Phosphatidylinositol-4-phosphate 5-Kinase 1α Modulates Ribosomal RNA Gene Silencing through Its Interaction with Histone H3 Lysine 9 Trimethylation and Heterochromatin Protein HP1-α*

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Background: Cell cycle-specific localization and nuclear function of Phosphatidylinositol-4-phosphate 5-kinase1α (PIP5K) is unclear.

Results: SUMOylation of PIP5K at Lys-244 and Lys-490 directs its nuclear entry and its interaction with H3K9me3/HP1-α, respectively.

Conclusion: PIP5K functions as a member of the rDNA silencing complex.

Significance: Our results indicate a possible novel epigenetic role of PIP5K per se in silencing of rDNA.

Phosphoinositide signaling has been implicated in the regulation of numerous cellular processes including cytoskeletal dynamics, cellular motility, vesicle trafficking, and gene transcription. Studies have also shown that nuclear phosphoinositide(s) regulates processes such as mRNA export, cell cycle progression, gene transcription, and DNA repair. We have shown previously that the nuclear form of phosphatidylinositol-4-phosphate 5-kinase 1α (PIP5K), the enzyme responsible for phosphatidylinositol 4,5-bisphosphate synthesis, is modified by SUMOylation at Lys-244 and Lys-490, it is unable to localize in the nucleus and nucleolus, respectively. Furthermore, by using chromatin immunoprecipitation assays, we have observed that PIP5K associates with the chromatin silencing complex constituted of H3K9me3 and heterochromatin protein 1α at multiple ribosomal DNA (rDNA) loci. These interactions followed a definite cyclical pattern of occupancy (mostly G1) and release from the rDNA loci (G1/S) throughout the cell cycle. Moreover, the immunoprecipitation results clearly demonstrate that PIP5K SUMOylated at Lys-490 interacts with components of the chromatin silencing machinery, H3K9me3 and heterochromatin protein 1α. However, PIP5K does not interact with the gene activation signature protein H3K4me3. This study, for the first time, demonstrates that PIP5K, an enzyme actively associated with lipid modification pathway, has additional roles in rDNA silencing.

Phosphoinositide, a specific phospholipid, serves as a potent signal transducer in multiple cellular events. Reversible phosphorylation of its inositol ring at positions 3–5 generates several phosphoinositide species (1). One of the important phosphoinositide-modifying enzymes, phosphatidylinositol-4-phosphate 5-kinase 1α (PIP5K),5 adds a phosphate specifically to the inositol ring having its fourth position previously phosphorylated, generating PIP₃. PIP5K is a 61-kDa protein migrating at about 68 kDa in SDS-PAGE (2). Although classical phosphoinositide signaling is concentrated in the plasma membrane, recent studies have suggested the presence of a distinct phosphoinositide signaling in the nucleus (3).

PIP₃ in the nucleus has been mapped onto detergent-insoluble fractions co-localizing with small nuclear ribonucleoprotein particles in nuclear speckles (4) and at electron-dense structures (5), suggesting its involvement in pre-mRNA processing. Studies indicate that PIP₃ can bind to both histones H1 and H3 and is therefore involved in regulating the state of chromatin in the nucleus (6). Further observations showed that PIP₂ can regulate the interaction of the chromatin remodeling complex BRG1- or HRBM-associated factors (BAF) with the nuclear matrix (7). Recent evidence also has identified PIP₂ in the nucleolus interacting with Pol I and upstream binding factor, implicating a possible role in Pol I transcription by interacting with pre-rRNA production and processing machineries.

5 The abbreviations used are: PIP5K, phosphatidylinositol-4-phosphate 5-kinase 1α; PIP₃, phosphatidylinositol 4,5-bisphosphate; SUMO, small ubiquitin-like modifier; rDNA, ribosomal DNA; Pol I, polymerase I; FC, fibrillar center; qPCR, quantitative PCR; hTop I, human topoisomerase I; KD, kinase-dead.
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(8). Although there have been some reports on the role of PIP₂ in the nucleus, only a few studies have documented spatial and functional characterization of PIP5K in the nucleus. Using a yeast two-hybrid screen, it was demonstrated that speckle-targeted PIPKα regulated-poly(A) polymerase was a novel interacting partner of PIP5K, and together they could regulate pre-mRNA processing (9). Additionally, it was shown that the retinoblastoma protein Rb, which recruits the BAF complex to transcription sites, can interact with and activate PIP5Kα (10). Nuclear c-Fos associates with and activates PIP5K, leading to transcriptional changes (11). Most of these studies implicated PIP₂ in mediating the aforesaid nuclear functions. Studies from our laboratory demonstrated that the nuclear pool of PIP5K is modified by SUMO-1, and during apoptotic stress in addition to the increase in this SUMOylated pool, poly-SUMO-2-modified PIP5K also occurs in the nucleus (12).

In the present study, we document that SUMOylation of PIP5K at Lys-244 is essential for its nuclear targeting. It was shown to accumulate in the fibrillar center (FC) region of the nucleolus during the G₁/S phase of the cell cycle. The results also demonstrated that PIP5K, SUMOylated at Lys-490, is present in the chromatin silencing complex consisting of H3K9me3 and heterochromatin protein 1α (HP1-α) at the rDNA loci. Together, our results indicate a possible novel epigenetic role of PIP5K protein per se in the silencing of rRNA genes in particular.

