

Supplementary Table S1. Primer sequences for RT-qPCR

Primer name	Sequence
Gapdh-F	5'-GTG TTCCTACCCCAATGTGT-3'
Gapdh-R	5'-ATTGTCATACCAGGAAATGAGCTT-3'
Tet1-F	5'-CGAAAGAACAGCCACCAGAT-3'
Tet1-R	5'-TTGCTCTTCTTCCCATGAC-3'
Tet2-F	5'-GTTGCAAGAAGAAAGCGGAG-3'
Tet2-R	5'-CTCTGCCCTTGCTGAAGGT-3'
Tet3-F	5'-TCCGGATTGAGAAGGTCATC-3'
Tet3-R	5'-CCAGGCCAGGATCAAGATAA-3'
Nanog-F	5'-AACCAAAGGATGAAGTGCAAGCGG-3'
Nanog-R	5'-TCCAAGTTGGGTTGGTCCAAGTCT-3'
Oct4-F	5'-TGGCGTGGAGACTTTGCA-3'
Oct4-R	5'-GAGGTTCCCTCTGAGTTGCTTTC-3'
Zfp42-F	5'-TCACTGTGCTGCCTCCAAGT-3'
Zfp42-R	5'-GGGCACTGATCCGCAAAC-3'
Klf4-F	5'-CGAACTCACACAGGCGAGAA-3'
Klf4-R	5'-CGGAGCGGGCGAATTT-3'
Pax6-F	5'-GGGAAAGACTAGCAGCCAAA-3'
Pax6-R	5'-TGAAGCTGCTGCTGATAGGA-3'
Sox1-F	5'-GCAGCGTTTCCGTGACTTTAT-3'
Sox1-R	5'-GGCAGAACCACAGGAAAGAAA-3'
Nestin-F	5'-AGGACCAGGTGCTTGAGAGA-3'
Nestin-R	5'-TTCGAGAGATTCGAGGGAGA-3'

Supplementary Table S2. Summary of the quantitative hMeDIP-seq data of ESCs and NPCs

Sample name	Number of reads (total)	Alignment rate	Number of reads (aligned)
ESCs_hMeDIP	9734647	86%	8367222
NPCs_hMeDIP	2849614	82%	2328130
ESCs_Input	8938949	66%	5885173
NPCs_Input	9310138	82%	7591632

Supplementary Table S3. Primer sequences for hMeDIP-qPCR

Primer name	Sequence
Ankrd23-F	TAGTCCCGGAGCTTTCTCCT
Ankrd23-R	CCCACAGAAGCCAGGATCTA
Hist1h2aa-F	CAACAAGAAGACGCGCATT
Hist1h2aa-R	ACTTGGTCTGGGACTTGTGG
Ftl1-F	GCCCTTAGTGGAAGGGGTAG
Ftl1-R	TAGATGATGGGTGGGTGGAT
Irf2bp2-F	GCATCTTTTGAGGCGTGACT
Irf2bp2-R	CTTCCACAGAAGGGCTCAAG

Supplementary Table S4. Primer sequences for glucMS-qPCR

Primer name	Sequence
Ankrd23-F*	TAGTCCCGGAGCTTTCTCCT
Ankrd23-R*	CCCACAGAAGCCAGGATCTA
Hist1h2aa-F	CCAAGGTCAAGTCTCGCTCT
Hist1h2aa-R	GAGGAGTAATGCGCGTCTTC
Ftl1-F*	GCCCTTAGTGGAAGGGGTAG
Ftl1-R*	TAGATGATGGGTGGGTGGAT
Irf2bp2-F*	GCATCTTTTGAGGCGTGACT
Irf2bp2-R*	CTTCCACAGAAGGGCTCAAG

