Developmental Biology 345 (2010) 80–93

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Evolution of Developmental Control Mechanisms

Evolution of extracellular Dpp modulators in insects: The roles of *tolloid* and *twisted-gastrulation* in dorsoventral patterning of the *Tribolium* embryo

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ARTICLE INFO

Article history:
Received for publication 23 January 2010
Revised 9 May 2010
Accepted 12 May 2010
Available online 26 May 2010

ABSTRACT

The formation of the BMP gradient which patterns the DV axis in flies and vertebrates requires several extracellular modulators like the inhibitory protein Sog/Chordin, the metalloprotease Tolloid (Tld), which cleaves Sog/Chordin, and the CR domain protein Twisted gastrulation (Tsg). While flies and vertebrates have only one *sog/chordin* gene they possess several paralogues of *tld* and *tsg*. A simpler and probably ancestral situation is observed in the short-germ beetle *Tribolium castaneum* (*Tc*), which possesses only one *tld* and one *tsg* gene. Here we show that in *T. castaneum tld* is required for early BMP signalling except in the head region and *Tc-tld* function is, as expected, dependent on *Tc-sog*. In contrast, *Tc-tsg* is required for all aspects of early BMP signalling and acts in a *Tc-sog*-independent manner. For comparison with *Drosophila melanogaster* we constructed fly embryos lacking all early Tsg activity (tsg;;srw double mutants) and show that they still establish a BMP signalling gradient. Thus, our results suggest that the role of Tsg proteins for BMP gradient formation has changed during insect evolution.

Introduction

Morphogenetic gradients are essential to provide positional information during embryogenesis. Among the best studied morphogens are bone morphogenetic proteins (BMPs), which are involved in dorsoventral (DV) patterning in flies and vertebrates (reviewed in De Robertis and Kuroda, 2004; Little and Mullins, 2006; O'Connor et al., 2006). In *Drosophila melanogaster*, a BMP gradient with peak levels at the dorsal midline specifies a sequence of cell fates along the DV axis. High BMP signalling levels induce the dorsalmost tissue, the extraembryonic amnioserosa, lower levels the dorsal epidermis and very low levels or absence of signalling are required to allow the development of the central nervous (Dorfman and Shilo, 2001; Ferguson and Anderson, 1992a,b; Mizutani et al., 2006; Sutherland et al., 2003). Similarly, a ventral-to-dorsal BMP activity gradient patterns the DV axis in vertebrates. Like in flies high levels of signalling correspond to the epidermis and low levels or absence to the central nervous system (reviewed in De Robertis and Kuroda, 2004; Little and Mullins, 2006).

In *D. melanogaster* and vertebrates the graded distribution of BMPs depends on the action of at least three conserved extracellular modulators, the BMP inhibitor Short-gastrulation (Sog, Chordin in vertebrates), the metalloprotease Tolloid (Tld) (BMP1 or Xolloid in vertebrates) and Twisted-gastrulation (Tsg) with a less defined biochemical function (reviewed in Little and Mullins, 2006). The spatially restricted expression of Sog/Chordin is crucial for BMP gradient formation. In *D. melanogaster*, *sog* is expressed in ventrolateral regions of the embryo, where Sog protein is secreted and diffuses to the dorsal side (Srinivasan et al., 2002). Sog forms complexes with two BMPs, Decapentaplegic (Dpp) and Screw (Scw), which prevents the interaction with their cell surface receptors and, at the same time, transports them to the dorsal side. Here, Sog is cleaved by Tolloid, the BMPs are released and activate their receptors (Eldar et al., 2002; Mizutani et al., 2005; Shimmi et al., 2005b; Wang and Ferguson, 2005; reviewed in O'Connor et al., 2006). Similarly, in vertebrates the DV gradient of BMP signalling depends on the formation of an opposite gradient of the Sog ortholog Chordin, which by complex formation with at least two different BMPs (BMP2/4 and BMP7) prevents signalling. Cleavage of Chordin by Tolloid homologues (BMP1, Xolloid) is required to release the active ligands (De Robertis and Kuroda, 2004).

While the roles of Sog/Chordin and Tld in BMP gradient formation are fairly well understood, the function of the third conserved extracellular modulator, Tsg, is still controversial (Oelgeschlager et al., 2000; Ross et al., 2001). In vertebrates experimental evidence has been presented for both pro- and for anti-BMP functions of *tsg* (for review see Little and Mullins, 2006). In zebrafish and *Xenopus* two *tsg* genes have been identified (Oelgeschlager et al., 2004), that might act

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doi:10.1016/j.ydbio.2010.05.019

redundantly. In flies three Tsg-like proteins exist, displaying a clear pro-BMP function. In the early fly embryo, *tsg* (*tsg1*) and the related gene *shrew* (*tsg3*) are each required to generate high levels of BMP signalling at the dorsal side (Bonds et al., 2007; Ross et al., 2001). Intermediate signalling levels are still attained in the absence of *tsg1* or *shrew*. So far it has not been investigated whether the simultaneous loss of both *tsg1* and *shrew* leads to a more severe reduction in BMP signalling. While *tsg1* and *shrew* are specific for the early embryo later processes in which Dpp is involved either are independent of Tsg proteins or use another *tsg* paralogue, *crossveinless 1* (*cv1*, also called *tsg2*), (Shimmi et al., 2005a; Vilmos et al., 2005 reviewed in O'Connor et al., 2006).

Given the complex picture which emerges for the network of BMP signalling components in vertebrates and flies, we have addressed the question whether an organism with a less derived mode of embryogenesis might have a simpler network architecture and therefore be more suited to unravel the basic interactions of the components. Previously, we showed that the red flour beetle *Tribolium castaneum*, which has a type of embryogenesis more typical for basal insects (short-germ type), uses Sog-mediated BMP transport to establish all ectodermal DV polarity (van der Zee et al., 2006). The system is different from that of *D. melanogaster* in at least two respects. First, only one ligand, Tc-Dpp, appears to be involved in early DV patterning (Van der Zee et al., 2008; van der Zee et al., 2006; Weber, 2006, RNdF, MvdZ and SR, unpublished results). *Tc-dpp* is evenly expressed along the DV axis in early embryos (van der Zee et al., 2006), in contrast to *D. melanogaster* where *dpp* is ventrally repressed by maternal and zygotic transcription factors (Jazwinska et al., 1999). Thus in *D. melanogaster* even in the absence of *sog* the ectoderm is patterned along the DV axis, while in *T. castaneum* all ectodermal DV polarity depends on ventral *sog* expression. Similar results with the spider *Achaearanea tepidariorum* indicate that this mode of DV patterning is ancestral for arthropods (Akiyama-Oda and Oda, 2006).

Second, the formation and refinement of the embryonic BMP activity gradient is different between *D. melanogaster* and *T. castaneum*. In flies, where all segments are specified at the same time, a broad region of dorsal BMP activation quickly refines into a narrow zone of high signalling activity straddling the dorsal midline (Dorfman and Shilo, 2001; Mizutani et al., 2005; Sutherland et al., 2003, Fig. 1B,C). This refinement process involves a positive feedback loop in which the *zerknüllt* (*zen*) gene participates to enhance ligand binding as a function of previous signalling strength (Wang and Ferguson, 2005). The refinement occurs evenly along the AP axis and leads to a bistable distribution of BMP signalling with very high levels in dorsalmost and low levels in dorsolateral regions (reviewed in O'Connor et al., 2006). In *T. castaneum* a flat gradient of BMP activity is established in the dorsal half of the blastoderm embryo which, however, does not refine to a narrow zone of high activity at the dorsal midline (Fig. 1G,K; van der Zee et al., 2005). In contrast to *Drosophila*, *Tc-zen* is not involved in Tc-Dpp gradient formation as *Tc-zen1* knockdown embryos show normal DV patterning (van der Zee et al., 2005). In addition, during early blastoderm stages BMP activity is restricted to the anterior-dorsal region, the prospective dorsal serosa (Fig. 1D,E,F,G—undifferentiated blastoderm). One to two hours later, BMP activity extends towards a dorsal-posterior region (Fig. 1H,I,J,K—differentiated blastoderm). Thus, in *T. castaneum* Dpp activity (pMad) does not only change along the DV axis, but also along the AP axis (Fig. 1F,J and cross sections in Fig. 1G,K). Given these differences between *D. melanogaster* and *T. castaneum*, we wondered how the extracellular modulators of BMP gradient formation, *tsg* and *tld*, act in *T. castaneum* embryos.

We uncovered several aspects where early DV patterning in *T. castaneum* is different, and probably simpler, than in *D. melanogaster*. First, the *T. castaneum* genome contains only one representative each of *tld* and *tsg* in contrast to several paralogues, which act during DV patterning in *D. melanogaster*. Second, phylogenetic analyses indicate that *T. castaneum tld* and *tsg* are related to *D. melanogaster tolkin* (*tok*) and *crossveinless1* (*cv1*/*tsg2*), respectively, which act mainly during later developmental processes in flies. The paralogues involved in *D. melanogaster* early embryonic patterning (*Dm-tld*, *Dm-tsg1* and *Dm-srw*) are derived forms. Despite their greater similarity in sequence to *Dm-tok* and *Dm-cv1* (*Dm-tsg2*), respectively, *Tc-tld* and *Tc-tsg* are required for early Dpp signalling during *T. castaneum* embryogenesis. Third, loss of *Tc-tsg* completely abolishes Dpp activity, suggesting an essential pro-BMP function that is independent of *Tc-sog*, as shown by double knock-down experiments. This major requirement of *Tc-tsg* for Dpp signalling in *T. castaneum* led us to analyse *D. melanogaster* embryos completely lacking *tsg* activity during early embryogenesis (*tsg*;;*srw* double mutants). Such embryos still show local Dpp signalling, pointing to different roles of Tsg proteins for Dpp signalling in both insects. Altogether our results suggest that the network involved in the regulation of the early BMP/Dpp gradient has been modified from a simple system with fewer modulators as found in *T. castaneum* to a complex system as seen during *D. melanogaster* early DV patterning.

Methods

Cloning of Tc-tsg and Tc-tld and phylogenetic analyses

A survey for possible *tsg* and *tld* orthologous sequences was performed in the recently published *Tribolium castaneum* genome (Richards et al., 2008). Primer design, RNA extraction, cDNA synthesis and RT-PCR were carried out using standard protocols. To obtain the complete mRNA sequences, 3′- and 5′-RACE reactions were done using the SMART RACE cDNA Amplification Kit (Clontech) following the manufacturer instructions. *Tc-tsg* corresponds to the GLEAN gene model Tc-03620 (NCBI accession number—XM_001812724) and *Tc-tld* to Tc-11197 (NCBI accession number—XM_965069.2). Phylogenetic analyses were performed as previously described (Van der Zee et al., 2008) and the Gene bank accession numbers can be found in the Supplemental data. Alignment files are available upon request. Specific motifs of Tolloid and Tsg-like proteins are graphically represented with the help of Weblogo (Crooks et al., 2004, shown in Supplemental data).

