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Oncopeltus fasciatus zen is essential for serosal tissue function in katatrepsis

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Abstract

Unlike most Hox cluster genes, with their canonical role in anterior–posterior patterning of the embryo, the Hox3 orthologue of insects has diverged. Here, we investigate the *zen* orthologue in *Oncopeltus fasciatus* (Hemiptera:Heteroptera). As in other insects, the *Of-zen* gene is expressed extraembryonically, and RNA interference (RNAi) experiments demonstrate that it is functionally required in this domain for the proper occurrence of katatrepsis, the phase of embryonic movements by which the embryo emerges from the yolk and adjusts its orientation within the egg. After RNAi knockdown of *Of-zen*, katatrepsis does not occur, causing embryos to complete development inside out. However, not all aspects of expression and function are conserved compared to grasshopper, beetle, and fly orthologues. *Of-zen* is not expressed in the extraembryonic tissue until relatively late, suggesting it is not involved in tissue specification. Within the extraembryonic domain, *Of-zen* is expressed in the outer serosal membrane, but unlike orthologues, it is not detectable in the inner extraembryonic membrane, the amnion. Thus, the role of zen in the interaction of serosa, amnion, and embryo may differ between species. *Of-zen* is also expressed in the blastoderm, although this early expression shows no apparent correlation with defects seen by RNAi knockdown.

Keywords: Hemiptera; Extraembryonic membrane; Serosa; Amnion; Katatrepsis; Blastokinesis; *zen*; *Hox3*; RNA interference; Evolution of gene function

Introduction

In the field of evolutionary developmental genetics, the bilaterian-wide conservation of the Hox genes' role in anterior–posterior axis specification is well known. Against this backdrop, the evolutionary history of *Hox3* in the arthropods is a striking case study of changes in gene function. The arthropod groups of the chelicerates and myriapods possess canonical *Hox3* orthologues, similar in sequence to those seen in deuterostomes (McGinnis and Krumlauf, 1992; Wada et al., 1999). They also retain Hox-like expression, with the primary site of expression between that of *Hox2* and *Hox4*, in the anterior part of the segmented embryo. However, within the insect lineage this locus is particularly rapidly evolving, with multiple instances of change in gene function and of gene duplication (Fig. 1).

At some point during insect evolution, *Hox3* lost its function in axial patterning and took on roles in the extraembryonic tissues. For example, in holometabolous insects such as the beetle *Tribolium castaneum* and the fruit fly *Drosophila melanogaster*, *zen*, the *Hox3* homologue, is required to pattern the extraembryonic tissues of the amnion and serosa but not the embryo itself (Rushlow et al., 1987; van der Zee et al., 2005; Wakimoto et al., 1984). Exactly when the Hox3-to-zen change in function occurred is uncertain. Available data suggest that it occurred after the divergence of the hexapod–crustacean clade (Cook et al., 2001) from the chelicerates and myriapods (Abzhanov et al., 1999; Damen and Tautz, 1998; Hughes and Kaufman, 2002; Telford and Thomas, 1998). The basal apteryogote insect *Thermobia domestica* (Thysanura) may represent a potential intermediate stage in the change in function, with a dual Hox-like (embryonic) and zen-like (extraembryonic) expression pattern (Hughes et al., 2004). Expression is zen-like and no longer embryonic in the neopteran insect order Orthoptera, as seen in the grasshopper *Schistocerca*

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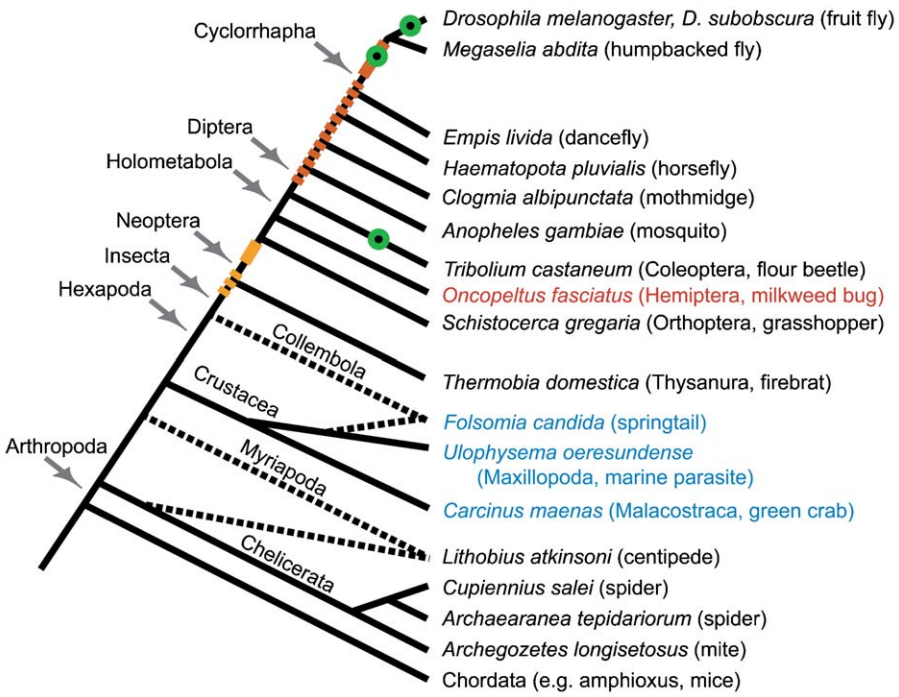


Fig. 1. Evolution of the Hox3 locus mapped onto a phylogeny of the relevant taxa. Green circles denote instances of gene duplication, leading to: the *bicoid* gene in cyclorrhaphan flies, and the two independent *zen* genes in *Drosophila* species and in *T. castaneum*. Orange and red indicate a change in function. The solid orange line indicates where, in the lineage leading to the neopteran insects, the change in function from a homeotic Hox-like role to an extraembryonic zen-like role could have taken place. The solid red line represents the divergence in function of zen and bicoid in the cyclorrhaphan fly lineage. The dashed orange and red lines encompass the lineages of species with composite expression patterns between old and new functions. Species listed in blue are those for which no expression data have been reported. The dashed black lines denote uncertainty in the phylogenetic position of the myriapods (Cook et al., 2001; Giribet et al., 2001; Hwang et al., 2001; Mallatt et al., 2004; Negrisolo et al., 2004), and of the collembolans (Cook et al., 2005; Kjer, 2004; Kristensen, 1991; Mallatt et al., 2004; Nardi et al., 2003), within the arthropods. Please see text for Hox3/zen/bcd references.

gregaria (Dearden et al., 2000; Falciani et al., 1996). Extraembryonic expression has been retained in all of the holometabolous insects studied (Doyle et al., 1986; Goltsev et al., 2004; Stauber et al., 1999, 2002; Terol et al., 1995; van der Zee et al., 2005). More specific pinpointing of the change in function, or tracking of progressive changes, will have to await further data, possibly from relevant taxa for which there is some sequence data (Cook et al., 2001; Mouchel-Vielh et al., 1998).

Subsequently, the ancestral *zen* gene has undergone at least three independent duplication events within the Holometabola. Independent duplications in the beetle *Tribolium castaneum* (Coleoptera) and in the *Drosophila* genus generated a second *zen* gene (*Tc-zen2*: Brown et al., 2001; *Drosophila z2*: Negre et al., 2005; Pultz et al., 1988). In the beetle, a recent study shows that these paralogues have diverged in function (van der Zee et al., 2005). In contrast, *zen* and *z2* in *Drosophila melanogaster* are apparently redundant: they have overlapping expression patterns and *zen* alone is sufficient for normal function; deletion of *z2* produces no embryonic defects (Rushlow et al., 1987). The other dipteran *zen* duplicate, with a rather more interesting fate, is the gene *bicoid*, encoding the maternal, anterior determinant of cyclorrhaphan flies, which has been treated extensively elsewhere (see, e.g., Hsia and McGinnis, 2003).

Here, we address the product of the original change in gene function: *zen* and its role in extraembryonic tissue. In most insects, there are two distinct extraembryonic membranes: the amnion, surrounding the embryo, and the serosa, surrounding the whole egg. During the process of katatrepsis, these membranes fuse and contract so that the embryo emerges from the yolky interior to occupy the surface of the egg. This embryonic movement is not seen in cyclorrhaphan flies, where the extraembryonic membrane is a derived, single, fused tissue: the amnioserosa (Schmidt-Ott, 2000). In either situation, during later embryogenesis the fate of the extraembryonic tissue is the same: at dorsal closure the embryonic flanks extend to make an intact body and replace this tissue, which subsequently dies.

We report *zen* expression and function in the milkweed bug *Oncopeltus fasciatus*. This bug is a hemimetabolous insect with well-developed extraembryonic membranes and a single known *Hox3* orthologue (described here and denoted *Of-zen*). As a member of the order Hemiptera, it derives from a lineage that probably occupies a phylogenetic position intermediate to the Orthoptera and Holometabola (Gullan and Cranston, 2000, p. 188, and references therein). Thus, it can be readily compared with previously examined species. Furthermore, the ability to specifically block gene function by RNA interference (RNAi) in *Oncopeltus* enabled us to functionally assay of the role of *zen* (Hughes and Kaufman, 2000; Liu and Kaufman, 2004a).

The evolution of *zen* within the insect lineage is an intriguing story, but with gaps in our knowledge concerning the ancestral function of zen and its evolutionary trajectory. At present, we have only expression data for many species, and these data

show several interspecific differences. Functional data are only available from the unrepresentative system of the fruit fly, and a study of recently diverged beetle paralogues (van der Zee et al., 2005). The *Oncopeltus* results presented here provide the first functional data on *zen* outside the Holometabola. Overall we contribute an additional data point to the existing comparative framework, providing material for addressing the evolution of *zen*, and for consideration of extraembryonic tissue function and of embryonic movements (katatrepsis) in a distinct insect order.

Materials and methods

Oncopeltus cultures

These experiments made use of two different laboratory cultures of *Oncopeltus fasciatus*: one in the laboratory of T. Kaufman, Department of Biology, Indiana University, Bloomington, USA, and one in the Department of Zoology, University of Cambridge, UK. Husbandry conditions are similar at both sites (Hughes and Kaufman, 2000). Collected eggs were incubated at 25°C. Both cultures were used for *zen* gene sequence determination, in situ hybridization to wild type embryos, and for maternal RNA interference experiments.

Isolation, identification, and analysis of the Of-zen gene

The *Of-zen* coding sequence was isolated by degenerate PCR targeting the homeobox, followed by 3′ and 5′ RACE PCR (BD SMART RACE cDNA amplification kit, BD Bioscience). In addition to standard degenerate homeobox primers (Cook et al., 2001), we used a Hox3-specific forward degenerate primer: KRARTAYT (5′-AGC GGG CCC GGa cng cnt way c-3′). The sequence of RACE PCR clones was confirmed by amplification of the coding sequence from cDNA template with gene specific primers. The complete coding sequence has been deposited in GenBank under the accession number DQ248972.

Embryo fixation

Embryos of all stages were fixed as described previously for blastoderm stage (Liu and Kaufman, 2004a), except that formaldehyde was used at 4% in PBS/0.1% Tween-20 for both prefixation before dechorionation (at least 5 min) and for subsequent fixation (1–1.5 h).

