Evolutionary origin of the amnioserosa in cyclorrhaphan flies correlates with spatial and temporal expression changes of *zen*

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Higher cyclorrhaphan flies including *Drosophila* develop a single extraembryonic epithelium (amnioserosa), which closes the germband dorsally. In most other insects two extraembryonic epithelia, serosa and amnion, line the inner eggshell and the ventral germband, respectively. How the two extraembryonic epithelia evolved into one is unclear. Recent studies have shown that, in the flour beetle *Tribolium* and in the milkweed bug *Oncopeltus*, the homeobox gene *zerknüllt* (*zen*) controls the fusion of the amnion with the serosa before dorsal closure. To understand the origin of the amnioserosa in evolution, we examined the expression and function of *zen* in the extraembryonic tissue of lower Cyclorrhapha. We show that *Megaselia abdita* (Phoridae) and *Episyrphus balteatus* (Syrphidae) develop a serosa and a dorsal amnion, suggesting that a dorsal amnion preceded the origin of the amnioserosa in evolution. Using *Krüppel* (*Kr*) and *pannier* (*pnr*) homologues of *Megaselia* as markers for serosal and amniotic tissue, respectively, we show that after *zen* RNAi all extraembryonic tissue becomes indistinguishable from amniotic cells, like in *Tribolium* but unlike in *Drosophila*, in which *zen* controls all aspects of extraembryonic development. Compared with *Megaselia* and *Episyrphus*, *zen* expression in *Drosophila* is extended to cells that form the amnion in lower Cyclorrhapha and is down-regulated at the developmental stage, when serosa cells in lower Cyclorrhapha begin to expand. These expression differences between species with distinct extraembryonic tissue organizations and the conserved requirement of *zen* for serosa development suggest that the origin of an amnioserosa-like epithelium was accompanied by expression changes of *zen*.

Megaselia | *Episyrphus* | *Drosophila* | EvoDevo | homology

The amnioserosa is a unique extraembryonic epithelium of higher flies (1). Because it has no direct equivalent in other insects, it provides a model to study the evolution of new morphology in connection with the underlying developmental gene network. Detailed comparisons of extraembryonic development between fly species with and without an amnioserosa would help to understand the mechanism by which this tissue evolved. In *Drosophila*, the amnioserosa develops from a small portion of the dorsal blastoderm into a squamous polyploid epithelial cell layer that closes the dorsal side of the gastrulating embryo (2). Later in development, epidermis replaces the amnioserosa, which disintegrates. Although the amnioserosa does not contribute embryonic tissue, it controls two vital morphogenetic movements of the embryo: germband retraction and dorsal closure. Germband retraction shortens the embryo and transforms the u-shaped germband into an essentially straight line of body segments. The amnioserosa mediates this process by signaling and physical interactions with the underlying yolk sac (3–5). Dorsal closure, a developmental process that follows germband retraction, seals the epidermis along the dorsal midline. The amnioserosa guides this process in conjunction with the yolk sac and the leading edge of the dorsal epidermis by providing contractile force (6–9). During dorsal closure, some of the amnioserosa cells segregate into the yolk, but the bulk of this tissue invaginates before it degrades, transiently forming a tube-shaped "dorsal organ" (5, 6, 10), similar to extraembryonic tissue in other insects (11). Unlike *Drosophila*, however, most insects develop two distinct extraembryonic epithelia, called amnion and serosa (11–15). Typically, these epithelia develop from an amnioserosal fold, which closes about the ventral side of the gastrulating embryo. The outer cell layer of the amnioserosal fold becomes the serosa. This epithelium detaches from the amnion and encloses the embryo. The inner cell layer of the amnioserosal fold becomes the amnion and retains continuity with the dorsal epidermis of the embryo. The distinct developmental trajectory of the amnioserosa prompts comparative developmental and genetic investigations that could reveal the mechanism that generated the amnioserosa in evolution.

Previous studies show that a wide range of insects require *zen* activity for extraembryonic development, but several variants in expression and function have been described (references in ref. 15). In *Drosophila*, *zen* controls all aspects of amnioserosa development; a second copy of *zen* (*z2*) is expressed in an identical pattern but is dispensable (16, 17). The cells of Zen-deficient *Drosophila* embryos either die or acquire an embryonic fate. Conversely, overexpression of *zen* causes an expansion of the amnioserosa (18). Zen-deficient *Drosophila* embryos also develop head defects, consistent with an expression domain of *zen* in the embryonic blastoderm (19, 20). Other insects express *zen* only in extraembryonic tissue (references in ref. 15). In the flour beetle *Tribolium*, *zen* controls the specification of serosal but not amniotic cells and later in development the fusion of the ventral amnion with the serosa, which precedes the dorsal contraction of the fused extraembryonic epithelium (14). In the milkweed bug *Oncopeltus*, *zen* activity may not be required for the specification of serosal blastoderm but controls the fusion of the amnion with the serosa, as in *Tribolium* (15). In this article we describe the expression and function of *zen* during extraembryonic development in two lower cyclorrhaphan taxa and propose a model for the evolutionary origin of the amnioserosa that integrates our findings and relevant published data from other species.

