

## CHAPTER 10

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# *Hox3/zen* and the Evolution of Extraembryonic Epithelia in Insects

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### Abstract

Insects have undergone dramatic evolutionary changes in extraembryonic development, which correlate with changes in the expression of the class-3 Hox gene *zen*. Here, we review the evolution of this gene in insects and point out how changes in *zen* expression may have affected extraembryonic development at the morphological and the genetic level.

### Introduction

During the early radiation of insects, *Hox3* abandoned its ancestral role in specifying segmental identity along the anteroposterior axis of the embryo and acquired a new role in extraembryonic tissue.<sup>1</sup> This evolutionary transition may have occurred in the stem lineage of modern Pterygota (winged insects), as indicated by expression data and protein comparisons. Canonical Hox proteins share a hexapeptide or YPWM motif,<sup>1</sup> which enables them to interact with the Hox-cofactor Exd/Pbx.<sup>2,3</sup> In the context of overlapping Hox gene expression patterns, e.g., during axis-specification, this interaction is important because it contributes indirectly to the DNA-binding specificity of individual Hox proteins.<sup>4</sup> The *Hox3* gene of the apterygotan firebrat *Thermobia* is expressed in a nested arrangement with other Hox genes in the prospective gnathocephalon and in the growth zone and encodes a protein with the YPWM motif.<sup>5,6</sup> In contrast, *Hox3* genes of Pterygota (named *zen* after their *Drosophila* prototype)<sup>7</sup> are expressed in extraembryonic tissue, lack expression in the germband and encode proteins without YPWM motif.<sup>6</sup> The present chapter is devoted to the evolutionary history of extraembryonic *zen* expression in insects. We cover data from five orders including the silverfish *Thermobia* (Thysanura), the grasshopper *Schistocerca* (Orthoptera), the bug *Oncopeltus* (Hemiptera), the beetle *Tribolium* (Coleoptera) and various fly species (Diptera). Yet, the focus of this review is on Diptera as this is the only insect order for which data on the expression and function of *zen* are available from multiple distantly related species. Even in this order, some relevant traits have been poorly sampled. Despite the 'patchiness' of the data matrix, we hope that as current working hypotheses our phylogenetic inferences will help to advance the field. To set the stage, we briefly review morphological aspects of extraembryonic development. In the following sections, we focus on variants of *zen* expression and their evolutionary significance. We propose that changes in *zen* expression underlie major reorganizations in extraembryonic development of pterygote insects.

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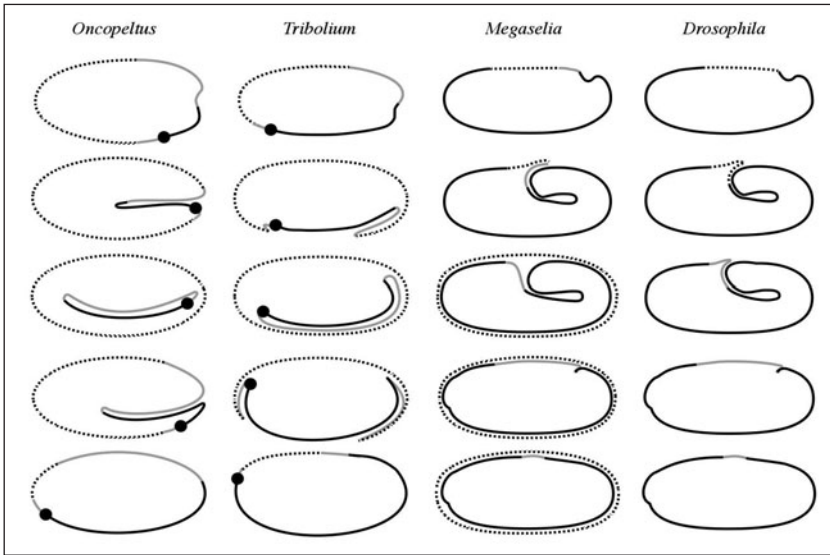


Figure 1. Schematic illustrations of extraembryonic developmental trajectories. Embryonic (black line), amniotic (grey) and serosal tissues (broken line) are indicated at consecutive developmental stages. Sketches are based on *Oncopeltus*,<sup>9,10</sup> *Tribolium*,<sup>23</sup> *Megaselia*<sup>18</sup> and *Drosophila*.<sup>19</sup> A filled circle indicates the position of the head in *Oncopeltus* and *Tribolium* embryos.

