Macrophage-Derived \( \text{upd3} \) Cytokine Causes Impaired Glucose Homeostasis and Reduced Lifespan in \emph{Drosophila} Fed a Lipid-Rich Diet

**Graphical Abstract**

**Highlights**

- Chronic lipid-rich diet results in JAK-STAT activation in \emph{Drosophila}
- Chronic JAK-STAT activation reduces lifespan and insulin sensitivity
- Lipid-rich diet induces JNK pathway-dependent production of \( \text{upd3} \) by macrophages
- Macrophage \( \text{upd3} \) controls JAK-STAT activation, survival, and insulin sensitivity

**In Brief**

Consumption of fatty foods is associated across species with inflammation, metabolic imbalance, and reduced lifespan. Woodcock et al. use \emph{Drosophila} fed a lipid-rich diet to demonstrate that an evolutionarily conserved “scavenger receptor-JNK-type 1 cytokine” cassette in macrophages controls glucose metabolism and reduces lifespan via activation of the JAK-STAT pathway.

**Accession Numbers**

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Macrophage-Derived upd3 Cytokine Causes Impaired Glucose Homeostasis and Reduced Lifespan in Drosophila Fed a Lipid-Rich Diet

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SUMMARY

Long-term consumption of fatty foods is associated with obesity, macrophage activation and inflammation, metabolic imbalance, and a reduced lifespan. We took advantage of Drosophila genetics to investigate the role of macrophages and the pathway(s) that govern their response to dietary stress. Flies fed a lipid-rich diet presented with increased fat storage, systemic activation of JAK-STAT signaling, reduced insulin sensitivity, hyperglycemia, and a shorter lifespan. Drosophila macrophages produced the JAK-STAT-activating cytokine upd3, in a scavenger-receptor (crq) and JNK-dependent manner. Genetic depletion of macrophages or macrophage-specific silencing of upd3 decreased JAK-STAT activation and rescued insulin sensitivity and the lifespan of Drosophila, but did not decrease fat storage. NF-kB signaling made no contribution to the phenotype observed. These results identify an evolutionarily conserved “scavenger receptor-JNK-type 1 cytokine” cassette in macrophages, which controls glucose metabolism and reduces lifespan in Drosophila maintained on a lipid-rich diet via activation of the JAK-STAT pathway.

INTRODUCTION

A lipid-rich diet has been associated with obesity and a reduced lifespan from Drosophila to humans (Birse et al., 2010; Driver and Cosopodiotis, 1979; Reilly and Kelly, 2011; Szendroedi and Roden, 2009; van Herpen and Schrauwen-Hinderling, 2008; Wagenner et al., 2013). This represents a worldwide concern as societal factors that include activation of the innate immune system, and in particular of macrophages, and the disruption of homeostasis (Biswas and Mantovani, 2012; Jin and Flavell, 2013; Moore and Tabas, 2011; Szendroedi and Roden, 2009; van Herpen and Schrauwen-Hinderling, 2008). Macrophage activation via innate pattern recognition receptors such as Toll-like receptor 4 (TLR4) and nuclear factor “kappa-light-chain-enhancer” of activated B cells (NF-κB) and the production of cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) is proposed to mediate insulin resistance and other complications of obesity (Arkan et al., 2005; Biswas and Mantovani, 2012; Jin and Flavell, 2013; Moore and Tabas, 2011; Olefsky, 2009; Reilly et al., 2013). Nevertheless, the effects of the genetic deletion of Toll-like receptors such as TLR4 on insulin resistance are controversial (Jin and Flavell, 2013; Orr et al., 2012; Saberi et al., 2009; Tanti et al., 2012), and fat uptake by murine macrophages in vivo was also associated with suppression, rather than activation, of NF-κB-dependent inflammatory gene expression (Spann et al., 2012). The cJun NH2-terminal kinase (JNK), in macrophages, was also recently shown to be important for the establishment of diet-induced inflammation and insulin resistance in mice (Han et al., 2013; Hirosumi et al., 2002).

