

Targeting of CTCF to the nucleolus inhibits nucleolar transcription through a poly(ADP-ribosyl)ation-dependent mechanism

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Summary

Multiple functions have been reported for the transcription factor and candidate tumour suppressor, CTCF. Among others, they include regulation of cell growth, differentiation and apoptosis, enhancer-blocking activity and control of imprinted genes. CTCF is usually localized in the nucleus and its subcellular distribution during the cell cycle is dynamic; CTCF was found associated with mitotic chromosomes and the midbody, suggesting different roles for CTCF at different stages of the cell cycle. Here we report the nucleolar localization of CTCF in several experimental model systems. Translocation of CTCF from nucleoplasm to the nucleolus was observed after differentiation of K562 myeloid cells and induction of apoptosis in MCF7 breast cancer cells. CTCF was also found in the nucleoli in terminally differentiated rat trigeminal ganglion neurons. Thus our data show that nucleolar localization of CTCF is associated with growth arrest. Interestingly, the 180 kDa poly(ADP-ribosyl)ated isoform of CTCF was predominantly found in the nucleoli

fractions. By transfecting different CTCF deletion constructs into cell lines of different origin we demonstrate that the central zinc-finger domain of CTCF is the region responsible for nucleolar targeting. Analysis of subnucleolar localization of CTCF revealed that it is distributed homogeneously in both dense fibrillar and granular components of the nucleolus, but is not associated with fibrillar centres. RNA polymerase I transcription and protein synthesis were required to sustain nucleolar localization of CTCF. Notably, the labelling of active transcription sites by *in situ* run-on assays demonstrated that CTCF inhibits nucleolar transcription through a poly(ADP-ribosyl)ation-dependent mechanism.

Supplementary material available online at <http://jcs.biologists.org/cgi/content/full/119/9/1746/DC1>

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Introduction

A growing body of evidence reveals the complexity of function of the transcriptional factor CTCF (Klenova et al., 2002; Ohlsson et al., 2001). CTCF is involved in the transcriptional regulation of genes implicated in a variety of functions, such as *MYC* (Filippova et al., 1996; Klenova et al., 1993), the chicken lysozyme (Burcin et al., 1997), β -amyloid precursor protein (APP) genes (Vostrov and Quitschke, 1997) and others (Dunn and Davie, 2003; Ohlsson et al., 2001). CTCF is the only protein identified so far that mediates enhancer-blocking activity of vertebrate insulators (Bell et al., 1999). Remarkably, this enhancer-blocking activity is conserved from *Drosophila* to man (Moon et al., 2005). Other functions of CTCF include the control of imprinted genes (Lewis and Murrell, 2004) and X-chromosome inactivation (Lee, 2003).

CTCF contains a DNA-binding domain composed of 11 zinc-fingers. The DNA target sequences recognized by CTCF

are fairly long (about 50 bp) and strikingly diverse; no single consensus sequence can be ascribed to all CTCF binding sites. CTCF employs different combinations of individual zinc fingers for its binding within promoters, silencers and insulators (reviewed by Dunn and Davie, 2003; Klenova et al., 2002; Ohlsson et al., 2001). Another complexity to understand CTCF functions is the fact that CTCF protein undergoes post-translational modifications. It can be phosphorylated by the protein kinase CK2 (El-Kady and Klenova, 2005; Klenova et al., 2001), as well as poly(ADP-ribosyl)ated, and this latter modification regulates its activity as a chromatin insulator (Klenova and Ohlsson, 2005; Yu et al., 2004).

CTCF is a candidate tumour suppressor gene because of the loss of heterozygosity involving the *CTCF* locus at the chromosome band 16q22 in different malignancies, the finding of tumour-specific CTCF zinc-finger mutations in various cancers (Klenova et al., 2002; Ohlsson et al., 2001) and growth-

suppressive features of CTCF (Qi et al., 2003; Rasko et al., 2001). However, elevated levels of CTCF in breast cancer cells and tumours have been found to be associated with resistance to apoptosis (Docquier et al., 2005).

In the majority of cells, CTCF is localized in the nucleus, independently of its phosphorylation state (Klenova et al., 2001; Klenova et al., 1993). The CTCF subcellular distribution during the cell cycle is dynamic and it has been found associated with mitotic chromosomes (Burke et al., 2005), mitotic centrosomes as well as the midbody of the cytokinesis (Zhang et al., 2004a), suggesting different roles for CTCF at different stages of the cell cycle. However, these studies did not focus on the nucleolus as a possible subnuclear target for CTCF. The nucleolus is the subnuclear compartment where ribosomal RNAs are synthesized, processed and assembled with ribosomal proteins (Andersen et al., 2002; Grébane-Younès et al., 2005; Lam et al., 2005; Raska et al., 2004). Apart from ribosome biogenesis, it is known that the nucleolus has additional functions such as cell cycle regulation, storage of nuclear factors, regulation of tumour suppressor and oncogene activities, and processing of spliceosomal small nuclear U6 RNA, telomerase RNA and signal recognition particle RNA (Arabi et al., 2005; Carmo-Fonseca et al., 2000; Grandori et al., 2005; Grébane-Younès et al., 2005; Hernandez-Verdun and Roussel, 2003; Lam et al., 2005; Olson et al., 2002).

The diversity of the nucleolus function is mirrored by its ultrastructural complexity. The three basic components of the nucleolus defined by electron microscopy are: (1) the fibrillar centre, in which the transcriptional machinery of RNA pol I concentrates, (2) the surrounding dense fibrillar component into which nascent rRNA extends and rRNA processing begins, and (3) the granular component, which contains partially processed rRNA and where the final stages of ribosome assembly occur (reviewed by Huang, 2002; Leung and Lamond, 2003; Raska et al., 2004). Interestingly, CTCF has been shown to co-purify with the protein of the granular component B23 (nucleophosmin), and both proteins are present at the insulator sites *in vivo* (Yusufzai et al., 2004). However, it is unknown whether CTCF can be localized in the nucleolus and if so, whether the subnucleolar localization of CTCF is regulated and the dynamics of CTCF in the nucleolus have a functional relevance. In the present study we show that in response to different stimuli CTCF localizes to the nucleolus in several human cell lines in culture, and also in rat mature trigeminal ganglion neurons *in vivo*. We have identified the CTCF domain responsible for such localization and found that the CTCF nucleolar distribution depends on rDNA transcription and protein synthesis. Finally, we demonstrated that CTCF inhibits nucleolar transcription and this function is regulated by poly(ADP-ribosylation).

Results

CTCF is targeted to the nucleolus upon different stimuli

We first explored CTCF localization upon induced differentiation by taking advantage of the ability of K562 leukaemia cells to undergo pluripotent myeloid differentiation (Munoz-Alonso et al., 2005). In these experiments, K562 were differentiated into an erythroid lineage with 1- β -D-arabinofuranosylcytosine (Ara-C) or into a megakaryocytic lineage with staurosporine (STA), and undifferentiated K562 were used as control. Cell differentiation was assessed using

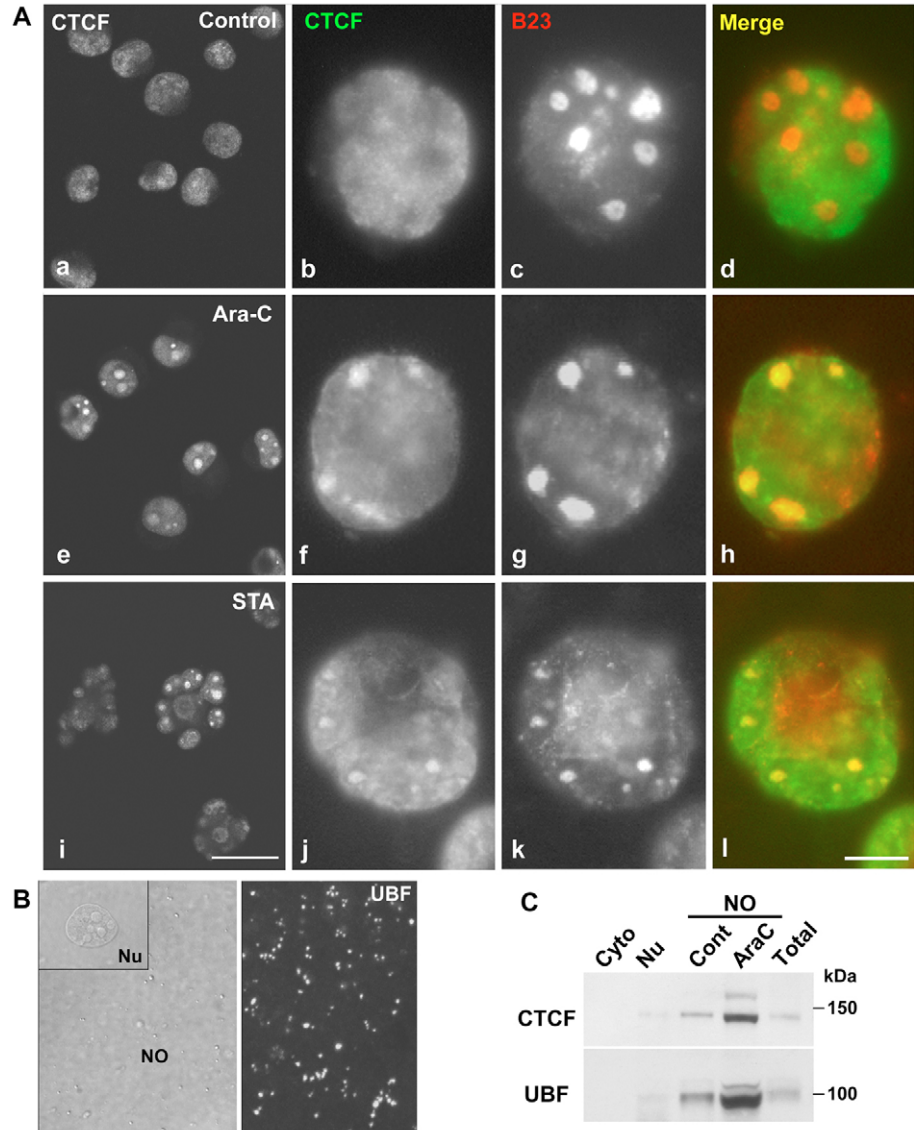
previously established morphological and cytochemical criteria (Torrano et al., 2005). We then analyzed the distribution of endogenous CTCF during differentiation of K562 by immunofluorescence using the anti-CTCF antibody. A low magnification image (Fig. 1Aa) shows that CTCF is diffusely distributed throughout the nucleoplasm in undifferentiated K562 cells. However, CTCF appears in nucleoli following cell differentiation. Both erythroid (Ara-C-treated) and megakaryocytic (STA-treated) differentiated cells exhibited an intense nucleolar CTCF immunostaining (Fig. 1Ae,i). The nucleolar localization of the CTCF was confirmed in double immunostaining experiments with antibodies against the protein B23, a marker of the granular component of the nucleolus. Thus, in undifferentiated K562 cells, CTCF was distributed throughout the nucleoplasm, but not concentrated in nucleoli, which appeared intensely labelled with the anti-B23 antibody (Fig. 1Ab-d). Erythroid and megakaryocytic differentiation of K562 cells led to the accumulation of CTCF in the nucleoli, as illustrated by co-localization of CTCF and B23 proteins (Fig. 1Af-h,j-l).

