Torso-like functions independently of Torso to regulate *Drosophila* growth and developmental timing

Travis K. Johnson[a,b], Tova Crossman[a], Karyn A. Foote[a,b], Michelle A. Henstridge[a], Melissa J. Saligari[a], Lauren Forbes Beadle[a], Anabel Herr[a,b], James C. Whisstock[b,c,1,2], and Coral G. Warr[a,1,2]

[a]School of Biological Sciences, [b]Department of Biochemistry and Molecular Biology, and [c]Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Monash University, Clayton, VIC 3800, Australia

Edited by Lynn M. Riddiford, Howard Hughes Medical Institute Janelia Farm Research Campus, Ashburn, VA, and approved July 26, 2013 (received for review May 26, 2013)

Activation of the *Drosophila* receptor tyrosine kinase Torso (Tor) only at the termini of the embryo is achieved by the localized expression of the maternal gene Torso-like (Tsl). Tor has a second function in the prothoracic gland as the receptor for prothoracicotropic hormone (PTTH) that initiates metamorphosis. Consistent with the function of Tor in this tissue, Tsl also localizes to the prothoracic gland and influences developmental timing. Despite these commonalities, in our studies of Tsl we unexpectedly found that *tsl* and *tor* have opposing effects on body size; *tsl* null mutants are smaller than normal, rather than larger as would be expected if the PTTH/Tor pathway was disrupted. We further found that whereas both genes regulate developmental timing, *tsl* does so independently of *tor*. Although *tsl* null mutants exhibit a similar length delay in time to pupariation to *tor* mutants, in *tsl:tor* double mutants this delay is strikingly enhanced. Thus, loss of *tsl* is additive rather than epistatic to loss of *tor.* We also find that phenotypes generated by ectopic PTTH expression are independent of *tsl*. Finally, we show that a modified form of *tsl* that can rescue developmental timing cannot rescue terminal patterning, indicating that Tsl can function via distinct mechanisms in different contexts. We conclude that Tsl is not just a specialized cue for Torso signaling but also acts independently of PTTH/Tor in the control of body size and the timing of developmental progression. These data highlight surprisingly diverse developmental functions for this sole *Drosophila* member of the perforin-like superfamily.

MACPF | growth rate | ecdysis | heterochrony

Terminal patterning in the *Drosophila* embryo involves secretion of the membrane attack complex/perforin-like (MACPF) protein Torso-like (Tsl) from specialized follicle cells at the anterior and posterior ends of the oocyte into the perivitelline space (1–3). Following its secretion, Tsl remains at the embryo poles through association with the vitelline membrane (4–6). Through a poorly understood mechanism that involves the eggshell proteins fs(1) Nasrat, *fs(1)Polehole*, and Closca, Tsl likely permits localized activation of the cysteine knot-like growth factor Trunk (Trk), possibly by proteolytic cleavage (7, 8). Activated Trk then binds to Tor and activates signaling at the embryo poles (9).

Tsl is the only MACPF-like protein that can be identified in the *Drosophila* genome (10). The majority of MACPF proteins characterized to date play roles in pore formation in mammalian immunity (including perforin itself and Complement C9) or in bacterial pathogenesis (11, 12). Currently, it is unclear how Tsl functions at the embryo poles, and a simple pore forming function is difficult to reconcile with a central role in activation of the Tor-signaling pathway.

Tor has a second major developmental role, in the prothoracic gland (PG), where it functions as the receptor for prothoracicotropic hormone (PTTH), a brain-derived neuropeptide hormone required for initiation of metamorphosis (13). Recently, it was shown that *tsl* is also expressed in the PG and that RNAi knockdown of *tsl* results in a significant (48 h) developmental delay (14). Given that PTTH and Trk belong to the same superfamily of cysteine knot-like growth factors (13), it seemed likely that Tsl plays a role in Tor activation in the PG. Here we confirm that loss of *tsl* leads to a delay in development. Remarkably, however, we discover that Tsl regulates body size and developmental timing independently of Tor. Our results show that Tsl has diverse functions, some of which are independent of Torso, and is thus not just a specialized cue for Torso signaling.

Results

Torso-Like Null Mutants Have a Developmental Delay. Previous studies have shown that RNAi knockdown of *torso* (*tor*) or *tsl* specifically in the PG results in an extended developmental period and delayed metamorphosis (13, 14). To begin dissecting the role of Tsl in the PG, and to understand its functional relationship with Tor, we first tested whether loss of *tsl* in our hands caused a developmental delay phenotype. As available stocks carrying *tsl* mutant alleles are likely hypomorphic and/or have additional genetic mutations, we used ends-out gene replacement to generate a *tsl* null mutant allele (tsl^{Δ}) in which the entire coding region is removed (Fig. S1 *A* and *B*). tsl^{Δ} homozygotes are viable and females are sterile, laying embryos that fail to specify terminal cell fate (Fig. S1*C*).

