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Conserved and divergent elements in Torso RTK activation in *Drosophila* development

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The repeated use of signalling pathways is a common phenomenon but little is known about how they become co-opted in different contexts. Here we examined this issue by analysing the activation of *Drosophila* Torso receptor in embryogenesis and in pupariation. While its putative ligand differs in each case, we show that Torso-like, but not other proteins required for Torso activation in embryogenesis, is also required for Torso activation in pupariation. In addition, we demonstrate that distinct enhancers control *torso-like* expression in both scenarios. We conclude that repeated Torso activation is linked to a duplication and differential expression of a ligand-encoding gene, the acquisition of distinct enhancers in the *torso-like* promoter and the recruitment of proteins independently required for embryogenesis. A combination of these mechanisms is likely to allow the repeated activation of a single receptor in different contexts.

The Torso (Tor) pathway is responsible for the specification of the most anterior and posterior regions of the *Drosophila* embryo. The Tor receptor itself is present all around the membrane of the early embryo but is activated only at its poles by a mechanism thought to involve the cleavage of its putative ligand, the Trunk (Trk) protein. Trk, which is expressed in the germ line, appears to be synthesised by the early embryo and secreted into the perivitelline space between the embryo membrane and the vitelline membrane, the latter a component of the eggshell that covers the developing embryo. There, in the perivitelline space, Trk is thought to be specifically cleaved at the poles by an unknown mechanism that is dependent on the *torso-like* (*tsl*) gene, which encodes a product anchored at the two poles of the vitelline membrane. Tsl is the only known component of the Tor pathway that is locally restricted and its expression domain is precisely what is responsible for the spatially restricted activation of the Tor receptor. However, Tsl is not synthesised in the early embryo, where the Tor protein is produced and accumulates at the membrane. Conversely, Tsl is synthesised by a subset of the somatic cells surrounding the oocyte that are responsible for eggshell production. This process is elaborate and a group of genes that are required for eggshell assembly are also responsible for anchoring Tsl on the vitelline membrane surrounding the embryo (e.g. *fs(1)Nasrat* (*fs(1)N*), *fs(1)polehole* (*fs(1)ph*) and *closca* (*clos*)) (for revisions on eggshell morphogenesis and on Tor signalling in embryonic pattern see[1] and[2], respectively).

Recently the Tor signalling also has been found to be responsible for the production of the ecdysone peak that triggers the larva to pupa transition; as a result, pupariation is delayed in mutants for the Tor pathway[3]. For this function, the Tor receptor accumulates in the prothoracic gland[3] and is activated by a distinct putative ligand, the Prothoracicotropic hormone (Ptth), which is delivered to the prothoracic gland by two pairs of neurons that innervate the gland[4]. Thus, the scenario is that of activation of a single receptor at two sites by two different ligands. These two putative ligands are similar at the sequence level -Trk and Ptth form a separate cluster among the cysteine-knot proteins and Ptth is the closest paralog of Trk- and ectopic *ptth* expression in the germ line partially rescues the lack of *trk* activity[3] and Fig. 1A,B. Here we further analyse Tor activation in the prothoracic gland and compare it to Tor activation in the embryo in order to identify common and specific elements. We discuss the implications of our results for the dual activation of the signalling pathway.

Results

First, to assess whether Trk can also trigger Tor activation in the prothoracic gland if appropriately expressed, we took advantage of the GAL4/UAS system[5] to induce general *trk* expression (see methods). For this purpose, we used the same driver as previously used to assess whether general expression of *ptth* advances the time of

