The transcription factor Gbx2 induces expression of Kruppel-like factor 4 to maintain and induce naïve pluripotency of embryonic stem cells

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The transcription factor Gbx2 (gastrulation brain homeobox 2) is a direct target of the LIF/STAT3 signaling pathway, maintains mouse embryonic stem cell (mESC) self-renewal, and facilitates mouse epiblast stem cell (mEpiSC) reprogramming to naïve pluripotency. However, the mechanism by which Gbx2 mediates its effects on pluripotency remains unknown. Here, using an RNA-Seq approach, we identified Klf4 (Kruppel-like factor 4) as a direct target of Gbx2. Functional studies indicated that Klf4 mediates the self-renewal–promoting effects of Gbx2, because knockdown of *Klf4* expression abrogated the ability of Gbx2 to maintain the undifferentiated state of mESCs. We also found that Gbx2 largely depends on *Klf4* to reprogram mEpiSCs to a mESC-like state. In summary, our study has uncovered a mechanism by which Gbx2 maintains and induces naïve pluripotency. These findings expand our understanding of the pluripotency control network and may inform the development of culture conditions for improved ESC maintenance and differentiation.

Mouse embryonic stem cells (mESCs)[3] are isolated from the inner cell mass of the preimplantation embryos (1, 2). They can be maintained indefinitely as self-renewing populations while retaining the pluripotency to differentiate into the cells of all three primary germ layers when cultured under appropriate conditions *in vitro* (3). In the past, the maintenance of mESCs rely on feeder cells *in vitro*, including mitotically inactivated fibroblasts or buffalo rat liver cell (1, 2, 4, 5). Now, however, the simultaneous addition of serum and leukemia inhibitory factor (LIF) can support the long-term self-renewal of mESCs in feeder-free conditions (6–8). LIF is able to activate three intracellular signaling pathways: the JAK/STAT3 pathway, the PI3K/AKT pathway, and the SH2 domain-containing tyrosine phosphatase/MAPK pathway (4, 9–11). LIF mainly through the JAK/STAT3 signaling pathway maintains mESC naïve pluripotent state (12–14). LIF initiates signaling cascade by binding to a heterodimer receptor complex, LIF receptor, and subunit glycoprotein 130 (6, 15), which will activate JAKs family members. JAKs stimulate cytoplasmic STAT3 (11, 16, 17). STAT3 then enters into the nucleus, where they act as a transcription factors to induce the expressions of the target genes (18).

Many downstream targets of the LIF/STAT3 signaling have been identified, such as *Gbx2* (19), *Klf4* (20, 21), *Sp5* (22), and *Tfcp2l1* (23, 24), whereas current exploring of these genes-mediated mechanisms in promoting mESC self-renewal is very limited. For example, elevated expression of *Gbx2* (*Gastrulation brain homeobox 2*), a LIF/STAT3 signaling downstream target, not only can allow long-term expansion of the undifferentiated mESCs in the absence of LIF but also is sufficient to improve the efficiency of reprogramming from mouse epiblast stem cells (mEpiSCs) to ground state mESC (19). The exact molecular mechanism of Gbx2 exerting these effects, however, remains elusive. Here, we identified that *Klf4*, as a key direct target of Gbx2, is critical for Gbx2 to promote mESC self-renewal and convert mEpiSCs to mESC state.

Results

Identification of downstream targets of Gbx2 in 46C mESCs

Overexpression of *HA*-tagged *Gbx2* through PiggyBac system (PB-*Gbx2*) is able to promote mESC self-renewal in the absence of LIF; these cells could be continually passaged while retaining typical mESC morphology and positive alkaline phosphatase (AP) activity, whereas empty vector (PB) control cells differentiated (Fig. 1, *A* and B, and supplemental Fig. S1, *A* and *B*), indicating that overexpressing *Gbx2* can recapitulate the effect of LIF to support mESC self-renewal. These results are consistent with our previous report (19). To screen out the functional targets of Gbx2, we performed a RNA-sequence analysis in PB and PB-*Gbx2* 46C mESCs and got 175 genes up-regulated by 2-fold or greater in PB-*Gbx2* cells compared with PB cells (GEO accession no. GSE98387) (Fig. 1*C*). To survey possible biological roles of these differentially expressed genes, we next performed gene ontology analysis and filtered

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This article contains supplemental Tables S1–S4 and Figs. S1–S5.

Data and details of the method used for the RNA-sequence analysis are available in the Gene Expression Omnibus (GEO) database under accession number GSE98387.

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[3] The abbreviations used are: mESC, mouse embryonic stem cell; mEpiSC, mouse epiblast stem cell; LIF, leukemia inhibitory factor; PB, PiggyBac system; AP, alkaline phosphatase; qRT-PCR, quantitative real-time PCR; 4-OHT, 4-hydroxytamoxifen; CHX, cycloheximide.

