DNA damage-dependent interaction of the nuclear matrix protein C1D with translin-associated factor X (TRAX)

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Summary

The nuclear matrix protein C1D is an activator of the DNA-dependent protein kinase (DNA-PK), which is essential for the repair of DNA double-strand breaks (DSBs) and V(D)J recombination. C1D is phosphorylated very efficiently by DNA-PK, and its mRNA and protein levels are induced upon γ-irradiation, suggesting that C1D may play a role in repair of DSBs in vivo. In an attempt to identify the biological function of C1D, we have employed the yeast two-hybrid system and found that C1D interacts specifically with Translin-associated factor X, TRAX. Although the biological function of TRAX remains unknown, its bipartite nuclear targeting sequences suggest a role for TRAX in the movement of associated proteins, including Translin, into the nucleus. We show that C1D and TRAX interact specifically in both yeast and mammalian cells. Interestingly, however, interaction of these two proteins in mammalian cells only occur following γ-irradiation, raising the possibility of involvement of TRAX in DNA double-strand break repair and providing evidence for biological functions of the nuclear matrix protein C1D and TRAX. Moreover, we show, using fluorescently tagged proteins, that the relative expression levels of TRAX and Translin affect their subcellular localization. These results suggest that one role for C1D may be to regulate TRAX/Translin complex formation.

Key words: C1D gene, Nuclear matrix, Translin, TRAX, DNA-PK

Introduction

Higher order organization of genomic DNA is one of the important mechanisms involved in control of cell-type-specific gene expression. Polypeptides involved in higher order chromatin folding are non-histone proteins and are tightly bound to DNA, even after treatment with harsh denaturants (Neuer et al., 1983). Although the biological functions for these proteins are still largely obscure, some of them have been reported to be associated with highly repetitive DNA sequences and to be involved in targeting a subset of genomic DNA to the nuclear matrix (Neuer and Werner, 1985; Neuer-Nitsche et al., 1988; Pfutz et al., 1992; Werner and Neuer-Nitsche, 1989). The nuclear matrix protein C1D is the first of such non-histone proteins to have been characterized at the sequence level (Nehls et al., 1998). Independently, C1D was found to be associated with the transcriptional repressor RevErb and the nuclear coreceptors N-Cor and SMRT, indicating that it could act as a component of the complex involved in transcriptional repression (Zamir et al., 1997). C1D is also an activator of DNA-dependent protein kinase (DNA-PK) (Yavuzer et al., 1998), which plays an important role in DNA double-strand break (DSB) repair and V(D)J recombination, a process specific to lymphocytes that is required for development of the immune system (Smith and Jackson, 1999). Interestingly, C1D is induced upon γ-irradiation and proposed to play a role in targeting DNA-PK to specific nuclear regions in response to DNA damage. Indeed, the *xrs5* cells deficient in one of the subunits of DNA-PK, Ku-80, exhibit irregularly shaped nuclear envelope and altered nuclear matrix compared with their wild-type controls (Korte and Yasui, 1993; Yasui et al., 1991). In connection with its role to regulate DNA-PK, C1D has also been shown to induce apoptosis in a p53-dependent manner (Rothbarth et al., 1999). Although the physiological function for C1D is not yet clear, accumulating evidence indicates that C1D is a multifunctional protein and, possibly by influencing chromatin structure, is capable of regulating different cellular events, such as DNA-repair, transcription and apoptosis.

In an attempt to identify the possible biological functions of C1D and the nuclear matrix, we have applied the yeast two-hybrid system (Fields and Song, 1989) to screen for polypeptides that interact with C1D. This approach has led us to identify a specific interaction between C1D and a recently identified protein, Translin-associated factor X (TRAX). TRAX has been shown to interact with Translin through yeast two-hybrid screening (Aoki et al., 1997) and exhibits extensive amino acid homology to Translin. Translin was first identified as a single-strand DNA binding protein that recognizes the consensus sequences, ATGCAG and GCCC(A/T)-(G/C)(G/C)(A/T), found at the breakpoint junctions of various chromosomal translocations seen in many lymphoid and solid tumours (Aoki et al., 1995; Chalk et al., 1997). Interestingly, homologues of TRAX and Translin are found in various other

organisms including mouse, fission yeast, frogs, insects and plants (Devon et al., 2000), suggesting that these molecules have important biological functions**.** Indeed, numerous functions have been proposed for Translin, ranging from mRNA transport and translational regulation to DNA recombination and repair.

In this paper we report that C1D and TRAX are capable of interacting specifically in yeast and in mammalian cells. However, in mammalian cells their interaction could be detected only after γ-irradiation and interestingly, only the dimeric but not the monomeric form of C1D can interact with TRAX. In addition, C1D and Translin interact with TRAX in a mutually exclusive fashion, and co-expression of TRAX and Translin results in their altered subcellular localization. The potential implications of these results in regard to the biological roles of C1D and TRAX in DNA repair and recombination are discussed.

