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Original Article

Regulation of PKCi-mediated pluripotency and gene expression by polycomb complex 1 in mouse embryonic stem cells

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Abstract: Objectives: Epigenetic modification of Polycomb repressive complex 1 (PRC1) plays an important role in regulating the pluripotency and self-renewal of embryonic stem cells (ES). Inhibition of protein kinase C (PKCi) can capture and maintain the pluripotency of mouse ES (mES). Here, we characterized the dynamic expression pattern of PRC1 and its mechanism of action in PKCi-derived mES (PKCi-mES). Methods: The expression of PRC1 components in PKCi-mES, 2iL-derived mES (2iL-mES), and mouse embryonic fibroblasts (MEF) was comparatively analyzed using Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot to identify differentially expressed subunits. Subsequent knockdown of these differential components was performed to examine their effects on cellular differentiation status, along with the expression of pluripotency genes and differentiation genes. Results: We found that PKCi-mES showed high expression of the PRC1 functional catalytic subunit RING1B, canonical PRC1-specific component CBX7, and non-canonical PRC1 specific component RYBP at both gene and protein levels in comparison with control. Knocking down *Ring1b* and *Cbx7* accelerated the differentiation of PKCi-mES and reduced the expression of core pluripotency genes and naïve marker genes. Genes associated with mesoderm, ectoderm, and endoderm differentiation were broadly upregulated after *Ring1b* knockdown, while *Cbx7* knockdown upregulated the expression of *Cbx8* and some ectoderm genes but downregulated the expression of some endoderm and mesoderm genes. Conclusions: These results indicate that PKCi activates both the canonical and non-canonical PRC1 pathways, finely regulates the expression of mES pluripotent and developmental genes, and helps maintain mES in a poised state between self-renewal and lineage commitment.

Keywords: Embryonic stem cells, polycomb, PRC1, epigenetics, PKC inhibition, mouse

Introduction

The capture, maintenance, and differentiation of embryonic stem cells (ES) are closely related to alterations in epigenetic mechanisms [1]. Accumulating evidence suggests that epigenetic modifications also play a crucial role in regulating stem cells properties [2-4]. Epigenetic modifications regulate the expression of developmentally relevant regulatory factors, support stem cell growth and pluripotency, and maintain balance in stem cell differentiation decisions and cell fate [5]. Regarding chromatin modification, Polycomb repressive complexes (PRC) normally act as repressors of gene expression by inhibiting cell differentiation-related genes involved in the regulation of stem cell properties [6, 7]. PRC-mediated gene silencing occurs mainly through the regulation of chromatin structure and post-translational modifications of histones [8-11]. In mammals, Polycomb complexes are divided into Polycomb repressive complex 1 (PRC1) and 2 (PRC2). Core components of PRC1 include E3 ubiquitin ligases RING finger protein 1 (RING1A/RING1B), six Polycomb group RING finger proteins (PCGF1-PCGF6), Chromobox (CBX), Polyhomeotic (PHC), and YY1 binding protein or YY1 associated factor 2 (RYBP/YAF2) [12]. PRC1 can be further divided into canonical Polycomb repressive complex 1 (cPRC1) and non-canonical Polycomb repressive complex 1 (ncPRC1)

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based on the presence or absence of CBX protein [13]. cPRC1 is assembled by PCGF2/4 and contains CBX proteins (CBX2, CBX4, CBX6, CBX7, and CBX8) that can bind H3K27me3, cognate proteins (PHC1-PHC3), and the SAM domain of PHC protein, which is required for Polycomb repression [14]. ncPRC1 has a RYBP or its homolog YAF2, which binds to PCGF1, PCGF3, PCGF5, and PCGF6 to form ncPRC1.1, ncPRC1.3, ncPRC1.5, and ncPRC1.6, respectively [15]. PCGF1-6 acts as RING1A/B binding proteins, and RYBP enhances E3 ubiquitin ligase activity [16]. RING1A and RING1B mediate the ubiquitin ligase activity, which are present in both cPRC1 and ncPRC1. PRC1 catalyzes H2AK119ub1 through ubiquitin ligases RING1A and RING1B to achieve gene repression [17]. RING1B plays a major regulatory role in ES [18].

The pluripotency of ES is regulated not only by epigenetic modifications but also by different signaling pathways. The 2iL culture system is routinely used to derive mES, it can stably capture and maintain the self-renewal and pluripotency of mES [19, 20]. Leukemia inhibitory factor (LIF) is routinely used in this system, as the LIF/JAK/STAT3 signaling pathway regulates ES pluripotency [21]. Inhibiting the ERK pathway with the small molecule inhibitor PD0325901 prevents mES from differentiating into ectoderm and mesoderm and maintains expression of the pluripotency genes *Nanog*, *Oct4*, and *Rex1* [22]. CHIR99021 inhibits GSK3 in the Wnt signaling pathway and releases β-catenin in its complex, which promotes the expression of pluripotency genes and self-renewal of mES [23-25]. On the other hand, protein kinase C (PKC) is involved in a variety of biological regulation processes such as immunity [26], cell differentiation [27], and development [28]. Meanwhile, mouse and rat ES cell lines have been successfully established by inhibiting PKC signaling (PKCi), which serves to maintain their pluripotency and self-renewal [29-31]. In particular, PKCi promotes ES self-renewal by inhibiting microRNA-21/29 [30] and regulates the pluripotency of ES through the nucleosome remodeling and deacetylase (NuRD) complex [31].

Although PRC1 is known to play an important role in maintaining the self-renewal and differentiation of ES [7], the mechanism by which it regulates PKCi-mediated self-renewal of ES is unclear. Most studies of Polycomb complexes in ES have employed LIF plus serum or 2iL systems [32, 33]. However, the regulatory role of PRC1 in the maintenance of ES pluripotency and self-renewal under PKCi has not been studied. Here, we examined the expression patterns of each core subunit of PRC1 in PKCi-derived mES (PKCi-mES) and the mechanism by which PRC1 core subunits regulate mES self-renewal and pluripotency by knocking down expression of the specific PRC1 components RING1B and CBX7 in PKCi-mES. This study is the first to elucidate the dynamic expression patterns of PRC1 core subunits (RING1B and CBX7) under PKCi conditions and their specific regulation of pluripotency-associated genes, providing a novel paradigm for understanding the crosstalk between signaling pathways and epigenetic complexes.

Materials and methods

Chemicals and reagents

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental animal feeding, superovulation, and blastocyst collection

C57BL/6J mice aged 6-8 weeks were purchased from the Comparative Medicine Center of Yangzhou University. Mice were housed in the Laboratory Animal Center of Nanjing Normal University (18-22°C, 50-60% humidity, lights on at 7:00 and off at 19:30). All animal experimental protocols were approved by the Animal Care and Use Committee of Nanjing Normal University (IACUC-20201209) and performed according to guidelines from the United States National Institutes of Health. Female mice (C57BL/6J, aged 6-8 weeks) were intraperitoneally injected with 7.5 IU pregnant mare serum gonadotropin (Ningbo Second Hormone Factory, China) followed by 7.5 IU human chorionic gonadotropin (Ningbo Second Hormone Factory) 48 h later. Female mice were mated with the normal male, and the vaginal plug was detected the next morning. On day 3.5 after the plug was seen, mice were euthanized through cervical dislocation, and blastocysts were collected by flushing the uterus with M2 medium.

