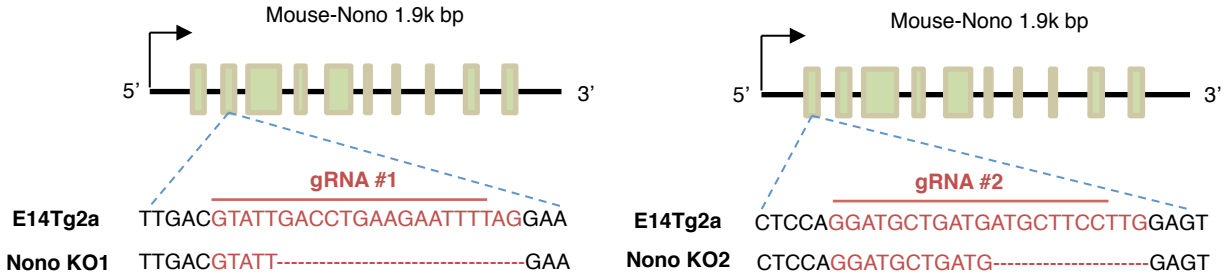
Supplemental Figures

Figure S1 Related to Figure 1-2

A



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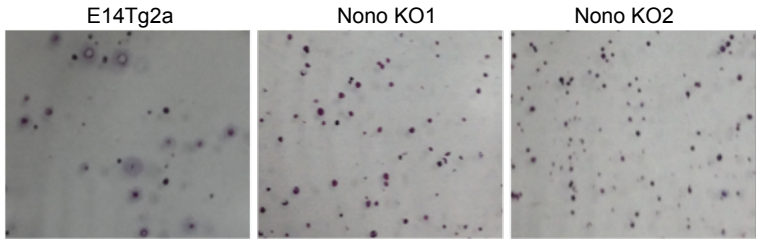
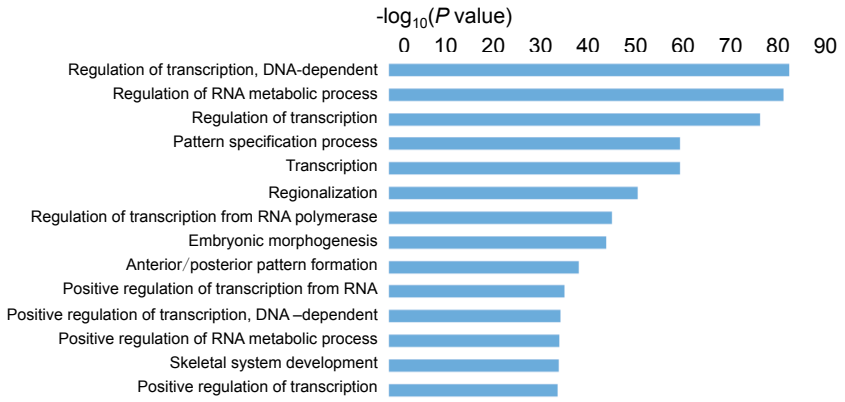


