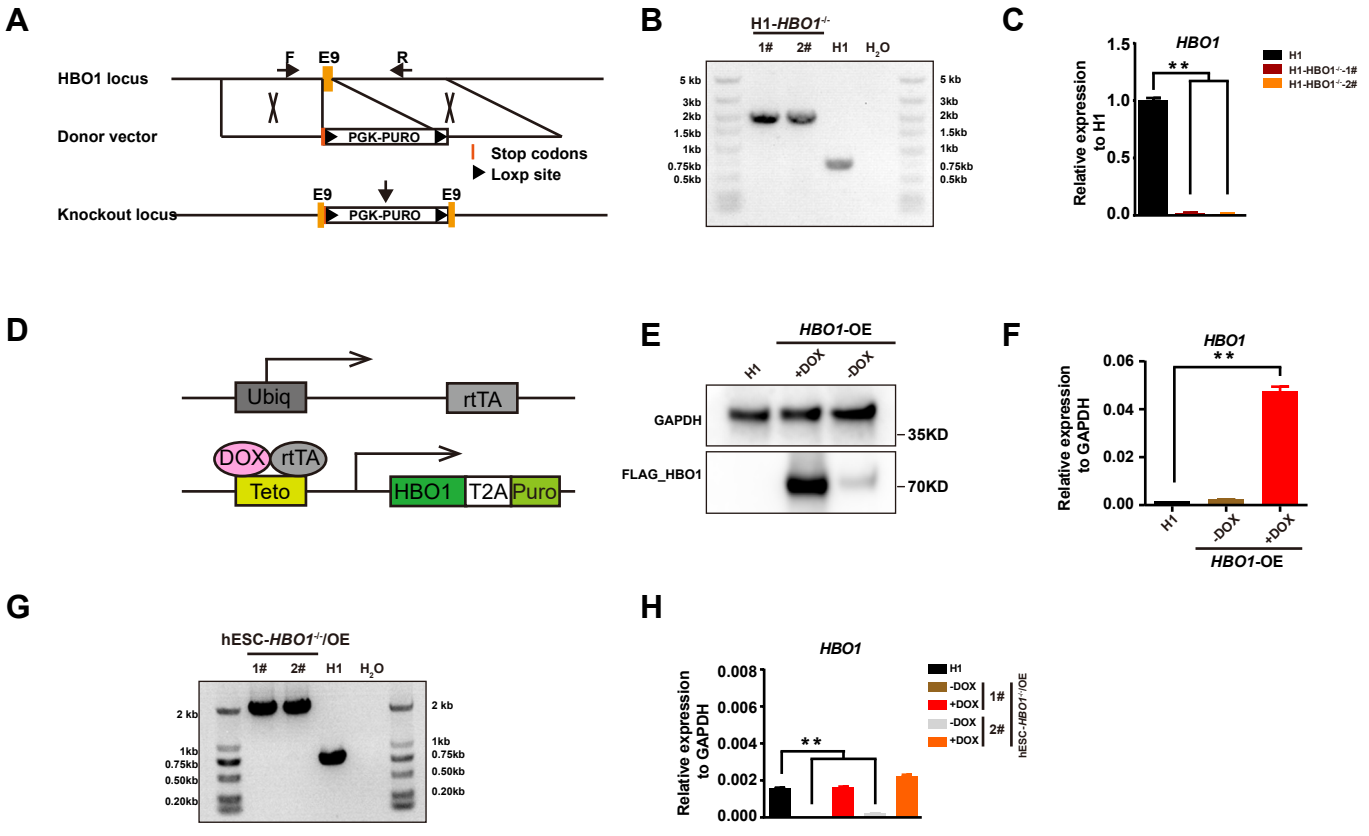
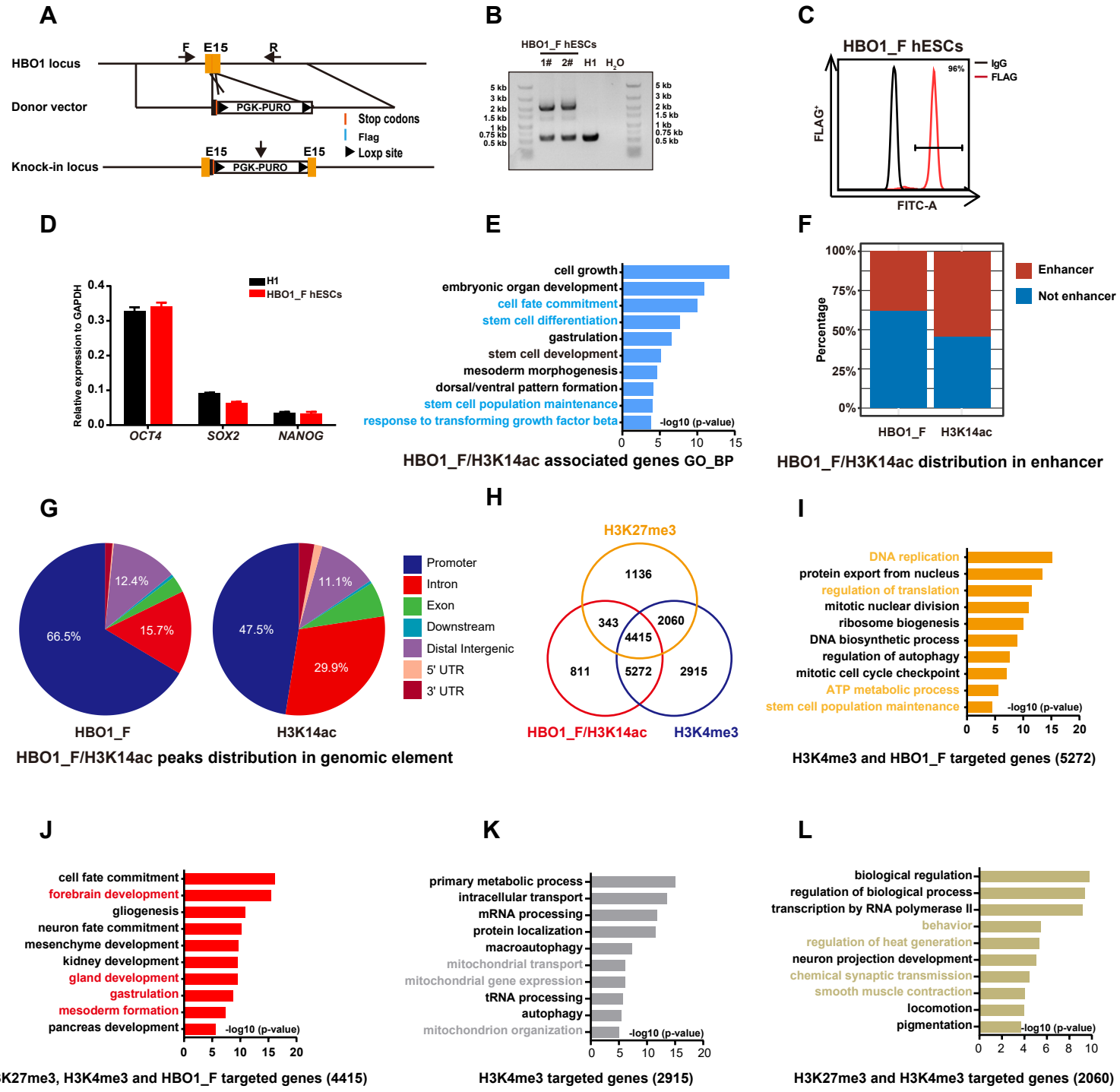
Supplementary Figure 1