The nucleolar localization of CTCF was confirmed by western analysis of nucleolar extracts from K562 cells. Fig. 1B shows isolated nucleoli examined with phase-contrast microscopy and immunostained for the nucleolar protein UBF, confirming the purity of the nucleolar fraction. As expected, CTCF was found in the nuclear fraction and whole cell extracts, but not in the cytoplasmic fraction obtained from untreated K562 cells (Fig. 1C). CTCF was detected in nucleolar fractions from both untreated and Ara-C-treated K562 cells (Fig. 1C), although it was more abundant in nucleoli from the latter, thus confirming the results obtained by immunofluorescent staining (Fig. 1A). Interestingly, in addition to the prevalent CTCF band of 130 kDa, an extra band of approximately 180 kDa was also detected in the fraction enriched in nucleoli. This band is likely to correspond to the previously described poly(ADP-ribosyl)ated form of CTCF (CTCF-180) (Klenova and Ohlsson, 2005; Yu et al., 2004).

To extend our observations to another model system, we treated MCF7 breast cancer cells with sodium butyrate (NaBu), which is a known inhibitor of HDAC activity and inducer of G2/M growth arrest (Joseph et al., 2005) (D.F., unpublished data), and apoptosis in MCF7 cells (Chopin et al., 2002; Chopin et al., 2004). In untreated MCF7 cells CTCF was diffusely distributed in the nucleoplasm, but strongly accumulated in nucleoli after NaBu treatment (Fig. 2A). Nucleolar localization of CTCF in MCF7 cells treated with NaBu was further confirmed by western analysis of nucleolar fractions of MCF7 cells (Fig. 2B). Notably, the 180 kDa poly(ADP-ribosyl)ated form of CTCF, described earlier (Yu et al., 2004), was the predominant form found in nucleoli of MCF7 cells, whereas the 130 kDa form of CTCF was more abundant in the nucleoplasm (Fig. 2B).

Having established the nucleolar localization of CTCF in human haematopoietic and breast cancer cell lines, we aimed to further confirm this distribution in cells that model more closely the *in vivo* situation. For this study, we selected primary sensory neurons of rat trigeminal ganglia, which are post-mitotic differentiated cells with prominent nucleoli (Pena et al., 2001). Squash preparations of trigeminal ganglion neurons immunostained with the anti-CTCF and anti-B23 antibodies revealed co-localization of CTCF and B23 in the nucleolus in

Fig. 1. Nucleolar localization of endogenous CTCF in human myeloid cells induced to differentiate. (A) Indirect immunofluorescence showing CTCF nucleolar localization during induced differentiation of K562 cells. (a-d) Control undifferentiated K562 cells; (e-h) K562 cells treated with 1 μ M 1- β -D-arabinofuranosylcytosine (Ara-C) for 3 days to induce erythroid differentiation; (i-l) K562 cells treated with 100 nM staurosporine (STA) for 3 days to induce megakaryocytic differentiation. After induction of differentiation, cells were immunostained with the anti-CTCF monoclonal antibody. The images at low magnification show accumulation of CTCF in nucleoli after induction with Ara-C or STA (a,e,i). The detailed images show co-localization of CTCF (b,f,j; green channel) and B23 (c,g,k; red channel); d,h,l are the merged images of b and c, f and g and j and k, respectively. Bars, 40 μ m in the low magnification images and 10 μ m in the high magnification images. (B) Differential interference contrast (DIC) images showing the morphology of the nucleus (Nu) and isolated nucleoli (NO) from K562 cells (left panel). The purity of the nucleolar fraction was assessed by immunostaining with the anti-UBF antibody (right panel). (C) Western analysis of nucleolar fractions isolated from undifferentiated K562 cells and K562 cells induced into erythroid differentiation. Cell fractions were obtained, resolved on the SDS-PAGE, blotted and probed. Western analysis of nucleolar fractions isolated from undifferentiated K562 cells (Cont) and cells treated with 1 μ M Ara-C for 5 days was performed with the anti-CTCF antibody (upper panel) and the anti-UBF antibody (lower panel). The developed films were scanned and quantified. In the NO fractions, the ratio of the intensity of the CTCF bands over the intensity of the corresponding UBF bands revealed a 2.4-fold increase of CTCF expression in the Ara-C fraction with respect to control. Cyto, cytoplasmic fraction; Nu, nuclear fraction; NO, nucleolar fraction; Total, whole cell extract.



these cells (Fig. 3A). To investigate the ultrastructural distribution of CTCF in the nucleolar compartments we performed immunogold electron microscopy with the rabbit polyclonal anti-CTCF antibody. As shown in Fig. 3B, gold particles, indicating CTCF immunoreactivity, decorated the reticulated strands of dense fibrillar and granular components of the neuronal nucleolus (Pena et al., 2001), with a preferential localization on the dense fibrillar component.

CTCF is targeted to the nucleolus by signals within its zinc-finger domain

To investigate which region of the CTCF protein was responsible for its nucleolar targeting, we cloned CTCF cDNA fragments in frame with the C terminus of GFP. The GFP-CTCF full-length construct was previously shown to have similar functional characteristics as the wild-type CTCF protein (Burke et al., 2005). In this study we further confirmed

this by demonstrating that the full-length fusion protein (GFP-CTCF) was able to effectively inhibit cell proliferation and cloning efficiency in K562 cells (Fig. S1 in supplementary material) similarly to the CTCF protein (Rasko et al., 2001; Torrano et al., 2005).

K562 cells were then transfected with the different deletion variants and examined by confocal microscopy 24 hours post-transfection. In order to avoid possible artefacts due to high levels of CTCF overexpression, only cells with moderate to low levels of GFP expression were chosen. Fig. 4A illustrates the expression pattern of different CTCF variants tagged with GFP. The full-length GFP-CTCF showed a nuclear distribution, with higher expression level in the nucleolus (Fig. 4Aa). The N-terminal portion of CTCF failed to localize in the nucleolus (Fig. 4Ab), the C-terminal portion showed a diffuse nucleoplasmic distribution of the fusion protein, including the nucleolar compartment (Fig. 4Ad), whereas the central domain

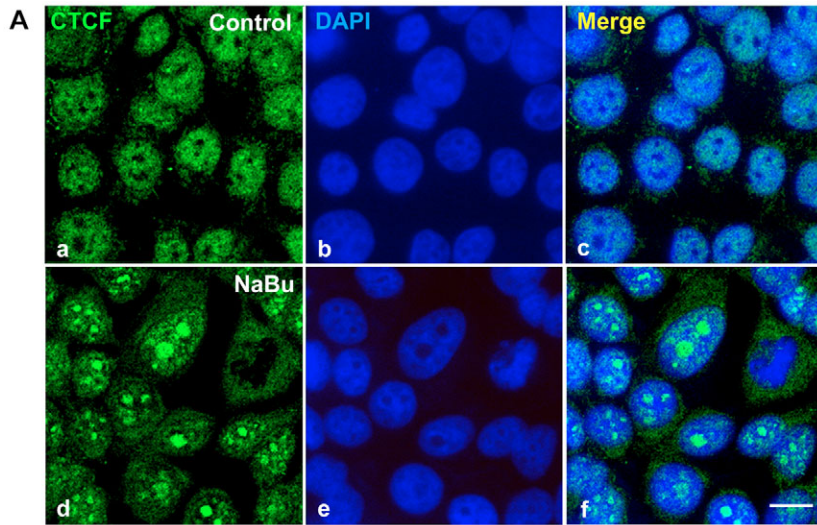
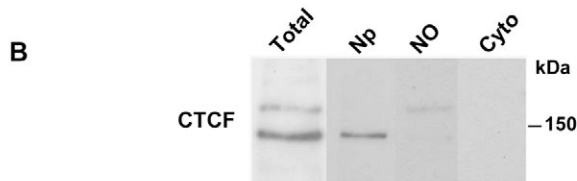


Fig. 2. Nucleolar localization of endogenous CTCF in human breast cancer cells treated with sodium butyrate. (A) Indirect immunofluorescent staining showing CTCF nucleolar localization following treatment of MCF7 cells with sodium butyrate (NaBu). After induction with 5 mM NaBu for 8 hours, MCF7 cells were immunostained with the anti-CTCF rabbit polyclonal antibody (a,d) or stained with DAPI (b,e); (c,f) merged images of CTCF and DAPI staining. (a-c) Untreated MCF7 cells (control); (d-f) MCF7 cells treated with NaBu. Bar, 10 μ m. (B) Nucleolar fractionation and western analysis of MCF7 cells treated with NaBu. MCF7 cells were treated with 5 mM sodium butyrate for 8 hours, nucleolar fractions were isolated and western analysis with the rabbit polyclonal anti-CTCF antibody was performed. Total, whole cell extract; Np, nucleoplasmic fraction; NO, nucleolar fraction; Cyto, cytoplasmic fraction.



containing the 11 zinc fingers (ZF) accumulated in the nucleolus (Fig. 4Ac). Deletion of the N terminus resulted in strong accumulation of the fusion protein in the nucleolus (Fig. 4Ae), and deletion of the C terminus led to a weak nucleolar

expression of the fusion protein (Fig. 4Af). In cells transfected with the empty vector, control GFP signal was distributed throughout the cytoplasm and nucleoplasm, but the nucleoli were excluded (data not shown).

