Loss of Polycomb Group Protein Pcgf1 Severely Compromises Proper Differentiation of Embryonic Stem Cells

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The Polycomb repressive complex 1 (PRC1) is essential for fate decisions of embryonic stem (ES) cells. Emerging evidence suggests that six major variants of PRC1 complex, defined by the mutually exclusive presence of Pcgf subunit, regulate distinct biological processes, yet very little is known about the mechanism by which each version of PRC1 instructs and maintains cell fate. Here, we disrupted the Pcgf1, also known as Nspc1 and one of six Pcgf paralogs, in mouse ES cells by the CRISPR/Cas9 technology. We showed that although these mutant cells were viable and retained normal self-renewal, they displayed severe defects in differentiation in vitro. To gain a better understanding of the role of Pcgf1 in transcriptional control of differentiation, we analysed mRNA profiles from Pcgf1 deficient cells using RNA-seq. Interestingly, we found that Pcgf1 positively regulated expression of essential transcription factors involved in ectoderm and mesoderm differentiation, revealing an unexpected function of Pcgf1 in gene activation during ES cell lineage specification. Chromatin immunoprecipitation experiments demonstrated that Pcgf1 deletion caused a decrease in Ring1B and its associated H2AK119ub1 mark binding to target genes. Altogether, our results suggested an unexpected function of Pcgf1 in gene activation during ES cell maintenance.

Polycomb group (PcG) proteins are known as epigenetic chromatin modifiers that regulate gene expression in multiple cell types and tissue contexts and are critical for cell fate decisions and development1–2. PcG-mediated gene silencing is associated with specific post-translational histone modifications3. Polycomb proteins participate in two major multiprotein complexes: the Polycomb repressive complexes 1 and 2 (PRC1 and PRC2)4. PRC2 consists of three core subunits: Eed, Suz12, and the histone methyltransferases Ezh1/2, which is responsible for catalysing both di-methylation and tri-methylation on lysine 27 of the histone H3 (H3K27me3)5,6. PRC1 mediates the monoubiquitylation of histone H2A at lysine 119 (H2AK119ub1)7,8. In mammals, all PRC1 complexes contain Ring1A/B which catalyse H2AK119ub1 and Pcgf1-6 which regulates PRC1 enzymatic activity9–12. On the basis of the presence or absence of Cbx proteins which can recognize and bind H3K27me3, PRC1 complexes can be grouped as canonical PRC1 and non-canonical PRC1 respectively13. Recently, six major groups of PRC1 complexes named PRC1.1–1.6, distinguished by the distinct member of the Pcgf (Polycomb group RING finger protein) family have been reported14. Although it is well documented that Polycomb complexes are implicated in stem cell maintenance, very little is known about the functions of each Pcgf family member in ES cells.

Pcgf1 is also known as Nspc1 which is mainly expressed in nervous system15. The Pcgf1-containing PRC1 (PRC1.1), also known as the dRing-associated factor (dRAF) complex in D. melanogaster16 and the BcoR complex in mammals17, has been reported to be important for the deposition of H2AK119ub1. Notably, recent studies revealed that Kdm2b, another component in this complex, plays a critical role in regulating the recruitment of PRC1.1 proteins Ring1B and Pcgf1 to its target genes and most H2AK119 ubiquitylation in ES cells18,19. Moreover, Knockdown Kdm2b in ES cells led to the failure of proper differentiation20,21. However, the role of Pcgf1 in regulating H2A monoubiquitylation and pluripotency maintenance in ES cells is not clear.

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Here, we use CRISPR/Cas9 strategy to establish Pcgf1 null ES cells. Although the deletion of Pcgf1 does not impair ES cell proliferation and EB formation, mutant cells display profound defects in differentiation. Importantly, RNA-seq analysis reveals that Pcgf1 plays an unexpected role in transcriptional activation, in contrast to the repressive role of canonical PRC1. Mechanistically, this process is initiated by Pcgf1-mediated noncanonical PRC1.1 complex assembly.

