A complex relationship between immunity and metabolism in Drosophila diet-induced insulin resistance

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A Complex Relationship between Immunity and Metabolism in Drosophila Diet-Induced Insulin Resistance

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ABSTRACT Both systemic insulin resistance and tissue-specific insulin resistance have been described in Drosophila and are accompanied by many indicators of metabolic disease. The downstream mediators of insulin-resistant pathophysiology remain unclear. We analyzed insulin signaling in the fat body studying loss and gain of function. When expression of the sole Drosophila insulin receptor (InR) was reduced in larval fat bodies, animals exhibited developmental delay and reduced size in a diet-dependent manner. Fat body InR knockdown also led to reduced survival on high-sugar diets. To look downstream of InR at potential mediators of insulin resistance, transcriptome sequencing (RNA-seq) studies in insulin-resistant fat bodies revealed differential expression of genes, including those involved in innate immunity. Obesity-associated insulin resistance led to increased susceptibility of flies to infection, as in humans. Reduced innate immunity was dependent on fat body InR expression. The peptidoglycan recognition proteins (PGRPs) PGRP-SB2 and PGRP-SC2 were selected for further study based on differential expression studies. Downregulating PGRP-SB2 selectively in the fat body protected animals from the deleterious effects of overnutrition, whereas downregulating PGRP-SC2 produced InR-like phenotypes. These studies extend earlier work linking the immune and insulin signaling pathways and identify new targets of insulin signaling that could serve as potential drug targets to treat type 2 diabetes.

KEYWORDS Drosophila, diabetes, innate immunity, insulin receptor, metabolism

Insulin signaling plays an essential role in the control of glucose homeostasis in all animals. After a sugary meal, insulin or insulin-like peptides are secreted into the circulation. These small-peptide hormones bind to (and activate) a highly conserved insulin receptor (InR) in a variety of target tissues. InR activation leads to a signal transduction cascade, including phosphorylation of the kinase Akt and consequent inactivation of the transcription factor FOXO, among other targets. Still, many insulin targets remain unknown. Depending on the target tissue, InR activation can promote glucose uptake, lipogenesis, and glycogen synthesis and can also regulate mitochondrial size and number, cell size, cell division, and differentiation (1–7).

Type 2 diabetes (T2D) is characterized by systemic insulin resistance and exhibits a range of pathophysiology in various target tissues. T2D patients are at increased risk for cardiovascular disease, nonalcoholic fatty liver disease, neuropathy, nephropathy, and retinopathy, although the mechanisms underlying these comorbidities are not fully understood. Another comorbidity in some T2D patients is increased inflammation. Type 2 diabetics exhibit increased infiltration of macrophages into adipose tissue (8, 9) and increased susceptibility to infection. Conversely, patients with some chronic infections

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are more likely to develop insulin resistance (10, 11). To reduce the incidence of these conditions, it is important to understand the genes linking these two pathways.

*Drosophila* is an efficient and highly sensitive model organism in which to study insulin signaling. Flies express only one InR and one insulin receptor substrate (encoded by *chico*), which are required for both growth and metabolic homeostasis. Insulin-deficient *Drosophila* and insulin-resistant *Drosophila* exhibit reduced size, developmental delay, and hyperglycemia and serve as models of type 1 and type 2 diabetes, respectively (12–14). In *Drosophila*, as in other organisms, a complex relationship between insulin signaling and the immune response exists (15–20). In some settings, increased immunity and increased insulin signaling promote healing: the immune response contributes to the repair of epidermal DNA damage in an InR-dependent manner (21), and epidermal wound healing requires InR (22). In other situations, decreased insulin signaling promotes increased immunity. Chronic FOXO activation (which occurs with decreased insulin signaling) leads to immune activation in the gut (23), and *chico* mutants exhibit increased infection resistance and a reduced bacterial load (24, 25). *Drosophila* FOXO plays a direct role by promoting antimicrobial peptide (AMP) gene expression during starvation, whereas its paralog, Forkhead, directly promotes AMP expression downstream of the target of rapamycin (TOR) (26, 27). Interestingly, overexpressing a constitutively active Toll receptor in the *Drosophila* fat body reduced insulin signaling (17). Therefore, the fly represents a conserved model in which to dissect the interplay between dietary excess, insulin signaling, and the immune response.

Diet-induced insulin resistance can be produced in *Drosophila* by chronic overfeeding using high-calorie diets (28–32). High-sugar (HS)-fed larvae and adult flies develop obesity, dyslipidemia, insulin resistance, decreased fertility, and cardiovascular disease (29–34). Previous studies showed a role for the fat body, a lipid storage tissue akin to mammalian adipose tissue, especially in animals on HS diets. Fat bodies increased lipid storage and developed insulin resistance after HS feeding, compared with control-fed animals. Fat body lipid storage is required for tolerance of HS feeding; genetically lean animals typically exhibit poor survival on HS diets (33, 34). However, roles for the fat body other than lipid storage have been poorly characterized in the context of overnutrition.

