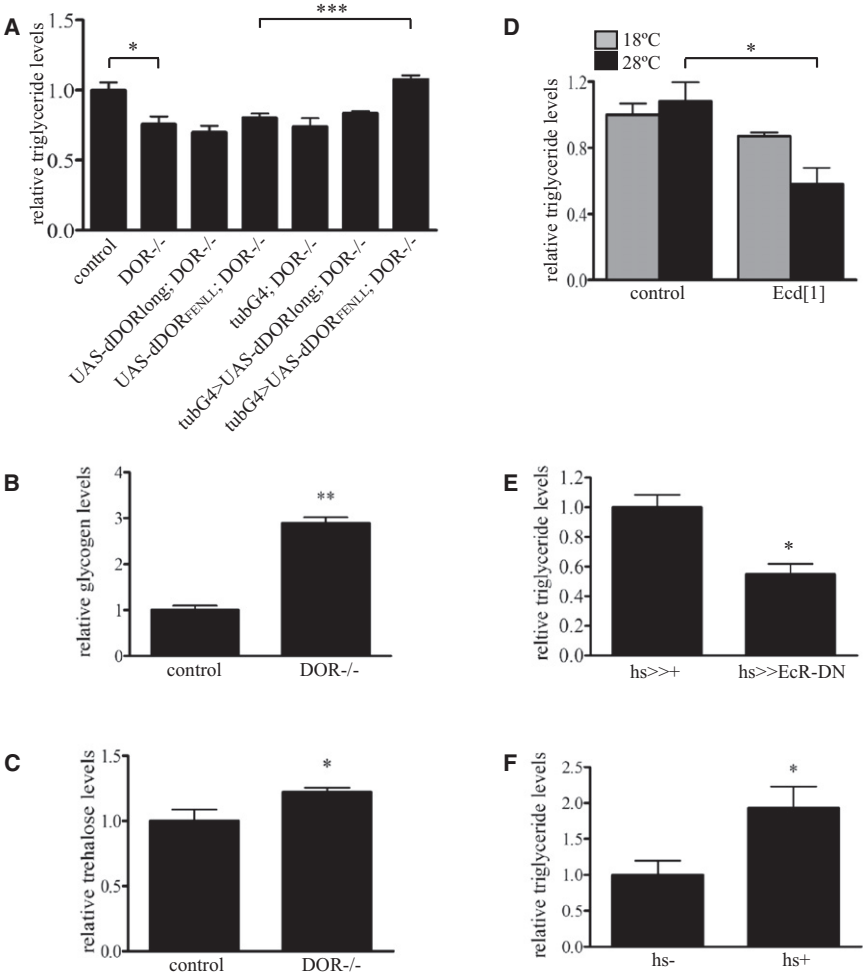

Figure 5. *dDOR* Knockout Flies Are Lean

(A) *dDOR* knockout flies are lean. This phenotype is rescued specifically by the $dDOR_{FENLL}$ isoform. Relative triglyceride levels, normalized to total body protein, for 1-day old adult flies of indicated genotypes, were measured. Error bars represent SEM. *t test = 0.015; ***t test = 0.0004.

(B) dDOR knockout flies have increased glycogen. Relative glycogen levels, normalized to total body protein, for 1-day-old control and knockout adult flies, were measured. Error bars represent SEM. **t test = 0.001.

(C) dDOR knockout flies have increased trehalose levels. Error bars represent SEM. *t test = 0.04.

(D) *ecd[1]* mutants have reduced total body triglycerides at semirestrictive temperature. Control and *ecd[1]* homozygous larvae were grown at 18°C and then shifted to 28°C at the wandering third-instar stage (black bars) or reared continuously at 18°C (gray bars). Relative triglyceride levels, normalized to total body protein, of the resulting 1-day old adults were measured. Error bars represent SEM. *t test = 0.03.

