HOKKAIDO UNIVERSITY

Title	Calcium Wave Promotes Cell Extrusion
Author(s)	Takeuchi, Yasuto; Narumi, Rika; Akiyama, Ryutaro et al.
Citation	Current biology, 30(4), 670-681 https://doi.org/10.1016/j.cub.2019.11.089
Issue Date	2020-02-24
Doc URL	https://hdl.handle.net/2115/80478
Rights	© 2019. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	Supplemental Figures.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

SUPPLEMENTAL INFORMATION

Supplemental Figures 1-7

Calcium Wave Promotes Cell Extrusion

Takeuchi *et al.*

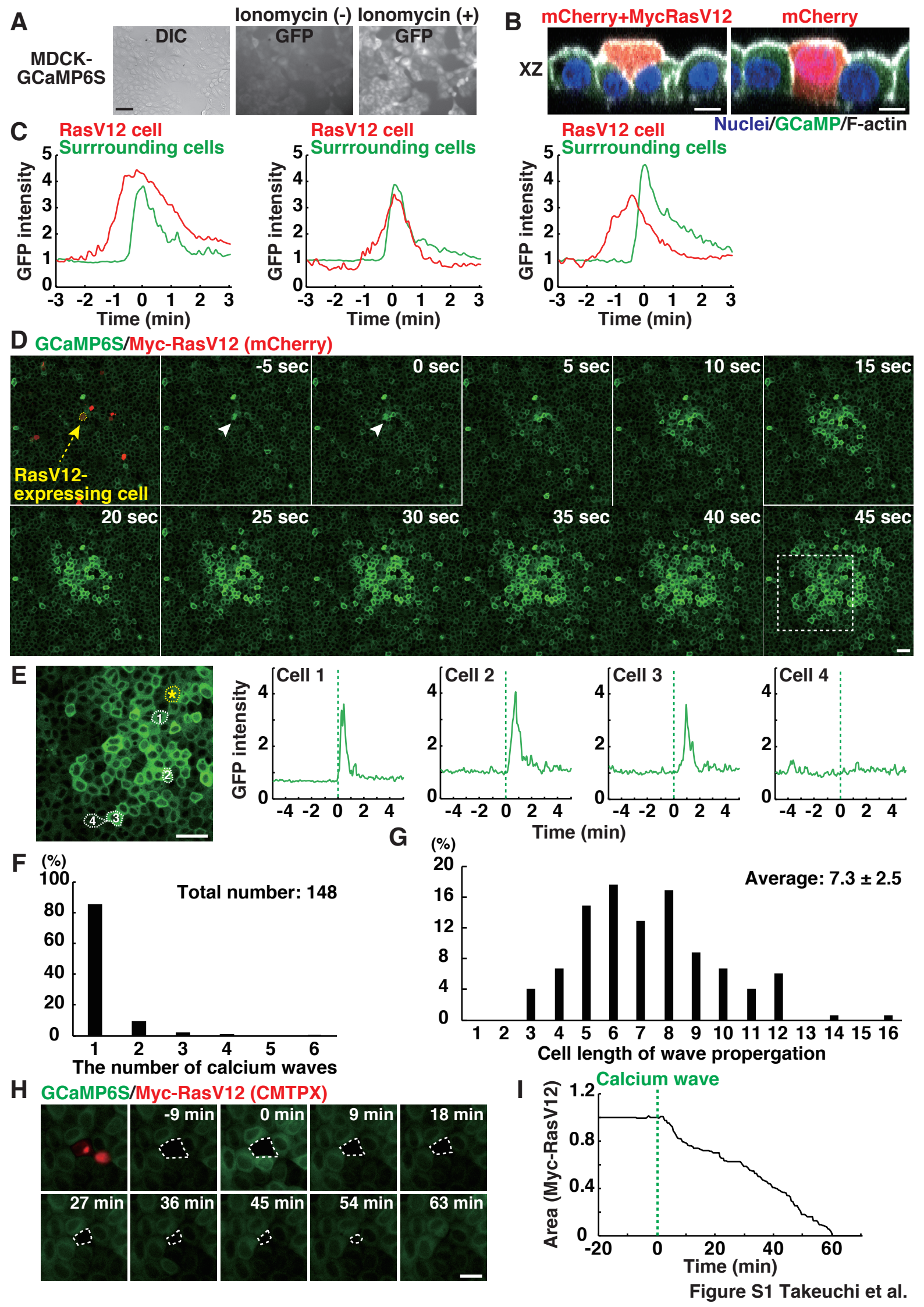

Figure S1 Takeuchi et al.

