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Location and translocation of CTCF in distinct cell lines

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I declare this thesis is original author work, which I disposed by myself. All used literature and others sources are mentioned in schedule of references and all sources are correctly cited in work.

Abstract

Location and translocation in distinct cell lines

CCCTC-binding factor (CTCF) is a versatile zinc finger protein with diverse regulatory functions such as cell growth, differentiation, apoptosis, enhancer-blocking activity and control of imprinted genes. The present work is aimed to clarify the contribution of poly(ADP-ribosylation) in CTCF regulation. CTCF is expressed in various isoforms, with two prominent bands at 130 kDa and 180 kDa in Raji, ARO, NPA and HeLa tumour cells untreated or treated with sodium butyrate. In particular, the latter band at 180 kDa represents a poly(ADP-ribosyl)ated isoform of CTCF which is significantly evident in all treated tumour cell lines. CTCF is usually localized in the nucleus and its subcellular distribution during the cell cycle is dynamic. Redistribution of CTCF in the nucleus may be important to trigger and sustain necessary metabolic changes leading to cell growth arrest and, further, to terminal differentiation and apoptosis. We demonstrated that CTCF was present all over the nuclei in untreated Raji cells, whereas CTCF is also concentrated in the nucleoli in treated ones. Translocation of CTCF was not evidenced in treated thyroid tumour and HeLa cells and was distributed in the cytosol, predominantly concentrated at the nuclear periphery in both untreated and treated ARO and NPA cells. Our preliminary data suggest that CTCF migrates in nucleoli through a poly(ADP-ribosyl)ation-dependent mechanism in Raji cells, but more experiments are needed further to elucidate this phenomenon.

Abstrakt

Lokace a translokace CTCF v různých buněčných liniích

CCCTC-vazebný faktor (CTCF) je polyfunkční protein s doménou zinkového prstu. Svými rozličnými regulačními funkcemi zasahuje do regulace buněčného růstu, diferenciace a apoptózy, má aktivitu blokující funkci funkci zesilovačů a kontroly imprintovaných genů. Tato práce kladla za cíl objasnit příspěvek poly(ADP-ribosyl)ace v regulaci CTCF. V buňkách ARO, NPA, Raji a HeLa inkubovaných za kontrolních podmínek a s butyrátem sodným jsme zjistili expresi CTCF v různých isoformách se dvěma významnými proužky o velikosti 130 kDa a 180 kDa. Zejména druhý zmiňovaný proužek v 180 kDa představuje poly(ADP-ribosyl)ovanou isoformu CTCF, která je zvláště patrná ve všech buňkách vystavených butyrátu sodnému. CTCF je obvykle lokalizován v jádře a jeho subcelulární distribuce během buněčného cyklu je dynamická. Redistribuce CTCF v jádře může být důležitá pro spuštění a udržení potřebných metabolických změn vedoucích k zastavení buněčného růstu, terminální diferenciaci a apoptóze. V této práci jsme prokázali, že u neovlivněných Raji buněk je CTCF přítomný v jádře, zatímco u ovlivněných butyrátem sodným je koncentrován také v jadérku. Translokace CTCF nebyla prokázána u kontrolních tyroidních nádorových a HeLa buněk, byl zde distribuován v cytosolu, u kontrolních a butyrátem sodným ovlivněných ARO a NPA buněk převážně koncentrovaný v jaderné periferii. Předběžně ze získaných dat usuzujeme, že CTCF v Raji buňkách přechází do jadérka na poly(ADP-ribosyl)aci závislém mechanismu. K objasnění tohoto jevu budou provedeny další experimenty.

Contents

Introduction

The genome of eukaryotes is partitioned into transcriptionally active and transcriptionally inactive domains. Insulators are DNA elements that maintain this partition, and they can be subdivided into two functional classes: barrier elements, which stop the spread of heterochromatin, and enhancer blockers, which prevent an enhancer from activating transcription in a neighbouring repressed region.

At present, research groups have focused on CTCF, new discovered transcription factor, which could be involved in processes associated with cancer triggering.

CTCF was originally isolated as a transcription factor, which recognized a CTC-rich sequence in the *c-myc* promoter. Over the years, CTCF has been shown to have complex and important roles in the control of gene expression. CTCF binds many different DNA target sequences through the combinatorial use of its 11 zinc fingers, and is capable of both activating and repressing gene transcription.

An additional role of CTCF is to act as an enhancer blocker that prevents communication between an enhancer and promoter of target gene. This process is known as transcriptional insulation. CTCF and YY1 are the main vertebrate proteins known to act as enhancer blockers. CTCF exerts this critical function at many loci. For example, enhancer blocking by CTCF permits correct expression of the imprinted genes H19 and IGF2.

In the past, significant advances were made in our understanding of how CTCF functions as an enhancer blocker at the 5´chicken β-globin insulator. At this insulator site, CTCF interacts with nucleophosmin, nuclear matrix protein that is concentrated at the surface of the nucleolus. This is thought to result in the formation of physically separated DNA loops, which would then prevent an enhancer element in one chromatin loop from acting on a gene in the neighbouring chromatin loop. It is likely that this model also accounts for CTCF action at other insulators, and yet alternative mechanisms cannot be ruled out at this point.

The activity of enhancer blockers is not static but can turn on and off. Recent experiments with CTCF have shown that post-translational modification of the protein plays an important regulatory role. In addition, the function of CTCF can also be regulated through interacting proteins.

Theory

CTCF

CCCTC-binding factor (CTCF), is a nuclear protein ubiquitously expressed in many different proliferating and differentiated cell types. CTCF is transcription factor that is highly conserved among distinct organisms.

CTCF plays important roles in the regulation of epigenetics and gene transcription. As a multifunctional protein, CTCF is also involved in the regulation of cell proliferation and of apoptosis.

Depending on the promoter context and cell background, CTCF may repress or activate transcription, however, its repression function predominates. CTCF was originaly found as a represor of the c-myc oncogene but was later characterized to be involved in enhancer blocking, chromatin insulation, and imprinting on diverse genes, such as the β –globin, c-Myc, and Igf2-H19 genes.

Zinc finger family

CTCF is a member of zinc finger superfamily. CTCF consists of central eleven zinc-finger domain, C-terminal domain, and N-terminal domain. A zinc finger consists of two antiparallel β sheets, and an α helix. The zinc ion is crucial for the stability of this domain type (Bulyk et al., 2001).

Central domain can bind to DNA. CTCF interacts with the major groove along the double helix of DNA in which case the zinc fingers are arranged around the DNA strand in such a way that the α-helix of each finger contacts the DNA (Bulyk et al., 2001).

The C-terminal zinc fingers are necessary for targeting of CTCF to mitotic chromosomes (Torrano et al., 2006).

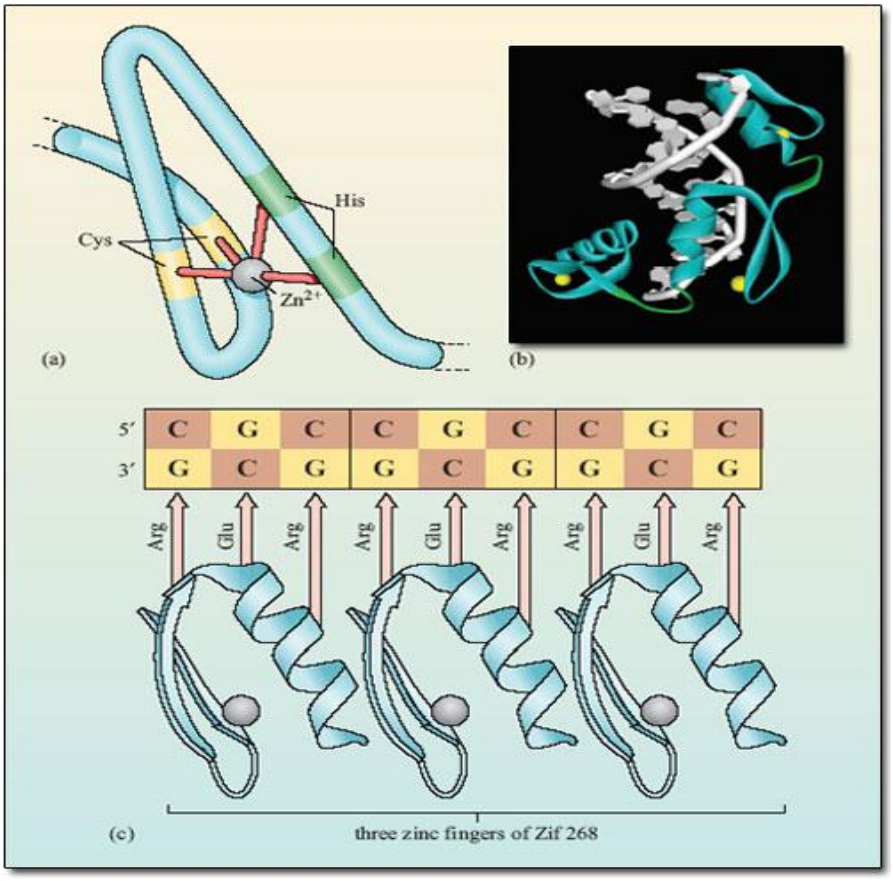

Figure 1: (a) Zinc finger domains coordinate a single zinc ion and are either Cys–His (as here) or Cys–Cys types. (b) Molecular model view of the binding of the mouse Zif 268 protein to DNA: three zinc fingers (cyan) lie within the major groove of the helix; the zinc atoms are shown in yellow. (c) Specific recognition of a target DNA by Zif 268 is a result of interactions between three amino acids and a triplet recognition sequence for each zinc finger (Bulyk et al., 2001).

CTCF binding sites

Combinatorial use of its multiple zinc finger allows CTCF to bind dissimilar target sites. CTCF binds to DNA sequences, each is 50 bp long, with high concentration of GC nucleotides. These target sites were characterized within promoters, silencers and insulators. It was investigated that CTCF also recognizes the 21 bp CpG- rich sequence repeats located within a 2 kb „imprinting control region“ (ICR) that lies between the Igf2 and H19 genes (Wolffe, 2000). These regions are also attractive for methyltransferases.

CTCF is sensitive to DNA methylation. In presence of DNA methylation CTCF is not able to bind to DNA. On the contrary, in anbsence of DNA methylation CTCF can bind to DNA (Wolffe, 2000).

Localization of CTCF

In the majority of cells, CTCF is localized in the nucleus, independently of its phosphorylation state. The CTCF subcellular distribution during the cell cycle is dynamic and it has been found to be associated with mitotic chromosomes, mitotic centrosomes as well as the midbody of the cytokinesis (Torrano et al., 2006).

In undifferentiated K562 cells (leukaemia cells), CTCF is diffusely distributed throughout the nucleoplasm, but not concentrated in nucleoli. Cellular differentiation leads to the accumulation of CTCF in the nucleoli (Torrano et al., 2006).

In another model system MCF7 breast cancer cells were used, that were treated with sodium butyrate, which is known inhibitor of histone deacetylase activity and inducer of G2/M growth arrest, and apoptosis in MCF7 cells. In untreated MCF7 cells CTCF was diffusely distributed in the nucleoplasm, but strongly accumulated in nucleoli after sodium butyrate treatment (Torrano et al., 2006).

The localizatin of CTCF in nucleoli may not only be signal mediated, but dependent on RNA- binding, which would involve other components such as RGG box and GAR box motifs (Torrano et al., 2006).

