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Inhibition of poly(ADP-ribosyl)ation induces DNA hypermethylation: a possible molecular mechanism

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ABSTRACT

The pattern of DNA methylation established during embryonic development is necessary for the control of gene expression and is preserved during the replicative process. DNA regions of about 1–2 kb in size, termed CpG islands and located mostly in the promoter regions of housekeeping genes, are protected from methylation, despite being about 6–10 times richer in the dinucleotide CpG than the rest of DNA. Their unmethylated state guarantees the expression of the corresponding housekeeping genes. At present, the mechanism by which CpG islands remain protected from methylation is not clear. However, some results suggest that poly(ADP-ribosyl)ation, an enzymatic process that introduces a postsynthetic modification onto chromatin proteins, might be involved. Here we show in L929 mouse fibroblast cells that inhibition of poly(ADP-ribose) polymerase(s) at different cell-cycle phases increases the mRNA and protein levels of the major maintenance DNA methyltransferase (DNMT1) in G1/S border. Increase of DNMT1 results in a premature PCNA-DNMT1 complex formation, which facilitates robust maintenance, as well as *de novo* DNA methylation processes during the G1/S border, which leads to abnormal hypermethylation.

Key words: DNA methylation • DNMT1-PCNA complex • poly(ADP-ribosyl)ation • oncosuppressor gene hypermethylation

DNA methylation is a specific postsynthetic modification of DNA that, in eukaryotic cells, appears to play an important role in the epigenetic modulation of gene expression (1). It is the major enzymatic DNA modification that results from the transfer of methyl groups from S-adenosyl methionine (S-AdoMet) to cytosine (C) with formation of 5-methylcytosine (5mC) (2). Although in vertebrates the presence of 5mC has occasionally been found in dinucleotide sequences CpC, CpA, and CpT (3–5). the best substrate for DNA methyltransferase is cytosine located in the CpG dinucleotide (6). About 80% of CpGs are methylated in human somatic cells, and these are distributed in a non-random fashion in genomic DNA (7), where methylated cytosines are present in bulk DNA while the unmethylated ones are located mainly

within particular DNA regions, termed CpG islands (8–10). The specific DNA methylation pattern results from the combination of maintenance methylation, *de novo* methylation, and demethylation processes (11–17) and is defined during the early stages of embryonic development (18).

As for the correlation between DNA methylation and gene expression, CpG islands—which range from 500 to 2000 base pairs in size—are usually found in the 5' promoter regions of the housekeeping genes (9) and overlap the genes to variable extents (19). There is evidence that transcription of many genes is inhibited when the CpG islands in their promoters are methylated (20, 21). CpG islands are also in tissue-specific and imprinted genes within the genes themselves; however, for at least some of these, methylation does not block transcription (19).

Although CpG frequency in the islands is 6–10 times higher than in bulk DNA (22), CpG dinucleotides are present in an unmethylated state in spite of *in vitro* experiments (23), showing that CpG islands are not by themselves unmethylable. A great deal of investigation has been and is being performed to clarify the mechanism by which CpG islands are protected from methylation during replication and why they remain untouched by the action of DNA methyltransferase in chromatin in spite of their localization in promoter region of housekeeping genes that are, by virtue of being present in decondensed structure, permanently accessible to transcription factors. Thus, an open question to clarify is how the CpG moieties in CpG islands become vulnerable or resistant to the action of DNA methyltransferase, and can thus lose or maintain their characteristic pattern of methylation (16).

Our previous data show that, following inhibition of poly(ADP-ribose) polymerases (PARPs), which bind ADP-ribose polymers in covalent and non-covalent way on chromatin proteins (24–27), DNMTs become capable of methylating cytosines on DNA. A block of poly(ADP-ribosyl)ation introduces in fact an anomalous hypermethylated pattern onto genomic DNA (28–30), and further experiments show that this inhibition also changes the methylation pattern of a plasmid transfected in its unmethylated form (31). As for the CpG islands, inhibition of PARPs leads to disappearance of “HpaII tiny fragments” after digestion of genomic DNA with methylation-dependent HpaII restriction enzyme and, at least for the *Htf9* promoter region, it allows the new methyl groups to position themselves on DNA (32).

