Novel functions for Dorsocross in epithelial morphogenesis in the beetle *Tribolium castaneum*

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**ABSTRACT**

Epithelial morphogenesis, the progressive restructuring of tissue sheets, is fundamental to embryogenesis. In insects, not only embryonic tissues but also extraembryonic (EE) epithelia play a crucial role in shaping the embryo. In *Drosophila*, the T-box transcription factor Dorsocross (Doc) is essential for EE tissue maintenance and therefore embryo survival. However, *Drosophila* possesses a single amnioserosa, whereas most insects have a distinct amnion and serosa. How does this derived situation compare with Doc function in the ancestral context of two EE epithelia? Here, we investigate the Doc orthologue in the red flour beetle, *Tribolium castaneum*, which is an excellent model for EE tissue complement and for functional, fluorescent live imaging approaches. Surprisingly, we find that Tc-Doc controls all major events in *Tribolium* EE morphogenesis without affecting EE tissue specification or maintenance. These macroevolutionary changes in function between *Tribolium* and *Drosophila* are accompanied by regulatory network changes, where BMP signaling and possibly the transcription factor Hindsight are downstream mediators. We propose that the ancestral role of Doc was to control morphogenesis and discuss how Tc-Doc could provide spatial precision for remodeling the amnion-serosa border.

**KEY WORDS: Dorsocross, Tribolium castaneum, Extraembryonic development, Epithelial morphogenesis, BMP signaling**

**INTRODUCTION**

The movement of cells and tissues is a prerequisite for morphogenesis and therefore for the development of all animals. Starting from a single cell – the zygote – different movements create the remarkable diversity of body plans and shapes between species. In the case of epithelial tissue sheets, their remodeling determines overall geometry and external form, as cells largely retain their neighbors and their polarity (St Johnston and Sanson, 2011). The final arrangement of embryonic tissues corresponds to the structures they build in the animal. In contrast, extraembryonic (EE) epithelia, generally called membranes, do not materially contribute to the embryo. However, while the EE membranes of amniotes (reptiles, birds, and mammals) become persistent compartments around the embryo until birth, insect EE membranes continue to actively reorganize and sculpt the embryo in late development (Panfilio, 2008).

Typically, insects have two extraembryonic membranes – the amnion and the serosa – which are both simple (monolayered) epithelia. In order to first provide protection to the embryo in the form of desiccation resistance (Goltsev et al., 2009; Jacobs et al., 2013; Vargas et al., 2014) and defense against pathogens (Jacobs et al., 2014), and later to leave the embryo uncovered, they perform a wide array of morphogenetic rearrangements. The degree to which EE reorganization affects the embryo ranges from small-scale effects on germ band extension and retraction in *Drosophila* (e.g. Lamka and Lipshitz, 1999) to whole-body axis inversions in hemimetabolous insects (Panfilio et al., 2006; Panfilio, 2008).

Underlying these global effects are morphogenetic events including reversible cell shape changes, invagination, epiboly, and less common behaviors such as fusion, rupture and whole tissue eversion.

Interactions on the gene and cell levels may differ dramatically between species even if the overall phenotypic outcome appears to be similar (Horn et al., 2015). Hence, the high diversity of EE membrane movements in insects is ideal, not only to explore different epithelial tissue behaviors, but also to disentangle which factors are conserved and which have evolved in the process of morphogenesis is regulated. Ultimately, this will lead to a more global understanding of tissue morphogenesis across the tree of life.

Despite the importance of EE membranes in insect development, little is known about their morphogenesis because *Drosophila* melanogaster, the most advanced insect model system, only possesses a single derived EE membrane, the amnioserosa. (Harden, 2002; Rafiqi et al., 2008; Schmidt-Ott, 2000). It is specified on the dorsal side of the blastoderm by the transcription factor Zerknillt (Zen; Wakimoto et al., 1984), and it essentially remains in this position until embryonic dorsal closure. Once established, the amnioserosal anlage requires input from the U-shaped group of genes throughout development (Frank and Rushlow, 1996). One of the U-shaped genes, *Dorsocross* (Doc), encodes a T-box transcription factor most closely related to the Tbx6 subfamily (Hamaguchi et al., 2004; Reim et al., 2003). *Dm-Doc* has three redundant paralogues. Loss of function of all three results in a failure of amnioserosal maintenance: although specified, the tissue develops defective morphology and the cells do not survive (Reim et al., 2003). As a consequence, germ band retraction fails, causing the characteristic U-shaped phenotype of the embryo.

