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

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Abstract

Transcription factors and epigenetic modulators are involved in the maintenance of self-renewal in embryonic stem (ES) cells. Here, we demonstrate the existence of a regulatory loop in ES cells between Sox2, an indispensable transcription factor for self-renewal, and Eed, an epigenetic modulator regulating histone methylation. We found that Sox2 and Eed positively regulate each other's expression. Interestingly, *Sox2* overexpression suppressed the induction of differentiation-associated genes in *Eed*-deficient ES cells without restoring histone methylation. This Sox2-mediated suppression was prevented by knockdown of the histone acetyltransferase (HAT), *Tip60* or *Elp3*, and Sox2 stimulated expression of these HATs. Furthermore, forced expression of either HAT resulted in repression of differentiation-associated genes in *Eed*-deficient cells. These results suggest that Sox2 overcame the phenotype of *Eed*-deficient ES cells by promoting histone acetylation. We also found that knockout of Eed and knockdown of these HATs synergistically enhanced the upregulation of differentiation-associated genes in ES cells. Taken together, our results suggest that the Eed/Sox2 regulatory loop contributes to the maintenance of self-renewal in ES cells by controlling histone methylation and acetylation.

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of the mammalian blastocyst (Evans and Kaufman, 1981; Martin, 1981). Mouse ES cells can self-renew and maintain their pluripotency when cultured in the presence of leukemia inhibitory factor (LIF). Several transcription factors play important roles in the self-renewal capacity of mouse ES cells (Niwa, 2007). STAT3 is a downstream transcription factor activated by LIF and is essential and sufficient for the maintenance of self-renewal (Niwa *et al*, 1998; Matsuda *et al*, 1999; Ying *et al*, 2008). Nanog is a homeobox transcription factor whose overexpression can bypass the requirement of LIF for self-renewal, although it is dispensable for self-renewal (Chambers *et al*, 2003; Mitsui *et al*, 2003; Chambers *et al*, 2007). The POU family transcription factor Oct3/4 plays a central role in ES cell self-renewal and ICM production (Nichols *et al*, 1998; Niwa *et al*, 2000). Another indispensable transcription factor is the SRY-related HMG-box protein Sox2. *Sox2*-deficient mouse embryos die shortly after implantation (Avilion *et al*, 2003), and a study using inducible *Sox2*-deficient ES cells revealed that Sox2 stabilizes ES cells in a pluripotent state by maintaining the requisite level of *Oct3/4* expression (Masui *et al*, 2007). It has been suggested that these transcription factors form networks and stimulate the expression of a set of self-renewal genes to

maintain the "stemness" of ES cells (Boyer *et al*, 2005; Loh *et al*, 2006; Chen *et al*, 2008).

In addition to transcription factors, histone modifiers also play an important role in ES cell self-renewal. The chromatin of self-renewing ES cells exhibits a characteristic structure of increased accessibility due to fewer and more loosely bound histones and architectural proteins (Meshorer and Misteli, 2006). When ES cells undergo differentiation, their chromatin structure changes dynamically in response to global histone modifications. Histone modifications have been shown to regulate gene activation and repression during development (Kouzarides, 2007). For example, acetylation of various residues of histone H3 (H3Ac) and histone H4 (H4Ac) are involved in transcriptional activation, whereas methylation of Lys-27 of histone H3 (H3K27me) is linked to transcriptional silencing.

Polycomb group (PcG) proteins are histone modifying proteins that participate in transcriptional repression. Three PcG proteins, enhancer of zeste 2 (Ezh2), embryonic ectoderm development (Eed) and suppressor of zeste 12 homolog (Suz12), comprise the core of the Polycomb repressive complex 2 (PRC2), which mediates H3K27me (Cao *et al*, 2002; Czermin *et al*, 2002; Kuzmichev *et al*, 2002; Müller *et al*, 2002). Ezh2 is a SET domain-containing histone methyltransferase and functions as

the catalytic subunit of PRC2. Eed exists in four isoforms (Eed1, Eed2, Eed3 and Eed4), which arise from alternate translation initiation sites in the same mRNA (Kuzmichev *et al*, 2004), and plays a crucial role in boosting the enzymatic activity of Ezh2. Finally, Suz12 is involved in nucleosome binding of PRC2 (Nekrasov *et al*, 2005). Genome-wide location analysis in ES cells has revealed that many PcG target genes encode transcription factors important in development (Boyer *et al*, 2006). In fact, mouse embryos deficient for *Suz12*, *Ezh2*, or *Eed* displayed embryonic lethality with gastrulation arrest (Faust *et al*, 1998; O'Carroll *et al*, 2001; Pasini *et al*, 2004), underscoring the importance of these PcGs in early embryogenesis.

PcG proteins are also involved in the repression of differentiation-associated genes in self-renewing ES cells. Previously, we and others showed that *Eed*-deficient ES cells exhibit de-repression of multiple differentiation-associated genes and are prone to differentiation (Boyer *et al*, 2006; Chamberlain *et al*, 2008; Ura *et al*, 2008). In wild-type ES cells, H3K27me accumulated in the promoter regions of differentiation-associated genes. In contrast, the H3K27me accumulation disappeared and multiple differentiation-associated genes were upregulated in *Eed*-deficient ES cells. Moreover, some self-renewal genes were downregulated, although the cells still proliferated and expressed other self-renewal genes, including Oct3/4. These findings

suggest that the molecular characteristics that define "stemness" are slightly but significantly decreased in *Eed*-deficient ES cells. Similarly, *Suz12*-deficient ES cells exhibited decreased H3K27me with increased expression of differentiation-associated genes (Pasini *et al*, 2007). *Ezh2*-deficient ES cells also exhibited reduced H3K27me, although differentiation-associated genes were not upregulated due to compensation by Ezh1 (Shen *et al*, 2008).

During ES cell differentiation, the downregulation of self-renewal genes and upregulation of differentiation-associated genes should occur in a simultaneous and coordinated manner. It is expected, therefore, that highly regulated cross-talk(s) may exist between transcription factors that stimulate the expression of self-renewal genes and PcG proteins that suppress the expression of differentiation-associated genes. Here, we report the discovery of cross-talk between Sox2 and Eed. We determined that these molecules each positively regulate the other's expression through a regulatory feedback loop. We also found that Sox2 blocks the induction of differentiation-associated genes in *Eed*-deficient ES cells through the upregulation of self-renewal genes by binding to their promoters and/or triggering histone acetylation. These data suggest that the Eed/Sox2 regulatory loop regulates self-renewal and differentiation of ES cells by controlling histone methylation and acetylation.

Results

Eed is a downstream target of Sox2

It has been suggested that the expression of differentiation-associated genes is repressed by PRC2-mediated H3K27me in self-renewing ES cells, but de-repressed by the loss of PRC2 activity in differentiating ES cells (Boyer *et al*, 2006; Lee *et al*, 2006; Pasini *et al*, 2007; Shen *et al*, 2008; Ura *et al*, 2008). In fact, a reduction of trimethylation of Lys-27 of histone H3 (H3K27me3) was observed in differentiating ES cells (Figure 1A), suggesting that PRC2 activity is reduced during ES cell differentiation. Among the three major components of PRC2, a decrease in *Eed* mRNA occurs earlier than in Ezh2 or Suz12 (Ura *et al*, 2008). Disruption of the *eed* gene resulted in the loss of H3K27me3 (Montgomery *et al*, 2005; Chamberlain *et al*, 2008; Ura *et al*, 2008), and overexpression of *Eed* was able to suppress the reduction of H3K27me3 during differentiation (Figure 1A). Taken together, these results suggest that the expression level of *Eed* determines the level of H3K27me3 during ES cell differentiation, and raise the possibility that *Eed* expression is strictly regulated by self-renewal transcription factors.

Among the three indispensable transcription factors for ES cell self-renewal, STAT3 and Oct3/4 have already been shown to directly regulate *Eed* expression (Ura *et*

al, 2008). Using 2TS22C ES cells, we examined whether Sox2 also regulates *Eed* expression. As reported previously by Masui *et al* (2007), *Sox2* expression in this cell line can be regulated by addition of tetracycline (Tet). Upon Tet treatment, the expression level of *Sox2* rapidly decreased, but recovered following Tet removal (Figure 1B). Similarly, expression of *Eed* was downregulated by Tet stimulation and recovered after Tet withdrawal. In agreement with *Eed* downregulation, the overall amount of H3K27me3 was diminished in Tet-treated 2TS22C ES cells (Figure 1C). These results suggest that Eed is downstream of Sox2. Interestingly, we found that the opposite is also true: the expression level of *Sox2* was reduced when *Eed* expression was suppressed by Tet treatment in Eed conditional knockout (cKO) ES cells (Ura *et al*, 2008), but was restored by Tet removal with re-expression of *Eed* (Figure 1D). These findings suggest the intriguing possibility that the self-renewal promoting transcription factor Sox2 and the differentiation-suppressing, epigenetic regulator Eed engage in tightly regulated cross-talk and form a regulatory loop in ES cells. In contrast, expression of *Stat3* and *Oct3/4* was not affected by *Eed* downregulation (Supplementary Figure S1). Therefore, the primary focus of this study is the relationship between Eed and Sox2.

First, it was determined whether Eed is a direct target of Sox2. The 2.6-kb

upstream region (-2600/-13) of the *eed* gene contains STAT3- and Oct3/4-binding sites (Ura *et al*, 2008). Since Sox2 often binds to a sequence adjacent to an Oct3/4-binding site, we searched for a putative Sox2 sequence near the Oct3/4-binding site (-2019/-2012) and found one such sequence (5'-AACAACAG-3') at -2037/-2030 (Figure 1E). A luciferase assay was then performed using the 2.6-kb upstream region to determine if the identified putative site is an authentic Sox2-binding site. As shown in Figure 1F, promoter activity of the 2.6-kb region was stimulated by the presence of Sox2, Oct3/4, or STAT3, but not by Nanog, suggesting that this region contains a Sox2-responsive element in addition to Oct3/4- and STAT3-responsive elements. Disruption of the putative Sox2-binding site by mutagenesis reduced promoter activity, which was further decreased when combined with additional mutations at the Oct3/4- and/or STAT3-binding sites (Figure 1G). Similarly, when *Sox2* was downregulated in 2TS22C ES cells, the promoter activity of the 2.6-kb region was reduced to a level comparable to that of the mutant lacking the Sox2-binding site (Figure 1H). Importantly, the promoter activity of the mutant 2.6-kb region was not reduced further by *Sox2* downregulation. These results suggest that Sox2 stimulates *Eed* expression through the Sox2-binding site at -2037/-2030.

To confirm the *in vivo* binding of Sox2 to the promoter region of the *eed* gene,

chromatin immunoprecipitation (ChIP) analysis was carried out. A DNA fragment (-2250/-1882) containing the identified Sox2-binding site in the *eed* gene was precipitated by anti-Flag antibody from *Flag-Sox2*–expressing ES cells, but not from control ES cells (Figure 1I). In contrast, another region (-382/-12) of the *eed* gene, which does not contain a Sox2-binding site, did not precipitate from *Flag-Sox2*–expressing ES cells. Taken together, these results indicate that Eed is directly regulated by Sox2 in ES cells.

Eed positively regulates Sox2 expression through repression of COUP-TFII

We next examined how *Sox2* expression is positively regulated by Eed, an epigenetic regulator that usually suppresses expression of its target genes. The *sox2* gene contains two enhancer regions, SRR1 and SRR2 (Tomioka *et al*, 2002) (Figure 2A). Withdrawal of LIF from the culture medium resulted in the rapid repression of *Sox2* expression (Supplementary Figure S1), as well as a reduction in the enhancer activities of SRR1 and SRR2 (Figure 2B), suggesting that these regions act as regulatory elements of *Sox2* expression in ES cells. Activity of these regions was also reduced in Eed cKO ES cells treated with Tet (herein referred to as *Eed*-deficient ES cells; Figure 2C). These results suggest that Eed regulates *Sox2* expression through the SRR1 and

SRR2 regions.

SRR2 contains one Sox2/Oct3/4-binding element and this element has been identified as a core element of SRR2 (Tomioka *et al*, 2002). Consistent with this, SRR2 containing mutations at the Sox2/Oct3/4-binding site (SRR2mt) showed negligible enhancer activity (Figure 2B and C), suggesting that the reduced enhancer activity of SRR2 in *Eed*-deficient cells was likely due to the downregulation of *Sox2*.

Next, SRR1 was analyzed for an Eed-responsive element. Deletion analysis revealed that the enhancer activities of the -2957/-2493 and -4074/-2685 regions of SRR1 were closely correlated with the expression level of *Eed* (Figure 2D), suggesting that an Eed-responsive element exists in the -2957/-2685 region of SRR1. This region contains a consensus-like sequence (5'-AGACCT-3') found in the binding site for the transcriptional repressors GCNF, COUP-TFI and COUP-TFII. Interestingly, the enhancer activity of an SRR1mt with mutations in this sequence was not affected by *Eed* downregulation (Figure 2D), suggesting that this putative binding site is the Eed-responsive element in SRR1.

