Supporting Information

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SI Materials and Methods

Stem Cell Lines Culture, Differentiation, and Treatment. The Shef-1 hESC line was obtained from the Servicio de Inmunologia, Hospital Universitario Central de Asturias, and H-181 pellets were from Centro Andaluz de Biología Molecular y Medicina Regenerativa–Andalusian Molecular Biology and Regenerative Medicine Centre, Seville, Spain. Each laboratory cultured these lines following established protocols. Shef-1 was cultured in hESC medium [KO-DMEM, 20% KO serum replacement (Gibco), 1× nonessential amino acids, 1× glutamine (both from Lonza), 0.1 mM β-mercaptoethanol, 4 ng/mL bFGF (Peprotech)] on a mouse MEF mitomycin C-treated (Sigma) feeder layer, whereas H-181 was cultured on Matrigel in feeder-conditioned hESC medium. Cell lines were established from individual embryos and maintained independently, thereby ensuring the independence of our results for type of line and culture conditions. These embryos were donated for research in accordance with the legal requirements of the country of origin. All donors gave written informed consent.

hESC were differentiated in vitro to EB and fibroblast-like cells (F-L) as described (1). Briefly, undifferentiated colonies were detached by treatment with collagenase IV (Gibco) and incubated as floating aggregates for 14 d (or as indicated) in ultra-low attachment flasks with hESC medium without bFGF or feeder conditioning. For further differentiation, they were attached to gelatin-coated plates and cultured in DMEM 15% FBS for an additional 15–20 d, passaging with trypsin two or three times. Cells finally appeared as a monolayer of fibroblasts and keratinocyte-like cells. In vitro neural differentiation of the Shef-1 cell line was carried out as described (2). For the mRNA stability assay, Shef-1 cells were treated with 5 μg/mL actinomycin D (Sigma) as indicated.

Mouse TC1 ES cells and the knockout ES line ($SIRT1^{\Delta ex4/\Delta ex4}$) (3) were cultured and differentiated to EB for 15 d by following protocols from the donating laboratory. Sirt1 transgenic ES cells were derived by the CNIO Transgenic Mouse Unit. Sirt1 transgenic ($^{+/+}$; tg/.) and WT ES cells were established de novo from blastocysts obtained by in vitro fertilization of WT C57BL/6 oocytes with sperm of a Sirt1 transgenic ($^{+/+}$; tg/.) male (4) by following standard protocols (5). After fertilization, embryos were cultured in KSOM at 37 °C for 3 d until they reached the blastocyst stage, then plated in ES cell medium with feeders until outgrowth of the inner cell mass was observed. ES cell medium is composed of DMEM (high glucose) supplemented with 15% FBS, 1,000 U/mL LIF, nonessential amino acids, Glutamax, and β-mercaptoethanol.

RNA Purification and Real-Time RT-PCR Analysis. RNA was isolated with TRIzol Reagent (Invitrogen) and reverse-transcribed by using the High Capacity cDNA Reverse Transcription Kit or the TaqMan MicroRNA Reverse Transcription Kit. Quantitative real-time RT-PCR (qPCR) was performed by using TaqMan Gene Expression Assays, SYBR green universal PCR master mix (see Dataset S5 for primers and assays) or the low-density TaqMan Human and Mouse Stem Cell Pluripotency Array and the ABI PRISM 7900 sequence-detection system (all from Applied Biosystems).

Western Blot Analysis. Cell lysates for protein analysis were prepared by SDS lysis extraction and analyzed by Western blot using anti-α-tubulin (Sigma), anti-SIRT1, anti-phospho-Ser27 SIRT1, anti-p53, anti-acetyl-lysine-382 of p53, anti-OCT4, and anti-SOX2 (all from Cell Signaling Technologies), anti-acetyl-K16 of histone H4 (Active Motif), anti-HuR and anti-V5 (Invitrogen), anti-methyl-HuR (6), anti-CARM1 (Biovision), anti-NANOG (R&D Biosystems), and anti-E-cadherin antibodies (Becton Dickinson). To purify nuclear and cytoplasmic fractions, cytosolic proteins were extracted with RSB buffer by using a complete protease inhibitor mixture (Roche), then separated from the nuclear pellet.