*Dm-Doc* also has additional developmental roles in the body proper: in the heart and hindgut during embryogenesis, and later in the wing imaginal discs during metamorphosis (Hamaguchi et al., 2012; Reim and Frasch, 2005; Sui et al., 2012). Given the essential roles that *Dm-Doc* plays, we asked how this would compare to the situation in an insect species with the typical EE complement of a distinct serosa and amnion that cover the embryo.

Here, we present our investigation of Dorsocross function and EE development in the red flour beetle, *Tribolium castaneum*. *Tribolium* is a tractable experimental model that is more representative for EE development across the insects (Handel et al., 2000; Panfilio, 2008), and it possesses a single orthologue, *Tc-Doc*. As in *Drosophila*, we
find that Tc-Doc is essential for EE development and some of the regulatory inputs and downstream effectors are conserved, but interactions among these genetic components and their precise EE functions differ. Tc-Doc seems not to share any of the specific functions of its fruit fly counterpart for patterning, specification, or maintenance of different tissues, but rather plays a major role in EE morphogenetic events throughout development, including early membrane formation over the embryo and later active membrane withdrawal and dorsal closure. In particular, using parental RNAi and timed single and double gene knockdown embryonic RNAi, we demonstrate the modular nature of early EE morphogenesis, where BMP signaling via Tc-decapentaplegic (Tc-dpp) is an important downstream factor. Phenotypic data also identify the fellow U-shaped group member Tc-hnt (Tc-hnt) as necessary for some of these events. By comparing the expression and function of Doc between Tribolium and Drosophila, we not only follow the evolution of the transcription factor Dorsocross but also of extraembryonic development itself.

RESULTS

Early Tc-Doc expression depends on Tc-Dpp in the amnion and Tc-Zen1 in the serosa

Early Tribolium development has been well characterized morphologically (Benton et al., 2013; Handel et al., 2000; Koelzer et al., 2014; Strobl and Stelzer, 2014). Landmark events are the differentiation of the blastoderm into serosa and germ rudiment (embryo and amnion); the flattening of the posterior pole at the primitive pit stage, marking the onset of morphogenesis; and subsequent EE fold migration to fully envelop the embryo during the serosal window (SW) closure stage (Movie 1, WT). The resulting egg topology consists of an inner amniotic cover over the embryo’s ventral surface and an outer serosal cover that encloses embryo, amnion and yolk (shown schematically in Fig. 8).

Tc-Doc mRNA is first detected at the undifferentiated blastoderm stage in a dorsal anterior domain. As the blastoderm differentiates, this domain expands throughout the entire serosa (Fig. 1A,B, Fig. S1). Earlier reports described strong dorsal serosal expression only (Nunes da Fonseca et al., 2008; van der Zee et al., 2005). We additionally detect weaker expression in the ventral serosa, probably reflecting the increased sensitivity of our longer, exon-only probe (Fig. S2). Shortly after the primitive pit stage, a new domain forms in the posterior amnion, which will fold over to cover the embryo (Fig. 1C, orange arrowhead). Meanwhile, serosal expression becomes restricted to the tissue border (Fig. 1C, blue arrowhead), the domain that will later form the rim of the serosal window (Fig. 2A).

In flies, extraembryonic expression of Doc requires the combined activity of Dpp and Zen (Rafiji et al., 2012; Reim et al., 2003). We therefore tested the expression of Tc-Doc in response to knockdown of Tc-dpp or Tc-zen1, the Tribolium orthologue of Dm-zen that is responsible for serosal specification (van der Zee et al., 2005). Tc-dppRNAI embryos show severe dorsal-ventral patterning defects, with a strongly ventralized fate map shift (van der Zee et al., 2006). In these embryos, Tc-Doc is absent from its undifferentiated blastoderm domain (Fig. 1D) and the posterior amniotic fold (Fig. 1F, orange arrowhead), which are both dorsal regions (Nunes da Fonseca et al., 2008). In contrast, expression in the (ventralized) serosa is still present and retracts to the tissue border during early morphogenesis (Fig. 1E,F, blue arrowheads; Fig. S3), similar to expression in the wild type. Conversely, after Tc-zen1 RNAi, Tc-Doc expression is still present at the undifferentiated blastoderm stage (Fig. 1G), but fails to expand ventrally into what would have been the serosal domain (Fig. S1), while amniotic expression is unaffected (Fig. 1H, orange arrowheads). The functionally distinct paralogue Tc-zen2 did not affect Tc-Doc expression (Fig. S4A-D). Thus, similar to the situation in Drosophila, in Tribolium, Tc-zen1 and Tc-dpp serve as positive regulators of Tc-Doc, although here their activities are spatially complementary (Tc-zen in the serosa, Tc-dpp in the amnion) rather than combinatorial, reflecting differences in the number of extraembryonic tissues.