These findings prompted exploration of the possibility that expression of *Sox2* is repressed by GCNF, COUP-TFI and/or COUP-TFII in *Eed*-deficient ES cells. We first examined whether Eed regulates expression of these repressors. Expression

analysis using Eed cKO ES cells revealed that *Eed* downregulation resulted in the induction of each of the repressors, as well as the downregulation of *Sox2* expression (Figure 2E), suggesting that the three repressors are downstream molecules of Eed. To identify the repressor(s) that binds to the Eed-responsive element, we compared the ratio of enhancer activity of SRR1 to that of SRR1mt (SRR1/SRR1mt) among the three repressors (Figure 2F). Forced expression of COUP-TFII significantly reduced SRR1/SRR1mt, suggesting that COUP-TFII represses the activity of SRR1 through the Eed-responsive element. On the other hand, neither GCNF nor COUP-TFI had an effect on this ratio. These results suggest that COUP-TFII binds to the Eed-responsive element in SRR1 to suppress *Sox2* expression in *Eed*-deficient ES cells. This interpretation is supported by the additional observation that the enhancer activity of SRR1 was not reduced in *Eed*-deficient ES cells when *COUP-TFII* was knocked down (Figure 2G). Furthermore, the expression level of *Sox2* in *Eed*-deficient ES cells was increased by knockdown of *COUP-TFII* (Figure 2H), and COUP-TFII was shown to bind to SRR1 *in vivo* (Figure 2I). We also observed that Eed, as well as K27-methylated histone H3, was associated with the promoter region of the *coup-tfII* gene, and both associations disappeared when *Eed* was repressed (Figure 2J). Taken together, these findings indicate that Eed positively regulates *Sox2* expression through

suppression of *COUP-TFII*, which binds to SRR1 and represses *Sox2* expression.

Sox2 overcomes the phenotype of Eed-deficient ES cells

The downregulation of *Sox2* in *Eed*-deficient ES cells (Figure 1D) prompted investigation of whether Sox2 can rescue the phenotype of *Eed*-deficient ES cells. When treated with Tet, Eed cKO ES cells underwent a morphological change and disruption of compact colony formation (Figure 3A and B). However, many *Sox2*-expressing, *Eed*-deficient ES cells formed compact colonies even in the presence of Tet. In addition, the expression of self-renewal genes (*Nanog*, *Rex1*, *Dax1, Fgf4* and *Lefty1)* was downregulated by Tet treatment, but was restored by *Sox2* overexpression (Figure 3C). Furthermore, induction of differentiation-associated genes (*Gata4*, *T*, *Fgf5* and *Cdx2*) in *Eed*-deficient ES cells was suppressed by Sox2 overexpression. In contrast, *Eed* overexpression appeared to have negligible effect on the phenotype of *Sox2*-deficient ES cells, as determined by cellular morphology and gene expression (Supplementary Figure S2).

Since H3K27me3 is absent in *Eed*-deficient ES cells, we next examined whether Sox2 expression can restore the loss of H3K27me3. Immunocytochemistry and western blot analysis revealed that the overall amount of H3K27me3 was reduced

in *Eed*-deficient ES cells, and *Sox2* overexpression had no effect on this reduction (Figure 4A and B; Supplementary Figure S3). Moreover, no increase in H3K27me3 in the promoter regions of differentiation-associated genes was detected in *Sox2*-expressing, *Eed*-deficient ES cells (Figure 4C). These results suggest that although Sox2 cannot restore H3K27me3, Sox2 can compensate for the absence of H3K27me3 in *Eed*-deficient ES cells.

Sox2 promotes histone acetylation in Eed-deficient ES cells

Next, we explored the molecular mechanism of how Sox2 suppresses the downregulation of self-renewal genes and the induction of differentiation-associated genes in *Eed*-deficient ES cells in an H3K27me3-independent manner. Genome-scale ChIP-chip analyses revealed previously that Sox2 binds to the promoter regions of many self-renewal genes, probably to stimulate their expression (Boyer *et al*, 2005; Chen *et al*, 2008). Since *Eed* deficiency leads to the downregulation of *Sox2* expression, the effect of *Eed* deficiency on Sox2 binding to the promoter regions of target genes was examined. We performed a ChIP assay for several known Sox2 target genes and found that the binding of Sox2 to the promoter regions was indeed reduced in *Eed*-deficient cells (Figure 3D). On the other hand, overexpression of *Sox2* restored

the amount of promoter-bound Sox2.

The involvement of histone acetyltransferases (HATs) in the self-renewal of ES cells has been suggested previously (Fazzio *et al*, 2008). We therefore examined the effect of Sox2 on histone acetylation, including H3Ac, H4Ac and acetylation at Lys-56 of histone H3 (H3K56Ac). Interestingly, immunostaining suggested that the overall amounts of H3Ac, H4Ac and H3K56Ac were reduced in *Eed*-deficient ES cells, while levels were maintained in *Sox2*-expressing, *Eed*-deficient ES cells (Figure 4A). Multiple quantitative analyses confirmed that levels of H3Ac, H4Ac and H3K56Ac were increased by Sox2 overexpression (Figure 4B and D). Furthermore, ChIP analysis demonstrated that reduced levels of H3Ac, H4Ac and H3K56Ac at the promoter regions of self-renewal genes in *Eed*-deficient ES cells were restored by *Sox2* overexpression (Figure 4E-G). These results suggest that Sox2 promotes histone acetylation in the promoter regions of self-renewal genes in *Eed*-deficient ES cells.

Sox2 positively regulates expression of HATs

Because *Sox2* overexpression maintained histone acetylation levels in *Eed*-deficient ES cells, the relationship between Sox2 and histone acetylation was further investigated. As shown in Figure 5A, western blot analysis revealed that overall levels of H3Ac,

H4Ac and H3K56Ac decreased in *Sox2*-deficient ES cells (*i.e.,* Tet-treated 2TS22C ES cells). ELISA also confirmed the reduction of H3Ac and H4Ac in *Sox2*-deficient ES cells (Figure 5B). These results suggest that Sox2 regulates histone acetylation in ES cells.

Analysis using GEO Profiles (http://www.ncbi.nlm.nih.gov/sites/entrez?db=geo) revealed that several HATs, including Elp3, Tip60, Myst3 and Myst4, are expressed in undifferentiated ES cells. We therefore examined whether these HATs are downstream molecules of Sox2 in ES cells. When LIF was withdrawn from the culture medium, *Sox2* expression was downregulated in ES cells, followed by the downregulation of *Elp3*, *Tip60*, *Myst3* and *Myst4* (Figure 5C), as well as a reduction in H3Ac, H4Ac and H3K56Ac (Figure 5A and B). When *Sox2* expression was downregulated by Tet treatment in 2TS22C ES cells, expression of these HATs was also downregulated, and this effect was reversed by the removal of Tet (Figure 5D). These results suggest that Elp3, Tip60, Myst3 and Myst4 are downstream targets of Sox2. Similarly, expression levels of these HATs decreased when *Eed* expression was suppressed and were restored after re-expression of *Eed* (Figure 5E). Moreover, reduced expression of HATs in *Eed*-deficient ES cells was restored by *Sox2* overexpression (Figure 5F). These data suggest that Eed regulates

the expression of *Elp3*, *Tip60*, *Myst3* and *Myst4* by controlling *Sox2* expression.

Role of histone acetylation in Sox2 activity in Eed-deficient ES cells

To evaluate the importance of histone acetylation in Sox2-mediated compensation for *Eed* deficiency, we first examined whether overexpression of HATs had an effect similar to that of Sox2 on *Eed*-deficient ES cells. First, *Elp3*- or *Tip60*-expressing Eed cKO ES cells were generated. An overall reduction in the amount of H3K27me3 in *Eed*-deficient ES cells was not reversed by *Elp3*- or *Tip60*-overexpression (Figure 6A and B). However, global levels of H3Ac, H4Ac and H3K56Ac were maintained in *Elp3*- or *Tip60*-expressing, *Eed*-deficient ES cells, and were comparable to those in undifferentiated control cells (Figure 6A and B; Supplementary Figure S4A and B).

When the effect of HAT expression on ES cell self-renewal was examined, more than half of the *Elp3*- or *Tip60*-expressing, *Eed*-deficient ES cells formed compact colonies (Figure 6A and C). Elp3 and Tip60 restored decreased expression of self-renewal genes and repressed the induction of differentiation-associated genes in *Eed*-deficient ES cells (Figure 6D). Importantly, expression of *Sox2* was still repressed in either *Elp3*- or *Tip60*-expressing, *Eed*-deficient ES cells, suggesting that the Elp3- or Tip60-mediated effect is independent of Sox2. As expected, loss of H3K27me3 at the

locus of differentiation-associated genes was not restored in *Elp3*- or *Tip60*-expressing, *Eed*-deficient ES cells (Supplementary Figure S4C), while a reduction in histone acetylation at the promoter regions of self-renewal genes in *Eed*-deficient ES cells was reversed by either *Elp3* or *Tip60* expression (Figure 6E-G). These results suggest that, similar to Sox2, Elp3 and Tip60 can compensate for the phenotype of *Eed*-deficient ES cells.

It was next determined whether the expression of HATs is required for Sox2 activity in *Eed*-deficient ES cells. When either *Elp3* or *Tip60* was knocked down, the expression of self-renewal genes was repressed in *Sox2*-expressing, *Eed*-deficient ES cells (Figure 7A). In addition, expression of differentiation-associated genes was increased in *Elp3*- or *Tip60*-knockdown cells, suggesting that expression of HATs is required for Sox2-mediated compensation for the loss of H3K27me3 in *Eed*-deficient ES cells.

H3K27me and histone acetylation are important for ES cell stemness

Finally, the relationship between histone acetylation and H3K27me in the regulation of self-renewal and differentiation-associated genes in ES cells was investigated (Figure 7B). As described above, either knockout of *Eed* or knockdown of *Elp3* or *Tip60*

reduced expression of self-renewal genes and increased expression of the differentiation-associated genes. When the two were combined, downregulation of self-renewal genes and upregulation of differentiation-associated genes were further enhanced, suggesting that both H3K27me and histone acetylation are required for maintenance of self-renewal genes and repression of differentiation-associated genes.

Discussion

STAT3, Oct3/4 and Sox2 have been shown to be indispensable for self-renewal of mouse ES cells. We demonstrated previously that STAT3 and Oct3/4 directly regulate the expression of *Eed* (Ura *et al*, 2008). In the present study, we showed that Sox2 also directly binds to the promoter region of the *eed* gene and positively regulates *Eed* expression. Therefore, it is likely that *Eed* expression in ES cells is strictly regulated by these three crucial factors. On the other hand, *Sox2* expression is regulated through SRR1 and SRR2 (Tomioka *et al*, 2002). Although it has been shown that SRR2 contains Oct3/4- and Sox2-binding sites and is regulated by these factors, the regulatory mechanism of SRR1 has not been elucidated yet. Our current study demonstrated that SRR1 contains a COUP-TFII-binding site, through which COUP-TFII can repress the enhancer activity of SRR1. We also found that Eed negatively controls *COUP-TFII* expression. These results indicate that SRR1 is positively regulated by Eed through suppression of *COUP-TFII*.

SRR2 has been shown to possess higher enhancer activity than SRR1 in undifferentiated embryonal carcinoma cells (Tomioka *et al*, 2002). Similarly, Figure 2B shows that SRR2 has much higher activity than SRR1 in ES cells. In addition, SRR2 is positively regulated by two key transcription factors, Oct3/4 and Sox2. It is

likely, therefore, that SRR2 plays a dominant role in induction of *Sox2* expression in ES cells. On the other hand, SRR1 contains a suppressive site that negatively controls the enhancer activity, such as the COUP-TFII binding site, suggesting that SRR1 is involved in regulation of *Sox2* expression. For example, we speculate that SRR1 region plays an important role in initiation of *Sox2* downregulation during ES cell differentiation. Although it is well established that Oct3/4 regulates *Sox2* expression, reduction in *Sox2* expression occurs earlier than that of *Oct3/4* during ES cell differentiation (Supplementary Figure S1), suggesting that *Sox2* downregulation during differentiation is not triggered by *Oct3/4* repression. Considering that *Eed* is downregulated during the early phase of differentiation (Supplementary Figure S1), suggestion of SRR1 by COUP-TFII, which is caused by *Eed* downregulation, appears to be the initial step of *Sox2* downregulation.

Several studies have suggested the importance of histone acetylation in ES cell self-renewal. Global reduction of acetylated histones is observed during differentiation of ES cells, and inhibition of histone deacetylase activity prevents differentiation (Lee *et al*, 2004). In human ES cells, approximately 1% of histone H3 is acetylated at Lys-56, and H3K56Ac is frequently observed in the promoter regions of self-renewal genes (Xie *et al*, 2009). In the present study, we demonstrated that total

levels of H3Ac, H4Ac and H3K56Ac were restored by *Sox2* overexpression in *Eed*-deficient ES cells. Sox2 also restored histone acetylation in the promoter regions of self-renewal genes. Furthermore, knockdown of HATs cancelled the effect of Sox2 without significant reduction of *Sox2* expression, and expression of HATs exhibited an effect similar to that of Sox2 on the phenotype of *Eed*-deficient cells. Taken together, these results indicate that Sox2 overcomes the loss of H3K27me by stimulating HAT activity.

