Biochemical and Biophysical Research Communications 437 (2013) 101–107

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

CHD4/NuRD maintains demethylation state of rDNA promoters through inhibiting the expression of the rDNA methyltransferase recruiter TIP5

Te Ling [a,b,1], Wenbing Xie [b,1], Min Luo [c,1], Meili Shen [b], Qiaoyun Zhu [b], Le Zong [b], Tingting Zhou [b], Jun Gu [b], Zhigang Lu [d,*], Feixiong Zhang [a,*], Wei Tao [b,*]

[a] College of Life Sciences, Capital Normal University, Beijing 100048, China
[b] Key Laboratory of Cell Proliferation and Differentiation of the Ministry of Education, College of Life Sciences, Peking University, Beijing 100871, China
[c] Shenzhen Center for Disease Control and Prevention, Shenzhen 518055, China
[d] Laboratory of Chemical Genomics, School of Chemical Biology and Biotechnology, Shenzhen Graduate School of Peking University, Shenzhen 518055, China

ARTICLE INFO

Article history:
Received 10 June 2013
Available online 22 June 2013

Keywords:
rRNA genes
TIP5
NoRC
DNA methylation
Reprogramming

ABSTRACT

Despite the well-established fact that NuRD (nucleosome remodeling and histone deacetylase) is incapable of actively demethylating DNA, the complex is surprisingly showed to be required for the establishment of unmethylated state at promoters of ribosomal genes. But the molecular mechanism underlying how NuRD mediates unmethylation at rDNA promoters remains obscure. Here we show that NuRD directly binds to the promoter of rDNA transcription silencer TIP5 (TTF-I interacting protein 5), one of the components of nucleolar remodeling complex NoRC that silences rRNA genes by recruiting DNA methyltransferase to rDNA promoters and increasing DNA methylation. NuRD negatively regulates TIP5 expression, thereby inhibiting rDNA methylation and maintaining demethylation state of rDNA promoters. The deficiency of NuRD components in reprogrammed cells activates TIP5 expression, resulting in the increased fraction of heterochromatic rRNA genes and transcriptional silencing. Thus, NuRD is able to control methylation status of rDNA promoters through crosstalking with NoRC complex.

1. Introduction

NuRD (nucleosome remodeling and histone deacetylase) is a multisubunit complex containing nucleosome remodeling and histone deacetylase activities [1,2]. MBD3 (methyl-CpG binding domain protein 3), another subunit of the NuRD complex, does not directly bind to methylated DNA but is highly related to MBD2 (methyl-CpG binding domain protein 2), a polypeptide that binds to methylated DNA [3]. NuRD provides a means of gene silencing by DNA methylation in gene transcription [3]. Surprisingly, CHD4/NuRD has been shown to activate rRNA synthesis and result in the increase of unmethylated rRNA gene promoters [4,5]. Mechanism investigation demonstrates that CHD4/NuRD binds to a subset of rRNA genes being unmethylated but not transcribed, so called poised state of rDNA promoters, and functional interplay with CSB to mediate the transition of rRNA genes from the permissive to the active state [5,6]. Strikingly, CHD4 is directly involved in the establishment of the poised state, and enables rDNA promoters to maintain unmethylated state [5,7]. It seems that one of the components of NuRD might be an enzyme capable of actively demethylating DNA. However, numerous studies failed to confirm the demethylase activity of NuRD [8–11]. Thus, the finding that NuRD complex maintains the unmethylated state of poised promoters raises a new question about the molecular mechanism underlying how NuRD maintains the hypomethylated state at rDNA promoters.

Ribosomal RNA (rRNA) genes exist in three different epigenetic states, active rDNA promoters being unmethylated and marked by euchromatic histone modifications, silent ones are methylated and exhibit heterochromatic features, and poised ones display bivalent chromatin modifications which are permissive for active transcription [5,12,13]. Several ATP-dependent chromatin remodeling complexes play an important role in establishing these three states and distinguishing silent heterochromatin and active euchromatin from the poised bivalent chromatin [13,14]. NoRC (nucleolar remodeling complex) consisting of SNF2h (sucrose nonfermenting protein 2 homologue) and TIP5 (TTF-I interacting protein 5) maintains the silent state of rDNA by repositioning of the promoter-bound nucleosomes, alternation in histone modifications, and increased DNA methylation, thereby establishing heterochromatic features at a subset of rDNA repeats [12,15–17]. NuRD has been shown to establish the poised state of rRNA genes being unmethylated and marked by bivalent histone modifications [5]. Poised rDNA promoters are permissive for transcription and can

* Corresponding authors.
E-mail address: weitao@pku.edu.cn (W. Tao).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.06.045

be further activated by another chromatin remodeler CSB (Cockayne syndrome group B), whose function is to maintain the active rDNA state [5,6]. Thus, different chromatin remodelers establish/modulate the ratio of active, poised and silent clusters of rRNA genes, and control the rDNA transcription level to meet the various demands for ribosome production and protein synthesis in cell activities such as reprogramming and differentiation.