In nucleolus CTCF was found homogenously distributed throughout the dense fibrillar and granular components. These results suggest that CTCF function in the nucleoli may be associated with synthesis and processing of pre-rRNA (dense fibrillar component) and pre-ribosomal (granular component) assembly (Torrano et al., 2006).

Translocation of CTCF from nucleoplasm to nucleolus

CTCF is predominantly nucleoplasmic protein in the majority of cells and its translocation to the nucleolus is likely to be a dynamic process and consequence of functional interactions with other macromolecules. This resembles the situation with MYC, which is usually a nucleoplasmic transcription factor rarely found in nucleoli in normal cells. Nevertheless, MYC plays an important role in regulation of rDNA transcription. A number of nuclear factors have been found to be transiently present in the nucleoli, continously exchanging with the nucleoplasm. Such dynamic interaction with the nucleolus often depend on the metabolic state of the cell (Torrano et al., 2006).

CTCF shifts from nucleoplasm to the nucleolus in response sodium butyrate treatment. Such redistribution of CTCF in the nucleolus may be important to trigger and sustain necessary metabolic changes leading to cell growth arrest and, further, to terminal differentiation and apoptosis (Torrano et al., 2006).

Experiments have demonstrated that the region responsible for nucleolar targeting of CTCF is localized within the central zinc-finger domain. The N-terminal portion of CTCF failed to localize in the nucleolus, the C-terminal portion of CTCF showed a diffuse nucleoplasmic distribution of the fusion protein, including the nucleolar compartment. Interestingly, when the zinc finger domain is divided in two parts they can still mediate nucleolar targeting, thus indicating that there may be two regions in the DNA-binding zing-finger domain of CTCF that are important for such localization (Torrano et al., 2006).

Targeting of CTCF to the nucleolus requires on going rDNA transcription and protein synthesis. This suggests a dynamic exchange of CTCF between the nucleolus and the nucleoplasm rather than the passive storage of this factor in the nucleolar compartment and also points to existence of a protein interaction network important for CTCF translocation in the nucleolus (Torrano et al., 2006).

Current study has shown the involvement of CTCF in the inhibition of the nucleolar transcription. It was investigated that full-length CTCF, but not the CTCF- zinc finger domain, dramatically inhibits nucleolar transcription. Results showing nucleolar accumulation of endogenous CTCF in growth-arrested cells. Thus we can suggest that nucleolar location of CTCF may be an important mechanism to simultaneously block cell proliferation and transcription from rDNA (Torrano et al., 2006).

Regulation of Igf2/H19 imprinted locus

The ICR of Igf2/H19 is differentially methylated between paternal and maternal chromosomes and is a key regulatory element of the Igf2-H19 locus. These genes lie in the same transcriptional orientation, separated by 90 kb. The maternal copy of the Igf2 gene is normally silent whereas the paternal copy is active. In contrast, the maternal H19 gene is active and the paternal copy is repressed. The silent paternal H19 gene is heavily methylated upstream of the promoter (Wolffe, 2000).

Methylation of CpG dinucleotides within the imprinting control region directly prevents CTCF binding. This methylation is necessery both for repression of the H19 gene and for activation of the Igf2 promoter (Wolffe, 2000).

At the 3´ end of the H19 gene is an enhancer that is required for the trascription of both Igf2 and H19. In this model, methylation of the H19 promoter on the paternal chromosome would silence the H19 gene and release the enhancer to activate the Igf2 promoter (Wolffe, 2000).

When the imprinting control region within the Igf2-H19 locus is unmethylated, as found on maternal chromosomes, CTCF binds to the insulator element between the two genes. The insulator-CTCF complex acts to block the Igf2 gene from the enhancer that is positioned 3´of H19. As a consequence, only the H19 gene is active (Wolffe, 2000).

When the ICR and the H19 gene are methylated, as found on paternal chromosomes, CTCF fails to bind to the insulator and so the 3´enhancer can

activate the Igf2 promoter. Also, the H19 promoter and flanking sequences are silenced by methylation, potentially via the assembly of specialized repressive chromatin (Wolffe, 2000).

Methylated DNA is known to recruit methyl CpG binding proteins that interact with histone deacetylase, which modifies the histones and directs the dominant silencing of genes. Both the transcription of the H19 gene and the activity of the insulator are controlled by methylation patterns that are parent-of-origin specific (Wallace and Felsenfeld, 2007).

It has been demonstrated that CTCF protects the maternal allele ICR againts DNA methylation (Recillas-Targa et al., 2006).

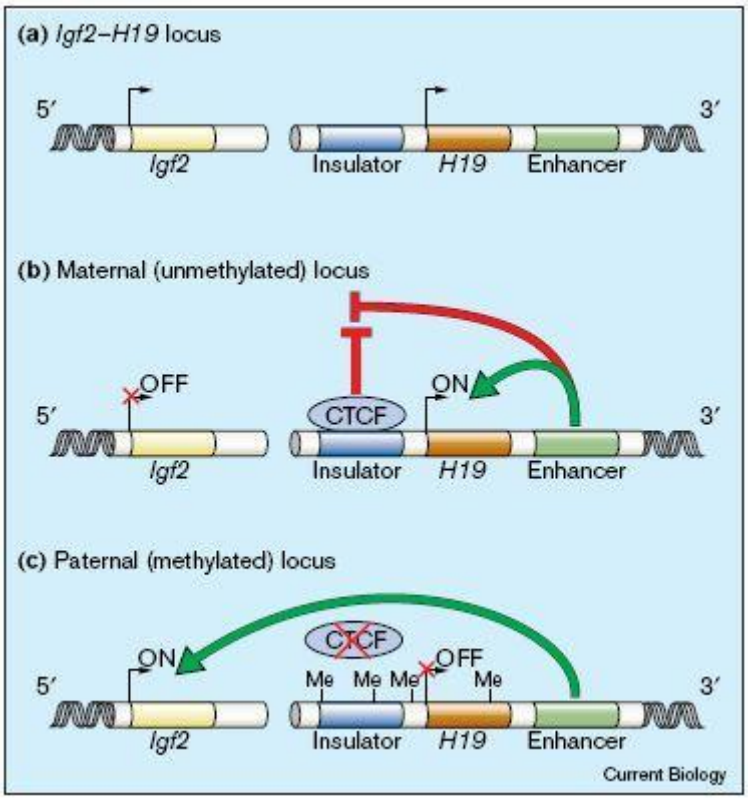

Figure 2: Regulation of Igf2/H19 locus (Wolffe, 2000).

CTCF and non-coding transcripts

Normal individuals have between 5 to 38 CTG repeats whereas in pathological condition this can rise up to thousands of repeats and the lenght of such amplified region correlates with the severity of disease. An outstanding

observation showed that on wild-type alleles the CTG repeat region is flanked by CTCF binding sites which define heterochromatin conformation strictly to the CTG repeats (Recillas-Targa et al., 2006).

In aberrant conditions, when there is expansion of the CTG repeats, the heterochromatin spreads and silence adjacent genes. Such abnormal chromatin spreading involves DNA methylation that prevents binding of CTCF thereby disrupting insulator function what may contribute to CTG repeat expansion. The spreading will cause the epigenetic silencing of gene expression without presence of any genetic defects (Recillas-Targa et al., 2006).

CTCF could have the capacity to block the spreading of repressive chromatin marks that originate in non-coding regions of the genome corresponding to repetitive elements. Displacement of CTCF will cause the expansion of abnormal epigenetic silencing over the promoter and beyond the transcription initiation site. In the abnormal expanded allele CTCF is not able to bind its recognition sequence, mainly because there is an increase on DNA methylation, causing the expansion of CTG repeats and associated heterochromatin marks (Recillas-Targa et al., 2006) .

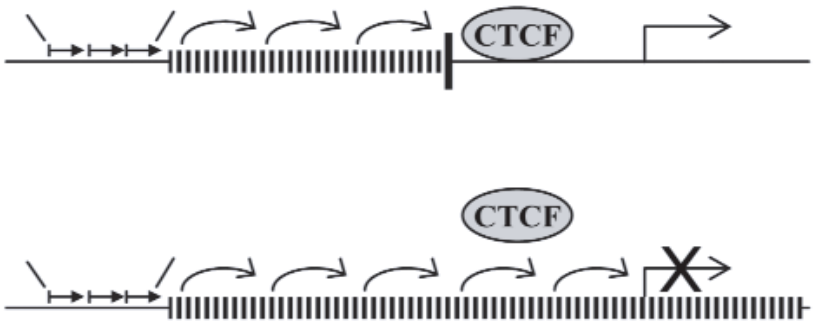

Figure 3: Model of promoter protection againts spreading of epigenetic silencing by CTCF (Recillas-Targa et al., 2006).

CTCF and escape genes

CTCF creates particular chromosomal boundaries on the inactive X chromosome allowing the expression of some genes that escape to X

chromosome inactivation known as escape genes (between 10 to 20% of human genes located on the X chromosome escape inactivation) (Recillas-Targa et al., 2006).

The incorporation of CTCF over such escape genes correlates with some chromatin marks presumably affecting gene expression. There is an unusual high level of histon H3 acetylation at the transition regions between distinct domains, what is consistent with the proposal that CTCF acts as a chromatin insulator. CTCF binding sites at escape domains protect, through enhancer-blocking properties, againts inappropriate activation of silent genes adjacent or in proximity to the active escape domains (Recillas-Targa et al., 2006).

The most attractive observation deals with the fact that CTCF influences the DNA methylation status of such particular domains embedded in the inactivated X chromosomes. CTCF plays a more structural role contributing to the formation of an escape gene domain, thus facilitating a particular topological organization (Recillas-Targa et al., 2006).

Long-distance regulation of gene expression

CTCF has been located coinciding with DNase I hypersensitive sites. DNase I hypersensitive sites come together and associate between them to create spatial clustering of chromatin, forming through multiple loop formation the so-called active chromatin hub, with regulatory consequences like differential expression during cellular differentiation (Recillas-Targa et al., 2006).

The convergent point is that the great majority of the DNase I hypersensitive sites implicated in the active chromatin hub formation bind CTCF. CTCF can multimerize, probably through some of the zinc- fingers that are not involved in contacting DNA. CTCF binds to itself or to other partner proteins in distinct places, thus facilitating the spatial clustering of distal genomic sites leading to the creation of an active chromatin hub. In this way CTCF can form inter- and intrachromosomal loop formation. This topological higher order chromatin organization seems to occur with consequences in gene expression coordination (Recillas-Targa et al., 2006).

Regulation of CTCF activity

One of the first clear evidences of long-distance action of insulators was the discovery of a CTCFdependent enhancer blocker element that is hormon regulated. CTCF binding sites are often flanked by thyroid hormone response elements (TREs). In the absence of thyroid hormone, an enhancer-blocking activity is turned on. In contrast, in the presence of thyroid hormone, CTCF-dependent enhancer-blocking function is interrupted, leading to gene expression. This evidence showing a thyroid hormone regulated CTCF-dependent enhancer-blocking activity (Recillas-Targa et al., 2006).

The enhancer-blocking activity is abolished in a T3 thyroid hormone dependent way, with a concominant remarkable increase of histone acetylation over the CTCF binding site. Therefore, T3 thyroid hormone and CTCF seem to collaborate creating a molecular complex, probably including various co-factors, which in turn could influence the recruitment of histone acetylase activities, allowing the CTCF-dependent enhancer-blocking activity to be controlled (Recillas-Targa et al., 2006).

The loss of enhancer-blocking activity in the presence of thyroid hormone is not caused by the dissociation of CTCF from chromatin. Thus, the combinatorial of factors and co-factors directly associated, or in close proximity, to CTCF clearly dictates its enhancer-blocking capabilities (Recillas-Targa et al., 2006).

