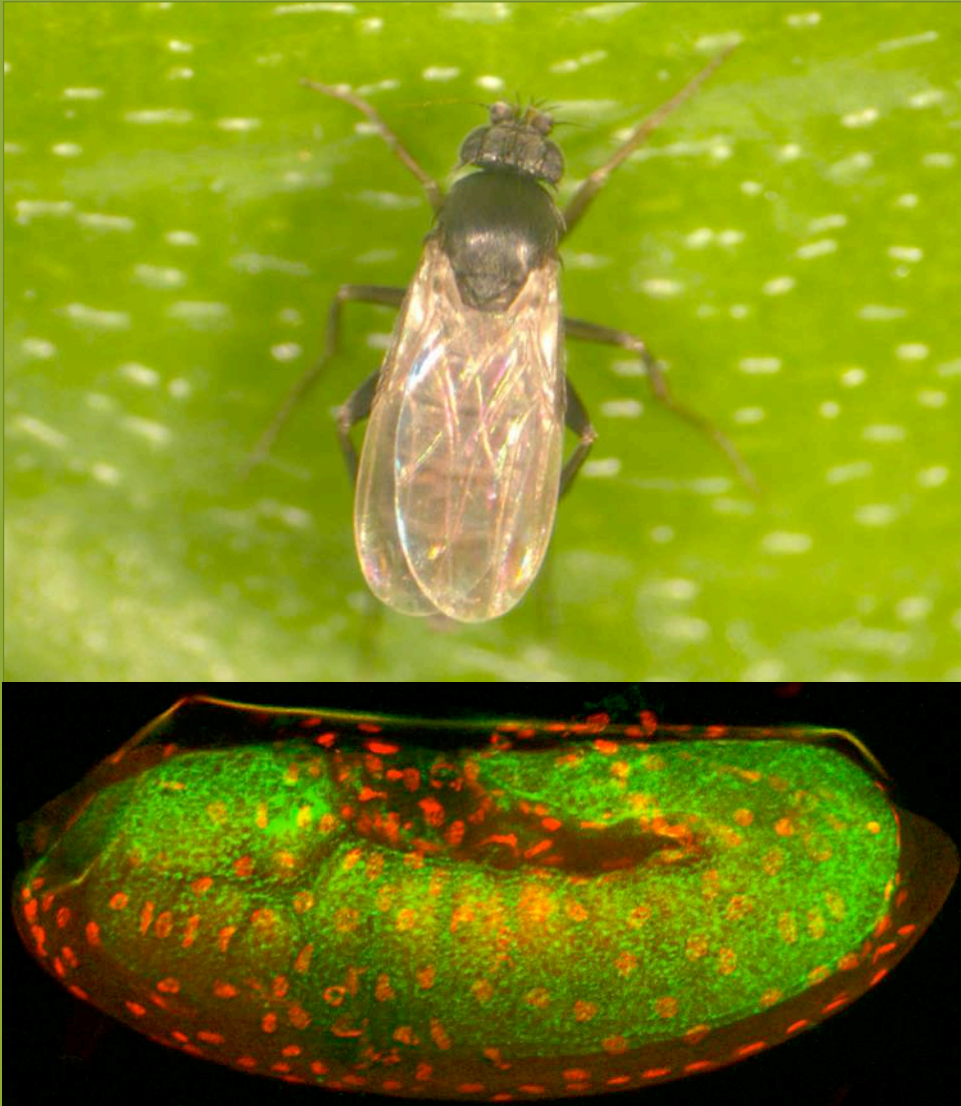# Morphological transitions and the genetic basis of the evolution of extraembryonic tissues in flies

Abdul Matteen Rafiqi

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the evolution of extraembryonic tissues in flies

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Introduction

Chapter 1: Introduction

Introduction

Understanding the genetic basis of evolutionary change that occurred a long time ago is a challenge. Because evolutionary changes become irreversible with the accumulation of secondary genetic change over time (Dollo's law, (1)). However, this difficulty is partially overcome when examining taxa that can interbreed. Interbreeding populations provide the possibility of linking specific portions of the genome to phenotypic change. The following [and other (5)] examples show that even large morphological changes can have simple genetic basis.

For example, marine sticklebacks typically have a continuous row of 32 to 36 armor plates (a bony exoskeleton) extending from head to tail. In contrast, many fresh water populations that are derived from the marine populations retain only zero to nine armor plates. This reduction in armor plates is mainly caused by allelic variation in a single gene, *ectodysplasin (eda)* (2). A similarly simple genetic change is observed between the *Drosophila* species that differ in larval morphology. The first instar larvae of many *Drosophila* species possess lawns of denticles and 'hair' like trichomes in the cuticle of abdominal segments. In *D. seccellia* however, the trichomes are entirely missing in the abdominal segments. These differences in morphology between *D. sechellia* and its sibling species are attributed to evolution at a single gene locus (*ovo/shaven-baby*) (3, 4).

The causal relationship of genetic and morphological differences between higher taxa is much more difficult to assess. The discovery of Hox genes (6-9) renewed interest in this problem. Hox genes are evolutionarily very stable homeobox containing genes that are organized in one or multiple gene complexes (10). Many of these genes have homeotic mutant phenotypes, in which one body part is transformed into another (11).

A role of homeotic mutations in evolution was proposed as early as the late nineteenth century (12) but there has been increased interest in their roles in evolution since genes causing these mutations have been observed to be conserved across morphologically different animals. Several studies comparing expression patterns of Hox genes in species with distinct morphologies show that Hox gene expression patterns often correlate with distinct morphologies. The number of neck vertebrae in birds provides one example of this correlation. Geese have a larger number of neck vertebrae than chickens; in turn, chickens have a larger number of neck vertebrae than mammals. In correlation with this morphological difference, in each species the anatomical boundary between the neck and the thorax is marked by the position of the expression of *Hoxc-6*. *Hoxc-6* expression starts at somite 21 in the goose embryo, at somite 19 in the chick embryo, and at somite 12 in the mouse embryo (13). In snakes, the entire trunk vertebrae exhibit morphology similar to the thoracic vertebrae of limb bearing vertebrates. Several of the Hox genes including *Hoxc-6* are expressed in the entire trunk, indicating loss of distinction between cervical, thoracic and lumbar vertebrae (14).

The fossil record suggests that primitive crustaceans had a rather uniform series of thoracic segments bearing appendages for locomotion. However, in some crustaceans, limbs from the anterior thorax have been modified for the manipulation of food. These modified thoracic appendages, termed maxillipeds, are morphologically and functionally more similar to the feeding appendages (maxillae) of the more anterior segments than to the rest of the thoracic limbs. In a comparison of crustaceans of six different orders, occurrence of maxillipeds invariably correlates with a loss of expression of Hox protein(s) Ultrabithorax/abdominalA (Ubx/AbdA) (15). Moreover, several of these limb modifications appear to be convergent, but the correlation with Ubx/AbdA expression pattern still holds. These correlations indicate that all of these changes may be caused by expression changes in the same gene(s). These

examples show that Hox gene expression correlates with morphological distinction in evolution. However, they do not definitively prove a causal relationship between the two.

In this thesis, I examine the evolution of extraembryonic tissues in flies and provide evidence that simple genetic changes caused major reorganizations of this tissue ~100 and ~150 million years ago. Extraembryonic epithelia are cellular envelopes that cover the embryo during development but do not become part of the embryo after development. These epithelia mostly serve a protective function during development [Reviewed in (16)]. In particular, the extraembryonic serosa secretes a protective cuticle called the serosal cuticle (17, 18). Further highlighting the protective role of the serosa, chemically induced degeneration of the serosa in moths leads to aborted embryos (19). In endoparasitic wasps the serosa protects the parasitic embryo from encapsulation (20, 21). In some of these species, the serosa also contributes cells that reside in the host hemolymph throughout the development of the wasp. These cells (Teratocytes) facilitate successful parasitism through signaling molecules (22, 23). In stick insects, the serosa plays a role in transcytosis of molecules from the yolk to the perivitelline fluid (24).

Ecologically, the presence of extraembryonic membranes correlates with the appearance of arthropod species that exploited terrestrial habitats for egg laying (16, 18, 25). A similar correlation seems to exist in vertebrates. In this group the evolution of an extraembryonic amnion around the developing embryo facilitated embryonic development outside aquatic habitats. The amnion occurs in *Amniotes* (reptiles, birds and mammals) but not in their amphibious relatives, whose embryonic development occurs in aquatic habitats. Fossil evidence suggests that terrestrial amniote embryos precede terrestrial amniote adults, highlighting the role the amnion played in their transition from aquatic to terrestrial environments (26).
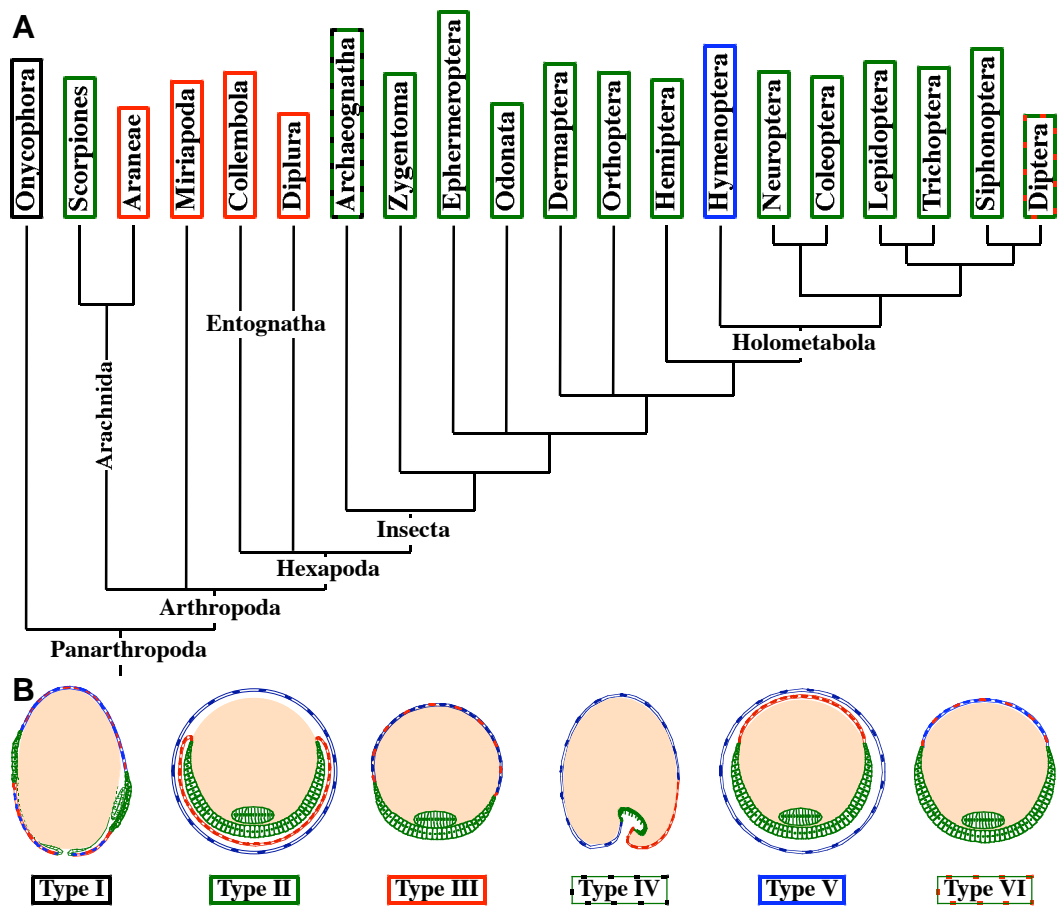
Fig. 1.1 Phylogenetic relationship and extraextraembryonic organization in Panarthrpoda. (A) Phylogenetic relationship between Panarthropod orders, in which extraembryonic tissue organizations has been described. Colors of text boxes indicate different types of extraembryonic tissue organization. Boxes with more than one color indicate presence of more than one type in the same order (see text for details) (B) Cartoons depicting transverse or sagittal (TypeI and TypeIV) sections of embryos with different types of extraembryonic organization. The embryos are depicted in green, the serosa in blue, the amnion in red and the yolk in ochre. Red and blue implies ambiguous or unknown homologies. Drawings based on (29) & (27) (Type I) . Dorsal is up.

Extraembryonic epithelia have been described in many terrestrial *Arthropods* and related velvet worms (*Onycophora*) (Fig. 1.1). Two extraembryonic epithelia are formed in velvet worms, one on the dorsal side and one on the ventral side. However, the embryonic germband remains laterally exposed (27). In scorpions (*Scorpiones*), two extraembryonic epithelia are formed, outer serosa and inner amnion. The serosa envelops the embryo while the amnion covers the ventral side of the embryo. Later in development, as the embryo undergoes dorsal closure, the amnion closes about the entire embryo (28). In spiders (*Araneae*), centipedes (*Scolopendromorpha*), springtails (*Collembola*), and *Diplura*, only a single dorsal extraembryonic epithelium, variably called extraembryonic ectoderm (27), amnioserosa (29) or serosa (18), is formed.

Most insects (*Insecta*) form an outer serosa that completely envelops the embryo, and an inner amnion that covers the ventral side of the embryo enclosing a fluid filled cavity called amniotic cavity [reviewed in (29) and (18)]. However, there are a few exceptions. In the most basal insects, bristletails (*Archaeognatha*), both serosa and amnion are formed but exist very briefly (30). In a few bristletail species two extraembryonic zones of different cell density are formed that hold the embryo in the middle without enclosing it; the posterior dense zone has been termed proamnion and the anterior thinner zone proserosa (29).

In ants, bees and wasps (*Hymenoptera*), the serosa forms a complete envelope but the amnion is dorsal and never covers the ventral side of the embryo. Hence, in this group an amniotic cavity is not formed (31-33). Most flies (Diptera) develop an amnion and a serosa but a few derived taxa (e.g., *Drosophila*) form a single extraembryonic epithelium (called amnioserosa) on the dorsal side covering the yolk but not the embryo [Reviewed in (29)] (Fig. 1.2). This thesis examines the developmental and genetic changes underlying the origin of the dipteran amnioserosa.

Fig. 1.2 Cartoons depicting development of extraembryonic tissue in flies. (A) Consecutive stages in development of extraembyonic serosa (blue) and amnion (red) in a typical basal fly. The embryo is shown in green, the yolk in ochre. The cellular blastoderm stage, the beginning of gastrulation and extended germband stages are shown. (B) The reduced extraembryonic amnioserosa (Black) is shown in a derived fly. Drawings based on (40) & (41). Anterior is to the left, dorsal up.
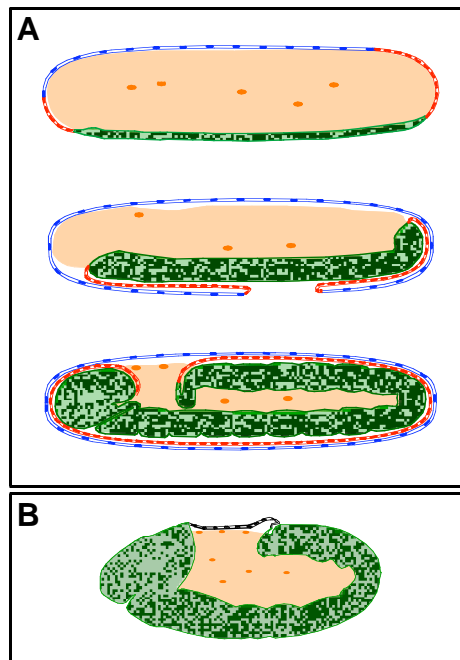
In chapter 2, I report the discovery of a novel type of extraembryonic tissue organization in dipterans. This tissue organization is similar to that found in hymenopterans where the amnion remains restricted to the dorsal side throughout development. Because this type of organization occurs consistently in independent basal branches of higher flies (Cyclorrhapha), I propose that this extraembryonic tissue organization preceded the evolution of the amnioserosa of more derived flies including *Drosophila*. In addition, I propose a genetic mechanism for both evolutionary transitions, that is, from ventral amnion to dorsal amnion and from dorsal amnion and serosa to amnioserosa.

In chapter 3, I test the proposed evolutionary mechanism for the second transition, from a dorsal amnion and serosa to the amnioserosa, by reproducing the genetic change in one of the "primitive" species. Experiments which tried to reverse the genetic change in the "derived" species, proved only partially successful, consistent with Dollo's law, that due to closure of certain evolutionary pathways that were possible in the ancestor, over an extended period of time, evolution is irreversible (1, 34 and references therein).

In chapter 4, I explore how the position of the extraembryonic anlage (precursor of (amnio-) serosa in the blastoderm stage) may affect the genetic mechanism of embryonic development. In some dipterans the extraembryonic anlage extends from the anterior pole along the dorsal midline, while in other dipterans it is confined to the dorsal blastoderm and does not extend to the anterior pole. This difference correlates with a patterning mechanism for head development in cyclorrhaphan flies that involves the gene *bicoid (bcd)* (35, 36). Maternal mRNA of *bcd* is localized at the anterior pole of the egg and translated in the syncytial embryo, in which Bcd forms a gradient with maximum concentration at the anterior pole. Bcd-deficient embryos lack the head and the thorax, exhibit variable segmentation defects in the abdomen, and develop a posterior abdomen with inverted anteroposterior polarity at the anterior pole. Conversely, injection of *bcd* mRNA into the posterior pole of early embryos can induce a mirror image duplication of the head and thorax. *bcd* is thus necessary

and sufficient for head development and contributes to the activation of many segmentation genes in the trunk [reviewed in (37, 38)]. The presence of *bcd* in the anterior pole should interfere with serosa specification at the anterior pole of the blastoderm and conversely *bcd* should be modified or absent in fly species in which the serosa anlage extends to the anterior pole (35). Under this hypothesis, *bcd* must be either absent or at least modified in *Episyrphus balteatus,* a cyclorrhaphan fly in which the serosa anlage extends to the anterior pole. In the genome, *bcd* is present in close proximity of its sister gene *zerknüllt (zen)* (39). To test whether *Episyrphus* lacks *bcd* or contains a modified *bcd* gene, I cloned and sequenced about 79kbp of the *zen* region in *Episyrphus balteatus*. This contig did not include a *bcd* ortholog supporting the hypothesis that in *Episyrphus, bcd* may not be conserved.

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Chapter 1: Introduction

Chapter 2

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

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Abbreviations: *zen*: *zerknüllt*; *pnr*: *pannier*; *Kr*: *Krüppel*; *dorsocross*: *doc*; *tup*: *tail-up*; dsRNA: double-stranded RNA; RNAi: RNA interference; PCR: polymerase chain reaction; nt: nucleotide; ORF: open reading frame; UTR: untranslated region of cDNA

Chapter 2: Evolutionary origin of the amnioserosa

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

Abstract

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 prior to 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 following *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 to *Megaselia* and *Episyrphus*, *zen* expression in *Drosophila* is extended to cells that form the amnion in lower Cyclorrhapha and is downregulated 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*.

Introduction

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: germ band 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 amnio-serosal fold, which closes about the ventral side of the gastrulating embryo. The outer cell layer of the amnio-serosal fold becomes the serosa. This epithelium detaches from the amnion and encloses the embryo. The inner cell layer of the amnio-serosal 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 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 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 paper, 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.