As candidate downstream targets of Sox2, we identified the HATs Elp3 and Tip60. Elp3 is the catalytic subunit of a histone acetyltransferase elongator complex (Svejstrup, 2007), and Tip60 is a subunit of a chromatin remodeling complex, the Tip60-p400 complex, which is involved in DNA damage response and cell cycle regulation (Squatrito *et al*, 2006). Both HATs were downregulated when *Sox2* expression was reduced, and their expression was recovered when *Sox2* expression was restored. In addition, reduced expression levels of these HATs in *Eed*-deficient cells were restored by Sox2 expression. These results suggest that Sox2 promotes HAT activity in *Eed*-deficient ES cells by upregulating *Tip60* and *Elp3*. This hypothesis is supported by recent reports suggesting that Tip60 is involved in ES cell self-renewal (Fazzio *et al*, 2008; Hu *et al*, 2009).

Analyses using *Eed*- or *Suz12*-deficient ES cells have revealed that although H3K27me is important for repression of differentiation-associated genes, loss of H3K27me is not sufficient for complete differentiation of ES cells (Montgomery *et al*, 2005; Azuara *et al*, 2006; Bernstein *et al*, 2006; Chamberlain *et al*, 2008; Ura *et al*, 2008). One possible explanation for the incomplete differentiation of H3K27me-deficient ES cells is that H3K27me plays an important role in ES cell differentiation (Pasini *et al*, 2007). However, the data presented here show that knockdown of *Elp3* or *Tip60* further enhanced repression of self-renewal genes and induction of differentiation-associated genes in *Eed*-deficient ES cells. These observations suggest the possibility that ES cell differentiation requires the reduction of histone acetylation in addition to the loss of H3K27me.

Several reports have described the phenotype of *Eed*-deficient ES cells (Morin-Kensicki *et al,* 2001; Montgomery *et al*, 2005; Azuara *et al*, 2006; Boyer *et al*, 2006; Schoeftner *et al*, 2006; Chamberlain *et al*, 2008; Shen et al., 2008; Ura *et al*, 2008). Basically, all the reports including ours have reached the same conclusion. For example, H3K27me is lost by *Eed* deficiency. Although some differentiation-associated genes are upregulated and some self-renewal genes are downregulated, *Eed*-deficient ES cells can be maintained in culture. The phenotype of

Eed deficiency is reversible (*Ura et al*, 2008). *Eed*-deficient ES cells contribute to all lineages in chimeric embryos (Morin-Kensicki *et al,* 2001; Chamberlain *et al*, 2008). Taken together, these observations indicate that Eed is dispensable for ES cell self-renewal and pluripotency. The observed discrepancies in the level of some marker genes among reports are probably due to difference in culture condition, clonal variation and/or adaptation. Despite dispensability, the fact that several differentiation-associated genes are upregulated by *Eed* deficiency indicates the importance of Eed in maintenance of "complete" self-renewal.

Recently, it was reported that ES cells comprise a heterogeneous population (Toyooka *et al*, 2008), suggesting the possibility that the observed change of gene expression may occur in not all but a portion of *Eed*-deficient ES cells. Although immunostaining has suggested that an endodermal marker Gata4 is upregulated in most of *Eed*-null ES cells (Boyer *et al,* 2006), further detailed analyses, such as a single-cell PCR analysis, should be done to clarify this point.

In this study, we discovered a regulatory loop between Eed and Sox2 in ES cells. The existence of this loop allows us to hypothesize the following model for the molecular mechanism of ES cell self-renewal (Figure 7C). In self-renewing ES cells, Eed represses the expression of differentiation-associated genes through H3K27me, and

Sox2 positively regulates self-renewal genes through direct binding and histone acetylation. Eed and Sox2 positively feedback to each other and maintain both H3K27me and histone acetylation at high levels. The finding that Sox2 can inhibit the induction of differentiation-associated genes in *Eed*-deficient ES cells without increasing H3K27me3 suggests that self-renewal gene products can somehow suppress the induction of differentiation-associated genes, even in the absence of H3K27me. Upon differentiation, the Eed/Sox2 loop is inactivated and both H3K27me and histone acetylation are downregulated simultaneously, leading to upregulation of differentiation-associated genes and downregulation of self-renewal genes. This mechanism allows the expression of self-renewal and differentiation-associated genes to be coordinately regulated in ES cells.

The discovery of the Eed/Sox2 regulatory loop also raises the intriguing possibility that the differentiation process in ES cells may consist of two stages. In the first stage, the Eed/Sox2 regulatory loop is inactivated even though Oct3/4 expression is maintained, and ES cells may begin to lose stemness and stay in an "incomplete" differentiated state. In the second stage, "complete" differentiation is accomplished by *Oct3/4* downregulation. We will explore these possibilities in future studies.

Materials and methods

Cell culture and plasmid transfection

ES cell lines, A3-1 (Azuma and Toyoda, 1991), 2TS22C (Masui *et al*, 2007) and Eed cKO, were cultured on gelatin-coated dishes with Dulbecco's modified Eagle's medium (DMEM), as described previously (Ura *et al*, 2008). Eed1 cKO and Eed4 cKO ES cells are *Eed*-null ES cells that express Myc-tagged Eed1 and Eed4 isoforms, respectively, under the control of "Tet-Off" system (Ura *et al*, 2008). Sox2- or HATs-expressing Eed cKO ES cells were established by introducing pCAGIHisDR-Flag-Sox2, -Elp3, or -Tip60 into Eed cKO ES cells, and cultured in the presence of 3 mM histidinol (Sigma, St Louis, MO, USA). Human embryonal kidney (HEK) 293 cells were cultured in DMEM containing 10% fetal bovine serum. Plasmids were introduced into cultured cells by lipofection with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or calcium phosphate-mediated transfection. The medium was replaced with fresh medium one day after transfection and samples were analyzed two days after transfection.

Plasmid construction

Construction of the expression vectors pCAG-IP, pCAGIP-STAT3, pCAGIP-mycOct3/4, pCAGIP-mycNanog, pCAGIP-mycGCNF, and pCAGIP-mycCOUP-TFI was described previously (Yoshida-Koide *et al*, 2004; Akagi *et al*, 2005; Kinoshita *et al*, 2007; Takao *et al*, 2007). The expression vector pCAG-IHisDR was constructed by replacing the IRES-puromycin resistance-poly A cassette of pCAG-IP with the IRES-histidiol resistance-DsRedT4-poly A cassette of pBRCAG-cHA-IRES-HisDsRedT4. The coding regions of mouse Sox2, COUP-TFII, Elp3 and Tip60 were amplified by PCR using a cDNA library of A3-1 cells as a template with the following primers: 5'- GAA TTC ATG TAT AAC ATG ATG GAG ACG-3' and 5'- GCG GCC GCT CAC ATG TGC GAC AGG GGC AG-3' for Sox2, 5'-GCG GCC GCT TAT TGA ATT GCC ATA TAT GGC CAG TTA AAA CTG CTG CCG-3' and 5'-GCG GCC GCT TAT TGA ATT GCC ATA TAT GGC CAG TTA AAA CTG CTG CCG-3' for COUP-TFII, 5'-GAA TTC ATG AGG CAA AAG AGG AAA GG-3' and 5'-GCG GCC GCT TAT TTT AGC ATC TTT ACC A-3' for Elp3, and 5'-GAA TTC ATG GCG GAG GTG GGG GAG AT-3' and 5'-GCG GCC GCT CAC CAC TTT CCT CTC TTG C-3' for Tip60. The plasmids pCAGIHisDR-Flag-Sox2, pCAGIHisDR-Flag-Elp3 and pCAGIHisDR-Flag-Tip60 were constructed by inserting the corresponding cDNAs into pCAG-IHisDR. pCAGIP-Flag-Sox2, pCAGIP-mycSox2, pCAGIP-mycEed1,

pCAGIP-mycEed4 and pCAGIP-mycCOUP-TFII were generated by inserting the corresponding cDNAs into pCAG-IP. The siRNA expression vectors pFIV-control, pFIV-COUP-TFI, pFIV-COUP-TFII#1 and pFIV-COUP-TFII#2 were constructed by inserting 5'- TGC GTT GCT AGT ACC AAC T -3', 5'-GCA GTT TCA ACT GGC CTT A-3', 5'-CCA CAT ACG GAT CTT CCA A-3' and 5'-CCG AGT ATA GCT GCC TCA A-3' into pFIV-H1/U6-puro (System Biosciences, Mountain View, CA, USA), respectively.

Construction of the three reporter plasmids, pGL2-Eed(-2600/-13), pGL2-Eed(-2600/-13)STAT3mt and pGL2-Eed(-2600/-13)Oct3/4mt, was described previously (Ura *et al*, 2008). To produce pGL2-Eed(-2600/-13)Sox2mt, mutations (AACAACAG to AACCCCAG) at the Sox2-binding site were introduced into pGL2-Eed(-2600/-13) by PCR. To generate pGL4-promoter, the SV40 promoter sequence of pGL2-promoter (Promega, Madison, WI, USA) was transferred into the Bgl II and Hind III sites of pGL4.10 (Promega). SRR1 and SRR2 were amplified by PCR and subcloned into pGL4-promoter to obtain pGL4pro-SRR1 and pGL4pro-SRR2, respectively. The SRR2 mutant (SRR2mt) containing mutations (CATTGTGATGCATAT to CCTGGGGCTTCCTCT) at Sox2- and Oct3/4-binding sites, the deletion mutants of SRR1 (-2957/-2493, -2666/-2493, -4074/-3205 and

-4074/-2685), and the SRR1 mutant (SRR1mt) carrying mutations (AGACCT to CGCCAT) at COUP-TFII–binding site were generated by PCR and inserted into pGL4-promoter.

Luciferase reporter assay

ES cells in a 6-cm dish were transfected with various combinations of plasmids by calcium phosphate-mediated transfection. Two days after transfection, ES cells were harvested and lysed in cell lysis buffer [20 mM Hepes-NaOH (pH 7.2), 10 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium fluoride, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1% Nonidet P-40 and 10% glycerol]. Luciferase activity was measured using a luciferase assay system (Promega) in a luminometer (Luminescencer AB-2200, ATTO, Tokyo, Japan).

Western blot analysis, immunostaining and ELISA

ES cells were harvested, lysed in 1x Sample buffer (6% glycerol, 10 mM Tris-HCl, 2% SDS, 50 mM DTT, 2 mM EDTA, 0.002% Coomassie Brilliant Blue R250), and heat denatured. Samples were subjected to SDS-PAGE and

electrophoretically transferred to a nitrocellulose membrane. The membranes were then probed with antibodies against H3K27me3, H3Ac, H4Ac, H3K56Ac (Millipore, Billerica, MA, USA), and lamin B1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The signals were visualized using enhanced chemiluminescence reagents (Perkin Elmer, Norwalk, CT, USA) with an LAS-1000 image analyzer (Fuji Film, Tokyo, Japan), and signal intensity was normalized to lamin B1.

For immunostaining, ES cells were fixed with 4% paraformaldehyde at 4°C for 30 min. After permeabilization with 0.5% Triton X-100 in phosphate-buffered saline (PBS) (-), the cells were pre-incubated with 1% BSA in PBS (-). The cells were then incubated with 1 μg/ml monoclonal anti-Flag-antibody (Sigma), or 300-fold diluted rabbit anti-H3K27me3, anti-H3Ac, anti-H4Ac, or anti-H3K56Ac antibodies at 4°C overnight, followed by incubation with 1000-fold diluted goat anti-rabbit or anti-mouse IgG FITC conjugate (Santa Cruz Biotechnology). Signal intensity was determined with Image J software. ELISA was performed using an EpiQuik global histone acetylation kit (Epigentek Group Inc., Brooklyn, NY, USA) according to the manufacturer's protocol.

Knockdown of target genes

Double-stranded small interfering RNAs (siRNAs) were purchased from Operon Biotechnologies (Huntsville, AL, USA). The target sequences used were 5'-CUA UCC GUG CUA GAU AUG ACC-3' for *Elp3*, 5'-CUA CGU AAU GAC GGA GUA UGA-3' for *Tip60*, and 5'-GCC ACA ACG UCU AUA UCA UGG-3' for *EGFP*. ES cells (1 x 10^5 cells) in a 6-cm dish were transfected with siRNA or the siRNA expression vector using Lipofectamine 2000. Two days after transfection, ES cells were harvested and subjected to gene expression analysis.

