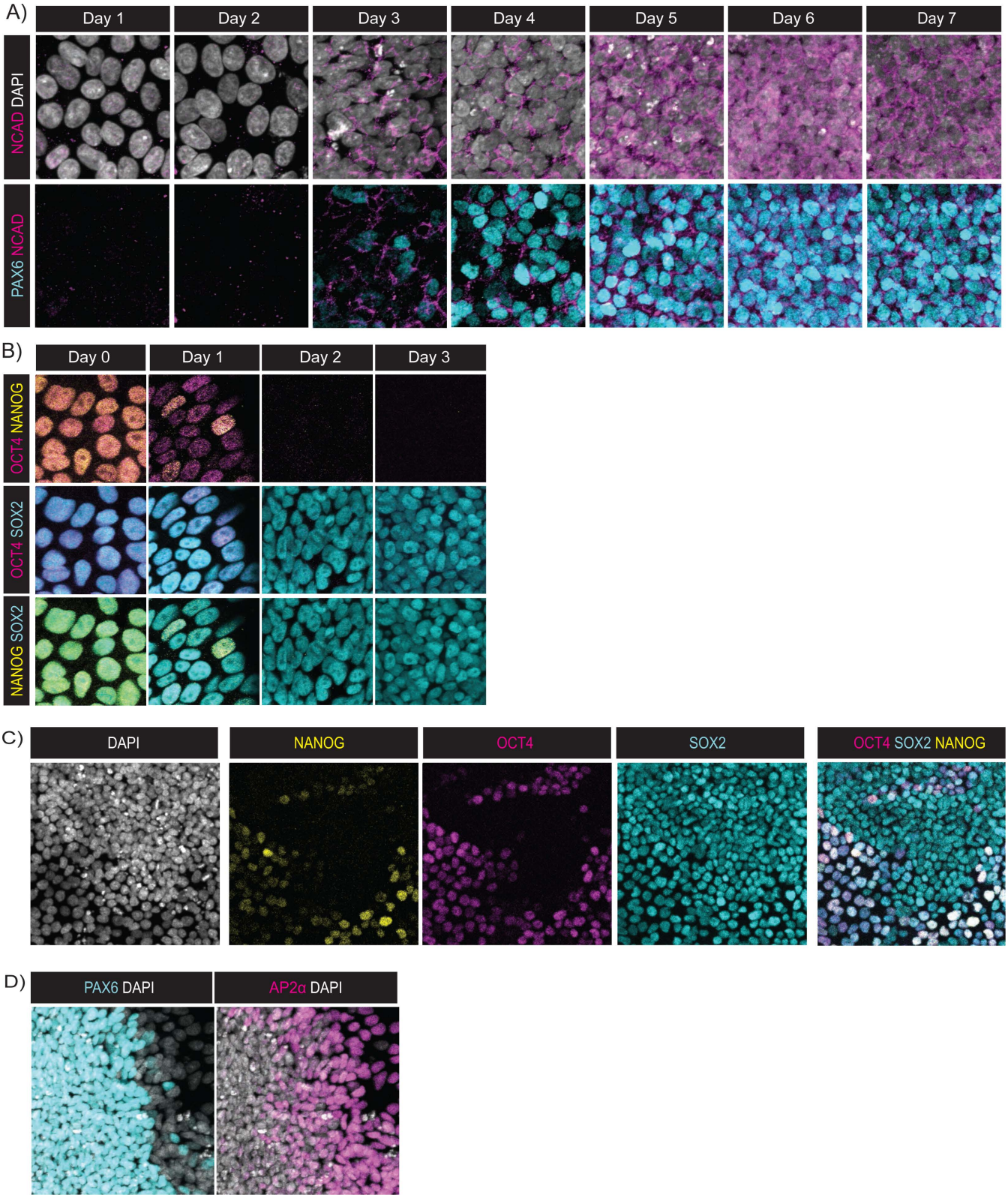


Figure S1: Nodal inhibition suppresses NANOG and OCT4 and then instructs expression of NCAD and PAX6 (related to Figure 1)
(A-B) Representative images of cells in standard culture fixed on the indicated days during the course of Nodal inhibition and immunostained for the neural progenitor marker NCAD and PAX6 (A) or SOX2, OCT4 and NANOG (B) at the conclusion of the induction. Experiment replicated 3 times. (C-D) Representative images of cells immunoflourescently co-stained for AP2/PAX6 or SOX2/NANOG/OCT4 at the conclusion of a two-phase induction protocol. Cells were initially differentiated in phase 1 for either 2 (C) or 4 (D) days in Nodal inhibition media and then treated with the indicated culture conditions for the subsequent 6 (C) or 4 (D) days in phase 2. Experiment replicated 3 times. Scalebar in A and B = 25 μm; in C and D = 50 μm.