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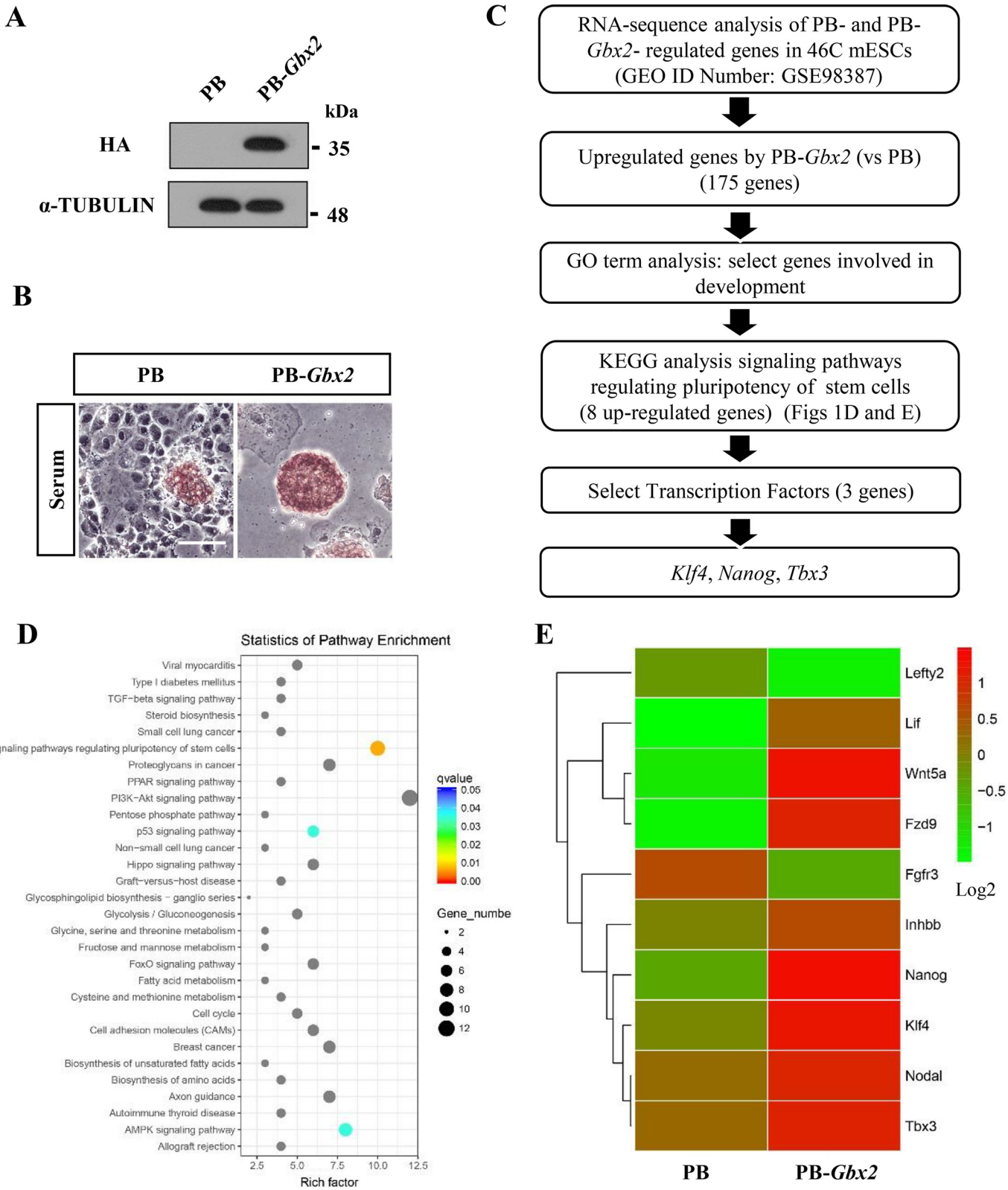

Figure 1. RNA-sequence analysis of the genes up-regulated by Gbx2 in 46C mESCs. *A*, *HA*-tagged *Gbx2* was introduced into 46C mESCs, and the protein level of HA-tagged Gbx2 was determined by Western blot. α-Tubulin is used as a loading control. *B*, AP staining of 46C mESCs overexpressing *Gbx2* cultured in the absence of LIF for 8 days. *Bar*, 100 μM. *C*, flow chart illustrating the approach used to identify the candidate genes regulated by Gbx2 in 46C mESCs. Cells were maintained in serum medium supplemented with LIF. *D*, the Kyoto Encyclopedia of Genes and Genomes analysis of signal pathways classified and enriched by *Gbx2*-induced genes. The data are presented in a scatter diagram. *E*, heat map showed the expression pattern of the indicated genes in PB and PB-*Gbx2* mESCs cultured in serum/LIF condition. Genes were ranked according to the level of log2 fold change. *Red*, expression above average; *green*, below average.

out those involved in development. The Kyoto Encyclopedia of Genes and Genomes method was then carried out to select those enriched in signaling pathways regulating pluripotency of stem cells. These gave a short list of eight candidates (*Wnt5a*, *Klf4*, *Nanog*, *Nodal*, *Inhbb*, *Tbx3*, *Lif*, and *Fzd9*) (Fig. 1, *D* and *E*). Among these genes, three transcription factors were finally identified: *Klf4*, *Nanog*, and *Tbx3*. Previous reports have shown that these targets are closely relevant to mESC pluripotency (20, 21, 25–29).