De novo derivation of mES

Both 2iL and PKCi culture systems were used. Collected mouse blastocysts were placed in

0.1% gelatin-coated culture dishes (ES-006-B, Millipore, USA) containing mouse fetal fibroblast feeder layers that were prepared in advance. PKCi medium contained KNOCKOUT Dulbecco's Modified Eagle Medium (DMEM) as basal medium (10829018, Gibco, USA), 15% KNOCKOUT serum replacement (10828028, Gibco), 7.5 μM Gö6983 (33053-19-7, Selleck, USA), 0.1 mM β-mercaptoethanol (ES-007-E, Millipore), 2 mM GlutaMax (35050-061, Gibco), 1 mM sodium pyruvate (11360088, Gibco), and 1% penicillin/streptomycin (SV30010, HyClone, USA). 2iL medium contained KNOCKOUT DMEM as basal medium, 15% KOSR, 10^3 IU/mL mLIF (ESG1107, Millipore), 1 μM PD0325901 (S1036, Selleck), 3 μM CHIR99021 (CT99021, Selleck), 0.1 mM β-mercaptoethanol, 2 mM GlutaMax, 1 mM sodium pyruvate, and 1% penicillin/streptomycin. After 3-4 days of cell culture, the medium was replaced with fresh medium, and the culture was continued until 1 week prior to subculture. Each ES cell colony was digested with Accutase (09720, STEMCELL, CA) and inoculated into a well of a new 24-well plate containing a feeder layer. The medium was replaced with fresh medium every 1-2 days, followed by subculture at a ratio of 1:3 every 3-5 days in 35-mm dishes containing a feeder layer. ES cells were frozen in 90% fetal bovine serum and 10% dimethylsulfoxide.

Lentivirus supernatant preparation and infection

293T cells were cultured in DMEM high glucose medium (C11995500BT, Gibco) supplemented with 10% fetal bovine serum (v/v) and prepared for transfection when their density reached 70-80%. Cells were placed in serum-free medium before transfection. Cells were transfected with shRING1B and shCBX7 plasmids (Tsingke, Beijing, China) along with the viral packaging plasmids psPAX and pMD2.G (5:3:2) with Lipofectamine 2000 reagent (11668019, Invitrogen, USA) at a 1:2 ratio of DNA (μg) to Lipofectamine 2000 (μL). The medium was replaced with fresh medium after 6 h, and the supernatant was collected at 48 h and 72 h, filtered (0.45-μm pore size, Millipore), concentrated, and temporarily stored at -80°C for later use. mES were inoculated in the cell culture plate and then cultured in antibiotic-free medium to 70-80% cell density. After 24 h of infection with shRING1B and shCBX7 interfering lentivirus supernatant, cells were placed in fresh medium without lentivirus, and cell samples were collected after 48 h of culture. Lentivirus supernatant contained shRNA targeting mouse *Ring1b* mRNA (shRING1B) and *Cbx7* mRNA (shCBX7). The target sequences for shRING1B and shCBX7 were GCAGACAAATGGAACTCAACC and CCTCAAGTGAAGTTACCGTGA, respectively.

Total RNA extraction from cells

Total RNA was extracted from mouse ES cells using VeZol reagent (R411, Vazyme, China) according to the manufacturer's instructions. Briefly, cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS), after which 1 mL VeZol reagent was added to each well, followed by shaking and digestion for 3-5 min. Cell lysates were collected into a 1.5-mL Eppendorf tube treated with diethyl pyrocarbonate, after which 0.2 mL chloroform was added and mixed well for 15 s. After standing for 2-3 min, cells were incubated at 4°C and 13,400 g for 15 min. The colorless aqueous phase from the supernatant was placed in a new Eppendorf tube, and an equal volume of isopropanol was added and mixed thoroughly. After standing for 10 min, the white precipitate of total RNA at the bottom of the tube was observed by centrifugation at 13,400 g for 10 min at 4°C, and the supernatant was discarded. Pre-cooled 1 mL of 75% ethanol was slowly added to wash precipitate along the tube wall, the supernatant was discarded after centrifugation at 13,400 g for 5 min at 4°C, and the wash was repeated once. The total RNA concentration was measured (NanoDrop spectrophotometer 2000, Thermo Fisher Scientific) after adding 20-30 μL precooled diethyl pyrocarbonate water and mixing evenly.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA was reverse transcribed into cDNA using a reverse transcription kit ABScript III RT Master Mix for qPCR (RK20429, ABclonal, China). According to the instructions, 1 μg total RNA and 4 μL ABScript III RT Mix were added to the PCR tube, which was supplemented with nuclease-free H_2O to 20 μL and mixed. The reverse transcription reaction was carried out in the PCR apparatus as follows: 25°C for 5 min, 42°C for 15 min, and 85°C for 5 s. qPCR was per-

formed using the Genious 2X SYBR Green Fast qPCR Mix kit (RK21207, ABclonal). The 10 μM forward and reverse primers, 10-fold diluted cDNA template, Genious 2X SYBR Green Fast qPCR Mix, and nuclease-free H_2O were mixed, gently shaken, and briefly centrifuged. The qPCR reaction was carried out as follows: 95°C for 3 min, 95°C for 5 s, and 60°C for 30 s for 40 cycles. Individual gene expression was normalized to *β-actin* expression, and results were analyzed using the $2^{-\Delta\Delta Ct}$ method. The qPCR primers used are listed in **Table 1**.

Total protein extraction and western blotting

Protein was extracted from mouse ES cells using radio immunoprecipitation assay (RIPA) lysis buffer (C500005, Sangon, China). Briefly, cells were washed three times with precooled DPBS, the appropriate volume of protein lysate was added, and cells were left on ice for 3-5 min. The cells were then scraped and transferred to a precooled 1.5-mL Eppendorf tube. After cells were vortexed and lysed thoroughly for 10 min, they were centrifuged at 13,400 g at 4°C for 15 min, and the supernatant was collected for determination of protein concentration using the bicinchoninic acid (E112-01, Vazyme) method.

Total protein (6 μg) was added to Sample Loading Buffer (RM00001, ABclonal) and then denatured at 100°C for 10 min. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at different concentrations of separating gels according to the molecular weights of target proteins. After electrophoresis, separated proteins were transferred to a polyvinylidene fluoride membrane (03010040001, Roche, Basel, Switzerland) by electro-transfer. Membranes were blocked with 5% non-fat milk (A600669, Sangon) in tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature for 1 h. After washing with TBST, membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies used are listed in **Table 2**. Membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000, BS13278, Bioworld) at room temperature for 2 h. After washing, membranes were processed using a SuperPico ECL Chemiluminescence Kit (E422-01, Vazyme), and protein bands were visualized using a Tanon 4600 Detection system. The intensity of images was analyzed with ImageJ software, and β-actin was used as an internal control.

Alkaline phosphatase (AP) staining

Mouse ES cells were washed three times with DPBS and fixed with 4% paraformaldehyde at room temperature for 15 min. The fixate solution was discarded, and cells were washed three times with DPBS. 1x BCIP/NBT chromogenic solution was prepared according to kit instructions (REF 11745832910, Roche, Switzerland). Cells were stained and incubated at room temperature in the dark for 30-40 min. Cells were then washed three times with DPBS, and the appropriate amount of DPBS was added to cover the cells. The cells were photographed under a microscope.

Statistical analysis

All experiments were conducted in biological triplicates for each group unless otherwise stated. Data on RT-qPCR were analyzed using GraphPad prism 6. Differences between the two groups were analyzed using independent samples *t*-tests. Differences among three or more groups were analyzed using one-way analysis of variance (ANOVA). Data are expressed as mean ± standard error (SEM). The letters 'a', 'b', and 'c' indicate significant differences among groups ($P < 0.05$).

