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Evolution of Developmental Control Mechanisms

Postgastrular *zen* expression is required to develop distinct amniotic and serosal epithelia in the scuttle fly *Megaselia*

Ab. Matteen Rafiqi, Steffen Lemke, Urs Schmidt-Ott *

University of Chicago, Dept. of Organismal Biology and Anatomy, CLSC 921B, 920 E. 58th Street, Chicago, IL 60637, USA

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ABSTRACT

The amnioserosa is an extraembryonic epithelium that evolved in higher cyclorrhaphan flies from distinct serosal and amniotic epithelia. The underlying genetic mechanism of this evolutionary transition is unknown. Amnioserosa development of *Drosophila* correlates with novel expression characteristics of the homeobox gene *zerknüllt* (*zen*), including a broad *zen* expression domain in the syncytial blastoderm and the complete absence of postgastrular *zen* expression. Here we examine the functional significance of these features by altering the activity profile of *zen* in *Megaselia* (a lower cyclorrhaphan fly with distinct serosal and amniotic epithelia) and *Drosophila*, and by examining in *Megaselia* the function of u-shaped group (ush-group) genes, which in *Drosophila* maintain the amnioserosa after gastrulation when *zen* is no longer expressed. In *Megaselia*, loss of postgastrular *zen* expression abrogates serosa development but allows amnion development. Ectopic expression of *zen* in early *Megaselia* embryos allows serosa formation but perturbs amnion development. *Megaselia* homologues of u-shaped group genes are not essential for serosa formation but mediate germband retraction and dorsal closure. Finally, ectopic postgastrular *zen* expression in *Drosophila* causes an enlargement of amnioserosa cells and interferes with the morphogenetic functions of the amnioserosa. Our results suggest that the origin of the amnioserosa involved the loss of postgastrular *zen* expression from extraembryonic tissue, that the early broad expression domain of *Drosophila zen* evolved afterwards, and that the ush-group genes ancestrally played a role in morphogenetic functions of the amnion.

Introduction

Higher cyclorrhaphan flies (Schizophora), such as *Drosophila*, develop a single extraembryonic epithelium, called amnioserosa (Grimaldi and Engel, 2005). This tissue closes the germband dorsally until the flanks of the embryo meet at the dorsal midline (dorsal closure) and the amnioserosa is internalized and disintegrates in the yolk (Campos-Ortega and Hartenstein, 1997; Kiehart et al., 2000; Reed et al., 2004). The amnioserosa mediates germband retraction, which aligns the elongated u-shaped embryo with the anterior–posterior (AP) axis of the egg, through interactions with the underlying yolk sac (Lamka and Lipshitz, 1999; Reed et al., 2004; Yip et al., 1997), and dorsal closure by providing contractile force to the leading edge of the epidermis (Hutson et al., 2003; Narasimha and Brown, 2004; Scuderi and Letsou, 2005; Solon et al., 2009). Other dipteran embryos undergo the same morphogenetic movements but generate distinct amniotic and serosal epithelia instead of an amnioserosa (Fig. 1) [reviewed in (Schmidt-Ott et al., in press)]. Lower cyclorrhaphan flies, such as the scuttle fly *Megaselia* (Phoridae), develop a continuous sheet of cells, which resembles the amnioserosa of schizophoran flies during early development but, after gastrulation, resolves into two extraembryonic cell layers: an amnion that closes the dorsal wall of the embryo and a serosa that envelopes the embryo (Fig. 1) (Rafiqi et al., 2008). How these differences in extraembryonic tissues evolved, and how extraembryonic tissues mediate germband retraction and dorsal closure in flies with an amnion and serosa is not known.

The *Drosophila* homeobox gene *zerknüllt* (*zen*) is necessary for the specification and early development of the amnioserosa (Pultz et al., 1988; Rushlow et al., 1987a,b), and over expression of *zen* causes an expansion of this tissue (Rushlow and Roth, 1996). Zen-deficient *Drosophila* embryos specify embryonic tissue along the dorsal midline instead of the amnioserosa and have fused optic lobes (Arora and Nusslein-Volhard, 1992; Chang et al., 2001). After gastrulation, when *zen* is no longer active, the amnioserosa is maintained by genes of the u-shaped group (ush-group) (Frank and Rushlow, 1996; Reim et al., 2003; Yip et al., 1997). The late amnioserosa plays an important role in germband retraction and dorsal closure, and mutations that impair ush-group genes perturb these processes. The ush-group includes *u-shaped* (*ush*), *serpent* (*srp*), *hindsight* (*hnt*), *dorsocross* (*doc*), and *tail-up* (*tup*). *srp*, *ush*, *doc* and *hnt* mutant embryos exhibit programmed cell death of the amnioserosa resulting in germband retraction and dorsal closure defects (Frank and Rushlow, 1996; Reim et al., 2003).

* Corresponding author. Fax: +1 773 702 3172.
E-mail address: uschmid@uchicago.edu (U. Schmidt-Ott).

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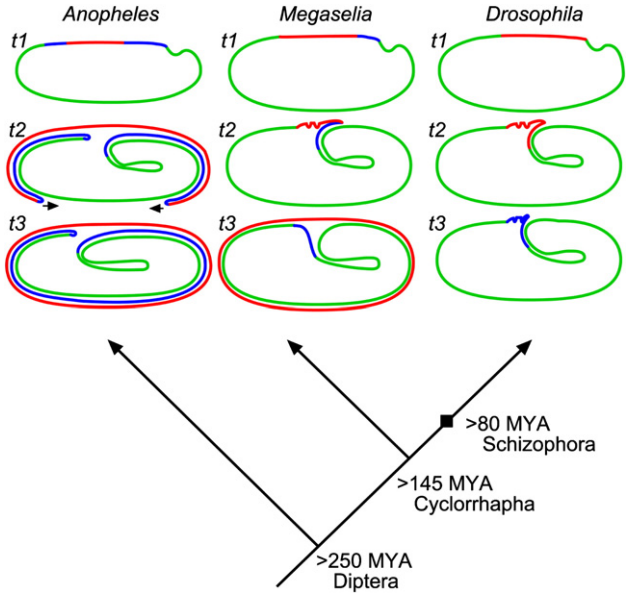

Fig. 1. Schematic comparison of extraembryonic development in dipteran insects. Early (t1), mid (t2) and late (t3) germband extension stages are shown for a non-cyclorrhaphan species (*Anopheles*), a lower cyclorrhaphan species (*Megaselia*) and a schizophoran species (*Drosophila*). The serosa/*zen*-expressing amnioserosa is depicted in red, the amnion/*zen*-negative amnioserosa in blue and the embryo in green. Anterior is left and dorsal up. The putative origin of the amnioserosa (black square) coincides with the beginning of the radiation of extant Schizophora. MYA = Million Years Ago. Drawings of *Anopheles* are based on (Goltsev et al., 2009). Drawings of *Megaselia* and *Drosophila* are reproduced with permission from (Rafiqi et al., 2008); Copyright (2008) National Academy of Sciences, U.S.A.

Tup-deficient embryos do not exhibit apoptosis in the amnioserosa but nonetheless produce a reduced amnioserosa and exhibit germband retraction and dorsal closure defects (Frank and Rushlow, 1996). The function of the homologues of the ush-group genes in species with an amnion and a serosa is not known.