In situ hybridization

An in situ probe to *Of-zen* was synthesized from a 776-bp fragment generated by 3′ RACE (from the 3′ portion of the homeobox through the 3′ UTR and poly-A tail: see Fig. 2c). We synthesized probes and used a hybridization protocol based on Liu and Kaufman (2004a), with the following modifications: the initial soak in RIPA detergent was optional; prehybridization at 60°C was at least 1 h; hybridization with probe was overnight (~15.5–18.5 h). Probes were used at 0.07–0.4 ng/μl.

Maternal RNAi

Double-stranded RNA (dsRNA) was synthesized as described previously (Liu and Kaufman, 2004a, 2005). Single-stranded RNA (ssRNA) was transcribed from linearized plasmid template with the Ambion MEGAscript kit except that synthesis from an SP6 promoter was with the Roche SP6 RNA polymerase enzyme and buffer and Ambion reagents. Annealing to produce the dsRNA was either performed before or after ssRNA purification. As reported previously, the latter method gives a cleaner product when inspected by gel electrophoresis; however, biologically there did not seem to be a difference in potency or toxicity. dsRNA was resuspended in 1× injection buffer at 2 μg/μl.

Three dsRNA constructs were used for *Of-zen*: a 3′ RACE fragment (ds i: 776 bp; as used for in situ hybridization), a 354-bp fragment including 13 bp of the 5′ UTR and the homeobox (ds ii), and a 400-bp fragment extending from the coding sequence into the 3′ UTR (ds iii; see Fig. 2c). Additionally, dsRNA to *hunchback* (1124 bp 3′ RACE fragment) and *decapentaplegic* (325 bp) were used as controls in one of the experiments. In all experiments, injection of 1× injection buffer was used as a negative control.

Virgin adult females were injected as described previously (Liu and Kaufman, 2004a). Injections were performed with a Hamilton 801RNE 10μl syringe with either a 32 or 26s gauge point #2 removable needle. The needle was cleaned between treatments by washing with ELIMINase (Decon Laboratories), or, more effectively, with Original Fairy Liquid (Procter and Gamble), and then rinsing with dH_20. The control treatment was injected after other treatments to confirm that the needle had been cleaned thoroughly. Females were provided with two males and tended to lay a single daily clutch averaging 14 eggs. Checks for egg laying were conducted approximately every 4 h, and collected eggs were incubated at 25°C. RNAi experiments were run for 15 to 21 days. Females that molted to adulthood shortly before injection (≤1 week before) tended to take longer after injection to begin egg laying. On the other hand, females that were older at the time of injection (≥10 days after molt) tended to lay more wild type eggs before the effects of RNAi could be seen in the progeny. This is probably due to the completion of more oocytes prior to injection in older females, such that deposition of dsRNA in the oocytes was relatively delayed compared to younger females.

Nuclear staining

Counter staining for nuclei (1.5 μg/ml SYTOX Green, Molecular Probes, or 1 μg/ml DAPI, Sigma; in PBS/0.1% Tween-20 for 1 h followed by washing for 1–2 h) was visualized under UV light. For confocal microscopy, embryos were stained for nucleic acid material (100 μg/ml propidium iodide, Sigma, staining as above; 0.6 μg/ml YO-PRO-1 iodide, Molecular Probes, staining for 30 min, as above) and simultaneously treated with RNase A (Qiagen, 1 μg/ml).

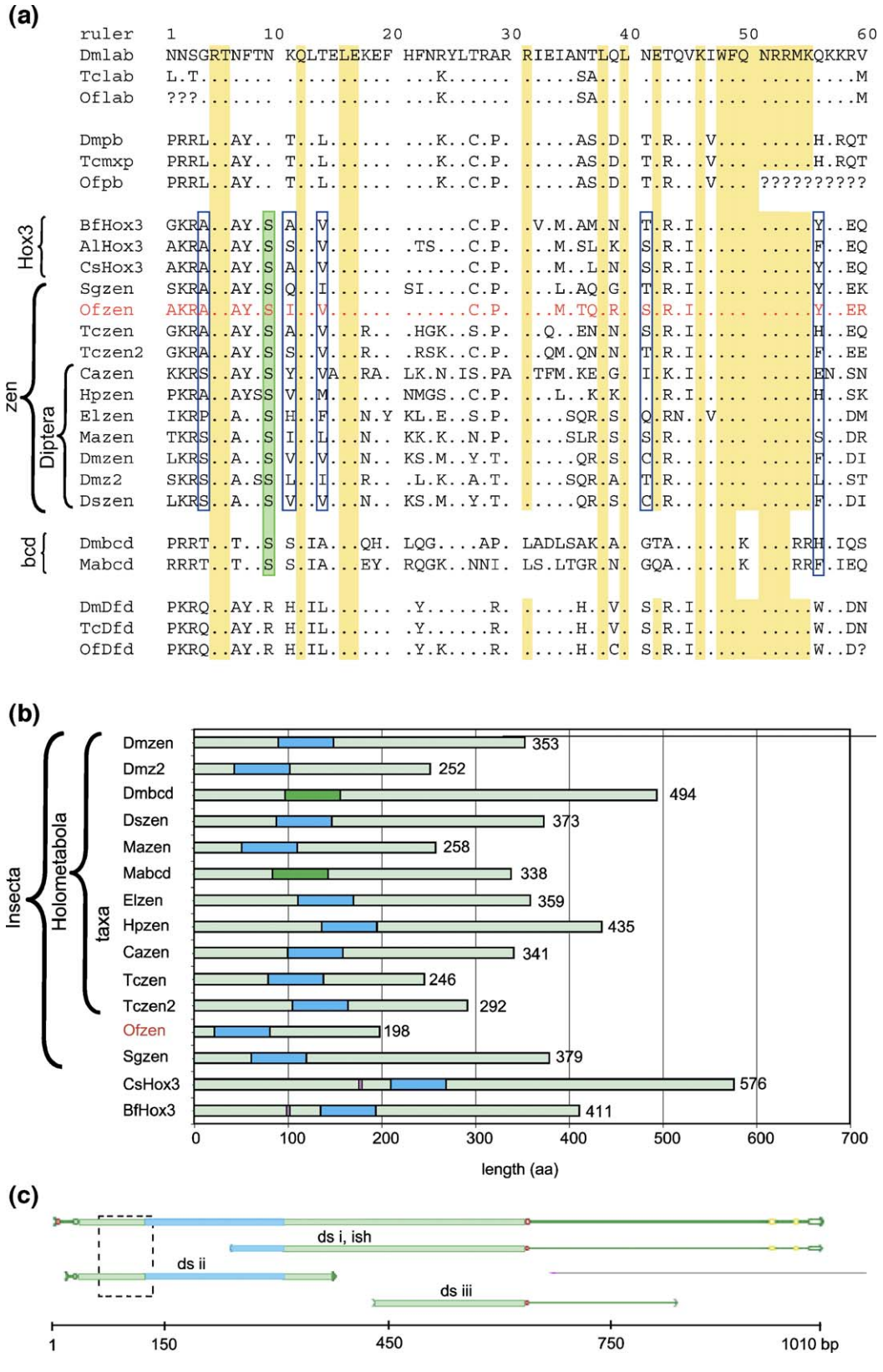
Cryosectioning

Embryos were stained with 2% methylene blue/1% boric acid, embedded in O.C.T. compound (Miles Diagnostics), frozen at −20°C, and sectioned manually on a glass slide with a razor blade.

Fig. 2. (a) Comparison of homeoboxes of Hox3 orthologues and of other anterior Hox cluster genes from *Drosophila*, *Tribolium*, and *Oncopeltus*. Amino acid residues are highlighted according to the degree of conservation: yellow = conserved (with the possible exception of bicoid), green = unique to Hox3 orthologues, blue outline = uniquely varies in Hox3 orthologues. Of-zen is highlighted in red. Other Hox gene name abbreviations: lab, labial; pb, proboscipedia; mxp, maxillopedia; bcd, bicoid; Dfd, Deformed. All pb orthologues are identical, as are Dm-Dfd and Tc-Dfd, Dm-zen and Ds-zen, and Tc-lab and Of-lab. Hox3 orthologue sequences included here are those of relevant taxa (black text in Fig. 1) for which the complete homeobox sequence is known. GenBank accession numbers: *Drosophila melanogaster*: Dm ANT-C (lab, pb, zen, z2, bcd, Dfd) AE001572-4; *Tribolium castaneum*: Tc-lab AF231104; Tc-mxp (pb) AF187068; Tc-zen, zen2, Dfd AF321227; *Oncopeltus fasciatus*: Of-lab AY627364; Of-pb AF279337; Of-zen DQ248972; Of-Dfd AY856073. *Branchiostoma floridae* (amphioxus) Hox3 X68045; *Archegozetes longisetosus* Hox3 AF085352; *Cupiennius salei* Hox3 AJ005643; *Schistocerca gregaria* zen X92654; *Clogmia albipunctata* zen AJ419659; *Haematopota pluvialis* zen AJ419660; *Empis livida* zen AJ419661; *Megaselia abdita* zen AJ133025; Ma-bcd AJ133024; *D. subobscura* X78058. (b) Whole protein comparison of Hox3/zen orthologues, with the homeodomain (Hox3/zen: cyan, bicoid: green), hexapeptide motif (where present: purple), and length of coding sequence indicated. Of-zen is highlighted in red. Species abbreviations as above. (c) Schematic of the *Of-zen* gene structure. Top line represents the complete cDNA transcript. The three different regions from which dsRNA was prepared are indicated below (ds i–iii), as is the region from which an in situ hybridization probe was synthesized (ish). Colored motifs: stop codons (red), start codon (first dark green box), coding sequence (light green), homeobox (cyan), ribosome drop-off signals (yellow), poly-A tail (final dark green box). Dashed box indicates the 98-bp region in which a large intron is probably located. Graphic based on Sequencher v. 4.2.2 overview diagram.

Microscopy and image processing

Images taken on dissecting and compound microscopes were captured with digital cameras driven by commercial software. Where indicated, manipulation consisted of manually stacking images taken at different focal depths to produce a single image. Embryos viewed with a confocal microscope were mounted in Vectashield (Vector Laboratories) under coverslips positioned with blu-tack. Specimens were visualized with a Leica TCS SP confocal



microscope with Ar 488 nm and Kr 568 nm lasers, and image data captured with Leica Confocal Software version 2.5 (Leica Microsystems, Heidelberg GmbH). Except where otherwise noted, optical sections of 10 μm over a 160-μm range were compiled into single image files using the 'projection' mode. Fluorescent images were adjusted for hue for the sake of consistency of images as well as best preservation of information in conversion from RGB to CMYK color modes.

Results

A single Of-zen gene has a typical Hox3/zen homeobox, and encodes an unusually small protein

There appears to be a single class 3 Hox gene in the milkweed bug. Two independent degenerate PCR screens with different primers recovered only a single sequence. One of the screens, where the forward primer was designed specifically to detect Hox3 orthologues, was particularly productive, generating 241 clones of 19 different homeobox containing genes. Among these, nine identical clones were determined to be the Hox3 orthologue, while the other 18 genes were identified as orthologues of genes belonging to other homeobox classes (Supplementary Information: Table S1). While these screens do not preclude the possibility of additional class 3 Hox genes in *Oncopeltus*, there is no expectation of additional genes based on phylogeny: a single class 3 Hox gene is known for most insect species, and lineages with two copies seem to have acquired them by independent instances of gene duplication (Fig. 1 and Supplementary Information).