Results

PKC inhibition increased the expression of Ring1b and cPRC1 core subunit Cbx7 in mES

RING1A and RING1B catalyze histone ubiquitination and are the key subunits responsible for the catalytic activity of PRC1, and are present in both cPRC1 and ncPRC1 [34]. We analyzed the mRNA expression of *Ring1a* and *Ring1b* in 2iL-derived mES (2iL-mES) and PKCi-mES using RT-qPCR and found that *Ring1a* and *Ring1b* expression were significantly higher in mES than in differentiated somatic cells such as mouse embryonic fibroblasts (MEF). There was no significant difference in the expression of *Ring1a* between 2iL-mES and PKCi-mES, however, the expression of *Ring1b* was significantly higher to 55% in PKCi-mES than in 2iL-mES (**Figure 1A**, $P < 0.05$).

Table 1. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) primer sequences and the size of amplified DNA products

Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
Bmp4	ATCACGAAGAACATCTGGAG	GAGATCACCTCATTCTCTGG	100
Cbx2	GCAAGCTGGAGTACCTGGTC	CTGGTTCCTTGAGCTTGGAG	222
Cbx4	GCATCGAAAAGAAGCGGATA	ACCGGACCTCTCCTTGCTAT	374
Cbx6	GCCGAATCCATCATTAAACG	TTGGGTTTAGGTCCCCTCTT	194
Cbx7	GAGGCAGAAGCAGACCTGAC	GCCGCTATTCACAGCTTCTC	170
Cbx8	AGGACGCATGGAATATCTCG	GGTTTTAGGCTTGGGTCCTC	166
cTnT	AGACTGGAGTGAAGAAGAGGAGGAC	CTGGGCTTGGGTTTGGTGT	186
Desmin	AGAAAGTGCATGAAGAGGAG	CCTCAGAGATGTTCTTAGCC	156
Esrrb	AACAGCCCCT ACCTGAACCT	CTCATCTGGT CCCCAAGTGT	245
Fgf4	GTGGTGAGCATCTTCGGAGTGG	GCGTAGGATTCGTAGGCGTTGT	146
Fgf5	GCTCGGAACATAGCAGTTTC	CCGTAAATTTGGCTTAACACAC	151
Foxa2	CACCTGAGTCCGAGTCTGAG	CGAGTTCATGTTGGCGTAGG	84
Gata4	TCTCACTATG GGCACAGCAG	GCGATGTCTG AGTGACAGGA	136
Gata6	CGGTCTCTACAGCAAGATGAAT	TGGTTGTGGTGTGACAGTTG	113
Klf4	ACTGTCACCCTGGCCTGCCTCT	CCCTCTTTGGCTTGGGCTCCT	165
Mixl1	TCTTCCGACAGACCATGTACC	GGCTGAAATGACTTCCCACTC	142
Nanog	GTCTGATTCAGGGCTCAGCA	AAGGCTTCCAGATGCGTTCA	92
Nestin	CTCGAGCAGGAAGTGGTAGG	TTGGGACCAGGGACTGTTAG	353
Oct4	GAAGCAGAAGAGGATCACCTTG	TTCTTAAGGCTGAGCTGCAAG	129
Pax6	CGGAAGCTGCAAAGAAATAG	CCTGTATTCTTGCTTCAGGT	145
Pcgf1	TGCCACCACCATCACAGAG	CTGCGTCTCGTGGATCTTGA	112
Pcgf2	CGGACCACACGGATTAAAATCA	CGATGCAGGTTTTGCAGAAGG	124
Pcgf3	CAGGTAAGCATCTGTCTGGAATG	GTAACAACCACGAACTTGAGAGT	206
Pcgf4	CAATGAAGACCGAGGAGAAGTT	TCCGATCCAATCTGCTCTGAT	107
Pcgf5	GCCTGGACTACGAGAACAAGA	TCATCACCTTCCTCATCTGCTT	126
Pcgf6	TCGTATTCCACCTGAACTTGAT	CCGATAGTTGCTTCTCCTGAA	141
Phc1	TAGCACAGATGTCCCTGTATGA	TTGCTGGAGCATGAACTGGTG	104
Phc2	CCGACTCAGAGATGGAGGAG	AAAGTCCCACTCGTTTGGTG	183
Phc3	TACCAGCGGCAGTATTACCC	TGCAGACTGACAGGAAGGTG	187
Rex1	GCCAGTCCAGAATACCAGAGT	AGCCATCTTCCTCAGTCTCG	92
Ring1a	GGAGTGCCTGCATAGGTTCTG	TAGGGACCGCTTGGATACCA	106
Ring1b	GAGTTACAACGAACACCTCAGG	CAATCCGCGCAAAACCGATG	158
Rybp	GAAGGTCGAAAAGCCTGACAA	AGCTTCACTAGGAGGATCTTTCA	130
Sox1	GGCCGAGTGGAAGGTCATGT	TCCGGGTGTTCCTTCATGTG	93
Sox2	ACAGCATGTCCTACTCGCAG	ATGCTGATCATGTCCCGGAG	160
Sox17	GCGTGGAGCAGGACCCGGCTTTCTT	GGACACTGCATAGTCCGAGACTGGA	101
T	ACCTATGCGGACAATTCATC	CAGACCAGAGACTGGGATAC	155
Yaf2	AAGACCGAGTAGAGAAGGACAA	GATCTCCAACAGTGACTTCCAA	132
β-actin	TGTTACCAACTGGGACGACA	GGGGTGTTGAAGGTCTCAAA	165

Among the five CBX proteins, CBX7 is mainly involved in regulating the self-renewal of stem cells and inhibits their differentiation, and CBX2/4/8 is primarily related to cell differentiation [32, 35]. By contrast, CBX6 transcriptional inhibitory activity is independent of PRC1, with CBX6 target genes mainly involved in cellular, metabolic, and protein modification processes [32]. We found that the mRNA expression of *Cbx2*, *Cbx6*, *Cbx7*, and *Cbx8* was significantly higher in mES than in differentiated somatic MEF cell controls ($P < 0.05$), but with no signifi-

Table 2. Antibody resources used for western blotting

Antibodies	Source	Identifier
Rabbit monoclonal anti-CBX2	ABclonal Technology	Catalog No.: A3294
Rabbit polyclonal anti-CBX4	Immunoway Biotechnology	Catalog No.: YT7583
Rabbit polyclonal anti-CBX7	Servicebio	Catalog No.: GB114894
Rabbit polyclonal anti-CBX8	ABclonal Technology	Catalog No.: A6222
Rabbit polyclonal anti-RING1B	ABclonal Technology	Catalog No.: A5563
Rabbit polyclonal anti-RYBP	ABclonal Technology	Catalog No.: A14605
Rabbit monoclonal anti-β-actin	ABclonal Technology	Catalog No.: AC026

cant difference in the expression of *Cbx2*, *Cbx6*, and *Cbx8* between 2iL-mES and PKCi-mES. However, the expression of *Cbx7* was significantly higher to 41% in PKCi-mES than in 2iL-mES (**Figure 1A**, $P < 0.05$). In addition, the expression of *Cbx4* was significantly lower in PKCi-mES than in 2iL-mES and MEF (**Figure 1A**, $P < 0.05$). We further measured the protein levels of RING1B, CBX7, and CBX4 by Western blot and found that RING1B (46% higher) and CBX7 (26% higher) expression were significantly higher in PKCi-mES than in 2iL-mES, consistent with their mRNA expression levels (**Figure 1B**, $P < 0.05$). However, there was no difference in the expression of CBX4 (**Figure 1B**).