Figure S1. The Mode of Calcium Wave Propagation around RasV12-Transformed Cells, Related to Figure 1

(A) Effect of Ionomycin on GCaMP6S fluorescence. (B) Fluorescence images of xz sections of an mCherry/Myc-RasV12-expressing cell or mCherry-expressing cell. (C) Three examples for the GFP intensity of GCaMP6S in mCherry/Myc-RasV12-expressing cells (red line) or surrounding cells (green line) upon calcium wave. For surrounding cells, the average GFP intensity of cells directly contacting the mCherry/Myc-RasV12-expressing cell is calculated. Values are expressed as a ratio relative to that at -3 min. (D) Another time-lapse observation of calcium wave by 5-s intervals. The arrowheads indicate a RasV12 cell in which the initial elevation of calcium occurred. The area in the white box is shown at higher magnification in the next figure. (E) Temporal changes of GCaMP6S fluorescence upon calcium wave propagation in a cell indicated by dotted lines (Cell 1-4). The asterisk indicates a Myc-RasV12 cell from which calcium wave originates. Time 0 indicates the occurrence of calcium wave. Note that calcium wave does not reach Cell 4. (F and G) Quantification of the number of calcium waves (F) and the cell length of wave propagation (G). 148 calcium waves were analyzed from 17 independent time-lapse analyses. (H and I) Another example of area reduction of an extruding Myc-RasV12 cell after calcium wave (cf. **Figures 1**G and 1H). Note that in this experiment, RasV12 cells did not express GCaMP6S. Scale bars, 100 μm (A, D, and E), 20 μm (B), 50 μm (H).

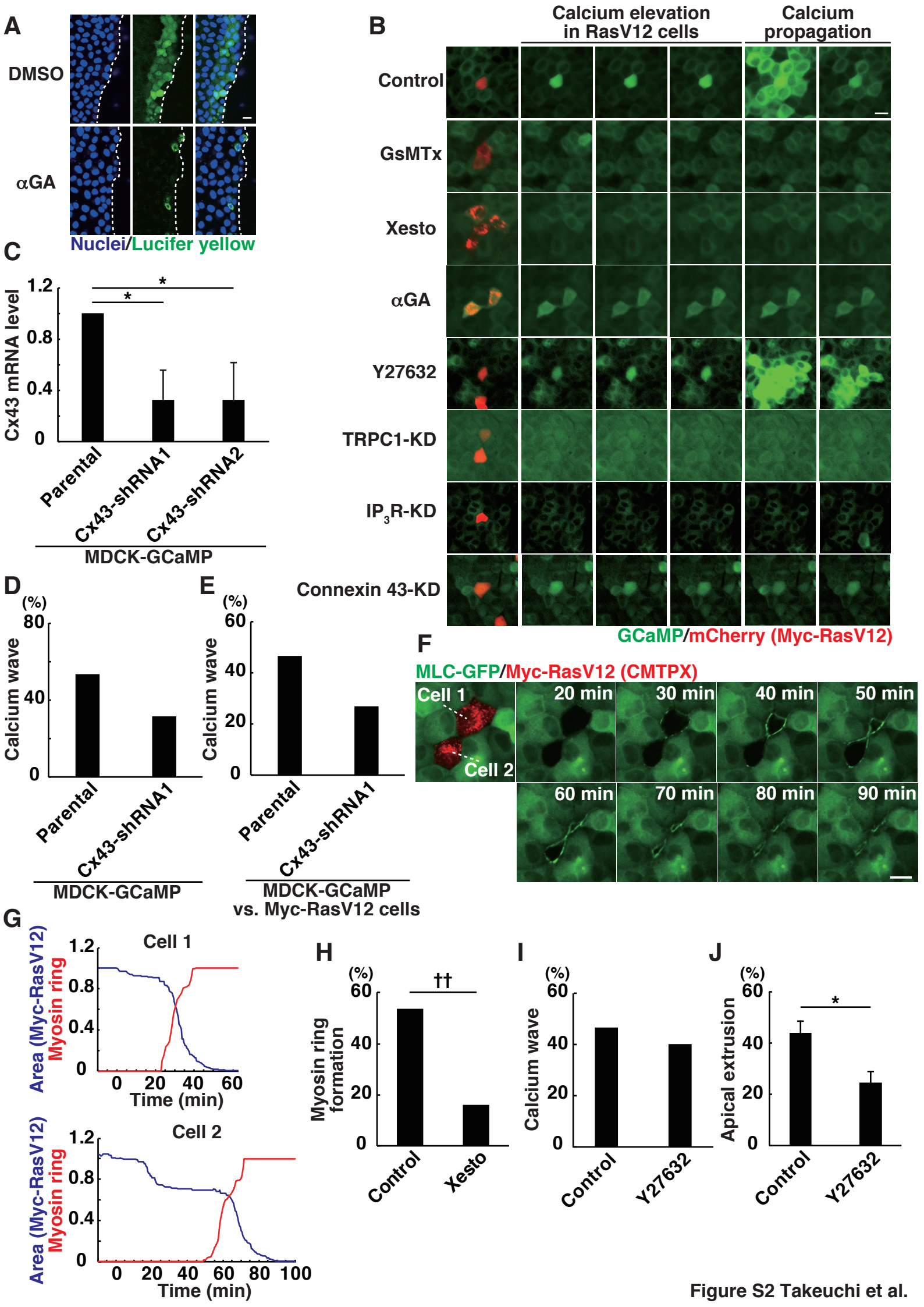

Figure S2 Takeuchi et al.

