BMP-dependent serosa and amnion specification in the scuttle fly Megaselia abdita

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SUMMARY
Bone morphogenetic protein (BMP) signaling is an essential factor in dorsoventral patterning of animal embryos but how BMP signaling evolved with fundamental changes in dorsoventral tissue differentiation is unclear. Flies experienced an evolutionary reduction of extra-embryonic tissue types from two (amniotic and serosal tissue) to one (amnionserosal tissue). BMP-dependent amnioserosa specification has been studied in Drosophila melanogaster. However, the mechanisms of serosal and amniotic tissue specification in less diverged flies remain unknown. To better understand potential evolutionary links between BMP signaling and extra-embryonic tissue specification, we examined the activity profile and function of BMP signaling in serosa and amnion patterning of the scuttle fly Megaselia abdita (Phoridae) and compared the BMP activity profiles between M. abdita and D. melanogaster. In blastoderm embryos of both species, BMP activity peaked at the dorsal midline. However, at the beginning of gastrulation, peak BMP activity in M. abdita shifted towards prospective amnion tissue. This transition correlated with the first signs of amnion differentiation laterally adjacent to the serosa anlage. Marker-assisted analysis of six BMP signaling components (dpp, gbb, scw, tkv, sax, sog) by RNA interference revealed that both serosa and amnion specification of M. abdita are dependent on BMP activity. Conversely, BMP gain-of-function experiments caused sharpened expression boundaries of extra-embryonic target genes indicative of positive feedback. We propose that changes in the BMP activity profile at the beginning of gastrulation might have contributed to the reduction of extra-embryonic tissue types during the radiation of cyclorrhaphan flies.

KEY WORDS: Evolutionary development, Bone morphogenetic protein, Diptera

INTRODUCTION
Bone morphogenetic proteins (BMPs) are important players in the dorsoventral differentiation of animal embryos (Nieuwkoop, 1948), but how BMP signaling evolves with changes in tissue specification is not well understood. Flies (Diptera) provide an excellent opportunity to address this question because the BMP pathway of Drosophila melanogaster embryos has been studied in much detail (O’Connor et al., 2006; Umulis et al., 2009) and because extra-embryonic tissue specification, presumably under the control of the BMP pathway, has changed in dipteran evolution (Fig. 1) (Schmidt-Ott et al., 2010). Schizophoran flies, which include D. melanogaster, develop a single extra-embryonic tissue called the amnioserosa. This tissue is specified along the dorsal midline of the blastoderm and closes the dorsal side of the germband. In non-schizophoran dipterans, dorsal blastoderm folds over the gastrulating embryo and differentiates into two tissues: a cuticle-secreting serosa underneath the eggshell and an amnion that either lines the ventral side or closes the dorsal side of the germband (Goltshev et al., 2007; Rafiqi et al., 2008; Goltshev et al., 2009). Dorsal patterning of the dipteran embryo must have changed with the evolutionary transition from two extra-embryonic tissue types to one, but whether the transition involved altered BMP activity or genetic changes downstream or independent of this signaling pathway is unknown.

In D. melanogaster, high levels of BMP activity are required to induce amnioserosa formation (Ray et al., 1991; Arora and Nusslein-Volhard, 1992; Ferguson and Anderson, 1992a; Wharton et al., 1993). This activity is induced by the extracellular ligands Decapentaplegic (Dpp) and Screw (Scw) (Padgett et al., 1987; Arora et al., 1994; Shimmi et al., 2005), which are secreted into the perivitelline space and transported in the presence of antagonists towards the dorsal midline (Francois et al., 1994; Ashe and Levine, 1999; Decotto and Ferguson, 2001; Eldar et al., 2002; Shimmi et al., 2005; Wang and Ferguson, 2005). Dorsally, BMP dimers are released from their antagonists to the transmembrane receptor proteins Thickveins (Tkv) and Saxophone (Sax), triggering phosphorylation of the transcription factor Mad and thereby BMP-dependent transcriptional gene regulation (Shimell et al., 1991; Ferguson and Anderson, 1992b; Marques et al., 1997; Shimmi et al., 2005; Wang and Ferguson, 2005). The BMP transport and release mechanism results in a shallow gradient of BMP activity, which broadly influences patterning in the dorsal ectoderm (Mizutani et al., 2006). Through a positive-feedback loop, the initially shallow gradient of BMP activity is transformed into a narrow and sharply delineated domain of high BMP activity (Wang and Ferguson, 2005; Umulis et al., 2006; Umulis et al., 2010), which, at this stage, becomes crucial for the expression of zerknüllt (zen) and hence amnioserosa specification (Rushlow et al., 2001; Liang et al., 2008). Dpp is essential for BMP activity and therefore controls the specification of all tissues that develop under the control of the BMP pathway in the early embryo, including the amnioserosa and dorsal ectoderm (Ferguson and Anderson, 1992b; Wharton et al., 1993). scw, a diverged paralog of glass bottom boat (gbb) (Van der Zee et al., 2008; Fritsch et al., 2011), boosts BMP activity along the dorsal midline and is required to generate high BMP activity for amnioserosa specification (Arora et al., 1994).
Functional data on the BMP pathway components of dipterans other than *D. melanogaster* are currently unavailable, but orthologs of many specific BMP signaling components of *D. melanogaster* have been identified in other cyclorrhaphan flies (Fritsch et al., 2011; Lemke et al., 2011). Using an antibody against pMad (the phosphorylated, activated BMP signal transducer), it has been shown that, in the mosquito *Anopheles gambiae*, BMP activity covers a much broader area of the dorsal blastoderm than in *D. melanogaster* (Goltsev et al., 2007). This difference correlates with clearly distinct expression patterns of short gastrulation (sog) and tolloid (tld), which encode extracellular BMP signaling components. Sog (a homolog of vertebrate chordin) functions as an antagonist of BMP dimers (Francois et al., 1994; Bielie et al., 1996; Marques et al., 1997; Shimmi et al., 2005). Tld, a metalloprotease, cleaves Sog and allows BMP dimers to bind their receptors (Marques et al., 1997; Shimmi et al., 2005; Wang and Ferguson, 2005).

