Torso-Like Is a Component of the Hemolymph and Regulates the Insulin Signaling Pathway in *Drosophila*

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ABSTRACT In *Drosophila*, key developmental transitions are governed by the steroid hormone ecdysone. A number of neuropeptide-activated signaling pathways control ecdysone production in response to environmental signals, including the insulin signaling pathway, which regulates ecdysone production in response to nutrition. Here, we find that the Membrane Attack Complex/Perforin-like protein Torso-like, best characterized for its role in activating the Torso receptor tyrosine kinase in early embryo patterning, also regulates the insulin signaling pathway in *Drosophila*. We previously reported that the small body size and developmental delay phenotypes of *torso-like* null mutants resemble those observed when insulin signaling is reduced. Here we report that, in addition to growth defects, *torso-like* mutants also display metabolic and nutritional plasticity phenotypes characteristic of mutants with impaired insulin signaling. We further find that in the absence of *torso-like*, the expression of insulin-like peptides is increased, as is their accumulation in insulin-producing cells. Finally, we show that Torso-like is a component of the hemolymph and that it is required in the prothoracic gland to control developmental timing and body size. Taken together, our data suggest that the secretion of Torso-like from the prothoracic gland influences the activity of insulin signaling throughout the body in *Drosophila*.

KEYWORDS Torso-like; Insulin signaling; Body size; Developmental timing; *Drosophila melanogaster*

IN holometabolous insects such as *Drosophila*, pulses of the steroid hormone ecdysone regulate the timing of developmental transitions, including the metamorphic transition, and hence growth duration (Henrich *et al.* 1999; Mirth and Riddiford 2007). During the larval and pupal stages of development, ecdysone is produced and released by the major endocrine organ, the prothoracic gland (PG), in response to multiple environmental and developmental stimuli [for review see Danielsen *et al.* (2013)]. Accordingly, there are many complex cellular signaling pathways involved in coordinating the responses to these signals. A well-studied example is prothoracicotropic hormone (PTTH), a brain-derived neuropeptide that regulates the production and release of

ecdysone in response to developmental cues (McBrayer *et al.* 2007). Prior to each larval molt, PTTH is secreted and activates the Torso (Tor) receptor tyrosine kinase (RTK) in the PG, which signals via the Ras/mitogen-activated protein kinase pathway to upregulate a set of ecdysone biosynthesis genes (Rewitz *et al.* 2009). Ablation of the PTTH neurons, or loss of function mutations in *tor*, prolongs the growth period between each developmental transition and results in an overall increase in body size (McBrayer *et al.* 2007; Rewitz *et al.* 2009).

Another critical signaling pathway that regulates ecdysone production is the evolutionarily conserved insulin signaling pathway, which acts in the PG to regulate ecdysone biosynthesis in response to nutrition (Caldwell *et al.* 2005; Colombani *et al.* 2005; Mirth *et al.* 2005). In particular, this pathway regulates larval growth rate and the timing of a developmental checkpoint known as critical weight, thereby controlling the timing of the onset of metamorphosis (Mirth *et al.* 2005; Koyama *et al.* 2014). In *Drosophila*, the insulin-like receptor (InR) is activated by a family of insulin-like peptides (dILPs) (Brogiolo *et al.* 2001). A subset of these (dILPs 2, 3, and 5) are expressed

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in neurons that innervate the corpora cardiaca, a group of cells neighboring the PG, and ablation of these neurons causes developmental delays and decreased body size (Rulifson *et al.* 2002).

We and others recently reported that mutant alleles of the Membrane Attack Complex/Perforin-like (MACPF) protein Torso-like (Tsl) exhibit a developmental delay phenotype indicative of defects in ecdysone production (Grillo *et al.* 2012; Johnson *et al.* 2013). Tsl is best known for its role in embryonic patterning, where it functions upstream of the Tor receptor to control its activity for patterning the embryo termini (Stevens *et al.* 1990; Savant-Bhonsale and Montell 1993; Martin *et al.* 1994). Unexpectedly, however, we reported evidence that Tsl does not act similarly with Tor in the PG to control developmental transitions and body size. Specifically, *tsl* and *tor* have opposing effects on body size, and the developmental delay phenotype observed in *tsl;tor* double mutants is strikingly enhanced compared to either mutation alone, suggesting an additive rather than epistatic interaction (Johnson *et al.* 2013).