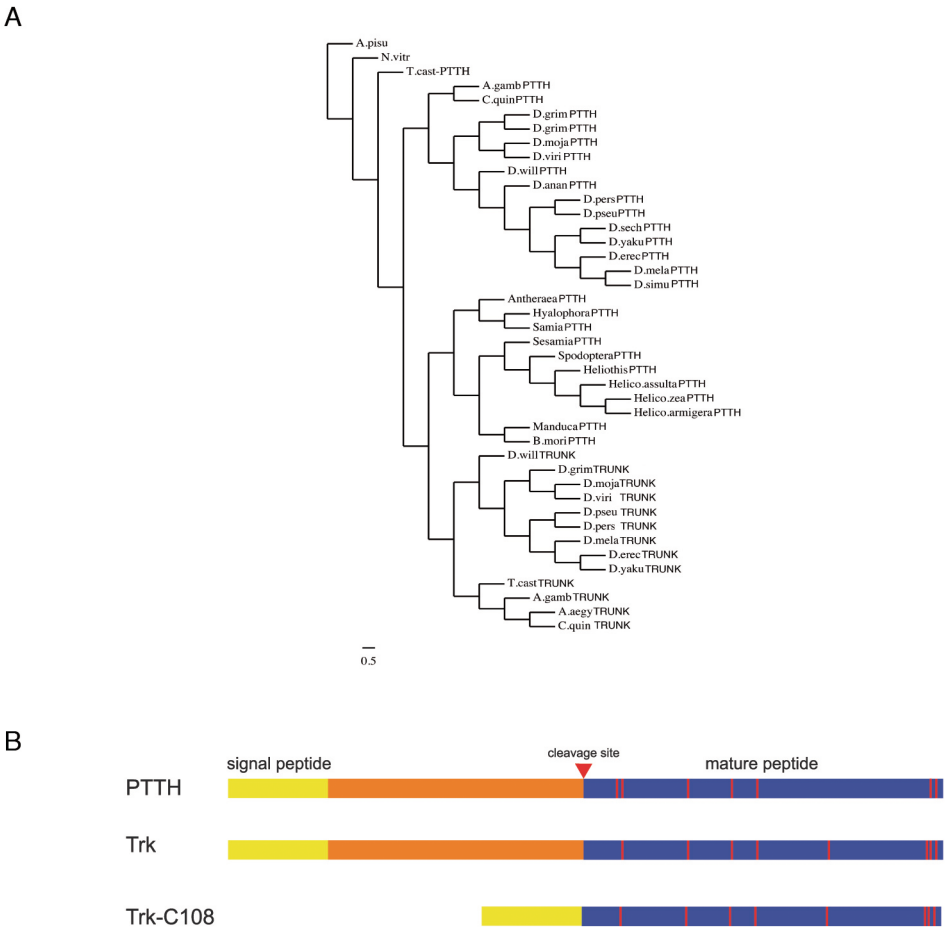

Figure 1 | Torso ligands are structurally and phylogenetically related. (A) Phylogenetic tree for Ptth and Trk from different insect species generated with the SATCHMO software[38] and the Drawgram viewer (http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=drawgram). (B) Diagram of *Drosophila* Ptth, Trk, and Trk^{C108}. Processing of Ptth and Trk is believed to release the mature C-terminal peptides (blue boxes). For details on Trk^{C108} see[7]. Red vertical lines indicate cysteines.

pupariation[4]. In this experiment we obtained similar results with *trk* and *ptth*, which in our hands did not produce a substantial effect (Fig. 2A). Of note, time of pupariation depends on many extrinsic conditions (temperature, food quality, etc.) as well as the genetic background. In order to be sure about the significance of the differences we have always referred our measurements to internal controls performed in parallel and in the same conditions as the actual experiment. Thus, while showing an internal consistence it is nevertheless difficult to compare the data from different set of experiments, which does not allow making a direct comparison between results obtained in different set of experiments in the same or different laboratories. The finding that general expression of *trk* did not produce a substantial effect on pupariation is in agreement with the observation that extra copies of *trk* do not increase Tor signalling in the embryo[6] and other observations, suggesting that the processing and not the overall amount of the Trk protein is the limiting factor for Tor activation[2,7]. Consistently, we found that general expression of Trk^{C108} (Fig. 1B), a truncated version of the protein that acts as an active form of Trk in embryonic patterning[7], has a mild but statistically significant effect in advancing the time of pupariation (Fig. 2A). This result is consistent with the observation that even expression of a constitutive form of the Tor receptor produces a rather minor advance in the time of pupariation[3]. Thus, the Tor receptor can be activated in both settings by either ligand, provided they are appropriately expressed and activated. While it has not been possible to generate a stable active form of Ptth[3], these results together with the partial rescue of the *trk* mutants by germ-line expression of *ptth*[3] suggest that a full-length Ptth is also likely to be cleaved.

Since Tsl is required to shift Trk to an active form in the germ-line, we addressed whether Tsl could have a similar role in the prothoracic gland. First we analysed if Tsl accumulates in the prothoracic gland. Expression was detected by the use of an anti-Tsl antibody, which shows Tsl accumulation in the prothoracic gland (Fig. 3A,B). Specificity of the prothoracic gland signal is confirmed by its absence in the transheterozygote combination for two deficiencies uncovering the *tsl* gene (Fig. 3C). Curiously, antibody staining also showed staining in the corpora cardiaca, which appears to be non-specific as it is also observed in the above-mentioned null condition for *tsl*. (Fig. 3A',C). These results were confirmed by the use of a *tsl-HA* tag form expressed under the control of the *tsl* promoter[8], which we found expressed in the prothoracic gland at least from the second larval instar (data not shown), but neither in the corpora cardiaca nor in the corpus allatum (Fig 3D). Finally, the anti-Tsl antibody also specifically detects Tsl accumulation in the ovarian cells known to express *tsl* (data not shown). Tsl accumulation in the prothoracic gland prompted us to analyse whether *tsl* mutants present a delay in pupariation. Since pupariation time can be greatly affected by the genetic background or even by second site mutations in the chromosomes bearing the particular *tsl* alleles, we analysed several tsl mutant combinations. In spite of some variation between *tsl* mutants, larvae presented a significant delay in pupariation (Fig. 2B). Finally, to study whether *tsl* function is specifically required in the prothoracic

