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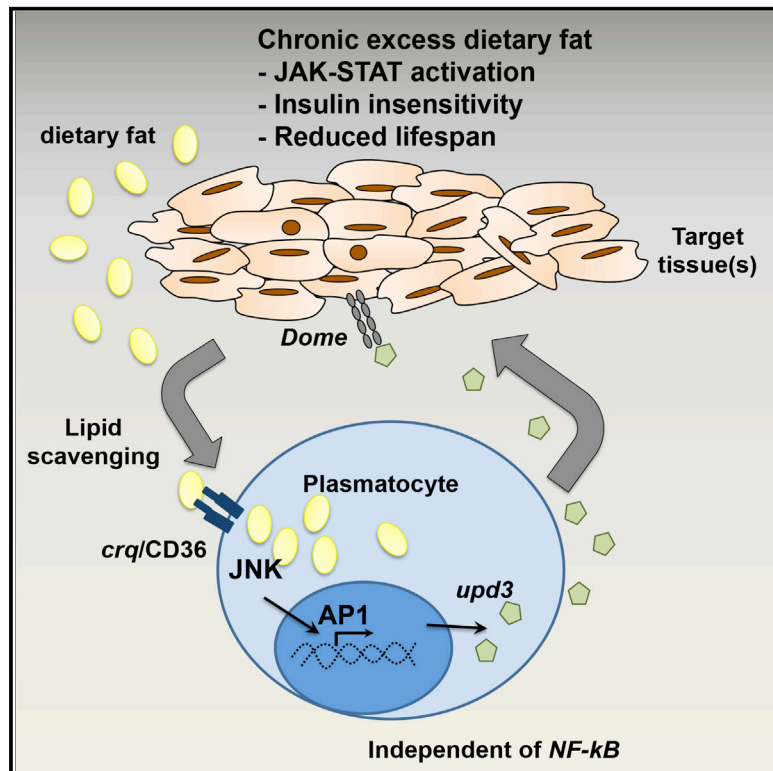
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# Immunity

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

### Graphical Abstract



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### In Brief

Consumption of fatty foods is associated across species with inflammation, metabolic imbalance, and reduced lifespan. Woodcock et al. use *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.

### Highlights

- Chronic lipid-rich diet results in JAK-STAT activation in *Drosophila*
- Chronic JAK-STAT activation reduces lifespan and insulin sensitivity
- Lipid-rich diet induces JNK pathway-dependent production of *upd3* by macrophages
- Macrophage *upd3* controls JAK-STAT activation, survival, and insulin sensitivity

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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- $\kappa$ B 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.

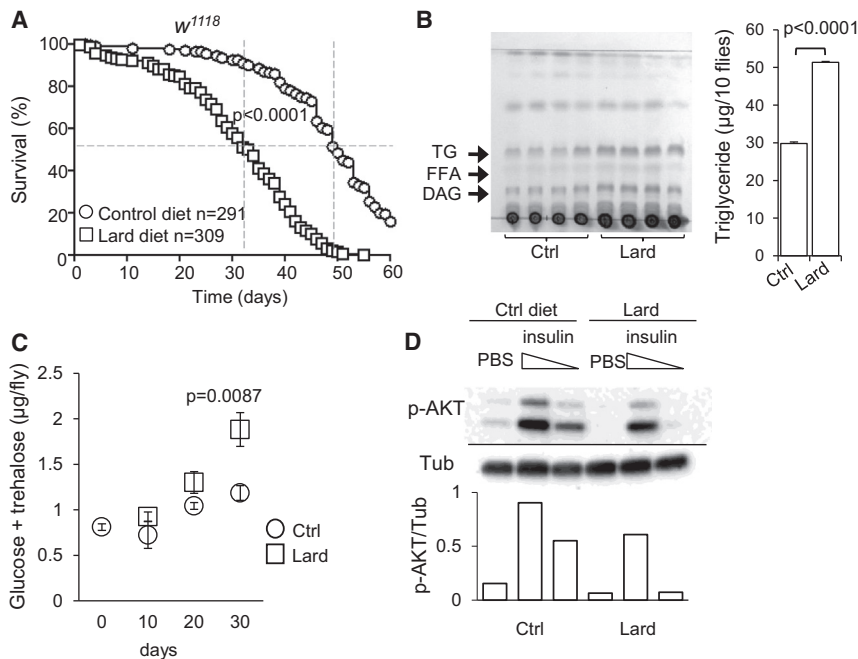
## INTRODUCTION

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 changes have led to an increase in dietary lipid intake. Lipid-rich diets and obesity are associated with worldwide “epidemics” of cardiovascular diseases, type 2 diabetes, cancer, and inflammatory diseases in humans (Alikhani et al., 2013; Biswas and Mantovani, 2012; Kroenke et al., 2013; Moore and Tabas, 2011). Diseases associated with lipid-rich diets share common general

features 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- $\kappa$ B) and the production of cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), 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- $\kappa$ B-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 knockout 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 (Lemaitre et al., 1996), and the adult fruit fly has a simple and genetically tractable myeloid immune system consisting of phagocytic macrophages, termed plasmatocytes (Agaïsse et al., 2003;



**Figure 1. Reduced Lifespan and Systemic JAK-STAT Activation in *Drosophila* on Lipid-Rich Diet**

(A) Survival of adult male flies (*w<sup>1118</sup>*) fed a control diet (circles) or lard diet (squares). Log-rank test,  $\chi^2 = 340.0$ ,  $p < 0.0001$ ; Wilcoxon test,  $\chi^2 = 271.9$ ,  $p < 0.0001$ . Data are pooled from six independent experiments. Dotted lines indicate 50% survival.