*Same to the primers used in hMeDIP-qPCR

Supplementary Table S5. GREAT ontology enrichment for “gain of 5hmC” peaks

Ontology	# Term Name	Binom Rank	Binom Raw P-Value	Binom FDR Q-Val	Binom Fold Enrichment	Binom Observed Region Hits	Binom Region Set Coverage	Hyper Rank	Hyper FDR Q-Val	Hyper Fold Enrichment	Hyper Observed Gene Hits	Hyper Total Genes	Hyper Gene Set Coverage
GO Biological Process	nephron tubule formation	105	2.97539e-8	2.43217e-6	3.3099	30	1.09%	504	7.29661e-3	3.6647	8	14	0.25%
	regulation of muscle cell differentiation	115	1.50379e-7	1.12235e-5	2.0118	67	2.44%	238	4.59330e-5	2.6054	26	64	0.82%
	oligodendrocyte differentiation	118	1.84751e-7	1.34383e-5	2.2720	50	1.82%	460	4.63199e-3	2.4228	17	45	0.54%
	nephron tubule morphogenesis	123	2.50707e-7	1.74944e-5	2.9266	31	1.13%	493	6.88666e-3	3.3953	9	17	0.29%
	segmentation	127	3.96756e-7	2.68138e-5	2.0166	62	2.26%	328	4.03400e-4	2.2446	28	80	0.89%
	limb bud formation	133	7.33052e-7	4.73066e-5	2.6769	33	1.20%	397	2.05029e-3	4.2755	8	12	0.25%
	renal tubule morphogenesis	134	7.82199e-7	5.01016e-5	2.6232	34	1.24%	532	9.04867e-3	3.0539	10	21	0.32%
	regulation of striated muscle cell differentiation	138	8.91190e-7	5.54282e-5	2.0791	54	1.97%	337	4.93412e-4	2.6177	20	49	0.63%
	lens fiber cell differentiation	141	1.16053e-6	7.06441e-5	3.1177	25	0.91%	267	1.11566e-4	4.0505	12	19	0.38%
	regulation of skeletal muscle fiber development	145	1.35093e-6	7.99658e-5	2.2926	42	1.53%	620	1.58856e-2	2.4521	13	34	0.41%
	nephron tubule development	146	1.39799e-6	8.21844e-5	2.2638	43	1.56%	495	6.99072e-3	2.6894	13	31	0.41%
	lens development in camera-type eye	148	1.60379e-6	9.30090e-5	2.0530	53	1.93%	329	4.06748e-4	2.5195	22	56	0.70%
	renal tubule development	151	1.65453e-6	9.40454e-5	2.2485	43	1.56%	593	1.19477e-2	2.5264	13	33	0.41%
	nephron epithelium morphogenesis	153	2.51941e-6	1.41334e-4	2.3450	38	1.38%	286	2.18686e-4	3.4533	14	26	0.44%
	regulation of dendrite development	154	2.78759e-6	1.55363e-4	2.2013	43	1.56%	582	1.15901e-2	2.2250	17	49	0.54%
	regulation of skeletal muscle tissue development	162	4.52897e-6	2.39952e-4	2.0408	49	1.78%	582	1.15901e-2	2.2250	17	49	0.54%
	metanephric nephron development	163	4.63424e-6	2.44022e-4	2.2274	40	1.46%	308	3.14458e-4	3.2066	15	30	0.48%
	nephron morphogenesis	167	6.09204e-6	3.13101e-4	2.2539	38	1.38%	343	5.55597e-4	3.2066	14	28	0.44%
	regulation of stem cell differentiation	172	7.32042e-6	3.65297e-4	2.8705	24	0.87%	727	3.26825e-2	3.2066	7	14	0.22%
	neural precursor cell proliferation	177	7.82643e-6	3.79516e-4	2.0317	47	1.71%	223	2.19033e-5	2.8923	23	51	0.73%

The test set of 2,748 genomic regions picked 3,153 genes (16%) of all 20,221 genes.	
GO Biological Process has 8,583 terms covering 15,210 (75%) of all 20,221 genes.	8,583 ontology terms were tested (100%) using an annotation count range of [1, Inf].
GREAT version 2.0.2	
Species assembly: mm9	
Association rule: Basal+extension: 5000 bp upstream, 1000 bp downstream, 100000 bp max extension, curated regulatory domains included	