The growth defects of *tsl* mutants more closely resemble those observed when insulin signaling is reduced; however, Tsl has not previously been implicated in the insulin signaling pathway. Furthermore, it is not clear whether the growth and developmental timing phenotypes of *tsl* null mutants are due to a role for Tsl in the PG. Here, we report that in addition to growth defects, *tsl* mutants also display several other physiological and biochemical characteristics of impaired insulin signaling. We further show that Tsl is required in the PG to control developmental timing and body size, and that it influences the expression of dILPs and their accumulation in the insulin-producing cells (IPCs). Finally, we show that Tsl is present in the larval hemolymph, strongly supporting the idea that Tsl is secreted from the PG into circulation, where it acts to regulate systemic insulin signaling.

Materials and Methods

Drosophila stocks

The following stocks were used: *w¹¹¹⁸* (BL5905), *chico¹* (BL10738), *Df(2L)BSC143* [*chico^{df}* (BL9503), a chromosomal deficiency that deletes the *chico* coding region], *InR^{A1325D}* (BL8263, a constitutively active form of InR), and *dilp2-3^Δ, dilp5³* (BL30889) from the Bloomington *Drosophila* stock center; *c7-Gal4*, obtained from FlyView (Janning 1997); *tsl^Δ*, a null mutant of *tsl* (Johnson *et al.* 2013); *phm-Gal4* (ch2) and *UAS-dicerII*; *phm-Gal4-22*, gifts from Michael O'Connor, University of Minnesota, Minneapolis (Ono *et al.* 2006); and *UAS-tsl^{RNAi}* and *gHA:tsl*, gifts from Jordi Casanova, Institute for Research in Biomedicine, Barcelona (Jimenez *et al.* 2002; Furriols *et al.* 2007). All flies were maintained at 25° on fly media containing, per liter: 7.14 g potassium tartrate, 0.45 g calcium chloride, 4.76 g agar, 10.71 g yeast, 47.62 g dextrose, 23.81 g raw sugar, 59.52 g semolina, 7.14 ml Nipagen (10% in ethanol), and 3.57 ml propionic acid.

Tsl constructs and generation of transgenic lines

To generate the *UAS-Tsl:HA* and *UAS-Tsl:RFP* constructs, the open reading frame of *tsl* followed by a short linker encoding the peptide SAGSAS and either three tandem hemagglutinin (HA) epitopes (for *UAS-Tsl:HA*) or the open reading frame for RFP (for *UAS-Tsl:RFP*) was synthesized and subcloned (GenScript) into pUASTattB via *Bgl*II and *Xho*I sites. To generate the *phm:Tsl* construct, a 1.1 kb fragment of the *phm* promoter region [from Ono *et al.* (2006)] was first cloned from genomic DNA (F – 5'-CTG CAG TGA TGC GCT GCT CCT TTG T-3', R – 5'-AGA TCT CAC TTT CGA TTT CCT CCT GC-3') into the pGEM-T Easy vector (Promega, Madison, WI), before being sequenced and subcloned into pUASTattB-*Tsl:eGFP* (Johnson *et al.* 2017) via *Pst*I and *Bgl*II sites to delete the UAS sequence. Transgenic lines were made (BestGene) via ΦC31 integrase-mediated transformation (Bischof *et al.* 2007), using the ZH-51CE attP-landing site.

Developmental timing and body size analysis

At 24 hr after a 4 hr lay on apple juice agar supplemented with yeast paste, first-instar larvae were sorted by GFP (on a balancer chromosome) into 8–10 groups of 15 or 20 individuals (depending upon experiment) per genotype. Larvae were placed into vials containing fly media (see recipe above) and scored every 8 hr for the time taken to reach pupariation. Following their eclosion, adult flies were sorted by sex and weighed in groups on a microbalance (Mettler Toledo).

Nutritional plasticity

At 24 hr after a 4 hr lay on apple juice agar supplemented with yeast paste, first-instar larvae were sorted by GFP (on a balancer chromosome) and placed into vials containing either standard fly media or one of three low nutrient diets (either 50, 25, or 10% nutrients of standard media). These diets were made by diluting standard fly media with 0.5% non-nutritional agar to the appropriate concentration. Adult flies were collected within 24 hr of eclosion, sorted, and weighed in groups on a microbalance (Mettler Toledo). For each genotype, 10 replicates of 15 larvae were raised on each food type. Because size increases exponentially with increasing nutritional quality, male and female weight data were log10 transformed and analyzed by fitting the log10 transformed weights with linear models, using food concentration and genotype as fixed effects, in R-studio. Significant interactions between food concentration and genotype on body weight indicates that the two genotypes show significant differences in nutritional plasticity for body weight.