### Setting the Stage: Morphological Evolution of Extraembryonic Development

Extraembryonic development of insects begins at the blastoderm stage, when a single cell layer forms around the yolk. At this stage, a portion of anterior or dorsal blastoderm—depending on the species—is specified to become the serosa, an epithelium underneath the eggshell, which secretes a cuticle.<sup>8</sup> Typically, serosa formation occurs by invagination of the posterior blastoderm (e.g., *Oncopeltus*, Fig. 1), or by closure over the ventral blastoderm (e.g., *Tribolium*, Fig. 1). In both cases, the internalized blastoderm pinches off from the serosa as a flattened hollow body, which is composed of a thin cell layer (amnion) and the gastrulating embryo. The fold through which part of the blastoderm is internalized is called ‘amnioserosal fold’ and the yolk-free space between the amnion and the embryo is called ‘amniotic cavity’. Later in development, while the flanks of the embryo close along the dorsal midline (dorsal closure), the process of serosa and amnion formation is reversed. The serosa and the amnion generate a continuous serosa-amnion epithelium (‘serosa-amnion fusion’), which retracts towards the anterior or dorsal side of the egg where both epithelia are resorbed by the yolk. This process ruptures the amniotic cavity and regenerates an opening in the extraembryonic epithelia (‘serosal window’). In hemimetabolous insects (lower Pterygota<sup>a</sup>), serosa-amnion fusion seems to be widely conserved, as it is required for realigning the inverted anteroposterior (A-P) axis of the early embryo with the A-P axis of the egg (katatrepsis).<sup>9,10</sup> In holometabolous insects (higher Pterygota), serosa-amnion fusion has been observed as well, but major variants of extraembryonic development have been found in all the large orders, including Hymenoptera, Coleoptera, Lepidoptera and Diptera.<sup>8,11–13</sup> Here we limit the discussion to variants within the dipteran order. In lower Diptera (noncyclotrhaphan flies), serosa and amnion

<sup>a</sup> Throughout this review, we use the term ‘lower’ in conjunction with a taxon name to designate basal branches of monophyletic taxa. Conversely, the term ‘higher’ is used when the intention is to exclude species on basal branches.

are generated essentially in the same way as in the beetle *Tribolium* (Fig. 1).<sup>14</sup> However, it seems that in many lower dipterans the two mature epithelia fail to fuse again and persist or degenerate independently, while dorsal closure proceeds.<sup>15-17</sup> More radical departures from the ancestral trajectory have been reported for higher dipterans (Cyclorrhapha). Lower cyclorrhaphan flies such as the phorid fly *Megaselia abdita* or the syrphid fly *Episyrphus balteatus* initiate the formation of an amnioserosal fold, but while their serosa expands ventrally, their amnion disjoins from the leading edge of the serosa and grows over the dorsal side of the yolk sac (e.g., *Megaselia*, Fig. 1).<sup>18</sup> In these species, serosa-amnion fusion does not occur and only the amnion is resorbed by the yolk. Finally, in higher cyclorrhaphan flies, while a rudimentary amnioserosal fold is transiently visible at the morphological level, the extraembryonic tissue does not disjoin at the edge of this fold and does not expand over the germband (e.g., *Drosophila*, Fig. 1). Instead, the extraembryonic anlage gives rise to a single epithelium, called amnioserosa, which is later resorbed by the yolk,<sup>19,20</sup> like the dorsal amnion in lower Cyclorrhapha. Taken together, the data suggest that extraembryonic development in the dipteran lineage evolved in three distinct steps. First, mature serosal and amniotic epithelia failed to fuse and retract as a continuous serosa-amnion epithelium during the process of dorsal closure. Second, ventral closure of the amnion was suppressed and substituted by closure of the amnion over the dorsal yolk sac. Third, serosa and dorsal amnion were transformed into an amnioserosa. All these evolutionary transitions seem to correlate with changes in the activity pattern of *zen*, which will be reviewed in the next section.

### Variants of *zen* Expression and Function in Insects and Possible Morphological Correlates

Variants of *zen* expression and function in insects (Fig. 2) suggest that this gene played an important role in the evolution of extraembryonic tissue. The apterygotan insect *Thermobia* expresses *Hox3* not only in the embryo (see Introduction) but also in the mature (completed) amnion.<sup>5,b</sup> Hemimetabolous Pterygota (e.g., *Schistocerca*, *Oncopeltus*) exhibit *zen* expression transiently during blastoderm formation and subsequently in the serosa and parts of the amnion.<sup>9,21,22</sup> In the grasshopper *Schistocerca gregaria*, extraembryonic *zen* expression begins in the leading edge of the developing serosa ('necklace cells') but in other species comparable *zen* expression has not been observed. Hence, *zen* expression in necklace cells might have evolved in the *Schistocerca* lineage. During later stages, *Schistocerca* expresses *zen* throughout the completed serosa and in adjacent parts of the completed amnion. In the bug *Oncopeltus fasciatus*, *zen* expression has been observed throughout the completed serosa and in a rim of amniotic cells, which connect with the contracting serosa. RNA interference (RNAi) against *Oncopeltus zen* (*Of-zen*) prevents rupture of the amniotic cavity and formation of a continuous serosa-amnion epithelium, as well as katatrepsis and dorsal closure.<sup>9,10,c</sup> To explain this phenotype, *zen* activity in the serosa has been invoked as a regulator of global contractions,<sup>10</sup> but an essential complementary role of amniotic *zen* expression in this process has not been ruled out.

