Figure S1 Related to Figure 1.

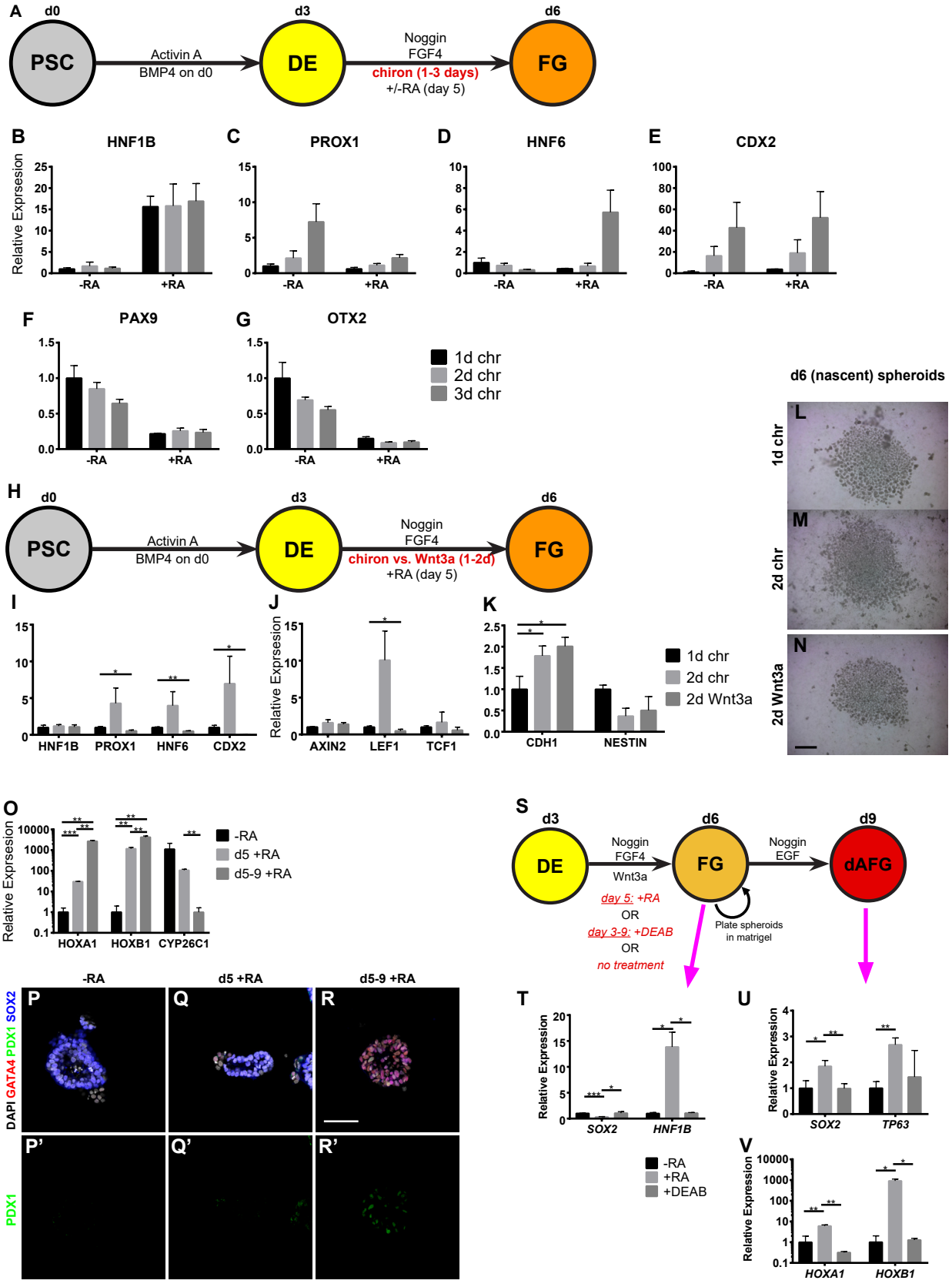

Figure S1 Related to Figure 1: Modulating the duration of Wnt and retinoic acid signaling to coordinate foregut patterning across the anterior-posterior axis.
(A) Schematic depicting the experimental protocol to pattern foregut spheroids along the anterior-posterior axis using CHIR99021 (chiron, or chr) and retinoic acid (RA). **(B-G)** qPCR analysis of day 6 spheroids resulting from varying the duration of chiron treatment with or without retinoic acid treatment for **(B)** the anterior and posterior foregut marker *HNF1B*, **(C-D)** the posterior foregut markers *PROX1* and *HNF6*, **(E)** the hindgut marker *CDX2*, **(F-G)** and the pharyngeal markers *PAX9* and *OTX2*. **(H-N)** A comparison of chiron versus Wnt3a treatment on endoderm by qPCR analysis of **(H)** the foregut marker *SOX2*, **(I)** *HNF1B*, *PROX1*, *HNF6*, and *CDX2*, **(J)** Wnt target genes *AXIN2*, *LEF1*, and *TCF1*, and **(K)** epithelial and neural marker *CDH1* and *NESTIN*, respectively. Spheroids generated with one day of chiron treatment have same gene expression profile as those generated with 2 days of Wnt3a. **(L-N)** Brightfield imaging of nascent spheroids resulting from chiron versus Wnt3a treatments show that the efficiency of generating spheroids is unaffected in the different conditions. **(O-R)** Analysis of day 9 spheroids resulting from altering the duration of retinoic acid treatment starting on day 5. qPCR analysis of **(O)** retinoic acid targets *HOXA1*, *HOXB1*, *CYP26C1*. **(S)** Schematic depicting experimental protocol for modulating retinoic acid signaling using the synthesis inhibitor DEAB. **(T-U)** qPCR analysis of **(T)** foregut markers at day 6, **(U)** dorsal anterior foregut markers *SOX2* and *TP63* (ΔN isoform) at day 9, and **(V)** RA targets *HOXA1* and *HOXB1* at day 9. Scale bar = 500 μm in **(L-N)** and 50 μm in **(P-R)**. Error bars indicate SD. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ for two-tailed t-test.