Experimental Procedures

Materials—The following antibodies were used: anti-FLAG (Sigma, F3165), β-actin (Cell Signaling Technology, 4970), and anti-nucleophosmin (Abcam, FC82291). Anti-mouse and anti-rabbit secondary antibodies conjugated with Alexa Fluor 488 (A11001 and A11008, respectively) and 568 (A11004 and A11011, respectively) were purchased from Life Technologies. Secondary antibodies conjugated with alkaline phosphatase (A3562 (mouse) and A3687 (rabbit)) were from Sigma. Anti-H3K4Me3 (39159) and anti-H3K9Me3 (39161) were from Active Motif, and anti-H3 (ab1791) antibody was from Abcam. Antibody for endogenous PIP5K was generated in rabbit as described previously (13). The specificity of the antisera was verified by Western blot analysis (see “Results”).

Cell Culture, Transfection, and Treatments—HEK-293, MCF-7, and A549 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂. HEK-293, MCF-7, and A549 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂. HEK-293, MCF-7, and A549 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂.

Site-directed Mutagenesis—Point mutations of PIP5K were generated using the QuikChange site-directed mutagenesis procedure (Stratagene) according to the manufacturer’s protocol.

Cell Synchronization and Cell Cycle Analysis—Cells were synchronized at G₁/S phase using the thymidine double block- ing method (15). Briefly, cells were plated in 60-mm Petri dishes and thymidine was added to a final concentration of 2 mM after cell adherence. The cells were cultured for 16 h. After removal of the thymidine and incubation for 10 h in fresh DMEM solution, thymidine was added to a final concentration of 2 mM for an additional 16 h.

The synchronized cells were collected and washed twice with PBS solution. Cells were fixed with chilled 70% alcohol at −20 °C for at least 24 h. The cell sediment was collected by centrifugation (1000 rpm for 3 min), washed twice with PBS solution, incubated with RNase A (20 mg/ml) for 30 min at 37 °C, and stained with 25 μg/ml propidium iodide (Sigma) for 30 min at room temperature. The cell cycle distribution was then evaluated using flow cytometry (BD FACVerse).

Cell Lysate Preparation and Western Blotting—Cell lysate preparation and Western blotting were performed as described previously (12).

Immunofluorescence and Microscopy—Immunofluorescence was done as described previously (12). Mounted cells were imaged using Nikon inverted research microscope ECLIPSE Ti-U and Olympus FV1000 LSM confocal microscope. Images were processed using NIS-Elements, Adobe Photoshop CS5, and Fluoview 1000 software.

Transcription Analysis by RT-qPCR—Total RNA was isolated by TRI Reagent (Sigma) extraction according to the manufacturer’s protocol. One microgram of total RNA was reverse transcribed using random hexamer primers and RevertAid reverse transcriptase kit (Fermentas). The synthesized cDNA was used for RT-qPCR using Maxima SYBR Green/ROX™ qPCR Master Mix (2×) (Fermentas) using the following primer sequences: β-actin forward, 5’-AGGCCACAGGGCGGTGAT-3’; β-actin reverse, 5’-GCCCACTAGGAATCTTCTGAC-3’; 18S rRNA forward, 5’-GTAAACCCTTGGAACCCCTT-3’; 18S rRNA reverse, 5’-CCATCCAATCGGTAGTAGCG-3’; 45S pre-rRNA forward, 5’-CTCGGTTATGGTACGGCTG-3’; 45S pre-rRNA reverse, 5’-CGGAAACCGCTGCTTTC-3’.

Chromatin Immunoprecipitation (ChIP), Re-ChIP, and q-PCR—ChIP assays were performed according to a standard protocol (16). Briefly, after cross-linking the cells with 1% formaldehyde and stopping the reaction by 0.125 mM glycine, cell lysis was done in cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40 (with fresh protease inhibitor)). After centrifugation, the nuclear pellet was lysed with nucleus lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS (with fresh protease inhibitor)). After sonication and preclearing of the chromatin, immunoprecipitation was subsequently performed with anti-FLAG, anti-H3K9me3, anti-H3, anti-H3K4me3, and IgG. Dynabeads, which were previously blocked, were used to pull down the chromatin complex. Beads were then washed with radiolabeled nucleic acid precipitation assay buffer, high salt buffer, LiCl buffer, and Tris-EDTA consecutively followed by RNase A and Proteinase K digestion at 37 °C. The beads were kept overnight, and the next day the samples were extracted with phenol-chloroform and chloroform fol-
showed a normal nuclear occupancy of the mutant protein, signifying that SUMOylation at Lys-244 is sufficient for nuclear import of PIP5K (Fig. 1A, panels p–r). To verify whether Lys-244 is modified by SUMO-1, we subjected anti-SUMO-1 immunoprecipitates from nuclear and cytosolic lysates of cells expressing WT-PIP5K, PIP5K-K33A, PIP5K-K490A, PIP5K-K244A, PIP5K-K33A/K490A, PIP5K-K244A/K490A, and PIP5K-K33A/K244A/K490A to Western blotting with anti-FLAG antibody. Mutants that had unmodified Lys-244 showed an immunoreactive band in the nuclear fraction, whereas PIP5K-K244A, PIP5K-K244A/K490A, and PIP5K-K33A/K244A/K490A did not (Fig. 1B). The presence of the immunoreactive band in the cytosolic fraction of PIP5K-K244A and PIP5K-K244A/K490A suggests that although other lysines in these two mutants could be modified by SUMO-1 it is not sufficient to translocate the protein into the nucleus. Moreover, analysis of nuclear and cytosolic fractions from HEK cells expressing PIP5K-K33A/K244A/K490A revealed that SUMOylation is abolished in the triple mutant (Fig. 1B). The presence of the immunoreactive band in the nuclear fraction of the double mutant (PIP5K-K33A/K490A) and absence of the nuclear form in the case of Lys-244-modified mutants suggests that Lys-244 is indeed SUMOylated and is necessary and sufficient for nuclear import of PIP5K. Thus, it can be concluded that SUMOylation at Lys-244 is instrumental in regulating the nuclear import of PIP5K.

