The *Drosophila* NR4A Nuclear Receptor DHR38 Regulates Carbohydrate Metabolism and Glycogen Storage

Anne-Françoise Ruaud, Geanette Lam, and Carl S. Thummel

Department of Human Genetics, University of Utah School of Medicine, Salt Lake City, Utah 84112-5330

Animals balance nutrient storage and mobilization to maintain metabolic homeostasis, a process that is disrupted in metabolic diseases like obesity and diabetes. Here, we show that DHR38, the single fly ortholog of the mammalian nuclear receptor 4A family of nuclear receptors, regulates glycogen storage during the larval stages of *Drosophila melanogaster*. DHR38 is expressed and active in the gut and body wall of larvae, and its expression levels change in response to nutritional status. *DHR38* null mutants have normal levels of glucose, trehalose (the major circulating form of sugar), and triacylglycerol but display reduced levels of glycogen in the body wall muscles, which constitute the primary storage site for carbohydrates. Microarray analysis reveals that many metabolic genes are mis-regulated in *DHR38* mutants. These include *phosphoglucomutase*, which is required for glycogen synthesis, and the two genes that encode the digestive enzyme amylase, accounting for the reduced amylase enzyme activity seen in *DHR38* mutant larvae. These studies demonstrate that a critical role of nuclear receptor 4A receptors in carbohydrate metabolism has been conserved through evolution and that nutritional regulation of DHR38 expression maintains the proper uptake and storage of glycogen during the growing larval stage of development. **(*Molecular Endocrinology* 25: 83–91, 2011)**

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Nuclear receptors are ligand-regulated transcription factors that are defined by a conserved zinc finger DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD). Functional studies have shown that many of these factors play a central role in maintaining metabolic homeostasis, often by acting as metabolic sensors that drive feedback or feed-forward transcriptional programs that act on the compound bound by the receptor (1). A number of ligand-independent orphan nuclear receptors have also been implicated in metabolic control, although their mechanisms of action remain more poorly understood.

In this study, we focus on the nuclear receptor 4A (NR4A) family of orphan nuclear receptors, a subclass that is represented by three paralogs in mammals: Nur77 (NR4A1), Nur-related protein 1 (Nurr1; NR4A2), and neuron-derived orphan receptor 1 (NOR-1; NR4A3). Crystallographic studies have shown that the ligand-binding pocket of NR4A receptors is filled with bulky hydrophobic amino acid side chains and that the LBD adopts a canonical protein fold characteristic of the agonist-bound, transcriptionally active state (2, 3). As a result, the activity of NR4A receptors is determined largely by their expression level and posttranslational modifications rather than a specific ligand (4, 5). A number of recent studies have revealed pleiotropic metabolic functions for these receptors (6). Nur77 and NOR-1 promote glucose utilization and oxidative metabolism, respectively, after β-adrenergic stimulation in skeletal muscle (7, 8). Nur77, Nurr1, and NOR-1 also participate in the up-regulation of hepatic gluconeogenesis in response to glucagon signaling and can increase blood glucose levels (9). In contrast to this diabetes-promoting role, Nur77

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Abbreviations: AKH, Adipokinetic hormone; DBD, DNA-binding domain; LBD, ligand-binding domain; NOR-1, neuron-derived orphan receptor 1; NR4A, nuclear receptor 4A; Nurr1, Nur-related protein 1; PA/S, periodic acid/Schiff; qRT-PCR, quantitative RT-PCR.

and NOR-1 have been shown to increase insulin sensitivity in adipocytes (10). These studies thus reveal distinct organ-specific functions for NR4A receptors in modulating glucose homeostasis and diabetes progression and raise the question of their role in controlling the systemic physiology of the animal. One study to date has explored this topic, showing that mice lacking *Nur77* develop hepatic steatosis and increased insulin resistance when challenged with a high-fat diet (11). This phenotype, however, is difficult to interpret due to the compensatory overexpression of Nurr1 and NOR-1 that is observed in *Nur77* mutants. It is not possible to investigate systemic functions in animals that are completely deprived of NR4A signaling because of the early lethality associated with double mutant combinations (12).

We are using *Drosophila* as a simple model system to investigate the metabolic functions of the NR4A nuclear receptors. The fly genome encodes a single NR4A ortholog, DHR38, with 48% overall amino acid identity to Nur77 and 64% identity in the DBD (13). *DHR38* null mutants die during metamorphosis, with developmental defects in the formation of the adult cuticle (14). This late lethality provides an opportunity to define the metabolic dysfunction associated with a complete loss of NR4A function. Here, we show that, like its mammalian counterparts, *DHR38* expression in larvae is modulated by the nutritional status of the animal. *DHR38* is expressed in the larval gut and body wall, the major tissues involved in nutrient uptake and energy expenditure. *DHR38* null mutants have normal levels of glucose, trehalose, and triacylglycerol, but display significantly reduced levels of glycogen. Consistent with this, microarray studies identify a number of key genes involved in carbohydrate metabolism that are mis-regulated in *DHR38* mutants. These include significant down-regulation of the two genes that encode amylase, the enzyme responsible for digesting complex carbohydrates, consistent with the reduced amylase enzyme activity seen in *DHR38* mutant larvae. *Pgm*, which encodes phosphoglucomutase, a key enzyme in glycogenesis, is also significantly underexpressed in *DHR38* mutants. We conclude that increased levels of *DHR38* expression in feeding larvae promote the appropriate uptake and storage of carbohydrates. In addition, our results demonstrate that carbohydrate metabolism represents an evolutionarily ancestral function for the NR4A subclass of nuclear receptors.