In this study, we show that NuRD complex binds directly to the promoter of TIP5 gene in mouse embryonic fibroblast (MEF) cells, and negatively controls TIP5 expression. Knockdown of NuRD components activates TIP5 expression, resulting in the increased level of DNA methylation and heterochromatic histone modification at rDNA promoters. Reprogramming of MEFs into induced pluripotent stem cells correlates with decreased level of NuRD and accordingly up-regulated expression levels of TIP5, thereby triggering rDNA promoter methylation, heterochromatin formation and transcriptional silencing. Thus, NuRD establishes the unmethylated state of rDNA promoter through repression of TIP5 expression which in return inhibits methylation at rDNA promoters. The results uncover a novel mechanism by which the functional interplay of two chromatin remodelers regulates the methylation status for rDNA transcription.

2. Materials and methods

2.1. Plasmids and antibodies

The retroviral encoding CHD4 and shRNA vectors are based on pBabe and pQsupR published by Ramirez-Carrozzi et al. respectively. The shRNA sequences are listed in Supplementary Table S1. Plasmids encoding mouse *Oct4*, *Sox2*, *Klf4* and *c-Myc* were from Addgene. The following antibodies were used: anti-*SSEA-1* (480, Santa Cruz, USA), anti-*Nanog* (30329, Santa Cruz, USA), anti-CHD4 (11378x, Santa Cruz, USA), anti-MBD2 (3754, Abcam, UK), anti-MBD3 (3755, Abcam, UK), anti-H3K9me3 (8898, Abcam, UK), anti-H3K4me3 (8580, Abcam, UK), anti-HDAC1 (7872x, Santa Cruz, USA), anti-HDAC2 (7029, Abcam, UK), anti-TIP5 (73303, Abcam, UK & polyclonal antibody provided by Ingrid Grummt Lab), anti-α-tubulin (T9026, Sigma, USA), anti-α-smooth muscle actin (N1584, Dako, Denmark), anti-α-fetoprotein (8108, Santa Cruz, USA), and anti-nestin (21249, Santa Cruz, USA).

2.2. Cell culture and iPSCs generation

Plat-E cells and MEFs were cultured in DMEM medium (Gibco, USA) containing 10% FBS (Gibco, USA), 50 U/ml penicillin and 50 mg/ml streptomycin (Gibco, USA). Mouse embryonic stem cells (mESCs) (R1) and induced pluripotent stem cells (iPSCs) were maintained in mESCs culture medium (DMEM/F12, 20% FBS, 1% NEAA, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 50 U/ml penicillin, 50 mg/ml streptomycin, 1000 U/ml LIF) (Gibco, USA) on feeder layers of mitomycin C (Roche, USA)-treated MEFs. For, retroviruses expressing *Oct4*, *Sox2*, *Klf4*, *c-Myc*, MBD2-shRNA, CHD4-shRNA or MBD3-shRNA were produced by transfecting vectors into Plat-E cells using the calcium phosphate method (OSKM for iPSCs induction). For iPSCs induction, MEFs were infected with the respective retroviruses supplemented with 4 μg/ml polybrene (Sigma, USA) and cultured in mESC medium or iSF1 medium (DMEM/high glucose, 10% KSR, 1/200 N2, 1 mM L-glutamine, 1% NEAA, 50 U/ml penicillin, 50 mg/ml streptomycin, 1000 U/ml LIF, 5 ng/ml bFGF) (Gibco, USA). For OSKM mediated reprogramming, iPSCs generation was monitored 16 post infection in mESC medium and 12 days post infection in iSF1 medium.

2.3. Northern blot hybridization

Non-isotopic labeling of the DNA probes with digoxigenin (DIG) was achieved using DIG-11-dUTP (Roche, USA). Specific probes for 45S pre-rRNA were obtained by amplification of the sequences from the pMr600 provided by Ingrid Grummt Lab. The primers used for Northern blotting hybridization are shown in Supplementary Table S1. Total RNA was electrophoresed on a 1.2% formaldehyde agarose gel, capillary-blotted onto a Hybond membrane (Amersham, USA), and UV cross-linked. Blots were hybridized with 45S pre-rRNA probes. The signal was developed with DIG High Prime DNA Labeling and Detection Starter Kit (Roche, USA).

