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Supplemental Information

A Chemical Biology Study of Human Pluripotent Stem Cells Unveils HSPA8 as a Key Regulator of Pluripotency

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SUPPLEMENTAL DATA

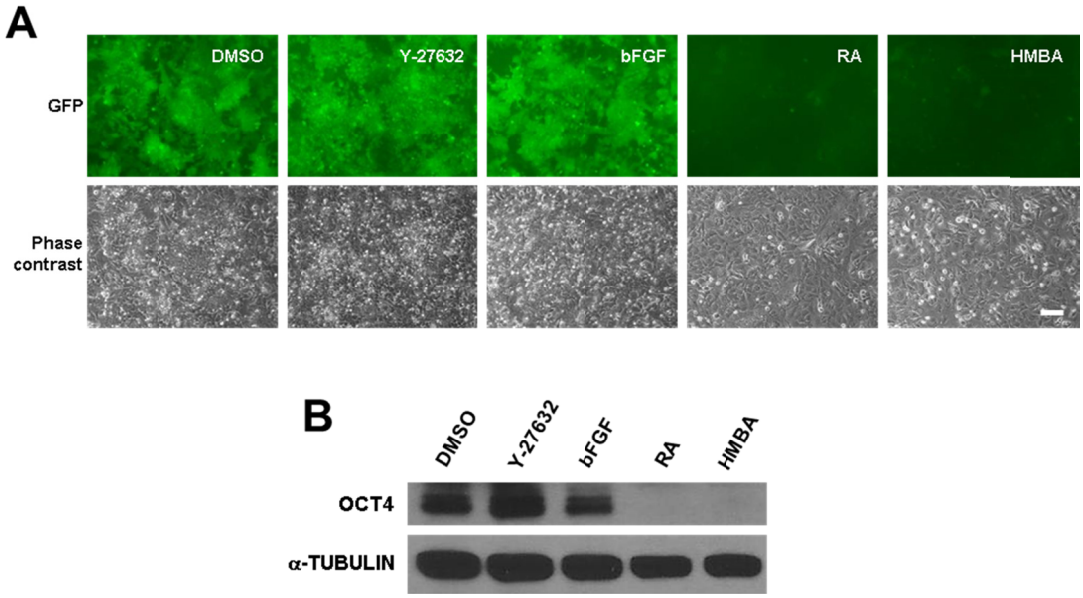


Figure S1, related to Figure 1. (**A**) Fluorescent (top panel) and phase contrast (bottom panel) images of NTERA-2-OP4k cells treated with DMSO, Y-27632 (10 μM), bFGF (4 ng/ml), retinoic acid (RA; 10 μM) and HMBA (3 mM) for 6 days. Scale bar: 100 μm. (**B**) Western blotting of OCT4 in NTERA-2-OP4k cells treated with DMSO, Y-27632, bFGF, RA, and HMBA for 7 days. α-TUBULIN was used as a loading control.

