Biochem. J. (2012) **441**, 645–652 (Printed in Great Britain) doi:10.1042/BJ20111417

online data

ADP-ribose polymers localized on Ctcf–Parp1–Dnmt1 complex prevent methylation of Ctcf target sites

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PARylation [poly(ADP-ribosyl)ation] is involved in the maintenance of genomic methylation patterns through its control of Dnmt1 [DNA (cytosine-5)-methyltransferase 1] activity. Our previous findings indicated that Ctcf (CCCTC-binding factor) may be an important player in key events whereby PARylation controls the unmethylated status of some CpG-rich regions. Ctcf is able to activate Parp1 [poly(ADP-ribose) polymerase 1], which ADP-ribosylates itself and, in turn, inhibits DNA methylation via non-covalent interaction between its ADP-ribose polymers and Dnmt1. By such a mechanism, Ctcf may preserve the epigenetic pattern at promoters of important housekeeping genes. The results of the present study showed Dnmt1 as a new protein partner of Ctcf. Moreover, we show that Ctcf forms a complex with Dnmt1 and PARylated Parp1 at specific Ctcf target sequences and that PARylation is responsible for the maintenance of the unmethylated status of some Ctcf-bound CpGs. We suggest a mechanism by which Parp1, tethered and activated at specific DNA target sites by Ctcf, preserves their methylation-free status.

Key words: CCCTC-binding factor (Ctcf), DNA methylation, DNA methyltranferase 1 (Dnmt1), epigenetics, poly(ADP-ribosyl)ation (PARylation), poly(ADP-ribose) polymerase 1 (Parp1).

INTRODUCTION

PARylation [poly(ADP-ribosyl)ation] is a post-synthetic modification catalysed by a family of enzymes known as PARP [poly(ADP-ribose) polymerases] [1] and made reversible by the activity of PARG [poly(ADP-ribose) glycohydrolase] [2]. This post-synthetic modification introduces PARs [poly(ADP-ribose) polymers] on to chromatin proteins and is peculiar since target proteins can be modulated by two distinct mechanisms: covalent and non-covalent PARylation [3–5]. It has been shown that fine coordination of PARP/PARG activities represents an important step in the modulation of gene expression [6–10]. In such a context we have envisaged a mechanism whereby PARylation regulates gene expression through its control of DNA methylation patterns [11,12].

It has been suggested previously that misregulation of PARylation may allow the introduction of anomalous methyl groups on to some CGIs (CpG islands) [11–13]. In fact, it has been found that Parp1 activity controls the unmethylated status and expression of *Dnmt1* [DNA (cytosine-5)-methyltransferase 1] [14], *p16* [15] and thrombomodulin (*THBD*) genes [16], whose altered expression is often characteristic of tumorigenic patterns. We have proposed previously that PARs present on PARylated Parp1, or on other transcription factors, are able to bind to Dnmt1 non-covalently thus preventing enzyme access to DNA and consequently DNA methylation [17]. Moreover, we have demonstrated previously that cross-talk between Parp1 and Dnmt1 can be mediated by Ctcf (CCCTC-binding factor). Ctcf promotes Parp1 automodification, accumulation of PARs and ultimately the inhibition of Dnmt1 activity [18].

Ctcf binds CpG-rich sequences both *in vitro* and *in vivo* in a methyl-sensitive fashion [19], and a role of Ctcf in protecting unmethylated DNA regions located on both CGI and imprinting control regions has been demonstrated [20–28]. The PARylated form of Ctcf has been found involved with insulator function at several Ctcf target sites [29]. Whether and how PARylation is important for the maintaining of the unmethylated status of Ctcf target sequences has not been sufficiently studied.

In the present study we focused on a well-characterized unmethylated target site of Ctcf, showed that Ctcf associates with PARylated Parp1 and Dnmt1 on this region, and that PARP activity is required to preserve the DNA methylation profile. In fact, depletion of PARs, obtained by PARG overexpression, causes loss of binding of both Ctcf and Parp1 from DNA associated with de novo methylation of Ctcf-bound CpGs. These observations allow for the possibility that Ctcf plays a role in the PARP-mediated protection of the unmethylated state of specific DNA sequences.

MATERIALS AND METHODS

Cell culture

L929 mouse fibroblasts and A1 mouse embryonic fibroblasts (from Parp1$^{-/-}$ mice) were maintained as sub-confluent culture in high-glucose Dulbecco's modified Eagle's medium, supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin and 50 mg/ml streptomycin. All culture solutions were from International PBI.

Abbreviations used: Ab, antibody; CGI, CpG island; ChIP, chromatin immunoprecipitation; Ctcf, CCCTC-binding factor; DMR1, differentially methylated region 1; Dnmt1, DNA (cytosine-5)-methyltransferase 1; dsDNA, double-stranded DNA; DTT, dithiothreitol; H1, histone 1; H2B, histone 2B; IP, immunoprecipitation; PARG, poly(ADP-ribose) glycohydrolase; Parp1, poly(ADP-ribose) polymerase 1; PARs, poly(ADP-ribose) polymers; PARylation, poly(ADP-ribosyl)ation; RE-ChIP, sequential ChIP; TBST, Tris-buffered saline plus 0.05 % Tween 20.

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Co-IP (immunoprecipitation)

Nuclei were collected from trypsinized and PBS-washed cells by centrifugation (at 16000 ***g*** for 10 s at 4 °C) following incubation (30 min) in isolation buffer [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 50 mM NaF and 0.5 mM DTT (dithiothreitol)]. Nuclear fraction was lysed in RIPA buffer [50 mM Tris/HCl (pH 8), 150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 % Nonidet P40 and 1mM EDTA), and protein concentration was determined using the Bradford Protein Assay Reagent (Bio-Rad Laboratories) with BSA (Promega) as the standard. Both buffers were supplemented with protease inhibitors (Complete EDTA-free, Roche Applied Science). Lysates (1.5 mg) were pre-cleared with protein A (for IP anti-Ctcf and anti-Parp1) or G (for IP anti-Dnmt1 and anti-PAR) agarose beads (Upstate Laboratories) on a rotative shaker at 4 °C for 2.5 h. Pre-cleared lysates were incubated with specific antibodies [rabbit polyclonal anti-Ctcf Ab (antibody) (Upstate Laboratories), mouse monoclonal anti-Dnmt1 Ab (Imgenex), rabbit polyclonal anti-Parp1 Ab (Alexis) and mouse monoclonal anti-PAR Ab (Trevigen)] and with normal rabbit or mouse IgG (Santa Cruz Biotechnology) on a rotative shaker at 4 °C. The agarose beads, previously saturated with BSA (1 μg/μl) overnight, were added to the lysate/Ab solutions and incubated for 2 h on a rotative shaker at 4 °C. Subsequently, beads were washed in IP buffer and boiled in SDS/PAGE sample buffer. The eluted proteins were analyzed by SDS/PAGE (8 % gel) and Western blotting. The Abs used were: goat polyclonal anti-Ctcf (Santa Cruz Biotechnology), mouse monoclonal anti-Dnmt1 (Imgenex) and mouse monoclonal anti-Parp1 (C2-10, Alexis Biochemicals).