Quantitative RT-PCR (qRT-PCR) analysis

Total RNAs were isolated from ES cells with Sepasol reagent (Nacalai tesque, Kyoto, Japan) and converted to cDNAs using Revertra Ace (Toyobo, Osaka, Japan) with oligo$(dT)_{12-18}$ primers (Nippon Gene, Tokyo, Japan). The amount of each cDNA was evaluated by quantitative PCR using MxPro Mx3005P (Stratagene, La Jolla, CA, USA). All samples were tested in triplicate, and the results from each sample were normalized relative to *Gapdh* expression. The sequences of the primer sets are shown in Supplementary Table S1.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using antibodies against the Myc epitope, Sox2 (sc-17319) (Santa Cruz Biotechnology), Flag epitope (Sigma), Lys27-methylated histone H3, acetylated histone H3, acetylated histone H4, or Lys56-acetylated histone H3 (Millipore), as described previously (Ura *et al*, 2008). For the detection of precipitated genomic DNA, quantitative PCR was performed. All samples were tested in triplicate and the results from each sample were normalized relative to input DNA. The sequences of the primer sets are shown in Supplementary Table S2.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1 Eed is a downstream target of Sox2. (**A**) *Eed* overexpression restores H3K27me3 during differentiation. ES cells transfected with a control or Eed1 expression vector were cultured with or without LIF for 4 days. The total amount of H3K27me3 in cell lysates was examined by western blot analysis (lower panels). Signal intensity was presented as the fold change relative to the sample in the presence of LIF (upper panel). (**B**) *Eed* expression is reduced by repression of *Sox2*. 2TS22C ES cells were cultured with or without Tet for the indicated periods. Expression levels of *Sox2* and *Eed* were quantified by qRT-PCR and presented as the fold change relative to an untreated control sample. Asterisk, significant difference from untreated control cells ($P<0.05$). Hash, significant difference from cells cultured with Tet for 24 hr and then without Tet for 24hr ($P<0.05$). (**C**) Repression of *Sox2* induces reduction of H3K27me3. 2TS22C ES cells were cultured with or without Tet for 4 days, and levels of H3K27me3 were detected by western blot analysis. (**D**) *Sox2* is reduced following *Eed* depletion. Eed1 cKO ES cells were cultured with or without Tet for the indicated periods. Expression of *Sox2* and *Eed* was examined by qRT-PCR. Asterisk, significant difference from untreated control cells ($P<0.05$). Hash, significant difference from cells cultured with Tet for 24 hr and then without Tet for 24 hr ($P<0.05$).

(**E**) Schematic representation of the promoter region of the *eed* gene. (**F-H**) Regulation of the *Eed* promoter region by STAT3, Oct3/4 and Sox2. (**F**) The reporter plasmid pGL2-Eed(-2600/-13) was transfected into HEK293 cells together with a control empty vector or expression vectors for Sox2, Oct3/4, STAT3, or Nanog. After 2 days in culture, cells were harvested and subjected to luciferase assay. (**G**) ES cells were transfected with pGL2-Eed(-2600/-13) derivatives carrying mutations at Sox2-, Oct3/4-, and/or STAT3-binding sites and cultured for 2 days. In each experiment, the data was normalized by setting the value of the wild-type promoter as 1.0. (**H**) 2TS22C ES cells were transfected with pGL2-Eed(-2600/-13) (wild-type) or pGL2-Eed(-2600/-13)Sox2mt (Sox2 mt) and cultured with or without Tet for 2 days. (**I**) Sox2 binds to the promoter region of the *eed* gene *in vivo*. ES cells transfected with a control or Flag-Sox2 expression vector were subjected to ChIP assay using an anti-Flag antibody, followed by qPCR using primers for the Sox2-binding site in the *eed* gene. The promoter region of the *eed* gene from -382 to -12 was used as a negative control. Asterisk, significant difference from sample precipitated with control IgG ($P<0.05$). In all experiments, error bars indicate standard deviation ($n=3$).

Figure 2 *Sox2* is regulated by Eed through COUP-TFII. (**A**) Schematic

representation of SRR1 and SRR2 in the *sox2* gene. (**B**) Enhancer activities of SRR regions decrease after LIF removal. After transfection with pGL4pro-SRR1, pGL4pro-SRR2 or pGL4pro-SRR2mt, ES cells were cultured with or without LIF for 2 days and subjected to a reporter assay to examine the enhancer activities of SRR1, SRR2 and SRR2mt. (**C**) The enhancer activities of the SRR regions decrease in *Eed*-deficient ES cells. Enhancer activities of SRR1, SRR2, and SRR2mt were examined in Eed4 cKO ES cells treated with or without Tet for 2 days. (**D**) Identification of the Eed-responsive element in SRR1. Luciferase assay was performed for SRR1 and its derivatives (shown in upper panel) in Eed4 cKO ES cells treated with or without Tet for 2 days. (**E**) Expression of *GCNF*, *COUP-TFI*, *COUP-TFII* (left panel) and *Sox2* (right panel) in *Eed*-deficient ES cells. Eed1 cKO ES cells were cultured with or without Tet for the indicated times. Expression of each gene was examined by qRT-PCR. Asterisk, significant difference from untreated cells ($P<0.05$). (**F**) COUP-TFII suppresses the enhancer activity of SRR1 through the Eed-responsive element. Enhancer activities of SRR1 and SRR1mt were examined in ES cells transfected with control, GCNF, COUP-TFI, or COUP-TFII expression vectors. Note that all values are presented as the ratio of the enhancer activity of SRR1 to that of SRR1mt. (**G**) Decreased enhancer activity of SRR1 in *Eed*-deficient ES cells is

restored by knockdown of *COUP-TFII*. The enhancer activity of SRR1 was examined in Eed1 cKO ES cells transfected with pFIV-control, pFIV-COUP-TFI, pFIV-COUP-TFII#1, or pFIV-COUP-TFII#2 in the presence or absence of Tet. (**H**) Knockdown of *COUP-TFII* induces *Sox2* expression. After transfection with pFIV-COUP-TFII, Eed4 cKO ES cells were cultured in the presence of Tet, and mRNA expression of *COUP-TFII* and *Sox2* was examined by qRT-PCR. Asterisk, significant difference from control cells ($P<0.05$). (**I**) *In vivo* binding of COUP-TFII to SRR1. ES cells transfected with either a control or a Myc-COUP-TFII expression vector were subjected to a ChIP assay using control IgG (IgG) or anti-Myc antibody (α-Myc), followed by qPCR using primers for the COUP-TFII–binding site of SRR1. The upstream region (-3917/-3713) of the COUP-TFII–binding site in SRR1 was used as a negative control. Asterisk, significant difference from sample precipitated with control IgG ($P<0.05$). (**J**) Eed regulates H3K27me3 at the promoter region of the *coup-tfII* gene. Eed4 cKO ES cells were cultured with or without Tet and subjected to a ChIP assay using anti-Myc (Eed) and anti-H3K27me3 (H3K27me3) antibodies, followed by qPCR using primers for the promoter region of the *coup-tfII* gene. Note that Eed4 cKO ES cells express Myc-tagged Eed4 in the absence of Tet. In all experiments, error bars represent standard deviation ($n=3$).

Figure 3 Sox2 overcomes the phenotype of *Eed*-deficient ES cells. (**A**) The morphology of *Sox2*-expressing, *Eed*-deficient ES cells. Eed4 cKO ES cells were transfected with either a control or Flag-Sox2 expression vector and cultured in the presence or absence of Tet. Immunostaining with anti-Flag antibody confirmed the expression of transgenes. Bars, 50 μm. (**B**) *Sox2*-expressing, *Eed*-deficient ES cells form compact colonies. Eed4 cKO ES cells transfected with a control or Flag-Sox2 expression vector were cultured with Tet for 4 days, and the percent that formed compact colonies was calculated by dividing the number of compact colonies by the total number of colonies. Results represent three independent experiments. (**C**) Sox2 suppresses downregulation of self-renewal genes and induction of differentiation-associated genes induced by *Eed* depletion. The indicated cells were cultured with or without Tet for 4 days. Expression of the indicated genes was examined by qRT-PCR. Asterisk, significant difference from Eed4 cKO cells cultured in the absence of Tet ($P<0.05$). Hash, significant difference from Tet-treated Eed4 cKO cells ($P<0.05$). (**D**) *Sox2* overexpression restores Sox2 binding to promoter regions of self-renewal genes in *Eed*-deficient ES cells. The indicated cells were

cultured with or without Tet for 4 days and subjected to ChIP assay using an anti-Sox2 antibody, followed by qPCR using primers for the Sox2-binding site of the indicated genes. Asterisk, significant difference from Eed4 cKO cells cultured without Tet ($P<0.05$). Hash, significant difference from Tet-treated Eed4 cKO cells ($P<0.05$). In all experiments, error bars indicate standard deviation ($n=3$).

Figure 4 Sox2 promotes histone acetylation in *Eed*-deficient ES cells. Eed4 cKO ES cells transfected with either control or Flag-Sox2 expression vectors were cultured in the presence or absence of Tet for 4 days. (**A, B**) Global amounts of H3Ac, H4Ac and H3K56Ac are maintained in *Sox2*-expressing, *Eed*-deficient ES cells. Control or *Sox2*-expressing, *Eed*-deficient ES cells were subjected to immunostaining (**A**) or western blot analysis (**B**) using anti-H3K27me3, H3Ac, H4Ac, and H3K56Ac antibodies. Bars, 50 μm. (**C**) Sox2 cannot restore H3K27me3 in the promoter regions of differentiation-associated genes in *Eed*-deficient ES cells. After culturing with or without Tet, the indicated cells were subjected to ChIP assay using an anti-H3K27me3 antibody, followed by qPCR using primers for the promoter region of the indicated genes. Asterisk, significant difference from Eed4 cKO cells cultured without Tet ($P<0.05$). (**D**) The relative amounts of H3Ac and H4Ac were

quantitatively determined by ELISA. (**E-G**) Sox2 restores histone acetylation in the promoter regions of self-renewal genes in *Eed*-deficient ES cells. The indicated cells were subjected to ChIP assay using anti-H3Ac (**E**), H4Ac (**F**) and H3K56Ac (**G**) antibodies, followed by qPCR using primers for the promoter regions of the indicated genes. Asterisk, significant difference from Eed4 cKO cells cultured without Tet ($P<0.05$). Hash, significant difference from Eed4 cKO cells cultured with Tet ($P<0.05$). In all experiments, error bars indicate standard deviation ($n=3$).

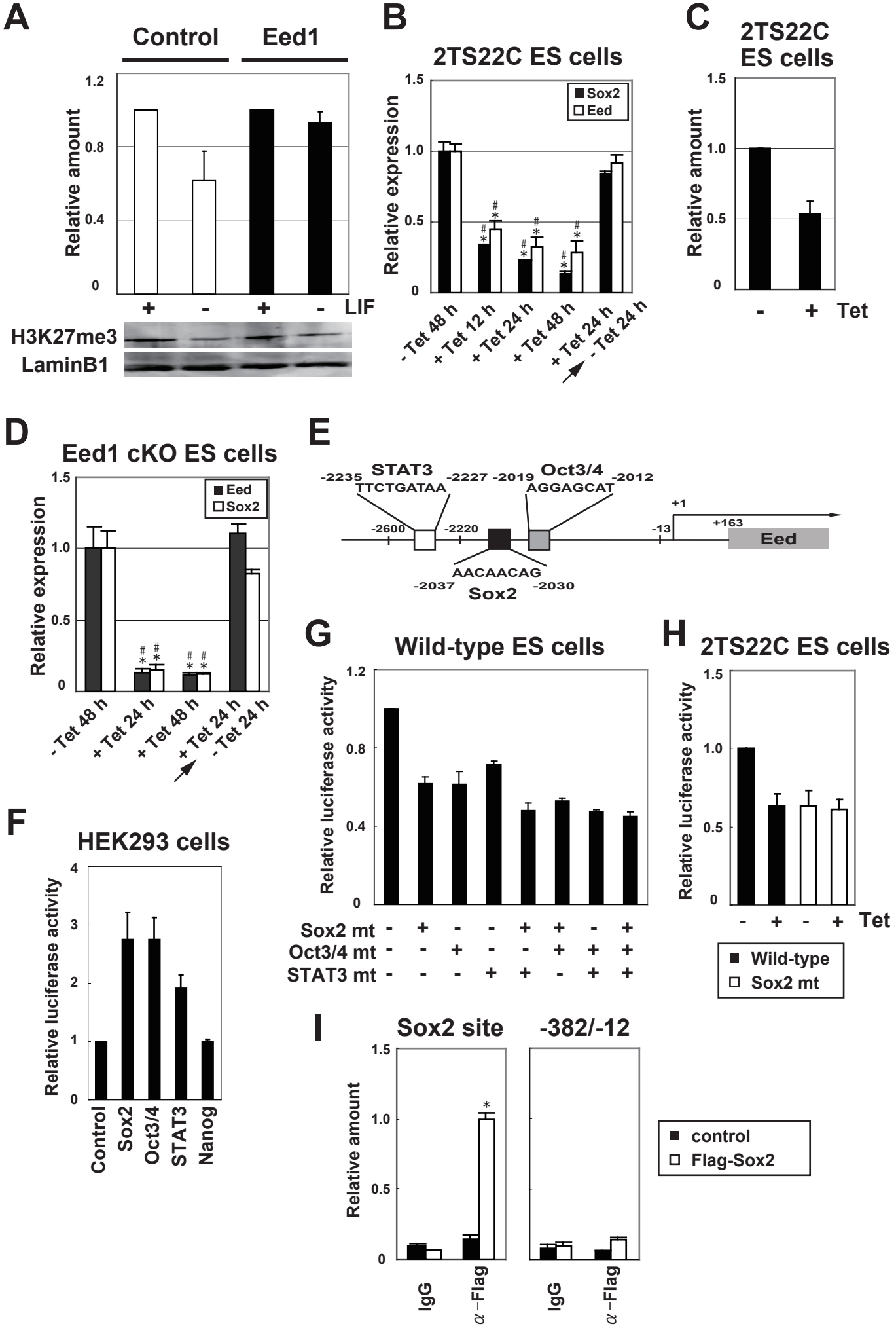
Figure 5 Histone acetyltransferases are downstream effectors of Sox2. (**A, B**) Global levels of H3Ac, H4Ac and H3K56Ac are reduced during ES cell differentiation and by *Sox2* downregulation. Wild-type and 2TS22C ES cells were cultured for 0 or 3 days without LIF and with Tet, respectively. The total amounts of modified histones were determined by western blot analysis (**A**) and ELISA (**B**). (**C**) Expression levels of HATs are reduced after the removal of LIF. Wild-type ES cells were cultured without LIF for the indicated number of days, and the expression of each HAT gene was examined by qRT-PCR. Asterisk, significant difference from wild-type cells at day 0 ($P<0.05$). (**D**) Expression of HATs is regulated by Sox2. 2TS22C ES cells were cultured with or without Tet for the indicated period, and the expression levels of HATs