This finding is of great interest, considering that an anomalous DNA methylation of CpG islands induces the silencing of correlated genes and that the mechanism involved in protecting these DNA regions against methylation is still unknown.

Thus, at a time when researchers’ attention is focused on deacetylation as the posttranslational modification tightly associated with DNA methylation (33, 34), other posttranslational modifications must be considered, including those catalyzed by PARPs (24–27).

The data reported here provide clues to understanding the molecular mechanism(s) connecting poly(ADP-ribosyl)ation with DNA methylation, giving a possible explanation as to how the inhibition of poly(ADP-ribosyl)ation leads to *in vivo* DNA hypermethylation. The suggested molecular mechanism is based on the fact that a) inhibition of poly(ADP-ribose) polymerase(s) at G1/S border increases the mRNA and protein levels of the major maintenance DNA

methyltransferase (DNMT1) and that b) this increase of DNMT1 results in a premature PCNA-DNMT1 complex formation, which facilitates robust maintenance as well as *de novo* DNA methylation processes during G1/S, when CpG islands are replicated (35).

MATERIALS AND METHODS

Cell cultures and synchronization techniques

L929 mouse fibroblasts cells, growing exponentially, were divided into subcultures that were treated in different ways in order to have cells synchronized at different stages of the cell cycle (36). To obtain inhibition of poly(ADP-ribosyl)ation, we treated cells with 2 mM 3-aminobenzamide (3-ABA) for different times. We used untreated cells as a control (–).

Cells were synchronized in G0 phase by starvation for 36 h in Glasgow minimal essential medium (GMEM) ,with the addition of 0.5% newborn calf serum and without glutamine. G0 (+) corresponds to cells treated with 3-ABA during the 36 h of starvation.

Cells were synchronized at the G1/S border by replacing the starvation medium with the normal growing medium for 9 h and subsequently incubating with 3 mM hydroxyurea (Fluka, Buchs, Switzerland) for 15 h. G1/S (+) and G1/S (++) correspond to cells treated with 3-ABA, respectively, for 24 h (after replacing the starvation medium) and for 60 h (during and after replacing the starvation medium).

Cells were synchronized at late S phase starting from cells synchronized in G0 phase. The starvation medium was replaced with normal medium, and cells were cultivated for another 16 h. Late S(+) and late S (++) correspond to cells treated with 3-ABA, respectively, for 22 h (6 h before replacing the starvation medium *plus* 16 h in normal medium growth) and for 52 h (during and after replacing the starvation medium). We used cytometric analysis to monitor different cell cycle phases (35).

Northern blot analysis

We performed analysis of *Dnmt*1 expression on 30 μg of total RNA purified from different samples. The blot was hybridized by using full-length cDNA for murine DNA methyltransferase (*Sma*I fragment of the pMG plasmid, T. Bestor construct). The probe was labeled by random priming by using α[^{32}P] dNTPs (3000 mCi/mole; Amersham Pharmacia Biotech, Uppsala, Sweden).

RT-PCR analysis

Total cellular RNA was quantitated by using a spectrophotometer, and 20 ng of total RNA was used (final volume of 25 μl) for reverse transcriptase polymerase chain reaction (RT-PCR) analyses. Every RT-PCR was performed by using the One-Step RT-PCR system (Life Technologies, Gaithersburg, MD), according to the following experimental scheme: 30 min at 50˚C; 2.5 min at 90˚C (experimental conditions for reverse-transcriptase reaction step); 40 cycles of 1 min at 94˚C, 50 s at the annealing temperature, which is specific for the DNA fragment

