The chromatin remodeler Chd4 maintains embryonic stem cell identity by controlling pluripotency- and differentiation-associated genes

The unique properties of embryonic stem cells (ESCs), including unlimited self-renewal and pluripotent differentiation potential, are sustained by integrated genetic and epigenetic networks composed of transcriptional factors and epigenetic modulators. However, the molecular mechanisms underlying the function of these regulators are not fully elucidated. Chromodomain helicase DNA-binding protein 4 (Chd4), an ATPase subunit of the nucleosome remodeling and deacetylase (NuRD) complex, is highly expressed in ESCs. However, its function in ESC regulation remains elusive. Here we report that Chd4 is required for the maintenance of ESC self-renewal. RNAi-mediated silencing of Chd4 disrupted self-renewal and up-regulated lineage commitment-associated genes under self-renewal culture conditions. During ESC differentiation in embryoid body formation, we observed significantly stronger induction of differentiation-associated genes in Chd4-deficient cells. The phenotype was different from that caused by the deletion of Mbd3, another subunit of the NuRD complex. Transcriptomic analyses revealed that Chd4 secured ESC identity by controlling the expression of subsets of pluripotency- and differentiation-associated genes. Importantly, Chd4 repressed the transcription of T box protein 3 (Tbx3), a transcription factor with important functions in ESC fate determination. Tbx3 knockdown partially rescued aberrant activation of differentiation-associated genes, especially of endoderm-associated genes, induced by Chd4 depletion. Moreover, we identified an interaction of Chd4 with the histone variant H2A.Z. This variant stabilized Chd4 by inhibiting Chd4 protein degradation through the ubiquitin-proteasome pathway. Collectively, this study identifies the Chd4-Tbx3 axis in controlling ESC fate and a role of H2A.Z in maintaining the stability of Chd4 proteins.

Embryonic stem cells (ESCs), derived from the inner cell mass of blastocyst-stage embryos, possess two unique properties: unlimited self-renewal under proper culture conditions in vitro and pluripotent differentiation potential toward all cell types of an organism (1). These cells are powerful tools for exploring the knowledge of mammalian embryonic development and provide renewable cell sources for regenerative medicine. Investigation of the mechanisms governing ESC self-renewal and stimuli inducing ESCs to differentiate to specific cell types is an essential step for the clinical use of ESC derivatives. Years of research in ESCs have demonstrated that the major regulation networks in ESCs are composed of intracellular regulators and extracellular signal-mediated pathways (2). The transcription factors Oct4, Sox2, and Nanog constitute the core regulatory circuitry for the maintenance of ESCs at an undifferentiated state, whereas leukemia inhibitory factor (LIF) is one of the major signaling pathways required for mouse ESCs to self-renew (3, 4). Moreover, researchers from different groups have identified additional self-renewal-associated transcriptional factors, such as Esrrb (5), Tbx3 (5), Tcl1 (5), Cnot3 (6), Trim28 (6), and Klf6 (7–9), forming expanded pluripotency-associated transcriptional networks. Recently, the epigenetic mechanism has attracted increasing attention and added more layers to ESC regulatory networks (10). It becomes necessary to understand how the cross-talk between epigenetic and genetic factors contributes to the control of ESC identity.

The NuRD complex is an epigenetic regulation-related protein complex functioning as a key factor in various important biological processes. It has the unique composition of two distinct functional enzymatic activities exercised by the

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2The abbreviations used are: ESC, embryonic stem cell; Chd, chromodomain helicase DNA-binding protein; LIF, leukemia inhibitory factor; NuRD, nucleosome remodeling and deacetylase; MEF, mouse embryonic fibroblast; Tc, tetracycline; Dox, doxycycline; KD, knockdown; eGFP, enhanced GFP; qRT-PCR, quantitative real-time PCR; PI, propidium iodide; EB, embryoid body; DEG, differentially expressed gene; GO, gene ontology; IP, immunoprecipitation; FCA, flow cytometry analysis.

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Embryonic stem cells (ESCs), derived from the inner cell mass of blastocyst-stage embryos, possess two unique properties: unlimited self-renewal under proper culture conditions in vitro and pluripotent differentiation potential toward all cell types of an organism (1). These cells are powerful tools for exploring the knowledge of mammalian embryonic development and provide renewable cell sources for regenerative medicine. Investigation of the mechanisms governing ESC self-renewal and stimuli inducing ESCs to differentiate to specific cell types is an essential step for the clinical use of ESC derivatives. Years of research in ESCs have demonstrated that the major regulation networks in ESCs are composed of intracellular regulators and extracellular signal-mediated pathways (2). The transcription factors Oct4, Sox2, and Nanog constitute the core regulatory circuitry for the maintenance of ESCs at an undifferentiated state, whereas leukemia inhibitory factor (LIF) is one of the major signaling pathways required for mouse ESCs to self-renew (3, 4). Moreover, researchers from different groups have identified additional self-renewal-associated transcriptional factors, such as Esrrb (5), Tbx3 (5), Tcl1 (5), Cnot3 (6), Trim28 (6), and Klf6 (7–9), forming expanded pluripotency-associated transcriptional networks. Recently, the epigenetic mechanism has attracted increasing attention and added more layers to ESC regulatory networks (10). It becomes necessary to understand how the cross-talk between epigenetic and genetic factors contributes to the control of ESC identity.