Supplementary Figure 1. Deletion of *HBO1* in human embryonic stem cells.

(**A**) Overview of *HBO1* targeting strategy. sgRNA and a homologous targeting plasmid containing puromycin-resistant cassette were constructed according to the *HBO1* gene. (**B**) PCR identification of WT H1 and two *HBO1*$^{-/-}$ hESC clones. WT H1 hESCs served as negative control. (**C**) Expression of *HBO1* by RT-qPCR in WT H1 and *HBO1*$^{-/-}$ hESCs. WT H1 hESCs serve as a control. (**D**) Diagram of a lentiviral-based inducible system for HBO1 expression controlled by DOX treatment, referred to as *HBO1*-OE. (**E**) Western blot analysis of FLAG (HBO1) in WT H1 and *HBO1*-OE with or withdrawal of DOX (three days). GAPDH was used as a loading control. (**F**) Expression of *HBO1* by RT-qPCR in the indicated cell lines. WT H1 hESCs serve as a control. (**G**) PCR identification of WT H1 and two hESCs-*HBO1*$^{-/-}$/OE clones. WT H1 hESCs served as a negative control. (**H**) Expression of *HBO1* by RT-qPCR in the indicated cell lines. WT H1 hESCs serve as a control. In C, F and H, the data represent mean ± SD from three biological repeats with three technical replicates. The significance level was determined using unpaired two-tailed Student's t-tests. **,$P<0.01$.

