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Article

Inheritance of Silent rDNA Chromatin Is Mediated by PARP1 via Noncoding RNA

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SUMMARY

Faithful propagation of specific chromatin states requires re-establishment of epigenetic marks after every cell division. How the original epigenetic signature is inherited after disruption during DNA replication is still poorly understood. Here, we show that the poly(ADP-ribose)-polymerase-1 (PARP1/ARTD1) is implicated in the maintenance of silent rDNA chromatin during cell division. We demonstrate that PARP1 associates with TIP5, a subunit of the NoRC complex, via the noncoding pRNA and binds to silent rRNA genes after their replication in mid-late S phase. PARP1 represses rRNA transcription and is implicated in the formation of silent rDNA chromatin. Silent rDNA chromatin is a specific substrate for ADP-ribosylation and the enzymatic activity of PARP1 is necessary to establish rDNA silencing. The data unravel a function of PARP1 and ADP-ribosylation that serves to allow for the inheritance of silent chromatin structures, shedding light on how epigenetic marks are transmitted during each cell cycle.

INTRODUCTION

Maintenance and transmission of proper chromatin organization is fundamental for genome stability and function in eukaryotes. During DNA replication, both heterochromatin and euchromatin are disrupted ahead of the replication fork and are then reassembled into their original epigenetic states behind the fork. How chromatin domains are restored on new DNA and transmitted through mitotic cell division remains a fundamental question in biology, with implications for development and complex diseases like cancer (Jasencakova and Groth, 2011). In higher eukaryotes, the repeated ribosomal RNA (rRNA) genes represent a striking example of how specific chromatin states are propagated during the cell cycle. In each cell, a fraction of rRNA genes is transcriptionally silent, replicates in late S phase, and is organized in heterochromatic structures by epigenetic mechanisms, including silent histone marks and CpG methylation (Li et al., 2005; Santoro, 2005, 2011; Santoro and Grummt, 2001; Santoro et al., 2002). By contrast, the "active" euchromatic rDNA fraction that replicates in early S phase represents rRNA genes competent for transcription whose activity is modulated according to the requirement of cell metabolism (Moss et al., 2007). Inheritance of silent rDNA chromatin is controlled by NoRC, the nucleolar remodeling complex comprising TIP5 and the ATPase SNF2h (Guetg et al., 2010; Santoro and Grummt, 2005; Santoro et al., 2002; Zhou et al., 2002). In mid-late S phase, TIP5 binds to silent rRNA genes after the passage of the replication fork and recruits DNA methyltransferases and histone modifier enzymes to re-establish silent rDNA chromatin (Li et al., 2005; Santoro et al., 2002; Zhou et al., 2002). Knockdown of TIP5 impairs rDNA silencing and induces genome instability at the rDNA locus and at the nearby centric and pericentric sequences (Guetg et al., 2010; Santoro et al., 2009). NoRC function requires the association of TIP5 with the noncoding RNA pRNA, a transcript originating from an RNA polymerase I (Pol I) promoter located 2 kb upstream of the pre-rRNA transcription start site (Mayer et al., 2006; Santoro et al., 2010). pRNA is synthesized by active rRNA genes during early S phase and then processed during mid S phase into a 250–300 nt fragment that matches the rDNA promoter sequences from −220 to +1 (Mayer et al., 2006; Santoro et al., 2010). Nucleolar retention of TIP5, rDNA methylation, and silent histone modifications at rDNA depend on pRNA (Mayer et al., 2006). Importantly, a TIP5 mutant with impaired RNA-binding activity (W531G,Y532A; $TIP5_{\Delta RNA}$) failed to establish rDNA heterochromatin (Mayer et al., 2006). pRNA sequences from nucleotides −127 to −49 in mouse forms a conserved hairpin structure that is specifically recognized by the TIP5-TAM domain. Upon pRNA binding, TIP5 undergoes a conformational change that was proposed to facilitate the interaction with other proteins required for rDNA silencing (Mayer et al., 2008). We have now examined the mechanism of NoRC-pRNA interaction that modulates recruitment of chromatin modifier enzymes to propagate rDNA heterochromatin during cell division.

RESULTS

PARP1 Associates with TIP5 and Binds to Silent rRNA Genes

To dissect the mechanisms of NoRC function in rDNA heterochromatin formation, we identified TIP5 interaction partners in HEK293T cells expressing HA-FLAG-TIP5 in association with proteomics and immunoblot analyses. As shown in Figure 1A, we identified the poly(ADP-ribose)-polymerase-1 (PARP1, also known as ARTD1) (Hottiger et al., 2010) as a TIP5-interacting protein. PARP1 is an enzyme possessing NAD^+-dependent

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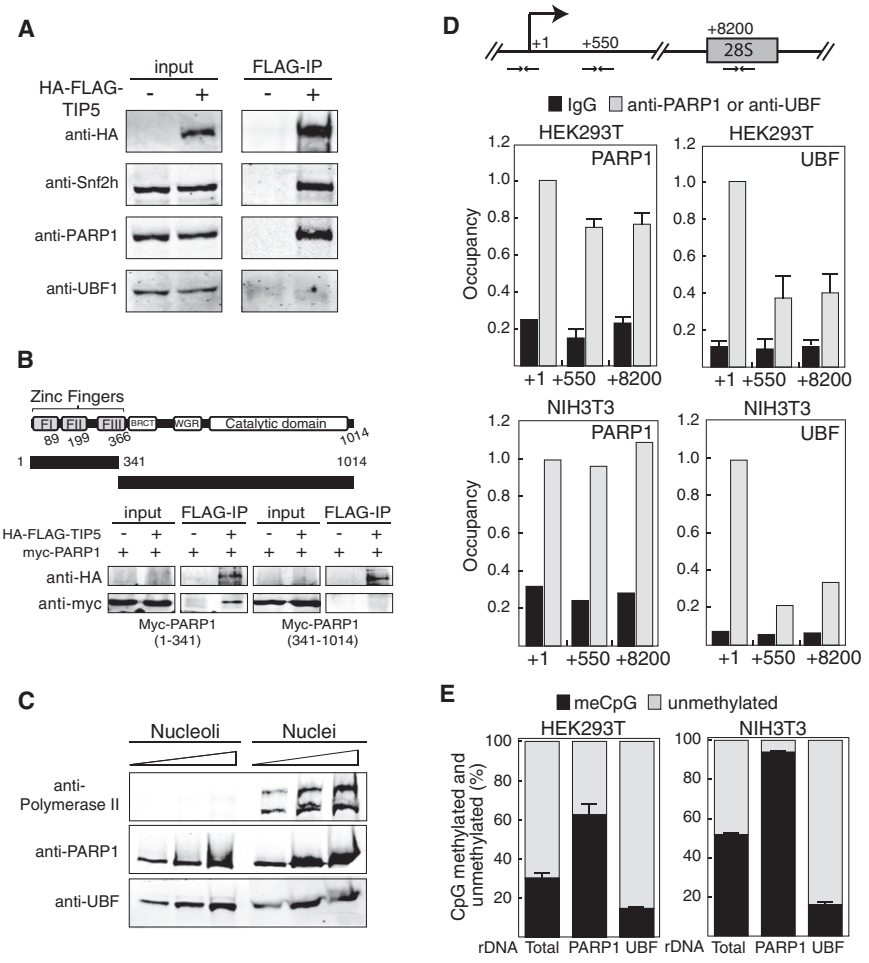

Figure 1. PARP1 Associates with TIP5 and Silent Methylated rRNA Genes

(A) FLAG immunoprecipitation from HEK293T cells expressing FLAG-HA-TIP5. Immunoblots show association of TIP5 with SNF2h (a component of NoRC complex) and PARP1 but not with the rDNA transcription factor UBF.