In *D. melanogaster*, sog is expressed laterally in the neurogenic ectoderm, whereas in *A. gambiae* it is expressed ventrally in the prospective mesoderm [as in beetles (van der Zee et al., 2006)]. The difference in sog expression could explain the comparatively broad pMad domain in *A. gambiae* embryos. Furthermore, in *D. melanogaster*, tld is expressed on the dorsal side of the blastoderm embryo in non-neurogenic ectoderm (Shimel et al., 1991), whereas in *A. gambiae* it is expressed more broadly but, notably, is repressed in a region that roughly corresponds to the serosa anlage. As the activity pattern of tld, in *D. melanogaster* at least, seems to be tied to its local transcription (Wang and Ferguson, 2005), the difference in tld expression between *D. melanogaster* and *A. gambiae* suggests that the prospective amnion, rather than the serosa, receives peak levels of BMP activity in the *A. gambiae* embryo. Yet, according to the authors of the mosquito study, pMad in the mosquito blastoderm peaks along the dorsal midline. They therefore proposed that laterally released BMP dimers accumulate along the dorsal midline, generating peak levels there. The resulting BMP activity gradient would specify serosal tissue at peak levels and amniotic tissue at a lower activity threshold (Goltsev et al., 2007). However, this model does not account for the fact that, immediately posterior to the serosa anlage, prospective amnion tissue straddles the dorsal midline.

Here we provide an analysis of BMP signaling in the scuttle fly *Megaselia abdita* (Phoridae). We chose this species because it is a close outgroup of amnioserosa-forming schizophoran flies, it is amenable to functional studies and easily maintained in culture, and because its egg size and embryonic development are very similar to those of *D. melanogaster*. We describe dynamic changes in BMP activity at the beginning of gastrulation that correlate with early signs of amnion specification. Furthermore, we show that both amnion and serosa patterning are dependent on BMP activity, and that BMP activity in *M. abdita* embryos is subject to positive feedback. Our findings are summarized in a new model of serosa and amnion specification.

**MATERIALS AND METHODS**

**Flies and cloning procedures**

*Megaselia abdita* Schmitz and *Drosophila melanogaster* (Oregon strain) were reared in the laboratory. Partial open reading frames of *M. abdita* (Mab) homologs were cloned into pCRII-TOPO vector (Invitrogen) using degenerate and specific PCR primers as follows (5′-3′): *Mab-dpp*, TTTYTGCAGCAGATGCTGNGGCNA and GGNNCGAASSTCCATAC; *Mab-gbb*, AARTCNGCNCCATTGTTY and NGCTRTGRNTGTNGCRCTATRTG; *Mab-scw*, TTCTCAATCCGTCAACAAGGC and CACAAATTATTAAACTTTAAAGCC; *Mab-sat*, TGYCARAAYGNCNATHCARTGYTGG and RTTYTGRGTCARCAYT-

**CYTTCACT*; *Mab-dkv*, GGNARGTNTGGYTNGCAARTG and TGCCCARCAYTCYTGCATDATYTT, Mab-sog, CCNCCNTTYGGNNTAT-GTAYTG and YTCYARAYNGARCTRAANARYTC; following by a half nested reaction using as a substitute for the first primer CARTGYMGNAAYTAANAAAAYGA.

