Figure S1, related to Figure 1

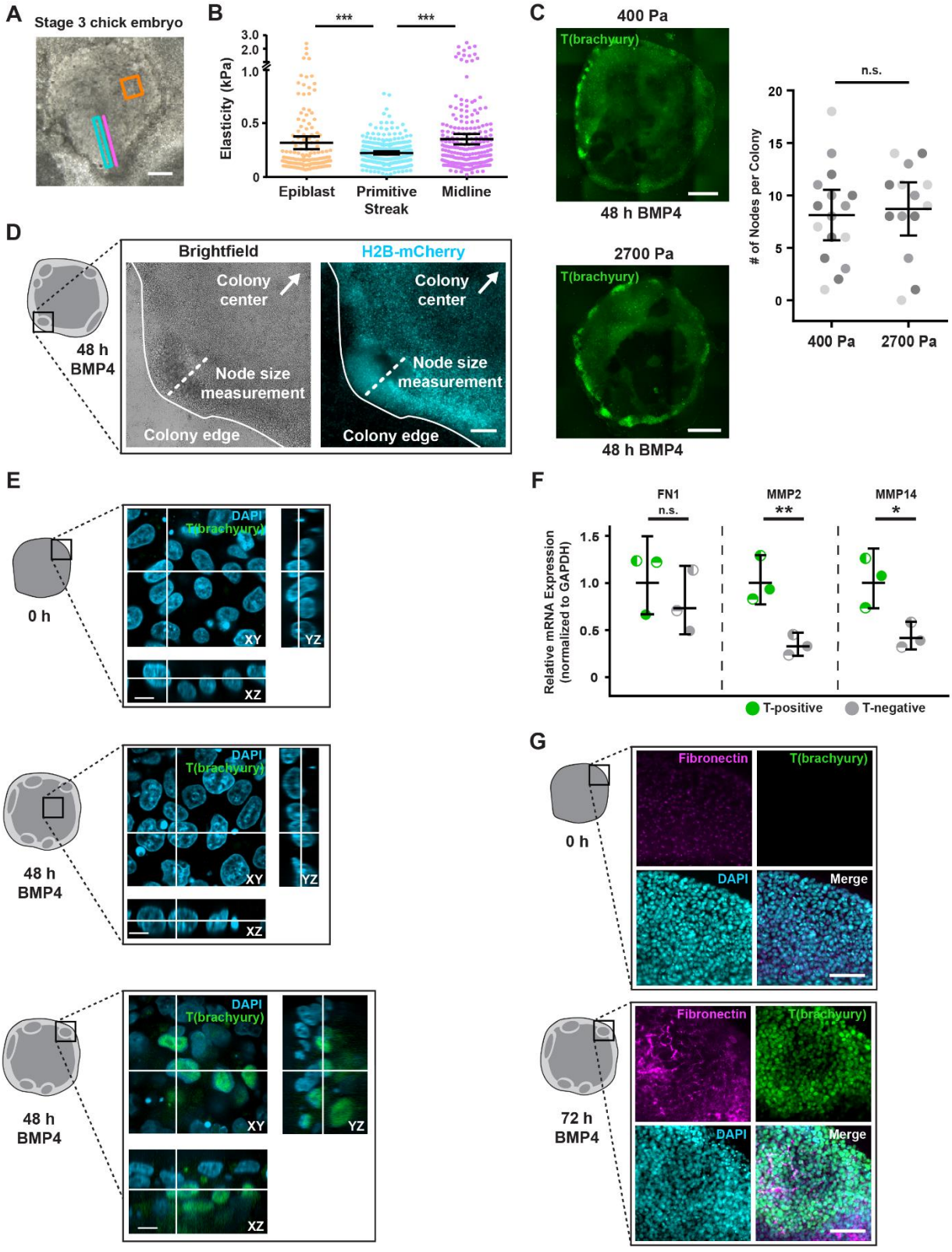


Figure S1, related to Figure 1: Compliance of the gastrulation-stage chicken embryo and additional characterization of the hESC "gastrulation-like" nodes.

(A) Representative brightfield image of an HH stage 3 chicken embryo extracted and prepared for atomic force microscopy measurements. Colored rectangles and line on the brightfield image indicate regions within the embryo where measurements were taken, corresponding to the data presented in (B). Scale bar = 500 μm.

(B) Atomic force microscopy measurements of HH stage 3 chicken embryos, grouped by regions of the embryo within which the measurements were taken. Data was collected from embryos prepared on four separate days (N = 4, 2, 1, and 3 embryos measured each day). Epiblast n = 140, primitive streak n = 260, and midline n = 236 elasticity measurements.

(C) Representative immunofluorescent images showing T(brachyury) expression within "gastrulation-like" nodes 48 h following BMP4 stimulation of hESC colonies cultured on 400 Pa and 2700 Pa polyacrylamide hydrogels. Scatter plot (right) displaying quantification of the number of "gastrulation-like" nodes observed per hESC colony at 48 h BMP4 on 400 Pa and 2700 Pa hydrogels. n = 16 (4, 6, 6) colonies from 400 Pa gels and n = 14 (5, 4, 5) colonies from 2700 Pa gels. Scale bars = 500 μm.

(D) Representative images of an H2B-mCherry hESC colony after 48 h BMP4 stimulation. The dashed white line indicates the size measurement of the identified "gastrulation-like" node taken along the radial axis of the colony. Scale bar = 200 μm.

