SUPPLEMENTAL FIGURE LEGENDS

Figure S1, Related to Figure1. Expression of EMT-Related Factors in hESCs-derived Neural Cells. Western blot to show expression of EMT factors with indicated antibodies in cells at different stages of neural differentiation from hESCs or indicated overexpression HEK293T cells. β-TUBULIN is a loading control. n=3.

Figure S2, Related to Figure 2. Construction of *ZEB1*-Depleted H9ES Cell lines. (A) Cas9/sgRNA-targeting site in *ZEB1* locus. sgRNA targeting sequences (in bold) was selected in the third exon (blue box) of *ZEB1*, and BfuC1 restriction sites were used for RFLP analysis. Protospacer adjacent motif (PAM) sequences are in orange. Cas9 cleavage sites are indicated by red arrow heads. Restriction sites are underlined. (B) RFLP analysis upon *ZEB1* sgRNA transfection in H9ESCs. The asterisk indicates the uncut PCR fragment used to quantify Indel frequency by RFLP. Ctrl: control, from wild-type H9ES cells. (C) RFLP analysis for picked monoclonal hESCs using BfuC1. Numbers in red are monoclonal hESCs without cut at *ZEB1* sgRNA locus. (D) Representative sequences of one homozygous *ZEB1* mutant clone (clone 9) with two different copy (one inserted "T" and one 7 base pairs deletion). KO: knockout, WT: wild-type.

