METHYLATION *STATUS* OF *DNMT*1 PROMOTER DEPENDS ON POLY(ADP-RIBOSYL)ATION

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Abstract

Research is focused on CpG islands and on the mechanism that poly(ADP-ribosyl)ation uses to defend the unmethylated state of these important DNA sequences which are located in the promoter regions of the housekeeping genes having a role of transcription regulators.

Data here reported show that inhibition of PARP activity allows the diffuse insertion of methyl groups onto some CpG islands and in particular on the CpG island which is located in the promoter region of *Dnmt*1 gene. Hence, following inhibition of PARPs activity, this promoter loses its protection against methylation becoming silenced through methylation as shown by analyses with Methylation Sensitive PCR (MS-PCR) and sequencing after bisulphite treatment.

Analyses of Western Blotting, RT-PCR and Real-time RT-PCR confirm that the gene has undergone silencing. The role of ADP-ribose polymers in silencing *Dnmt*1 has been demonstrated by additional experiments in which overexpression of poly(ADP-ribose) glycohydrolase leads to reduction of ADP-ribose polymers in nuclei associated to a sharp decrease of Dnmt1 level respect to control. A parallel genome-wide methyl-sensitive restriction assay demonstrates that the variation of Dnmt1 level is followed by a bimodal alteration of DNA methylation pattern. In fact, the inhibition of poly(ADP-ribosyl)ation initially causes an increase in methyl-group insertion onto DNA while this phenomenon is reversed after prolonged treatments and demethylation is detected within Alu sequences.

Considering the important role played by Dnmt1 in the epigenetic scenario, these data lead us to think about what happens in tumor cells where both anomalous methylation of some CpG islands and diffuse hypomethylation are present. These findings open up a new path into epigenetic research in tumors. What is remarkable is that the demethylated pattern found in Alu sequences after treatment of cells with 3-ABA for 96 hours is very similar to the one found on DNA from cells treated with 5-AZA for the same time. The discovery of a DNA demethylating activity dependent on the use of inhibitors of poly(ADP-ribosyl)ation process increases the knowledge of mechanism by which these inhibitors enhance the cytotoxicity of other anticancer agents.

Introduction

The DNA methylation machinery

DNA methylation (for reviews see Costello and Plass, 2001; Bird, 2002; Zlatanova *et al*., 2004; Caiafa and Zampieri, 2005) is the post-synthetic modification that by transferring a methyl group from S-adenosylmetionine to carbon-5 of cytosine ring introduces 5mC as new base on DNA. Enzymes involved in this reaction are DNA methyltransferases and their preferential target for methylation are cytosines located in CpG dinucleotides. The mCpG dinucleotides are distributed in a non-random fashion in genomic DNA so that methylation pattern is characterized by the presence of methylated cytosines on the bulk of DNA while the unmethylated ones are mainly located within particular regions termed CpG islands. Specific DNA methylation pattern results from the combination of maintenance and *de novo* methylation and demethylation processes. Maintenance methylation, which occurs within a minute or two after replication, recognizes and modifies hemimethylated sites generated during DNA replication thus preserving the tissue-specific methylation pattern. DNA methyltransferase 1 (Dnmt1) is considered primarily responsible for maintenance methylation due to its preference for hemimethylated DNA, its maximum level of expression being in S cell-cycle phase (Szyf *et al*.,1991) and not least because of its association with proliferating cell nuclear antigen (PCNA) during DNA replication at DNA replication foci (Chuang *et al*., 1997). Dnmt3a and Dnmt3b are considered responsible for the *de novo* methylation process which explains how the pattern of methylation can be changed by introducing new methyl groups onto DNA at sites in which neither strand was previously methylated. These enzymes play an important role during early stages of embryonic development when a bimodal pattern of methylation is defined (Kafri *et al.*, 1992). It is important to say that it is too simplistic to consider that only these enzymes are involved in DNA methylation as for most of them there are several isoforms and new Dnmts have been identified (for review see Robertson, 2002). A great deal of research is being carried out to establish their roles. The original distribution of roles mentioned above for the main Dnmts may have to be reconsidered in light of the fact that some of them have been seen cooperating in maintaining DNA methylation and gene silencing in human cancer cells during DNA replication (for review see El-Osta, 2003).

The thermodynamic problem, concerning the removal of methyl groups from cytosine makes the understanding of DNA demethylation mechanism somewhat complicated. A DNA demethylase activity, capable of catalyzing replacement of the methyl group with a hydrogen

derived from water and of releasing the methyl group in the form of methanol, has been proposed as well as the alternative possibility of the removal of 5mC or 5mCpG dinucleotide by glycosylase activity and their substitution with the unmethylated base or dinucleotide. Whatever the enzymatic mechanism(s) may be, DNA demethylation is an active process in cells and plays a role not only in the definition of the methylation pattern in early stages of development but also in the passage towards pathological events as tumorigenesis (for reviews see Cedar and Verdine, 1999; Bird, 2003).

Histone modifications, DNA methylation and proteins able to bind methylated DNA play a dynamic role in determining chromatin structure suitable for gene expression or silencing. At present, much information is available as to how several epigenetic modifications work together in determining regions of chromatin that have to be more or less suitable for transcription (for review see Jaenisch and Bird, 2003). An unresolved problem is the timing with which the various epigenetic events occur in establishing gene expression. On this subject at least two scenarios can be suggested. One foresees DNA methylation as the first event as it was observed that histone deacetylase can reach chromatin through its association with proteins able to recognize and bind methylated DNA (Nan *et al.*, 1998; Jones *et al.*, 1998). The second scenario considers DNA methylation to be the final stabilizing moment which occurs only after epigenetic events associated with histones (i.e. K9H3 methylation) have already shut down gene expression (for review see Bird, 2001).

There is also considerable evidence that chromatin dynamics involve the action of small RNAs (siRNAs and miRNAs) in all eucaryotes including mammals (for review see Lippman and Martienssen, 2006). Using human tissue culture cells, it has been shown that promoter-directed siRNA inhibits transcription of an integrated, proviral elongation factor 1alpha (EF1A) promoter–green fluorescent protein reporter gene and of endogenous EF1A by DNA methylation–mediated silencing (Morris *et al.*, 2004). Similar results were obtained with siRNA targeted to E-Cad and *erB2/HER2* promoters colocalizing with CpG islands (Kawasaky and Taira, 2004).

Distribution of mCpG and CpG dinucleotides on genomic DNA

The frequence with which CpG dinucleotides are found on genome is much lower than expected except for CpG island regions where their number is nearer to the expected one. This happened during evolution due to the presence of spontaneous deaminase activity in nuclei (for review see Jones and Baylin, 2002). This enzymatic reaction transforms

methylated cytosine into thymine while the unmethylated cytosine is transformed into uracil. Subsequent control and repair mechanisms recognize uracil as an extraneous base on DNA and thus substitute it while this substitution does not happen where thymine is concerned as it is a common base on DNA.

Going back to CpG islands being enriched in CpG dinucleotides, the explanation lies in the non methylation of their normal state which preserves their regions from deamination-dependent mutations.

Summarizing some characteristics of CpG islands (Antequera and Bird, 1993; Antequera and Bird, 1994; Antequera, 2003) there are about 30,000 generally located in the 5' promoter region of housekeeping genes sometimes overlapping the coding region to variable extents (usually the first exon). Although their sequence is enriched in CpG dinucleotides, which are the best substrates for DNA methyltransferase, the CpG islands are unmethylated. There is evidence that the transcription of genes associated with them is active when these regions are in unmethylated state while it is inhibited when these regions undergo methylation (for reviews see Jones and Baylin, 2002; Robertson, 2002) (Fig. 1). It is to note that CpG islands have recently been found in several tissue-specific and imprinted genes within the genes themselves without their methylation blocking gene expression (for review see Jones, 1999).

Along DNA other discrete regions, enriched in CpG dinucleotides, must be considered. They are repetitive elements termed B elements (for review see Turker, 2002) in mice and Alu elements in humans (Takai and Jones, 2002). Analysis of sequence of human chromosomes 21 and 22 has led to the definition of more stringent parameters which allow the distinguishing of CpG islands and Alu sequences (Takai and Jones, 2002). The new parameters for definition of CpG islands are fixed in this way: minimum size ≥ 0.5 kbp, C+G content ≥ 55% and observed CpG/expected CpG ≥ 0.65. The important difference between CpG islands and Alu repeats lies in the fact that the latter are a good substrate for the DNA methylation that occurs in these regions that are generally methylated. B and Alu sequences are defined cis-acting methylation centers both as suspected of signalling de novo DNA methylation and as - in absence of a boundary - they may be able to spread methylation to the adjacent DNA regions.

A strong association is shown between CpG islands and Alu repeats. A detailed analysis of the sequence of human chromosomes 21 and 22 showed that 54.4 % of CpG islands occur between Alu regions and the number of CpGs that are found in 5' regions of genes is about the same as the number of Alu-associated CpGs (Takai and Jones, 2002).

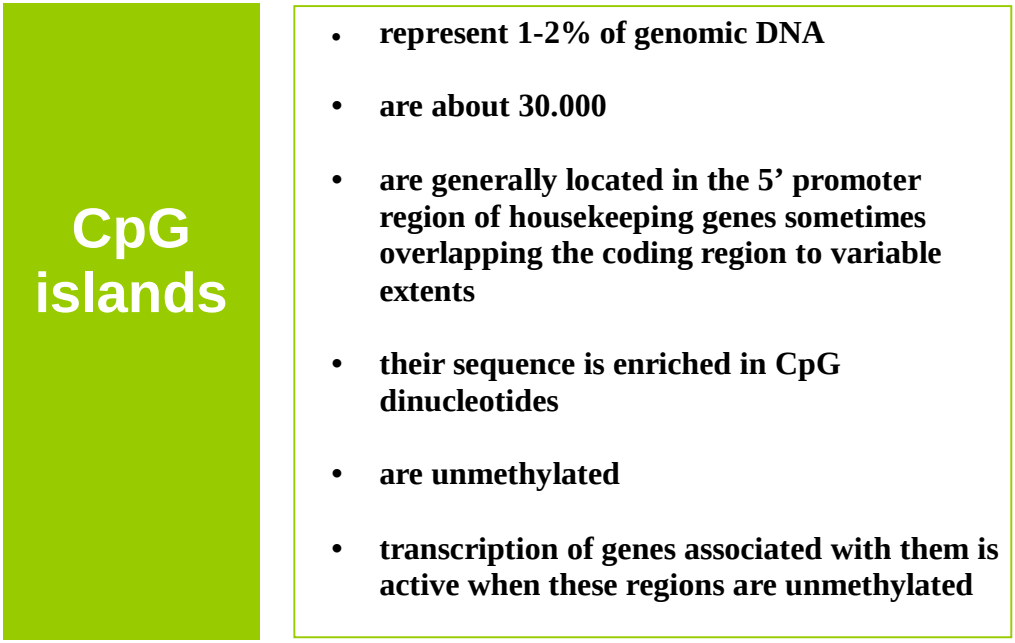


Fig. 1: Main features of CpG island regions

The jigsaw of unmethylated state of CpG islands

Concerning the regulation of DNA methylation process, two unresolved questions are:

a) to clarify the mechanism by which CpG islands are protected from methylation during replication and in chromatin;

b) to understand how CpG islands, located in the promoter regions of tumor suppressor genes, become methylated during tumorigenesis while they are unmethylated in normal cells.