Immunofluorescence. hESC and EB cells were fixed and stained (7) by using rabbit anti-SIRT1 (Cell Signaling Technologies), mouse anti-OCT4 or anti-TUBB3 antibodies (Millipore), followed by Alexa448 goat anti-rabbit IgG or Alexa594 goat anti-mouse IgG (Molecular Probes). SIRT1-TUBB3 costaining is presented as maximum projection of 10 *z*-stacked images (1.5 μm thick) to show the whole axon path.

Flow Cytometry. EB were disaggregated by incubation in 0.4 U/mL Collagenase B (Roche; 2 h) and in enzyme-free cell dissociation buffer (Gibco; 10 min). A single-cell suspension was obtained by filtration through a 40-μm pore strainer and Histopaque 1077 (Sigma) separation of live mononuclear cells. Cells were fixed, permeabilized, and stained with the Intraprep kit (Beckman Coulter). We used anti-SIRT1 (Santa Cruz), anti-OCT4, anti-MAP2 antibodies (Cell Signaling), followed by FITC-conjugated anti-mouse IgG_{2B} and PE-anti-rabbit IgG (H+L) (Southern Biotech). Cells were analyzed in a Cytomics FC 500 (Beckman Coulter). Because the distribution for SIRT1 staining was continuous (no positive or negative populations were distinguishable), we arbitrarily defined two subpopulations: cells expressing high SIRT1 levels (SIRT1-high, FITC intensity $> 1.5 \times 10^2$, and SIRT1-low, FITC intensity $2–4 \times 10^1$). To study SIRT1-associated expression of pluripotency and neuroectodermal development markers, we analyzed OCT4 and MAP2 expression and plotted the two cell subpopulations by PE fluorescence intensity. Overlapped histograms are normalized for peak height.

ChIP-on-chip, qChIP, and Data Analysis. The chromatin immunoprecipitation assay was carried out as described (8) by using anti-acK9H3 (Millipore), anti-AcK16H4, anti-SIRT1, and anti-H3 (Abcam) antibodies. For the ChIP-on-chip assay, we used the Agilent Human Promoter Array. SIRT1-immunoprecitated DNA fragments and the corresponding unbound fractions were labeled and hybridized by following manufacturer's instructions. Results were analyzed by using the Agilent DNA Analytics program (9). Gene ontology was examined with DAVID (10, 11), a Web-based tool; results are shown in Dataset S2 as a GO chart and in Dataset S3 as GO clustering. For ChIP-on-chip validation, we selected the following genes: δ-like 4 (*DLL4*), a Notch ligand required for normal embryonic vascular development; LIM homeobox 1 (*LHX1*), a homeodomain-containing transcription factor (TF) involved in developmental processes such as axon guidance; paired box 6 (*PAX6*), a TF involved in neuroectodermal definition and oculogenesis; member 6 of the wingless-type MMTV integration site family (*WNT6*), a secreted glycoprotein important in myocardial and neural crest development; bone morphogenetic protein 1 (*BMP1*), a metalloprotease that participates in embryonic patterning by cleaving matrix proteins and morphogens; Hairy/enhancer of split 7 (*HES7*), a TF involved in somite segmentation; T-box 3 (*TBX3*), a TF that affects developmental events such as sinoatrial node determination, limb bud positioning and retinal dorso-ventral patterning; serpin peptidase inhibitor, clade E member 1-plasminogen activator inhibitor type 1 (*SERPINE1*), a secreted protease inhibitor that regulates TGFβ and EGF signaling; homeobox A5 (*HOXA5*) a homeobox-containing TF important in lung, intestinal, and thyroid morphogenesis and blood cell differentiation; and TIMP metal-

lopeptidase inhibitor 1 (*TIMP1*), a matrix protein that regulates proteases in development.

For qChIP, PCRs were carried out with SYBR-green PCR master mix and analyzed by using the 7900HT Fast Real-Time PCR System (all from Applied Biosystems). Primers for each promoter are shown in Dataset S5. The enrichment factor refers to the copy number of a gene of interest in the bound fraction after ChIP with the appropriate antibody, divided by the copy number of that gene in the unbound form for that antibody. For SIRT1, ChIP data are expressed as the percent enrichment of the SIRT1 immunoprecipitated sample relative to the negative control. For histone mark ChIP, where two samples were compared, data were further normalized for the total histone H3 signal.