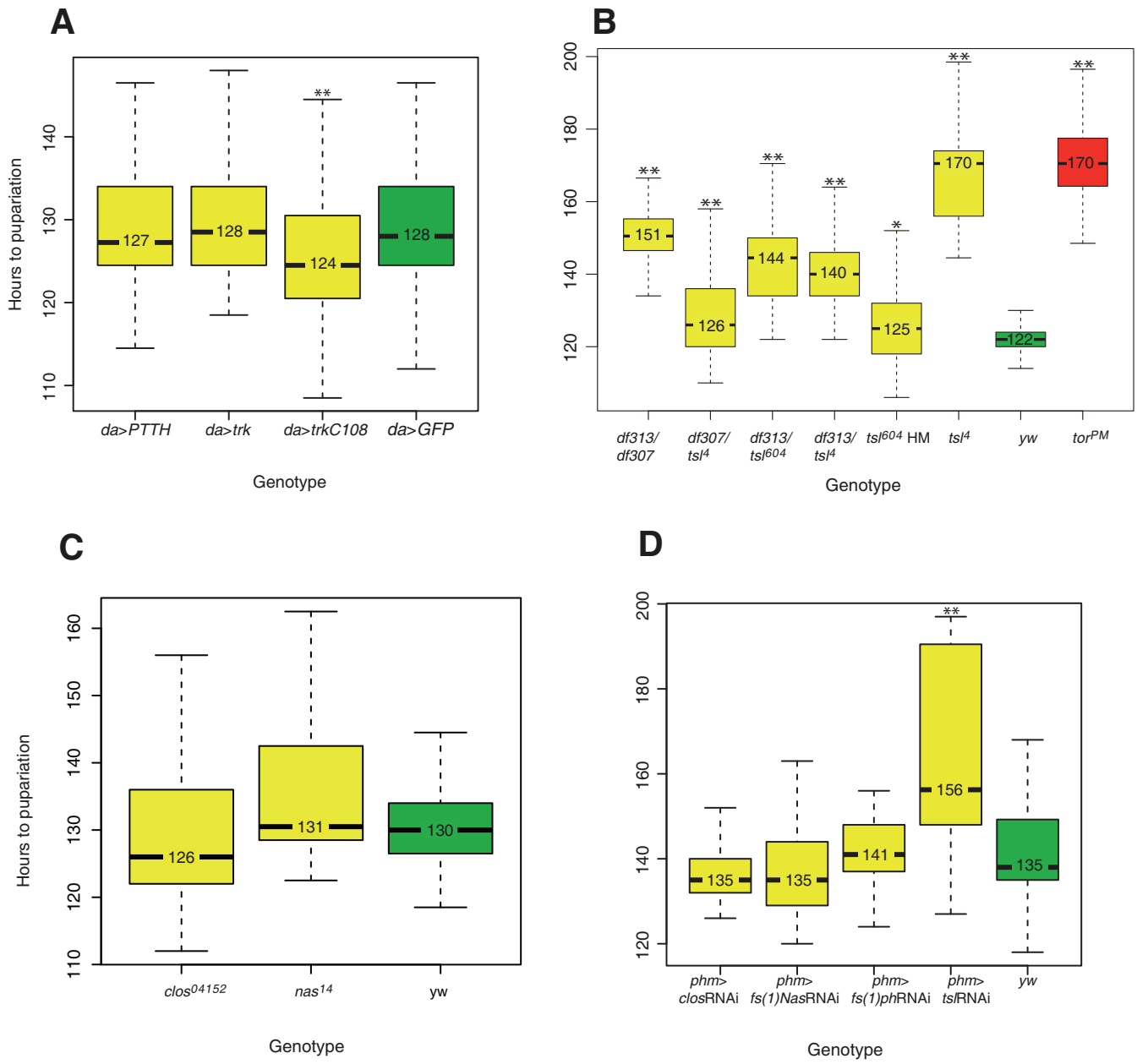

Figure 2 | Analysis of pupariation time. (A) Overexpression of *ptth* and full-length *trk* does not significantly advance pupariation as compared to GFP overexpression, while overexpression of a cleaved form of *trk* (*trk*^{C108}) slightly but significantly advances pupariation (individuals examined: *da>trk*,110; *da>trk*^{c108},58; *da>Ptth*,72; *da>GFP*,86). (B) All *tsl* mutant combinations analysed show significant developmental delays when compared to a *yw* control (individuals examined *df313/df307*: 96; *tsl*^{313}/*tsl*^{604}: 121; *df307/tsl*^{4}: 97; *df313/tsl*^{4}: 71; *tsl*^{604}: 90; *tsl*^{4}: 35; *tor*^{PM}: 75; *yw*: 164). (C) Conversely, *fs(1)N and clos mutants* do not show any significant delay (individuals examined: *fs(1)N*^{14}: 116; *clos*^{04152}: 117; *yw*: 138). (D) Prothoracic gland knockdown of *fs(1)N, clos* and *tsl* produce the same outcome as that of their corresponding mutants; knock-down of *fs(1)ph* also behaves as those for *fs(1)N* and *clos* (individuals examined: *phm>tslRNAi*,22; *phm>fs(1)phRNAi*: 30; *phm>fs(1)NRNAi*: 41; *phm>closRNAi*: 32; *yw*: 56). Boxplots were used to represent data: black lines represent medians, colour boxes represent the interquartile range and small bars at the ends of dotted lines represent the upper and the lower values. * is used to indicate a p-value <0.05 and ** is used to indicate a p-value <0.01.