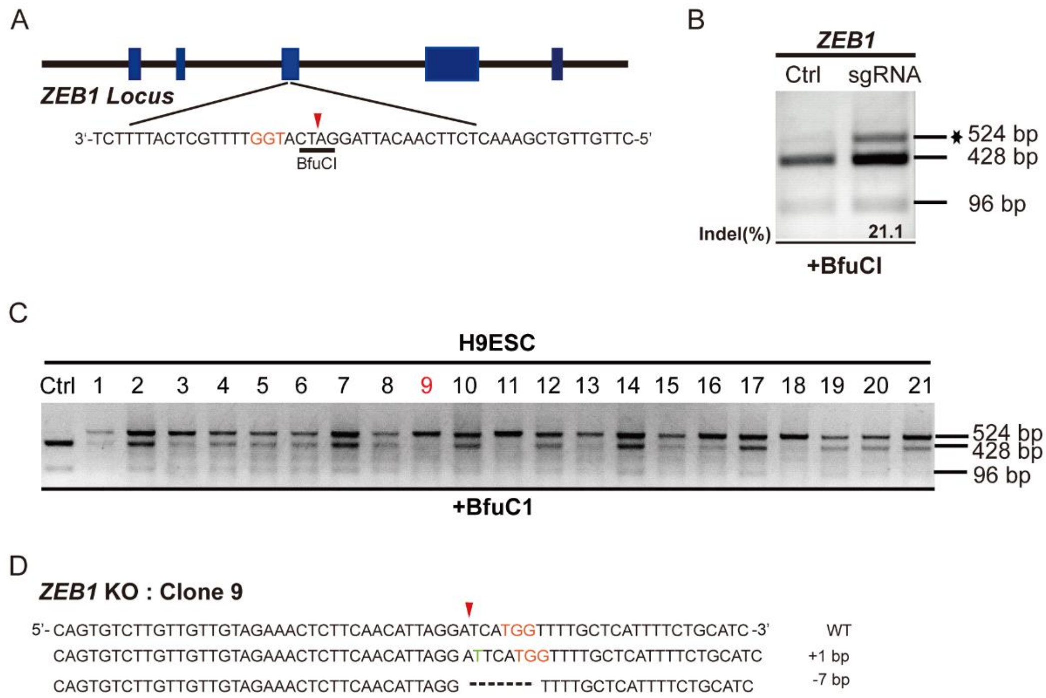


Figure S3, Related to Figure 3. Establishment of A H9ESC Line with Inducible Expression of *ZEB1*. (A) Vector construction for inducible expression of *ZEB1*. The CMV promoter of pLVX-Tet3G plasmid was replaced by elongation factor-1α (EF1α) to optimize transgene expression in human ESCs. The expression plasmid of pLVX-TRE3G was modified by replacing the puromycin fragment with mNeonGreen-P2A-Blasticidin (NPB) to facilitate observation, selection and monoclonal hESCs picking. 3×Flag-*ZEB1* and 3×Flag-EGFP fragments were inserted, respectively after TRE3G promoter, which could induce the expression of inserted genes when supplemented with doxycycline in the medium. The pLVX-TRE3G-3×Flag-EGFP ES cell lines were used as a control to verify whether the inducible system works. (B) Representative images showing a visible ES cell colony from a single hESC sorted by flow cytometry due to inserting the fragment of mNeonGreen. n=3. Scale bar: 50 μm. (C) Western blot analyses on the 6 lines of monoclonal H9ES cells (3×Flag-EGFP) cells with or without Dox using indicated antibodies. Administration of doxycycline in hESCs did not affect the pluripotency of the cells. (D) Western blot showing that inducible expression of *ZEB1* in the six lines of monoclonal hESCs did not promptly affect the expression of OCT4 and NANOG. GAPDH is a loading control in C and D. n=3. (E) Representative Immunostaining images showing TUJ1-positive neurons detected in ESC for long-term cultivation with *ZEB1* overexpression. n=3. Neo^r: Neomycin, mNG: mNeonGreen, 2A: self-cleaving 2A peptide, BSD: Blasticidin, Dox: Doxycycline, C1, C1, C2, C3, C4, C5 and C6: six H9ES cell lines from six single hESC, respectively.

