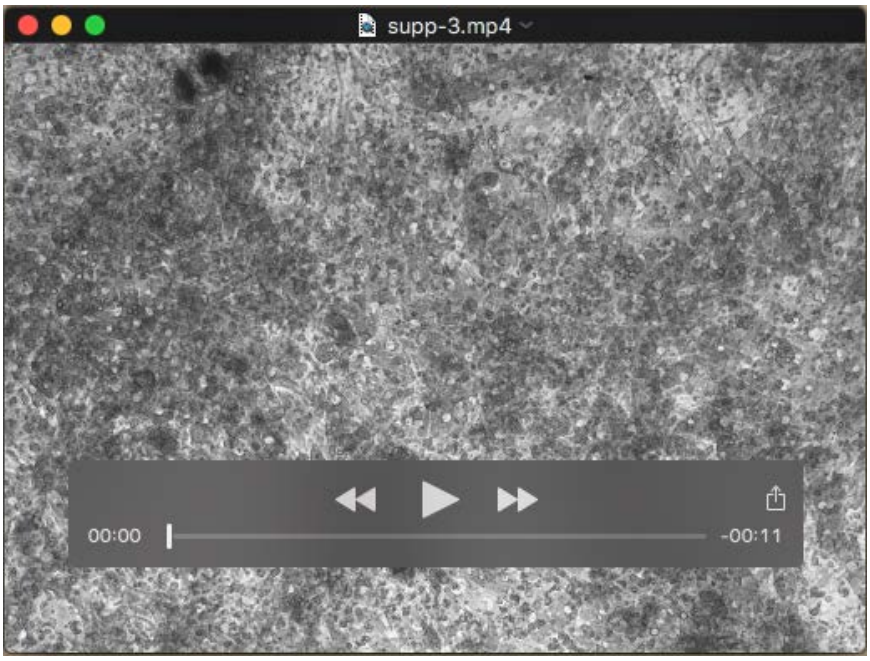
SUPPLEMENTARY INFORMATION



Movie 1. Reset cR-H9-EOS cells after formative transition and differentiation to myotubes for 40 days.

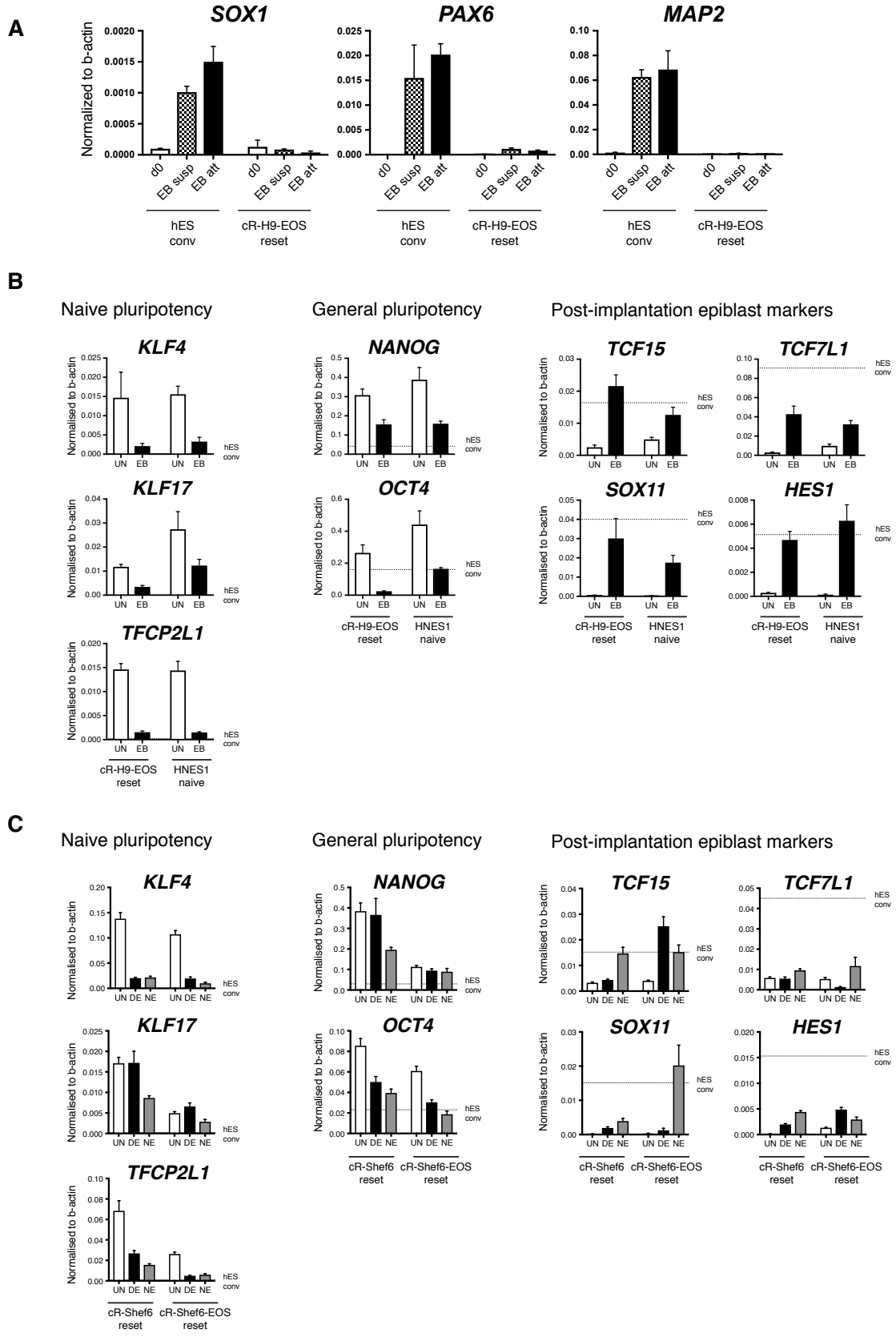


Figure S1. Human naïve PSC do not respond to somatic differentiation protocols. (A) Neuroectodermal markers are not induced directly from the naïve state. RT-qPCR analysis of neuroectodermal markers in aggregates cultured in suspension for 14 days (susp) or plated after 7 days in suspension and outgrown in adherent culture until day 14 (att). (B, C) Expression of markers after human naïve PSC were exposed to differentiation conditions: (B) embryoid bodies for 14 days, (C) monolayer

induction of definitive endoderm for 3 days and neuroectoderm for 10 days. Expression level in conventional hESC is indicated as dotted line.
UN – undifferentiated, EB – embryoid bodies, DE – definitive endoderm, NE – neuroectoderm.
The data in Fig. S1B, S1C are derived from experiments also presented in Figure 1B, 1D, 1F. Expression of NANOG and OCT4 in Fig. S1B is the same as in Figure 1B.