(B) Thin-layer chromatography (TLC) analysis of group of ten flies fed a control or lard diet for 10 days. TG, triglycerides; FFA, free fatty acids; DAG, diacyl glycerol. Histogram represents quantification of TG;  $n = 4$ ; mean  $\pm$  SEM.

(C) Glucose and trehalose content of flies fed control (circles) or lard diet (squares). Histograms represent mean  $\pm$  SEM from four or five samples of three flies per diet.

(D) Amount of phospho-AKT 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 30 days. Histogram represents quantification p-AKT over *Tubulin*, a representative experiment of three is shown. Please also see Figure S1.

Charroux and Royet, 2009; Gold and Brückner, 2014; Holz et al., 2003; Krzemiński 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 NF- $\kappa$ B pathway, in *Drosophila* maintained under a lipid-rich diet, while *Drosophila* macrophages became foamy and produced the type 1 cytokine *unpaired3* (*upd3*), which activates the JAK-STAT pathway. Using a combination of genetic approaches, we found that the production of *upd3* by macrophages required the scavenger receptor *croquemort* (*crq*) and 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

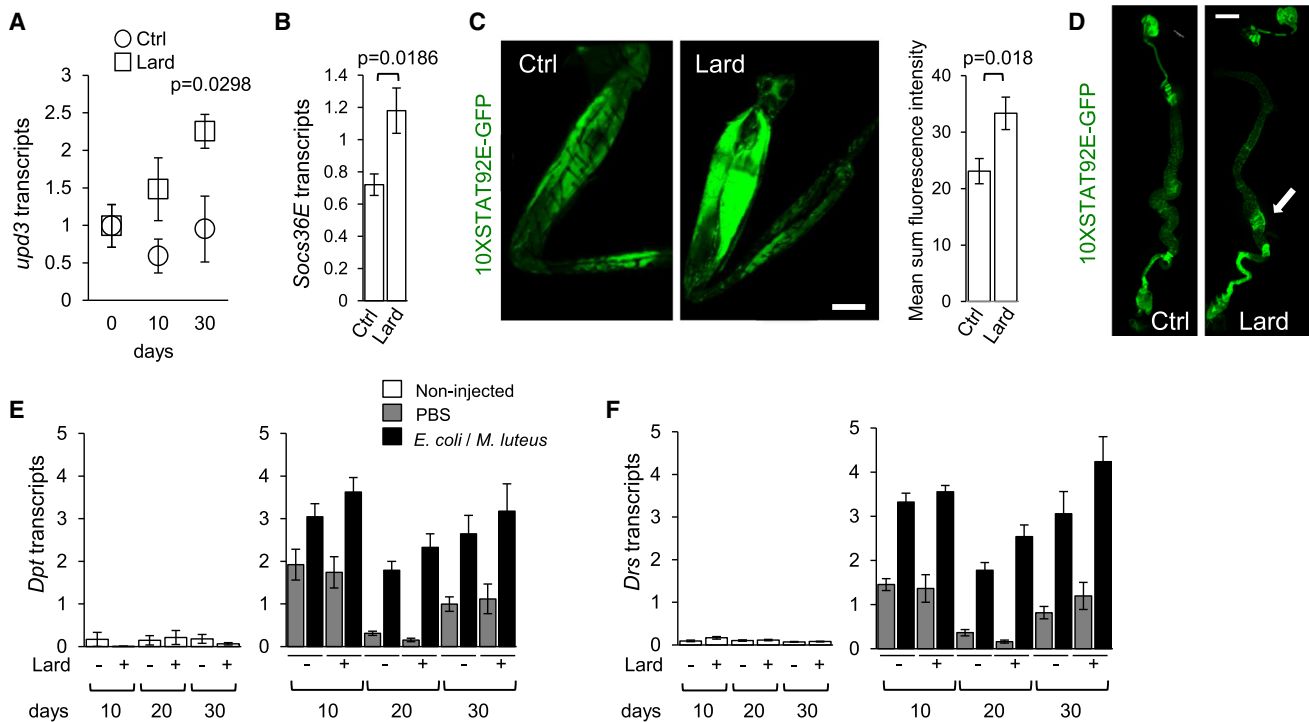
### Reduced Lifespan and Systemic JAK-STAT Activation in *Drosophila* on Lipid-Rich Diet

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<sup>1118</sup>* 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). *Ilp2*, *Ilp3*, and *Ilp5* (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- $\kappa$ B 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,



**Figure 2. Systemic Activation of the JAK-STAT Pathway in Chronic Lipid-Rich Diet**

(A) *upd3* transcription in flies fed a control or lard diet, three flies per sample, histogram represents mean  $\pm$  SEM of four or five independent samples per diet and per time point.

(B) As in (A), *Socs36E* transcription in flies fed for 20 days.

(C) Confocal imaging analysis of GFP expression in legs from 10xSTAT92E-GFP flies fed control or lard diets for 10 days. Bar is 100  $\mu$ m; a representative experiment is shown out of six. Histogram represents quantification of the mean sum fluorescence intensity  $\pm$  SEM for six flies per diet.

(D) As in (C), STAT92E-GFP expression in guts. Arrow represents area of enhanced STAT92E-GFP expression in the midgut. Bar is 300  $\mu$ m. Representative experiment is shown out of eight flies per diet. See also Figure S2.

