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Hedgehog signaling pathway function conserved in *Tribolium* segmentation

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Abstract In *Drosophila*, maintenance of parasegmental boundaries and formation of segmental grooves depend on interactions between segment polarity genes. Wingless and Engrailed appear to have similar roles in both short and long germ segmentation, but relatively little is known about the extent to which Hedgehog signaling is conserved. In a companion study to the *Tribolium* genome project, we analyzed the expression and function of *hedgehog*, *smoothened*, *patched*, and *cubitus interruptus* orthologs during segmentation in *Tribolium*. Their expression was largely conserved between *Drosophila* and *Tribolium*. Parental RNAi analysis of positive regulators of the pathway (*Tc-hh, Tc-smo*, or *Tc-ci*) resulted in small spherical cuticles with little or no evidence of segmental grooves. Segmental Engrailed expression in these embryos was initiated but not maintained. Wingless-independent Engrailed expression in the CNS was maintained and became highly compacted during germ band retraction, providing evidence that derivatives from every segment were present in these small spherical embryos. On the other hand, RNAi analysis of a negative regulator (*Tc-ptc*) resulted in embryos with ectopic segmental grooves visible during germband elongation but not discernible in the first instar larval cuticles. These transient grooves formed adjacent to Engrailed expressing cells that encircled wider than normal *wg* domains in the *Tc-ptc* RNAi embryos. These results suggest that the *en–wg–hh* gene circuit is functionally conserved in the maintenance of segmental boundaries during germ band retraction and groove formation in *Tribolium* and that the segment polarity genes form a robust genetic regulatory module in the segmentation of this short germ insect.

Keywords *Hedgehog* · *Smoothened* · *Cubitus interruptus* · *Patched* · Segmental groove · Segmentation

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Introduction

The ontogenic stage at which the body plans of animals belonging to the same phylum reach maximum morphological similarity is called the phylotypic stage. In insects and other arthropods, the phylotypic stage is the elongated germband at which the three germ layers have formed and segments are morphologically evident along the entire anterior–posterior axis (Sander 1997). In *Drosophila*, segment polarity genes, most of which are components of two major signal transduction pathways (the Wingless and Hedgehog signaling pathways), control the formation of grooves between segments and anterior–posterior patterning within each segment. Initially, pair-rule genes activate *engrailed* (*en*) expression at the anterior boundary of each parasegment and *wingless* (*wg*) at the posterior boundary DiNardo and O'Farrell (1987; Howard et al. 1988; Ingham et al. 1988). Engrailed protein activates expression of *hedgehog* (*hh*), which encodes a secreted protein that signals to surrounding cells (Hidalgo and Ingham 1990; Ingham and Hidalgo 1993). Hh signaling leads to the continued activation of *wg*, whose secreted protein product is necessary for the continued activation of *en*. This positive feedback loop ensures the interdependence of these three genes for the maintenance of each other's expression until embryonic stage 9–10 (Forbes et al. 1993). At the end of stage 10, *en* expression becomes independent of *wg*, and it

is around this stage that segmental boundaries are morphologically visible at the posterior edge of cells expressing *en* and *hh*.

Evidence that this segment polarity network might be conserved is primarily based on the expression patterns of *en*, *wg*, and more recently, Hh pathway component genes in other insects as well as non-insect arthropods and annelids (Patel et al. 1989; Brown et al. 1994; Kraft and Jackle 1994; Nagy and Carroll 1994; Grbic et al. 1996; Peterson et al. 1998; Damen 2002; Dhawan and Gopinathan 2003; Simonnet et al. 2004). There is also limited data suggesting this network is functionally conserved among insects. Ectopic expression of *Drosophila wg* in *Tribolium* induces ectopic *en* in the anterior half of the parasegment suggesting functional conservation of the *wg–en* interaction (Oppenheimer et al. 1999). *wg* and/or *en* have been implicated by RNAi analyses in the proper formation of segmental boundaries in the milkweed bug *Oncopeltus faciatus* (Angelini and Kaufman 2005), the honey bee *Apis mellifera* (Beye et al. 2002), the blowfly *Lucilia sericata* (Mellenthin et al. 2006) and the beetle *Tribolium* (Ober and Jockusch 2006). While RNAi analysis of *wg* and *hh* in the cricket *Gryllus* (Miyawaki et al. 2004) failed to reveal significant effects on segmentation, RNAi analysis of *armadillo*, a *wg* pathway component, does implicate *wg* signaling in segmentation in this short germ insect. In the long germ embryo of *Drosophila*, where all segments initiate virtually simultaneously, loss of any one of these three genes destabilizes the expression of the others and they eventually fade, resulting in shorter embryos that, in addition to the loss of segmental grooves, also show misspecified epidermal cell fates (Ingham et al. 1991; Forbes et al. 1993). We have investigated genes that encode the Hh-signaling pathway components *hedgehog* (*hh*), *patched* (*ptc*), *smoothened* (*smo*), and *cubitus interruptus* (*ci*) to determine whether they function with *en* and *wg* in the formation of segmental boundaries in this short germ band insect.

The Hh-signaling pathway is well-conserved between insects and vertebrates (Huangfu and Anderson 2006) and is thus likely to be conserved in *Tribolium*. The main components were first elucidated in *Drosophila,* where Hh is secreted by cells in the posterior compartment of embryonic segments and larval imaginal discs. It diffuses to the anterior compartment (Lee et al. 1992; Tabata et al. 1992; Tashiro et al. 1993) where the signal is controlled by two membrane proteins: Patched (Ptc), a twelve pass transmembrane protein (Hooper and Scott 1989; Nakano et al. 1989) and Smoothened (Smo), a seven pass transmembrane protein (Alcedo et al. 1996; van den Heuvel and Ingham 1996). In the absence of Hh signal, Ptc represses Smo activity (Chen and Struhl 1996). Signaling is initiated by binding of Hh to its receptor Ptc, which relieves this repression and allows Smo to signal to a multimeric complex inside the cell. This complex is composed of the serine threonine kinase Fused (Alves et al. 1998), the kinesin related protein Costal-2 (Sisson et al. 1997), a novel cytoplasmic protein Suppressor of fused (Monnier et al. 1998) and a zinc finger transcription factor *Cubitus Interruptus* (*Ci*; Motzny and Holmgren 1995). In unstimulated cells, this complex sequesters *ci*, inhibits nuclear import of the full-length 155 kDa protein and promotes its cleavage to generate an N-terminal 75-kDa form containing the Zn finger DNA-binding domain, which can enter the nucleus and repress transcription of Hh target genes (Aza-Blanc et al. 1997). When Hh signal is transduced, activation of Smo inhibits *ci* cleavage and activates the full-length protein, which then translocates to the nucleus, resulting in the transcription of Hh-responsive target genes including *wg*, *ptc*, *gooseberry*, and *decapentaplegic* (Alexandre et al. 1996; Dominguez et al. 1996; Hepker et al. 1997; Ingham and McMahon 2001).

