Embryonic stem cells (ESCs) possess the capacity to proliferate indefinitely in an undifferentiated state and to differentiate into various cell types in an organism. However, the critical question of how self-renewal and differentiation are precisely regulated in ESCs is not entirely understood at present. Here, we report the essential role of Tbx3, a pluripotency-related transcription factor of the T-box gene family, for both the maintenance of self-renewal of mouse ESCs and for their differentiation into extraembryonic endoderm (ExEn). We show that Tbx3 is highly expressed in ExEn cells in addition to undifferentiated ESCs. Knockdown of Tbx3 expression using tetracycline-regulated Tbx3 siRNA resulted in the attenuation of ESC self-renewal ability and aberrant differentiation processes, including reduced ExEn differentiation but enhanced ectoderm and trophectoderm differentiation. Conversely, inducible forced expression of Tbx3 triggered ExEn lineage commitment. Mechanistically, Tbx3 directly activated the expression of Gata6, an essential regulator of ExEn. Interestingly, Tbx3 modulated H3K27me3 modification and the association of the PRC2 complex with the promoter region of Gata6. Taken together, the results of this study revealed a previously unappreciated role of a pluripotency factor in ExEn differentiation. Additionally, our data reveal that Tbx3 may function through direct binding and epigenetic modification of histones on the Gata6 promoter to maintain the ExEn differentiation potential of ESCs.

The mouse blastocyst contains a trophectodermal epithelium surrounding an inner cell mass that later gives rise to primitive endoderm and epiblast cells. The primitive endoderm will develop into extraembryonic endoderm (ExEn), which ultimately contributes to the yolk sac, whereas the epiblast contains pluripotent cells that generate all of the cell types of the three embryonic germ layers (ectoderm, endoderm, and mesoderm) and some extraembryonic tissues (1, 2). Mouse embryonic stem cells (ESCs) derived from the inner cell mass of blastocyst-stage embryos retain the capacity for unlimited self-renewal and their full developmental potential when cultured in vitro in the presence of leukemia inhibitory factor (LIF). Withdrawal of LIF leads to the disruption of ESC self-renewal and extensive differentiation into various lineages. Understanding the mechanisms governing self-renewal and early lineage commitment is an essential step toward the future utilization of ESC derivatives in clinical applications.

The unique properties of ESCs are conferred by a set of pluripotency-associated transcription factors, including Oct4, Sox2, and Nanog (3–6). Recent studies of the transcriptome and protein interactome of ESCs have further revealed the key role of Oct4/Sox2/Nanog-centered transcriptional and protein interaction networks in controlling ESC identity (7–11). However, the precise molecular mechanisms through which ESCs determine the cell fate between self-renewal and lineage commitment remain largely unknown. In addition to the distinct transcriptional hierarchy in ESCs, a poised epigenetic state also contributes their identity. It has been demonstrated that the chromatin of development-associated genes are occupied by histones (H3) with both permissive lysine-4 and repressive lysine-27 trimethylation (H3K4me3 and H3K27me3) signatures of gene expression that prime these genes for subsequent activation (12, 13). It is an interesting and critical question how these bivalent modifications are established and regulated in the regulatory sequences of key players in lineage commitment. A number of factors and complexes including components of polycomb repressive complex 2 (PRC2) have been implicated in the epigenetic control of ESC self-renewal and differentiation. The identification and investigation of such factors are important in the efficient differentiation of ESCs into desired cell types as well as to reprogram somatic cells to a pluripotent state.

The T-box transcription factor family is important for a variety of developmental processes, and the interaction of complex 2; Tc, tetracycline; EB, embryoid body; qPCR, quantitative real-time PCR; RA, retinoic acid; XEN, extraembryonic endoderm; TS, trophoblast stem; O.E., overexpression.
these factors with H3K27-demethylase and H3K4-methyl-transferase was reported recently (14). Among T-box family members, Tbx3 is the earliest expressed gene in mouse inner cell mass cells, and it is later expressed in ExEn cells (15). Its depletion results in embryonic lethality and defects in the mammary gland, limbs, and yolk sac (16). In ESCs, Tbx3 has been reported to maintain ESC self-renewal (17), and sustained expression of Tbx3 is sufficient to maintain ESCs in an undifferentiated state in the absence of LIF (18). Interestingly, Tbx3 was also shown to improve the germline competency of induced pluripotent stem cells (19). Despite the importance of Tbx3, however, the precise roles of Tbx3 in ESC self-renewal and differentiation processes as well as possible mechanisms underlying its functions have not been well characterized.