Figure S2. Effect of Various Inhibitors or Knockdown on Calcium Wave and Myosin Ring Formation, Related to Figure 2

(A) Validation of the effect of the gap junction inhibitor αGA. Note that αGA suppresses the intercellular diffusion of Lucifer Yellow from the wound edge. (B) Effect of various inhibitors or knockdown on the initial calcium elevation in RasV12-expressing cells and the following calcium propagation. Images were captured every 30 s. (C) Effect of connexin 43 (Cx43)-shRNA expression on the Cx43 mRNA level in MDCK-GCaMP6S cells. Data are mean ± SD from three independent experiments. $^{*}P<0.05$ (two-tailed Student's *t*-tests). (D and E) Effect of connexin 43 (Cx43)-knockdown on calcium wave. (D) Expression of Myc-RasV12 was transiently induced in MDCK-GCaMP6S or MDCK-GCaMP6S Cx43-shRNA1 cells. n=30 and 54 cells from three independent experiments. (E) MDCK-pTRE3G Myc-RasV12 cells were surrounded by MDCK-GCaMP6S or MDCK-GCaMP6S Cx43-shRNA1 cells. n=30 and n=61 cells from three independent experiments. The comparable effect of Cx43-knockdown on calcium wave was also observed using Cx43-shRNA2 cells. Note that the expression level of connexin 43 is not influenced by the RasV12 expression (data not shown). (F) Time-lapse analyses of myosin accumulation in the surrounding cells. MDCK-pTRE3G Myc-RasV12 cells stained with CMTPX (red) were surrounded by MDCK-MLC (myosin-II light chain)-GFP cells. (G) Two examples for area reduction of an extruding Myc-RasV12 cell (blue line) and myosin ring formation (red line). Time 0 denotes the point where the area reduction of Cell 1 starts. Values of myosin ring are quantified as a relative ratio to the whole perimeter (myosin-II-positive perimeter/the whole perimeter) around the respective extruding Myc-RasV12 cell. (H) Effect of Xestospongin C (Xesto) on the myosin accumulation in the surrounding cells. n=39 and 37 cells from three independent experiments. $^{\dagger\dagger}P$

<0.01 (chi-square test). (I and J) Effect of the ROCK inhibitor Y27632 on calcium wave (I) and apical extrusion (J). (I) n=30 and 75 cells from three independent experiments. (J) Data are mean ± SD from three independent experiments. n≧50 cells for each experimental condition. *P<0.05 (two-tailed Student's t-tests). (A, B, and F) Scale bars, 20 μm.

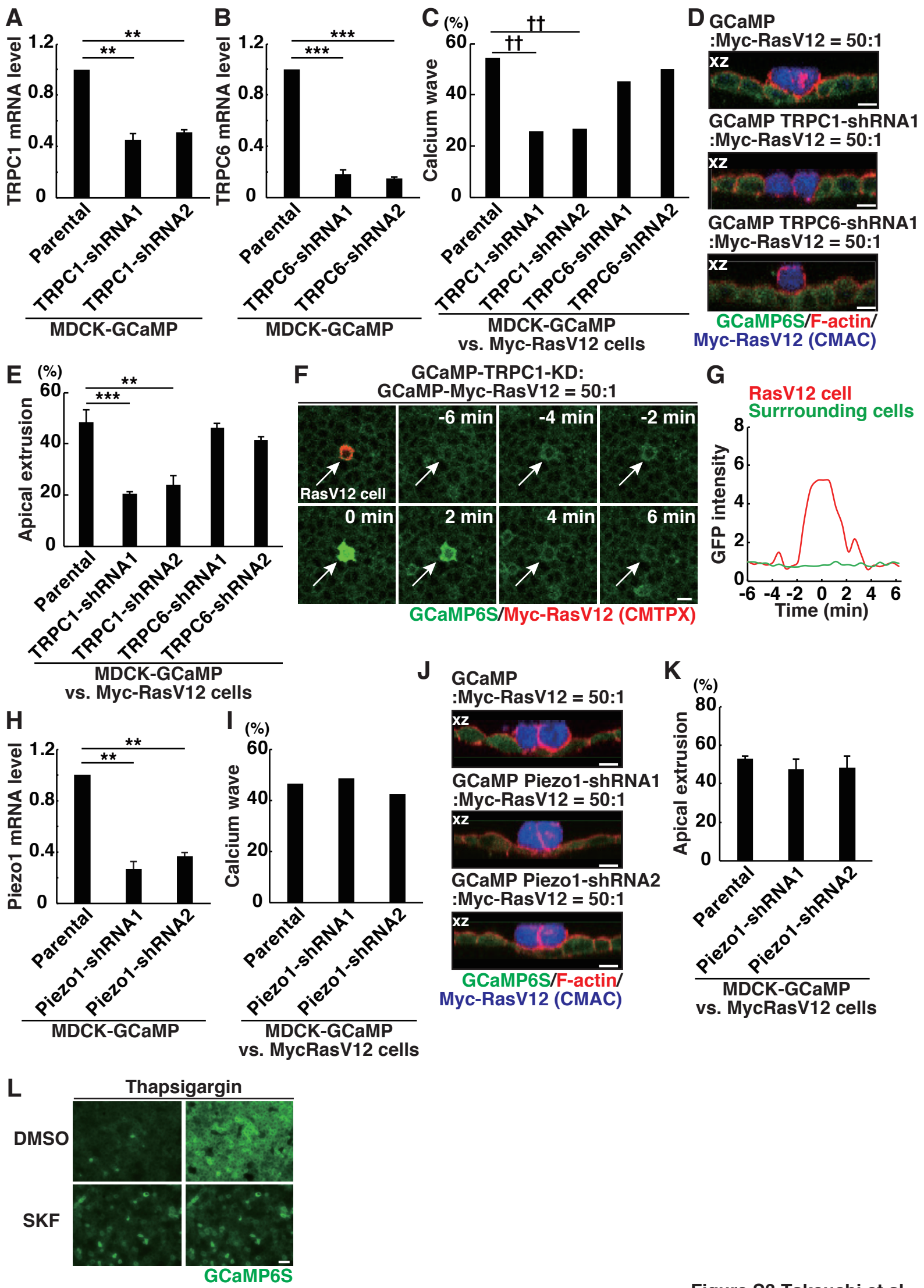

Figure S3 Takeuchi et al.

