Extracellular vesicle formation mediated by local phosphatidylserine exposure promotes efficient cell extrusion

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Article

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Extracellular vesicle formation mediated by local phosphatidylserine exposure promotes efficient cell extrusion

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Abstract:

Numerous unwanted cells are removed from epithelial and endothelial tissues—in which cells are tightly connected to one another—without disturbing tissue integrity and homeostasis. Cell extrusion is a unique mode of cell removal from tissues, and it plays an important role in regulating cell numbers and the eliminating unwanted cells, such as apoptotic cells, cancer cells, and cells with a lower fitness in cell competition [1-3]. During this process, cells delaminate from the cell layer, to which they initially used to adhere, through communication with the neighboring cells [4,5]. Defects in cell extrusion are believed to associate with inflammation and cancer in epithelium as well as blood vessel dysfunction [6,7]. However, the correlation between them has not yet been evaluated owing to a lack of knowledge of the underlying mechanisms. In particular, the process whereby the cell exit from the tissue remains to be elucidated. Here, we report a novel and conserved execution mechanism of cell extrusion—common to mammalian cells and *Drosophila* epithelia—i.e., spatiotemporally regulated extracellular vesicle formation in extruding cells at a site opposite to the direction of extrusion. Particularly, we found that a lipid-scramblase– mediated local exposure of phosphatidylserine is responsible for extracellular vesicle formation and is crucial for the execution of cell extrusion, and inhibition of this process disrupted prompt cell delamination as well as tissue homeostasis. Furthermore, we revealed the mechanism underlying vesicle formation. Importantly, our results reveal that membrane dynamics is the driving force by a "rocket launch"-like mechanism behind the extrusion of cells from tissues, a fundamental cell behavior in multicellular organisms that is also observed in other contexts including cancer cell invasion and neural cell differentiation. Our understanding of this new mechanism of cell extrusion enables us to

examine the relationship between cell extrusion abnormalities and the onset of various diseases.

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Main Text:

5   Although the contraction of the actomyosin complex produces the driving mechanical force for execution of cell extrusion [8,9], how the cell delaminates from the tissue remains a fundamental yet unanswered question. We hypothesized that membrane dynamics play a key role in the execution of cell extrusion. To investigate this, first, we visualized the cell membrane with membrane-bound GFP (membrane GFP) and analyzed the detailed membrane dynamics in

10   both extruding cells and their neighboring cells. In Mardin-Darby canine kidney II (MDCKII) cell monolayers, cell extrusion occurs spontaneously after confluence to adjust cell density [4].We found extensive budding of the membrane of extruding cells and the presence of many vesicles inside the neighboring cells via live-imaging analysis of membrane GFP-expressing MDCKII cells (92/92 extrusion events) (Fig. 1A and Movie S1). Immunostaining results revealed that

15   cleaved caspase3 is contained in the vesicles of the neighboring cells (110/113 extrusion events) in addition to the cytoplasm of extruding cells (Fig. 1B). This suggests that these vesicles are derived from extruding apoptotic cells and engulfed by their neighbors.

To verify this, we performed mosaic analysis via the co-culture of RFP-expressing and membrane GFP-expressing cells. The deformation process in extruding cells began with a slight

20   invagination of the apical surface, and after 15 min, the cells showed budding-like membrane dynamics (Fig. 1C and Fig. S1). Then, the budding was rapidly fragmented, and the shed fragments were engulfed by the neighboring cells, as shown by the GFP-positive vesicles containing RFP in GFP-expressing cells adjacent to RFP-expressing extruding cells (10/10

extrusion events) (Fig. 1D and Movie S2). Fragmentation and engulfment occurred transiently rather than continuously, and the remaining portion of the extruding cell after fragmentation delaminated from the cell layer to the apical direction (Fig. 1C and Fig. S1). Interestingly, fragmentation and engulfment occurred preferentially at the basolateral or basal parts of the

5   extruding cells (10/10 extrusion events) (Fig. 1E and Movie S3). In addition, the protrusion of the extruding cell from the monolayer began simultaneously as fragmentation and engulfment (Fig. 1C and F). These results suggest that fragmentation and/or engulfment are involved in the execution of cell extrusion.

Apoptotic cells expose phosphatidylserine (PS) as an "eat-me" signal and are engulfed by

10   phagocytes [10]. Staining results with Annexin V, a PS-binding protein, showed that extruding cells exposed PS, and signals were enriched at the basal and basolateral parts with a patched pattern (Fig. 1G), strongly suggesting that the cell fragments that exposed PS were then recognized and engulfed by the neighbors. Lysotracker staining results showed that the phagocytic vesicles fused with the lysosomes 30-60 min after the engulfment (Fig. S2).

15   To evaluate the function of engulfment during cell extrusion, we intended to disturb the engulfment process using the MFG-E8 mutant protein. Secreted MFG-E8 protein binds to both PS exposed on apoptotic cells and integrins on phagocytes to promote phagocytosis, whereas the MFG-E8-D89E mutant protein (D89E), which carries a mutation in the integrin-binding motif, inhibits PS-mediated phagocytosis in a dominant-negative manner [11]. Although we expected that

20   D89E-overexpression in MDCKII cells prevents engulfment after fragmentation, we observed that it impaired the fragmentation process. Half of the cell extrusion did not show fragmentation, even after 25 min following the deformation of the cell shape (Fig. 2A, Fig. S3, and Movie S4). These extruding cells with delayed fragmentation despite the continuous membrane deformation

showed impaired protrusion from the cell layer and prolonged time to complete extrusion (Fig.

2B and C). Furthermore, upon D89E expression, some cleaved caspase3-positive cells were

found in the cell layer without protrusion, indicating the impaired execution of cell extrusion

(Fig. 2D). These results suggest that fragmentation and/or engulfment is critical for prompt cell

5   extrusion.

Another mammalian epithelial cell line, EpH4, showed fragmentation during cell

extrusion (61/61 extruding cells) at the basal half of extruding cells (7/7 extruding cells).

However, the fragments were not rapidly engulfed by neighboring cells but remained in or were

moved around the basolateral intercellular space (Fig. 2E and Movie S5). The masking of PS by

10   the D89E mutant in EpH4 cells inhibited fragmentation and importantly, cell extrusion (Fig. 2F,

G, H, Fig. S4, and Movie S6). The results from these two cell lines indicated that fragmentation,

which is mediated by PS exposure, is a common process and promotes the protrusion of

extruding cells. PS exposure in apoptotic cells is governed by the Xk-related protein (Xkr)

scramblase family, particularly Xkr4, Xkr8, and Xkr9 [12]. The knockdown of *Xkr8*, the most

15   abundant among the three in EpH4 cells (Table S1), decreased PS exposure and caused abnormal

fragmentation, impaired protrusion, and longer extrusion (Fig. 2I, J, K, and L), whereas the

knockdown of *TMEM16F* (*Ano6*), the most abundant lipid scramblase belonging to the

TMEM16 family in EpH4 cells (Table S1), did not affect fragmentation nor extrusion (Fig. S5).

Fragmentation was also observed in apoptotic cell extrusion induced by UV irradiation in

20   cultured cells (Fig S6). To further show that fragmentation occurs *in vivo*, time-lapse imaging

was performed on the pupal dorsal abdominal epidermis of *Drosophila*. During the early pupal

stage, all large-sized larval epithelial cells (LECs) extrude in the basal direction, whereas small

adult epithelial cells (histoblasts) replace the epithelial space through frequent cell division and

movement [13,14] (Fig. 3A). Upon extrusion of a lateral membrane-localized GFP (Lgl:GFP)-expressing LEC adjacent to other LECs, fragmentation of extruding cell was observed (23/23 extruding cells) (Fig. 3A and Movie S7). In mosaic analysis in which RFP was expressed in LECs only whereas Lgl:GFP was expressed in both LECs and histoblasts (Fig. 3B), RFP-positive vesicles derived from extruding LECs were detected in the neighboring histoblasts (5 of 5 extrusion events), indicating that fragmentation (and engulfment by the neighboring LECs and histoblasts) occurs during physiological cell extrusion *in vivo*. Fragmentation and engulfment were observed preferentially at the apical half of the extruding cells (4/5 extrusion events), which was in the opposite direction of extrusion (Fig. 3C). Apical fragmentation occurred after the apical surface, which was formerly occupied by extruding cells, was closed and covered by their neighbors (10/10 extrusion events) (Fig. 3D and Movie S8).

