What basal membranes can tell us about viscous forces in *Drosophila* ventral furrow formation

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ABSTRACT

Ventral furrow (VF) formation in *Drosophila melanogaster* is an important model of epithelial folding. Past studies of VF formation focus on the role of apical constriction in driving folding. However, the relative contributions of other forces are largely unexplored. When basal membrane formation is genetically blocked using RNAi-mediated anillin knockdown (*scra* RNAi), the VF is still capable of folding. *scra* RNAi and control embryos display quantifiable cell length differences throughout gastrulation, as well as qualitative differences in membrane integrity. To interpret our observations, we developed a computational model of VF formation that explicitly simulates the flows of the viscous cytoplasm. The viscosity included in our model is required for tissue invagination in the complete absence of basal membranes and explains the observed differences in membrane lengths across conditions. In the absence of basal membranes, epithelial folding requires the presence of viscous shear forces from cytoplasm. Our model characterizes folding during VF formation as a “swimming phenomenon”, where tissue deforms by pushing against the ambient viscous surroundings. Since VF formation is successful in *scra* RNAi embryos, we propose that models of gastrulation should also be tested for their ability to replicate folding in the absence of basal membranes.

INTRODUCTION

Epithelial folding occurs throughout many stages in animal development (Leptin, 1991; Martinez Arias & Stewart, 2003), including neurulation (Maruyama & Andrew, 2012) and lung bud formation (Kim et al., 2013). During *Drosophila* gastrulation, the initially-monolayered embryo becomes multilayered through epithelial folding (Campos-Ortega & Hartenstein, 1985; Kohler, 1994).

After fertilization of a *Drosophila* egg, the nuclei undergo 13 division cycles and migrate to the periphery of the embryo. Since nuclear division at this stage is not accompanied by cytokinesis, this results in an embryo that is essentially a large multinucleate cell encased in a rigid vitelline membrane (Leptin, 1991; Zalokar & Erk, 1976). Subsequently, lateral membranes grow inwards to compartmentalize peripheral nuclei into individual cells, forming the epithelium in a process known as cellularization (Campos-Ortega & Hartenstein, 1985; Lecuit & Wieschaus, 2000). Throughout the course of cellularization, cells remain open to the yolk sac such that there is a "hole" on the basal side of each cell (see schematic in Figure 1–figure supplement 1) (Krueger et al., 2019; Loncar & Singer, 1995; Mazumdar & Mazumdar, 2002).

As cellularization completes, gastrulation begins with the folding of the ventral furrow (VF) (Campos-Ortega & Hartenstein, 1985; Leptin & Grunewald, 1990; Sweeton et al., 1991; Turner & Mahowald, 1977). VF formation involves a sequence of shape changes in a subset of ventral cells, starting with constriction of their apical membranes (Kam et al., 1991; Leptin, 1991). As VF formation proceeds, lateral membranes lengthen before eventually shortening again as the basal membranes seal and the ventral tissue invaginates to form a furrow (Lye & Sanson, 2011; Sweeton et al., 1991; Turner & Mahowald, 1977). These shape changes are driven by acto-myosin contractility in the membrane-associated cytoskeleton (Martin et al., 2009, 2010). Since

cellularization is still ongoing during the initial apical constriction phase of VF formation, cells remain open and only start sealing basally as tissue invagination begins. The simultaneous occurrences of basal membrane formation and epithelial folding indicate one process may rely on the other. Past studies of VF formation focus on the role of apical constriction in driving epithelial folding (Sawyer et al., 2010). However, the relative contributions of forces in the various cellular domains remain elusive, and the contribution of basal membranes to VF formation is largely unexplored.

RESULTS AND DISCUSSION

VF formation in *scra* RNAi embryos occurs despite the absence of basal membranes

We began by exploring the role of basal membranes in VF formation. Loss-of-function mutations in Anillin (Scraps) – a component of the contractile ring scaffold that narrows to seal off cells and form basal membranes – delay basal membrane formation (Field et al., 2005; Thomas & Wieschaus, 2004; Xue & Sokac, 2016). However, gastrulation has not been characterized in this genetic background. For experimental simplicity, we first replicated this phenotype via maternal GAL4-driven expression of short hairpin RNA against *scra* (UAS-scraTRiP). Live imaging of embryos expressing maternal GAL4, UAS-scraTRiP, and a membrane marker (UAS-Nrt-GFP) showed that the effects of *scra* RNAi during cellularization were indistinguishable from those seen in Anillin loss-of-function mutants (Figure 1–figure supplement 2) (Field et al., 2005; Thomas & Wieschaus, 2004; Xue & Sokac, 2016). To better characterize gastrulation in this background, we prepared sections of heat-fixed gastrulas stained for Neurotactin, Snail, and DAPI (membrane, mesodermal, and nuclear markers, respectively), see Figure 1. As a control, embryos of similar genetic background without UAS-scraTRiP were