In holometabolous insects, serosal tissue expresses *zen* at all stages.<sup>18,21,23-25</sup> Amniotic *zen* expression has been reported for a beetle (*Tribolium*) but only at the site where the serosal window opens up prior to dorsal closure. In species that lack this process (e.g., the lower cyclorrhaphan flies *Megaselia* and *Episyrphus*), amniotic *zen* expression has not been observed. In *Tribolium castaneum*, *zen* is critical for both serosa specification and serosa-amnion fusion. The distinction of these functions is possible because of a recent duplication of the *zen* locus in the *Tribolium* lineage<sup>26</sup> and subfunctionalization of the resulting *Tribolium* paralogs, *Tc-zen1* and *Tc-zen2*.<sup>23</sup> Both genes are expressed throughout the serosal tissue, while only *Tc-zen2* is expressed in the

<sup>b</sup> Amniotic expression was observed at the torpedo stage, when extraembryonic epithelia have already formed.

<sup>c</sup> In *Of-zen* RNAi embryos, the serosa and the amnion form a 'serosal window pane' that fails to rupture, keeping the amniotic cavity closed and detracting the flanks of the embryo, which under these conditions close ventrally.

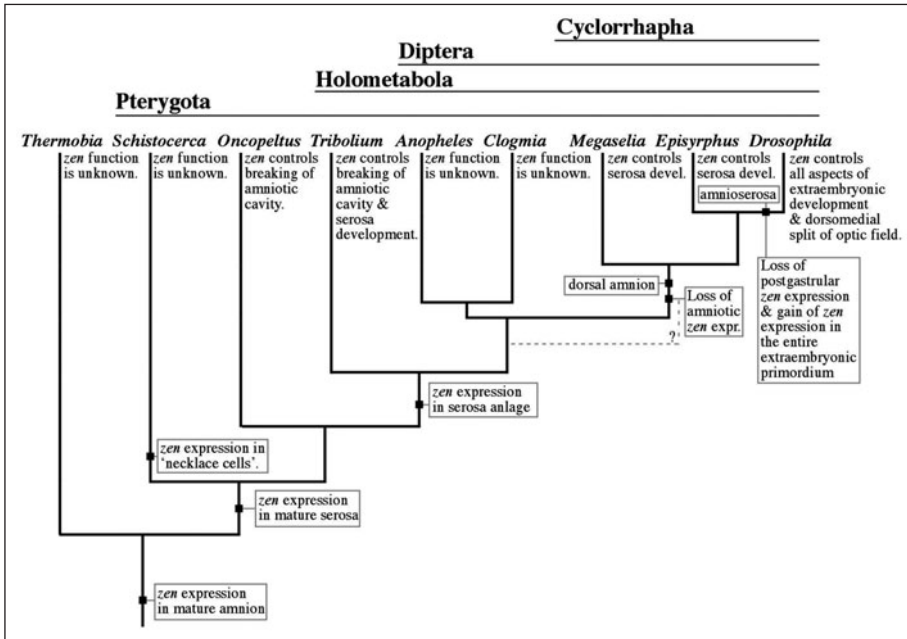


Figure 2. Evolution of extraembryonic *zen* expression and function. Phylogenetic relationships are shown according to Grimaldi and Engel.<sup>51</sup> For details see text. For the eggs of *Schistocerca* and diverse lower dipterans (including *Clogmia*), the presence of maternal *zen* transcripts has been documented.<sup>21,22,25</sup> *Anopheles*, *Megaselia*, *Episyrphus* and *Drosophila* lack maternal *zen* expression.<sup>7,18,24,25</sup>