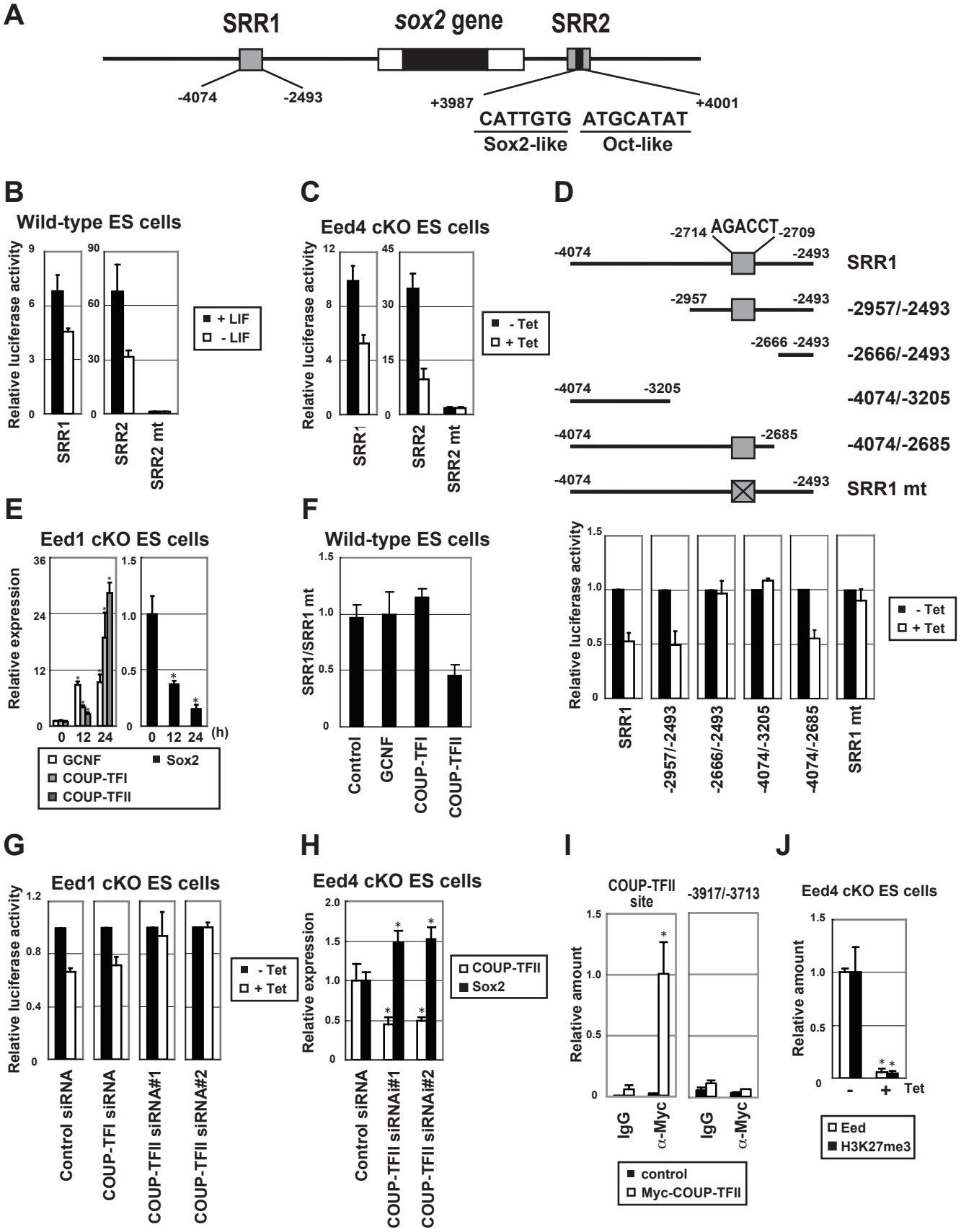
were determined. Asterisk, significant difference from untreated control cells (P<0.05). Hash, significant difference from cells cultured with Tet for 24 hr and then without Tet for 24 hr (P<0.05). (**E**) Expression of HATs is regulated by Eed. Eed4 cKO ES cells were cultured with or without Tet for the indicated period and the expression of HATs was examined. Asterisk, significant difference from untreated control cells (P<0.05). Hash, significant difference from cells cultured with Tet for 24 hr and then without Tet for 24 hr (P<0.05). (**F**) Reduced expression of HATs in *Eed*-deficient ES cells is restored by *Sox2* expression. The indicated cells were cultured with or without Tet for 4 days, and expression of the indicated HATs was measured. Asterisk, significant difference from Eed4 cKO cells cultured without Tet (P<0.05). Hash, significant difference from Tet-treated Eed4 cKO cells (P<0.05). In all experiments, error bars indicate standard deviation (n=3).

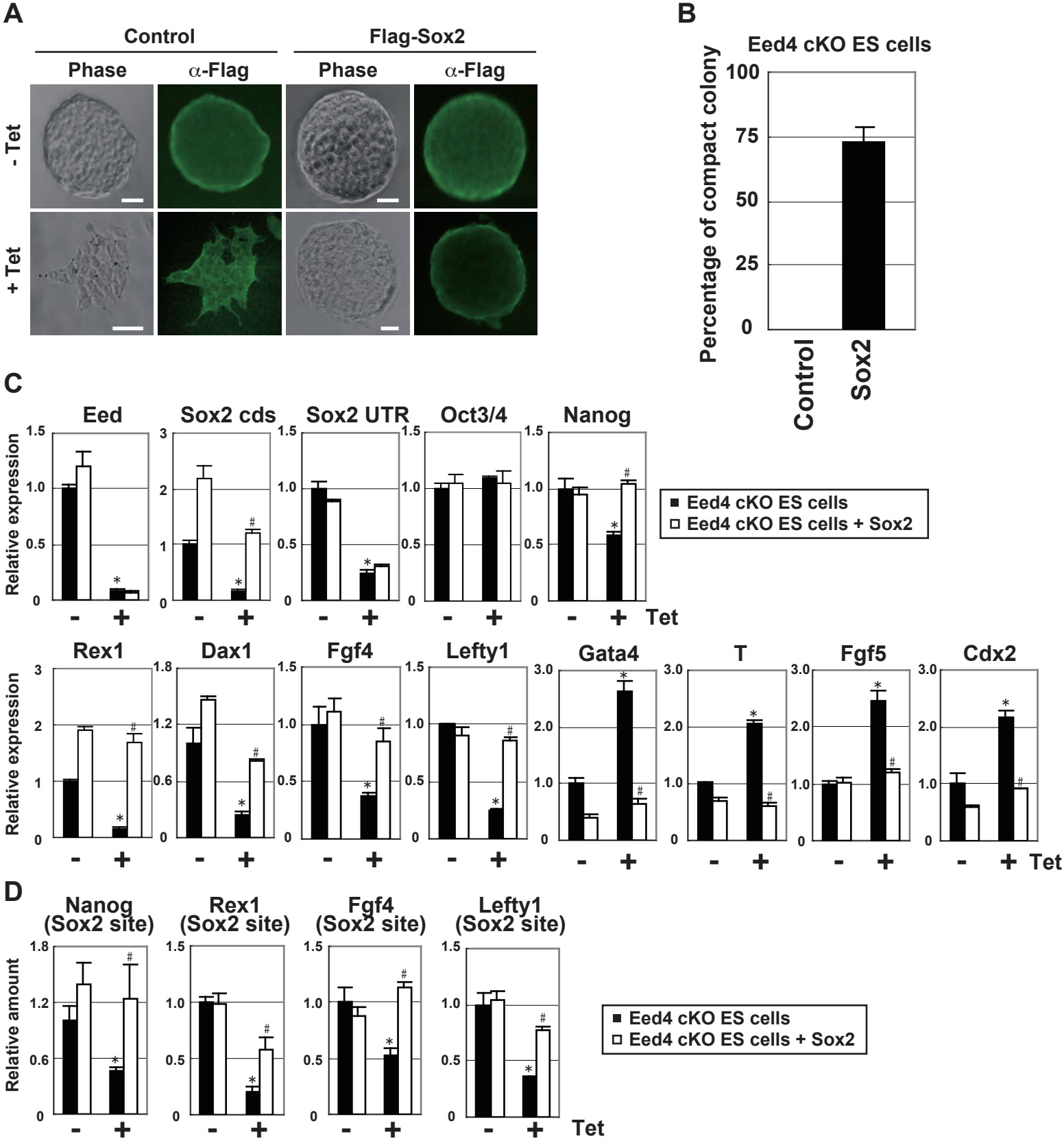
Figure 6 Elp3 and Tip60 overcome the phenotype of *Eed*-deficient ES cells. Eed4 cKO ES cells transfected with control, Flag-Elp3, or Flag-Tip60 expression vectors were cultured in the presence or absence of Tet for 4 days. (**A**) Immunostaining of *Elp3*- or *Tip60*-expressing Eed4 cKO ES cells using anti-Flag, H3K27me3, H3Ac, H4Ac and H3K56Ac antibodies. Bars, 50 μm. (**B**) Global amounts of H3Ac, H4Ac

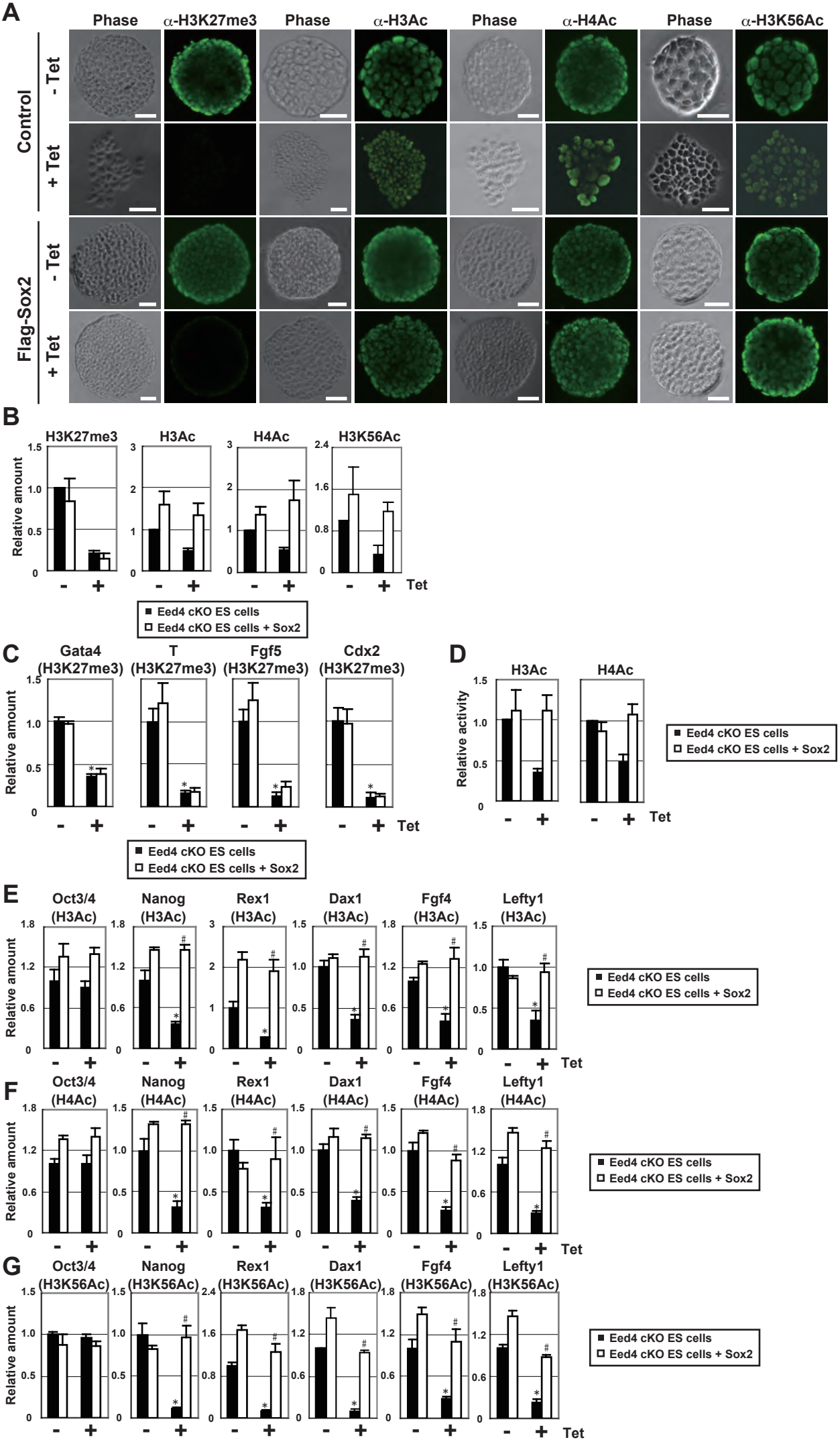
and H3K56Ac are restored by *Elp3* or *Tip60* expression in *Eed*-deficient ES cells. Overall amounts of the modified histones were examined by western blot analysis. (**C**) *Elp3*- or *Tip60*-expressing, *Eed*-deficient ES cells form compact colonies. The ratio of compact colonies was calculated by dividing the number of compact colonies by the total number of colonies. Results are representative of three independent experiments. (**D**) Elp3 or Tip60 suppresses downregulation of self-renewal genes and induction of differentiation-associated genes induced by *Eed* depletion. Expression of the indicated genes was examined by qRT-PCR. Asterisk, significant difference from Eed4 cKO cells cultured without Tet ($P<0.05$). Hash, significant difference from Eed4 cKO cells cultured with Tet ($P<0.05$). (**E-G**) Elp3 or Tip60 restores histone acetylation in the promoter regions of self-renewal genes in *Eed*-deficient ES cells. The indicated cells were subjected to ChIP assay with anti-H3Ac (**E**), anti-H4Ac (**F**) and anti-H3K56Ac (**G**) antibodies, followed by qPCR using primers for the promoter regions of the indicated genes. Asterisk, significant difference from Eed4 cKO cells cultured without Tet ($P<0.05$). Hash, significant difference from Eed4 cKO cells cultured with Tet ($P<0.05$). In all experiments, error bars indicate standard deviation ($n=3$).