2.4. Gene expression analysis and chromatin immunoprecipitation (ChIP)

cDNA was generated from 1 μg of total RNA using random primers dN6 (Promega, USA) and M-MLV reverse transcriptase (Promega, USA). qPCR was performed using the SYBR Premix Ex Taqkit (Takara, Japan) with primers listed in Supplementary Table S1. Crosslinked chromatin was sonicated, precleared and incubated with specific antibodies. Immunoprecipitated DNA was quantified by real-time PCR, calculating the ratio of DNA in the immunoprecipitates *versus* input chromatin. Data were normalized to control reactions with IgGs.

2.5. Cell characterization

Alkaline phosphatase (AP) staining was performed using NBT/BCIP substrates (Promega, USA). For *in vitro* differentiation, mESCs or iPSCs were harvested by trypsinization and transferred to bacterial culture dishes containing mESC medium without LIF. After 3 days, aggregated cells were plated onto gelatin-coated tissue culture dishes and incubated for another 3 days. Differentiation was determined by immunostaining with antibodies against smooth muscle actin, α-fetoprotein or nestin. For teratoma assays, 1×10^6 iPSCs were subcutaneously injected into nude mice. All animal studies were conducted at the AAALAC approved Animal Facility in the Laboratory Animal Center of Peking University. Experiments were undertaken in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals, with the approval of Peking University Laboratory Animal Center, Beijing. Tumors were surgically dissected after four weeks, fixed, embedded in paraffin, and sections were stained with hematoxylin and eosin.

2.6. Bisulfite sequencing

To monitor DNA methylation of the rDNA promoter, genomic DNA was modified by bisulfite using the EpiTect Bisulfite Kit (Qiagen, Germany), amplified by PCR and sequenced. The PCR product contains −143, −133 and +8 three CpG dinucleotide sites and the sequences are: for-5′-GAGTTTTTTTTCTTTTTCTTA-3′, rev-5′-CAATTATCACAAACTACCCAC-3′.

2.7. Statistical analysis

All experiments were performed at least three times. The results are expressed as the mean ± SE. Differences among groups were determined by the Student's *t*-test. $p < 0.05$ is considered statistically significant.

3. Results

3.1. NuRD binds to the promoter of TIP5 and represses its transcription

TIP5/NoRC recruits DNMTs and establishes a silent chromatin structure at rRNA genes, but it is transcribed by RNA polymerase II. Given that mainly acting as a broad repressor for RNA polymerase II transcriptional activity, NuRD is possibly involved in the regulation of TIP5 expression. To determine whether NuRD was able to interact directly with the TIP5 promoter, we performed chromatin immunoprecipitation assays in MEFs. PCR primer sets were designed to amplify ChIP amplicons spanning 2 kb upstream to 26 kb downstream of the transcription start site (TSS) of TIP5 (Fig. 1A). The ChIP result showed that NuRD strictly bound to the