The NuRD complex is an epigenetic regulation-related protein complex functioning as a key factor in various important biological processes. It has the unique composition of two distinct functional enzymatic activities exercised by the
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ATPase/helicase Chd3/4 (Mi-2α/β) and the histone deacetylases Hdac1/2 (11, 12). Additional proteins included in this complex are proteins with a methyl-DNA-binding domain (Mbd2/3), WD40 repeat proteins (Rbbp7/4), metastasis-associated proteins (Mta1/2/3), and poorly defined proteins (Gata2a/b) (11). Previous studies have revealed a role of the NuRD complex in the regulation of ESC identity, especially the function of Mbd3, a core functional component essential for the stable formation of the complex (13). Interestingly, Mbd3−/− ESCs can self-renew normally and maintain an undifferentiated state even in the absence of LIF (13). However, Mbd3-depleted ESCs cannot make lineage specification properly in response to differentiation stimuli (13, 14).

Compared with Mbd3, the role of Chd4, the largest component of the NuRD complex, in the regulation of ESC identity is poorly defined. Chd4, also known as Mi-2β, belongs to the class II CHD subfamily. It was originally identified as an autoantigen in dermatomyositis (15). Limited studies of Chd4 in stem cells show that CHD4 is essential for planarian stem cell differentiation and tissue regeneration by contributing to neoblast formation (16) and that it is required for the self-renewal and multilineage differentiation potential of mouse hematopoietic stem cells (17). In ESCs and during somatic cell reprogramming, Chd4 is usually considered as a partner of Mbd3, acting consistently with the NuRD complex as a whole (18, 19). However, increasing studies indicate that there are submodules of large protein complexes in a context-dependent manner. The possibility exists that Chd4 may function as a peripheral subunit of the NuRD complex in different cell types such as ESCs (20, 21).

In this study, we evaluated the specific function of Chd4 in mouse ESCs and found that, differently from Mbd3, Chd4 is a gene required for ESC self-renewal. Chd4-deficient ESCs have an attenuated self-renewal ability. Interestingly, Chd4 acts as a repressor for the expression of the transcription factor Tbx3, thereby inhibiting lineage specification, especially toward the endoderm. Further, our study reveals that the protein stability of Chd4 is controlled postranslationally. The histone variant H2A.Z interacts with Chd4 and stabilizes Chd4 proteins by preventing 26S proteasome-mediated Chd4 protein degradation. Taken together, this study defines the role of Chd4 in ESC fate determination and identifies the Chd4–Tbx3 axis for the precise control of ESC fate.

Results

Chd4 is required for the self-renewal of ESCs

We began with comparing the expression level of Chd4 between mouse ESCs (CGR8 and J1 ESC lines) and mouse embryonic fibroblast (MEF) cells and found that both mRNA and protein levels of Chd4 were significantly higher in ESCs than in MEF cells (Fig. 1, A and B). The finding implies that Chd4 may have a role in ESCs.

To determine the role of Chd4 for ESC self-renewal, we constructed Tet-On Chd4 RNAi ESC lines stably integrated with inducible Chd4 shRNAs responsive to doxycycline (Dox) treatment. To ensure knockdown (KD) specificity, two stable cell lines (Chd4i-1 and Chd4i-8) with distinct shRNA sequences targeting Chd4 were established. An enhanced GFP RNAi (eGFPi) cell line with an eGFP shRNA sequence constructed in the same system was used as a negative control. After treating the inducible cells of Chd4i lines with Dox, Chd4 expression levels drastically decreased at both mRNA and protein levels, whereas the treatment did not alter the Chd4 level in eGFPi cells (Fig. 1, C and D), confirming the specificity of our induction system. To exclude the effect of Chd4 shRNA on the homolog of Mi-2, Chd3 (also known as Mi-2α), we examined the expression level of Chd3 in Tc-inducible RNAi cell lines (eGFPi, Chd4-i-1, and Chd4-i-8) treated with or without Dox for 4 days. In accordance with a previous report (22), we found that Chd3 expression was much less abundant compared with Chd4 in undifferentiated ESCs (supplemental Fig. 1). Intriguingly, Chd3 mRNA levels were elevated to some extent in Chd4-depleted cells, implying a potential compensatory role. However, the elevated Chd3 level was lower than that of Chd4 after RNAi treatment and not sufficient to affect the effect of Chd4 depletion.

