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RESEARCH ARTICLE

Adult Functions for the *Drosophila* DHR78 Nuclear Receptor

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Background: The Testicular Receptors 2 and 4 (TR2, TR4) comprise a small subfamily of orphan nuclear receptors. Genetic studies in mouse models have identified roles for *TR4* in developmental progression, fertility, brain development, and metabolism, as well as genetic redundancy with *TR2*. Here we study the adult functions of the single *Drosophila* member of this subfamily, *DHR78*, with the goal of defining its ancestral functions in the absence of genetic redundancy. Results: We show that *DHR78* mutants have a shortened lifespan, reduced motility, and mated *DHR78* mutant females display a reduced feeding rate. Transcriptional profiling reveals a major role for DHR78 in promoting the expression of genes that are expressed in the midgut, suggesting that it contributes to nutrient uptake. We also identify roles for *DHR78* in maintaining the expression of genes in the ecdysone and Notch signaling pathways. Conclusions: This study provides a new context for linking the molecular activity of the TR orphan nuclear receptors with their complex roles in adult physiology and lifespan. *Developmental Dynamics 000:000–000, 2017*. © 2017 Wiley Periodicals, Inc.

Key words: metabolism; nuclear receptor; transcription; gene expression

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Introduction

Nuclear receptors are ligand-regulated transcription factors that play central roles in growth, development, and metabolism (Chawla et al., 2001; Mullican et al., 2013). They are defined by a highly conserved zinc finger DNA binding domain and a C-terminal ligand binding domain, which can accommodate small lipophilic chemical compounds. Although the transcriptional activity of many nuclear receptors is regulated by ligand binding, a large number remain classified as orphan receptors, with no known ligand. In this study, we focus on a small subfamily of orphan nuclear receptors, the Testicular Receptors 2 and 4 (TR2, TR4; NR2C1/2). Although these receptors were named based on their abundant expression in the testes and brain, this receptor subfamily is also expressed in the kidneys, liver, adipose tissues, and muscle, suggesting that they may have broad functions (Lin et al., 2014).

Initial phenotypic studies of *TR4* null mutant mice revealed clear postnatal phenotypes, with significant growth retardation and a shortened lifespan (Collins et al., 2004). *TR4* mutants also display reproductive defects consistent with their abundant expression in the testes and ovary. These include defects in spermatogenesis and reduced male fertility, as well as reduced female fertility, smaller ovaries, and defects in follicle development (Collins et al., 2004; Mu et al., 2004; Chen et al., 2008a). *TR4* mutants also display defects in motor coordination and abnormal development of the cerebellum, consistent with its abundant expression in neuronal tissues (Chen et al., 2008b). Several more recent studies have extended the roles of *TR4* to metabolism and mitochondrial function. These include smaller adipocytes with reduced triglyceride accumulation, protection against hepatic steatosis, and resistance to diet-induced obesity in *TR4* mutant mice (Kang et al., 2011).

In contrast to these broad roles for *TR4* in development and metabolism, *TR2* mutant mice were reported as having no detectable phenotypes (Shyr et al., 2002), although a recent study identified defects in vision and retinal development (Olivares et al., 2017). *TR2, TR4* double-knockout mice, however, are early embryonic lethal, indicating that these paralogs have redundant functions (Shyr et al., 2009). Moreover, most studies of these widely expressed nuclear receptors have been restricted to whole body knockouts, leaving us with a poor understanding of their tissue-specific functions. In contrast, the fruit fly *Drosophila* has only a single copy of the TR2/4 receptor subfamily, DHR78. Functional studies of *DHR78* in *Drosophila* thus provides an ideal opportunity to define the mechanisms by which the TRs regulate development and metabolism without the complications of genetic redundancy.

DHR78 was originally identified in molecular screens for new nuclear receptors (Fisk and Thummel, 1995; Zelhof et al., 1995). Two EMS-induced point mutations were generated that behave as

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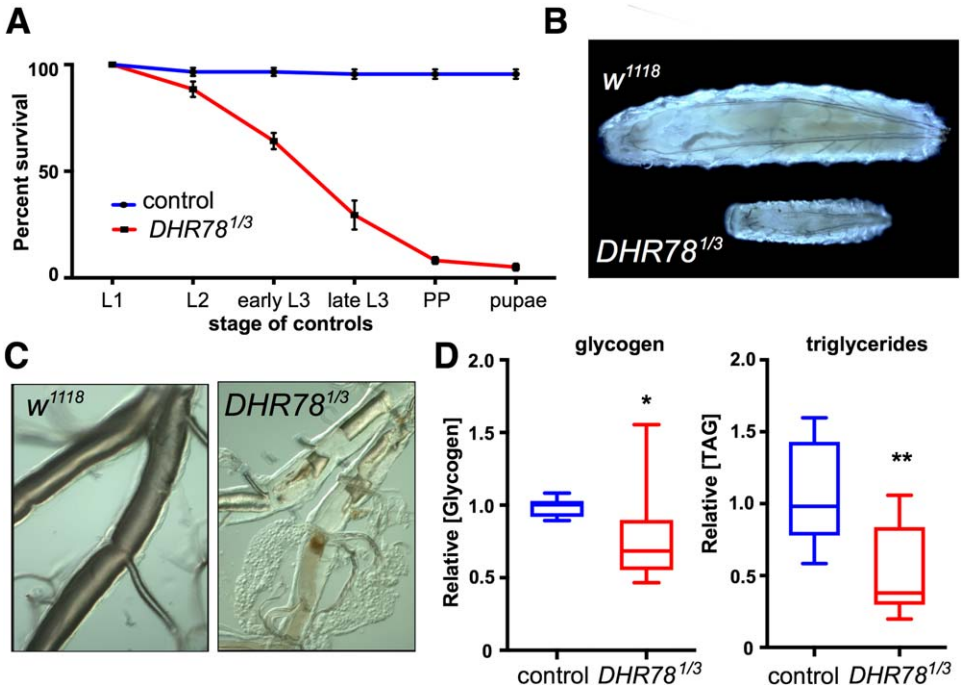

