## Supplemental Experimental Procedures

*Quantitative RT-PCR*

TaqMan inventoried primers and probes (Applied Biosystems):

| **Gene** | **Catalog number** |
|---|---|
| Nanog | Mm02384862_g1 |
| Rex1 | Mm01194089_g1 |
| Brachyury | Mm00436877_m1 |
| Foxa2 | Mm00839704_mH |
| Gata4 | Mm00484689_m1 |
| Gata6 | Mm00802636_m1 |
| Sox17 | Mm00488363_m1 |
| Shh | Mm00436528_m1 |
| Pax6 | Mm01334068_m1 |
| Gata1 | Mm00484678_m1 |
| Myf5 | Mm00435125_m1 |
| Pax8 | Mm00440623_m1 |
| Thyroglobulin | Mm00447525_m1 |
| Nkx2.1 or Ttf1 | Mm00447558_m1 |
| Pdx1 | Mm00435565_m1 |
| Afp | Mm00431715_m1 |
| Alb1 | Mm00802090_m1 |
| Sox7 | Mm00776876_m1 |
| Tshr | Mm00442027_m1 |
| Ifabp | Mm00433188_m1 |
| Hnf6 | Mm00839394_m1 |
| Ptf1α | Mm00479622_m1 |
| 18S rRNA | 4319413E |

Sequences of primers used with SYBRgreen system:

| Gene | 5' → 3' | 5' → 3' |
|---|---|---|
| Gtl2/Meg3 | TTGCACATTTCCTGTGGGAC | AAGCACCATGAGCCACTAGG |
| Dlk1 | CCCAGGTGAGCTTCGAGTG | GGAGAGGGGTACTCTTGTTGAG |
| Rian | TCGAGACACAAGAGGACTGC | ATTGGAAGTCTGAGCCATGG |
| Oct4 | TAGGTGAGCCGTCTTTCCAC | GCTTAGCCAGGTTCGAGGAT |
| Aat | AATGGAAGAAGCCATTCGAT | AAGACTGTAGCTGCTGCAGC |
| GAPDH | TGCACCACCAACTGCTTATC | TGCATGGACTGTGGTCATGAG |

*ES and iPS cell pancreatic differentiation*

ES and iPS cell pancreatic differentiation was performed as previously described (1). Briefly, ES and iPS cells were differentiated to primitive streak-like cells using Wnt3a (25ng/ml, R&D systems) and Activin-A (50ng/ml, R&D systems) for 1 day, followed by definitive endoderm differentiation using Activin-A (50ng/ml) for 5 days. The resulting cells were then exposed to Fgf10 (50ng/ml, R&D systems) and KAAD-Cyclopamine (0.75uM, Stemgent), for 3 days, followed by Fgf10 (50ng/ml), KAAD-Cyclopamine (0.75uM) and Retinoic Acid (2uM, Sigma) for 3 days. Day 12 cells were harvested for RNA extraction and RT-PCR analysis.

*Kidney capsule transplants*

All animal studies were approved by the Institutional Animal Care and Use Committee of Boston University School of Medicine. Kidney capsule transplantation was performed on recipient SCID mice (Taconic, CB17SCRF-M), anesthetized with ketamine/xylazine. A small flank incision was made to expose the right kidney in each mouse, and 500,000 cells were injected beneath the kidney capsule, followed by 2-layered flank wound closure. Four weeks later kidneys from euthanized mice were harvested, fixed overnight in 4% paraformaldehye, and embedded in paraffin for sectioning. The tumor area was defined by the elliptical area formula π*A*B (A=horizontal diameter, B=vertical diameter). 5 um thick tissue sections of all tumors were stained with hematoxylin and eosin (H&E) reviewed by an attending anatomical pathologist. Structures deriving from transplanted cells considered to be characteristic of each germ layer (2) were scored based on morphology, H&E

staining, and, where indicated in the text, immunostaining with antibodies against Foxa2, Tuj1, or smooth muscle actin (see below). Average germ layer structure numbers per low power field per group were calculated based on review of 5 random fields per tissue section.

*Immunohistochemistry and immunostaining*

Paraffin sections of kidney capsule transplants were processed for immunohistochemistry by standard methods after antigen retrieval using antigen unmasking solution (Vector Laboratories), and quenching of endogenous peroxidase. CAS block buffer (Invitrogen) was applied for 1 hour at room temperature. Primary antibody was applied for 2hrs using: anti-smooth muscle actin (Neomarkers, MS-113-B1) d1/50; Foxa2 (Santa cruz, sc-6554) d1/50; Tuj1 (R&D systems, BAM1195) d1/200. Secondary antibodies conjugated to biotin (Vector Laboratories) or HRP (Vector Laboratories) were applied followed by Vectastain ABC reagent (Vector Laboratories), and methyl green counterstaining (TACS) where indicated.

