# SHPRH regulates rRNA transcription by recognizing the histone code in an mTOR-dependent manner

Deokjae Lee[a,b,1], Jungeun An[c,2], Young-Un Park[c], Hungjiun Liaw[a,3], Roger Woodgate[d], Jun Hong Park[c], and Kyungjae Myung[a,b,c,4]

[a]Genome Instability Section, Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892; [b]Department of Biological Sciences, School of Life Sciences, Ulsan National Institute of Science and Technology, Ulsan, 689-798, Korea; [c]Center for Genomic Integrity, Institute for Basic Science, Ulsan, 689-798, Korea; and [d]Laboratory of Genomic Integrity, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-2371

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**Many DNA repair proteins have additional functions other than their roles in DNA repair. In addition to catalyzing PCNA polyubiquitylation in response to the stalling of DNA replication, SHPRH has the additional function of facilitating rRNA transcription by localizing to the ribosomal DNA (rDNA) promoter in the nucleoli. SHPRH was recruited to the rDNA promoter using its plant homeodomain (PHD), which interacts with histone H3 when the fourth lysine of H3 is not trimethylated. SHPRH enrichment at the rDNA promoter was inhibited by cell starvation, by treatment with actinomycin D or rapamycin, or by depletion of CHD4. SHPRH also physically interacted with the RNA polymerase I complex. Taken together, we provide evidence that SHPRH functions in rRNA transcription through its interaction with histone H3 in a mammalian target of rapamycin (mTOR)-dependent manner.**

SHPRH | rRNA transcription | histone H3 methylation | mTOR

Human ribosomal DNA (rDNA) is composed of hundreds of tandem repeats of 42.9-kb rDNA units that are organized into transcribed and intergenic regions (1). About one-half the 47S precursor ribosomal RNA (pre-rRNA) genes are actively transcribed, and the other half remain silent (2, 3). Transcription, processing of rRNA, and the assembly of ribosomes take place in the nucleoli (2, 4). Once transcribed in the nucleoli, pre-rRNA is immediately processed into small mature 28S, 18S, and 5.8S rRNAs that, together with ribosomal proteins, make a ribosome. Tight regulation of ribosome biogenesis, including rRNA transcription and synthesis of ribosomal proteins, is important in many biological processes such as cell proliferation, apoptosis, and autophagy (5–7), and is closely associated with metabolic processes. Because of its importance in many metabolic pathways, dysregulation of ribosomal biogenesis is linked to aging and diverse diseases, including anemia and cancers (8–12). 47S pre-rRNA is transcribed by the RNA polymerase I complex, whose activity is controlled by cellular responses to nutritional states, cellular stresses, growth, differentiation, and cell cycle (9). Posttranslational modifications of transcription factors, for example, phosphorylation of upstream binding factor (UBF), help regulate rRNA transcription (13). In addition to posttranslational modifications of transcription factors, nucleolar remodeling complex, NuRD (nucleosome remodeling and deacetylation) complex, and energy-dependent nucleolar silencing complex also affect rRNA transcription by modifying epigenetic signatures of rDNA, as well as histones in the rDNA promoter (14–16). In addition to conventional active and silent histone signatures, the rDNA promoter has another histone signature called a poised state. CHD4 and CSB-containing NuRD complex establish a poised chromatin signature of rDNA that represses but primes rRNA transcription by marking histone H3 with both active (H3 K4me3) and inactive (H3 K27me3) modifications (16). However, it is unclear how these epigenetic changes control the transcription of rRNA.

The mammalian target of rapamycin (mTOR) pathway is a master pathway that controls overall cellular activities, including autophagy, macromolecule biosynthesis, and cell cycle in response to nutrients, stress, and growth factors (17, 18). Indeed, the mTOR pathway controls rRNA transcription and production of ribosomal proteins in response to nutrient, growth factors, and serum (19–21).

Previously, we found SHPRH as a mammalian RAD5 homolog (22, 23). RAD5 functions to avoid the collapse of the DNA replication fork by promoting the bypass of DNA damage that would normally stall the DNA replication fork. RAD5 and its mammalian homolog SHPRH polyubiquitylate proliferating cell nuclear antigen (PCNA) to promote DNA damage bypass via an uncharacterized recombination-dependent pathway (24, 25). Furthermore, there are two RAD5 homologs in mammals, which suggests that functions other than DNA damage bypass might have been developed during evolution (26–28).

Although the epigenetic signature and mTOR-dependent regulation for rRNA transcription are important, it is not clearly understood how RNA polymerase I can be directed to recognize such signatures to determine rRNA transcription. In this study, we report that SHPRH promotes rRNA transcription in a nutrient/mTOR-dependent manner by recruiting RNA polymerase I to the active rDNA promoter. SHPRH localized at the rDNA promoter

## Significance

**Transcription of ribosomal RNA (rRNA), which composes the ribosome with other proteins, is tightly regulated to maintain the right number of ribosomes. Many DNA repair proteins have functions in addition to their role in DNA repair. We provide evidence that SHPRH functioning in DNA repair at stalled DNA replication forks recognizes epigenetic histone codes of rDNA through its plant homeodomain (PHD) and modulates 47S rRNA transcription. SHPRH bound to rDNA promoters and promoted RNA polymerase I recruitment for rRNA transcription. SHPRH localization to the rDNA promoter was inhibited by trimethylation of histone H3 lysine 4, which is a mark of the rDNA promoter at poised status on starvation. Collectively, we suggest a mechanism controlling 47S rRNA transcription by SHPRH in a histone methylation-dependent manner.**

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[1]Present address: Medytox Inc., Bundang-gu, Seongnam-si, Gyeonggi-do, 463-400, Korea.

[2]Present address: Department of Biological Sciences, Chonbuk National University, Jeonju, Chonbuk, 561-756, Korea.

[3]Present address: Department of Life Sciences, National Cheng Kung University, Taiwan City, Taiwan 70101.

[4]To whom correspondence should be addressed. Email: kmyung@ibs.re.kr.

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in the nucleoli through the interaction between its plant homeodomain (PHD) and a specifically modified histone H3. Localization of SHPRH is redistributed into foci in the nucleoli under starvation or exposure to rapamycin that inhibits the mTOR pathway.

## Results

**SHPRH Forms Starvation-Induced Foci in the Nucleoli in an mTOR-Dependent Manner.** SHPRH and HLTF, homologs of yeast Rad5, were previously identified as E3 ubiquitin ligases that catalyze the polyubiquitylation of PCNA and participate in methyl methane sulfonate (MMS)-induced DNA damage repair (26–28). In contrast to yeast in which Rad5-deficiency causes high sensitivity to DNA-damaging agents, silencing expression of SHPRH or HLTF in human cells or knockouts of SHPRH and HLTF in mice showed mild or no severe sensitivity to DNA damaging agents, respectively (29–31) (Fig. S1 *A* and *B*). Therefore, we hypothesized that SHPRH would have a function other than DNA damage repair in mammals. To explore alternative functions, we first determined cellular localization of SHPRH. SHPRH was detected in the nucleus, as well as in the chromatin-bound fraction, when stained with an antibody (3F8) that specifically detects SHPRH (Fig. 1*A* and Fig. S2 *A*–*C* and S3). When cells were cultured for several days without changing media, SHPRH formed nucleolar foci, as judged by colocalization with a nucleolar protein, Fibrillarin, and these foci were not detected in fresh growth media (Fig. 1 *A*–*C*).

