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Supplemental Information

Gastruloid Development Competence Discriminates Different States of Pluripotency

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Supplemental Experimental Procedures

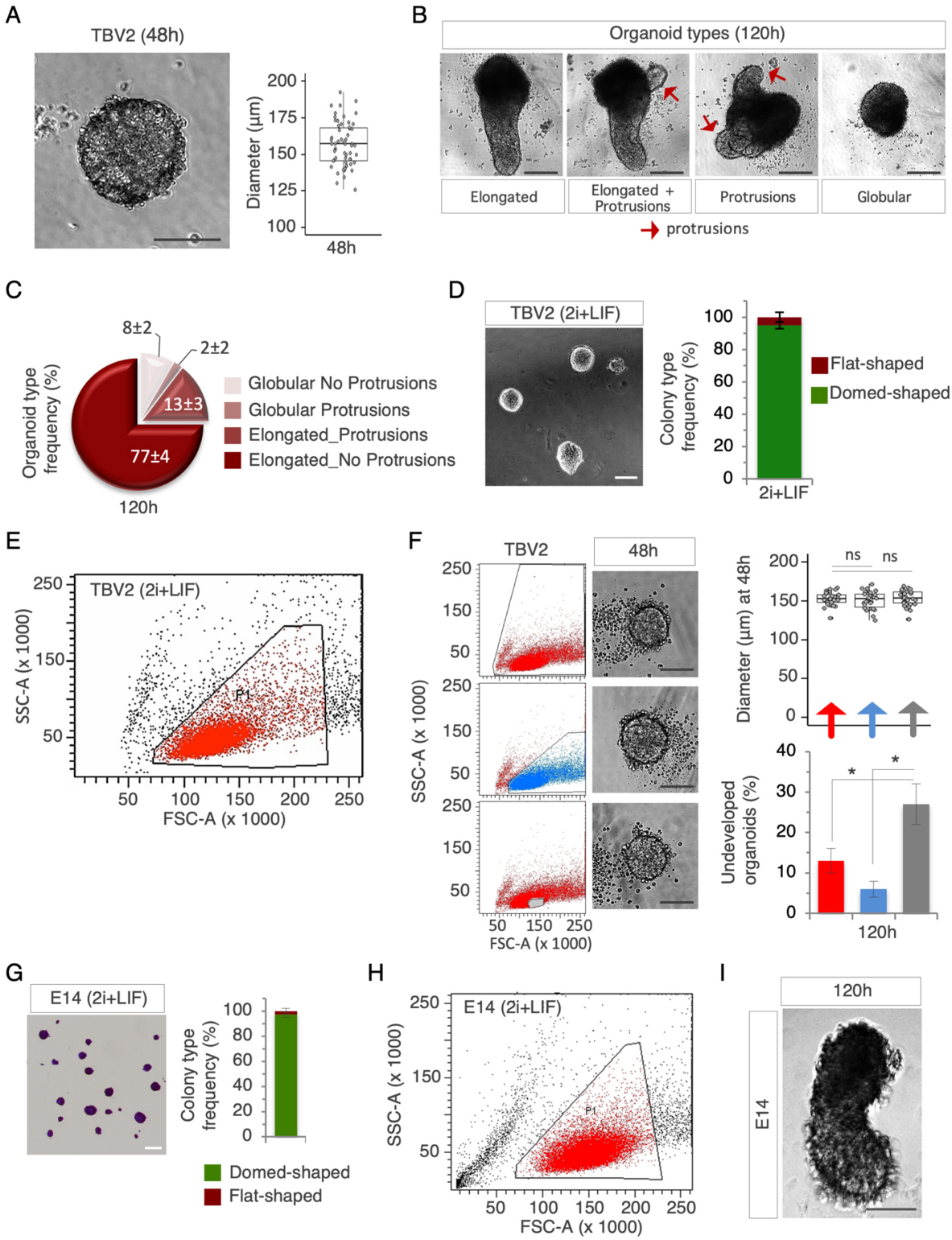

Figure S1. Experimental set up of the optimised gastruloid assay, Related to Figure 1. (**A**) Representative picture of TBV2 mESCs (WT) aggregates at 48h after aggregation (left) and box plot diagram (right) of the distribution of the aggregates' diameter (n=3; 60 gastruloids analysed).