For immunostaining cells in tissue culture, ES or iPS cells were fixed with 4% paraformaldehyde for 30min at room temperature, followed by permeabilization with 0.2% Triton and protein blocking with serum-free Dako Protein Block (DakoCytomation). Foxa2 goat anti-mouse antibody (Santa Cruz, sc-6554) was applied for 2hrs (1:50 dilution; room temperature), followed by donkey anti-goat IgG-Alexa 546 secondary antibody (Invitrogen; 1:50 dilution; 1.5 hours; RT). Alb1 rabbit anti-human antibody (DAKO, A001) was applied for 2hrs (1:100 dilution; 37 degrees), followed by donkey anti-rabbit IgG-Cy3 secondary antibody (Jackson ImmunoResearch; 1:200 dilution; 2 hrs; 37 degrees). DAPI staining was used for nucleus detection.

*Glycogen storage assay*

Glycogen storage assays of undifferentiated (day 0) and differentiated (day 19) hepatocyte-like ES and iPS-derived cells was performed as previously described (3). Briefly, ES/iPS cells were differentiated for 5 days to endoderm, sorted for ckit+/Sox2dim and replated

under hepatocyte differentiation conditions until day 19. After trypsinization and resuspension in 200ul of distilled water, total protein content of cell lysates was measured by Bradford assay. 60ul of each cell lysate was treated with 240ul of KOH at 100$^{o}$C for 20min. 125ul of this treated cell suspension was used to produce a colometric reaction with yellow anthrone solution. The OD was read at 620nm. The glycogen content of each sample was estimated using a glycogen standard curve. MEF-depleted undifferentiated ES/iPS cells were used as a control. All cells were grown in basal high glucose-containing media (450mg/dl).

*DNA CpG methylation mapping*

Genomic DNA was extracted and purified using DNeasy Blood & Tissue Kit (Qiagen). 100ng of DNA was processed with EpiTect Bisulfite Kit (Qiagen) followed by nested PCR amplifying the proximal promoter regions of Oct4 and Foxa2, using the following primers: Oct4 F1:5'GTAAGTAAGAATTGAGGAGTGG3' R1:5'TCCAAACCCACCTAAAAACC3' F2:5'GATGGTTGAGTGGGTTGTAAGG3' R2:5'CCAACCCTACTAACCCATCACC3', FoxA2 F1:5'GTTTTGTTTGGGGTAGATAAGG3' R1:5'CCTAACACTCCCAAAACC3' F2:5'GATAGTTTTGGTTTTTGTAGG3' R2:5'CCTAATAAAATCCCTTCC3'. Resulting PCR products were purified using QIAquick gel extraction Kit (Qiagen) and ligated to the PGEM®-T vector (Promega). Ligation products were used to transform JM109 competent cells (Promega) followed by plating on Ampicillin supplemented agar plates. At least 10 colonies from each plate were picked, mini-cultured, miniprepped (QIAprep Spin Miniprep Kit, Qiagen) and analyzed by digestion using SpeI and SacII (New England Biolabs). Five positive clones were sequenced using T7 oligo. For pyrosequencing of IG-DMR CpG islands, DNA extracts were processed with EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. Quantitative methylation analysis of 29 CpG islands in the IG-DMR region of the Dlk1/Gtl2 locus was performed using the ADS935 (Mouse Gtl2) Assay by EpigenDx Inc. (Worcester, MA).

**Supplemental References:**

1. D'Amour, K.A., Bang, A.G., Eliazer, S., Kelly, O.G., Agulnick, A.D., Smart, N.G., Moorman, M.A., Kroon, E., Carpenter, M.K., and Baetge, E.E. 2006. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol* 24:1392-1401.
2. Gertow, K., Przyborski, S., Loring, J.F., Auerbach, J.M., Epifano, O., Otonkoski, T., Damjanov, I., and Ahrlund-Richter, L. 2007. Isolation of human embryonic stem cell-derived teratomas for the assessment of pluripotency. *Curr Protoc Stem Cell Biol* Chapter 1:Unit1B 4.
3. Roelandt, P., Sancho-Bru, P., Pauwelyn, K., and Verfaillie, C. 2010. Differentiation of rat multipotent adult progenitor cells to functional hepatocyte-like cells by mimicking embryonic liver development. *Nat Protoc* 5:1324-1336.

Figure S1, related to Figure 2

**Figure S1: In vivo testing of pluripotency and germline transmission of iPS cell clone ST8**. Following injection of iPS cells (B6/129 strain) into blastocysts (albino strain) and transfer into pseudopregnant mouse mothers, high level coat color chimerism of the resulting 4 chimeric mice is shown (left panel). After breeding of a chimeric male with an albino female (middle panel), germline transmission was achieved, with generation of several black and agouti mice derived from iPS clone ST8 (right panel) in each litter. A representative example of three independent experiments is shown.