If SHPRH foci were formed as a result of DNA damage in long culture condition, they should colocalize with other DNA damage-induced foci. However, we found no evidence that SHPRH colocalized with DNA damage response proteins, including phospho-RPA32, γH2AX, pol η, pol κ, PCNA, phospho-CHK1, 53BP1, XPA, and BRCA1 (Fig. S4 *A* and *B*). In addition, there were no SHPRH foci after treatment of cells with 60 J/m$^2$ UV irradiation or 0.01% MMS treatment unless cells were cultured for several days before treatment (Fig. S4 *C* and *D*). HP1β is a major heterochromatin protein associated with nucleolar regions (32). The location of nucleolar SHPRH foci did not overlap with HP1β (Fig. S4*E*).

SHPRH foci became more distinct in cells cultured for several days without changing growth media. We therefore hypothesized that cells experiencing nutrient restriction would generate SHPRH foci in the nucleolus. Consistent with our hypothesis, when cells were starved by incubating in HBSS, or treated with 2.5 μg/mL rapamycin that blocks the mTOR pathway and mimics starvation conditions (17, 18, 33), SHPRH foci were clearly detected in the nucleoli (Fig. 1 *B* and *C*). Starvation in HBSS did not increase DNA damage responses, including phosphorylation of RPA32 and CHK1; thus, the nucleolar SHPRH foci were not induced by DNA damage responses in starved condition (Fig. S4*F*). Starvation or rapamycin treatment did not change SHPRH level, suggesting SHPRH foci formation on starvation was not a result of the induction of SHPRH protein level (Fig. S4 *G* and *H*). We then investigated kinetics of nucleolar foci formation on starvation condition. SHPRH began forming nucleolar foci 2 h after cells were incubated in HBSS media (Fig. 1*D*). When HBSS media was replaced with normal growth media, the number of nucleolar SHPRH foci decreased in a time-dependent manner (Fig. 1*E* and Fig. S4*I*). Collectively, SHPRH forms nucleolar foci in response to inhibition of the mTOR pathway.

**SHPRH Interacts with the Ribosomal DNA Promoter in an mTOR-Dependent Manner.** The mTOR-dependent pathway promotes ribosomal biogenesis and inhibits autophagy (17, 18, 20). Multiple repeated ribosomal DNA (rDNA) clusters are localized in the nucleoli, and some of them are actively transcribed (Fig. 2*A*) (2, 34, 35). Because SHPRH was found in the chromatin-bound fraction and localized in the nucleoli (Fig. 1*A* and Fig. S3), we investigated whether SHPRH would bind to specific regions of ribosomal DNA (Fig. 2*A*). When SHPRH was cross-linked with chromatin and immunoprecipitated, a ribosomal DNA promoter (H42.9) was enriched in precipitates (Fig. 2*B*). We did not detect the enrichment of the H42.9 region in precipitates of cells whose SHPRH expression was silenced by shRNA, suggesting the enrichment of SHPRH was specific (Fig. 2*B* and Fig. S2*C*). Chromatin immunoprecipitation (ChIP) experiments with two different human cell lines, K562 and HEK293, showed enrichment of SHPRH on the rDNA promoter (Fig. S5 *A* and *B*). Because starvation or rapamycin treatment induced the formation of nucleolar SHPRH foci (Fig. 1 *B* and *C*), we next investigated whether enrichment of SHPRH at the rDNA promoter is also affected by starvation. SHPRH enrichment at the promoter of rDNA was reduced on starvation (Fig. 2*C*). Instead, starvation as well as rapamycin treatment caused SHPRH enrichment at different locations in rDNA, including H8 (Fig. S5*C*). Simultaneously, a modified chromatin marker, H3 K4me2, was increased at rDNA H8 and decreased at rDNA H42.9 (Fig. S5 *C*–*E*), suggesting SHPRH is redistributed from the rDNA promoter to a different region in the rDNA cluster and forms foci structures when cells were starved. In addition, we compared the location of SHPRH foci with UBF, which binds to active rDNA (16). Although fewer nucleolar proteins and foci of SHPRH were found compared with those of UBF, which is an essential factor for rDNA transcription (36), SHPRH foci were partially colocalized with or outside the UBF positive regions (Fig. S5 *F*–*H* and Movie S1). Relative occupancies of UBF in the rRNA transcribed region (H1–H13) were decreased in starved cells where SHPRH was redistributed from the rDNA promoter (Fig. S5*I*). Thus, starvation induces relocalization of SHPRH to other regions from the active rDNA promoter. SHPRH also interacted with other promoters that are not related to rDNA transcription, although no significant change in their mRNA level by SHPRH depletion was detected (Fig. S5 *J* and *K*). Collectively, when the mTOR pathway is inhibited by starvation or rapamycin, SHPRH is released from the rDNA promoter and forms nucleolar foci at a different location in the nucleoli.

**The PHD of SHPRH Interacts with Histone H3 When Lysine 4 of H3 Is Not Trimethylated.** In contrast to yeast Rad5 or human HLTF, SHPRH has a PHD (22, 28), which is generally known to interact with specifically modified histone H3 (Fig. 3*A*) (37–39). To determine specificity of the interaction between SHPRH and histones, individually purified histone proteins were incubated with purified SHPRH (Fig. S6*A*). Similar to other proteins containing a PHD, only histone H3 was coprecipitated with a full-length SHPRH (Fig. 3*B* and Fig. S6*B*). To examine the interaction between SHPRH and histone H3 in more detail, interactions between GST-conjugated SHPRH PHD (GST-PHD) and differentially methylated histone H3 peptides were monitored. Unlike GST protein, which did not bind to any H3 peptide, GST-PHD interacted with various histone H3 peptides except the lysine 4 trimethylated H3 (H3 K4me3) peptide (Fig. 3*C*). Trimethylation of lysine enhances the hydrophobicity of lysine. We therefore hypothesized that the hydrophilic lysine 4 of H3 would be important for the interaction with SHPRH. To test this hypothesis, mutant H3 peptides (H3 K4A and H3 K9A) lacking hydrophilicity in their lysine residue were used to study in vitro interactions (Fig. 3*D*). In contrast to the interaction between H3 K9me3 and H3 K9A peptides with GST-PHD, both H3 K4me3 and H3 K4A did not interact with GST-PHD, suggesting that trimethylation at K4 with a high hydrophobicity inhibited the interaction between H3 and SHPRH.