In vitro protein-binding assay: pull down

ProBond™ resin (50 μl; Invitrogen) precharged with Ni^{2+} was added to 1.2 ml of native purification buffer (Invitrogen) in the presence or absence of 0.8 pmol of bvCTCF (baculovirus recombinant Ctcf) recombinant His-tagged protein [18]. The mix was incubated overnight on a rotative shaker at 4 °C. Then the resin was collected and incubated with 0.4 pmol of human recombinant PARP1 (Alexis) and/or 0.2 pmol of human recombinant DNMT1 (New England Biolabs) in 300 μl of native purification buffer for 3 h on a rotative shaker at 4 °C. After washing the resin with native purification buffer, proteins were eluted from the beads by boiling in SDS/PAGE sample buffer. Protein–protein interactions were analysed by SDS/PAGE (6 % gel) and Western blot analysis. The Abs employed were: rabbit polyclonal anti-Ctcf (Upstate Laboratories), goat polyclonal anti-Ctcf (Santa Cruz Biotechnology), mouse monoclonal anti-Dnmt1 (Imgenex) and mouse monoclonal anti-Parp1 (C2-10, Alexis Biochemicals).

Polymer binding assay: polymer blot

Recombinant proteins (10–0.5 pmol) were dotted on to nitrocellulose membranes (Hybond ECL Amersham Pharmacia Biotech). The blots were treated as described previously [17]. Briefly, the blots were incubated in 5 ml of TBST {TBS [Tris-buffered saline; 10 mM Tris and 0.15 M NaCl (pH 7.4)] plus 0.05 % Tween 20} containing either PARylated Parp1 or ADP-ribose polymers with/without dsDNA (double-stranded DNA) of salmon sperm as competitor, at a PAR:dsDNA ratio of 1:25 (w/w). After incubation for 1 h at room temperature (21 °C), the membranes were extensively washed with TBST and subjected to immunostaining using mouse monoclonal anti-PAR Ab (10 HA, Trevigen) and goat anti-mouse horseradish peroxidase-conjugated Abs (Santa Cruz Biotechnology). PARylated Parp1 and free PARs were obtained as described previously [17,18]. Recombinant H2B (histone 2B) and H1 (histone 1) (Sigma–Aldrich) were used as positive controls for PARs binding, with DNase I (Roche Applied Science) as the negative control.

ChIP (chromatin immunoprecipitation) and RE-ChIP (sequential ChIP)

ChIP was performed as described previously [14] with the following modifications. Starting sample consisted of 2×10^6 cross-linked cells. To obtain fragmented cross-linked chromatin with an average fragment length of 750 bp, a sonication step was performed consisting of about 30 pulses of 10 s each (with 10 s rest between each) at 50 % power in an ice bath (Labsonic M sonifier, Braun Biotech international). Each washing step of ChIP complexes was performed twice.

In RE-ChIP assays the primary ChIP (for Ctcf) was performed accordingly to the standard protocol starting from 2×10^6 cells for each IP. The ChIP complexes elution step was performed in 100 μl of TE buffer [10 mM Tris/HCl and 1 mM EDTA (pH 8)] containing 20 mM DTT by incubating the samples at 37 °C for 30 min. DTT was then removed using Microcon YM-10 filters (Millipore) and the ChIP complexes were eluted in 100 μl of 1 % SDS, 10 mM EDTA and 50 mM Tris/HCl (pH 8.0), supplemented with protease inhibitors (complete EDTA-free, Roche Applied Science). Samples were then diluted by adding 900 μl of IP dilution buffer [0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris/HCl (pH 8.0), 167 mM NaCl, 5 mg/ml BSA and protease inhibitors). An aliquot (10 %) of the sample was taken as the input. Secondary IPs (for Parp1, Dnmt1 and PAR) were then performed according to the standard ChIP protocol by adding the second Abs. Control secondary IPs with purified normal rabbit total IgGs (Santa Cruz Biotechnology) were also performed. Immunoprecipitated DNA was resuspended in 50 μl of water and real-time PCR amplifications were performed from 12 μl of the purified DNA. Linearity of PCR amplifications was checked by making standard curves generated using 1:1 serial dilutions (from 100 to 12.5 %) of the input. To exclude the presence of primer dimers or PCR products from misannealed primers in the amplifications, a melting curve was performed after each PCR run. PCR reactions in the absence of template DNA were performed as a negative control. The Abs used for IPs were: mouse monoclonal anti-PARs (10 HA, Trevigen), rabbit polyclonal anti-Parp1 (Alexis), rabbit polyclonal anti-Ctcf (Upstate Laboratories), rabbit polyclonal anti-Dnmt1 (New England Biolabs) and normal rabbit IgGs (Santa Cruz Biotechnology). Primers used for the amplification are listed in the Supplementary Materials and methods section (at http://www.BiochemJ.org/bj/441/bj4410645add.htm).

PARG overexpression

Transfection experiments were performed as described previously [14]. For the ChIP assays 0.7×10^6 cells were seeded in 100×15 mm culture dishes and transfected with Lipofectamine™ Plus Reagent (Invitrogen) using the manufacturer's protocol. The assays were performed with 11 μg/dish of purified plasmid DNA of either empty myc-vector (pCS2) as the control or a myc–PARG construct (pCS2–Myc–PARG) together with 1.1 μg/dish of pBabe-puro vector (Addgene) for puromycin selection of transfected cells. After 24 h the cells were incubated for a further 24 or 72 h in culture medium supplemented with puromycin (2 μg/ml; Calbiochem). Western blot assays of nuclear cell lysates were run to prove transfection of myc-PARG. The Abs used were: mouse monoclonal anti-Myc (9E10 clone, hybridoma-conditioned

medium), mouse monoclonal anti-PAR (10 HA, Trevigen) and rabbit polyclonal anti-Sp1 (H-225, Santa Cruz Biotechnology).

