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Maternal Torso Signaling Controls Body Axis Elongation in a Short Germ Insect

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Summary

In the long germ insect *Drosophila*, all body segments are determined almost simultaneously at the blastoderm stage under the control of the anterior, the posterior, and the terminal genetic system [1]. Most other arthropods (and similarly also vertebrates) develop more slowly as short germ embryos, where only the anterior body segments are specified early in embryogenesis. The body axis extends later by the sequential addition of new segments from the growth zone or the tail bud [2–4]. The mechanisms that initiate or maintain the elongation of the body axis (axial growth) are poorly understood [5–8]. We functionally analyzed the terminal system in the short germ insect *Tribolium*. Unexpectedly, Torso signaling is required for setting up or maintaining a functional growth zone and at the anterior for the extraembryonic serosa. Thus, as in *Drosophila*, fates at both poles of the blastoderm embryo depend on terminal genes, but different tissues are patterned in *Tribolium*. Short germ development as seen in *Tribolium* likely represents the ancestral mode of how the primary body axis is set up during embryogenesis. We therefore conclude that the ancient function of the terminal system mainly was to define a growth zone and that in phylogenetically derived insects like *Drosophila*, Torso signaling became restricted to the determination of terminal body structures.

Results and Discussion

In *Drosophila*, the anterior- and posterior-most terminal body regions of the embryo depend on the maternal terminal-group genes. One of them, the *torso-like* (*tsl*) gene is expressed in somatic follicle cells located at the anterior and posterior pole of the oocyte. In the embryo, *tsl* contributes to the local activation of the receptor tyrosine kinase *Torso* at the egg poles. The signal is transduced to the nucleus via a Ras-Raf-MAP-K/Erk phosphorylation cascade, and leads to the expression of the zygotic target genes *tailless* (*tll*) and *huckebein (hkb)* at the posterior terminus of the embryo. Failure to activate *Torso* signaling results in defects in the head skeleton and loss of all segments posterior to abdominal segment 7, in addition to loss of the hindgut and posterior midgut anlagen [9–13].

Whether an anteriorly acting terminal system is a general feature of all insects has been challenged because under certain conditions, *Torso* function at the anterior is dispensable for head development in *Drosophila* [14]. This hypothesis is supported by the expression of the *Tribolium* ortholog of *tll* at the posterior, but not at the anterior pole of blastoderm stage embryos [15]. Thus, in *Tribolium*, posterior terminal cells appear to be determined before the onset of abdomen formation. It is unknown, however, whether these cells specify posterior fate after axis elongation and abdomen formation is completed or whether they also contribute to earlier steps of segmentation.

Isolation and Functional Characterization of Beetle *torso* and *torso-like* Orthologs

We have isolated the orthologs of the key components of the *Torso* pathway in the short germ beetle *Tribolium torso* (*Tc-tor*) and *torso-like* (*Tc-tsl*). As in *Drosophila*, *Tc-torso* mRNA is maternally inherited by the embryo and expressed ubiquitously in freshly laid eggs (Figures 1A–1C), and *Tc-tsl* is expressed during oogenesis anteriorly and posteriorly in the follicle cells of the oocyte (Figure 1D).

Knocking down the function of *Tc-torso* or *Tc-tsl* using parental RNA interference [16] leads to identical embryonic phenotypes. Whereas the head and the anterior thorax are unaffected (Figures 1F, 1G, and 1J), unexpectedly the most extreme *Tc-torso*^{RNAi} and *Tc-tsl*^{RNAi} embryos lack all structures that develop during postblastodermal abdominal growth (Figures 1G and 1J; see Table S1 available online with this article). Thus, the head and thoracic segments that form in *torso* or *tsl* RNAi embryos likely represent the structures, which are determined already during the *Tribolium* blastoderm stage [17]. Less strongly affected embryos fail to form the full number of abdominal segments (Figure 1F).

To determine whether the *Tc-torso* RNAi phenotype does not reflect a late function of maintaining abdominal fate prior to cuticularization, we analyzed the expression of Engrailed protein in *Tc-torso*^{RNAi} embryos at a stage when abdominal segments should already have developed. Indeed, in strongly affected embryos, Engrailed stripes corresponding to the head and thorax, but not to abdominal segments, are present (Figure 1I).

