nature cell biology

SUPPLEMENTARY INFORMATION

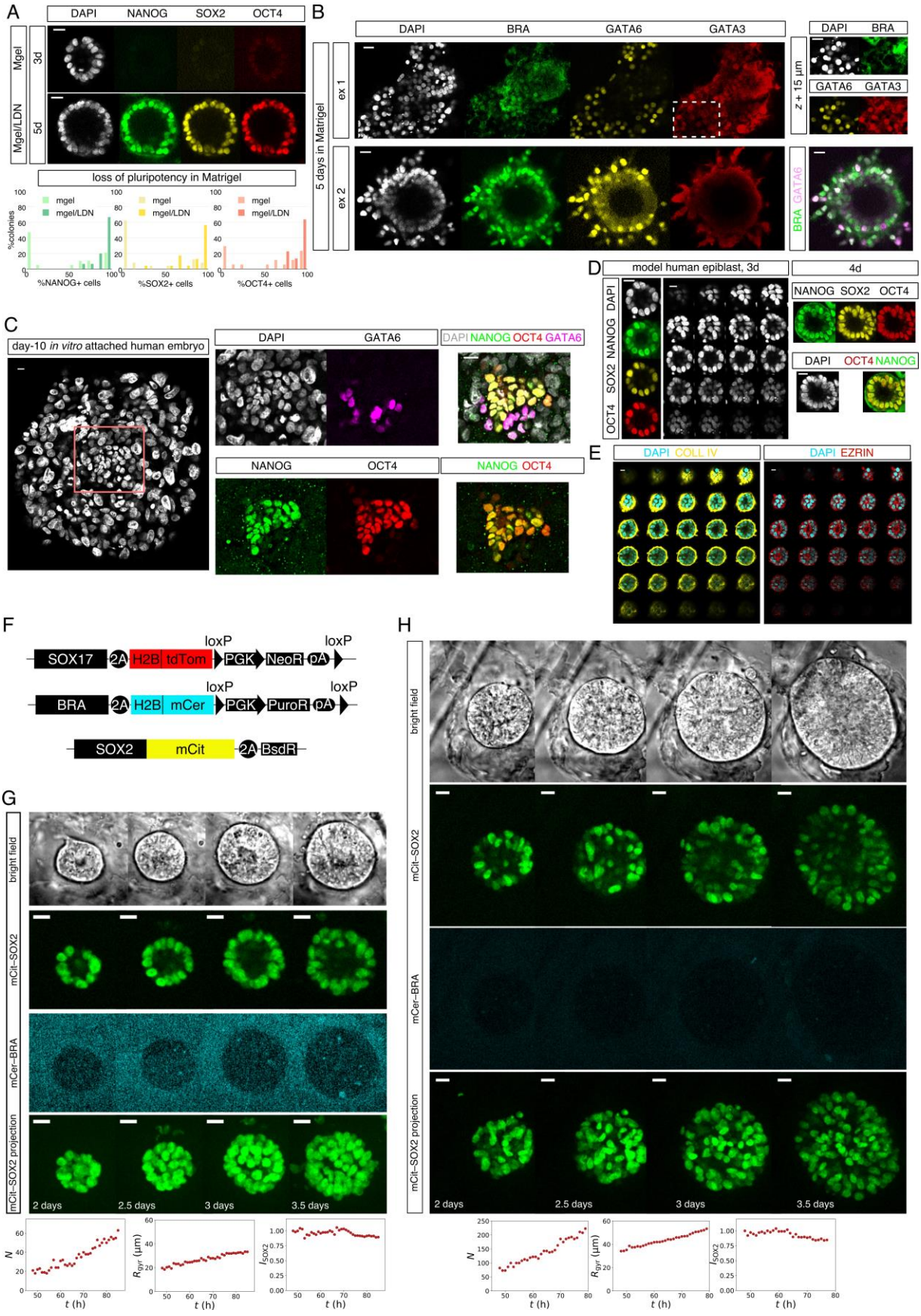
https://doi.org/10.1038/s41556-019-0349-7

In the format provided by the authors and unedited.

A 3D model of a human epiblast reveals BMP4-driven symmetry breaking

Mijo Simunovic[1,2], Jakob J. Metzger[1,2], Fred Etoc[1,2], Anna Yoney[1,2], Albert Ruzo[1], Iain Martyn[1,2], Gist Croft[1], Dong Shin You, Ali H. Brivanlou[1*] and Eric D. Siggia[2*]