We found that colonies of Chd4-deficient ESCs were smaller than those of control cells and brighter, especially at the edge of the colonies (Fig. 1E). The phenotype could be observed after 4 days of Dox treatment and became more obvious after cell passaging. Moreover, the attachment of ESC colonies to dishes seemed looser than that of control cells, as indicated by the fact that Chd4-deficient ESCs floated during examination of the expression of alkaline phosphatase, an often-used molecular marker of undifferentiated ESCs (data not shown). The loose attachment of Chd4 KD ESCs prevented us from conducting colony formation assays to assess their capacity to self-renew. Instead, we carried out a fluorescence-based competitive assay, which has also been used in determining the self-renewal ability of ESCs (5). For this assay, stable cell lines expressing GFP and shRNAs targeting Chd4 or luciferase were generated and mixed with control ESCs stably expressing non-targeting shRNA (GFP-negative) cells at a 4:1 ratio. The percentages of GFP-positive cells were determined at every passage for six continuous generations. A progressive reduction of GFP-positive cells was observed in Chd4 KD cells for both Chd4i lines (Fig. 1F), indicating that Chd4 depletion impaired the ability of ESCs to self-renew.

To characterize Chd4 depletion-induced phenotypes at a molecular level, we examined the expression levels of pluripotency- and differentiation-associated genes in established inducible RNAi cell lines treated with or without Dox for 4 days. Our qRT–PCR assay results showed that expression of self-renewal-associated genes responded to the silencing of Chd4 differentially. The transcript levels of Oct4 and Rex1 remained unchanged, whereas the levels of Tbx3 and Klf5 increased. Moreover, Chd4 depletion led to reduced Nanog and Klf4 expression (Fig. 1G), suggesting a unique and complicated transcription-regulatory role of Chd4 in ESCs. As for differentiation-associated genes, the expression levels of markers for the mesoderm (T and Mixl1), endoderm (Gata6 and Sox17), trophoblast (Cdx2 and Hand1), and ectoderm (Fgf5) increased to various degrees in Chd4-depleted cells (Fig. 1H), implying that Chd4 might repress the expression of lineage master genes to maintain ESCs at an undifferentiated state. As a negative
control, Dox treatment did not alter expression levels of any genes tested in eGFPi cells (Fig. 1I). Collectively, these results reveal that Chd4 is required for the maintenance of ESC self-renewal, probably through controlling the expression of various sets of important genes.

**Chd4 maintains ESC proliferation and survival**

Usually, disruption of self-renewal genes in ESCs would give rise to differentiated cell morphologies, including enlarged cell size, decreased ratio of the nucleus to cytoplasm, and the loss of compact ESC colonies. In contrast, Chd4-depleted ESCs had reduced self-renewal ability with smaller and tight colonies (Fig. 1E). To determine whether the smaller size of colonies was due to fewer cells in colonies, we seeded the same number of cells in different groups and counted the cells for nine consecutive passages, with 3 days for one passage. Cell growth curves showed that Chd4-deficient ESCs (Chd4i in the presence of Dox) had significantly slower cell growth rates than control cells (Chd4i in the absence of Dox) (Fig. 2A and B). The similar cell growth rate of eGFPi cells in the absence and presence of Dox excluded an influence of Dox on cell growth (Fig. 2C). This result indicated that Chd4 was needed for the normal growth of ESCs. We then examined whether the reduced cell growth of Chd4 KD cells was a result of decreased cell proliferation or increased cell apoptosis. Cell proliferation and apoptosis were examined by BrdU incorporation assay and Annexin V-PI staining assay, respectively. Our results indicated that Chd4 deficiency decreased ESC proliferation ability (Fig. 2D) and increased the proportions of early apoptotic cells significantly (Fig. 2E). These results indicate that Chd4 plays a role in maintaining the normal growth of ESCs, probably by ensuring cell survival and proliferation.

**Chd4 deficiency promotes ESC differentiation**

The abovementioned studies were conducted under culture conditions supporting ESC self-renewal. We wanted to know the role of Chd4 during ESC differentiation. To this end, we performed differentiation assays both in vitro and in vivo. We cultured ESCs in suspension for the formation of embryoid bodies (EBs), a widely used ESC differentiation strategy (23),...
and compared the expression profiles of lineage marker genes between control (in the absence of Dox) and Chd4 KD cells (in the presence of Dox) on days 0, 3, 6, and 9 of EB formation. Specific and efficient KD of Chd4 expression by Dox treatment during EB formation was first validated (Fig. 3, A and C). Moreover, the normal induction of marker genes for various lineages, including Fgf5, T, Gata6, and Cdx2, during EB formation was indicated by a gradual up-regulation of these genes in control cells, similar to the expression pattern reported previously during EB formation (24). Significantly stronger induction of all these lineage marker genes was found in Chd4 KD cells compared with control cells at all time points examined (Fig. 3 B).

However, Dox treatment did not alter the expression profiles of these genes in eGFPi cells, excluding the possibility that changes of gene expression in Chd4i cells were caused by the Dox treatment (Fig. 3 D). The finding indicated that depletion of Chd4 promoted ESC differentiation during EB formation. In addition, we examined the expression patterns of Oct4 and Tbx3 in the same differentiation system. Both genes were quickly and substantially down-regulated on day 3 of EB formation and maintained at low levels until day 9 in control cells (supplemental Fig. 2, A–D), consistent with their known expression patterns during differentiation (24). Similar to ESCs cultured under self-renewal conditions, Chd4 KD cells did not alter Oct4 levels while significantly elevating Tbx3 levels on different days of EB formation, further supporting the notion that Chd4 has a repressive effect on Tbx3 expression.