There has been a lot of research to discover the mechanism that normally keeps CpG islands unmethylated, some of which has tried to pinpoint *cis*-acting sequences able to conduct such an important mechanism. As CpG island sequences have been seen to be methylatable in *in vitro* experiments the hypothesis that they are intrinsically unmethylatable has fallen through (Bestor *et al.*, 1992).

Attention of many researchers has been diverted to consensus binding sites for transcription factor Sp1 (Brandeis *et al.*, 1994; Macleod *et al.*, 1994; Mummanemi *et al.*, 1998). The mouse *Aprt* housekeeping gene – whose CpG island includes the promoter, the first and second exons and the first intron – has been chosen for the study of this mechanism as Sp1 consensus sites are present in its promoter region. Their connection to the mechanism that keeps the CpG islands unmethylated is being researched with interest as these Sp1 sites often occupy a strategic position both in the mouse B1 (for review see Turker, 2002) and in the human Alu repetitive elements that often flank CpG islands (Takai and Jones, 2002). B1 and Alu sequences - because of their high methylation level - are considered methylation centers and Sp1 sites in these patterns are seen as boundaries able to stop the spreading of methylation from B1 and Alu elements to unmethylated CpG islands (Fig. 2).

The diffusion of methylation from methylated CpG dinucleotides toward the adjacent unmethylated ones has previously been amply shown (for review see Lindsay and Adams, 1996) but without explaining how CpG islands are protected from the spreading of methylation. As concerns the expression of mouse *Aprt* gene, it has been shown that among the Sp1 consensus sites, sites three and four are required for gene transcription while site two is the specific one in the blocking of methylation spreading and is reinforced in this role by sites three and four (Mummaneni *et al.*, 1998). Their silencing is a gradual process in differentiated cells (Yates *et al.*, 2003). If it is the boundary caused by Sp1 which isolates the islands from a general state of methylation it can be presumed that in tumorigenesis this mechanism is, in some way, lost (Fig. 2).

However, data in literature are not unanimous as while some experiments show that the gene-correlated CpG island is methylated when Sp1 binding sites, in correspondence with gene, are disrupted by direct mutagenesis (Brandeis *et al.*,1994; Macleod *et al.*, 1994; Mummanemi *et al.*, 1998), no anomalous methylation of CpG islands is found in mice in which Sp1 is not expressed following homozygous deletion (Marin *et al,*. 1997). Anyway this mechanism cannot be considered universal as Sp1 consensus sites are not always present in promoter regions of housekeeping genes. It is reasonable to think that some other transcription factor(s), through their ties with active promoters containing CpG islands, can act as *trans*-acting elements able to protect the unmethylated state of these DNA regions. Experiments, carried out on T24 bladder carcinoma cell line, in which the remethylation kinetics was examined for *p16* gene after it had been demethylated by treatment with 5-aza 2'-deoxycytidine, have shown that the timing of remethylation varied, being longer for promoter region than for coding regions of gene, i.e. exon 2. This could be due to the presence

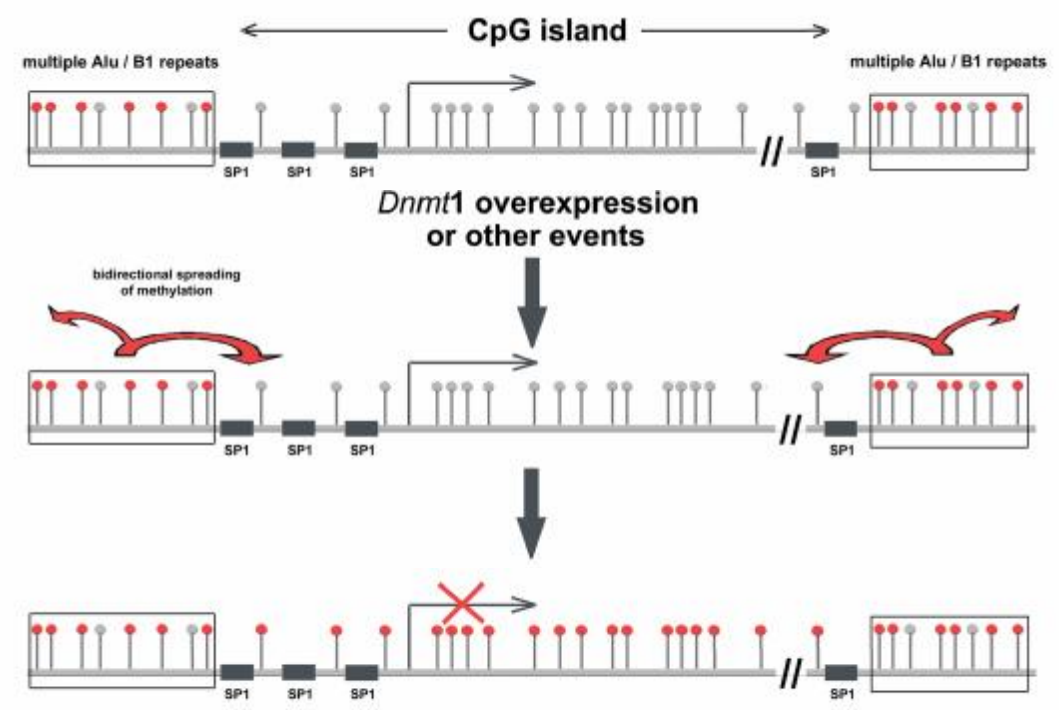


Fig. 2: Alu repeats and CpG islands are physically and functionally correlated on genomic DNA: a role for Sp1 elements. In the scheme CpG island is juxtaposed to multiple Alu elements which have been proposed as "*de novo* methylation centers". Sp1 sites, located upstream and downstream of transcription start in the island, play a role in preventing methylation. Cluster of Sp1 elements is suggested as boundary region capable of protecting CpG island from the spreading of methylation arising from Alu elements. Grey and red lollipops represent non-methylated and methylated CpGs, respectively (Adapted from Caiafa and Zampieri, 2005).

of transcription factor(s) on promoter region capable of impeding Dnmt1 from entering DNA (Bender *et al.*, 1999).

An important point in determining variation of methylation patterns is the nuclear level of Dnmt1 as it has often been seen to be high in tumor cells (Baylin *et al.*, 2001). Thus anomalous increased level of the enzyme could be involved in determining aberrant

introduction of new methyl groups onto DNA. Housekeeping gene promoters are hypermethylated, with the silencing of correlated genes, in human cell lines expressing 50-fold increased level of Dnmt1 (HMT) (Vertino *et al.*, 1996) and further research, performed on the same cells, has shown that not all *loci* containing CpG rich regions are equally affected by methylation in cells overexpressing Dnmt1 but about 70% of them were resistant to *de novo* methylation and only a few of them (3,8%) were methylation-prone (Feltus *et al.*, 2003). Recently, it has been shown that RNA interference (RNAi)-mediated silencing of Dnmt1 leads to promoter demethylation and re-expression of some tumor suppressor genes in several cancer cells (Robert *et al.*, 2003; Suzuki *et al.*, 2004). This confirms the important role played by Dnmt1 in tumorigenesis although additional data have shown that CpG island hypermethylation is maintained in human cancer cells after homozygous deletion (Rhee *et al.*, 2000) or RNAi-mediated depletion of Dnmt1 (Ting *et al.*, 2004).

As not all tumor cells in which there is hypermethylation of tumor suppressor genes show an overexpression of Dnmt1 (Eads *et al.*, 1999), and as the expression of Dnmt1 is cell-cycle dependent (Szyf *et al.*, 1991), attention has been diverted to the idea that the expression of Dnmt1 can occur in an anomalous cell-cycle phase - i.e. in G1/early S phase - when CpG rich regions and active genes replicate (Delgado *et al.*, 1998). It has been observed that remethylation process can also occur in cells blocked in G1 (Bender *et al.*, 1999).

In this scenario the competition existing between p21 and Dnmt1 for the same binding domain on PCNA (Chuang *et al.*, 1997) may play an important role. PCNA is the protein to which Dnmt1 binds when, immediately following replication, the maintenance methylation intervenes to guarantee that methylation pattern of the parent strand is conserved on the newly synthesized one. In early S phase, when CpG island regions replicate (Delgado *et al.*, 1998), the level of Dnmt1 is too low to compete with p21 for the binding site on PCNA and so replication occurs without subsequent methylation. It has been suggested that an as yet unidentified biological event, able to induce Dnmt1 expression in early S phase, could make the binding to PCNA easier thus allowing the methylation of early replicating genes in tumorigenesis (Baylin, 1997). Moreover, Dnmt1 seems to be a cofactor capable of regulating negatively expression of p21 through a direct or indirect link with Sp1 (Milutinovic *et al.*, 2004). Thus, an anomalous overexpression of Dnmt1 in the early S phase could further facilitate the formation of PCNA-Dnmt1 complex.

Another suggested mechanism sees the action of a protein which, linking Dnmt1, inhibits its catalytic activity, thus preventing the introduction of new methyl groups onto DNA. This role is played by Rb (Pradhan *et al.*, 2002), a protein involved in cell-cycle control and

therefore having an important role as tumor suppressor gene. It has been shown that Rb links through its pockets B and C the regulatory domain of Dnmt1 and that following this association the bond existing between Dnmt1 and DNA is destabilized. Overexpression of Rb leads to DNA hypomethylation. All this suggests that this mechanism plays a role in the hypermethylation of oncosuppressor genes in tumors where often Rb is absent or mutated (Hanahan and Weinberg, 2000).

The outcome that ORIs map within or immediately adjacent to CpG islands has been seen as a mechanism probably involved in maintaining the unmethylated state of CpG islands. Analysis of very short DNA fragments (< 1.5 Kbp), which are formed at the beginning of S phase, allowed the observation that their sequence is similar to that of unmethylated CpG island regions. Replication time of CpG islands is about 2-2.5 hours while the bulk of DNA needs more time to replicate. Thus origins of replication colocalize frequently with CpG islands so that the great number of proteic factors involved in these processes could be enough to make access difficult to Dnmt1 that, as mentioned above, is less expressed in early S-phase.

Experiments performed on human X-linked hypoxantine phosphoribosyltransferase gene (HPRT) have shown that colocalization of CpG islands and ORIs is dependent on the methylation state of CpG island. In fact the CpG island, which is methylated in the inactive allele, replicates in late S phase (Schmidt and Migeon, 1990). Thus, unmethylated CpG island regions should be particularly suitable for starting DNA replication because of their uncondensed chromatin structure (Tazi and Bird, 1990) and this colocalization has been suggested as a mechanism by which CpG islands are protected from methylation during replication (Antequera and Bird, 1999), (Fig. 3).

Finally, there are proteins that targeting Dnmts on specific DNA regions mediate transcriptional silencing (for Reviews see Robertson, 2002; El-Osta, 2003).

At present much information is available but not enough to allow the elaboration of a final model(s) explaining how CpG islands are protected from methylation. In spite of the great effort put into solving this scientific problem, much still has to be done to provide definite proof which solves this fascinating puzzle.