**Results**

**Lys-244 SUMOylation Is Essential for PIP5K Nuclear Import—**

PIP5K is a soluble enzyme that has been shown to be present in both the nucleus and cytosol. Previous reports from our laboratory confirmed that nuclear PIP5K is modified by SUMO-1 (12). Primary sequence analysis of PIP5K by SUMOplot™ displays six possible sites for SUMOylation, three of which (Lys-33, Lys-244, and Lys-490) have a probability score higher than 0.50 (Table 1). To identify the specific SUMOylation site(s) responsible for its nuclear targeting, we generated Lys to Ala mutants in the above sites. Expression of FLAG-tagged WT and mutant PIP5Ks in HEK-293 cells showed that the mutants having PIP5K-K244A failed to get imported into the nucleus (Fig. 1A, panels j–l, panels m–o, and panels s–u), whereas PIP5K-K33A (Fig. 1A, panels d–f) and PIP5K-K490A (Fig. 1A, panels g–i) mutants had normal nuclear distribution. To determine whether SUMOylation at Lys-244 is sufficient for nuclear import of PIP5K, we created double point mutations at Lys-33 and Lys-490 residues. Expression of this construct, which only had Lys-244 unmodified, failed to get imported into the nucleus (Fig. 1A, panels p–r). Re-ChIP assays were performed as described elsewhere (18). Briefly, the immunocomplex pulled down by the first antibody (anti-FLAG) was treated with 0.05 m DTT at 30 °C for 60 min followed by a 20-fold dilution before performing the pulldown with the second antibody (anti-H3K9me3, anti-HP1-, or IgG). The -fold enrichment values were calculated and normalized with respect to input DNA and IgG control. Briefly, the calculations were as follows for the input (Ct1), IgG_IP (Ct2), Antibody_IP (Ct3) values.

\[
\text{Adjusted input (A)} = Ct1 - 6.644
\]

\[
\Delta \text{IgG_IP (B)} = Ct2 - A
\]

\[
\Delta \text{Antibody_IP (C)} = Ct3 - A
\]

\[
\Delta \Delta \text{Antibody_IP (D)} = C - B (\text{normalization with IgG})
\]

\[
\text{Fold enrichment} = 2^{-D}
\]

Re-ChIP assays were performed as described elsewhere (18). Briefly, the immunocomplex pulled down by the first antibody (anti-FLAG) was treated with 0.05 m DTT at 30 °C for 60 min followed by a 20-fold dilution before performing the pulldown with the second antibody (anti-H3K9me3, anti-HP1-, or IgG). The -fold enrichment values were calculated and normalized with values obtained from pulldown with anti-FLAG antibody and IgG.

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**TABLE 1**

| Sl. no. | Position | Sequence | Score |
|--------|----------|----------|-------|
| 1      | Lys-244 | SVKMKHJKDGLGST | 0.96  |
| 2      | Lys-33  | SSASGKRFIASEV  | 0.84  |
| 3      | Lys-490 | TSTTLKTEVAESE  | 0.50  |
To verify whether PIP5K occupied the FC region, after actinomycin D treatment, the cells were doubly labeled with antibodies against FLAG and hTop I. We observed a very strong co-localization of PIP5K and hTop I in the nucleolar caps, confirming PIP5K occupancy in the FC region (Fig. 3, panels b–d). The FC region is known to contain Pol I-enriched rDNA transcription units (22). Thus, PIP5K localization in the FC region suggests a possible role of this protein in rDNA transcription.

**PIP5K Associates with H3K9me3 during rDNA Silencing—** To investigate the role of PIP5K in rDNA transcription, we monitored the occupancy pattern of modified histone H3 in the context of rRNA gene in cells expressing either wild type or kinase-dead PIP5K. We chose to use kinase-dead PIP5K (PIP5KD) as a negative control because our previous studies (12) and results from other laboratories (23) have shown that this mutant fails to get translocated into the nucleus. We sought to study the relative occupancy pattern of an activation mark, H3K4me3, and a repression mark, H3K9me3, throughout the human rDNA loci using ChIP assays (Fig. 4).