(B) TIP5 interacts with PARP1-zinc-finger FI/FII. FLAG immunoprecipitation from HEK293T cells cotransfected with myc-PARP1$_{1-341}$ and myc-PARP1$_{341-1014}$ expressing plasmids in the presence or absence of vectors expressing HA-FLAG-TIP5 is shown. Coprecipitated proteins were visualized using HA and Myc antibodies. The schema represents PARP1 domains analyzed for the interaction studies. FI/FII are zinc finger, FIII is zinc-binding domain.

(C) PARP1 is present in the nucleoli. Identical NIH 3T3 cell equivalents of purified nucleoli and nuclei were analyzed for PARP1, UBF, and Pol II enrichment with the corresponding antibodies. The purity of nucleoli was assessed by the lack of Pol II signals.

(D) PARP1 associates with rRNA genes. ChIP shows PARP1 and UBF occupancy at the rRNA gene in HEK293T and NIH 3T3 cells. Data are represented as bound/input values normalized to the occupancy at the rDNA promoter (+1).

(E) PARP1 associates with the promoter of methylated silent rRNA genes. ChIP-chop analysis showing meCpG content of rDNA promoter sequences from total rDNA and of chromatin immunoprecipitated with PARP1 and UBF antibodies. CpG methylation was assayed by digestion with HpaII (NIH 3T3) or SmaI (HEK293T). The bars indicate the relative level of methylated silent rDNA (HpaII/SmaI-resistant, black) compared to unmethylated active rDNA (light) measured by qPCR. Error bars indicate the SD of three independent experiments.

catalytic activity to synthesize ADP-ribose (PAR) polymers bound to itself or to other proteins, including histones (Messner and Hottiger, 2011). Mapping of TIP5-PARP1 interaction domains by coexpression of myc-tagged PARP1 mutants and immunoprecipitation of HA-FLAG-TIP5 revealed that this association is mediated by the first N-terminal 341 aa of PARP1 that comprises the two zinc fingers FI/FII (Figure 1B). Although historically studied in the context of DNA genotoxic stress signaling, PARP1 has recently been linked to the regulation of chromatin structure, transcription, and chromosome organization (Hassa and Hottiger, 2008; Krishnakumar and Kraus, 2010). Accumulation of PARP1 in the nucleolus of interphase cells was documented (Meder et al., 2005; Rancourt and Satoh, 2009) (Figure S1A). Consistent with this, we detected a large fraction of PARP1 in purified nucleoli of NIH 3T3 cells (Figure 1C). The role of PARP1 in the nucleolus remained unclear. To determine whether PARP1 associates with rRNA genes, we performed ChIP assays in NIH 3T3 and HEK293T cells. PARP1 bound to both rDNA promoter and coding regions (Figure 1D). A similar, but not identical rDNA binding profile was determined for UBF. As TIP5 binding is restricted to the promoter of silent rRNA genes (Figure S1B) (Santoro et al., 2002), we investigated whether PARP1 associates either with the silent or active rDNA fraction. To test this, we performed a ChIP-chop assay (Santoro et al., 2002); we isolated chromatin associated with PARP1 and UBF and monitored rDNA promoter methylation (the epigenetic mark characterizing the promoter of silent rRNA genes) by methylation-sensitive restriction analysis. Consistent with previous results, UBF bound to unmethylated active rRNA genes (Figure 1E) (Santoro and Grummt, 2001; Santoro et al., 2002). In contrast, PARP1 was preferentially associated with the methylated, silent rDNA fraction. Taken together, the results indicated that PARP1 associates with TIP5, the subunit of the rDNA repressor NoRC complex, and binds to the promoter of silent rRNA genes.

Association of PARP1 with TIP5 Is Mediated by pRNA

To investigate the relationship between PARP1 and TIP5 in rDNA binding, we performed ChIP assays in HEK293T expressing shRNA-control, *-Parp1*, and *-Tip5* sequences (Figures 2A and S2). In TIP5-depleted cells, binding of PARP1 to the rDNA promoter decreased while the association with the coding region was inversely proportional to the distance from the rDNA promoter. In turn, in PARP1-depleted cells, the association of

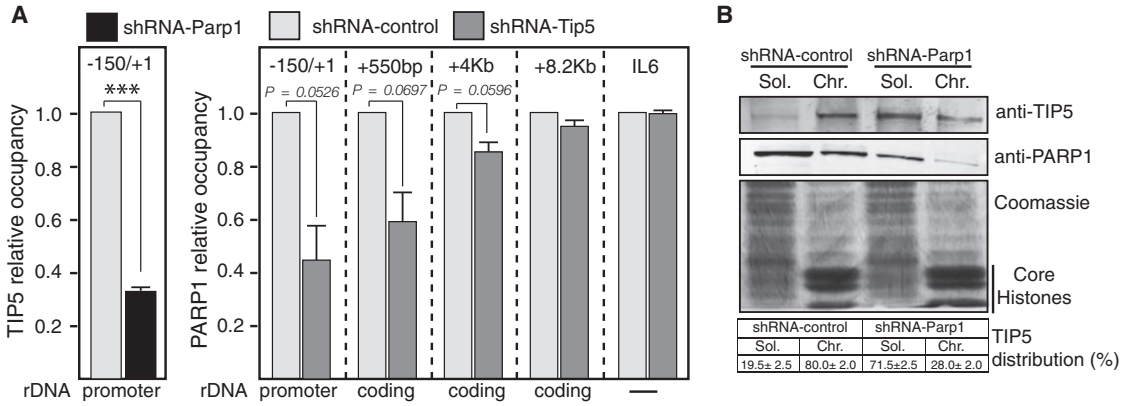

Figure 2. Recruitment of PARP1 to rDNA Promoter Is Interdependent with That of TIP5

(A) ChIP shows occupancy at rDNA regions in HEK293T cells after depletion of PARP1 and TIP5 by shRNA. Values (bound/input) are normalized relative to rDNA occupancy of cells expressing shRNA-control sequences. IL6 represents a control gene that associates with PARP1 but is not regulated by TIP5. Error bars indicate the SD of two independent experiments (***, $p < 0.001$ versus Control).

(B) PARP1 is required for the association of TIP5 with chromatin. Chromatin-bound (Chr.) and soluble (Sol.) fractions of HEK293T cells expressing shRNA-control and *-Parp1* sequences were analyzed with anti-TIP5 and -PARP1 antibodies. Coomassie staining of the membrane showed equivalent protein loading and efficiency of chromatin extraction. Relative values of chromatin-bound and soluble TIP5 are indicated (two independent experiments).

