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Supplementary Information for

Asf1a resolves bivalent chromatin domains for the induction of lineage-specific genes during mouse embryonic stem cell differentiation

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Supplementary Materials and Methods

Cell culture. Mouse E14 embryonic stem (ES) cells, kindly provided by Dr. Tom Fazzio, were cultured in ES medium, containing DMEM with 15% FBS (Sigma), 2mM L-glutamine (Cellgro), 1 X MEM nonessential amino acids (Cellgro), 1mM sodium pyruvate (Cellgro), 55uM beta-mercaptoethanol (Gibco) and mLIF, on gelatin coated plates. For CRISPR/Cas9 targeting, sgRNAs were cloned in pSpCas9BB-2A-Puro (PX459) vector purchased from Addgene, and transfected in ES cells using Lipo3000 (Invitrogen). After puromycin selection, single clones were screened using Surveyor mutation detection kits (IDT), and genotypes of candidate clones were confirmed by Sanger sequencing of PCR products amplified at target loci from genomic DNA.

Plasmids and antibodies. Mouse Asf1a and Asf1b cDNA were purchased from Open Biosystems and cloned in lentivirus-based vector pWPXL (Addgene) with one FLAG tag at the C terminus. Lenti-virus was produced in HEK293T cells with standard protocol. After virus infection, single clones were screened for protein expression level. The following antibodies were used in this study: Polyclonal anti-Asf1a (homemade, rabbit, antigen: LEDAESSNPNC); anti-FLAG (Sigma, F1804); anti-tubulin (DSHB, 12G10); FITC mouse anti-BrdU (BD Biosciences, 556028); anti-HIRA (Millipore, WC119); anti-p60 (Santa cruz， sc-393662); anti-H3.3 (Millipore, 09-838); anti-H3 for WB (homemade as previously described) (1); anti-H3 for ChIP (ab1791); anti-Nanog (Cell signaling, 4903); anti-Oct4 (Cell signaling, 2840); anti-H3K4me3 (Ab8580); anti-H3K9me3 (Active motif, 61013); anti-H3K27me3 (Cell signaling, 9733); anti-H3K27Ac (homemade, rabbit, antigen: AARK(Ac)SAPC); anti-Suz12 (Cell signaling, 3737); anti-Ezh2 (Cell signaling, 5246); anti-UTX (Bethyl, A302-374A); anti-JMJD3 (Ab154126).

Mouse ES cell characterization. 1,000 ES cells were plated in each well of 96-well plate with 100 μL of normal ES medium. Cells were counted every 24h by the cell titer blue assay kit (Promega, G8080) according to manufacturer's instructions. Alkaline phosphatase (AP) staining kit was from System Biosciences (AP-100R-1). To analyze ES cell cycle profile, cells cultured in 60mm dish were pulsed with 10 μM BrdU for 15min at 37°C. Cells were then harvested and fixed in 75% ethanol at -20°C overnight. Incorporated BrdU was stained using FITC BrdU flow kit (BD, 559619) according to the manufacturer's instructions.

Immunoprecipitation (IP). ES cells were infected with viruses expressing Empty Vector (EV), WT FLAG-Asf1a/b or FLAG-Asf1a mutants. About 2×10^8 cells for each sample were suspended in 1ml of lysis buffer (50 mM HEPES-KOH, pH 7.4, 200 mM NaCl, 0.5% Triton X-100, 10% glycerol, 100 mM EDTA and proteinase inhibitors), incubated on ice for 15min and dounced for 50 times using tissue grinder (Sigma, D8938-1SET). After clarification by centrifugation, the lysate was incubated with 30 μL of Anti-FLAG M2 agarose beads (Sigma, A2220) at 4°C overnight. The beads were then washed five times using lysis buffer for 5 min each. Proteins were eluted twice by 2 mg/ml FLAG peptide (Sigma, F4799) at 16 °C for 30 min. The eluted proteins were then precipitated by trichloroacetic acid (TCA), dissolved with 1× SDS sample buffer and analyzed by Western blot.

***In vitro* differentiation assay and RT-PCR analysis.** Embryoid bodies (EBs) were formed in ES medium without mLIF using the hanging drop method (300 cells per 30 μL drop) on dish lids for 3 days. EBs were collected and cultured without mLIF in 10 cm petri dishes on a horizontal rotator at a speed of 30 rpm, with medium changed every other day for up to 10 days. At each time point, the diameter of 50 EBs for each line was quantified using ImageJ.

The neural differentiation of ES cells was performed in serum free adherent monolayer culture as previously described(2, 3). Briefly, ES cells were plated at high density (1.6-2.4 $\times 10^6$ per 60mm dish) onto gelatin-coated dish and cultured for 24hrs in standard ES medium with mLIF. The differentiation was initiated by seeding ES cells in N2B27 medium (3×10^5 cells/60mm dish). The medium was changed every other day up to 6 days. Cells were re-plated in N2B27 medium supplied with EGF (10 ng/ml, R&D) and FGF-2 (10 ng/ml, R&D) at day 6, and thereafter cultured in the same condition with medium changed every other day. Neural precursors were collected at day 10 for the following analysis.

To analyze gene expression using RT-PCR, total RNA was extracted from around 1×10^6 cells or 100 EBs using the miRNeasy Mini kit (Qiagen). cDNAs were synthesized from 0.5 μg of RNA using random hexamers (Invitrogen). Quantitative PCR was performed in 20 μL reactions containing 0.1 μM primers and SYBR Green PCR Master Mix (Bio-Rad Laboratories). Mouse GAPDH was used as a control to normalize the expression of each gene. Table S1 lists primer sequences for RT-PCR.