Fig. 1. *DHR78* mutants display larval lethality and tracheal defects. **A**: The percent of surviving w^{1118} controls and $DHR78^{1/3}$ mutants are shown at different time points representing the stage of control animals as either first instar larvae (L1), second instars (L2), early or late third instars (L3), prepupae (PP), or pupae. Most *DHR78* mutant larvae are dead when controls pupariate. *DHR78* mutants progress normally into the second instar, when they begin to display roving behavior, developmental asynchrony, and lethality as second or early third instar larvae (Fisk and Thummel, 1998). **B,C**: *DHR78* mutants that reach the third instar are significantly smaller than w^{1118} controls (B) and display tracheal molting defects (C). **D**: Late second instar w^{1118} controls (blue) and $DHR78^{1/3}$ mutants (red) display reduced glycogen and triglyceride stores, likely due to a starvation response from their roving behavior. Data were normalized to protein levels to account for size differences between controls and mutants, then graphed relative to control levels. Data are graphically represented as box plots. $^{*}P < 0.05$, $^{**}P < 0.005$.

genetic null alleles (Fisk and Thummel, 1998). $DHR78^{1}$ carries a G-to-A transition at the splice acceptor site in the second intron of *DHR78*, while $DHR78^{3}$ contains a C-to-T transition that changes an arginine codon in the ligand-binding domain coding region to a stop codon (Fisk and Thummel, 1998). *DHR78* mutants die as larvae with reduced size and developmental asynchrony. Their tracheal system appears highly disrupted, with defects in gas filling and remnants of tracheal cuticle from previous molts (Fisk and Thummel, 1998). All of these phenotypes, including the larval lethality, can be rescued by tracheal-specific expression of wild-type *DHR78* in a mutant background (Astle et al., 2003).

In addition, tracheal-specific *DHR78* RNAi recapitulates the major defects seen in the whole animal mutant. Taken together, these studies indicate that *DHR78* is necessary and sufficient for proper tracheal development during larval stages. This is consistent with the expression pattern of DHR78 protein, which is abundant in the trachea and salivary glands, along with expression in the imaginal discs, intestine, and Malpighian tubules (Fisk and Thummel, 1998).

Here, we exploit our ability to rescue the larval lethality of *DHR78* mutants to study the functions of this nuclear receptor in adults. We show that mutants rescued to adulthood with tracheal-specific expression of wild-type *DHR78* appear normal but display reduced motility and a shortened lifespan. Stored energy in the forms of glycogen and triglycerides, as well as glucose, are unaffected. Mated *DHR78* mutant females, however, feed less than controls. Transcriptional profiling by RNA-seq reveals a major effect on genes expressed in the intestine, including reduced expression of endopeptidases, transmembrane transporters, and components of the peritrophic matrix. Taken together, our results parallel those seen in studies of *TR4* mutant mice and provide a molecular framework for understanding the roles of DHR78 in larval and adult development.

Results

DHR78 Mutants Die as Larvae With Tracheal Defects

The $DHR78^{1}$ and $DHR78^{3}$ mutants were recovered from our lab stocks and outcrossed to w^{1118} for several generations. As expected, the resulting $DHR78^{1}/DHR78^{3}$ transheterozygous null mutants display the range of phenotypes reported in our earlier studies (Fisk and Thummel, 1998; Astle et al., 2003). These include larval lethality and developmental asynchrony, with most mutants arresting their development during the second and third larval instars (Fig. 1A,B). These animals display defects in tracheal molting and reduced size (Fig. 1B,C). We also noted that mutant larvae were often on the sides of the vials, displaying a classic anoxic escape response consistent with their disrupted tracheal system (Wingrove and O'Farrell, 1999). Metabolite measurements in second instar larvae revealed low levels of glycogen and triglycerides, suggesting that these small wandering animals survive on stored nutrients (Fig. 1D).

DHR78 Mutant Adults Display Reduced Motility and a Shortened Lifespan

The early lethality of *DHR78* null mutants prevents a broader understanding of its regulatory roles outside of tracheal development. In