CTCF and cancer

In human cells, CTCF gene has been mapped to chromosome band 16q22.1. Such chromosomal region frequently contains deletions found in sporadic breast and prostate tumours. For that reason loss of heterozygosity at chromosome 16q22.1 and CTCF integrity has attracted the attention of several groups. It is thought that genetic loss of CTCF may be associated with miss-

regulation of large number of genes involved in the control of cell cycle progression (Recillas-Targa et al., 2006).

CTCF has been found to regulate transcription of multiple genes including $p19^{ARF}$, $p16^{INK4a}$, PIM-1, PLK, BRCA1, p53, p27, *Ecadherine*, E2F1, TERT and IGF2 among others. What seems very attractive is that the promoter sequence of the majority of these genes is abnormally methylated in different tumours. Therefore, the list of aberrantly methylated CTCF DNA targets in different human cancers is not restricted to imprinted genes ICR sites (Recillas-Targa et al., 2006).

Recillas-Targa et al. (2006). has demostrated that the CTCF-DNA interaction is methylation-sensitive and that the loss of CTCF from the retinoblastoma promoter correlates with the incorporation of a methyl-CpG-binding protein, with its cognate chromatin repressing components, which in turn could induce epigenetic silencing of this cell cycle regulator. Thus, it is proposed that CTCF is a critical component of a growing list of tumour suppressor genes. In addition CTCF may protect such genes againts undesired DNA methylation

Today it is generally accepted that genetic mutations are not only way for the loss of BRCA1 expression observed in breast tumours. Suggestion that altered patterns of DNA methylation influates breast cancer is supported by observation of abnormally methylated BRCA1 tumour supressor gene in 15 to 20% of sporadic breast cancer cases. The existence of CTCF binding sites creating a transition site between methylated and unmethylated state in the BRCA1 promoter strongly supports the model that was envisioned for the CTCF site identified at the human retinoblastoma promoter. It is proposed that CTCF binding creates a protective barrier againts the spreading of DNA methylation over the core promoter sequences of BRCA1 gene (Recillas-Targa et al., 2006).

Proposed capacity of CTCF to protect againts DNA methylation do not allow to propose a single mechanism for such a protection. In any case, the basic concept has to do with the capacity of CTCF and assosiated chromatin remodelers to act as a barrier and block the spreading of epigenetic silencing effects of the surrounding chromatin over CpG-island (Recillas-Targa et al., 2006).

Therefore,CTCF is an appealing candidate to protect tumour suppressor gene promoters and introns against DNA methylation, possibly in collaboration with other factors, and that could represent a key epigenetic factor in carcinogenesis (Recillas-Targa et al., 2006).

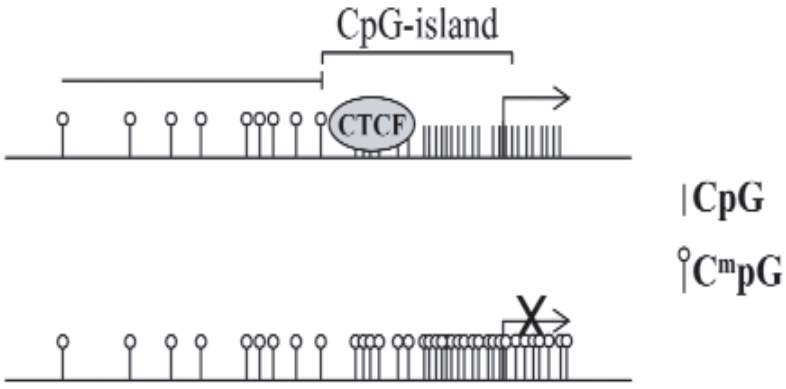

Figure 4: Protection against DNA methylation of CpG- island by CTCF (Recillas-Targa et al., 2006).

CTCF and BORIS

D'Arcy and co-laborators (2008) identified a novel factor that binds to the CTCF target sequence. Such factor is the testis-specific expressing CTCF paralogue, named Brother of the Regulator of Imprinted Sites (BORIS), which encodes a protein highly conserved on the central 11 zinc-finger domain but with divergent amino- and carboxy-terminal domain.

BORIS was classified as a protein belonging to the cancer testis antigen (CTA) family. The CTA gene products exhibit highly tissue-restricted expression and are immunogenic in cancer patients. The function of the majority of the CTA is still unknown, however, some CTAs are thought to be implicated in the regulation of the gene expression and others may control gametogenesis (D'Arcy et al., 2008).

While CTCF is expressed both in cancer and normal somatic tissues, BORIS is only expressed in human cancers and germ cells. Aberrant

expression of BORIS in human cancer has been proposed to lead to displacement of CTCF from its normal target sites, re-patterning of chromatin insulator boundaries, and widespread epigenetic deregulation. This model is supported by the observation that overexpression of BORIS in mammalian cells induces the expression of CG antigens, including BORIS itself and MAGE-A1 (D'Arcy et al., 2008).

Promoters of CTA genes are methylated and repressed in normal somatic cells that express CTCF and not BORIS, but are specifically demethylated and activated in testicular germ cells and in cancer cells that express BORIS (Renaud et al., 2007).

Moreover high BORIS levels correlated with high levels of progesterone receptor and oestrogen receptor. Both of them could promote breast cancer (D'Arcy et al., 2008).

Abnormal expression of BORIS is associated with majority of cancer cell lines, including primary breast, prostate, colorectal and lung tumours. BORIS is not normally expressed in females, interestingly, D'Arcy et al. (2008) demonstrated that BORIS protein indeed appears in all breast cancer lines tested and in 70,7% of breast tumours. BORIS was not detected in primary normal breast cells, which suggest that BORIS is likely to be associated with the immortalised and malignant cells (D'Arcy et al., 2008).

BORIS expression in DNMT deficient cells directly correlates with promoter DNA hypomethylation and an altered histone H3 modification pattern, in a region encompassing the transcriptional start site (Woloszynska-Read et al., 2007).

DNA methylation, and expression of CTCF and p53 represent three mechanisms involved in the negative regulation of BORIS transcription (Renaud et al., 2007).

Recent study has identified three transcriptional starts of BORIS. The study has shown that all three BORIS promoters contain CTCF binding sites, suggesting that CTCF acts directly or indirectly to regulate the BORIS promoters (Renaud et al., 2007).

p53 has been shown to repress transcription by indirect interactions with a target promoter through a promoter-bound transcriptional activator as the

intermediate (Renaud et al., 2007).

Treatment of cancer cell lines that express BORIS at low levels with the demethylating agent, 5-aza-dC, resulted in demethylation of the promoter CpG island and substantial increases in BORIS transcription (Renaud et al., 2007).

Post-translational modification of CTCF

CTCF´s functions may be regulated through its genomic location and/or by the choice of zinc fingers used in DNA binding. In addition, CTCF is post-translationally modified, it can be phosphorylated in its C-terminus and poly(ADP-ribosyl)ated in its N-terminus (it is likely that more modifications will be identified). These modifications probably play a key role in regulating CTCF binding and/or in mediating CTCF´s different functions.

Phosphorylation

Phosphorylation appears to be one the most important and most studied forms of post-translational modifications involved in the regulation of transcription factor providing a link between signal transduction and expression of genes. Phosphorylation controls the function of transcription factors at different levels. It can affect, either positively or negatively, DNA binding affinity, trans-activating/-repressing function or compartmentalisation of transcription factors (El-Kady and Klenova, 2005).

Protein kinase CK2 (CK2) is known for phosphorylation of proteins. CK2 is highly conserved in evolution and has been found in all eukaryotic cells. CK2 is a dynamic molecular complex composed of two subunits a and a´ (representing the catalytic domain of CK2) and a dimer of the b-subunits (representing the regulatory domain of CK2) (El-Kady and Klenova, 2005).

It phosphorylates serines and threonines immersed in acidic sequences within proteins and peptides and the minimum requirement for phosphorylation is the sequence S*/T*XXDE (in which asterisk denotes the phosphorylated serine or threonine and X represents any non-basic amino acid) (El-Kady and Klenova, 2005).

El-Kady and Klenova investigated that CK2 phosporylation is implicated in regulation of activity of CTCF. In the context of the chicken c-myc promoter, they demostrated the coexpression of CTCF with protein kinase. CK2 change CTCF activity from a transcriptional repressor to an activator (El-Kady and Klenova, 2005).

Previous studies have shown that most phosphorylation sites in CTCF are placed in the C-terminal region. Phosphorylation residues in CTCF are restricted to the motif spanning Ser-604 to -612. In the contexts of all the c-myc promoter-based constructs, substitution of all serines, 604, 609, 610 and 612, with Ala create CTCF isoforms that exerted stronger inhibitory effects than the wild-type protein (El-Kady and Klenova, 2005).

Further tests revealed that phopshorylation of CTCF does not occur when Ser-612 is mutated. Importance of Ser – 612 is attributed to its role as „gate-keeper“ in controlling phosphorylation constructs of other upstream residues (e.g., Serines 604, 609 and 610) (El-Kady and Klenova, 2005).

The introduction of the negative charge at the phosphorylation sites by replacement of the serines (604, 609, 610 and 612) with the glutamic acid residues created a phopsho-mimetic CTCF mutant. The inherent transcriptional activating role of this acidic mutant is weaker than that mediated by synergistic action of the wild-type protein and CK2. Overexpression of this ‘‘weak’’ acidic mutant at higher levels (3.0 lg) compensated for the partial weakness of its charge (El-Kady and Klenova, 2005).

The substitutions of the CK2 sites could affect cell growth inhibition by CTCF. El Kady´s´ and Klenova´s´ results have shown that the growth suppressive effects of the wild-type CTCF in COS7 were significant, but not very strong. Substitution of four and five serines in $pAla^{604,609,610,612}$ and $pAla^{578,604,609,610,612}$ caused remarkable growth inhibition. This is however not surprising because CTCF controls transcriptional activity of many genes

involved in the regulation of cell proliferation growth inhibition (El-Kady and Klenova, 2005).

Given that CK2 levels are often elevated in cancers it is tempting to speculate that overexpression of CK2 in cancer cells could lead to excessive phosphorylation of CTCF, which may result in non-controllable overproduction of c-myc and thus may be one of the mechanisms of maintenance and evolution of tumour cell population (El-Kady and Klenova, 2005).

Poly(ADP-ribosyl)ation

The poly(ADP-ribose) polymerase (PARP) and poly(ADP-ribose) were discovered 40 years ago by Pierre Chambon and Paul Mendel (1963). It was only recently recognized that PARPs are a family of enzymes with 18 members in human genome (Ame et al., 2004, Otto et al., 2005).