Previously, we identified three features of *zen* expression in *Drosophila*, which have no equivalent in *Megaselia* or other insects with an amnion (Figs. 2A–H) (Goltsev et al., 2007; Rafiqi et al., 2008; Stauber et al., 2002; van der Zee et al., 2005). In *Megaselia*, *zen* expression begins during blastoderm cellularization in a narrow stripe along the dorsal midline while in *Drosophila zen* expression begins at an earlier blastoderm stage in a much broader domain. Furthermore, *Megaselia zen* is specifically expressed in the serosa and is excluded from the amnion while *Drosophila zen* is expressed in the entire extraembryonic tissue. Finally, *Megaselia zen* continues to be expressed in the expanding serosa while *Drosophila zen* is repressed immediately after gastrulation [see also (Rafiqi et al., 2008)]. The sudden repression of *zen* in *Drosophila* embryos between the end of gastrulation and the beginning of stomodeum formation occurs at the same developmental stage as the expansion of the serosa and its disjunction from adjacent tissue in *Megaselia*. Since *zen* is essential for serosa development, we hypothesize that the loss of postgastrular *zen* expression in an ancestor of *Drosophila* led to the origin of a single dorsal extraembryonic epithelium. This implies that early developmental stages of the ancestral amnioserosa would have been composed of *zen*-positive serosal and *zen*-negative amniotic tissue while postgastrular developmental stages of the ancestral amnioserosa would have been equivalent to a *zen*-negative dorsal amnion. Subsequently, after the distinction between amniotic and serosal tissue became obsolete, gain of *zen* expression in the early blastoderm could have inhibited amnion development and homogenized the tissue. The *Drosophila* amnioserosa may thus reflect termination of serosa development during germband extension and delayed amnion specification until that stage (Rafiqi et al., 2008). This hypothesis leads to the following predictions. (1) Postgastrular *Mab-zen* expression should be required for serosa development. (2) Since ush-group genes are active in the late amnioserosa and are required for germband retraction and dorsal closure in *Drosophila*, they should likewise be active in the late amnion of *Megaselia* and be required for germband retraction and dorsal closure. (3) Ectopic *Mab-zen* expression should interfere with amnion development and its putative morphogenetic functions in germband retraction and dorsal closure. We tested these predictions by examining the phenotypic effects of repressing late *Mab-zen* expression, by repressing *Megaselia* ush-group genes and by injecting *Mab-zen* mRNA into early blastoderm embryos. We also examined the effect of over expressing *zen* in postgastrular embryos of *Drosophila*.

Materials and methods

Cloning procedures

A fragment of *Megaselia hindsight* (*Mab-hnt*) was obtained by PCR on cDNA using degenerate primers (5′- ACNACNAAYGGNAAYATGCA/ 5′- CAYTTRAANGGYTTYTGNCC);

Megaselia u-shaped (*Mab-ush*) using degenerate primers (5′- MGNCAYATGMGNATGCAYCAR/5′- YTTRCARAANACDATRTTRCAYTC);

Megaselia dorsocross (*Mab-doc*) using degenerate primers (5′- GGNACNGAGATGRTCATHACNAAR/5′- GAANCCYTTNGCRAANGG RTTRTT); *Megaselia tail-up* (*Mab-tup*) using degenerate primers (5′- CARATHCAYGAYCARTAYAT/5′- RTANGTNACRTANSWRTCNG);

Megaselia dopa decarboxylase (*Mab-ddc*) using degenerate primers (5′- ATHATGCCNGGNGTNACNCAYTGGCA/5′- GGNGCNGANCCYTG-CATRTCGTGYTT). *Mab-doc* 3′ end sequence was extended using Rapid Amplification of cDNA ends (RACE) using the primer (5′- AATTCCATGG-GCGCCACAACAATC). The *Mab-hnt* probe comprised 948 nucleotides of the ORF (open reading frame); *Mab-ush* probe 1032 nucleotides of the ORF; *Mab-doc* probe 492 nucleotides of the ORF; *Mab-tup* probe 1083 nucleotides of the ORF and *Mab-ddc* probe 837 nucleotides of the ORF. All probes were labeled with digoxigenin as described (Kosman et al., 2004; Tautz and Pfeifle, 1989). To create the template for capped *Mab-zen* mRNA, the ORF was PCR-amplified from cDNA with the primer pair 5′- ACCATGGGCACTTTTGACAACGATTTC/5′- TGTCGACTTAAAGCTCCA-AGATATCAATG, digested with NcoI and SalI, and cloned into pSP35 (Amaya et al., 1991). The predicted protein sequence of all sequenced clones ($n=6$) displayed polymorphisms compared to the previously published sequence of *Mab-zen* (Stauber et al., 1999) at positions Y2G, R110M, R132P, and T251S. To create a template for *Mab-zen* mRNA with a stop codon in front of the homeodomain (K44Stop), the *Mab-zen* ORF was amplified from cDNA by two overlapping PCR fragments with the primer pairs 5′- ACCATGGGCACTTTTGACAACGATTTC/5′- GGGCTGCTG-GACTAGTCACTATCACTTCCATTGTC and 5′- GTGATAGTGACTAGTCCAG-CAGCCCTGCAAAG/5′- TGTCGACTTAAAGCTCCAAGATATCAATG, and reconstituted from the two fragments using only terminal primers. The product was digested with NcoI and SalI, and cloned into pSP35. Apart from the introduced stop codon, the predicted protein sequence of these independently cloned *Mab-zen* fragments carried the same polymorphisms as the *Mab-zen* wildtype clone. To create template for double-stranded RNA, pCRII-TOPO (Invitrogen) cloned fragments were PCR-amplified using the primer pair 5′-TAATACGACTCACTATAGGGA-GACCA-CAGTGTGCTGGAATTCGCCCTT/5′-TAATACGACTCACTATAGG-GAGACCA-CTGGATATCTGCAGAATTCGCCC that are specific to the vector sequence flanking insert.

RNAi and mRNA injections

Injections of *Mab-zen* double-stranded RNA (dsRNA) prior to gastrulation were done as described (Rafiqi et al., 2008). After gastrulation embryos were carefully staged by discarding disparate stages from a 15-minute egg collection. Double-stranded RNA was injected, at a concentration of about 5 μM, through the ventral side of dechorionated embryos after the pole cells had migrated to the