Causation studies in humans are challenging to conceive and to perform. Comprehensive genetic studies in mice are also arduous, in part due to their long duration, given rodent lifespans (Robertson et al., 2011), and difficulties in the interpretation of conventional knock-out and cell depletion experiments (Clementi et al., 2009; Feng et al., 2011; Jin and Flavell, 2013; Orr et al., 2012; Saberi et al., 2009; Tanti et al., 2012). Therefore, the causative role of macrophages and the basic molecular mechanisms that may underlie their contribution to the disruption of homeostasis and lifespan reduction remain difficult to elucidate.

In contrast, Drosophila melanogaster is well suited to genetic analyses and survival studies (Dionne et al., 2006; Driver and Cosopodiotis, 1979; Heinrichsen and Haddad, 2012) and is an excellent model to explore homeostatic responses to stress, and in particular metabolic responses to diet (Birse et al., 2010). Drosophila has also proven to be a powerful model to decipher conserved mechanisms of innate immunity in metazoans (Le maître et al., 1996), and the adult fruit fly has a simple and genetically tractable myeloid immune system consisting of phagocytic macrophages, termed plasmacytes (Agaisse et al., 2003;
Charroux and Royet, 2009; Gold and Brückner, 2014; Holz et al., 2003; Krzemie et al., 2010; Lebestky et al., 2000; Meister, 2004), allowing for genetic analysis of macrophage functions. We thus reasoned that Drosophila may be a favorable model to dissect evolutionarily conserved immune pathway(s) that may be at play in the macrophage response to excess dietary lipids and metabolic stress in vivo and to identify the roles of macrophages in longevity, glucose homeostasis, and fat storage.

We found that shorter lifespan, increased fat storage, reduced insulin sensitivity, and hyperglycemia were associated with systemic activation of the Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway, but not of the JNK and was responsible for the systemic JAK-STAT activation, decreased insulin sensitivity, and the reduced lifespan of flies maintained on a lipid-rich diet. Fat storage itself was independent of macrophage activation and did not influence survival. These data demonstrate a causal role of macrophages in reducing insulin sensitivity and lifespan in flies maintained on a lipid-rich diet, via a pathway conserved in vertebrates.

RESULTS

Reduced Lifespan and Systemic JAK-STAT Activation in Drosophila on Lipid-Rich Diet

Extending earlier studies on the effect of dietary fat on metabolism and longevity in Drosophila melanogaster (Birse et al., 2010; Driver and Cosopodiotis, 1979; Heinrichsen and Haddad, 2012), we found that wild-type w^{1118} and Oregon-R Drosophila fed diets supplemented with lard and maintained at either 25°C or 29°C start dying 15–20 days earlier than flies on a control diet and thus show a decrease in lifespan of ~30% (Figure 1A; Figures S1A and S1B). Thin-layer chromatography (TLC) (Al-Anzi and Zinn, 2010) indicated that the total triglyceride content of lipid-rich diet-fed flies was increased compared to controls (Figure 1B), whereas food consumption was comparable (Figure S1C). Drosophila also presented with a progressive increase in glucose/trehalose, which doubled after 30 days of lipid-rich diet exposure (Figure 1C). lip2, lip3, and lip5 (encoding insulin-like peptides) transcripts were unchanged (Figure S1D), but we observed a blunted phosphorylation of AKT in response to insulin, indicating that the lipid-rich diet resulted in impaired insulin sensitivity (Figure 1D; Figure S1E). Lipid-rich diet was also associated with an early and sustained increase of the cytokine upd3 detectable at the whole-fly level (Figure 2A) and increased expression of the endogenous JAK-STAT target gene Socs36E (Figure 2B). Systemic JAK-STAT activation was confirmed by the analysis of 10XSTAT92E-GFP flies (Bach et al., 2007) where increased GFP expression was detected in muscle (Figure 2C) and midgut (Figure 2D; Figures S2A and S2B). In contrast, we did not detect activation of the transcriptional targets of the Toll and Imd NF-κB pathways in whole flies, although both the Toll and Imd pathways were responsive to sterile and septic injury (Figures 2E and 2F; Figure S2C). These data indicated that reduced lifespan and impaired glucose metabolism are associated with a systemic JAK-STAT activation in response to a lipid-rich diet in Drosophila.