Klf4 is a direct downstream target of Gbx2

To confirm that the expression levels of these three candidates are really regulated by PB-*Gbx2*, we used quantitative real-time PCR (qRT-PCR) to detect their mRNA levels and found that only the *Nanog* and *Klf4* transcripts obviously increased upon *Gbx2* overexpression (Fig. 2*A*). To further investigate the effect of Gbx2 on *Nanog* and *Klf4* expression, we used three different approaches. First, PB or PB-*Gbx2* mESCs were routinely cultured in LIF/serum condition, and then LIF was withdrawn from 1 to 24 h. qRT-PCR analysis revealed that enforced *Gbx2* could inhibit the further down-regulation of *Klf4* caused by LIF withdrawn, but not *Nanog* (Fig. 2, *B* and *C*). Second, we introduced a *Gbx2-ERT2* transgene into 46C mESCs (Fig. 2*D*). Administration of 4-hydroxytamoxifen (4-OHT) to *Gbx2-ERT2*-expressing cells results in the translocation of GBX2-ERT2 into the nucleus to activate Gbx2 targets to support the mESC pluripotency (Fig. 2*E*). As a result, 4-OHT treatment gradually up-regulated *Klf4* expression, but not *Nanog*, in mESCs overexpressing *Gbx2-ERT2* within 120 min (Fig. 2*F*). Finally, to test whether *Gbx2* knockdown has an effect on *Klf4* expression, we infected 46C mESCs with lentiviruses encoding three shRNAs specific for *Gbx2* mRNA (*Gbx2* sh#1, *Gbx2* sh#2, and *Gbx2* sh#3). Stable knockdown (80–90%) of *Gbx2* transcript levels was observed following drug selection (Fig. 2*G*). As expected, the transcript of *Klf4* decreased in *Gbx2* shRNA cells compared with *scramble* control mESCs (Fig. 2*G* and supplemental Fig. S2). Together, these data suggest that Gbx2 engages in *Klf4* expression to support the mESC pluripotency, and the latter is a downstream target of Gbx2.

To determine whether *Klf4* is a direct target of Gbx2, we analyzed the Gbx2-binding consensus motifs (Fig. 2*H*) and predicted two potential binding sites within *Klf4* promoter regions (from −5000 to +1) from the JASPAR CORE database (motif 1, $^{-3677}$TGATCTAATTAGATCCT^{-3661}; and motif 2, $^{-3927}$GCAGCTAATGAGTCTAG^{-3911}). We then performed ChIP-qRT-PCR in PB-*Gbx2* 46C mESCs and found the direct interaction of Gbx2 with the *Klf4* promoter containing the sequence of motif 2 (Fig. 2*I*). To further determine whether Gbx2 is a functional activator of the Klf4 promoter, *Gbx2* was co-transfected with reporter vectors that drive the expression of luciferase under the control of *Klf4* promoter fragments (pGL6-*Klf4*), comprising our validated *Gbx2* consensus motif 2 or mutated motif 2 sequences (Mut) (Fig. 2*J*). Under these conditions, we observed a 3.6-fold increase in wild-type promoter activity relative to the mutant sequence in PB-*Gbx2* cells (Fig. 2*J*). These results further verify direct transcriptional activation of *Klf4* by Gbx2. Moreover, the induction of *Klf4* expression by *Gbx2-ERT2* cells was similar with or without cycloheximide (CHX) (Fig. 2*K*), an inhibitor of protein biosynthesis, which excludes the possible regulation of *Klf4* by other *Gbx2*-induced targets. In conclusion, these results collectively indicate that *Klf4* is a direct target of Gbx2.

Klf4 mediates the effect of Gbx2 in promoting mESC self-renewal

Both Gbx2 and Klf4 are able to promote 46C mESC self-renewal in the absence of LIF when overexpressed (Fig. 1*B* and supplemental Fig. S3, *A* and *B*). To examine whether Gbx2 depends on Klf4 to maintain mESC pluripotency, *Klf4* transcript was knocked down (*Klf4* sh#1 and *Klf4* sh#2) in PB-*Gbx2* 46C mESCs and validated by Western blot and qRT-PCR methods (Fig. 3, *A* and *B*). Knockdown of *Klf4* led to PB-*Gbx2* mESC differentiation in the absence of LIF, as indicated by the flat cell morphology, decreased AP activity (Fig. 3, *C–E*), and low expression level of pluripotency marker NANOG (Fig. 3*F*). Conversely, they exhibited high-level expression of the endoderm markers, such as *FoxA2*, *Gata4*, and *Gata6* (supplemental Fig. S4), whereas PB-*Gbx2* cells infected with *scramble* control lentivirus kept undifferentiated state (Fig. 3, *C–F*), suggesting that deletion of *Klf4* abolished the defining ability of Gbx2 to confer LIF-independent self-renewal. These results together indicate that the function of Gbx2 for the acquisition of naïve pluripotency in mESCs is dependent on Klf4.