Results

DHR38 is expressed in the larval gut and body wall

As a first step toward functional characterization of *DHR38*, we determined its spatial pattern of expression. Although earlier studies have shown that *DHR38* is expressed throughout embryonic and larval stages, these efforts were confounded by its low level of expression, which is near background levels of detection (13, 14). To circumvent this difficulty, we performed quantitative RT-PCR (qRT-PCR) on dissected organs, including the brain complex, gut, fat body, and carcass from fed third instar larvae, and measured levels of *DHR38* mRNA relative to an internal *rp49* mRNA control. We found that *DHR38* transcripts are highly enriched in the larval carcass, which includes the epidermis and body wall muscle (Fig. 1A). To determine whether the low levels of *DHR38* expression detected in the gut and the fat body are above background levels, we measured *DHR38* mRNA in these two tissues dissected from either control or *DHR38* null mutant larvae, which carry the molecularly defined $DHR38^{Y214}$ null allele in combination with a deficiency that removes the *DHR38* locus (15). This revealed that *DHR38* is expressed at low levels in the gut but not in the larval fat body (Fig. 1B).

DHR38 expression is regulated by the nutritional status of the animal

Nuclear receptors of the NR4A subgroup are orphan receptors, the activity of which is determined largely by their expression level (4, 5). In addition, the expression of all three mammalian NR4A receptors is responsive to the

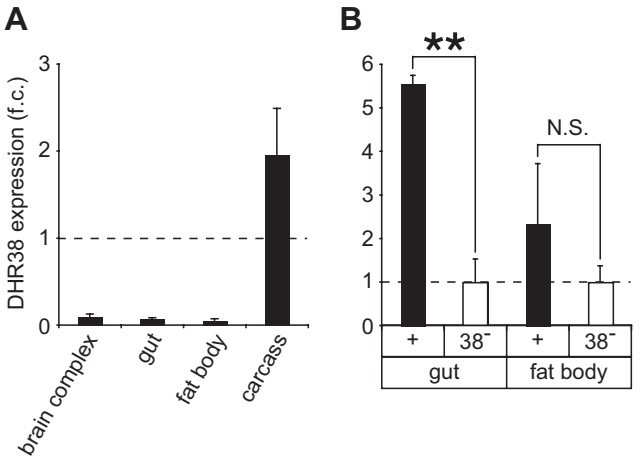

FIG. 1. *DHR38* expression profile. A, *DHR38* is most abundantly expressed in the larval carcass. *DHR38* transcript levels were measured by qRT-PCR using RNA samples isolated from dissected third instar larval brain complexes, gut, fat body, or carcass. The fold-change (f.c.) in these mRNA levels relative to the level in the whole animal is depicted. B, *DHR38* is expressed at low levels in the larval gut but not in the fat body. Levels of *DHR38* mRNA were determined by qRT-PCR using RNA isolated from dissected guts or fat bodies of control or $DHR38^{Y214}/Df(2)Ketel^{RX32}$ mutant third instar larvae (38^{-}). The amount of *DHR38* mRNA in wild-type tissue is presented relative to the level detected in null mutant animals. The region amplified by PCR is fully deleted in the *DHR38* mutant. *Error bars* represent SEM, $n \geq 3$ independent samples of 3–10 animals each; **, $P < 0.01$. N.S., Not significant, Student's *t* test.

feeding status of the animal (9). To determine whether a similar mode of regulation exists in *Drosophila*, *DHR38* mRNA levels were measured in larvae that were maintained for 24 h either in the absence of food or on a rich diet consisting of yeast paste. This study revealed that *DHR38* expression is significantly higher in fed larvae (Fig. 2A). These results are consistent with published microarray data, which indicate that *DHR38* mRNA levels are reduced upon starvation (16).

In an effort to identify specific nutritional cues that might be responsible for this regulation, larvae were transferred to media that contained either sugars, starch, fatty acids, or proteins. None of these dietary conditions, however, was sufficient to up-regulate *DHR38* expression, although mRNA levels are significantly reduced in animals maintained on a pure protein diet (Fig. 2A). These results are consistent with the inability of these individual nutrients to support larval growth and suggest that changes in *DHR38* expression are linked to the availability of a complete diet (17). These observations also raise the interesting possibility that *DHR38* might transduce nutritional signals in *Drosophila* larvae.

Several pathways have been identified in *Drosophila* that signal changes in nutritional status, including a conserved insulin/IGF system counterbalanced by the glucagon analog adipokinetic hormone (AKH) (18–20). In vertebrates, NR4A expression is induced by insulin and glucagon in a tissue-specific manner, with insulin stimulating NOR-1 and Nur77 expression in adipocytes and glucagon inducing the three paralogs in hepatocytes (9, 10). To test whether a similar form of regulation might exist in *Drosophila*, we assayed *DHR38* mRNA levels in larvae that carry a strong loss of function mutation in the single insulin/IGF receptor homolog *InR* (Fig. 2B). *DHR38* mRNA levels were also measured in larvae that specifically overexpress AKH in the fat body, which shares functions with the mammalian liver (Fig. 2C). This expression is sufficient to promote hyperglycemia and decrease levels of fat body triacylglycerol (20). *DHR38* expression, however, is not significantly altered under either of these conditions, suggesting that it is controlled independently of these metabolic sensors.