Lipid-Rich Diet Promotes Upd3 Production by Macrophages in Drosophila

Tissue macrophages are professional phagocytes across the animal kingdom. Drosophila macrophages, termed plasmatocytes, are found throughout tissues of the adult fly (Meister,
2004; Figure 3A) and are left over from embryonic and larval hematopoiesis (Holz et al., 2003; Krzemien et al., 2007, 2010). They express lectins and scavenger receptors such as Hemolectin (Hml) (Goto et al., 2001) and crq, a fly CD36 homolog (Franc et al., 1999; Stuart et al., 2005; Figure S3). Drosophila macrophages changed morphology on lipid-rich diet (Figure 3B). Their numbers were not modified as a result of the diet (Figure 3C), but analysis of fluorescence-activated cell sorting (FACS)-sorted plasmatocytes indicated they become foamy, accumulating neutral triglycerides and lipids within large Oil red O-positive lipid vacuoles (Figure 3D), a feature also typical of the tissue macrophage response to a lipid-rich diet in vertebrates (Li and Glass, 2002). Whole-genome expression array analysis indicated that expression of a large number of genes was upregulated or downregulated in macrophages from flies on a lipid-rich diet after 30 days, in comparison to cells from control diet-fed flies (Figure 3E). Transcription-factor binding site representation analysis (Clark et al., 2013) identified an overrepresentation of AP-1, Fox, and ATF-CREB binding sites, but not NF-κB binding sites, in the vicinity of upregulated genes (Figure 3F). A qPCR analysis of FACS-sorted plasmatocytes did not detect activation of the transcriptional targets of the Toll and Imd NF-κB pathways, Drosomycin (Drs) and Diptericin; but indicated that macrophages from flies on a lipid-rich diet produced the JAK-STAT activating cytokine upd3, but not upd and upd2 (Figure 3G). Drs and Dpt transcripts in unchallenged (white bars), PBS-injected (gray bars), and E. coli- and M. luteus-infected flies (black bars) after 10, 20, and 30 days of control or lard-enriched diets. Histograms represent mean ± SEM of 4–5 independent samples per diet, per time point. Please also see Figure S2.

Macrophage-Derived Upd3 Is Responsible for the Metabolic and Lifespan Phenotype on Lipid-Rich Diet

Upd3 was previously shown to be produced by macrophages in response to infection (Agaisse et al., 2003), but upd3 is also produced by several other cell types and tissues in Drosophila, including midgut epithelial cells, where it plays an essential role in gut infection and homeostasis (Buchon et al., 2009; Jiang et al., 2009; Osman et al., 2012). To investigate whether upd3 produced by macrophages or from other sources might be responsible for the phenotype of flies under a lipid-rich diet, we generated tissue-specific upd3 knockdown flies. Silencing of upd3 in the midgut or muscle did not rescue fly longevity (Figures S4A and S4B). However, silencing of upd3...
in macrophages improved fly longevity (Figure 4A) and decreased upd3 induction in response to lipid-rich diet at whole-fly level (Figure 4B). Furthermore, macrophage-specific upd3 knockdown also decreased glucose/trehalose almost to control concentrations (Figure 4C), and restored AKT phosphorylation in response to insulin (Figure 4D; Figures S4C–S4E). Itp2, Itp3, and Itp5 transcription was stable (Figure 4E), and plasmatocyte numbers were unchanged (Figure S4F). In addition, macrophage-specific upd3 knockdown did not affect fly fat content (Figure 4F).