Consistent with reports on other arthropods, we found that the expression patterns of *hh*, *ci*, *smo*, and *ptc* were largely conserved in *Tribolium.* Using RNAi to study the function of these genes during segmentation in *Tribolium*, we followed embryonic development in these embryos using En as a marker of segment development and integrity. When the Hh signal was depleted by RNAi, segments were specified normally in the posterior growth zone and the embryos elongated as fully as wild-type. En and *wg* expression in these embryos, although properly initiated, was not maintained and defects appeared during germ band retraction, resulting in tiny, sphere-shaped embryos lacking segmental grooves. On the other hand, overactivation of the pathway by *ptc* RNAi produced embryos with transient ectopic segmental grooves and embryonic cuticles with enlarged heads and thoracic appendages. All together, these results indicate that Hedgehog signaling is an essential component of the segment polarity network in *Tribolium*, which is necessary to maintain segmental integrity during germband retraction after the segments have been enumerated in the growth zone. The conserved function of an *en–wg–hh* gene circuit during segmentation suggests that the segment polarity genes constitute a robust gene regulatory module in this short germ insect.

Materials and methods

Beetle husbandry

Tribolium castaneum strain GA-1 was reared in whole wheat flour supplemented with 5% dried yeast at 30 C (Beeman et al. 1989).

Identification of hedgehog pathway component genes in the *Tribolium* genome

Partial cDNAs of *tc-hh*, *tc-ci*, *tc-smo* and *tc-ptc*, cloned into the pCR4TOPO vector (Invitrogen), were obtained from Y. Tomoyasu. Orthologs of each gene were identified in the annotation of the *Tribolium* genome (the *Tribolium* genome consortium, in review). The sequences of the partial cDNAs matched those deduced from the gene models with minor differences. *hh*, glean gene number tc01364 or NCBI mRNA accession number XM961615, is located on LG 2; *smo*, TC05545 or XM966834, is on LG 8; *ci*, TC03000 or XM965017, is on LG 3 and *ptc*, TC04745 or XM962700 is on LG 1=X.

In situ hybridization and immunostaining

Whole mount *in situ* hybridizations were performed according to established protocols (Tautz and Pfeifle 1989). Expression of Engrailed in *Tribolium* embryos was determined using the α-Invected antibody, 4D9 which cross-reacts with Tc-En (Brown et al. 1994). Double staining for the different mRNAs in addition to En protein was performed simultaneously according to the protocol of Nagaso et al. (2001).

RNA interference (RNAi)

Templates for dsRNA synthesis were amplified as described (Tomoyasu et al. 2007). Double stranded RNA was synthesized using the T7 MEGAscript kit (Ambion) and purified using the MEGAclear kit (Ambion). Different amounts of dsRNA (Table 1) were mixed with injection buffer (5 mM KCl, 0.1 mM KPO_4 pH 6.8) prior to injection. Parental RNAi was performed and affected embryos were analyzed as previously described (Bucher et al. 2002).

Microscopy and imaging

Stained embryos and larval cuticles were documented with a Nikon Digital DXM 1200F camera on an Olympus BX50 microscope using Nikon ACT-1 version 2.62 software. Brightness and contrast of all images were adjusted and some were placed on a white background using Adobe Photoshop 7.0.1 software.

Results

Expression patterns of *Tc-hh, Tc-smo*, and *Tc-ci*

Tc-hh transcripts are first detected in the presumptive head lobes on either side of the ventral mesoderm (arrowhead in Fig. 1a) and at the posterior end of the embryo. As the embryonic rudiment condenses, faint stripes of *Tc-hh* expression appear immediately posterior to the intense stripes in the head lobes and in the presumptive mandibular segment (arrowheads in Fig. 1b). Gnathal and trunk stripes appear in an anterior to posterior progression (Fig. 1c). Double staining for En expression revealed that *Tc-hh* and Tc-En are coexpressed in cells of the posterior compartment in each segment (Fig. 1d). During germband elongation, expression at the posterior end of the embryo resolves into spots on either side of the mesoderm and eventually into a ring surrounding the proctodeum (Fig. 1b–d and arrowhead in f). In the head, twin spots appear on either side of the presumptive stomodeum. As the head lobes mature, expression in the anterior region of the developing stomodeum increases (Fig. 1c,d and black arrowhead in e), while the anterior-most stripes of *Tc-hh* expression resolve into spots in the brain that overlap with the Tc-En-expressing cells (Fig. 1c,d and blue arrowhead in e).

Table 1 Summary of RNAi effects

Gene	DsRNA μg/μl	Class I (%)	Class II (%)	Class III (%)	Undeveloped (%)	Wild type (%)	Totals (*n*)
Tchh	3.0	0	6	83	11	0	464
	1.5	18	73	0	9	0	477
Tcsmo	3.0	0	0	89	11	0	579
	1.5	0	43	43	14	0	477
	0.75	38	44	0	20	0	230
Tcci	3.0	0	33	61	5	0	694
	1.5	0	30	63	7	0	405
	0.75	73	19	0	8	0	293
Tcptc	4.0	0	76	–	24	0	271
	2.0	13	68	–	19	0	189

Embryos were collected every 48 h for the first 2 weeks after injection. Phenotypic classes are unique for each gene. See text for details. Percentages have been rounded up.