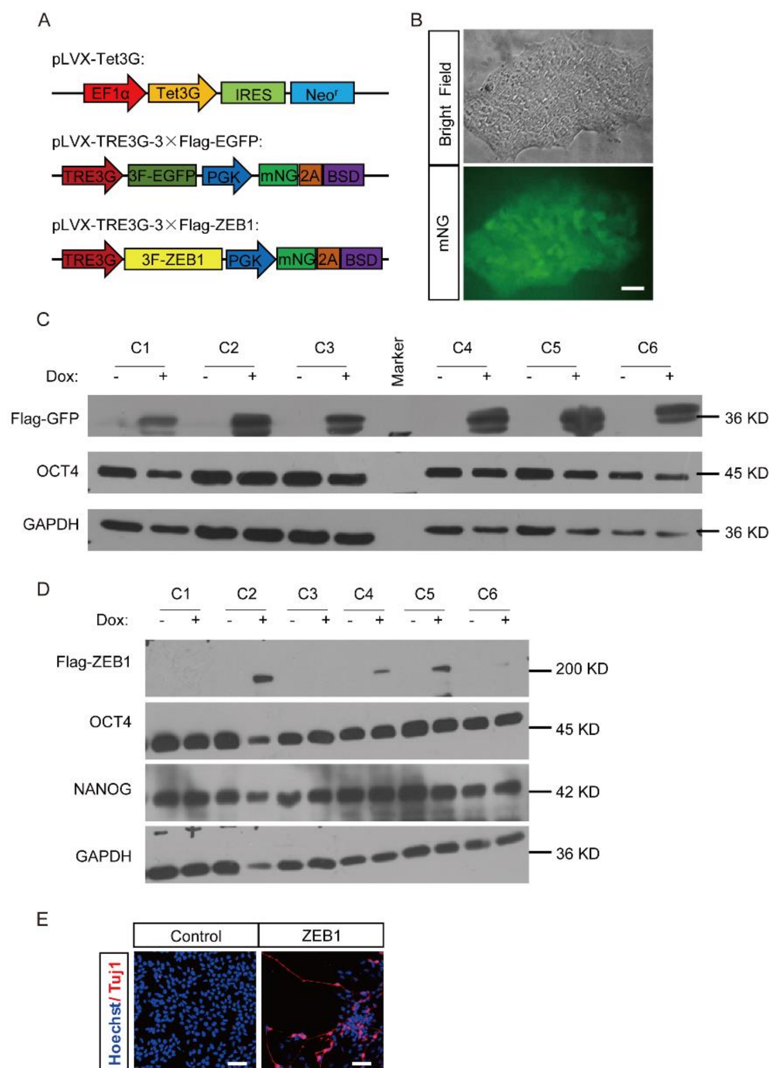


Figure S4, Related to Figure 3. Immunostaining for pluripotency markers. Immunostaining images showing the expression of ZEB1 and the pluripotency markers including SOX2, OCT4 and NANOG. n=3. Scale bar: 50 μm. Hoechst staining was to show the nuclei in (C) (hereafter).