Results

**Pcgf1 knockout ES cell line is established by CRISPR/Cas9 technology.** Although it has been reported that PRC1.1 complex is mainly responsible for the H2AK119ub modification in HeLa cells and NT2 cells, the physiological function of this complex is still not well understood. Recently, study from Barbara Dupret group showed that Pcgf1, the core component of PRC1.1 complex is involved in cell proliferation during early embryogenesis by generating Pcgf1−/− zebra fish. To investigate the role of Pcgf1 in the maintenance and differentiation of mouse ES cells, we established the Pcgf1 knockout ES cell line utilizing the CRISPR/Cas9 genomic editing tool. In order to knockout the region encoding RING finger motif and introduce a frame shift, we designed two sgRNAs specifically targeting exon 2 and exon 3 in the mouse Pcgf1 gene and cloned them into the PX330 vector which encodes the Cas9 nuclease. The two sgRNAs-Cas9 encoding vectors were co-transfected into ES cells. Colonies with successful genome editing were selected by puromycin. To further identify the genomic change of targeting Pcgf1 by CRISPR/Cas9 system, the genomic DNA of cells was extracted and amplified using the designed primer sets flanking the two cleavage sites by PCR reaction. Sanger sequencing of the truncated transcript showed the deletion of 259 bp in Pcgf1 mRNA and also introduction of a frame shift to the truncated coding sequence. Furthermore, truncated transcript of Pcgf1 was assessed by reverse transcription and polymerase chain reaction (RT-PCR) amplification. Sanger sequencing of the truncated transcript showed the deletion of 259 bp in Pcgf1 mRNA and also introduction of a frame shift to the truncated coding sequence. To verify whether the Pcgf1 gene was completely knocked out, we examined Pcgf1 protein expression in the positive clones using Western blot analysis with a monoclonal antibody.
which specifically recognizes an epitope encoded by exon 4–9 (Fig. 1D). Our results clearly indicated that Pcgf1 protein expression was totally ablated in Pcgf1−/− ES cells and truncated proteins were not detected.

**Pcgf1 is dispensable for ES cell self-renewal.** One of the main characteristics of ES cells is self-renewal, which is the capacity to propagate indefinitely while retaining the cellular potential of differentiation into multiple cell types26. To elucidate the role of Pcgf1 in the maintenance of ES cell self-renewal, wild-type and Pcgf1−/− ES cells were cultured on mitomycin-C inactivated MEF feeder layer. The Pcgf1−/− ES cells displayed the ability to form ES cell colonies. These colonies exhibited morphology similar to those from wild-type ES cells. Furthermore, Pcgf1−/− ES colony size was comparable to wild-type (Fig. 2A). Consistent with this, we found that ES cells loss of Pcgf1 had no difference in the fraction of cells in G1, G2 and S phase compared to wild-type by using flow cytometry analysis (Fig. 2B). To check pluripotency status, we performed alkaline phosphatase (AP) assay with wild-type and Pcgf1−/− cell colonies on MEF feeder cell layer. Pcgf1−/− ES cells showed high AP activity (Fig. 2C). In agreement with these observations, overall expression levels of ES cell core pluripotency factors (Oct4, Nanog and Sox2) were not significantly altered upon knockout of Pcgf1 (Fig. 2D). Taken together, these results indicated that Pcgf1 was dispensable for self-renewal of mouse ES cells. Western blot analysis also showed the protein levels...
of other components of PRC1.1 (Ring1B and Rybp), PRC2 member Suz12 as well as other PCGF family member (Pcgf5) were not changed in Pcgf1\(^{-/-}\) ES cells (Fig. 2D). Interestingly, Western blot also showed that the expression level of BcoR protein was reduced in Pcgf1\(^{-/-}\) ES cells, suggesting that Pcgf1 regulated the stability of BcoR protein (Fig. 2D). This is in agreement with those obtained by other authors who reported that knockdown of Pcgf1 resulted in reduced levels of the BcoR in NT2 cells\(^{23}\).