We next asked whether nucleolar localization of CTCF, as observed in K562 cells, also occurred in other cell lines. For this purpose we used the UR61 neuron-like cells, derived from the rat pheochromocytoma PC12 cell line (Guerrero et al., 1988). Differentiated UR61 cells have a sympathetic neuron-like phenotype with inhibited proliferation and prominent nucleoli (Navasques et al., 2004) making them a suitable model for the detailed analysis of CTCF localization. In these experiments, UR61 cells were transfected with the indicated GFP-CTCF constructs; after 24 hours, cells were fixed, permeabilized and counterstained with propidium iodide (PI) to detect nucleic acids (Fig. 4B). Owing to the dispersed chromatin configuration of the UR61 cells, the nucleolus appears intensely stained with the PI and stands out on the pale nucleoplasm (Fig. 4Be-h). In UR61 cells distribution of the different deletion variants of GFP-CTCF was identical to those observed

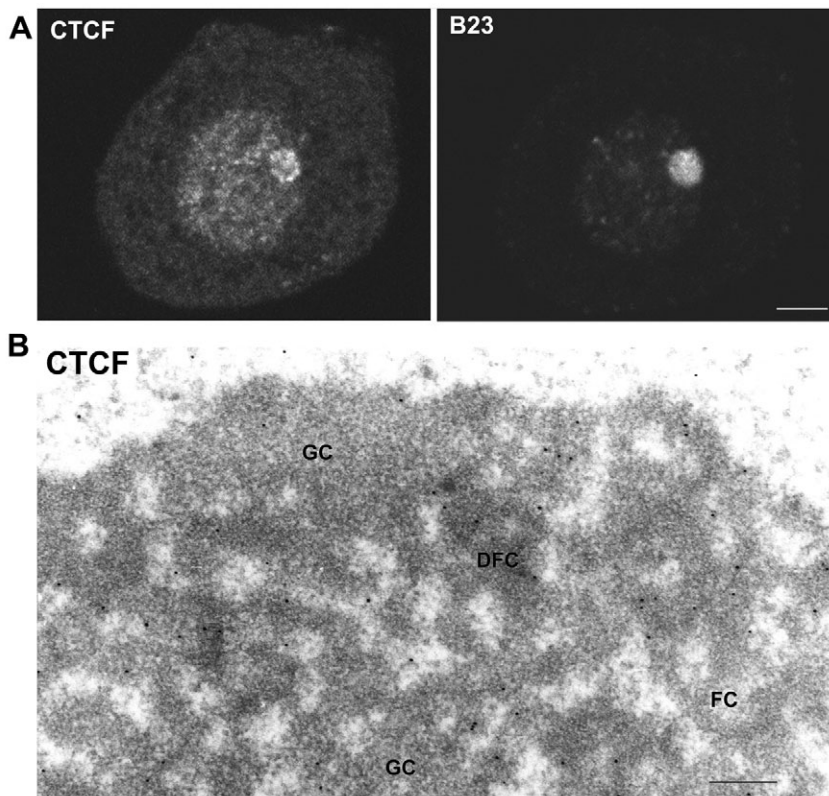


Fig. 3. Nucleolar localization of endogenous CTCF in rat neurons in vivo. (A) Confocal laser microscopy following double immunofluorescent staining with anti-CTCF rabbit polyclonal antibody (left panel) and anti-B23 antibody (right panel) in differentiated neurons from rat trigeminal ganglia. Bar, 10 μ m. (B) Immunoelectron localization of CTCF in the nucleolus of trigeminal ganglion neurons. Note the typical reticulated configuration of the nucleolus with its granular component (GC), dense fibrillar component (DFC) and fibrillar centres (FC). Gold particles indicating CTCF immunoreactivity preferentially decorated the dense fibrillar component of the nucleolus of rat neurons. Bar, 200 nm.

in K562 cells, with clear nucleolar localization of both the full-length GFP-CTCF and GFP-CTCF-ZF domain fusion proteins (Fig. 4Ba,c), diffuse nucleolar staining of the GFP-CTCF-C-terminal domain (Fig. 4Bd), and absence of nucleolar localization when the GFP-CTCF-N-terminal domain was expressed (Fig. 4Bb).

Analysis of subnuclear localization of different CTCF domains fused with GFP was also performed in HeLa cells; the results were identical to those obtained in K562 and UR61 cell lines (Fig. S2 in supplementary material). The data on the nucleolar targeting of GFP-CTCF constructs expressed in the three different cell lines studied are summarized in Fig. 4C. Taken together, these experiments demonstrate that the region responsible for nucleolar targeting of CTCF is localized within the central zinc-finger domain.

Subnuclear localization of the GFP-CTCF full-length in UR61 cells

To define the possible nucleolar subdomains targeted by CTCF, UR61 cells were transfected with the full-length GFP-CTCF construct (Fig. 5A). GFP detection was either combined with PI staining for nucleic acids or with immunostaining for (1) UBF, a transcription factor for rDNA that is preferentially located in fibrillar centres, (2) fibrillarin, a marker of the dense fibrillar component, and (3) B23, a marker of the granular component. Analysis of UR61 cells expressing the GFP-CTCF showed a homogeneous distribution of the CTCF fusion protein throughout the nucleolus, which was counterstained with PI (Fig. 5Aa-c). It is noteworthy that nucleolar CTCF signal did not overlap with the small nucleolar dots, fibrillar centres, characteristic for UBF immunostaining (Fig. 5Ad-f). However, a significant co-localization of CTCF with both fibrillarin (Fig. 5Ag-i) and B23 (Fig. 5Aj-l) was observed in the nucleolus. These findings indicate that full-length CTCF is not selectively associated with the fibrillar centres, but rather distributed more homogeneously in both the dense fibrillar and granular components of the nucleolus in UR61 cells.

Nucleolar localization of the full-length CTCF protein requires RNA pol I transcription

To determine a possible role for active rDNA transcription in the nucleolar distribution of CTCF, we added