Materials and Methods

Cloning, mutagenesis and sequencing

Wild-type TRAX, Translin and C1D open reading frames (ORFs) were isolated by PCR with primers placing suitable restriction sites to the 5′ and 3′ ends and cloned into various yeast and mammalian expression plasmids. The yeast expression plasmid is pACT. The mammalian expression constructs include a HA-epitope (a peptide derived from influenza haemagglutinin protein)-tagged expression vector pCMV 5′2N3T (kindly provided by T. Kouzarides), and plasmids expressing various chromophores of the *Aequorea victoria* green fluorescent protein (GFP) obtained from Clontech: pEGFP (enhanced green fluorescent protein), pEYFP (enhanced yellow fluorescent protein), pECFP (enhanced cyan fluorescent protein) and pDsRed1 (red fluorescent protein). All other C1D constructs have been described previously (Yavuzer et al., 1998). MutLZ-Trax (the mutated LZ motive of TRAX) was generated in two steps. First, the region between amino acids 1-194 was isolated by PCR with mismatch primers placing *Sal*I/*Bam*HI sites at the 5′ end and a *Bgl*II site at the 3′ end. The region between amino acids 194-272 was then amplified via PCR, using primers that place a *Bgl*II site at the 5′ end and *Sal*I/*Bam*HI sites at the 3′ end. The two PCR fragments were digested with *Sal*I/*Bgl*II and cloned in a triple ligation into the *Xho*I site of pACT. This construct produced a mutant TRAX where a *Bgl*II site is inserted, which changes the 194th amino acid leucine to arginine and the preceding lysine to serine. The whole fragment was then cut out with *Bam*HI and cloned into T7-Myc/plink; it was transcribed and translated in vitro using TnT Coupled Reticulocyte Lysate system (Promega) to confirm that it gives a similar size product to wild-type TRAX. Sequencing of PCR fragments, point mutations and cDNA inserts from positive clones of the two-hybrid screen was performed with an automatic sequencer (ABI 377, version 2.1.1). Sequence comparisons were conducted using GenBank database searches.

Yeast two-hybrid screen

The yeast two-hybrid screen was performed as described previously using a B-lymphocyte cDNA library in pACT (Harper et al., 1993) and full-length C1D in fusion with the DNA-binding domain of bacterial LexA protein (DBD-C1D) (Yavuzer et al., 1998). The yeast strains and the control plasmids (DBD-Lamin and DBD-Daughterless) were kindly provided by S. Hollenberg. *Saccharomyces cerevisiae* L40 (*MAT**a** trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*) was grown at 30°C in YPD medium (1% yeast extract, 2% polypepton and 2% glucose) and was transformed with DBD-C1D. Human B-lymphocyte cDNA library was then transformed into this strain using the lithium acetate method. Double transformants were plated on selective medium lacking Trp, Leu, His, Lys and uracil and were grown at 30°C for 3 days. Colonies were transferred on Whatman 40 filters to test for β-galactosidase activity using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Positive clones were rescued and tested for specificity by mating analysis or retransforming into L40 together with the control plasmids mentioned above. For the mating assay, DNA from positive clones was extracted using glass beads and then transformed by electroporation into the bacterial strain HB101, which has a defect in the leuB gene that can be complemented by LEU2 from yeast, and therefore provides selection of the library plasmids. The transformants were plated onto M9 medium containing ampicillin and proline and incubated at 37°C for 24 hours. DNAs of five colonies from each plate were then isolated by the alkaline lysis method, transformed into the yeast strain AMR70 (*MATα his3 lys2 trp1 leu2 URA3::(lexAop)$_8$-lacZ*) and were mated with the L40 strain carrying the bait plasmid. For liquid β-galactosidase assays using ONPG (o-nitrophenyl β-D-galactopyronoside) as a substrate, a yeast colony was grown at 30°C to mid-log phase (OD_{600}) and the cells were resuspended in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) and lysed by freeze-thaw cycles. Seven hundred μl of Z buffer and 160 μl of ONPG (4 mg/ml) were added to 100 μl of lysate and incubated at 30°C until a yellow colour appeared. The reaction was stopped by the addition of 400 μl 1 M Na_2CO_3 and OD_{420} of the samples were read. β-Galactosidase units were calculated according to the formula: $1000\times OD_{420}/(t\times V\times OD_{600})$ where t is elapsed time (in minutes) of incubation and V is 0.1 ml×concentration factor.

Ni-NTA pull-down assays

His-tagged C1D was expressed in bacteria and bound to Ni-NTA column as described previously (Yavuzer et al., 1998). His-tagged hepatitis-C virus core protein, p16 (kindly provided by Mehmet Ozturk), was purified under denaturing conditions and immobilized on Ni-NTA beads. TRAX was transcribed and translated in vitro using TnT-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. Ten μg of radiolabelled translation product and the immobilized fusion proteins were incubated in 200 μl binding buffer (20% glycerol, 100 mM NaCl, 0.1×TE pH 8.0, 0.1% NP-40, 1 mM DTT, 30 μg BSA) at room temperature for 20 minutes followed by four washes using wash buffer (20% glycerol, 100 mM NaCl, 0.1×TE pH 8.0 and 0.5% NP-40). The beads were then resuspended in 20 μl of 2×SDS sample buffer, boiled, centrifuged at 10,000 ***g*** for 5 minutes and loaded on 10% SDS-PAGE. The gel was soaked in Amplify solution (Amersham) for 30 minutes, dried and visualized by autoradiography.

Cell culture, transient transfections and co-immunoprecipitations

COS 7 cells were maintained in DMEM supplemented with 10% fetal calf serum and grown at 37°C with 5% CO_2 as monolayers. Twenty four hours before transfections, cells were plated into 35 mm 6-well plates at a density of 3×10^4. Transfections were performed using FuGENE 6 reagent (Roche) with 0.5 μg of DNA according to the manufacturer's instructions. Cells were harvested 24 hours after transfection and lysed in lysis buffer (PBS containing 0.5% Triton-X, 5 mM EDTA and protease inhibitor cocktail tablets; Roche, 1873580). Cells that were subjected to 20 Gy of gamma (Cs^{137} source) or 50 j/m^2 of UV irradiation (Stratagene) were incubated for 1 hour at 37°C before harvesting. Thirty μl of Protein G agarose beads per sample was used and washed twice in PBS. The lysates were incubated with either an anti-GFP (Clontech) or anti-HA monoclonal antibody (Roche) for 2 hours at 4°C and then with protein G agarose beads for another 2 hours. The beads were washed five times with PBS followed by five washes with the lysis buffer. Precipitates were then separated

by 10% SDS-PAGE and blotted onto nitrocellulose. Western blotting was performed with anti-GFP antibody or a polyclonal antiserum raised against the full-length C1D and immunoreactive bands were visualized using ECL (Amersham) according to the manufacturer's instructions.