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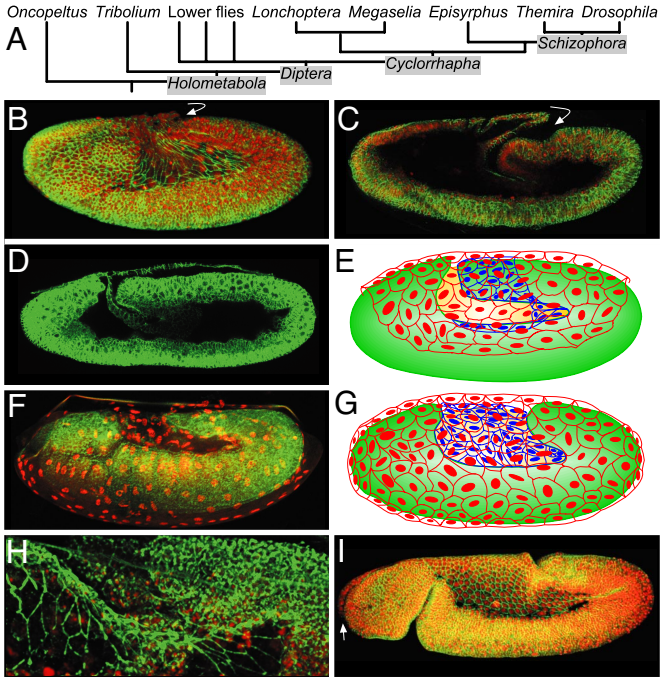

Fig. 1. Phylogenetic position and extraembryonic development of *Megaselia* and *Episyrphus*. (*A*) Phylogenetic tree of species mentioned in the text. (*B–H*) Confocal Z-stacks of *Megaselia* embryos during germband extension (*B–D*) and germband retraction (*F* and *H*). Cartoons (*E* and *G*) depict serosa cells (red), amnion cells (blue), and the germband (green) at stages shown in *D* and *F*. Note the amnioserosal fold (curved arrows in *B* and *C*), the expansion of the preserosa (*D–G*), and the large amnion cells (*H*) of an embryo in which the serosa has been removed. (*I*) *Episyrphus* embryo showing the anterior edge of the preserosa (arrow). Embryos were labeled with anti-phosphotyrosine antibodies (green) and Topro-3 or DAPI (red in *B, C, F, H,* and *I*). Anterior is left, and dorsal is up.

Results

Serosa and Dorsal Amnion in Lower Cyclorrhaphan Flies. Previous work suggests that the amnioserosa evolved in the lineage of cyclorrhaphan flies (21). To map the origin of this tissue more precisely, we determined the occurrence of a serosa in four cyclorrhaphan dipterans (*Lonchoptera*, *Megaselia*, *Episyrphus*, and *Themira*) by nuclear staining (Fig. 1*A*). In *Themira*, the only representative of higher Cyclorrhapha (Schizophora) in our sample, we identified extraembryonic tissue resembling the amnioserosa of *Drosophila* [supporting information (SI) Fig. 7]. In the lower cyclorrhaphan species *Lonchoptera*, *Megaselia*, and *Episyrphus*, which represent the two branches most closely related to Schizophora (22), we observed a complete serosa. To test whether lower Cyclorrhapha also develop an amnion, we labeled the cell membranes and nuclei of *Megaselia* and *Episyrphus* embryos (*Lonchoptera* embryos were not available in sufficient numbers) with a mixture of anti-phosphotyrosine antibodies and DNA stain, and we examined different stages under a confocal microscope. In both species we observed a serosa and an amnion (Fig. 1 and SI Fig. 8). Prospective serosa cells flatten and become polyploid during germband extension (Fig. 1*B*). While the germband continues to extend, the serosa expands over the germband and fuses on the ventral side. At the posterior and lateral margin of the serosa, this process involves the formation of an amnioserosal fold, which disjoins at its edge (Fig. 1 *C–E* and SI Fig. 8*A*). The cells of the outer layer of the amnioserosal fold (preserosa) contribute to the serosa (Fig. 1 *F* and *G* and SI Fig. 8 *B* and *C*). Cells of the inner cell layer of the amnioserosal fold (preamnion) form the amnion. After disjoining from the edge of the preserosa, the preamnion develops into a dorsal cell layer (Fig. 1*H*). Thus, throughout development the amniotic cells remain on the dorsal side. Removing the vitelline layer after completion of the serosa damages both the amnion and the serosa (SI Fig. 8 *D* and *E*). This observation suggests that the amnion attaches to the serosa and that the serosa attaches to the vitelline layer. During dorsal closure, the amnion is replaced by dorsal epidermis. The serosa disappears during dorsal closure, but we did not observe the formation of a dorsal organ (contracted and invaginated serosa tissue), suggesting that the serosa disintegrates underneath the vitelline layer without contracting. In *Episyrphus*, unlike in *Megaselia*, the amnioserosal fold is shallow and the preserosa extends to the anterior pole (Fig. 1*I*). Our findings in *Megaselia* and *Episyrphus* suggest that the evolution of a dorsal amnion preceded the evolutionary origin of the amnioserosa (see *Discussion*).