These data suggested that Drosophila macrophages were responsible for reduced lifespan and the disruption of glucose homeostasis, via their production of upd3. To confirm whether the production of upd3 was indeed responsible for systemic JAK-STAT activation and for the survival and metabolic phenotypes, we examined Drosophila genetically deficient for upd3 (upd3 null) (Osman et al., 2012). The lifespan of upd3 null Drosophila was identical on control and lipid-rich diets (Figure 5A). Upd3 null flies did not present with increased JAK-STAT activation in the course of a lipid-rich diet (Figure 5B).
Figure 4. Macrophage-Derived Upd3 Controls Survival and Insulin Sensitivity in Lipid-Rich Diet

(A) Survival of plasmatocyte (HmlΔ-Gal4)-driven upd3 knockdown flies (HmlΔ-Gal4,UAS-2xeGFP/UAS-upd3-IR, closed symbols) and control flies (UAS-upd3-IR/+, open symbols) on control or lard diet. Data are pooled from five independent experiments. Log-rank, \( \chi^2 = 68.79, p < 0.0001 \); Wilcoxon, \( \chi^2 = 65.83, p < 0.0001 \) for flies under lard diet.

(B) Upd3 transcription in flies as in (A) at days 0 and 20. Histogram shows mean ± SEM of four or five independent samples of three flies per diet and genotype.

(C) Total glucose and trehalose content of flies as in (A) on a control or lard diet for 40 days. Histogram represents mean ± SEM from four or five samples of three flies per diet.

(D) Amount of phospho-AKT in flies as in (A) 10 min after injection of PBS, 320 pg/fly (high) insulin, and 64 pg/fly (low) insulin in samples of three flies fed a control or lard diet for 40 days. Histogram represents quantification p-AKT over total AKT, \( n = 3 \); see also Figure S4.

(E) Ilp2, Ilp3, and Ilp5 transcription in upd3-IR and control flies fed a lard diet. Three flies per sample; histogram represents mean ± SEM of four or five independent samples per diet and per time point.

(F) TLC for TG contents of groups of ten flies as in (A) fed a control or lard diet for 20 days. Histogram represents quantification of TG; \( n = 4 \), mean ± SEM. Please also see Figure S4.
and did not develop hyperglycemia (Figure 5C), although they stored triglycerides when fed lipid-rich diets (Figure 5D). Plasmaocyte-specific expression of \textit{upd3} in \textit{upd3 null} flies reduced their lifespan on a control diet (Figure S5), and ectopic inducible expression of \textit{upd3} or of \textit{upd2}, in plasmatocytes or in the fat body, was also sufficient to reduce \textit{Drosophila} lifespan (Figure 5E).

Altogether, these data suggested that, in \textit{Drosophila} maintained on lipid-rich diet, macrophages are a major source of \textit{upd3} production, which is responsible for systemic activation of the JAK-STAT pathway, reduced lifespan, and the disruption of glucose homeostasis.

**Drosophila Macrophages Control Glucose Homeostasis and Survival of Flies on Lipid-Rich Diet**

To further challenge this hypothesis, we generated inducible macrophage-depleted adult flies (Charroux and Royet, 2009). Cell death was triggered in adult flies by inducible expression of the proapoptotic protein \textit{reaper} (\textit{rpr}) (White et al., 1996) in macrophages under the control of \textit{Hml} \textit{D} combined with a temperature-specific repressor (McGuire et al., 2004). Injected fluorescent lipids (DiI-LDL) are rapidly scavenged by plasmacytes, and there was a strong correlation between the DiI and \textit{Hml} \textit{D}+ cells 1 hr after injection (Figure 6A). Furthermore, expression of \textit{upd3-GFP} increased in plasmacytes of flies fed a control diet. Please also see Figure S5.
injected with DiI-LDL (Figure 6B). Following rpr induction, macrophage depletion was complete as assessed by the absence of lipid scavenging in the flies (Figure 6C; Figure S6A). Macrophage depletion extended the longevity of flies on lipid-rich diet (Figure 6D), and decreased upd3 and Socs36E expression (Figure 6E). Macrophage-depleted flies were also protected from hyperglycemia in comparison to controls (Figure 6F). Macrophage depletion did not affect the triglyceride content of the flies (Figure 6G), and Ilp2, Ilp3, and Ilp5 transcription remained stable (Figure S6B). Our data thus indicated that macrophages are responsible for the reduced lifespan of Drosophila under lipid-rich diet, via the production of upd3. We therefore investigated the pathways and molecules that drive upd3 production in Drosophila macrophages.