Quantification of food intake

Early feeding third-instar larvae were transferred to fresh dyed food (4.5% blue food dye) and allowed to feed for 1 hr. After feeding, larvae were removed from food using 20% sucrose solution, washed in distilled water and dried. Replicates of 10 larvae were homogenized in 80 μl of cold methanol and centrifuged for 10 min at 4°. A total of 60 μl of

supernatant from each sample was analyzed in a spectrophotometer at 600 nm. As standards, a twofold dilution series of food dye, using a starting concentration of 4 μ l dye/ml methanol was used. Five biological replicates were analyzed per genotype.

Hemolymph glucose and trehalose measurements

Hemolymph was pooled from 15 wandering third-instar larvae to obtain duplicate samples of 1 μ l for assay. Five biological replicates were performed per genotype. Glucose was measured by adding 99 μ l of Thermo Infinity Glucose Reagent (Thermo Scientific) to each sample and processing as per the manufacturer's instructions. Trehalose was measured using the same reagent after digestion to glucose using trehalase, with a 10-fold dilution due to higher levels of trehalose compared to glucose. For trehalose digestion, 1 μ l of hemolymph was incubated in 25 μ l of 0.25 M sodium carbonate at 95° for 2 hr, cooled to room temperature, and 8 μ l of 1 M acetic acid and 66 μ l of 0.25 M sodium acetate (pH 5.2) were added to make digestion buffer. A total of 1 μ l porcine trehalase (T8778; Sigma, St. Louis, MO) was added to 40 μ l of this mixture and incubated at 37° overnight. The resulting glucose was analyzed using 10 μ l of reaction and 90 μ l Thermo Infinity Glucose Reagent as above. Glucose and trehalose standards were treated together with samples to quantify sugar levels in hemolymph.

Whole body triglyceride measurements

Triglycerides were quantified in whole wandering third-instar larvae as per Musselman *et al.* (2011). Ten larvae were homogenized in PBS + 0.1% Tween and then diluted 1:100 with PBS. Samples were heated for 5 min at 65° to inactivate lipases and 2 μ l of each sample was mixed with 198 μ l Thermo Infinity Triglyceride Reagent (Thermo Scientific) and processed as per the manufacturer's instructions. The absorbance of samples at 500 nm was used as a relative measure of triglyceride content and was normalized to larval weight. Five biological replicates of 10 larvae were analyzed in duplicate for each genotype.

Immunoblotting

Hemolymph was extracted from ~80 wandering third-instar larvae on ice. Following centrifugation at $16,000 \times g$ for 5 min at 4°, supernatant was heat-inactivated at 60° for 10 min, re-spun and the remaining supernatant was combined with 1 mM DTT, 10 mM NaF, and complete EDTA-free protease inhibitor cocktail (Roche). For phosphorylated Akt (pAkt) blots, five third-instar larvae were homogenized in 80 μ l of lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2.5 mM EDTA, 0.2% Triton X, 5% glycerol, complete EDTA-free protease inhibitor cocktail (Roche)] and spun at $500 \times g$ for 5 min at 4°. Reducing buffer (containing 6 M urea for Tsl immunoblots) was added to all samples before boiling and separation by SDS-PAGE (any kDa TGX; Bio-Rad, Hercules, CA) followed by transfer onto an Immobilon-P membrane (Millipore, Bedford, MA). Membranes were probed with either 1:1000 anti-HA

(12CA5; Roche), 1:1000 anti-phosphorylated *Drosophila* Akt (4054S; Cell Signaling), or 1:1,000,000 anti- α -tubulin (B-5-1-2; Sigma), washed and incubated with HRP-conjugated secondary antibody (1:10,000; Southern Biotech). Immunoblots were developed using ECL Prime (GE Healthcare) and imaged using a chemiluminescence detector (Vilber Lourmat). pAkt blot images were quantified using ImageJ and differences between genotypes were determined by unpaired *t*-tests from five biological replicates.