The activity of mammalian NR4A receptors is known to be modulated by a number of factors, including post-translational modification and dimerization with other nuclear receptors (4). We therefore examined whether we could detect changes in the activity of the DHR38 LBD in response to the nutritional status of the animal. For this purpose, we used transgenic animals that carry a heat-inducible construct that encodes the yeast GAL4 DNA-binding domain fused to the DHR38 LBD (*hsp70-GAL4-DHR38*) in combination with a *UAS-nlacZ* reporter gene

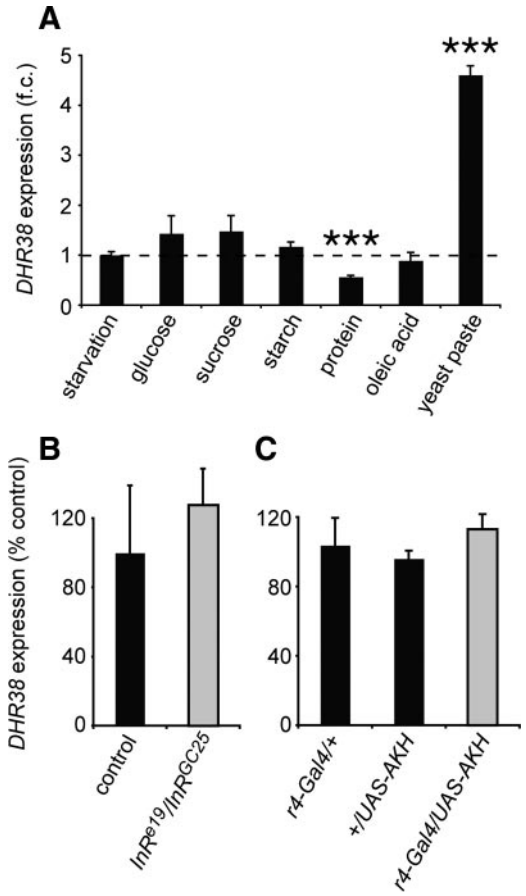

FIG. 2. *DHR38* expression is regulated by the nutritional status of the animal. A, *DHR38* expression is regulated by dietary factors. Late second instar larvae were either starved or maintained on different food sources, as listed, for 24 h. RNA was then extracted, and the levels of *DHR38* and *rp49* mRNA were determined by qRT-PCR. Levels of *DHR38* mRNA normalized to the levels of *rp49* mRNA are presented as a fold-change (f.c.) between the amount in fed animals and the amount in starved animals. The qRT-PCR signal detected in starved *DHR38* null mutant larvae is less than 0.05% that seen in starved control animals, indicating that it is significantly above background levels (data not shown). B and C, *DHR38* expression is not affected by changes in insulin or AKH signaling. Total RNA from control, *InR* mutant, or AKH-overexpressing larvae was analyzed by qRT-PCR for changes in *DHR38* mRNA levels relative to the levels of an internal *rp49* mRNA control. B, Second instar InR^{e19}/InR^{GC25} larvae raised at 25 C that display a strong loss of *InR* function (*gray bar*) (38) have the same levels of *DHR38* expression as those in w^{1118} control larvae (*black bar*). *InR* inactivation was verified by the small size of *InR* mutant larvae relative to controls (data not shown). C, Control third instar larvae that carry either the fat body-specific *r4-GAL4* driver or the *UAS-AKH* transgene (*black bars*) have the same level of *DHR38* mRNA as larvae that carry both transgenes and thus overexpress AKH in the fat body (*gray bar*). The efficiency of AKH overexpression was confirmed by measuring circulating trehalose levels, which are higher in AKH-overexpressing larvae when compared with the controls (20). *Error bars* represent SEM, $n \geq 3$ independent samples of 3–10 animals each; ***, $P < 0.001$, Student's *t* test.

that directs the synthesis of nuclear-localized β-galactosidase (2, 21). This system provides a faithful means of following LBD activity within the animal. No changes in DHR38 LBD activity were detected upon comparing fed and starved larvae at several developmental stages (data

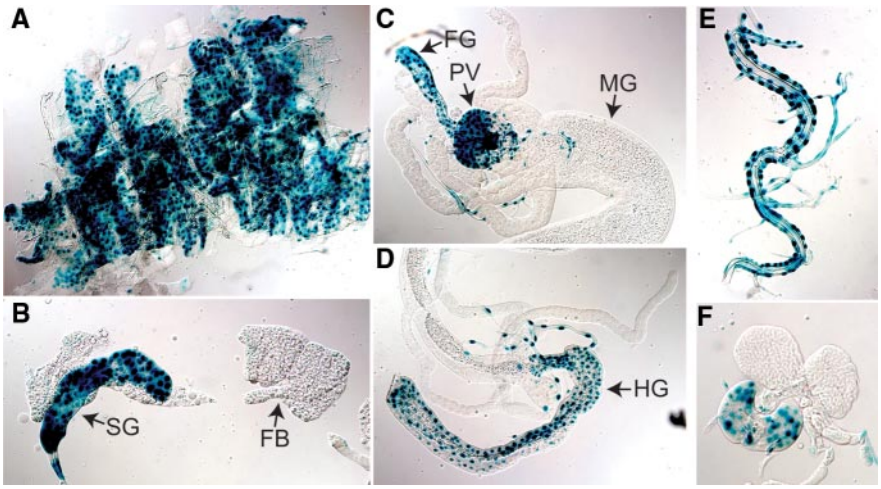

FIG. 3. Activation of the DHR38 LBD is spatially restricted. Fed second instar larvae that carry both the *hsp70-GAL4-DHR38* and *UAS-nlacZ* transgenes were heat treated and allowed to recover for approximately 3 h, after which organs were dissected and stained with X-gal to detect β-galactosidase activity (21). High levels of DHR38 LBD activity are restricted to the carcass (A), the salivary glands (SG) (B), the foregut (FG) and proventriculus (PV) (C), and hindgut (HG) (D), as well as the tracheae (E) and prothoracic gland (F). In contrast, no activity is detected in the fat body (FB) (B) or larval midgut (MG) (C). Control experiments have shown that the *UAS-nlacZ* reporter is capable of being expressed in all tissues at this stage in development (39).

