STAT3 and Oct-3/4 Control Histone Modification through Induction of Eed in Embryonic Stem Cells

Received for publication, August 30, 2007, and in revised form, January 16, 2008 Published, JBC Papers in Press, January 16, 2008, DOI 10.1074/jbc.M707275200

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Mouse embryonic stem (ES) cells can self-renew in the presence of leukemia inhibitory factor (LIF). Several essential transcription factors have been identified for the self-renewal of mouse ES cells, including STAT3, Oct-3/4, and Nanog. The molecular mechanism of ES cell self-renewal, however, is not fully understood. In the present study, we identified Eed, a core component of Polycomb repressive complex 2, as a downstream molecule of STAT3 and Oct-3/4. Artificial activation of STAT3 resulted in increased expression of Eed, whereas expression of a dominant negative mutant of STAT3 or suppression of Oct-3/4 expression led to down-regulation of Eed. Reporter, chromatin immunoprecipitation, and electrophoretic mobility shift assays revealed that STAT3 and Oct-3/4 directly bind to the promoter region of Eed, suggesting that Eed is a common target molecule of STAT3 and Oct-3/4. We also found that suppression of STAT3, Oct-3/4, or Eed causes induction of differentiation-associated genes as well as loss of Lys27-trimethylated histone H3 at the promoter regions of the differentiation-associated genes. Suppression of STAT3 and Oct-3/4 also resulted in the absence of Eed at the promoter regions. These results suggest that STAT3 and Oct-3/4 maintain silencing of differentiation-associated genes through up-regulation of Eed in self-renewing ES cells.

Embryonic stem (ES) cells are derived from the inner cell mass of the mammalian blastocyst and have two major characteristics, pluripotency and self-renewal (1, 2). Previous studies have identified several essential transcription factors for the self-renewal of mouse ES cells, such as Oct-3/4, Nanog, and STAT3 (3). Oct-3/4 is a POU-family transcription factor involved in inner cell mass formation (4). A precise level of Oct-3/4 expression is required for maintenance of ES cells: repression of Oct-3/4 leads to trophectodermal differentiation, and overexpression of Oct-3/4 stimulates differentiation, mainly to extraembryonic endoderm (5). Nanog is a homeodomain transcription factor whose overexpression sustains ES cell self-renewal (6). Targeted disruption of the nanog gene results in ES cell differentiation, primarily along the primitive endoderm lineage, suggesting that Nanog prevents ES cells from endoderm differentiation (7).

The pluripotency and self-renewal of mouse ES cells can be maintained by the presence of leukemia inhibitory factor (LIF). LIF stimulation leads to the activation of transcription factor STAT3. Previously, using a fusion protein consisting of STAT3 and the ligand-binding domain of estrogen receptor (STAT3ER), we demonstrated that the self-renewal of ES cells can be maintained by activation of STAT3ER with a synthetic estrogen receptor ligand, 4-hydroxytamoxifen (4HT), even in the absence of LIF (8). Another study showed that expression of a dominant negative mutant of STAT3 causes differentiation of ES cells (9). These observations indicate that the activation of STAT3 is essential and sufficient for the self-renewal of mouse ES cells.

Despite having found the essential transcription factors, the molecular mechanism behind ES cell self-renewal is poorly understood. When ES cells undergo differentiation, chromatin modification changes dynamically, and expression of a set of differentiation-associated genes is induced, suggesting that in self-renewing ES cells, the expression of the differentiation-associated genes is suppressed, at least partially, through chromatin modification (10). It is well known that the N-terminal domain of histone is subject to multiple post-translational modifications, such as acetylation, methylation, phosphorylation, and sumoylation (11, 12). These modifications are believed to influence the transition between the open and compacted chromatin structures and to correlate with the activated or repressed status of gene promoters. It is easy to speculate, therefore, that the essential transcription factors in ES cell self-renewal suppress differentiation-associated genes through regulation of histone modification.

