Development 134, 2415-2424 (2007) doi:10.1242/dev.02863

Evolution of the dorsal-ventral patterning network in the mosquito, *Anopheles gambiae*

Yury Goltsev[1], Naoyuki Fuse[2], Manfred Frasch[3], Robert P. Zinzen[1], Gregory Lanzaro[4] and Mike Levine[1,*]

The dorsal-ventral patterning of the *Drosophila* embryo is controlled by a well-defined gene regulation network. We wish to understand how changes in this network produce evolutionary diversity in insect gastrulation. The present study focuses on the dorsal ectoderm in two highly divergent dipterans, the fruitfly *Drosophila melanogaster* and the mosquito *Anopheles gambiae*. In *D. melanogaster*, the dorsal midline of the dorsal ectoderm forms a single extra-embryonic membrane, the amnioserosa. In *A. gambiae*, an expanded domain forms two distinct extra-embryonic tissues, the amnion and serosa. The analysis of approximately 20 different dorsal-ventral patterning genes suggests that the initial specification of the mesoderm and ventral neurogenic ectoderm is highly conserved in flies and mosquitoes. By contrast, there are numerous differences in the expression profiles of genes active in the dorsal ectoderm. Most notably, the subdivision of the extra-embryonic domain into separate amnion and serosa lineages in *A. gambiae* correlates with novel patterns of gene expression for several segmentation repressors. Moreover, the expanded amnion and serosa anlage correlates with a broader domain of Dpp signaling as compared with the *D. melanogaster* embryo. Evidence is presented that this expanded signaling is due to altered expression of the *sog* gene.

KEY WORDS: Mosquito, *Anopheles gambiae*, Embryo, Gastrulation, Amnion, Serosa, Gene network, Sog, Dpp, Zen, Dorsal-ventral patterning

INTRODUCTION

The dorsal-ventral patterning of the *Drosophila* embryo is controlled by a well-defined gene regulation network that is deployed by Dorsal (reviewed by Moussian and Roth, 2005), a sequence-specific transcription factor related to mammalian NF-kB (also known as Nfkb1) (Lenardo and Baltimore, 1989). The Dorsal protein is distributed in a broad nuclear gradient in precellular embryos. This transient gradient leads to stable circuits of cell differentiation that control gastrulation (Stathopoulos and Levine, 2004), including the invagination and patterning of the mesoderm, and the establishment of diverse cell types within the ectoderm.

The Dorsal gradient regulates over 50 target genes in a concentration-dependent manner (Stathopoulos and Levine, 2002). Approximately 40 of the genes encode sequence-specific transcription factors (TF) or components of signal transduction (ST) pathways that impinge on the activities of the TFs. Dorsal target enhancers have been identified for about half of these genes, and the DNA binding sites recognized by many of the TFs have been determined (reviewed by Stathopoulos and Levine, 2005). This information has permitted the construction of a detailed gene network, or circuit diagram, containing nearly 200 functional interconnections among the 40 TF and ST Dorsal target genes (Levine and Davidson, 2005).

It is our long-term goal to understand how changes in the *Drosophila* dorsal-ventral (DV) patterning network produce diverse gastrulation profiles in different insects. In the present study we compare dorsal-ventral patterning in *Drosophila melanogaster* and the malaria mosquito, *Anopheles gambiae*. Both insects are members of the same order, Diptera, but are highly divergent and last shared a common ancestor ~200 million years ago (Gailey et al., 2006). Genome turnover is so extensive that homologous enhancers do not display any vestige of sequence similarity. By contrast, sequence conservation is readily detected among extensively divergent vertebrates such as humans and pufferfish (Santini et al., 2003). Despite the turnover in the noncoding sequences of divergent insects, there is extensive conservation of the segmentation gene network, which serves to establish broadly similar body plans. For example, altered patterns in gap gene expression are balanced by compensatory changes in the regulation of downstream pair-rule genes (Goltsev et al., 2004).

Classical embryological studies revealed broad similarities in DV patterning among diverse Diptera (see Sander, 1975). However, notable differences were detected in the formation of the extraembryonic membranes (EMs). Specifically, higher dipterans such as *D. melanogaster* contain one EM, the amnioserosa (Demerec, 1950), whereas lower dipterans, such as mosquitoes, contain distinct amnion and serosa tissues (Christophers, 1960; Davis, 1967; Guichard, 1971; Idris, 1960; Ivanova-Kazas, 1949). Indeed, most insects contain separate tissues, suggesting that the formation of the single aminoserosa is a derived characteristic (Schmidt-Ott, 2000; Stauber et al., 1999). The analysis of segmentation gene expression in *A. gambiae* and specifically the repression of individual *eve* stripes in the presumptive serosa suggested early divergence in the DV patterning of the EMs of *D. melanogaster* and *A. gambiae* (Goltsev et al., 2004).

Here, we extend the previous analysis of segmentation to obtain a detailed picture of early dorsal-ventral patterning in *A. gambiae*. Particular efforts focus on the analysis of Dorsal target genes governing mesoderm invagination and the patterning of the ectoderm. Evidence is presented that the patterning of the ventral

[1]Department MCB, Division of GGD, Center for Integrative Genomics, University of California, Berkeley, CA 94720, USA. [2]Department of Developmental Genetics, National Institute of Genetics, 1111 Yata, Mishima 411-8540, Japan. [3]Brookdale Department of Molecular, Cell and Developmental Biology, Box 1020, Mount Sinai School of Medicine, New York, NY 10029, USA. [4]Department of Entomology, University of California, Davis, CA 95616, USA.

*Author for correspondence (e-mail: mlevine@berkeley.edu)

Accepted 3 April 2007

half of the embryo, the mesoderm and ventral neurogenic ectoderm, is highly conserved in *A. gambiae* and *D. melanogaster*. By contrast, the patterning of the dorsal ectoderm exhibits many differences.

The dorsal ectoderm of *D. melanogaster* produces just two cell types, dorsal epidermis and the amnioserosa. The latter tissue arises from a restricted region of the dorsal-most ectoderm, along the dorsal midline. In *A. gambiae*, the dorsal ectoderm is significantly expanded, and the dorsal midline is subdivided into distinct amnion and serosa lineages. The expansion of the dorsal ectoderm can be explained by a broadening in the domain of Dpp (BMP) signaling (reviewed by Podos and Ferguson, 1999) in the early *A. gambiae* embryo. Evidence is presented that this expansion results, in part, from the restricted expression of the Dpp inhibitor, Sog, within the presumptive mesoderm. In *Drosophila*, *sog* is expressed in a broad pattern that encompasses the entire neurogenic ectoderm (Francois et al., 1994). This broad *sog* pattern restricts Dpp signaling to the dorsal midline (Ashe and Levine, 1999; Decotto and Ferguson, 2001; Eldar et al., 2002; Holley et al., 1995; Marques et al., 1997; Mizutani et al., 2005; Shimmi et al., 2005), whereas the ventrally restricted *sog* pattern in *A. gambiae* appears to produce a broader domain of Dpp signaling. The broad *sog* pattern in *Drosophila* is driven by an intronic enhancer containing optimal Dorsal binding sites (Markstein et al., 2002). An analogous enhancer in *A. gambiae* contains low-affinity sites, and when expressed in transgenic *D. melanogaster* embryos it recapitulates restricted expression in the mesoderm, similar to the endogenous *A. gambiae* pattern. Thus, the interconversion of high- and low-affinity Dorsal binding sites appears to produce altered threshold responses to the Dorsal gradient. We discuss how subtle changes in the Dorsal patterning network can convert separate serosa and amnion tissues into a single tissue.

