SUPPLEMENTARY IMFORMATION

Eed/Sox2 regulatory loop controls ES cell self-renewal through histone methylation and acetylation

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Table S1. Primers for RT-PCR analysis

Gene name	Forward primer	Reverse primer
Eed	caacaccagccaccctctat	gagaaggtttgggtctcgtg
Oct3/4	caaggcaagggaggtagaca	caaaatgatgagtgacagacagg
Nanog[1]	cacccacccatgctagtctt	accctcaaactcctggtcct
STAT3	cacaggattgatgcccaag	cgaaagtcaggttgtggtc
Sox2, Sox2 cds[2]	gagtggaaacttttgtccgaga	gaagcgtgtacttatccttcttcat
Sox2 UTR[2]	ccccttttattttccgtagttgtat	gaagcgtgtacttatccttcttcat
Rex1[3]	gagattagccccgag	cgccctgaggaagc
Dax1[4]	tcctgtaccgcatctatgtg	atctggaagcagggcaagta
Fgf4[2]	gggaggctacagacagcaag	ctgtgagccaccagacagaa
Lefty1[2]	tgtgtgtgctctttgcttcc	ggggattctgtccttggttt
Fgf5[5]	gctgtgtctcaggggattgt	cactctcggcctgtcttttc
T[5]	ctccaacctatgcggacaat	ccattgctcacagaccagag
Gata4[1]	cccttccctcttcaaattcc	cttttccagagctccacctg
Cdx2[2]	aggctgagccatgaggagta	cgaggtccataattccactca
Eomes[1]	cctggtggtgttttgttgtg	tttaatagcaccgggcactc
Elp3	acaggggccatactgtgaag	accagggttgggtagagctt
Tip60	ccaaggaaaaggaatccaca	tctgacaggggtttctcagg
Myst3	gtcattctccggctgtgact	agcagaggtgacgtgaggtt
Myst4	tctcggaacagctggaactt	tccaatccacattgcgtaga
GCNF[2]	acagtcctatgtaggaatcgaatga	catgcatttcatactaattggtcac
CouptfI[2]	agagactaagaggactctccctgac	tcctttccaatgtacttacagatca
CouptfII[2]	gtccaagacacaagctgaggt	aatcacgttacctataagtgccaac
GAPDH	tgatgacatcaagaaggtggtgaag	tccttggaggccatgtaggccat

1. Toyooka et al. (2008) Development.135, 909-918.
2. Masui et al. (2007) Nat Cell Biol.9, 625-635.
3. Ura et al. (2008) J Biol Chem. 283, 9713-9723.
4. Sun et al. (2009) Mol Cell Biol. 29, 4574-4583.
5. Shimosato et al. (2007) BMC Dev Biol. 3, 80.

Table S2. Primers for ChIP analysis

Gene name	Forward primer	Reverse primer
Eed (Sox2 binding site)[1]	gcataggaggagatttctga	cccaaaacacctctcatcgt
Eed (-382/-12)[1]	tcagaaaccggtggaaagac	tgcaaacgaacgaaagtctg
CouptfII promoter	gcatccgagatgcttcattt	caaaatgatgagtgacagacagg
SRR1 (couptfII binding site)	cataaacaccagccaccatt	ggggtctggctaggtctctt
SRR1 (-3917/-3783)	tttggaacccacagttgaca	cattccgaggaagagcagac
Oct4 promoter	cctaagggttgtcctgtcca	tcacctagggacggtttcac
Nanog promoter	ccaatgtgaagagaagcaa	tggcgatctctagtgggaag
Fgf4 promoter	ctgctgtcctgaatgtcct	gtcacactgtggcttggcta
Lefty1 promoter	ttctagacagcccctcctca	tcttgagtctgcggaggaat
Rex1 promoter	ggcatttgcataactgagca	cttggacccctccctttta
Dax1 promoter	gtgctgagactctcccttgg	cgccgcttgggacttattta
Nanog (Sox2 binding site)[2]	gtctttagatcagaggatgcccc	ctacccacccctattctccca
Fgf4 (Sox2 binding site)[2]	gggaggctacagacagcaag	ctgtgagccaccagacagaa
Lefty1 (Sox2 binding site)[2]	aagctgcagacttcattcca	cgggggatagatgaagaaac
Rex1 (Sox2 binding site)	gcgatgggacgaaagtgtaa	gggcaagactcttgctcag
Gata4 promoter[1]	taatagggccctgtgattgctc	aagcgctcttttctccttccc
T promoter[1]	gctgttgggtagggagtcaa	cagcgggaagaaacaaag
Fgf5 promoter[1]	atggggtcagagagga	aagggaaccaaaaactga
Cdx2 promoter[1]	acaatgccgacttttgaacc	acctccccagtttctccact
Eomes promoter	ttctgtattgtgccgcagag	attccctctgctcggttttt