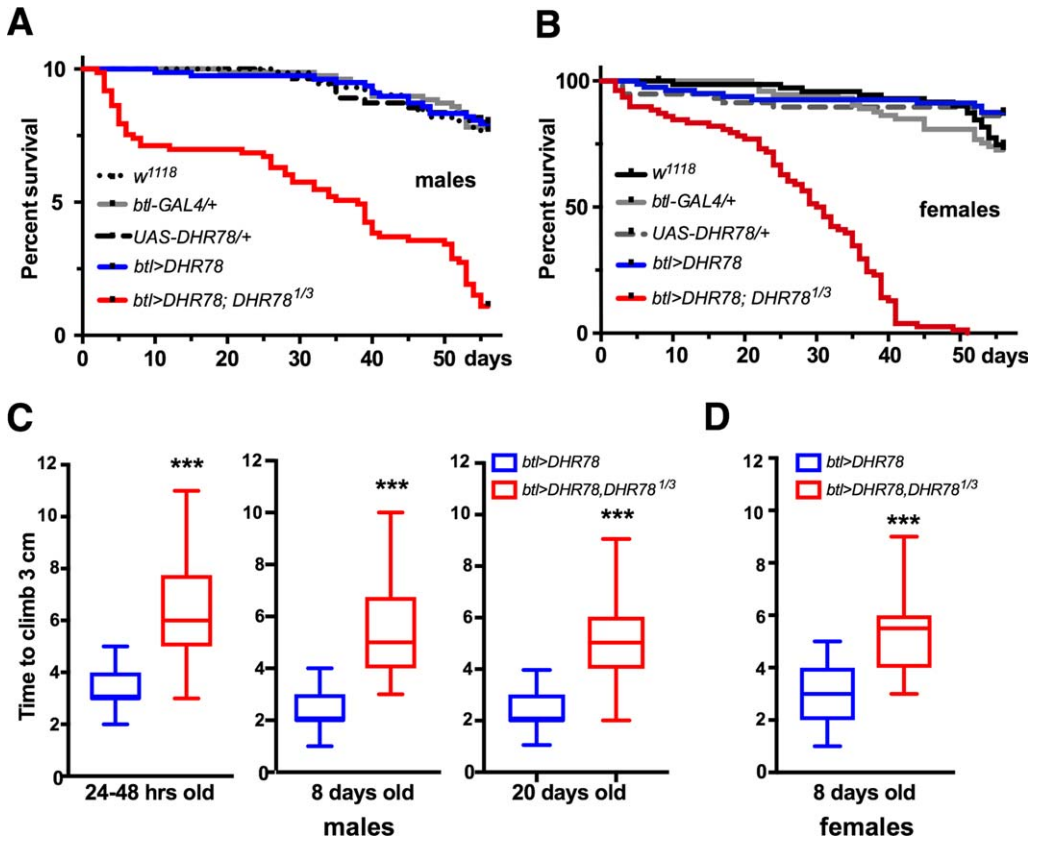

Fig. 2. Rescued *DHR78* mutants display shortened lifespans and motility defects. **A,B**: Lifespan was measured in four control genotypes (black, gray, gray dotted line, and blue) and rescued *DHR78* mutant (red) males (A) and females (B). n > 55 flies per genotype. $P < 0.001$ for both lifespan experiments, comparing *btl>DHR78* controls (blue) to rescued *DHR78* mutants (red). **C,D**: *btl>DHR78* controls (blue) and rescued *DHR78* mutants (*btl>DHR78; DHR78$^{1/3}$*, red) as either males (C) or females (D) display reduced motility when tested with a negative geotaxis assay. Data are graphically represented as box plots. n = 15–35 flies per genotype. $^{***}P < 0.0005$.

addition, their wandering behavior complicates an assessment of possible metabolic functions for this nuclear receptor. To overcome these problems, we used tracheal-specific expression of wild-type *DHR78* in a *DHR78^{1}/DHR78^{3}* transheterozygous mutant background to rescue their lethality through to adulthood (Astle et al., 2003). We generated five stocks for our studies of *DHR78* function during adult stages, all in a common *w^{1118}* genetic background: (1) a *w^{1118}* control stock, (2) *w^{1118}*; *btl-GAL4*, (3) *w^{1118}*; *UAS-DHR78*, (4) *w^{1118}*; *btl-GAL4*; *UAS-DHR78*, and (5) these two transgenes in *DHR78^{1}/DHR78^{3}* mutants. We refer to the latter stock (*w^{1118}*; *btl-GAL4/+*; *UAS-DHR78 DHR78^{3}/DHR78^{1}*) as "rescued mutants" throughout this study.

Initial analysis of *DHR78* rescued mutants revealed that they all survive to adulthood and form normal-appearing animals, as reported in our earlier study (Astle et al., 2003). These mutant adults, however, die significantly more rapidly than controls, with males displaying a more prolonged lifespan than females (Fig. 2A,B). Approximately one-third of mutant males survive to 45 days, when almost all mutant females have died. We also noticed that rescued *DHR78* mutant males and females have reduced motility. This was quantified by tapping *btl-GAL4*; *UAS-DHR78* controls and *btl-GAL4*; *UAS-DHR78*; *DHR78^{1}/DHR78^{3}* rescued mutants to the bottom of a vial and measuring the time it takes for them to climb 3 centimeters. This study revealed that the mutants display significantly reduced negative geotaxis (Fig. 2C,D). Many mutant animals fail to climb at all, remaining motile at the bottom of the vial. Of interest, these defects are similar in both males and females, as well as across all three time points examined (Fig. 2C,D).

DHR78 Mutant Females Display a Reduced Feeding Rate

In an effort to assess the metabolic health of *DHR78* rescued mutants, we measured stored energy reserves in the forms of glycogen and triglycerides (TAG) as well as glucose in 1-week-old males or mated females maintained on a normal diet (Fig. 3A–F). No significant differences were observed in any of these metabolites, although glucose levels appear to be more variable in males (Fig. 3A). We also assayed for feeding rates in rescued *DHR78* mutant males and females to determine if this might contribute to their overall nutrient levels. Both virgin females and males displayed no significant changes in feeding rate over a 2-hr period (Fig. 3G). Mated females, however, consume approximately 50% less than controls. Taken together, these results suggest that mated *DHR78* mutant females can compensate for their reduced feeding rate to maintain their stored energy reserves on a normal diet.