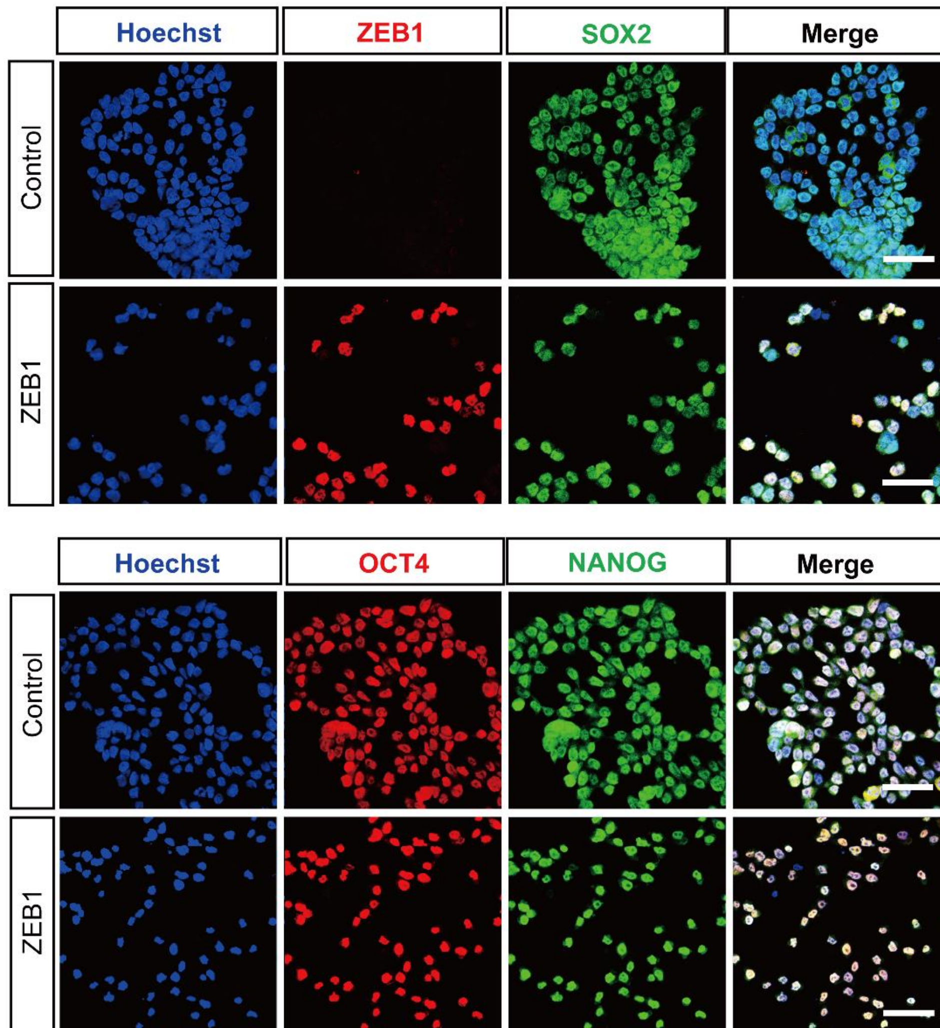
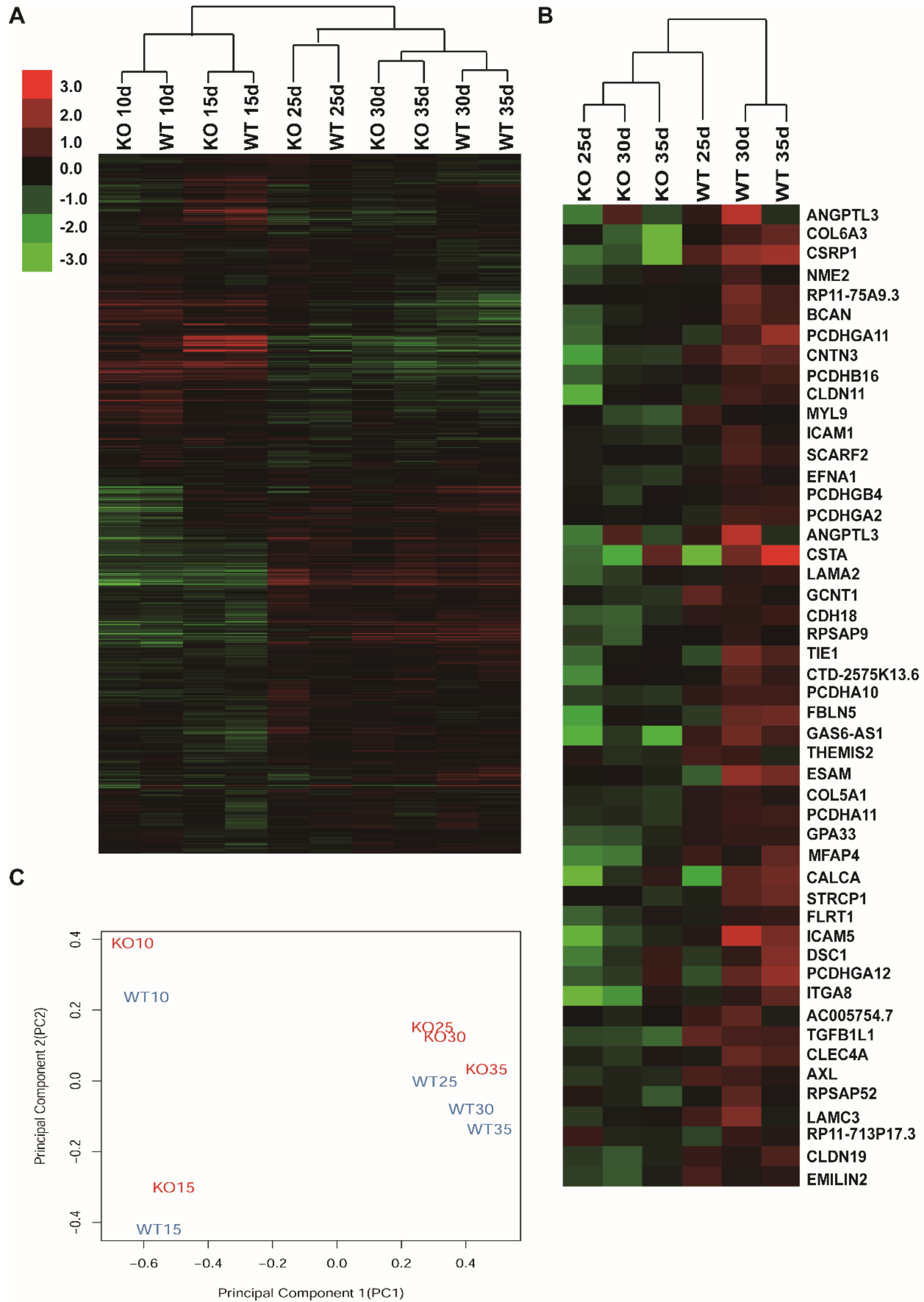


Figure S5, Related to Figure 7. RNA-seq analysis. (A) Hierarchical clustering analysis of the whole-genome profiles of different time points during the differentiation. (B) Principle component analysis (PCA) of the indicated samples. (C) Hierarchical clustering analysis showed gene profiles of different time points.



