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Supplemental information

A combined human gastruloid model of cardiogenesis and neurogenesis

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Figure S1

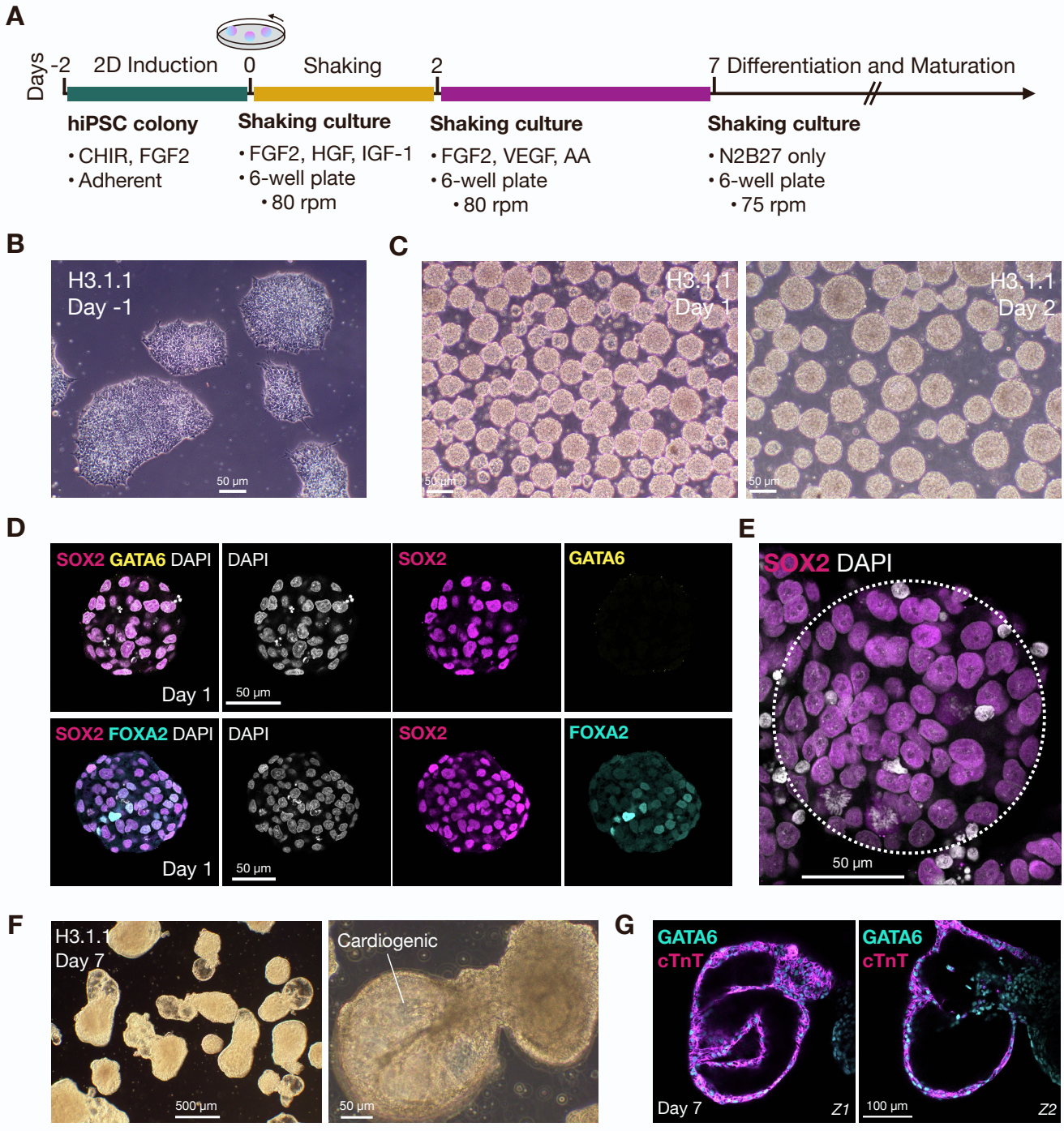
A
Days
-2
2D Induction
0
Shaking
2
7 Differentiation and Maturation
hiPSC colony
• CHIR, FGF2
• Adherent
Shaking culture
• FGF2, HGF, IGF-1
• 6-well plate
• 80 rpm
Shaking culture
• FGF2, VEGF, AA
• 6-well plate
• 80 rpm
Shaking culture
• N2B27 only
• 6-well plate
• 75 rpm
B
H3.1.1
Day -1
50 µm
C
H3.1.1
Day 1
H3.1.1
Day 2
D
SOX2 GATA6 DAPI
DAPI
SOX2
GATA6
Day 1
SOX2 FOXA2 DAPI
FOXA2
E
SOX2 DAPI
F
H3.1.1
Day 7
500 µm
Cardiogenic
G
GATA6
cTnT
Day 7
Z1
100 µm
Z2