(E) Flies expressing a dominant-negative version of EcR have reduced total body triglycerides. Flies carrying either heat shock-GAL4 alone (hs > > +) or heat shock-GAL4 with UAS-EcR [W650A] (hs > > EcR-DN) were heat shocked (25 min, 38°C) at wandering third-instar stage to induce expression. Relative triglyceride levels, normalized to total body protein, of the resulting 1-day old adults were measured. Error bars represent SEM. *t test = 0.01.

(F) *EcR-B2*-overexpressing flies are fat. Larvae carrying a transgene for heat-shock-inducible *EcR* expression (*hs-EcR-B2*) were grown at 25°C and either not heat shocked ("hs-") or heat shocked during feeding and wandering third-instar larval stages (37°C, 45′) to induce *EcR-B2* expression ("hs+"). Relative triglyceride levels, normalized to total body protein, for resulting 1-day-old adults were measured. Error bars represent SEM. *t test = 0.04.

a consequence of the diabetes, or it plays a causative role in development of the disease. To distinguish these two possibilities, animals lacking DOR activity need to be analyzed to test whether modulation of DOR activity leads to metabolic consequences. With this in mind, we asked whether *dDOR* knockout flies have metabolic defects. We measured total body triglycerides, normalized to total body protein, and found that *dDOR* knockouts are lean, containing 25% less fat than controls (*t test = 0.015, Figure 5A). *dDOR* knockouts also had significantly increased glycogen stores (**t test = 0.001, Figure 5B) and circulating trehalose levels (*t test = 0.04, Figure 5C) compared to controls, indicating that dDOR regulates metabolic balance. Focusing on the triglyceride phenotype, we found that it was rescued by expression of $dDOR_{FENLL}$, but not of $dDOR_{long}$ (***t test = 0.0004, Figure 5A, columns 6 and 7), indicating that the leanness of *dDOR* mutants results from impaired ecdysone signaling.

In order to test the metabolic consequences of modulating ecdysone signaling during pupal development, we performed three experiments. First, to reduce ecdysone signaling during pupal development, we employed the widely used *ecdysoneless[1]* mutation (*ecd[1]*), which is a temperature-sensitive mutation that disrupts ecdysone production at the restrictive temperature of 29°C [31]. We reared control and *ecd[1]* mutant animals at permissive temperature (18°C) until they started wandering and then shifted the animals to the semirestrictive temperature of 28°C . This allowed animals to survive to adulthood but reduced their ecdysone signaling, as could be ascertained by reduced levels of the EcR target genes *BR-C* and *E75* in the *ecd[1]* mutants (Figure S4A). At 1 day of adult age, we measured the fat of these *ecd[1]* and control animals and found that *ecd[1]* animals were 46% leaner than their controls (*t test = 0.03, Figure 5D, black bars). In contrast, *ecd[1]* and control animals reared continuously at permissive temperature had no significant difference in fat levels (Figure 5D, gray bars). This suggests that reduced ecdysone signaling during pupal development leads to leaner adults, similar to what we observe in *dDOR* knockouts. As a second approach to decreasing ecdysone signaling, we created flies carrying a heat-shock-inducible dominant-negative EcR construct (heat shock-GAL4, UAS-EcR[W650A]) ("hs > > EcR-DN"). These animals, as well as control animals carrying only heat shock-GAL4 ("hs > > +"), were heat shocked at wandering L3 (25 min, 38°C). These heat-shock conditions allowed hs > > EcR-DN animals to survive to adulthood but reduced their ecdysone signaling, as could be ascertained by reduced levels of the EcR target genes *BR-C* and *E75* 4 hr after heat shock (Figure S4B). At 1 day of adult age, we measured the