We then visualized the emergence of segments in embryos with impaired Torso signaling by analyzing the *Tc-even-skipped* (*Tc-eve*) expression pattern (Figure 2). In wild-type embryos, *Tc-eve* is initially expressed in a double segmental pattern that later resolves into secondary segmental stripes [18]. *Tc-tsl*^{RNAi} does not interfere with the formation of the first two primary *Tc-eve* stripes that give rise to the gnathal and the first thoracic (T1) segments (Figure 2B). However, although the third primary *Tc-eve* expression domain (*Tc-eve* stripe 3) forms

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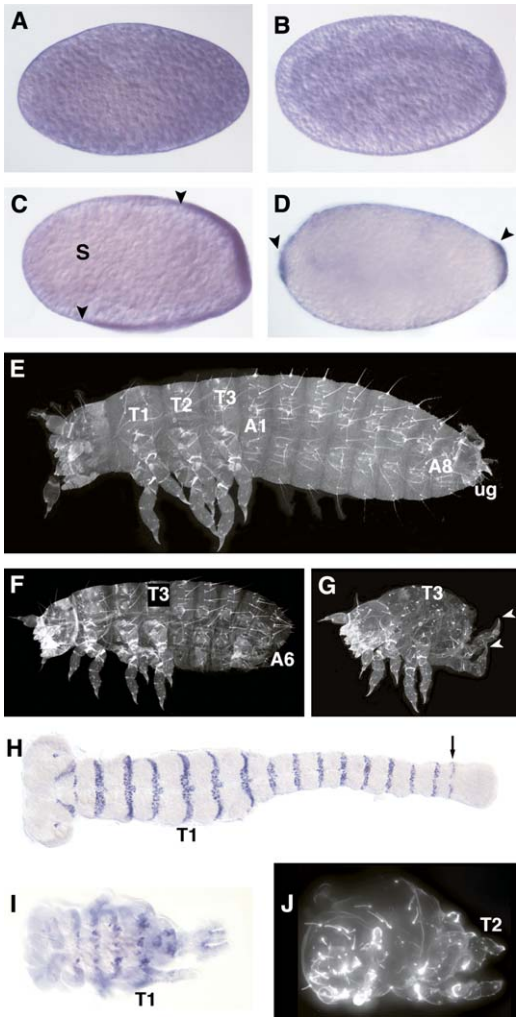

Figure 1. Expression and Function of Terminal Genes in *Tribolium*

(A–D) Expression pattern of *Tc-torso* and *Tc-torso-like*. (A and B) *Tc-torso* mRNA is ubiquitously expressed during early embryogenesis of *Tribolium*. (C) At the posterior pit stage, *Tc-Torso* expression is found only in the anlagen giving rise to the embryo. Arrowheads mark the border of extraembryonic serosa (S) and the embryo. The darker staining at the posterior pole reflects that more layers of tissue developed at this stage, when gastrulation had started. Anterior points to the left, posterior to the right; ventral is down in (C) and cannot be distiguished in (A and B). (D) *torso-like* expression during late oogenesis is seen at the anterior and posterior pole in the follicle cells of the oocyte (arrowheads), but not within the oocyte as judged by the analysis of different focal planes. The focal plane is on the posterior follicle cells and therefore anterior expression is out of focus. Anterior is to the left.

(E–J) Axis elongation is affected by *Tc-torso* and *Tc-tsl* RNAi. (E and H) Wild-type, (F, G, and I) *Tc-torso*^{RNAi} embryo, (J) *Tc-torso-like*^{RNAi} larva, (E–G and J) cuticle preparations, (H and I) immunohistochemical detection of the Engrailed protein. (E) The wild-type cuticle of a first instar larva constists of the head, three thoracic segments (T1-3), eight abdominal segments, and the posterior terminus bearing the urogomphi (ug). (F) Weak *Tc-torso* RNAi phenotype with six developed abdominal segments. No terminal structures have developed. (G) Strong *Tc-torso* RNAi phenotype. Thorax but no abdomen or terminal structures have developed (arrowheads point to the claws belonging to the legs of thoracic segment 3). The strongest phenotypes found contain a normal head and two thoracic segments T1 and T2 (see also Figure 1J).

