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Supplemental Information

Capturing Cardiogenesis in Gastruloids

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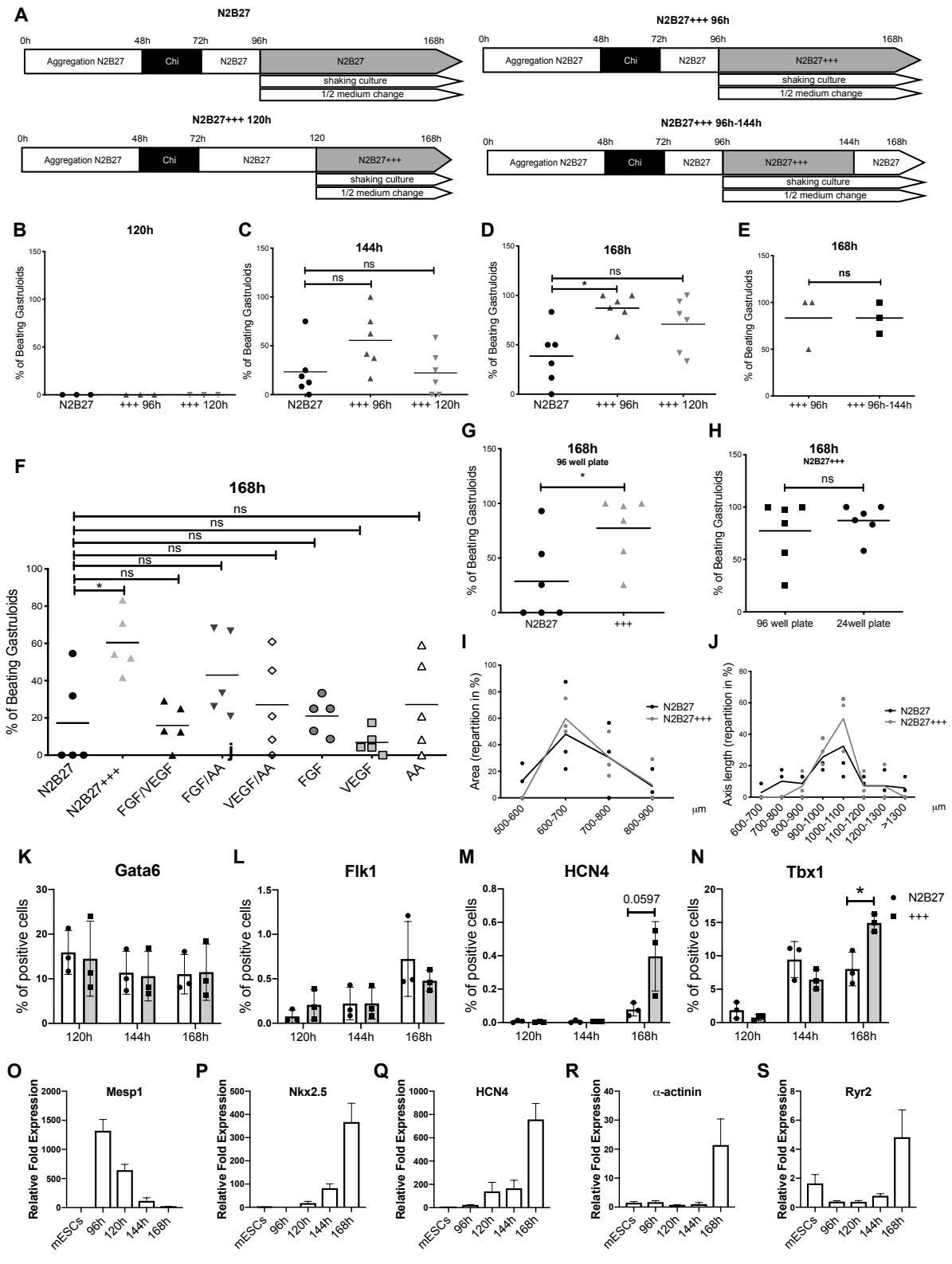

