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# Integrin-Dependent Apposition of *Drosophila* Extraembryonic Membranes Promotes Morphogenesis and Prevents Anoikis

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## Summary

**Background:** Two extraembryonic tissues form early in *Drosophila* development. One, the amnioserosa, has been implicated in the morphogenetic processes of germ band retraction and dorsal closure. The developmental role of the other, the yolk sac, is obscure.
**Results:** By using live-imaging techniques, we report intimate interactions between the amnioserosa and the yolk sac during germ band retraction and dorsal closure. These tissue interactions fail in a subset of *myospheroid* (*mys*: βPS integrin) mutant embryos, leading to failure of germ band retraction and dorsal closure. The *Drosophila* homolog of mammalian basigin (EMMPRIN, CD147)—an integrin-associated transmembrane glycoprotein—is highly enriched in the extraembryonic tissues. Strong dominant genetic interactions between *basigin* and *mys* mutations cause severe defects in dorsal closure, consistent with basigin functioning together with βPS integrin in extraembryonic membrane apposition. During normal development, JNK signaling is upregulated in the amnioserosa, as midgut closure disrupts contact with the yolk sac. Subsequently, the amnioserosal epithelium degenerates in a process that is independent of the *reaper*, *hid*, and *grim* cell death genes. In *mys* mutants that fail to establish contact between the extraembryonic membranes, the amnioserosa undergoes premature disintegration and death.
**Conclusions:** Intimate apposition of the amnioserosa and yolk sac prevents anoikis of the amnioserosa. Survival of the amnioserosa is essential for germ band retraction and dorsal closure. We hypothesize that during normal development, loss of integrin-dependent contact between the extraembryonic tissues results in JNK-dependent amnioserosal disintegration and death, thus representing an example of developmentally programmed anoikis.

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## Introduction

The role of extraembryonic tissues in regulating embryonic development has only recently begun to be appreciated in *Drosophila*. Two cell types that arise at the *Drosophila* cellular blastoderm stage are extraembryonic (i.e., do not contribute to the mature embryo). The first, the amnioserosa, is an epithelium derived from the dorsalmost region of the blastoderm [1]. The second, the yolk sac, originates during cellularization of the blastoderm: membrane fusion basal to the blastoderm nuclei forms both the basal membrane of each somatic cell and a single continuous plasma membrane—the yolk sac membrane—that envelops the yolk [1]. Within the yolk syncytium, there are some 200 nuclei; thus, the yolk sac is a large, membrane bound, multinucleate cell.

The amnioserosa plays a key role in germ band retraction and dorsal closure. It is likely to function both in cell signaling [2, 3] and in generating the forces that drive these morphogenetic processes [4–6]. The role of the yolk sac during development has remained obscure. The expression of several genes in the yolk nuclei, including *serpent* [7], *sisterlessA* [8], *D-ret* [9], *forkhead* [10], and those encoding imaginal disc growth factors (IDGFs) [11], suggests that the yolk sac may play important roles in processes other than nutrition. The developmental defects produced by loss-of-function alleles of *sisterlessA*, which is expressed exclusively in the yolk nuclei from blastoderm stages on, have led to speculation that the yolk may play a role in morphogenesis [8]. However, the functions of the yolk sac in morphogenesis—if any—are unknown.

Here, we show that physical interaction of the amnioserosa and yolk sac plays a crucial role in both germ band retraction and dorsal closure of the embryo. We demonstrate that βPS integrin mediates extraembryonic membrane interactions that are required for survival of the amnioserosa. Anoikis of the amnioserosa occurs during normal development after closure of the midgut disrupts integrin-dependent apposition of the amnioserosa and yolk sac. In *mys* mutants, failure to establish apposition of extraembryonic membranes leads to premature anoikis of the amnioserosa. We investigate a possible role for JNK signaling and the *reaper/hid/grim* cell death genes in amnioserosal anoikis during normal development.

## Results

### The Amnioserosal and Yolk Sac Membranes Establish Intimate Contact during Germ Band Retraction and Dorsal Closure

In fixed, sectioned material it can be seen that as germ band retraction commences, there is a gap between the amnioserosa and the yolk sac membrane (Figures 1A–1C). Membrane projections from both the basal side of the amnioserosa and the dorsal region of the yolk sac can be seen to penetrate this space (Figures 1B