Figure S2 Related to Figure 1.

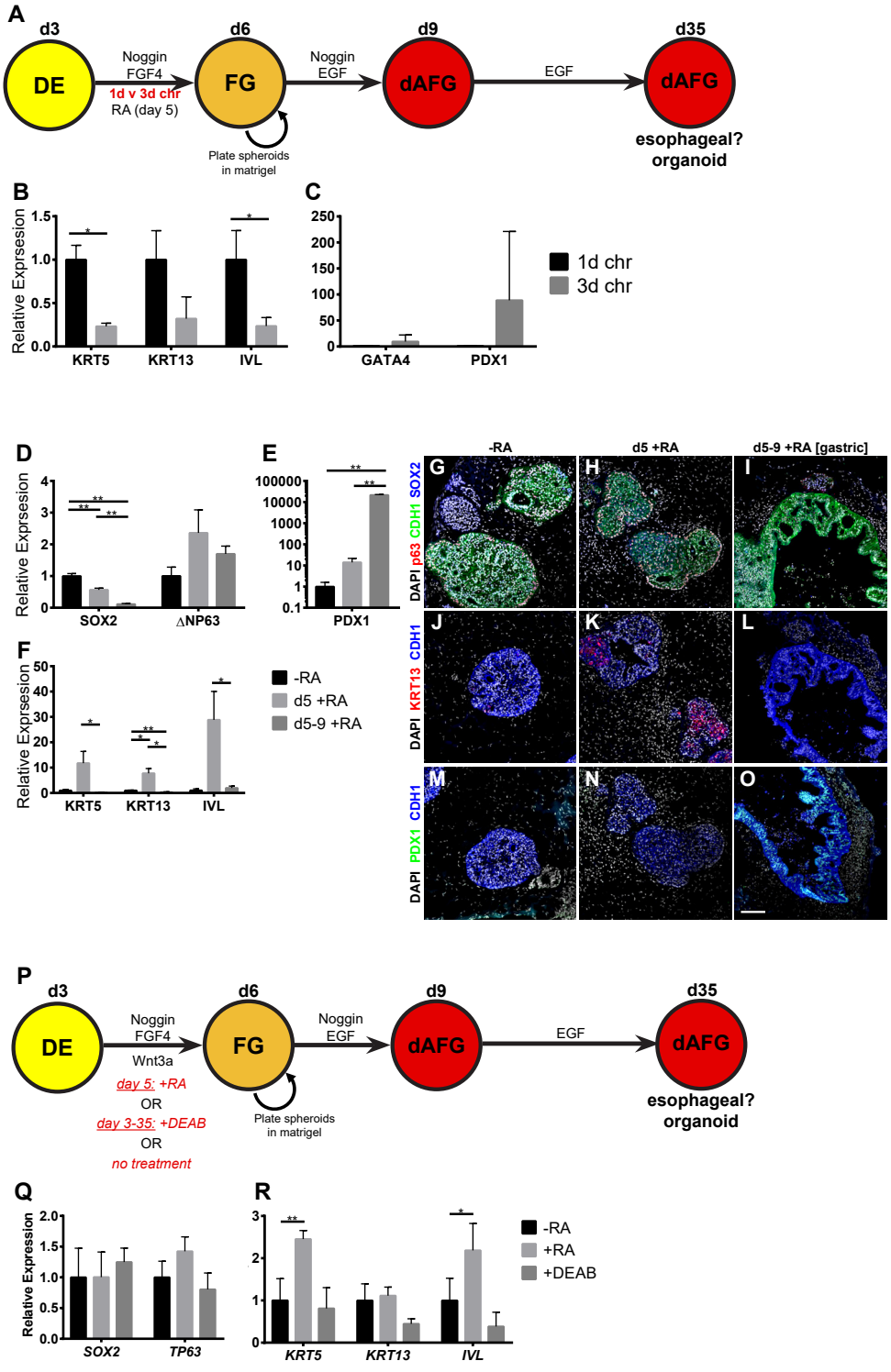

Figure S2 Related to Figure 1: Early modulation of Wnt and retinoic acid signaling affects later differentiation into human esophageal organoids.
(A) Schematic depicting the experimental protocol to generate organoids starting with foregut spheroids treated with chiron for 1 or 3 days. **(B-C)** qPCR analysis of day 35 (1 month old) organoids for **(B)** stratified squamous epithelial markers *KRT5*, *KRT13*, and *KRT13*, and **(C)** posterior foregut markers *GATA4* and *PDX1*. **(D-O)** Analysis of day 35 (1 month old) organoids resulting from altering the duration of retinoic acid treatment starting on day 5. qPCR analysis of **(D)** anterior foregut basal transcription factors *SOX2* and ΔN isoform of *TP63*, **(E)** antral stomach and pancreas marker *PDX1*, and **(F)** stratified squamous epithelial markers *KRT5*, *KRT13*, and *IVL*. Immunofluorescence analysis of one month old organoids with **(G-I)** SOX2 and p63, **(J-L)** KRT13, and **(M-O)** PDX1. **(P)** Schematic depicting experimental protocol for modulating retinoic acid signaling using the synthesis inhibitor DEAB. **(Q-R)** qPCR analysis of day 35 organoids of **(Q)** esophageal basal markers *SOX2* and *TP63*, **(R)** stratified squamous epithelial markers *KRT5*, *KRT13*, and *IVL*. These data is representative of 2 separate experiments with n=3 wells in each experiment. Scale bar = 100 μm. Error bars indicate SD. *$p \leq 0.05$ and **$p \leq 0.01$ for two-tailed t-test.