Expression Differences of *zen* Between Lower Cyclorrhapha and *Drosophila*. To assess whether the differences in *zen* expression could account for some of the differences in extraembryonic development, we compared the expression of *zen* in *Megaselia*, *Episyrphus*, and *Drosophila*. Previously we reported that *Megaselia zen* expression is strictly zygotic and marks developing extraembryonic tissue (23, 24). However, we inadvertently removed the expanded serosa together with the vitelline membrane and did not distinguish serosal and amniotic tissue. We therefore reexamined the expression of *Megaselia zen*. The first expression occurs at the beginning of blastoderm cellularization (Fig. 2 *A* and *A′*). As cell membranes grow inwards, the expression narrows and is subsequently restricted to the developing serosa, which unlike the prospective amnion does not invaginate with the proctodeum (Fig. 2 *B*, *B′*, and *C*). This expression persists during the expansion of the serosa (Fig. 2 *D* and *D′*). To determine which features of *Megaselia zen* expression are characteristic for lower Cyclorrhapha, we examined the expression of a newly identified *zen* homologue from *Episyrphus* (SI Fig. 9). As in *Megaselia*, maternal *zen* transcript could not be detected in ovaries or early embryos of *Episyrphus* (data not shown). Zygotic expression begins in the syncytial blastoderm (Fig. 2 *E*, *E′*, *F*, and *F′*). Later in development *Episyrphus zen* expression occurs in the developing serosa but not in the amnion or any embryonic tissue (Fig. 2 *G*, *H*, and *H′*). The comparison with *Drosophila*, in which transcript and protein patterns of *zen* closely match (17), reveals several expression differences. First, the strong and broad dorsal expression of *zen* during blastoderm formation in *Drosophila* (Fig. 2*I*) has no equivalent in the expression patterns of the identified *zen* homologues of *Megaselia* and *Episyrphus*. Second, *zen* expression in *Drosophila* extends to the proctodeum (Fig. 2 *J* and *K*), unlike in *Megaselia* and *Episyrphus*, where the amnion separates *zen* expression from the proctodeum. Third, unlike in *Megaselia* and *Episyrphus*, *zen* expression in *Drosophila* is downregulated during the late phase of germband extension [stage 8 (2)] (Fig. 2*L*). In addition, *Drosophila zen* is expressed in the head and germ line (17, 20) but appears to be strictly extraembryonic in *Megaselia* and *Episyrphus*.

Phenotypic Effects of *zen* RNAi in *Megaselia* and *Episyrphus*. We used RNAi to analyze the function of *zen* during embryogenesis in *Megaselia* and *Episyrphus*. We injected preblastoderm embryos with *zen* dsRNA and analyzed them at different time points (SI Fig. 10). *Megaselia* embryos injected with a 4.7 μM solution of dsRNA consistently developed a single extraembryonic epithelium on the dorsal side (Fig. 3 *A* and *B*). To test whether *Megaselia zen* is required for dorsal closure, we repeated the RNAi experiment and allowed the embryos to develop a cuticle. Approximately half of the developed embryos exhibited a "dorsal open" cuticle (Fig. 3*C*), whereas the other half was indistinguishable from wild type (Fig. 3*D*). The absence of dorsal closure defects in a large proportion of RNAi embryos could reflect

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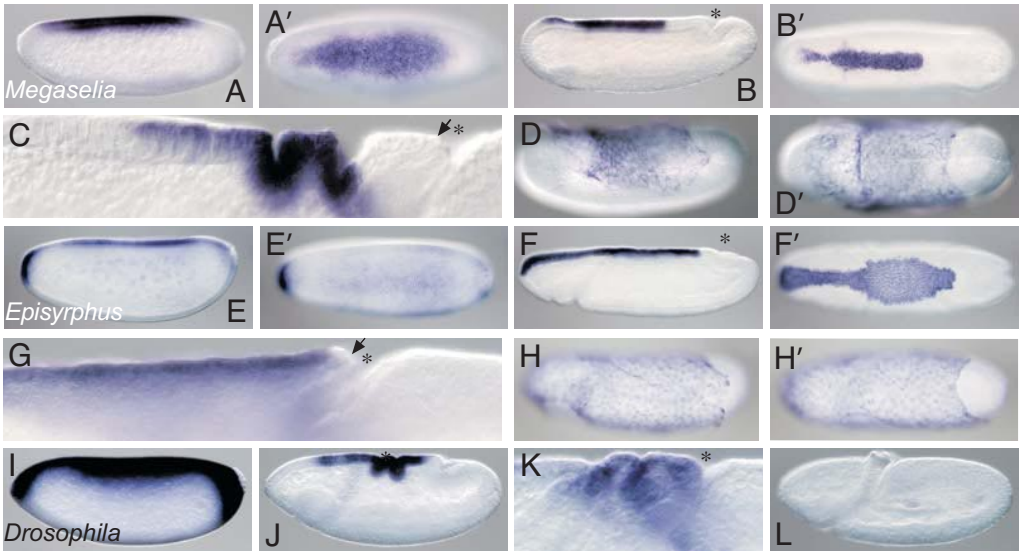