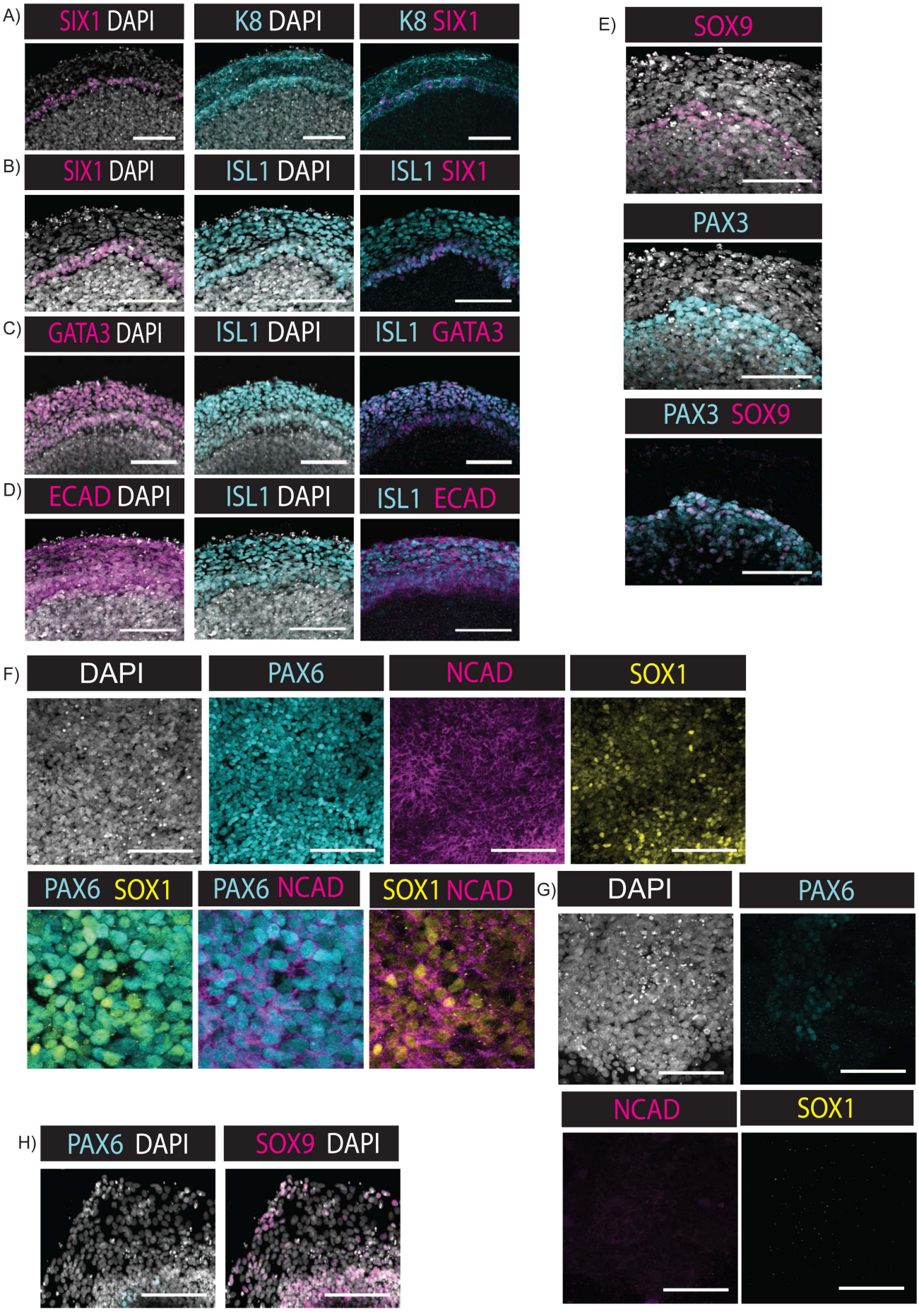


Figure S2: Two-step ectoderm induction protocol instructs gene expression patterns associated with anterior fates (related to Figure 2)
(A-E) Representative images of the colony edge following a two-step ectoderm induction protocol and co-stained for SIX1/K8 (A), SIX1/ISL1 (B), GATA3/ISL1 (C), ECAD/ISL1 (D) and SOX9/PAX3 (E). (F) Representative images of the colony center following a two-step ectoderm induction protocol and co-stained for PAX6, NCAD and SOX1. (G-H) Representative images of the colony center (G) and edge (H) with the indicated antibodies following 3 days of induction in N2B27 alone and 3 days in BMP+SB. Experiment replicated at least 3 times. Colony diameter = 700 μm. Scalebar = 100 μm.