Genomic bisulfite sequencing

Genomic bisulfite sequencing analysis was performed as described previously [14]. DMR1 amplicon was obtained by nested PCR with the second PCR performed on 5 μl of the initial amplification using the condition described by Lopes et al. [30]. The amplified DNAs were cloned into the TOPO TA-Cloning Vector (pCR 2.1-TOPO kit, Invitrogen). Forty independent bacterial clones for each sample were cultured in selective LB (Luria–Bertani) medium and the corresponding recombinant plasmids were extracted (PureLink Quick Plasmid Miniprep Kit, Invitrogen) and sequenced. Sequencing was performed by the Value Read sequencing service (Eurofins MWG Operon). The primers and annealing temperatures are indicated in the Supplementary Materials and methods section.

ChIP-CHOP assay

For ChIP-CHOP analysis of the DMR1 b and c fragments, the Ctcf-, Parp1- and Dnmt-pulled down DNA (20 μl), and input DNA (20 μl after 1:100 and 1:200 dilutions) from ChIP assays were digested for 1 h at 37 °C with 10 units of either the methylation-sensitive HpaII restriction enzyme or its methylation-insensitive isoschizomer MspI (New England Biolabs). Digestion was followed by heat inactivation. As a control, the IgG ChIP fractions were also restricted. As an uncut control, ChIP DNA fractions were prepared for HpaII digestion and immediately heat inactivated to prevent restriction. Samples (2.5 μl) were than subjected to PCR analysis with the same primers used in ChIP assays. Amplification was performed using the Platinum PCR SuperMix (Invitrogen) with 0.5 μM of the primers. PCR was run for 40 (for input samples) or 45 (for ChIP DNA fractions) cycles adopting the thermal conditions used for ChIP assays.

RESULTS

Dnmt1 is a new partner of Ctcf and is present in the Ctcf-PARylated Parp1–Dnmt1 complex

The association between Parp1 and Ctcf, as well as the interaction between Parp1 and Dnmt1, were reported previously [17,18,31,32]. In the present paper we show by co-IP assay that Ctcf, Dnmt1 and Parp1 form a complex in L929 mouse fibroblasts nuclei (Figure 1a). Parp1 has been shown to interact both with Ctcf and Dnmt1. To test the possibility that interactions in the complex are mediated by Parp1, Ctcf–Dnmt1 co-IP assays were performed on Parp1 knockout mouse fibroblasts (Parp1$^{-/-}$ A1 cells). The results, shown in Figure 1(b), indicate that Ctcf and Dnmt1 associate with each other even in the absence of Parp1, thus excluding Parp1 as a mediator of interaction. Such a conclusion was further corroborated by *in vitro* binding assays between recombinant proteins. In fact, such experiments showed that Ctcf directly binds both Parp1 and Dnmt1 and that the ability of Ctcf to interact with Dnmt1 is not influenced by the presence of Parp1 and *vice versa* that Parp1–Dnmt1 interaction occurs independently of Ctcf (Figure 1c). Thus Dnmt1 adds to the list of Ctcf protein partners [32,33], a finding that has not been reported so far. Comparing the amount of each protein present in the nuclear extract with the amount found in the co-IP, it appears that most of cellular Dnmt1 is associated with Ctcf whereas only a fraction of total Parp1 is engaged in such a complex (Figures 1a and 1b).

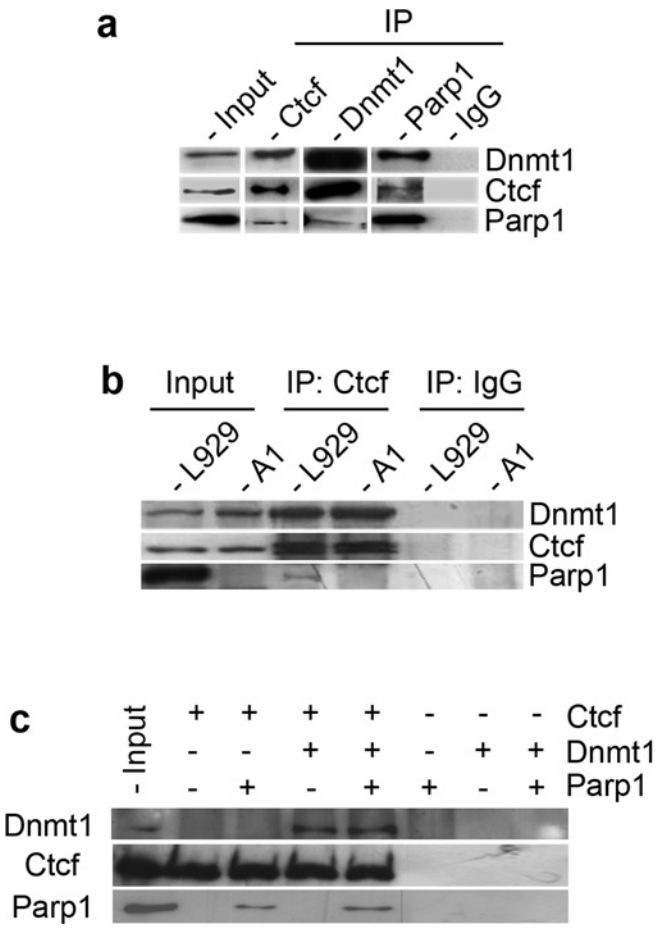

Figure 1 Analysis of Ctcf interaction with Parp1 and Dnmt1

(**a**) Reciprocal co-IP of Ctcf, Parp1 and Dnmt1 in nuclear lysates. Non-specific rabbit IgGs were used as negative control. (**b**) Immunoprecipitation of Ctcf in nuclear lysates from L929 (Parp1 proficient) and A1 (Parp1$^{-/-}$) cells. (**c**) Pull-down assay with recombinant brCtcf (His-tagged), Parp1 and Dnmt1 proteins.