The ectopic expression of secreted Annexin V-EGFP fusion proteins (Annexin V:GFP) in histoblasts detected PS exposure in a speckled manner at the apical sites of extruding LECs, which are the likely sites to be fragmented, after the cell started to deform (13/13 extruding cells) (Fig. 3E, movie S9), whereas the expression of the mutant Annexin V:GFP (unable to bind to PS) did not (0/22 extruding cells). This result suggests that spatio-temporally regulated PS exposure triggers fragmentation. Annexin V:GFP-expression impaired both fragmentation and extrusion (data not shown) owing to the function to mask the exposed PS as reported in [15]. Furthermore, when Annexin V:GFP was expressed in LECs, the adult dorsal abdomen showed loss of continuity of the epidermis and bristles at the midline (Fig. 3F), a severe defect in cell death or extrusion as previously reported [16]. The knockdown of the *Drosophila Xk* gene (*Xk* or CG32579), a single homolog of the mammalian Xkr family, showed abnormality in the fragmentation of extruding cells (Fig. 3G, Fig. S7). The progression of cell extrusion after the

closure of the apical surface of the extruding cells was impaired in the *Xk*-knockdown epidermis as evidenced by the persistence of extruding cells in the cell layer for an extended time (Fig. 3H). The replacement of larval cells by histoblasts was delayed in the *Xk*-knockdown pupal epidermis (Fig. 3I).

5   In another physiological cell extrusion, during cell turnover in the adult intestinal epithelium, fragmentation and engulfment occurred in the *Drosophila* midgut as indicated by the formation of some vesicles in the neighboring cells (Fig. S8), as well as in the mouse intestinal organoid as shown by time-lapse imaging results (Fig. S9 and Movie S10). When *Xk* was knocked down in an enterocyte-specific manner in the *Drosophila* midgut, an increased tissue

10   width and shorter lifespan were observed (Fig. S10). These results indicate that PS exposure-induced fragmentation of the extruding cells is a universal process that plays a significant role in the execution of cell extrusion to ensure tissue development and homeostasis.

Lastly, we speculated that fragmentation involves the local formation and release of extracellular vesicles (EVs), which are observed in blood coagulation, intercellular signal

15   transduction, and various pathological processes [17-19]. The size of fragments formed during cell extrusion ranges between 0.5 and > 2 μm in diameter, and a small part of them were positive for DNA staining (a positive example: Fig. S11 and a negative example: Fig. 1B). In EVs, including exosomes, microvesicles, and apoptotic bodies, PS exposure and actomyosin accumulation at the budding sites, mediated by the function of the Arf family, Phospholipase D (PLD), and

20   extracellular regulated kinase (ERK), have been reported in microvesicle formation [20,21]. In addition to the requirement of PS exposure for the fragmentation (Fig. 2 and 3), actin or myosin accumulated around the root of the budding sites of the fragments in MDCKII cells and *Drosophila* LECs (Fig. 4A and B).

When *Arf6, Arf1,* or *Pld1* was knocked down in EpH4 cells, impaired fragmentation and delayed extrusion with defective protrusion were observed (Fig. 4C–E, Fig S12). Extruding EpH4 cells with *Arf6* or *Pld1*-knockdown showed local PS exposure (control; 77%, *Arf6*-knockdown; 55%, and *Pld1*-knockdown; 58% of extruding cells), consistent with that the PS exposure precedes EV formation. Arf51F (homologous to mammalian Arf6) accumulated at the apical part of extruding cells in *Drosophila* pupa during fragmentation (6/6 extrusion events) (Fig.4F). *Arf51F*-knockdown in the pupal epidermis showed impaired fragmentation and delayed progression of cell extrusion (Fig. 4G and H). Furthermore, the knockdown of *Arf79F* (homologous to mammalian *Arf1*)*,* which is more abundant than *Arf51F,* in the *Drosophila* midgut (Table S1) in an enterocyte-specific manner resulted in enlarged midguts and shorter lifespans (Fig. S13). These data indicate that the fragmentation of the extruding cells is spatio-temporally restricted EV formation governed by a similar mechanism as microvesicle formation and promotes cell protrusion, thus the execution of cell extrusion.

Taken together, our findings reveal that local PS exposure and subsequent EV formation mediated by PLD and the ARF family in extruding cells is a conserved mechanism that promotes efficient cell extrusion. Prolonging this process caused defects in epithelial tissue, suggesting that prompt execution of extrusion is critical for tissue homeostasis including such as its barrier function. EV formation occurs at the site opposite the direction of extrusion in the apico–basal axis and simultaneously with cell protrusion. Thus, membrane dynamics in extruding cells produce a kind of driving force of extrusion, in addition to the actomyosin complex formed by the neighboring cells and/or extruding cells [8,9], and might control the directionality of extrusion. The polarized formation and/or release of the vesicles may produce force for cell protrusion by touching the neighboring cell membrane or the extracellular matrix similar to the propulsion of

rockets (Fig. 4I). Alternatively, vesicle release might contribute to the promotion of the invasion of neighboring cells by opening the space, thus facilitating cell extrusion. Promoting cell extrusion is a novel physiological function of EVs and PS exposure. Whether the released vesicles function in signal transduction via this engulfment, as in other contexts [22], has to be investigated. Future studies on EV formation in extruding cells will provide new insights into EV biology and increase the understanding of the relationship between impaired cell extrusion and epithelial diseases, including cancer and inflammation.

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Supplementary Materials:

Materials and Methods

Figures S1-S13

Tables S1-S2

Movies S1-S10

References 1-7

Figures:

A

0 min   10 min

20 min   30 min   60 min

Membrane GFP

10μm

B

Membrane GFP   cleaved Caspase3   DAPI   Merge

X-Y   10μm

X-Z   Apical   Basal

C

• Invagination of apical surface (start)   (n=11~17)

Recovery from apical invagination

Protrusion of apical surface

Fragmentation and engulfment

Detachment from cell layer

Time (min)