In situ hybridisation, immunohistochemistry

T. castaneum eggs were fixed as previously described (Chen et al., 2000). The one-colour *in situ* hybridisation protocol for *T. castaneum* was essentially the one described by Tautz and Pfeifle, 1989 without the proteinase K treatment step. For two-colour *in situ* hybridisation the method described by Liu and Kaufman, 2004 in *Oncopeltus fasciatus* was adapted for *T. castaneum*. Immunohistochemistry and fluorescent antibody stainings were done essentially as previously described (Nunes da Fonseca et al., 2008; Roth et al., 1989).

dsRNA synthesis and beetle injections

PCR fragments containing T7 promoter initiation sites on both sides were used as templates for dsRNA synthesis with the Ambion Megascript Kit. Two non-overlapping dsRNA constructs for each gene (*Tc-tsg* and *Tc-tld*) were used for injections and led to identical phenotypes (details available upon request). For simultaneous injection of two different dsRNA, primers leading to similar size amplicons were designed and used as templates for dsRNA synthesis reaction. A mixture of two dsRNA using equal concentrations (1 μg/μl) was injected as previously described into pupae (Bucher et al., 2002) or adults (van der Zee et al., 2005). Only embryos obtained from the two first egg lays (3 days—30 ºC) were analysed during double-injection experiments. The efficiency of the double knock-down was at least 80% in all double-injection experiments (*Tc-dpp*/*Tc-sog*, *Tc-*

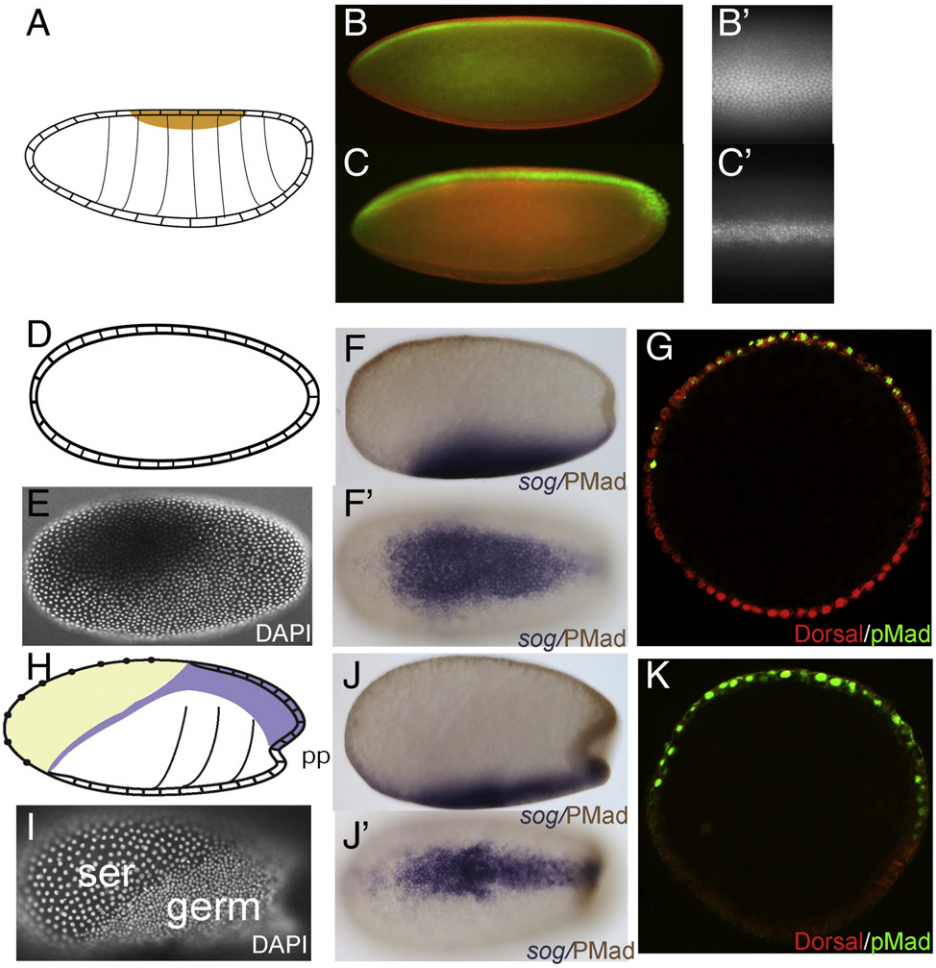

Fig. 1. In *Drosophila melanogaster*, Dpp activity (pMad) narrows over time along the DV axis, whereas pMad appears in an AP progression in *Tribolium castaneum*. (A) Scheme of a *Drosophila melanogaster* embryo, lateral view. The lines represent seven double segments, corresponding to 14 segment anlagen, which are simultaneously established. The orange area represents the amnioserosa. (B,C) Phalloidin (red) highlighting cell outlines and Dpp activity (green) during beginning of cellularisation (B) and shortly before gastrulation (C). (B′,C′) Dorsal views from the middle region (white box) of the same embryos showed in B and C. Dpp activity narrows from an early broad gradient (B′) towards a 5–6 cell wide domain of high activity as observed in C. In *D. melanogaster* Dpp activity (pMad) is constant along the AP axis. (D,H) Schematic drawings of *Tribolium castaneum* development at undifferentiated blastoderm stage (D) and differentiated blastoderm stage, shortly before gastrulation (H). In H serosal cells are represented in yellow and amniotic cells in purple; and the primitive pit (pp) is indicated. Oblique lines represent three double segments (six segment anlagen) which are specified before gastrulation. (E,I) Nuclear staining (DAPI) at undifferentiated blastoderm stage (E), where all nuclei appear identical, and at differentiated blastoderm stage (I), where the larger serosa nuclei (ser) can be distinguished from the smaller nuclei of the germ rudiment (germ). (F,F′,J;J′) Head to the left. *In situ* hybridisation for *Tc-sog* (blue) and antibody staining the phosphorylated form of Mad (pMAD-brown). (F,J) Lateral views. (F) At undifferentiated blastoderm stage, pMad (Dpp activity) is detected in the dorsal serosa. (J) At differentiated blastoderm stage, pMad is detected along the whole AP axis in the dorsal region of the embryo. (F′J′) Ventral views. In blue *Tc-sog* expression domain, this extends laterally beyond the mesodermal border at undifferentiated blastodermal stage (F′), but shrinks to the mesoderm shortly before gastrulation (J′). (G,K) Cross sections showing Dorsal protein (red) and pMad (Dpp activity—green). While the nuclear Dorsal gradient is present at uniform blastoderm stage (E′), it disappears during blastoderm differentiation (F′). In contrast, pMad DV extent does not refine to a dorsal narrow stripe as described above for *D. melanogaster*.

sog/*Tc-tsg*, *Tc-sog*/*Tc-tld*) as judged by *in situ* hybridisations performed in parallel using WT embryos at similar embryonic stages.

Drosophila melanogaster stocks

The following fly stocks were used in our studies: tsg^{XB}/*FM7 ftz-lacZ*, srw^{10K28}/*TM3 hb-lacZ*, srw^{10K28}/*TM3 ftz-lacZ*, tsg^{B117}/*FM7 ftz-lacZ*;; srw^{10K28}/*TM3 hb-lacZ*, tsg^{B117}/*FM7 ftz-lacZ*;; srw^{10K28}/*TM3 ftz-lacZ*, tld^{B4}/*TM3 hb-lacZ*, dpp^{H46}/*CyO23 P[dpp^{+}]*.

Results

The Tribolium castaneum genome contains only one tld and one tsg gene

Tolloid-like (Tld) proteins contain one metalloprotease, two EGF-like and five CUB domains, resembling the BMP1 protein in vertebrates (Scott et al., 1999; Shimell et al., 1991, Figure S1). Drosophilids possess two *tld* genes: *tld* and, *tolkin* (*tok* or *tolloid-related*) which map close to each other in the genome. In *Drosophila melanogaster*, Tld is the major factor required for Sog cleavage during embryonic DV patterning (Marques et al., 1997), while Tok has a minor function in the same process (Srinivasan et al., 2002). In contrast, only Tok is required for Sog cleavage during wing formation (Finelli et al., 1995, Serpe et al., 2005). Both genes are adapted to their respective functions in Sog processing and cannot substitute for each other (Serpe et al., 2005). In addition *tok* has been shown to cleave and thereby activate the activin-like ligand Dawdle in *D. melanogaster* (Parker et al., 2006; Serpe and O'Connor, 2006). Also, in vertebrates Tolloid-like genes regulate the activity of TGF-ß ligands both by cleaving the Sog homologue Chordin and by processing the N-terminal pro-domains of ligands (for review see Hopkins et al., 2007). Thus, Tok might be functionally more similar to vertebrate Tolloids. Only one Tolloid gene is found in syntenic chromosomal regions of *Anopheles gambiae*, *Apis mellifera* and *Tribolium castaneum* (data not shown, available at Ensembl browser). Phylogenetic analyses (Fig. 2A) and specific amino acid signatures (Figure S1) suggest that the unique