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, Themira*) by nuclear staining (Fig. 2.1A). In *Themira*, the only representative of higher Cyclorrhapha (Schizophora) in our sample, we identified extraembryonic tissue resembling the amnioserosa of *Drosophila* (Fig. S1). 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 cocktail of anti-phosphotyrosine antibodies and DNA stain, and examined different stages under a confocal microscope. In both species, we observed a serosa and an amnion (Fig. 2.1, Fig. S2). Prospective serosa cells flatten and become polyploid during germband extension (Fig. 2.1B). 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 amnio-serosal fold, which disjoins at its edge (Fig. 2.1C-E, Fig. S2A). The cells of the outer layer of the amnio-serosal fold (preserosa) contribute to the serosa (Fig. 2.1F, G, Fig. S2B, C). Cells of the inner cell layer of the amnio-serosal fold (preamnion) form the amnion. After disjoining from the edge of the preserosa, the preamnion develops into a dorsal cell layer (Fig. 2.1H). 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 (Fig. S2D, E). This observation suggests that the amnion attaches to the serosa and the serosa 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 amnio-serosal fold is shallow and the preserosa extends to the anterior pole (Fig. 2.1I). Our findings in *Megaselia* and *Espiryphus* suggest that the evolution of a dorsal amnion preceded the evolutionary origin of the amnioserosa (see Discussion).
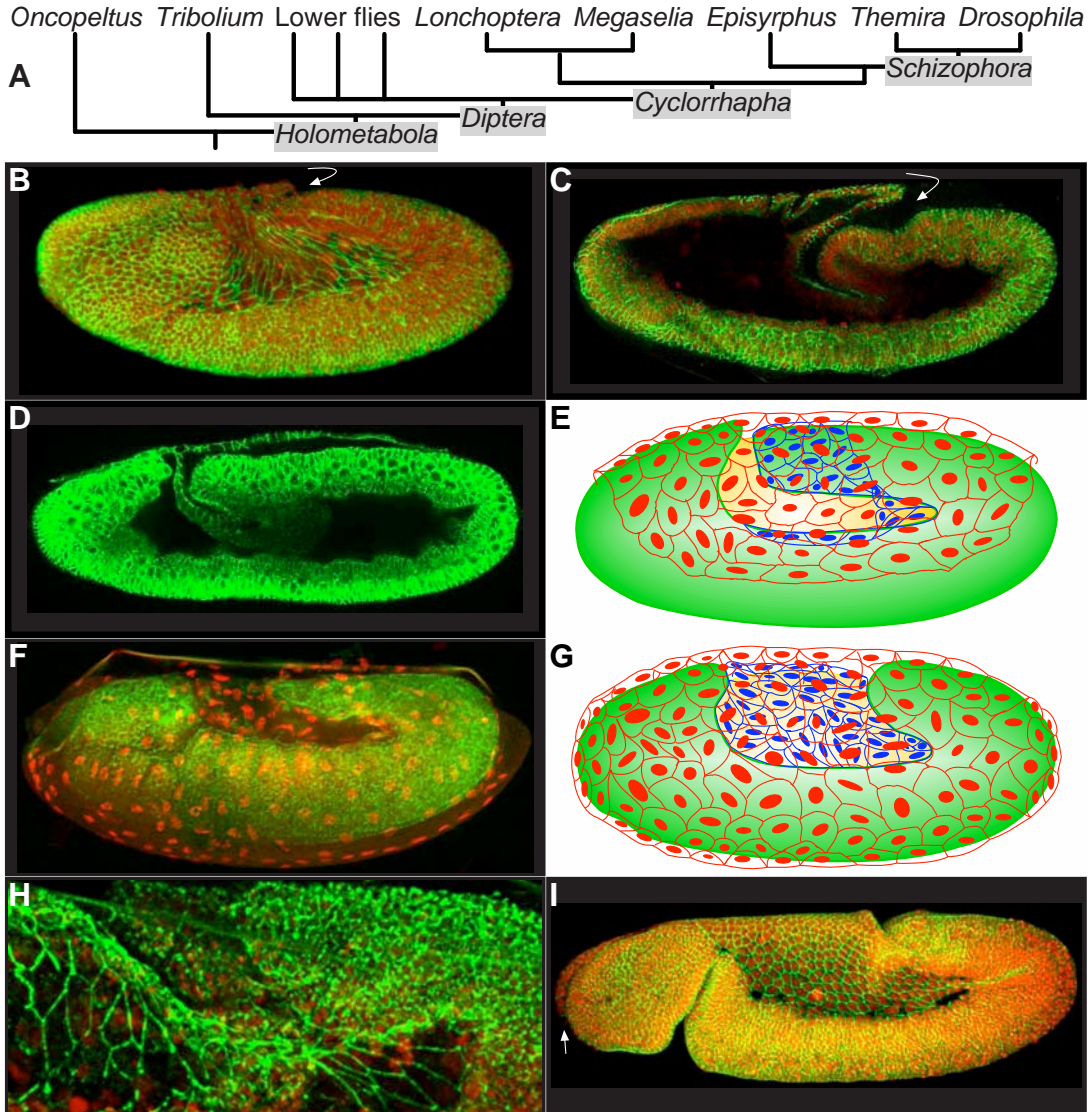
Fig. 2.1. Phylogenetic position and extraembryonic development of *Megaselia* and *Episyrphus*. (A) Phylogenetic tree of species mentioned in the text. (B-H) Confocal Z-stacks of *Megaselia* embryos during germband extension (B-D) and germband retraction (F, H). Cartoons (E, G) depict serosa cells (red), amnion cells (blue) and the germband (green) at stages shown in D and F. Note the amnio-serosal 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 preseorsa (arrow). Embryos were labeled with anti-phosphotyrosine antibodies (green) and Topro-3 or DAPI (B, F, I; red). Anterior is left and dorsal up.
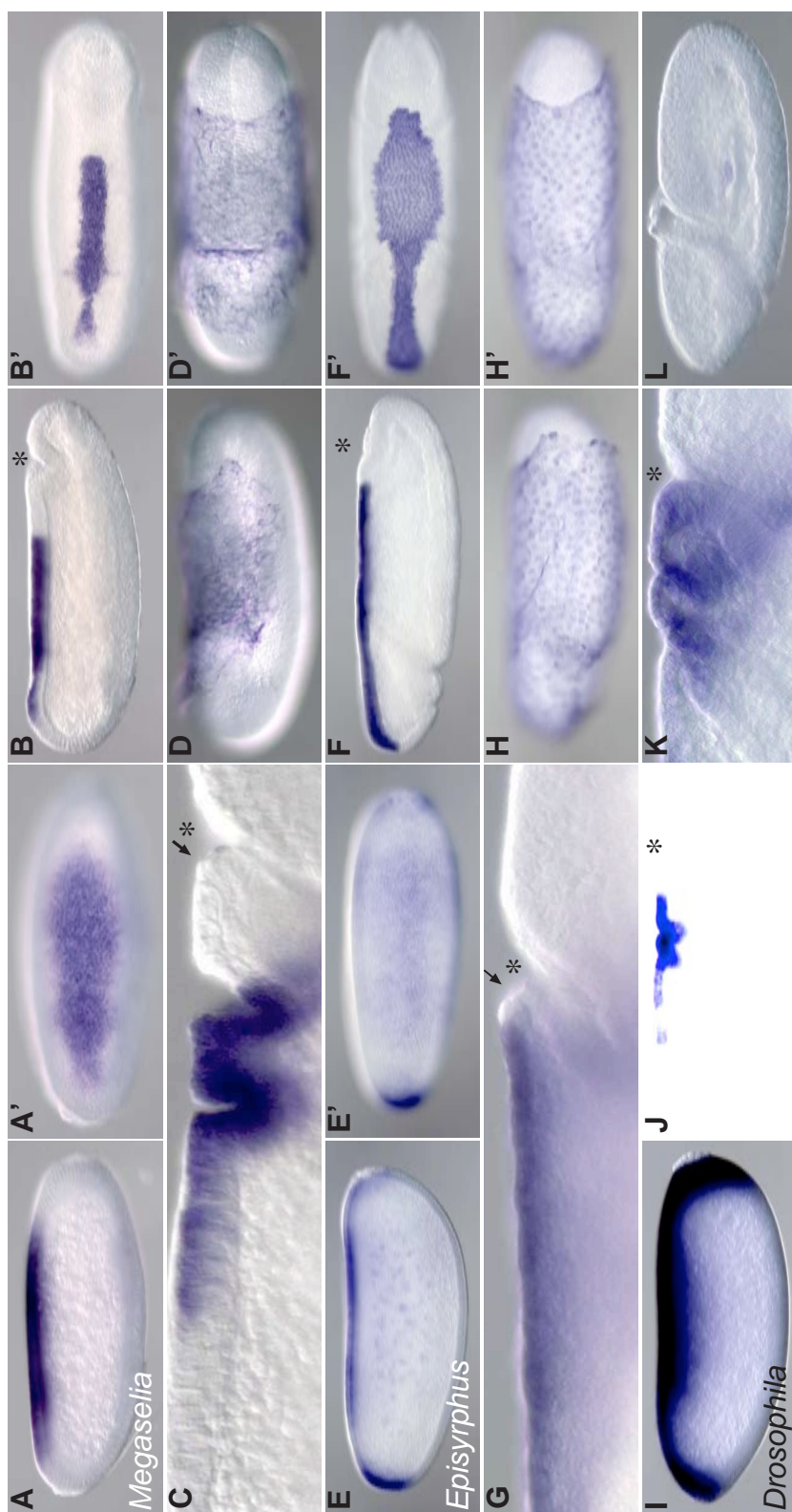
Fig. 2.2. Comparison of *zen* expression between *Megaselia, Episyrphus* and *Drosophila*. RNA in situ hybridizations of (A-D) *Megaselia,* (E-H) *Episyrphus* and (I-L) *Drosophila* embryos. Embryos are at the syncytial blastoderm stage (A, E, I), early gastrulation (B, F, J), germ band extension (C, G, K), or the extended germband stage (D, H, L). Anterior is left. The proctodeal invagination is marked by an asterisk. In *Megaselia* and *Episyrphus,* the amnio-serosal fold (arrow) migrates during the progression of the fold to the posterior expression boundary of *zen.*

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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. 2.2A, 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. 2.2B, B', C). This expression persists during the expansion of the serosa (Fig. 2.2D, 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* (Fig. S3). As in *Megaselia*, maternal *zen* transcript could not be detected in ovaries or early embryos of *Episyrphus* (not shown). Zygotic expression begins in the syncytial blastoderm (Fig. 2.2E-F'). Later in development, *Episyrphus zen* expression occurs in the developing serosa but not in the amnion or any embryonic tissue (Fig. 2.2G-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. 2.2I) 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. 2.2J, K), unlike in *Megaselia* and *Episyrphus*, where the amnion separates *zen* expression form 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. 2.2L). In addition, *Drosophila zen* is expressed in the head and germline (17, 20) but appears to be strictly extraembryonic in *Megaselia* and *Episyrphus*.
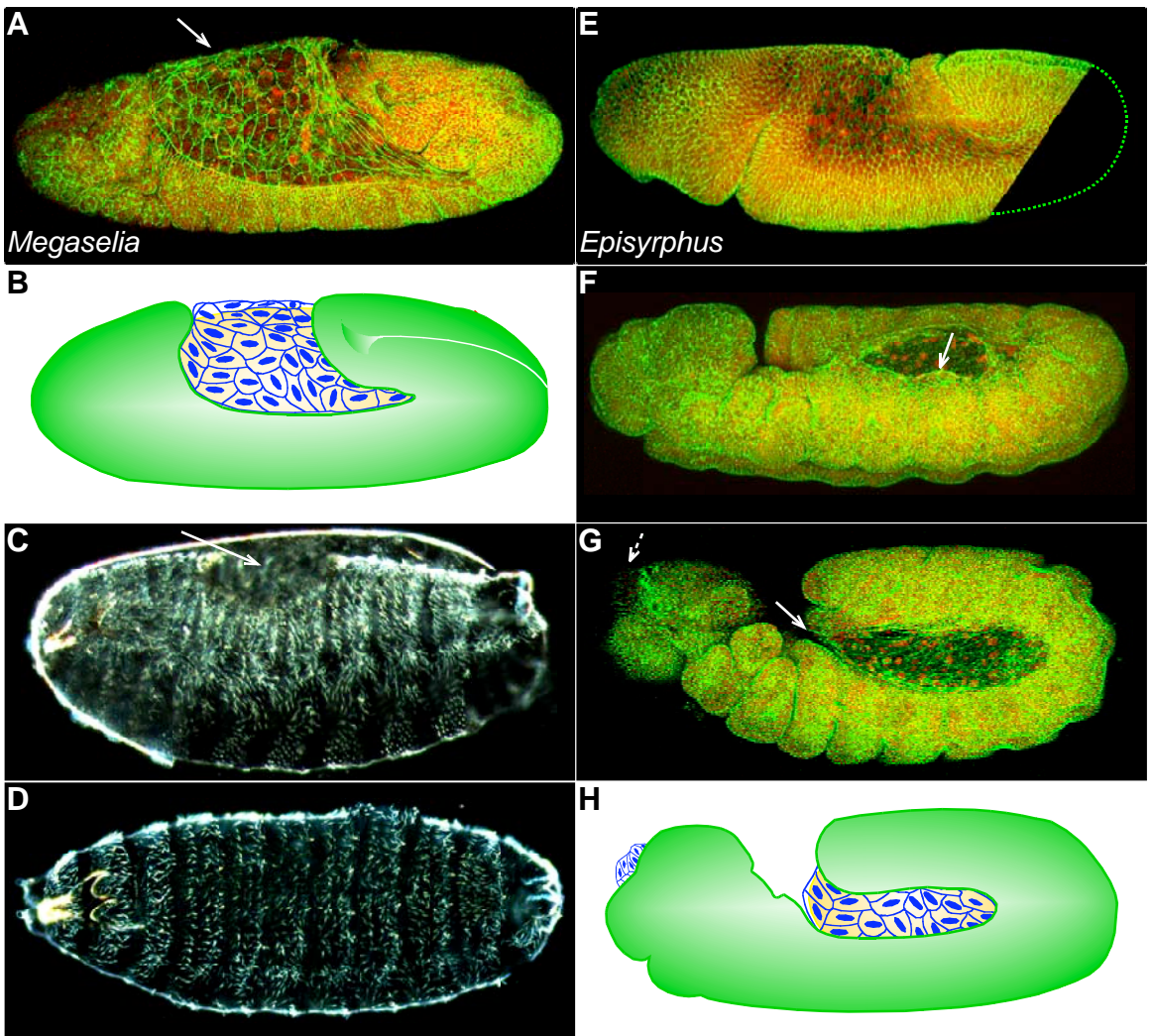
Fig. 2.3. *zen* RNAi in *Megaselia* and *Episyrphus* causes the formation of a single extraembryonic epithelium on the dorsal side. (A) Single extraembryonic epithelium (arrow) of a *Megaselia* RNAi embryo during germband retraction in dorsolateral view. (B) Schematic representation of the RNAi phenotype in *Megaselia* depicting extraembryonic tissue (blue) and the germband (green). (C, D) RNAi cuticles of *Megaselia* larvae showing a 'dorsal open' phenotype (arrow) in lateral view (B) and a 'dorsal closed' phenotype in dorsolateral view (C). (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 wildtype embryos (cf. panel E with Fig. 1I). 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 of the RNAi phenotype in *Episyrphus*. Anterior is left and dorsal up unless specified otherwise. Embryos were stained with anti-phospho-tyrosine antibodies and Topro-3 (A, F, G) or DAPI (E).
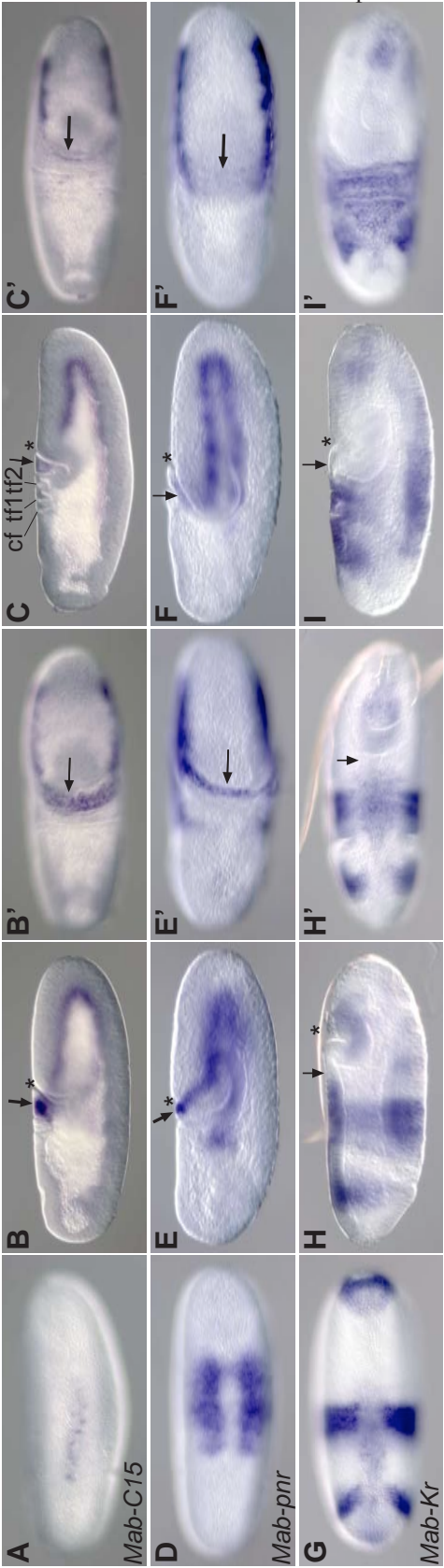
Fig. 2.4. Expression of *C15*, *pnr*, and *Kr* in *Megaselia*. (A-C) *Megaselia C15*. (D-F) *Megaselia C15*. (G-I) *Megaselia pnr*. (G-I) *Megaselia Kr*. All embryos are shown in dorsal view (A, D, G, B', -I') or in lateral view with dorsal up. Anterior is left. Arrows point to the prospective amnion. The proctodeal invagination is marked by an asterisk.
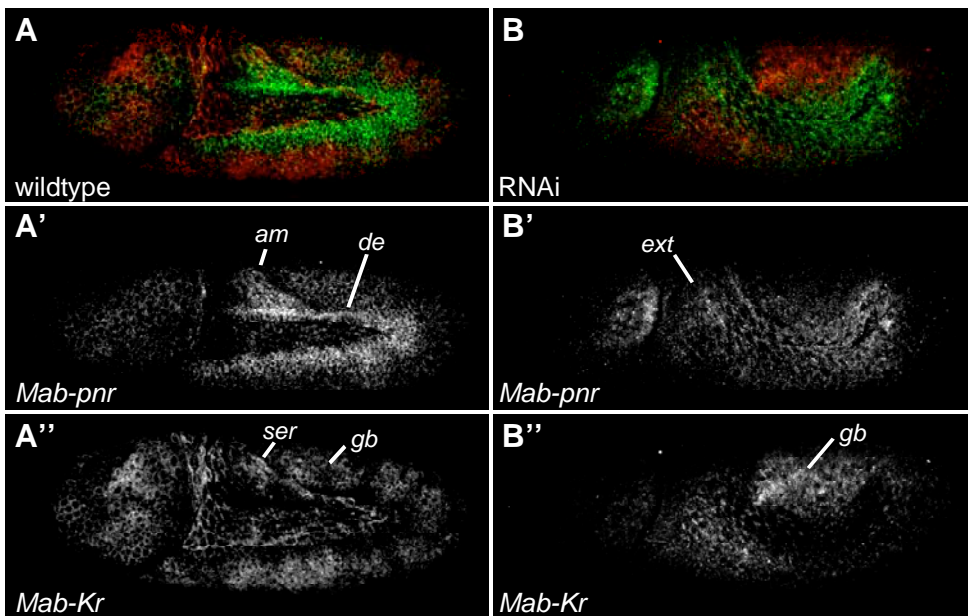
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Fig. 2.5. Extraembryonic primordium of *Megaselia zen* RNAi embryos expresses *pnr* but not *Kr*. (A) Wildtype. (B) RNAi phenotype. Expression of *pnr* is shown in green or as monochrome image of the green channel (A', B'), *Kr* in red or as monochrome image of the red channel (A'', 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.

Phenotypic effects of *zen* RNAi in *Megaselia* and *Episyrphus*. To analyze the function of *zen* during embryogenesis in *Megaselia* and *Episyrphus*, we used RNA interference (RNAi). We injected preblastoderm embryos with *zen* double-stranded RNA (dsRNA) and analyzed them at different time points (Fig. S4). *Megaselia* embryos injected with a 4.7 μM solution of dsRNA consistently developed a single extraembryonic epithelium on the dorsal side (Fig. 2.3A, B). To test whether *Megaselia zen* is required for dorsal closure, we repeated the RNAi experiment and allowed the embryos to develop a cuticle. About half of the developed embryos exhibited a 'dorsal open' cuticle (Fig. 2.3C), while the other half was indistinguishable from wildtype (Fig. 2.3D). The absence of dorsal closure defects in a large proportion of RNAi embryos could reflect residual *zen* activity. Alternatively, the 'dorsal open' morphology could reflect a hypomorphic phenotype caused by an oversize 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 (Fig. S4). In *Episryphus*, all embryos developed a single extraembryonic epithelium on the dorsal side when injected with a 1.7 μM solution of *Episyrphus zen* dsRNA (Fig. 2.3E-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, while 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* (Figs. S5)**.** 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 about 60% of the trunk region (not shown). This expression disappears during blastoderm cellularization. At the beginning of gastrulation, some cells of the serosa primordium weakly express *C15* (Fig. 2.4A), 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. 2.4B, B'). In a few embryos, we noticed *C15* expression also in the posterior serosa primordium, suggesting that during early gemband 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 (not shown). In the preamnion, we observed an attenuation of *C15* expression shortly before these cells begin to stretch (Fig. 2.4C, 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. 2.4D). During germband extension, we consistently observed expression in the ectoderm and in the preamnion (Fig. 2.4E-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 amnio-serosal fold until high expression levels remain only in the leading edge of the dorsal epidermis (Fig. 2.4F, 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 (not shown). Here, we consider only the extraembryonic expression of *Megaselia Kr*, which starts with gastrulation (Fig. 2.4G). During germband extension, we observed *Kr* expression in the expanding preserosa but not in the amnion (Fig. 2.4H, H', I, I'). Thus, *Megaselia Kr* is a serosa marker.

We used the *Megaselia* homologues of *pnr* and *Kr* to examine the identity of extraembryonic cells following *zen* RNAi. As a time window, we chose germband extension stages, when *pnr* expression in *Megaselia* wildtype embryos is up-regulated in the prospective amnion and downregulated in the prospective serosa. We performed double in situ hybridization experiments to examine simultaneously the expression of *Kr* and *pnr* (Fig. 2.5A, A', A''). *Megaselia zen* RNAi embryos lacked extraembryonic but not embryonic *Kr* transcripts, and expressed *pnr* evenly throughout the extraembryonic primordium and dorsal ectoderm (Fig. 2.5B, B', 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 prior to 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 towards the end of the Cretaceous period in the stemgroup 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. 2.6).
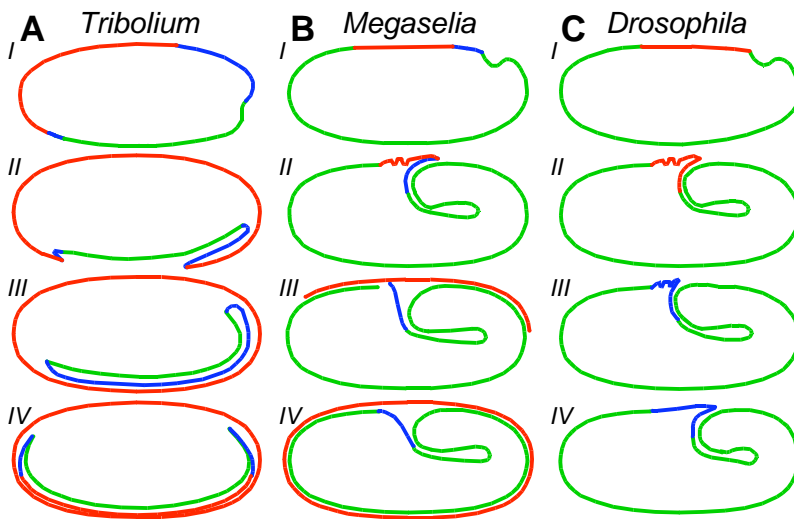
Fig. 2.6. Schematic comparison of extraembryonic *zen* expression between holometabolous insects. Successive developmental stages of *Tribolium* (A), *Megaselia* (B), and *Drosophila* (C) are shown with *zen* expressing extraembryonic tissue depicted in red. Prospective extraembryonic tissue that does not express *zen* is depicted in blue, and embryonic tissue 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 up.