(**B**) Representative pictures of different organoids' phenotypes at 120h after aggregation. Red arrows indicate protrusions (bar=100 μm). (**C**) Pie chart representing the percentage of the different phenotypic outcomes showed in panel B. Data are mean ± SD (n=3; 60 gastruloids analysed). (**D**) Representative pictures (left) and colony type quantification (right) of colonies generated from 2i+LIF cells. Data are mean ± SD (n=3; ~ 450 colonies analysed). (**E**) Flow cytometry scatter plot (FSC *vs* SSC) of 2i+LIF ESCs. Dead cells and debris are excluded based on FSC/SSC parameters. (**F**) Flow cytometry histograms of the different sorted populations from untreated FBS+LIF ESCs (left); bright field representative pictures of cell aggregates (middle), diameter distribution (right, top graph) at 48h after aggregation and percentage of undeveloped organoids at 120h after aggregation (right, bottom graph). Total population=red; population without dead cells and debris=light blue; narrow population = grey (n=3; 30 gastruloids/condition analysed; *$p<0.01$) (bar= 100μm). (**G**) Representative picture (left) and colony type quantification (right) of colonies generated from 2i+LIF E14 ESCs (n=3; ~ 450 colonies analysed). (**H**) Flow cytometry scatter plot (FSC *vs* SSC) of 2i+LIF E14 ESCs (bar= 100μm). (**I**) Representative picture of elongated-shaped gastruloids at 120h (bar=100 μm).