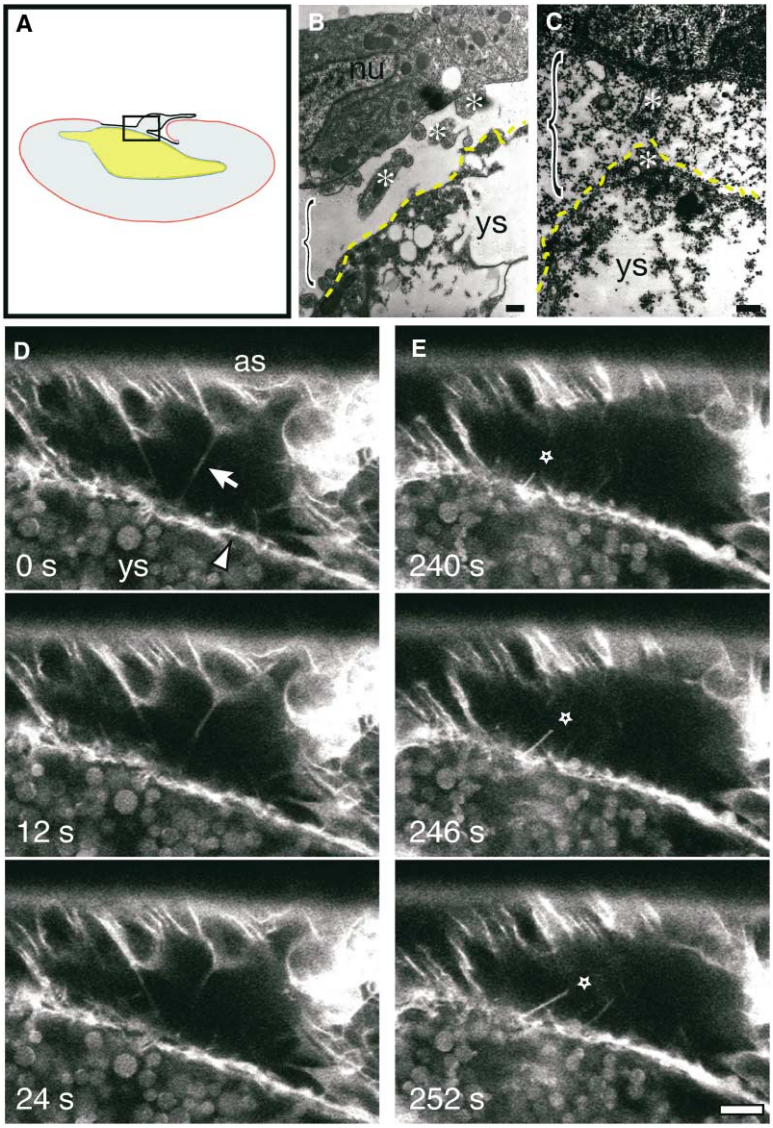


Figure 1. Phase I of Amnioserosa Yolk Sac Membrane Interaction: Transient Contacts

(A) Diagram of an early stage 12 embryo in sagittal view depicting the germ band (gray with red outline), amnioserosa (gray with black outline), and yolk sac (yellow with blue outline). The box indicates the region shown in transmission electron micrographs (B and C) and live image sequences in (D) and (E). (B) Transmission electron micrographs of the region of the amnioserosa that overlies the yolk. The yolk sac membrane is marked by the yellow dotted line. Abbreviations: nu, nucleus of amnioserosal cell; ys, yolk sac. Asterisks mark some of the membrane protrusions from the amnioserosa; the gap between the amnioserosa and the yolk sac is indicated with a bracket. (C) Ruthenium red-stained embryos reveal that the cleft between amnioserosa and yolk sac (indicated with a bracket) is enriched in glycoconjugates. Note the membrane bound processes (asterisks) from both the amnioserosa and the yolk sac, which come into close contact. Scale bars in (B) and (C), 500 nm. Filopodia-like extensions ([D], arrow) emanate from the basal side of the amnioserosal epithelium and make transient contact with the yolk sac membrane (arrowhead). Extensions ([E], star) from the apical side of the yolk sac membrane make transient contact with the basal side of the amnioserosa. Frames were sampled at 6 s intervals from a live-imaging movie (see Movie 1). The embryo carries Ubi-DE-cadherin-GFP, ftz-GAL4, UAS-actin-GFP, and *G289*, the protein trap transposon (PTT) that reports basigin expression as a basigin-GFP fusion protein. The basigin-GFP signal is particularly strong on the yolk sac membrane (which can be seen to overlie the yolk, ys) but is also present on the amnioserosa (as). In all images, anterior is to the left and dorsal toward the top of the page. Scale bar represents 10 μm.

and 1C). This space is enriched in glycoconjugates as assayed by ruthenium red staining (Figure 1C). Since the bulk of the extracellular matrix is not laid down at this developmental stage [12], these polysaccharides may be associated with transmembrane glycoproteins rather than an elaborate extracellular matrix (ECM) per se.

Live imaging of germ band retraction and dorsal closure revealed that contacts between the yolk sac membrane and the amnioserosa initiate at the beginning of germ band retraction and are remarkably dynamic. Imaging was carried out by using combinations of three different GFP fusion proteins that serve as markers of the F actin-based cytoskeleton (actin-GFP) [13]; the amnioserosal and yolk sac membranes (DE-cadherin-GFP) [14]; and *G289*, a homozygous viable PTT line [15] that reports basigin expression as a basigin-GFP fusion protein (see Experimental Procedures).

The initial, transient contacts between the amnioserosa and the yolk sac membrane—referred to here as phase I interactions—occur as germ band retraction initiates and are accomplished by two classes of cellular extensions: filopodia that emanate from the amnioserosa and contact the yolk sac membrane (marked by GFP-DE-cadherin + actin-GFP; Figure 1D and Movie 1), and membrane bound projections emanating from the yolk sac, which contact the amnioserosa (marked by basigin-GFP; Figure 1E and Movie 1). Their lack of stable association with their target cells and their highly dynamic character suggest that neither the amnioserosal nor the yolk sac projections generate the mechanical forces that drive morphogenesis. Instead these projections may facilitate a chemosensory or signaling function between the amnioserosa and yolk sac membrane, as discussed below.