RNA Interference. RNA interference was performed by using a protocol slightly modified from that of Braam et al. (12). Briefly, confluent hESC colonies were disaggregated by using Accutase (1:10 in PBS) and filtered through a 40-μm strainer to obtain a single-cell suspension, then seeded in Matrigel-coated plates with mTESR1 medium (Stem Cell Technologies). The siRNA complex was prepared with Lipofectamine RNAiMAX (Invitrogen) by following the manufacturer's protocol and mixed with the cells at the time of seeding. Cells were maintained in mTESR1, which was changed daily, and collected at the indicated time. For EB formation, cells were scraped 2 d after transfection and aggregates resuspended in EB formation medium, as above.

RNA Immunoprecipitation. mRNA was immunoprecipitated as described (13) by using anti-HuR (Santa Cruz), anti-methyl-HuR, and anti-V5 antibodies (Invitrogen). Cell pellets were homogenized in lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes at pH 7.0, 0.5% Nonidet P-40, 1 mM DTT, RNase OUT and complete protease inhibitor mixture), precleared with IgG1 mouse control (BD Pharmingen) and Protein A Sepharose beads (Sigma), then incubated with beads and IgG1 or with anti-HuR antibody, washed with NT2 buffer, and incubated with RNase-free DNase I (Invitrogen; 15 min, 37 °C). RNA was examined by qPCR.

HuR Phosphorylation Assay. Phosphorylated-HuR (Ser) was immunoprecipitated from 100 μg of total protein by using an anti-phosphoserine–agarose-conjugated antibody (Sigma). Immunoprecipitated phospho-HuR was detected by using total HuR antibody (Santa Cruz).

Cloning of HuR Wild-Type, HuR(R217K) and HuR(R217A), Plasmid Construction, and Transfection. Full-length HuR cDNA was obtained from the RZPD Deutsches Ressourcenzentrum für Genomforschung. HuR(WT)-V5 was constructed by PCR amplification using the 5′ oligonucleotide containing the V5 tag sequence, and subcloned into pCDNA 3.3 TOPO vector (Invitrogen). HuR (R217K)-V5 and HuR(R217A)-V5 mutants were constructed by using the QuikChange kit for site-directed mutagenesis (Stratagene) with two complementary oligonucleotides, and the pCDNA-HuR (WT)-V5 plasmid as the template. Primers are shown in Dataset S5. For plasmid transfection, Shef-1 hESC was prepared as a single-cell suspension (see RNA Interference above). Cells were pelleted for each reaction and transfected with plasmid by using the Amaxa Nucleofector kit 2 (Lonza), following manufacturer's protocols.

Bisulphite Sequencing of Multiple Clones. DNA methylation was quantified by PCR analysis following bisulphite modification of DNA. Bisulphite genomic sequencing was carried out as described (14). Primers were designed by using Methyl Primer Express Software (Applied Biosystems); primer sequences are shown in Dataset S5.