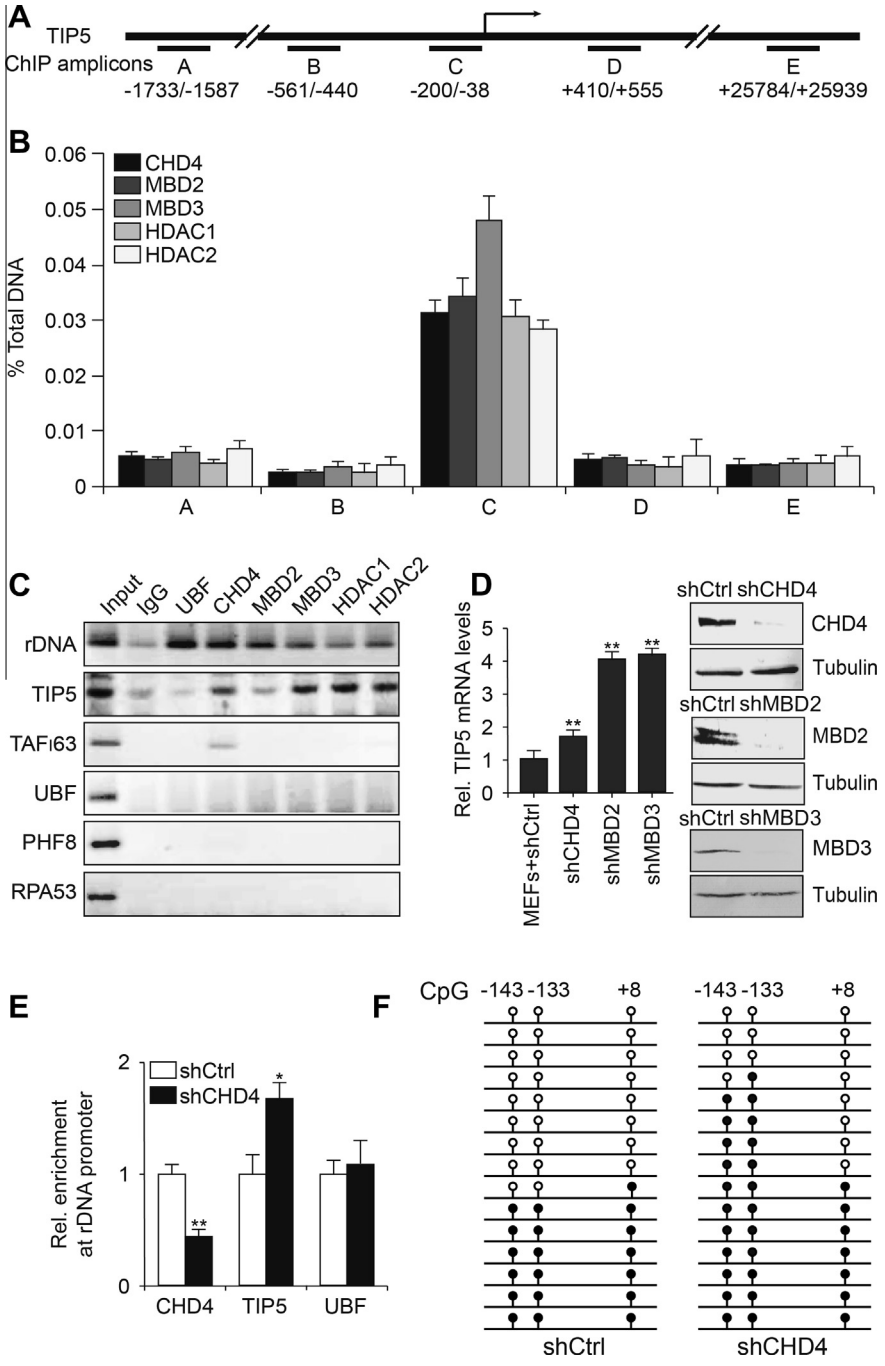

Fig. 1. CHD4/NuRD interacts with TIP5/NoRC. (A) Schematic of locations of primer sets used in (B) throughout TIP5 gene. Primer sets cover 2 kb upstream and 26 kb downstream of the transcription start site (arrows). The locations of primer pairs are shown below. (B) Cross-linked chromatin from MEFs was incubated with antibodies against CHD4, MBD2, MBD3, HDAC1 and HDAC2, subunits of NuRD complex. The precipitated DNA was analyzed by qPCR using primer pairs that amplify the indicated regions of TIP5 indicated in the scheme above. Error bars represent s.d. from at least three independent experiments. (C) PCR performed with ChIPed DNA from (A) and primer sets of indicated genes' promoter region. rDNA is a positive control that NuRD binds directly. (D) RT-qPCR measuring TIP5 transcription level in MEFs upon knockdown CHD4, MBD2 or MBD3. Data are normalized to GAPDH mRNA ($^{**}p < 0.01$, $n = 3$). Error bars denote standard deviation ($n = 3$). The western blots (WB) show the level of CHD4, MBD2 and MBD3 in MEFs infected with retroviruses encoding gene specific shRNA or control shRNA. Analyses were performed 8 days post-infection. (E) Quantitative PCR detects the enrichment of CHD4 and TIP5 on rDNA promoters upon depletion of CHD4 in (D) ($^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$). (F) Bisulfite sequencing data show methylation of rDNA promoter from depletion of CHD4 in MEFs. Methylated CpGs are represented by black circles, unmethylated CpGs by white ones.