The results of the ChIP assay showed significantly increased enrichment of the H3K9me3 mark at different regions of rRNA gene in the cells expressing the wild type PIP5K as compared with the PIP5KD (Fig. 4B), whereas the level of occupancy for the activation mark H3K4me3 remained almost unchanged in both cases (Fig. 4B). Thus, it was evident that nuclear expression of PIP5K was associated with the transcriptionally repressed state of rDNA. Furthermore, looking at the transcription profile of rRNA gene in asynchronous cells, it was evident that overexpression of PIP5K inhibited expressions of 18S (Fig. 4D) and 45S pre-rRNA (Fig. 4E).
endogenous nuclear PIP5K in HEK, HEK-PIP5K, and HEK-PIP5KD cells. Western blot analysis of nuclear lysate from these cells, PIP5K was mostly found out of the chromatin material (panel c). The scale bar represents 5 μm. B, asynchronous (ASYN) (panels a–d) and double thymidine-blocked (panels e–h) HEK-293-FLAG-PIP5K cells were double stained for PIP5K (green) and B23 (nucleolar marker) (red). Panels a, e, d, and h) contain DAPI-stained nuclei (blue). PIP5K was enriched in the nucleolus of G1/S phase cells (panels f and h, arrowheads). The scale bar represents 5 μm. C, HEK-293, MCF-7, and A549 cells were blocked at G1/S and stained for endogenous PIP5K (green) and B23 (red). Panels a, e, i, and merged also contain DAPI-stained nuclear images (blue). Endogenous PIP5K was found enriched in the nucleolus of all the cell types. The scale bar represents 5 μm.

4E) as compared with the parental HEK, whereas both their expressions were increased considerably in cells expressing PIP5KD (Fig. 4, D and E). We were intrigued by the fact that the absence of PIP5KD in the nucleus should not have modulated rRNA expression as observed here (Fig. 4, D and E). Because it has been demonstrated by others that the PIP5KD has dominant negative activity (24), we wanted to verify whether or not in our case it acts in a dominant negative manner by suppressing the nuclear translocation of the endogenous PIP5K. To address this, we sought to determine the relative amounts of endogenous nuclear PIP5K in HEK, HEK-PIP5K, and HEK-PIP5KD cells. Western blot analysis of nuclear lysate from these cells using anti-PIP5K antibody revealed that HEK-PIP5KD cells contained a reduced (about 35% as compared with untransfected HEK cells) amount of endogenous nuclear PIP5K (Fig. 4F). Therefore, indeed expression of PIP5KD partly suppresses the nuclear translocation of endogenous PIP5K, resulting in an enhanced synthesis of rRNA in these cells.

To further understand whether this effect was due to an alteration in the signal transduction cascade of PIP5K or a direct recruitment of the protein to the rDNA loci, we carried out ChIP studies using anti-FLAG antibody in HEK cells expressing FLAG-PIP5K. Results revealed that there was indeed a substantial recruitment of PIP5K in almost all the rDNA loci with greater recruitment at the intergenic sequence loci (H27) (Fig. 4G). To verify whether the recruitment of PIP5K was on the repressed regions of rDNA loci, a ChIP experiment was carried out separately with anti-H3K9me3, anti-histone H3, and anti-HP1-α antibodies. HP1-α is another well documented member of the gene silencing complex where it binds to and works in conjunction with H3K9me3, anti-histone H3, and anti-HP1-α antibodies. HP1-α is another well documented member of the gene silencing complex where it binds to and works in conjunction with H3K9me3 (25, 26). It is indeed interesting to note that the increased occupancy of PIP5K was concomitant to the elevated levels of both H3K9me3 (Fig. 4H), HP1-α (Fig. 4I), and histone H3 (Fig. 4J) at the respective rDNA loci. Moreover, the occupancy of PIP5K was greatly reduced at the above mentioned regions of the rDNA with the onset of S phase (Fig. 4G). H3K9me3 (Fig. 4H), HP1-α (Fig. 4I), and histone H3 (Fig. 4J) occupancy at similar sites also followed an identical pattern. It is notable that although in asynchronous phase 18S and 45S pre-rRNA synthesis was repressed in cells expressing WT-PIP5K as compared with the untransfected HEK-293 cells the initiation of S phase showed that synthesis of both 18S and 45S pre-rRNAs in cells expressing WT-PIP5K was elevated in a manner similar to that of untransfected HEK-293 cells. This all the more indicated that, despite its enrichment in
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The nucleolus, PIP5K was not associated with the rRNA genes during the G1/S phase (Fig. 4, D and E). Although the similar occupancy pattern of PIP5K, H3K9me3, and HP1-α over the rRNA gene suggested a role of PIP5K in rDNA repression, it was still not clear whether PIP5K is a component of the heterochromatin complex comprising H3K9me3 and HP1-α. We carried out re-ChIP assays where the DNA-protein complexes from HEK cells overexpressing PIP5K were first pulled down by anti-FLAG antibody. This immunoprecipitate was subjected to a second pulldown by either anti-H3K9me3 antibody or anti-