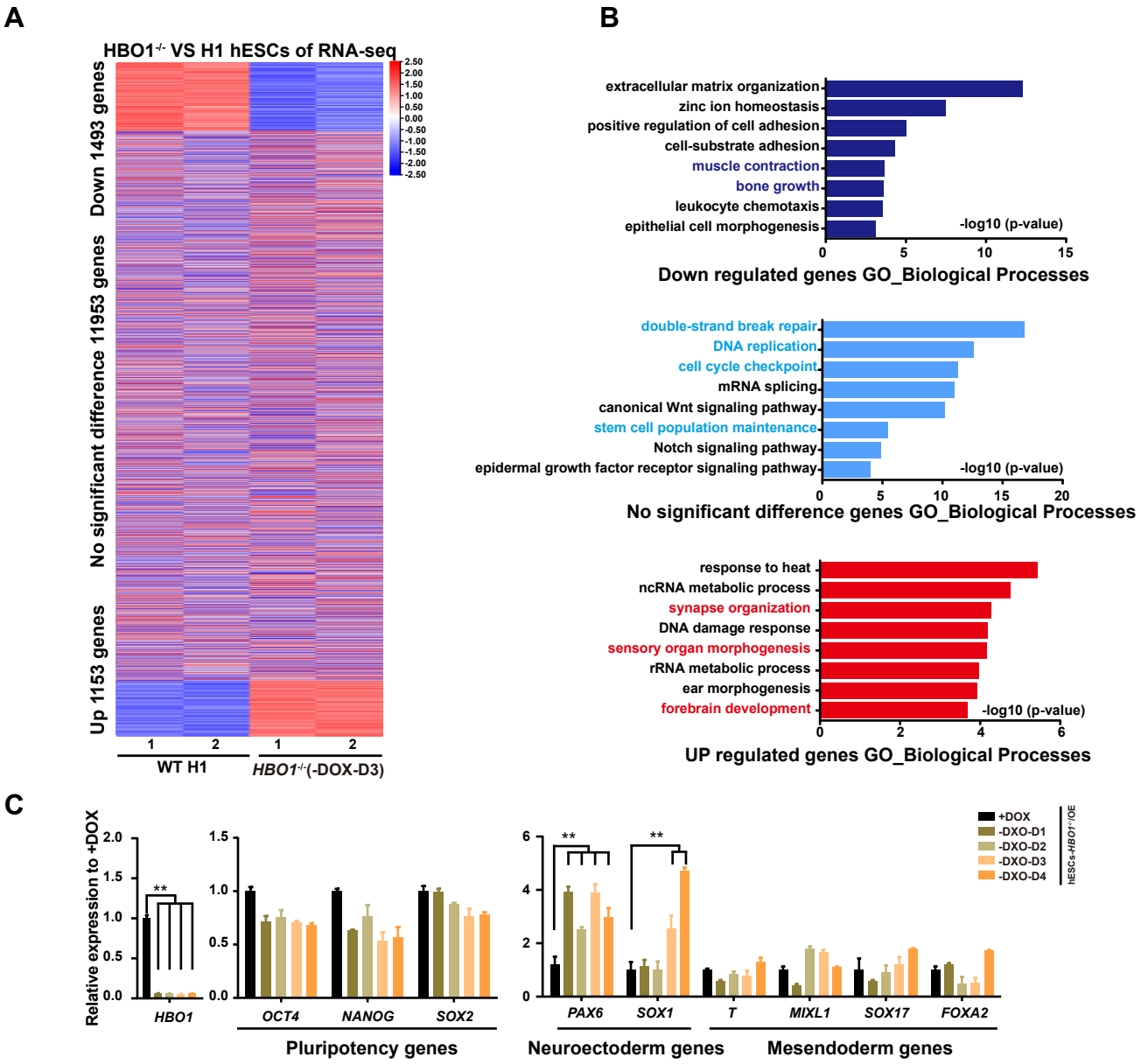
Supplementary Figure 2



Supplementary Figure 2. Knock in triple-FLAG into HBO1 locus in hESCs.