gland, we inactivated the function of this gene by an RNAi construct under the control of a *phm*-gal4 driver[9]. Under these conditions, pupariation was delayed in a similar way (Fig. 2D).

We next addressed whether Tsl activity in the prothoracic gland is indeed required for Tor activation. We monitored MAPK/ERK diphosphorylation as a readout of Tor activity. In the wild-type, dpERK strongly accumulated in the cells of the prothoracic gland (Fig. 3E) in a Tor-dependent manner, as dpERK was barely detected in the prothoracic gland of *tor* mutants (Fig. 3F). Similar results were obtained for the prothoracic gland in *tsl* mutant larvae (Fig. 3G). All together, these data indicate that *tsl* is specifically expressed in the prothoracic gland and that its activity is required for Tor activation. A previous analysis of the *tsl* promoter identified two enhancer regions responsible for its expression in distinct groups of somatic follicle cells in the ovary[10]. Using the same kind of reporter construct analysis, we revealed that a different region of the *tsl* promoter drives its expression in the prothoracic gland, thus indicating the presence of a separate prothoracic gland enhancer (Fig. 4).

Another set of genes is required for embryonic Tor activation, upstream of Trk. In particular, the products of the genes *fs(1)N*,

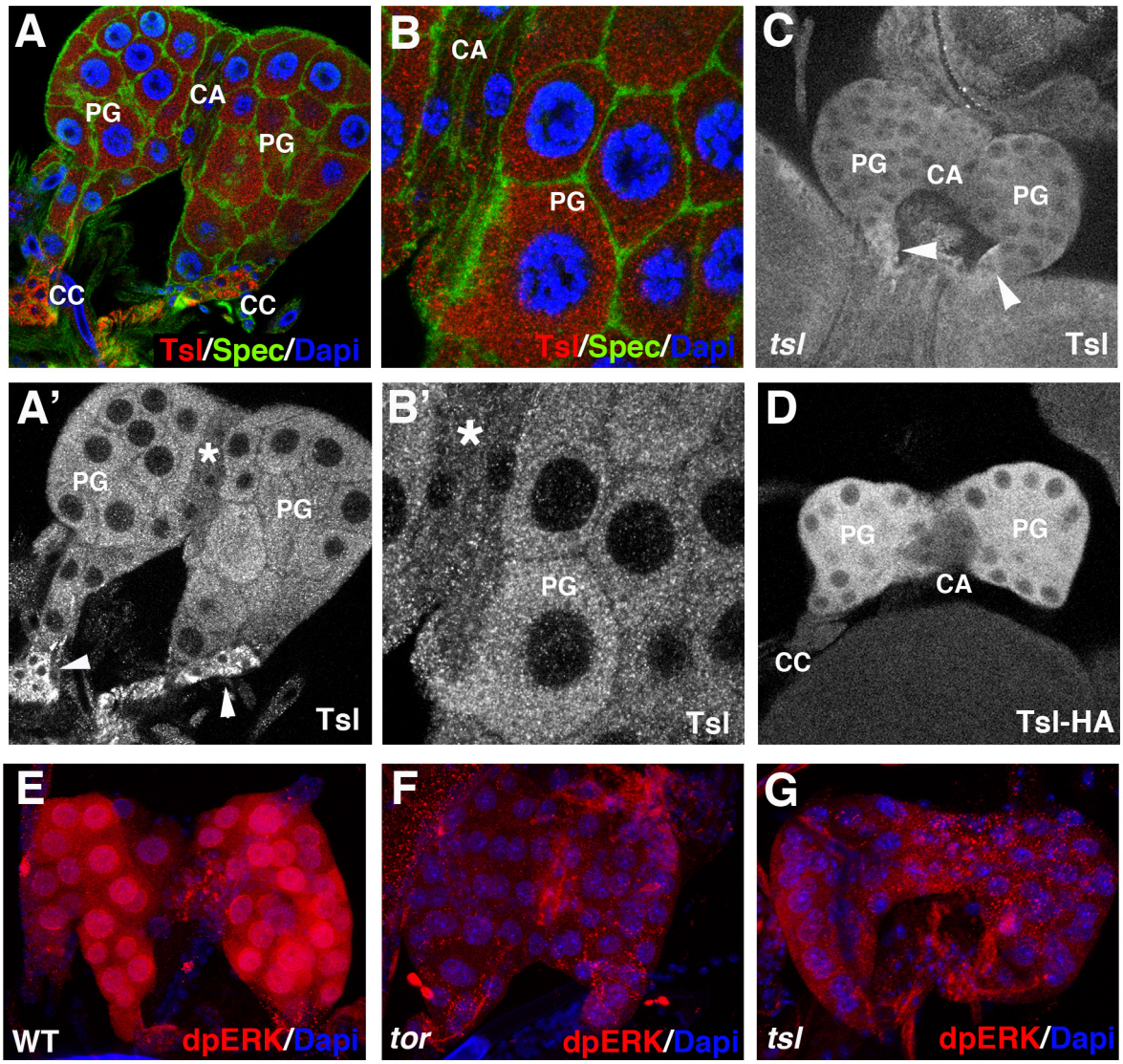

Figure 3 | ***tsl* expression and patterns of dpERK in the prothoracic gland.** (A,B) A ring gland from a 3rd instar larva stained with anti-Tsl (red) and anti-Spectrin (green) antibodies and DAPI (blue). (A',B') Same pictures showing only Tsl accumulation; antibody signal is detected at the prothoracic gland (PG) and in the corpora cardiaca (CC) (arrowheads) but not in the corpus allatum (CA) (asterisk). (C) Specificity of the signal at the PG is shown by its absence in 3rd instar larvae mutant for a *tsl* null combination (*Df(3R)cakiX-313/CASKX-307*); conversely, signal at the CC appears to be unspecific because it is also detected in the *tsl* null combination (arrowheads). (D) A ring gland from a 3rd instar larva bearing a *tsl-HA* construct stained with an anti-HA antibody; signal is detected in the PG but not in the CA nor in the CC. All images correspond to single stacks. (E–G) Anti-dpERK staining (red) at the PG from wild type (E), *torXR* (F) and *tsl4* (G) 3rd instar larvae; dpERK is detected only in the wild type.