Figure S3 Related to Figure 2.

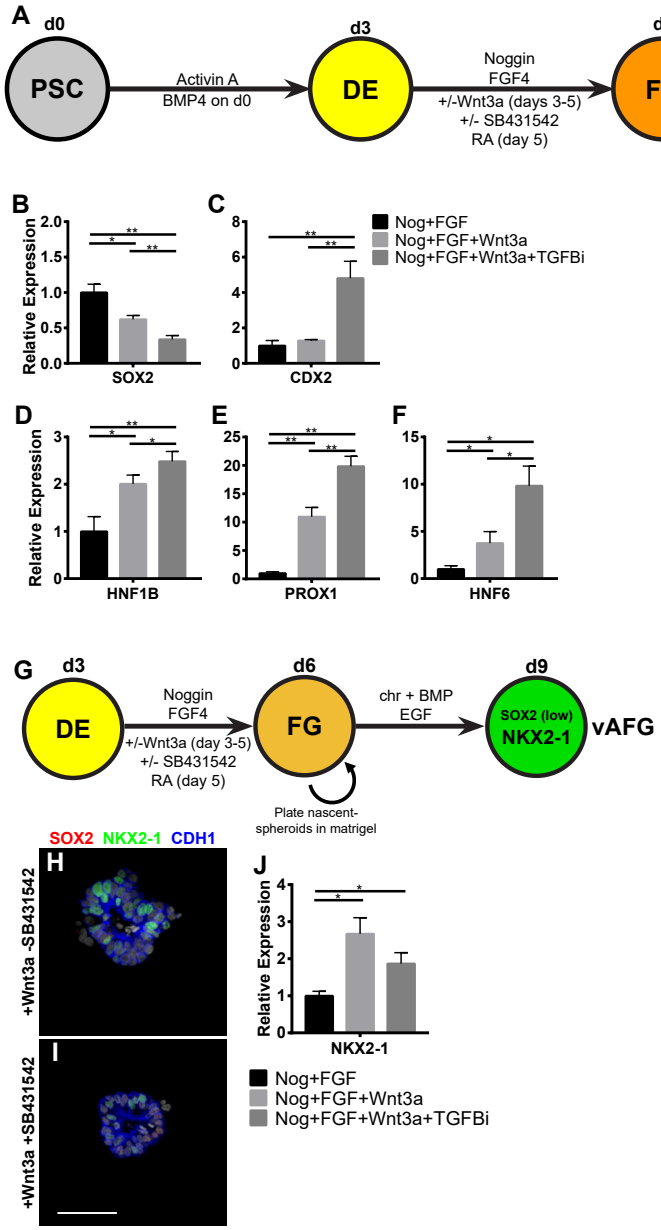

Figure S3 Related to Figure 2: TGFβ inhibition is not required to pattern foregut into anterior foregut spheroids in these culture conditions.
(A) Schematic depicting the experimental protocol to test the requirement of TGFβ signaling in anterior-posterior patterning of the foregut. **(B-F)** qPCR analysis on day 6 anterior foregut spheroids treated with and without Wnt3a and TGFβ inhibitor (SB431542, 10 μM) treatment for **(B-C)** foregut marker *SOX2* and hindgut marker *CDX2*, **(D)** foregut marker *HNF1B*, and **(E-F)** posterior foregut markers *PROX1* and *HNF6*. **(G)** Schematic depicting the experimental protocol to test the competency of anterior foregut spheroids treated with and without Wnt3a or the TGFβ inhibitor SB431542 to respond to respiratory induction. **(H-J)** Analysis of day 9 spheroids for NKX2-1 by (**H-I)** immunofluorescence and **(J)** qPCR. Scale bar = 50 μm. Error bars indicate SD. *$p \leq 0.05$ and **$p \leq 0.01$ for two-tailed t-test.

Figure S4 Related to Figure 3.