The intimate and persistent interaction between the amnioserosa and yolk sac—phase II—initiates in the dorsal-anterior region of the amnioserosa (Figures 2A and 2B; Movie 2). This contact is maintained and further contact is established in an anterior-to-posterior direction as retraction progresses (Figures 2C and 2D; Movie 3). Close apposition of the amnioserosa and yolk sac

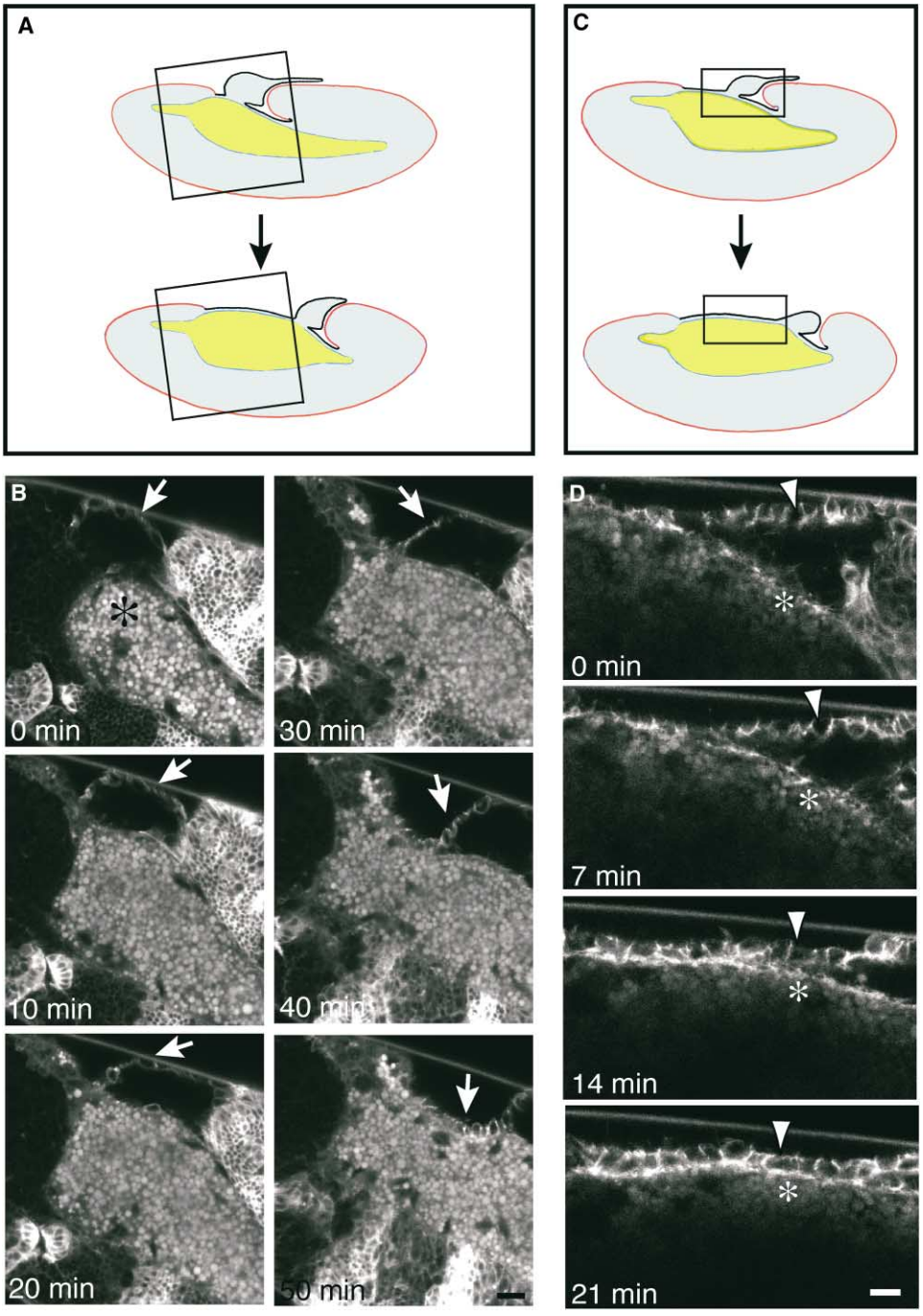


Figure 2. Phase II of Amnioserosa Yolk Sac Membrane Interaction: Intimacy

(A and C) Schematic diagrams showing early (A) and late (C) stages of germ band retraction. The color scheme is as in Figure 1A. The boxes in (A) and (C) indicate the positions of the images shown in (B) and (D), respectively. (B) Intimate apposition of the amnioserosa (arrow) and yolk sac (asterisk) initiates in the dorsal-anterior (0 min) and then progresses posteriorly (10 to 50 min). Frames were sampled at 10 min intervals from a live-imaging movie (see Movie 2). The embryo carries Ubi-DE-cadherin-GFP, ftz-GAL4, and UAS-actin-GFP and is viewed parasagittally over the gap between the cephalic region and the retracting caudal germ band. Scale bar represents 20 μm. (C) Late phase II apposition of the amnioserosa (arrowhead) and the yolk sac membrane (asterisk). In this case, frames were sampled every 7 min from a live-imaging movie (see Movie 3). The embryo carries Ubi-DE-cadherin-GFP, ftz-GAL4, UAS-actin-GFP, and the *G289* PTT. The basigin-GFP signal is particularly strong on the yolk sac membrane (asterisk), indicating that the amnioserosa achieves close intimacy with this membrane. The embryo is viewed parasagittally. Scale bar represents 10 μm.

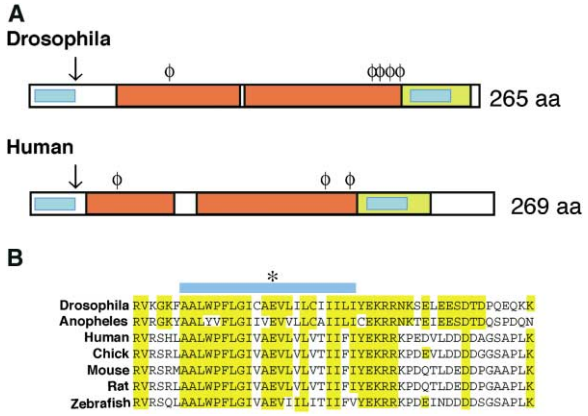


Figure 3. Evolutionary Conservation of Basigin Structure

(A) Domain structure of *Drosophila* and human basigin: Ig domains (red), transmembrane domains (blue), basibox (yellow), predicted O-linked glycosylation sites ($\phi$), and predicted signal peptide cleavage site (arrow).

