Long title: A revised understanding of Tribolium morphogenesis further reconciles short and long germ development

Short title: A major revision to our understanding of short germ embryogenesis

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Abstract

In *Drosophila melanogaster*, the germband forms directly on the egg surface and solely consists of embryonic tissue (termed long germ development). In contrast, most insect embryos undergo a complicated set of tissue rearrangements to generate a condensed, bilayered germband (termed short/intermediate germ development). The ventral side of the germband is embryonic, while the dorsal side is thought to be an extraembryonic tissue called the amnion. While this tissue organisation has been accepted for decades, and has been widely reported in insects, its accuracy has not been directly tested in any species.

Using live cell tracking and differential cell labelling in the short germ beetle *Tribolium castaneum*, I show that most of the cells previously thought to be amnion actually give rise to large parts of the embryo. This process occurs via the dorsal-to-ventral flow of cells and contributes to germband extension. In addition, I show that true ‘amnion’ cells in *Tribolium* originate from a small region of the blastoderm. Together, my findings show that development in the short germ embryos of *Tribolium* and the long germ embryos of *Drosophila* is more similar than previously proposed. Dorsal-to-ventral cell flow also occurs in *Drosophila* during germband extension, and I argue that the flow is driven by a conserved set of underlying morphogenetic events in both species. Furthermore, the revised *Tribolium* fatemap that I present is far more similar to that of *Drosophila* than the classic *Tribolium* fatemap. Lastly, my findings show that there is no qualitative difference between the structures of the blastoderm and the short/intermediate germ germband. As such, the same tissue patterning mechanisms could function continuously throughout the cellularised blastoderm and germband stages, and easily shift between them over evolutionary time.
Introduction

Insects are the most speciose phylum of animals and display remarkable diversity in adult morphology (1). Insect embryo development is also very diverse, particularly in the stages leading to the formation of the elongated, segmented embryo (called the germ band) (2). The molecular and morphogenetic basis of this process is best understood in the fly *Drosophila melanogaster*. In this species, a predominantly hierarchical chain of patterning events specifies nearly all segments more-or-less simultaneously at the syncytial blastoderm stage (3). Cellularisation takes place near the end of this process, after which point morphogenetic events such as germ band extension (GBE) occur (see Fig 1 for schematic summary). The *Drosophila* mode of development is termed long germ development and is fairly representative of most true flies (4). In contrast, the vast majority of insects undergo short or intermediate germ development, meaning that only a handful of segments are specified at the blastoderm stage and the remaining segments are specified sequentially as the germ band elongates (5).

Short germ development has been best studied in the beetle *Tribolium castaneum*, and recent research has shown that development in this species is more similar to *Drosophila* than previously thought. For example, while previous molecular studies have suggested that *Tribolium* segment specification occurs in a qualitatively different way to *Drosophila* (6,7), recent detailed analyses have shown that a relatively conserved patterning network may underlie segment specification in both species (8–10).

The morphogenetic events occurring during GBE are also more conserved than previously thought. In *Drosophila*, GBE is predominantly driven by the mediolateral intercalation of ectodermal cells (i.e. convergent extension), although cell deformation along
the anterior-posterior (AP) axis and cell divisions are also involved (11–16). In contrast to this, *Tribolium* germband elongation was previously thought to be driven by the so-called ‘growth zone’ at the posterior of the germband (17). Now, however, it is clear that *Tribolium* germband elongation is also predominantly driven by mediolateral cell intercalation (see Fig 1 for schematic summary of *Tribolium* development) (7,18,19). Furthermore, in both *Tribolium* and *Drosophila*, this intercalation requires the striped expression of a specific group of Toll genes (so-called Long Toll/Loto class genes) (20,21).

It is highly likely that germband elongation mediated by cell intercalation is homologous in these two species, and probably in other arthropods, as well (21). As such, I will hereafter refer to *Tribolium* ‘germband elongation’ as ‘germband extension’/GBE, unifying the *Drosophila*/*Tribolium* terminology. In addition, as there is no evidence for a qualitatively different ‘growth zone’ in *Tribolium* (i.e. a specialised zone of volumetric growth), I will refer to the posterior unsegmented region as the ‘segment addition zone’ (8,23,24).

Despite the similarities described above, substantial differences in the embryonic fatemaps of these species exist (Fig 1). In *Drosophila*, almost the entire blastoderm is fated as embryonic tissue, and only a small dorsal region is fated as extraembryonic tissue (termed the amnioserosa) (22). In contrast, in *Tribolium*, roughly the anterior third of the blastoderm gives rise to an extraembryonic tissue called the serosa (25). Of the remaining blastoderm, a large dorsal region is thought to give rise to a second extraembryonic tissue called the amnion, with only the remaining ventral tissue giving rise to the embryo itself (26–28). Like the amnioserosa, the serosa and the amnion are proposed to support the embryo during development, but are thought to degenerate prior to hatching and not contribute to any larval or adult structures (29,30).
*Drosophila* and *Tribolium* also exhibit dramatic differences in the morphogenetic events occurring during early development (Fig 1). When GBE occurs in *Drosophila*, the germband stays at the surface of the egg and the amnioserosa largely remains in place. In *Tribolium*, on the other hand, germband extension begins with a complicated process called embryo formation (see Fig 1; for a detailed description see (18,31)). During this process, the presumptive embryonic ectoderm condenses towards the ventral side of the egg via both mediolateral cell intercalation and a cuboidal-to-columnar cell shape transition.

Simultaneously, epithelial folding and tissue involution occurs, causing the presumptive amnion to fold over the embryo to form the multi-layered germband. During these movements, the serosa cells undergo a cuboidal-to-squamous transition and spread over the entire egg surface.

The differences in fatemap and tissue folding described above show that both fatemap shifts and reductions in early morphogenetic events have contributed to the evolution of the long germ mode of development. Understanding how these differences evolved is integral to understanding the short-to-long germ transition, but in order to study how this occurred, we first need to understand how these tissues develop in each species.