PCGF1-6 are core components of PRC1 and can enhance the activity of E3 ubiquitin ligase [36]. Of the six PCGF homologs, only PCGF2 and PCGF4 exist in cPRC1 [16, 18]. We found that mRNA expression of *Pcgf2* and *Pcgf4* was significantly higher in mES than in differentiated somatic cells, MEF ($P < 0.05$), but there was no significant difference in their expression between 2iL-mES and PKCi-mES (**Figure 1A**). PHC1/2/3 are main cPRC1 components in mES [16] and the SAM domain of PHC is required for PRC1 clustering, which links to chromatin condensation and gene silencing [14, 37]. When we measured the mRNA expression of *Phc1*, *Phc2*, and *Phc3*, we found that *Phc1* and *Phc3* expression were significantly higher in mES than in MEF ($P < 0.05$). However, *Phc2* expression was significantly lower in mES than in somatic MEF, but there was not significantly different *Phc1*, *Phc2*, or *Phc3* expression between mES, 2iL-mES and PKCi-mES (**Figure 1A**).

PKC inhibition induced the expression of ncPRC1 core subunit Rybp in mES

When we examined the gene expression of core components of ncPRC1, we found that *Rybp*, *Yaf2*, *Pcgf1*, *Pcgf3*, *Pcgf5*, and *Pcgf6* were significantly higher in mES than in somatic cells, MEF (**Figure 1C**, $P < 0.05$). RYBP stimulates PRC1-mediated H2AK119ub1 and is essential for ES differentiation [16]. Among ncPRC1 components, only *Rybp* expression was significantly higher in PKCi-mES than in 2iL-mES ($P < 0.05$), whereas there was no significant difference in the expression of *Yaf2*, *Pcgf1*, *Pcgf3*, *Pcgf5*, or *Pcgf6* between 2iL-mES and PKCi-mES (**Figure 1C**). Furthermore, protein expression of RYBP was significantly higher in PKCi-mES than in 2iL-mES (**Figure 1D**, $P < 0.05$), consistent with its mRNA expression level.

In addition, RT-qPCR results showed that the mRNA expression of core pluripotency genes (*Sox2* and *Nanog*) and naïve state-specific markers (*Fgf4*, *Esrrb*) was significantly higher in PKCi-mES than that in 2iL-mES, while *Oct4* and *Rex1* expression was similar, and *Klf4* expression was lower in PKCi-mES (Figure S1).

Ring1b knockdown inhibits expression of pluripotency genes and affects expression of three germ layer genes in PKCi-mES

We found that RING1B, a major functional component of PRC1, is activated by PKCi, but its role in PKCi-mES remains unclear. We performed a shRNA interference experiment to determine the role of RING1B in PKCi-mES. Knockdown of *Ring1b* by lentivirus infection significantly decreased *Ring1b* mRNA by 64% and protein by 62% compared with that in the control group (**Figure 2A**, **2B**, $P < 0.05$). AP staining showed that *Ring1b* knockdown did not affect the total number of AP-positive mES colonies but increased the proportion of differentiated colonies from 16% to 21% and significantly decreased the proportion of undifferentiated colonies from 50% to 41% (**Figure 2C**, $P < 0.05$). RT-qPCR results showed that the mRNA expression of core pluripotency genes (*Oct4*,

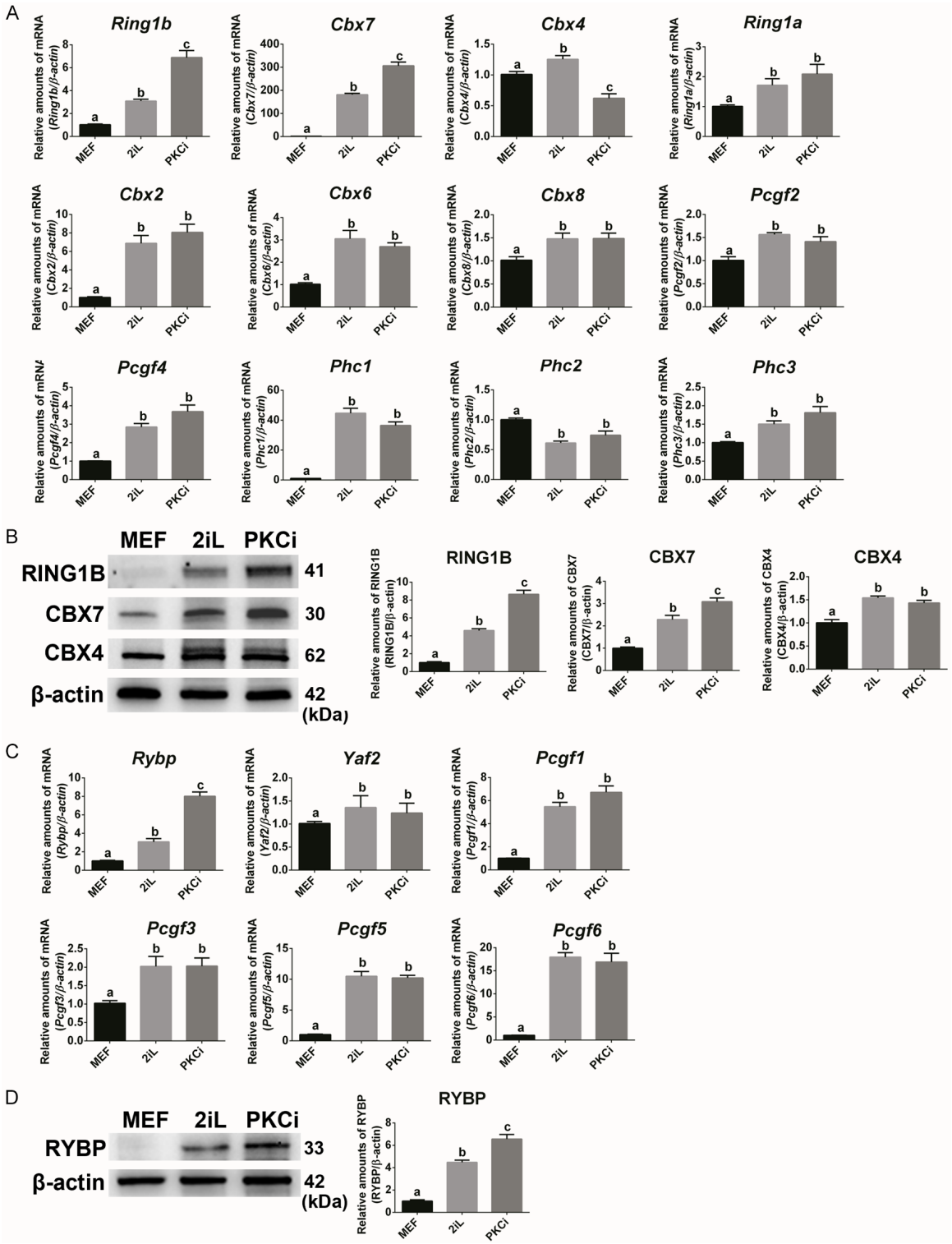

Figure 1. Gene expression of PRC1 core components. A. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) results showing that mRNA expression of *Ring1b*, *Cbx7*, *Ring1a*, *Cbx2*, *Cbx6*, *Cbx8*, *Pcgf2*, *Pcgf4*, *Phc1*, and *Phc3* was significantly higher in mES than in mouse embryonic fibroblast cells (MEF), whereas *Cbx4* and *Phc2* expression was significantly lower in PKCi-mES than in MEF. *Ring1b* and *Cbx7* expression was significantly higher in PKCi-mES than in 2iL-mES, and *Cbx4* expression was significantly lower in PKCi-mES than in 2iL-mES. There were no significant differences in the expressions of *Ring1a*, *Cbx2*, *Cbx6*, *Cbx8*, *Pcgf2*, *Pcgf4*, *Phc1*, *Phc2*, and *Phc3* between PKCi-mES and 2iL-mES. B. Western blot results showed that the expression of RING1B and CBX7 was sig-