Microscopy and microphotography

Vectors expressing C1D, TRAX and Translin in fusion with GFP and various GFP variants were transiently transfected into COS 7 cells as described above. Twenty four hours after transfections, expression of the fusion proteins were examined in the control and γ-irradiated living cells for 24 hours. Fluorescence microscopy was performed on a Zeiss Axiovert 135TV microscope equipped with a CCD camera (Hitachi Denshi, Ltd) and appropriate filter sets, and the images were processed by using OpenLab software (Improvision).

Results

Identification of proteins interacting with the nuclear matrix protein C1D

A yeast two-hybrid screen was employed in an attempt to identify proteins interacting with C1D. To this end, we generated the yeast expression plasmid DBD-C1D, which directs the synthesis of full-length open reading frame of C1D, fused C-terminal to the bacterial LexA DNA binding protein. DBD-C1D was then introduced into the yeast reporter strain L40 (Hollenberg et al., 1995) that contains both the *HIS3* and *lacZ* genes under the control of LexA operators. Strain L40 containing DBD-C1D is His^- and does not express detectable β-galactosidase activity, indicating that C1D is transcriptionally inert when fused to LexA.

Strain L40 containing DBD-C1D was then transformed with a human B-lymphocyte cDNA library in the vector pACT. A total of ~1×10^8 transformants were obtained, of which ~3×10^3 were capable of growing on medium lacking histidine. Of these, 280 were found to produce elevated β-galactosidase activity. False positives were eliminated by using a mating assay and 120 transformants were identified as containing cDNAs that conferred a His^+, $LacZ^+$ phenotype in a fashion that was specific to L40 cells expressing the DBD-C1D fusion protein. The cDNA expression vectors from 80 of these transformants were then rescued, and sequencing revealed five cDNA groups, one of which comprised C1D. This finding served as a positive control for the screening because C1D homodimerisation has been demonstrated previously (U.Y., unpublished). A search of the GenBank database revealed that a second group of overlapping sequences that derived from the same cDNA encodes a recently identified protein termed TRAX (GenBank accession no. X95073) (Aoki et al., 1997).

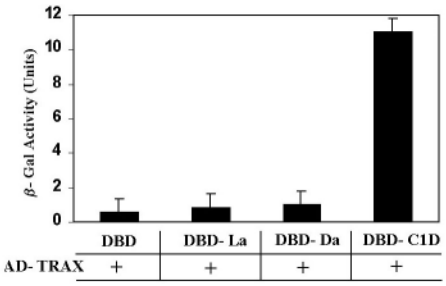


Fig. 1. C1D and TRAX interact specifically. TRAX fused to the Gal4 activation domain (AD-TRAX) was transformed into the reporter yeast strain together with DBD alone, DBD-lamin (La), DBD-Daughterless (Da) or DBD-C1D and interactions were measured by β-galactosidase activity using ONPG.

C1D interacts with TRAX specifically

To establish the specificity of the two-hybrid interaction between C1D and TRAX, the full-length open reading frame of TRAX was expressed in yeast as an activation domain tagged fusion protein (this fusion is referred to as AD-TRAX). AD-TRAX was transformed into L40 together with baits comprising the LexA DNA-binding domain (DBD) alone, DBD-Lamin, DBD-Daughterless or DBD-C1D; the level of activity from the integrated *LacZ* reporter was determined in each case using the ONPG assay. TRAX interacts specifically with DBD-C1D given that no interaction is detected with DBD alone, DBD-Lamin or DBD-Daughterless (Fig. 1). In reciprocal two-hybrid experiments where C1D was fused to the activation domain and TRAX to the C-terminal DNA-binding region of LexA and introduced into the strain L40, a similar level of β-galactosidase activity was also determined (data not shown). Therefore, we concluded that C1D and TRAX interact specifically.

C1D and TRAX interact in vitro

Next, we determined whether C1D is able to bind TRAX in vitro. For this, C1D was expressed in *E. coli* as a His-tagged fusion protein and was purified by use of Ni-NTA beads. Hepatitis C core protein, which has a similar size to C1D, was also expressed and purified under the same conditions as C1D and was used as control. The immobilized fusion proteins were then incubated with the in vitro transcribed and translated TRAX and pull-down assays were performed. As shown in Fig. 2, although no interaction is observed between TRAX and pQE alone or hepatitis C core protein, a substantial proportion of the input TRAX (one-tenth) is bound by C1D, indicating that C1D and TRAX also interact specifically in vitro.

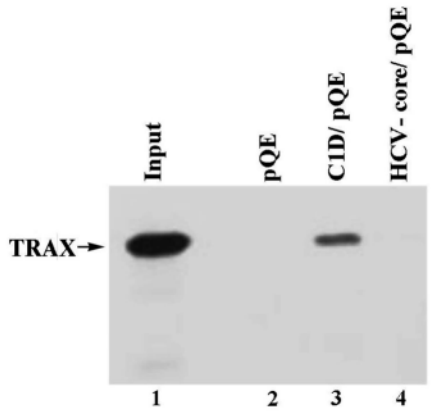


Fig. 2. C1D and TRAX interact in vitro. C1D and hepatitis C virus core proteins (HCV-core/pQE) were expressed in bacteria using the plasmid pQE30 (Qiagen) and purified by Ni-NTA column under denaturing conditions. One μg of each of the immobilized fusion proteins and pQE alone were incubated with In vitro Transcribed and Translated (IVTT) TRAX product and pull-down assay was performed. One tenth of the input TRAX was bound by C1D.