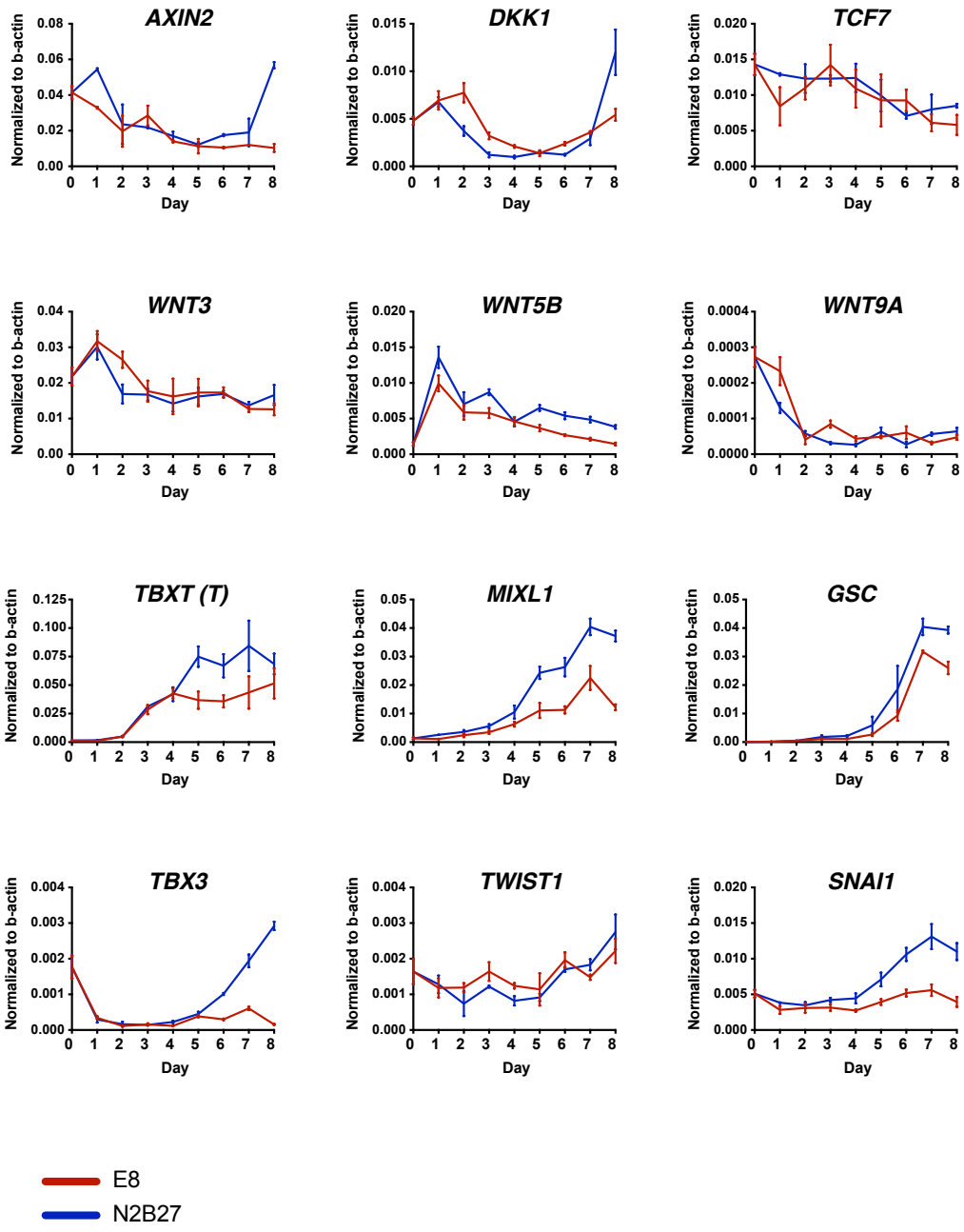


Figure S2. Expression of WNT signalling pathway members and targets in N2B27 or E8
RT-qPCR analysis

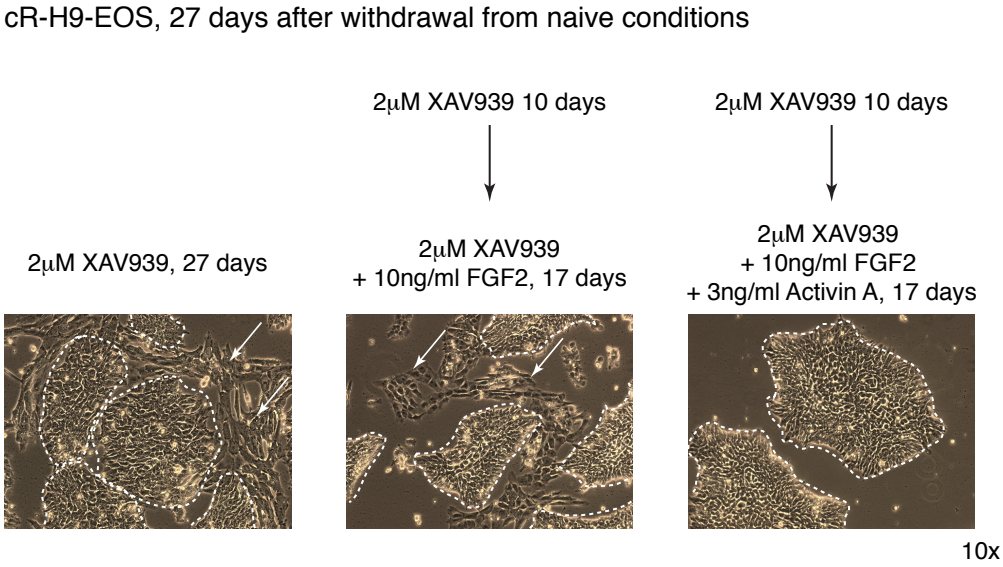


Figure S3. hPSC can be maintained after formative transition
Images of cR-H9EOS cells cultured after 10 days transition in XAV939 for 27 days in total in the indicated conditions, 10x. Arrows indicate spontaneously differentiated cells, dotted contours outline undifferentiated colonies.

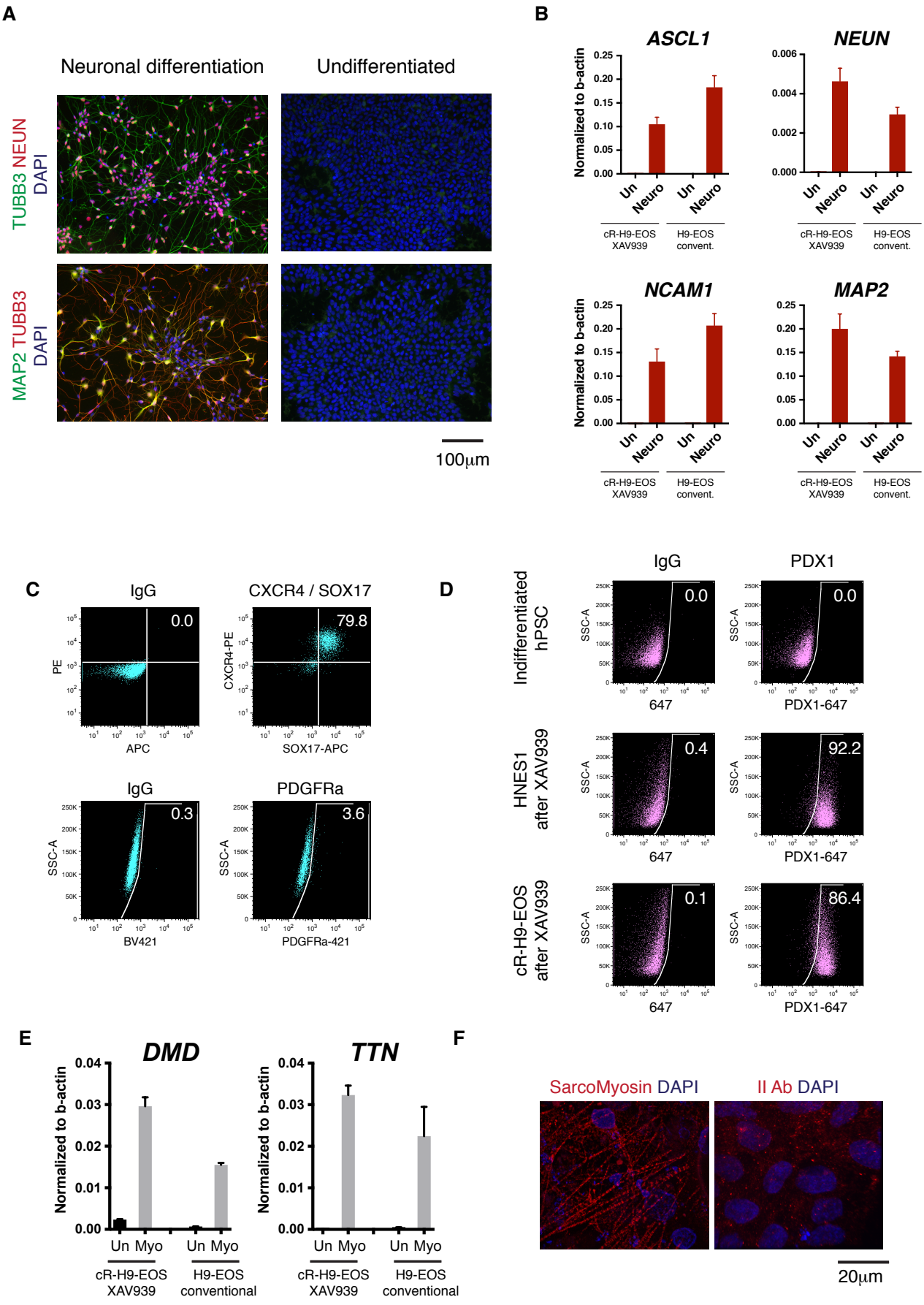


Figure S4. Further differentiation after formative transition in XAV939.