Figure S1 | Comparison between N2B27 and N2B27+++ culture conditions. ***Related to Fig. 1.*** **A**, Schematics of the different culture conditions tested. **B-E**, Percentage of beating gastruloids from 120 to 168h in the different conditions. **F**, Exposure to N2B27+++ induces beating at higher frequencies compared to exposure to single factors or couples. **G, H,** Frequencies of beating gastruloids grown in 96well plates (**G**) and comparison with those grown in 24 well plates from 144h (**H**). Each dot in **B-H** represents an independent experiment. **I-J**, Quantification of area and axis of gastruloids at 168h grown in N2B27 or N2B27+++. Mean of *n*=3 independent experiments. **K-N**, FACS analysis graphs showing the percentages of reporter positive

cells in N2B27 or N2B27+++ at different time points. *n*=3 independent experiments. **O-S**, qPCR gene expression profiles of cardiac genes in gastruloids from 96 to 168 h. Data are expressed as relative fold expression compared to mESCs in $n = 4$ independent experiments. Data are represented as individual data points or mean ± SD.

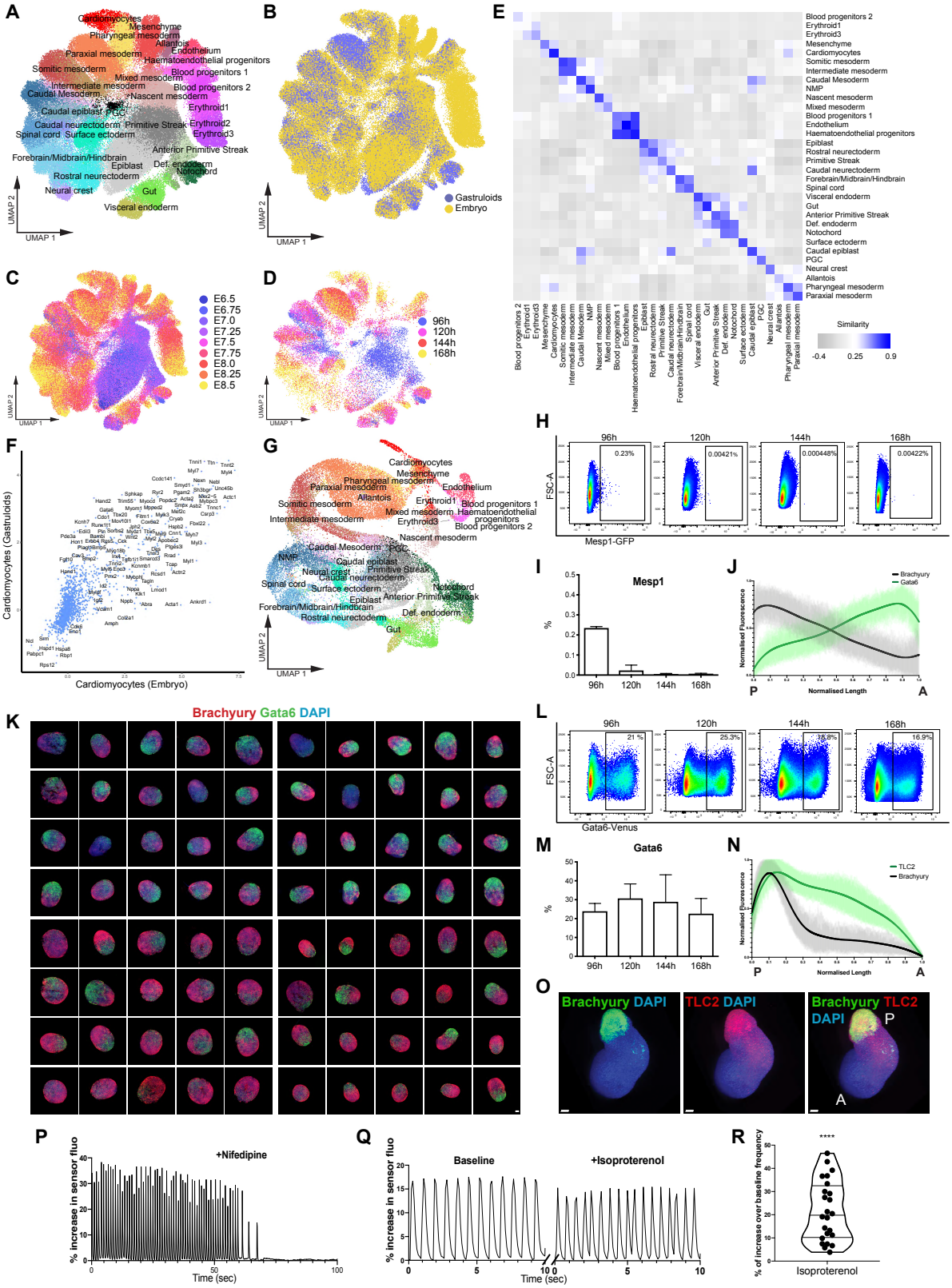

Figure S2 | Development of cardiovascular progenitors in gastruloids. ***Related to Fig. 1 and Fig. 2.*** **A**, **B**, UMAP plots showing the alignment of embryonic and gastruloid cells. The *in vivo* cell identities as annotated by (Pijuan-Sala et al., 2019) were transferred to their analogs in gastruloids based on the alignment (**A**) and the relative distribution of gastruloid and embryo cells within the UMAP is shown in (**B**). **C**, **D**, Correspondence between embryonic stages (**C**) and gastruloid time points (**D**) based on the aligned view. **E**,