In this work, we show a novel role for insulin in controlling the fat body’s immune response in the setting of overnutrition. Diet and genotype were manipulated to probe the insulin signaling pathway in the fly. First, a genomic approach identified potential mediators of insulin signaling in the fat body and found a number of differentially expressed (DE) stress, immune, and metabolic genes as well as the transcription factor Seven-up. We show that fat body insulin signaling controls circulating glucose homeostasis, fat storage, and the immune response. In addition, the immune response exerts a surprising influence on diabetes-like phenotypes. Our data support a model in which the fat body serves to balance the competing pathways of inflammation and insulin signaling during overnutrition.

**RESULTS**

*Fat body insulin signaling promotes glucose clearance and lipid homeostasis.* Previously, we and others showed that HS feeding leads to insulin resistance in the *Drosophila* fat body (30–32). Therefore, we took a genetic approach to study the role of fat body insulin signaling during overnutrition by targeting the sole *Drosophila* InR. We used the GAL4/upstream activation sequence (GAL4/UAS) system to elicit transgenic RNA interference (RNAi) in the larval fat body using r4-GAL4 (38). Fat body *InR* knockdown phenotypes (called InRi throughout) were markedly affected by dietary sugar concentrations. A 1 M concentration of sucrose was almost entirely lethal to r4-GAL4; UAS-InRi flies (Fig. 1A). Therefore, we focused on 0.7 M sucrose (HS) for our InRi overnutrition studies. On control diets (0.15 M sucrose), r4-GAL4; UAS-InRi transgenic larvae showed no significant difference in hemolymph glucose concentrations when compared with *w*1118; r4-GAL4 genetically matched controls (Fig. 1B). Upon HS feeding,
however, r4-GAL4; UAS-InR larvae exhibited significant increases in hemolymph glucose compared with w1118; r4-GAL4 larvae (Fig. 1B). Increased hemolymph glucose or fly “hyperglycemia” correlated with reduced Akt phosphorylation, a downstream marker of InR signaling, in insulin-stimulated fat bodies from r4-GAL4; UAS-InR larvae fed HS diets.

**FIG 1** Fat body loss of InR exacerbates type 2 diabetes-like phenotypes in overfed Drosophila. r4-GAL4 was used to express double-stranded RNA (dsRNA) encoding a UAS-dependent RNAi transgene, targeting InR in the fat body. (A) Wild-type controls survive on 1 M sucrose, whereas very few fat body InRi adults eclose after rearing on this HS diet (n = 6 for each genotype). (B) HS (0.7 M) feeding leads to hyperglycemia in fat body InR loss-of-function wandering third-instar larvae, compared with controls. No effect on hemolymph glucose concentration is observed when InR is reduced in fat bodies of larvae fed the control diet (0.15 M sucrose) (n = 36 for each genotype and diet combination). (C) Fat body reduction of InR expression eliminates insulin-stimulated Akt phosphorylation at serine 505, as expected (n = 9 for each genotype). (D) HS (0.7 M) feeding leads to developmental delay in fat body InR loss-of-function larvae, compared with controls. No developmental delay occurs when InRi larvae are fed the control diet (0.15 M sucrose) (n = 3 for each genotype and diet combination). (E) InR RNAi in fat body reduces whole-animal size in both control and 0.7 M HS-fed wandering third-instar larvae (n = 10 for each genotype and diet combination). (F) No difference in whole-animal triacylglycerides (TAG) is observed upon fat body InR RNAi after rearing on the 0.7 M HS diet (n = 11 for each genotype). (G to I) Nile Red staining of lipid storage droplets in the larval fat body shows increased droplet size upon fat body InR RNAi, compared with controls. Bars, 50 μm. Thirty-nine different fat body fields were measured for each genotype and diet combination. A two-tailed Student t test was used to derive P values. Error bars show standard errors of the means (SEM).
Fig. 1C). The r4-GAL4; UAS-InRi larvae exhibited a significant developmental delay on HS diets (Fig. 1D) as well as a decrease in stage-matched weights on both diets (Fig. 1E), consistent with previously reported phenotypes in insulin-resistant larvae (13, 14, 30, 39). Although no difference in percent body fat was observed (Fig. 1F), fat body lipid droplet size increased when InR was reduced (Fig. 1G, H, and I). Thus, decreased insulin signaling in the fat body is sufficient to exacerbate type 2 diabetes-like phenotypes in Drosophila challenged with overnutrition.

A genomic approach to identify molecular pathways downstream of InR. Because defects in peripheral tissue insulin signaling during caloric overload are associated with a variety of complications, we set out to discover novel factors or effectors downstream of the insulin receptor. Taking a differential expression approach, we characterized the transcriptomes of control and UAS-InRi fat bodies using transcriptome sequencing (RNA-seq). Conversely, we measured differential expression in control and UAS-InRCA fat bodies with transient expression of a constitutively active mutant InR (InRA1325D) (5) (Fig. 2A). Control and InRi-expressing fat bodies were isolated from wandering third-instar larvae, when animals could be synchronized due to the developmental delay of r4-GAL4; UAS-InRi larvae (n = 3 for each genotype). (B) Overlap between the data sets generated by the two strategies, i.e., the common set of InR target genes that we considered to be of interest for follow-up studies. DAVID was used to identify potentially interesting genes and pathways in this overlap.