Figure S1. Directed developmental cardiogenesis and spontaneous contractility in modified neuro-gastro-cardiac EMLOs (EMLOCs), related to Figure 1. (**A**) Overview of protocol for EMLOC gastruloid generation. Cardiogenesis was induced at 48 h post-aggregation by addition of VEGF and ascorbic acid (AA). (**B**) Phase contrast image of 2D hiPSC colonies 24 h after induction with N2B27 + CHIR/FGF2. (**C**) EMLOC suspension cultures 24 h and 48 h after dissociation and spontaneous aggregation on the orbital shaker. (**D**) Day 1 aggregates do not express GATA6 (yellow) and exhibit non-uniform FOXA2 expression (cyan) by immunofluorescence. (**E**) Immunofluorescence in a single day 1 aggregate (white dotted line) demonstrates uniform, non-polarized expression of SOX2 (magenta). Cells are counterstained with DAPI (grey). (**F**) Phase contrast images of polarized gastruloids with spontaneously contracting cardiogenic chambers (day 7). Annotated EMLOC is shown (right, see also **Movie S2**). (**G**) Two immunofluorescence Z-slices with cardiac biomarkers GATA6 (blue) and cardiac Troponin-T (cTnT, magenta) in a day 7 gastruloid demonstrating chamber cytoarchitecture. Images are representative of the general EMLOC population at these stages. Individual scale bars provided.

Figure S2

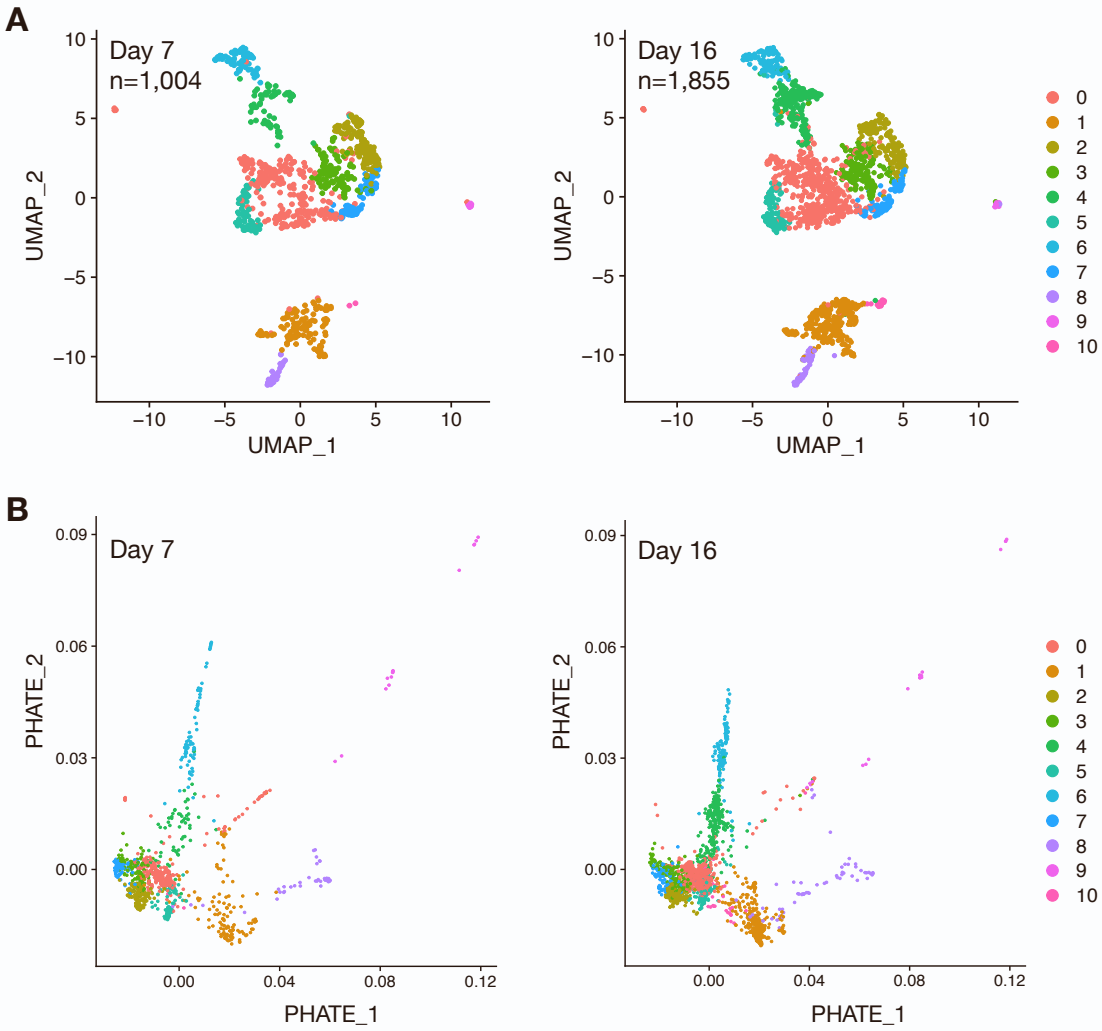
A
Day 7
n=1,004
Day 16
n=1,855
UMAP_2
UMAP_1
10
5
0
−5
−10
0
1
2
3
4
5
6
7
8
9
10
B
Day 7
Day 16
PHATE_2
PHATE_1
0.09
0.06
0.03
0.00
0.04
0.08
0.12