As Mbd3 and Chd4 are both in the NuRD complex, and our data indicated that silencing of Chd4 induced phenotypes entirely different from those reported for Mbd3 under both self-renewal and differentiation culture conditions, it became necessary to re-examine the function of Mbd3 to verify the difference. To this end, we generated two ESC lines (Mbd3i-1 and Mbd3i-2) stably transduced by lentiviruses carrying Mbd3 shRNAs along with one control line (Ni) using non-targeting shRNA. The Mbd3 KD efficiency was first validated (Fig. 3 E). With these lines, we evaluated marker gene expression patterns in the same EB formation system. In accordance with reported results, the induction of all differentiation genes tested was blocked in Mbd3 KD cells on day 3 of EB formation. Significantly impaired induction of Gata6 and Cdx2 expression was observed at all time points examined (Figs. 3 F). These data indicated that, different from Chd4, Mbd3 KD impaired ESC differentiation. Therefore, Chd4 and Mbd3 have distinct functions in ESCs both at the undifferentiated state and during differentiation.

Furthermore, we assessed the developmental potential of Chd4 KD ESCs in vivo by teratoma formation assays. We injected Chd4 KD ESCs (Chd4i-1 and Chd4i-8) stably transduced by lentiviruses carrying Chd4 shRNAs, along with Ni ESCs stably transduced by non-targeting shRNA, into immune-deficient mice. For each mouse, we injected the Ni cells into the left leg and one Chd4 KD cell line into the right leg. After 3 weeks, we observed teratomas in all injected mice, and there was no obvious difference in the size of teratomas in the two legs of the same mouse. At the fourth week after injection, we isolated teratomas and found that teratomas derived from Chd4 KD ESCs were bigger than those derived from Ni ESCs (Fig. 3 G and supplemental Fig. 2 F). This is in agreement with the results of our in vitro assays, which indicated that Chd4 KD promoted ESC differentiation.
KD and Ni ESCs contained various cell types or tissues of all three germ layers, including pigmented neural rosettes (ectoderm), cartilage (mesoderm), and goblet cells (endoderm) (Fig. 3H and supplemental Fig. 2F). These results support the notion that Chd4-deficient ESCs sustain and even have more robust developmental potentials.

**Chd4 recruits Suz12 at the Tbx3 promoter to prevent aberrant expression of Tbx3**

To understand how Chd4 KD induced the up-regulation of lineage marker genes, we tested the role of Tbx3 in Chd4 KD-induced phenotypes, as Tbx3 expression increased significantly in Chd4-depleted cells, and our previous study showed that Tbx3 has dual functions in both ESC self-renewal maintenance and endoderm gene induction (25). To answer the question of whether Chd4 regulated Tbx3 expression directly, we first searched published Chd4 ChIP sequencing data (18) and found the enrichment of Chd4 at the Tbx3 promoter region. Our ChIP-qPCR results validated the binding of Chd4 at the Tbx3 gene (Fig. 4A), implying a direct regulatory role of Chd4 for Tbx3 transcription. Additionally, to obtain more evidence that Tbx3 is indeed a target of Chd4, we cloned three genomic DNA fragments from the Tbx3 promoter region (each about 1 kb surrounding Chd4-binding sites) into a luciferase reporter plasmid, respectively, and transfected these reporter plasmids into 293FT cells together with different amounts (0, 25, 50, and 100
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Figure 4. Chd4 inhibits Tbx3 transcription to prevent lineage specification of ESCs. A, ChIP-qPCR was performed to test the specific binding of Chd4 at the Tbx3 promoter region. The schematic indicates the position of amplified genomic DNA fragments by related primers. The fold enrichment of each group was normalized by control (ctl) IgG values. B, luciferase assays using a luciferase reporter plasmid containing Tbx3 promoter sequences. Three Tbx3 promoter regions around Chd4-binding sites were cloned into a luciferase reporter plasmid and transduced into 293FT cells together with different quantities of a Chd4 expression construct. O.E., overexpression. C, a ChIP-qPCR assay was conducted to examine Suz12 binding at the Tbx3 promoter region in Chd4i-1 ESCs with or without Dox treatment for 3 days. D and G, the transcript levels of Tbx3 and Chd4 in two Dox-inducible cell lines in the absence or presence of Tbx3 siRNA were tested by qRT-PCR. Chd4i-1 and Chd4i-8 ESCs were cultured with or without Dox treatment for 3 days before passaging. A specific Tbx3 siRNA oligo was introduced into Dox-treated Chd4 knockdown cells on the fourth day. RNA samples were collected 2 days later for qRT-PCR assays. E and H, the expression levels of pluripotency markers in the cells described in D and G were examined. F and I, the expression levels of lineage markers in cells described in D and G were examined to test the rescue effect of Tbx3 RNAi on Chd4 deficiency. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