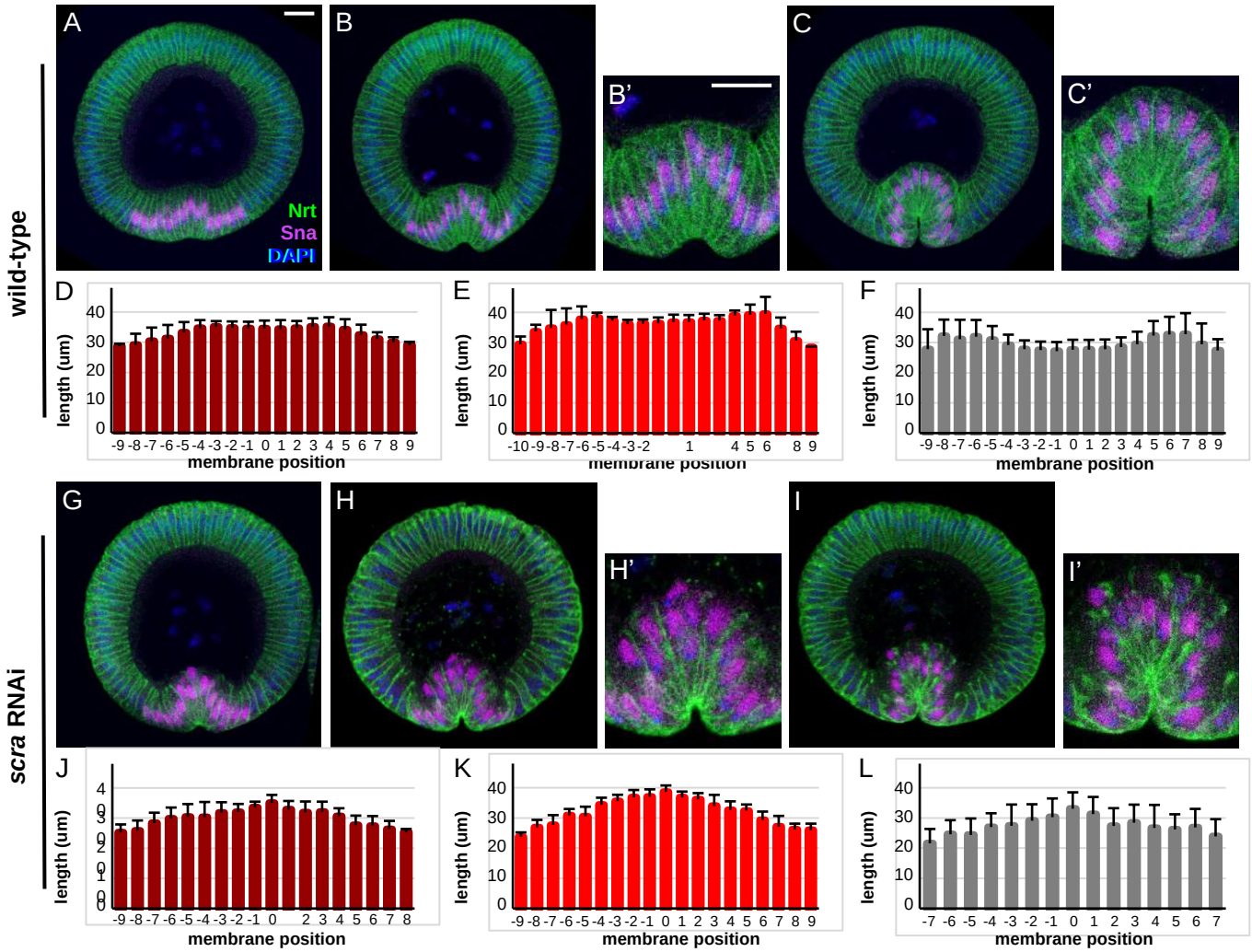

Figure 1. Basal membranes are not required for VF formation.

(A-C) Confocal immunofluorescence of heat-methanol fixed wild-type embryos during (A) apical constriction, (B) invagination, and (C) late VF formation. (D-F) Mesoderm lateral membrane lengths in wild-type embryos during (D) apical constriction (n = 9 embryos), (E) invagination (n = 7 embryos), and (F) late VF formation (n = 10 embryos). (G-I) Confocal immunofluorescence of heat/methanol-fixed *scra* RNAi embryos during (G) apical constriction, (H) invagination, and (I) late VF formation. (J-L) Mesoderm lateral membrane lengths in *scra* RNAi embryos during (J) apical constriction (n = 8 embryos), (K) invagination (n = 8 embryos), and (L) late VF formation (n = 6 embryos). (B'), (C'), (H'), and (I') are 2x magnifications of (B), (C), (H), and (I), respectively. All scale bars are 20µm. Data are represented as mean ± s.d. Membrane lengths in each of the three stages of VF formation are all significantly different across conditions (wt vs. *scra* RNAi, $p < 0.001$).

Figure supplement 1. Schematic of membrane shape changes throughout VF formation.

Figure supplement 2. Acto-myosin ring closure is delayed in *scra* RNAi embryos.

Figure supplement 3. Central mesodermal cells lengthen more, peripheral mesodermal cells shorten less throughout VF formation in *scra* RNAi embryos compared to wild-type.

Figure supplement 4. Non-mesodermal cells are shorter during invagination in *scra* RNAi embryos.

Figure supplement 5. Membranes in *scra* RNAi embryos deteriorate more over time.

stained according to the same protocol (Figure 1A-C). Basal membranes never form in *scra* RNAi gastrulas, yet the VF is remarkably still capable of folding to its usual depth (Figure 1G-I).

Membrane lengths are altered in *scra* RNAi embryos

We were interested to see if there were any morphological differences between *scra* RNAi and control embryos. To this end, we used bar charts to compare the distribution of lateral membrane lengths along transverse sections of *scra* RNAi (Figure 1D-F) and control (Figure 1J-L) embryos at various stages of VF formation. In control embryos, distributions exhibit a curve with two peaks along the mesoderm: peripheral cells are shortest, followed by the most central cells, while cells at the boundary between these two regions are longest (Figure 1D-F). As apical constriction proceeds, lateral membranes lengthen (Figure 1D-E), then shorten throughout invagination as the VF forms more fully (Figure 1E-F). While the temporal lengthen-then-shorten pattern of morphological changes remains relatively unaffected in *scra* RNAi embryos (Figure 1–figure supplement 3), the spatial distributions of membrane lengths along the anillin depleted-VF have only one peak (Figure 1J-L). This difference arises in the degree to which various regions of the VF lengthen and shorten. Between apical constriction and invagination, central VF cells in *scra* RNAi embryos lengthen more than those of control embryos (Figure 1–figure supplement 3B). Over the same period, peripheral VF cells in *scra* RNAi embryos shorten less than those of control embryos (Figure 1–figure supplement 3BC).