Pcgf1 is required for ES cell differentiation. Majority of PcG components has been identified as necessary for proper ES cell differentiation\(^{27}\). We next examined the differentiation properties of Pcgf1\(^{-/-}\) ES cells. We first generated embryoid bodies (EBs) in hanging drops at the first three days and subsequently maintained them in rotating conditions in the absence of LIF (Fig. 3A), and examined EBs morphology by microscopy. EBs mimic, to some extent, early embryonic development and are often utilized as an in vitro differentiation assay to test ES cell pluripotency\(^{28}\). Our results showed that null ES cells retained the ability to differentiate into EBs. From days 3–12, Pcgf1\(^{-/-}\) EBs were macroscopically very similar to wild-type EBs; however, these mutant EBs were smaller than their wild-type counterparts. We randomly chose 20 EBs at 3, 7 or 12-day culture and scored their relative diameters microscopically (Fig. 3B). Our data indicated that Pcgf1\(^{-/-}\) ES cells formed EBs with an average size half that of the wild-type. These proliferation defects in the Pcgf1\(^{-/-}\) EBs suggested a delayed differentiation of Pcgf1 deficient ES cells. Of note, lentiviral expression of FLAG-tagged Pcgf1 in Pcgf1\(^{-/-}\) entirely rescued normal EB size (Fig. 3B). RT-qPCR analysis of 12-day EBs demonstrated that the expression of ES cell marker genes Oct4 and Nanog were dramatically decreased during the process of wild-type EB differentiation (Fig. 3C). Consistent with their aberrant EB formation, however, the Pcgf1\(^{-/-}\) ES cells maintained high levels of Oct4 and Nanog mRNA over the 12 days of culture, displayed severe misregulation of the differentiation marker genes in comparison to the wild-type. Although all lineage genes were upregulated after EB induction, the mesoderm- and ectoderm-specific genes (Flk1, Brachyury, Fgf5 and Nestin) were markedly downregulated in EBs derived from Pcgf1 knockout ES cells (Fig. 3C). In particular, we detected significant decreased Flk1 expression in undifferentiated Pcgf1\(^{-/-}\) ES cells and during EB culture, indicating that Pcgf1 activates Flk1 gene expression which is
consistent with our RNA-seq analysis (Supplementary Table 2). Interestingly, the endoderm differentiation might not completely be disturbed in the absence of Pcgf1 because Gata4 was almost normally induced in EBs derived from knockout ES cells (Fig. 3C). It has been reported that Ring1B represses the expression of Gata4 via direct binding to its promoter regions 29. Moreover, the data in this manuscript demonstrated that Pcgf1 is required for the ordered chromatin recruitment of Ring1B (see the proposed model below). Therefore, we propose that Pcgf1 displays specificity toward endoderm formation through modulating Ring1B activity. Notably, trophectoderm marker Eomes was also reduced in knockout ES-derived EBs (Fig. 3C). Remarkably, lentiviral expression of FLAG-tagged Pcgf1 was able to restore the pattern of expression of differentiation markers to levels similar to those in control cells. Collectively, these data suggest that Pcgf1 deletion impairs ES cell differentiation in vitro, likely by preserving expression of high levels of Oct4 and Nanog, which in turn perturb the differentiation process.