The form and function of the *Tribolium* serosa has been analysed in several studies (25,32,33), and comparative research in several fly species has revealed how the loss/reduction of the serosa in flies may have occurred (reviewed in (29)). The amnion, on the other hand, has proven harder to analyse, and the precise embryo/amnion boundary at the blastoderm stage is unknown. However, a clear boundary between embryo and amnion has been proposed to exist at the germband stage (Fig 1) (26). Cells in the ventral half of the germband are thought to give rise to all embryonic structures, while cells in the dorsal half of the germband are thought to form the amnion (28,34,35). This germband structure has been
described in many insects over the past century and is proposed to represent the core conserved structure of short/intermediate germ embryos (reviewed in (2,36,37)). However, the proposed boundary between cells fated to become embryo and those fated to become amnion has not been directly tested.

I here set out to investigate the development of the presumptive amnion in *Tribolium* using a combination of fluorescent live imaging and fate mapping techniques. To my great surprise, I find that the majority of the cells previously described as ‘amnion’ actually form large parts of the embryo proper. Using fate-mapping experiments, I show that true ‘amnion’ cells originate from a very small domain of the blastoderm, just as the *Drosophila* amnioserosa cells do. I also show that the movement of cells from the ‘amnion’ side of the germband to the ‘embryo’ side of the germband occurs via the large scale flow of the ectodermal epithelium. Lastly, I describe the underlying causes of this flow, and show how this tissue movement is likely homologous to the dorsal-to-ventral tissue flow that occurs during *Drosophila* GBE.

This discovery forces a major shift in our view of development in *Tribolium* (and of short/intermediate germ insects in general) and demonstrates that there is no qualitative difference in germband tissue structure between *Tribolium* and *Drosophila*. Furthermore, this discovery has significant consequences for how we must study short/intermediate germ development going forward, as what was previously thought to be amnion (but is actually a large region of the embryo) is routinely discarded or ignored during sample preparation.

**Results**
Live cell tracking reveals movement of ‘amnion’ cells into the embryo

To examine the development of the *Tribolium* presumptive amnion in detail, I carried out high resolution live imaging of embryos transiently labelled (18) with a fluorescent histone marker (H2B-venus) to label nuclei. My goal was to track presumptive amnion cells from the blastoderm stage onwards. However, it was not possible to accurately track the majority of cells throughout embryo condensation and GBE, due to the extensive morphogenetic rearrangements that take place during this process. Instead, I focused on the stage immediately following condensation, when the multi-layered germband has formed, and the embryonic region where the presumptive amnion is closest to the surface of the egg.

Specifically, I tracked over 200 presumptive amnion cells in the central region of the embryo from the closure of the serosa window until after the formation of the thoracic segments (over 11 hours of development; Fig 2 Supplemental Movie 2). As previously described (18), the germband and yolk exhibit pulsatile movements during this period, as well as rotating within the serosa (Supplemental Movie 1).

The presumptive amnion initially consisted of many tightly packed cells that became increasingly spread out during GBE (Supplemental Movie 2, Fig 2(A-C)). However, rather than remaining restricted to the ‘amnion territory’, many of the tracked cells moved around the edge of the germband in to the ‘embryo territory’. Differential labelling of tracked cells clearly showed that these cells that moved around the germband edge became part of the embryo proper (Supplemental Movie 2 and Fig 2(A-C)). The cells that joined the ‘embryo territory’ became tightly packed, continued to divide, and formed embryonic structures (Supplemental Movie 3 and Fig 2(D-F)). In contrast, cells that remained in the ‘amnion territory’ became squamous and stopped dividing. The nuclei of these latter cells became enlarged (Supplemental Movie 3 and Fig 2(D-F)), suggesting that they underwent
endoreplication to become polyploid, as seen in the *Tribolium* serosa and in the *Drosophila* amnioserosa (22,27). In addition, several germ band nuclei underwent apoptosis (Supplemental Movie 3) as has been described in fixed embryos (38). These results show that many of the cells previously thought to be extraembryonic amnion give rise to embryonic structures. As such, it is not accurate for the entire epithelium to be called ‘amnion’ and I will instead refer to it as the ‘dorsal epithelium’ as this region is made up of dorsal/dorsolateral ectoderm.

Differential cell labelling confirms widespread dorsal-to-ventral cell movement

My next question was whether the movement of cells from the dorsal epithelium to the ventral epithelium occurs throughout the AP axis or is just limited to the thoracic region. The extensive movements of the germ band made it difficult to track individual cells accurately at the anterior and posterior poles. To overcome this problem, I combined differential cell labelling with long term fluorescent live imaging to follow small groups of nuclei throughout development. Specifically, I microinjected mRNA encoding a nuclear-localised photoconvertable fluorescent protein (NLS-tdEos) into pre-blastoderm embryos to uniformly label all nuclei, then photoconverted a small patch of nuclei at different positions along the AP axis at the final uniform blastoderm stage. I then performed long term confocal live imaging of both the unconverted and photoconverted forms of the fluorescent protein throughout the period of GBE (or longer). Unlike that of *Drosophila*, the *Tribolium* egg shell does not show any dorsoventral (DV) polarity, and I was therefore unable to specifically target particular locations along the DV axis. Instead, I opted for a brute-force approach and performed the photoconversion experiment for 50-150 embryos at each AP position (75% egg length [EL] from the posterior pole, 50% EL, 25% EL, and close to the posterior pole), and
then used the subsequent live imaging to determine the DV position of the photoconverted cells. Using a new live imaging set up (see Materials and Methods), I obtained the same range of hatching rates as I typically obtain for other microinjection experiments (approximately 80%, REF), even after continuous confocal live imaging for almost the entirety of Tribolium embryonic development (3.5 days; Supplemental Movie 4). Both unconverted and photoconverted protein persisted throughout germ band extension and retraction, although fluorescent signal faded over time. I have included various examples from this data set in Supplemental Figures 1-3. In addition, I have made the raw confocal data for a large number of timelapses available online (>300 embryos, >700 GB of data, upload in progress at time of submission) for the benefit of the community. This data will likely prove valuable for a wide range of research projects.

During my live imaging, ectodermal cell clones became elongated along the AP axis over time, as previously reported in a Tribolium study that used a non-live imaging cell clone method (19). However, Nakamoto and colleagues stated that “labelled ectodermal cells ... rarely mix with unlabelled cells” even as clones became greatly elongated. In contrast, I frequently observed non-converted nuclei in the midst of labelled nuclei (Fig 3(A-F), Supplemental Figures 1-3), confirming that extensive mediolateral cell intercalation occurs throughout GBE. This difference in clone behaviour between the two approaches may be due to differences in the size of the initial group of photoconverted cells, or in the resolution of the subsequent imaging.

