**Drosophila HNF4 Directs a Switch in Lipid Metabolism that Supports the Transition to Adulthood**

**Graphical Abstract**

**Highlights**
- Lipid stores are consumed rapidly after *Drosophila* transitions into adulthood
- *Drosophila* HNF4 directs fatty acid conversion to VLCFA and hydrophobic hydrocarbons
- VLCFA/hydrocarbon synthesis ensures adaptation to dry conditions and dietary sugars
- The role for HNF4 in regulating VLCFA synthesis is conserved between flies and mice

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**In Brief**
Storelli et al. show that the *Drosophila* HNF4 nuclear receptor couples lipid metabolism with development to meet the needs of adult life. *Drosophila* HNF4 directs the rapid conversion of lipid stores into waterproofing hydrocarbons at the onset of adulthood, allowing the animal to adapt to dry conditions and dietary sugars.

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Drosophila HNF4 Directs a Switch in Lipid Metabolism that Supports the Transition to Adulthood

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SUMMARY

Animals must adjust their metabolism as they progress through development in order to meet the needs of each stage in the life cycle. Here, we show that the dHNF4 nuclear receptor acts at the onset of Drosophila adulthood to direct an essential switch in lipid metabolism. Lipid stores are consumed shortly after metamorphosis but contribute little to energy metabolism. Rather, dHNF4 directs their conversion to very long chain fatty acids and hydrocarbons, which waterproof the animal to preserve fluid homeostasis. Similarly, HNF4α is required in mouse hepatocytes for the expression of fatty acid elongases that contribute to a waterproof epidermis, suggesting that this pathway is conserved through evolution. This developmental switch in Drosophila lipid metabolism promotes lifespan and desiccation resistance in adults and suppresses hallmarks of diabetes, including elevated glucose levels and intolerance to dietary sugars. These studies establish dHNF4 as a regulator of the adult metabolic state.

INTRODUCTION

All organisms undergo a series of distinct biological programs as they advance through their life cycle. Changes in nutrition and systemic physiology must be coupled with these transitions in order to meet the specific metabolic needs of each stage in development (Li and Tennesen, 2017; Miyazawa and Aulehla, 2018). For example, the mammalian heart undergoes a switch from using glucose as an energy source during fetal stages to fatty acids after birth, as this organ increases oxidative energy metabolism (Lehman and Kelly, 2002). In Drosophila, a mid-embryonic transcriptional switch establishes an aerobic glycolytic metabolic state that supports larval growth (Tennesen et al., 2011). Stage-specific metabolic and physiological states may also render organisms more susceptible to nutritional stress, such as during puberty in humans (Catalano, 2010; Kelsey and Zeitler, 2016), and metabolic perturbations can feedback on developmental progression. For example, it is well known that childhood obesity alters the timing of sexual maturation (Burt Solorzano and McCartney, 2010). Moreover, disease can reverse developmental changes in physiological state, demonstrating the importance of understanding the links between development and metabolism for potential therapeutic applications (Lehman and Kelly, 2002).

Hepatocyte Nuclear Factor 4α (HNF4α, NR2A1) is a member of the nuclear receptor superfamily of ligand-regulated transcription factors. It binds long-chain fatty acids (LCFAs) and acts as a transcriptional activator, although the role of the ligand in receptor activation remains unclear (Duda et al., 2004; Wisely et al., 2002; Yuan et al., 2009). HNF4α is expressed in the intestine, liver, kidneys, and pancreas and is required for early embryonic development in mice (Chen et al., 1994; Li et al., 2000). Loss of HNF4α in the liver leads to hepatic lipid accumulation (steatosis) and to systemic physiological defects, including reduced levels of plasma lipid, defects in hepatic acylcarnitine production in response to cold stimulation, progressive weight loss, and a shortened lifespan (Hayhurst et al., 2001; Simcox et al., 2017). This is accompanied by changes in the expression of genes involved in lipid metabolism, suggesting that this factor plays an important role in maintaining lipid homeostasis (Hayhurst et al., 2001; Simcox et al., 2017; Yin et al., 2011). Similar phenotypes are seen in C. elegans and Drosophila mutants for homologs of HNF4α, suggesting that these functions are conserved through evolution (Palanker et al., 2009; Van Gilst et al., 2005). Human HNF4α loss-of-function mutations are also responsible for an autosomal dominant form of diabetes called Maturity Onset Diabetes of the Young 1 (MODY1) (Yamagata et al., 1996). Taken together, these observations suggest that HNF4α plays tissue-specific roles in coordinating glucose and lipid metabolism during development.

The basic mechanisms of metabolic control and intertissue communication are conserved between invertebrates and vertebrates, allowing functions discovered in the fruit fly Drosophila to be directly applied to our understanding of mammalian physiology and human disease (Musselman and Kuhnlein, 2018; Owusu-Ansah and Perrimon, 2014; Telemann et al., 2012). The Drosophila genome encodes a single HNF4α ortholog called Drosophila HNF4 (dHNF4) that is widely expressed in the animal (Palanker et al., 2009; Zhong et al., 1993). dHNF4 mutants progress normally through embryogenesis, larval stages, and metamorphosis. However, most animals die upon emergence from their pupal case, and the remaining escapers succumb within
the first days of adulthood (Barry and Thummel, 2016; Palanker et al., 2009). Interestingly, this early adult lethality can be partly rescued if dHNF4 mutants are reared on a low-sugar diet (Barry and Thummel, 2016). This study also demonstrated a role for dHNF4 in maintaining glucose homeostasis and mitochondrial function in adults. In spite of these advances, however, the metabolic basis for the adult-onset lethality and sugar toxicity in dHNF4 mutants remains unknown.

Here, we show that specialized lipid-metabolizing cells called oenocytes become steatotic after the emergence of dHNF4 mutant adults. This lipid accumulation progresses independently of feeding and parallels the developmental clearance of larval adipose cells that occurs during the first days of adult life (Aguila et al., 2007). Improper lipid metabolism in oenocytes does not compromise the starvation response of newly emerged dHNF4 mutants, indicating that the fate of these lipid stores is not for energy production. Rather, oenocytes are the primary site for the production of very long chain fatty acids (VLCFA) and VLCFA-derived hydrocarbons, which are secreted and transported through the epidermis and the overlaying cuticle to act as waterproofing agents and pheromones (Makki et al., 2014; Wicker-Thomas et al., 2015; Wigglesworth, 1998). We show that dHNF4 supports the transcriptional induction of genes involved in VLCFA and hydrocarbon synthesis shortly after adult emergence and is required in oenocytes to suppress developmental steatosis and maintain resistance to dehydration. These roles appear to be conserved through evolution since HNF4α is required in mouse hepatocytes for the proper expression of genes encoding fatty acid elongases, which are required to suppress hepatic steatosis and allow the survival of newborn pups in a dry environment (Aidahmesh et al., 2011; Sassa et al., 2013; Westerberg et al., 2004). Finally, we demonstrate that dHNF4 acts through hydrocarbon production in oenocytes to preserve glucose homeostasis and suppress dietary sugar toxicity. Taken together, our work demonstrates an essential role for dHNF4 in coupling metabolism with developmental progression by directing the rapid conversion of free fatty acids into VLCFAs and hydrocarbons at the onset of adult life.

RESULTS

dHNF4 Mutants Are Short-Lived, Sensitive to Dietary Sugar, and Hyperlipidemic

All genetic studies used a transheterozygous combination of dHNF4 null alleles (w; dHNF4<sup>177/dHNF4<sup>239>, referred to as dHNF4 mutants) and genetically matched controls that are transheterozygous for precise excisions of the EP2449 and KG08976 P elements (referred to as controls), as described previously (Palanker et al., 2009). To investigate the physiological defects in dHNF4 mutant adults, we reared animals during larval stages on a yeast diet devoid of sugar (0% sugar) and switched them to a 0% or a 15% sugar diet after adult emergence (Figure 1A). dHNF4 mutant adults are short-lived compared to controls regardless of their diet. Consistent with earlier reports, adult-specific exposure to dietary carbohydrates further reduces their lifespan (Figure 1A) (Barry and Thummel, 2016). The overall short lifespan of dHNF4 mutants and their sensitivity to dietary sugar suggests that they suffer from metabolic defects. Consistent with this, dHNF4 mutants have low glucose and glycogen levels at 1 week of age on a sugar-free diet, and slightly lower glycogen and markedly elevated glucose levels when switched to a 15% sugar diet at emergence (Figures 1B and 1C). In contrast, 1-week-old dHNF4 mutants are hyperlipidemic on both diets (Figures 1B and 1C). These results led to the hypothesis that defects in lipid homeostasis contribute to the short lifespan in dHNF4 mutant adults on both the 0% and 15% sugar diets.

dHNF4 Suppresses Adult-Onset Hyperlipidemia

In order to examine lipid metabolism in more detail, we measured triglyceride (TAG) levels in staged control and dHNF4 mutants that were either kept on the sugar-free diet or transferred to a 15% sugar diet at emergence. On the sugar-free diet, controls lose TAG during early adulthood and then recover their stored lipids between days 3 and 7 (Figure 2A). This trend is also observed in controls shifted to the 15% sugar diet at emergence, although the overall changes are reduced (Figure 2B). The early drop in TAG is consistent with the developmental clearance of lipid-containing larval adipose cells, which occurs during the first 2 days of adulthood (Aguila et al., 2007), followed by a recovery of stored lipids due to dietary uptake and de novo lipogenesis. Interestingly, dHNF4 mutants begin their adult life with normal levels of TAG, demonstrating that the defects in lipid homeostasis develop after emergence (Figures 2A and 2B). They then display either a drop and recovery in TAG levels on the sugar-free diet, or a steady increase in TAG on the 15% sugar diet, with a gradual onset of hyperlipidemia on both diets. Taken together, these observations indicate that dHNF4 is required to suppress excess lipid accumulation in newly emerged adults.