Developmental timing analyses of tsl^{Δ} homozygotes revealed a significant delay of 26 h to reach pupariation compared with heterozygous controls (Fig. 1*A*, $P < 0.001$). This length of delay is less than but consistent with that observed by Grillo et al. (14) using RNAi. To ensure that the tsl^{Δ} delay was solely due to loss of *tsl* activity, we showed that it could be rescued by ubiquitous

Significance

Torso-like (Tsl) is the sole *Drosophila* member of the membrane attack complex/perforin-like protein superfamily, generally known for pore-forming function and immune defence roles. Tsl, however, has a well-characterized developmental role in controlling activation of the receptor tyrosine kinase Torso (Tor) to achieve patterning of the termini of the early embryo. Here we report that the second known role of Tor, as the receptor for the hormone that induces metamorphosis, does not require Tsl. Instead, we find that Tsl controls developmental timing and growth independently of Tor. We conclude that Tsl plays a broader than expected role during development and is not merely a specialized cue for Tor signaling.

Author contributions: T.K.J., A.H., J.C.W., and C.G.W. designed research; T.K.J., T.C., K.A.F., M.A.H., M.J.S., and L.F.B. performed research; T.K.J., J.C.W., and C.G.W. analyzed data; and T.K.J., J.C.W., and C.G.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]J.C.W. and C.G.W. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: coral.warr@monash.edu or james.whisstock@monash.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1309780110/-/DCSupplemental.

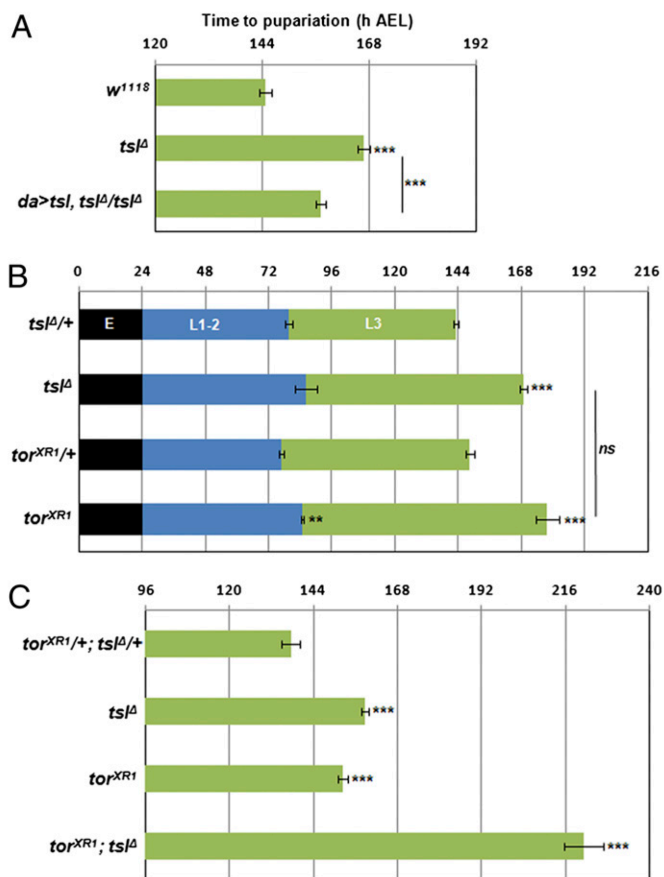

Fig. 1. *tsl* functions in the PG independently of *tor* to regulate developmental timing. (*A*) tsl^{Δ} homozygotes pupariate ~1 d later than controls. Weak ubiquitous expression (*da-Gal4*) of a UAS-*tsl* transgene results in a significant reduction (~10 h, $P < 0.001$) in developmental delay compared with tsl^{Δ}. (*B*) tor^{XR1} and tsl^{Δ} larvae exhibit a similar delay to pupariation compared with controls and a similar delayed transition from the L2 to the L3 stage. (*C*) Larvae deficient for both *tor* and *tsl* display a greatly extended delay in time to pupariation (83 h) compared with controls ($P < 0.001$). h AEL, hours after egg lay. Error bars represent ±1 SEM for all graphs. ***$P < 0.001$, **$P < 0.01$ from two-tailed *t* tests in all cases. $n = 6$ or greater for all means with no fewer than 52 individuals tested for each genotype.

expression of a UAS-*tsl* transgene in the tsl^{Δ} background (Fig. 1*A*, $P < 0.001$). In further support, we found that transheterozygotes carrying the tsl^{Δ} allele and a strongly hypomorphic allele of *tsl*, tsl^{5}, also exhibited a developmental delay (Fig. S2).

The *tor* null allele tor^{XR1} was previously shown to have a delay to pupariation of ~30 h (13); however, as developmental timing depends on many extrinsic conditions (e.g., temperature, media type), it was important to compare it to the *tsl* mutant under our laboratory conditions. When we compared homozygotes for tor^{XR1} and for tsl^{Δ}, we found that they exhibited a similarly lengthened developmental period ($P = 0.065$, Fig. 1*B*). Furthermore, both tsl^{Δ} and tor^{XR1} homozygotes displayed a similar ($P = 0.760$) L2–L3 transition delay of 7 h ($P = 0.006$ for tor^{XR1}; not significant for tsl^{Δ}).