Figure S3. Effect of Knockdown of TRPC1, TRPC6, or Piezo1 on Calcium Wave and Apical Extrusion, Related to Figure 2

(A and B) Effect of TRPC1-shRNA (A) or TRPC6-shRNA expression (B) on the respective mRNA levels in MDCK-GCaMP6S cells. Data are mean ± SD from three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$ (two-tailed Student's *t*-tests). (C-E) Effect of TRPC1- or TRPC6-knockdown in the surrounding cells on calcium wave (C) or apical extrusion (D and E) of Myc-RasV12-expressing cells. MDCK-pTRE3G Myc-RasV12 cells stained with CMAC (blue) were co-cultured with MDCK-GCaMP6S, MDCK-GCaMP6S TRPC1-, or TRPC6-knockdown cells at a ratio of 1:50. (C) n=30, 31, 51, 51, and 50 cells from three independent experiments. $^{\dagger\dagger}P < 0.01$ (chi-square test). (D) Fluorescence images of xz sections. (E) Data are mean ± SD from three independent experiments. n≧50 cells for each experimental condition. $^{**}P<0.01$, $^{***}P<0.001$ (two-tailed Student's *t*-tests). (F) Time-lapse analyses of calcium imaging of RasV12-expressing MDCK-GCaMP6S cells surrounded by MDCK-GCaMP6S TRPC1-shRNA1 cells. MDCK GCaMP6S-pTRE3G Myc-RasV12 cells stained with CMTPX (red) were co-cultured with MDCK-GCaMP6S TRPC1-shRNA1 cells at a ratio of 1:50. After incubation with doxycycline and tetracycline for 10 h, we performed time-lapse observation for 16 h. Images are extracted from a representative time-lapse analysis. The arrows indicate a RasV12-expressing cell. (G) The GFP intensity of GCaMP6S is quantified in a RasV12-expressing cell (red line) or the surrounding TRPC1-knockdown cells (green line). For surrounding cells, the average GFP intensity of cells directly contacting the RasV12-expressing cell is calculated. Values are expressed as a ratio relative to that at -6 min. (H) Effect of Piezo1-shRNA expression on the Piezo1 mRNA level in MDCK-GCaMP6S cells. Data are mean ± SD from three independent experiments. $^{**}P<0.01$ (two-tailed

Student's *t*-tests). (I-K) Effect of Piezo1-knockdown in the surrounding cells on calcium wave (I) or apical extrusion (J and K) of Myc-RasV12-expressing cells. MDCK-pTRE3G Myc-RasV12 cells stained with CMAC (blue) were co-cultured with MDCK-GCaMP6S or MDCK-GCaMP6S Piezo1-shRNA1, or Piezo1-shRNA2 cells at a ratio of 1:50. (I) n=30, 33, and 33 cells from three independent experiments. (J) Fluorescence images of xz sections. (K) Data are mean ± SD from three independent experiments. n≧50 cells for each experimental condition. (L) Validation of the effect of the STIM1 inhibitor SKF96365. Note that SKF96365 strongly suppressed Thapsigargin-mediated SOCE. Scale bars, 20 μm (D, F, and J), 100 μm (L).