There are two major classes of PHDs (38). The first class of PHD found in BPTF and ING2 interacts with histone H3 carrying K4me3. The second class of PHD found in CHD4 and PHF21A interacts with unmodified, K4me, or K4me2 H3. Negatively charged amino acids such as glutamate or aspartate in the PHD

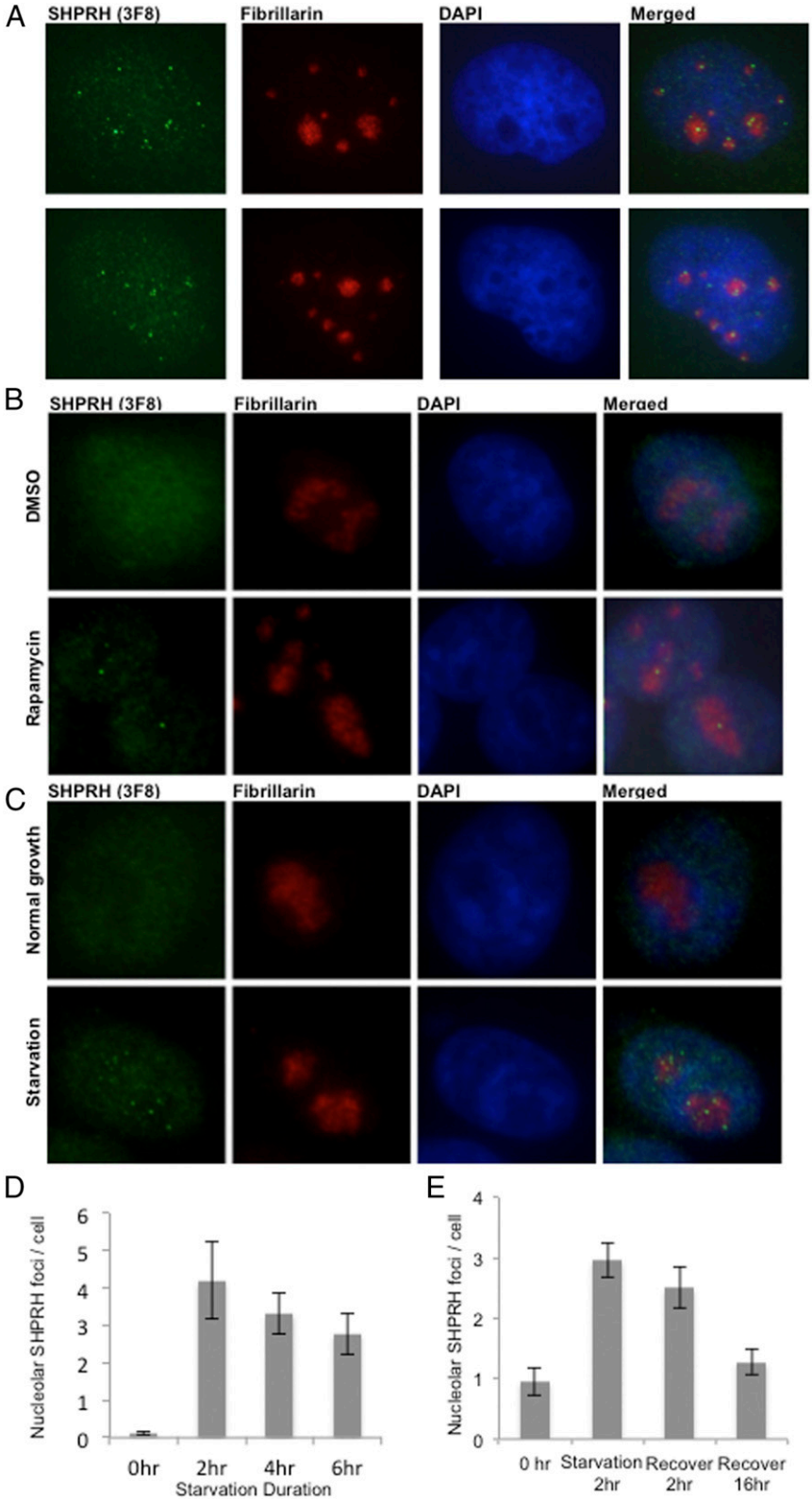


**Fig. 1.** Starvation or rapamycin treatment induce the nucleolar SHPRH foci. (*A*) Nucleolar SHPRH foci were detected in HeLa cells. HeLa cells were immunostained with anti-SHPRH 3F8, anti-Fibrillarin antibody, and DAPI. In addition to broad staining of SHPRH detected in the nuclei, large and dense SHPRH foci were colocalized with the nucleolar marker Fibrillarin. (*B* and *C*) Nucleolar SHPRH foci were induced on treatment with 2.5 μg/mL rapamycin for 24 h (*B*) or starvation with HBSS for 2 h (*C*) in HeLa cells. Endogenous SHPRH (green) and Fibrillarin (red) were shown. (*D*) Nucleolar SHPRH foci were sustained during starvation. After starvation, the number of nucleolar SHPRH foci per nucleus was counted in HeLa cells after fixation at indicated times. The averages of foci numbers from two independent experiments were plotted with mean ± SEM. (*E*) SHPRH foci disappeared when cells were recovered from starvation. After starvation in HBSS for 2 h, cells were recovered by supplying normal growth media for indicated times. Foci were counted from more than 74 nuclei. Graphs are presented with mean ± SD.

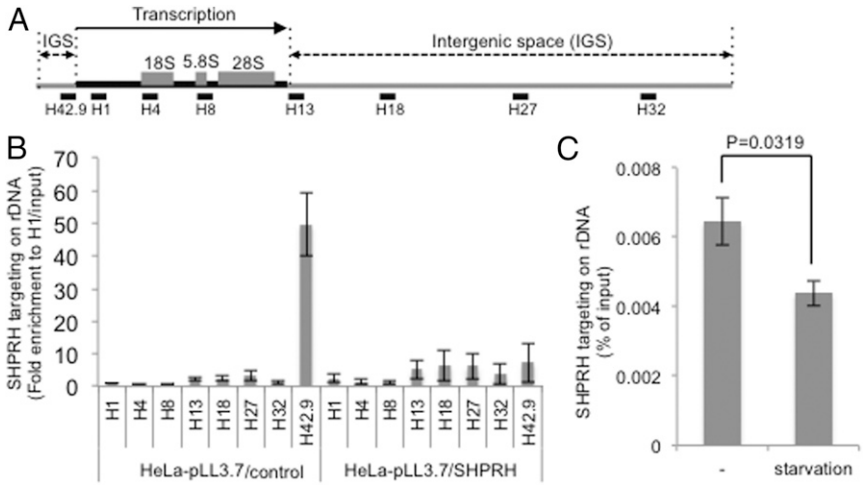


**Fig. 2.** SHPRH is recruited to the 47S rRNA gene promoter. (*A*) Schematic representation of an individual rDNA unit. The black bar and number indicate the amplified regions for ChIP-qPCR (quantitative PCR) assay. (*B* and *C*) ChIP assay with anti-SHPRH 3F8 antibody showed that SHPRH binds to the rDNA promoter. (*B*) HeLa cells stably transduced with control or shSHPRH lentivirus (HeLa-pLL3.7 control and HeLa-pLL3.7/SHPRH, respectively) were used for ChIP assay with anti-SHPRH 3F8 antibody. Bars indicate the relative values of each rDNA region normalized to input DNA and SHPRH-interacting H1, and represented as mean ± SEM from two independent experiments. (*C*) Starvation reduced the enrichment of SHPRH at the rDNA promoter (H42.9). HeLa cells were starved with HBSS for 4 h. Precipitated DNA was amplified with H42.9 primers. Data are represented as mean ± SEM from five independent experiments.

are critical for its interaction with histones (40, 41). To examine whether this requirement for a negatively charged amino acid in the PHD is conserved in SHPRH, we aligned the SHPRH PHD with other PHDs. We found a critical amino acid, a glutamate at amino acid residue 660 (E660) in SHPRH, aligned with negatively charged amino acids of PHDs that interact with unmodified, K4me, or K4me2 H3 (Fig. 3*E*). When E660 of SHPRH was mutated to a neutral amino acid, alanine (A) or tyrosine (Y), the mutant SHPRH PHDs no longer interacted with histone H3 in vitro (Fig. 3*F*). Similarly, the exogenously expressed SHPRH PHD mutant protein (E660A) showed less interaction with H3 K4me2 (Fig. S6*C*). Consistent with the in vitro interactions between H3 and SHPRH, in vivo immunoprecipitation with crosslinking by a histone H3 K4me2 antibody coprecipitated SHPRH, but not with a H3 K4me3 antibody (Fig. 3*G*).