Torso-Like Acts Independently of Torso in Developmental Timing and Body Size. We next asked whether *tsl* exerts its effects on developmental timing by acting through the *tor* pathway or whether *tsl* has a role separate from that of *tor* in developmental timing. To address this, we tested tor^{XR1}; tsl^{Δ} double mutants to assess whether the delay caused by loss of *tsl* was epistatic or additive to loss of *tor*. We found that the double mutants displayed a drastically extended larval period, pupating 83 h after the controls ($P < 0.001$, Fig. 1*C*), and significantly later than either tor^{XR1} or tsl^{Δ} alone (both $P < 0.001$). Thus, *tsl* and *tor* show additive effects on developmental timing, indicating different roles.

In further support of *tsl* having a developmental role that is independent of *tor*, we also observed a distinct phenotypic difference between *tsl* and *tor* mutant adults. Loss of *tor* or ablation of PTTH-producing neurons results in larger-than-normal adults due to prolonged duration of feeding as larvae (13, 15). Intriguingly, despite the developmental delay observed for *tsl* null mutants, adults are not larger as would be expected if the PTTH/Tor pathway had been perturbed. Instead, tsl^{Δ} homozygotes are 28% smaller than controls ($P < 0.001$, Fig. 2 *A* and *C*, *Inset*). tor^{XR1}; tsl^{Δ} double-mutant flies are also smaller than normal ($P < 0.001$) and similar in size to tsl^{Δ} alone ($P = 0.076$), indicating that for this phenotype *tsl* is epistatic to *tor*. Furthermore, tsl^{Δ} larvae are notably smaller than heterozygous controls when the controls have reached the wandering stage (Fig. 2*B*). Indeed, tsl^{Δ} larvae measured throughout their entire third instar stage revealed a severe growth rate defect (ANCOVA $F_{1,11} = 8.55$, $P = 0.014$, Fig. S3), likely initiated in the preceding larval stages.

To determine whether the reduced size is due to loss of cells or to smaller cells, we counted wing-hair density in adults. These results showed that the decreased body size of tsl^{Δ} is due to a reduction in cell size (females, $P < 0.001$; males, $P = 0.011$; Fig. 2*C*). This contrasts with results obtained from manipulation of PTTH, which affects cell number rather than size (15). Taken together, these data indicate that the *tor* and *tsl* mutant delays are likely to have separate mechanistic bases and suggest an earlier and *tor*-independent role for *tsl* in growth regulation.

To try and determine if the PG is the site of action of *tsl* in control of our observed developmental timing and body size defects, we expressed a *tsl* transgene specifically in the PG using *phm-Gal4*. This was unable to rescue the developmental delay or small size of tsl^{Δ} mutants (Fig. S4 *A* and *B*). We also did not observe a significant delay or small size phenotype when we knocked down *tsl* in the PG using RNAi (Fig. S4 *C* and *D*). However, driving RNAi constructs with the strong ubiquitous driver *Actin-Gal4* also did not produce the delay or body-size difference (Fig. S4 *E* and *F*); thus in our hands the RNAi constructs are unable to achieve sufficient knockdown to produce *tsl* loss-of-function phenotypes. From these results we cannot conclude if the observed phenotypes are due to *tsl* expression in the PG or a different tissue.

Torso-Like Is Not Required for Ectopic PTTH Function. In terminal patterning, Tsl is thought to mediate conversion of Trk into a form capable of activating Tor signaling (7, 8, 16). Similarities between the Trk and PTTH sequences, including in the relative positions of putative cleavage sites, have led to the suggestion that PTTH might also require Tsl function for its activity (13, 14). As our results for *tor;tsl* double mutants indicated otherwise, we explored this further. We reasoned that, if Tsl is acting independently of PTTH/Tor signaling in controlling developmental timing and body size, then any effects of PTTH overexpression might not be suppressed by removal of Tsl. To this end, we drove expression of a UAS-*ptth* transgene directly in the PG using *phm-Gal4*. This resulted in a marked advancement in the time of pupariation by 19 h compared with the control, consistent with previous observations (15) ($P < 0.001$, Fig. 3*A*). Removal of *tsl* did not restore normal developmental timing ($P < 0.001$). Furthermore, the reduction in adult size caused by ectopic PTTH expression was enhanced, rather than suppressed, by removal of *tsl* (females, $P = 0.003$; males, $P < 0.001$; Fig. 3*B*).