Late Tc-Doc expression is amniotic and mesodermal

At the serosal window stage, distinct bilateral Tc-Doc expression domains emerge over the posterior-lateral part of the head lobes (Fig. 2A). Fluorescent in situ hybridization shows that these domains encompass both inner and outer tissue layers of the SW rim.
Within the dorsal mesoderm (Fig. 2E,F, red arrows), where the specification or maintenance of the EE tissues, early mesoderm specification or embryonic segmentation (Fig. S6A-D, Movies 1,2). As Doc is a key component for cardiogenesis in Drosophila (Reim and Frasch, 2005), we also tested Tc-Doc function in Tribolium heart development, using an enhancer trap line that marks the cardioblast cell row and in situ hybridization against the cardioblast marker Tc-midline (Koelzer et al., 2014). However, no obvious defects in heart development could be detected (Fig. S6E,F, Movie 2; see Discussion), consistent with the absence of Tc-Doc expression from this domain (Fig. S4E).

Rather than the EE maintenance defects known from Drosophila, in Tribolium, we found several temporally distinct defects linked to the morphogenetic movements of the extraembryonic tissues (Fig. 3B).

**Early Tc-Doc RNAi phenotype: impaired closure of the serosal window**

SW closure is the first major EE rearrangement. Both the serosa and the amnion must undergo intra-tissue fusion and inter-tissue detachment in order to create complete, distinct EE covers (Fig. 8D-F, schematic). While the amnion then extends with the embryo, the serosa remains largely static under the eggshell, to which it becomes attached through its secreted cuticle (Hilbrant et al., 2016; Jacobs et al., 2013).
After Tc-Doc RNAi, serosal window closure often does not occur (Fig. 3B, 49%, n=108). Typically, the posterior amniotic fold slows down and stops moving anteriorly (Movie 1). As a consequence, the embryo remains tethered to the serosa via the amnion, anchoring the head at the anterior ventral position where the SW usually closes (Fig. 4A,B, Movie 1). This physical constraint causes diverse posture defects during germ band extension as the body twists and curls, occasionally with the gnathal segments protruding through the open SW (Fig. 4C). Remarkably, embryos can – at least partially – recover from many of these posture defects during germ band retraction.

Mid-development Tc-DocRNAi phenotype: impaired extension of the posterior germ band
To test whether the germ band posture defects are exclusively secondary effects of the head being anchored at a ventral position, we used embryonic RNAi (eRNAi) to circumvent the early SW defect (see Fig. S7 for an overview on eRNAi timing). When injected at 11.4-12.4 h after egg lay (hAEL), none of the embryos showed a defect in SW closure or the associated head anchoring phenotype. However, many embryos (15 of 32) still exhibited a transiently curled abdomen. Abdominal extension itself seems normal but lacks guidance over the posterior pole of the egg (Fig. 4D, Movie 3). This shows that Tc-Doc is necessary for proper extension of the abdomen independent of the serosal window defect.

Tc-Doc is upstream of specific Tc-decapentaplegic and Tc-iroquois domains
We next inspected the Tribolium orthologues of several developmental genes associated with Doc or involved in EE development in Drosophila (Hamaguchi et al., 2004, 2012; Reim et al., 2003; Reim and Frasch, 2005; Sui et al., 2012). For example, Drosophila Doc has a morphogenetic role in the wing imaginal disc, where it regulates extracellular matrix remodeling through Matrix metalloproteinase 2 (Mmp2, Sui et al., 2012). However, we found that parental RNAi against the Tribolium orthologue was lethal prior to the SW stage, in contrast to results from Knorr et al. (2009) (Fig. S8). RT-qPCR analysis also showed no change in the low levels of Tc-Mmp2 during early embryogenesis after Tc-Doc RNAi (Fig. S5C). Meanwhile, although the EE marker genes Tc-zen1, Tc-zen2 and Tc-pannier (van der Zee et al., 2005, 2006) were...
unaffected by Tc-Doc RNAi, we did find altered expression profiles for Tc-dpp and Tc-iroquois (Tc-iro).