**Upd3 Production by Macrophages Is Controlled by Croquemort and JNK, but Not by NF-κB Signaling**

The above data suggested that lipid uptake by macrophages may regulate upd3 production. We thus screened the fly genome for putative scavenger receptors involved in lipid uptake and expressed by macrophages (Figure S7A) and tested their roles in upd3 production and survival. Plasmatocyte-specific knockdown of two scavenger receptors, a fly CD36 homolog crq (Franc et al., 1999; Stuart et al., 2005) and the EGF-like repeat containing scavenger receptor Nimrod C1 (NimC1) (Kurucz et al., 2007) (Figures S7A and S7B), resulted in a 30%–40% reduction in macrophage lipid uptake (Figure 7A). Knockdown of crq decreased upd3 and Socs36E expression in the whole fly (Figure 7B) and extended Drosophila lifespan (Figure 7C; Figure S7C), yet Ilp2, Ilp3, and Ilp5 transcription was not significantly changed
Figure 7. Control of Upd3 Expression by Macrophages during Lipid-Rich Diet

(A) Percentage reduction in Dil-LDL uptake as compared to controls in plasmatocyte-specific scavenger receptor knockdown flies (crq and NimC1) and in Hemese (He) and CG10345 knockdown flies (see Figure S7A and Experimental Procedures). Histograms represent mean ± SEM; n = 4 flies per genotype.

(B) upd3 and Socs36E transcripts in plasmatocyte (HmlJ > crq-IR, gray) and control (UAS-crq-IR/+, white) flies fed lard diets for 20 days; three flies per sample; histogram represents mean ± SEM of four or five independent samples per diet.
(Figure S7D). However, knockdown of NimC1 in macrophages did not influence the lifespan of flies on a lipid-rich diet (Figure S7E).

The lack of effect of NimC1 knockdown on survival despite its effect on lipid uptake suggested that the effect of crq knockdown on survival may be linked to signaling. We thus investigated the roles of signaling pathways that may be involved in the expression of upd3 by macrophages. Silencing of myeloid differentiation primary response gene 88 (Myd88), immune deficiency (Imd), and TGF-β-activated kinase 1 (Tak1) in macrophages (Figure 7D) did not rescue Drosophila lifespan on lipid-rich diet. These data were in accordance with the lack of detectable NF-κB activation in macrophages (see Figures 2E, 2F, 3F, and 3G). In contrast, we found that silencing of JNK (bsk) in macrophages (Hml-J-Gal4 > bsk-IR) extended the longevity of flies maintained under a lipid-rich diet (Figure 7E) and decreased expression of upd3 (Figure 7F) and Socs36E (Figure 7G) in the whole fly, but did not affect plasmatocyte numbers (Figure S7F). This result was consistent with the observed overrepresentation of AP-1 but not NF-κB binding sites in the vicinity of genes upregulated in plasmatocytes from the observed overrepresentation of AP-1 but not NF-κB binding sites in the vicinity of genes upregulated in plasmatocytes from the lipid-rich diet-fed flies (Figure 3F). Additionally, silencing of signal-transducer and activator of transcription protein at 92E (Stat92E) in macrophages did not rescue Drosophila longevity on lipid-rich diet (Figure 7D), suggesting that JNK acts downstream of crq, but upstream of upd3 (Figure S7H). Indeed, regulation of the JNK transcriptional target puckered (puc) on a lipid-rich diet was lost in crq knockdown flies (Figure S7G). The regulation of upd3 expression by JNK in macrophages is reminiscent of the previously described regulation of upd3 expression by JNK (Jiang et al., 2009) but not NF-κB signaling (Buchon et al., 2009) in enterocytes from the Drosophila midgut.

