Stem Cell Reports, Volume *3*

Supplemental Information

Transient p53 Suppression Increases Reprogramming of Human Fibroblasts without Affecting Apoptosis and DNA Damage

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Figures

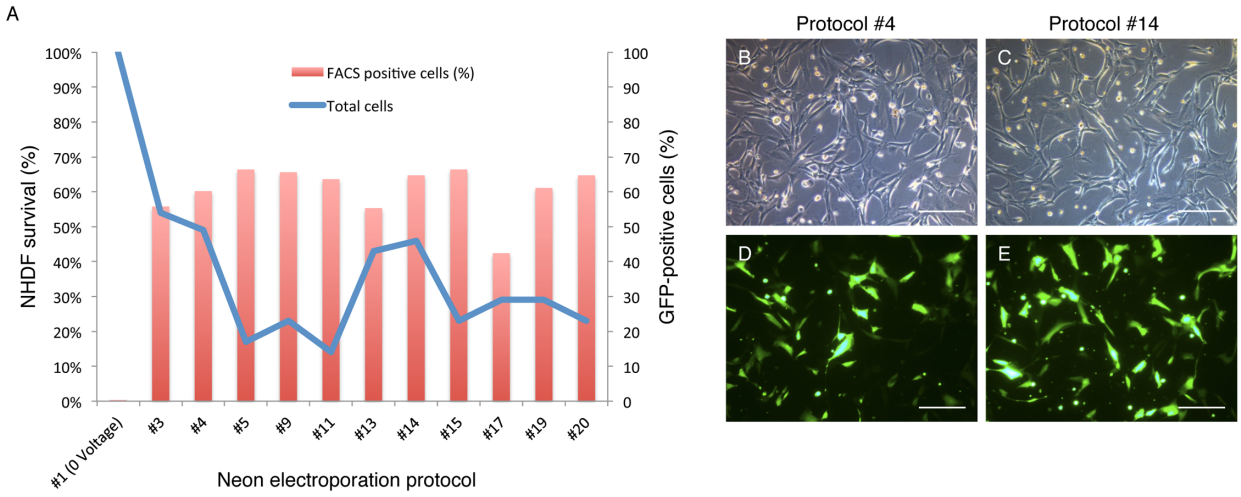


Figure S1. Optimization of non-integrative reprogramming procedure, related to Figure 1. A) Survival and flow cytometry analyses of normal human dermal fibroblasts (NHDFs) one day after electroporation with a GFP plasmid using a Neon™ electroporation device at different conditions. B-E). Phase contrast morphology and green fluorescent protein (GFP) fluorescence of NHDFs electroporated with a single pulse at 1600 V for 20 ms (Protocol #4; B, D) or two pulses at 1200 V for 20 ms (Protocol #14; C, E). Scale bars correspond to 200 µm.