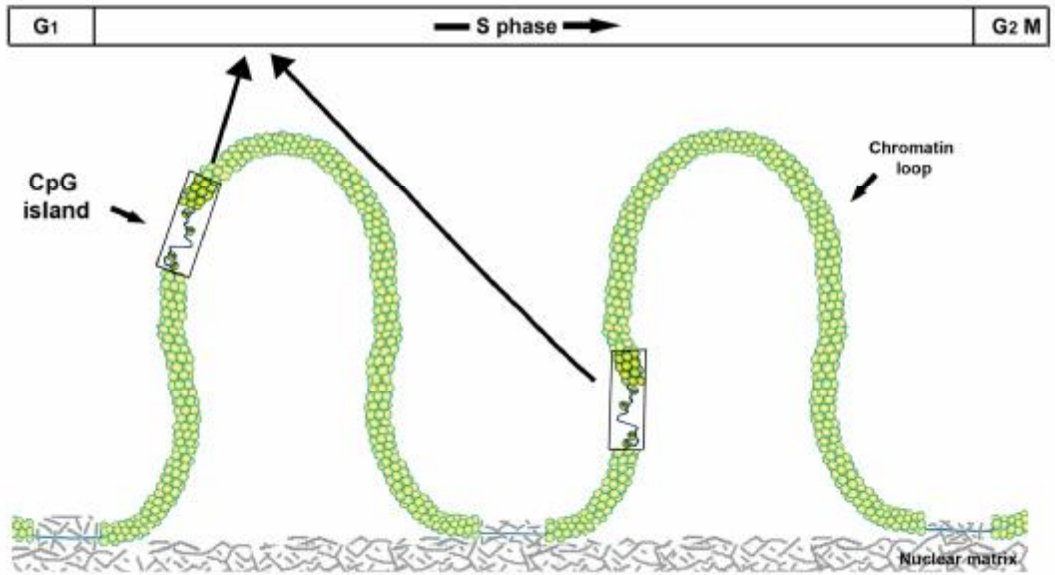


Fig. 3: **ORIs are often located at CpG island regions *in vivo.*** The fact that ORIs map within or immediately adjacent to CpG islands has been seen as a mechanism probably involved in maintaining the unmethylated state of CpG islands. CpG islands, because of their localization in decondensed chromatin regions, could offer an easier replication starting point than condensed chromatin. Thus CpG islands and correlated active genes replicate in early S phase while silent genes in more advanced S phase. The lower level of Dnmt1 in early S phase than during the S phase favours the maintaining of the unmethylated state of CpG islands (Adapted from Caiafa and Zampieri, 2005).

Poly(ADP-ribosyl)ation

Poly(ADP-ribosyl)ation, an enzymatic mechanism catalyzed by a family of ADP-ribose polymerases, leads to the transfer of ADP-ribose unit from nicotinamide adenine dinuleotide (NAD) to chromatin proteins (D'Amours *et al.*, 1999, Kim *et al.*, 2005). Chains of different sizes ranging from 2 to 200 units are built on a protein acceptor and moreover these chains are branched and so rich in negative charges that they become molecular adaptors of the protein acceptor capable of interfering with the link between protein-protein and protein-DNA. The dynamics of poly(ADP-ribosyl)ation process is guaranteed by the presence of many

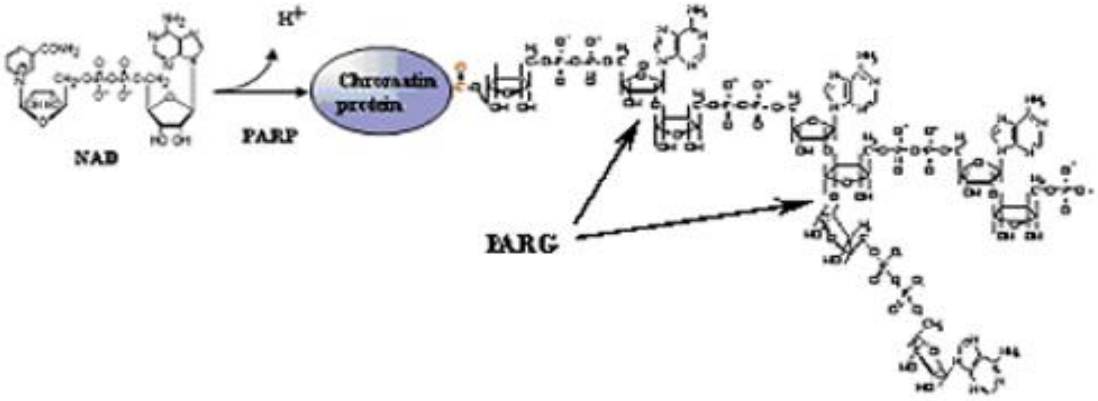


Fig. 4: Poly(ADP-ribosyl)ation reaction and enzymes. Synthesis of linear and branched chains of poly(ADP-ribose) by PARP on chromatin protein using NAD as substrate. The action of poly(ADP-ribose) glycohydrolase (PARG) is directed to linear and branched ADP-ribose polymers (adapet from Zardo *et al.*, 2003).

poly(ADP-ribose) polymerases and one poly(ADP-ribose) glycohydrolase (PARG), an enzyme which hydrolizes and detaches ADP-ribose units from the polymers (Fig. 4).

A shuttle mechanism has been proposed to explain how PARG, which is a cytoplasmatic enzyme, can work in nuclei where most PARPs are located (Davidovic *et al.*,. 2001).

Degradation of polymers starts when their nuclear level exceeds 15 μM and their half-life is calculated at one minute (Alvarez-Gonzalez and Althaus, 1989).

Recently it has been recognized that PARPs are a family of enzymes composed of 18 members (Amé *et al.*, 2004). Their different subcellular localization suggest specific biological roles for these enzymes wich are still to be defined.

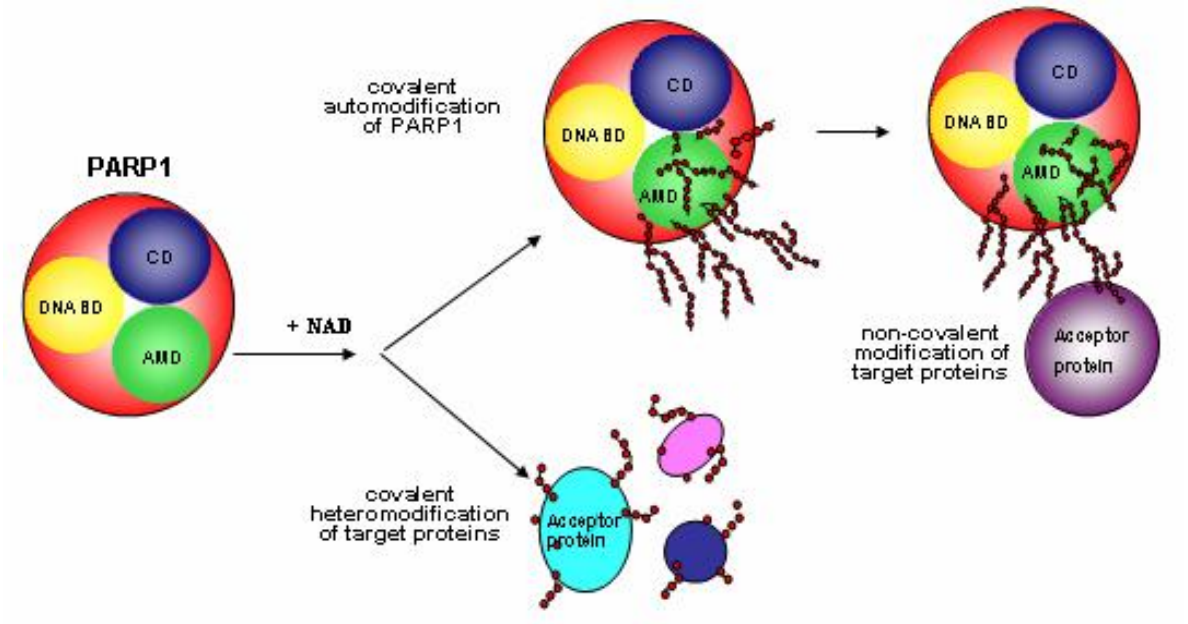


Fig. 5: Covalent and non-covalent modification of a protein acceptor by PARP1. DNA BD: DNA binding domain; CD: Catalytic Domain; ADM; Automodification Domain (adapted from Zardo *et al.*, 2003).

The best known is PARP1 which is responsible for about 90 % of all the ADP-ribose polymers found in cells (D'Amours *et al.*, 1999). This enzyme is ubiquitously expressed although at tissue and cell-type specific level. PARP1 is involved in many physiological and pathological processes. The physiological roles of this enzyme include apoptosis, transcription, maintenance of genomic stability, DNA-base excission repair and aging while in pathological conditions, an important role has been attributed to PARPs' inhibitors in the cure of diabetes mellitus, ischaemia-reperfusion damage in heart, brain, kidney and bowel, acute and chronic inflammatory disorders and septic and haemorrhagic shock (Smulson *et al.*, 2000; Burkle, 2001). An increased catalytic activity by about 500 times is observed following nicks in both single and double strand DNA. Three domains, having three different functional roles, are present on the enzyme (Fig. 5).

The N-terminal domain, which contains zinc-finger motifs, recognizes nicks and successively links damaged DNA independently of the sequences involved (Ménissier-de Murcia *et al.,* 1989). The central domain is termed automodification domain. There are 28 sites of modification on it and it is on them that ADP-ribose polymers are built until long and branched chains are formed (Desmarais *et al.,* 1991). The C-terminal tail contains the catalytic domain. The mechanism by which PARP1 modifies itself is particular. Around nicked DNA the enzyme works in dimeric form, and it is the catalytic site of one enzymatic molecule which introduces ADP-ribose units onto the central domain of the other one. This process is called automodification. Heteromodification occurs when ADP-ribose polymers are introduced onto different chromatin proteins. *In vitro* experiments suggest that automodified PARP1 or ADP-ribose polymers are by themselves able to interact with chromatin proteins also through uncovalent binding. The link is so strong that the association between protein and ADP-ribose polymers cannot be reversed either by drastic chemical conditions or by elevated concentration of single or double strand DNA *vs* polymers (Panzeter *et al.,* 1992). Thus important chromatin proteins undergo covalent and uncovalent poly(ADP-ribosyl)ation (Fig. 5) and sometimes the same protein - see H1 histone (Panzeter *et al.,* 1992, 1993) - is substrate for both modifications. Chromatin decondensation, induced by PARP activation was shown many years ago (Poirier *et al.,* 1982). Recent research on Drosophila has demonstrated that active puff loci are rich in PARP1 and ADP-ribose polymers, this being the necessary condition to induce chromatin decondensation and gene expression (Tulin and Spradling, 2003). Mechanism of PARP1-mediated puffing and chromatin remodelling reinforces the idea previously suggested by Althaus to explain the role played by PARP1 in DNA repair (Althaus, 1992).