(B) The basibox: perfectly conserved amino acids are highlighted in yellow. The predicted transmembrane domain is shown in blue with the conserved glutamic acid residue (E) indicated (asterisk).

membranes persists during dorsal closure (data not shown).

### Basigin Structure and Expression Is Highly Conserved in Mammals and *Drosophila*

In mammals, basigin has been reported to be expressed and to function in extraembryonic tissues during early development, when it is required for embryo implantation [16, 17]. Basigin also functions in retinal epithelial morphogenesis [18] and during invasive growth of tumors [19]. Since *Drosophila* basigin is highly enriched on the extraembryonic membranes prior to and during their close apposition (Figures 1 and 2), we directed our attention to the structure and function of *Drosophila* basigin.

The *Drosophila* basigin transcription unit (*CG31605*, FBgn0051605) encodes multiple transcript variants (for details see http://www.fruitfly.org/). The transcripts encode two distinct protein isoforms: a long, 298 amino acid (aa) isoform and a short, 265 aa isoform (only the latter is shown in Figure 3A). The long and short isoforms differ only at their amino and carboxy termini: the first 50 aa of the long form are substituted by 25 aa in the short form; the long form also has an 8 aa carboxy-terminal extension. The distinct N-terminal regions each contain their own unique transmembrane domains and signal peptide cleavage sites. The long isoform's N-terminal region is glycine rich. Database searches show that long and short isoforms also exist for human basigin.

*Drosophila* and mammalian basigin exhibit strong conservation of immunoglobulin (Ig) domain organization, location of predicted O linked glycosylation sites, as well as extracellular and cytoplasmic tail length (Figure 3A). Both mammalian and *Drosophila* basigin have two extracellular Ig domains, the C-terminal of which appears to be representative of a "primordial" Ig domain [19]. There is an additional, more C-terminal 50 amino acid stretch of conservation, which will be referred to as the "basibox" and which includes the predicted transmembrane domain (Figure 3B). One of the defining features of the basibox is a glutamic acid residue in the middle of the transmembrane domain. The basibox is 52%–54% identical between *Drosophila* and vertebrates; the central 27 amino acids show 78%–81% identity.

### Basigin and βPS Integrin (*mys*) Mutants Show Dominant Genetic Interactions

There are multiple P element inserts in or near the *basigin* gene. One, the *NP6293* GAL4 P element insertion, is in the 5′UTR of a predicted *basigin* transcript. This insertion causes leaky postembryonic lethality when homozygous and is referred to here as $bsg^{NP6293}$. Homozygous $bsg^{NP6293}$ embryos show no defects in germ band retraction and dorsal closure. A P element insert that causes male sterility has previously been referred to as *gelded* [20].

Basigin and integrins associate physically in mammals, possibly through direct contacts between basigin and the $\beta_1$ integrin subunit [21]. In *Drosophila* there is a single β integrin, called βPS integrin, which is encoded by the *myospheroid* (*mys*) gene [22]. $mys^1$ mutants show germ band retraction and dorsal closure defects [23–25].

Basigin and βPS integrin mutants show striking dominant genetic interactions: while $bsg^{NP6293}$ mutants show no defects in dorsal closure and $mys^1$ mutant embryos show only weak dorsal closure defects—evidenced by a small dorsal hole—$mys^1$ mutant embryos from females in which the dose of the *basigin* gene is reduced by 50% show a striking increase in the size of the dorsal hole, while double mutant embryos show an even greater increase in dorsal hole size (Figure 4A). The dominant genetic interaction of *bsg* and *mys* mutants is consistent with the possibility that basigin and integrin proteins interact physically in *Drosophila*.

### βPS Integrin Is Required for Intimate Apposition of the Amnioserosa and Yolk Sac

Live imaging shows that those $mys^1$ mutant embryos that fail germ band retraction exhibit apparently normal phase I interactions (for example, yolk sac projections are produced and contact the amnioserosa; Movie 4). However, phase II membrane apposition fails completely. Most striking is a failure of the dorsal-anterior region of the amnioserosa to initiate contact with the yolk sac membrane (Figure 4B; Movie 4). In those $mys^1$ mutant embryos that complete germ band retraction, there is failure to maintain the apposition of the amnioserosa and yolk sac membrane, with subsequent high penetrance failure of dorsal closure. Narasimha and Brown [26] (this issue of *Current Biology*) provide independent evidence supporting a role for integrin in apposition of amnioserosal and yolk sac membranes during dorsal closure.