Tables

Table S1, Related to Figure 2. Three sgRNAs in *ZEB1* Target Loci.

Site	**Sequences**
sgRNA1	ATCTTGTGGAAAGGACGAAACACCGACTCTTCAACATTAGGATCAGTTTTAG AGCTAGAAATAGCAAGT
sgRNA2	ATCTTGTGGAAAGGACGAAACACCGTGGTCCTCTTCAGGTGCCTCGTTTTAG AGCTAGAAATAGCAAGT
sgRNA3	ATCTTGTGGAAAGGACGAAACACCGTCATCATGACCACTGGCTTCGTTTTAG AGCTAGAAATAGCAAGT

Table S2, Related to Figure 2. T7-*ZEB1*-sgRNA in vitro transcription (IVT) template and PCR primers.

T7-*ZEB1*-sgRNA IVT template	TAATACGACTCACTATAGGGACTCTTCAACATTAGGATCAGTTTTA GAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACT TGAAAAAGTGGCACCGAGTCGGTGCTTTT
T7 Forward Primer	TAATACGACTCACTATAGGG
Tracr Reverse Primer	AAAAGCACCGACTCGGTGCC

Table S3, Related to Figure 2. Primer Oligonucleotides Used for PCR Amplification of *ZEB1*-sgRNA1 Target.

Site	**Forward Primer**	**Reverse Primer**
ZEB1-sgRNA1	ACATTTGAAAGGGATACTG	GTTAAGATCCATTTACCAT

Table S4, Related to Figure 2. Potential Off-Targets Site of *ZEB1*-sgRNA1 Locus.

sequence	**score**	**mismatches**	**UCSC gene**	**locus**
TCTCTTCACCATT AGGATCACGG	4.6	2MMs [1:9]		chr4:-158491201
AATAGTCAACAT TAGGATCAAGG	2.4	3MMs [2:4:5]		chr20:-6602701
ATGCCTCAACATT AGGATCAAGG	2.3	3MMs [2:3:5]		chr14:-33856226
AATGATCAATATT AGGATCAAAG	1.3	4MMs [2:4:5:10]		chr8:+77571849
TCTATTCAACATT AGGCTCATGG	1.3	3MMs [1:4:17]		chr6:-118201102
GCTTTTTTACATT AGGATCAAAG	0.9	4MMs [1:4:7:8]		chr6:-118491217
ACTATTCAAAATT	0.9	3MMs [4:10:19]		chr18:-53780793

AGGATAATGG				
ACTCCTCAAAATT AGGATGAAAG	0.9	3MMs [5:10:19]		chr2:-18484900
AGTTTTGAATATT AGGATCAGAG	0.9	4MMs [2:4:7:10]		chr8:-80576773
GTTTTTCAGCATT AGGATCAGGG	0.9	4MMs [1:2:4:9]	NM_001130675	chr4:+141317254
AGACTTCACAATT AGGATCAAAG	0.8	4MMs [2:3:9:10]		chr14:-67900203
ACTCAACAACAC TAGGATCAGGG	0.8	3MMs [5:6:12]		chr5:-135573194
ACTATTCCACATT ATGATCAAAG	0.8	3MMs [4:8:15]		chr5:-52126506
ACTATCCCAGATT AGGATCAAAG	0.8	4MMs [4:6:8:10]		chr7:-127538077
ACTATTCATCATT AGGCTCAGAG	0.7	3MMs [4:9:17]		chr6:-145091069
TGTGTTCAACATT AGGCTCAAAG	0.6	4MMs [1:2:4:17]		chr15:-96804920
AATCTGAGACAT TAGGATCACAG	0.6	4MMs [2:6:7:8]		chr7:+21316232
AATCTGAAATATT AGGATCACGG	0.5	4MMs [2:6:7:10]		chr13:+110870621
TCTCTTAAAAGTT AGGATCATGG	0.5	4MMs [1:7:10:11]		chr6:+26235931
ATGCTTAAACACT AGGATCATAG	0.5	4MMs [2:3:7:12]		chr3:+6900245
TTTTTTCAACATT AAGATCACAG	0.4	4MMs [1:2:4:15]		chr1:+78166804
ACTCATCTGCAGT AGGATCAAAG	0.4	4MMs [5:8:9:12]		chr3:+85464932
AATGTTAAACATT AGGGTCATGG	0.4	4MMs [2:4:7:17]		chr7:+141131343