Fig 2 Comparison of InR loss- and gain-of-function strategies. (A) Fat body loss- and gain-of-function strategies differed in stage and duration. (Left) Constitutively active InR was expressed during the mid-third instar via a heat shock promoter and was compared to InR in heat-shocked wild-type (yw−) controls. Samples used for RNA-seq consisted of yw− or InRCA fat bodies isolated from larvae actively feeding on control (5% sugar) diets for 6 h after the heat shock. (Right) InR loss-of-function was elicited by dsRNA (RNAi) expressed in the fat body throughout larval development on a 0.7 M sucrose diet, compared to fat body driver r4-GAL4 crossed with w1118 controls. Control or chronic InR fat bodies were isolated from wandering third-instar larvae, when animals could be synchronized due to the developmental delay of r4-GAL4; UAS-InRi larvae (n = 3 for each genotype). (B) Overlap between the data sets generated by the two strategies, i.e., the common set of InR target genes that we considered to be of interest for follow-up studies. DAVID was used to identify potentially interesting genes and pathways in this overlap.
twenty-seven genes were differentially expressed in fat bodies after transient InR activation, with a false-discovery rate of less than 5%. One thousand six hundred eighty-four genes were differentially expressed in InR\textsuperscript{i} fat bodies from larvae fed HS diets, whereas only 176 genes were differentially expressed in InR\textsuperscript{i} fat bodies from larvae on control diets, consistent with the increased severity of phenotypes observed on HS versus control feeding in these larvae. To refine our list of target genes, we considered the overlap between the HS-fed InR\textsuperscript{i} and the InR\textsuperscript{CA} differentially expressed (DE) gene data sets, which consisted of 220 genes (see Table S4 in the supplemental material). Some of these overlapping DE genes encode endoplasmic reticulum (ER) stress response proteins and other proteins with roles in electron transfer as well as in nuclear hormone receptor and adipokinetic hormone (Akh) signaling (Table 1). In particular, AkhR expression was increased (1.8-fold) in InR\textsuperscript{i} fat bodies and decreased (−2.3-fold) in InR\textsuperscript{CA} fat bodies. Likewise, expression of the nuclear receptor gene Seven-up (Svp) was increased (2.4-fold) in InR\textsuperscript{i} fat bodies and decreased (−1.8-fold) in InR\textsuperscript{CA} fat bodies (Table S4). Mechanistic studies have established important roles for both AkhR and Svp in fat body metabolic homeostasis (40), suggesting that our list of targets contains physiologically relevant regulators of insulin signaling in the fat body. Other pathways were highlighted by gene ontology analysis using DAVID (36), including redox biochemistry, metabolism, membrane transport, and the immune response (Fig. 2B). Targets of InR included several metabolic enzymes and translational regulators, similar to what others have shown previously (41–43) (Tables 1 and S4).

As in humans, ER stress may play a role in HS-induced hyperglycemia and insulin resistance. Focusing on the set of 83 genes that were coordinately regulated with increased expression in HS-fed, InR\textsuperscript{i} fat bodies and reduced expression when InR was constitutively active, we noticed two genes encoding proteins with roles in the ER stress response. ER oxidoreductin 1-like (encoded by CG1333/Ero1L) helps the ER make disulfide bonds and was increased to 235% in InR\textsuperscript{i} fat bodies and reduced to 46% in InR\textsuperscript{CA} fat bodies (Table 1). P58IPK (encoded by CG8286) acts via protein-protein interactions with ER stress kinases and was increased to 198% in InR\textsuperscript{i} fat bodies and reduced to 39% in InR\textsuperscript{CA} fat bodies (Table 1). Because these ER stress response genes were transcriptionally upregulated in previous studies by the ER stressor tunicamycin (44) and ER stress is associated with type 2 diabetes in mammalian adipose tissues (45–47), we set out to define a role for ER stress in HS-induced fly diabetes-like phenotypes. To test a role for ER stress under HS feeding conditions, we used 0.8 M sucrose, which was the maximum that the larvae could tolerate. This 0.8 M HS diet was supplemented with ER stress relief to look for physiological improvements in larvae. The chemical chaperone 4-phenylbutyrate (PBA) was added to fly food at 5 mM, a concentration previously shown to extend life span and ameliorate ER stress in Drosophila (48, 49). Larvae were reared to maturity on 0.8 M sucrose HS plus vehicle or PBA food, and we measured insulin-resistant phenotypes. A modest but significant reduction in hemolymph glucose was observed with PBA feeding, consistent with an improvement in insulin signaling (Fig. 3A). In addition, triacylglycerides (TAG) increased modestly in males fed PBA in addition to the 0.8 M HS diet (Fig. 3B). However, no differences in weight were observed between control and PBA-fed HS larvae (Fig. 3C) and we saw no change in insulin signaling at the PO4-Akt level in isolated fat bodies (Fig. 3D). Next, we used the protein kinase R-like ER kinase (PERK) inhibitor GSK2606414, which has previously been shown to reduce ER stress and to have therapeutic value via dietary supplementation at 10 μM in flies (50, 51). PERK functions in the ER stress response by phosphorylating the α subunit of eukaryotic initiation factor 2 (eIF2α) and thereby inhibits translation (52, 53). A modest improvement in hemolymph glucose concentrations was observed in larvae reared on GSK2606414-supplemented food (Fig. 3E). No significant improvements in growth or TAG homeostasis were detected, although a trend toward reduced TAG concentrations was observed (Fig. 3F and G), consistent with a role for ER stress in HS-induced metabolic disease. Finally, we overexpressed an activated form of the transcription factor X-box binding protein 1 (Xbp1s), which protects against ER stress (54, 55), in the fat body. We hypothesized that this might protect tissues from
HS-induced metabolic damage, but r4-GAL4; UAS-Xbp1s larvae fared poorly. At a concentration of 0.7 M sucrose, we were able to collect significant numbers of these transgenic larvae but observed no improvements (Fig. 3H to J). Thus, while ER stress may play a role in our model of insulin resistance during caloric overload, it does not seem to play a major role in the fat body.