The *Oncopeltus* Hox3 homeobox is typical of class 3 Hox genes (Fig. 2a). Outside of the homeobox, there are no well conserved coding sequence motifs in the *zen* genes of insects. Beyond the immediate flanking region of the homeobox, orthologues quickly become unalignable—even in the *Drosophila melanogaster* duplicates—except in the case of the recently duplicated paralogues in *Tribolium*. Previous suggestions of other conserved motifs (Falciani et al., 1996) are not borne out by alignments of more complete data sets (data not shown). However, these additional data do support two other previous observations (Falciani et al., 1996). Firstly, the hexapeptide motif typical of Hox proteins is only present in those orthologues that retain Hox-like (rather than zen-like) expression. Secondly, the homeobox of zen-like orthologues occupies a more N-terminal position within the coding sequence. Thus, sequence level identification of zen-like orthologues is limited to these two criteria and also a general tendency to encode proteins smaller than Hox-like orthologues (Fig. 2b).

The *Oncopeltus* orthologue, designated *Of-zen*, encodes the smallest Hox3/zen protein yet described, with only 198 amino acids in the coding sequence (Fig. 2b). The coding sequence is interrupted by a single intron that is located near the 5′ end of the homeobox and is probably large, as attempts to amplify across this region were unsuccessful (Fig. 2c). Constructs used for in situ hybridization and RNAi are also indicated in Fig. 2c.

Oncopeltus embryology: Blastokinesis and orientation of axes

In common with other species of the Heteroptera (Johannsen and Butt, 1941, pp. 271–280), *Oncopeltus* displays a complex mode of blastokinesis, the embryonic movements within the egg termed anatrepsis and katatrepsis (Fig. 3).

Development from the cellular blastoderm proceeds to immersion anatrepsis (for a description and definition of this process, see Heming, 2003, pp. 126–128), beginning at about 22% of embryogenesis (note time line in Fig. 3l). This mode of anatrepsis occurs by invagination of the germ rudiment (embryo proper and amnion) wholly into the yolk, in a caudad-first orientation at the posterior pole of the egg (Figs. 3b–c). The invaginating tissue then continues to grow within the yolk through germband extension and elongation of the posterior growth zone (Figs. 3d–f). The amniotic membrane is closely associated with the developing embryo and creates a ventral, fluid-filled cavity around the early embryo. The serosal membrane, which has the distinctive morphology of large, flattened, polyploid cells, wraps around both the yolk and the embryo (Johannsen and Butt, 1941, p. 272; Roth, 2004).

At about 50% of embryogenesis, the second phase of blastokinesis, katatrepsis, commences, reversing the movements of anatrepsis (Figs. 3g–j). Katatrepsis begins with a rupturing of the amnion as an intact membrane covering the embryo, and its fusion with the outer serosal membrane. The serosa then contracts towards the anterior of the egg, followed by amnion and embryo. The germband embryo emerges, head-first, from the yolk through the original site of invagination, and then migrates up the ventral side of the egg until the head of the embryo is at the anterior pole of the egg. Pulling by the serosa or amnion during contraction and also advance of the embryo have been described as active forces in this process (Butt, 1949; Dorn, 1976; Heming, 2003, p. 128). Katatrepsis is rapid: once amnion–serosa fusion has occurred, it is complete within 2% of embryogenesis (4 h).

After katatrepsis is completed, dorsal closure takes place. The lateral flanks of the embryo grow dorsally over the yolk and replace the amnion, which had served as a provisional dorsal covering. Dorsal closure is completed at the dorsal midline, in a posterior-to-anterior progression. Thus, the final location of the extraembryonic serosa (and possibly the anterior region of the amnion) is dorsal and posterior to the embryo's head (Fig. 3k), where the tissue continues to contract and sinks into the primary dorsal organ—itself becoming the secondary dorsal organ (Johannsen and Butt, 1941, p. 49), before degenerating by apoptosis (data not shown).

In describing embryogenesis we follow the convention of Liu and Kaufman (2004a), defining the axes of the egg as the same as those of the embryo in its final position prior to hatching. However, due to the nature of blastokinesis, the invaginated germband embryo rests in an opposite orientation: the anterior of the embryo is at the posterior pole of the egg, and the ventral surface of the embryo faces into the yolky interior of the egg, or toward the dorsal surface of the egg (Fig. 3f). It is only after katatrepsis has occurred about half-way through embryogenesis that axes of egg and embryo correspond. The

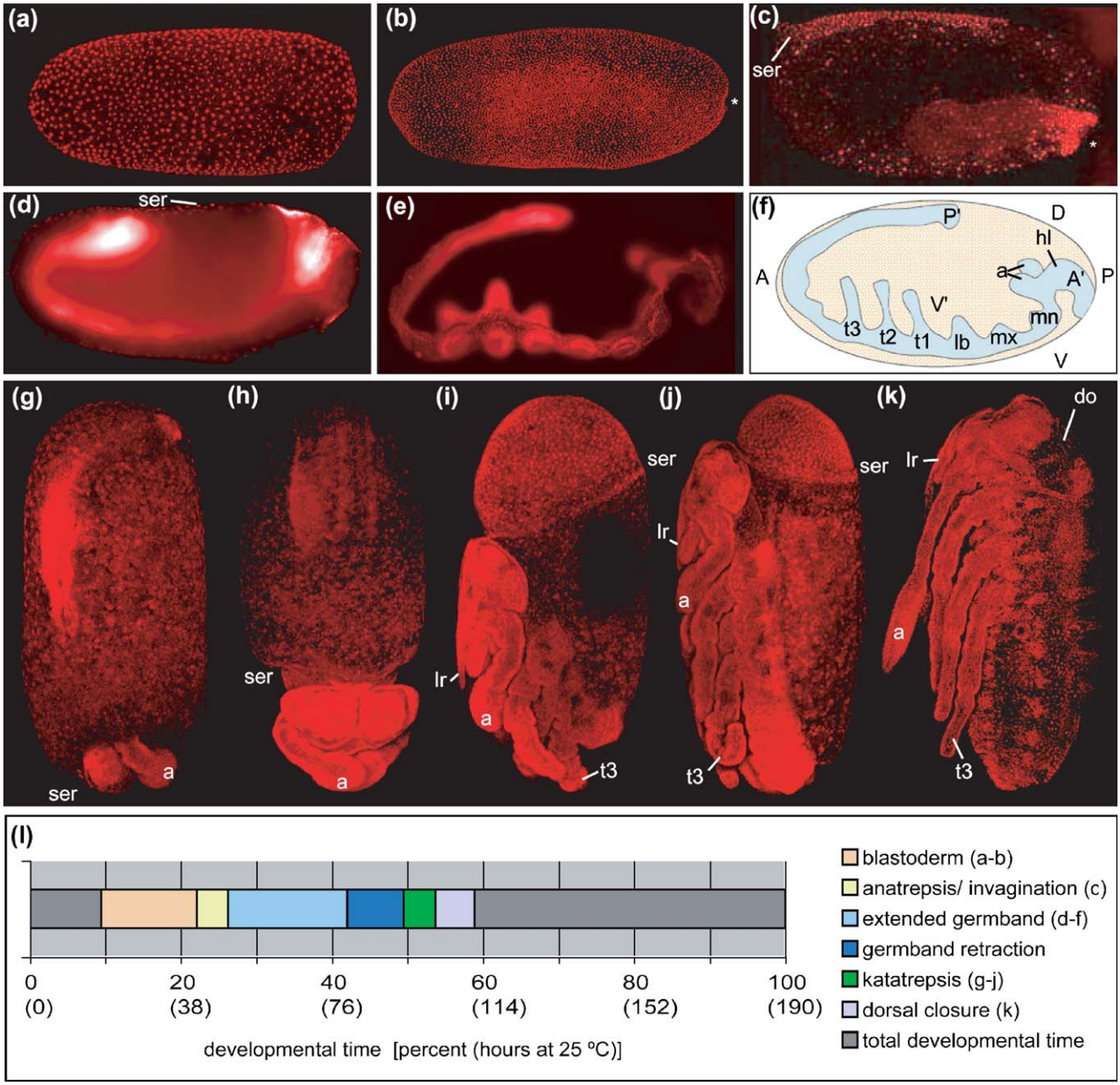


Fig. 3. Illustration of blastokinesis during *Oncopeltus* development by nuclear staining: (a) early blastoderm (18 h), (b–f) anatrepsis and early germband extension (42–54 h), (g–j) katatrepsis (94–102 h), (k) dorsal closure (102–116 h). (l) A developmental time line illustrating the duration of stages examined in this study (letters of embryo images corresponding to given stages are indicated). The size and density of nuclei on the surface of the blastoderm are initially uniform (a), but by the initiation of invagination lateral plates show increased nuclear density (b). Larger yolk nuclei can be seen below the surface (b, c). Anatrepsis occurs by invagination of the germ rudiment at a site just ventral to the posterior pole (asterisk). The developing germband embryo extends within the yolk, doubling back on itself around the anterior pole of the egg. The head lobes remain at the site of invagination and develop at the posterior pole (c–e, lateral views of successive time points). Thus, the axes of egg and embryo are reversed (f). This reverse orientation of egg and embryo is corrected during katatrepsis (g: initiating, h: early, i: mid, j: late): as the serosa (labeled at the amnion–serosa border) contracts toward the anterior pole, the amnion and embryo emerge from the yolk such that the embryo moves up the ventral surface of the egg while the amnion serves as a provisional dorsal covering. As the embryo emerges head-first at the posterior pole, head and antennae, then labrum, and lastly legs and abdomen exit the yolk. After katatrepsis, dorsal closure (k, late stage) proceeds as the embryonic flanks grow dorsal–laterally, replacing the amnion. The serosa retreats into the dorsal organ before degenerating (Butt, 1949). In anatrepsis and germband images (a–f), egg-anterior is to the left (views are lateral, with dorsal up, for stages at which dorsal–ventral orientation can be determined: b–f). In katatrepsis and dorsal closure images (g–k), anterior is to the top and dorsal is right (views are lateral except the ventral view in h). Images are single focal depths (c–e) and confocal projections (a–b, g–k; incomplete in i). The serosa has mostly been removed in (c), such that the yolk nuclei and germ rudiment are visible. The germband embryo in (e) has been dissected free of yolk and membranes. The retracted germband embryo can be seen below the surface in (g–h). Abbreviations: A–P and D–V, anterior–posterior and dorsal–ventral axes of the egg; A′–P′, V′ axis and side of the embryo; a, antenna; do, dorsal organ; hl, head lobe; lb, labial segment/labium; lr, labrum; mn, mandibular segment; mx, maxillary segment; ser, serosa; t1–3, thoracic segments/legs.

anterior of the egg is clearly distinguished throughout development by the presence of a ring of micropyles on the exterior of the chorion and a more pointed shape to that pole of the egg. Unfortunately, the dorsal–ventral axis of the egg is much less clear, and at blastoderm stages is not distinguishable without staining for marker genes (Liu and Kaufman, 2004a). Morphological distinction—either by light microscopy or inspection of a nuclear stain for nuclear density—cannot be made until the onset of invagination. Though the anterior, posterior, and ventral regions of the embryo are distinct throughout the germband stages, the dorsal surface does not properly exist until after dorsal closure; the lateral margins of the embryonic flank are the presumptive dorsal territory.