Figure S2, related to Figures 2 and 3

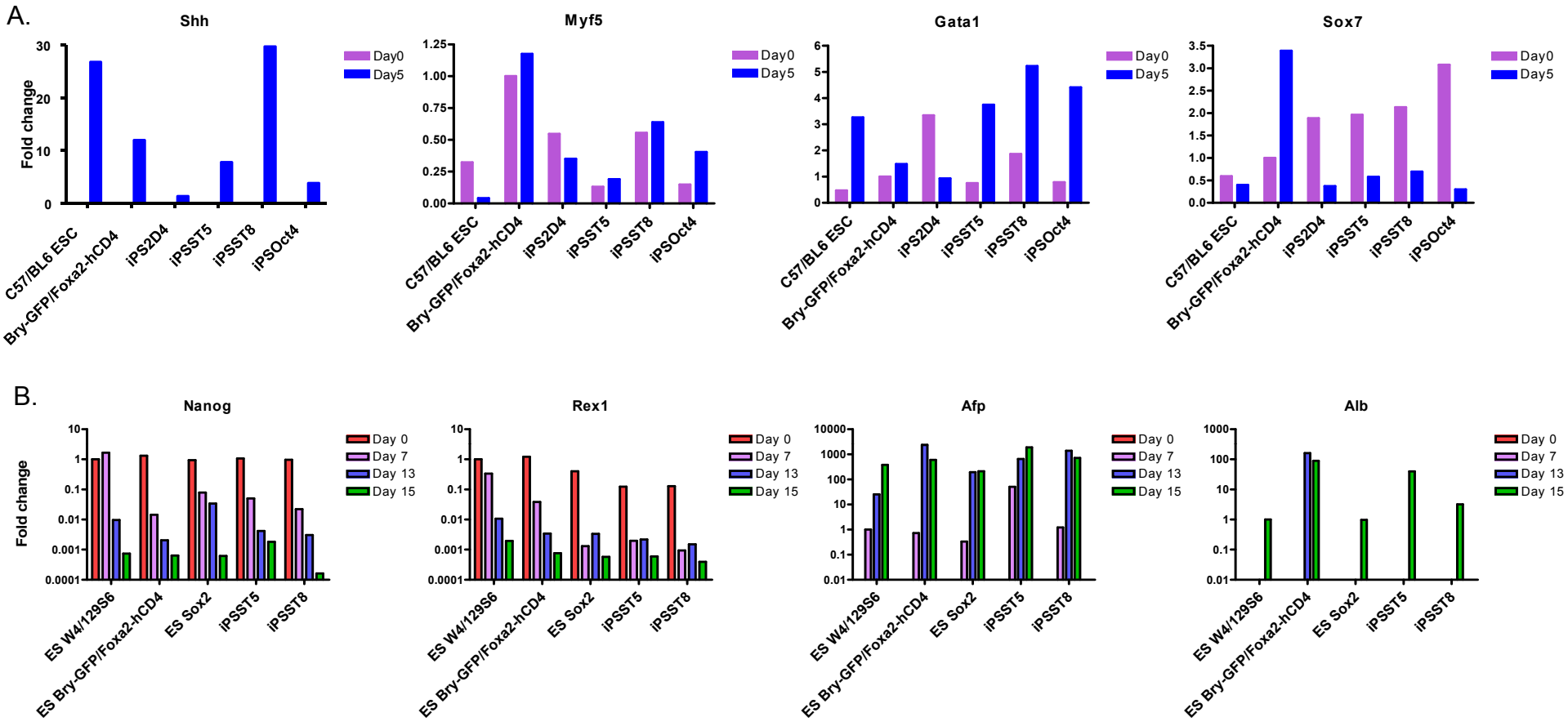



**Figure S2: Comparison of multiple ES and iPS cells undergoing endodermal directed differentiation.** (A) Gene expression analysis (qRT-PCR) of ST8 iPS cells (Sox2-GFP), Oct4 iPS (Oct4-GFP), 2D4 iPS (Nanog-GFP), and 129/Ola ES cells (Bry-GFP/Foxa2-hCD4) on day 0 vs 5. Shh, used as an endodermal marker, was upregulated with differentiation while mesodermal marker gene expression was inconsistent, being either minimally detected (Myf5) or suggestive of low level induction (Gata1). Sox7, used as an extraembryonic endoderm marker was downregulated in most samples (B) Gene expression analysis (qRT-PCR) of ES W4/129S6, 129/Ola ES (Bry-GFP/Foxa2-hCD4), ES Sox2, ST8 and ST5 iPS (Sox2-GFP) cells during hepatocyte differentiation. Different ES cell lines (of different genetic backgrounds) exhibit different differentiation kinetics; note slightly earlier (albumin expression by day 13) compared to other non-isogenic ES cell lines. (C) Summary of cell counts during endodermal differentiation indicates iPS cell lines generated with reprogramming vectors that have high level residual transgene overexpression driven by constitutively active promoters (SEF4 and SEF11) do not proliferate in this protocol, in contrast to ST5 and ST8 iPS cell lines. Results represent 3 repeated experiments.