Figure S2. UMAP and PHATE visualization of scRNAseq for day 7 and day 16 EMLOC time points, related to Figure 1. (**A**) UMAP representation of day 7 (1,004 cells) and day 16 (1,855 cells) according to the integrated dataset with ten clusters. (**B**) PHATE representation of samples in (**A**).

Figure S3

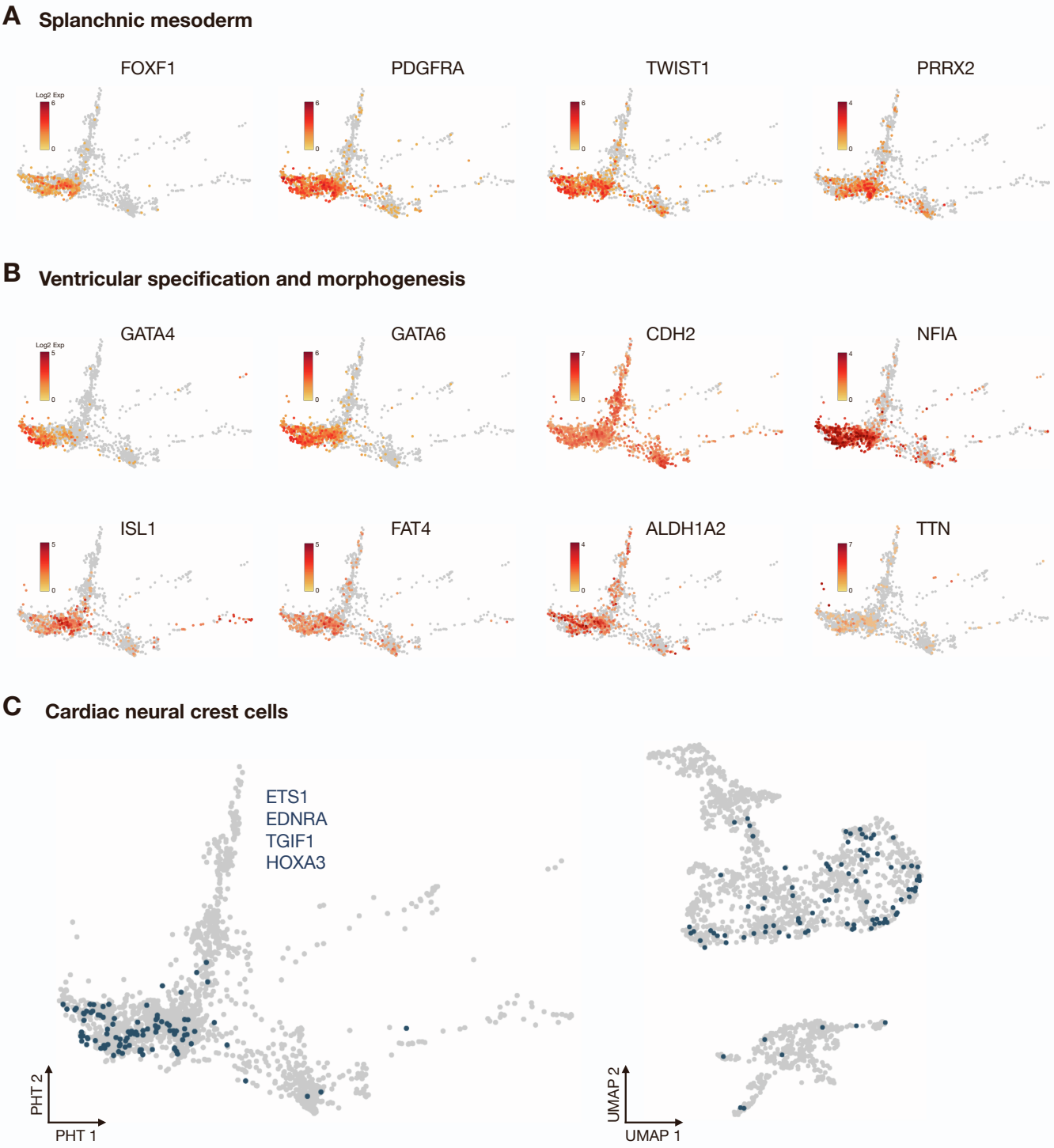
A
Splanchnic mesoderm
FOXF1
PDGFRA
TWIST1
PRRX2
Log2 Exp
B
Ventricular specification and morphogenesis
GATA4
GATA6
CDH2
NFIA
ISL1
FAT4
ALDH1A2
TTN
C
Cardiac neural crest cells
ETS1
EDNRA
TGIF1
HOXA3
PHT 2
PHT 1
UMAP 2
UMAP 1