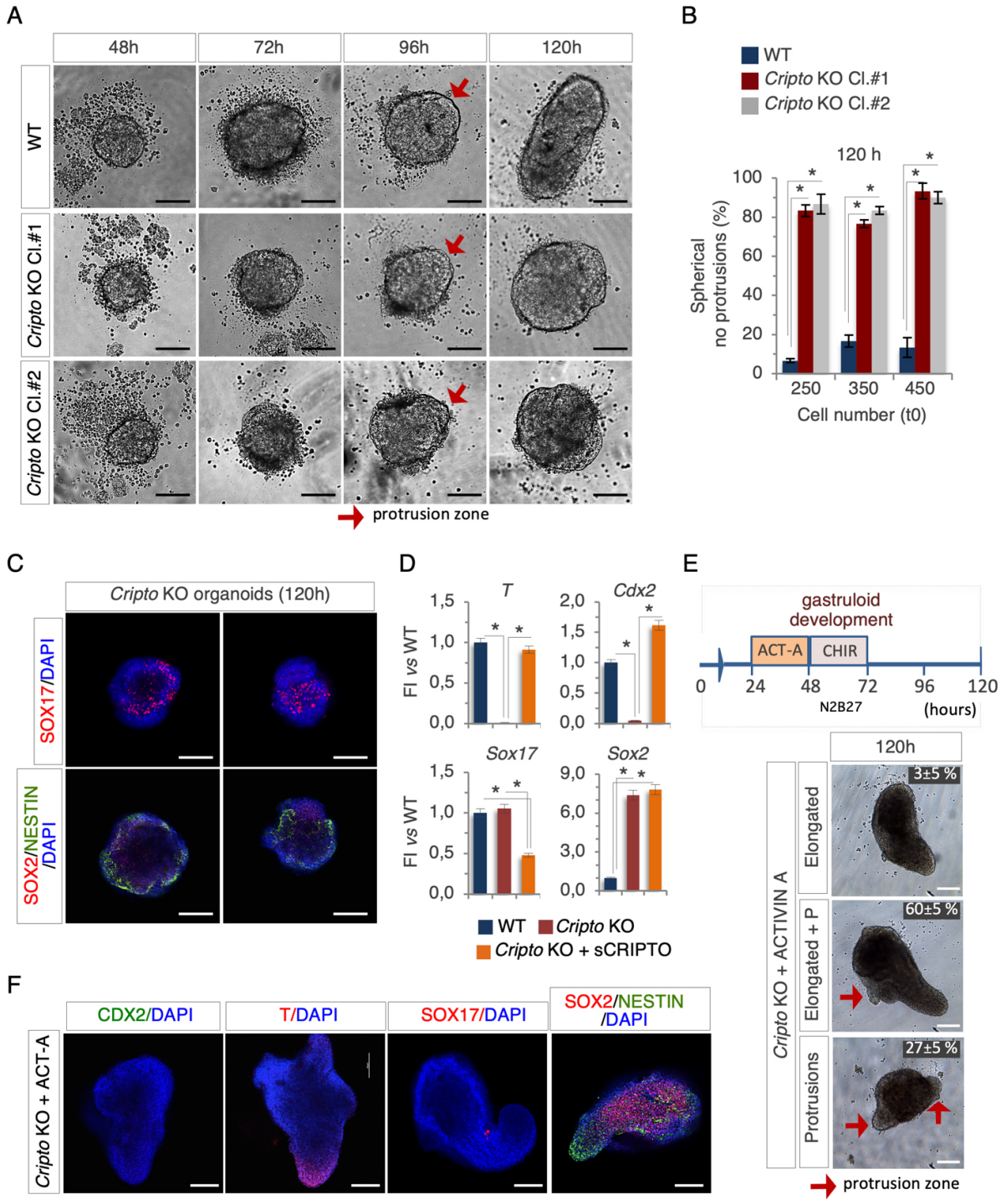

Figure S2. Validation of the gastruloid formation assay using *Cripto* KO ESCs, Related to Figure 2. (**A**) Time course bright field representative pictures of WT and *Cripto* KO (Cl.#1 and Cl.#2) aggregate to gastruloid transition. Light red arrows indicate the protrusion zone (bar=100

μm). (**B**) Percentage of spherical organoids without protrusions derived from WT (blue) and *Cripto* KO ESCs clones #1 (Cl.#1, red) and #2 (Cl.#2, grey). Data are mean ± SD (n=3; 30 gastruloids/condition analysed; *p<0.01). (**C**) Representative confocal pictures of immunofluorescence with SOX17 (red) or SOX2/NESTIN (red/green) on *Cripto* KO-derived organoids at 120h. Nuclei were counterstained with DAPI (blue) (bar=100 μm). (**D**) qRT-PCR analysis of *T*, *Cdx2*, *Sox17* and *Sox2* in WT (blue), *Cripto* KO (red) and *Cripto* KO + sCRIPTO (orange) organoids at 120h. Data are normalized to *Gapdh* and are mean ± SD of fold change (FI) *vs* WT (n≥3; *p<0.01). (**E**) Schematic representation of the experimental design (top). Representative bright field pictures and percentage distribution (bottom) of the different organoids generated from *Cripto* KO + ACT-A at 120h. Red arrows indicate the protrusion zone. Data are mean ± SD (bar=100 μm). (**F**) Representative confocal images of CDX2 (green), T (red), SOX17 (red) and SOX2/NESTIN (red/green) on *Cripto* KO + ACT-A organoids (120h). Nuclei were counterstained with DAPI (blue) (bar=100 μm).

