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Systemic Activin signaling independently regulates sugar homeostasis, cellular metabolism, and pH balance in *Drosophila melanogaster*

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The ability to maintain cellular and physiological metabolic homeostasis is key for the survival of multicellular organisms in changing environmental conditions. However, our understanding of extracellular signaling pathways that modulate metabolic processes remains limited. In this study we show that the Activin-like ligand Dawdle (Daw) is a major regulator of systemic metabolic homeostasis and cellular metabolism in *Drosophila*. We find that loss of canonical Smad signaling downstream of Daw leads to defects in sugar and systemic pH homeostasis. Although Daw regulates sugar homeostasis by positively influencing insulin release, we find that the effect of Daw on pH balance is independent of its role in insulin signaling and is caused by accumulation of organic acids that are primarily tricarboxylic acid (TCA) cycle intermediates. RNA sequencing reveals that a number of TCA cycle enzymes and nuclear-encoded mitochondrial genes including genes involved in oxidative phosphorylation and β-oxidation are up-regulated in the *daw* mutants, indicating either a direct or indirect role of Daw in regulating these genes. These findings establish Activin signaling as a major metabolic regulator and uncover a functional link between TGF-β signaling, insulin signaling, and metabolism in *Drosophila*.

dIlp2 | hormone | acidosis

Regulation of cellular metabolism and metabolic homeostasis is crucial for maintaining cellular and organismal physiology. Consequently, robust regulatory networks have evolved in most organisms to adapt and maintain a desired metabolic state depending on the environment and/or developmental stage (1, 2). Deciphering how components of these networks function to achieve homeostasis is essential for understanding normal physiology and the underlying mechanisms behind multifaceted disorders like metabolic syndrome and diabetes. Our understanding of these regulatory networks is largely confined to known metabolic pathways like the insulin signaling (IS) pathway, cellular target of rapamycin (TOR) pathway, and neuroendocrine signals. Nevertheless, additional extracellular signaling mechanisms that can regulate cellular and physiological metabolism must exist to allow integration of environmental, physiological, and developmental cues. In accordance with this possibility, involvement of classical developmental pathways in regulating physiological homeostasis has recently gained much attention (3, 4).

One such developmental pathway that has been shown to affect several aspects of metabolism, including nutrient and energy homeostasis, is TGF-β signaling (5, 6). TGF-β signaling has recently been proposed to regulate mitochondrial biogenesis in mammals based on the observation that aberrant Activin signaling in mice can lead to changes in energy metabolism and mitochondrial gene expression (7). However, the study used a gene-replacement strategy where the mature domain of Activin-A was replaced with that of Activin-B (*Inhiba*^{BK}), thereby changing the relative levels of these two ligands. TGF-β ligands signal through distinct combinations of type I and type II receptors that are specific to different classes of ligands (8). Although both Activin-A and -B can signal redundantly through the type I receptor ALK4, Activin-B can also signal through ALK7 initiating unique biological responses (9). Hence, studies involving the *Inhiba*^{BK} mouse fail to distinguish individual contributions of Activin-A and Activin-B in the manifestation of the energy metabolism defects. Additionally, both Activin-A and -B have been implicated in regulation of IS (10). Because IS is known to impinge on both the TOR and AMPK signaling pathways, aberrant Activin signaling can potentially affect mitochondrial metabolism by affecting IS. Parsing out these mechanistic details is crucial for understanding the role of TGF-β/Activin signaling in metabolism and devising strategies to manipulate this pathway for therapeutic gain.

In this study, we use *Drosophila melanogaster* to investigate the role of TGF-β/Activin signaling in regulation of metabolic homeostasis. *Drosophila* contains a highly conserved TGF-β signaling pathway. However, the number of signaling components in *Drosophila* is much smaller than vertebrates, allowing easy genetic manipulation of the pathway and reduces complications arising from functional redundancies between signaling molecules (8). Canonical TGF-β/Activin signaling in the fly is mediated by just three ligands, Activinβ (Actβ), Dawdle (Daw) [also called Alp (Activin-like peptide)], and Myoglianin (Myo), that signal through a single type I receptor, Babo, and a single intracellular R-Smad homolog, Smox (11). In addition, considerable parallels exist in the regulation of metabolic homeostasis between *Drosophila* and mammals providing a powerful genetic

Significance

Deciphering the systemic signaling mechanisms that modulate metabolic activity has important implications owing to the central role that metabolism plays in regulating organismal adaptability and survival. Here, we show that loss of *Drosophila* TGF-β/Activin-like ligand Dawdle (Daw) causes major alterations in larval metabolic activity, including accumulation of tricarboxylic acid cycle intermediates, acidification of hemolymph pH, and misregulation of insulin signaling and nuclear-encoded mitochondrial gene expression. These metabolic defects lead to a food-dependent lethality phenotype, suggesting that Daw likely influences environmental adaptability via its modulation of several central metabolic processes. These observations, coupled with previous findings in mammals and *Caenorhabditis elegans*, highlight a potentially conserved role for TGF-β/Activin signaling in regulating important metabolic processes across the animal kingdom and may have clinical implications.