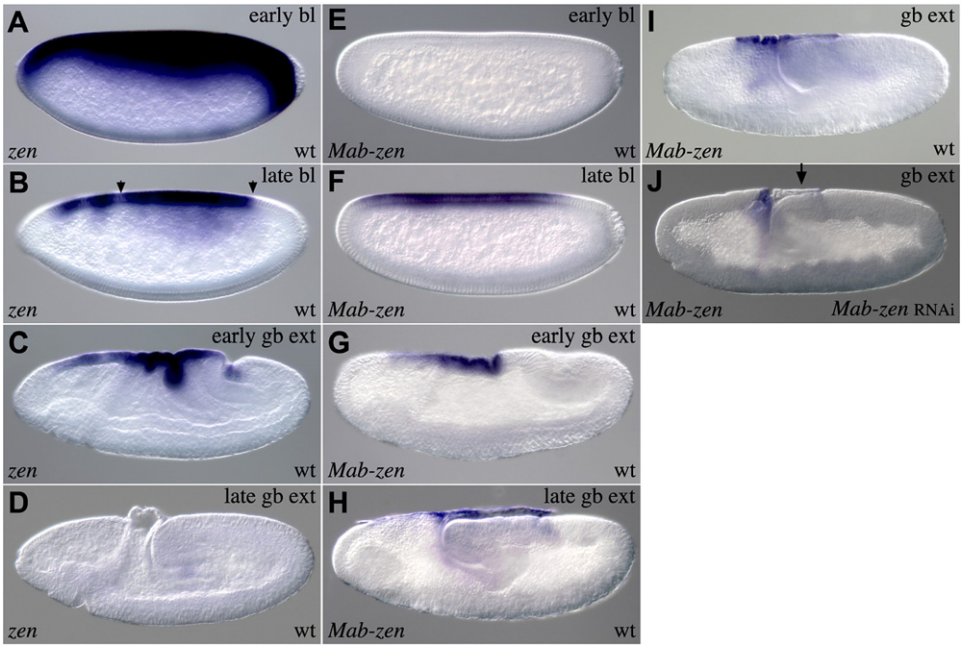

Fig. 2. Comparison of *zen* expression between *Drosophila* and *Megaselia* and effect of late *zen* RNAi on *zen* expression in *Megaselia*. (A–H) RNA in situ hybridizations of *Drosophila* (A–D) and *Megaselia* (E–H) embryos at early (A, E) and late (B, F) blastoderm stages and consecutive germband extension stages (C, D, G, H). The limits of the amnioserosa anlage along the anteroposterior axis are marked by arrowheads. (I, J) *Mab-zen* expression in a wildtype embryo (I) and a *Mab-zen* RNAi embryo (J) at mid germband extension stage. *Mab-zen* RNAi was induced at the beginning of germband extension. Note the absence of *Mab-zen* expression in the posterior serosa (arrow). Embryos are shown in lateral view with anterior to the left. Abbreviations: bl, blastoderm stage; gb ext, germband extending stage.

dorsal side and the proctodeum had invaginated. Capped mRNA was prepared as described (Lemke and Schmidt-Ott, 2009) and injected at a concentration of 5 µg/µl (14.35 µM) unless mentioned otherwise.

Staining procedures and cuticle preparations

Megaselia embryos were heat fixed as described (Rothwell and Sullivan, 2000) with modifications. The embryos were treated with a boiling solution of 0.7% NaCl and 0.05% Triton X-100 followed by a heptane and methanol devitellinization step. Postfixation was done either with 5% formaldehyde in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.1% Tween20 pH 7.4) or with 5% (of aqueous phase) formaldehyde in 1:1:2 mixture of PBS, Methanol and Heptane respectively. Postfixation in either case was followed by a second heptane and methanol devitellinization step. *Drosophila* embryos were fixed by Slow-Formaldehyde-Fixing-Method (Rothwell and Sullivan, 2000). In situ hybridizations, immunohistochemistry and cuticle preparations were done as described (Rafiqi et al., 2008; Stern and Sucena, 2000). Rat monoclonal Tubulin antibody YOL1/34 (Kilmartin et al., 1982) was used in 1/100 dilution.

Staging of Megaselia embryos

At 25 °C, germband extension starts ca. 3:20 h to 3:50 h after egg deposition and ends prior to serosa completion (at 5 h to 5:30 h) and stomodeum formation (at 5:30 h to 6:30 h). Germband retraction begins roughly 7:30 h to 8 h after egg deposition.

Heat shock experiments with Drosophila

pCaSpeRHS plasmid containing 46 nucleotides of the 5′ UTR and the *zen* open reading frame (with the last codon changed from AAC to ATC) and the SV40 small t antigen 3′ UTR (Rushlow and Roth, 1996) was used to generate homozygous *hsp70-zen* insertions on the second and third chromosome. For heat shock experiments, egg depositions were collected over 30 min on agar plates and covered with a thin film of 27-halocarbon oil (Sigma H773) to select appropriate developmental stages. Agar plates with staged embryos were submerged without lid in a water bath at 38 °C. The embryos were subsequently allowed to develop at 25 °C.

Results

Repression of zen during germband extension abrogates serosa development in Megaselia

The functional significance of *zen* expression after gastrulation in the developing serosa of insects is unknown. As the late phase of *zen* expression is not conserved in *Drosophila*, we examined in *Megaselia* whether this phase of *zen* expression is critical for the formation of distinct serosal and amniotic epithelia. First, we tested how fast and until what stage *Megaselia* embryos respond to *Mab-zen* RNAi. We injected embryos with *Mab-zen* dsRNA at blastoderm cellularization stages or later and fixed batches after incubation at 23 +/− 2 °C in 10-minute time intervals. These embryos were subsequently analyzed using RNA in situ hybridization for *Mab-zen*. Blastoderm stage embryos at the beginning or during cellularization responded within 10 min of injection with an almost complete loss of *Mab-zen* expression (64%; $n = 14$) (Figs. S1A, B). After 1 h, *Mab-zen* transcripts were completely absent (95%, $n = 62$). In embryos injected at the beginning of pole cell migration (during gastrulation; t1 in Fig. 1) the *Mab-zen* RNAi response was as fast, but was observed in fewer embryos and only in posterior parts of the serosa anlage (30%, $n = 10$). One hour after injection, we observed *Mab-zen* repression in the entire extraembryonic fold but no repression adjacent to the cephalic furrow (23%, $n = 147$) (Figs. 2I and J). Embryos that were injected during mid germband extension stage did not respond to RNAi even up to 2 h after injections ($n = 59$, data not shown). Together, these results indicate that cell membranes act as barriers for RNAi effector molecules but that in posterior serosa cells *Mab-zen* RNAi can still be induced during gastrulation. We took advantage of this fact to assess the late function of *Mab-zen* in extraembryonic development using in situ probes against *Megaselia Krüppel* (*Mab-Kr*)

(Rafiqi et al., 2008) and *Megaselia dopa decarboxylase* (*Mab-ddc; this study*) as extraembryonic markers.

Mab-ddc is specifically expressed in the serosa (Figs. 3A and B). *Mab-Kr* is also expressed throughout the developing serosa (Figs. 3C and D) [see also (Rafiqi et al., 2008)]. Later, *Mab-Kr* is also expressed in the posterior and lateral parts of the amnion (Figs. 3E and F). This expression domain appears after germband extension (when the serosa has been completed).

In *Mab-zen* RNAi embryos injected prior to blastoderm formation, serosal *Mab-Kr* (Rafiqi et al., 2008) and *Mab-ddc* expression (this study) was suppressed (Fig. 3G; 100%, $n=26$). However, after stomodeum formation *Mab-zen* RNAi embryos expressed *Mab-Kr* in the posterior portion of their single extraembryonic epithelium (Fig. 3H; 100%, $n=17$), consistent with the proposed amniotic identity of all extraembryonic tissue in *Mab-zen* RNAi embryos (Rafiqi et al., 2008).