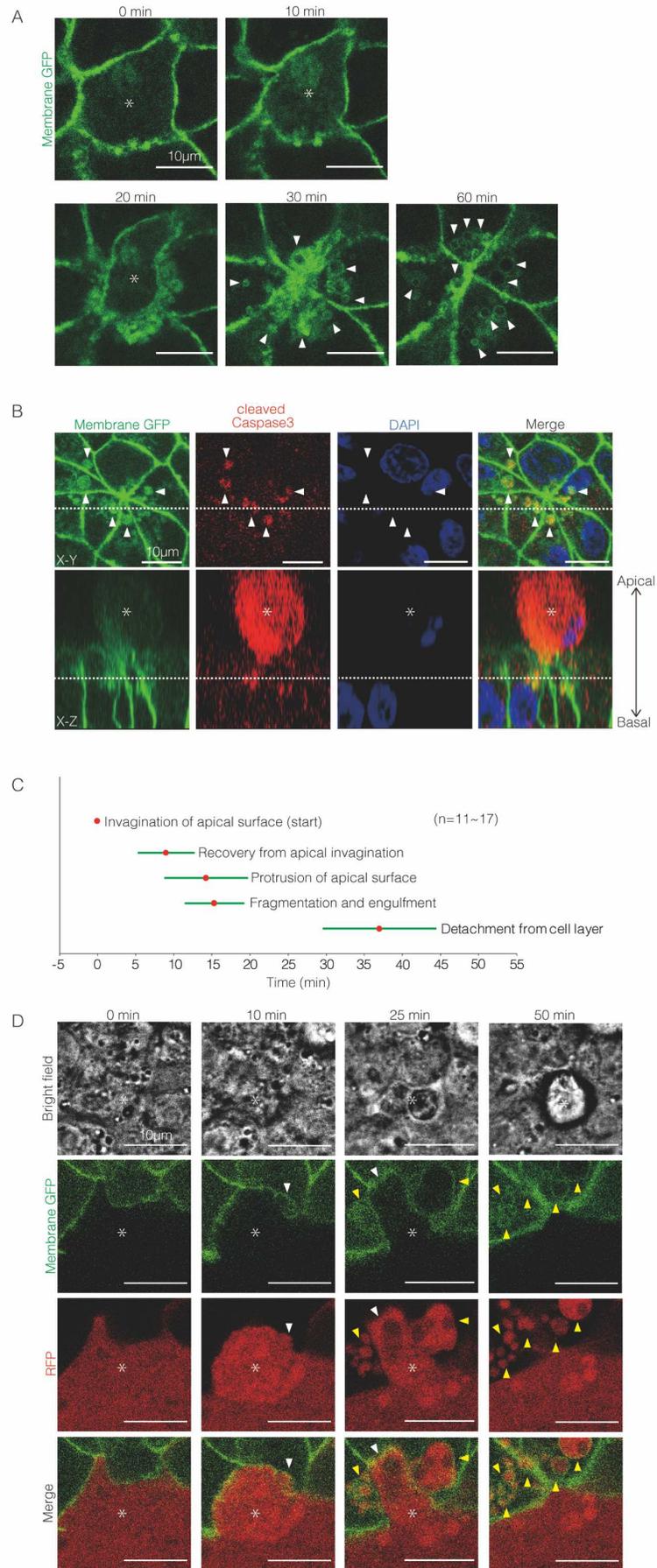
D

0 min   10 min   25 min   50 min

Bright field   10μm

Membrane GFP

RFP

Merge

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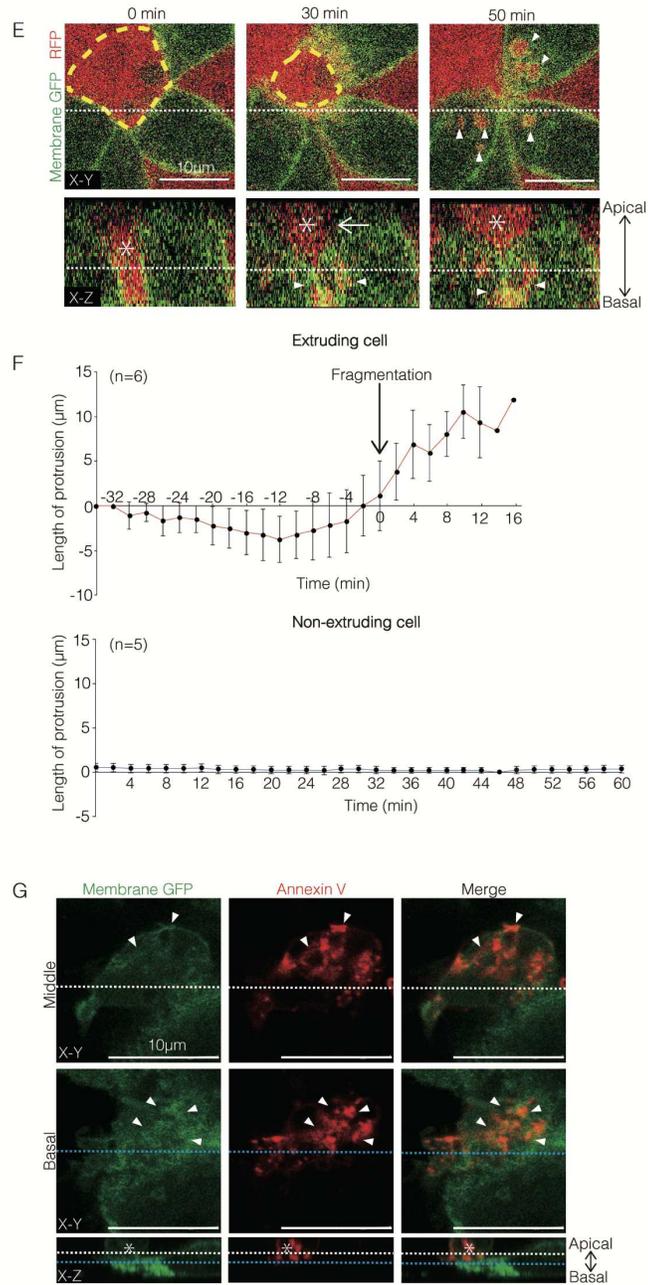
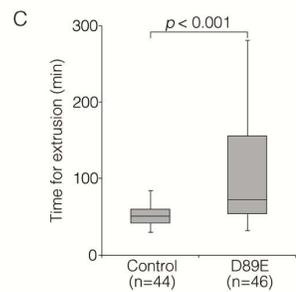
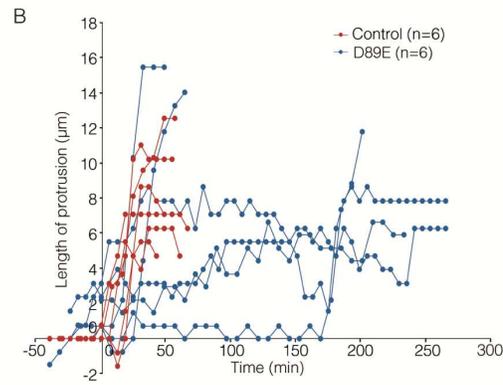
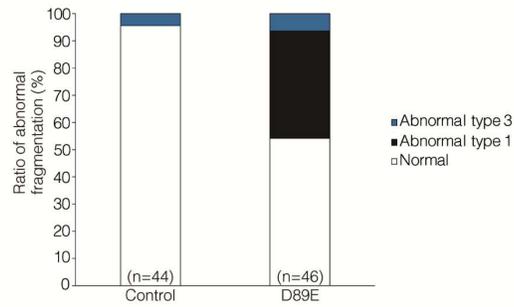
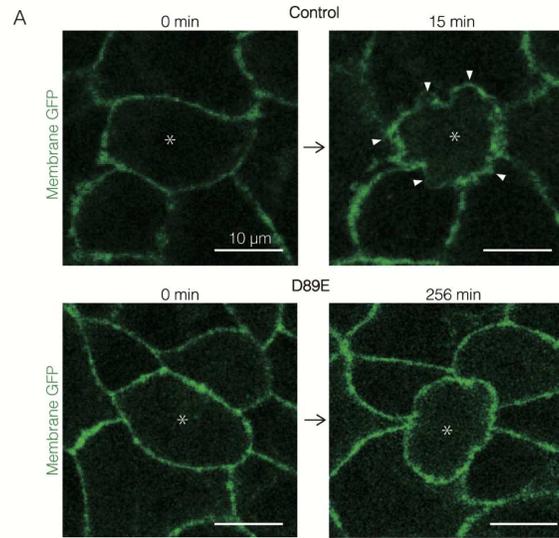


Fig. 1E-G

Fig. 1. Fragmentation of extruding cells and engulfment of the fragments by the neighboring cells in mammalian cultured cells. (**A**) Vesicles (arrowheads) in the neighboring cells of an extruding cell (asterisks, here and elsewhere) in palmitoylated GFP (membrane GFP)-expressing MDCKII cells. (**B**) Immunostained membrane GFP-expressing MDCKII cells for cleaved caspase3 with DAPI. Arrowheads indicate vesicles in the neighboring cells of an extruding cell and dashed lines (here and elsewhere) indicate the corresponding position in another angled view (x-y or x-z images). (**C**) Schematic of processes during cell extrusion in MDCKII cells. Average (circles) and standard deviation (lines) of the time after the start of cell extrusion (defined as the invagination of apical surface) are shown. (**D**) Co-culture between membrane GFP- and RFP-expressing MDCKII cells. White arrowheads indicate membrane budding in a RFP-expressing extruding cell (asterisks), whereas yellow arrowheads indicate the vesicles containing RFP with GFP-labeled membrane in GFP-expressing neighboring cells. (**E**) Fragmentation at the basal part (arrowheads) and concurrent apical protrusion (arrow) in an RFP-expressing extruding cell (yellow dotted lines) under co-culture conditions. (**F**) Quantification of the length between the apical surface of the extruding or non-extruding cell and the apical surface of the cell layer. Time 0 in extruding cell indicates the start of fragmentation. (**G**) An Annexin V-stained membrane GFP-expressing extruding MDCKII cell (asterisks) in the middle and basal x-y sections with x-z sections.