Early Tc-dpp expression peaks in a stripe domain along the serosa/germ rudiment border (Chen et al., 2000; van der Zee et al., 2006). We found that subsequent wild-type expression of Tc-dpp largely resembles Tc-Doc expression, including increasing expression in the posterior amnion during the SW stage and expression in the lateral SW domains over the head lobes (compare Fig. 5A,C with Fig. 2A,C). After Tc-Doc RNAi we found two distinct changes in Tc-dpp expression. Firstly, the early posterior amniotic expression was much stronger (Fig. 5B,D, orange arrow). Secondly, Tc-dpp was initially absent in the lateral SW domains (Fig. 5B, orange arrowheads), but was then expressed throughout the whole SW at the time when it would already have closed in the wild type (Fig. 5D). We confirmed the local activity of Tc-Dpp in these domains with antibody staining against its downstream effector pMAD (Persson et al., 1998). In the wild type, strong pMAD accumulation is restricted to the lateral SW domains and adjacent regions of the serosa (Fig. 5E). In contrast, Tc-DocRNAi embryos showed pMAD in the posterior amnion and posterior SW, but without distinct lateral SW domains (Fig. 5F).

The earliest changes in expression of Tc-dpp and pMAD in response to Tc-Doc RNAi occur just when the Tc-DocRNAi phenotype first manifests. We therefore asked if loss of Dpp signaling could also affect SW closure. The profound ventralization resulting from pRNAi of Tc-dpp precludes SW formation (van der Zee et al., 2006). We therefore used eRNAi to avoid these early patterning defects and injected dsRNA against Tc-dpp at various time points (Fig. S7). Even when injecting at 7-8 hAEL, the phenotype was similar to that after pRNAi (Fig. S3). However, with injections at 11.3-12.4 hAEL, all embryos managed to close the SW phenotype was similar to that after pRNAi (Fig. S3). However, with time points (Fig. S7). Even when injecting at 7-8 hAEL, the patterning defects and injected dsRNA against Zee et al., 2006). We therefore used eRNAi to avoid these early

However, parental Tc-iro RNAi did not produce any defect in closing the SW (data not shown).

**Knockdown of Tc-hindsight resembles the Tc-DocRNAi phenotype**

In Drosophila, one of the main downstream targets of Doc in the amnioserosa is hindsight (hnt), another member of the U-shaped group (Reim et al., 2003). We therefore tested its expression and function with respect to Doc in Tribolium. Wild-type expression of Tc-hnt is exclusively serosal (Fig. 6A-D) and upon Tc-Doc RNAi we did not detect a clear change in its expression pattern by in situ hybridization or its transcript levels by RT-qPCR (Fig. S5C). However, after Tc-zen1 RNAi, Tc-hnt is ectopically expressed in amniotic tissue folds (Fig. 6F), which is highly reminiscent of Tc-Doc expression after Tc-zen1 RNAi (Fig. 2G) and is spatially distinct from an early, Tc-zen1-independent terminal domain of Tc-hnt (Fig. 6E). In later stages, Tc-hnt is also expressed in an actin-rich amniotic crease in the Tc-zen1RNAi background (Fig. 6G, arrowhead; Panfilio et al., 2013).

As pRNAi of Tc-hnt led to fertility, we used eRNAi to investigate its function. Although Tc-Doc expression examined by in situ hybridization was unaffected, early injections for Tc-hnt RNAi (7.8-8.8 hAEL, see Fig. S7) produced a similar phenotype to that of Tc-DocRNAi embryos: open SW, anchored head and posture defects (Fig. 6H, Movie 5, hnt). An embryonic double knockdown of Tc-hnt and Tc-Doc did not significantly worsen the early phenotype (Fig. 6I, Movie 5, Doc/hnt). Thus, Tc-hnt and Tc-Doc show a highly similar early RNAi phenotype despite being largely expressed in different tissues at the SW closure stage (see Discussion).

**Late Tc-DocRNAi phenotype: ectopic extraembryonic membrane rupture and failure of dorsal closure**

In the wild type, the EE membranes must actively withdraw from the embryo to facilitate dorsal closure. This begins when the amnion and the serosa rupture underneath the head when the embryo has undergone 72% of development (Koelzer et al., 2014). The membranes then pull back to the dorsal side, where the serosa forms the characteristic dorsal organ and sinks into the yolk while the amnion provides a temporary epithelial cover until the flanks of the embryo join at the dorsal midline. Having completed