Next, we injected 264 embryos with *Mab-zen* dsRNA during gastrulation and analyzed the embryos at germband retraction stages using a *Mab-Kr* in situ probe and DAPI ($n=136$) or a *Mab-ddc* in situ probe and DAPI ($n=128$). About a third of the embryos were excluded from the analysis because of unspecific defects (improper wound healing, incomplete embryos) or because they had failed to reach the stage of germband retraction and could not be unambiguously scored for serosa completion. Most of the remaining embryos were indistinguishable from wildtype embryos. However, of the *Mab-ddc*-stained embryos, 9% lacked a complete serosa ($n=88$). In these embryos, anterior extraembryonic tissue that had disjoined and expanded over parts of the embryo consistently expressed *Mab-ddc*, while the posterior portion of the same extraembryonic epithelium, which had not disjoined from the adjacent epidermis, did not express *Mab-ddc* (Fig. 3I). Of the *Mab-Kr* stained embryos, 6% lacked a complete serosa ($n=87$). These embryos consistently exhibited a bipartite extraembryonic expression pattern of *Mab-Kr*. Anterior extraembryonic tissue that had disjoined and expanded over parts of the embryo and the posterior portion of the same extraembryonic epithelium expressed *Mab-Kr* while the middle portion of this epithelium was *Mab-Kr* negative (Fig. 3J). The modified extraembryonic expression patterns of *Mab-ddc* and *Mab-Kr* can be explained with the spatial pattern of *Mab-zen* repression in posterior serosa tissue following *Mab-zen* RNAi during gastrulation (Fig. 2J). Together the data therefore suggest that postgastrular *Mab-zen* expression in the serosa is necessary for the disjunction of the serosa from adjacent tissue and its subsequent expansion over the embryo but is dispensable for amnion development.

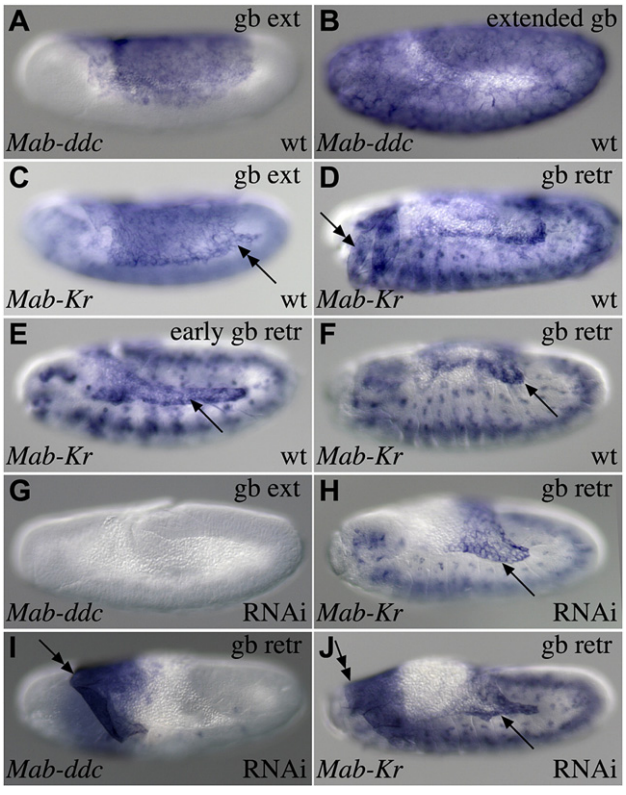

Fig. 3. Late *Mab-zen* expression is required for serosa development. (A, B) Expression of *Mab-ddc* in the serosa of wildtype embryos. (C–F) Expression of *Mab-Kr* in wildtype embryos. Note expression in the serosa (double arrow) and in the amnion (single arrow). (G) Absence of *Mab-ddc* expression following early *Mab-zen* RNAi. (H) Expression of *Mab-Kr* following early *Mab-zen* RNAi. Expression in the posterior amnion is marked (arrow). (I, J) Following late *Mab-zen* RNAi, *Mab-Kr* and *Mab-ddc* are expressed in the anterior serosa-like cells (which develop due to incomplete *Mab-zen* knockdown; double arrows); *Mab-Kr* is also expressed in the posterior portion of the extraembryonic epithelium (J; single arrow). In (D) the serosa has broken open at the anterior end during fixation; in (E, F) the serosa has been removed. Embryos in (B, D–F) were post-fixed in a PBS, methanol and heptane mixture while the other embryos were post-fixed in PBS (see Materials and methods). Anterior is left and dorsal up. Abbreviations: gb ext, germband extending stage; gb retr, germband retracting stage.

Megaselia homologues of hnt, ush, tup and doc control germband retraction and dorsal closure but are not required for the formation of the serosa

To understand how the developmental functions of the ush-group genes evolved with extraembryonic morphology, we cloned *Megaselia* homologues of four ush-group genes, including *hnt* (*Mab-hnt*), *ush* (*Mab-ush*), *tup* (*Mab-tup*) and *doc* (*Mab-doc*) (Fig. S2) and examined their transcript expression and function. *Mab-hnt* was expressed in a stripe of variable width, which included prospective amniotic, serosal and proctodeal tissue (Fig. 4A). In the amniotic tissue, we detected *Mab-hnt* transcript until the stage of amnioserosal fold formation (Fig. 4B). *Mab-hnt* was also expressed in the anterior and posterior midgut primordia and in ectodermal cells at germband retraction stages that we tentatively assigned to the tracheal placodes and the peripheral nervous system but was not expressed in the dorsal epidermis (Fig. 4C, C'). In the serosa, *Mab-hnt* transcript was detected until amnioserosal fold formation and reappeared during germband retraction, i.e., after the completion of serosa epithelium (data not shown).

Mab-ush was ubiquitously expressed in the early blastoderm (data not shown). During mid cellularization of the blastoderm, *Mab-ush* was expressed in a dorsal stripe slightly broader than the extraembryonic blastoderm; expression was also detected in yolk nuclei (Fig. 4D). During germband extension stages, *Mab-ush* expression was detected in the anterior midgut and in all extraembryonic cells and the bordering epidermis (Figs. 4E–F'). In the amnion, *Mab-ush* was detected until the stage when the stomodeum had formed (data not shown). While *Mab-ush* was not detected in the serosa during its expansion, it was detected in the completed serosa at germband retraction stages (data not shown). In addition, *Mab-ush* was expressed in cells that we tentatively assign to the tracheal placodes and the developing tracheal system (Fig. 4F).

Mab-tup expression started in a dorsal stripe that appeared to be broader than the *Mab-hnt* and *Mab-ush* expression (Fig. 4G). *Mab-tup* expression in the serosa faded during germband extension ahead of expression in the amnion, which could be detected until early stages of serosa expansion (Fig. 4H). From this stage onwards, *Mab-tup* was expressed in the dorsal epidermis (Fig. 4I). In addition, *Mab-tup* was expressed in the mid-dorsal head ectoderm, epipharynx, optic lobes, blocks of mesodermal cells and, following germband retraction, in a small mid-dorsal patch of the hindgut (data not shown).