The putative leucine zipper region of TRAX is involved in interaction with C1D

TRAX contains a putative leucine zipper (LZ) (Aoki et al., 1997) motif between amino acids 73-208. Because this motif may be involved in protein-protein interactions, we tested whether this region has any role in its interaction with C1D. We introduced mutations into the putative LZ motif of TRAX within an otherwise intact full-length protein (this mutant is termed mutLZ-TRAX). We then subcloned mutLZ-TRAX to a yeast expression plasmid as a fusion to the Gal4 activation domain (AD-mutLZ-TRAX) and transformed into strain L40 together with the plasmid expressing DBD-C1D and determined the β-galactosidase activity. As expected, a significant activity was observed with AD-TRAX, whereas a low level of β-galactosidase activity was detected when mutLZ-TRAX-AD was tested for a two-hybrid interaction with DBD-C1D (Fig. 3A). To confirm the requirement of the putative LZ region of TRAX in C1D interaction, we used the N-terminal region of TRAX (between amino acids 1-210) and expressed this truncated protein as a fusion with the Gal4 activation domain (AD-N-TRAX) and determined the β-galactosidase activity. As seen in Fig. 3A lane 2, the truncated version of TRAX protein, which contains the N-terminal region including the putative LZ, interacted with C1D as strongly as the wild-type TRAX protein.

Because TRAX can interact with both Translin and C1D, and the putative LZ region is important in its interaction with C1D, it was important to determine whether the same region also plays a role in Translin/TRAX interaction. The yeast two-hybrid assay employed for this purpose showed that the putative LZ region of TRAX is also important for TRAX/Translin interaction, as the mutLZ-TRAX did not interact with Translin (Fig. 3B). However, unlike the C1D/TRAX interaction, which is mainly governed through the N-terminal region of TRAX containing the LZ motif, TRAX/Translin interaction is likely to require the full-length TRAX protein with an intact LZ region, as neither the N-terminal nor the C-terminal regions of TRAX alone interacted with Translin (Fig. 3B, lanes 3,4). Because the mutLZ-TRAX did not interact with either C1D or Translin, it was important to check whether it is expressed in yeast. As an HA-epitope was tagged to the N-terminal region of mutLZ-TRAX, we were able to detect the protein levels by western blotting in total yeast extracts, and we showed that the mutLZ-TRAX was expressed in both of the strains carrying DBD-C1D and DBD-Translin (data not shown).

C1D and translin bind to TRAX in a mutually exclusive manner

According to the yeast two-hybrid results, TRAX binds to both C1D and Translin. Therefore, we wanted to determine the relative interaction between TRAX and C1D or Translin when three molecules are found together. His-tagged C1D was immobilized on Ni-NTA beads, and in vitro transcribed and translated TRAX and Translin were incubated with the immobilized fusion protein either alone (Fig. 4, lanes 3,4) or together (lanes 5, 6 and 7). Similar to the previous result (Fig. 2), around 10% of input TRAX was bound to C1D (lane 3), whereas binding of Translin to C1D was barely detectable (lane 4). When TRAX and Translin were incubated simultaneously with the immobilized C1D, the binding of TRAX to C1D was similar to the levels obtained when TRAX was incubated with C1D alone (compare lanes 3 and 5). By contrast, when TRAX and Translin were pre-incubated for 1 hour and then added to the immobilized C1D, only a small fraction of TRAX was able to bind to C1D (lane 6). When TRAX was incubated with C1D for 1 hour prior to the addition of Translin (lane 7), binding of TRAX to C1D was increased almost twofold with respect to its binding without pre-incubation in the absence of Translin (compare lanes 3 and 7). Taken together with the fact that both C1D and Translin require the putative LZ region of TRAX to bind (Fig. 3), these results would suggest that C1D and Translin bind TRAX in a mutually exclusive fashion.

C1D and TRAX interaction is stimulated by γ-irradiation in mammalian cells

Because C1D mRNA and protein levels have been shown to

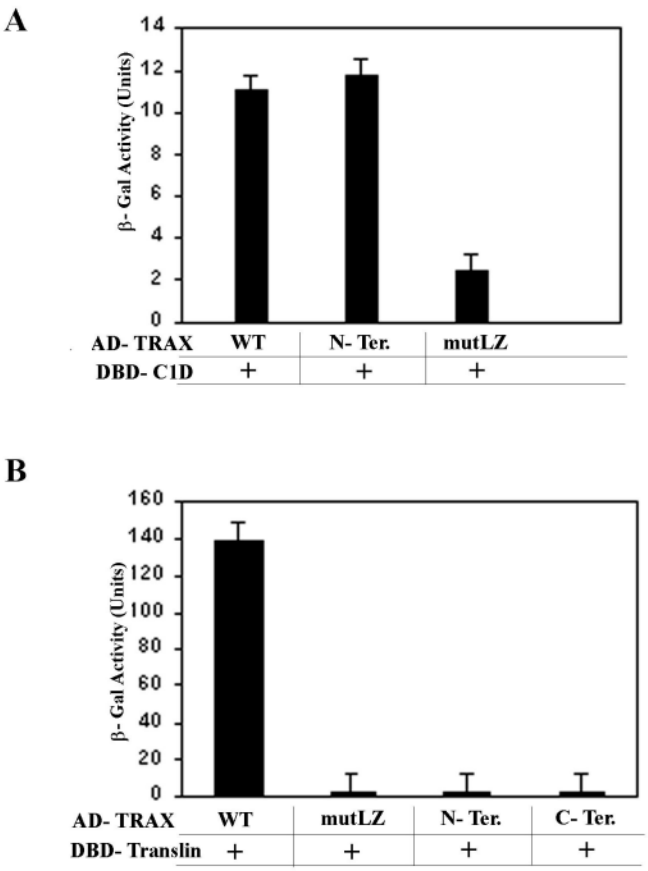


Fig. 3. The putative LZ region of TRAX is essential for interactions with C1D and Translin. (A) Yeast two-hybrid assay was performed using DBD-C1D and an activation domain (AD) tagged forms of TRAX; wild-type (WT), N-terminal region containing the LZ motif (N-Ter) or mutLZ where the LZ region of TRAX has been mutated within an otherwise intact protein. The interactions were measured by β-galactosidase activity using ONPG. (B) Constructs as in (A) were used to detect interaction with the Translin protein expressed as a fusion to the bacterial LexA protein (DBD-Translin). Mutations that disrupt the LZ region of TRAX abolish its interaction with Translin and neither the N-terminal region of TRAX carrying an intact LZ nor the C-terminal region alone (C-Ter) are sufficient for interaction with Translin.