(H) Engrailed protein expression in a wild-type embryo undergoing segmentation. The penultimate abdominal Engrailed stripe 9 (arrow) has just formed. I *Tc-torso*^{RNAi} embryo of a similar stage as the embryo shown in (H). The legs have considerably elongated. The posteriormost segment bears a tripartite leg presumably formed as a result of a regeneration event. (J) *torso-like*^{RNAi} phenotype (cuticle preparation). As for the strong *Tc-torso* RNAi phenotype, the head and the first thoracic segments form without any obvious defects. Posterior to T2, however, no additional segments or terminal structures developed. Anterior is to the left. Abbreviations: T, thoracic segments; ug, urogomphi (leg-like appendages on abdominal segment 10).

normally, this domain does not resolve into segmental stripes, and no additional primary *eve*-stripes form (Figure 2F, I). In the wild-type, *Tc-eve* stripe 3 covers the region where the second (T2) and third thoracic (T3) segment will develop. Although *Tc-eve* stripe 3 does not split in *Tc-tsl* ^{RNAi} embryos, this domain gives rise to the second thoracic segment. Thus, Torso signaling is required for the initiation of axial growth or maintaining the segmentation process.

The Integrity of the Growth Zone Is Affected in *Torso*^{RNAi} Embryos

As revealed by DAPI staining and by morphology (Figures 2D, 3B, and 4D), posterior invagination of cells is abolished in both *Torso*- and *tsl* ^{RNAi} embryos, and as a consequence, no posterior pit forms. To understand how Torso signaling is propagated at the posterior pole and to test whether downstream gene activity is affected in the growth zone in *Tc-torso*^{RNAi} embryos, we analyzed the activity of the Map-kinase (Figure 3) and the expression of *Tc-wingless* (*Tc-wg*), *Tc-tailless* (*Tc-tll*), *Tc-caudal* (*Tc-cad*), and *Tc-forkhead* (*Tc-fkh*) RNA in early embryos (Figure 4 and Supplemental Data).

The active state of the Torso receptor is transduced to the nucleus via the Ras-Raf signal transduction pathway and leads to the activation of zygotic target genes. The activity of this pathway can be visualized with an antibody that recognizes ErkPP [19] but does not discriminate between the different pathways that involve ErkPP signaling. In nontreated embryos, ErkPP can be detected in a subpopulation of the serosa, a single row of cells at the border of the serosa and the embryonic anlage; at the rims of the mesoderm; and at the posterior pole (Figure 3A). In *torso*^{RNAi} embryos posterior ErkPP expression is lost, further indicating that ErkPP is involved in propagating terminal signaling (Figure 3B). ErkPP expression in the serosa is mildly affected whereas the other sites where ErkPP activity is detected in the wild-type are normal. ErkPP activity in the amnion appears not to be reduced; however, the amnion itself does also not form properly (Figure 3D). Whether this is a direct or indirect consequence of *Torso* reduction is unclear.

In addition to segmental stripes, a terminal *wingless* (*wg*) expression domain first seen at the blastoderm stage is present throughout the phase of body elongation in the growth zone of the wild-type (Figures 4A–4C). In *Tc-torso*^{RNAi} embryos, the posterior terminal *Tc-wg* domain is missing at the blastoderm stage, as well as in older embryos corresponding in age to wild-type embryos undergoing body axis extension (Figures 4D–4F). *Drosophila-torso* mutant embryos also lack the posterior terminal *wg* expression domain [20], indicating, that the