MATERIALS AND METHODS

Fly and mosquito stocks

Anopheles gambiae Kisumu strain was reared at 26°C, 75% humidity, with a 12-hour light/dark cycle. Adults were maintained on a 10% sucrose solution and females were blood-fed on anesthetized hamsters. *Drosophila melanogaster* strain yw^{67} was used for in situ hybridizations, as described previously (e.g. Stathopoulos et al., 2002). P-element-mediated transformation was performed using standard methods (Rubin and Spradling, 1982). Flies of the genotype P{*Kr-zen-Gal4*} were mated to those of the P{*UAS-A.gzen*}/+. The resulting embryos were of the genotype P{*Kr-zen-Gal4*}/+;*UAS-A.g.zen*/+; or P{*Kr-zen-Gal4*}/+;+/+. The embryos bearing P{*UAS-A.g.zen*} exhibit ectopic *Race*-expressing cells.

Cloning and injection of DNA fragments

Mosquito DNA was derived from the *Anopheles gambiae* Kisumu strain. We partially resequenced the *sog* locus to correct for the gaps in the mosquito genome assembly (Fig. S3 in supplementary material). The *A. gambiae sog* enhancer fragments were amplified from genomic DNA with the following primers:

Fragment 1: ACCAGGTCGTGTGCAGCTCGCGTATGGTCTT, GGCGTGCGAGCTCTTGCGTCTCCTACGCAG;

Fragment 2: GAGAACCGGTAATGGTCTAGCCGCCAA, GCAACCCCAACAACAACTCTGTTCACA;

Fragment 3: TGGTAGCACTTCGCACATTCGAGTTAG, TCAGCATCGACGATGCAATACCATACG;

Fragment 4: GCCGGTACGTGGTAGAGTGGCAGAGTA, CTGACCAGACGGCAGACCACGGTAGAA;

Fragment 5: TCTGATATGTCTGGGACGGTGTGTTGT, CTGGATGTTCGCATCACGTCTTCCTCT.

PCR products were cloned into a [-42evelacZ]-pCaSpeR vector (Small et al., 1992). The exact coding sequence for the *A. gambiae zen* gene was determined by RACE using an *A. gambiae* embryonic cDNA Marathon library. The coding sequence was amplified with the following pair of primers: ATAAAGTTTCTGTTAAGCAACTGCAGTAA, CCAGATGTCGTAGTACCCATTATATGGTAA.

PCR products were cloned into the pUAST (Brand and Perrimon, 1993) expression vector. Constructs were introduced into the *D. melanogaster* germline by microinjection as described previously (Ip et al., 1992). Between three and nine independent transgenic lines were obtained for each construct.

Whole-mount in situ hybridization

Mosquito embryos were collected and fixed as described previously (Goltsev et al., 2004). Hybridization probes were prepared against specific *A. gambiae* genes identified by reciprocal BLAST analyses. The hybridization probes were generated by RT-PCR amplification from embryonic RNA. A 26 bp tail encoding the T7 RNA polymerase promoter (TAATACGACTCACTATAGGGAGA) was included on the 5′ side of the reverse primer. PCR products were purified with the Qiagen PCR purification kit and used directly as templates for in vitro transcription reactions. The following primer pairs were used to amplify each of the indicated *A. gambiae* segmentation genes. (The T7 promoter sequence is denoted by the symbol [T7].):

twi: CTTATACTGGACATTAGTGGAGCCGGTT, AAG[T7]GGAAGCTAGCCGGAGCGTCTGTATCTT;

sna: CCACACCTCGTTCAACTCGTACCTTTCGTC, AAG[T7]TGGCATGAAGCTGTCCTCCGAGATGTT;

sim: AGCGTCAATCATACGACTCACCACCTCGTA, AAG[T7]TAGAACATAGTTGACGCTAACGATACA;

vnd: CCGGTGCTGACCTGGTCGCCGCTGCTGTTT, AAG[T7]GGACCGCCGTCAGCAGGTCGTGCGGTT;

brk: CCAGTTCAAGCTGCAGGTGCTCGACTCGTA, AAG[T7]TCCGGCTAATGTTGTACTTGGTCGCGA;

ind: TTCTAGTGGACTCGTTAATCAGTGATAAGC, AAG[T7]AGTGCGTACGATCTTCTGCTGATCGTT;

dpp: ACGCTAGTCGAGATAGAGAAGAACCTTCT, AAG[T7]CCGCAGCCAACGACCGTCATGTCCTGGTA;

tkv: CTGCTACTGCGAGGGCCACTGTCCGGGCAA, AAG[T7]GAGTCCGTCTCGAGCTTGACGAGCGTT;

zen: TCGCTGCTGACAGTTATATTGGTTCAACTA, AAG[T7]ATCATTATCGAGAGATGTGCTACAAGCCT;

hnt: CAGATGCAGGATGTGCCGCCCACGCCGGCC, AAG[T7]GGCGGTAGCTCAGCATCGCCCGACACCACGC;

tup: CGCTTATCCTTGTGCGTTGGATGCGGCGGTC, AAG[T7]CCATGTGCGAACCGATCGGAGGACCTGGCC;

Doc1: ACCGTCAGCAAATGTTGCAACGGATACCAG, AAG[T7]CGAGGAGGAGGTGTTGTTGCAGCCCATCTT;

ems: CTGGCGGCCCAGTTCCAGGCGGCCGCCCTT, AAG[T7]TCGGACAGTCGTCCATGTCGATGAACT;

hb: GGCTCGGACTGTGAGGATGGCTCGTACGAT, AAG[T7]CAGGTACGGGAACAGTGGCAGACTGCCGTT;

ttk: ATGGTGCAAACGAATCCGCTGCTCGGTACT, AAG[T7]CGCGAACGGACATCTCTGTGAGTGCTT;

sog: TGCCAGTTTGGCAAGACCATACGCGAGCTG, AAG[T7]CTTCTCGCACTTGTACTGCTGGTGGTCGCA;

tld: TGCTTGCGGAGGTCAGCTGGACACGCCGAA, AAG[T7]CTGATGTGGCTCAATATCGAACACATTGAA;

rho: CGGGTTCTTCGTCTACCACTCACTCACGTT, AAG[T7]ATACCTCTTCACTTTCCTCCTCTAGCCTCT.

Antibodies and staining for pSMAD

Rabbit anti-pMad antibody was kindly provided by P. ten Dijke (Leiden University Medical Center, Leiden, Netherlands). *D. melanogaster* and *A. gambiae* embryos were fixed as described previously (Goltsev et al., 2004). Primary antibodies were used at a dilution of 1:200. Secondary anti-rabbit antibodies conjugated to alkaline phosphatase (Jackson ImmunoResearch) were used for staining.

Scanning electron microscopy

Fly and mosquito embryos were fixed as for in situ hybridization. The embryos were subsequently post-fixed in 25% glutaraldehyde for 30 minutes, dehydrated and dried. Embryos were coated with gold-palladium and observed with a JOEL JSM 5800LV scanning electron microscope.

Computational identification of shared motifs and enhancers

A Dorsal position weighted matrix (Papatsenko and Levine, 2005) was used to identify potential Dorsal binding clusters at the *Anopheles sog* locus. The recently developed ClusterDraw software was used for this analysis (Zinzen et al., 2006).