(E) Representative spinning-disk confocal z-stack reconstructions before and 48 h after BMP4 stimulation showing T(brachyury) expression and nuclei within pluripotent colonies (top), the central regions of BMP4 treated colonies (middle), and within the "gastrulation-like" nodes (bottom). Scale bars = 10 μm.

(F) Relative mRNA expression levels of fibronectin and matrix metalloproteinases (MMPs) in T-positive and T-negative cells isolated from geometrically-confined hESC colonies on compliant hydrogels following 36 h of BMP4 stimulation.

(G) Representative immunofluorescent images of fibronectin, nuclei, and composite in the "gastrulation-like" nodes prior to and after 72 h BMP4. Scale bars = 100 μm.

For (B), (C), (F): Line and bars represent mean ± 95% CI. Rectangles on colony cartoons indicate imaged regions. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. kPa = kilopascals. n.s. = not significant.

Figure S2, related to Figure 2

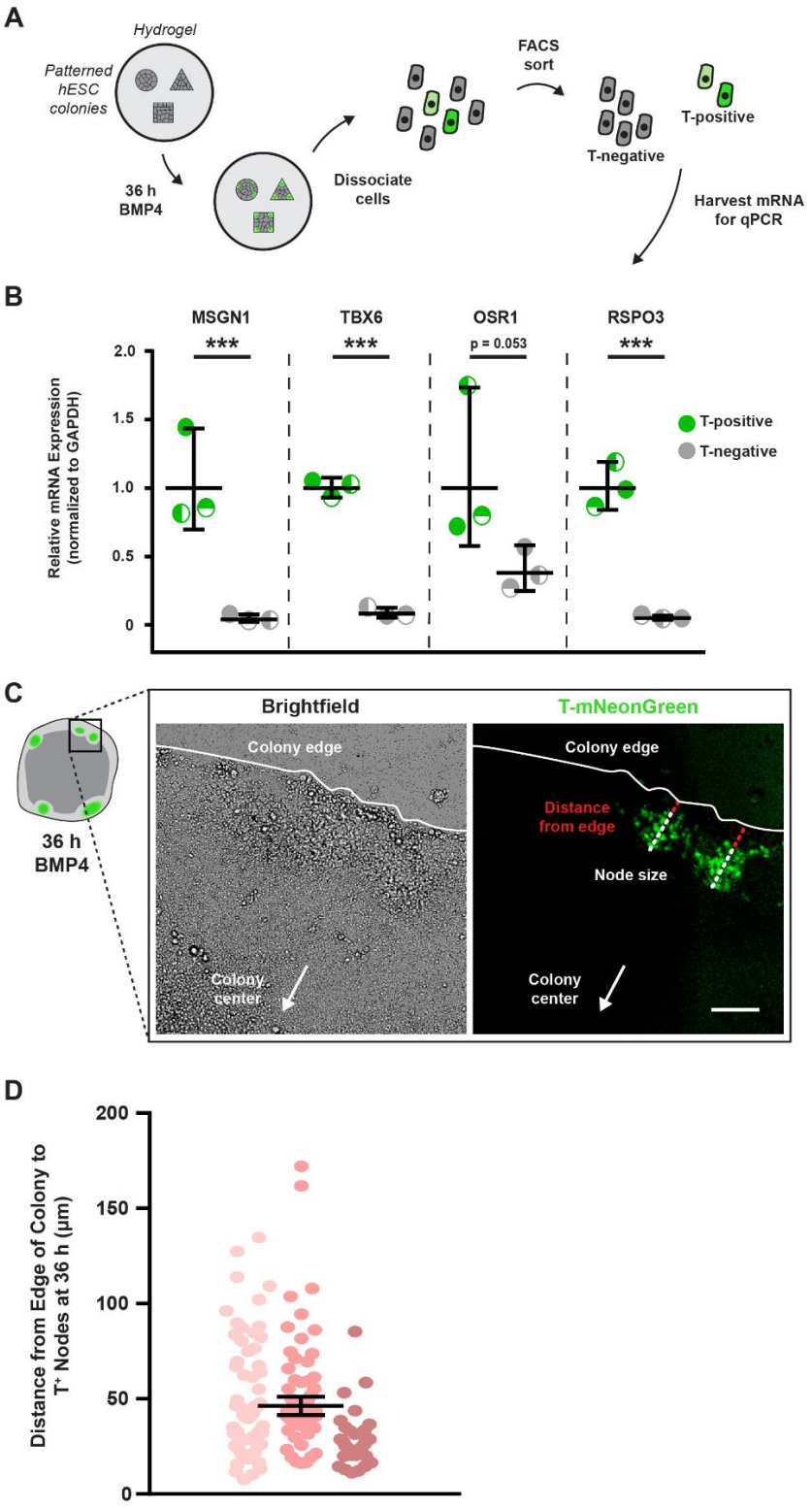


Figure S2, related to Figure 2: Additional characterization of the T-mNeonGreen hESCs.

(A) Cartoon of isolation protocol used to compare gene expression between T-mNeonGreen-positive and T-mNeonGreen-negative cells following 36 h of BMP4 stimulation.

(B) Relative mRNA expression levels of direct transcriptional targets of T(brachyury) in T-positive and T-negative cells.