In situ hybridization, immunohistochemistry, RNAi and mRNA injections

For single-color in situ hybridization, *M. abdita* embryos were collected, heat fixed and hybridized as described (Rafiqi et al., 2008; Rafiqi et al., 2011). Antisense RNA probes were prepared from the linearized pCRIT-TOPO plasmids of the PCR clones and labeled with digoxigenin or FITC as described (Tautz and Pfiffner, 1989; Kosman et al., 2004). For immunostaining and double in situ hybridization, *D. melanogaster* and *M. abdita* embryos were fixed by the slow formaldehyde fixing method using PEMS (100 mM PIPES, 2 mM MgSO4, 1 mM EGTA (pH 6.9) (Rothwell and Sullivan, 2000). For *M. abdita*, a 3:1 PEMS:methanol solution was used instead of PEMS, and formaldehyde was used at a final concentration of 5% instead of 4%. These embryos were treated with proteinase K in PBS at a final concentration of 0.08 U/ml (1 hour on ice for in situ hybridizations; 2 minutes at room temperature for pMad immunohistochemistry) and postfixed in 5% formaldehyde in PBT (0.1% Tween 20 in PBS) for 25 minutes. The proteinase K treatment was necessary to reduce species-specific background of the pMad antisera in *M. abdita* embryos. Double in situ hybridizations with probes against Mab-z and Mab-hnt transcripts were performed with digoxigenin (DIG)-labeled and fluorescein (FITC)-labeled probes, and detected with Fab fragments from anti-FITC or anti-DIG antibodies conjugated with alkaline phosphatase (AP; Roche, Indianapolis, IN, USA). AP activity was detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Roche), which produce a dark blue precipitate, or with VectorRed (Vector Labs, Burlingame, CA, USA), which produces red precipitate and a fluorescent signal in the Cy3 channel. Double in situ hybridizations with probes against Mab-ve and Mab-z or Mab-hnt transcripts were performed with biotin-labeled and FITC-labeled probes, respectively. Biotin was detected with mouse anti-biotin IgG and Alexa Fluor 488-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA). FITC was detected with rabbit anti-FITC IgG and Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch). pMad was detected with rabbit antiserum against the phosphopeptidase NH2-CNPS-S[PO3]-V-S[PO3]-COOH, which was generated by Dan Vasiiliauskas, Susan Morton, Tom Jessell and Ed Laufer and kindly provided by Ed Laufer (Columbia University, New York, NY, USA), biotinylated goat anti-rabbit IgG (Vector Labs), and AP-conjugated antibiotin Fab fragments (Roche). For Mab quantifications, we used Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch).

Capped mRNAs of tkv, skv-a and noggin were prepared from available pSP35T plasmids (Holley et al., 1996; Neul and Ferguson, 1998) using SP6 polymerase with the mMessage mMachine Kit (Ambion). mRNA was prepared at 3 µg/µl (tkv, skv-a) or 5 µg/µl (noggin). Embryos were injected at the syncytial blastoderm stage (~1:40 to 2:45 hours after egg deposition), and fixed at late blastoderm stage or shortly after the onset of gastrulation. RNA interference (RNAi) was performed as described (Rafiqi et al., 2008; Rafiqi et al., 2010).

**Staging of embryos**

For consistent staging of both *Megaselia* and *Drosophila* embryos, morphological landmarks were measured from sagittal views of Nomarski images using ImageJ software (Schneider et al., 2012). Progression of blastoderm cellularization was approximated by dividing the distance from the apical cell membrane to the cellularization front (d1) by the distance of the apical cell membrane to the basal side of the nuclear envelope (d2) and dividing the d1/d2 coefficient by 2. Accordingly, the time point when the apicobasal level of the cellularization front coincided with the basal side of blastoderm nuclei was treated as 50% blastoderm cellularization. Ventral furrow formation (mesoderm invagination) and cephalic furrow formation were used as markers to determine the beginning of gastrulation. The onset of pole cell migration was variable. After cephalic furrow formation, we
Serosa and amnion specification

RESULTS

Distinction of serosa and amnion in early *M. abdita* embryos

In *M. abdita*, serosa specification can be visualized by studying the expression of the zen homolog *Mab-zen* (Stauber et al., 1999). *Mab-zen* is activated at the beginning of cellularization and appears in a dorsal domain that spans ~16 cell diameters of the dorsoventral perimeter at 50% egg length (supplementary material Fig. S1). During early cellularization, the width of this domain narrows until it precisely coincides with the anlage of the serosa, which at 50% egg length is ~6-7 cell diameters in width (Fig. 2A,D). Specific markers for amniotic tissue have not been identified. Some markers, including *Mab-pnr*, *Mab-tup* and *Mab-doc*, that are activated in the dorsal blastoderm are later differentially repressed in the serosa, but they are not suitable to distinguish amongniotic and embryonic tissue because they are also expressed in the dorsal epidermis (Rafiqi et al., 2008; Rafiqi et al., 2010). Another marker, *Mab-hnt* (a homolog of *hindsight*, also known as *pebbled*), is expressed in the serosa and the amnion but not in the adjacent lateral epidermis of the germ band (Rafiqi et al., 2010). To test whether earlier *Mab-hnt* expression occupies a broader domain than *Mab-zen* expression (serosa anlage), we performed double in situ hybridization experiments. Our method of detecting *Mab-zen* transcript (NBTCIP) precluded the detection of the *Mab-hnt* transcript in cells in which both genes are expressed owing to quenching of the *Mab-hnt* signal by NBTCIP, thereby highlighting *Mab-hnt*-positive cells in which *Mab-zen* is repressed. Both in the cellular blastoderm and during gastrulation, the dorsal expression domain of *Mab-hnt* was ~1-2 cell diameters broader than the expression domain of *Mab-zen* (Fig. 2A-D'). These observations show that *Mab-hnt* expression is broader than the serosa anlage.