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**Fig. 5. Expression of Tc-dpp, pMAD and Tc-iro are altered after Tc-Doc RNAi.** (A,C) In the wild type, Tc-dpp is expressed in the same bilateral domains (orange arrowheads) as Tc-Doc (Fig. 2A). (B,D) After Tc-Doc RNAi, these expression domains are absent, although Tc-dpp is expressed earlier in the posterior amnion (orange arrow) and more strongly throughout the late serosal window (D). (E,F) Similarly, antibody staining against the downstream effector pMAD shows a loss of the bilateral domains and an expansion of signaling throughout the serosal window and the posterior amnion. (G-H) Transcript enrichment of the amnion marker Tc-iro specifically in the bilateral region is also lost. All images are ventral views with anterior left. (A’-H’) Nuclear counterstains. Scale bar: 100 µm.
their roles, both EE tissues then undergo apoptosis (Panfilio et al., 2013).

After Tc-Doc RNAi, we found that EE membrane rupture was frequently impaired (Fig. 3B, 69%, n=137). Clearly, no rupture took place when the SW was still open (Fig. S5E, 27%; Movie 2). Furthermore, rupture often occurred ectopically: either posteriorly only or from both poles (Fig. 7A,B, Fig. S5E, 42%; Movie 6). Remarkably, posterior rupture resulted in successful dorsal closure in a few cases, highlighting the high plasticity of late EE development, also seen after Tc-zen1 RNAi (Panfilio et al., 2013). However, rupture and withdrawal are highly dynamic and require tight coordination between the two EE membranes. Consequently, the vast majority of Tc-DocRNAi embryos showed withdrawal defects that compromised tissue integrity, resulting in a fatal outflow of yolk (Movie 2).

As Tc-Doc parental RNAi efficiency waned with time after injection, we occasionally observed a failure to complete dorsal closure, despite apparently normal rupture, withdrawal and dorsal organ formation (17%, n=23, 13 days after injection). In these cases, the zipper of the flanks (Panfilio et al., 2013) initiated, but then arrested and reversed (Fig. 7C,D, Movie 7).

**DISCUSSION**

Extraembryonic development in Tribolium is highly dynamic, with key events being serosal window closure, membrane rupture and withdrawal, and dorsal closure. These events are also the most morphogenetically challenging ones, and therefore particularly susceptible to perturbation. Nonetheless, it is striking that knockdown of Tc-Dorsocross interferes with all of these events without introducing any detectable defect in EE tissue specification or maintenance. Here, we first discuss the different Tribolium RNAi phenotypic features before we consider how Dorsocross and its developmental context have evolved within the insect lineage.

**Tribolium Dorsocross is a key regulator of serosal window closure**

Closure of the serosal window involves extensive epithelial reorganization to produce two separate EE covers over the embryo, and Tc-Doc plays a major role in this process. Tc-Doc is expressed throughout the posterior amniotic fold and at the border of the serosal window (Fig. 1C), and after Tc-Doc RNAi the earliest defect is the slowing down and stagnation of morphogenesis in these regions (Movie 1). We further show that Tc-Doc regulates BMP signaling in these domains, preventing early signaling in the posterior amniotic fold (Fig. 5A-F). Due to its role in dorsal-ventral patterning, we could not directly test the contribution of BMP...
signaling to early fold progression (Fig. 8A,C) but suggest that restriction of BMP signaling is important based on the expression patterns we observed. This is also consistent with the observation that parental Tc-dpp RNAi leads to greater invagination of embryonic tissue than in the wild type (van der Zee et al., 2006).

With our investigations of Tc-hnt, we also identified a second potential mediator of Tc-Doc function. It has been reported that a structure reminiscent of a supracellular actomyosin cable at the amnion-serosa border is responsible for final closure of the SW (Benton et al., 2013). The regulation of such a cable could help to explain the specific role of Tc-Doc and the high similarity of the Tc-Doc and Tc-hnt knockdown phenotypes (compare Movie 1 with Movie 5), despite the fact that Tc-hnt expression is serosal while Tc-Doc is predominantly amniotic at this stage. An actin cable would require input from upstream activators only in a small, defined domain at the rim of the early window, and indeed, this is the region where Tc-hnt and Tc-Doc expression coincides (compare Fig. 1C, Fig. 2A-C with Fig. 6B,C). Furthermore, simultaneous RNAi of both genes did not lead to a more severe phenotype (Fig. 6I, Movie 5), suggesting that they act in the same pathway.