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system to investigate the role of TGF-β signaling in metabolism and homeostasis (1, 2, 5).

Here, we demonstrate that canonical TGF-β/Activin signaling, mediated by the *Drosophila* Activin homolog Daw, is a central metabolic regulator that impacts both mitochondrial metabolism and IS. Interestingly, we find that although Daw regulates IS by positively regulating release of *Drosophila* insulin-like peptides (Dilps), the effect of Daw on mitochondrial metabolism is independent of insulin/insulin-like growth factor signaling (IIS) and may be mediated by changes in expression of nuclear-encoded mitochondrial genes.

Results

Absence of Daw Leads to Food-Dependent Lethality and Loss of Insulin Signaling. Null *daw* mutants showed a food-dependent larval lethality phenotype where greater than 90% of mutant larvae died before the third instar wandering stage when grown on standard cornmeal food (CMF) (Fig. 1 *A* and *B*). Mutant larvae that did reach the pupal stage (<10%) showed significant developmental delay compared with *yw* controls (Fig. 1*A*), and the mutant pupae never reached the adult stage. Interestingly, both larval lethality and developmental delay were significantly rescued when mutants were grown on 60% yeast paste food (YPF) (Fig. 1 *A* and *B*). Moreover, when grown on YPF, a significant number of *daw* mutants eclosed as adults that were of comparable size to control animals (Fig. S1*A*). Similar to *daw* mutants, loss of *babo* or *Smox* also showed a food-dependent larval lethal phenotype. The majority of either *babo* or *Smox* mutant larvae also failed to reach the pupal stage on CMF but formed prepupae on YPF. Notably, unlike *daw*, null mutations in either *myo* or *actβ* do not significantly affect larval viability on CMF food. These results show that canonical TGF-β signaling, initiated by Daw, is necessary for survival on the CMF.

To determine the cause of this food-dependent lethality, we looked for metabolic defects in the *daw* mutants by measuring steady-state levels of key metabolites in feeding third-instar mutant larvae grown on YPF and comparing them to stage matched controls. *daw* mutant larvae showed a significant increase in total triacylglycerol (TAG), glucose, and glycogen stores compared with controls (Fig. 1 *C*–*E*). These observations indicate a role for Daw in IIS as a number of previous studies in *Drosophila* have shown an association between elevated TAG and glycogen with loss of IIS (12–14). Because IIS is also known to negatively regulate circulating sugar concentration in *Drosophila* larvae, we examined and measured the circulating sugar concentration of *daw* mutant and control larvae and found a significant increase in the *daw* mutants compared with relevant controls confirming a strong loss of IIS in *daw* mutants (Fig. 1*F*). *daw* mutant larvae also showed a strong reduction in phospho-Akt level and a strong increase in InR expression in the peripheral tissues, further supporting the loss of IIS in these mutants (Fig. S1 *B* and *C*). A similar increase in circulating sugar concentration (about 40%) was also observed in *Smox* null mutant larvae, indicating that Daw acts through the canonical Smad pathway to regulate IIS (Fig. 1*G*).

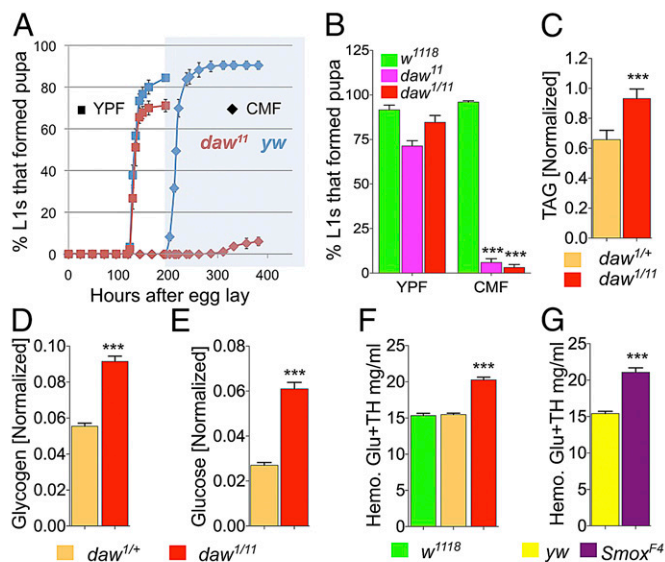

Fig. 1. Loss of canonical Daw signaling leads to food dependent larval lethality and loss of IIS. (*A*) Developmental curves showing percentage of larvae that formed pupae and the time required to reach pupariation on CMF and YPF (*n* = 3–4; all viability assays contained 60 animals per sample). (*B*) Viability of homozygous (daw^{11}) and transheterozygous ($daw^{1/11}$) mutants and wild-type controls on CMF and YPF (*n* = 6). (*C*–*E*) Total TAG, glycogen, and glucose content (normalized to total protein) of *daw* mutant and control larvae grown on YPF (*n* = 20–36). (*F* and *G*) Hemolymph glucose plus trehalose (Glu+TH) concentration of *daw* and *Smox* mutant and control larvae (*n* = 36 for *daw* and controls and *n* = 8–12 for *Smox* mutants and controls). All results are reported as means ± SEM.