1. Ura et al. (2008) J Biol Chem. 283, 9713-9723.
2. Masui et al. (2007) Nat Cell Biol.9, 625-635.

Figure legends

Figure S1 Expression levels of *Eed* and *Sox2* are reduced in differentiating and *Eed*-deficient ES cells. Wild-type ES cells were cultured for 0 to 6 days in the absence of LIF, and Eed1 cKO ES cells were cultured with or without Tet for 4 days. Expression levels of the indicated genes were measured by qRT-PCR.

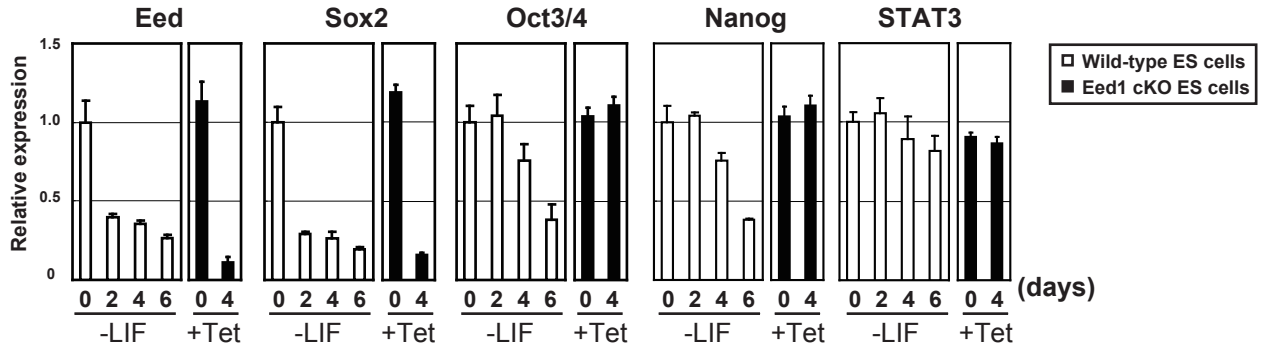
Figure S2 Eed cannot suppress ES cell differentiation induced by *Sox2* downregulation. (**A**) Morphology of *Eed*- and *Sox2*-expressing 2TS22C cells. 2TS22C cells transfected with *Eed1* or *Sox2* were cultured with or without Tet for 4 days. Scale bar = 50 μm. (**B**) Ectopic expression of *Sox2*, but not *Eed*, induces compact colony formation of *Sox2*-deficient ES cells. *Sox2*-, *Eed1*-, or *Eed4*-expressing 2TS22C cells were cultured for 4 days in the presence of Tet. The number of cell colonies was counted manually, and the ratio of compact colonies to total colonies was determined. Three independent experiments were performed and more than 100 colonies were counted in each experiment. Bars represent the means and standard deviations. (**C**) Neither Eed1, nor Eed4 suppresses the downregulation of self-renewal genes or induction of trophectodermal genes induced by *Sox2* depletion. Cells were cultured in the presence or absence of Tet for 4 days, and expression of the indicated genes was examined by qRT-PCR. Note that most Tet-treated 2TS22C ES cells differentiated into trophectoderm-like cells (Masui *et al*, 2007). (**D**) Eed restores H3K37me3 in the promoter regions of differentiation-associated genes. Cells were cultured with or without Tet for 4 days and subjected to ChIP assay using an anti-H3K37me3 antibody, followed by qPCR using primers for the promoter regions of