Although we could not detect a role for either gene in regulating the other, subtle changes in expression at the SW rim after RNAi may have escaped our detection methods, masking a potential epistatic relationship between these genes of the U-shaped group. We suggest that Tc-Doc may be upstream of Tc-hnt. Firstly, Drosophila Doc is upstream of hnt in the amnioserosa (Reim et al., 2003). More importantly, the Tribolium genes have the same expression pattern after Tc-zen1 RNAi (Fig. 2G, Fig. 6F). Despite the complete loss of the serosa, Tc-hnt – like Tc-Doc – is found in the amniotic rim. While Tc-Doc expression still occurs in its wild-type domain in amniotic tissue bordering the embryo, Tc-hnt, which would be expected to be absent in serosa-less Tc-zen1 RNAI eggs, is now present in the same domain. Assuming an interaction between the two genes, the most parsimonious explanation would be that input from Tc-Doc induces Tc-hnt ectopically in the amnion. Consistent with this hypothesis and with a specific morphogenetic
role, Tc-hnt is specifically expressed at the site of an amniotic actin cable at later stages (Fig. 6G, see also Panfilio et al., 2013). Furthermore, given that Tc-hnt is expressed throughout the serosa (Fig. 6B-D), it is more likely to be input from the spatially restricted Tc-Doc (Fig. 2A-B) that governs SW closure.

In summary, we suggest that Tc-Doc plays an essential role in SW closure by suppressing BMP signaling in the early amnion while activating Tc-hnt in the rim of the SW, thereby facilitating both fold progression and final constriction for window closure (Fig. 8A,C,E). Future elucidation of which actin binding proteins may be upregulated by Tc-Doc will clarify the link between transcriptional regulation and morphogenetic output.

Intra-tissue fusion and inter-tissue detachment are distinct extraembryonic events

Several events must occur for successful SW closure. After the posterior amniotic fold migrates anteriorly, the amnion and serosa must undergo intra-tissue fusion concomitant with inter-tissue separation (Fig. 8D-F). Although wild-type detachment of the amnion and serosa has not been documented directly, rotation of the embryo shortly after closure shows that the serosa and the amnion are free to move independently (Hilbrant et al., 2016; Koelzer et al., 2014). One frequent early defect in Tc-DocRNAi embryos is that the head does not extend anteriorly (Fig. 4B,C) as a result of its indirect anchoring to the serosa via the amnion. This corresponds to the site of bilateral SW expression of Tc-Doc and Tc-dpp, and we show that BMP signaling is delayed and no longer tightly localized to this domain after Tc-Doc RNAi (Fig. 5B,F). Furthermore, even when intra-tissue fusion occurred after late injection of RNAi of Tc-dpp, some embryos (20%) still exhibited mild abnormalities consistent with an amnion-serosa detachment defect (Movie 4). Hence, we distinguish detachment as a separate event (Fig. 8A,F).

The role of Dm-Doc in bending of the wing imaginal disc provides an explicit example of this transcription factor acting upstream to promote cell shape change via reorganization of the cellular microtubule web and a reduction of integrins and extracellular matrix components, probably through activation of Matrix metalloproteinase 2 (Sui et al., 2012). While such remodeling events would also be important for epithelial reorganization during Tribolium SW closure, it remains unclear if subtle changes in Tc-Mmp2 expression after its essential early role (Fig. S8) can contribute to the morphogenetic function of Tc-Doc, or whether other effectors are used here.

Tc-Doc contributes to tissue coordination throughout late morphogenesis

Even when SW closure and membrane detachment are successful, almost half of the late injected Tc-DocRNAi embryos still showed an abdominal posture defect (Fig. 4D, Movie 3) that correlates with amniotic expression (Fig. 1C, Fig. 2C,D). In addition, late Tc-dpp eRNAi resulted in a similar abdominal phenotype (Movie 4). This suggests that regulation of BMP signaling by Tc-Doc in the amnion ensures proper extension of the posterior germ band, distinct from the earlier event of posterior fold progression (Fig. 8A, asterisk; see below). As germ band extension requires the coordination of the amnion with the embryo, extracellular signaling is likely to be important in regulating cellular rearrangements in both tissues (e.g. Nakamoto et al., 2015).

Our results further identify Tc-Doc as a new essential component for late EE morphogenesis. Impaired EE withdrawal after Tc-Doc RNAi (Fig. 7A-B) suggests that there is an underlying defect in biomechanical competence of the serosa and amnion to rupture at the endogenous anterior position (Hilbrant et al., 2016). It will be interesting to see whether Tc-Doc interacts with known, late EE-specific regulators such as Tc-zen2 (van der Zee et al., 2005). Specific perturbations in the late dorsal closure event of epidermal zipping (Fig. 7C,D) also highlight a potential role of Tc-Doc in embryonic-extraembryonic tissue coordination.