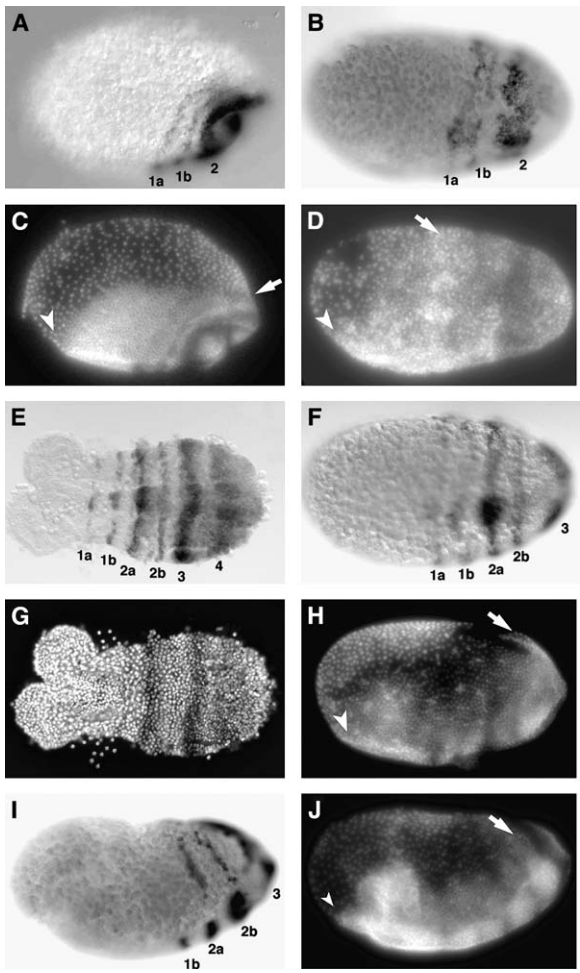

Figure 2. Segmentation Is Disturbed in *Tc-torso-like*^{RNAi}-Treated Embryos

Expression of *Tc-even-skipped* in *Tc-tsl* ^{RNAi} embryos. Wild-type (A, C, E, and G) and *Tc-tsl*^{RNAi} embryos (B, D, F, H, I, and J) stained for *Tc-eve* by in situ hybridization and for DAPI. The panels show bright field (A, B, E, F, and I) and epi-fluorescence images of the same embryos (C, D, G, H, and J) respectively. (A and C) Gastrulating wild-type embryo. The nuclei of the serosa are larger and wider spaced than those of the germ rudiment. The primary *Tc-eve* domain (*Tc-eve* stripe 1) has split into two secondary segmental stripes (1a and 1b) corresponding to the mandibular and maxillary segment, in addition the second primary *Tc-eve* stripe (*Tc-eve* stripe 2) is visible. (B and D) *Tc-tsl* RNAi embryo of the corresponding age to (A). The head anlage is extended toward the anterior (D, arrowhead), extraembryonic tissue is reduced, and the germ rudiment is less compact (D, arrow). (E and G) flat-mounted, wild-type embryo. Additional primary and secondary *Tc-eve* expression is visible. *Tc-eve* stripe 2 has split (2a and 2b) representing the labial and first thoracic segment. (F and H) *Tc-tsl*^{RNAi} embryo of the corresponding age to (E). As judged by the absents of the primary *Tc-eve* stripe 4 posterior development is blocked. Still the germ rudiment is less compact (H, arrow) and extended toward the anterior pole (H, arrowhead). As in (D and J) no invaginations of cells at the postrior pole takes place. (I and J) During further development the germ band of *Tc-tsl*^{RNAi} embryos is compacting, however nor additional *Tc-eve* domains appear neither the *Tc-eve* stripe 3 splits confirming that only the blastoderm derived segments from. Arrowheads (C, D, H, and J) point to the anterior end of the germ rudiment, and arrows (C, D, H, and J) point to the dorsal margin of the embryo proper. In all panels anterior points to the left and embryos are viewed laterally except for (E, F, G), that show ventral views.

dependence of *wg* on *torso* is conserved. The segmental *wg* stripes that were built prior to the growth process, form close to the posterior end. This shows that a presegmented region (PSR) normally separating the last segment formed at the posterior end of the embryo is strongly reduced or absent in *torso*^{RNAi} embryos (Figures 4E and 4F). The absence of *Tc-cad* (Figures 4H and 4I) and *Tc-tll* (Figure 4K) in *torso*^{RNAi} embryos establishes these genes as potential targets of terminal signaling also in *Tribolium*.

As judged from the lack of the posterior *Tc-forkhead* (*Tc-fkh*) expression domain, *Tc-torso*^{RNAi} embryos do not develop a hindgut (Supplemental Data, Figure 2B). *Tc-fkh* itself, which is also expressed in the growth zone throughout most of the segmentation process [15], seems not to be required in the axis elongation process because *Tc-fkh* RNAi leads only to malformation of the hindgut (Figure S2D, Table S1).