(A) Immunostaining for neuronal markers of capacitated cells differentiated to neurons and undifferentiated (capacitated cells which were not induced to differentiation). (B) RT-qPCR analysis of neuronal marker expression. (C) Flow cytometry analysis for CXCR4, SOX17 and PDGFRa upon induction of definitive endoderm following capacitation in XAV939. (D) Intracellular flow cytometry analysis for PDX1 in capacitated naïve hPSC subjected to definitive endoderm induction and subsequent differentiation to foregut endoderm and undifferentiated (capacitated cells which were not induced to differentiation). (E) Expression of sarcomeric markers in myotubes differentiated from capacitated naïve and conventional hPSC. (F) Immunostaining for sarcomeric myosin (MF20 antibody) in myotubes differentiated from capacitated naïve hPSC; control staining with secondary antibody only.

Un – undifferentiated, Neuro – neurons, Myo – myotubes.

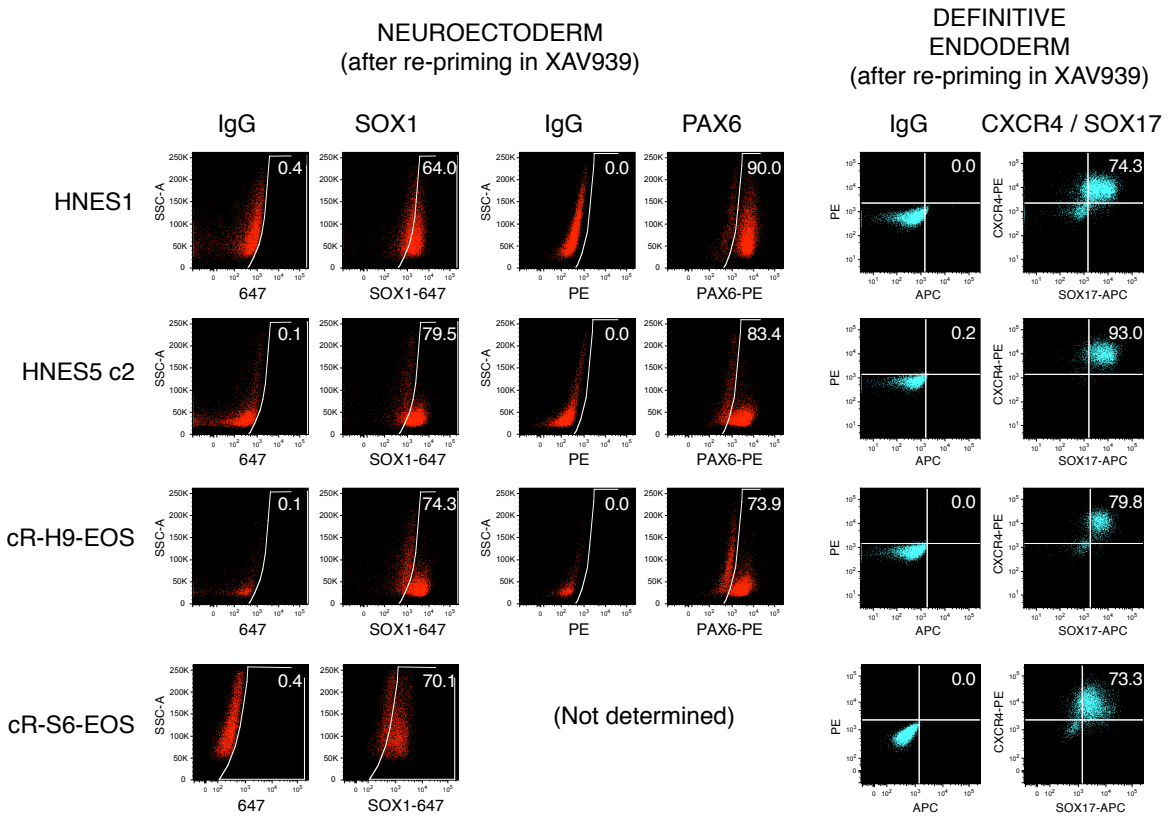


Figure S5. Differentiation competence of various naïve hPSC after capacitation in XAV939.

Neuroectoderm induction was analysed by flow cytometry for PAX6 and SOX1. Definitive endoderm induction was analysed by flow cytometry for CXCR4 and SOX17.

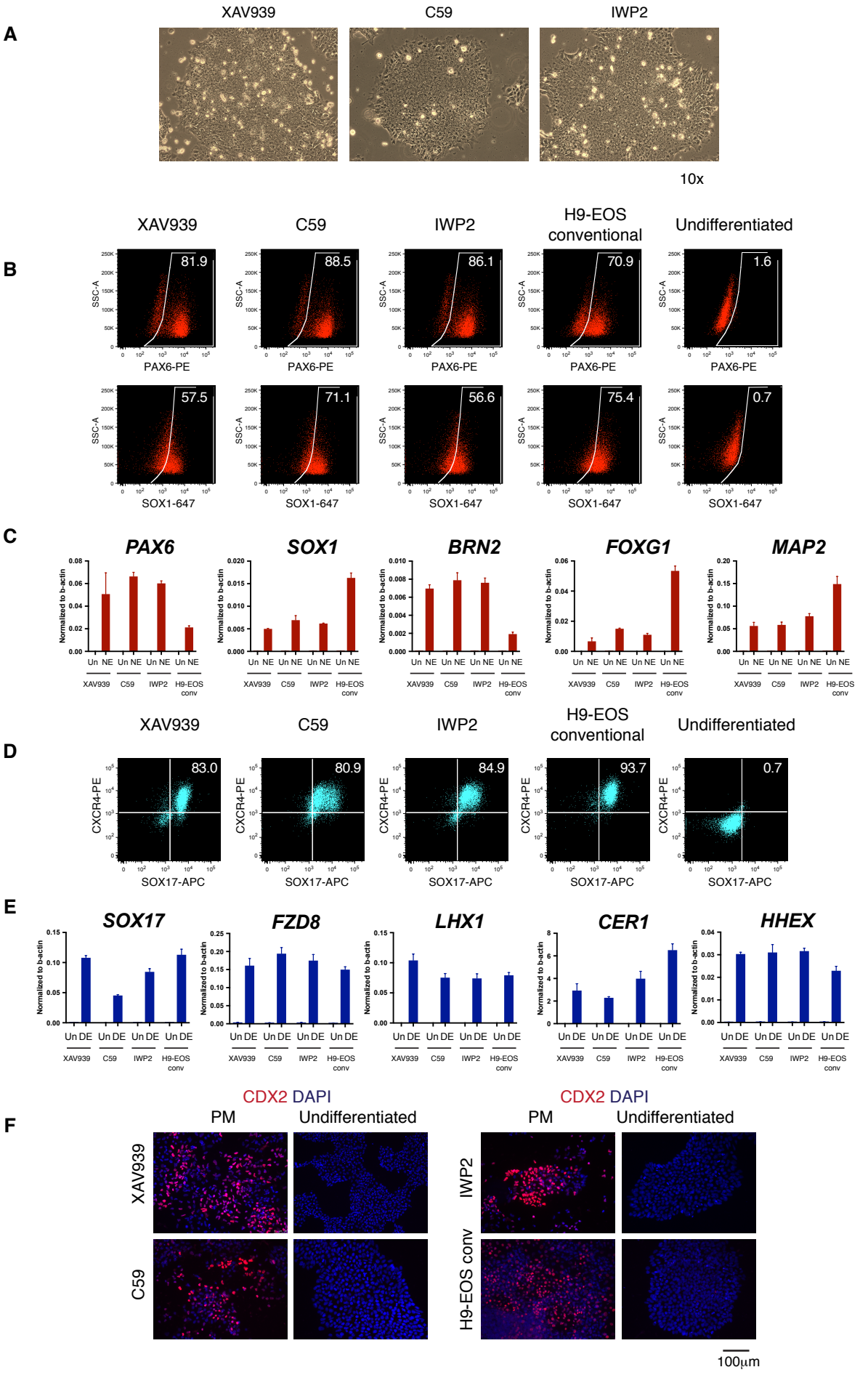


Figure S6. Formative transition using different WNT pathway inhibitors.

(A) Cell morphology after formative transition in XAV939, C59 or IWP2 for 12 days (bright field, 10x). (B,C) Neuroectoderm induction after capacitation in XAV939, C59 or IWP2, analysed by: (B) flow cytometry for PAX6 and SOX1; (C) RT-qPCR. (D,E) Definitive endoderm induction after capacitation in XAV939, C59, IWP2, assayed by: (D) flow cytometry for CXCR4 and SOX17; (E) RT-qPCR. (F) Immunostaining for CDX2 after paraxial mesoderm induction of cells capacitated in XAV939, C59, IWP2. Undifferentiated cells (capacitated cells which were not induced to differentiation) were used as a control in (B-F).