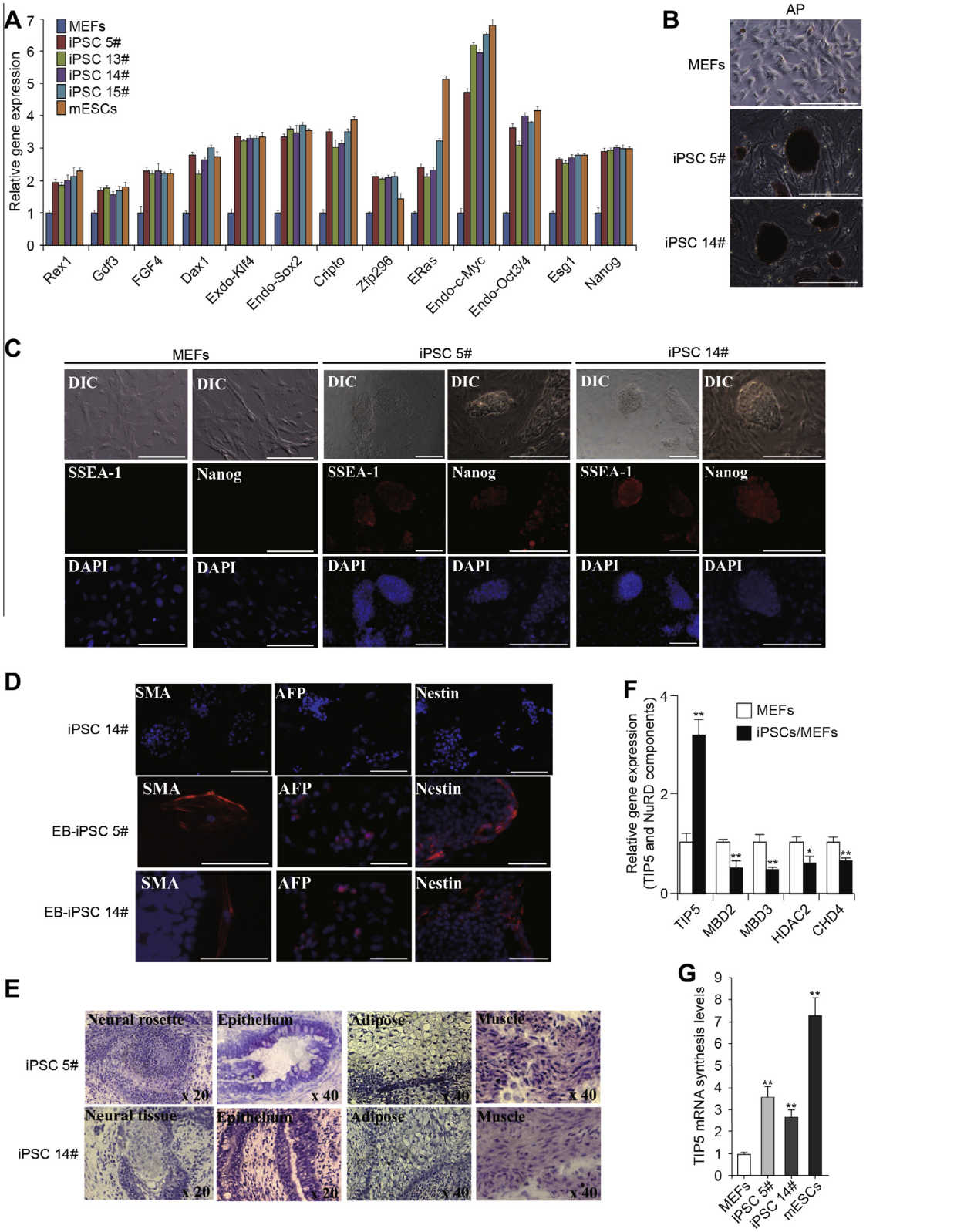

Fig. 2. Deficiency of NuRD components in reprogrammed cells accompanied by the increase of TIP5. Characterization of iPSCs derived from MEFs transfected with *Oct4*, *Sox2*, *Klf4* and *c-Myc* (OSKM). RT-qPCR analysis of mESC marker genes (A), AP staining (B), immunofluorescence of mESC marker genes (*SSEA-1* and *Nanog*) (C), immunofluorescence showing embryoid body (EB)-mediated differentiation of iPSCs (EB-iPSC 5# and EB-iPSC 14#) (D) and hematoxylin and eosin staining showing teratoma formation of iPSCs (E). Scale bars = 100 μm. (F) Comparison of TIP5 and NuRD transcriptional level in iPSCs and mouse embryonic fibroblasts (MEFs). The bars represent the relative levels of pre-rRNA normalized to GAPDH mRNA ($^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$). (G) RT-qPCR analysis shows the expression level of TIP5 in MEFs, mESCs and iPSC lines. The bars represent the relative levels of pre-rRNA normalized to GAPDH mRNA ($^{**}p < 0.01$, $n = 3$).