PAR interaction with Ctcf

The capability of the three proteins to be covalently and/or non-covalently modified by PARylation was addressed by IP assays performed using specific Abs against PARs (Figure 2a). The results indicate that both Ctcf and Dnmt1 interact with polymers or, at least, that they are complexed with other PARylated proteins. Notably, the upward smear characterizing Parp1 immunostaining (Figure 2a, middle panel) indicates that the auto-modified Parp1 (PARylated Parp1) can be immunoprecipitated by anti-PAR Abs.

Ctcf was reported to be covalently PARylated *in vivo* [29,31,34]. In the present paper we show that the 130 kDa Ctcf co-purifies with PARs (Figure 2a). Although this isoform could be covalently modified by oligo(ADP-ribose) polymers [31], the presence of a putative consensus sequence for non-covalent PAR binding on the Ctcf protein [11] suggested that non-covalent interactions might occur as well. In fact, we found by *in vitro* polymer-binding assays that Ctcf does indeed interact both with PARylated Parp1 and free PARs (Figure 2b and Supplementary Figure S1 at http://www.BiochemJ.org/bj/441/bj4410645add.htm). It is noteworthy that the affinity of PARs for Ctcf is strikingly higher than that of H2B taken as positive control [35] and that the interaction persists even in the presence of competitor DNA [PARs:DNA ratio of 1:25 (w/w)]; the latter

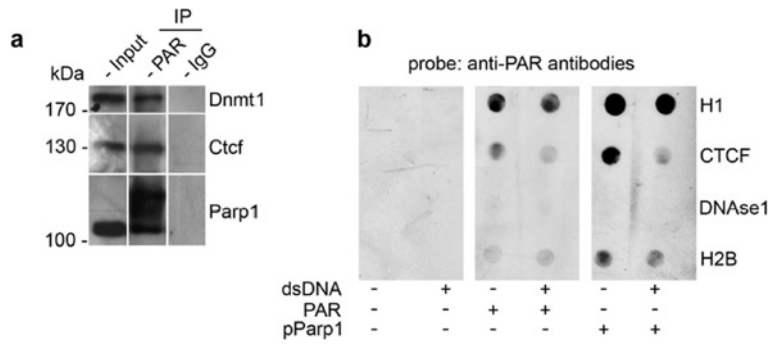

Figure 2 Analysis of Ctcf interaction with PARs

(**a**) Immunoprecipitation of PARs in nuclear lysates. Molecular mass is given in kDa on the left-hand side. (**b**) *In vitro* PAR binding assay with recombinant brCtcf. Blots were incubated with either *in vitro* PARylated Parp1 (pParp1) or free PARs with or without competitor dsDNA. Non-covalent association between PARs and proteins was detected by anti-PAR Abs. Recombinant H2B and H1 were used as positive control for PAR binding, whereas DNAse1 served as the negative control.

was used to rule out non-specific interactions due to the highly negatively charged nature of PARs.

Co-localization of PARs, Parp1 and Dnmt1 with Ctcf on to Ctcf DNA target sequences

We then asked whether the complex associates with well-known DNA target sequences for Ctcf. By ChIP analysis we checked the differentially methylated region located upstream of the *Igf2* promoter 1 (DMR1) (Figure 3a) for occupancy by Ctcf, Parp1, Dnmt1 and PARs.

Figure 3(B) clearly shows that Ctcf-specific DNA targets within the DMR1 core region (fragments b and c) [23] are also specifically occupied by Parp1 with its catalytic product (PARs) and by Dnmt1. These fragments are significantly enriched in the ChIP fractions if compared to the *Actb* promoter taken as negative control.

The simultaneous presence of Parp1, PARs and Dnmt1 with Ctcf on DMR1 region was further confirmed by RE-ChIP. A first round ChIP, in which anti-Ctcf Abs were used, was followed by RE-ChIP with anti-Parp1, anti-PARs or anti-Dnmt1 Abs. The results show the presence of a Ctcf–Parp1–PARs–Dnmt1 complex at the Ctcf consensus regions. No signal was detected in the controls when anti-IgG Abs were used in the second round of ChIP analysis (Figure 3c).

Depletion of PARs delocalizes the Ctcf–Parp1 complex from Ctcf target sites

Depletion of PARs was obtained by overexpression of PARG. As reported previously [14], the semistable overexpression of PARG in L929 mouse fibroblasts led to a sharp decrease of endogenous PARs (Figure 4a).

Co-IP carried out following PARG overexpression shows that the Ctcf–Parp1–Dnmt1 complex is maintained in the nucleus (Figure 4b). This indicates that PARs are not required for the complex stability. We then asked if the presence of the complex at the Ctcf DNA target sites was compromised after the depletion of PARs. ChIP experiments show that DMR1 b and c fragments co-purifying with PARs were notably reduced after PARG overexpression with the associated loss of DNA binding of both Ctcf and Parp1. By contrast, the overexpression of PARG did not affect significantly the localization of Dnmt1 (Figure 4c).

Depletion of PARs leads to *de novo* methylation of Ctcf-bound CpGs

We have shown previously that inhibition of PAR synthesis leads to *de novo* introduction of methyl groups on to DNA [14,36–38]. These findings, and the well-known property of Ctcf to bind selectively to unmethylated target sites, prompted us to test the hypothesis that the displacement of Ctcf–Parp1 complex from the DMR1 was associated with changes in the methylation pattern of its DNA targets in PARs-depleted L929 cells.

We determined by bisulfite sequencing the methylation status of CpGs within the DMR1 regions encompassing the Ctcf target sequences assayed by ChIP experiments upon PARG overexpression as shown in Figure 5.

Of great interest was the highly evident increase of methylation detectable after PARG overexpression. In particular, CpGs number 4, 5 and 6 underwent significant *de novo* methylation with CpG 5 and 6 reaching full methylation. Notably, these CpGs are the ones resident in the regions involved in the binding with the Ctcf-PARylated Parp1–Dnmt1 complex, as shown by the ChIP assays. This suggests that the loss of Ctcf binding upon PARG overexpression is associated with DNA hypermethylation of Ctcf target sequences.