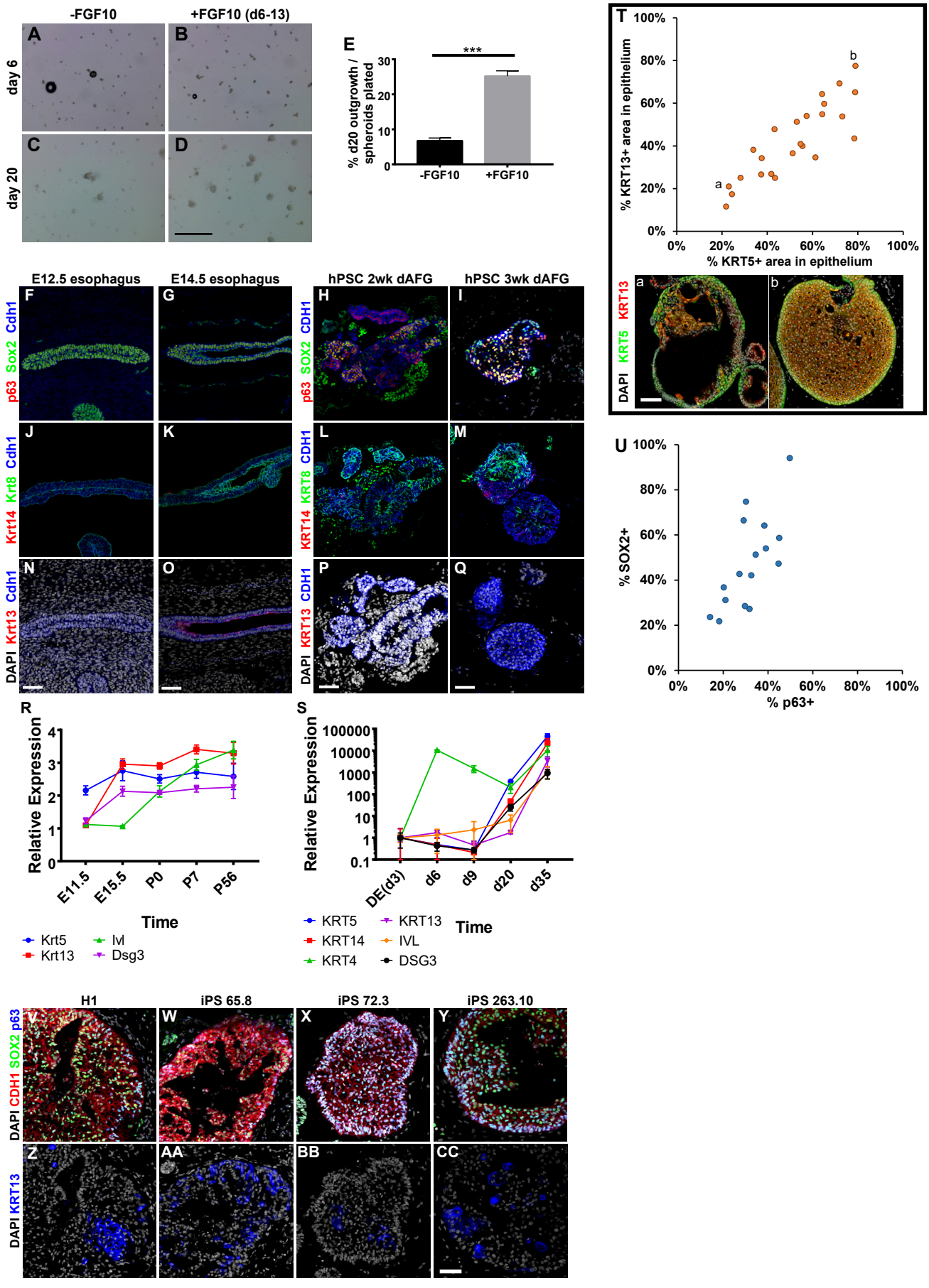

Figure S4 Related to Figure 3: Robust outgrowth of human esophageal organoids and a comparison to mouse embryonic esophageal development.
(A-E) Improved spheroid outgrowth efficiency of organoids treated with FGF10 from day 6-13 as analyzed by **(A-D)** brightfield imaging and **(E)** quantification of the images. **(F-Q)** Comparative analysis by immunofluorescence staining of mouse embryonic esophagi at E12.5 **(F,J,N)** and E14.5 **(G,K,O)** and human esophageal organoids (HEOs) at 2 weeks **(H,L,P)** and 3 weeks **(I,M,Q)**. **(R)** Gene expression of stratified squamous epithelial markers in mouse esophagi across time; public data acquired from GEO dataset GSE34728 (Chen et al. 2012). **(S)** qPCR analysis of various time points during differentiation of definitive endoderm to human esophageal organoids for stratified squamous epithelial markers. **(T)** Quantification of day 62 HEOs for % area of epithelia positive for KRT5 and KRT13. Each point is an individual organoid, and subpanels "a" and "b" are representative images of different organoids depicted in this graph. **(U)** Quantification of day 62 HEOs for % epithelial nuclei positive for SOX2 and p63. Each point is an individual organoid. **(V-CC)** Immunofluorescence analysis of 1-month old HEOs across different cell lines tested, examining esophageal enriched markers SOX2 and p63 **(V-Y)**, and KRT13 **(Z-CC)**. Scale bar = 500 μm in **(A-D)**, 50 μm in **(F-Q, V-CC)**, and 100 μm in **(T-U)**. Error bars indicate SD. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ for two-tailed t-test.

Figure S5 Related to Figure 4.