We also measured the length of ectodermal membranes (outside the VF) at the same three stages of VF formation (Figure 1–figure supplement 4). In both cases, ectodermal membranes lengthen during VF formation. However, ectodermal lengthening is delayed in *scra* RNAi embryos (Figure 1–figure supplement 4B-C).

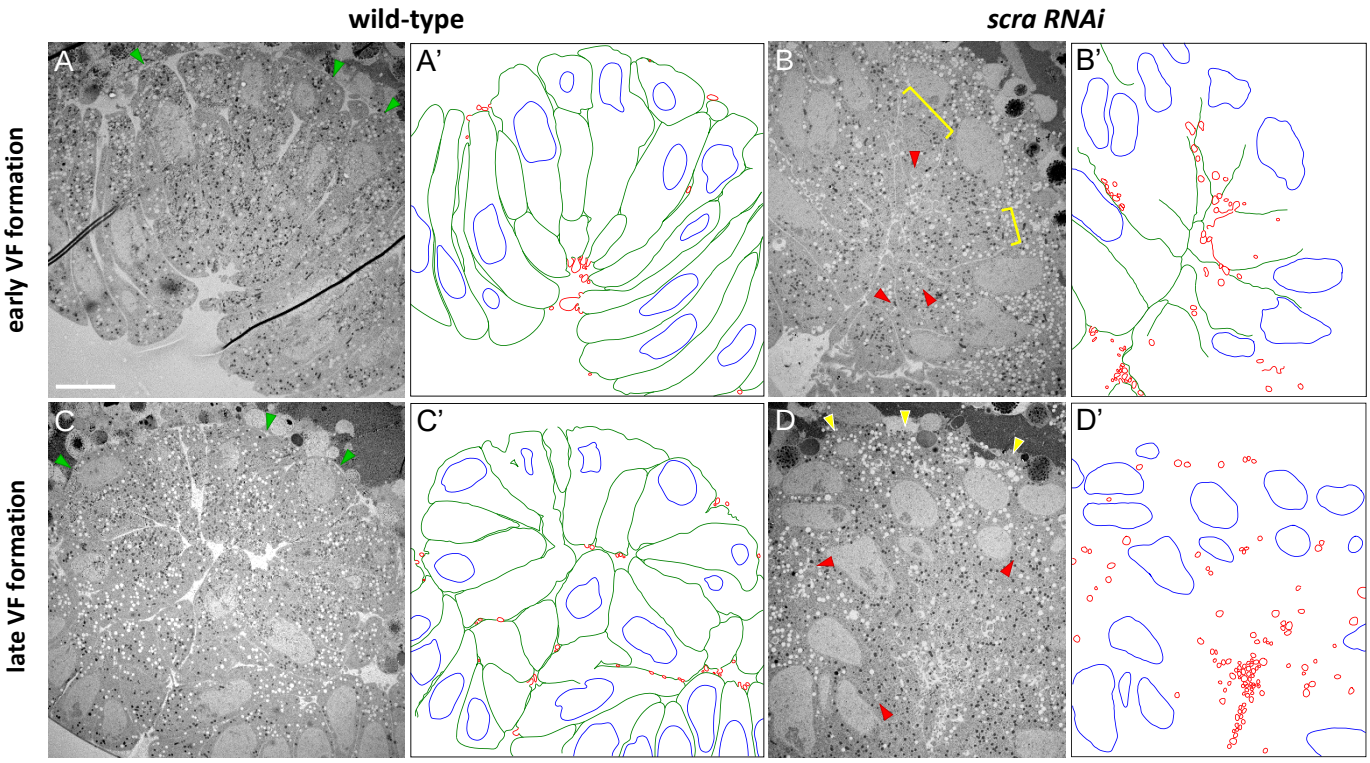

Figure 2. Basal membranes never form in *scra* RNAi embryos.

(A-D) Low mag TEM of embryo sections prepared using a combined high pressure freezing/freeze substitution method during invagination (A,B) or late VF formation (C,D) in wild-type (A,C) and *scra* RNAi (B,D) embryos. Interstitial spaces are visible on the basal sides of VF cells (green arrows) in wild-type embryos during both early (A) and late (B) VF formation. Basal interstitial spaces are absent (yellow brackets) and lateral membranes are starting to degrade into vesicles (red arrows) in early VF formation *scra* RNAi embryos (B). Basal interstitial spaces are still absent (yellow arrows) during late VF formation in *scra* RNAi embryos (D) and lateral interstitial spaces are no longer visible, replaced by an increased number of vesicles (red arrows). (A'-D') are hand-drawn traces of (A-D), respectively, highlighting intact interstitial spaces (green), nuclei (blue), and vesicles (red). All scale bars are 10μm.

VFs in *scra* RNAi embryos remain folded despite degradation of lateral membranes

Anillin mutants additionally present with membrane degradation and nuclear displacement (Field et al., 2005). Depletion of anillin in *scra* RNAi gastrulas also results in the gradual disintegration of lateral membranes, which eventually form vesicles (Figure 1–figure supplement 5A-D). Membrane degradation increases in severity over time. Some VF cells become multinucleated, and VF nuclei are intermittently displaced into the yolk sac (Figure 1G-I). These findings suggest that Anillin plays a role in VF formation – not only during actin ring closure and basal membrane formation, but also in maintaining membrane stability.