Early adulthood is characterized by the clearance of lipid-containing larval adipose cells (Aguila et al., 2007), and our previous work demonstrated that dHNF4 regulates lipid catabolism (Palanker et al., 2009). Thus, the progressive hyperlipidemia in dHNF4 mutants could be explained, at least in part, by reduced clearance of lipid stores. To test this, we analyzed TAG consumption in control and dHNF4 mutants starved after emergence. We transferred newly emerged flies to empty vials sealed with a dense-weave cellulose acetate stopper saturated with water. Remarkably, under these hydrated conditions, control flies survive starvation for up to 10-fold longer than has been reported in the literature (Figure 2C; Aguila et al., 2007; Chatterjee et al., 2014). This suggests that current starvation protocols shorten lifespan by limiting their access to water. Similar results are seen when other control fly lines are subjected to starvation with constant access to hydration, although there are strain-dependent differences in lifespan (Figure S1A). Unexpectedly, dHNF4 mutant adults are resistant to starvation compared to controls, contrary to what has been shown in larvae (Figure 2C; Palanker et al., 2009). In addition, stored lipids appear to play little role in supporting young adult survival under starvation conditions. In starved controls, TAG is cleared after 3 days even though these animals survive for several weeks (Figures 2C and 2D). In contrast, TAG is incompletely cleared in starved dHNF4 mutants and remains stable between days 3 and 7 (Figure 2D). Thus, dHNF4 is required for lipid catabolism, but stored fat is not limiting for the survival of young adults upon starvation.
Analysis of other key metabolites revealed that protein levels decline by about 25% during the first week of starvation, while glucose and glycogen levels drop during the first days of starvation and then partially recover to maintain homeostasis in controls (Figures S1B–S1D). These results suggest that unlike mammals that depend on lipid-derived ketogenesis to survive starvation, fruit flies depend primarily on amino acids to support gluconeogenesis and survival (Cahill, 2006). dHNF4 mutants emerge with less protein, glucose, and glycogen, but these metabolites follow similar trends as controls and normalize by day 7 (Figures S1B–S1D). The relatively normal use of protein and carbohydrates in starved dHNF4 mutants likely explains the ability of these animals to persist under starvation conditions and highlights the central role of dHNF4 in maintaining lipid homeostasis.

dHNF4 Suppresses Developmental Steatosis in Oenocytes

We examined the lipid stores of young adults to identify the tissue(s) contributing to hyperlipidemia in dHNF4 mutants. The larval adipose cells that are free-floating in the hemolymph of

Figure 1. dHNF4 Mutants Display a Reduced Lifespan, Sensitivity to Dietary Carbohydrates, and Defects in Metabolic Homeostasis

(A) Lifespan was scored for controls (blue) and dHNF4 mutants (red) reared on a 0% sugar diet as larvae and transferred to a 0% (dotted line) or a 15% (solid line) sugar diet after adult emergence. Control median lifespan—0% sugar diet: 46 days (n = 46); 15% sugar diet: 75 days (n = 38); p < 0.0001. dHNF4 mutant median lifespan—0% sugar diet: 11 days (n = 31); 15% sugar diet: 6 days (n = 41); p < 0.0001.

(B and C) Glucose, glycogen, and triglyceride levels were measured in 7-day-old control and dHNF4 mutants reared on a 0% sugar diet (B) or a 15% sugar diet (C) after emergence. Metabolite levels are normalized to total protein and presented relative to the amount in control animals.
newly emerged adults are eliminated normally in starved dHNF4 mutants (Figures S2A and S2B). Similarly, the rate of TAG clearance in the hemolymph of both starved and fed young adults appears largely unaffected (Figure S2C). Staining with the lipophilic dye Oil Red O revealed that neutral lipids are progressively lost in larval adipose cells and the fat body of starved controls (Figure 3A, upper and middle panels). A similar pattern is seen in the fat bodies of dHNF4 mutants; however, lipids accumulate rapidly in oenocytes, which are specialized lipid metabolizing cells embedded in the adult fat tissue (Figure 3A, lower panels) (Gutierrez et al., 2007; Makki et al., 2014). This steatosis persists for at least 14 days of starvation (Figure S2D). In contrast, the oenocytes in controls contain small lipid droplets at emergence, which are cleared within 2 days of starvation (red arrowhead, Figure 3A, middle panels). Lipid accumulation in the oenocytes of dHNF4 mutants occurs independently of nutritional status, as it is also observed in animals fed a 0% or a 15% sugar diet (Figure 3B).

**dHNF4 Functions in Oenocytes to Maintain Lipid Homeostasis**

Antibody stains reveal that dHNF4 is expressed at high levels in oenocytes, with low levels of expression in the neighboring adult adipose tissue (Figure S3A). Silencing of dHNF4 expression in oenocytes using the PromE-Gal4 driver and a UAS-dHNF4 RNAi transgene results in lipid accumulation in oenocytes in both starved and fed animals (Figures 4A and S3B). A similar result is seen in the oenocytes of starved females, indicating that steatosis is not a remote effect from dHNF4 RNAi in male accessory glands, where the PromE-Gal4 driver is also expressed (Figure S3C) (Billeter et al., 2009; Bousquet et al., 2012). Conversely, expression of wild-type dHNF4 in the oenocytes of dHNF4 mutants rescues lipid accumulation in these cells, as well as TAG clearance upon starvation (Figures 4B, 4C, and S3D). These results demonstrate that dHNF4 acts in oenocytes to suppress lipid accumulation in these cells and to maintain systemic lipid homeostasis in starved animals.

**Impaired Fatty Acid Elongation Contributes to Developmental Steatosis in dHNF4 Mutant Oenocytes**

Oenocyte-specific RNAi directed against key genes in the fatty acid β-oxidation pathway does not lead to steatosis, demonstrating that this phenotype does not arise from a defect in lipid catabolism (Figure S4A). Rather, dHNF4 might be required for the proper production of VLCFAs and their derivatives called hydrocarbons, which are the primary products of adult oenocytes (Billeter et al., 2009; Makki et al., 2014; Parvy et al., 2012; Wicker-Thomas et al., 2015). Hydrocarbons are secreted to the overlying epidermis and cuticle where they act as pheromones and waterproofing agents (Makki et al., 2014; Wicker-Thomas et al., 2015; Wigglesworth, 1988). Briefly, VLCFA/hydrocarbon biosynthesis starts with palmitate, which is synthesized by ACC and FASN (Figure 5A). This fatty acid is elongated by the successive action of elongase, KAR, HACD, and TER enzymes, before being converted to hydrocarbons (Cinnamon et al., 2016; Qiu et al., 2012; Wicker-Thomas et al., 2015). Analysis of previous RNA sequencing (RNA-seq) data indicates that the transcription of genes involved in VLCFA and hydrocarbon biosynthesis, including KAR, Cyp4g1, Cpr, and many elongases, is reduced in mature adults lacking dHNF4 function (Figure S4B) (Barry and Thummel, 2016). We validated these results in young starved adults by RT-qPCR (Figure S5B). The expression of

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**Figure 2. dHNF4 Suppresses Hyperlipidemia in Young Adults**

(A and B) Triglyceride (TAG) levels were measured in 0 day, 3 day, or 7-day-old controls (blue) and dHNF4 mutants (red) reared on a 0% sugar diet and transferred to a 0% (A) or a 15% (B) sugar diet after adult emergence. TAG levels are normalized to total protein and represented relative to the level in 0-day controls. Black asterisks represent statistically significant differences between controls and mutants at a given time point, while red or blue asterisks represent statistically significant differences between two time points for either controls or mutants.