amnion. RNAi against *Tc-zen1*, which is epistatic to serosal expression of *Tc-zen2*, suppresses serosa development and causes the formation of a single extraembryonic epithelium in which all cells are amnion-like. *Tc-zen2* RNAi suppresses serosa-amnion fusion and timely rupture of the amniotic cavity, indicating a possible function of amniotic *zen* expression in this process. The function of *Tc-zen1* is probably conserved across a wide range of holometabolous insects. For example, in *Megaselia abdita*, RNAi against *zen* suppresses serosa development and results in the formation of a single, amnion-like extraembryonic epithelium, which is sufficient to support germband retraction and dorsal closure,<sup>18</sup> just like in *Tribolium*. In summary, *zen* expression in the mature serosa has been reported for a wide range of (pterygote) insects, while *zen* expression in the serosa anlage and *zen*-dependent serosa specification might be characteristic of Holometabola. *zen* expression in the late amnion may have evolved before the radiation of Pterygota but seems to be restricted to species that undergo serosa-amnion fusion prior to katartripsis (Hemimetabola only) and dorsal closure and we suspect that the amniotic domain is required for the formation of a continuous serosa-amnion epithelium.

Among holometabolous insects the role of *zen* in *Drosophila* stands out. Unlike the beetle *Tribolium* and the lower cyclorrhaphan flies *Megaselia* and *Episyrphus*, the higher cyclorrhaphan (schizophoran) fly *Drosophila* is unable to specify any extraembryonic tissue in the absence of *zen* activity.<sup>d</sup> Also the expression pattern of *Drosophila zen* appears diverged. Its novel features

<sup>d</sup> *Drosophila melanogaster* contains two copies of the *zen* locus (*zen*, *zen2*), which are expressed similarly. We do not consider *zen2* separately because available mutants of *zen* also remove the activity of *zen2* and because *zen2* was shown to be dispensable for normal development.

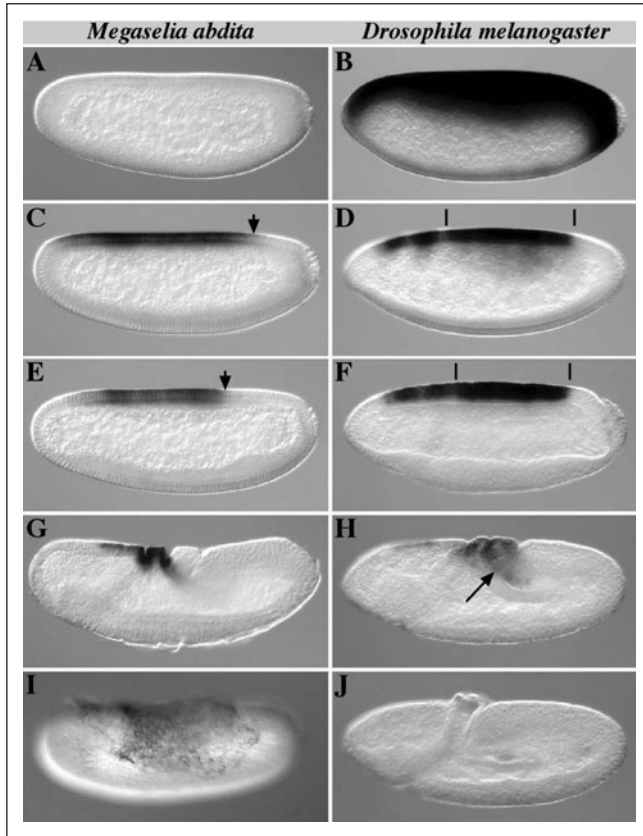


Figure 3. Expression of *Mab-zen/zen* transcripts in *Megaselia abdita* and *Drosophila melanogaster*. Matching consecutive stages of *Megaselia* (left) and *Drosophila* (right) are shown before cellularization (A,B), during cellularization (C,D), at the beginning of gastrulation (E,F), during gastrulation (G,H) and after gastrulation (I,J). Horizontal bars indicate the position of the amnioserosa anlage. Posterior shortening of the *Mab-zen* domain is indicated by arrowheads and the *zen*-positive invaginated portion of the amnioserosa is indicated by an arrow. Panels C, I and J from reference 18. Anterior is left and dorsal up.

include a broad dorsal expression domain at early blastoderm stages, a posteriorly expanded and laterally compressed expression domain of *zen* at late blastoderm stages and absence of *zen* expression after gastrulation (Fig. 3). Below, we discuss the evolutionary significance of these *Drosophila*-specific features.