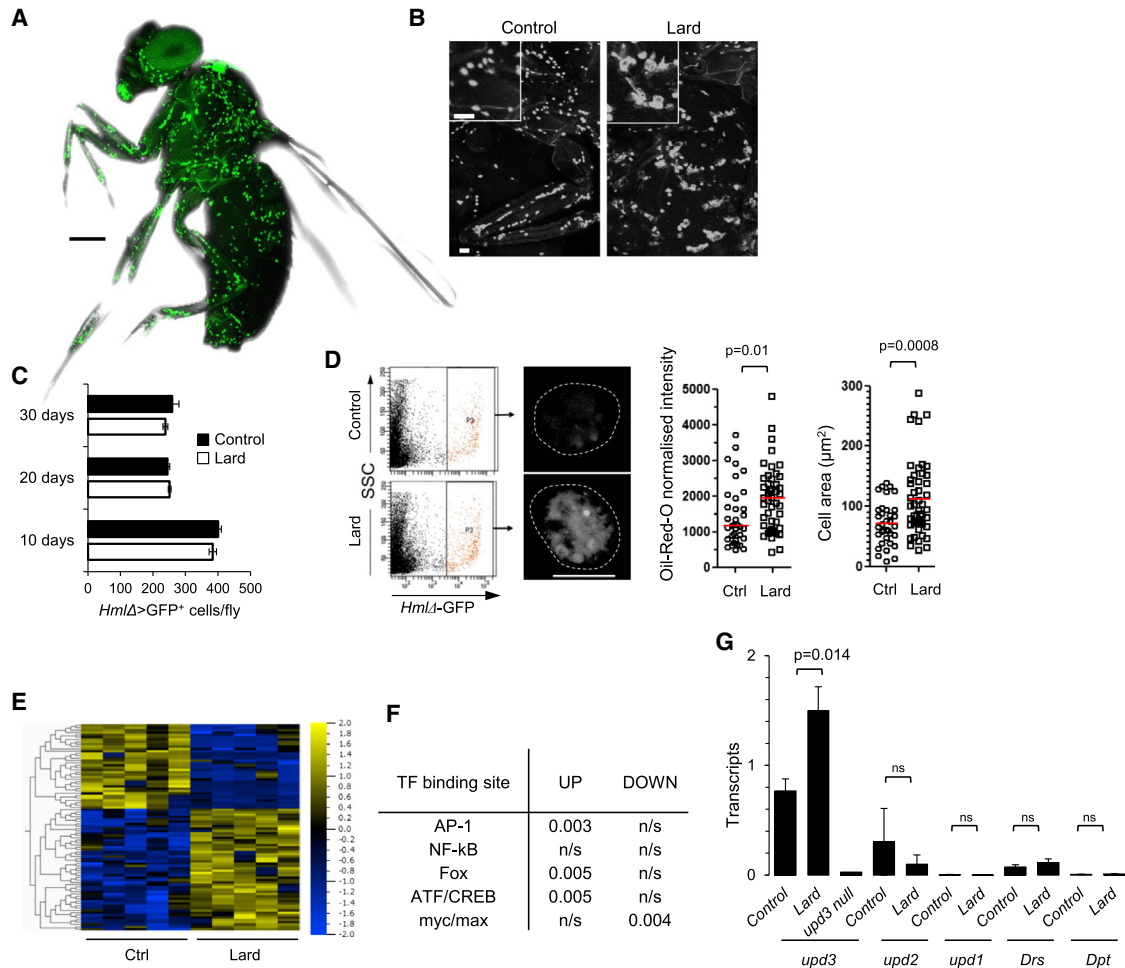
(E and F) *Drs* and *Dpt* transcripts in unchallenged (white bars), PBS-injected (gray bars), and *E. coli*- and *M. luteus*-infected flies (black bars) after 10, 20, and 30 days of control or lard-enriched diets. Histograms represent mean  $\pm$  SEM of 4–5 independent samples per diet, per time point. Please also see Figure S2.

2004; Figure 3A) and are left over from embryonic and larval hematopoiesis (Holz et al., 2003; Krzemień 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- $\kappa$ B 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- $\kappa$ B pathways, *Drosomycin* (*Drs*) and *Diptericin*, but indi-

cated that macrophages from flies on a lipid-rich diet produced the JAK-STAT activating cytokine *upd3*, but not *upd* and *upd2* (Figure 3G). *Upd*, *upd2*, and *upd3* share a common gp130-like receptor, *domeless* (*dome*), that signals via a single JAK (*hop*) and a single STAT (*Stat92E*) (Agaisse and Perrimon, 2004; Binari and Perrimon, 1994; Brown et al., 2001; Hou et al., 1996; Yan et al., 1996). These data indicated that lipid-rich diet promotes *Drosophila* macrophage activation characterized by the production of *upd3*.

### 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*



### Figure 3. Macrophage Activation in Response to Chronic Lipid-Rich Diet

(A) Confocal microscopy image (tiled/z stack, see [Supplemental Experimental Procedures](#)) of GFP<sup>+</sup> plasmatocytes in 2-week-old *w<sup>1118</sup>;HmlΔ-Gal4,UAS-2xeGFP* male. Representative of more than ten flies. Bar is 250 µm. See also [Figure S3](#).

(B) As in (A), in flies maintained on control or lard diet for 10 days. Bars, 50 µm.

(C) Number of GFP<sup>+</sup> plasmatocytes in flies (as in A) fed a control or lard diet for 10, 20, or 30 days. Histograms show mean ± SEM from eight flies per diet and time point.