Of-zen transcripts are expressed in a complex, dynamic pattern at blastoderm stages

The course of *Of-zen* transcript expression in blastoderm stage embryos was assayed by in situ hybridization on embryos collected every 4 h, from 18 to 42 h (9–22% development), corresponding to stages from the early cellular blastoderm to the initiation of invagination (Fig. 3l: orange, Fig. 4). Compared to later stages of embryogenesis (see below), transcript expression in the blastoderm is weak, and development of the detection reaction was typically allowed to proceed for 30 h (four to twelve times longer than for later stages). Under these conditions, *zen* expression is first detected after cellularization is complete (22–26 h), with faint expression in the cytoplasm surrounding surface nuclei in the central region of the egg (Fig. 4b). It is not detectable near the anterior or posterior poles. This sparse, punctate expression then becomes stronger (26–34 h; Figs. 4c–d), but no specific pattern is discernible until 34–38 h, when a complex and apparently dynamic pattern becomes apparent.

There are three components of this blastoderm expression pattern. The first, and most strongly staining, features are the two bilateral plates of more densely packed cells (Figs. 3b, 4e–f). In Butt's (1949) descriptions these lateral plates are identified as the presumptive germ rudiment. The second feature of the *Of-zen* blastoderm expression pattern is a broad, ventral band that links the two lateral plates, but with somewhat fainter expression and more restricted extent along the anterior–posterior axis (the central ~35–40% of egg length; Fig. 4g). Finally a thin, transverse strip of staining is seen on the dorsal surface of the egg (2–3% egg length), which extends to the edge of the lateral plates (Figs. 4h–i). This region of staining corresponds to an area of increased cell density (Fig. 4i′), though the fate of these cells is unknown. Also at this stage, punctate expression can be seen below the surface of the blastoderm, associated with yolk nuclei (Figs. 4e–i). No extraembryonic expression of *Of-zen* is detected at this stage (see below for interpretation of *zen* expression in the light of fate map information).

Of-zen blastoderm expression (lateral plates, ventral band, dorsal strip) is transient. The expression in the lateral plates is detectable only beginning at 30–34 h, and the entire pattern already begins to fade at 38–42 h. The dorsal strip can only be detected in some 34–38-h embryos and at no other stage, suggesting that it is the briefest feature of expression, persisting for less than 4 h (less than 2% of embryogenesis).

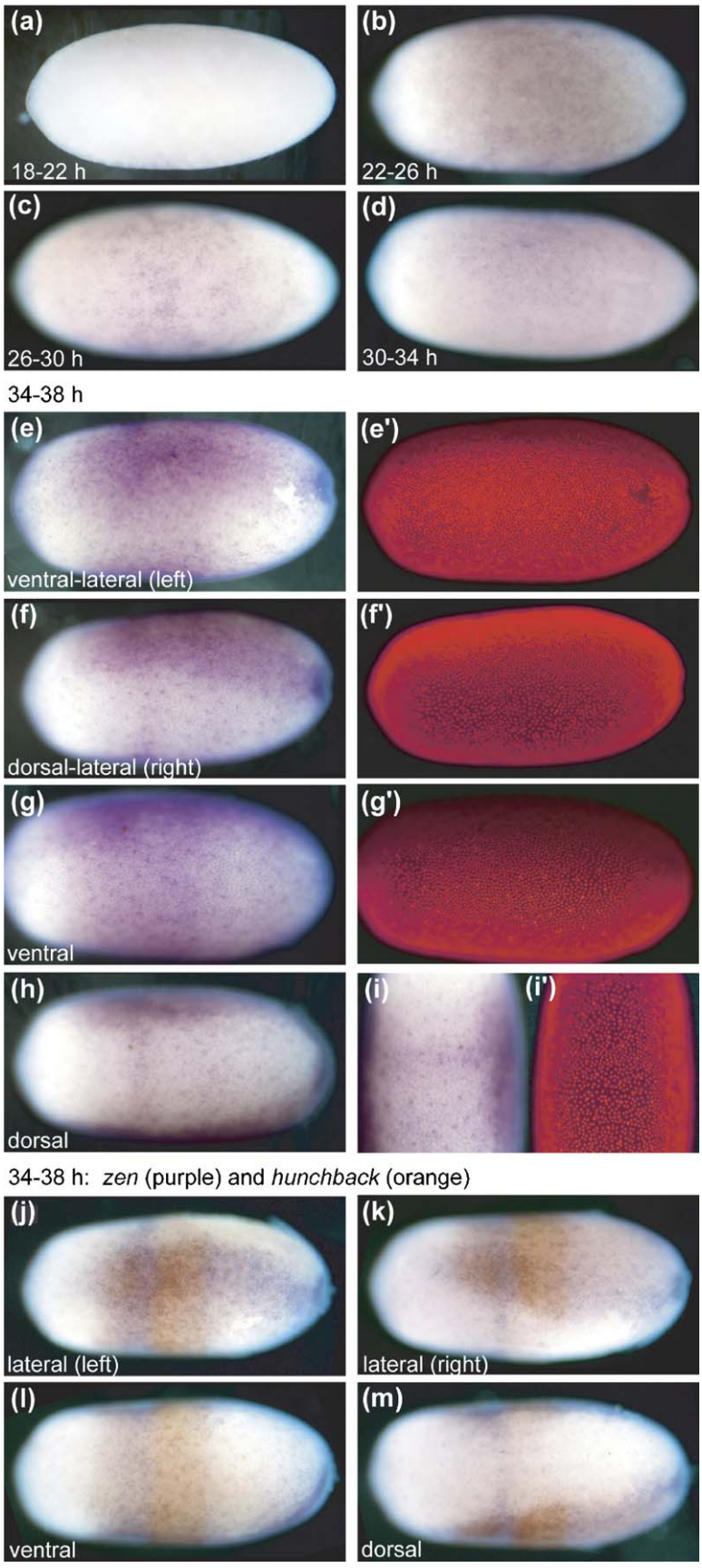
Some features of *Of-zen* expression, particularly the ventral band and central region of the lateral plates, resemble the blastoderm expression of genes involved in the early patterning of the germ rudiment, such as *hunchback* and *engrailed* (Liu and Kaufman, 2004a). However, a direct comparison of the expression of *Of-zen* and *Of-hb* indicates that though these genes have extensively overlapping expression domains, they have distinct boundaries (Figs. 4j–m). *Of-hb* expression occurs in a ventral saddle-shaped domain encompassing about 60% of egg circumference and from 25% to 60% egg length from the anterior pole, but it is cleared from the dorsal surface. The ventral expression consists of two bands: an anterior band and a more strongly staining, broader, posterior band. The *hunchback* expression is in general much more crisply defined than the *zen* expression. Together, both ventral bands of *hunchback* expression seem to occupy the same region as the *zen* ventral band. The *hunchback* bands extend dorsal–laterally to a slightly more dorsal position than the *zen* lateral plates, and with a different shape to the dorsal border of the domain. The *hunchback* bands occupy a narrower region of egg length than the *zen* lateral plates. Lastly, *hunchback* expression, similar to other embryonic patterning genes, is entirely cleared on the dorsal side of the egg, in the region of the *zen* dorsal strip.

Of-zen expression occurs in the serosa from germband stages

As the germ rudiment invaginates into the yolk, the blastoderm expression pattern of *Of-zen* fades (compare Figs. 5a with b). Strong staining is seen in the invaginating germ rudiment, but this is observed with both antisense and sense strand in situ probes (Figs. 5a–c), and is presumably nonspecific background. No clear expression is seen through the early germband stage (54 h or 28% development; data not shown).

From mid germband stages onwards (64 h, 34% development) *zen* transcript is expressed throughout the serosa but no specific expression is seen in the amnion (Figs. 5d–e, 6). The germband stains strongly after in situ hybridization to whole eggs, but this is likely to be nonspecific, as it is also observed

Fig. 4. Expression of *Of-zen* transcript at blastoderm stages: (a–d) early blastoderm stages, (e–i) peak expression in a complex pattern at 34–38 h, (j–m) expression of *zen* (purple) and *hunchback* (orange) in 34–38 h embryos. No transcript is detected at the time of cellularization (a, 18–22 h), though shortly thereafter transcript can be detected at low levels throughout the central region along the anterior–posterior axis (b–c, 22–30 h). This weak expression refines into lateral plates (d, 30–34 h; e–i, 34–38 h). Peak expression includes bilateral plates of unequal size (e: left, f: right; there is no clear bias for one side always being larger), a fainter ventral band between the lateral plates (g), and a thin strip on the dorsal surface (h, magnified in i). The features of the expression pattern, particularly the dorsal strip, seem to correspond to regions of increased cell density (corresponding nuclear stains in e′–g′, i′). Although the expression patterns of *Of-zen* and *Of-hb* are similar, they observe different dorsal and lateral boundaries (j: left, k: right, l: dorsal), and the *Of-zen* dorsal strip occurs in a region cleared of *Of-hb* transcript (m). Anterior is to the left in all views except in (i) where it is up for side-by-side comparison of the light micrograph and fluorescent nuclear stain. In later stages where it can be determined, dorsal is up except in right-side views (f, k) where ventral is up.


(a)
18-22 h
(b)
22-26 h
(c)
26-30 h
(d)
30-34 h
34-38 h
(e)
ventral-lateral (left)
(e')
(f)
dorsal-lateral (right)
(f')
(g)
ventral
(g')
(h)
dorsal
(i)
(i')
34-38 h: zen (purple) and hunchback (orange)
(j)
lateral (left)
(k)
lateral (right)
(l)
ventral
(m)
dorsal

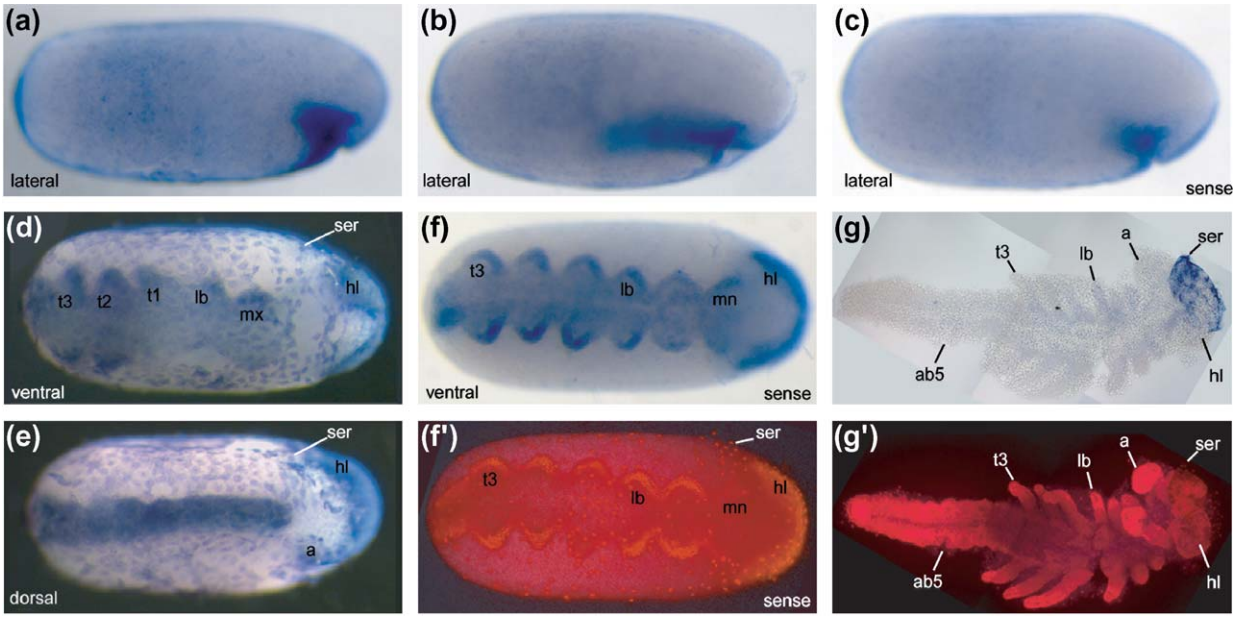


Fig. 5. Germband stage expression of *Of-zen* transcript: (a–c) embryos undergoing invagination of the germ rudiment (46–48 h), (d–f) mid germband stage (64–66 h), (g) late germband stage (70.5–76.5 h). The blastoderm stage expression in lateral plates and yolk nuclei can still be seen at the onset of invagination before stain fades to low background levels (compare a with b–c). Strong stain is seen in the invaginating germ rudiment, but is also seen with the sense strand probe (c). Later, strong nuclear staining for *zen* is seen throughout the serosa from mid germband stage (though the tissue damages easily and in some regions has been lost, d–e). The strong stain throughout the germband at this stage is also obtained with the sense strand probe, but not staining of the serosa (f). Background stain of the germband is greatly reduced when embryos are dissected free of the yolk before in situ hybridization (g). Note that the mandibular segment is not visible in (d) as the embryo curves into the yolk at that point (seen to a lesser extent in f). Orientation is egg-anterior to the left. Abbreviations are as in previous figures. Additional: ab1–10, abdominal segments.

with the sense strand probe, whereas the serosal expression is specific to the antisense strand probe (Figs. 5d–f). No *Of-zen* transcript is detected after hybridization to the dissected germ rudiment (Fig. 5g).