(C) Representative images of a T-mNeonGreen hESC colony after 36 h BMP4 stimulation. The dashed white lines indicate the size measurement of the identified "gastrulation-like" nodes taken along the radial axis of the colony. The dashed red lines indicate the "distance from edge" measurements, also taken along the radial axis of the colony. Scale bar = 100 μm.

(D) Scatter plot of the "distance from edge" measurements taken for "gastrulation-like" nodes identified after 36 h of BMP4 stimulation. n = 15 (5, 6, 4) colonies. Data points from each experiment are represented by different shades of red.

For (B), (D): Line and bars represent mean ± 95% CI. ***$p < 0.001$. MSGN1 = Mesogenin 1, TBX6 = T-Box Transcription Factor 6, OSR1 = Odd-Skipped Related Transcription Factor 1, RSPO3 = R-Spondin 3.

Figure S3, related to Figure 3

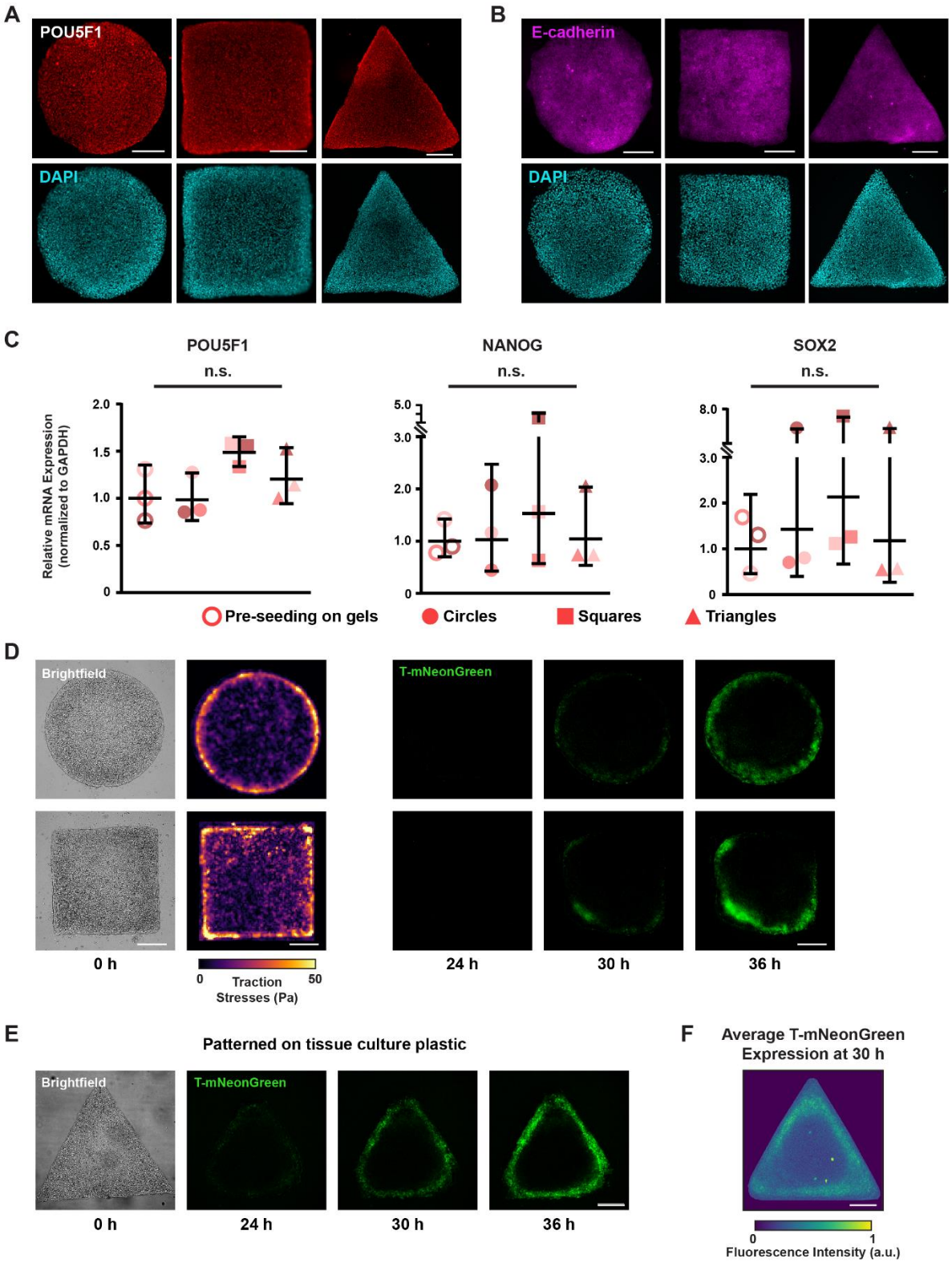


Figure S3, related to Figure 3: Geometrically-confined hESCs remain pluripotent in maintenance conditions and additional data illustrating the relationship between regions of high tension and mesoderm specification.

(A) Representative immunofluorescent images of POU5F1 and nuclei in geometrically-confined colonies of hESCs on compliant hydrogels in maintenance conditions.

(B) Representative immunofluorescent images of E-cadherin and nuclei in geometrically-confined colonies of hESCs on compliant hydrogels in maintenance conditions.

(C) Relative mRNA expression levels of pluripotency genes in geometrically-confined colonies of hESCs on compliant hydrogels in maintenance conditions, relative to levels prior to seeding on hydrogels. Line and bars represent mean ± 95% CI.