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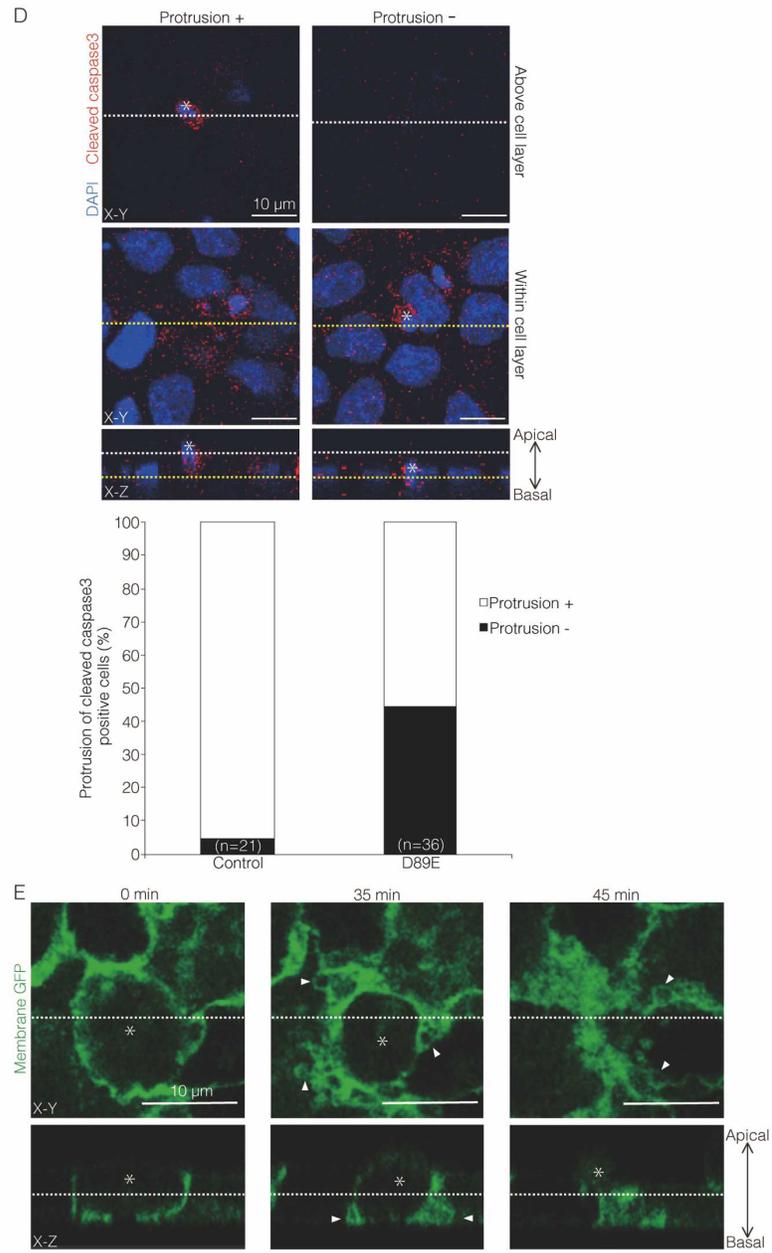
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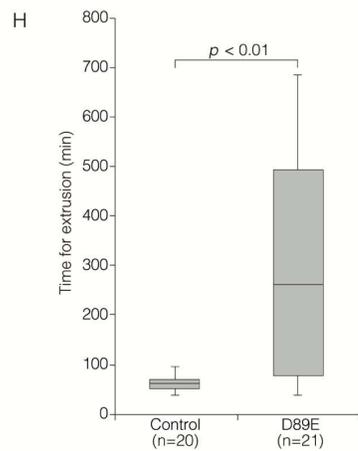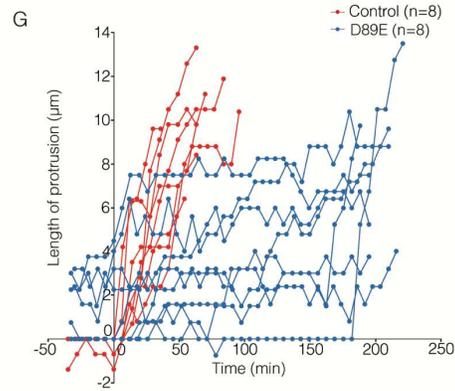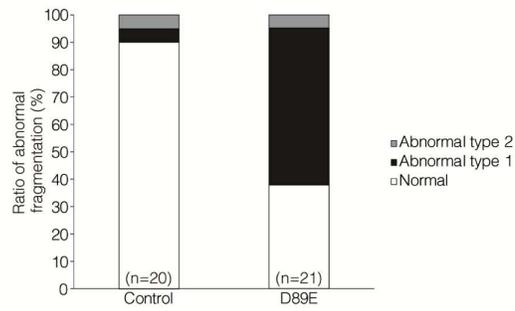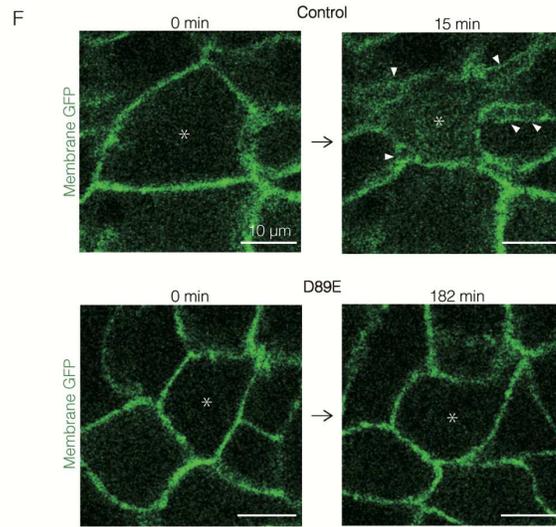
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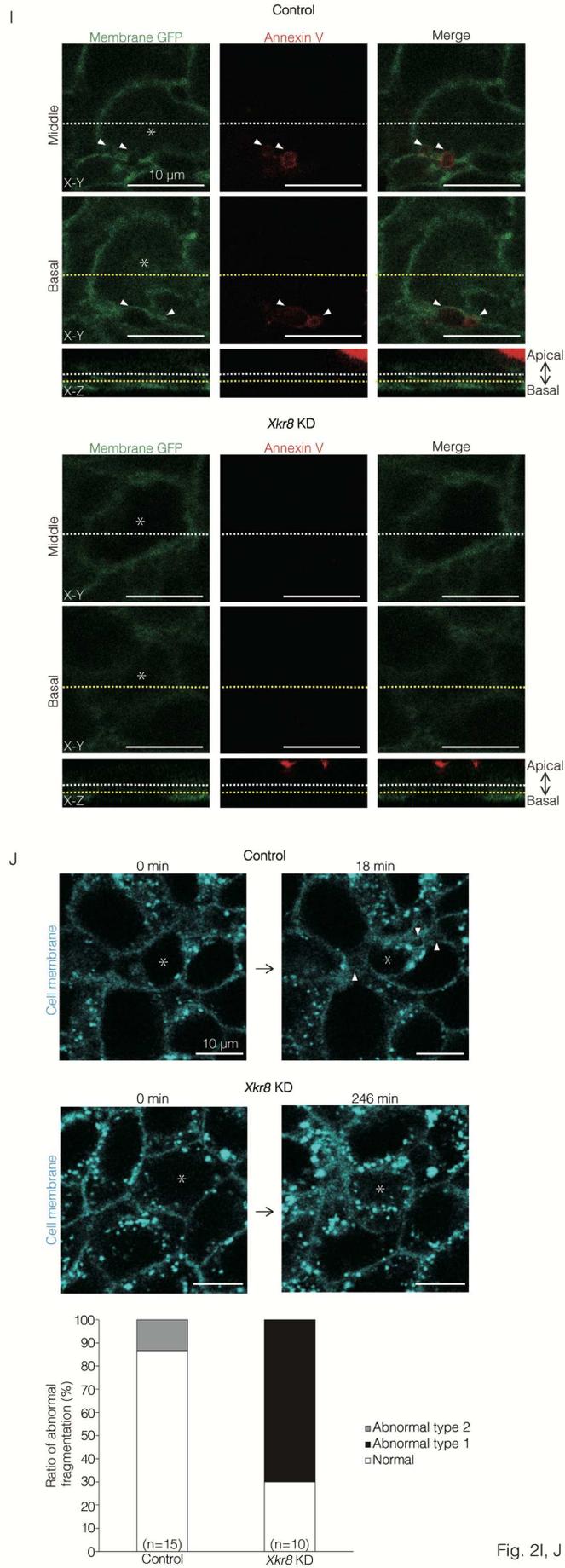
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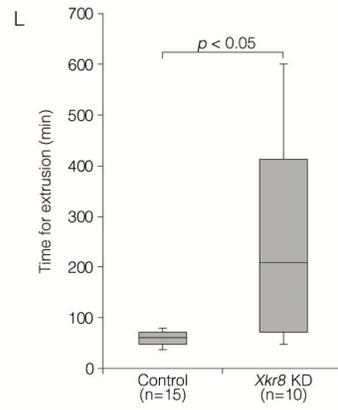
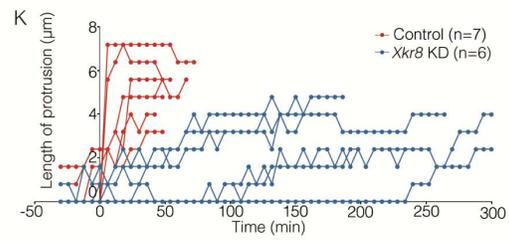
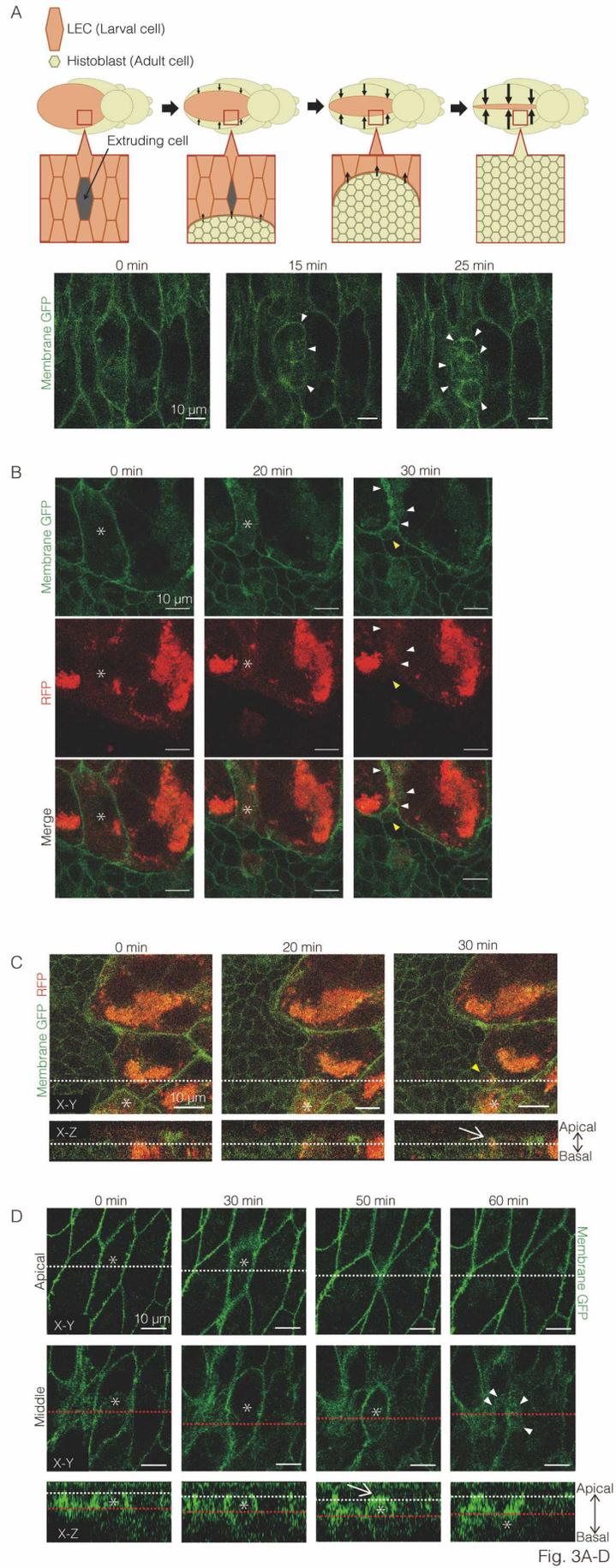