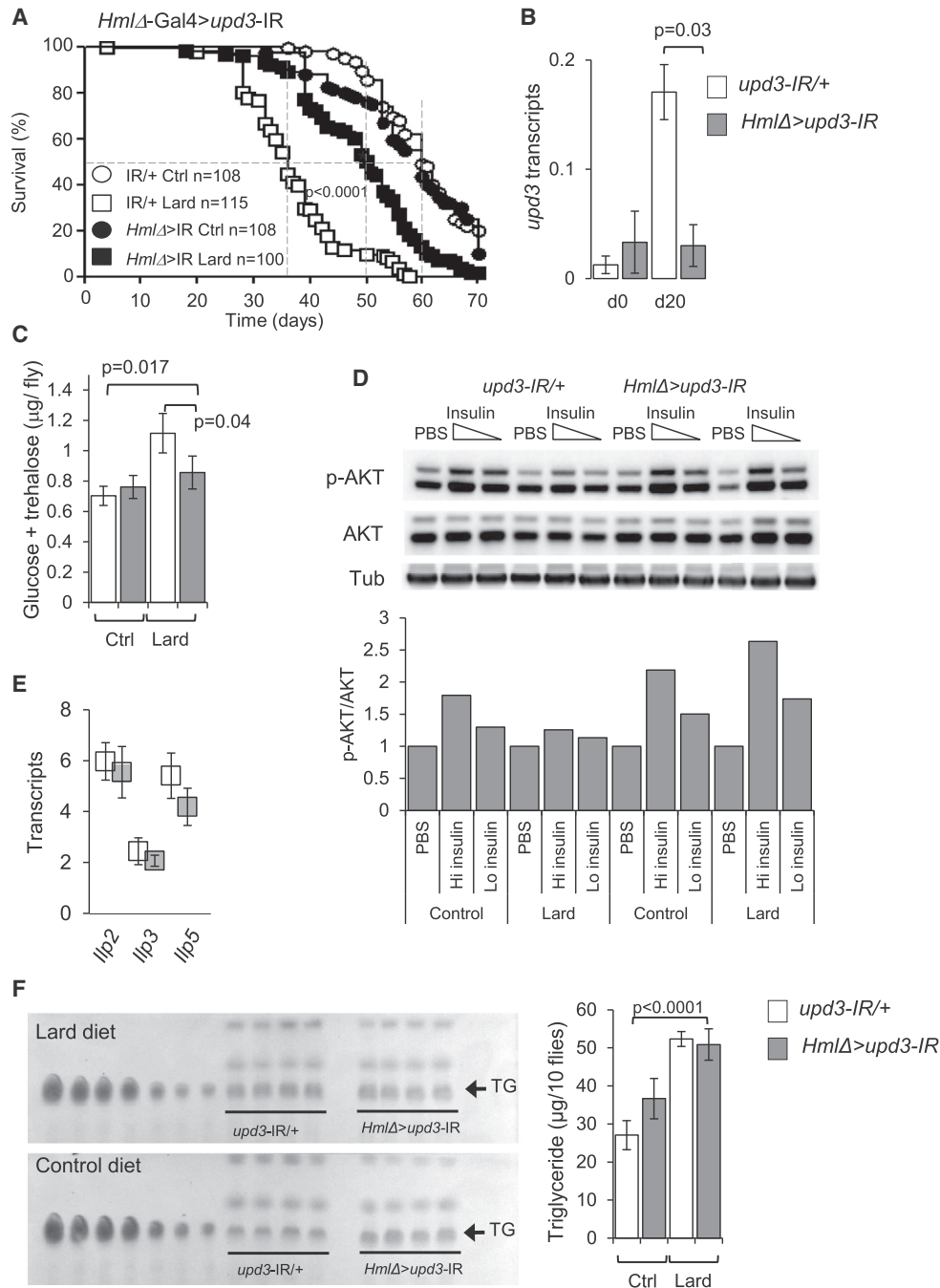
(D) Analysis of oil red O lipid staining intensity and cell area of FACS-sorted plasmatocytes from flies fed a control (n = 35 cells) or lard diet (n = 49 cells) for 20 days. Images are analyzed by confocal microscopy; bar is 7.5 µm. Symbols represent values for individual cells; red bar indicates the mean. Plasmatocytes analyzed were collected from > 4 independent experiments.

(E and F) Whole-genome expression array (Agilent) analysis of plasmatocytes from flies fed a normal or lard diet for 30 days. (E) Heat map represents up- and downregulated annotated genes across five independent biological replicates (fold change > 2, p < 0.01). (F) Transcription-factor binding sites identified as overrepresented in groups of genes shown in (E). “Fox” refers to a generic FOX (winged-helix) binding site.

(G) *upd3*, *upd2*, *upd1*, *Drs*, and *Drs1* transcripts in FACS-sorted plasmatocytes from *w<sup>1118</sup>;HmlΔ-Gal4,UAS-2xeGFP* flies fed a control or lard diet for 20 days. Histogram represents mean ± SEM from four or five samples of 20,000 cells per diet and genotype. *Upd3* null represents *upd3* transcription in *upd3* null sorted plasmatocytes under control diet. Please also see [Figure S3](#).

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](#)). *Ilp2*, *Ilp3*, and *Ilp5* 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*)-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.