In the present study we generated inducible Tbx3 knockdown and Tbx3-overexpressing ESC lines and systemically investigated the functions of Tbx3 in the control of ESC self-renewal and differentiation processes. Our data show that knockdown of Tbx3 disrupted ESC self-renewal and impaired ExEn differentiation while enhancing ectoderm and trophectoderm differentiation. Conversely, overexpression of Tbx3 activated ExEn-specific genes and induced ESC differentiation into ExEn-like cells. Mechanistically, we found that Tbx3 directly regulates Gata6 expression and controls epigenetic modifications at its promoter. Our data reveal a novel function of Tbx3 in sustaining the potential of pluripotent cells to differentiate into ExEn in addition to its known role in maintaining ESC self-renewal and provide new insights into epigenetic regulation of ESC properties.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Culture, and Cell Differentiation—Information for plasmids and cell lines used in this study is provided in the supplemental data. The colony-forming assay procedures, embryoid body formation protocols, and cell differentiation protocols are also described in the supplemental Experimental Procedures.

Inducible Tbx3 Knockdown ESC Lines—siRNA Tbx3 (5′)-, siRNA Tbx3 (7′), and siRNA EGFP-inducible cell lines were established in CGR8 ESCs as previously described (20). Briefly, The CGR8-TetR host ESCs were transfected with the specific siRNA plasmids (see supplemental Experimental Procedures) by electroporation and selected using puromycin (1 μg/ml) and zeocin (60 μg/ml). Single clones were picked and tested for knockdown efficiency. All stable clones were cultured with 0.5 μg/ml puromycin and 30 μg/ml zeocin. siRNA expression was induced after adding 0.1 μg/ml tetracycline (Tc) to the culture medium.

Inducible Tbx3-overexpressing ESC Lines—The inducible Tbx3- and Tbx3 Y149S-overexpressing cell lines were generated in CGR8 cells using a Rosa-Tet system (see supplemental Experimental Procedures for plasmids) as previously described (21). The puromycin-resistant colonies were selected and maintained by 1 μg/ml puromycin and 0.3 μg/ml Tc. Exogenous expression was induced after the removal of Tc from cell culture.

Transient siRNA Knockdown Experiments—Tbx3 was knocked down using Stealth RNAi Duplex Oligonucleotides (Invitrogen) in CGR8 ESCs or F9 embryonal carcinoma cells. siRNA sequences and detailed information are provided in the supplemental Experimental Procedures.

Semi-quantitative RT-PCR and Quantitative Real-time PCR (qPCR)—Total RNA was isolated with TRIzol reagent (Invitrogen) and transcribed into cDNA using oligo(dT)₁₅ and ReverTra Ace reverse transcriptase (Toyobo). qPCR was performed using the ABI PRISM 7900 Fast Real-Time PCR system (Applied Biosystems) and the Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. The primer sequences used for RT-PCR and qPCR are provided in supplemental Table 1.

Western Blotting—Western blot analysis was performed under standard denaturing conditions using the following antibodies at 1:1000 dilution: Tbx3 (Santa Cruz), FLAG (Sigma), α-tubulin (Sigma), Oct4, and Nanog (rabbit polyclonal antibodies raised and affinity-purified in our laboratory). All experiments were performed at least three times with similar results.

Immunofluorescence—ESCs and XEN cells were stained as previously described (22). The confocal images were captured using confocal microscopy (TCS SP5, Leica). The primary antibodies were used with following dilutions: Tbx3 (1:1000, provided by Hitoshi Niwa (23)), Gata4 (1:100, Santa Cruz), and Cdx2 (1:200, BioGenex).

Luciferase Reporter Assays—Luciferase reporter assays were performed using the Dual-Luciferase Assay System (Promega) according to the manufacturer’s instructions. Details are provided in the supplemental Experimental Procedures.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed with CGR8 ESCs using the Fast ChIP method (24) with some modifications. Briefly, cells were cross-linked in 1% formaldehyde, and nuclear extracts were sonicated. Immunoprecipitation was conducted using control IgG or specific antibodies against FLAG (Sigma), H3K4me3 (Abcam), H3K27me3 (Millipore), EZH2 (Cell Signaling Technology), Suz12 (Abcam), and EED (Millipore). Immunoprecipitated DNA and input DNA were recovered using Chelex-100 resin (Bio-Rad) as described (24). The recovered DNA was used for qPCR analysis with specific primers provided in supplemental Table 1.