### TABLE 1 Differential expression in fat bodies undergoing short-term InR activation (InRCA) or chronic InR knockdown (InRk)

<table>
<thead>
<tr>
<th>Gene function and product abbreviation</th>
<th>Gene product full name</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ER stress</strong></td>
<td></td>
<td>InRCA/+</td>
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<tr>
<td>PS8IPK</td>
<td>PS8IPK</td>
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<tr>
<td>Ero1L</td>
<td>Endoplasmic reticulum oxidoreductin 1-like</td>
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<tr>
<td>Hsp70Bb</td>
<td>Heat shock protein 70Bb</td>
<td>5.48</td>
</tr>
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<td><strong>Immune response, antimicrobial peptides</strong></td>
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<td>InRCA/+</td>
</tr>
<tr>
<td>Dif</td>
<td>Dorsal-related immunity factor</td>
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</tr>
<tr>
<td>AttaA</td>
<td>Attacin-A</td>
<td>−4.65</td>
</tr>
<tr>
<td>AttaB</td>
<td>Attacin-B</td>
<td>−7.19</td>
</tr>
<tr>
<td>IM3</td>
<td>Immune-induced molecule 3</td>
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</tr>
<tr>
<td>DptB</td>
<td>Diptericin B</td>
<td>−4.97</td>
</tr>
<tr>
<td><strong>CYP450 enzymes</strong></td>
<td></td>
<td>InRCA/+</td>
</tr>
<tr>
<td>Cyp28a5</td>
<td>Cyp28a5</td>
<td>−2.56</td>
</tr>
<tr>
<td>Cyp28d1</td>
<td>Cyp28d1</td>
<td>−2.55</td>
</tr>
<tr>
<td>Cyp309a1</td>
<td>Cyp309a1</td>
<td>−3.37</td>
</tr>
<tr>
<td>Cyp4d1</td>
<td>Cytochrome P450-4d1</td>
<td>−2.53</td>
</tr>
<tr>
<td>Cyp4d14</td>
<td>Cyp4d14</td>
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<tr>
<td>Cyp4g1</td>
<td>Cytochrome P450-4g1</td>
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<td>Cyp4p1</td>
<td>Cytochrome P450-4p1</td>
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<td>Cyp6d5</td>
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<tr>
<td>Cyp6t1</td>
<td>Cyp6t1</td>
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<tr>
<td>Cyp9b1</td>
<td>Cytochrome P450-9b1</td>
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<tr>
<td>Cyt-b5r</td>
<td>Cytochrome b$_5$-related</td>
<td>−2.32</td>
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<td><strong>Metabolic enzymes, inversely regulated</strong></td>
<td></td>
<td>InRCA/+</td>
</tr>
<tr>
<td>IP3K2</td>
<td>Inositol 1,4,5-triphosphate kinase 2</td>
<td>−2.04</td>
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<td>Odc2</td>
<td>Ornithine decarboxylase 2</td>
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<tr>
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<td>CG1969</td>
<td>Glucosamine-phosphate N-acetyltransferase 1</td>
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<tr>
<td>CG30463</td>
<td>Polypeptide N-acetylgalactosaminyltransferase</td>
<td>2.33</td>
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<td><strong>Metabolic enzymes, coordinately regulated</strong></td>
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<tr>
<td>Sodh-1</td>
<td>Sorbitol dehydrogenase 1</td>
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</tr>
<tr>
<td>Tpi</td>
<td>Triose phosphate isomerase</td>
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<tr>
<td>Pgm</td>
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</tr>
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<tr>
<td>Got2</td>
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<td>Mdh2</td>
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<td>Mdh1</td>
<td>Malate dehydrogenase 1</td>
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</tr>
<tr>
<td>Aldh</td>
<td>Aldehyde dehydrogenase</td>
<td>−2.41</td>
</tr>
<tr>
<td>AKHR</td>
<td>Adipokinetic hormone receptor</td>
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<td><strong>Ribosome</strong></td>
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<td>RpL4</td>
<td>Ribosomal protein L4</td>
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*aInR target genes in the fat body indicate conserved biology between human and fly insulin resistance, including ER stress, the immune response, redox biochemistry, translation, and key metabolic pathways.*
A yin and yang relationship exists between insulin signaling and immunity. Another pathway highlighted by RNA-seq analyses that has been shown to intersect with insulin signaling in other studies is the immune response (17, 26). Hemolymph from larvae fed control diets melanizes quickly, but HS-fed hemolymph demonstrated a marked reduction in melanization, consistent with a reduced innate immune response (Fig. 4A). Differential expression studies in larval fat body and whole larval and adult *Drosophila* flies also supported a role for the immune response: many immune genes were differentially expressed in the contexts of HS feeding, InR reduction, or InR activation (Table 1; see also Tables S1, S2, S3, S4, S5, and S6 in the supplemental material) (30, 33). Therefore, we asked whether the immune response was affected in HS-fed or *r4-GAL4*; *UAS-InR* larvae. We induced septic injury using the Gram-negative