RESULTS

Mesoderm invagination

D. melanogaster and *A. gambiae* embryos exhibit distinct patterns of mesoderm invagination (Fig. 1A). Scanning electron photomicrographs of the ventral surface of the *D. melanogaster* embryo (top panel) show that the presumptive mesoderm constricts at the apical surface. Mesectodermal cells, and possibly some of the lateral mesoderm cells, have processes oriented towards the ventral furrow. It is conceivable that these protrusions contribute to mesodermal tube closure and mesectoderm intercalation (Leptin et al., 1992; Leptin and Grunewald, 1990). The presumptive mesoderm of *A. gambiae* also have apical constrictions (Fig. 1A, bottom panel), however, they lack the oriented protrusions mentioned above. There is a shallow groove at the midline in place of the deep furrow seen in *D. melanogaster*. These observations suggest that the mesoderm does not undergo the same type of coherent involution in *A. gambiae* as seen in *D. melanogaster*.

Mesoderm invagination has been studied by analyzing the expression of *twist*, an early determinant of mesoderm fate (Boulay et al., 1987; Thisse, 1987). This approach was used in staged *A. gambiae* embryos which were hybridized with a digoxigenin-labeled *twist* antisense RNA probe, and then mounted in plastic and sectioned (Fig. 1B). *twist* staining is restricted to the ventral-most 25% of the embryo circumference, as seen in *D. melanogaster*. There is transient apical constriction of the mesoderm plate (Fig. 1Bc), followed by the appearance of a shallow groove along the ventral midline. There is no organized involution of the mesoderm, but instead, individual mesoderm cells undergo progressive ingression during germband elongation (Fig. 1Bd-f). This ingression is similar to that seen in mutant *D. melanogaster* embryos lacking *fog-concertina* signaling. In these mutants, there is a severe reduction of the ventral furrow and mesoderm cells fail to invaginate (Costa et al., 1994; Dawes-Hoang et al., 2005). Nonetheless, many of the mutant embryos survive because of ingression of the mesoderm during elongation. Interestingly, the *A. gambiae* genome lacks a clear homologue of the *fog* gene (see Discussion).

Conservation of the neurogenic ectoderm

A number of marker genes were analyzed to determine whether there have been significant changes in the DV patterning network responsible for the mesoderm and neurogenic ectoderm in flies and mosquitoes (Fig. 2). Despite the different modes of mesoderm invagination, the overall limits of the presumptive mesoderm are quite similar in flies and mosquitoes (Fig. 2A-D). In both cases, the *twist* and *snail* expression patterns are restricted to the ventral-most regions destined for later ingression during elongation. As in *D. melanogaster*, the *snail* pattern has somewhat sharper lateral borders than those seen for *twist* (Fig. 2C; compare with A). *sim* expression is restricted to single lines of cells immediately straddling the *snail* borders (Fig. 2E,F). These lines coincide with the ventral-most regions of the neurogenic ectoderm, and the cells will form specialized mesectodermal derivatives along the ventral midline of the nerve cord (Martin-Bermudo et al., 1995).

In *D. melanogaster*, intermediate and low levels of the Dorsal gradient lead to sequential patterns of *vnd* and *ind* expression, which pattern the medial and lateral portions of the future nerve cord

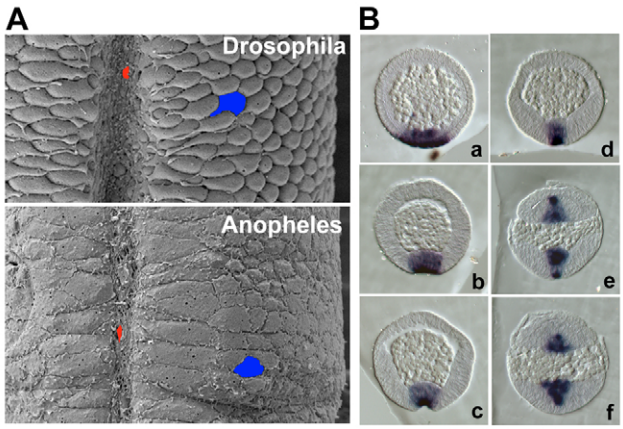

Fig. 1. Gastrulation morphology in *D. melanogaster* and *A. gambiae* embryos. (**A**) Scanning electron micrographs of *D. melanogaster* and *A. gambiae* embryos, ventral views. The apical surface of a lateral neuroectoderm cell is colored blue and a mesoderm cell is colored red. Note the constriction of the apical cell surface during gastrulation. (**B**) Cross sections of gastrulating mosquito embryos after hybridization with a digoxigenin-labeled *twist* antisense RNA probe. The different panels show progressive time points starting with the stage immediately preceding gastrulation (a) and ending with the completion of gastrulation (f). Ingression of the mesoderm is best seen in e when some of the mesoderm progenitors are inside the blastocoel while the rest are on the surface. Staining in both top and bottom regions of e and f is due to germband elongation.

(McDonald et al., 1998; Weiss et al., 1998). Similar sequential patterns are seen for the corresponding genes in *A. gambiae* (Fig. 2I,J; data not shown). The *ind* pattern has a segmental periodicity in *A. gambiae* (Fig. 2I), but is otherwise similar to the expression pattern seen in *D. melanogaster* (Fig. 2J).

The *brinker* gene encodes a transcriptional repressor that is a component of the Dpp (BMP) signaling pathway in the *D. melanogaster* embryo (Campbell and Tomlinson, 1999; Jazwinska et al., 1999). It is activated in ventral and lateral regions of the neurogenic ectoderm, in a pattern similar to *vnd* (Fig. 2H). Once again, a comparable pattern is seen in *A. gambiae* (Fig. 2G). Overall, the preceding results suggest that the initial patterning of the mesoderm and neurogenic ectoderm depend on similar mechanisms in the fly and mosquito embryos. The only clear difference is the formation of a coherent ventral furrow and invaginated mesodermal tube in *D. melanogaster*.

Distinct patterning of the dorsal ectoderm

There is a clear difference in the dorsal ectoderm of *D. melanogaster* and *A. gambiae* embryos. The *A. gambiae* embryo is enclosed by the serosa, an external cuboidal layer of cells that forms an extraembryonic membrane (Fig. 3B,D,E,F; blue arrows in B and D, pseudo-colored blue in F). There is also a separate amnion that connects the embryo proper to the serosa (e.g. Fig. 3D,F – red arrow in D and pseudo-colored red in F) and therefore resides between the external serosa and the germband. The establishment of a double-layered extraembryonic envelope is a highly dynamic process, well described for a number of diverse insects (reviewed by Schmidt-Ott, 2005) (see also van der Zee et al., 2005). The electron micrograph in Fig. 3A and the DIC image in Fig. 3D show the initial phases of germband elongation in the mosquito. At this stage the caudal regions of the germband begin