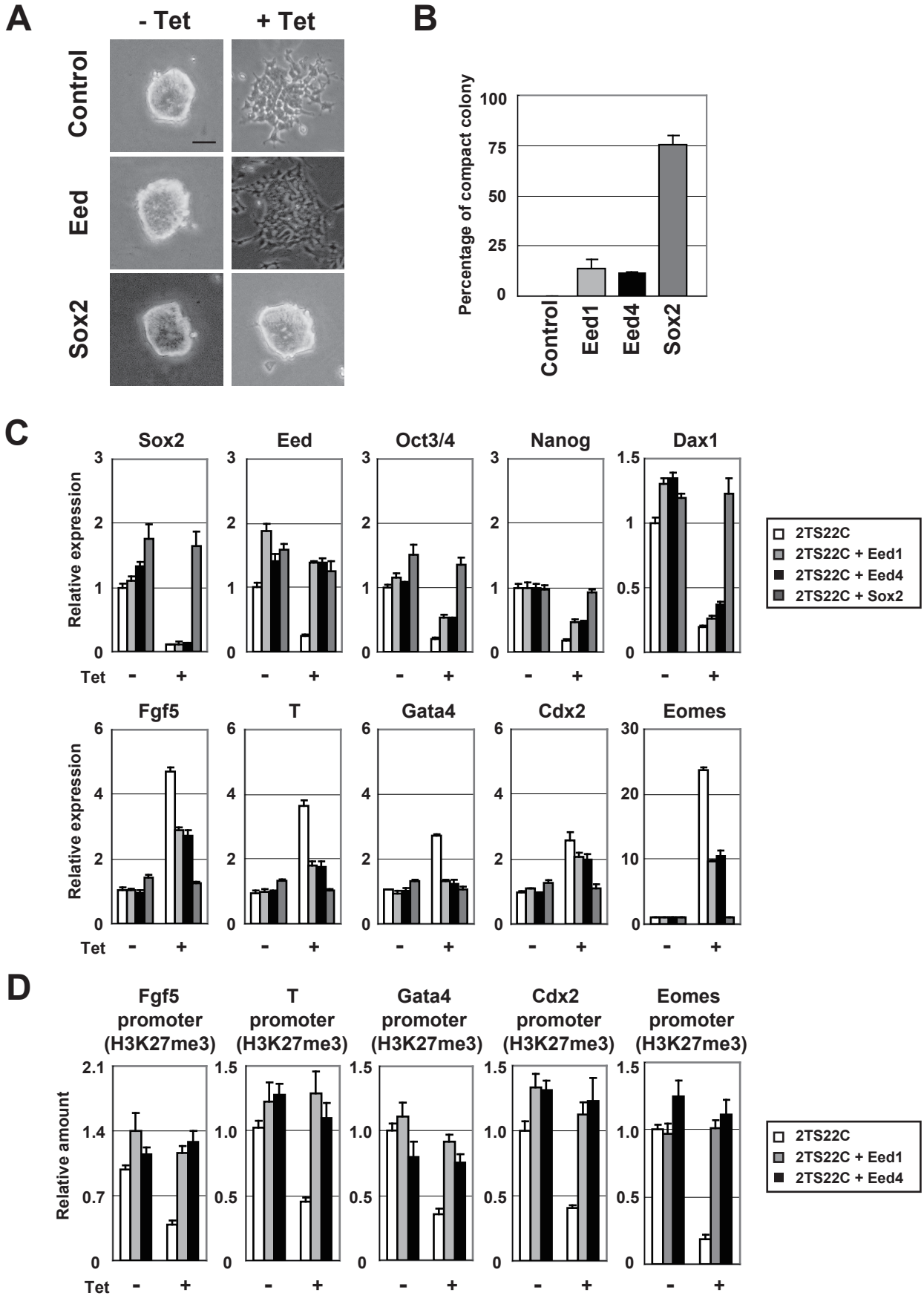
the indicated genes. It should be noted that the failure to suppress trophectodermal differentiation is not due to insufficient functional levels of *Eed* expression in 2TS22C ES cells, since the expression level of *Eed* is high enough to restore the reduced level of H3K37me3.