(C) Lifespan was scored for control and dHNF4 mutants upon starvation under humid conditions. Animals were reared on a 0% sugar diet as larvae and starved at emergence. Median lifespans—controls: 16 days (n = 102); dHNF4 mutants: 23 days (n = 74); p < 0.0001.

(D) Triglyceride (TAG) levels were measured in 0-, 1-, 2-, 3-, 5-, or 7-day-old controls (blue) and dHNF4 mutants (red) reared on a 0% sugar diet and starved at emergence under humid conditions. TAG levels are not normalized to protein because protein diminishes upon starvation (Figure S1B). Data are presented relative to the level in 0-day-old controls. The asterisks represent statistically significant differences between controls and dHNF4 mutants at a given time point.

See also Figure S1.
dHNF4, ACC, KAR, Cpr, Cyp4g1, and three genes encoding elongases (CG30008, CG18609, and CG16904) is induced after emergence and peaks at day 2 in controls, indicating that VLCFA/hydrocarbon biosynthesis is activated shortly after this developmental transition. This induction is blunted in the absence of dHNF4, with major effects on KAR, Cpr, CG30008, and CG18609. dHNF4 is thus required for the developmental induction of key genes in the VLCFA/hydrocarbon biosynthetic pathway at the onset of adulthood. Consistent with this, oenocyte-specific expression of dHNF4 in dHNF4 mutants is sufficient to rescue KAR and CG18609 transcript levels (Figure S4C). The expression of CG30008, however, remains unaffected, suggesting that other tissues can also direct fatty acid elongation in a dHNF4-dependent manner.

The reduced expression of genes involved in VLCFA/hydrocarbon synthesis could contribute to oenocyte steatosis in dHNF4 mutants. Consistent with this possibility, we observed lipid accumulation in the oenocytes of young adults that carry

Figure 3. Oenocytes Become Steatotic at the Onset of Adulthood in dHNF4 Mutants

(A) Oil Red O stains are depicted for larval adipose cells of starved control adults (upper panels), oenocytes and adult fat bodies of starved controls (middle panels), and dHNF4 mutants (lower panels). Animals were reared on a 0% sugar diet as larvae and starved after emergence under humid conditions.

(B) Oil Red O stains are depicted for oenocytes and fat bodies of control and dHNF4 mutants at emergence (day 0) or in 3-day-old animals that were either starved or fed a 0% or 15% sugar diet after emergence.

(A and B) Oenocytes are outlined with a blue dotted line. Larval adipose cells (individual cells or aggregates) are outlined with a yellow dotted line and indicated with a yellow arrowhead. The red arrowhead in day 1 controls indicates small persisting lipid droplets in control oenocytes at this time. Scale bars: 50 μm.

See also Figure S2.
the PromE-Gal4 driver in combination with a UAS-KAR RNAi transgene (Figure 5C). PromE-Gal4-driven expression of ACC, FASN_{CG17374}, Cpr, and Cyp4g1 RNAi transgenes was fully lethal between larval stages and adult emergence, as previously reported (Garrido et al., 2015; Gutierrez et al., 2007; Parvy et al., 2012). To circumvent this lethality, we used the PromE-Gal4, Tub-Gal80ts driver, which allows temperature-inducible RNAi expression in oenocytes. As expected, no developmental lethality was observed when animals were left to develop at the permissive temperature (21°C). Shifting newly emerged adults to the restrictive temperature (29°C) led to mild oenocyte steatosis upon ACC RNAi, indicating that malonyl-CoA production and downstream fatty acid elongation is required to suppress lipid accumulation (Figure 5D). Adult-specific oenocyte RNAi for FASN_{CG17374}, Cpr, and Cyp4g1, however, did not lead to oenocyte steatosis, while RNAi for KAR led to reduced but detectable lipid accumulation relative to our results with the constitutively active PromE-Gal4 driver (Figures 5C and 5D). Steatosis is also observed with a second KAR RNAi transgene (KAR RNAi#2), which was lethal prior to adulthood when driven with PromE-Gal4. In addition, adult-specific dHNF4 RNAi led to abundant lipid accumulation in oenocytes, demonstrating that dHNF4 is required after emergence to suppress steatosis at the onset of adulthood (Figure 5D). Similar results were seen with adults fed a 0% sugar diet (Figure S4D). Taken together, these results suggest that the reduced expression of genes involved in VLCFA elongation (ACC and KAR) is responsible for the steatosis observed in the oenocytes of dHNF4 mutants following adult emergence.

**dHNF4 Mutants Have Reduced Levels of Hydrocarbons and Are Sensitive to Dehydration**

If the VLCFA/hydrocarbon biosynthetic pathway is impaired in newly emerged dHNF4 mutants, then we should see reduced hydrocarbon production. To test this hypothesis, we used small-molecule gas chromatography-mass spectrometry (GC-MS) to appreciate...
measure hydrocarbon levels in controls and dHNF4 mutants at emergence, and in 3-day-old fed and starved animals (Figures 6A and S5A). Consistent with the temporal profile of VLCFA/hydrocarbon biosynthetic gene expression, most hydrocarbons are either low or absent at emergence but increase in 3-day-old controls. This can be seen for 7(Z)-tricosene, tricosane, and 7(Z)-pentacosene, which are abundant in males, along with 7(Z), 11(Z)-heptacosadiene, and a number of other hydrocarbons (Figures 6A and S5A) (Everaerts et al., 2010). Importantly, hydrocarbons are produced in starved controls, suggesting that pre-existing larval fat stores provide an important source of precursors for this pathway. Dietary input, however, is required for maximal hydrocarbon biosynthesis, as levels increase in fed adults (Figures 6A and S5A). Interestingly, the synthesis of some hydrocarbon species could rely specifically on dietary lipids, as suggested by the major increases in tricosane 3, heneicosane, and hydrocarbon 2;Figures 6A and S5A). Impor-
carried over into the young adult (7(Z), 11(Z)-heptacosadiene, starved animals (Figure S5A). In addition, a few hydrocarbons are already present at moderate levels at emergence, suggesting that they are synthesized at an earlier developmental stage and carried over into the young adult (7(Z), 11(Z)-heptacosadiene, nonacosene, and hydrocarbon 2;Figures 6A and S5A). Importantly, many hydrocarbons are significantly reduced in fed or starved dHNF4 mutants, demonstrating that this nuclear receptor is required for proper hydrocarbon biosynthesis. In contrast, heneicosane levels are unaffected by a loss of dHNF4 function suggesting that this hydrocarbon is produced through a distinct pathway (Figure S5A).

The hydrophobic properties of hydrocarbons reduce trans- epidermal water loss and play a major role in desiccation resistance (Qiu et al., 2012; Wicker-Thomas et al., 2015). Consistent with this function, dHNF4 mutants die significantly faster than controls when challenged by dry starvation in the absence of both food and water (Figures 6B and S5B). dHNF4 silencing in oenocytes recapitulates this desiccation sensitivity, and oenocyte-specific expression of wild-type dHNF4 in mutants rescues this phenotype (Figures 6C and 6D). Moreover, dHNF4 mutants lose more water than controls when exposed to dry conditions, but fluid homeostasis is rapidly restored when these animals are returned to a humid environment (Figure 6E). We further tested for defects in the hydrophobic coating of the cuticle by collecting dead dry control and dHNF4 mutant flies following dry starvation and incubating them in water. While control carcasses remain at the surface of the liquid following a 2.5-hr incubation, dHNF4 mutant carcasses sink below the surface (Figure S5C). After a 24-hr incubation in water, dHNF4 mutant carcasses swell because of passive incorporation of liquid, while only mild fluid incorporation is seen in control carcasses (Figure 6F). In addition, the expression of dHNF4 in the oenocytes of dHNF4 mutants blocks passive water incorporation in dried carcasses (Figure S5D). Taken together, these results demonstrate that dHNF4 is required in oenocytes for cuticular hydrophobicity, reducing trans-epidermal water loss, and providing tolerance to dry conditions.