Supplementary Figure Legends

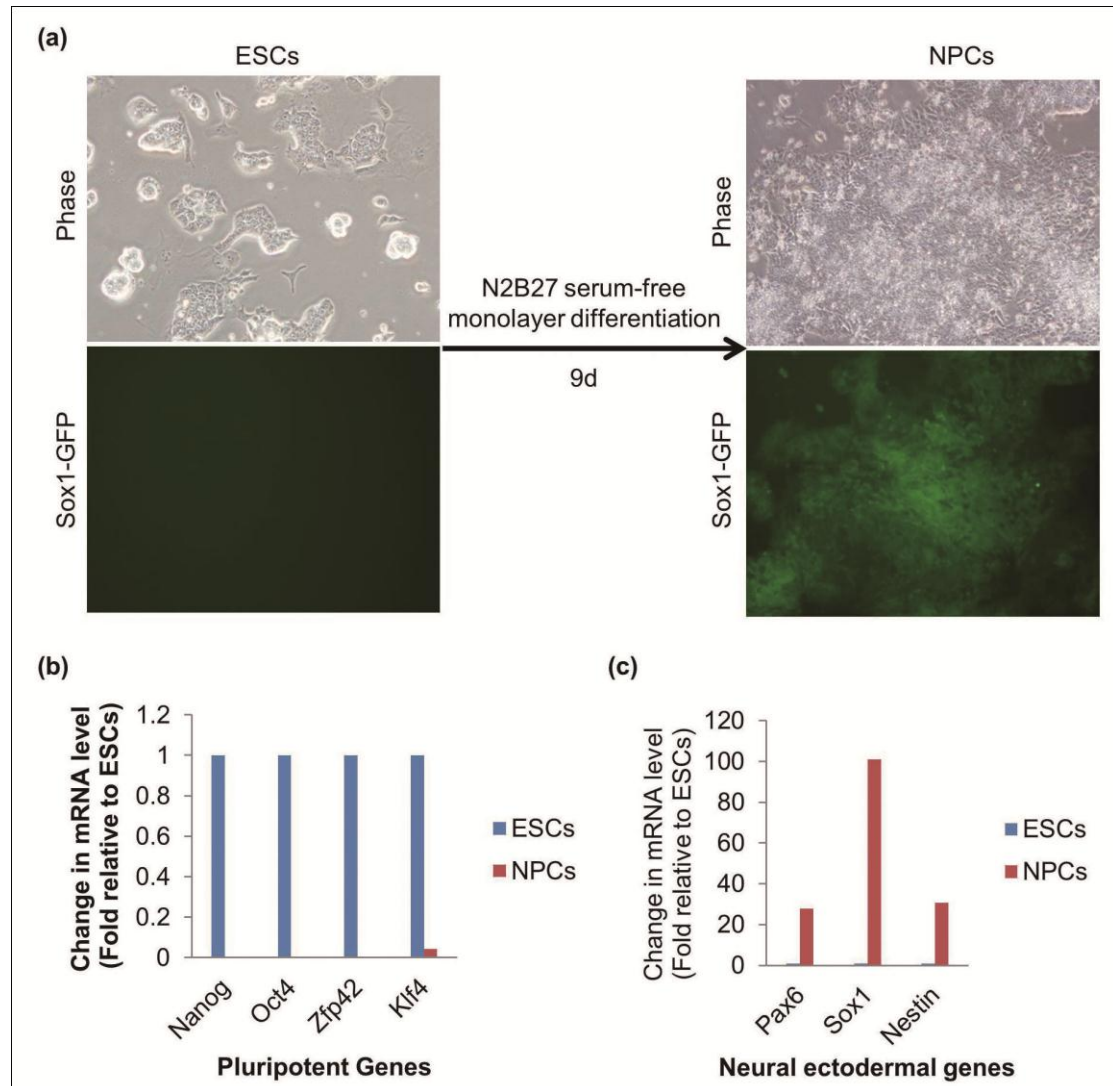
Supplementary Figure S1. Differentiation of mouse ESCs into NPCs via N2B27 serum-free monolayer differentiation *in vitro*. (a) Phase and *Sox1*-GFP of undifferentiated mouse ESCs and ES-derived NPCs. (b) RT-qPCR analysis of mRNA expression of pluripotent marker *Nanog*, *Oct4*, *Zfp42*, and *Klf4* in ESCs and NPCs. (c) RT-qPCR analysis of mRNA expression of neural ectoderm marker *Pax6*, *Sox1*, and *Nestin* in ESCs and NPCs.