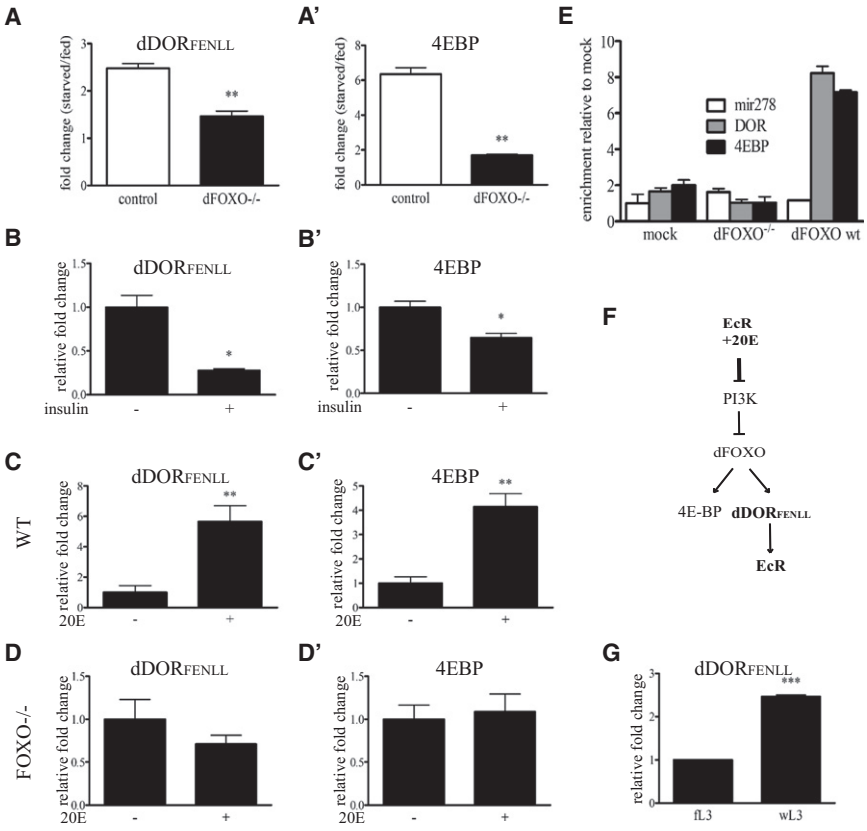

Figure 6. *dDOR$_{FENLL}$* Expression Is Regulated Directly by FOXO, Forming a Feed-Forward Regulatory Loop between Insulin and Ecdysone Signaling

(A and A′) *dDOR$_{FENLL}$* expression is upregulated in a FOXO-dependent manner upon nutrient withdrawal in fat body of third-instar larvae. Control (white bars) or *FOXO* mutant larvae (black bars) were either fed or deprived of food for 18 hr. Expression of *dDOR$_{FENLL}$* (A) and *4E-BP* (a direct FOXO target, A′) in adipose tissue was measured by quantitative RT-PCR, normalized to *rp49*. Fold induction of expression in starved relative to fed larvae is indicated. Error bars represent SEM. **t test < 0.01.

(B and B′) Insulin signaling represses *dDOR-FENLL* expression in fat bodies. *dDOR$_{FENLL}$* (B) and *4E-BP* (B′) mRNA levels in wandering third-instar larvae fat bodies treated in the presence or absence of 5 μg/ml insulin for 4 hr in Grace's insect medium were measured by quantitative RT-PCR relative to *rp49*. Error bars represent SEM. *t test < 0.03.

(C and C′) *dDOR$_{FENLL}$* and *4EBP* expression increase in fat body explants treated with ecdysone. Fat bodies of third-instar larvae were cultured in Grace's insect medium in the presence or absence of 20E (10 ug/ml for 4 hr), and expression of *dDOR$_{FENLL}$* (C) and *4E-BP* (C′) was measured by quantitative RT-PCR relative to *rp49*. Note the different y axis scale in (D) and (D′) versus (C) and (C′). Error bars represent SEM. **t test < 0.01.

(D and D′) *dDOR$_{FENLL}$* and *4EBP* expression do not increase in fat body explants of *FOXO* mutants treated with ecdysone. Fat bodies of third-instar-larvae *FOXO$^{21/25}$* mutants were treated as in (C), and expression of *dDOR$_{FENLL}$* (D) and *4E-BP* (D′) was measured by quantitative RT-PCR relative to *rp49*. Error bars represent SEM.