Immunostaining and fluorescence quantification

Newly molted L3 larvae were collected and allowed to age on standard media for 24 hr before brains were dissected and fixed in PBS containing 4% paraformaldehyde for 40 min. Tissues were extensively washed in PBS containing 0.3% Triton X-100 and then blocked for 1 hr in PBT containing 2% normal goat serum (Sigma-Aldrich). Primary antibodies [rat anti-dILP2 and rabbit anti-dILP5; gifts from Dr. Pierre Léopold (Géminard *et al.* 2009)] were diluted to 1:800 in fresh block solution and incubated overnight at 4°. After extensive washing with PBS containing 0.3% Triton X-100, secondary antibodies (anti-rat Alexa Fluor 488 and anti-rabbit Alexa Fluor 568 conjugated; Molecular Probes, Eugene, OR) were diluted to 1:500 and incubated overnight at 4°. Brains were mounted in Fluoromount-G mounting medium (Southern Biotech) and Z series of the IPCs were obtained using a spinning disk confocal microscope (CV1000; Olympus), maintaining a 1 μ m step size and identical imaging settings across all genotypes. ImageJ software was used to generate maximum projection images of the Z stacks and to quantify total fluorescent intensity across the IPCs. This was achieved by drawing an area of interest around each group of IPCs and calculating the raw grayscale values in this region of interest. The total fluorescence was normalized to IPC area to account for the size discrepancy between genotypes.

dILP gene transcript quantification

For each biological replicate, 10–15 third-instar larvae (anterior end only) or 20–25 dissected third-instar larval brains were snap frozen before RNA was extracted using TRIreagent (Bioline) and treated with DNase (Promega). Complementary DNA was synthesized using Tetro reverse transcriptase (Bioline) by priming either 5 μ g (for anterior ends) or 1 μ g (for dissected brains) of RNA with oligo (dT) and random hexamers. Quantitative PCRs were performed in triplicate on a Light Cycler 480 (Roche), using SensiMix SYBR (Bioline) and primers specific for *dilp2* (forward: 5'-ACG AGG TGC TGA GTA TGG TGT GCG-3'; reverse: 5'-CAC TTC GCA GCG GTT CCG ATA TCG-3'), *dilp5* (forward: 5'-TGT TCG CCA AAC GAG GCA CCT TGG-3'; reverse: 5'-CAC GAT TTG CGG CAA CAG GAG TCG-3'), and *Rp49* (forward: 5'-GCC GCT TCA AGG GAC AGT ATC T-3'; reverse: 5'-AAA CGC GGT TCT GCATGA G-3'). Fold changes relative to *Rp49* were determined using the $\Delta\Delta$ CT method and means and SE calculated from three to five biological replicates per genotype.

Figure 1 *torso-like* null mutants phenocopy mutants with reduced insulin signaling. (A–C) *tsl^Δ* larvae have significantly elevated hemolymph glucose levels (A, $P = 0.0002$), unaltered hemolymph trehalose levels (B, $P = 0.7232$), and increased total triglyceride content (C, $P = 0.0040$) compared to heterozygous controls (*tsl^{Δ/+}*). $n = 5$ groups of 10 larvae for all means. (D and E) The variation of both male (D) and female (E) adult body weight over different food concentrations is significantly smaller for *tsl^Δ* animals compared to heterozygous controls (*tsl^{Δ/+}*). Regression lines that differ significantly in their slopes, indicating differences in nutritional plasticity for body size between genotypes, are marked with different letters. $n = 6–10$ groups of at least three individuals for each food type. (F) *tsl^Δ* larvae show reduced levels of pAkt. (G) Levels of pAkt were quantified from four biologically independent experiments, using Tubulin as a loading control. pAkt/Tubulin densities were standardized by fixing the values of *tsl^{Δ/+}* to 1. For all bar graphs, error bars represent ± 1 SEM and genotypes sharing the same letter indicate that they are statistically indistinguishable from one another ($P < 0.05$, two-tailed t -tests). The data used to generate each graph can be found in Supplemental Data File 1 in File S3.