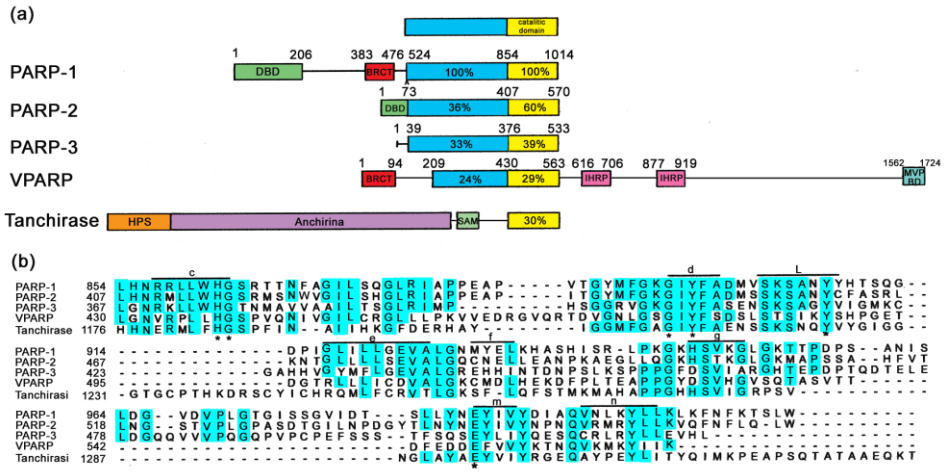

Figure 5: (a) Schematic primary structures of five members of the poly(ADP-ribose)polymerase (PARP) family. Percentage indicate homology to PARP-1. (b) Alignment of the catalytic domains of the five human PARPs. Amino acid identities between three or more PARPs are shaded. The secondary structures indicated by lines are based on the crystal structure. β-strands are indicated by c, d, e, f, g, m, n and L indicates an α helix (Tempera, 2006).

Poly(ADP-ribosyl)ation can be defined as the post-translational modification of a protein with a homopolymeric chain composed of linear and branched sequences of repeating ADP-ribose units linked together in a complex polymer. The synthesis of poly(ADP-ribose) (pADPr) as a consequence of PARP activation plays a role in many processes with direct roles in cell survival. For years, owing to its induction by DNA strand breaks, poly(ADP-ribosyl)ation has been thought to have functions related to the genomic integrity and cell death pathways. However, recent studies suggest that PARPs and pADPr are important in much broader spectrum of cellular functions such as recombination, inflammation, cancer and also in the regulation of gene transcription (Bryant et al., 2005).

The poly(ADP-ribose)polymerase structure

Poly(ADP-ribosyl)ation marks are established by poly(ADP-ribose) polymerase enzymes. Biochemically, PARP uses nicotinamide adenine dinucleotide (β-NAD^+) to form polymers of ADP-ribose. The enzyme operates in a processive manner, adding ADP-ribose sequentially to a protein acceptor. The PARPs superfamily, as mentioned before, includes 18 members but it is the founding family member, PARP-1, that has been best studied (De Murcia et al.,1994, Kurosaki et al. 1987).

Poly(ADP-ribose)polymerase-1 (PARP-1; EC 2.4.2.30) is a nuclear enzyme present in all eukaryotes. PARP-1 is a 116-kDa protein consisting of three main domains: the N-terminal DNA-binding domain containing two zinc fingers, the automodification domain, and the C-terminal catalytic domain (De Murcia et al.,1994, Kurosaki et al., 1987).

The primary structure of the enzyme is highly conserved in eukaryotes (human and mouse enzyme have 92% homology at the level of aminoacid sequence) with the catalytic domain showing the highest degree of homology between different species; the catalytic domain contains the so-called PARP signature sequence, a 50-aminoacid block showing 100% of homology between vertebrates (Kurosaki et al., 1987).

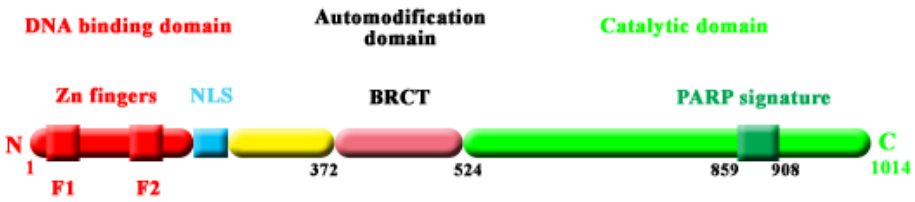

Figure 6: Schematic representation of the domain structure of PARP-1. The major protein domain, as determined by limited proteolyc digestion, are delineated on top. Important structural and functional elements are shown at the bottom. F1, first zinc finger; F2, second zinc finger; NLS, nuclear location signal; BRCT, "BRCA1 C-terminus". Numbers refer to amino-acid positions (Tempera, 2006).

The N-terminal DNA-binding domain (DBD) extends from the initiator methionine to threonine-373 in human PARP-1. The first zinc-finger (F1) starts at cysteine-21 and ends at cysteine-56, while the second zinc finger (F2) is found between cysteine-125 and cysteine-162. PARP zinc fingers are structurally and functionally unique, since: (i) they co-ordinate zinc molecules with Cys-Cys-His-Cys motif, (ii) they contain 28 and 30 residues, whereas most other zinc finger usually contain 12-13 aminoacids, and (iii) they recognize altered structures in DNA rather than particular sequences. The only know protein that has a zinc finger similar of those of PARP-1 is DNA ligase III (Lindahl, et al., 1995, Caldecott et al., 1996).

The automodification domain of PARP-1 is located in the central region of the enzyme, between residues 374 and 525 (human protein) (Kurosaki et al., 1987). This basic domain contains the majority of the 15 glutamic acid residues that would be involved in PARP-1 automodification (Marsischky et al., 1995). The automodification domain of PARP-1 contains a leucine-zipper motif in the N-terminal part and also a BRCT (BRCA1 C-terminus: breast cancer susceptibility protein C terminus) domain (from aminoacids 384 to 479). This domain consists of approximate 95 (weakly conserved) amino acids found in several proteins that regulate cell-cycle checkpoints and DNA repair (Bork et al., 1995). There is a growing amount of evidence suggesting that BRCT domain is

a protein-protein interaction modules that allows BRCT-motif-containing proteins to establish strong and specific association (Masson et al., 1998).

The catalytic domain of human PARP-1 is localized in the C-terminal part of the enzyme. It has a molecular mass of approximately 55 kDa and spans residues 526-1014 in human PARP-1 (Kurosaki et al. 1987). The catalytic activity of this fragment cannot be stimulated by DNA strand breaks and corresponds to the basal activity of the native enzyme (Simonin et al., 1993). Residues spanning position 859-908 in human PARP-1 are phylogenetically well conserved (Ruf et al., 1996) and comprise what is called as “PARP signature”. The catalytic domain of chicken PARP-1 has been crystallized and then resolved by X-ray diffraction. The active site of this domain consists of a β-α-loop-β-α structural motif which is responsible for NAD^+ binding and it is found in several mono(ADPr) transferases. This motif differs significantly from the Rossman fold (β-β motif) found in other NAD^+-utilizing enzymes and appears to be representative of a new family of ADP-ribosyltransferases (Althaus, 1992).

PARP-1 functions

PARP-1 functions as a DNA damage sensor and signalling molecule binding to both single- and double-stranded DNA breaks. Upon binding to damaged DNA mainly through the second zinc-finger domain, PARP-1 forms homodimers and catalyzes the cleavage of β-NAD^+ into nicotinamide and ADP-ribose and then uses the latter to synthesize branched nucleic acid-like polymers of ADP-ribose covalently attached to nuclear acceptor proteins (Fig. 3). Glutamic acid and aspartic acid residues are most likely to be modified by poly(ADP-ribosyl)ation. The level of pADPr in mammalian cells can transiently increase 500-fold after DNA damage (D’Amours et al., 1999).

The size of branched polymer varies from a few to 200 ADP-ribose units. Because of its high negative charge, the covalently attached ADP-ribose polymer dramatically affects the function of target proteins. *In vivo*, the most abundantly poly(ADP-ribosyl)ated protein is PARP-1 itself, and auto-poly(ADP-ribosyl)ation represents a major regulatory mechanism for PARP-1 resulting in

the down-regulation of the enzyme activity. In addition to PARP-1, histones are also considered to be major acceptors of poly(ADP-ribose). Poly(ADP-ribosyl)ation confers negative charge to histones, leading to electrostatic repulsion between DNA and histones. This process has been implicated in chromatin remodelling, DNA repair, and transcriptional regulation. Several transcription factors, DNA replication factors and signaliing molecules [NF-κB (Oliver et al., 1999), Ap-2 (Kannan et al., 1999), Oct-1 , YY1 (Oei et al., 2001), B-MYB (Cervellera et al., 2000), p53 (Wersierska-Gadek et al., 2001), topoisomerase I] have been show to become poly(ADP-ribosyl)ated by PARP-1.

The effect of PARP-1 on the function of these protein is carried out by noncovalent protein-protein interactions and by covalent poly(ADP-ribosyl)ation. However, pADPr is not only involved in the DNA damage response and apoptosis but also has important regulatory functions in the normal physiology of the cell. New findings make clear that the regulation of poly(ADP-ribosyl)ation and its dynamics should be viewed in a cell-wide context rather than being in confined to the nucleus, which has been the focus of most studies to date. The recent discovery of unanticipated functions of PARPs and poly(ADP-ribose) glycohydrolase (PARG) are clearly in line with the concept of multifunctional enzymes. The PARPs dogma stating that synthesis of pADPr is totally dependent on the presence of a DNA strand breaks has been strongly challenged by recent reports from Potaman et al. (2005) and Lonskaya et al. (2005) showing that PARP-1 catalytic activity by non-B DNA structures in the absence of DNA strand breaks.

The regulation of PARP-1 activity is established through different mechanism. The best characterized mechanism is the down regulation of enzyme activity through auto-poly(ADP-ribosyl)ation (Kawaichi et al., 1981). Furthermore, nicotinamide, the smaller cleavage product of NAD^+, also exerts inhibitory effects on PARP-1, allowing negative feedback regulation. Phosphorylation of PARP-1 by protein kinase C also results in enzyme inhibition (Bauer et al., 1992). The abundance of PARP-1 may also change under certain condition, suggesting a transcriptional or post-transcriptional regulation (Tramontano et al., 2006). It is not yet clear whether PARP-1 induction significantly alters the poly(ADP-ribosyl)ating capacity of the cells, because

PARP-1 is one of the most abundant nuclear protein [(0.2-2.0) x 10^6 molecules per cell; 1.0 x 10^6 molecules per cell is the average amount found in most cells] .

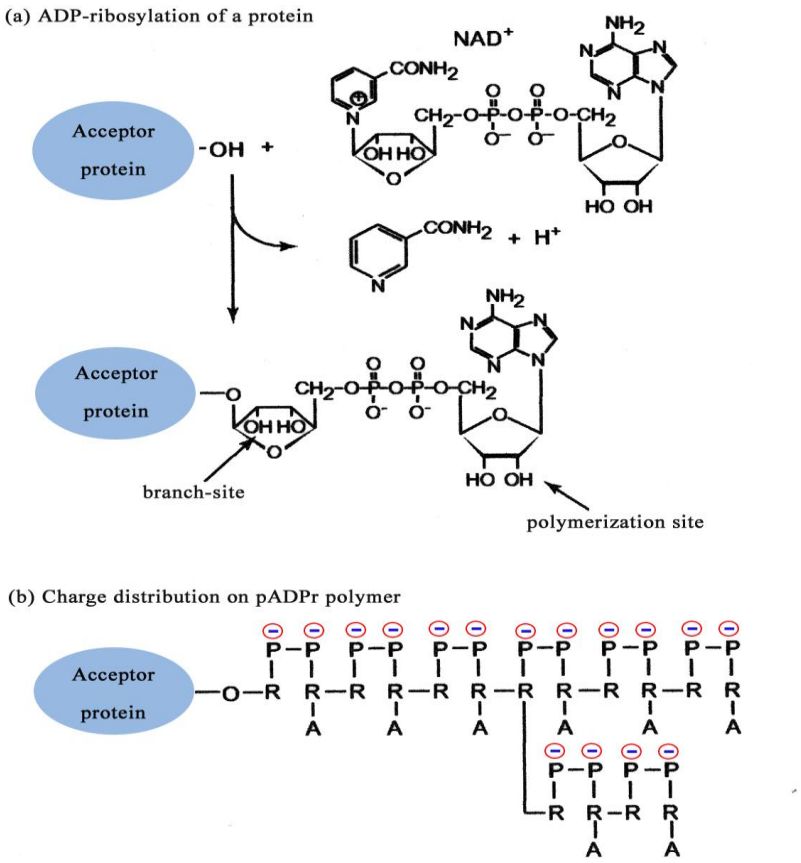

Figure 7: (a) ADP-ribosylation of a protein acceptor using NAD^+ as substrate. (b) Synthesis of a negatively charged ADP-ribose polymer (Tempera, 2006).