In addition, because PTTH has been shown to be able to act in place of Trk if it is artificially expressed in the early embryo (13), we asked whether maternal Tsl is required for this action of PTTH. We drove expression of PTTH in the germ line

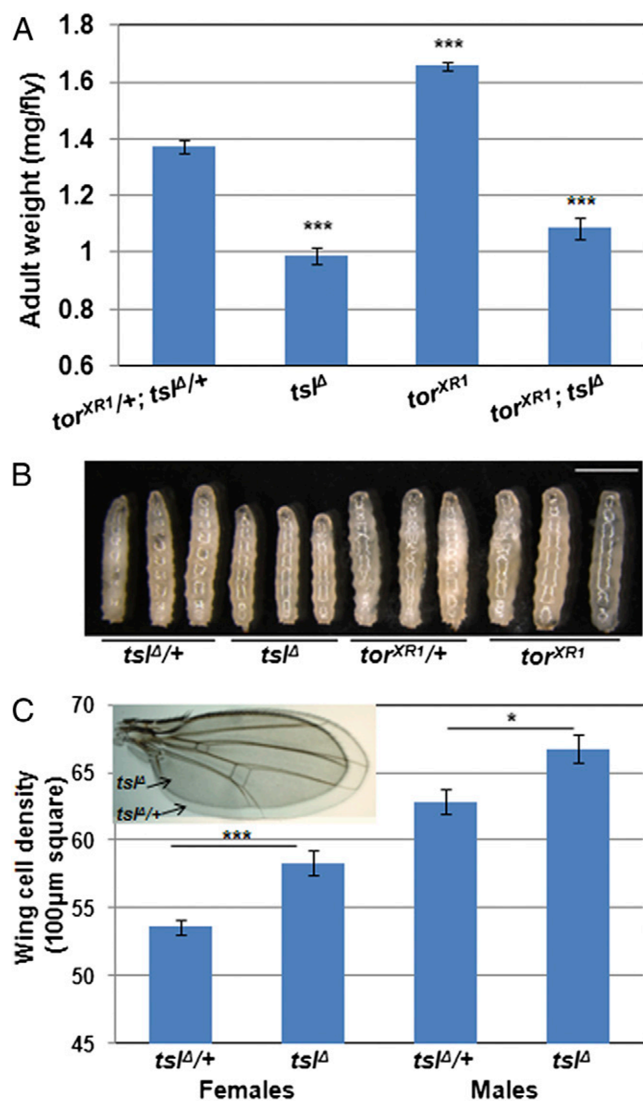

Fig. 2. *tsl* and *tor* have opposing effects on body size. (*A*) tsl^{Δ} adult flies weigh 28% less than controls ($P < 0.001$) and 40% less than tor^{XR1} individuals. Flies mutant for both genes weigh the same as flies lacking *tsl* alone (21% reduction compared with control, $P = 0.08$ compared with tsl^{Δ}). Note that only females were tested here due to insufficient recovery of adult males mutant for both genes; however, effects of *tor* and *tsl* on male weight were highly consistent with the female data. $n = 5$ or greater for each genotype with more than 17 flies weighed per genotype. (*B*) Third-instar larvae at 127 h AEL. At this stage, control larvae have begun wandering but *tsl* and *tor* mutants remain feeding. tsl^{Δ} larvae are notably reduced in size at this point, suggesting that *tsl* is acting earlier than *tor* to regulate growth (Scale bar, 2 mm.) (*C*) Wing-cell density is significantly higher in tsl^{Δ} flies compared with controls, indicating that cell size is reduced in tsl^{Δ} wings [tsl^{Δ} wings are smaller than the control (*Inset*)]. $n = 10$ for all wing-cell density measurements for each genotype. Error bars represent ±1 SEM for all graphs. ***$P < 0.001$, *$P < 0.05$ from two-tailed *t* tests in all cases.

(*Gal4:VP16-nos.UTR*) and observed ubiquitous over-activation of the Tor pathway (Fig. 4*C*) as indicated by near ubiquitous *tailless* (*tll*) transcription (normally restricted to precise domains at the poles of the embryo). This phenotype suggests that, unlike Trk, PTTH is able to activate Tor in the embryo without a requirement for Tsl, which here is expressed only at the embryo poles. We note that in a previous study overexpression of PTTH in the germ line did not result in Tor over-activation (13); however, this study used a construct in the pUAST vector, and we alternatively used the pUASp vector, which expresses far more strongly in the germ line (17).

Tagged Form of Tsl Rescues the Developmental Delay and Small Size but Not Terminal Patterning. Finally, in further support of Tsl having different modes of action in patterning and developmental timing, we observed that a Tsl genomic rescue construct that carries ∼3 kb of promoter and the *tsl* coding sequence tagged with three tandem hemagglutinin (HA) epitopes at the N terminus (*HA:tsl*) (4) can completely rescue the developmental delay and reduced size of the tsl^{Δ} mutation (Fig. 4 *A* and *B*), but intriguingly does not rescue the terminal patterning defect (Fig. 4*C*). The lack of rescue of the terminal patterning defect is not due to incorrect localization of the tagged Tsl protein, as we and others have confirmed that the HA:Tsl protein is indeed secreted into the perivitelline space (4). We therefore reason that the HA tag is disrupting the mechanism of action of Tsl in the early embryo but not in the developing larva, which again is indicative of different protein activities in different tissues.

Discussion

Our observations define a role for *torso-like* in the regulation of developmental timing and body size that is independent of the Tor pathway. The recent discoveries that (*i*) *tor* encodes the receptor for PTTH (13) and that (*ii*) *tsl* is also expressed in the PG (14) prompted us to investigate whether Tsl plays a role in the activation of PTTH similar to the one it plays for Trk in the embryo. Although *tsl* is similar to *tor* in terms of expression in the PG and in one aspect of its larval development mutant phenotype (13, 14, and herein), here we show that these similarities are coincidental rather than indicative of cooperative function.