(D) Representative brightfield images and corresponding traction stress maps of geometrically-confined circle and square hESC colonies prior to BMP4 stimulation, and time-lapse images of T-mNeonGreen expression in the same colonies after BMP4 stimulation.

(E) Representative brightfield image and time-lapse T-mNeonGreen images of a geometrically-confined triangle hESC colony on tissue culture plastic before and after BMP4 stimulation.

(F) Normalized average intensity map of T-mNeonGreen expression within the geometrically-confined triangle hESC colonies on tissue culture plastic 30 h after BMP4 stimulation. n = 28 (7, 9, 12) colonies.

All scale bars = 250 μm. n.s. = not significant.

Figure S4, related to Figure 3

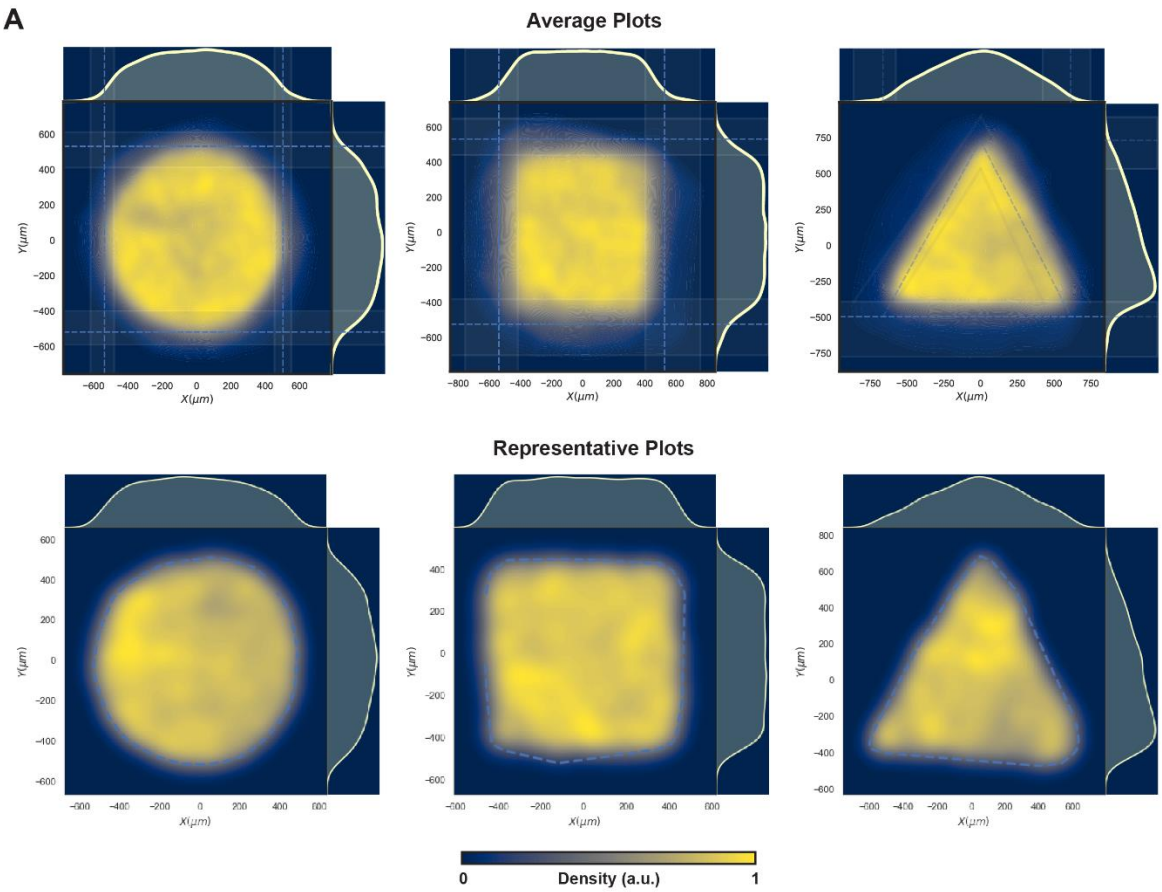


Figure S4, related to Figure 3: Geometrically-confined hESC colonies exhibit uniform cell density and a concentration gradient of apically-secreted inhibitors is insufficient to explain observed mesoderm specification patterns.

(A) Average (top) and representative (bottom) kernel density estimator (KDE) plots of cell density within geometrically-confined hESC colonies. For average plots, dashed blue lines represent the average X and Y positions of colony boundaries, transparent shaded regions indicate the range. For representative plots, dashed blue lines indicate the colony boundary as determined by brightfield images. n = 29 (6, 5, 18) circle colonies, n = 29 (7, 3, 19) square colonies, and n = 24 (6, 4, 14) triangle colonies.

(B) Representative brightfield and immunofluorescent images of nuclei and T-mNeonGreen expression for geometrically-confined triangle-Pac-Man hESC colonies on tissue culture plastic 36 h after BMP4 stimulation.

(C) Normalized average intensity map of T-mNeonGreen expression within the geometrically-confined triangle-Pac-Man hESC colonies on tissue culture plastic 36 h after BMP4 stimulation. n = 23 (8, 5, 10) colonies.

All scale bars = 250 μm.