The catabolism of poly(ADP-ribose)

Two enzymes, poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase, are involved in the catabolism of poly(ADP-ribose), with PARG cleaving ribose-ribose bonds of both linear and branched portions of poly(ADP-ribose) and lyase removing the protein proximal ADP-ribose monomer (Davidovic et al., 2001).

PARG degrades long polymers extremely rapidly in vitro, whereas short polymers are processed much less efficiently under the same conditions. This is

well illustrated by the fact that the K_m of PARG is low for long polymers (< 0.3 μM) and increases significantly for shorter polymers (10 μM). Another factor affecting polymer degradation of pADPr by PARG is association on acceptor protein. Indeed, pADPr is degraded more rapidly when it is covalently associated with proteins such as PARP-1 and histone H1. The endoglycosylase activity of PARG is physiologically important, because it is responsible for the generation of protein-free ADPr polymers that can interact with histones and other nuclear proteins. In addition, it is important to note that even a low level of endoglycolytic activity, such as 10%, would result in a significant increase in the degradation kinetics of polymers. Therefore the endoglycosidic hydrolysis of long polymers would efficiently prevent the hyper-modification of nuclear proteins with very long chain of ADPr. In addition, this type of hydrolysis could allow PARP-1 to remain active, by loosening the polymers that prevent its interaction with DNA (Zahradka and Ebisuzaki, 1982).

Poly(ADP-ribosyl)ation and epigenetic

Epigenetic regulation is commonly believed to involve certain aspects of heritable changes in gene activity without a change in DNA sequences. There is a considerable contemporary interest in epigenetic marks, since they are essential for the correct implementation of temporal, tissue-specific and parent-of-origin-dependent gene expression and they are reprogrammable. Epigenetic states impinge on chromatin conformations, which not only are faithfully replicated, but also directly or indirectly control the accessibility of transcription factors to the chromatin fibre. In this regard, a link between epigenetics and PARP-1 should not have come as a surprise, since poly(ADP-ribosyl)ation of histone proteins has long been associated with an extended and open chromatin conformation believed to facilitate the access of DNA repair factors to the damage chromatin (Kraus and Lis, 2003).

However, recurrent observation that a certain population of PARP-1 and PARP-2 are normally associated with centromeres (Saxena et al., 2002) and that many centromere-associated chromatin factors, such as Cenpa, Cenpb

and Bub3, can be found to be normally poly(ADP-ribosyl)ated (Saxena et al., 2002), are not easily reconciled with a function for these factors in DNA repair only. Moreover PARP-1 is able to interact directly with DNA via common *cis* regulatory motif, such as TGTTG, to repress a range of target genes (Huang et al., 2004), hinting a more direct involvement in epigenetic/gene regulation unlinked to genotoxic stress.

Another twist in the epigenetic-poly(ADP-ribosyl)ation saga came with the observation that PARP-1 is found deposited throughout the *Drosophila* chromatin. Upon stimulus, such as heat-shock, the PARP-1 enzyme is activated to open up the chromatin conformation to generate the well-known chromosomal puffs linked with transcriptionally active loci (Tulin and Spradling, 2003).

Taken together, these data suggest a link between poly(ADP-ribosyl)ation and epigenetic process. A further insight into potential molecular mechanism of these events comes from the observation that apart from histones, a number of key transcription factors, such as p53 (Wersierska-Gadek et al., 2001), YY1 (Oei et al., 2000) and CTCF (Yu et al., 2004) have been documented to be poly(ADP-ribosyl)ated both, *in vivo* and *in vitro*. Poly(ADP-ribosyl)ation of p53 and YY1 abolishes their interaction with DNA hinting at yet another, albeit indirect, mode of epigenetic regulation of PARP enzymes. On the other hand, poly(ADP-ribosyl)ation of chromatin insulator protein, CTCF, does not noticeably impede its binding to a range of target sites, potentially reflecting the fact that CTCF-dependent chromatin insulation is neutralized by inhibition for PARPs (Yu et al., 2004). Indeed one of the models proposing a poly(ADP-ribosyl)ation-dependent mechanism of epigenetic control is based on reversible poly(ADP-ribosyl)ation of a DNA bound CTCF (Yu et al., 2004).

Aim of the research

It is well known that poly(ADP-ribosylated) proteins such as H1 histone, topoisomerase 1, p53, EBNA1, YY1 and CTCF play an important role in the apoptosis, viral activation, cancerogenesis and modulation of chromatin structure.

Recently CTCF has been found to be associated with PARP- 1. Poly(ADP-ribose) can link two distinct molecules of CTCF (or CTCF and another partner protein) and, thereby can make dimers (or even polymers). The dimers linked by a poly(ADP-ribose) are more resistant to poly(ADP-ribose) glycohydrolase. This implies that CTCF can potentially form homo-polymer complex stabilized by poly(ADP-ribose) polymer. By this ability CTCF implicates in higher order chromatin conformation such as formation of loops, which bring different domains of DNA and also particular sets of proteins together in nuclear compartments (Klenova and Ohlsson, 2005)

It was reported that CTCF could play an important role in the arrest of cell proliferation by blocking nucleolar gene transcription and possibly by affecting other unknown nucleolar functions. This assumption was based on the observation that CTCF is targeted to nucleoli in the course of inducted differentiation of cultured erythroid cells (line K562) and induction of apoptosis in MCF7 breast cancer cells.

Torrano et al. (2006) have shown that inhibition of poly(ADP-ribosyl)ation impaired the translocation of the full-length CTCF into the nucleolus and restored nucleolar transcription, thus indicating that inhibition of nucleolar transcription by CTCF depends on active poly(ADP-ribosyl)ation (Torrano et al., 2006).

Moreover, several models that describe possible mechanisms for multiple functions of CTCF in establishment and maintenance of epigenetic programs are now emerging. CTCF participation in the control of the cell cycle could include an anti-apoptotic function affecting varied target genes,

suggesting the involvement of CTCF in neoplasic processes.The possibility of CTCF to exert a protective role in several tumour could correlate with a control of epigenetic mechanisms such as DNA methylation, poly(ADP-ribosylation), acetylation etc.

Therefore, this work was aimed to demonstrate during the differentiation in distinct tumour cell lines:

- whether CTCF expression could be modulated
- whether poly(ADP-ribosyl)ated isoforms of CTCF are present
- whether a possible alteration of CTCF compartmentalization occurs

To achieve this goal, four different tumour cell lines were used: Raji, HeLa, ARO and papillary cell (NPA). The Raji are Burkitt lymphoma derived-EBV positive, HeLa are derived from cervical cancer cells. ARO, thyroid tumour cell line, has high capability of proliferation but do not express hormone. Papillary, thyroid tumour cell line, actively proliferates and expresses hormone.

Cell differentiation was induced by sodium butyrate that acts as an inhibitor of histone deacetylases leading to stimulation of gene expression. The presence of CTCF was be proved by immunoprecipitation and Western blot analysis. The compartmentalization of CTCF was be observed by indirect immunofluorescence microscopy.

Materials

RPMI 1640 medium, Dulbecco´s modified Eagle´s/F12 medium (DMEM/F12), penicilin and streptomycin were purchased from Sigma Aldrich. Molecular weight marker PageRuler was purchased from Fermentas. Protein A agarose and Protein G agarose were purchased from Upstate Biotechnology. Primary antibody against PAR was purchased from Trevigen, primary against CTCF from Upstate Biotechnology, primary C23 antibody from Santa Cruz Biotechnology. Secondary antibody against rabbit polyclonal antibody was obtained from Amersham, GE Healthcare, normal mouse IgG from Upstate Biotechnology. Secondary FITC anti-rabbit antibody and Texas Red-conjugated secondary anti-mouse antibody was a gift from Prof. Patricia Mancini from Univ. „La Sapienza“ Polyvinilidene difluoride membranes (PVDF), autoradiography film and ECL Advance Western Blotting detection kit were purchased from, Amersham - GE Healthcare. All the material was reagent grade.

Experimental procedures

Cell differentiation

Principle:

HeLa, Aro and NPA cells were treated with sodium butyrate for 24 hours, Raji cells were treated with sodium butyrate, phorbol 12,13-dibutyrate and TGF β2 for 48 hours in incubator under 5% CO_2 at 37°C. Sodium butyrate induce cell differentiation. Differentiated cells change their morphology.

Cell culture:

1. Aro, HeLa, NPA and Raji cells were obtained from Univ. „La Sapienza“
2. HeLa cells were maintained in culture DMEM/F12 medium supplemented with penicillin (100 U/ml), streptomycin (0,1 mg/ml), 5% foetal bovine serum and glutamine (2mM)
3. NPA and ARO cells were maintained in RPMI 1640 medium supplemented with 1% sodium pyruvate
4. Raji cells were maintained in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (0,1 mg/ml), 5% foetal bovine serum and glutamine (2mM)
5. steps 5-7 only for adherent cell lines (ARO, HeLa, NPA): old culture medium was discarded from the flask and cells were washed with 5 ml sterile PBS to prevent serum antitryptic affect
6. 2 ml of 0,05% trypsine was added into the flask to detach cells from the bottom
7. 6 ml of serum was added into the flask to stop trypsin activity
8. cells were transferred into falcon and were centrifuged (900*g* for 10 min)
9. cells were resuspended with appropriate medium supplemented with 5% foetal bovine serum

Cell differentiation:

1. Raji cells were treated by mixture of 5 mM sodium butyrate, 20 ng/ml phorbol 12,13-dibutyrate and 0,04 ng/ml TGF β2
2. ARO, HeLa and papillary cells were treated by 5 mM sodium butyrate
3. cells were transferred into culture flasks
4. culture flasks with ARO, NPA, HeLa and Raji were maintained in humified incubator under 5% CO_2 at 37°C for 24 hours and 48 hours, respectively.
5. cells after 24 and 48 hours respectively checked under microscope
6. cells were detached by trypsinization and collected as described in steps 5–8

Immunoprecipitation

Principle: Specific protein is separated from whole cell lysate by reaction antigen-antibody. The protein complex, once bound to the specific antibody, is removed from the bulk solution by capture with an antibody-binding protein attached to protein A and G, as a solid support. The solid support is washed to remove any proteins not specifically and tightly bound through the antibody. Components of the bound immune complex (both antigen and antibody) are eluted from the support by ion strength and detergents. Precipitated proteins are analyzed using gel electrophoresis and western blotting technique.