Across holometabolous insects, early *zen*-expression is confined 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. 2.6). 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, Kr*) together with *C15, pnr, dorsoccross* (*doc*) and *tail-up (tup)* (25, 32, 33), which have been described as amnion markers in other species (14, 31) (Fig. 2.4). 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 de-repression 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.

The amnioserosa of Doc-deficient embryos also fails to fold properly during germband extension, while *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 downregulated. However, the folds in the amnioserosa of *Drosophila* occur likewise in the preserosa of *Megaselia* (compare to figure 2.8A in 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. 2.3). 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*, *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 degenerate primer

pairs: 5'ATHGGNCAYCCITAYCARAAY / 5'TTNACYTGIGCRTCNG-
TCATYTT for C15, 5'RTNATGATGDSNWSNTGGMG / 5'CCRCANGCR-
TTRCANARRTARTG for *pnr*, 5'AARCAYGTRYTKMARAAYCAYGA /
5'YTTYARYTGRTTRSWRTCRSWRAA and 5'GATCATCAYYTSAARAC-
NCA / 5'TTMAGGTGRTTSGAGTCRSYRAA for *Kr*. A fragment of
Episyrphus zen was obtained with degenerate primers for *bicoid* (38) due to
mispriming of the lower primer. The cDNA amplification Kit SMART
(Clontech) was used. cDNA was prepared from 0-5 hour old embryos
(collected at room temperature). *zen dsRNA from Megaselia and Episyrphus
was prepared from* cDNA nucleotides (nt) 34-760, and nt 50-909,
respectively (position 1 being the first nucleotide of the open reading frame
(ORF). essentially as described (39). For microinjection, embryos were
aligned on a glass slide along a 0.2mm glass capillary, briefly desiccated, and
covered with halocarbon oil (one part 27-oil, Sigma H773; 3 parts 700-oil
Sigma H8898 for *Episyrphus* and 27-oil only for *Megaselia*). Roughly 65 pl
of dsRNA solution were 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*, *Megaselia pnr*), Biotin (*Megaselia Kr*) or Digoxigenin
(*Megaselia C15*, *Megaselia zen*, *Episyrphus zen*). The *Megaselia zen* probe
was complementary to nt 120 to 1071 of the ORF. The *Megaselia C15* probe
was complementary to nt 362-973 of the partial ORF and included 62 bases
of 3'untranslated region (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 DIG, FITC and BIO (Roche) were
used for probe detection. For Fluorescent double in situs, 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 cocktail from ZYMED) and anti-α-actin
(Actin-4; gift of W. Sullivan, UC Santa Cruz) primary antibodies and Alexa-

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 using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA).

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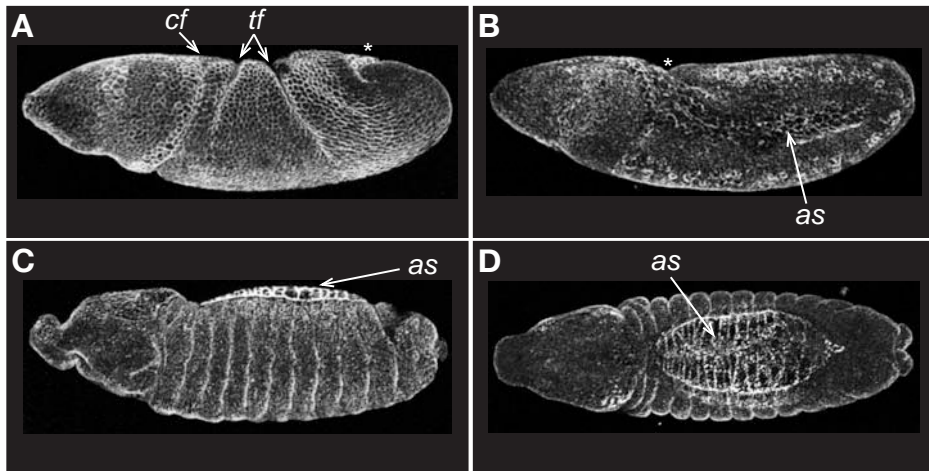41. Tautz D, Pfeifle C (1989) *Chromosoma* 98, 81-85.

Fig. S2.1. Amnioserosa development in *Themira biloba*. (A) Embryo at the beginning of germband extension. (B) Extended germband. (C and D) Embryos after germband retraction. The embryos were stained with a mixture of anti-phosphotyrosine and anti-α-actin antibodies to mark cell membranes. The vitelline membrane has been removed. Lateral (A-C) and dorsal (D) views are shown. Anterior is left. cf, cephalic furrow; tf, transverse folds; as, amnioserosa; *, proctodeal/hindgut invagination
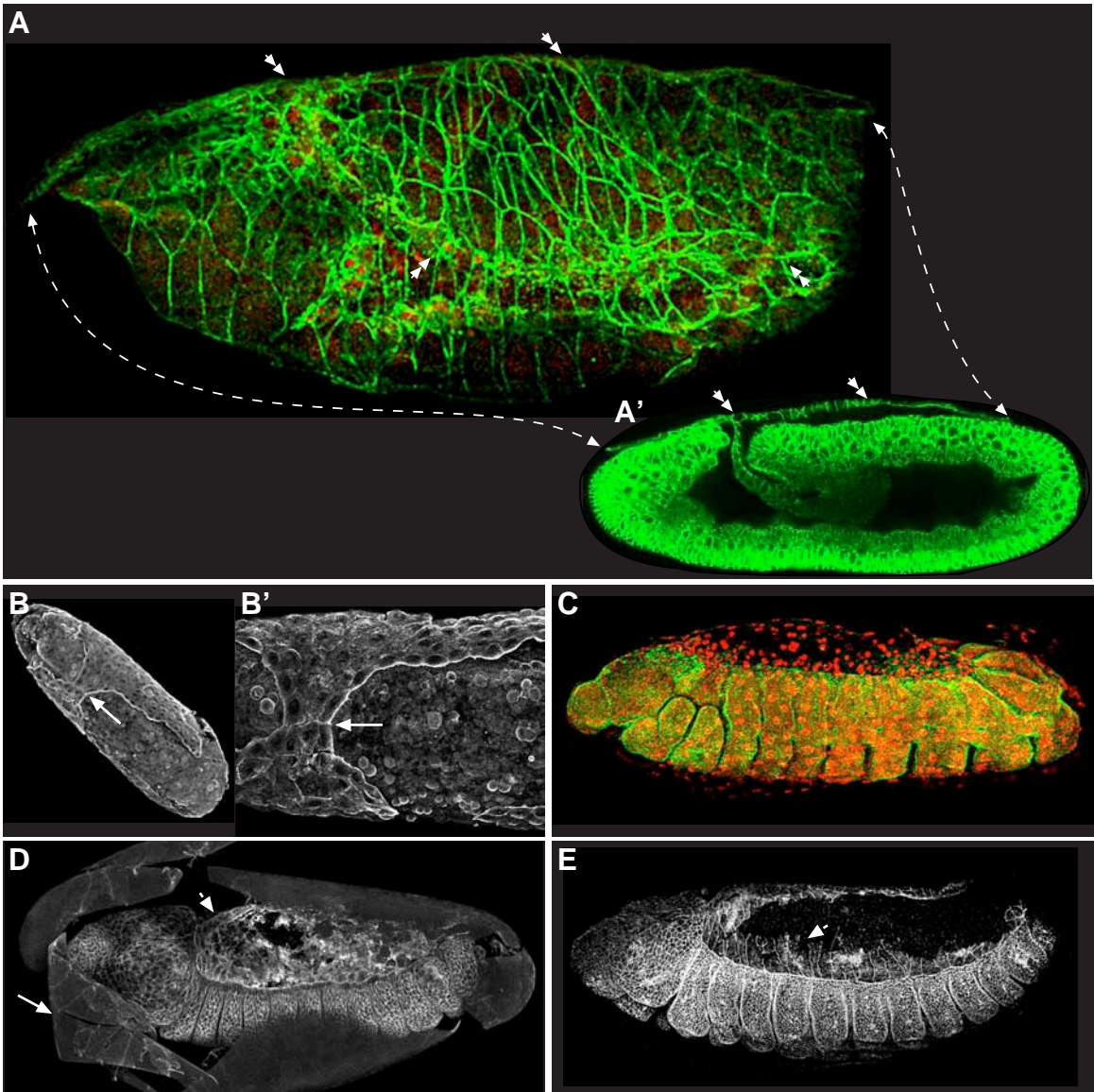
Fig. S2.2. Extraembryonic development in *Megaselia* and *Episyrphus*. (A) Confocal Z-stacks showing the extraembryonic tissue in *Megaselia* during the early expansion phase of the serosa. (A') Overview of the same embryo. Note the extent of the double layer of amnion and serosa (double arrows) compared to the extent of the preserosa (outer arrows). (B, B') Ventral fusion of the serosa (arrow) in *Episyrphus*. (C) *Episyrphus* embryo after germband retraction showing large serosa nuclei (red) surrounding the embryo. (D, E) Confocal Z-stacks of *Megaselia* (D) and *Episyrphus* (E) embryos after germband retraction. The removal of the vitelline layer and the serosa (arrow) damages the amnion (broken arrow). The embryos were labeled with anti-α-actin (B, D), or anti-phosphotyrosine antibodies (A, C and E) and Topro3 (A, C).

Fig. S2.3. Protein alignment of Zen homologues. Predicted amino acid sequences of *zen* orthologues from *Anopheles* (Aga), *Clognia* (Cal), *Haematopota* (Hpl), *Empis* (Eli), *Episyrphus* (Eba), *Megaselia* (Mab), and *Drosophila* (Dme) are shown. Amino acids that are conserved in the majority of the sequences are shaded in grey; dashes denote gaps. The homeodomain and the MAD-ZEN interaction domain of the *Drosophila* protein are underlined (1, 2). Numbers refer to the last amino acid in each row. GenBank accession numbers of *zen* homologues: Aga AAAB01008846; Cal AJ419659; Hpl AJ419660; Eli AJ419661; Mab AJ295635; Eba DQ323932; Dme NM_057445.
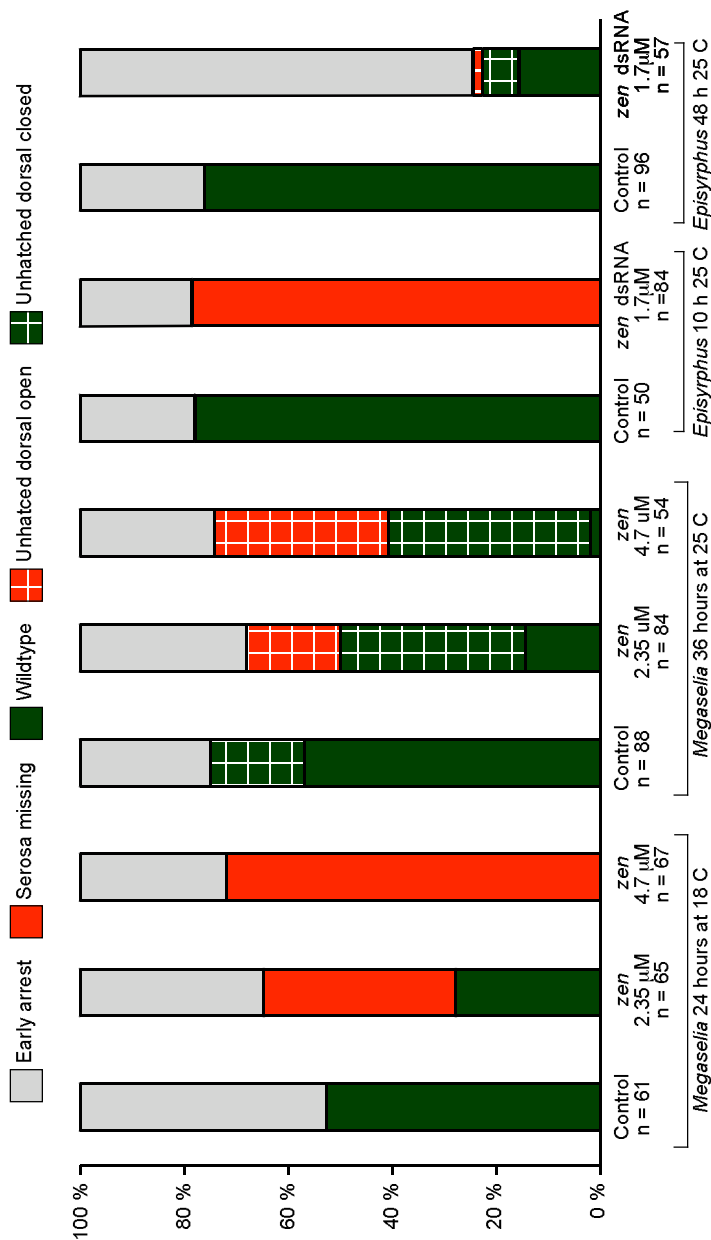
Fig. S2.4. Frequencies of *zen* RNAi phenotypes in *Megaselia* and *Episyrphus*.

C15 alignment

```
Tca MS------------DHELTEDDHEINV-LDTDSRLS-HSDSEARSSRSRC---------STPENSGSHHNSTTVLPFSISNLLGKNFF--------QKSEPDQLYPSPG------  79
Mab MSSVHEEDDRDFDGDINVD--------DVDSDSRISCESDIDLD---GSCYDDNEMHLREHIQPSEHTRSSNENLPFSISRLLSKPYETTFKNNNNNNNDDKDLIRGGGGSSSAG 104
Dme MSS-HEEDDHEHENEHGEEVEEQEIHVDVDSDSRMSCGSGSDVDMDGGSCYDESETPLSESLQSEQTRSSSSENLPFSISRLLSKPFETS----HHHHNNNNHLLSSSPGSSSNN 110

Tca -----------LFPQRG------LGGLMPSGFYGQGVLRVPAHRPLGPGSPAGGVFS--------PWTLGLDPVLQRSAAAAAFATQVVKDRLAATFPMTRRIGHPYQNRTPPKR 168
Mab SDCERETSSS--------HHITTSLAGS-----YGSAAAAIYSYP-VYAHAPG-HVLRVPAQNSPLSWSI---PPLHH---AALAHQAVKDRLAAAFPIARRIGHPYQNRTPPKR 198
Dme NNSGEKGEEKELLQQEDHDLAYKLATSIANSTYGSAAA-LYSYPHLYPSAAGGHVLRVPPQRTPLTWAL---PPLHH---AALAHQAVKDRLAAAFPIARRIGHPYQNRTPPKR 217

Tca KKPRTSFTRLQIAEELEKRFHKQKYLASAERAALAKTLKMTDAQVKTWFQNRRTKWRRQTAEEREAERQAANRLMLSLQAEALSKGYMSEGPPPSSG---------PGDTALSALQ 274
Mab KKPRTSFTRIQVAEELEKRFHKQKYLASAERAALARGLKMTDAQVKTWFQNRRTKWRRQTAEEREAERQAANRLMLSLQAEALSKGF---GPPPNSGLSSVQSPSSAPGAPLAALH 310
Dme KKPRTSFTRIQVAEELEKRFHKQKYLASAERAALARGLKMTDAQVKTWFQNRRTKWRRQTAEEREAERQAANRLMLSLQAEAISKGF---APPS----APLGSQGGVNGAPLAALH 325
    Homeodomain
Tca NLQPWAGPYSAPQPALAESMC                                                                                             295
Mab GLQPWAEQ-HSTP-------C                                                                                             323
Dme GLQPWAEASHAAG-------C                                                                                             339
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Pnr alignment

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Tca MFHTSG-------------GGGGYSDGNAGFHQHLQQSPVYVPSSRAVPQYPSPASGHFGAAAHQSSWAHAGGTYGEMAAPSAHSLGTSPHAGALSAGQFYAQN--MMMSSWR-A  99
Mab MFHST--SAYPDI-------SAGTGNVVSSYHQQASAAPVYVPSNRAL----SSQYSHFGTSAAQNAWT--ADSFS-----SAHS------------QFYAQNA-MMMGSWRTA  81
Dme MYHSSAVAAYTDLAAAGSAASAGVGVGVSGYHQQAVNAPVYVPSNRQY----NHVAAHFGSAAAQNAWT--TEGFG-----SAHA------------QFYSPNAAVMMGSWRSA  91

Tca YDGTG--FQRTSPYDGSMEFQFGEGRECVNCGAISTPLWRRDGTGHYLCNACGLYHKMNGMNRPLIKPSKRL---TATRRLGLCCTNCGTRTTTLWRRNNDGEPVCNACGLYFKLH 210
Mab FDPTAAFHQANPYDSSMDFQFGEGRECVNCGAISTPLWRRDGTGHYLCNACGLYHKMNGMNRPLIKPSKRL---TATRRLGLSCTNCGTRTTTLWRRNNEGEPVCNACGLYFKLH 193
Dme YDPSG--FQRSSPYESAMDFQFGEGRECVNCGAISTPLWRRDGTGHYLCNACGLYHKMNGMNRPLIKPSKRLVSATATRRMGLCCTNCGTRTTTLWRRNNDGEPVCNACGLYYKLH 205
                                          Zinc Finger Domain
Tca GVNRPLAMRKDGIQTRKRKPKK-----PVGGERDDSSSASVEEIVFYL--------LPLREQKL-RLKSRSVPEMSPQTHILARRI----------------------HQFC-N 287
Mab GVNRPLAMRKDGI                                                                                                     206
Dme GVNRPLAMRKDGIQTRKRKPKKTGSGSAVGAGTGSGTGSTLEAIKECKEEHDLKPSLSLERHSLSKLHTDMKSGTSSSSTLMGHHSAQQQQQQQQQQQQQQQQQQQSAHQQCFP 320
    GVNRPLAMRKDGIQTRKRKPKK
Tca LLSETKSLVHQSSQQHS-----SQSQSQTHHNH-----QL----DKSSPVERPYLGQTSLLPATSTSAIKSE-------------------------PGYEYSCIQNQP 357
Mab                                                                                                                   206
Dme LYGQTTTQQQHQQHGHSMTSSSGQAHLSARHLHGAAGTQLYTPGSSSGGGSASAYTSHSAETPALSNGTPSPHYQHHHLGGTHGHHVTAAAAHHHFHAAAAVAAYGVKTEASAT 435

Tca SYPYQQ------IFGFPGSANSSGEVA---------YHHQHHVTAAAKLMASS                                                             395
Mab                                                                                                                   206
Dme NYDYVNNCYFGGTFGALGGAATTTAMAGGAASELAGYHHQHNVIQAAKLMATS                                                           488
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Kr alignment

