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Supplemental Information

Tension Creates an Endoreplication Wavefront that Leads Regeneration of Epicardial Tissue

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SUPPLEMENTAL INFORMATION

Figures S1-S7

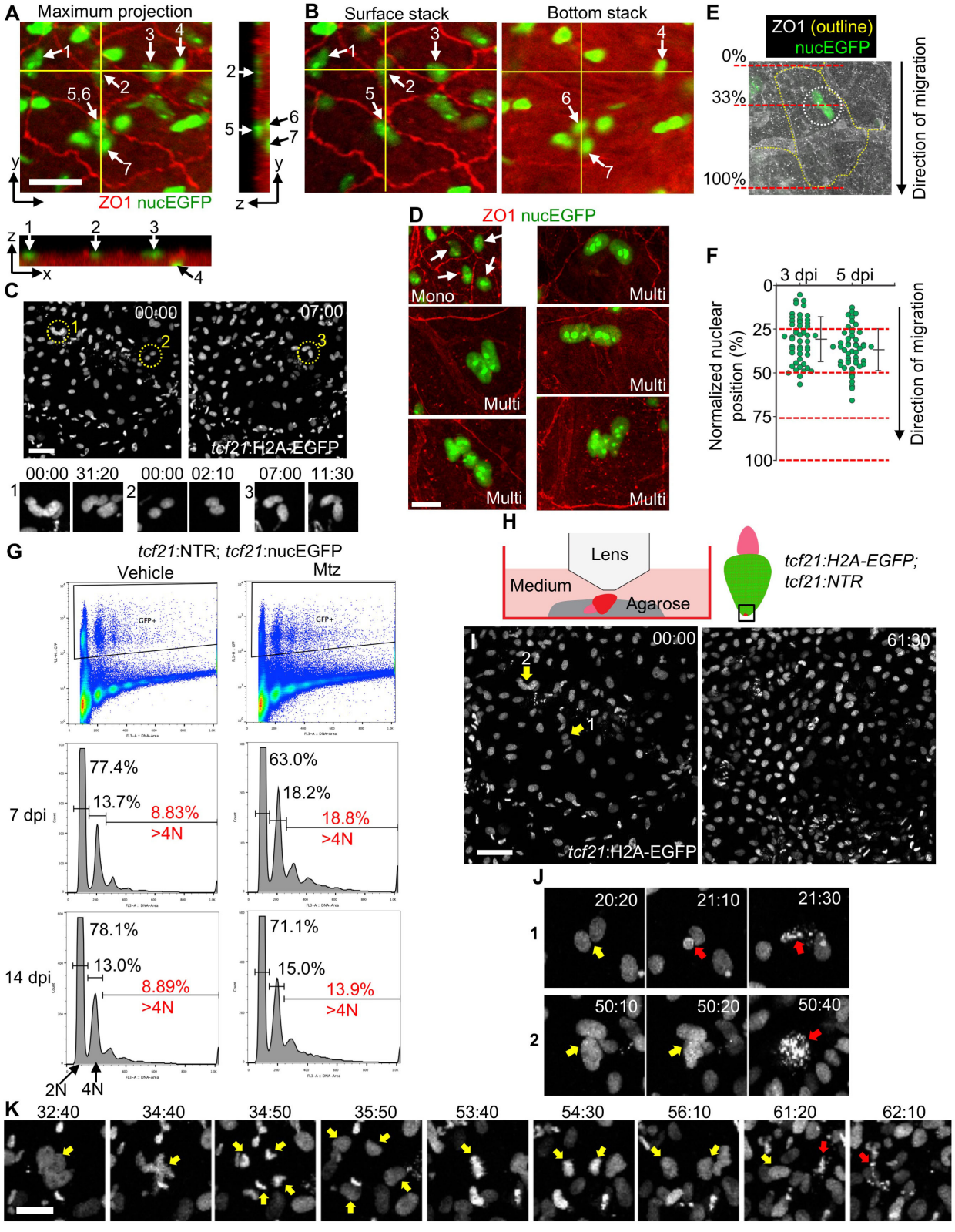
A
Maximum projection
ZO1 nucEGFP
B
Surface stack
Bottom stack
C
00:00
07:00
tcf21:H2A-EGFP
00:00 31:20
00:00 02:10
07:00 11:30
D
ZO1 nucEGFP
Mono
Multi
E
ZO1 (outline)
nucEGFP
0%
33%
100%
Direction of migration
F
3 dpi
5 dpi
Normalized nuclear position (%)
Direction of migration
G
tcf21:NTR; tcf21:nucEGFP
Vehicle
Mtz
GFP+
7 dpi
77.4%
13.7%
8.83%
>4N
63.0%
18.2%
18.8%
>4N
14 dpi
78.1%
13.0%
8.89%
>4N
71.1%
15.0%
13.9%
>4N
2N
4N
H
Lens
Medium
Agarose
tcf21:H2A-EGFP;
tcf21:NTR
I
00:00
61:30
tcf21:H2A-EGFP
J
20:20
21:10
21:30
50:10
50:20
50:40
K
32:40
34:40
34:50
35:50
53:40
54:30
56:10
61:20
62:10

Figure S1. Analysis of Nucleation and Apoptosis in Polyploid Cells (Related to Figure 1)

(A) Orthogonal view of a z-stack image. A maximum projection image of the x-y plane is shown with nucEGFP in green and ZO1 staining in red. Yellow lines indicate positions for views of the y-z plane (right) and the x-z plane (bottom), respectively. Arrows and numbers denote nuclei. Scale bar, 20 μm.