Figure S3. Developmental features of cardiogenesis in EMLOCs, related to Figure 2. (**A**) Characteristic gene biomarkers for splanchnic mesoderm (*FOXF1, PDGFRA, TWIST1, PRRX2*). (**B**) Gene biomarkers of ventricular cell specification and morphogenesis (*GATA4, GATA6, CDH2, NFIA, ISL1, FAT, ALDH1A2, TTN*). (**C**) PHATE (left) and UMAP (right) visualization of cells co-expressing *ETS1, EDNRA, TGIF1, HOXA2* consistent with a cardiac neural crest cell phenotype.

Figure S4

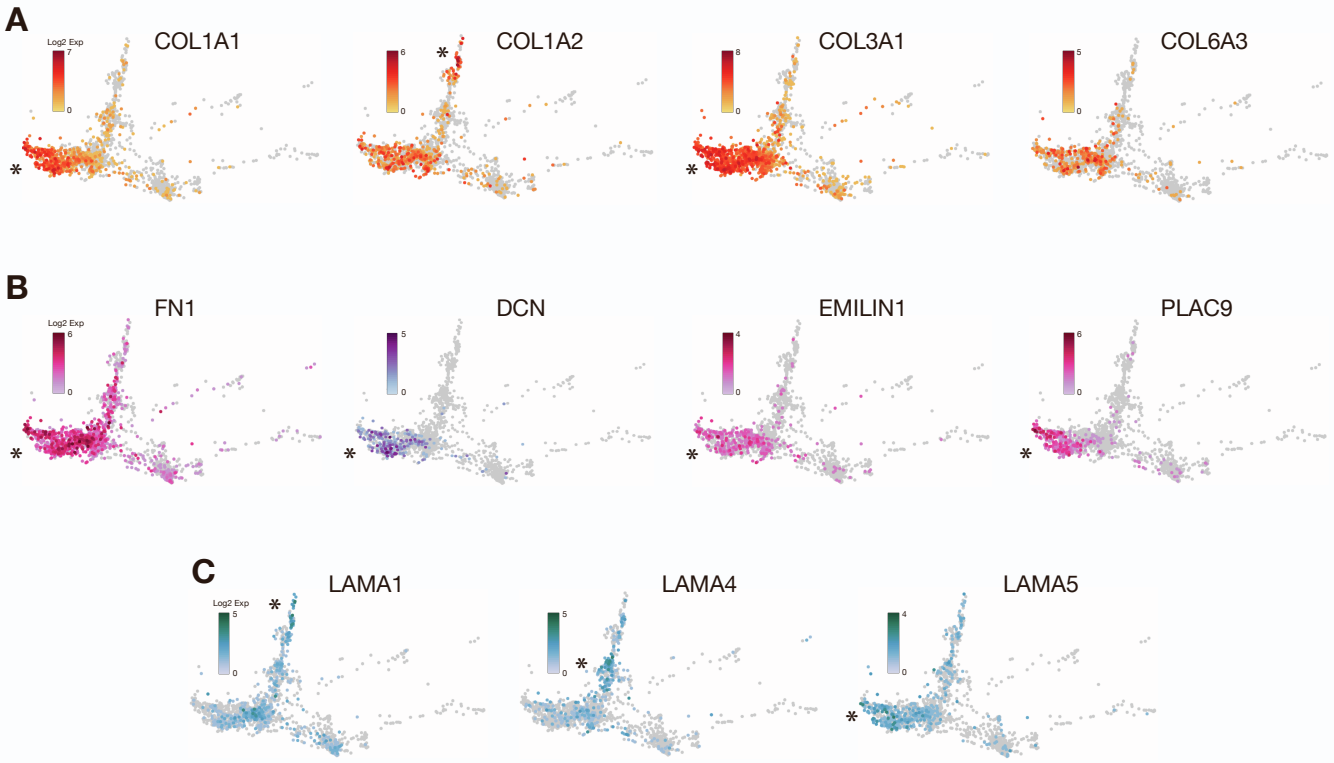
A
COL1A1
COL1A2
COL3A1
COL6A3
B
FN1
DCN
EMILIN1
PLAC9
C
LAMA1
LAMA4
LAMA5
Log2 Exp

Figure S4. EMLOCs generate the appropriate cardiac ECM milieu, related to Figure 2. (**A**) Four collagen genes (*COL1A1, COL1A2, COL3A1, COL6A3*). (**B**) Glycoprotein (*FN1*) and proteoglycan (*DCN*) genes along with *EMILIN1, PLAC9*. (**C**) Three laminin genes (*LAMA1, LAMA4, LAMA5*) show distinct distributions by PHATE. Asterisk (*) indicates region of highest expression (Cui et al., 2019).