Mab-doc expression in the blastoderm was detected along the dorsal midline, in two lateral patches of the head region and in a dorsal wedge marking the anterodorsal rim of the proctodeum (Fig. 4J). *Mab-doc* expression in the serosa faded during early germband extension (Fig. 4K); expression in the amnion faded just

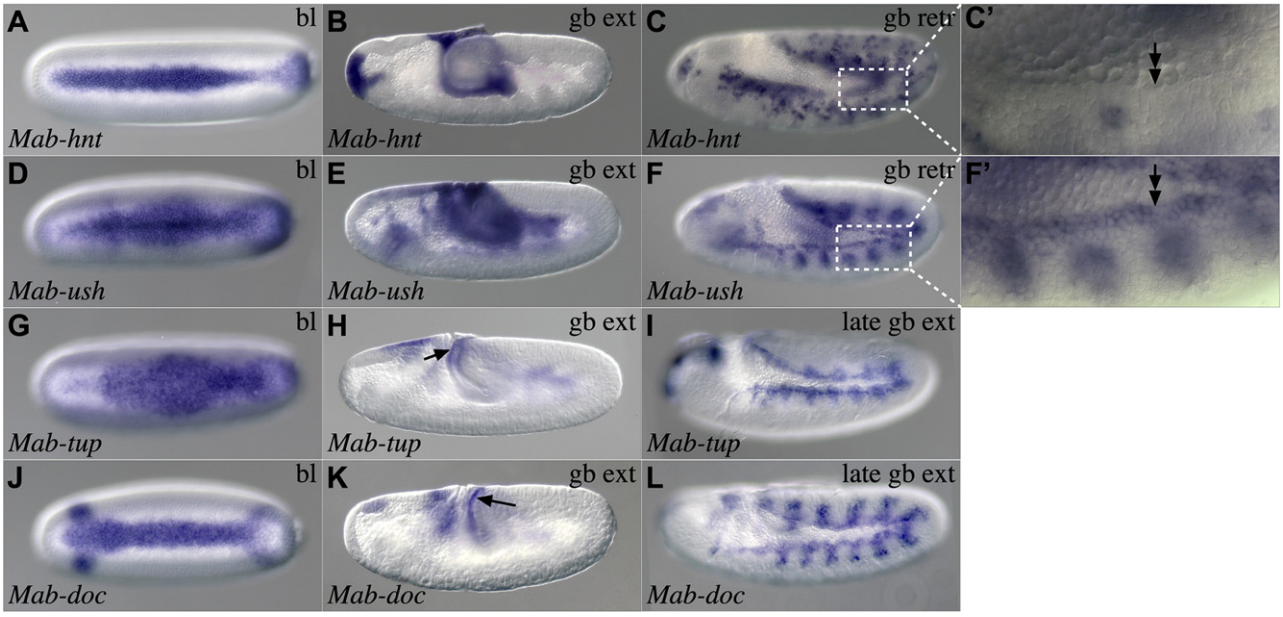

Fig. 4. Expression of *Mab-hnt*, *Mab-ush*, *Mab-tup* and *Mab-doc*. (A–C') *Mab-hnt*. (D–F') *Mab-ush*, (G–I) *Mab-tup*, (J–L) *Mab-doc*. Note that *Mab-hnt* expression is excluded from dorsal epidermis (double arrowhead in C', magnification of area in dotted rectangle) and that *Mab-ush* is expressed in the dorsal epidermis (double arrowhead in F', magnification of area in dotted rectangle). Single arrows point to amniotic expression. Embryos are shown in dorsal view (A, D, G and J) or in lateral view with dorsal up. Anterior is left. Abbreviations: bl, blastoderm stage; gb ext, germband extending stage; gb retr, germband retracting stage.

before the expansion phase of the amnioserosal fold (Fig. 4L). *Mab-doc* was also expressed in the (prospective) optic lobes, a small medial patch of head blastoderm (later part of the epipharynx), and in a metameric pattern of mesodermal and epidermal cells, presumably including the tracheal placodes (Fig. 4L and data not shown).

Next, we examined the function of *Mab-hnt*, *Mab-ush*, *Mab-tup* and *Mab-doc* by RNAi. *Mab-hnt* RNAi cuticles lacked the anterodorsal region of the head and thorax, and were open and u-shaped ($n=37$; Figs. 5A–C). *Mab-ush* RNAi cuticles were open on the dorsal side and were u-shaped. Most of these cuticles (70%) also had reduced head structures and a reduced number of anterior trunk segments ($n=16$; Fig. 5D). *Mab-tup* RNAi cuticles (64%, $n=25$) and *Mab-doc* RNAi cuticles (33%, $n=39$) had reduced head structures and were open in the anterior trunk region but u-shaped cuticles were not observed in either case (Figs. 5E and F). However, when both *Mab-tup* and *Mab-doc* were targeted by RNAi, all cuticles exhibited a u-shaped morphology in addition to the defects observed in individual gene knockdowns (Fig. 5G; $n=61$). In each experiment, we also noticed small spherical or otherwise highly abnormal cuticles. We attributed these cuticles to embryos with unspecific defects and did not analyze them further. Taken together, our results suggest that *Mab-hnt*, *Mab-ush*, *Mab-tup* and *Mab-doc* support germband retraction and dorsal closure.

We suspected that the RNAi-induced u-shaped phenotypes are caused by a precocious loss of amnion cells, but in the absence of a specific marker for the entire amnion we were unable to test this hypothesis directly. We therefore sought to increase the number of amnion cells in *Mab-doc*/*Mab-tup* double RNAi background (which causes less severe head defects than *Mab-hnt* or *Mab-ush* RNAi background) by also repressing *Mab-zen* to allow serosa cells to acquire an amnion-like identity. When repressing *Mab-tup* and *Mab-doc* together with *Mab-zen* by triple RNAi, germband retraction was rescued (90%, $n=12$). Dorsal closure was rescued as well, albeit with alignment defects along the dorsal midline in nearly half of the cuticles (Fig. 5H). This result suggests that germband retraction and dorsal closure defects in response to double *Mab-doc*/*Mab-tup* RNAi might indeed be caused by a precocious reduction of the amnion.

To test whether *Mab-hnt*, *Mab-ush*, *Mab-tup* or *Mab-doc* are required for serosa formation, we stained the respective RNAi embryos as well as *Mab-doc*/*Mab-tup* double RNAi embryos with the nuclear marker DAPI and examined them for serosa presence. In most embryos, the serosa was present (*Mab-hnt*: 79%, $n=39$; *Mab-ush*: 73%, $n=62$; *Mab-tup*: 88%, $n=50$; *Mab-doc*: 96%, $n=116$; *Mab-tup*+*Mab-doc*: 78%, $n=72$). In the remaining embryos, early developmental arrest (or delayed development) precluded examination of the serosa. In summary, *Mab-hnt*, *Mab-ush*, *Mab-tup* and *Mab-doc* might not be required for serosa formation but control the same morphogenetic movements as their putative orthologues in *Drosophila*.

Ectopic Mab-zen expression interferes with amnion development

In *Drosophila*, *zen* is broadly expressed in early blastoderm embryos. This expression domain is absent in *Megaselia* (and other insects with an amnion). To test whether ectopic *zen* expression in *Megaselia* interferes with extraembryonic development, we injected syncytial embryos with capped *Mab-zen* mRNA and examined serosa formation (with DAPI staining) and cuticular phenotypes. At the stage when the stomodeum had formed but the germband had not retracted, the serosa was present in 92% of the embryos ($n=140$), the remaining embryos showed a stalled dorsal serosa together with non-specific embryonic defects. Cuticles of *Mab-zen* injected embryos exhibited variable head defects (44%), together with dorsal closure (25%) or germband retraction defects (19%) ($n=16$) (Fig. 5I). A mutated version of the *Mab-zen* mRNA with a stop codon in front of the homeobox had no effect on germband retraction or dorsal closure (96%, $n=69$) except in 3 embryos (4%), which exhibited defects in dorsal closure.