The possibility remained that the phenotypes seen in immunostainings reflected differences in Neurotactin localization and not the presence or absence of membranes. To substantiate our findings, we examined the fine structure of epithelial membranes using transmission electron microscopy (TEM). Specifically, we froze gastrulas of various stages under high pressure, subjected them to freeze substitution, made 60-70nm transverse cross sections, and imaged them under TEM. At early stages of VF formation, interstitial spaces between ventral cells are visible in control embryos at low magnification (Figure 2A) indicating the presence of intact membranes (traced in green). In early stage *scra* RNAi gastrulas, these interstitial spaces are less intact, and lateral membranes have started disintegrating into vesicles (traced in red) (Figure 2B). Ventral cells in late stage control gastrulas have intact lateral and basal membranes (Figure 2C), all of which are absent in late stage *scra* RNAi gastrulas. Specifically, at the late stage, interstitial spaces are no longer visible and have been replaced by an increased number of vesicles (Figure 2E). These TEM results confirm that the VF is indeed capable of forming in *scra* RNAi embryos, and the furrow retains its shape in the absence of basal, and subsequently, lateral membranes.

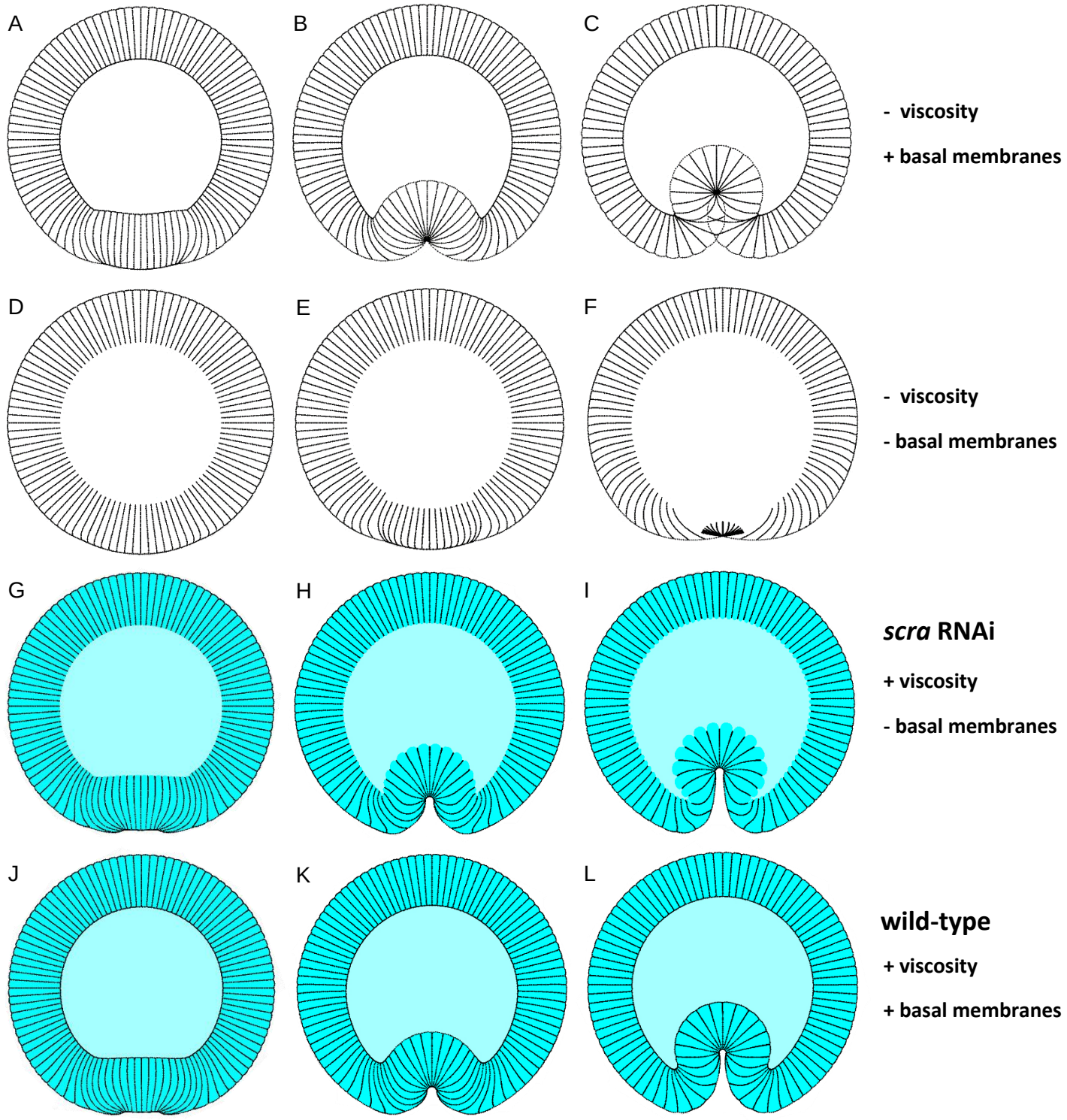

Figure 3. Apical constriction and lateral tensions alone are not sufficient to form the VF.

Simulation results from a mathematical model of ventral furrow formation. Cells are elastic along all edges and constrained apically by modeled vitelline membrane. Simulation conditions differ as follows: (A-C) inviscid interior, basal stresses present, (D-F) inviscid interior, basal stresses absent, (G-I) viscous interior, basal stresses absent, and (J-L) viscous interior, basal stresses present.

Figure supplement 1. Model of VF formation with low basal stressed failed to replicate cell length distribution seen *in vivo.*

VF formation without basal membranes is explained by viscosity

Many existing models of VF formation require volume conservation within cells (Rauzi et al., 2013). It is unclear how such models could describe a mutant in which basal membranes are missing. A mechanistic question remains: how are *scra* RNAi embryos capable of forming a furrow while cells remain open? To explore this question, we developed a series of computational models of gastrulation, in which a set of initially rectangular model "cells" represents a transverse cross-section of the embryonic epithelium. In these models, each cell is exclusively elastic along its surface. Dynamics are driven by contractile stresses in the different membranes (note that when we refer to membranes in this section, we imply the membrane-associated cytoskeleton instead of the lipid bilayer). Specifically, we assume that all membranes are subjected to a relatively small amount of constitutive contractile "pre-stress". The 16 mesodermal cells in our model have additional stress in their apical and lateral membranes. Technical details of model implementation are described in Supplemental Methods; the code used to generate the results is publicly available under https://github.com/doubrovinskilab/anillin_code.