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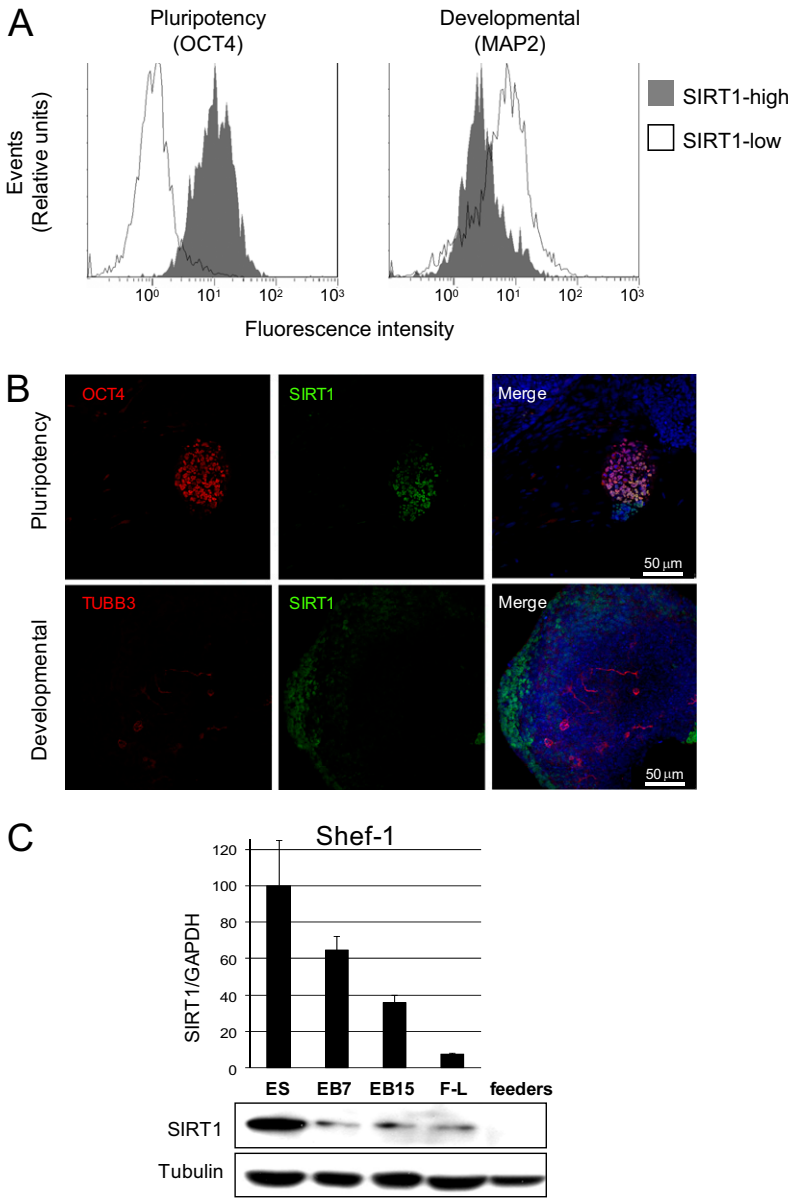

Fig. S1. Characterization of SIRT1 down-regulation during EB differentiation. (*A*) Flow cytometry analysis of pluripotency (OCT4) and neural differentiation (MAP2) markers in disaggregated EB cells. We arbitrarily defined a gate for cells with FITC intensity $> 1.5 \times 10^2$ (SIRT1-high subpopulation) and for cells with a FITC intensity from 2 to 4×10^1 (SIRT1-low), and plotted these subpopulations by PE fluorescence intensity. Graphs show the overlap of these histograms, scaled to the same total intensity, to visualize peak displacement. (*B*) Confocal fluorescence microscopy assay showing cell localization of SIRT1 (green) and OCT4 or TUBB3 (red) in Shef-1 15-d EB. (*C*) Time-course expression study of SIRT1 by qPCR (relative to GAPDH) and WB during Shef-1 hESC differentiation. α-tubulin was used as loading control. Mitomycin C-treated mouse embryonic fibroblasts used as feeder cells showed no murine Sirt1. Real-time PCR values are mean ± SD of three independent experiments.