Interestingly, mice mutants for Elov1 and Elov4, which encode fatty acid elongases, die shortly after birth from acute dehydration and display a loss of epidermal hydrophobic barrier function (Cameron et al., 2007; Sassa et al., 2013). This observation raises the possibility that the regulatory link between VLCFA biosynthesis and HNF4 may be conserved through evolution. To test this hypothesis, we examined the expression levels of several fatty acid elongase genes in both control and HNF4α mutant mouse hepatocytes, where they are normally abundantly expressed (Moon et al., 2009, 2014; Wang et al., 2006). Specific loss of HNF4α in mature hepatocytes results in significant reductions in KAR, Elov3, and Elov5 mRNA levels (Figure 6G). Interestingly, the transcriptional level of Elov6 is elevated in HNF4α mutant hepatocytes, suggesting that the receptor regulates specific steps in the VLCFA biosynthetic pathway. We conclude that the regulation of VLCFA biosynthesis is an evolutionarily conserved function for HNF4.

dHNF4 Acts in Oenocytes to Suppress Sugar Toxicity

The central role of dHNF4 in oenocyte function and lipid metabolism at the onset of adulthood raises the possibility that these activities could contribute to the reduced lifespan of dHNF4 mutants (Figure 1). As a first step toward testing this hypothesis, we examined the lifespan of either control adults (PromE>mCherry RNAi) or animals with oenocyte-specific dHNF4 RNAi (PromE>dHNF4 RNAi) maintained on a sugar-free diet, or raised on this diet and then transferred to 15% sugar after emergence (Figure 7A). As seen in our earlier study of dHNF4 mutants (Figure 1A), controls display an increased lifespan in response to dietary sugar (solid blue curve, Figure 7A). In contrast, dHNF4 RNAi animals display a major reduction in lifespan, demonstrating that dHNF4 is required in oenocytes to suppress sugar toxicity (solid red curve, Figure 7A). Of note, controls and dHNF4 RNAi animals have similar survival curves on a sugar-free diet (dotted blue and red curves, median lifespans: 31 days and 36 days, respectively, p = 0.502; log-rank Mantel-Cox test) (Figure 7A). Moreover, oenocyte-specific expression of wild-type dHNF4 in mutants dramatically prolongs the lifespan of adults transferred to a 15% sugar diet and partly rescues their metabolic defects (purple in Figures 7B and 7C). Consistent with the absence of lipid accumulation in oenocytes (Figure 4B), oenocyte-specific dHNF4 rescue reduces triglyceride levels in mutants (Figure 7C). These levels, however, remain elevated relative to controls, suggesting that dHNF4 acts in multiple tissues to maintain systemic lipid homeostasis. Similarly, oenocyte-specific dHNF4 rescue partly rescues the elevated glucose levels seen in mutants (Figure 7C). dHNF4 is thus required in oenocytes for maintaining triglyceride and glucose homeostasis, as well as suppressing the deleterious effects of a sugar diet.

dHNF4 is required in oenocytes for hydrocarbon production, suppressing dietary sugar toxicity and maintaining glucose homeostasis, raising the possibility that these physiological processes are linked. In support of this hypothesis, adult-specific silencing of several genes involved in hydrocarbon biosynthesis (PromE-Gal4, tub-Gal80ts, >ACC, >KAR, or >Cyp4g1 RNAi) leads to elevated glucose levels and reduces the longevity of animals raised on a 15% sugar diet (Figures 7D, 7E, S6A, and S6B). Importantly, these physiological defects appear to be linked to the inability to maintain fluid homeostasis. Raising animals with oenocyte-specific Cyp4g1 RNAi induced at emergence in high humidity restores both their longevity and systemic glucose levels when fed a 15% sugar diet (Figures 7D and 7E). Similarly, humidity is sufficient to rescue the sensitivity of dHNF4 mutants to a sugar diet. Increasing humidity on a sugar-free diet has little
Figure 5. *dHNF4* Is Required for the Proper Expression of Genes Involved in VLCFA and Hydrocarbon Synthesis

(A) A simplified representation of the VLCFA and hydrocarbon biosynthetic pathway is depicted along with the *Drosophila* genes (in blue) that encode key enzymes in this pathway. Elongase, HACD, and TER are each encoded by multiple genes.

(B) RT-qPCR analysis of *dHNF4* mRNA and transcripts encoding key steps in the VLCFA/hydrocarbon metabolic pathway in starved controls (blue bars) and starved *dHNF4* mutants (red bars) after adult emergence. Transcript levels were analyzed at 0, 1, 2, and 3 days or 0 and 2 days after adult emergence. Transcript levels are normalized to *rp49* mRNA and presented relative to the level in day 0 controls. Asterisks represent statistically significant differences with control transcripts at the same time point.

(C) Oil Red O stains are depicted for oenocytes and adult fat bodies from 7-day-old starved controls (*PromE>mCherry RNAi*) and animals with oenocyte-specific *KAR RNAi* (*PromE>KAR RNAi*) reared at 25°C.
effect on the lifespan of dHNF4 mutants (Figure 7F, pink curves), while the same conditions on a 15% sugar diet dramatically prolong mutant lifespan (Figure 7F, red curves), with animals surviving longer than those fed a sugar-free diet (Figure 7F, right panel).

In addition, humidity prolongs the lifespan of animals with oenocyte-specific loss of dHNF4 function on a 15% sugar diet, while it has no significant effect on the lifespan of mutants with oenocyte-specific dHNF4 rescue (Figures S6C and S6D). This indicates that increased humidity specifically compensates for the defects associated with the loss of dHNF4 in the oenocytes of animals with systemic dHNF4 loss-of-function. Finally, we found that while increased humidity has no effect on glycogen or TAG levels in dHNF4 mutants transferred to a 15% sugar diet at emergence, it restores their elevated glucose levels (Figures 7G and S6E). Taken together, our results support the model that the inability of dHNF4 mutants to produce hydrocarbons and maintain systemic fluid homeostasis contributes to their dietary sugar sensitivity and defects in glucose homeostasis.

DISCUSSION

dHNF4 Is Required to Coordinate VLCFA/Hydrocarbon Production with Adult Emergence

Drosophila breeds and feeds on rotting fruits, which are ephemeral ecosystems. Thus, while the Drosophila larva lives inside a semi-liquid nutritive substrate and feeds extensively to support its rapid increase in mass, the mature adult fly emerges into an unpredictable environment where it uses flight for dispersal and the colonization of more favorable niches. Similar to all insects, the large surface-to-volume ratio of Drosophila adults makes them vulnerable to dehydration (Albers and Bradley, 2004). In addition, the metabolic demands of flight require a high rate of gas exchange via the respiratory system, increasing water loss (Lehmann, 2001). We show here that the successful transition from a stationary and protected pupa to a motile adult that can survive in a dry environment is dependent on the dHNF4 nuclear receptor, which acts in the oenocytes to direct a transcriptional program that supports the rapid production of VLCFAs and cuticular hydrocarbons. This activity is consistent with earlier studies, which have demonstrated an important role for oenocytes in VLCFA/hydrocarbon synthesis and desiccation resistance, and places dHNF4 as a central transcriptional regulator for oenocyte function (Chatterjee et al., 2014; Chiang et al., 2016; Cinnamon et al., 2016; Köhler et al., 2009; Makki et al., 2014; Qiu et al., 2012; Wicker-Thomas et al., 2015).

One of the few larval tissues to escape autophagic cell death during metamorphosis is the larval fat body, which dissociates into individual adipose cells in pupae (Nelliott et al., 2006). These cells persist through metamorphosis as free-floating larval cells within the hemolymph of the animal and begin to clear at adult emergence, with complete loss by day 2 of adulthood due to apoptosis (Agüla et al., 2007; Butterworth, 1972; Figures S2A–S2C). The larval adipocyte cells have been proposed to act as an energy reserve for the newly emerged adult, prior to feeding (Agüla et al., 2007). In support of this, newly emerged adults are more starvation resistant than mature adults (Agüla et al., 2007). We show, however, that whole-body and hemolymph TAG levels drop to low levels between emergence and day 3 of adulthood, on either a sugar-free diet or a rich 15% sugar diet (Figures 2A, 2B, and S2C). The lipids contained in larval adipose cells thus appear to be consumed independently of nutritional input, suggesting that they contribute to non-energetic functions. Moreover, control adults can survive for weeks in the absence of stored lipids, further indicating that they provide a relatively minor source of energy at this stage in development (Figures 2C and 2D).