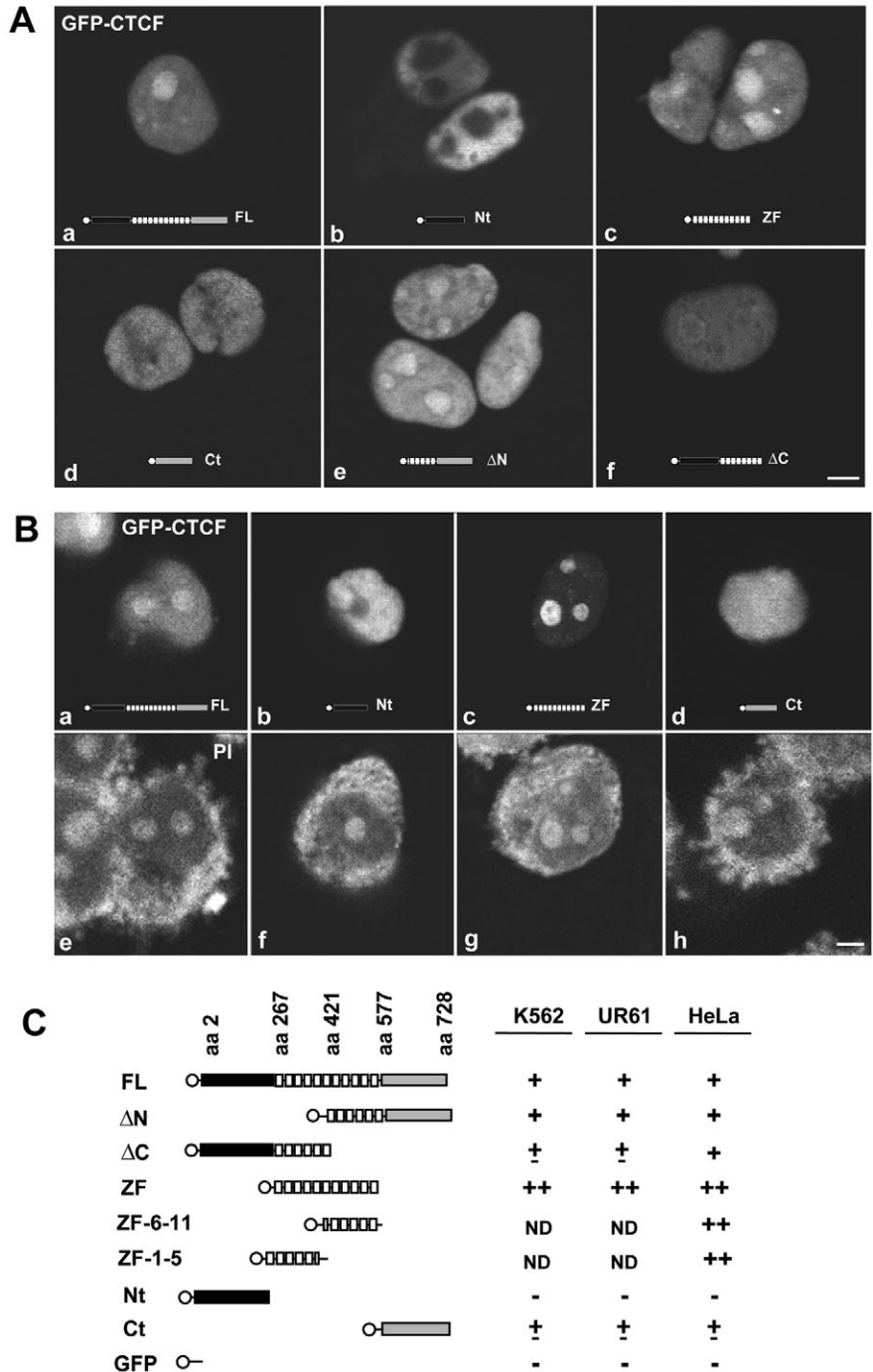


Fig. 4. The zinc-finger region of CTCF targets it to the nucleolus. (A) The distribution of the various GFP-CTCF fusion proteins, as described in C, in K562 cells. K562 cells were transfected with the indicated GFP-CTCF truncated variants, cells were fixed 24 hours post-transfection and green fluorescence was analyzed by confocal microscopy. Bar, 5 μ m. (B) The distribution of GFP-CTCF fusion proteins in UR61 cells. UR61 cells were transfected with the indicated GFP-CTCF truncated variants (as in C), cells were fixed 24 hours post-transfection and stained with propidium iodide. (a-d) green fluorescence from GFP-CTCF fusion proteins; (e-h) propidium iodide staining labelling nucleoli. Bar, 5 μ m. (C) Schematic representation of the full-length and truncated versions of CTCF fused to the C terminus of GFP. Numbers denote amino acid positions. The summary of the nucleolar localization for the different GFP-CTCF fusion proteins in K562, UR61 and HeLa cells is shown. Fusion proteins were present (+) or not (-) in the nucleolus as indicated; (++) , strong nucleolar accumulation; (±), weak signal; ND, not determined.

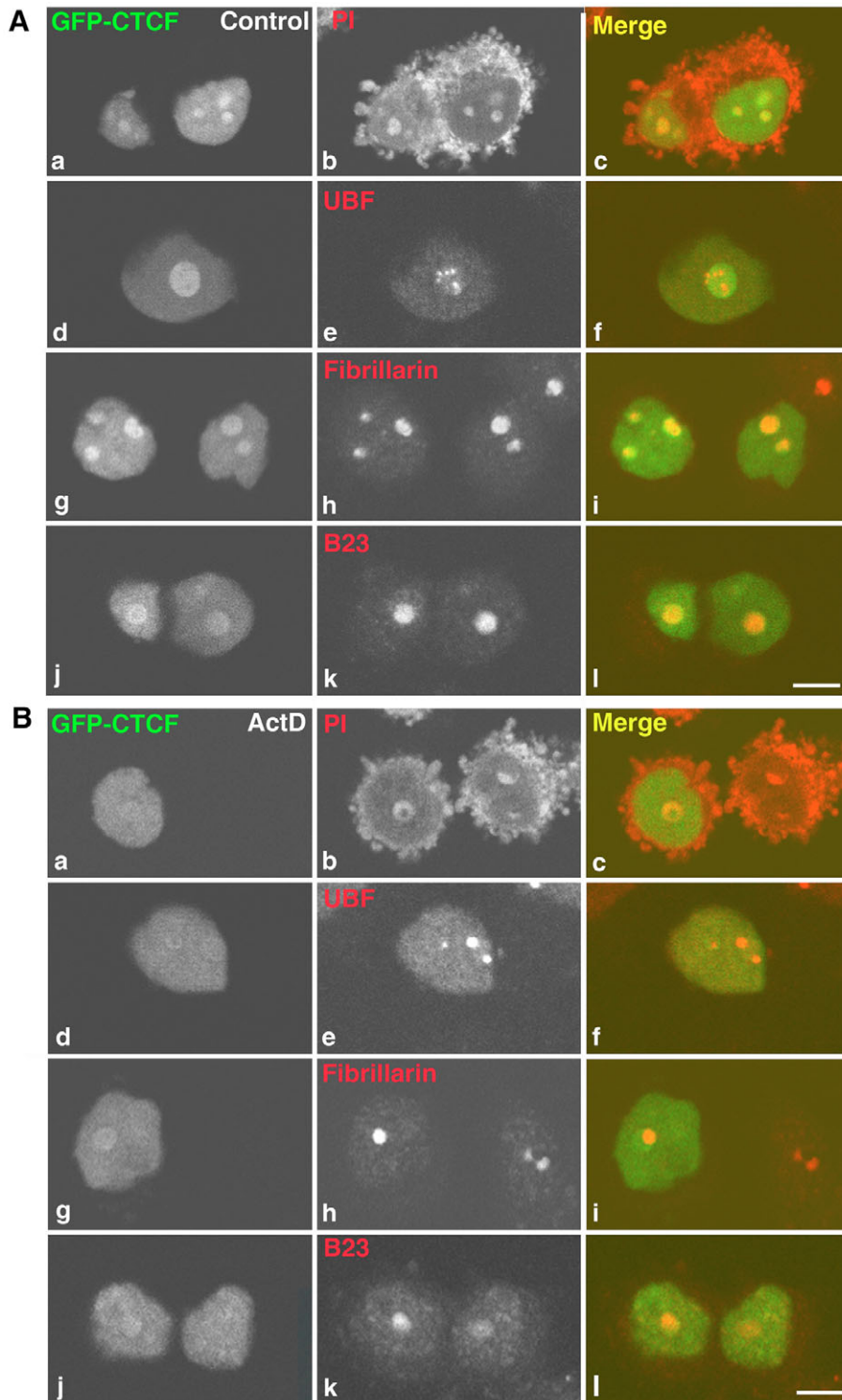


Fig. 5. Nucleolar localization of CTCF is dependent on the transcription from rDNA. UR61 cells were transfected with the full-length CTCF fused with the GFP (GFP-CTCF) and either left untreated (A) or treated 24 hours post-transfection with 0.05 $\mu\text{g/ml}$ actinomycin D (ActD) for 1 hour to specifically inhibit RNA pol I (B). Cells were fixed, immunostained for UBF, fibrillarin or B23 as indicated. Nucleic acids were visualized with propidium iodide (PI). The localization of the GFP-CTCF fusion protein (a,d,g,j; green channel), the markers for the different nucleolar compartments (b,e,h,k; red channel) and the merged images (c,f,i,l) are shown. Confocal images show that GFP-CTCF is released from the nucleolus upon inhibition of transcription by RNA pol I. Bar, 20 μm in a; 10 μm in b-l.

actinomycin D (Act D) to the culture medium of UR61 transfected cells at concentration sufficient to selectively inhibit RNA pol I transcription (Christensen et al., 2004). Treatment with Act D induced the segregation of the nucleolar components, as observed after PI staining (Fig. 5Bb), in agreement with previous reports (Puvion-Dutilleul et al., 1992). It is noteworthy that most of the cells treated with Act D exhibited a marked reduction of GFP-CTCF labelling in the nucleolus compared with untreated cells (Fig. 5Ba,d,g,j), indicating that targeting of CTCF to the nucleolus is dependent on nucleolar transcription.

We then analyzed the localization of CTCF in the nucleoli of UR61 cells treated with Act D by using immunofluorescent staining for the nucleolar markers UBF (Fig. 5Bd-f), fibrillarin (Fig. 5Bg-i) and B23 (Fig. 5Bj-l). Following treatment with Act D, fibrillar centres immunostained with the anti-UBF antibody were reorganized to form one or two larger dots free of CTCF (Fig. 5Bd-f). Furthermore, the nucleolar remnant of GFP-CTCF showed a diffuse distribution and was not specifically segregated with the fibrillarin-positive (Fig. 5Bg-i) or B23-positive (Fig. 5Bj-l) nucleolar domains.

In order to investigate whether the Act D-induced inhibition of the nucleolar targeting of CTCF is dependent on the full-length form of this protein, we next transfected UR61 cells with the construct harbouring the central zinc-finger domain of CTCF tagged with GFP (GFP-ZF), and exposed these cells to low doses of the RNA pol I inhibitor Act D (Fig. 6). As described above, GFP-ZF signal was strongly concentrated in nucleoli counterstained with PI in untreated cells (Fig. 4Bc,g; Fig. 6Aa-c). The nucleolar distribution pattern of this fusion protein in nucleolar domains immunostained for UBF, fibrillarin and B23 was similar to that observed in UR61 cells transfected with the GFP-CTCF construct (Fig. 6Ad-l). As expected, Act D treatment for 1 hour induced nucleolar segregation in most nucleoli. In these cases, the fusion protein was segregated with the rRNA-enriched domains of the nucleolus stained with PI (Fig. 6Ba-c). Interestingly, the GFP-ZF fusion protein remained in the nucleoli after

inhibition of RNA pol I (Fig. 6Ba,d,g,j). Moreover, this fusion protein was not concentrated in the larger fibrillar centres immunolabelled with the anti-UBF antibody (Fig. 6Bd-f) or in the segregated masses of dense fibrillar component stained with the anti-fibrillarin antibody (Fig. 6Bg-i). However, the nucleolar distribution pattern of GFP-ZF was similar to that of the protein B23 (Fig. 6Bj-l).