Anterior Extraembryonic Tissue Is Reduced in *Tc-tsl* RNAi

The irregular expression of ErkPP in the serosa of embryos deficient for Torso signaling (Figure 3D) suggests a function for this pathway also in patterning this extraembryonic tissue. Indeed, in *Tc-tsl*^{RNAi} embryos, the serosa is severely reduced in size whereas the presumptive head region appears enlarged and extended toward the anterior (Figure 2D). This finding is corroborated by the expanded expression domain of the head marker gene *Tc-006A12* in *Tc-tsl*^{RNAi} embryos (Figures 3F and 3H), which in wild-type embryos is expressed just posterior of the serosa in a wedge-shaped domain (Figures 3E and 3G) [21, 22]. Nevertheless, embryos develop with normal head structures (Figures 1F, 1G, 1I, and 1J), similarly as in embryos where serosa reduction results from reduction of *zen-1* activity [21].

The anterior phenotype, however, differs among embryos depleted for either *Tc-torso* or *Tc-tsl*. Compared with the *Tc-tsl* RNAi, the serosa is less affected with *Tc-torso* RNAi (Figure 3D), indicating that *Tc-tsl* has been more sufficiently downregulated via RNAi than *Tc-torso*.

Thus, in a short germ embryo Torso signaling is—like in *Drosophila*—required for patterning both the anterior and the posterior region of the embryo. Due to the difference in the anlagenplan of short and long germ embryos, however, different tissues and different processes depend on the Torso pathway.

The Evolution of the Terminal System

We have presented here the first functional characterization of the terminal-class genes *torso* and *torso-like* outside the dipterans. At the anterior, Torso signaling is involved in the specification of the anterior-most structure in the *Tribolium* egg, the extraembryonic serosa. At the posterior, *Tc-torso-like* and *Tc-torso* are required for terminal fates, including the posterior gut primordial and for body axis growth.

Our results show that the terminal system in *Tribolium* functions to establish the growth zone and to initiate rostrocaudal growth. In the absence of the terminal signal, *caudal* expression and pair-rule patterning is not maintained during later stages of development. It is not known how the loss of posterior patterning and the