TIP5 promoter (Fig. 1B). As expected, NuRD was associated with the rDNA promoter [5], however we did not detect association of NuRD with promoters of Pol I transcription initiation complex genes, i.e., TAF 63, UBF and RPA53, as well as PHF8 that is an activator for Pol I transcription (Fig. 1C). We then examined the role of NuRD in the regulation of TIP5. We measured TIP5 transcription upon short-hairpin RNA (shRNA)-mediated depletion of NuRD. We found that depletion of NuRD components, i.e., CHD4, MBD2 and MBD3, increased the level of TIP5 transcription (Fig. 1D). Given that TIP5 is a well-known key player in silencing rDNA promoters and knockdown NuRD results in increased methylation at rDNA promoters [5,7,17] (Fig. 1E and F), these findings imply that NuRD maintains the unmethylated state of rDNA promoter through down-regulating TIP5 expression level.

3.2. NuRD deficiency in reprogrammed cells activates TIP5 expression

To investigate the mechanism underlying rDNA transcription regulation through the crosstalk between NuRD and NoRC, we firstly reprogrammed mouse embryonic fibroblast (MEF) cells into induced pluripotent stem (iPS) cells as previously described [18], mouse embryonic stem (mES)-like clones were generated with the infection of pMXs based retroviral vectors containing defined four factors, i.e., *Oct4*, *Klf4*, *Sox2* and *c-Myc*. RT-qPCR examination showed that all the selected ESC marker genes were activated in iPSC 5#, iPSC 13#, iPSC 14# and iPSC 15# clones (Fig. 2A). Two mESC-like clones, 5# and 14#, were chosen as replicates for the following study. Immunocytochemistry staining verified that these two clones were alkaline-phosphatase (AP) staining positive (Fig. 2B), expressed typical pluripotency marker genes including *Nanog* and *SSEA-1* (Fig. 2C). iPSCs formed embryoid bodies in non-coated plastic dishes. When grown in tissue culture dishes, the embryoid bodies from iPSC 5# and iPSC 14# cells attached to the dish bottom and initiated differentiation. After 3 days, immunostaining detected cells positive for α-smooth muscle actin (mesoderm marker), α-fetoprotein (endoderm marker), and nestin (ectoderm marker) (Fig. 2D). Importantly, the established iPSC lines could form embryonic body (EB) efficiently and differentiate into three germ layers specific cell type *in vitro* (Fig. 2E). These data confirmed the pluripotency of iPSC 5# and iPSC 14# *in vitro*.

Then we analyzed gene expression of CHD4/NuRD and TIP5 in MEFs and iPSCs. Specifically, CHD4/NuRD components were down-regulated, whereas rDNA specific repressor TIP5 was significantly up-regulated (Fig. 2F). Therefore, these findings demonstrated that the decreased level of NuRD is accompanied by increased TIP5, strongly suggesting that rDNA promoters could transited to silent state during reprogramming. Indeed, RT-qPCR result showed that TIP5 was up-regulated after reprogrammed as well as in ESCs (Fig. 2G).

3.3. The fraction of silent rRNA genes is increased during reprogramming

We monitored the levels of rDNA transcription in MEFs, two iPSC clones and mouse embryonic stem (mES) cells. Both Northern blot and RT-qPCR analyses demonstrated that rDNA transcription is down-regulated upon iPSCs generation (Fig. 3A and B). Expression of Pol I transcription machinery such as RPA53 (subunit of