(B) Two z-axis stacks of the image shown in (A). The numbers indicate the same nuclei shown in (A). The nuclei 1, 2, 3, and 5 are in the outermost layer, while nuclei 4, 6, and 7 are in the inner layer, embedded within the muscle (red autofluorescence).

(C) Video frames from Movie S1 with *tcf21*:H2A-EGFP shown in grayscale. Nuclei of three cells are circled in low-magnification images (top), and two frames of each are displayed below. Nuclei within individual cells frequently overlap. Scale bar, 50 μm.

(D) Examples of mononucleate cells (top left, arrows) and multinucleate cells. Scale bar, 20 μm.

(E, F) Quantification of nuclear positions within each multinucleate cell at 3 and 5 dpi, respectively. The cell length was measured parallel to the direction of epicardial tissue regeneration. The nuclear position was defined as the center of an area encircling all of the nuclei in each cell and was normalized in percentiles to cell length. n = 50 for each timepoint. Bars indicate mean ± S.D.

(G) FACS analysis of purified epicardial cells. Epicardial cells were purified from uninjured (Vehicle, left) and regenerating samples (Mtz, right) and stained with PI for DNA content. *tcf21:NTR; tcf21:nucEGFP* ventricles were collected at 7 and 14 dpi. Percentages of 2N, 4N and >4N cells are indicated. Samples from regenerating hearts

have 113% (7 dpi) or 56% (14 dpi) more >4N cells (red) than those from uninjured hearts.

(H) Schematic for experiments in (I-K).

(I) Video frames acquired at the ventricular apex at the final stages of epicardial regeneration, covering 61.5 h. *tcf21*:H2A-EGFP is shown in grayscale. Arrows and numbers denote nuclei shown in (J). Scale bar, 50 μm. See also Movie S1.

(J) High-magnification view of nuclear doublets indicated in (I) undergoing nuclear fragmentation (red arrows). Scale bar, 20 μm.

(K) Video frames of an unconventional division of a nuclear doublet undergoing fragmentation. Yellow arrows denote multinucleate cell and daughter nuclei. Red arrows indicate nuclear fragmentation. One daughter nucleus divided again before fragmentation. Scale bar, 20 μm. Timing, hh:mm.

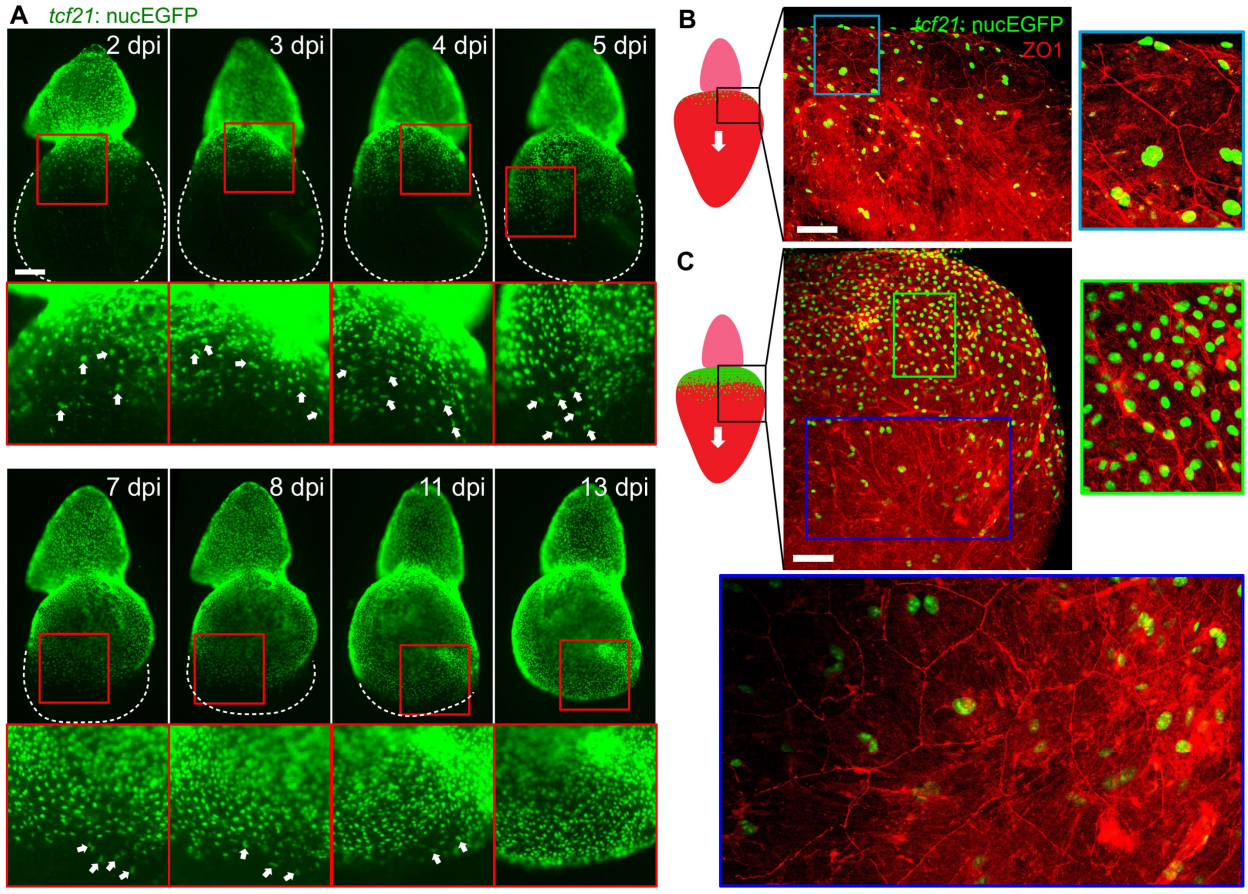
A
tcf21: nucEGFP
2 dpi
3 dpi
4 dpi
5 dpi
7 dpi
8 dpi
11 dpi
13 dpi
B
tcf21: nucEGFP
ZO1
C