Loss of Daw Leads to Accumulation of Tricarboxylic Acid Cycle Intermediates and Systemic Acidosis. To identify potential additional metabolic roles for Daw in larvae, we performed a high-throughput GC/MS-based metabolomics analysis of *daw* mutant and control larvae comparing abundances of small cellular metabolites. We found that loss of Daw led to a significant increase in the abundance of multiple intracellular metabolic acids. The majority of these organic acids are intermediates in the tricarboxylic acid (TCA) cycle, indicating a role of Daw in the regulation of intracellular sugar/mitochondrial metabolism (Fig. 2*A*). Interestingly, some of these metabolites, such as citrate and succinate, are found in high concentrations in the hemolymph of Lepidopterans and are proposed to play an important role in regulating pH balance (15, 16). We therefore checked defects in pH homeostasis in the *daw* mutants. We found that accumulation of acidic metabolites in the *daw* mutants is indeed associated with significant acidification of *daw* larval hemolymph compared with controls (Fig. 2*B*). Interestingly, CMF is substantially more acidic than YPF owing to addition of propionic acid (PA) as a mold inhibitor. Because *daw* mutants exhibit internal acidosis, we explored the possibility that the mutants may be vulnerable to acidic food conditions. We found that PA-induced acidity is one of the major components of CMF that leads to lethality of *daw* mutants on this food (Figs. S2 and S3 and *SI Results and Discussion*).

The accumulation of acidic metabolites in *daw* mutants indicates that the observed acidosis is likely metabolic in origin. We tested whether the acidosis phenotype of *daw* mutants can be rescued by activating TGF-β signaling in major metabolic tissues of the larvae. We found that expression of an activated type I receptor, $babo^{CA}$, in large metabolically active tissues like the gut, muscle, or fat body (FB), was able to significantly rescue the acidosis phenotype (Fig. 2*C*). Interestingly, overexpressing $babo^{CA}$ in the FB most strongly affected hemolymph pH, making the hemolymph significantly more basic than normal, and also caused a significant reduction in levels of metabolic acids in the *daw* mutants (Fig. 2 *C* and *D*). These observations indicate that Daw affects hemolymph pH and production of metabolic acids by primarily targeting the FB and also indicates a direct correlation between acidosis and the level of metabolic acids.

Daw Dose-Dependently Regulates Hemolymph Sugar Concentration and pH. TGF-β signaling in *Drosophila* is mediated by three ligands, Daw, Actβ, and Myo. Because loss of Daw alone leads to the acidosis phenotype, we checked whether Daw is the primary TGF-β ligand acting on the larval FB. Western blots performed using FB and muscle/epidermis tissue showed that endogenous p-Smox signal is specifically lost in the larval FB in absence of Daw, indicating that Daw is the primary TGF-β ligand acting on the FB (Fig. 3*A*). Consistently, overexpression of *daw* in the FB caused significant alkalinization of the *daw* mutant

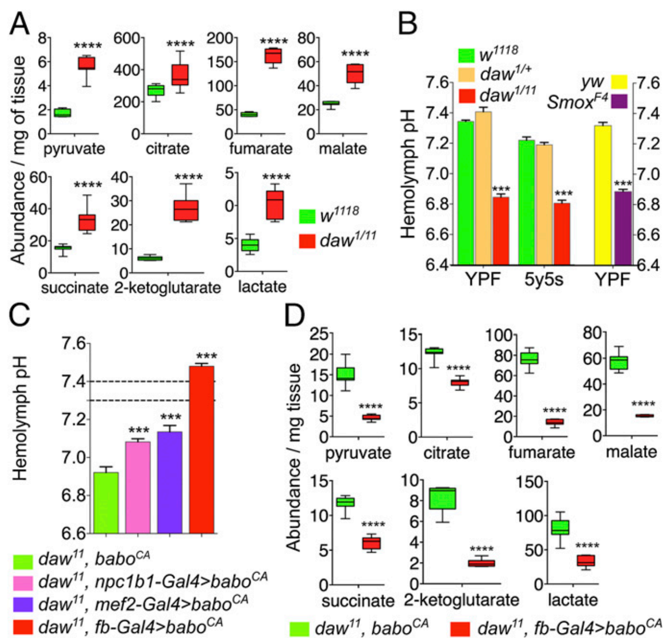

Fig. 2. Loss of canonical Daw signaling leads to cellular metabolic defects and systemic acidosis. (*A*) High-throughput GC/MS-based measurement of cellular metabolic intermediates show significant increase in multiple sugar metabolism and TCA cycle intermediates (n = 8.) (The experiment was repeated twice. Results remained consistent and box plots from only one set of data are shown here.) (*B*) Hemolymph pH of *daw* mutant, *Smox* mutant, and control larvae grown on YPF and a semidefined 5y5s food (n = 8–12). (*C*) Hemolymph pH of *daw* mutant expressing $babo^{CA}$ in the gut (*npc1b1-Gal4*), muscle (*mef2-Gal4*), and FB (*fb-Gal4*) and relevant control larvae (n = 8–12). (Dashed lines indicate range for hemolymph pH of wild-type animals YPF.) (*D*) Relative abundance of metabolites that were up-regulated in *daw* mutants in control (*daw* mutant) larvae and mutant larvae overexpressing $babo^{CA}$ in the larval FB (box plot; n = 8 per value; with 15 animals per sample).