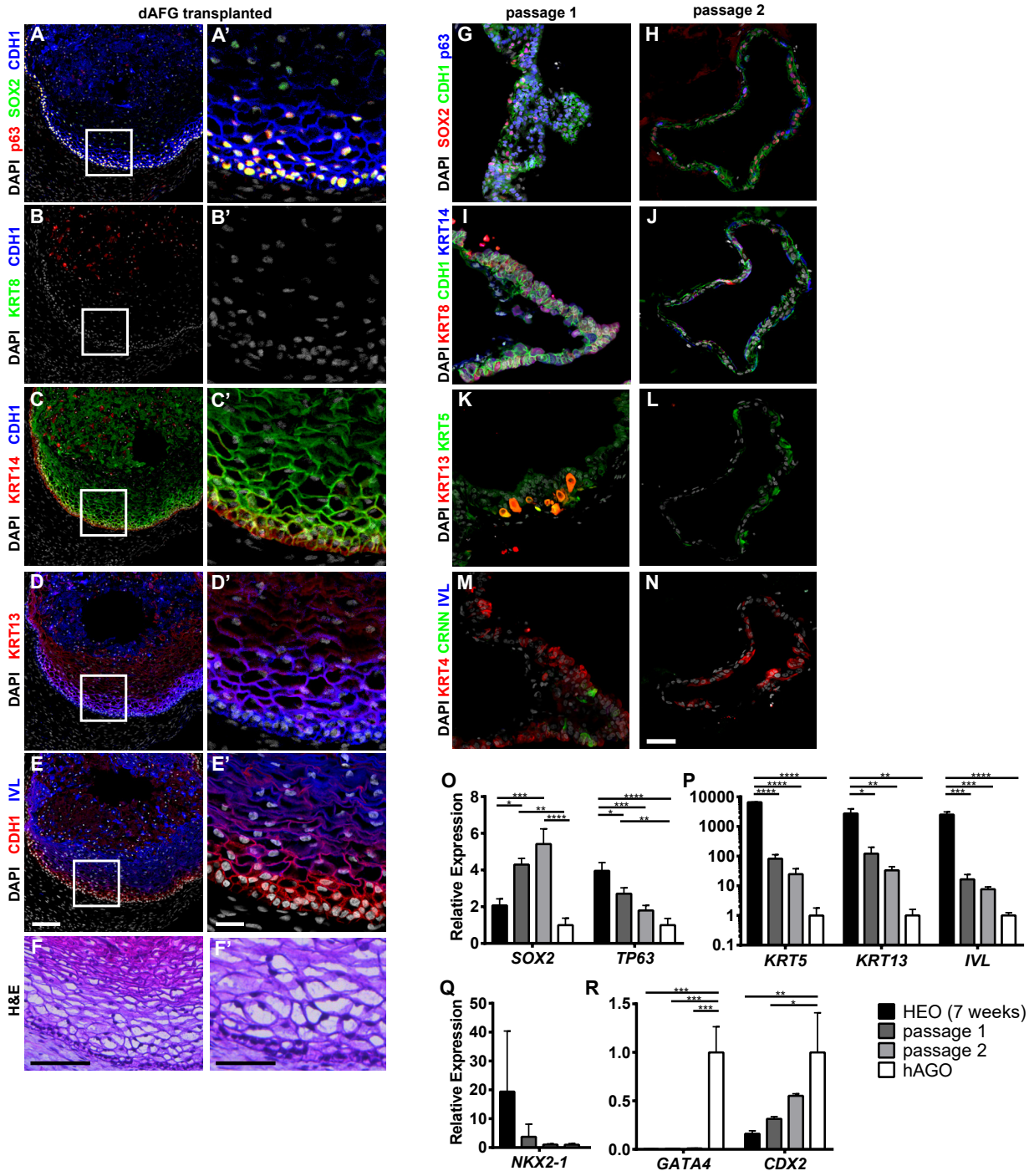

Figure S5 Related to Figure 4: Alternate methods of human esophageal organoid maturation and expansion. **(A-F)** Analysis of HEOs grown for 2 months after transplantation into immunodeficient mice's kidney capsules by **(A-E)** Immunofluorescence images for early (KRT8) and differentiated (KRT13, KRT14, and IVL) esophageal specific markers, and **(F)** H&E. **(G-R)** Analysis of HEOs mechanically passaged (dissociated and re-cultured) twice. **(G-N)** IF images of passaged organoids for **(G-H)** transcription factors SOX2 and p63, **(I-J)** immature (KRT8) and basal marker (KRT14), **(K-L)** basal (KRT5) and suprabasal (KRT13) markers, and **(M-N)** suprabasal differentiated markers KRT4, CRNN, and IVL. **(O-R)** qPCR analysis comparing passaged HEOs to normal HEOs and gastric organoids (hAGO) for **(O)** transcription markers *SOX2* and *TP63*, **(P)** stratified squamous markers *KRT5*, *KRT13*, *IVL*, and **(Q-R)** patterning markers for lung (*NKX2-1*), stomach (*GATA4*), and intestine (*GATA4* and *CDX2*). *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ for two-tailed t-test.

Figure S6 Related to Figure 5.