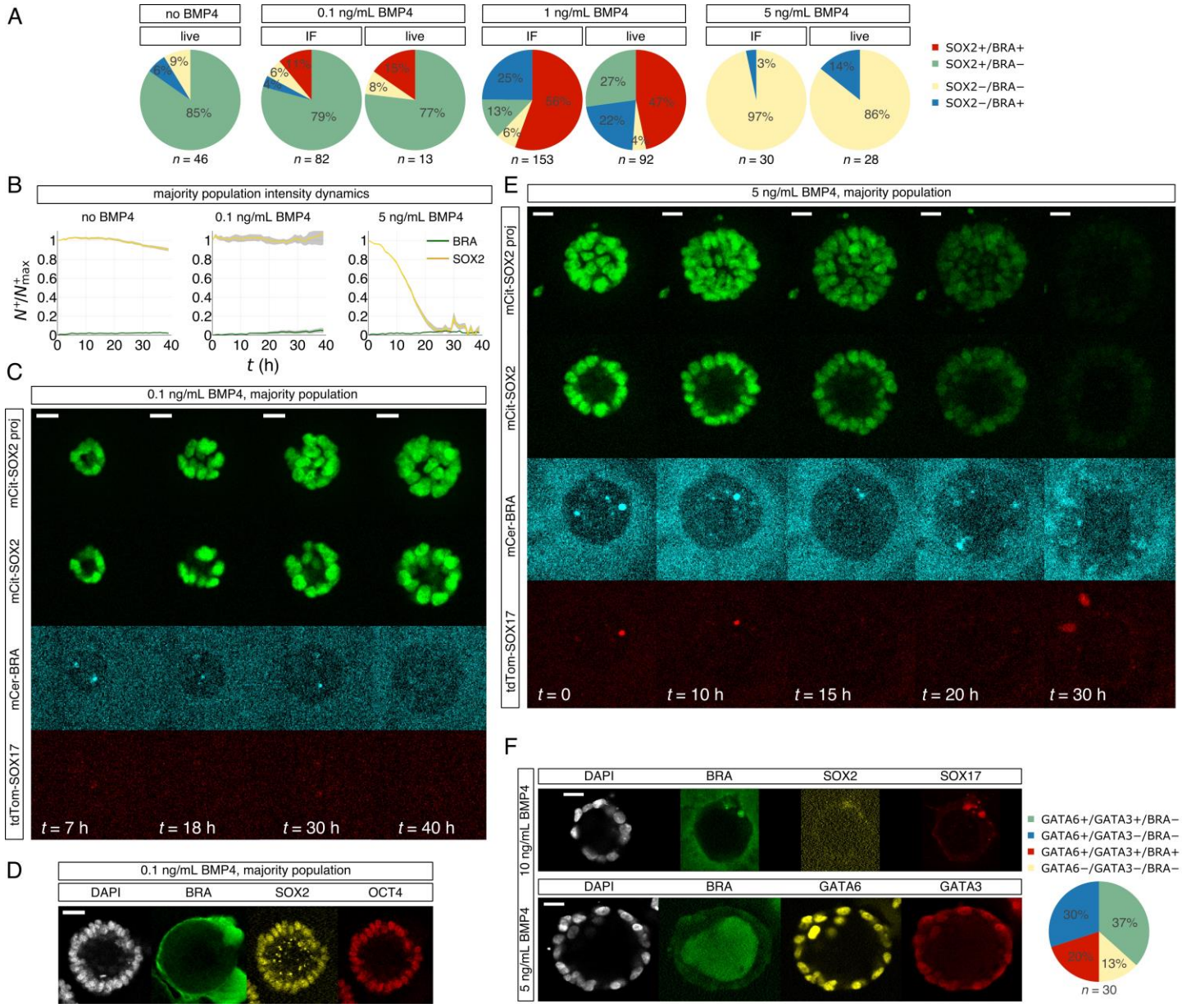
[1]Laboratory of Stem Cell Biology and Molecular Embryology, The Rockefeller University, New York, NY, USA. [2]Center for Studies in Physics and Biology, The Rockefeller University, New York, NY, USA. *e-mail: brvnlou@rockefeller.edu; siggiae@rockefeller.edu


A
DAPI NANOG SOX2 OCT4
Mgel
Mgel/LDN
3d
5d
loss of pluripotency in Matrigel
%colonies
mgel
mgel/LDN
%NANOG+ cells
%SOX2+ cells
%OCT4+ cells
B
DAPI BRA GATA6 GATA3
5 days in Matrigel
ex 1
ex 2
z + 15 µm
DAPI BRA
GATA6 GATA3
BRA GATA6
C
day-10 in vitro attached human embryo
DAPI GATA6
DAPI NANOG OCT4 GATA6
NANOG OCT4
NANOG OCT4
D
model human epiblast, 3d
4d
DAPI
NANOG
SOX2
OCT4
NANOG SOX2 OCT4
DAPI OCT4 NANOG
E
DAPI COLL IV
DAPI EZRIN
F
SOX17 2A H2B tdTom loxP PGK NeoR pA loxP
BRA 2A H2B mCer loxP PGK PuroR pA loxP
SOX2 mCit 2A BsdR
G
bright field
mCit–SOX2
mCer–BRA
mCit–SOX2 projection
2 days
2.5 days
3 days
3.5 days
N
R_{gyr} (µm)
I_{SOX2}
t (h)
H
bright field
mCit–SOX2
mCer–BRA
mCit–SOX2 projection
2 days
2.5 days
3 days
3.5 days
N
R_{gyr} (µm)
I_{SOX2}
t (h)

Supplementary Figure 1

Supplementary analysis of an *in vitro* 3D model of a pre-gastrulation human epiblast.