Figure S2 Related to Figure 3-4

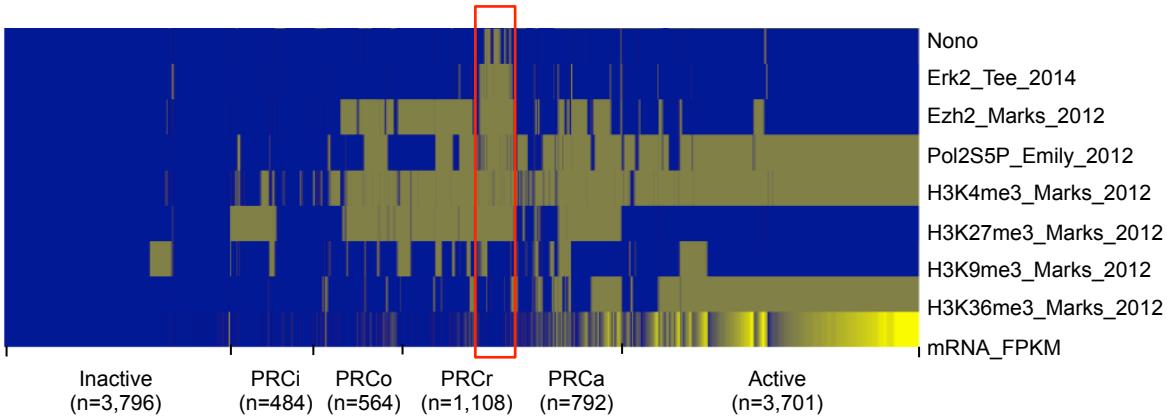
A



B

Developmental genes	Nono/Erk2 target genes
Ectoderm markers	*Fgf5, Sox1, Cdx2, Eomes……*
Mesoderm markers	*Brachyury/T, Gata6, Nkx2.5, Tbx6, Tbx20……*
Endoderm markers	*Pax9, Sox17, Gata4, Foxa2……*
All *Hox* clusters	*Hoxa, Hoxb, Hoxc, Hoxd……*

C



D

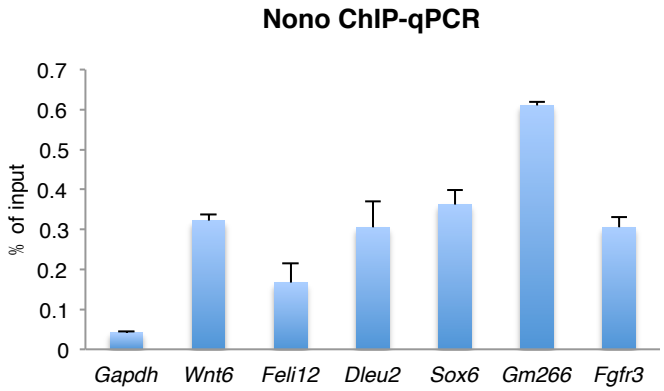
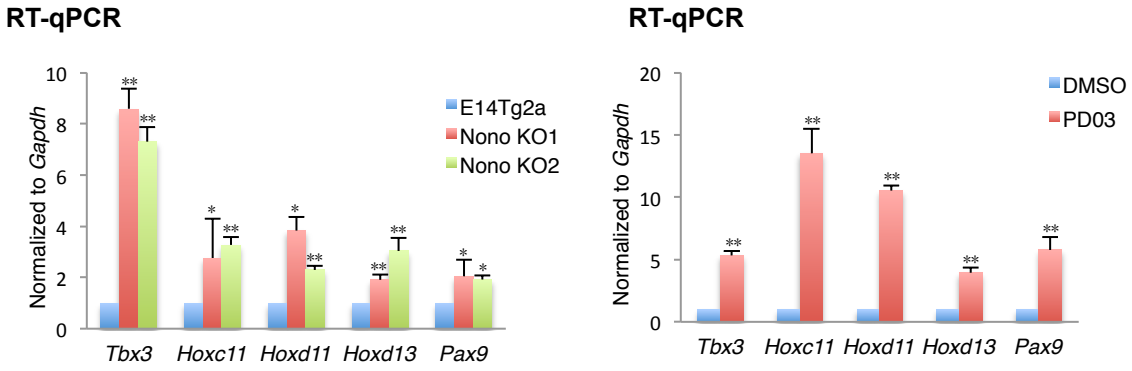


Figure S3 Related to Figure 5

A



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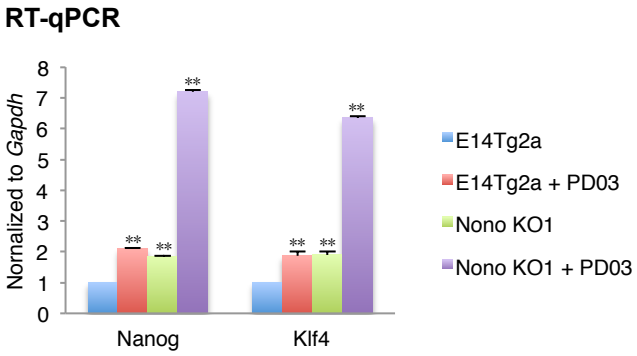