NE – neuroectoderm, DE – definitive endoderm, PM – paraxial mesoderm, Un – undifferentiated.

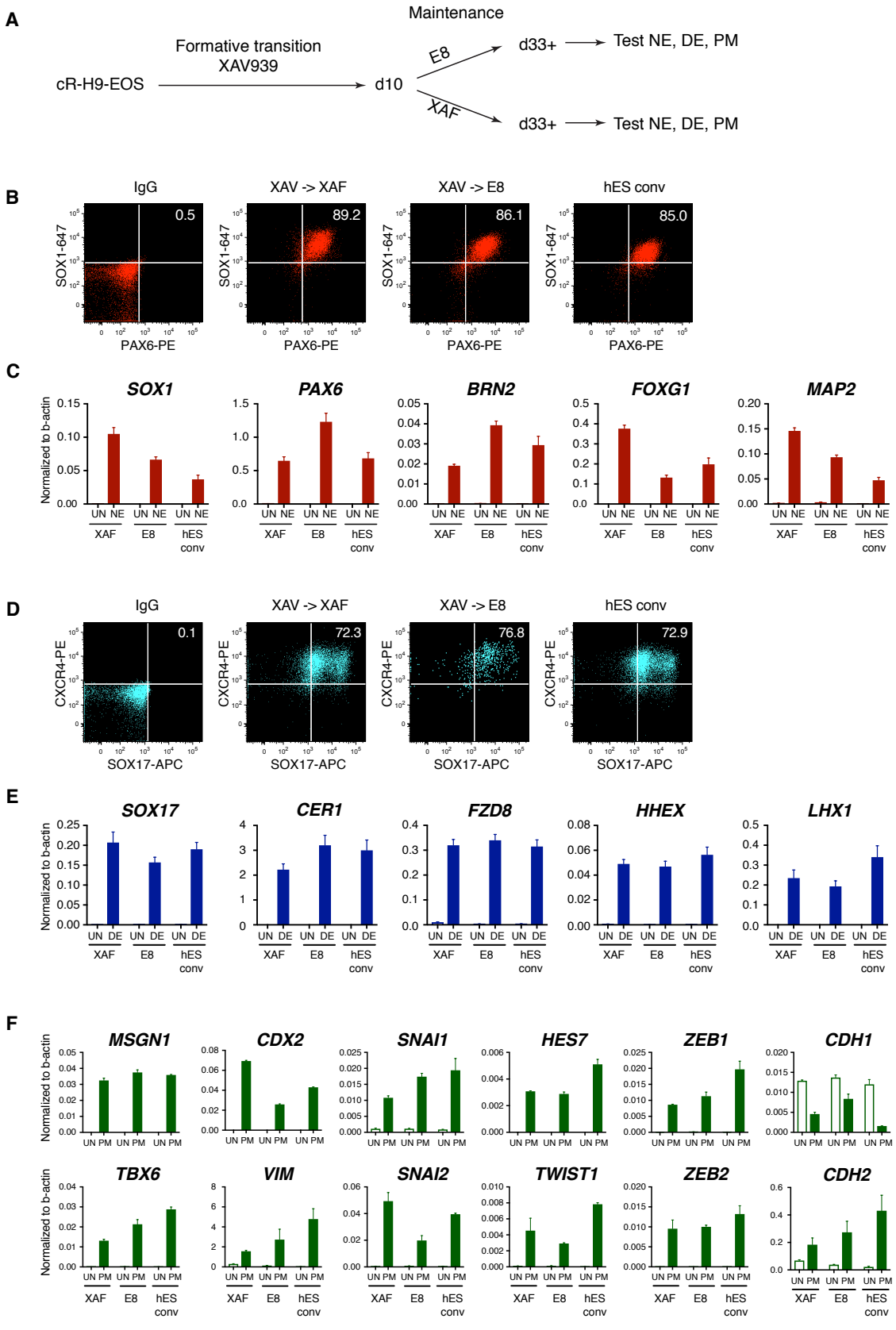


Figure S7. Differentiation capacities of PSC after capacitation in XAV939 followed by extended maintenance.

(A) Experimental setup. (B, C) Neuroectoderm differentiation analysed by: (B) flow cytometry for PAX6 and SOX1 and (C) RT-qPCR. (D,E) Definitive endoderm differentiation analysed by: (D) flow cytometry for SOX17 and CXCR4 and (E) RT-qPCR. (F) Paraxial mesoderm induction analysed by RT-qPCR.

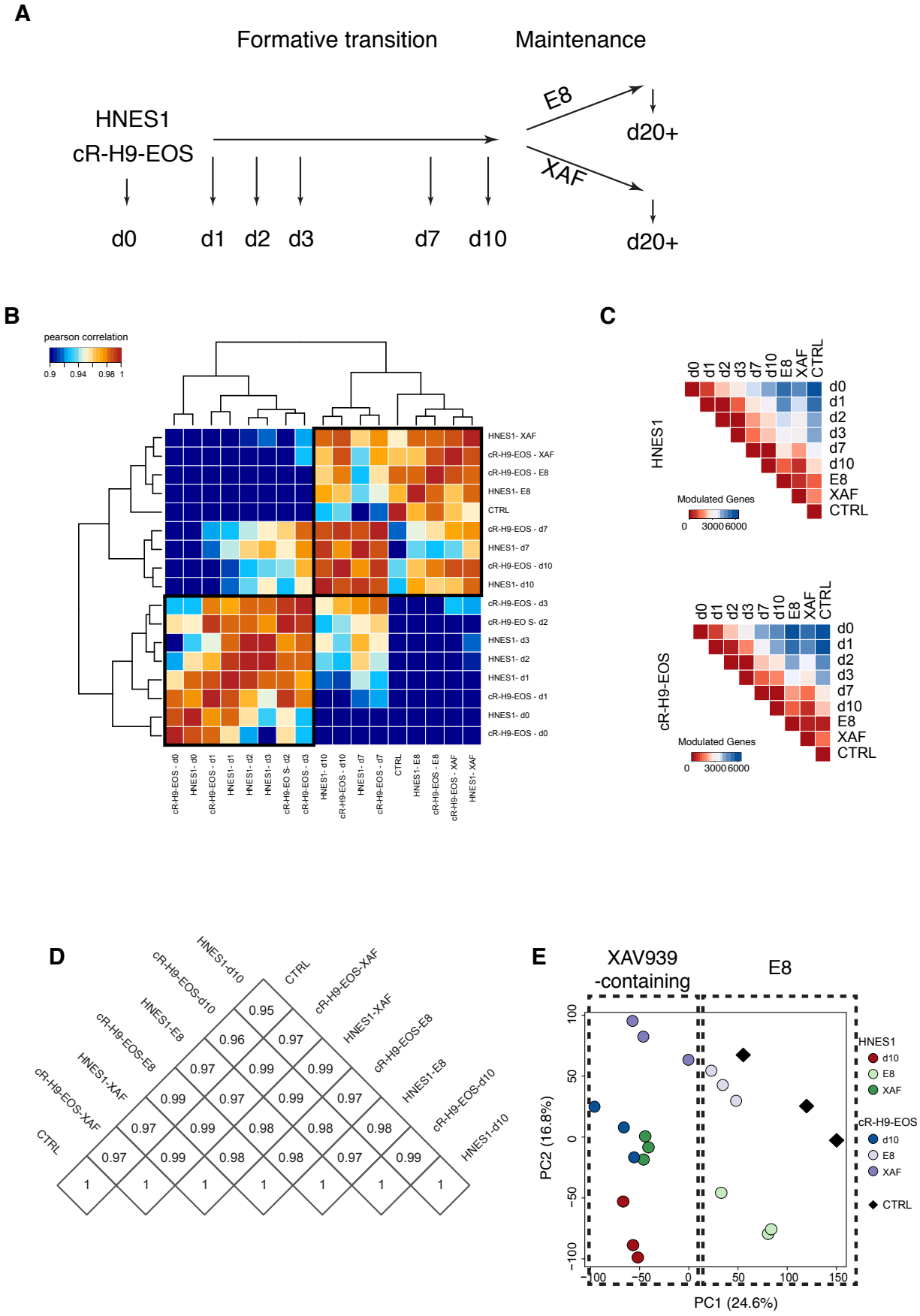


Figure S8. Transcriptome profiling of naïve hPSC during formative transition and maintenance.