RNA-seq. Total RNA was extracted the same way as RT-PCR. RNA-seq libraries and deep sequencing were conducted by Novogene mRNA next-generation sequencing services. Two replicates for each sample were sequenced. Raw data were aligned to the mouse genome mm9 and to gene annotations from Refseq gene using TopHat v2.05. Cufflinks v2.0.2 was used to quantify FPKM values, and Cuffdiff for differential expression analysis use FDR < 0.01. Gene ontology analysis was conducted using GO TermFinder (http://go.princeton.edu/cgi-bin/GOTermFinder).

Chromatin immunoprecipitation (ChIP)-deep sequencing (ChIP-seq) and ChIP-real time PCR. Both adherent cells and EBs were collected by digesting to single cells and cross-linked with 1% formaldehyde for 10 min, quenched with 125 mM glycine for 5 min at room temperature and washed twice with cold TBS. For Actinomycin D (Sigma, A1410) treatment, the drug was added at the concentration of 1μg/ml for 30 min before crosslinking. About 2×10^7 fixed cells were re-suspended in cell lysis buffer (10 mM Tris-HCl, pH7.5, 10 mM NaCl, 0.5% NP-40) and incubated on ice for 10 min. Pellets were washed once with MNase digestion buffer (20 mM Tris-HCl, pH7.5, 15 mM NaCl, 60 mM KCl, 1 mM $CaCl_2$). After re-suspended in 500 μL MNase digestion buffer containing proteinase inhibitor cocktail (Sigma), the lysates were incubated in the presence of 1,000 units of MNase (NEB, M0247S) at 37 °C for 20 min with continuous mixing in a thermal mixer. After adding the same volume of sonication buffer (100 mM Tris-HCl, pH8.1, 20 mM EDTA, 200 mM NaCl, 2% Triton X-100, 0.2% sodium

deoxycholate), the lysates were sonicated for 15 min (30 sec on / 30 sec off) using Bioruptor (Diagenode) and centrifuged at top speed for 10 min. The chromatin content was estimated by Qubit assay. Equal amount of chromatin were incubated with 2 μg of antibody or 30 μL of M2 beads on a rocker at 4 °C overnight. After adding 30 μL protein G beads (Pierce, 20398), the reactions were rocked for another 3 hours at 4 °C. The beads were washed twice with ChIP buffer (50 mM Tris-HCl, pH8.0, 10 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate), once with high salt buffer (ChIP buffer with 500 mM NaCl), LiCl buffer (10 mM Tris-HCl, pH8.0, 0.25 M $LiCl_2$, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA), and TE buffer. Bound chromatin was eluted and reverse-crosslinked at 65°C overnight. DNA was purified using PCR purification kit (Qiagen) after the treatment of RNase A and proteinase K. ChIP DNA was analyzed by real-time PCR using primers listed in Table S1. For ChIP-seq, 10ng of ChIP and Input DNA were processed for library preparation by following Ovation ultralow DR multiplex kit (NuGEN). The ChIP-seq library DNA was sequenced from single end using an Illumina NextSeq 500 instrument.

ChIP-seq data analysis. Sequencing reads from H3K4me3 and H3K27me3 ChIP-seq were aligned to the mouse genome (mm9) using the Bowtie2 software (4). After removal of PCR duplication reads by SAMtools (5), genome-wide read coverage was calculated by BEDTools (6) and in-house Perl programs. In order to calculate the ChIP-seq read density at promoters, the UCSC annotation genes were selected and then read density (normalized to Reads Per Kilobase per Million mapped reads) was calculated across the promoter regions. The promoters used in this study were defined as 2kb upstream and 500bp downstream of TSS (base on UCSC annotation genes transcription start sites).

Histone acid extraction. For histone acid extraction, around 5×10^6 cells were used for each sample. EBs were digested into single cells using 0.25% trypsin (Gibco, 25200) before extraction. Total histones were extracted as previously described (7).