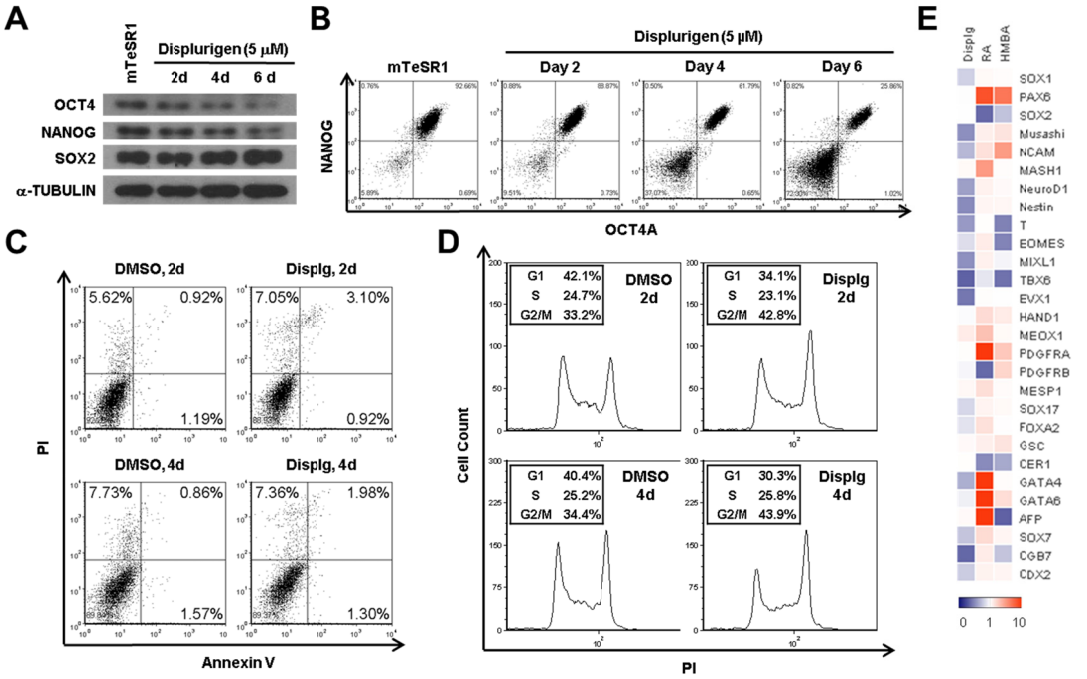


Figure S2, related to Figure 2. (**A**) Western blotting of OCT4, NANOG, and SOX2 in H1 hESCs untreated (mTeSR1) or treated with NSC375009 (Displurigen, 5 μM) for 2, 4, and 6 days (d). α-TUBULIN was used as a loading control. (**B**) Intracellular FACS analysis of OCT4A and NANOG in H1 hESCs untreated (mTeSR1) or treated with 5 μM NSC375009 (Displurigen) for 2, 4, and 6 days. (**C**) FACS analysis of propidium Iodide (PI) and Annexin V in H1 hESCs treated with DMSO or Displg (10 μM) for 2 days (2d) and 4 days (4d). (**D**) FACS analysis of PI in H1 hESCs treated with DMSO or Displg (10 μM) for 2 days (2d) and 4 days (4d). (**E**) Heatmap showing quantitative-PCR analysis of neuroectoderm (NE), mesoderm (MESO), endoderm (ENDO), and trophectoderm (TE) markers in H9 hESCs treated with Displg (10 μM), RA (10 μM), and HMBA (3 mM) for 6 days under differentiation condition. All values were normalized to the level (=1) of mRNA in DMSO control. Each bar represents mean ± SD (error bars). Overall average value of relative mRNA expression from n = 3 independent experiments were shown. *ACTB* (*β-actin*) was used as a loading control.

A

Hit Description	Score ($p < 0.05$ when score > 45)
keratin 1	278
keratin 10	179
heat shock 70 kDa protein 8 (HSPA8)	155

B

MSKGPAVGIDLGTTYSCVGVFQHG**KVEIIANDQGNRT**TPSYVAFTDTE**RLIGDA**
AKNQVAMNPTNTVFDAKRLIGRRFDDAVVQSDMKHWPFMVVNDAGRPKVQV
EYKGETKSFYPEEVSSMVLT**KMKEIAEAYLGKT**VTNAVVTVPAYFNDSQRQAT
KDAGTIAGLNVLRIINEPTAAAIAYGLDKKVGAERNVLIFDLGGGTFDVSILTIED
GIFEVKSTAGDTHLGGEDFDNRMVNHFIAEFKRKHKKDISENKRAVRRLRTAC
ERAKRTLSSSTQASIEIDSLYEGIDFYTSITRARFEELNADLFRGTLDPVEKALRD
AKLDKSQIHDIVLVGGSTRIPKIQ**KLLQDFFNGKE**LNKSINPDEAVAYGAAVQAA
ILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIK**RNTTIPTKQ**TQTFTTYSDN
QPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIEVTFDIDANGILNVS
AVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDEKQRDKVSS**KNSL**
ESYAFNMKATVEDEKLQGKINDEDKQKILDKCNEIINWLDKNQTAEKEEFEHQ
QKELEKVCNPIITKLYQSAGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD

C

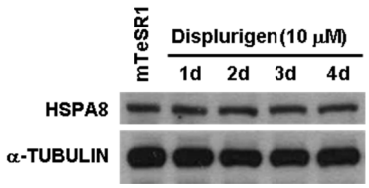


Figure S3, related to Figure 3. (**A**) List of all human proteins identified by mass spectrometry in the distinct 70 kDa band of Displg-biotin pull-down samples. (**B**) 7 unique peptide sequences in HSPA8 protein were identified by mass spectrometry. (**C**) Western blotting of HSPA8 in H1 hESCs untreated (mTeSR1) or treated with 10 μM Displg for 1 - 4 days (d). α-TUBULIN was used as a loading control.

