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The dynamic expression of extraembryonic marker genes in the beetle *Tribolium castaneum* reveals the complexity of serosa and amnion formation in a short germ insect

Rahul Sharma [a], Anke Beermann [b], Reinhard Schröder [a,*]

[a] University of Rostock, Institute of Biology, Department of Genetics, Albert-Einstein Str. 3, 18059 Rostock, Germany
[b] University of Tübingen, Department of Animal Genetics, Auf der Morgenstelle 28, 72076 Tübingen, Germany

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ABSTRACT

Most insect embryos develop with two distinct extraembryonic membranes, the serosa and the amnion. In the insect beetle *Tribolium* the early origin of the serosa within the anterior blastoderm is well established but the origin of the amnion is still debated. It is not known whether this tissue develops from a blastodermal precursor or originates *de novo* later from embryonic tissue during embryogenesis.

We undertook an in-depth analysis of the spatio-temporal expression pattern profile of important extraembryonic membrane marker genes with emphasis on early blastoderm development in *Tribolium*.

The amnion marker *iroquois* (*Tc-iro*) was found co-expressed with the serosa marker *zerknüllt1* (*Tc-zen1*) during early blastoderm formation in an anterior cap domain. This domain later resolved into two adjacent domains that likely represent the precursors of the serosa and the amnion. In addition, we found the *hindsight* ortholog in *Tribolium* (*Tc-hnt*) to be a serosa-specific marker. Surprisingly, *decapentaplegic* (*Tc-dpp*) expression was not seen as a symmetric cap domain but detected asymmetrically first along the DV- and later also along the AP-axis. Moreover, we found a previously undescribed domain of phosphorylated MAD (pMAD) protein in anterior ventral serosal cells.

This is the first study showing that the anterior-lateral part of the amnion originates from the anterior blastoderm while the precursor of the dorsal amnion develops later *de novo* from a dorsal-posterior region within the differentiated blastoderm.

Embryonic development in insects has been studied for a long time and by now is best understood at the genetic and at the cellular level in the model organism *Drosophila melanogaster*. In contrast, tissues that do not contribute to the embryo proper, the extraembryonic membranes, have long been neglected. Still, these membranes fulfill important functions like assisting the morphogenetic movements, protecting the embryo against physical or mechanical stress and acting as immunological barriers (Jacobs et al., 2013; Jacobs and van der Zee, 2013; Panfilio, 2008). One main reason for the negligence could be the presence of only a single and highly reduced extra embryonic tissue, the amnioserosa in *Drosophila*. However, the presence of two distinct extraembryonic tissues, the serosa and the amnion is a key feature for most insects like *Anopheles*, *Tribolium*, *Nasonia* and *Oncopeltus* (Buchta et al., 2013; Goltsev et al., 2007; Panfilio, 2008; van der Zee et al., 2005). Recently, there has been an increased interest in understanding the processes and mechanisms involved in the formation of extraembryonic membranes (Dearden et al., 2000; Garcia-Solache et al., 2010; Panfilio, 2008; Panfilio et al., 2006; Panfilio and Roth, 2010; Rafiqi et al., 2008, 2012; Schmidt-Ott, 2000; van der Zee et al., 2005).

Whereas the amnioserosa in *Drosophila* derives from the dorsalmost region of the cellular blastoderm under the control of dorsal–ventral patterning system (Moussian and Roth, 2005; Reeves and Stathopoulos, 2009), the extraembryonic tissue serosa in *Tribolium* originates from an anterior region under the control of the anterior–posterior and the terminal patterning systems (Kotkamp et al., 2010; Lynch and Roth, 2011; Schoppmeier and Schröder, 2005). However, the developmental origin of the other extraembryonic tissue – the amnion – remains obscure. Currently it is discussed that the amnion is mainly of embryonic origin and forms undistinguishable from the embryonic anlage late in development (Anderson, 1972; Handel et al., 2000; Panfilio, 2008).