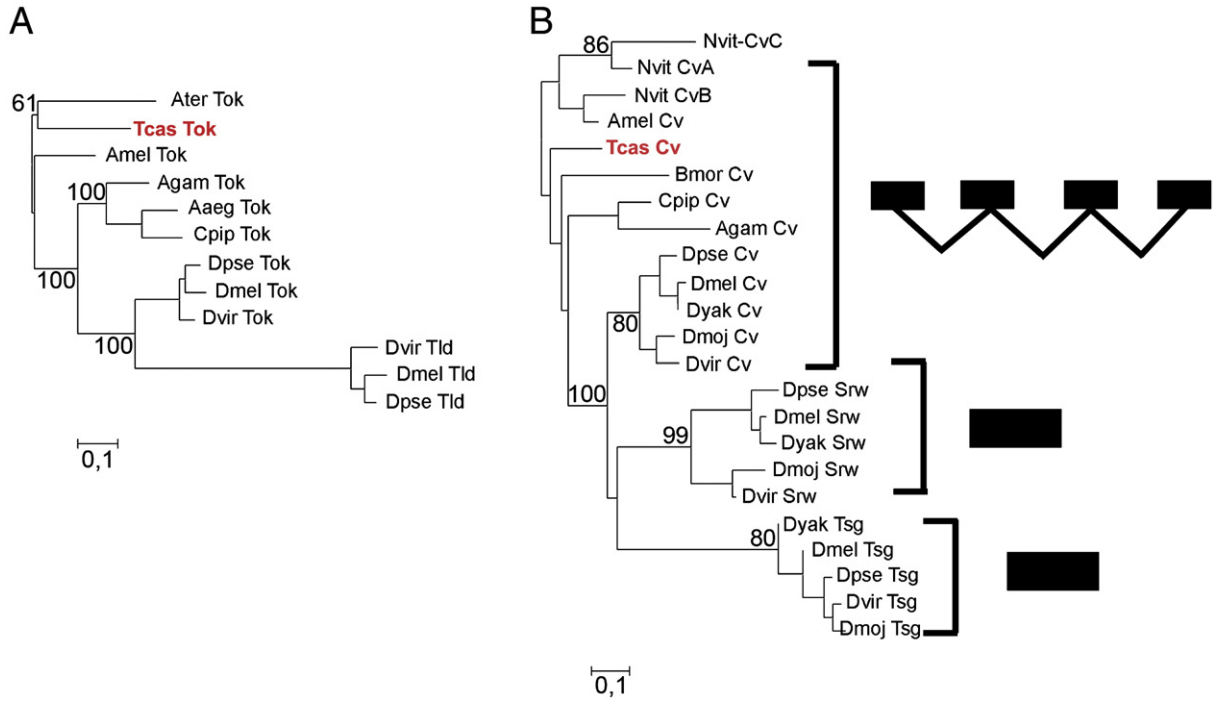

Fig. 2. Tld and Tsg proteins involved in early embryonic patterning arose via gene duplication in the lineage giving rise to higher Diptera. (A) Maximum likelihood tree of all retrievable arthropod Tolloid-like proteins. *tolloid* (*tld*) probably arose by a duplication event from *tolkin* (*tok*), since only the latter molecule is found in all non-Drosophilid arthropods. The *T. castaneum* Tok protein (Tcas Tok) is displayed in red. Amino acid substitution model: WAG + i + g. (B) Maximum likelihood tree of insect Twisted-gastrulation (Tsg) proteins. *srw* and *tsg* probably have arisen from a *cv1* gene which is present in all insect genomes sequenced so far. The intron–exon structure (black boxes) of *cv1* genes is different from *srw* and *tsg*, which are intronless genes (unique black box). The *T. castaneum* Cv protein (Tcas Cv) is displayed in red. Amino acid substitution model: JTT + i + g. Species names used in the phylogenetic trees. Tcas = *Tribolium castaneum*; Amel = *Apis mellifera*; Cpip = *Culex pipiens*; Aaeg = *Aedes aegypti*; Agam = *Anopheles gambiae*; Dvir = *Drosophila virilis*; Dmel = *Drosophila melanogaster*; Dpse = *Drosophila pseudoobscura*; Dyak = *Drosophila yakuba*; Dmoj = *Drosophila mojavensis*; Nvit = *Nasonia vitripennis*; Bmor = *Bombyx mori*; Ater = *Achaearanea tepidariorum*; Dpse = *D. pseudoobscura*.

tld gene found in the genome of *T. castaneum* is more closely related to *tok* than to *tld*.

Drosophilids possess three *tsg*-like genes: *tsg1*, *shrew* (*tsg3*) and *crossveinless 1* (*cv1* or *tsg2*), while only one *tsg* gene was found in the *A. gambiae*, *A. mellifera* and *T. castaneum* genomes. In *D. melanogaster* *tsg1* and *shrew* are small, intronless genes required for DV patterning in the embryo, while *cv1* is required for wing venation (crossvein formation) and is dispensable for embryonic patterning (Bonds et al., 2007; Shimmi et al., 2005a; Vilmos et al., 2005). Interestingly, the single *T. castaneum tsg* gene and its homologues in *Apis mellifera* and *A. gambiae* are closely related to *cv1* from Drosophilids (Fig. 2B). This conclusion is corroborated by specific amino acid signatures found in Cv1 and the Tsg proteins of *T. castaneum*, *A. mellifera* and *A. gambiae*, as depicted for the second cysteine rich region (CR) (Figure S2 and Vilmos et al., 2005) and by a common intron–exon organisation (Fig. 2B). Based on these analyses, we infer that the unique *tsg* gene in the *T. castaneum* is a representative of the ancestral version of *tsg* in insects and that this version is retained in Drosophilids as *cv1* (*tsg2*), while *tsg1* and *shrew* (*tsg3*), which act in the early embryo, have independently arisen from a *cv1*-like ancestral gene (Fig. 2B).

Taken together, both in the case of *tld* and of *tsg* Drosophilids, and maybe higher dipterans, possess lineage specific paralogues which are dedicated to early embryonic patterning while those paralogues acting mainly during later development have retained ancestral characteristics. The question thus arises whether the *T. castaneum* genes resembling the *D. melanogaster* genes acting during later development possess early embryonic functions.

Tc-tld and Tc-tsg expression suggest a role in early embryo patterning

Tc-tld transcripts are first visible at the undifferentiated blastoderm stage (Fig. 3A, A′ for DAPI), where they are broadly detected in almost all cells, with the exception of the egg poles. At the same stage, *Tc-sog* transcripts are broadly detected ventrally (Fig. 3A″). During the differentiated blastoderm stage (Fig. 1H,I), *Tc-tld* is observed in the presumptive germ rudiment with highest levels in a broad anterior domain (Fig. 3B, B′ for DAPI). At this stage expression is uniform along the DV axis. In *D. melanogaster*, *Dm-tld* (like *Dm-dpp* and *Dm-zen*) is repressed at the ventral side of the blastoderm embryo by the NF-κB transcription factor Dorsal (Kirov et al., 1994; Shimell et al., 1991). Although nuclear Tc-Dorsal is present at the ventral side of the *T. castaneum* embryo during early differentiated blastoderm stages, it appears not to repress *Tc-tld*. Similar results were obtained for *Tc-dpp* and *Tc-zen* (Chen et al., 2000) and together support the idea that Tc-Dorsal lacks a repressor function. During further development the germ rudiment condenses to the ventral side; *Tc-tld* is up-regulated in presumptive ectoderm and repressed in the presumptive mesoderm (Fig. 3C). Thus, the *Tc-tld* pattern is complementary to that of *Tc-sog*, *Tc-twist* and *Tc-snail* (*Tc-sna*) which at this stage are largely confined to the mesoderm (Handel et al., 2005; van der Zee et al., 2006 and see Fig. 3D for a double-staining *Tc-twi*/*Tc-tld*).

During gastrulation, *Tc-tld* remains expressed broadly in the ectoderm and repressed in the mesoderm (Fig. 3E, arrow). This is different from *D. melanogaster*, where *tld* expression is confined to the non-neurogenic ectoderm (Shimell et al., 1991), and similar to *A. gambiae*, where *tld* also has broad ectodermal expression (Goltsev et al., 2007). During germ band extension *Tc-tld* is up-regulated in the posterior growth-zone from which the abdominal segments are formed (data not shown). In the mature anterior segments *Tc-tld* expression is found in the CNS, close to the ventral midline (data not shown) in a pattern reminiscent of *Dm-tok* CNS expression in *D. melanogaster* (Finelli et al., 1995). A *Tc-tld* expression domain between the head lobes might represent the presumptive stomodeum (Fig. 3F, arrow).

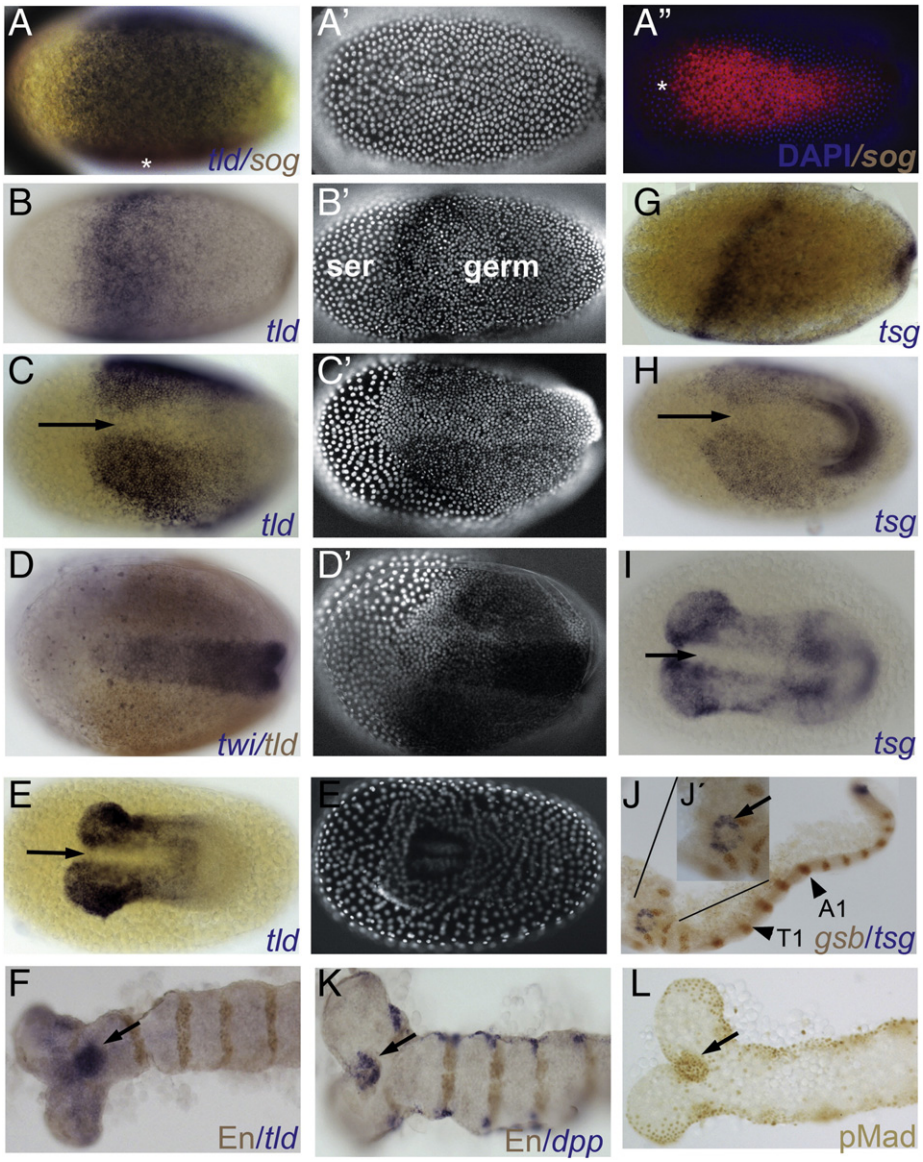

Fig. 3. *Tc-tld* and *Tc-tsg* expression suggest a role in early embryo patterning. (A–F) *Tc-tld* (G–J) *Tc-tsg* expression patterns during embryogenesis. (K) *In situ* for *Tc-dpp* plus antibody staining against Engrailed (En). (L) Dpp activity (pMad antibody staining). A,A′,G,J are lateral views; the remaining panels are ventral views. Anterior points to the left in all panels. A′,B′,C′,D′,E′ are DAPI stainings of A,B,C,D,E respectively. (A) During undifferentiated blastoderm stage (see A′-DAPI), *Tc-tld* is expressed in a broad domain and excluded from the egg poles, while *Tc-sog* is detected ventrally. Ventral view of the same embryo in A″ shows *Tc-sog* expression (red) and DAPI nuclear staining (blue). (B) *Tc-tld* and (G) *Tc-tsg* expression during blastoderm differentiation. (C) *Tc-tld* and (H) *Tc-tsg* expression during early gastrulation. Arrow denotes the absence of transcripts of both genes in the mesoderm. (D) A double *in situ* hybridisation using the mesodermal marker *Tc-twi* (blue) and *Tc-tld* (orange). (E, E′,I) During the serosal window stage (Handel et al., 2000), both transcripts are detected in the ectoderm, but are absent from the mesoderm (arrow). (F,K,L) Anterior part of embryos during germ band elongation. Arrow points to the putative stomodeum. (F) *Tc-tld in situ* hybridisation (blue) and anti-Engrailed antibody staining (brown, segmental marker). (K) *Tc-dpp in situ* hybridisation (blue) and anti-Engrailed antibody (brown, segmental marker). (L) pMad (Dpp activity). (J,J′) Double *in situ* hybridisation of *Tc-tsg* (blue) and *Tc-gsb* (segmental marker, red) during germ band elongation. (J′) Arrow points to *Tc-tsg* expression in the putative stomodeum.