Figure S5, related to Figure 4

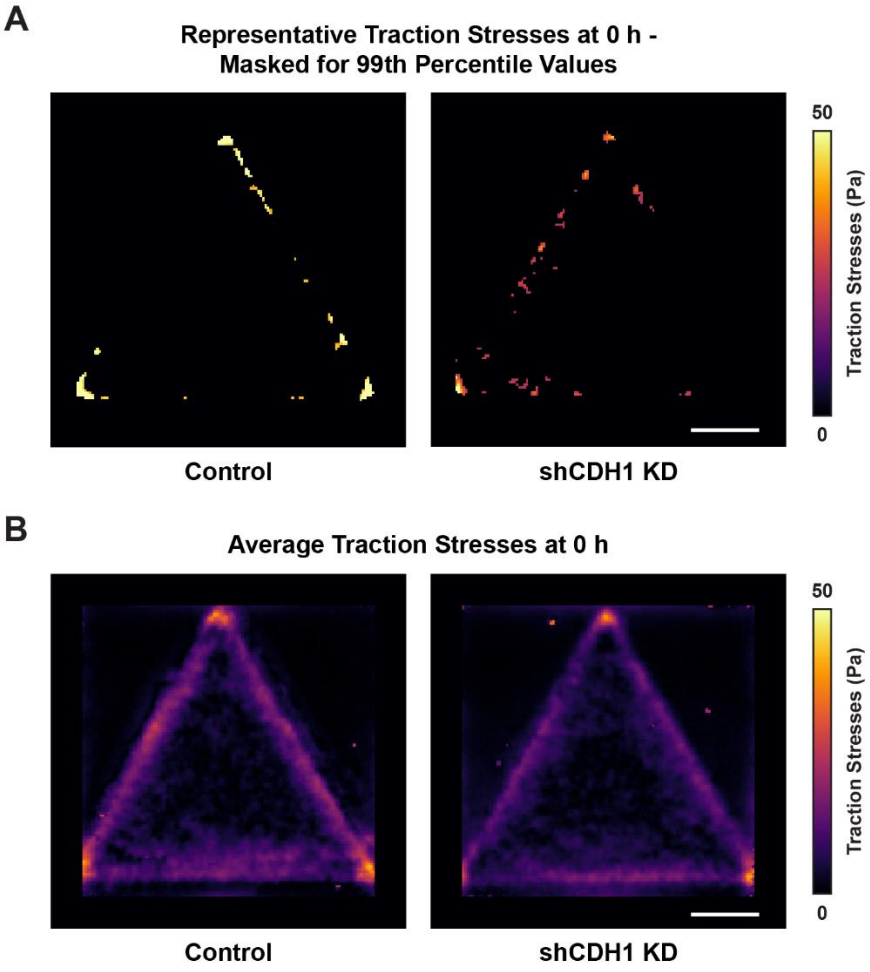


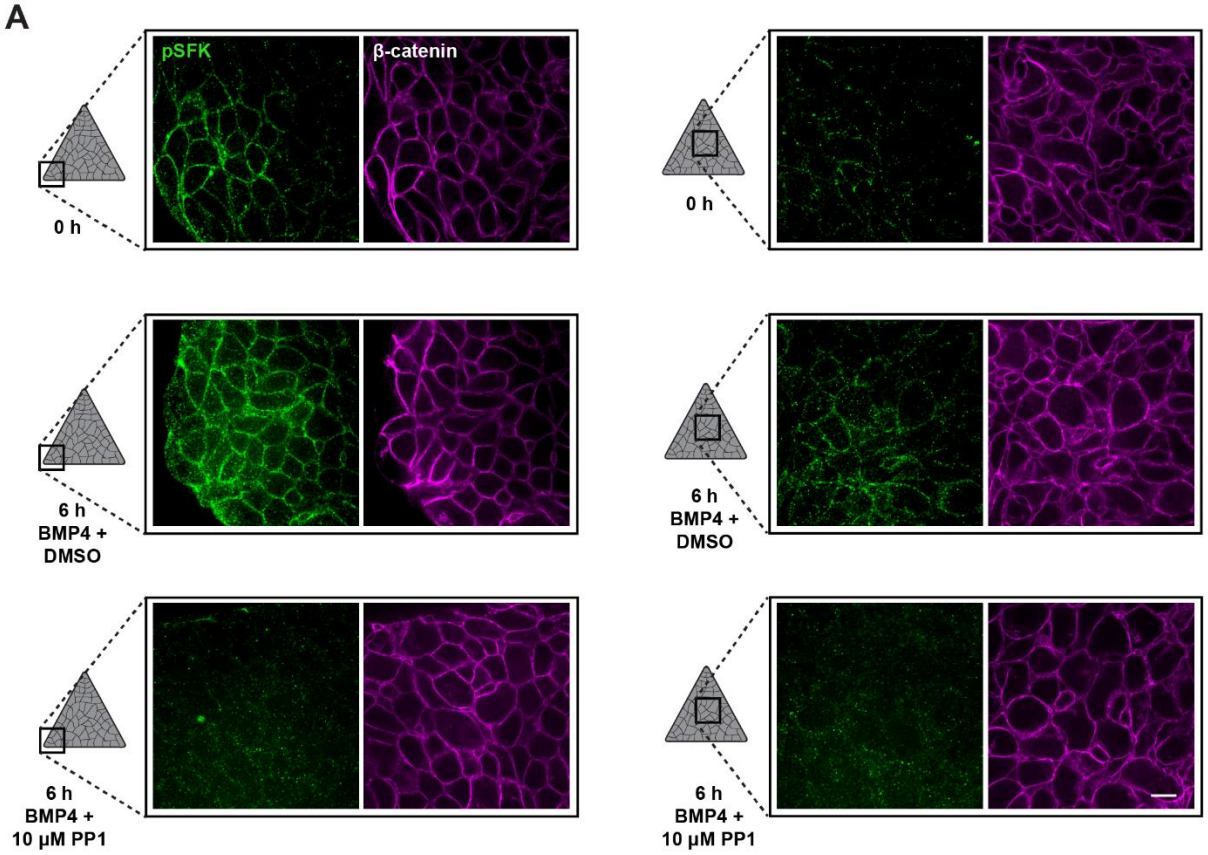
Figure S5, related to Figure 4: Additional quantification of traction stresses in shCDH1 control and knockdown conditions.