Taken together, these results indicate that the full-length form of CTCF can accumulate in both the dense fibrillar component, where rRNA transcription and pre-rRNA processing take place, and the granular component, the site of ribosome assembly. This nucleolar localization is dynamic, depends on the transcription of rDNA and necessitates the full length CTCF protein.

Nucleolar localization of the full-length CTCF requires de novo protein synthesis

We next asked whether inhibition of protein synthesis could have an effect on the localization of CTCF. The blockage of protein synthesis can be achieved with cycloheximide (Chx); it leads to a rapid depletion of short half-life transcription factors of the RNA polymerase I complex and inhibition of rDNA transcription and pre-rRNA processing (Gokal et al., 1986; Stoykova et al., 1985). In these experiments, UR61 cells were transfected with GFP-CTCF or GFP-ZF constructs and, 24 hours post-transfection, treated with Chx for 3 hours (Fig. 7b,d). Whereas untreated UR61 cells exhibited typical nucleolar accumulation of GFP-CTCF fusion protein (Fig. 7a,e), Chx treatment prevented the nucleolar targeting of GFP-CTCF, which was completely excluded from the nucleolus counterstained with PI (Fig. 7b,f). This effect was not so apparent in cells transfected with the zinc-finger domain, since a moderate GFP-ZF signal remained in the nucleolus after exposure to Chx (Fig. 7d,h), in comparison with the higher nucleolar signal detected in untreated transfected cells (Fig. 7c,g). In summary, the Cyclohexamide-induced inhibition of de novo protein synthesis interferes with the targeting of CTCF to the nucleolus.

Nucleolar transcription is inhibited by full length CTCF

To gain an insight into the functional significance of the nucleolar CTCF, we

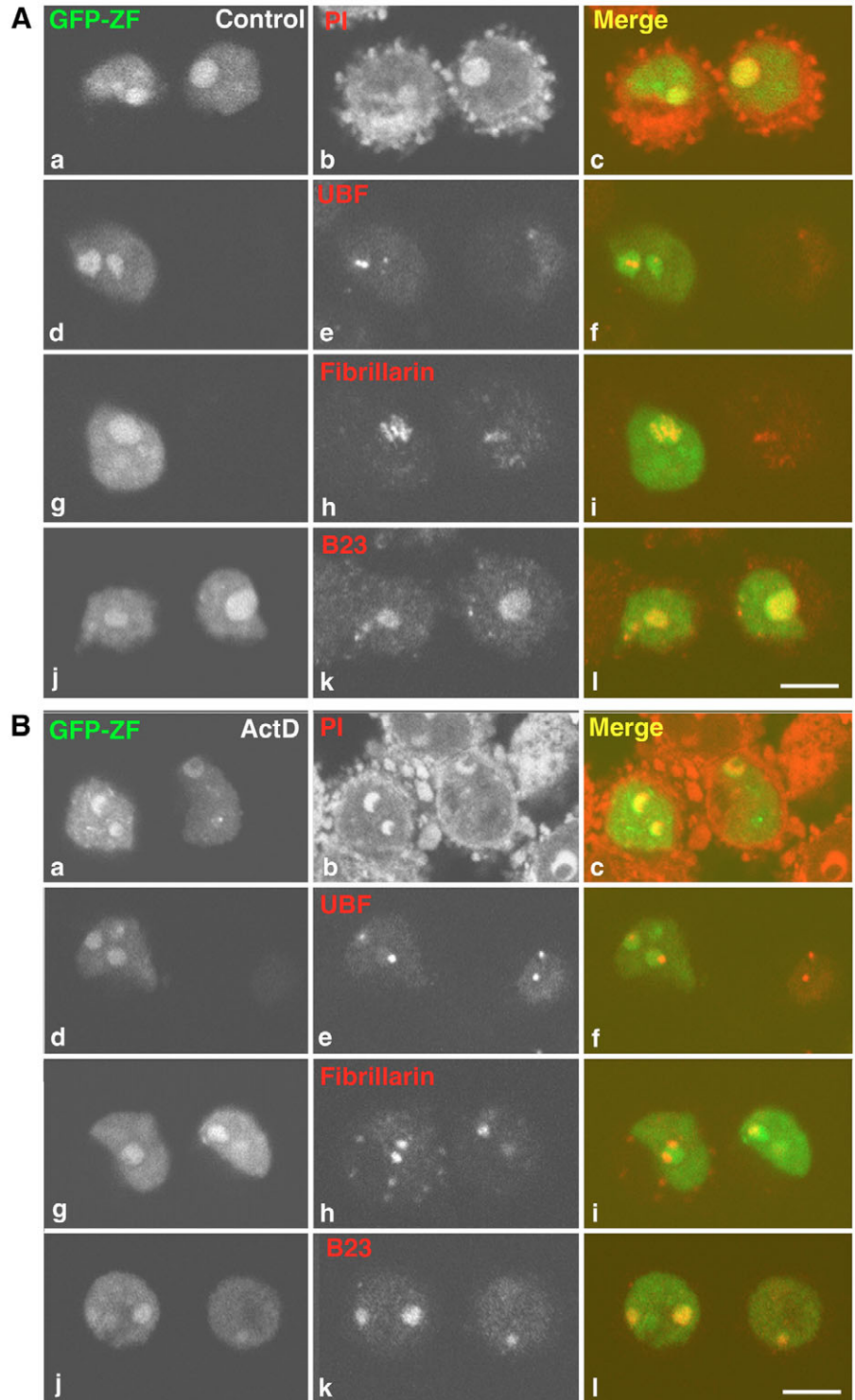
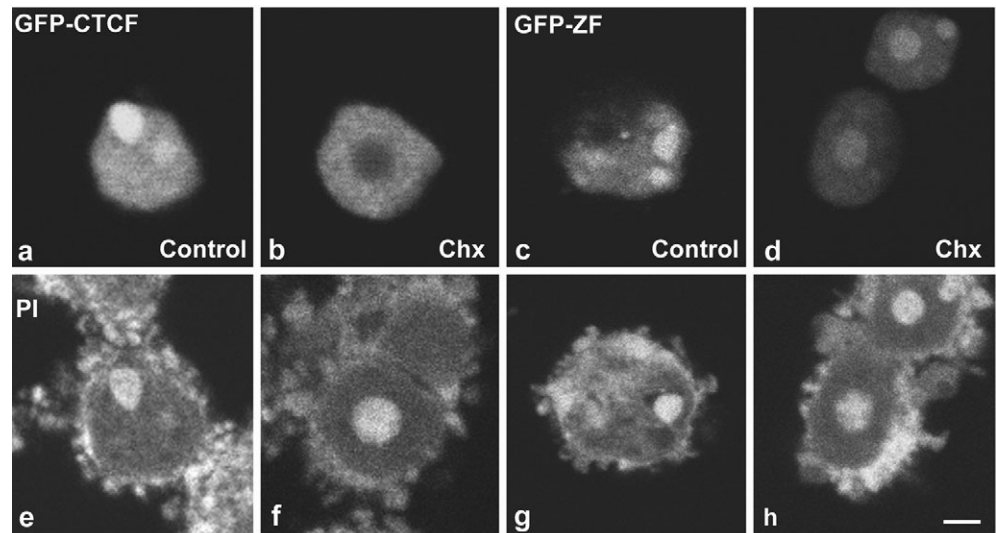


Fig. 6. Nucleolar localization of the zinc-finger region of CTCF is independent of the transcription from rDNA. UR61 cells were transfected with the construct harbouring the central zinc-finger domain of CTCF fused with the GFP (GFP-ZF) and either left untreated (A) or treated 24 hours post-transfection with 0.05 μ g/ml actinomycin D (ActD) (B) for 1 hour to specifically inhibit RNA pol I. Cells were fixed and immunostained for UBF, fibrillarin or B23 as indicated. Nucleic acids were visualized with propidium iodide (PI). The localization of the GFP-ZF fusion protein (a,d,g,j; green channel), the markers for the different nucleolar compartments (b,e,h,k; red channel) and the merged images (c,f,i,l) are shown. Confocal images show that GFP-ZF remains in the nucleolus following the inhibition of RNA pol I transcription. Bars, 10 μ m.

Fig. 7. Full-length CTCF, but not CTCF-ZF localization in the nucleolus requires protein synthesis. UR61 cells were transfected with the GFP-CTCF full length (GFP-CTCF; a,b,e,f) or with the GFP-CTCF-zinc-finger (GFP-ZF; c,d,g,h). 24 hours post-transfection cells were treated with 20 μ g/ml cycloheximide (Chx) for 3 hours to inhibit protein synthesis. Untreated cells served as control. Cells were then fixed and analyzed by confocal microscopy for green fluorescence (a-d). Nucleoli were stained with propidium iodide (e-h). GFP-CTCF fusion protein, but not GFP-ZF, is delocalized from the nucleolus upon protein synthesis inhibition with cycloheximide. Bar, 5 μ m.



investigated the possible involvement of CTCF in the regulation of nucleolar transcription. We therefore performed an in situ run-on assay based on the incorporation of 5'-fluorouridine (5'-FU) into nascent RNA, which labels active transcription sites (Boisvert et al., 2000). UR61 cells were transfected with full-length GFP-CTCF (Fig. 8a,d), the GFP-ZF domain (Fig. 8g) or the GFP vector (Fig. 8j). Twenty-four hours post-transfection, cells were exposed to pulses of 5'-FU, and subsequently immunostained for the detection of the halogenated nucleotide. In agreement with the high level of transcription in nucleoli, short pulses of 5'-FU for 5 minutes (Fig. 8b) and 10 minutes (Fig. 8e,h,k) resulted in the incorporation of 5'-FU predominantly into nascent pre-rRNA at the nucleolar transcription sites, and foci of 5'-FU signal were clearly observed within the nucleolus (arrow in Fig. 8b). However, in cells transfected with GFP-CTCF, the incorporation of 5'-FU into nucleoli was undetectable or very weak (Fig. 8a-c,d-f). By contrast, the nucleolar patterns of 5'-FU incorporation in cells transfected with GFP-ZF or GFP were similar to that observed in non-transfected cells (Fig. 8g-i,j-l and Fig. 9, upper panel). We conclude that full-length CTCF, but not the CTCF-ZF domain, dramatically inhibits nucleolar transcription.