(A) Experimental setup of global gene expression analysis during formative transition. (B) Heatmap showing correlations between samples during capacitation, after prolonged culturing, and with conventional H9EOS hPSC. Clusters of similar samples are outlined. (C) Heatmaps showing numbers of modulated genes in pair-wise comparisons between all points of the time course, CTRL – conventional H9-EOS. (D) Analysis of correlation coefficients after further culture in XAF or E8, and H9-EOS conventional cells and (E) PCA plot performed with all expressed genes.

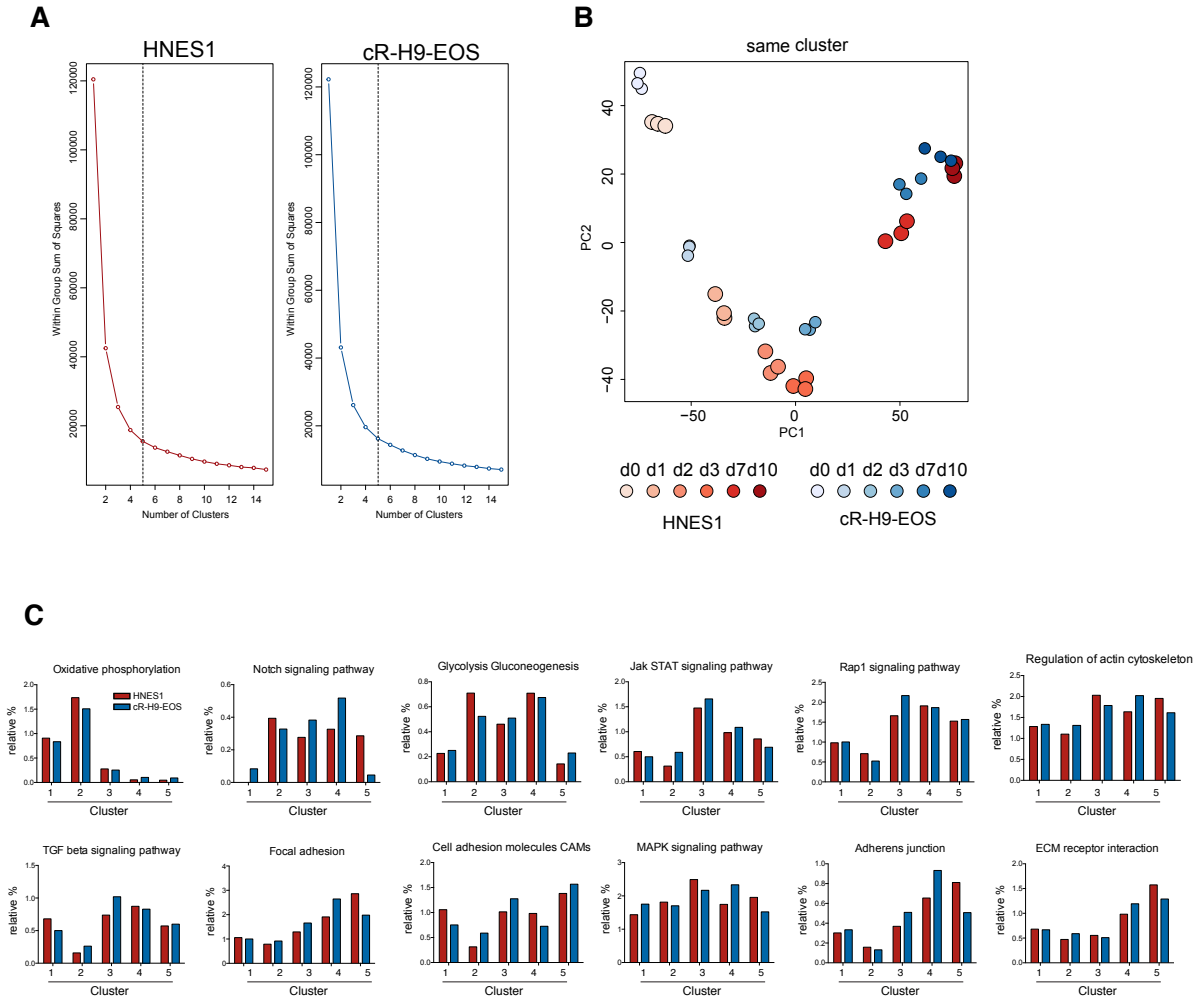


Figure S9. Clustering analysis of variable genes during formative transition
(A) Soft clustering analysis was performed for the set of genes variable between any two time points during the transition and in at least one of the cell lines. Number of clusters was defined using elbow method. (B) PCA of samples during formative transition, performed with genes belonging to the same clusters in HNES1 and cR-H9EOS. (C) Representation of KEGG pathways within clusters of variable genes. The bars indicate proportions of genes of each cluster belonging to indicated pathways.

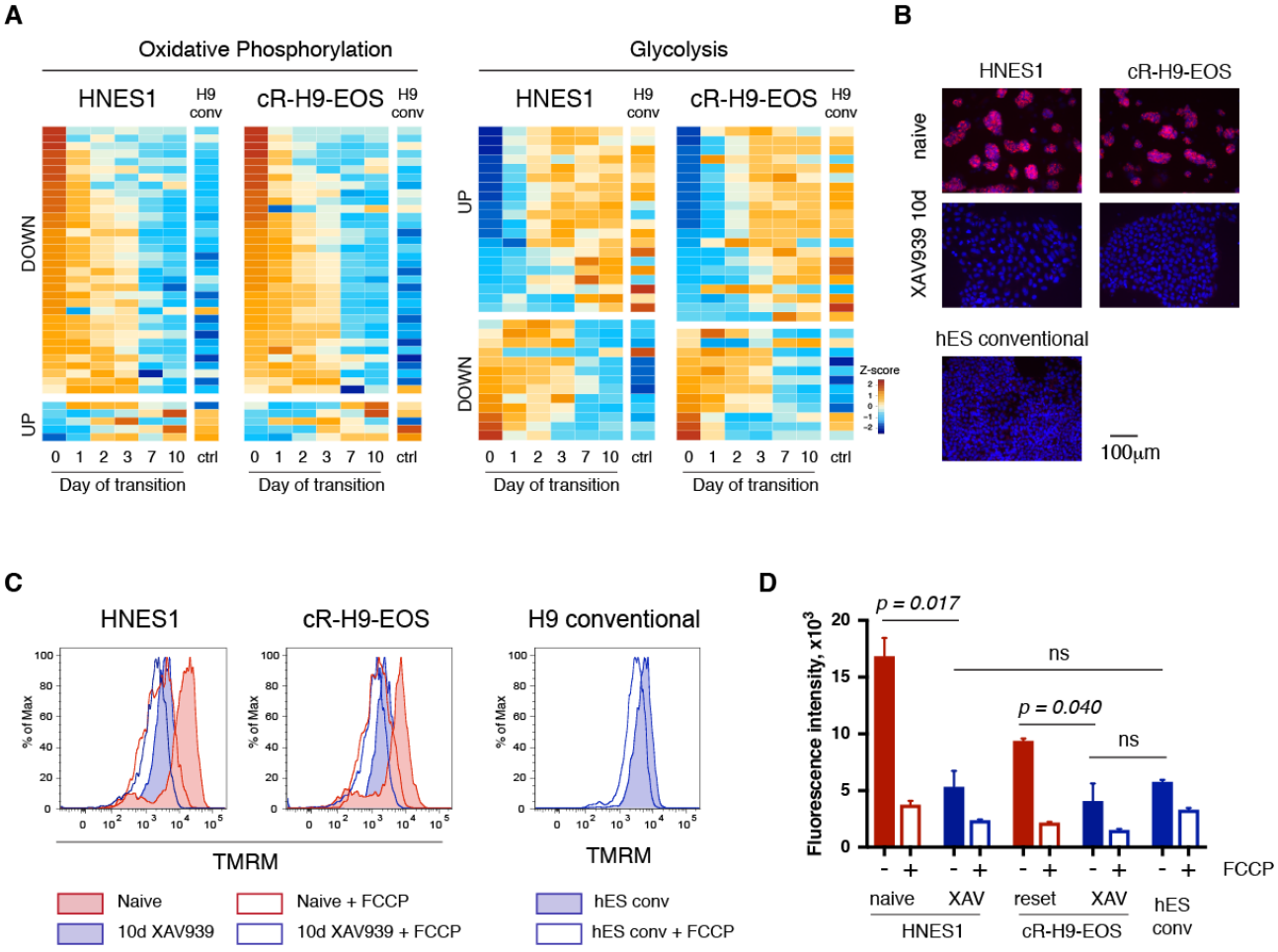


Figure S10. Mitochondrial membrane potential reduces during formative transition.