We propose that lipids in the newly emerged adult are shunted toward a purpose that is more profound than energy production at this stage in development—the formation of a waterproof cuticle—and that this occurs in a dHNF4-dependent manner. Stored lipid levels are unaffected in newly emerged dHNF4 mutant adults relative to controls (Figures 2A and 2B). Lipids, however, accumulate rapidly after this stage in oenocytes, in the presence or absence of nutrients, representing a developmental role for dHNF4 in maintaining lipid homeostasis in this cell type (Figures 3A and 3B). The timing of lipid accumulation in the oenocytes of starved and fed dHNF4 mutants coincides with the timing of lipid loss and larval adipose cell death (Figures 3A, 3B, and S2A–S2C). Our genetic studies demonstrate that dHNF4 is specifically required in oenocytes to suppress developmental steatosis (Figures 4A and 4B). This appears to be due to its role in inducing the expression of genes involved in VLCFA elongation. Oenocyte-specific silencing of KAR or ACC recapitulates the developmental steatotic phenotype, while silencing genes that encode downstream or upstream steps in the hydrocarbon biosynthetic pathway (FASN<sup>Cpr</sup>, Cpr, or Cyp4g1) has no effect on the lipid levels in oenocytes (Figures 5C, 5D, and S4D). Further studies are needed to determine the molecular mechanisms that link VLCFA elongation with oenocyte lipid homeostasis. Finally, adults normally emerge with low hydrocarbon levels that increase dramatically over 3 days, even in the absence of food, in agreement with the hypothesis that the larval adipose cells provide the primary source of precursors for hydrocarbon production (Figures 6A and S5A). These results are consistent with previous reports, which have shown that dietary manipulation during larval life affects the blend of hydrocarbons in young adults (Stefana et al., 2017; Wicker-Thomas et al., 2015). Altogether, these observations support the model that dHNF4 mediates the rapid conversion of persisting larval fat stores into hydrocarbons shortly after adult emergence.

Drosophila continuously shed and produce hydrocarbons throughout adulthood, indicating that this biosynthetic pathway is active at later stages to maintain a hydrophobic cuticle (Stefana et al., 2017). Consistent with this, we find that oenocyte-specific dHNF4 RNAi in mature adults leads to a similar degree
Figure 6. dHNF4 Is Required for Hydrocarbon Production and Tolerance to Desiccation in Newly Emerged Adults
(A) Hydrocarbon levels are depicted as determined by GC-MS at emergence (day 0), in 3-day-old starved flies, and in 3-day-old flies fed a 0% sugar diet for both controls and dHNF4 mutants. Values are presented relative to the amount in 3-day-old fed controls. The identity of each hydrocarbon was confirmed by comparison with purified standards.
(B) Longevity was scored for controls and dHNF4 mutants starved in the absence of water. Median lifespans—controls: 13 hr (n = 132) (extrapolated using control data from Figure S5B); dHNF4 mutants: 9 hr (n = 48); p < 0.0001.
(C) Longevity was scored for controls (PromE>mCherry RNAi, blue) and animals with oenocyte-specific dHNF4 RNAi (PromE>dHNF4 RNAi, red) in the absence of water. Median lifespans—controls: 10 hr (n = 95); PromE>dHNF4 RNAi animals: 7 hr (n = 113); p < 0.0001.
(D) Longevity was scored for dHNF4 mutants (dHNF4−, UAS-dHNF4, red) and dHNF4 mutants with oenocyte-specific dHNF4 rescue (dHNF4−, PromE>dHNF4, blue) in the absence of water. Median lifespans—dHNF4 mutants: 8 hr (n = 141); dHNF4−, PromE>dHNF4 animals: >10 hr (n = 254); p < 0.0001.

(legend continued on next page)
of lipid accumulation as that seen upon inducing RNAi at emergence (Figure S7E). Further studies are required to address this continuing role for dHNF4 in mature adults. In addition, the mechanisms that partition stored lipids toward VLCFA/hydrocarbon production or energy production remain to be determined. Finally, the key functions we describe for dHNF4 in oenocytes are shared between males and females, indicating that they are not sex specific (Figures S3C and S7A–S7D).

**Humid Conditions Are Required to Assess the Starvation Resistance of Adult Drosophila**

Our studies of starvation resistance indicate that newly emerged *Drosophila* adults can tolerate the absence of nutrients for at least a week. During the non-feeding period of metamorphosis, pupae consume about half of their glycogen and TAG stores and most of the trehalose gathered during the larval stages (Matsuda et al., 2015). In spite of this depletion of energy reserves, sufficient nutrients persist to allow the newly emerged adult to survive and disperse in their new environment. Under hydrated conditions, control lines emerge with the ability to survive from 1 to 3 weeks in the absence of nutrients (Figures 2C and S1A). This contrasts with published data, most of which report survival of *Drosophila* adults for up to 3 days (Aquilla et al., 2007; Chatterjee et al., 2014). These assays use either moist filter paper or PBS in 1% agar as a medium for starvation (Tennesen et al., 2014). We suggest that these conditions provide insufficient humidity to accurately distinguish the effects of dehydration from the effects of nutrient depletion. Without moisture or food, *Drosophila* die in less than a day, with a median lifespan of 13 hr (Figures 6B and S5B). Thus, as is well known in mammals, sufficient hydration is critical for assessing the ability of animals to properly mobilize stored nutrients for survival during starvation.

**dHNF4 Regulates the Expression of Genes in the VLCFA/Hydrocarbon Biosynthetic Pathway**

Changes in metabolism must accompany each stage in development in order to allow normal progression through the life cycle. Although little is known about how development and metabolism are coupled, nuclear receptors appear to play a central role in this process (Li and Tennesen, 2017). Consistent with this, our previous study of *dHNF4* showed that it is highly up-regulated at the end of metamorphosis as the fly begins its adult life, coordinately inducing genes involved in glucose homeostasis and oxidative phosphorylation (Barry and Thummel, 2016). Functional studies showed that *dHNF4* acts at this stage in the insulin-producing cells to maintain glucose-stimulated insulin secretion and acts in the fat body to promote glucose clearance. Here, we expand upon these activities for *dHNF4* at the onset of adulthood to include an essential role in supporting VLCFA and hydrocarbon production in the oenocytes to allow adult survival and dispersion.

Key genes involved in VLCFA/hydrocarbon production are induced at the onset of adulthood in control adults and reduced in expression in *dHNF4* mutants, including *KAR, Cpr, Cyp4g1*, and several genes encoding predicted elongases (Figures 5A and 5B). In addition, *dHNF4* transcripational activity can be activated by LCFA and VLCFA, which appear to act as ligands for this nuclear receptor (Palanker et al., 2009). This is consistent with studies of mammalian HNF4*α*, which have identified LCFA as ligands that can trigger the conformational changes required for coactivator recruitment (Duda et al., 2004; Wisely et al., 2002; Yuan et al., 2009). Thus, the free fatty acids generated by lipolysis from the larval adipose cells could act as ligands for *dHNF4* in oenocytes, driving the transcription of genes in the VLCFA/hydrocarbon pathway. The resulting VLCFAs might further activate *dHNF4*, providing a feed-forward loop to enhance VLCFA/hydrocarbon production. These levels of regulation could ensure that free fatty acids are rapidly and efficiently converted into VLCFAs and hydrocarbons to provide an effective waterproof barrier for the young adult.

**dHNF4 Links Systemic Fluid Homeostasis with Tolerance to Dietary Sugars**

Simple carbohydrates are required for optimal *Drosophila* longevity, yet *dHNF4* and VLCFA/hydrocarbon biosynthesis are required in oenocytes to provide tolerance to these nutrients (Figures 7A, 7B, 7D, and S6A). In addition, increased humidity is sufficient to rescue sugar toxicity and hyperglycemia in *dHNF4* mutants, suggesting that defects in fluid homeostasis are contributing to these phenotypes (Figures 7F and 7G). Defects in fluid homeostasis could occur in *dHNF4* mutants because of impaired waterproofing of the cuticle or the trachea. Parvy and colleagues demonstrated that, besides providing precursors for hydrocarbon synthesis, VLCFA metabolism in oenocytes is required to remotely waterproof the respiratory system in larvae (Parvy et al., 2012). Notably, disrupting VLCFA production in oenocytes via ACC, KAR, or FASN*CG17374* RNAi in this cell type results in defects in tracheal waterproofing and larval lethality. We also observed lethality between larval and pupal stages when we drove these RNAi constructs with a constitutive oenocyte-specific GAL4 driver. However, we do not see defects in tracheal air filling or lethality in *dHNF4* mutant larvae or in animals with constitutive *dHNF4* RNAi expression in oenocytes, suggesting that there are no significant effects on tracheal waterproofing in these animals. In addition, altering cuticular hydrocarbon production while leaving VLCFA synthesis intact (by silencing *Cyp4g1* in oenocytes) induces sugar toxicity and defects in glucose homeostasis (Figures 7D and 7E). We thus postulate that reduced hydrocarbon production by oenocytes and altered...
Figure 7. dHNF4 Is Required in Oenocytes for Suppressing Dietary Sugar Toxicity and Maintaining Systemic Glucose Homeostasis

(A) Lifespan was scored for controls (PromE>mCherry RNAi, blue) and animals with oenocyte-specific dHNF4 RNAi (PromE>dHNF4 RNAi, red) reared on a 0% sugar diet as larvae and transferred to a 0% or a 15% sugar diet after adult emergence (dotted and solid lines, respectively). Control median lifespan—0% sugar diet: 31 days (n = 49); 15% sugar diet: 48 days (n = 41); p < 0.0001. PromE>dHNF4 RNAi median lifespan—0% sugar diet: 36 days (n = 49); 15% sugar diet: 20 days (n = 50); p < 0.0001.