Heat map showing the similarity by cell type between embryo and gastruloid cells in **A** (cosine similarity based on the scaled expression of all variable genes). **F**, Scatterplot showing the scaled variable gene expression in gastruloids vs. embryo cardiomyocytes. **G**, Transferred annotations from (Pijuan-Sala et al., 2019) shown on the UMAP projection of **Fig. 1D. H**, **I**, FACS analysis of *Mesp1-GFP* gastruloids from 96 to 168h (**H**) and relative quantification (**I**). *n*=2 independent experiments. **J**, **K**, Unbiased collection of gastruloids from 4 independent experiments showing *Gata6* and Brachyury signals along the A/P axis of gastruloids at 96h (**K**) and relative quantification (**J**). *n*=104 gastruloids from 4 independent experiments. **L**, **M**, FACS analysis plots **(L)** and quantification (**M**) of *Gata6-Venus* gastruloids from 96 to 168. *n*=2 independent experiments. **N**, **O**, Representative pictures of TLC2 gastruloids at 168h, showing expression of Brachyury and TLC2 (*TCF/LEF-mCherry)* (**O**) and relative quantification of the signals along the A/P axis (**N**). *n*=54 gastruloids from 4 independent experiments. **P-R**, Treatment of 168 h gastruloids with Nifedipine (*n*=9 gastruloids) (**P**) or Isoproterenol (**Q, R**) abolishes or fastens calcium spiking in the gastruloid cardiac portion, respectively. Graph in (**Q**) shows representative calcium spikes of gastruloids before and after Isoproterenol treatment, and graph in (**R**) the percentage of increase over baseline frequency of gastruloids after Isoproterenol treatment. *n*=24 gastruloids. A: anterior; P: posterior. Scale bars, 100μm. Data are represented as individual data points or mean ± SD.