To test whether the dorsal *Mab-hnt* expression domain overlaps with tissue that gives rise to the dorsal epidermis, we performed double in situ hybridizations with *Mab-eve*, a homolog of the pair-rule segmentation gene *even-skipped*, which is expressed in transverse stripes of the blastoderm (Bullock et al., 2004). Probes against *Mab-eve* and *Mab-zen* revealed that *Mab-eve* is gradually repressed in the serosa anlage (Fig. 3A-A''). By the time the embryo entered gastrulation, a narrow gap had formed between the *Mab-zen* expression domain and the dorsalmost ends of the *Mab-eve* stripes (Fig. 3B-B''). By contrast, the expression domains of *Mab-hnt* and *Mab-eve* abutted each other (Fig. 3C-D''). We expected this result for gastrulating stages but not for blastoderm stages, given that *Mab-hnt* is activated in a broader domain than *Mab-zen* (Fig. 2A-B'). At blastoderm stages, *Mab-hnt* expression lateral to the *Mab-zen* domain might be too weak to be revealed by fluorescent in situ hybridization. Nevertheless, our observations suggest that in gastrulating embryos a rim of *Mab-hnt*-positive cells has formed around the serosa anlage, in which the expression of markers of the embryo proper is repressed. We suspect that these cells give rise to the amnion and propose that amnion specification occurs, at least partially, after the specification of the serosa at the beginning of gastrulation.

BMP activity in early *M. abdita* embryos

Sequential patterning of the serosa and amnion raises the question of whether this process correlates with changes in the activity profile of BMPs. To address this question, we analyzed the distribution of pMad in *M. abdita* embryos at blastoderm and early gastrulation stages. The earliest embryos in which we could detect pMad were at the beginning of cellularization (Fig. 4A,A'). During

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**Fig. 1. Evolution of extra-embryonic tissue in flies (Diptera).**

Embryo sketches (anterior left, dorsal up) depicting the anlagen of the serosa (red) and amnion (orange) or amnio-serosa (brown) as well as their topology upon differentiation in various fly families (taxa examined are in bold print) (reviewed by Rafiqi et al., 2011) are shown above the phylogeny of Diptera (Wiegrann et al., 2011).

staged embryos based on the position of the proctodeum. To quantify this position we projected the dorsalmost point of the proctodeum (the anterior-dorsal kink) onto the length axis of the embryo and determined its distance to the posterior pole relative to total egg length (proctodeum position in percent).

Image analysis

Fluorescent images for pMad profiles were taken using a Zeiss LSM510 confocal microscope. Microscope settings were adjusted to avoid saturation but were kept constant for embryos stained on the same day. Subsequently, the images were further analyzed using ImageJ. Profile plots of 'summary' z-projections (40 pixels thick) were obtained using the Plot Profile function; the data were exported into spreadsheets. The profiles were calibrated with reference to background fluorescence intensity, which was calculated by averaging intensities in the non-expressing lateral region of the embryo. To pool profile data of different embryos at the same developmental stage, amplitudes were adjusted by subtracting the average background value from all the values for a given embryo. Confocal imaging of NBT/BCIP precipitate was performed using a long-pass background value from all the values for a given embryo. Confocal microscope. Microscope settings were adjusted to avoid saturation fluorescence was imaged with a Cy3 filter set.

Sequence analysis

Sequences were analyzed using the Geneious platform. Alignments were performed using ClustalW with a gap penalty of 3.0 and an extended gap penalty of 1.8 as recommended (Hall, 2001). Positions that were not conserved and represented in less than 20% of the sequences were removed from the alignment. Gene trees were generated using the maximum likelihood method in PHYML with the Jones-Taylor-Thornton substitution model and 500 bootstrap iterations (Guindon and Gascuel, 2003). The sequences reported in this paper have been deposited in the GenBank database (accession numbers: *Mab-dpp*, JQ712977; *Mab-gbb*, JQ712978; *Mab-Sax*, JQ712979; *Mab-scw*, JQ712980; *Mab-sog*, JQ712981; and *Mab-skv*, JQ712982).
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Fig. 2. Double in situ hybridizations for Mab-zen and Mab-hnt. (A–D’) Differential interference contrast (A,C) and confocal (B–B’,D–D’) images of M. abdita embryos (anterior left, dorsal view) at cellular blastoderm stage (A–B’) and gastrulation (C–D’). Transcripts were detected using alkaline phosphatase in the presence of NBT/BCIP (Mab-zen, dark blue in A,C) or VectorRed (Mab-hnt, red in A,C). Confocal images of NBT/BCIP precipitate were obtained using a long-pass emission filter (LP 615, Zeiss); fluorescence of VectorRed was detected in the Cy3 channel. B and D are merged false-color images of B,’B” and D,’D”, respectively. Note that the fluorescent signal is quenched by NBT/BCIP.

blastoderm cellularization, we recorded a stronger signal with sharper boundaries, measuring at 50% egg length ~8-10 cell diameters in width (Fig. 4B,B’). The pMad domain varied in width along the anteroposterior axis and excluded the pole cells. During early gastrulation, the central portion of the pMad domain gradually broadened, concomitant with an apparent depletion of pMad levels in the center of the domain (Fig. 4C-F’). The lateral expansion of the pMad domain resulted primarily from pMad in additional cells, rather than from changes in cell shape.