Data and reagent availability statement

Data and reagents are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

In addition to its role in regulating growth and developmental timing, the insulin signaling pathway is also critical for regulating glucose and lipid metabolism in *Drosophila* [for review see Garofalo (2002)]. Thus, in addition to growth defects, mutants with reduced insulin signaling are unable to regulate their blood sugar levels (Rulifson *et al.* 2002). This results in increased levels of glucose in the hemolymph (but not of trehalose, a glucose disaccharide that is synthesized from intracellular glucose in the fat body and secreted into circulation), and increased triglyceride content (Böhni *et al.* 1999; Rulifson *et al.* 2002; Ugrankar

et al. 2015). To determine if *tsl* mutants also have such defects we performed metabolic analyses. This revealed that *tsl* null mutant (*tsl^Δ*) larvae have significantly elevated hemolymph glucose levels (Figure 1A, $P = 0.0002$), despite consuming less food than heterozygous controls over a 1 hr period (Supplemental Material, Figure S1 in File S1, $P = 0.0040$). By contrast, the concentration of circulating trehalose was unaltered in *tsl^Δ* larvae (Figure 1B, $P = 0.7232$). In addition to the observed increase in circulating glucose, *tsl^Δ* larvae had increased triglyceride content per milligram of body weight compared to heterozygous controls (Figure 1C, $P = 0.0040$). Taken together, the metabolic phenotype of *tsl^Δ* larvae is consistent with previous studies on *chico* and other insulin pathway mutants (Böhni *et al.* 1999; Rulifson *et al.* 2002; Ugrankar *et al.* 2015), and supports the idea that Tsl regulates the insulin signaling pathway.

Insulin signaling is also required for coupling nutrition and growth, such that body size is adjusted according to nutritional

Figure 2 Torso-like genetically interacts with the insulin signaling pathway. (A) Larvae deficient for both *tsl* and *dilps* 2, 3, and 5 (*dilp2-3 Δ , dilp5³, tslⁱ*) show a similar delay in time to pupariation (~325 hr) to loss of *dilps* 2, 3, and 5 alone ($P = 0.2192$). (B) The reduced time to pupariation caused by expression of *InR^{CA}* in the prothoracic gland (~42 hr, $P < 0.0001$) is not suppressed by removal of *tsl* ($P = 0.5015$). Error bars represent ± 1 SEM for all graphs. Genotypes sharing the same letter indicate that they are statistically indistinguishable from one another ($P < 0.05$, ANOVA and pairwise *t*-tests). $n \geq 10$ for all means and ≥ 37 individuals were tested per genotype. The data used to generate each graph can be found in Supplemental Data File 1 in File S3. hAEL, hr after egg lay.

availability (Tang *et al.* 2011). For example, in wild-type flies kept under low nutritional conditions insulin signaling is downregulated, resulting in a reduced larval growth rate and decreased adult body size. Accordingly, mutants with impaired insulin signaling are unable to adjust their body size in response to nutrition to the same extent as wild-type flies, as signaling is downregulated even in highly nutritious environments (Tang *et al.* 2011). To determine whether *tsl* mutants also share this feature with insulin signaling mutants, we examined their adult body size when grown as larvae on foods with varying nutrient content. Diluting the larval diet to 50, 25, and 10% of control food resulted in progressively smaller adults for both male and female heterozygous controls (Figure 1, D and E). At the lowest food concentration *tslⁱ* animals showed little difference in body size compared to heterozygous controls. However, as the food concentration increased, *tsl* mutants exhibited significantly reduced plasticity for body size (Figure 1, D and E; $P = 0.0104$ for males, $P = 0.0038$ for females; Table S1 in File S2) as would be expected if insulin signaling is impaired. The reduction in plasticity for *tslⁱ* animals was not found to be as severe as that observed in *chico* mutant animals, which appeared smaller across all food concentrations (Figure S2 in File S1, $P < 0.0001$ for both males and females; Table S2 in File S2).

Next, we used immunoblotting to measure the levels of pAkt, a biochemical readout of insulin signaling pathway activity. Consistent with a reduction in insulin signaling, we found that *tslⁱ* larvae had significantly lower pAkt levels compared to heterozygous controls (Figure 1, F and G, $P = 0.0440$). Taken together, these data show that many aspects of the *tsl* mutant

phenotype parallel those seen in insulin signaling mutants, supporting the idea that Tsl is required for insulin signaling in response to nutrition.

To provide further evidence that Tsl acts in the insulin signaling pathway, we conducted genetic interaction studies. We first asked if the delay caused by loss of *tsl* is epistatic or additive to that caused by loss of the dILPs. Consistent with previous studies (Grönke *et al.* 2010), removing dILPs 2, 3, and 5 resulted in a severe developmental delay, with a delay of ~341 hr compared to heterozygous controls (Figure 2A, $P < 0.0001$). Loss of *tsl* alone resulted in an 18 hr delay (Figure 2A, $P < 0.0001$). In larvae mutant for *tsl* and *dilps* 2, 3, and 5, the observed developmental delay was similar to the delay seen for *dilp2-3 Δ , dilp5³* mutants alone (Figure 2A, $P = 0.2192$). This result suggests that Tsl and dILPs 2, 3, and 5 act via the same signaling pathway to regulate developmental timing.