(A) Heatmaps showing genes involved in oxidative phosphorylation and glycolysis (according to KEGG pathways annotation), that are differentially expressed during the formative transition. The genes were ordered according to Z score. (B) Fluorescent images showing TMRM staining in naïve HNES1 and reset cR-H9-EOS before and after the formative transition, as compared to conventional hPSC. Counterstaining Hoechst33342. (C) Flow cytometry results of TMRM staining in naïve and reset PSC, capacitated PSC, conventional hPSC, measured in the presence or absence of FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone), an uncoupling agent of mitochondrial oxidative phosphorylation. (D) Quantitation of flow cytometry results as in (C), shown as fluorescence intensity, the values were derived from independent formative transitions for each cell line. *P*-values were calculated using Student's two-tailed t-test.

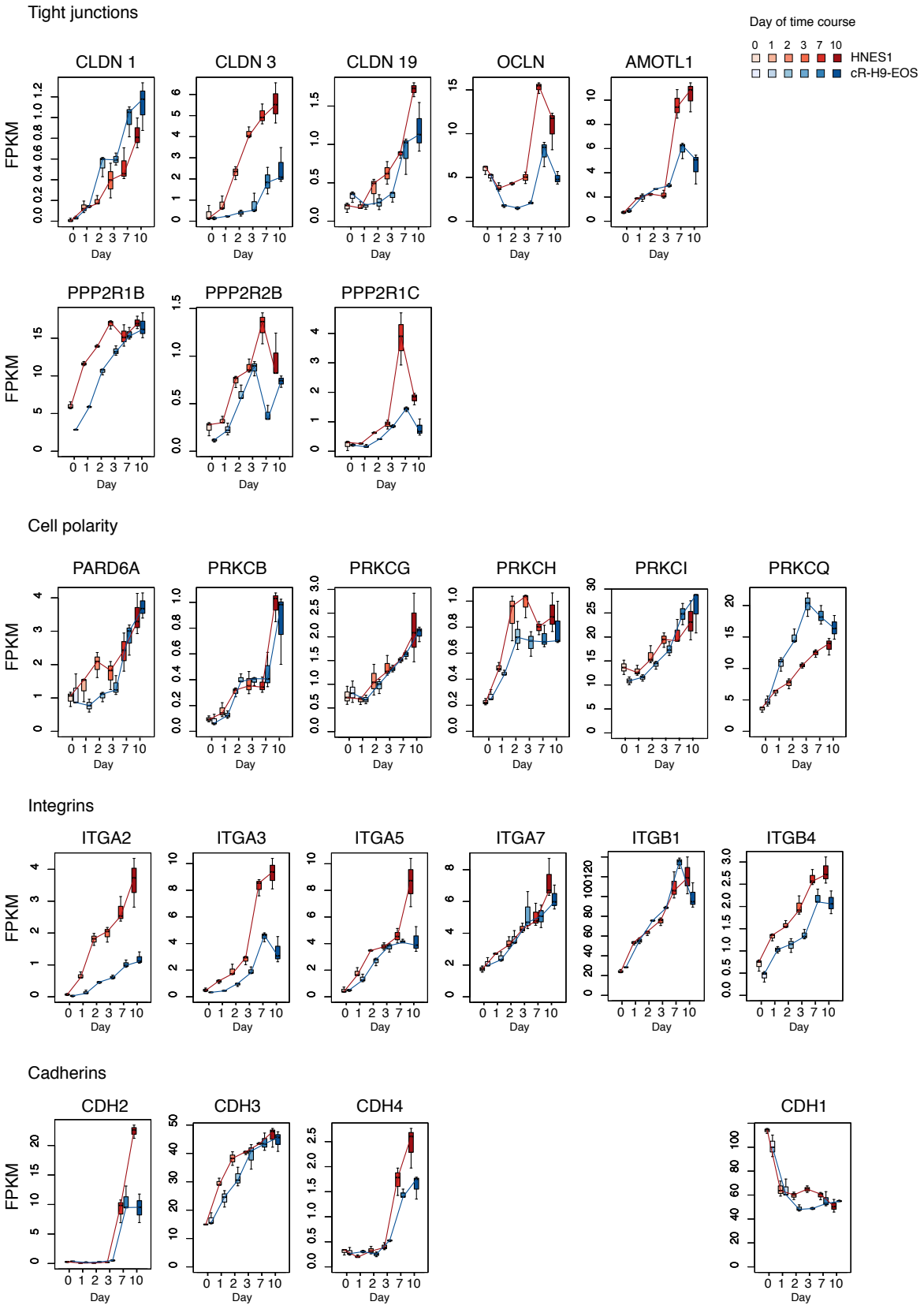


Figure S11. RNAseq expression values for epithelial markers during the formative transition.

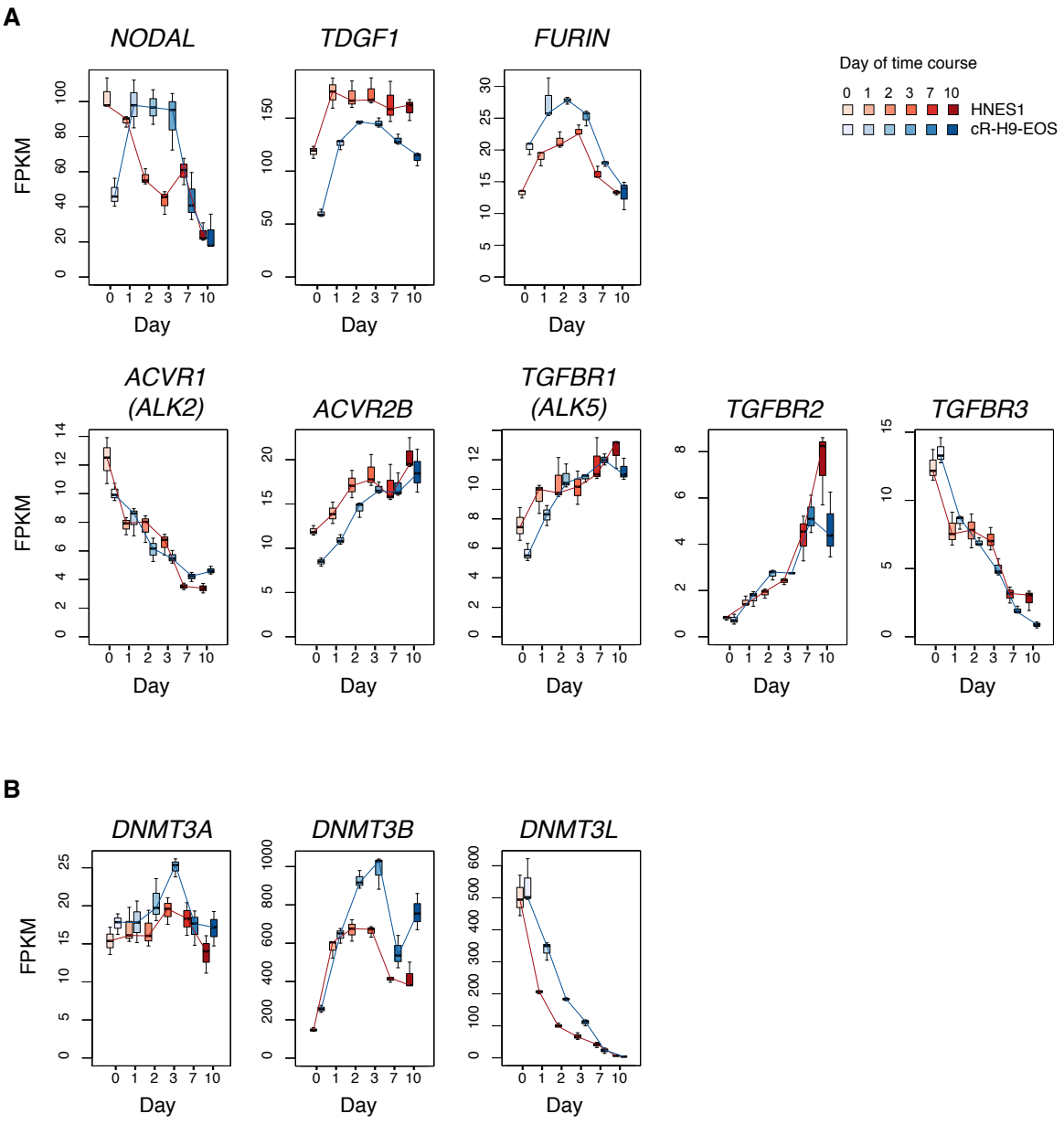


Figure S12 Expression of selected genes during the formative transition
(A) RNAseq expression values for genes associated with TGFb pathway. *INHBA*, *INHBB*, *TGFB1*, *PACE4*, *ACVR1B* (*ALK4*), *ACVR1C* (*ALK7*), *ACVR2A* showed very low or undetectable levels. (B) *DNMT3A*, *DNMT3B* and *DNMT3L* expression during formative transition.