Supplementary Figure S2. 5hmC distribution at enhancers and DNase I hypersensitive sites (DHS). (a) 5hmC distribution at ESC enhancers (H3K4me1 & H3K27ac) in ESCs and NPCs. (b-d) 5hmC distribution at DHSs of three classes: (b) DHSs lacking H3K4me1 and H3K27ac (left); (c) DHSs with a poised enhancer chromatin signature; and (d) DHSs with an active enhancer chromatin signature.

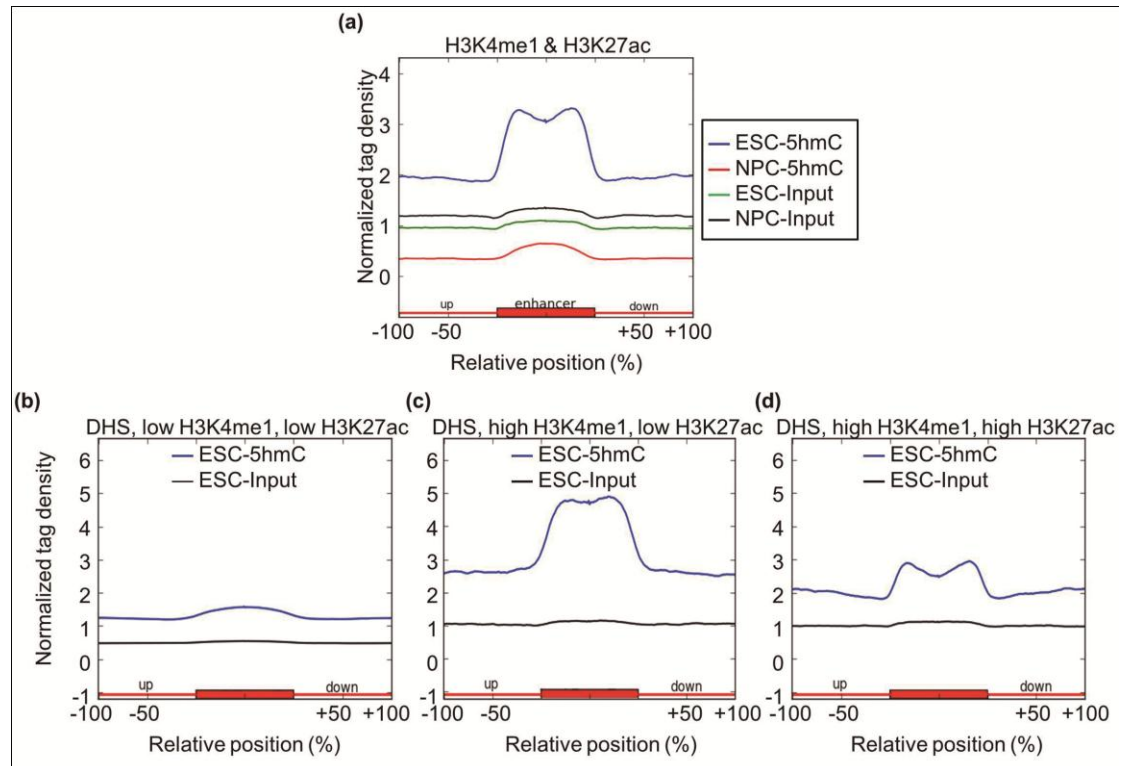
Supplementary Figure S3. Comparison of the hMeDIP-seq data of ESCs generated by quantitative and conventional hMeDIP-seq. (a) Distribution of 5hmC across gene body regions. (b) UCSC browser screen shots of 5hmC enrichment at *Gli1* and *Scnn1a*. (c) The distributions of 5hmC peaks in different genomic features detected by conventional and quantitative hMeDIP-seq. (d) Venn diagram shows the overlapping 5hmC peaks detected by quantitative and conventional hMeDIP-seq.