In this study, we searched for a molecule that would regulate histone modification in self-renewing ES cells, identifying embryonic ectoderm development (Eed) as a downstream mol-
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cule of STAT3 and Oct-3/4. Eed is a major component of Polycomb repressive complex (PRC)-2, which is involved in methylation of histone H3 at Lys27. We found that both STAT3 and Oct-3/4 directly bind to the promoter region of Eed and regulate its expression at the transcriptional level. We also found that suppression of STAT3, Oct-3/4, or Eed triggers induction of differentiation marker genes and loss of Lys27-trimethylated histone H3 at their promoter regions. Suppression of STAT3 and Oct-3/4 also resulted in disappearance of Eed from the promoter regions. These results suggest that STAT3 and Oct-3/4 stimulate the expression of the eed gene to silence the expression of differentiation-associated genes in self-renewing ES cells.

EXPERIMENTAL PROCEDURES

Cell Culture—ES cell lines A3-1 (13), ZHBTc4 (5), and A3-1 expressing STAT3 (8) were cultured on gelatin-coated dishes with Dulbecco’s modified Eagle’s medium (DMEM) (Nacalai Tesque, Kyoto, Japan) containing 15% fetal bovine serum, 2 mM L-glutamine, 1x nonessential amino acid (Chemicon, Temecula, CA), 1x nucleoside mix (Chemicon), 40 μM β-mercaptoethanol (Sigma), and 0.1% mouse LIF, produced as conditioned media from human embryonic kidney 293 (HEK293) cells expressing LIF. HEK293 cells were cultured in DMEM containing 10% fetal bovine serum.

Plasmid Construction—The mammalian expression vector pCAG-IP was constructed by inserting the sequence of internal ribosomal entry site and the puromycin-resistance gene into pCAGGS (14, 15). Plasmid pCAG-dnSTAT3/IP was constructed by inserting cDNA for a dominant negative mutant of STAT3 and Eed, and Oct-3/4 directly bind to the promoter region of Eed and regulates its expression at the transcriptional level. We also found that suppression of STAT3, Oct-3/4, or Eed triggers induction of differentiation marker genes and loss of Lys27-trimethylated histone H3 at their promoter regions. Suppression of STAT3 and Oct-3/4 also resulted in disappearance of Eed from the promoter regions. These results suggest that STAT3 and Oct-3/4 stimulate the expression of the eed gene to silence the expression of differentiation-associated genes in self-renewing ES cells.
pCAG-IP. The TRE sequence of pTRE-myc (Clontech), the sequence of the Myc epitope tag, and the rabbit β-globin poly(A) sequence of pCAGGS were then combined and inserted into the SspI site of pCAGIP-tTA2 to produce pTRE-tTA2p-myc, to which each cDNA for the Eed isoform was inserted. To establish Eed conditional knock-out ES cells, linearized pBS-Eed-IRES-Hygro was introduced into A3-1 cells by electroporation (240 V, 500 microfarads). Cells were selected with 300 μg/ml hygromycin and subjected to genomic Southern blot analysis. Two of 11 hygromycin-resistant clones were Eed+/− ES cell clones. Tetracycline-inducible vector of each Eed isoform was then transfected into Eed+/− ES cells. After selection with 0.5 μg/ml puromycin, cells were analyzed by Western blotting. Eed+/− ES cells that express Eed in a Tet-dependent manner were electroporated with linearized pBS-Eed-IRES-β-Geo and then selected with 375 μg/ml G418. Genomic Southern blot analyses revealed that, in each case, 1–2% of clones were Eed conditional knock-out ES cell clones.

Genomic Southern Blot Analysis—A 670-bp probe was isolated by PCR with 5′-GGGCCAAACCTTCTCCTGT-3′ and 5′-GTGGCTACTCTGGGATACA-3′. The amplified fragment was cloned into pCRII (Invitrogen) and verified by sequencing. Isolated genomic DNA was digested with Scal and subjected to 1.0% agarose gel electrophoresis. The gel was soaked in 0.25 mM HCl and neutralized in 0.4 n NaOH. The separated genomic DNAs in the gel were then transferred to Hybond-N+ nylon membrane. After UV cross-linking, the membrane was hybridized overnight with 32P-labeled probe in QuikHyb at 68 °C. The membrane was washed in 0.2× SSC, 0.1% SDS at 68 °C, and radiolabeled bands were visualized with BAS-2000.