During katatrepsis, *zen* is expressed strongly in the contracting serosa (first seen after 1 h detection; Fig. 6) while the amnion remains clear of stain (although see Figs. 6b–c). Stain accumulation in the mandibular and maxillary stylets is most likely nonspecific, as stain accumulates with all tested probes. *Of-zen* expression is not uniform throughout the serosa. At early stages, staining appears stronger in 'creases' (Figs. 6a, c) that correspond to regions where cells are more closely packed in the contracting tissue (Fig. 6d). Throughout katatrepsis expression is stronger on the ventral than on the dorsal surface, which is the surface along which the embryonic body emerges (compare Figs. 6a and b). However, particularly clearly from mid katatrepsis onward (Figs. 6m–n), this does not correspond to variation in cell density in the tissue (Fig. 6o). Even when dorsal expression is faint, the serosal boundary with the amnion is stained for transcript. The amnion–serosa boundary becomes a thick brim of tissue in late katatrepsis, but otherwise the tightly packed cells of the serosal cap do not show variation in density. By mid katatrepsis the difference in nuclear spacing of embryo, amnion, and serosa is very distinct.

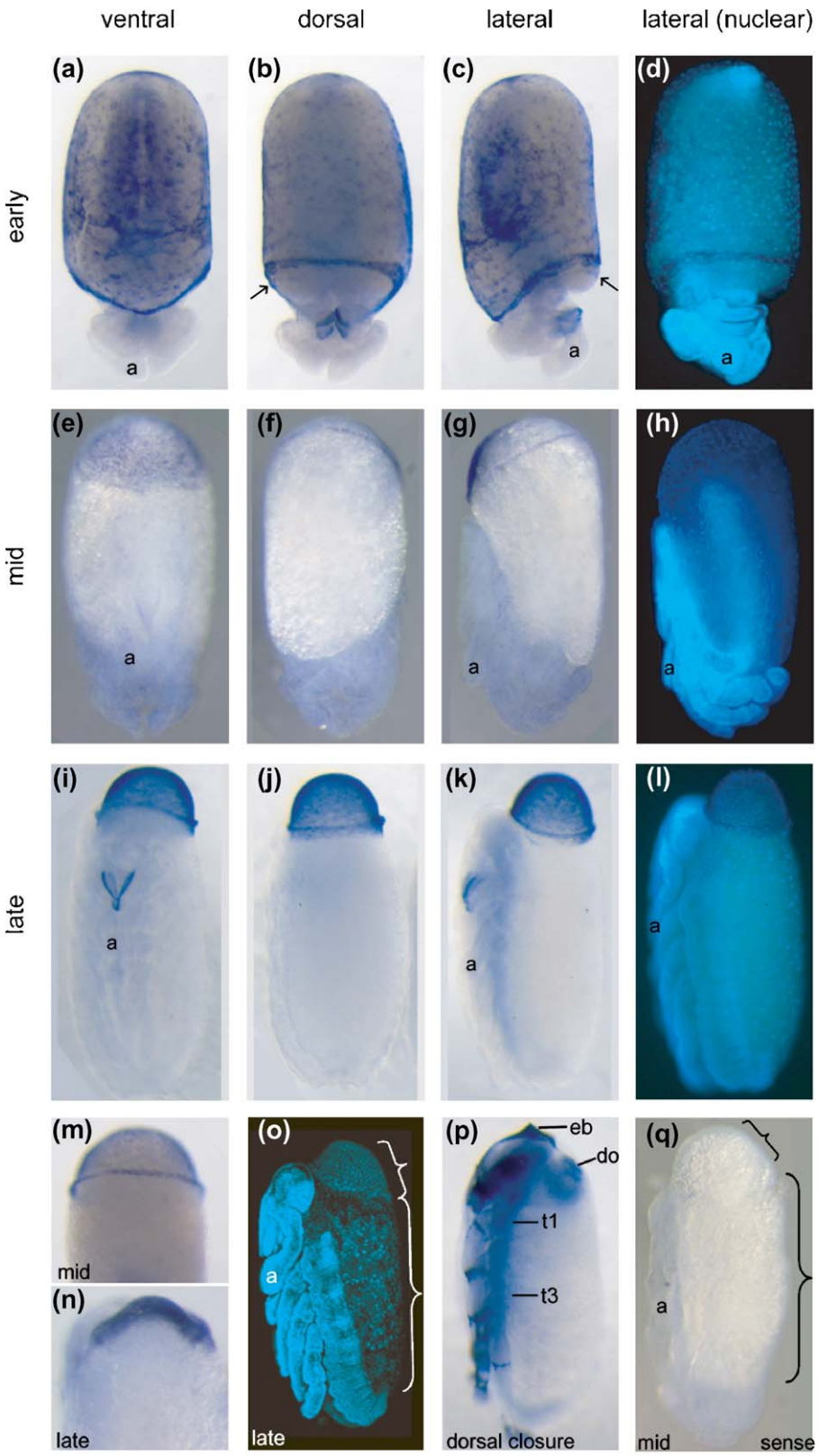
After katatrepsis, when the extraembryonic tissue has retreated into the dorsal organ, this structure stains for *Of-zen* transcript (Fig. 6p). It is not possible to follow expression further by whole egg in situ hybridization, as already at the time of dorsal closure, embryonic cuticle has begun to form on the ventral surface. This accounts for the nonspecific stain associated with the joints and distal tips of the appendages, the accessory mouthparts, and the 'egg burster'—a thickening of cuticle on the top of the head to aid in hatching (Johannsen and Butt, 1941, p. 270).

Maternal RNAi targeted to Of-zen results in a failure of katatrepsis and complete eversion of the embryo

We used maternal RNA interference to examine the embryonic function of *Of-zen*. The *Of-zen* RNAi phenotype is robust (quantitative results below), with a single suite of characteristics. Embryos fail to undergo katatrepsis and,

Fig. 6. *Of-zen* transcripts are strongly expressed in the serosa throughout katatrepsis: (a–d) early, (e–h) mid, (i–l) late stages, (p) advanced dorsal closure stage (with nonspecific background due to cuticle deposition). Variation in expression within the serosal cap (m–n) does not correspond to differences in cell density within the tissue (o), except at the thick boundary with the amnion. No signal is obtained with the sense strand probe in either the serosa or the amnion (q: mid katatrepsis stage). No expression is seen in the amnion (except possibly at the earliest stages: arrow in b–c). Embryonic background is reduced as the embryo emerges from the yolk (except for the artifact of staining in some embryos of the stylets [a–c, e, i, k]; see text). Views of each stage are of a single embryo. The antennae are labeled in ventral and lateral views as a reference point. Fluorescent images are manual stacks (composites) of single images at different focal depths (d, h, l), or a confocal projection (o). The nuclear counter stain seen in lateral view (d, h, l) is not precisely the same angle as the lateral light micrograph. In specimens not stained for *Of-zen* (o, q), curly brackets demarcate the serosa (smaller bracket) and the amnion (larger bracket). Anterior is up in all views, and in lateral views dorsal is to the right (including o–q). Images specifically of the serosal cap are in dorsal view. Abbreviations are as in previous figures. Additional: eb, egg burster.

therefore, complete development inside out, *i.e.* they are fully everted (Fig. 7). The cuticular body of the embryo is much smaller than wild type, only occupying about half of the total egg volume (wild type embryos occupy the entire space) (compare Figs. 7a and c, b and d). The head of the hatching-stage embryo remains at the posterior pole of the egg (opposite pole to the micropyles on the chorion, Figs. 7c–d), which is the normal orientation prior to katatrepsis.