Figure S5

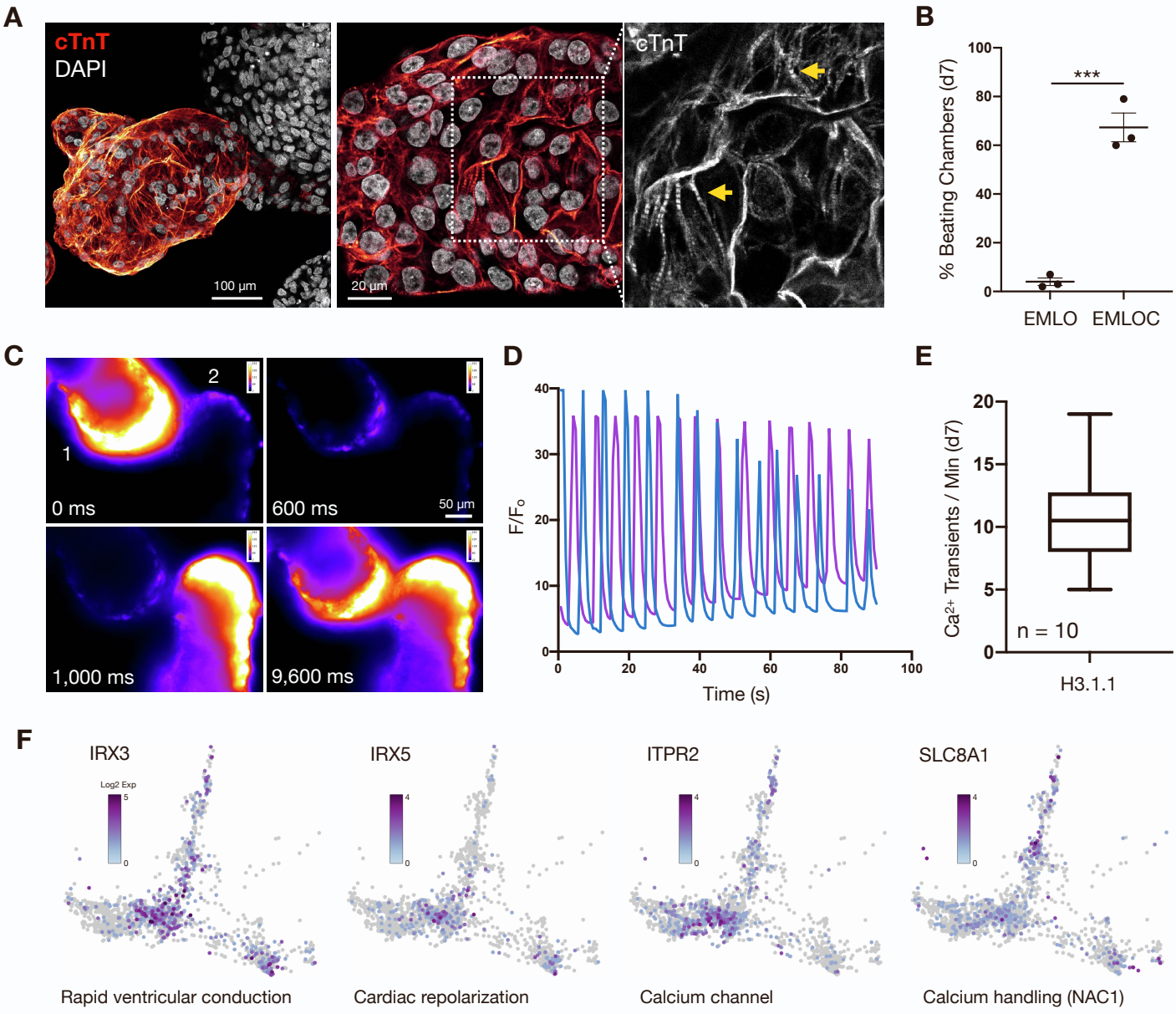
A
cTnT
DAPI
100 µm
20 µm
cTnT
B
% Beating Chambers (d7)
***
EMLO
EMLOC
C
0 ms
600 ms
50 µm
1,000 ms
9,600 ms
D
F/Fo
Time (s)
E
Ca2+ Transients / Min (d7)
n = 10
H3.1.1
F
IRX3
IRX5
ITPR2
SLC8A1
Log2 Exp
Rapid ventricular conduction
Cardiac repolarization
Calcium channel
Calcium handling (NAC1)