Correlation between poly(ADP-ribosyl)ation and DNA methylation

The Caiafa's research group gave the evidence of a crosstalk between poly(ADP-ribosyl)ation and DNA methylation as they found that competitive inhibition of poly(ADP-ribose) polymerases (PARPs) leads to introduction of anomalous methyl groups onto DNA (Zardo *et al.,* 1997; de Capoa *et al.,* 1999; Zardo *et al.,* 1999) and in particular onto some CpG islands (Zardo and Caiafa, 1998). To explain this phenomenon two possible molecular mechanisms have been proposed. The first one based on the observation that inhibition of PARP activity leads to over-expression of Dnmt1 in the anomalous G1/early S phase *in vivo* (Zardo *et al.,* 2002), thus allowing the premature formation of PCNA-Dnmt1 active complex in the phase in

which CpG islands undergo replication (Delgado *et al.*, 1998). The second based on the observation that modified PARP1 and Dnmt1 coimmunoprecipitate *in vivo* and on data showing that ADP-ribose polymers, present on modified PARP1, almost completely inhibit Dnmt1 activity *in vitro* (Reale *et al.*, 2005). Thus, inhibition of PARP activity could allow new methyl groups to be inserted onto DNA both during replication and in chromatin: during replication because inhibition of PARP activity induces Dnmt1 overexpression in G1/S phase and increases the formation of the active complex PCNA-Dnmt1 in this anomalous phase (Fig. 6, panel A) and in chromatin because unmodified PARP1 is unable to inhibit Dnmt1 (Fig. 6, panel B). Although suggested mechanisms offer a possible explanation of how inhibition of PARP activity affects DNA methylation patterns, they need to be further investigated to make clear the connection between these two epigenetic modifications.

Considering the great deal of research focused on the study of variation of Dnmt1 level in tumour cells, where an over-expression of Dnmt1 has often been seen as the cause of tumour suppressor gene hypermethylation and silencing, research has been focused on the previous finding showing that inhibition of PARP activity induces over-expression of Dnmt1.

Data here reported show that expression and level of Dnmt1 are time-dependent on the treatment of cells with 3-aminobenzamide - a competitive inhibitor of PARP activity - so much so that short times of treatment induce an over-expression of *Dnmt1* while longer times lead to its silencing. Investigation of a possible correlation between Dnmt1 level and DNA methylation pattern shows that the island that is present in the promoter of *Dnmt1* loses its protection against methylation becoming silenced through methylation following inhibition of PARPs activity. The silencing of the enzyme goes with introduction of diffuse demethylation of those Alu sequences whose methylation is characteristic of normal cells.

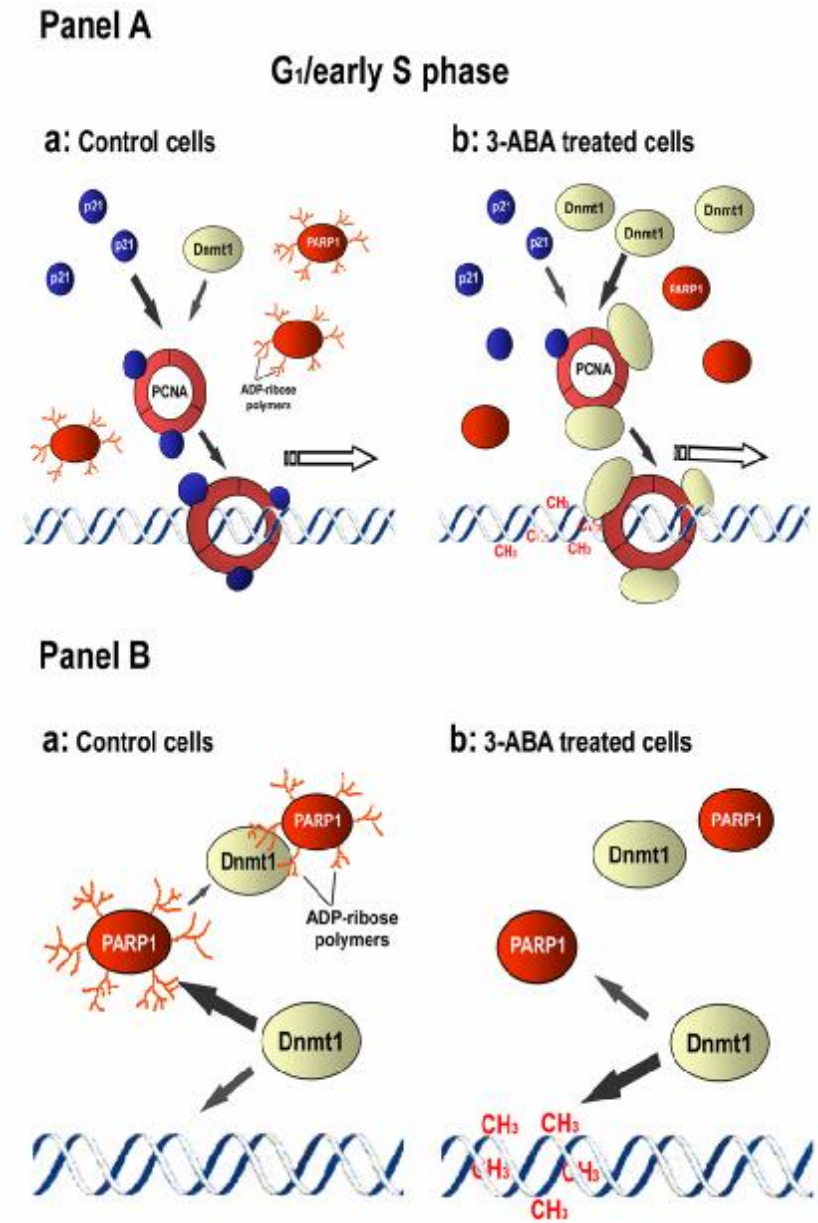


Fig. 6 : Inhibition of PARP activity induces DNA hypermethylation. Panel A reports the scheme proposed to explain DNA hypermethylation dependent on PARP inhibition during DNA replication: (a) In normal cells, modified PARP1 plays a role in controlling the expression of Dnmt1 in the appropriate cell-cycle phase, and thus the level of Dnmt1-PCNA active complex is low in G1/S phase. (b) Inhibition of PARP activity induces overexpression of Dnmt1 in G1/S phase, causing anomalously high level of the active Dnmt1-PCNA complex in this phase. Panel B reports the scheme proposed to explain DNA hypermethylation dependent on PARP inhibition in chromatin. (a) In normal cells, ADPR-polymers, present on PARP1, link in uncovalent way Dnmt1. Affinity of Dnmt1 for ADPR-polymers is so high that they compete with dsDNA 30 times more concentrated for the binding with the enzyme and the presence of ADPR-polymers almost completely inhibits the Dnmt1 activity *in vitro.* (b), inhibition of poly(ADP-ribosyl)ation frees Dnmt1 from its tie with PARP1 and in absence of ADPR-polymers Dnmt1 activity is restored. Thus, following inhibition of poly(ADP-ribosyl)ation, the two mechanisms could cooperate in inducing anomalous DNA hypermethylation (adapted from Caiafa and Zampieri, 2005).

Experimental procedures

Cell culture and drug treatment of cells

L929 mouse fibroblasts and human primary fibroblasts – from passage 19 to 32 - were maintained in Dulbecco's modified Eagle's medium (Cambrex) supplemented with 10 % fetal calf serum (Cambrex) at 37°C with 5% CO_2. For 3-aminobenzamide (3-ABA) treatments 0,3 – 0,5 x 10^6 cells were seeded in 10 cm Petri dishes and, after 24 hours, the culture medium was added of 3-ABA (Sigma) at a final concentration of 1 mM. The medium was replaced every 5 hours and the treatment was carried out up to 96 hours. Cell proliferation was monitored by counting viable cells (trypan blue exclusion) with a Burker chamber at each treatment time. For 5-aza-deoxycytidine (5-AZA) treatment, the growth medium was supplemented with 5 μM 5-AZA (Sigma) every 24 hours over 48 or 96 hours. Cells were then harversted by trypsinization, washed with PBS and eventually stored at -80°C for further analysis.

SDS-PAGE and Western Blot

To obtain nuclear lysates, fresh PBS-washed cell pellets were resuspended in ice-cold isolation buffer (10 mM TRIS-HCl pH 7.9, 4 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.25 M Sucrose, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin) and incubated on-ice for 30 min. Nuclei were than harvested by 1000 x g centrifugation and lysed in ice cold lysis buffer (50 mM TRIS-HCl pH 7.8, 2 mM EDTA, 0.5 M Nacl, 0.1 % NP-40, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin). To obtain total cell lysates PBS-washed cell pellets were resuspended in lysis buffer and incubated on-ice for 30 min. After sonication and high-speed centrifugation, both cellular and nuclear lysates, normalized for total protein content (50 – 100 μg) were resolved by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech) by electro-blotting. Western-blot analyses were performed according to standard procedures. Immunocomplexes were visualized by ECL-Plus chemiluminescent detection (Amersham Pharmacia Biotech) after reaction with the following primary antibodies: polyclonal antibody to human Dnmt1 (New England Biolabs), monoclonal antibody to mouse Dnmt1 (Imgenex), polyclonal antibody to Sp1 (H-225, Santa

Cruz), polyclonal antibody to Tubulin (E-19, Santa Cruz), monoclonal antibody to Poly(ADP-ribose) (10H, Alexis).

Quantitative and semi-quantitative RT-PCR

Total RNA (1 μg), purified from human fibroblasts by RNeasy Mini Kit (Qiagen), was subjected to retrotrascription using Superscript First-Strand Synthesis system (Invitrogen). Real Time PCR reactions were carried out in a final volume of 25 μl of iQ SYBR Green Supermix (Bio-Rad), added of 150 nM specific primers and of an amount of cDNA corresponding to 10 ng of total RNA. To evaluate PCR efficiency for each couple of primers, a standard curve was generated using 1-fold serial dilutions (from 40 to 2,5 ng) of the cDNA control at 24 hours. Efficiency was in the range between 95 and 100%. The amount of Dnmt1 cDNA was calculated from the standard curve and normalized with respect to that one of 18S rRNA. The following PCR primers (Ghoshal *et al.*, 2005) were used for the analysis: Dnmt1, 5'-AGGGAAAAGGGAAGGGCAAG-3' sense, 5'-AGAAAACACATCCAGGGTCCG-3' antisense and 18S rRNA, 5'-TCAAGAACGAAAGTCGGAGG-3' sense, 5'-GGACATCTAAGGGCATCACA-3' antisense. PCR amplification was performed on a iCyclerIQ (Bio-Rad) thermal cycler adopting the following conditions: the initial denaturation at 95°C for 10 min; 45 cycles of: 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. Dissociation at 95°C for 1 min and 55°C for 30 sec was performed to check for the formation of a primer dimer. The dissociation profile for the amplified products indicated that none of the primer pairs generated a dimer. For semi-quantitative RT-PCR analysis, PCR reactions were carried out in a final volume of 25 μl of PCR buffer (Invitrogen) added of 1.5 mM MgCl2, 0.2 mM dNTPs, 0.5 μM of the gene- specific primers, 1 U of Taq DNA polymerase (Invitrogen) and an amount of cDNA corresponding to 20 ng of total RNA. After denaturation at 94°C for 4 min, PCR was done for 32 cycles (Dnmt1), 35 (Dnmt3a and Dnmt3b) or 26 cycles (Gus and β-Actin) of 45 sec at 94°C, 45 sec at the primer specific temperature of annealing and 1 min at 72°C followed by one step of 7 min at 72°C. Primer sequences and annealing temperatures were as follows: Dnmt1 (ac. num.: NM001379) 5'-AGGAGGAGGAAGCTGCTA-3' sense, 5'-CCACTCATACAGTGGTAGATTTG-3' antisense, annealing temperature 53.7°C; Dnmt3a (ac. num.: NM022552) 5'-TGGAAAGCGGTGACACGCC-3' sense, 5'-CGCTTCTGCAGGGGCT-3' antisense, annealing temperature 54,6°C; Dnmt3b (ac. num.: XM047018) 5'-AGGATCTTTGGCTTTCCTGTGC-3' sense, 5'-AATGAGCACCGTGTTAGGCTG-3'

antisense, annealing temperature 57,7°C; (Gus (ac. num.: NM 000181) 5'-GCGGCATTTTGTCGGCTGGGTGTG-3' sense, 5'-TGGCAGGGTGGTGGGGGTGAGTGTG-3' antisense, annealing temperature 64°C; β-Actin (ac. num.: NM001101) 5'-CCCCGTGCTGCTGACCGAGGCC-3' sense, 5'-CCGCTCGGCCGTGGTGGTGAA-3' antisense, annealing temperature 60°C. Amplification products were visualized by 2% agarose gel electrophoresis and ethidium bromide staining of the whole PCR reaction mix.