being amplified, 1 min at 70°C; and finally 6 min at 70˚C. *Dnmt*1 (accn no: X14805): annealing temperature, 62˚C; amplified fragment size, 467 bp; primers, 5'-GTGAAACGCCCAAAGAAGG-3' sense/5'-TTCCCTTTGTTCCCAGGGCT-3' antisense. *p21* (accn no: U24173): annealing temperature, 55˚C; amplified fragment size, 640 bp; primers, 5'-GTCAGAGTCTAGGGGAATTG-3' sense/5'-TAAGACACACAGAGTGAGGG-3' antisense. β-*actin* (accn no: AW212307): annealing temperature, 55˚C; amplified fragment size, 435 bp; primers, 5'GGCATAGAGGTCTTTACGG-3' sense/5'-CACAGGCATTGTATGGACTC-3' antisense. c-*fos* (accn no: 50399): annealing temperature, 52˚C, amplified fragment size, 319 bp; primers, 5'-GGTCCTTTTCTTCTATAG-3' sense/5'GTTTTTCCTTCTCTTTCAG-3' antisense.

Co-immunoprecipitation and immunoblot analysis

Co-immunoprecipitation was performed on 0.5 mg of cellular lysates from cells synchronized at the G1/S border or late S phase by using anti amino-terminal domain of DNMT1 rabbit polyclonal antibodies (New England BioLabs Inc. Beverly, MA) cross-linked to dynabeads protein A (Dynal, Oslo, Norway). Pre-immune serum was used in immunoprecipitation experiments to control for non-specific protein interactions with the antibody. SDS/PAGE and transfer of proteins to nitrocellulose membranes were performed according to standard procedures. Immunostaining of PCNA and p21 co-precipitated with DNMT1 was performed by using anti p21 (F-5) and anti PCNA (PC10) monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and detected by using ECL Chemiluminescent detection system (Amersham). The same antibodies were used to detect DNMT1, PCNA, and p21 proteins in nuclear lysates (40 μg). Sp1 protein was quantified by specific antibodies (1C6, Santa Cruz Biotechnology) in each nuclear lysate and was used as proteic internal control.

Densitometric analysis

RT-PCR amplified DNAs or proteins detected by immunoblot assay were quantified by desitometric scanning of the bands (Bio Image, Milllipore, Vimodrone, Italy).

RESULTS

In our experiments, treatment of cells with 2 mM 3-ABA was used to inhibit PARPs with the same method used in our previous published data (28–32).

Experiments were conducted at different cell-cycle phases to determine whether the poly(ADP-ribosyl)ation process modulates DNA methylation through the expression of *Dnmt*1 in a specific phase. Cytometric analysis was used to monitor different cell-cycle phases (data not shown). Northern blot (data not shown) and RT-PCR analysis show that inhibition of poly(ADP-ribose) polymerase(s) increases the expression of *Dnmt*1 (Fig. 1a). Densitometric analysis confirms that, following cell treatment with 3-ABA, the amount of *Dnmt*1 that is expressed in cells at the G1/S border, is the same as or greater than that normally expressed in S phase control cells. The influence of poly(ADP-ribosyl)ation on *Dnmt*1 gene expression seems to be gene-specific, because parallel experiments have shown that 3-ABA treatment does not influence the expression of both β-*actin* and c-*fos* and *p21* genes (Fig. 1a). Furthermore, using rabbit polyclonal antibodies against the amino terminal domain of DNMT1, we showed (Fig. 1b) that

the increased expression of the *Dnmt*1 gene is associated with a higher amount of cellular enzyme. Our results have shown that competitive inhibition of PARPs increases the mRNA (four times) and protein levels (one time) of the major maintenance DNMT1, in respect to control cells.

It has been reported that DNMT1 and p21 compete for the same binding site (37) on proliferating cell nuclear antigen (PCNA) during DNA replication (38–40). This prompted us to study the expression of the *p21* gene. Results in Figure 1a show that, in untreated control cells, the expression of *p21* gene is correlated inversely with that of *Dnmt*1 gene in G0 cell-cycle phase, as reported in literature (37).