Fig. 2K, L

Fig. 2. The PS exposure-mediated fragmentation of extruding cells promotes the execution of cell extrusion in mammalian cultured cells. (**A**) Delayed fragmentation of membrane GFP-expressing extruding MDCKII cells with MFG-E8 D89E-overexpression. Representative images (Abnormal type 1 for D89E) and the quantification of abnormal fragmentation in the control and D89E-expressing extruding cells. Asterisks indicate extruding cells (here and elsewhere). Characterization of each abnormal type is shown in Fig. S3. (**B and C**) Quantification of the apical protrusion in extruding MDCKII cells (B) and the time for the completion of cell extrusion (C) in the control and D89E-expressing extruding cells. (**D**) Immunostaining for cleaved caspase3 with DAPI. Representative images of cleaved caspase3-positive cells with protrusion (protrusion +) or within the cell layer (protrusion -) and the distribution of cleaved caspase3-positive cells with or without protrusion in the control and D89E-expressing extruding cells. (**E**) Fragmentation of membrane GFP-expressing extruding EpH4 cells. Fragments (arrowheads) were not engulfed but remained in the intercellular space. (**F**, **G**, and **H**) Similar abnormalities in D89E-expressing EpH4 cells and D89E-expressing MDCKII cells: fragmentation (abnormal type1 for D89E) (F), protrusion (G), and time for the completion of extrusion (H) in control or D89E-expressing EpH4 cells. (**I**, **J**, **K**, and **L**) Phenotypes of *Xkr8*-siRNA EpH4 cells: PS exposure shown by Annexin V staining (I), fragmentation shown by staining with CellMask (abnormal type1 for *Xkr8*-siRNA) (J), protrusion (K), and time for the completion of extrusion (L). Arrowheads indicate budding or shed vesicles.

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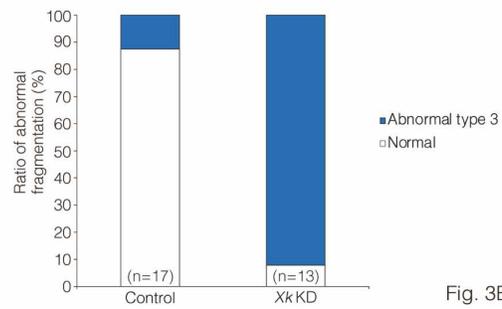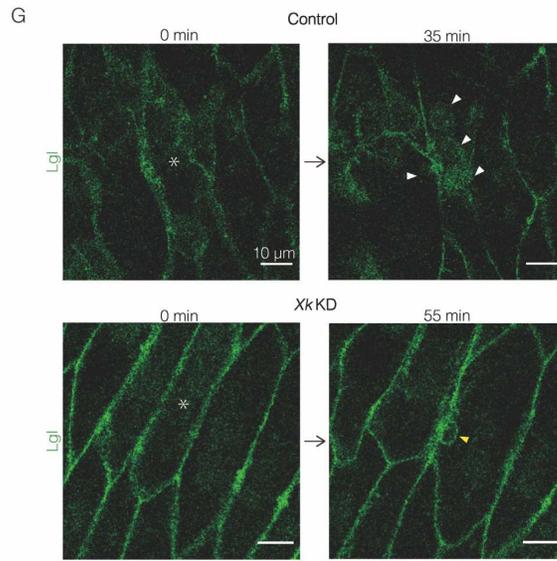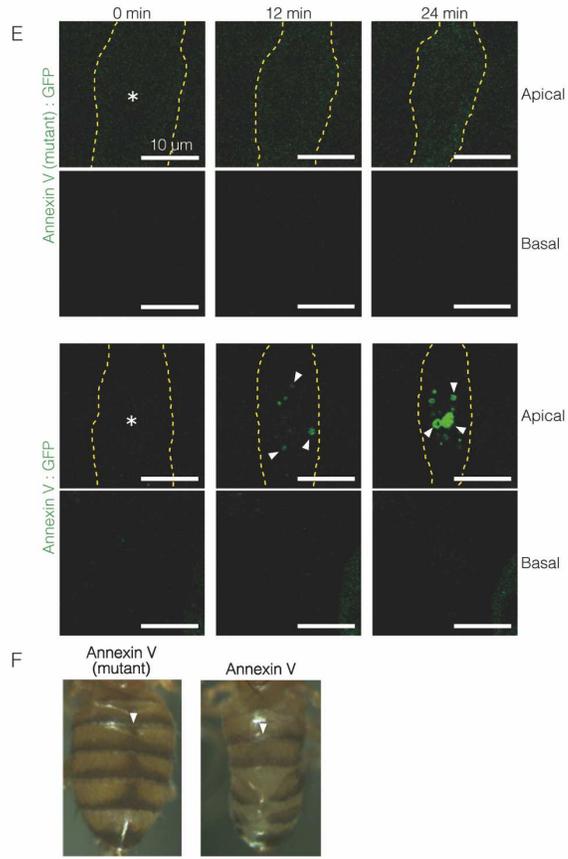
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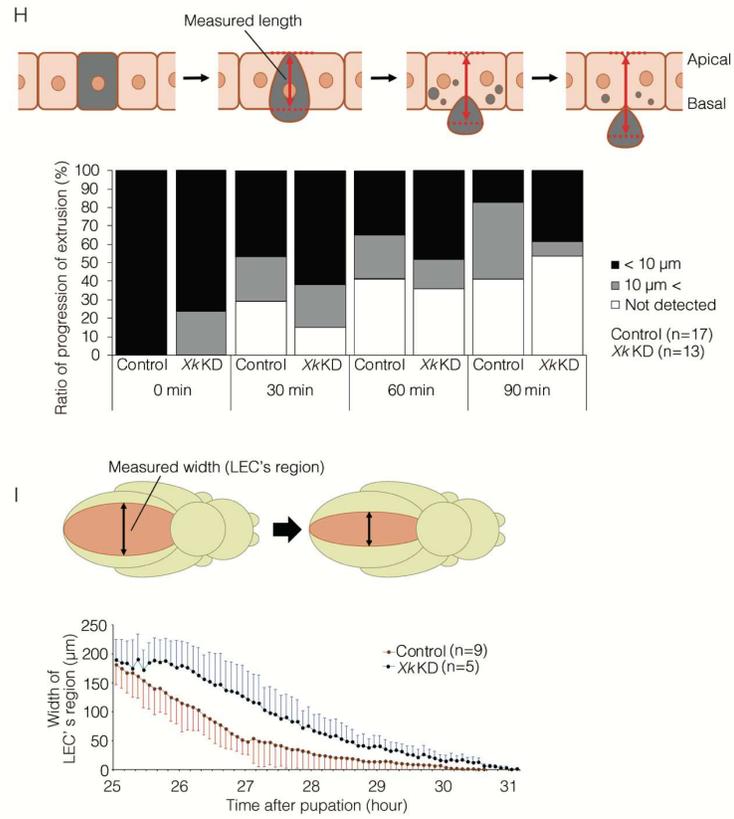