In the most minimal version of this model, the mesoderm constricts apically and then invaginates (Figure 3A-C). When this model is modified so that cells exchange their internal contents freely with the yolk sac, (in other words basal membranes are absent to mimic *scra* RNAi embryos), the furrow does not form although apical constriction does occur (Figure 3D-F). We then modified this model to include a viscous cellular interior. Strikingly, this modification resulted in complete mesoderm invagination in the absence of basal membranes, mimicking our observations in *scra* RNAi embryos (Figure 3G-I). Importantly, the assumption of a highly viscous interior is based on physical measurements of embryonic *Drosophila* epithelium

(Doubrovinski et al., 2017). Furthermore, a model which includes both viscosity and basal membranes (Figure 3J-L) results in very similar dynamics to the model with basal membranes but no viscosity (Figure 3A-C).

Finally, we asked if our model could reproduce consistent differences in the distribution of membrane lengths between *scra* RNAi and control. Beginning mid-way through gastrulation in control embryos, ventral cells that immediately flank the center of the mesoderm are the longest (Figure 1E). Our model including viscosity indeed reproduces this observation (compare Figure 3I and 3L). Remarkably however, this result was not reproduced with the parameter set we initially chose (Figure 3–figure supplement 1), since the longest membranes in the control model were found in the middle. However, our *in vivo* observations were readily replicated *in silico* by simply increasing the magnitude of active stress in the basal domain of all cells; these are the results shown in Figure 3. This demonstrates that the magnitude of active stresses on the basal surface can control the fine details of furrow shape, despite not being required for invagination. Our computational result can also be understood intuitively: when a curved surface is subject to in-plane stress, it tends to flatten. This effect is strongest where curvature is greatest. In our case, the curvature of the basal side is greatest precisely where mesoderm is flanked by the ectoderm. In this way, those flanking cells become significantly longer along the lateral direction if basal stress is increased.

To summarize, our minimal model accounts for both the invagination of the mesoderm in the absence of basal membranes as well as for the qualitative differences in mesodermal thickness between *scra* RNAi and control gastrulas. The key feature of our model is accounting for the viscous drag (i.e. shear) on the membranes from the ambient cytoplasm. In the absence of this effect, no invagination will result in the case when cells are not sealed basally. Basal membranes

help confine the cytoplasm to the cellular interior, but viscous shear forces can have a similar effect. If viscosity is high enough, shear forces will prevent any appreciable amount of cytoplasm from escaping through the basal opening before invagination completes. Since invagination is largely unperturbed in the absence of basal membranes, viscous shear must be a key physical effect involved in driving tissue folding.

METHODS

Drosophila genetics

For anillin depletion experiments (Figures 1-2), we used the following genotype: UAS-Nrt-eGFP; P{y[+t7.7] v[+t1.8]=TRiP.GL01269}attP2/mat15-GAL4. TRiP.GL01269 was derived from RRID:BDSC 41841 (Bloomington Drosophila Stock Center); mat15-GAL4 was derived from RRID:BDS_80361 (Bloomington Drosophila Stock Center). To generate UAS-Nrt-eGFP, full-length Nrt-RB was cloned by PCR from LD22004 (Drosophila Genomics Resource Center stock #5736) and inserted into pPWG (Drosophila Genomics Resource Center stock #1078). This plasmid was injected by BestGene using P-element insertion. Embryos were collected from female progeny on grape agar plates supplemented with yeast paste after ≥3.5hrs.

Fluorescent immunohistochemistry

Fly embryos were heat-methanol fixed as described previously (Müller & Wieschaus, 1996). The block, primary antibody, and secondary antibody steps were all done overnight with nutation at 4°C. Primary and secondary antibodies were diluted in block solution (1x PBS, 0.1% Triton X-100, 5% heat shocked goat serum or 0.2% BSA). Antibodies used include mouse anti-Neurotactin (1:50) (DSHB), guinea pig anti-Snail (1:2000) (gift from Eric Wieschaus), goat anti-mouse IgG-Alexa Fluor 488 (1:500) (Invitrogen), and goat anti-guinea pig IgG-Alexa Fluor 568 (1:500) (Invitrogen). Nuclear staining was done using DAPI (1µg/mL) (Invitrogen).

Confocal fluorescence imaging of embryo sections

Immunostained fly embryos were staged in 1x PBS with 0.1% Triton X-100 under bright field on an Accu-Scope dissection scope. A coverslip was prepared by cutting off ~1cm of the short edge and placing it in the center of the coverslip, perpendicular to the long edge, then adding a linear pool of AquaPolymount along the supporting glass strip. Selected gastrulas were transferred to pool of mounting medium and sectioned along the dorsal-ventral axis

using a 22g needle. Embryo halves were positioned cut side down, leaning against the supporting glass strip. Z-stacks were imaged using a Plan-Apochromat 63x/1.40 Oil objective on a Zeiss LSM 700 confocal microscope. For immunohistochemistry experiments, we fixed and stained samples over the course of several weeks, until we had at least 40 separate gastrulas imaged. From these, we selected all samples in which the morphology had not been significantly distorted by sectioning damage or angle – these results are collected in Figure 1.