Thus, in cells treated with 3-ABA, the inhibition of poly(ADP-ribosyl)ation increases the amount of DNMT1 relative to that of p21—whose level remains unchanged—and this shift in the intracellular equilibrium between these two proteins could favor the formation of the DNMT1-PCNA complex, as happens in transformed cells (37).

This phenomenon was investigated further by co-immunoprecipitation experiments by using anti-DNMT1 antibodies and analyzing the presence of PCNA and p21 in DNMT1 immunoprecipitates by using anti-PCNA and anti-p21 antibodies (Fig. 2).

The amount of DNMT1 present in G0 phase cells, even following treatment with 3-ABA, was too low to be detected. Densitometric analysis of immunoprecipitate from cells at the G1/S border has shown that the inhibition of PARPs increases by 2.5-fold the amount of PCNA, which co-immunoprecipitates with DNMT1, whereas the amount of immunoprecipitated p21 decreases by half (Fig. 2). These data indicate that the inhibition of poly(ADP-ribosyl)ation, by increasing *Dnmt*1 expression, favors the premature formation of PCNA-DNMT1 at the G1/S border.

PCNA clamps the DNA helix in a circular trimeric complex, where each one of the three units can bind to either DNMT1 or p21. Here we have assumed that, following treatment of cells with 3-ABA, the ratio of p21-PCNA-DNMT1 changes inside the trimer and that some p21 can still remain bound to PCNA in spite of the increased amount of bound DNMT1. The high level of DNMT1 gets the better of p21 during the competition for binding to PCNA.

Experiments were performed to establish whether poly(ADP-ribosyl)ation modulates *Dnmt*1 expression through an indirect mechanism. It is known that in c-*fos*-transfected cells (CMV-c-*fos*), where the expression of c-*fos* is increased 25-fold, the amount of DNMT1 is 10 times higher than in control cells with a parallel 20% increase in the number of methyl groups present in the DNA (41). It was considered that changes in poly(ADP-ribosyl)ation might affect the levels of c-*fos* and thereby affect *Dnmt*1 expression. However, treatment of cells with 3-ABA does not affect the expression of c-*fos* proto-oncogene (Fig. 1a), excluding the possibility that the increased *Dnmt*1 expression is due to this protein.

DISCUSSION

Previously, methods have been described to induce DNA hypomethylation—for example, treatment of cells with 5-azacytidine (42)—but the treatment of cells with 3-ABA is the first treatment that has been shown to induce *in vivo* DNA hypermethylation.

Our previous data have shown that inhibition of poly(ADP-ribose) polymerases is involved *in vivo* in modifying the unmethylated state of CpG islands (32). This finding is of great interest considering that an anomalous DNA methylation of CpG islands induces the silencing of correlated genes. The mechanism involved in protecting these DNA regions against methylation is still unknown (9, 10, 16). To explain how the inhibition of poly(ADP-ribosyl)ation leads to DNA hypermethylation, we performed additional experiments on cells at different cell-cycle phases. Our data have shown that competitive inhibition of PARPs, through treatment of cells with 3-ABA, increases the mRNA and protein levels of the major maintenance DNMT1. This favors the expression of the enzyme at the G1/S border to such an extent that the level becomes similar or higher than in S phase control cells—the phase in which DNMT1 is typically expressed. In fact, it is well known that different DNMTs are expressed differently during the cell-cycle (43–45), and various studies have shown that DNMT1 is up-regulated in S phase when the enzyme is involved in the maintenance reaction. Recently, it has been shown that antisense constructs, which down-regulate cellular DNMT1 levels, induce a rapid and corresponding increase in the level of the cell-cycle regulator p21 (46, 47).