### ***Suppression of Postgastrular zen Expression May Have Triggered the Origin of the Amnioserosa***

In holometabolous insects, serosal *zen* expression is maintained from the time of serosa specification until after the completion of the serosa epithelium.<sup>18,23</sup> However, in *Drosophila*, *zen* expression (transcript and protein) fades during stage 8, shortly after a pseudo-amnioserosal fold has formed. We found in *Megaselia* that during early gastrulation *zen* expression is still critical for the formation of distinct serosal and amniotic epithelia.<sup>28</sup> Suppression of *zen* expression at this stage alters the developmental trajectory of the extraembryonic epithelia: the serosa fails to expand over the germband and does not disjoin from the edge of the amnion but rather becomes part

of it or disintegrates. Hence, the amnioserosa could have originated by the loss of postgastrular *zen* expression, which suppressed further serosa development while allowing the formation of a *zen*-negative dorsal amnion. This hypothesis implies that late developmental stages of the ancestral amnioserosa (after stage 8) were equivalent to a dorsal amnion, whereas early developmental stages of the ancestral amnioserosa (until stage 8) were composed of *zen*-expressing serosal and *zen*-negative amniotic tissue. However, in *Drosophila*, all cells of the early amnioserosa express *zen* (including cells that invaginate with the proctodeum and which formerly gave rise to the amnion) and hence, might best be compared with early serosa cells of lower cyclorrhaphan flies. This novel feature of extraembryonic development may have evolved after the origin of the amnioserosa because at this point in time the distinction of serosal and amniotic primordia had become obsolete. The genetic mechanisms by which *zen* gained control over all amnion-competent cells of the *Drosophila* blastoderm were probably different along the anteroposterior (A-P) and the dorsoventral (D-V) axis and will be discussed separately.

### ***Reduction of the Amniotic Anlage along the A-P Axis***

In *Megaselia*, the expression domain of *zen* in the cellular blastoderm is shortened at the posterior end. The cells, in which *zen* has been actively repressed, invaginate with the proctodeum and become part of the amnion (cf. Figs. 1 and 3). The repressor has not been identified but could be dependent on *Megaselia caudal* (*Mab-cad*), which is specifically expressed in amniotic blastoderm.<sup>29</sup> *Drosophila* lacks expression of *caudal* in this domain of the blastoderm and does not down-regulate *zen* there. As a result, *zen* is also expressed in cells that eventually invaginate with the proctodeum (cf. Figs. 1 and 3). These cells become part of the amnioserosa. Thus, the posterior expansion of *zen* expression in the cellular blastoderm of *Drosophila* may have caused a reduction of the amnion Anlage along the A-P axis. Consistent with this hypothesis, we recently found that the injection of capped *Mab-zen* mRNA into syncytial *Megaselia* embryos represses genes with amniotic expression and causes defects in germband retraction and dorsal closure, but does not interfere with the formation of the serosa (unpublished data).

### ***Reduction of the Amniotic Anlage along the D-V Axis***

While derepression of posterior *zen* activity in the *Drosophila* lineage may account for the reduction of the amniotic Anlage along the A-P axis, a different mechanism must account for the reduction of the amniotic Anlage along the D-V axis, because in the cellular blastoderm of *Drosophila*, the expression domain of *zen* is narrower than in other dipterans.<sup>24</sup> How was this transition achieved? Below, we argue that the gain of early broad *zen* expression (spanning about 40% of the D-V perimeter) in the *Drosophila* lineage was critical for the evolution of an all-*zen*-dependent extraembryonic Anlage. To make the argument, it is necessary to introduce another *Drosophila* gene, *decapentaplegic* (*dpp*).<sup>30</sup> This gene encodes a ligand of the bone morphogenetic protein (BMP) family. It is secreted into the space between the eggshell and the embryo and promotes pattern formation in the dorsal ectoderm in a concentration-dependent manner (reviewed in ref. 31). The dorsal blastoderm of *dpp*-deficient *Drosophila* embryos acquires a more ventral neurogenetic fate; moderate levels of *dpp* activity are sufficient to specify dorsal embryonic ectoderm (but insufficient for specifying the amnioserosa); and high-level overexpression of *dpp* converts all dorsal cells to an amnioserosa fate.<sup>32</sup>

The activity of *dpp* can be visualized with an antibody against the phosphorylated form of the intracellular protein Mad, which transmits the Dpp-dependent signal from the cytoplasm to the regulatory DNA sequences of target genes.<sup>33-35</sup> As secreted Dpp undergoes extracellular transport towards the dorsal midline, its activity range in the dorsal ectoderm is confined to a narrower portion than the distribution of *dpp* transcript (ca. 40% of the D-V perimeter) would suggest (reviewed in ref. 31). Long-range, extracellular transport of Dpp accounts for the shallow pMad gradient that is observed in the early *Drosophila* blastoderm and which spans about 20% of the D-V perimeter with peak levels at the dorsal midline. In older embryos (undergoing blastoderm cellularization), pMAD levels experience an additional and much more dramatic lateral contraction (accompanied by dorsal increase) in response to a positive feedback loop that is at least in part