Chromatin Immunoprecipitation (ChIP) Assay—For the ChIP assay, 1–5 × 106 ES cells were treated with DMEM containing 1% formaldehyde for 10 min at room temperature for cross-linking, which was stopped by a 10-min incubation with 1.5 M glycine. After washing twice, the cells were resuspended in 300 μl of SDS lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS, and protease inhibitors) by pipetting and kept on ice for 20 min. The chromatin was then sonicated into fragments with an average length of 0.5–3 kb. After centrifugation at 15,000 rpm for 10 min, the supernatants were diluted with dilution buffer (50 mM Tris-HCl (pH 8.0), 1.1% Nonidet P-40, 167 mM NaCl, and protease inhibitors). The extracts were pre-cleaned by incubation with 30 μl of protein G-Sepharose beads (Amersham Biosciences) for 6 h. The supernatants were mixed with antibodies for 16 h and incubated with protein G-Sepharose beads for 12 h. The incubated beads were then washed once with radioimmuno precipitation buffer (50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 2% Nonidet P-40, and 0.2% SDS) containing 150 mM NaCl, once with radioimmuno precipitation buffer containing 500 mM NaCl, and once with LiCl wash solution (10 mM Tris-HCl (pH 8.0), 250 mM LiCl, 1 mM EDTA, and 0.5% Nonidet P-40). The washed beads were incubated in elution buffer (10 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA, and 0.5% SDS) at 65 °C for 12 h, followed by phenol/chloroform treatment and ethanol precipitation. ChIP DNA was amplified by standard PCR using Taq polymerase (Ampli-
Expression of Eed is regulated by STAT3 and Oct-3/4. A, down-regulation of Eed protein upon LIF removal. ES cells were cultured in the presence (+) or absence (−) of LIF for the indicated periods. Cells were harvested, and cell lysates were subjected to Western blot analysis using antibodies against Eed and lamin B1. Note here that the anti-Eed antibody recognizes all Eed isoforms. Lamin B1 was used as an internal control. B, effect of LIF removal on mRNA levels of Eed, Ezh2, and Suz12. After culture with (+) or without (−) LIF for the indicated periods, ES cells were subjected to Northern blot analysis. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. C, activation of STAT3 induces Eed expression. STAT3ER-expressing ES cells were cultured with (+) or without (−) 4HT in the absence of LIF for 4 days and subjected to Northern blot analysis. D, expression of dominant negative STAT3 (dnSTAT3) reduces the expression level of Eed mRNA. After transfection with pCAG-IP (control) or pCAG-dnSTAT3-IP (dnSTAT3), ES cells were selected with puromycin for 2 days and subjected to Northern blotting. E, Oct-3/4 regulates Eed expression. ZHBTc4 cells were cultured in the presence of LIF with (+) or without (−) Tet for the indicated period. To restore the expression of Oct-3/4, the culture medium of Tet-treated cells was changed to a Tet-free medium, and cells were cultured for one more day (+ Tet1d/− Tet1d). The expression level of Eed was examined by Northern blotting. F, effect of Nanog knockdown on Eed expression. After transfection with pSilencer 3.1-H1 puro (control) or pSil-H1p-Nanog (Nanog RNAi), ES cells were cultured for 2 days in the presence of puromycin and subjected to Northern blotting.

To confirm that Eed is a downstream molecule of STAT3, we examined the effect of STAT3ER and dominant negative mutants of STAT3 (dnSTAT3) on Eed expression. In agreement with DNA chip analysis, the expression level of Eed did increase in ES cells expressing STAT3ER when cultured with 4HT (Fig. 1C). Ectopic expression of dnSTAT3, on the other hand, resulted in down-regulation of Eed expression (Fig. 1D). These results suggested that the expression of Eed is controlled by STAT3 in ES cells.