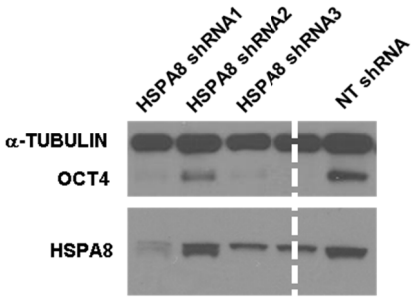


Figure S4, related to Figure 4. Western blotting of HSPA8-isoform-1 and OCT4 in H9 hESCs infected with lentivirus-particles containing Non-Target (NT) shRNA and HSPA8-1 shRNAs, shown as un-cropped blots. The lane which was cropped-off for Figure 4B is shown in this figure marked by a white dash line on top. The corresponding cropped blots are shown in Figure 4B.

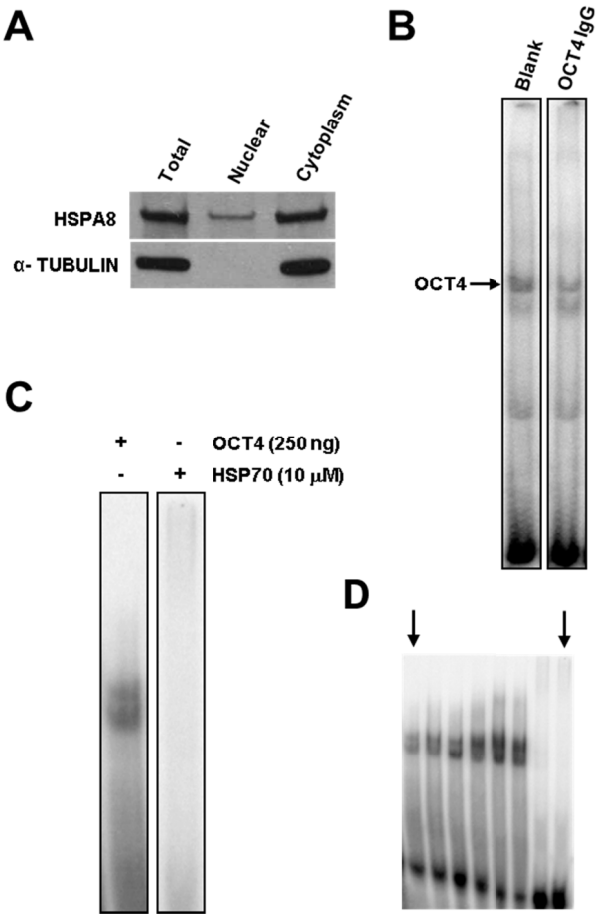


Figure S5, related to Figure 5. (**A**) Western blotting of HSPA8 in total lysate (Total), nuclear fraction (Nuclear), and cytoplasmic fraction (Cytoplasm) of undifferentiated H1 hESCs. Each lane was loaded with samples extracted from an equal number of cells. α-TUBULIN, an exclusively cytoplasmic protein, was used as a marker for the presence of cytoplasmic contents and to demonstrate clear separation of nuclear versus cytoplasmic fractions in this experiment. (**B**) EMSA using nuclear extracts of H9 hESCs treated with or without anti-OCT4 IgG showing the bindings of OCT4 proteins to the radio-labeled OCT4-binding element in *OCT4* promoter. The label "OCT4" points to the band sensitive to anti-OCT4 IgG treatment. (**C**) EMSA showing lack of DNA binding activity of purified recombinant HSP70 protein (10 μM) to a labeled OCT4-binding element *in vitro*. Purified recombinant OCT4 protein (250 ng) was used as a positive control. (**D**) The un-cropped image of Figure S5C. Black arrows point to the two lanes that were cropped and shown in Figure S5C.

Table S1. Antibody sources and dilutions

Antibody	Source	Catalog Number	Dilution
OCT4	Santa Cruz	sc-9081	1:1,000 (WB)
SOX2	Millipore	AB5603	1:500 (WB)
NANOG	Cell Signaling	4903P	1:500 (WB)
α-TUBULIN	Abcam	ab11304	1:10,000 (WB)
HSPA8-Isoform-I	R&D systems	MAB4148	1:100 (WB)
HSP70	Abcam	ab5439	1:1000 (WB)
OCT4A-Alexa647	BD Biosciences	562252	1:20 (FACS)
NANOG-PE	BD Biosciences	560483	1:5 (FACS)

(WB: Western blotting)