Methylation-dependent restriction analysis

Methylation-dependent restriction analysis of genomic DNA was performed as previously described (Zardo and Caiafa, 1998). Briefly, genomic DNAs were extracted by standard methods from human and mouse fibroblasts. DNA (1 μg) was subjected to digestion for 16 hours at 37°C with 20 units of HpaII or MspI (New England Biolabs). The digested fragments (100 ng) were labelled by end-fill reaction in the presence of 0.5 U of polymerase I Klenow fragment (Invitrogen) and 10 μCi of [α-32P]dCTP (Amersham Pharmacia Biotech) in a 30 μl reaction volume for 15 min at room temperature. After ethanol precipitation of the reaction mix, the labelled DNA restriction fragments were evidenced by 2% agarose gel electophoresis followed by autoradiography of the gel.

Methylation-sensitive Southern blot analysis

The DNA methylation level of Alu Sx sequences was evaluated by Southern blot analysis. DNAs (2 μg) were digested with 40 U of MspI or HpaII for 16 hours at 37°C. After 1.5% agarose gel electrophoresis, the digested DNA was blotted on Hybond-N nylon membrane (Amersham-Pharmacia) and the presence of new-HpaII cutting sites was individuated by using two probes (Yandava *et al.*, 1997) directed against Alu –Sx sequences: (Alu 1) 5'-CGACCTCGAGATCTCGGCTCACTGCAA-3' and (Alu 2) 5'-CGACCTCGAGATCTCGGCTCACTGCAA-3'. Labelling of probes and detection was performed using DIG Oligonucleotide 3'-End Labeling Kit and DIG Luminescent Detection Kit (Roche).

Sodium bisulphite reaction

Sodium bisulphite modification of genomic DNA was performed as previously described (Zardo and Caiafa, 1998) with minimal modifications. Briefly, genomic DNAs were extracted by standard methods from human fibroblasts both untreated and treated with 3-ABA. DNA (1 µg) was denatured by adding NaOH to a final concentration of 0.3 M for 15 min at 37°C. For the sulphonation reaction, the DNAs were incubated in the dark for 17 hours at 55°C in the presence of 3.1 M sodium bisulphite, 0.5 mM hydroquinone and 6.25 M urea in a final volume of 0.24 ml at pH 5.0. DNAs were then purified from the mixture reactions using the Wizard DNA Clean-Up system (Promega) and resuspended in 50 µl of water. Alkaline desulphonation of DNA was performed at 37°C for 15 min by the addition of NaOH to the final concentration of 0.3 M. This solution was neutralized by adding ammonium acetate (pH 7.0) to a final concentration of 3.0 M. After ethanol precipitation, the modified DNAs were dissolved in 20 µl of water and stored at -80°C.

Methylation-sensitive PCR

PCR amplification was carried out on 100 ng of bisulphite modified DNA in 25 µl of PCR buffer (Invitrogen) added of 1.5 mM MgCl2, 0.2 mM dNTPs, 0.5 µM of specific methylated (M: 5'-CGTCGTTTTCGTTTATCGTT-3' sense, 5'-ATACCTACCGCCTACGAACA-3' antisense) or unmethylated (U: 5'-GTTTTATTTTTTTGTATAGGGTATTG-3' sense, 5'-CCCCCATAATACCTACCACCT-3' antisense) primer pairs and 0.6 U of Taq DNA polymerase (Invitrogen). Cycling conditions were as follows: 94°C for 4 min followed by 50 cycles of 45 sec at 94°C, 60°C (reduced of 0.2°C after each cycle) for 30 sec, 72°C for 30 sec and a final extention of 10 min at 72°C. The amplification products were visualized by 2% agarose gel electrophoresis and ethidium bromide staining. Primer pairs were designed using MethPrimer software on *Dnmt*1 5' full lenght GpG island (accessed on line at www.urogene.org/methprimer/index1.html).

Genomic bisulphite sequencing

Genomic sequencing analysis of the human Dnmt1 promoter region spanning from -152 to +85 was performed on bisulphite modified DNA (100 ng) from human fibroblasts. The bisulphite modified promoter of Dnmt1 was amplified using the following primers: 5'-

TGGAATTGAGGATTTTATTTAAGG-3' sense and 5'-ATACCCTATACAAAAAAATAAAAC-3'antisense. The amplified DNAs were cloned into the TA-cloning vector (Invitrogen). Six independent clones for each sample were submitted to sequencing procedure (MWG-Biotech). Primer pairs for sequencing were designed using MethPrimer software.

Description of myc-PARG construct

Human PARG cDNA, containing the complete coding region, was isolated by PCR amplification using as template cDNA prepared from poly A+ selected RNA from human adult skeletal muscle. The following oligonucleotides were used in the PCR reaction: 5'-CCGGAATTCAATGAATGCGGGCCCCGGCTGTGAACCC-3' sense, -5'-GCCGCTCGAGTCAGGTCCCTGTCCTTTGCCCTGAATG-3' antisense. The amplified DNA fragment was cloned in the myc-tag expression vector pCS2-MT and sequenced by GeneLab Service (Enea-Casaccia).

Cell transfections

L929 mouse fibroblasts were transfected (using lipofectamine reagent) with either myc-vector empty or myc–PARG construct, together with pBabe Puro vector in a ratio 1:10. After 24 hours cells were incubated for further 72 hours in culture medium supplemented with puromycin (4μg/ml). Cells were then rinsed twice with PBS and lysed and subjected to SDS-PAGE and Western Blotting.

Results

Effect of PARP activity inhibition on Dnmt1 expression

In order to verify whether and how the level and the expression of Dnmt1 are dependent on poly(ADP-ribosyl)ation, inhibition of PARP activity was performed in parallel on mouse (Fig. 11 b and c) and human fibroblasts (Fig. 7) by using 1 mM 3-ABA, the competitive inhibitor of PARP activity and extending the time of treatment with the inhibitor up to 96 hours.

Experiments of Western Blot, carried out in human primary fibroblasts (Fig. 7a and b) show that Dnmt1 protein level increases three-fold *vs* control at early times of treatment with 3-ABA, confirming what had already been seen in mouse cells blocked at different cell-cycle phases (Zardo *et al.*, 2002). Unexpectedly, upon prolonged 3-ABA treatment, a sharp decrease of the protein can be observed. RT-PCR and Real-Time RT-PCR experiments show that the transitory induction of Dnmt1 level, followed by its remarkable decrease, depends on the modulation of *Dnmt*1 gene transcription (Fig. 7c and d).

Proliferation of cell cultures was controlled at different treatment times (up to 96 hours) both for human and mouse fibroblasts. The curves were superimposable thus excluding differences in growth rates between control and treated cells (Fig. 8).

Analysis of the methylation status of CpG islands and of Alu Sx sequences

*Hpa*II digestion of genomic DNA (Bird *et al.*, 1985) was used to investigate a possible correlation between Dnmt1 level and the DNA methylation pattern. The diminishing of radioactive incorporation after digestion of genomic DNA with *Hpa*II (Fig. 9 a and b), the restriction enzyme capable of cutting exclusively CCGG unmethylated sequences, was used to evaluate the anomalous insertion of new methyl groups onto CpG island regions. As control for the amount of DNA loaded in the restriction reactions, a parallel analysis was performed using *Msp*I, the methylation unsensitive isoschizomer of *Hpa*II. Tiny-fragments (100-300 bp), produced following digestion with *Hpa*II of genomic DNA purified from control cells, were quantified by densitometric analysis and their amount considered as 1.0. Fig. 9 c and d shows that the amount of tiny fragments decreases progressively until 72 hours of treatment reaching the values of 0.31 and 0.19 respectively in mouse and human fibroblasts, while it is possible to observe an inversion of the phenomenon at 96 hours of

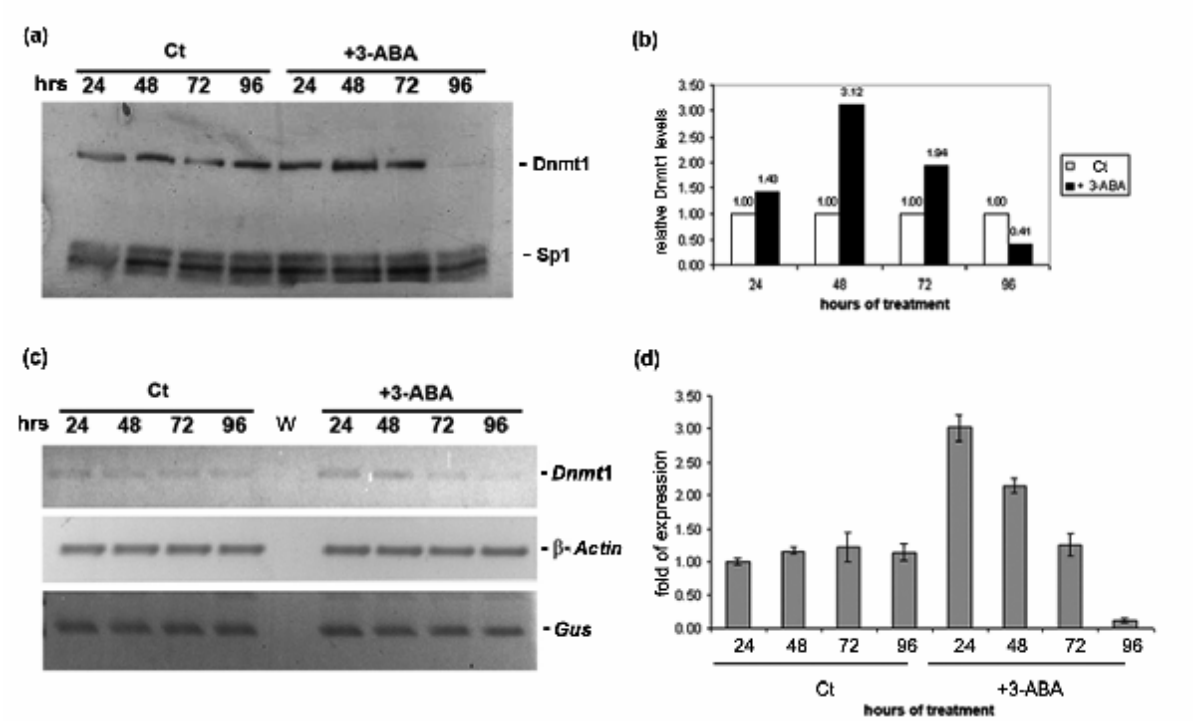
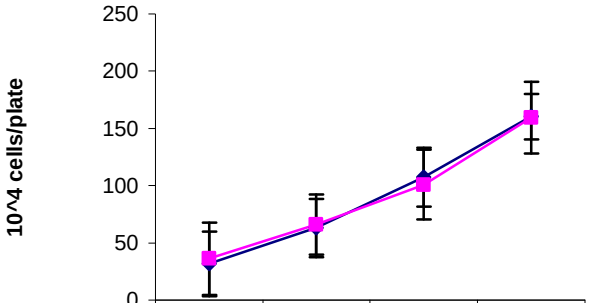