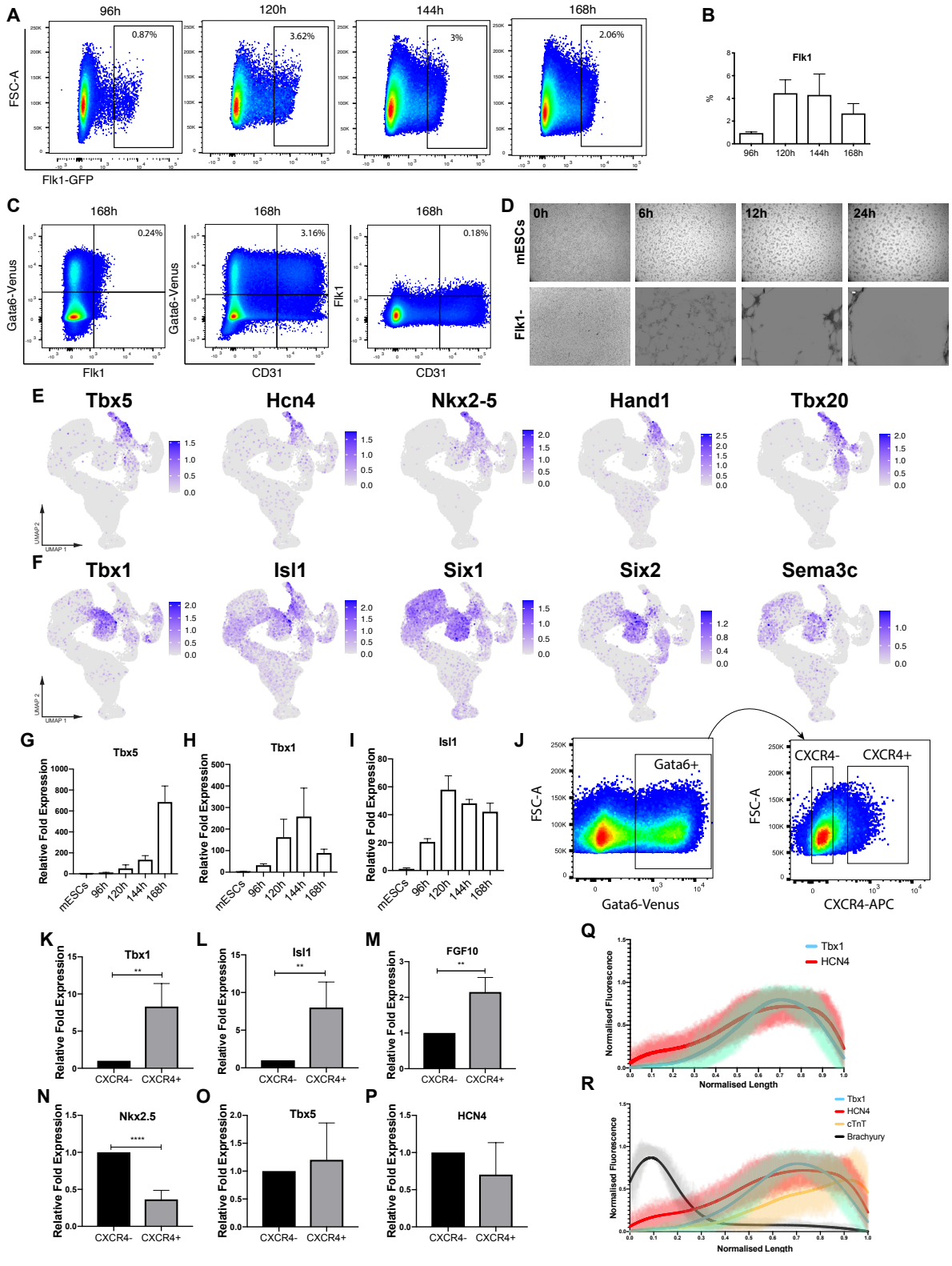

Figure S3 | Characterization of cardiovascular progenitors. ***Related to Fig. 3 and Fig. 4.*** **A, B,** FACS analysis of *Flk1-GFP* gastruloids from 96 to 168 (**A**) and relative quantification (**B**). *n*=2 independent experiments. **C**, FACS analysis of *Gata6-Venus* gastruloids showing co-expression of Flk1 and CD31. *n*=2 independent experiments. **D**, Angiogenesis assay showing inability of undifferentiated mESCs and *Flk1*$^{-}$ cells to form vascular-like tubes. **E**, **F**, Expression of FHF (**E**) and SHF (**F**) marker genes. **G-I**, qPCR showing expression levels of markers of FHF (**G**) and SHF (**H**, **I**) in gastruloids from 96 to 168 h. Data are expressed as relative fold expression compared to mESCs in $n = 4$ independent experiments. **J**, Representative plot

showing the gating strategy used to isolate SHF-enriched progenitors. **K-P,** Expression levels of markers of SHF (**K-M**) and FHF (**N-P**) in *Gata6*$^{+}$/CXCR^{-} and *Gata6*$^{+}$/CXCR^{+} cells isolated from gastruloids at 168 h. Data are expressed as relative fold expression compared to *Gata6*$^{+}$/CXCR^{-} cells in $n = 4$ independent experiments. **Q**, **R**, Quantification of *Tbx1*, *Hcn4*, cTnT and Brachyury signals along the A/P axis in gastruloids. *n*=42 gastruloids stained for *Tbx1*, *Hcn4* and cTnT and *n*=26 gastruloids stained for *Tbx1*, *Hcn4* and Brachyury. A: anterior; P: posterior. Scale bar, 100μm. Data are represented as mean ± SD.