Table S5, Related to Figure 1 and Figure7. Primers Used for Quantitative Reverse-Transcription PCR.

Gene	**Forward Primer**	**Reverse Primer**
ZEB1	CTGACTGTGAAGGTGTACCA	GTACATCCTGCTTCATCTGC
PAX6	ACCCATTATCCAGATGTGTTTGCCCGAG	ATGGTGAAGCTGGGCATAGGCGGCAG
SOX1	CAGCAGTGTCGCTCCAATTCA	GCCAAGCACCGAATTCACAG
OTX2	TCAACTTGCCCGAGTCGAGG	CAATGGTCGGGACTGAGGTG

DCX	CAAGTCTAAGCAGTCTCCCATC	ATAGCCCTGTTGGACACTTG
NESTIN	GGAAGAGAACCTGGGAAAGG	CTTGGTCCTTCTCCACCGTA
FGF8	GACCCCTTCGCAAAGCTCAT	CCGTTGCTCTTGGCGATCA
WNT8B	AAGGCCGAGAGTGCCTAAG	CTGCGCGGCTACAGAAGTA
TGFB1	CAATTCCTGGCGATACCTCAG	GCACAACTCCGGTGACATCAA
ATF5	TGGCTCGTAGACTATGGGAAA	ATCAACTCGCTCAGTCATCCA
SMAD7	GGACAGCTCAATTCGGACAAC	GTACACCCACACACCATCCAC
EMX2	CGGCACTCAGCTACGCTAAC	CAAGTCCGGGTTGGAGTAGAC
NKX2-1	AGCACACGACTCCGTTCTC	GCCCACTTTCTTGTAGCTTTCC
SIX3	CAAGGAGTCTCACGGCAAG	GCAATGCGTCTTCTGCTCG
POU3F2	AAGCGGAAAAAGCGGACCT	GTGTGGTGGAGTGTCCCTAC
RHOU	GCTACCCCACCGAGTACATC	GGCTCACGACACTGAAGCA
DLX1	ATGCACTGTTTACACTCGGC	GACTGCACCGAACTGATGTAG
MCM2	ATGATCGAGAGCATCGAGAACC	GCCAAGTCCTCATAGTTCACCA
FOXM1	ATACGTGGATTGAGGACCACT	TCCAATGTCAAGTAGCGGTTG

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Culture and Differentiation of Human ESCs

H9ESC line (WiCell Institute, Madison, WI, passages 29-50) were maintained on hESC-qualified Matrix (BD Matrigel™, 354277) with feeder-free media, Essential 8™ Medium (E8, Gibco, A1517001) (1). Neural differentiation from hESCs was performed as described previously. For neural induction, human ESCs were digested with Dispase (2 units, Gibco, 17105-041) and cultured with neural induced medium [DMEM/F12 (11330), 100× N2 (17502) and 100× nonessential amino acids (11140), all from Gibco]. For neuronal differentiation, neural progenitor clusters were dissociated with Accutase (1 unit/ml, Gibco, A11105-01) and cultured in neural differentiation medium [1:1 DMEM-F12/Neurobasal medium (21103), 100× N2 supplement, 50× B27 supplement (17504) and 100× GlutaMAX (35050), all from Gibco] supplemented with trophic factors, including brain-derived neurotrophic factor (BDNF, 10 ng/ml), glial-derived neurotrophic factor (GDNF, 10 ng/ml), insulin-like growth factor 1 (IGF1, 10 ng/ml), and cyclic adenosine monophosphate (cAMP) (1 μM) (all from R&D Systems).