Early *Tc-tsg* expression during undifferentiated blastoderm stage is uniform (data not shown). During blastoderm differentiation, *Tc-tsg* expression becomes restricted to the germ rudiment with higher levels along the anterior border abutting the serosa and at the dorsal side of the posterior pit (Fig. 3G). This expression pattern is similar to that of *Tc-dpp* (Chen et al., 2000; van der Zee et al., 2006), but different from that of *Dm-tsg1*, which is restricted to a narrow dorsal domain of the *D. melanogaster* blastoderm embryo (Mason et al., 1994). During early germ band stages *Tc-tsg* is expressed in the ectoderm and absent from the mesoderm, similar to *Tc-tld* (compare Fig. 3C,H and E,I). Extending germ band embryos also express *Tc-tsg* in the growth-zone (Fig. 3J) and in a ring-shaped domain between the head lobes (Fig. 3J′). This ring of expression probably surrounds the dot-like stomodeal domain of *Tc-tld* (Fig. 3J′, *Tc-tld* in Fig. 3F). *Tc-dpp* transcripts (Fig. 3K) and Dpp activity (Fig. 3L—pMad) are also detected in the stomodeum suggesting a function of BMP signalling in stomodeum specification. In completely extended germ bands, *Tc-tsg* transcripts can be detected in the dorsal ectoderm and in the leg buds (data not shown).

Comparison of tld and tsg function in Drosophila and Tribolium

To compare *tld* and *tsg* function in *Drosophila* and *Tribolium* we first describe the respective *Drosophila* mutants using three marker genes to characterise their DV pattern. *dorsocross 2* (*doc2*) served as marker for dorsalmost, *pannier* (*pnr*) for dorsolateral and *muscle specific homeobox* (*msh*) for lateral (neuroectodermal) cell fates (Fig. 4A–C).

Using this marker set *Dm-tld* and *Dm-dpp* show very similar, if not identical ventralised phenotypes during blastoderm stage. The

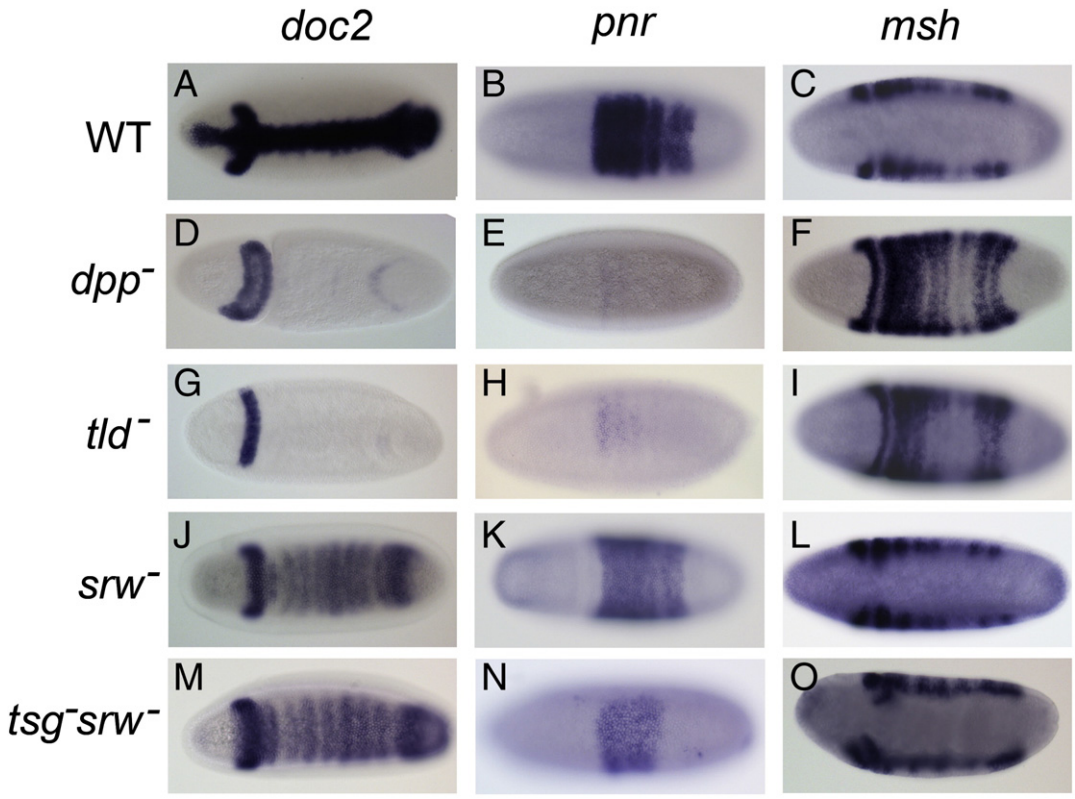

Fig. 4. *Drosophila* Dpp signalling in embryos lacking the function of both embryonic *tsg* genes. *Drosophila* embryos during blastodermal stages. All panels are dorsal views, anterior pole points to the left. (A,B,C) WT. (D,E, F) dpp^{H46}/dpp^{H46}. (G,H,I) tld^{B4}/tld^{B4}. (J,K,L) srw^{10K28}/srw^{10K28}. (M,N,O) tsg^{XB}/tsg^{XB};;srw^{10K28}/srw^{10K2}. *In-situ* hybridisation for *dorsocross-2* (*doc2*—A,D,G,J, M), *pannier* (*pnr*—B,E,H,K,N) and *muscle-segment-homeobox* (*msh*—C,F,I,L,O). *tld*, *tsg*, *srw* and *tsg*;;*srw* homozygous embryos were identified by the absence of *ftz-LacZ* or *hb-LacZ* expression derived from balancer chromosomes (*FM7 ftz-LacZ*, *TM3 ftz-LacZ*, *TM3 hb-LacZ*).

mutant embryos are characterised by a loss of dorsal and dorsolateral cell fates as inferred from the lack of *doc2* and *pnr* expression and by the dorsal expansion of neuroectodermal cell fates as inferred from the ectopic expression of *msh* (Fig. 4D–I). Weak dorsal *pnr* expression and *msh* repression was still detectable in *dpp* and *tld* mutant embryos probably due to very low levels of signalling by Scw (and Dpp) homodimers (Shimmi et al., 2005b; see Discussion).

In contrast to *dpp* and *tld*, *tsg1* and *srw* mutant *Drosophila* embryos are only weakly ventralised (Zusman and Wieschaus, 1985; Bonds et al., 2007; Mason et al., 1994). In *srw* mutants dorsal *doc2* expression is diminished and does not refine to a narrow dorsal stripe, while *pnr* and *msh* are not affected (Fig. 4J–L). The same applies for *tsg1* (Mason et al., 1994 and data not shown) indicating that in each of the two mutants only the peak levels of Dpp signalling are lost. The sequence similarity of *tsg1* and *srw* (Figure S2) could allow for functional redundancy. To investigate this possibility, we constructed *tsg1*;;*srw* double mutant embryos. The double mutant embryos did not show a more severe phenotype than each single mutant (Fig. 4M–O) indicating that *tsg1* and *srw* do not act redundantly. Since the phenotypic strength of the double mutant was not even slightly enhanced compared to the single mutants, we did not investigate the *tsg1*;;*srw cv1* triple mutant which genetically would remove all Tsg function. A further reason for not including *cv1* is that this gene shows only late embryonic expression and *cv1* mutant embryos are viable (Shimmi et al., 2005a; Vilmos et al., 2005). Thus, it is likely that *tsg1*;;*srw* double mutant embryos lack all early Tsg activity. This implies that different levels of Dpp signalling can be established in the early *D. melanogaster* embryo even in the absence of Tsg function.

We next analysed the functions of *tld* and *tsg* in *T. castaneum* employing the technique of parental RNAi (Bucher et al., 2002). As for *Tc-dpp* (van der Zee et al., 2006), injection of dsRNA for *tld* and *tsg* into pupae prevents egg production in adult females, suggesting a role for both genes during oogenesis. Therefore, we injected dsRNA into adult females. The injected females produced normal numbers of eggs, but they did not give rise to hatching larvae nor to larvae exhibiting cuticle formation. This suggests that like the loss of *Tc-dpp*, loss of *Tc-tld* or *Tc-tsg* leads to severe defects during early development. To analyse early development of the RNAi embryos we used DAPI stainings for marking the serosa (large, widely spaced nuclei; Fig. 5A, ser) and germ rudiment (small, densely packed nuclei, Fig. 5A, germ) as well as a number of molecular markers for different AP and DV territories. *Tc-dorsocross* (*Tc-doc*) served as a marker for the dorsal serosa (Fig. 5D, d. ser, van der Zee et al., 2006), *Tc-iroquois* (*Tc-iro*) as a marker for the anterior amnion (Fig. 5G, ant. am) and the dorsal amnion/dorsal ectoderm (Fig. 5G—am/d. ect, Nunes da Fonseca et al., 2008). Finally, *Tc-mille-pattes* (*Tc-mlpt*) was used to mark the head anlagen, which occupy a triangular region with the broad base at the ventral side and the tip at the dorsal side (Fig. 5J; Savard et al., 2006; van der Zee et al., 2005). We also visualised Dpp signalling with stainings for phosphorylated Mad (pMAD) (Dorfman and Shilo, 2001; Sutherland et al., 2003; Nunes da Fonseca et al., 2008; van der Zee et al., 2006, Figs. 1 and 5M,M′).