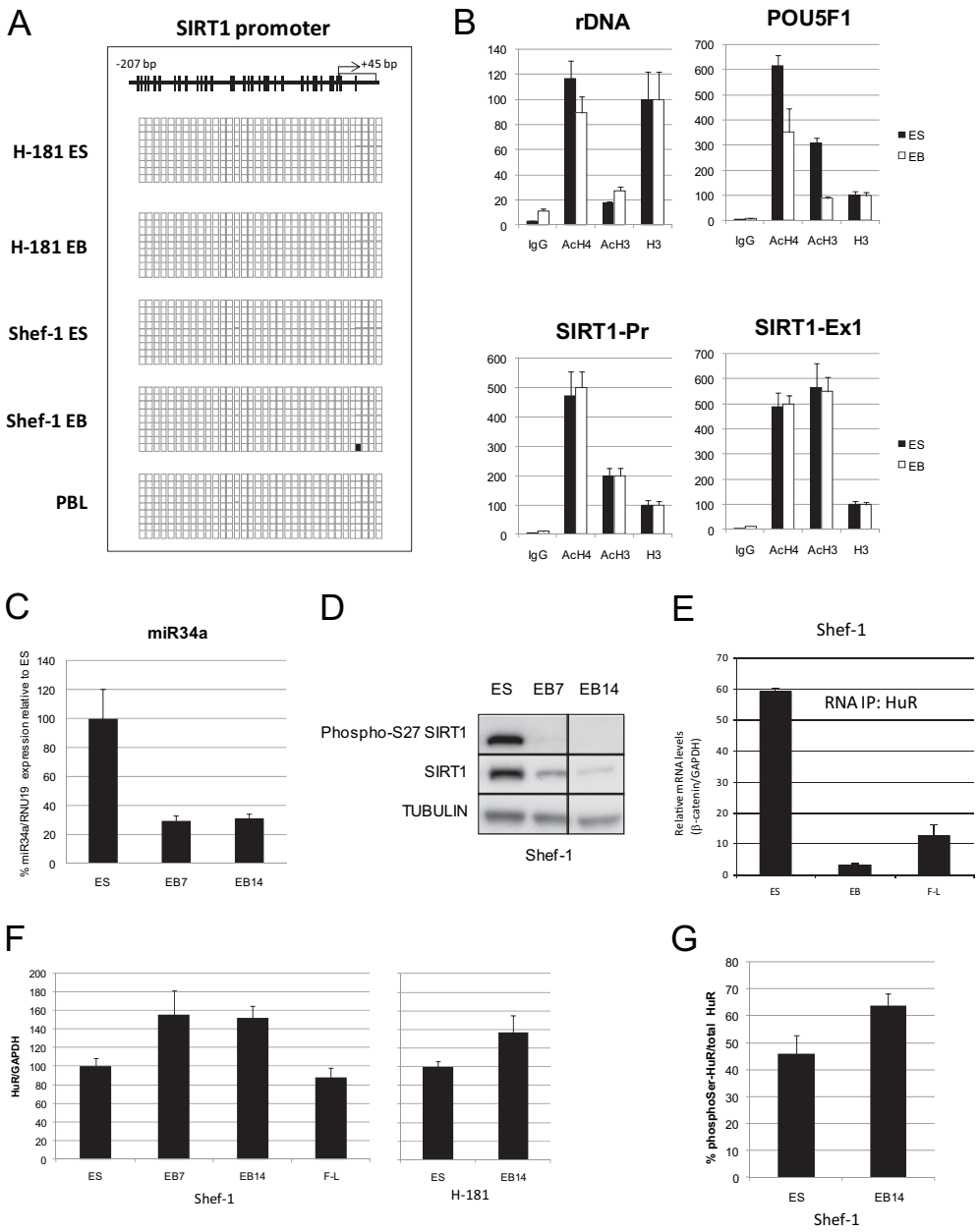

Fig. S2. Mechanisms of SIRT1 transcript and protein regulation. (*A*) Bisulfite genomic sequencing of multiple clones of the *SIRT1* promoter in two hESC lines (H-181, Shef-1), the corresponding differentiated sample (EB), and peripheral blood lymphocytes. Black box, methylated CpG; white box, unmethylated CpG. (*B*) qChIP of global acetylated histones H4 and H3 in Shef-1 hESC and EB. qPCR corresponding to promoters of *rDNA* (negative control), *POU5F1* (OCT4; positive control of down-regulation of activation markers in differentiation), and two regions of the *SIRT1* promoter located up- (SIRT1-Pr) and downstream (SIRT1-Ex1) of the transcriptional start site. IgG, negative IP control. Results are expressed as the percentage bound/unbound ratio of DNA copy number for each IP, further normalized with respect to total H3 enrichment (considered constant in chromatin). (*C*) Time-course expression study of miR34a by qPCR (relative to RNU19, a small RNA whose expression is considered constant) in hESC Shef-1 (ES) and in 7- and 14-d EB (EB7 and EB14). (*D*) Western blot analysis of hESC line Shef-1 (ES), and 7- and 14-d EB (EB7 and EB14) with anti-phosphorylated-Ser27 of SIRT1, -total SIRT1 and -α-tubulin (loading control) antibodies. (*E*) HuR binding to β-catenin RNA. RNA IP of HuR in hESC (ES), 15-d EB (EB) and fibroblast-like cells (F-L) of the Shef-1 hESC line. β-catenin levels were detected by qPCR (relative to GAPDH). (*F*) Time-course expression study of HuR by qPCR (relative to GAPDH) in hESC Shef-1 (ES), 7- and 14-d EB (EB7 and EB14), and fibroblast-like cells (F-L) (*Left*), and H-181 hESC and 14-d EB (*Right*). (*G*) Densitometric quantification of phosphorylated-HuR detected after IP with anti-phospho-Ser antibody and WB analysis in Shef-1 ES and 14-d EB. Data relative to total HuR levels. Data are expressed as mean ± SD of three independent experiments.