Figure S3, related to Figure 3

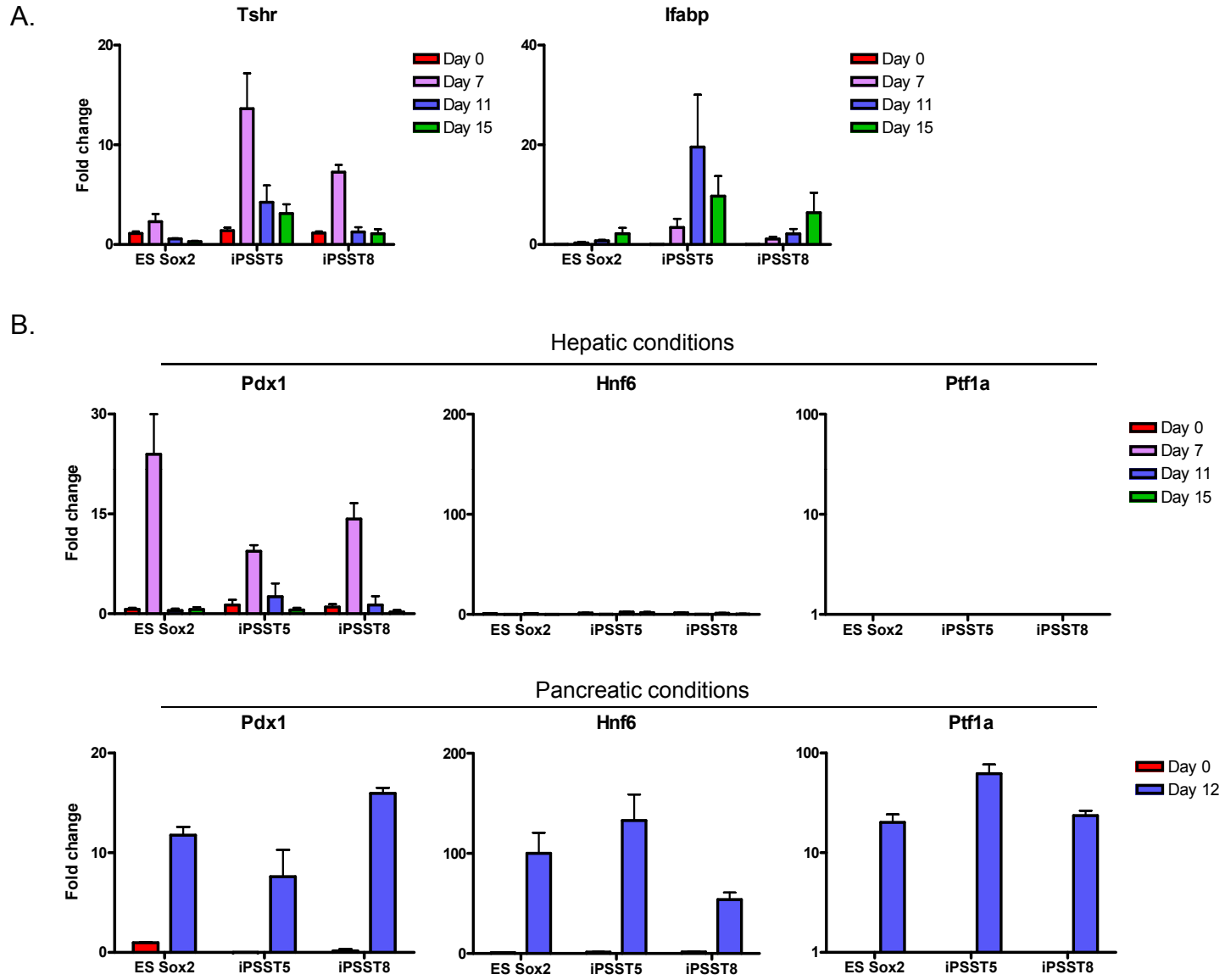
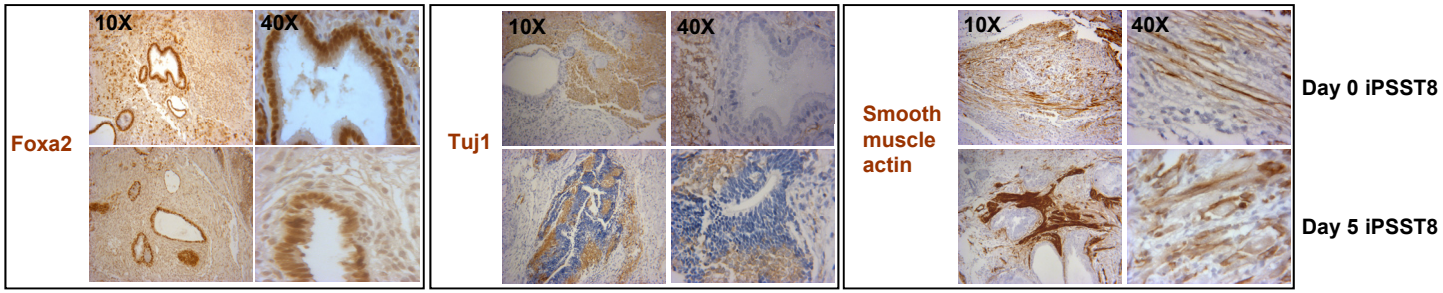
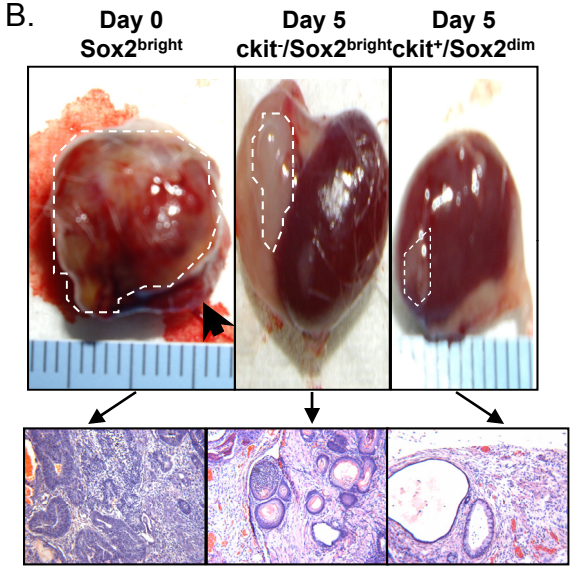
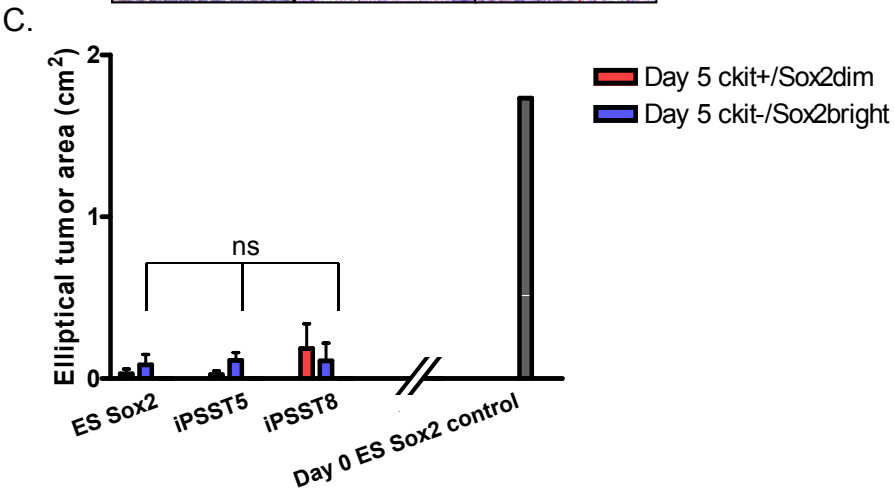