hemolymph, phenocopying the effect of FB-specific overexpression of $Babo^{CA}$ (Fig. 3*B*). Interestingly, FB overexpression of *daw* also completely rescued larval lethality of the *daw* mutants on CMF enabling them to eclose as adults (Fig. S4*A*) and also significantly rescued the diabetic phenotype observed in the *daw* mutants (Fig. 3*C*). The FB however is unlikely to be the target of *daw* signaling for the loss of IIS observed in the mutants because expression of smox RNAi in the FB does not induce a diabetic phenotype in wild-type larvae, nor does FB > $Babo^{CA}$ rescue the diabetic phenotype *daw* mutants (Fig. 4*E*). Therefore, we explored the possibility that Daw made by the FB can act on other tissues in the larvae in an endocrine fashion. To test this hypothesis, we tried rescuing the acidosis phenotype of the *daw* mutants by overexpressing *daw* in the insulin-producing cells (IPCs). Overexpression of *daw* in the IPCs was able to significantly rescue the acidosis phenotype of the *daw* mutants, indicating that Daw made by the IPCs can rescue TGF-β signaling in the FB that is responsible for the acidosis phenotype (Fig. 3*D*). We further confirmed the ability of Daw to function in an endocrine manner by overexpressing *daw* in a number of non-FB tissues (IPCs, oenocytes, and muscle) in *daw* mutants and by examining restoration of p-Smox in the FB using Western blots. Overexpression of *daw* in any of these tissues significantly restored p-Smox in the FB, and the strength of signal in the FB correlated with the size of the tissue in which *daw* was expressed (muscle > oenocytes> IPCs; Fig. 3*E*). Overexpression of *daw* in any of these tissues could also rescue lethality of *daw* mutants on CMF, and expression of the other two *Drosophila* TGF-β ligands, *actβ* and *myo*, did not increase in these rescued animals (Fig. S4 *B* and *C*). These results show that Daw expressed in one tissue can indeed signal systemically and likely in a dose-dependent manner much like a hormone. The dose-dependent effect of Daw on the strength of TGF-β signaling is also observed in our rescue experiments for metabolic phenotypes. Whereas expressing *daw* in the IPCs partially rescue both the acidosis and diabetic phenotype, expressing *daw* in the FB caused the hemolymph pH to become more alkaline than normal (Fig. 3*B*) and also caused the circulating sugar concentration to drop much below the normal range (Fig. 3*D*).

Daw Independently Regulates Dilp Release and Accumulation of Metabolic Acids in Peripheral Tissues. Because *daw* mutants show metabolic phenotypes such as an elevated circulating sugar concentration and TAG accumulation, similar to that produced by a reduction in IS, we sought to elucidate the mechanism by which Daw affects IIS. To do so, we examined various aspects of IIS such as *dilp* expression, Dilp release, and signal reception in peripheral tissues of *daw* mutants. We found that loss of Daw did not affect mRNA levels of *dilp1*, *dilp2*, *dilp3*, or *dilp5* in the IPCs of feeding mid–third-instar larvae (Fig. 4*A*). Under feeding conditions, larval IPCs release most of their Dilps into the hemolymph resulting in only faint signals within the neuron cell body when immunostained with anti-Dilp antibodies (17). However, immunostaining CNSs from feeding *daw* mutant larvae with anti-Dilp2 antibody showed significant residual Dilp2 peptide in the IPC cell bodies (Fig. 4 *B* and *B′*). In the absence of any change in mRNA levels of *dilp2*, this result likely indicates a deficiency in Dilp release from the IPCs. Similar accumulation was also observed for Dilp5 peptide when the ability of the mutant IPCs to release the peptide in response to feeding was assayed (Fig. S5*A*). These results suggest that Daw affects IIS by regulating release of Dilps from the larval IPCs.

Loss of IS is known to cause metabolic ketoacidosis in vertebrates. Therefore, we tested whether loss IIS is responsible for, or contributes to, the acidosis phenotype observed in the *daw* mutants. To this end, we generated chronically diabetic larvae by overexpressing a potassium channel *Ork1ΔC* in the larval IPCs thereby blocking Dilp release (Fig. S6*A*). However, these severely diabetic larvae showed normal hemolymph pH, indicating that loss of IIS alone does not induce metabolic acidosis in *Drosophila* larvae (Fig. 4*C*). To further test whether loss of IIS contributes to acidosis observed in the *daw* mutants, we rescued the diabetic phenotype of the *daw* mutants and checked whether