Fig. 2. Comparison of *zen* expression among *Megaselia*, *Episyrphus*, and *Drosophila*. RNA *in situ* hybridizations of *Megaselia* (*A–D*), *Episyrphus* (*E–H*), and *Drosophila* (*I–L*) embryos are shown in lateral and dorsal orientation (*A′–F′* and *H′*). Embryos are at the syncytial blastoderm stage (*A*, *E*, and *I*), early gastrulation (*B*, *F*, and *J*), germband extension (*C*, *G*, and *K*), or the extended germband stage (*D*, *H*, and *L*). Anterior is left. The proctodeal invagination is marked by an asterisk. In *Megaselia* and *Episyrphus* the amnioserosal fold (arrow) migrates during the progression of the fold to the posterior expression boundary of *zen*.

residual *zen* activity. Alternatively, the dorsal open morphology could reflect a hypomorphic phenotype caused by an oversized dorsal epithelium that resulted from incomplete suppression of serosa development. At present we cannot distinguish between these possibilities. Although we observed protruding extraembryonic tissue in some of the open cuticles, lowering the concentration of dsRNA to 2.35 μM resulted in a moderate increase of embryonic viability rather than an increase in the proportion of RNAi embryos with a dorsal open phenotype (SI Fig. 10). In *Episyrphus*, all embryos developed a single extraembryonic epithelium on the dorsal side when injected with a 1.7 μM solution of *Episyrphus zen* dsRNA (Fig. 3 *E–H*). Other aspects of development, including germband retraction and dorsal closure, were not affected, except in two embryos in which incomplete dorsal closure was observed together with an oversized dorsal extraembryonic epithelium. We conclude that in both species *zen* is necessary for serosa development, whereas the serosa may not be essential for embryonic development including germband retraction and dorsal closure.

To characterize the extraembryonic tissue that persists in RNAi embryos of lower cyclorrhaphan flies we searched for amnion and serosa markers in *Megaselia*. We cloned *Megaselia* homologues of *C15*, *pnr*, and *Kr* (SI Fig. 11). In *Drosophila*, the transcripts and proteins of all three genes are expressed throughout the developing amnioserosa, albeit in different time windows. Extraembryonic *C15* expression starts in the blastoderm and continues until after germband retraction (25, 26). Extraembryonic *pnr* expression starts in the blastoderm and fades during the extended germband stage (stage 10) (27). Finally, *Kr* expression in the amnioserosa begins during the extended germband stage (stage 9) and persists until after germband retraction (28). *C15* and *pnr*, but not *Kr*, are also expressed in the dorsal epidermis abutting the amnioserosa.

In *Megaselia*, *C15* is first expressed during the formation of the syncytial blastoderm, spanning ≈60% of the trunk region (data not shown). This expression disappears during blastoderm cellularization. At the beginning of gastrulation, some cells of the serosa primordium weakly express *C15* (Fig. 4*A*), but this expression disappears shortly after the germband has started to extend. During germband extension, *Megaselia C15* is activated in preamnion cells and the leading edge of the dorsal epidermis (Fig. 4 *B* and *B′*). In a few embryos we noticed *C15* expression also in the posterior serosa primordium, suggesting that during early germband extension extraembryonic *C15* expression is either dynamic or somewhat variable between individuals. In addition, we detected weak *C15* expression in creases of the preserosa that transiently form during germband extension (data not shown). In the preamnion we observed an attenuation of *C15* expression shortly before these cells begin to stretch (Fig. 4 *C* and *C′*).

Megaselia pnr expression starts in the mid-dorsal blastoderm but fades at the beginning of gastrulation in cells along the dorsal midline, leaving two parallel stripes that are joined at their posterior end (Fig. 4*D*). During germband extension we consistently observed expression in the ectoderm and in the preamnion (Fig. 4 *E*, *E′*, *F*, and *F′*). In two embryos at the stage of early germband extension, we also detected expression in the posterior portion of the serosa primordium, suggesting that *pnr* expression in this tissue is initially either dynamic or variable between individuals, like the expression of *Megaselia C15*. In the preamnion expression levels of *Megaselia pnr* attenuate with the progression of the amnioserosal fold until high expression levels remain only in the leading edge of the dorsal epidermis (Fig. 4 *F* and *F′*). In summary, the expression patterns of *pnr* and *C15* in germband-extending *Megaselia* embryos are similar, and high expression levels of both genes transiently mark the developing amnion.

Megaselia Kr expression in the blastoderm is similar to *Kr* expression in *Drosophila* except for a gap along the dorsal midline (data not shown). Here we consider only the extraembryonic expression of *Megaselia Kr*, which starts with gastrulation (Fig. 4*G*). During germband extension we observed *Kr* expression in the expanding preserosa but not in the amnion (Fig. 4 *H*, *H′*, *I*, and *I′*). Thus, *Megaselia Kr* is a serosa marker.