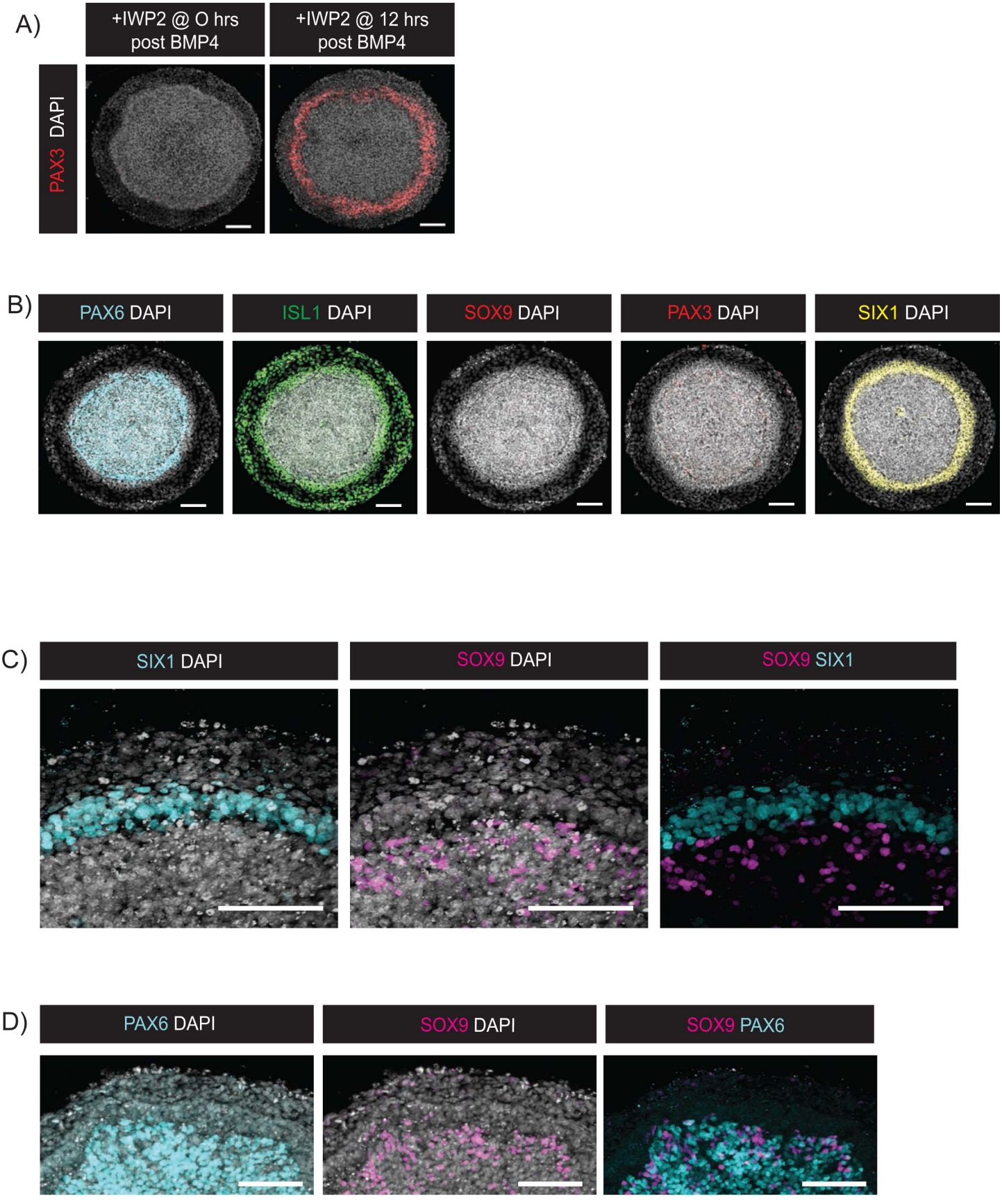


Figure S3: WNT inhibition before or at the time of BMP4 treatment inhibits neural crest emergence (related to figure 3)
(A) Representative images of colonies immunostained for PAX3 at the conclusion of the experiment. Colonies were initially induced for 3 days in ectoderm induction media and then subsequently differentiated for 3 days in N2B27 media containing BMP4 and SB. The time between BMP4 and IWP2 addition is indicated in the banner above the corresponding image. Experiment replicated 4 times. Colony diameter = 700 µm. (B) Representative images of colonies immunostained for PAX6, ISL1, SOX9, PAX3 or SIX1 at the conclusion of induction. Colonies were initially induced for 3 days in ectoderm induction media with IWP2 and then subsequently differentiated for 3 days in N2B27 media containing BMP4, SB and IWP2. Experiment replicated 4 times. (C-D) Representative images of the colony edge co-stained for SIX1/SOX9 (C) and PAX6/SOX9 (D). Colonies were initially induced for 3 days in ectoderm induction media and then subsequently differentiated by IWP2 introduced 12 hours post BMP+SB treatment. Experiment replicated 4 times. Colony diameter = 700 µm. Scalebar = 100 µm.