Statistical Analysis—All values are shown as the means ± S.D. Student’s t-test was used to determine the significance of differences in comparisons. The 0.05 level of confidence was considered statistically significant. All the experiments with statistical analysis were independently performed three times.

RESULTS

Tbx3 Is Highly Expressed in Undifferentiated ESCs and ExEn Cells—Previous studies have reported that Tbx3 expression is down-regulated for several days after LIF withdrawal (18, 25). To determine the expression profiles of Tbx3 during the early stages of LIF withdrawal-induced ESC differentiation, we measured Tbx3 transcript levels from 12 to 36 h after LIF withdrawal. qPCR analysis showed that the level of Tbx3 expression decreased dramatically at 12 h and continued to decrease up to 36 h after LIF withdrawal, indicating that Tbx3 expression responds rapidly to the LIF signal (Fig. IA). To test
whether Tbx3 expression was regulated by other differentiation stimuli, ESCs were induced to differentiate through embryoid body (EB) formation in the presence of LIF. As shown in Fig. 1B, Tbx3 mRNA levels decrease significantly from day 3 to day 6 and remained at a low level until the 9th day of EB formation. Therefore, our results confirmed and extended the previous finding that Tbx3 expression is markedly down-regulated during ESC differentiation.

Because the expression of Tbx3 in other cell types has not been carefully examined, we compared the level of Tbx3 mRNA and proteins in various cell lines, including mouse ESCs (CGR8 and E14T), mouse fibroblast cells (mouse embryonic fibroblasts (MEF) and NIH 3T3), ExEn cells (XEN), and trophoblast stem (TS) cells (26, 27) and found that the Tbx3 expression level was highest in ESC lines and lowest in MEF, NIH3T3, and TS cells, similar to that of Oct4 and Nanog. Unlike Oct4 and Nanog, however, Tbx3 was also expressed in XEN cells at a relatively high level (Fig. 1C and D). This unique expression pattern of Tbx3 implies that it may function differently from Oct4 and Nanog in the control of ESC self-renewal and differentiation.

Knockdown of Tbx3 Compromises ESC Self-renewal and ExEn Lineage Commitment Potential in Undifferentiated ESCs—To systemically investigate the role of Tbx3 in ESCs, we established ESC lines that were stably integrated with Tc-inducible siRNAs targeting Tbx3 sequences. Two isoforms of Tbx3 exist in mammalian cells. Tbx3 isoform 2 (NM_198052.1) differs from Tbx3 isoform 1 (NM_011535.2) in its DNA binding domain, where it lacks 20 amino acids (28). To silence the expression of both isoforms, our siRNA sequences targeted their overlapping regions. To ensure the specificity of the siRNA, two independent sequences of Tbx3, siRNA Tbx3 (5) and siRNA Tbx3 (7), were selected, and multiple single clones were amplified for each sequence. Stable ESC lines of siRNA Tbx3 were also generated to act as a Tc treatment control. As shown in Fig. 2, A and B, the addition of Tc knocked down Tbx3 mRNA and protein levels efficiently, whereas it did not affect Tbx3 expression in siRNA EGFP-expressing cells, validating the specificity and efficiency of Tbx3 silencing using Tc treatment.

Once these ESC lines were available, we first evaluated the role of Tbx3 in the control of the ESC self-renewal ability using colony-forming assays (6, 29). In both Tbx3 siRNA cell lines, the total number of ALP-positive colonies decreased significantly in the presence of Tc compared with that in the absence of Tc (Fig. 2C and D). Furthermore, the percentage of differentiated colonies increased in Tbx3 knockdown cells (Fig. 2C and D). In contrast, treatment with Tc altered neither the total number nor the percentage of differentiated colonies in siRNA EGFP-expressing ESCs. This finding is consistent with previous reports that Tbx3 is essential for the maintenance of ESC self-renewal (17, 19). It is worth mentioning that the number of colonies in siRNA Tbx3 (7) cells in the absence of Tc was higher than that in siRNA EGFP or Tbx3 (5) cells, implying the variation in the capacity of colony-forming among various cell lines. To characterize the