We next investigated whether the SHPRH PHD is important for the enrichment of SHPRH at the promoter of rDNA. The localization of ectopically expressed wild-type and mutant SHPRH (E660A) proteins at the rDNA promoter was monitored by ChIP. As expected, although the ectopically expressed wild-type SHPRH protein was enriched at the rDNA promoter, the E660A SHPRH mutant was not (Fig. 4*A* and Fig. S6*D*).

Unlike most promoters where H3 K4me3 represents an active state, H3 K4me3 at the rDNA promoter denotes a poised state, whereas H3 K4me2 with acetylated H4 represents the active state (16, 42). SHPRH is enriched at the rDNA promoter and released from it on starvation. Thus, we hypothesized that SHPRH depletion or starvation would change the status of methylation in H3 from K4me2 to K4me3. H3 K4me3 at the rDNA promoter was increased and H3 K4me2 decreased after depletion of SHPRH, or on starvation (Fig. 4 *B* and *C*). Consistent with our data, it has been reported that the occupancy of H3 K4me3 at the rDNA promoter is increased by serum deprivation (16, 43). Thus, we conclude that the PHD of SHPRH is important for the enrichment of SHPRH at the rDNA promoter by recognizing histone codes.

**SHPRH Up-Regulates 47S rRNA Transcription.** Because SHPRH is recruited to the rDNA promoter, we hypothesized that SHPRH would affect the level of pre-rRNA transcription. Indeed, depletion of SHPRH with two individual siRNAs that target the 3′ UTR of SHPRH reduced 47S pre-rRNA levels (Fig. 5*A* and Fig. S7*A*). Conversely, SHPRH overexpression increased 47S pre-rRNA levels (Fig. 5*B*). The inhibition of the mTOR pathway by starvation or by the treatment with rapamycin similarly reduced 47S pre-rRNA levels, and there was no additive or synergistic reduction of pre-rRNA levels by SHPRH depletion (Fig. 5*C* and Fig. S7*B*). Thus, SHPRH enhances the level of 47S pre-rRNA in an mTOR-dependent manner. There are two possible ways that SHPRH could increase 47S pre-rRNA levels. One is to enhance pre-rRNA transcription, so as to produce newly synthesized rRNA, and the other is to increase the stability of existing pre-rRNA. To determine which process is promoted by SHPRH, we measured newly transcribed nascent rRNA by pulse-labeling cells with BrUTP that were detected in situ (44) after SHPRH knockdown. We found that depletion of SHPRH reduced the level of newly transcribed rRNA in situ (Fig. 5*D* and Fig. S7*C*). Similarly, starved cells showed low levels of newly transcribed nascent rRNA. There was no obvious synergistic or additive effect on the level of pre-rRNA or newly transcribed nascent rRNA on starvation and depletion of SHPRH (Fig. 5 *C* and *D* and Fig. S7 *B* and *C*). Thus, our observations indicate that SHPRH promotes pre-rRNA transcription and is directly inhibited by starvation.

**SHPRH Interacts with and Regulates RNA Polymerase I Complex.** RNA polymerase I transcribes 47S pre-rRNA (13). rRNA transcription is directly controlled by transcription factors, as well as indirectly controlled by epigenetic factors (3, 13). Because SHPRH interacted with the active rDNA promoter and increased rRNA transcription, it is possible that SHPRH could directly control rRNA transcription through an interaction with the RNA polymerase I complex. We therefore examined an interaction between SHPRH and the RNA polymerase I complex by coimmunoprecipitation. SHPRH-myc-His was induced in a cell line containing a stable doxycycline-inducible SHPRH construct and was immunoprecipitated with anti-6xHis antibody. The RNA polymerase I subunit, RPA194, UBF that functions to activate RNA polymerase I (13, 45) and histone H3, but not Fibrillarin, was specifically coprecipitated with SHPRH (Fig. 6*A*). Serial deletion mutant analysis of SHPRH revealed that amino acids between 784 and 1,288 amino acids were responsible