Considering that inhibition of PARPs increases the expression of DNMT1 without affecting that of p21 and the fact that these two proteins compete for the same binding site on PCNA, the results presented here explain how the inhibition of poly(ADP-ribosyl)ation leads to DNA hypermethylation. Although it is well known that DNMT1 is primarily expressed in S-phase in normal cells (43–45), the increased expression of this enzyme following PARPs inhibition induces an early formation of the DNMT1-PCNA complex at the G1/S border. The precocious presence of a DNMT1-PCNA complex may modify the unmethylated state of the promoter regions in housekeeping genes (CpG islands) that are present in early replicating DNA (35).

However, additional mechanism(s) cannot be ruled out to explain the involvement of poly(ADP-ribosyl)ation in the control of the methylation process. Poly(ADP-ribosyl)ated isoforms of both PCNA (48) and p21 (49) have been described, and it would seem important to ascertain whether this postsynthetic modification can modulate the competition between p21 and DNMT1 for the PCNA binding domain. However, some papers have shown that DNMT1 is induced by the Ras-Jun pathway (50–51) or by c-*fos* (52). Although our results exclude a direct involvement of c-*fos* hyperexpression in causing DNMT1 abundance, they do not exclude the possibility that a posttranscriptional mechanism or other pathways can activate c-*fos*, and this is worth further research.

The 3-ABA induced up-regulation of DNMT1, and the following premature formation of PCNA-DNMT1 complex in G1/S border is just a part of the molecular mechanism through which inhibition of poly(ADP-ribose) polymerases introduces anomalous hypermethylation onto DNA (Fig. 3).

As for the existing correlation between the cellular level of DNMTs and the cellular transformation, results reported in the literature do not agree (46, 47, 53–56).

If we consider the possibility that cellular transformation could be due at a hyper-expression of DNMT1 in a phase of the cell-cycle in which the enzyme is not normally expressed (44, 45, 56), our results could lead to a new line of research aimed at clarifying the mechanism through which anomalous hypermethylation of CpG islands occurs during neoplasia (56–60).