(A) Representative maps of average traction stresses measured within geometrically-confined triangle hESC colonies with and without shCDH1 knockdown, masked to only display traction stresses greater than the 99^{th} percentile value for each map. The unmasked representative maps are shown in Figure 4D. Note that the voxels corresponding to the top 1% of traction stress values are primarily located in the corners of each map.

(B) Maps of average traction stresses measured within geometrically-confined triangle hESC colonies with and without shCDH1 knockdown. n = 13 (3, 2, 8) colonies from each condition.

Scale bars = 250 μm.

Figure S6, related to Figure 6



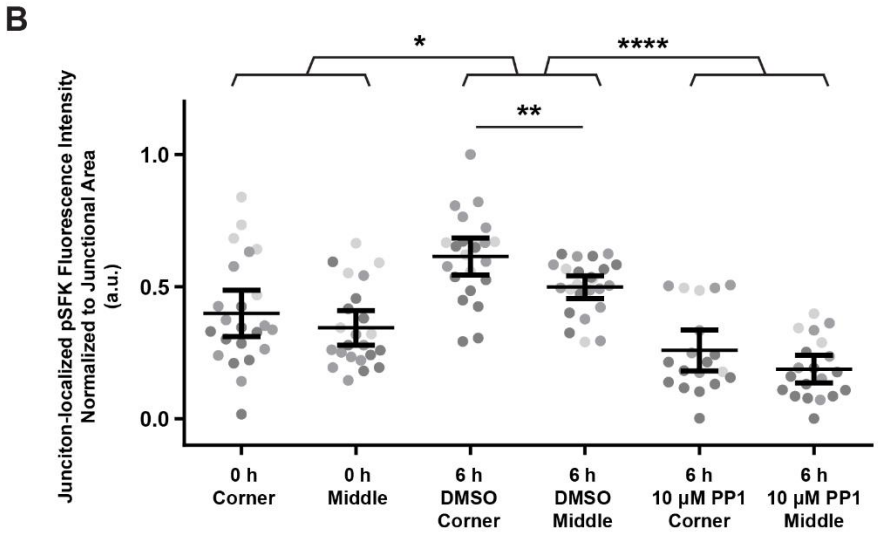


Figure S6, related to Figure 6: BMP4 upregulates junction-localized phosphorylated Src-family kinases specifically in high-tension regions of hESC colonies.

(A) Representative immunofluorescent images of phosphorylated Src-family kinases and β-catenin in the corner and middle of typical geometrically-confined triangle hESC colonies prior to and after 6 h stimulation with BMP4 plus either vehicle (DMSO) or Src inhibitor (PP1; 10 μM). Rectangles on colony cartoons indicate imaged regions. Scale bar = 10 μm.

(B) Scatter plot quantifying junction-localized phosphorylated Src-family kinases fluorescence intensity, normalized to junctional area measured using β-catenin fluorescence in the corner and middle of geometrically-confined triangle hESC colonies prior to and after 6 h stimulation with BMP4 plus either vehicle (DMSO) or Src inhibitor (PP1; 10 μM).

*$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$. a.u. = arbitrary units.