Klf4 is critical for Gbx2 to reprogram mEpiSCs to naïve pluripotent state

Recent reports have established that the reprogramming efficiency from primed-state mEpiSCs to naïve-state mESCs can be facilitated by activation of STAT3 signaling or its downstream targets, such as *Gbx*2 (19), *Tfcp2l1* (22), and *Klf4* (25). These promote us to examine whether Klf4 has ability to mediate the function of Gbx2 in reprogramming mEpiSCs toward naïve pluripotency. First, we overexpressed *HA*-tagged *Gbx2* (PB-*Gbx2*) in CD1 mEpiSCs (Fig. 4*A*) and then infected these cells with *Klf4* shRNA lentivirus. After selection, the mRNA level of *Klf4* decreased significantly in PB-*Gbx2* CD1 EpiSCs when compared with PB cells or PB-*Gbx2* cells infected with *scramble* lentivirus (Fig. 4*B*). These cells were cultured in the conventional mESC medium supplemented with LIF plus 2i. After 12 days, we observed that the PB-*Gbx2* cells expressing *scramble shRNA* formed many dome-shaped colonies and showed strong AP activity (Fig. 4, *C* and *D*). Two ESC-like colonies were picked up and could be continuously expanded in LIF/serum. They expressed high levels of the naïve pluripotency markers *Nanog*, *Stella*, *Klf2*, and *Rex1* but a low level of the mEpiSC-specific marker *Fgf5* (Fig. 4*E*). However, *Klf4* shRNA cells died or differentiated; similar results were observed in PB mEpiSCs (Fig. 4, *C* and *D*). Therefore, knockdown of *Klf4* is capable of eliminating the reprogramming-promoting effect of Gbx2. Previous report shows that Klf4 interacts directly with other reprogramming factors (Oct4 and Sox2) to induce induced pluripotent stem cells (iPS cells) (30, 31). To investigate whether KLF4 protein and GBX2 protein interact during the mEpiSC reprogramming, we overexpressed *Flag*-tagged *Klf4* and *HA*-tagged *Gbx2* in one CD1 mEpiSC line and then performed co-immunoprecipitation experiments after the cells were transferred into reprograming conditions (LIF/2i) for