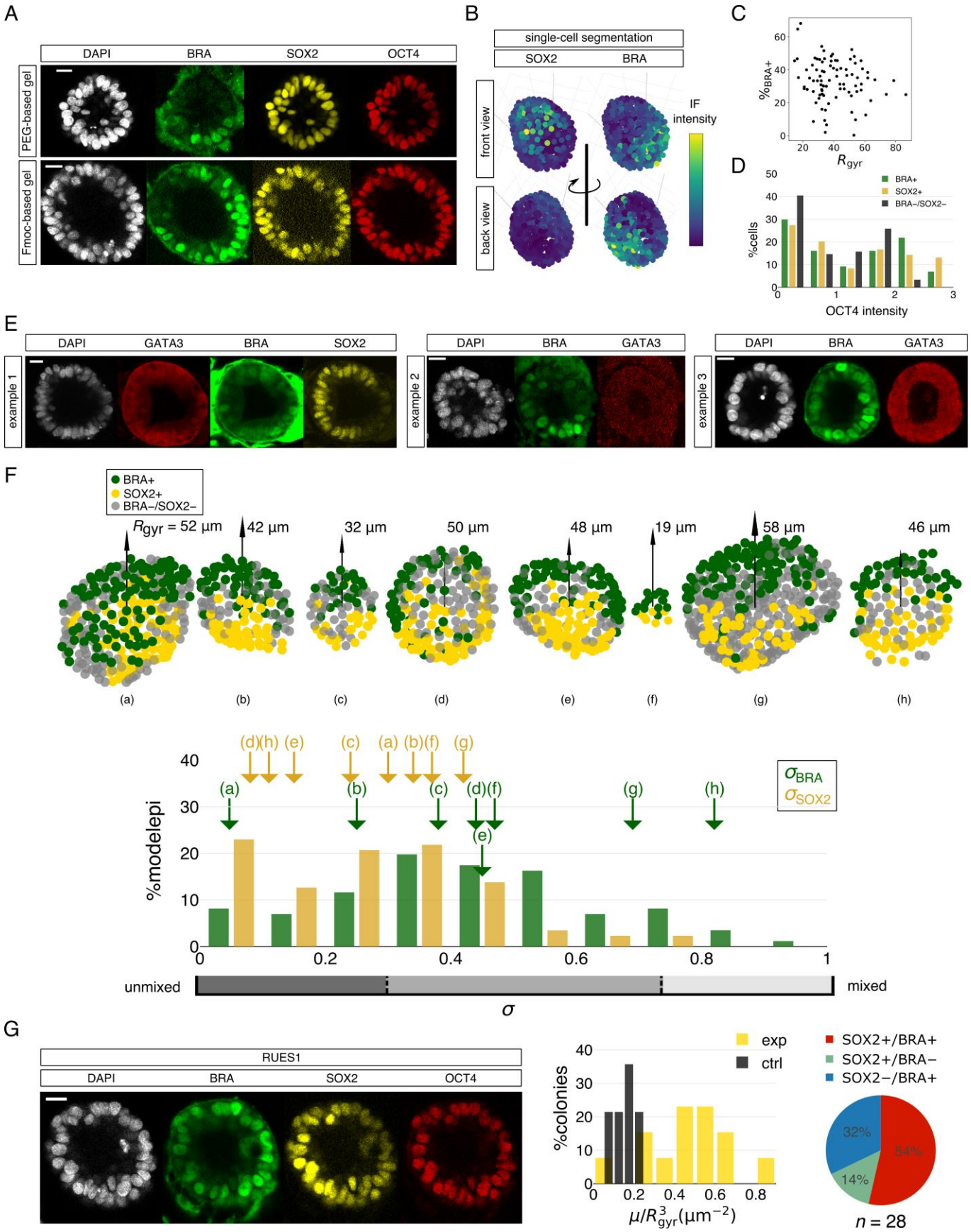
(A-B) Spontaneous differentiation of spherical hESC epithelia grown in pure Matrigel from single cells. (A) Shown are IF of pluripotency markers of a colony grown in Matrigel (Mgel) for three days and in Matrigel and LDN (Mgel/LDN) for five days. Plots quantify the pluripotency loss where each colony is binned based on the fraction of its cells expressing the pluripotency marker (*n*=31 independent colonies for Mgel; *n*=28 independent colonies for Mgel/LDN). The spontaneous differentiation of Matrigel-grown hESC colonies is BMP4-dependent, since colonies remain pluripotent in the presence of the BMP inhibitor LDN. (B) After five days in Matrigel, cells lose structural integrity and appear to invade into the surrounding gel (representative of 12 independent colonies). Shown are two different examples. Top right shows a detail in the dotted rectangle 15 µm displaced in *z* from the image on the left, where these nuclei are in focus. (C) Day-10 *in vitro* attached human embryo. *Left*: DAPI of an entire embryo; *right*: magnified epiblast (NANOG+ and OCT4+) and primitive endoderm (GATA6+). Shown is a different example from Figure 1 and representative of 4 embryos. (D) Additional data on human epiblast model (spherical hESC epithelia grown in hydrogel/Matrigel mix). Pluripotency markers in the human epiblast model grown for three (*left*) or four days (*right*). Left example shows a *z*-slice and the entire *z*-stack in DAPI. Both differ from Figure 1 and are based on 51 epiblast models from the main figure. (E) Surface markers *z*-stacks in a human epiblast model. Shown is the same example as in Figure 1. All scale bars, 20 µm. (F) Schematics of the RUES2-GLR line, expressing mCitrine-SOX2 (mCit-SOX2), mCerulean-BRACHYURY (mCer-BRA), and tdTomato-SOX17 (tdTom-SOX17). (G–H) Live-cell imaging of the human epiblast models made up of RUES2-GLR cells under pluripotency conditions, starting ~48h after seeding. The quantification shown is for the single examples shown here. Imaging frequency: 1 h^{-1}. Shown examples are different from the one in Figure 1 and representative of 39 model epiblasts that remained SOX2+ out of 48 total imaged independent model epiblasts. See also Video S1. All scale bars, 20 µm. Numerical source data are available in Supplementary Table 2.



Supplementary Figure 2

Patterning of a human epiblast model by high BMP4 concentration.