We used the *Megaselia* homologues of *pnr* and *Kr* to examine the identity of extraembryonic cells after *zen* RNAi. As a time window, we chose germband extension stages, when *pnr* expression in *Megaselia* wild-type embryos is up-regulated in the prospective amnion and down-regulated in the prospective serosa. We performed double *in situ* hybridization experiments to examine simultaneously the expression of *Kr* and *pnr* (Fig. 5

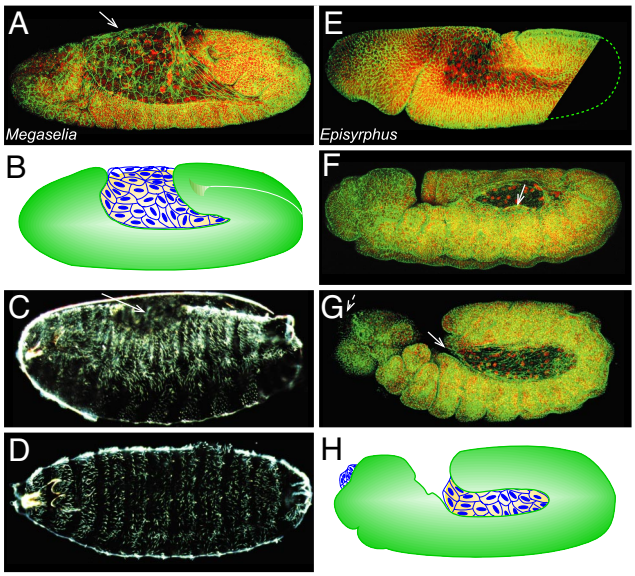

Fig. 3. *zen* RNAi in *Megaselia* and *Episyrphus* causes the formation of a single extraembryonic epithelium on the dorsal side. (*A*) Single extraembryonic epithelium (arrow) of a *Megaselia* RNAi embryo during germband retraction in dorsolateral view. (*B*) Schematic representation of the RNAi phenotype in *Megaselia* depicting extraembryonic tissue (blue) and the germband (green). (*C* and *D*) RNAi cuticles of *Megaselia* larvae showing a dorsal open phenotype (arrow) in lateral view (*C*) and a "dorsal closed" phenotype in dorsolateral view (*D*). (*E–G*) Consecutive developmental stages of RNAi phenotypes in *Episyrphus* in lateral view. Midway through germband extension, extraembryonic cells are morphologically indistinguishable from embryonic cells, unlike in wild-type embryos (compare *E* with Fig. 1*I*). After germband extension, a single layer of extraembryonic cells extends from the edge of the epidermis (arrows in *F* and *G*). Note ectopic anterior cells (broken arrow in *G*). (*H*) Schematic representation of the RNAi phenotype in *Episyrphus*. Anterior is left, and dorsal is up (unless specified otherwise). Embryos were stained with anti-phosphotyrosine antibodies and Topro-3 (*A*, *F*, and *G*) or DAPI (*E*).

A, *A′*, and *A″*). *Megaselia zen* RNAi embryos lacked extraembryonic but not embryonic *Kr* transcripts and expressed *pnr* evenly throughout the extraembryonic primordium and dorsal ectoderm (Fig. 5 *B*, *B′*, and *B″*). These results suggest that the extraembryonic epithelium of *Megaselia zen* RNAi embryos has an amniotic identity.

Discussion

Two Major Transitions in Extraembryonic Morphology in Dipteran Evolution. Our survey of extraembryonic tissue organization in dipterans suggests three distinct trajectories of extraembryonic development. The transient formation of a serosa and a ventral amnion is common throughout lower (non-cylorrhaphan) Diptera and other insect orders and almost certainly reflects the primitive condition for extraembryonic development in Diptera (12, 29). The amnioserosa of schizophoran flies is therefore derived. *Megaselia* and *Episyrphus* develop a serosa and dorsal amnion without passing through a developmental stage with a ventral amnion. This developmental trajectory, hitherto unknown in dipterans, occurs at least in two paraphyletic taxa of the lower Cyclorrhapha (Aschiza): *Megaselia* (Phoroidea) and *Episyrphus* (Syrphoidea). Our findings therefore suggest that the last common ancestor of *Megaselia*, *Episyrphus*, and Schizophora shared this type. We conclude that the evolution of the amnioserosa was preceded by the evolution of a dorsal amnion and propose two major morphological transitions in the course of extraembryonic tissue evolution in flies. The first transition consisted in suppressing the formation of a ventral amnion, perhaps through a fusion of this tissue with the serosa before its ventral completion, and occurred most likely in the late Jurassic period after the cyclorrhaphan lineage had split from other extant dipterans. A similar transition occurred independently in the insect order Hymenoptera (30). The second transition consisted in the reorganization of the preserosa and the preamnion into a single dorsal epithelium, the amnioserosa, and occurred apparently toward the end of the Cretaceous period in the stem group of Schizophora.