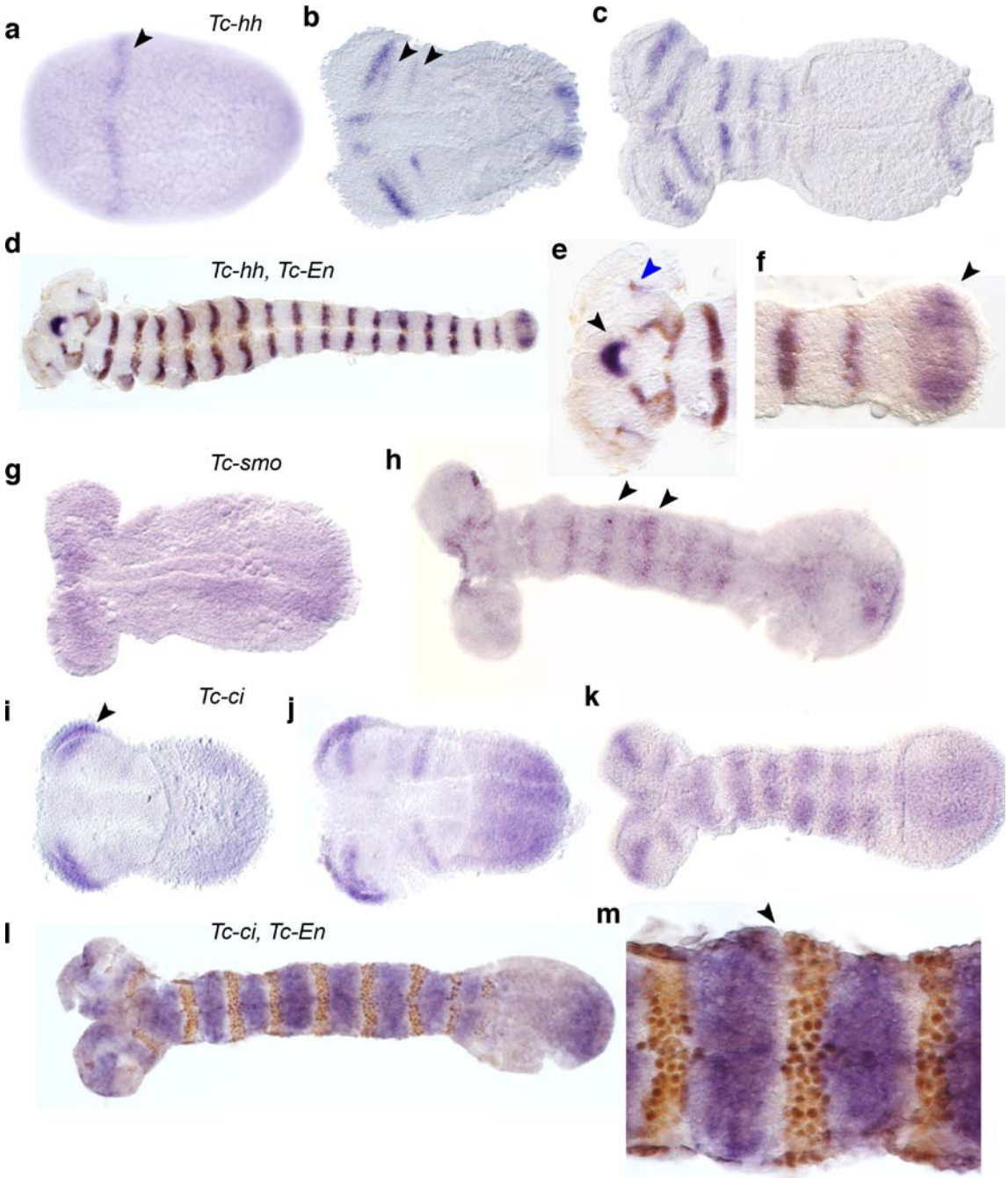

Fig. 1 Expression of *Tc-hh*, *Tc-ci*, and *Tc-smo* in *Tribolium* during segmentation. In these ventral views, anterior is to the left. **a**–**c** *Tc-hh*; **d**–**f** *Tc-hh* (*purple*) and *Tc-En* (*gold*); **g**–**h** *Tc-smo*; **i**–**k** *Tc-ci*; **l**, **m** *Tc-ci* (*purple*) and *Tc-En* (*gold*); **a** Anterior stripes of *Tc-hh* (*arrowhead*) in the presumptive head lobes of a blastoderm embryo. Expression at the posterior end of the embryo is not in the plane of focus. **b** Weak stripes appear in the antennal and mandibular segments (*arrowheads*) posterior to the dark stripes in the head lobes. Twin spots of expression flank the mesoderm at the posterior end of the embryo and near the stomodeum. **c** In addition to expression in the head lobes and antennae, three gnathal stripes and one trunk stripes appear in an elongating germband embryo. **d** Coexpression of Tc-En (*gold*) and *Tc-hh* (*purple*) in the posterior compartment of each segment. Note Tc-En expression at the ventral midline in the absence of *Tc-hh*. **e**–**f** *Tc-hh* expression in the stomodeum and the proctodeum (*arrowheads*) in the absence of Tc-En. Expression of *Tc-hh* in the head lobes has resolved into spots that overlap Tc-En expression (*blue arrowhead*). **g** Ubiquitous expression of *Tc-smo* in an early germ band embryo. **h** In an elongating germ band embryo, *Tc-smo* expression appears ubiquitous with some segmental modulation (*arrowheads*). **i** Expression of *Tc-ci* at the anterior edge of the head lobes (*arrowhead*) of a young germband embryo. **j** Gnathal stripes appear first in a slightly older embryo. Expression throughout the posterior region of the embryo fades anteriorly. **k** Wide *Tc-ci* stripes in the head lobes and antennal segments as well as in the gnathal and thoracic segments of an elongating germ band embryo. **l** Tc-En (*gold*) expression does not overlap *Tc-ci* (*purple*) expression in cells of the anterior compartment of each segment. **m** Enlarged view of a few thoracic segments from the embryo shown in **l** reveals **a** gap of two or three rows of cells anterior to the Tc-En stripes that do not express *Tc-ci* (*arrowhead*)

Expression at the posterior end of the embryo continues throughout germ band extension (Fig. 1b,d and f) eventually surrounding the proctodeum as it invaginates (Fig. 1f). As segments mature, *Tc-hh* expression at the ventral midline clears and *Tc-hh* is not expressed in the CNS. In total, there are three gnathal, three thoracic, and ten abdominal *Tc-hh* stripes with additional expression in the stomodeum, proctodeum, antennae, and brain (Fig. 1d).

Tc-smo is expressed ubiquitously in the germband (Fig. 1g) in *Tribolium*. As the germ band elongates, expression appears to modulate slightly within each segment (arrowheads in Fig. 1h). *smo* transcripts are expressed in a similar manner in *Drosophila* (Alcedo et al. 1996).

Tc-ci expression is first detected at the anterior edge of each head lobe in the early embryonic rudiment (arrowhead in Fig. 1i). Dynamic expression of *Tc-ci* in the head lobes eventually resolves to a wedge of cells in the lateral eye field (Fig. 1j). Expression is detected in the labrum (Fig. 1l) and in a broad posterior region, which fades toward the anterior (Fig. 1j). As the germ band elongates, *Tc-ci* is expressed in a broad stripe in every segment in an anterior to posterior progression (Fig. 1i–k). Double staining for Tc-En indicates that *Tc-ci* is expressed in the anterior compartment where the anterior-most *Tc-ci* expressing cells are immediately posterior to the Tc-En-expressing cells of the preceding segment (Fig. 1m). Segmentally reiterated stripes of *ci* expression are also observed in the mulberry silkworm *Bombyx mori* (Dhawan and Gopinathan 2002), the spider *Cupiennius salei* (Damen 2002), and the millipede *Glomeris* (Janssen et al. 2004), suggesting that the segmental expression of *ci* in the anterior region of each segment is conserved among arthropods. Closer examination of *Tc-ci* expression revealed a gap of two or three rows of cells immediately anterior of the Tc-En-expressing cells that do not express *Tc-ci* (Fig. 1m). In contrast, expression of *ci* throughout the anterior compartment of each segment in *Drosophila* is thought to be essential to the function of the Hh-signaling pathway in maintaining *wg* expression (Orenic et al. 1990; Hepker et al. 1997). Expression of *ci* immediately anterior to En-expressing cells is conserved in the spider (Damen 2002), but has not been reported for *Bombyx* or *Glomeris*. Thus, it is not clear whether this unusual expression pattern is unique to *Tribolium*.