```
Tca M--PLLQETTP----------KRDMLDSQEKTPLSS------------VSYPSM-------FTPSQLLMASHLMAASR---------LSLPT-NPAFF--HPGLLPLAWQA---  69
Mab MALSLLQDAQTRGLAAALAGIKRENV--DQCLQMTPPQGAHN-------LHP--GVNPAANFQAFGMLSPQQFLAANRTAAALMAAQIPMSHLA---FN---TALFGQWPGTPQQ  98
Dme MSISMLQDAQTRTLAAALAGIKQEDVHLDRSMSLSPPMSANTSATSAAAIYPAMGLQQAAAASAFGMLSPTQLLAANRQAAAFMA-QLPMSTLANTLFPHNPAALFGAWAAQQSL 114
                                                                N-terminal Repression Region                  * *
Tca -------NSPPSPPAPSELPALKSRK--LNNNNVVSSTNQEIRGPKRKTWKVEEDSPS-----------PTSSVSP----------------------EVKDSSRDRPFTCEVCNR 142
Mab GLLGT--NSPPASPISPL----------LNTTPATAMEPQ-LKKARKISIKKDLTSPSSMMDVSMDYMPAGPISPPSSGSSPNSNQEQSP-----VPQNKDGSRDKSFTCKICSR 195
Dme PPQGTHLHSPPASPHSPLSTPLGSGKHPLNSPNSTPQHHEPAKKARKLSVKKEFQTEISMSVNDMYHTSGGPISPPSSGSSPNSTHDGAGGNAGCVGVSKDPSRDKSFTCKICSR 229
                                                                       *NLS  *      *     *   *   *      *     *  *  *  * *
Tca SFGYKHVLQNHERTHTGEKPFECQECHKRFTRDDHHLKTHMRLHTGERPYCEHCDRQFVQVANLRRHLRVHTGERPYGCEHCSMKFSDSNQLKAHVLIHTNEKPFECDKCRGRFR 257
Mab SFGYKHVLQNHERTHTGEKPFECPECHKRFTRDDHHLKTHMRLHTGEKPYHCSHCDRQFVQVANLRRHLRVHTGERPYTCEICEQRFSDSNQLKTHMLVHNGEKPFECERCHLKFR 310
Dme SFGYKHVLQNHERTHTGEKPFECPECHKRFTRDDHHLKTHMRLHTGEKPYHCSHCDRQFVQVANLRRHLRVHTGERPYTCEICDGKFSDSNQLKSHMLVHNGEKPFECERCHMKFR 344
                                                               Zinc Finger Domain                                  *  *
Tca RRHHLVHHKCGGEEEAERAPAPAVRAA--GDAAAARGAARADGAG---GPLHDHRP------ALAQQRRVAAVQVP-QLAGGG------------RRGPGPARSRRHLPAHLVA 347
Mab RRHHLMSHKCGILGTSSPPPTPAISPVMS-DYALN--------------MCDQKSYSAGSESAE---SLHENDVPLDLSEEGEEKRSNSSAD-----SKNMKRIFPRLAPTQII 401
Dme RRHHLMNHKCGI----QSPPTPALSPAMSGDYPVAISAIAIEASTNRFAAMCA--TYGGSNESVDMEKATPEDDGPLDLSEDGASSVDGHCSNIARRKAQDIRRVF-RLPPPQIP 452
                                                                                                          C-terminal
Tca GTCFVQL--------IGMRVPP-------------EMMQAGDYC                                                              370
Mab HYPMPNLPVQTEPEDLSMHSPRSSTHDDDLDDLDEAATLNLKQHKQFIMDQQSTPLKRLHHEMMME                                        467
Dme HVP-SDMPEQTEPEDLSMHSPRSIGSHEQTDDID----------LYDLDDAPASYMGHQQH                                             502
    Repression Region
```

Fig. S2.5. Protein alignments of C15, Pnr, and Kr homologues. Predicted amino acid sequences of orthologues from *Tribolium* (Tca), *Megaselia* (Mab), and *Drosophila* (Dme) are shown. The homeodomain of C15 (3), the zinc-finger region of Pnr (4), the zinc-finger region, the N- and C-terminal repression domains, and nuclear localization signal (NLS) of Kr (5-7) are underlined. Amino acids that are conserved in the majority of the sequences are shaded in gray, and dashes denote gaps. GenBank accession numbers of C15, Pnr, and Kr homologues are as follows: for Tca, XM_969890, XM_967958, and NM_001039438; for Mab, EU287990, EU287991, and EU287992; for Dme, NM_057525, NM_057337, and NM_079143.

Chapter 2: Evolutionary origin of the amnioserosa

Chapter 3

Temporal expression differences of *zerknüllt (zen)* explain the origin of amnioserosa

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Submitted

Chapter 3: Temporal expression differences of *zen*

Temporal expression differences of *zerknüllt (zen)* explain the origin of amnioserosa

Abstract

Pinning down genetic changes that underlie morphological differences between higher taxa such as families or orders is a challenge because evolution is irreversible over an extended time. However, it should be possible to experimentally recapitulate some of these transitions if underlying genetic differences are simple and can be reproduced in an extant species that exhibits the primitive morphology. Here, we apply this rationale to the origin of the amnioserosa, a unique epithelium of higher flies that evolved approximately 100 million years ago. Using a transfection-based RNA interference technique, we show that a predicted temporal change in the regulation of the homeobox gene *zerknüllt* (*zen*) in *Megaselia abdita* (Phoridae), which split from other higher flies before the amnioserosa evolved, is sufficient to reorganize the ancestral amnion and serosa into a single amnioserosa-like epithelium.

Introduction

Recent work in animals and plants supports the idea that changes in single genes underlie important morphological change in adaptive evolution. The reduction of the pelvic girdle or armor plates in fish, trichome patterns in fly larvae, new macrochaetae in adult flies, and new leaf morphology in tomatoes are predominantly controlled by expression changes of single genes (1-7). Likewise, key adaptations in crop domestication, such as the origin of naked grains or plant architecture in maize, evolved primarily in response to changes in a single genes (8, 9). While these examples refer to morphological differences between closely related taxa, we suspect that modifications in single genes also underlie major innovations much deeper in the phylogeny. Subsequent genetic changes would be expected to obscure this fact, but less so in taxa retaining the primitive morphology than in the taxon with the diverged morphology, which undergoes genetic accommodation (10-12). Thus, it might be possible to recapitulate morphological transformations that occurred a long time ago by repeating the critical genetic change in an extant species with the ancestral morphology. The extraembryonic epithelia of higher flies provide an opportunity to functionally test this hypothesis.

Higher flies of the infraorder Schizophora, including Drosophila, develop a unique extraembryonic epithelium (the amnioserosa), which combines genetic and functional traits of the amnion and the serosa, two extraembryonic epithelia found in less derived flies and other insects (13, 14). Because the amnioserosa is a stable trait across Schizophora and has no direct equivalent in fly taxa that are basal to Schizophora, it represents an evolutionary novelty that originated deep in the phylogeny of flies. In Drosophila, the amnioserosa develops from a narrow strip of dorsal blastoderm into a squamous dorsal epithelium (15). Although the amnioserosa does not contribute embryonic tissue, it controls vital morphogenetic movements of the embryo, including germband retraction and dorsal closure. Germband retraction shortens the embryo, thereby aligning the folded germband along the anteroposterior axis, and dorsal

closure seals the embryonic epidermis along the dorsal midline. The amnioserosa mediates these movements through interactions with the underlying yolk sac and the germband (references in ref. 16, 17). Independent branches of cyclorrhaphan outgroups to Schizophora (lower Cyclorrhapha) develop a serosa and a dorsal amnion, suggesting that this extraembryonic tissue organization preceded the evolutionary origin of the amnioserosa (14). In lower cyclorrhaphan flies, a portion of the extraembryonic dorsal blastoderm folds over the germband. During germband extension, cells in the outer layer of this 'amnioserosal fold' (preserosa) stop dividing, become polyploid, and spread over the entire germband. Cells of the inner layer of the amnioserosal fold (preamnion) undergo polyploidization and flattening after the preserosa cells and disjoin from the leading edge of the preserosa to seal the embryo's dorsal side until dorsal closure is completed.

Results and Discussion

Recently, we proposed that the evolutionary transformation of the serosa and dorsal amnion into a single dorsal epithelium was achieved by suppressing the expansion of the preserosa and its disjunction from adjacent tissue in response to a temporal change in the expression of the homeobox gene *zerknüllt* (*zen*) (Fig. 3.1) (14). In holometabolous insects with an amnion and a serosa, *zen* is required to distinguish serosa cells from prospective amnion cells, and is continuously expressed in the developing serosa (14, 18). Extraembryonic *zen* expression in Drosophila differs temporally, in that it persists only until germband extension (stage 8), and spatially, in that it is expressed in all extraembryonic cells (19). Given these expression differences and the selectorgene-like function of *zen* for serosa cells in the context of amnion-competent tissue, the evolutionary transition from embryos with a dorsal amnion and a serosa to embryos with an amnioserosa could have been triggered by the precocious down-regulation of *zen* during germband extension in the preserosa of a schizophoran ancestor that lived about 100 million years ago. Provided that the distinction of serosa and

amnion cells at any developmental stage is dependent on the expression of *zen*, the temporal change in *zen* expression would have transformed the preserosa into a portion of the dorsal amnion, making the early distinction of amnion and serosa cells obsolete. This scenario suggests that the activation of *zen* in all extraembryonic cells of Drosophila (Fig. 3.1) was a consequence rather than a cause of the amnioserosa origin that may have stabilized the new morphology. The evolutionary mechanism that we propose for the origin of the amnioserosa predicts that down-regulation of *zen* during germband extension in a lower cyclorrhaphan taxon such as Megaselia is sufficient to transform the dorsal amnion and the serosa into a single amnioserosa-like epithelium, while prolonged *zen* expression in the amnioserosa of Drosophila would not be expected to recover the ancestral morphology because of secondary genetic changes in the lineage with the new morphology.

To examine the effect of prolonged *zen* expression in the amnioserosa, we crossed transgenic Drosophila lines carrying UAS-*zen* or UAS-*lac*Z (control) transgenes with Gal4 lines that drive expression of UAS-transgenes in the amnioserosa after stage 8, when endogenous *zen* is repressed in the amnioserosa. We stained offspring after germband retraction with an antibody against Hindsight, which labels the amnioserosa nuclei (20). Significant differences in cell number and cell size between the amnioserosa of these embryos and wildtype controls were not observed. In additional control experiments, we found that, unlike *lac*Z reporter transcripts (Fig. 3.2A, B) and endogenous *zen* transcripts (Fig. 3.2C), ectopic *zen* expression was repressed in the cytoplasm and nuclei of embryonic cells as well as in the cytoplasm of amnioserosa cells (Fig. 3.2D; Supplementary Fig. S3.1). This result suggests that *zen* repression in the late amnioserosa involves a mechanism that affects transcript stability. Consistent with this hypothesis, we identified sequence in the 3'UTR of *zen* mRNA (TTTTGAGTTAAAAAAGTTATA), which could be part of an unknown microRNA or a small interfering RNA precursor (Supplementary Fig. S3.2) (21, 22).
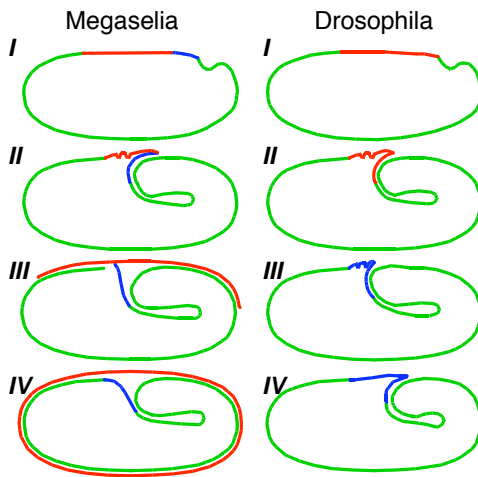
Fig. 3.1. Temporal and spatial differences of *zen* expression between *Megaselia* and *Drosophila*. Successive developmental stages are sketched with *zen*-expressing extraembryonic tissue depicted in red and other extraembryonic tissue depicted in blue. Anterior is left and dorsal up. In *Drosophila*, a second copy of *zen*, *z2*, is expressed in the same pattern (*26*). It has been shown that *z2* is dispensable for development (*27*) but a redundant function with *zen* has not been ruled out since the available null mutation of *zen* also removes *z2*.

Fig. 3.2. *zen* overexpression experiments in *Drosophila*. (A-D) Embryos heterozygous for *3734-Gal4* and *UAS-lac*Z stained with a *lac*Z antisense probe (A, B), or *3734-Gal4* and *UAS-zen* stained with 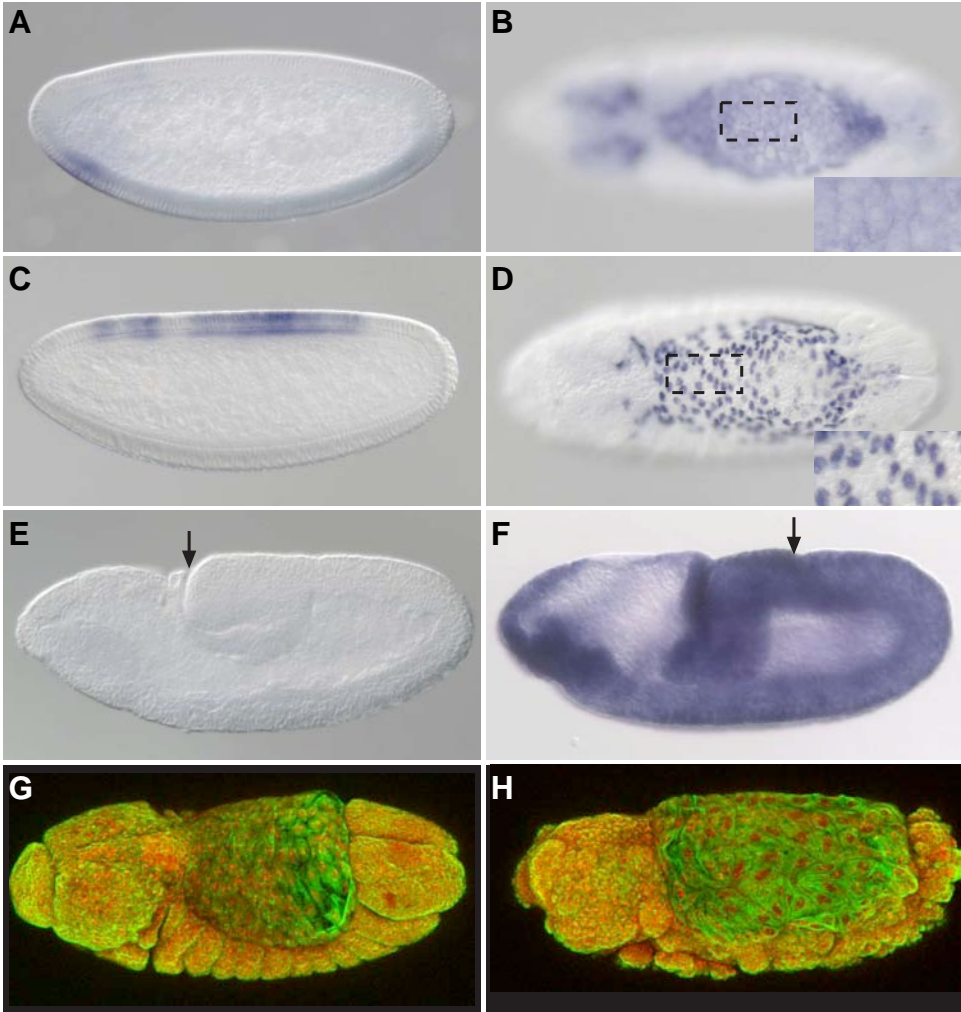a *zen* antisense probe (C, D). Note that endo-genous *zen* expression is cytoplasmic (C), whereas ectopic expression of *zen* in the amnioserosa is restricted to nuclei (D). (E, F) *Drosophila* w^{1118} embryos without (E) and homozygous for the *hsp70-zen* transgene (F) after a 45-minute heat shock stained with a *zen* antisense probe. Note differences in the extension of the pseudo-amnioserosal fold (arrow). (G, H) Drosophila w^{1118} embryos without (G) and homozygous for the *hsp70-zen* transgene (H) after a 90-minute heat shock followed by 7 hours at 25 ^{O}C stained with anti-tubulin antibody (green) and DAPI (red). Note the large size of cells and nuclei in the embryo carrying the *hsp70-zen* transgene. Embryos are shown in lateral (A, C, E, F), dorsal (B, D, H) or dorsolateral (G) view with anterior to the left.

To effectively override *zen* repression in the mature amnioserosa, we used an available heat shock construct (*hsp70-zen*) that lacks the endogenous 3' UTR of *zen* mRNA (23). We generated five independent homozygous *hsp70-zen* lines and selected two of them, which after heat shock treatment yielded strong ubiquitous *zen* expression and indistinguishable phenotypes. Unlike in control embryos lacking the *hsp70-zen* transgene, a 45-minute heat shock in the *hsp70-zen* lines administered to embryos at late stage 7 or early stage 8 (i.e. during germband extension) caused ubiquitous ectopic *zen* expression. In embryos that had reached stage 9, when *zen* expression in wildtype embryos is already repressed, we observed the formation of a prominent pseudo-amnioserosal fold (Fig. 3.2E, F). In a second set of experiments, we doubled the length of the heatshock and allowed embryos with and without the *hsp70-zen* transgene to develop until cuticle formation. All transgenic embryos failed to hatch and ~70% exhibited the tail-up phenotype indicative of defective dorsal closure often together with head involution defects (Supplementary Fig. S3.3). In contrast, most control embryos developed normally and hatched with only ~20% exhibiting a tail-up phenotype. In a third set of experiments, we allowed *hsp70-zen* and control embryos to develop after the 90-minute heat shock for 7 hours at 25°C, and stained them with an antibody against tubulin to visualize mircotubuli 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) (24), but the size of amnioserosa cells and nuclei was dramatically increased (Fig. 3.2G, H). Unlike in previous experiments with the same *hsp70-zen* construct in a transgenic line that is no longer available (23), 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, we may have prevented an expansion of the amnioserosa primordium in early embryos by precisely controlling the developmental stage of all embryos subjected to the heat shock procedure (see Materials and Methods). Our experiments show that prolonged *zen* expression causes an expansion of amnioserosa cells. However, the effect of prolonged extraembryonic *zen* expression in Drosophila appeared to be

limited. Serosa formation was not observed, and the edge of the amnioserosa did not disjoin from adjacent tissue, suggesting that Drosophila may have lost the ability to generate a complete serosa.

In Megaselia, repression of *zen* throughout development by RNA interference (RNAi) results in the formation of a dorsal amnion and the complete absence of the serosa (14). But this experiment does not mimic the evolutionary change of *zen* expression because species with and without an amnioserosa express *zen* during early development. We therefore thought to delay the effect of *Mab-zen* RNAi in Megaselia until the developmental stage when the amnioserosal fold has formed. First, we examined by whole mount in situ hybridization the time course of *Mab-zen* mRNA degradation following RNAi. We found that RNAi occurred within less than 10 minutes following injection of *Mab-zen* double-stranded RNA (dsRNA) into the yolk, with cells close to the injection site responding faster than cells further away from it (Supplementary Fig. S3.4). With the beginning of gastrulation, cells no longer responded to the injection of dsRNA into the yolk. These observations suggest that the effect of RNAi was limited by the diffusion rate of the RNAi effectors rather than the decay rate of targeted mRNA, and that cells pinch off from the yolk sac with gastrulation, thereby preventing the uptake of dsRNA. When we injected *Mab-zen* dsRNA after the cellularization front of the blastoderm had reached the yolk sac, i.e. within minutes before the onset of gastrulation, and examined *zen* expression 30 minutes later, *zen* expression was absent in 66%, partially reduced in 24%, and wildtype in the remaining 10% of the embryos (n=56). When embryos injected in the same way were allowed to develop until germband retraction and stained with DAPI to visualize nuclei of the serosa, 54% of them lacked the serosa and contained only a single extraembryonic epithelium on the dorsal side (n=55). In the remaining embryos we detected serosa tissue. These experiments indicate a requirement of *zen* expression for serosa development after the beginning of gastrulation.

Second, to delay *Mab-zen* RNAi until the formation of the amnioserosal fold, equivalent to the stage when in Drosophila *zen* is shut down, we adapted a new peptide-based cell culture transfection system for

use in Megaselia embryos. *Mab-zen* dsRNA was coated with N-TER peptide (Sigma) to allow cellular uptake. To test whether N-TER coated dsRNA was taken up by preserosa cells, we injected the dorsal perivitelline space of gastrulating embryos with peptide-coated biotin-labeled dsRNA of *Mab-zen*, and analysed these embryos 30 minutes or 60 minutes after the injection using an antibody against biotin. After 30 minutes the biotin-labeled RNA was detected within cells of the embryo including most or all extraembryonic tissue (Fig. 3.3A, A'). In embryos that were fixed and stained one hour after the injection the intensity of the signal was generally higher (data not shown). We observed some variability in the extent of RNA uptake but all of the injected embryos showed at least some, and most (87%, n=31) significant, uptake (Fig. 3.3A, A'). To examine the effect of *Mab-zen* RNAi during germband extension, we repeated this experiment with unlabeled *Mab-zen* dsRNA. We examined the expression of *Mab-zen* mRNA 31-37 or 49-54 minutes after the injection. 10% of the early batch (n=20) and 29% of the later batch (n=49) showed significant loss of *zen* transcripts (Fig. 3.3B, B'). Another batch of embryos injected with peptide-coated *Mab-zen* dsRNA was allowed to develop for seven hours after the injection and was then analyzed using antibody against Tubulin and DAPI. In about a quarter of these embryos, only a single extraembryonic epithelium had formed on the dorsal side (n=37) (Fig. 3.3C, C'). The same result was obtained with a batch of embryos (n=19) that was injected after the amnioserosal fold had formed.

To genetically characterize the extraembryonic epithelium that resulted from *Mab-zen* RNAi during germband extension, we examined the expression of *Megaselia pannier* (*Mab-pnr*) and *Megaselia Krüppel* (*Mab-Kr*) in transfected embryos that were also stained for *Mab-zen* expression. *Mab-pnr*, a marker of the dorsal epidermis and amnion (14), was expressed in preserosa cells in which *Mab-zen* was repressed (Fig. 3.4A-B''). Conversely, *Mab-Kr*, a marker for the preserosa (14), was repressed in preserosa cells that had lost cytoplasmic *Mab-zen* expression (Fig. 3.4C-D). At later stages, i. e. after the distal edges of the preamnion and preseosa have disjoined, when endogenous *Mab-Kr* is activated in the amnion (Fig. 3.4E-F), *Mab-zen* RNAi embryos always expressed *Mab-Kr* in the extraembryonic

tissue, even when *Mab-zen* was repressed throughout development (Fig. 3.4G). In Drosophila, *Kr* expression in the amnioserosa follows the same time course as amniotic *Mab-Kr* expression in Megaselia, being activated in the amnioserosa after *zen* expression is shut down (Fig. 3.4H-J) (25). The similarity of extraembryonic *Kr* expression in Drosophila and *Mab-zen* RNAi embryos of Megaselia supports the hypothesis that the amnioserosa evolved by transforming the preserosa into a portion of the dorsal amnion. To gain a more detailed understanding of the degree of similarity that persists to the present day between late amnioserosa cells in Drosophila and amniotic cells in Megaselia, as well as between early amnioserosa cells in Drosophila and preserosa cells in Megaselia, it will be necessary to systematically compare the extraembryonic functions of 'amnioserosa genes' of Drosophila and their Megaselia homologs.

The experiments described in this paper demonstrate that *zen* expression after formation of the amnioserosal fold is essential for the disjunction of the preserosa from adjacent tissue and its expansion over the Megaselia germband. Suppression of this late *Mab-zen* expression makes the preserosa indistinguishable from amniotic cells and similar to the late amnioserosa of Drosophila. Therefore, the loss of late *zen* expression can explain the reorganization of amnion and serosa into an amnioserosa-like epithelium in evolution. In summary, we have been able to functionally validate a genetic mechanism for the evolutionary origin of a novel morphology deep within the phylogenetic tree of the insect order Diptera. Our example suggests that pinning down simple genetic changes that underlie big morphological differences between higher taxa is possible when the preceding morphology survives, as is often the case (10, 11), in some extant species, and experimental tests are carried out in an experimental system that represents the primitive condition.
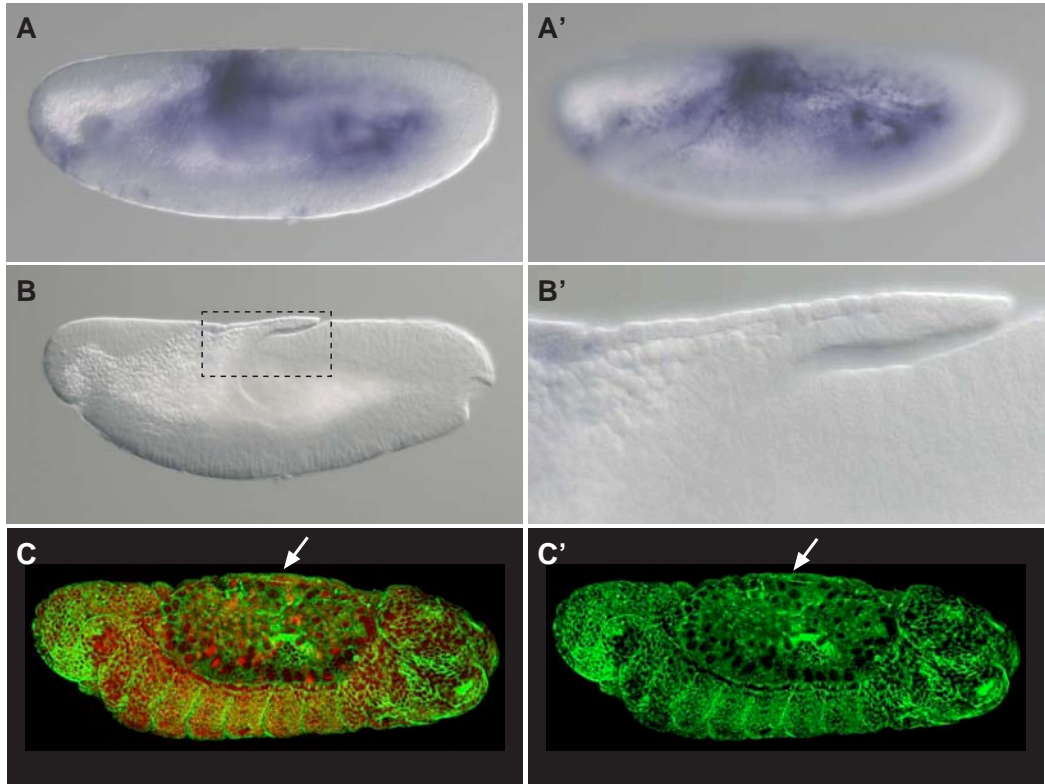
Fig. 3.3. *Mab-zen* RNAi in *Megaselia* germbands equivalent to stage 8 in *Drosophila* causes the formation of a dorsal extraembryonic epithelium. (A, A') *Megaselia* embryos transfected with biotin labeled *Mab-zen* dsRNA stained after 30 minutes with an antibody against biotin. (B, B') *Megaselia* embryos transfected with *Mab-zen* dsRNA stained after 60 minutes with a *Mab-zen* antisense probe. Note almost complete absence of *Mab-zen* transcripts and arrested amnioserosal fold, blowup in (B') corresponding to dashed window in (B). (C-C') *Megaselia* embryos transfected with *Mab-zen* dsRNA stained after germband retraction with anti-tubulin antibody (green) and DAPI (red). The vitelline layer was removed after confirming the absence of a serosa using DAPI staining. Note a single dorsal extraembryonic tissue is formed (Arrow). Lateral (A-B') and dorsolateral (C, C') views are shown with anterior to the left.
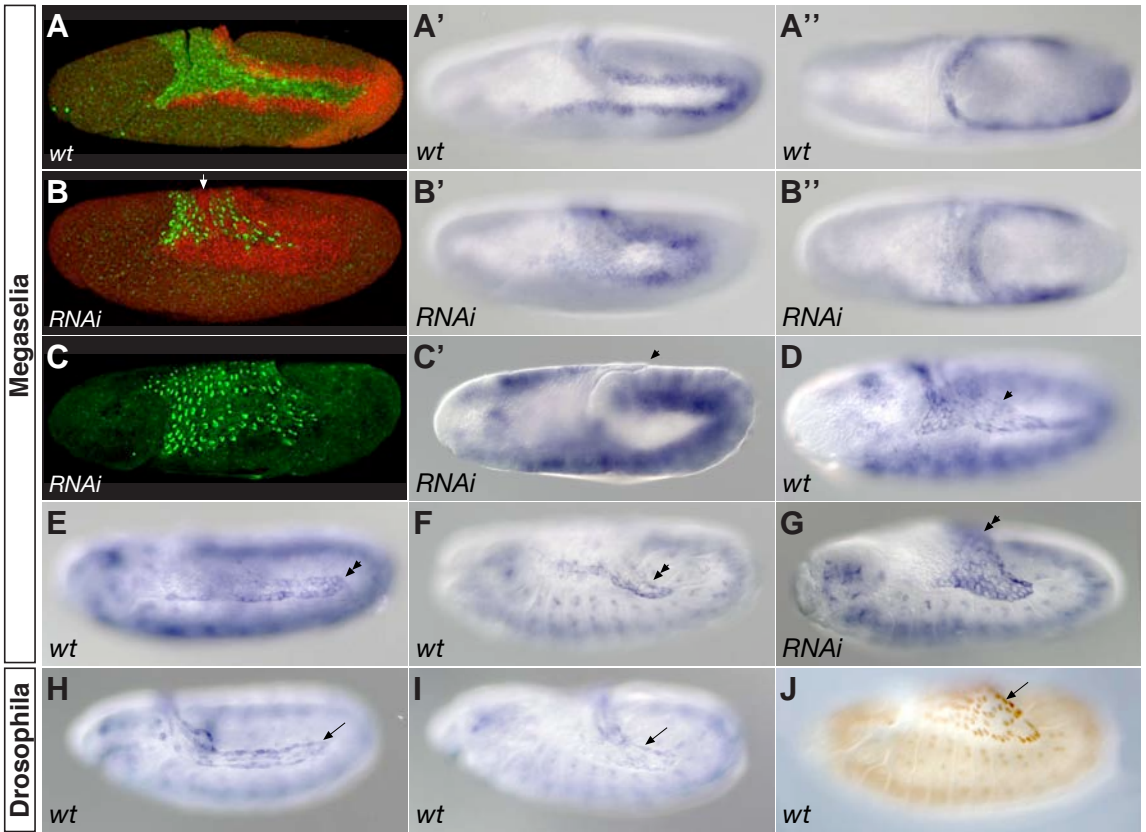
Fig. 3.4. Continuous *Mab-zen* requirement for serosa development. (A-B'') Expression of *Mab-zen* (green) and *Mab-pnr* (red, A', A'', B', B'') in wiltype (A-A'') and transfected (B-B'') *Mab-zen* RNAi embryos at the beginning of amnioserosal fold formation. Fluorescence at the posterior pole is background due to incomplete devitellinization. Note partial repression of *Mab-zen* and concomitant ectopic expression of *Mab-pnr* (white arrowhead). Cytoplasmic repression of *Mab-zen* precedes repression in nuclei (data not shown). (C, C') Transfected embryo showing strong nuclear but reduced cytoplasmic expression of *Mab-zen* (C), and repression of extraembryonic *Mab-Kr* expression (C', black arrowhead). (D) Wildtype *Megaselia* embryo showing *Mab-Kr* expression in the preserosa (black arrowhead). (E, F) *Mab-kr* expression in wildtype embryos after the completion of germband extension (E) and during germband retraction (F) showing expression in the amnion (double arrowheads). (G) *Mab-Kr* expression during germband retraction following early *Mab-zen* RNAi. (H-J) Transcript (H, I) and protein (J) distribution of *Kr* in the amnioserosa (arrow) of *Drosophila*. Lateral views with dorsal up and dorsal views (A'', B'') are shown with anterior to the left and dorsal up.

Materials and Methods

Drosophila fly work

Females of the Gal4 driver lines w^{1118}; P{w(+mW.hs)=GawB}c381 (Bloomington Stock Center) and Kr-Gal4 (26) were crossed with males of lines carrying either *UAS-lacZ·NLS* (Bloomington Stock Center) or *UAS-zen* (27) transgenes. 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 (23) was used to generate homozygous *hsp70-zen* insertions on the second and third chromosome. For heat shock experiments egg depositions were collected over 30 minutes 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.

RNAi experiments in Megaselia

Injections of *Mab-zen* dsRNA prior to gastrulation were done as described (14). For perivitelline injections, 0.78µl of 5 µM *Mab-zen* dsRNA was diluted with 2.22µl siRNA dilution buffer of the N-TER™ Nanoparticle siRNA Tansfection System from Sigma. 0.48 µl N-TER™ peptide was diluted with 2.52 µl RNase free water. Both solutions were mixed and incubated at room temperature for 20 minutes. The resulting nanoparticles of N-TER™ peptide and *Mab-zen* dsRNA were stored on ice until injection. Injections into the dorsal perivitelline space were carried out under oil from the ventral side, i.e. through the dechorionated embryo.

In situ hybridization, immunocytochemistry and microscopy

Drosophila embryos were fixed by Slow Formaldehyde Fixing Method (28). Rat monoclonal Tubulin antibody YOL1/34 (29) and mouse monoclonal Hindsight antibody 27B8-1G9 (20) were used in 1/100 and 1/10 dilutions, respectively. Megaselia embryos were heat fixed as described (28) with modifications. The embryos were treated with a boiling solution of 0.7%

NaCl and 0.05% Triton-X100 followed by a heptane and methanol devitellinization step. Postfixation was done with 5% formaldehyde in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.1% Tween20 pH 7.4) and was followed by a second heptane and methanol devitellinization step. In situ hybridization and cuticle preparations were done as described (14, 30).

Acknowledgements

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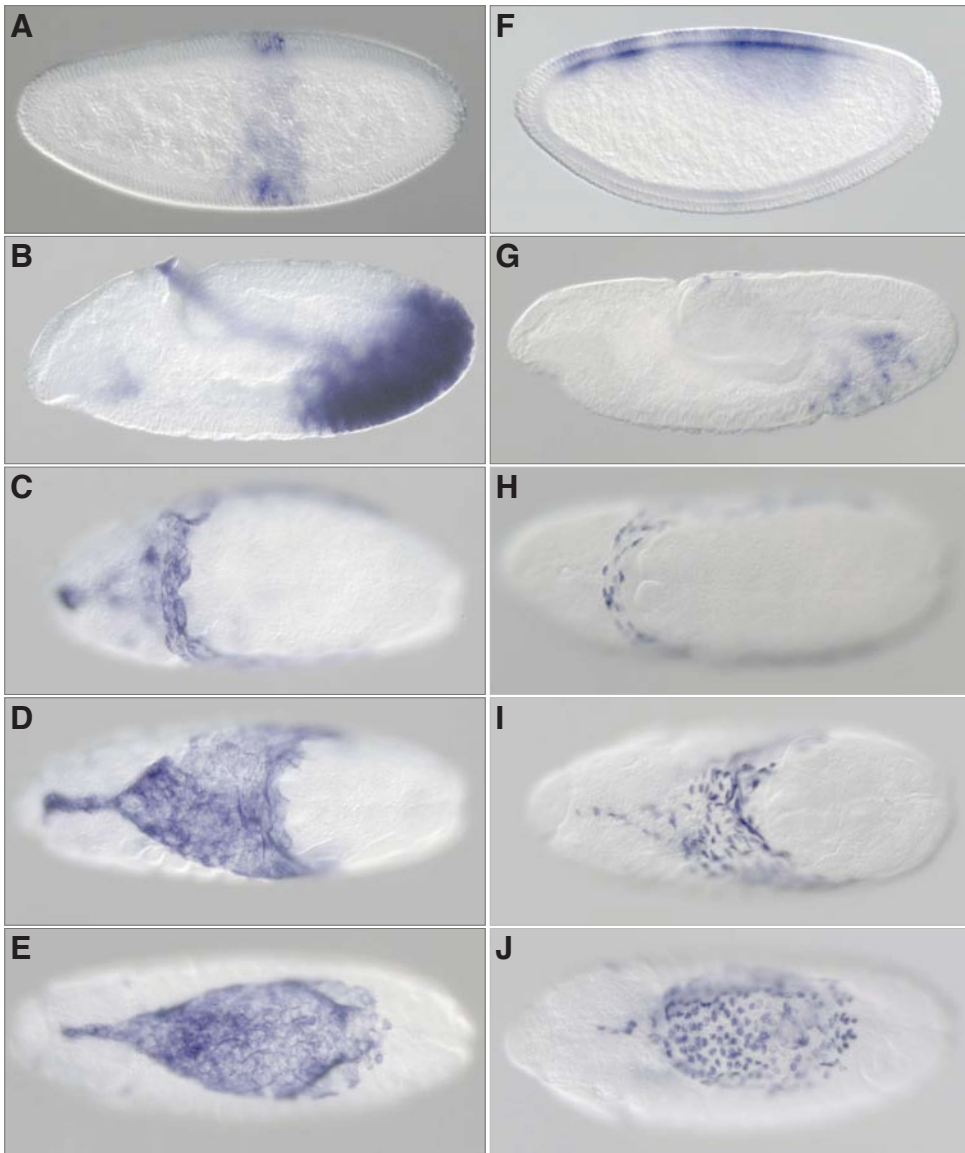
Fig. S3.1. *zen* overexpression with *Kr*-Gal4 and UAS-*zen*. (A-E) Embryos heterozygous for *Kr*-Gal4 and UAS-*lacZ* stained with a *lacZ* antisense probe. (F-L) Embryos heterozygous for *Kr*-Gal4 and UAS-*zen* stained with a *zen* antisense probe. Successive developmental stages are shown with anterior to the left in lateral view with dorsal up (A, B, F, G) or dorsal view (C-E, H-J). Note suppression of cytoplasmic *zen* expression in the amnioserosa, and of cytoplasmic and nuclear *zen* expression in the embryo.

A Nucleotides on Chromosome 2L identical to *zen* 3'UTR (Chromosome 3R)

5'TTTTGAGTTAAAAAAGTTATA

Putative stem loop in the 2L region