not shown). Interestingly, however, the activity of the DHR38 LBD is restricted to specific tissues, with high levels of activation in the body wall, salivary glands, regions of the gut, trachea, and prothoratic gland (Fig. 3). Two of these, the body wall and gut, correspond to tissues in which the receptor is normally expressed (Fig. 1, A and B). This pattern of LBD activation suggests that, like its mammalian counterparts, DHR38 activity is controlled posttranslationally. In addition, it indicates that changes in DHR38 expression level provide the primary means for its regulatory response to the nutritional status of the animal.

DHR38 mutants display reduced levels of glycogen

Many of the basic metabolic pathways that maintain homeostasis in vertebrates are conserved in the fly (reviewed in Refs. 2, 22). This includes the use of glycogen and triacylglycerol as the major intracellular stored forms of energy, whereas the disaccharide trehalose, and to a lesser extent glucose, function as circulating sugars. To uncover possible metabolic functions for *DHR38*, we measured the levels of these compounds under both feeding and starved conditions in control and *DHR38* mutant animals. Animals that carry the $DHR38^{Y214}$ mutation in combination with *Df(2)Ketel*, a deficiency that removes the *DHR38* locus, were used to provide a complete loss of *DHR38* function (15).

Control and *DHR38* mutant larvae were subjected to complete starvation, and the animals were followed for several days. No detectable difference was observed in the starvation response of control and mutant animals, with both strains surviving 2 d in the absence of food (Supplemental Fig. 1A, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). We went on to determine whether we could detect defects in the levels of basic metabolites in fed or starved *DHR38* mutants. The soluble protein content of fed control and mutant larvae was similar, although *DHR38* mutant larvae had slightly reduced amounts of soluble protein upon starvation (Fig. 4A). In contrast, the levels of triacylglycerol are unaffected in the mutant, under either fed or starved conditions (Fig. 4B). Glucose and trehalose levels are also unaffected in either fed or starved *DHR38* mutants (Fig. 4C and Supplemental Fig. 1B). Glycogen levels, however, are significantly reduced in both fed and starved mutants (Fig. 4D). This effect was confirmed by measuring glycogen levels in animals that carry two other *DHR38* mutant allele combinations, $DHR38^{56}$/*Df(2)Ketel* and $DHR38^{Y214}$ homozygotes (Fig. 4, E and F). In addition, the reduced glycogen levels observed in $DHR38^{Y214}$ mutants can be efficiently rescued by a *hs-DHR38* transgene that expresses the wild-type receptor (Fig. 4F). *DHR38* is thus required to promote appropriate glycogen storage in *Drosophila* larvae. In spite of this defect, however, the *DHR38* mutants are able to maintain relatively normal trehalose levels under both fed and starved conditions, although the mechanisms that underlie this response remain unclear (Fig. 4C).

Glycogen is stored in the larval muscle

The liver and skeletal muscle constitute the two major sites for glycogen storage in vertebrates. An analogous distribution of glycogen depots has been observed in adult *Drosophila*, with the highest levels of glycogen present in the fat body and lower levels in the halteres, flight muscle, and gut (23). Little work, however, has been done to examine the glycogen distribution in *Drosophila* larvae, with only a few reports describing low levels of glycogen in the fat body and salivary glands (24, 25). We therefore used iodine and periodic acid/Schiff (PA/S) staining on paraffin sections of fixed larvae to determine the spatial distribution of glycogen stores. This study revealed that glycogen is most abundant in the body wall muscles, the primary muscles used for larval movement, with much