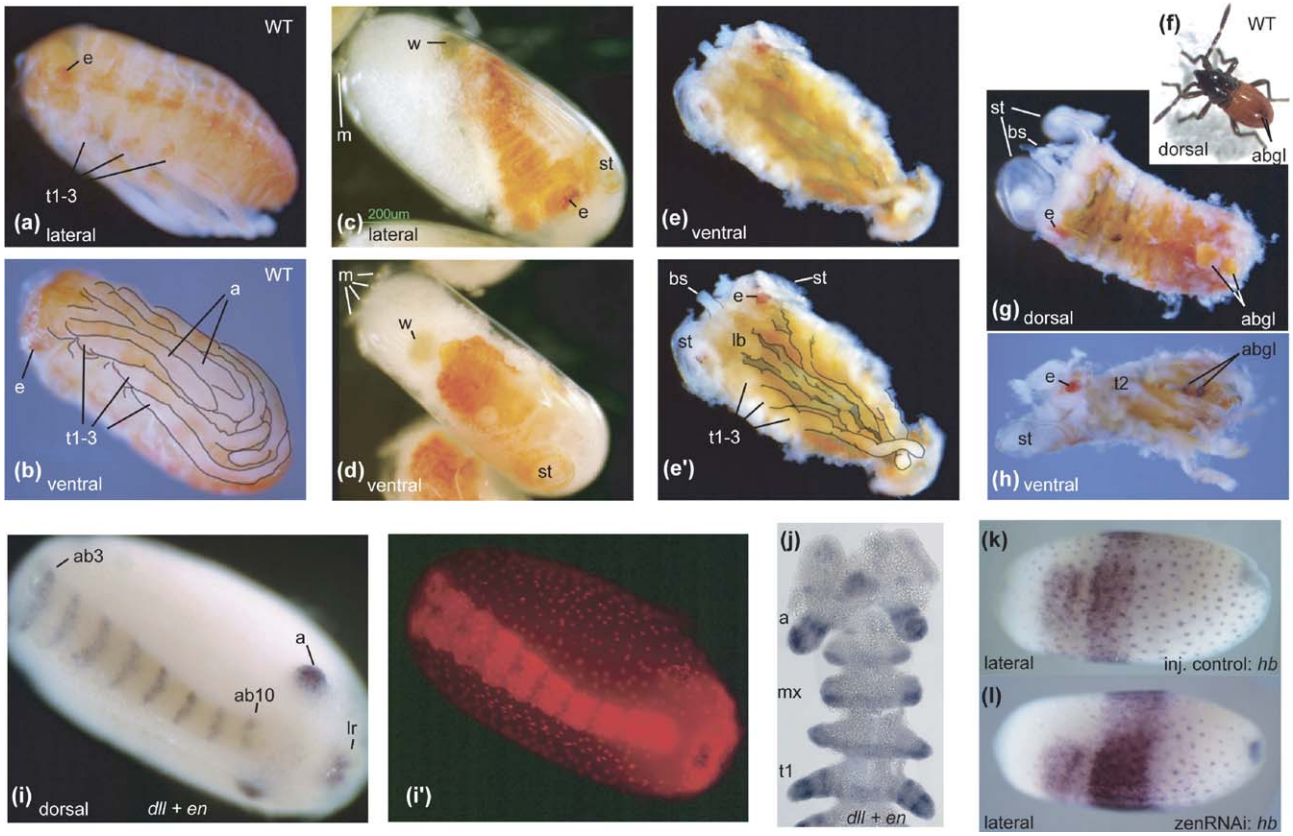


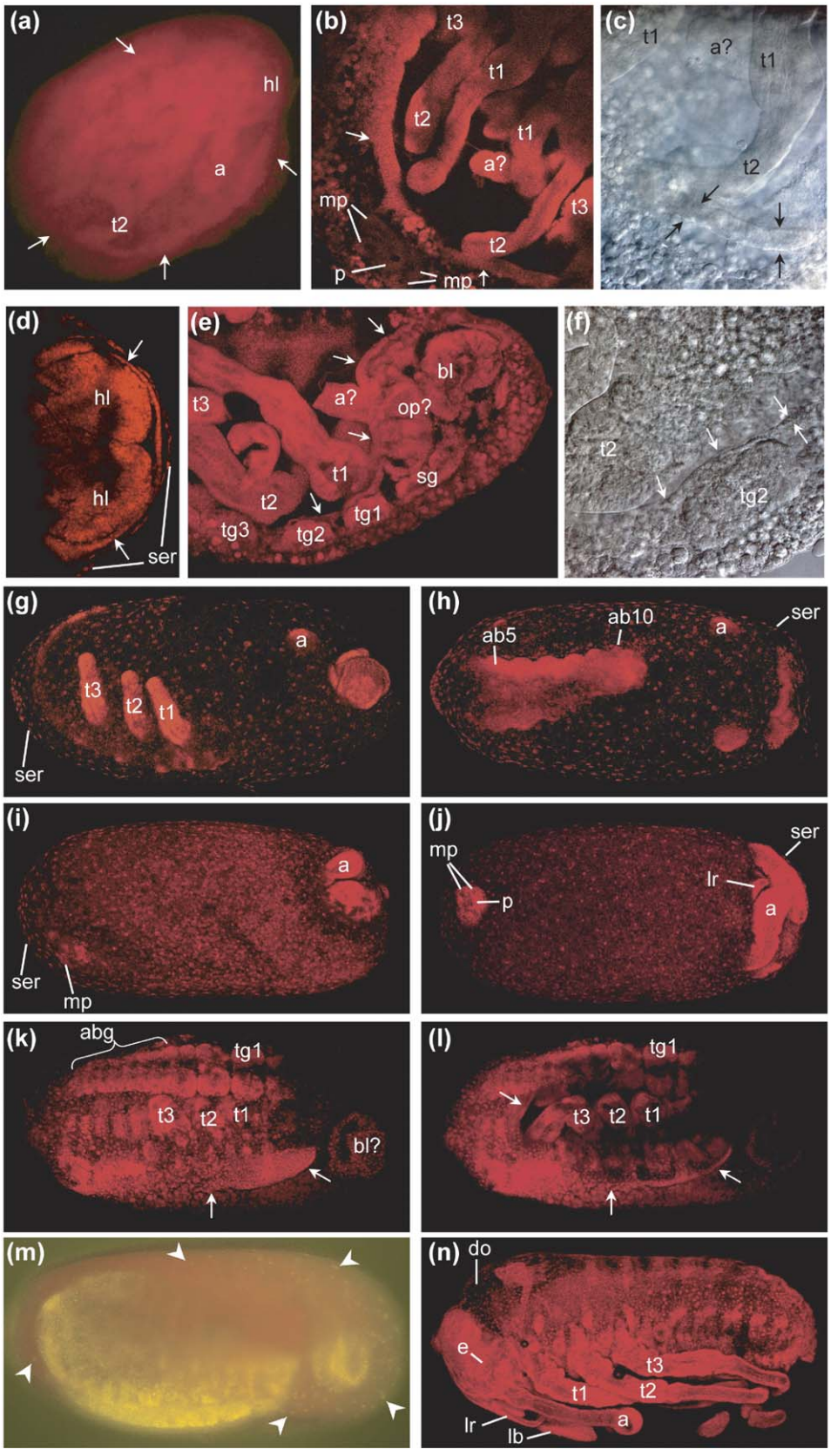
Fig. 7. *Of-zen*^{RNAi} embryos are everted. Wild type embryos shortly before hatching (a–b), are contrasted with *zen*^{RNAi} embryos at hatching age, still in the chorion (c–d). The cuticular body houses the appendages, as seen on dissection (e). The exterior of the cuticle has the outpocketings of glands (g), while on the interior the pigmentation is the same as on a wild type exterior (compare h and f). A single color in situ hybridization reaction for the marker genes *engrailed* and *distalless* indicates that in *zen*^{RNAi} embryos segmentation and appendage patterning appear normal (i–j, mid germband stage, 70–72 h). Similarly, the blastoderm expression pattern of *hunchback* does not appear to be affected by *zen*^{RNAi} (k injected control, l *zen*^{RNAi} embryo; 34–38 h). (The difference in strength of stain between the two embryos is not consistently seen across the two treatments.) Anterior is up/left in all views (egg-anterior for whole eggs, embryo-anterior for dissected specimens); dorsal is up/right in lateral views. Abbreviations are as in previous figures. Additional: abgl, abdominal glands; bs, brain stem; e, eye; m, micropyles; st, stylet; w, waste sac; WT, wild type.

Outside the cuticular body is a large white mass, which comprises both unincorporated yolk and externalized organ tissue (Figs. 7c–e, g, 8b, e, k). Dissection of the cuticular body reveals that the appendages are enclosed by the body wall (Fig. 7e). The mandibular and maxillary stylets, accessory mouthparts for piercing, are usually stored in bilateral pouches in the head. In everted embryos, these structures are clearly visible as external coils nested in organ tissue (Figs. 7c–e, g–h). On wild type hatchlings, two spots of black pigmentation on the dorsal abdomen indicate the site of two glands (Fig. 7f). These appear in reverse in everted embryos as orange out-pocketings of tissue (Fig. 7g). Turning everted embryos right-side-in reveals the corresponding black pigmentation on the internal surface of the cuticle (Fig. 7h). Thus, *Of-zen*^{RNAi} embryos have a wholly inside out topology.

Despite eversion the embryos are well developed. They show no clear signs of necrosis until after wild type hatching age (data not shown). Organ tissue appears complete, and includes Malpighian tubules, an extruded waste sac, ganglia of the central nervous system, and brain lobes, all of which are in the correct relative position despite being external (Figs. 7c–d,

Fig. 8. Fate of the extraembryonic membranes in *Of-zen*^{RNAi} embryos: the amnion (a–f) and the serosa (g–n). A thick ridge of tissue (arrows in a–f, k–l), presumably both the amnion and the embryonic epithelium, encircles the cuticular body (a), is below the serosa (d), and separates the enclosed appendages from external organ tissue (b–c, e–f). However, higher magnification microscopy of cryosectioned specimens (b and e: 20× fluorescent, c and f: 40× DIC) fails to resolve the ridge of tissue into individual cell layers. Meanwhile, the serosa disappears by the end of the dorsal closure stage. It initially appears wild type (g: lateral and h: dorsal views, 92–94 h), but later the nuclear signal is relatively weak and with higher background (i: lateral and j: dorsal, 98–106 h). Still later (k–n, 110–112.5 h), there is no trace of the serosa, although a few small, scattered nuclei remain on the surface (arrowheads in m). By this time the amnion and serosa ought to have retreated into the dorsal organ for degeneration, as indicated on a wild type clutch mate of an everted embryo (n, same stage as the embryo shown in k–m). The embryos in (a–c) and in (e–f) are cryosectioned preparations, where autofluorescent images (b, e) are confocal projections of 15–35 μm sections at the cut face. All other fluorescent images are nuclear stains of whole eggs. Confocal projections are seen in (d: z-section projection; g–k, m: full projections; l: projection of partial optical stack). (m) is a composite image from different focal depths on a compound microscope. Egg-anterior is to the left in all views. Abbreviations are as in previous figures. Additional: abg, abdominal ganglia; bl, brain lobe; mp, Malpighian tubules; op, optic plate; p, proctodaeum; sg, subesophageal ganglion; tg1–3, thoracic ganglia.

8b, e, k). Pigmentation of the cuticle is wild type in pattern, with red eyes and an orange thoracic region darkening to a red abdominal region. Strength of pigmentation also appears normal (in contrast to some embryos that arrested early and never acquired darker, red, coloration). Segments are well formed and distinguishable (Figs. 7c–d). Inspection of younger



Of-zen^{RNAi} embryos (germband stages, prior to the stage when katatrepsis would take place) indicates that both segmentation and appendage patterning appear normal, as assayed by in situ hybridization with the marker genes *engrailed* and *distalless* (Figs. 7i–j, *Of-dll*: Angelini and Kaufman, 2004; *Of-en*: Liu and Kaufman, 2004a). Also, despite the overlapping blastoderm expression patterns, blastoderm *hunchback* expression is also normal in *Of-zen*^{RNAi} embryos (Figs. 7k–l, Liu and Kaufman, 2004a).

The everted topology created by *Of-zen* RNA interference resembles that described as a consequence of physical obstruction of katatrepsis in other species:

> '[E]verted' embryos … result from suppression of katatrepsis in dragonflies (Ando 1955) and leaf hoppers (Sander 1959, 1960). In the absence of katatrepsis the amniotic cavity remains intact and, in these species, becomes lined by the flanks of the germband. These at the time of dorsal closure grow in ventral instead of dorsal direction, and face the lumen of the cavity with their outer sides which produce the cuticle. Legs and bristles then grow into the amniotic cavity, while the internal organs 'dangle' outside in the space originally occupied by the yolk system (Sander, 1976).

Thus, eversion is a consequence of the failure of katatrepsis, such that the embryo is confined by the amnion at the time of lateral outgrowth of embryonic flanks for what would otherwise be dorsal closure.

Of-zen^{RNAi}*-induced failure of katatrepsis is the consequence of inert extraembryonic membranes*

In the wild type situation, katatrepsis proceeds by fusion of the amnion and the serosa, rupturing of the amniotic cavity at the posterior pole of the egg, and then contraction of the serosa and emergence of the embryo from the yolk (Heming, 2003, p. 128). In *Of-zen*^{RNAi}-depleted embryos it appears that both amnion and serosa are specified correctly and persist throughout early embryogenesis, but are inert at the time of katatrepsis: neither rupture of the amniotic cavity nor contraction of the serosa is observed.