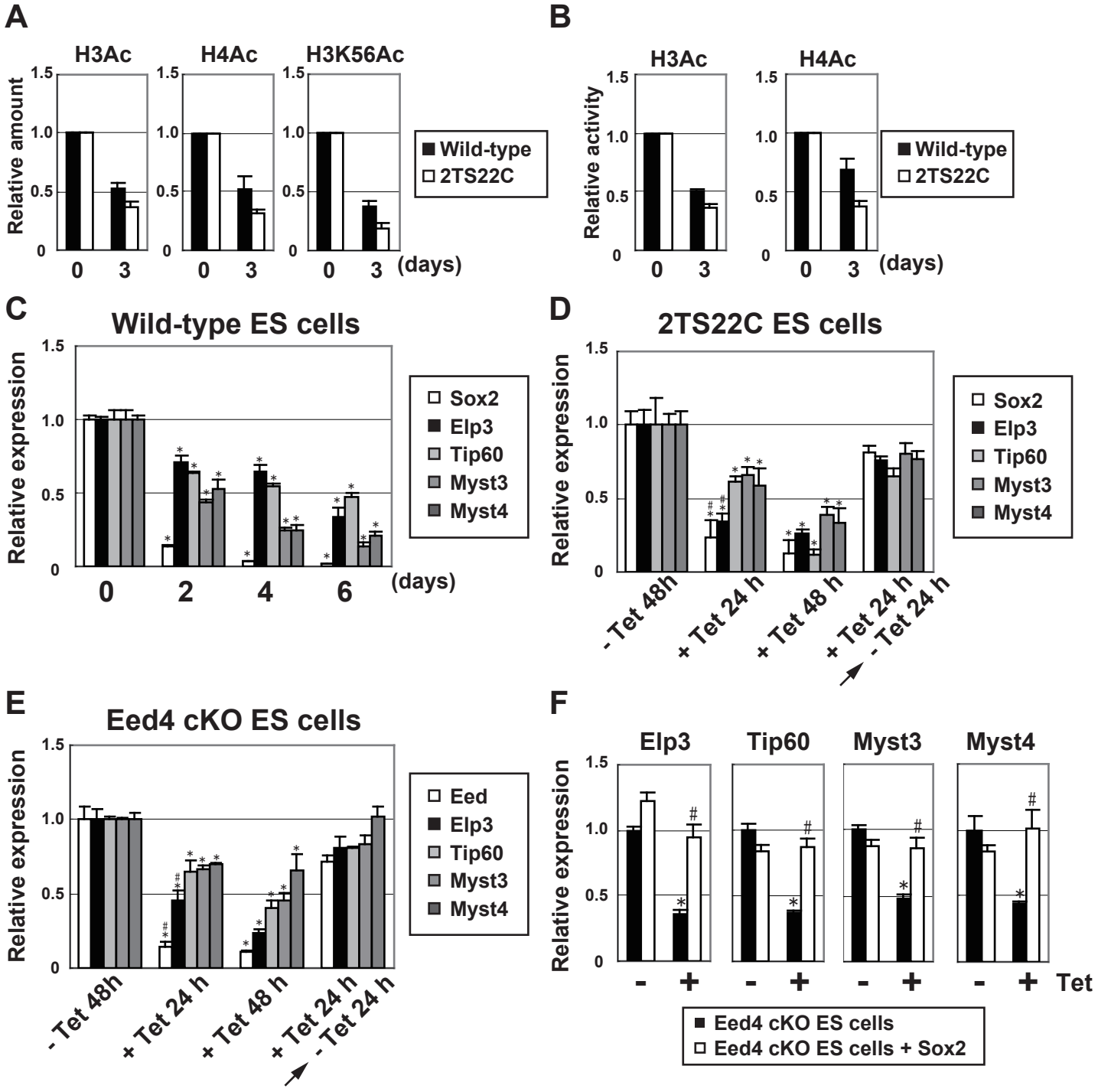
Figure 7 H3K27me and histone acetylation cooperatively regulate stemness in ES cells. (**A**) Knockdown of *Elp3* or *Tip60* reverses the effect of Sox2 on *Eed*-deficient ES cells. *Sox2*-expressing Eed4 cKO ES cells were transfected with EGFP siRNA (*siEGFP*), Elp3 siRNA (*siElp3*), or Tip60 siRNA (*siTip60*) and cultured with Tet for 2 days. Expression of the indicated genes was examined by qRT-PCR. Asterisk, significant difference from EGFP siRNA-transfected cells ($P<0.05$). (**B**) Synergistic effect of *Eed* depletion and HAT knockdown on ES cell differentiation. Wild-type ES cells and Eed4 cKO ES cells were transfected with EGFP siRNA, Elp3 siRNA, or Tip60 siRNA, and cultured with or without Tet for 2 days. The expression of the indicated genes was examined by qRT-PCR. Asterisk, significant difference from EGFP siRNA-transfected Eed4 cKO cells cultured with Tet ($P<0.05$). Hash, significant difference from Elp3 or Tip60 siRNA-transfected Eed4 cKO cells cultured without Tet ($P<0.05$). In all experiments, error bars represent standard deviation ($n=3$). (**C**) The proposed role of the Eed/Sox2 regulatory loop in ES cell self-renewal.


A
Control
Eed1
Relative amount
1.2
0.8
0.4
0
+ - + - LIF
H3K27me3
LaminB1
B
2TS22C ES cells
Relative expression
1.5
1.0
0.5
0
Sox2
Eed
- Tet 48 h
+ Tet 12 h
+ Tet 24 h
+ Tet 48 h
+ Tet 24 h
- Tet 24 h
C
2TS22C ES cells
Relative amount
1.5
1.0
0.5
0
- + Tet
D
Eed1 cKO ES cells
Relative expression
1.5
1.0
0.5
0
Eed
Sox2
- Tet 48 h
+ Tet 24 h
+ Tet 48 h
+ Tet 24 h
- Tet 24 h
E
STAT3
-2235
TTCTGATAA
-2227
-2019
Oct3/4
-2012
AGGAGCAT
+1
-2600
-2220
-13
+163
Eed
AACAACAG
-2037
-2030
Sox2
F
HEK293 cells
Relative luciferase activity
4
3
2
1
0
Control
Sox2
Oct3/4
STAT3
Nanog
G
Wild-type ES cells
Relative luciferase activity
1.2
0.8
0.4
0
Sox2 mt - + - - + + - +
Oct3/4 mt - - + - - + + +
STAT3 mt - - - + + - + +
H
2TS22C ES cells
Relative luciferase activity
1.5
1.0
0.5
0
- + - + Tet
Wild-type
Sox2 mt
I
Sox2 site
-382/-12
Relative amount
1.5
1.0
0.5
0
IgG
α-Flag
IgG
α-Flag
control
Flag-Sox2


A
SRR1
sox2 gene
SRR2
-4074
-2493
+3987
+4001
CATTGTG
ATGCATAT
Sox2-like
Oct-like
B
Wild-type ES cells
Relative luciferase activity
+ LIF
- LIF
SRR1
SRR2
SRR2 mt
C
Eed4 cKO ES cells
Relative luciferase activity
- Tet
+ Tet
SRR1
SRR2
SRR2 mt
D
-2714
AGACCT
-2709
-4074
-2493
SRR1
-2957
-2493
-2957/-2493
-2666
-2493
-2666/-2493
-4074
-3205
-4074/-3205
-4074
-2685
-4074/-2685
-4074
-2493
SRR1 mt
Relative luciferase activity
- Tet
+ Tet
SRR1
-2957/-2493
-2666/-2493
-4074/-3205
-4074/-2685
SRR1 mt
E
Eed1 cKO ES cells
Relative expression
(h)
GCNF
Sox2
COUP-TFI
COUP-TFII
F
Wild-type ES cells
SRR1/SRR1 mt
Control
GCNF
COUP-TFI
COUP-TFII
G
Eed1 cKO ES cells
Relative luciferase activity
- Tet
+ Tet
Control siRNA
COUP-TFI siRNA
COUP-TFII siRNA#1
COUP-TFII siRNA#2
H
Eed4 cKO ES cells
Relative expression
COUP-TFII
Sox2
Control siRNA
COUP-TFII siRNAi#1
COUP-TFII siRNAi#2
I
COUP-TFII site
-3917/-3713
Relative amount
IgG
α-Myc
IgG
α-Myc
control
Myc-COUP-TFII
J
Eed4 cKO ES cells
Relative amount
-
+
Tet
Eed
H3K27me3


A
Control
Flag-Sox2
Phase
α-Flag
Phase
α-Flag
- Tet
+ Tet
B
Eed4 cKO ES cells
Percentage of compact colony
100
75
50
25
0
Control
Sox2
C
Eed
Sox2 cds
Sox2 UTR
Oct3/4
Nanog
Relative expression
Eed4 cKO ES cells
Eed4 cKO ES cells + Sox2
Tet
Rex1
Dax1
Fgf4
Lefty1
Gata4
T
Fgf5
Cdx2
Relative expression
Tet
D
Nanog (Sox2 site)
Rex1 (Sox2 site)
Fgf4 (Sox2 site)
Lefty1 (Sox2 site)
Relative amount
Eed4 cKO ES cells
Eed4 cKO ES cells + Sox2
Tet


A
Phase
α-H3K27me3
Phase
α-H3Ac
Phase
α-H4Ac
Phase
α-H3K56Ac
Control
- Tet
+ Tet
Flag-Sox2
- Tet
+ Tet
B
H3K27me3
H3Ac
H4Ac
H3K56Ac
Relative amount
Tet
Eed4 cKO ES cells
Eed4 cKO ES cells + Sox2
C
Gata4 (H3K27me3)
T (H3K27me3)
Fgf5 (H3K27me3)
Cdx2 (H3K27me3)
Relative amount
Tet
Eed4 cKO ES cells
Eed4 cKO ES cells + Sox2
D
H3Ac
H4Ac
Relative activity
Tet
Eed4 cKO ES cells
Eed4 cKO ES cells + Sox2
E
Oct3/4 (H3Ac)
Nanog (H3Ac)
Rex1 (H3Ac)
Dax1 (H3Ac)
Fgf4 (H3Ac)
Lefty1 (H3Ac)
Relative amount
Tet
Eed4 cKO ES cells
Eed4 cKO ES cells + Sox2
F
Oct3/4 (H4Ac)
Nanog (H4Ac)
Rex1 (H4Ac)
Dax1 (H4Ac)
Fgf4 (H4Ac)
Lefty1 (H4Ac)
Relative amount
Tet
Eed4 cKO ES cells
Eed4 cKO ES cells + Sox2
G
Oct3/4 (H3K56Ac)
Nanog (H3K56Ac)
Rex1 (H3K56Ac)
Dax1 (H3K56Ac)
Fgf4 (H3K56Ac)
Lefty1 (H3K56Ac)
Relative amount
Tet
Eed4 cKO ES cells
Eed4 cKO ES cells + Sox2