When I examined clones initially located in the dorsal epithelium, I found that movement of cells from the dorsal epithelium to the ventral epithelium occurred throughout the posterior of the embryo during GBE (Fig 3(A-F), Supplemental Movie 5). I also observed the same movements at the anterior of the germ band (Fig 3(G-J)), although I have focused
my analysis on the middle and posterior parts of the embryo. Together with the cell tracking
data, these results show that most of what was previously thought to be ‘amnion’ is in fact
embryonic tissue, and that cells move from the dorsal epithelium to the ventral epithelium
throughout the germ band (summarised in Fig 3(K,L)).

Support for these findings also comes from comparing the expression pattern of the
transcription factor Caudal in both Tribolium and Drosophila. In both species, this gene is
initially expressed in a broad posterior domain that then retracts along the AP axis (39,40). In
Drosophila, expression persists for longer in dorsal tissue than ventral tissue (9). Similar
dorsoventral dynamics, although not previously remarked upon, are also evident in
Tribolium; Tc-cad expression does indeed persist longer in the dorsal epithelium than in the
ventral epithelium (Supplemental Fig 4).

Tribolium serpent may mark true ‘amnion’

As described earlier, cells that remained in the dorsal epithelium became squamous, and this
cell shape change occurred progressively along the AP axis (Fig 4(A)). This change in cell
shape may be a sign of maturation of true ‘amnion’. While characterising cell fate markers, I
found that the Tribolium ortholog of the GATA factor serpent (Tc-srp) exhibited spatial and
temporal expression dynamics that were very similar to those of the potential ‘amnion’ (Fig
4(B), Supplemental Fig 5). At the end of GBE, all but the posterior-most cells of the dorsal
epithelium were squamous (data not shown) and Tc-srp seemed to be expressed in dorsal
epithelium cells along nearly the full length of the germ band (Supplemental Fig 5(l2)).
However, this latter finding was difficult to confirm as most of the dorsal epithelium is lost
during embryo fixation at this embryonic stage (presumably due to the fragility of the
tissue). I also found Tc-srp to be expressed in several other domains, including in the
presumptive endoderm (Supplemental Fig 5). In *Drosophila, serpent* is also expressed in extraembryonic tissue (the amnioserosa) (41–44), and, therefore, Tc-*srp* may mark ‘true’ extraembryonic amnion. However, future work is required to confirm whether this putative amnion degenerates prior to hatching (as is required to be defined as extraembryonic). For simplicity, I will refer to this tissue as ‘amnion’ for the remainder of this text.

**A revised *Tribolium* fatemap**

To determine which blastoderm cells give rise to the amnion, I analysed 85 embryos in which the dorsal and dorsolateral blastoderm cells were labelled by NLStdEos photoconversion as described above. I found that amnion cells arose from a very small domain of dorsal-most cells (that tapers from its anterior to posterior extent) and from a narrow strip of cells between the presumptive embryo and presumptive serosa (summarised in Fig 5 and Supplemental Fig 6). At 50% EL, only approximately the 6 dorsal-most cells (approximately 8% of the circumference of the blastoderm) gave rise to all amnion cells stretching from one side of the thorax to the other (Fig 4(C), Supplemental Fig 1). Nearer to the posterior of the blastoderm (25% EL), even fewer cells gave rise to amnion (approximately 3 of the most dorsal cells; approximately 6% of the circumference; Fig 4(D), Supplemental Fig 2). The posterior limit of the amnion was difficult to define, as although some cells from approximately 5-10% EL appeared to become amnion (Fig 4(E)), these cells condensed posteriorly towards the hindgut during germ band retraction, and might have contributed to the hindgut tissue (Supplemental Fig 3, Supplemental movie 6). I was unable to unambiguously determine the fate of these cells. At the anterior of the embryo, I found that a narrow strip of 1-2 cells between the presumptive embryo and presumptive serosa also gave rise to amnion (Fig 3(G-J)). Substantial additional work is required to define a complete
blastoderm fate map for *Tribolium*, however, my findings show that the ‘amnion’ domain is drastically smaller than previously proposed.

**Discussion**

In this article, I have shown that the majority of cells that were previously thought to be extraembryonic amnion actually give rise to embryonic tissue. The movement of cells from the dorsal side of the germband to the ventral side was visible in live cell tracking and differential cell labelling experiments. My results also show that the true amnion region appears to differentiate progressively along the AP axis during GBE, as shown by differences in cell behaviour and the expression of the gene *Tc-srp*. Lastly, the cells that give rise to this presumptive amnion predominantly originate from a small domain on the dorsal side of the blastoderm.

**A revised understanding of the short germ embryo**

The revision to the blastoderm fate map is essentially a quantitative shift in our understanding of where cell fate boundaries lie along the DV axis. In the revised *Tribolium* blastoderm fate map (Fig 5, Supplemental Fig 6), the proportion of the blastoderm that gives rise to the presumptive amnion is much smaller than previously thought. The presumptive amnion domain is, therefore, remarkably similar in size to the amnioserosa domain of the *Drosophila* blastoderm fate map (22). However, it is important to recognize that fate maps such as those presented here show a static picture of a dynamic process. There is no evidence that the presumptive amnion is specified at the blastoderm stage in *Tribolium*. Instead, the progressive changes in cell shape and *Tc-srp* expression in the dorsal epithelium
of the germ band suggest that the amnion is specified progressively along the AP axis during GBE. Progressive specification of DV cell fates during GBE fits with previous hypotheses (34,45), and analysis of how this process occurs represents an exciting avenue of future research (possible mechanisms are discussed more later).

In contrast to the fatemap revision, the observation that cells move from the dorsal half of the germ band to the ventral half of the germ band represents a qualitative shift in our understanding of development in short/intermediate germ insects. In the classic model of short/intermediate germ development, the germ band was thought of as a more-or-less flat sheet of ectodermal cells (with mesoderm underneath) covered dorsally by the extraembryonic amnion. Because of this, the entire dorsal epithelium is routinely removed during embryo preparation, or not included in descriptions of gene expression patterns and embryonic phenotypes. Based on the new data presented here, it is obvious that we have been discarding or ignoring large parts of the embryo. Furthermore, the movement of cells from the dorsal epithelium into the ventral epithelium must be contributing to GBE, and is, therefore, a key aspect of the extension and overall development of the germ band that has thus far been missed.