In striking contrast to the results with *D. melanogaster*, knockdown of *Tc-tsg* in *T. castaneum* leads to complete loss of ectodermal polarity. The germ rudiment expands anteriorly and dorsally so that the embryo–serosa border becomes straight (Fig. 5C). The dorsal markers *Tc-doc* and *Tc-iro* are completely eliminated except for some *Tc-iro* expressing cells at the anterior border of the germ rudiment (residual anterior amnion, Fig. 5F,I), which are probably specified by the AP patterning system. The triangular *Tc-mlpt* domain entirely loses DV asymmetry and appears as a broad band of expression (Fig. 5L). No pMAD activity can be detected in the embryo (Fig. 5O), suggesting complete loss of Dpp signalling. During later development the embryos do not regain ectodermal DV polarity. They form radially

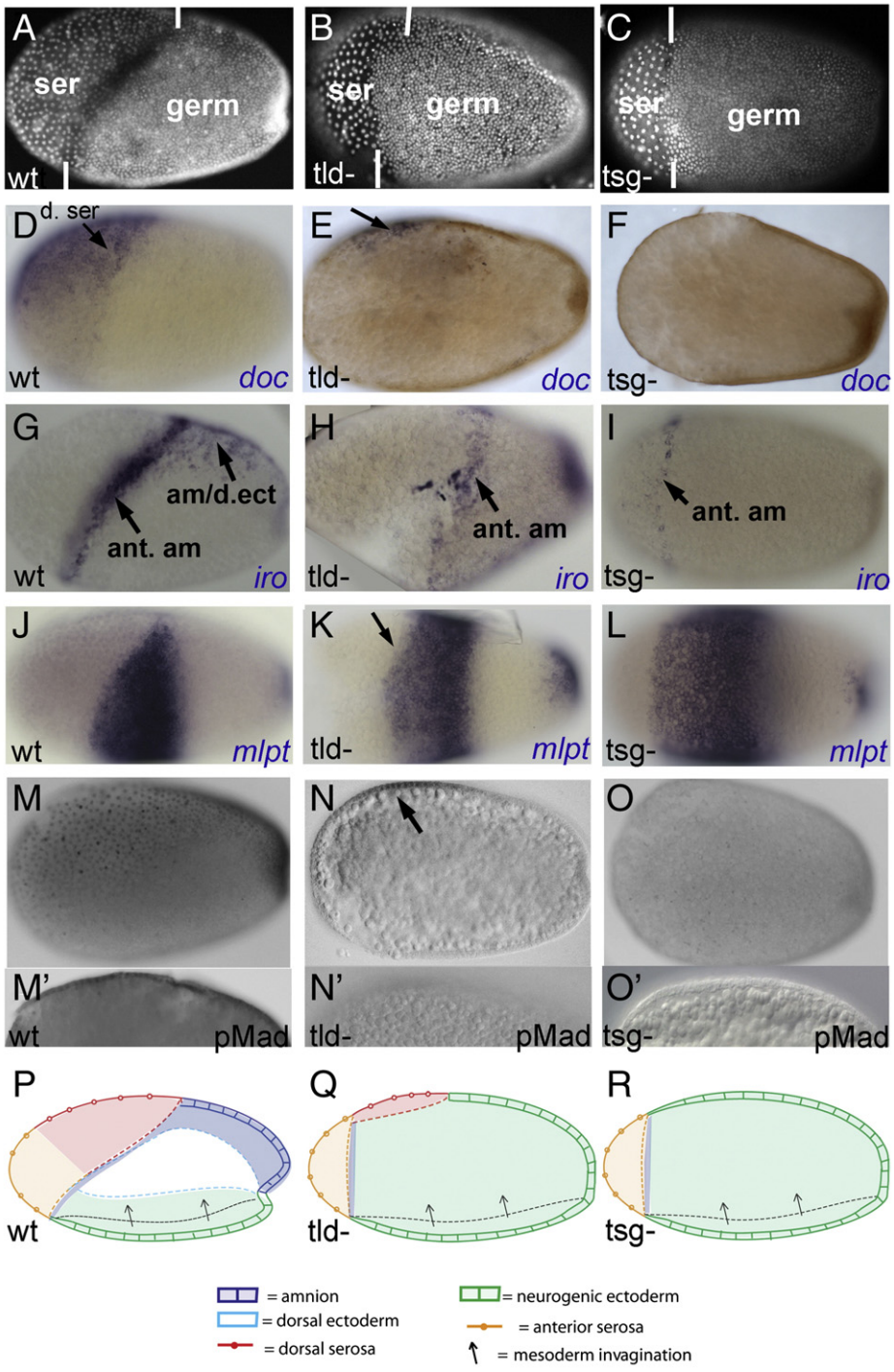

Fig. 5. Changes in early fate map and Dpp activity after *Tc-tld* and *Tc-tsg* RNAi. Differentiated blastoderm stage embryos, lateral views, anterior to the left. (A,D,G,J,M,P) WT; (B,E,H,K, N,Q) *Tc-tld* RNAi; (C,F,I,L,O,R) *Tc-tsg* RNAi embryos. (A–C) Nuclear stainings (DAPI) of (A) WT, (B) *Tc-tld* RNAi and (C) *Tc-tsg* RNAi embryos. ser: serosa, germ: germ rudiment (embryo plus amnion). (A–C) White bars demarcate the serosa–embryo boundary, both dorsally and ventrally. Embryo in A is a DAPI staining of the embryo in G, explaining the observed shadow. (D–F) *Tc-doc* expression in (D) WT, (E) *Tc-tld* RNAi and (F) *Tc-tsg* RNAi embryos. d. ser: dorsal serosa. Arrow in E points to a residual *Tc-doc* expression after *Tc-tld* RNAi. (G–I)) *Tc-iro* expression in (G) WT, (H) *Tc-tld* RNAi and (I) *Tc-tsg* RNAi embryos. ant. am: anterior amnion, am/d.ect: dorsal amnion and dorsal ectoderm. (J–L) *Tc-mlpt* expression in (J) WT, (K) *Tc-tld* RNAi and (L) *Tc-tsg* RNAi embryos. Arrow in K points to the region where *Tc-mlpt* is slightly narrower in *Tc-tld* RNAi embryos when compared to *Tc-tsg* RNAi. (M–O) Lateral views. Antibody staining against pMad (Dpp activity) in (M,M′) WT, (N,N′) *Tc-tld* RNAi and (O,O′) *Tc-tsg* RNAi embryos. M, N′ and O focuses at the egg surface, while M′, N and O′ are sagittal optical sections. Arrow in N points to the residual pMad present in *Tc-tld* RNAi embryos, while pMad is absent in *Tc-tsg* RNAi. (P–R) Schematic drawings of (P) WT, (Q) *Tc-tld* RNAi and (R) *Tc-tsg* RNAi fate maps at the differentiated blastoderm stage.

symmetric ectodermal tubes which show *Tc-gooseberry* (*Tc-gsb*) and *Tc-mlpt* expression evenly around the circumference (Fig. 6C,F). In WT germband stage embryos the *Tc-gsb* stripes are restricted to the ventral ectoderm (Fig. 6D; Nunes da Fonseca et al., 2008) and *Tc-mlpt* is expressed in asymmetric domains in the head region and posterior abdomen (Fig. 6A). The ectodermal cells of *Tc-tsg* knock-down embryos have neural identity, as inferred from the expression of *Tc-achaete-scute* (*Tc-ash*), a marker of the ventral neurogenic ectoderm (Wheeler et al., 2005) (Fig. 7A,B). Taken together, both the early and late *Tc-tsg* RNAi phenotypes indicate a complete loss of Dpp activity (Fig. 5R). Accordingly, these *Tc-tsg* RNAi phenotypes are indistinguishable from the knock-down of *Tc-dpp*, *Tc-Mad* or *Tc-Med* (van der

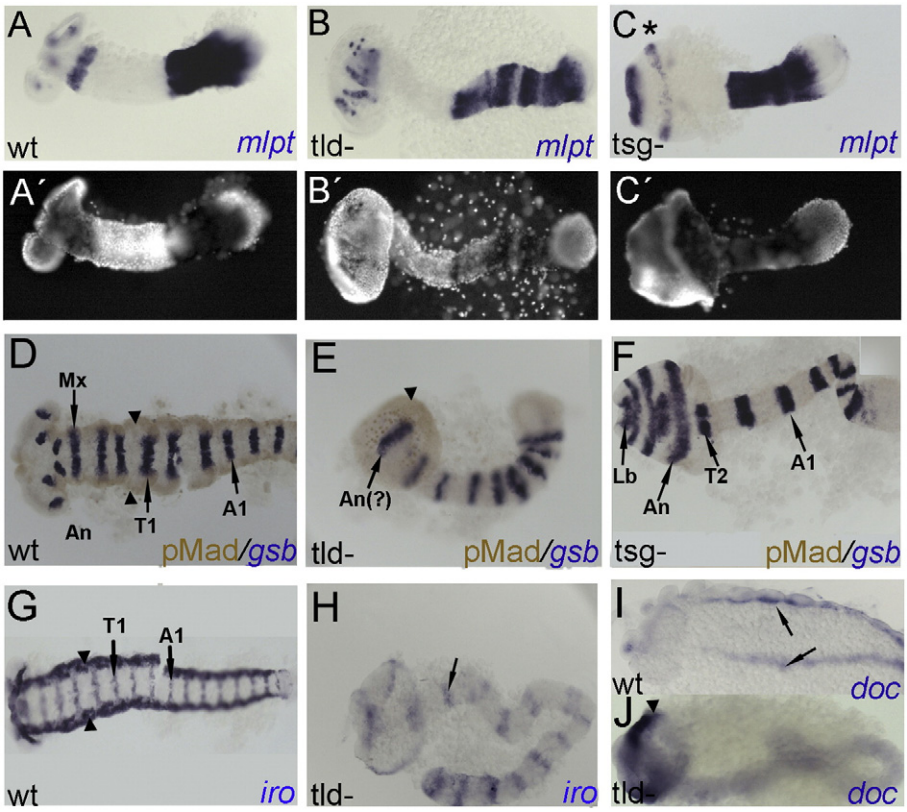

Fig. 6. Later development of *Tc-tld* and *Tc-tsg* RNAi embryos. (A–J) Germ band stage embryos, anterior to the left. (A,A′,D,G, I) WT, (B,B′,E,H,J) *Tc-tld* RNAi, (C,C′,F) *Tc-tsg* RNAi. (A–C) *Tc-mlpt in situ* hybridisation and corresponding nuclear stainings (DAPI), in (A,A′) WT, (B,B′) *Tc-tld* RNAi and (C,C′) *Tc-tsg* RNAi embryos. (C) Asterisk highlights the symmetric expansion of *Tc-mlpt* after *Tc-tsg* RNAi. (D–F) *in situ* hybridisation for *Tc-gsb* (blue) and pMad antibody staining (brown) in (D) WT, (E) *Tc-tld* RNAi and (F) *Tc-tsg* RNAi embryos. Arrowheads in D point to the lateral margins of pMad staining in the WT. In (E), after *Tc-tld* RNAi pMad is only detected in the head region (arrowhead), while in (F) after *Tc-tsg* RNAi pMad is not present at all. (G,H) *Tc-iro in situ* hybridisation. (G) In WT, *Tc-iro* is expressed in every segment (arrows in T1 and A1) and in two broad lateral domains (prospective dorsal regions, arrowheads). (H) After *Tc-tld* RNAi, the segmental expression of *Tc-iro* is still evident (arrow), while the broad lateral domains are absent. (I,J) *Tc-doc in situ* hybridisation. (I) In WT *Tc-doc* is expressed in the dorsal ectoderm (arrows). (J) After *Tc-tld* RNAi expression is only detected in the embryonic head (arrowhead). Mx—maxilla, Lb—labium, An—antenna, T1, T2—first and second thoracic segment; respectively, A1—first abdominal segment.

Zee et al., 2006 and Figure S4) and show that, in *T. castaneum*, the single *tsg* gene has a pro-BMP function, required for all levels of Dpp signalling activity during embryogenesis.