The Ctcf-PARylated Parp1–Dnmt1 complex binds to unmethylated DNA target sequences

Partial methylation of the Ctcf-binding targets within the DMR1 raised the question of whether the complex was preferentially associated with the unmethylated DNA targets. To address this issue, a methyl-sensitive ChIP-CHOP assay was performed for the DMR1 b and c regions. DNA pulled-down with anti-Ctcf, -Parp1 and -Dnmt1 Abs was digested with the isoschizomer endonucleases HpaII and MspI. Both enzymes digest the sequence CCGG containing CpG number 5 and 6 present in DMR1 b and c respectively (see Figure 5), but HpaII is blocked by the methylation of the CpG cytosine. The input DNA was digested in parallel. The proportion of HpaII-sensitive DNA, present in the input DNA and in the immunoprecipitated material, was evaluated by end-point PCR (Figure 6). As expected, MspI digestion destroyed the entire pool of DMR1 sequences, both in the input samples and in the fractions of Ctcf, Parp1 and Dnmt1 ChIPs. Under the same conditions, HpaII digestion only partially digested input DNA, which consists of both the methylated and unmethylated DNA molecules. By contrast HpaII digestion of ChIP fractions is undistinguishable from MspI digested DNA. This indicates that in cells where the PARP activity was not

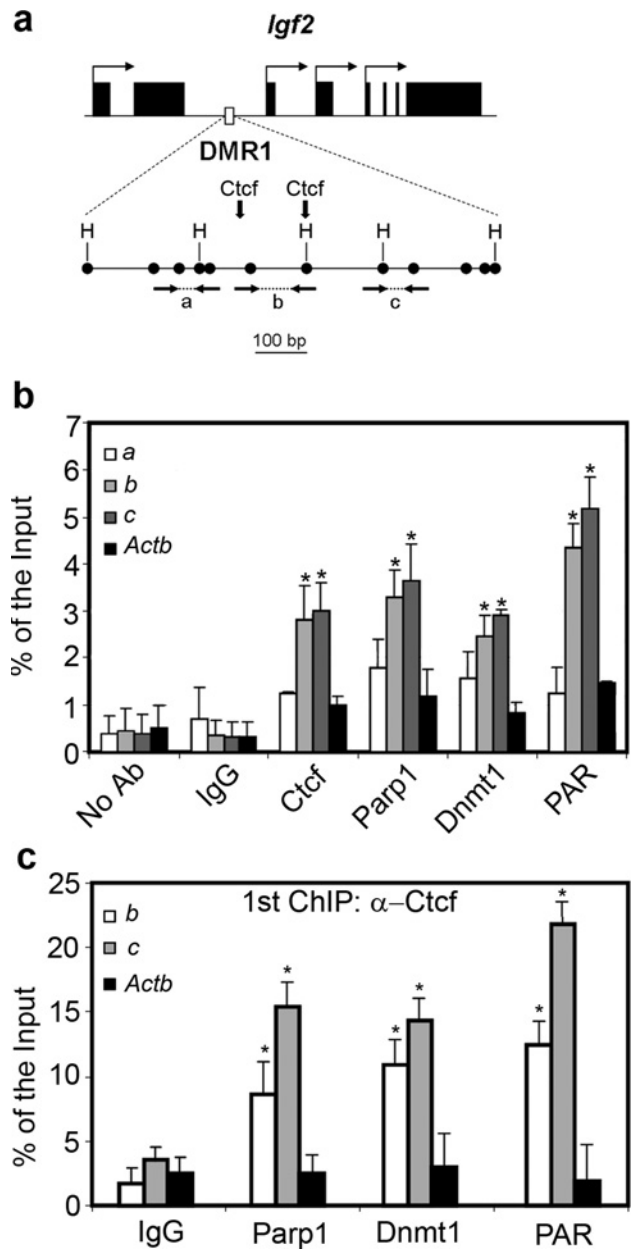

Figure 3 ChIP analysis of DMR1 occupancy by Ctcf, Parp1, PARs and Dnmt1

(**A**) Schematic map of the locus. The DMR1 region is expanded to show the approximate position of putative Ctcf-binding sites and of PCR primers used to detect the presence of specific DNA sequences in ChIP complexes. Circles represent CpG dinuclotides. H, HpaII sites. (**B**) ChIP analysis of DMR1 region carried out with anti-Ctcf, anti-Parp1, anti-Dnmt1 and anti-PAR Abs. Beads alone (No Ab) and anti-IgG Abs were used as negative controls. Real-time PCR data are expressed as percentage of the signal detected for the non-immunoprecipitated input (4 % of the chromatin subjected to immunoprecipitation) taken as 100 %. The *Actb* promoter served as a control. (**C**) Standard ChIP assay for Ctcf was followed by RE-ChIP to assess the co-occupancy of Ctcf, with Parp1, PARs and Dnmt1 at the Ctcf DNA target sites within the DMR1. Real-time PCR was performed for the DMR1 b and c fragments. The efficiency of RE-ChIP at each of the sites was calculated as a percentage of the chromatin input that co-purified with Ctcf in the first ChIP (10 % of the Ctcf ChIP fraction). Results are means ± S.E.M. calculated from three experiments. $^{*}P < 0.05$ compared with IgG controls (Student's *t* test).

inhibited, Ctcf, Parp1 and Dnmt1 are predominantly bound to DMR1 DNA fragments containing the unmethylated HpaII sites (Figure 6).

DISCUSSION

Ctcf is a Zn-finger transcriptional factor involved in several important biological roles [39], which range from the control of imprinting to the onset of X chromosome inactivation [40–44]. Genome-wide studies have provided evidence that Ctcf, for which thousands of binding sites scattered throughout the human and mouse genomes have been found [45–47], also plays an important role in the organization of chromatin architecture through formation of intra-chromosomal loops or inter-chromosomal bridges [48]. Ctcf uses these long-range interactions to mediate gene expression/repression. Ctcf is considered an insulator protein for its ability to bind insulator sequences which, according to the way they regulate gene expression, are termed enhancer blocking insulators or barrier insulators [40].