DHR78 Regulates Gene Expression in the Adult Intestine

Defining the regulatory functions of *DHR78* requires an understanding of its downstream target genes. Toward this goal, we

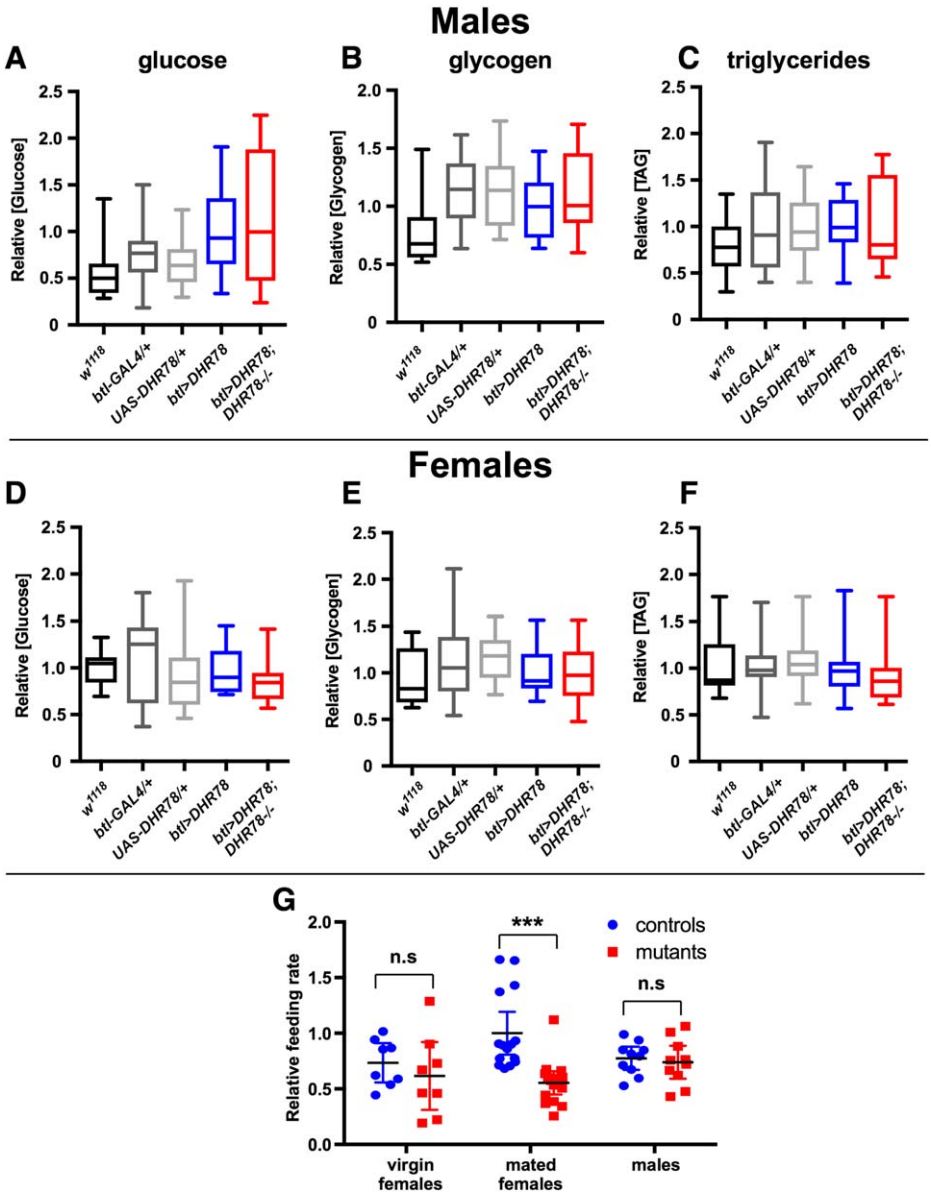

Fig. 3. *DHR78* mutant mated females display a reduced feeding rate. **A–F**: Samples were collected from four control genotypes (black, dark gray, light gray, and blue) and rescued *DHR78* mutants (*btl>DHR78; DHR78*$^{1/3}$; red) as either males (A–C) or mated females (D–F), and assayed for glucose (A,D), glycogen (B,E), or triglycerides (C,F; TAG). n = 11–24 samples for each group. Data are graphically represented as box plots, relative to the metabolite level in *btl>DHR78* controls (blue). **G**: *btl>DHR78* controls (blue) and rescued *DHR78* mutants (*btl>DHR78; DHR78*$^{1/3}$, red) were assayed for feeding rate by measuring the amount of radioactive food ingested after an overnight fast. Data are combined from two separate experiments for each condition. ***$P < 0.0005$.

analyzed the transcriptional profile of rescued *DHR78* mutants using RNA-seq. We selected 1-week-old female w^{1118}; *btl-GAL4*; *UAS-DHR78* animals as controls and w^{1118}; *btl-GAL4*; *UAS-DHR78 DHR78*^{1}/*DHR78*^{3} rescued mutants. We used mated females because of their apparent defects in feeding (Fig. 3G). A total of 510 genes are differentially expressed in *DHR78* mutants (≥1.5-fold change, 1% FDR), with 283 genes showing reduced abundance and 227 genes showing increased abundance (Supplementary Table S1, which is available online).