(B) Lifespan was scored for dHNF4 mutants (dHNF4–, UAS-dHNF4, red line) and dHNF4 mutants with oenocyte-specific dHNF4 rescue (dHNF4–, PromE>dHNF4, purple line) reared on a 0% sugar diet as larvae and transferred to a 15% sugar diet after emergence. Median lifespan—dHNF4 mutants: 3 days (n = 93), dHNF4–, PromE>dHNF4 animals: 54 days (n = 134); p < 0.0001.

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cuticular waterproofing play central roles in the physiological defects we observe in dHNF4 mutants. Our understanding of diabetes in humans provides possible models to explain the physiological mechanisms that link dHNF4 activity in oenocytes to fluid and carbohydrate homeostasis. In mammals, excess blood glucose cannot be effectively reabsorbed by the kidney and is excreted with urine (Lawrence, 1940). This induces osmotic diuresis (an increase in urination volume) to dilute the glucose osmolyte. Increased urination can lead to dehydration that further increases hyperglycemia and produces a hyperosmolar hyperglycemic state, which is a major contributor to diabetic mortality (Pasqual and Umpeirez, 2014; Rosenbloom, 2010). One possibility is that dHNF4 mutants provide a sensitized genetic context for the development of a hyperosmolar hyperglycemic state in Drosophila. Increased transepidermal water loss could reduce hemolymph volume, contributing to the development of hyperglycemia on a sugar-containing diet. This hypothesis could explain the elevated glucose levels observed in dHNF4 mutants and the normalization of glucose levels in high humidity. Further studies are required to test this possibility and dissect the mechanisms that couple dHNF4 function in oenocytes to systemic carbohydrate homeostasis.

A number of studies have focused on characterizing the signaling pathways that govern glucose sensing and metabolism in Drosophila (Ghosh and O’Connor, 2014; Havula et al., 2013; Matsuda et al., 2015; Owusu-Ansah and Perrimon, 2014; Telemann et al., 2012). These studies have provided evidence for glucose-induced cellular damage via oxidative stress or the formation of advanced glycation end-products (Garrido et al., 2015; Teesalu et al., 2017). In spite of these efforts, however, more remains to be learned about the mechanisms underling dietary sugar toxicity. Our work suggests unexpected roles for oenocyte lipid metabolism and hydration status in regulating glucose homeostasis and suppressing sugar toxicity in Drosophila.

Roles for HNF4 in the Synthesis of VLCFAs Are Conserved through Evolution

Interestingly, the role of VLCFAs in reducing transepidermal water loss is conserved through evolution (Nakamura et al., 2014). Mutations in genes encoding VLCFA acyl-CoA synthetases ACS-20 and ACS-22 result in disrupted cuticular barrier function and hypo-osmolarity in C. elegans (Kage-Nakadai et al., 2010). Similarly, mouse mutants for Elovl1 and Elovl4 are born normally but die shortly thereafter from acute dehydration, similar to dHNF4 mutant adults (Cameron et al., 2007; Li et al., 2007; McMahon et al., 2007; Sassa et al., 2013; Vasiredy et al., 2007). This lethality is accompanied by reduced epithelial barrier function, defects in the lamellar structure of newborn skin, reduced levels of VLCFAs, and the absence of key acylceramides that contribute to skin hydrophobicity. Genetic studies of the gene encoding ELOVL3, which elongates C20 fatty acids to C22 and C24, revealed similar functions. Elovl3 mutant mice have disrupted skin and hair morphology resulting in increased transepidermal water loss (Westerberg et al., 2004). In addition, Elovl5 and Elovl6 mouse mutants display hepatic steatosis, similar to the lipid accumulation we see in dHNF4 mutant oenocytes (Moon et al., 2009; Moon et al., 2014). Taken together, these results indicate that VLCFA production is critical for proper lipid metabolism and survival after birth in mouse, revealing a perinatal transition that parallels the role for VLCFAs and hydrocarbons in newly emerged Drosophila adults. Interestingly, our studies also link Elovl gene expression to HNF4α function in mice. We show that Elovl3, Elovl5, and KAR are all expressed at reduced levels in HNF4α mutant hepatocytes, indicating that this regulatory link is conserved through evolution (Figure 6G). Further studies are required to determine if HNF4α exerts a similar role in mammalian skin and if its activity is required for epithelial barrier function and desiccation resistance.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - **Drosophila Strains and Handling**
  - **Fly Diets**
  - **HNF4α Mutant Mouse Hepatocytes**
- **METHOD DETAILS**
  - **Lifespan Studies on 0% and 15% Sugar Diets**
  - **Studies on Diets with Increased Humidity**
  - **Starvation**
  - **Dry Starvation**

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(C) Triglyceride, glucose, and glycogen levels were measured in 7-day-old controls (Cont.), dHNF4*, UAS-dHNF4 mutants (dHNF4*) and dHNF4 mutants with oenocyte-specific dHNF4 rescue (Rescue) transferred to a 15% sugar diet after emergence. Metabolite levels are normalized to total protein and presented relative to the amount in control animals.

(D) Lifespan was scored for animals with oenocyte-specific Cyp4g1 RNAi induced at emergence in different dietary and humidity conditions. Animals were reared as larvae on a 0% sugar diet at room temperature and transferred to a 0% sugar diet (blue line) or a 15% sugar diet (red lines) with or without increased humidity (red dotted and solid lines, respectively) at 25°C to induce RNAi expression. Median lifespan: 0% sugar diet: 20 days (n = 50), 15% sugar diet: 13 days (n = 70), 15% sugar diet with increased humidity: >35 days (n = 67), p < 0.0001.

(E) Glucose levels were measured in 7-day-old PromEl-GAL4, tub-Gal80ts, UAS-mCherry RNAi controls (mCherry RNAi, blue box), and animals with oenocyte-specific Cyp4g1 RNAi induced at emergence (Cyp4g1 RNAi, red boxes) transferred to a 15% sugar diet with or without increased humidity after adult eclosion.

(F) Lifespan was scored for dHNF4 mutants reared on a 0% sugar diet as larvae and transferred to a 0% sugar diet (pink) or a 15% sugar diet (red) with or without increased humidity (dotted and solid lines, respectively) after adult emergence. The panel on the right depicts the median lifespans (numbers above each bar) and number of animals examined.

(G) Glucose levels were measured in 7-day-old controls and dHNF4 mutants reared on a 0% sugar diet and transferred to a 15% sugar diet at adult emergence, with or without increased humidity. See also Figures S6 and S7.
SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at https://doi.org/10.1016/j.devcel.2018.11.030.

AUTHOR CONTRIBUTIONS


SUPPLEMENTAL INFORMATION


ACKNOWLEDGMENTS

The authors would like to thank the Bloomington Stock Center, the Transgenic RNAi Project (TRiP), FlyBase, B. Dauwalter, A. Gould, and B. Mollereau for providing fly stocks; M. Peltier for help with the lifespan assays; and F. Leulier for comments on the manuscript. Metabolomics analysis was performed at the Metabolomics Core Facility at the University of Utah, which is supported by the NIH (S10 OD016232-01, S10 OD021505-01, and U54 DK110858-01). G.S. was supported by the NIH (R01DK108941, C.S.T.).

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AUTHOR CONTRIBUTIONS

C.S.T. supervised the work. G.S. designed and interpreted experiments with input from H.-J.N. G.S. and H.-J.N. performed the experiments. J.S. and C.J.V. generated the HNF4α mutant hepatocytes and produced cDNA. G.S. and C.S.T. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

References


genetic analysis identifies a role for the lipid desaturase Desat1 in starvation-induced autophagy in Drosophila. Autophagy 5, 980–990.