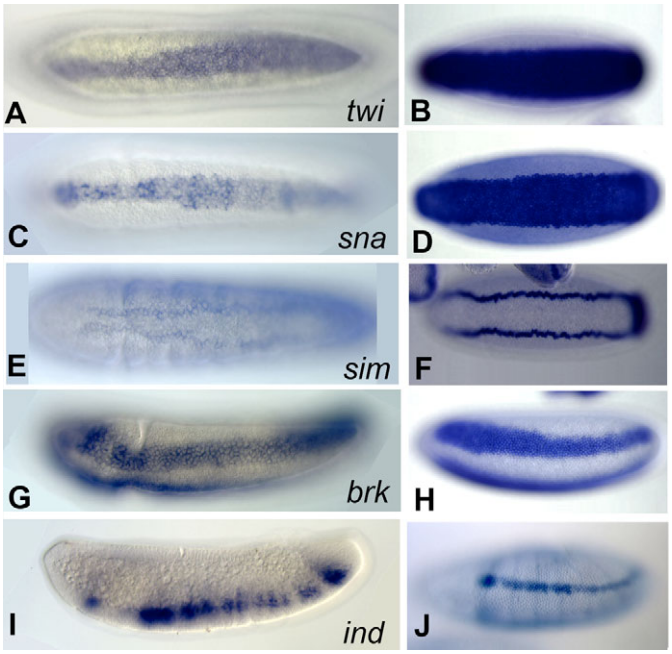

Fig. 2. Expression of mesoderm and neurogenic patterning genes in *D. melanogaster* and *A. gambiae* embryos. Gastrulating embryos (*A. gambiae*, left and *D. melanogaster*, right) were hybridized with different digoxigenin-labeled antisense RNA probes. (**A-F**) Ventral views; (**G-J**) lateral views. (A,B) *twist* (*twi*); (C,D) *snail* (*sna*); (E,F) *single-minded* (*sim*); (G,H) *brinker* (*brk*); (I,J) *intermediate neuroblasts defective* (*ind*).

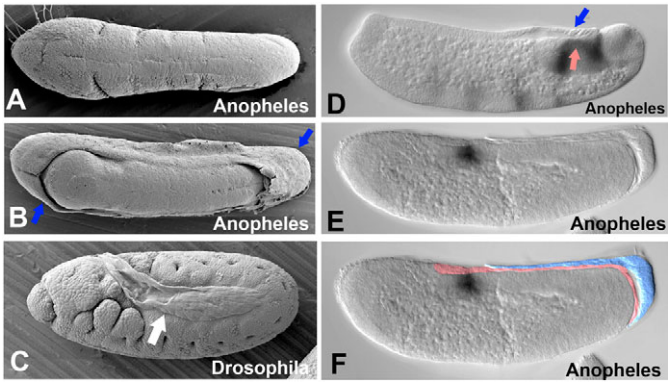

Fig. 3. Extra-embryonic membranes. Staged *A. gambiae* embryos were analyzed by scanning electron microscopy (**A**,**B**) and DIC microscopy (**D**,**E**,**F**). (**C**) Lateral view of *D. melanogaster* embryo at the end of germband extension stage; the amnioserosa is indicated by the white arrow. The embryos in the left panels are at the same relative stages as those shown in the right panels. The same embryo is shown in E and F, and was colored in F to better visualize the separate amnion (red) and serosa layers (blue). The embryos in D-F were hybridized with an *eve* RNA probe. Blue arrows indicate the serosa, red arrows indicate the amnion.

to migrate beneath the serosa and the double-layered topology of the extraembryonic membrane is established. The EMs have not yet extended to ventral regions (Fig. 3A). Later, the amnion continues to migrate over the germband stretching the serosa around the embryo (Fig. 3B,E,F). Finally, the *A. gambiae* embryo becomes fully enclosed, whereby the amnion and serosa fuse along the ventral midline of the germband.

The *D. melanogaster* embryo contains a single amnioserosa arising from the dorsal midline (white arrow, Fig. 3C). The formation of separate amnion and serosa lineages is probably ancestral for insect embryos, since these are seen in a broad range of insects, including flour beetles, bees and grasshoppers (Dearden et al., 2000; Panfilio et al., 2006; Schmidt-Ott, 2000; Stauber et al., 1999; Stauber et al., 2002; van der Zee et al., 2005). Higher Dipterans, such as *D. melanogaster*, are somewhat unique in containing just a single amnioserosa.

Early separation of serosa and aminon lineages

A variety of dorsal patterning genes were examined in *A. gambiae* embryos in an effort to determine the basis for the formation of distinct ectodermal derivatives. For example *hindsight* (*hnt*; also known as *peb* – Flybase) (Frank and Rushlow, 1996) is expressed along the dorsal midline of *D. melanogaster* embryos (Fig. 4B), while *tailup* (*tup*) (Thor and Thomas, 1997) is expressed in a broader pattern that encompasses both the presumptive amnioserosa and dorsolateral ectoderm (Fig. 4D). The *hnt* expression pattern seen in *A. gambiae* is similar to that detected in *D. melanogaster*, although there is a marked expansion in the dorsal-ventral limits of the presumptive extra-embryonic territory (Fig. 4A; prospective serosa is marked by red oval). By contrast, the *tup* pattern in *A. gambiae* is dramatically different from that seen in *D. melanogaster* – it is excluded from the prospective serosa and restricted to the future amnion (Fig. 4C).

The T-box genes *Dorsocross1* (*Doc1*) and *Doc2* are involved in amnioserosa development and expressed along the dorsal midline and in a transverse stripe near the cephalic furrow of gastrulating *D. melanogaster* embryos (Reim et al., 2003) (Fig. 4F). The *Doc1* and *Doc2* orthologues in *A. gambiae* exhibit restricted expression in the presumptive amnion (Fig. 4E,G; the white arrow in G indicates the amnion), similar to the *tup* pattern. The expression patterns of the two genes are identical but only *Doc1* is shown. They are initially expressed in a broad dorsal domain (data not shown) but come to be repressed in the serosa. There is also a head stripe of expression comparable to the *D. melanogaster* pattern (Fig. 4E). Additional dorsal-ventral patterning genes are also expressed in a restricted pattern within the developing amnion (see Fig. S1 in supplementary material). Overall, the early expression patterns of *tup*, *Doc1* and *Doc2* (and additional patterning genes) foreshadow the subdivision of the dorsal ectoderm into separate serosa and amnion lineages in *Anopheles*.

Altered expression of Dpp signaling components in *Anopheles* embryos

In *D. melanogaster*, the patterning of the dorsal ectoderm depends on Dpp and Zen, along with a variety of genes encoding Dpp signaling components, such as the Thickveins (Tkv) receptor. Most of the corresponding genes are expressed in divergent patterns in *A. gambiae* embryos (Fig. 5). For example, *dpp* and *tkv* are initially expressed throughout the dorsal ectoderm (data not shown), but become excluded from the presumptive serosa and restricted to the amnion (Fig. 5A,C). By contrast, both genes have broad, nearly uniform expression patterns in the dorsal ectoderm of *D. melanogaster* embryos (Fig. 5B,D).