Evolution of Dorsocross expression and function

We show that Dorsocross has multiple morphogenetic functions in Tribolium extraembryonic development. To understand the evolution of the functions of Doc within insects, we must first consider the changes in EE membrane configuration between the species we compare. In dipterans that possess two EE membranes, Doc is initially expressed in a dorsal domain that comprises the prospective amnion and serosa, but is later excluded from the serosa (Anopheles: Goltsev et al., 2007; Megaselia: Rafiqi et al., 2010). It has been suggested that the repression of Doc from the serosa is mediated by persistent zen expression and a switch in its role from initial activation (Rafiqi et al., 2010, 2012; Reim et al., 2003). Although Tc-Doc clears from the serosa during the SW stage (Fig. 2C), so too does Tc-zen1 (Sharma et al., 2013a), whereas Tc-zen2 does not affect Tc-Doc expression (Fig. S4A-D), indicating that such a repressive interaction is not conserved.

In Drosophila, Dm-Doc is necessary for the maintenance of the single amniopersosal tissue (Reim et al., 2003). Loss of function results in a defect in germ band retraction, causing the eponymous U-shaped embryonic phenotype. However, the dependence of germ band retraction on EE membranes evolved within the cyclorrhaphan fly lineage (Rafiqi et al., 2010). In Tribolium, the complete loss of the serosa and fundamental changes in amnion topology after Tc-zen1 RNAi do not disturb germ band retraction (Panfilio et al., 2013; van der Zee et al., 2005). Thus, the morphogenetic context of Tc-Doc function is fundamentally different from that of Dm-Doc. In Megaselia, knockdown of Ma-Doc leads to an end-stage dorsal open phenotype, which has been attributed to a loss of amniotic cells (Rafiqi et al., 2010). This could be consistent with the maintenance function seen in the Drosophila amniopersosa, or with a loss of tissue integrity due to morphogenetic defects, as in Tribolium. Future investigations into cell and tissue rearrangements are necessary to fully address the function of Doc in Megaselia. Meanwhile, we suggest that Doc’s ancestral function was to direct extraembryonic morphogenesis and that this role has been lost in the dipteran lineage.

Conversely, the diverse roles of Doc in the body proper appear to be an innovation within the Drosophila lineage. These roles include cardioblast specification (Reim et al., 2005), dorsal-ventral patterning of the hindgut (Hamaguchi et al., 2012) and fold progression of the wing imaginal disc (Sui et al., 2012). Of these, Tc-Doc expression in the early mesoderm (Fig. 2E,F) would be suggestive of a role in heart development in Tribolium as well. However, Tc-Doc is not detectable in the cardioblast cells themselves (Fig. S4E), and we do not observe a clear heart-specific defect after Tc-Doc RNAi (Fig. S6F). Nonetheless, future work examining Doc protein expression (Reim et al., 2003) and focusing on heart development in a genetic background rescued for EE-associated postural defects that could obscure heart morphology (Reim and Frasch, 2005) could more directly address this issue.

The evolution of BMP signaling deployment in relation to Dorsocross

Consistent with the changing roles of Doc between Tribolium and Drosophila, we also found differences in the corresponding gene
regulatory networks employed in EE development (Fig. 8A-B). While strictly upstream of Doc in Drosophila, BMP signaling through the ligand Dpp plays a major role in mediating several morphogenetic functions of Doc in Tribolium. It seems counterintuitive that both Tc-Doc RNAi, which causes an early increase in BMP signaling in the posterior amniotic fold (Fig. 5B-F), and loss of BMP signaling after Tc-dpp RNAi result in the same abdualm extension phenotype (Movies 3, 4). However, as seen for regulation of Dm-Doc by Dm-Zen, BMP signaling in Tribolium may play distinct, stage-specific roles. Alternatively, the precise dosage of BMP signaling may be important here, as has been suggested in the context of dorsal closure in Tribolium (Sharma et al., 2013b) and Drosophila (Wada et al., 2007). Indeed, similar mechanisms to control tissue sheet movements during dorsal closure may also apply to BMP’s morphogenetic role in the early posterior amnion. The lack of Dpp regulation by Doc in Drosophila could be because Drosophila EE morphogenesis is highly reduced compared with Tribolium, eliminating the need for morphogenetic BMP signaling in the amnioserosa. Further investigation of the regulatory context and feedback loops in which BMP signaling operates (e.g. Gavin-Smyth et al., 2013), including in additional insect species, will enhance our understanding of the role of this pleiotropic pathway in epithelial remodeling.