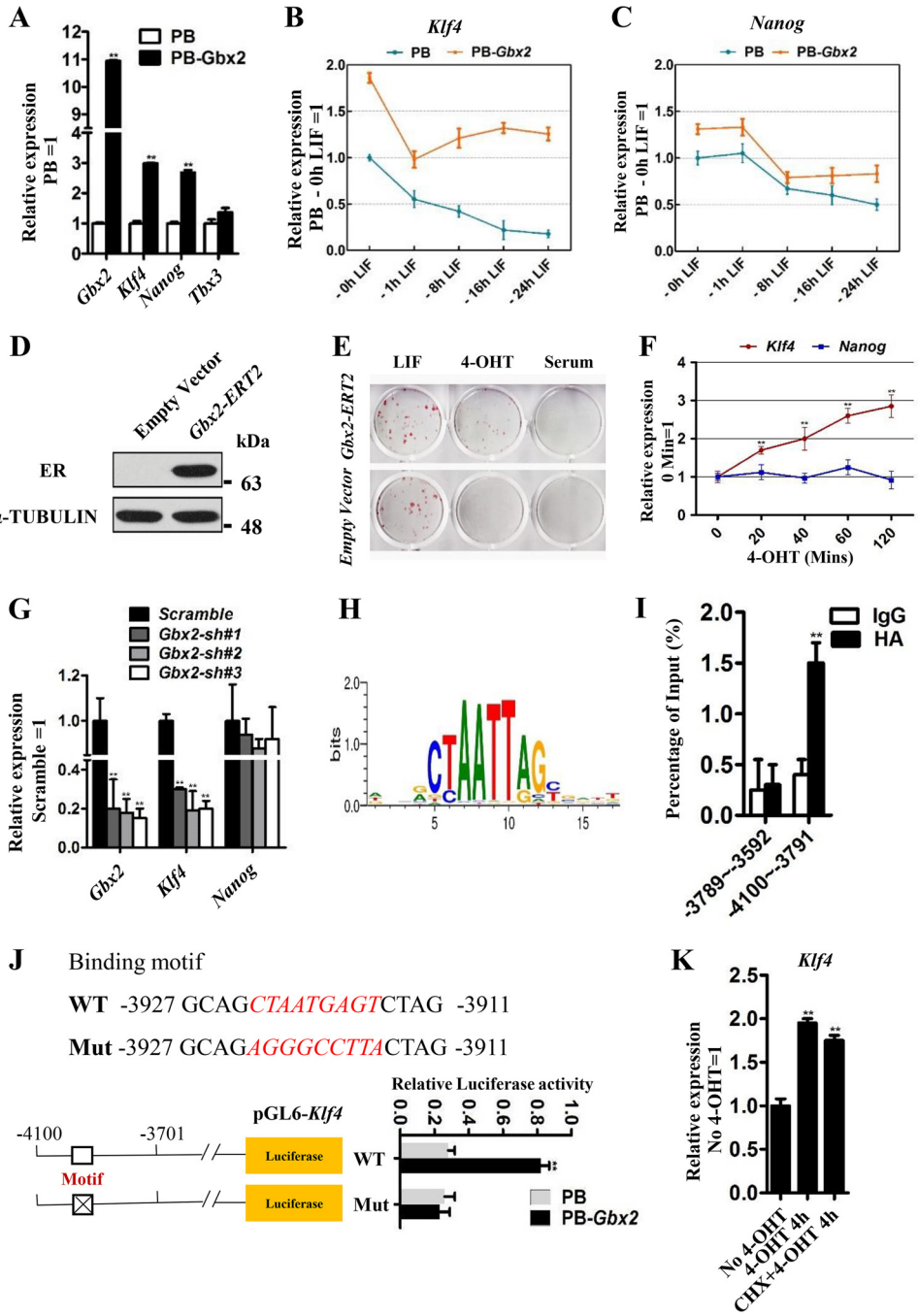

Figure 2. Gbx2 directly regulates *Klf4* expression in mESCs. *A*, qRT-PCR analysis of *Nanog*, *Klf4*, and *Tbx3* expression levels in PB and PB-*Gbx2* 46C mESCs. The data are presented as the means ± S.D. of three independent experiments. **, $p < 0.01$ *versus* PB. *B* and *C*, qRT-PCR analysis of *Klf4* and *Nanog* gene expression in PB or PB-*Gbx2* mESCs after withdrawal of LIF for 0, 1, 8, 16, and 24 h. *D*, Western blot analysis of ER protein in empty vector and Gbx2-ERT2 transfected mESCs cultured in LIF/serum. *E*, AP staining of 46C mESCs overexpressing *Gbx2-ERT2* or empty vector. The cells were cultured in serum medium supplemented with LIF or 1 μM 4-OHT for 8 days. *F*, qRT-PCR analysis of the expression levels of *Klf4* and *Nanog* in *Gbx2-ERT2 cells* treated with 4-OHT for the indicated time. The data are presented as the means ± S.D. of three independent experiments. **, $p < 0.01$ *versus* 0 min. *G*, qRT-PCR analysis of *Gbx2*, *Klf4*, and *Nanog* gene expression in 46C mESCs infected with *scramble* or *Gbx2 shRNA* lentivirus. The data are presented as the means ± S.D. of three independent experiments. **, $p < 0.01$ *versus* scramble. *H*, the Gbx2-binding consensus motifs defined from the JASPAR CORE database. *I*, *HA-Gbx2* transfected 46C mESCs were processed for ChIP with antibody for the HA epitope or IgG as control. Fold enrichments of precipitated DNA relative to the total input chromatin were assessed by qRT-PCR. −3789 to −3593 and −4100 to −3791 indicate two fragments of *Klf4* gene promoter. The data are presented as the means ± S.D. of three independent experiments. **, $p < 0.01$ *versus* IgG. *J*, sequence of the Gbx2 consensus motif within the *Klf4* promoter. The WT and mut sequences were used in the luciferase activity assay. The data are presented as the means ± S.D. of three independent experiments. **, $p < 0.01$ *versus* PB. *K*, qRT-PCR analysis of *Klf4* in *Gbx2-ERT2 mESCs* deprived of LIF overnight and 4-OHT stimulation for 4 h in the presence or absence of the protein-synthesis inhibitor CHX. CHX (50 mg/ml) was added to the medium 1 h before 4-OHT treatment. The data are presented as the means ± S.D. of three independent experiments. **, $p < 0.01$ *versus* No 4-OHT.

2 days. The results show that there is no direct interaction between GBX2 and KLF4 at protein levels (supplemental Fig. S5). Overall, these data thus imply that *Gbx2* is sufficient to convert mEpiSCs to naïve pluripotent state via up-regulating the Klf4 expression at transcriptional level but not through direct interaction with KLF4 protein.