Procedure:

1. at least 20 x 10^6 of control and induced cells were collected and centrifuged at 1300 rpm for 10 min
2. cells were washed twice with ice-cold PBS

3. cells were resuspended in RIPA buffer containing 1mM PMSF-proteinase inhibitors and 1% protease inhibitor cocktail (Roche) to lysate cell membranes
4. 30 µl of each cell solution (2 x 10^6 cells) was saved as control input,
5. cells were homogenized with 10 strokes by pestal A and rotated at 4°C for 30 min to solubilize the proteins
6. cell solutions were centrifuged at 13 000 rpm for 5 min and the supernatants were saved
7. the cell solution was aliquoted for immunoprecipitation with certain primary antibodies
8. supernatants were incubated with primary antibodies (anti IgG-monoclonal, anti PAR- monoclonal, anti CTCF- polyclonal) at 4°C with rotation for at least 2 hours (or over night)
9. protein A (beads for polyclonal primary antibody) and protein G (beads for monoclonal primary antibody) were washed three times with RIPA buffer with rotation at 4°C, the beads were collected by centrifugation at 3000 rpm for 1 min at 4°C
10. the supernatant after incubation with primary antibody was incubated with 100 µl of 50% slurry protein A or protein G for 2 hours
11. beads were collected by centrifugation at 3000 rpm for 1 min
12. beads were washed five times with RIPA buffer with rotation for 10 min
13. beads were resuspended with Laemmli (1982) sample buffer
14. beads were heated for 5 min at 60°C in block heater
15. samples were centrifuged at 3000 rpm for 1 min to the beads go in a pellet
16. all samples (20 µl) without beads were loaded onto SDS-PAGE
17. separated proteins were visualised by Western blot and by ECL Advance

Western blot analysis

Principle: To determine CTCF protein, the samples prepared by immunoprecipitation were resolved by 10% SDS-PAGE. Separated proteins were blotted onto PVDF membrane. The blot was incubated with primary antibody and horseradish peroxidase conjugated secondary antibody. Proteins were visualized by chemiluminescence detection with ECL Advance kit.

Procedure:

Preparation of SDS-Polyacrylamide gel

1. the glass plates were assembled according to the manufacturer´s instructions
2. 10% resolving SDS-polyacrylamide gel was prepared in an Erlenmeyer flask: 40% acrylamide/bis solution, 29:1 (BIO-RAD), upper buffer (Tris-HCl pH 8,8), distilled water, 10% sodium dodecyl sulfate (SDS), 10% ammonium persulfate solution, tetramethylethylenediamine
3. the mixture was swirled rapidly and poured into the gap between the glass plates
4. the acrylamide solution was overlayed with butanol by pasteur pipette to prevent oxygen diffusing into the gel and inhibiting polymerization
5. the overlay was poured off after polymerization
6. 5% stacking SDS-polyacrylamide gel was prepared in Erlenmeyer flask: 40% acrylamide/bis solution, 29:1 (BIO-RAD), lower buffer (Tris-HCl pH 6,8), distilled water, 10% sodium dodecyl sulfate (SDS), 10% ammonium persulfate solution, tetramethylethylenediamine
7. the mixture was swirled rapidly and poured onto polymerized resolving gel
8. a comb was immediately inserted into the stacking gel solution and removed after polymerization

Electrophoresis

1. the wells were washed with distilled water to remove any unpolymerized acrylamide
2. the gel was mounted in the electrophoresis apparatus
3. Tris-glycine buffer was added to the reservoir
4. the molecular weight marker (5 µl) and the samples (20 µl) were loaded into the bottom of the wells by Hamilton microliter syringe that was washed three times with distilled water and once with aceton
5. the electrophoresis apparatus was attached to an electric power supply and apllied a voltage of 8 V/cm to the gel (I_{max}= 20 mA)
6. the power supply was turned off when bromophenol blue reached the bottom of the resolving gel

Western Blot

1. wearing the gloves, six pieces of Whatman 3MM paper and one piece of PVDF were cut according to exact size of the gel
2. the PVDF membrane was pre-wetted by soaking in 100% methanol for 10 seconds than quickly washed in distilled water and 1% transfer buffer (10mM N-cyclohexyl-3-aminopropane sulphonic, pH=11)
3. 3MM papers were soaked in 1% transfer buffer
4. 3 sheets of soaked 3MM paper and PVDF membrane were stacked on anode, the gel was carefully transferred on membrane and three sheets of 3MM papers was placed on top of the stack
5. using a glass pipette as a roller, air bubbles and excess of transfer buffer were squeezed
6. the transfer apparatus was set up, upper electrode as cathode was placed on the stack
7. an electric current 0,8mA per cm^2 was applied for 1 hour
8. when the blot was finished, the gel was transferred to Coomasie Brillant Blue and was stained on slowly rocking platform over night (minimum 4

hours) at room temperature, the gel was destained on slowly rocking platform with destaining solution 1 (50% methanol + 12% acetic acid) for 10 min and then with destaining solution 2 (20% ethanol + 5% acetic acid) for 1hour, staining with Coomasie Brillant Blue allows to check retained protein in the gel

9. the PVDF membrane was removed from sandwich, the bottom left-hand corner of the membrane was cut off
10. the PVDF membrane was incubated with 5% non fat dried milk solution with gentle agitation for 1hour at room temperature to block nonspecific binding
11. the PVDF membrane was incubated with primary antibody (polyclonal anti-CTCF or monoclonal anti-PAR 1:1000) with gentle agitation for 2 hours at room temperature (or over night at 4°C)
12. the PVDF membrane was washed with agitation as follows: once with PBS for 5 min, twice with PBS/Tween 0,1% for 10 min, finally with PBS for 5 min
13. the PVDF membrane was incubated with secondary antibody (anti-rabbit or anti-mouse conjugated with horseradish peroxidase 1:5000 in 5% non-fat dried milk in PBS) for one hour with gentle agitation at room temperature
14. the PVDF membrane was washed with agitation as follows: once with PBS for 5 min, twice with PBS/ Tween 0,1% for 10 min, finally with PBS for 5 min

Ehnanced chemiluminscence detection

1. the detection solution were mixed in a ratio of 40:1 (2 ml solution A + 50 μl solution B)
2. the excess wash buffer was drain from the washed membrane and was placed protein side up on a clean straight surface, the mixed detection reagent was pipette on the membrane
3. the PVDF membrane was incubated with detection reagent for 5 min at room temperature in the dark

4. the excess detection reagent was drain off and the PVDF membrane was placed protein side up in an X-ray film cassette
5. the sheet of blue-light sensitive autoradiography film was placed on top of membrane, that was covered with foil and was exposed for 10 min in closed cassette
6. the film was removed and replaced with a second sheet of unexposed film (second exposure for 1 hour)
7. the film was developed immediately with developer and fixer solution in the dark
8. for data presentation, the films were scanned and processed with Adobe Photoshop CS software

Fluorescence microscopy

Principle: To visualize subcellular location of CTCF protein within differentiated and undifferentiated cells, biomolecule is labelled with primary anti-CTCF antibody and secondary FITC anti-rabbit antibody which is labelled with fluorophore.To visualize nucleolin, nucleolar protein, biomolecule is labelled with primary anti-nucleolin antibody and Texas Red-conjugated secondary anti-mouse antibody. Under fluorescence microscope two determined proteins have different colour of fluophore. The images can be merged and we are able to determine the subcellular localization of protein. Fluorescent dyes as propidium iodide (PI) and 4,6- diamino-2-phenylindole (DAPI) are used to recognize nucleus and nucleolus.

Procedure:

1. cells (at the density 0,5 x 10^6) were grown on cover slips in 12-well plate, differentiated cells were treated with 5 mM sodium butyrate see in chapter: cell differentiation
2. cells were washed twice with PBS

3. cells were fixed in 500 µl 4% paraformaldehyde and was incubated 20 min at room temperature
4. cells were washed three times (5 min each time) with PBS
5. cells were incubated with 40 µl of primary antibody anti-CTCF 1:50 supplemented with 60 µM digitonin in PBS for 1 hour
6. cells were washed three times (5 min each time) with PBS
7. cells were incubated with 40 µl of FITC secondary anti-rabbit antibody 1:50 supplemented with 60 µM digitonin in PBS in the dark for 30 min
8. cells were washed three times (5 min each time) with cold PBS
9. cells were incubated with 40 µl of primary antibody anti-nucleolin 1:20 supplemented with 60 µM digitonin in PBS for 1 hour
10. cells were washed three times (5 min each time) with PBS
11. cells were incubated with 40 µl of Texas Red- conjugated secondary antibody 1:10 supplemented with 60 µM digitonin in PBS in the dark for 30 min
12. cells were washed three times (5 min each time) with cold PBS
13. cells were incubated with fluorescent propidium iodide 1:100 in PBS in the dark for 2 min, or with fluorescent dye 4,6- diamino-2-phenylindole 1:35 000 in distilled water in the dark for 1 min
14. cells were washed three times (5 min each time) with PBS
15. preparates were assembled with blanching solution
16. results of immunostaining were observed by fluorescence microscope with AxioCam MRC Zeiss camera (Axiovision 3. 1 software), the images were processed with I.A.S. software

Results

Identification of poly(ADP-ribosyl)ated CTCF in different tumour cell lines

Because CTCF protein could be expressed in multiple forms and undergoes post-translation modification we investigated whether CTCF could be a substrate for PARP-1 and whether CTCF isoforms protein are present in different tumour cell lines.

To achieve these aims, the cell lysates obtained from Raji, HeLa, ARO, NPA cells treated or untreated with sodium butyrate were analyzed by Immunoprecipitation assay.

Fig. 8 shows the IP results with CTCF in treated and untreated Raji cells. Immunocomplexes analyzed by western blot using antibodies raised against the CTCF, evidenced that this protein migrates in different isoforms. In particular the prevalent bands were at 130 kDa and 180 kDa on SDS- PAGE in untreated Raji cells, whereas the 180 kDa protein band is significantly unlighted in treated ones.

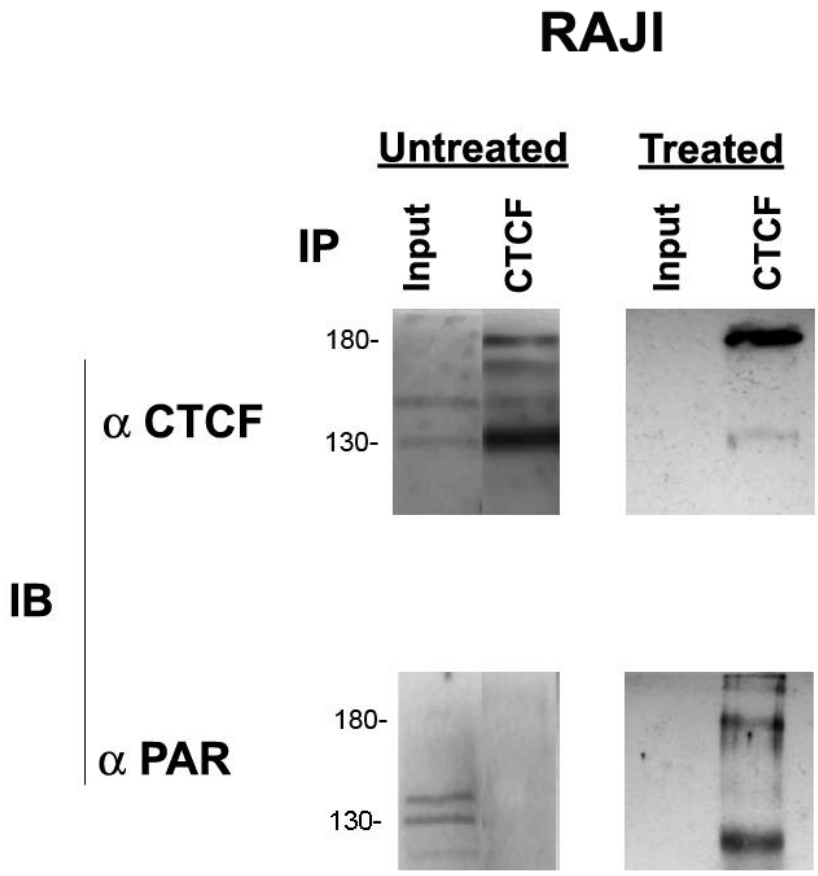

Figure 8. Western blots with anti-CTCF and anti-PAR in Raji cells. Cell lysates from untreated and treated Raji cells were immunoprecipitated with anti-CTCF. The samples were separated by 10% SDS-PAGE gel, transferred to PVDF membrane and probed with respective antibodies. Proteins were visualized by chemiluminescence detection with ECL Advance kit.