Table S2. qPCR primers

Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
ACTB	agagctacgagctgcctgac	cgtggatgccacaggact
OCT4 (isoform A)	cttctcgccccctccaggt	aaatagaacccccagggtgagc
NANOG	tttggaagctgctggggaag	gatgggaggaggggagagga
E-cadherin	tggaggaattcttgctttgc	cgctctcctccgaagaaac
N-cadherin	ctccatgtgccggatagc	cgatttcaccagaagcctctac
FN1	gacgcatcacttgcacttct	gcaggtttcctcgattatcct
SOX1	tacagccccatctccaactc	gctccgacttcaccagagag
PAX6	atttcccgctctggttcag	tagcgaagcctgacctctgt
SOX2	ggcagctacagcatgatgcaggagc	ctggtcatggagttgtactgcagg
Musashi	gagggttcgggtttgtcacg	ggcgacatcacctcctttgg
NCAM	atggaaactctattaaagtgaaccctg	tagacctcatactcagcattccagt
MASH1	cgacttcaccaactggttctg	atgcaggttgtgcgatca
NeuroD1	aagccatgaacgcagaggaggact	agctgtccatggtaccgtaa
Nestin	tccgatgggtttgcagat	cctcctcctgatcctcctct
T (Brachyury)	gctgtgacaggtacccaacc	catgcaggtgagttgtcagaa
EOMES	gtggggaggtcgaggttc	tgttctggaggtccatggtag
MIXL1	ggcgtcagagtgggaaatcc	gcagttcacatctacctcaagag
TBX6	gaacggcagaaactgtaagagg	gtgtgtctccgctcccatag
EVX1	ttcacccgagagcagattg	ccggttctggaaccacac
HAND1	aaaggccctacttccagagc	tgcgctgttaatgctctcag
MEOX1	aaagtgtcccctgcattctg	cactccagggttccacatct
PDGFRA	ccacctgagtgagattgtgg	tcttcaggaagtccaggtgaa
PDGFRB	aggctggccactacaccat	agcactcggacagggacat
MESP1	ctgttggagacctggatgc	cgtcagttgtcccttgtcac
SOX17	acgccgagttgagcaaga	tctgcctcctccacgaag
FOXA2	tgggagcggtgaagatggaagggcac	tcatgccagcgcccacgtacgacgac
GSC	gaggagaaagtggaggtctggtt	ctctgatgaggaccgcttctg
CER1	acagtgcccttcagccagact	acaactacttttttcacagccttcgt
GATA4	ggaagcccaagaacctgaat	gttgctggagttgctggaa
GATA6	aatacttcccccacaacacaa	ctctcccgcaccagtcat
AFP	agcttggtggtggatgaaac	ccctcttcagcaaagcagac
SOX7	ctcagggcagggaggtct	gcactcggataaggagagtcc
CGB7	tccttggcgctagaccac	cagggaggcacaggagtg
CDX2	gaccctcgccaccatgta	ctagggtacatgctcacgtcct

Table S3. shRNA sequences

Target	shRNA	Sequence (5' – 3')
HSPA8-1	HSPA8-1-1	ccgggcaactgttgaagatgagaaactcgagtttctcatcttcaacagttgctttttg
	HSPA8-1-2	ccggccaagacttcttcaatggaaactcgagtttccattgaagaagtcttggttttg
	HSPA8-1-3	ccgggcccgatttgaagaactgaatctcgagattcagttcttcaaatcgggctttttg

Table S4. EMSA probes

Probes	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
NANOG	tctgcagctacttttgcattacaatggccttggtgag	ctcaccaaggccattgtaatgcaaaagtagctgcaga
OCT4	cagacagcagagagatgcatgacaaaggtgccgtgatggttc	gaaccatcacggcacctttgtcatgcatctctctgctgtctg
SOX2	gccgtttgccttcatttccataagaagattaaga	tcttaatcttcttatggaaatgaaggcaaacggc

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture

H9 and H1 hESC lines (WiCell Research Institute, Madison, WI) were maintained under a feeder condition or a feeder-independent condition. For the feeder condition (Thomson et al., 1998), primary mouse embryonic fibroblasts (MEFs) prepared from embryos of pregnant CF-1 mice (day 13.5 of gestation; Charles River) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS (Hyclone), 1% non-essential amino acids (NEAA; Invitrogen), and penicillin/streptomycin, and mitotically inactivated by gamma irradiation. H9 and H1 hESCs were cultured on irradiated MEFs in media containing DMEM/F12, 20% knockout serum replacement (KSR; Invitrogen), 4 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen), 1% NEAA, 1 mM glutamine, and 0.1 mM β-mercaptoethanol. For the feeder-independent condition, hESCs were cultured on Matrigel (BD Biosciences)-coated plates in mTeSR1 medium (StemCell Technologies) as described (Ludwig et al., 2006). The experiments described in this study were conducted with H9 and H1 hESCs between passages 30 and 60. NTERA-2 cells (NTERA-2 cl.D1) were purchased from American Type Culture Collection (ATCC) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum.