In the beetle *Tribolium castaneum*, a detailed microscopic study of embryogenesis has revealed a tight interplay between the extraembryonic tissues, the serosa and the amnion, and the embryonic tissue during the morphogenetic movements of early embryogenesis (Handel et al., 2000).

The formation of the tissues (serosa, amnion and the embryo proper) and their morphogenetic movements in relation to each

* Corresponding author. Tel.: +49 381 498 6330; fax: +49 381 498 6302.
E-mail address: reinhard.schroeder@uni-rostock.de (R. Schröder).

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other is highly dynamic and therefore difficult to visualize in static pictures. However, the morphological dynamics can be followed by systematically analyzing the expression pattern of genes expressed in the extraembryonic membranes and their precursor cells (marker genes). Primarily, *zen1* expression in *Tribolium* marks serosal tissue (Falciani et al., 1996; Sanches-Salazar et al., 1996; van der Zee et al., 2005, 2006). The second *zen* gene in *Tribolium Tc-zen2* shows the same expression profile as *Tc-zen1* except for a late amniotic expression domain (van der Zee et al., 2005). For the amnion, so far, two marker genes *Tc-pannier* (*Tc-pnr*) and *Tc-iroquois* (*Tc-iro*) have been established (Nunes da Fonseca et al., 2010, 2008; van der Zee et al., 2005, 2006). In *Tribolium*, *Tc-pnr* is expressed in only a subset of the amnion, the dorsal amnion (van der Zee et al., 2005, 2006) whereas *Tc-iro* expression was described in the anterior and the dorsal primordium of the amnion (Nunes da Fonseca et al., 2010, 2008).

Among dipterans, the expression pattern of another marker gene *hindsight* in the extraembryonic tissues appears to be evolutionary conserved. In *Drosophila* the *hindsight* (*hnt*)/*pebbled* gene is expressed in the amnioserosa and is required for germ band retraction (Frank and Rushlow, 1996; Yip et al., 1997). In other flies, that develop with two distinct extraembryonic membranes like *Anopheles* (Goltsev et al., 2007) and *Megaselia* (Rafiqi et al., 2008), *hnt* is expressed early in the blastoderm in a broad domain that includes both the tissues, the prospective serosa and the prospective amnion (Goltsev et al., 2007; Rafiqi et al., 2010, 2012). To elucidate whether in *Tribolium* the *hindsight* ortholog also marks the extraembryonic tissues, we analyzed its expression pattern during early embryogenesis.

In *Drosophila* and *Tribolium*, the proper formation of extraembryonic membranes requires an input of the dorsal–ventral patterning system and Decapentaplegic (Dpp) is one crucial molecule that regulates the expression and activity of genes in these tissues (Ferguson and Anderson, 1992; Lynch and Roth, 2011; van der Zee et al., 2006). The active sites of Dpp-signaling within the embryo can be visualized by monitoring the expression of the downstream transducer of this pathway, phosphorylated MAD (Mothers against Dpp)/pMAD. In *Drosophila*, *dpp* mRNA expression and Dpp-activity coincide at the dorsal side of the early embryo and later at the dorsal epidermis and the amnioserosa (Dorfman and Shilo, 2001). In contrast, in *Tribolium*, the site of Dpp-activity (marked by the pMAD antibody) has been reported exclusively on the dorsal surface opposite to the ventral expression of *Tc-dpp* mRNA (Chen et al., 2000; Nunes da Fonseca et al., 2008; van der Zee et al., 2008, 2006). While the early *Tc-dpp* expression in a symmetric anterior polar cap has been described as serosa-specific, the Dpp-activity was found restricted to the dorsal serosal and embryonic tissue at late blastoderm stages (Chen et al., 2000; Sanches-Salazar et al., 1996; van der Zee et al., 2006).

To date, our current knowledge about the well-established extraembryonic-marker genes *Tc-zen1* and *Tc-iro* and their regulators Dpp and pMAD is based on the description of only few embryological stages from mostly lateral views, and is therefore incomplete. This is especially obvious for the expression of the amnion marker *Tc-iro* and the Dpp-activity marker pMAD that so far have been described only at the late differentiated blastoderm stage and during germband extension (Nunes da Fonseca et al., 2010, 2008). In this study we provide a comprehensive overview on the tissue-specific gene expression patterns of different marker genes in the early wildtype embryo that aims for a more complete understanding of the dynamics and topology of the extraembryonic membranes in relation to the embryo in *Tribolium*.