Figure S2. Cell Size and Nuclear Number during *ex vivo* Epicardial Regeneration (Related to Figure 1)

(A) A whole ventricular explant (with outflow tract at top) undergoing *ex vivo* epicardial regeneration. *tcf21:NTR; tcf21:nucEGFP* hearts were incubated in culture medium with 1 mM Mtz for 24 h. Explants were assessed daily for EGFP fluorescence. White dashed lines outline ventricles. Regions framed in red are enlarged to show details below each panel. White arrows denote nuclear doublets. Scale bar, 200 μm.

(B, C) Images of flattened explants stained with an anti-ZO1 antibody (red). Images of the framed region represented in the cartoons from 2 hearts are shown. Regions framed in cyan, green and blue were enlarged in the same scale at the right or the bottom. Optical sections are shown in enlarged images in (C) to more clearly indicate ZO1 staining. Scale bar, 100 μm.

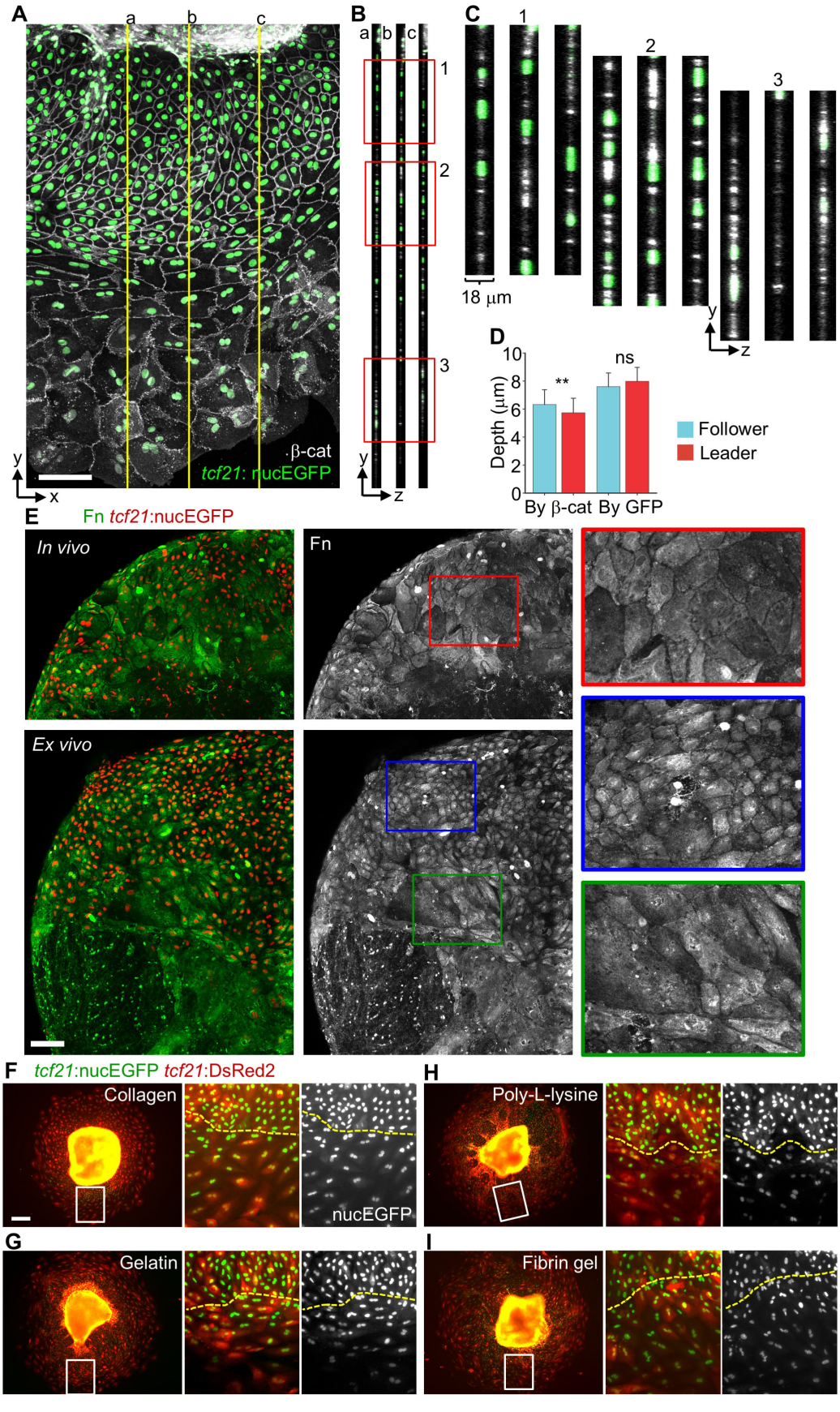
A
a
b
c
β-cat
tcf21: nucEGFP
y
x
B
a b c
1
2
3
y
z
C
1
2
3
18 μm
y
z
D
Depth (μm)
10
8
6
4
2
0
**
ns
By β-cat
By GFP
Follower
Leader
E
Fn tcf21:nucEGFP
In vivo
Fn
Ex vivo
F
tcf21:nucEGFP tcf21:DsRed2
Collagen
nucEGFP
H
Poly-L-lysine
G
Gelatin
I
Fibrin gel