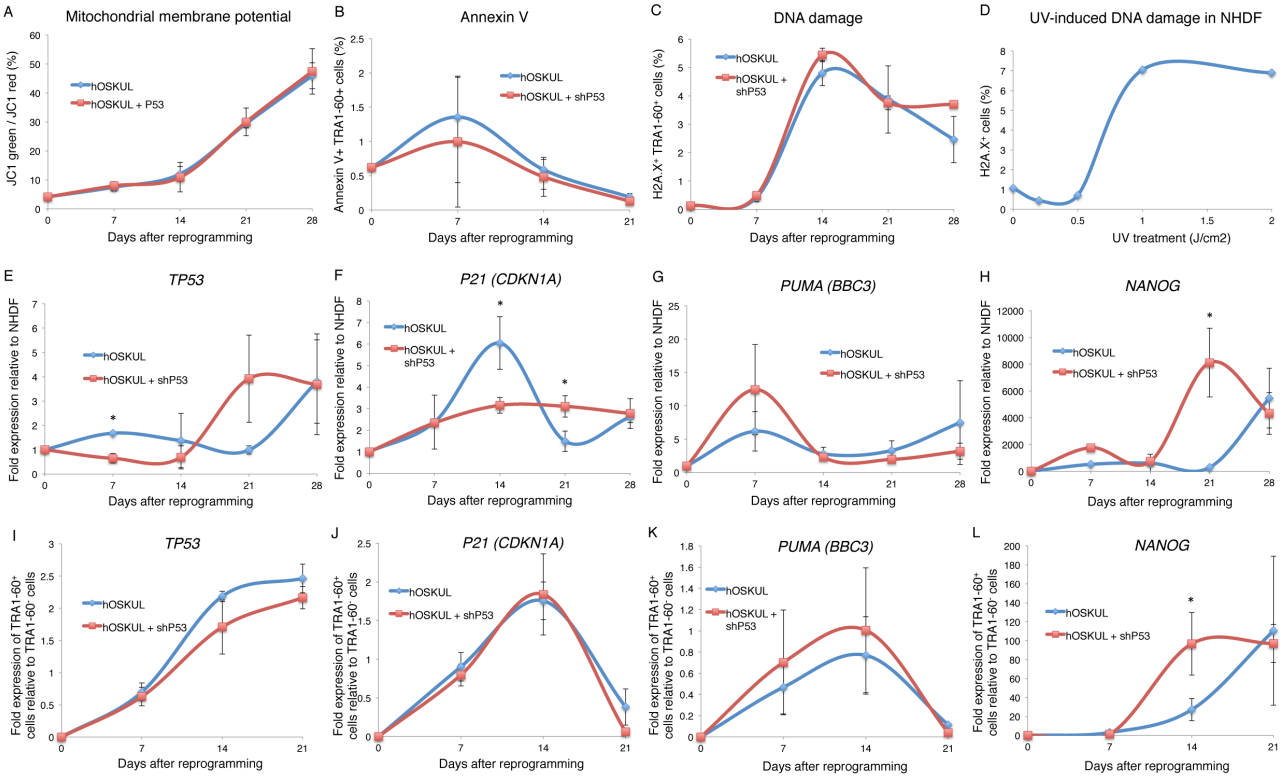
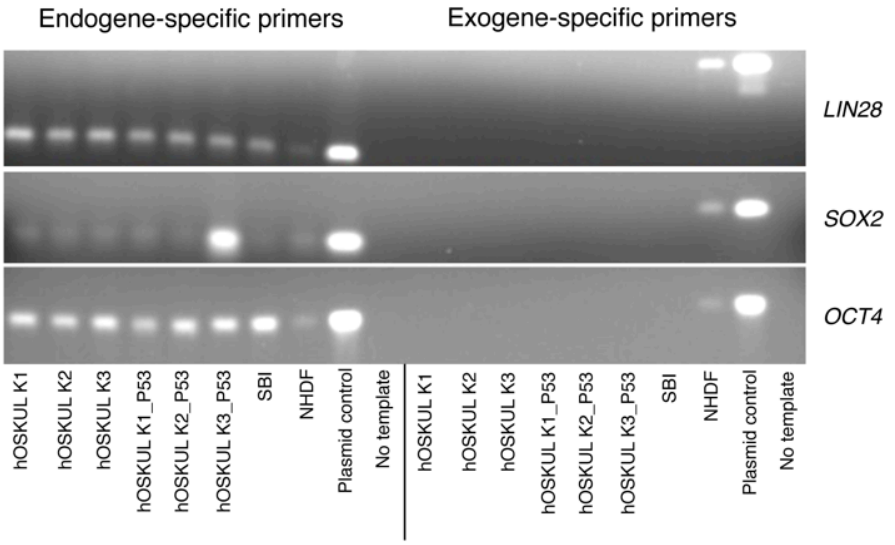


Figure S2. Apoptosis, DNA damage and expression of *P53*, *P21*, *PUMA* and *NANOG* during reprogramming with or without (w/wo) a short hairpin to p53 (shp53), related to Figure 2. Normal human dermal fibroblasts (NHDFs) were analyzed on day 0, 7, 14, 21 (and 28) after reprogramming with the episomal plasmids *hOCT4*, *hSOX2*, *hKLF4*, *hL-MYC* and *hLIN28* (hOSKUL) w/wo shp53. A) Mitochondrial membrane potential assay with the dyes JC-1 and DAPI. In apoptotic cells, the mitochondrial potential collapses and the monomeric form of JC-1 shows green fluorescence. B) Flow cytometry of cells double positive for Annexin V and TRA1-60. C) Flow cytometry of cells double positive for the DNA damage marker H2A.X and TRA 1-60. D) Flow cytometry with H2A.X of NHDFs subjected to different doses of ultraviolet radiation. E-L) Quantitative real-time PCR analyses in the total cell population (E-H) or in TRA1-60 sorted cells (I-L) after reprogramming w/wo shP53. E, I) *TP53*, F, J) *P21,* G, K) *PUMA* and H, L) *NANOG.* Relative expression is shown as the fold change ($2^{-\Delta\Delta Ct}$) with NHDFs as reference and GAPDH as reference gene. Y error bars depict standard deviation of three independent experiments. *p<0.05.