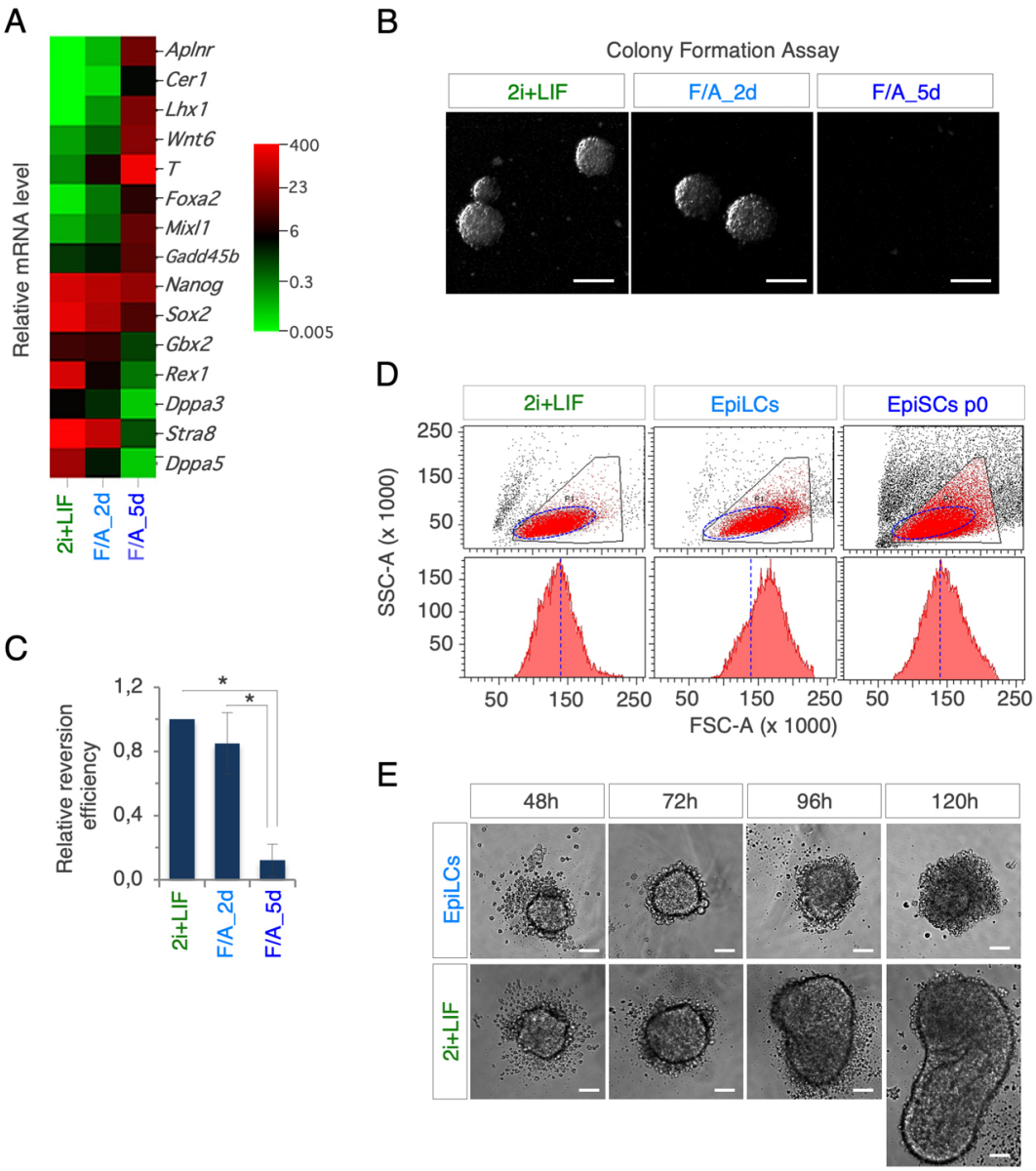

Figure S3. Abortive development of gastruloids derived from primed pluripotent states, Related to Figure 3. (**A**) Heat-map of mRNA levels of the indicated genes in naïve (2i+LIF) cells, F/A_2d (EpiLCs) and EpiSCs/p0 (F/A_5d). (**B**) Bright field representative pictures of colony formation assay of 2i+LIF, F/A_2d and F/A_5d cells (bar=100 µm). (**C**) Relative reversion efficiency of colony formation assay of 2i+LIF (naïve), F/A_2d and F/A_5d cells. Data are mean ± SD (n=3; ~ 450 colonies analysed). (**D**) Flow cytometry scatter plot (FSC *vs* SSC) and histogram of the naïve (2i+LIF) ESCs, EpiLCs and EpiSCs FACS-sorted populations. (**E**)