Fig. 3H-I

Fig. 3. PS exposure-mediated fragmentation of extruding cells promotes the execution of cell extrusion in *Drosophila* pupal epidermis to ensure tissue development. (**A**) Diagram of cell extrusion during the replacement of LECs with histoblasts (top) and fragmentation (arrowheads) of an extruding LEC (asterisk, here and elsewhere) adjacent to other LECs in Lgl:GFP (membrane GFP)-expressing pupal epidermis (bottom). (**B** and **C**) Pupal epidermis, in which only LECs express RFP and both LECs and histoblasts express Lgl:GFP (membrane GFP). White and yellow arrowheads indicate extruding cell-derived RFP-positive vesicles in LECs and histoblasts, respectively. Images of x-y sections (B) and those with x-z sections (C). Fragmentation occurred at the apicolateral parts (arrow) of LECs (asterisk), which extrude to the basal direction. (**D**) Fragmentation (arrowheads) occurred immediately after the apical surface closure (arrow) with neighboring cells in Lgl:GFP-expressing LECs. (**E**) Visualization of local PS exposure in Annexin V:GFP- or Annexin V mutant:GFP-expressing extruding LECs (dotted lines). (**F**) Dorsal abdomen of adult *Drosophila* expressing Annexin V:GFP or Annexin V mutant:GFP. Arrowheads indicate the midlines. (**G**) Abnormal fragmentation in *Xk*-RNAi LECs. Representative images (Abnormal type 3 for *Xk*-RNAi) and the quantification of the abnormal fragmentation in the control and *Xk*-RNAi Lgl:GFP-expressing LECs. Characterization of each abnormal type is shown in Fig. S7. (**H**) Quantification of the progression of cell extrusion after the sealing of the apical surface by the neighboring cells. Diagram shows that the distance from the apical surface of cell layer to a section in which the width of the extruding LEC is at the maximum (red double-sided arrows). The distance is classified whether more or less than 10 μm and its distribution at the indicated times and genotypes is shown in the graph (using same images in Fig. 3G). Not detected means the progressed or completed extrusion. (**I**) Reduction in

the width of the region covered by LECs (LEC's region) with time evolution in the control and

Xk-RNAi pupal epidermis.

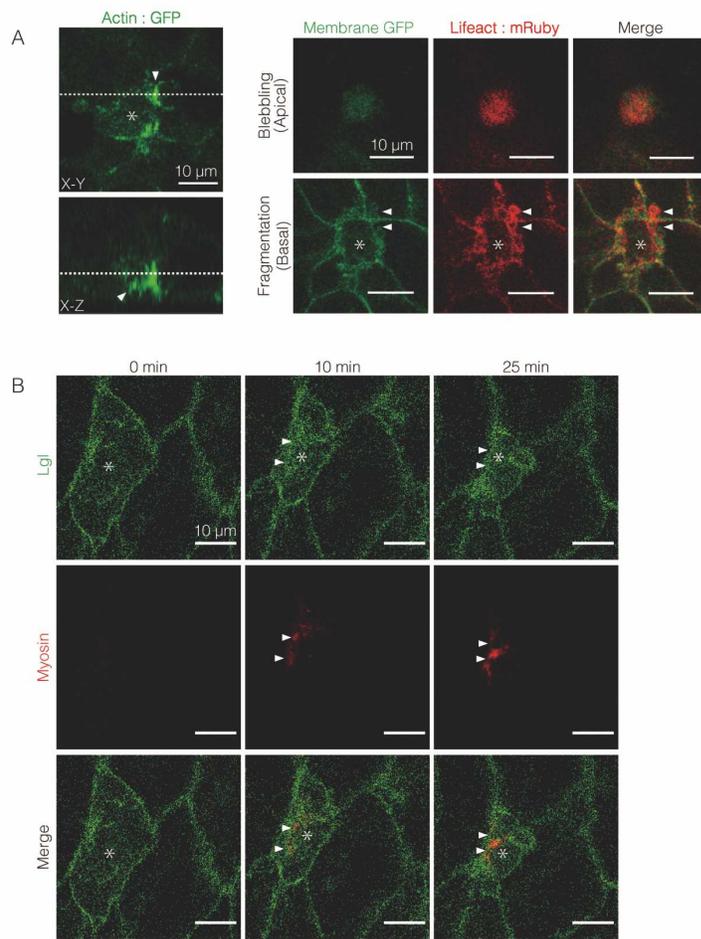
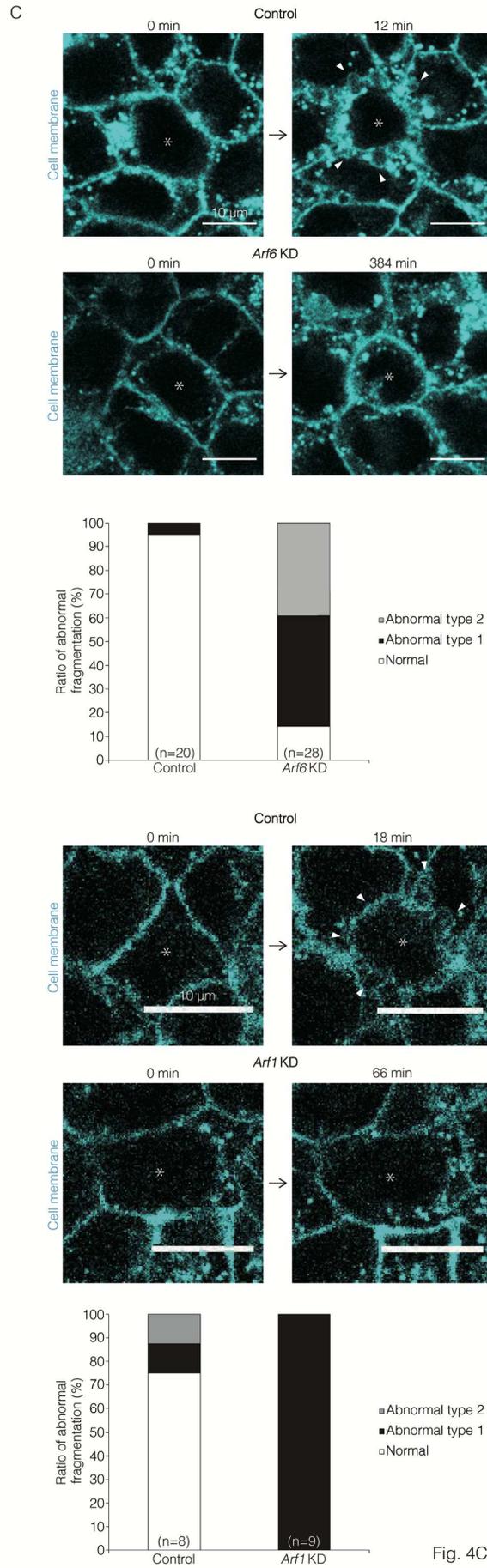