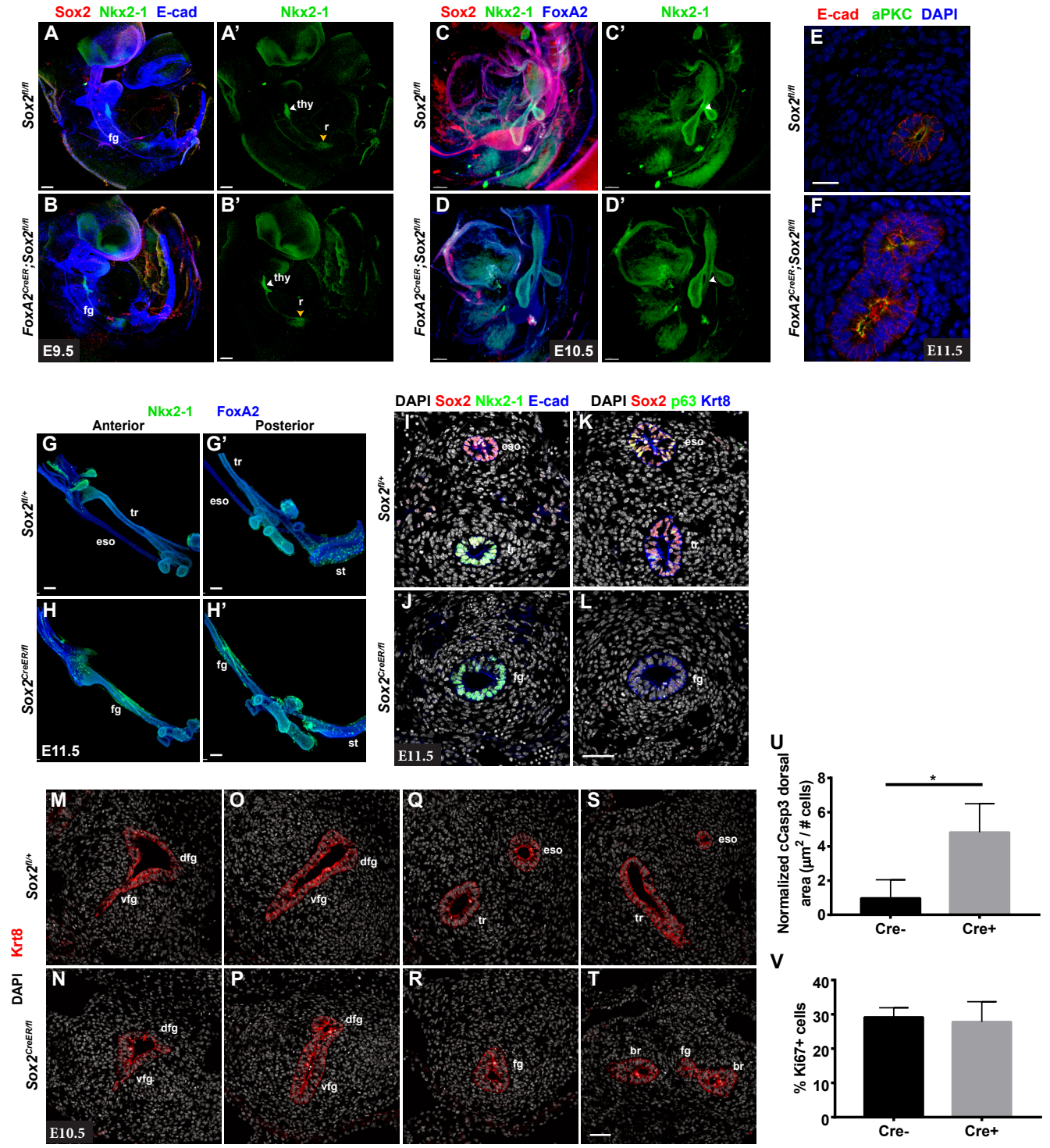

Figure S6 Related to Figure 5: Post-gastrulation endodermal or broad Sox2 knockout results in a similar phenotype of esophageal agenesis.
(A-B) Wholemount immunofluorescence (IF) analysis for dorsal marker Sox2 (red), respiratory marker Nkx2-1 (green) in E9.5 control (*Sox2*$^{fl/fl}$) and Sox2-DE-LOF (*FoxA2*^{CreER};*Sox2*$^{fl/fl}$) embryos. **(C-D)** Wholemount IF analysis for Sox2, Nkx2-1 in E10.5 control (*Sox2*$^{fl/fl}$) and Sox2-DE-LOF (*FoxA2*^{CreER};*Sox2*$^{fl/fl}$) embryos. White arrowheads highlight normal versus ectopic Nkx2-1 expression. **(E-F)** IF analysis of foregut sections of E11.5 embryos for the apical marker aPKC (green). **(G-H)** Wholemount IF analysis for Nkx2-1 of E11.5 control (*Sox2*$^{fl/+}$) and Sox2-cKO (Sox2$^{CreER/fl}$) embryos taken from dams gavaged at 8.5dpc. **(I-L)** Immunofluorescence analysis of E11.5 embryos (similar as in E-F) for **(I-J)** Sox2 and Nkx2-1, and **(K-L)** Sox2 and p63. **(M-T)** IF analysis of E10.5 control (*Sox2*$^{fl/+}$) and Sox2-cKO (Sox2$^{CreER/fl}$) embryos taken from dams gavaged at 8.5dpc for epithelial morphology across the anterior **(M,N**) to posterior **(S,T)** axis. **(S,T)** Quantification of **(U)** cleaved Caspase 3 and **(V)** Ki67 IF staining in E10.5 mouse embryonic foregut for cell death at the point of segregation of the dorsal and ventral foreguts in control (Cre-, *Sox2*$^{fl/+}$) and Sox2 cKO (Cre+, *Sox2*$^{CreER/fl}$) embryos taken from dams gavaged at 8.5dpc. Scale bar = 100 μm for **(A-D, G-H)**, 50 μm for **(I-T)**, and 25 μm for **(E-F)**. For Sox2-DE-LOF embryos, n=3 embryos of each genotype at E9.5, and n=2 embryos for each analysis for each genotype at E11.5 (a minimum of 2 litters were harvested for each analysis and time point). For Sox2-driven Sox2 cKO embryos, n=3 embryos for each genotype. Error bars indicate SD. *$p \leq 0.05$ for two-tailed t-test. fg = foregut, dfg = dorsal foregut, vfg = ventral foregut, ph = pharyngeal endoderm, eso = esophagus, r = respiratory progenitor, thy = thyroid, tr = trachea, br = bronchi, st = stomach.

Figure S7 Related to Figure 7.