**Figure S3: Gene expression changes during directed differentiation of ES/iPS cells in media designed to favor hepatic vs pancreatic lineage specification *in vitro*.** (A) Gene expression changes during a two-stage protocol for endodermal directed differentiation in conditions designed to favor hepatic specification. In comparison to robust hepatic marker gene induction (shown in Figure 3 of the text), induction of non-hepatic endodermal lineages was typically transient, as evidenced by expression of markers of thyroid (Tshr) and intestine (ifabp) by day 7-11 of differentiation in each ES and iPS cell clone tested. (B) Despite Pdx1 transient expression, hepatocyte nuclear factor 6 (Hnf6) and pancreas transcription factor 1α (Ptf1α) are not expressed at any time point in 'hepatic conditions'. In contrast, endoderm directed differentiation in 'pancreatic conditions' results in expression of Pdx1 along with Hnf6 and Ptf1α, demonstrating pancreatic commitment. Error bars indicate SEM, N=3.

Figure S4, related to Figures 3 and 4



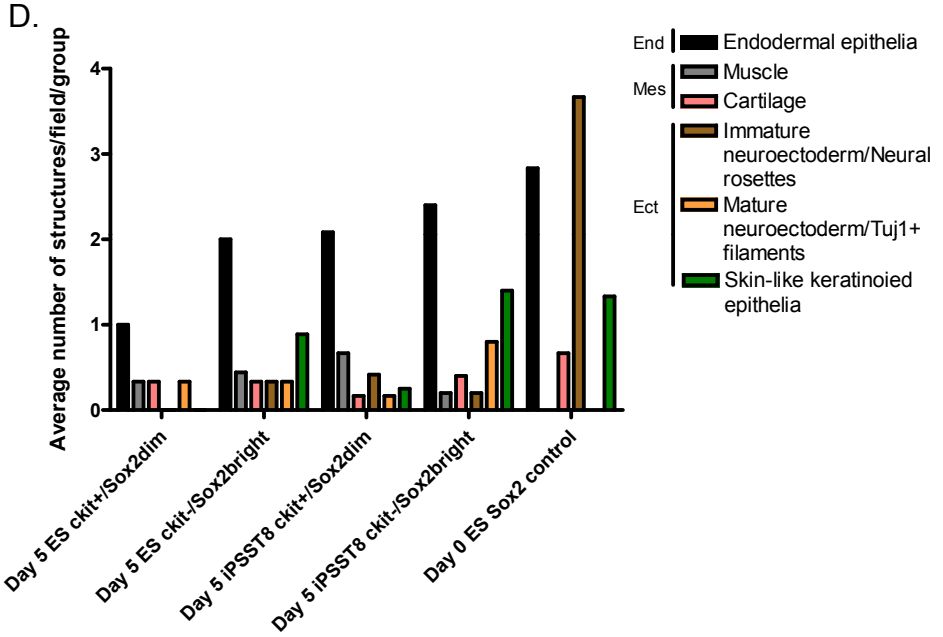




**Figure S4: Kidney capsule transplantation of day 0 or day 5 ES/iPS-derived cell populations.** (A) Both day 0 and 5 *unsorted* iPSST8-derived cells robustly formed endodermal epithelia with nuclear Foxa2 immunostaining (brown). Some degree of neuroectodermal and mesodermal differentiation was detectable after transplantation of unsorted day 5 cells, as demonstrated by Tuj1+ cells (brown) and smooth muscle actin (SMA) staining (brown), respectively. (B) Representative