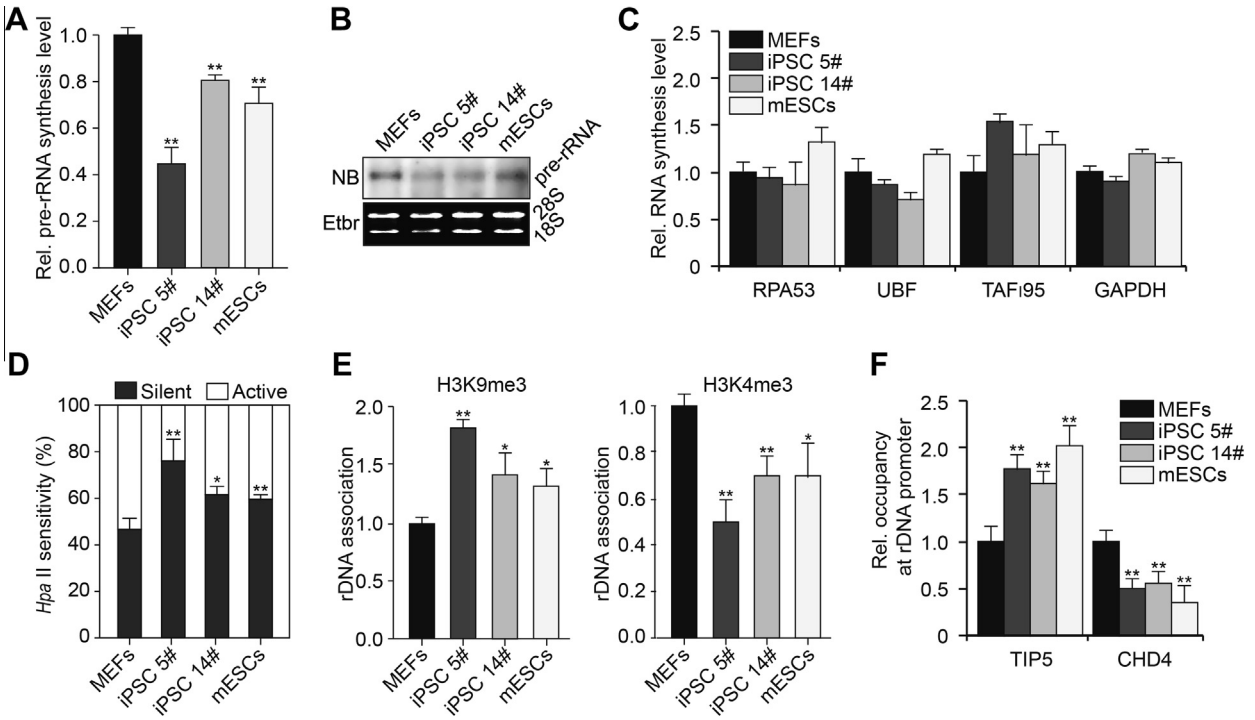

Fig. 3. pre-rRNA synthesis decreased during reprogramming as well as in mESCs. (A) RT-qPCR analysis shows the expression level of pre-rRNA in MEFs, mESCs and iPSC lines. The bars represent the relative levels of pre-rRNA normalized to GAPDH mRNA ($^{**}p < 0.01$, $n = 3$). (B) Northern blot (NB) shows the expression level of pre-rRNA in MEFs, mESCs, and two iPSC lines (iPSC 5# and iPSC 14#) induced by ectopic *Oct4*/*Sox2*/*Klf4*/*c-Myc* (OSKM). The panel below shows ethidium bromide (EtBr) stained cellular rRNA. (C) RT-qPCR analysis showing the expression of components of polymerase I initiation complex in MEFs, mESCs and iPSC lines. RPA53 is a subunit of Pol I; TAF 95 is a subunit of TIF-IB. Error bars represent standard deviation ($^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$). (D) Real-time PCR data showing the level of *Hpa* II-sensitive (white bars) and *Hpa* II-resistant (grey bars) rDNA promoter in MEFs, iPSCs and mESCs. PCR primers map upstream or downstream of the *Hpa* II site at -143 and normalized to internal primer without *Hpa* II site ($n = 3$). (E) ChIP assay monitoring rDNA enrichment of H3K4me3 (euchromatic mark) and H3K9me3 (heterochromatic mark) in MEFs, iPSCs or mESCs. Quantitative PCR (qPCR) uses specific primer for the promoter of mouse rDNA. Data are normalized to histone H3. Error bars denote standard deviation ($^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$). (F) ChIP assay shows the relative occupancy of TIP5 and CHD4 in iPSCs and mESCs compared to MEFs at rDNA promoter. Data are normalized to input chromatin. Error bars denote standard deviation ($^{**}p < 0.01$, $n = 3$).

Pol I), UBF and TAF_I 95 (subunit of TIF-IB) during reprogramming is not decreased, excluding the possibility that down-regulation of rDNA transcription was caused by the deficiency of Pol I transcription machinery (Fig. 3C). Consistent with the down-regulation of rDNA transcription, *Hpa*II digestion analysis revealed that methylation at rDNA promoters increased in iPSCs as well as in mESCs compared to MEFs (Fig. 3D). rDNA promoter in reprogrammed cells maintains higher level of heterochromatic mark (H3K9me3) and less euchromatic mark (H3K4me3), whereas rDNA promoter in MEFs features by more euchromatic histone modifications (Fig. 3E). Indeed, much more TIP5 but less CHD4 occupies at rDNA promoter in reprogrammed and stem cells (Fig. 3F). Collectively, these data indicates reprogramming from MEFs to iPSCs induced epigenetic resetting of rDNA promoter to heterochromatic silent state, revealing that cell programming negatively regulates rDNA transcription.