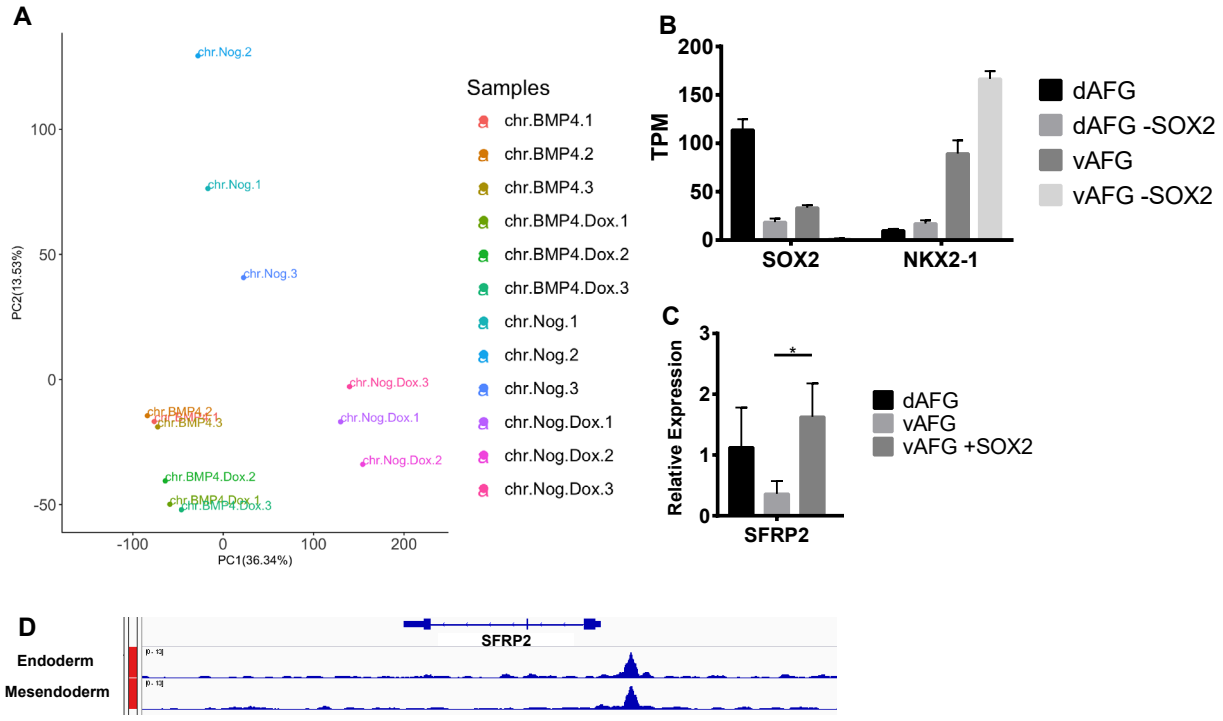

Figure S7 Related to Figure 7: Analysis of loss or gain of function on Sox2 in human cultures.
(A) Principal component analysis of the transcriptome resulting from day 9 anterior foregut cultures patterned along the dorsal-ventral axis with and without SOX2 (without or with Dox treatment to activate the CRISPR interference construct). Dorsal vs. ventral anterior foregut (dAFG vs. vAFG) is indicated as chr+Nog and chr+BMP4, respectively. Knockdown is indicated by the +Dox. **(B)** TPM values for *SOX2* and *NKX2-1*. **(C)** qPCR analysis for *SFRP2* on day 9 anterior foregut cultures patterned along the dorsal (dAFG) and ventral (vAFG) axis, including inducing exogenous HA-tagged SOX2 in ventral cultures by Dox treatment on day 8. **(D)** Genome browser view of Sox2 peaks at the *SFRP2* locus in hPSC-derived endoderm (GSM1505764) and mesendoderm (GSM1505767) from GEO dataset GSE61475 (Tsankov et al., 2015). Scale bar = 500 μm. Error bars indicate SD. *$p \leq 0.05$ for two-tailed t-test.

Table S1: List of primers used for qPCR analysis. Related to Figures 1-7.

Gene	Species	Strand	Sequence
DSG3	human	forward	CGT GGT TGT CTC CGC TAG AA
	human	reverse	CCG AGG TAG CAT TGA GGG TT
KRT5	human	forward	CTG GTC CAA CTC CTT CTC CA
	human	reverse	GGA GCT CAT GAA CAC CAA GC
FOXE1	human	forward	CGA CAA CCC CAA AAA GTG GC
	human	reverse	GCC CAG TAG TTG CCC TTA CC
TBX1	human	forward	GTC TAT GTG GAC CCA CGC AA
	human	reverse	CTG CGT GAT CCG ATG GTT CT
SFRP2	human	forward	CCA CCG AGG AAG CTC CAA A
	human	reverse	TTC AGG TCC CTT TCG GAC AC
SOX2	human	forward	GCT TAG CCT CGT CGA TGA AC
	human	reverse	AAC CCC AAG ATG CAC AAC TC
PAX9	human	forward	GGT AGG GTA AGG AGC CAT GC
	human	reverse	CTG GAG CAG GAA GCC AAG TA
GATA4	human	forward	TAG CCC CAC AGT TGA CAC AC
	human	reverse	GTC CTG CAC AGC CTG CC
KRT13	human	forward	AGG TGA AGA TCC GTG ACT GG
	human	reverse	GTT GTT TTC AAT GGT GGC G
KRT14	human	forward	GGC CTG CTG AGA TCA AAG AC
	human	reverse	TCT GCA GAA GGA CAT TGG C
IVL	human	forward	CTG CCT CAG CCT TAC TGT GA
	human	reverse	GGA GGA GGA ACA GTC TTG AGG
LEF1	human	forward	CAC TGT AAG TGA TGA GGG GG
	human	reverse	TGG ATC TCT TTC TCC ACC CA
TCF1	human	forward	GAC TTG ACC ATC TTC GCC AC
	human	reverse	CCT CAA AGA GCT GGA GAA CCT
HNF1B	human	forward	TCA CAG ATA CCA GCA GCA TCA GT
	human	reverse	GGG CAT CAC CAG GCT TGT A
PROX1	human	forward	GGC ATT GAA AAA CTC CCG TA
	human	reverse	ACA GGG CTC TGA ACA TGC AC
HNF6	human	forward	TGT TGC CTC TAT CCT TCC CA
	human	reverse	GGA GGA TGT GGA AGT GGC T
PDX1	human	forward	CGT CCG CTT GTT CTC CTC
	human	reverse	CCT TTC CCA TGG ATG AAG TC
(ΔN isoform) TP63	human	forward	AGC CAG AAG AAA GGA CAG CA
	human	reverse	TCG TGT ACT GTG GCT CAC TAA
RFX6	human	forward	CCA GTT TTT GAG CTA AGC GAA
	human	reverse	TGG CAT CAA AGA GAG CAG TG
MNX1	human	forward	CTG CCT AAG ATG CCC GAC T
	human	reverse	AGC TGC TGG CTG GTG AAG
NKX2-1 (TTF1)	human	forward	CTC ATG TTC ATG CCG CTC
	human	reverse	GAC ACC ATG AGG AAC AGC G