images of tumor size (outlined with white dashed line) 4 weeks after transplantation of *sorted* day 5 ES/iPS cell-derived populations. Note little residual kidney (black arrow head) in recipient of undifferentiated ES cells, in contrast to small tumors localized within kidney capsules after transplantation of sorted day 5 ckit+/Sox2dim cells. Representative H&E staining of sections through each tumor are shown. (C) Elliptical tumor area (cm2) of derived tumors 4 weeks after transplantation of each indicated sorted cell population. Error bars indicate SEM, N=4. (D) Quantification of lineages in kidney capsule grafts deriving from each sorted population was conducted by structure counting of 5 random tissue sections from each recipient. All tumors contain some degree of trilineage differentiation but with less immature and ectodermal strucures in D5 ckit+/Sox2dim sorted cells, compared to day 0 ES control transplanted cells. End=endoderm, Mes=mesoderm, Ect=ectoderm

Figure S5, related to Figures 3 and 4

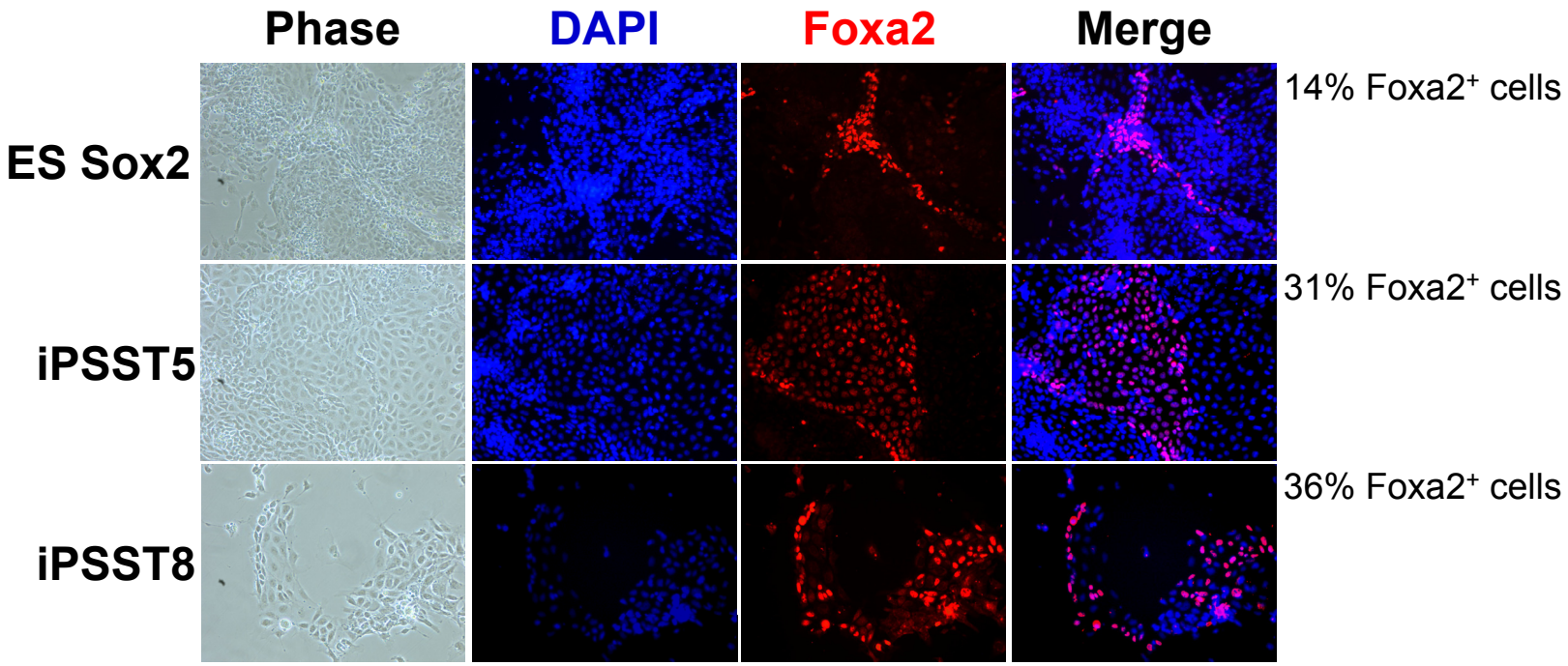



**Figure S5: Comparison of Foxa2 protein expression in day 7 outgrowth after replating *unsorted* day 5 endodermal ES and iPS-derived cells. Each indicated cell line** was differentiated to endoderm for 5 days and then plated on gelatin coated wells for 2 days to allow out growth of colonies. % of outgrowth cells staining positively for nuclear Foxa2 is indicated (right margin). Approximately 3000 cells were counted for each cell line in 2 parallel wells.