The u-shaped cuticles of *Mab-zen* injected embryos suggest that *Mab-zen* expression might interfere with amnion genes [the serosa is dispensable for germband retraction (Rafiqi et al., 2008)]. To test whether this is the case, we examined the effect of *Mab-zen* mRNA injection on the expression of *Mab-tup*, *Mab-doc*, *Mab-Kr*, *Mab-pnr* [a previously described amnion marker (Rafiqi et al., 2008)] and *Mab-ddc*. We observed repression of all genes that are expressed in the amnion. Repression was observed in every tissue of gastrulating embryos, including in the serosal *Mab-Kr* domain. However, at lower concentration, injected *Mab-zen* mRNA had no effect on *Mab-Kr* expression, while still repressing (at reduced frequency) the expression of *Mab-tup*,

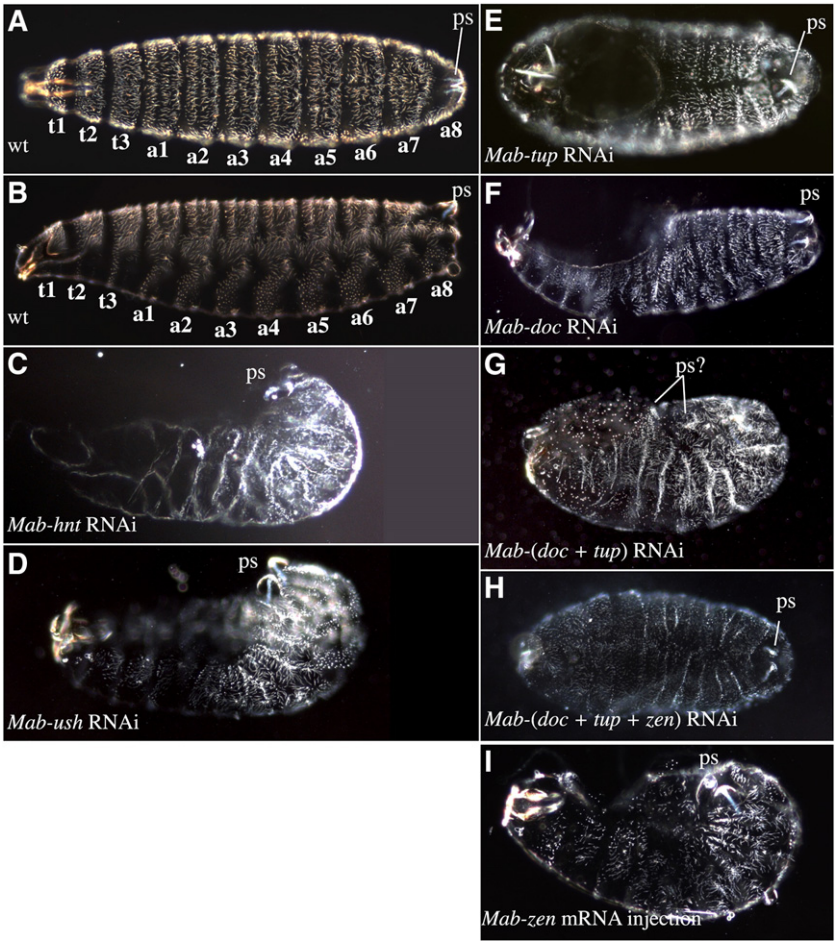

Fig. 5. *Megaselia* homologues of ush-group genes control germband retraction and dorsal closure. (A, B) Wildtype cuticles of *Megaselia*. Thoracic (t1–t3) and abdominal (a1–a8) segments and posterior spiracles (ps) are marked. (C–H) RNAi embryos following single knockdowns of *Mab-hnt* (C), *Mab-ush* (D), *Mab-tup* (E) and *Mab-doc* (F), double knockdown of *Mab-doc* and *Mab-tup* (G) and triple knockdown of *Mab-doc*, *Mab-tup* and *Mab-zen* (H). (I) Cuticle of an embryo that has been injected with *Mab-zen* mRNA. Cuticles are in lateral (B, C, F, G), dorso-lateral (D, I), or dorsal (A, E, H) views, anterior is left.

Mab-doc and *Mab-pnr*. Mutated *Mab-zen* mRNA had no effect on the expression of *Mab-pnr* ($n = 50$). In contrast, *Mab-ddc*, the only gene in our sample with strictly serosal expression, was activated upon injection of *Mab-zen* mRNA (Fig. 6). Together, these results suggest that ectopic *Mab-zen* expression may not interfere with serosa development but perturbs amnion development by repressing amnion specific genes.

Prolonged zen expression in Drosophila interferes with germband retraction and dorsal closure

Since the loss of postgastrular *zen* expression in *Megaselia* suppresses serosa development, we wondered whether the induction of postgastrular *zen* expression in *Drosophila* can interfere with the late functions of the amnioserosa and/or induce the formation of a serosa. In previous experiments, it was found that a heat-shock-inducible *zen* transgene (*hsp70-zen*) increases the number of amnioserosa cells two to threefold when activated in gastrulating (3–4 h old) embryos with a 45-minute heat shock (Rushlow and Roth, 1996). We carried out similar experiments with two newly generated *hsp70-zen* lines (the original line is no longer available). When heat shocked, both lines exhibited strong ubiquitous *zen* expression and developed a prominent pseudo-amnioserosal fold (Figs. 7A and B). Following *zen* over expression, the embryos failed to hatch, and most exhibited defects in germband retraction and dorsal closure often together with head defects (86%, $n = 148$), the remaining fraction also had mild retraction defects. Most heat shocked control embryos developed normally and hatched (75%, $n = 102$); 25% exhibited retraction defects (Figs. 7C and D). These results suggest that ectopic *zen* interferes with the functions of the amnioserosa during germband retraction and dorsal closure. Finally, we allowed *hsp70-zen* and control embryos to develop after a 90-minute heat shock for 7 h at 25 °C, and stained them with an antibody against tubulin to visualize microtubuli and cell size, as well as DAPI to visualize nuclei. The number of amnioserosa cells in these embryos was within the range observed in wildtype (~170, SD 10.2) (Li et al., 2005), but the sizes of amnioserosa cells and nuclei were dramatically increased (Figs. 7E and F). Unlike in previous experiments with the same *hsp70-zen* construct in a transgenic line that is no longer available (Rushlow and Roth, 1996), we did not observe an increase in the number of amnioserosa cells. The discrepancy could be due to cell death because we analyzed embryos at a slightly older stage. Alternatively, a bias for older embryos in our selection of germband extension embryos for heat shock treatment (see Material and methods) may have prevented a size increase of the amnioserosa anlage. Our results show that prolonged *zen* expression causes an enlargement of the amnioserosa cells. While this phenotype is reminiscent of the morphology of serosa cells, disjunction of the amnioserosa from adjacent tissue was not observed and the tissue remained confined to the dorsal side. Nonetheless, the occurrence of germband retraction and dorsal closure defects at high frequency show that ectopic *zen* expression interferes with functions of the late amnioserosa.