fs(1)ph and *clos* are needed for the proper anchoring/stabilisation of the Tsl protein on the vitelline membrane, which has been held responsible for their involvement in Tor activation[8,11,12]. In addition, Stevens and collaborators[11] have postulated a further requirement of the *fs(1)N* and *fs(1)ph* gene products in order for Tsl to exert its function, a not yet identified requirement not due simply to the amount of Tsl protein on the vitelline membrane. This proposal prompted us to analyse whether these genes are similarly required for Tor activation in the prothoracic gland. However, we did not detect any specific expression of Ha-tagged rescuing constructs for *fs(1)N*, *fs(1)ph* or *clos* in the prothoracic gland, any specific signal by *in situ* hybridization or any specific staining with a Clos antibody, the only one available for one of these three gene products (data not shown). Accordingly, we did not detect any consistent pupariation delay in either the *fs(1)N* or *clos* mutants (Fig. 2C) or upon expression of the *fs(1)N*, *fs(1)ph* or *clos* RNAi constructs under the control of the *phm*gal4 driver (Fig. 2D).

The structural similarity between Ptth and Trk suggest a possible shared origin probably due to a duplication of an ancestral gene. To address this question we sought those genes in different insects. Orthologues for both *trk* and *ptth* have been identified in *Tribolium castaneum* (*Tc-trk* and *Tc-ptth*) ([13,14] and our own results). Conversely, we have found only one ortholog in *Nasonia*, a member of the Hymenopthera, which are thought to be the most basal group of the holometabolous insects and in the aphid *Acyrthosiphon pisum,* a hemimetabolous insect. This finding indicates that the presumed duplication at the origin of the two putative ligands occurred at the base of the holometabolous insects (Fig. 1A). More interestingly, we have found that the *Tribolium* genes display the same differential regional expression pattern as their *Drosophila* homologues: *Tc-trk* is expressed in the oocyte and *Tc-ppth* in some neurons in the brain (Fig. 5A,D). Accordingly, injection of *Tc-trk* RNAi, but not *Tc-ptth* RNAi, in the abdomen of *Tribolium* females led to the production of larvae offspring with a terminal phenotype (Fig. 5C), similar to the