**DISCUSSION**

Collectively, our observations in a simple model organism demonstrate an essential role of macrophages in the loss of glucose homeostasis and lifespan reduction caused by a chronic lipid-rich diet, via the prolonged production of the type 1 cytokine upd3 that activates the JAK-STAT pathway. Further, we identify a genetic crq-JNK-upd3 cassette in macrophages critical for the longevity of “obese” flies. Our findings suggest that scavenging of excess lipids via crq, a CD36 analog (Stuart et al., 2005), results in the activation of a JNK-mediated stress response in macrophages, which in turn drives their production of upd3, activating the JAK-STAT pathway, which controls insulin sensitivity and lifespan in Drosophila.

It is interesting to note that this response is distinct from a NF-κB-dependent immune response, since reduced longevity and insulin resistance due to lipid-rich diet were not caused by transcription of NF-κB target genes in the whole fly or in macrophages. These data are consistent with recent evidence that lipid uptake by murine macrophages does not activate NF-κB (Spann et al., 2012). It is also noteworthy that the lipid storage phenotype was not affected by macrophage depletion or genetic reduction of upd3 production and JAK-STAT signaling. This suggests that, in the fly, the macrophage stress response mediated by cytokine production causes disease, rather than lipid storage itself.

The JAK-STAT pathway and upd3 in Drosophila are involved in the maintenance of tissue homeostasis and tissue repair in conditions of acute stress and infection via the control of stem cell proliferation in the Drosophila gut, testis, ovary, and kidney (Jiang et al., 2009; Kohlmaier and Edgar, 2008; Osman et al., 2012; Singh et al., 2007); the inhibition of apoptosis (Betz et al., 2008); and the regulation of Ilp secretion (Rajan and Perrimon, 2012) and insulin signaling (Karpac et al., 2011). Reducing insulin-TOR activity in Drosophila decreases cardiac dysfunction induced by short-term lipid-rich diet (Birse et al., 2010). Paradoxically, we found here that sustained production of upd3 in response to chronic excess dietary lipid caused impaired glucose metabolism and decreased lifespan. Thus JAK-STAT activation may contribute to the regulation of energy storage, at least in part through insulin receptor signaling, a protective effect that may nevertheless affect host fitness and cause pathogenesis, depending on its magnitude and duration (Jamieson et al., 2013; Medzhitov et al., 2012).

Vertebrate CD36 is involved in lipid scavenging (Stuart et al., 2005), and activates JNK (Baranova et al., 2008; Rahaman et al., 2006), while mice with JNK-deficient macrophages present with decreased production of cytokines, including IL-6, and are protected against insulin resistance (Han et al., 2013). Therefore, we hypothesize that a conserved mechanism by which excess lipids activate a chronic stress response mediated by macrophage scavenger receptors, JNK, and type 1 cytokine production may cause dysregulated metabolic homeostasis and reduced lifespan and represents a target for therapeutic interventions.

**EXPERIMENTAL PROCEDURES**

**Drosophila melanogaster Stocks**

See Supplemental Experimental Procedures.

**Lipid-Rich Diet Preparation**

Lipid-rich diets were prepared using the control diet as a base recipe and adding lard (protein, nil; carbohydrates, nil; saturated fat, 44%; monounsaturated, molecular weight 200-600 Da; Sigma) and casein (protein, 15.6%; carbohydrates, 0.5%; saturated fat, 2.5%; monounsaturated, molecular weight 200-600 Da; Sigma) to the control diet to provide a 1:1 ratio of lipids to proteins. The diets were autoclaved and stored at 4°C. The composition of the final diets was determined by gas chromatography (GC).
42%; polyunsaturated, 9.5%; salt, nil; fiber, nil; Sainsbury’s Basics Lard) in a weight-for-volume manner as described by Birse et al. (Birse et al., 2010), at either 6.3% or 15%. All experiments were carried out using the 15% lard-enriched diet except for experiments shown in Figures 3B and 3D, where a 6.3% lard-enriched diet was used.