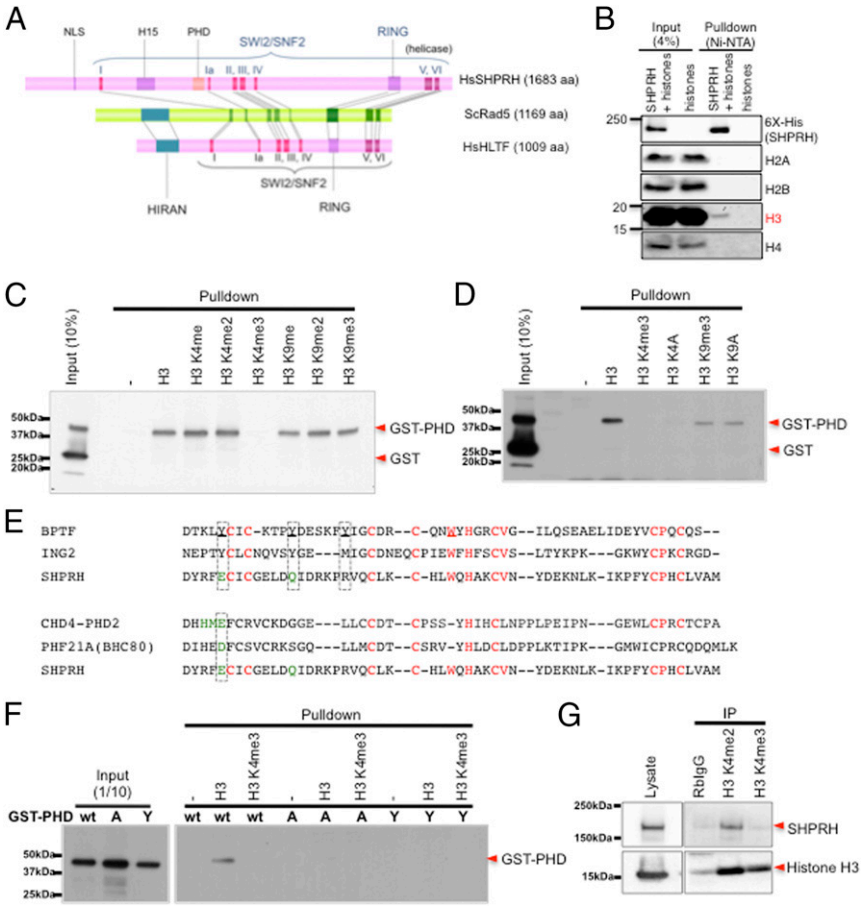


**Fig. 3.** The PHD of SHPRH is important for the interaction with histone H3. (*A*) Schematic representation of human SHPRH, HLTF, and yeast Rad5 protein structures (27). Nuclear localization signal sequence (NLS), linker histone H1/H5 domain (H15), PHD, HIRAN, SWI2/SNF2 helicase domains and RING domain are indicated. (*B*) SHRPH interacted with histone H3. Purified SHPRH-myc-His was incubated with calf histones. Protein complexes were precipitated with Ni-NTA bead and analyzed by Western blotting. (*C* and *D*) Mixture of GST and GST-PHD was incubated with indicated synthetic histone H3 peptides. Indicated lysine was mono-, di-, or trimethylated or substituted with alanine. Biotinylated peptides were pulled down with Streptavidin Sepharose. GST and GST-PHD were detected with anti-GST antibody. (*E*) Alignment of PHDs. Sequences of SHPRH PHD are aligned with PHDs of K4 trimethylated H3-binding BPTF and ING2 and PHDs of unmethylated H3-binding CHD4-PHD2 and PHF21A. Highly conserved sequences are indicated with red or green. Critical H3-binding residues are indicated with dotted box. (*F*) Changes of the conserved glutamate to alanine (E660A; A in *F*) or tyrosine (E660Y; Y in *F*) of SHPRH PHD abolished the interaction of the PHD of SHPRH with H3 peptides. (*G*) Endogenous SHPRH interacted with histone H3 K4me2, but not with H3 K4me3. 293T lysates were immunoprecipitated with indicated antihistone H3 antibodies or control rabbit antibody. Western blotting was performed with anti-SHPRH 3F8 and anti-histone H3 antibodies.

for the interaction with RNA polymerase I (Fig. S8 *A* and *B*). Consistently, SHPRH without this region could not induce pre-rRNA transcription (Fig. S8*C*). SHPRH harboring a point mutation in the PHD or really interesting new gene (RING) domain could not induce pre-rRNA transcription, although they still interacted with RNA polymerase I (Fig. S8 *B*, *D*, and *E*). In addition, ChIP experiment showed that RNA polymerase I recruitment to rDNA promoter was down-regulated by SHPRH depletion or starvation (Fig. 6 *B* and *C*). Conversely, SHPRH overexpression increased the recruitment of RNA polymerase I to the rDNA promoter (H42.9) and the level of pre-rRNA, which was not observed with the SHPRH PHD mutant (E660A) or RING mutant (C1432A; Fig. 6*D* and Fig. S8 *D* and *E*). Changes of SHPRH PHD mutant or RING mutant's localization would be a result of defects of these mutant proteins localizing to the rDNA promoter (Fig. 4). Under starved condition, the interaction between SHPRH and RNA polymerase I was decreased (Fig. 6*E* and Fig. S8 *F* and *G*). Thus, the PHD and RING domains of SHPRH and its potential cooperation with RNA polymerase I are important for rRNA transcription by recognizing the status of rDNA promoters.

**CHD4 Mediates the Relocalization of SHPRH at the rDNA Promoter.** CHD4/NuRD establishes a poised status of rDNA that pauses rDNA transcription by specific histone modifications. In starved cells, the amount of CHD4-occupied and poised rDNA marked by H3 K4me3 is increased and RNA polymerase I binding on the rDNA is reduced (16). Silencing of CHD4 expression decreases the level of H3 K4me3 and increases the CpG-methylation in the rDNA promoter, which results in the inactivation of rDNA (16). When CHD4 expression was silenced, targeting of SHPRH to the rDNA promoter was also diminished, which is consistent with the involvement of SHPRH at the active rDNA promoter (Fig. 7*A*). There was an increase of H3 K4me2 at H8 rDNA and decrease of H3 K4me2 at H42.9 rDNA when CHD4 was depleted, which would facilitate SHPRH relocalization (Fig. S9 *A* and *B*). The simultaneous knockdown of SHPRH and CHD4 did not further decrease pre-rRNA transcription (Fig. 7*B*), supporting the cooperation of pre-rRNA transcription by SHPRH and CHD4. To investigate whether blocking rRNA transcription alone would be enough to dissociate SHPRH from the rDNA promoter, SHPRH was monitored after cells were treated with the rRNA transcription inhibitor, actinomycin D. Although SHPRH did not

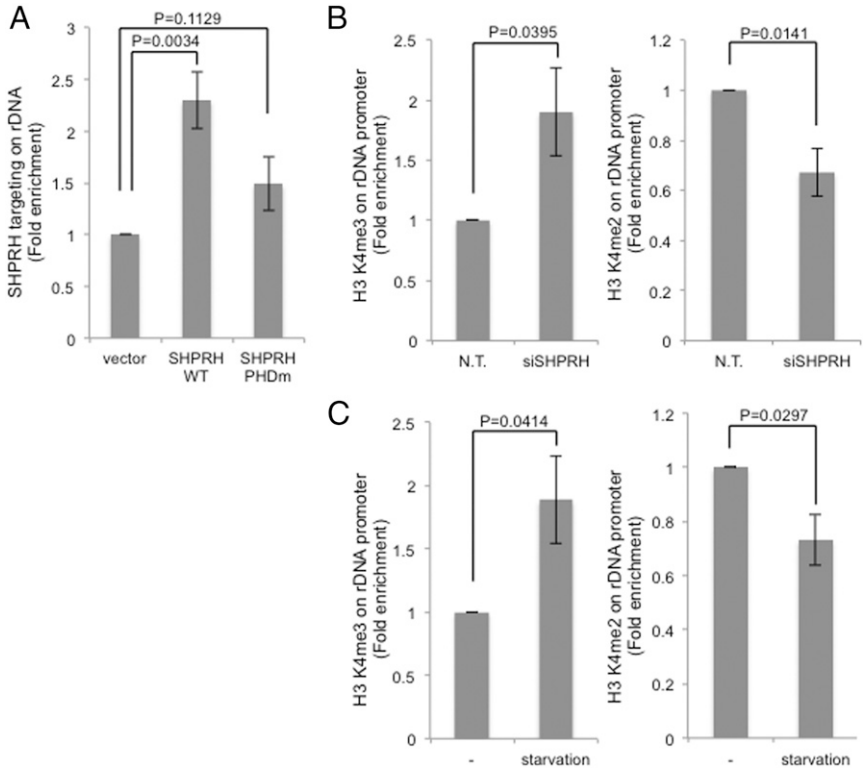


**Fig. 4.** rDNA promoter marked by H3 K4me3 was void of SHPRH. (*A*) SHPRH was targeted to rDNA through its PHD. HeLa cells were transfected with empty vector, SHPRH-myc-His wild-type, and SHPRH-myc-His PHD, which has an E660A mutation. Binding of SHPRH to rDNA promoter (H42.9) was analyzed with ChIP assay. SHPRH was precipitated with anti-6xHis antibody. Data are represented with mean ± SEM from four independent experiments. (*B*) HeLa cells were transfected with siRNA of nontargeting or SHPRH 3′UTR or (*C*) starved with HBSS for 6 h. ChIP assay was performed with anti-H3 K4me3 (*Left*) or anti-H3 K4me2 (*Right*) antibodies. Bars indicate the relative values amplified with H42.9 primers. Each targeting was normalized to input DNA. Graphs show mean ± SEM from five (*B*, *Left*) or four independent experiments.

significantly accumulate at the H8 region, SHPRH dissociated from rDNA promoter H42.9 by inhibition of rRNA transcription (Fig. S9*C*). Thus, epigenetic changes in rDNA, as well as inhibition of rRNA transcription, appear to affect the relocalization of SHPRH. Collectively, we concluded that CHD4 changes the status of the rDNA promoter to control rDNA transcription and the influences on SHPRH for rRNA transcription.