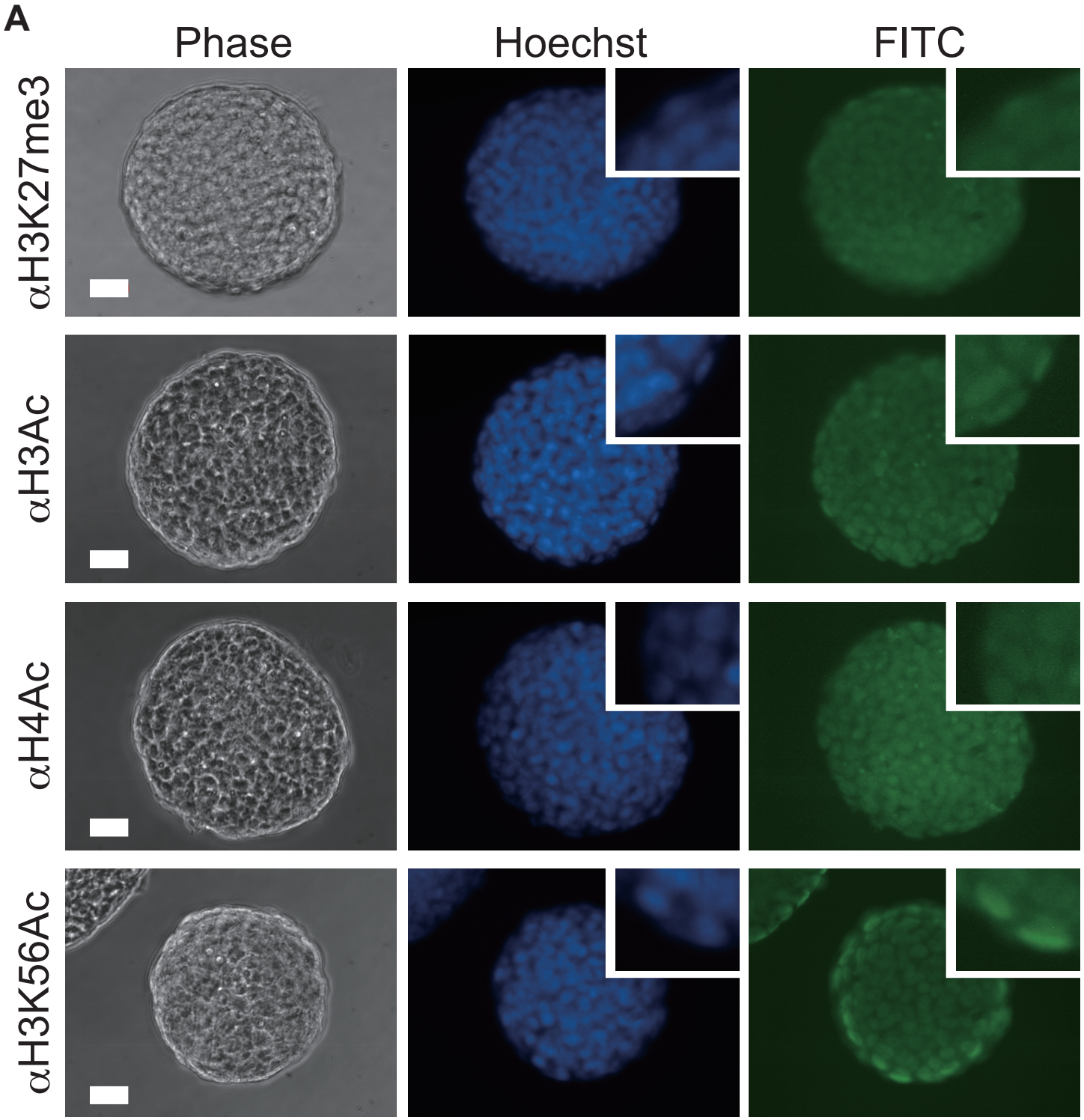
Figure S3 Sox2 promotes histone acetylation in *Eed*-deficient ES cells. (**A**) Immunostaining patterns of ES cells with antibodies against methylated or acetylated histone. Eed4 cKO ES cells were subjected to staining with Hoechst or immunostaining using anti-H3K27me3, H3Ac, H4Ac, and H3K56Ac antibodies. Inset, image with higher magnification. Note that the pattern of immunostaining shows a good correspondence with that of nuclear staining by Hoechst. Scale bar = 200 μm. (**B**) Fluorescence intensities of images shown in Figure 4A were measured using image analysis software, NIH image J.