A
H3Ac
H4Ac
H3K56Ac
Relative amount
1.5
1.0
0.5
0
0
3
(days)
Wild-type
2TS22C
B
H3Ac
H4Ac
Relative activity
(days)
C
Wild-type ES cells
Relative expression
0
2
4
6
(days)
Sox2
Elp3
Tip60
Myst3
Myst4
D
2TS22C ES cells
- Tet 48h
+ Tet 24 h
+ Tet 48 h
+ Tet 24 h
- Tet 24 h
E
Eed4 cKO ES cells
Eed
F
Elp3
Tip60
Myst3
Myst4
Tet
Eed4 cKO ES cells
Eed4 cKO ES cells + Sox2

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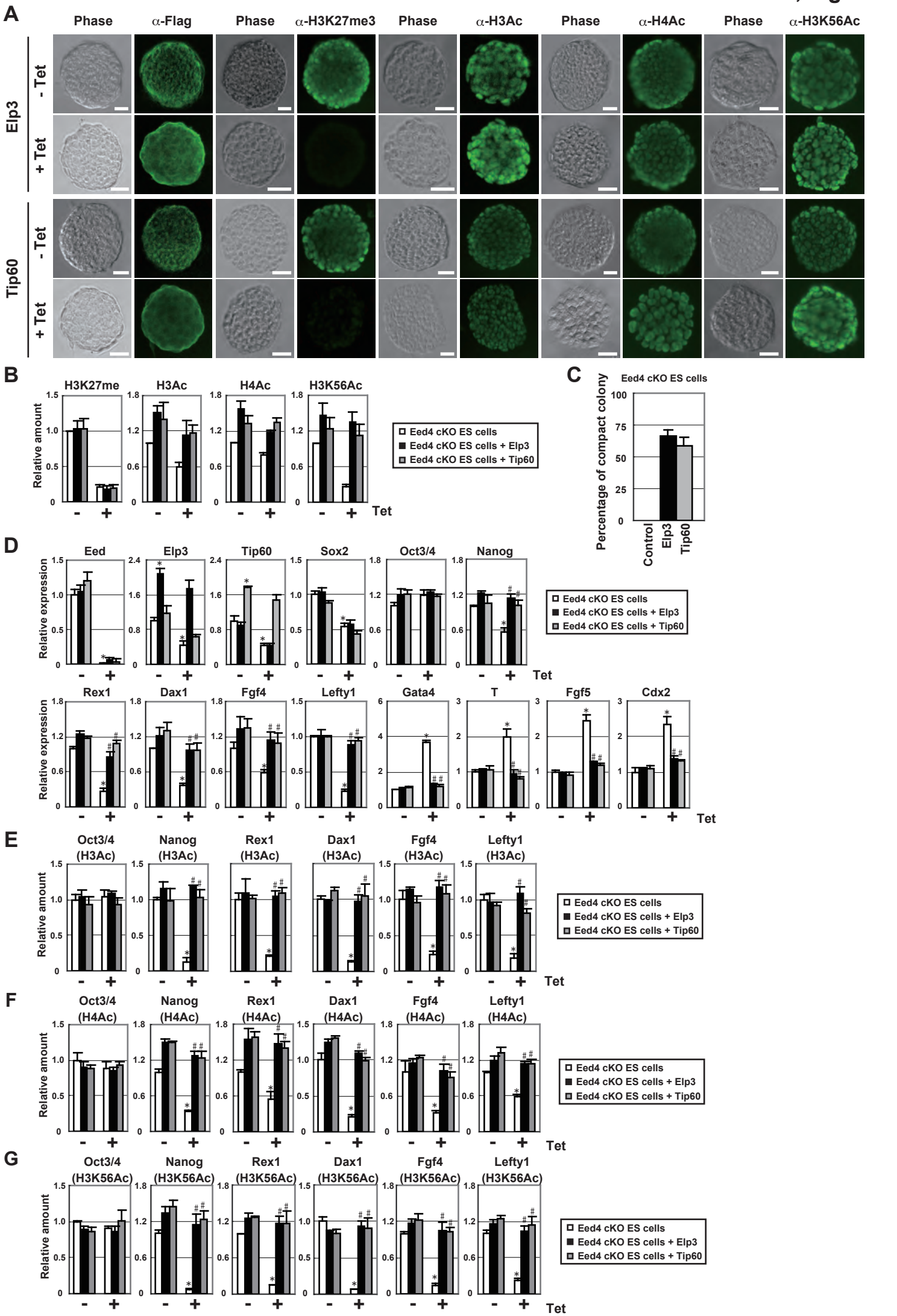
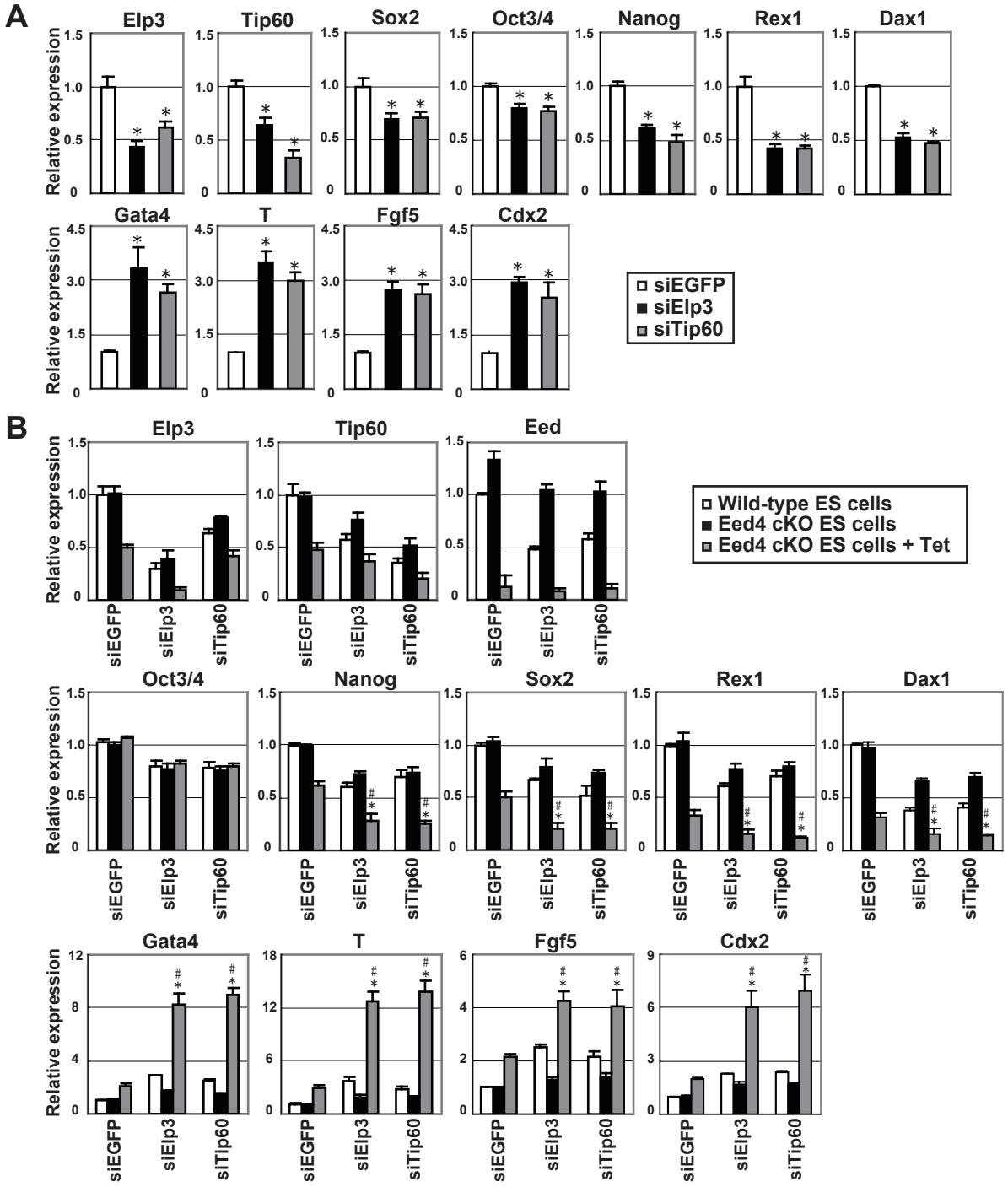
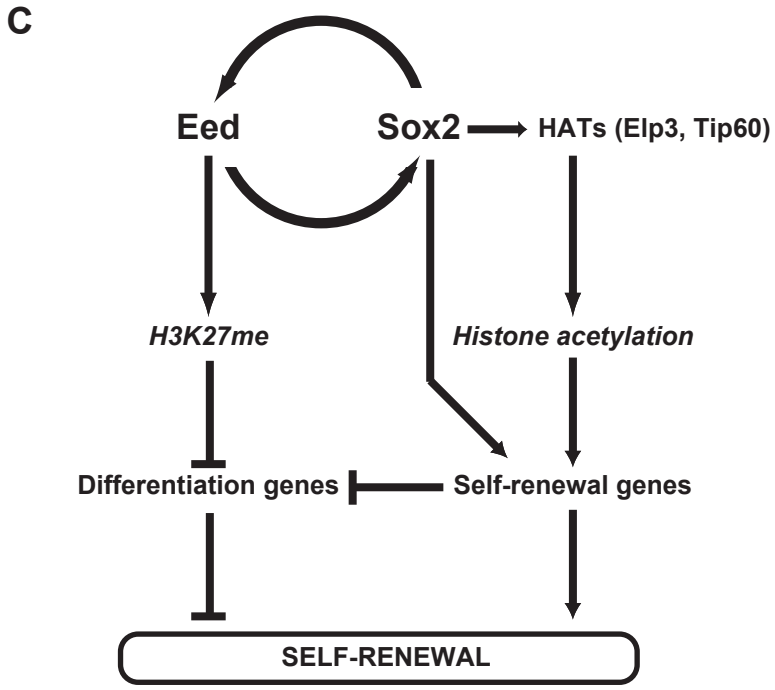



A
Elp3
Tip60
Sox2
Oct3/4
Nanog
Rex1
Dax1
Gata4
T
Fgf5
Cdx2
Relative expression
siEGFP
siElp3
siTip60
B
Eed
Wild-type ES cells
Eed4 cKO ES cells
Eed4 cKO ES cells + Tet


C
Eed
Sox2
HATs (Elp3, Tip60)
H3K27me
Histone acetylation
Differentiation genes
Self-renewal genes
SELF-RENEWAL

SUPPLEMENTARY IMFORMATION

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

Hiroki Ura, Kazuhiro Murakami, Tadayuki Akagi, Keita Kinoshita, Shukuro Yamaguchi, Shinji Masui, Hitoshi Niwa, Hiroshi Koide and Takashi Yokota

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	ctacccacccccctattctccca
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.

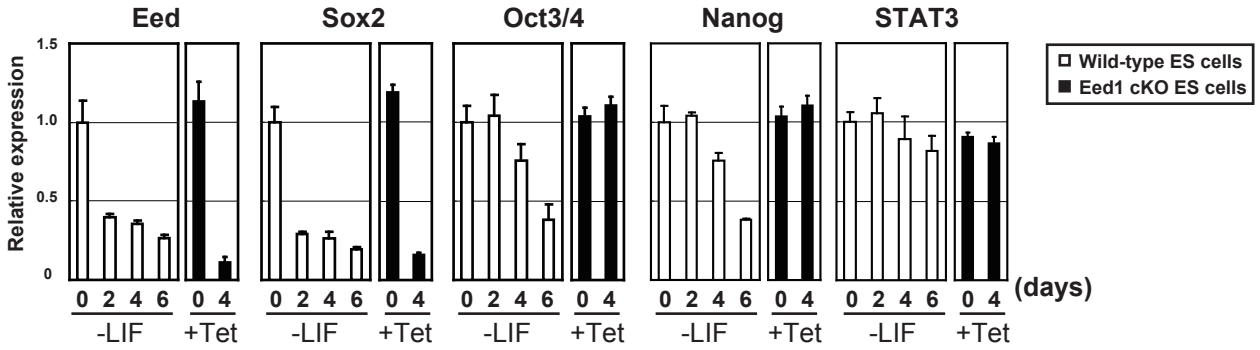
Figure S2 Eed cannot suppress ES cell differentiation induced by *Sox2* downregulation. (**A**) Morphology of *Eed*- and *Sox2*-expressing 2TS22C cells. 2TS22C cells transfected with *Eed1* or *Sox2* were cultured with or without Tet for 4 days. Scale bar = 50 μm. (**B**) Ectopic expression of *Sox2*, 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