Figure S7, related to Figure 7

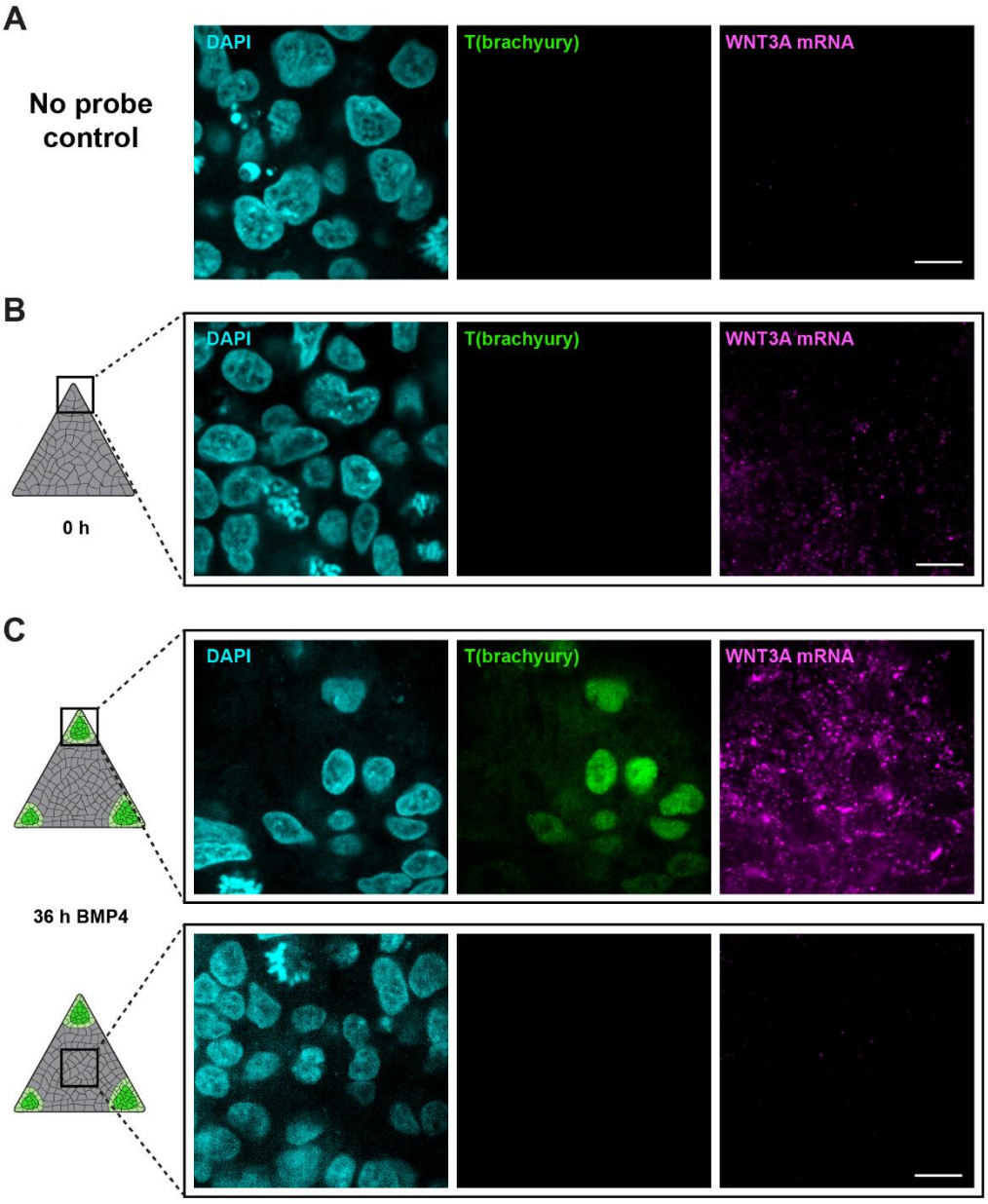


Figure S7, related to Figure 7: WNT3A *in situ* hybridization via hybridization chain reaction before and after BMP4 treatment.

(A) Representative immunofluorescent images of nuclei, immune-detected T(brachyury) protein, and *in-situ*-detected WNT3A mRNA in geometrically-confined triangle hESC colonies with no split initiator probes targeting WNT3A prior to amplification with fluorescent probes.

(B) Same as in (A), but including split initiator probes in geometrically-confined triangle hESC colonies prior to stimulation with BMP4.

(C) Same as in (B), but following 36 h of BMP4 stimulation.

Rectangles on colony cartoons indicate imaged regions. All scale bars = 10 μm.

Table S1, related to STAR Methods, Quantitative PCR (qPCR): Primers used for qPCR.

Gene	Forward Primer Sequence, 5' to 3'	Reverse Primer Sequence, 5' to 3'
CDH1	CGGCCTGAAGTGACTCGTA	GCCGCTTTCAGATTTTCATC
FN1	ACAGCTCATCCGTGGTTGTA	TCTTGGTGGGCTGACATTCT
GAPDH	CAGCCTCAAGATCATCAGCA	TGTGGTCATGAGTCCTTCCA
GSC	TCTCAACCAGCTGCACTGTC	CGTTCTCCGACTCCTCTGAT
MMP2	GAAGGATGGCAAGTACGGCT	GGAATGGAAACTTGCAGGGC
MMP14	CCCAACATCTGTGACGGGAA	TTGGTTATTCCTCACCCGCC
MSGN1	TGTTGGACCCACCAGAACAC	TTGCAAAGGATGAGCCTCCC
NANOG	AGATGCCTCACACGGAGACT	AAGTGGGTTGTTTGCCTTTG
POU5F1	AGTGAGAGGCAACCTGGAGA	ACACTCGGACCACATCCTTC
OSR1	TCCCTGGTTCCCTCATGTCA	CGGATCTTCTTGCGTTGCTG
RSPO3	ACTTGCGACTGATTTCTTGGC	TCCTTGGCAGCCTTGACTAA
SNAI2	TTGTGTTTGCAAGATCTGCGG	TGCAAATGCTCTGTTGCAGT
SOX2	AGGATAAGTACACGCTGCCC	TAACTGTCCATGCGCTGGTT
TBXT	CAGCAAAGTCAAGCTCACCA	TGGACCCCCAACTCTCACTA
TBX6	GAACCGGGAGCTATGGAAGG	AGAAACAAGTAGCGGGCCTC
WNT3A	GCCCCACTCGGATACTTCTT	GAGGAATACTGTGGCCCAAC
WNT4	CCCTCATGAACCTCCACAAC	ACCTCACAGGAGCCTGACAC
WNT8A	TGTGATGGGTCAAACAATGG	TCCTTCCCCTTCTCCAAACT

Table S2, related to STAR Methods, *In Situ* Hybridization via Hybridization Chain Reaction (ISH-HCR): Split initiator hybridization probe sequences used for WNT3A ISH-HCR.