In order to compare the lateral extent of the pMad domain with the serosa anlage, we double stained embryos for Mab-zen transcript (using NBT/BCIP) and pMad (using VectorRed). At the cellular blastoderm stage, the pMad domain was almost completely quenched along the dorsoventral perimeter, indicating that at this stage pMad levels are low along the lateral sides of the serosa anlage (Fig. 4G-G’). However, at early gastrulation stages the pMad domain was distinctly broader than the Mab-zen domain (Fig. 4H-H”). These observations suggest that, at the beginning of gastrulation, BMP activity increases in the lateral amnion.

Comparison of the BMP activity profiles between M. abdita and D. melanogaster

We next examined whether the difference in extra-embryonic tissue specification between M. abdita and D. melanogaster correlates with a difference in the BMP activity profiles of these two species. In D. melanogaster, our enzyme-based staining protocol for pMad produced a stronger signal than in M. abdita, and both before and after the onset of gastrulation we detected pMad in a slightly broader domain than zen (supplementary material Fig. S2). However, in these experiments potential saturation of the pMad staining might have obscured any temporal redistribution of pMad. To test whether M. abdita and D. melanogaster undergo comparable shifts in their respective pMad profiles during early gastrulation stages, we therefore examined the pMad levels of both species quantitatively using fluorophore-conjugated antibodies instead of enzyme-conjugated Fab fragments (antigen-binding fragments). In both species, the pMad profile of optical cross-sections (measured at 50% egg length) changed during early gastrulation, both in intensity and width. Specifically, we observed shallower and broader pMad profiles in gastrulating embryos than in late blastoderm embryos (Fig. 5A-G’; supplementary material Fig. S3). This transition occurred at slightly variable stages relative to our early gastrulation markers (mesoderm invagination, cephalic furrow formation, proctodeum position). To compare the profiles of multiple embryos within each species, we pooled similar pMad profiles and defined partially overlapping sequential developmental windows in which they occur (Fig. 5A’-G’). We then combined the average profiles of these groups in single graphs (Fig. S5-H,I). This procedure revealed that during early gastrulation stages the pMad profile of D. melanogaster peaks at the dorsal midline, whereas the pMad profile of M. abdita peaks laterally (forming a saddle). These observations indicate a specific increase of pMad level in prospective amnion tissue of M. abdita, which is quantitatively distinct from the broadening of the pMad domain in D. melanogaster at comparable stages.

Fig. 3. Double in situ hybridizations for Mab-eve and Mab-zen or Mab-hnt. (A–D”) Mab-eve and Mab-zen (A–B”) or Mab-eve and Mab-hnt (C–D”) expression at the cellular blastoderm stage (A–A”,C–C”) and during early gastrulation (B–B”,D–D”). Mab-eve was detected using Cy3 (green); Mab-zen and Mab-hnt were detected with Alexa Flou 488 (red). Note the gap between the dorsal edges of the Mab-eve stripes and the Mab-zen domain, in particular at the later stage (B). By contrast, the Mab-eve stripes abut the Mab-hnt domain of gastrulating embryos (D). Dorsal view, anterior to the left. Boxed regions in A–D are enlarged in A’-D”.

For the full text, please refer to the original publication.
Fig. 4. pMad and Mab-zen transcript in M. abdita. (A-F') pMad domains at consecutive blastoderm (A-B’) and early gastrulation (C-F’) stages. Note the reduced mid-central pMad levels in gastrulating embryos (black arrows). Lateral and dorsal view with anterior to the left. (G-H') pMad and Mab-zen transcript shortly before (G-G') and shortly after (H-H') the onset of gastrulation. Mab-zen transcript was detected using NBT/BCIP (LP615 channel) and pMad using VectorRed (Cy3 channel), which is quenched in the presence of NBT/BCIP precipitate. Note the increased pMad signal lateral to the Mab-zen domain after the beginning of gastrulation (white arrows). Dorsal view with anterior to the left.

Expression of BMP signaling components in the M. abdita embryo

To test whether BMP signaling in the prospective serosa and amnion involves the same set of BMP signaling components, we cloned M. abdita homologs of the BMP ligand genes dpp (Mab-dpp), scw (Mab-scw) and gbb (Mab-gbb) (supplementary material Fig. S4), the BMP receptor genes sax (Mab-sax) and tkv (Mab-tkv) (supplementary material Fig. S5), and the BMP antagonist gene sog (Mab-sog) (supplementary material Fig. S6). In this section we document their specific expression patterns as revealed by whole-mount in situ hybridization and show that all these genes are expressed during early development in M. abdita.