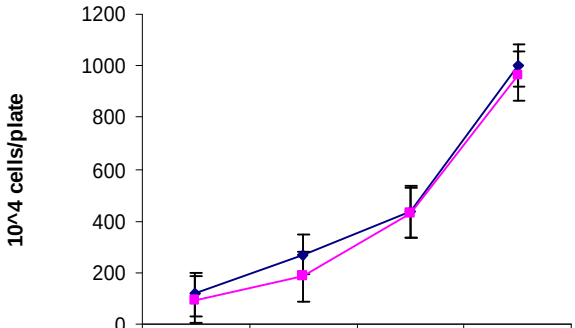
Fig. 7: Effect of PARP activity inhibition on Dnmt1 expression. Nuclear lysates from untreated (Ct) and treated (+ 3-ABA) human fibroblasts were analysed by SDS-PAGE and Western blotting with antibodies specific for Dnmt1 and for Sp1, **(a)**. For each sample, the level of Dnmt1 protein was quantified by the densitometry of the band, which was normalized considering the internal level of Sp1. Dnmt1 levels are represented respect to the level of the untreated samples taken as 1.0, **(b)**. RNAs, isolated from untreated (Ct) and treated (+ 3-ABA) human fibroblasts, were used to perform semi-quantitative RT-PCR in order to evaluate the mRNA levels of Dnmt1 using β-Actin and β-glucuronidase (Gus) as internal controls. (W) refers to the amplification performed in absence of template DNA **(c)**. Dnmt1 mRNA level relative to 18 S rRNA level has been further assessed by Real-Time PCR and results are displayed **(d)** as fold of expression relative to the level measured for Dnmt1 mRNA in the untreated sample at 24 hours (Ct 24) taken as 1.0.

(a)



	24 hrs	48 hrs	72 hrs	96 hrs
Ct	31,705	63	107,33	160,25
ABA	36,25	65,75	100,83	159,25

(b)



	24 hrs	48 hrs	72 hrs	96 hrs
Ct	116.33	269.55	435.35	999.5
ABA	95.75	184	431.75	961.5

Fig. 8: Effect of 3-ABA treatment on proliferation of cultures. Cell proliferation was evaluated at each treatment time for untreated (Ct) and treated (ABA) human (a) and mouse fibroblasts (b) by counting viable cells after trypan blue staining. Counts are expressed as the total number of cells x 10^4 in a 10 cm Perti culture plate. The graphics are an average of three independent experiments.

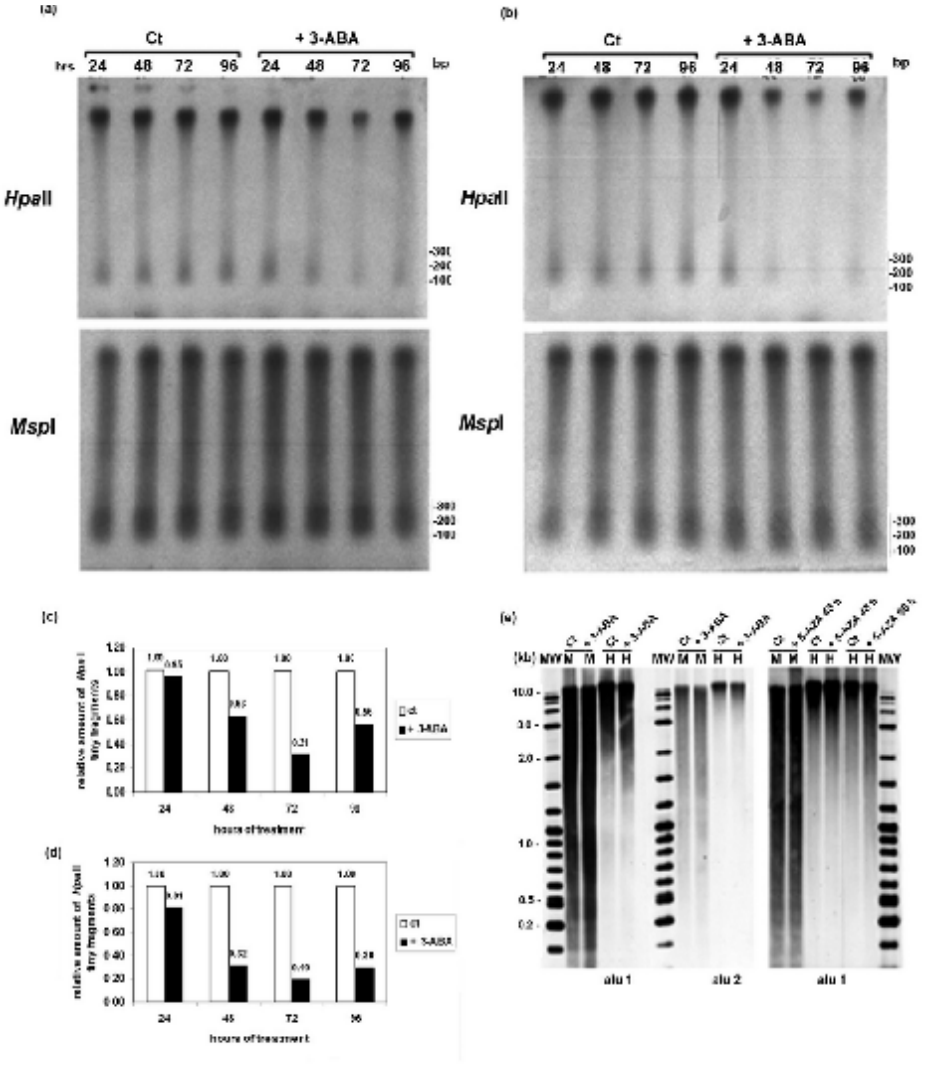


Fig. 9: Analysis of the methylation status of CpG islands and of Alu Sx sequences. Detection of anomalous methyl groups on *Hpa*II CpG island fragments by methylation-dependent restriction analysis was performed on genomic DNAs purified from cells treated with 1 mM 3-ABA (+3-ABA) for 24, 48, 72 and 96 hours *vs* DNAs purified from control cells (Ct). DNA samples from human fibroblasts **(a)** and from mouse fibroblasts **(b)** were digested with *Hpa*II and with *Msp*I and the obtained fragments were labelled by end-fill reaction. The autoradiographyc signal of the labelled fragments ranging from 100 to 300 bp, was quantified by desitometric analysis. Histograms for human **(c)** and mouse **(d)** fibroblasts show the amount of *Hpa*II tiny fragments *vs Msp*I fragments for each sample. The relative amount of digested fragments from untreated samples was taken as 1.0. The methylation *status* of Alu Sx repeated sequences was investigated by Southern blot **(e)** on DNAs purified from human fibroblasts both untreated (Ct) and treated for 96 hours in the presence of 3-ABA (+3-ABA). DNA samples were digested with the methyl-sensitive enzyme *Hpa*II (H) and its insensitive isoschizomer *Msp*I (M, control for the amount of DNA). The presence of new *Hpa*II cutting sites were visualized using two probes directed against two different Alu-Sx sequences (*Alu*1 and 2). As positive control for DNA demethylation, digestion was performed in parallel on DNA from cells treated for 48 and 96 hours with 5-AZA.

treatment. In fact, at this latter time the values are respectively 0.56 and 0.30. What we detect after shorter times of treatment confirms our previous data (Zardo and Caiafa, 1998) and the the lower quantity of inhibitor used during the cell treatment likely explains the need for longer times to see the decrease of tiny fragment level. The recovery of *Hpa*II tiny fragments observed at 96 hours is a novel finding of great interest because it reveals an unexpected demethylation event.

As further confirmation for demethylation, Southern-Blot analyses were carried out using two different probes against Alu Sx elements (Yandava *et al.*, 1997) since these are enriched in CpG dinucleotides. Southern-blotting was carried out on DNAs purified from cells treated and untreated for 96 hours with 3-ABA after digestion of both samples with *Hpa*II. Fig. 9e shows that DNA purified from 3-ABA treated cells has undergone increased digestion by *Hpa*II due to the presence of new demethylated cutting sites within the Alu sequences. The pattern observed was similar to the one observed after treatment of cells with 5 μM 5-aza-deoxycytidine (5-AZA) for 96 hours.

Effect of PARP activity inhibition on the methylation pattern of the CpG island located in 5' regulatory region of human Dnmt1

DNA demethylation (Bird, 2003) may depend on either an active process, played by a DNA demethylating enzyme that could intervene as consequence of the spreading of anomalous methylation on DNA, or on a passive process in which Dnmt1, the enzyme able to guarantee the correct maintenance DNA methylation, is silenced. Considering the above mentioned results, one molecular mechanism that could explain the silencing of *Dnmt*1 is the alteration of the methylation pattern of its promoter after inhibition of PARP activity. To verify such hypothesis the methylation pattern of the CpG island located at *Dnmt*1 5' region (Fig. 10 a) was analysed by bisulphite reaction (Frommer *et al.*, 1992). As preliminary approach methylation-sensitive PCR (MSP) was performed on the first exon of *Dnmt*1 human gene. The region to examine was selected according to "MethPrimer" software evaluation. MSP detects a partial methylation of the chosen sequences since both methylated and unmethylated specific primers give a PCR product when DNA was purified from cells treated for 96 hours with 3-ABA (Fig. 10 b). The sequencing of a DNA promoter region was selected as further investigation to analyze its methylation state. As shown in Fig. 10 c, all twelve CpG dinucleotides present in the fragment are almost fully methylated unlike respective controls, where they are completely unmethylated.