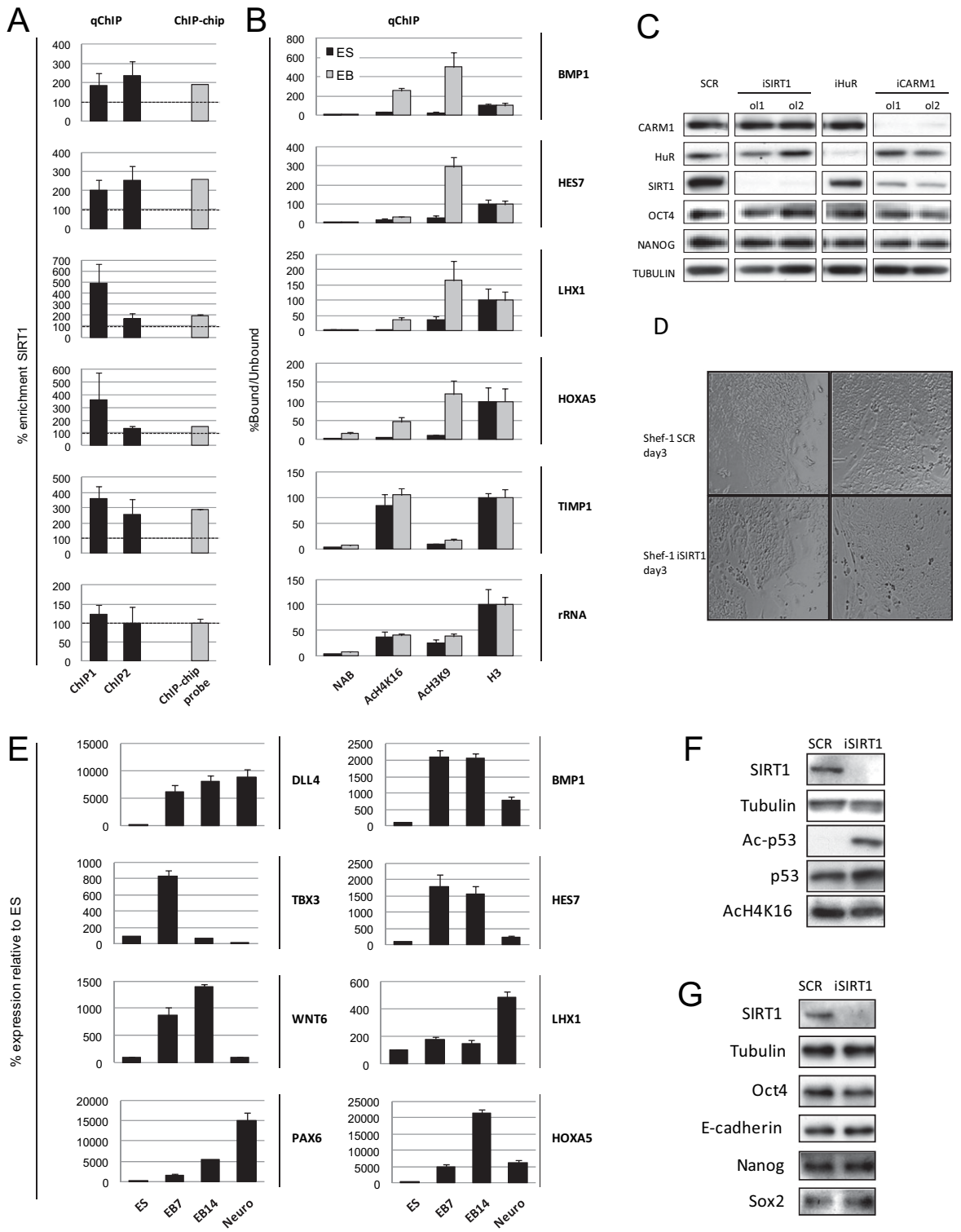

Fig. S3. SIRT1 ChIP-on-chip and RNAi validation. (*A*) qChIP of SIRT1 in Shef-1 hESC. qPCR was used to study enrichment relative to a chromatin sample immunoprecipitated without antibody (NAB) for the SIRT1-bound regions of *BMP1*, *HES7*, *LHX1*, *HOXA5*, and *TIMP1*. Results expressed as Fig. 3*A*. The ribosomal RNA gene (rDNA) promoter was included as a negative control for SIRT1 binding and histone modifications. (*B*) qChIP of acetyl-lysine 16 of histone H4 (AcH4K16) and acetyl-lysine 9 of histone H3 (AcH3K9) in Shef-1 hESC and EB. qPCR corresponds to the same genomic regions as above. Results are expressed as in Fig. 3*B*. (*C*) Western blot of CARM1, HuR, SIRT1, POU5F1 (OCT4), NANOG, and α-tubulin in Shef-1 cells, 3 d after transfection with control siRNA (SCR), SIRT1-specific siRNA (iSIRT1 ol1, ol2), HuR1-specific siRNA, or CARM1-specific siRNA (iCARM1 ol1, ol2). (*D*) Phase-contrast microscopy photographs of hESC Shef-1, 3 d after transfection with scrambled siRNA (SCR) or iSIRT1 ol1 siRNA (iSIRT1). (*E*) Expression of SIRT1-bound genes during EB and neural differentiation. Gene expression was measured by qPCR in Shef-1 hESC and 7- and 14-d EB (EB7 and EB14), and in the neuron-enriched sample derived from in vitro differentiation to neuroectodermal cells. Data are normalized to GAPDH expression, relative to the undifferentiated hESC sample (GAPDH expression is set to 100%). qPCR values are mean ± SD of three independent experiments.(*F*) WB of SIRT1, acetyl-lysine 382 of p53, total p53, acetyl-lysine 16 of histone H4, and α-tubulin in Shef-1 cells 3 d after transfection with control siRNA (SCR) or SIRT1-specific siRNA (iSIRT1). (*G*) Western blot using the same samples, for pluripotency markers Oct4, E-cadherin, Nanog, and Sox2.