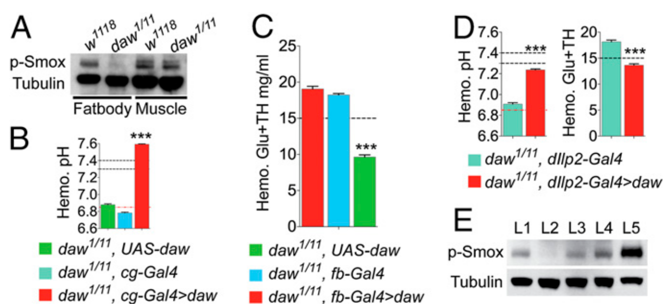

Fig. 3. Dose-sensitive endocrine role of Daw in regulating sugar and pH homeostasis. Black dashed lines in the figures indicate normal value/range of hemolymph sugar concentration or pH in wild-type animals. (*A*) Endogenous p-Smox signal in wild-type and daw mutants larval FB and muscle. (*B*) Hemolymph pH of *daw* mutant larvae expressing *daw* under the regulation of *cg-Gal4* driver and of relevant controls (n = 6). Red dashed line, hemolymph pH of *daw* mutants on YPF. (*C*) Hemolymph Glu+TH concentration of *daw* mutant larvae expressing a *daw* under the regulation of FB-specific driver *fb-Gal4* and of relevant controls (n = 12–20). (*D*) Hemolymph pH and Glu+TH concentration of *daw* mutant larvae expressing *daw* in the larval IPCs (*dilp2-Gal4* driver) and of relevant controls (n = 12–13 and 24, respectively). (*E*) Endogenous p-Smox levels in FB tissue dissected from wt, mutant control and rescued animals expressing *daw* in the IPCs, oenocytes, and muscle. L1: w^{1118}; L2: $daw^{1/11}$, *UAS-daw/+*; L3: $daw^{1/11}$, *dIlp2 > daw*; L4: $daw^{1/11}$, *OK72 > daw*; L5: $daw^{1/11}$, *mef2 > daw*. All quantitative results are reported as means ± SEM.

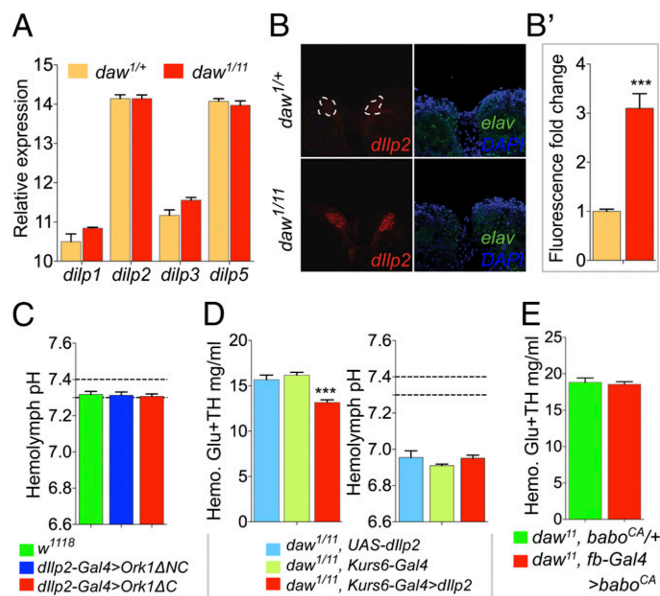

Fig. 4. Independent roles of Daw in Dilp release and metabolic acidosis in the *Drosophila* larvae. (*A*) Expression level of *dilp1*, *dilp2*, *dilp3*, and *dilp5* in dissected $daw^{1/11}$ and control larval CNSs reported as abundance relative to *rpl23* (n = 6). (*B*) Immunostaining of CNSs from stage matched feeding *daw* mutant and control larvae with anti-Dilp2 (red) antibody (stained and imaged under identical conditions). Anti-Elav (green) staining of same samples serves as internal control for staining intensity. (*B′*) Fluorescence-intensity quantification of Dilp2 staining of CNSs from *daw* mutant and control feeding larvae (n = 9 CNSs from three independent experiments). (*C*) Hemolymph pH of mid–third-instar larvae expressing a potassium channel *Ork1ΔC* in the IPCs (n = 8–11). (*D*) Hemolymph Glu+TH concentration and hemolymph pH of *daw* mutant larvae overexpressing *dilp2* under regulation of the *Kurs6-Gal4* driver and of relevant controls (n = 12 for both experiments). (*E*) Hemolymph sugar concentration of *daw* mutant larvae expressing $babo^{CA}$ under the regulation of *fb-Gal4* driver and of mutant controls (n = 10–14). All results are reported as means ± SEM.

acidosis was ameliorated. Overexpressing *dilp2* in a group of noninsulin producing neurosecretory cells using the *Kurs6-Gal4* driver has been shown previously to hasten developmental timing and produce smaller adults, as expected for Dilp2 overproduction (17). We found that overexpressing *dilp2* using the *Kurs6-Gal4* driver significantly reduced circulating sugar concentration in wild-type larvae (Fig. S6*B*) and also significantly rescued the diabetic phenotype of *daw* null mutants (Fig. 4*D*). However, *Kurs6-Gal4*–driven overexpression of *dilp2* did not rescue the acidosis phenotype observed in the *daw* mutants (Fig. 4*D*), indicating that loss of IIS does not contribute to acidosis observed in *daw* mutants. Conversely, activation of TGF-β signaling in the FB, which can rescue both acidosis and accumulation of TCA cycle intermediates in the *daw* mutants (Fig. 2*C*), does not rescue the diabetic phenotype observed in the mutants (Fig. 4*E*). Taken together, these results show that the effect of Daw on acidosis and accumulation of TCA cycle intermediates is independent of its effect on IIS.