Representative bright field pictures of aggregates to gastruloid transition of EpiLCs and naïve ESCs at the indicated time points (bar=50 μm).

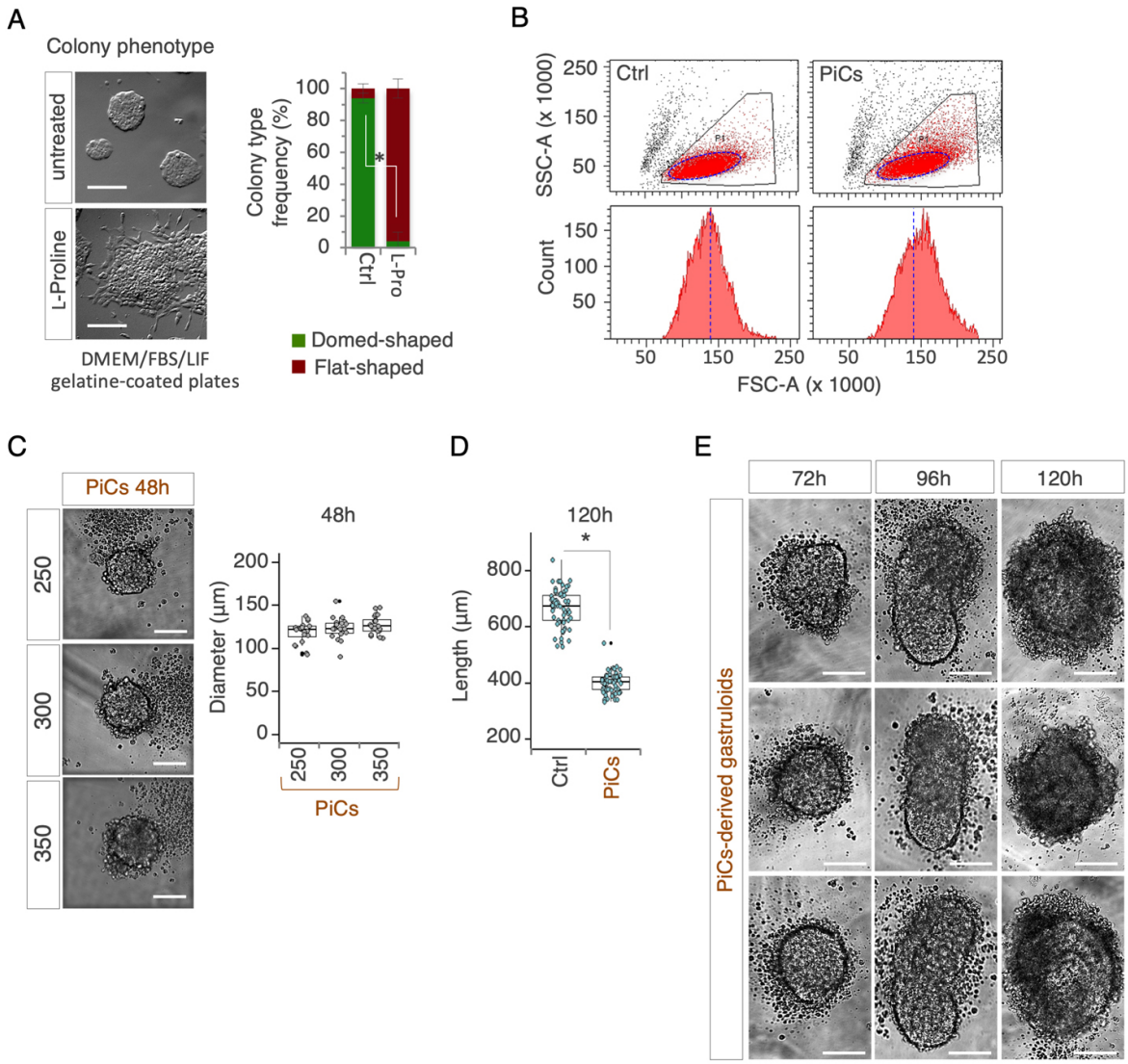

Figure S4. PiCs-derived organoids show a peculiar developmental process, Related to Figure 5. (**A**) Representative bright field pictures (left) and colony type quantification (right) of colonies generated from Control (untreated) ESCs and PiCs (n=3; ≈450 colonies/condition analysed; *p<0.01). (**B**) Flow cytometry 2D scatter plot (FSC *vs* SSC) and histogram (FSC) of Control (Ctrl) and PiCs sorted populations used for the gastruloid formation assay. (**C**) Representative bright field pictures (left) and diameter analysis (right) of aggregates (48h after aggregation) generated from PiCs seeded at different densities (250, 300 and 350 cells per well) (n=3, 30 gastruloids/condition analysed; bar=100 µm). (**D**) Length distribution of Control and

PiCs-derived gastruloids at 120h (n=3, 60 gastruloids/condition analysed; *p<0,01). (**E**) Representative bright field pictures of PiCs-derived gastruloids at the indicated time points (bar=100 μm).