There is an equally dramatic change in the *zen* expression pattern. In *A. gambiae*, expression is restricted to the presumptive serosa territory, even at the earliest stages of development (Fig. 5E,G). By contrast, *zen* is initially expressed throughout the dorsal ectoderm of

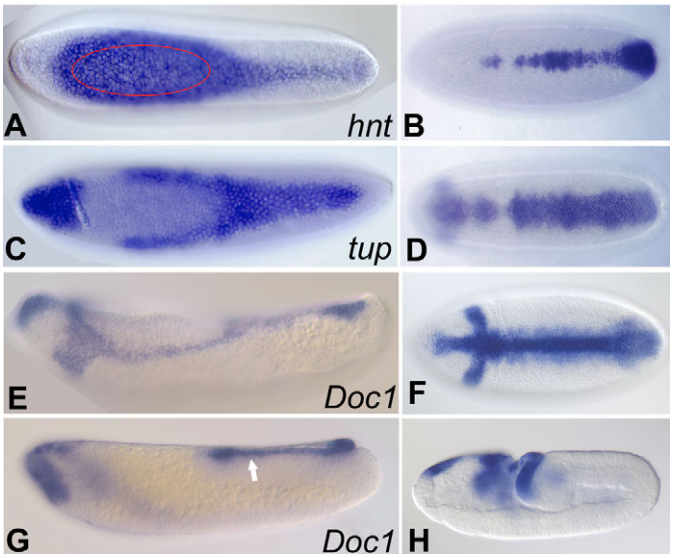

Fig. 4. Expression of dorsal patterning genes in *A. gambiae*. Embryos (*A. gambiae*, left and *D. melanogaster*, right) were hybridized with the indicated antisense RNA probes. A-D,F are dorsal views; E,G,H, are lateral views. (**A**,**B**) *hindsight* (*hnt*); (**C**,**D**) *tailup* (*tup*); (**E**,**F**) *Dorsocross1* (*Doc1*) before germband elongation; (**G**,**H**) *Doc1* after germband elongation. The area outlined by the red oval in A is the prospective serosa; the white arrow in G indicates the amnion.

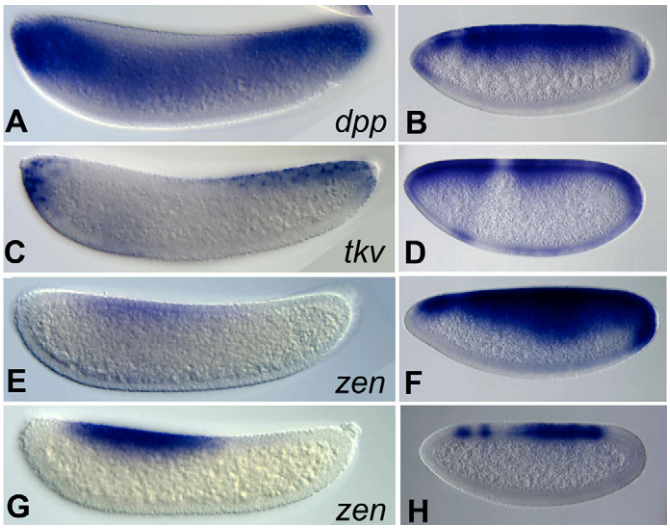

Fig. 5. Expression of Dpp signaling components in *A. gambiae*. Embryos (*A. gambiae*, left and *D. melanogaster*, right; all lateral views) were hybridized with the indicated antisense RNA probes. (**A**,**B**) *decapentaplegic* (*dpp*); (**C**,**D**) *thick veins* (*tkv*); (**E-H**) *zerknullt* (*zen*). All embryos are at the cellular blastoderm stage, except E, which is a precellular mosquito embryo.

cellularizing embryos in *D. melanogaster* (Fig. 5F), and becomes restricted to the dorsal midline by the onset of gastrulation (Fig. 5H). Thus, the *dpp/tkv* and *zen* expression patterns are essentially complementary in *A. gambiae* embryos, but extensively overlap in *Drosophila* (see below).

Serosa-specific repressors?

The loss of *dpp* (Fig. 5A), *tkv* (Fig. 5C), *Doc1* (Fig. 4E), *Doc2* (not shown) and *tup* (Fig. 4C) expression in the presumptive serosa of *A. gambiae* embryos raises the possibility that *zen* activates the expression of one or more repressors in the serosa. It is unlikely that Zen itself is such a repressor since the expression of the *A. gambiae zen* gene in transgenic *Drosophila* embryos does not alter the normal development of the amnioserosa (see Fig. S2 in supplementary material).

Different segmentation genes were examined in an effort to identify putative serosa-specific repressors. For example, the gap gene *hunchback* (*hb*) is initially expressed in the anterior regions of *A. gambiae* embryos, in a similar pattern to that seen in *D. melanogaster* (Bender et al., 1988; Lehmann and Nusslein-Volhard, 1987), but by the onset of gastrulation a novel pattern arises within the presumptive serosa (Goltsev et al., 2004). *hb* expression has also been seen in the developing serosa of other insects, including a primitive fly (*Clogmia*) and the flour beetle, *Tribolium* (Stauber et al., 2002; Wolff et al., 1995).

Two additional segmentation genes behave like *hb*, *empty spiracles* (*ems*) and *tramtrack* (*ttk*) (Fig. 6A,C). *ems* is involved in head patterning in *D. melanogaster* (Dalton et al., 1989). Its expression is limited to a single stripe in anterior regions of cellularizing *D. melanogaster* embryos (Fig. 6B). Staining is seen in a comparable anterior region of *A. gambiae* embryos (Fig. 6A), but a second site of expression – not seen in *Drosophila* – is also detected in the presumptive serosa.

Ttk is a maternal repressor that helps establish the expression limits of several pair-rule stripes (Read et al., 1992). It is ubiquitously expressed throughout the early *D. melanogaster* embryo (Fig. 6D), but has a tightly localized expression pattern within the presumptive serosa of *A. gambiae* embryos (Fig. 6C). Thus, novel patterns of *ems* and *ttk* expression are consistent with the possibility that serosa-specific repressors help subdivide the dorsal ectoderm into separate serosa and amnion lineages in *A. gambiae* embryos (see Discussion).

Altered *sog* and *tolloid* expression patterns

The analysis of dorsal-ventral patterning genes identified two critical differences between the pre-gastrular fly and mosquito embryos. First, there are separate serosa and amnion lineages in *A. gambiae*, but just a single amnioserosa in *D. melanogaster*. Second, there is an expansion in the limits of the dorsal ectoderm in *A. gambiae* as compared with the *D. melanogaster* embryo. Localized repressors might help explain the former observation of separate lineages, but do not provide a basis for the expansion of the dorsal ectoderm.

In *D. melanogaster*, the limits of Dpp signaling are established by the repressor Brinker (Jazwinska et al., 1999) and the inhibitor Sog (Francois et al., 1994). Genetic studies suggest that Sog is the more critical determinant in early embryos. It is related to Chordin, which inhibits BMP signaling in vertebrates (Francois and Bier, 1995), and is expressed in broad lateral stripes encompassing the entire neurogenic ectoderm (Fig. 7B) (Markstein et al., 2002). The secreted Sog protein directly binds Dpp, and blocks its ability to interact with the Tkv receptor (e.g. Shimmi et al., 2005). However, Sog-Dpp complexes are proteolytically processed by the Tolloid (Tld) metalloprotease (Mullins, 1998), which is expressed throughout the dorsal ectoderm of early *Drosophila* embryos (Fig. 7G) (Marques et al., 1997). Tld helps ensure that high levels of the Dpp signal are released at the dorsal midline located far from the restricted source of the inhibitor Sog (Shimmi et al., 2005).

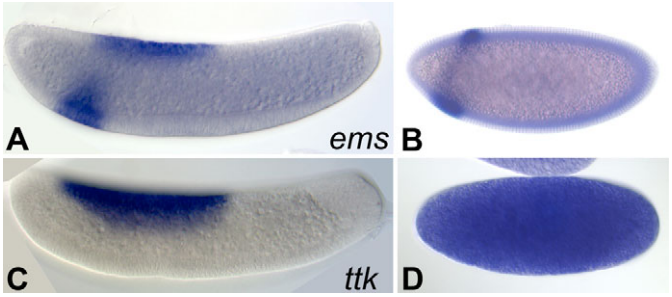

Fig. 6. Expression of putative repressors in the *A. gambiae* serosa. Embryos (*A. gambiae* left and *D. melanogaster* right; all lateral views) were hybridized with the indicated antisense RNA probes. Embryos are oriented to show lateral views. (**A**,**B**) *empty spiracles* (*ems*); (**C**,**D**) *tramtrack* (*ttk*).