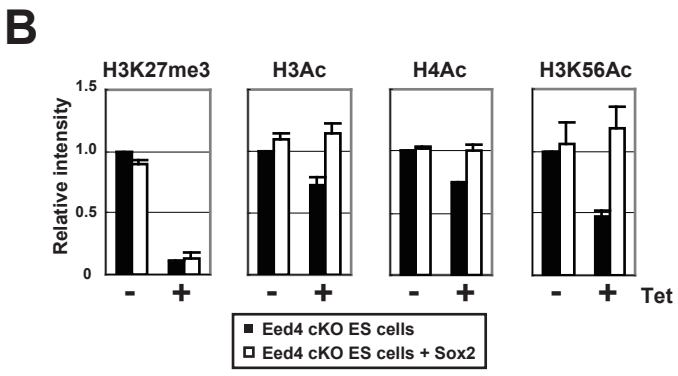
Figure S4 Elp3 and Tip60 overcome the phenotype of *Eed*-deficient ES cells.
Eed4 cKO ES cells transfected with control, Flag-Elp3, or Flag-Tip60 expression vector were cultured in the presence or absence of Tet for 4 days. (**A, B**) Histone acetylation levels are maintained in *Elp3*- or *Tip60*-expressing, *Eed*-deficient ES cells. **(A)** Fluorescence intensities of images shown in Figure 6A were measured using NIH image J. (**B**) The amounts of H3Ac and H4Ac in the indicated cells were determined by ELISA. Data are presented as fold changes relative to the untreated control sample. (**C**) Loss of H3K37me3 in the promoter regions of differentiation-associated genes in *Eed*-deficient ES cells is not restored by the expression of *Elp*3 or *Tip60*. The indicated cells were subjected to ChIP assay with an anti-H3K37me3 antibody,