Figure S6, related to Figures 5 and 6

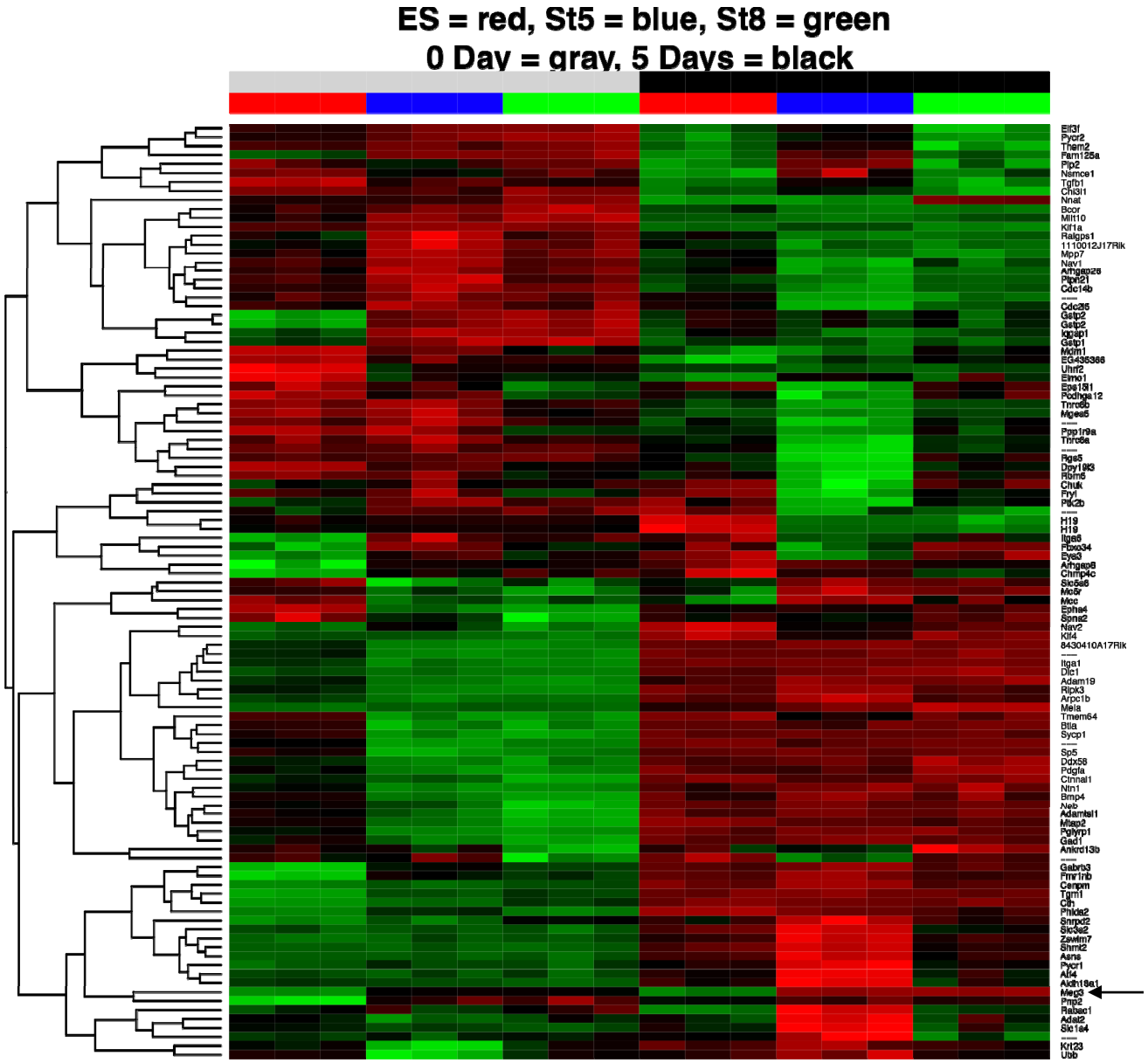



**Figure S6: Interaction effect of cell type and time on gene expression in ES and iPS cells undergoing endodermal directed differentiation.** Heat map clustering analysis across 18 samples of ES and iPS cells at day 0 and day 5 indicates genes differentially expressed based on the interaction of time and cell type (differences in gene expression with time that are modulated by cell type). 2-way ANOVA was employed to calculate the 105 genes passing an FDR<0.01 cutoff. Gtl2/Meg 3 (arrow) was the top most differentially expressed gene by FDR-adjusted p-value.