Fig. 4A, B

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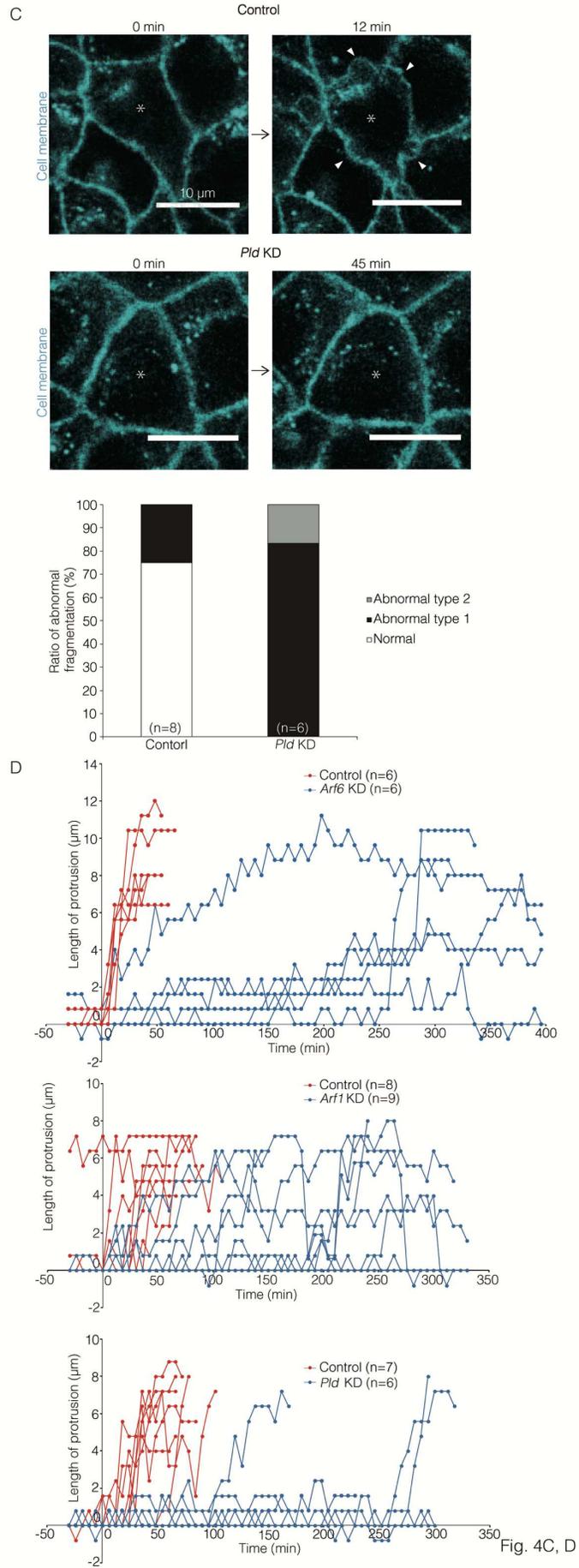
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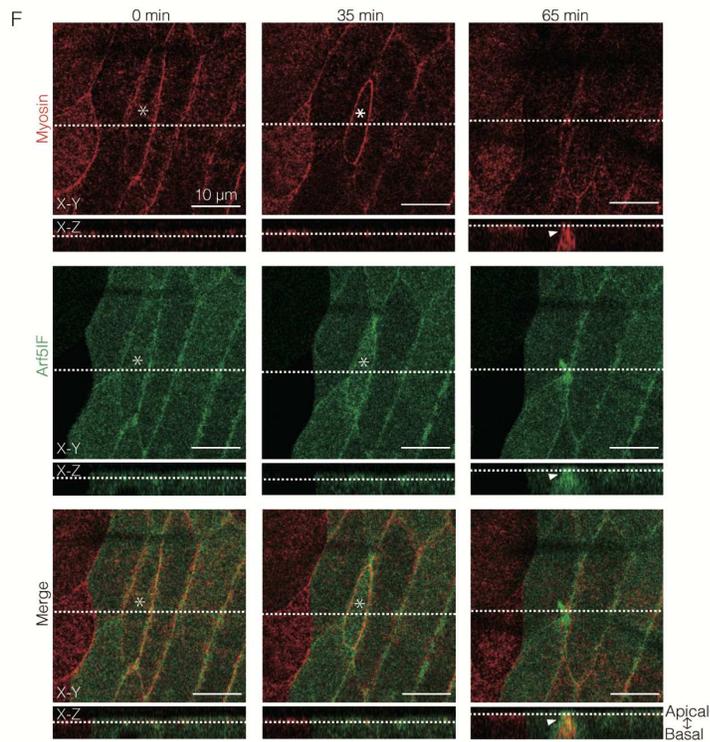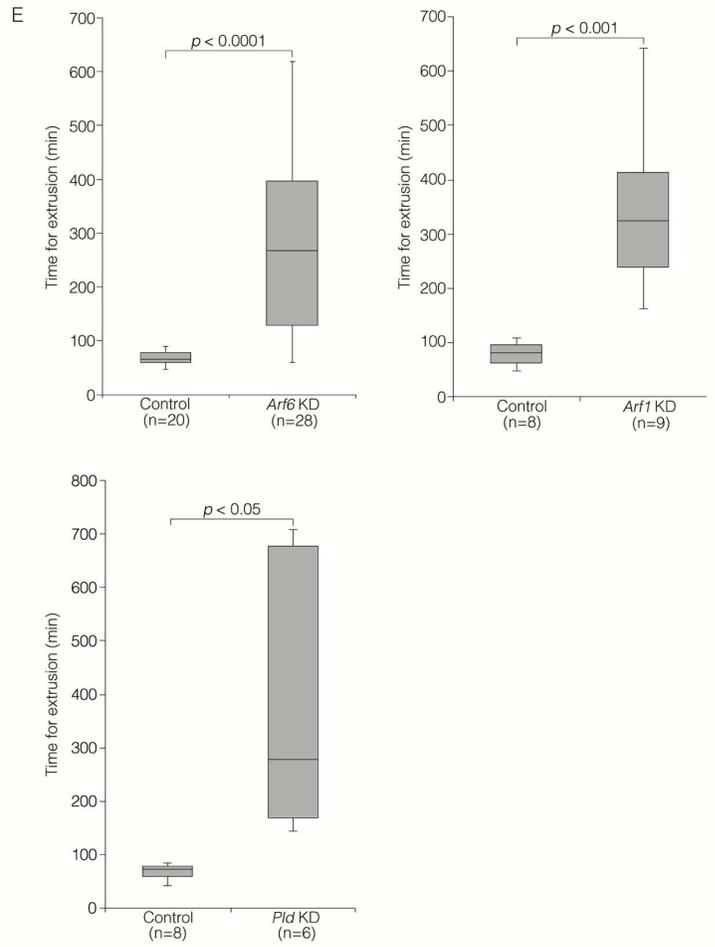
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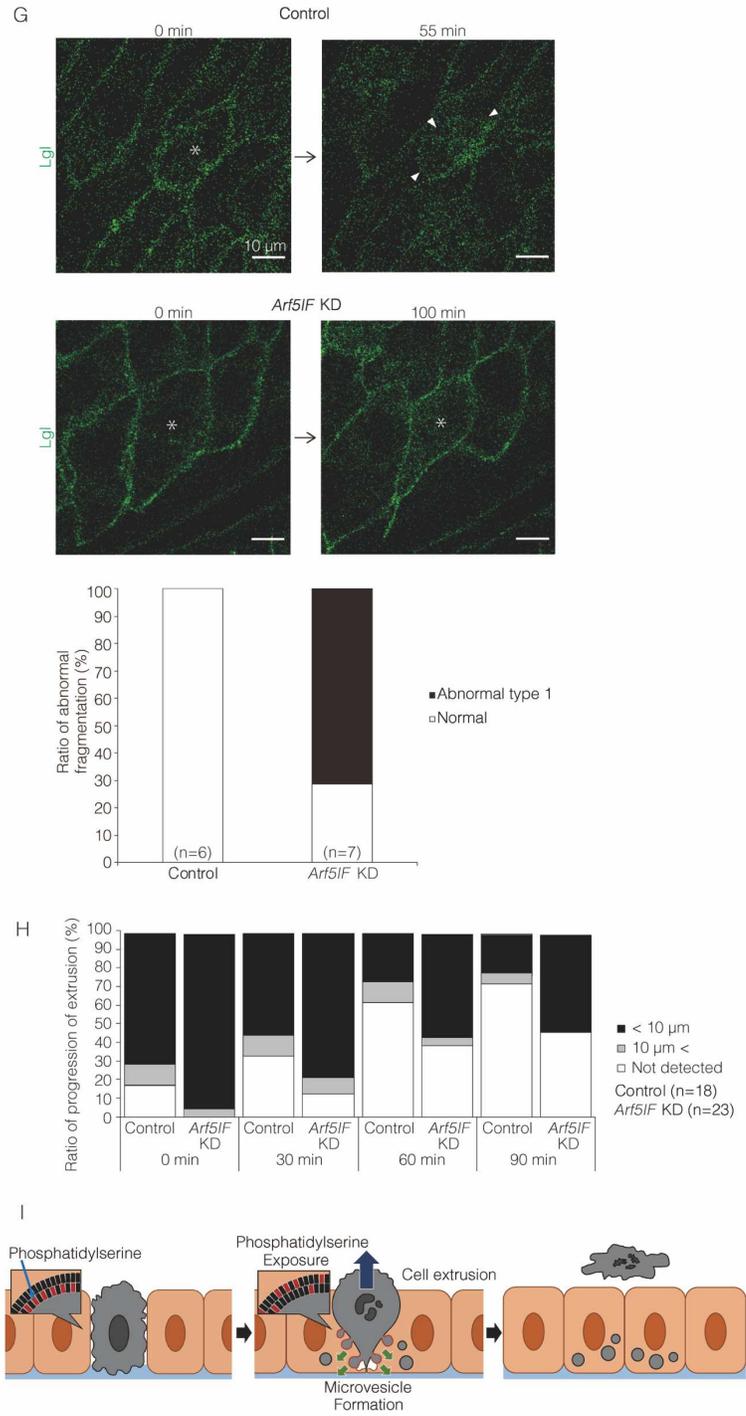
Fig. 4G-I