nificantly higher in PKCi-mES than in 2iL-mES, whereas there was no significant difference in CBX4 expression. C. RT-qPCR results showed that the mRNA expression of *Rybp*, *Yaf2*, *Pcgf1*, *Pcgf3*, *Pcgf5,* and *Pcgf6* was significantly higher in mES than in MEF. *Rybp* expression was significantly higher in PKCi-mES than in 2iL-mES, whereas there was no significant difference in the expression of *Yaf2*, *Pcgf1*, *Pcgf3*, *Pcgf5,* or *Pcgf6* between PKCi-mES and 2iL-mES. D. Western blot results showed that the expression of RYBP was significantly higher in PKCi-mES than in 2iL-mES. MEF: fibroblast cell control. 2iL: mES control. Data are shown as mean ± SEM (n = 3). The letters 'a', 'b', and 'c' indicated significant differences among groups ($P < 0.05$).

Sox2, and *Nanog*) and naïve state-specific markers (*Klf4*, *Rex1*, and *Esrrb*) was significantly downregulated after *Ring1b* knockdown compared with that in PKCi-mES without *Ring1b* knockdown (**Figure 2D**, $P < 0.05$). The expression of *Fgf4* was not affected by *Ring1b* knockdown. Basically, after *Ring1b* knockdown, some of the representative candidate genes for three germ layers were elevated in mRNA expression. For example, expression of *Gata4* and *Gata6* were upregulated (**Figure 2E**, $P < 0.05$), although *Foxa2* was downregulated in the endoderm. In the mesoderm, *Bmp4* and *Mixl1* were also upregulated (**Figure 2E**, $P < 0.05$), though *cTnT*, *Desmin* and *T* were not changed. The expression of ectodermal genes *Fgf5*, *Nestin*, and *Pax6* was significantly upregulated, whereas *Sox1* expression was unchanged.

Cbx7 knockdown inhibits expression of pluripotency genes and affects expression of three germ layer genes in PKCi-mES

As a core component of cPRC1, CBX7 is required for the recruitment of RING1B to chromatin [38] and plays an important role in maintaining mES pluripotency and inhibiting cell differentiation [32]. After knocking down *Cbx7* through lentiviral infection, we found that *Cbx7* mRNA decreased by 66% and protein decreased by 51% compared with that in the control group (**Figure 3A**, **3B**, $P < 0.05$). AP staining showed that knockdown of *Cbx7* did not affect the total number of positive colonies in PKCi-mES but increased the proportion of differentiation from 16% to 22% and mixed colonies from 34% to 42%, thus significantly reducing the proportion of undifferentiated colonies from 50% to 36% (**Figure 3C**, $P < 0.05$). RT-qPCR showed that the mRNA expression of pluripotency genes (*Oct4* and *Sox2*) and naïve state-specific markers (*Klf4*, *Rex1*, and *Esrrb*) was significantly downregulated after *Cbx7* knockdown compared with PKCi-mES without *Cbx7* knockdown (**Figure 3D**, $P < 0.05$), whereas the expression of *Nanog* and *Fgf4* was unchanged. Knockdown of *Cbx7* resulted in fluctuations of the expression of three germ layer genes, but mostly increased gene expression in the ectoderm (**Figure 3E**). For example, the expression of ectodermal genes *Fgf5*, *Nestin*, and *Pax6* was significantly upregulated, whereas *Sox1* expression was downregulated. Except for the downregulation of *Gata4*, there were no significant differences in the expression of other genes (*Gata6*, *Foxa2*, and *Sox17*) in the endoderm. Also, except for the downregulation of *Desmin* and *T*, there were no significant differences in the expression of other genes (*Mixl1*, *Bmp4*, and *cTnT*) in the mesoderm (**Figure 3E**, $P < 0.05$). In addition, knockdown of *Cbx7* significantly increased *Cbx8* mRNA and protein levels but decreased the expression of *Cbx2* and *Cbx4* (Figure S2).

Discussion

Our study clearly demonstrated that PRC1 played a pivotal role in regulating the pluripotency of mES under PKC inhibition, especially those PRC1 key subunits. We found that compared with 2iL-mES, PKCi-mES showed simultaneous upregulation of RING1B, the cPRC1 core component CBX7, and the ncPRC1 core component RYBP, indicating that PKCi promotes the biological activity of both PRC2-dependent cPRC1 and PRC2-independent ncPRC1. That is, cPRC1 and ncPRC1 were involved in the inhibition of developmental differentiation genes and the maintenance of mES self-renewal in PKCi-mES. Parallel PRC2/cPRC1 and ncPRC1 pathways are suggested to exist in mES that silence lineage-specific genes and maintain mES self-renewal. At least one pathway must be activated to repress lineage-related gene transcription, otherwise spontaneous differentiation will be triggered [33]. Although *Cbx4* mRNA level was lower in PKCi-mES, the level of CBX4 protein was not different between PKCi and 2iL ES conditions.

Our study confirmed that with the PKC inhibition by Gö6983, mouse ES can be successfully derived and maintain their ability for self-renewal and ES pluripotency. Core pluripotency fac-