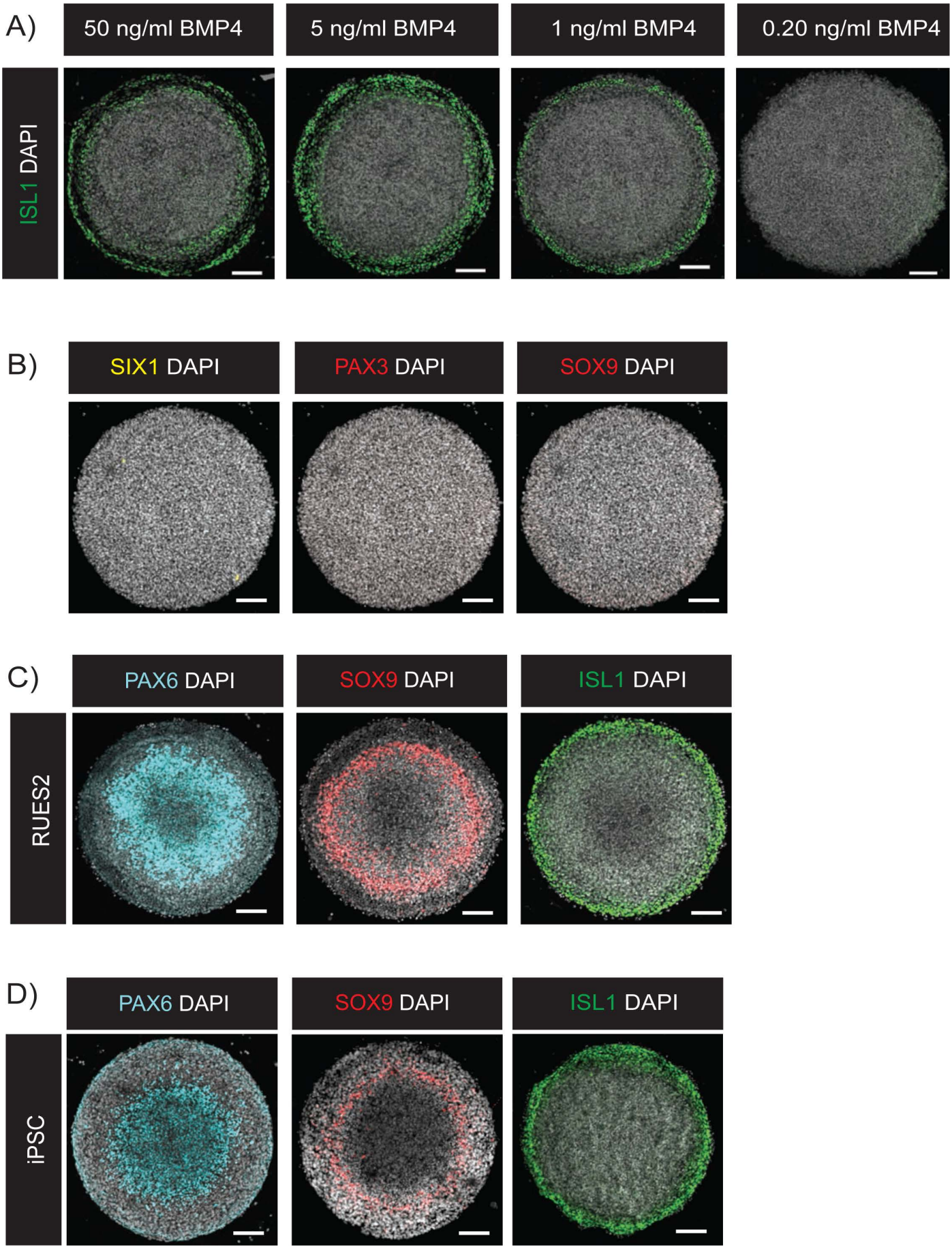


Figure S4: Surface ectodermal fates require a minimum concentration of BMP4 (related to Figure 4)
(A) Representative images of colonies immunostained for ISL1 at the conclusion of induction. Colonies were induced with a three-step ectoderm induction protocol and patterned with the indicated concentration of BMP4. Experiment replicated 3 times. Colony diameter = 700 µm. (B) Representative images of colonies immunostained for SIX1, PAX3 or SOX9 at the conclusion of the experiment. Colonies were induced for the first 3 days in ectoderm induction media with IWP2 and then differentiated for the subsequent 3 days in N2B27 media containing 0.20 ng/ml of BMP4, IWP2 and SB. Experiment replicated 2 times (C-D) Representative images of RUES2 (C) and WTC-11 (iPSC) (D) stem cell colonies induced with a three-step ectoderm induction protocol using 2 ng/ml of BMP4 and immunostained for PAX6, SOX9 and ISL1. Experiment replicated 3 times. Colony diameter = 700 µm. Scalebar = 100 µm.