CDH1 (E-CAD)	human	forward	GAC CGG TGC AAT CTT CAA A
	human	reverse	TTG ACG CCG AGA GCT ACA C
AXIN2	human	forward	CTG GTG CAA AGA CAT AGC CA
	human	reverse	AGT GTG AGG TCC ACG GAA AC
KRT4	human	forward	CCT GAG ATC CAG AAA GTC CG
	human	reverse	TTC CAT TTG GTC TCC AGG AC
CDX2	human	forward	CTG GAG CTG GAG AAG GAG TTT C
	human	reverse	ATT TTA ACC TGC CTC TCA GAG AGC
NESTIN	human	forward	GAG GGA AGT CTT GGA GCC AC
	human	reverse	AAG ATG TCC CTC AGC CTG G
HOXA1	human	forward	GTA CGG CTA CCT GGG TCA AC
	human	reverse	ACT TGG GTC TCG TTG AGC TG
HOXB1	human	forward	AAC CCA CCC AAG ACA GCG AA
	human	reverse	CGC GCT TCT TCT GCT TCA TTC
CYP26C1	human	forward	GTT CCC TTC AGT GGC CTA CG
	human	reverse	ACA GCC GAC TCC TTC AGC TC
OTX2	human	forward	GGA AGC ACT GTT TGC CAA GAC C
	human	reverse	CTG TTG TTG GCG GCA CTT AGC T
CRNN	human	forward	TGT GAT TGT GAA ACC CCA CGA
	human	reverse	GCA CTC TCG CTC AGT GTC TT
TMPRSS11A	human	forward	GTC TCC TGG TTC ACT TCC TAG T
	human	reverse	GTG TTG CTT TGT CCG AAA TTG T
TMPRSS11D	human	forward	GCA GTC ACC ATA GCT CTA CTT G
	human	reverse	CCA CTC AAA GTC CTG TAT TCC TG
CPHA (PPIA)	human	forward	CCC ACC GTG TTC TTC GAC ATT
	human	reverse	GGA CCC GTA TGC TTT AGG ATG A

Table S2: List of antibodies used for immunofluorescence staining. Related to Figures 1-7.

Antibody	Company	Catalog Number	RRID Number	Dilution
Goat anti-Sox2	Santa Cruz Biotechnology	#sc-17320 (Y-17)	RRID:AB_2286684	1:250
rabbit anti-Sox2	Abcam	#ab97959	RRID:AB_2341193	1:1000
rabbit anti-p63	Santa Cruz Biotechnology	#sc-8343	RRID:AB_653763	1:200
mouse anti-HNF1b	BD Transduction Laboratories	#612504	RRID:AB_399805	1:500
rat anti-E-cadherin	R&D Systems	#MAB7481	RRID:AB_2076679	1:1000
goat anti-E-cadherin	R&D Systems	#AF648	RRID:AB_355504	1:1000
mouse anti-E-cadherin	BD Transduction Laboratories	#610182	RRID:AB_397581	1:500
rabbit anti-Nkx2.1	Abcam	#ab76013	RRID:AB_1310784	1:1000
rat anti-Krt8	DSHB	#TROMA-I-S	RRID:AB_531826	1:100
mouse anti-Krt4	Abcam	#ab9004	RRID:AB_306932	1:200
rabbit anti-Krt13	Abcam	#ab92551	RRID:AB_2134681	1:1000
rabbit anti-Krt14	BioLegend	#905301 (PRB-155P)	RRID:AB_2565048	1:2000
rabbit anti-Ki67	Cell Marque	#275R-15 (SP6)	RRID:AB_1158037	1:100
rabbit anti-Ivl	Atlas Antibodies	#HPA055211	RRID:AB_2682739	1:250
rabbit anti-Dsg3	Cell Marque	#436R-15		1:200
goat anti-FoxA2	Santa Cruz Biotechnology	#sc-6554	RRID:AB_2262810	1:500
rat anti-HA-Biotin	Sigma-Aldrich (Roche)	#12158167001 (3F10)	RRID:AB_390915	1:300
goat anti-Pdx1	Abcam	#ab47383	RRID:AB_2162359	1:5000
goat anti-Gata4	Santa Cruz Biotechnology	#sc-1237	RRID:AB_2108747	1:200
rabbit anti-Caspase 3 (cleaved)	Cell Signaling	#9661	RRID:AB_2341188	1:200
rabbit anti-Cdx2	Cell Marque	#235R-15 (EPR2764Y)	RRID:AB_1516799	1:200
mouse anti-Cdx2	BioGenex	#cdx2-88	RRID:AB_2650531	1:300
rabbit anti-B-catenin	Santa Cruz	#sc-7199	RRID:AB_634603	1:100
mouse anti-Filaggrin	Santa Cruz	#sc-66192	RRID:AB_1122916	1:200
goat anti-Cornulin	R&D Systems	#AF3607	RRID:AB_2085498	1:200
anti-DIG alkaline phosphatase	Sigma-Aldrich	#11093274910	RRID:AB_514497	1:5000
AlexaFluor Donkey anti-goat 488	Thermo Fisher Scientific	#A11055	RRID:AB_2534102	1:500
AlexaFluor Donkey anti-goat 568	Thermo Fisher Scientific	#A11057	RRID:AB_2534104	1:500
AffiniPure Donkey anti-mouse 647	Jackson ImmunoResearch laboratories	#715-605-150	RRID:AB_2340862	1:500
AlexaFluor Donkey anti-mouse 568	Thermo Fisher Scientific	#A10037	RRID:AB_2534013	1:500
AlexaFluor Donkey anti-rabbit 647	Thermo Fisher Scientific	#A31573	RRID:AB_2536183	1:500
AlexaFluor Donkey anti-rabbit 546	Thermo Fisher Scientific	#A10040	RRID:AB_2534016	1:500
AffiniPure Donkey Anti-Rat 647 IgG	Jackson ImmunoResearch laboratories	#712-605-153	RRID:AB_2340694	1:500