**FIGURE 1.** Tbx3 is expressed in both undifferentiated ES and ExEn cells. A, Tbx3 expression levels in CGR8 ES cells after LIF withdrawal at the indicated time points were determined by qPCR. B, the expression pattern of Tbx3 in EBs aggregated from CGR8 ES cells at the indicated time points was analyzed by qPCR and Western blot (WB) analysis, respectively. β-Tubulin was used as a loading control. C and D, levels of Tbx3 mRNA and proteins in different cell lines were analyzed by qPCR and Western blot (WB) analysis, respectively. E, immunostaining of Tbx3 and Oct4 in CGR8 ES cells and XEN cells is shown. Scale bar, 75 μm. MEF, mouse embryonic fibroblasts; **, p < 0.01.
functional role of Tbx3 in the regulation of the differentiation potential of ESCs cultured under self-renewal conditions, we monitored the expression levels of pluripotency-associated and differentiation-associated marker genes by qPCR. After knockdown of Tbx3, the expression levels of ectoderm markers (Fgf5 and Pax6) and trophectoderm markers (Cdx2 and Psx1) increased obviously, whereas the expression of Oct4 and Nanog as well as mesoderm markers (T, Gsc, and Mixl1) remained unchanged, suggesting that Tbx3 may specifically suppress ectoderm and trophectoderm commitment. Notably, the expression of all of the ExEn genes examined (Dab2, Gata4, Sox17, and Gata6) was significantly repressed in Tbx3.
knockdown cells (Fig. 2, E and F), implying a potential role of Tbx3 in ExEn lineage commitment. As a negative control, Tc treatment did not alter the expression levels of any of the genes tested in the siRNA EGFP-expressing cells ( supplemental Fig. S1). To further validate the specific effect of Tbx3 on gene expression in ESCs, we repeated the experiments using two synthesized siRNA duplex oligonucleotides (oligos) against Tbx3, and similar results were obtained ( supplemental Fig. S2). These results indicate that Tbx3 is not only necessary for ESC self-renewal but is also probably required for maintaining appropriate levels of ExEn genes.

Down-regulation of Tbx3 Impairs the ExEn Differentiation Program in Differentiating ESCs—The functional role of Tbx3 described above was found when ESCs were cultured under the self-renewal conditions in the presence of LIF. To address the question of what roles Tbx3 plays during ESC differentiation processes, we examined the expression pattern of several differentiation-associated marker genes during the process of EB formation. LIF was included in the culture for the sake of maintaining Tbx3 expression and for more clearly displaying its effect. Data from qPCR analysis showed that knockdown of Tbx3 substantially compromised the induction of the endoderm marker Gata6, but it enhanced the activation of the trophectoderm marker Cdx2 and the ectoderm marker Fgf5 during EB formation in both siRNA Tbx3 (5)- and siRNA Tbx3 (7)-expressing cells (Fig. 3, A and B). This finding is consistent with the notion that silencing Tbx3 expression impairs ExEn differentiation while enhancing trophectoderm and ectoderm differentiation.

To exclude the possibility that the aberrant differentiation of one germ layer was due to altered differentiation processes occurring in another germ layer during EB formation in Tbx3 knockdown cells, we adopted lineage-specific differentiation models to clarify the effect of Tbx3 knockdown. First, we cultured the established inducible cell lines under trophectoderm differentiation conditions (27). As expected, without Tc treatment, few siRNA Tbx3 (5)- or siRNA Tbx3 (7)-integrated cells differentiated into Cdx2-positive trophectoderm cells. However, knockdown of Tbx3 markedly increased the number of Cdx2-positive cells (Fig. 3C). Consistent with this, the transcript levels of trophectoderm markers (Cdx2 and Hand1) were also significantly up-regulated in the Tbx3 knockdown cells (Fig. 3D).

Next, ESCs were induced to differentiate into endoderm cells by retinoic acid (RA) treatment. Successful differentiation was indicated by the appearance of Gata4-positive ExEn-like cells in the untreated siRNA Tbx3 (5/7) and siRNA EGFP cells. Clearly, the number of Gata4-positive cells was substantially reduced when Tc was added to silence Tbx3 expression (Fig. 4A). Data from qPCR analysis also showed reduced transcript levels of endoderm markers (Gata4 and Gata6) in Tbx3 knockdown cells (Fig. 4B).