In summary, phase II membrane intimacy is compromised in $mys^1$ mutants, implicating βPS integrin in the close apposition of amnioserosal and yolk sac membranes. The failure of both germ band retraction and dorsal closure in $mys^1$ mutants suggests that close ap-

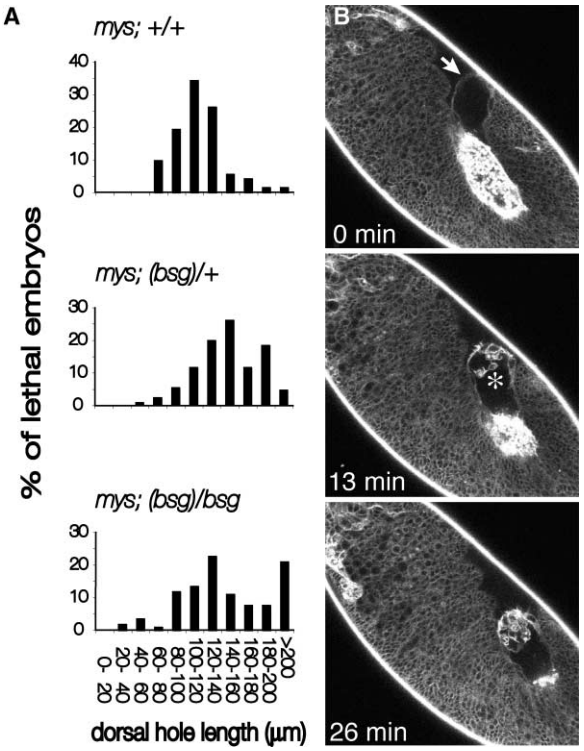


Figure 4. Quantification of Dorsal Closure Defects in *mys* Mutants with Varied Doses of *bsg* and Failure of Phase II Membrane Apposition and Anoikis in *mys* Mutants

(A) Histograms showing the size distribution of dorsal cuticular hole length (μm) in single and double mutant lethal embryos. Shown are the distributions for $mys^{1}$ (top; n = 73), embryos from a cross of $mys^{1}$/+ ; $bsg^{NP6293}$/+ females to wild-type males (middle; n = 131), and embryos from a cross of $mys^{1}$/+; $bsg^{NP6293}$/+ females to $bsg^{NP6293}$/+ males (bottom; n = 120). In the latter two crosses, the maternal dose of both *mys* and *bsg* is reduced. The embryonic genotype is indicated in each panel; parentheses indicate that it was not possible to distinguish those that carry the mutation from those that did not. The data in the middle panel show dominant dose-dependent maternal and/or zygotic interaction of *bsg* with *mys*.

(B) In $mys^{1}$ mutants, absence of phase II contact between the amnioserosa (arrow, 0 min) and the yolk sac membrane is shown in the 0, 13, and 26 min frames. Loss of epithelial integrity occurs progressively at 13 and 26 min (see Movie 5). Hemocytes (asterisk) can be seen engulfing the dead amnioserosal cells. The embryo is from a cross of $mys^{1}$/*FM7, Kr-GAL4 UAS-GFP* females to *FM7, Kr-GAL4 UAS-GFP/Y; G289*/+ males. Thus the lack of Kr-GAL4-driven GFP signal means that the genotype of the filmed embryo is unambiguously $mys^{1}$/*Y*, labeled with *G289* (basigin-GFP).

position of the extraembryonic membranes is required for these morphogenetic processes. The strong enhancement of $mys^{1}$ dorsal closure defects by $bsg^{NP6293}$ mutants suggests that basigin functions together with βPS integrin in these morphogenetic processes. Anterior-to-posterior "zipping up" of the membranes may generate forces that help push the germ band posteriorly. Alternatively, as shown below, the role of integrin-dependent membrane apposition may be indirect, promoting survival of the amnioserosa, which in turn directs retraction and closure via signaling and/or physical contacts.

## In Wild-type Embryos the Amnioserosa Disintegrates and Dies after Dorsal Closure is Complete

In wild-type embryos, the concomitant closure of the dorsal epidermis and midgut abrogate apposition of the amnioserosa and yolk sac. We therefore asked when during normal development the amnioserosa loses integrity and dies. It has been shown, by using live imaging, that a small subset of the amnioserosal cells drop out of the epithelium prior to completion of closure [4]. However, live imaging of the majority of amnioserosal cells—which remain in the epithelium—after dorsal closure [1] has not been attempted previously.

We therefore live-imaged embryos in which amnioserosal cells had been specifically labeled (see Experimental Procedures), thus definitively addressing the fate of the amnioserosa after dorsal closure: the amnioserosa invaginates to form a tube-like structure with its perimeter cells aligning on the dorsal side of the tube, beneath the dorsal midline of the embryo (Figures 5A and 5C; Movie 5). Over a period of 2–3 hr, individual nonperimeter cells round up and are extruded from the tube (Figure 5B; Movie 5). Finally, the amnioserosal perimeter cells also dissociate (Figure 5D). As amnioserosal cells are extruded, they are rapidly engulfed by hemocytes, which thus become GFP positive (Figure 5B; Movie 5). These results are fully consistent with those inferred from analysis of fixed sectioned embryos [27].

It is possible to visualize a subset of the amnioserosal cells as acridine orange positive either before they leave the tube or shortly thereafter (Figures 5E and 5F). Both acridine orange staining and engulfment by hemocytes are hallmarks of dying cells. To determine whether death of amnioserosal cells might be *reaper* dependent, we asked whether we could visualize *reaper* expression in the amnioserosal cells prior to or after extrusion. No *reaper*-expressing cells were detected (data not shown). To further test whether amnioserosal cell death might be *reaper* dependent, we used the *H99* deficiency (*Df(3L)H99*), which removes the *reaper*, *head involution defective* (*hid*), and *grim* genes [28], and visualized the amnioserosa with anti-HNT antibody [2, 29]. If amnioserosal death were *reaper* dependent, one would expect HNT-positive cells to persist in *H99* mutants when compared with wild-type. Such persistence does not occur (Figures 5G and 5H). While it is conceivable that HNT expression is downregulated in a persistent amnioserosa, the simplest interpretation of these data is that death of the amnioserosa is *reaper* independent. This conclusion is consistent with the recent suggestion that *Drosophila* embryos have a caspase-independent cell engulfment system, which is still operative in *H99* mutants [30].