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Fig. 1

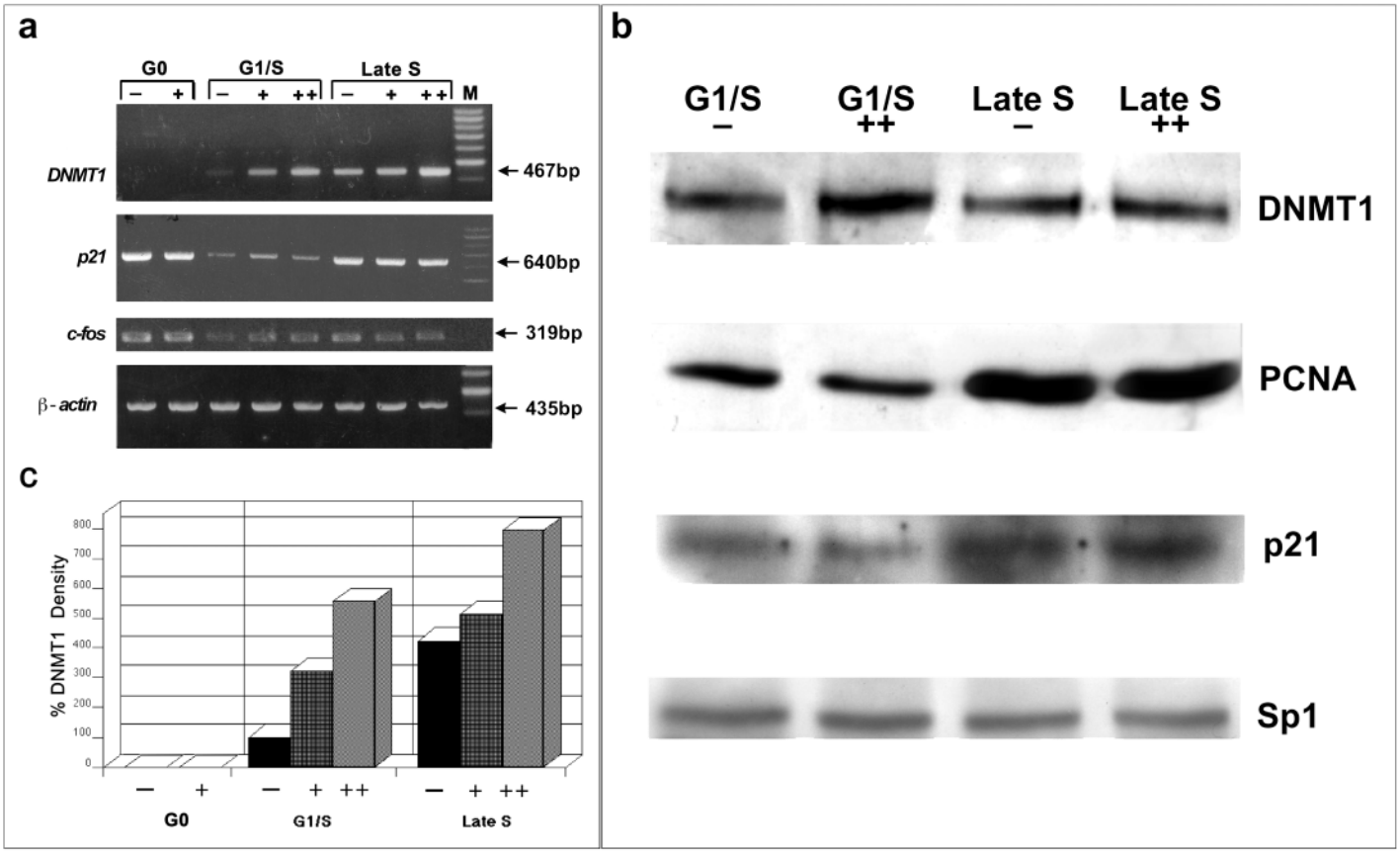

Figure 1. a) RT-PCR analyses of *Dnmt*1, *p21*, c-*fos* were performed by using β-*actin* as a control for mRNA levels.Total cellular RNA was quantified by using a spectrophotometer and 20 ng of total RNA was used (final volume of 25 µl) for RT-PCR analyses. Number of RT-PCR cycles to perform was chosen according to the minimum number of cycles necessary to obtain a quantifiable amplification of mRNA samples obtained from untreated control cells in G1/S border. **b)** Immunoblot showing the amount of DNMT1, p21, and PCNA present in 40 µg of nuclear lysates from cells synchronized at the G1/S border or in late S phase. Blots were developed with purified rabbit polyclonal antibodies against the N-terminal domain of DNMT1 (New England BioLabs), anti-p21 (F-5) and anti-PCNA (PC10) monoclonal antibodies (Santa Cruz Biotechnology) and were detected by using ECL chemiluminescent detection system (Amersham). Sp1 protein was used as an internal control and quantified by specific antibodies (1C6, Santa Cruz Biotechnology). Proteins detected by immunoblot assay were quantified by desitometric scanning of the bands (Bio Image, Millipore). Control cells (–) and cells treated with 3-ABA for 60 h (cells synchronized at the G1/S border) or 52 h (cells synchronized in late S phase) as indicated on top (++). **c)** *Dnmt*1 quantitative densitometric analysis of RT-PCR: Data represent the average of two independent experiments.