Finally, we utilized F9 cells, which have been often used as a model to study RA-induced ExEn differentiation (30), to further investigate the role of Tbx3 in the ExEn differentiation process. As expected, RA treatment significantly activated the expression of ExEn genes (Gata4, Gata6, and Dab2), whereas Oct4, Sox2, and Nanog were gradually down-regulated and became undetectable after RA treatment for 3 or 4 days. Intriguingly, unlike Oct4, Sox2, and Nanog, the expression of Tbx3 was first activated, reached a peak at day 2, and then decreased to a level slightly higher than in cells without RA treatment ( supplemental Fig. S3A). This unique expression pattern again argues for a distinct role of Tbx3 during ExEn lineage commitment. To obtain additional experimental evidence of the involvement of Tbx3 in ExEn differentiation in F9 cells, we knocked down Tbx3 expression using two of the above-described synthesized siRNA duplex oligos against Tbx3 and found that knockdown of Tbx3 significantly suppressed the activation of Gata4, Gata6, and Dab2 during RA-induced F9 cell differentiation into ExEn lineages ( supplemental Fig. S3B). Collectively, our data strongly indicate that Tbx3 is required for ExEn commitment and also functions to suppress trophectoderm and ectoderm differentiation during differentiation processes in pluripotent stem cells.

 Forced Expression of Tbx3 Is Sufficient to Induce ESC Differentiation into ExEn Lineages—To determine whether forced expression of Tbx3 is sufficient to induce ExEn differentiation in ESCs, we overexpressed Tbx3 in ESCs using an episomal expression system that confers high expression levels of the transgene (6). Consistent with a previous report (18), we failed to obtain any ESC colonies in the transfectants, implying that extremely high dosages of Tbx3 were not compatible with ESC survival. To precisely control Tbx3 expression in ESCs, we established ESC lines carrying a Tc-controlled Tbx3 cDNA unit (Tbx3 isoform 1) (21). In such cell lines, exogenous Tbx3 expression was suppressed in the presence of Tc. Withdrawal of Tc resulted in up-regulation of Tbx3 expression to 4–6-fold of the endogenous level, as measured at both mRNA and protein levels (Fig. 5, A and B). Two independent inducible ESC lines, specifically, overexpression (O.E.) Tbx3 (101) and O.E. Tbx3 (102), were utilized in the following experiments.

When cultured at a regular cell density, ESCs overexpressing Tbx3 exhibited differentiated cell morphology, even in the presence of LIF, whereas control ESCs remained as compact undifferentiated ESC colonies (Fig. 5C). Similarly, when cultured in a clonal density, ESCs overexpressing Tbx3 showed substantially lower ALP activities and significantly higher percentages of fully differentiated colonies (~80%) (Fig. 5, D and E). Notably, we found that the differentiated cell morphology of Tbx3-overexpressing cells was similar to that of cells overexpressing Gata6, which is considered a typical morphology of ExEn cells (31) (Fig. 5C). Results from qPCR analysis revealed that ExEn markers (Gata6, Sox17, Nr2f2, and Dab2) were all dramatically activated by forced expression of Tbx3 (Fig. 5, F and G). In addition, a slight increase in the levels of the ectoderm marker Fgf5 and the mesoderm marker T was also observed, whereas we did not observe significant changes in the expression of the trophectoderm marker Cdx2 in Tbx3-overexpressing cells. With respect to pluripotency-associated genes, overexpression of Tbx3 caused a small decrease in the mRNA levels of Oct4 and Nanog. These observations demonstrate that the most prominent phenotype caused by forced Tbx3 expression in ESCs is massive differentiation into ExEn-like cells.
Because Tbx3 isoform 2 is also expressed in ESCs (data not shown), we were interested in determining whether Tbx3 isoform 2 had an effect similar to Tbx3 in ESCs. To this end we introduced Tbx3 isoform 2 into ESCs through the same inducible overexpression system, and similar induction of the expression of exogenous isoform 2 of Tbx3 was obtained by

![Graphs showing expression levels of various markers over time]

**FIGURE 3. Silencing Tbx3 expression results in abnormal ESC differentiation processes.** A and B, qPCR was performed to estimate the expression levels of differentiation markers of ectoderm (Fgf5), trophectoderm (Cdx2), and ExEn (Gata6) in EBs derived from siRNA Tbx3 (5/7) ESCs. The cells were suspended to form EBs with LIF in the presence or absence of Tc at indicated time points. C, immunostaining of Cdx2 in siRNA Tbx3 (5/7) and siRNA EGFP cells cultured under trophoblast stem cell conditions for 4 days in the presence or absence of Tc is shown. Scale bar, 250 μm. D, qPCR analysis of the levels of trophectoderm markers (Cdx2 and Hand1) in cells shown in C at indicated time points. *, p < 0.05; **, p < 0.01.
Tc withdrawal. Forced expression of Tbx3 isoform 2 also triggered ESC differentiation and activated the expression of ExEn marker genes, although to a lesser extent than that of Tbx3 (supplemental Fig. S4). This result is in agreement with similar roles of Tbx3 isoforms that have been found in other physiological contexts (32).