The revised model of the germ band does present some technical challenges for future work on short/intermediate germ embryo. The flattened geometry of the germ band makes it difficult to image both the dorsal and ventral epithelium using bright-field microscopy approaches. However, this problem can be overcome either by using fluorescence based techniques and confocal microscopy or by mechanical sectioning of the germ band. Both approaches have been shown to work well in Tribolium (for example see (7,46) and results in this manuscript). In the rest of this article, I discuss why the revised fatemap and cell flow accord well with what we know about Tribolium development, and
outline the implications of this discovery on our understanding of the evolution of insect development.

The cellular and molecular causes of tissue flow unify the blastoderm and the germband

The revised model of the Tribolium germband reconciles the blastoderm and germband stages. The ectoderm of the germband is a continuous epithelium, which means that the movement of cells from the dorsal epithelium to the ventral epithelium occurs as a tissue level ‘flow’. Such dorsal-to-ventral tissue flow also occurs during embryo condensation in Tribolium (18), and I propose that the flow is caused by largely the same morphogenetic processes at both stages. The evidence for this hypothesis is summarised here, but for an extended discussion see Appendix section 1.

Three morphogenetic processes contribute to dorsal-to-ventral cell flow in Tribolium, and at least two of the three occur at both the blastoderm and germband stages. First, mediolateral cell intercalation occurs at both stages and causes tissue-wide convergence (along the DV axis) and extension (along the AP axis). This process requires two Toll genes that are expressed in rings around the entire blastoderm and germband epithelium (21).

Second, tissue specific cell shape changes occur at both stages such that ventral/lateral cells become columnar and dorsal/dorsolateral cells become thinner (during condensation (Supplemental Fig 7)) or squamous (during GBE). The tissue level effect of these changes is contraction of the ventral/lateral tissue and spreading of the dorsal tissue. The flattening of dorsal/dorsolateral cells is likely regulated by BMP signalling, as not only does BMP activity correlate with the cell shape changes (see Appendix section 1), but functional disruption of BMP signalling components leads to uniform cell shape changes along the DV axis (28,47). A third major morphogenetic event is gastrulation of the mesoderm. This occurs along the
ventral midline, and as gastrulation occurs, the ectoderm moves ventrally to seal the gap left in the epithelium (46). At the stage when a complete germ band has formed, gastrulation is complete along most of the embryo. However, current data suggests mesoderm gastrulation may be ongoing in the segment addition zone (46). If true, the ongoing invagination would contribute to tissue flow in this region.

It is important to note that while each of the events described here is involved in the dorsal-to-ventral tissue flow, no single event is absolutely required for it. In the absence of cell intercalation, embryo condensation and thinning of dorsal/dorsolateral ectoderm still takes place yielding abnormally wide and short germ bands (21). In the absence of tissue specific cell shape changes, condensation occurs in a more radially symmetrical manner yielding a tube-shaped germ band that undergoes segment specification and convergent extension (28,47). Finally, both condensation and GBE are only mildly affected in the absence of mesoderm specification (48). This functional independence comes from each of the three process being specified by different pathways (intercalation via segment specification, dorsal thinning via dorsal tissue specification, and gastrulation via ventral tissue specification). There may be further, as yet undiscovered morphogenetic events that also contribute to the dorsal-to-ventral tissue flow.

Reconciling long and short germ development

I propose that the dorsal-to-ventral tissue flow occurring during embryo condensation and GBE in *Tribolium* is homologous to the dorsal-to-ventral tissue flow that occurs during gastrulation and GBE in *Drosophila* (Fig 1). This conclusion is based on the flow being driven by a conserved set of morphogenetic events.
As described above, tissue flow in *Tribolium* is caused by (1) mediolateral cell intercalation, (2) tissue specific cell shape changes along the DV axis, and (3) gastrulation at the ventral side of the embryo. Equivalent processes are all observed in *Drosophila* as well.

In *Drosophila*, Toll-mediated mediolateral cell intercalation causes the tissue wide convergence (along the DV axis) and extension (along the AP axis) of the ectoderm during GBE (20). As in *Tribolium*, the periodic expression of the Toll genes is driven by the pair-rule genes. Conservation at the level of tissue identity, morphogenetic process, and molecular control strongly suggest Toll-mediated cell intercalation to be homologous.

Cell shape changes are harder to compare between these species, as unlike most insects, cellularisation in *Drosophila* leads to the direct formation of columnar cells (22,49). However, tissue specific cell shape changes along the DV axis do occur and are dependent on BMP signalling (50,51); for a detailed description see Appendix section 2). While the intracellular effectors of these cell shape changes are unknown, the dorsal patterning function of BMP signalling is homologous in *Drosophila* and *Tribolium*, and many dorsal cell specification genes are conserved between these two species.

Last, *Drosophila* mesoderm gastrulation also occurs along the ventral midline and it causes lateral/dorsolateral ectoderm to move ventrally (50). As above, the intracellular effectors of *Tribolium* mesoderm gastrulation are unknown, but the upstream patterning events and the tissue specification genes are highly conserved (34,48). Furthermore, gastrulation of mesoderm at the ventral region is widely conserved within the insects, and is undoubtedly a homologous process in each species (52).

The conservation of the tissue-level flow and the underlying processes shows that development in *Drosophila* and *Tribolium*, which are themselves models for long and short germ development respectively, are far more similar than previously thought. This similarity
is further emphasised when the *Tribolium* germband is represented as a cylinder rather than its normal *in vivo* flattened shape (Fig 6). This finding helps explain how the long germ mode of development has evolved independently many times, as it takes fewer evolutionary ‘steps’ to go from one form to another than previously thought.