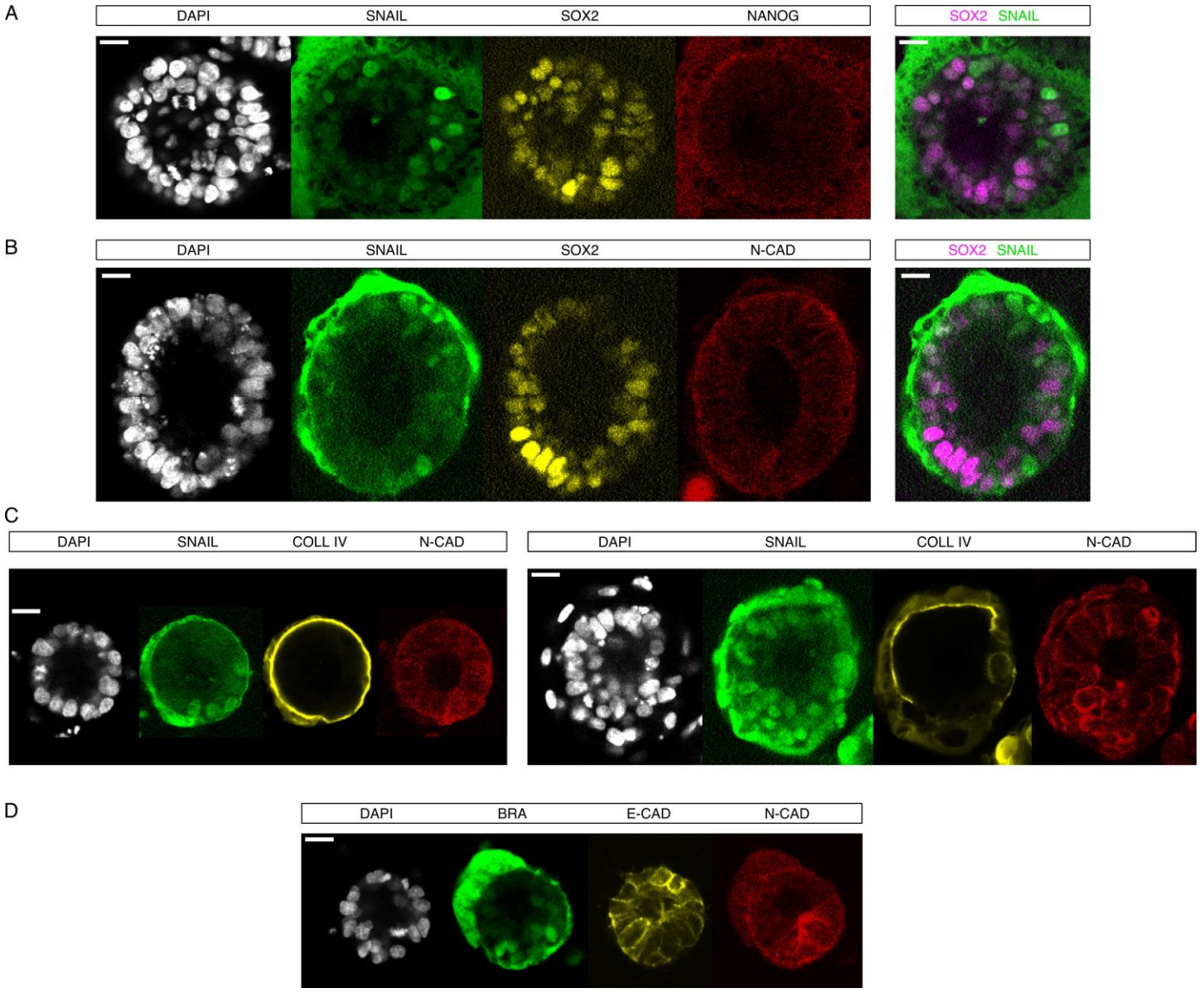
(A) Population fractions of colonies at various BMP4 concentrations from IF staining and from live-cell imaging data, presented separately. The statistics was deduced from seven independent experiments for 1 ng/mL (four IF stained, three imaged live), from two independent experiments for 5 ng/mL (one IF stained, one imaged live), and from three independent experiments for 0.1 ng/mL (one IF stained, two imaged live). The data for each BMP4 concentration from independently run experiments was pooled, IF and live imaging separately, and the *n* indicated in each pie chart represents the number of total individual 3D colonies. (B) BRA and SOX2 intensity change of the majority population under pluripotency conditions and with BMP4 for 2 days at different concentrations. From left to right: *n*=38, *n*=8, and *n*=23 individual 3D colonies. Thick line is mean, grey line is SE. (C) Live-cell imaging of a human epiblast model stimulated with 0.1 ng/mL BMP4 added at time zero, representative of majority population from 13 imaged 3D colonies. See also Video S2. (D) IF staining of epiblast models stimulated for two days with 0.1 ng/mL BMP4, showing epiblast markers (representative of the majority population of 82 3D colonies). (E) Live-cell imaging of a model human epiblast stimulated with 5 ng/mL BMP4 added at time zero, representative of majority population from 28 imaged 3D colonies. See also Video S3. (F) *Left*: IF of representative colonies after high (5 or 10 ng/mL) BMP4 dose for two days, showing the expression of germ layer and extraembryonic markers (top, representative of 20 3D colonies; bottom, 30 3D colonies). *Right*: population fractions of colonies two days after 5 ng/mL BMP4 in terms of differentiation markers (*n*=30 individual 3D colonies).


A
DAPI
BRA
SOX2
OCT4
PEG-based gel
Fmoc-based gel
B
single-cell segmentation
SOX2
BRA
front view
back view
IF intensity
C
%BRA+
R_{gyr}
D
BRA+
SOX2+
BRA−/SOX2−
%cells
OCT4 intensity
E
DAPI
GATA3
BRA
SOX2
example 1
example 2
example 3
F
BRA+
SOX2+
BRA−/SOX2−
R_{gyr} = 52 µm
42 µm
32 µm
50 µm
48 µm
19 µm
58 µm
46 µm
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
%modelepi
σ_{BRA}
σ_{SOX2}
unmixed
mixed
σ
G
RUES1
DAPI
BRA
SOX2
OCT4
%colonies
exp
ctrl
$\mu/R_{gyr}^3(\mu m^{-2})$
SOX2+/BRA+
SOX2+/BRA−
SOX2−/BRA+
32%
54%
14%
n = 28

Supplementary Figure 3

Breaking the AP symmetry: further examples and analyses.