**Transcription Factor Gata6 Is a Direct Target of Tbx3** — To elucidate the molecular mechanism underlying the function of Tbx3 in ESCs, we investigated whether activation of ExEn genes by Tbx3 was dependent on its DNA binding activity. A non-DNA binding mutant of Tbx3 (Y149S) was introduced into ESCs through the same inducible overexpression system, designated Tbx3 Y149S (301), because the Tyr-149 mutant form of Tbx3 (Fig. 6A) is known to impair its DNA binding activity (33). Tbx3 Y149S protein was induced to a level similar to exogenous wild type Tbx3 after withdrawal of Tc (Fig. 6B). However, the Tbx3 mutant failed to induce ESC differentiation, as determined by both cell morphology (Fig. 6C) and marker gene expression (Fig. 6D), indicating that the DNA binding activity is indispensable for Tbx3 to induce ExEn differentiation in ESCs. Subsequently, to identify the key downstream target of Tbx3 for its regulatory function in ExEn differentiation, we focused on the ExEn marker genes that rapidly responded to exogenously expressed Tbx3. Among the marker genes examined, the expression of Gata6 was induced immediately at day 1, whereas Sox17 and Gata4 were up-regulated from day 2 and day 3, respectively, after Tbx3 overexpression (Fig. 6E). Strikingly, up-regulation of Gata6 was observed even before the morphological change of the ESCs, which occurred 2 days after Tbx3 overexpression was induced (supplemental Fig. S5). This observation suggests that early activation of Gata6 could be a cause rather than a consequence of ESC differentiation.

To verify that Gata6 is indeed a target gene of Tbx3, we searched the 6-kb upstream sequence of Gata6 and found a conserved Tbx3 binding site at the −1-kb putative promoter sequence of Gata6 and created a mutation at the Tbx3 binding site (Fig. 6F). Luciferase reporter assays showed that knockdown of Tbx3 significantly reduced Gata6 pro-
moter activity, whereas the activity of the mutant promoter was not affected by Tc treatment (Fig. 6G). Importantly, the activity of the mutant Gata6 promoter was markedly lower than that of the wild type promoter. These results indicate that Tbx3 and its binding sites are critical for normal expression of Gata6 in ESCs. Furthermore, we went on to examine the activity of the promoter in cell lines overexpressing wild type Tbx3 (101) and the mutant Tbx3 Y149S (301). Overexpression of wild type Tbx3, but not the mutant form of Tbx3, markedly activated the Gata6 promoter (Fig. 6H), further validating the importance of Tbx3 and its DNA binding activity in the control of Gata6 expression. In addition, as anticipated, neither the wild type nor the mutant form of Tbx3 exhibited any effect on the mutant promoter. Finally, we performed ChIP assays to examine the interaction of Tbx3 with the endogenous Gata6 promoter in vivo using an anti-FLAG antibody in the cell line overexpressing Tbx3 (101). After induction of the expression of FLAG-tagged Tbx3, an obvious association of Tbx3 with the Gata6 promoter, but not with the 3’-UTR sequence of Gata6, was detected (Fig. 6I), indicating the recruitment of Tbx3 to the Gata6 promoter. Taken together, our data clearly demonstrate that Gata6 is a direct target of Tbx3.