TIP5 with the rDNA promoter decreased. Consistent with this, after extraction of HEK293T chromatin with Triton X-100, the large majority of TIP5 in shRNA-control cells remained associated with chromatin (Mayer et al., 2006), whereas TIP5 was easily extracted in PARP1-depleted cells and enriched in the soluble fraction (Figure 2B). These results suggest that recruitment of TIP5 and PARP1 to rDNA promoter is dependent on each other while the fraction of PARP1 bound to the second half of the coding region is independent of TIP5.

Previous results showed that nucleolar retention of TIP5 is disrupted after RNase A treatment (Mayer et al., 2006). Similarly to TIP5, treatment of cells with RNase A displaced PARP1 from nucleoli while the localization of UBF, as previously reported (Mayer et al., 2006), remained unaffected (Figure 3A), indicating that PARP1 nucleolar localization is dependent on RNA. To test whether PARP1 associates with pRNA, we measured pRNA content of immunoprecipitated HA-TIP5, -$TIP5_{\Delta RNA}$ or -PARP1 (Figure 3B). As expected, TIP5 but not $TIP5_{\Delta RNA}$ associated with pRNA. Importantly, although a large portion of PARP1 is involved in nonnucleolar activities, we detected a 2-fold enrichment of pRNA after PARP1 immunoprecipitation relative to control-IP, suggesting that PARP1 associates with NoRC/pRNA complex. Northwestern analysis determined that the region mapped as TIP5-interaction domain of PARP1 (Figure 1B) and containing zinc fingers FI/FII (aa 1–214) bind to RNA (Figure 3C). A similar binding to RNA was detected for the zinc-binding domain FIII, while the region comprising BRCT, WGR, and the catalytic domain showed low or no affinity for RNA. To analyze whether RNA mediates TIP5-PARP1 association, we performed FLAG immunoprecipitation of HA-FLAG-TIP5 and $TIP5_{\Delta RNA}$ in HEK293T cells (Figure 3D). The association of $TIP5_{\Delta RNA}$ with PARP1 was greatly reduced, suggesting that RNA, possibly pRNA, might mediate TIP5-PARP1 interaction. Consistent with this, treatment of bead-bound TIP5 complexes with ethidium bromide (EtBr), which by virtue of its ability to alter nucleic structure upon intercalation destabilizes protein associations mediated by DNA and RNA (Lai and Herr, 1992), strongly reduced TIP5-PARP1 association without affecting the interaction with Dnmt1, a known TIP5-interacting protein (Santoro et al., 2002) (Figure 3E). These results indicated that TIP5-PARP1 association is mediated by nucleic acids. As nuclear extracts were treated with DNase I and the DNA binding properties of $TIP5_{\Delta RNA}$ are not affected (Mayer et al., 2006), we reasoned that TIP5-PARP1 interaction is most probably mediated by RNA. To determine whether pRNA directly mediates TIP5-PARP1 association, we performed GST pull-down assays using DNA/RNA-free purified recombinant GST-$TIP5_{1-598}$, containing the TAM domain, and His-$PARP1_{1-214}$, comprising FI/FII domains. Binding reactions were performed in the absence or presence of in vitro transcribed RNAs. In the absence of RNA, TIP5 and PARP1 did not associate, underscoring the role of RNA in this interaction (Figure 3F, lane 2). In contrast, TIP5-PARP1 interaction was detected in the presence of rRNA −232/−1 in sense orientation (lane 3). This RNA comprises the sequences (−127/−49) forming the conserved loop structure that is necessary for the binding to TIP5 (Mayer et al., 2008). The antisense rRNA (−232/−1), the sense RNA (−232/−140) that lacks sequences required to form the loop, and the control RNA showed a marked reduction in their ability to mediate TIP5-PARP1 binding. These results indicate that pRNA sequences, and specifically the region implicated in the formation of the loop structure (Mayer et al., 2008), directly mediate TIP5-PARP1 association.

PARP1 Establishes rDNA Silencing via Its ADP-Ribosylation Activity

To determine whether PARP1 is implicated in the formation of silent rDNA chromatin, we measured rRNA transcription and rDNA methylation levels in NIH 3T3 cells selected for stable expression of shRNA-control, *-Tip5*, or *-Parp1* sequences (Figures 4A–4C). Consistent with previous results, rRNA synthesis increased and methylated rDNA levels reduced in

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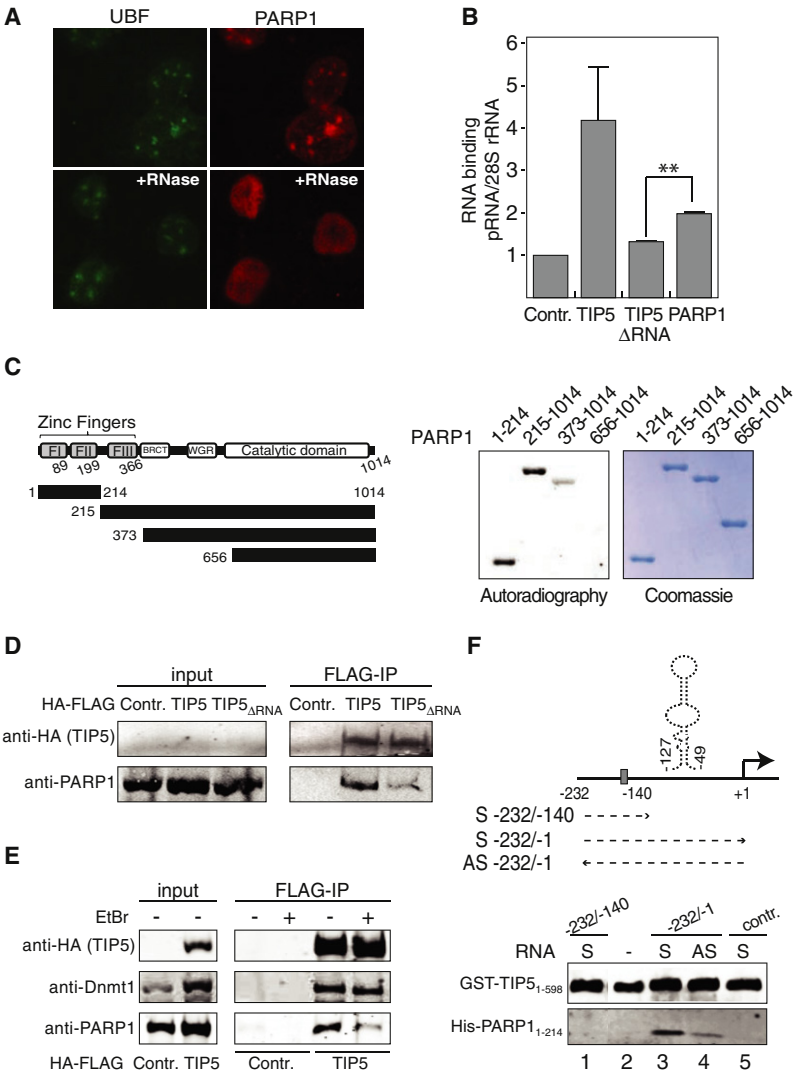

Figure 3. PARP1-TIP5 Association Is Mediated by pRNA

(A) PARP1 nucleolar localization is sensitive to RNase A treatment. Permeabilized NIH 3T3 cells were treated with RNase A, and localization of PARP1 and UBF was visualized by immunofluorescence.