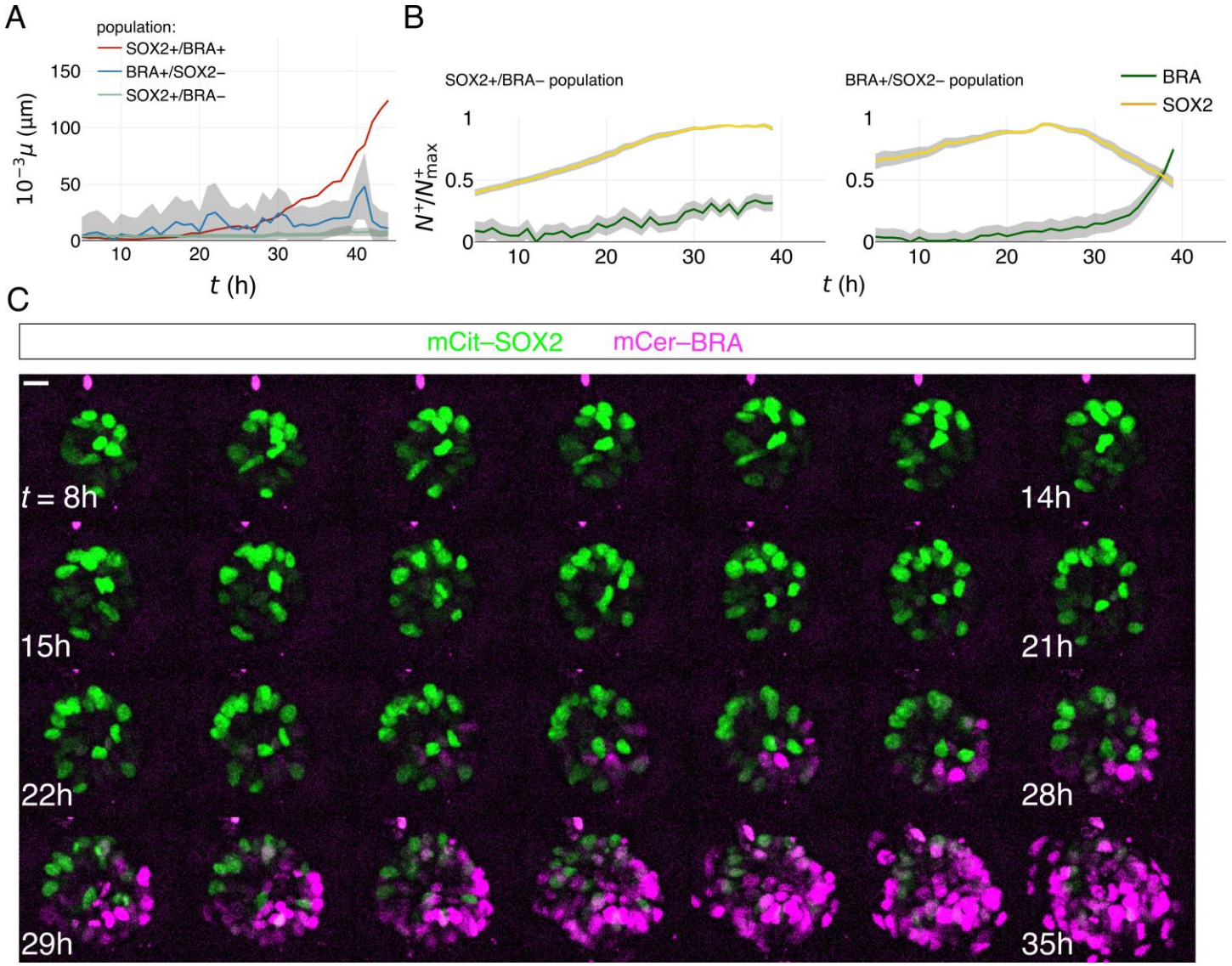
(A) Symmetry breaking was observed in two tested chemically different hydrogels (PEG-based, *n*=66 BRA+/SOX2+ individual 3D colonies; and Fmoc-based, representative of 21 BRA+/SOX2+ individual 3D colonies). Shown are examples from IF confocal slices and the single-cell IF quantification. Both examples are different from the ones in Figure 3. (B) 3D single-cell segmentation of the example in Figure 3A where the 3D image is rendered by drawing a sphere centered at the centroid of each segmented cell. Spheres are color-coded based on their BRA or SOX2 IF intensity. (C) %BRA+ cells as a function of colony radius (*n*=87 BRA+/SOX2+ individual 3D colonies, the same population as in Figure 3). (D) Histogram of OCT4 expression levels for three different cell populations in symmetry broken colonies (*n*=87, the same population as in Figure 3). (E) Symmetry-broken colonies stimulated with 1 ng/mL BMP4 do not show appreciable GATA3 expression (7 individual 3D colonies with the BRA/SOX2/GATA3 combination and 8 with the BRA/GATA3 combination using an alternative (rabbit) GATA3 antibody). (F) Additional examples of symmetry-broken 3D colonies, with various S values indicated below in the S distribution. The colonies have been rotated so that the dipole moment points up relative to a common origin for all the colonies. All dipole moments were divided by R_{gyr}^{3} to compare different size colonies and, when rendering the images, multiplied by 50 for clarity. (G) IF staining, dipole quantification, and population analysis of BMP4-differentiated 3D colonies composed of an alternative hESC line, RUES1, with 1 ng/mL BMP4 for 2d. 28 individual 3D colonies were imaged regardless of BRA/SOX2 expression and used to generate the population fraction pie chart. Dipole quantification was done on the BRA+/SOX2+ subpopulation as with all data with RUES2, i.e., on *n*=15 individual colonies.



Supplementary Figure 4

Molecular signatures of symmetry breaking and EMT.