RNAi analysis of *Tc-hh*, *Tc-smo,* and *Tc-ci*

The conserved expression patterns of *Tc-hh* and *Tc-smo* are consistent with the hypothesis that the Hh function in segmentation is conserved in *Tribolium*, but the lack of *Tc-ci* gene expression in cells immediately anterior to *Tc-hh* is not. To determine whether the Hh-signaling pathway is required for proper segmentation, we performed parental RNAi analysis of these Hh pathway components. Three different amounts of *Tc-hh* and *Tc-smo* dsRNA were injected, which uncovered a range of hypomorphic phenotypes (Table 1). Similar RNAi phenotypes were produced for both genes, and the RNAi cuticles were classified into three different categories (Class I, II, and III) based on severity as listed in Table 1. Regardless of the severity, none of the RNAi embryos hatched and the cuticle had to be dissected out of the vitelline membranes. Class I cuticles display the weakest phenotypes, in which the head is severely reduced and contains only rudimentary limb structures. The legs are present but slightly warped, and segmental grooves in the abdomen are occasionally fused but all eight abdominal segments are present (Fig. 2b (*Tc-hh*), f (*Tc-smo*)). Class II cuticles are small and spherical, with a large protuberance at the anterior end and three small warped pairs of limbs. The presence of the spiracle on the second thoracic segment (arrowhead in Fig. 2c and g) allowed us to identify these structures as rudimentary legs. The small amount of cuticle posterior to these small warped legs is smooth and lacked any abdominal features (Fig. 2c (*Tc-hh*), g (*Tc-smo*)). The most severely affected embryos, Class III, produced small spherical cuticles with a large protuberance at the anterior end and no obvious head structures or thoracic limbs (Fig. 2d (*Tc-hh*), h (*Tc-smo*)). These cuticles are very smooth on all sides, with no sign of grooves and are quite small relative to the size of similarly aged wild-type cuticles (Fig. 2a *Tc-hh*, e *Tc-smo*). The head and gnathal appendages appear to be more sensitive to the depletion of *Tc-hh* or *Tc-smo* than the legs, as even in the weakly affected individuals the head, including gnathal segments, failed to form properly (Fig. 2b,f).

The phenotypes of *Tc-ci* RNAi embryos are not as severe as those observed for *Tc-hh* or *Tc-smo*. The most weakly affected, Class I *Tc-ci* RNAi cuticles (Fig. 2j), are shorter and fatter than wild-type with severely reduced heads, but contain all thoracic and abdominal segments. The Class II *Tc-ci* RNAi cuticles are also shorter than wild-type with a protuberance at the anterior end and fairly normal thoracic limbs, but little to no sign of segmental grooves in the abdomen (Fig. 2k). Similar to Class III RNAi embryos of *Tc-hh* and *Tc-smo*, Class III *Tc-ci* RNAi embryos produced smooth unsegmented cuticles lacking heads and gnathal appendages (Fig. 2l) that are considerably smaller than wild-type cuticles (Fig. 2i). Unlike *Tc-hh* and *Tc-smo* Class III RNAi embryos, they still produced fairly normal legs (Fig. 2l). Interestingly, loss of function mutants of *ci* in *Drosophila* also produce milder limb phenotypes than do *hh* mutants (Methot and Basler 1999).

To understand how the segmentation process is affected by loss of Hh signaling, we followed Tc-En expression during elongation and retraction in Class III RNAi embryos (Fig. 3). In RNAi embryos for all three genes, segmental stripes of Tc-En initiate normally during germ band

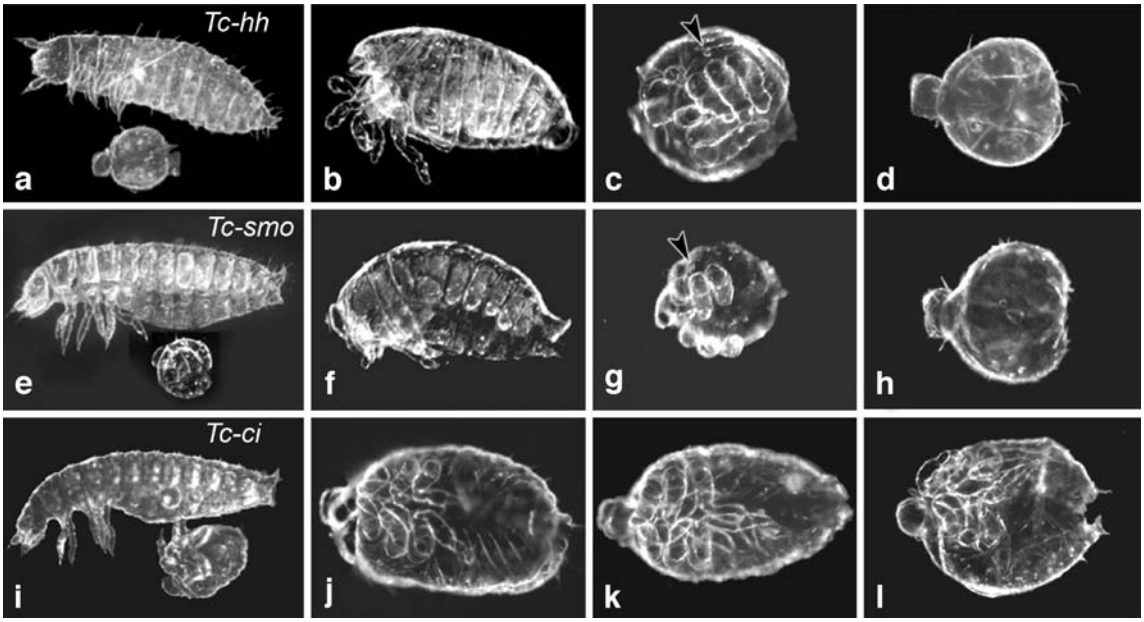

Fig. 2 *Tc-hh*, *Tc-smo*, and *Tc-ci* RNAi phenotypes in first instar larval cuticles. **a**–**d** *Tc-hh* RNAi; **e**–**h** *Tc-smo* RNAi; **i**–**l** *Tc-ci* RNAi. **a** Comparison of a severely affected small spherical *Tc-hh* RNAi cuticle and a wild-type cuticle at the same magnification. **b** Lateral view of a mildly affected Class I cuticle containing reduced gnathal appendages, normal legs and all abdominal segments. **c** Ventral view of a Class II cuticle that is similar in size and shape to more severely affected Class III cuticles but retains three pairs of thoracic appendages. Note spiracle on second thoracic segment (*arrowhead*). **d** Ventral view of a small spherical Class III cuticle with rudimentary head structures, no appendages and no segmental grooves. **e** Comparison of a severely affected small spherical *Tc-smo* RNAi cuticle and a wild-type cuticle at the same magnification. **f** Class I cuticle with highly reduced head structures and mouthparts but a full complement of body segments. **g** Class II cuticle with three pairs of legs and rudimentary head structures. Note spiracle on second thoracic segment (*arrowhead*). **h** A severely affected Class III *Tc-smo* RNAi cuticle phenotypically similar to the most severely affected *Tc-hh* RNAi cuticle. **i** Comparison of a severely affected small spherical *Tc-ci* RNAi cuticle and a wild-type cuticle at the same magnification. **j** Mildly affected Class I cuticle with reduced head structures, three pairs of legs and mildly fused abdominal segments. **k** Class II cuticles are slightly longer than intermediate *Tc-hh* or *Tc-smo* RNAi cuticles with highly fused abdominal segments. l A severely affected Class III *Tc-ci* RNAi spherical cuticle with three pairs of legs