A



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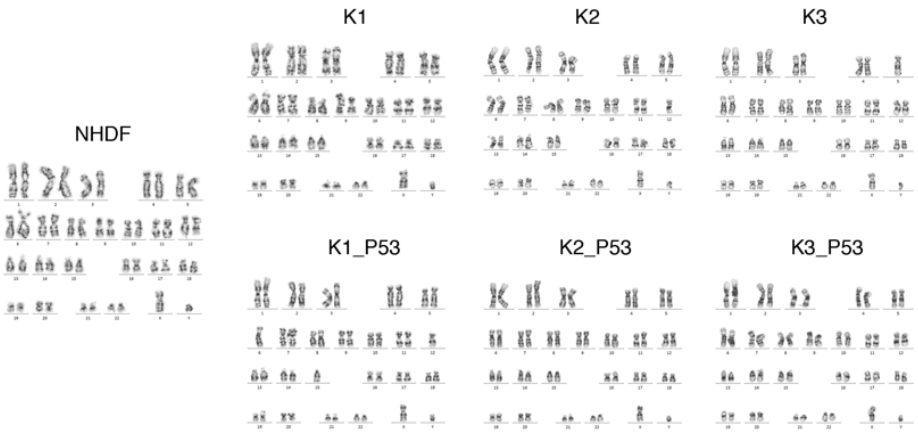

Figure S3. Expression analysis of episomal vectors in iPSC lines and karyotyping of induced pluripotent stem cell lines, related to Figure 3. A) Reverse-transcriptase PCR at passage 10 with the endogenous and exogenous pluripotency genes *LIN28*, *SOX2,* and *OCT4* in iPSC lines generated with or without a short hairpin to p53. Normal human dermal fibroblasts (NHDFs) transfected two days before with hOSKUL served as a positive control and a sample without template served as negative control. An iPSC line (SBI) generated by lentivirus was included as a reference. B) Representative karyotypes of the founder normal human dermal fibroblasts (NHDFs) and induced pluripotent stem cell (iPSC) lines generated with or without a short hairpin to p53 (shp53).

Tables

Table S1. Copy number variations in induced pluripotent stem cell lines, related to Figure 3.

iPSC line[1]	Type	Chr[2]	Band	Start position	End position	Size (kb)[3]	Genes within the CNV[4]	DGV[5]
K1	Gain	1	q44	245500158	245787005	287	*KIF26B*	Variation
K1	Gain	3	p12.2	81531958	82762047	1230	*GBE1*	Variation
K3	Loss	2	q33.3	205758866	205984167	225	*PARD3B*	Variation
K1_shp53	Gain	5	q34	167784954	167951642	167	*WWC1, RARS*	
K1_shp53	Loss	7	q11.22	69672679	70023881	351	*AUTS2*	
K1_shp53	Loss	7	q31.1	110709365	110842032	133	*IMMP2L, LRRN3*	Variation
K1_shp53	Gain	10	q21.1	53966718	54049359	83	*PRKG1*	Variation
K1_shp53	Loss	12	q23.1	100658499	100697244	39	*DEPDC4, SCYL2*	Variation
K2_shp53	Loss	13	q31.3	94337996	94506524	169	*GPC6*	Variation
K3_shp53	Loss	X	q21.33	94049304	94129700	80		

[1]Induced pluripotent stem cell line, [2]chromosome, [3]kilobases, [4]copy number variation, [5]database of genomic variance.

Table S2. Primers used for methylation analyses, related to Figure 3.

Gene relation	Primer names	Primer sequences[1] 5'-3'	Product sizes (bp)	Products chromosomal extend[2]
NANOG	ConNRR-F1	ttATATTttTGATTTAAAAGTTGGAAA	298	Chromosome 12
	ConNRR-R1	aaCAACCAaCTCAaTCCAaCAaAAC		701.877 -702.174
POU5F1	ConOCT4-3-F	AttTGttTTTTGGGtAGTTAAAGGt	221	Chromosome 6
(OCT4)	ConOCT4-3-R	CCAaCTaTCTTCATCTTaaTaaCATCC		31.080.784-31.080.564
RAB25	ConRAB25-F1	AGGGGtTATTTtTTGtTtATTAGTTG	178	Chromosome 1
	ConRAB25-R1	TaaaTaTCTaCTCTCAaCCTaaaCTCCC		7.519.337-7.519.514
SALL4	ConSALL4-F1	GGttAATtAGtTGTtAGGGtTtATGA	110	Chromosome 20
	ConSALL4-R2	TCCTaaAaTTaaaAAATTTACCCCC		20.615.097-20.615.206
UBE1L	ConUBE1L-F1	GtTTGGttTTGGTTttTGTTTGtAt	169	Chromosome 3

ConUBE1L-R1	aAATACAaCTCCTCATCCAaTAaCTTC	49.791.174-49.791.342

[1]Lower case ‘t’ corresponds to a cytosine in the unconverted target sequence. Lower case ‘a’ corresponds to a guanine in the unconverted target sequence. [2]Genome Reference Consortium Human Build 37.