While I have focused on *Tribolium* and *Drosophila* here, evidence exists that my new findings in *Tribolium* also apply to other short/intermediate germ insects. For example, in the intermediate germ bug *Oncopeltus fasciatus*, the dorsal epithelium of the germband initially consists of a thick epithelium that then progressively becomes squamous late during GBE ((53) and data not shown). These tissue specific cell shape changes are likely the same as those occurring during *Tribolium* GBE. Furthermore, *Oncopeltus* pair-rule genes, *Lotol Toll* genes and even segment polarity genes are expressed in rings around the entire germband prior to thinning of the dorsal epithelium (shown but not described in (21,54,55)). The expression of these genes in the dorsal epithelium provides additional evidence that much of the *Oncopeltus* dorsal epithelium is made up of embryonic tissue. Future analyses of the molecular and morphogenetic drivers of GBE need to analyse the entire germband, rather than focusing on the ventral half.

The *Drosophila* posterior gut fold may represent an evolutionary remnant of short germ development

Despite the deep conservation in morphogenetic events described above, a very obvious difference exists between the germbands of *Drosophila* and *Tribolium*. While the *Drosophila* germband develops mostly on the egg surface with the apical side of the epithelium facing outwards, the epithelium of the *Tribolium* germband folds onto itself such that the apical side faces inwards (Fig 1). The last common ancestor of *Tribolium* and *Drosophila* almost
certainly exhibited short/intermediate germ development (56), which would include the formation of a bi-layered germ band like that of *Tribolium*. As such, in the lineage leading to *Drosophila*, the epithelial folding that previously generated the bi-layered germ band must have become heavily reduced during evolution.

I propose that remnants of the folding seen in *Tribolium* still exist in *Drosophila* today, in what is now termed the “posterior gut fold”. The epithelial folding that causes the formation of the bi-layered germ band of *Tribolium* initiates with a deep infolding of tissue at the posterior of the embryo (classically termed the posterior amniotic fold) (18,27).

Epithelial infolding also occurs at the posterior of the *Drosophila* blastoderm during posterior gut formation (57), and the similarities between these folds have been pointed out before (27). However, the evolutionary relationship of these two structures has been unclear due to the belief that the dorsal half of the fold in *Tribolium* gave rise to the amnion. Now, with the revised fatemap, it is clear that these folds form from the same region of the embryo.

Therefore, I propose that the epithelial fold that forms during posterior gut formation in *Drosophila* is homologous to the posterior epithelial fold that forms during embryo condensation in *Tribolium*. This hypothesis is supported by two pieces of molecular data. First, a recent report found that a gene essential for hindgut development (the *Tribolium* ortholog of *senseless*) is already expressed at the posterior pole of the *Tribolium* embryo at the blastoderm stage (58). Second, the *Tribolium* posterior fold is controlled at the morphogenetic level by the Fog signalling pathway, just like it is in *Drosophila* (Frey et al. manuscript in preparation).

Due to this revised evolutionary relationship and the new *Tribolium* fatemap, I propose that the posterior fold in *Tribolium* be renamed from ‘posterior amniotic fold’ to ‘posterior fold’.
New insights in tissue patterning

While I have predominantly focused on morphogenetic events in this manuscript, the revised model of short germ embryo development also has impacts for how we understand tissue patterning to occur in *Tribolium*. Here, I present one example of this, but for descriptions of pair-rule gene expression, posterior terminal gene expression, and of how DV patterning may be occurring in the germband, see Appendix section 3.

Segment polarity genes are expressed in a single stripe in each segment, extending from near the ventral midline of the embryo to the lateral edge of the germband (59–61). Under the classic model, these domains appear to be static. However, the revised model shows that cells at the lateral edges of the germband move ventrally as cells from the dorsal epithelium move around the edges of the germband. This means that for each stripe of a segment polarity gene to ‘stay’ at the edge of the germband, cells at the correct AP location must activate segment polarity gene expression when they move from the dorsal epithelium to the ventral. This can be thought of like Lewis Carroll’s Red Queen’s race, where “it takes all the running you can do, to keep in the same place” (62). These dynamics mean that each expression domain spreads from ventral-to-dorsal across the ectoderm, but that this occurs simultaneously with the dorsal-to-ventral flow of tissue. A similar ventral-to-dorsal activation of segment polarity gene expression can be seen in the *Drosophila* ectoderm (Supplemental Fig 8), indicating that the DV position of expression is controlled by a conserved upstream mechanism.

While I have only described a handful of examples in this manuscript, other genes are likely to have important expression domains in both the ventral and dorsal epithelia of the
germband. Accurately characterising these domains is necessary for understanding how these genes function in *Tribolium* and for elucidating how their functions may have evolved.

Conclusions

The deep conservation of morphogenetic events that I am proposing here may seem unlikely, but I would argue that such conservation should have been expected from the beginning. The vast majority of morphogenetic events are controlled by upstream patterning networks. As such, morphogenesis can be viewed as a physical readout of such networks.

Decades of research has shown that many of the key patterning genes that function during germband formation and extension in *Drosophila* have conserved roles in *Tribolium* and other insects. As such, without evidence to the contrary, conservation of the cellular events driven by these patterning genes makes perfect sense.

Materials and methods

*Tribolium* animal husbandry, egg collection, and RNA *in situ* hybridisation was performed as previously described (21). The *Tc-srp* ortholog was previously described (63) and was cloned into pGEM-t (Promega Reference A1360) with primers TCCGCTGTGGATCTAGT and TGC GAT GACT GT GACG T G TA. The *Tc-cad* ortholog was as previously used (18).

The *H2B-ven* fusion was created by fusing the *D. melanogaster* histone *H2B* coding sequence (without the stop codon) from the published H2B-RFP (18) to the *venus* fluorescent protein (64) and cloning into the pSP64 Poly(A) (Promega Reference P1241) expression vector. The *NLS-tdEos* fusion was kindly provided by Matthias Pechmann.

Additional details and both plasmids are available upon request to M. Pechmann or myself.
Capped mRNA synthesis was performed as previously described (18). H2B-ven capped mRNA was injected at 1 µg/µL, NLS-tdEos capped mRNA was injected at 2-3 µg/µL.