## Discussion

In this study, we present an unexpected role for SHPRH in rRNA transcription. SHPRH was enriched at the rDNA promoter in the nucleoli, where SHPRH promoted pre-rRNA transcription. Enhanced pre-rRNA transcription by SHPRH depends on the nutrient state of the cell and CHD4. SHPRH accumulates at the active rDNA promoters to assist the recruitment of RNA polymerase I for pre-rRNA transcription, whereas SHPRH is displaced from the rDNA promoter when histone H3 is trimethylated at K4 and relocalized to other rDNA sites including the H8 region of rDNA (Fig. S10).

The majority of DNA damage repair proteins were identified and studied in depth after cells were challenged with DNA-damaging agents (46, 47). After DNA damage, many DNA repair proteins are redistributed in cells in response to posttranslational modifications, including phosphorylation, ubiquitylation, and acetylation, or their level is increased (48, 49). However, some repair proteins are nevertheless expressed at high levels in the absence of DNA damage, suggesting they may be required for other cellular functions in the absence of DNA damage. Indeed, the nonhomologous end joining DNA repair pathway functions during the development of lymphocytes (50) and variable, diversity and joining gene segments recombination that is required to produce antibody and T-cell receptor diversity (51). Likewise, nucleotide excision repair proteins participate in transcriptional activation in the absence of DNA damage (52). Embryonic lethality through the inactivation of many DNA repair proteins suggests those proteins are important for rapid proliferation. For example, knockout of the Nijmegen breakage syndrome gene leads to early embryonic lethality in mice (53). Although human ATAD5 regulates the level of ubiquitylated PCNA in response to DNA damage (54), ATAD5 also regulates the lifespan of DNA replication factories by modulating the level of PCNA on chromatin (55, 56). SHPRH is also expressed in most tissues regardless of exogenous DNA damage (57). Furthermore, although SHPRH and HLTF catalyze the ubiquitylation of PCNA, which is important for DNA damage bypass, disruption of SHPRH and HLTF genes in mice did not cause a significant cellular sensitivity to DNA damaging agents (29, 30). Thus, our current study suggests that the regulation of pre-rRNA transcription by SHPRH would be an alternative function of SHPRH in addition to the DNA damage response and repair. For this function of SHPRH, a ubiquitylating activity is also required, as the RING domain mutation of SHPRH no longer recruited RNA polymerase I to the rDNA promoter, although the RING domain itself was not required for interaction (Fig. 6*D* and Fig. S8 *B* and *C*). Thus, the regulation of pre-rRNA transcription by SHPRH requires protein–protein interaction, as well as ubiquitylation activity of SHPRH.

Histone codes, also known as epigenetic codes, have begun to emerge as an additional important signature affecting gene expression. The loci of rDNA are also modified with diverse histone methylations and acetylation (45, 58). Regulation of pre-rRNA transcription by SHPRH through the interaction between the PHD of SHPRH and the histone codes suggests rRNA expression

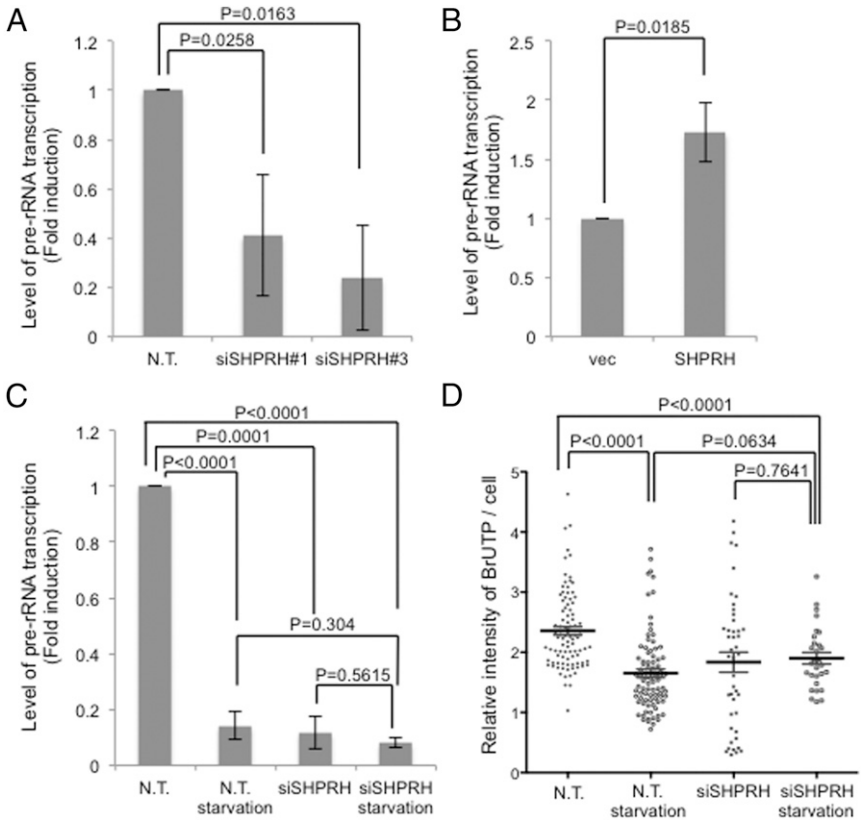


**Fig. 5.** SHPRH regulates 47S ribosomal RNA transcription. (*A*) SHPRH was depleted in HeLa cells by transfecting individual siRNA targeting the 3′UTR of SHPRH for 48 h. Nontargeting (N.T.) siRNA was used as a control. (*B*) Empty vector or SHPRH-myc-His-expressing vector ectopically expressed in HeLa cells for 24 h. (*C*) HeLa cells were transfected with nontargeting siRNA (N.T.) or siRNA targeting the SHPRH 3′UTR. Forty-eight hours after transfection, cells were starved with HBSS for 2 h and harvested. (*A*–*C*) The level of pre-rRNA was analyzed by reverse transcription and qPCR. RNA levels of pre-rRNA were normalized to β-actin. Data show mean ± SEM from independent two independent experiments for *A*, five experiments for *B*, and three experiments for *C*. (*D*) HeLa cells were transfected with nontargeting siRNA or siRNA targeting the SHPRH 3′UTR for 48 h. For staining of nascent rRNA, cells were permeabilized and rRNA was synthesized in vitro with Br-UTP. Amount of nascent rRNA was stained with anti-BrdU (BU1/75) antibody, and images were taken with a fixed exposure time. Intensities of nascent rRNA were analyzed with ImageJ (NIH).