Table S3. Antibodies used for confirmation of pluripotency and *in vitro* differentiation, related to Figure 3 and 4.

	Antibody and host species	**Dilution**	**Company and catalog number**
Pluripotency	rabbit anti-NANOG	1:500	Peprotech, 500-P236
	goat anti-OCT4	1:500	Santa Cruz, sc-8628
	rat anti-SSEA3	1:100	Biolegend, 330302
	mouse anti-SSEA4	1:100	Biolegend, 330402
	mouse anti-Tra-1-81	1:200	Biolegend, 330702
	mouse anti-Tra-1-60	1:200	Biolegend, 330602
In vitro	mouse anti-Smooth muscle actin (SMA)	1:500	DAKO, M0851
differentiation	rabbit anti-Alpha-1-fetoprotein (AFP)	1:500	DAKO, A0008
	mouse anti-Beta-III-tubulin (TUJI)	1:4000	Sigma-Aldrich, T8660
	mouse anti-SOX2	1:100	RD systems, MAB2018
	rabbit anti-NESTIN	1:4000	Millipore, ABD69
	mouse anti-VIMENTIN	1:500	DAKO M0725
	sheep anti-VGLUT1	1:200	Abcam, AB79774

Supplemental Experimental Procedures

Unless otherwise stated, consumables and reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA. Plasmids were purchased from Addgene, Cambridge, MA, USA.

Reprogramming of normal human dermal fibroblasts and establishment of iPSC lines

NHDFs (Lonza, CC-2511) from an 18-year-old male (XY NHDF) and a 32-year-old female (XX NHDF) were cultured in fibroblast medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin and streptomycin (Pen/Strep) and 2 ng/ml basic fibroblast growth factor (bFGF; Miltenyi Biotech, Bergisch Gladbach, Germany).

At passage 5-6, the NHDFs from a 18-year old healthy male were trypsinized and 10^5 cells were electroporated with a total of 1μg of the episomal plasmids *hOCT4* (Addgene plasmid #27076) or *hOCT4* with a short hairpin to *TP53* (shp53; Addgene plasmid #27077) in combination with *hSOX2* and *hKLF4*, (Addgene plasmid #27078) and *hL-MYC* and *hLIN28* (Addgene plasmid #27080), abbreviated *hOSKUL* or *hOSKUL* + shp53*,* respectively. As a control, a fibroblast line from the same NHDF line was electroporated with a PLK.O-shp53 plasmid (Godar et al., 2008) and stable clones expressing shp53 were selected using puromycin. The electroporation was performed using a Neon™ electroporation device (Life Technologies, Carlsbad, CA, USA) with a single pulse at 1600 V for 20 ms or two pulses at 1200 V for 20 ms. Optimization of the electroporation procedure was performed with an episomal GFP plasmid (Addgene plasmid #27082).

Immediately after electroporation, the cells were transferred to a single well of a 6-well culture dish containing warm fibroblast medium without Pen/Strep, which was changed the day after to fibroblast medium with Pen/Strep. Seven days after electroporation, NHDFs were trypsinized and split 1:2 onto hESC-qualified Matrigel-coated dishes (BD Biosciences, Franklin

Lakes, NJ, USA) and cultured in mTeSR1 medium (Stem Cell Technologies, Vancouver, BC, Canada) in 5% O_2, 5% CO_2 in N_2 with the medium replenished every other day.

On day 28, six iPSC lines were established from the same XY NHDF donor using the plasmids *hOSKUL* (named K1, K2 and K3) or *hOSKUL* + shp53 (named K1_shp53, K2_shp53 and K3_shp53). The primary iPSC colonies were dissected out manually by needles and transferred to new Matrigel-coated 6-well dishes and cultured in mTeSR1. After a second manual passage, the iPSC lines were split 1:6 every 5-6 days with Dispase (Stem Cell Technologies, Vancouver, BC, Canada). At passage 10, the iPSC lines were harvested for subsequent analyses or frozen in CryoStem freezing medium (Stemgent, Cambridge, MA, USA).