We next examined the relationship of Eed to Oct-3/4 and Nanog, other important transcription factors for ES cell self-renewal. To examine the involvement of Oct-3/4 in Eed expression, we used ZHBTc4 ES cells, in which tetracycline (Tet) stimulation leads to down-regulation of Oct-3/4 expression (5). ZHBTc4 ES cells were treated with or without Tet, and the expression levels of Eed and Oct-3/4 were determined. Consistent with a previous report (5), Tet stimulation repressed the expression of Oct-3/4 in ZHBTc4 ES cells, and its expression was recovered by removal of Tet (Fig. 1E). In good correspondence with Oct-3/4 expression, the expression level of Eed was decreased by Tet stimulation and restored after Tet removal, suggesting that Eed is a downstream molecule of Oct-3/4 in ES cells.

To find out whether Nanog is also involved in the regulation of Eed expression, we knocked down Nanog by RNA interference and examined its effect on Eed expression. Knockdown of Nanog resulted in induction of GATA4 and GATA6 (data not shown), as reported previously (7). In contrast, the expression level of Eed was not altered, regardless of down-regulation of Nanog (Fig. 1F), suggesting that Nanog is not a major regulator of Eed. However, we could not exclude the possibility that suppression of Nanog was not enough to influence Eed expression.

STAT3 and Oct-3/4 Directly Bind to the Promoter Region of Eed—To clarify whether Eed is a direct target of STAT3 and Oct-3/4, we first performed a reporter assay. Since there are several putative binding sites for STAT3 and Oct-3/4 in a 2.6-kb upstream region (−2600/−13) of the eed gene, we cloned this region into a reporter plasmid carrying the luciferase gene (Fig. 2A). Consistent with the results of Northern blot analysis, either expression of dnSTAT3 or suppression of Oct-3/4 expression led to the reduction of promoter activity, whereas knockdown of Nanog had no effect (Fig. 2B). Moreover, combined expression of dnSTAT3 and Oct-3/4 siRNA resulted in further reduction of promoter activity. These results suggested that the 2.6-kb fragment contains the binding sites for STAT3 and Oct-3/4. To search for these sites in this fragment, we constructed three deleted mutants of the promoter (−2220/−13, −1400/−13, and −400/−13) and examined the effect of dnSTAT3 and Oct-3/4 siRNA on their promoter activities. dnSTAT3 showed no effect on any of the deleted mutants. Oct-3/4 siRNA reduced the activity of −2220/−13 as compared with the control siRNA but showed no effect on −1400/−13 and −400/−13. These results suggest that the binding sites for STAT3 and Oct-3/4 are located in −2600/−2220 and −2220/−1400, respectively. Based on several target sequences of STAT3 that have been reported (17, 18, 19), the consensus binding sequence of STAT3 seems to be 5′-TTC(C/T)N(A/G)GAA-3′, where N represents any nucleotide. When we looked for this sequence in −2600/−2220, one similar sequence (5′-TTCTGATAA-3′) was found at −2235/−2227. Disruption of this sequence by mutagenesis reduced promoter activity, suggesting that STAT3 binds with this sequence (Fig. 2C). Similarly, we found two candidate sequences for the Oct-3/4-binding site in −2220/−1400, and mutation analysis suggested that Oct-3/4 binds to 5′-AGGAG-
CAT-3’ at −2019/−2012 but not to 5’-GAATGCAT-3’ at −2089/−2082. In good correspondence with the synergistic effect of dnSTAT3 and Oct-3/4 siRNA on the Eed promoter (Fig. 2B), disruption of both STAT3- and Oct-3/4-binding sites further reduced promoter activity.

We next conducted a ChIP assay with antibodies against STAT3 and Oct-3/4. As shown in Fig. 3, A and B, the DNA fragment encompassing from −2250 to −1882 was co-precipitated with both STAT3 and Oct-3/4. On the other hand, the DNA fragment from −382 to −12, which does not contain any STAT3- and Oct-3/4-binding site, was not co-precipitated, suggesting the in vivo association of STAT3 and Oct-3/4 with the promoter region of Eed through their putative binding sites.