Figure S3. Analysis of Cell Volume and the Relevance of ECM Components to Epicardial Regeneration (Related to Figure 2)

(A) A maximum projection view of an explant culture, showing *tcf21*:nucEGFP in green and β-catenin staining in grayscale. Three yellow lines (a, b and c) denote positions of views for the y-z planes in (B).

(B, C) y-z views for the planes denoted in (A). The framed regions in (B) are enlarged to show details in (C).

(D) Quantification of the cell depths by measuring junctions (β-catenin signals, By β-cat) or nuclei (nucEGFP signals, By GFP). n = 50 (By β-cat, follower), 64 (By β-cat, leader), 25 (By GFP, follower) and 10 (By GFP, leader), respectively. ** $P < 0.01$; ns, not significant; Mann-Whitney Rank Sum Test. Bars indicate mean ± S.D.

(E) Images of fibronectin (Fn) staining of regenerating hearts both *in vivo* and *ex vivo*. Fn is shown in green (merged images) or grayscale (single-channel images) and tcf*21*:nucEGFP is shown in red. The framed regions are enlarged to show details on the right. Scale bar, 100 μm.

(F-I) *tcf21:nucEGFP; tcf21:DsRed2* epicardial explants were plated in differently coated dishes for 5 days. Dishes were coated with 0.01% type I collagen (F), 0.1% gelatin (G) or 0.01% poly-L-lysine (H) solutions overnight. Fibrin gel (I) was freshly made as described previously (Kim, et al., 2012). The framed regions of left panels are enlarged to show as middle (merged) and right panels (nucEGFP only). The yellow lines approximately separate follower (top) and leader cell regions (below). Scale bar, 100 μm.