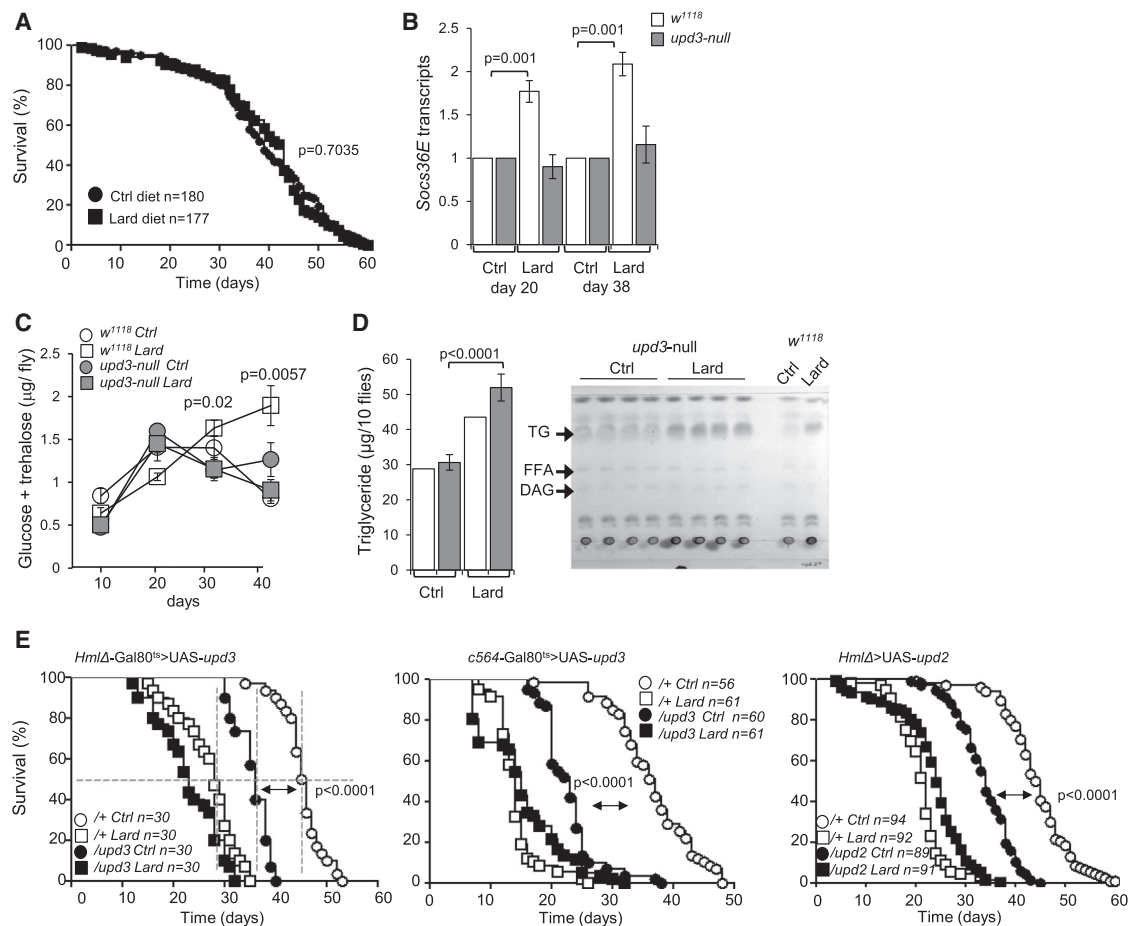
(B) *Upd3* transcription in flies as in (A) at days 0 and 20. Histogram shows mean  $\pm$  SEM of four or five independent samples of three flies per diet and genotype.

(C) Total glucose and trehalose content of flies as in (A) on a control or lard diet for 40 days. Histogram represents mean  $\pm$  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) *llp2*, *llp3*, and *llp5* transcription in *upd3-IR* and control flies fed a lard diet. Three flies per sample; histogram represents mean  $\pm$  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  $\pm$  SEM. Please also see Figure S4.



**Figure 5. Chronic Lipid-Rich Diet Does Not Affect Lifespan and Glucose Level in *Upd3* Null *Drosophila***

(A) Survival of *upd3* null on control and lard diets. Log-rank,  $\chi^2 = 0.2264$ ,  $p = 0.6342$ ; Wilcoxon,  $\chi^2 = 0.1449$ ,  $p = 0.7035$ . Data are pooled from eight independent experiments.

(B) *Socs36E* transcripts in *upd3* null flies and *w<sup>1118</sup>* controls. Three flies per sample; histogram represents mean  $\pm$  SEM of four or five independent samples per diet and per time point.

(C) Time course of glucose and trehalose concentrations in flies as in (B) on a control or lard diet. Histograms represent mean  $\pm$  SEM from four or five samples of three flies per diet.

(D) TLC analysis of TG content of *upd3* null flies and *w<sup>1118</sup>* controls fed a control or lard diet for ten days. Histogram represents quantification of TG;  $n = 4$ , mean  $\pm$  SEM.