Tbx3 Regulates H3K27me3 Modification and PRC2 Complex Recruitment at the Gata6 Promoter—Based on reports that the genomic locus of Gata6 as well as other development-associated genes is bivalently modified by histone modifications (12) and that members of the T-box transcription factor family are associated with H3K27-demethylase and H3K4-methyltransferase to activate the expression of certain developmental genes (14), we sought to determine whether Tbx3 regulates Gata6 expression through affecting these histone modifications. For this purpose we conducted ChIP assays using H3K4me3 and H3K27me3 antibodies in siRNA Tbx3 (5) cells or O.E. Tbx3 (101) cells. Consistent with previous reports (12, 34), the Gata6 promoter was indeed enriched with H3K4me3 and H3K27me3 marks (Fig. 7, A and B). Notably, the association of H3K27me3, but not H3K4me3 was reg-
Dual Functions of Tbx3 in Mouse ESCs

**FIGURE 6.** Tbx3 directly activates Gata6 expression via its DNA binding activity. A, shown is a schematic illustration of wild type Tbx3 (Tbx3) and its DNA binding activity mutant (Tbx3 Y149S). B, expression levels of Tbx3 and Tbx3 Y149S were examined by Western blot (WB) analysis in inducible O.E. Tbx3 (101) and Tbx3 Y149S (301) cells by Tc withdrawal for 3 days. C, shown are phase contrast images of ESCs overexpressing Tbx3 or Tbx3 Y149S, as indicated in B. Scale bar, 100 μm. D, expression levels of endoderm markers were determined by qPCR in O.E. Tbx3 Y149S (301) cells in the presence or absence of Tc for 4 days are shown. WB, Western blot. E, shown is the time course of Gata4, Gata6, Sox17, and Tbx3 expression levels at the indicated time points in O.E. Tbx3 (101) cells induced by Tc withdrawal. F, shown is a schematic illustration of the putative Gata6 promoter-luciferase reporter containing the Tbx3-binding sequence. G, luciferase assays of the 1-kb upstream fragment of the Gata6 reporter in siRNA Tbx3 (5) and siRNA EGFP (enhanced green fluorescent protein) cells with or without Tc treatment for 72 h are shown. These cells were pretreated with Tc for 48 h before transfection. H, luciferase assays of the 1-kb upstream fragment of the Gata6 reporter in O.E. Tbx3 (101) and Tbx3 Y149S (301) cells with or without Tc treatment for 72 h are shown. These cells were pretreated with Tc for 48 h before transfection. I, ChIP assays were performed using rabbit IgG and anti-FLAG antibodies in O.E. Tbx3 (101) cells were cultured with or without Tc for 3 days, and antibody-associated DNA fragments were detected by RT-PCR using Gata6 promoter and 3’UTR primers. mut, mutant; *, p < 0.05; **, p < 0.01.
Depletion of Tbx3 enhanced the H3K27me3 association at the Gata6 promoter, whereas forced expression of Tbx3 reduced this association, although neither manipulation altered H3K4me3 at the same genomic locus (Fig. 7, A and B). This finding suggests that Tbx3 may promote Gata6 expression via inhibiting H3K27me3 modification at the Gata6 promoter in addition to its direct activation of the Gata6 gene.

Given that the H3K27me3 marker is catalyzed by PRC2 complex proteins (EZH2, SUZ12, and EED), we hypothesized that Tbx3 might play a role in the recruitment of PRC2 proteins to the Gata6 locus. To test this hypothesis, we carried out ChIP assays using antibodies against components of the PRC2 complex. Interestingly, knockdown of Tbx3 resulted in an obvious enhancement of EZH2, Suz12, and EED proteins associated with the Gata6 locus (Fig. 7C). Conversely, overexpression of Tbx3 significantly decreased the recruitment of these PRC2 proteins to the Gata6 promoter region (Fig. 7D). Therefore, we speculate that Tbx3 may maintain Gata6 expression in undifferentiated ESCs as well as during ESC differentiation through both its transcriptional activation activity and its ability to influence the chromatin environment by interference with the association of PRC2 proteins to the promoter.

**DISCUSSION**

In this study, by taking advantage of inducible siRNA and overexpression strategies, we systematically defined the role of Tbx3 in the control of self-renewal and early cell fate decisions in mouse ESCs under self-renewal culture conditions as well as during differentiation processes induced by various stimuli. In addition to its known role in ESC self-renewal, we uncovered a previously unrecognized function of Tbx3 in ExEn lineage commitment both under ESC culture conditions and during differentiation processes (Fig. 7E). Moreover, our results revealed that Tbx3 exerts this function through directly activating Gata6 expression as well as modulating histone modifications at the epigenetic level. Therefore, our findings not only reveal a novel role of Tbx3 but also provide
new insights into how pluripotency is maintained from the perspective of genetics as well as epigenetics.