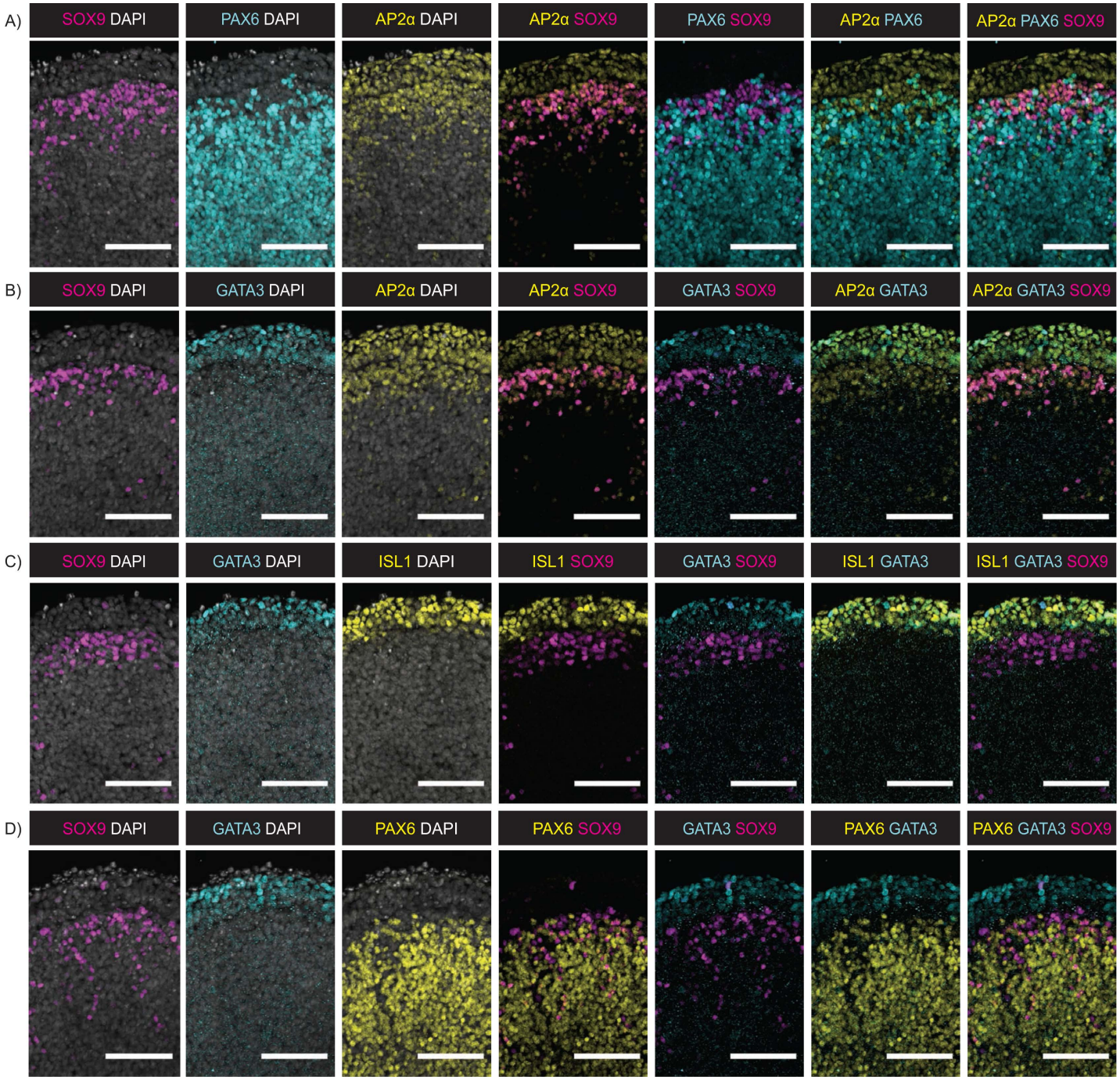
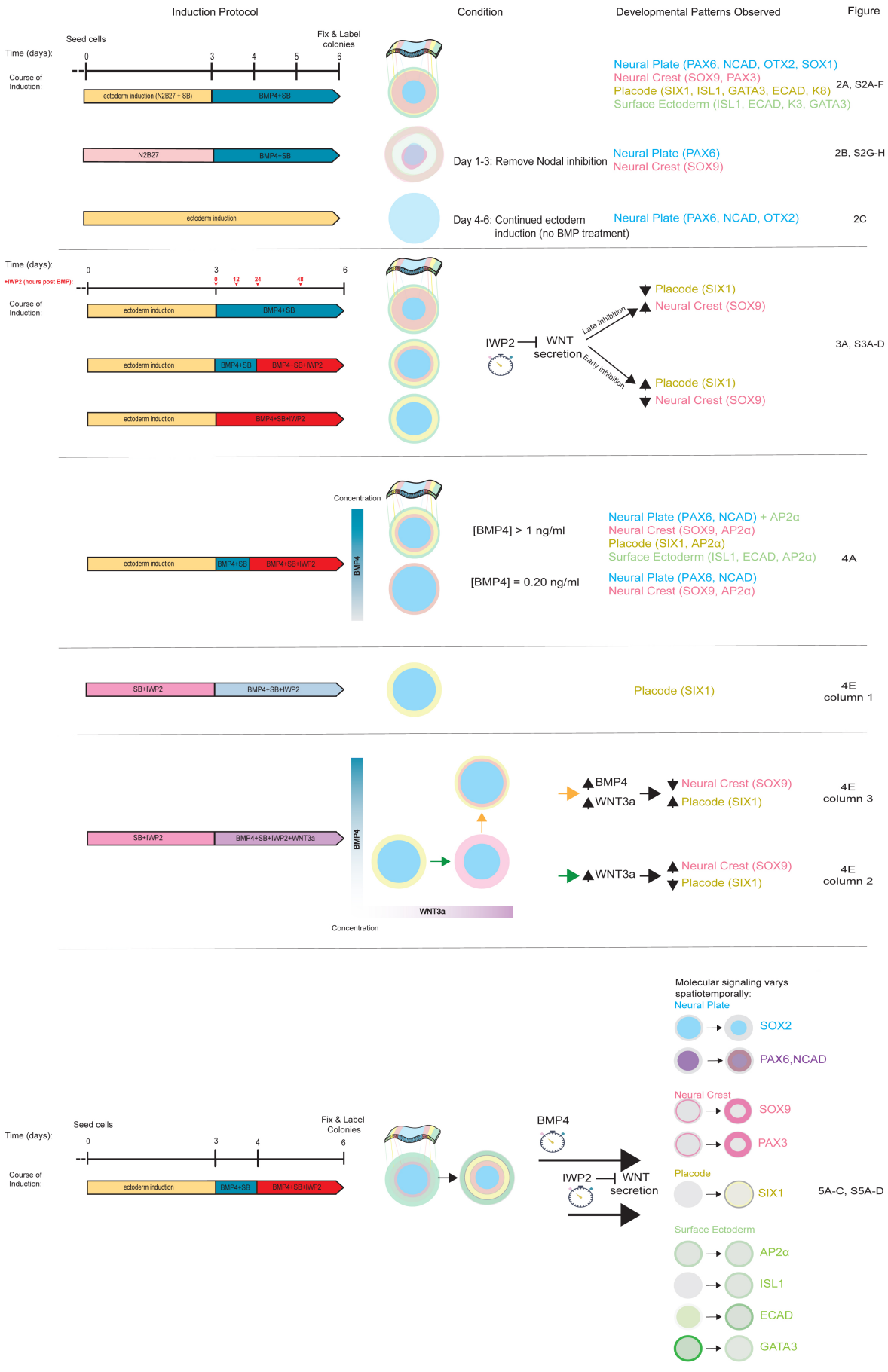


Figure S5: Separation of human surface and neural ectoderm following a three-step induction protocol using 1 ng/ml of BMP4.
(A-D) Representative images of day 6 hESC colonies immunostained for the indicated labels. Colonies were induced with a three-step ectoderm induction protocol using 1 ng/ml of BMP4. Experiment replicated 2 times. Colony diameter = 700 µm. Scalebar = 100 µm.