g) of the Chd4 expression construct. The luciferase reporter assay results indicated that Chd4 overexpression significantly inhibited Tbx3 promoter-controlled luciferase activities (Fig. 4B). These data support the notion that Chd4 may directly repress the expression of Tbx3. Second, as it was reported that the NuRD complex could recruit PRC2 to the genomic regulatory region (26), we examined the association of the key component of PRC2, Suz12, at the promoter region of Tbx3 in control and Chd4-deficient cells to address the questions of whether PRC2 would be recruited to the Tbx3 gene and whether Chd4 could regulate PRC2 recruitment. As anticipated, Suz12 was recruited to the Tbx3 locus, and Chd4 KD significantly reduced the recruitment (Fig. 4C). Finally, we tested whether abnormally activated Tbx3 expression caused by Chd4 KD could account for the aberrant induction of differentiation-associated genes. To this end, we transfected Tc-inducible RNAi cells (Chd4i-1 and Chd4i-8) with specific Tbx3 siRNAs after the cells were treated with or without Dox for 4 days to knock down Tbx3 in Chd4 KD cells and collected cells for experiments 2 days after Tbx3 siRNA transfection (Fig. 4, D and G). Our qRT-PCR results showed that Tbx3 KD could rescue Chd4-KD induced down-regulation of the pluripotency gene Nanog but not other tested pluripotency genes (Fig. 4, E and H). This finding is in line with a previous report that Tbx3 overexpression could induce ESC differentiation via repressing Nanog transcription (27). On the other hand, Tbx3 could alle-
violate Chd4 KD-induced up-regulation of marker genes, including T, Gata4, Gata6, Sox17, and Cdx2. Among Chd4 KD-induced genes, elevated expression of Gata6, a master regulator of the endoderm, was most markedly inhibited by Tbx3 deficiency (Fig. 4, F and I), consistent with our previous finding that Gata6 is a direct downstream target of Tbx3 (25). In contrast, Chd4 KD-induced up-regulation of Hand1 and Mixl1 was not affected by Tbx3 RNAi. Therefore, the restriction of Tbx3 expression and, in turn, the maintenance of Nanog expression as well as inhibition of a subset of lineage marker gene expression could underlie the function of Chd4 in maintaining ESCs at an undifferentiated state.

Chd4 controls the transcription program of ESCs

To further elucidate the molecular mechanism of Chd4 to maintain the ESC state and obtain a global view of Chd4 functions, we performed microarray assays using samples from Chd4i-1 cells treated with Dox for 3 days as the experimental group and the same cells without Dox treatment as the control group. Two biological duplicates for each group were used (Fig. 5A). Of all differentially expressed genes (DEGs) with the -fold changes equal or larger than 1.5, 1418 and 1297 genes were up- and down-regulated, respectively, in Chd4-depleted cells. Specific gene ontology (GO) analysis of DEGs revealed that Chd4 activated proliferation-related genes and repressed cell death-related genes, in line with reduced cell proliferation and increased cell apoptosis in Chd4-deficient ESCs. Moreover, DEGs were associated with biological processes such as developmental processes, neuron differentiation, and regulation of signaling, indicating roles of Chd4 in safeguarding stemness by regulating subsets of functional genes (Fig. 5, B and C).

To verify DEGs identified by microarray analyses, we performed qRT-PCR for genes with the enriched GO term of developmental processes, including those involved in neural
specification, such as Chd5 (28), Otx2 (29), Zic2 (30), Zic5 (31), and Rufy3 (32) and the endoderm marker gene Sox7, in Chd4-inducible KD cell lines (Fig. 5D). Consistent with the microarray data, the expression of these genes was up-regulated by Chd4 KD (Fig. 5, E and F). Moreover, the expression of genes enriched in the term of embryonic development with known functions in ESC stemness maintenance, such as Tet genes (33), Foxd3 (32), Gli2 (34), Jak1 (3), and Myc (35), was examined. Indeed, the expression levels of these genes were attenuated after Chd4 was knocked down (Fig. 5, E and F). In contrast, Dox treatment did not change expression levels of these tested genes in eGFPi cells (Fig. 5D). The transcriptome profile analyses indicate that Chd4 could ensure ESC self-renewal by activation of self-renewal-associated genes and repression of lineage specifiers.

**Chd4 proteins are stabilized by the histone variant H2A.Z**

Our experimental data identified Chd4 as an important protein for maintaining the ESC state. Thus, we wanted to know which proteins would associate with Chd4 in ESCs. To find Chd4-associated proteins, we carried out immunoprecipitation experiments using Chd4-specific antibodies and mouse ESC extracts, followed by mass spectrometry analyses. Among the identified proteins, Chd4 ranked high, and there were other subunits of the NuRD complex, including Hdac1, Hdac2, Mta2, and Rbbp4 (supplemental Table 2), proving the specificity and efficiency of our identification system. Unexpectedly, we found some histone variants, including H2A.Z. As H2A.Z was reported to maintain ESC self-renewal (36), we further characterized the interaction between Chd4 and H2A.Z.