Inhibition of PAR polymerases impairs the targeting of CTCF to the nucleolus

Since the poly(ADP-ribosyl)ated form of CTCF (CTCF-180) was detected in nucleolar fractions of K562 (Fig. 1C) and MCF7 (Fig. 2B) cells, we investigated the possible involvement of poly(ADP-ribosyl)ation of this factor in its nucleolar targeting. We analyzed the effect of the PAR polymerase inhibitor 3-aminobenzamide (ABA) on the nucleolar localization of GFP-CTCF and GFP-ZF variants, as well as on the nucleolar transcription, in UR61 cells. Following ABA treatment, nucleoli preserved the normal staining pattern with PI (Fig. 10b) and with the anti-UBF antibody (Fig. 10e). Interestingly, most of the transfected cells did not accumulate GFP-CTCF in nucleoli after ABA treatment (Fig. 10a-i and Fig. 9, lower panel). On the contrary, ABA did not interfere with the nucleolar localization of the GFP-ZF variant (Fig. 10j). Furthermore,

following ABA treatment, a similar pattern of 5'-FU incorporation in nucleoli was observed in non-transfected cells and cells transfected with GFP-CTCF (Fig. 10g-i and Fig. 9). This indicates that treatment with ABA interferes with nucleolar localization of CTCF leading to activation of transcription in the nucleolus. As expected, the patterns of 5'-FU incorporation in cells transfected with the GFP-ZF (Fig. 10j-l) or GFP (data not shown) constructs were similar to that detected in non-transfected cells. In summary, inhibition of poly(ADP-ribosyl)ation impairs CTCF nucleolar translocation, resulting in the restoration of active nucleolar transcription.

Discussion

CTCF accumulates in nucleoli of mammalian cells upon different stimuli

In this report we show that CTCF accumulates in the nucleoli upon induction of differentiation in human myeloid cells and induction of growth arrest and apoptosis in human breast carcinoma cells. Furthermore, CTCF has been found in the nucleoli of differentiated adult rat neurons in vivo. The common characteristic between these cell models is inhibition of cell proliferation, which may be linked to the presence of CTCF in nucleoli. By using K562 cells, we previously demonstrated that CTCF is differentially regulated during myeloid differentiation (Delgado et al., 1999) and has a functional role in the regulation of the erythroid pathway (Torrano et al., 2005). In this report, we show that differentiation of K562 cells into erythroid and megakaryocytic lineages is accompanied by relocation of CTCF to the nucleolus. Similarly, in MCF7 cells, CTCF shifts from nucleoplasm to the nucleolus following NaBu 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.

CTCF is a predominantly nucleoplasmic protein in the majority of cells and its translocation to the nucleolus is likely to be a dynamic process and a consequence of functional interactions with other macromolecules. This resembles the situation with MYC, which is usually a nucleoplasmic

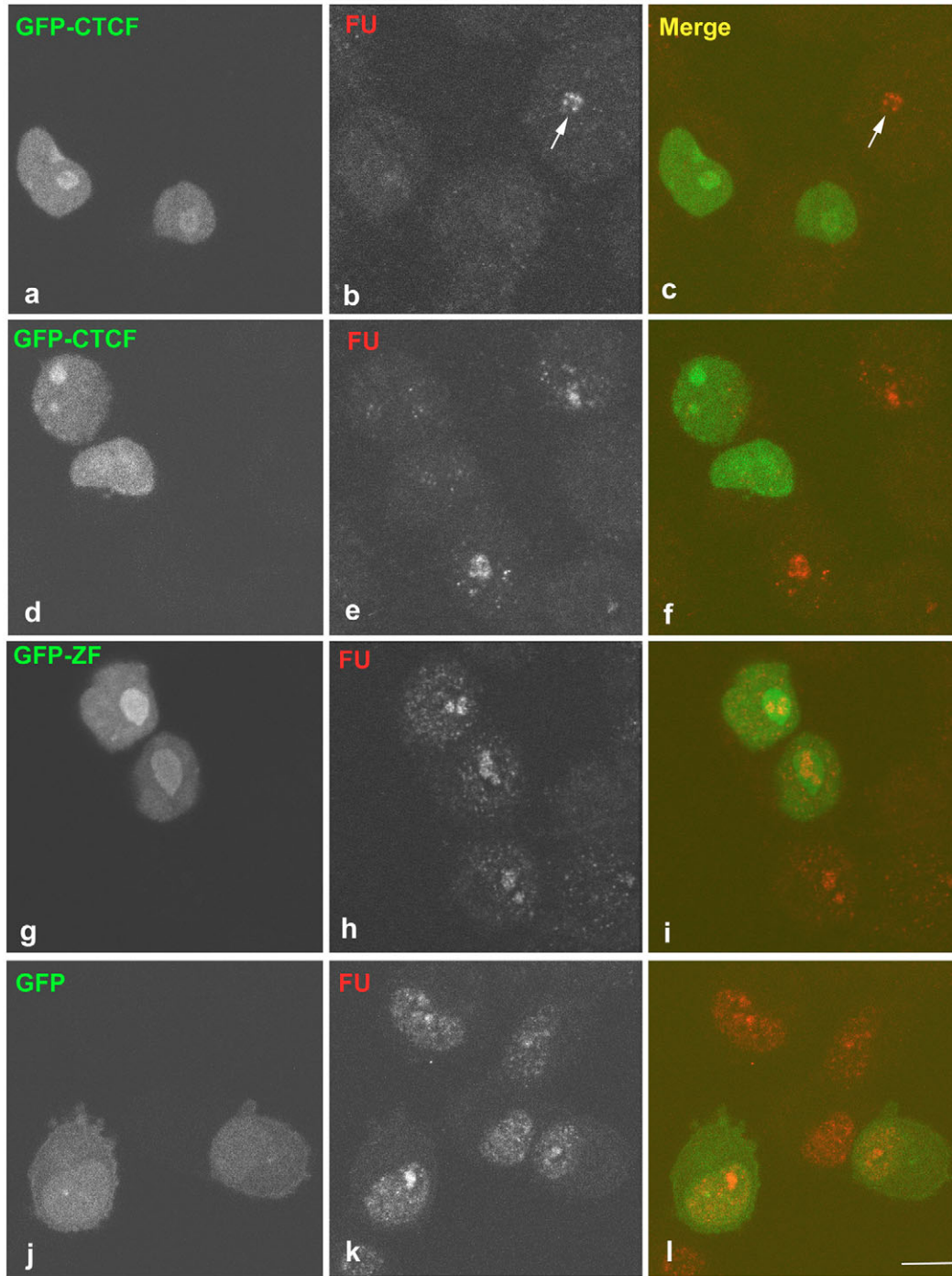


Fig. 8. Full-length CTCF, but not CTCF-ZF, inhibits nucleolar transcription. UR61 cells were transfected with the GFP-CTCF full length (GFP-CTCF; a-c,d-f), the GFP-CTCF-zinc-finger (GFP-ZF; g-i) or the pEGFP vector (GFP; j-l). 24 hours post-transfection cells were pulse-labelled with 5'-fluorouridine (FU) for 5 minutes (b) or 10 minutes (e,h,k). Cells were then fixed and the sites of 5'-FU incorporation revealed with an anti-BrdU antibody and a Texas Red-conjugated secondary antibody. The localization of the GFP-fusion proteins (a,d,g,j; green channel), the 5'-FU incorporation (b,e,h,k; red channel) and the merged images (c,f,i,l) are shown. Confocal images show that GFP-CTCF, but not GFP-ZF or GFP inhibits 5'-FU incorporation into nascent RNA. Bar, 10 μ m.

transcription factor rarely found in nucleoli in normal cells (Arabi et al., 2003). Nevertheless, MYC plays an important role in the regulation of rDNA transcription (Arabi et al., 2005; Grandori et al., 2005). A number of nuclear factors have been found to be transiently present in the nucleoli, continuously exchanging with the nucleoplasm (Dundr and Misteli, 2002). Such dynamic interactions with the nucleolus often depend on the metabolic state of the cell (Andersen et al., 2002). These findings may explain why CTCF has not been identified in the nucleolar proteome in HeLa cells (Andersen et al., 2005; Andersen et al., 2002).

The zinc-finger domain targets CTCF to the nucleolus
In this study, by using recombinant proteins of full-length and truncated versions of CTCF fused to the GFP, we have demonstrated that the central zinc-finger region of CTCF, which is responsible for DNA binding in the silencing function of CTCF, is also responsible for its nucleolar targeting. This has been demonstrated in three cell lines of different origin (K562, UR61 and HeLa). In previous studies, CTCF over-expression was found to be strongly associated with growth suppression (El-Kady and Klenova, 2005; Qi et al., 2003; Rasko et al., 2001; Torrano et al., 2005). Here, we found that

transiently over-expressed GFP-CTCF protein was targeted into nucleoli in the population of proliferating cells. It may be significant for initiation of the processes required for growth inhibition caused by ectopic CTCF.