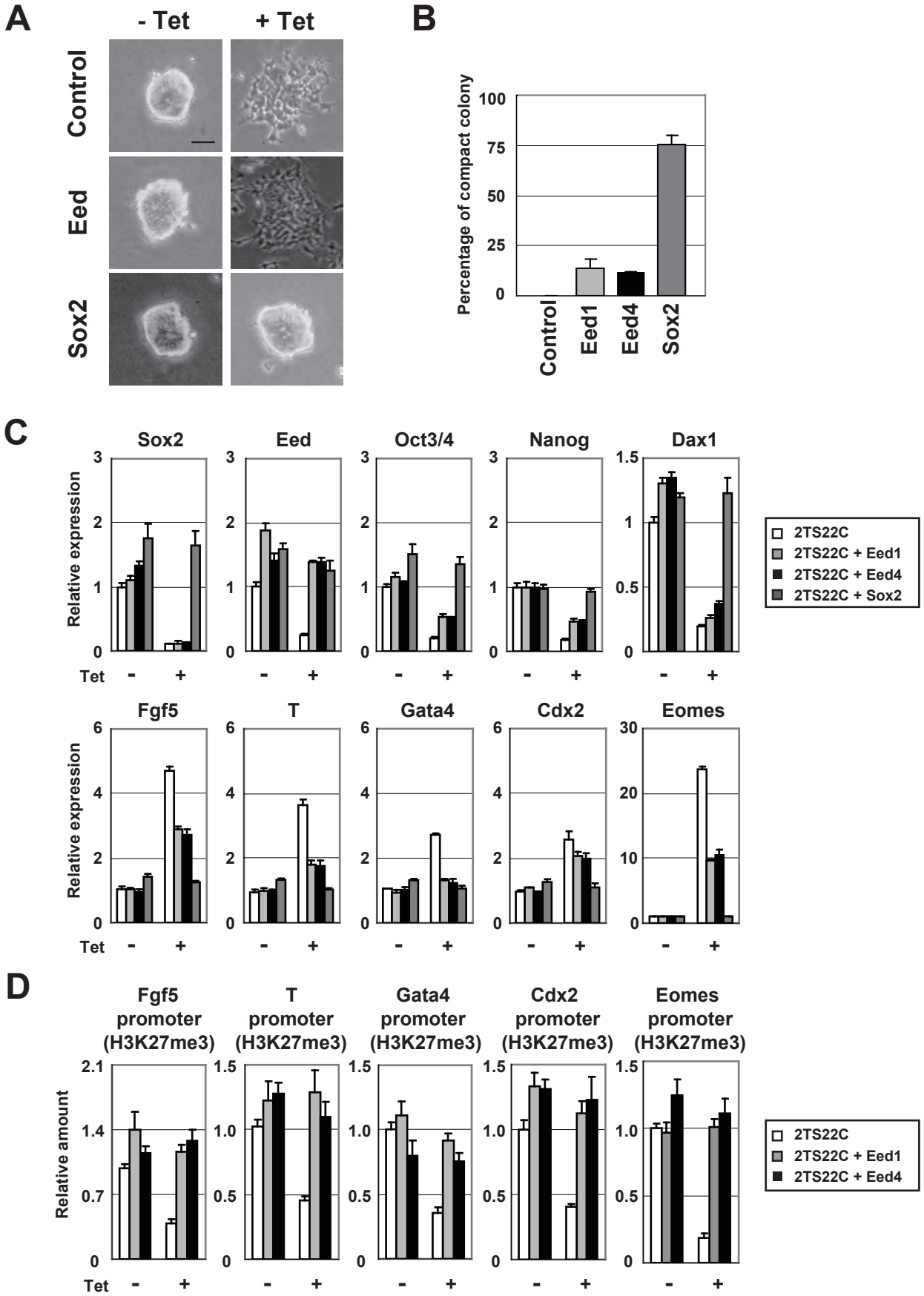
the indicated genes. It should be noted that the failure to suppress trophectodermal differentiation is not due to insufficient functional levels of *Eed* expression in 2TS22C ES cells, since the expression level of *Eed* is high enough to restore the reduced level of H3K37me3.

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.

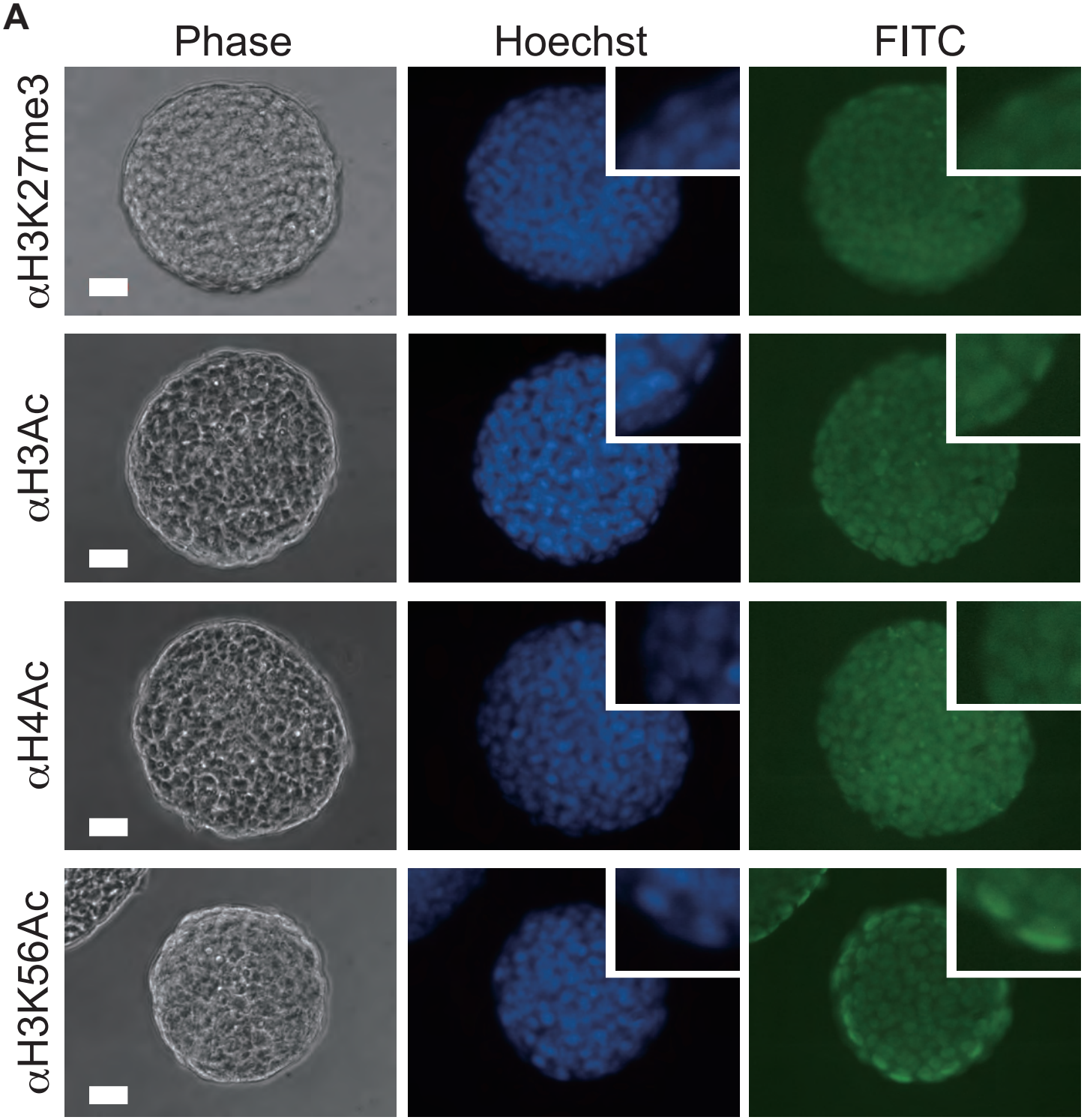
Figure S4 Elp3 and Tip60 overcome the phenotype of *Eed*-deficient ES cells. Eed4 cKO ES cells transfected with control, Flag-Elp3, or Flag-Tip60 expression vector were cultured in the presence or absence of Tet for 4 days. (**A, B**) Histone acetylation levels are maintained in *Elp3*- or *Tip60*-expressing, *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.

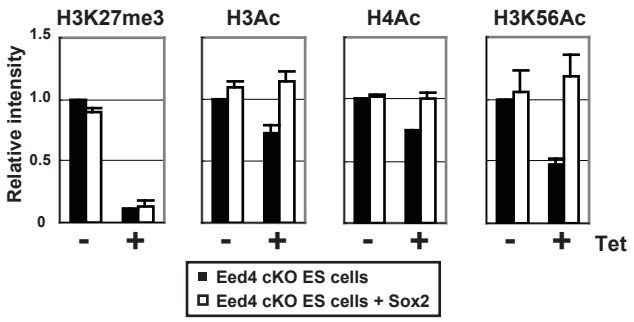

Eed
Sox2
Oct3/4
Nanog
STAT3
Relative expression
1.5
1.0
0.5
0
Wild-type ES cells
Eed1 cKO ES cells
0 2 4 6 0 4
-LIF
+Tet
(days)

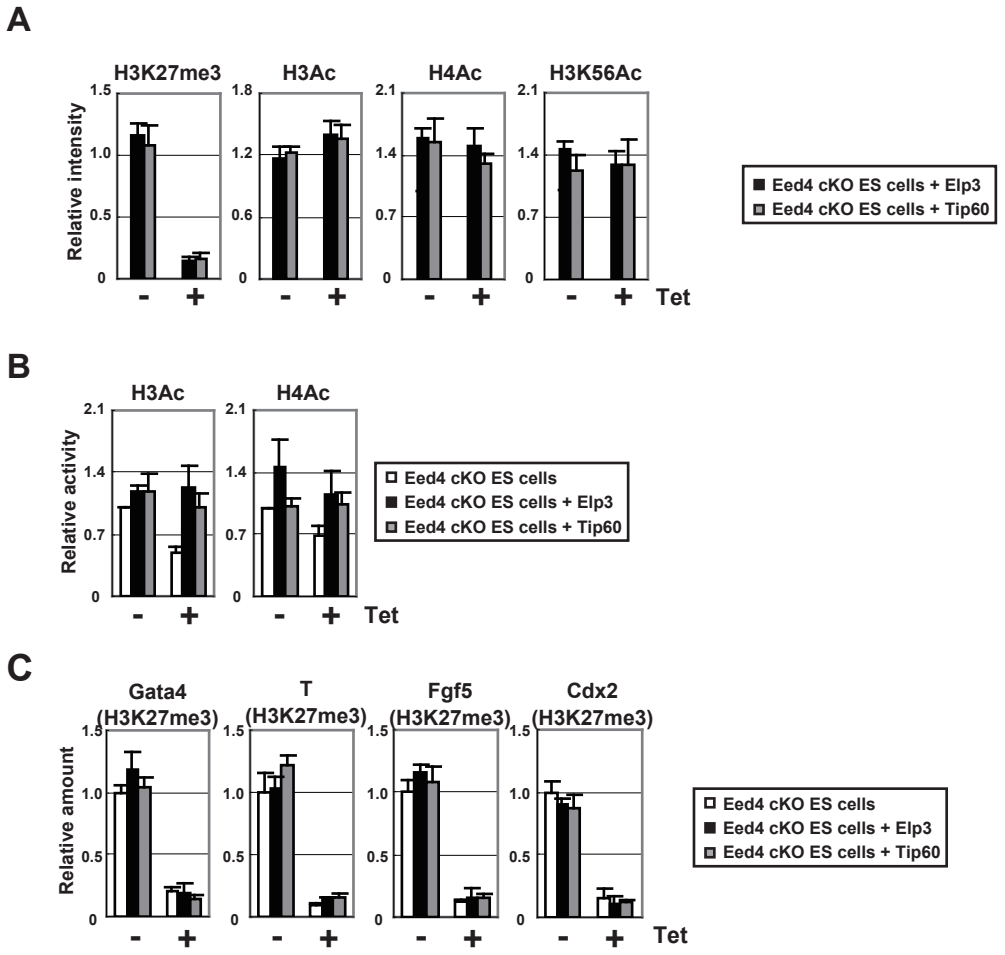

A
- Tet
+ Tet
Control
Eed
Sox2
B
Percentage of compact colony
100
75
50
25
0
Control
Eed1
Eed4
Sox2
C
Sox2
Eed
Oct3/4
Nanog
Dax1
Relative expression
Tet
-
+
2TS22C
2TS22C + Eed1
2TS22C + Eed4
2TS22C + Sox2
Fgf5
T
Gata4
Cdx2
Eomes
D
Fgf5 promoter (H3K27me3)
T promoter (H3K27me3)
Gata4 promoter (H3K27me3)
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
Eed4 cKO ES cells + Elp3
Eed4 cKO ES cells + Tip60
Tet
B
H3Ac
H4Ac
Relative activity
Eed4 cKO ES cells
Eed4 cKO ES cells + Elp3
Eed4 cKO ES cells + Tip60
Tet
C
Gata4 (H3K27me3)
T (H3K27me3)
Fgf5 (H3K27me3)
Cdx2 (H3K27me3)
Relative amount
Eed4 cKO ES cells
Eed4 cKO ES cells + Elp3
Eed4 cKO ES cells + Tip60
Tet