Table S1: List of Primers used in this study, Related to Figure 6 and Figure 7.

Gene symbol	Forward (5'-3')	Reverse (5'-3')
Blimp1	GGTGAATCAGGGTGCCTTTA	GAGAGGTGCAGGGAAGCAC
Cdx2	AGGAAGCCAAGTGAAAACCA	CAGCCAGCTCACTTTTCCTC
Cer1	CAGGCCGTGACTCAGCCAGCAG	CCGGGAAAACGAATGGAACTGC
Esrrb	GGCCACCAATGAATGTGAG	AGCCGTCGCTTGTACTTCTG
Fgf5	CAAAGTCAATGGCTCCCACGAAG	CTACAATCCCCTGAGACACAGCAAATA
Gapdh	TGCACCACCAACTGCTTAGC	TCTTCTGGGTGGCAGTGATG
Klf4	TAGTGGCGCCCTACAGCGGT	TCGTGTGTGTTGGGCCGGTG
Nanog	AAGTACCTCAGCCTCCAGCA	GTGCTGAGCCCTTCTGAATC
Nanos3	GGGTGCTGTGTCCCATTTTG	ACCTGCATAGACACCTGCTG
Oct3-4	TCAGCTTGGGCTAGAGAAGG	TGACGGGAACAGAGGGAAAG
Prdm14	AAGGCACACAGGGACAACTC	CTGGTTCCGCTGGATGTCTC
Rex1	TTGCCTCGTCTTGCTTTAGG	AAAATGAATGAACAAATGAAGAAAA
Sox17	AGCTAAGCAAGATGCTAGGCAAG	TCTCTGCCAAGGTCAACGC
Sox2	CACAACTCGGAGATCAGCAA	CTCCGGGAAGCGTGTACTTA
T/Bra	GAACCTCGGATTCACATCGT	TTCTTTGGCATCAAGGAAGG
Tfcp2l1	TGCCCATCTTCAAGC	CCAGCAGCCGGATTT

Table S2: List of primary and secondary antibodies used in this study, Related to Figure 1, 2, 5, 6 and 7.