![Figure 3](http://mcb.asm.org/)

**FIG 3** ER stress may play a role in HS-induced hyperglycemia and insulin resistance. Modulation of ER stress during development had mixed effects on wandering third-instar larvae reared on a 0.8 M sucrose HS diet (A to G) or a 0.7 M sucrose HS diet (H to J). (A) Hemolymph glucose concentrations were reduced by feeding HS supplemented with 5 mM 4-phenylbutyrate (PBA) (*n* ≥ 44). (B) Percent body fat in female (F) and male (M) larvae reared on both diets (*n* ≥ 11). (C) Larval weight at maturity after rearing on HS or PBA-supplemented HS diets (*n* ≥ 11). (D) Akt phosphorylation was quantified in the presence of 0.5 μM insulin relative to loading control in fat bodies from larvae reared on HS or PBA-supplemented HS diets (*n* ≥ 9). (E) Hemolymph glucose concentrations in wild-type larvae fed 0.8 M sucrose supplemented with 10 μM PERK inhibitor (inh.) GSK2606414 (*n* ≥ 11). (F) PERK inhibitor seems to modestly decrease stored larval TAG (*n* ≥ 6). (G) No effect of PERK inhibitor on larval weight was observed (*n* ≥ 6). (H) Fat body overexpression of the spliced Xbp1 cDNA (*Xbp1s*) was unable to reduce hyperglycemia in larvae fed 0.7 M HS diets, compared with driver-crossed controls (*n* ≥ 24). (I) Fat body overexpression of Xbp1s led to modest effects on TAG accumulation in male larvae (*n* ≥ 10). (J) Fat body overexpression of Xbp1s reduced larval weight in both sexes (*n* ≥ 10). A two-tailed Student *t* test was used to derive *P* values. Error bars show SEM.
bacterium *Pseudomonas aeruginosa* (S) at a concentration at which many but not all animals typically die (see Materials and Methods for details). A significant fraction of larvae died after a clean pricking injury (data not shown) as reported by others (57). Therefore, we continued with adult infection studies. Consistent with a diminished immune response indicated by reduced immune gene expression after HS feeding (Tables S5 and S6), HS-reared adults exhibited increased infection-based mortality after 1 week (79%), compared with control-diet-reared flies (59%) (Fig. 4B), consistent with a reduction in immune function. Clean pricking (without the pathogen) produced a negligible reduction in 7-day adult survival regardless of diet (Fig. 4). Given the finding that loss-of-function in insulin receptor signaling increased the expression of immune response genes (Tables 1, S1, and S2), we hypothesized that the *r4-GAL4*; UAS-*InRi* loss-of-function flies would be protected from death after infection. Indeed, this was the case, as 23% of *r4-GAL4*; UAS-*InRi* loss-of-function flies survived acute infection, compared with a 12% survival for control flies fed the same moderately high-sugar diet (0.7 M sucrose) (Fig. 4C). Therefore, reducing InR in the fat body can partially rescue the increased infection susceptibility observed during overnutrition.

**Peptidoglycan recognition protein RNAi produces insulin-like phenotypes.** Several members of the immune gene family encoding the peptidoglycan recognition proteins (PGRPs) were differentially expressed along with insulin signaling (Table 1). We chose two of these for further study. *PGRP-SB2* is selectively expressed in the larval fat body according to FlyAtlas (58) and is downregulated by HS feeding and by *InR* RNAi in the fat body. We knocked down *PGRP-SB2* and observed a significant improvement in growth and increase in the number of flies surviving 1 M sucrose (Fig. 5A and B). *PGRP-SC2*, in contrast to *PGRP-SB2*, was upregulated by expressing *InR* RNAi in the fat body. Knockdown of *PGRP-SC2* led to the opposite phenotypes of *PGRP-SB2*, displaying sugar-specific lethality and reduced size during caloric overload, compared with controls (Fig. 5C and D). Other HS-associated phenotypes showed less striking influences of PGRP knockdown (see Fig. S1 in the supplemental material). There was not a rescue of the H5-induced developmental delay in *r4-GAL4*; UAS-*PGRP-SB2* larvae (Fig. S1A). Fat body *PGRP-SB2* RNAi did not improve hemolymph glucose homeostasis or triglyceride content (Fig. S1B and C), and *r4-GAL4*; UAS-*PGRP-SB2* fat bodies did not have improved insulin signaling, at least as measured by phospho-Akt level (Fig. S1D). *PGRP-SC2* RNAi did have minor effects on TAG content (Fig. S1E) and infection susceptibility (Fig. S1F). As in fat body *InR* RNAi adult flies, *PGRP-SC2* RNAi improved infection resistance despite reducing growth and survival on HS diets, although the data were significant only at one time point (Fig. S1F). On the other hand, *PGRP-SB2* RNAi adults seemed to exhibit