Transition from a Serosa and Dorsal Amnion to an Amnioserosa. The evolutionary transformation of the serosa and dorsal amnion into a single dorsal epithelium could have been achieved by suppressing the expansion of the preserosa and its disjunction from adjacent tissue. Similar to the amnioserosa of *Drosophila*, the preserosa of *Megaselia* and *Episyrphus* depends on the activity of *zen* and acquires its distinct cellular morphology during germband extension, ahead of amniotic cells. Importantly, the expansion of the preserosa in *Megaselia* and *Episyrphus* correlates with persisting *zen* expression in the developing serosa. In contrast, *zen* expression in the amnioserosa is downregulated at about the same developmental stage when the presorsa of lower Cyclorrhapha begins to expand. This change in *zen* expression could have been critical for redirecting extraembryonic development in such a way that only a single dorsal epithelium forms. In other words, *zen* expression in the developing serosa could be essential for maintaining developmental properties of serosal and repressing properties of amniotic cells. Functional studies in other species are consistent with this hypothesis (Fig. 6).

Across holometabolous insects, early *zen* expression is con-

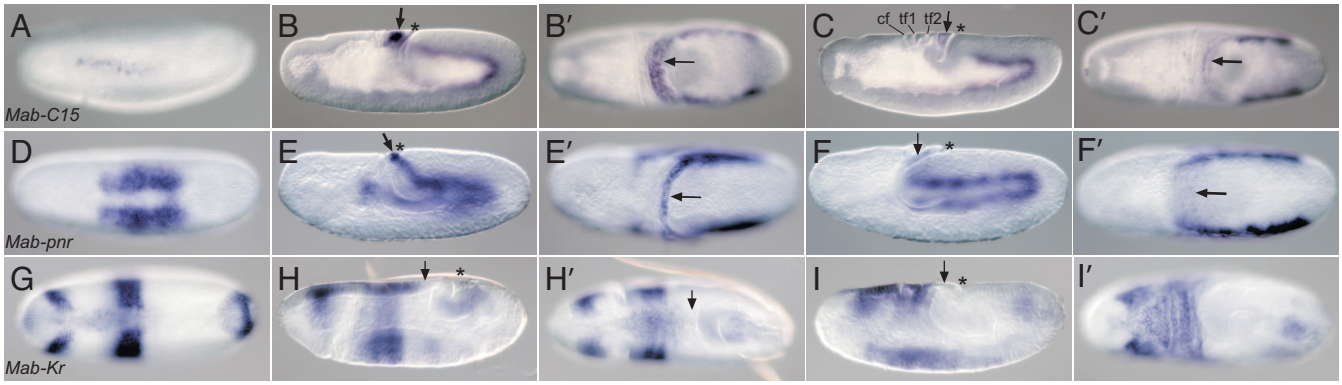

Fig. 4. Expression of *C15*, *pnr*, and *Kr* in *Megaselia*. (*A–C*) *Megaselia C15*. (*D–F*) *Megaselia pnr*. (*G–I*) *Megaselia Kr*. All embryos are shown in dorsal view (*A*, *B′*, *C′*, *D*, *E′*, *F′*, *G*, *H′*, and *I′*) or in lateral view with dorsal up. Anterior is left. Arrows point to the prospective amnion. The proctodeal invagination is marked by an asterisk. cf, cephalic furrow; tf1 and tf2, transverse folds in the preserosa.

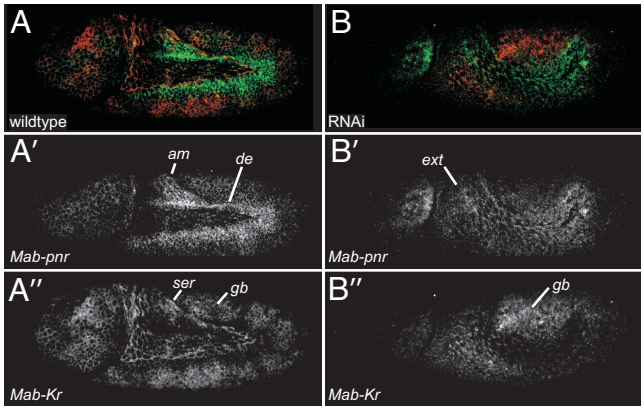

Fig. 5. Extraembryonic primordium of *Megaselia zen* RNAi embryos expresses *pnr* but not *Kr*. (*A*) Wild type. (*B*) RNAi phenotype. Expression of *pnr* is shown in green or as a monochrome image of the green channel (*A′* and *B′*), *Kr* in red, or as a monochrome image of the red channel (*A″* and *B″*). In the RNAi embryo, note the suppression of extraembryonic *Kr* expression and the uniform expression of *pnr* in dorsal ectoderm including extraembryonic tissue (ext). am, preamnion; de, dorsal ectoderm; ser, preserosa; gb, posterior germband.