Expression of Cellular Metabolic and Nuclear-Encoded Mitochondrial Genes Is Significantly Altered in the *daw* Mutants. RNA-sequencing analysis of FB-specific RNA was used to examine the molecular basis of the metabolic changes that lead to the acidosis phenotype in *daw* mutants. A total of 986 genes deregulated ≥1.3-fold in the *daw* mutant FB were detected. A gene ontology (GO)-enrichment analysis shows that majority of the significantly enriched GO classes belongs to cellular metabolic processes (Fig. S7). We found that, consistent with acidosis observed in *daw* mutants, genes involved in carboxylic and organic acid metabolic processes were significantly more enriched among the up-regulated genes (Fig. S7). Notably, a large number of TCA cycle enzymes were significantly up-regulated in the *daw* mutants, indicating an increase in TCA cycle activity and output (Fig. 5*A*). Apart from TCA cycle enzymes, a large number of known and predicted proteins involved in the respiratory electron transport chain (ETC) were also significantly up-regulated (Fig. 5*B* and Fig. S8). Additionally, enzymes involved in β-oxidation, lipid mobilization, and ketogenesis were also up-regulated (Fig. 5*C* and Fig. S8). Because the TCA cycle, ETC, and β-oxidation are all housed in the mitochondrion, these results indicate a general increase in the expression of nuclear-encoded mitochondrial genes in the *daw* mutants. Up-regulation of β-oxidation and lipid mobilization genes also indicate an increased mobilization and degradation of fatty acids that can fuel the TCA cycle and mitochondrial metabolism by producing more acetyl-CoA. Consistently, a liquid chromatography/MS-based quantification of acetyl-CoA showed a significant increase in acetyl-CoA content of *daw* mutant larvae (Fig. S9*B*).

Discussion

In this study, we investigated the involvement of the *Drosophila* Activin-like ligand Daw in regulating systemic and cellular metabolism. Our results indicate that Daw is a major regulator of cellular and mitochondrial metabolism and IIS in *Drosophila* larvae. We also find that loss of Daw leads to a significant increase in the expression of numerous known and predicted nuclear-encoded mitochondrial genes (heretofore referred to as mitochondrial genes) that are involved in the ETC, TCA cycle, and β-oxidation, indicating a potential role of Daw in regulating mitochondrial gene expression.

We report that loss of Daw leads to significant internal acidosis that is associated with increased abundance of metabolic acids like pyruvate and lactate along with citrate, malate, succinate, α-ketoglutarate, and fumarate that are intermediates of TCA cycle. Because activating TGF-β signaling in the FB could rescue both the acidosis phenotype and accumulation of metabolic acids in the *daw* mutants, our observations strongly suggest that acidosis observed in *daw* mutants is metabolic in nature and is caused by the accumulation of metabolic acids produced by the larval FB. Accumulation of TCA cycle intermediates is of particular interest because it indicates an involvement of TGF-β signaling in regulating either directly or indirectly the TCA cycle. We observe that loss of Daw leads to significant increase in the expression of multiple TCA cycle enzymes in the larval FB,

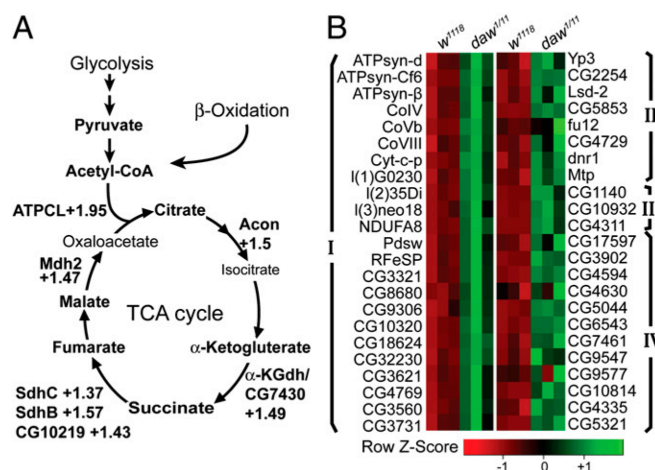

Fig. 5. Daw is a major regulator of metabolic gene expression in the *Drosophila* larval FB. (*A*) Enzymes involved in the TCA cycle are up-regulated in the *daw* mutants. (*B*) Heat map of selected gene groups that were significantly up-regulated in the *daw* mutants. Group I, known and predicted ETC genes; group II, lipid mobilization; and group III, Ketogenesis and Group IV: β-oxidation.