Statistical analysis of immunostaining results

For the purposes of all experiments, all samples are biological replicates. We binned data from bar charts in Figure 1 (19 positions, -9 to 9) in sequential groups of 3, producing a plot with 6 average values of membrane lengths along the VF. We then compared experimental groups pairwise using Fisher's linear discriminant analysis. Specifically, we produced two sets of projections on the direction normal to the discriminant hyperplane, each set corresponding to one of the experimental groups being compared. Finally, the significance of the difference between the two sets of projections was assayed using the pairwise Kolmogorov-Smirnov test. An exception was made in the case of late VF formation *scra* RNAi embryos, where we only binned data from 15 positions (-7 to 7) – this was due to a smaller sample size for this group. For Figure 1–figure supplement 4, all data were used in analysis.

Transmission electron microscopy

A combined high pressure freezing and freeze substitution (HPF/FS) method was used to fix fly embryos as described elsewhere (Zhang & Chen, 2008). Briefly, a Wohlwend Compact 03 high pressure freezer was used to fix the embryos. Samples were freeze substituted in 1% osmium tetroxide, 0.1% uranyl acetate in 98% acetone and 1% methanol using a Leica EM AFS2. The embryos were embedded in Embed-812 resin and polymerized in a 60°C oven overnight. Blocks were sectioned with a diamond knife (Diatome) on a Leica Ultracut 7 ultramicrotome (Leica Microsystems) and collected onto slot grids and post stained with 2% aqueous uranyl acetate and lead citrate. Images were acquired on a JEOL JEM-1400 Plus TEM equipped with a LaB_6 source operated at 120 kV using an AMT-BioSprint 16M CCD camera. For TEM experiments, we continuously staged and fixed embryos over the course of 6-9 hours. Embryo sections shown in Figure 2 are representative of 5 (Figure 2A), 3 (Figure 2B), 11 (Figure 2C), or 4 (Figure 2D) samples. From these, we selected all samples in which the morphology had not been significantly distorted by ice/mechanical damage or other staining artifacts.

Mathematical modeling

For a detailed description, please see the Supplemental Materials.

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COMPETING INTERESTS

The authors declare that no competing interests exist.

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SUPPLEMENTAL FIGURES

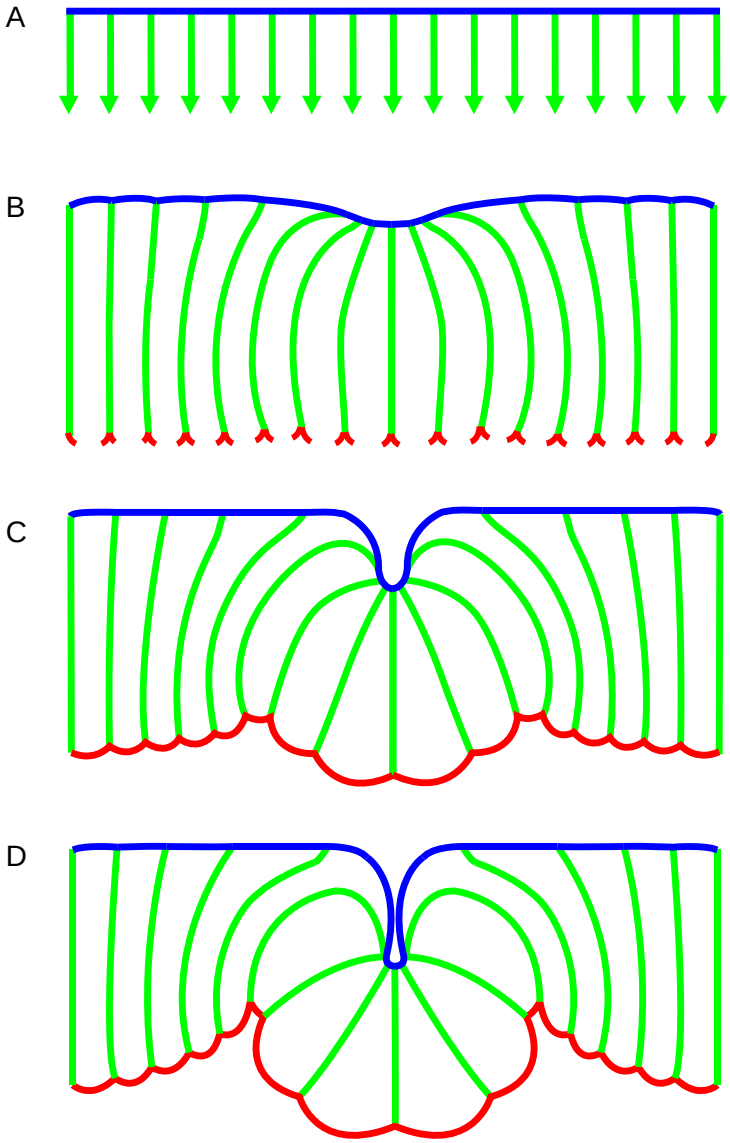

Figure 1–figure supplement 1. Schematic of membrane shape changes throughout VF formation.

(A-D) Illustration of VF formation in cross section showing shape changes in apical (blue), lateral (green), and basal (red) membranes throughout tissue folding. Lateral membranes lengthen (arrows) throughout cellularization (A). Lateral membranes of VF cells lengthen more as apical membranes constrict to initiate VF formation (B). Lateral membranes shrink back down as tissue invaginates and basal membranes form. Basal membranes seal and expand (C) as the VF continues to fold inward (D).

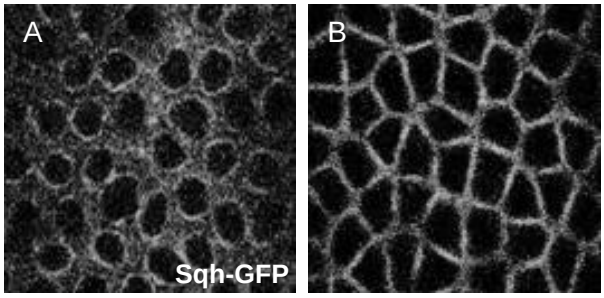

Figure 1–figure supplement 2. Acto-myosin ring closure is delayed in *scra* RNAi embryos.