Through co-immunoprecipitation assays, we detected H2A.Z in protein complexes precipitated by Chd4 KD (Fig. 6A). A specific interaction between endogenously expressed Chd4 and H2A.Z in ESCs was indicated by Chd4 antibody-mediated immunoprecipitation followed by Western blot analysis using Chd4 and H2A.Z antibodies, respectively. IgG was used as a control for immunoprecipitation. B and C, the protein and mRNA levels of Chd4 and H2A.Z were detected after transfection of the indicated siRNA oligos for 2 days by qRT-PCR and Western blotting, respectively. D, the protein levels of Chd4 and H2A.Z were detected. Tubulin was used as a loading control. ESCs were transfected with the indicated plasmids or siRNA oligos for 2 days and then treated with MG132 or DMSO for 3 h. E, the effect of H2A.Z knockdown on the ubiquitination level of Chd4 was examined. ESCs transfected with the indicated siRNA oligos for 2 days were treated with MG132 for an additional 3 h and harvested for immunoprecipitation assays using Chd4 antibody, followed by Western blotting using ubiquitin antibody. F, the RNAi efficiency of H2A.Z siRNA oligos was tested by Western blotting using H2A.Z antibody, and tubulin was used as a loading control. **, p < 0.01.
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C). These results indicate that H2A.Z may regulate Chd4 at the posttranscriptional level.

We next asked whether Chd4 proteins in ESCs would be controlled by the 26S proteasome-mediated degradation pathway. To answer this question, we compared the protein levels of Chd4 between DMSO-treated and proteasome inhibitor MG132-treated ESCs. Western blot analysis results showed that MG132 treatment obviously reduced the Chd4 protein levels (Fig. 6D), suggesting that Chd4 proteins were probably subject to degradation through 26S proteasomes. Intriguingly, overexpression of H2A.Z also increased Chd4 protein levels in DMSO-treated ESCs to a level comparable with that in MG132-treated ESCs. Importantly, MG132 treatment abrogated the H2A.Z KD-induced reduction in the Chd4 protein level (Fig. 6D). These results indicated that H2A.Z repressed the degradation of Chd4 through the 26S proteasome pathway. As the 26S proteasome usually degrades ubiquitinated proteins (37), we set out to test whether Chd4 proteins could be modified by ubiquitin and, if so, whether the ubiquitination level of Chd4 would be regulated by H2A.Z. By determining ubiquitinated Chd4 protein levels with Chd4-specific antibody-mediated immunoprecipitation and Western blot analysis with an ubiquitin antibody, we were able to show that Chd4 could be ubiquitinated and that H2A.Z KD increased the ubiquitin-modified Chd4 proteins (Fig. 6, E and F), which could lead to enhanced degradation through proteasomes. Therefore, it is likely that H2A.Z stabilizes Chd4 proteins via repressing Chd4 ubiquitination and degradation.

Discussion

NuRD is a bifunctional protein complex combing histone deacetylation and chromatin remodeling activities. Both Mbd3 and Chd4 are important subunits of the complex. The role of Mbd3 in ESC self-renewal and differentiation has been extensively investigated. However, much less is known about the function of Chd4 in ESC fate determination. Here we defined the function of Chd4 in both undifferentiated ESCs and during their differentiation, and investigated the molecular mechanisms by which it functions and is stabilized. Preeminent is the finding that silencing of Chd4 impairs the ability of ESCs to self-renew and enhances differentiation processes. Consistently, a previously conducted genome-wide RNAi screen identified Chd4 as a self-renewal candidate gene whose depletion would reduce Nanog expression (38). We also found reduced Nanog expression in Chd4-depleted cells. Moreover, Chd4 KD reduced the Klf4 transcript level. Therefore, it appears that Chd4 is needed for the normal expression of subsets of self-renewal-associated genes in ESCs. Interestingly, the expression levels of Tbx3 and Klf5 were up-regulated by Chd4 KD, although they are also considered pluripotency-associated genes (8, 39). Thus, Chd4 also has a role in preventing the overexpression of certain pluripotency-associated genes. As overexpression of Tbx3 could lead to ESC differentiation into extraembryonic endoderm lineages (25), suppression of aberrantly activated Tbx3 expression is consistent with Chd4 as a gene functioning to sustain ESC identity. However, it is not known whether Klf5 could act as a lineage specifier or not. An alternative explanation for the up-regulated Klf5 expression is that there might be a negative transcriptional feedback between Klf4 and Klf5, with down-regulation of Klf4 leading to up-regulation of Klf5 in Chd4 KD cells. Further experiments are required to test these hypotheses. Different from our results is that Chd4 was found to be required for Oct4 depletion-induced ESC differentiation (18). We repeated the study and found that silencing of Chd4 did not significantly affect ESC differentiation induced by Oct4 depletion (data not shown). Thus, we consider that Chd4 acts to safeguard ESC identity.