Tbx3 is considered an important component of the self-renewal regulatory circuitry in ESCs, as supported by previous reports and our own data (17, 19). However, the role of Tbx3 in the maintenance of pluripotency and control of ESC differentiation programs is less well understood. We found that, similar to other self-renewal regulators, knockdown of Tbx3 led to the activation of certain lineage marker genes, including ectoderm and trophectoderm markers, suggesting that it acts to repress the expression of these lineage regulators to sustain ESCs in an undifferentiated state. A surprising finding of our experiments is that the expression of endoderm markers was significantly reduced when Tbx3 was silenced, suggesting an essential role for Tbx3 in the maintenance of a basal level of ExEn lineage markers. This finding supports the notion that Tbx3 is indispensable for the maintenance of pluripotency in ESCs as well as self-renewal.

Our data show that down-regulation of Tbx3 compromised ExEn lineage commitment in several differentiation models and that forced expression of Tbx3 was sufficient to induce ESC differentiation into ExEn lineages. These findings correspond well with the expression of Tbx3 in the ExEn lineages during early embryo development and the yolk sac defect in Tbx3 knock-out mice (15, 16). In fact, several other pluripotency-associated factors have also been reported to participate in the differentiation of distinct lineages during development. For example, Oct4 is known to be required for neuroectoderm differentiation in ESCs and for mesoderm and cardiac commitment (35, 36). Sox2 has been reported to contribute to the development of embryonic and extraembryonic ectoderm (37). Forced expression of these genes leads to ESC differentiation (3, 38), which has been attributed to their failure to activate self-renewal-associated genes due to the abnormally high gene dosages in these experiments. Alternatively, this could also be explained as targeted activation of lineage-specific genes. Another remarkable example is Sall4. Similar to Tbx3, it is highly expressed in both ES and ExEn cells, and it is required for the maintenance of stem cells for these two lineages (34). Interestingly, Tbx3 has been shown to be significantly down-regulated in Sall4-depleted ExEn cells (34), suggesting that Tbx3 might be a downstream factor of Sall4 in ExEn development. Further investigation is required to clarify the similarity and differences between the functions of Tbx3 and Sall4 in the cell fate decisions of stem cells.

Tbx3 controls ESC fate decision in a tightly controlled dosage-dependent manner. Our data showed that overexpression of Tbx3 caused ESC differentiation in the presence of LIF, whereas Niwa et al. (18) reported that sustained expression of Tbx3 in the absence of LIF maintains ESC self-renewal. These phenomena could be explained by different dosages of Tbx3 and the presence or absence of LIF in two studies. In fact, our inducible overexpression system resulted in an approximately 4–6-fold increase in the expression of Tbx3 in the presence of LIF, whereas Niwa et al. (18) were able to generate a stable ESC line with a normal level of Tbx3 in the absence of LIF. In further support of this explanation, we found that overexpression of Tbx3 in the absence of LIF could partially rescue the ESC differentiation induced by LIF withdrawal (supplemental Fig. S6). Therefore, it is reasonable to speculate that Tbx3 at different dosages might associate with different partners and activate or suppress distinct downstream genes.

The transcriptional networks of pluripotency factors for maintaining self-renewal have been extensively analyzed. However, understanding how they promote distinct lineage differentiation remains elusive. Here, we show that Tbx3 positively regulates the expression of the master ExEn regulator Gata6 and demonstrate that Tbx3 is both necessary and sufficient to directly activate the transcription of Gata6. Nevertheless, we do not exclude the possibility that other factors in addition to Gata6 also play roles in this process. Interestingly, we observed that Tbx3 inhibited the repressive H3K27me3 modification and the enrichment of PRC2 proteins at the Gata6 gene, providing the first experimental evidence for the modulation of gene expression by Tbx3 through an epigenetic mechanism in ESCs. It is of note that Tbx3 did not affect the level of H3K4me3 at the Gata6 promoter, supporting the possibility that the H3K27- and H3K4-methyl modification complexes could be uncoupled, allowing a multistep model to act to finely tune the epigenetic status of individual downstream genes in a context-specific manner.

In conclusion, our data reveal that Tbx3 is essential for both ESC self-renewal and ExEn differentiation and identify Gata6 as a direct target of Tbx3. Our results provide new insight into the bifunctional role of the pluripotency factor Tbx3. Further investigation of how these key factors contribute to cell fate decisions in a spatial and temporal manner will contribute to efficient differentiation of ESCs into desired cell types as well as reprogramming of adult somatic cells into stem cells or other cell types.

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