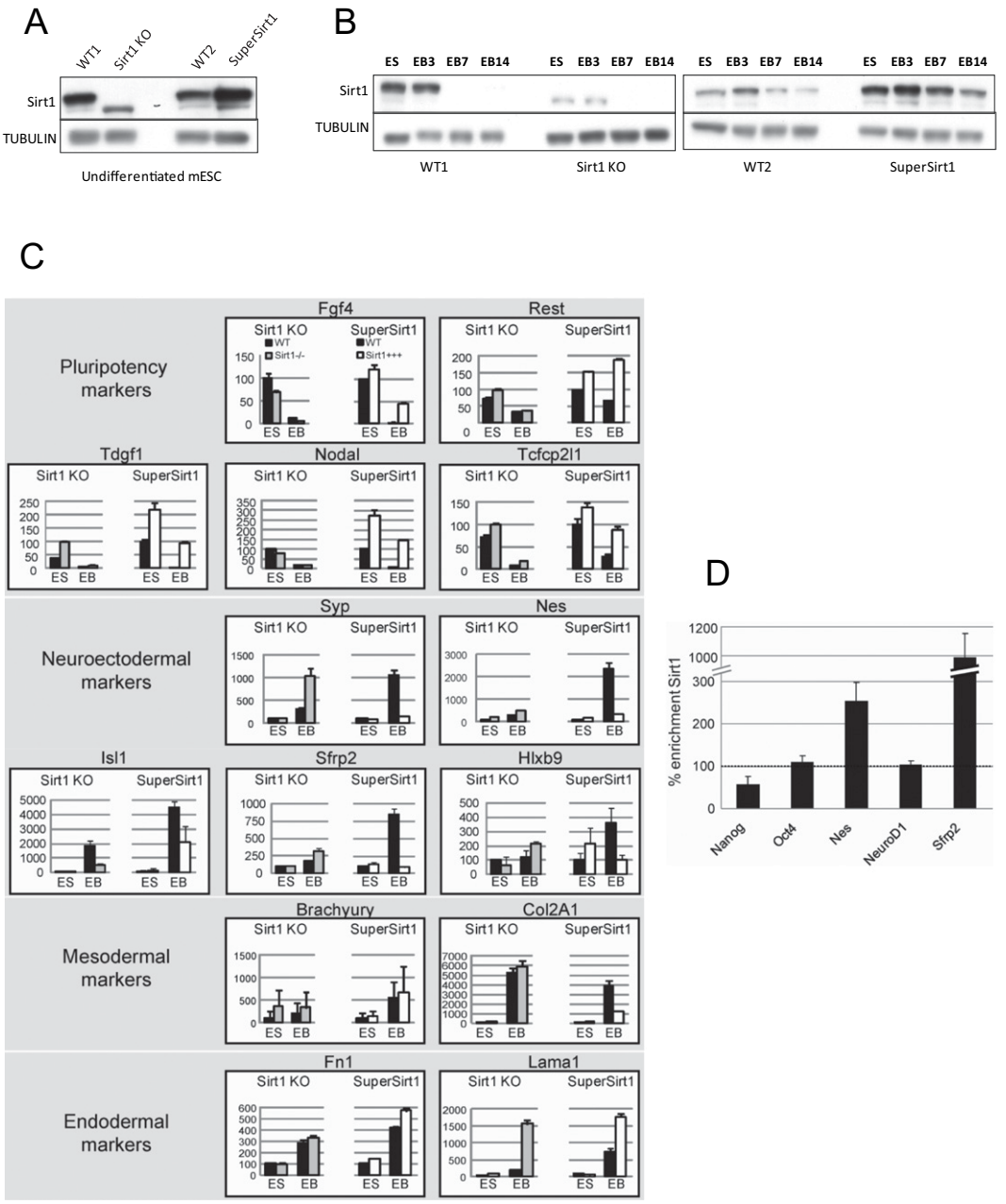

Fig. S4. mES cell lines characterization. (*A*) WB analysis of Sirt1 levels in mES lines of TC1 (WT1), TC1 with deletion of Sirt1 exon 4 (SIRT KO), mES clone with the normal copy number of the Sirt1 gene (WT2; *Materials and Methods*) and an ES clone bearing extra copies of the *Sirt1* gene (SuperSirt1). (*B*) WB of Sirt1 in the same mESC lines differentiated into EB for 3, 7, and 14 d (EB3, EB7, EB14). α-tubulin was used as a loading control. (*C*) Pluripotency and differentiation marker expression in Sirt1 KO ($Sirt1^{-/-}$) or SuperSirt1 ($Sirt1^{+++}$) mESC differentiation. Five pluripotency (Fgf4, Rest, Tdgf1, Nodal, Tcfcp2l1), five neuroectodermal (Syp, Nes Isl1, Sfrp2, Hlxb9), two mesodermal (Brachyury, Col2A1), and two endodermal (Lama, Fn1) cell markers were measured by qPCR in mESC (ES) and 15-d EB. Data are normalized with respect to Gapdh expression, relative to the undifferentiated ES sample for each line. qPCR values are mean ± SD of three independent experiments. (*D*) qChIP of Sirt1 in TC1 mESC. qPCR was used to analyze Sirt1 enrichment relative to a chromatin sample immunoprecipitated without antibody, for the *Nanog*, *Oct4*, *Nes*, *Neurod1*, and *Sfrp2* promoters. Results are expressed as percent enrichment of the bound/unbound ratio (DNA copy number) of Sirt1 relative to the NAB immunoprecipitate.

Other Supporting Information Files

Dataset S1 (XLS)
Dataset S2 (XLS)
Dataset S3 (XLS)
Dataset S4 (XLSX)
Dataset S5 (XLSX)