A strong nucleolar accumulation of CTCF was only observed when the 11 zinc-finger region was expressed, while the N-terminal or the C-terminal regions were dispensable for the nucleolar targeting of the CTCF (Fig. 4C). Interestingly, when the zinc finger domain is divided in two halves (constructs ZF-1-5 or ZF-6-11) they can still mediate nucleolar targeting in HeLa cells, thus indicating that there may be two regions in the DNA-binding zinc-finger domain of CTCF that are important for such localization (Fig. S2 in supplementary material). It is noteworthy that this central zinc-finger region is capable of binding not only DNA targets, but also proteins, for example the Y-box DNA/RNA-binding factor YB-1 which may also be involved in the nucleolar targeting of CTCF (Chernukhin et al., 2000; Klenova et al., 2004; Ohlsson et al., 2001). Interestingly, the C-terminal zinc fingers (ZF-6-11) are necessary for targeting of CTCF to mitotic chromosomes (Burke et al., 2005) thus pointing to the importance of the DNA binding domain for targeting into different nuclear compartments.

Inspection of the protein sequence of the CTCF zinc-finger domain revealed three possible nucleolar localization signals

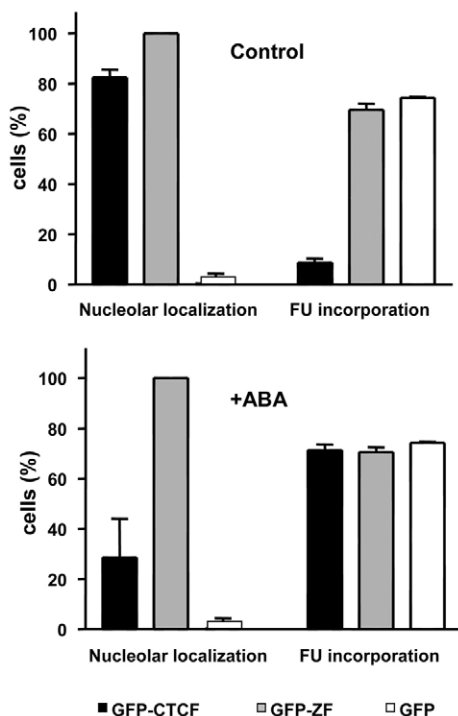


Fig. 9. Quantification of nucleolar localization of the GFP fusion proteins and FU incorporation in nucleoli, in untreated cells and cells treated with ABA. UR61 cells, untreated (Control, upper panel) and treated with 3-aminobenzamide for 8 hours (ABA, lower panel) were transfected with GFP-CTCF, GFP-ZF and GFP. 24 hours post-transfection, cells were pulse-labelled with 5'-FU for 10 minutes and cells showing nucleolar localization of the GFP fusion proteins (bars on the left) and cells showing 5'-FU incorporation in nucleoli (bars on the right) were scored. Data show the percentage of cells (mean \pm s.e.m.) from three experiments.

(NuLS) containing a characteristic arginine hinge flanked by basic amino acids, proposed to be a consensus for some NuLS (Henderson et al., 1995) (not shown). Evidence for this short sequence being a consensus for NuLS are, however, conflicting (Schmidt-Zachmann and Nigg, 1993) and the exact sequences responsible for nucleolar targeting of CTCF within the zinc-finger domain remain to be established. In addition, it is conceivable that localization 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 (Siomi and Dreyfuss, 1997).

CTCF is associated with several components of the nucleolus

To determine the subnucleolar distribution of CTCF within the nucleolus we have analysed the co-localization of the CTCF with UBF, fibrillarin or B23. The rDNA transcription factor UBF is associated with rDNA loci within the nucleolus, which can be observed as discrete foci corresponding to fibrillar centres (Roussel et al., 1993). In contrast, CTCF labelling is much more homogeneous and does not show the characteristic foci labelling seen with UBF. CTCF is therefore not selectively associated with the fibrillar centres, but homogeneously distributed throughout the dense fibrillar and granular components of the nucleolus. 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.

Interestingly, B23 and CTCF were found to be present at the insulator sites *in vivo* and exogenous insulator sequences tethered to the nucleolus in a CTCF-dependent manner (Yusufzai et al., 2004). Our results of double immunofluorescence analysis for CTCF and B23 are consistent with the co-localization of both proteins in nucleolar subdomains.

The nucleolar accumulation of CTCF depends on active RNA pol I transcription and protein synthesis

Our results indicate that CTCF fails to accumulate in the nucleolus after inhibition of pol I transcription with low doses of Act D. Treatment with Chx, which leads to a depletion of short-lived transcription factors required by the pol I transcription machinery and severe nucleolar dysfunction (Cavanaugh et al., 2002 and references therein), also prevents translocation of CTCF to the nucleolus. Collectively, these findings indicate that targeting of CTCF to the nucleolus requires ongoing 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 the existence of a protein interaction network important for CTCF translocation in the nucleolus. Similar networks have been proposed for other factors that accumulate in the nucleolus in the same way as CTCF (Desterro et al., 2003; Straight et al., 1999; Zhang et al., 2004b).

In contrast to the CTCF-full length form, the central zinc-finger domain accumulates in the nucleolus after treatment with Act D and Chx. Thus, Act D-induced depletion from the nucleoli of CTCF depends on the full-length protein, whereas the nucleolar localization of the DNA binding zinc-finger domain is not affected by this treatment. We hypothesize that translocation of the full-length CTCF in the nucleolus or/and

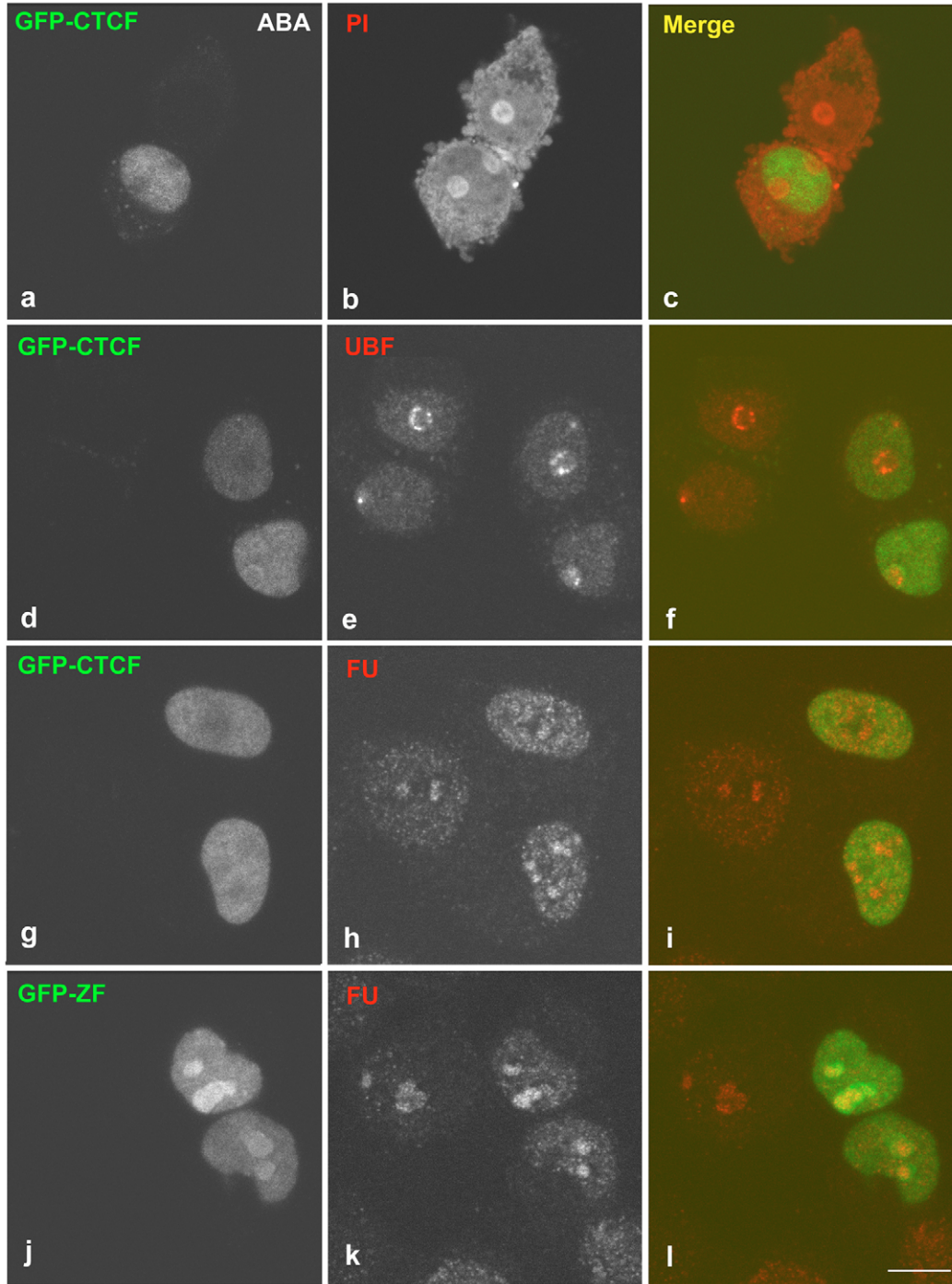


Fig. 10. Inhibition of PARPs impairs nucleolar localization of CTCF-full length, but not CTCF-ZF. UR61 cells were treated for 8 hours with 3-aminobenzamide (ABA) and then transfected with the GFP-CTCF full length (GFP-CTCF; a-c,d-f,g-i), or with the GFP-CTCF-zinc-finger domain (GFP-ZF; j-l). 24 hours post-transfection cells were analyzed by confocal microscopy for green fluorescence (a,d,g,j). Nucleoli were stained with propidium iodide (PI; b), or immunostained for UBF (e). Cells were also pulsed with 5'-FU for 10 minutes (h,k); merged images are also shown (c,f,i,l).

its retention there may be regulated processes that require the N- and C-terminal domains and depend on the presence of particular types of RNA and/or proteins. Interestingly, the full-length CTCF, but not the zinc-finger fusion protein, was functionally active in inhibiting cell growth in a clonogenic assay (Fig. S1 in supplementary material), in agreement with previous reports showing cell growth inhibition by CTCF (Rasko et al., 2001; Torrano et al., 2005).