Mab-dpp expression was detected on the dorsal side of the late blastoderm and gastrula (Fig. 6A-D’). After gastrulation, Mab-dpp expression was most prominent in a band of dorsal epidermis but not in its leading edge (Fig. 6E). However, after stomodeum formation [corresponding roughly to stage 10 in D. melanogaster (Campos-Ortega and Hartenstein, 1997)], a prominent stripe of Mab-dpp expression was observed ~1 cell diameter away from the leading edge of the epidermis. In part, the transcripts forming this expression domain seemed to belong to leading edge cells but their subcellular localization was opposite to the side of the amnion (Fig. 6F). We also noticed expression in more lateral epidermal and presumably mesodermal cells of the trunk segments, and in parts of the gnathal segments and clypeolabrum. After pharynx differentiation (corresponding roughly to stage 11 in D. melanogaster) a new, presumably mesodermal domain was observed in the first and second abdominal segments (Fig. 6G). Mab-scw expression was nearly ubiquitous, but weak and only detected during blastoderm stages (Fig. 6H-J). Mab-gbb was expressed in a broad dorsal domain of the blastoderm but not at the poles (Fig. 6K,K’). During gastrulation, Mab-gbb expression was strong in the serosa and amnion (Fig. 6L), but at the end of gastrulation Mab-gbb expression was stronger in the amnion than in the serosa (Fig. 6M). After stomodeum formation, Mab-gbb was expressed in the leading edge of the epidermis, in more lateral portions of the trunk and head epidermis, and in a cellular band of the mesoderm (Fig. 6N,N’). In summary, all three BMP ligand genes were expressed in the blastoderm but Mab-scw was downregulated prior to gastrulation and only Mab-dpp and Mab-gbb were expressed in older embryos.

Mab-sax was ubiquitously expressed until early gastrulation. Subsequently, the transcript was predominantly detected in the mesoderm (Fig. 7A-C). Mab-tkv transcript was also detected ubiquitously until gastrulation. During gastrulation, Mab-tkv expression shifted to the ventral side, including the mesoderm (Fig. 7D-F). Mab-sog expression started during the cellular blastoderm stage as a pair of broad lateral stripes (Fig. 7G,G’). During mesoderm invagination, the Mab-sog expression domains converged towards the ventral midline (Fig. 7H,H’). At this stage, Mab-sog expression was also detected in a number of cells in the gap between the Mab-sog stripes. By the time the embryo started germ band extension, the two stripes were close to each other as a result of ventral mesoderm invagination but, with the exception of a row of Mab-sog-positive cells at the ventral midline, a small gap remained throughout gastrulation (Fig. 7J,J’). In the head region, the stripes extended dorsally.

Role of specific BMP signaling components in serosa and amnion specification

To test whether serosa and amnion specification require BMP signaling and depend on the same set of BMP signaling components, we repressed Mab-dpp, Mab-scw, Mab-gbb, Mab-sax, Mab-tkv or Mab-sog by RNAi and tested for suppression of Mab-zen and Mab-hnt activation and for ectopic dorsal expression of Mab-eve. All components of the BMP pathway that we tested in this way affected dorsal patterning but did so with varying efficiency (Fig. 8; Fig. 9A-L). RNAi against Mab-dpp most efficiently suppressed the dorsal expression of Mab-zen (27/27) and Mab-hnt (25/25) and prevented the dorsal repression of Mab-eve (45/55) (Fig. 9A-F). RNAi against Mab-scw or Mab-gbb caused suppression of Mab-zen (20/41 or 29/77) and Mab-hnt (9/51 or 27/77) activation less frequently and almost never interfered with the dorsal repression of Mab-eve stripes (1/50 or 4/82). Double RNAi against Mab-scw and Mab-gbb proved slightly more efficient (26/40 for Mab-zen; 13/40 for Mab-hnt; 5/23 for Mab-eve) but was still much less efficient than RNAi against Mab-dpp.

Among the receptors, RNAi against Mab-sax suppressed the activation of Mab-zen (24/25) and Mab-hnt (23/31) and caused ectopic dorsal expression of Mab-eve (21/29) in most embryos (Fig. 9G-I), whereas Mab-tkv RNAi was less efficient in suppressing the activation of Mab-zen (26/40) and Mab-hnt (13/39) and almost never interfered with the dorsal repression of Mab-eve stripes (1/32). Mab-sog RNAi resulted in suppression or in reduction and broadening of Mab-zen (57/57) and Mab-hnt (44/44) activation (Fig. 9J,K) and interfered with the dorsal repression of Mab-eve (18/26) (Fig. 9L).
Evidence for a positive-feedback loop in BMP signaling

In *D. melanogaster*, a positive-feedback loop refines BMP activity along the dorsal midline (Wang and Ferguson, 2005). This feedback response transforms the initially broad and shallow gradient of BMP activity in blastoderm embryos into a much narrower and steeper gradient. In *D. melanogaster*, the mechanism was discovered by monitoring extracellular BMP-receptor interactions and intracellular pMad in blastoderm embryos that had been injected with mRNA of constitutively active Tkv (*tkv-a*). Tkv-a induces a correlated wave front of BMP-receptor interactions and intracellular pMad, which is accompanied by a decrease of receptor-bound ligand and pMad in adjacent cells. None of these effects is observed following injection of wild-type *tkv* mRNA, indicating that a positive-feedback mechanism is crucial to achieve the local boost in BMP activity (Wang and Ferguson, 2005).