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**A**

**B**

**C**

**D**

**E**

**F**

**G**

**H**

**I**

**J**

**K**

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| Image Tag | Description |
|-----------|-------------|
| A         | Schematic diagram of nucleolus and PIP5K localization |
| B         | Bar graph showing H3K9me3 ChIP enrichment for different primers |
| C         | Bar graph showing H3K4me3 ChIP enrichment for different primers |
| D         | Bar graph showing 18S expression for different cell lines |
| E         | Bar graph showing 45S pre-rRNA expression for different cell lines |
| F         | Western blot images of PIP5K and B23 proteins |
| G         | Bar graph showing FLAG (PIP5K) ChIP enrichment for different primers |
| H         | Bar graph showing H3K9me3 ChIP enrichment for different primers |
| I         | Bar graph showing HP1-α ChIP enrichment for different primers |
| J         | Bar graph showing Histone H3 ChIP enrichment for different primers |
| K         | Bar graph showing Re-ChIP assay enrichment for different primers |
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HP1-α antibody. The precipitated DNA from the above immunoprecipitates was analyzed by qPCR, which showed considerable recruitment of PIP5K-H3K9me3 and PIP5K-HP1-α complexes at the rDNA loci (Fig. 4K). Because HP1-α and H3K9me3 are established interacting proteins of the repression complex (25, 26), we reasoned that it is likely that at least a tripartite complex comprising PIP5K, HP1-α, and H3K9me3 exists on the rDNA loci. These results for the first time demonstrated the occupancy of PIP5K (a phosphoinositide kinase) throughout the rDNA loci during its transcriptional repression.

**PIPK Interacts with H3K9me3 and HP1-α in a DNA-dependent Manner**—The aforesaid results suggest that PIP5K is recruited to various rDNA loci having elevated levels of H3K9me3 and HP1-α occupancy. So far, the primary sequence of PIP5K has not been shown to possess any canonical DNA binding domain; therefore, only a physical interaction of PIP5K with proteins associated with these loci can account for this recruitment of PIP5K to DNA. To ascertain the association of PIP5K with transcription repression signature(s), immunoprecipitation was performed from HEK-293 cells stably expressing FLAG-tagged PIP5K with either anti-H3K9me3 or anti-HP1-α, and immunoblotting was done with anti-FLAG antibody. The results indicated that the binding preference of H3K9me3 (Fig. 5A, panel I) and HP1-α (Fig. 5A, panel II) for FLAG-tagged PIP5K was quite strong. To verify whether or not the interaction was due to overexpression of FLAG-PIPK5, a similar experiment was performed with an antibody raised against PIP5K in the laboratory. The specificity of the antibody was checked by Western blot analysis using purified His-tagged PIP5K (Fig. 5B, panel I) and protein lysates of parental HEK cells and HEK cells overexpressing FLAG-PIPK5 (Fig. 5B, panel II). Interaction of endogenous PIP5K with H3K9me3 and HP1-α was observed, suggesting that the binding was not due to the overexpression of FLAG-PIPK5 (Fig. 5C). Moreover, this result also demonstrated that, although anti-H3K9me3 antibody could pull down PIP5K and HP1-α, anti-HP1-α antibody co-immunoprecipitated both H3K9me3 and PIP5K (Fig. 5C). This observation corroborates the result of the re-ChIP assays (Fig. 4K). Partial co-localization of FLAG-PIPK5 and HP1-α in HEK cells expressing FLAG-PIPK5 also confirmed the interaction between PIP5K and HP1-α (Fig. 5D, panels b–d).

To investigate whether the association of PIP5K is specific for the transcription repression mark, we checked its interaction with the activation mark H3K4me3. Our result indicated poor binding of FLAG-tagged PIP5K with H3K4me3 (Fig. 5E)

Furthermore, to verify whether the association of PIP5K with H3K9me3 and HP1-α is DNA-dependent or not, we performed DNase I digestion prior to respective immunoprecipitation experiments. In these assays, the lysates were treated with or without DNase I and then subjected to immunoprecipitation with either anti-H3K9me3 (Fig. 5F, panel I) or anti-HP1-α (Fig. 5F, panel II) antibody and immunoblotted with anti-PIPK5 antibody (Fig. 5F, panels I and II,upper). The respective interaction is indeed compromised in the presence of DNase I, implicating that DNA is the critical assembly platform for these protein interactions to occur. It is also evident from all these data that the recruitment of PIP5K onto the rDNA loci is via its interaction with H3K9me3 and HP1-α.

**SUMOylation at Lys-490 of PIP5K Is Essential for Its Interaction with the Silencing Proteins**—All our immunoprecipitation/Western blotting results have shown that both H3K9me3 and HP1-α interacted with the 90-kDa form of PIP5K that is SUMOylated (Fig. 5A and Ref. 12). Other studies have shown that even recruitment of HP1-α to pericentric heterochromatin required it to be modified by SUMO. Moreover, our *in vitro* assay showed that unmodified PIP5K bound weakly with the core histones, suggesting that unmodified PIP5K is inefficient for its binding to modified histones (Fig. 6A). Thus, our results indicate that the SUMOylation status of PIP5K could be crucial in mediating the interaction of PIP5K with the silencing proteins. A closer observation of the spatial distribution of PIP5K-SUMO mutants in the nucleus revealed that K490A did not