Embryo microinjection was performed as previously described (18), with the following changes. Up to 100 dechorionated embryos were mounted on a rectangular coverslip (24 mm by 50 mm) that rested on a microscope slide. Water was allowed to dry off the embryos before they were covered in Voltalef 10S halocarbon oil and injected as usual. The coverslip (still resting on the slide) was then placed in a petri-dish (92 mm) containing a base layer of 1% agarose (dissolved in water) and placed at 30-32°C until the embryos were at the appropriate stage for imaging. The coverslip was then removed from the slide, inverted (so that embryos were face down), and quickly but gently placed on a lumox dish (50 mm; Sarstedt Reference 94.6077.410) that was sitting upside down. The corners of the coverslip rested on the raised plastic lip of the dish such that the membrane and embryos were close to each other but not touching. To ensure lateral stability of the coverslip during the timelapse recording, approximately 5-10 µL of heptane glue (made by soaking parcel tape in heptane) was placed at each corner. Additional Voltalef 10S halocarbon oil was then added to fill any remaining space between the coverslip and the oxygen permeable membrane. This contraption was then stuck to a microscope slide (using double sided tape) for imaging on an upright microscope. This last step may be unnecessary depending on the microscope stage and orientation.

Live imaging was performed on an upright Zeiss SP8 confocal microscope equipped with Hybrid detectors in the Biocentre Imaging facility (University of Cologne). Image stacks of 15-50 focal planes with z-steps ranging from 2-10 µm were taken with a 10x/0.3NA dry objective or a 20x/0.7NA multi-immersion objective at intervals of 5-45 minutes. The temperature of the sample during imaging could not be carefully regulated, but was typically
between 25-28 degrees. While this lack of temperature control is not ideal, it does not affect the findings presented in this manuscript.

Photoconversion of NLS-tdEos protein was performed by constantly scanning the region of interest for 20-30 seconds with the 405 wavelength laser at low power (5%). These settings were manually determined on the above microscope, and need to be determined independently on different systems. Photoconversions were performed during the final uniform blastoderm stage, as photoconversion prior to this resulted in substantial diffusion of the photoconverted protein during nuclei division. The positions of the different regions of the embryo (75% EL etc.) were determined by measuring the length of each embryo in the LASX software and selecting the appropriate region. Photoconversions were performed on all embryos on the coverslip before setting up the timelapse, which led to a 0.5-2 hour delay between performing the photoconversion and beginning the timelapse. As such, the positions of the photoconverted region at the first time point in the timelapses in this manuscript do not reflect the original region of photoconversion.

Imaging of fixed material was performed on an upright Zeiss SP8 confocal, an upright Zeiss SP5 confocal microscope and an inverted Zeiss SP5 confocal microscope. The Drosophila gooseberry expression patterns were kindly provided by Erik Clark and acquired as in (65). Images and timelapses were analysed using FIJI (66) and Photoshop CS5. Manual cell tracking was performed on confocal hyperstacks with MTrackJ (67). The figures were arranged and the schematics created using Inkscape.

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Fig 1. Schematics of development in *Drosophila* and *Tribolium*. The two left columns show schematics of *Drosophila* embryos from the uniform blastoderm stage to the extended germ band stage. The right three columns show schematics of *Tribolium* embryos at comparable stages. The schematics in the right-most column show dissected, flat mounted embryos. Red arrows display cell/tissue movement. The question marks highlight two regions where the tissue boundaries are unknown/undescribed. Several features have been omitted, including the yolk, mesoderm gastrulation, anterior gut formation and appendage
formation. The *Drosophila* fatemap is based on data from (22) and the references therein.

Refer to text for additional details.

**Fig 2. Live cell tracking reveals contribution of ‘amnion’ cells to embryonic tissue.** (A-F)

Time series from fluorescent live imaging of a *Tribolium* embryo expressing H2B-venus. The serosa nuclei located above the germ band have been manually removed from these frames, but left in the surrounding territory (arrowhead in (A+A')). (A'-C') show optical transverse sections of the respective frame at the position shown by the dashed line (the surface of the egg is to the left). In (A-C), all nuclei that lie in a region of the ‘amnion territory’ in (A) have been tracked and differentially labelled depending on whether they become part of the embryo (magenta; labels disappear when nuclei join the germ band), become located at the edge of the germ band (yellow) or remain in the ‘amnion territory’ (cyan). In (D-F), a line of nuclei that lie in the ‘amnion territory’ in (D) have been tracked and differentially labelled depending on whether they become part of the embryo (coloured points; daughter cells are labelled in same colour as parent) or remain in the ‘amnion territory’ (cyan; no division takes place). Note that in panel (D), the orange spot is mostly hidden below the yellow spot because the nuclei in that region are partially overlapping when viewed as projections. The first frame of the timelapse was defined as timepoint 0. In (A-F), embryos are oriented based on the AP/DV polarity of the egg with anterior to the left and dorsal to the top. (A-C) are maximum intensity projections of one egg hemisphere. (D-F) are average intensity projections of 46 microns to specifically show the germ band. Scale bars are 50 µm.

**Fig 3. Differential cell labelling reveals widespread movement of cells from the dorsal epithelium to the ventral epithelium.** (A-J) Time series from fluorescent live imaging of two
Tribolium embryos expressing NLS-tdEos showing unconverted protein (magenta) and photoconverted protein (cyan). In (A-F') a patch of nuclei at the posterior-dorsal region of the blastoderm were photoconverted. Panels (A-F) show the posterior region of the germ band during late GBE and panels (A'-F') show optical transverse sections made at the position of the dashed line at each timepoint (roughly following the same nuclei). Serosa nuclei are marked by white crosses in the transverse sections. In (G-J), a patch of nuclei at the anterior-lateral region of the blastoderm were photoconverted. Panels (G-J) show the anterior of the germ band during condensation and GBE. In both embryos, all converted nuclei are initially located in the dorsal epithelium, but most move into the ventral epithelium during GBE. (K-L) Schematics showing the classic and revised models of the Tribolium germ band (presumptive amnion is shown in purple, presumptive embryo is shown in grey, red arrows show the newly discovered tissue flow). The first frame of the timelapses was defined as timepoint 0. In (A-I), embryos are oriented based on the AP/DV polarity of the egg with anterior to the left and dorsal to the top. In (A'-F'), the surface of the egg is oriented to the left. In (K-L), schematics show flatmounted germ bands with the focus on the dorsal epithelium, the anterior to the left and the orthogonal sections are oriented with the dorsal half of the germ band to the left. (A-J) are maximum intensity projections of one egg hemisphere. Scale bars are 50 µm.