Generation and validation of NTERA-2-OP4k reporter cells

The plasmid containing EGFP driven by a fragment of OCT4 promoter (~4 kb) was kindly provided by Dr. Wei Cui (Imperial College London) and was as described (Gerrard et al., 2005). We established NTERA-2-OP4k cells containing the OCT4-EGFP construct by transfecting cells

using Amaxa Nucleofection System (Nucleofector Kit L; Program X-001), selecting transfected cells with G418 (500 μg/ml, 2 weeks), and enriching EGFP-positive cells using fluorescence-activated cell sorting (FACS) (Cytomation Plus, Dako).

For validation of reporter activity, NTERA-2-OP4k cells were plated at a density of 0.1 – 0.25 × 10^5 cells/cm^2 and incubated with RA (Sigma; 10 μM), HMBA (Sigma; 3 mM), bFGF (Invitrogen; 4 ng/ml), and Y-27632 (Calbiochem; 10 μM). After 6 - 7 days of incubation, changes in the level of EGFP signals were examined using fluorescent microscopy (Zeiss), flow cytometry (BD Biosciences LSR II), and fluorescent plate reader (Analyst HT, Molecular Devices).

Large-scale chemical screening

Large-scale chemical screening was conducted at the High-Throughput Screening Facility (HTSF) at the University of Illinois at Urbana−Champaign (http://www.scs.illinois.edu/htsf/index.html). The HTSF hosts 171,077 compounds from several compound libraries, which include the Marvel Library, the HTSF House Library, the ChemBridge MicroFormat Library, and the National Cancer Institute (NCI) library.

For large-scale chemical screening, NTERA-2-OP4k cells were trypsinized and seeded onto 96/384-well plates (at 0.1 – 0.25 × 10^5 cells/cm^2) using a WellMate Microplate Dispenser (Matrix). Compounds were added immediately after plating using a 96-well or a 384-well pin-tool. The first two and last two columns of 384-well plates and the first and last column of

96-well plates were used for DMSO treatment as negative controls. Cells were incubated for 6 - 7 days without medium change. EGFP expressions of individual wells were recorded by the fluorescence plate reader (Analyst HT, Molecular Devices). EGFP signal detected with compound treatments were compared to the DMSO control, and those with significant reduction (>30%; average level of background signals subtracted before comparison) were marked as potential hits and were subsequently inspected visually using a fluorescence microscope (Zeiss). The final compound concentrations applied to the screening plates were 5 - 10 μM (Marvel library), 500 nM - 1 μM (NCI library), 5 - 10 μM (House library), and 10 - 20 μg/ml (ChemBridge MicroFormat library).

Western blotting

Cultured cells were lysed directly by 2× Laemmli buffer (Bio-Rad), boiled for 5 min, and analyzed using SDS-PAGE electrophoresis followed by wet-transfer onto nitrocellulose membranes using a system manufactured by Bio-Rad. The membranes were blocked using blocking solution (5% BSA in Tris-buffered saline containing 0.1% Tween-20 [TBST]), and then incubated with primary antibodies, diluted in TBST, at 4°C overnight. The membranes were then washed by TBST for 3 × 5 min, and incubated with horseradish peroxidase (HRP) conjugated secondary antibodies at room temperature for 1 hr. Finally, the membranes were washed 5 min each time for 3 – 5 times by TBST and developed using Super-Signal West Pico Chemiluminescent Substrate (Pierce). Antibody information are described in Table S1.

RNA extraction, reverse transcription, and quantitative-PCR

Total RNA were isolated using RNeasy mini kit (QIAGEN). cDNAs were synthesized from the purified RNAs using Reverse Transcription System (Promega). Quantitative-PCR was performed using QuantiTech SYBR Green PCR kit (QIAGEN). Signals were analyzed using the comparative C_T method, and ACTB gene was used as an internal control. The designs of gene-specific primers are described in Table S2.

Intracellular FACS analysis

Single cell suspensions were acquired through Accutase (Invitrogen) treatment. Cells were fixed and stained using Transcription Factor Buffer Set (BD Biosciences) following the manufacturer's instructions. Conjugated antibodies including OCT4A-Alexa647 and NANOG-PE (Table S1) were used. Cells were resuspended in PBS supplemented with 1% BSA and analyzed using a BD Biosciences LSR II flow cytometry analyzer and BD FACSDiva software.