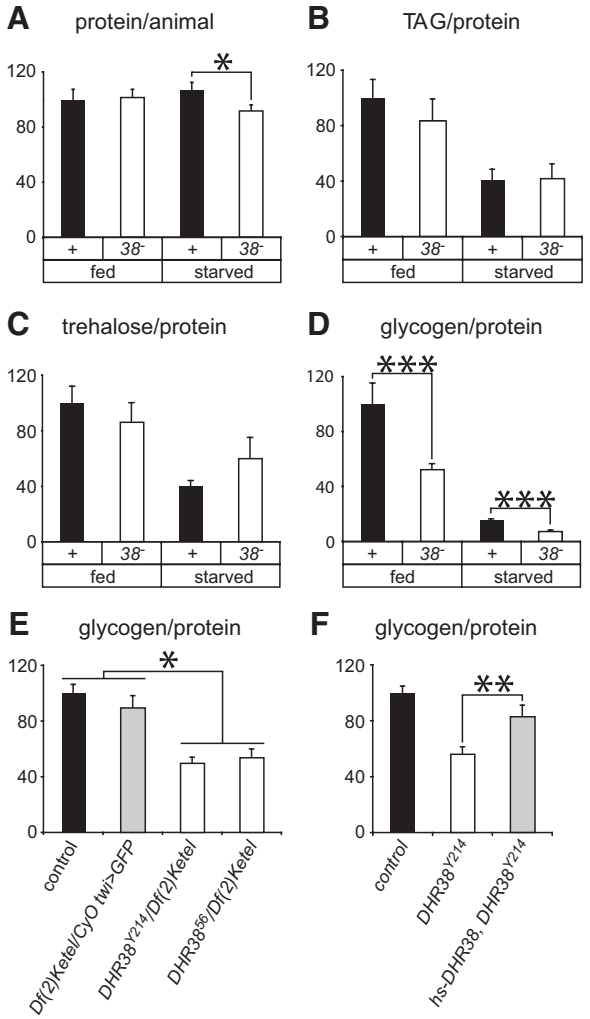

FIG. 4. *DHR38* mutants display reduced levels of glycogen. A–D, control and *DHR38*^{Y214}/*Df(2)Ketel* (*38*$^{-}$) late second instar larvae were either collected or starved for 24 h, and homogenates were assayed for protein (A), triacylglycerol (TAG) (B), trehalose (C), or glycogen (D). A, The amount of protein per larva is presented relative to a control level of 100% and is slightly reduced in starved *DHR38* mutants. B–D, Triacylglycerol, trehalose, and glycogen levels were normalized to the amount of protein and are presented relative to the level in fed controls. The differences in trehalose levels between control and *DHR38* mutant animals are not significant. E, Reduced glycogen levels are present in two different *DHR38* null mutant combinations. Glycogen levels were measured in starved control larvae, larvae that are heterozygous for the *Df(2)Ketel* deficiency, or null mutant larvae that carry either the *DHR38*^{Y214} or *DHR38*^{56} allele in combination with the *Df(2)Ketel* deficiency. F, The reduced glycogen levels in homozygous *DHR38*^{Y214} mutant larvae are rescued by constitutive expression of a *hs-DHR38* transgene. *Error bars* represent SEM, n ≥ 6 independent samples of 5–40 animals each; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Student's *t* test.

lower levels in the fat body and gut (Fig. 5, A and B). These observations are consistent with the spatial expression pattern of many genes involved in glycogen metabolism, which are most abundantly expressed in the larval carcass (26). In addition, glycogen levels are detectably lower in the muscles of *DHR38* mutant larvae, as revealed by PA/S staining (Fig. 5, C and D). Taken together, those results indicate that glycogen is primarily stored in

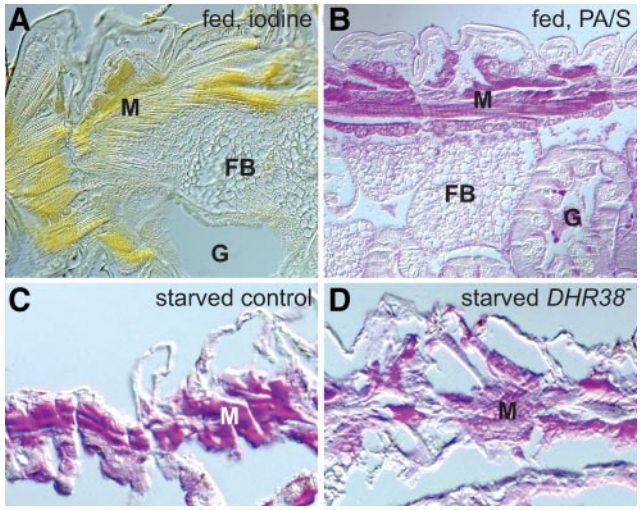

FIG. 5. *DHR38* mutants display reduced levels of glycogen in the muscle, which is the primary tissue for glycogen storage in larvae. A and B, Fed control third instar larvae were dissected and stained with either iodine vapor (A) or PA/S reagent (B), revealing high levels of glycogen in the muscle (M) but not the fat body (FB) or gut (G). C and D, PA/S staining of starved control or *DHR38*^{Y214}/*Df(2)Ketel* third instar larvae reveals that glycogen levels are reduced in the muscles of *DHR38* mutant larvae.

the larval muscle and that this process depends on *DHR38* function.

DHR38 regulates carbohydrate metabolism gene expression

Microarray studies were conducted in an effort to determine the molecular mechanisms by which *DHR38* regulates glycogen levels. RNA was extracted from fed control and *DHR38* mutant larvae, labeled, and hybridized to Agilent *Drosophila* 44K microarrays (Agilent Technologies, Santa Clara, CA). All experiments were conducted in triplicate to facilitate statistical analysis. The raw data were quantile-normalized, and gene expression changes were determined using GeneSifter (1.75-fold cutoff, adjusted $P \leq 0.02$). This study identified 437 genes that are down-regulated in *DHR38* mutants and 431 genes that are up-regulated (Supplemental Table 1).

Comparison of the *DHR38*-regulated genes with a list of genes that comprise the major metabolic pathways in *Drosophila* revealed a significant overlap in these data sets (χ^2 test, $P = 6 \times 10^{-5}$) (Supplemental Table 2). These include genes that act in a number of processes, including amino acid catabolism and lipid metabolism. We focused our attention on those genes that might impact carbohydrate uptake or glycogen synthesis, because these are the primary pathways in feeding larvae that could contribute to the changes in glycogen content seen in *DHR38* mutant animals. Two genes that encode amylases are among the most highly down-regulated genes in *DHR38* mutants: *Amy-p* (−11.4-fold) and *Amy-d* (−11.0-fold). These