be induced upon γ-irradiation (Yavuzer et al., 1998), we wished to determine whether γ-irradiation would affect TRAX/C1D interaction in mammalian cells in vivo. C1D protein is insoluble and remains tightly bound to DNA during whole-cell or nuclear extract preparations and therefore cannot be detected by western blotting of soluble cell extracts (Yavuzer et al., 1998). Therefore, COS 7 cells were transiently transfected with vectors expressing HA-epitope-tagged version of C1D (HA-C1D) and YFP-tagged TRAX (YFP-TRAX). Cells expressing YFP only and untransfected cells were also included as controls. Transfection of cells with C1D-expression vectors enables the production of sufficient soluble C1D and thus allows co-immunoprecipitation assays to be performed (Yavuzer et al., 1998). Twenty four hours following transfection, 20 Gy of γ-irradiation was applied to a set of transiently transfected cells and after incubating for an additional hour, lysates were prepared from the irradiated and unirradiated cells. Immunoprecipitations were performed with anti-GFP, which also recognizes YFP, or anti-HA antibodies, followed by immunoblotting with an antibody against C1D. In both unirradiated and irradiated cells, C1D monomers (16 kDa) and SDS-resistant dimers (32 kDa) (Nehls et al., 1998) were detected in immunoprecipitations using anti-HA antibody followed by immunoblotting with anti-C1D (Fig. 5A, lanes 1,4). As expected, C1D was detected in lysates from HA-C1D-transfected cells but not in lysates from untransfected or YFP-expressing cells (lanes 6 and 5, respectively). When HA-C1D and YFP-TRAX were cotransfected and immunoprecipitated with anti-GFP antibody and probed with anti-C1D antibody, we were not able to detect C1D interaction with TRAX from unirradiated cells (lane 2). However, the 32 kDa SDS-resistant C1D homodimer, but not the 16 kDa monomer, was readily detectable upon γ-irradiation (lane 3), indicating that γ-irradiation induces TRAX/C1D interaction.

Because γ-irradiation results in DNA double-strand breaks, we next determined whether the interaction between C1D and TRAX was specific to DNA double-strand breaks or whether it could also be induced by other types of DNA damage. To test this, we used UV irradiation (50 j/m^2), which is known to activate mainly the nuclear excision repair (NER) pathways. COS 7 cells were transiently transfected with vectors expressing GFP-TRAX and HA-C1D and immunoprecipitations were performed using antibodies raised against GFP peptide or HA-epitope. As for controls, lysates from untransfected and HA-C1D- or GFP-TRAX-transfected cells were also immunoprecipitated using the same antibodies

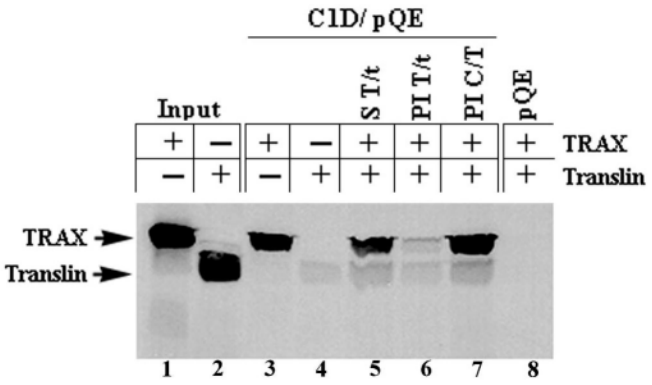


Fig. 4. Binding of C1D and Translin to TRAX is mutually exclusive. C1D was expressed in bacteria and immobilized on Ni-NTA column as explained in the legend to Fig. 2. IVTT TRAX and Translin products were incubated with the immobilized C1D either alone (lanes 3 and 4) or together. TRAX (T) and Translin (t) IVTT products were incubated simultaneously (S) with the immobilized C1D for 2 hours before washing (lane 5), TRAX and Translin IVTT products were pre-incubated (PI) for 1 hour before adding to immobilized C1D and incubated for another hour (lane 6), IVTT product of TRAX was incubated with the immobilized C1D (C) for 1 hour and then Translin was added and incubated for an additional hour (lane 7). The plasmid expressing His only (pQE) was expressed in bacteria, purified and immobilized under the same conditions as C1D and was used in lane 8 as a negative control.