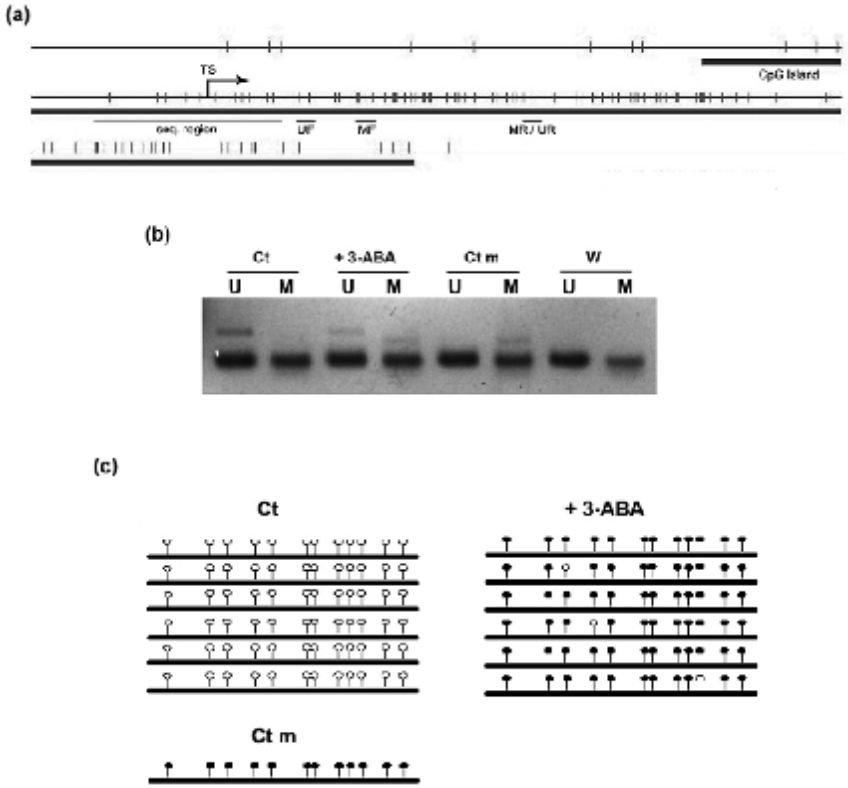


Fig. 10: Effect of PARP activity inhibition on the methylation pattern of the CpG island located in 5' regulatory region of human *Dnmt*1. Graphycal representation **(a)** of the CpG island region (dark gray line) colocalizing with the transcription start site (TS) of *Dnmt*1 ("CpG Island Searcher", on-line at http://cpgislands.usc.edu/) and GpG (dashes) distribution within the 5' region of human *Dnmt*1 gene. Genomic DNAs were extracted from human fibroblasts both untreated (Ct) and treated for 96 hours with 3-ABA (+3-ABA), and bisulphite reaction was performed to obtain a methylation specific modification of the DNA sequence. **(b)** The methylation *status* of CpG dinucleotides within *Dnmt*1 promoter was assayed by methylation-sensitive PCR analysis (MSP). Amplification was carried out adopting methylation (MF and MR) or unmethylation (UF and UR) specific primer pairs. MSP analyses of DNA purified from untreated cells (Ct) and the same DNA sample methylaled with bacterial Sss1 *in-vitro* (Ct m) were taken respectively as negative and positive controls of methylation. (W) refers to amplification performed in absence of template DNA. The amplification products were visualized by 2% agarose gel electrophoresis and ethidium bromide staining.

The methylation *status* of *Dnmt*1 promoter region spanning from -152 to +85 **(a)** was further evaluated by bisulphite sequencing **(c)** following 3-ABA treatment**.** Six independent clones for each sample were analysed by sequencing procedure. Each row of circles represents the sequence of an individual clone. Open circle, unmethylated CpG site; filled circle, the methylated one.

Therefore, a deregulation of PARP activity allows the introduction of anomalous methyl groups on the CpG island present on *Dnmt*1 promoter.

Dnmt1 silencing depends on both PARG over-expression and PARP activity inhibition in mouse fibroblast cells

Experiments of poly (ADP-ribose) glycohydrolase (PARG) over-expression - the enzyme responsible for dynamic ADP-ribose polymer degradation in nuclei (D'Amours *et al.*, 1999) – were performed on mouse cells to evaluate a direct involvement of ADP-ribose polymers in the regulation of Dnmt1 expression excluding secondary effects due to treatment of cells with 3-ABA.

Fig. 11 a shows that over-expression of this enzyme causes a sharp degradation of ADP-ribose polymers associated with a remarkable reduction of Dnmt1 protein level. This result is in agreement with the decrease of Dnmt1 observed in mouse cells treated for prolonged times with 3-ABA (Fig. 11 b and c) and suggests that ADP-ribose polymers are involved in the control of Dnmt1 expression.

PARP activity inhibition does not affect the mRNA level of other *de novo* DNA methyltrasferases

An mRNA level time course of the known members of the DNA methyltrasferase enzyme family (Dnmt1, Dnmt3a and Dnmt3b) was performed following inhibition of poly(ADP-ribosyl)ation in human fibroblasts by semi-quantitative RT-PCR. As shown in fig. 12, 3-ABA treatment of cells does not affect Dnmt3a and 3b mRNA levels, which remain comparable to untreated controls at each treatment time, while an initial induction followed by down-regulation is detectable as already shown for Dnmt1 mRNA level.

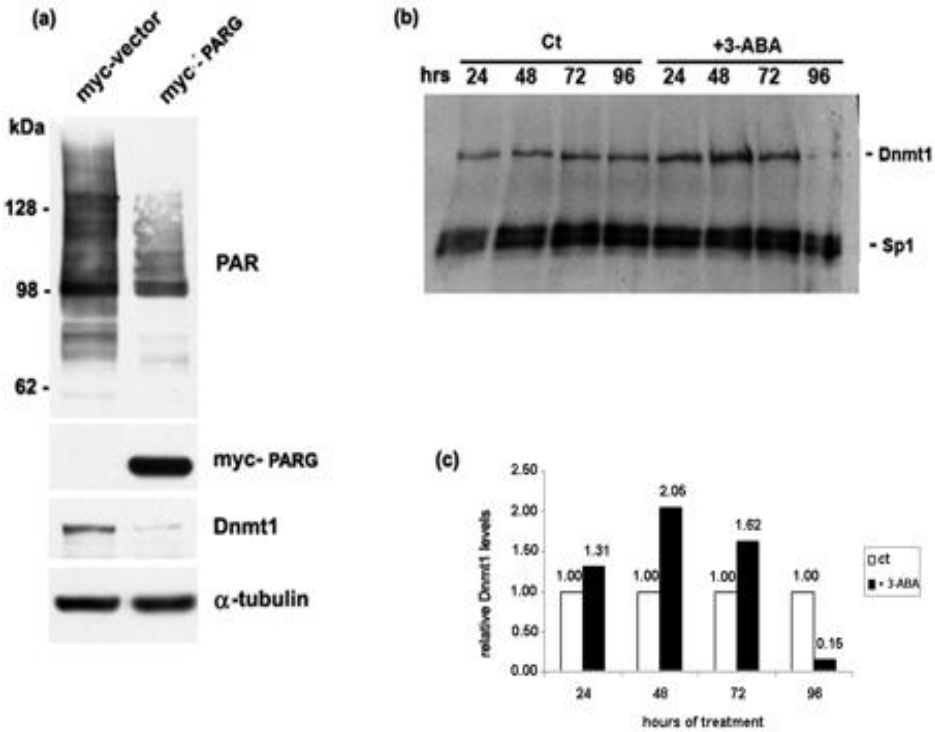


Fig. 11: Dnmt1 silencing depends on both PARG over-expression and PARP activity inhibition in mouse fibroblast cells.

Whole-cell extracts of L929 cells trasfected with myc-vector and myc-PARG analysed by Western blotting after 3-days selection in puromycin. The blot was incubated with antibodies specific for poly(ADP-ribose) (PAR), myc, Dnmt1 and α-tubulin **(a)**. Nuclear lysates from untreated L929 cells (Ct) and from the treated ones (+ 3-ABA) were analysed by SDS-PAGE and Western blotting with antibodies specific for Dnmt1 and for Sp1, **(b)**. For each sample, the level of Dnmt1 protein was quantified by the densitometry of the band, which was normalized considering the internal level of Sp1. Dnmt1 levels are represented respect to the level of the untreated samples taken as 1.0, **(c)**.

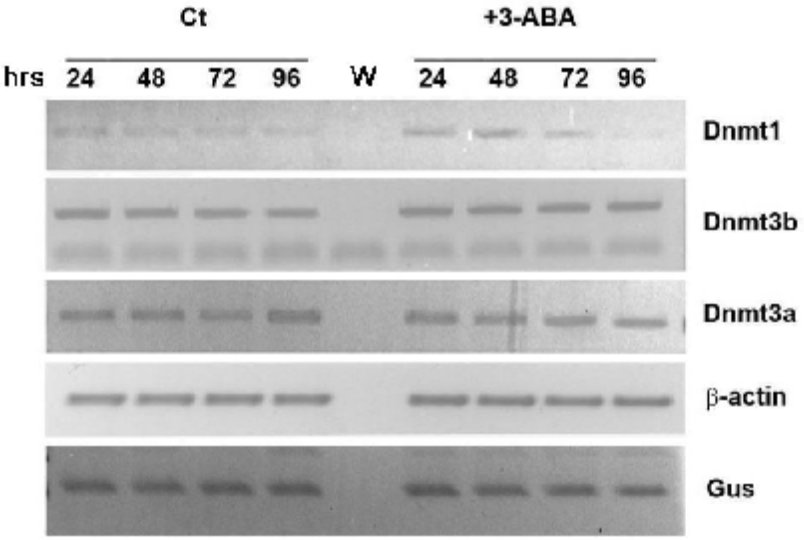


Fig. 12: Effect of PARP activity inhibition on mRNA levels of the most important DNA methyltransferases.

RNAs isolated from untreated (Ct) and treated (+ 3-ABA) human fibroblasts were used to perform semi-quantitative RT-PCR in order to evaluate the mRNA levels of Dnmt1, Dnmt3a, Dnmt3b using β-actin and β-glucuronidase (Gus) as internal controls. (W) refers to the amplification performed in absence of template DNA.

Discussion

In conclusion these results confirm that by reducing PARP activity (after 24-48 hours of treatment with 3-ABA) there is a threefold increase in Dnmt1 level (Zardo *et al.*, 2002) and modification of the genomic methylation pattern (Zardo *et al.*, 1997; de Capoa *et al.*, 1999; Zardo *et al.*, 1999; Zardo and Caiafa, 1998). Additional data also indicate that, following inhibition of PARP activity, the promoter of *Dnmt*1 is no longer protected from anomalous methylation.

These novel findings suggest a complex pathway focused on Dnmt1 in which the decreased activity of PARPs leads, in turn, to induction of *Dnmt*1 expression, to insertion of anomalous methyl groups onto CpG island located in the promoter region of *Dnmt*1 and to its transcriptional silencing that seems, therefore, to be responsible for a diffuse hypomethylation of genomic DNA.

To have identified a CpG island protected from aberrant *de novo* methylation by poly(ADP-ribosyl)ation gives us the chance to put forward a mechanism by which PARP activity takes part in the protection of CpG islands. As has been said in the introduction, several models of mechanism(s) by which CpG islands are protected from the action of DNA methytransferases have been greatly debated. DNA methylation has the propensity to spread from methylated sites to adjacent unmethylated ones unless “boundary elements” are present (Turker, 2002). It has been suggested that a static barrier, protecting GpG islands from methylation, could be formed by transcription factors that could compete with Dnmts for sites within the CpG island preventing their methylation. This barrier action of trascription factors would be partially overcome by abnormal high levels of Dnmts (Vertino *et al.*, 1996). Considering the inhibitory effect played by ADP-ribose polymers on Dnmt1 activity, the high affinity of this enzyme for ADP-ribose polymers and the existence *in vivo* of a complex between poly(ADP-ribosyl)ated PARP1 and Dnmt1 (Reale *et al.*, 2005), a poly(ADP-ribosyl)ated structural protein, or a transcription factor or an automodified member of the PARP family could be the element able to inhibit the Dnmt1 activity and block Dnmt1 access to CpG islands. Thus, following inhibition of poly(ADP-ribosyl)ation Dnmt1 loses its inhibitory tie with ADP-ribose polymers and gains access to unmethylated CpG dinucleotides (fig. 13). There is a growing list of DNA binding protein acceptors of poly(ADP-ribose) (D’amours *et al.*, 1999) and the evaluation of the occupancy of Dnmt1 promoter by a poly(ADP-ribosyl)ated isoform of such a protein could be a favorable approach to the