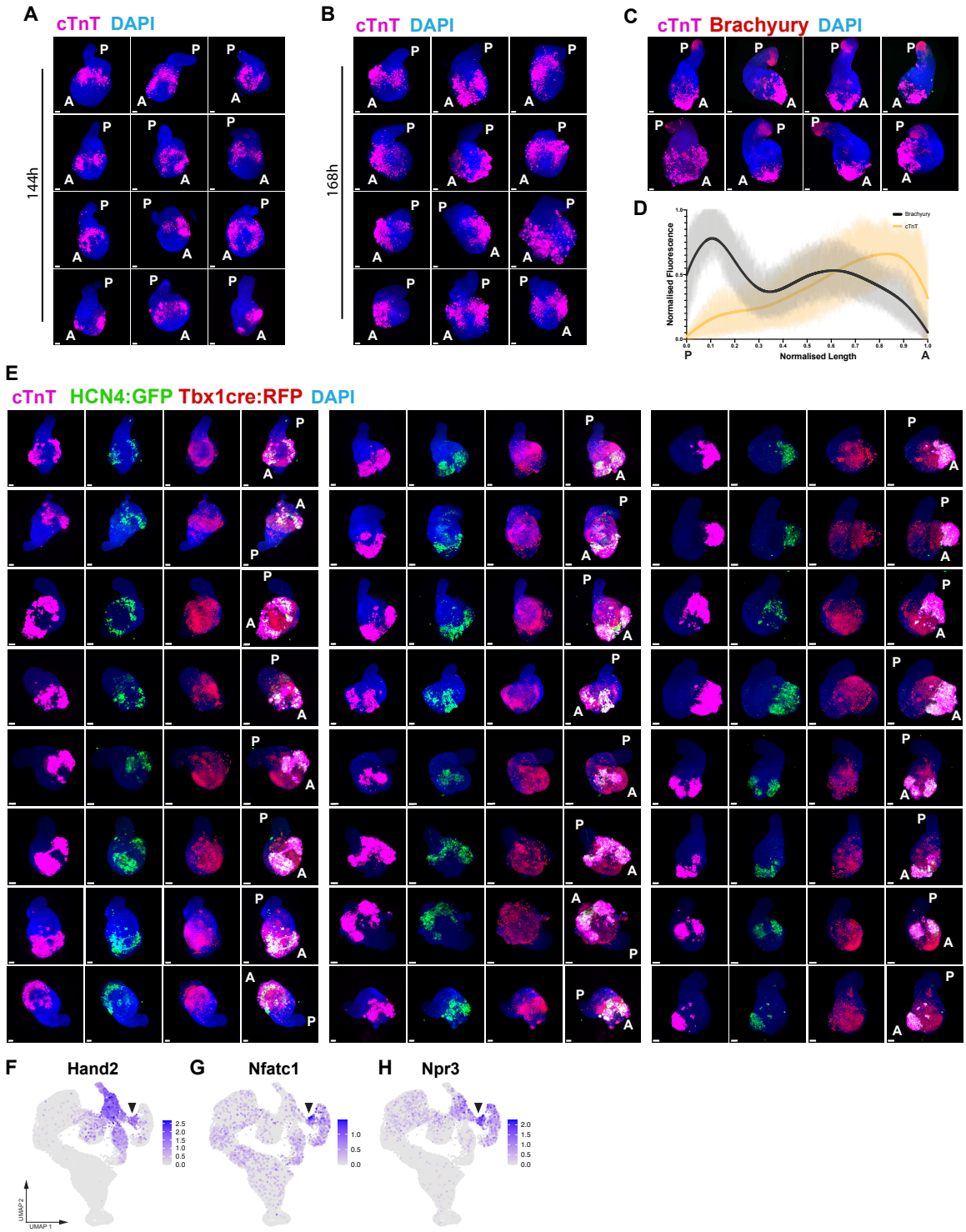

Figure S4 | Characterization of the gastruloid cardiac portion. ***Related to Fig. 4 and Fig. 5.*** **A, B,** Representative collection of *Sox1-GFP::Brachyury-mCherry* gastruloids at 144h (**A**) and 168h (**B**), showing expression of cTnT in the anterior domain. *n*=4 independent experiments. **C**, **D,** Staining (**C**) and relative quantification (**D**) of cTnT and Brachyury signals along the A/P axis of gastruloids at 168h. *n*=38 gastruloids from 2 independent experiments. **E**, Representative collection of *Hcn4-GFP::Tbx1Cre-RFP* gastruloids at 168h, showing the cardiac portion in the anterior domain. *n*=4 independent experiments. **F-H**, Expression of endocardial genes. A: anterior; P: posterior. Scale bars, 100μm.