(B) PARP1 associates with pRNA. Values were measured by qRT-PCR. RIP assay monitoring levels of pRNA associated with immunoprecipitated HA-TIP5, -TIP5$_{\Delta RNA}$, and -PARP1 in HEK293T cells. pRNA levels were normalized to 28S rRNA and IP from cells transfected with empty vector (Contr.). Error bars indicate the SD of three independent experiments (**, $p < 0.01$ versus TIP5$_{\Delta RNA}$).

(C) Northwestern analysis was performed. Schema representing PARP1 proteins were analyzed for RNA binding. Membrane-bound recombinant PARP1 proteins (see Coomassie) were incubated with radiolabeled pRNA sequences (−232/−1), and bound RNA was visualized by autoradiography.

(D) RNA mediates association of PARP1 with TIP5. FLAG immunoprecipitation of HA-FLAG-TIP5 and -TIP5$_{\Delta RNA}$ in HEK293T cells was performed.

(E) Bead-bound FLAG-TIP5 immunoprecipitates from HEK293T cells were incubated with or without EtBr (10 μg/ml). After being washed, coprecipitated proteins were visualized with anti-HA, -DNMT1, and -PARP1 antibodies. The data show one representative experiment out of two independent experiments.

(F) pRNA mediates association of PARP1 with TIP5. Schema representing mouse rDNA promoter sequences (upper panel), including the region −127 to −49 forming the conserved hairpin structure. In vitro transcribed rRNAs are shown below (rRNA −232/−140, −232/−1; 200 bases non-rRNA sequences, contr.). Sense (S) and antisense (AS) orientation are indicated. RNA/DNA-free purified recombinant bacterial expressed GST-TIP5$_{1-598}$ and His-PARP1$_{1-214}$ (lower panel) were incubated with the indicated 5 pmoles of renaturated RNAs. GST pull-down proteins were visualized with anti-GST and anti-His antibodies.

the absence of TIP5 (Guetg et al., 2010; Santoro et al., 2009). Similar results were detected in PARP1-depleted NIH 3T3 (Figures 4B and 4C) and HEK293T cells (Figure S3A), implying a role of PARP1 in rDNA silencing. Moreover, knockdown of PARP1 decreased the levels of H3K9me2 bound to rDNA, a histone mark associated with silent rDNA chromatin (Santoro et al., 2002) (Figure S3B). To support these data, we monitored rRNA transcription and rDNA promoter methylation levels in HEK293T cells overexpressing TIP5, PARP1, or PARP1$_{E988K}$ (a mutant lacking the ability to generate PAR polymers) (Rolli et al., 1997) (Figures 4D, 4E, and S4A–S4C). Consistent with previous data, elevated levels of TIP5 repress rRNA transcription (Figure 4D) due to de novo methylation of rRNA gene copies (Figure 4E) (Santoro and Grummt, 2005; Santoro et al., 2002). A similar transcriptional repression and increased methylated rDNA fraction was detected after PARP1 overexpression, indicating that elevated PARP1 levels promote de novo rDNA silencing. Taken together, the results show that PARP1 plays a central role in the formation of silent rDNA chromatin.

PARP1 is responsible for the majority of cellular PAR formation. During genotoxic stress, binding of PARP1 to DNA strand breaks catalyzes synthesis of PAR from NAD^+ and modifies many nuclear proteins, including itself and histones (Quénet et al., 2009). CoIP and ChIP assays revealed that the PARP1$_{E988K}$ mutant binds to TIP5 and rDNA similarly to PARP1, indicating that PARP1-dependent parylation did not affect PARP1-TIP5 association and recruitment to rDNA (Figures 4F and 4G). Of note is that the PARP1$_{E988K}$ mutant was less efficient in repressing rRNA transcription and in methylating rDNA (Figures 4D, 4E, and S4D). Consistent with this, the levels of H3K9me2 at rDNA were increased by overexpression of PARP1 but not by the PARP1$_{E988K}$ mutant (Figure S3C), suggesting that PARP1-mediated parylation plays a role in the formation of silent rDNA chromatin.

Silent rDNA Chromatin Is Substrate of Parylation

The enzymatic activity of PARP1 is stimulated by binding to DNA strand breaks (Krishnakumar and Kraus, 2010). The experiments

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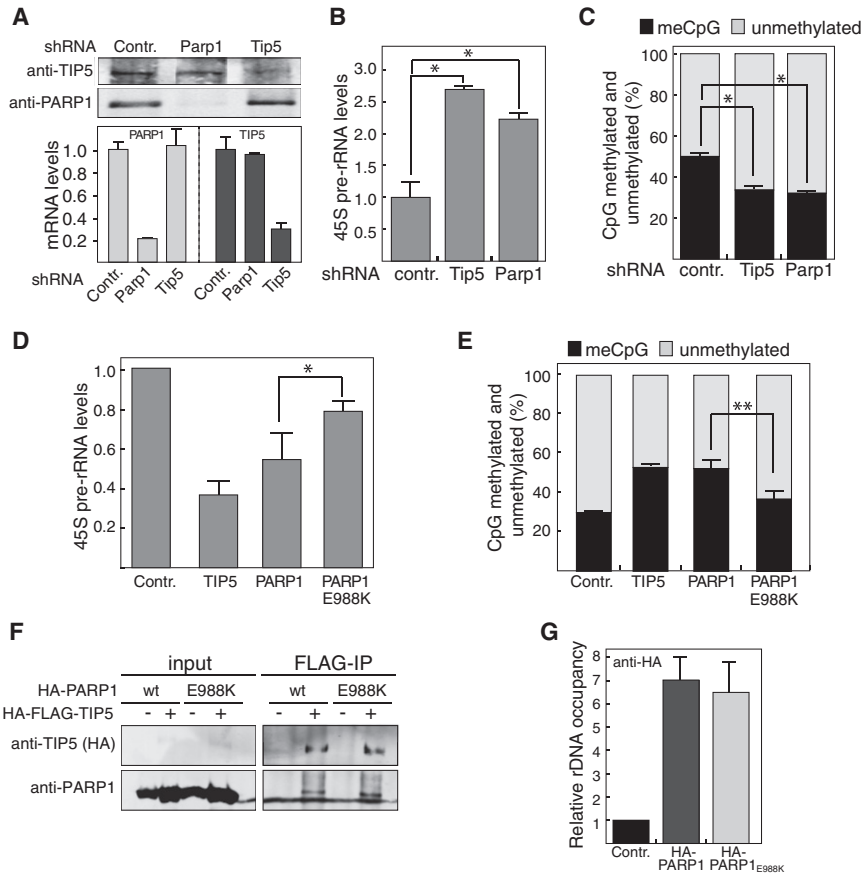

Figure 4. PARP1 Mediates rDNA Silencing

(A) Immunoblot (upper panels) and mRNA levels (lower panel) show depletion of TIP5 and PARP1 in NIH 3T3 cells. mRNA levels were normalized against rsp12 mRNA.