Generation of Inducible Cas9 Expressing (iCas9) H9 hESC Line

A knockin vector targeting human AAVS1 locus was built, which contained a Tet-On3G system controlling Cas9 expression. A constitutive promoter (CBh) transcribes Tet3G was designed to be in the opposite direction of TRE3G element to ensure no leaky of Cas9 expression without doxycycline. The vectors also contained a splice-acceptor (SA) site followed by “2A-puromycin” resistance gene cassette. All parts were built on puc19 (Addgene: #50005) as the backbone using Gibson Assembly.

To generate iCas9 expressing H9 hESC line, 10 mM Y27632 was added to E8 medium for 2 hr before

nucleofection. For the nucleofection, cells were harvest using Accutase and resuspended (2 million cells) in 100 µl P3 reagent (Lonza, V4XP-3024) with each AAVS1 pairs (2 µg), knockin vector (5 µg), and nucleofected using program CB-150 (Lonza 4D-nucleofector system). After nucleofection, cells were plated on matrigel-coated 6-well-plate in E8 medium supplemented with 10 mM Y27632 for the first 24 hr. Puromycin selection (0.5 µg/ml) began at day 2 and 10-14 days later. Individual colonies were picked and expanded for further analysis.

sgRNAs Design

For sgRNA design, we used the CRISPR design tool developed by Dr. Zhang's group at MIT (http://crispr.mit.edu/). We designed three sgRNAs in *ZEB1* target loci (Table S1), inserted into PX458 (Addgene: #48138) and identified the indel mutation efficiency with T7EI assays for each target in HEK293T cells (cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, 11965) supplemented with 10% FBS (Gibco, 10099), and selected the sgRNA1 with the highest targeting specificity to transfect in hESCs.

sgRNA Production Through In Vitro Transcription

For production of sgRNA, we used the in vitro transcription (IVT) methods, which have been reported faster and more cost-effective for knockout studies (2,3). We firstly designed a nucleotide oligo that included the T7 promoter sequence and sgRNA1 target of *ZEB1* loci followed by a constant chimeric sgRNA sequence (Table S2). Then PCR to amplify the T7-*ZEB1*-sgRNA1 oligo using the the T7 F and Tracr R universal primers (Table S2). The PCR-amplified T7-sgRNA IVT template was transcribed with the MEGAshortscript T7 Transcription kit (Life Technologies, AM1354) to generate sgRNAs. The resulting sgRNAs were purified using the MEGAclear kit (Life Technologies, AM1908) following manufacturer's instructions, eluted in RNase-free water at a final concentration of 10 µM, and stored at -80 °C until use.

Generation of the *ZEB1*-Knockout hESC Line using iCRISPR

Before and during transfection, the iCas9 hESCs were treated with doxycycline (Dox, 2 µg/ml, Sigma, D9891) and ROCK inhibitor (1 µM, Calbiochem, 688001) for 24 hr. For transfection, cells were dissociated using Accutase, and transfected in suspension with sgRNAs using Lipofectamine RNAiMAX (Invitrogen, 13778) following the manufacturer's instructions. Briefly, *ZEB1*-sgRNA1 (10 nM final concentration) and Lipofectamine RNAiMAX were diluted separately in Opti-MEM (Life Technologies). Then mixed the Lipofectamine RNAiMAX diluent into *ZEB1*-sgRNA diluent, incubated for 5 min at RT, and added dropwise into suspended iCas9 hESCs. A second transfection was performed 24 hr later in adherent culture. Two days after the last sgRNA transfection, hESCs were dissociated into single cells until colonies from single cells became visible. Each colony was picked in duplicates, one for the assessment analysis and the other for passage.