CTCF has a functional role inhibiting nucleolar transcription that depends on active PARP

In this study we demonstrate the involvement of CTCF in the

inhibition of the nucleolar transcription. To identify the functional role of CTCF in the nucleolus, we performed run-on transcription assays, which provide a powerful tool to investigate the overall activity of gene expression in situ, in both cell cultures (Boisvert et al., 2000) and animal models (Casafont et al., 2006). Following short pulses of 5'-FU incorporation, a high concentration of nascent 5'-FU-labeled RNA was found in the nucleolus, consistent with the intense transcriptional activity of ribosomal genes (Russell and Zomerdijk, 2005). 5'-FU-incorporation clearly delineated the nucleolar sites of transcription in non-transfected UR61 cells or cells transfected with the GFP-ZF-fusion protein, whereas a

weaker or no detectable signal was found in cells transfected with the full-length GFP-CTCF protein (see Fig. 8). These findings strongly suggest CTCF involvement in the control of rDNA transcription. Intriguingly, CTCF binding sites in the rDNA of *Xenopus* have been found (West et al., 2002) although further studies are required to define the precise role of CTCF in the regulation of rDNA transcription.

The importance of the fine balance between the proliferation status of the cell and the accumulation of rRNAs, which is mainly controlled at the level of rDNA transcription, has already been recognized (Russell and Zomerdijk, 2005). In line with these findings, our results, showing nucleolar accumulation of endogenous CTCF in growth-arrested cells, suggest that nucleolar location of CTCF may be an important mechanism to simultaneously block cell proliferation and transcription from rDNA.

Recent studies have shown that poly(ADP-ribosylation) of CTCF regulates its activity as a chromatin insulator (Klenova and Ohlsson, 2005; Yu et al., 2004). Increasing numbers of roles of poly(ADP-ribosylation) in critical cellular processes such as DNA damage, chromatin modification, transcription regulation and many others have been described (Kim et al., 2005; Rouleau et al., 2004). In the present report, significant levels of the 180 kDa CTCF isoform, likely to represent poly(ADP-ribosylated) CTCF, were found in the nucleoli in K562 and MCF7 cell lines. Using a PARP inhibitor (ABA) we found that inhibition of poly(ADP-ribosylation) 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-ribosylation). Interestingly, PARP-1 and PARP-2, have been found to accumulate in nucleoli in a complex with B23 (Meder et al., 2005). It is conceivable that CTCF may be a part of the same functional network, as interaction with PARP-1 and B23 has been documented (Yusufzai et al., 2004). In summary, we conclude that the nucleolar localization of CTCF, associated to growth inhibition, cell differentiation or apoptosis has a functional significance, inhibiting the transcription of the nucleolus through mechanisms involving CTCF poly(ADP-ribosylation).

Materials and Methods

Cell culture, induction of differentiation and drugs treatment

The myeloid K562 cells (from the American Type Culture Collection) were grown in suspension in RPMI 1640 medium (Invitrogen) supplemented with 8% foetal calf serum (Biochrome). The erythroid and megakaryocytic differentiation was induced with 1 μ M 1- β -D-arabinofuranosylcytosine (Ara-C; Upjohn) and 100 nM staurosporine (STA) (Roche), respectively, and monitored as previously described (Munoz-Alonso et al., 2005). Human breast carcinoma MCF7 cells were maintained in RPMI 1640 supplemented with 10% foetal calf serum. Growth arrest in MCF7 cells was induced with 5 mM sodium butyrate (NaBu) for 8 hours. Longer exposures to NaBu leads to apoptotic cell death (Chopin et al., 2002; Chopin et al., 2004; Docquier et al., 2005). HeLa cells, from human cervical carcinoma, were grown in DMEM medium supplemented with 10% foetal calf serum. UR61 cells, derived from the rat pheochromocytoma PC12 cell line (Guerrero et al., 1988), were grown attached to the plates in RPMI 1640 medium supplemented with 10% foetal calf serum and 500 μ g/ml of G418. Where indicated, UR61 cells were incubated with 0.05 μ g/ml actinomycin D (ActD) (Sigma) for 1 hour to selectively inhibit RNA pol I transcription (Christensen et al., 2004), with 20 μ g/ml cycloheximide (Chx) for 3 hours to inhibit protein synthesis (Mattsson et al., 2001) or with 8 mM 3-aminobenzamide (ABA) (Sigma) to inhibit poly(ADP-ribose) polymerases (Yu et al., 2004).

Transfections and clonogenicity assays

Cells were transfected with full-length and truncated versions of CTCF fused to the C terminus of EGFP. The construction of the GFP-CTCF plasmids has been previously described (Burke et al., 2005). For transient transfections, 5×10^6 K562

cells in exponential growth were resuspended in 0.8 ml of RPMI-8% FCS containing 10 μ g of the different expression constructs and electroporated at 260 V and 1 mF with a Bio-Rad electroporator. UR61 cells were transiently transfected with 1 μ g of plasmid DNA using FuGene 6 transfection reagent (Roche). Cells were analyzed 24 hours post-transfection.

For clonogenicity assays, K562/S, a derivative of the K562 cell line able to grow attached to plastic (Delgado et al., 2000), was used. 2×10^6 cells growing in p60 plates were electroporated with 5 μ g of pEGFP-C2-derived constructs: pEGFP vector (GFP), full-length CTCF (GFP-CTCF) or CTCF-zinc-finger region (GFP-ZF). 36 hours post-transfection, G418 (500 μ g/ml) was added and the colonies were counted after 12 days of selection.

Isolation of nucleoli and western analysis

Extracts enriched in nucleoli were prepared from K562 cells untreated or treated for 4 days with 1 μ M Ara-C, essentially as previously described (Andersen et al., 2002).

For western analysis, samples were resuspended in Laemmli buffer, separated on a 8% SDS-PAGE gel, transferred onto a nitrocellulose membrane (Millipore) and probed. Antibodies used were: anti-CTCF monoclonal antibody (1:500; BD Biosciences), anti-CTCF polyclonal antibody (1:500; Abcam, Cambridge, UK) and anti-UBF monoclonal antibody (1:500; Santa Cruz Biotechnology). Immunocomplexes were detected by a chemiluminescent method (ECL, Amersham) following the manufacturer's instructions.

Preparation of trigeminal ganglion neurons

Young adult male Sprague-Dawley rats were used. The animals were housed, supervised and handled according to the approved national guidelines for animal care. For immunofluorescence, animals were fixed with 3.7% paraformaldehyde, trigeminal ganglia were removed and squash preparations of neurons were made as previously described (Pena et al., 2001).

Immunofluorescence and confocal and immunoelectron microscopy

The UR61 cells grown on glass coverslips were fixed for 10 minutes in 3.7% paraformaldehyde in PBS. Cells were permeabilized with 0.5% Triton X-100 for 10 minutes, and successively incubated with primary (overnight incubation at 4°C) and secondary antibodies. Propidium iodide staining (4 μ g/ml final concentration) of fixed and permeabilized cells was performed for 5 minutes. K562 cells were routinely fixed with methanol for 10 minutes at -20°C and immunostaining was performed as above. For endogenous nucleolar CTCF detection of K562 or MCF7, an additional modification involving a step of microwave heating after fixation in formaldehyde was included (Docquier et al., 2005).

Primary antibodies used were: anti-CTCF mouse monoclonal (1:100; BD Biosciences), anti-CTCF rabbit polyclonal (1:30; Abcam), anti-UBF (1:200; Santa Cruz), mouse monoclonal anti-fibrillarin (Reimer et al., 1987), anti-B23/nucleophosmin (1:50; Santa Cruz). Secondary antibodies (Jackson Laboratories) were conjugated with Texas red or FITC. For detection of GFP-CTCF fusion proteins, transfected cells were fixed in 3.7% paraformaldehyde, permeabilized with 0.5% Triton X-100 for 10 minutes and mounted with anti-fading mounting medium Vectashield (Vector Laboratories) with or without DAPI to visualize the nucleus. Cell samples were examined using a Zeiss 63 \times NA 1.4 PlanApo objective. Images were recorded using a Bio-Rad MRC 1024 confocal laser microscope equipped with argon (488 nm) and HeNe (543 nm) lasers.

Immunoelectron microscopy of rat trigeminal ganglia neurons was performed as described previously (Pena et al., 2001). Briefly, animals were fixed with 4% paraformaldehyde and small segments of trigeminal ganglia were dehydrated and embedded in Lowicryl K4M (Sigma) at -20°C. Ultra-thin sections were sequentially incubated with 0.1 M glycine in PBS, the primary anti-CTCF rabbit polyclonal antibody and the secondary antibody conjugated to 10 nm or 15 nm gold particles (1:25; Biocell Technology). The sections were stained with uranyl acetate and lead citrate. As controls, sections were treated as described but the primary antibody was omitted.

Run-on transcription assay

For immunodetection of nascent RNA, pulses of 5'-fluorouridine (5'-FU) (Sigma) were administered essentially as described previously (Boisvert et al., 2000). UR61 cells were transfected with different pEGFP-derived constructs and 24 hours post-transfection 5'-FU was added, to a final concentration of 2 mM in the culture medium. After 5 or 10 minutes, cells were fixed with 3.7% paraformaldehyde in HPEM buffer (30 mM Hepes, 65 mM Pipes, 2 mM EGTA, 2 mM MgCl₂) containing 0.5% Triton X-100 for 10 minutes. The incorporation of 5'-FU into nascent RNA was detected with an antibody against halogenated UTP (1:50, anti-BrdU clone BU-33; Sigma) and a Texas Red-conjugated secondary antibody (Jackson Laboratories).

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