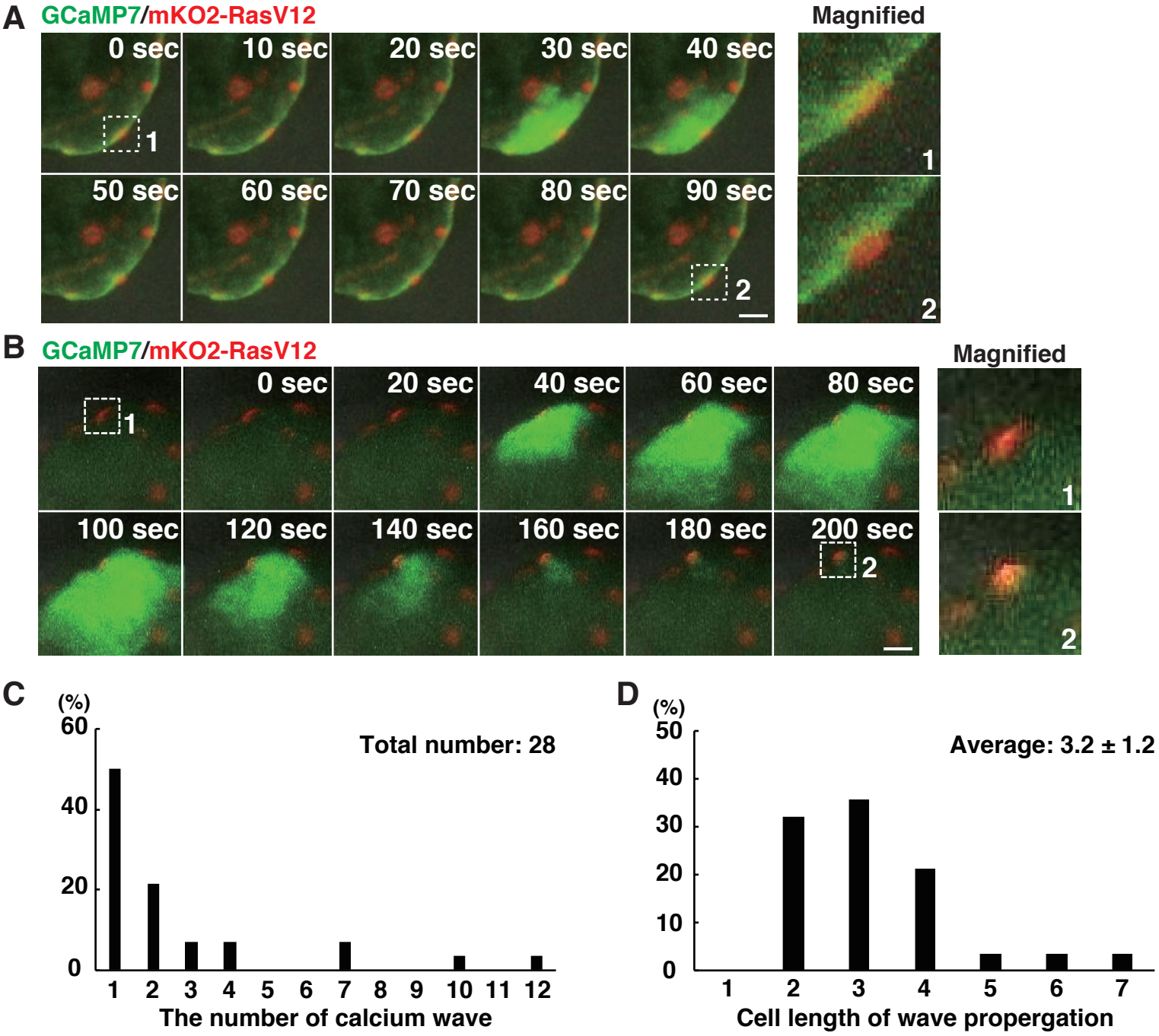

Figure S4 Takeuchi et al.

Figure S4. Calcium Wave Occurs Prior to Apical Extrusion in Zebrafish Embryos as Well, Related to Figure 3

(A and B) Other presentations of time-lapse images of calcium wave from extruding mKO2-RasV12-expressing cells in zebrafish embryos (cf. Figure 3C). Note that RasV12 cells are apically extruded at 5-15 min after calcium wave. The area in the white box is shown at higher magnification in the right panel, presenting a lateral view of an extruding RasV12 cell. Scale bars, 50 μm. (C and D) Quantification of the number of calcium waves (C) and the cell length of wave propagation (D) in the zebrafish system. 28 calcium waves were analyzed from 10 independent time-lapse experiments.