Figure S5. Spontaneous contractility and calcium handling in the EMLOC cardiac region, related to Figure 1. (**A**) Sarcomeres are visible with cTnT at day 7 in EMLOC formation. Progressive zoom with high magnification black and white image (right). Yellow arrows depict striations. (**B**) Percentage of EMLOCs with beating chambers versus trunk biased gastruloids formed by the original EMLO protocol (Olmsted and Paluh, 2021a, 2021b). (N = 3 repeat experiments; ***p = 0.0005, t = 10.40 df = 4 by unpaired two-tailed t-test). (**C**) Fluo-4 AM calcium imaging time course in two adjacent H3.1.1 EMLOCs. Individual scale bars provided. Images shown with fire LUT (ImageJ) and calibration bar. Individual scale bars provided. (**D**) Quantified F/F_o time series from (**C**). The two EMLOCs shown were captured in the same field and are representative of the population. (**E**) Box-and-whisker plot quantification of Fluo-4 AM Ca^{2+} transients/min in H3.1.1 day 7 EMLOCs (n = 10 EMLOCs, max = 19, min = 5, median = 10.5, q1 = 8, q3 = 12.75) from N = 2 separate differentiations. (**F**) Genes involved in rapid ventricular conduction (*IRX3*), repolarization (*IRX5*), calcium flux (*ITPR2*) and handling (*SLC8A1*/NAC1) are upregulated in EMLOCs. Day 16 scRNAseq data visualized by PHATE.

Figure S6

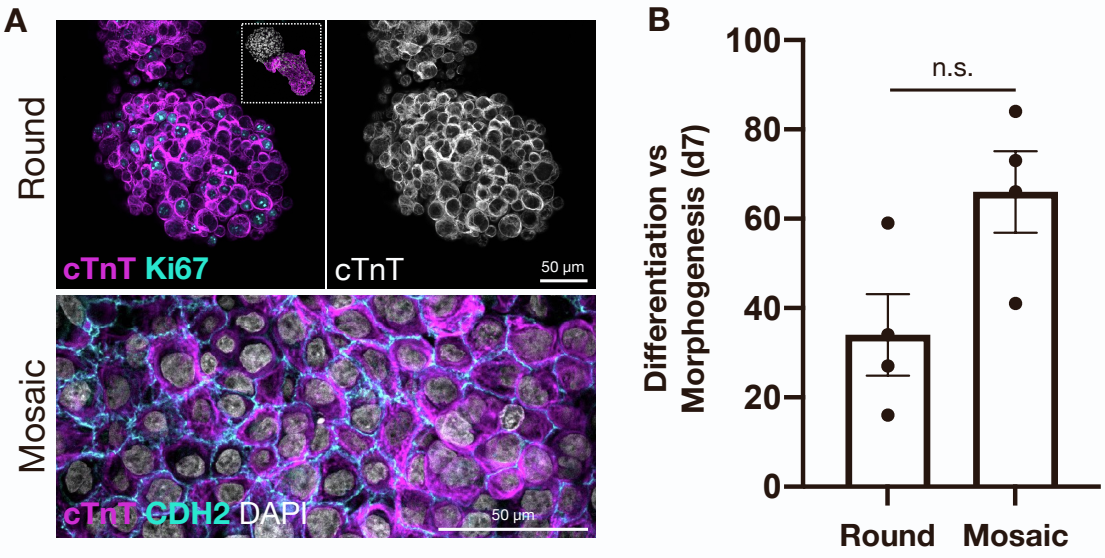
A
Round
cTnT Ki67
cTnT
50 µm
Mosaic
cTnT CDH2 DAPI
50 µm
B
Differentiation vs Morphogenesis (d7)
100
80
60
40
20
0
n.s.
Round
Mosaic

Figure S6. Assessment of cardiomyocyte morphology with differentiation and morphogenesis states, related to Figure 5. (**A**) Relative proportions of day 7 EMLOCs with rounded, proliferating cardiomyocyte progenitors versus flattened, mosaic cardiomyocyte progenitors suggests co-existing phases of differentiation and morphogenesis as previously described for mouse cardiogenesis (N = 4 replicate experiments; n.s. $p = 0.1776$, $t = 1.754$, $df = 3$ by paired two-tailed t-test). (**B**) Immunofluorescence examples of rounded, proliferating progenitors (top: cTnT, Ki67) versus flat, mosaic progenitors (bottom: cTnT, CDH2, DAPI). Left inset is example of whole EMLOC with rounded cardiomyocytes. Individual scale bars provided.

