Supplementary information, Data S1 Materials and Methods

Plasmid construction, antibodies, and reagents

Plasmids encoding Smek1 and Smek2 were generated by PCR. To knockdown *Smek1*, *Smek2*, and *PP4c*, pLKO.1 vectors expressing the shRNAs targeting following sequences were used (Open Biosystem): for *Smek1*, GCACAACAGAATGATGATGAT; for *Smek2*, GCTTGGATCTAACACAACCA; for *PP4c*, GACCGTGGTTTCTACAGTGTT. To construct plasmids encoding Flag-Smek1 and Flag-Smek2, cDNA fragments were generated by PCR and then cloned into pCAG vector. For mouse HA-Tcf3 expression constructs, cDNAs were obtained by RT-PCR using mRNA isolated from ESCs and specific primer pairs (available upon request). The PCR products were cloned into the pCS2-HA vector. All constructs were sequenced to verify their authenticity.

The following primary antibodies were used: rabbit anti-Smek1, anti-Smek2 (Sigma), anti-PP4c (Bethyl Laboratories), anti-acetyl histone H3, anti-acetyl histone H4 (Upstate); goat anti-HDAC1 (C-19), anti-brachyury (C-19; Santa Cruz Biotechnologies); and mouse anti-HA (F-7), anti-Oct3/4 (C-10; Santa Cruz Biotechnologies), anti-β-catenin (BD Biosciences), anti-Flag M2 (Sigma), anti-Islet1 (Developmental Studies Hybridoma Bank). Anti-Flag M2 antibody-conjugated agarose beads (Sigma) were used for immunoprecipitation. Corresponding secondary antibodies were from Jackson Laboratories. The recombinant Wnt3a and Dkk1 were from R&D SYSTEMS. BIO was from Sigma.

Western blotting, immunoprecipitation, and HDAC activity assays

ESCs were lysed in hypotonic buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM phenylmethylsulphonylfluide (PMSF), and 1 mM dithiothreitol (DTT)). After centrifugation at 8000xg for 1 min, nuclear pellets were extracted with 10 mM HEPES, pH 7.8, 400 mM NaCl, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 12.5% glycerol, 0.2 mM PMSF, 1mM DTT, and protease inhibitor mixture (Roche). HEK293T cells were lysed in a lysis buffer containing 25 mM Tris-HCl,

pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% glycerol, and protease inhibitors (Roche). ESC nuclear extracts or HEK293T cell lysates were immunoprecipitated using appropriate antibodies and Protein A/G agarose beads (Pierce). Rabbit IgG was used as a control. Immunoprecipitates were eluted using SDS sample buffer and separated by 8 or 10% SDS-PAGE. After blocking, the blots were incubated with a primary antibody and then with a peroxidase-conjugated secondary antibody. Blots were developed by enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology).

HDAC activity assays were performed by using the HDAC fluorimetric assay kit (BIOMOL). Anti-Smek1, -Smek2, and -PP4c and rabbit IgG immunoprecipitates from ESC nuclear extracts were incubated with Fluor de Lys substrate with or without Trichostatin A (TSA) for 1 hr. Fluorescence signals were measured by using SpectraMax M5 (Molecular Devices). Activities were normalized based on the amount of HDAC1 immunoprecipitate.

Immunofluorescence

ESCs and EBs were fixed with 4% paraformaldehyde and washed in phosphate-buffered saline (PBS), then permeabilized with 0.05% Triton X-100 in PBS. Fixed cells were incubated with blocking solution containing 1% normal goat or rabbit serum (Jackson Laboratories) and 2% BSA (Sigma). Cells were incubated overnight at 4^{o}C with primary antibodies. After washing with PBS, the cells were incubated with secondary antibody, and were counterstained with Hoechst dye (Molecular Probes). Images were obtained using a fluorescence microscope with an AxioCam camera (Zeiss). For study of differentiation into cardiomyocytes and endothelial cells, EB was dissociated into single cells between day 6 and 8 after aggregation and plated in 0.1% gelatin-coated 24 wells in triplicate. After 12 days, cells were immunostained and quantified by counting the number of antibody-labeled cells in each well. Data were obtained from at least three independent experiments.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described [1]. Briefly, cells were chemically cross-linked by the addition of 37% formaldehyde at a final concentration 1% for 10 min at room temperature. Fixation was terminated by adding 2 M glycine. Cells were lysed and sonicated to generate 300-1000 bp DNA fragments. Samples were immunoprecipitated with appropriate antibodies and rabbit IgG as a control. Immunoprecipitated DNA was purified by a PCR purification kit (Qiagen) and analyzed by PCR and quantitative real-time PCR.

RT-PCR and quantitative real-time PCR

Total RNA was prepared using an RNeasy kit (Qiagen) with on-column DNase digestion. Two μg of total RNA was reverse-transcribed using Superscript II (Invitrogen). PCR amplification was performed using specific primer pairs (available upon request). Quantitative real-time PCR was carried out using SYBR Green I (BD Biosciences) and samples were quantified by amplification of *Gapdh* (for gene expression analysis), and 0.1% input control or *actin*-promoter amplification (for ChIP analysis), as internal controls for each sample. The data are presented as relative expression and enrichment (n-fold) with standard errors of the mean ± S.D with respect to control samples. All experiments were performed in duplicate at least three separate times.

Supplemental Reference

1 Boyd KE, Wells J, Gutman J, Bartley SM, Farnham PJ. c-Myc target gene specificity is determined by a post-DNA binding mechanism. *Proc Natl Acad Sci USA* 1998; **95**:13887-13892.