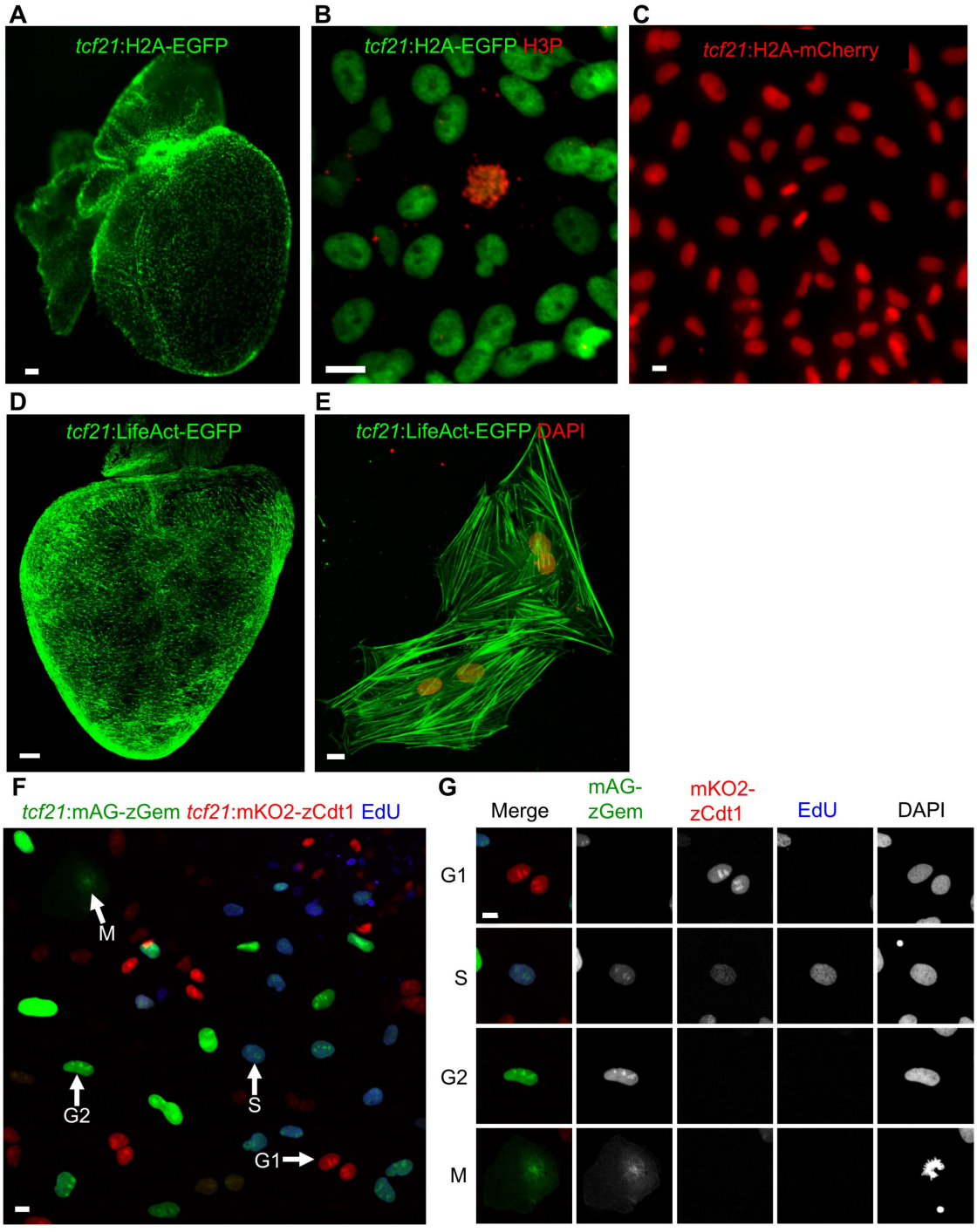
A
tcf21:H2A-EGFP
B
tcf21:H2A-EGFP H3P
C
tcf21:H2A-mCherry
D
tcf21:LifeAct-EGFP
E
tcf21:LifeAct-EGFP DAPI
F
tcf21:mAG-zGem tcf21:mKO2-zCdt1 EdU
M
G2
S
G1
G
Merge
mAG-zGem
mKO2-zCdt1
EdU
DAPI
G1
S
G2
M

Figure S4. New Reporter Lines for Live Imaging (Related to Figure 3)

(A, B) A *tcf21:H2A-EGFP* reporter line indicates chromatin in epicardial cells of a whole-mounted heart (A) and in epicardial explant culture (B). Anti-phospho-Histone H3 staining (H3P) was performed to label condensed chromatin (red). Scale bars, 100 μm (A) or 10 μm (B).

(C) High-magnification view of *tcf21:H2A-mCherry* epicardial cells. Scale bar, 10 μm

(D, E) A *tcf21:LifeAct-EGFP* reporter labels F-actin in whole-mounted (D) or isolated epicardial cells (E). DAPI stains nuclei red in (E). Scale bars, 100 μm (D) or 10 μm (E).

(F, G) Epicardial explant culture from a *tcf21:FUCCI* heart, stained after a 1 h pulse of EdU to mark S phase. Arrows in (F) denotes different cell cycle phases, also highlighted in (G). Scale bars, 10 μm.