### Key Resources Table

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### Experimental Models: Cell Lines

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### Experimental Models: Organisms/Strains

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- *D. melanogaster: w 1; HNF4α-CyO, twi-Gal4, UAS-GFP*  
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  This paper  
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(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Carl Thummel (carl.thummel@genetics.utah.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Drosophila Strains and Handling**

A detailed list of fly strains and genotypes used for these studies is provided in the Key Resources Table. dHNF4 mutants are transheterozygotes for dHNF4 null alleles (w-; dHNF4D17/dHNF4D33), which were generated previously by imprecise excision of P-element insertions (EP2449 and KG08976) as described (Palanker et al., 2009). Genetically-matched controls throughout the paper are transheterozygotes for precise excisions of the EP2449 and KG08976 P-elements (w-; EP2449/KG08976). For tissue-specific transgene expression, we used the PromE-Gal4 driver (also known as P{Desat1-GAL4.E800}) and the temperature-sensitive PromE-Gal4, Tub-Gal80ts driver. PromE-Gal4 drives expression in larval and adult oenocytes, as well as in the male accessory gland (Billeter et al., 2009; Bousquet et al., 2012). For gene silencing, we used RNAi transgenes from the Transgenic RNAi Project (TRiP, https://fgr.hms.harvard.edu/fly-in-vivo-rnai), available through the Bloomington Drosophila Stock Center (BDSC). Tissue-specific expression of UAS-mCherry RNAi was used as control. Fly strains were routinely kept at room temperature on a standard diet composed of yeast, cornmeal, corn syrup, and malt. For experiments, parents were crossed in six ounce square bottom plastic bottles (Genesee Scientific Cat# 38354) containing the 0% sugar diet (recipe below) at 25°C unless otherwise stated. Progeny were collected at emergence and transferred to Drosophila polystyrene vials (Genesee Scientific Cat# 32-110) at a density of
5-10 animals/vial. Files were kept at different temperatures for different experiments as described in the figure legends or as stated below. Males were used for all experiments, except for Figures S3C and S7A–S7D.

**Fly Diets**

0% sugar diet: For 1L: 80g active dry yeast, 10g agar, 0.5g MgSO4, 0.5g CaCl2. 15% sugar diet: For 1L: 80g active dry yeast, 10g agar, 50g sucrose, 100g glucose, 0.5g MgSO4, 0.5g CaCl2. Ingredients were mixed with water and brought to boil in a microwave oven. After cooling, 4 mL of 99% propionic acid and 5.2 g methyl 4-hydroxybenzoate sodium salt (Sigma-Aldrich Cat# 85265-500G) was added to the mixture and the solution was poured into vials or bottles.

**HNF4α Mutant Mouse Hepatocytes**

All mouse procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah. Animals were maintained in 12 hr light/12 hr dark cycles at 22°C–24°C and given constant access to water and Teklad global soy protein-free diet (Cat# 2920x-030917M). Food was withdrawn on the day of experimentation. HNF4αlox/lox mice were bred to C57BL/6J mice for five generations before crossing to homozygosity (Hayhurst et al., 2001). At 12 weeks of age, male HNF4αlox/lox mice received an intravenous injection of 400 µL of adeno-associated virus 8 (AAV8) encoding either eGFP (AAV8-TBG-eGFP) or Cre recombinase (AAV8-TBG-Cre) regulated by thyroid hormone binding globin (TBG) to drive hepatocyte specific expression (titre 10^{12} genome copies/mL) (Hayashi et al., 1993; Simcox et al., 2017). One week after virus administration, the mice were fasted for 5 hours starting at 10am. At 3pm the mice were anesthetized using 5% isoflurane until unresponsive to pedal reflex test and then sacrificed by cervical dislocation. Tissues were harvested and flash frozen in liquid nitrogen for later processing.

**METHOD DETAILS**

**Lifespan Studies on 0% and 15% Sugar Diets**

Animals were collected at adult emergence (eclosion) and transferred to polystyrene vials (Genesee Scientific Cat# 32-110) containing fly food at a density of 5-10 males per vial at 25°C unless otherwise stated. Vials were closed with a dense weave cellulose acetate stopper (Flug; Genesee Scientific Cat# 49-101) to reduce water evaporation from the food. Animals were transferred weekly to vials containing fresh media and lethality was assayed every 1-2 days. Experiments were repeated two to three times and a representative experiment was selected for the figures.

**Studies on Diets with Increased Humidity**

Animals were collected at emergence and transferred to polystyrene vials (Genesee Scientific Cat# 32-110) containing fly food at a density of 5-10 males per vial. To increase humidity, a dense weave cellulose acetate stopper (Genesee scientific Cat# 49-101) was saturated with water and covered with parafilm. The lifespan and metabolite studies in increased humidity shown in Figures 7F, 7G, S6C, S6D, and S6E were performed at room temperature (approximately 21°C) to avoid excessive condensation in the vials. For the lifespan and metabolite studies performed with the PromE>, tub-Gal80ts> driver in increased humidity (Figures 7D and 7E), animals were raised at room temperature during development and transferred to 25°C (instead of 29°C) to avoid excessive condensation in the vials while allowing PromE-Gal4 driven expression of UAS-transgenes. Non-humid controls for these experiments were kept at room temperature or 25°C depending on the experiment. Experiments were repeated two to three times, pooled data from these experiments is depicted in Figures 7F, S6C, and S6D, while a representative experiment is presented in Figure 7D.

**Starvation**

Polystyrene vials (Genesee Scientific Cat# 32-110) were half filled with water and a dense weave cellulose acetate stopper (Genesee Scientific Cat# 49-101) was pushed to the bottom of the vial allowing saturation of this artificial substrate with water. Excess water was discarded and newly-emerged flies were transferred to these vials at a density of 5-10 males per vial. Vials were sealed with a second dense weave cellulose acetate stopper to reduce water evaporation. For most starvation experiments, animals were kept at room temperature (approximately 21°C) and transferred to a new starvation vial twice a week. Some starvation experiments were conducted at 29°C (Figure 5D). In this case, animals were transferred daily to fresh starvation vials and collected at 3-5 days after emergence for Oil Red O stains. We noticed that despite daily transfers to fresh hydrated vials, the lifespan of starved animals at 29°C is dramatically reduced compared to animals kept at room temperature.

**Dry Starvation**

For starvation in dry condition (Figures 6B–6D, S7B, and S7C), adults were collected at emergence and kept in standard (hydrated) starvation conditions at a density of 5-10 animals/vial for three days, to provide time for hydrocarbon synthesis (Figures 5B, 6A, and S5A). Animals were then transferred to empty polystyrene vials sealed with a dense weave cellulose acetate stopper and kept at room temperature. Lethality was scored hourly for 10 hours. Experiments were conducted at least three times. Pooled data from these experiments are shown in the figures.
**Fly Water Content Determination**

Animals were collected at emergence and kept in standard (hydrated) starvation conditions at a density of 5-10 males/vial for three days before being anesthetized using CO2. Pools of ten animals were weighed using a precision scale (T=0 hour). Animals were then exposed to dry conditions by transferring them to empty polystyrene vials sealed with a dense weave cellulose acetate stopper at room temperature. After 2.5 hrs of dry starvation, animals were anesthetized and weighed with a precision scale (T=2.5 hours). Animals were then transferred to humid starvation conditions and weighed after a 2 hr recovery period (T=4.5 hours) before being rapidly killed by exposing them to -20°C. The following day, carcasses were exposed to the open air and left to dry for a period of 4 days, after which they were weighed in pools of 10 to determine their dry weight. Fly water content (in percentage of body weight) at each time point was calculated using the formula: Water content = ((WeightT – average dry weight)/WeightT) x 100. The dry weight was assumed to be constant between T=0 and T=4.5 hours.

**Metabolite Assays**

Whole body glycogen and glucose levels were determined using a hexokinase assay kit (Sigma GAHK20) and amyloglucosidase (Sigma A1602-25MG). Whole body triglyceride levels were determined using the triglyceride reagent (Sigma T2449-10ML) and the free glycerol reagent (Sigma F6428-40ML). Metabolite assays were performed as described with minor modifications (Tennesen et al., 2014). Unless otherwise stated, glucose, glycogen and triglyceride levels were normalized to protein to compensate for variations in sample homogenization and/or animal size. Protein levels were determined using a Bradford assay (BioRad Cat# 5000006). At least four replicates of 4-5 males were used for each sample in metabolite assays. Independent experiments were performed 2-3 times. Figures illustrate a representative experiment (Figures 1B, 1C, 4C, 7C, 7E, 7G, S6B, and S6E) or pooled data from all experiments (Figures 2A, 2B, 2D, S1B–S1D, and S2C). For triglyceride measurements in hemolymph (Figure S2C), the abdomens of five males were opened in 120 μL PBS. Animals were gently shaken to facilitate the diffusion of hemolymph as well as free-floating larval adipose cells in PBS. Carcasses were discarded and the remaining solution was homogenized and used for triglyceride assays. Triglyceride levels were normalized to the number of animals rather than protein for this experiment.