Pcgf1 works as a transcriptional activator. To understand the underlying mechanism by which Pcgf1 affects ES cell differentiation, it is critical to characterize Pcgf1 regulated transcripts. To this end, we performed RNA-seq analysis on Pcgf1−/− and wild-type ES cells. RNA-seq analysis identified 2331 genes with >2-fold altered expression levels in Pcgf1−/− compared to wild-type ES cells (Fig. 4A, Supplementary Table 2). Importantly, re-expression of Pcgf1-FLAG was accompanied by 82% of these genes altered >2-fold in the opposite direction (Pcgf1-FLAG infected cells compared with Pcgf1−/−). Together, these criteria revealed a set of 1929 Pcgf1 target genes. 1491 (77%) genes were downregulated in the absence of Pcgf1 while only 438 genes (23%) were upregulated (Fig. 4B). Expression of some of the transcripts identified as downregulated by RNA-seq analysis was evaluated independently by RT-quantitative PCR (RT-qPCR) (see figure below). The RNA-seq data have been deposited at the Gene Expression Omnibus under accession number GSE95383. Thus, Pcgf1 generally functioned as a transcriptional activator in ES cells. Next, we used gene ontology (GO) analysis to identify the functions of the significantly downregulated genes. These genes were enriched in many functional categories which conformed to the differentiation phenotype we observed, like the development of mesoderm (muscle contraction, blood circulation) and ectoderm (regulation of neurotransmitter levels and synaptic signaling) (Fig. 4C). Figure 4D showed 36 genes downregulated with >24-fold decrease. As expected, these genes were mainly associated with mesoderm and ectoderm differentiation or related to pathways essential for these two germ layer differentiation (e.g. Pla2g4f, C06sa5, C01a2, Rnls and Chrnd for mesoderm; Pclo, Ryr3, Pde6b, Calb2, Atp2b2 and Kcnj2 for ectoderm). Thus, Pcgf1 acts predominantly as a transcriptional activator which regulates mesoderm and ectoderm differentiation in ES cells.
Pcgf1 is essential for the recruitment of PRC1.1. Previous studies demonstrated that Pcgf1 associates with Kdm2b, Rybp, Ring1A/B and BcoR in MEL, HeLa S3 and HEK293 cells and together they form a non-canonical PRC1 complex in human HEK293T cells, referred to as PRC1.1. To determine that Pcgf1 can indeed associate with PRC1 in ES cells, we performed immunoprecipitation using protein extracts derived from Pcgf1−/− ES cells rescued with Flag-tagged Pcgf1 (Pcgf1+/−+Pcgf1) (lane 4). Antibodies for western blots were indicated to the left. 5% of the total cell lysate used for each immunoprecipitation was loaded in lanes 1 and 2. (B) H2AK119ub1 and H3K27me3 levels in Pcgf1−/− ES cells. WT and Pcgf1−/− ES cells nuclear extractions were used to examine H2AK119ub1 and H3K27me3 levels by Western blot, H3 was used as a loading control. (C) Expression of six selected target genes (Klf4, Hhip, Flk1, Neurod1, Hes2 and Nptx1) in WT and Pcgf1−/− ES cells. Hprt1 was a negative control. (D) Flag ChIP-qPCR analysis was performed in the designated ES cells. (E) ChIP-qPCR was used to analyse the occupancy of BcoR, Rybp, Ring1B, H2AK119ub1, Suz12 and H3K27me3 on Pcgf1 targeting genes in WT and Pcgf1−/− ES cells. Error bars indicated ± SD. Bar graphs represented the mean of 3 independent biological repeats. *p < 0.05, **p < 0.01, ***p < 0.001 by two-tailed Student’s t-test.
also reduced in Pcgf1−/−. The occupancy of PRC2 component Suz12 and its chromatin modification H3K27me3 on Pcgf1 targets are therefore reduced in Pcgf1−/− ES cells, which was consistent with RNA-seq analysis (Fig. 5C). ChiP-qPCR analysis using Flag antibody in the Pcgf1−/− ES cells rescued with Flag-tagged-Pcgf1 and parental control cells confirmed the specific binding of Pcgf1 at the promoters of these identified targets genes (Fig. 5D). This suggested Pcgf1 regulates its targeting genes by direct binding.