**FIGURE 4. PIP5K associates with H3K9me3 during rDNA silencing.** A, schematic representation of a human rDNA repeat. The positions of qPCR amplicons in ChIP assays are indicated with *solid square dots*. IGS, intergenic spacer. B, ChIP experiments were done with parental HEK-293 cells (black bars) and HEK-293 cells expressing WT-PIPK5 (white bars) and kinase-dead PIP5K (gray bars) using anti-HP1-α antibody. The precipitated DNA was analyzed by qPCR using primers indicated in A and (ii) upstream regulatory region of p53 gene. The *-fold enrichment of p53* that was normalized with *that of histone H3 and IgG* is plotted. Each *bar* is an average of biological triplicates. Error bars show S.E. C, ChIP experiments were done with parental HEK-293 cells (black bars) and HEK-293 cells expressing WT-PIPK5 (white bars) and kinase-dead PIP5K (gray bars) using anti-H3K9me3 antibody. The precipitated DNAs were analyzed by qPCR using primers designed for amplicons as shown in A, (i) upstream regulatory region of p53 gene, and (ii) upstream regulatory region of p53 gene. The *-fold enrichment of p53* that was normalized with *that of histone H3 and IgG* is plotted. Each *bar* is an average of biological triplicates. Error bars show S.E. D, total RNA was isolated from parental HEK, HEK transfected with empty pCMV-Tag2B (HEKVECTOR), HEK-293-FLAG-PIPK5 (HEK_PIP5K), and HEK-293-FLAG-PILS5KD (HEK_PILS5KD) cells (asynchronous [Asyn] black bars) and G1/S [gray bars]) and quantitated by RT-qPCR analysis for 45S pre-rRNA expression. Data were normalized to the amount of β-actin mRNA. Each *bar* is an average of biological triplicates. Error bars show S.E. E, total RNA was isolated from parental HEK, HEK transfected with empty pCMV-Tag2B (HEK_VECTOR), HEK-293-FLAG-PIPK5 (HEK_PIP5K), and HEK-293-FLAG-PILS5KD (HEK_PILS5KD) cells (asynchronous [black bars] and G1/S [gray bars]) and quantitated by RT-qPCR analysis for 45S pre-rRNA expression. Data were normalized to the amount of β-actin mRNA. Each *bar* is an average of biological triplicates. Error bars show S.E. F, 50 μg of nuclear protein lysates from untransfected HEK cells, HEK cells overexpressing WT-PIPK5 (HEK_Wt-PIPK5), and HEK cells overexpressing kinase-dead mutant (HEK_PK5KD) were analyzed by Western blotting (WB) using anti-PIPK5 and anti-B23 antibodies as indicated. G, ChIP experiments were done with asynchronous (ASYN) [black bars] and G1/S [gray bars]-blocked HEK-293-FLAG-PILS5KD cells using anti-FLAG antibody. The precipitated DNA was analyzed by qPCR using primers indicated in A. The *-fold enrichment of H3K9me3* that was normalized with *that of histone H3 and IgG* is plotted. Each *bar* is an average of biological triplicates. Error bars show S.E. H, ChIP experiments were done with asynchronous (black bars) and G1/S [gray bars]-blocked HEK-293-FLAG-PIPK5 cells using anti-H3K9me3 antibody. The precipitated DNA was analyzed by qPCR using primers indicated in A. The *-fold enrichment of H3K9me3* that was normalized with *that of histone H3 and IgG* is plotted. Each *bar* is an average of biological triplicates. Error bars show S.E. I, ChIP experiments were done with asynchronous (black bars) and G1/S [gray bars]-blocked HEK-293-FLAG-PILS5KD cells using anti-H3K9me3 antibody. The precipitated DNA was analyzed by qPCR using primers indicated in A. The *-fold enrichment of HP1-α* that was normalized with *that of IgG* is plotted. Each *bar* is an average of biological triplicates. Error bars show S.E. J, ChIP experiments were done with asynchronous (black bars) and G1/S [gray bars]-blocked HEK-293-FLAG-PIPK5 cells using anti-H3K9me3 antibody. The precipitated DNA was analyzed by qPCR using primers indicated in A. The *-fold enrichment of histone H3* that was normalized with *that of IgG* is plotted. Each *bar* is an average of biological triplicates. Error bars show S.E. K, re-ChIP experiments were done with HEK cells expressing FLAG-PILS5KD with consecutive pulldowns of FLAG followed by either H3K9me3 (black bars) or HP1-α (gray bars) as indicated. The *-fold enrichment values presented were normalized with *that of FLAG* followed by IgG pulldown and plotted (*Experimental Procedures*). Error bars show S.E.
FIGURE 5. Interaction of PIP5K with H3K9me3 and HP1-α is DNA-dependent. A, panel I, whole cell lysates from HEK-293-FLAG-PIP5K cells were immunoprecipitated with anti-H3K9me3 antibody. These immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-FLAG and anti-H3K9me3 antibodies as indicated. NC represents the negative control for immunoprecipitation (IP) where only protein A-agarose beads but no corresponding IgG was used. 40 and 150 μg of protein lysates were used as input and for immunoprecipitation, respectively. Protein molecular weight markers (M) were used to show the relative position of the protein bands. Panel II, whole cell lysates from HEK-293-FLAG-PIP5K cells were immunoprecipitated with anti-HP1-α antibody. These immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-FLAG and anti-HP1-α antibodies as indicated. NC represents the negative control for immunoprecipitation where only protein A-agarose beads but no corresponding IgG was used. 40 and 150 μg of protein lysates were used as input and for immunoprecipitation, respectively. B, panel I, uninduced (UN) and induced (IN) (0.5 mM isopropyl 1-thio-β-D-galactopyranoside at 37 °C for 2 h) DE-3(pET33-PIP5K) lysate and purified HIS-PIP5K were separated by 10% SDS-PAGE and immunoblotted with the affinity-purified IgG fraction of PIP5K antiserum (1:1000). Panel II, 50 μg of whole cell protein lysates from untransfected HEK cells and HEK cells overexpressing WT-PIP5K (HEK_PIP5K) were analyzed by Western blotting (WB) using anti-PIP5K and anti-FLAG antibodies as indicated. Whole cell protein lysates from both cell types were immunoblotted with anti-β-actin antibody (as indicated) as loading controls. C, 150 μg of whole cell protein lysates from HEK-293 cells were immunoprecipitated with anti-H3K9me3 and anti-HP1-α antibodies as indicated. These were then immunoprobbed with anti-PIP5K antibody (upper), anti-H3K9me3 antibody (middle), and anti-HP1-α antibody (lower) as indicated. Neg Ctrl, negative control where only protein A-agarose beads but no corresponding IgG was used for immunoprecipitation. D, FLAG-PIP5K co-localizes with HP1-α. HEK-293-FLAG-PIP5K cells were doubly stained for FLAG (green) and HP1-α (red) using the respective antibodies. The merged panel (panel d) containing a DAPI-stained nuclear image shows appreciable co-localization of FLAG-PIP5K and HP1-α (arrowheads). The scale bar represents 5 μm. E, whole cell lysates from HEK-293-FLAG-PIP5K cells were incubated with or without DNase I prior to immunoprecipitation with anti-H3K9me3 as indicated. F, panel I, DNase I treatment impairs PIP5K interaction with H3K9me3. Whole cell protein lysates from HEK-293 cells were incubated with or without DNase I prior to immunoprecipitation with anti-H3K9me3 as indicated. The immunoprecipitates from both sets were subjected to Western blot analysis using anti-PIP5K antibody and anti-H3K9me3 antibody. NC, negative control as described in A.
show nuclear targeting (Fig. 1, panels g–i). PIP5K-K490A even failed to accumulate in the nucleolus when these cells were synchronized at G1/S phase (Fig. 6B, panels b and c). Immunoblot analysis of anti-H3K9me3 (Fig. 6C, lanes 3 and 4) and anti-H1-α (Fig. 6C, lanes 5 and 6) immunoprecipitates from lysates of HEK-293 cells expressing FLAG-PIP5K-K490A mutant with anti-FLAG antibody revealed a null interaction of both the silencing proteins with PIP5K-K490A mutant as compared with the WT-PIP5K. To demonstrate whether Lys-490 is modified by SUMO-1, we expressed a K33A/K244A double mutant form of PIP5K that had Lys-490 unmodified. Analysis of anti-SUMO-1 immunoprecipitates from nuclear and cytosolic lysates of these cells revealed a 90-kDa immunoreactive band when probed with anti-FLAG antibody (Fig. 6D). Due to the loss of SUMOylation at 244, the protein failed to be imported into the nucleus, but the cytosolic fraction showed the presence of SUMOylated PIP5K, primarily modified by SUMO-1 at Lys-490. Thus, the overall results suggested that the interaction of PIP5K with H3K9me3 and H1-α was dependent on the SUMOylation of the kinase at Lys-490.