Figure S7

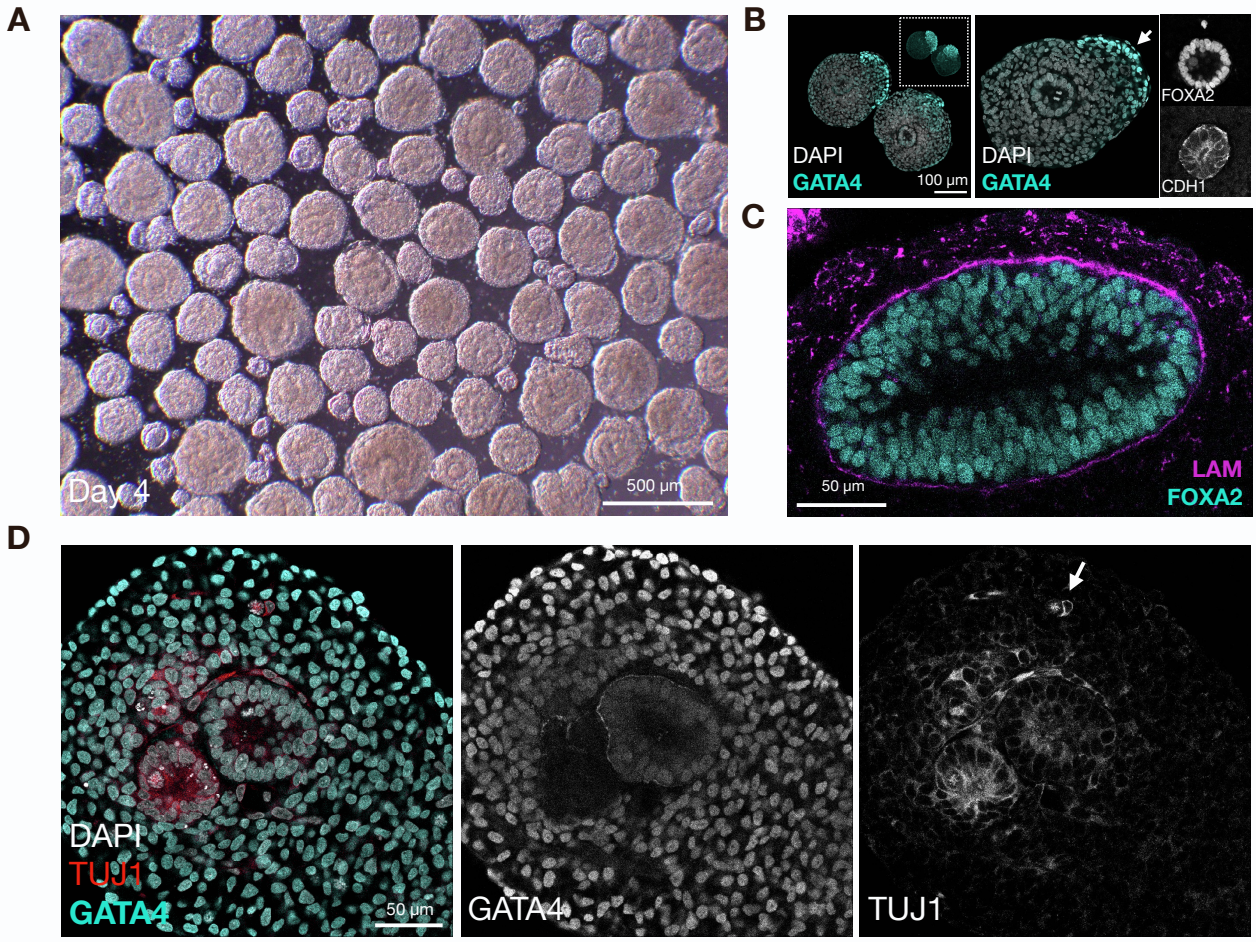
A
Day 4
500 µm
B
DAPI
GATA4
100 µm
DAPI
GATA4
FOXA2
CDH1
C
50 µm
LAM
FOXA2
D
DAPI
TUJ1
GATA4
50 µm
GATA4
TUJ1

Figure S7. Additional characterization of the gut tube in EMLOCs, related to Figure 4. (**A**) Day 4 EMLOCs with visible gut tube endoderm. (**B**) FOXA2+/CDH1+ gut tube endoderm is self-organized posterior to the GATA4+ cardiac crescent. White arrow points to early serous lining of the cardiac crescent. (**C**) FOXA2+ gut tube endoderm is laminated, where has TUJ1+/GATA4- neural rosettes are more continuous with surrounding cells, labeled in (**D**). TUJ1 was also observed in mitotic spindle MTs (white arrow). Individual scale bars provided.