ChiP-qPCR with antibodies directed against each PRC1.1 component (BcoR, Ring1B and Rybp) and PRC2 core subunit Suz12 was performed on these targets. Additionally, ChiP-qPCR was done on the Pcgf1−/− ES cells to determine the effect of Pcgf1 deletion on PRC1.1 and PRC2 occupancy at specific target promoters. PRC1.1 component (BcoR, Ring1B and Rybp) and PRC2 subunit Suz12, as well as their associated H2AK119ub1 and H3K27me3, were enriched at these 6 targets. The enrichment of BcoR, Ring1B and Suz12 were greatly reduced in Pcgf1−/− ES cells. In contrast, deletion of Pcgf1 did not alter the binding of Rybp (Fig. 5E). Notably, the reduction of BcoR occupancy might partially due to the instability of this protein in Pcgf1−/− ES cells as mentioned before. Finally, despite our observation that global H2AK119ub1 and H3K27me3 was not affected by loss of Pcgf1, we observed that enrichment of these two histone modifications were reduced by 3 to 17-fold in the Pcgf1−/− ES cells at these specific targets of Pcgf1. These results are consistent with recent findings that PRC1-dependent H2AK119ub1 is a recruitment cue for PRC2 and H3K27me3. Collectively, our results demonstrated that Pcgf1 has a key role in regulating Ring1B recruitment to its target genes in ES cells.

Discussion

The PRC1 family can be divided into at least six groups, referred to as PRC1.1−1.6, based on the identity of the Pcgf1 subunit. However, the biological function of each group is still unclear. In this study, we were focused on the Pcgf1 which is a core component of PRC1.1 complex. We generated Pcgf1 gene deficient ES cells by CRISPR-Cas9. We found that Pcgf1 is not essential for the self-renewal of ES cells (Fig. 2). However, Pcgf1 can promote the development of mesoderm and ectoderm during differentiation process in vitro (Fig. 3C). Notably, loss of Pcgf1 results in reduced levels of the BcoR protein. Interestingly, it has been reported that BcoR plays a role in the differentiation of ES cells into mesoderm and ectoderm.

Genetic evidence indicate that the role of PRC1 function as a transcriptional repressor through epigenetic mechanisms. Nonetheless, multiple lines of evidence exist to support a role for Pcg in transcriptional activation. Recently, it has been reported that many unmethylated CpG islands that are targeted by Kdm2b, one component of PRC1.1, are found at the promoters of actively transcribed genes, hinting that this complex could act as transcriptional activators to promote differentiation by targeting early lineage-specific genes in ES cells. Our RNA-seq analysis showed that 1491 target genes were downregulated in Pcgf1−/− ES cells compared to wild-type ES cells, whereas only 438 target genes were upregulated in all 1929 target genes with >2-fold altered expression levels in Pcgf1−/−. Therefore, Pcgf1 primarily works as a transcription activator in ES cells (Fig. 4). However, our observations are contradictory to previously published data demonstrating that Pcgf1 represses transcription when fused to the GAL4 DBD in COS-7 cells. Therefore, Pcgf1 might impact transcriptional activity in a cell type-dependent manner. Additionally, Zhonghua Gao et al. recently reported that another noncanonical PRC1 complex, PRC1.5, can activate transcription through recruiting CK2 and co-activator P300 by one of its core components, Aut2. In the future study, we will further explore which component in PRC1.1 complex can recruit transcriptional co-activator to render this complex capable of transcription stimulation.