genes encode enzymes that are predicted to perform the first step in the breakdown of complex dietary carbohydrates (26). One other gene encodes an amylase in *Drosophila*, *Amyrel* (27). It is, however, less abundantly expressed than the *Amy* genes and is unaffected in *DHR38* mutants. We also identified one gene that is mis-regulated in *DHR38* mutants and that plays a role in glycogen metabolism, *Pgm* (−1.8-fold). This gene encodes phosphoglucomutase, which catalyzes the interconversion of glucose-1-phosphate, the precursor for glycogen synthesis, and glucose-6-phosphate, the primary form of intracellular sugar (Fig. 6A). Northern blot hybridization confirmed the results of our microarray study, showing markedly reduced expression of the *Amy* genes and *Pgm* (Fig. 6B). A similar effect is seen in animals that carry other *DHR38* mutant allele combinations (Supplemental Fig. 2). We also examined the expression of three other genes that play a critical role in glycogen metabolism: *UGP* (*CG4347*), which encodes uridine diphosphate-glucose pyrophosphorylase; *CG6904*, which encodes glycogen synthase (the key enzyme in glycogen synthesis); and *GlyP* (*CG7254*), which encodes glycogen phosphorylase, the rate-limiting enzyme in glycogenolysis (Fig. 6A). None of these genes, however, are significantly or reproducibly mis-regulated in *DHR38* mutant larvae (Fig. 6B and Supplemental Table 1). Taken together, these data indicate that DHR38 regulates a subset of genes involved in carbohydrate metabolism.

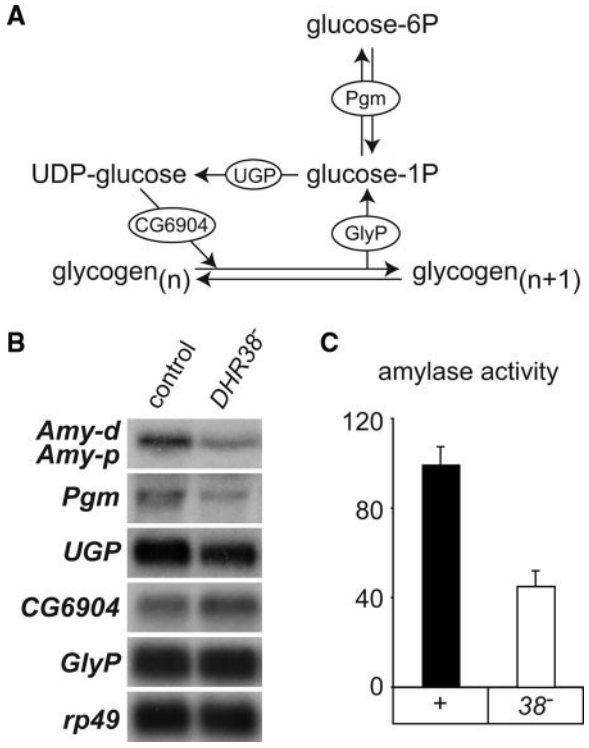

FIG. 6. *DHR38* regulates the expression of genes involved in carbohydrate metabolism. A, Schematic representation of glycogen metabolism in *Drosophila*. Glycogen synthesis involves the conversion of glucose-6-phosphate to glucose-1-phosphate through the action of phosphoglucomutase (Pgm). The production of uridine diphosphate-glucose (UDP) by UGP (CG4347) provides the building blocks for glycogen synthesis, mediated by glycogen synthase (CG6904). Glycogen phosphorylase (GlyP) is the rate-limiting enzyme that controls glycogen breakdown. B, *Amy* and *Pgm* expression are markedly reduced in *DHR38* mutants. RNA isolated from fed control and *DHR38*^{Y214}*/Df(2)Ketel* (*38*$^{-}$) larvae was analyzed by Northern blot hybridization to detect *Amy-p* and *Amy-d*, *Pgm*, *UGP*, *CG6904*, and *GlyP* expression. Blots were hybridized with *rp49* as a control for loading and transfer. The *Amy-p* and *Amy-d* genes are highly similar in sequence and length and thus cannot be distinguished by Northern blot analysis. C, Amylase activity is reduced in *DHR38* mutant larvae. Amylase activity was measured in dissected guts from third instar feeding larvae using a fluorescent substrate.

We next tested the possibility that the reduced expression of either *Amy-p*/*Amy-d* or *Pgm* in *DHR38* mutant larvae contributes to their reduced levels of glycogen. Measuring amylase enzyme activity in both control and *DHR38* mutants revealed that amylase levels are reduced approximately 2-fold, reflecting the reduced levels of *Amy* mRNA seen in these animals (Fig. 6, B and C). Efficient disruption of *Amy* expression by RNAi, however, has no effect on whole-animal glycogen levels (Supplemental Fig. 3A). Similarly, inactivation of *Pgm* expression by RNAi does not impact the glycogen content of these animals (Supplemental Fig. 3B). Our results suggest that no single DHR38 target gene is sufficient to explain the reduced levels of glycogen in these animals. Rather, it is likely that DHR38 coordinates the expression of multiple genes that act together to maintain glycogen homeostasis.

Discussion

The physiological functions of NR4A nuclear receptors have remained unclear due to the presence of multiple redundant vertebrate paralogs that perform divergent organ-specific roles in regulating glucose homeostasis and diabetes progression (9, 10). Here, we characterize the metabolic functions of *DHR38*, the single ancestral NR4A nuclear receptor in *Drosophila*, and demonstrate that it is required for proper glycogen storage in the feeding larva. These results add DHR38 to the growing list of invertebrate nuclear receptors controlling energy homeostasis and confirm that a key role for NR4A receptors in carbohydrate metabolism has been conserved during evolution.