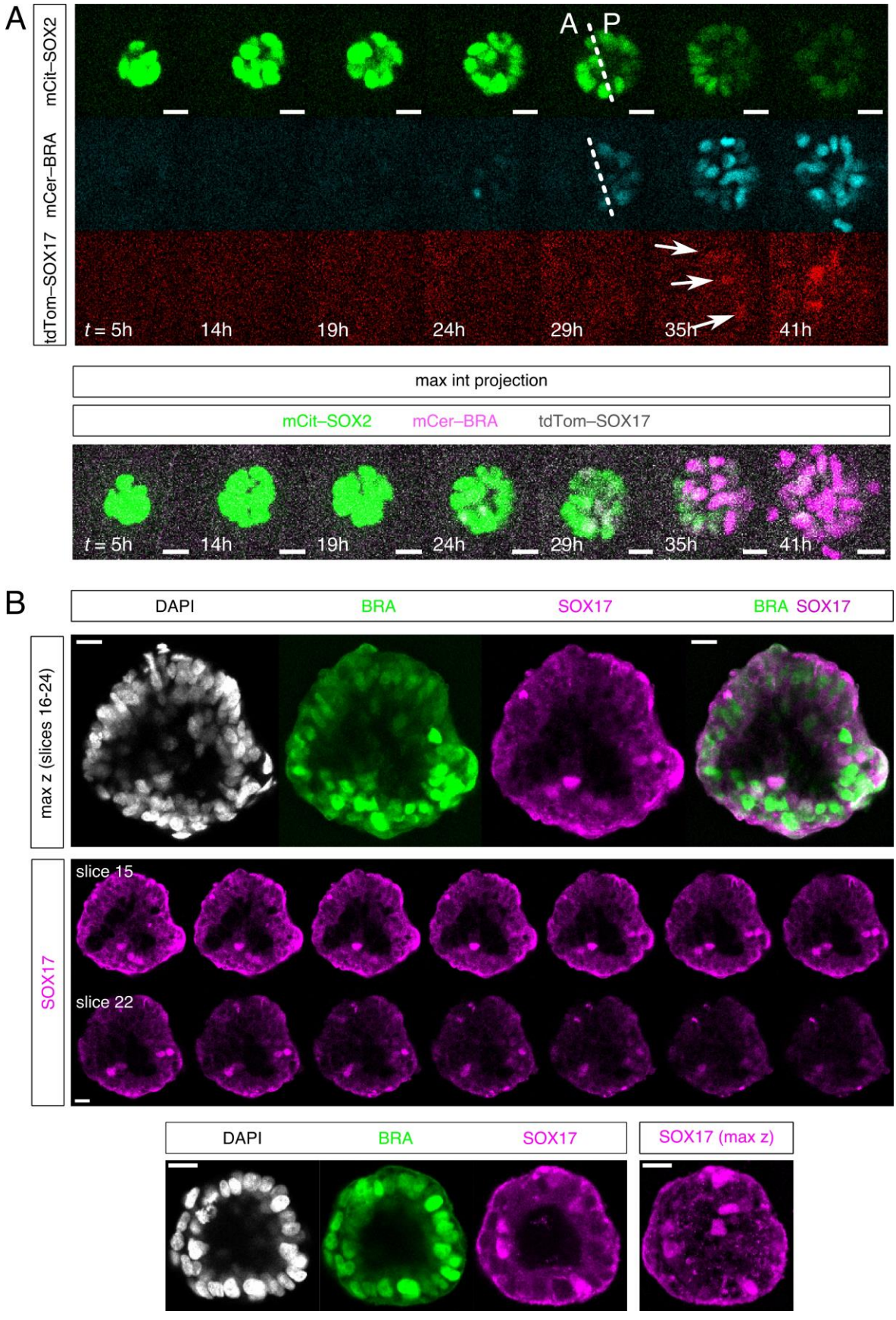
(A-B) Further examples of SNAIL expression, co-stained with SOX2 and NANOG or N-CAD (11 individual 3D colonies with the SNAIL/SOX2 combination, of which 9 were also co-stained for NANOG). (C) Further examples of COLL IV downregulation. *Left*: thinning of COLL IV, co-expressed with SAIL and N-CAD; *right*: partial disappearance of COLL IV in the SNAIL+ and N-CAD+ region. The example on the right has prominent EMT marked by cells leaving the tissue (5 individual 3D colonies with the SNAIL/COLL IV/N-CAD combination). (D) Further example of E-CAD downregulation and N-CAD expression in the BRA-rich region (3 individual 3D colonies with the BRA/E-CAD/N-CAD combination). All scale bars, 20 μm.



Supplementary Figure 5

Dynamics of patterning at 1 ng/mL BMP4.

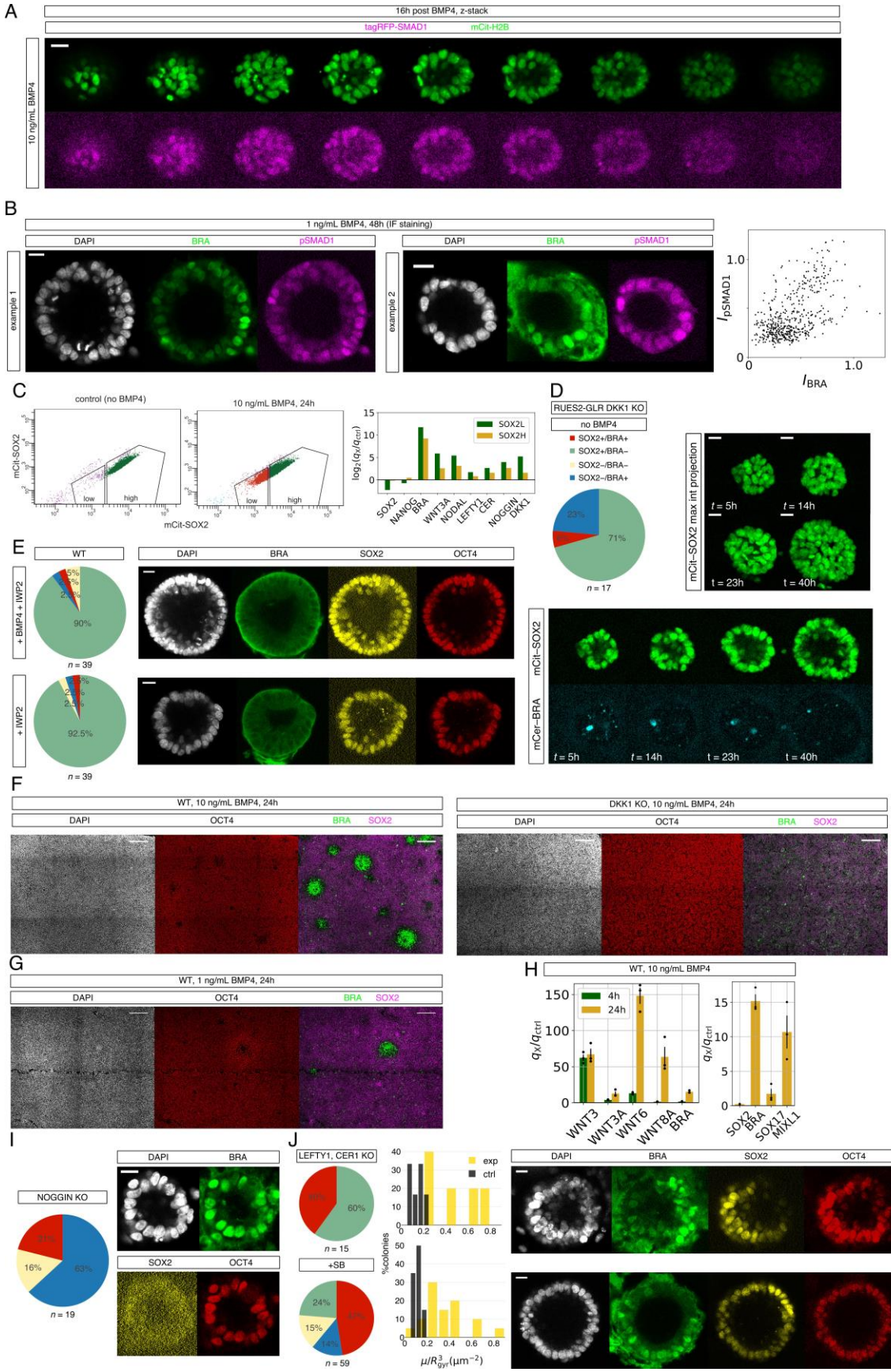
(A-B) Symmetry-breaking and expression quantification for the pure SOX2+ (*n*=23 individual 3D colonies imaged live) and pure BRA+ (*n*=13 individual 3D colonies imaged live) population. Cell segmentation and analysis done on two out of three independent live imaging experiments. (A) Volume uncorrected AP dipole for the SOX2+/BRA− and the BRA+/SOX2− population, compared to the BRA+/SOX2+ population. Only the BRA+/SOX2+ population shows dipole activation. (B) Protein expression analysis, measured as BRA+ or SOX2+ voxel number, N^+, normalized by the maximum number of expressing voxels of individual colony, N^+_{max}. The SOX2+ voxel number initially increases due to growth of the colony and finally declines to background. Red line copied from data in Figure 5B for comparison, thick lines are mean, shadows are SE. (C) Full trajectory of live imaging of symmetry breaking of the example shown in Figure 5A.