is also tightly regulated via different histone codes and their interacting proteins. Lysine 4 trimethylation of histone H3, which is typically coded for active transcription by RNA polymerase II, is differently recognized in rRNA transcription as a poised epigenetic marker. The CHD4/NuRD is associated with such poised rRNA genes and protects the methylation-dependent inactivation of rDNA (16). The NuRD complex also has activities of ATP-dependent nucleosome disruption and histone deacetylation activities that usually result in the deactivation of transcriptional activity (59, 60). Starvation turns off rRNA transcription by dissociating SHPRH together with the RNA polymerase I complex from the rDNA promoter. Indeed, when cells are differentiated or in serum depletion, the level of H3 K4me3 in rDNA is increased (16). Although CHD4 establishes the poised rDNA, a depletion of CHD4 causes the up-regulation of H4 acetylation and rDNA methylation, which result in the down-regulation of rDNA transcription (16). Therefore, CHD4 depletion might make rDNA devoid of rRNA transcription machinery despite histone acetylation, which leads to the dissociation of SHPRH from the rDNA promoter (Fig. 7*A*). Moreover, in CHD4-depleted cells, the H3K4me2 was increased at a nonpromoter rDNA region, including H8, and decreased at rDNA promoter H42.9 (Fig. S9 *A* and *B*), which can drive the redistribution of SHPRH. Therefore, the role of SHPRH at the rDNA promoter for rRNA transcription can be suppressed through the establishment of constitutive heterochromatins in association with CHD4 depletion.

NuRD complexes and CHD4 are important during embryonic development, carcinogenesis, and starvation. In addition, theses complexes are recruited to damaged DNA and appear to regulate DNA damage responses (61–63). On the basis of such diverse effects of the CHD4 and NuRD complexes in modulating histone signatures, it is possible that the methylation-dependent localization of SHPRH would not be restricted to rRNA gene expression. SHPRH might affect gene regulation in DNA that has differential histone signatures caused by DNA damage, gene silencing, development, or differentiation. Indeed, SHPRH is widely distributed in the nucleus, and most of the SHPRH was detected in the chromatin bound fraction (Fig. 1*A* and Fig. S3) and localized at the promoters of several genes, although the significance of such localization is not understood at the present time (Fig. S5 *J* and *K*).

Starvation or rapamycin treatment induced nucleolar SHPRH foci (Fig. 1 *B* and *C*). The foci represent SHPRH relocalization and accumulation outside of the rDNA promoter because SHPRH dissociates from the rDNA promoter either on starvation or on treatment of rapamycin without changing the level of SHPRH protein (Fig. 2*C* and Fig. S4 *G* and *H*). Depletion of SHPRH increased the frequency of MMS-induced chromosomal breaks (22, 26). In yeast, the number of rDNA repeats is regulated during aging (64). Thus, it is possible that accumulation of SHPRH on starvation could suppress genomic instability in repeated rDNA through recombination. Although it is not clear whether the number of rDNA repeats in mammals are changed

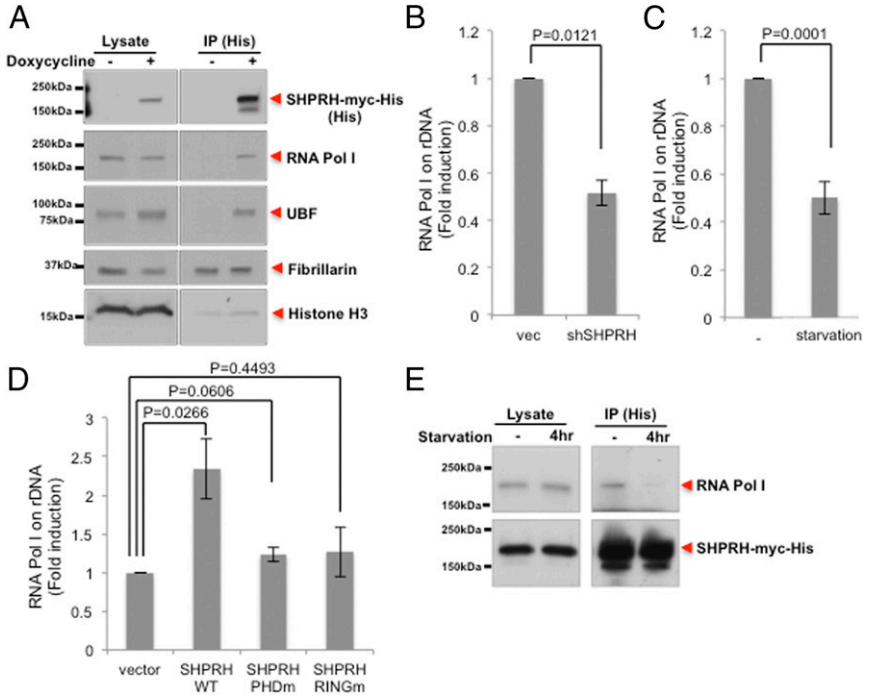


**Fig. 6.** SHPRH is associated with RNA polymerase I complex in a starvation-dependent manner. (*A*) SHPRH interacted with RNA polymerase I complex. The expression of SHPRH-myc-His was induced by treatment of doxycycline to 293-TetOn-SHPRH-myc-His cells. Precleared lysates were immunoprecipitated with anti-6xHis antibody. Precipitates were analyzed by Western blotting with anti-6xHis conjugated with HRP, anti-RPA194, anti-UBF, anti-Fibrillarin, and anti-histone H3 antibodies. (*B–D*) RNA polymerase I binding on rDNA was regulated by SHPRH or starvation. (*B*) SHPRH was stably depleted by infecting HeLa cells with lentivirus expressing shRNA of SHPRH. (*C*) HeLa cells were starved in HBSS for 4 h. (*D*) HeLa cells were transfected with empty vector, SHPRH-myc-His wild-type, or SHPRH mutant having an E660A mutation in PHD or a C1432A mutation in RING domain. ChIP assay was performed with anti-RPA194 antibody. Data are represented with mean ± SEM: *B*, $n = 2$; *C*, $n = 6$; and *D*, $n = 3$. A relative amount of binding was normalized to input DNA. (*E*) The interaction between SHPRH and RNA polymerase I was down-regulated in starved cells. 293-TetOn-SHPRH-myc-His cells were treated with 1 μg/mL doxycycline for 2 d to induce SHPRH-myc-His expression. Cells were starved for 4 h in HBSS and harvested for immunoprecipitation with anti-6xHis antibody.

during aging, it is possible that starvation-induced SHPRH foci could reflect a structure of antirecombination in the nucleoli. It is an intriguing idea, as such regulatory mechanisms could indirectly connect to the longevity regulation of the mTOR pathway (65). Alternatively, starvation-induced SHPRH foci could be a simple reservoir of unnecessary proteins before its degradation. Indeed, during starvation or autophagy, nonessential proteins lose their activity and are degraded (66). Consistent with this notion, on 24 h-starvation, the level of SHPRH protein was slightly decreased, suggesting it is being degraded.