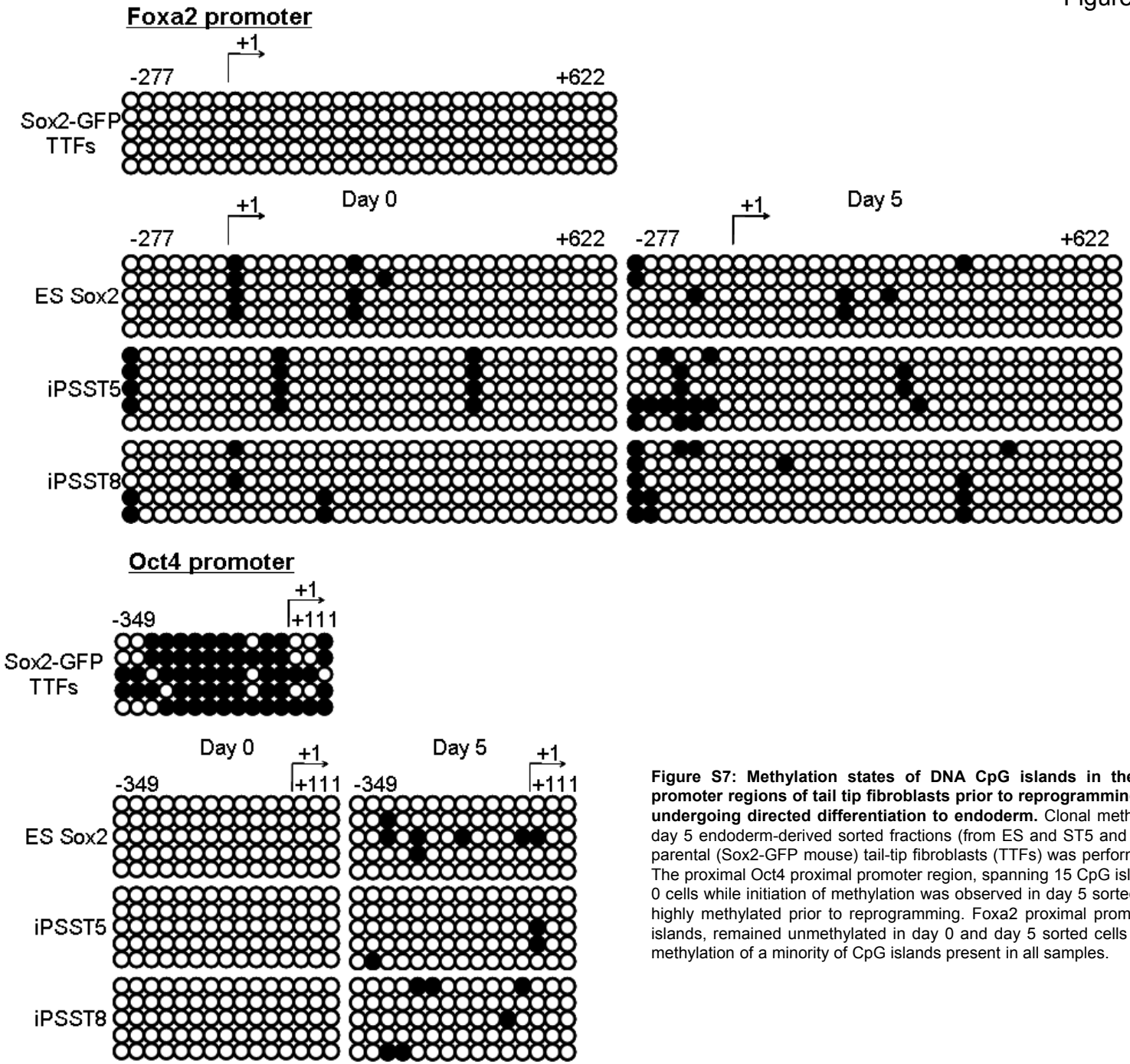



**Figure S7: Methylation states of DNA CpG islands in the Oct4 and Foxa2 proximal promoter regions of tail tip fibroblasts prior to reprogramming as well as ES and iPS cells undergoing directed differentiation to endoderm.** Clonal methylation analyses of Day 0 and day 5 endoderm-derived sorted fractions (from ES and ST5 and ST8 cell lines) as well as their parental (Sox2-GFP mouse) tail-tip fibroblasts (TTFs) was performed using bisulfite sequencing. The proximal Oct4 proximal promoter region, spanning 15 CpG islands, was unmethylated in day 0 cells while initiation of methylation was observed in day 5 sorted endodermal cells; TTFs were highly methylated prior to reprogramming. Foxa2 proximal promoter region, spanning 33 CpG islands, remained unmethylated in day 0 and day 5 sorted cells as well as TTFs, with variable methylation of a minority of CpG islands present in all samples.