Because, previously studies by Klenova and Ohlsson (2005) have demonstrated that this latter band correspond to a poly(ADP-ribosyl)ated CTCF isoform, CTCF immunocomplexs were submitted to western blot with PAR antibody. The results shown in Fig. 8 demonstrated that the CTCF band at a180 kDa were poly(ADP-ribosyl)ated in treated cells, whereas these modified isoforms are almost absent in untreated cells. Similarly, results were obtained with treated and untreated HeLa cells (Fig. 9).

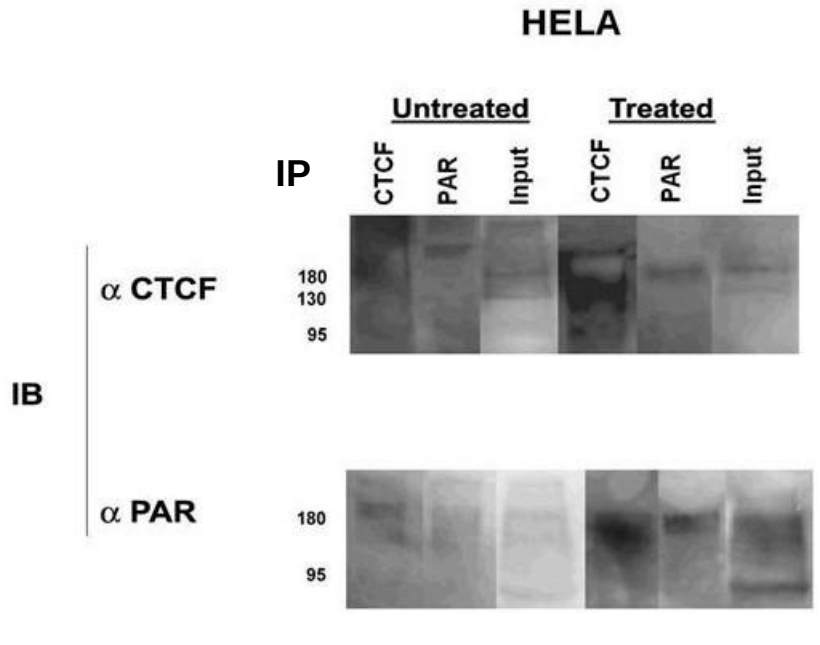

Figure 9. Western blots with anti-CTCF and anti-PAR in HeLa cells. Cell lysates from untreated and treated HeLa cells were immunoprecipitated with anti-CTCF and anti-PAR antibodies. The samples were separated by 10% SDS-PAGE gel, transferred to PVDF membrane and probed with respective antibodies. Proteins were visualized by chemiluminescence detection with ECL Advance kit.

Immunoprecipitation analysis of thyroid tumor cells such as ARO cells-derived undifferentiated carcinoma and NPA cells-derived papillary carcinoma demonstrated that CTCF migrates as 130 kDa and 180 kDa in the treated and untreated cells (Fig. 10). Furthermore, CTCF was co-immunoprecitated with PAR immunocomplex. Both the bands were detected in the untreated in ARO cells, whereas only the band at 180 kDa was present in the treated ones (Fig. 10).

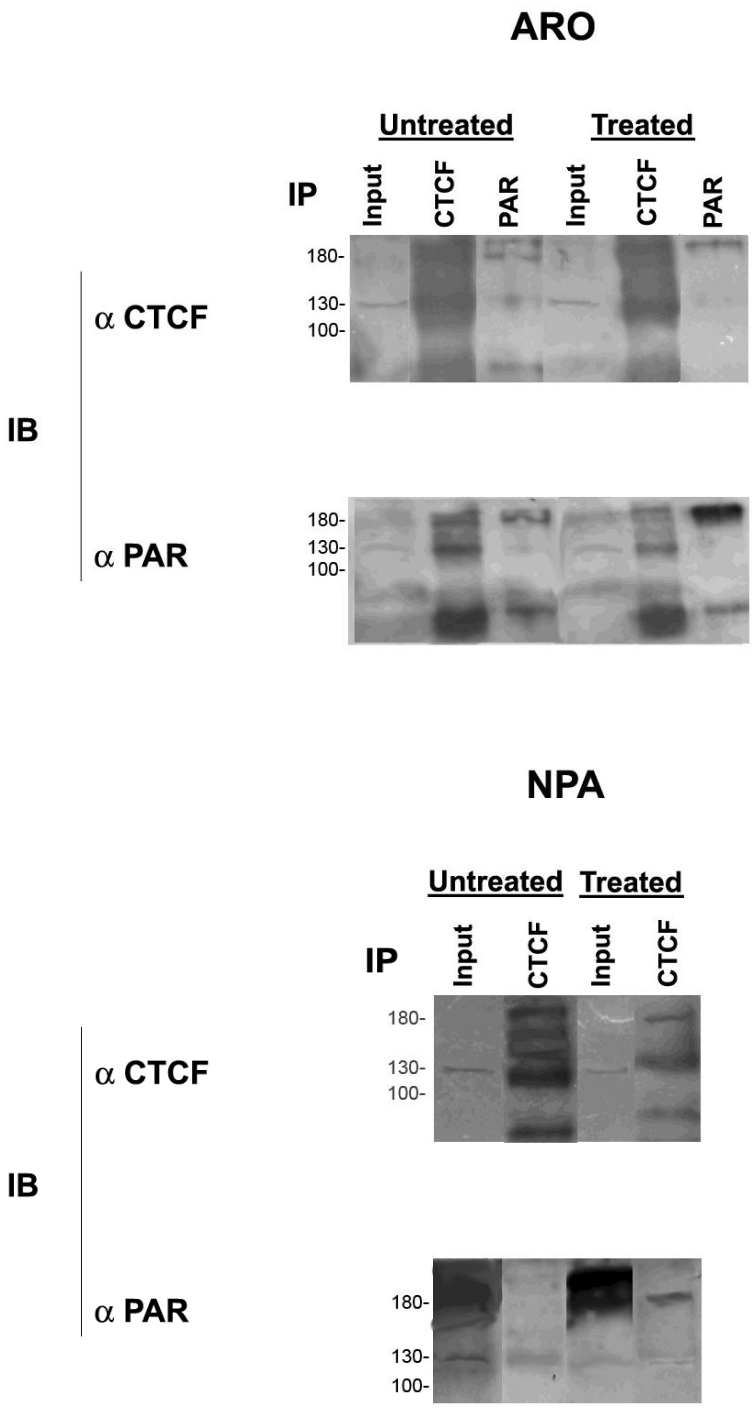

Figure 10. Western blots with anti-CTCF and anti-PAR in NPA and ARO cells. Cell lysates from untreated and treated ARO and NPA cells were immunoprecipitated with anti-CTCF and anti-PAR antibodies. The samples were separated by 10% SDS-PAGE gel, transferred to PVDF membrane and probed with respective antibodies. Proteins were visualized by chemiluminescence detection with ECL Advance kit.

To confirm that CTCF underwent to poly(ADP-ribosyl)ation process the western blotting was performed with anti-PAR antibodies. Fig. 10 shows that CTCF immunocomplex obtained from ARO and NPA cell lysates was poly(ADP-ribosyl)ated. Notably, the 180 kDa poly(ADP-ribosyl)ated CTCF was abundant in both treated tumour cell lines.

CTCF distribution in different cellular compartments

In the majority of cells, CTCF is localized in the nucleus, but recently its distribution has been correlated to the different stages of cell cycle.
Hence, in this study it has been investigated whether the ability of CTCF to migrate in different cellular compartment was dependent on the form of neoplasia.

Fig. 11 shows that CTCF was present all over the nuclei in untreated Raji cell, whereas in treated cells CTCF is also concentrated in the nucleoli. To confirm whether CTCF is targeted to nucleoli, the cells were also immunostained with antibodies against nucleolin.

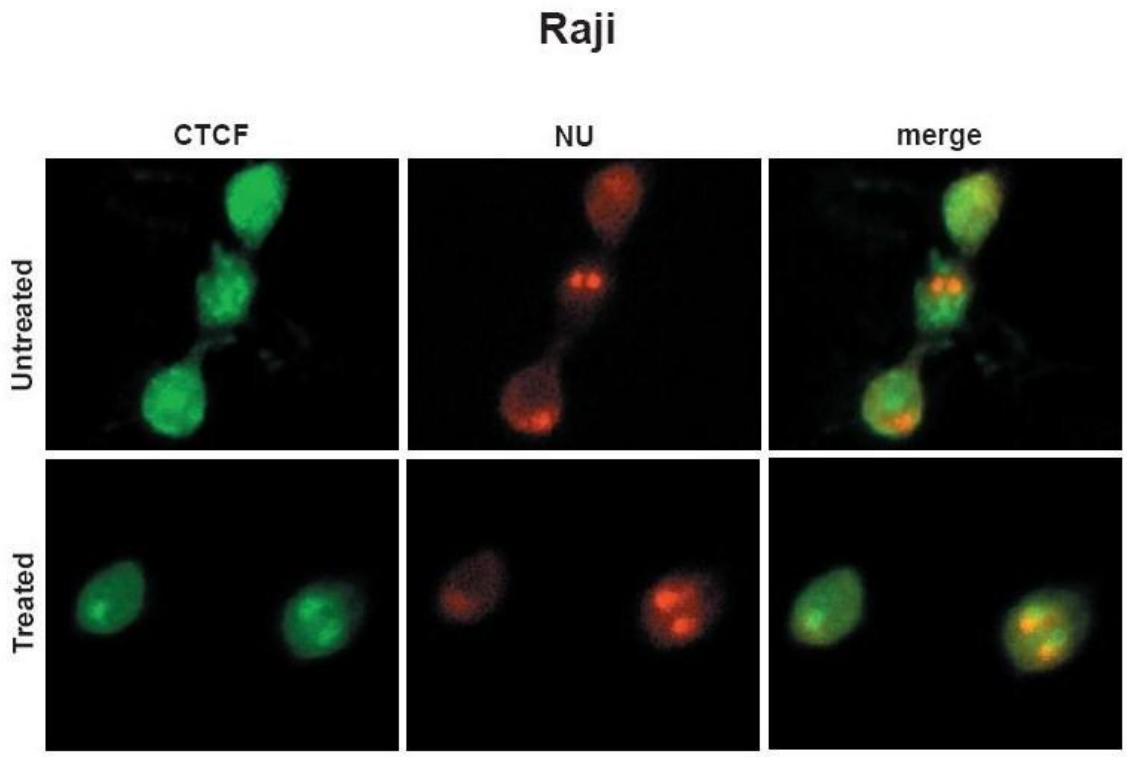

Figure 11.Indirect immunofluorescence of CTCF and nucleolin. Untreated and treated Raji cells were immunostained with primary anti-CTCF antibody (1:50) then with secondary FITC anti-rabbit antibody or with primary anti-nucleolin antibody (1:20) and then with Texas Red-conjugated secondary anti-mouse antibody. The results of immunostaining were observed by fluorescence microscope with AxioCam MRC Zeiss camera (Axiovision 3. 1 software). The images were processed with I.A.S. software.