Altogether, our results open the way for a better understanding of the diverse epithelial reorganization events observed in Tribolium and in animals in general. They also provide an excellent case study for investigating how transcription factors can change function during evolution, including switching from morphogenetic instruction to a tissue maintenance function, and changing expression from extraembryonic to embryonic territories to acquire novel roles in the developing embryo.

MATERIALS AND METHODS

Tribolium castaneum (Herbst) stocks

San Bernardino (SB) wild type (Brown et al., 2009), nuclear GFP (nGFP) (Sarrazin et al., 2012), and heart (Gβ4609) and serosa (GI2424) enhancer trap (Koelzer et al., 2014) lines were kept under standard culturing conditions (Brown et al., 2009) at 30°C, 40% RH. SB and nGFP were used for in situ hybridization, while the nGFP and enhancer trap lines were used for live imaging.

Gene-specific knockdown, histology, in situ hybridization and immunohistochemistry

Parental RNAi was performed as described (van der Zee et al., 2005), with double-stranded RNA (dsRNA) resuspended in double-distilled water (ddH2O). The total mass of injected dsRNA was typically 0.3-0.4 µg per adult female and 0.1-0.15 µg per pupa. For Tc-Doc a total of 16 independent parental RNAi experiments were conducted, and offspring embryos were analyzed within 14 days after injection. Embryonic RNAi was typically performed with 1 µg/µl dsRNA in ddH2O. Three rounds of injections were performed to confirm qualitatively similar phenotypes for Tc-Doc, Tc-hnt and Tc-Doc/Tc-hnt knockdowns: dsRNA concentration was lowered to 0.6 µg/µl for single knockdowns to achieve lower penetrance (SW open: 61% for Tc-Doc and 17% for Tc-hnt, n=23 each). Double knockdown was then performed with 0.6 µg/µl dsRNA for both genes, which restored penetrance (89%, n=28) but did not qualitatively change the phenotype. The numbers of eRNAi experiments per gene and time point are listed in Fig. S7. Fuchsin staining (‘histology’), Fig. 3A, Fig. S5A) and cuticle preparation (Fig. 3A, Fig. S5B) were performed based on standard protocols (van der Zee et al., 2005; Wigand et al., 1998). In situ hybridization was performed as described (Koelzer et al., 2014), except for fluorescence detection, where Fast Red (Roche) was used instead of NBT-BCIP, and images were acquired with a Zeiss LSM 700 confocal microscope. All probes were tested at least three times in independent experiments with the sense probe as a negative control. Image projections were generated with Zen2 (Zeiss) or HeliconFocus 5.3 (Helicon Soft). All primer sequences for probes and dsRNA are listed in Table S1. Antibody staining was performed using standard protocols (Brown et al., 2009) with an anti-phospho-Smad1/5 rabbit antibody (Cell Signaling, 41D10, used at 1:100) and secondary detection with anti-rabbit alkaline phosphatase (Cell Signaling, 7054, used at 1:200). After in situ hybridization or antibody staining, embryos were mounted in Vectashield mountant containing DAPI for nuclear counterstaining (Vector Laboratories).

RT-qPCR

RNA was extracted using TRizol reagent (Ambion) according to the manufacturer’s instructions with subsequent phenol/chloroform extraction to increase purity. Total RNA quality was confirmed by agarose gel electrophoresis and spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific). cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) after DNase treatment with the TURBO DNA-free Kit (Applied Biosystems). Absence of genomic DNA contamination was tested by agarose gel electrophoresis and melting curve analysis after RT-qPCR amplification with intron-spanning primers. RT-qPCR was performed on a 7500 Fast cycler (Applied Biosystems) using Fast SYBR Green Master Mix (Life Technologies). All samples were measured in three biological replicates. Ribosomal protein S3 (RpS3) was chosen as a reference gene after testing eight candidate genes using NormFinder (Andersen et al., 2004). Raw data were analyzed using LinReg (Ruijter et al., 2009) and calculated in Excel (Microsoft) using the ΔΔCt method. All primer sequences for RT-qPCR are listed in Table S1.

Live imaging

Time-lapse imaging was performed as described (Panfilio et al., 2013) using Zeiss Axioplan2 and Applied Precision DeltaVision RT microscopes with post-acquisition handling in ImageJ (NIH) and Photoshop (Adobe).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

T.H. and K.A.P. designed and performed research, analyzed data and wrote the paper.

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Supplementary information

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