Fig 4. Development of the putative amnion. (A-A'') Tribolium embryo transiently expressing the membrane marker GAP43-YFP. (A) shows an overview of the whole egg, (A') shows the dorsal epithelium of the same embryo at the position of the white box, (A'') is an optical sagittal section at the position of the dashed line in (A') showing the apical-basal height of cells of the dorsal epithelium. (B) Tc-srp (red) expression in a flatmounted Tribolium
germband also showing nuclei (DAPI, blue). The strong Tc-srp signal in nuclei may suggest nuclear or peri-nuclear localisation of the transcript, or it may be due to the cell body being flattened. The strong patch of expression at the anterior medial region is from cells beneath the ventral ectoderm (see Supplemental Fig 1 for a full expression series). (C-E) Extended germband stage Tribolium embryos transiently expressing NLS-tdEos showing unconverted protein (magenta) and photoconverted protein (cyan). In each embryo, the clone of converted cells spans the entire amnion. (C1-2) show both sides of the same embryo in which a 6 nuclei wide patch of dorsal-most cells located at 50% EL were photoconverted at the blastoderm stage. (E) shows an embryo in which a 3 nuclei wide patch of dorsal-most cells located at 25% EL were photoconverted at the blastoderm stage. (F) shows an embryo in which a 3 nuclei wide by 6 nuclei long patch of dorsal-most cells located at roughly 2-10% EL were photoconverted at the blastoderm stage. In (A) and (C-E), embryos are oriented based on the AP/DV polarity of the egg with anterior to the left and dorsal to the top. In (A’’), the surface of the egg is oriented to the bottom. In (B), the anterior of the germband is to the left. (A) is an average intensity projection of one egg hemisphere. (A’) is an average intensity projection of 6 µm to specifically show the dorsal epithelium. (B) is a maximum intensity projection of the whole germband. (C-E) are maximum intensity projections of one egg hemisphere. Abbreviations are: ventral ectoderm (VE) and dorsal ectoderm (DE). Scale bars are 50 µm.

**Fig 5. Schematics showing the classic and revised Tribolium fatemaps and germband models.** Schematics drawn as in Fig 1 to show the classic fatemap and germband model and the revised fatemap and model based on the results of this manuscript. Note that the posterior amnion/embryo boundary is unclear. The schematics of the flatmounted
germbands are drawn with the focus on the dorsal epithelium. See text for additional details, and Supplemental Fig 6 for an extended figure.

**Fig 6. Representation of the Tribolium germ band as a cylinder.** (A₁) the germ band of *Tribolium* (and other insects) is usually drawn as it is in *in vivo*, that is, as a flattened shape with the ventral cell fates to the top and the dorsal cell fates to the bottom. However, the embryo can be unflattened (A₂) without changing the planar dimensions of the tissue itself, and rotated (A₃) such that the dorsal-ventral axis is oriented in the widely accepted standard of dorsal to the top and ventral to the bottom. When this is done, and third dimension is incorporated to show the AP axis (A₄), the similarities in tissue boundaries and tissue rearrangements in *Tribolium* and *Drosophila* (B) become obvious. Note that while both (A₄) and (B) are cylindrical, the epithelium of the *Tribolium* germ band inverts during embryo condensation so the apical surface of the embryo faces inwards. Both (A) and (B) are sections from roughly the middle of the respective embryo during GBE. Possible flattening of dorsal tissue in *Drosophila* has been omitted due to a lack of detailed data at this stage.

**Supplementary Figure 1. Results of photoconversions at 50% egg length.** NLS-tdE was labelled extended germband stage *Tribolium* embryos in which a patch of blastoderm nuclei were photoconverted at 50% egg length (from the posterior pole) at different DV positions. The approximate DV position of the patch and the approximate DV width of the clone (in terms of nuclei number) are shown. The dorsal labelled embryo is shown from both sides to demonstrate the photoconverted nuclei cover the full DV extent of the amnion (arrows). Unconverted protein is shown in magenta, converted protein is shown in cyan. Images are
maximum intensity projections of one egg hemisphere. All eggs are oriented with the
anterior to the left and ventral to the bottom. Scale bars are 100 µm.

Supplementary Figure 2. Results of photoconversions at 25% egg length. NLS-tdEos labelled
extended germband stage Tribolium embryos in which a patch of blastoderm nuclei were
photoconverted at 25% egg length (from the posterior pole) at different DV positions. The
approximate DV position of the patch and the approximate DV width of the clone (in terms
of nuclei number) are shown. Unconverted protein is shown in magenta, converted protein
is shown in cyan. Images are maximum intensity projections of one egg hemisphere. All eggs
are oriented with the anterior to the left and ventral to the bottom. Scale bars are 100 µm.

Supplementary Figure 3. Results of photoconversions near the posterior pole. NLS-tdEos
labelled extended germband stage Tribolium embryos in which a patch of blastoderm nuclei
were photoconverted near the posterior pole at different DV positions. The approximate DV
position of the patch and the approximate DV width of the clone (in terms of nuclei number)
are shown. The second dorsally labelled embryo is shown at high magnification at two
timepoints and with a transverse section (at the position of the dashed green line) to show
the movement of tissue from the dorsal epithelium into the hindgut. Unconverted protein is
shown in magenta, converted protein is shown in cyan. Images are maximum intensity
projections of one egg hemisphere except for the bottom three embryos, which are shown
as maximum intensity projects through the germband in order to better show the labelled
nuclei. All eggs are oriented with the anterior to the left and ventral to the bottom except
for the second timepoint of the second dorsal view, which is shown with the posterior of the
germband to the left. Scale bars are 100 µm.
Supplementary Figure 4. *Tc-cad* is expressed further to the anterior in the dorsal epithelium than in the ventral epithelium. (A-A’’) show maximum intensity projections of the posterior region of a flatmounted *Tribolium* germband shortly after serosa window closure stained to show nuclei (DAPI, blue) and the mRNA expression pattern of *Tc-cad* (red). The panels beside or below each main panel show optical sections through the germband at the position of the respective coloured line in the main panel. (B-B’’) show maximum intensity projections of approximately one half of the germband from the lateral side (i.e. projections of optical sagittal sections from the midline to the lateral edge of the germband). Scale bar is 100 µm.