```
        10          20          30            40
  5'.-UUAU     C    U    AGU               U   UG-  G---    C
        AUAG UUUU UUUG   UAAAAAAGUUA AU    AU      UGG U
        UGUU AAAA AAAU   GUUUUUUCAGU UA    UA      ACC G
  3'\ ----    C    U    G--           C   UAA  AACA   A
                80          70          60          50
```
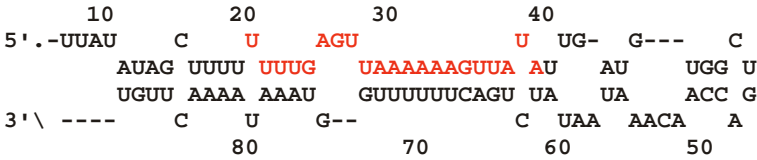
B Nucleotides in zen 3'UTR complimentary to *kuzbanian* (*kuz*) intron 2

5'TTTGAGTTAAAAAAGTTA

Putative stem loop in *kuz* intron

```
         150         160         170          180
  5'.-UU    U         UUU    UAU      U-   U-   UCUUU--    A
        UUU GGUUAAAG     UUG   UGUUG  UG   UA        UGC U
        AAA UCAAUUUU     AAU   ACAAU  AU   GU        ACG G
  3'\ --    C         UUC    UC-      UU   UU   UGCAUUU    U
        230         220         210          200         190
```
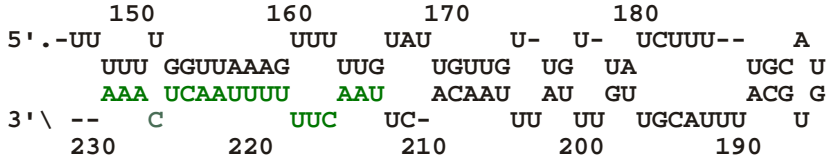
Fig. S3.2 MicroRNA/siRNA candidates mediating *zen* transcript degradation in *Drosophila*. (A) 21 nucleotides identical to *zen* 3'UTR on chromosome 2 are part of a putative stem loop structure. (B) 18 nucleotides in *zen* 3'UTR are part of a putative stem loop structure in the second intron of *kuzbanian*. Stem-loops have been predicted using the Mfold program (1).

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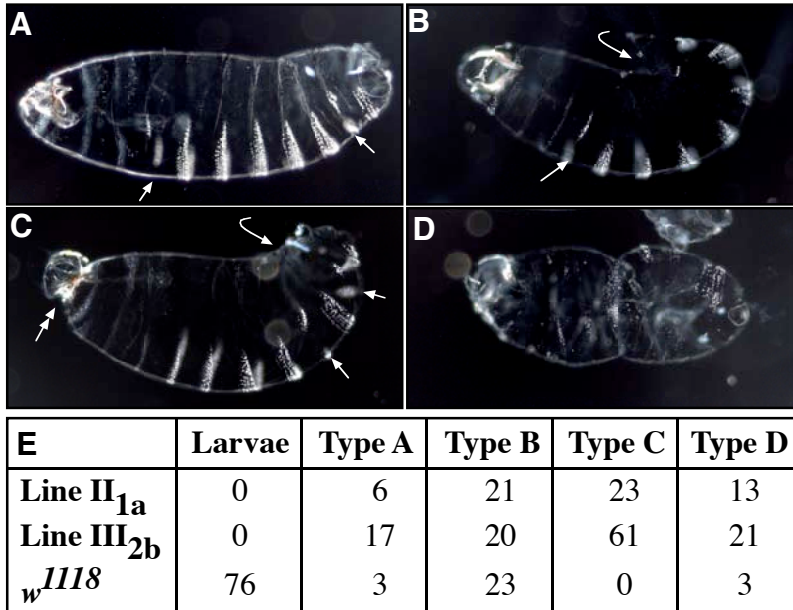| E	Larvae	Type A	Type B	Type C	Type D
Line II$_{1a}$	0	6	21	23	13
Line III$_{2b}$	0	17	20	61	21
w^{1118}	76	3	23	0	3

Fig. S3.3 Heat shock experiments in *Drosophila*. (A-D) Cuticles of *hsp70-zen* larvae heat shocked for 90 minutes starting at germband extension. Type A (A) shows minor segmentation defects (arrows). Type B (B) shows segmentation defects and u-shaped morphology. Type C (C) shows in addition head involution defects. Type D (D) exhibits severe non-specific defects. (E) Frequencies of occurrence of different phenotypes in heat shocked wildtype controls, and two independent *hsp70-zen* transgenic lines.

Fig. S3.4 Limits of conventional RNAi in *Megaselia*. (A) Blastoderm embryo injected during cellularizytion with *Mab-zen* dsRNA, fixed after 10 minutes, and stained with a *Mab-zen* antisense probe. (B, C) Early gastrulating embryos injected with *Mab-zen* dsRNA, fixed after 10 minutes (B), and 20 minutes (C), and stained with a *Mab-zen* antisense probe. Injection sites are marked (asterisks). Note that completion of cellularization at the beginning of gastrulation prevents RNAi, and that cytoplasmic transcript responds faster to RNAi than the nuclear transcript (arrow).
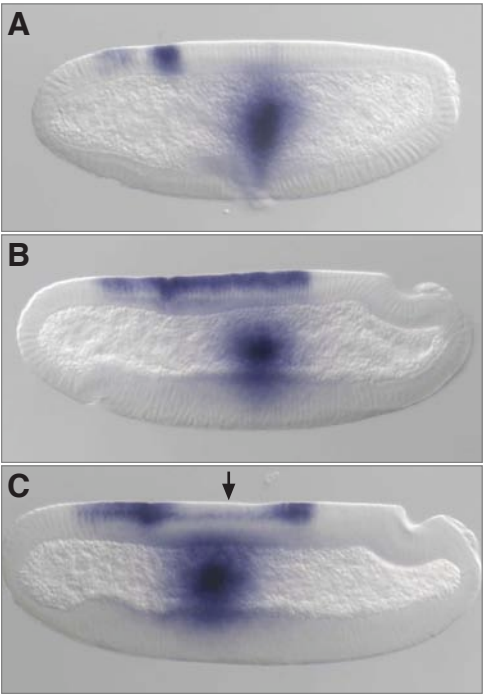
Chapter 4

Seven *zen*-like genes in the Hox3 locus of the hover fly *Episyrphus balteatus* (Syrphidae)

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Submitted

Chapter 4: *Zerknüllt* (*zen*) locus of *Episyrphus balteatus*

Seven *zen*-like genes in the Hox3 locus of the hover fly

Episyrphus balteatus (Syrphidae)

Abstract

The Hox 3 locus of *Drosophila* includes three genes, *zerknüllt* (*zen*), *zerknüllt 2* (*z2*), and *bicoid* (*bcd*). Phylogenetic surveys suggest that all three genes evolved from a single *zen*-like progenitor, with the origin of *bcd* in the early lineage of higher flies (Cyclorrhapha) preceding the duplication that resulted in *zen* and *z2*. Zen activity is required for specifying the extraembryonic anlage on the dorsal side of the blastoderm. In contrast, *bcd* activity is necessary and sufficient for development of anterior structures. In many species without *bcd*, anterior blastoderm contributes to the extraembryonic serosa, suggesting that the origin of *bcd* caused a relocation of the extraembryonic anlage in evolution. Recently, we discovered a cyclorrhaphan fly, *Episyrphus balteatus* (Syrphidae), in which the serosa anlage and *zen* expression extend to the anterior pole. This observation raises the question, whether *bcd* is modified or absent in *E. balteatus*. To address this question, we cloned and sequenced ~79 kbp of the Hox3 locus of *E. balteatus*. We discovered 6 new *zen*-like genes upstream of *E. balteatus zen* (*Eba-zen*). Four of these genes are expressed during early development, two of which contribute to the specification of serosa cells. None of the six genes exhibited similarities to *bcd*.

Introduction

The Hox-gene-complex of most insects contains 10 paralogous homeobox genes, most of which function in the specification of segment identities during early development (1). The Hox3 gene of insects, *zerknüllt* (*zen*), is an exception. Most insects require *zen* activity for extraembryonic development rather than for specifying individual segments of the embryo (referenced in 2). In particular, higher insects (Holometabola) appear to require *zen* for all aspects of development of serosa, an extraembryonic epithelium that envelops the embryo. For example, in embryos of the red flour beetle Tribolium (3) or lower cyclorrhaphan flies (*Episyrphus*, *Megaselia*) (4) that have been subjected to *zen* RNAi, all extraembryonic cells are indistinguishable from cells of the amnion, a distinct extraembryonic epithelium that develops from the margins of the embryonic rudiment (5). Tribolium contains two copies of *zen* (*Tc-zen1*, *Tc-zen2*), both of which are expressed in the serosa. However, *Tc-zen2* expression in the serosa is dispensable and dependent on *Tc-zen1*. After germband retraction, *Tc-zen2* is activated in the amnion (where *Tc-zen1* is never expressed) and controls the fusion of the amnion with the serosa, which precedes dorsal closure. In *Drosophila*, in which serosa and amnion are combined into a single extraembryonic epithelium (amnioserosa), *zen* controls all extraembryonic development. In addition, *zen* splits the eye field into left and right optic lobes (6), and may function in a subset of the primordial germ cells (pole cells), where it is also expressed (7). It has been shown that *z2,* a copy of *zen* with very similar expression, is dispensable in the presence of *zen* (8, 9). The possibility that *zen* is likewise dispensable in the presence of *z2* has not been ruled out as mutations deleting only *zen* have not been described. Hence, both genes might function in a redundant manner. *zen* and *z2* are conserved across the genus *Drosophila* (10) but this gene-pair evolved independently of the duplication that generated *Tc-zen1* and *Tc-zen2* in

Tribolium (11). In the malaria mosquito and other dipterans only a single *zen* gene has been found (12-14), consistent with the assumption that a single copy of *zen* was present throughout most of the dipteran radiation.

In addition to *zen* and *z2*, the Hox3 locus of *Drosophila* contains a second type of class 3 Hox gene, *bicoid* (*bcd*), ca. 2 kb upstream of *zen* (15). This gene is necessary and sufficient to induce the formation of a complete embryonic head (16-21), and contributes to the activation of many segmentation genes in the trunk (reviewed in 22, 23). Maternal mRNA of *bcd* is localized at the anterior pole of the egg and translated in the syncytial embryo, in which Bcd forms a gradient with maximum concentration at the anterior pole. Bcd-deficient embryos lack the head and the thorax, exhibit variable segmentation defects in the abdomen, and develop a posterior abdomen with inverted anteroposterior polarity at the anterior pole. Conversely, injection of *bcd* mRNA into the posterior pole of early embryos can induce a mirror image duplication of the head and thorax. Thus, *bcd* functions as anterior determinant of the *Drosophila* embryo and does not share any known functions with *zen* or *z2*.

Expression data and RNAi experiments in the housefly *Musca* and *Megaselia* indicate an essential role of *bcd* in specifying head development across a wide range of cyclorrhaphan flies (24-27). The genomes of non-cyclorrhaphan insects seem to lack *bcd* but contain Hox3 genes of the *zen* type. Thus, *bcd* originated in the cyclorrhaphan lineage evolving most of its developmental functions *de novo*. Previously, we proposed that with the emergence of *bcd* the extraembryonic anlage was relocated from an anterior-dorsal to a strictly dorsal portion of the blastoderm, which is characteristic of cyclorrhaphan flies (14, 28). More recently, however, we discovered a cyclorrhaphan fly, *E. balteatus* (Syrphidae), with an anterior-dorsal serosa anlage (4), raising the question whether in this species *bcd* is absent or modified. PCR experiments with degenerate *bcd* primers did not yield any *bcd*–like sequence (S. Lemke, M. Stauber, U. Schmidt-Ott, manuscript in

preparation). Given that a diverged *bcd* homologue could have escaped detection by PCR, we initiated a genomic walk starting out from *Eba-zen* and focusing on its upstream region, where *bcd* is located in other flies (10, 11). Here, we describe a 79 kb contig including *Episyrphus zen* (*Eba-zen*) and six paralogs, none of which bears similarity to *bcd* in sequence, expression or function. Our results are consistent with the hypothesis that *E. balteatus* lacks *bcd*.

Results and Discussion

A 79 kb contig with seven *zen* homologues

Screening of a BAC Library of *E. balteatus* with *Eba-zen* yielded two clones, IIc1B (51 kbp insert) and IVa4C (46 kbp insert). The longer clone, IIc1B, was fully sequenced, clone IVa4C was sequenced partially. Downstream of *Eba-zen*, the ends of the two clones coincided. At the other end, IVa4C (although ca. 5 kbp shorter than IIc1B) extended beyond IIc1B by about 1.6 kbp, indicating polymorphism between the two clones. Gene prediction indicated in total 16 genes in the sequence of IIc1B, out of which 11 (with predicted coding sequence between 57 and 585 bp) did not show significant similarity to any known gene or other DNA sequence in the NCBI database (as of February 2008). One of the predicted genes exhibited homology to *CG11686* of *Drosophila melanogaster* (E value = 9.9 e-05) (Supplementary Fig. S4.3). Three of the newly predicted genes, all upstream of *Eba-zen*, showed significant similarity to *Eba-zen* and other *zen* homologues. These genes were named *Eba-z2*, *Eba-z3*, and *Eba-z4* (Fig. 4.1).

The coding sequence of *Eba-z4* was used to isolate a genomic phage clone (ph-625) from a Lambda-Fix II phage library of *E. balteatus*. The insert of this clone (15.5 kbp) provided 12.4 kbp of new sequence. Gene prediction indicated five additional genes in this sequence of which two with short coding

sequences (132 bp and 234 bp) did not show any similarity to known genes or DNA sequences. The other three predicted genes showed significant similarity to *zen* homologues and were named *Eba-z5, Eba-z6,* and *Eba-z7,* respectively (Fig. 4.1). In addition, we detected a partial homeodomain sequence related to *Eba-z3* and *Eba-z6,* interrupted by several stop codons, to which we refer as pseudogene (*Eba-zps*). Downstream of the homeodomain, *Eba-z2*, *Eba-z3* and *Eba-z6* show some sequence conservation among themselves, which is not conserved in *Eba-zen* or *zen* homologues from other species. Conversely, two conserved motifs that are found in *zen* homologues of most other flies (A-box, B-box) are not conserved in any of the *Eba-z* paralogues (Supplementary Fig. S4.1, data not shown). In pair-wise comparisons all *zen* related sequences in the contig showed higher conservation at the nucleotide level (23.3%-81.1%) than at the amino acid level (13.1%-68.4%; Supplementary Fig. S4.2), suggesting that the paralogues of *Eba-zen* diverged with limited, or no constraints.

Fig. 4.1. *zen* paralogues of *E. balteatus*. (A) Genomic organization of the ~79 kbp contig. Arrows indicate the orientation of individual genes. Triangles indicate introns (faded grey). (**B**) Homeodomain alignment of Zen and Bcd homologues from *Drosophila melanogaster* (Dme), *Megaselia abdita* (Mab), *Episyrphus balteatus* (Eba), *Lonchoptera lutea* (Llu), and *Platypeza consobrina* (Pco). The amino acid sequence of Eba-Zps has been obtained by omitting two small insertions between residues 39 and 40 (5 base pairs) and 51 and 52 (4 base pairs). Eba-Z6 coding sequence starts with the forth amino acid of the homeodomain. Eba-Z5 contains only a partial homeodomain. Identical (dark grey) amino acids are highlighted. Conserved differences in the amino acid sequence between Zen or Bcd homologues are shaded light grey and black, respectively. Amino acid positions are given above the alignment, and residues that make contact with the major grove (asterisks), the minor groove (circles) and the sugar-phosphate backbone (triangles) are indicated. (**C**) Homeobox tree. Relationships between the homeoboxes are depicted in an unrooted phylogram based on nucleotide sequences of cyclorrhaphan *zen* and *bcd* homologues. The tree was generated using PAUP (version 4) software. The optimality criterion was set to parsimony. Numbers indicate bootstraps. Bootstrap analysis was performed in 1000 replicates. Gaps were treated as missing data.