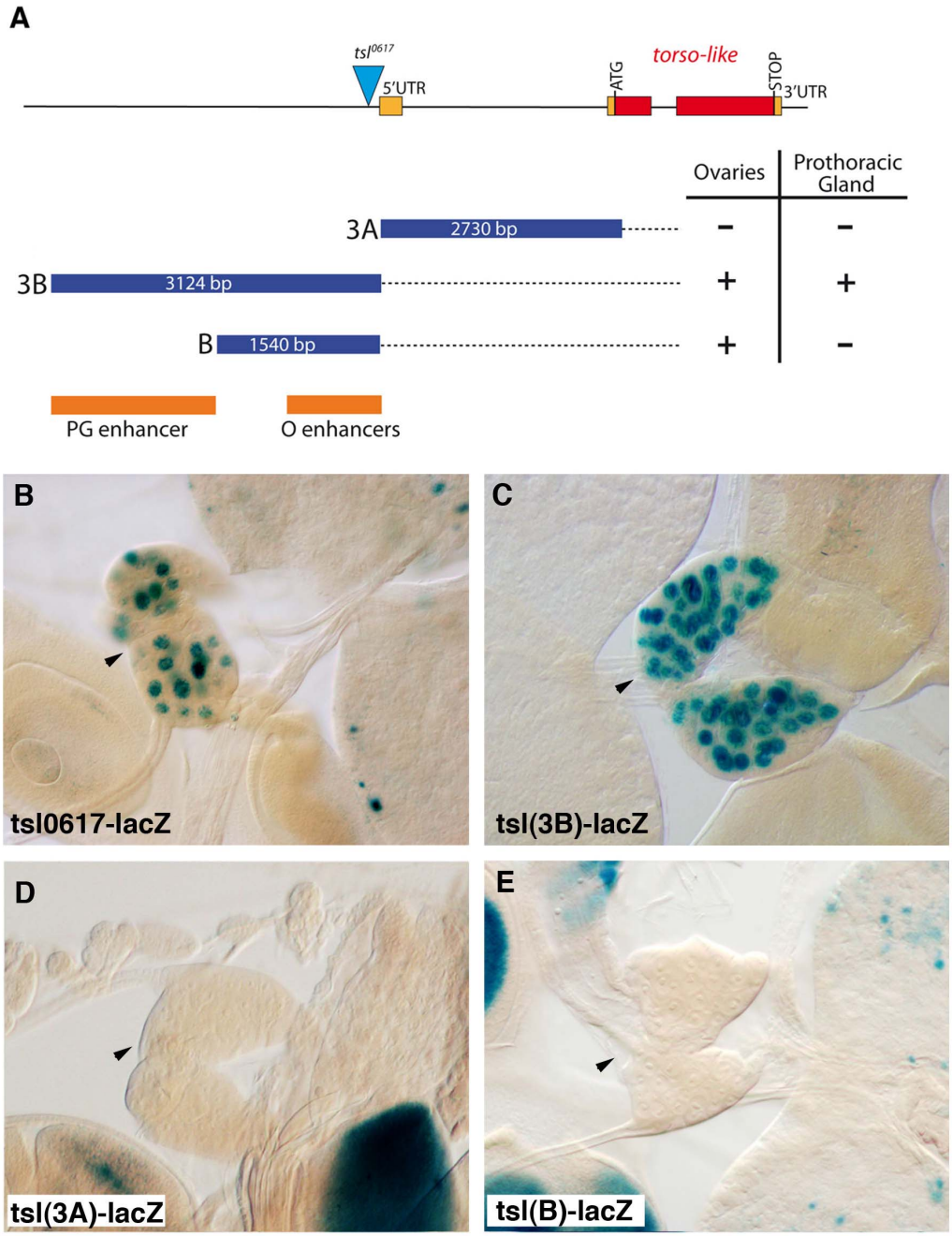

Figure 4 | Distinct enhancers regulate *tsl* expression in the prothoracic gland and in the ovarian follicular epithelium. (A) The *tsl* gene and fragments tested for enhancer activity[10]. Red and orange rectangles are translated and untranslated exons respectively. The triangle is the P element in tsl^{0617}. Blue rectangles are the fragments in the *in vivo* enhancer assay. Dark orange rectangles show the identified enhancers, PG for the prothoracic gland and O for the ovaries (for expression in ovaries see[10]). (B) Expression of the P-enhancer trap tsl^{0617} is detected in the PG (arrowhead). (C–E) While *tsl*(3B) drives lacZ expression in the PG, *tsl*(3A) and *tsl*(B) do not (arrowheads).

ones reported for *Tc-tor* or *Tc-tsl* RNAi (our own data and[15]). However, we could not assess the contribution of either *Tc-tor* or *Tc-ptth* to pupariation as we were not able to establish an accurate system for timing *Tribolium* pupariation comparable to that established for *Drosophila*. We must highlight that the timing of pupariation in *Drosophila* is highly variable and susceptible to many environment inputs, which we were able to overcome using the techniques available for this species.