PARP activity has been shown previously to be essential for mediating both enhancer blocking and barrier functions of Ctcf [15,29,49]. In particular, Yu et al. [29] showed that the repressed status of the maternal *Igf2* allele was lost in cells treated with 3-aminobenzamide, a general inhibitor of PAR polymerases. On the other hand, it has been shown that Ctcf and Parp1 co-localize in the vicinity of a DNA boundary region located 2 kbp upstream of the *p16* gene transcriptional start site preventing the spreading of heterochromatin on to the promoter region. Interestingly, in cells treated with 3-aminobenzamide, the promoter becomes repressed. This indicates that PARP activity drives the boundary activity of Ctcf at this locus. Furthermore, loss of Ctcf binding and hypermethylation of *p16* promoter CGI are associated in cancer cells where *p16* expression is silenced [15]. These results, together with the fact that Parp1 localizes on Dnmt1 promoter protecting its unmethylated state and expression by its activity [14], suggest a role for PARylation in maintaining some regulatory CpG-rich sequences in the unmethylated state.

The mechanism(s) by which Ctcf prevents the methylation of specific CpG-rich DNA regions is unknown. However, we demonstrated that Ctcf can bind Parp1 inducing its automodification and that PARylated Parp1, in turn, inhibits Dnmt1 activity through the polymers [17,18]. This evidence prompted us to speculate that such a mechanism may underlie the ability of Ctcf to maintain the proper methylation pattern of its target binding sites.

At first, we searched for physical interaction between Parp1, PARs, Dnmt1 and Ctcf. Co-IP and pull-down experiments demonstrate a direct interaction between Ctcf, Dnmt1 and Parp1, and provide information about the existence *in vivo* of a complex between Ctcf-PARylated Parp1 and Dnmt1. Data of co-IP on *parp1*$^{-/-}$ cells together with pull-down assays show that PARs are not required for the complex formation between the three proteins and that the interaction between Ctcf and Dnmt1 occurs independently of the presence of Parp1 in the complex. Nonetheless, PARs, the catalytic product of PARP activity, take part in the complex as shown by IP with anti-PAR Abs. In the present paper we show that the 130 kDa Ctcf, co-purifies with PARs. Although the130 kDa Ctcf was shown to be covalently modified by oligo(ADP-ribose) polymers [31], the presence of a putative consensus sequence for non-covalent PAR binding on the Ctcf protein [11] suggested that a non-covalent interaction of Ctcf with PARs might also occur. By polymer-blot analysis, the present study demonstrates, for the first time, that Ctcf, besides being a covalent acceptor of PARs [29,31], can also bind non-covalently with PARs either free or Parp1-bound.

On the basis of the evidence of Ctcf interaction with Parp1, PARs and Dnmt1, we determined whether these proteins and PARs coexist at specific Ctcf DNA targets. The results of the present study that DMR1, located upstream the *Igf2* promoter

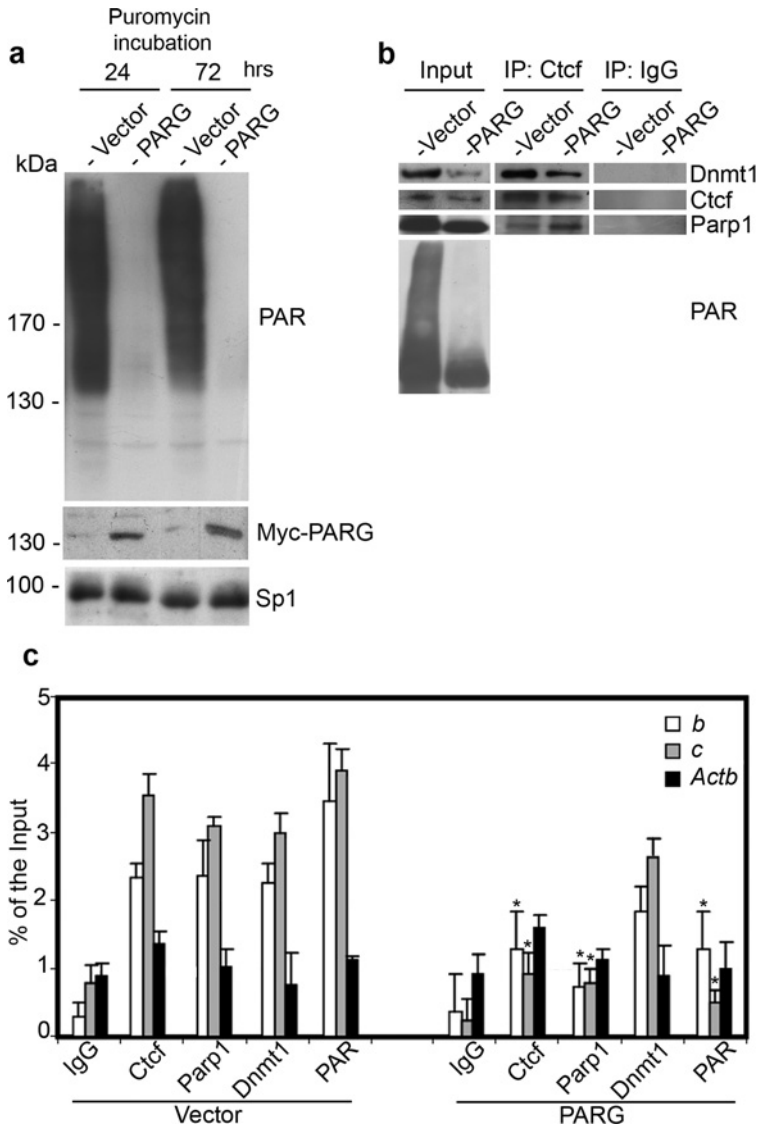

Figure 4 Analysis of Ctcf–Parp1–Dnmt1 interactions and DMR1 region occupancy following PARG overexpression

(**A**) Western blot analysis of nuclear lysates from L929 cells overexpressing PARG after 24 and 72 h of puromycin selection. Analyses were performed by anti-PARs, anti-Myc Abs for exogenous PARG and anti-Sp1 Ab as a control. (**B**) Immunoprecipitation of Ctcf in nuclear lysates from L929 cells overexpressing PARG 48 h after transfection. (**C**) ChIP analysis was carried out with anti-Ctcf, anti-Parp1, anti-Dnmt1 and anti-PAR Abs in L929 cells overexpressing PARG compared with controls after 72 h of puromycin selection. Real-time PCR data are expressed as the percentage of the signal detected for the non-immunoprecipitated input (4 % of the chromatin subjected to immunoprecipitation) taken as 100 %. Results are means ± S.E.M. for three experiments. The *Actb* promoter was used as negative control. $*P < 0.05$ compared with controls (Student's *t* test). Vector, L929 cells transfected with pCS2 empty vector; PARG, cells transfected with pCS2–Myc–PARG vector containing full-length cDNA for human PARG.