Supplementary Figure S4. GO analysis of the genes with *de novo* DNA hydroxymethylation (NPCs vs. ESCs, \log_2 5hmC density ratio ≥ 1) at promoters (TSS-/+1kb) or gene body regions (from TSS+1kb to TES).

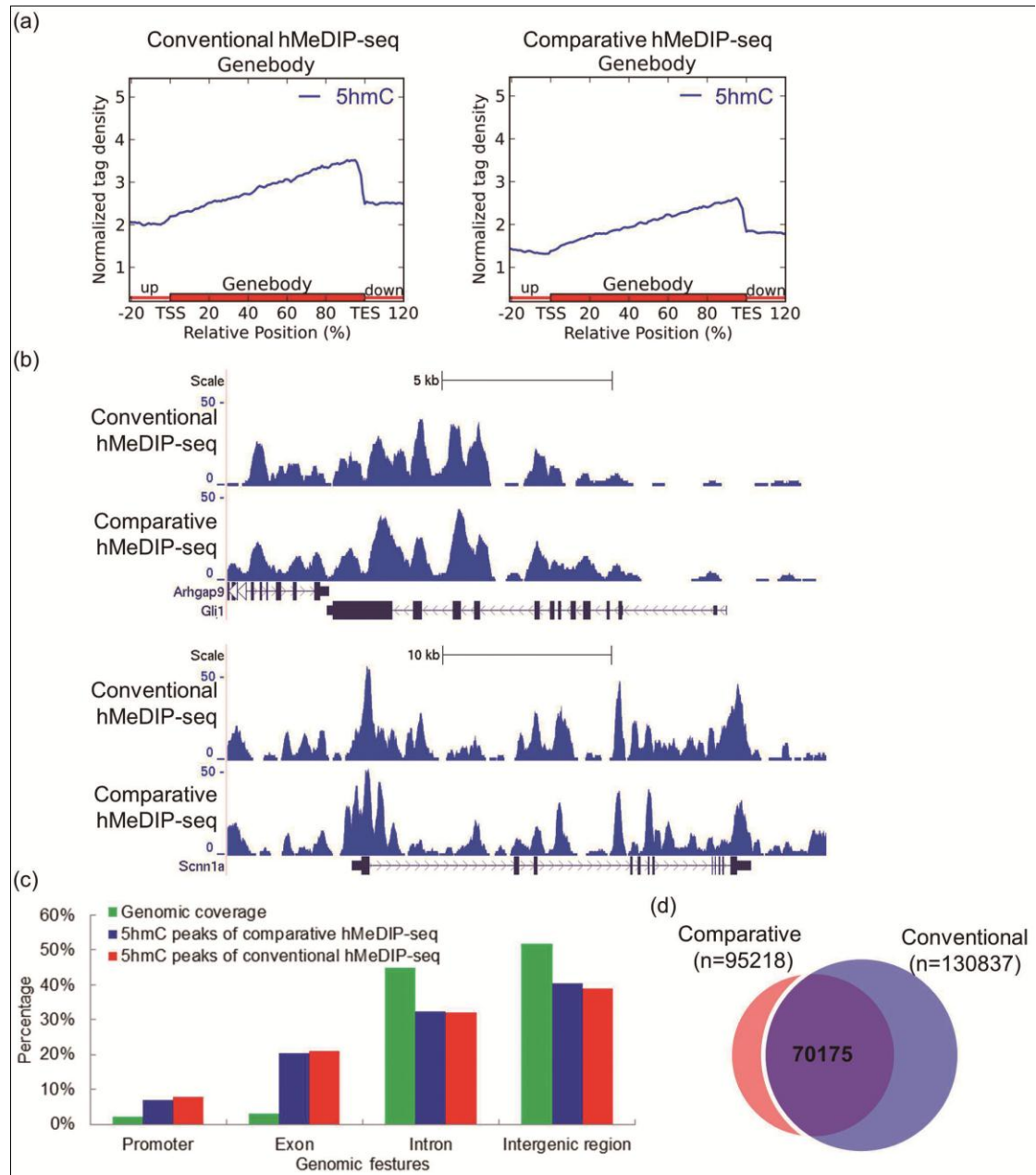
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4