4. Discussion

The association with histone deacetylases and diverse transcriptional repressors is consistent with the function of NuRD complex in gene silencing. However, NuRD is also associated with active genes, suggesting a versatile use of NuRD in transcriptional regulation [4,19–21]. Regarding to rDNA transcription, NuRD has been shown to activate rRNA genes expression through mediating the unmethylation of rDNA promoters. Such unmethylated rDNA promoters bound by NuRD are poised for transcription and act as reservoir for ready-to go rRNA genes [5]. Overexpression of NuRD results in increase of unmethylated promoters, indicating that NuRD directly involves in maintaining unmethylation. Though MBD2 was shown to activate genes by removing the methyl residues [11], numerous studies failed to confirm the demethylase activity of MBD2 [8–10]. However, several studies confirmed that NuRD complex is capable of specifically maintaining unmethylation state of rDNA promoters, and activating rDNA transcription [4,5,7], but the underlining mechanism remains unclear. The identification of the crosstalk between NuRD and TIP5 in this study demonstrated that NuRD mediates the unmethylation through repressing expression of TIP5. Though NuRD itself does not perform as a demethylase, it can drive rDNA promoters to unmethylated state by inhibiting the recruitment of DNMTs to rDNA promoters. Thus, the establishment of unmethylated rDNA promoters in poised state can be achieved through repression of TIP5 by NuRD.

Cell differentiation rapidly down-regulate the rate of rRNA synthesis, the reduced rate of rRNA formation during differentiation is not regulated at the level of pre-rRNA processing instead due to a decrease in rDNA transcription [22]. In contrast to differentiation, somatic cells can be reprogrammed into induced pluripotent stem (iPS) cells by defined transcription factors [18,23]. The reprogramming cells undertake dramatically modulation on epigenetic resetting and gene expression pattern including rDNA transcription. But the alteration of rDNA transcription during reprogramming and underlying regulation mechanism, however, remains poorly understood. Given that differentiation induces down-regulation of rDNA transcription, accordingly, reprogramming was thought to up-regulate rRNA synthesis. However, in this study, our results demonstrated that cell programming in fact negatively influenced rRNA synthesis, suggesting that the two processes are not simply reversal ones. It is worthwhile to note that the decreased capacity of embryonic stem cells for pluripotent differentiation correlates in time with activation of ribosomal genes [24]. It is likely that the activation of ribosomal genes correlates with the early stage of differentiation, but eventually the transcription down-regulates at terminal differentiation, suggesting that rDNA transcriptional level undergoes undulating regulation process during cell development.

Most importantly, in contrast to the increased methylation at rDNA promoter in reprogrammed cells, there is no change in methylation at rDNA promoter upon differentiation [5], suggesting that distinct mechanisms are applied to silence rDNA transcription in reprogramming and differentiation process. During cell differentiation, the up-regulated expression level of NuRD results in the accumulation of poised rDNA promoters without changes of rDNA methylation. While in reprogramming, decreased expression level of NuRD in mESCs and iPSCs induced elevated expression of TIP5 that triggers the increased DNA methylation and accumulation of silent state rDNA promoters. Thus, our finding of crosstalk between NuRD and NoRC demonstrates functional interplay between two distinct ATP-dependent chromatin remodeling complexes in controlling rDNA transcription during cell reprogramming and differentiation.

Author contributions

Conceived and designed the experiments: ZL, FZ, WT. Performed the experiments: TL, WX, ML, QZ, LZ, MS, TZ. Analyzed the data: TL, ZL, FZ, WT. Contributed reagents/materials/analysis tools: JG. Wrote the manuscript: WT.

Acknowledgments

We gratefully acknowledge Prof. Ingrid Grummt for kindly providing antibodies. Work in WT lab has been supported by the National Natural Science Foundation of China (NSFC, grant 30871377, 31171255 and 31071116) and the National Basic Research Program of China (973 Program, Grant No. 2013CB530700). F.Z. has been supported by the National Natural Science Foundation of China Grant 30971453.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.045.

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