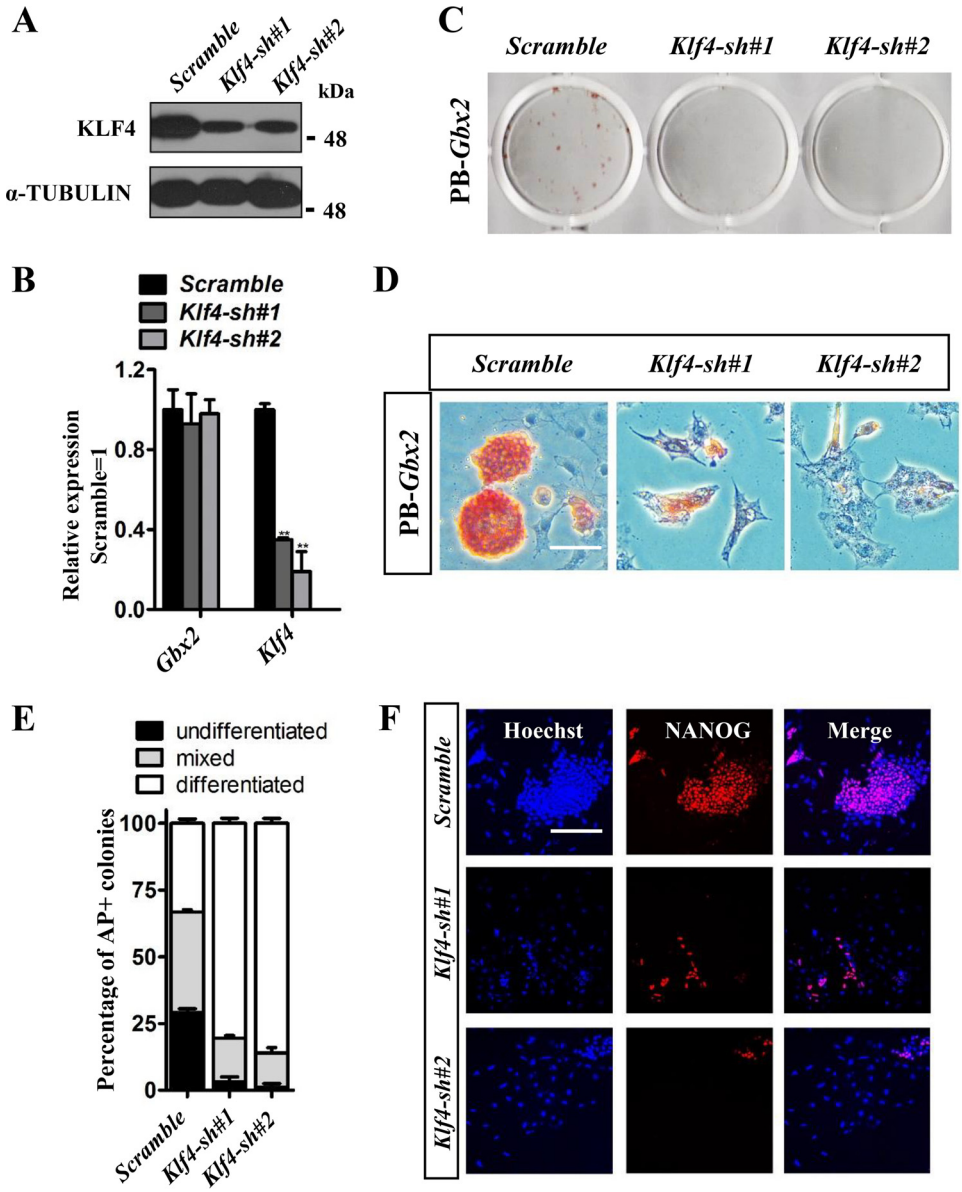

Figure 3. Knockdown of *Klf4* abrogates the self-renewal-promoting effect of Gbx2 in mESCs. *A* and *B*, Western blot or qRT-PCR analysis of *Klf4* and *Gbx2* expression in PB-*Gbx2* 46C mESCs infected with *Klf4 shRNA* lentivirus. The data are presented as the means ± S.D. of three independent experiments. **, $p < 0.01$ *versus* scramble. *C* and *D*, AP staining of colonies arising from *Klf4* knockdown mESCs carrying PB-*Gbx2* transgenes cultured in serum medium without LIF for 7 days. *Bar*, 100 μm. *E*, quantification of AP positive colonies in Fig. 3*D*. The data represent means ± S.D. from triplicate experiments. *F*, immunofluorescence staining of NANOG in *Klf4* knockdown 46C mESCs overexpressing PB-*Gbx2*. *Bar*, 100 μm.

Discussion

LIF/STAT3 signaling pathway maintains mESC pluripotency through activation of many downstream transcription factors including *Gbx2* (19), *Tfcp2l1* (24), *Klf4* (21), etc. As one of the direct targets of STAT3, Gbx2 plays pivotal roles during embryogenesis and is required for the development of the anterior hindbrain, spinal cord, inner ear, heart, and neural crest cells (32, 33). Previous research shows that the overexpression of *Gbx2* can facilitate the maintenance of mESC self-renewal and the reprogramming of mEpiSCs to naïve pluripotent state (19). However, how Gbx2 input is integrated with the pluripotency gene regulatory network has remained unclear.

To resolve this issue, we sought the key target of Gbx2 and identified the transcription factor Klf4. Gbx2 markedly increases the *Klf4* expression level, and this is likely the key contribution of Gbx2 to the maintenance of mESC self-renewal, because knockdown of *Klf4* is able to abrogate the self-renewal-promoting effect of Gbx2 in mESCs (Fig. 3, *A*–*F*). This is unsurprising, given that Klf4, a member of the Kruppel-like factor family, has been shown to be a direct target of STAT3 (20, 21). *Klf4* transfectants were able to produce undifferentiated colonies in the absence of LIF (supplemental Figs. S3, *A* and *B*), consistent with previous reports (20, 21). Klf4 functions may largely rely on Nanog, because Klf4 directly binds to the promoter region of *Nanog* and regulates its expression (34). On the other hand, knockdown of *Nanog* expression induces differentiation of mESCs that overexpress *Klf4* (34). Accordingly, we also observed that enforced *Gbx2* could slightly up-regulate *Nanog* expression (Fig. 2*A*). Nanog is a key naïve pluripotency factor and has ability to support mESC self-renewal when over-