(E) FOXO binds the *dDOR* promoter. Quantification by qPCR of genomic DNA in ChIP material from wild-type animals with preimmune serum ("mock," a negative control), from *FOXO* mutant animals with anti-FOXO antibody ("dFOXO$^{-/-}$," a negative control), and from wild-type animals with anti-dFOXO antibody ("dFOXO wt"). Promoter regions assayed were those of *4E-BP* (a direct FOXO target, black bars), *mir-278* (negative control, white bars), and *dDOR* (gray bars).

(F) Schematic representation of the feed-forward regulatory mechanism comprising ecdysone signaling, insulin signaling, and dDOR$_{FENLL}$. In the fat body, ecdysone signaling blocks insulin signaling, thereby activating FOXO. FOXO then induces expression of dDOR$_{FENLL}$, which potentiates ecdysone receptor signaling.

(G) *dDOR$_{FENLL}$* expression is induced from feeding to wandering third-instar larvae. *dDOR$_{FENLL}$* mRNA levels measured by quantitative RT-PCR relative to *rp49* for feeding ("fL3") and wandering ("wL3") third-instar larvae. Error bars represent SEM. ***t test < 0.0001.

fat of hs > > EcR-DN and control animals and found that hs > > EcR-DN animals were 45% leaner than controls (*t test = 0.01, Figure 5E), consistent with the results obtained with *ecd[1]* animals. Finally, as a third complementary approach, we increased ecdysone signaling during pupal development to test the effect on animal metabolism. Flies with heat-shock-inducible expression of the ecdysone receptor (*hs-EcR-B2*) were heat shocked at the end of larval development and compared to animals of the same genotype that were not heat shocked. We first tested whether *EcR* induction increased ecdysone signaling by measuring mRNA levels of *BR-C* and *E75* in the resulting 1-day-old adults, and we found that they were indeed elevated relative to uninduced animals (Figure S4C). We then measured triglyceride levels in the resulting 1-day-old adults and found that animals with increased ecdysone signaling were almost twice as fat as the uninduced animals (*t test = 0.04, Figure 5F). In sum, both the ecdysone gain-of-function and loss-of-function experiments indicate that ecdysone signaling promotes adiposity during pupal development. This is in agreement with the differential ability of dDOR$_{FENLL}$ and dDOR$_{long}$ to rescue the leanness of *dDOR* knockout animals (Figure 5A), considering that dDOR$_{FENLL}$ potentiates EcR signaling but dDOR$_{long}$ does not (Figure 4A). Nonetheless, it is possible that dDOR$_{FENLL}$ rescues the leanness of *dDOR* knockouts by interacting with other unknown factors.

dDOR Is a Direct Target of the FOXO Transcription Factor

Because expression of human *DOR* is misregulated, via an unknown mechanism, in rats upon development of diabetes, we asked whether expression of *Drosophila DOR* is also regulated by nutritional conditions. Given that the FENLL isoform of dDOR is responsible for the metabolic defects of *dDOR* mutants (Figure 5A), we focused our attention on the FENLL isoform. We either fasted or fed third-instar larvae for 18 hr and then assayed *dDOR$_{FENLL}$* mRNA levels in fat body by quantitative RT-PCR. When control larvae were fasted, *dDOR$_{FENLL}$* expression in fat body increased > 2-fold (Figure 6A, white bar). One important signaling pathway that is inhibited upon fasting is insulin [32]. We therefore asked whether *dDOR$_{FENLL}$* expression is inhibited by insulin, because this would explain its upregulation upon fasting. We treated explanted fat bodies in the presence or absence of 5 μg/ml insulin and assayed *dDOR$_{FENLL}$* expression levels by

quantitative RT-PCR. In the presence of insulin, $dDOR_{FENLL}$ expression decreased by 73% (*t test = 0.03, Figure 6B). $dDOR_{FENLL}$ expression levels also decreased by 59% in S2 cells treated with 1 μM insulin for 2 hr (**t test = 0.006, Figure S5A).