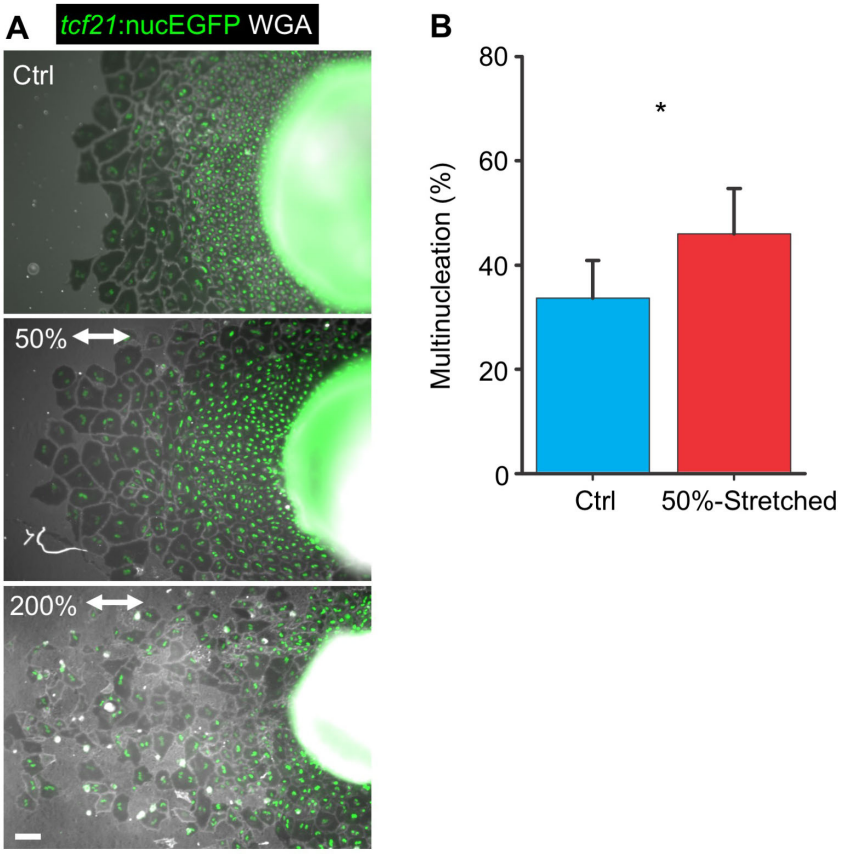

Figure S5. Mechanical Stretching of Epicardial Sheets (Related to Figure 5)

(A) The same experiment as shown in Figure 5, using *tcf21:nucEGFP* tissue with cell shapes outlined with a WGA staining (grayscale). (Top) Unstretched control. (Middle) Explants after 50% stretch. (Bottom) Explants after 200% stretch, which typically damaged the tissue. The dual arrow indicates stretch direction. Scale bars, 100 μm.

(B) Quantification of epicardial cell multinucleation within a quadrant of the epicardial sheet shown in (A) after 50% stretch. n = 8 (Ctrl) and 7 (Stretched) explants. * $P < 0.05$, Mann-Whitney Rank Sum Test. Bars indicate mean ± S.D.

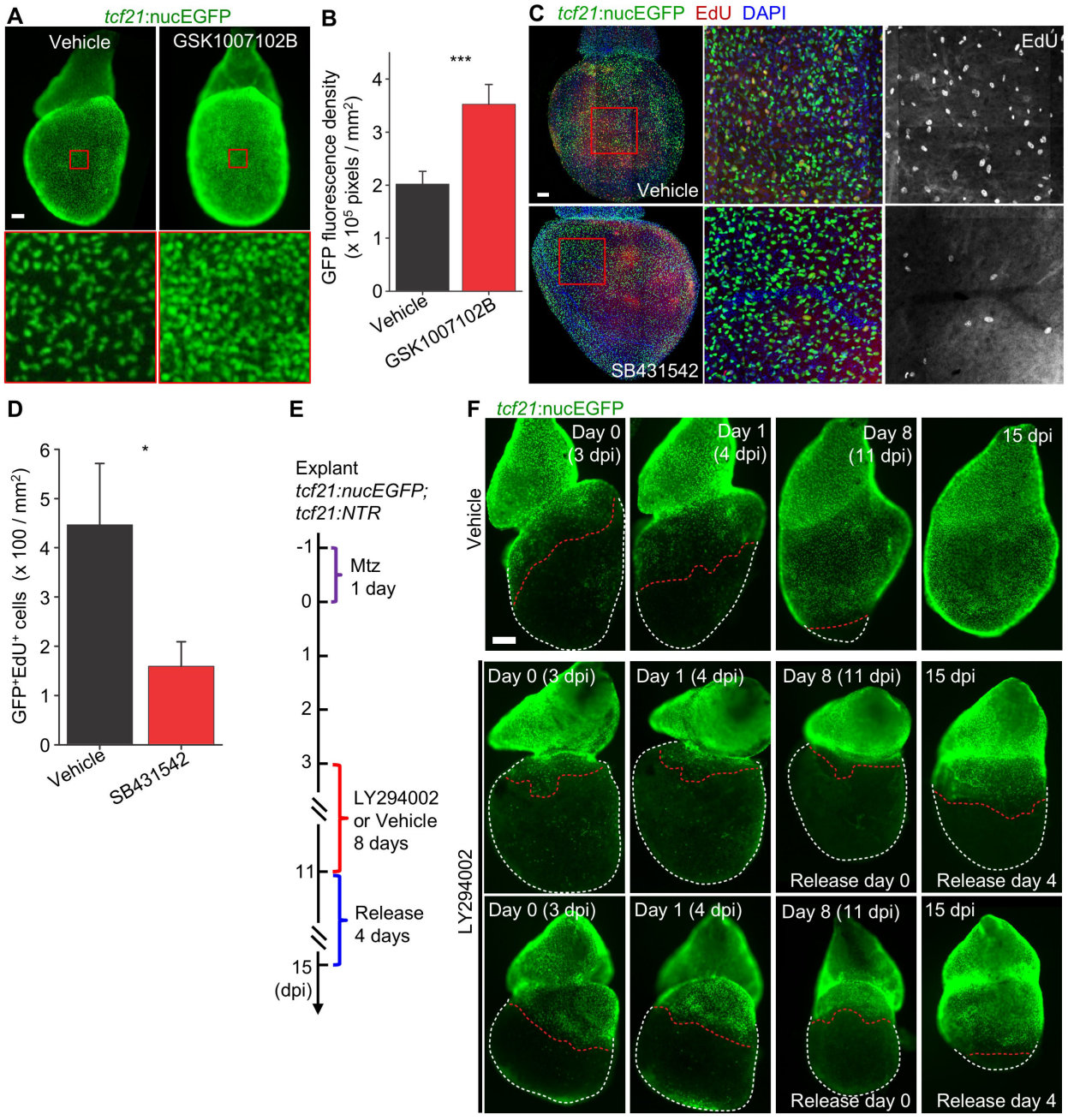
A
tcf21:nucEGFP
Vehicle
GSK1007102B
B
GFP fluorescence density (x 10^5 pixels / mm^2)
***
Vehicle
GSK1007102B
C
tcf21:nucEGFP EdU DAPI
EdU
Vehicle
SB431542
D
GFP+EdU+ cells (x 100 / mm^2)
*
Vehicle
SB431542
E
Explant
tcf21:nucEGFP;
tcf21:NTR
Mtz
1 day
LY294002
or Vehicle
8 days
Release
4 days
(dpi)
F
tcf21:nucEGFP
Vehicle
LY294002
Day 0
(3 dpi)
Day 1
(4 dpi)
Day 8
(11 dpi)
15 dpi
Release day 0
Release day 4