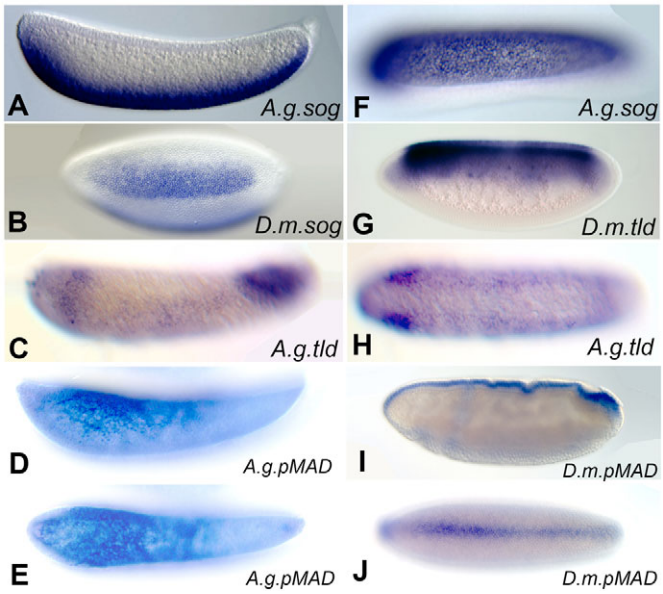

Fig. 7. Expansion of Dpp signaling in *A. gambiae* dorsal ectoderm. Mosquito (*A.g.*) and fruitfly (*D.m.*) embryos were hybridized with *short gastrulation* (*sog*; **A**,**B**,**F**) or *tolloid* (*tld*; **C**,**G**,**H**) antisense RNA probes. A,B,C, and G are lateral views; F and H are ventral views. Mosquito (**D**,**E**) and fruitfly (**I**,**J**) embryos were also stained with an antibody that recognizes the active, phosphorylated form of Mad, pMad. (D,I) Lateral views; (E,J) dorsal views. The resulting staining patterns indicate a much broader domain of Dpp signaling in the dorsal ectoderm of *A. gambiae* embryos as compared with *D. melanogaster*.

The expression patterns of the *sog* and *tld* genes in *A. gambiae* are very different from those seen in *D. melanogaster* (Fig. 7). *sog* expression is primarily detected in the ventral mesoderm, although low levels of *sog* transcripts might extend into the ventral-most regions of the neurogenic ectoderm (Fig. 7A,F). This pattern is more restricted across the dorsal-ventral axis than the *D. melanogaster sog* pattern (Fig. 7B). *tld* expression is restricted to lateral regions of *A. gambiae* embryos (Fig. 7C,H) and is excluded from the dorsal ectoderm, which is the principal site of expression in *Drosophila* (Fig. 7G). These significant changes in the *sog* and *tld* expression patterns might account, at least in part, for the expanded limits of Dpp signaling in the dorsal ectoderm of *A. gambiae* embryos (see Discussion).

Direct evidence for broader Dpp signaling was obtained using an antibody that detects phosphorylated Mad (pMad) (Persson et al., 1998), the activated form of Mad obtained upon induction of the Tkv receptor. In *D. melanogaster* pMad expression is restricted to the dorsal midline (Fig. 7I,J). This is the domain where Sog-Dpp complexes are processed and peak levels of Dpp interact with the receptor Tkv. The spatial limits of the *sog* expression pattern are decisive for this restricted domain of pMad activity. Just a twofold reduction in the levels of Sog (*sog*/+ heterozygotes) causes a significant expansion in pMad expression (Mizutani et al., 2005).

There is a marked expansion of the pMad expression domain in *A. gambiae* embryos as compared with *Drosophila* (Fig. 7D,E). The domain encompasses the entire presumptive serosa and extends into portions of the presumptive amnion. The *dpp* and *tkv* expression patterns are downregulated in the presumptive serosa (Fig. 5A,C), nonetheless, the pMad staining pattern clearly indicates that this is the site of peak Dpp signaling activity. The early expression of both *dpp* and *tkv* encompasses the entire dorsal ectoderm. It would appear that peak Dpp signaling is somehow maintained in the developing serosa even after the downregulation of *dpp* and *tkv* expression in this tissue (see Discussion). A similar scenario is seen in the *Drosophila* embryo, in that there is downregulation of both *dpp* and *tkv* expression along the dorsal midline of gastrulating embryos (e.g. Affolter et al., 1994).

The *A. gambiae sog* enhancer

To determine the basis for expanded Dpp signaling we identified and characterized a *sog* enhancer in *A. gambiae*. The *D. melanogaster* enhancer is located in the first intron of the *sog* transcription unit (Fig. 8D). It is ~300 bp in length and contains four evenly spaced, optimal Dorsal binding sites (Markstein et al., 2002). These sites permit activation of *sog* expression by low levels of the Dorsal gradient; however, closely linked Snail repressor sites inactivate the enhancer in the ventral mesoderm. A putative *A. gambiae* enhancer was identified by scanning the *sog* locus for potential clusters of Dorsal binding sites. The recently developed cluster-draw program was used for this purpose since it successfully identified a *sim* enhancer in the honeybee, *Apis mellifera*, which is even more divergent than *Anopheles* (Zinzen et al., 2006). The best putative Dorsal binding cluster was identified within the first intron of the *A. gambiae sog* locus (Fig. 8C). Several genomic DNA fragments were tested for enhancer activity, but only this cluster was found to activate gene expression in transgenic *Drosophila* embryos (summarized in Fig. 8D).

Two different genomic DNA fragments, 3.7 kb and 1.1 kb, that encompass the intronic binding cluster were tested in transgenic embryos (see Fig. 8D). Both fragments were attached to a *lacZ* reporter gene containing the core *eve* promoter from *D. melanogaster*, and both direct *lacZ* expression in the presumptive mesoderm (Fig. 8A,B; data not shown). They exhibit the same restricted dorsal-ventral limits of expression as that seen for the endogenous *sog* gene in *A. gambiae*, although the smaller fragment produces ventral stripes whereas the larger fragment directs a more uniform pattern (not shown). The change in the dorsal-ventral limits – broad expression in *D. melanogaster* and restricted expression in *A. gambiae* – might be due to the quality of individual Dorsal binding sites in the two enhancers (see Discussion).