Discussion

Here we provided evidences that Tsl participates in Tor activation both in the embryo and in the prothoracic gland. In the embryo, the Trk^{C108} cleaved form activates Tor in the absence of *tsl* function[7], thereby suggesting that the latter is directly or indirectly involved in the processing of the Trk protein. Given the similarity between Trk and Ptth, the effect of Tsl in dpERK accumulation in the prothoracic gland and the effect of Trk^{C-108} and Tsl in advancing and delaying pupariation respectively, we propose that Tsl is similarly involved in Ptth processing in the prothoracic gland. It should be noted that in the prothoracic gland Tsl and Tor proteins are produced in the same cells while during embryogenesis Tor accumulates in the embryo upon synthesis while Tsl is synthesized and secreted from cells surrounding the oocyte. However, *tor* and *tsl* expression in distinct cell types is not an absolute requirement for Tor activation in

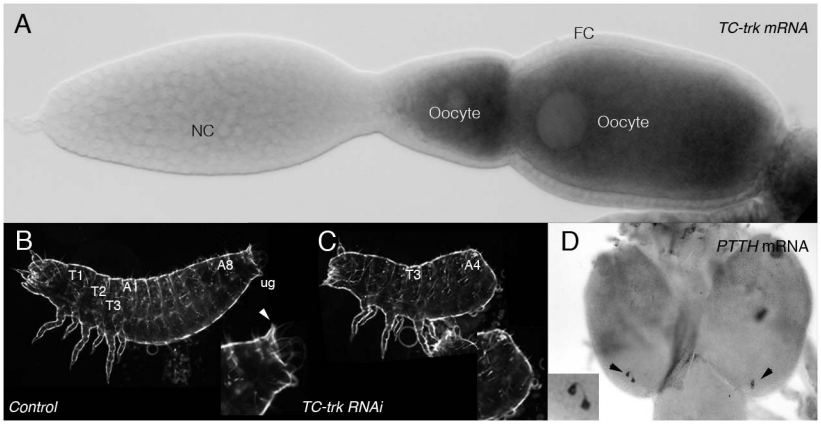

Figure 5 | *trk* and *ptth* in *Tribolium castaneum*. (A) *Tc-trk* mRNA is detected in the developing oocyte; no expression is found in nurse (NC) and follicular cells (FC). (B) *Tribolium* larva from control injection. (C) *Tc-trk* RNAi injections impair terminal patterning. (D) *Tc-ptth* mRNA is detected in two pairs of neurons in the larval brain.

embryogenesis as we have previously shown that *tsl* expression in the germline is also functional in Tor activation[16]. Thus, Tsl is detected in the cytoplasm of the cells where it is synthesised both in the ovary[8,12] and in the prothoracic gland (Fig. 3), although the presence of a signal peptide in the protein suggests that it is secreted in both cases. Indeed, secreted Tsl is detected, upon specific processing, in the vitelline membrane, a particular type of extracellular matrix in the early embryo[11]; yet, we have not been able to detect Tsl at the extracellular matrix of the prothoracic gland cells.

As Tsl lacks any feature indicating that it has protease activity[17,18], it has been suggested that this protein participates in the activation or nucleation of such an enzymatic complex[2]. In this scenario, similar proteins that could equally be activated/nucleated by Tsl could carry out the processing of Trk and Ptth. In this case, Tsl would be the common module in both events of Tor activation. Alternatively, the same players could be involved in both Trk and Ptth processing, in which case, the common module for Tor activation should be enlarged to also encompass the same processing complex. Final clarification of these two possibilities awaits the identification of the Trk (and Ptth) processing mechanism, which still remains elusive.

Conversely, *fs(1)N*, *fs(1)ph* and *clos* are required for Tor activation only in the early embryo indicating that Tsl does not need the function of these gene products to exert its function outside the embryo. Indeed, a relevant function of these three proteins is in vitelline membrane morphogenesis[8,11,12,19,20]. Therefore, it is likely that these proteins are recruited to anchor Tsl at the vitelline membrane and thus they participate in Tor activation exclusively in embryonic patterning. Of note, several observations have led to the proposal that anchorage of Tsl in the vitelline membrane serves to store it in a restricted domain until Tor activation in the early embryo[21,22].

As for Tor, Toll signalling is a transduction pathway that was initially thought to act only in early embryonic patterning[23] but does in fact participate in other signalling events[24,25]. However, in the case of Toll signalling, a single putative ligand, Spätzle (spz), acts both during embryonic patterning and in immunity (reviewed in[26]), while for Tor signalling different putative ligands are responsible for its activation in embryonic patterning and in the control of pupariation. Spz triggers Toll activation in many scenarios because the *spz* promoter drives its expression in several groups of cells[27], possibly by distinct enhancers. In contrast, in the case of the Tor pathway a likely duplication might have generated two genes each with a distinct expression pattern and encoding the corresponding ligand for one of the two Tor activation events. The observation that Coleoptera but not Hymenopthera possess both *trk* and *ptth* orthologues suggest the putative duplication to have occurred at the origin of holometabolous insects. However and regarding *tsl* as the key element in ligand activation, multiple usage of the Tor pathway appears to have evolved by recruiting independent enhancers responsible for the distinct expression of the same gene.

In summary, Tor activation in oogenesis and in the prothoracic gland is linked to the following: a duplication and subsequent differential expression of *trk* and *ptth*; the acquisition of independent specific oogenesis and prothoracic gland enhancers in the *tsl* promoter; and the recruitment of proteins independently required for organ morphogenesis, in particular for eggshell assembly. The *Drosophila* EGFR resembles the case of Tor as another example of the repetitive use of the same receptor by different ligands in different contexts: Gurken in oogenesis and Spitz, Vein, and Keren during other stages of development (reviewed in[28]). Thus, we propose a combination of gene duplication, enhancer diversification and cofactor recruitment to be common mechanisms that allow the co-option of a single receptor-signalling pathway in distinct developmental and physiological functions.