The phenotypes produced by RNAi for *Tc-tld* are similar to that of *Tc-tsg*. However, the ventralisation is consistently weaker in the head region in comparison to *Tc-tsg* RNAi embryos. DAPI staining of *Tc-tld* RNAi embryos reveals a strong expansion of the germ rudiment towards anterior-dorsal positions (Fig. 5B). However, the border between serosa and germ rudiment retains some obliqueness similar to the anterior border of *Tc-mlpt* expression (Fig. 5K—arrow). The deletion of the dorsal amnion/dorsal ectoderm domain of *Tc-iro* suggests a loss of dorsal cell fates (Fig. 5H). However, *Tc-doc* expression is not completely eliminated from the serosa (Fig. 5E see also schematic drawing Fig. 5Q; van der Zee et al., 2006) and residual pMAD (Fig. 5M,M′,N, N′) is found in narrow dorsal-anterior region overlapping with the *Tc-doc* expression domain (Fig. 5N—arrow). Thus, early *Tc-tld* RNAi embryos retain residual DV polarity in the head region and the serosa. This residual polarity can be well recognised when the serosa expands asymmetrically during gastrulation (see Supplemental data, Figure S3). During germ band extension all trunk segments including those derived from the posterior growth-zone lack ectodermal DV polarity. This is evident from the lateral expansion of *Tc-gsb* stripes (Fig. 6E), the lack of *Tc-iro* and *Tc-doc* lateral expression domains (Fig. 6H, J), and absence of all pMAD activity in the trunk region (Fig. 6E). All above markers demarcate the dorsal ectoderm in WT (Fig. 6G, I). However, in the head region *Tc-mlpt* is expressed asymmetrically (Fig. 6B) and residual *Tc-doc* expression (Fig. 6J—arrowhead) as well as pMAD activity (Fig. 6E—arrowhead) are retained.

In summary, *Tc-tld* RNAi embryos show a loss of ectodermal DV polarity in the trunk region while they maintain residual DV polarity in the head and serosa (Fig. 5Q). Residual BMP activity after *Tc-tld* RNAi might be due to a partial knock-down. However, injection of two dsRNA constructs targeting non-overlapping regions lead to identical results. No endogenous *Tc-tld* transcripts could be detected in the RNAi knockdown embryos (data not shown). The identical outcome with two different dsRNA constructs also argues against off-target effects.

Since the *Tc-tld* RNAi phenotype is clearly different from the partial *Tc-dpp* knock-down phenotypes previously described (Ober and Jockusch, 2006; van der Zee et al., 2006), we considered the possibility whether *Tc-tld*, like *tok* in *D. melanogaster* (Parker et al., 2006; Serpe and O'Connor, 2006) and BMP1 in vertebrates (reviewed in Hopkins et al., 2007) is involved in regulating the activity of other TGFß ligands, like Activin. However, this scenario is unlikely since Activin does not contribute to patterning in the early *T. castaneum* embryo (Figure S4). Moreover, there is no difference between the knock-down phenotypes of *Tc-Mad*, the cytoplasmic signal transducer downstream of BMPs, and *Tc-Medea*, the common Smad, involved in transducing all TGFß signals (Figure S4). We assume, therefore, that *Tc-tld* primarily acts via *Tc-dpp* in the early embryo and that in contrast to *D. melanogaster*, loss of *Tc-tld* differentially affects BMP signalling along the AP axis.

The tolloid, but not tsg, function depends on the presence of sog

T. castaneum embryos are severely dorsalised upon loss of *sog* (van der Zee et al., 2006). The clear morphological distinction between the

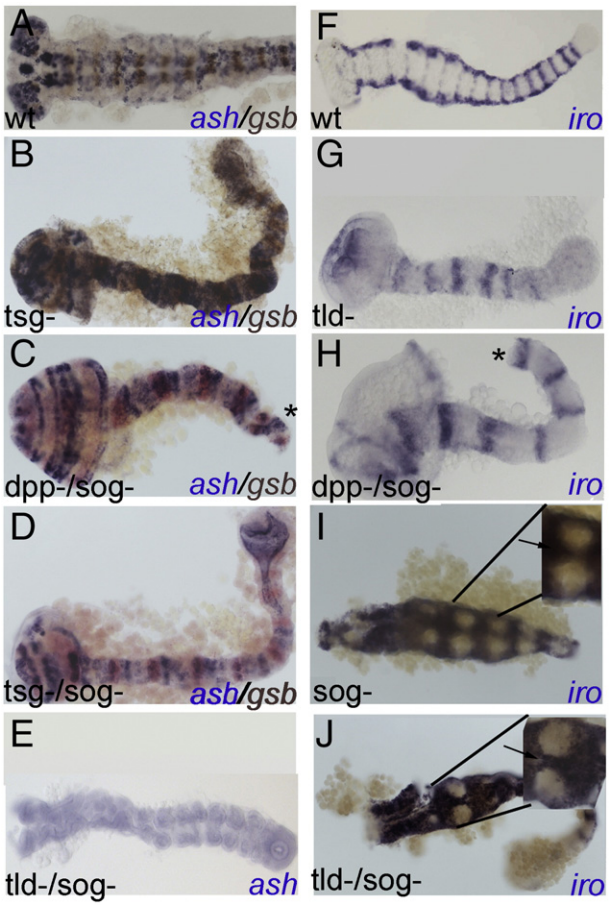

Fig. 7. Analysis of *Tc-sog* expression in *Tc-sog*/*Tc-tsg* double RNAi embryos. (A,B,A′,B′) Lateral views, head to the left. *In situ* hybridisation for *Tc-sog* and antibody staining against pMad (Dpp activity) in WT (A) and *Tc-tsg*/*Tc-sog* double RNAi embryos (B). White box demarcates area is shown enlarged in C,D. (A′, B′) Nuclear stainings (DAPI) of (A) WT and (B) *Tc-tsg*/*Tc-sog* double RNAi embryos. Arrowheads indicate the serosa–germ rudiment border. (C, D) Ventral views—*Tc-sog* expression in the regions boxed in A and B. (C) WT embryo displaying high concentrations of *Tc-sog* transcripts in the cytoplasm. (D) In *Tc-tsg*/*Tc-sog* double RNAi embryos, transcripts are mainly present in nuclei (see text for details).

dorsalised RNAi phenotype of *Tc-sog* and the severely ventralised RNAi phenotypes of *Tc-tld* or *Tc-tsg* enables us to perform epistasis experiments by double RNAi (Table 1). Corresponding double mutant experiments in *D. melanogaster* have been difficult due to the weakness and similarity of the mutant phenotypes (Marques et al., 1997; Wang and Ferguson, 2005).

Table 1
Epistasis analysis of *Tc-dpp*, *Tc-sog* and its modulators, *Tc-tsg* and *Tc-tld*.

RNAi	Embryos Analysed	WT	Dorsalised (head absent)	Ventralized (big head)
tld-	52	4 (7,7%)	1 (1,9%)	47 (90,4%)
Dpp-	55	1 (1,8%)	0	54 (98,2%)
tsg-	40	0	0	40 (100%)
sog-	50	2 (4%)	48 (96%)	0
Dpp-/sog-	50	5 (10%)	1 (2%)	44 (88%)
tsg-/sog-	48	4 (8,3%)	2 (4,2%)	42 (87,5%)
tld-/sog-	47	6 (12,8%)	41 (87,2%)	0

Blue boxes highlight the most frequent phenotypes recovered after dsRNA injections.

To perform double knock-down experiments the two respective dsRNAs (for details see Methods) were simultaneously injected into adult females. Batches of egg lays derived from the injected females were first scored for gross-morphological changes. While ventralised embryos (*tsg*, *tld* and *dpp* RNAi) have enlarged head regions, dorsalised embryos (*sog* RNAi) completely lack the head region (Table 1). The initial phenotypic characterisation was confirmed with molecular markers.

As a control we performed the double knock-down for *Tc-sog* and *Tc-dpp*. Embryos derived from *Tc-sog*/*Tc-dpp* double RNAi females showed a ventralised phenotype indistinguishable from that of *Tc-dpp* RNAi alone (van der Zee et al., 2006, Fig. 7C). This can be seen from the enlarged head region, uniform expansion of the *Tc-ash* expression throughout the ectoderm (Fig. 7C), and the lack of the *Tc-iro* dorsal ectodermal domain (Fig. 7H). To confirm the double knock-down, *in situ* hybridisation for *Tc-sog* was performed with the embryos derived from *Tc-sog*/*Tc-dpp* double RNAi injected females. The double knock-down was highly penetrant (more than 80% of the ventralised embryos lacked *Tc-sog* expression, data not shown). The epistasis of the *dpp* phenotype in *sog*;*dpp* double mutants was also described for *D. melanogaster* (Biehs et al., 1996) and agrees with the biochemical and phenotypic data which so far have implicated Sog/Chordin only in the modulation of BMP signalling.

A prediction for the phenotype caused by simultaneous knock-down of *Tc-sog and Tc-tld* is less straightforward. In *D. melanogaster* so far only a role of Tld in Sog processing has been described, implying that *sog* acts downstream of *tld*. Indeed, the *sog*;*tld* double mutant has a *sog*-like phenotype in *D. melanogaster* (Marques et al., 1997). However, *Tc-tld* is more closely related to *Dm-tok*, which also has *sog*-independent functions. Nonetheless, embryos recovered from *Tc-sog*/*Tc-tld* double RNAi displayed the same phenotype as *Tc-sog* RNAi alone (van der Zee et al., 2006, Fig. 7E,I–J). In this case the double knock-down was confirmed by *Tc-tld in situ* hybridisation (data not shown). Besides the lack of the head region *Tc-sog* RNAi embryos have a very characteristic feature: they exhibit a duplication of the dorsal *Tc-iro* or *Tc-pnr* expression domain along the ventral midline (van der Zee et al., 2006, Fig. 7I,J). This “double dorsal” phenotype was clearly discernable after *Tc-sog*, *Tc-tld* double RNAi (Fig. 7I,J—inset arrow), indicating that the ventralisation caused by *Tc-tld* is completely suppressed by additional loss of *Tc-sog*. Thus, *Tc-tld* acts in the early *Tribolium* embryo largely via *Tc-sog* (Fig. 9B). This is in agreement with our interpretation of the *Tc-tld* knockdown phenotype and suggests that, in line with biochemical data from other systems, *Tc-tld* is only required to cleave and thereby inactivate *Tc-Sog*.

Like Tld, Tsg has been shown to act in a complex with Sog suggesting that its function might also depend on the presence of Sog. However, in *D. melanogaster* careful epistasis analysis of *tsg* and *sog* has provided evidence for *sog*-independent functions of *tsg* (Wang and Ferguson, 2005). This analysis was hampered by the similarity of the *Dm-tsg* and *Dm-sog* phenotypes (both result in a loss of dorsal most fates). In *T. castaneum* the *Tc-sog*/*Tc-tsg* double RNAi leads to a complete suppression of the *Tc-sog* phenotype (Fig. 7D). The double knock-down was confirmed by *Tc-sog in situ* hybridisation. In these experiments, more than 80% of the double RNAi embryos at differentiated blastoderm stage did not show any *Tc-sog* expression (24 out of 29). In the remaining double RNAi embryos *Tc-sog* expression was observed only in the nuclei (Fig. 8C,D), suggesting that only primary transcripts are present. A similar RNA distribution—disappearance of cytoplasmic and maintenance of nuclear mRNA—was seen in RNAi experiments with *delta* in the spider *Achaearanea tepidariorum* (*At*) (Oda et al., 2007).