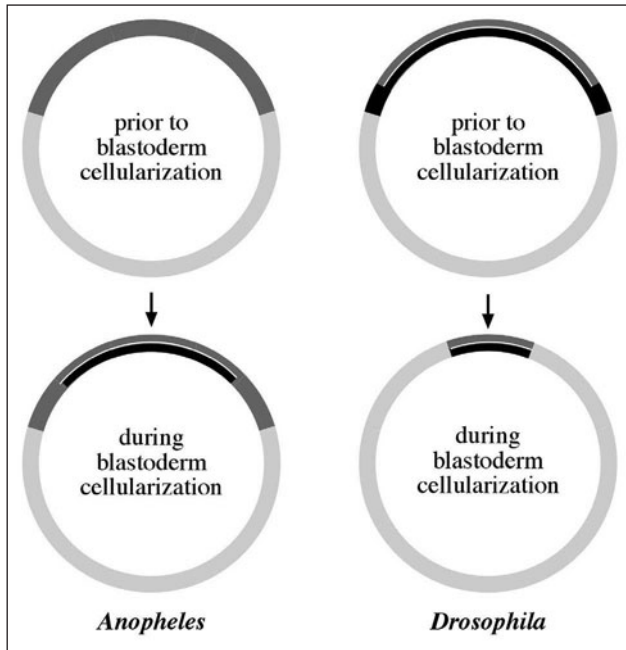


Figure 4. Evolution of the relationship of *zen* and *dpp* activities in the blastoderm. Blastoderm embryos of *Anopheles* (left) and *Drosophila* (right) are depicted as transverse sections at two consecutive stages with *dpp* activity in dark grey and *zen* activity in black. Note that in *Anopheles*, *dpp* activity extends beyond the expression domain of *zen*, while in *Drosophila*, *dpp* activity does not extend beyond the activity range of *zen*. For details and references see text.

dependent on *zen*.<sup>36</sup> The result of the refinement is a high-level pMad domain that now spans only about 10% of the D-V perimeter, precisely the width of the amnioserosa anlage.<sup>c</sup> The important point is that throughout blastoderm development, *dpp* activity of the *Drosophila* embryo does not extend beyond the expression domain of *zen*. This is different in lower dipterans.

Compared to *Drosophila*, the spatial relationship of Dpp activity and *zen* expression in the mosquito *Anopheles* appears to be inverted (Fig. 4). In *Anopheles*, the pMad domain is much broader than in *Drosophila* and extends at all blastoderm stages beyond the boundaries of the *zen* domain.<sup>24</sup> Provided that *Anopheles dpp* is essential for the specification of amnion-competent blastoderm, as it probably is<sup>f</sup>, and considering that *zen* represses amnion development in amnion-competent blastoderm,<sup>18,23</sup> the gain of early broad *zen* expression in the *Drosophila* lineage after the origin of the amnioserosa might well have delayed amnion specification until the end of gastrulation by promoting serosa development at early stages.

<sup>c</sup> The evolutionary trend towards bistable Dpp activity in the late blastoderm could have gradually increased the ratio of serosal to amnion-competent blastoderm and might have prevented ventral amnion closure in the stem lineage of schizophoran flies.

<sup>f</sup> In *Tribolium*, the effect of *dpp* RNAi on extraembryonic development has been assessed using early markers for the amnion (*Tc-pnr*) and a dorsal sector of the serosa anlage (*Tc-doc*). *Tc-dpp* RNAi abolishes *Tc-pnr* as well as *Tc-doc* expression. However, only amnion development is suppressed, while the serosa primordium is merely reduced. In *Tribolium*, the specification of the serosa strongly depends on anterior signaling-input from a receptor tyrosine kinase ('terminal system'). In *Oncopeltus*, *dpp* RNAi suppresses the invagination of the germ rudiment, which might depend on the specification of amniotic tissue, but does not interfere with serosa development.

### Expression of *zen* in the Optic Field

*Drosophila zen* has also an embryonic function. In wildtype embryos of *Drosophila*, regulatory genes that establish the optic field, such as *sine oculis* (*so*) and *eyes absent* (*eya*), are repressed at the dorsal midline in response to *zen*, which prevents a cyclopic phenotype.<sup>40</sup> However, in *Megaselia* and *Episyrphus*, *zen* RNAi does not affect the visual system. In these species, the specification of amnion-competent blastoderm under the control of *dpp* might be sufficient for splitting the optic field into left and right parts. In *Drosophila*, genes of the optic field may have acquired Zen binding sites in their *cis*-regulatory DNA. This model implies a repressor function of Zen. Alternatively, *Drosophila* genes of the optic field may have retained the ancestral, Dpp-dependent regulatory mechanism. In this case, it should be possible to rescue the phenotype of *zen*-mutant embryos in the optic field by increasing Dpp activity along the dorsal midline.