(**A**) Overview of HBO1 locus knock-in triple-FLAG strategy in hESCs. (**B**) PCR identification in WT and two HBO1_F hESCs clones. WT hESCs served as a negative control. (**C**) FACS analysis of FLAG expression in HBO1_F hESCs. (**D**) Expression of pluripotency genes by RT-qPCR in WT H1 and HBO1_F hESCs. WT H1 hESCs serve as a control. the data represent mean ± SD from three biological repeats with three technical repeats. The significance level was determined using unpaired two-tailed Student's t-tests. (**E**) GO terms of biological processes analysis of FLAG (HBO1_F) and H3K14ac co-binding genes. (**F**) Histogram analysis of FLAG (HBO1_F) and H3K14ac peaks distribution in enhancer. (**G**) Pie chart analysis of FLAG (HBO1_F) and H3K14ac peaks distribution in genomic element. (**H**) Overlap between FLAG (HBO1_F)/H3K14ac, H3K4me3 and H3K27me3 targeted genes in WT hESCs. H3K4me3 (23) and H3K27me3 (24) ChIP-seq data were published. (**I**) GO terms of biological processes analysis of H3K4me3 and HBO1_F targeted genes (5272). (**J**) GO terms of biological processes analysis of H3K27me3, H3K4me3 and HBO1_F targeted genes (4415). (**K**) GO terms of biological processes analysis of H3K4me3 targeted genes (2915). (**L**) GO terms of biological processes analysis of H3K27me3 and H3K4me3 targeted genes (2060).