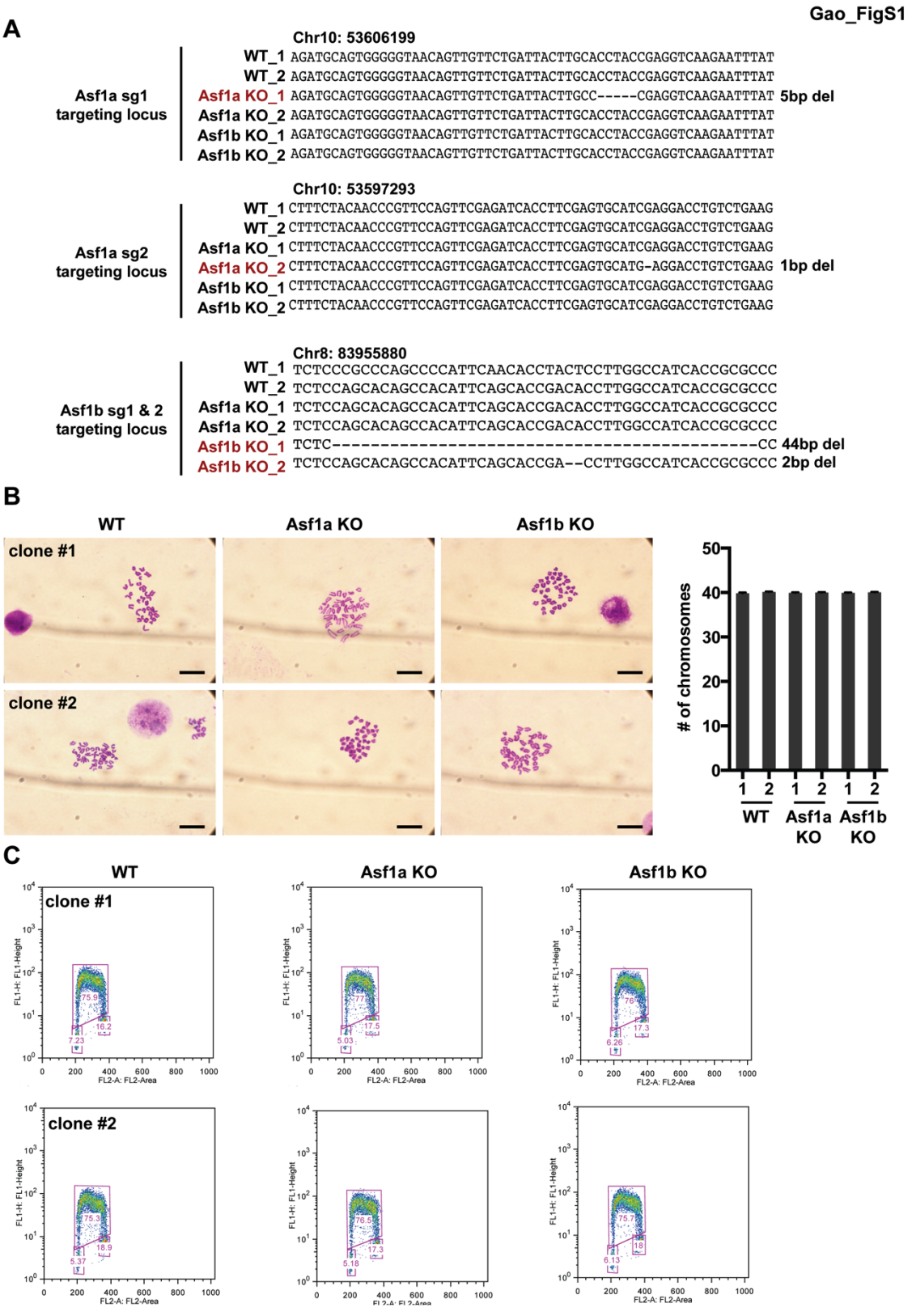

Fig. S1. Asf1a or Asf1b is not required for mouse ES cell growth and identity. (**A**) Sanger sequencing results of two different Asf1a KO and Asf1b KO clones generated

from different sgRNAs show frame-shift deletion at Asf1a and Asf1b loci. (**B**) The karyotypes of Asf1a and Asf1b KO clones are normal. Representative images of chromosome spread from two independent lines show normal karyotype (left panel). Scale bar; 10 μm. The average of chromosome number counted from 15 chromosome spreads for each line (right panel). (**C**) Analysis of cell cycle progression of mouse ES cells by measuring BrdU incorporation. Cell populations at different cell cycle stages were gated and quantified. The quantification was shown in Fig. 1C. **Related to Fig. 1**

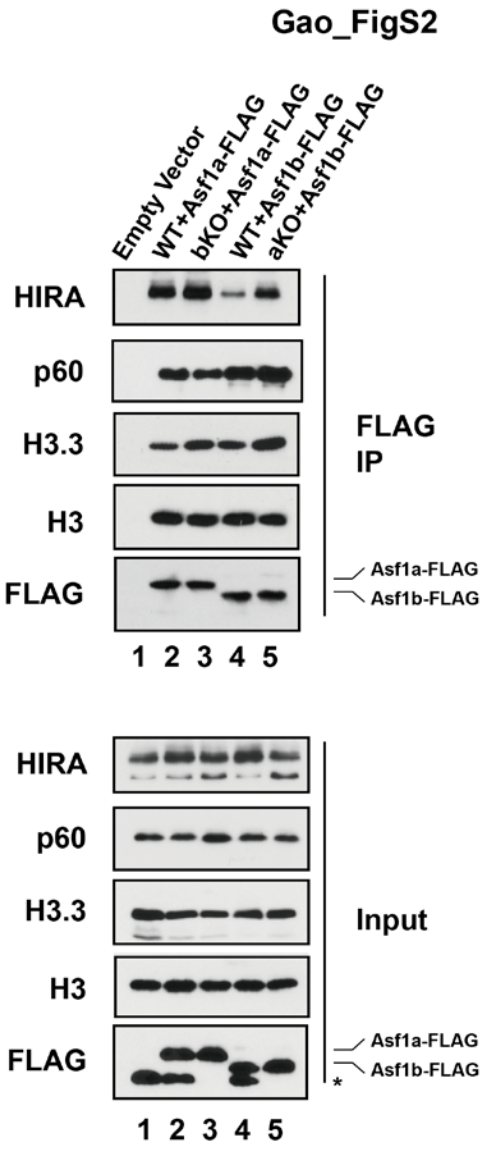

Fig. S2. In the absence of Asf1a, the interaction between Asf1b and HIRA increases. Empty vector (EV), FLAG-Asf1a or FLAG-Asf1b were stably expressed in WT, Asf1a KO (aKO) and Asf1b KO (bKO) cells. Exogenously expressed Asf1a and Asf1b were immuno-precipitated using antibodies against the FLAG epitope (M2 beads). **Related to Fig. 1**.