### The Amnioserosa Gene-Network in Evolutionary Perspective

Above, we argued that in the *Drosophila* lineage, the loss of postgastrular *zen* expression abrogated serosa development and resulted in the formation of a single extraembryonic epithelium, while boundary conflation of the amnion-competent cellular blastoderm and the *zen* expression domain (serosa anlage) generated a uniform extraembryonic primordium with early serosal features. This simple model provides an evolutionary framework for understanding gene functions in the amnioserosa. In closing this chapter, we discuss a few examples that may serve to illustrate this point.

After stage 8 (when *zen* is shut off), the maintenance of the amnioserosa depends on genes of the *u-shaped* group (*ush*-group), such as *u-shaped* (*usb*), *dorsocross* (*doc*), *hindsight* (*hnt*), *tail-up* (*tup*) or *serpent* (*srp*).<sup>41-43</sup> These genes are unrelated but share similar germband-retraction and dorsal-closure phenotypes due to the precocious disintegration of the amnioserosa. All of them are co-expressed with *zen* (see Berkeley *Drosophila* Genome Project at <http://www.fruitfly.org/cgi-bin/ex/insitu.pl>). However, none of them is required for the specification of the amnioserosa. In *Anopheles*<sup>24</sup> and *Megaselia* (our unpublished data), *doc*, *tup* and *hnt* are expressed in the amnion. Thus, it is possible that the maintenance functions of *ush*-group genes in the amnioserosa relate to functions of their homologs in the amnion of less derived dipterans.

Two of these genes, *doc* and *hnt*, activate *Krüppel* (*Kr*) in the late amnioserosa.<sup>42,43</sup> In *Megaselia*, the putative ortholog of *Kr* (*Mab-Kr*) is expressed in the early serosa and later in the amnion. In the early serosa, *Mab-Kr* transcripts appear with gastrulation and persist at least until the formation of the amnioserosal fold. In the amnion, expression starts with stomodeum formation (like in *Drosophila*), i.e., after the completion of the serosa and persists at least until germband retraction (Fig. 5). The temporal correlation suggests that *Kr* expression in the amnioserosa is homologous to the amniotic expression in *Megaselia*. Consistent with this hypothesis, *Mab-zen* RNAi embryos lack the early (serosal),<sup>18</sup> but not the late (amniotic) phase of extraembryonic *Mab-Kr* expression.<sup>28</sup> Conversely, serosal *Kr* expression in *Megaselia* (and *Episyrphus*, S.L. unpublished observation) has no equivalent in *Drosophila* and may have been lost in this lineage because postgastrular serosa development is suppressed.

Another example, *pannier* (*pnr*), encodes a GATA transcription factor, which is expressed in the early amnioserosa and in the dorsal epidermis.<sup>44-46</sup> However, in the amnioserosa, the activity of *pnr* appears to be blocked and both the *pnr* transcript and the *pnr* protein disappear during stage 9.<sup>47,48</sup> In *Megaselia* and *Tribolium*, *pnr* is expressed in the early amnion and adjacent embryonic tissue.<sup>18,23</sup> In *Megaselia*, amniotic *pnr* transcript (protein data are not available) lasts until the early expansion phase of the serosa, which is roughly comparable to the stage when *pnr* is down-regulated in *Drosophila*. Thus, it is possible that the ancestral extraembryonic function of *pnr* was restricted to the early phase of amnion development. According to our model, such a function should have been lost with the conflation of the boundaries of amnion-competent and serosal blastoderm. Hence, we propose that *pnr* expression expanded into the amnioserosa without consequences because another factor in this tissue repressed its activity.