To demonstrate that STAT3 and Oct-3/4 directly bind to their putative binding sites in the Eed promoter, we carried out electrophoretic mobility shift assay. When the STAT3 probe, an oligonucleotide containing the putative STAT3 binding site at −2235/−2227, was incubated with the

FIGURE 2. Identification of binding sites for STAT3 and Oct-3/4 in the promoter region of Eed. A, schematic view of the Eed promoter. B, effect of dnSTAT3 and Oct-3/4 RNA interference on promoter activity. ES cells were transfected with the indicated reporter plasmid and pCAG-dnSTAT3-IP (black bar), pSi-Oct-3/4 (white bar), pSi-H1p-Nanog (dark gray bar), or pCAG-dnSTAT3-IP plus pSi-Oct-3/4 (gray bar). All values are presented as a percentage of the control experiment, in which ES cells were transfected with the empty and/or control RNA interference vector. C, mutational analysis of Eed promoter. ES cells were transfected with the indicated reporter plasmid. Bottom, sequences of one putative STAT3 site and two putative Oct-3/4 sites in each reporter plasmid are shown. Lowercase letters indicate mutated nucleotides. In each experiment, bars represent the means and S.D. (n = 3). WT, wild type.
nuclear extracts isolated from STAT3-expressing HEK293 cells, the mobility of the probe was retarded (Fig. 3C, lane 2). In contrast, no retardation was observed in the case of nuclear extracts prepared from HEK293 cells (lane 6), suggesting that exogenous STAT3 in the extracts binds with the STAT3 probe. This binding was competed by an excess amount of unlabeled STAT3 competitor, whereas mutated STAT3 competitor gave no effect on the binding, suggesting that exogenous STAT3 in the extracts binds with the DNA probe. Treatment of the cells with LIF enhanced the binding of endogenous and exogenous STAT3 to the probe (Fig. 3C, lanes 10 and 12). Prior to harvest, the STAT3ER-expressing HEK293 cells were stimulated with LIF (lanes 10 and 12). Prior to incubation, the extracts from STAT3ER-expressing HEK293 cells were incubated with 4HT to activate the DNA binding ability of STAT3ER (lanes 13–16). Nonspecific binding was determined using a 100-fold molar excess of unlabeled competitor DNA (lanes 3, 7, 15, and 19) and mutant competitor DNA (lanes 8, 11, and 20). D, direct binding of Oct-3/4 with its putative binding site. 32P-Labeled Oct-3/4 probe was incubated with recombinant MBP-Oct-3/4. Competitor oligonucleotides were added at a 100-fold molar excess. Arrows, specific complex positions. Results are representative of at least three independent experiments.
these transcription factors should involve the methylation of differentiation-associated genes through Eed. If so, suppression by STAT3 and Oct-3/4 maintain the silencing of differentiation genes through Eed.

Furthermore, as in the case of Eed1, reversible change in cellular morphology and gene expression was observed for all isoforms (Fig. 7).

To prove the involvement of Eed in the STAT3- and Oct-3/4-mediated gene silencing, we examined whether Eed associates with the promoters of the differentiation marker genes. ChIP analysis using anti-Eed antibody showed that the promoter regions of all marker genes but Nodal are indeed occupied with Eed in the presence of LIF stimulation, STAT3 activation, or Oct-3/4 expression (Fig. 8, B–D). When Eed expression was repressed by Tet treatment, association of histone H3 at Lys37 (H3K27), since Eed is a component of PRC2 that catalyzes the H3K27 methylation. To examine this possibility, we carried out the following experiments for several differentiation-associated markers (Pax3, Fgf5, T, Tbx5, GATA4, GATA6, and Cdx2) whose expression is suppressed by STAT3 and/or Oct-3/4 (Fig. 8A). Nodal, whose expression is not regulated by STAT3 and Oct-3/4, was used as a negative control. First, we determined whether the induction of differentiation markers during ES cell differentiation involved the loss of H3K27 methylation. When the methylation status of H3K27 in total lysates was compared between the presence and absence of LIF, we found that self-renewing ES cells contain a higher level of methylated histone than differentiated cells (Fig. 8B). ChIP assay revealed that LIF stimulation leads to the association of H3K27 methylation with ~1-kb upstream regions of the transcription start sites for Pax3, Fgf5, T, Tbx5, GATA4, GATA6, and Cdx2 (Fig. 8B). In contrast, LIF-dependent association of H3K27 methylation was not observed for Nodal. These results suggested that the H3K27 methylation plays an important role in LIF-stimulated silencing of these genes. We next examined whether STAT3 and Oct-3/4 are involved in the regulation of this modification. Either suppression of STAT3 activity or repression of Oct-3/4 expression resulted in the reduction of H3K27 methylation activity in total cell lysates (Fig. 8, C and D), suggesting that histone methylation is regulated by these transcription factors.