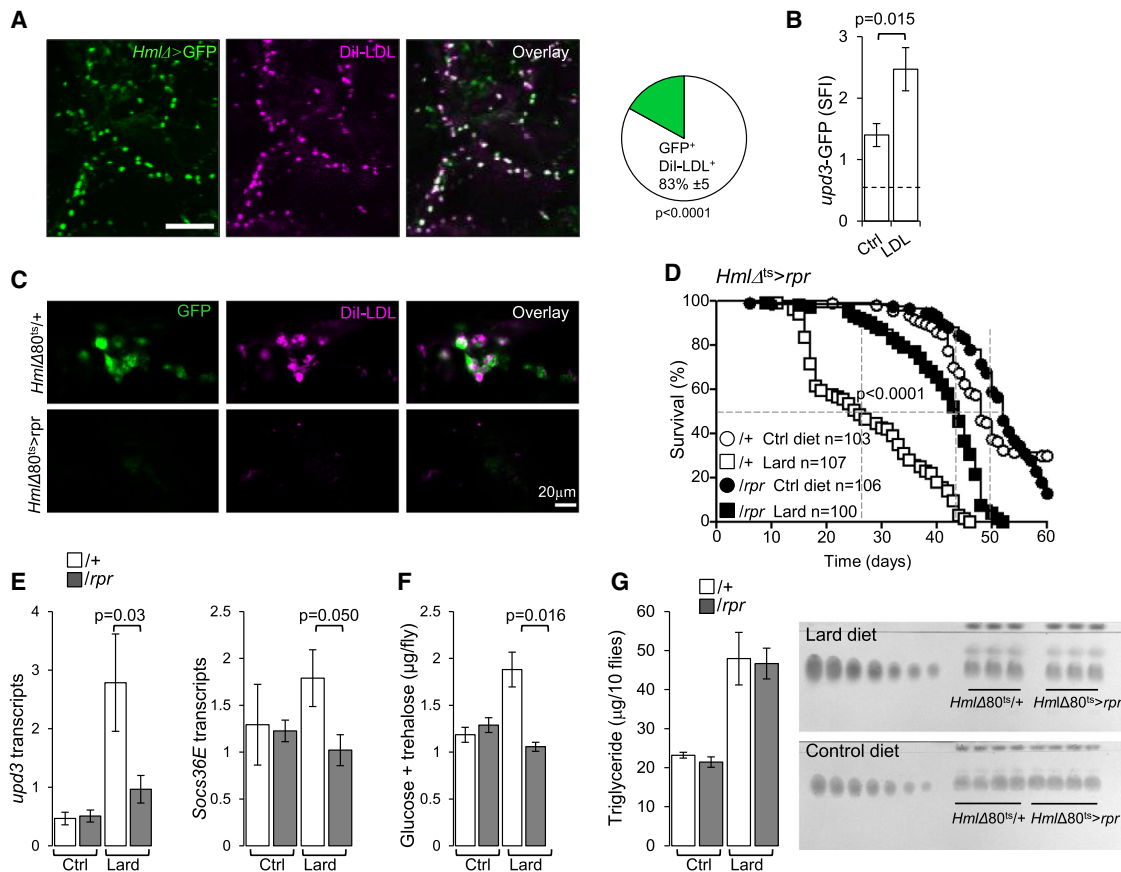
(E) Left: Survival of inducible plasmacyte (*HmlΔ*)-driven overexpression of *upd3* (*HmlΔGal80<sup>ts</sup>* > *upd3*, closed shapes) compared to control flies (*UAS-upd3/+*, open shapes). Log-rank,  $\chi^2 = 56.16$ ,  $p < 0.0001$ ; Wilcoxon,  $\chi^2 = 46.46$ ,  $p < 0.0001$  for flies fed a control diet. Center: Survival of inducible fat body (*c564Gal80<sup>ts</sup>* > *upd3*, closed shapes) compared to controls (*UAS-upd3/+*, open shapes). Data are pooled from two independent experiments. Log-rank,  $\chi^2 = 97.38$ ,  $p < 0.0001$ ; Wilcoxon,  $\chi^2 = 88.73$ ,  $p < 0.0001$  for flies fed a control diet. Right: Survival of plasmacyte (*HmlΔ*)-driven overexpression of *upd2* (*HmlΔ* > *upd2*, closed shapes) compared to controls (*UAS-upd2/+*, open shapes). Data are pooled from three independent experiments. Log-rank,  $\chi^2 = 119.5$ ,  $p < 0.0001$ ; Wilcoxon,  $\chi^2 = 102.7$ ,  $p < 0.0001$  for flies fed a control diet. Please also see Figure S5.

and did not develop hyperglycemia (Figure 5C), although they stored triglycerides when fed lipid-rich diets (Figure 5D). Plasmacyte-specific expression of *upd3* in *upd3* null flies reduced their lifespan on a control diet (Figure S5), and ectopic inducible expression of *upd3* or of *upd2*, in plasmacytes or in the fat body, was also sufficient to reduce *Drosophila* lifespan (Figure 5E).

Altogether, these data suggested that, in *Drosophila* maintained on lipid-rich diet, macrophages are a major source of *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 *reaper* (*rpr*) (White et al., 1996) in macrophages under the control of *HmlΔ* 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 *HmlΔ<sup>+</sup>* cells 1 hr after injection (Figure 6A). Furthermore, expression of *upd3*-GFP increased in plasmacytes of flies