1, required binding to Ctcf to maintain its methylation pattern [23]. ChIP and RE-ChIP analyses of DMR1 sequence support the proposition of co-occupancy by Ctcf, Parp1, PARs and Dnmt1. These findings are consistent with previous reports showing co-localization of Ctcf with Parp1 at the *H19* DMR [31] and *p16* promoter [15], and colocalization of Ctcf with both Parp1 and Dnmt1 at the *p53* promoter [50]. Ectopic PARG overexpression in L929 cells allowed us to demonstrate that the Ctcf–Parp1 complex is released from Ctcf target sites in cells depleted of PARs. In addition, the evidence that protein–protein interactions between Ctcf, Parp1 and Dnmt1 are maintained in the nucleus of cells depleted of PARs indicates that PARs are not required for the complex stabilization, whereas they are necessary for the localization of the complex at Ctcf target sites. All this suggests that what is regulated by PARP activity is the ability of Ctcf to bind its DNA target sites.

Since it is well known that the ability of Ctcf to bind its target sites is methylation sensitive and that the inhibition of PARP activity leads to CpG methylation, as a follow-up step we analysed the DMR1 methylation pattern in PAR depleted and control L929 cells. It is noteworthy that the hypermethylation detected following PAR depletion in DMR1 occurs only on those Ctcf consensus regions on which the Ctcf–Parp1–PARs and Dnmt1 complex is located. By contrast, the methylation level of other CpGs, residing in DNA regions refractory to Ctcf binding in ChIP assays (i.e. those contained in fragment a of DMR1), remains unchanged. Preferential occupancy by Dnmt1 within Ctcf-positive target sites supports the intriguing hypothesis of an instructive *de novo*

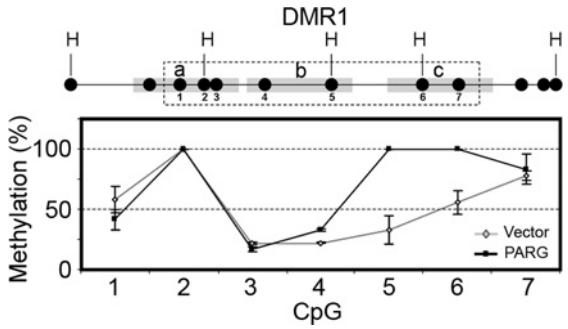

Figure 5 Analysis of DNA methylation of DMR1 following PARG overexpression

The methylation *status* of the DMR1 core region was evaluated by bisulfite sequencing in L929 cells overexpressing PARG compared with control cells after 72 h of puromycin selection. Results are the mean frequency of methylation ± S.E.M. found for each CpG from three independent PARG overexpression experiments. Vector, L929 cells transfected with pCS2 empty vector; PARG, cells transfected with pCS2–Myc–PARG vector containing full-length cDNA for human PARG. The open box in the map represents the approximate extent of the sequenced region (H, HpaII sites). Grey boxes indicate the position of fragments assayed in ChIP experiments.

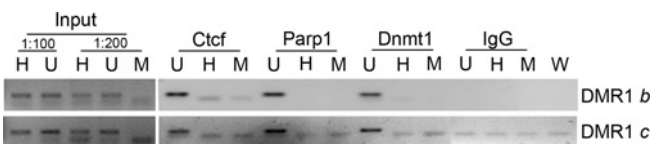

Figure 6 Ctcf, Parp1, and Dnmt1 bind to non-methylated DNA molecules within the DMR1

Ctcf, Parp1, and Dnmt1 ChIPs were performed for the DMR1 b and c fragments. End-point PCR was performed after digestion of ChIP fractions DNA and inputs with either HpaII (H, methylation sensitive) or MspI (M, methylation insensitive) following heat inactivation of the restriction enzymes. The input (4 % of the chromatin subjected to immunoprecipitation) was diluted 1/100 or 1/200 before restriction. The uncut (U) fractions consisted in HpaII digestions preventively blocked by heat inactivation. W, PCR performed in the absence of added template.

methylation that may occur when PAR synthesis is antagonized and affect the association of Ctcf with its target sequences; the consequence of such events is the destabilization of the complex in Ctcf target regions. The evidence that DMR1 CpG 5 is one of the target sites responsible for the binding of methyl-sensitive Ctcf [23], gives an intriguing rationale to the loss of binding of the complex that we detected in these regions upon overexpression of PARG. This possibility is strengthened by the finding that the binding of Ctcf, Parp1, Dnmt1 and PARs within the DMR1 is selective towards the DNA molecules containing unmethylated CpGs, as indicated by ChIP-CHOP assays.

Globally, our results support a model envisaged in previous reports [11] which proposed a mechanism of epigenetic control of Ctcf boundary function based on PARylation. In this model, Ctcf harbouring PARP activity at its DNA target sites prevents *de novo* methylation of CpG dinucleotides by inhibiting Dnmt1 activity. Thus defective PARP activity would cause hypermethylation of Ctcf DNA targets through de-repression of Dnmt1.

A future aim is be to establish if PARylated Parp1, by itself and/or following PARylation of Ctcf and/or other transcription factors, may introduce an epigenetic code on chromatin, by marking those DNA sequences that must be maintained in an unmethylated state in normal cells and preventing Dnmt1 access to these sequences.

Consistent with this, new interesting and emerging additional findings are: (i) the identification of Dnmt1 among the protein partners of Ctcf; (ii) the non-covalent binding between PARs and Ctcf; (iii) the presence of Ctcf–Parp1–Dnmt1 complex, whose stability is independent of PARP activity in the nucleus. The complexity of the scenario where the players are Ctcf, Parp1 and Dnmt1 goes in parallel with the complexity of Ctcf, which is defined as multifunctional for the polyhedric roles in which it is involved and for which thousands of target sites are present in the genome [45–47].