A
mCit–SOX2
mCer–BRA
tdTom–SOX17
A
P
t = 5h
14h
19h
24h
29h
35h
41h
max int projection
mCit–SOX2
mCer–BRA
tdTom–SOX17
t = 5h
14h
19h
24h
29h
35h
41h
B
DAPI
BRA
SOX17
BRA SOX17
max z (slices 16-24)
SOX17
slice 15
slice 22
DAPI
BRA
SOX17
SOX17 (max z)

Supplementary Figure 6

Dynamics of symmetry breaking and SOX17 activation.

(A) Live-cell imaging of 3D epiblast models composed of the RUES2-GLR line in all three channels, showing the activation of SOX17 (observed in 10 out of 11 BRA+/SOX2+ individual 3D colonies imaged live, 19 total imaged colonies in all three channels). Showing a different example from Figure 5. Arrows point to SOX17+ cells. Bottom row is an overlay of all three markers above. Confocal slices among different channels are different, but same for each channel. (B) IF staining for SOX17 48h after 1 ng/mL BMP4 treatment, showing two different examples from the partial BRA+ population. There is sporadic SOX17 activation seen in examples with prominent EMT (SOX17 observed in 6 out of 8 of imaged 3D colonies with the BRA/SOX17 combination). Shown either confocal slice, partial, or full max *z* projections for clarity, so to show all SOX17+ cells. All scale bars, 20 μm.


A
16h post BMP4, z-stack
tagRFP-SMAD1
mCit-H2B
10 ng/mL BMP4
B
1 ng/mL BMP4, 48h (IF staining)
DAPI
BRA
pSMAD1
example 1
example 2
I_{pSMAD1}
I_{BRA}
C
control (no BMP4)
10 ng/mL BMP4, 24h
mCit-SOX2
low
high
$\log_2(q_X/q_{ctrl})$
SOX2L
SOX2H
SOX2
NANOG
BRA
WNT3A
NODAL
LEFTY1
CER
NOGGIN
DKK1
D
RUES2-GLR DKK1 KO
no BMP4
SOX2+/BRA+
SOX2+/BRA−
SOX2−/BRA−
SOX2−/BRA+
23%
71%
n = 17
mCit–SOX2 max int projection
t = 5h
t = 14h
t = 23h
t = 40h
E
WT
DAPI
BRA
SOX2
OCT4
+ BMP4 + IWP2
+ IWP2
90%
92.5%
n = 39
mCit–SOX2
mCer–BRA
F
WT, 10 ng/mL BMP4, 24h
DKK1 KO, 10 ng/mL BMP4, 24h
G
WT, 1 ng/mL BMP4, 24h
H
WT, 10 ng/mL BMP4
4h
24h
q_X/q_{ctrl}
WNT3
WNT3A
WNT6
WNT8A
BRA
SOX2
BRA
SOX17
MIXL1
I
NOGGIN KO
21%
16%
63%
n = 19
J
LEFTY1, CER1 KO
40%
60%
n = 15
+SB
24%
15%
n = 59
exp
ctrl
%colonies
$\mu/R^3_{gyr}(\mu m^{-2})$

Supplementary Figure 7

Further evidence for the mechanism of symmetry breaking in the model epiblast.