Fig. 2

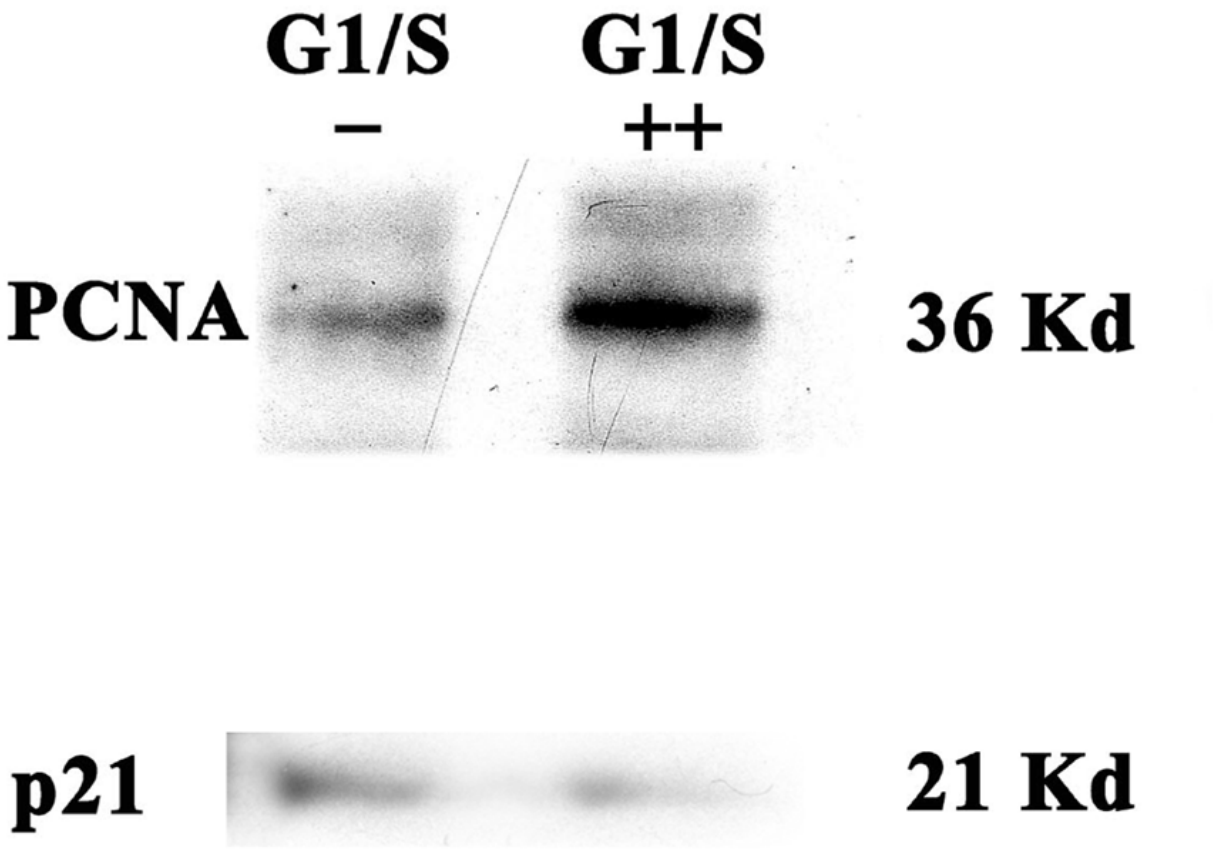

Figure 2. Levels of PCNA and p21 which co-immunoprecipitate with DNMT1. The figure refers to results obtained from cells synchronized at the G1/S border. Immunoblot of PCNA and p21 co-precipitated from 0.5 mg of cellular lysates by using anti amino-terminal domain of DNMT1 rabbit polyclonal antibodies (New England BioLabs) cross-linked to dynabeads protein A (Dynal). SDS/PAGE and transfer of proteins to nitrocellulose membranes were performed according to standard procedures. Immunostaining of PCNA and p21 co-precipitated with DNMT1 was performed by using the same antibodies reported for the detection of these proteins in nuclear lysates. Immunoprecipitation experiments have been performed three times, each time with similar results.