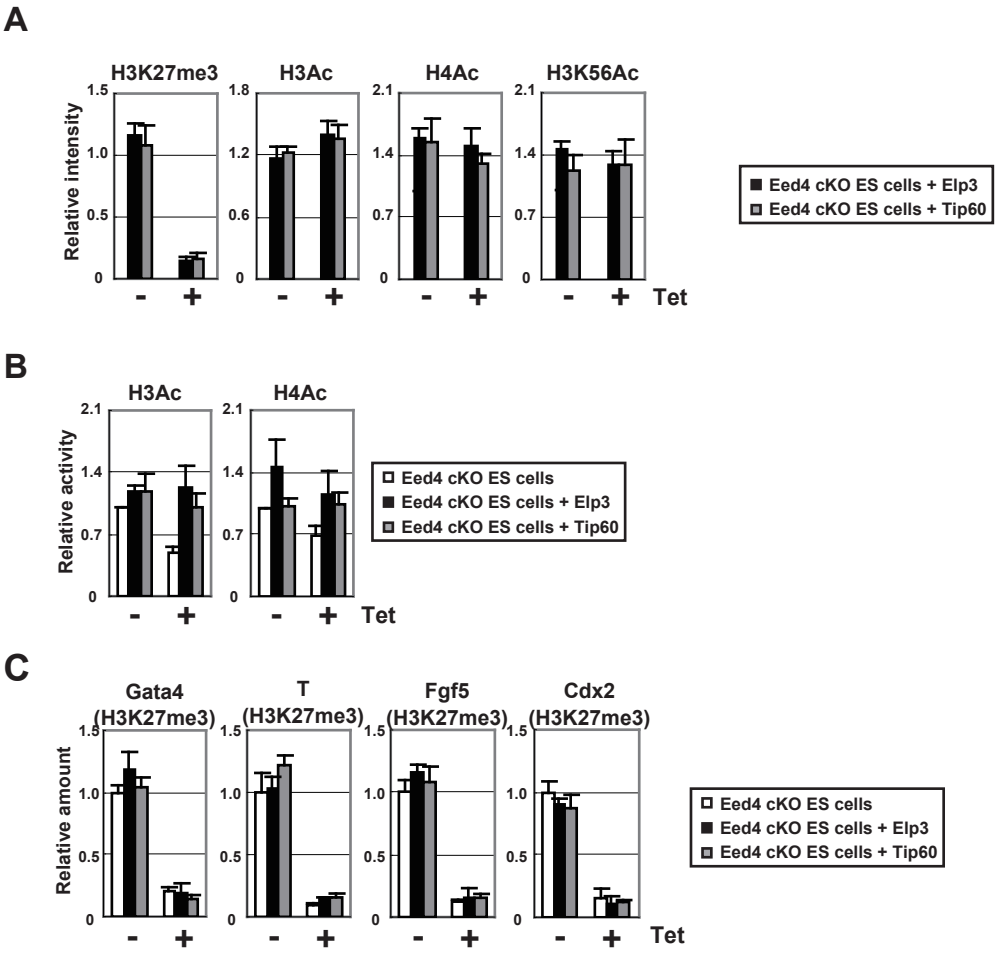
followed by qPCR using primers for the promoter regions of the indicated genes.


Eed
Sox2
Oct3/4
Nanog
STAT3
Relative expression
1.5
1.0
0.5
0
0 2 4 6 0 4
-LIF +Tet
(days)
Wild-type ES cells
Eed1 cKO ES cells


A
- Tet
+ Tet
Control
Eed
Sox2
B
Percentage of compact colony
100
75
50
25
0
Control
Eed1
Eed4
Sox2
C
Sox2
Eed
Oct3/4
Nanog
Dax1
Relative expression
Tet
- +
2TS22C
2TS22C + Eed1
2TS22C + Eed4
2TS22C + Sox2
Fgf5
T
Gata4
Cdx2
Eomes
D
Fgf5 promoter (H3K27me3)
T promoter (H3K27me3)
Gata4 promoter (H3K27me3)
Cdx2 promoter (H3K27me3)
Eomes promoter (H3K27me3)
Relative amount
2TS22C
2TS22C + Eed1
2TS22C + Eed4


A
Phase
Hoechst
FITC
αH3K27me3
αH3Ac
αH4Ac
αH3K56Ac


B
H3K27me3
H3Ac
H4Ac
H3K56Ac
Relative intensity
1.5
1.0
0.5
0
- + - + - + - +
Tet
Eed4 cKO ES cells
Eed4 cKO ES cells + Sox2


A
H3K27me3
H3Ac
H4Ac
H3K56Ac
Relative intensity
1.5
1.0
0.5
0
1.8
1.2
0.6
2.1
1.4
0.7
- + Tet
Eed4 cKO ES cells + Elp3
Eed4 cKO ES cells + Tip60
B
H3Ac
H4Ac
Relative activity
2.1
1.4
0.7
0
Eed4 cKO ES cells
Eed4 cKO ES cells + Elp3
Eed4 cKO ES cells + Tip60
C
Gata4 (H3K27me3)
T (H3K27me3)
Fgf5 (H3K27me3)
Cdx2 (H3K27me3)
Relative amount
1.5
1.0
0.5
0