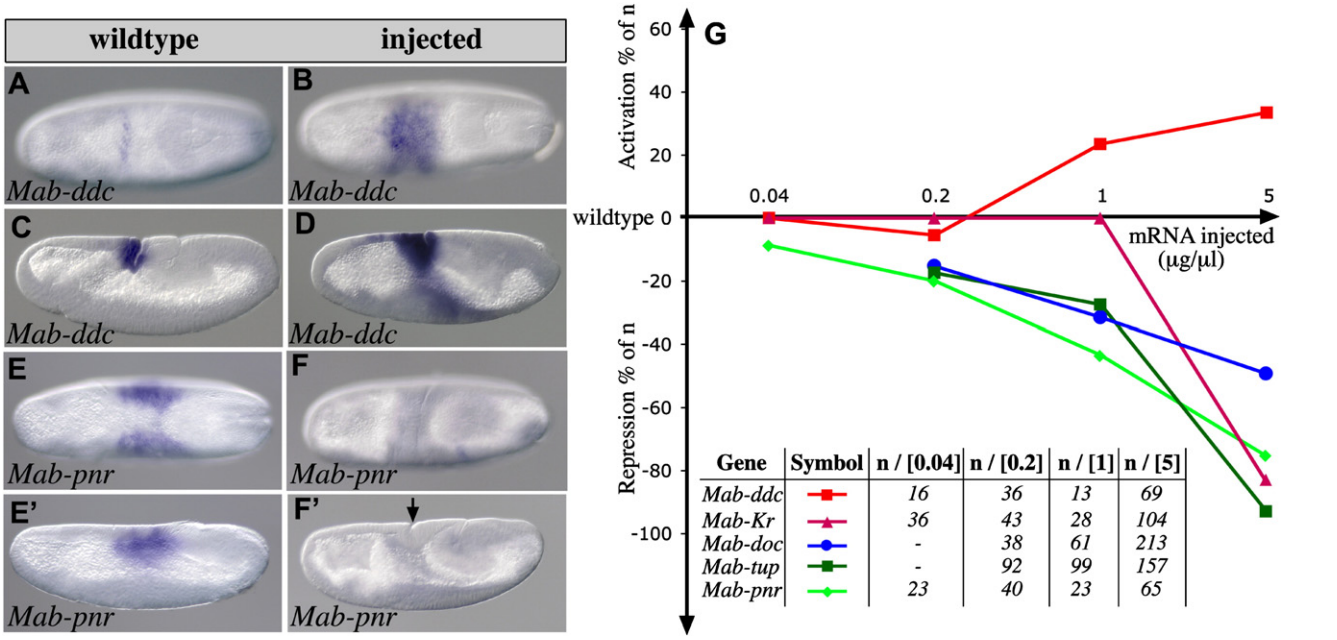

Fig. 6. Over expression of *Mab-zen* causes repression of amnion genes and activation of serosa genes. (A–D) Expression of *Mab-ddc* in (A, C) wildtype and (B, D) *Mab-zen* mRNA injected embryos. (E–F') Expression of *Mab-pnr* in wildtype (E, E') and *Mab-zen* mRNA injected (F, F') embryos. *Mab-zen* mRNA injections frequently induced the formation of an ectopic fold (arrow in F'). (G) Line diagram depicting the number of embryos (n) in % that show activation or repression of *Mab-ddc* (red line with squares), *Mab-Kr* (purple line with triangles), *Mab-doc* (blue line with circles), *Mab-tup* (dark green line with squares) and *Mab-pnr* (light green line with lozenges) following *Mab-zen* mRNA injection. Embryos are shown in dorsal (A, B, E, F) or lateral (C, D, E', F') view with anterior to the left.

Discussion

Two alternative genetic mechanisms have been proposed for explaining the evolutionary origin of the amnioserosa. One model posits that the loss of a repressor downstream of *zen* caused an expansion of amniotic expression domains to serosal tissue and thereby the origin of the amnioserosa in dipteran evolution (Goltsev et al., 2007). Alternatively, we proposed that the origin of the amnioserosa was triggered by the loss of postgastrular *zen* expression, and that genetic changes at pregastrular stages, including the potential loss of repressors downstream of *zen* and expression of *zen* in the entire amnion-competent blastoderm, evolved later, essentially to accommodate the new morphology (Rafiqi et al., 2008). Here, we have examined the functional significance of specific differences in *zen* expression between *Megaselia* and *Drosophila*. By inducing *Mab-zen* RNAi in *Megaselia* during gastrulation we demonstrated an essential role of postgastrular *zen* expression in (i) promoting disjunction of the leading edge of the serosa from adjacent tissue, (ii) controlling the expansion of the serosa, and (iii) maintaining serosal tissue. Conversely, by ectopically expressing *Mab-zen* in syncytial *Megaselia* embryos we obtained evidence that ectopic *zen* activity in gastrulating *Megaselia* embryos interferes with amnion development, thereby disrupting germband retraction and dorsal closure. These results show that the loss of postgastrular *zen* activity can trigger the morphological changes that accompanied the evolution of the amnioserosa by abrogating serosa development and allowing the formation of a dorsal amnion, while early ectopic *zen* expression can interfere with amnion development and may thus explain the loss of distinct serosa-amnion boundaries in early *Drosophila* embryos. Hence, the net effect of *zen* expression changes in the *Drosophila* lineage appears to have resulted in the loss of the serosa and delayed amnion specification. Any alternative scenario that does not involve the loss of postgastrular *zen* expression would have to account for how amnioserosa development was possible after gastrulation in the presence of extraembryonic *zen* expression.

Our model is also consistent with the analysis of ush-group genes in *Megaselia*. In *Megaselia*, the homologues of the ush-group genes *Mab-ush*, *Mab-hnt*, *Mab-tup* and *Mab-doc* promote germband retraction and dorsal closure, like ush-group genes in *Drosophila*. This indicates a postgastrular role of all four genes in maintaining the dorsal amnion functions of *Megaselia*. Like *Drosophila hnt*, *Mab-hnt* expression is excluded from the dorsal epidermis [Fig. 4 and (Yip et al., 1997)] ruling out any implication of this tissue in the *Mab-hnt* RNAi phenotype. The amniotic functions of *Mab-tup* and *Mab-doc* might be subtler than the amniotic function of *Mab-hnt* and *Mab-ush* because defects in germband retraction were only observed when these genes were knocked down together. It may reflect the presence of undiscovered paralogues of *Mab-tup* and *Mab-doc* with partly redundant functions or differences in the regulation of these genes between *Megaselia* and *Drosophila*. Alternatively, these genes might function downstream of *Mab-hnt* and *Mab-ush*. Taken together, the RNAi data for *Mab-hnt*, *Mab-ush*, *Mab-tup* and *Mab-doc* indicate that *Megaselia* homologues of late amnioserosa maintenance genes are responsible for maintaining a functional amnion in *Megaselia*.