T7EI and RFLP Assay

T7EI and RFLP analysis were executed for assessment of genome modification. Two or three days after the last sgRNA transfection, cells were lysed in the following lysis buffer: 0.2 mg/ml protein K, 10 mM Tris pH 7.5, 1mM CaCl2, 3 mM MgCl2, 1 mM EDTA and 1% Triton ×100. The lysed cells were incubated at 65 °C for 10 min and at 95 °C for 15 min. PCR were conducted with 1 µl of cell lysate as template using 2×Taq MasterMix (Tiangen, KT204) in a total volume of 20 µl reactions. The primers

were listed in Table S3. For T7EI assays, 12 μl of PCR products were hybridized in NEB Buffer 2 (New England Biolabs) using the following protocol: 95 °C, 5 min; 95 °C – 85 °C at - 2 °C /s; 85°C-25 °C at -0.1 °C /s; hold at 4 °C. Then, 1 μl T7 Endonuclease I (New England Biolabs, M0302S) was added in the hybridized PCR products and the reactions were incubated at 37 °C for 20 min. The products were then separated on 2 % agarose gel. Quantification of DNA bands based on relative band intensities was done using ImageJ. Indel frequencies were calculated using the formula $100 \times (1- (1- (b + c) / (a + b + c))^{1/2})$, where a is the integrated intensity of the undigested PCR product, and b and c are the integrated intensities of each cleavage product (4). For RFLP analysis, 12 μl PCR products were digested with 1μl BfuC1 Restriction Endonuclease and analyzed on 2 % agarose gel. Indel frequencies were determined by the formula $100 \times a / (a + b + c)$.

Potential off-target sites were identified using the software CRISPR tool (Table S4). And the most likely off-targets falling in gene coding sequences (four sites of gRNA-mediated targeting experiment) were analyzed through PCR and sequencing.

Generation of Plasmid Constructs

To overexpress *ZEB1* in H9ESCs and *ZEB1*-depleted H9ESCs, the tetracycline inducible tet-on lentiviral-expression vectors were used. The plasmid of pLVX-Tet3G (Clontech, 631358) was modified by replacing the CMV promoter with elongation factor-1α (EF1α) to optimize transgene expression in human ESCs. The expression plasmid of pLVX-TRE3G (Clontech, 631193) was modified by replacing the puromycin fragment with mNeonGreen-P2A-Blastidin (BSD). The CDs domain of *ZEB1* with 3× Flag tag at the 5' end was inserted in multiple clone sites of pLVX-TRE3G. The pLVX-TRE3G-3×Flag-EGFP vector was constructed as a control. All the constructs have been verified by DNA sequencing before being used.

Lentivirus Production

Lentivirus production was described previously (5). Briefly, medium containing the lentiviruses pLVX-Tet3G, pLVX-TRE3G-3×Flag-EGFP and pLVX-TRE3G-3×Flag-ZEB1 respectively were harvested after 48 hr and 72 hr of transfection in HEK293T cells.

Immunofluorescence Staining (Cultured Cells)

Immunostaining experiments were performed as described previously (6). Cells were fixed in 4% paraformaldehyde in PBS at room temperature for 30 min, and permeablized with PBST (PBS with 0.3% Triton X-10) for 15 min (3 × 5 min) and incubated with blocking buffer [3% (v/v) normal donkey serum in PBST] for 45 min. Primary antibodies were diluted in antibody buffer [1% (v/v) normal donkey serum in PBST] and incubated with cells overnight at 4 °C. Cells were washed in PBST, three times each for 5 min. The secondary antibodies were donkey anti-rabbit, mouse, or goat IgG conjugated with Alexa-488, -594, or -647 (Invitrogen) and incubated in the dark at room temperature for 1 h. The DNA was labeled by Hoechst 33342.

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