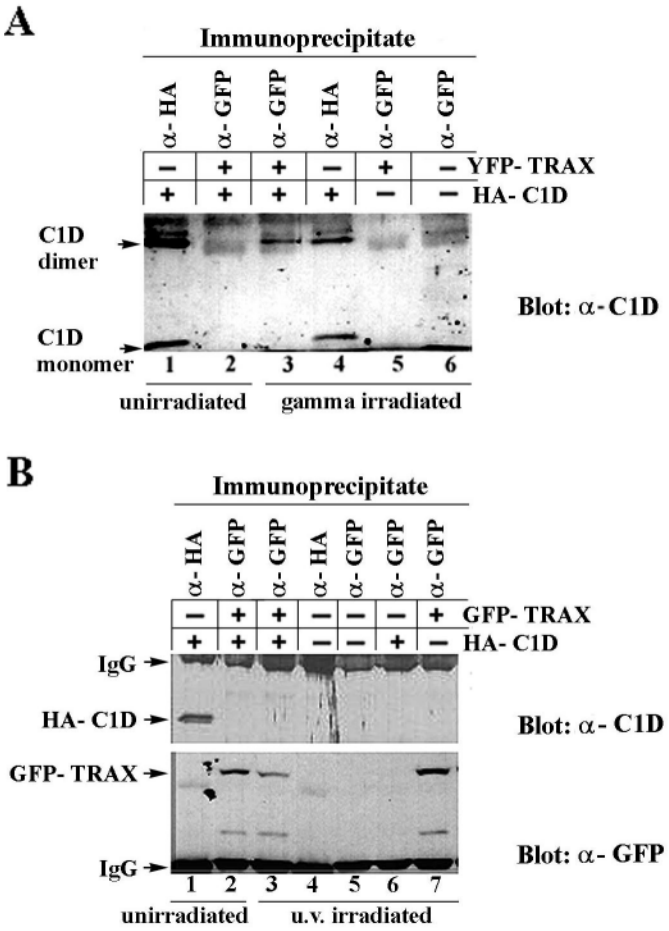
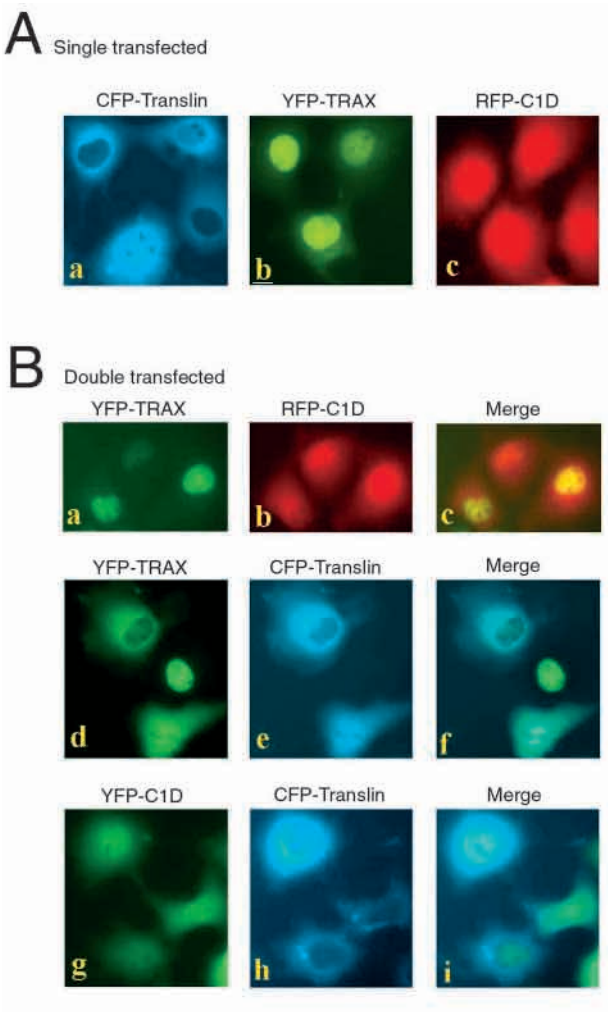


Fig. 5. SDS-resistant C1D homodimers interact with TRAX upon γ-irradiation. (A) Transiently transfected COS 7 cells were immunoprecipitated using the antibodies indicated. For lanes 3-6 the cells were treated with 20 Gy of ionizing irradiation before lysate preparation. Western blotting was performed by an anti-C1D antibody. The monomer and dimer forms of C1D are indicated. (B) Transiently transfected COS 7 cells were immunoprecipitated using the indicated antibodies. For lane 3-7, the cells were treated with 50 j/m^2 UV irradiation. The top panel shows the western blotting using an anti-C1D antibody, and in the bottom panel, the blot was stripped and reprobed with an anti-GFP antibody to demonstrate the presence of GFP-TRAX. For each lane, immunoprecipitations were performed using the cell lysates obtained from 3×10^5 cells.



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Transfection	% Nuclear cytoplasmic: Translin pre	Translin post	TRAX pre	TRAX post	C1D pre	C1D post	% nuclear: TRAX pre	TRAX post	Irradiation
Single	6	4.5	—	—	100	100	100	100	
Double	10	11	—	—	—	—	—	—	
	34	43	58	61	—	—	—	—	
	19	15	—	—	100	100	—	—	
	—	—	—	—	100	100	100	100	
Triple	30	46	44	65	100	100	—	—	

(Fig. 5B, lanes 4-7). The immunoprecipitates were then blotted with an anti-C1D antibody (Fig. 5B, top panel). We were able to detect the 32 kDa dimeric form of C1D only in cell lysates from HA-C1D-transfected cells (lane 1). However, C1D was not detectable when cell lysates from GFP-TRAX- and HA-C1D-transfected cells were immunoprecipitated with an anti-GFP antibody (lane 2). In contrast to γ-irradiation, treatment of these double-transfected cells with UV irradiation did not induce interaction of TRAX with C1D (lane 3). To confirm that the lack of interaction between C1D and TRAX upon UV irradiation is not due to the absence of TRAX expression, we stripped the blot and reprobed with an antibody raised against GFP-peptide (Fig. 5B, bottom panel). As expected, a protein of ~60 kDa, which is the expected size for GFP-TRAX protein, was detected in all the cell lysates from GFP-TRAX-transfected cells (lanes 2, 3 and 7) but not in lysates from untransfected or HA-C1D-transfected cells (lanes 1, 4, 5 and 6). We conclude therefore that C1D and TRAX interact in mammalian cells in response to agents inducing DNA-double strand breaks but not in response to UV irradiation.