fined to the developing serosa and controls a developmental switch that allows the *zen*-expressing portion of the blastoderm to acquire serosal fate (Figs. 2–5) (14, 24, 31). In strong *zen* RNAi phenotypes of *Tribolium* and *Megaselia*, all extraembryonic cells are indistinguishable from amniotic cells, indicating that the specification of amniotic cells is independent of *zen* activity. In contrast, *zen* expression in *Drosophila* occurs in the entire extraembryonic primordium and is essential for the formation of all extraembryonic tissue (17). Together, the data from holometabolous insects suggests that in the Schizophora lineage *zen* expanded its expression domain to the entire amniotic primordium and became essential for all extraembryonic development (Fig. 6). Genetic changes upstream of *zen* must have occurred in the *Drosophila* lineage to allow these changes in *zen* expression and function (31). In addition, genetic changes immediately downstream of *zen* must have occurred to allow the coexpression of "serosa genes" (*zen* and *Kr*) together with *C15*, *pnr*, *dorsocross* (*doc*), and *tail-up* (*tup*) (25, 32, 33), which have been described as amnion markers in other species (14, 31) (Fig. 4). The coexpression of *zen* and amnion genes could have resulted from the loss of a transcriptional repressor of amnion genes downstream of *zen* (31). However, despite the apparent derepression of amnion markers in the amnioserosa, their *Drosophila* homologues have either no or only a subtle role in the initial differentiation of the amnioserosa when *zen* is expressed. *pnr* activity in the amnioserosa appears altogether blocked (34). *C15* null mutations do not interfere with larval hatching, indicating that their amnioserosa is essentially intact (35). Doc- and Tup-deficient embryos exhibit defects in the maintenance of amnioserosa cells only after stage 8 (25, 36). Thus, *doc* and *tup* unfold their function in the amnioserosa, with one exception (see below), after *zen* expression has been suppressed. Our model implies that these functions of *doc* and *tup* are comparable to the function of their homologues in the amnion of lower Diptera.

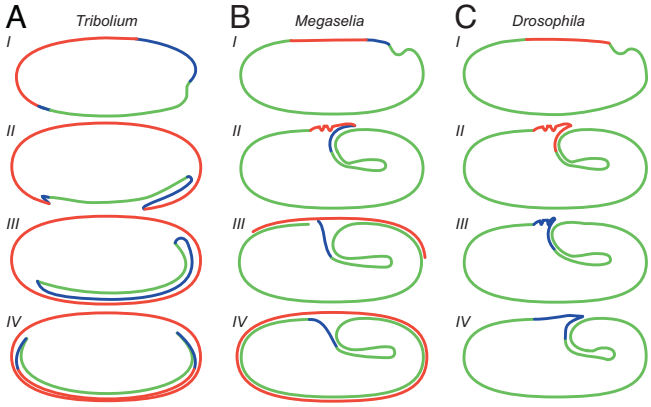

Fig. 6. Schematic comparison of extraembryonic *zen* expression between holometabolous insects. Successive developmental stages of *Tribolium* (*A*), *Megaselia* (*B*), and *Drosophila* (*C*) are shown with *zen*-expressing extraembryonic tissue depicted in red. Prospective extraembryonic tissue that does not express *zen* is depicted in blue, and embryonic tissue is in green. Note spatial differences of *zen* expression between species and the down-regulation of *zen* in *Drosophila* at about the same developmental stage that the preserosa of *Megaselia* begins to expand beyond the distal edge of the amnion. Anterior is left, and dorsal is up.

The amnioserosa of Doc-deficient embryos also fails to fold properly during germband extension, whereas *zen* is still expressed (25). This phenotype of a putative "amnion gene" in the early amnioserosa could suggest that some amnion properties of the amnioserosa unfold before *zen* expression is down-regulated. However, the folds in the amnioserosa of *Drosophila* occur likewise in the preserosa of *Megaselia* (compare to figure 2.8A in ref. 2) and probably constitute a preserosa rather than an amnion trait of the amnioserosa. Accordingly, we speculate that a *doc* homologue controls fold formation in the preserosa of *Megaselia*.

Finally, it has been suggested that only marginal cells of the late amnioserosa are equivalent of the amnion in less derived species (37). This idea, which is based on the observation that marginal amnioserosa cells are genetically distinct from other parts of this tissue, does not contradict our model because it refers to late properties of the amnioserosa. In addition, it does not rule out the possibility that central parts of the late amnioserosa are also amnion-like because the genetic differences between peripheral and central cells of the amnioserosa during dorsal closure could correspond to differences between peripheral and central cells of the dorsal amnion in lower cyclorrhaphan flies. More work in lower cyclorrhaphan species is needed to settle this question.

In summary, genetic modifications of extraembryonic development in the schizophoran lineage of *Drosophila* include changes upstream and downstream of *zen* as well as major changes in the expression pattern of *zen* itself both early and late in development. It is unlikely that all these changes happened at the same time. We propose that the early down-regulation of extraembryonic *zen* expression triggered the suppression of the expansion of the preserosa and the rift between this and adjacent tissue, thereby generating a single and strictly dorsal extraembryonic epithelium. At the same time, the early down-regulation of extraembryonic *zen* expression could have allowed preserosa tissue to acquire amniotic properties and to become essential for germband retraction and dorsal closure, which do not require the serosa (14) (Fig. 3). A more uniform amnioserosa anlage may have evolved gradually, perhaps to stabilize the developmental trajectory of the new morphology.