Recent observation showed that H2AK119ub1 is essential for PRC1 mediated gene repression. Previously, Pcgf1 has been shown to enhance H2AK119ub1 and knock down of Pcgf1 by siRNAs reduces H2A ubiquitylation level in HeLa cells. In contrast, our study showed that the global level of H2AK119ub1 is unchanged in Pcgf1−/− ES cells (Fig. 5B), which is consistent with a recent study in zebra fish. Therefore, the global level of H2AK119ub1 is probably mediated by other component of PRC1.1. Notably, the unchanged levels of H2AK119ub1 in Pcgf1−/− might also be due to a result of redundant and compensatory mechanisms that have evolved to maintain global H2AK119ub1 levels. Experiments examining the effects of combinatorial deletion mutants of Pcgf5 may further clarify the specific roles that different version of PRC1 play in the maintenance of H2AK119ub1 levels. Indeed, knockdown of Kdm2b results in an approximately 40% global reduction of H2AK119ub1. Furthermore, we found that deletion of Pcgf1 resulted in the reduction of Ring1B and its associated histone mark H2AK119ub1 at Pcgf1 target genes. This result is similar to those observed for Pcg6−/− ES cells, suggesting a common molecular mechanism controlling Polycomb recruitment by Pcgf1 family in ES cells. Of note, the occupancy of BcoR (another component of PRC1.1) at these targets is also decreased. The reduction of BcoR enrichment in Pcgf1−/− ES cells is probably partially due to the destabilization of its protein level (Fig. 2D). Therefore, Pcgf1 is required for the recruitment of Ring1B and/or BcoR to its target genes (Fig. 5E). Interestingly, the occupancy of PRC2 component Suz12 and its chromatin modification H3K27me3 on Pcgf1 targets are also reduced in Pcgf1−/− ES cells. This is consistent with recent studies which suggest that PRC1-dependent H2AK119ub1 acts as cue for the downstream H3K27me3 deposition by PRC2 complex. As mentioned before, Pcgf1 works as a transcription activator in ES cells. The co-localization of Pcgf1 and H2AK119ub1 on Pcgf1 targets suggests that Pcgf1-mediated gene expression in ES cells might be H2AK119ub1-independent.

Although the Pcgf1-deleted ES cells do not show detectable proliferation defects and form EBs with an efficiency similar to that of wild-type cells, mutant cells exhibit severe defects in differentiation in vitro. These phenotypes are very similar to the ones recently reported for Kdm2b knockout ES cells, suggesting there could be
overlapping functions among the PRC1.1 complexes. The failure of Pcgf1−/− ES cells to undergo proper differentiation is consistent with the inability to fully inactivate core pluripotency genes (Oct4 and Nanog) and the absence of transcriptional activation of lineage marker genes during differentiation in the same cells. Accordingly, our results indicate that Pcgf1 and Kdm2b share common biological functions. Moreover, Kdm2b knockdown in ES cells also demonstrates a critical function of Kdm2b in recruiting PRC1 to CpG Islands of developmental regulators19,21. Future study need to address how Pcgf1 and Kdm2b cooperatively contribute to the PRC1.1 chromatin recruitment and establishment of a specialized chromatin state. Based on these findings, we propose a model for Pcgf1-mediated PRC1.1 chromatin recruitment. We propose that Pcgf1 can interact with Kdm2b which can recognize unmethylated CpG islands and then recruit other components of PRC1.1 to target genes. H2AK119ub1 deposition by this complex, in turn, recruits PRC2 (Fig. 6).

We observed in vitro that ES cells deficient in Pcgf1 display severe defects in ectoderm and mesoderm differentiation. Additionally, Pcgf1 has been involved in the proliferation and differentiation of tumor cells45. Further studies using Pcgf1 conditional mice will shed light about the in vivo contributions of Pcgf1 during early development, homeostasis of the tissue and tumorigenesis.

Methods

ES cell culture. ES cells were co-cultured with mitomycin-inactivated murine embryonic fibroblasts (MEFs) on gelatinized tissue culture plates in DMEM (Gibico) supplemented with 15% fetal calf serum (Gibico), non-essential amino acids (Gibico), leukemia inhibitory factor (LIF), penicillin/streptomycin (Sunshine Biotechnology), L-glutamine (Sunshine Biotechnology) and 0.1 mM β-mercaptoethanol (sigma) as described46 at 37 °C with 5% CO2.