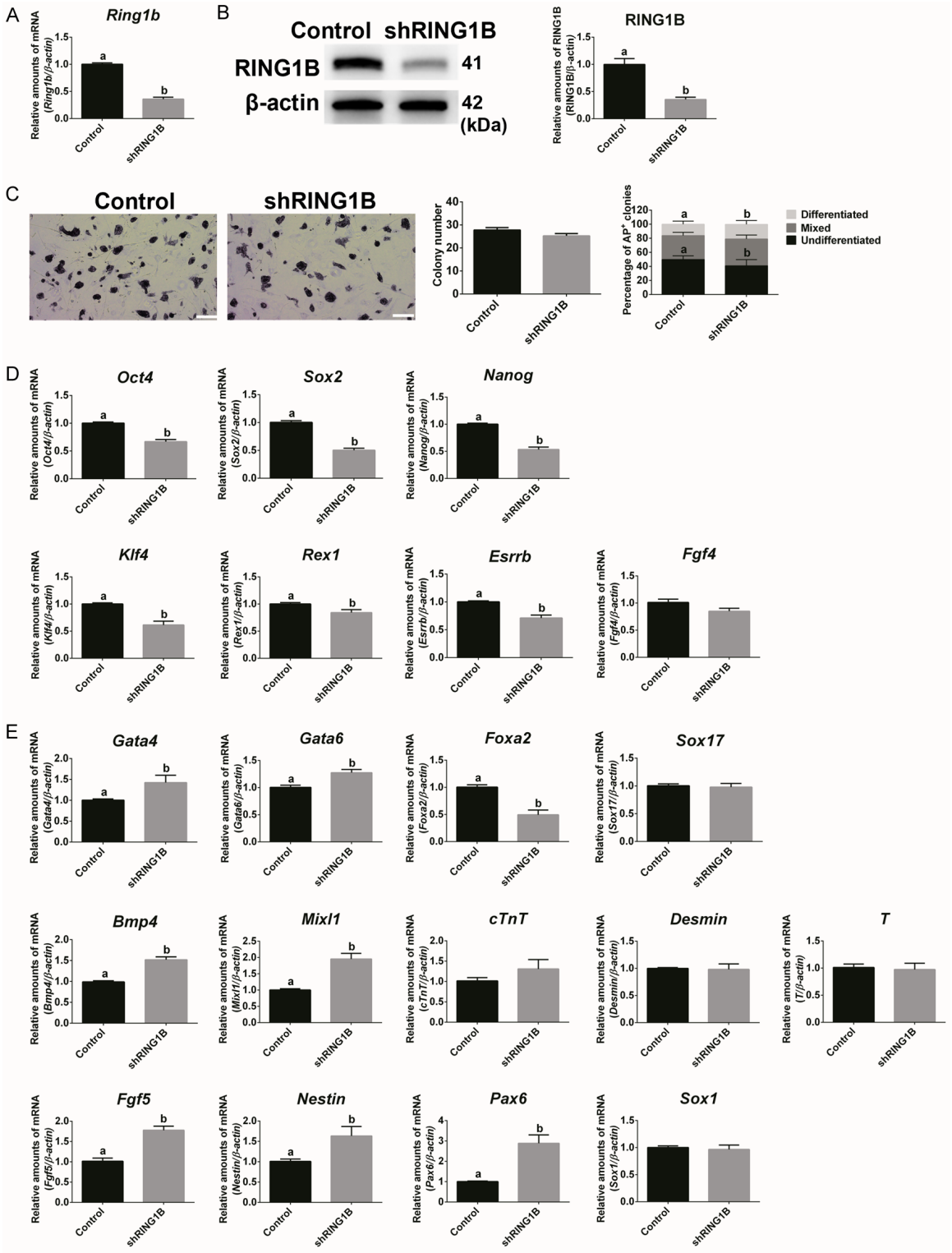

Figure 2. Effect of *Ring1b* knockdown on the expression of pluripotency and differentiation genes in PKCi-mES. A. RT-qPCR results showed that *Ring1b* mRNA expression was significantly decreased after *Ring1b* knockdown to 36%. B. Western blot results showed that *Ring1b* knockdown significantly reduced its protein expression to 38%. C. *Ring1b* knockdown did not affect the total number of AP-positive colonies but increased the proportion of differentiated colonies from 16% to 21% ($P < 0.05$), and decreased the proportion of undifferentiated colonies in PKCi-mES from 50% to 41% ($P < 0.05$). Scale bar, 200 μm. D. RT-qPCR results showing that the mRNA expression of pluripotency genes (*Oct4*, *Sox2*, and *Nanog*) and naïve state-specific marker genes (*Klf4*, *Rex1*, and *Esrrb*) was significantly

downregulated after *Ring1b* knockdown compared with control. There was no effect of *Ring1b* knockdown on the expression of *Fgf4*. E. Among endodermal genes, *Foxa2* expression was downregulated, while *Gata4* and *Gata6* expression were upregulated, and there was no significant difference in *Sox17* expression after *Ring1b* knockdown compared with control. Among mesodermal genes, *Bmp4* and *Mixl1* expression was upregulated while there was no significant difference in *cTnT*, *Desmin*, or *T* expression after *Ring1b* knockdown compared with control. Among ectodermal genes, *Fgf5*, *Nestin*, and *Pax6* expression was upregulated, but there was no significant difference in *Sox1* expression after *Ring1b* knockdown compared with control. Control: PKCi-mES without *Ring1b* knockdown. shRING1B: PKCi-mES with *Ring1b* knockdown. Data are shown as mean ± SEM (n = 3). The letters 'a' and 'b' indicate significant differences among groups ($P < 0.05$).

tors OCT4, SOX2, and NANOG and naïve state-specific markers KLF4, FGF4, ESRRB, and REX1 are involved in the self-renewal and maintenance of ES [39-44]. Compared with 2iL-mES, expression of the core pluripotency genes *Sox2* and *Nanog* and naïve markers *Fgf4* and *Esrrb* was significantly increased in PKCi-mES, whereas expression of *Oct4* and *Rex1* was unchanged. Expression of the naïve marker *Klf4* was significantly lower in PKCi-mES than in 2iL-mES (Figure S1). Some studies show that *Klf4* and *Rex1* are not essential for the pluripotency of ES [39, 44]. It was reported that inhibition of the PKCζ-NF-κB-microRNA-21/microRNA-29 axis is key to maintaining ES self-renewal and naïve pluripotency [29, 30]. We also found that regulatory NuRD complex and its MBD3 subunit influence the naïve pluripotency of mES cultured in a PKCi ES system [31]. Our further studies show that the expression of related genes is regulated by the PKC-CREB phosphorylation signaling pathway under PKCi (Du et al, unpublished data). In this study, it is apparent that higher levels of pluripotency genes are expressed in mES under PKCi than that in 2iL conditions, in order to maintain their self-renewal and stemness.

The epigenetic regulator PRC1 is involved in repressing the expression of differentiation-related genes, thereby maintaining the self-renewal and pluripotency of ES [7]. RING1B is a common functional subunit of cPRC1 and ncPRC1 that catalyzes H2AK119ub1 [45-47]. We found that RING1B plays a more important role in PRC1 than RING1A under PKCi, since *Ring1a* expression was similar between PKCi-mES and 2iL-mES (**Figure 1**). In mES, RING1B is important for repressing developmental regulators, and Polycomb silencing is downstream of the core transcriptional regulatory circuit [48], further indicating that RING1B is important for maintaining ES cell identity. In mES, RING1B knockout reduces ubiquitination without affecting cell morphology [46]. Only when RING1A/B is knocked out simultaneously do mES lose their normal morphology, stop proliferating, and exhibit de-repression of genes related to differentiation and development [48]. These results indicate that RING1A and RING1B may have complementary and alternative roles in ES [46]. Since RING1B showed more expression in PKCi-mES than 2iL-mES in our study, we attempted to knockdown its expression in PKCi-mES and to explore its function in mES stemness. When RING1B was knocked down by 62% under PKC inhibition, the number of both differentiated and mixed colonies was significantly increased, resulting in a dramatic drop of undifferentiated PKCi-mES colonies. After RING1B knockdown, many development genes in the three germ layers tested were upregulated in RNA expression, such as *Gata4*, *Gata6*, *Bmp4*, *Mixl1*, *Fgf5*, *Nestin* and *Pax6* (**Figure 1**). We assumed this derepressed expression of development genes due to the alleviation of PRC1 repression, in turn, will repress the expression of core pluripotency genes, *Oct4*, *Sox2*, *Nanog*, and naïve markers *Klf4*, *Rex1* and *Esrrb* in a wide range, finally resulting in the differentiation of mES cells after RING1B knockdown. This is in accordance with the findings that stem cells are poised at the transition phase between self-renewal and differentiation which is finely orchestrated by expression regulating circuitry of transcription factors, such as pluripotency and differentiation genes [32, 49]. The catalytic decline of RING1B in PRC1 for H2AK119ub1 led to de-repression of genes related to development and differentiation, which fully demonstrated the role of PRC1 in maintaining ES self-renewal [18, 48]. On the other hand, the fact that expression of differentiation genes inhibits the expression of pluripotent genes, which will lead to the cell fate determination and differentiation of cell lineages in ES cells [48, 50], indicates that ES are in a delicate balance between self-renewal and differentiation that is directed by PRC1 [48, 51]. This reflects that stem cells possess variable cellu-