Positive feedback of BMP signaling could be essential for the dynamic changes in the pMad domain that occur during blastoderm and gastrulation stages (see Discussion). To test whether such a feedback loop exists in early *M. abdita* embryos, we injected *tkv-a* mRNA into *M. abdita* embryos at the syncytial blastoderm stage and monitored its effect on the expression of *Mab-zen* and *Mab-hnt* (we used these markers instead of pMad because of background problems with the detection of pMad in the injected embryos). The injection of *tkv-a* mRNA consistently induced ectopic expression of *Mab-zen* (40/44) and *Mab-hnt* (34/35) anywhere along the dorsoventral axis, and often caused a reduction of endogenous expression, especially in the immediate vicinity of induced dorsal expression domains (Fig. 9M,N). None of these effects was observed when injecting *tkv* mRNA and staining for *Mab-zen* (*n=20*) or *Mab-hnt* (*n=31*).

Conversely, injection of *Xenopus* *noggin* mRNA, which has been shown to repress BMP signaling in *D. melanogaster* (Holley et al., 1996), caused a reduction of *Mab-zen* (28/48) and *Mab-hnt* (12/40) expression in *M. abdita* (Fig. 9O). From these experiments, we conclude that BMP signaling in early *M. abdita* embryos is subject to a positive-feedback loop that refines the dorsal expression domains of *Mab-zen* and *Mab-hnt*.

DISCUSSION

The role of BMP signaling in serosa and amnion specification in *M. abdita*

Flies experienced an evolutionary reduction of extra-embryonic tissue types from two to one, which must have been accompanied by changes in dorsal embryonic patterning. Here we have examined BMP signaling, a key regulator of dorsal patterning, in early embryos of *M. abdita*, a dipteran fly with distinct serosal and amniotic tissues and a close outgroup of schizophoran flies that develop a single amnioserosa. We found that in *M. abdita*, serosa and amnion patterning is dependent on a range of BMP signaling components. Furthermore, we identified a conspicuous difference in the dynamic BMP activity profile between *M. abdita* and *D. melanogaster*, which coincides in time and space with early signs of lateral amnion specification in gastrulating *M. abdita* embryos. Here we discuss our findings in the light of potential models of serosa and amnion specification and propose a synthesis of the available data.

Previously, it has been proposed that serosal and amniotic tissue types are specified by distinct threshold responses to a BMP activity gradient, which peaks along the dorsal midline of the blastoderm (Goltsev et al., 2007). Consistent with this idea, we found in *M. abdita* blastoderm embryos that pMad levels form a gradient that peaks at the dorsal midline, and that *Mab-hnt*, a marker of prospective serosa and amnion tissues, is activated in a broader domain than *Mab-zen*. However, although amnion specification might begin at the blastoderm stage, the distinction of lateral amnion cells from embryonic cells is still unclear at this stage as indicated by the confluent expression...
domains of the embryonic marker \textit{Mab-eve} and the serosa marker \textit{Mab-zen}. We therefore believe that lateral amnion specification is delayed until early gastrulation, when the \textit{Mab-eve} stripes have receded from the \textit{Mab-zen} domain and now abut the dorsal \textit{Mab-hnt} expression domain. This observation suggests that the specification of serosa tissue and that of the amnion occur, at least in part, at different time points: serosa specification at the cellular blastoderm stage and (lateral) amnion specification at the beginning of gastrulation.

Amnion patterning during early gastrulation correlates with a quantitative shift of the peak pMad level away from the serosa towards the region that gives rise to the amnion (Fig. 4G,H). In early gastrulation stages of \textit{D. melanogaster}, this change in the pMad profile appeared attenuated and was quantitatively clearly distinct from that in \textit{M. abdita} (Fig. 5H,I). The redistribution of pMad during early gastrulation might be important for specifying the amnion in \textit{M. abdita}.