Eversion is explained by the persistence of an intact amniotic cavity at the time of dorsal closure, confining the embryo and dictating the surface along which the embryonic flanks grow. We have found it difficult to visualize the amnion directly at these stages, probably due to apposition of the much thicker embryonic tissue. The mature amnion is a single layer of attenuated cells (Dorn, 1976), while the flanks of the embryo are several cell layers thick, with dense packing of cuboidal cells in the epidermis. Inspection of *Of-zen*^{RNAi} progeny from the time of katatrepsis reveals a thick layer of tissue that encapsulates the entire embryonic body (Fig. 8a: arrows). It has not been possible to distinguish distinct cell layers in this tissue, but we presume it to be the amnion with an embryonic lining (ventral to the legs, arrows in Figs. 8b, c) continuous with the ventral embryonic epidermis (dorsal to the legs, arrows in Figs. 8e, f). From the time that dorsal closure would be complete, this layer can be seen between the enclosed appendages and the external organ tissue (Figs. 8b, e). We infer that the amniotic cavity remains intact during outgrowth of the embryonic flanks, because ventral closure and eversion of the head are complete. (The head is apposed to the site of wild type amnion–serosa fusion.) The subsequent fate of the amniotic tissue is unclear, but it may degenerate at the same developmental stage as in wild type embryos. No cells with large nuclei can be seen along the body wall by the end of dorsal closure (arrows in Figs. 8k–l). What is clear is that this amnion layer is below the surface of the egg, and separate from the serosa, as seen around the head lobes of an embryo at late katatrepsis/early dorsal closure stage (Fig. 8d), and even more clearly when organogenesis is more advanced (Figs. 8a–b). There is no observed contact of the amnion with the serosa, which is required for wild type amnion–serosa fusion at the initiation of katatrepsis.

However, we can observe the fate of the serosa. It appears that in *Oncopeltus zen*^{RNAi} embryos the serosa persists throughout early embryogenesis, and then disappears as wild type katatrepsis and dorsal closure would normally take place (Figs. 8g–m). Fluorescent nuclear staining just prior to katatrepsis and after it should have commenced (Figs. 8g–h, and data not shown), shows apparently wild type germband embryos within an outer serosal membrane with large nuclei (compare to germband stage in Fig. 7i′). By the time when katatrepsis should have completed and dorsal closure initiated, the serosa persists on the surface, but fluorescent nuclear signal is less distinct from background, possibly indicating a loss of integrity in the tissue or increased proliferation of sub-surface, embryonic tissue (Figs. 8i–j). Inspection shortly after dorsal closure should have completed reveals embryos with a full complement of external nervous ganglia and with enclosed legs (Figs. 8k–l, compare with Fig. 8n). There is no trace of the normal dorsal cap of contracted serosa, but an almost complete loss of surface nuclei: only a few, small and scattered nuclei remain of the former serosal tissue (Fig. 8m). As the disappearance of the serosa occurs at about the same time in development as in wild type embryos, it is probable that the serosa degenerates on the surface of everted embryos at the same developmental stage as degeneration of the serosa within the dorsal organ of wild type embryos. However, we have not been able to visualize apoptosis of the serosa in everted embryos, although patterns of apoptotic cell death within embryonic tissues are comparable to those in wild type embryos (data not shown). Nuclear inspection reveals that within a single clutch of everted embryos, some do have an intact serosa while others do not, suggesting that degeneration of the tissue is rapid. Although we cannot rule out loss of the tissue due to necrosis, its abrupt disappearance suggests coordinated death by apoptosis.

The Of-zen^{RNAi} *phenotype is all-or-nothing: embryos cannot be somewhat inside out*

Eversion was the single phenotypic result obtained after treatment with three different *Of-zen* dsRNA fragments (Fig. 2c); progeny from control females injected with buffer only were wild type and hatched (Table 1). No other phenotypic class

Table 1
Quantitative results from *Of-zen* RNAi experiments

Treatment totals (no. females, egg batches)	[% (*n*)]			
	Wild type	RNAi phenotype	Other defects	Scored total
(a) Experiment 1: Of-zen ds i only				
Zen (ds i) (5, 63)	0.8 (3)	97.2 (349)	2.0 (7)	359
Injected control (2, 22)	84.0 [a]	15.4 [a, b]	0.6 (1)	N/A [a]
(b) Experiment 2: three different Of-zen dsRNA constructs				
ds i (2, 18)	16.5 (52)	82.0 (259)	1.6 (5)	316
ds ii (2, 16)	40.2 (53)	54.6 (72)	5.3 (7)	132
ds iii (3, 27)	25.1 (97)	72.9 (282)	2.1 (8)	387
Zen total (7, 61)	24.2 (202)	73.4 (613)	2.4 (20)	835
Injected control (2, 15)	99.4 (331)	0.0 (0)	0.6 (2)	333
(c) Experiment 3: dilution series of Of-zen ds i				
Neat (=10 μg) (2, 8)	53.2 (59)	45.0 (51)	0.9 (1)	111
1/10th (=1 μg) (2, 9)	13.8 (15)	86.2 (94)	0.0 (0)	109
1/100th (=0.1 μg) (2, 25)	31.6 (56)	67.8 (120)	0.6 (1)	177
Zen total (6, 42)	32.8 (130)	66.8 (265)	0.5 (2)	397
Injected control (2, 34)	98.7 (381)	0.3 (1) [c]	1.0 (4)	386
Hunchback (1, 24)	0.0 (0)	100.0 (144)	0.0 (0)	144
Decapentaplegic (2, 8)	20.4 (29)	79.6 (113)	0.0 (0)	142

The following double-stranded RNA constructs were used: (a) Experiment 1: a single dsRNA construct, ds i only, (b) Experiment 2: the original dsRNA construct (ds i) and two non-overlapping constructs (ds ii and ds iii), and (c) Experiment 3: a dilution series of *Of-zen* ds i, and other genes as positive controls.

[a] In the original experiment wild type hatchlings from injected controls were scored by egg batch, rather than individual embryos.

[b] The presence of embryos with the RNAi phenotype in the injected control is explained by contamination of the syringe plunger after injection with *Of-zen* dsRNA (despite cleaning with ELIMINase; no subsequent problems of contamination were observed when the needle was cleaned with Fairy Liquid).

[c] Possible operator error in handling of dishes with collected eggs.

was observed, although some everted embryos showed defects of cuticular closure (experiment 2 only: 3 embryos with such defects, 0.5% of total everted embryos, n = 613). Furthermore, in an experiment where dsRNA specific to other *Oncopeltus* genes was also used as positive controls, RNAi phenotypes were gene specific, with knockdown phenotypes for other genes corresponding to published descriptions (Table 1c, *Of-dpp*: Angelini and Kaufman, 2005; *Of-hb*: Liu and Kaufman, 2004a). In fact across experiments, five of the 18 females injected with *Of-zen* dsRNA had 99–100% everted progeny.

In contrast to other genes examined by RNAi, both in *Oncopeltus* and elsewhere (parental/maternal and embryonic RNAi in *Oncopeltus*, *Gryllus*, *Tribolium*: Angelini and Kaufman, 2004, 2005; Herke et al., 2004; Hughes and Kaufman, 2000; Liu and Kaufman, 2004a,b, 2005; Mito et al., 2005; Schröder, 2003; van der Zee et al., 2005), there was no observed variation in severity of the *Of-zen* RNAi phenotype. The only other *Oncopeltus* gene for which this has been reported to be the case is with maternal RNAi for *Of-dpp* (Angelini and Kaufman, 2005, and this paper), where developmental arrest (though not death) occurs very early, leaving little scope for variation from a morphological perspective. In contrast, with *Of-zen* the entire suite of features described for eversion (small size, orientation in egg, stylets, organ tissue, everted glands, appendages within cuticle) occurs in all affected embryos, these embryos having developed for the complete duration of wild type embryogenesis.

The lack of variation in phenotypic severity for *Of-zen* pRNAi is unusual. We, thus, performed additional experiments in an attempt to produce hypomorphic phenocopies. An RNAi experiment using lower concentrations of dsRNA (1/10th and 1/100th: Table 1c) failed to produce weaker phenotypes. This might suggest that the dilution series did not use low enough concentrations to see a biological effect (especially compared to the degree of variation in uptake of dsRNA due to wound leakage). However, additional data support the alternative interpretation that there is no hypomorph for *Of-zen* RNAi.

With maternal RNAi the penetrance of the phenotype can wane over time, producing hypomorphic phenocopies presumably due to depletion of the double-stranded molecule from the mother's hemolymph (Liu and Kaufman, 2004a, 2005). We found that with *Of-zen* the RNAi effect also wanes in some females, but without an effect on the phenotypic classes of progeny (Figure S1 and Table S2). With these females, 100% of progeny in the earlier egg batches were everted, and 100% of progeny from the final egg batches were wild type and hatched (Figure S1). Thus, the data collected encompass the entire course of the RNAi effect. Further, in several egg batches, wild type and fully everted embryos co-occur (42% of egg batches, n = 24). This phenomenon of co-occurrence was also observed for egg batches of females who do not show a trend of waning effect. Across experiments, this was observed for 30 egg batches (18.3%, n = 164) from 12 females. That eggs fertilized and laid at the same time go on to develop either normally or in fully everted fashion strongly suggests that there is no hypomorph possible, that eversion is an all-or-nothing phenotype (see also Supplementary Information). Embryogenesis must be sensitive to even small amounts of *Of-zen* dsRNA.

The sensitivity of embryos to *Of-zen* dsRNA suggests that katatrepsis is easily perturbed. To examine whether defects in katatrepsis and eversion are common, we scored wild type, untreated embryos from the Cambridge culture for the occurrence of natural defects (see also Supplementary information). Most embryos developed normally. Of the 3% with defects, half of these showed features of eversion (e.g. legs inside, external stylets). However, none of those with features of eversion completely resembled an *Of-zen*^{RNAi} embryo, as all spontaneously everted embryos had head defects, some sufficiently severe that anterior morphology was unidentifiable. Some were partially everted, such as having an un-everted head but everted thorax, or an un-everted anterior with everted abdomen. In comparison, defects of dorsal closure were much less frequent.

Discussion

Of-zen is not required for formation of extraembryonic tissues

Our knockdown experiments clearly show that the *Of-zen* gene is required for normal function of the extraembryonic membranes during katatrepsis. In other winged insects where *zen* genes play a role in the functioning of the extraembryonic

membranes, *zen* expression serves as a marker for extraembryonic tissues from the earliest stages of embryogenesis. However, this does not seem to be the case in *Oncopeltus*. The earliest stages of *zen* expression overlap substantially with the expression of other genes, including *hunchback* (Figs. 4j–m) and *engrailed* (Liu and Kaufman, 2004a), which presumably mark embryonic tissues. At present we have no evidence for any role of *Oncopeltus zen* in embryonic tissue: the knockdown phenotype shows no early defects in embryonic patterning (segment formation or appendage patterning, Figs. 7i–j). On the other hand, in *Of-zen*^{RNAi} embryos extraembryonic tissue morphology appears normal prior to the stage when katatrepsis would take place (Figs. 8g–h), demonstrating that *Of-zen* is not required for formation of the extraembryonic membranes. These results raise the question of what, if any, role *Of-zen* might play during the blastoderm stage.