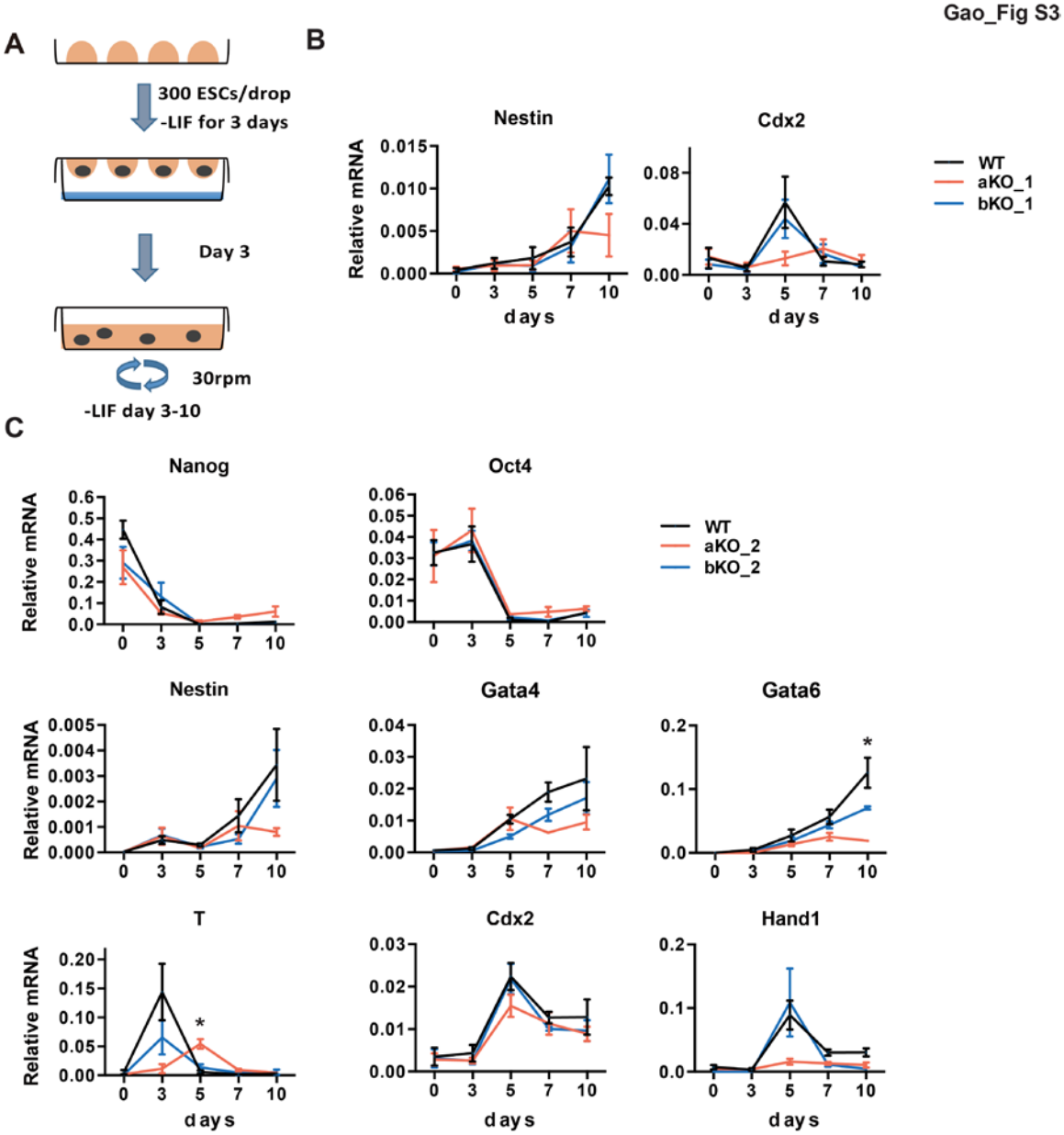

Fig. S3. Asf1a deletion affects the expression of lineage-specific genes. (**A**) The hanging drop method was used for embryoid body (EB) formation. (**B**) RT-PCR analysis of additional lineage-specific genes of Fig. 2. (**C**) Analysis of gene expression of EBs formed by another independent line (clone #2) by RT-PCR. Results were from three different experiments and error bars represent mean ± SEM. P value was calculated between WT and Asf1a KO. * $p<0.05$. **Related to Fig. 2.**