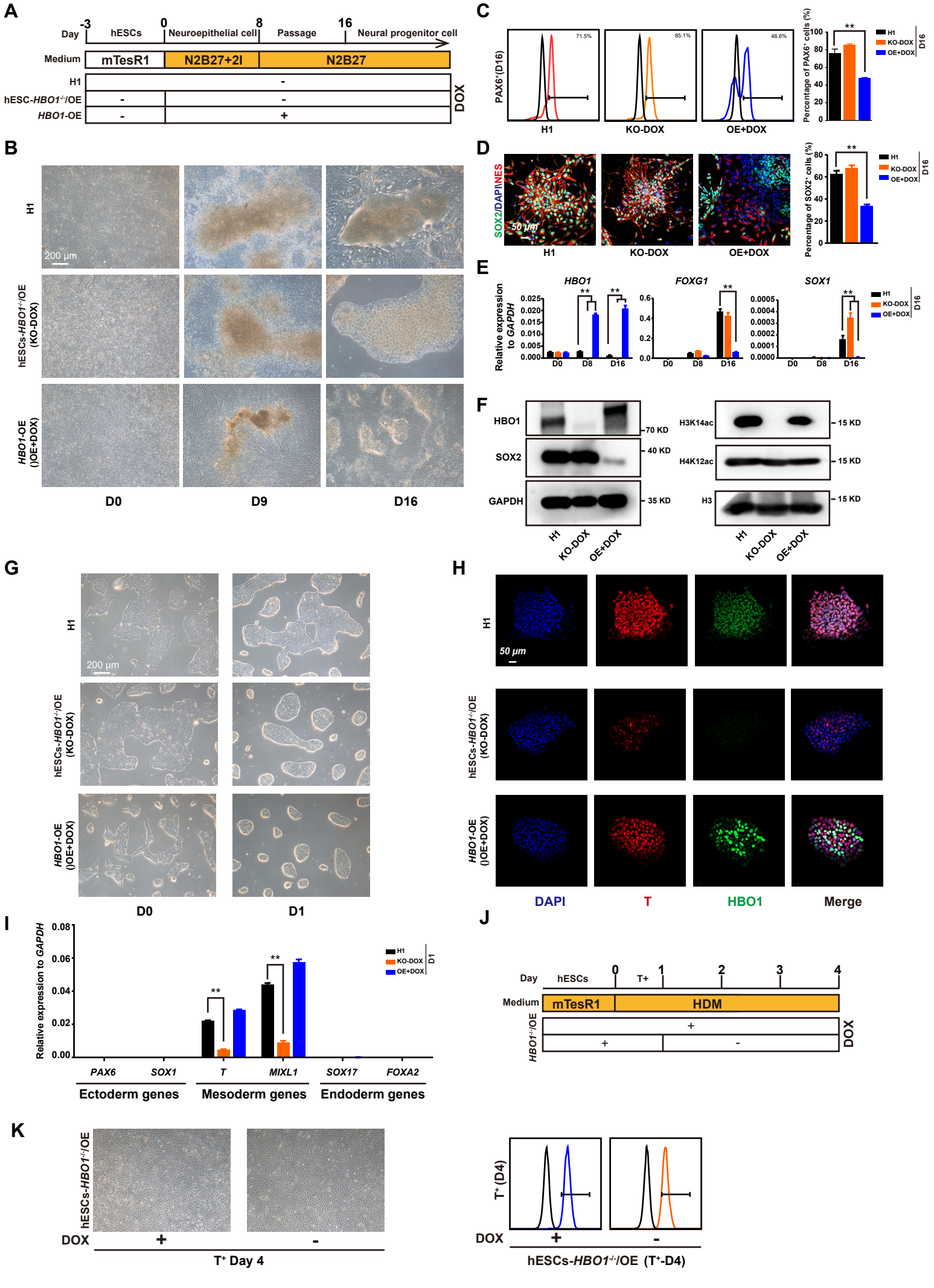
Supplementary Figure 3



Supplementary Figure 3. After three days withdrawal DOX, hESCs-*HBO1*$^{-/-}$/OE cells still maintained undifferentiated state.

(**A**) Data from RNA-seq. Heatmap analysis of up- or down-regulated and no significant genes from hESCs-*HBO1*$^{-/-}$/OE (-DOX-day 3) compared with WT H1 hESCs. (**B**) GO terms of biological processes analysis of down-regulated, no significant and up-regulated genes, as described in A. (**C**) RT-qPCR analysis of *HBO*1, pluripotency, ectoderm and mesendoderm associated genes in hESCs-*HBO1*$^{-/-}$/OE with or withdrawal of DOX. hESCs-*HBO1*$^{-/-}$/OE with DOX serve as a control. The data represent mean ± SD from three biological repeats with three technical repeats. The Significance level was determined using unpaired two-tailed Student's t-tests. **, $P<0.01$.