During blastoderm stages, double knock-down embryos (*Tc-sog*/*Tc-tsg*) showed all features of ventralised *T. castaneum* embryos, an expanded germ rudiment and a smaller anterior serosa (Fig. 8A′,B′—

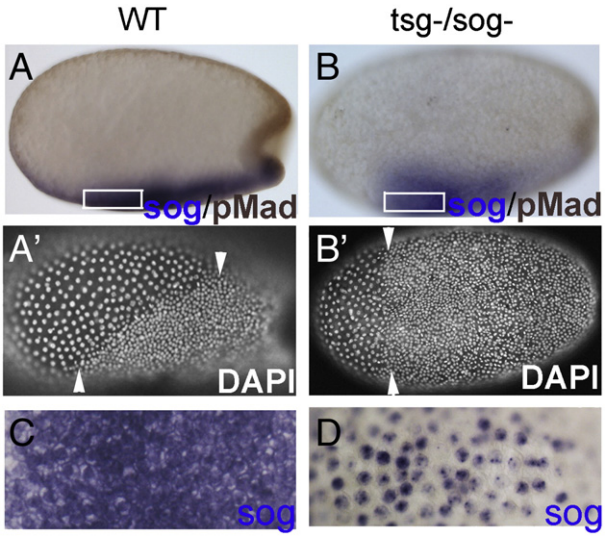

Fig. 8. Double RNAi experiments suggest that Tc-Tld acts on Tc-Sog, while Tc-Tsg acts independently of Sog. (A–J) Germ band stage embryos, anterior to the left. (A–D) Double *in situ* hybridisation using the neurogenic marker *Tc-ash* (blue) and the segmental marker *Tc-gsb* (red) in (A) WT, (B) *Tc-tsg* RNAi, (C) *Tc-dpp*/*Tc-sog* double RNAi, and (D) *Tc-tsg*/*Tc-sog* double RNAi. (E) *Tc-ash* expression in *Tc-tld*/*Tc-sog* double RNAi embryos. (F–J) *Tc-iro in situ* hybridisations in (F) WT, (G) *Tc-tld* RNAi, (H) *Tc-dpp*/*Tc-sog* double RNAi, (I) *Tc-sog* RNAi and (J) *Tc-tld*/*Tc-sog* double RNAi.

DAPI). During germ band elongation, these knock-down embryos were also indistinguishable from *Tc-tsg* or *Tc-dpp* single knock-down with regard to head morphology and *Tc-ash* expression (Fig. 7D). This suggests that the pro-BMP function of *Tc-tsg* is independent of *Tc-sog* (see discussion).

Discussion

Our study provides insights into mechanistic and evolutionary aspects of extracellular BMP modulation in insects. Mechanistically, the differential requirement of *Tc-tld* along the AP axis and the strong Sog-independent pro-BMP activity of *Tc-tsg* are most interesting. Phylogenetically, it comes as a surprise that the single *Tc-tld* and *Tc-tsg* genes acting during early development in *T. castaneum* are more closely related to *D. melanogaster* homologues that mainly act during late development (*Dm-tok* and *Dm-cv1*, respectively). In the following sections we first discuss the roles of *Tc-tld* and *Tc-tsg* in early *T. castaneum* development and suggest ways to integrate our observations on Tsg function with current biochemical findings. Second, we address the evolutionary changes in the network of BMP signalling which occurred in the lineage leading to *D. melanogaster*.

Tolloid function

In *D. melanogaster*, Sog is cleaved by Tld, which releases Dpp/Scw heterodimers from Sog-Dpp/Scw complexes and allows them to bind to their receptors (reviewed in O'Connor et al., 2006). In *tld* mutants Dpp/Scw heterodimers are sequestered by Sog, which largely abolishes Dpp signalling. Sog binds also to Dpp and Scw homodimers, but with a much lower affinity (Shimmi et al., 2005b), which might allow the formation of free homodimers even in absence of *tld*. The homodimers, however, are known to have only very low signalling activity (Shimmi et al., 2005b). These facts might explain the weak dorsal *pnr* expression and *msh* repression which was still detectable in *Dm-dpp* and *Dm-tld* mutant embryos (Fig. 4). In *Dm-dpp* mutant embryos Scw homodimers are still present while in *tld* mutant embryos Dpp and Scw homodimers not bound by Sog might lead to low level signalling.

With our marker set we cannot distinguish the strength of ventralisation observed in early embryos mutant for *Dm-dpp* and *Dm-tld*. A similar conclusion was drawn on the basis of pMad staining (Ross et al., 2001). However, it is well known that during later development *tld* mutants show a weaker ventralisation than those of *dpp* (Arora and Nusslein-Volhard, 1992; Ferguson and Anderson, 1992b; Shimell et al., 1991). This might be due to minor differences in early BMP signalling, which we cannot detect with our marker genes or to postblastoderm functions of *dpp* which are independent from *tld* and *sog* (Shimmi et al., 2005b; Wang and Ferguson, 2005). The possibility of a dorsalising influence of Dpp during postblastoderm stages has been demonstrated by late ectopic Dpp expression (Staehling-Hampton et al., 1994) and heterotopic cell transplantations (Technau and Campos-Ortega, 1986; Udolph et al., 1995).

However, irrespective of the reasons for the weaker phenotype, residual dorsal structures are found along the entire AP axis of late *Dm-tld* mutant embryos which are indistinguishable from embryos mutant for hypomorphic *Dm-dpp* alleles. In contrast, *Tc-tld* knock-down embryos are completely ventralised along most of the AP axis displaying residual dorsal structures only in the head and serosa region. (Fig. 6E,H), a phenotype we have so far have not observed by partial knock-down of *Tc-dpp* (Ober and Jockusch, 2006; van der Zee et al., 2006). The differential requirement for Tc-Tld activity along the AP axis might be related to the strong temporal gradient with which BMP activity appears during blastoderm stages. The BMP gradient is established about 2 h earlier in the anterior compared to the posterior half of the embryo (Fig. 1D–K). This appears to be a consequence of *Tc-dpp* and *Tc-sog* expression in the early embryo (van der Zee et al., 2006). Expression of *Tc-dpp* is first slightly stronger towards the anterior pole and later is up-regulated in an anterior stripe of cells along the serosa–germ rudiment boundary. In this region *Tc-sog* is repressed (van der Zee et al., 2006). Thus, locally produced Tc-Dpp might not be fully inhibited by Tc-Sog (Fig. 9A). In addition, it is possible that the affinity of Tc-Sog for Tc-Dpp homodimers is lower than the affinity of Dm-Sog to Scw/Dpp heterodimers allowing for higher levels of Tc-Dpp homodimers even in the presence of Tc-Sog. Together this might explain the observed *Tc-tld* independent Dpp activity in anterior regions of the embryo.

Twisted gastrulation function

Although the function of Tsg has been extensively studied in *D. melanogaster* and vertebrates, contradictory results have been reported regarding the role of Tsg with support for anti-BMP (Blitz et al., 2003; Chang et al., 2001; Ross et al., 2001; Wills et al., 2006) and pro-BMP (Little and Mullins, 2004; Oelgeschlager et al., 2000; Oelgeschlager et al., 2003; Xie and Fisher, 2005; Zakin and De Robertis, 2004) activity. Biochemically, Tsg can bind to Sog and BMP ligands independently or in a Sog-BMP-Tsg complex (Oelgeschlager et al., 2000; Ross et al., 2001). In order to reconcile pro- and anti-BMP functions, it has been proposed that Tsg has a dual role in modulating BMP/Dpp signalling. First, Tsg would enhance Sog/Chordin binding to Dpp/BMP, resulting in an inhibitory function for BMP signalling. Second, Tsg would enhance Tld cleavage of Sog/Chordin, enabling Dpp/BMP to signal (Larrain et al., 2001; Ross et al., 2001; Scott et al., 2001). Both proposed biochemical functions are incompatible with our observations that Tsg has only pro-BMP functions in *T. castaneum*, which are, moreover, independent of Sog. Also in *D. melanogaster* and zebrafish epistasis experiments have provided evidence for Sog/Chordin independent functions of Tsg (Finelli et al., 1995; Little and Mullins, 2004; Wang and Ferguson, 2005). However, the relevant phenotypic effects were fairly subtle due to the weak pro-BMP function of Tsg in both organisms.

Three mechanisms could account for a Sog-independent pro-BMP effect of Tsg which, as observed in *T. castaneum*, affects all levels of BMP signalling. The first mechanism assumes that the BMPs and Tsg

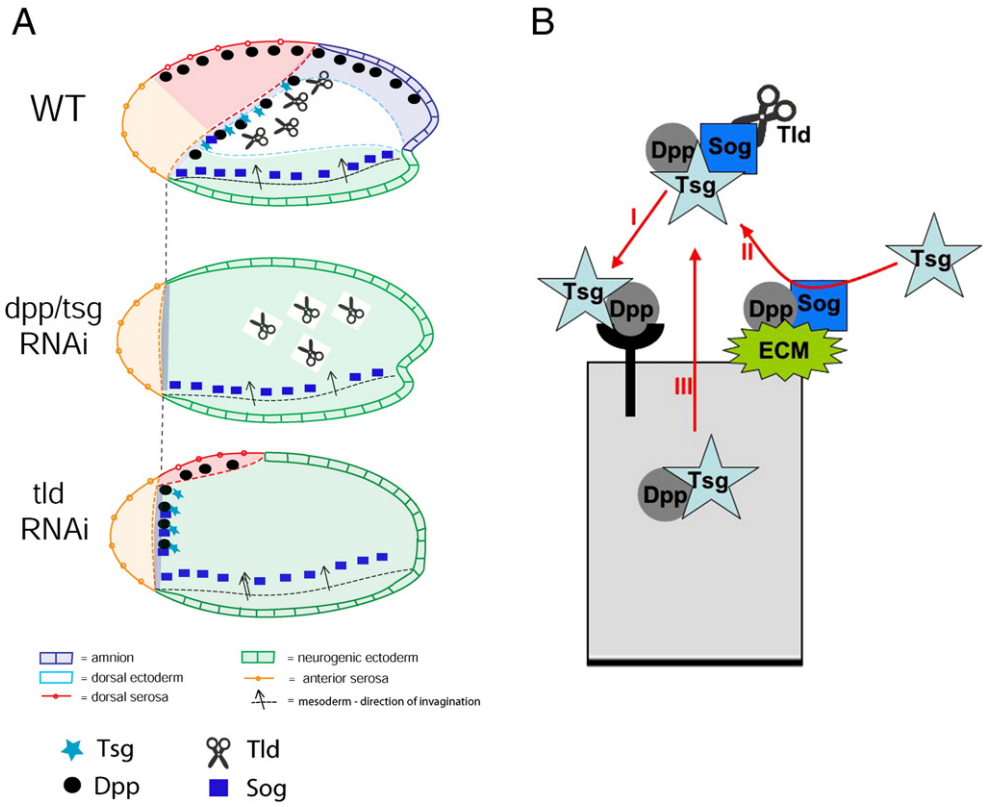

Fig. 9. A model for Dpp transport and activation in the *Tribolium* early embryo. (A) Dpp (black circle), Tsg (light blue star), Sog (dark blue square) and Tld (scissors) are required to concentrate Dpp activity in the dorsal side of the *Tribolium* embryo. (B) Putative interactions among Dpp and its modulators obtained by epistatic analyses (for details see Discussion). Grey square represents a cell. I, II and III refer to the models proposed in the discussion session. ECM—extracellular matrix.