**Larval Adipose Cell Counts**

An abdomen from a single male was dissected in 10μL PBS. The gut was removed and the carcass was gently shaken to allow the larval adipose cells to disperse in PBS. The carcass was discarded and the larval adipose cells were harvested with a pipet and deposited into an Invitrogen Countess cell counting chamber slide (ThermoFisher Cat# C10228). Cell counting slides were placed upside-down and observed by bright field microscopy using a Zeiss Axioskop2 Plus microscope. Pictures were taken to capture the entire slide and thus capture all the larval adipose cells collected from a single fly. The cumulative surface of larval adipose cells (in pixels) was determined using ImageJ software (https://imagej.nih.gov/ij/download.html). A macro was created to automate the process. Cumulative cell surface was determined rather than larval adipose cell number because these cells can form aggregates that impair automated counting. Six to twelve animals were analyzed per condition. Each dot in Figure S2B represents a single animal.

**GC/MS Hydrocarbon Analysis**

Males were collected at emergence, or after being starved or fed a 0% sugar diet for 3 days. Five samples of 20 animals were collected per condition. Sample preparation and gas chromatography/mass spectrometry (GC/MS) analysis were performed by the Metabolomics Core Research Facility at the University of Utah School of Medicine. The identity of some hydrocarbon species was confirmed by comparison with purified standards (7(Z)-tricosene (Cayman Chemical Cat# 9000313), tricosane (Sigma-Aldrich Cat# 263850), 7(Z)-pentacosene (Cayman Cat# 9000530) and 7(Z), 11(Z)-heptacosadiene (Cayman Chemical Cat# 10012567). The identity of other hydrocarbon species detected in the samples was determined by their ionization mass.

**Oil Red O Stains**

Oil red O stains were performed as described with minor modifications (Tennesen et al., 2014). Briefly, abdominal cuticles (containing adipose tissue and oenocytes) of 10 animals were dissected in PBS and fixed in 4% formaldehyde for one hour at room temperature. They were washed twice in PBS and twice in propylene glycol before being incubated for one hour at 60°C in Oil red O diluted in propylene glycol. Cuticles were then washed twice in propylene glycol and twice in PBS. Fat body and oenocytes were dissected by gently scraping the inner side of the abdominal cuticle with forceps in a drop of glycerol on a microscopy slide. A coverslip was then deposited on top of the dissected tissues before observation by bright field microscopy. Pictures were taken with a Zeiss Axioskop Plus microscope equipped with an Infinity 3 Lumenera camera and the Infinity Analyze software. Independent Oil red O stains were performed two to three times on sets of 10 cuticles and a representative image is shown in each figure. For larval adipose cells, male abdomens were dissected in PBS, the gut was removed and the carcass was shaken gently to allow larval adipose cells to disperse in PBS. Larval adipose cells were then collected by pipetting and transferred to a 1.5mL Eppendorf tube. Larval adipose cells were washed and stained with Oil red O as described except that they were collected by centrifugation between each step.

**Immunostaining to Detect dHNF4 Protein**

Affinity-purified guinea pig anti-dHNF4 antibodies were used for immunofluorescence studies as described (Palanker et al., 2009). Abdominal cuticles were dissected in cold PBS, fixed in 4% formaldehyde for 30 min at room temperature, and washed three times.
for 10 min each in PBS, 0.2% Triton-X100. Samples were blocked using 5% normal goat serum for 1.5–2 hours at room temperature and incubated with primary antibodies for a minimum of 12 hours at 4°C. They were washed and incubated with secondary antibody at room temperature (approximately 21°C) for two hours. Abdominal cuticles were then mounted in glycerol. Images were acquired using a Zeiss Axioskop2 Plus microscope equipped with a X-Cite 120 LEDBoost from Excelitas Technologies.

**RT-qPCR**

Real Time-qPCR (RT-qPCR) experiments were conducted with an Applied Biosystem Quantstudio 3 device. SYBR GreenER qPCR SuperMix Universal (ThermoFisher Scientific Cat# 11762100) was used for RT-qPCR reactions. ROX was diluted ten times and 0.1 μL was added to the mix for a final reaction volume of 20 μL. Fold inductions in transcript level were determined using the ΔΔCt method. For the Drosophila samples, transcript levels were normalized to rp49. For the mouse samples, transcript levels were normalized to Rpl13. RT-qPCR experiments were conducted on 4 to 5 independent Drosophila cDNA samples per condition, and 6 independent mouse hepatocyte cDNA samples per condition. The figures represent the combined data from all samples. A list of primers used in our RT-qPCR experiments is available in Table S1. We used primers for RT-qPCR experiments after confirming an efficiency between 90 and 110%.

**cDNA Synthesis from Drosophila Tissues**

RNA was extracted from samples of 5 to 10 males using the Nucleospin RNA kit from Macherey-Nagel (Cat# 740955.50). Reverse transcription was performed on 0.25–1 μg RNA using the Superscript Reverse Transcriptase II from ThermoFisher Scientific (Cat# 18064022) and oligo(dT) primers. cDNA was used as a template for qRT-PCR as described above.

**cDNA Synthesis from Mouse Hepatocytes**

RNA was isolated from the left lobe of the liver using Trizol reagent (Invitrogen Cat# 15596026). The only deviation from the manufacturers protocol was an extra centrifugation at 7,500xg to remove excess lipids directly after homogenization with the TissueLyser II (Qiagen Cat# 85300). SuperScript VILO Master Mix (Thermofisher Cat# 11755050) was used for reverse transcription with a starting concentration of 1 μg RNA for each sample, after which cDNA samples were diluted 1:10 with nuclease-free water (Thermofisher Cat# R0581). cDNA was used as a template for qRT-PCR as described above.

**Information Related to Experimental Design**

Blinding was not used in the course of our study. No data or subjects were excluded from our analyses.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Graphical Representation and Statistical Analysis of the Data**

Graphical representation and statistical analysis of the data were performed using GraphPad Prism 6 software (www.graphpad.com).

For lifespan studies, data is presented as Kaplan-Meier survival plots. Short black vertical lines on the plot indicate when animals were removed from the experiment, for example when flies escaped during their transfer to a new vial (Figures 7A and 7B). The number of animals used in lifespan experiments and the median lifespan in each condition are indicated in the figure legends. A Log-Rank (Mantel-Cox) test was used for statistical comparison of survival plots. The p-value is indicated in the figure legends when the difference is statistically significant.

For glucose, glycogen and triglyceride assays scored at a single time point, data are represented by box and whiskers plots. Data are represented as XY graphs when metabolite levels were scored at multiple time points (Figures 2A, 2B, 2D, S1B–S1D, and 4C). For box and whiskers plots, the box extends from the 25th to 75th percentiles, the line represents the median, and the whiskers represent minimum and maximum values. For XY graphs, the dots represent mean values and the error bars represent standard deviation. We used a Mann-Whitney test for pairwise comparisons with both types of data/representations. Asterisks illustrate statistically significant differences between conditions. ****p<0.0001; 0.0001<***p<0.001; 0.001<**p<0.01; 0.01<p<0.05.

To analyze cumulative larval adipose cell surfaces after emergence (Figure S2B), the data is presented as grouped individual values. Each dot represents the cumulative larval adipose cell surface from a single male. The black horizontal line in the dot plot represents the mean, and the black error bars represent the standard deviation. We used a Mann-Whitney test for pairwise comparisons. Asterisks illustrate statistically significant differences between controls and dHNF4 mutants at a given timepoint: ****p<0.0001. For the analysis of fly water content upon dry starvation (Figure 6E), dots represent average water content at each time point (11<n<13), and the error bars represent the standard deviation. We used a Mann-Whitney test for pairwise comparisons. Asterisks illustrate statistically significant differences between controls and dHNF4 mutants: ****p<0.0001. Experiments were repeated three times, one representative experiment is shown in the figure.

For transcript analysis by RT-qPCR (Figures 5B, 6G, and S4C), the data is presented as histograms. Each bar represents the mean and the error bars represent the standard deviation. We used a Mann-Whitney test for pairwise comparisons. Asterisks illustrate statistically significant differences between controls and dHNF4 mutant Drosophila, or between controls and HNF4α mutant mouse hepatocytes: 0.001<**p<0.01; 0.01<*p<0.05.
For hydrocarbon levels (Figures 6A and S5A), the data is presented as interleaved box and whiskers plots. The box extends from the 25th to 75th percentile, the line represents the median, and whiskers represent minimum and maximum values. For pairwise comparisons between controls and dHNF4 mutants at each time point (Day 0, Day 3 starv. and Day 3 0% sugar diet) we performed multiple t-tests. We did not assume consistent standard deviations, and set a desired false discovery rate (Q) value of 1.000%. A single asterisk represents a p-value below the cutoff (p<0.01).

No specific method was used to determine whether the data met the assumptions of the statistical approach.