We next overexpressed a constitutively active and ligand-independent form of InR (*InR^{CA}*) in the PG (using *phm*-Gal4) and asked whether Tsl is required for its function. We chose to manipulate InR activity specifically in the PG because it is well established as the key tissue involved in the InR-mediated regulation of ecdysone production (Mirth *et al.* 2005), and because we know that *tsl* is expressed there (Grillo *et al.* 2012). As expected (Walkiewicz and Stern 2009), overexpression of *InR^{CA}* in the PG markedly reduced the time to pupariation (Figure 2B, $P < 0.0001$). This phenotype was not suppressed by loss of *tsl* (Figure 2B, $P = 0.5015$), suggesting that Tsl activity is not required for insulin signaling downstream of InR in the PG. Taken together, the results of these two genetic interaction experiments further support the idea

Figure 3 *torso-like* mutants show increased insulin-like peptide expression and accumulation in the insulin-producing cells. (A–D) Removal of *tsl* increases the accumulation of dILP2 (A and B) and dILP5 (C and D) in the IPCs. dILP protein levels were standardized by fixing the values of *w¹¹¹⁸* to 1. $n \geq 35$ for all means. (E and F) Expression of *dilp2* (E, $P = 0.0240$) and *dilp5* (F, $P = 0.0016$) is significantly increased in *ts^A* larvae compared to controls (*ts^A/+*). (G and H) *dilp2* expression in the larval brain is not significantly altered in *tsl* mutants (G, $P = 0.4243$); however, expression of *dilp5* is significantly elevated (H, $P = 0.0009$). Expression levels were normalized using an internal control, Rp49, and then standardized by fixing the values of *ts^A/+* larvae to 1. $n = 3–5$ for all means and ≥ 75 individuals were tested per genotype. For all graphs, error bars represent ± 1 SEM and genotypes sharing the same letter indicate that they are statistically indistinguishable from one another ($P < 0.05$, ANOVA and pairwise *t*-tests). The data used to generate each graph can be found in Supplemental Data File 1 in File S3.

that Tsl acts in the insulin signaling pathway and, if so, it does so either upstream or at the level of InR.

In the early embryo our work has led us to hypothesize that Tsl is required for the secretion of the Tor ligand, Trunk (Henstridge *et al.* 2014; Johnson *et al.* 2015). We therefore reasoned that it might regulate secretion of the dILPs, the ligands for InR. In mutants that affect dILP secretion an accumulation of dILP2 and dILP5 is observed in the IPCs

(Géminard *et al.* 2009; Rajan and Perrimon 2012; Sano *et al.* 2015; Koyama and Mirth 2016). We therefore immunostained the IPCs in *ts^A* larvae for both dILP2 and dILP5. This revealed that *ts^A* larvae had a significant increase in the accumulation of both dILP2 (Figure 3, A and B; $P = 0.0019$) and dILP5 (Figure 3, C and D; $P < 0.0001$) in the IPCs compared to controls, with dILP2 accumulating to a lesser extent than dILP5.

Figure 4 Torso-like is required in the prothoracic gland to regulate both developmental timing and body size. (A) Knockdown of *tsl* specifically in the prothoracic gland (using *phm*-Gal4) results in a significant developmental delay (~11 hr, $P = 0.0018$) that is similar to the delay observed for *tsl^Δ*. (B) No developmental delay is observed when *tsl* is knocked down specifically in the fat body (using *c7*-Gal4). (C and D) Expression of an *UAS-Tsl:RFP* (*UAS-Tsl*) transgene specifically in the prothoracic gland (using *phm*-Gal4) rescues both the developmental delay (C) and reduced adult body size (D) of *tsl^Δ* homozygotes ($P < 0.0001$ for both delay and body size compared to *tsl^Δ*). (E and F) The developmental delay (C) and reduced adult body size (D) or *tsl* mutants is partially rescued by the *phm:Tsl:3Myc:eGFP* (*phm:Tsl*) construct ($P < 0.0001$ for both delay and body size compared to *tsl^Δ*). Error bars represent ± 1 SEM for all graphs. Genotypes sharing the same letter indicate that they are statistically indistinguishable from one another ($P < 0.05$, ANOVA and pairwise *t*-tests). $n \geq 10$ for all means and ≥ 37 individuals were tested per genotype. The data used to generate each graph can be found in Supplemental Data File 1 in File S3. hAEL, hr after egg lay.