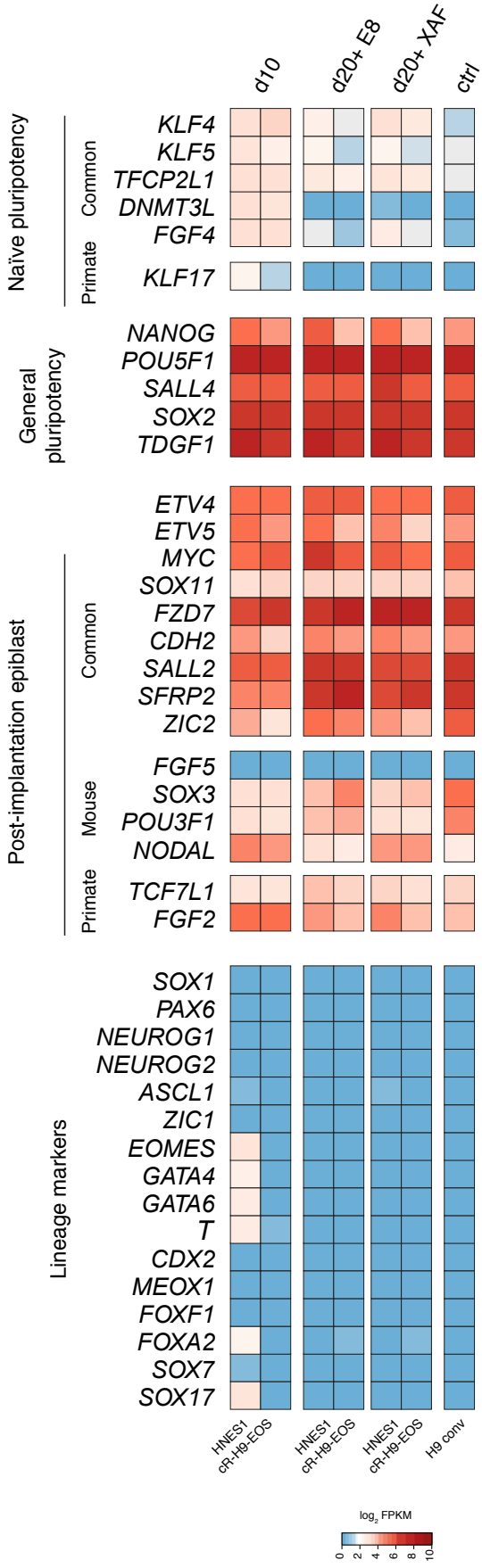


Figure S13. Expression of selected genes in PSC after 10 days of capacitation in XAV939, after further maintenance in E8 or XAF, and conventional H9 hES.

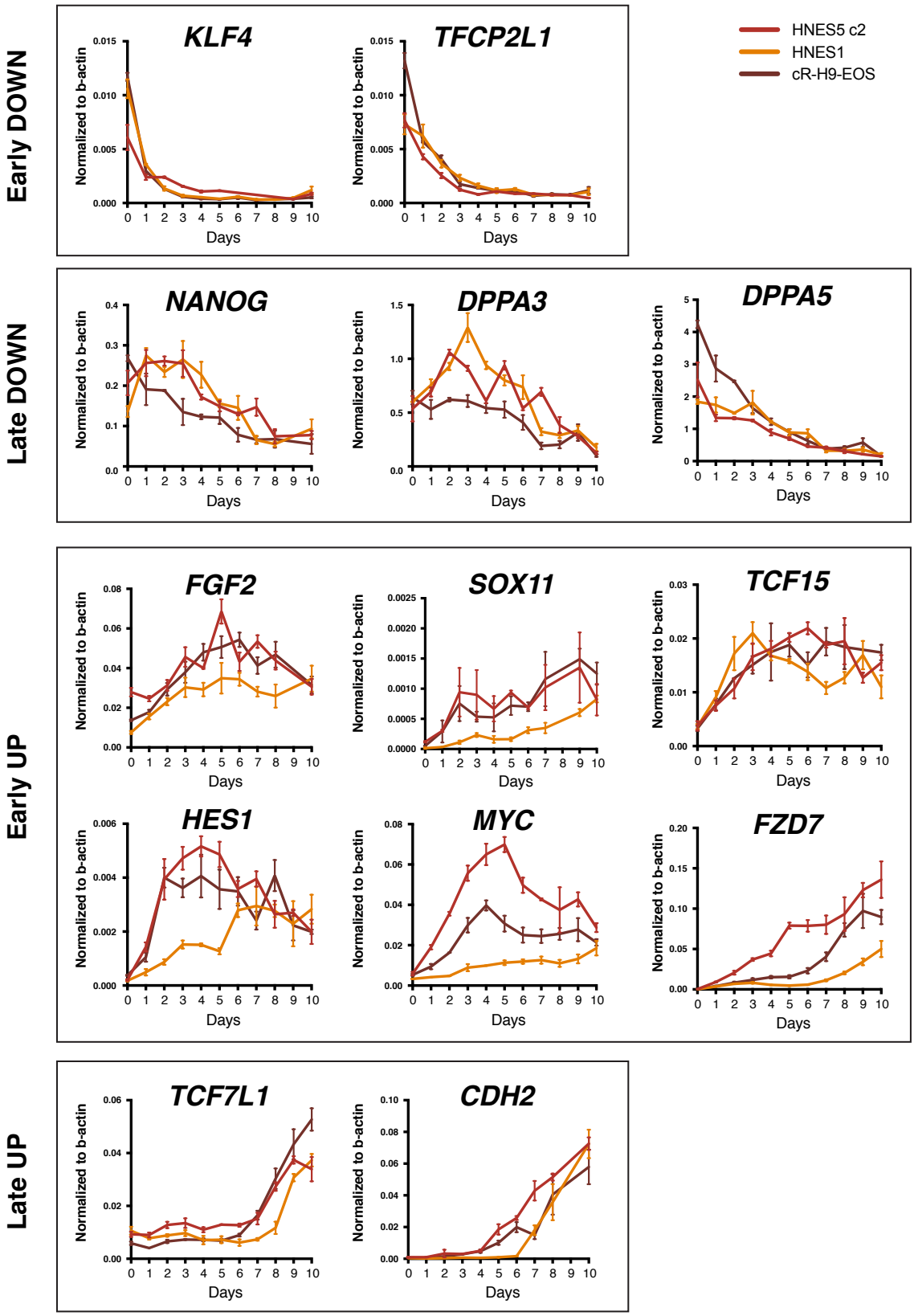


Figure S14. Confirmation of selected gene expression dynamics during formative transition

Expression of genes with different dynamics according to RNAseq was evaluated by RT-qPCR in an independent experiment using HNES1, HNES5c2 and cR-H9-EOS.