An abnormality of ribosome biogenesis is linked with many genetic diseases, including Diamond-Blackfan anemia and

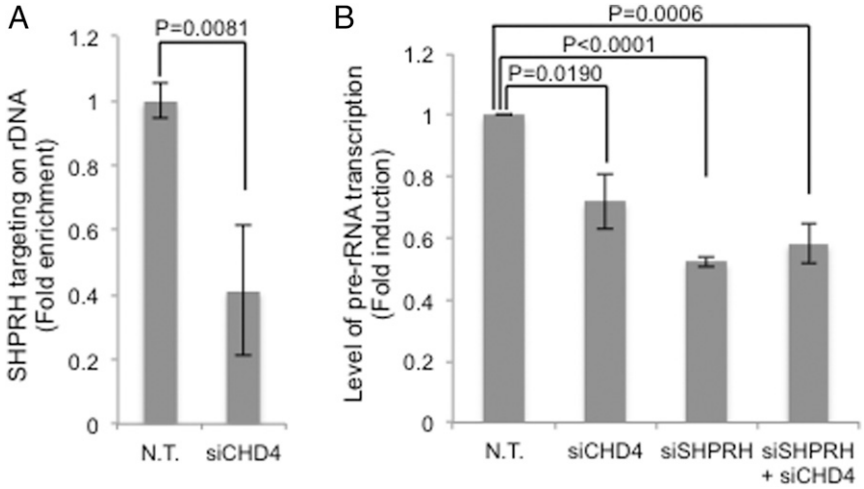


**Fig. 7.** SHPRH is associated with CHD4 in rRNA transcription. (*A*) CHD4 controlled the targeting of SHPRH on rDNA. To deplete CHD4, HeLa cells were transfected with siRNA of CHD4 or nontargeting siRNA as control. ChIP assay was completed with anti-SHPRH 3F8 antibody, and precipitates were analyzed by qPCR in triplicate. (*B*) CHD4 and SHPRH cooperated in pre-rRNA transcription. CHD4 or SHPRH was depleted with siRNA in HeLa cells. The amount of pre-rRNA normalized to β-actin was measured by RT-qPCR. Data show mean ± SEM from four independent experiments.

5q− syndrome (8, 67). Mutations in genes encoding ribosomal proteins would directly affect ribosomal functions in protein translation and could result in anemia. However, the causative mutations of a large portion of these anemic diseases have not been identified (68). Therefore, it is possible that mutations in genes regulating rRNA transcription or expression of ribosomal protein would be causative mutations affecting the remaining anemic patients whose mutations have not been identified. Although there was no clear anemic phenotype in *Shprh*$^{-/-}$ mice under normal growing conditions, it is possible that stress or nutrient challenge could induce an anemic condition or nonanemic diseases in *Shprh*$^{-/-}$ mice; for example, Treacher Collins syndrome. In the hematopoietic system, differential methylation on H3 lysine 4 also marks active or poised hematopoietic genes and determines the lineage of hematopoietic development (69). Thus, it is possible that SHPRH could function in hematopoietic systems as well.

Taken together, we demonstrate a regulatory function of SHPRH in 47S ribosomal RNA transcription through the specific recognition of histone codes in the rDNA promoter, with the cooperation of the chromosomal remodeler, CHD4.

## Materials and Methods

**Cell Culture, Reagents, Proteins, and Antibodies.** HeLa cells were maintained in DMEM supplemented with 10% FBS. To induce starvation conditions, cells were incubated in HBSS, including $Ca^{2+}$ and $Mg^{2+}$ (Invitrogen). The sources of antibodies and reagents are provided in *SI Materials and Methods*.

**In Vitro Peptide-Binding Assay.** Twenty nanograms of purified GST and GST-tagged PHD of SHPRH was incubated in pulldown buffer (50 mM Tris·HCl at pH 7.5, 150 mM NaCl, 0.1% Tween-20, protease inhibitor mixture) for 1 h at 4 °C with rotation with 0.5 μg biotinylated synthetic H3 peptides (Upstate or Invitrogen; *SI Materials and Methods*) that were differentially methylated or mutated, followed by a further 30-min incubation with 10 μL Streptavidin Sepharose (GE Healthcare). Precipitates were washed 3 times with pulldown buffer and subjected to Western blot analysis.

**Chromatin Immunoprecipitation.** Chromatin immunoprecipitation was performed as previously described (70). Cells were cross-linked with 1% formaldehyde at room temperature for 10 min, rinsed with PBS twice, and collected into collection buffer (100 mM Tris·HCl at pH 9.4, 10 mM DTT). Cells were incubated for 15 min at 30 °C and collected by centrifugation for 5 min at 2,000 × *g*. Pellets were washed sequentially with PBS, buffer I (10 mM Hepes at pH 6.5, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100) and buffer II (10 mM Hepes at pH 6.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA). Pellets were then lysed in lysis buffer (50 mM Tris·HCl at pH 8.1, 10 mM EDTA, 1% SDS, protease inhibitor mixture) by sonication. Cell debris was removed by centrifugation of lysates for 10 min at 13,000 × *g*, and supernatants were diluted in 10 volume dilution buffer (20 mM Tris·HCl at pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, protease inhibitor mixture). Cross-linked DNA and protein complexes were immunoprecipitated by incubation with 2 μg specific antibody overnight after immunoclearing with 2 μg salmon sperm DNA, 2 μg control IgG, and Protein G Sepharose for 2 h at 4 °C. Soluble chromatins were further incubated with Protein G Sepharose, 2 μg salmon sperm DNA for 1 h. Immunocomplexes were washed sequentially in TSE I (20 mM Tris·HCl at pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), TSE II (20 mM Tris·HCl at pH 8.1, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), and buffer III (10 mM Tris·HCl at pH 8.1, 250 mM LiCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate), and three times in TE buffer. DNA–protein complexes were eluted two times with elution buffer (0.1 M $NaHCO_3$, 1% SDS) and reverse-cross-linked by incubating at 65 °C for 6 h. DNA fragments were purified by ethanol precipitation and analyzed by quantitative PCR, using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen). Primer sequences for qPCR are referred from ref. 71.

**Nascent rRNA Staining.** Staining of Nascent rRNA was performed as described previously (44). HeLa cells were washed with PBS and with permeabilization buffer (20 mM Tris·HCl at pH 7.5, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM PMSF). Cells were treated with permeabilization buffer containing 0.5% Triton X-100 for 5 min and washed with permeabilization buffer. Synthesis of rRNA was achieved by incubation of permeabilized cells in synthesis buffer (50 mM Tris·HCl at pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 25 U/mL RNasin, 1 mM PMSF, 0.5 mM ATP, CTP and GTP, 0.2 mM Br-UTP) for 30 min at 37 °C. The cells were then washed three times with PBS containing 25 U/mL RNasin and fixed with 10% formaldehyde in PBS containing 25 U/mL RNasin and 0.1% BSA for 20 min. After washing twice with PBS containing 0.1% BSA for 5 min each, nascent rRNA in the cells were stained with anti-BrdU (BU1/75) antibody (Abcam) and Alexa488-conjugated anti-rat secondary antibody. Images were analyzed using ImageJ (NIH).

Other materials and methods are provided in *SI Materials and Methods*.

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