self-renewal of Eed-null ES cells. By introduction of a Tet-dependent expression system of Eed2, -3, and -4 into Eed+/− ES cells, we established the Eed cKO cell lines 2C9, 3D9, and 4D2. Northern (C) and Western (D) blot analyses of Eed cKO ES cells. Parental ES cells were cultured in the presence or absence of LIF for 4 days, and Eed cKO ES cells were cultured in the presence of LIF with or without Tet for 4 days. Note here that all exogenous Eed isoforms are tagged with the Myc epitope.
H3K27 methylation with these promoters was dramatically inhibited even in the presence of LIF (i.e., in the presence of STAT3 activation and Oct-3/4 expression) (Fig. 9). This was true for all isoforms. Moreover, in good correspondence with the reversibility of gene expression, H3K27 methylation was also recovered by re-expression of Eed. These results suggested that Eed is involved in STAT3- and Oct-3/4-dependent gene silencing of differentiation markers.

**DISCUSSION**

Lysine methylation of histone H3 plays a critical role in epigenetic inheritance of chromatin states (25). For example, methylation at Lys 

in ES cells resulted in inducible expression of the eed gene and demonstrated that Eed is a direct target gene of these transcription factors. Using an inducible expression system, we confirmed that knock-out of the eed gene resulted in induction of several differentiation-associated genes as well as loss of histone methylation. Re-expression of Eed restored these phenotypes, indicating that Eed-mediated silencing of
differentiation markers is reversible. Furthermore, we showed that LIF removal, repression of STAT3 activity, or suppression of Oct-3/4 expression triggers the induction of differentiation-specific genes and the disappearance of Eed from the promoter regions of these genes. Taken together, our present data indicate that in self-renewing ES cells, STAT3 and Oct-3/4 control the expression of Eed to maintain the silencing of multiple differentiation-associated genes.

In addition to Eed, PRC2 contains Ezh2 and Suz12, and these three components are all essential for PRC2 activity. Ezh2 is a catalytically active component, and establishment of Ezh2-deficient ES cells has not been successful (28). As in the case of Eed, although Suz12-null ES cells can be established, loss of this molecule leads to global loss of H3K27 trimethylation and destabilization of Ezh2 (27). To estimate the importance of STAT3- and Oct-3/4-mediated transcriptional regulation of the eed gene in the control of PRC2 activity, we compared the time course of Eed down-regulation during ES cell differentiation with those of Ezh2 and Suz12 (Fig. 1B). When LIF was removed from the culture medium, reduction of Eed mRNA occurred more rapidly than those of the other components. These data suggest that, among the three components, Eed is a primary target for STAT3- and Oct-3/4-mediated PRC2 regulation.

During embryo development, Oct-3/4 expression is restricted in inner cell mass and germ cells, whereas STAT3 is expressed in various kinds of cells. In addition, STAT3 and Oct-3/4 have been reported to be involved in tumor development (29, 30). Since the two transcription factors control Eed expression through direct binding to its promoter region, these factors may utilize Eed for gene silencing, not only in the maintenance of ES cells, but also in embryogenesis and tumorigenesis.

Recent reports have suggested that Eed isoforms may play distinct roles (e.g. in brain development) (24). Although all of the isoforms contain the Ezh2-binding domain, each isoform forms different PRCs; the largest form (Eed1) is predominantly present in PRC2, whereas the two shortest forms (Eed3 and Eed4) are present in PRC3, and Eed2 is in PRC4 (23, 31). From the standpoint of ES cell self-renewal, it was reported previously that the levels of Eed1, Eed2, and Eed4 declined during the differentiation process, whereas that of Eed3 was sustained (31). It was also observed that down-regulation of Eed2 occurred more rapidly among the isoforms. Based on these observations, it was suggested that Eed2 might play an important role in self-renewal. In this study, however,
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we could not find any significant difference among Eed isoforms in the maintenance of self-renewal. All of the isoforms maintained compact colony formation and suppressed expression of differentiation-associated markers, suggesting that all isoforms play a similar role in self-renewing ES cells. Since Eed2, Eed3, and Eed4 are shorter than Eed1 at their N terminus, our data also suggest that the N-terminal portion of Eed is not important for Eed-dependent compact colony formation or gene regulation.