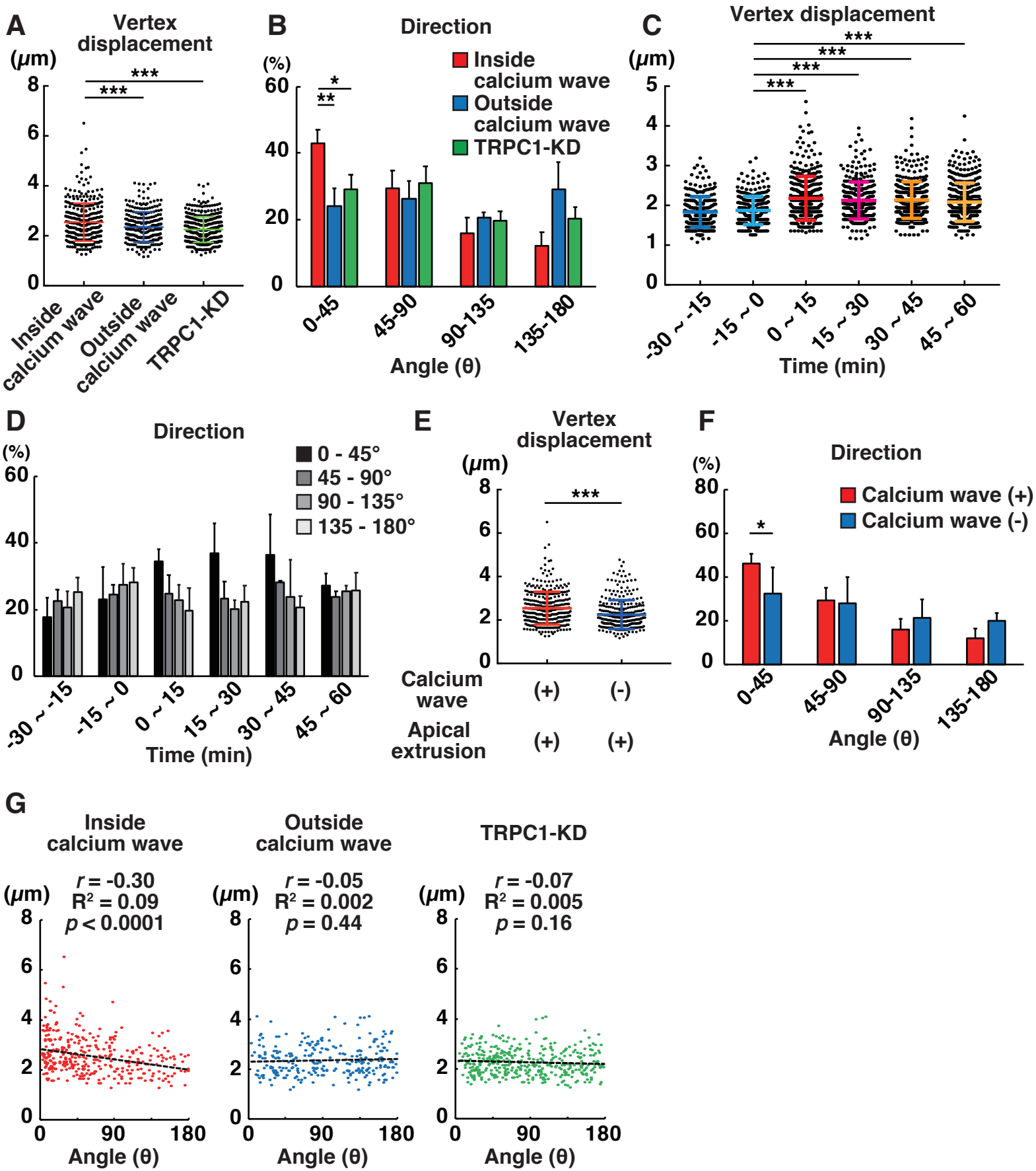

Figure S5 Takeuchi et al.

Figure S5. Calcium Wave Induces the Polarized Movement of the Surrounding Cells toward the Extruding Transformed Cells, Related to Figure 4

(A-D) Analyses of vertex movement of the surrounding cells that reside inside or outside of calcium wave during apical extrusion of Myc-RasV12-expressing cells. MDCK-pTRE3G Myc-RasV12 cells were co-cultured with MDCK-GCaMP6S cells or MDCK-GCaMP6S TRPC1-knockdown cells with the SiR-actin probe. After incubation with doxycycline for 10 h, we performed time-lapse observation for 16 h. All vertices inside calcium wave were quantified in these analyses (Inside calcium wave). The vertices outside calcium wave reside within 3-6 cells from the border of calcium wave (Outside calcium wave). The TRPC1-KD vertices reside within 7 cells away from RasV12 cells (TRPC1-KD). (A) Quantification of the displacement of the vertex movement. The displacement of each vertex movement is depicted as a dot. Data are mean ± SD. n=346, 273 and 399 from three independent experiments. ***P<0.001 (unpaired t-tests). (B) Quantification of the direction of the vertex movement. Arrow angle (θ) was classified into 4 categories (0-45, 45-90, 90-135, 135-180), and the ratio of each category was quantified. Data are mean ± SD from three independent experiments. n=346, 273 and 399 from three independent experiments. *P<0.05, **P<0.01 (unpaired t-tests). (C and D) Analyses of vertex displacement (C) or direction (D) of the surrounding cells that reside inside of calcium wave within the narrower time windows (15 min) before and after calcium wave. Time 0 denotes the occurrence of calcium wave. Data are mean ± SD from three independent experiments. n=265, 289, 344, 332, 365, and 378 from three independent experiments. Note that the extrusion time of Myc-RasV12 cells in these analyses was around 90 min after calcium wave. (C) The displacement of each vertex movement is depicted as a dot. ***P<0.001 (unpaired t-tests). (D) Quantification of

the direction of the vertex movement. (E and F) Analyses of vertex displacement (E) or direction (F) around apically extruding cells with or without calcium wave. For vertices without calcium wave, the vertices within 7 cells away from RasV12 cells were analyzed. (E) The displacement of each vertex movement is depicted as a dot. Data are mean ± SD. n=346 and 311 from three independent experiments. ***$P<0.001$ (unpaired *t*-tests). (F) Data are mean ± SD from three independent experiments. n=346 and 311 from three independent experiments. *$P<0.05$ (unpaired *t*-tests). (G) Correlation between the displacement and direction of vertices. The displacement (y-axis) and direction (x-axis) of each vertex movement is depicted as a dot. n=346, 273 and 399 from three independent experiments. Pearson *r* correlation is used to quantify the degree of correlation between displacement and arrow angle (θ) of vertices (two-tailed Pearson *r* correlation).