leading to the possibility that overproduction of TCA cycle intermediates is caused by an increased TCA cycle activity. Similar effects of TCA cycle enzyme overexpression on overproduction of metabolic acids have been documented before (18–20). For instance, overexpression of mitochondrial malate dehydrogenase has been shown to increase both production and exudation of citrate, oxalate, malate, succinate, and acetate by up to 4.2-fold and 7.1-fold, respectively, in Alfalfa root tips (20). Similarly, overexpression of citrate synthase has been documented to increase citrate production in both *Saccharomyces cerevisiae* and *Arabidopsis* (18, 19). We checked whether a similar effect could be seen in *Drosophila* larvae by overexpressing *Drosophila* mitochondrial malate dehydrogenase (*mdh2*) using a tub-Gal4 driver. Pan larval overexpression of *mdh2* was able to cause significant acidification of larval hemolymph, supporting the possibility that loss of *daw* leads to acidosis by up-regulating expression of TCA cycle enzymes (Fig. S9*A*). Nevertheless, our data do not rule out the possibility that overexpression of mitochondrial genes is rather an outcome and not the cause for the metabolic changes seen in *daw* mutants.

Although the effects of Daw on acidosis and accumulation of mitochondrial metabolites is caused primarily by its action on the larval FB, we could rescue these phenotypes by overexpressing *daw* in the IPCs, indicating that Daw can act in an endocrine fashion. This conclusion is further supported by our data showing that loss of endogenous p-Smox signal in *daw* mutant FB is rescued by overexpressing *daw* either in the IPCs, oenocytes, or muscle. Additionally, our finding that exogenously expressed Daw influences circulating sugar concentration, hemolymph pH, and FB-specific p-Smox signal in a tissue size (dose)-dependent manner further suggests that Daw is likely released into the hemolymph from tissues of origin and exerts its effect in an endocrine manner like a hormone. Consistent with this view, recent MS data reveal the presence of Daw in the hemolymph (21). Curiously, unlike most hormones whose expression is limited to one tissue or gland, *daw* is expressed in a wide array of tissues including muscle, FB, gut, imaginal discs, and surface glia of the CNS (22, 23). One possible purpose for such a wide distribution in hormone expression could be that, being a metabolic regulator, expression or release of Daw is regulated by local nutritional or stress responses and Daw in turn signals to other tissues to allow systemic integration of these stimuli.

In vertebrates Activin-A has been proposed to positively regulate insulin release based on in vitro experiments showing increased Ca^{2+} influx and insulin release from isolated β-cells upon stimulation with Activin-A (10, 24, 25). Whereas numerous receptor and TGF-β–antagonist loss/gain-of-function studies support a physiological role for TGF-β signaling in β-cell function and glucose tolerance (26, 27), evidence for any in vivo role of Activin-A in insulin release is lacking. Our finding, that Daw positively regulates Dilp release and IIS in *Drosophila* larvae, provides compelling evidence for the in vivo role of an Activin-like ligand in insulin release. Recent work in *Caenorhabditis elegans* suggests a similar role of TGF-β signaling in insulin release where loss of the worm R-Smad homolog *daf-8* blocks release of a surrogate neuropeptide (human atrial natriuretic factor–GFP) from the insulin-producing ASI neurons (28). Mechanistically, DAF8 was shown to regulate polarity of the neurosecretory neurons by up-regulating expression of a potassium channel (*exp-2*). However, such a cell-autonomous mechanism may not be the case in *Drosophila* because neither *SmoxRNAi* nor $babo^{CA}$ overexpression in the IPCs affected circulating sugar concentration in the wild-type or *daw* mutants, respectively (Fig. S5 *B* and *C*). Moreover, the *Drosophila exp-2* homolog, *shab*, did not show any expression change in *daw* mutants, indicating potential mechanistic differences in the way TGF-β signaling regulates insulin release in worms and flies (Fig. S5*D*). Nevertheless, our work demonstrates a functional conservation of the role of TGF-β/Activin signaling in regulating insulin release and provides previously unidentified direct evidence for an in vivo role of a TGF-β/Activin ligand in the process.

Intriguingly, although loss of IS is known to cause metabolic ketoacidosis in vertebrates, we found that chronic loss of IIS did not lead to acidosis in *Drosophila* larvae, nor did loss of IIS contribute to the acidosis observed in the *daw* mutants. Lack of a ketoacidosis response in *Drosophila* compared with vertebrates upon loss of IIS could be attributable to differences in the way IIS regulates metabolism in insect larvae. Unlike vertebrates, where loss of insulin leads to fat mobilization and production of ketone bodies, in *Drosophila* and *Bombyx* larvae, loss of insulin leads to accumulation of TAG, suggesting that the modes of alternative energy production upon loss of IIS are likely different in insect larvae and vertebrates (13, 14, 29).

Another interesting point is that we could also completely rescue acidosis in *daw* mutants without ameliorating the diabetic phenotype. This finding indicates that acidosis does not contribute to loss of IIS in the *daw* mutants, as has been proposed by a few vertebrate studies where metabolic acidosis seems to lead to insulin resistance (30, 31). It is important to note that acidosis always correlates with the relative amount of metabolic acids. Because we could reduce the output of metabolic acids without affecting the diabetic phenotype, we also conclude that Daw regulates peripheral cellular and mitochondrial metabolism independent of IIS.