Apoptosis analysis

Cells were collected and washed in cold PBS. Cells were then stained using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) following the manufacturer's instructions. Cells were analyzed using a BD BD Biosciences LSR II flow cytometry analyzer and BD FACSDiva software.

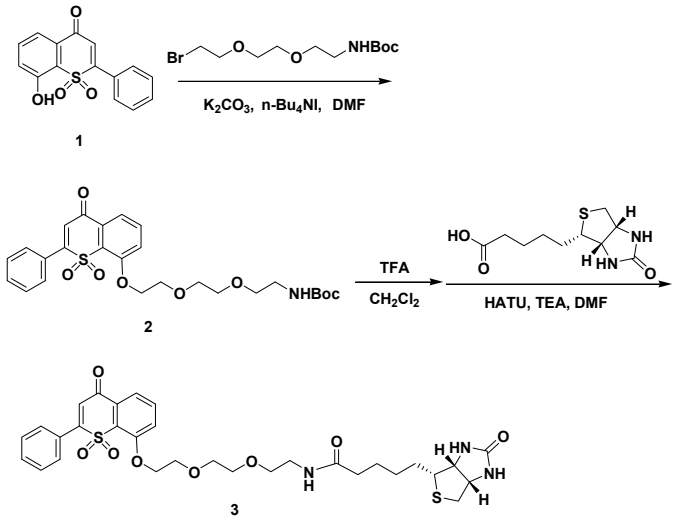
Cell cycle analysis

Cells were collected and washed in cold PBS, and fixed in chilled ethanol overnight at 4°C. Cells were then washed and resuspended in PBS with 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase A for 30 min at 37°C. DNA content was measured by flow cytometry.

Differentiation assay

H9 and H1 hESC were seeded onto Matrigel coated plates in single cell in mTeSR1 medium. Cells were then incubated with a basal differentiation medium containing Advanced RPMI 1640 (Invitrogen), 2% B-27 supplement (Invitrogen), and 1% Glutamax (Invitrogen) with compound treatments (DMSO, 10 μM Displg, 10 μM RA, and 3 mM HMBA). Medium was changed every other day. Samples were collected on day 6 for analysis.

Synthesis of Displurigen-biotin



Synthesis of Compound 2: Compound 1 (0.0055 g, 0.019 mmol), 2-[2-(2-tertbutoxycalbonyl

aminoethoxy) ethoxy]ethyl bromide (0.0071 g, 0.023 mmol), K_2CO_3 (0.0030 g, 0.022 mmol) and *n*-Bu_4NI (0.0014 g, 0.0038 mmol) were suspended in DMF (1 mL). The suspension was heated at 60 °C for 12 h. After evaporation of the solvent, crude product was purified by flash chromatography on silica gel (EtOAc : Hexanes = 1 : 1) and then EtoAc to give **2** as solid (0.0086 g, 0.0166 mmol, 86.5 %). ^{1}H NMR (300MHz, $CDCl_3$, 25 ^{o}C) δ 1.43 (s, 9H), 3.29 (m, 2H), 3.53 (t, *J* = 5.1 Hz, 2H), 3.58 (m, 2H), 3.76 (m, 2H), 3.99 (t, *J* = 4.5 Hz, 2H), 4.40 (t, *J* = 4.8 Hz, 2H), 4.98 (bs, 1H), 6.73 (s, 1H), 7.42-7.62 (m, 4H), 7.68 (t, *J* = 6.9 Hz, 1H), 7.83-7.90 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 ^{o}C) 28.4, 40.4, 69.3, 70.2, 70.4, 70.5, 71.4, 119.9, 120.4, 127.8, 129.1, 130.0, 131.6, 133.8, 154.5, 155.9, 156.6, 178.4