Most nuclear receptors involved in metabolic regulation function as sensors for small metabolites, which modulate their transcriptional activity (1). In contrast, structural studies have demonstrated that NR4A family members do not possess a ligand-binding cavity and function as constitutively active receptors (2, 3). The activity of NR4A receptors is thus primarily determined by their expression level. In line with this observation, *DHR38* expression is induced by favorable nutritional conditions,

whereas the activity of its LBD, as determined using the GAL4-DHR38 ligand sensor system (21), is unaffected by the nutritional status of the animals (data not shown). Therefore, similar to its vertebrate counterparts, DHR38 activity appears to be primarily regulated at the transcriptional level. This response is most likely indirect and could represent a novel signaling pathway for favorable nutritional conditions because it is not affected by the two major nutrient-sensing systems in *Drosophila*, the insulin and AKH pathways (Fig. 2, B and C).

DHR38 is essential for efficient glycogen storage in the muscle, which we identified as the primary storage site for glycogen in larvae. Similarly, skeletal muscle glycogen represents 90% of the total stored complex carbohydrates in vertebrates. Glycogen is used *in situ* in glycolytic muscle fibers to provide sugar precursors for glycolysis during exercise, a function conserved in *Drosophila* where it provides the primary source of energy for adult muscle function (23, 28). Glycogen stored in the larval muscle may therefore perform a similar role, providing the primary energy source to support muscle function during starvation. Indeed, larval movement is critical for the hyperactivity and dispersal behavior that is seen upon nutrient deprivation (29). This is thought to be an adaptive behavior that allows animals to explore their environment in search of new food sources. A specific function for glycogen in adaptive muscle physiology fits with the metabolic profile of DHR38. Levels of glycogen, triacylglycerol, and trehalose all drop in starved larvae, consistent with their use as an energy source to maintain viability (Fig. 4, B–D). The reduced levels of glycogen in *DHR38* mutants, however, do not impair their ability to survive a period of starvation (Supplemental Fig. 1A), consistent with a more specific function of glycogen in larval muscle physiology. Finally, it is interesting to note that Nur77 is preferentially expressed in fast-twitch glycolytic muscles, where it acts to promote glucose utilization and glycogenolysis, hallmarks of high muscle glycogen content (7). This result raises the possibility that NR4A receptors exert an evolutionarily conserved role in maintaining muscle physiology through their effects on carbohydrate metabolism and glycogen homeostasis.

Our microarray analysis identified candidate metabolic genes that could mediate *DHR38* function in regulating glycogen storage, including two amylases (*Amy-d* and *Amy-p*) and the converting enzyme phosphoglucomutase (*Pgm*). *Amy-d* and *Amy-p* are most abundantly expressed in the larval gut, whereas *Pgm* is highly expressed in the larval carcass, reflecting the patterns of *DHR38* expression and suggesting that they may be direct regulatory targets of the receptor (26). This possibility is consistent with the identification of potential NR4A binding sites in sequences adjacent to *Amy-d* and *Amy-p* (Supplemental Fig. 4). This binding site has been well defined and can be recognized by DHR38 *in vitro* (13, 30). A related sequence located downstream from *Amy-p* can also be bound by DHR38 protein, although at lower affinity (data not shown), suggesting that DHR38 could directly regulate *Amy-d* and *Amy-p* transcription.

Individually inactivating these putative target genes, however, was not sufficient to phenocopy the low glycogen accumulation observed in *DHR38* mutants (Supplemental Fig. 3). For the phosphoglucomutase gene *Pgm*, this finding was unexpected, because changes in *Pgm* activity have been correlated with glycogen content in natural *Drosophila* populations (31). It is possible that undetected variation at additional loci participates in controlling glycogen levels in those animals. Alternatively, another study reached the opposite conclusion based on analysis of the two common electrophoretic variants at the *Pgm* locus (32). We conclude that DHR38 coordinates the expression of multiple genes that act together to maintain glycogen homeostasis. Similarly, vertebrate nuclear receptors of the NR4A family coregulate a number of genes to control common metabolic processes. In the liver, for example, NR4As stimulate the expression of the glucose-6-phosphatase *G6pc*, the fructose bisphosphatases *Fbp1* and *Fbp2*, the enolase *Eno3*, and the glucose transporter Glut2 (*Slc2a2*) to promote gluconeogenesis (9).

The identification of a critical metabolic role for DHR38 is consistent with our overall understanding of NR4A function. Mammalian NR4A receptors contribute to a wide range of biological pathways, including apoptosis, neurological disease, inflammation, carcinogenesis, and atherogenesis (5). Similarly, in addition to its role in muscle carbohydrate homeostasis described here, DHR38 has an essential developmental function in late pupae, when it is required for the proper integrity of the adult cuticle. This function may be related to the ability of the DHR38 LBD to be activated by ecdysteroids, which control cuticle deposition (2). *DHR38* null mutants die at the end of metamorphosis with rupturing of the cuticle at leg joints and consequent hemolymph leakage (15). Several adult cuticle genes are expressed at reduced levels in *DHR38* mutant pupae, one of which, *Acp65A*, appears to be directly regulated by the receptor and specifically expressed at the joints (33). No changes in glycogen content or ATP levels, however, are detected in these pupae (Supplemental Fig. 5), suggesting that this represents a specific developmental function for the receptor. In addition, *DHR38* is expressed at high levels in the adult brain and regulates DOPA (3,4-dihydroxyphenylalanine) decarboxylase expression in several tissues, suggesting that roles for NR4A receptors in dopaminergic neuron func-

tion have been conserved through evolution (26, 34). Characterization of the tissue-specific functions of *DHR38*, as well as its roles in physiology and metabolism during adult stages, should provide further insights into its regulatory activities and provide a framework for understanding how NR4A receptors integrate their widespread developmental and metabolic functions in the animal.