DISCUSSION

A comprehensive analysis of dorsal-ventral patterning genes in the *A. gambiae* embryo reveals elements of conservation and divergence in the gastrulation network of *D. melanogaster*. There is broad

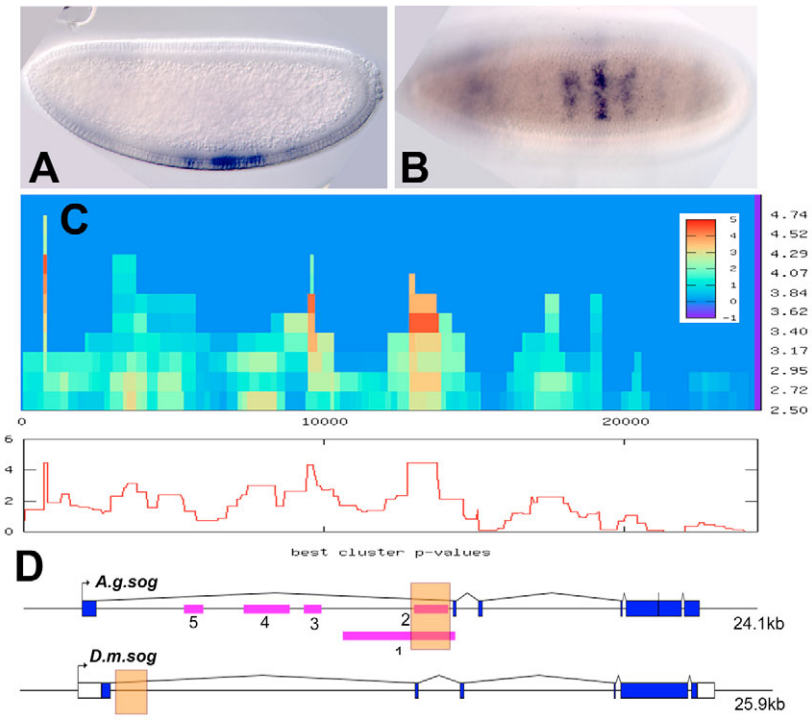

Fig. 8. The *A. gambiae sog* enhancer directs restricted expression in transgenic *D. melanogaster* embryos. (**C**) The Cluster-Draw program (Zinzen et al., 2006) identified several potential Dorsal binding clusters in the *A. gambiae sog* locus. (**D**) The best cluster is located at the 3′ end of intron 1 (top schematic). Different genomic DNA fragments (numbered pink bars) were attached to a *lacZ* reporter gene, inserted into the *D. melanogaster* genome (lower schematic) and expressed in transgenic embryos. Only fragments 1 and 2 exhibited any activity as measured by in situ hybridization with a *lacZ* antisense RNA probe. (**A**,**B**) Lateral and ventral views, respectively, of embryos after staining with the larger fragment (the fragment 1-*lacZ* fusion gene).

conservation in the expression of regulatory genes responsible for the patterning of the mesoderm and neurogenic ectoderm, including sequential expression of *sim*, *vnd* and *ind* in the developing nerve cord. By contrast, there are extensive changes in the expression of regulatory genes that pattern the dorsal ectoderm. These changes foreshadow the subdivision of the dorsal ectoderm into separate serosa and amnion lineages in *A. gambiae*.

Evolution of mesoderm invagination

The major difference in the early patterning of the mesoderm in flies and mosquitoes concerns the manner in which mesoderm cells enter the blastocoel of gastrulating embryos. In *D. melanogaster*, there is a coherent invagination of the mesoderm through the ventral furrow, much like the movement of bottle cells through the blastocoel of *Xenopus* embryos (Keller, 1981). By contrast, there is no invagination of the mesoderm in *A. gambiae*. Instead, the mesoderm undergoes progressive ingression during germband elongation. This type of ingression is seen in *D. melanogaster* mutants lacking *fog* signaling (Costa et al., 1994; Dawes-Hoang et al., 2005). The *A. gambiae* genome lacks a clear homologue of *fog*, and it is therefore conceivable that *fog* represents an innovation of the higher Diptera that was only recently incorporated into the *D. melanogaster* dorsal-ventral patterning network.

Evolution of extra-embryonic morphology

D. melanogaster is somewhat unusual in having an amnioserosa, rather than separate serosa and amnion tissues as seen in most insects (Dearden et al., 2000; Panfilio et al., 2006; Stauber et al., 2002; van der Zee et al., 2005). In certain mosquitoes the serosa secretes an additional proteinaceous membrane that provides extra protection against desiccation (Harwood, 1958; Harwood and Horsfall, 1959). The changes in gene expression in the *D. melanogaster* and *A. gambiae* dorsal ectoderm provide a basis for understanding the evolutionary transition of two dorsal tissues in *A. gambiae* into a novel single tissue in higher dipterans.

The *D. melanogaster* amnioserosa expresses a variety of regulatory genes, including *Doc1/2* and *tup*. The expression of most of these genes is restricted in the presumptive amnion of the *A. gambiae* embryo. *zen* is the only dorsal patterning gene, among those tested, that exhibits restricted expression in the serosa. Several segmentation genes have a similar pattern, and one of these, *ttk*, encodes a known repressor. Ectopic expression of Ttk causes a variety of patterning defects in *Drosophila* embryos, including disruptions in head involution and germband elongation that might arise from alterations in the amnioserosa (Read et al., 1992). We propose that *zen* activates *ttk* in the serosa of *A. gambiae* embryos. The encoded repressor might subdivide the dorsal ectoderm into separate serosa and amnion tissues by inhibiting the expression of *Doc1/2* and *tup* in the serosa. The loss of this putative *zen-ttk* regulatory linkage might be sufficient to allow Dpp signaling to activate *tup* and *Doc1/2* throughout the dorsal ectoderm, thereby transforming separate serosa and amnion tissues into a single amnioserosa. According to this scenario, the loss of *zen* binding sites in *ttk* regulatory sequences might be responsible for the evolutionary transition of the amnioserosa (summarized in Fig. 9; see below).

Expansion of the dorsal ectoderm territory

The formation of separate amnion and serosa tissues is not the only distinguishing feature of *A. gambiae* embryos when compared with *D. melanogaster*. There is also a significant expansion in the overall limits of the dorsal ectoderm. This can be explained, in part, by distinct patterns of *sog* expression.

The broad expression limits of the Sog inhibitor are responsible for restricting Dpp/pMad signaling to the dorsal midline of the *D. melanogaster* embryo (summarized in Fig. 9A). This pattern depends on a highly sensitive response of the *sog* intronic enhancer to the lowest levels of the Dorsal gradient. The Dorsal binding sites in the *sog* enhancer are optimal sites, possessing perfect matches to the idealized position weighted matrix of Dorsal recognition sequences (Papatsenko and Levine, 2005). By contrast, the *A.*