Synthesis of Compound 3: Deprotection of **2** (0.0086 g, 0.0166 mmol) was performed with 0.5 mL of CH_2Cl_2 and 0.2 mL TFA at 0 °C. After stirring at 0 °C for 3 h, the reaction mixture was concentrated. Toluene (0.5 mL) was added to the residue and then evaporated to remove TFA. The procedure was repeated three times. The resulting TFA salt of the deprotected amine was dissolved in DMF (1 mL) and D-biotin (0.0071 g, 0.029 mmol). HATU (0.012 g, 0.0316 mmol) was added followed by a triethylamine (25 μL, 0.18 mmol). After stirring at rt overnight, the mixture was concentrated under reduced pressure and the residue was purified by flash chromatography on silica gel ($CHCl_3$: EtOH = 10 : 1), which gave **3** (0.0077 g, 0.012 mmol, 72.1 %) as solid. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C) δ 1.37 (m, 2H), 1.68 (m, 4H), 2.15 (t, *J* = 7.5 Hz, 2H), 2.70 (d, *J* = 12.9 Hz, 1H), 2.88 (dd, *J* = 5.4 Hz, *J* = 12.9 Hz, 1H), 3.09 (m, 1H), 3.40 (m, 2H), 3.56 (t, *J* = 5.1 Hz, 2H), 3.67 (m, 2H), 3.78 (m, 2H), 3.98 (t, *J* = 4.8 Hz, 2H), 4.23 (m,

1H), 4.40 (m, 2H), 4.47 (m, 1H), 4.93 (s, 1H), 5.77 (s, 1H), 6.50 (t, 1H), 6.75 (s, 1H), 7.42-7.60 (m, 4H), 7.66 (t, J = 7.5 Hz, 1H), 7.84 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 ^{o}C) 25.8, 28.3, 36.1, 39.4, 40.8, 55.7, 60.4, 62.0, 69.4, 70.2, 70.6, 70.7, 71.2, 120.0, 120.7, 128.2, 129.4, 130.3, 131.9, 134.2, 154.6, 156.7, 164.0, 173.5, 178.6; MS (ESI^+) (m/z): $[M + H]^+$ Calcd. For $C_{31}H_{38}N_3O_8S_2$: 644.2095; Found: 644.2104.

Affinity chromatography, electrophoresis, and silver staining

hESCs were cultured under the feeder-independent condition with or without the presence of displurigen-biotin (10 μM) overnight, washed in PBS three times and lysed in Ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors cocktail (Sigma). The lysates were then diluted two-fold by Tris buffer (50 mM Tris-HCl, pH 7.5).

For affinity chromatography ("pull-down"), diluted lysates were incubated with streptavidin−agarose beads (Sigma) for 5 hr at 4°C. The beads were collected using centrifugation and washed with wash buffer (75 mM NaCl, 0.5 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, 50 mM Tris-HCl, pH 7.6). The washed beads were suspended in 2× Laemmli buffer (Bio-Rad) and heated at 100°C for 5 min.

For electrophoresis and silver staining, 10 μl samples were loaded on polyacrylamide gel. Silver staining was performed using ProteoSilver Plus Silver Stain Kit (Sigma). Protein bands detected by silver staining were selectively excised for mass spectrometry analysis.

Mass spectrometry analysis

Gel slices were dehydrated and destained in 50% Acetonitrile + 25 mM NH4HCO3 and gently crushed using a plastic pestle inside a 1.5 ml eppendorf tube. The crushed gel was dried briefly using Speedvac (Thermo) before digestion with Trypsin (mass spectrometry grade, G-Biosciences at 1:50 w/w) in 25 mM ammonium bicarbonate. Digestion was performed using a CEM Discover Microwave Digestor (Mathews, NC) for 15 min at 75 watts, 55°C. The digested peptides were extracted using 50% acetonitrile and 5% formic acid, lyophilized and reconstituted in 5% acetonitrile and 0.1% formic, 10 µl of which was used for LC/MS analysis. Mass spectrometry was performed using a Waters Q-ToF connected to a Waters nanoAcquity UPLC (Milford, MA). Column was a Waters Atlantis dC18 nanoAcquity UPLC column 75 µm x 150 mm (3 µm particle size) running at 250 nl/min. Gradient was from 100% A to 60% B (A: water and 0.1% formic acid; B: acetonitrile and 0.1% formic acid). Data collection was performed using MassLynx 4.1 (Waters). The top 4 intensive precursor ions from each survey scan were subjected to MS/MS by Collision Induced Dissociation (CID). The raw mass spectrometric data were processed using ProteinLynx Global Server 2.2.5 (Waters). The refined peaklists were analyzed using Mascot 2.2 (Matrix Science, London, UK) with a tolerance of ± 0.4 Da for both the precursor ions and fragment ions. Searches were carried out using the NCBI non-redundant protein database.

Embryoid body (EB) formation

H9 and H1 hESC colonies were dissociated from the culture surface by 20 min treatment of Dispase (Invitrogen). Suspended colonies were pooled by brief centrifugation (1000 rpm, 1 min),

resuspended in medium containing Advanced RPMI 1640 (Invitrogen), 2% B-27 supplement (Invitrogen), and 1% Glutamax (Invitrogen), and then plated into ultra-low attachment plates (Corning). Medium was changed every other day.