**Life Span Assays**

Male flies were collected soon after eclosion, and groups of approximately 20–30 age-matched flies per genotype were placed on the given control and lipid-rich diets. All survival experiments were conducted at 25°C or 29°C. For temperature-sensitive plasmatocyte-depleted survival assays, fly crosses were carried out at 18°C to prevent Gal4 activity during development. Flies were collected soon after eclosion and were then placed at 29°C for 48 hr to initiate the inhibition of Gal80ts, and therefore the transcription of reaper, to induce plasmatocyte cell death in adult flies. Once the plasmatocytes had been depleted in these flies, they were put in groups, per genotype, and placed on the given diets, and the survival assay was carried out at 25°C. In all other experiments utilizing Gal80ts, crosses were also carried out at 18°C to prevent Gal4 activity during development. Flies were collected soon after eclosion over 2–3 days and were placed at 29°C for the entire survival assay, in order to ensure the overexpression or knockdown of upd3 was maintained throughout the fly lifespan. In all survival assays carried out, vials were checked each day for any fly deaths, which were recorded. Food vials were kept on their sides to minimize the possibility of fly death from becoming stuck to the food, and vials were changed for fresh food every other day. Flies were transferred into new vials without using CO2.

**Plasmatocyte Deletion**

Plasmatocyte cell death was triggered in adult flies by inducible expression of the proapoptotic protein reaper (rpr) (McGure et al., 2004). Rpr expression was controlled by the temperature-sensitive driver Tubulin-Gal80ts and the plasmatocyte-expressed driver Hml-A-Gal4. In these flies the ubiquitous Tub-Gal80ts promoter represses Gal4 activity in all tissues at temperatures below 29°C.

**Insulin Sensitivity Experiment**

Flies were injected with 50 nl of PBS or insulin-resuspended in PBS at a high dose (6.4 µg/µl) and a low dose (1.28 µg/µl). Flies from each condition were smashed in groups of three, in triplicate, 10 min postinjection in 75 µl of 2x Laemmlı loading buffer (100 mM Tris [pH 6.8], 20% glycerol, 4% SDS, 0.2 M DTT). Samples were stored at −80°C until required. Eight microtubes of this lysate were loaded per lane. Protein was transferred to nitrocellulose membranes. Antibodies used were as follows: from rabbit, anti-S505-phosphorylated Akt (CST #4054, 1:1,000); from mouse, anti-Akt (CST #4691, 1:1,000); from mouse, anti-α-tubulin (12G10 Developmental Studies Hybridoma Bank, used as an unpurified supernatant at 1:10,000). Secondary antibodies were HRP anti-rabbit IgG (CST #7074) and HRP anti-mouse IgG (KPL), both used at 1:5,000. Proteins were detected with Supersignal West Pico Chemiluminiscent Substrate (Thermo Scientific) using a BioRad Molecular Imager, and band densitometry was analyzed using Bio-Rad Image Lab software.

**Glucose/Trehalose Measurement Assay**

Flies were starved for 1 hr before being smashed in 75 µl TE + 0.1% Triton X-100 (groups of three flies per genotype and per diet were smashed per sample). After smashing, samples were immediately stored at −80°C. Samples were thawed when required and incubated at 90°C for 20 min to inactivate fly enzymes. Standards were prepared (ten series 1:2 dilution) using glucose for quantification. A total of 5 µl of sample was loaded per condition into flat-bottom 96-well tissue culture plates. Each fly sample was run three times, first alongside water for calculation of background fly absorbance, second with glucose reagent (Alere) for glucose, and third with glucose reagent plus trehalase for trehalose and glucose measurement. Plates were then incubated at 37°C for 1 hr before reading with a Wallac spectrophotometer at 490 nm.

**Thin-Layer Chromatography Triglyceride Measurement**

See Supplemental Experimental Procedures.

**Feeding Assay**

See Supplemental Experimental Procedures.

**Quantitative Real-Time PCR**

See Supplemental Experimental Procedures.

**Confocal Microscopy**

See Supplemental Experimental Procedures.

**Dil-LDL Injection**

See Supplemental Experimental Procedures.

**Bacterial Infection**

See Supplemental Experimental Procedures.