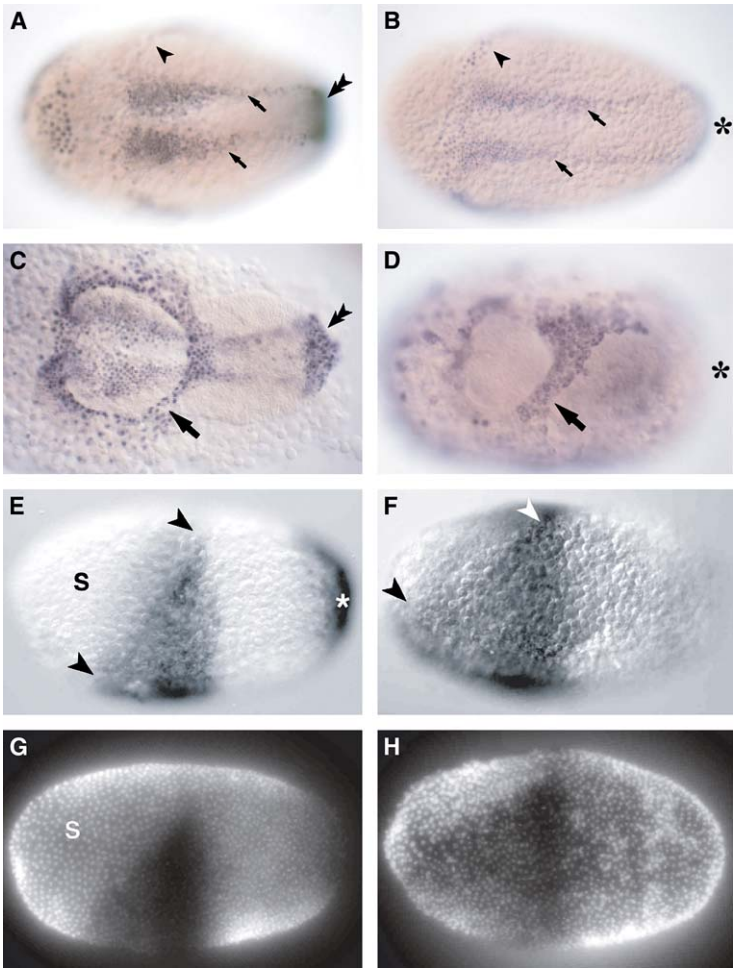

Figure 3. Torso Signaling Is Required for Anterior Extra embryonic Structures

Activated Map-K and expression of the marker gene *Tc-006A12* in the growth zone depend on Torso signaling. (A, C, E, and G) wild-type embryos (B and D) *torso*^{RNAi} embryos (ventral views) (F and H), *tsl*^{RNAi} embryos. (A) In wild-type embryos active Map kinase is seen at the rims of the mesoderm (arrows) at the border between serosa and embryo (arrowhead) in patches in the serosa and at the posterior pole (double arrowhead). (C) Slightly later Map-K-PP is active also at the edges of the amnion window (arrow) while it closes over the germ rudiment. (B and D) In *torso*^{RNAi} embryos, Map-K-PP activity is absent in the growth zone (asterisk), the amnion window and the serosa are irregularly formed but essentially present. (E and G) Expression of the marker gene *Tc-006A12* in wild-type embryos (in situ hybridization [E] DAPI staining of the same embryo [G]). The nuclei of the serosa (S) are larger and wider spaced than those of the germ rudiment. The anterior margin of *Tc-006A12* expression (arrowheads) marks the posterior border of the serosa (S). In addition a posterior domain is visible (asterisk). (F and H) *Tc-tsl*^{RNAi} embryo of the corresponding age as shown in (E). *Tc-006A12* expression has expanded into anterior direction (arrowheads), the serosal anlagen (S) is reduced to small anterior-dorsal portion. The posterior *Tc-006A12* expression domain is lost, possibly due to the absence of the growth zone. Arrowheads (E and F) point to ventral and dorsal border of the *Tc-006A12* expression domain. In all panels anterior points to the left and embryos are viewed laterally.

loss of posterior growth relate to each other. Since the growth zone specific expression domain of *wg* depends on *torso* one could speculate that the posterior domain of *Tc-wg* is required for both, regulation of cell proliferation and coordination of continued segmental patterning. The involvement of the Wg pathway in the axis elongation process has already been demonstrated for *Gryllus* [23].

Insects like *Drosophila* that develop as long germ embryos have short life cycles and are thus perfectly adapted to quickly changing environments like rotting fruits. During *Drosophila* embryogenesis, the posterior-most Engrailed stripe corresponding to abdominal segment 9 forms under the influence of *Torso* slightly later than the other stripes [10]. This could be seen as a rudimentary elongation of the body axis in *Drosophila* and likely reflects the ancient function of Torso signaling.

In contrast to long germ embryos, the formation of the complete trunk occurs in a secondary growth process in short germ embryos of arthropods and vertebrates. We propose that the involvement of Torso signaling in body axis growth likely represents the ancient function of this gene in the development of short germ insects. Thus, during the evolution from short to long germ development, Torso signaling lost its major function in axial growth and was recruited as an additional gradient system to specify the position of posterior abdominal segmentation gene domains during the blastoderm stage. Whether Torso signaling is involved in body axis growth also in other short germ animals remains to be shown.

Experimental Procedures

For isolation and phylogenetic analysis of Tc-torso and Tc-tsl (see Supplemental Data).

RNA Interference, In Situ Hybridization, Immunohistochemistry, and Cuticle Analysis

Double-stranded RNA (dsRNA) was produced as described [24] using the Maxi-Script-Kit (Ambion). Two clones containing parts of the *Tc-torso* dsRNA corresponding to the 5′ end of the complementary-DNA were used as templates for the in vitro transcriptions. One clone (*Tc-Tor*1.6, nucleotide positions 133–1715) includes sequences coding for the RTK domain A. The second clone (*Tc-Tor*0.9, nucleotide positions 86–1006) only contains sequences located 5′to the RTK domain. RNA preparation of both clones yielded the extreme *torso* RNAi phenotype, excluding the possibility that an unrelated gene coding for a similar RTK was affected. As template for the *tsl* dsRNA the 481bp fragment was used. *Tc-fkh* dsRNAs was produced from a previously described clone (accession number: AF217810) [15, 25]. Parental and embryonic RNAi was performed as described [16, 26] and dsRNA at a concentration of 500 ng μl^{-1} was injected into pupae. In control experiments using *Sp8* dsRNA at the same concentrations, only the appendage-specific phenotype [27] could be obtained, and no defective abdominal outgrowth phenotype was observed (Table S1).