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**FIG 4** Attenuation of the immune response by HS feeding. (A) Hemolymph isolated from wandering third-instar larvae reared on 0.15 M sucrose melanizes within 5 min at room temperature (left), whereas little color is observed in 5-min-old hemolymph isolated from larvae reared on 1 M sucrose (right). (B) Seven-day survival of adults reared on 0.15 M or 1 M sucrose diets. Three- to 7-day-old wild-type (w*1118*; *r4-GAL4* transheterozygous) flies were infected with a semilethal dose of *Pseudomonas aeruginosa*, and survival was monitored for 7 days (n ≥ 23 vials). (C) Seven-day survival of adults reared on a 0.7 M sucrose diet. Three- to 7-day-old control (w*1118*; *r4-GAL4* transheterozygous) and *InR* (w*11022*; UAS-*InRi*) fly survival over 7 days after inoculation with *P. aeruginosa* (n ≥ 19 vials). Control flies pricked without *P. aeruginosa* are shown at the top of each graph (n ≥ 13 vials for each). A two-tailed Student *t* test was used to derive *P* values. Error bars show SEM.
a slight impairment in infection resistance, which approached statistical significance only in 0.7 M-reared animals (Fig. S1G and H). These data support a model in which PGRP-SB2 impairs insulin signaling and PGRP-SC2 promotes it.

Identification of novel potential InR-dependent promoters. To search for genomic elements that confer insulin sensitivity, we characterized potential regulatory mechanisms for two gene lists: (i) genes decreased in InRi fat bodies and increased in InRCA fat bodies (45 genes) and (ii) genes decreased in InRCA fat bodies and increased in InRi fat bodies (83 genes). Known TF binding sites, as well as de novo 5- to 12-base sequences, were identified in promoters (-700 to +100 with respect to the transcriptional start site [TSS]) of the genes in each of the two lists. Several such sequence elements were identified as enriched in these data and may serve as sensors of insulin signaling (Fig. 6).

DISCUSSION

In this work, we link two established functions of the fat body, insulin signaling and the immune response, and show that they vary reciprocally to one another at the transcriptional level. Increasing insulin signaling led to reduced immune gene expression, and decreasing insulin signaling led to increased immune gene expression and increased resistance to infection. Surprisingly, we found that HS diet-induced obesity and growth defects could be regulated by fat body misexpression of immune response genes. This cell-autonomous molecular relationship complements previous studies in both flies and mammals (16, 19, 20, 59) and supports a counterregulatory model in which insulin signaling and the immune response negatively regulate each other to maintain energy balance.
Using differential expression to search for potential downstream regulators of insulin signaling in the fat body, we focused on selected gene ontology categories based on the literature. A number of known FOXO targets were identified, as expected (60–62). Ribosomal subunits were also overrepresented, consistent with previous studies showing a direct relationship between insulin signaling and ribosome biogenesis (41, 63). This DE gene list was therefore likely to include additional, novel targets of physiological importance. Most (58%) of the overlapping DE genes were inversely regulated between constitutively active and reduced InR function. Many, however, were regulated the same direction in both paradigms, which was unexpected. The former are likely to be more-direct targets and/or sensors of insulin pathway activity. The latter group of genes are likely to have more-complicated relationships with the insulin signaling pathway, such as stage- or diet-specific roles. Changes in gene expression might represent downstream effects of insulin signaling or counterregulatory attempts to fix cellular growth or metabolic defects during insulin resistance. One counterregulatory attempt might be the observed increase in fat body \textit{PGRP-SC2} expression in InRi fat bodies, which might be expected to improve downstream aspects of insulin signaling. Another potential compensatory mechanism for insulin resistance in InRi fat bodies is the gene \textit{ilp6}, which encodes a growth promoter during metamorphosis (64) and increases in expression in InRi fat bodies on both control and HS diets (351% and 385%, respectively; for both comparisons, \( P < 0.001 \)). The promoter elements identified using bioinformatics analysis may serve as key markers for and/or mediators of insulin signaling. Future studies will explore these possibilities.

Many fat body DE genes represent conserved pathways that are of clinical importance in the pathophysiology of type 2 diabetes. Both HS and InR knockdown in the fat body increased the expression of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting step of gluconeogenesis (Table 1) (33). The insulin-resistant fat body may therefore act somewhat like the insulin-resistant liver, which exhibits an increase in hepatic gluconeogenesis in type 2 diabetes in some rodent and human studies (65, 66). InR also led to increased pyruvate dehydrogenase kinase (PDK) and decreased ATP citrate lyase expression, both of which could inhibit glycolytic flux. Reducing glycolysis contributes to hyperglycemia and insulin resistance in some genetic forms of type 2 diabetes (67, 68) and is the basis of the competitive fatty acid versus glucose utilization model known as the Randle cycle (69, 70). In addition, we observed significant changes in the expression of cytochrome P450 enzymes and well as regulators of fatty acid and polyamine metabolism, protein glycosylation, and the immune response. We set out to understand which of these InR DE genes represented physiological changes in insulin-resistant flies.

First, we looked at ER stress, also known as the unfolded protein response. During
ER stress, the accumulation of aberrant proteins leads to compensatory increases in degradation or refolding chaperones. ER stress is relieved by clearing the ER lumen of misfolded proteins and by reducing translation (71). ER stress increases in obese, high-calorie-fed, T2D patients, and reducing ER stress can improve T2D in rodents (45, 47, 72). We saw a transcriptional upregulation of genes that promote the ER stress response along with a downregulation of ribosomal genes in insulin-resistant fat bodies. Surprisingly, there was only a minor role for ER stress in fat body insulin signaling, if any. One possibility is that the upregulation of ER stress response genes can offset an early increase in ER stress, such that healthy levels of stress can be maintained.