The phenotype of Eed-null ES cells seems to be complicated. Despite expression of various differentiation-associated genes, expression levels of Oct-3/4 and Nanog, critical genes for self-renewal, were not reduced in Tet-treated Eed cKO ES cells. Although Tet-treated Eed cKO ES cells failed to form compact colonies, their morphology was different from that of differentiated ES cells. Eed-mediated change in compact colony formation and gene silencing was reversible. In addition, we were able to culture Eed cKO ES cells in the presence of Tet for more than 1 month (data not shown). On the other hand, our attempt to establish Eed-null ES cells without a Tet-inducible system was unsuccessful (data not shown). From these data, we conclude that Eed-deficient ES cells are not completely differentiated but remain between an undifferentiated and a differentiated state. One reason for this phenotype must be that Eed down-regulation does not lead to induction of all the differentiation-associated genes. For example, the expression level of Nodal remained constant even in the absence of Eed, and induction of T and Fgf5 upon Eed suppression was relatively weak when compared with that upon LIF removal. It is therefore possible that a gene(s) that is essential for ES cell differentiation is not up-regulated in Eed-null cells. Since the expression level of Oct-3/4 was not reduced in Tet-treated Eed cKO cells, good candidates for such genes are Oct-3/4 repressors (GCNF, Coup-tfII, and Coup-tfI) that inhibit Oct-3/4 expression through binding with its promoter region (32, 33). When we examined the expression of these repressors, however, all of them were up-regulated in Eed-null ES cells (data not shown), suggesting the existence of another essential factor for suppression of Oct-3/4.

Identification of essential factors for Oct-3/4 repression and/or differentiation would help explain why Eed-null ES cells stay in the "incomplete" undifferentiated state. Another possible explanation for incomplete self-renewal of Eed-null ES cells is that Eed is required for ES cell differentiation, as in the case of Suz12 (27). It has been reported that Eed mutant embryos died at gastrulation (34). The present study demonstrated that, although Eed was down-regulated upon ES cell differentiation, differentiated ES cells still expressed significant amount of Eed (Fig. 1, A and B). These observations suggest the involvement of Eed in mouse development and probably in ES cell differentiation.

Abolishment of Eed expression resulted in loss of H3K27 methylation and induction of Eed target genes, such as Pax3, Tbx5, and GATA4. Interestingly, although H3K27 methylation at the promoter regions of the T and Fgf5 genes was also strongly decreased in Eed-null ES cells, induction of these genes was relatively small. In addition, we were not able to detect H3K27 methylation at the promoter region of the nodal gene. Considering that expression of many genes is regulated by their transcriptional activators and/or repressors, it is possible that up-regulation of activators and/or down-regulation of repressors, in addition to abrogation of H3K27 methylation, is required for induction of these genes.

In conclusion, we demonstrated that STAT3 and Oct-3/4 regulate Eed expression, through which these two factors control PRC2 activity in self-renewing ES cells and prevent induction of differentiation-associated genes. Although we were not able to find a relationship between Eed and Nanog in the present study, genome-wide location analysis has revealed that Oct-3/4 and Nanog occupy promoter regions of many differentiation-associated genes (35). It is therefore likely that in self-renewing ES cells, critical transcription factors for self-renewal control the differentiation-specific genes by direct association with their promoter regions and/or by expression regulation of proteins that are involved in histone modification.

Acknowledgment—We thank Hitoshi Niwa (RIKEN Center for Developmental Biology, Japan) for ZHBTc4 cells, pGT1.8 IRES-hygro-pA2, and pGT1.8 IRES-β-Geo-pA.

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