Alkaline Phosphatase and TRA1-81 live staining

On day 28 after reprogramming, iPSC colonies were live stained for 30 min with a TRA1-81 antibody (Stemgent, Cambridge, MA, USA) or fixed in 4% paraformaldehyde (PFA) and stained for Alkaline phosphatase (AP) using an AP detection kit (Merck-Millipore, Billerica, MA, USA). The total numbers of AP-positive iPSC colonies were quantified using a stereomicroscope.

Flow cytometry

On day 0 (NHDFs) and on days 7, 14, 21 and 28 after reprogramming, cells were trypsinized and washed in flow buffer containing 1% bovine serum albumin (BSA) in Dulbecco's Phosphate-Buffered Saline (DPBS). Cells were either incubated for 45 min with a combination of APC conjugated anti-SSEA4 (BD Biosciences, Franklin Lakes, NJ, USA) and PE conjugated anti-TRA-1-60 (BD Biosciences, Franklin Lakes, NJ, USA or fixed for 15 min with 4% PFA, incubated for 30 min in 90% ice-cold methanol and incubated with Alexaflour 488-conjugated anti-p53, anti-p21, anti-phospho-histone H2A.X (Ser139) antibody (Cell Signaling Technology, Danvers, MA, USA)

or DyLight 488-conjugated anti-PUMA (Novus Biologicals, Littleton, USA) and PE-conjugated anti-SSEA4 and 647-conjugated anti-TRA-1-60 (BD Biosciences, Franklin Lakes, NJ, USA). After washing twice in flow buffer, flow cytometric analysis was performed with a BD FACSarray Bioanalyzer or a BD Accuri C6 (BD Biosciences, Franklin Lakes, NJ, USA). Unlabeled and isotype labeled NHDF's were used as controls for gating. As a positive control, NHDFs were exposed to different ultraviolet (UV) treatments at 254 nm, ranging from 0, 0.2, 0.5, 1 and 2 J/cm^2.

Apoptotic measurements

Apoptosis was measured by a mitochondrial membrane potential assay (Chemometec, Alleroed, Denmark). Briefly, cells were stained with the dyes JC-1 and DAPI. In healthy cells, JC-1 forms aggregates showing red fluorescence, whereas, in apoptotic cells the mitochondrial potential collapses and JC-1 localizes to the cytosol in its monomeric form showing green fluorescence. Quantification of green and red JC-1 fluorescence was carried out by use of a Nucleocounter NC-3000 (Chemometec Alleroed, Denmark).

Immunofluorescence staining

At passage 10, all iPSC lines were fixed with 4% PFA in PBS for 15 min and stained by standard immunofluorescence staining procedures. The primary antibodies (Supplementary table 1) were visualized with the secondary antibodies Alexa 488 or Alexa 594 diluted 1:400 (Life technologies, Carlsbad, CA, USA) and counterstained with Hoechst bisbenzimide 33258. Images were acquired on a Leica DMRB-fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was purified from unsorted and TRA1-60 sorted cells during reprogramming as well as

from six iPSC lines at passage 10 and NHDFs by RNeasy kit (Qiagen, Hilden, Germany). Conversion to cDNA and quantitative real-time PCR (qRT-PCR) were performed using TaqMan RNA-to-C_T™ 1-step kit (Life Technologies, Carlsbad, CA, USA). Primers for qRT-PCR (Life Technologies, Carlsbad, CA, USA) included *SOX2*, *ZFP42*, *NANOG*, *POU5F1 (OCT4)*, *DNMT3B*, *TP53*, *P21* (*CDKN1A*), *PUMA* (*BBC3*) and *GAPDH*. Relative quantification was calculated using $2^{-\Delta\Delta Ct}$ with NHDFs as a reference and GAPDH as reference gene. A commercial iPSC line (named SBI; System Biosciences, Mountain view, CA, USA), generated by viral transduction of human foreskin fibroblasts with *OCT4*, *SOX2*, *KLF4* and *C-MYC*, was used as a positive control.

In vitro differentiation

Embryoid body (EB) formation was performed by transferring Dispase-treated clumps of iPSC to ultra-low attachment plates (Corning, Corning, NY, USA) in mTeSR1. After 2 days of culturing, the medium was changed to DMEM/F12 containing 20% knockout serum replacement (Life Technologies, Carlsbad, CA, USA), 1x non-essential amino acid, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol and 1% pen/strep. After 7 days, the EBs were plated on 0.1% gelatin-coated culture dishes and cultured in DMEM supplemented with 10% FBS, 2 mM L-gultamine and 1% pen/strep for up to three weeks. The cells were fixed for 15 min in 4% PFA for immunocytochemical analyses with antibodies of TUJI, SMA and AFP (Supplementary table 1).