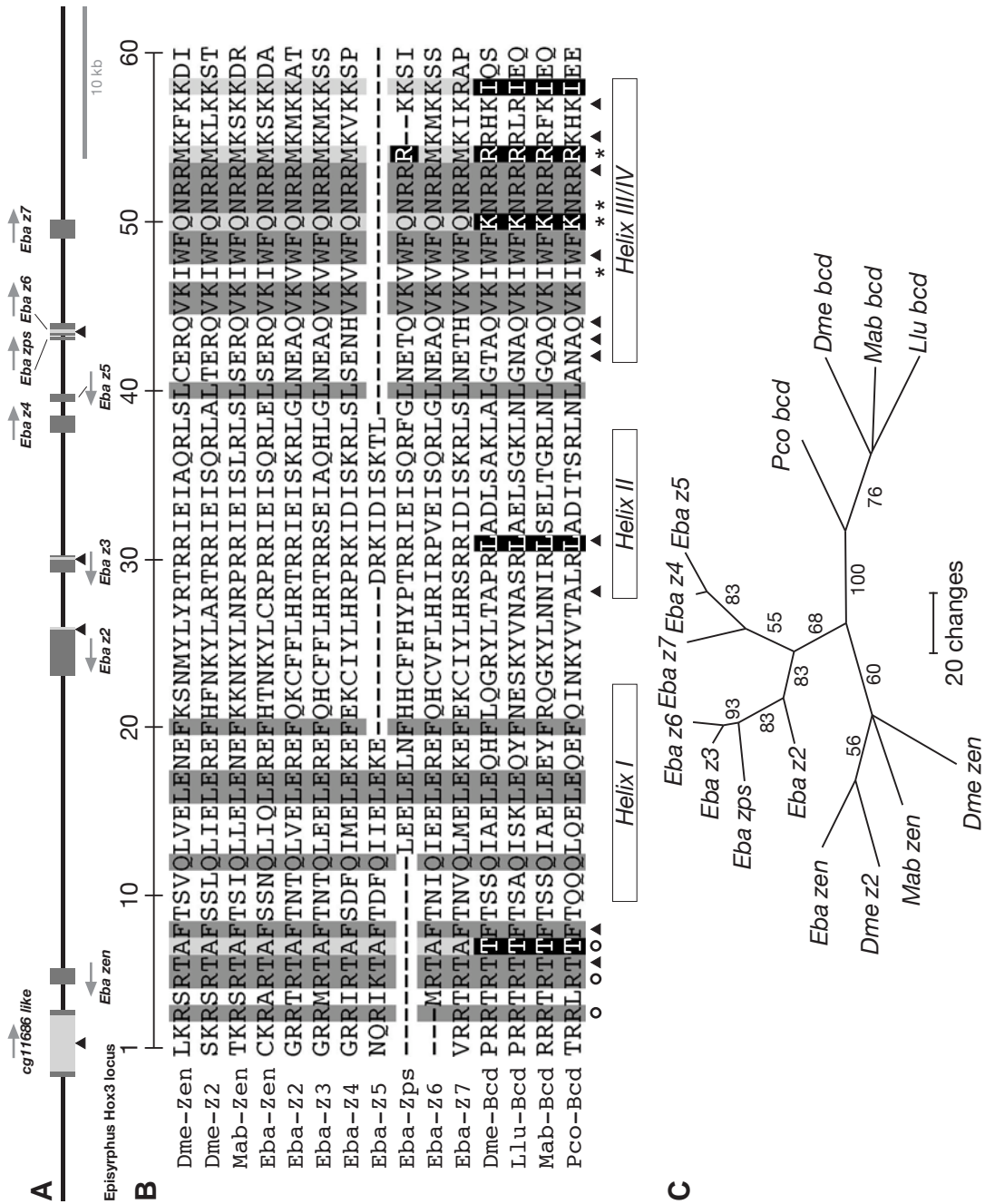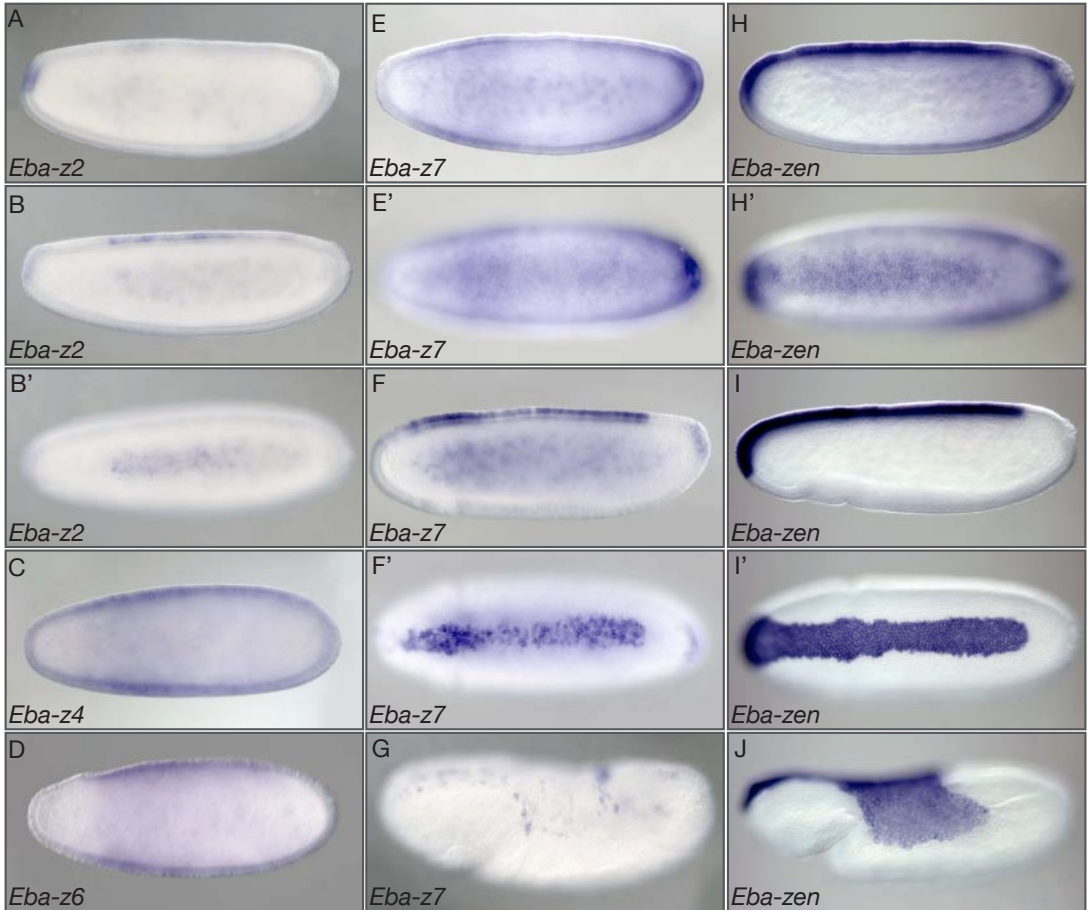
Fig. 4.2. Expression of *zen* homologues in *E. balteatus*. RNA in situ hybridizations with probes against (A-B) *Eba-z2*, (C) *Eba-z4*, (D) *Eba-z6*, (E-G) *Eba-z7*, and (H-J) *Eba-zen* whole mount embryos are shown in lateral (A-B, E-J) and dorsal view (B'-I', C and D). Anterior is left and dorsal is up in lateral views.

Using *Eba-z7* as probe we obtained BAC clone, 37K7. The complete insert sequence of 37K7 (18.8 kbp) overlapped with ph-625 by 5.1 kbp and revealed many polymorphisms, including a 1.8 kbp fragment between *Eba-z6* and *Eba-z7* that was present only in the BAC clone. Except *Eba-z7*, the 37K7 sequence included only very short predicted coding sequences (39 to 156 bp), which did not exhibit significant similarity to any known gene or DNA. The distal 7 kb of the 37K7 sequence contained repetitive and AT-rich sequence, and prevented further genomic walking. To test for linkage with *proboscipedia (Hox2)* or *Deformed (Hox4)*, we cloned partial cDNAs of *Episyrphus proboscipedia (Eba-pb)* and *Episyrphus Deformed (Eba-Dfd)* (Supplementary Fig. S4.3). None of these sequences overlapped with the *Eba-zen* contig.

In summary, we cloned and sequenced a ~79 kbp contig that contained in addition to *Eba-zen*, six *Eba-zen* paralogues, one *Eba-zen* related pseudogene, and at least one additional conserved gene of unknown function (Fig. 4.1A). None of the predicted protein sequences exhibited significant homology to Bcd or Bcd orthologues (Fig. 4.1B, C, and data not shown).

Expression and function of *Eba-zen* paralogues

Previously, we reported the expression and function of *Eba-zen* (4). To examine the expression of the newly identified *Eba-zen* paralogues, we performed whole mount *in situ* hybridization experiments on embryos. None of the transcripts could be detected in freshly laid eggs, indicating that these genes lack maternal expression. *Eba-z2* transcripts were detected during blastoderm cellularization but only in a few cells of the serosa anlage (Fig. 4.2A, B, B'). In older embryos, *Eba-z2* was not expressed. *Eba-z3 and Eba-z5* transcripts were not detected at any embryonic stage. *Eba-z4* and *Eba-z6* were weakly expressed in about 10% of the embryos at the syncytial blastoderm stage, suggesting that these genes might be subject to expression polymorphism (Fig. 4.2C-D). In embryos at earlier or later stages, *Eba-z4* and *Eba-z6* expression was not detected. *Eba-z7*

expression appeared at the onset of cellularization in the yolk nuclei and throughout the blastoderm except at the anterior pole (Fig. 4.2E, E'). In late blastoderms and early gastrulas, irregular expression was detected in the serosa anlage (Fig. 4.2F, F', G). In addition, we observed expression around yolk nuclei and in some of the pole cells. In summary, out of the six newly identified *Eba-zen* paralogues, only four (*Eba-z2*, *Eba-z4*, *Eba-z6*, *Eba-z7*) were expressed in embryos.

To examine the function of *Eba-z2*, *Eba-z4*, *Eba-z6*, and *Eba-z7*, we injected early embryos with double-stranded RNA of *Eba-z2*, *Eba-z4*, *Eba-z6* or *Eba-z7*, and scored embryos for serosa development. Embryos injected with double-stranded RNA of *Eba-z4* or *Eba-z6* were indistinguishable from wildtype embryos. *Eba-z2* RNAi or *Eba-z7* RNAi increased the frequency of discontinuous serosa sheets during germband extension (Fig. 4.3). However, defects in the mature serosa were not observed (*Eba-z2* n=23, *Eba-z7* n=28), indicating that early defects are compensated during later stages of development. Cuticles from *Eba-z2* (n=64) and *Eba-z7* (n=83) RNAi embryos showed no scorable defects in germband retraction, dorsal closure or rates of hatching. In summary, all *Eba-zen* paralogs appear to be dispensable for embryonic development but *Eba-z2* and *Eba-z7* contribute *zen* activity to developing serosa cells.

Evolutionary history of the Hox3 locus of *E. balteatus*

All *Eba-zen* paralogs originated most likely from a single copy of *Eba-zen* (Fig. 4.1C). Taking into account sequence similarities between the paralogs (Fig. 4.1C; Supplementary Figures S1, S2), and the relative positions of the paralogs in the contig, six duplications and two inversions can most parsimoniously explain the gene complex in its current form (Fig. 4.4). According to this model, the *Eba-z2/3/4/5/6/7* progenitor duplicated and gave rise to *Eba-z2/3/6* and an inverted *Eba-z4/5/7*, both of which duplicated to generate *Eba-z2* and *Eba-z3/6*,

and *Eba-z4/5* and *Eba-z7,* respectively. At that point, a tandem duplication of *Eba-z3/6* and *Eba-z4/5* concomitant with, or followed by an inversion of the distal copy could have generated the current gene complex. The pseudogene *Eba-zps* is most similar to *Eba-z3 and Eba-z6*. Based on its orientation and position in the contig it more likely constitutes a copy of *Eba-z6* than of *Eba-z3*. In summary, recent gene duplications and inversions in the *E. balteatus* lineage may account for all Hox3 related genes in the contig.

Evidence for absence of *bcd* in *E. balteatus*

The absence of any *bcd*-like gene in the contig is surprising given the conserved position of *bcd* 2-16 kbp upstream of *zen* in other flies (10, 11). Likewise, it is surprising that degenerate PCR experiments in *E. balteatus* did not yield any *bcd*-like sequence because the same approach identified clear-cut *bcd* orthologues in three basal cyclorrhaphan taxa (Phoridae, Lonchopteridae, Platypezidae) (13, 24). Finally, the specification of serosa cells at the anterior pole of the *E. balteatus* blastoderm seems to argue against the presence of high *bcd* activity at the anterior pole because such activity would be expected to induce head development. Given that the three data sets are independent we speculate that *E. balteatus* lacks the *bcd* gene. The most widely accepted dipteran phylogeny places *E. balteatus* in the sister taxon of, or at least close to higher Cyclorrhapha (Schizophora) (29, 30). Accordingly, *bcd* may have been lost in the *E. balteatus* lineage. Alternatively, the *E. balteatus* lineage split from other Cyclorrhapha before the origin of *bcd* and would a priori lack this gene. A reassessment of the phylogenetic position of *E. balteatus* relative to other Cyclorrhapha will be necessary to answer this question.
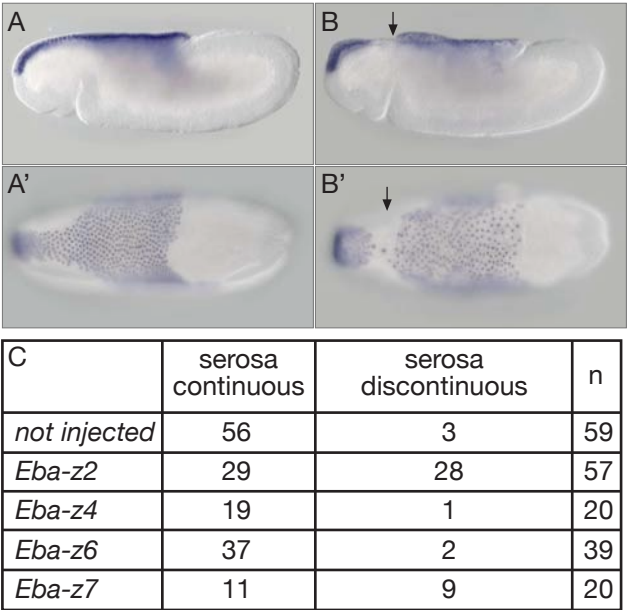
Fig. 4.3. RNAi phenotypes of *Eba-z2, Eba-z4, Eba-z6* and *Eba-z7*. (A-B') Alternative *Eba-zen* expression patterns in *Eba-z2* RNAi embryos. Anterior is left and dorsal up. In about half of the embryos undergoing germband extension *Eba-zen* expression was detected in a continuous sheet of serosa cells, indistinguishable from wildtype (A, A'). In the other half *Eba-zen* expression was discontinuous (B, B'). (D) Frequencies of continuous and discontinuous *Eba-zen* expression patterns in uninjected controls and RNAi embryos undergoing germband extension. Note that in later embryos *Eba-zen* expression was always continuous.
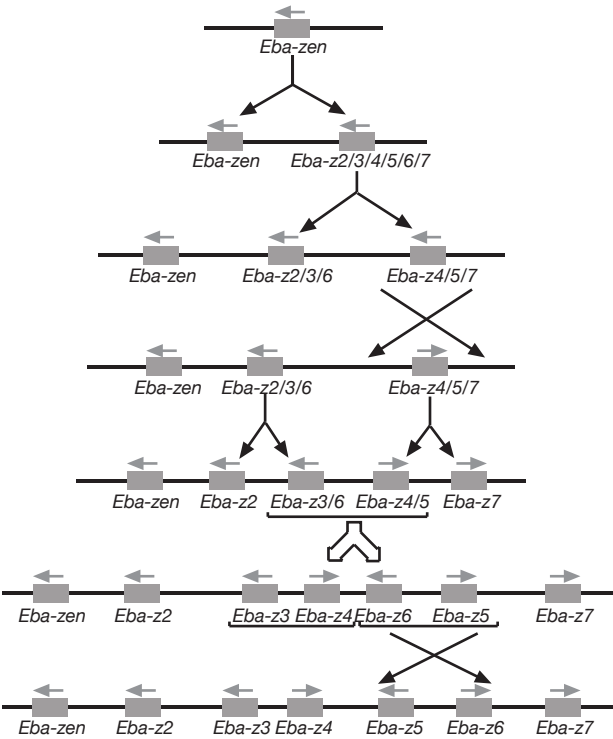
C	serosa continuous	serosa discontinuous	n
not injected	56	3	59
Eba-z2	29	28	57
Eba-z4	19	1	20
Eba-z6	37	2	39
Eba-z7	11	9	20

Fig. 4.4. Probable duplication and inversion events that led to the modified *zen* locus of *E. balteatus*. Grey rectangles indicate genes and arrows their orientation. Brackets designate tandem duplications and crossed arrows inversions.

Materials and Methods

Cloning

Genomic fragments of *Episyrphus* homologues of *proboscipedia (pb)* and *Deformed (Dfd)* were PCR-amplified from genomic DNA using degenerate primer pairs:

5' ACNGCNTAYACNAAYACNCA/ 5'RTTYTGRAACCANACYTTNAC for *pb* and 5'WSITAYCARCCBGGIATGGARC / 5'CKICKRTTYTGRAACCAR-ATYTTRAT for *Dfd*. To obtain longer cDNA fragments we used the cDNA amplification Kit SMART (Clontech) on cDNA that was prepared from 0-5 hour old embryos (collected at room temperature). Genomic DNA from the *E. balteatus* Degeer Hox3 locus was obtained from two Bacterial Artificial Chromosome (BAC) libraries and a Lambda-Fix II (Stratagene) library (primary titer 7.1 x 10^5, once amplified) packaged in Gigapack III XL-11 packaging extract (Stratagene) (31). The BAC libraries were constructed as described (32). Genomic DNA was prepared from ovaries and partially digested either with restriction enzyme *Bam*HI or with restriction enzyme *Hin*dIII. About 9000 size selected *Bam*HI inserts (40-70 kb) and the same number of *Hin*dIII inserts (10-50 kb) were cloned into pIndigoBAC-5 vector (Epicentre). The *Bam*HI BAC clones were spotted in a 4 x 4 pattern on nylon membrane using a Q-bot (Genetix) (32). *E. balteatus* material for the *Bam*HI BAC library and the Lambda-FixII library was collected in the surroundings of Göttingen (Germany). All other *E. balteatus* material was obtained from P. Katz (Katz Biotech AG, Baruth, Germany).

Screening and sequencing

The BAC libraries were screened using PCR pooling technique. 96 pools of 96 clones each were prepared from each BAC library. PCR screening was performed in 96-well format using the primer pair

5'GAGTCACCCTCACCGAATAAG/ 5'TGAAAGTTCACGCTGTGGAC within the *Eba-zen* open reading frame and the primer pair 5'AAACTATTTCCCGCCGCCTT/ 5'TGATCTTCACGGAAGGATTTCG within the *Eba-z7* open reading frame and 114 bp upstream of it. The spotted *Bam*HI BAC library was also screened with radio-isotope labeled probes. The lambda-FixII library was screened with a radio-isotope labeled probe for *Eba-z4* ORF following instructions of the Stratagene user manual. BAC clones IIc1B and IVa4C were shot-gun sequenced and assembled at Department of Genome Analysis, Braunschweig, Germany. The insert of the phage clone Ph-625 (15.5 kbp) and BAC clone 37K7 (18.8 kbp) were sequenced using stepwise sequence-walking.

Sequence analysis

Gene structures were predicted by the Fgenesh program using the Web server at http://kaikogaas.dna.affrc.go.jp (33). Gene trees were generated using PAUP (version 4) (34). Alignments were generated using Clustal method of MegAlign (DNASTAR) software.

In situ hybridization

For *in situ* hybridization, injected embryos were fixed as described (35) with modifications: The embryos were treated with a boiling solution of 0.7% NaCl and 0.05% Triton-X100 and a subsequent heptane and methanol devitellinization step. This procedure was followed by a 5% formaldehyde fix in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.1% Tween20 pH 7.4) and a second heptane and methanol devitellinization step. In situ hybridization and RNAi was done as described (4). RNA probes were labeled with digoxigenin (Roche). The *z2* probe was complementary to 944 bases starting from position 74 (position 1 is the predicted translation start), the *z3* probe was complementary to 898 bases

starting from position -26, the *z4* probe was complementary to 727 bases starting from position 9, the *z6* probe was complementary to 672 bases starting form position 1, and the *z7* probe was complementary to 1002 bases starting from position -114. Double-stranded RNA was generated by combining sense and anti-sense strands spanning 859 bp of *z2* starting at position 160, 854 bp of *z3* starting at position 19, 727 bp of *z4* starting at position 9, 673 bp of *z6* starting at position 1, and 879 bp of *z7* starting at position 10.

Acknowledgments

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Homeodomain

Eba-Zen, Eba-Z2, Eba-Z3, Eba-Z4, Eba-Z5, Eba-Zps, Eba-Z6, Eba-Z7

A-box

B-box

Fig. S4.1. Alignment of the deduced amino acid sequences of *zen* homologues from *Episyrphus balteatus*. Identical amino acids are underlaid in yellow; dashes indicate gaps. The homeodomain is boxed in black. Two motifs of Eba-Zen that are conserved in dipteran Zen homologues of other species but not in Eba-Zen paralogs are boxed in red. Conserved regions between Eba-z2, Eba-z3 and Eba-z6 are boxed in blue. The numbers at the right margin refer to the last amino acid in each row.

Chapter 4: *Zerknüllt* (*zen*) locus of *Episyrphus balteatus*

Eba-z2 **Eba-Z2**	*Eba-z3* **Eba-Z3**	*Eba-z4* **Eba-Z4**	*Eba-z5* **Eba-Z5**	*Eba-zps* **Eba-Zps**	*Eba-z6* **Eba-Z6**	*Eba-z7* **Eba-Z7**	
28.5 **21.8**	*26.0* **19.8**	*28.2* **21.8**	*26.2* **16.7**	*23.3* **24.8**	*28.9* **23.2**	*29.7* **21.1**	*Eba-zen* **Eba-Zen**
	49.8 **43.0**	*30.9* **22.8**	*30.2* **21.4**	*38.6* **31.2**	*48.2* **48.2**	*30.9* **24.9**	*Eba-z2* **Eba-Z2**
		27.7 **22.1**	*25.4* **21.4**	*50.6* **35.5**	*81.7* **68.4**	*26.5* **20.6**	*Eba-z3* **Eba-Z3**
			45.2 **35.7**	*26.1* **21.3**	*33.6* **26.3**	*39.3* **26.8**	*Eba-z4* **Eba-Z4**
				25.4 **13.1**	*25.8* **19.0**	*41.7* **29.8**	*Eba-z5* **Eba-Z5**
					26.7 **24.8**	*26.9* **23.4**	*Eba-zps* **Eba-Zps**
						34.8 **25.4**	*Eba-z6* **Eba-Z6**

Fig. S4.2. Pairwise sequence comparisons between Zen homologues of *E. balteatus*. Amino acid similarities are indicated in bold, and nucleotide similarities are indicated in italics.