The function of other components in the NuRD complex has been studied. For example, Hdac1 and Hdac2, despite being functionally redundant, were reported to be essential for ESC proliferation and self-renewal by positive regulation of key pluripotency-associated transcription factors (40). Moreover, Mta1, a member of both NuRD and an ESC-specific complex referred as NODE (Nanog and Oct4-associated deacetylases) containing Nanog, Oct4, and NuRD components (Hdac1/2 and Mta1/2 but not Rbbp7 and Mbd3), was shown to be a gene required for ESC self-renewal (41). In contrast, Mbd3 was shown to be dispensable for ESC self-renewal but required for differentiation (13, 42). The required role of Mbd3 for ESC differentiation was verified in this study. Therefore, distinct subunits of the same complex could exhibit opposite functions in ESC fate determination. It is not uncommon that the same protein contributes to the composition of different protein complexes at the same time. For example, the class I Hdacs, Hdac1 and Hdac2, the core subunits of the NuRD complex, can also serve as the catalytic core of the Sin3A and CoREST complexes in mammalian cells (43). Our finding suggests that Chd4 might have a function independent of the classical NuRD complex, its silence therefore giving rise to a phenotype different from that of Mbd3 deletion in ESCs. Supporting this notion, recent studies indicate that Chd4 might only be a peripheral component of the NuRD complex (20) and that it dynamically interacts with the core components of NuRD (21). Taken together, this study reveals a role of Chd4 different from that of the classical NuRD complex.

Our transcriptome profiling analyses reveal that the number of genes up-regulated and down-regulated after Chd4 KD was comparable, implying that Chd4 might also have an active role in gene transcription in addition to being a component of the repressive NuRD complex in ESCs. Indeed, Chd4 KD down-regulated a set of genes essential for the stemness maintenance in ESCs and up-regulated another set of genes promoting lineage commitment. For instance, the expression levels of neural specification- and endoderm-associated genes were significantly elevated in Chd4 KD cells. Moreover, a recent study demonstrated that Chd4 was involved in both RNA polymerase II recruitment at H3K4me3-marked active promoters and also in transcriptional repression at bivalent promoters (44). This is in accordance with our results and explains the functional role of Chd4 in ESC self-renewal maintenance.

Moreover, our study provides evidence of the cross-link between the chromatin remodeler and transcriptional factor in ESCs. We found that Chd4 could directly bind the Tbx3 promoter and repress its transcription, functioning by affecting the recruitment of Suz12, a component of the repressive PRC2 complex, at the locus. Interestingly, the reduction in the ele-
vated Tbx3 expression through Tbx3 RNAi treatment could rescue the Chd4 KD-caused reduction in the expression of the pluripotency gene Nanog and also significantly revert the increased expression of a subset of lineage markers, especially those of the endoderm. The finding indicates that Chd4 may repress differentiation, at least partially, through preventing aberrant activation of Tbx3 expression. The experimental results of our study and previous studies by others support this notion. Overexpression of Tbx3 in ESCs triggered extraembryonic endoderm commitment, whereas silencing Tbx3 disrupted ESC self-renewal and attenuated the ability of ESCs to differentiate into the endoderm (25). Additionally, Weidgang et al. (45) found that overexpression of Tbx3 in ESCs could enhance mesendoderm lineage specification by both direct activation of related master genes and indirect regulation of the Nodal-Smad signaling pathway. Thus, similar to Oct4 and Sox2, Tbx3 plays an important role in ESC self-renewal and also promotes specific lineage commitment. Recently, Waghrey et al. (46) reported that Tbx3 inhibited mesoderm differentiation by directly repressing Wnt signaling members. It seems that the function of Tbx3 is highly dependent on developmental stages. Identification of the regulatory role of Chd4 for Tbx3 establishes a link between a chromatin remodeler and transcription factor and explains how Chd4 maintains the balance of self-renewal and differentiation. However, the regulation of Tbx3 transcription can only partially account for the role of Chd4 in controlling ESC fate. Further investigation is needed to understand how Chd4 safeguards ESC identity through control of other important genes at both the epigenetic and genetic levels.

Additionally, this study uncovers the interaction of Chd4 with the histone variant H2A.Z in ESCs, reporting an important role of this histone variant in maintaining the protein stability of Chd4. Through comparison of the whole-genome binding profiles of Chd4 and H2A.Z using published datasets (18, 36), we found that 89.8% of genes having Chd4 occupancy are also bound by H2A.Z (data not shown). This analysis supports the existence of interactions between Chd4 and H2A.Z at the chromatin level. Intriguingly, a role of H2A.Z for inhibition of Nanog protein degradation through the ubiquitin-proteasome pathway was previously observed (47). Therefore, stabilizing proteins encoded by pluripotency-associated genes could be one of the important mechanisms by which H2A.Z sustains ESC self-renewal.

Collectively, we propose a working model in which Chd4 ensures self-renewal of ESCs through controlling pluripotency- and differentiation-associated genes; particularly through directly repressing the expression of Tbx3. In addition, the histone variant H2A.Z contributes to the maintenance of ESC identity, probably by stabilizing key self-renewal proteins such as Chd4 (Fig. 7). The study establishes a direct and functional link between a chromatin remodeler and a dually functional transcription factor and highlights a function of Chd4 independent of the classical NuRD complex.