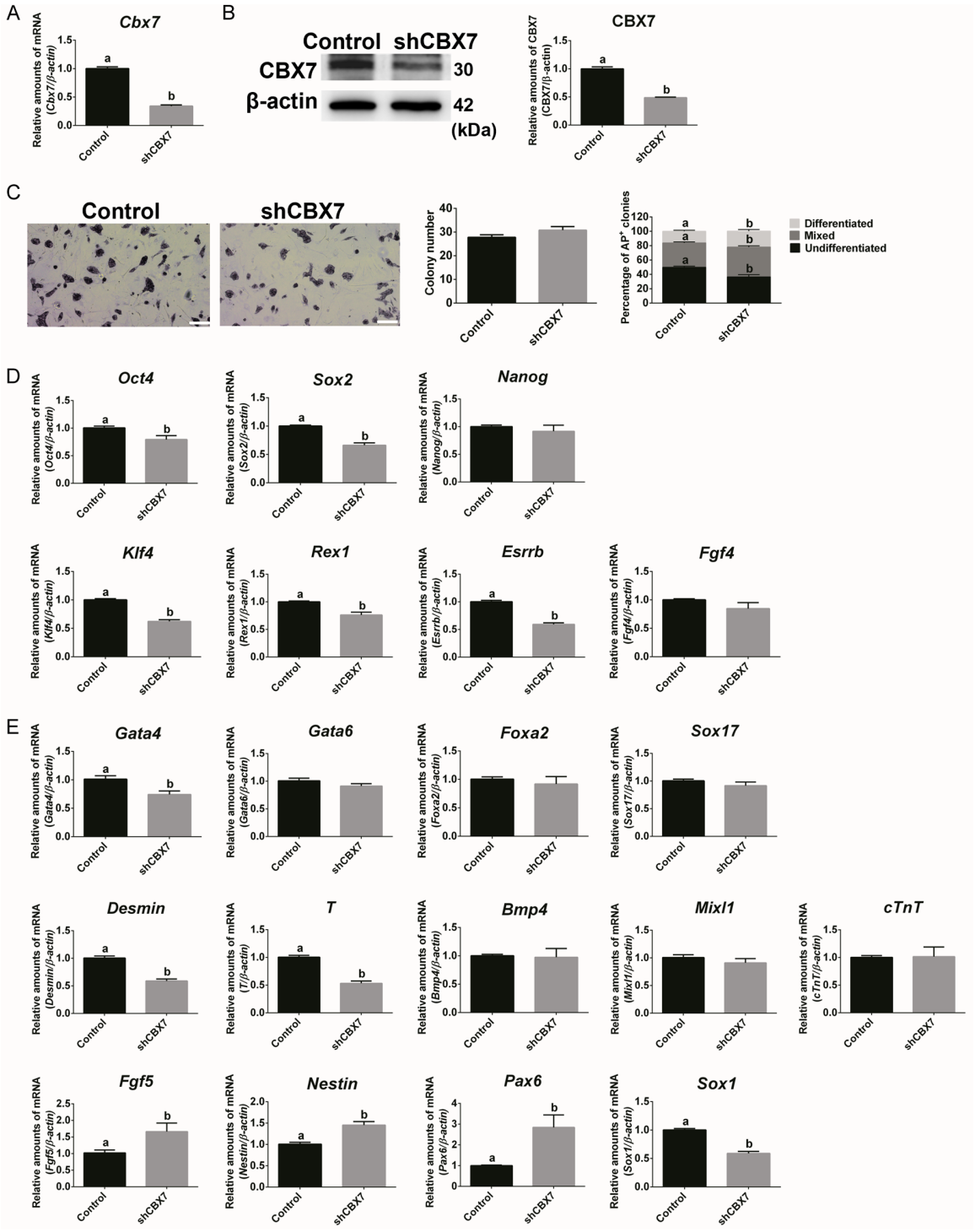

Figure 3. Effect of *Cbx7* knockdown on the expression of pluripotency and differentiation genes in PKCi-mES. A. RT-qPCR results showed that *Cbx7* mRNA expression was significantly reduced after *Cbx7* knockdown to 34%. B. Western blot results show that the protein level of *Cbx7* was significantly decreased after *Cbx7* knockdown to 49%. C. Knockdown of *Cbx7* did not affect the total number of AP-positive colonies in PKCi-mES but increased the proportions of differentiation from 16% to 22% ($P < 0.05$) and mixed colonies and reduced the proportion of undifferentiated colonies from 50% to 36% ($P < 0.05$). Scale bar, 200 μm. D. RT-qPCR results showed that the expression of pluripotency genes (*Oct4* and *Sox2*) and naïve state-specific markers (*Klf4*, *Rex1*, and *Esrrb*) was significantly downregulated after *Cbx7* knockdown, whereas *Nanog* and *Fgf4* gene expression were unchanged. E. Among endodermal genes, *Gata4* expression was downregulated while there was no significant difference in *Foxa2*, *Gata6*,

or *Sox17* expression after *Cbx7* knockdown compared with control. Among mesodermal genes, *Desmin* and *T* expression was downregulated, but there was no significant difference in *Bmp4*, *Mixl1*, or *cTnT* expression after *Cbx7* knockdown compared with control. Among ectodermal genes, *Fgf5*, *Nestin*, and *Pax6* expression were upregulated while *Sox1* expression was downregulated after *Cbx7* knockdown compared with control. Control: PKCi-mES without *Cbx7* knockdown. shCBX7: PKCi-mES with *Cbx7* knockdown. Data are shown as mean ± SEM (n = 3). The letters 'a' and 'b' indicate significant differences between groups ($P < 0.05$).

lar plasticity and developmental capacity [48, 52].

The CBX subunit of cPRC1 can recognize H3K27me3 catalyzed by EZH1/2 and recruit PRC2 through binding to H3K27me3, which together exerts transcriptional repression with PRC2, forming the classical Polycomb recruitment model [53]. CBX7 is a unique component of ES that plays a distinct role in maintaining stem cell self-renewal and stemness and inhibits the cell fate determination and further differentiation of mES [32]. In mES, CBX7 is necessary for recruiting RING1B and the knockdown of *Cbx7* diminished the amount of RING1B localized to chromatin [38]. *Cbx7* knockdown induces differentiation and derepresses lineage-specific markers, while ectopic expression of *Cbx7* inhibits differentiation and X chromosome inactivation and enhances mES self-renewal [35]. CBX7 inhibits especially the expression of homologs CBX2, CBX4, and CBX8 [32, 54], whereas the expression of CBX2, CBX4, and CBX8 promote the differentiation of stem cells into three germ layers [32, 35]. In the present study, we found significantly greater expression of CBX7 in PKCi-mES than in 2iL-mES, indicating that PKCi promotes more expression of core cPRC1subunit CBX7. It was reported that knockdown of CBX7 leads to derepression of developmental genes and the expression of CBX4 and CBX8 [35]. In our study and under PKCi conditions, after knockdown of CBX7, there was a clear trend in the differentiation of stem cells, with an increased number of differentiated colonies. The mRNA levels of pluripotency core genes *Oct4* and *Sox2* and naïve marker genes *Klf4*, *Rex1*, and *Esrrb* were significantly downregulated. Notably, the expression of ectoderm marker genes *Fgf5*, *Nestin*, and *Pax6* was preferentially and significantly increased after CBX7 knockdown, indicating that CBX7 plays a dominant role in the regulation of ectoderm genes, and especially that *Cbx8* expression was promoted in both RNA and protein levels after CBX7 knockdown (Figure S2), consistent with previous findings [32, 35], *Cbx8* increased expression in the absence of *Cbx7*. On the other hand, after knockdown of CBX7, the expression of some endoderm (*Gata4*) and mesoderm (*Desmin* and *T*) genes decreased, along with decreased expression of CBX2 and CBX4 (Figure S2). This suggests that there are differences in regulatory mechanism specificity or the regulatory circuit between PKCi-induced mES and LIF- or 2iL-induced mES. Likewise, there may be some deviations in the selection of representative gene markers of the three germ layers and the spatiotemporal expression patterns of specific genes, which need to be comprehensively analyzed through a panoramic gene expression profile [55, 56]. We found that *Cbx6* expression was not different between PKCi and 2iL conditions (**Figure 1**). It was found that the transcriptional repression activity of CBX6 is independent of PRC1 [32] and its role in maintaining ES identity is mainly based on LIF/serum conditions [57].