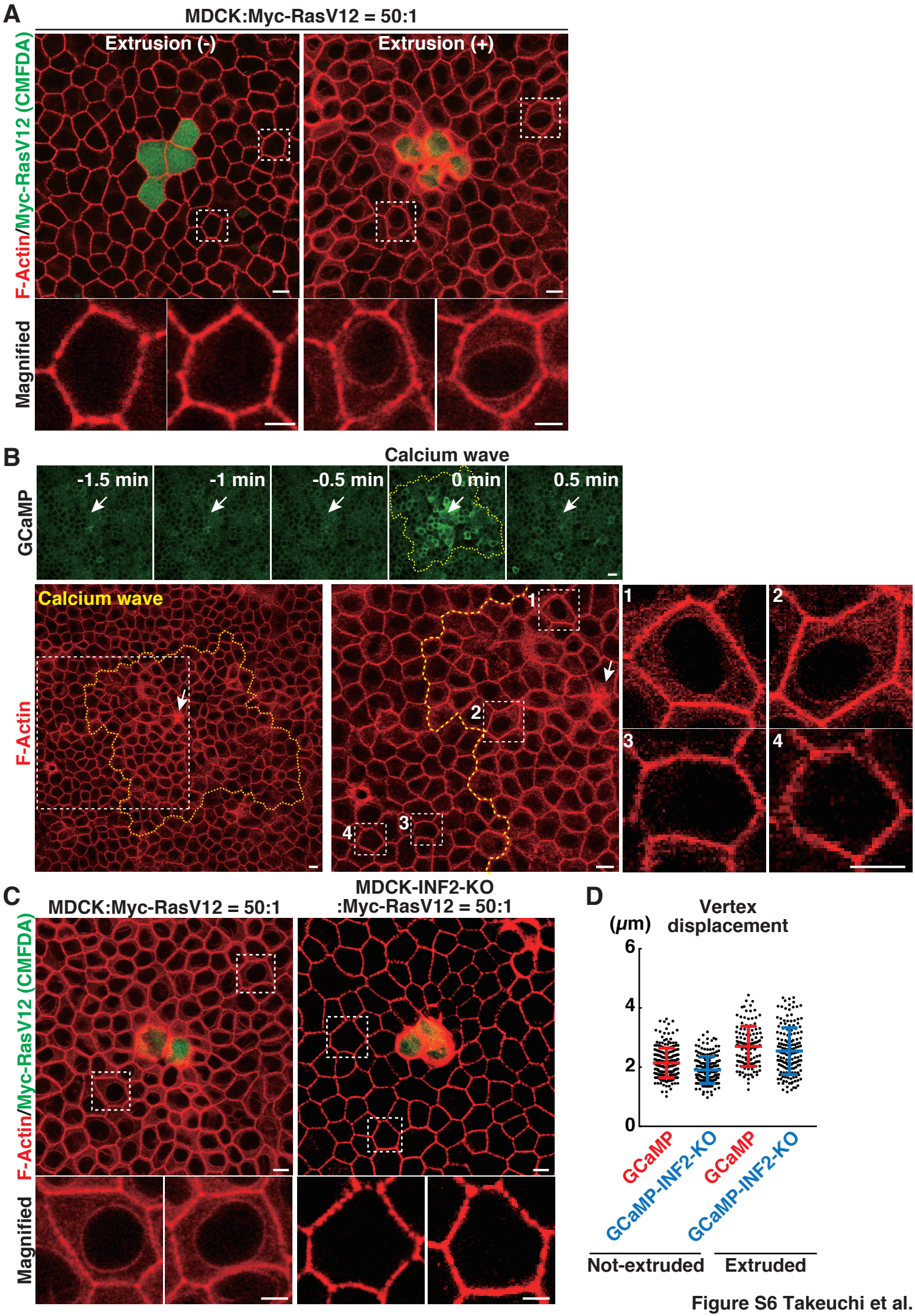

Figure S6 Takeuchi et al.

Figure S6. The Actin Rearrangement around Apically Extruding Cells and Effect of Knockout of Inverted Formin 2 (INF2) in the Surrounding Cells on the Actin Phenotype, Related to Figures 5 and 6

(A) Fluorescence images of F-actin (red) with Alexa-Fluor-568-conjugated phalloidin in the mix culture of MDCK-pTRE3G Myc-RasV12 cells stained with CMFDA (green) and MDCK cells. Larger images of Figure 5A (center and right) are shown. The status of Myc-RasV12 cells is not-extruded (left) or extruding (right). The area in the white box is shown at higher magnification in the lower panel. (B) Time-lapse analysis followed by F-actin staining using grid-chamber glass-bottom dishes. MDCK-pTRE3G Myc-RasV12 cells were co-cultured with MDCK-GCaMP6S cells. After incubation with doxycycline for 10 h, we performed time-lapse observation for 16 h followed by phalloidin staining. Upper images (green) are extracted from time-lapse observation of calcium wave. The arrows indicate an extruding RasV12 cell. Yellow line indicates the border of calcium wave. Lower panels show the fluorescence images of F-actin (red). The area in the white box is shown at higher magnification in the right panel. Note that F-actin accumulation in the cytosol and perinuclear region occurs in cells that calcium wave has reached. (C) Fluorescence images of F-actin (red) in the mix culture of MDCK-pTRE3G Myc-RasV12 cells stained with CMFDA (green) and MDCK or MDCK-INF2-knockout cells. Larger images of Figure 6C are shown. The area in the white box is shown at higher magnification in the lower panel. (D) Quantification of the displacement of the vertex movement of MDCK-GCaMP6S or MDCK-INF2-knockout cells. MDCK-pTRE3G Myc-RasV12 cells were co-cultured with MDCK-GCaMP6S or MDCK-GCaMP6S INF2-knockout cells. After incubation with doxycycline for 10 h, we performed time-lapse observation for 16 h. The displacement of each vertex movement during apical

extrusion is depicted as a dot. The vertices reside within 7 cells away from RasV12 cells were analyzed. Data are mean ± SD. n=225, 191, 126, and 171 from three independent experiments. Scale bars, 10 μm (A, C), 20 μm (B),