**Fluorescence-Activated Cell Sorting**

FACS samples were prepared as per Clark et al. (Clark et al., 2011). Approximately 90 adult male Drosophila (w; Hml-A-Gal4, UAS-2xGFP) were homogenized through a 70 µm cell strainer (BD Biosciences) with ice-cold 1x PBS-EDTA (2 mM) into a 50 ml Falcon. The filtered sample was then centrifuged at 4°C for 15 min at 150 x g, the supernatant was discarded, and the pellet was resuspended in 5 ml of PBS-EDTA (2 mM) and passed once again through a 70 µm cell strainer; this wash step was repeated. GFP-positive Hml-A-positive plasmatocytes were sorted through a 100 µm nozzle at 20 psi pressure using a FACS Aria II. For every sort, a GFP-negative sample was first analyzed in order to calibrate the sorter for detection of GFP-positive cells. Cells were sorted into RLT buffer from the RNAeasy Plus Micro Kit (QIAGEN) in order to extract RNA for cDNA generation and perform RT qPCR, or cells were sorted into PBS in order to fix and stain them for imaging.

**Oil Red O Staining of FACS-Sorted Plasmatocytes**

See Supplemental Experimental Procedures.

**Plasmatocyte Gene Expression Arrays**

Plasmatocytes were isolated from w; Hml-A-Gal4, UAS-2xGFP flies that were either kept for 30 days on lard-supplemented food or normal food as described above. Five thousand to ten thousand GFP-positive plasmatocytes were sorted from five independent samples per group and sorted directly into 6.4 µl preheated SuperAmp Lysis buffer (Milenyi). Samples were incubated for 10 min at 42°C according to the manufacturer’s guidelines and stored at −20°C until shipping. Samples were further analyzed and processed by the Miltenyi Genomic Service. For each of the cDNAs, 250 ng was used as template for Cy3 labeling according to the manufacturer’s protocol. The Cy3-labeled cDNAs were finally hybridized to an Agilent Whole Drosophila Genome Oligo Microarrays Custom 8x60K. The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. The software determines feature intensities (including background subtraction), rejects outliers, and calculates statistical confidences. Normalized data sets were compared using an unpaired t test (p < 0.01) and a fold change > 2 between normal diet and lard-supplemented food. Annotated significantly differentially regulated genes were depicted in a heat map. Heat map of differentially regulated genes represents the log2 of the fold change between normal diet and lard-supplemented diet.

**Statistical Analysis**

For real-time quantitative PCR, TLC and the colorimetric assay for glucose and trehalose levels standard sets were used in order to calculate values for each sample. An unpaired t test was used to calculate statistical significance for all experiments, excluding survival assays, where log-rank and Wilcoxon tests were performed using GraphPad Prism.

Transcription factor-binding site analysis on plasmatocyte microarray data was performed using Clover precisely as previously described (Clark et al., 2013). All genes up- or downregulated more than 2-fold and with p < 0.01 were used in this analysis. Further analysis of the plasmatocyte microarray data was done with Glucore.


AUTHOR CONTRIBUTIONS

F.G. and K.J.W. designed the study and wrote the manuscript. K.J.W., K.K., C.A.P., and V.V. performed experiments. K.J.W., K.K., M.S.D., and F.G. designed the experimental data. Authors are indebted to Celine Trouillet and Marisol Gonzalez Melendez for help with cell sorting and maintaining the fly room; Dr. Claire Pean and Dr. Werner Wolfgang for help with experiments; Dr. Brian Stramer, Dr. Brigitte Senechal, and Andrew Page for critical reading of the manuscript and support; and Prof. Bruno Lemaire, Dr. Nicolas Buchon, and Dr. James C.G. Hombría for helpful suggestions and the kind gift of upd3 null, mex1-Ga4 flies, and UAS-upd2 flies. K.J.W. was a PhD fellow of the MRC (Medical Research Council, UK). C.A.P. is a fellow of the École de l’INSERM Liliane Bettencourt. K.K. was funded by a research fellowship by the DFG (German Research Foundation). This work was supported by a Wellcome Trust Senior Investigator award (WT101853MA) and ERC Investigator award (2010-StG-261299) from the European Research Council to F.G.

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