Figure S6. Chemical Screening for Regulators of Epicardial Cell Proliferation (Related to Figure 7)

(A) Whole mounted *tcf21:nucEGFP* ventricles after 6 days of culture as explants, treated with DMSO (Vehicle) or GSK1007102B. Framed regions are enlarged and shown below.

(B) Quantification of EGFP fluorescence density. n = 8 (Vehicle) and 5 (GSK1007102B) explants, respectively. *** $P < 0.001$, Student's *t*-test. Bars indicate mean ± S.D.

(C) Images of fixed and flattened *tcf21:nucEGFP* explants after 2 days of culture with DMSO (Vehicle) or SB431542. Samples were incubated with EdU for 1 h before fixation (shown in red and grayscale). DNA was stained with DAPI (blue). Scale bars, 100 μm.

(D) Quantification of $EGFP^+EdU^+$ nuclei per mm^2 on the ventricular surface, from hearts in (C). n = 3 explants for each. * $P < 0.05$, Student's *t*-test. All error bars indicate S.D.

(E, F) PI3K regulates epicardial regeneration. (E) Schematic for experiments in (F). *tcf21:NTR; tcf21:nucEGFP* animals were used. LY294002 (50 μM) was added for 8 days (3 - 11 dpi). Then, explants were rinsed twice with PBS and further cultured for 4 days. (F) Epicardial regeneration *ex vivo* in the presence of DMSO (Vehicle) or 50 μm LY294002. All vehicle-treated explants (16 of 16) displayed regeneration. All LY294002-treated explants displayed a block of regeneration (23 of 23), which resumed in each case after drug wash-out. Scale bar, 200 μm.