Although the observed accumulation of dILP2 and dILP5 could reflect a defect in their secretion, it was also possible that the observed accumulation reflects elevated expression of these peptides. Increased *dilp2/5* expression is commonly observed when there is a systemic reduction in insulin signaling caused by insulin resistance in peripheral tissues (Musselman *et al.* 2011; Pasco and Leopold 2012). To determine whether the observed accumulation of dILP2 and dILP5 results from their elevated expression we quantified

dilp2 and *dilp5* messenger RNA levels. This showed that the expression of both *dilp2* (Figure 3E, $P = 0.0240$) and *dilp5* (Figure 3F, $P = 0.0016$) was elevated in *tsl^Δ* larvae compared to heterozygous controls. As low levels of *dilp* expression have previously been detected in larval tissues other than the IPCs (Brogiolo *et al.* 2001), we also quantified *dilp2* and *dilp5* messenger RNA levels specifically in the larval brain. We found no significant difference in the expression of *dilp2* (Figure 3G, $P = 0.4243$) in *tsl^Δ* brains compared to

heterozygous controls; however, expression of *dilp5* was significantly elevated (Figure 3H, $P = 0.0009$). Taken together, these findings suggest that Tsl influences *dilp* expression during larval development.

We next asked if the role of Tsl in insulin signaling is due to a function in the PG. Previously, Grillo *et al.* (2012) showed that *tsl* is expressed in the PG and that RNA interference (RNAi) knockdown of *tsl* specifically in this tissue results in a significant developmental delay. Although we were unable to reproduce this result using the publicly available RNAi lines (Johnson *et al.* 2013), when we used the same RNAi line used in the Grillo *et al.* (2012) study [originally generated by Furriols *et al.* (2007)], we did observe a developmental delay phenotype (Figure 4A, $P = 0.0018$). By contrast, no phenotype was observed when we knocked down *tsl* expression specifically in the fat body using *c7-Gal4* (Figure 4B, $P = 0.9201$).

We also asked if PG-specific expression of *tsl* could rescue the growth defects of *tsl* mutants. When we expressed an *UAS-Tsl:RFP* transgene (*UAS-Tsl*) in the PG using *phm-Gal4*, we found this completely rescued both the developmental delay (Figure 4C, $P < 0.0001$) and small body size (Figure 4D, $P < 0.0001$) of *tsl* mutants. However, this transgene also partially rescued the *tsl^Δ* phenotypes in the absence of the *Gal4* driver, most likely due to leaky transgene expression. To overcome this problem, we generated a genomic rescue construct in which the *phm* promoter sequence (from *phm-Gal4*) was fused to the *tsl* coding sequence C-terminally tagged with three tandem Myc epitopes and the eGFP coding sequence (*phm:Tsl*). We found that this transgene partially rescued both the developmental delay (Figure 4E, $P < 0.0001$) and small body size (Figure 4F, $P < 0.0001$) of *tsl* null mutants. Together with the RNAi experiments, these data strongly suggest that *tsl* expression is required in the PG for regulating developmental timing and body size. However, we are unable to rule out the possibility that *tsl* is also required in other tissues for these roles.

How might Tsl function in the PG to regulate systemic insulin signaling? Because Tsl is a secreted protein, this could be explained if Tsl is secreted from the PG into the hemolymph. We therefore asked if Tsl is found in the larval hemolymph. To do this we used a genomic rescue construct that carries ~3 kb of promoter and the *tsl* coding sequence N-terminally tagged with three tandem HA epitopes (*gHA:Tsl*; Jimenez *et al.* 2002). This construct has previously been shown to completely rescue the developmental delay and reduced body size of *tsl^Δ* animals (Johnson *et al.* 2013). Using immunoblotting, we were able to clearly detect *gHA:Tsl* in the larval hemolymph (Figure 5A). We further asked if PG-produced Tsl enters the hemolymph by expressing a functional C-terminally tagged Tsl transgene (*UAS-Tsl:HA*) in the PG (*phm-Gal4*) and performing Western blots on protein extracted from larval hemolymph. This revealed that *Tsl:HA* protein was present in the hemolymph (Figure 5B). Although this is an overexpression situation, we reason that because Tsl is endogenously expressed in the PG, it is likely that at least a

Figure 5 Torso-like is secreted from the prothoracic gland into the larval hemolymph. (A) Immunoblot (anti-HA) of Tsl expression in the larval hemolymph. (B) Tsl is also detected in the hemolymph via immunoblotting (anti-HA) when expressed in the prothoracic gland, using *phm-Gal4*.

proportion of the total Tsl in circulation originates from the PG. However, it remains possible that the Tsl we detect in the hemolymph with the genomic construct is produced and secreted from another tissue.