Gene ontology (GO) analysis revealed that many genes encoding enzymes with predicted oxidoreductase activity are expressed at increased levels in *DHR78* mutants (Table 1). Most of these are cytochrome P450 enzymes that could represent a detoxification response. Other major up-regulated gene categories correspond to defense response pathways suggesting that *DHR78* mutants have an active stress response. GO analysis of the down-regulated dataset revealed a predominance of endopeptidases along with transmembrane transporters and components of the peritrophic matrix (Table 1). Of interest, many of these genes are normally abundantly expressed in the *Drosophila* intestine. To examine this more closely, we analyzed the expression levels of *DHR78*-regulated genes in wild-type tissues as reported in FlyAtlas (Robinson et al., 2013). By depicting these results in a heat map, it became apparent that the genes that are up-regulated in

TABLE 1. Top Gene Ontology Categories for DHR78-Regulated Genes[a]

GO category	No. of genes (total)	*P*-Value	No. of additional similar categories
Down-regulated genes (283)			
Endopeptidase activity	25 (398)	1e-10	9
Urea cycle intermediate	3 (4)	1.7e-3	1
Transmembrane transporter activity	11 (150)	2.4e-3	9
Peritrophic matrix	5 (24)	2.7e-3	
Lysozymes	4 (14)	4.1e-3	1
Hydrolase acting on glycosyl bonds	8 (102)	1.2e-2	1
Arginine metabolism	2(3)	3.0e-2	
Up-regulated genes (227)			
Oxidoreductase activity	24 (541)	3.9e-10	
Defense response	16 (164)	1.3e-8	
Electron carrier (P450s)	13 (166)	3.9e-6	4
Defense response to bacteria	7 (64)	3.8e-4	8
Response to stimulus	25 (982)	1.0e-3	
Peptidase activity	17 (557)	1.1e-3	5

[a]Determined using GOstat: http://gostat.wehi.edu.au/cgi-bin/goStat.pl

DHR78 mutants are expressed in a range of different tissues in wild-type animals while many down-regulated genes are normally expressed in the midgut (Fig. 4). This includes many genes that are abundantly and specifically expressed in this tissue, such as the eleven *Jonah* peptidase genes that are down-regulated in *DHR78* mutants (Supplementary Table S1) (Carlson and Hogness, 1985).

In contrast, comparisons of our data with published datasets revealed no significant overlaps with genes regulated by the DHR38, DHR96, or dHNF4 nuclear receptors (Sieber and Thummel, 2009; Ruaud et al., 2011; Barry and Thummel, 2016). Taken together, these data suggest that DHR78 plays a major role in regulating genes in the adult midgut, including enzymes that promote nutrient uptake. They also have an active stress response, consistent with their reduced survival as adults.

Discussion

DHR78 represents the single *Drosophila* ortholog of the TR2/TR4 orphan nuclear receptor subfamily, providing an ideal simple model for functional analysis. In this study, we use genetic rescue to overcome the larval lethality of *DHR78* null mutants, allowing us to perform phenotypic characterization during the adult stage. We show that *DHR78* mutants have a shortened lifespan and reduced motility. Of interest, these phenotypes resemble those seen in *TR4* mutant mice, which have a shortened lifespan and defects in motor coordination (Collins et al., 2004; Chen et al., 2008b). In contrast, *TR4* mutant mice display a slight but significant increase in feeding rate, the opposite of what we report here in mated *Drosophila* females (Kang et al., 2011). In addition, several genes encoding digestive enzymes are expressed at reduced levels in *DHR78* mutant females, providing another potential level for reduced nutrient uptake. It is also interesting to speculate that the reduced feeding behavior could arise from an overall defect in neuronal function, which could also explain the reduced motility.

This motility defect likely occurs before adult development because there is no change with age as is normally seen in progressive forms of neuronal or muscle degeneration in *Drosophila*. A defect in neuronal development would be consistent with studies of *TR4* in mouse models (Chen et al., 2008b). In addition, several genes involved in *Drosophila* neuronal function are expressed at reduced levels in *DHR78* mutants (Supplementary Table S1). These include *zydeco*, which is involved in glial-neuronal interactions, *Dscam4*, which regulates dendritic targeting, and several genes involved in early neuronal development, including *miranda, ventral nervous system defective*, and *robo2* (Melom and Littleton, 2013; Tadros et al., 2016; Gramates et al., 2017). Future studies of the tissue-specific functions of *DHR78* could address this possible association between neuronal development and the reduced motility and feeding rate in mutants.

Analysis of the RNA-seq data revealed that several genes in the ecdysone signaling pathway are expressed at reduced levels in *DHR78* mutant females (Supplementary Table S1). These include *disembodied*, which encodes a key ecdysone biosynthetic enzyme, *SMRTER*, which acts as a corepressor for EcR, *broad*, which encodes an ecdysone-inducible zinc finger transcription factor, and *Eip71CD* (DiBello et al., 1991; Tsai et al., 1999; Chavez et al., 2000). Of interest, our original study of *DHR78* mutants centered on its role in coordinating ecdysone-regulated gene expression in larvae (Fisk and Thummel, 1998). Future studies could address the possibility that ecdysone levels are reduced in *DHR78* mutant females. In this regard, we also note that the ovaries of mated *DHR78* mutant females are reduced in size—another phenotype associated with both *TR4* mutant mice and ecdysone signaling defects (Chen et al., 2008a; Sieber and Spradling, 2015).