Both differential expression and septic injury studies pointed to an important and conserved relationship between insulin resistance and the immune response. Type 2 diabetic patients exhibit increased susceptibility to infection, and some infections increase insulin resistance (11, 73–75). Likewise, in the Drosophila HS diet-induced T2D model, systemic insulin resistance and obesity caused by HS feeding also corresponded with reduced survival after septic injury. This parallel between flies and humans is consistent with a recently established model in which diet-induced obesity paradoxically increases inflammation but reduces insulin function (8, 9, 18). The increase in immune gene expression in InR fat bodies corresponded to improved resistance to infection in these animals, which is perhaps a surprising result, considering the increased susceptibility to infection in HS-reared flies. Reducing InR in this context seems to negate the detrimental effects of an HS diet, such that genetically engineered insulin resistance acted protectively, boosting the immune response. Our results are consistent with a previously reported role for the transcription factor FOXO, a negative regulator of insulin signaling, which can directly induce immune gene expression in flies and mammalian cells (26, 76). Interestingly, previous studies in Drosophila have shown that infection or activation of the tumor necrosis factor (TNF) receptor or Toll pathway can inhibit insulin secretion and signaling (16, 17, 77), whereas eliminating macrophages improves insulin sensitivity (78). Our results support a negative-feedback model in which insulin signaling inhibits innate immunity and immunity inhibits insulin signaling, a relationship that is strained on an HS diet. This mechanism would help to maintain metabolic homeostasis when nutrients are limited via reallocation of fat body resources to fighting infection when necessary instead of to promotion of growth or energy storage. Such a model has been proposed (18, 79).

A wide variety of antimicrobial peptides and peptidoglycan recognition proteins (PGRPs) are secreted by the fat body during the Drosophila immune response, which typically recognizes the presence of infection via the Toll or IMD pathways, converging on the transcription factor NF-κB to promote pathogen recognition and elimination, as in humans (80, 81). Taking a candidate gene approach, we focused on two genes in the PGRP family, PGRP-SB2 and PGRP-SC2. PGRP-SB2 expression was increased in InRCA fat bodies, whereas PGRP-SC2 mRNA was increased in InRi fat bodies, suggesting opposing functions for these two proteins. Reducing PGRP-SB2 in the fat body led to increased growth and survival when challenged with HS feeding, suggesting that it exerts a negative effect on insulin signaling. In contrast, fat body RNAi targeting PGRP-SC2 gave phenotypes similar to those induced by InR RNAi, including increased lethality and reduced size on HS diets, suggesting that PGRP-SC2 is protective against HS-induced metabolic toxicity. An increase in infection resistance, albeit at a single time point (Fig. S2F), is consistent with previous reports that PGRP-SC2 negatively regulates IMD/Relish-dependent immune gene expression (23, 82). Interestingly, other studies have found no role for PGRP-SB or PGRP-SC in susceptibility to infection despite their effects on immune gene expression (23, 83, 84). Although the mechanisms underlying the different phenotypes in these PGRP-SB2i and PGRP-SC2i flies are still poorly understood, they are likely to fit into the narrative established by previous investigators showing a complex relationship between immunity and insulin signaling. Future studies will explore the roles of these and other InR target genes and putative InR target promoters in the insulin response.

These studies represent a simple paradigm in which to identify insulin targets in
different cells, organs, and tissues that exhibit insulin-dependent phenotypes, such as the heart, eye, brain, gut, gonad, or muscle. Because the complications of type 2 diabetes affect many organs and systems, probing transcriptional targets of InR in these and other tissues may reveal additional, tissue-specific genes and mechanisms that contribute to insulin resistance.

MATERIALS AND METHODS

**Fly lines.** Controls and RNAi lines were obtained from the Vienna Drosophila Resource Center (VDRC). VDRC’s w^1118 line (stock line number 60000) and RNAi-TK landing site control (60100) were used as genetic background controls for RNAi transgenes targeting InR (number 992), PGRP-SB2 (number 106538), and PGRP-SC2 (number 104578). hs-GAL4 (number 2077) and UAS-Inr.A1325D (number 8263) lines were from the Bloomington Drosophila stock center. The r4-GAL4 line was from reference 38, and the UAS-Dcr2 line was used in all crosses to amplify RNAi (85). Offspring of the control cross between line 60100 and the UAS-Dcr2; r4-GAL4 line were used to test the ER stress drugs (Fig. 3). UAS-Xbp1s flies were a gift from Diego Rinson-Limas (54).

**Drosophila husbandry.** The lab maintenance diet was a standard corn meal-dextrose-agar diet. Experimental diets consisted of Bloomington’s Semidefined food with sucrose used for all sugar at concentrations of 0.15 M (5%), 0.7 M (24%), 0.8 M (27%), and 1 M (34% sucrose), as used in previous studies (30, 33); 5 mM 4-phenylbutyrate (PBA; Sigma number P21005) and 10 μM PERK inhibitor (GSK2606414) were added to the diet as indicated, and these diets were compared to diets using an equivalent amount of the appropriate solvent or diluent (water or dimethyl sulfoxide [DMSO]). Heat shock treatments were done for 30 min in empty fly vials using a 37°C water bath. Six hours of recovery after heat shock gave robust phosphorylation of Akt, and this duration was therefore used for the RNA-seq experimental samples.