Subcellular localization of C1D, TRAX and Translin

To determine the subcellular localization of Translin, TRAX and C1D, we expressed these proteins in COS 7 cells as fusions with different GFP variants either alone or in combination, and determined the expression patterns in the living cells. The fluorescence results are shown in Fig. 6A-C with a summary shown in Fig. 6D. In agreement with previous results (Kasai et al., 1997; Chennathukuzhi et al., 2001), CFP-Translin when expressed alone was found to be cytoplasmic in over 90% of cells examined (Fig. 6Aa). In around 6% of cells however, CFP-Translin exhibited both nuclear and cytoplasmic localization. γ-Irradiation or other DNA damaging agents that induce DNA double-strand breaks did not cause any alteration in its subcellular localization in agreement with previous observations (Chennathukuzhi et al., 2001). By contrast, YFP-TRAX was localized to the nucleus in all cells examined,

Fig. 6. Subcellular localizations of TRAX, Translin and C1D. (A) Plasmids expressing CFP-Translin, YFP-TRAX and RFP-C1D were transfected into COS 7 cells and subcellular localizations were determined in the living cells. Translin was mainly cytoplasmic; however, 6% of cells exhibited nuclear/cytoplasmic staining (a). TRAX was nuclear (b) and C1D was mainly nuclear, but a cytoplasmic staining was also evident (c). (B) COS 7 cells were double transfected with the indicated plasmids. (a-c) TRAX/C1D double-transfected cells showing that TRAX is solely nuclear (a) whereas C1D is showing a nuclear/cytoplasmic staining pattern (b), and colocalizes with TRAX in the nucleus (c). In TRAX/Translin double-transfected cells (d-f), TRAX and Translin colocalize in the cytoplasm and in the nucleus. The middle cell in d and f expresses TRAX only. In C1D/Translin double-transfected cells (g-i), C1D is mainly nuclear (g) and Translin is cytoplasmic (h). (C) COS 7 cells triple transfected with TRAX-, Translin- and C1D-expressing plasmids. (a-c) A single cell in which a diffuse nuclear/cytoplasmic staining pattern is observed. In d-f, another field is shown where two of the cells were triple transfected and in which the upper cell TRAX and C1D were expressed in the nucleus (d and f) when Translin was mainly cytoplasmic (e); the lower cell shows a diffuse nuclear-cytoplasmic staining pattern as in a-c. (D) Summary of the results demonstrating the percentage of nuclear/cytoplasmic and solely nuclear localizations of Translin, TRAX and C1D.

whereas C1D was observed in a diffuse nuclear (predominantly)/cytoplasmic staining pattern as observed using anti-C1D antibody (U.Y., unpublished) (Fig. 6Ab and Fig. 6Ac, respectively). As with Translin, the localization of TRAX and C1D did not change following γ-irradiation (data not shown). Because Translin exhibited nuclear/cytoplasmic localization in 6% of the cells when expressed alone, we wanted to see whether an alteration would occur in its nuclear localization when expressed together with C1D or TRAX. To constitute a basal level for the percentage of cells exhibiting nuclear Translin in double transfections, Translin was co-expressed with a plasmid expressing YFP only and was found to show nuclear/cytoplasmic localization in around 10% of the cells (Fig. 6D). A different picture emerged with TRAX or C1D double transfections: co-expression of Translin with TRAX resulted in an almost 3.5-fold increase in the proportion of cells in which Translin was observed to be nuclear/cytoplasmic (34%) colocalizing with TRAX, and a striking alteration in the location of TRAX, with around 58% of cells expressing TRAX both in cytoplasm and nucleus (compared to 100% nuclear when expressed alone). In 42% of the cells TRAX was solely cytoplasmic and colocalized with Translin. Importantly, in these cells where TRAX was cytoplasmic, Translin was never found to be nuclear. In Fig. 6Bd-f, a representative area consisting of three cells is shown; two of the cells contain both TRAX and Translin. In one of the cells, TRAX and Translin are co-localized to cytoplasm, whereas in the other, a diffuse nuclear/cytoplasmic staining pattern is observed. The third cell expresses TRAX only and, as expected, when Translin is not co-expressed, TRAX is solely nuclear. This single TRAX-expressing cell in this particular area also acts as an internal control demonstrating that the filters used to detect YFP or CFP are highly specific. These alterations in the compartmentalization of TRAX and Translin presumably reflect interactions between these two proteins and indicates that the relative expression levels of these proteins is important in determining their subcellular localization. γ-Irradiation did not cause a significant alteration in the subcellular localizations of TRAX and Translin. On the contrary, when TRAX was expressed with C1D, neither C1D nor TRAX changed their localizations either before or after γ-irradiation and TRAX stayed in the nucleus (Fig. 6Ba) whereas C1D showed its usual nuclear/cytoplasmic staining pattern (Fig. 6Bb), colocalizing with TRAX in the nucleus (Fig. 6Bc). Co-expression of Translin and C1D (Fig. 6Bg-i) resulted in a slight increase in the proportion of the cells containing nuclear Translin (19%); however, once again γ-irradiation did not have any effect on the subcellular localizations of either Translin or C1D. These results demonstrate that γ-irradiation does not affect the localization of TRAX, Translin or C1D, but the otherwise nuclear TRAX changes its localization when Translin is co-expressed and stays in the nucleus when C1D is co-expressed.