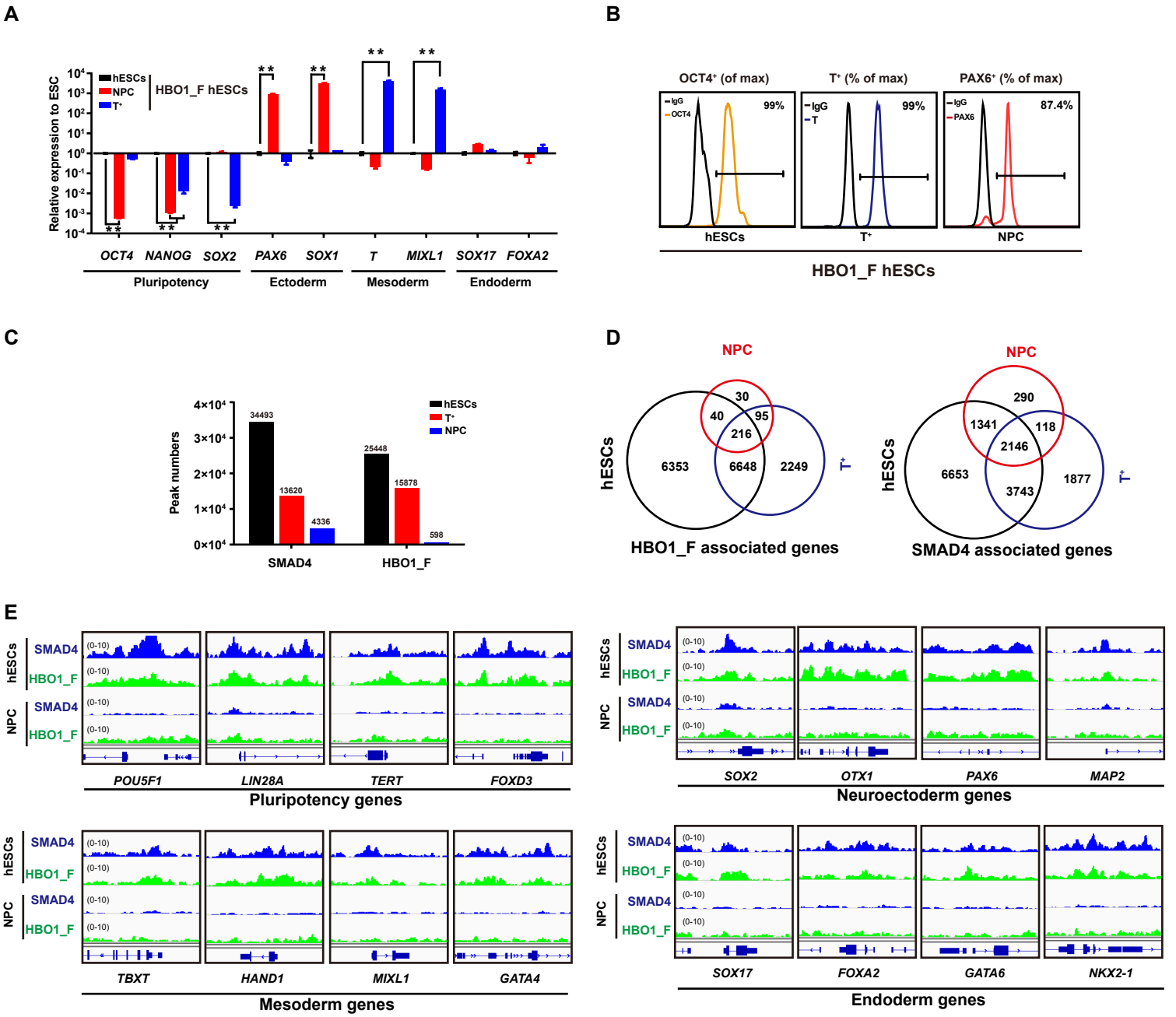
Supplementary Figure 4



Supplementary Figure 4. HBO1 inhibits neural differentiation but is essential for mesodermal differentiation.

(**A**) Scheme for Neural progenitor cell differentiation from hESCs. (**B**) Representative images of Neural progenitor cells differentiation from WT H1 hESCs, hESCs-*HBO1*$^{-/-}$/OE withdrawal of DOX (KO-DOX) and HBO1-OE with DOX (OE+DOX). The scale bar represents 200 μm. (**C**) FACS analysis of PAX6 expression in the indicated cell lines at day 16 of neural differentiation. (**D**) Immunostaining analysis of neuroectoderm markers SOX2/NES expression in the indicated cell lines. The scale bar represents 50 μm. (**E**) RT-qPCR analysis of *HBO1*, *FOXG1* and *SOX1* expression in the indicated cell lines. H1 serve as a control. (**F**) Western blot analysis of FLAG (HBO1), SOX2, H3K14ac and H3K23ac in the indicated cell lines. GAPDH and H3 serve as a loading control. (**G**) Representative images of T^+ cells differentiation from WT H1 hESCs, hESCs-*HBO1*$^{-/-}$/OE withdrawal of DOX (KO-DOX) and HBO1-OE with DOX (OE+DOX). (**H**) Immunostaining analysis of T and HBO1 expression in the indicated cell lines. The scale bar represents 50 μm. (**I**) RT-qPCR analysis of ectoderm, mesoderm and endoderm genes expression in the indicated cell lines. H1 serve as a control. (**J**) Scheme for T^+ cell differentiation from hESCs-*HBO1*$^{-/-}$/OE with DOX, and then these cells were cultured using same medium with or withdorw DOX for 3 days. (**K**) Left: Representative images of T^+ cells differentiation from hESCs-*HBO1*$^{-/-}$/OE with or withdrawal of DOX for 3 days. Right: FACS analysis of T expression in the indicated cell lines at day 4 of T^+ cells differentiation. In C and D, the data represent mean ± SD from three biological repeats. In E and I, the data represent mean ± SD from three biological repeats with three technical repeats. The Significance level was determined using unpaired two-tailed Student's t-tests. **,$P<0.01$.

Supplementary Figure 5



Supplementary Figure 5. HBO1 and SMAD4 bind in the chromatin of mesoderm genes upon pluripotency exit.