ANTIBODY	COMPANY	CAT. NO.	APPLICATION
AP2γ	Santa Cruz	sc-53162	IF 1:100
BLIMP1	Santa Cruz	sc-47732	IF 1:50
CDX2	Cell Signaling	3977	IF 1:100
Ki67	ThermoFisher Scientific	MA5-14520	IF 1:50
NANOG	Cell Signaling	8822	IF 1:400
NESTIN	Santa Cruz	sc-33677	IF 1:100
OCT4	Santa Cruz	sc-8628	IF 1:100
SOX17	R&D	AF1924	IF 1:200
SOX2	Cell Signaling	D1C7J	IF 1:100
T	Santa Cruz	sc-17745	IF 1:200
Alexa Fluor 488 Donkey anti-Goat IgG	Invitrogen	A11055	IF 1:400
Alexa Fluor 488 Donkey anti-Rabbit IgG	Invitrogen	A21206	IF 1:400
Alexa Fluor 594 Donkey anti-Rabbit IgG	Invitrogen	A21207	IF 1:400
Alexa Fluor 594 Donkey anti-Goat IgG	Invitrogen	A11058	IF 1:400
Alexa Fluor 594 Donkey anti-Mouse IgG	Invitrogen	A21203	IF 1:400
Alexa Fluor 647 Goat anti-Rat IgG	Invitrogen	A-21247	IF 1:400

Supplemental Experimental Procedures

EpiLCs, EpiSCs, L-Proline- induced cells (PiCs) and Primordial Germ Cell-like cells (PGCLCs) generation.

To generate EpiLC and EpiSCs (p0), 2i+LIF mESCs were seeded at 1500 cells/cm^2 onto FBS-coated plates in N2B27 supplemented with ACTIVIN A (20 ng/ml; Invitrogen) and bFGF (12 ng/ml; Provitro) and cultured for 2 and 5 days, respectively.

To generate PiCs (Comes et al, 2013), ESCs were plated at low density (50–250 cells/cm^2) on gelatin-coated plates in DMEM/15%FBS/LIF supplemented with L-Pro (250–500 μM) (Sigma-Aldrich) and grown for 4-5 days. Crystal Violet cell colony staining and colony type analysis (domed vs flat) was performed as previously described (Comes et al., 2013). The analysis was performed blinded by two investigators on at least 400 colonies per condition.

For PGCLCs differentiation, 2.5x10^4 cells/well were plated in U-shaped ultra-low attachment 96-multiwell (Corning Costar) in serum-free GK15 (GMEM [Invitrogen]) medium containing 15% KSR, 0.1 mM NEAA, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine. To induce PGCLC differentiation, complete medium was supplemented with BMP4 (500 ng/ml; R&D Systems), LIF (1000 u/ml; ESGRO, Millipore), SCF (100 ng/ml; R&D Systems), and EGF (50 ng/ml; R&D Systems). After 4 days, the cell aggregates were dissociated with Trypsin and analysed.

Immunofluorescence analysis

Stem cell colonies were dissociated with either accutase or trypsin-EDTA (5 min at 37°C), cells were re-suspended in 15% FBS/1x PBS and centrifuged (800 rpm for 8 min) onto glass slides (5x10^5

cells/spot) using a Thermo Shandon Cytocentrifuge (CytoSpinTM 4). Cytospin cell samples were fixed in 4% paraformaldehyde (PFA), permeabilized in 0.1% Triton X-100 (10min at RT) and incubated overnight at 4°C with primary antibodies. After washing (0,5% Tween-1x PBS), cells were incubated with appropriate secondary antibodies. Nuclei were counterstained with DAPI (Invitrogen). Images were obtained using the DMI6000B microscope and the DFC 350FX B/W digital camera (Leica Microsystems). Confocal images were obtained on a Nikon A1 microscope. The AF6000 (Leica Microsystems) and NIS Element C (Nikon, Tokyo) software were used for image acquisition/elaboration. Primary and secondary antibodies are listed in Supplementary Table 2.