In the case of *Tc-fkh* RNAi only a 1:20 dilution resulted in embryonic survival. In situ hybridizations, the 4,6-diamidino-2-phenylindole (DAPI) [17] staining, and immunohistochemistry were essentially

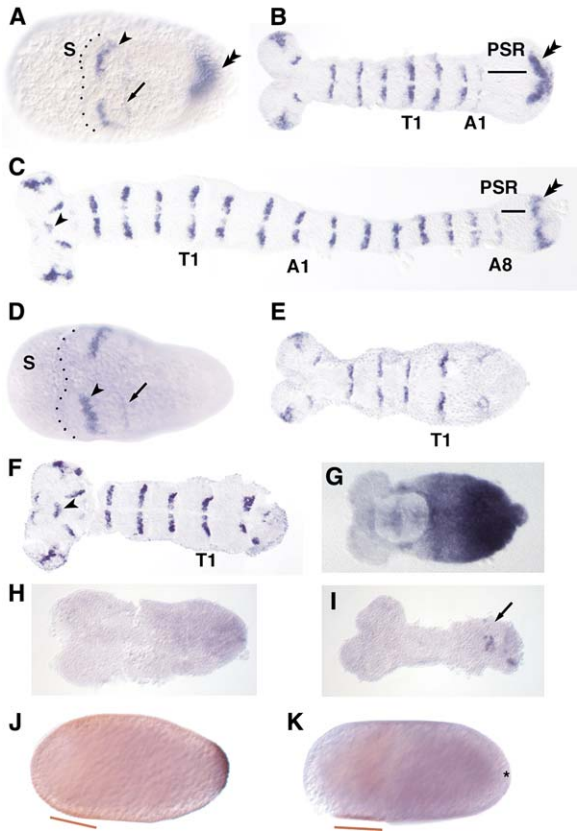

Figure 4. Expression of *Tc-wingless*, *Tc-caudal*, and *Tc-tailless* in Wild-Type and *Tc-torso*^{RNAi} Embryos

(A–C) Successively older stages of wild-type embryos are stained for *Tc-wg* expression. (A) Embryo undergoing gastrulation. The arrowhead points to the anterior and the double arrowhead to the posterior terminal *Tc-wg* expression domain, the arrow marks the first segmental *Tc-wg* stripe. Dots denote the anterior margin of the embryonic head. S, extraembryonic serosa. (B) The *Tc-wg* stripe corresponding to abdominal segment 1 (A1) is forming anterior to the posterior terminal *Tc-wg* expression domain (double arrowhead) leaving the presegmented region (PSR) free of *Tc-wg* expression. (C) Wild-type embryo with eight abdominal *Tc-wg* stripes (arrowhead points to the stomodeal *wg* domain). (D–F) *Tc-torso*^{RNAi} embryos. The posterior terminal expression domain of *Tc-wg* is not formed in embryos treated with *Tc-torso* dsRNA, and no abdominal *Tc-wg* stripes are seen in these embryos. Embryos of similar developmental stages (as judged by the *Tc-wg* stripes developed) are paired in (A) and (D), (B) and (E), and (C) and (F).

(G–I) Staining for *Tc-caudal* expression in wild-type (G) and *torso*-RNAi embryos (H and I). In the wild-type, *Tc-caudal* is expressed in the posterior half of the germ rudiment (G), and remains expressed in the growth zone throughout axis elongation [5, 33]. Only remnants of *caudal* expression is detectable in *torso*^{RNAi} embryos ([H], arrow in [I]) indicating that *torso* acts upstream of *caudal* in *Tribolium*.

(J and K) *Tc-orthodenticle*/*Tc-tailless* double staining in wild-type (J) and *torso*^{RNAi} (K) embryos. The similar stage of both embryos is shown by the concommitant staining for *orthodenticle* expression that has resolved to a stripe (bar). Posterior *tailless* expression cannot be detected in in *torso*^{RNAi} embryos (K, asterisk). All panels, anterior is to the left.

performed as described [15, 26, 28–31]. Cuticles of first-instar larvae were embedded in Hoyers mounting medium [32] mixed with lactic acid (1:1) and analyzed with differential interference contrast (DIC) or confocal laser scanning microscopy (Leica).

Supplemental Data

Supplemental Data include Experimental Procedures, two figures, and one table and are available at http://www.current-biology.com/cgi/content/full/15/23/2131/DC1/.

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Accession Numbers

The cDNA sequence for *Tribolium castaneum torso* and *tsl* have been deposited in the GenBank database under the accession numbers AY618898 and AJ969955, respectively.