Discussion

PIP5K is mainly a cytosolic enzyme that has been well studied for its role in membrane dynamics and regulation of the actin cytoskeleton including cell migration (27, 28). Recent reports have associated PIP5K with poor prognosis in prostate cancer (29). Additionally, the γ-isofrom of the kinase has been implicated in anchorage-independent growth of tumor cells (30). PIP5K is spatially organized to “nuclear speckles” that are distinct from known membrane structures (4). Nuclear targeting of phosphoinositide-metabolizing enzymes is achieved through various mechanisms. Although some possess a bona fide nuclear localization signal, others like PIPKIβ are targeted by a “kinase insert region” (31). PIP5K has neither a nuclear localization signal nor any kinase insert region. We have shown previously that the nuclear form of PIP5K is mostly modified by SUMO-1 in normal cells (12). In the present study, we confirmed that SUMOylation at Lys-244 is required for its nuclear translocation and that SUMOylation at Lys-490 is required for its nucleolar localization (Fig. 1A). Although Lys-33 can be SUMOylated, it plays no part in either nuclear targeting or nucleolar occupancy. Furthermore, we showed that PIP5K physically associates with rDNA loci through its interaction with the transcription repression machinery components H3K9me3 and HP1-α in a SUMOylation-dependent manner. This is possibly the first report of the involvement of PIP5K protein per se with the chromatin silencing complex, unraveling yet an unknown facet of this enzyme associated with lipid biosynthesis pathway.