One transcription factor mediating much of the transcriptional output of the insulin pathway is FOXO [32, 33]. FOXO activity is suppressed by insulin signaling [34]. We tested whether regulation of $dDOR_{FENLL}$ expression is mediated by FOXO by studying animals containing the $FOXO^{21/25}$ null allele combination. We starved $FOXO^{21/25}$ mutants and found that the fasting-induced upregulation of $dDOR_{FENLL}$ expression in fat body was strongly impaired (**t test = 0.005, Figure 6A, black bar), indicating that this transcriptional regulation is FOXO dependent. The transcriptional regulation of $dDOR_{FENLL}$ is analogous to that of a canonical FOXO target gene, *4E-BP*. *4E-BP* expression is suppressed by insulin in vivo in fat bodies (*t test = 0.01. Figure 6B′) and in S2 cells (**t test = 0.006, Figure S6A′) and increases in vivo in fat body upon fasting of wild-type animals (Figure 6A′, white bar) but does not increase upon fasting of *FOXO* mutant animals (**t test = 0.006, Figure 6A′, black bar). We therefore asked whether *dDOR* is also a direct transcriptional target of FOXO. In *Drosophila*, FOXO targets sites are preferentially located within 1 kb of the target promoter [32]. We screened the *dDOR* promoter region and found a perfect consensus FOXO binding site (GTAAACAA) 230 nt upstream of the transcription start site of the *–RA* and *–RB* transcripts. To test whether FOXO binds this site in vivo, we performed chromatin immunoprecipitation (ChIP) of endogenous FOXO from third-instar larvae. We performed two negative controls: a mock ChIP using preimmune serum from wild-type animals, and a ChIP using anti-FOXO antibody [35] from $FOXO^{21/25}$ null mutant animals (Figure 6E) [36]. Quantitative PCR (qPCR) on the immunoprecipitated material revealed that the promoter region of *4E-BP*, an established direct target of FOXO [35, 37], was strongly enriched in the FOXO ChIP from wild-type animals, but not in the negative controls (Figure 6E, black bars). Strikingly, the promoter region of *dDOR-RA/B* was also strongly enriched in the FOXO ChIP (Figure 6E, gray bars) but not in the negative controls, indicating that FOXO binds the *dDOR* promoter in vivo. As a negative control, the genomic region of *mir-278* was not enriched in the FOXO ChIP (Figure 6E, white bars). Together, these data indicate that expression of $dDOR_{FENLL}$ is inhibited by insulin signaling as a direct target of FOXO (Figure 6F), and identify a molecular mechanism by which insulin signaling inhibits ecdysone signaling in the fat body. Because dDOR is involved in linking nutrient signaling to EcR signaling, we tested whether *dDOR* mutants have impaired fitness upon nutrient deprivation. Upon removal of food (but not water), *dDOR* knockout animals died more rapidly than controls (Figure S5B).

dDOR Forms a Feed-Forward Regulatory Loop of Ecdysone Signaling

Insulin signaling in the fat body is inhibited by ecdysone signaling [18, 21–23]. Activation of the ecdysone pathway in vivo in the fat body causes a reduction in PI3K activity and increased FOXO activity [18, 23]. Because our data indicate that $dDOR_{FENLL}$ is a direct FOXO target, this raises the possibility that $dDOR_{FENLL}$ is part of a feed-forward mechanism whereby ecdysone, via insulin signaling, amplifies expression of components required for its own signaling (Figure 6F). We tested this possibility by treating explanted fat bodies in the presence or absence of 20E for 4 hr. In the presence of 20E, expression of the two FOXO targets *4E-BP* and $dDOR_{FENLL}$ both increased, as assayed by quantitative RT-PCR (**t test < 0.01, Figures 6C and 6C′). As expected, in fat bodies explanted from FOXO mutants, treatment with 20E no longer increased expression of either $dDOR_{FENLL}$ or *4E-BP* (Figures 6D and 6D′). Consistent with this notion, expression of $dDOR_{FENLL}$ also increases in vivo when third-instar larvae transition from feeding to wandering as a consequence of an ecdysone pulse (***t test < 0.0001, Figure 6G). These data indicate that ecdysone signaling increases expression of $dDOR_{FENLL}$ in a FOXO-dependent manner. Because $dDOR_{FENLL}$ is required for efficient ecdysone signaling, this forms a feed-forward regulatory loop (Figure 6F).