Discussion

Our data presented here provide compelling evidence that Tsl is secreted into the hemolymph and regulates growth and developmental timing via the insulin signaling pathway. Although the *tsl* mutant phenotypes described here closely resemble those observed when insulin signaling is reduced in the entire organism, it should be noted that loss of *tsl* has a less severe effect on the pathway compared to mutations in other genes. For example, loss of function mutations in *InR* are homozygous lethal, and only a few heteroallelic combinations produce viable adults in which growth defects can be observed (Chen *et al.* 1996). By comparison, mutations in the adaptor protein Chico do not result in lethality, but rather cause severe growth and metabolic defects (Böhni *et al.* 1999). Here, we find that the defect in nutritional plasticity for body size is not as severe in *tsl* mutants as it is in *chico* mutants. Our findings therefore suggest that Tsl regulates, but is not essential for insulin signaling.

How might Tsl regulate insulin signaling throughout the body? One possibility is that Tsl regulates the insulin response in all tissues by acting in conjunction with InR (Figure 6A). Loss of insulin response in *tsl* mutants could then result in the increased *dilp* expression that we observe. Alternatively, Tsl may act to influence the activity of the dILPs, which in turn

Figure 6 Possible mechanisms of Torso-like function from the prothoracic gland. (A) Tsl is secreted from the PG into circulation where it acts in conjunction with InR in all tissues to modulate insulin signaling. (B and C) Tsl influences the activity of the dILPs in the IPCs, which in turn regulate systemic insulin signaling. In this model, Tsl could act on either (B) the fat body (FB), which is responsible for producing and releasing important regulators of dILP secretion (R, brown oval) such as CCHamide-2, Unpaired-2, Growth-blocking peptides 1 and 2 and Stunted, or (C) the IPCs to directly control dILP activity.

regulate systemic insulin signaling. There are two main tissues that are known to regulate dILP activity [for a complete review of the regulation of dILP production and secretion see Nässel and Vanden Broeck (2016)]. One is the fat body, which produces and releases important regulators of dILP secretion in response to intracellular nutrient levels (Figure 6B; Géminard *et al.* 2009; Rajan and Perrimon 2012; Sano *et al.* 2015; Delanoue *et al.* 2016; Koyama and Mirth 2016). Interestingly, a recent study found that knocking down *tor* specifically in the fat body led to a decreased body size, leading the authors to suggest that Tor acts in the fat body to influence insulin signaling via an unknown mechanism (Jun *et al.* 2016). Given the key role of Tsl in the regulation of Tor activity during early embryogenesis, it is therefore possible that Tsl acts in the Tor pathway in the fat body. However, fat body-specific knockdown of *tor* does not result in a developmental delay (Jun *et al.* 2016), thus this would seem unlikely to be the only role for Tsl in regulating growth and developmental transitions. In addition, experiments that we have performed to detect Tsl or knockdown its expression in the fat body have not provided any evidence of Tsl expression or function in this tissue. Determining if Tsl acts in the fat body to regulate dILP activity, either with Tor or with another pathway, requires further investigation.

An alternative possibility is that Tsl acts directly on the IPCs to control the activity of the dILPs (Figure 6C). Given the close proximity of the IPCs to the PG, this perhaps fits better with the known role of Tsl in the early embryo, where it is secreted from the follicle cells and acts locally on Tor signaling (Jimenez *et al.* 2002; Stevens *et al.* 2003). It is therefore possible that the systemic effects of Tsl are due to a role in regulating dILP expression and/or secretion in the IPCs. These ideas could be tested in future by experiments such as examining the kinetics of dILP secretion in *tsl* mutants following starvation, or testing if artificially stimulating dILP

release can rescue the *tsl* mutant phenotype. Understanding the exact role of Tsl in this system will provide fundamental insights into the mechanisms that regulate the evolutionarily conserved insulin signaling pathway, as well as the role of MACPF proteins in developmental signaling events.

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