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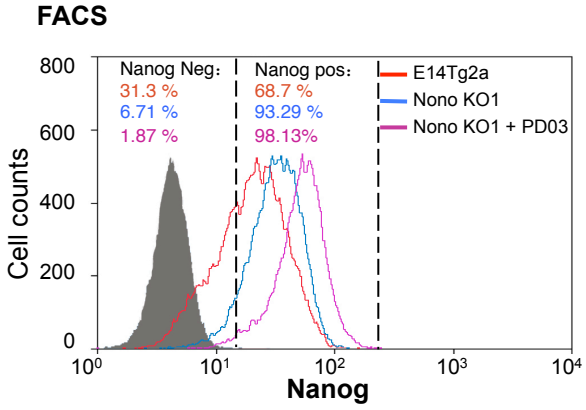
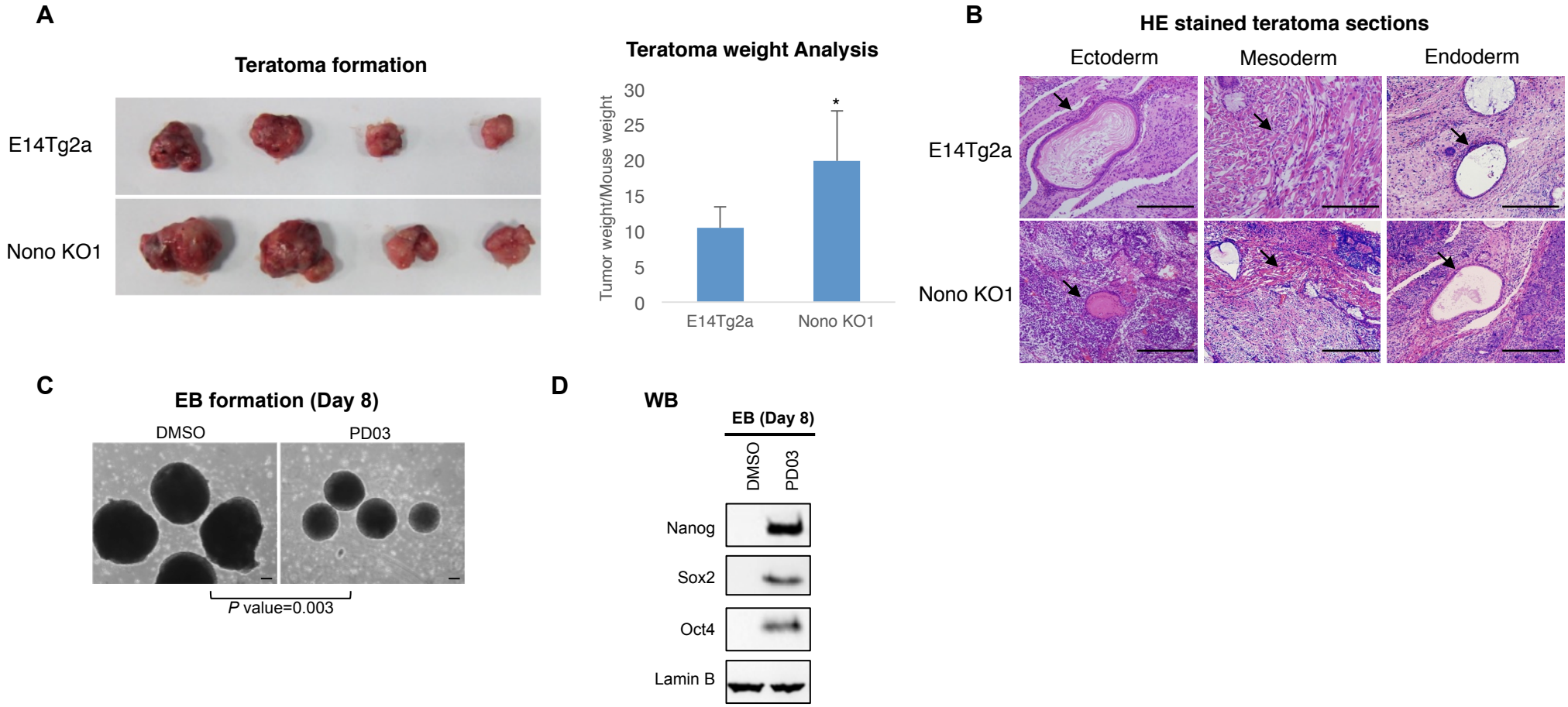


Figure S4 Related to Figure 7



Supplementary Figure Legends

Figure S1. Related to Figure 1

(A) Schematic illustrating how the positions and sequences of the two gRNAs used to generate Nono KO mouse ESCs. Note Nono is in the X chromosome thus E14Tg2a (male) cells only have one copy of Nono.

(B) Alkaline phosphatase (AP) staining of colonies formed 7 days post plating from indicated cell lines.