***In vitro* ATPase assay**

The HSP70 ATPase rates were determined as described (Freeman et al., 1995). In brief, ATP hydrolysis was determined by measuring the release of [^{32}P]Pi from [γ-^{32}P]ATP. The ATPase rates were calculated utilizing an average [γ-^{32}P]ATP hydrolysis rate at each time point (5, 10, 15 and 20 min) from three separate experiments after the background hydrolysis was subtracted. The data were visualized and quantified by PhosphorImager analysis (Molecular Dynamics). The effect of Displurigen on the ATPase rate of HSP70 was measured by incubating HSP70 (5 min) with varying levels of Displurigen prior to the introduction of ATP.

Lentivirus production and infection

For viral packaging, expression vectors were co-transfected with pCMV-dR8.91 and pCMV-VSV-G into 293T cells by $CaPO_4$ precipitation. After overnight incubation, culture medium was replaced by virus-packaging medium containing DMEM, 30% FBS, and 1 mM sodium pyruvate. Supernatants were collected 48 hr later and concentrated approximately 100× by ultracentrifuge (20,000 rpm, 1 hr). H1 and H9 hESCs were infected by virus concentrates in the presence of Polybrene (6 μg/ml), and then subjected to puromycin (0.5 μg/ml) selection for 3-5 days prior to analysis.

HSPA8 overexpression plasmid construction

HSPA8-isoform-1 cDNA clone was purchased from OriGene (SC322471), and subcloned into a pSin-EF2 plasmid (Addgene; modified with a short adaptor sequence) at the MluI/NdeI site.

Immunoprecipitation

Cells treated with DMSO or Displg (100 μM, 2 hours) were lysed in ice-cold RIPA buffer (Pierce) supplemented with a protease inhibitor cocktail (Sigma). After clarification, cell lysates were pre-cleared with Protein A/G magnetic beads (Pierce) and thereafter incubated with 2 μg of anti-OCT4 antibody (Santa Cruz) and with DMSO or 1 mM Displg, respectively. After 2 hours of incubation at 4°C, Protein A/G magnetic beads were added to the cell lysates. The resulting immunoprecipitates were washed with ice-cold PBS and analyzed by Western blotting.

Electrophoretic mobility shift assay (EMSA)

EMSA analysis was performed using Buffer C nuclear extracts (10 μg) from hESCs. The extracts were incubated with poly dI-dC (Sigma) and ^{32}P-end labeled oligonucleotides: *OCT4*, *NANOG* and *SOX2* binding sequence probes along with the complimentary primers. The protein-DNA complexes were resolved by native polyacrylamide gel (4%) electrophoresis and the dried gels were visualized using a PhosphorImager (Molecular Dynamics). Sequences of *OCT4*, *NANOG* and *SOX2* probes are listed in Table S4.

In vitro OCT4-DNA binding assay

OCT4 cDNA was cloned from the pSin-EF2-OCT4-Pur vector (Addgene) and inserted into the PET-24a protein expression vector, which contains a C-terminal His Tag. OCT4 and HSP70 proteins were expressed in Rosetta cells and captured with the Talon metal affinity resin (Clontech). The proteins were further purified using Resource Q and Superdex 200 columns (GE Healthcare). OCT4 protein binding to the *OCT4* response element (Supplemental Experimental Procedures) was assessed by the use of EMSA analysis in the presence or absence of purified HSP70 protein.

Statistical analysis

Statistical analyses were performed using Microsoft Excel. Student's *t*-test was used to compare two experimental groups, assuming unequal variances. Differences are considered significant when $p < 0.05$.

SUPPLEMENTAL REFERENCES

Freeman, B.C., Myers, M.P., Schumacher, R., and Morimoto, R.I. (1995). Identification of a Regulatory Motif in Hsp70 That Affects Atpase Activity, Substrate-Binding and Interaction with Hdj-1. Embo J *14*, 2281-2292.

Gerrard, L., Zhao, D., Clark, A.J., and Cui, W. (2005). Stably transfected human embryonic stem cell clones express OCT4-specific green fluorescent protein and maintain self-renewal and pluripotency. Stem Cells *23*, 124-133.

Ludwig, T.E., Levenstein, M.E., Jones, J.M., Berggren, W.T., Mitchen, E.R., Frane, J.L., Crandall, L.J., Daigh, C.A., Conard, K.R., Piekarczyk, M.S.*, et al.* (2006). Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol *24*, 185-187.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. In Science, pp. 1145-1147.