Methods

Fly strains. The following strains were used: yw as a wild-type, UAS-*trk*[29] UAS-*trk*$^{C108\ 7}$, UAS-*ptthHA*[4], *phm*-Gal4[9], *da*Gal4 (Bloomington #8641), *tsl*$^{0617\ 17}$, *tsl*(3B)-lacZ, *tsl*(3A)-lacZ, *tsl*(B)-lacZ[16], *tsl* RNAi[10], *tsl*^{H17}, *tsl*$^{604\ 18}$, *tor*^{XR}, *tor*$^{PM\ 30}$, *fs(1)N*$^{14\ 8}$, *clos*$^{c04152\ 12}$, and *fs(1)N* (#45489), *clos* (#39697) and *fs(1)ph* (#14413) RNAi from the VDRC[31]. *Df(3R)caki*$^{X\text{-}313}$ and *CASK*$^{X\text{-}307}$ have been molecularly characterised[32] and our previous work on the terminal system has shown the transheterozygote combination to behave as null allele for *tsl*. We used GFP balancers and mutant larvae selected accordingly.

Developmental timing analysis. Fertilised eggs were collected on peach juice agar plates. Collections were done at 2 h intervals during 2 days, generating at least 6-8 replicates per experiment. For each experiment, newly hatched first instar larvae were collected 24 hours after egg laying and transferred into vials with regular cornmeal food. Larvae were raised in groups of 25 to prevent overcrowding and the drying of fly food, at 25°C in a 12-h light/dark cycle. Pupariation was scored at continuous 2 h intervals, and the total time to pupariation for scored individuals was introduced on a Microsoft Excel spreadsheet.

Statistical analysis and data representation. In order to choose the most appropriate statistical tests to compare data samples we run the Shapiro-Wilk test[33] on the single datasets. For all the tested datasets, the p-value was inferior to the alpha level of 0.05, indicating that data deviate significantly from a normally distributed population. Since normality of the data is one of the requirements of parametric statistics (i.e. Student's *t*-test), we decided to apply non-parametric statistics for pair wise comparisons. Mutant and control datasets were compared with the Mann-Whitney U test[34], which is the non-parametric alternative to the *t*-test for independent samples. The R software package was used to perform all the tests and to generate the plots.

Immunostaining and in situ hybridization. Larval tissues were dissected in ice-cold PBS and fixed in 4% PBS-formaldehyde for 20 min (8% PBS-formaldehyde for detection with anti dpERK antibody). Incubations were o/n at 4°C with primary antibodies and for 2 h at r.t. with secondary antibodies. Samples were mounted in Fluoromount (Southern Biotech) or Vectashield with DAPI (Vectorlabs). We used

anti-Spectrin (D.S.H.B. Iowa), anti-Tsl (see below), anti-dpERK (Cell signalling), anti-HA (3F10, Roche) and fluorescent conjugated secondary antibodies (Jackson ImmunoResearch). *Tribolium* whole mount *in situ* were performed according to[35].

Generation of anti-Tsl polyclonal antibody. A fragment corresponding to aminoacids 20–353 was PCR amplified and cloned into pProEX-HTa. Transfected BL21 cells were grown in ZYP-5052[36] at 37°C o/n, the culture centrifuged and resuspended in a 8M urea buffer, the protein extract sonicated and purified with a Ni column (Sigma), the purified protein loaded in a 10% acrylamide gel and the corresponding band used to immunise rats. Immunopurification was performed in a Ni column and elution with 4M MgCl2. Eluted antibody was dialysed with PBS 1X and used at 1:50.

Tribolium RNAi injections. Double-stranded RNA (dsRNA) was produced as described[37]. A clone containing the whole *trk* cDNA (700 nt.) was used as a template for *in vitro* transcription. Parental RNAi was performed as described[37] and *trunk* dsRNA at a concentration of 0.5 µg/µl was injected into pupae. A total of 23 larvae out of 26 hatched (88.4%) displayed axis extension defects. A *ptth* cDNA (558nt.) was used as a template to generate dsRNA. No phenotype was observed upon *ptth* dsRNA injection (78/78). H_2O was injected as control and no defective abdominal outgrowth phenotype was observed in the offspring (40/40). Cuticles of first-instar larvae were embedded in Hoyers mounting medium mixed with lactic acid (1:1) and analysed with differential interference contrast (DIC).

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Author contributions

MG, MF, XFM and JC designed the experiments. MG and MF performed the main experiments. MG, CM and XFM did the *Tribolium* experiments. JC wrote the manuscript and all authors reviewed it.

Additional information

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