elongation (blue arrowheads in Fig. 3a,d and g). Tc-En expression fades as the segments matured (black arrowheads Fig. 3a,d and g), suggesting that Hh signaling is required for the maintenance of Tc-En. However, Tc-En expression in cells along the midline, presumably in the developing CNS (wild-type, Fig. 3i), is maintained in the RNAi embryos, which allowed us to follow segmentation in RNAi embryos. *Tc-hh*, *Tc-smo*, and *Tc-ci* RNAi embryos completed elongation more or less normally (Fig. 3a,d and g), but abnormalities became evident during retraction. The head lobes fail to mature, and there is no evidence of antennal Tc-En stripes. There is also a protuberance at the anterior end of the embryo that is likely to correspond to the anterior protuberance seen in the RNAi cuticles (Fig. 2d, h and l, and arrow in 3 h). In embryos that completed retraction, the unsegmented germbands are highly compacted, and Tc-En expression pattern is very irregular (Fig. 3c,e and h). Compared to wild-type germ bands at a similar stage, these germ bands occupy only a portion of the egg (Fig. 3j,k). In addition, loss of early Hh signaling at the ventral midline affected cell fate, as indicated by the loss of Tc-En expression here (Fig. 3b,e). Loss of Hh signal in *Drosophila* similarly affects En expression in midline cells (Bossing and Brand 2006). We also found that *Tc-wg* expression was initiated but not maintained in these embryos (data not shown), suggesting *Tc-wg* is a target of the Hh pathway.

hedgehog was first isolated in a screen for mutations that disrupt the *Drosophila* larval cuticle pattern and identified as one of the segment polarity genes with a 'lawn of denticles' phenotype (Nusslein-Volhard and Wieschaus 1980). In segment polarity mutants (e.g., *wg*, *hh*, and *smo*), deletion of a portion of the larval epidermis in each segment is accompanied by a mirror image duplication of the remaining structures. As a result, they contain the normal number of segments, but are smaller than wild-type due to partial deletion of each segment. In our study, we found *Tc-hh* and *Tc-smo* RNAi embryos to be smaller in size than the wild-type *Tribolium* larva (Fig. 2a,e), which may be due to cell death, fusion, or failure of cell division in segments during germ band retraction. The few random bristles produced in severely affected RNAi cuticles do not have any definable polarity suggesting that, unlike *Drosophila*, loss of function of these genes in *Tribolium* does

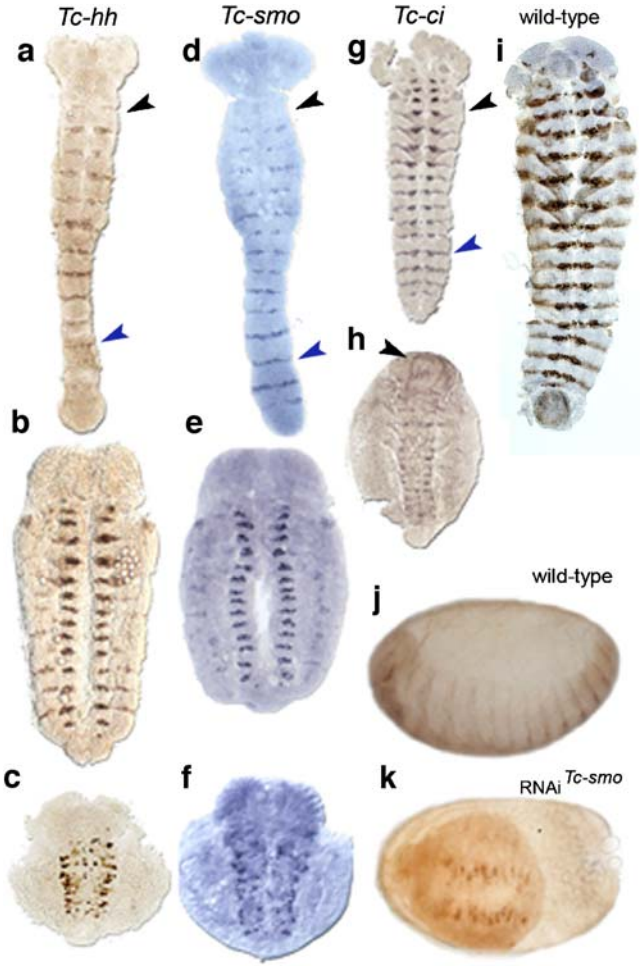

Fig. 3 En staining in wild-type and severely affected *Tc-hh*, *Tc-smo* and *Tc-ci* RNAi embryos during elongation and retraction. **a**–**c** *Tc-hh* RNAi; **d**–**f**, **k** *Tc-smo* RNAi; **g**, **h** *Tc-ci* RNAi; **i**, **j** wild-type. **a** Tc-En expression initiated normally in the posterior segments of an elongating *Tc-hh* RNAi germband embryo (*blue arrowhead*) but did not persist in older, more anterior segments (*black arrowhead*). **b** During germband retraction, patches of Tc-En expression are visible near the ventral midline, and laterally in the posterior segments. **c** In a highly compacted germ band with fused segments, Tc-En expression is disrupted along the ventral midline. **d** Expression of Tc-En in an elongating *Tc-smo* RNAi embryo initiated normally (*blue arrowhead*) but failed to persist in older, more anterior segments (*black arrowhead*). **e** During germband retraction, Tc-En is expressed in patches along the ventral midline, but has faded laterally. **f** In a highly compacted germ band, persists in a disrupted pattern along the ventral midline. **g** In an elongating *Tc-ci* RNAi embryo, similar to *Tc-hh* and *Tc-smo* RNAi, Tc-En initiated normally (*blue arrowhead*) but is not maintained (*black arrowhead*). **h** Tc-En expression at the ventral midline of a retracted germband embryo. Note the extended stomodeum (*arrowhead*) near the anterior end. **i** Tc-En expression in the CNS along the ventral midline and in the lateral ectoderm of each segment of a wild-type embryo during germband retraction. **j** Lateral view of a wild-type embryo inside the vitelline membrane at the end of germ band retraction. Tc-En expression persists laterally to the edge of the germband. **k** Ventral view of a severely affected *Tc-smo* RNAi embryo located near one end of the egg

not appear to produce mirror image duplications or affect polarity within the segments.