Reports from several laboratories have demonstrated the role of PIP3 in modulating various nuclear functions (3, 31). It might be argued that the nuclear presence of PIP5K is obvious to produce PIP3. However, it was shown that PIP5K interacts with speckle-targeted PIPKια regulated-poly(A) polymerase independently of PIP3 to regulate pre-mRNA processing (9). Moreover, PIP2 has been shown to be present in the nuclear
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chromatin during mitosis (32), although our results suggest that PIP5K is not associated with the mitotic chromatin (Fig. 2A, panel c). Previously, the nuclear PIP2 had been shown to be regulated by cell cycle stages (33) and to interact with Pol I and upstream binding factor in the nucleolar cap region (8). Interestingly, our study revealed an enhanced accumulation of PIP5K in the nucleolar cap region upon G1/S arrest in multiple cell types (Figs. 3 and 2C). Thus, we speculated that the nucleolar PIP5K per se may have a direct role in modulating the biosynthesis of rRNA. Results from our ChIP experiments indeed revealed that nuclear expression of WT-PIP5K is associated with high occupancy of the repression signature H3K9me3 along with PIP5K and HP1-α throughout the rDNA gene loci in asynchronous cells. It is to be noted that the recruitment of PIP5K was lowest at the promoter region and highest at the intergenic spacer region (intergenic sequence loci/H27). This indicates that PIP5K might well be implicated in maintaining the overall epigenetic state of rDNA genes as intergenic transcripts have been shown to regulate the same (34). Considerable -fold enrichment in the re-ChIP assays (Fig. 4K) coupled with a direct physical association of PIP5K with the proteins of the heterochromatin complex, namely H3K9me3 and HP1-α, in a DNA-dependent manner (Fig. 5) makes it a probable component of the chromatin silencing machinery.

Notably, although the rDNA loci were shown to be occupied by enhanced levels of WT-PIP5K, H3K9me3, and HP1-α in asynchronous (mostly G1) cells, these levels showed a considerable decrease in the early S phase (Fig. 4, G, H, and I). Interestingly, the decreased association of PIP5K with rDNA loci in the early S phase was quite opposite to its enhanced nucleolar localization in the same phase (Fig. 2, B and C). This discrepancy in enhanced nucleolar localization of PIP5K and its reduced association with rDNA during G1/S phase is partly clarified by our results that suggest that, as opposed to the asynchronous phase, in the early S phase synthesis of both 18S and 45S pre-rRNAs in cells expressing WT-PIP5K is derepressed to a level similar to that in untransfected HEK-293 cells (Fig. 4, D and E). This corroborates the ChIP data, which showed decreased recruitment of PIP5K on the rRNA genes during the early S phase. This result signifies that PIP5K enriched in the nucleolus during the G1/S phase had reduced association with the rRNA genes to allow its expression.

Furthermore, it has been shown that upstream binding factor is continuously present in the nucleolus both in its inactive (G1 phase) and active states (early S phase) (35). It has also been shown that although the transcription of rRNA genes is activated during late G1 to G2 phases it is repressed in the M phase and most of the G1 phase (35). Moreover, our results demonstrate that SUMOylation of PIP5K at Lys-490 plays a major role in mediating its association with H3K9me3 and HP1-α (Fig. 6C). Because the nucleolus is an active site for deSUMOylation (36), it is likely that PIP5K SUMOylated at Lys-490 in particular remains bound to rDNA loci in the majority of the G1 phase, but with the onset of G1/S transition, deSUMOylation of PIP5K at Lys-490 leads to its reduced association with the rRNA genes in the nucleolus. In fact, our preliminary data reveal gradual to complete deSUMOylation of PIP5K at around S/G2 phase. A recent study indeed showed that inhibition of SUMOylation relieved repression of 5S rRNA genes and is correlated with a decrease in trimethylation of H3K9 (37). This indicated that PIP5K, H3K9me3, and HP1-α followed a definite cyclical pattern of occupancy and release from the rDNA loci throughout the cell cycle.

Based on the fact that PIP2 binds histones (6), one might argue that the PIP5K interaction with the silencing complex proteins is PIP2-mediated. Our in vitro studies have clearly demonstrated that PIP5K can bind to core histones albeit weakly (Fig. 6A). Moreover, PIP5K-K490A mutant failed to bind to both H3K9me3 and HP1-α (Fig. 6C). Even PIP5K did not interact with H3K4me3 (Fig. 5E). Thus, it can be ascertained that PIP5K binding to histones is irrespective of the presence of any PIP2 and depends on its SUMOylation status at Lys-490.

Taken together, our study demonstrates a distinct role of the phosphoinositide kinase in rRNA gene silencing through its physical association with H3K9me3 and HP1-α, the proteins of the chromatin silencing complex. The present study also delineates a differential role of SUMOylation of PIP5K at Lys-244 for nuclear targeting and at Lys-490 for its interaction with the above silencing proteins. We thus establish a novel association of a lipid biosynthesis enzyme with chromatin and its yet unexplored role in regulating Pol I-mediated transcription.

Author Contributions—A. S. and C. D. conceived the study and designed the experiments. R. C. and S. S. performed the experiments. A. G. and K. B. were involved in raising the antibody against endogenous PIP5K, affinity purification of His-tagged PIP5K, and other technical assistance. A. S., C. D., R. C., and S. S. analyzed the data and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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