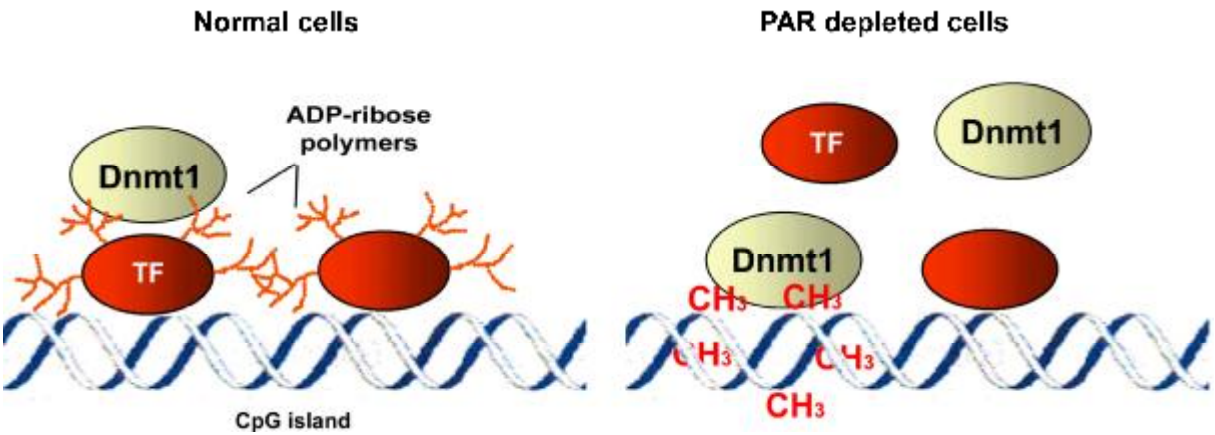


Fig. 13: Mechanism suggested for the ADP-ribose-mediated protection of the unmethylated *status* of the CpG island located in *Dnmt*1 promoter region. In normal cells a poly(ADP-ribosyl)ated transcription factor (TF) bound to the CpG island located on the *Dnmt*1 promoter, links in a non covalent way Dnmt1 thus preventing its access GpG dinucleotides. In fact, Dnmt1is attracted to them rather than to DNA. Following this binding the catalytic activity of Dnmt1 is inhibited (Reale *et al.*, 2005). Decrease of ADP-ribose polymers (PAR) both by treatment of cells with inhibitors of PARP activity and by over-expression of PARG allows the introduction of new methyl groups onto the CpG island since low level of PAR de-represses Dnmt1 catalytic activity.

understanding of the mechanism(s) by which ADP-ribose polymers mediate the protection of CpG islands.

Data here reported demonstrate that the decrease of ADP-ribose polymers leads to a deregulation of the genome-wide methylation pattern suggesting that both hypermethylation of CpG islands and widespread hypomethylation could depend on poly(ADP-ribosyl)ation.

This has a particular relevance considering that hypermethylation of CpG island promoter regions of tumour suppressor genes (Costello *et al.*, 2000; Robertson, 2002; Jones and Baylin, 2002; Baylin and Ohm, 2006) and widespread DNA hypomethylation (Hoffmann and Schulz, 2005) are a relevant epigenetic event occurring in cancer (Fig. 14). Moreover, a great amount of research has been carried out over the years to see if the nuclear level of Dnmt1 is by itself capable of causing the aberrant methylation pattern in tumour cells (Vertino *et al.*, 1996;

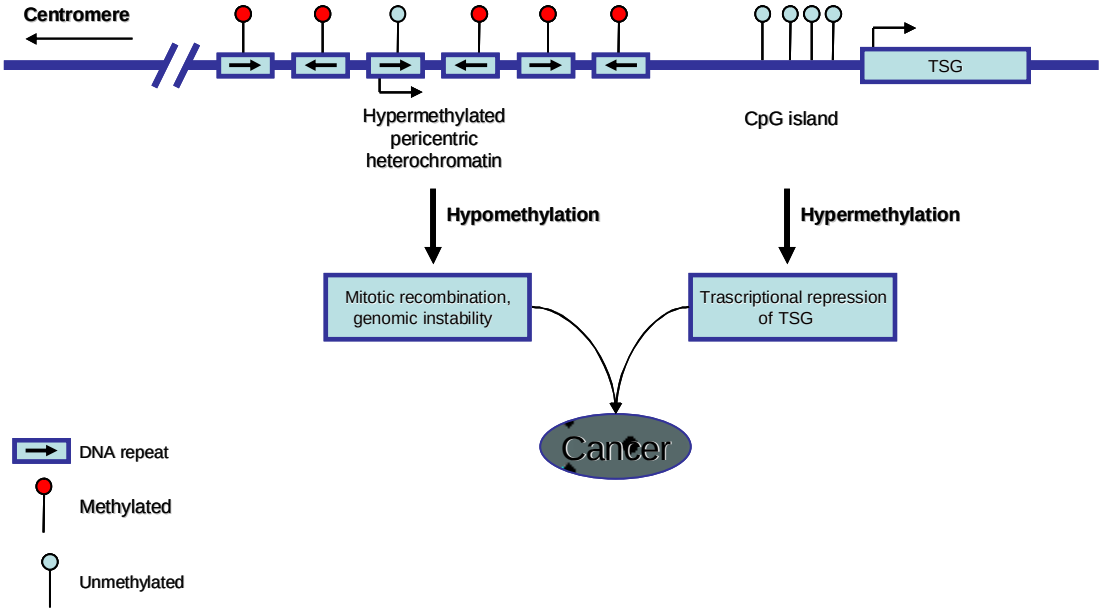


Fig. 14: DNA methylation and cancer. Graphical display of a representative region of genomic DNA in a normal cell and tumour cells where repeat-rich heterochromatin becomes hypomethylated and this contributes to genomic instability, through increased mitotic recombination events. *De novo* methylation of CpG islands also occurs in cancer cells, and this can result in the transcriptional silencing of some tumor suppressor genes (TSG).

Baylin, 1997; Eads *et al.*, 1999; Feltus *et al.*, 2003; Rhee *et al.*, 2000; Rhee *et al.*, 2002; Leu *et al.*,2003; Robert *et al.*, 2003; Ting *et al.*, 2004¸ Suzuki *et al.*, 2004) and in cells where Dnmt1 was stably over-expressed (Vertino *et al.*, 1996) it has been possible to observe anomalous methylation of some tumour-suppressor genes. Subsequent research, carried out on the same cells, has shown that not all the CpG islands undergo methylation but only a few are prone to methylation when Dnmt1 was over-expressed (Feltus *et al.*, 2003). The silencing

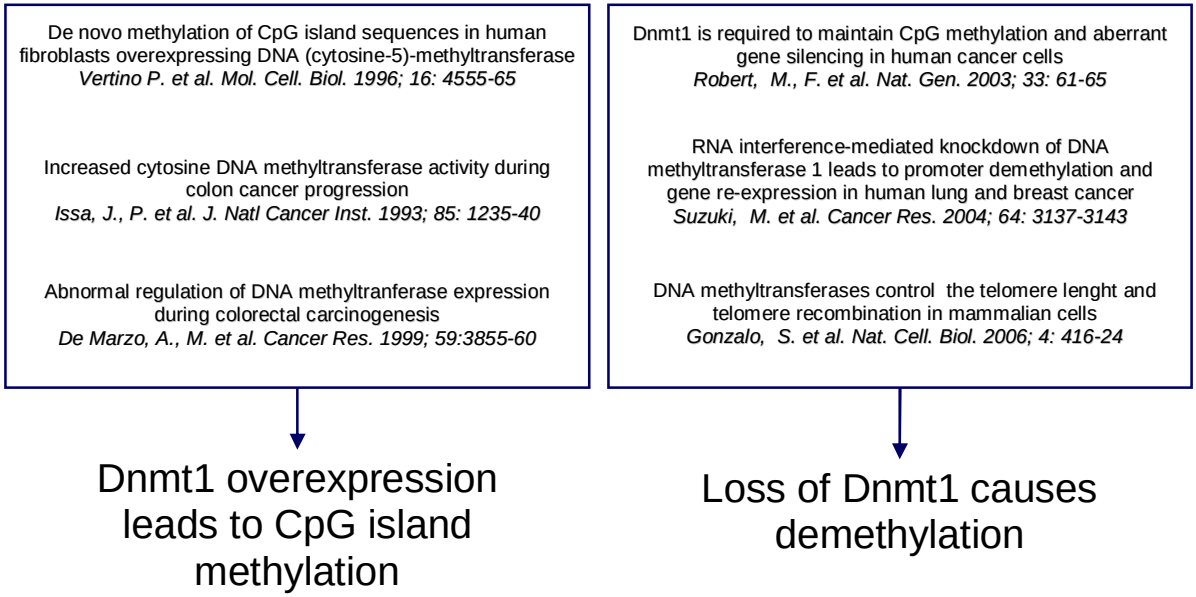


Fig. 15: Levels of Dnmt1 control DNA methylation pattern of genome

of Dnmt1 by RNA interference has often been performed to establish if it leads to demethylation and re-expression of oncosuppressor genes in tumor cells (Leu *et al.*,2003; Robert *et al.*, 2003; Ting *et al.*, 2004; Suzuki *et al.*, 2004). Of great interest are findings showing that Dnmt1 silencing allows also demethylation and re-expression of some germ-line specific genes whose repression is methylation-dependent in somatic cells (Loriot *et al.*, 2006; Fang *et al.*, 2006). As the promoters of these genes become demethylated in many tumour cells, this observation open up the possibility that a passive demethylation, due to silencing of Dnmt1, is involved in determining diffuse genome-wide hypomethylation . Of great interest is the recent finding that demethylation of sub-telomere CpG rich regions in Dnmt1-depleted cells lead to telomeres elongation (Gonzalo *et al.*, 2006) (Fig. 15)

In this context the silencing of Dnmt1 dependent on inhibition of PARP activity could represent a new experimental approach for the understanding of the role played by Dnmt1 in determining the aberrant methylation patterns in tumour cells. What is remarkable is that the demethylated pattern found in Alu sequences after treatment of cells with 3-ABA for 96 hours

is very similar to the one found on DNA from cells treated with 5-AZA for the same time. The discovery of a DNA demethylating activity dependent on the use of inhibitors of poly(ADP-ribosyl)ation process increases the knowledge of mechanism by which these inhibitors enhance the cytotoxicity of other anticancer agents (Jagtap and Szabo, 2005).

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During the Ph.D. course

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Oral presentations to scientific meetings

Reale, A., **Zampieri, M.,** De Matteis, G., Caiafa, P. **La poli(ADP-ribosilazione) è coinvolta nella modulazione dell'attività di Dnmt1**. XVII Convegno "I processi di Poli ADP- ribosilazione"**.** Università degli Studi di Roma «La Sapienza», 17-18/12/2004 Roma, Italia.

Zampieri, M., Guastafierro, T., Cecchinelli, B., Reale, A., Caiafa, P. **PARylation controls the expression level of Dnmt1.** XVIII Convegno "I processi di Poli ADP-ribosilazione"**.** Università degli Studi di Verona, 04-05/10/2005 Verona, Italia.

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Zampieri, M., Passananti, C., Corbi, N., Reale, A., Guastafierro, T., Cecchinelli, B., Calabrese, L., Caiafa, P. **La poli-ADP-ribosilazione protegge lo stato non metilato della regione promotrice di *DNMT1***. XIX Convegno "I processi di Poli ADP-ribosilazione"**.** Università degli Studi di Napoli Federico II, Complesso Universitario di M.S. Angelo. 09-10/11/2006 Napoli, Italia.