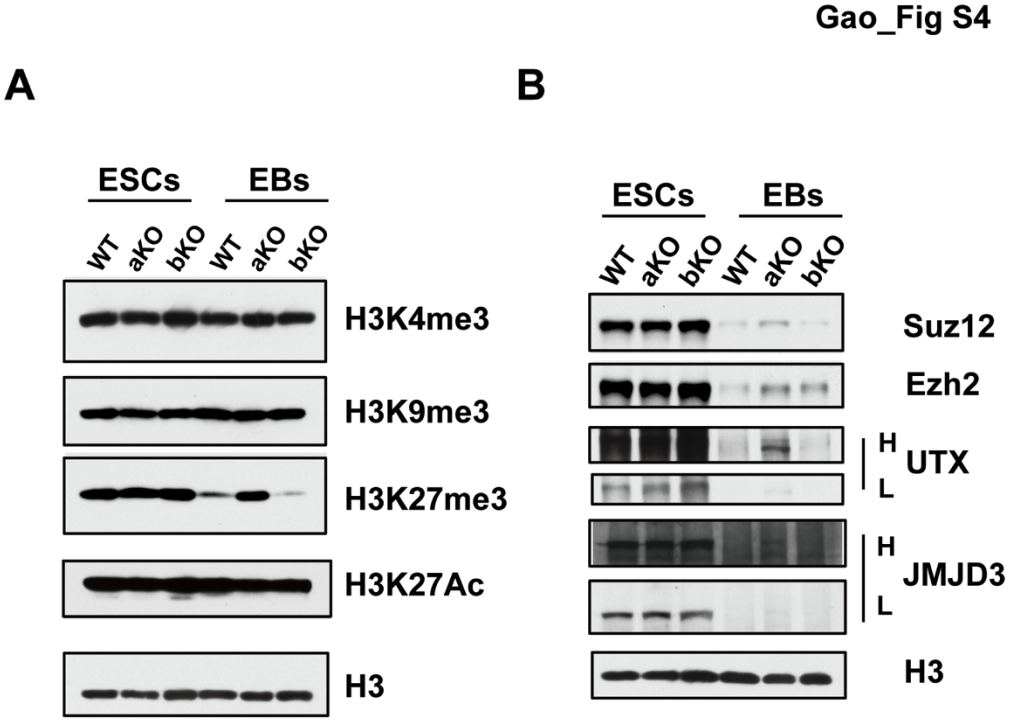

Fig. S4. A global reduction of H3K27me3 during ES cell differentiation is compromised in Asf1a deletion cells. (**A**) Analysis of global histone modifications in in WT, Asf1a KO (aKO) and Asf1b KO (bKO) ES cells and day 10 EBs by Western blot. Histones were isolated using acid extraction. (**B**) Western blot analysis of histone H3K27 modifying enzymes in ES cells and day 10 EBs. H3 was used as a loading control.

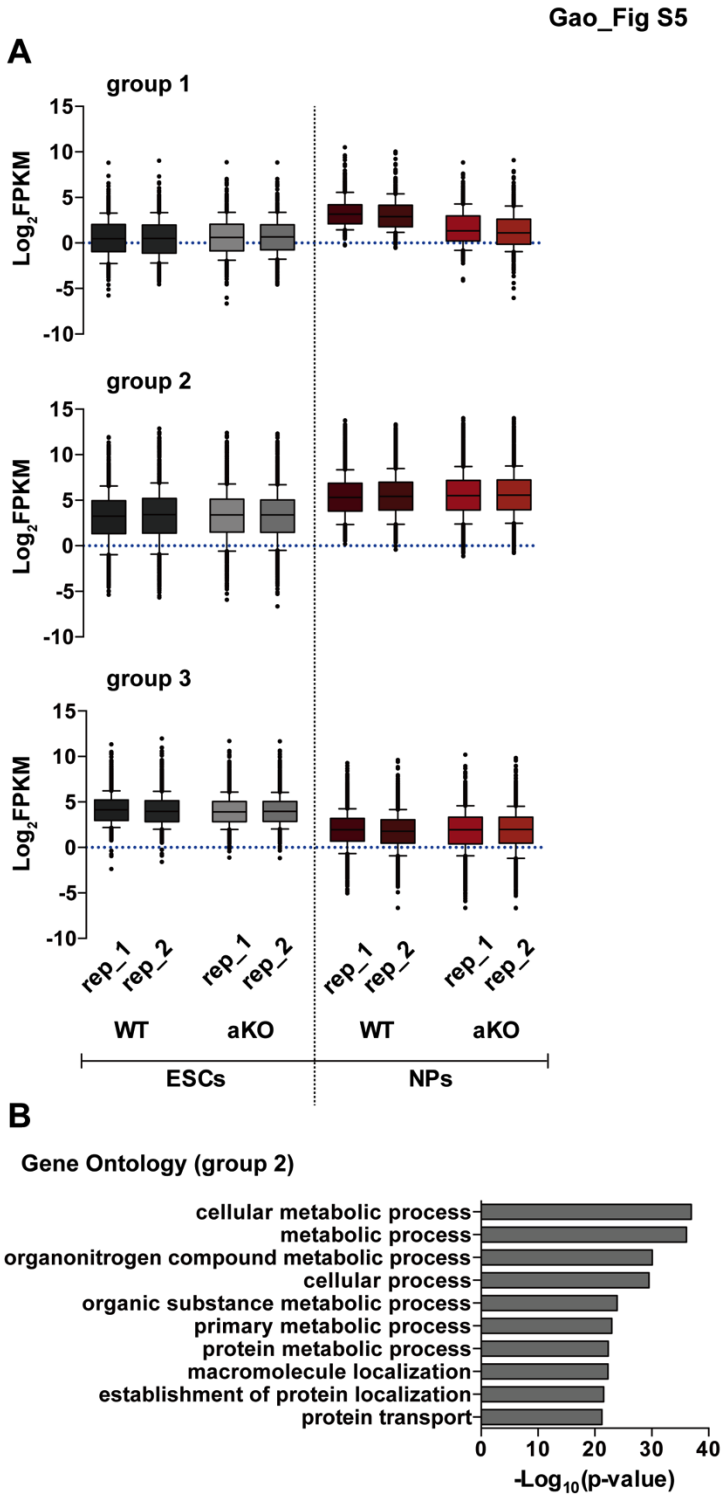

Fig. S5. Asf1a is important for ES neural differentiation. (**A**) Box plot showing the average expression level of the three sub-groups of genes identified in Fig. 4D. Data were from two independent repeats of RNA-seq experiments (rep1 and rep2). (**B**) Gene ontology analysis of group 2 of genes. **Related to Fig. 4.**