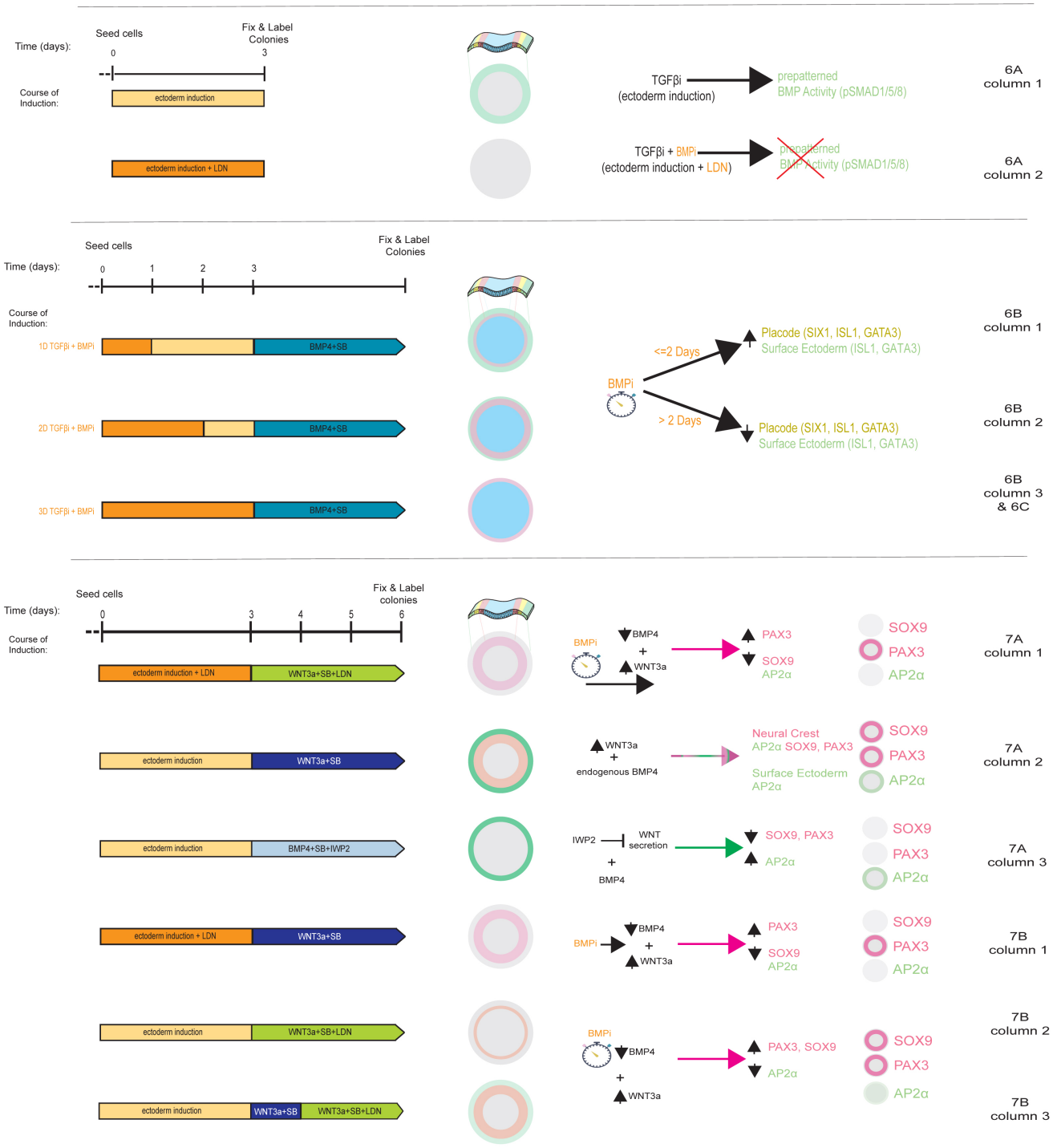


Table S1: An overview linking spatiotemporal and combinatorial pathway activity to the emergence and position of fates within human ectodermal tissue.