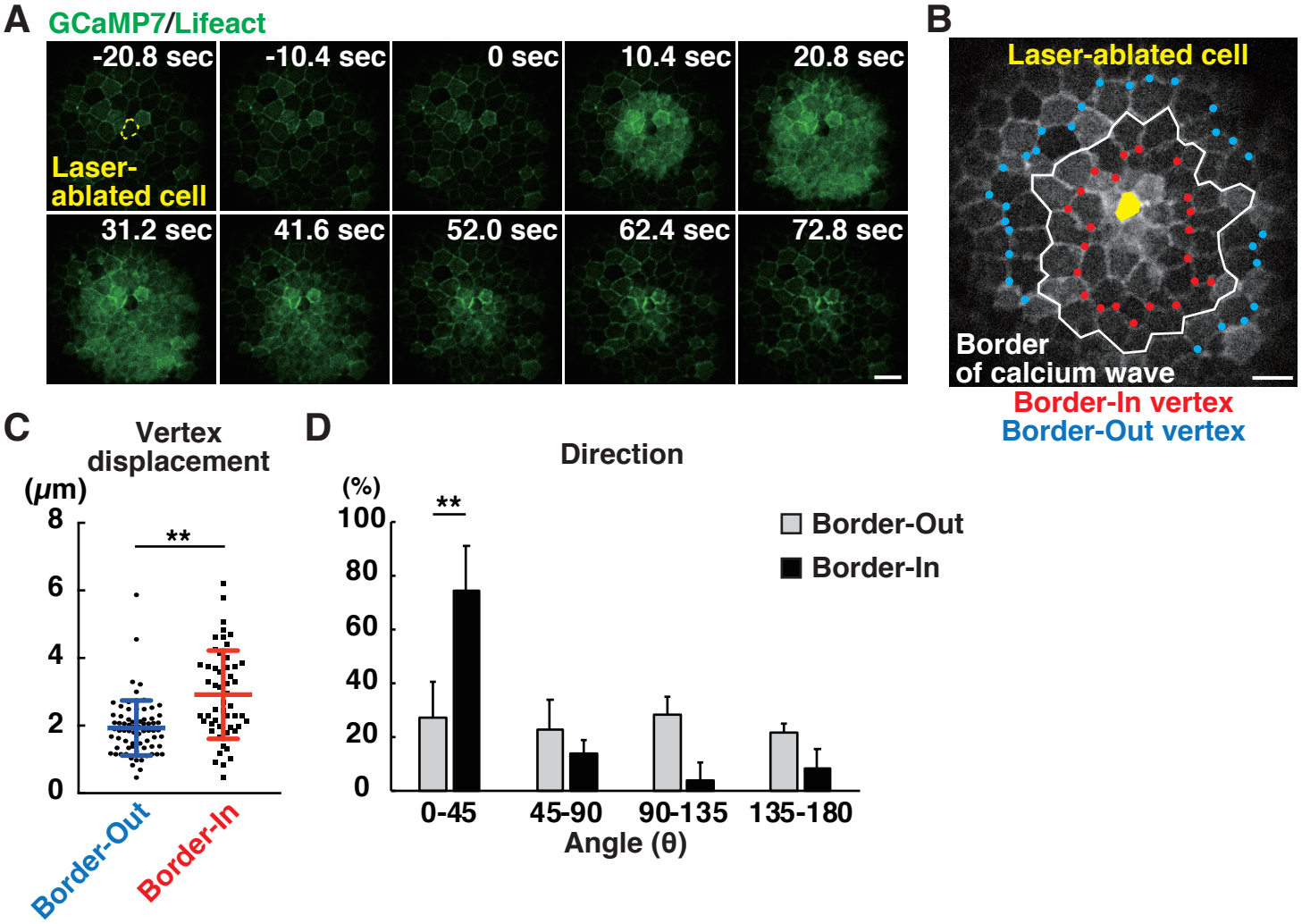

Figure S7 Takeuchi et al.

Figure S7. Calcium Wave Occurs around Laser-Ablated Dying Cells in Zebrafish Embryos, Related to Figure 7

(A) Time-lapse images of calcium wave from an extruding laser-ablated dying cell in the enveloping layer of zebrafish embryos. Note that in addition to calcium wave (GCaMP7, green), much weaker Lifeact-GFP fluorescence is also observed in the background. (B) A representative image of GFP-Lifeact (Start). The red dot (Border-In vertex) or blue dot (Border-Out vertex) is one-row inside or outside vertex from the border of calcium wave, respectively. (A and B) Scale bars, 50 μm. (C and D) Quantification of the displacement (C) or direction (D) of the vertex movement. n=74 and 52 from three independent experiments. (C) The displacement of each vertex movement is depicted as a dot. Data are mean ± SD. $^{**}P<0.01$ (unpaired *t*-tests). (D) Data are mean ± SD from three independent experiments. $^{**}P<0.01$ (unpaired *t*-tests).