**Gene expression.** RNA-seq experiments were done as described previously (33) using three biological replicates for each genotype and/or diet. Briefly, fat bodies were separated from the rest of the animal after bisecting, inverting, and placing the insect into phosphate-buffered saline (PBS). Fat bodies were dislodged by pipetting and separated by centrifugation; the purity of fat body tissue was checked on a slide before freezing in TRIzol. RNA was extracted and DNase and Ribozyme treated, and then libraries were prepared and Illumina Hi-seq sequenced and data were processed by the Washington University Genome Technology Access Center. The Tuxedo Suite (86) and EdgeR (35) were used to detect expressed transcripts with a P value of <0.01 and a false-discovery rate of <0.05. DAVID was used to characterize the gene ontology of differentially expressed gene lists with a P value cutoff of <0.05 (36). Microarray studies on adult Canton-S flies were done as previously described (30).

**Hemolymph glucose assays.** Hemolymph glucose assays were done as described previously (33). Briefly, larvae were rinsed in a sieve to remove any food from the cuticle surface and then dried. Jeweler’s fine forceps were used to injure the animal, and hemolymph was collected and then added to frozen Infinity glucose reagent (Thermo number TR22421), and the mixture was incubated at 37°C for 5 to 10 min and then quantified using a microplate spectrophotometer at OD_{340} and a standard curve and blank well.

**Triglyceride assays.** Triglyceride assays were done as described previously (33). Briefly, wandering third-instar larvae were rinsed and dried and then weighed and frozen at −80°C until the assay. PBS−0.1% Tween was used to homogenize larvae. Tubes were incubated at 65°C for 5 min and then cooled to room temperature. An aliquot was added to Thermo Infinity triglyceride reagent (number TR22421), and the mixture was incubated at 37°C for 5 to 10 min and then quantified using a microplate spectrophotometer at OD_{570} and a standard curve and blank well.

**Insulin stimulation and Western blotting.** Insulin stimulation experiments were done as previously described (33). Briefly, larvae were rinsed, bisected, inverted, and placed in Schneider’s medium. Insulin (1 μM; Sigma S6243) or dilution buffer (10 mM HEPES) was added, and larvae were cultured for 15 min. Fat bodies were harvested and frozen in sample buffer until Western blotting was performed. The PO_{4}-Akt band intensity was normalized to syntaxin as a loading control. Primary antibodies used were from Cell Signaling (anti-PO_{4}-Akt at Ser505, number 4054) and the Developmental Studies Hybridoma Bank (antisyntaxin, number 8C3).

**Nile Red staining and lipid droplet quantification.** Larvae were inverted to expose fat bodies, fixed for 30 min in 4% paraformaldehyde in PBS, and then washed 3 times, 10 min each time, in PBS and 0.1% Triton X (PBS-TX) and stained in PBS-TX + 0.001% Nile Red (Acros number 200007-168) for 1 to 1.5 h. Larvae were washed again in PBS-TX and mounted for confocal microscopy using a laser excitation of 543 nm. ImageJ software was used to quantify droplet sizes.

**Infection assays.** *Pseudomonas aeruginosa* strain PA14 was grown overnight and diluted to an A_{600} of 0.005, which corresponded to approximately 3.0 × 10^7 CFU/ml. Adults were anesthetized in CO2 and injured using a 0.001-mm-tip tungsten needle (Fine Science Tools number 10130-05) dipped in culture and then allowed to wake, and viability was monitored for 7 days. Larval infection susceptibility was measured by septic injury during the wandering third instar, and then viability was monitored for 10 days, spanning metamorphosis and eclosion. Animals stabbed without inoculum were used as a control and determined to die of death not associated with the pathogen.

**Bioinformatics motif discovery.** We used the MatInspector program (37, 87) in Genomatix Genome Analyzer (GGA) v3.2, to identify known transcription factor binding sites common to at least 85% of the sequences in each gene list. We searched the putative promoter sequences (lengths optimized by GGA)
against Genomatix’s Matrix Family Library version 9.1, including all vertebrate matrices, insect matrices, and Core Promoter Elements (where a match required 0.75 similarity to the core, most conserved positions of the binding site, as well as similarity above a matrix-specific cutoff). We used the HOMER (Hypergeometric Optimization of Motif EnRichment) suite of tools (88) to perform de novo motif discovery, i.e., identify novel binding sites within the putative promoter sequences of genes in each list. We defined the promoter sequences as bases \(-700\) to \(+100\) of the TSS and looked for motifs of length \(5\) through \(12\) that are enriched relative to the rest of the genome.

Accession number(s). Complete RNA-seq and microarray data are accessible at GEO (https://www.ncbi.nlm.nih.gov/geo/) via accession numbers GSE97447 (InRi data), GSE96763 (InRCA data), and GSE105448 (adult Canton-S data).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/MCB.00259-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 3, XLSX file, 0.2 MB.
SUPPLEMENTAL FILE 4, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 5, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 6, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 7, XLSX file, 0.1 MB.

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