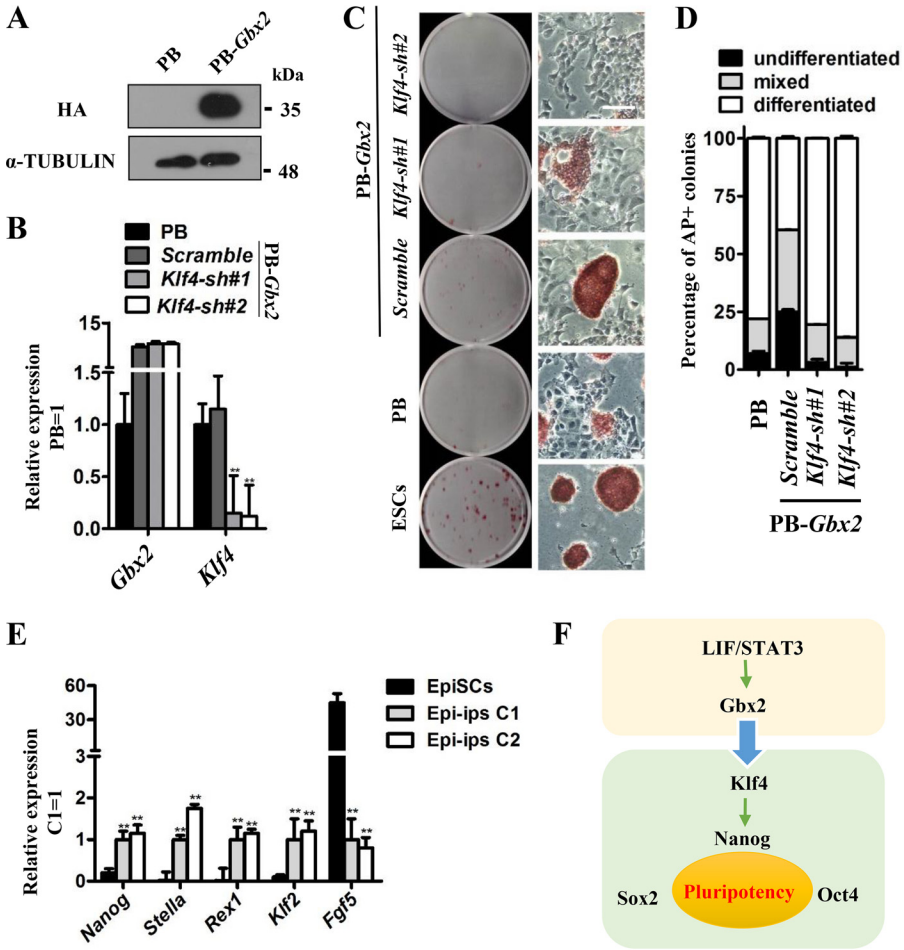

Figure 4. Klf4 is indispensable for Gbx2 to reprogram mEpiSCs to naïve pluripotent state. *A*, *HA*-tagged *Gbx2* (PB-*Gbx2*) was introduced into CD1 mEpiSCs and the protein level of HA-tagged Gbx2 was determined by Western blot. The cells were maintained in medium supplemented with activin A, basic FGF, and IWR-1. *B*, qRT-PCR analysis of *Gbx2* and *Klf4* expression levels in *Klf4* knockdown mEpiSCs overexpressing PB-*Gbx2*. The data are presented as the means ± S.D. of three independent experiments. **, $p < 0.01$ *versus* PB. *C*, AP staining of colonies generated from *Klf4* knockdown mEpiSCs carrying PB-*Gbx2* transgene. The cells were cultured in serum medium supplemented with LIF/2i for 12 days. *Bar*, 100 μm. *D*, quantification of the AP-positive colonies in *C*. *E*, comparison of gene marker expression in PB-*Gbx2*/*scramble* Epi-iPS cells and CD1 mEpiSCs. *Nanog*, *Stella*, *Rex1*, and *Klf2* are mESC markers, whereas *Fgf5* is a mEpiSC marker. The data are presented as the means ± S.D. of three independent experiments. **, $p < 0.01$ *versus* mEpiSCs. *Epi-iPS*, induced pluripotent stem cells generated from mEpiSCs; *C1*, clone 1; *C2*, clone 2. *F*, schematic diagram of Gbx2 input to the pluripotency network. Gbx2 and Klf4 integrate the LIF/STAT3 signal pathway into the core transcription factor network of mESCs.

expressed (11, 35). Therefore, Klf4 may function as a bridge in linking the LIF/STAT3/Gbx2 signaling into the core pluripotency network (Nanog, Oct4, and Sox2) to guard the ground state of mESCs (Fig. 4*F*).

Apart from its role in promoting mESC self-renewal, Gbx2 also acts as a transcription factor capable of reprogramming mEpiSCs to naïve pluripotency state (19). mEpiSCs are isolated from the epiblasts of early postimplantation mouse embryos and have a different and even opposite expression pattern with mESCs (36, 37). *Gbx2* is highly expressed in the mESCs but is barely detectable in mEpiSCs (19), similar to the dynamic change of *Klf4* expression (25). Klf4, one of the four canonical Yamanaka factors that direct somatic cell reprogramming (30), has been shown to be capable of reprogramming mEpiSCs to an ESC state (25). Gbx2 binds to the promoter regions of Klf4 and positively regulates its transcription (Fig. 2, *H–J*). Thus, knockdown of *Klf4* eliminates the reprograming function of Gbx2 in mEpiSCs (Fig. 4, *B–D*), indicating that Klf4 is essential for Gbx2 to induce the generation of naïve pluripotency. Notably, human ESCs share many similar features with the primed mEpiSCs (4), which are truly differentiated from the ground state mESCs (38). Although overexpression of *STAT3* or its target *Gbx2* fails to promote human ESC self-renewal (39, 40), the reinforcement of *STAT3* or its other target *Klf4* in combination with *Klf2* or *Oct4* was capable of reprogramming human ESCs into naïve-like pluripotency (41, 42). It will be of great interesting to investigate whether Gbx2 and the Gbx2/Klf4 axis contribute to establish and maintain the naïve pluripotent state of human ESCs.