```
Dme MQEVCSSLDTTSMGTQIKSESPLNPLQVQTGQTSLPV----GGCGGAGVVGGVGGVGVSVGQPGIGQQGV 66
Eba MQEVCSSLDPQ-MGAQIKSESPLNPLHIQTGQTIIPPVVLGGNTPGGPVVVSGGGSGGGNGGGGIGNATP 69

Dme PPVPSVLMVNKMTPNCDKRSADTAYWMTASEGGFINSQPSMAEFLNHLSPESPK-IGTPVGSGAIGGVGV 135
Eba N--------NKMAPNCDKR-ADTAYWM-GTESGFINSQPSMAEFLNHLSPESPKMVGTP----------- 119

Dme NVNVNVGVGVGYPVGVVPQTPDGMDSVPEYPWMKEKKTSRKSSNNNNQE--NGLPRRLRTAYTNTQLLEL 203
Eba ----------GYPVV---QAVDSMDSVPEYPWMKEKKTSRKNNNQGETSIKNGLPRRLRTAYTNTQLLEL 175

Dme EKEFHFNKYLCRPRRIEIAASLDLTERQVKVWFQNRRMKHKRQTLSKTDDEDNKDSLKGDDDQSDSNSNS 273
Eba EKEFHFNKYLCRPRRIEIAASLDLTERQV                                         204
```

Proboscipedia alignment

```
Dme GYGPAANVPNTSNGGGGGGSGAVLGGGAVGGSANGYYGGYGGGYGTANGSVGSTHSQGHSPHSQMMDLPL 244
Eba -----------SN------------------SNGGYYGGY---YGASNG------------HHQMLDLPI 26

Dme QCSSTEPPTNTALGLQELGLKLEKRIEEAVPAGQQLQELGMRLRCDDMGSENDDMSEEDRLMLDRSPDEL 314
Eba QCPNTEP-TNTVLGLQELGLKLEKRIEEAVPAGQQLQELGMRLRCDDTSSEHDDMLEEDRLMLDRSPDEM 95

Dme GSNDNDDDLGDSDSDEDLMAETTDGERIIYPWMKKIHVAGVANGSYQPGMEPKRQRTAYTRHQILELEKE 384
Eba GSHENDDDLCETDSDDDLNCETTDGERVIYPWMKKIHVAGVANGSFQPGMEPKRQRTAYTRHQILELEKE 165

Dme FHYNRYLTRRRRIEIAHTLVLSERQIKIWFQNRRMKWKKDNKLPNTKNVRKK-TVDANGNPTPVAKKPTK 453
Eba FHYNRYLTRRRRIEIAHTLVLSERQIKIWFQNRRMKWKKDNKLPNTKNVRKKNTTDANGQPVP-NKKPKR 234

Dme RAASKKQQQAQQQQQSQQQQTQQTPVMNECIRSDSLESIGDVSSSLGNPPYIPAAPETTSSYPGSQQHLS 523
Eba TSNSKKQQAVQ----------------NECIRSDSLESIGDVSSSLGNPPYIPAAGGEAANLIPQQQQQQ 288

Dme NNNNNGSG-----NNNNNNNNNNSNLNNNNNNNQMGHTNLHGHLQQ--QQSDLMTNLQLHIKQDYDLTAL 586
Eba HPTYQGSQHQTNHPSNNNNNNNTSTANNNNNN-----LALHNQMHTGMHQHPVMTNLS-HIKQDYDLTSL 352
```

Deformed alignment