Overview schematic summarizing the induction protocols and experimental conditions presented in this work and the resulting developmental patterns observed. The self-organizing embryonic stem cell system experimental platform presented here highlights the regulatory role of endogenous WNT signaling (Figures 3, 4, 5 and 7) and defines the signaling logic that guide human ectoderm development (Figures 5 and 7) in a spatiotemporal manner.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat Anti-SOX9 (1:100)	R&D Systems	Cat# AF3075
Goat Anti-BRACHYURY (1:300)	R&D Systems	Cat# AF2085
Mouse Anti-CDX2 (1:50)	Biogenex	Cat# MU392A
Goat Anti-NANOG (1:100)	BD Biosciences	Cat# 560482
Mouse Anti-PAX3 (1:30)	Developmental Studies Hybridoma Bank	Antibody Registry ID# AB528426
Mouse Anti-TFAP2α (1:100)	Developmental Studies Hybridoma Bank	Antibody Registry ID# AB2313948
Rabbit Anti-phosphoSMAD1/5/8 (1:200)	Cell Signaling Technologies	Cat# 13820
Rabbit Anti-SOX2 (1:200)	Cell Signaling Technologies	Cat# 5024S
Rabbit Anti-SIX1 (1:300)	Cell Signaling Technologies	Cat# 12891
Rabbit Anti-GATA3 (1:200)	Thermo Fisher Scientific	Cat# PA1-101
Rabbit Anti-E-Cadherin (1:300)	Cell Signaling Technologies	Cat# 3195S
Mouse Anti-N-Cadherin (1:200)	Sigma-Aldrich	Cat# C2542
Mouse Anti-ISL1 (1:75)	Developmental Studies Hybridoma Bank	Antibody Registry ID# AB2314683
Rabbit Anti-OTX2 (1:300)	Abcam	Cat# 21990
Mouse Anti-K8 (1:50)	Developmental Studies Hybridoma Bank	Antibody Registry ID# AB531826
Mouse Anti-PAX6 (1:50)	Developmental Studies Hybridoma Bank	Antibody Registry ID# AB528427
Rabbit Anti-PAX6 (1:300)	Biolegend	Cat# 901301

Bacterial Strain		
5-alpha Competent E.coli	New England Biolabs	Cat# C2987H
Chemicals, Peptides, and Recombinant Proteins		
Human BMP4 Recombinant Protein	Fisher Scientific	Cat#314BP050
Human Wnt3a Recombinant Protein	Amsbio	Cat#AMS.rhW3aL-002-stab
cOmplete™ Lysis-M	Sigma-Aldrich	Cat#04719956001
cloneR	STEMCELL Technologies	Cat#05889
DAPI (4,6-diamidino-2-phenylindole, dihydrochloride)	Fisher Scientific	Cat#D1306
IWP2	Stemgent	Cat#04-0034
Laemmli Sample Buffer	Bio-Rad	Cat#1610737
LDN193189	Thermo Fisher Scientific	Cat#04-0074-02
mTeSR1	STEMCELL Technologies	Cat#85875
Dulbecco's PBS Without calcium and magnesium	Caisson Labs	Cat# PBL01-6X500ML
Dulbecco's PBS with calcium and magnesium	Caisson Labs	Cat# PBL02-6X500ML
Human recombinant laminin521 protein	Biolamina	Cat# R021599/X0086842
Neurobasal Medium (L-glutamine)	Life Technologies	Cat# 21103-049
Glutamax 100x	Life Technologies	Cat# 35050061
N-2 Supplement (100x)	Life Technologies	Cat# 17502048
B-27 Supplement (50x), minus vitamin A	Life Technologies	Cat# 12587010

β-Mercaptoethanol	Fisher Scientific	Cat# 21985023
DMEM/F12	VWR	Cat# 45000-344
Puromycin	Fisher Scientific	Cat#A1113803
ROCK inhibitor Y-27632	Fisher Scientific	Cat#50-175-998
SB431542	Stemgent	Cat#04-0010-05
96-well CYTOOplates	CYTOO	Cat#20-950-00
Arena EMB CYTOOchips	CYTOO	Cat#10-021-00-18
Ibidi µ-Plate 24 well black	Ibidi	Cat#82406
Ibidi µ-Slide 8 well	Ibidi	Cat#80826
Accutase	Fisher Scientific	NC9839010
Critical Commercial Assays		
DNeasy Blood and Tissue Kit	Qiagen	Cat#69504
HiSpeed Plasmid Midi Kit	Qiagen	Cat#12643
P3 Primary Cell 4D-Nucleofector® X Kit L	Lonza	Cat# V4XP-3024
Experimental Models: Cell Lines		
ESI-017	ESI BIO	RRID:CVCL_B854
ESI017 GFP-β-catenin	(Massey *et al.*, 2018)	
RUES2	Gift from Brivanlou lab (Rockefeller University)	
WTC-11 GFP- β-catenin, iPSC	Paul Allen Institute	Cell Line ID: AICS-0058 cl.67

Software and Algorithms		
Benchling		https://benchling.com/
MATLAB		https://www.mathworks.com/products/matlab.html
Matlab scripts for quantifying, analyzing data	This paper	https://github.com/warmflasha/CellTracker

Table S2: Key Resource Table