Supplementary Figure 5. RNA expression of the *Tribolium* ortholog of the GATA factor *serpent*. (A-F) whole mount and (G-J) flatmount *Tribolium* embryos from the pre-blastoderm to the retracting germband stage stained for *Tc-srp* mRNA (red) and nuclei (DAPI, blue). (G₁) and (G₂) show the same embryo imaged from both sides. (H₁) and (H₂) show projections from the dorsal epithelium (H₁) and the ventral epithelium (H₂) of the same embryo. *Tc-srp* mRNA is maternally provided (A), and expression is ubiquitous until the late blastoderm stage (B-C) when expression clears from the blastoderm but persists in the yolk nuclei (scattered spots in [D-E]). During embryo condensation, *de novo* expression arises in a patch of blastoderm cells at the anterior medial region (arrowhead in F). This patch of *Tc-srp* expressing cells invaginates as part of the ventral furrow and becomes located beneath the ectoderm (arrowhead in G₁-H₃). This expression domain is likely homologous to the anterior ventral expression domain in *Drosophila* that marks the prohemocytes. During serosa window closure, expression appears in a ring of dorsal epithelium cells (G₁). After serosa
window closure, expression persists in the dorsal epithelium (H₁) and (H₃). Unlike Drosophila, there is no expression domain at the posterior of the blastoderm (E) or the early germband (G₂). After germband elongation, a de novo expression domain appears at the posterior most point of the embryo (I₁). Given the location of this domain at the base of the forming hindgut, this is likely the posterior endoderm primordium. Expression can also be seen in a patch of amnion that has remained attached to the germband (arrow in I₂), but most of the rest of the amnion has been lost. Several other regions of expression can be seen, including in the presumptive fat body (the segmental domains running down the body), in presumptive hemocyte clusters (the two side-by-side domains in the anterior), and in an anterior domain that may mark the anterior endoderm primordium. Expression also persists in the yolk nuclei (data not shown). All embryos are shown with anterior to the left. (E-E”) is oriented with the ventral to the bottom, (F-F”) is oriented as a ventral view. (H₁-H₃”) is an optical sagittal section at approximately the midline of the embryo in (H₂). (A-F) are maximum intensity projections of one egg hemisphere. (G₁-G₂”), (I₁-I₁”) and (J-J”) are maximum intensity projections of flatmounted germbands. (I₂-I₂”) is a maximum intensity projection of part of the germband to better show the amnion/dorsal epithelium. Scale bars are 50 µm.

Supplementary Figure 6. Extended schematics showing the classic and revised Tribolium fatemaps and germband models. Refer to Fig 5 and the text for details.

Supplementary Figure 7. Tissue specific cell shape changes during Tribolium condensation. Stills from timelapses of two Tribolium embryos transiently expressing GAP43YFP to label membranes. The second panel of each timepoint shows optical transverse sections at the
position of the dashed line in the related panel. Ventral and lateral ectoderm becomes

columnar, while dorsal ectoderm becomes flattened. The non-columnar cells at the bottom
of the left hand embryo are likely the presumptive mesoderm. The first frame of the
timelapses was defined as timepoint 0. Both embryos are oriented with the anterior to the
left and ventral to the bottom. Abbreviations are: Dorsal (Dor), Lateral (Lat), Ventral (Ven)
and Ectoderm (Ect). Scale bars are 100 µm.

Supplementary Figure 8. DV dynamics of the segment polarity gene *gooseberry* in
*Drosophila*. Whole mount *Drosophila* embryos from the blastoderm stage (late stage 5) until
the extending germband stage (late stage 7/early stage 8). The red arrows in the 2\textsuperscript{nd} and 3\textsuperscript{rd}
panels highlight the dorsal-to-ventral tissue flow and the GBE. The vertical arrow is at
parasegment boundary 8/9, and is the same size in both panels. All embryos are
ventrolateral views with anterior to the left and dorsal to the top. Scale bar is 100 µm.
Images were kindly provided by Erik Clark.

Supplementary Movie 1. Confocal timelapse of a *Tribolium* embryo transiently expressing
H2B-ven to mark nuclei. A maximum intensity projection of one egg hemisphere is shown.
Anterior is to the left, the ventral side of the egg is to the bottom.

Supplementary Movie 2. Same timelapse as Supplementary Movie 1, but with nuclei of the
dorsal epithelium tracked until they join the ventral epithelium. Nuclei that join the ventral
epithelium are labelled magenta, nuclei that become located at the edge of the germband
are labelled yellow, nuclei that remain in the dorsal epithelium are labelled cyan. Anterior is
to the left, the ventral side of the egg is to the bottom. See Fig 2(A-C) for more details.

Supplementary Movie 3. Same timelapse as Supplementary Movie 1, but with a line of nuclei of the dorsal epithelium tracked. Nuclei that remain in the dorsal epithelium are labelled cyan. Anterior is to the left, the ventral side of the egg is to the bottom. See Fig 2(D-F) for more details.

Supplementary Movie 4. Confocal timelapse of a *Tribolium* embryo transiently expressing NLS-tdEos (magenta) with a line of blastoderm cells photoconverted (cyan). The brightness increases approximately halfway through the movie due to a manual increase in laser power at this point. A maximum intensity projection of one egg hemisphere is shown. Anterior is to the left, the ventral side of the egg is to the bottom.

Supplementary Movie 5. Confocal timelapse of a *Tribolium* embryo transiently expressing NLS-tdEos (magenta) with a patch of blastoderm cells photoconverted (cyan). (A) shows a maximum intensity projection of one egg hemisphere is shown. (B) shows an optical transverse section. (C) shows an average intensity projection of 10 optical transverse sections. Anterior is to the left, the ventral side of the egg is to the bottom. See Fig 3(A-F) for more details.

Supplementary Movie 6. Confocal timelapse of a *Tribolium* embryo transiently expressing NLS-tdEos (magenta) with a patch of blastoderm cells photoconverted (cyan). Towards the end of the timelapse, the cyan nuclei in the dorsal epithelium appear to condense posteriorly to the hindgut. A maximum intensity projection of one egg hemisphere is shown.
Anterior is to the left, the ventral side of the egg is to the bottom.
A1. Ventral → Dorsal

A2. Ventral → Dorsal (Unflatten)

A3. Dorsal → Ventral (Rotate)

A4. Drosophila

Tribolium