Analysis of *Tc-ptc* and overactivation of the Hh pathway

Transcripts of *Tc-ptc* are first detected in a broad domain in the posterior regions of the head lobes in the embryonic rudiment encompassing the antennal segments and the stomodeum, and at the posterior end of the embryo (Fig. 4a). Broad segmental stripes of *Tc-ptc* appear in an anterior to posterior progression during germband elongation (Fig. 4b). Expression fades in the middle of each initial stripe resulting in two narrow *Tc-ptc* stripes per segment (Fig. 4c). Double staining with Tc-En and *Tc-ptc* indicates that in each segment, one of the narrow *Tc-ptc* stripes marks the anterior boundary of a segment while the other is located immediately anterior to Tc-En-expressing cells (Fig. 4c and arrowhead in inset). This pattern persists even after germ band retraction; the anterior stripe appears stronger than the posterior stripe in each segment. *ptc* expression during segmentation in *Drosophila* is similarly dynamic (Nakano et al. 1989; Hidalgo and Ingham 1990).

As discussed above, Ptc is a negative regulator of the Hh-signaling pathway in *Drosophila*. Depletion of a negative regulator of the pathway would ectopically activate the pathway. To understand what happens when the Hh pathway is overactivated in *Tribolium*, we performed parental RNAi using two different amounts of *Tc-ptc* dsRNA (Table 1). The resulting cuticles (Fig. 4d,e,f) are very different from the ones described above for the other three genes. In mildly affected embryos (Class I), the head appendages are misshapen, while the legs are relatively normal (Fig. 4d). In the most severely affected embryos (Class II), all segments are present, but the head and thoracic appendages are enlarged and misshapen (Fig. 4e,f).

To understand the phenotype of the *Tc-ptc* RNAi embryos, we examined the expression of *Tc-wg* and Tc-En. In *Tc-ptc*-depleted embryos, segmental expression of Tc-En and *Tc-wg* is initiated normally (Fig. 4h), although the domains of *Tc-wg* expression in the head and at the posterior end of the embryo are expanded. Closer inspection revealed ectopic Tc-En expression at the ventral midline (Fig. 4k). In slightly older embryos, *Tc-wg* is expressed more intensely than normal in enlarged domains (Fig. 4i,l). Cells surrounding the expanded *Tc-wg* domains appear to be invaginating, as if beginning to form grooves. Closer inspection revealed a row of non-*Tc-wg*-expressing cells between the *Tc-wg* domain and the grooves (Fig. 4l). Tc-En is expressed in ectopic stripes that, in combination with the normal Tc-En stripes, would surround the expanded *Tc-wg* domains (Fig. 4j,m). In addition to the normal grooves that form posterior to the normal Tc-En stripe, ectopic grooves initiate anterior to the ectopic Tc-En