Importantly, the data presented here show that the genes required for activation of Tor in the early embryo are not used in its activation in the PG. We and others (13, 15) have shown that ectopic expression of PTTH in cells that do not normally produce it, in the early embryo and the PG, results in active PTTH that is capable of activating Tor. These data suggest that these cells produce all proteins necessary for activation of PTTH. In contrast, ectopic Trk driven in the PG (where it is not normally produced) has no effect on developmental timing or adult size despite expression of Tsl in this tissue (14). Taken together, we conclude that the activation requirements of Trk and PTTH are quite different. Specifically, *tsl* expression is unnecessary for PTTH activity and insufficient for ectopic Trk activity.

Why would Tor be activated differently in the embryo and in the PG? We reason that the answer to this question possibly lies with differences in the two ligands. During early embryogenesis Trk is secreted in an inactive form that requires Tsl and other terminal class genes for activation (7–9). In this situation Tor activation requires spatial constraint that is achieved by restricted Tsl expression controlling spatially localized Trk activation. In contrast, because the PG is directly innervated by PTTH-producing neurons that synapse within the gland (15, 18), spatial control of Tor activation might not be necessary in this context. We hypothesize that PTTH may simply be secreted from these neurons in a form that does not require further local activation and thus does not require Tsl.

How might Tsl act in controlling body size and development timing? The *tsl* mutant phenotypes presented here resemble those observed when insulin signaling is reduced (19, 20). Specifically, overexpression of a dominant negative form of the insulin-like receptor (InR) causes reduced size and delayed development (21). Additionally, larvae carrying heat-sensitive alleles of *InR* also display developmental delays and effects of size (22). However, despite these phenotypic similarities, experiments in our laboratory have not yielded evidence for interactions between *tsl* and the InR pathway.

Another possibility is that *tsl* regulates developmental timing and growth earlier in development or in a tissue distinct from the PG. The growth rate defect that we observe in the *tsl* mutants may indicate that it acts earlier in development than *tor*. It must

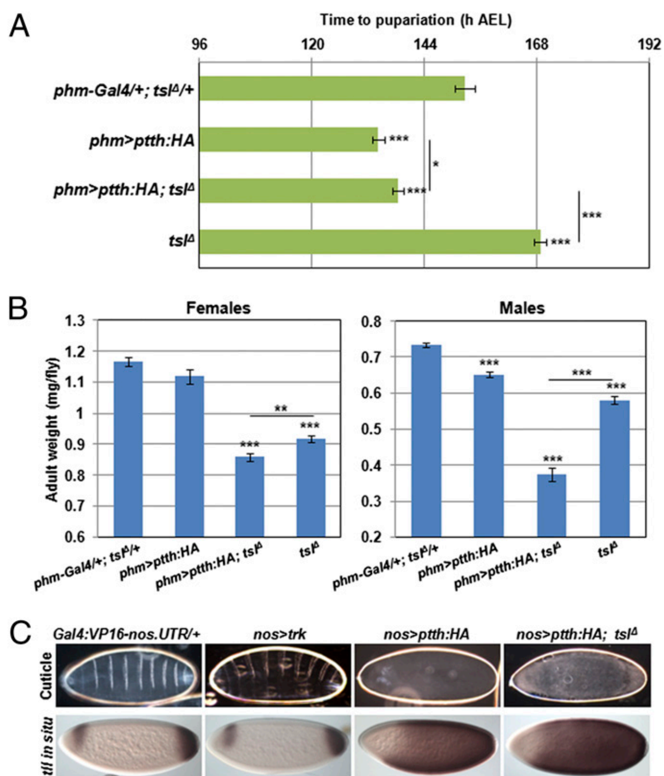

Fig. 3. Ectopic PTTH activity is independent of *tsl*. (*A*) Overexpression of PTTH in the prothoracic gland (*phm*-Gal4) results in a decreased time to pupariation (19 h less than the control, $P < 0.001$; 35 h less than tsl^{Δ}, $P < 0.001$). Removal of *tsl* under these conditions marginally slowed pupariation ($P = 0.034$) but not to close to normal or tsl^{Δ} developmental timing ($P = 0.034$). Means are calculated from $n = 10$ with a minimum of 108 individuals tested per genotype. (*B*) Overexpression of PTTH using *phm*-GAL4 gives a strong reduction in adult weight in males that is further reduced by loss of *tsl* (all means significantly different from each other; $P < 0.001$). Females followed a similar but nonsignificant trend due to overexpression of PTTH having little affect on adult weight. $n = 7$ or greater with at least 28 flies weighed per genotype. (*C*) Overexpression of Trk in the germ line (*nos*-Gal4) has no effect on terminal patterning because Trk requires Tsl activity, which is present only at the poles. PTTH overexpression, however, results in a grossly disrupted embryonic cuticle due to ubiquitous Tor signaling throughout the whole embryo, which is also shown by the greatly expanded *tailless* expression. This is unaffected by removal of *tsl*. Anterior is to the left and maternal genotypes are as indicated. Images are representatives of more than 60 embryos in each case. Error bars represent ±1 SEM for all graphs. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ from two-tailed *t* tests in all cases.