Figure S2. Related to Figure 3-4

(A) GO term analysis of all Nono target genes in mESCs.

(B) Representatives of Nono/Erk2 co-bound genes with known functions in lineage specification and development.

(C) Hierarchical clustering analyses of Nono, Erk2, RNAPIIS5P, Ezh2 and indicated histone modifications for 10,445 non-overlapping RefSeq genes. Four major groups of PRC occupied genes were identified based on the occupancies of H3K27me3, H2A ubiquitination, H3K4me3, RNAPII (S2P, S5P and S7P) and mRNA expression level (Brookes et al., 2012). Most Nono occupied genes (boxed) are within PRCr group (H3K27me3, H2A ubquitination, H3K4me3, RNAPIIS5P occupied but without RNAPIIS2P and S7P). Erk2, RANPIIS5P, Ezh2 and histone modification data are adapted from published data. (Brookes et al., 2012; Marks et al., 2012; Tee et al., 2014)

(D) Nono ChIP-qPCR analyses at 6 selected Erk2 targets without detectable Nono ChIP-seq signal in WT (E14Tg2a) mESCs. *Gapdh* locus was used as a control.

Figure S3. Related to Figure 5

(A) RT-qPCR analyses of the expressions of selected Nono target genes in WT (E14Tg2a), two Nono KOs (left) and PD03 treated WT (E14Tg2a) (right) mESCs.

(B) RT-qPCR analyses of Nanog and Klf4 levels in WT (E14Tg2a), PD03 treated WT (E14Tg2a), Nono KO1 and PD03 treated Nono KO1 (E14Tg2a) (right) ESCs.

(C) Quantitative flow cytometry analysis of Nanog expression in WT (E14Tg2a), Nono KO1 and PD03 treated Nono KO1 cells.

In panel A and B, all q-PCR data are represented as mean ± SD (n=3), * $p < 0.05$; ** $p < 0.01$, T test.

Figure S4. Related to Figure 7

(A) Left, teratomas derived from WT (top) and Nono KO1 cells (bottom). Right, weights (mg) of teratomas from WT and Nono KO cells. Data are represented as mean ± SD (n=4), * $p < 0.05$, T test.

(B) Images of hematoxylin/eosin stained sections of wild E14Tg2a and Nono KO1 ESCs formed teratomas; arrows point to represented tissues from all three germ layers. The scale bar represents 100 μm.

(C) Morphological analysis of the day 8 EBs formed from DMSO and PD03 treated mESCs. The scale bar represents 100 μm. *P* values by T test.

(D) Western blot analyses of Nanog, Sox2, Oct4 and Lamin B (as loading control) in the day 8 EBs formed from DMSO and PD03 treated mESCs.

Extended Experimental Procedures

Cell culture and differentiation procedures

mESC cells were cultured in standard mESC medium with leukemia inhibitory factor (LIF, Millipore #ESG1107, 100 Units/ml) in DMEM containing 10% fetal calf serum (Gibco #16000-044), 1% combination of the antibiotics penicillin and streptomycin (Hyclone #SV30010), 1% NEAA (Gibco #11140) and 0.1% 2-Mercaptoethanol (Gibco#21985-023). For single inhibitor or 2i treatments, MEK inhibitor PD0325901 (1 mM) and GSK3β inhibitor CH99021 (3 mM) were used. 293T cells were used for the Erk shRNA virus packaging and were cultured in DMEM containing 10% fetal calf serum and 1% combination of the antibiotics penicillin and streptomycin. Plasmids were transfected using Lipofectamine 2000 (Invitrogen #11668-019).

For EB differentiation, embryoid bodies (EBs) were allowed to form in the absence of LIF by hanging drops containing ~1,000 mES cells/drop on petri dish lids for 48h, and then collected and transferred to standard mES culture (without LIF) in non-coated petri dishes for further development. The EB samples were harvested at different time points indicated in the figures for further examinations.

Neuronal differentiation was induced by LIF withdrawal with the established protocol (Ying et al., 2008).

For RA-induce differentiation assays, ESCs were grown in the absence of LIF and supplemented with 0.1 mM all-trans retinoic acid (RA) for indicated days.

In colony formation assay, ~600 mESC cells were seeded in 6-well plate (60 cells/cm^2), cultured in standard condition for 7 days and then stained using an Alkaline Phosphatase Detection Kit (Sigma #85L3R-1).