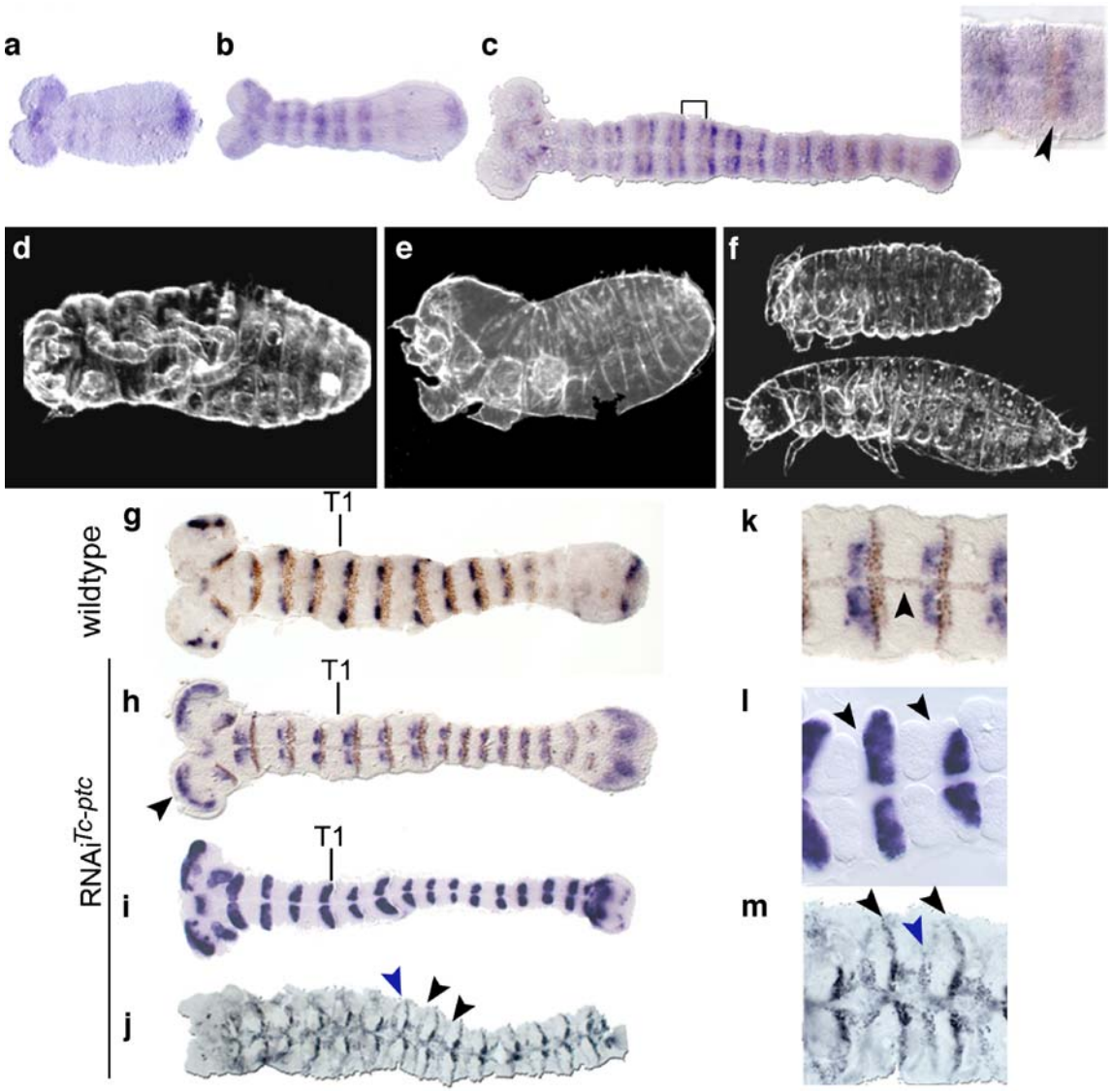

Fig. 4 Analysis of *Tc-ptc* in *Tribolium.* **a**–**c** *Tc-ptc* expression in wild-type embryos; **d**–**f** *Tc-ptc* RNAi cuticles; g wild-type; **h**–**m** *Tc-ptc* RNAi. **a** Expression of *Tc-ptc* in three gnathal stripes, in a broad posterior region of the head lobes and in the posterior region of an early germ band embryo. **b** Segmental stripes appear sequentially during elongation and resolve in double stripes. **c** Tc-En (*gold*) expression in the posterior compartment abuts, but does not overlap the double *Tc-ptc* stripes (extent denoted by *black lines*) in the anterior compartment of each segment. *Inset*: enlarged view of *Tc-ptc* stripes adjacent to a stripe of Tc-En (*arrowhead*). **d** A mildly affected Class I *Tc-ptc* RNAi cuticle with deformed legs and head appendages. **e** A more severely affected Class II cuticle with enlarged misshapen legs but a normal complement of abdominal segments. **f** Comparison of a Class II *Tc-ptc* RNAi cuticle and a wild-type cuticle at the same magnification. **g** Expression of Tc-En (*gold*) and *Tc-wg* (*purple*) in a wild-type embryo during elongation. **h** Segmental expression of Tc-En and *Tc-wg* initiated fairly normally in a *Tc-ptc* RNAi germband. *Tc-wg* expression domains in the head lobes and around the proctodeum are expanded. **i** Expanded expression of *Tc-wg* in a slightly older embryo. **j** Ectopic stripes of Tc-En expression (*blue arrowhead*) in a germband undergoing retraction. **k** Ectopic expression of Tc-En at the ventral midline (*arrowhead*) in a close up of the embryo in **h**. **l** Transient grooves (*arrowheads*) around expanded *Tc-wg* expression domains in a close up of the embryo in **i**. **m** Ectopic Tc-En stripes (*blue arrowhead*) between each set of normal Tc-En stripes (*black arrowhead*) in a close up of the embryo in **j**. T1, first thoracic segment

stripes. Thus, overactivation of the Hh-signaling pathway in *Tribolium* leads to overexpression of *Tc-wg*, and ectopic expression of Tc-En, which results in ectopic groove formation. In *Drosophila*, binding of Hh to Ptc receptor relieves repression of *wg* and allows expression of target genes. In *Drosophila ptc* mutants, ectopic induction of *wg* and En result in the formation of extra grooves (Nakano et al. 1989), suggesting that the functional role of Ptc is highly conserved between these two species. However, unlike *Drosophila*, the ectopic grooves in *Tribolium* are transient and it appears that a late regulatory action restores the normal number of segments in this insect.

Discussion

Expression patterns suggest that the function of the Hh-signaling pathway is conserved in several developmental pathways in *Tribolium*.

In *Tribolium*, the expression patterns of the four Hh-signaling pathway components we examined are highly similar to those of their *Drosophila* counterparts. In both *Drosophila* and *Tribolium,* the domains of *Tc-hh* are very similar to those of Tc-En. However, there are some significant differences in their expression dynamics. For example, the antennal Tc-En stripes appear after the three gnathal Tc-En stripes, whereas antennal *Tc-hh* stripes appear before the gnathal *Tc-hh* stripes. Each metameric stripe of *Tc-hh* is laterally continuous across the width of the germband after mesoderm invagination. Later, *Tc-hh* expression has disappeared at the ventral midline in anterior segments, while in posterior segments the stripes are still continuous. In contrast, Tc-En expression continues in the CNS after *Tc-hh* expression has faded there. *Tc-hh* expression in the stomodeum and at the posterior end of the blastoderm embryo in the absence of Tc-En suggests that, similar to *hh* in *Drosophila* (Lee et al. 1992; Mohler and Vani 1992; Tashiro et al. 1993), *Tc-hh* is involved in some En-independent processes in these regions.

In both *Drosophila* and *Tribolium*, expression of segment polarity genes is initiated by pair-rule genes (recently reviewed by Damen 2007). Soon thereafter, their expression is controlled by interactions between the segment polarity genes themselves. In *Drosophila*, Ptc is constitutively active unless or until Hh represses Ptc activity. In the absence of Hh, unbound Ptc keeps the pathway switched off. One of the targets of the Hh pathway is *ptc* itself. In cells where its activity is antagonized upon binding of Hh, *ptc* continues to be expressed, but *ptc* expression disappears in cells that do not receive the signal. Thus, an initially broad domain of *ptc* expression resolves into two narrow stripes flanking the En expression domain. *Tc-ptc* expression pattern is similar: each broad stripe splits into two, such that each En stripe is bracketed by two *Tc-ptc* stripes, suggesting that *Tc-ptc* itself might also be a target of the Hh pathway in *Tribolium*.

The expression patterns of Hh pathway component genes are highly conserved between *Drosophila* and *Tribolium*, except for that of *Tc-ci* (Fig. 1l,m). In *Drosophila*, *ci* transcripts are initially expressed uniformly in the early cellular blastoderm and persist until the end of germ band elongation. At that point, *ci* expression is directly repressed by En in cells of the posterior compartment in each segment. In *Tribolium*, expression of *Tc-ci* is somewhat different in that there is a narrow region of two to three cells between the *Tc-ci* and En-expressing cells that does not express *Tc-ci*. In *Tribolium*, the absence of *Tc-ci* transcripts in cells just anterior to En-expressing cells might suggest the existence of an En-independent mechanism regulating *Tc-ci* expression. Alternatively, it is possible that *Tc-ci* transcripts turn over rapidly in these cells, but the expression pattern is conserved at the protein level. In *Drosophila*, *ci* is regulated post-transcriptionally. At stage 11, *ci* transcripts are localized throughout the anterior compartment of each segment whereas protein levels are lower at the center of each transcriptional stripe and higher in cells that bracket the En-expressing cells (Motzny and Holmgren 1995; Slusarski et al. 1995) much like what we describe here for *ptc* transcription in *Tribolium* and previously for Ptc protein levels in *Drosophila*. It will be interesting to see if *Tc-ci* is also post-transcriptionally regulated in *Tribolium*.

Functional analysis of Hh pathway component genes supports conserved roles in segment boundary formation.