Karyotyping

At passage 10, all iPSC lines and NHDFs were treated for 45 min with KaryoMAX colcemid (Life Technologies, Carlsbad, CA, USA) and harvested in fresh fixative containing 25% acetic acid and 75% methanol. Karyotyping was performed on G-banded metaphase chromosomes using standard

procedures. At least 10 metaphases were analyzed per sample with an approximate resolution of 550 to 600 bands per haploid genome.

Genome wide transcriptome analysis

For whole transcriptome microarray analysis, RNA was isolated from the iPSC lines K1 and K1_shp53 at passage 16, K2 and K2_shp53 at passage 12 and K3 and K3_shp53 at passage 13, as well as NHDFs and SBI iPSC with RNeasy Kit (Qiagen, Hilden, Germany). cDNA was hybridized to a HT12 v4 BeadChip microarray (Illumina, San Diego, CA, USA), which contains more than 47.000 probes for known human genes. Raw data were analyzed with the PluriTest algorithm (www.pluritest.org) or processed in Genome Studio data analysis software (Illumina, San Diego, CA, USA) using quantile normalization and a detection p-value of < 0.01 and subsequently analyzed in Multi Experiment Viewer (www.tm4.org) using a comparative t-test with Bonferroni correction and $p < 0.05$.

Copy number variation (CNV) analyses

DNA was purified from all iPSC lines at passage 10 and founder NHDFs using Wizard genomic DNA purification kit (Promega, Madison, WI, USA). CNV analysis was performed using the high resolution CytoScan HD chromosome microarray platform (Affymetrix, Santa Clara, CA, USA), which provides 750.000 polymorphic (SNP, single nucleotide polymorphism) and 1.900.000 non-polymorphic (CNV) markers. Raw data were processed using Affymetrix Chromosome Analysis Suite (ChAS) and manually corrected for false positive hits. To prevent false positive CNVs arising due to inherent microarray “noise”, gains and losses of > 30kb (and >30 consecutive probes) were taken into consideration (confidence limit > 90%). The identified CNVs were compared with the Database of Genomic Variants (DGV; http://projects.tcag.ca/variation/) and 1038 phenotypically

healthy samples run on the same platform to exclude common variants. The data were interpreted using the UCSC Genome Browser (http://genome.ucsc.edu). Data is available upon request.

Directed neural differentiation

Directed neural differentiation was carried out according to Shi and colleagues (Shi et al., 2012), with minor modifications. Briefly, iPSC lines generated w/wo shp53 (n=6) were cultured in mTeSR1 and ESC-qualified Matrigel until they reached 70-80% confluence. The medium was then changed to neural induction medium (NIM) consisting of DMEM/F12 and Neurobasal medium (1:1; Life Technologies, Carlsbad, CA, USA), 1 x N2 and 1 x B-27 minus Vitamin A (both from Life Technologies Carlsbad, CA, USA), 1mM Glutamax and Pen/Strep including the dual SMAD inhibitors LDN193189 (0.2 μM) and SB431542 (10 μM). After 10-12 days, the cells were split 1:3 with Dispase onto poly-L-ornithine (20ug/ml) and laminin (5 μg/ml)-coated dishes and cultured in neural maintenance medium (NMM) consisting of NIM without LDN193189 and SB431542. When rosette structures were observed, 20 ng/ml bFGF was added for 4 days followed by a second split (1:3) using Dispase. Hereafter, the cells were split 1:3 by Accutase upon confluence. At days 21 and 35, the cells were fixed in 4% PFA for immunocytochemical analyses with neuron-specific antibodies (Supplementary table 1) and on day 80, recordings of intracellular calcium kinetics were performed. Cells in 96-well plates were incubated for 1h at RT with the fluorescent calcium indicator, Calcium 5 (Molecular Devices, Sunnyvale, CA, USA) dissolved in Hanks Balanced Salt Solution (HBSS; Life Technologies). Following loading, recordings were performed with an FDSS 7000 fluorescence kinetics plate reader (Hamamatzu Photonics K.K., Hamamatsu city, Japan). The excitation wavelength was 480 nm; the emission wavelength was 540 nm and the sampling frequency 1Hz.