AUTHOR CONTRIBUTION

Michele Zampieri carried out ChIP analysis, drafted the manuscript and participated in the design of the study. Tiziana Guastafierro carried out the IP, polymer blot and pull-down assays, drafted the manuscript and participated in the design of the study. Roberta Calabrese, Fabio Ciccarone and Maria G. Bacalini performed DNA methylation analyses. Anna Reale and Mariagrazia Perilli drafted the manuscript and participated in the design of the study. Claudio Passananti produced overexpression vectors. Paola Caiafa conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was partially financed by Ministero della Salute (to P.C.) and by Ministero dell'Istruzione, dell'Università e della Ricerca (to P.C. and M.P.).

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Received 2 August 2011/29 September 2011; accepted 11 October 2011
Published as BJ Immediate Publication 11 October 2011, doi:10.1042/BJ20111417

Biochem. J. (2012) **441**, 645–652 (Printed in Great Britain) doi:10.1042/BJ20111417

online data

SUPPLEMENTARY ONLINE DATA

ADP-ribose polymers localized on Ctcf–Parp1–Dnmt1 complex prevent methylation of Ctcf target sites

Michele ZAMPIERI*†[1], Tiziana GUASTAFIERRO*†[1], Roberta CALABRESE*†, Fabio CICCARONE*†, Maria G. BACALINI*†, Anna REALE*†, Mariagrazia PERILLI‡, Claudio PASSANANTI§ and Paola CAIAFA*†[2]

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Materials and Methods

ChIP and RE-ChIP

The following primers were used for the amplification in ChIP and RE-ChIP assays: DMR1a, sense 5′-TGGCTCTTCAATGGACACCTT -3′ and antisense 5′-TCTCTGGAGAAGCCGCTGA -3′; DMR1b, sense 5′-TCCAGAATCGGGACTCTGTT -3′ and antisense 5′-CCTCTGCTAAGGGTCTCCTTT-3′; DMR1c, sense 5′-TGGAATGAGGAACATCACCA-3′ and antisense 5′-TCTATCCCTGGCTTTTCTGG-3′; and *Actb*, sense 5′-TTGGCTCCGCGTCGCTCACTCAC-3′ and antisense 5′-CCCCAGAATGCAGGCCTAGTAACCGAGAC-3′.

Genomic bisulfite sequencing

The following primers and annealing temperatures were used for the amplifications in the genomic bisulfite sequencing experiments: DMR1, first sense 5′-GGTTAGGTGAAGGTTTTGTGGGTAGTTATA-3′ (annealing temperature = 50 °C) and antisense 5′- ATATTCCCCTTTCAAATTCCAATCTACATC-3′; and DMR1, second sense 5′-GGTGGTTTTTTAATGGATATTTTAAGGTGA-3′ and antisense 5′-CCAACCTCTATCCCTAACTTTTCTAACCTC-3′ (annealing temperature = 52 °C).

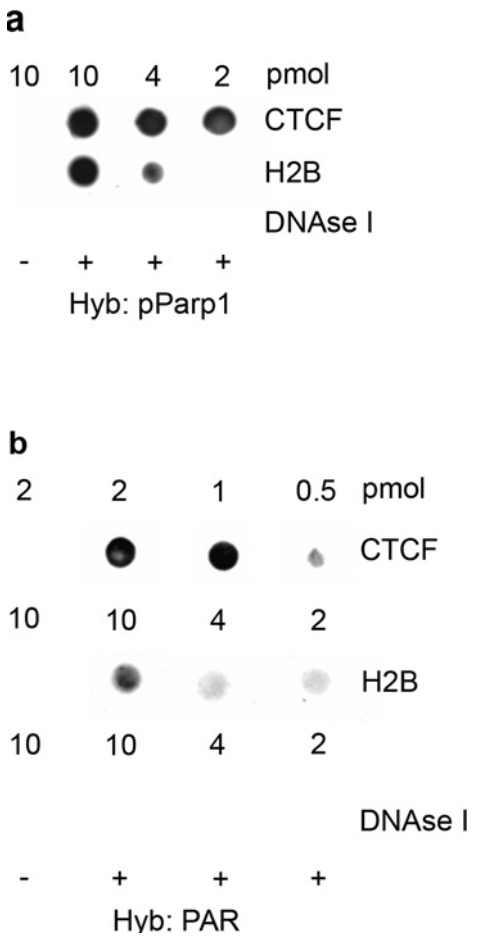

Figure S1 Analysis of Ctcf interaction with PARs

PAR-binding assays performed on different amounts of recombinant Ctcf protein. Blots were incubated with either *in vitro* PARylated Parp1 (pParp1) (**a**) or free polymers (**b**). Non-covalent association between PARs and proteins was detected by anti-PAR Abs. Equimolar amounts of recombinant H2B and DNaseI were also assayed as positive and negative controls respectively.

Received 2 August 2011/29 September 2011; accepted 11 October 2011
Published as BJ Immediate Publication 11 October 2011, doi:10.1042/BJ20111417

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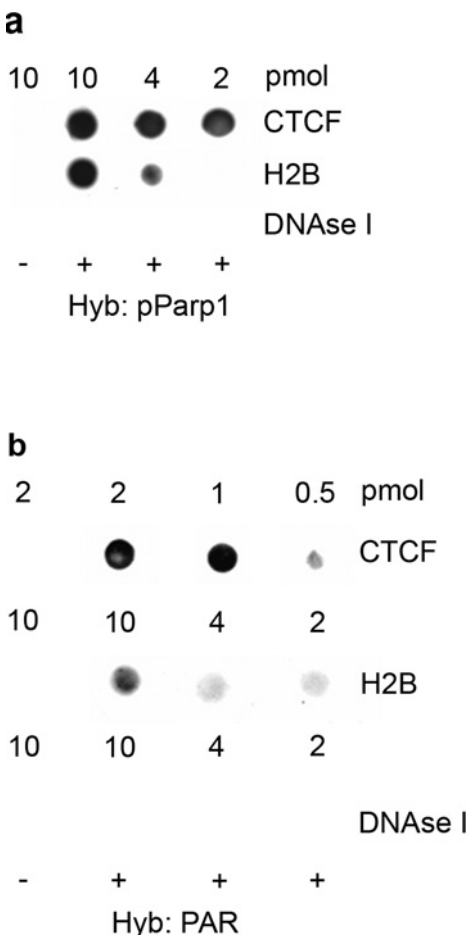

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