(B and C) 45S pre-rRNA levels (B) and rDNA promoter methylation (C) in NIH 3T3-shRNA-control, -*Tip5*, and -*Parp1* cells. rRNA levels were measured by qRT-PCR and normalized to rsp12 mRNA and to shRNA-control cells. meCpG content was measured after digestion with HpaII. Error bars indicate the SD of two independent experiments (*, $p < 0.05$ versus Control).

(D) 45S pre-rRNA levels of HEK293T cells over-expressing HA-TIP5, -PARP1, and -PARP1$_{E988}$. Values were measured by qRT-PCR as described above. Error bars indicate the SD of four independent experiments. *, $p < 0.05$ versus PARP1WT.

(E) rDNA promoter methylation was analyzed by SmaI digestion. The SmaI-resistant methylated rDNA fraction was measured by qPCR. Error bars indicate the SD of four independent experiments (**, $p < 0.01$ versus PARP1WT).

(F and G) PARP1-TIP5 association (F) and PARP1 recruitment to rDNA (G) are independent of PARP1-activity. (F) FLAG immunoprecipitation of HEK293T cells expressing HA-PARP1 or HA-PARP1$_{E988K}$ with or without HA-FLAG-TIP5. Immunoprecipitates were detected with anti-HA antibodies. (G) ChIP analysis of HA-PARP1 and HA-PARP1$_{E988K}$ in HEK293T cells depleted of endogenous PARP1 by shRNA. Analysis was performed with anti-HA antibodies. Cells transfected with empty vectors were used as control. Error bars indicate the SD of three independent experiments.

described so far were performed in the absence of genotoxic stress and induced DNA breaks, raising the question of how PARP1 activity can be promoted to establish rDNA silencing. To test whether RNA can activate PARP1, we measured PARP1 activity by monitoring automodification of an RNA/DNA-free recombinant PARP1 in the presence of dsDNA or rRNAs (Figure 5A). As expected, no signal was detected in the absence of NAD^+ or nucleic acids, while incubation with DNA strongly stimulated PARP1 activity. rRNA also stimulated PARP1 activity, although to a lesser degree, but not in a sequence-dependent manner, indicating that RNA might activate PARP1 when bound to TIP5. In support of this, incubation of the tandem affinity purified (TAP)-TIP5 complex with radiolabeled NAD^+ revealed automodification of PARP1, indicating that PARP1 bound to pRNA-TIP5 complex is enzymatically active (Figure 5B).

To determine whether components of the TIP5 complex are PARP1 substrates, we enhanced the parylation reaction by incubating bead-bound purified TAP-TIP5 or TAP-TIP5$_{\Delta RNA}$ complexes with recombinant PARP1 (rPARP1), radiolabeled NAD^+, and dsDNA (Figure 5C). After washing, bead-bound TAP-TIP5 or TAP-TIP5$_{\Delta RNA}$ complexes were separated by gel electrophoresis and labeled proteins were analyzed by gel autoradiography. As shown in Figure 5C, rPARP1 efficiently binds to TIP5 complex and parylates itself, TIP5, and other TIP5-interacting protein(s). In contrast, rPARP1 incubated with TIP5$_{\Delta RNA}$ complex was unable to parylate TIP5$_{\Delta RNA}$ and displayed a reduced automodification activity that was probably due to its low binding efficiency to the TIP5$_{\Delta RNA}$ mutant. These results indicate that PARP1 parylates components of TIP5 complex but not TIP5$_{\Delta RNA}$ complex.

Next, we investigated whether rDNA chromatin is parylated. To date, the available antibodies recognizing PAR can only detect specific parylated proteins and do not recognize some of the well-characterized targets of parylation, thus limiting their use in parylated protein detection (Dani et al., 2009). Indeed, we did not detect specific signals, not even PARP1, by immunoblot of nucleolar extracts with 10H antibody, which is generally used to identify long PAR polymers that form under genotoxic stress (Kawamitsu et al., 1984). This indicated that either nucleolar PAR levels were too low to be detected and/or polymers were too short. To overcome this technical limitation, we purified nucleolar parylated proteins that associate with the GST-macrodomain module *m*Af1521 (which potently and selectively binds parylated proteins) (Figures 5D and S5A) (Dani et al., 2009; Karras et al., 2005). This strategy has been recently used to identify parylated proteins in mammalian cells (Dani et al., 2009). As shown in Figure 5D, nucleolar histone H3 and PARP1 bound to *m*Af1521. This association was impaired in cells treated with the PAR inhibitor PJ34 or depleted of PARP1, indicating that nucleolar PARP1 is parylated and

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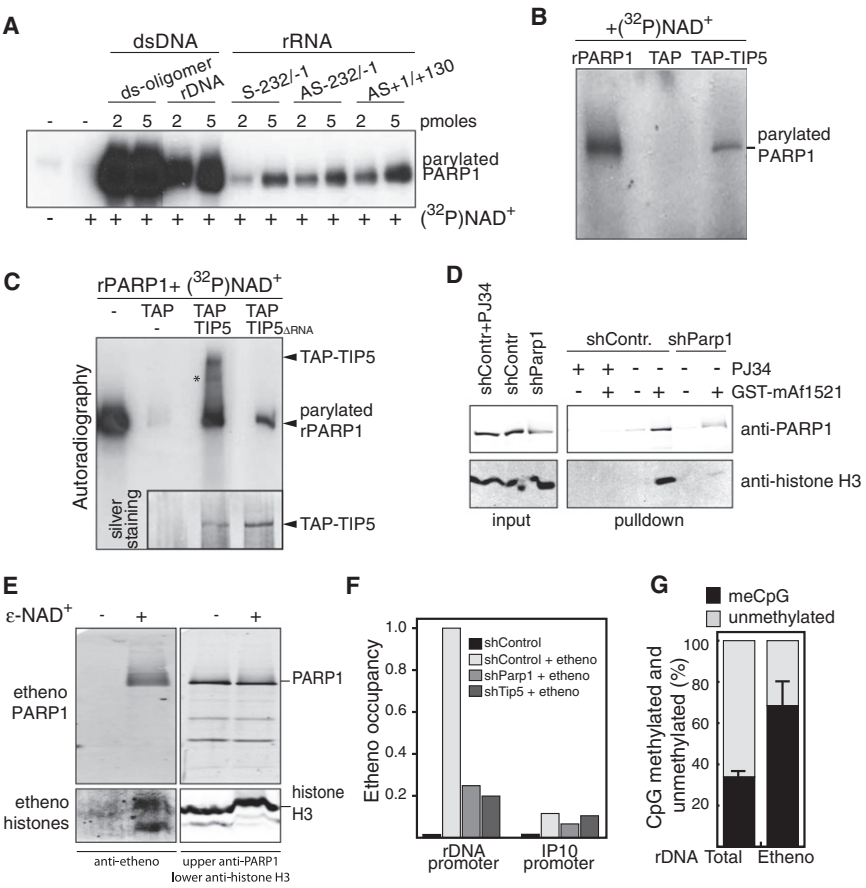

Figure 5. Silent rDNA Chromatin Is Parylated

(A) RNA stimulates PARP1 enzymatic activity. Automodification of DNA/RNA-free PARP1 incubated with radiolabeled NAD^+ in the presence or absence of DNAs and rRNAs.