(**A**) Expression of pluripotency, neuroectoderm, mesoderm, and endoderm associated genes by RT-qPCR in the indicated cell lines. HBO1_F hESCs serve as control. The data represent mean ± SD from three biological repeats with three technical repeats. The significance level was determined using unpaired two-tailed Student's t-tests. **, $P<0.01$. (**B**) FACS analysis of OCT4, T and PAX6 expression in the indicated cell lines. (**C**) Bar diagram analysis of the number of SMAD4 and FLAG (HBO1_F) peaks from CUT&Tag-seq data in hESCs, T^+ cells, and NPC. (**D**) Left: overlap of FLAG (HBO1_F) binding genes between hESCs, T^+ cells, and NPC. Right: overlap of SMAD4 binding genes between hESCs, T^+ cells, and NPC. (**E**) Genomic views analysis of the SMAD4 and FLAG (HBO1_F) binding in hESCs and NPC cells.

Supplementary Table 1. List of sgRNAs and primers for gene targeting

Knock-out					
Gene	sgRNA sequence	Primers for donor DNA construction(5'-3')		Primers for validation(5'-3')	
HBO1	ACCAGGTATCAAGCTCATAG	*HBO1*-5'-LA-F	AGAGGCAGGGTTTGCTGGCTAC	KO-F	CCTCTGCCTCTTGGGCGATTC
		HBO1-5'-LA-R	CTCCTATTGCCAAGAAATCAAAAGTCA		
		HBO1-3'-RA-F	GCACATGGTGAGTTGTCTTGGGTT	KO-R	GAAATGAAGAGCAGTAGGCAGCAAG
		HBO1-3'-RA-R	CTGGAGAACCTGAGTCAAAGCAACA		
Knock-in					
Gene	sgRNA sequence	Primers for donor DNA construction(5'-3')		Primers for validation(5'-3')	
HBO1	AAATGACAGACAGATCCCTA	*HBO1*-5'-LA-F	GTTTCCCCTGAATGTGAGAACGG	KI-F	TCACTGAGGGTAAAGTAGGAAGAATGG
		HBO1-5'-LA-R	AGTGCCCTTGGGAGGGGTCC		
		HBO1-3'-RA-F	AGTGACCTGTCATTCCGAGCCA	KI-R	AGTACCATAACAGAACTCCTTGGGTCAG
		HBO1-3'-RA-R	AGCCACAAACCACATCCCTCAAC		

Supplementary Table 2. List of primers

Gene	Forward primer(5'-3')	Reverse primer(5'-3')
FUW-HBO1	ATGCCGCGAAGGAAGAGGAA	AGTGCCCTTGGGAGGGGTCC
pSIN-HBO1	ATGCCGCGAAGGAAGAGGAAT	TTAAGTGCCCTTGGGAGGGGT
pSIN-H_NTD^{-}	ATGACAGAGGGAAGCAACATGATTAAAACA	TTAAGTGCCCTTGGGAGGGGT
pSIN-H_MYST^{-}	ATGCCGCGAAGGAAGAGGAAT	TTAGATTTGGCCTTGCAGCCTTAACT
pSIN-H_HAT^{-}	ATGCCGCGAAGGAAGAGGAAT	TTAGGGCTCCACATCATAATATAATGTCT TGT
Q-H_NTD^{-}	AAGAGCCAAACGATACTCCGCC	TGTTGTCCGCCTCTGTCATAACATAG
Q-H_MYST^{-}	TCTGGACTGAGCAAAGAACAGAAAGAG	GAATTGGCCGCCCTAGATGC
Q-H_HAT^{-}	TTTAGAAAACCTGACAAGCGAGTATGAC	ATGTTGCTTCCCTCTGTGATTTGG
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
OCT4	CCTCACTTCACTGCACTGTA	CAGGTTTTCTTTCCCTAGCT
SOX2	CCCAGCAGACTTCACATGT	CCTCCCATTTCCCTCGTTTT
NANOG	TGAACCTCAGCTACAAACAG	TGGTGGTAGGAAGAGTAAAG
PAX6	ATGTGTGAGTAAAATTCTGGGCA	GCTTACAACTTCTGGAGTCGCTA
FOXG1	GAGCGACGACGTGTTCATC	GCCGTTGTAACTCAAAGTGCTG
SOX1	AATTTTATTTTCGGCGTTGC	TGGGCTCTGTCTCTTAAATTTGT
MAP2	TGAAGCAAAGGCACCTCAC	TATGGGAATCCATTGGCG
NGN2	AGGAAGAGGACGTGTTAGTGC	GCAATCGTGTACCAGACCCAG
TUJ1	GGCCAAGGGTCACTACACG	GCAGTCGCAGTTTTCACACTC
OTX2	CAAAGTGAGACCTGCCAAAAAGA	TGGACAAGGGATCTGACAGTG
T (BRACHYURY)	GAGAGCGAGCTGTGGCTGCG	CGTACTTCCAGCGGTGGTTGTC
RUNX1	CTGCCCATCGCTTTCAAGGT	GCCGAGTAGTTTTCATCATTGCC
EOMES	ATTCCACCGCCACCAAACTGAG	CAGTGGGATTGAGTCCGTTTATGTTG
MIXL1	GCAAGCGCACGTCTTTCAGC	CGAGACTTGGCACGCCTGTTC
FOXA2	CAGTATGCTGGGAGCGGTGAAG	TGTTGCTCACGGAGGAGTAGCC
SOX17	ACGCTTTCATGGTGTGGGCTAAG	CGCTCTGCCTCCTCCACGAAG
HBO1	TTTAGAAAACCTGACAAGCGAGTATGAC	ATGTTGCTTCCCTCTGTGATTTGG
TGFB1	GGCCGGGGAGAGTGCAGAAC	TGAGCCTCAGCAGACGCAGC
NODAL	TCCCCTCTGGCGTACATGCTG	TGAAGCCTGCTCTGTGTCGGG
LEFTY1	TGCAGCTGTGCCTCGGATGG	CAGTCATCGCCAGCTCTCCTGG
LEFTY2	TCCATTGAGCCCTCTAACTGAACG	GGGTGATGGACGGGAAAGACAG
SMAD6	ATCTCCGCCACCTCCCTACTCTC	TGGTCGTACACCGCATAGAGGC
SMAD7	ACTGTCCAGATGCTGTGCCTTCC	GCTGACTCTTGTTGTCCGAATTGAG