Of interest, several genes in the Notch signaling pathway are also expressed at reduced levels in *DHR78* mutants (Supplementary Table S1). These include *Notch* itself, *pigs*, which is a direct target of Notch signaling that restricts Notch activation, *Rhabconnectin* (*Rbcn-3A*), and *uninflatable* (*uif*) (Siebel and Lendahl, 2017). Notably, *pigs* mutants display reduced motility, suggesting that the reduced expression of this gene may contribute to this

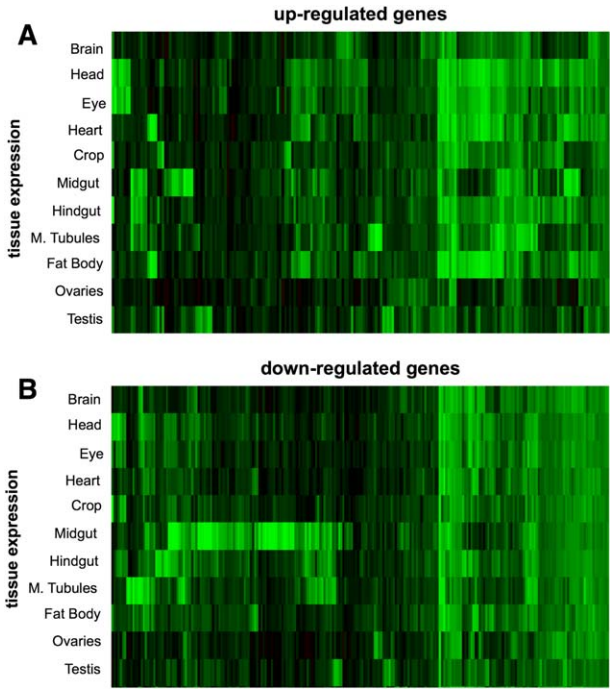

Fig. 4. Many intestinal genes are expressed at reduced levels in *DHR78* mutants. Heat maps are depicted for genes that change their expression level in *DHR78* mutant females. **A,B**: Each row of the heat map represents a specific tissue, and each column represents an individual gene either up-regulated (A) or down-regulated (B) in mutants. The color corresponds to the expression level for that gene in wild-type tissues as reported by FlyAtlas (Robinson et al., 2013), with black representing little or no expression and green representing higher expression. More broadly expressed genes are shown on the right side of the heat map. Many genes that are abundantly expressed in the midgut of wild-type flies are down-regulated in *DHR78* mutants.

DHR78 mutant phenotype (Pines et al., 2010). Similarly, *uif* mutants display defects in tracheal development, tracheal growth, and tracheal molting, with lethality during larval development—phenotypes that resemble those of *DHR78* mutants (Zhang and Ward, 2009). Taken together, these observations raise the interesting possibility that DHR78 acts to support proper levels of Notch signaling and that the reduced expression of *uif* may contribute to the lethal phenotypes seen in mutant larvae.

We also note that Notch lies next to another gene that is underexpressed in *DHR78* mutants, *kirre*. Many other genes that are underexpressed in *DHR78* mutants lie adjacent to each other. These include the *LysC, LysD*, and *LysE* gene cluster, the *CG6295* and *CG6296* predicted lipases, and the *Jon25Bi, Jon25Bii*, and *Jon25Biii* gene cluster. In contrast, only a few of the up-regulated genes lie next to one another. This observation suggests that DHR78 is binding directly to these loci and acting as a transcriptional activator, similar to its mouse ortholog TR4 (Lin et al., 2014). Molecular studies of the regulatory functions of DHR78 are required to allow a direct test of this model.

In conclusion, by moving our studies of *DHR78* mutants into adults we have provided a new context for understanding the molecular and developmental roles of this orphan receptor. Further studies of *DHR78* mutants will complement the characterization of *TR2* and *TR4* in mice, and will provide more insights into the molecular pathways that lie downstream from these orphan nuclear receptors, linking their transcriptional regulatory functions to the complex developmental and metabolic phenotypes in these animals.

Experimental Procedures

Drosophila Strains and Media

Flies were raised at 25 °C on media containing 8% yeast, 6% glucose, 3% sucrose, and 1% agar for all adult studies. Larvae were raised on grape juice media containing yeast paste at 25 °C. All genetic studies used a transheterozygous combination of $DHR78^{1}$ and $DHR78^{3}$ null alleles generated previously by EMS-induced mutation (Fisk and Thummel, 1998). Both strains were outcrossed for three generations to w^{1118} to establish a uniform genetic background. The *UAS-DHR78* and *btl-GAL4* transgenic lines (Astle et al., 2003) were also outcrossed for three generations to w^{1118}, and then crossed to the $DHR78^{3}$ and $DHR78^{1}$ mutant lines, respectively. The *DHR78* stocks used in this study are: (1) w^{1118}; *btl-GAL4;* +/+, (2) w^{1118};+/+; *UAS-DHR78*, (3) w^{1118}; *btl-GAL4;* $DHR78^{1}$/*TM3 Dfd-GMR-nvYFP3* Sb^{1}, (4) w^{1118};+/+; *UAS-DHR78* $DHR78^{3}$/*TM3 Dfd-GMR-nvYFP3* Sb^{1}. Stocks 1 and 2 were crossed to generate the w^{1118}; *btl-GAL4/+; UAS-DHR78/+* control line, and stocks 3 and 4 were crossed to generate the w^{1118}; *btl-GAL4/+;* $DHR78^{1}$/ *UAS-DHR78* $DHR78^{3}$ rescued mutants.

Lifespan Studies

The lethality of *DHR78* mutants was analyzed by transferring 20–30 newly hatched first instar larvae to fresh media with yeast paste and scoring daily for survival. At least three replicates were analyzed per condition. For adult lifespan studies, newly eclosed flies were sorted based on sex and 20–25 flies were transferred to vials containing 8% yeast, 9% sugar for a total of 55–80 flies per condition. Five control males were added to each vial containing female flies. Flies were transferred daily to fresh food and scored for lethality.