Fig. 4. Arf family- and PLD-mediated EV formation promotes efficient cell extrusion in mammalian cultured cells and *Drosophila* pupal epidermis. (A) Accumulation of actin (arrowheads) at the budding sites at the basal parts but not blebbing sites (rarely shed) of the apical surface in Actin:GFP-expressing (10/10 extruding cells) and Lifeact:mRuby2- and membrane GFP-expressing (33/39 extruding cells) extruding MDCKII cell. (B) Accumulation of myosin (arrowheads) in Lgl:GFP- and sqh (myosin regulatory right chain):mKate2-expressing extruding LECs (5/5 extruding cells). (C) Quantification of abnormal fragmentation according to the criteria in fig. S3 (Abnormal type 1 for representative images of siRNA) in *Arf6-*, *Arf1-*, or *Pld1-* siRNA extruding Eph4 cells stained with CellMask. Arrowheads indicate cell fragments. (D) Quantification of protrusion of the indicated EpH4 cells. (E) Time for completion of cell extrusion for the indicated EpH4 cells in Fig. 4C. (F) Localization of Arf51F visualized in Arf51F:GFP- and sqh:mKate2-expressing pupal epidermis. (G) Abnormal fragmentation in *Arf51F*-RNAi LECs. Representative images (Abnormal type 1 for *Arf51F*-RNAi) and the quantification of the abnormal fragmentation in the control and *Arf51F*-RNAi Lgl:GFP-expressing LECs according to the criteria in Fig. S7. Arrowheads indicate cell fragments. (H) Distribution of extruding LECs for the indicated times in the control and *Arf51F*-RNAi pupal epidermis. (I) Proposed model for the execution of cell extrusion.

Figures
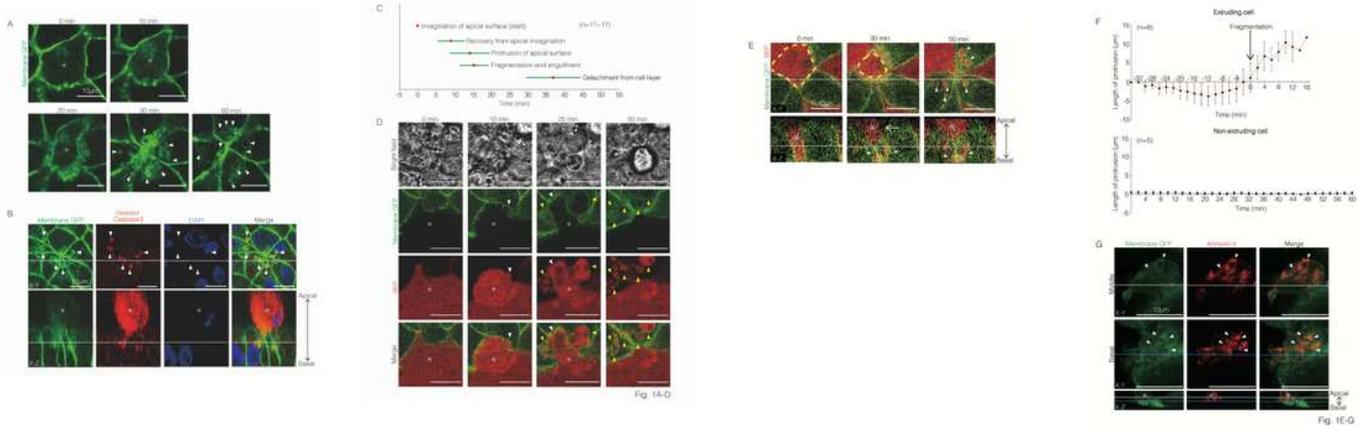
Figure 1

Fragmentation of extruding cells and engulfment of the fragments by the neighboring cells in mammalian cultured cells.
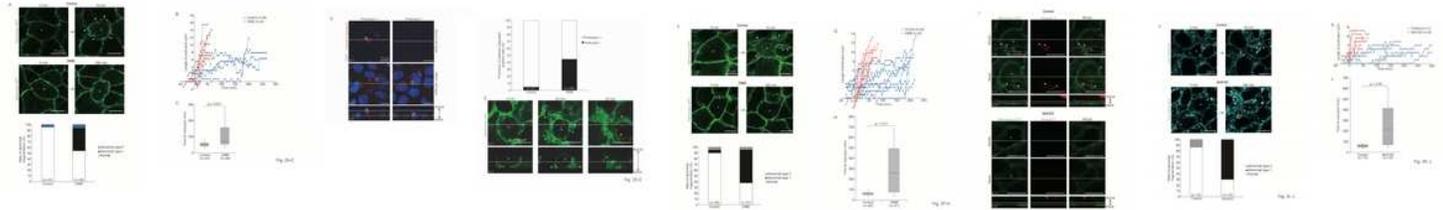
Figure 2

The PS exposure-mediated fragmentation of extruding cells promotes the execution of cell extrusion in mammalian cultured cells.
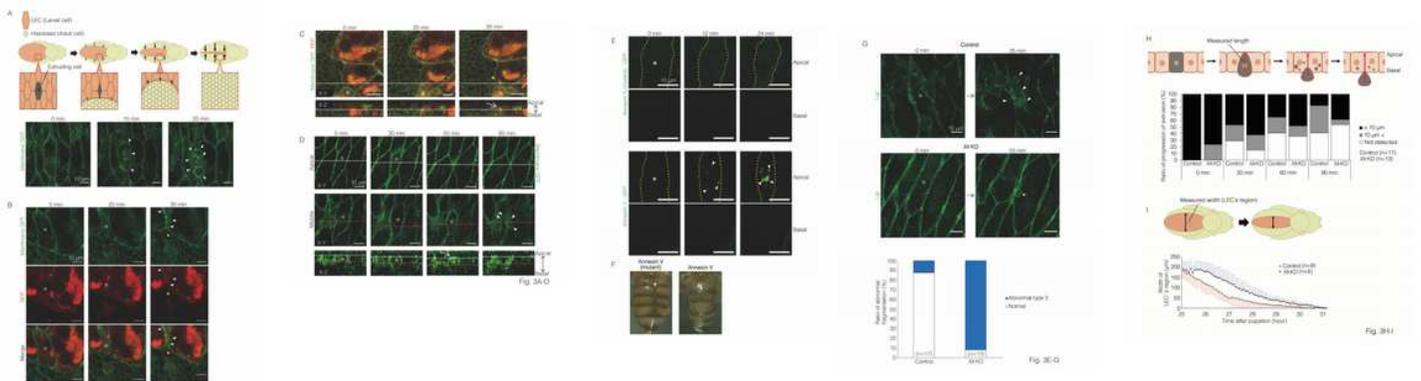
Figure 3

PS exposure-mediated fragmentation of extruding cells promotes the execution of cell extrusion in Drosophila pupal epidermis to ensure tissue development.

Figure 4

Arf family- and PLD-mediated EV formation promotes efficient cell extrusion in mammalian cultured cells and Drosophila pupal epidermis.