Figure S8

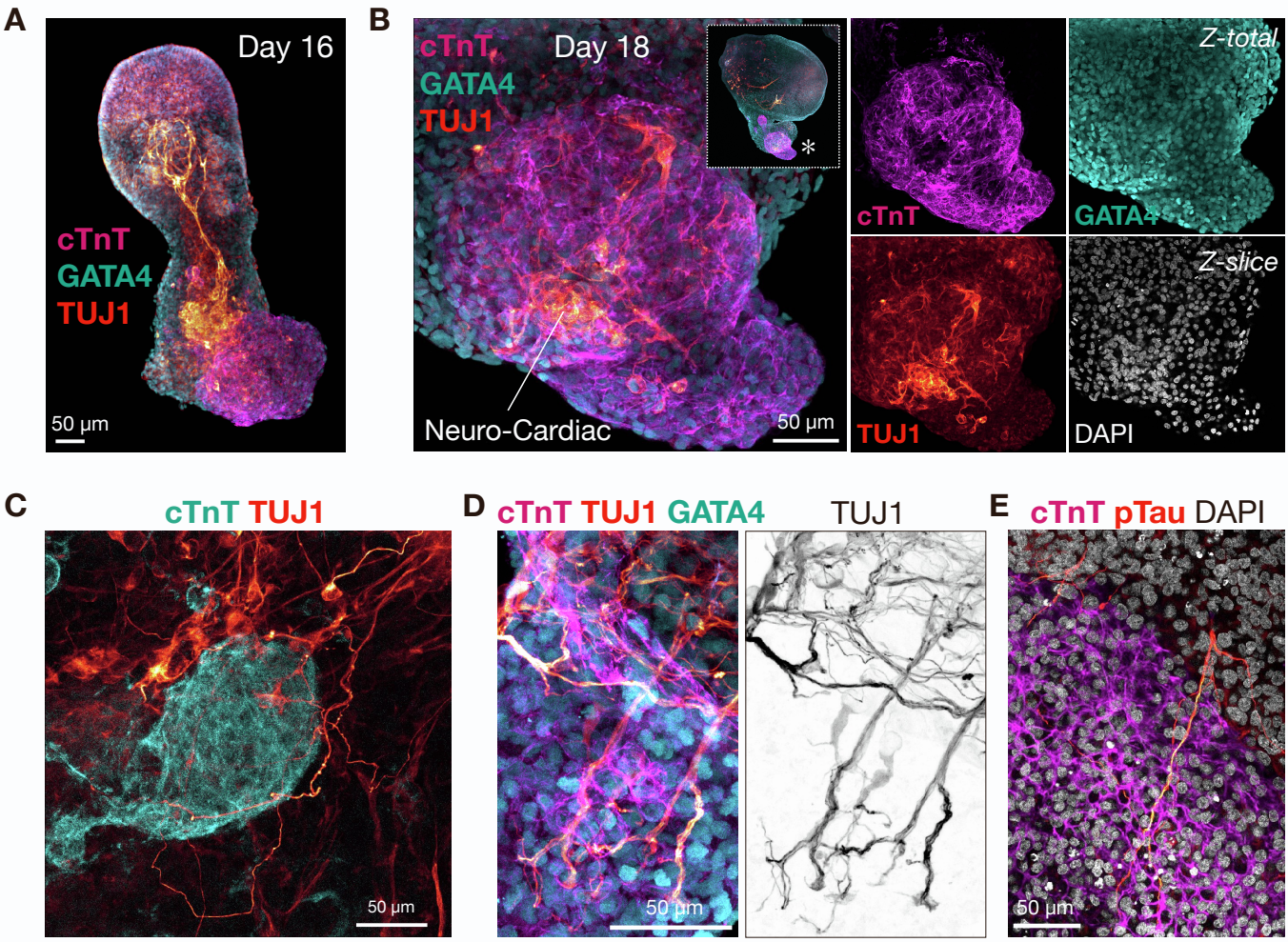
A
Day 16
cTnT
GATA4
TUJ1
50 µm
B
cTnT,
GATA4
TUJ1
Day 18
*
Neuro-Cardiac
50 µm
cTnT
Z-total
GATA4
TUJ1
Z-slice
DAPI
C
cTnT TUJ1
50 µm
D
cTnT TUJ1 GATA4
TUJ1
50 µm
E
cTnT pTau DAPI
50 µm

Figure S8. Additional characterization of neuronal fibers in EMLOCs, related to Figure 7. (**A**) Immunofluorescence of cardiac biomarkers cTnT (magenta) and GATA4 (cyan) along with TUJ1 (red) in day 16 H3.1.1 EMLOCs demonstrate the emergence of neurons proximal to the cardiac region. (**B**) Immunofluorescence of cTnT (magenta) and TUJ1 (red) at day 18 depicts ganglionated neuronal plexus-like structures in the cardiac region. Merge is shown with individual cTnT, GATA4 (cyan), TUJ1 channels. Left inset is low magnification EMLOC image. (**C**) TUJ1+ neuronal fiber termination (red) onto cTnT+ cells (cyan) reminiscent of nodal innervation. (**D**) Cardiac biomarkers cTnT (magenta) and GATA6 (cyan) with TUJ1. (**E**) Axons identifiable by pTau within the cTnT+ myocardium (right). Individual scale bars provided.