The interpretation of the early expression pattern of *Of-zen* is complicated by the fact that there is no clear understanding of the *Oncopeltus* blastoderm fate map. Two potential blastoderm fate maps have been posited. The expression pattern of *engrailed* (Liu and Kaufman, 2004a) implies that the ventral and lateral areas of the blastoderm, in a saddle shape that girdles the egg, provide the cells that invaginate at the posterior pole to form the germ rudiment (embryo and amnion). As invagination proceeds, the surface *engrailed* stripes are carried into the yolk as a cell sheet. This indicates a high level of organization in the blastoderm and corroborates the idea that there is no cell mixing during invagination (Roth, 2004). The regions not stained for *engrailed*, the anterior pole and dorsal surface of the egg, would be presumed extraembryonic territories corresponding to the serosa (with a possible contribution of the posterior dorsal cells to the invaginating amnion). An alternative fate map based on classical, descriptive studies identifies the presumptive germ rudiment as arising from the bilateral plates of cells (Fig. 3b), which will converge ventral to the posterior pole of the egg and then invaginate at the pole (Johannsen and Butt, 1941, p. 271; Roth, 2004). Such a fate map also leaves the anterior pole as extraembryonic territory, and posits that the dorsal surface is extraembryonic and specifically even the presumptive serosa (Johannsen and Butt, 1941, p. 271). However, the fate of the ventral side of the blastoderm is unclear, and it may contribute to the germ rudiment (posteriorly) and/or to the serosa (anteriorly). However, there is no clear correlation in blastoderm stage embryos between *Of-zen* expression and either putative extraembryonic territory: *zen* staining in the dorsal and anterior regions is transient, confined to a thin strip of cells spanning the dorsal surface and of unknown fate. Certainly an explicit fate map of the blastoderm is needed, including identification of early marker genes for distinct tissue types, particularly the amnion.

Serosal expression and function of Of-zen

Of-zen serosal expression commences from the mid germband stage (34% development; Fig. 5), and then persists for the life of this tissue (Fig. 6). *Zen* is not expressed in the mature amnion (Fig. 6), and we have no evidence that it is expressed at any earlier stage in cells fated to become the amnion. Serosal tissue specification, persistence, and appropriate timing of degeneration are unaffected by *zen* knockdown by RNA interference (Fig. 8). Thus, *zen* is not required for making a polyploid, extraembryonic tissue that surrounds yolk and embryo, and that retains its developmental 'clock' for degeneration. However, as knockdown of *zen* renders the serosa inert, although it is morphologically normal, *zen* is clearly required for functional competence of this tissue. Both wild type transcript expression and the RNAi phenotype suggest that *Of-zen* is specifically required for the process of katatrepsis to take place. We believe that the first step of katatrepsis, amnion–serosa fusion, does not occur in *zen*^{RNAi} embryos. As *zen* expression is specific to the serosa, these data suggest that any autonomous role of *zen* in the extraembryonic membranes, and in amnion–serosa fusion in particular, is mediated by the serosa.

The evolution of zen expression and function: comparison with other insect species

Extraembryonic expression of *Hox3/zen* is a feature unique to insects, and it is known to be functionally required in this domain in some of the species investigated. However, inference of the ancestral state or the evolutionary trajectory of this gene is complicated by the details of interspecific data.

Differences in Hox3 orthologue expression between the two extraembryonic tissues, the amnion and the serosa, are extensive, possibly due to interspecific differences in the structure and function of these tissues relative to the embryo itself. In the primitively wingless *Thermobia domestica,* extraembryonic expression of *Hox3* occurs in the early amnion (Hughes et al., 2004). *Oncopeltus fasciatus zen, Tribolium castaneum zen1* (van der Zee et al., 2005), and *Anopheles gambiae zen* (Goltsev et al., 2004) are only expressed in the serosa. *Schistocerca gregaria zen* and *Tribolium castaneum zen2* are both initially expressed in the serosa, but expression also includes a later, weaker domain in the mature amnion (Dearden et al., 2000; Falciani et al., 1996; van der Zee et al., 2005). The fused amnioserosa of *Drosophila melanogaster* appears to express both *zen* and *z2* uniformly (Rushlow et al., 1987), but the derived state of the tissue makes direct comparison difficult; various arguments have been used to describe the amnioserosa as essentially either an amnion (Dearden et al., 2000; Roth, 2004) or a serosa (Heming, 2003, p. 131; Patel et al., 2001; S. Roth, personal communication). Only in *Thermobia* is *Hox3/zen* expression amniotic-only (although expression at later stages of embryogenesis are unknown), while in all other taxa the principal extraembryonic domain is in the serosa. Possibly *Thermobia* represents an ancestral state prior to the acquisition of a serosal expression domain, or it may represent an instance of loss of serosal expression from an ancestor with expression in both tissues. On the other hand, *zen* expression in the late amnion is seen in *Thermobia*, *Schistocerca*, and with *Tribolium zen2*. This suggests that if *Of-zen* as described here is the single orthologue in *Oncopeltus*, it might represent

a somewhat derived state in which an amniotic domain of expression has been lost.

The borders where the embryo and amnion, and the amnion and serosa meet seem important, and are demarcated by *Hox3/zen* expression. *Thermobia Hox3* extraembryonic expression is limited to weak, heterogenous expression in a single ring of cells at the edge of the germband embryo (Hughes et al., 2004). In *Schistocerca*, extraembryonic zen protein expression originates from 'necklace cells' at the amnion–serosa border (Dearden et al., 2000). In *Oncopeltus*, *zen* transcript expression is heterogenous in the serosa during katatrepsis, but is consistently stronger in the serosal cells at the amnion–serosa border. Although identification of cells at the boundary of tissue types is difficult, the *Thermobia Hox3*-expressing cells are identified as amniotic, while the border cells expressing zen in *Schistocerca* and *Oncopeltus* appear to belong to the serosa. Thus, it may be easy for *zen* orthologues to change domain of expression between extraembryonic tissues, but these expression data suggest an evolutionarily early role for zen at these tissue boundaries. Zen may function in defining and/or maintaining the boundaries themselves, or in propagating information from the boundary into the extraembryonic tissue.

One way in which *Oncopeltus zen* seems to differ from other *zen* orthologues is in the timing of onset of expression in the serosa. As mentioned above, *zen* expression usually serves as an early marker for the serosa. In addition to the species described above, this is also seen for the *zen/bcd* composite gene of lower flies (Stauber et al., 1999, 2002). *Of-zen* expression in the serosa begins relatively late in development, only detectable after the serosa has matured to polyploidy and the germ rudiment has invaginated into the interior of the egg.

Functional data in *Oncopeltus, Tribolium* and *Drosophila* enable further evaluation of zen orthologues. Comparison of the single *Oncopeltus* zen with the two paralogues of *Tribolium* (van der Zee et al., 2005) is complex. Although *Tc-zen1* and *Tc-zen2* are both expressed in the serosa, only *Tc-zen2* is expressed in the amnion. Thus, comparison of expression suggests that *Of-zen* and *Tc-zen1* might be more similar due to serosa-specific expression. However, comparison of functional data from RNA interference suggests Of-zen is more similar to Tc-zen2. Loss of Tc-zen1 results in respecification of presumptive extraembryonic tissue to an embryonic fate. In contrast, loss of Of-zen or Tc-zen2 results in a serosa that persists but is inert, causing failure of amnion–serosa fusion and ultimately eversion of the embryo. As the need for amnion–serosa fusion is abrogated in the absence of a serosa, the simultaneous knockdown of Tc-zen1 and Tc-zen2 resembles the phenotype of Tc-zen1 alone. Clearly, the two *Tribolium* genes together do not represent a comparable situation to that seen for Of-zen.

Evaluating functional similarity is made still more difficult by consideration of *Drosophila* zen. Functional data suggest that the role of Of-zen corresponds to that of Tc-zen2, while Tc-zen1 and Dm-zen are comparable, as loss of Dm-zen also results in respecification of tissue to an embryonic fate (Irish and Gelbart, 1987; Wakimoto et al., 1984). This is possible if the ancestral zen gene functioned both in early differentiation of the serosa and in amnion–serosa fusion mediated by both the amnion and serosa. Then, *Oncopeltus* would have lost the former function and the need for the amnion in the latter, while *Drosophila*, with its derived anatomy, would have lost the latter function altogether, and the *Tribolium* paralogues would represent an instance of subfunctionalization.

Functional implications for interactions of amnion, serosa, and embryo

Expression data in *Oncopeltus* suggest that amnion–serosa fusion is mediated by zen function in the serosa alone, while the *Tribolium* data imply that zen is required in both amnion and serosa (or possibly only the amnion, if *Tc-zen2* in the serosa is redundant to *Tc-zen1*). In this beetle, the timing (prior to dorsal closure) and location (a restricted domain near the site of fusion with the serosa) of *Tc-zen2* expression in the amnion correlate specifically with a possible role in amnion–serosa fusion (van der Zee et al., 2005). In the bug, expression of *Of-zen* is exclusive to the serosa, implying that the serosa is an active party in initiating amnion–serosa fusion.

A second difference between *Oncopeltus* and *Tribolium* concerns the physical relationship of the amnion and serosa after they have fused. In *Oncopeltus* these membranes retain distinct morphologies throughout katatrepsis, sharing a clear border (Figs. 3i–j, 6). In *Tribolium* there is an increase in nuclear density in the region where the membranes meet, but the membrane surface remains smooth and fusion may involve intercalation of cells of the two membranes (van der Zee et al., 2005).

RNAi experiments also suggest differences between *Tribolium* and *Oncopeltus* regarding the context in which zen functions. Knockdown experiments in *Tribolium* reveal a high degree of regulation and compensation in the beetle embryo. RNAi of *Tc-zen1* alone or simultaneously of *Tc-zen1* and *Tc-zen2* result in regulation by the embryo proper (for size) and compensation by the amnion (to provide a dorsal covering), leading to a viable, wild type larva. Further, knockdown of either paralogue singly or together produces a range of severity in phenotype. In contrast, in *Oncopeltus* the lack of an *Of-zen*^{RNAi} hypomorph and the high occurrence of eversion-related defects in wild type embryos implies that katatrepsis is an easily perturbed process. As small quantities of double-stranded RNA are sufficient to generate the *Of-zen*^{RNAi} phenotype, there may be a threshold requirement for zen. Above such a threshold development proceeds normally; below it there seems to be little buffering of the system at the cellular level, and a lack of plasticity in the respective function of embryonic, serosal, and amniotic tissues. Processes mediated by *Oncopeltus zen* may act as a bottleneck in extraembryonic membrane function.

What is clear from RNAi knockdowns for *Oncopeltus* and *Tribolium*, and from *Drosophila* mutants, is that the outgrowth of the embryonic flanks for dorsal closure is initiated independently in the embryonic body, irrespective of relative positioning of the extraembryonic tissue(s), and occurs at the appropriate time in development. However, in all three cases the extraembryonic membranes are needed for the correct progression and positioning of this process.

Further investigation of extraembryonic membrane functions at katatrepsis will require consideration of the larger molecular context. Eversion-related defects in wild type *Oncopeltus* progeny showed a high level of variation between embryos, and all included at least minimal defects in head formation and other processes. None of these embryos precisely resembled the *Of-zen*^{RNAi} phenotype. This suggests the expected situation: that there is more to katatrepsis than zen function in the serosa. Other factors may interact with or function independently of zen. Partial eversion suggests that such factors may function downstream of zen. Also, the interaction of the amnion and serosa necessary for fusion probably requires signaling from and morphogenetic changes within both tissues, and may even require signaling input from the embryo. Data from holometabolous insects suggest many potential candidate genes for these functions (Frank and Rushlow, 1996; Patel et al., 2001; Reim et al., 2003; Tatei et al., 1995; van der Zee et al., 2005). Although the functional requirement for orthologous genes in extraembryonic tissues can vary considerably, it is encouraging for the candidate gene approach that eversion due to failure of katatrepsis has also been seen elsewhere in the Hemiptera and even in the Odonata (Sander, 1976).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.12.028.

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