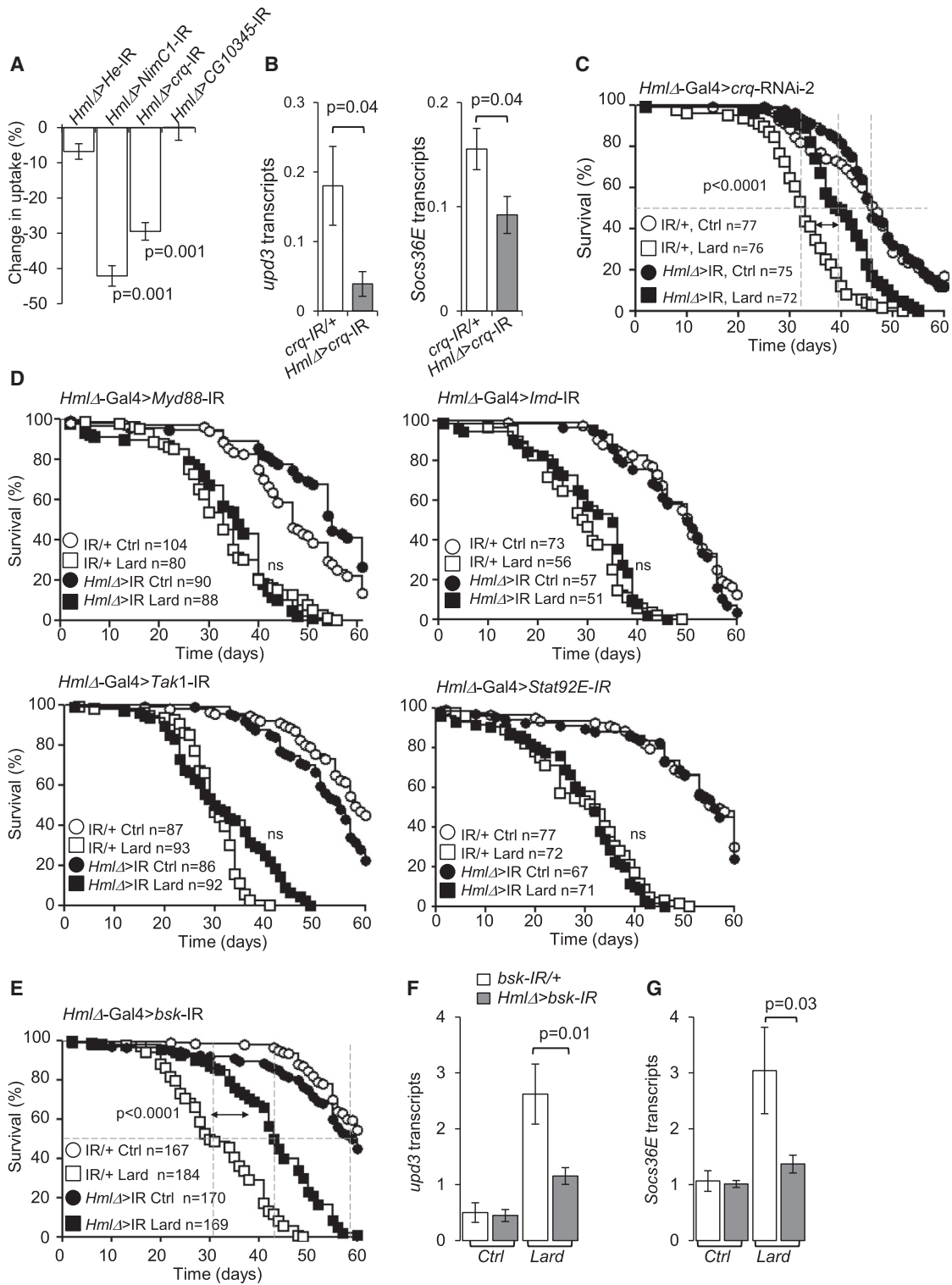
**Figure 6. Macrophage Depletion in Lipid-Rich Diet Rescues *Drosophila* Lifespan and Glucose Metabolism**

(A) Dil-LDL (magenta) uptake 1 hr after injection by *HmlΔ*<sup>+</sup> plasmacytes (green). White cells represent the overlay of GFP and Dil-LDL. Bar, 100 μm. Pie chart: percent colocalization of *HmlΔ*<sup>+</sup> cells with Dil-LDL, mean ± SD,  $p < 0.0001$ ,  $n = 3$ .  
 (B) Sum GFP fluorescence intensity in *upd3*-Gal4,UAS-GFP reporter flies injected with Dil-LDL or PBS. Mean ± SEM,  $n = 4$ .  
 (C) Confocal images of plasmacyte-depleted and control flies injected with Dil-LDL (see [Experimental Procedures](#)).  $n = 4$ .  
 (D) Survival of plasmacyte-depleted (*HmlΔGal80<sup>ts</sup> > rpr*, closed shapes) and control flies (*HmlΔGal80<sup>ts</sup>/+*, open shapes) fed a control or lard diet. Data are pooled from four independent experiments. Log-rank,  $\chi^2 = 75.53$ ,  $p < 0.0001$ ; Wilcoxon,  $\chi^2 = 68.46$ ,  $p < 0.0001$  for flies fed a lard diet.  
 (E) *upd3* and *Socs36E* transcripts in flies as in (D) fed lard or control diets for 20 days; three flies per sample; histogram represents mean ± SEM of four or five independent samples per diet.  
 (F) Total glucose and trehalose content of flies as in (D) on control or lard diet for 30 days. Histograms represent mean ± SEM from four or five samples of three flies per diet.  
 (G) TLC analysis of triglyceride contents of groups of ten flies as in (D) fed control or lard diets for 20 days. Histogram represents quantification of triglyceride (TG);  $n = 4$ , mean ± SEM. Please also see [Figure S6](#).

injected with Dil-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 *llp2*, *llp3*, and *llp5* 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. Plasmacyte-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 *llp2*, *llp3*, and *llp5* 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 plasmacyte-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  $\pm$  SEM; n = 4 flies per genotype.