At present, it is unclear which factor or factors cause the dynamic change of the pMad profile during early gastrulation in \textit{M. abdita}. The broadening of the pMad domain at this stage could be dependent on a target of the Toll signaling pathway, which presumably functions as a conserved dorsoventral pattern organizer on the ventral side (Lynch and Roth, 2011), or a factor downstream of BMP signaling. Alternatively, the positive-feedback loop of BMP signaling, for which we obtained evidence in \textit{M. abdita}, could explain the dynamics of BMP activity both in the blastoderm and during early gastrulation. In the blastoderm, analogous to the role of positive feedback of BMP signaling in \textit{D. melanogaster}, this mechanism might refine and enhance BMP signaling in the \textit{M. abdita} embryo and thereby influence the expression boundaries of target genes such as \textit{Mab-zen}, \textit{Mab-hnt} and \textit{Mab-eve}. At the beginning of gastrulation, positive feedback could drive the dorsoventral depression and lateral shift of BMP activity. In line with this idea, experimental
perturbation of BMP activity in early *D. melanogaster* embryos and mathematical modeling suggest that, in the presence of hyperactive positive feedback, an imbalance of receptor-bound BMP and Sog-mediated transport can change the directionality of extracellular BMP flux to the extent that a single domain of high BMP activity splits into two distinct peaks (Umulis et al., 2010). In wild-type *M. abdita* embryos, the balance of receptor-bound BMPs and Sog-mediated transport could be such that, under the influence of continuous positive BMP signaling feedback, the center of BMP activity shifts towards the amnion. Accordingly, suppression of positive BMP signaling during blastoderm stages should affect both serosa and amnion specification, whereas suppression of this feedback loop at the beginning of gastrulation would perturb amnion development. It should be possible to test this hypothesis once the molecular details of the positive-feedback loop are better understood.

In summary, our findings are consistent with a mixed model of serosa and amnion specification involving threshold responses to a BMP gradient, autoregulation of the BMP profile and differential response to high BMP activity over time (Fig. 10).

**Evolution of BMP signaling in the early fly embryo**

Our interpretation of the dynamic change of BMP activity during early gastrulation as an important factor in amnion specification raises the question of whether the increase of BMP activity in prospective amnion of early gastrula stages is conserved in other species. Functional data on extra-embryonic tissue specification in
the beetle *Tribolium castaneum* provide material for an outgroup comparison, which allows us to assess the directionality of the evolutionary change that we observe between cyclorrhaphan flies. In *T. castaneum*, the serosa is a derivative of anterior and dorsal blastoderm (Falciani et al., 1996) and is dependent on two signaling pathways: Torso (Tor) and BMP. Tor is a MAP kinase blastoderm (Falciani et al., 1996) and is dependent on two

- In *T. castaneum*, downregulation of the Tor pathway by RNAi causes a severe reduction of the serosa anlage (Schoppmeier and Schröder, 2005), whereas downregulation of the BMP pathway by RNAi against *T. castaneum* dpp (Tc-dpp) only suppresses a dorsal portion of the serosa anlage (van der Zee et al., 2006). Whereas Tc-dpp appears to control serosa specification only in part, its role in amnion specification appears to be crucial. Tc-dpp RNAi suppresses the expression of Tc-pnr, an early amnion marker, which indicates that Tc-dpp is required for amnion specification.

- The BMP activity profile of early *T. castaneum* embryos is consistent with its predominant role in the amnion. Initially, *T. castaneum* pMad accumulates along the whole dorsal side of the blastoderm embryo and tapers off laterally, but with the beginning of gastrulation, peak levels of pMad shift to the amnion (van der Zee et al., 2006; Nunes da Fonseca et al., 2008). The accumulation of pMad at the onset of gastrulation in the prospective amnion in *T. castaneum* is reminiscent of the shifting pMad peak levels in *M. abdita* embryos during early gastrulation. The apparent conservation of this feature in distantly related insects with serosal and amniotic tissues but distinct blastoderm fate maps suggests that BMP-dependent amnion specification requires BMP signaling in the prospective amnion at the beginning of gastrulation. Therefore, BMP-dependent amnion specification could be an ancient evolutionary heritage of insects, whereas the essential requirement of high BMP levels for serosa specification might have evolved in dipterans.

**BMP signaling and the evolution of sog expression**

The blastodermal pMad domain is much broader in the mosquito *A. gambiae* than in *D. melanogaster* or in *M. abdita*. The broad pMad domain of *A. gambiae* correlates with ventral (mesodermal) sog expression, which we found to be conserved in the harlequin fly *Chironomus riparius* (Chironomidae), a distant relative of *A. gambiae* (supplementary material Fig. S7A–B’). Mesodermal sog expression has also been reported for *T. castaneum* (van der Zee et al., 2006). By contrast, the comparatively narrow pMad domains of *D. melanogaster* (supplementary material Fig. S7C–E’) and *M. abdita* (Fig. 7G,G’) correlate with predominantly lateral (neuroectodermal) sog expression. This correlation is also conserved in *Episyrphus balteatus* (Syrphidae), another cyclorrhaphan fly with serosal and amniotic tissue (supplementary material Fig. S7G–I’). In summary, a shift in sog expression from ventral to lateral blastoderm might have been an important factor in narrowing the early embryonic activity range of BMP signaling in dipteran evolution, as proposed previously (Goltsev et al., 2007). However, the phenotypic consequences of shifting the center of sog expression from the mesoderm anlage to the neurogenic ectoderm are still unclear. Our data suggest that neuroectodermal sog expression evolved in the stem lineage of cyclorrhaphan flies possibly in conjunction with the evolutionary transition from ventral to dorsal amnion closure (Fig. 1).

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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