```
Dme MPNPLWFVFWLLVFWFVSFFVAFFCAFFYIWVYAFASCIPALTGISDILLQGVQFPFYCGKAMLEGKQAF 70
Eba M-NPIWAIFWLIIFFFISFVVAGFSAFCYVFLYVLVVCIPGLSGVTDILLQGIQFPHFCAKAMMEGKPLC 69
```

CG11686 alignment

Fig. S4.3. Amino acid alignments of Proboscipedia, Deformed, and CG11686. *D. melanogaster* (Dme) sequences are shown above *E. balteatus* (Eba) sequences. Identical amino acids are underlaid in gray.

Chapter 5

General Discussion

Chapter 5: General Discussion

General Discussion

Two evolutionary transitions in extraembryonic tissue of flies. The goal of this thesis is to better understand the evolutionary origin of the amnioserosa. The amnioserosa is a unique extraembryonic epithelium of higher flies that has no direct equivalent in less derived flies or insects. Most insects develop two extraembryonic tissues, amnion and serosa, each of which shares different aspects with the amnioserosa.

I have distinguished three types of extraembryonic morphologies in flies. One of them was previously unknown in dipterans, although a similar type evolved independently in hymenopterans (ants, bees and wasps). The formation of a serosa and a ventral amnion reflects the primitive condition in flies, while the formation of a single dorsal epithelium represents the most derived condition. The third type, described in this thesis for the first time, consists of a serosa and a dorsal amnion.

Based on the phylogenetic relationship of fly species with different morphologies, I argue that the dorsal amnion is intermediate between the primitive and derived types within flies. Thus, the extraembryonic tissue of flies may have evolved in at least two steps. In the first step, the amnion remained dorsal rather than growing ventral, in the second step, the dorsal amnion and serosa fused and reduced to give rise to the amnioserosa.

I argue that expression changes in *zen*, a selector gene for serosal identity, can explain both transitions. Under this hypothesis, *zen* expression of the serosa anlage expanded at the expense of the amnion anlage, thus reducing the amnion. This prevented the amnio from expanding ventrally, giving rise to the intermediate type, the dorsal amnion. In a second step, expression was suppressed in the expanding preserosa, causing it to revert to an amnion-like tissue.

To reproduce the temporal change of *zen* expression in species with serosa and dorsal amnion, I developed a technique to deliver double-stranded RNA for RNA interference to embryonic cells. The technique also allows to tightly control for time. I used this method to knock down *zen* after gastrulation to reproduce the transition from the intermediate to the derived extraembryonic tissue organization in *Megaselia*. With this experiment I demonstrated that a temporal change in the expression of *zen* can explain the origin of the amnioserosa, which evolved more than 100 million years ago (1).

Adaptive advantage of evolutionary transitions in extraembryonic tissue of flies. The serosa and amnion of insects are thought to have evolved primarily as protective layers for the embryo and may have facilitated their enormous ecological success. Yet, the ventral amnion was lost independently in two lineages of insects, Hymenoptera and Diptera, and the serosal envelope was lost in higher flies. Could there have been an adaptive advantage of losing these tissues in evolution?

A hint for the possible advantage of the loss of the ventral amnion comes from the observation that in a laboratory population of the hemipteran bug *Oncopeltus,* which forms a ventral amnion, 1.7% of the embryos develop as everted (2), a condition in which the limbs develop inside the body wall and the internal body parts develop outside the body wall (3). Everted embryos occur when the amnion fails to disrupt and retract before dorsal closure. In insects with a dorsal amnion there is no requirement for such a disruption in the amnion, as it remains dorsal throughout development. Evolution of the dorsal amnion could therefore have reduced the risk of eversion.

In the same way, transforming serosa and dorsal amnion into an amnioserosa might be linked to the role of the amnioserosa in dorsal closure (4, 5). Laser ablation of the amnioserosa leads to disruption of the process of dorsal closure indicating that the physical integrity of the amnioserosa is required for

proper dorsal closure (6, 7). In *Megaselia*, the dorsal amnion disjoins from the expanding serosa during germband extension and extends as a free edge, which flips upside down to fuse with the cephalic ectoderm (Fig. 5.1). This process might be vulnerable to disruption, in which case dorsal closure defects would ensue. Conversely, the amnioserosa does not undergo this developmental transition and remains contiguous with the embryonic ectoderm throughout development. The evolution of the amnioserosa may therefore have added to the robustness of the process of dorsal closure, reducing the occurrence of spontaneous 'dorsal open' phenotypes. However, cases of spontaneous eversions or dorsal open defects in Diptera have not yet been documented. To sustain these arguments requires a thorough comparison of the frequency of naturally occurring developmental defects in dipterans with a ventral or dorsal amnion or an amnioserosa.

Evolution of temporal *zen* regulation. As discussed in chapters 2 and 3, suppression of *zen* activity prior to the expansion of serosa was probably key for transforming the serosa and dorsal amnion into an amnioserosa. What could have caused this change in *zen* expression? And was this change gradual or sudden? My gain-of-function experiments in *Drosophila* (Chapter 3) suggest that in the late amnioserosa *zen* is repressed at the post-transcriptional level. Exogenous expression of *zen* containing the coding sequence and the native *zen* untranslated region (3'UTR) in the late amnioserosa was restricted to the nucleus and was completely absent from embryonic tissues. The late amnioserosa normally does not express *zen*. Control transcripts using the same driver lines were detected in the late amnioserosa nuclei and cytoplasm and in several regions in the embryo. In another experiment, where the 3'UTR had been replaced by a different (SV40) 3'UTR, the transcripts were found in nuclei and cytoplasm of both the amnioserosa and embryonic cells. A sequence analysis of the *zen* 3'UTR revealed two possible regulatory mechanisms. The

first mechanism involves small interfering-RNA (si-RNA) and the second mechanism involves micro-RNA (mi-RNA). si-RNAs cause suppression of genes with complimentary sequences (8, 9) by forming an RNA duplex, which is processed through the mechanism of RNA interference [Reviewed in (10)]. In the *zen* 3'UTR I found a sequence that was complimentary to the second intron of gene *kuzbanian (kuz)*. *kuz* is expressed throughout development in high levels in the nervous tissue but at low levels throughout the embryo (11). The *zen* 3'UTR and *kuz* intron duplex could therefore be responsible for regulating *zen* transcripts by affecting their survival. While si-RNAs match their targets perfectly, mi-RNAs have an imperfect match with the target sequence. They involve formation of stem loop structures that are encoded separately in the genome. These structures are recognized by a different set of proteins and processed to cause gene silencing through transcript degradation [Reviewed in (10)]. In the *zen* 3'UTR, a sequence of 21 nucleotides that partially overlaps with the sequence of the putative si-RNA matched a potential mi-RNA sequence on a different chromosome. However, this sequence did not match any known mi-RNA in the current database. Experimental deletions in the *zen* 3'UTR or in the putative si-RNA or mi-RNA regions in *Drosophila* would be needed to understand the causal mechanism involved in temporal regulation of *zen*.

Evolution of spatial *zen* regulation. The early *Drosophila* amnioserosa resembles the *Megaselia* preserosa, while the late amnioserosa resembles the *Megaselia* dorsal amnion. This implies that the early amnion and the late serosa were "lost" in the course of amnioserosa evolution. Above, I have discussed how the late serosa was lost in response to a temporal change in the expression of *zen*. However, *zen* expression changed also spatially. In *Drosophila,* all extraembryonic cells express *zen* and seem to suppress early amnion development. In this thesis I propose that the spatial expansion of early *zen*

expression to all extraembryonic cells evolved after the temporal change, because such a change on its own, would not explain the arrest of preserosa expansion. In *Drosophila, zen* is expressed in the dorsal half of the preblastoderm embryo, much earlier than in *Megaselia* (see Fig. 2.2). Since the presence of *zen* promotes a serosa fate of amnion competent tissue, it is possible that this change suppressed the early amnion. Injecting capped *zen* mRNA into early *Megaselia* embryos to recapitulate this early expression did not cause any phenotype (preliminary observation, data not shown) consistent with the hypothesis that temporal change in the expression of *zen* triggered the origin of amnioserosa, while spatial expression changes of *zen* (and amnion genes, see below) followed later.

Evolution of amnioserosa gene network. I have shown that a knockdown of *zen* in *Megaselia* causes amnion marker *pannier (pnr)* to be expressed in the entire extraembryonic tissue and simultaneously the preserosa marker *Krüppel* (*Kr*) to be down regulated. I have also shown that the late expression of *Kr* in the amnion is maintained in the amnion after knocking down *zen*. While the significance of *pnr* and *Kr* expression in the amnioserosa is not understood (*pnr* is not essential for proper amnioserosa development (12) and the function of *Kr* in the amnioserosa has not been analyzed) several other genes are known to be required for proper development of the amnioserosa. The most studied of these genes are those in the U-shaped group, which includes *u-shaped* (*ush*), *hindsight* (*hnt*), *serpent* (*srp*), *tailup* (*tup*) and three *Dorsocross* paralogs (*Doc1*, *Doc2*, *Doc3*)(13). The genes of u-shaped group may be acting in two or more different pathways in the amnioserosa (14-16). Mutations in these genes produce several degrees of defects in the morphogenetic processes of germband retraction and dorsal closure. These processes include genetic and physical interactions between the amnioserosa and the adjoining dorsal ectoderm. Since the *Megaselia* ectoderm is attached to the amnion and not to the serosa during these

processes, it is possible that paralogs of these genes in *Megaselia* play a similar role in the amnion during these morphogenetic processes. However, since the disjunction of the serosa and amnion during development may involve genetic interactions between the two tissues at their boundaries, it cannot be ruled out that serosa development is also affected when the activity of these genes is suppressed. A good starting point for future studies would be to examine homologs of the u-shaped group genes such as *Doc* and *hnt,* which have been examined in detail in *Drosophila* (5, 13, 15), with respect to their expression and extraembryonic function in *Megaselia*. With my method of nano-particle mediated RNAi that allows gene knockdown in *Megaselia* after cellularization, it should be possible to target these and other genes in specific time windows thereby reducing pleiotropic effects that would obscure the analysis.

Evolution of the extraembryonic anlagen. In a separate project, I asked whether the position of the serosa anlage in the blastoderm affects embryonic patterning mechanism. The position of the serosa anlage varies among dipterans. It has been previously proposed that cyclorrhaphan species containing localized transcripts of the anterior determinant *bicoid* (*bcd*) have a strictly dorsal serosa anlage; conversely, *bcd* or *bcd*-like transcripts are absent in non-cyclorrhaphan species where the serosa anlage extends to the anterior (17, 18). Since the position of *bcd* in the genome is highly conserved (19, 20), it is possible to test the presence or absence of *bcd* by looking at the genomic locus that usually contains *bcd*. In my analysis of extraembryonic anlagen in Cyclorrhapha, I found that *Episyrphus balteatus* has an anterior-dorsal serosa anlage. Thus, *Episyrphus* is exceptional among cyclorrhaphan flies with respect to the extraembryonic anlage and prompts the question, whether this species contains a *bcd* ortholog. To test for the presence or absence of *bcd* in this species, I cloned and sequenced c.a. 79 kb of genomic DNA in the *hox3* region of *Episyrphus* where *bcd* is normally found. I did not find *bcd* or a *bcd*-like

gene, supporting the hypothesis that absence of *bcd* correlates with extraembryonic development in the anterior. However, I cannot exclude the possibility that close linkage of *bcd* and *zen* was abandoned in *Episyrphus,* in which case I may have missed this gene. Serendipitously, my sequence analysis revealed presence of seven *zen* paralogs in this contig. Sequence homology between these paralogs and known *zen* and *bcd* homologues from other dipterans suggests that these duplications arose independently within the *Episyrphus* lineage. Thus it is unlikely that any of these *zen*-like genes is orthologous to *bcd.*

Phylomimicking mutations. Mutations that affect development of the embryo might have played analogous roles during evolution (21). Phylomimicking mutations, as they are called, are of special interest when they produce large effects in morphology. Such large effect phylomimicking mutants have been termed "hopeful monsters" by Richard Goldschmidt, who proposed that these could be produced through disturbances in the process of development (22). Our observation that *zen* RNAi phenotype in *Megaselia* and *Episyrphus* resembles the wild type in *Drosophila* is consistent with Goldschmidt's hypothesis because I was able to show that a simple change in expression of *zen* can explain the evolutionary origin of an amnioserosa-like epithelium. Such tests should, therefore, also be possible for other examples of phylomimicking mutations.

Conclusion. The research described in this thesis demonstrates that change in expression pattern of a single gene caused a morphological transition deep within the phylogeny. It corroborates evo-devo studies that predict a correlation between gene expression and morphology. But this work extends evo-devo further by genetically recapitulating the proposed evolutionary change and demonstrating its efficacy. My example shows that even at macro evolutionary

scale, i.e. over large phylogenetic distances, evo-devo can provide insight into the causal relationship between gene expression and morphology.

Represented on the phylogenetic tree, the three morphological types of extraembryonic tissue organization in flies; a serosa and ventral amnion ('primitive'), a serosa and dorsal amnion ('intermediate'), and the amnioserosa ('derived'), suggest a transition in two steps in evolution. Apparently, all three morphological types occur in extant species in agreement with the proposed transitions and their phylogenetic position. This is an indication that these traits represent a morphological series that recapitulates the chronological series that produced them in evolution (Fig. 5.2). This phenomenon is known as the "principle of identity of morpho- and chrono-clines", stated by Maslin as, "morpho-clines are partially or entirely identical to chrono-clines" (23). In the case of extrambryonic tissues, I was able to recapitulate the evolutionary change in the 'intermediate' species to attain the 'derived' state but my attempt to reverse this transition was not successful. Thus knowing the direction of change is important to achieve artificial transformation of one form to another. Maslin's principle might thus prove useful in experimental attempts to recapitulate evolutionary events.
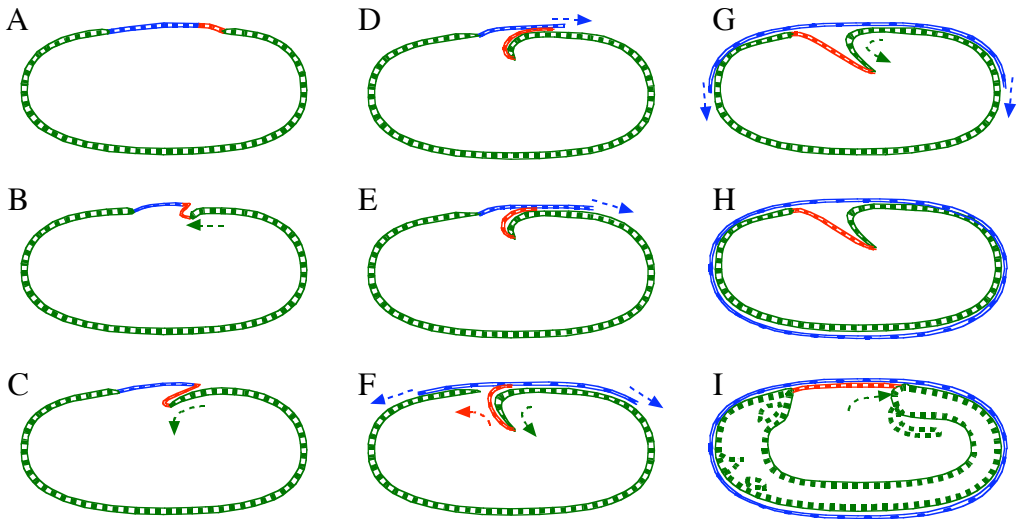
Fig. 5.1 Morphogenetic movements of the amnion in *Megaselia*. (A-I) Cartoons of consecutive stages in *Megaselia* depicting the serosal tissue (blue), amniotic tissue (red), and embryonic tissue (green) with anterior to the left and dorsal up. Arrows indicate directions of morphogenetic movements, and colors of arrows correspond to the tissue that undergoes the movement.
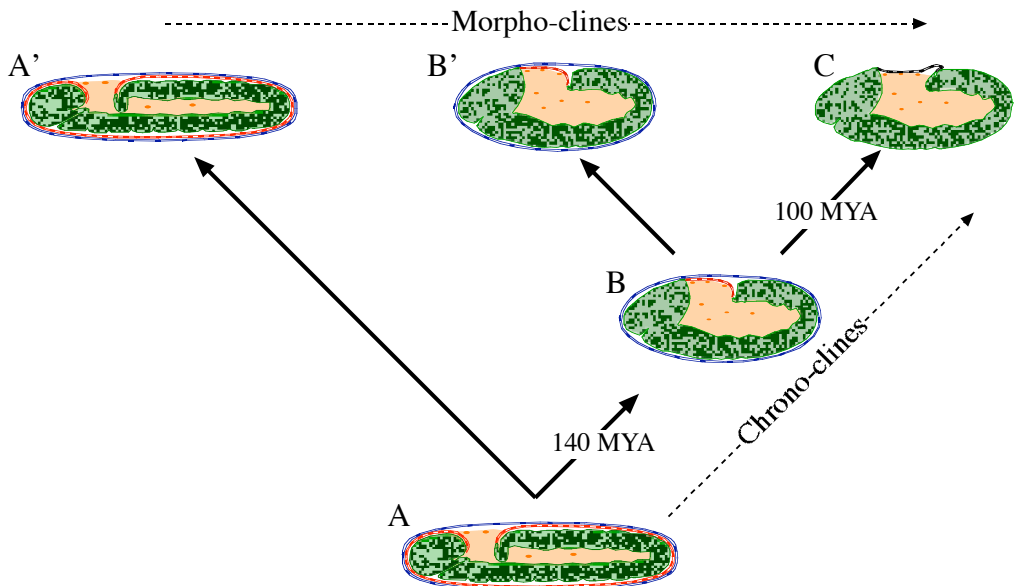

Fig. 5.2 Maslin's principle of identity of morpho-clines and chrono-clines applies to extraembryonic epithelium in dipterans. (A, A') the primitive state (B, B') the intermediate (or intervenient) state and (C) the derived state. Serosa (blue) amnion (red), embryo (green) and yolk (ochre) are highlighted. Drawings based on (24) & (25).

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Chapter 5: General Discussion

Summary

Changes in the genotype influence changes in morphology during evolution, giving rise to the vast diversity of morphological features that we observe. The ability to describe how genetic change causes morphological transformation is key for a mechanistic understanding of evolutionary change. This thesis examines morphological and genetic aspects of the origin of the amnioserosa, a unique tissue of higher flies (Cyclorrhapha), which combines different aspects of the amnion and the serosa, two protective extraembryonic epithelia commonly found in lower flies and other insects.

Typically, extraembryonic epithelia develop from an amnio-serosal fold, which closes about the ventral side of the gastrulating embryo. The outer cell layer of the amnio-serosal fold becomes the serosa. This epithelium disjoins from the amnion and encloses the embryo. The inner cell layer of the amnio-serosal fold becomes the amnion and retains continuity with the dorsal epidermis of the embryo. Higher flies of the taxon Schizophora differ: they develop a single extraembryonic epithelium on the dorsal side called the amnioserosa. Here, a third type is reported in close relatives of Schizophora (lower Cyclorrhapha), in which the amnion is dorsal and the serosa complete. Based on the occurrence of these three extraembryonic tissue organizations in flies two major morphological transitions in the organization of the extraembryonic epithelia of flies are proposed, one that occurred before the cretaceous radiation of flies some 150 million years ago in which the amnion got relocated from the ventral to the dorsal side of the embryo, and another that occurred before the tertiary radiation of flies some 100 million years ago in which the dorsal amnion and the serosa combined into a single epithelium, the amnioserosa.

The genetic analysis focuses on the homeobox gene *zerknüllt* (*zen*). Most insects express *zen* in their extraembryonic tissues. In the milkweed bug

Summary

Oncopeltus, zen controls the fusion of the amnion with the serosa. In the flour beetle *Tribolium*, *zen* controls not only the fusion of the amnion with the serosa but also the distinction of serosal from amniotic cells. In the fruitfly *Drosophila*, *zen* controls the distinction of amnioserosal from embryonic cells. Here, it is shown that in lower cyclorrhaphan flies, *zen* controls serosal cell-fate, like in *Tribolium*. Furthermore, spatial and temporal differences in the extraembryonic expression of *zen* are reported between *Drosophila* and lower cyclorrhaphan flies. Extraembryonic *zen* expression in *Drosophila* abuts the posterior and lateral germ rudiment, unlike in lower cyclorrhaphans in which the prospective amnion is interspersed. Ectopic expression of *zen* in *Megaselia* through injection of capped mRNA had no effect on development, suggesting that spatial expression difference may not explain the morphological reorganization into an amnioserosa-like tissue in evolution. Temporally, extraembryonic *zen* expression in *Drosophila* is downregulated during the late phase of germband extension, unlike in lower cyclorrhaphans in which it persists. A new transfection-based technique for delivery of double-stranded RNA to embryonic cells was developed and used to mimic the time course of *zen* expression of *Drosophila* in *Megaselia*. This procedure was sufficient to create an amnioserosa-like tissue in Megaselia. In addition, differences in extraembryonic *zen* expression were observed between lower cyclorrhaphan species. In one species (*Megaselia*) it was restricted to dorsal balstoderm, like in *Drosophila*. In another species (*Episyrphus*), it extended to the anterior tip of the blastoderm egg. This difference cannot explain the origin of the amnioserosa but may reflect differences in embryonic pattern formation through *bicoid*, which determines the position of the head by means of localized mRNA. To test this prediction, ca. 60 kb upstream of *zen* (spanning the expected genomic position of *bicoid*) were sequenced in *Episyrphus*. This region did not contain a *bicoid* homologue.

The research described in this thesis demonstrates that temporal change in the expression pattern of a single gene can explain the origin of the amnioserosa. This result shows that even at a macroevolutionary scale, i.e. over large phylogenetic distances, evo-devo studies can provide insight into the causal relationship between gene expression and morphology. In addition, the unique *zen* expression in *Episyrphus* and *zen* locus data from this species point to a possible difference in embryonic patterning in this taxon.

Summary

Samenvatting

Gedurende de evolutie staan genetische veranderingen aan de basis van morfologische veranderingen. Daarom is inzicht in hoe en welke genetische veranderingen bijdragen aan morfologische veranderingen van belang om de processen die tot evolutie leiden beter te begrijpen. Het onderzoek in dit proefschrift richt zich op de morfologische ontwikkeling van de cellaag die het embryo van insecten beschermt. Dit epitheel wordt geen onderdeel van het organisme als dit zich verder ontwikkelt, maar heeft slechts een beschermende functie. Gedurende de evolutie is een dergelijk beschermend epitheel ontstaan bij de overgang van water- naar landleven.

De meeste insecten vormen een buitenste epitheel, de serosa, die het embryo volledig omsluit en een binnenste epitheel, amnion, dat alleen de buikkant beschermt. De meeste vliegen (Diptera) vormen een amnion en een serosa. Echter sommige taxa (bijvoorbeeld Drosophila) vormen een enkel epitheel, dat amnioserosa genoemd wordt. In dit proefschrift wordt bestudeerd welke genetische veranderingen ten grondslag liggen aan het ontstaan van de amnioserosa gedurende de evolutie.

Daartoe werd een derde vorm van weefselorganisatie van het beschermende epitheel onderzocht, die voorkomt bij een bepaalde groep van vliegen (lagere Cyclorrapha). Hierbij omringt het serosa het embryo volledig maar is het amnion aan de rugkant gelegen. Deze organisatie van amnion en serosa is een tussenvorm tussen de 2 eerder beschreven organisaties bij vliegen en biedt daarom extra mogelijkheden om de vorming van het amnioserosa tijdens de evolutie beter te begrijpen.

Eerdere studies hadden aangegeven dat mogelijk de transcriptiefactor Zerknullt (Zen) een cruciale rol bij de vorming van de amnioserosa gespeeld zou kunnen hebben. In dit proefschrift is de expressie van Zen tijdens de vorming van de 3 verschillende weefselorganisaties van het amnion/serosa bestudeerd. Dit gaf aan

dat de plaats waar het *Zen*-gen tot expressie komt heel goed zou kunnen verklaren hoe de organisatie van amnion en serosa van lagere Cyclorrapha zich ontwikkelde in vergelijking met het amnioserosa zoals dat bij Drosophila voorkomt. Echter als het (ruimtelijke) expressiepatroon van *Zen* in een lagere Cyclorrapha zo werd veranderd dat het lijkt op dat van Drosophila, dan vormde deze Cyclorrapha geen amnioserosa. Een ander verschil in de expressie van *Zen,* betreft de timing van expressie. In Drosophila wordt de expressie van Zen tijdens de ontwikkeling van het amnioserosa namelijk tijdelijk verlaagd. Wanneer dit in de bestudeerde Cyclorrapha-soort wordt opgewekt dan leidt dit tot de vorming van een amnioserosa. Met andere woorden: een belangrijke morfologische overgangsstap in de evolutie van amnioserosavorming kan nagebootst worden door het expressiepatroon (in de tijd) van een enkel gen, namelijk Zen, te veranderen.

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Curriculum Vitae

Abdul Matteen Rafiqi was born on 7th of August 1975 in the valley of Kashmir in India. He attended St. Joseph's High School, Baramulla and Tyndale Biscoe Memorial High School, Srinagar, from where he graduated in 1994. He then joined Amar Singh Memorial College in Srinagar to obtain a Bachelor in Biological Sciences (BSc) but switched after one year to Regional Research Station and Faculty of Agriculture, Wadura, Kashmir. After graduating from Wadura in 1999, he studied at Jammu University, Jammu, for a Master's program in Biotechnology. It was during this time that he started his training in molecular biology in collaboration with the Regional Research Laboratory, Jammu. In 2000, he received a fellowship from The Netherlands Organization for International Cooperation in Higher Education (NUFFIC) to study at Wageningen University for a Master's in Biotechnology. He moved to The Netherlands in August 2000 to pursue this program. After graduating from Wageningen in January 2002, he briefly worked as a teaching assistant with Prof. Ton Bisseling at Wageningen University. Shortly after that, in June 2002, he started research for a PhD at the laboratory of Dr. Urs Schmidt-Ott at the Max Planck Institute for Biophysical Chemistry, in Göttingen, Germany. In November 2003, he moved with this laboratory to The University of Chicago, to continue his research for a PhD.

List of Publications

Rafiqi, A. M., Lemke, S. J., Ferguson, S., Stauber, M. & Schmidt-Ott, U. (2008) *Proc. Natl. Acad. Sci. U S A* **105,** 234-239.

Lemke, S. J., Stauber, M., Shaw, P. J., **Rafiqi, A. M.,** Prell, A. & Schmidt-Ott, U. (2008) *Evol. Dev.* **10,** 413-420.

Rafiqi, A. M. & Schmidt-Ott, U. Temporal expression differences of *zerknüllt* (*zen*) explain the origin of amnioserosa. (Submitted)

Rafiqi, A. M., Raedts, J., Schön, O., Blöcker, H., & Schmidt-Ott, U. Seven *zen*-like genes in the Hox3 locus of the hover fly *Episyrphus balteatus* (Syrphidae). (Submitted)

Meeting Publications

Rafiqi A.M., Lemke S.J. and Schmidt-Ott U. "Mapping the evolutionary origin of *bicoid* gene" *Drosophila* research conference, Washington DC (poster presentation, March 2004)

Rafiqi A.M., Lemke S.J. and Schmidt-Ott U. "*Episyrphus balteatus* has four *zerknullt* genes and a serosa" *Drosophila* research conference, San Diego, CA (Platform presentation, April 2005)

Cover Photos:
Front: *Megaselia abdita* adult photo by Sean Ferguson. *M. abdita* germband retraction stage embryo.
Back: *Episryphus balteatus* adult with eggs laid over Fava bean plant infested with aphids.