In *Drosophila*, mutations in genes encoding positive regulators of the Hh pathway including *hh*, *smo*, and *ci* produce smaller than wild-type embryos with asegmental phenotypes (Nusslein-Volhard and Wieschaus 1980). In *Tribolium*, *Tc-hh*, and *Tc-smo* RNAi embryonic phenotypes are nearly identical; both produce highly compacted spherical cuticles with no evidence of appendages or segmental grooves. The most severe *Tc-ci* RNAi phenotypes are not as severe as those of *Tc-hh* or *Tc-smo* RNAi. Interestingly, the *ci94* allele in *Drosophila* is a null allele (Slusarski et al. 1995; Methot and Basler 1999) and these mutant embryos differ considerably from *hh* mutants. *hh* mutants are much shorter in length and have a continuous 'lawn of denticles' phenotype whereas *ci94* mutants are almost normal in size and have alternating naked cuticle and denticle belts on the ventral surface. Target genes of *hh* are partially derepressed in the absence of *ci*, producing a *ci* phenotype that is milder than that of *hh* (Methot and Basler 2001). An analogous situation has been described for the Wingless signaling pathway in *Drosophila* where derepression of target genes in the absence of *pangolin* results in a milder segment polarity phenotype compared to that of *wg* null mutants (Cavallo et al. 1998; Waltzer and Bienz 1998). In *hh* and *smo* mutants, only the repressor function of *ci* remains, producing the catastrophic phenotype. In contrast, loss of both the activating and repressor forms results in the milder phenotype seen in *ci* null mutants. In *Tribolium*, the most severe *Tc-ci* RNAi phenotype is milder than the most severe *Tc-hh* RNAi phenotype, suggesting similar regulation of the Hh pathway in the beetle.

In *Drosophila*, loss of Wg or Hh-signaling results in larvae that are smaller than wild-type due, at least in part, to epidermal cell death during and after germband retraction (Martinez-Arias and Lawrence 1985) or a combination of

transformation and cell death (Klingensmith et al. 1989). In *Tribolium*, *hh*, *smo*, and *ci* RNAi individuals are greatly reduced in size compared to the wild-type (Fig. 2). These embryos go through the events of early embryogenesis normally, producing the full complement of segments and initiating Tc-En and *Tc-wg* expression. Tc-En and *Tc-wg* expression fade and the segmental remnants become highly compacted along the anterior–posterior axis during retraction. *Tribolium* embryos lacking *Tc-wg* also elongate normally but fail to maintain En expression and form shorter than wild-type embryos during retraction (Ober and Jockusch 2006). While it is likely that cell proliferation and programmed cell death both contribute to shaping the embryo in *Tribolium*, during normal development cells divide randomly throughout the elongating germ band (Brown et al. 1994); organized patterns of cell division or cell death have not been reported. Similarly, it is likely that excessive cell death or the lack of cell proliferation contributes to the severely compacted terminal phenotype of *Tc-hh*, *Tc-smo* or *Tc-ci* RNAi embryos, but closer examination will be required to determine if this is so.

In *Drosophila ptc* mutants, deletion of the midregion of each segment is accompanied by a mirror image duplication of the remaining denticles (Nusslein-Volhard and Wieschaus 1980). In contrast, *Tribolium Tc-ptc* RNAi embryos, which also display the correct number of segments, are characterized by distended gnathal and thoracic appendages. Although there are some random bristles, we could not identify any noteworthy difference in the polarity of these bristles that could be attributed to a characteristic loss of function phenotype for genes belonging to this class. This suggests that unlike *Drosophila*, the loss of segment polarity gene function in *Tribolium* does not result in any morphologically identifiable polarity defect in the cuticle.

In the absence of *ptc* in *Drosophila* (DiNardo et al. 1988) and *Tribolium,* En expression is established properly; but later, de novo En stripes appear between the normal stripes. Ectopic expression of En in *Drosophila* is not due to regulation of *ptc* by pair-rule factors (DiNardo et al. 1988). In the absence of *ptc*, the *wg* expression domain broadens anteriorly. Expanded *wg* expression induces ectopic En stripes, which cause ectopic groove formation, suggesting that the principal function of Ptc is to repress *wg*. Similar expression of *Tc-ptc* in *Tribolium*, considered with the expanded *Tc-wg* domains that are surrounded by ectopic *Tc-En*-expressing cells and the ectopic grooves transiently formed in *Tc-ptc* RNAi embryos suggest that the role of *Tc-ptc* is likely to be functionally conserved between *Drosophila* and *Tribolium*. However, in *Tc-ptc* RNAi embryos, ectopic Tc-En is also detected along the ventral midline, a phenotype that has not been described for *Drosophila ptc* mutants. *hh* RNAi has been attempted in the orthopteran *Gryllus bimaculatus* (Miyawaki et al. 2004). Unfortunately, this organism seems to be resistant to *hh* dsRNA. In the RNAi embryos, the level of *hh* is not reduced. The embryos develop normally and hatched larvae show no cuticular defects. Lack of *ptc* analysis in other insects makes it difficult to speculate as to whether this novel expression of Tc-En along the ventral midline is specific to *Tribolium*, or a general feature related to short germ development. Finally, although ectopic grooves appear to form around the expanded *Tc-wg* domains, they are not detected in the terminal cuticles, implying that events late in embryogenesis restore the normal number of segmental grooves.

Conservation of the *hh–wg–en* gene circuit in short germ segmentation

Several lines of evidence suggest that the segment polarity gene circuit, in which the expression of the *hh*, *wg*, and *en* genes are dependent upon one another in the long germ mode of segmentation elucidated in *Drosophila*, is conserved in the short germ mode of segmentation found in *Tribolium*. As in *Drosophila*, in the absence of *smo*, *hh*, *ci* (this paper), or *wg* (Ober and Jockusch 2006) in *Tribolium*, En expression is not maintained. In addition, *Tc-wg* mRNA fails to persist in *Tc-hh*, *Tc-smo and Tc-ci* RNAi embryos (data not shown). Furthermore, expression patterns and RNAi phenotypes of the Hh pathway components we examined (*Tc-hh*, *Tc-smo*, *Tc-ci*, and *Tc-ptc*) suggest that the regulation of the Hh pathway is also conserved. Segment polarity genes, which function last in the segmentation gene hierarchy, are expressed in segmental fields that have been predefined by genes at higher levels (gap and pair rule). Conservation of the segment polarity gene circuit in *Tribolium* suggests that that segment polarity genes form a robust regulatory module in the short germ mode of segmentation in this beetle and their expression patterns in numerous insects and chilicerates suggest that this module is likely to be conserved among the Insecta, and perhaps the Arthropoda.

While the expression patterns of segment polarity orthologs are highly conserved, the expression patterns of pair-rule gene orthologs vary greatly among insects and other arthropods (recently reviewed in Tautz 2004; Peel et al. 2005; Damen 2007). Functional analysis in *Tribolium* indicates that interactions among pair-rule gene homologs differ from those of their *Drosophila* counterparts (Choe et al. 2006) and that some secondary pair-rule genes function in opposite parasegmental registers in *Drosophila* and *Tribolium* (Choe and Brown 2006). These findings suggest that the inputs from pair-rule genes to the segment polarity gene module are likely to be quite different in each of these insects. Functional analysis of how pair-rule genes regulate the alternating expression of *en* and *wg* in *Tribolium* will

provide insight into these differences, which will ultimately help us understand the evolution of genetic regulatory networks. Interestingly, the segment polarity gene module seems to be resilient enough to withstand such evolutionary changes in input from the pair-rule gene module.

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