also be noted that our data do not implicate the PG as the source of timing and growth defects observed in *tsl* null mutants. Consistent with the latter possibility, we have been unable to rescue the *tsl* null phenotypes by specifically expressing *tsl* in the PG using *phm-Gal4*. In addition, we were unable to replicate the delay observed by Grillo et al. (14) using RNAi knockdown of *tsl* in the PG. As technical difficulties and experimental variations between laboratories can underlie such differences, however, the PG remains a candidate tissue given its crucial role in regulation of developmental transitions in response to nutritional inputs (23, 24). Further experiments to manipulate Tsl function in different tissues and at different times during development will be required to determine the specific tissues and pathways underlying these phenotypes. Taken together, however, our current results reveal the surprising finding that the function of Tsl in its maternal patterning role is mechanistically distinct from its zygotic role in the developing larva.

Materials and Methods

***Drosophila* Stocks.** The following stocks were used: w^{1118} (BL5905); *hs-FLP hs-I-SceI* (BL9634) ; *ey-FLP* (BL5580); *da-Gal4* (BL12429); *phm-Gal4* (gift from Leonie Quinn, University of Melbourne, Melbourne, originally from Michael O'Connor, University of Minnesota, Minneapolis); *Gal4::VP16-nos.UTR* (BL7293); tor^{XR1}, a protein null allele of *torso* (25); *HA:tsl* (gift from Jordi Casanova, IRB Barcelona, Barcelona); tsl^{5}, a strong EMS-induced loss-of-function mutation (Y148N); *UAS-*tsl^{RNAi} (v14430); and *ZH-96E attP* (BL24487) for ΦC31 integrase-mediated germ-line transformation.

Transgenics and Generation of *tsl* Null Mutants. A genomic fragment containing the coding sequence of *ptth* including introns and a C-terminal single HA tag was synthesized and cloned (Genscript) into pUASP to make UASP-*ptth*-HA. UASP-*trk* was similarly constructed using the full-length *trk* cDNA. Purified plasmid DNA was injected into w^{1118} embryos (BestGene Inc.) using standard *P*-element transformation methods (26) to yield transgenic flies. For UAS-*tsl*, the coding sequence of *tsl* was first cloned from cDNA (F-AGA TCT ATG CTG AGC GAT GCG GTC GTG CCT, R-CTC GAG CTA TCG GGT GGG ATG ACT CTG CGG) into the pGEM-T Easy vector (Promega), then sequenced, and then subcloned into pUASTattB. Transgenics were made via ΦC31 integrase-mediated transformation (27, 28) (UAS-*tsl*) using the ZH-96E attP landing site. For all transgenes, several independent transgenic lines were established and tested.

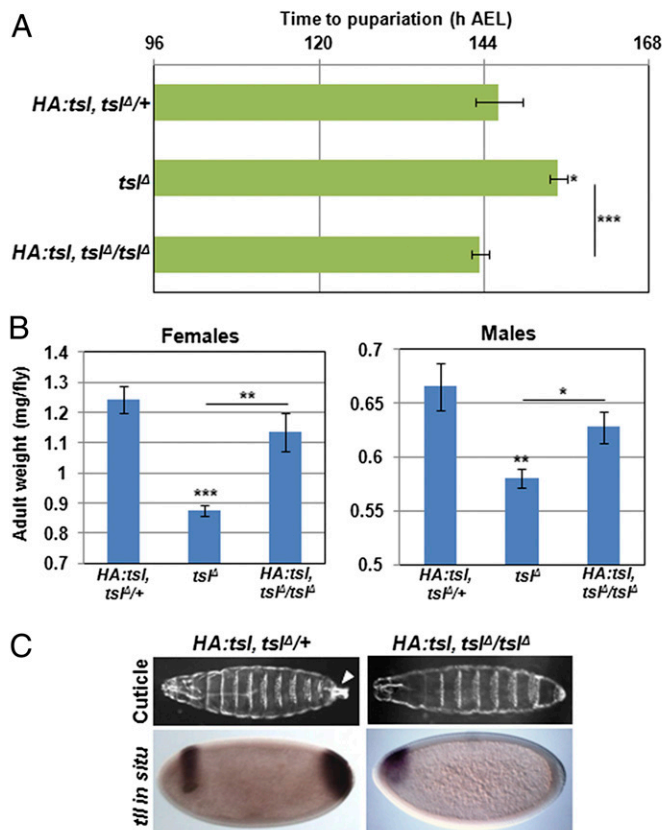

Fig. 4. A tagged form of Tsl functions in developmental timing but not in terminal patterning. The developmental delay (*A*) and reduced size (*B*) of tsl^{Δ} homozygotes was rescued by the *HA:tsl* construct ($P < 0.001$ for delay, $P < 0.01$ for female size, and $P < 0.05$ for males all compared with tsl^{Δ}). *HA:tsl* does not rescue the *tsl* maternal null terminal phenotype (*C*). Note that structures posterior to abdominal segment 8, including the filzkorper (arrowhead), are absent in larval cuticles, and posterior *tll* expression is absent in early embryos from *HA:tsl*, tsl^{Δ} mothers. Pupariation times are means representative of $n = 8$ or greater with at least 65 individuals scored for each genotype. Means for adult weight are calculated from $n = 6$ or greater with no fewer the 24 flies weighed for each genotype. Error bars represent ±1 SEM for all graphs. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ from two-tailed *t* tests in all cases.