(B) TIP5-associated PARP1 is enzymatically active. Bead-bound purified TAP and TAP-TIP5 complex from HEK293T cells were incubated with radiolabeled NAD^+. Automodified PARP1 was detected by autoradiography. As control, recombinant (r)PARP1 was included in the analysis.

(C) TIP5 is substrate for PARP1 parylation. Bead-bound purified TAP, TAP-TIP5, and -$TIP5_{\Delta RNA}$ complexes from HEK293T cells were incubated with recombinant (r)PARP1, radiolabeled NAD^+, and dsDNA. Bead-bound parylated peptides were visualized by SDS-PAGE gel autoradiography. Silver staining showed equivalent TAP-TIP5 and -$TIP5_{\Delta RNA}$ amounts used for the assay. (*) indicates additional parylated protein(s).

(D) Parylated proteins from purified nucleolar extracts of HEK293T cells, treated with or without the PARP inhibitor PJ34 and expressing shRNA-control and -*Parp1* sequences, were pulled down with GST or GST-mAf1521 domain. PARP1 and histone H3 were visualized by immunoblots.

(E) Purified nucleoli from NIH 3T3 cells were incubated with and without etheno-NAD^+. Histone and PARP1 incorporation of etheno moieties was monitored with anti-ethenoadenosine antibodies. Histone H3 and PARP1 were detected with the corresponding antibodies.

(F) ChIP assay showing incorporation of etheno moieties at rDNA and IP10 promoters in HEK293T cells expressing shRNA-Control, -*Parp1* and -*Tip5* sequences. Values (bound/input) are normalized to the association of etheno with the rDNA promoter of control cells.

(G) Methylated silent rDNA chromatin is substrate for parylation. Anti-etheno ChIP samples were subjected to ChIP-chop analysis. The bars indicate the relative level of SmaI-resistant, methylated silent genes (black) and unmethylated active genes (light). Error bars indicate the SD of three independent experiments.

nucleolar chromatin is parylated by PARP1. Similarly, parylated nucleolar PARP1 and histone H3 were detected by aminophenyl boronate affinity chromatography, commonly used to purify parylated proteins (Figure S5B) (Adamietz et al., 1979; Okayama et al., 1978; Rosenthal et al., 2011). To test whether parylation is a specific marker of silent rDNA chromatin, we measured incorporation of the NAD^+ analog etheno-NAD^+ in nucleolar extracts (Figure S5C) using anti-ethenoadenosine antibodies. As expected, both histones and PARP1 were the major acceptor molecules of etheno moieties (Figure 5E). To determine whether silent rDNA chromatin is indeed a substrate for parylation, we measured binding of ethenoparylated proteins to rDNA by incubating nuclei with etheno-NAD^+ and by performing ChIP assays using anti-ethenoadenosine antibodies (Figures 5F and S5D). Ethenoparylated proteins were highly enriched at the rDNA promoter when compared to the promoter of the control gene IP10. The levels of etheno-PAR incorporation were reduced in cells depleted of PARP1 and TIP5, underscoring the role of PARP1 as the enzyme responsible for rDNA chromatin ADP-ribosylation and the role of TIP5 in recruiting PARP1 to the rDNA promoter. Notably, rDNA promoter sequences bound by ethenoparylated proteins were enriched in CpG methylation (Figure 5G). Taken together, the results indicate that nucleolar chromatin is parylated by PARP1 and that silent rDNA chromatin is a specific substrate of parylation.

PARP1 Binds to Silent rRNA Genes after the Passage of the Replication Fork

Previous data showed that NoRC complex is implicated in the inheritance of silent rDNA chromatin replicating in mid-late S phase (Guetg et al., 2010; Li et al., 2005). Timing of synthesis and maturation of pRNA through S phase correlates with NoRC binding to silent rRNA genes at mid-late S phase (Santoro et al., 2010), linking pRNA to propagation of silent rDNA chromatin during the cell cycle. To determine whether binding of PARP1 to silent rRNA genes occurs at rDNA replication, we synchronized T24 cells into the cell cycle and incubated with BrdU 1 hr before sample collection (Figures 6A and S6). Anti-BrdU immunoprecipitation showed the replication timing of active (early S phase) and silent genes (mid S phase) (Figures 6B and S7A). ChIP analysis indicated an increase in PARP1 binding to rDNA chromatin at mid-late S phase and a decrease at a later time in the cell cycle (G2/M, M/G1) (Figures 6C, left panel, and S7B). Anti-BrdU ChIP of PARP1-associated rDNA

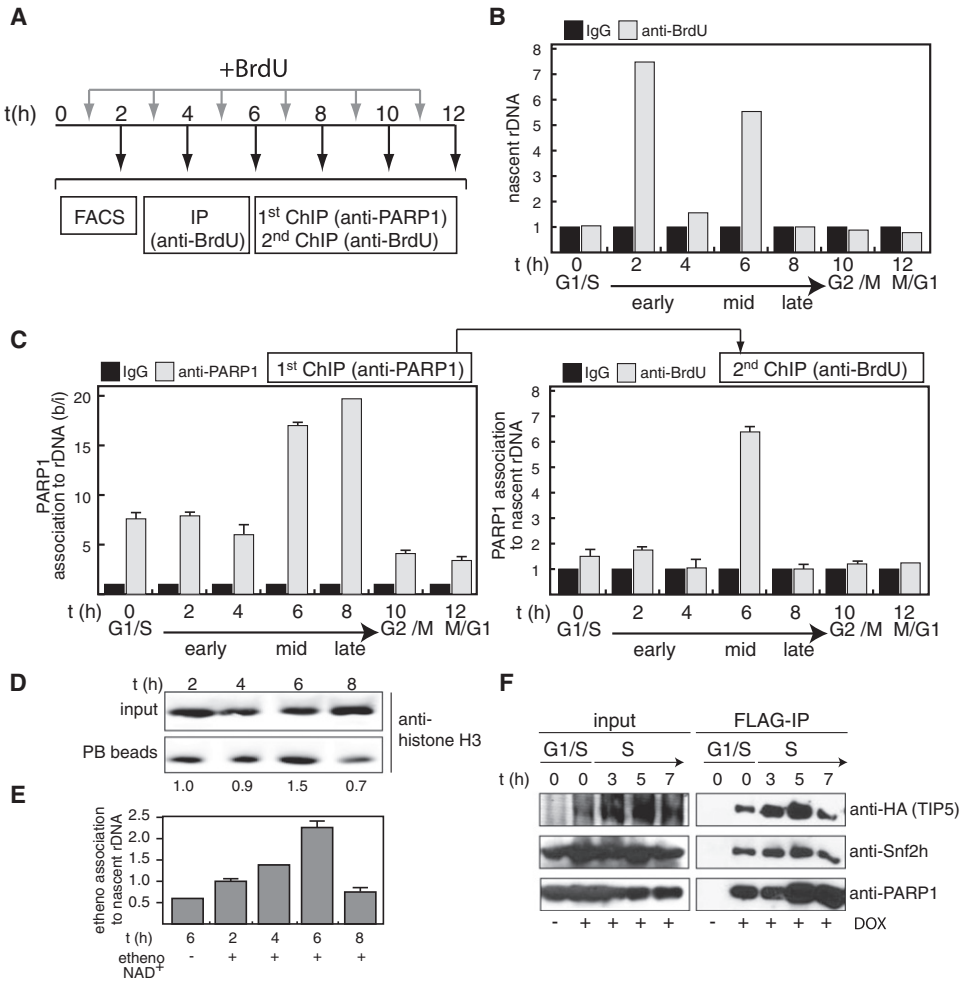

Figure 6. PARP1 Binds to Newly Replicating Silent rRNA Genes

(A) Protocol for synchronization of the cell cycle and BrdU labeling in T24 cells.