## Disintegration and Death of the Amnioserosa Is Preceded by Loss of Integrin-Dependent Contact with the Yolk Sac and Upregulation of JNK Signaling

It has been shown that loss of integrin-dependent contact between cells and the extracellular matrix leads to cell death, a process referred to as anoikis [31]. Anoikis

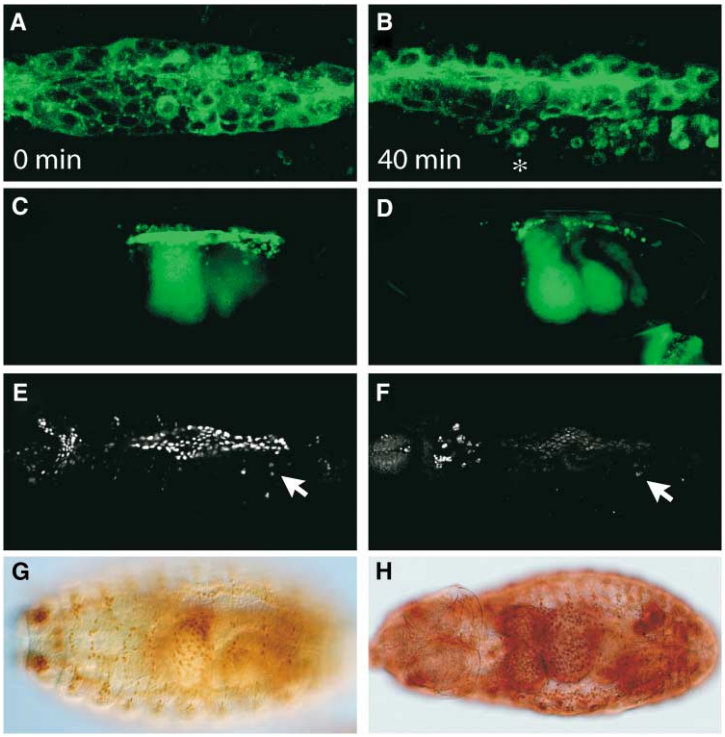


Figure 5. Disintegration and Death of the Amnioserosa Occurs after Dorsal Closure and Is *reaper/hid/grim* Independent

(A and B) The amnioserosa (GFP-labeled with *LP1* GAL4 and UAS-actin-GFP) comes to underlie the epidermis after dorsal closure (A) and subsequently disintegrates (B). Cells that are extruded from the amnioerosa are engulfed by hemocytes, which become GFP positive (asterisk). Frames were sampled from a live-imaging movie (see Movie 5).

(C and D) The *YET1* vital enhancer trap labels the perimeter cells of the amnioserosa, which align beneath the dorsal epidermal midline after closure (C). The *YET1*-labeled cells dissociate in the final stages of amnioserosal disintegration (D). Standard epifluorescence images are shown. The yolk enveloped by the gut autofluoresces in these images.

(E and F) Amnioserosal cells become acridine orange positive after dorsal closure. The amnioserosa is labeled with *LP1* GAL4-driven nuclear β-galactosidase (E). These cells are also acridine orange positive (F); one such double-labeled cell is highlighted with an arrow.

(G and H) The amnioserosa does not persist in *H99* mutant embryos (H) when compared to wild-type (G). Embryos were immunostained for HNT, which can be seen to label the nuclei of the midgut, peripheral nervous system, and tracheae. *H99* mutants were identified on the basis of failure of head involution.

is promoted by the Jun amino-terminal kinase (JNK) pathway [31]. Our previous analyses showed that JNK signaling in the amnioserosa is downregulated prior to dorsal closure [3]. In those analyses, *puckered-lacZ* expression was used as a read-out of JNK signaling, and it was shown that relocation of JUN and FOS proteins from the nucleus to the cytoplasm of amnioserosal cells correlates with downregulation of JNK signaling. While JNK signaling is downregulated in the amnioserosa prior to dorsal closure, we have found that JNK signaling is upregulated in this tissue as dorsal closure approaches completion (Figure 6). Thus, reactivation of JNK signaling in the amnioserosa follows loss of integrin-dependent apposition of the amnioserosa and yolk sac membrane and precedes amnioserosal disintegration and death. These data are consistent with the hypothesis that midgut closure disrupts integrin-dependent apposition of the amnioserosa and yolk sac, thus inducing JNK signaling in the amnioserosa and its subsequent anoikis.

## Discussion

We have shown that in the *Drosophila* embryo, intimate apposition of the extraembryonic membranes is integrin

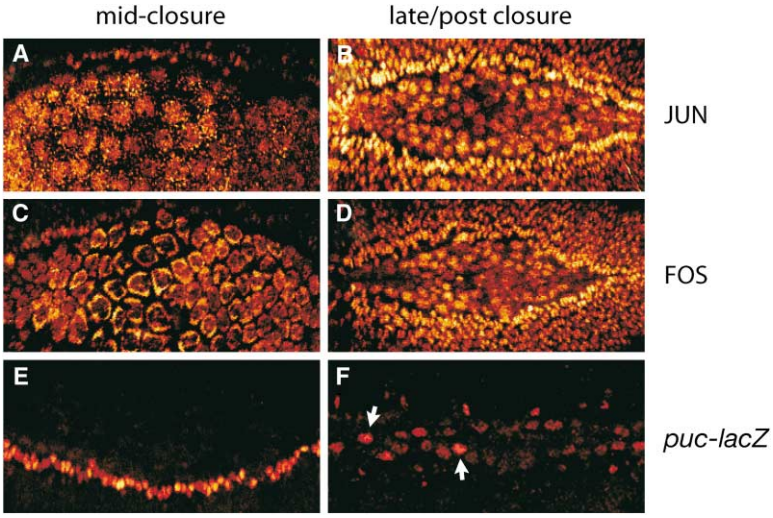


Figure 6. JNK Signaling Is Upregulated in the Amnioserosa toward the Completion of Dorsal Closure