Generation of Pcgf1−/− ES cells. Pcgf1−/− ES cells were generated by Cas9 technology as described25. Briefly, we designed two sgRNAs by using online tool (http://crispr.mit.edu/). sgRNAs were cloned into the pX330-U6-Chimeric-BB-CBh-hSpCas9 (pX330; Addgene plasmid ID 42230) vector. The sgRNA expression constructs were verified by sequencing. sgRNA-Cas9 vectors were co-transfected with a plasmid encoding puromycin (Puro) resistance into ES cells. After 24 hours, ES cells were treated with puromycin for 48 hours and then seeded on MEF feeder to form single colony. The Pcgf1−/− ES cell colonies were identified via DNA-PCR, RT-PCR and Western blot.

Generation of Pcgf1 expression vector and establishment of a stable Pcgf1−/− rescued (Pcgf1−/−/Pcgf1) ES cell line. The Pcgf1 full-length cDNA (NM_197992) was modified by adding N-terminal Flag-tag (DYKDDDDK) sequence into the forward PCR-primer, followed by cloning into pBluescript KS (-). The complete coding sequence was verified by sequencing. The correct inserts were cloned into lentiviral vector46. Lentiviral supernatants were produced as described46. Briefly, lentivirus was packaged in 293T cells and concentrated in lentiviral supernatant was used to infect Pcgf1−/− ES cells with polybrene (Sigma, final concentration of 8 μg/ml). Puromycin was used to screen positive Pcgf1−/− ES cell line and the FLAG-tagged Pcgf1 expression levels were examined by Western Blot. The primers used for PCR are shown in Supplementary Table 1.

Alkaline phosphatase (AP) staining. ES cell cultures were fixed with 4% PFA (Solarbio) on ice-cold 75% ethanol (drop-wise, while vortexing) for 30 minutes, and stored at −20 °C for at least 4 hours. Subsequently, cells were washed twice with PBS, harvested and incubated for 30 min at 37 °C with RNase A (100 μg/ml, Vazyme, A411-01/02), and stained with the propidium iodine (20 μg/ml) protected from light for 60 min at 37 °C followed by analysis on a FACS LSRFortessa (BD Biosciences) as described46.

Cell cycle analysis (Flow cytometry). ES cells were trypsinized, washed three times with PBS, fixed in ice-cold 75% ethanol (drop-wise, while vortexing) for 30 minutes, and stored at −20 °C for at least 4 hours. Subsequently, cells were washed twice with PBS, harvested and incubated for 30 min at 37 °C with RNase A (100 μg/ml, Vazyme, A411-01/02), and stained with the propidium iodine (20 μg/ml) protected from light for 60 min at 37 °C followed by analysis on a FACS LSRFortessa (BD Biosciences) as described46.

Embryoid body (EB) formation and analysis. ES cells were trypsinized and resuspended in medium without LIF46. 30 μL (500–1000 cells/drops) was pipetted onto the Petri-dish plate lid, and 10 mL of PBS was placed on a plate to prevent the drops from desiccation. EBs were grown in hanging drops and were cultured for 3 days (37 °C, 5% CO2). Three days later, EBs were harvested and cultured on a rotating shaker (37 °C, 5% CO2).
Fresh medium was replaced every 2 days to avoid medium exhaustion. Total RNA was collected from day 3, 7 and 12 (Trizol, Invitrogen) and analysed by RT-qPCR.

Global gene expression analysis, RNA preparation and RT-qPCR. Total RNA collected from ES cells and EBs was purified using Trizol (Invitrogen). RNA was reverse transcribed into cDNA with oligo(dT) or random primers using the HiScript™TM 1st Strand cDNA Synthesis Kit (Vazyme Biotech). Quantitative real-time PCR (RT-qPCR) was performed using PowerUp™ SYBR® Green Master Mix (Invitrogen) on a StepOne™ Software v2.3 (Applied Biosystems). The relative expression of genes was analysed based on the $2^{-\Delta\Delta Ct}$ method using the Actin gene as a control. The primers used for RT-qPCR are shown in Supplementary Table 1.