The *tsl* null mutant was generated using ends-out gene replacement (29). First, the donor plasmid pW25-tsl was built by cloning 4.2 kbp of upstream (F-AGC AGG CGC GCC TGC AAT CAG CTG TCT TGA CC, R-TAC CCG TAC GCC TTT TAA CCC ACA GCC AAA) and 2.0 kbp of downstream (F-AGC AGC GGC CGC CAT CAA GAA GGT GGC CCT TA, R-TAC CGG TAC CTC CGA CTT GCA TTT CAG CTA) sequence flanking the *tsl*-coding region into the appropriate sites in the ends-out transformation vector pW25. Purified pW25-*tsl* was injected into w^{1118} embryos (BestGene Inc.). The donor targeting element was mobilized by crossing to hs-FLP and hs-I-SceI and applying a heat shock for 80 min at 37 °C daily for days 3–5 of development. Mosaic- and white-eyed virgins were crossed to *ey*-FLP, and stable lines were established from w+ progeny (~1/6 vials yielded a w+ individual). Lines were characterized for targeting events via PCR (Fig. S2, A-GCA ATC TGT GAG GTT CAT CTG TAA GGT CAA GAC AG, B-CGG GTA GAG CCG TTT ACC CGT TTC GAT TAC CGG TTC, C-GCA ACT GAA GGC GGA CAT TGA CGC TAT CGA CCT ATT CAG, D-CAG CTG GTA CTC CGG CAA TTG TTG TGC CC). Of the 22 w+ lines that we generated, 4 underwent gene replacement. *Tsl* transcriptional activity was assessed in the mutants by RT-PCR from ovary-derived cDNA using primers specific for *tsl* (detail above) and a control gene *CyclinK* (F-GAG CAT CCT TAC ACC TTT CTC CT, R-TAA TCT CCG GCT CCC ACT G).

RNA in Situ Hybridizations. A digoxigenin-labeled (Roche) probe against *tll* was hybridized with 0–4 h old embryos using standard protocols (30) before imaging with differential interference contrast optics.

Cuticle Preparations. Adults were allowed to lie on media containing apple juice supplemented with yeast paste for 24 h before being removed. Embryos developed for a further 24 h before dechorionation in 50% (vol/vol) bleach and mounting on slides in a mixture of 1:1 (vol/vol) Hoyer's solution:lactic acid. Slides were incubated overnight at 65 °C and imaged using dark-field optics (Leica).

Phenotypic Measurements. All stocks were maintained at 25 °C on standard media for all experiments. First-instar larvae were sorted by GFP (on balancer chromosome) 24 h following a 3-h lay into 6–10 groups of 10 or 20 individuals (depending upon experiment) per genotype. Larvae were placed into vials containing standard fly media and scored every 8 h for the time taken to reach pupariation. L2–L3 transition was scored by inspecting larval spiracle and mouthhook morphology every 3 h on three food plates, each containing 20 per genotype. Adults were sorted several days following eclosion and weighed in groups on a microbalance (Mettler Toledo). Wing-cell density was calculated from wing-hair counts within a rectangle (147×198 μm) averaged across two zones per wing for 10 wings per genotype. Growth rates were determined by weighing four groups of five larvae for each time point until pupariation. Linear regression was used to calculate growth rates and ANCOVA to assess whether rates were different (Prism 6.0b).

ACKNOWLEDGMENTS. We thank James Heaney and the Australian *Drosophila* Biomedical Research Facility (OzDros) for technical support; Jordi Casanova for providing the N-terminally tagged Tsl construct; Marc Freeman, Peter Dearden, and Richard Burke for discussions; and two anonymous reviewers for their valuable feedback. This work was supported by the National Health and Medical Research Council of Australia (NHMRC), the Australian Research Council (ARC), and an Interdisciplinary Research Grant from Monash University. J.C.W. is an ARC Federation Fellow and an honorary NHMRC Principal Research Fellow.