Teratoma Formation

A total of $5x10^6$ E14Tg2a and Nono KO mouse ESCs were injected subcutaneously into NOD-SCID mice. Five weeks later, mice were euthanized and tumors were removed and fixed in formalin for several days and then subsequently imbedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological analysis.

RNAi and CRISPR

Erk1/2 RNAi was performed using two shRNA combinations (Erk1-1: 5'-GCCATGAGAGATGTTTACATT-3', Erk2-1: 5'-GCGCTTCAGACATGAGAAC-3') and shErk1-2/Erk2-2 (Erk1-2: 5'-GACCGGATGTTAACCTTCA-3', Erk2-2: 5'-GTACAGAGCTCCAGAAATT-3'). The stable cell lines with pLKO-puro shErk1 and pLKO-blasticidin shErk2 double KD were selected by puromycin (2 mg/ml) and blasticidin (10 mg/ml), respectively.

CRISPR-Cas9 gene targeting was carried out as previously described (Maeder et al., 2013) and the single knockout clones were isolated and then confirmed by Western blot showing undetectable Nono protein. Guiding RNA sequences used are: Nono KO1, Nono exon2: 5'-GTATTGACCTGAAGAATTTT-3', Nono KO2, exon1: 5'-GGATGCTGATGATGCTTCCT-3'. Frameshifts around the targeted regions in both KO cell lines were also sequence verified.

RNA analyses

Total RNA samples were isolated using TRIzol (Invitrogen #15596-018) and then reverse transcribed into cDNAs using a kit from Takara (#RR047A).

Co-Immunoprecipitation

Nuclear extracts of mESCs were prepared as described previously (Mendez

and Stillman, 2000), with the following minor modifications. Nuclear extracts were lightly sonicated for 15 s and then centrifuged at 12,000 rpm for 10 min at 4° C. Supernatants were incubated with 2 μg of Nono antibody, Flag antibody, RNAPIIS5P antibody and control IgG, respectively, followed by the addition of 15 μl of Protein A/G agarose beads (Millipore). After overnight incubation at 4° C, beads were washed 5 times with washing buffer (50 mM Tris-HCl pH 7.9, 150 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 5% glycerol, 0.1% NP-40, 3 mM β-ME with protease inhibitors), and boiled for 10 min in 50 μl of SDS loading buffer for Western blot analyses.

ChIP and ChIP-seq

ChIP assays were performed as described elsewhere (Lan et al., 2007). Briefly, chromatin samples were incubated with specific antibodies in the ChIP Lysis buffer (20 mM Tris-HCl pH8.1, 150 mM NaCl, 2 mM EDTA, 1% TritonX-100 and 0.05% SDS) overnight at 4° C. The protein-DNA complexes were immobilized on pre-washed protein A/G beads (15 μl per reaction). The bound fractions were washed 3 times with the Lysis buffer, and twice with the Low Salt Wash buffer (10 mM Tris-HCl, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% Na-deoxylcholate), and once with 10 mM Tris-HCl pH8.0. Elution and reverse crosslinking were carried out in the Elution buffer (50 mM Tris-HCl pH8.0, 10 mM EDTA and 1% SDS) at 65° C for 4 hours. After 1 hour of RNase A and Proteinase K digestion at 55℃, DNA samples were then purified using PCR extraction kit (QIAGEN #28006). The precipitated DNA samples were either analyzed by qPCR (Roche) or prepared for DNA deep sequencing according to manufacture's guidelines (Guo et al., 2014 and Illumina) as described in Methods and Procedures.

mRNA-seq

mRNA seq were carried out according to manufacture's guidelines and our previous protocol (C-10365, Life Technologies, (Guo et al., 2014)). For

bioinformatics analyses, the sequence reads were mapped to mouse transcripts and genome databases using the software TopHat (Trapnell et al., 2009) allowing 2bp mismatches per 25bp seed.

Hierarchical clustering analysis

Hierarchical clustering analysis in Figure S1C was performed after binary classification of Nono, Erk2, RNAPIIS5P, Ezh2 and histone modifications for 10,445 non-overlapping RefSeq genes. The enrichment of each mark at TSSs or TESs is normalized to the binary classification threshold (Brookes et al., 2012; Marks et al., 2012; Tee et al., 2014).