(A,B) Maximum intensity projections of the cellularizing ventral epithelium in live control (A) and *scra* RNAi (B) embryos during late cellularization. Acto-myosin rings remain open and are misshapen in *scra* RNAi embryos, preventing the formation of basal membranes.

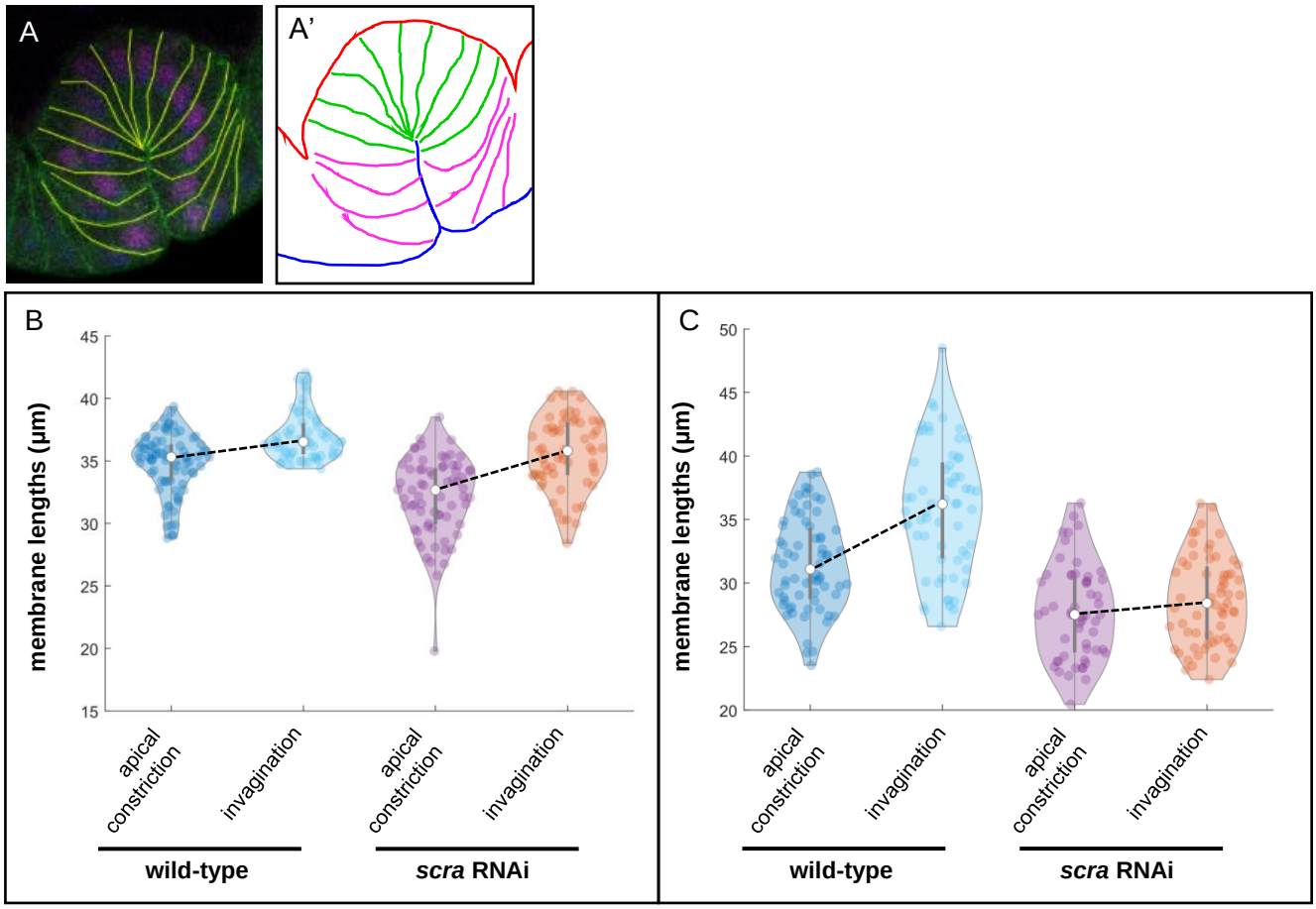

Figure 1–figure supplement 3. Central mesodermal cells lengthen more, peripheral mesodermal cells shorten less throughout VF formation in *scra* RNAi embryos compared to wild-type.

Example of designated central (green) and peripheral (pink) mesoderm regions shown in (A,A'). Violin plots show changes in average central (B) and peripheral mesodermal membrane lengths (C) between apical constriction (ac) and invagination in wild-type (n_{ac} = 9 embryos, n_{invag} = 7 embryos) and *scra* RNAi embryos (n_{ac} = n_{invag} = 8 embryos).