1. Stevens LM, Frohnhöfer H-G, Klingler M, Nüsslein-Volhard C (1990) Localized requirement for torso-like expression in follicle cells for development of terminal anlagen of the Drosophila embryo. *Nature* 346(6285):660–663.
2. Savant-Bhonsale S, Montell DJ (1993) *torso-like* encodes the localized determinant of *Drosophila* terminal pattern formation. *Genes Dev* 7(12B):2548–2555.
3. Martin J-R, Raibaud A, Ollo R (1994) Terminal pattern elements in *Drosophila* embryo induced by the *torso-like* protein. *Nature* 367(6465):741–745.
4. Jiménez G, González-Reyes A, Casanova J (2002) Cell surface proteins Nasrat and Polehole stabilize the Torso-like extracellular determinant in *Drosophila* oogenesis. *Genes Dev* 16(8):913–918.
5. Stevens LM, Beuchle D, Jurcsak J, Tong X, Stein D (2003) The *Drosophila* embryonic patterning determinant torsolike is a component of the eggshell. *Curr Biol* 13(12): 1058–1063.
6. Ventura G, Furriols M, Martín N, Barbosa V, Casanova J (2010) *closca*, a new gene required for both Torso RTK activation and vitelline membrane integrity. Germline proteins contribute to *Drosophila* eggshell composition. *Dev Biol* 344(1):224–232.
7. Casanova J, Furriols M, McCormick C-A, Struhl G (1995) Similarities between trunk and spätzle, putative extracellular ligands specifying body pattern in *Drosophila*. *Genes Dev* 9(20):2539–2544.
8. Casali A, Casanova J (2001) The spatial control of Torso RTK activation: A C-terminal fragment of the Trunk protein acts as a signal for Torso receptor in the *Drosophila* embryo. *Development* 128(9):1709–1715.
9. Sprenger F, Nüsslein-Volhard C (1992) Torso receptor activity is regulated by a diffusible ligand produced at the extracellular terminal regions of the *Drosophila* egg. *Cell* 71(6):987–1001.
10. Rosado CJ, et al. (2007) A common fold mediates vertebrate defense and bacterial attack. *Science* 317(5844):1548–1551.
11. Hadders MA, Beringer DX, Gros P (2007) Structure of C8α-MACPF reveals mechanism of membrane attack in complement immune defense. *Science* 317(5844):1552–1554.
12. Law RHP, et al. (2010) The structural basis for membrane binding and pore formation by lymphocyte perforin. *Nature* 468(7322):447–451.
13. Rewitz KF, Yamanaka N, Gilbert LI, O'Connor MB (2009) The insect neuropeptide PTTH activates receptor tyrosine kinase torso to initiate metamorphosis. *Science* 326(5958):1403–1405.
14. Grillo M, Furriols M, de Miguel C, Franch-Marro X, Casanova J (2012) Conserved and divergent elements in Torso RTK activation in *Drosophila* development. *Sci Rep* 2:762.
15. McBrayer Z, et al. (2007) Prothoracicotropic hormone regulates developmental timing and body size in *Drosophila*. *Dev Cell* 13(6):857–871.
16. Furriols M, Casali A, Casanova J (1998) Dissecting the mechanism of torso receptor activation. *Mech Dev* 70(1–2):111–118.
17. Rørth P (1998) Gal4 in the *Drosophila* female germline. *Mech Dev* 78(1–2):113–118.
18. Ghosh A, McBrayer Z, O'Connor MB (2010) The *Drosophila* gap gene *giant* regulates ecdysone production through specification of the PTTH-producing neurons. *Dev Biol* 347(2):271–278.
19. Nijhout HF (2003) The control of body size in insects. *Dev Biol* 261(1):1–9.
20. Edgar BA (2006) How flies get their size: Genetics meets physiology. *Nat Rev Genet* 7(12):907–916.
21. Slack C, Giannakou ME, Foley A, Goss M, Partridge L (2011) dFOXO-independent effects of reduced insulin-like signaling in Drosophila. *Aging Cell* 10(5):735–748.
22. Shingleton AW, Das J, Vinicius L, Stern DL (2005) The temporal requirements for insulin signaling during development in Drosophila. *PLoS Biol* 3(9):e289.
23. Layalle S, Arquier N, Léopold P (2008) The TOR pathway couples nutrition and developmental timing in *Drosophila*. *Dev Cell* 15(4):568–577.
24. Mirth CK, Truman JW, Riddiford LM (2005) The role of the prothoracic gland in determining critical weight for metamorphosis in *Drosophila melanogaster*. *Curr Biol* 15(20):1796–1807.
25. Sprenger F, Stevens LM, Nüsslein-Volhard C (1989) The *Drosophila* gene *torso* encodes a putative receptor tyrosine kinase. *Nature* 338(6215):478–483.
26. Rubin GM, Spradling AC (1983) Vectors for P element-mediated gene transfer in *Drosophila*. *Nucleic Acids Res* 11(18):6341–6351.
27. Groth AC, Fish M, Nusse R, Calos MP (2004) Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. *Genetics* 166(4):1775–1782.
28. Bischof J, Maeda RK, Hediger M, Karch F, Basler K (2007) An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases. *Proc Natl Acad Sci USA* 104(9):3312–3317.
29. Gong WJ, Golic KG (2003) Ends-out, or replacement, gene targeting in *Drosophila*. *Proc Natl Acad Sci USA* 100(5):2556–2561.
30. Tomancak P, et al. (2002) Systematic determination of patterns of gene expression during *Drosophila* embryogenesis. *Genome Biol* 3(12): research0088.