RYBP is an important core component of ncPRC1 that, unlike cPRC1, does not rely on PRC2 and the modification of H3K27me3 to enhance the catalytic function of RING1B catalytic H2AK119ub1 on the target gene [58]. It was reported that RYBP increases the expression of genes related to the cell cycle and cell metabolism, and RYBP and CBX7 have different binding sites in mES with non-overlapping functions [38]. We found that the expression of RYBP was significantly higher in PKCi-mES than in 2iL-mES, indicating that RYBP has a more regulatory effect on mES cell division and metabolism under PKC inhibition. It was reported that RYBP knockout alone does not affect mES self-renewal; however, when both RYBP and PRC2 components EED or SUZ12 are simultaneously knocked out, H2AK119ub1 of mES is lost, and mES lose their self-renewal ability and spontaneously differentiate [33]. This indicates that both cPRC1 and ncPRC1 play an important role in maintaining mES pluripotency and self-renewal. In this study, we did not perform a separate and individual RYBP

knockdown analysis for PKCi-mES, warranting future investigation of the mechanism by which the combination of ncPRC1 and PRC2 maintains the pluripotency of PKCi-mES.

Conclusions

In summary, our study results indicate that mES under PKCi have two regulatory modes, classical cPRC1 and non-classical ncPRC1, which jointly regulate the pluripotency and self-renewal of stem cells and inhibit the expression of developmental genes. PKCi-mES exhibited higher RING1B, CBX7, and RYBP expression than 2iL-mES. The high degree of binding of RING1B to RYBP indicates strong catalytic activity of H2AK119ub1, whereas the CBX7 regulatory pathway assumes a suppressive function of cell lineage differentiation. Knockdown of RING1B or CBX7 accelerated the differentiation of PKCi-mES and increased the expression of some three germ layer genes. These results reveal the important role of PRC1 in maintaining the stemness and self-renewal of PKCi-mES. Moreover, PKCi-mES has a high degree of cell plasticity and exists in a dynamic balance between pluripotency and cell differentiation.

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Disclosure of conflict of interest

None.

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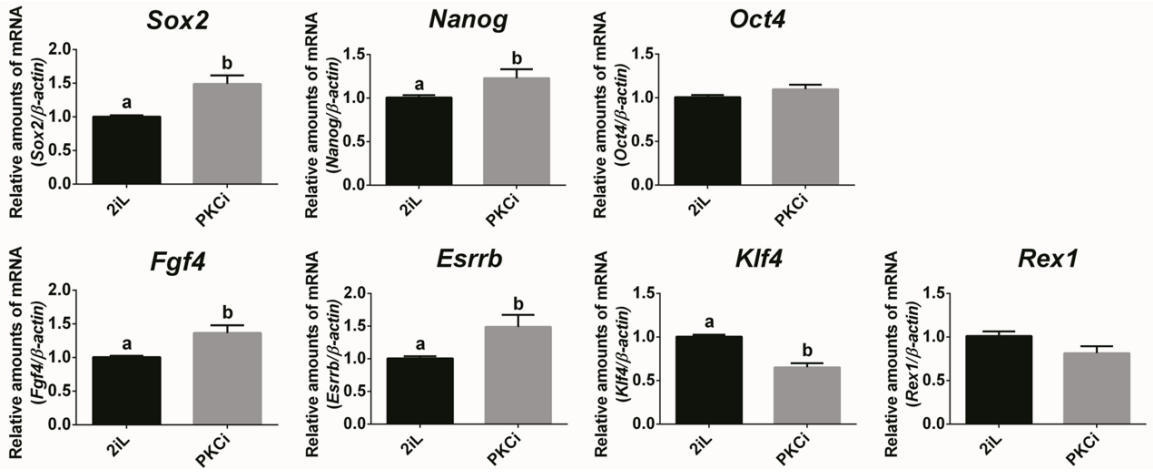

Figure S1. Expression of pluripotency genes in 2iL-mES and PKCi-mES. RT-qPCR results showed that the expression of *Sox2*, *Nanog*, *Fgf4*, and *Esrrb* was significantly higher in PKCi-mES than in 2iL-mES. The expression of *Klf4* was significantly lower in PKCi-mES than in 2iL-mES, whereas *Oct4* expression was unchanged. Data are shown as mean ± SEM (n = 3). 2iL: mES control. The letters 'a' and 'b' indicate significant differences between groups ($P < 0.05$).

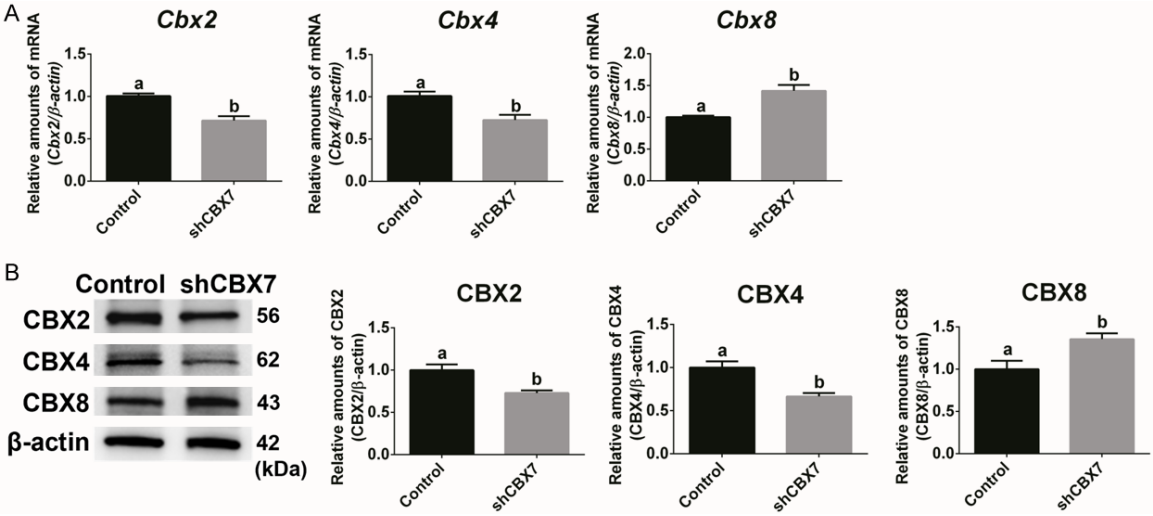

Figure S2. Effect of *Cbx7* knockdown on *Cbx2/4/8* gene expression. (A) RT-qPCR results showed that *Cbx8* mRNA expression was significantly increased by 29% and *Cbx2* and *Cbx4* mRNA expression was significantly decreased by 29% and 28%, respectively, after *Cbx7* knockdown. (B) Western blot results show that CBX8 protein expression was significantly increased from 1.0 to 1.34, and CBX2 (from 1.0 to 0.73) and CBX4 (from 1.0 to 0.67) protein expression was significantly decreased after *Cbx7* knockdown. Data are shown as mean ± SEM (n = 3). Control: PKCi-mES without *Cbx7* knockdown. shCBX7: PKCi-mES with *Cbx7* knockdown. The letters 'a' and 'b' indicate significant differences between groups ($P < 0.05$).