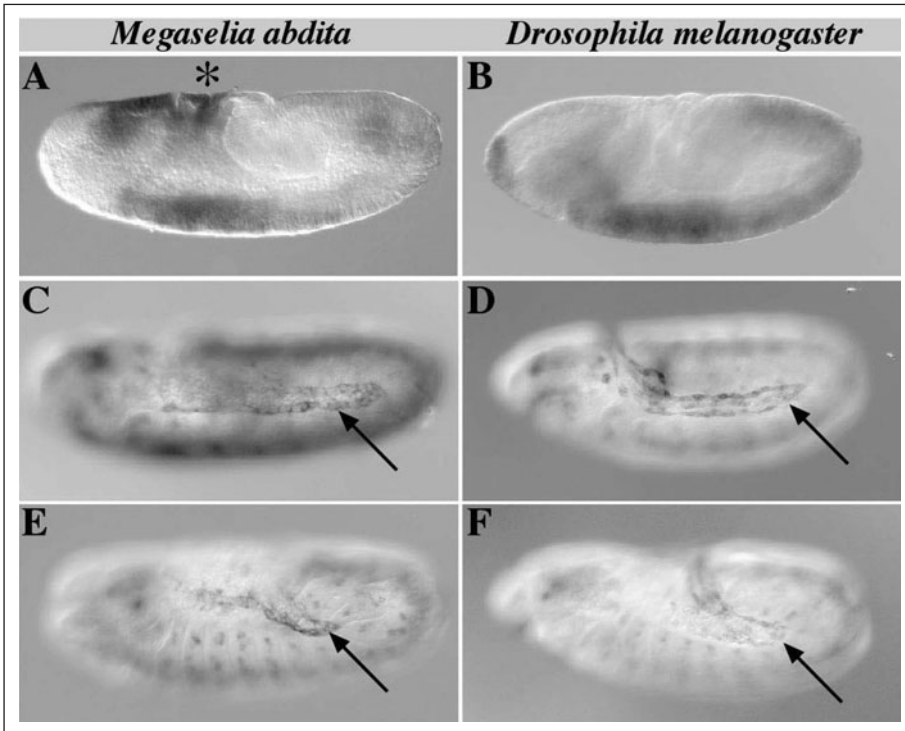


Figure 5. Expression of *Mab-Kr/Kr* transcripts in *Megaselia abdita* and *Drosophila melanogaster*. Matching consecutive stages of *Megaselia* (left) and *Drosophila* (right) are shown during gastrulation (A,B), shortly before germband retraction (C,D) and during germband retraction (E,F). Early serosal (asterisk) and amniotic/amnioserosal *Kr* expression domains (arrows) are marked. Anterior is left and dorsal up.

As a final example of how our hypothesis for the origin of the amnioserosa can provide context for the function of genes in the amnioserosa, we turn to the homeobox gene *C15*, which is activated in the amnioserosa anlage and a narrow strip of adjacent dorsal ectoderm by direct *cis*-regulatory input of *dpp* effectors and Zen.<sup>49</sup> *C15* protein persists throughout the lifetime of the amnioserosa,<sup>43</sup> but *C15* null mutations do not interfere with the formation of viable larvae.<sup>50</sup> Thus, *C15* might affect amnioserosa development in minor ways. In *Megaselia*, *C15* is predominantly expressed in the early amnion and in the dorsal epidermis.<sup>18</sup> Transcript expression in the amnion fades before the expansion of the serosa, which is consistent with a developmental role of this gene in the early amnion. As in the case of *pnr*, the early amniotic function may have been lost in the *Drosophila* lineage as the boundary between amnion-competence and *zen* expression dissolved.

## Conclusion

Changes in the expression of *zen* accompanied major reorganizations in extraembryonic development both at the morphological and the regulatory level. At this point, however, the data matrix for any molecular feature of extraembryonic development in insects remains very patchy. With this limitation in mind our tentative conclusions from the above discussion are as follows:

1. The ancestral extraembryonic role of *zen* in pterygote insects was in the mature serosa and in parts of the mature amnion, mediating katatrepsis or a related blastokinetic movement. Holometabola abandoned katatrepsis but retained the functions of *zen* in controlling

- rupture of the amniotic cavity and in generating a continuous serosa-amnion epithelium. The loss of amniotic *zen* expression may underlie the suppression of this process in some holometabolous lineages.
2. Zen-dependent serosa specification evolved before or during the early radiation of Holometabola.
  3. In the course of dipteran evolution, the ratio of extraembryonic to embryonic blastoderm was reduced and the ratio of *zen*-positive to *zen*-negative amnion-competent blastoderm was increased. The underlying mechanisms involved posterior derepression of *zen* and enhanced transport of Dpp towards the dorsal midline.
  4. In schizophoran flies, postgastrular *zen* expression was lost. This change in *zen* expression may have triggered the origin of the amnioserosa in the schizophoran lineage by suppressing postgastrular serosa development, while allowing the completion of a dorsal amnion.
  5. After the origin of the amnioserosa, extraembryonic development became entirely dependent on *zen*. This change in the function of *zen* may have evolved in concert with a novel broad dorsal expression domain of *zen* in the early blastoderm and bistable Dpp signaling activity in the cellular blastoderm.
  6. The requirement for *zen* activity in the optic field evolved with the loss of *zen*-independent specification of amnion-competent blastoderm.

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