(B) Replication timing of rRNA genes in T24 cells. Anti-BrdU immunoprecipitations of DNA from synchronized cells.

(C) Left panel. ChIP (1st) showing binding of PARP1 to rDNA promoter during cell cycle. Data are presented as bound/input and normalized to IgG control. Right panel. Anti-BrdU ChIP (2nd) showing association of PARP1 with rDNA after replication in mid S phase.

(D) Enrichment of parylated nucleolar histone H3 at mid S phase. Aminophenyl boronate (PB) affinity chromatography of nucleolar extracts purified from T24 cells at the indicated times of S phase. Quantifications are shown.

(E) ChIP assay showing incorporation of etheno moieties at rDNA in T24 cells during S phase. Values (bound/input) are normalized to the association of etheno with rDNA promoter at 2 hr.

(F) PARP1 and TIP5 associate during S phase. Dox-inducible HA-FLAG-TIP5/HEK293T cells were synchronized at G1/S, collected postrelease and FLAG-immunoprecitated samples were analyzed using anti-HA, -Snf2h and, -PARP1 antibodies.

showed that PARP1 binds to rRNA genes that incorporated BrdU at mid S phase, indicating that PARP1 binds to silent rRNA genes after the passage of the replication fork (Figure 6C, right panel). At this time point, we detected an enrichment of parylated nucleolar histone H3 and an increased incorporation of etheno moieties at rDNA when compared to other times of S phase (Figures 6D, 6E, and S8B). Of note is that parylation decreased close to the end of S phase, suggesting that newly replicated rDNA chromatin is transiently parylated at this time point of the cell cycle. Consistent with the timing of PARP1 binding to rDNA, CoIP experiments revealed that TIP5-PARP1 association levels increased at mid-late S phase (Figures 6F and S8A). Taken together, these results indicated that PARP1 is recruited to silent rRNA genes after the passage of the replication fork. On the basis of our results, we propose that PARP1 and its associated enzymatic activity play a central role in the epigenetic inheritance of silent rDNA chromatin (Figure 7).

DISCUSSION

The key finding of our work is that PARP1 is a critical component of the machinery that establishes and maintains silent rDNA

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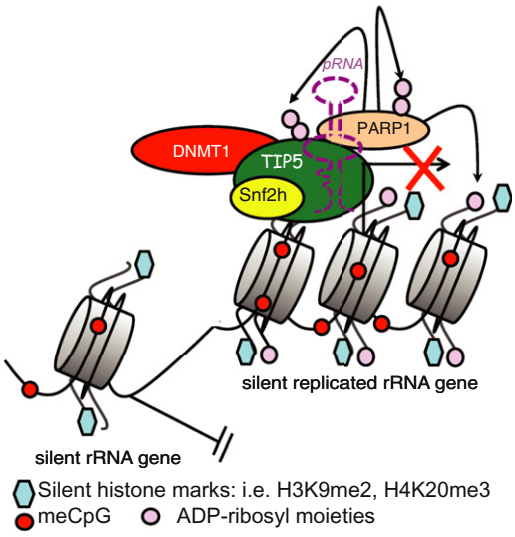

Figure 7. Model Showing the Inheritance of Silent rDNA Chromatin Mediated by TIP5, pRNA, and PARP1

After the passage of the replication fork in mid S phase, TIP5-pRNA-PARP1 complex binds to nascent rRNA genes. pRNA mediates association of TIP5 and PARP1 and activates the enzymatic activity of PARP1 to parylate PARP1 itself, TIP5, or histones. PARP1 enzymatic activity facilitates formation of silent rDNA chromatin and transcriptional silencing.

chromatin during cell division. We found that PARP1: (i) binds to TIP5 and that this interaction is mediated by pRNA; (ii) associates with silent rRNA genes after the passage of the replication fork; and (iii) represses rRNA transcription and establishes silent rDNA chromatin via its ADP-ribosyltransferase activity (Figure 7).

pRNA Mediates TIP5-PARP1 Association

Binding of PARP1 to silent rRNA genes after the passage of the replication fork (mid-late S phase) correlates well with the timing of production of the mature pRNA and TIP5 association with rDNA (Li et al., 2005; Santoro et al., 2010). After replication of silent rRNA genes, mature pRNA might guide TIP5 to rDNA via the recently proposed triple helix formation (Schmitz et al., 2010) or by stabilizing TIP5 association with rDNA chromatin after recruitment mediated by the transcription terminator factor TTF-1, a known TIP5-interacting protein that binds to rDNA promoter in a sequence-specific manner (Strohner et al., 2001). In both cases, the reported TIP5 conformational change induced upon binding to pRNA (Mayer et al., 2008) might favor the association of PARP1 and subsequent recruitment to newly synthesized rDNA. Similar to pRNA, the lincRNA HOTAIR was recently shown to act as a scaffold by providing binding surfaces to assemble the polycomb repressive complex 2 (PRC2) and the histone demethylase LSD1 at target genes (Tsai et al., 2010). The role of pRNA sequences in mediating the association of PARP1 with TIP5 and binding to newly synthesized silent rDNA copies strongly supports the idea that specific noncoding RNA can potentially direct complex patterns of chromatin states at specific genes in a spatially and temporally organized manner.

PARP1 Is a Critical Component of the Machinery that Maintains Silent rDNA Chromatin during Cell Division

Early studies determined a link between PARP1 and the DNA replication process. PARP1 was shown to colocalize with replication foci throughout S phase and to interact with several DNA replication proteins, many of which were poly ADP-ribosylated (Dantzer et al., 1998; Simbulan et al., 1993; Simbulan-Rosenthal et al., 1996; Sugimura et al., 2008). In addition, PARP activity was found to be enhanced in replicating cells (Lehmann et al., 1974), in the vicinity of replication forks (Jump et al., 1979) and in newly replicated chromatin (Anachkova et al., 1989). Notably, the role of PARP1 in DNA replication was mainly described in combination with DNA repair and recombination. PARP1 was shown to collaborate with the repair protein Mre11 to promote replication fork restart after release from replication block (Bryant et al., 2009), and PARP1 and PARP2 were described to be required for hydroxyurea-induced homologous recombination to promote cell survival after replication blocks (Bryant et al., 2009; Yang et al., 2004). Whether PARP1 plays a role for the inheritance of specific chromatin states during DNA replication was so far not addressed. Binding of PARP1 to silent rRNA genes after the passage of the replication fork and its ability to establish rDNA silencing strongly suggest a critical role in the inheritance of silent rDNA chromatin during cell division. In support of this, recent results identified PARP1 as SMARCAD1 interacting protein in several human cell lines (Rowbotham et al., 2011). SMARCAD1 is recruited to sites of DNA replication and ensures that silenced loci, such as pericentric heterochromatin, are correctly re-established. Although the role of PARP1 in the maintenance of pericentric heterochromatin mediated by SMARCAD1 was not explored by this study, we considered that this hypothesis could not be excluded. Recently, we showed that TIP5 binds to major and minor satellite DNA and that TIP5 knockdown impairs heterochromatin formation at these repeats and induces genome instability (Guetg et al., 2010). Interestingly, we found that PARP1 binds to mouse centric repeats and human alpha satellite DNA (Figures S7B, S9A, and S9B) and that depletion or overexpression of PARP1 affects the H3K9me2 levels at the alpha satellite DNA (Figure S3). Whether the function of PARP1 in the propagation of silent rDNA chromatin is linked to the maintenance of centric-pericentric heterochromatin will be subject to future investigation.