Deregulation of mitochondrial metabolism and function is associated with a number of both inherited and acquired human disorders, including but not limited to type 2 diabetes, neurodegenerative disorders, and tumorigenesis (32, 33). Consequently, therapeutic interventions that can restore or increase mitochondrial activity are thought to be essential for our ability to treat these complex and often debilitating diseases (32). Our study showing that the *Drosophila* Activin-like ligand Daw is involved either directly or indirectly in regulating mitochondrial gene expression and TCA cycle activity suggests that TGF-β/Activin signaling could be a potential therapeutic target for modulating metabolism. Additionally, our results suggest the possibility that there could be a functional relationship between altered TGF-β signaling and the manifestation of certain human diseases. For instance, both acidosis and TGF-β signaling are known to play critical roles in tumor progression by facilitating angiogenesis and metastasis (34–38). Our observation that TGF-β signaling can affect production of metabolic acids, including lactate, presents a unique perspective for understanding the role of TGF-β signaling in tumor progression. The effect of Daw on TCA cycle activity and expression of oxidative phosphorylation genes also has implications for tumor growth. Reduction of TCA cycle activity and oxidative phosphorylation coupled with an increased flux through glycolysis (Warburg effect) is characteristic of most tumors and is considered essential for production of cellular building blocks required for sustaining rapid cellular growth (39, 40). Involvement of Daw in the regulation of the TCA cycle and oxidative phosphorylation, along with the strong reported association of TGF-β with tumor growth, raises the possibility that TGF-β affects tumor growth by impinging on Warburg metabolism. Interestingly, a recent paper demonstrating the involvement of TGF-β signaling in promoting Warburg metabolism in breast tumors is consistent with this view (41). In conclusion, our study showing the involvement of the *Drosophila* TGF-β/Activin ligand Daw in regulating metabolism and IS expands our understanding of the role of TGF-β signaling in physiology and disease. Additionally, our work opens up the possibility of using *Drosophila* genetics for better understanding the role of TGF-β signaling in regulating metabolism.

Materials and Methods

Fly Strains and Food. *daw* alleles daw^{11} and daw^{aact1} (referred as daw^1) and $Smox^{F4}$, $babo^{Fd4}$, and $babo^{CA}$ alleles are described elsewhere (22, 42–45). Other fly lines used were as follows: *UAS-daw* (22), *UAS-dilp2* (46), *UAS-Ork1ΔC* and *UAS-Ork1ΔNC* [Bloomington Stock Center (BSC)], *UAS-SmoxRNAi3B3* (43), *cg-Gal4* (BSC), *fb-Gal4* (T. Neufeld, University of Minnesota, Minneapolis), *mef2-Gal4* (BSC), *npc1b1-Gal4* (L. Pallanck, University of Washington, Seattle), *UAS-mdh2* (47), and *Kurs6-Gal4* (G. Korge, Freie University Berlin, Berlin).

For overexpression experiments, a single *Gal4* element was used to drive a single UAS construct. Controls contained either a single copy of the *Gal4* driver (Gal4 control) or a single copy of the UAS element (UAS control). Mutant and control larvae were grown on YPF or semidefined food from the time of hatching onwards, unless mentioned otherwise. Refer to *SI Materials and Methods* for more details on growth conditions; 5y15s [high-sugar diet (HSD)] food consisted of 5% (wt/vol) yeast, 10% (wt/vol) dextrose, and 5% (wt/vol) sucrose.

Metabolite and pH Assay. TAG, glycogen, and hemolymph sugar assays were performed as described elsewhere (17, 48). For hemolymph pH assay, 3 μL of hemolymph was mixed with 2 μL of pyranine dye (Invitrogen; 1.2 mM final concentration). The mixture was briefly centrifuged, and the absorbance spectrum of the supernatant was measured using a multichannel nanodrop spectrophotometer (NanoDrop 8000). The ratio of absorbance at 450 nm and 405 nm was plotted against a standard curve to determine the pH. pH standards ranging between pH 6.6 and pH 7.6 were prepared by titrating 50 mM Tris·HCl. See *SI Materials and Methods* for more details.

Statistics. All graphs and statistical analyses were generated using GraphPad Prism4. Standard Student *t* test or ANOVA followed by a post hoc Tukey's honest significant difference test was used for statistical analysis. *P* values are designated using the following convention: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, and $****P \leq 0.0001$.

Antibodies and Immunostaining. Antibodies used were rat anti-Dilp2 (1/500), mouse anti-Dilp5 (1/800) (gift from the P. Leopold laboratory, Institut Valrose Biologie, Nice, France), and mouse anti-Elav (1/500) (9F8A9; Developmental Studies Hybridoma Bank). Antibody straining was performed as described elsewhere (17). Samples were imaged using a confocal laser-scanning microscope (Zeiss LSM710).

Fluorescence Quantification. Confocal Z series of the IPCs were obtained using a 1-μm step size and identical laser power and scan settings. Zeiss Zen software was used to generate multiple intensity projection images. Subsequently, background signal was subtracted and fluorescence intensity was measured by drawing selections on the IPCs.

RNA Sequencing. RNA sequencing (Illumina) of FB RNA was performed by Genewiz. Data analysis was done using the Galaxy tool set made available by the Minnesota Supercomputing Institute.

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