In thyroid tumour cells the distribution of CTCF was strikingly different. CTCF was distributed in the cytosol, predominantly concentrated at the nuclear periphery in both untreated and treated ARO and NPA cell.

Curiously, a similar distribution of nucleolin was observed in treated ARO and NPA cells (Fig. 12).

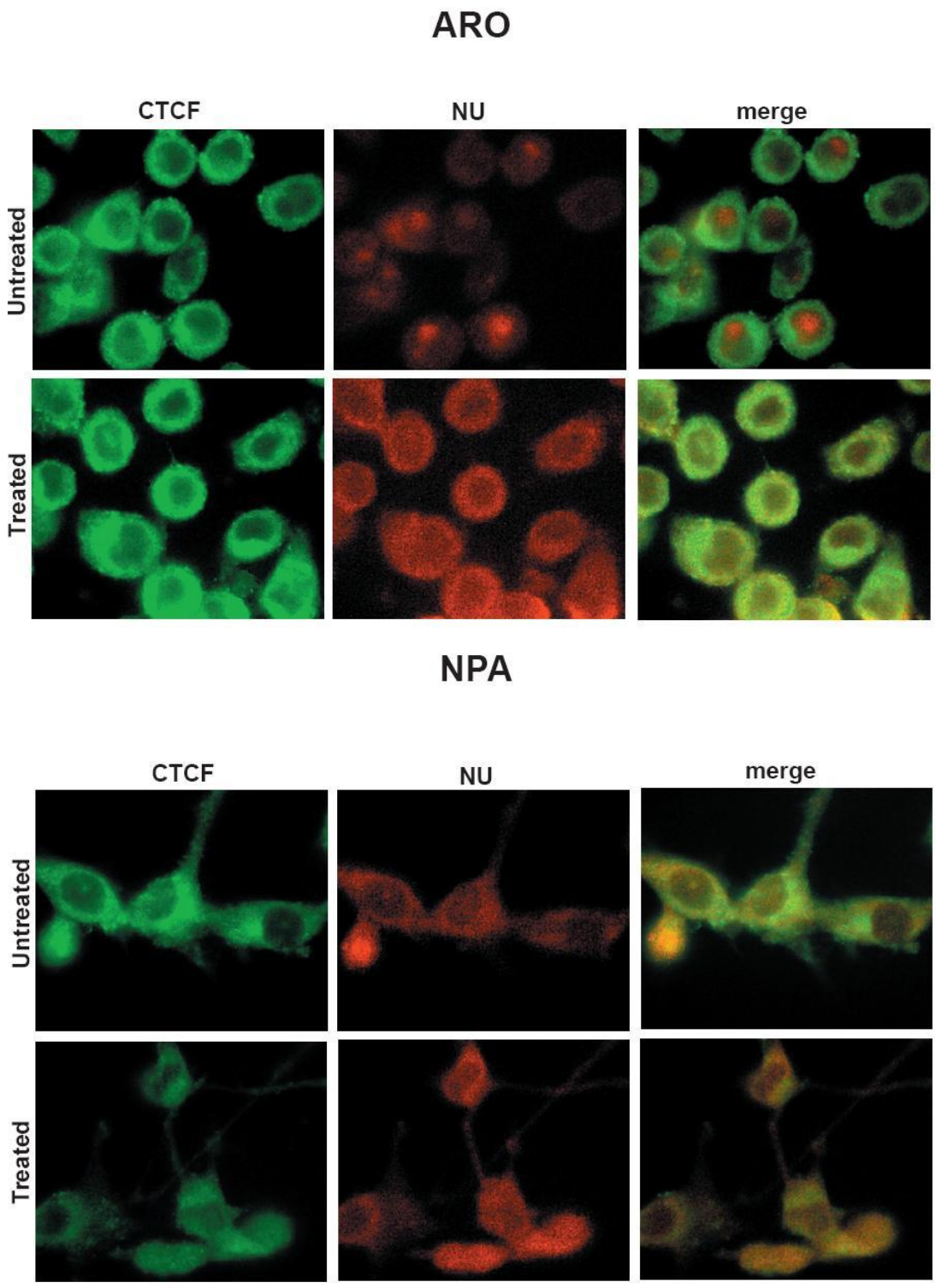

Figure 12.Indirect immunofluorescence of CTCF and nucleolin, nucleolar protein. Untreated and treated ARO and NPA cells were immunostained with primary anti-CTCF antibody (1:50) and then with secondary FITC anti-rabbit antibody or with primary anti-nucleolin antibody (1:20) and then with Texas Red-conjugated secondary anti-mouse antibody. The results of immunostaining were observed by fluorescence microscope with AxioCam MRC Zeiss camera (Axiovision 3. 1 software). The images were processed with I.A.S. software.

Fig. 13 shows that CTCF also localizes in the cytosol in untreated and treated HeLa cells stained with DAPI. In particular, the protein was more concentrated in a point very close to the nucleolus in treated cells. It looks like a gem.

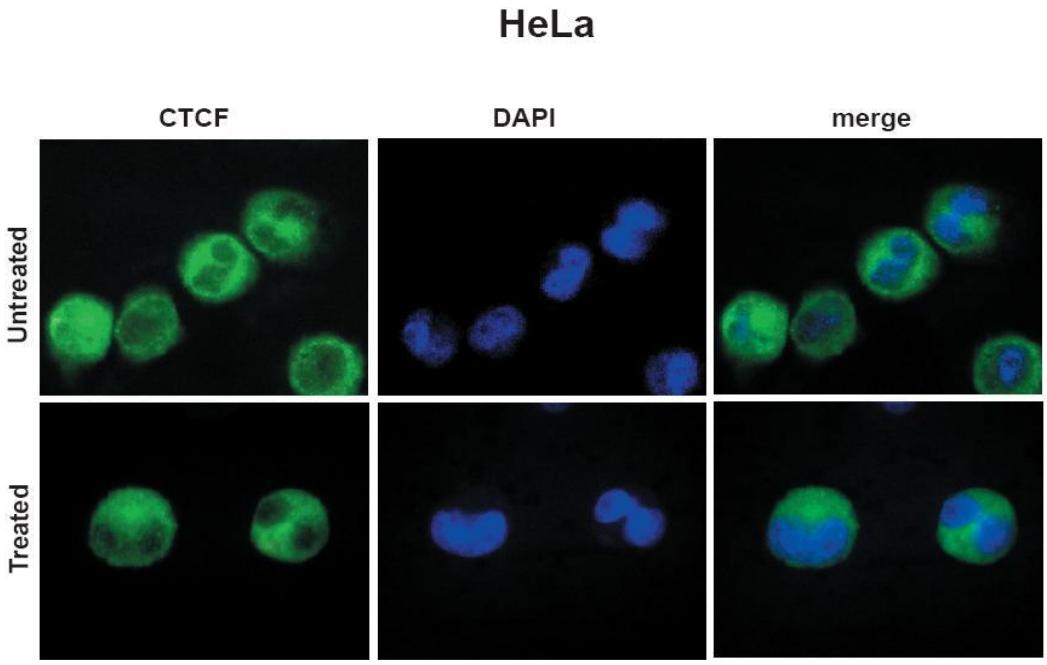

Figure 13.Indirect immunofluorescence of CTCF, nucleoplasm stained with DAPI. Untreated and treated HeLa cells were immunostained with primary anti-CTCF antibody (1:50) then with secondary FITC anti-rabbit antibody and with DAPI. The results of immunostaining were observed by fluorescence microscope with AxioCam MRC Zeiss camera (Axiovision 3. 1 software). The images were processed with I.A.S. software.

Discussion

CTCF, a DNA-binding protein, regulates genome activity through its capacity to act as an enhancer blocker. Moreover, it contributes to protect tumour suppressor gene promoters and introns against DNA methylation and to regulate the expression of cell-cycle related genes. All these features suggest an involvement of CTCF in neoplasia processes. Yu et al. (2004) found a poly(ADP-ribosyl)ated CTCF isoform by investigating the interaction between the maternal H19 ICR allele and CTCF. Therefore, we have analyzed whether CTCF underwent post-translational modifications in different tumour species treated or untreated with sodium butyrate a well known differentiating agent.

In Raji, ARO, NPA and Hela tumour cells CTCF was expressed in various isoforms with two prominent bands at 130 kDa and 180 kDa. The latter band was significantly poly(ADP-ribosyl)ated in tumour cells treated with sodium butyrate. In addition, this poly(ADP-ribosyl)ated CTCF isoform was found in other tumour sources like MCF-7 breast cancer cells and K562 myeloid cells suggestive of any specific tumour differences in the turnover of the poly(ADP-ribosylation) mark. (Yu et al., 2004 and Torrano et al., 2006). Moreover, CTCF can make dimers (or even polymers) linked by a poly(ADP-ribose), which are more resistant to poly(ADP-ribose) glycohydrolase. The loss of poly(ADP-ribosyl)ation could lead to epigenetic lesions at the key growth-promoting genes with subsequent misregulation and tumour development (Klenova et al., 2005).

Modification of CTCF by poly(ADP-ribosy)lation can influence not only its insulator function, but also the regulation of transcription by CTCF. Indeed, it has been demonstrated that treatment with 3-aminobenzamide, a well known PARP inhibitor, leads to de-regulation of CTCF-dependent transcription of the *c-myc* and p^{19ARF} genes.

It becomes increasingly evident that targeting of transcription factors to particular nuclear compartments plays an important role in the control of

genome activity.

Data obtained by immunofluorescence experiments showed that in Raji cells, CTCF shifts from nucleoplasm to the nucleolus following sodium butyrate treatment. Such redistribution of CTCF in the nucleus may be important to trigger and sustain necessary metabolic changes leading to cell growth arrest and, further, to terminal differentiation and apoptosis. These results are in agreement with those of Torrano et al. (2006), who has demonstrated that CTCF is targeted to nucleoli in differentiating erythroid lineage.

Moreover, preliminary data obtained incubating the Raji cells with 3-ABA known PARP inhibitor, suggested that CTCF migrated in nucleoli through a poly(ADP-ribosyl)ation-dependent mechanism (data not shown).

Interestingly, PARP-1 and PARP-2 have been found to accumulate in nucleoli in a complex with nucleophosmin/B23. It is conceivable that CTCF may be a part of the same functional network, as interaction with PARP-1 and B23 has been documented (Torrano et al., 2006).

Conversely, CTCF traslocation was not evidenced in treated thyroid tumor cells lines. In spite of the observed sodium butyrate-induced morphological changes, CTCF was localized mainly in the cytoplasm, close to the nuclear periphery.

Surprisingly, nucleolin was present throughout the nucleus and cytoplasm in ARO and NPA cells. This finding correlates with the work of Otake et al. (2007) who has reported the presence of nucleolin in cytoplasm and plasma membrane in some tumour cells. In HeLa cells CTCF was not identified in nucleolar proteome in agreement with the data obtained by Andersen et al. (2002, 2005).

Taking into the account the findings by Klenova and Ohlsson (2005), present data indicate that CTCF could form dimers (or even polymers) linked by a poly(ADP-ribose) moiety, thus affecting high order chromatin structure. Morover, the CTCF distribution in different cellular compartments could be responsible for an epigenetic misregulation leading to the development of neoplasia. More experiments will be devoted to elucidate these phenomena.

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