(B) *upd3* and *Socs36E* transcripts in plasmacyte (*HmlΔ* > *crq-IR*, gray) and control (*UAS-crq-IR/+*, white) flies fed lard diets for 20 days; three flies per sample; histogram represents mean  $\pm$  SEM of four or five independent samples per diet.

(legend continued on next page)

(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- $\beta$ -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- $\kappa$ B activation in macrophages (see Figures 2E, 2F, 3F, and 3G). In contrast, we found that silencing of JNK (*bsk*) in macrophages (*Hml $\Delta$* -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 plasmacyte numbers (Figure S7F). This result was consistent with the observed overrepresentation of AP-1 but not NF- $\kappa$ B binding sites in the vicinity of genes upregulated in plasmacytes from 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 pucker (*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- $\kappa$ B 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- $\kappa$ B-dependent immune response, since reduced longevity

and insulin resistance due to lipid-rich diet were not caused by transcription of NF- $\kappa$ B 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- $\kappa$ B (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 Perimon, 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,

(C) Survival of plasmacyte-specific *crq* knockdown flies compared to controls as in (B) on control or lard diet. Data pooled from four independent experiments. Log-rank,  $\chi^2 = 31.15$ ,  $p < 0.0001$ ; Wilcoxon,  $\chi^2 = 32.75$ ,  $p < 0.0001$  for flies on a lard diet.

(D) Survival of plasmacyte (*Hml $\Delta$* )-specific *Myd88* (top left), *Imd* (top right), *Tak1* (bottom left), and *Stat92E* (bottom right) knockdown flies (closed shapes) and controls (open shapes) on a control or lard diet. *Hml $\Delta$*  > *Myd88*-IR data were pooled from three independent experiments. Log-rank,  $\chi^2 = 0.00000002953$ ,  $p = 1.0000$ ; Wilcoxon,  $\chi^2 = 1.124$ ,  $p = 0.2891$  for flies on a lard diet. *Hml $\Delta$*  > *Imd*-IR: data were pooled from three independent experiments. Log-rank,  $\chi^2 = 2.104$ ,  $p = 0.1469$ ; Wilcoxon,  $\chi^2 = 2.872$ ,  $p = 0.0901$  for flies on a lard diet. *Hml $\Delta$*  > *Tak1*-IR data were pooled from three independent experiments. Log-rank,  $\chi^2 = 1.124$ ,  $p = 0.2891$ ; Wilcoxon,  $\chi^2 = 1.138$ ,  $p = 0.2862$  for flies on a lard diet. *Hml $\Delta$*  > *Stat92E*-IR data are pooled from three independent experiments. Log-rank,  $\chi^2 = 1.011$ ,  $p = 0.3146$ ; Wilcoxon,  $\chi^2 = 0.03924$ ,  $p = 0.8430$  for flies fed a lard diet.

(E) Survival of plasmacyte-specific *bsk* knockdown flies (*Hml $\Delta$*  > *bsk*-IR, closed shapes) and control flies (*UAS-bsk*-IR/+, open shapes) on control and lard diets. Data are pooled from six independent experiments. Log-rank,  $\chi^2 = 112.6$ ,  $p < 0.0001$ ; Wilcoxon,  $\chi^2 = 91.36$ ,  $p < 0.0001$  for flies on a lard diet.

(F and G) *upd3* and *Socs36E* transcription in flies as in (E) on lard or control diets for 30 days; three flies per sample; histogram represents mean  $\pm$  SEM of four or five independent samples per diet. Please also see Figure S7.

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 plasmacyte-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 Gal80<sup>ts</sup>, and therefore the transcription of *reaper*, to induce plasmacyte cell death in adult flies. Once the plasmacytes 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 Gal80<sup>ts</sup>, 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 CO<sub>2</sub>.

#### Plasmacyte Deletion

Plasmacyte cell death was triggered in adult flies by inducible expression of the proapoptotic protein *reaper* (*rpr*) (McGuire et al., 2004). *Rpr* expression was controlled by the temperature-sensitive driver *Tubulin-Gal80<sup>ts</sup>* and the plasmacyte-expressed driver *HmlΔ-Gal4*. In these flies the ubiquitous *Tub-Gal80<sup>ts</sup>* 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/ml) and a low dose (1.28 μg/ml). Flies from each condition were smashed in groups of three, in triplicate, 10 min postinjection in 75 μl of 2x Laemmli 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 microliters of this lysate was loaded per lane. Protein was transferred to nitrocellulose membranes. Antibodies used were as follows: from rabbit, anti-S505-phosphorylated *Drosophila* Akt (CST #4054, 1:1,000) and anti-pan-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 Chemiluminescent 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*; *HmlΔ-Gal4*, *UAS-2xeGFP*) 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 × 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 *HmlΔ*-positive plasmacytes 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 RNeasy 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 Plasmacytes

See Supplemental Experimental Procedures.

#### Plasmacyte Gene Expression Arrays

Plasmacytes were isolated from *w*; *HmlΔ-Gal4*, *UAS-2xeGFP* 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 plasmacytes were FACS sorted from five independent samples per group and sorted directly into 6.4 μl preheated SuperAmp Lysis buffer (Miltenyi). 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 log<sub>2</sub> 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 plasmacyte 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 plasmacyte microarray data was done with Qlucore.

## ACCESSION NUMBERS

MIAME-compliant raw data (gene expression profile of hemocytes from *Drosophila melanogaster* on lipid-enriched diet compared to normal diet) were deposited in the GEO database and can be accessed under the reference GSE63254.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2014.12.023>.

## 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 experiments and analyzed the experimental data.

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