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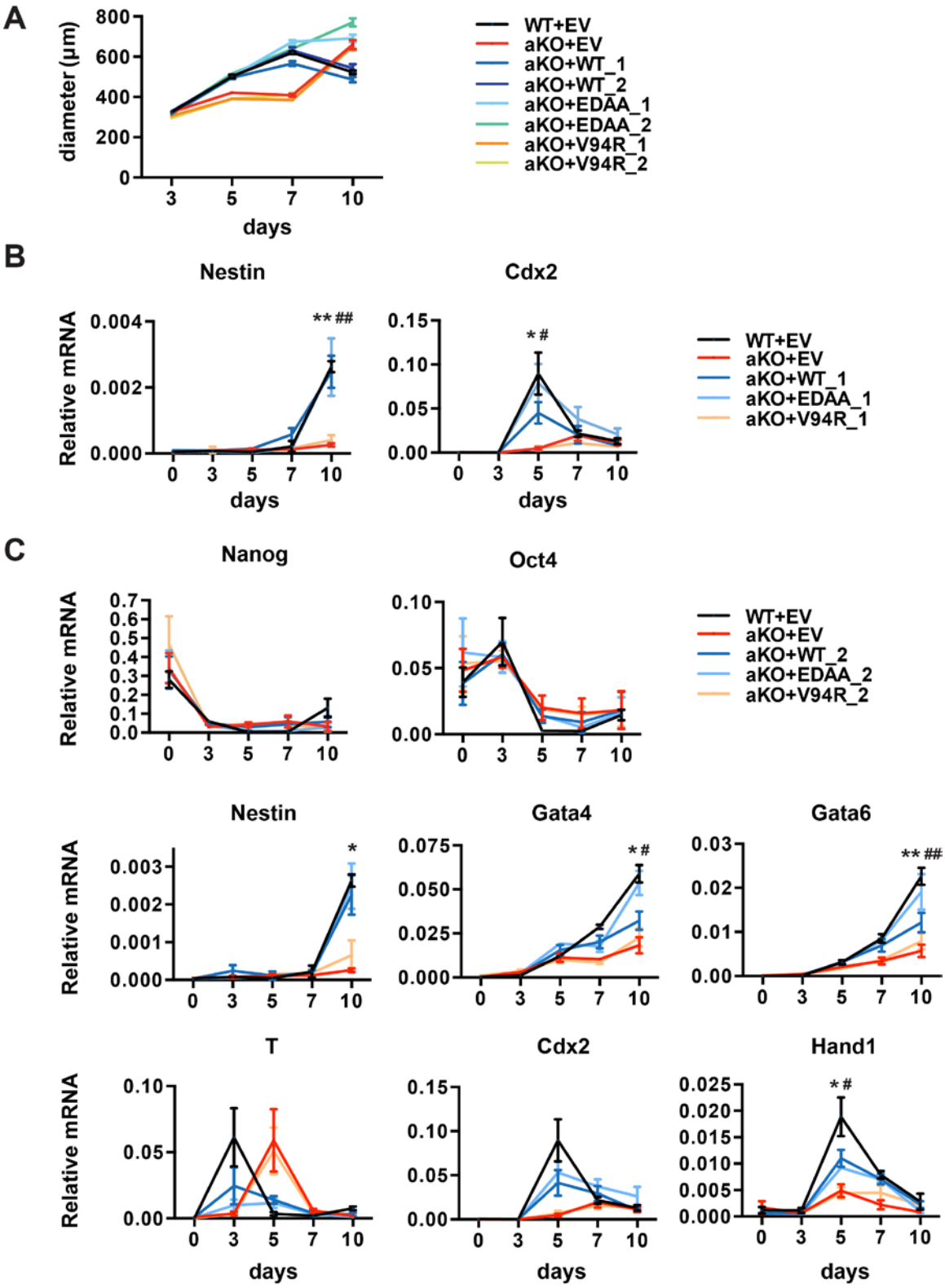
A
diameter (μm)
800
600
400
200
0
3
5
7
10
days
WT+EV
aKO+EV
aKO+WT_1
aKO+WT_2
aKO+EDAA_1
aKO+EDAA_2
aKO+V94R_1
aKO+V94R_2
B
Nestin
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Relative mRNA
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0.15
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aKO+EDAA_1
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aKO+EDAA_2
aKO+V94R_2
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Fig. S6. The interaction between Asf1a and H3 is important for its function in differentiation. (**A**) Growth curve of EBs formed by two independent clones. The diameter of 50 EBs for each line at each time point was measured by ImageJ. Error bars represent mean ± SEM. (**B**) RT-PCR analysis of additional lineage-specific genes of Fig. 5. (**C**) Gene expression analysis by RT-PCR using another independent line (#2) during EB differentiation. Data were from three different experiments and error bars represent mean ± SEM. * ($p<0.05$) and ** ($p<0.01$) represent the comparison between (WT+EV) and (aKO+EV). # ($p<0.05$) and ## ($p<0.01$) represent the comparison between (WT+EV) and (aKO+V94R). aKO: Asf1a KO line. **Related to Fig. 5**