Metabolic Assays

Larval metabolic studies shown in Figure 1D were conducted using 25 staged late second instar larvae from at least three independent collections. Metabolite levels were normalized to protein levels and combined to determine average concentration and standard error of the mean, relative to the metabolite levels found in w^{1118} controls. TAG measurements were performed using a coupled colorimetric assay (Sigma T2449) as described (Palanker et al., 2009; Tennessen et al., 2014). Glycogen and glucose concentrations were determined using the hexokinase (HK) and/or glucose oxidase (GO) assay kits (Sigma GAHK20, GAGO20) as described (Tennessen et al., 2014). Total protein levels were determined in parallel by Bradford assay. For adults, samples of five flies per replicate were collected at one week of age and homogenized in 100 µl of 1 × phosphate buffered saline. Each assay was repeated at least three times for a combined total of 11–24 replicates per genotype, per sex. Metabolite levels were normalized to protein levels and combined to determine average concentration and standard error of the mean, relative to the metabolite levels found in *btl-GAL4; UAS-DHR78* controls. Both control and tracheal-rescued mutant females were maintained with w^{1118} males to ensure that mating took place.

Motility Assay

Flies were anesthetized at eclosion and sorted based on sex into fresh vials of food for a total of 10–15 flies per vial, with four vials per experiment. Motility was assayed no earlier than 24 hr postanesthetization and flies were not anesthetized again after the initial sorting. The motility assay was performed by transferring flies to an empty vial placed in front of graph paper and a ruler. The vial was tapped down until all flies fell to the bottom. The time it took for individual flies to climb 3 cm was recorded by video and later analyzed. This experiment was repeated three times with similar results.

Feeding Rate Assay

Control and mutant flies were sorted by sex at eclosion and placed into separate vials, with several w^{1118} males added to the vials with females. Flies were collected at 1 week of age, fasted overnight on 1% agar, and then allowed to feed for 2 hr on radioactive media containing ~5,000 cpm/μl α-^{32}P-dCTP in 8% yeast, 9% sugar in 1% agar (Tennessen et al., 2014). Animals were then transferred to unlabeled food for 30 min and sorted into samples of five flies on ice. A scintillation counter was used to measure the radioactivity in each sample, and this value was used to determine the relative volume of media consumed, as described (Tennessen et al., 2014). Concentrations were normalized to mated control females. This experiment was repeated 2–3 times with a total of 8–10 flies per genotype, per condition.

RNA-Seq

One week old female *DHR78* rescued mutants and controls (*btl>DHR78*) were raised on a 8% yeast, 9% sugar diet. Eight biological replicates per genotype (n = 12 females per sample) were collected. RNA was extracted using Trizol (ThermoFisher 15596026) and pairs of biological replicates were pooled to obtain four biological replicates for further purification using an RNeasy Mini kit (Qiagen 74104). RNA quality was analyzed by using an Agilent Bioanalyzer RNA 6000. Library generation (Illumina TruSeq RNA Sample Preparation Kit v2 with oligo dT selection) and sequencing (HiSeq 50 Cycle Single Read Sequencing v3) were performed by the High-Throughput Genomics core facility at the University of Utah. Standard replicate RNA-seq analysis was performed using USeq and DESeq analysis packages with alignment to the *Drosophila melanogaster* dm3 genome assembly. Transcripts meeting a cutoff of ≥1.5-fold difference in mRNA abundance and 1% FDR were considered as differentially expressed genes. Heat maps were generated in R using ggplots2 combined with tissue-specific expression data from FlyAtlas (Robinson et al., 2013). Genes were separated into lists based on whether they were up- or down-regulated in *DHR78* rescued mutants and analyzed based on the abundance of gene expression in wild-type dissected tissues. RNA-seq data from this study can be accessed at NCBI GEO (accession number: GSE106866).

Statistics

Data and statistical analysis were performed using GraphPad PRISM 7 software. Pairwise comparisons were calculated using a two-tailed Student's *t*-test comparing w^{1118} controls and *DHR78* mutants (Fig. 1D) or *btl*-rescued *DHR78* mutants with genetically matched *btl>DHR78* controls (Figs. 2C, 3). Error bars are ± 1 SEM unless otherwise noted. Box plots display the upper and lower quartiles and the median, with the bars representing the 5–95% confidence intervals. Feeding data are presented as a dot plot showing individual data points and the median.

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References

Astle J, Kozlova T, Thummel CS. 2003. Essential roles for the Dhr78 orphan nuclear receptor during molting of the Drosophila tracheal system. Insect Biochem Mol Biol 33:1201–1209.

Barry WE, Thummel CS. 2016. The Drosophila HNF4 nuclear receptor promotes glucose-stimulated insulin secretion and mitochondrial function in adults. Elife 5:e11183.

Carlson JR, Hogness DS. 1985. Developmental and functional analysis of Jonah gene expression. Dev Biol 108:355–368.

Chavez VM, Marques G, Delbecque JP, Kobayashi K, Hollingsworth M, Burr J, Natzle JE, O'Connor MB. 2000. The Drosophila disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. Development 127:4115–4126.

Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. 2001. Nuclear receptors and lipid physiology: opening the X-files. Science 294:1866–1870.

Chen LM, Wang RS, Lee YF, Liu NC, Chang YJ, Wu CC, Xie S, Hung YC, Chang C. 2008a. Subfertility with defective folliculogenesis in female mice lacking testicular orphan nuclear receptor 4. Mol Endocrinol 22:858–867.

Chen YT, Collins LL, Chang SS, Chang C. 2008b. The roles of testicular orphan nuclear receptor 4 (TR4) in cerebellar development. Cerebellum 7:9–17.

Collins LL, Lee YF, Heinlein CA, Liu NC, Chen YT, Shyr CR, Meshul CK, Uno H, Platt KA, Chang C. 2004. Growth retardation and abnormal maternal behavior in mice lacking testicular orphan nuclear receptor 4. Proc Natl Acad Sci U S A 101:15058–15063.