The Enzymatic Activity of PARP1 Is Required for rDNA Silencing

Much evidence exists to support a paradoxical dual contribution of PARP1 in transcription regulation. PARP1 has been implicated in the formation of chromatin structures that are permissive to transcription. In MCF-7 breast cancer cells, PARP1 localizes to the promoters of almost all actively transcribed genes and acts to exclude linker histone H1 from a subset of PARP1-stimulated promoters (Kim et al., 2004; Krishnakumar et al., 2008). On the other hand, our study showed that PARP1 preferentially binds to the promoter of silent rRNA genes and participates in the establishment of rDNA silencing. Similarly, PARP1 was reported to bind to constitutive heterochromatin regions, including the centromeres (Kanai et al., 2003) and telomeres (Beneke et al.,

2008). In *Drosophila*, genetic studies implicated PARP1 in organizing the chromatin structure of nucleoli and heterochromatin domains and silencing retrotransposable elements (Kotova et al., 2010; Tulin et al., 2002). The enzymatic activity of PARP1 was proposed as the switch event that might distinguish between PARP1 with corepressor and coactivator function (Ji and Tulin, 2010). The ability to disrupt chromatin structure by parylating histones and destabilizing nucleosomes was one of the earliest functional effects of PARP1 to be characterized (Huletsky et al., 1989; Kim et al., 2004; Mathis and Althaus, 1987; Messner and Hottiger, 2011; Poirier et al., 1982; Wacker et al., 2007). The role of parylation in decondensing chromatin finds its best example in the rapid accumulation of PAR at heat shock loci in response to heat shock in *Drosophila* (Tulin and Spradling, 2003). dPARP is required for heat shock-induced "puffing" (i.e., chromatin decondensation), and knockdown of dPARP or treatment with a PARP inhibitor prevents heat shock-induced nucleosome loss and enhanced transcription at the Hsp70 gene (Petesch and Lis, 2008). However, examples exist where PARP1, when acting as coactivator, does not require its enzymatic activity (Hassa and Hottiger, 2002; Kraus and Lis, 2003; Pavri et al., 2005). Our data indicated that the enzymatic activity of PARP1 is not only limited to processes where PARP1 acts as coactivator, but it can function as corepressor. We showed that PARP1-mediated parylation affects formation of rDNA silencing and that silent rDNA chromatin is a substrate for parylation. These results are consistent with previous studies showing that many of the *Drosophila* parylated proteins were particularly enriched in nucleoli and in the heterochromatic chromocenter regions (Tulin et al., 2002). Our data indicate that nucleolar histones are parylated by PARP1 and that PARP1 can parylate itself and other components of the NoRC complex, including TIP5. The increase in nucleolar histone parylation found when rRNA genes are replicated and bound by PARP1 strongly suggests a functional link between parylation and the re-establishment of silent rDNA chromatin. Consistent with this, $PARP1_{E988K}$ (a mutant lacking the ability to generate PAR polymers) was less efficient in repressing rRNA transcription and in establishing silent rDNA chromatin. Taken together, these results suggest that the propagation of silent marks at the rDNA locus requires PARP1 activity. Moreover, our results demonstrate that RNA has the ability to activate PARP1. Thus, pRNA might not only mediate the association of PARP1 with TIP5 but may also modulate the enzymatic activity of PARP1.

There are many possible ways that parylation can act to establish silent rDNA chromatin. PARP1 could covalently modify another protein to activate the rDNA silencing process. We showed that components of the NoRC complex, including TIP5, are parylated by PARP1. Recent advances in PAR-mass spectrometry (Messner et al., 2010) will allow determination of whether and how parylated NoRC complex affects the formation of rDNA heterochromatin. Alternatively, histone parylation might serve to destabilize nucleosomes to gain accessibility to the action of DNA methyltransferases and/or of histone-modifying enzymes. Moreover, parylation of histones might facilitate the deposition of silent histone modifications by docking chromatin enzymes. The transient parylation of newly synthesized rDNA chromatin detected in our assays (Figures 6D and 6E) suggests that PAR is required only for a short time to initiate formation of silent rDNA chromatin.

The identification of PARP1 and parylation as regulators of rDNA silencing adds a further layer of complexity in the readout of PAR signaling. We did not detect formation of long PAR polymers, typically forming upon genotoxic signaling or in "puff" formation. Whether the length or the structure of PARs might represent a critical mark that distinguishes PARP1 as coactivator or corepressor remains yet to be elucidated. The contribution of PARP1 in both activating and repressing transcription can be also appreciated in the nucleolus. In addition to our studies showing binding of PARP1 to the rDNA repressor TIP5, previous results identified the association of PARP1 with B23 and nucleolin, nucleolar proteins involved in several processes including rDNA transcription and elongation, ribosome assembly, and rRNA processing (Leitinger and Wesierska-Gadek, 1993; Meder et al., 2005). Based on these results and the fact that a fraction of PARP1 that associates with the second half of the rDNA coding region is TIP5-independent (Figure 2A), we predict that PARP1 might play additional roles in regulating nucleolar activities. Moreover, PARP2, the PAR member closest to PARP1, was also identified within the nucleolus (Meder et al., 2005). Whether its nucleolar role is overlapping or functionally different from that of PARP1 is yet to be analyzed. The generation of antibodies specific to parylated histones and recent advances in PAR-mass spectrometry will soon afford us a better understanding of how the code of parylated histones or other chromatin and transcription regulators is mechanistically interpreted.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell-Cycle Synchronization

T-Rex™-293 stable cell line that expresses HA-Flag-TIP5 under doxycycline induction was generated according to the manufacturer's protocol (Invitrogen). Expression was induced with 1 μg/ml doxycycline (Sigma) for 24 hr before harvesting. For synchronization, cells were collected 2 hr postrelease from a thymidine (2 mM) / mimosine (400 μM) double block. NIH 3T3 cells were selected for stable expression for 10 days after transduction with retroviruses expressing shRNA-control, -*Tip5*, or -*Parp1* sequences. T24 bladder tumor cells were arrested in G0 by contact inhibition (Jin et al., 1997). After 2–3 days of confluence, cells were split by seeding multiple 100 mm dishes at a concentration of $\approx 3 \times 10^6$ cells per dish. After 14 hr, cells reached G1/S phase (here referred to as t = 0 hr). Cell-cycle synchronization and progression was confirmed by flow cytometry (FACS).

SUPPLEMENTAL INFORMATION

Supplemental Information includes nine figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.molcel.2012.01.024.

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