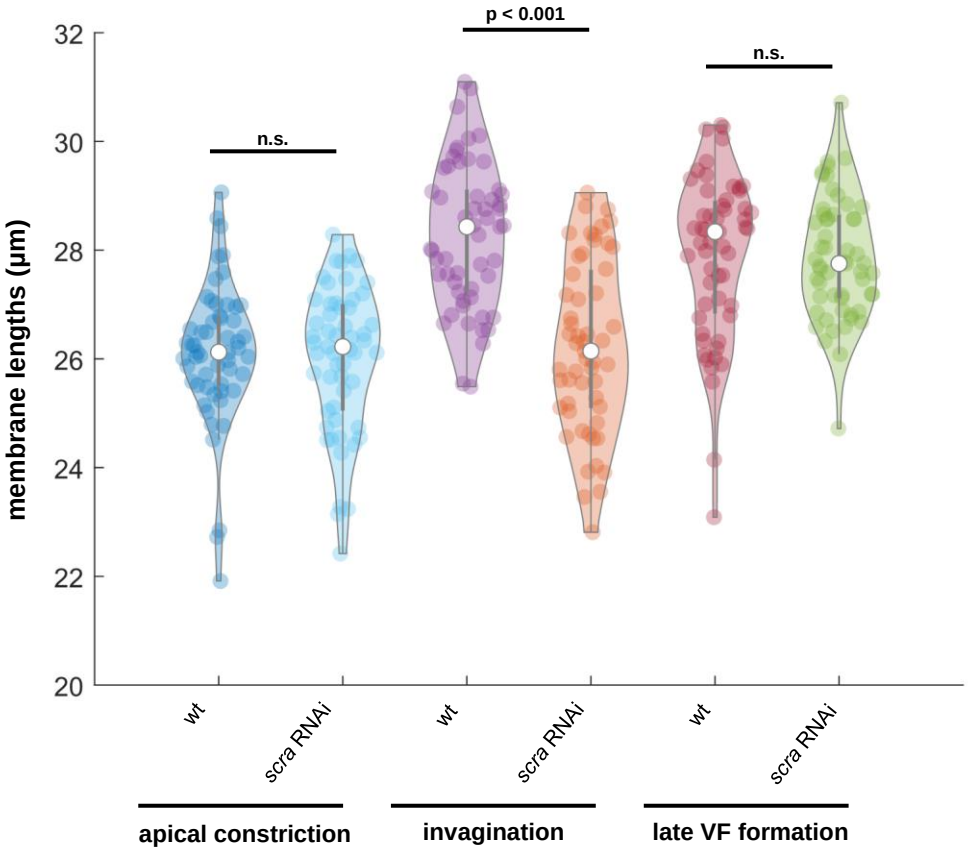

Figure 1–figure supplement 4. Non-mesodermal cells are shorter during invagination in *scra* RNAi embryos.

Violin plots of non-mesodermal cell lengths during apical constriction, invagination, and late VF formation in wild-type (wt) and *scra* RNAi embryos. Lateral cells of *scra* RNAi embryos do not lengthen as quickly as those in control, resulting in shorter epithelium mid VF-formation. By late VF formation, lateral cells of *scra* RNAi embryos have reached lengths similar to those in wild-type embryos.

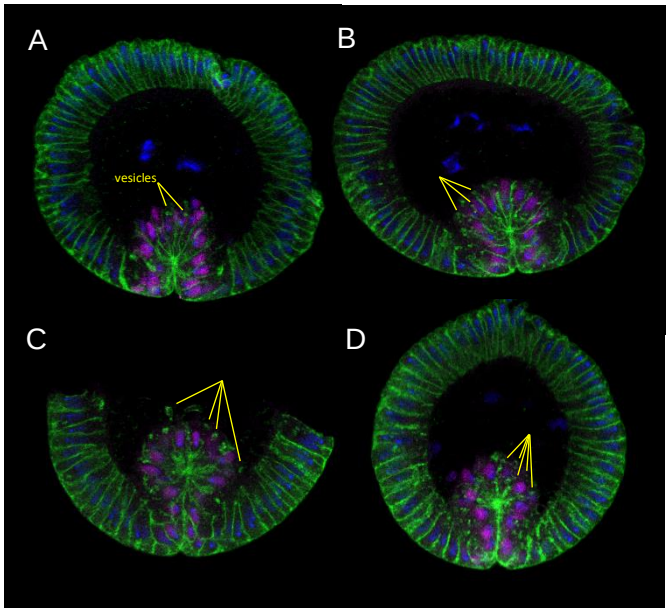

Figure 1–figure supplement 5. Membranes in *scra* RNAi embryos deteriorate more over time.

Confocal immunofluorescence of heat/methanol-fixed anillin RNAi embryos at increasingly older stages of gastrulation (A-D). Membrane degradation increases with age, and vesicles become more numerous. Note: the embryo section in C is incomplete due to how it was cut, not any depleted protein expression or lack of antibody staining.

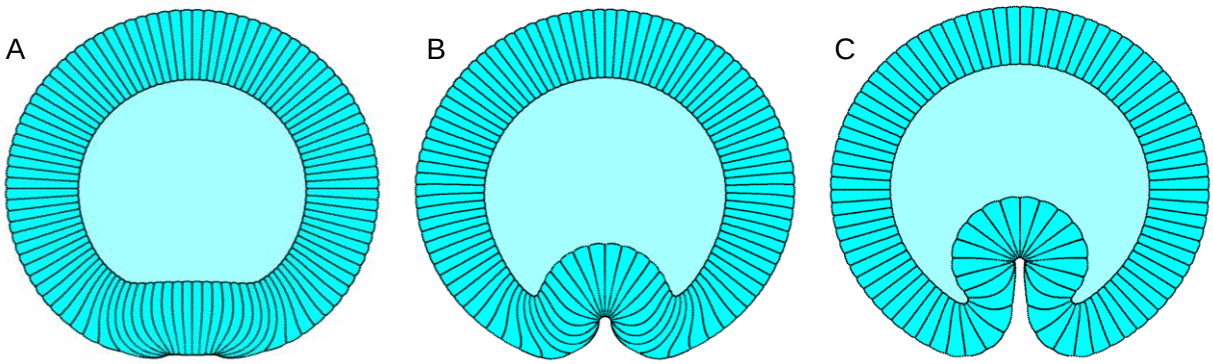

Figure 3–figure supplement 1. Model of VF formation with low basal stressed failed to replicate cell length distribution seen *in vivo.*

(A-C) Simulation results from a mathematical model VF formation. Cells are elastic along all edges and constrained apically by modeled vitelline membrane.