ChIP Primers

Gapdh-ChIP-F	5'-GAGCCTCCTCCAATTCAACC-3'
Gapdh-ChIP-R	5'-TGAGACTTACAAACACTCTCCT-3'
Hoxd11-ChIP-F	5'-GAACAGAGCGGAAAAACCTG-3'
Hoxd11-ChIP-R	5'-CTGGGAGCTTGTTGCTTCTT-3'
Pax9-ChIP-F	5'- ACGTTGTCAGATCCGAGGAG-3'
Pax9-ChIP-R	5'- TCCCTTAAATCGCAAAATGG-3'
Tbx3-ChIP-F	5'-TGAGGCCTTTCAGACGTAGG-3'
Tbx3-ChIP-R	5'-GCCTGTCCTGCGTTTATGTC-3'

qPCR Primers

Gapdh-F	5'-GCTACACTGAGGACCAGGTTGTCT-3'
Gapdh-R	5'-CCTGTTGCTGTAGCCGTATTCA-3'
Tbx3-F	5'-AGGAGCGTGTCTGTCAGGTT-3'
Tbx3-R	5'-GCCATTACCTCCCCAATTTT-3'
Hoxc11-F	5'-GAGAACACGAATCCCAGCTC-3'
Hoxc11-R	5'-TCACTTGTCGGTCTGTCAGG-3'
Hoxd11-F	5'-AGAGGTCCCGGAAAAAGC-3'

Hoxd11-R	5’-TGGAACCAGATTTTGACTTGC-3’
Hoxc5-F	5’-CCCCAATATCCCTGCCTATAAC-3’
Hoxc5-R	5’-CAGCCATGTCTACCCCGTG-3’
Gata4-F	5’-TTCCTCTCCCAGGAACATCAAA-3’
Gata4-R	5’-GCTGCACAACTGGGCTCTACTT-3’
Pax6-F	5’-GACCTCCTCATACTCGTGCA-3’
Pax6-R	5’-CCATGGGCTGACTGTTCATG-3’
Hoxd13-F	5’-TGGAACAGCCAGGTGTACTG-3’
Hoxd13-R	5’-TGTCTCTCCGAAAGGTTCGT-3’
Cdx2-F	5’-AGGCTGAGCCATGAGGAGTA-3’
Cdx2-R	5’-CGAGGTCCATAATTCCACTCA-3’
Sox17-F	5’-CACAACGCAGAGCTAAGCAA-3’
Sox17-R	5’-CGCTTCTCTGCCAAGGTC-3’
Klf4-F	5’-CCAGCAAGTCAGCTTGTGAA-3’
Klf4-R	5’-GGGCATGTTCAAGTTGGATT-3’
OCT4-F	5’-GTTGGAGAAGGTGGAACCAA-3’
OCT4-R	5’-CTCCTTCTGCAGGGCTTTC-3’
Nanog-F	5’-CCTCCAGCAGATGCAAGAA-3’
Nanog-R	5’-GCTTGCACTTCATCCTTTGG-3’
Rex1-F	5’-AGGCCAGTCCAGAATACCAG-3’
Rex1-R	5’-GGAACTCGCTTCCAGAACCT-3’
Tuji-F	5’-GCGCATCAGCGTATACTACAA-3’
Tuji-R	5’-TTCCAAGTCCACCAGAATGG-3’
Nestin-F	5’-CTGCAGGCCACTGAAAAGT-3’
Nestin-R	5’-TTCCAGGATCTGAGCGATCT-3’
TBP-F	5’-GGGGAGCTGTGATGTGAAGT-3’
TBP-F	5’-CCAGGAAATAATTCTGGCTCA-3’

Antibodies

Nono	sc-166702	Santa cruz	WB
Nono	ab70335	Abcam	ChIP/IP
Erk1/2	05-1152	Millipore	WB
p-Erk1/2	#4370S	Cell Signaling	WB
Erk2	05-157	Millipore	ChIP
Nanog	eBioMLc-51	eBioscience	IF/WB
Oct3/4	sc-5279	Santa cruz	WB
H3K4me3	#9751S	Cell Signaling	ChIP/WB
H3K27me3	#9733S	Cell Signaling	ChIP/WB
Lamin B	66095-1-lg	Protein-tech	WB
Sox2	sc-17320	Santa cruz	WB
Total RNAPII	ab817 (8WG16)	Abcam	ChIP
RNAPIIS5P	Ab5131	Abcam	ChIP/IP

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