Fig. 3

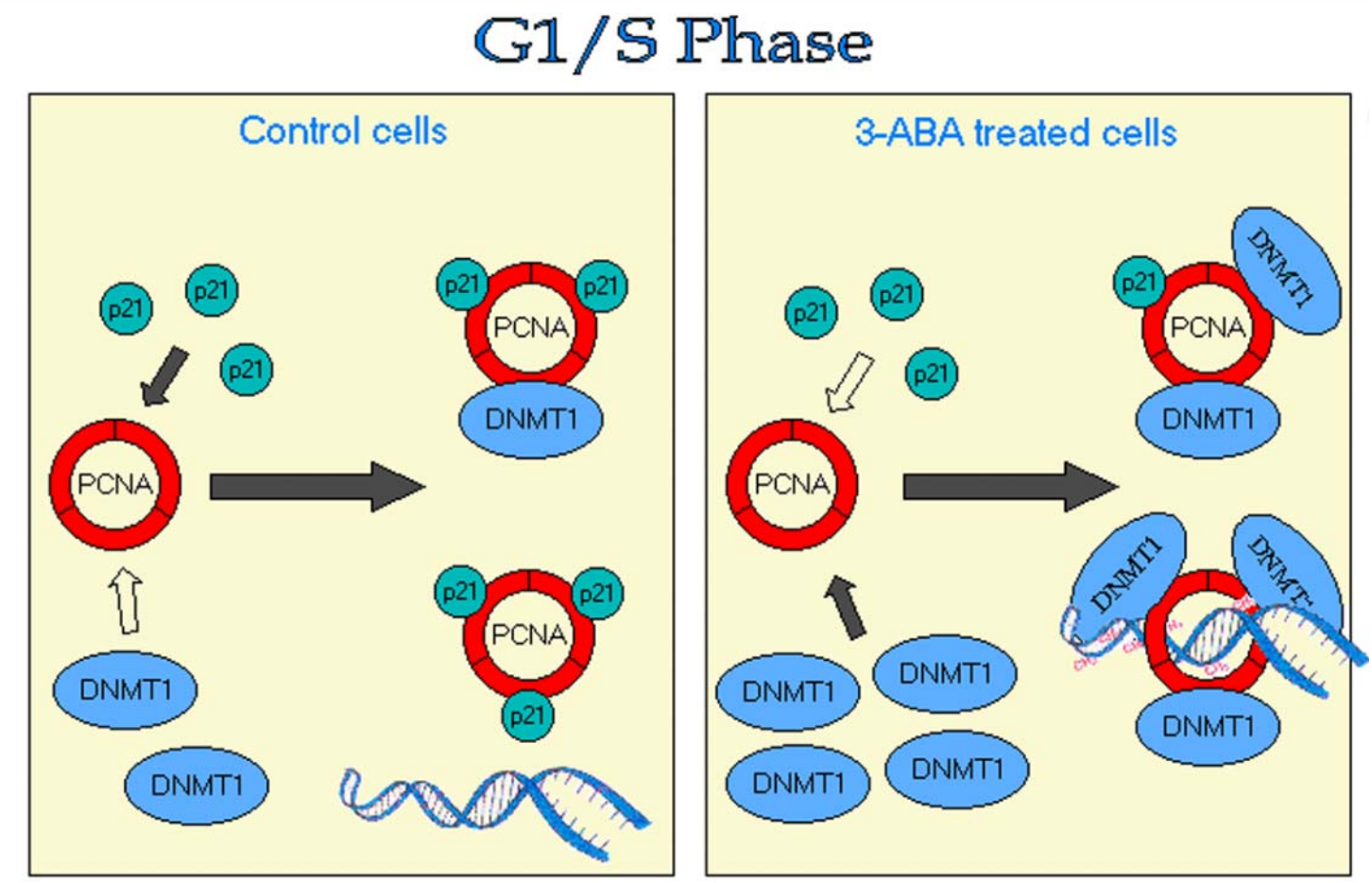

Figure 3. Schematic model suggesting that the 3-ABA induced up-regulation of DNMT1 and the following premature formation of active PCNA-DNMT1 complex in G1/S border may be part of the molecular mechanism through which inhibition of poly(ADP-ribose) polymerases introduces anomalous hypermethylation onto DNA.