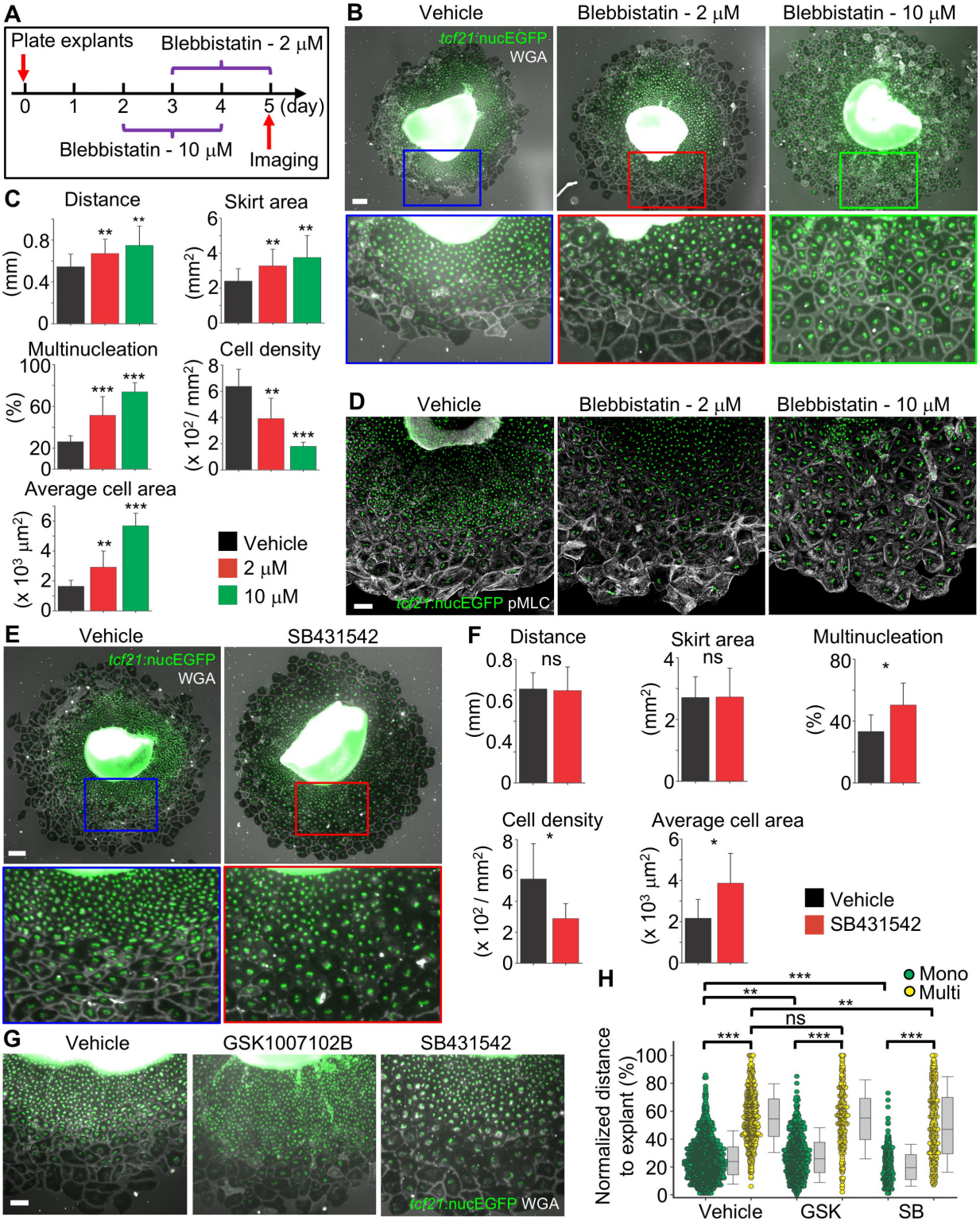
A
Plate explants
Blebbistatin - 2 μM
0 1 2 3 4 5 (day)
Blebbistatin - 10 μM
Imaging
B
Vehicle
Blebbistatin - 2 μM
Blebbistatin - 10 μM
tcf21:nucEGFP
WGA
C
Distance
(mm)
Skirt area
(mm²)
Multinucleation
(%)
Cell density
(x 10² / mm²)
Average cell area
(x 10³ μm²)
Vehicle
2 μM
10 μM
D
Vehicle
Blebbistatin - 2 μM
Blebbistatin - 10 μM
tcf21:nucEGFP pMLC
E
Vehicle
SB431542
tcf21:nucEGFP
WGA
F
Distance
ns
Skirt area
ns
Multinucleation
(%)
Cell density
(x 10² / mm²)
Average cell area
(x 10³ μm²)
Vehicle
SB431542
G
Vehicle
GSK1007102B
SB431542
tcf21:nucEGFP WGA
H
Mono
Multi
ns
Normalized distance to explant (%)
Vehicle
GSK
SB

Figure S7. Chemical Treatment of Epicardial Explant Cultures (Related to Figure 7)

(A) Schematic for experimental design in (B-D). *tcf21:nucEGFP* explants were plated for 5 days. Blebbistatin was added from day 3 to day 5 (2 μM) or from day 2 to day 4 (10 μM). Images were acquired and analyzed at day 5.

(B) Epicardial explant culture assays with Vehicle or Blebbistatin (2 μM or 10 μM) treatment as shown in (A). Framed regions are enlarged below to show details. *tcf21*:nucEGFP is shown in green, WGA staining is shown in grayscale. Scale bar, 200 μm.

(C) Quantification of epicardial tissue growth in (B). For migration distance and skirt area, n = 20 (Vehicle), 20 (2 μM), and 18 (10 μM) explants, respectively. For nucleation, cell density, and average cell area, n = 10 explants for each treatment. *** $P < 0.001$; ** $P < 0.01$; Mann-Whitney Rank Sum Test. All comparisons were versus Vehicle-treated group. Bars indicate mean ± S.D.

(D) Epicardial explant culture in (A) was stained with pMLC (Ser19, grayscale). Scale bar, 100 μm.

(E) Epicardial explant culture assays with Vehicle or SB431542 (10 μM) treatment. *tcf21:nucEGFP* explants (shown in green) were plated for 5 days. WGA staining is used to outline cells (grayscale). Drug was added from day 0 to day 5. Framed regions are enlarged below to show details. Scale bar, 200 μm.

(F) Quantification of epicardial tissue growth in (E). For migration distance and skirt area, n = 20 explants for each. For nucleation, cell density, and average cell area, n = 10 explants for each treatment. * $P < 0.05$; ns, not significant; Mann-Whitney Rank Sum Test. Bars indicate mean ± S.D.

(G) Epicardial explant culture assays with Vehicle, GSK1007102B (0.5 μM) or SB431542 (10 μM) treatment. *tcf21:nucEGFP* explants (shown in green) were plated for 5 days. WGA staining is used to outline cells (grayscale). Drugs were added from day 0 to day 5. Scale bar, 100 μm.

(H) Spatial distribution of nucleation after treatment with Vehicle, GSK1007102B (GSK), or SB431542 (SB) as shown in (G). n = 1,481 (Vehicle), 819 (GSK), and 439 (SB) cells, respectively. *** $P < 0.001$; ns, not significant; Mann-Whitney Rank Sum Test. Bars indicate S.D.