Table S1. Summary of capacitation experiments on different cell lines

Naïve cell line	Parental cell line	WNT inhibitor	No. of independent experiments	Differentiation tests after capacitation
Embryo-derived HNES				
HNES1	N/A	XAV939	14	NE, DE, PM
		C59	2	NE, DE, PM
		IWP2	1	NE, DE, PM
HNES5c1	N/A	XAV939	1	DE
HNES5c2	N/A	XAV939	5	NE, DE
Reset hESC				
cR-S6-EOS-vpc	Shef6-EOS*	XAV939	1	NE, DE
cR-S6-EOS-vpcx	Shef6-EOS*	XAV939	1	NE, DE
cR-S6-EOS-vpcy	Shef6-EOS*	XAV939	1	NE, DE
cR-H9-EOS-PC25B	H9-EOS-1*	XAV939	2	NE, DE
cR-H9-EOS-vpc	H9-EOS-2*	XAV939	1	NE, DE
cR-H9-EOS-vpcx	H9-EOS-2*	XAV939	11	NE, DE, PM
		C59	2	NE, DE, PM
		IWP2	2	NE, DE, PM
Reset hiPSC				
cR-LQT1 #26	LQT1 #26	XAV939	1	-
cR-LQT1 #26-9.3-C6	LQT1 #26-9.3-C6	XAV939	1	DE

* chemically reset lines derived from the same parental cell line in independent resetting experiments are shown separately
N/A – not applicable

NE – neuroectoderm, DE – definitive endoderm, PM – paraxial mesoderm

Table S2. List of primers.

mRNA	Forward (5'-3')	Reverse (5'-3')	Product size	UPL probe
OCT4	cttcgcaagccctcatttc	gagaaggcgaaatccgaag	88	66
NANOG	agatgcctcacacggagact	tttgcgacactcttctctgc	127	31
SOX2	tgctgcctctttaagactaggac	cctggggctcaaacttctct	75	35
KLF4	gggagaagacactgcgtca	ggaagcactgggggaagt	88	52
TFCP2L1	gctcttcaacgccatcaaa	caggggcactcgattctg	88	17
KLF17	ctcctgctgctggtccttag	acagttgccacgtccagtg	74	64
DPPA3	aatgctagaatagggaatcaagaca	agcatagagtagctttctcaacctg	114	41
AXIN2	tctgtggggaagaaattccata	caaactcatcgcttgctttttt	129	88
DKK1	ttctccctcttgagtccttctg	ctaccatcgcgacaaagacc	76	21
TCF7	ccctgacctctctggcttct	tcaaggatgggtgggtga	89	27
WNT3	cctgcaagtagggcacca	cccatgagacttcgctgaat	74	5
WNT5B	ctgctgctgctgttcacg	ccgggttcaaagctaatgac	91	56
WNT9A	cgtcttgtgggtgtcctga	cctgtatccataccagcaacc	64	23
MIXL1	ggtaccccgacatccactt	gcctgttctggaaccatacct	87	32
GSC	cctccgcgaggagaaagt	cgttctccgactcctctgat	92	29
TBX3	gcagctttcaactgcttcg	accctcgctgggacataaat	90	19
TWIST	gggccggagacctagatg	tttccaagaaaatctttggcata	112	50
TCF7L1	ccatgaacgcctcgatgt	gagccaccatgtgaggaga	60	54
FGF2	cacttcaaggaccccaagc	cctctctcttctgcttgaagttg	137	4
SOX11	tccgtgctggtgattttatg	acgttgccaacaagtttcg	61	84
TCF15	tgttccgggacactctgg	caggctgaatggatcctcac	78	80
HES1	gtgaagcacctccggaac	gtcacctcgttcatgcactc	113	60
MYC	tgctccatgaggagacacc	cttttccacagaaacaacatcg	92	77
FZD7	gccagcttgtgcctaatagaa	agccgggagaaactcacag	61	54
SOX17	acgccgagttgagcaaga	tctgcctcctccacgaag	82	61
HHEX	cggacggtgaacgactaca	agaaggggctccagagtagag	76	61
LHX1	atgcaacctgaccgagaagt	caggtcgctaggggagatg	121	80
CER1	gccatgaagtacattgggaga	cacagccttcgtgggttatag	69	41
FZD8	cgccacgcgttaatttct	ccggttctggaaccacac	135	19
SOX1	accaggccatggatgaag	cttaattgctggggaattgg	67	37
PAX6	ggcacacacacattaacacactt	ggtgtgtgagagcaattctcag	71	9
BRN2	aataaggcaaaaggaaagcaact	caaaacacatcattacacctgct	72	57

ASCL1	gctcttacgacccgctca	atgcaggttgtgcgatca	129	15
MAP2	actgcagctctgcctttagc	gacagtctgttctgaggcaggt	84	16
NEUN	ccaccattttcccaggtct	attttccccgaggcactct	96	1
NCAM1	cgaccatccacctcaaagtc	cggaggcttcacaggtaaga	105	68
FOXG1	atgatccccaagtcctcgtt	gtggtggttgtcgttctgg	69	64
VIM	tgagattgccacctacaggaa	gagggagtgaatccagattagttt	113	11
TBX6	gaacggcagaaactgtaagagg	gtgtgtctccgctcccatag	101	5
MSGN1	agctcaggatgaggaccttg	ctggcctctctggctgtaga	80	87
HES7	gcagcctggaagagctga	acggcgaactccaatatctc	100	78
CDH1	ggtctgtcatggaaggtgct	gatggcggcattgtaggt	95	5
CDH2	tgcacagatgtggacaggat	ccacaaacatcagcacaagg	106	15
SNAI1	gcgagctgcaggactctaat	cggtggggttgaggatct	102	62
ZEB1	agcacttaagaattcacagtggag	catttcttactgcttatgtgtgagc	106	36
ZEB2	cgcagtgacacagccattat	gttccaggtggcaggtca	104	17
TBXT	gctgtgacaggtacccaacc	catgcaggtgagttgtcagaa	106	23
TTN	ccaagttacccatggaaaggt	actcgggcctggtctactg	84	41
DMD	ctgcgtggatatgtgtctgaa	acacggatcctccctgttc	69	66

Table S3. Taqman probes (all from ThermoFisher Scientific)

KLF4	Hs00358836_m1
KLF17	Hs00703004_s1
TFCP2L1	Hs00232708_m1
DPPA5	Hs00988349_g1
NANOG	Hs04399610_g1
ACTB	Hs01060665_g1
GAPD (GAPDH)	4352934E

Table S4. Antibodies for flow cytometry.

Antibody	Cat. Number	Company	Dilution
PE conjugated mouse IgG2a anti-human CD184 (CXCR4)	555974	BD Pharmingen	1:25
APC conjugated goat polyclonal anti-human SOX17	IC1924A	RnD Bio-Techne	1:100
BV421 conjugated mouse IgG2a anti-human CD140a (PDGFRa)	562799	BD Biosciences	1:100
Alexa647 conjugated mouse IgG1 anti-human SOX1	562224	BD Biosciences	1:100
PE conjugated mouse IgG2a anti-human PAX6	561552	BD Biosciences	1:100
Mouse monoclonal IgG2a anti-human PDX1	MAB2419	RnD Bio-Techne	1:1000

Table S5. Antibodies for immunostaining.

Antibody	Cat. number	Company	Dilution
Polyclonal goat IgG anti-human HNF-3b/FOXA2	AF2400	RnD Bio-Techne	1:50
Polyclonal goat IgG anti-human SOX17	AF1924	RnD Bio-Techne	1:100
Polyclonal goat IgG anti-human SOX1	AF3369	RnD Bio-Techne	1:100
Polyclonal rabbit IgG anti-PAX6	AB2237	Merck Millipore	1:500
Polyclonal rabbit IgG anti-human MAP2	4542	Cell Signaling Technology	1:100
Polyclonal rabbit IgG anti-human NEUN	ABN78	Merck Millipore	1:100
Monoclonal mouse IgG2a anti-human β-Tubulin 3 (TUBB3)	T8578	Sigma-Aldrich	1:1000
Polyclonal rabbit IgG anti-human CDX2	3977S	Cell Signaling Technology	1:100
Polyclonal rabbit IgG anti-human TBX6	ab38883	Abcam	1:400
Polyclonal rabbit IgG anti-human NANOG	ab21624	Abcam	1:100
Monoclonal mouse IgG1 anti-human NANOG	14-5768-82	eBiosciences ThermoFisher Scientific	1:300
Polyclonal goat IgG anti-human/mouse Brachyury (TBXT)	AF2085	RnD Bio-Techne	1:250
Monoclonal mouse IgG2b anti-human OCT4, C-10	sc-5279	Santa Cruz	1:100
Polyclonal rabbit IgG anti-human GKLF (KLF4), H-180	sc-20691	Santa Cruz	1:300
Polyclonal rabbit IgG anti-human KLF17	HPA024629	Atlas Antibodies	1:300
Monoclonal mouse IgG2b anti-sarcomere myosin	MF20	DSHB	1:10

Table S6.
Excel spreadsheet showing expression values for genes involved in glycolysis and oxidative phosphorylation, derived from KEGG annotation. Log FPKM values.

Click here to Download Table S6