(A, C, and E) Midclosure. (B and D) Late closure. (F) Postclosure stage embryo. (A and B) JUN. (C and D) FOS. (E and F) *puc-lacZ*. Both JUN and FOS are largely cytoplasmic in midclosure stages but become predominantly nuclear during late closure. *puc-lacZ* is not expressed during midclosure (only epidermal leading edge cells are seen) but is activated in the amnioserosa (arrows) during late closure.

dependent and promotes the integrity and survival of the amnioserosa. During normal development, closure of the midgut abrogates contact between the amnioserosa and yolk sac. JNK signaling is then upregulated in the amnioserosa, which subsquently disintegrates and dies, consistent with this being an example of developmentally programmed anoikis. In a subset of *mys* (βPS integrin) mutant embryos, apposition of the extraembryonic membranes never occurs, and the amnioserosa undergoes premature anoikis. The strong genetic interaction of *mys* and *basigin* mutants is consistent with the known physical interaction of these molecules in mammals [21] and suggests that basigin might act together with integrin to promote extraembryonic membrane interaction and to prevent anoikis of the amnioserosa. Failure of germ band retraction and dorsal closure occurs in integrin mutants and is greatly enhanced when basigin levels are reduced. Together, these results suggest that extraembryonic membrane interaction promotes survival of the amnioserosa, which in turn directs germ band retraction and dorsal closure through physical contacts [4–6] and/or intercellular signaling [2, 3].

The hypothesis that amnioserosal anoikis is triggered during normal development by loss of integrin-mediated contact with the yolk sac membrane makes several testable predictions. First, that in mutants in which the amnioserosa undergoes premature apoptosis prior to germ band retraction (e.g., *hindsight* [2, 32]), phase II apposition of the amnioserosa and yolk sac membrane may fail. Second, that premature amnioserosal apoptosis in these mutants is a consequence, rather than a cause of loss of amnioserosal epithelial integrity. Third, that the amnioserosa may persist in mutants lacking a midgut or in those defective for midgut closure.

It remains to be determined whether disintegration and death of the amnioserosa during normal development is caused solely by loss of contact with the yolk sac (i.e., is nonautonomously induced) versus whether signals from cell types other than the yolk—or even an amnioserosa-autonomous program—also play a role. For example, it is possible that upregulation of JNK signaling in the amnioserosa is independent of loss of contact with the yolk sac. Analysis of mutants lacking a midgut provide a test of this possibility: if disintegration and death of the amnioserosa occur even when apposition with the yolk sac is maintained, signals from other cell types or amnioserosa-autonomous processes would be implicated.

The specific role of JNK signaling in amnioserosal anoikis is difficult to assess because downregulation of JNK signaling in the amnioserosa and up-regulation of JNK signaling in the leading edge of the epidermis are required for dorsal closure [3]. Thus JNK pathway mutants stall morphogenesis prior to dorsal closure, making it impossible to assess a possible later role. Expression of dominant-negative JNK specifically in the amnioserosa only later in development, when closure is almost complete, will be necessary to rigorously test the role of JNK activation in amnioserosal anoikis.

All of the data presented above support the hypothesis that phase II amnioserosa-yolk sac membrane association is necessary for maintenance of the amnioserosal epithelium and, thus, the morphogenetic processes of germ band retraction and dorsal closure. However, the role of the transient phase I interaction is less clear. As described above, it is unlikely that the phase I interactions play a role in generation of the forces that lead to close apposition of these extraembryonic membranes. It seems more likely that the transient interactions play a role in communication between the yolk sac and the amnioserosa. It has recently been reported that the ecdysone receptor and active ecdysteroids are present in the amnioserosa and required for germ band retraction [33]. Expression of a dominant-negative form of the ecdysone receptor worsens germ band retraction defects in *mys* (βPS integrin) mutants [33]. Furthermore, it has been speculated that enzymes residing in the yolk might participate in conversion of ecdysone to its active forms [33]. We have observed dynamic invaginations of the yolk sac membrane, which dive into the yolk mass and transiently contact the yolk spheres (Movie 6). Thus, one tantalizing possibility is that these invaginations transport active forms of ecdysone—as well as other key signaling molecules—from the yolk spheres to the yolk sac membrane. Phase I amnioserosa-yolk membrane contacts and/or phase II intimate membrane apposition might subsequently bring these molecules to the amnioserosa.

## Conclusions

The extraembryonic tissues of *Drosophila* play a crucial role in directing embryonic morphogenesis. Close apposition of the yolk sac membrane and the basal cell membranes of the amnioserosa is dependent on βPS integrin. This intimate membrane association is required to promote survival and to prevent anoikis of the amnioserosa. The amnioserosa then directs germ band retraction and dorsal closure through physical contacts and/or signaling. Disintegration and death of the amnioserosa after closure of the epidermis and midgut correlates with upregulation of JNK signaling in the amnioserosa, is independent of *reaper/hid/grim* function, and is likely to represent the first example of developmentally programmed anoikis in *Drosophila*.