In summary, our study reveals an unrecognized mechanism of Gbx2 in mESC self-renewal. Gbx2 may exert this function by acting as a fast responding mediator to the LIF/STAT3 signal changes, eventually modulating the expression of *Klf4*, a well established key factor in maintenance of mESC stemness. In the future, understanding how Gbx2 and Klf4 cooperate with other downstream targets of STAT3 to maintain the pluripotent state of mESCs might greatly facilitate the development of better culture conditions for the maintenance and derivation of authentic ESCs from species other than mice and rats.

Experimental procedures

Cell culture

46C mESCs were routinely maintained on 0.1% gelatin-coated plates in DMEM (HyClone) containing 10% FBS (HyClone), 1× sodium pyruvate (Gibco), 1× non-essential amino acids (Gibco), 2 mM GlutaMAX (Gibco), 0.1 mM β-mercaptoethanol, and 100 units/ml LIF (Millipore). Mouse CD1 mEpiSCs (43) were maintained in the mESC medium without LIF but supplemented with 10 ng/ml activin A (Peprotech), 10 ng/ml basic FGF (Peprotech), and 2 μM IWR-1 (Sigma).

Overexpression and knockdown plasmid construction

The coding regions of *Gbx2* and *Klf4* were cloned from mESC cDNA and inserted into the PiggyBac vector (PB). The *shRNA*-expressing plasmids were generated according to pLKO.1-TRC protocol (Addgene). The target-specific *shRNA* sequences for *Gbx2* and *Klf4* used in this study are listed in supplemental Table S1.

Alkaline phosphatase staining and qRT-PCR

The AP activity assay and qRT-PCR analysis were performed according to our previous report (24). The primers used for the qRT-PCR analysis are listed in supplemental Table S2.

Western blotting

Western blotting was performed according to a standard protocol. The primary antibodies used for probing were α-tubulin (SC-8035, Santa Cruz, 1:1000), HA (C29F4, Cell Signaling Technology, 1:1000), KLF4 (D1F2, Cell Signaling Technology, 1:1000), and ER (MC-20, Santa Cruz, 1:500).

Immunofluorescence staining

Immunostaining was performed via standard protocols. Primary antibody used was against NANOG (14295-1-AP, Proteintech, 1:500). Hoechst 33342 (Hoechst) was used for nuclear staining.

Chromatin immunoprecipitation assay

ChIP was performed using ChIP assay kit (P2078; Beyotime, Haimen, China) according to the manufacturer's protocol. Anti-HA antibody (C29F4; Cell Signaling Technology) was used for immunoprecipitation and IgG (2729S; Cell Signaling Technology) as a control antibody. ChIP enrichment was performed by qRT-PCR. The primer sequences and locations in regions of *Klf4* gene are listed in supplemental Table S3.

Luciferase assay

The *Klf4* gene promoter regions (from −4100 to −3701) carrying the wild-type binging sites or mutant sequences were analyzed by amplified PCR and then cloned into pGL6-basic plasmid, named pGL6-*Klf4*. The primers sequences are listed in supplemental Table S4. Plasmids were co-transfected with a *Renilla* luciferase plasmid into 46C mESCs overexpressing *Gbx2* or empty vector. After 48 h, luciferase activities were measured with the dual luciferase assay kit (Transgen, Beijing, China).

Reprogramming

For reprogramming, 1×10^5 transfectants were seeded on a 0.1% gelatin-coated 6-well plate and cultured in serum medium supplemented with LIF/2i (3 μM CHIR99021 and 1.5 μM PD0325901). Medium were refreshed every other day.

Co-immunoprecipitation

Cell extracts were prepared using Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors). The supernatant was collected and incubated with 10 μl of anti-FLAG affinity gel (SG4110-16; GNI, Tokyo, Japan) for 2 h at 4 °C. The beads were then washed six times with lysis buffer and resuspended in 100 μl of 1× SDS sample buffer for Western blotting analysis.

Accession numbers

Data and details of the method for the RNA-sequence analysis are available in the Gene Expression Omnibus under accession number GSE98387.

Statistical analysis

All data are reported as the mean ± S.D. A Student's *t* test was used to determine the significance of differences in comparisons. Values of $p < 0.05$ were considered statistically significant.

Author contributions—M. W., L. T., and S. Y. conceived and designed the experiments. M. W. and L. T. performed the experiments. D. L. analyzed the data. Q.-L. Y. contributed reagents or materials. M. W. and S. Y. wrote the paper.

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