Nuclear extraction, immunoprecipitation, Western blot analysis and histone extraction. ES cells were harvested and lysed in hypotonic lysis buffer (10 mM Tris–Cl pH 8.0, 1 mM KCl, 1.5 mM MgCl$_2$ and 0.5 mM β-mercaptoethanol, 10 mg/ml PMSF, Protease Inhibitor Mix (Sigma)). Nuclear Extracts were prepared from nuclei using lysis buffer (20 mM Tris–HCl pH 8.0, 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 1% Triton-X-100, 25% Glycerol, 0.5 mM β-mercaptoethanol, 10 mg/ml PMSF, Protein inhibitors). Nuclear extracts from the Pcgf1 Flag-tagged ES cells were incubated with M2 agarose beads (A2220, Sigma), in binding buffer (2/3 volume dilution buffer (20 mM Tris–HCl pH 8.0, 0.2 mM EDTA) and 1/3 volume nuclei lysis buffer) overnight at 4°C. The beads were washed 3 times with washing buffer (20 mM Tris–HCl at pH 8.0, NaCl 450 mM and 0.2 mM EDTA) and subsequently the bound proteins were dissolved in gel loading buffer. Total proteins were separated by SDS-PAGE. The proteins were transferred on polyvinylidene fluoride (PVDF) membrane and the membrane was blocked with 5% (w/v) non-fat milk for one hour at room temperature and then incubated overnight at 4°C with antibodies against Pcgf1 (sc-515371, Santa Cruz Biotechnology, 1:1,000), Flag (sc-807, Santa Cruz Biotechnology, 1:1,000), BcoR (12107-1-AP, Proteintech, 1:1000), Ring1B (09–723, Millipore, 1:1000), Nanog (sc-134218, Santa Cruz Biotechnology, 1:1,000), Oct4 (sc-5297, Santa Cruz Biotechnology, 1:1,000), Sox2 (sc-17320, Santa Cruz Biotechnology, 1:1,000), Pcgf5 (ab201511, Abcam, 1:1000), β-Actin (A01010-1, Abbkine, 1:1000), Suz12 (sc-46264, Santa Cruz Biotechnology, 1:1,000), H2AK119ub1 (#8240, Cell Signaling Technology, 1:1,000), H3K27me3 (#9733, Cell Signaling Technology, 1:1,000), H3 (17168-1-AP, Proteintech, 1:1000). Then, the membrane was incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG-HRP (sc-2005, Santa Cruz Biotechnology, 1:5000), goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz Biotechnology, 1:5000) for 1 h. Chemiluminescence was detected using the ECL blot detection system. Histone Extraction was performed as described47.

RNA-seq. Total RNA was isolated using Trizol reagent (Gibco, 15596–018) according to the manufacturer’s protocol. The preparation of whole RNA-seq libraries and deep sequencing were performed by the Annoroad Gene Technology Corporation (Beijing, PR China). RNA integrity number (RIN) and the concentration were measured using a 2100 RNA Nano 6000 Assay Kit (Agilent Technologies, CA, USA). The mRNA was enriched with Oligo (dT) mRNA magnetic beads. RNA-seq libraries were prepared using 6 bp random primers and librar-
Y. Y. genotyped and characterized Pcgf1 knockout ES cells with help of W. Z. and Y. Y. performed RT-qPCR and ChIP-qPCR and analysed all experiments with help of Y. H.; H. T. performed Western blot analysis; X. Y. provided technical advice. This project was supported by grants from the National Natural Science Foundation of China (31471387 and 31671532) and the 2015 Shuangchung Program of Jiangsu Province to J. Q.

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**Author Contributions**

Y.Y. genotyped and characterized Pcgf1 knockout ES cells with help of W.Z. and Y.Y. performed RT-qPCR and ChIP-qPCR and analysed all experiments with help of Y.H.; H.T. performed Western blot analysis; X.Y. provided...
advice on experimental design and collected and organized data, with assistance of Q.J.; J.Q. designed, analysed, supervised research and wrote the manuscript with help of Y.Y. The manuscript was critically reviewed and approved by all authors.

**Additional Information**

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