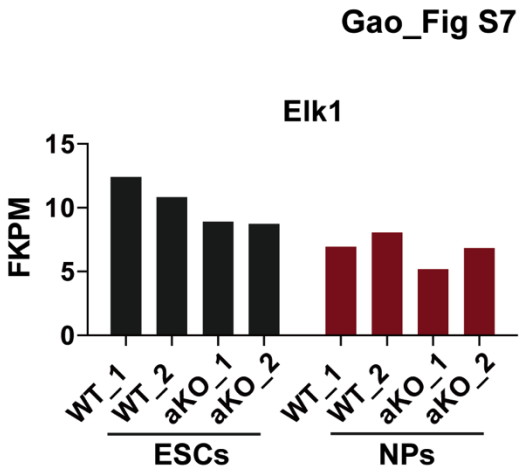

Fig. S7. The expression level of Elk1 in ES cells and NPs during neural differentiation identified by RNA-seq. Related to Fig. 6.

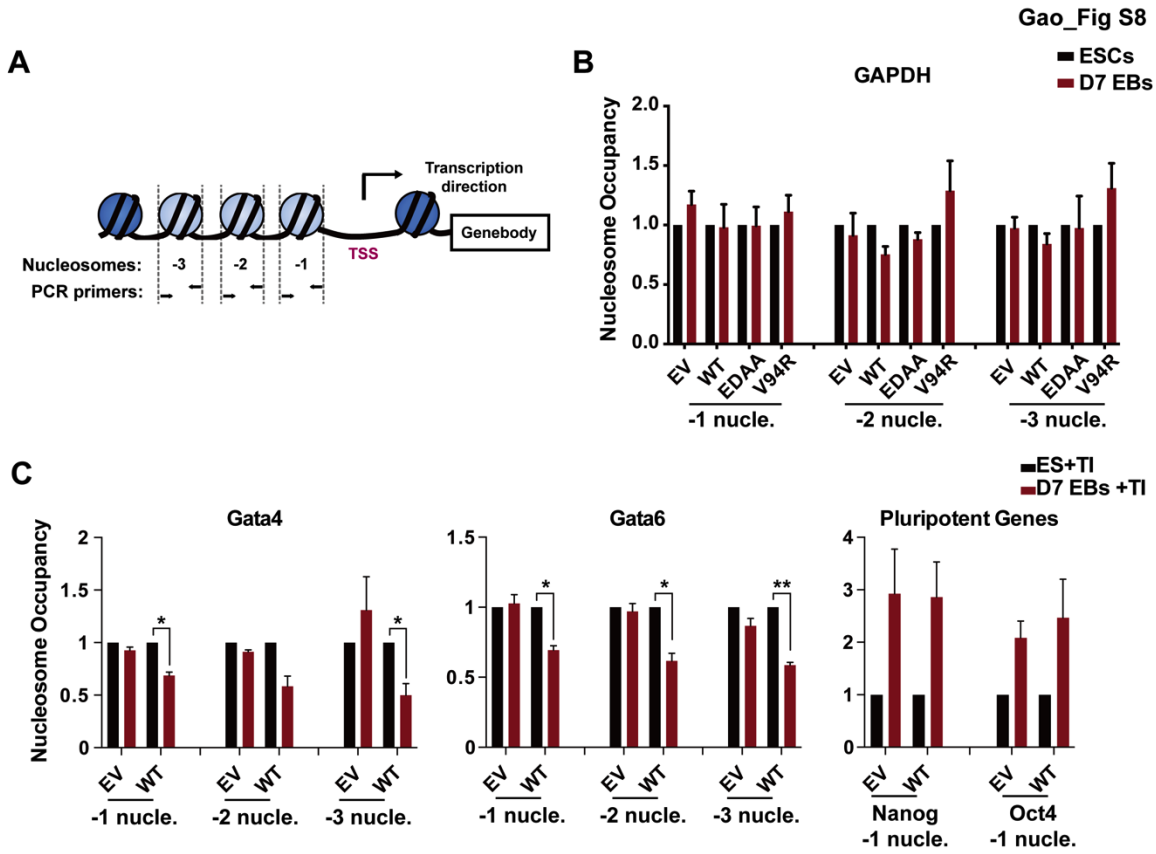

Fig. S8. Asf1a is involved in evicting histones at bivalent gene promoters. (**A**) Primer designs to analyze nucleosome occupancy at three nucleosomes (-1 to -3 nucleosomes) upstream of gene TSS. (**B**) Nucleosome occupancy analysis at the housekeeping gene GAPDH promoter using the same samples as in Fig. 7A-B. (**C**) Nucleosome occupancy analysis in presence of transcription inhibitor (TI), Actinomycin D. Asf1a KO ES cells and day 7 EBs expressing EV or WT Asf1a were treated with 1ug/mL Actinomycin D for 30min before crosslinking for H3 ChIP. The H3 ChIP enrichment was normalized against its enrichment at the gene body of GAPDH and the nucleosome occupancy of ES cells in each line was set as 1. Results were from three different experiments and error bars represent mean ± SEM. * $p<0.05$. **Related to Fig. 7.**