Supplementary Table 3. List of antibodies used in this study

Name of Antibody	Company (Cat. No.)	Application
HRP-Conjugated GAPDH Monoclonal Antibody	Proteintech (HRP-60004)	WB
Rabbit anti-MYST2 (HBO1)	Cell Signaling Technology (58418S)	WB/IF
Mouse anti-OCT-3/4	Santa Cruz Biotechnology (sc-5279)	WB
Rabbit anti-NANOG	Cell Signaling Technology (3580S)	WB
Rabbit anti-Sox2	Cell Signaling Technology (23064S)	WB/IF
Mouse anti-SOX2	R&D (mab2018)	WB/IF
Rabbit anti-HIST3H3 (H3)	Abcam (ab1791)	WB
Rabbit anti-Histone H3 (acetyl K23)	Abcam (ab177275)	WB
Rabbit anti-Histone H3 (acetyl K14)	Cell Signaling Technology (7627S)	WB
Rabbit anti-Histone H4 (acetyl K5)	Cell Signaling Technology (8346T)	WB
Rabbit anti-Histone H4 (tri methyl K12)	Cell Signaling Technology (8346T)	WB
mouse Monoclonal ANTI-FLAG	Sigma (F1804)	WB/FACS/Co-IP/CUT-Tag
mouse anti-HA	Sigma (H3663)	Co-IP
Rabbit anti-SMAD4	Cell Signaling Technology (46535)	CUT-Tag
Rabbit anti-TUJ1	Genetex (GTX130245)	IF
Rabbit anti-MAP2	Cell Signaling Technology (8707)	IF
Mouse anti-NES	Cell Signaling Technology (33475)	IF
Rabbit anti-GATA3	Cell Signaling Technology (5852)	IF
Goat anti-T	R&D (AF2085)	IF
Goat anti-SOX17	R&D (AF1924)	IF
Rabbit anti-GATA6	Cell Signaling Technology (5851T)	IF
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific (A-11004)	IF

Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific (A-11011)	IF
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific (A-11008)	IF
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific (A-11001)	FACS
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Thermo Fisher Scientific (A-21447)	IF
Donkey anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific (A-21206)	IF
Mouse anti-PAX6	BD Biosciences (561664)	FACS
Anti-Brachyury-FITC Antibody	Sigma (FCMAB302F)	FACS
Alexa Fluor®488 Mouse IgG2a, κ Isotype control	BD biosciences (558055)	FACS