Loss of the Ventral Amnion in Evolution. Currently, a mechanistic understanding of the evolutionary origin of the dorsal amnion in cyclorrhaphan flies is limited by the lack of functional data on extraembryonic development in lower dipterans. However, the comparison of our results in *Megaselia* and *Episyrphus* with data from *Tribolium* (14) and *Oncopeltus* (15) suggests a tentative model that is based on spatiotemporal changes in the expression of *zen*. In *Tribolium*, serosa and ventral amnion are completed during germband extension. Subsequently, both epithelia fuse and retract dorsally, such that a single extraembryonic epithelium closes the embryo. Initially, only the serosa expresses *zen*

(*Tc-zen1* and *Tc-zen2*), but, preceding the fusion of the amnion with the serosa, *zen* (*Tc-zen2*) is activated also in the amnion. RNAi against *Tc-zen2* suppresses the fusion of the amnion with the serosa. Such embryos retain an intact amnion and close ventrally about the appendage buds. Likewise in *Oncopeltus*, the fusion of the completed amnion with the serosa is critical for normal dorsal closure and depends on the activity of *zen*, which is expressed throughout the serosa and at the site of fusion in the amnion. We speculate that *zen*-expressing amnion cells of *Tribolium* and *Oncopeltus* become indistinguishable from serosa cells and arrange to form a single epithelium, thereby causing the fusion of both tissues. To explain the transition from a ventral amnion to a dorsal amnion in dipteran evolution we propose a precocious expression of *zen* in the developing amnion. The earliest time point for *zen* expression in the developing amnion would be the blastoderm stage. Therefore, the boundaries of *zen* expression in the blastoderm relative to the boundaries of amnion-competent blastoderm might control the morphological difference between a ventral and a dorsal amnion perhaps not only in dipterans but also in hymenopterans, which modified the topology of the amnion in a similar manner (30).

Materials and Methods

Flies, Cloning Procedures, and RNAi. *Megaselia abdita* Schmitz, *Episyrphus balteatus* Degeer, and *Drosophila melanogaster* (Oregon strain) were reared in the laboratory. Genomic fragments of *Megaselia* homologues of *C15*, *pnr*, and *Kr* were PCR-amplified from genomic DNA using the following degenerate primer pairs: 5′-ATHGGNCAYCCITAYCARAAY/5′-TTNACYTGIGCRTCNGTCATYTT for C15, 5′-RTNATGATGDSNWSNTGGMG/5′-CCRCANGCRTTRCANARRTARTG for *pnr*, and 5′-AARCAYGTRYTKMARAAYCAYGA/5′-YTTYARYTGRTTRSWRTCRSWRAA and 5′-GATCATCAYYTSAARACNCA/5′-TTMAGGTGRTTSGAGTCRSYRAA for *Kr*. A fragment of *Episyrphus zen* was obtained with degenerate primers for *bicoid* (38) because of mispriming of the lower primer. The cDNA amplification Kit SMART (Clontech) was used. cDNA was prepared from 0- to 5-h-old embryos (collected at room temperature). *zen* dsRNA from *Megaselia* and *Episyrphus* was prepared from cDNA nucleotides 34–760 and nucleotides 50–909, respectively (position 1 being the first nucleotide of the ORF; essentially as described in ref. 39). For microinjection, embryos were aligned on a glass slide along a 0.2-mm glass capillary, briefly desiccated, and covered with halocarbon oil [one part 27-oil (Sigma H773) and three parts 700-oil (Sigma H8898) for *Episyrphus* and 27-oil only for *Megaselia*]. Approximately 65 pl of dsRNA solution was injected per embryo.

***In Situ* Hybridization, Immunocytochemistry, and Microscopy.** *In situ* hybridization was done as described with minor modifications (40, 41). Protocols are available on request. RNA probes were labeled with fluorescein (*zen* and *Megaselia pnr*), biotin (*Megaselia Kr*), or digoxigenin (*Megaselia C15*, *Megaselia zen*, and *Episyrphus zen*). The *Megaselia zen* probe was complementary to nucleotides 120-1071 of the ORF. The *Megaselia C15* probe was complementary to nucleotides 362–973 of the partial ORF and included 62 bases of 3′ UTR. The *Megaselia pnr* probe was complementary to the ORF and 111 nucleotides of 5′ UTR. The *Megaselia Kr* probe was complementary to the entire ORF, 44 nt of the 5′ UTR, and 79 nt of the 3′ UTR. Fab fragments against digoxigenin, fluorescein, and biotin (Roche) were used for probe detection. For fluorescent double *in situ* hybridizations, Tyramide Signal Amplification TSA (Molecular Probes) was used following the instructions of the manufacturer. To label cell membranes we used monoclonal mouse anti-phosphotyrosine (PY-plus mixture from Zymed) and anti-α-actin (Actin-4; gift of W. Sullivan, University of California, Santa Cruz, CA) primary antibodies and Alexa Fluor 488 or Cy3-conjugated secondary antibodies (Molecular Probes). The nuclei were stained with DAPI (Molecular Probes) or Topro-3 (Molecular Probes). Confocal scans were done with a Leica SP2 AOBS Spectral Confocal Microscope or a Zeiss Axiovert 200 Microscope. The 3D projection of image stacks was done with ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD).

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