Probe	Sequence, 5' to 3'
WNT3A_1a	AAAGTCTAATCCGTCCCT TT AGTAAGAAGTATCCGAGTGGGGCCA
WNT3A_1b	CCCAGAGCCTGCTTCAGGCTGCAGA TT GCCTCTATATCTCCACTC
WNT3A_2a	AAAGTCTAATCCGTCCCT TT CAGCGACCACCAGATCGGGTAGCTG
WNT3A_2b	CAGGGAGGAATACTGTGGCCCAACA TT GCCTCTATATCTCCACTC
WNT3A_3a	AAAGTCTAATCCGTCCCT TT TAGTTCCTGCAGAAGCGGAGCTGCT
WNT3A_3b	TCGGCCACGCTGGGCATGATCTCCA TT GCCTCTATATCTCCACTC
WNT3A_4a	AAAGTCTAATCCGTCCCT TT CCGAAGATGGCCAGGCTGTCGTGGA
WNT3A_4b	TCCCTGGTAGCTTTGTCCAGCACGG TT GCCTCTATATCTCCACTC
WNT3A_5a	AAAGTCTAATCCGTCCCT TT CTGAGGCAATGGCGTGGACAAAGGC
WNT3A_5b	AGCGTGTCACTGCAAAGGCCACACC TT GCCTCTATATCTCCACTC
WNT3A_6a	AAAGTCTAATCCGTCCCT TT ACTTCCAGCCCTTGCCTGGTGAGCC
WNT3A_6b	ACTCGATGTCCTCGCTACAGCCACC TT GCCTCTATATCTCCACTC
WNT3A_7a	AAAGTCTAATCCGTCCCT TT GGTTGCGACCACCAGCATGTCTTCA
WNT3A_7b	AGGAAGTCACCGATGGCGCGGAAGT TT GCCTCTATATCTCCACTC
WNT3A_8a	AAAGTCTAATCCGTCCCT TT ACCTTGAAGTAGGTGTAGCGCGGCC
WNT3A_8b	TAGTAGACCAGGTCGCGCTCCGTGG TT GCCTCTATATCTCCACTC
WNT3A_9a	AAAGTCTAATCCGTCCCT TT AGTGGAACACGCAGCGGCACTTCTC
WNT3A_9b	ACTCCTGGCAGCTGACGTAGCAGCA TT GCCTCTATATCTCCACTC
WNT3A_10a	AAAGTCTAATCCGTCCCT TT CAGGGAAAAGCCCACCCTCAGGCAG
WNT3A_10b	CCGTTTAGGTGGGAGTCCTGCTCCA TT GCCTCTATATCTCCACTC
WNT3A_11a	AAAGTCTAATCCGTCCCT TT AGCCCTGCCTTCAGGTAGGAGTTCT
WNT3A_11b	AGAGAGGAGACACTAGCTCCAGGGA TT GCCTCTATATCTCCACTC
WNT3A_12a	AAAGTCTAATCCGTCCCT TT TGGAGCTCCGCCTCATTCAGGAGCA
WNT3A_12b	AAGCCAACGCAGAGCCCCTCCCCAT TT GCCTCTATATCTCCACTC
WNT3A_13a	AAAGTCTAATCCGTCCCT TT GCCCAATCTGTAGCCCCGCCTCTGT
WNT3A_13b	AGCCCTGTCCCACCCAAGAGAAGCC TT GCCTCTATATCTCCACTC
WNT3A_14a	AAAGTCTAATCCGTCCCT TT ACCCAGAGCCACGCCCTTACTGGGA
WNT3A_14b	GGTAGAAGCCTACCTAGTGCCCCGC TT GCCTCTATATCTCCACTC
WNT3A_15a	AAAGTCTAATCCGTCCCT TT ATAAAACCCCACTCCTAAGGAGGCG
WNT3A_15b	CCCATCCAGGAAGAAGCCTCATCCA TT GCCTCTATATCTCCACTC
WNT3A_16a	AAAGTCTAATCCGTCCCT TT AAGGAGCCTATGCAGGCCACGCCCA
WNT3A_16b	TGGTCCCAGAGAAGCCCCACCCACA TT GCCTCTATATCTCCACTC
WNT3A_17a	AAAGTCTAATCCGTCCCT TT GAAGAGTCCCACCCGCGGAGAGAAG
WNT3A_17b	GCCTTAATCAGGAGGGCGGTTCCCA TT GCCTCTATATCTCCACTC
WNT3A_18a	AAAGTCTAATCCGTCCCT TT TCTGGAGCCGGGATTCCTGCAGAAG
WNT3A_18b	AGGTGGCTGGTGGGCTGAATTTCCT TT GCCTCTATATCTCCACTC
WNT3A_19a	AAAGTCTAATCCGTCCCT TT ATGGAACCTTACAGGGGGTTGGGGA
WNT3A_19b	AACCTTCCCAGCTCGACGCAGGGGT TT GCCTCTATATCTCCACTC
WNT3A_20a	AAAGTCTAATCCGTCCCT TT TGGGTGGTCAAACCCCAGGCTGAGG
WNT3A_20b	TTTCCCCAGGTAGGGCCCCTGGTCA TT GCCTCTATATCTCCACTC