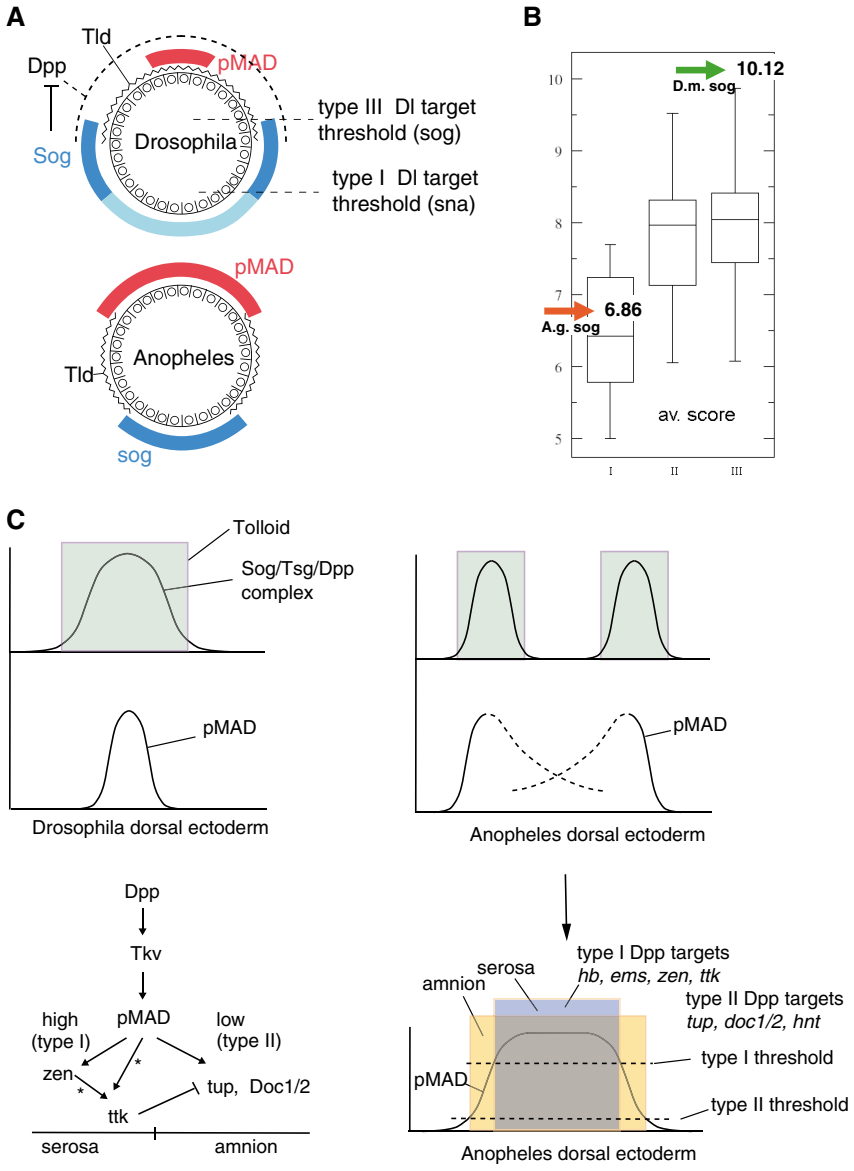

Fig. 9. Model for the patterning of the *A. gambiae* dorsal ectoderm. (**A**) The diagrams represent cross-sections of early mosquito (bottom) and fruit fly (top) embryos. The *sog* expression pattern (blue) is restricted to ventral regions of mosquitoes, but is broadly distributed in lateral regions of *D. melanogaster*. The ventralization of the *sog* pattern in *A. gambiae* might cause the indicated expansion of Dpp signaling and pMad expression (red). There is sequential expression of Sog and Tld in both the fruitfly and mosquito embryo. However, the two patterns are shifted towards ventral regions in the mosquito embryo. (**B**) Quality of Dorsal binding sites in the *Anopheles sog* enhancer (average score of 6.7) as compared with those in the *Drosophila* enhancer (average score of 10.1). The score range covered by the box contains 50% of all data points (the second and third quartiles of distribution). The bottom and top marks correspond to maximal and minimal score values, respectively (see Papatsenko and Levine, 2005). The Roman numerals beneath the plots indicate each of the three major patterning thresholds. For example, the *htl* and *sna* enhancers are type 1 enhancers that are activated only by high levels of the Dorsal gradient. (**C**) Tld is responsible for generating a peak of Dpp signaling at the dorsal midline, resulting in a spike of pMad activity in the *Drosophila* dorsal ectoderm (left panels). By contrast, the altered patterns of *tld* and *sog* expression in *A. gambiae* embryos are expected to generate two peaks of Dpp signaling activity, resulting in the broad plateau of pMad staining in the dorsal ectoderm (right panels). The subdivision of the dorsal ectoderm into distinct amnion and serosa lineages can be explained on the basis of the expanded pMad staining pattern, and the recruitment of the repressor Ttk into the Dpp signaling network. The asterisks indicate specific regulatory linkages that are lost in *D. melanogaster*. Only one of these linkages is required for the expression of *ttk*, or some other serosa-specific repressor in *A. gambiae*.

gambiae intronic *sog* enhancer contains low-quality Dorsal binding sites, similar to those seen in the regulatory sequences of genes activated by peak levels of the Dorsal gradient, such as *twist*. The binding sites in the *D. melanogaster sog* enhancer have an average score of ~10. By contrast, the best sites in the *A. gambiae sog* enhancer have scores in the 6.5-7 range, typical of enhancers that mediate expression in the mesoderm in response to high levels of the Dorsal gradient (Fig. 9B). Although we did not explicitly test every potential regulatory sequence in the *A. gambiae sog* locus, none of the putative Dorsal binding clusters in the vicinity of the gene possess the quality required for activation by low levels of the Dorsal gradient in the neurogenic ectoderm. Thus, the narrow limits of *sog* expression in *A. gambiae* embryos can be explained by the occurrence of low-quality Dorsal binding sites, along with the loss of Snail repressor sites.

The altered *sog* expression pattern is probably not the sole basis for the expansion of the dorsal ectoderm. *A. gambiae* embryos also exhibit a significant change in the *tld* expression pattern. *tld* is expressed throughout the dorsal ectoderm in *D. melanogaster*, but restricted to the neurogenic ectoderm of *A. gambiae*. Tld cleaves inactive Tsg-Sog-Dpp complexes to produce peak Dpp signaling along the dorsal midline of *Drosophila* embryos (Fig. 9C). We propose that the altered *tld* pattern in combination with altered *sog* leads to two dorsolateral sources of the active Dpp ligand in mosquito embryos. The sum of these sources might produce a step-like distribution of pMad across dorsal regions of mosquito embryos (Fig. 9C). This broad plateau of pMad activity might be responsible for the observed expansion of the dorsal ectoderm territory, and the specification of the serosa.

In *Drosophila*, *tld* is regulated by a 5′ silencer element that prevents the gene from being expressed in ventral and lateral regions in response to high and low levels of the Dorsal gradient. This silencing activity is due to close linkage of Dorsal binding sites and recognition sequences for ‘co-repressor’ proteins (e.g. Ratnaparkhi et al., 2006). Our preliminary studies suggest that Dorsal activates the *A. gambiae tld* gene, possibly by the loss of co-repressor binding sites in the 5′ enhancer (Kirov et al., 1993).

We propose that there are at least two distinct threshold readouts of Dpp signaling in the dorsal ectoderm of *A. gambiae* embryos. Type 1 target genes, such as *hb*, *ems*, *ttk* and *zen*, are activated by

high levels and thereby restricted to the presumptive serosa. Type 2 target genes, such as *tup* and *Doc1/2*, can be activated – in principle – by both high and low levels of Dpp signaling in the presumptive serosa and amnion. However, these target enhancers contain binding sites for one or more type 1 repressors expressed in the serosa. Our favorite candidate repressor is Ttk. Perhaps the type 2 *tup* enhancer contains optimal pMad activator sites as well as binding sites for the localized repressor Ttk, which keeps *tup* expression off in the serosa and restricted to the amnion (see diagram in Fig. 9C). As discussed earlier, the simple loss of *ttk* regulation by the Dpp signaling network might be sufficient to account for the evolutionary conversion of separate serosa and amnion tissues into a single amnioserosa. Localization of this single tissue within a restricted domain along the dorsal midline would arise from concomitant dorsal shifts in the *sog* and *tld* expression patterns.

We are grateful to Peter ten Dijke for generously providing the pMad antibody, and Dmitri Papatsenko for help with the computer analysis of *sog* enhancers. We also thank members of Levine lab for helpful discussions. We are deeply grateful to Lanzaro lab members and specifically to Claudio Menesis for maintaining the mosquito colony. This work was funded by grants from the NIH (GM46638 and GM34431).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/13/2415/DC1

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