1. Results

1.1. Expression dynamics of the amnion marker iroquois (Tc-iro) during blastoderm development

In *Tribolium*, the expression pattern of *iroquois* (*Tc-iro*) has been used as a molecular marker to describe the anterior and the dorsal amnion at late developmental embryonic stages (Kotkamp et al., 2010; Nunes da Fonseca et al., 2010, 2008). Whether *Tc-iro* expressing amnion precursor cells can be found earlier during embryogenesis is still unknown. We therefore describe the spatio-temporal expression profile of *Tc-iro* during blastoderm maturation.

No maternal contribution of *Tc-iro* mRNA was seen in freshly laid eggs (Fig. 1A and A'). The first very weak, ubiquitous *Tc-iro* expression was detected at the start of the early embryonic nuclear divisions (Fig. 1B and B') and became confined to a crescent shape domain at the anterior pole in older stage embryos (Fig. 1C and C'). At the uniform blastoderm stage, the *Tc-iro* expression domain broadened and developed into a rotationally symmetric cap (bars, Fig. 1D, 1–3'). Later, this *Tc-iro* domain receded (asterisks in Fig. 1E) from the anterior pole to transform into a ring-like domain (Fig. 1E, 1–3'). When viewed laterally this domain was slightly oblique (Fig. 1E, 2–2'), while the ventral and the dorsal domain of that ring remained perpendicular to the anterior-posterior axis of the embryo (Fig. 1E, 1–1' and 3–3'). Quickly thereafter, no *Tc-iro* expression was found at the anterior pole except in a ring domain (Fig. 1F, 1–3'). The ring domain persisted only for a short time as at an older stage a loss of *Tc-iro* expression at the dorsal surface was observed (Fig. 1G, 3–3') that resulted in a horseshoe-shape domain (Fig. 1G, 1–2').

After the differentiation of the blastoderm, a weak expression of *Tc-iro* mRNA was detected in a new domain of dorsal-posterior cells (black arrowheads; Fig. 1H, 3–3'). This new *Tc-iro* domain became more profound later in development (black arrowheads; Fig. 1I, 3–3'). At the same time, the lateral *Tc-iro* domain receded, leaving a gap between the anterior-lateral and the dorsal domain (black arrow; Fig. 1I, 2–2').

During posterior invagination of the blastoderm, *Tc-iro* expression was seen as a thin stripe at the anterior-lateral border between the serosa and the germ rudiment (Fig. 1J, 1–2'). At this stage, the broad dorsal domain of *Tc-iro* expression followed the posterior invagination (Fig. 1J, 3–3').

Later at the beginning of germband extension, *Tc-iro* transcripts were visible in extraembryonic cells at the edges around the serosal window that are morphologically indistinguishable from embryonic cells (Handel et al., 2000) (black arrowheads; Fig. S1, A–A'). In addition, *Tc-iro* transcripts were also detected in the emerging segmental stripes in an older extending germband (arrows, Fig. S1, D–D') (Nunes da Fonseca et al., 2010).

In summary, we have shown the blastodermal domain of *Tc-iro* expression for the first time and revealed that how this early anterior cap domain progressively resolved into a stripy domain present at the border between the serosa and the germ anlage that likely represents the precursor of the anterior-lateral amnion.

1.2. Expression dynamics of the serosa marker zerknüllt-1 (Tc-zen1) during blastoderm development

The *zerknüllt-1* gene in *Tribolium* (*Tc-zen1*) is an exclusive serosa marker (Falciani et al., 1996) and has been used in various functional studies to judge the fate of the serosa (Fu et al., 2012; Kotkamp et al., 2010; Nunes da Fonseca et al., 2008; van der Zee et al., 2005). Here we aim to provide a complete understanding of