DiBello PR, Withers DA, Bayer CA, Fristrom JW, Guild GM. 1991. The Drosophila Broad-Complex encodes a family of related proteins containing zinc fingers. Genetics 129:385–397.

Fisk GJ, Thummel CS. 1995. Isolation, regulation, and DNA-binding properties of three Drosophila nuclear hormone receptor superfamily members. Proc Natl Acad Sci U S A 92:10604–10608.

Fisk GJ, Thummel CS. 1998. The DHR78 nuclear receptor is required for ecdysteroid signaling during the onset of Drosophila metamorphosis. Cell 93:543–555.

Gramates LS, Marygold SJ, Santos GD, Urbano JM, Antonazzo G, Matthews BB, Rey AJ, Tabone CJ, Crosby MA, Emmert DB, Falls K, Goodman JL, Hu Y, Ponting L, Schroeder AJ, Strelets VB, Thurmond J, Zhou P, the FlyBase Consortium. 2017. FlyBase at 25: looking to the future. Nucleic Acids Res 45:D663–D671.

Kang HS, Okamoto K, Kim YS, Takeda Y, Bortner CD, Dang H, Wada T, Xie W, Yang XP, Liao G, Jetten AM. 2011. Nuclear orphan receptor TAK1/TR4-deficient mice are protected against obesity-linked inflammation, hepatic steatosis, and insulin resistance. Diabetes 60:177–188.

Lin SJ, Zhang Y, Liu NC, Yang DR, Li G, Chang C. 2014. Minireview: pathophysiological roles of the TR4 nuclear receptor: lessons learned from mice lacking TR4. Mol Endocrinol 28:805–821.

Melom JE, Littleton JT. 2013. Mutation of a NCKX eliminates glial microdomain calcium oscillations and enhances seizure susceptibility. J Neurosci 33:1169–1178.

Mu X, Lee YF, Liu NC, Chen YT, Kim E, Shyr CR, Chang C. 2004. Targeted inactivation of testicular nuclear orphan receptor 4 delays and disrupts late meiotic prophase and subsequent meiotic divisions of spermatogenesis. Mol Cell Biol 24:5887–5899.
Mullican SE, Dispirito JR, Lazar MA. 2013. The orphan nuclear receptors at their 25-year reunion. J Mol Endocrinol 51:T115–T140.
Olivares AM, Han Y, Soto D, Flattery K, Marini J, Molemma N, Haider A, Escher P, DeAngelis MM, Haider NB. 2017. The nuclear hormone receptor gene Nr2c1 (Tr2) is a critical regulator of early retina cell patterning. Dev Biol 429:343–355.
Palanker L, Tennessen JM, Lam G, Thummel CS. 2009. Drosophila HNF4 regulates lipid mobilization and beta-oxidation. Cell Metab 9:228–239.
Pines MK, Housden BE, Bernard F, Bray SJ, Roper K. 2010. The cytolinker Pigs is a direct target and a negative regulator of Notch signalling. Development 137:913–922.
Robinson SW, Herzyk P, Dow JA, Leader DP. 2013. FlyAtlas: database of gene expression in the tissues of Drosophila melanogaster. Nucleic Acids Res 41:D744–D750.
Ruaud AF, Lam G, Thummel CS. 2011. The Drosophila NR4A nuclear receptor DHR38 regulates carbohydrate metabolism and glycogen storage. Mol Endocrinol 25:83–91.
Shyr CR, Collins LL, Mu XM, Platt KA, Chang C. 2002. Spermatogenesis and testis development are normal in mice lacking testicular orphan nuclear receptor 2. Mol Cell Biol 22:4661–4666.
Shyr CR, Kang HY, Tsai MY, Liu NC, Ku PY, Huang KE, Chang C. 2009. Roles of testicular orphan nuclear receptors 2 and 4 in early embryonic development and embryonic stem cells. Endocrinology 150:2454–2462.
Siebel C, Lendahl U. 2017. Notch signaling in development, tissue homeostasis, and disease. Physiol Rev 97:1235–1294.
Sieber MH, Spradling AC. 2015. Steroid signaling establishes a female metabolic state and regulates SREBP to control oocyte lipid accumulation. Curr Biol 25:993–1004.
Sieber MH, Thummel CS. 2009. The DHR96 nuclear receptor controls triacylglycerol homeostasis in Drosophila. Cell Metab 10: 481–490.
Tadros W, Xu S, Akin O, Yi CH, Shin GJ, Millard SS, Zipursky SL. 2016. Dscam proteins direct dendritic targeting through adhesion. Neuron 89:480–493.
Tennessen JM, Barry WE, Cox J, Thummel CS. 2014. Methods for studying metabolism in Drosophila. Methods 68:105–115.
Tsai CC, Kao HY, Yao TP, McKeown M, Evans RM. 1999. SMRTER, a Drosophila nuclear receptor coregulator, reveals that EcR-mediated repression is critical for development. Mol Cell 4:175–186.
Wingrove JA, O'Farrell PH. 1999. Nitric oxide contributes to behavioral, cellular, and developmental responses to low oxygen in Drosophila. Cell 98:105–114.
Zelhof AC, Yao TP, Evans RM, McKeown M. 1995. Identification and characterization of a Drosophila nuclear receptor with the ability to inhibit the ecdysone response. Proc Natl Acad Sci U S A 92:10477–10481.
Zhang L, Ward REt. 2009. uninflatable encodes a novel ectodermal apical surface protein required for tracheal inflation in Drosophila. Dev Biol 336:201–212.