Table S1. Oligonucleotides used in this study

	Forward	Reverse
Asf1a sg1	CACCGCTGATTACTTGCACCTACCG	AAACCGGTAGGTGCAAGTAATCAGC
Asf1a sg2	CACCGGATCACCTTCGAGTGCATCG	AAACCGATGCACTCGAAGGTGATC
Asf1b sg1	CACCGCGCGGTGATGGCCAAGGTGT	AAACACACCTTGGCCATCACCGCGC
Asf1b sg2	CACCGCACATTCAGCACCGACACCT	AAACAGGTGTCGGTGCTGAATGTGC
Nanog RT	TCTTCCTGGTCCCCACAGTTT	GCAAGAATAGTTCTCGGGATGAA
Oct4 RT	CACCATCTGTCGCTTCGAGG	AGGGTCTCCGATTTGCATATCT
Sox2 RT	GCGGAGTGGAAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
Nestin RT	CCCTGAAGTCGAGGAGCTG	CTGCTGCACCTCTAAGCGA
Fgf5 RT	GCAAGTGCCAAATTTACGGATGAC	TTGCTCGGACTGCTTGAACCTG
Gata4 RT	CCCTACCCAGCCTACATGG	ACATATCGAGATTGGGGTGTCT
Gata6 RT	TGCCTCGACCACTTGCTATGAAAA	CACTGATGCCCCTACCCCTGAG
T RT	TCCCGGTGCTGAAGGTAAATGTGT	TTGGGCGAGTCTGGGTGGATGTAG
Cdx2 RT	GCGGCTGGAGCTGGAGAAGGAGTT	CGGCGGCTGTGGAGGCTGTTGT
Hand1 RT	ATGTGCCCGCCGACACCAAG	CGGGCTGCTGAGGCAACTCC
Sox21 RT	GCGGTGCTTTACGATACGTTG	CCGAACATCAGAACCGAGCT
Zfpm2 RT	TGAAGACAACTCGCATCAGG	TAGCTCCCTCTGGGTCTGAA
GAPDH RT	CTGACGTGCCGCCTGGAGAAAC	CCCGGCATCGAAGGTGGAAGAGT
Nanog pro.	GGGTAGGGTAGGAGGCTTGA	CGGCTCAAGGCGATAGATT
Oct4 pro.	GGGGTGAGAGGACCTTGAA	GGACAGGACAACCCTTAGGAC
Gata4 pro.	AAGCGCTCTTTTCTCCTTCC	GTGAGGGCTACAGGGAGTGA
Gata6 pro.	CCTTCCCATACACCACAACC	CCCCTCCTTCCAAATTAAGC
Sox21 pro.	GTCATCTCTCCCCTCCCTCTAT	AGTTACCTCCCTAGCCTTCCAG
Zfpm2 pro.	GGATGAAGTTCTCAGAGCTGGT	GCGCGAACTTTTACACCTACTT
Sox9 pro.	CTACGAGGGGCGTCAGAGTA	GGGGAATCAATGAAAACCAA
Nanog -1 nuc.	TGTCTTTAGATCAGAGGATGCCC	ACCATGGACATTGTAATGCAAAAGA
Nanog -2 nuc.	ACCAACTTACTAAGGTAGCCCG	ATCTTCCCTCCAAAAGTGCGG
Nanog -3 nuc.	TCTTCCATTGCTTAGACGGC	CCATGCCCAATTTAAGGAGTGT
Oct4 -1 nuc.	CATCCGAGCAACTGGTTTGTG	ACAACCCTTAGGACGGGACC
Oct4 -2 nuc.	TGGGCTGAAATACTGGGTTCA	TCCATTGAATGTTCGTGTGCC
Oct4 -3 nuc.	ATCTTGAGGAAAGAGGCCCCG	TCCATCTTGTGTTGTCCAGGTTG
Gata4 -1 nuc.	CTCCGTTCTGCCCTCAGTG	CTTGAACAGGGCGCTGGA
Gata4 -2 nuc.	TCTTTTCTCCTTCCCACGAGC	GGGGTGCGCGTTTTGTAT
Gata4 -3 nuc.	CTAACCTTAGAGCCCATTCAAGGA	ACTGACGCCGACTCCAAACT
Gata6 -1 nuc.	CTGGATGATTATGGGTCGCTG	TCCTCAGAGTAGGAGCGAGTC
Gata6 -2 nuc.	GAGCGTTGAATTCCAAAGGACC	AATTAAGCCCCGCGCAGT
Gata6 -3 nuc.	AGGGAAGGTACCCCGCTC	GCTAGTTTGGTTGTCGCGTTC
Sox21 -1 nuc.	CTCCCAACACTCCTCCTCCT	CACCAAGCTCCAAACAGTGC
Sox21 -2 nuc.	TGAAAGCGGTACACCAGAGG	AAGACTCTTCCCCCACTCCC

Sox21 -3 nuc.	TCTTGCCTCATGGTTGCACT	GGGCTCAACCTAGGCTCATT
Zfpm2 -1 nuc.	CCTTGCACTCCACGCGA	GCCATCAGCGAAAGGAGGAT
Zfpm2 -2 nuc.	CTCCAGGAGGGGAGCAGTAA	GCCTCTTCCAATGGACCCTC
Zfpm2 -3 nuc.	CGACTCTTGTGGGCCTAGTG	TGGGCTCTCAGGAGACCATT
GAPDH -1 nuc.	GGAGCCAGGGACTCTCCTTT	TCCTGTGTTCTCCCCTCACT
GAPDH -2 nuc.	TCTTCACTCCGAAGGGTAGGG	AGCAAAGAGGCGCCAGAGT
GAPDH -3 nuc.	TGCTTAAAAGGCAGGGAGCA	GGTAGGGCAATCTGGAAGGG
GAPDH genebody	GAGGATGGAACGGGGAGAAG	AGGCCCTATAGGCCAGGATG

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