(A) *Z*-stack of epiblast model composed of the SMAD1 reporter line stimulated with 10 ng/mL BMP4 at 16h (from the same sample as in Figure 6 with 16 individual 3D colonies). (B) IF staining two days after 1 ng/mL BMP4, showing examples with partial BRA expression and a heterogeneous pSMAD1 expression, with no apparent correlation between pSMAD1 and BRA expression. Plot shows no strong correlation between pSMAD1 and BRA IF intensity levels in partial BRA+ examples (*n*=6 imaged 3D colonies showing partial BRA expression). (C) FACS sorting dot plot of dissociated RUES2-GLR epithelium grown on filters under pluripotency conditions (*left*) and after 24h of 10 ng/mL BMP4 (*center*). Right: gene expression analysis from Figures 7B-C of sorted cells compared (in log2 fold change) to untreated cells. The measurement was done by pooling *n*=10 individual samples (separate filters) and compared to *n*=5 untreated samples, thus the variance was not measured. (D) Monitoring pluripotency maintenance of epiblast models made from the RUES2-GLR DKK1 KO line. Pie chart shows end-point patterning statistics. Snapshots show an example of live-cell imaging of the majority population under pluripotency conditions (*n*=12 3D colonies for the majority population). Scale bar, 20 μm. (E) Patterning statistics and examples of WT RUES2 epiblast models stimulated with 2 μM IWP2 or 2 μM IWR1-endo along with 1 ng/mL BMP4 for two days. For each case, a representative example of a colony from the majority population is shown (i.e., no differentiation). In both cases, *n*=39 3D colonies were imaged. Scale bar, 20 μm. (F) Patterning of filter cultures, comparing RUES2 WT (3 independent experiments) and RUES2 DKK1 KO (2 independent experiments), stimulated for 24h with 10 ng/mL BMP4 from the bottom compartment. Scale bar, 200 μm. (G) Filter colony stimulated with 1 ng/mL from the bottom compartment for 24h (2 independent experiments). (H) Gene expression analysis from bulk cells at 4h and 24h, of filters stimulated with 10 ng/mL from the bottom compartment (*n*=3 independent experiments, box centered at mean, error is SE, each dot represents a mean of three technical replicas). (I) Differentiation of RUES2 NOGGIN KO epiblast models with 1 ng/mL BMP4 for two days. *Left*: end-point statistics; *right*: IF stain of an example 3D colony from the majority population (total *n*=19 imaged 3D colonies). Scale bar, 20 μm. (J) Checking for NODAL influence on symmetry breaking. Shown are stimulation with 1 ng/mL BMP4 for two days of RUES2 LEFTY1 & CERBERUS 1 (CER1) KO epiblast models (top) and the WT with the NODAL inhibitor SB (bottom). Pie charts show end-point statistics (*n* represents total imaged 3D colonies) and AP dipole quantification (the measured and the random population are statistically different, *p*=0.008 calculated with the two-sided Mann-Whitney U test; *n*=6 and *n*=20 segmented BRA+/SOX2+ colonies for the LEFTY1/CER1 KO and WT with SB cases, respectively); *right*: IF stains of symmetry-broken 3D colonies. All scale bars, 20 μm.

Supplementary Table 1

A list of primary and secondary antibodies used.

Supplementary Table 2

Numerical source data for figures.

Supplementary Video 1

Growth of RUES2-GLR model epiblasts under pluripotency conditions. Showing three examples representative of 39 model epiblasts that remained SOX2+ out of 48 total imaged independent model epiblasts. Scale bars, 20 μm.

Supplementary Video 2

Live-cell imaging of epiblast models under low BMP4 concentration (0.1 ng/mL).

Showing two examples representative of the majority population from 13 imaged 3D colonies. Scale bars, 20 μm.

Supplementary Video 3

Live-cell imaging of epiblast models under high BMP4 concentration (5 ng/mL).

Showing three examples representative of majority population from 28 imaged 3D colonies. Scale bars, 20 μm.

Supplementary Video 4

Live-cell imaging of epiblast models at 1 ng/mL BMP4.

Shown is an example from the BRA+/SOX2+ population (from data presented in Figures 2 and S2), with BRA activation at ~22h and is restricted to one area of the colony, following by its expansion. EMT (cell escape) can be seen at 34h. The experiment ends at around ~40h with cells escaping into the matrix. The same example as in Figure S5C. BRA is magenta in the overlays for clarity. MIP, maximum intensity projection. A total of 92 individual 3D colonies were imaged live of which 43 were classified as BRA+/SOX2+, of which 34 were selected for segmentation and analysis due to higher image quality. Scale bar, 20 μm.

Supplementary Video 5

Live-cell imaging of epiblast models at 1 ng/mL BMP4.

Shown are four examples from the BRA+/SOX2+ population. BRA is magenta in the overlays for clarity. MIP, maximum intensity projection. The experiment ends at around ~40h with cells escaping into the matrix. A total of 92 individual 3D colonies were imaged live of which 43 were classified as BRA+/SOX2+, of which 34 were selected for segmentation and analysis due to higher image quality. Scale bars, 20 μm.

Supplementary Video 6

Live-cell imaging of epiblast models at 1 ng/mL BMP4 showing SOX17 activation following symmetry breaking.

Showing two examples from the BRA+/SOX2+ population. SOX17 activation observed in 10 out of 11 BRA+/SOX2+ 3D colonies, 19 total imaged colonies using all three channels BRA. is magenta and SOX17 in gray scale in the overlays for clarity. MIP, maximum intensity projection. Scale bars, 20 μm.

Supplementary Video 7

Live-cell imaging of epiblast models composed of RUES2 with fluorescently tagged SMAD1.

Top: 1 ng/mL BMP4; *center*: 10 ng/mL BMP4; *bottom*: control, untreated cells. Sampling: no BMP4, 6 individual 3D colonies; 1 ng/mL BMP4, 24 individual 3D colonies; and 10 ng/mL BMP4, 6 individual 3D colonies. Green: H2B; magenta: tagRFP-SMAD1. Scale bars, 20 μm.

Supplementary Video 8

Live-cell imaging of epiblast models stimulated with 1 ng/mL BMP4, comparing the WT (RUES2-GLR) with RUES2-GLR DKK1 KO lines.

The majority population in the RUES2-GLR DKK1 case is the one that uniformly deactivates SOX2 and activates BRA, i.e., BRA+/SOX2− (16 imaged individual 3D colonies).