Discussion

We discover here a novel coactivator of the ecdysone receptor, dDOR, which plays an important role during metamorphosis. Clearly not all EcR functions are impaired in *DOR* mutants. For instance, we see very little lethality during larval stages of development, indicating that larval molts are occurring properly. It is possible that different EcR coactivators are important for different aspects of EcR signaling, for instance with rigor mortis playing an important role in the regulation of larval molts [16]. Alternatively, because induction of EcR target genes is reduced but not completely eliminated in *dDOR* knockout animals, this could reflect the differential sensitivity of various biological processes to the degree of EcR activation. Future work may shed more light on this issue. Interesting in this context is that we were able to rescue the lethality of *DOR* knockouts by feeding 20E. This suggests that either *DOR* knockouts also have low ecdysone titers due to impaired expression of *E75A*, which is involved in an ecdysone feed-forward production pathway [38], or because the elevated ecdysone titers achieved by supplying exogenous 20E allow other coactivators to compensate for *DOR* loss of function.

This work identifies a new link between ecdysone signaling and insulin signaling. It was previously known that ecdysone signaling inhibits insulin signaling in the fat body [18, 21–23]. We show here that, conversely, insulin signaling also inhibits ecdysone signaling. When insulin signaling is high, FOXO activation is low and *dDOR* expression is low. Conversely, when insulin signaling drops, this allows FOXO to become active, resulting in elevated levels of *dDOR* expression and maximal activation of EcR target genes. In sum, we find that there is a mutual antagonistic relationship between insulin signaling and ecdysone signaling in the fat body, possibly creating a system with two equilibrium states—high ecdysone/low insulin and low ecdysone/high insulin. This makes biological sense because insulin plays an anabolic role in the fat body, whereas ecdysone plays a catabolic role, encouraging lipid mobilization and autophagy. By identifying *dDOR* as a direct FOXO target, we shed light on the molecular mechanism by which part of this antagonistic relationship is achieved.

A second consequence of the regulation of *dDOR* by FOXO is the creation of a feed-forward regulatory mechanism. When ecdysone signaling is activated, it inhibits insulin signaling and activates FOXO, causing increased expression of *dDOR*. This results in potentiation of the ecdysone signal. This type of mechanism may be important for the dramatic activation of the ecdysone pathway at the end of larval development. Indeed, ecdysone signaling has several autoregulatory positive feedback loops, including EcR-dependent transcription

of the EcR gene and downregulation of a microRNA, miR-14, which inhibits EcR expression [39].

DOR was first identified as a gene whose expression is aberrant in Zucker diabetic rats [4]. Until *DOR* knockout mice are analyzed, it is possible that this aberrant regulation is either a cause or a consequence of the diabetes. Because *dDOR* knockout flies have reduced triglyceride and elevated glycogen stores (Figures 5A and 5B), it is tempting to speculate that aberrant *DOR* expression in mammals might actually cause metabolic defects and not simply be a consequence of them. Although *DOR* expression was downregulated in muscle of diabetic rats [4], we found a 2-fold increase in *hDOR* expression in adipose tissue of type 2 diabetic patients (A.Z., unpublished data). This indicates that regulation of *DOR* expression—and hence the effect on metabolism—in conditions of metabolic disease in mammals is likely to be tissue specific and complex. The reduction in triglycerides in *dDOR* knockout flies is also interesting in light of the antagonistic relationship between ecdysone signaling and insulin signaling in the fly. Previous work has shown that flies with systemically reduced insulin signaling have elevated triglyceride levels [40, 41]. Therefore, the leanness of *dDOR* knockouts would be consistent with increased systemic insulin signaling in *dDOR* knockout animals.