While an amniotic function of ush-group genes might be conserved across Diptera, including *Drosophila* [(Goltsev et al., 2007; Rafiqi et al., 2008), this study], regulatory interactions among ush-group genes and *zen* might have changed. In *Drosophila*, the expression patterns of ush-group genes and *zen* overlap until the end of gastrulation, when *zen* is repressed and ush-group genes become essential for maintaining the amnioserosa. *Drosophila zen* activates ush-group genes in the amnioserosa (Goldman-Levi et al., 1996; Reim et al., 2003). In *Megaselia*, the expression domains of *zen* and ush-group genes overlap as well, but *zen* is dispensable for the activation of these genes, and it mediates the repression of *Mab-doc* and *Mab-tup* during germband extension (A. M. Rafiqi, unpublished observations) or at other stages, when over expressed (Fig. 6). Thus, *Mab-zen* may be acting as a context-dependent repressor of ush-group genes in *Megaselia*. To explain this possibility, we suggest that in the *Megaselia* blastoderm, a repressive effect of Zen on ush-group genes might be

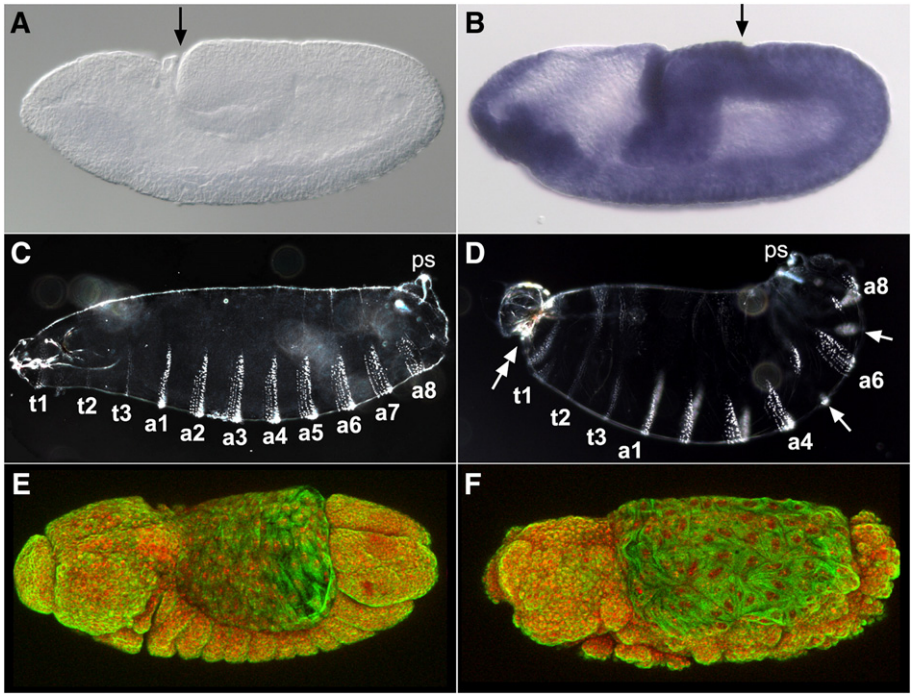

Fig. 7. Over expression of *zen* in *Drosophila* interferes with morphogenetic amnioserosa functions and leads to an enlargement of amnioserosa cells. (A–F) *Drosophila w1118* embryos without the *hsp70-zen* transgene (A, C, E) and homozygous for the *hsp70-zen* transgene (B, D, F). (A, B) Embryos fixed immediately after a 45-minute heat shock and stained with a *zen* antisense probe. Note differences in the extension of the pseudo-amnioserosal fold (arrow). (C, D) Larval cuticles of heat shocked embryos. Head defects (double arrow), segmentation defects (arrows), the position of the posterior spiracles (ps), thoracic segments (t1–t3) and abdominal segments (a1–a8) are marked. (E, F) Embryos after a 90-minute heat shock followed by 7 h at 25 °C stained with anti-tubulin antibody (green) to visualize microtubules and cell size, and DAPI (red) to visualize nuclei. Both embryos are of the same age but ectopic *zen* expression interferes with normal development. Note the large size of cells and nuclei in the amnioserosa of the embryo carrying the *hsp70-zen* transgene. Embryos and cuticles are shown in lateral (A–D) or dorso-lateral (E, F) view with anterior to the left.

overridden by signaling activity of Decapentaplegic (Dpp), a ligand of the BMP pathway, which patterns the dorsal blastoderm [reviewed in (O'Connor et al., 2006)]. Genetic data suggest that effectors of the Dpp pathway activate ush-group genes and other targets, including *zen*, *race* and *C15* synergistically with Zen (Ashe et al., 2000; Lin et al., 2006; Rushlow et al., 2001; Xu et al., 2005). However, using reporter genes under the control of a minimal enhancer of *C15*, it was also shown that some binding sites of Zen in this enhancer mediate repression (Lin et al., 2006). This finding seems to suggest that Zen functions as a context-dependent repressor in *Drosophila* as well. To further explore this idea, it might be useful to examine the spatiotemporal activity pattern of *dpp* in *Megaselia* and to test whether over expression of *zen* in *Drosophila* represses ush-group genes.

The paleontological record of cyclorrhaphan flies suggests that the last common ancestor of *Megaselia* and *Drosophila* lived about 145 million years ago (Grimaldi and Engel, 2005). Although a serosal expression domain of *hunchback* may have been lost in the *Megaselia* lineage [referenced in (Lemke and Schmidt-Ott, 2009)], the extraembryonic gene network of *Megaselia* appears to be similar to that of other dipterans (Goltsev et al., 2007) and hence similar to the ancestral dipteran condition. The amnioserosa gene network has diverged from this ancestral state not only by bringing all aspects of early extraembryonic development under the control of *zen* and by relieving the repression of amnion genes (e.g. *doc*, *tup*, *pnr*) in what used to be the serosa primordium, but also by the massive loss of extraembryonic expression domains of serosa genes (Goltsev et al., 2007, 2009), including the serosa domains of *ddc* and *Kr*. Therefore, *Drosophila* has probably lost the ability to revert to the ancestral mode of extraembryonic development. In contrast, our finding that *Megaselia* responds to the loss of late zen expression with the reorganization of serosal and amniotic tissues into a single amnion-like epithelium is consistent with the idea that this genetic change might have triggered the reorganization of extraembryonic tissue in the schizophoran lineage (>80 million years ago). Accordingly, the ability to respond to the loss of postgastrular *zen* expression with the origin of an amnioserosa-like extraembryonic tissue organization may have been present 65 million years prior to the origin of the amnioserosa, and was retained in the *Megaselia* lineage, together with the ancestral morphology, for a total of 145 million years or more (Fig. 1). We suspect that a long time period over which the same genetic mechanism could have generated an amnioserosa-like epithelium is linked to the preservation of the ancestral morphology. Therefore, a species with a trait in its primitive form might allow insight into the genetic mechanism of evolutionary change of this trait in related species, which the more diverged species cannot provide because they accumulated secondary genetic changes at a faster pace.

Note added in proof

The sequences in this paper have been submitted to Genbank under accession numbers: Megaselia-dopa-decarboxylase GU725005, Megaselia-dorsocross GU725001, Megaselia-hindsight GU725002, Megaselia-tailup GU725003, and Megaselia-u-shaped GU725004.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.01.040.

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