Supplemental Methods:

Western blot

Cells were rinsed in PBS and lysed with RIPA buffer (50 mM TRIS pH 7.4, 1% NP40, 0.25% sodium deoxycolate, 1 mM EDTA and 1 mM sodium chloride). The following inhibitors were added prior to lysis: 1 mM PMSF, 1 mM NaF, 1 mM activated Na_3VO_4, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 10 µg/ml pepstatin (Sigma). Lysates were incubated at 4˚C for 15 minutes, and supernatant was collected after a 15-minute centrifugation at 4˚C. Samples were boiled for 5 minutes in SDS-sample buffer (Boston BioProducts) and run on Tris-HCL Criterion Precast Gels (Bio-Rad Laboratories) for two hours at 100V. Proteins were transferred unto a nitrocellulose membrane (Bio-Rad) overnight at 20V at 4˚C. Blots were blocked in 5% non-fat milk in TBST (Tris Buffered Saline from Bio-Rad Laboratories with 0.1% Tween-20) for 1 hour. Primary antibodies were incubated in 5% BSA in TBST overnight. The following antibodies from Cell Signaling Technology were used at 1:1000: PTEN, p-AKT (S473), p-GSK3ß (S9), p-S6 (S235/236), OCT4, NANOG, and SOX2. GAPDH (1:1000, Abcam) was used as a loading control. HRP conjugated secondaries (1:2500, Promega) were added for 1 hour in 5% non-fat milk in TBST.

Quantitative Real Time PCR (qPCR)

RNA (1 µg) was extracted using the RNeasy Mini Kit (Qiagen) and the first strand was generated using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen). QPCR was performed using FastStart SYBR Green Master mix (Roche). Details of the primer sequences used in this study are provided in the Supplemental Information (Table S1).

Immunofluorescence

To generate paraffin-embedded sections, tissues were fixed in 4% PFA for 24 hours, rinsed in PBS and sectioned at 4 µm. Prior to immunostaining, sections were deparaffinized according to methods described in the immunofluorescence protocol from Cell Signaling Technology (http://www.cellsignal.com/support/protocols/IF.html). To perform antigen unmasking, slides were brought to a boil and then maintained in 10 mM sodium citrate buffer (pH 6.0) for 10 minutes at a sub-boiling temperature (95-99º C). Slides were allowed to cool on the bench top for 30 minutes. Sections were incubated

with blocking buffer (0.3% Triton-X 100, 5% normal goat serum in PBS) for an hour and incubated with primary antibody in antibody diluent (1% BSA, 0.3% Triton-X 100 in PBS) overnight. Primary antibodies used were: MAP2 (1:200, Millipore), NESTIN (1:100, Santa Cruz), and P-Histone H3 (1:100, Santa Cruz). Sections were incubated in TRITC- and/or FITC-conjugated secondary antibodies (Jackson Immunoresearch and Pierce, respectively). Slides were mounted in Vectashield Mounting Medium for Fluorescence with DAPI (Vector Labs).

Similarly, for immunocytochemistry, cells were fixed in 4% PFA for 30 minutes, blocked for an hour in blocking buffer, and incubated in primary antibody diluent overnight. Primary antibodies used were: OCT4 (1:100, Santa Cruz), ß- Catenin (1:100, Cell Signaling), FOXA2 (1:100, Cell Signaling), PAX6 (1:100, Covance), and TUJ1 (1:1000, Covance). Cells were then incubated with secondary antibody. To achieve nuclear staining, Hoescht 33342 (1:1000, Invitrogen) was used.

FACS analysis

HESCs were dissociated into single cells using 0.05% trypsin-EDTA, rinsed with PBS, and resuspended in FACS buffer (1% BSA in PBS) for 10 minutes at 4°C. Primary antibodies were added to cells for 20 minutes at 4°C. After a FACS buffer wash, secondary antibodies were added for 20 minutes at 4°C. For intracellular staining, cells were fixed with 2% PFA for 10 minutes at 37˚C and then permeabilized using 90% methanol for 30 minutes at 4˚C. Subsequently, primary antibodies were added for one hour at room temperature. After FACS wash, cells were incubated with secondary antibody (Promega) for 30 minutes at room temperature. Cells were stained with the following extracellular antibodies: TRA-1-81 (1:100, Santa Cruz Biotechnology), CD34 (1:50, BD Biosciences), PECAM (CD31) (1:100, BD Biosciences). Nuclear staining was achieved using the following antibodies from Cell Signaling Technology: OCT4 (1:600), NANOG (1:200), SOX2 (1:200), p-AKT S473 (1:100). FACS acquisition was performed using a LSR II FACS sorter and analyzed using the instrument's FACS DIVA software (BD Biosciences).