are secreted independently and associate in the extracellular space (Fig. 9B). After complex formation Tsg would be essential for targeting BMPs to their receptors. The interaction of Sog/Chordin with Tsg (Larrain et al., 2001) could interfere with of Tsg's pro-BMP function explaining the inhibitory role of Sog/Chordin. However, Tc-Tsg would, even in the absence of Sog/Chordin, be required for binding of BMPs to their receptors. Mediating ligand–receptor interactions represents a very basic step of BMP signalling. Therefore, one would expect that such a mechanism is fairly constrained in evolution. The second mechanism also assumes that the BMPs and Tsg are secreted independently. However, here a crucial step is the binding of secreted BMPs to extracellular matrix components like type IV collagen (Wang et al., 2008) and proteoglycans (Belenkaya et al., 2004; Akiyama et al., 2008; for review Kerszberg and Wolpert, 2007). It is the release of BMPs from their binding to the ECM which requires Tsg (Wang et al., 2008). Evolutionary changes in requirement for Tsg can be explained easily by alterations of the amount and composition of the ECM and/or the affinity with which BMPs are sequestered by ECM components. Changes in the amounts of proteoglycans have recently been shown to be important for BMP gradient formation in the *Drosophila* embryo (Bornemann et al., 2008). To test whether similar mechanisms apply to *Tribolium* it will be interesting to study the function of type IV collagen and proteoglycans in the early embryo. The last mechanism is based on the co-expression of BMPs and Tsgs in all systems studied so far, in contrast to the non-overlapping expression of BMP and Sog/Chordin. The co-expression of BMPs and Tsgs provides the possibility that the two proteins can bind to each other prior to secretion. This has been ruled out for *Dm-tsg1* as the sole mechanism of action since *tsg1* ectopically expressed in a ventral domain can diffuse to the dorsalmost region and rescue the dorsal midline cells in *tsg* mutant embryos (Mason et al., 1997). However, *D. melanogaster* might represent a rather special case (see next section). The fact that all Dpp activity is lost after *Tc-tsg* knock-down in *T. castaneum* could be explained by assuming that Tsg is already required prior to Dpp secretion for aspects of Dpp processing or folding. It might be interesting for future biochemical approaches to include tissue culture experiments which are not restricted to secreted proteins derived from supernatants, but test the potential interaction of proteins co-expressed prior to secretion.

The evolution of BMP signalling

Phylogenetic analysis shows that *Dm-tld*, *Dm-tsg1* and *Dm-srw* are lineage specific paralogues whose functions are largely restricted to the DV patterning of the *D. melanogaster* blastoderm embryo (Fig. 2). Recruitment of lineage-specific paralogues for early development is also seen for the BMP ligands in *D. melanogaster*. Phylogenetic analyses have previously shown that *Dm-scw* is derived from a *gbb*-like ancestor (Van der Zee et al., 2008). *Dm-dpp* acts together with *Dm-scw* only in the early embryo while during later development *Dm-dpp* interacts with *Dm-gbb*.

Three aspects might be relevant to explain the observed evolutionary changes. First, the speed of pre-gastrula development in *D. melanogaster* (at least twice as fast as *T. castaneum*) favours the transcription of genes with short pre-mRNAs. This was first demonstrated for the *knirps* and *knirps-related* genes (Rothe et al., 1992). Recent genome-wide analyses showed that the majority of the early expressed zygotic genes in *D. melanogaster* are intronless (De Renzis et al., 2007). Thus, the evolutionary trend towards fast development apparently has favoured the employment of intronless paralogues that have arisen by retrotransposition events (reviewed in Swinburne and Silver, 2008). Both *Dm-tsg1* and *Dm-srw* are encoded by small intronless genes (Bonds et al., 2007; Mason et al., 1994) while *Dm-cv1* and the other insect *tsg* genes have a common gene

structure with three introns (Fig. 2). Likewise, *Dm-tld* has a shorter transcript and smaller introns than *Dm-tok* (Finelli et al., 1995; Nguyen et al., 1994). In the case of *Dm-scw* and *Dm-gbb* both genes code for intronless transcripts (Arora et al., 1994; Khalsa et al., 1998). However, there are also marked exceptions to this rule. E.g. *Dm-sog* possesses large introns and produces large transcripts (Francois et al., 1994). The same applies for *u-shaped*, *pannier*, *ventral nervous system defective* and *dpp* itself. The introns of these genes may contain regulatory elements required for their multiple functions in later development which precludes their elimination.

Second, the higher dipterans evolved features of extraembryonic development which are unique among insects (Panfilio, 2008; Rafiqi et al., 2008). Whereas all other insect orders retain two extraembryonic membranes, the amnion and the serosa, the higher dipterans have only one membrane, the amnioserosa, which is derived from a narrow stripe of cells straddling the dorsal midline. Amnioserosa specification requires high (peak) levels of BMP signalling provided with high temporal and spatial precision (Fig. 1). Much of the complexity of the network observed in *D. melanogaster* appears to be dedicated or adjusted to this spatiotemporal refinement process, which does not occur in *T. castaneum*. Thus, the two embryo-specific Tsgs (*tsg1* and *srw)* are mainly required to generate peak levels of BMP signalling (reviewed in O'Connor et al., 2006; Fig. 4). *Dm-tld* function has adapted to this context since *Dm-tok* is unable to replace *Dm-tld* (Serpe et al., 2005), although both cleave Sog *in vitro*. This suggests that following gene duplication protein evolution has fine-tuned the binding properties and enzymatic activity of Dm-Tld to optimise the fast formation of BMP peak levels in the early embryo. The second BMP ligand Dm-Scw, which forms heterodimers with Dm-Dpp, is also mainly required for peak-levels of BMP signalling. Scw/Dpp heterodimers activate receptor complexes containing two type I receptors (Tkv and Sax). This leads to synergistic signalling. In addition, a positive feedback is initiated as increased receptor signalling enhances ligand binding (Wang and Ferguson, 2005; Umulis et al., 2006, 2010). In *T. castaneum* neither a second BMP ligand nor a second type I receptor are required for early BMP gradient formation (R.N.F and S.R.; unpublished results, Weber, 2006). Although functional studies have not been performed, a less complex system of extracellular BMP modulators is also found in the basal Diptera *Anopheles gambiae* (Goltsev et al., 2007), which like *T. castaneum* still possesses two extraembryonic membranes. This suggests that much of the specific complexity found in the BMP signalling in the early *D. melanogaster* embryo can be correlated with the invention of the amnioserosa. Indeed, amnioserosa formation needs to be tightly controlled since even minor reduction in the amount of this tissue (due to slightly reduced BMP levels) has lethal consequences for the embryo (e.g. Arora and Nusslein-Volhard, 1992).

Third, several observations indicate that the Dorsal/Toll signalling pathway has a more prominent function for DV axis formation in *D. melanogaster* compared to *T. castaneum*. (1) In *D. melanogaster* Dm-Dorsal is directly involved in the establishment of ventral cell fates, while in *T. castaneum* the Tc-Dorsal gradient disappears before these cell fates are specified (Chen et al., 2000; Nunes da Fonseca et al., 2008; Roth et al., 1989, Cowden and Levine, 2003, Wheeler et al., 2005). (2) Dm-Dorsal acts as a direct transcriptional inhibitor of *Dm-dpp*, *Dm-zen* and *Dm-tld* within the neurogenic region (Huang et al., 1993; Kirov et al., 1994), while in *T. castaneum* Tc-Dorsal does not repress *Tc-dpp*, *Tc-zen* and *Tc-tld* (Chen et al., 2000; Nunes da Fonseca et al., 2008, this paper). (3) Dm-Dorsal activates *brinker* in the neurogenic ectoderm, leading to transcriptional repression of *Dm-dpp* and Dpp target genes (Jazwinska et al., 1999). In contrast, in *T. castaneum* a *brinker* homologue exists, but it is not expressed in the early embryo (van der Zee et al., 2006). Together it is possible that these changes have relaxed the requirement for extracellular BMP inhibitors, like Dm-Sog, in DV patterning of the ectoderm and in particular in establishing the neurogenic region. *Dm-sog* mutant embryos have only minor defect in the CNS. In contrast, *sog* plays a crucial role in DV patterning and is essential for neural development in *T. castaneum* (van der Zee et al., 2006) and the spider *Achaearanea tepidariorum* (Akiyama-Oda and Oda, 2006). The same applies for *sog* homologues in vertebrates (De Robertis and Kuroda, 2004), echinoderms (Lapraz et al., 2009) and hemichordates (Lowe et al., 2006) indicating that sog's role in BMP inhibition during DV patterning is ancestral within bilateria. Thus, we believe that *T. castaneum* represents the ancestral situation with regard to the basic function of *Tc-sog*. Phylogenetic reconstruction suggests that at least within insects this also holds true for the *Tc-tsg* and *Tc-tld*. However, with regard to other specific features of the BMP signalling network we cannot rule out that the early *T. castaneum* embryo uses a derived system with a reduced number of components. For instance, *T. castaneum* might represent an exception regarding the number of ligands and receptors employed in early patterning. Both *D. melanogaster* and vertebrates employ at least two types of BMP ligands (BMP4/2–Dpp and BMP7–Gbb) and two type I receptors, similar to Tkv and Sax for DV patterning (Little and Mullins, 2009). *T. castaneum* also uses Gbb and Sax, however not during early, but rather during late DV patterning in the growth zone (R.N.F and S.R.; unpublished results, Weber, 2006). This observation might indicate that the early BMP network of *T. castaneum* is secondarily simplified. However, a broader species sampling is required to substantiate this conclusion. This should be possible in the near future since several insect species covering a wide phylogenetic range are now accessible for molecular and functional studies (Lynch et al., 2010).

Acknowledgments

We are grateful to Jessica Cande and Mike Levine (UC Berkeley), and Ginés Morata (Universidad Autonoma de Madrid, Spain) for the gift of Tc-Dorsal and pMad antibody, respectively. Jeremy Lynch and Kristen Panfilio provided insightful comments on the manuscript. R.N.d.F and M.v.d.Z were members of the International Graduate School in Genetics and Functional Genomics, Cologne. This project is part of the DFG CRC680, University of Cologne. R.N.d.F acknowledges the support of CNPQ, FAPERJ, FUNEMAC and INCT-Entomologia Molecular. Author contributions: R.N.d.F. and S.R. designed research; R.N.d.F., M.v.d.Z., and S.R performed research; R.N.d.F., M.v.d.Z., and S.R. analysed data; and R.N.d.F. and S.R. wrote the paper. All authors approved the final version to be published. The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.05.019.

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