Materials and Methods

Fly stocks and culture conditions

The w^{1118} strain was used as a control in all experiments. Two *DHR38* mutant alleles were used in this study: $DHR38^{Y214}$ (15) and $DHR38^{56}$ (14). $DHR38^{Y214}$ is an excision mutant that deletes the coding region for both the DBD and LBD, whereas $DHR38^{56}$ is a mutant that introduces a premature stop codon in the three *DHR38* transcripts. Mutant phenotypes were examined in animals carrying a *DHR38* mutation in combination with $Df(2)Ketel^{RX32}$ (35), a small deficiency that removes the *DHR38* locus. The heat-inducible *P[w+; hs-DHR38]* transgene was used for rescue experiments (14). Fly stocks were maintained at 18–25 C on standard cornmeal-agar-yeast food. For metabolic experiments, larvae were grown on agar/molasses and/or agar egg caps supplemented with fresh yeast paste at low density (50–100 larvae per egg cap) at 25 C, unless noted otherwise. For controlled feeding experiments, larvae were placed on filter paper humidified with distilled water (starvation), 20% glucose, 20% sucrose, 20% starch (S9765; Sigma, St. Louis, MO), 20% casein, or 5 mg/ml oleic acid.

Quantitative RT-PCR and Northern blot analysis

Animal and tissue samples were dissected and/or collected in TriPure isolation reagent (Roche, Basel, Switzerland) and frozen in liquid nitrogen. Total RNA was isolated following the manufacturer's instruction. cDNA was prepared from 0.5 μg of RNA in a 25 μl reaction using the Protoscript cDNA kit (New England Biolabs, Ipswich, MA). PCRs of 25 μl were prepared in 96-well plates using SYBR Green master mix (Bio-Rad, Hercules, CA), with a primer concentration of 3 mM (DHR38f-GCAACATAACTACAACTCGCACA, DHR38r-AGCTTCGACAGCAGCAGTG, rp49f-ATGCTAAGCTGTCGCACAAA, and rp49r-CGATGTTGGGCATCAGATACT). Quantitative PCR was performed using a Bio-Rad iCycler (MyiQ Single Color). Data were collected from at least three independent samples. To determine the relationship between mRNA abundance and PCR cycle number, all primer sets were calibrated using serial dilutions of cDNA preparations. Relative abundance is reported as *DHR38* mRNA levels relative to *rp49* mRNA levels. The data were analyzed using efficiency-corrected comparative quantitation. For Northern blot analysis, total RNA samples were fractionated by formaldehyde agarose gel electrophoresis, transferred to a nylon membrane, and cross-linked by UV irradiation, as described (36). Blot hybridization and washing was performed as described (36).

Metabolic assays

For trehalose assays, larvae were homogenized in 100 μl ice-cold trehalase buffer [5 mM Tris (pH 6.6), 137 mM NaCl, and 2.7 mM KCl] (see Ref. 37), centrifuged at maximum speed (15,000 $\times$ *g*) for 3 min, and the resulting supernatant was immediately incubated at 70 C for 5 min; 30 μl of 1/10 diluted samples were treated with or without trehalase (11 mU/μl, T8778; Sigma) overnight at 37 C. Resulting glucose levels were assayed (GAGO-20; Sigma). Trehalose levels were obtained by subtracting the amount of free glucose in the untreated sample from the total glucose present in the sample treated with trehalase and were normalized to protein amounts in each homogenate using a Bradford assay (Bio-Rad). Glucose and glycogen assays were performed as described (16).

Glycogen staining

Larvae were pinned down, fixed in Carnoy's fixative for 2 h at room temperature, and transferred to 4 C overnight. Animals were then dehydrated in an ethanol series followed by xylene, embedded in paraplast, and 6-μm sections were collected. After deparaffinization and hydration, sections were either exposed to iodine vapor for 3 min and mounted in glycerol or stained using a PA/S stain (395B-1KT; Sigma) following the manufacturer's instruction.

Amylase activity measurement

Three dissected guts were homogenized in 100 μl ice-cold 10 mM Tris (pH 7.4) and centrifuged at maximum speed for 3 min. A 50-μl sample of supernatant was used to measure amylase activity using the EnzCheck kit (E33651; Molecular Probes, Eugene, OR), following the manufacturer's instructions. *Bacillus sp.* α-Amylase (A6380; Sigma) was used to establish the standard curve.

Microarrays

Control w^{1118} and $DHR38^{56}/Df(2)Ketel$ mutant larvae were collected 18 h after the second to third instar molt. RNA was isolated from these animals using TriPure isolation reagent (Roche) and purified on RNeasy columns (QIAGEN, Valencia, CA). Samples were prepared in triplicate to facilitate subsequent statistical analysis. Probe labeling, hybridization to two-color Agilent *Drosophila* 44K arrays, and scanning were performed by the University of Utah Microarray Core Facility. The data were quantile-normalized using R, and the fold changes in gene expression and *t* statistics were determined using GeneSifter (VizX Labs, Seattle, WA). *P* values were calculated using the Benjamimi and Hochberg correction for false-discovery rate. Comparison between microarray datasets was performed with Microsoft Access. Microarray data from this study can be accessed at National Center for Biotechnology Gene Expression Omnibus (accession no. GSE23047).

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Address all correspondence and requests for reprints to: Dr. Carl S. Thummel, University of Utah, 15 North 2030 East Room 2100, Salt Lake City, Utah 84112-5330. E-mail: carl.thummel@genetics.utah.edu.

Present address for A.F.R.: Max Planck Institute for the Biology of Aging, Köln 50931, Germany.

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