### Experimental Procedures

#### *Drosophila* Stocks

In this study we used mutants in *myospheroid* (*mys*$^{1}$, Bloomington *Drosophila* Stock Center), *basigin* (*bsg*$^{NP6293}$, from NP Consortium, provided by the *Drosophila* Genetic resource center in Kyoto Institute of technology; *bsg*$^{B39.1M2}$, Bloomington), and *twisted gastrulation* (*tsg*$^{2}$, Bloomington). Various GFP markers were used to label either the yolk membrane (*G289*, a PTT line from X. Morin [15]—see RT-PCR section below), the cell membrane in general (Ubi-DE-cadherin-GFP#5, from H. Oda [14]), or actin-based cellular projections (UAS-actin5C-GFP5.2, from H. Oda [13]). The *YET1* vital enhancer trap line expresses specifically in the perimeter cells of the amnioserosa and was provided by A. Michelson. A GFP-tagged balancer chromosome *FM7c, P{GAL4-Kr.C}DC1, P{UAS-GFP.S65T}DC5* (Bloomington) was used to unambiguously identify living *mys* mutant embryos. For this study we recombined a ftz-GAL4 driver (from J.P. Vincent) onto a chromosome with UAS-actin5C-GFP5.2 and Ubi-DE-cadherin-GFP#5. A *w*$^{1118}$ stock was used as a control for quantitative dorsal hole experiments, while PTT lines *G280* (His-2AV-GFP) and *G262* (lamin-GFP) (from X. Morin [15]) were used as controls for RT-PCR analysis of *G289* (see RT-PCR section below). The *LP1* GAL4 driver is specifically expressed in the amnioserosa [34]. Use of the *puc-lacZ* enhancer trap *A251* to assess JNK signaling in the

amnioserosa has been described previously [3]. The UAS-nuc-lacZ transgenic line and *Df(3L)H99*, a small deficiency that removes the *reaper*, *hid*, and *grim* cell death genes [28], were obtained from the Bloomington *Drosophila* Stock Center.

### Transmission Electron Microscopy

Embryos were chemically fixed by n-heptane permeabilization [35]: after dechorionation, embryos were fixed for 20 min in heptane saturated with 25% glutaraldehyde and 50 mM sodium cacodylate (pH 7.4). After hand devitellinization, the embryos were fixed for 2h in 1% osmium tetroxide, 2% glutaraldehyde, and 50 mM sodium cacodylate (pH 7.4). To visualize glycoconjugates, 0.5% ruthenium red was added at this step [36]. Then the embryos were postfixed in 1% osmium tetroxide and 50 mM sodium cacodylate (pH 7.4) for 1 hr and stained with 2% uranyl acetate for 30 min. Dehydrated embryos were infiltrated with a 1:1 mixture of Epon/Araldite and acetone overnight. After two more changes of Epon/Araldite and incubations overnight, embryos were embedded in Epon/Araldite plus DPM-30 and polymerized for 48 hr at 65°C. Sections (60–80 nm) were cut on a Reichert Ultracut S ultramicrotome mounted on net grids and stained with uranyl acetate and lead citrate. Sections were analyzed with a Jeol JEM-1200 EX electron microscope.

### Time-Lapse Confocal Microscopy

Embryos were collected for 2 hr and aged at 25°C on grape juice agar medium with an automated *Drosophila* egg collector (Flymax Scientific Equipment Ltd.). Approximately 30 min prior to imaging, appropriately staged embryos were hand-dechorionated with forceps and mounted in halocarbon oil (1:1 mix of series 56:series 700) on a gas permeable membrane (BioFoil 25, Heraeus, Inc.) attached to the bottom of a polystyrene Petri dish into which a hole had been cut. Embryos were covered with a coverslip and the dish was kept covered in the presence of tissue moistened with water to protect embryos from dehydration. Time-lapse images were captured on a Zeiss Axiovert 100 confocal microscope by using LSM510 software. NIH ImageJ was used to process image stacks and Volocity 2.0.1 (Improvision, Inc.) software was used to compile QuickTime movies. Adobe Photoshop and Illustrator were used to process the images presented in figures.

### Cuticle Preparations and Quantitation of Dorsal Hole Size

Methods were as described [3]. The longest dimension of the dorsal cuticle holes was measured in μm with Openlab 3.1.5 (Improvision, Inc.) software and the data graphed with Microsoft Excel.

### Reverse Transcription (RT)-PCR

RT-PCR was used to confirm that the *G289* PTT inserted 3′ of the *basigin* transcription unit fuses GFP to basigin. Total RNA was extracted from embryos utilizing Trizol (Gibco BRL) according to the manufacturer's instructions. SuperScript one-step RT-PCR with Platinum *Taq* (Invitrogen) was then utilized to synthesize cDNA following the manufacturer's instructions. RNA was extracted from the following fly lines: *G289*, *G280*, *G262* and $w^{1118}$. Gene-specific oligonucleotides (oligos) were used in various combinations in PCR amplification reactions. Only *G289* gave the predicted product, showing that *G289* results in a chimeric *basigin-GFP* mRNA. RT-PCR was used to confirm that the *G289* PTT fuses GFP to basigin. The sequences of the oligos used are available upon request.

### Analysis of *Drosophila* Basigin Protein Sequence

BLAST (NCBI) was used to identify homologous protein sequences. Prediction of protein domains (Ig and transmembrane) was done with MotifScan (ProSite database), signal peptide cleavage sites with SignalP V2.0, and glycosylation sites with YinOYang on the Expasy server (R. Gupta, J. Hansen, and S. Brunak, unpubished data; see http://www.cbs.dtu.dk/services/YinOYang/).

### Whole-Mount Immunohistochemistry and Acridine Orange Staining

Immunohistochemistry for β-galactosidase, HNT, FOS and JUN, and acridine orange staining was carried out as previously described [3, 37].

### Supplemental Data

Supplemental Movies and Results are available with this article online at http://www.current-biology.com/cgi/content/full/14/5/372/DC1/.

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