Experimental procedures

Cell culture

CGR8 (provided by Austin Smith) and J1 (provided by Xiaohua Shen) mouse ESCs were cultured under feeder-free conditions on 0.1% gelatin-coated dishes with 10% and 15% FBS, respectively, as described previously (6, 25). 293FT cells (Invitrogen) were cultured according to the manual of the manufacturer.

Inducible Chd4 KD ESC lines

Chd4i-1-, Chd4i-8-, and eGFPi-inducible ESC lines were established in J1 ESCs as described previously (25). Specific
shRNA-containing plasmids (supplemental Table 1) were transfected into J1-TetR host ESCs and selected with 1 μg/ml puromycin and 400 μg/ml zeocin. Single colonies were selected and tested for RNAi efficiency in the Chd4i-1 and Chd4i-8 lines, and the pooled eGFPi cells were used. The stable lines were maintained with 0.5 μg/ml puromycin and 100 μg/ml zeocin.

**Transient siRNA KD assays**

Lipofectamine 2000 reagent (Invitrogen) was used for ESC transfection. The sequences of Tbx3 stealth RNAi oligos are provided in supplemental Table 1.

**Virus packaging and transduction**

Virus packaging was conducted in 293FT cells. After transfection of specific shRNAs (the sequences are shown in supplemental Table 1) and control shRNA plasmids with lentivirus packaging plasmids, supernatants were collected after 48 h. Filtered supernatants were used to infect ESCs for subsequent experiments.

**Quantitative real-time RT-PCR (qRT-PCR)**

Total RNA was extracted using TRIzol reagent (Invitrogen) and purified by phenol/chloroform and isopropyl alcohol. Reverse transcription was conducted with a Fastquant reverse kit (Tiangen). All qRT-PCR assays were carried out on ABI Prism 7900 or Viia7. The primers used are provided in supplemental Table 1.

**Western blotting and immunoprecipitation (IP)**

Cells were lysed in co-IP buffer (50 mM Tris–HCl (pH7.5), 150 mM NaCl, 10% glycerol, 2 mM EDTA, and 0.5% Nonidet P-40) with fresh addition of 1 mM PMSF (Sigma) for Western blotting and protease inhibitor mixture (Selleck) according to the instructions of the manufacturer for immunoprecipitation. The primary antibodies used included Chd4 (Bethyl), tubulin (Sigma), H2A.Z (Proteintech), H2A.X (Proteintech), Gapdh (Bioworld), and ubiquitin (Cell Signaling Technology). In the IP assays, protein G magnetic beads (Millipore) were added to the cell lysate after overnight incubation with specific antibodies for an additional 2 h.

**Flow cytometry analysis**

Inducible RNAi cell lines (eGFPi, Chd4i-1, and Chd4i-8) were treated with or without 5 ng/ml doxycycline for 3 days, and then the cell proliferation rate was measured by BrdU incorporation as described previously (48). Early apoptosis was measured by staining with Annexin V and propidium iodide (PI) according to the protocol described previously (48). Treated cells were analyzed by BD Accuri C6 flow cytometers.

**Competitive assays**

The experiment was conducted as published previously (49).

**EB formation**

Tc-inducible RNAi cells were seeded on gelatin-coated dishes and treated with or without Dox for 2 days. Then cells were transferred to Petri dishes of low adhesion (Qingdao α) and cultured in suspension using the ESC medium without LIF.

**Teratoma formation**

For the generation of Ni, Chd4i-1, and Chd4i-8 ESCs, lentivirus supernatant was prepared. After J1 ESCs were transfected with the specific virus supernatant, cells were expanded for one passage and digested on the fifth day after transfection. About 1.5 × 10⁶ cells of each group were injected into a leg of NOD/SCID mice. For each mouse, Ni cells and Chd4 KD cells were injected into the left and the right legs, respectively.

**RNA microarray analysis**

Total RNA was extracted from Chd4i-1 cells with or without doxycycline treatment for 3 days. Two biological replicates for each group were prepared and sent to the Shanghai Biochip Co. to perform the Affymetrix mouse 430 2.0 array. The GO analysis was conducted online using Gene Ontology Consortium website.

**Luciferase reporter assays**

The assay was performed using the Dual-Luciferase assay system kit (Promega) according to the manual of the manufacturer.

**ChIP**

ChIP assays were conducted as described previously (25). An equal quantity of IgG (Cell Signaling Technology) belonging to the same isotype was used as the control for Chd4 ChIP antibody (Abcam). The verification primers used are provided in supplemental Table 1.

**Statistical analysis**

All experiments were performed three times to obtain quantitative results. Data are shown as means ± S.D. Unpaired Student’s t test was used to determine the statistical significance of differences between two groups. For all figures, the significance is indicated as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Author contributions**—Y. J. and H. Z. designed the research and wrote the paper. H. Z. performed the major experiments and analyzed data. Z. H. and G. W. analyzed data. X. L. performed the cell culture. J. G. and F. T. performed the teratoma injection and H&E staining work.

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