Intriguingly, dDOR shares a number of features with its mammalian homolog. Like hDOR, dDOR functions as a nuclear hormone coactivator. Whereas hDOR binds $TR_{\alpha 1}$, dDOR binds EcR. $TR_{\alpha 1}$ and EcR are similar in that they both form heterodimeric complexes with RXR/USP. In fact, EcR can form a functional complex with the human USP homolog RXR in mammalian cells [9, 10]. Furthermore, EcR and $TR_{\alpha 1}$ both play catabolic roles in some contexts. For instance, ecdysone signaling induces autophagy and lipid mobilization in the fat body and programmed cell death in salivary glands during metamorphosis [23, 42]. Likewise, thyroid hormones increase basal metabolic rates, induce fat mobilization, and enhance fatty acid oxidation [43]. A second similarity between dDOR and hDOR is that both are transcriptionally regulated by nutritional inputs. *DOR* expression is misregulated in diabetic rats, whereas *dDOR* expression changes depending on whether the animals are feeding or fasting. Because we find that regulation of *dDOR* expression is insulin and FOXO dependent, this raises the possibility that the transcriptional effect on *DOR* in diabetic rats may also be insulin dependent. A third similarity is that both hDOR and dDOR have two separable functions—as a nuclear hormone receptor coactivator, and as a regulator of autophagy (this work and [5, 6]). This makes particular biological sense within the context of the fat body, where ecdysone signaling induces autophagy during metamorphosis. Therefore, the dual functions of dDOR work in parallel, both by potentiating ecdysone signaling and by interacting with the autophagy proteins Atg8a/b.

In sum, this work discovers dDOR as a novel EcR coactivator required during fly metamorphosis. Furthermore, it identifies *dDOR* as a novel component of a gene regulatory network integrating ecdysone and insulin signaling to regulate fly development and metabolism.

Experimental Procedures

Expression Constructs, Fly Lines, and qPCR

All oligos used for cloning, for quantitative RT-PCR, and for qPCR are listed in the Supplemental Experimental Procedures, as is a detailed description of plasmid constructions (overexpression and knockout constructs). Pal1sx-188-cc-Luc and s-188-cc-RLuc plasmids with the EcRE-luciferase construct and Renilla control were obtained from the DGRC collection. Flies containing *tubulin-Gal4*, *hs-GAL4*, *hs-EcR-B2*, *UAS-EcR$_{W650A}$*, and *ecd[1]* were from the Bloomington Stock Center (BSD); FOXO^{21} and FOXO^{25} were previously described in [36].

Antibodies and Immunoprecipitation

Anti-FLAG (Sigma, F1804), anti-HA (Roche, 11867423001), and anti-EcR (DSHB, DDA2.7) were used. Anti-dDOR was raised in guinea pigs against the full-length dDORlong protein expressed in *E. coli*. Protein G-agarose was from Roche.

Metabolic Analyses

Flies were grown at a controlled density of 50 larvae per vial on food consisting of 10 liters water, 80 g agar, 800 g corn powder, 100 g soya powder, 180 g dry yeast, 800 g malt extract, 220 g syrup, 62.5 ml propionic acid, 6.25 ml phosphoric acid, and 24 g nipagin. Lipids, glycogen, and trehalose were quantified in 1-day-old males as described in the Supplemental Experimental Procedures.

Staging of Animals for qRT-PCR on Ecdysone Response Genes

L1 larvae hatching within a tight 4 hr time window were picked and seeded in vials containing food (as above) supplemented with 0.05% bromophenol blue at a density of 50 per vial and grown at 25°C. This procedure was repeated on multiple subsequent days. "Feeding" L3 animals are animals 24 hr prior to pupation (based on the pupation behavior of siblings). Animals 18 hr, 8 hr, and 4 hr prior to pupation were based on clearance of blue food from the guts as described [44]. "Wandering" L3 animals are animals 0 to 6 hr prior to pupation.

Ecdysone Treatment of Fat Body Explants

Fat bodies were dissected from wandering third-instar larvae and incubated for 4 hr in Grace's insect medium (Sigma) in the presence or absence of 1 μM 20-hydroxyecdysone (Sigma) as indicated. RNA was then extracted with Trizol as previously described [32].

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at doi:10.1016/j.cub.2010.08.055.

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