In cells triple transfected with YFP-TRAX, CFP-Translin and RFP-C1D expression vectors, we were not able to detect any significant alteration in the subcellular localizations of TRAX or Translin compared to TRAX/Translin double-transfected cells (Fig. 6D). In other words, C1D over expression did not cause an overall alteration in the localization of TRAX or Translin. In Fig. 6Cd-f, a group of cells, of which two were triple transfected, is shown. C1D again exhibits a nuclear/cytoplasmic staining (Fig. 6Cf), whereas TRAX is solely nuclear in one of these cells (Fig. 6Cd, upper cell) and nuclear/cytoplasmic in the lower cell. Translin, however, is also nuclear/cytoplasmic in this lower cell, but is mainly cytoplasmic in the upper cell and therefore does not colocalize with TRAX. In the bottom, left-hand side, a third cell is seen (Fig. 6Cd,e) which only expresses nuclear/cytoplasmic TRAX and Translin, and a fourth cell (Fig. 6Ce) expressing Translin only, again acting as internal controls; this shows that the different filters used for GFP, YFP or DsRed are specific. Although in a few cells expression of C1D appears to maintain TRAX in the nucleus, whereas Translin was mainly cytoplasmic, the majority of cells showed a nuclear/cytoplasmic staining (Fig. 6Ca-c), making it difficult to determine whether TRAX was complexed with Translin or C1D. However, we noted a slight increase in the percentage of cell expressing nuclear TRAX or Translin after irradiation in the presence of C1D, compared to no increase in the double-transfected cells (Fig. 6D).

Discussion

The protein and mRNA levels of the nuclear matrix protein C1D increase upon γ-irradiation (Yavuzer et al., 1998). In addition, C1D interacts with DNA-PK and is an efficient substrate for this kinase, which plays an essential role in the repair of DNA double-strand breaks. The interaction between C1D and DNA-PK is significant in the sense that C1D is the first substrate identified that is capable of activating DNA-PK in the absence of free DNA-ends (Yavuzer et al., 1998). These findings raised the possibility that C1D might play a role in DSB repair. Given the role of the nuclear matrix in the regulation of important cellular events, such as DNA replication, transcription, RNA splicing, topoisomerase activity, nucleotide excision and DSB repair (Berezney, 1984; Cockerill and Garrard, 1986; Jackson, 1991; Johnston and Bryant, 1994; Kaufman and Shaper, 1991; Koehler and Hanawalt, 1996; Korte and Yasui, 1993; Nelson et al., 1986; Verheijen et al., 1988; Yasui et al., 1994; Yasui et al., 1991), it is clearly important to reveal the role of C1D in any of these events.

Using the yeast two-hybrid system to screen a human B-lymphocyte library we showed that C1D can interact specifically with the Translin-interacting protein, TRAX. Although the putative LZ region of TRAX is important not only in interaction with C1D but also in its interaction with Translin, C1D and Translin show different requirements for interaction with TRAX outside the LZ region. The N-terminal region of TRAX that contains an intact LZ region is sufficient for its interaction with C1D, whereas Translin requires full-length TRAX with an intact LZ region for interaction. However, consistent with Translin and C1D sharing an interaction motif within TRAX, we were able to show that binding of Translin and C1D to TRAX appears to be mutually exclusive and that stable interaction between C1D and TRAX in mammalian cells occurs upon γ-irradiation. Although the biological functions of these proteins are not yet clear, these findings suggest that both C1D and TRAX might play a role in the repair of DNA DSBs.

Cells use various mechanisms to protect their genome against genotoxic insults. One obvious mechanism is to detect

the damage and activate DNA repair machinery to repair the damage before cell division takes place. When the damage is beyond repair, cells can activate their suicide programs, and via apoptosis the damaged cell destroys itself. C1D may play a role in both types of mechanism as overexpression of C1D is capable of inducing apoptosis in tumour cell lines (Rothbarth et al., 1999) and C1D interacts with DNA-PK, which has an established role in double-strand DNA break repair.

An important feature of tumour cells is that they carry specific chromosomal abnormalities, including translocations, that result in the altered expression or structure of cellular gene products giving rise to functional activation which contributes to the initiation or progression of cancer (Rabbitts, 1994). Chromosome translocations in malignant tumours may arise by several mechanisms. The translocations seen in lymphomas and leukaemias involve the immunoglobulin or T-cell receptor genes and the Ig/TCR recombinase signal sequences are present on both affected chromosomes. In some other lymphoid malignancies, the breakpoint junctions are characterized by another consensus sequence, which is recognized by the Translin protein (Aoki et al., 1995). Translin recognition site sequences have also been identified in solid tumours (Chalk et al., 1997) indicating that Translin may be important in the genesis of chromosomal translocations in some types of neoplasms. As well as being a sequence-specific DNA-binding protein, Translin is also capable of binding to RNA, and the mouse homologue of human Translin, the testis brain RNA-binding protein (TB-RBP), was identified as a RNA-binding protein capable of recognizing conserved elements in the 3′ untranslated regions of specific mRNAs in brain and testis (Han et al., 1995; Wu et al., 1997; Wu and Hecht, 2000).

Although the role of TRAX in any of these events is not yet clear, both human and mouse TRAX can form heterodimers with Translin/TB-RBP (Aoki et al., 1997; Taira et al., 1998; Wu et al., 1999) and the TRAX/Translin heterodimer is capable of binding to both single-stranded DNA and RNA, raising the possibility that TRAX/Translin heterodimer is multifunctional. However, little is known about the biological functions of Translin and TRAX.

Previous data demonstrated that Translin is primarily cytoplasmic in many cell lineages but it is found in the nucleus in haematopoietic cell lines. Interestingly, however, treatment of HeLa cells with mitomycin C or etoposide, agents that induce DNA double-strand breaks, was reported to cause translocation of Translin into the nucleus (Kasai et al., 1997). The subcellular localization of TB-RBP changes in meiotic and post-meiotic cells (Gu et al., 1998; Morales et al., 1998), but agents causing DNA DSBs did not affect localization of TB-RBP (Chennathukuzhi et al., 2001). In agreement with this latter study, and consistent with Translin containing a REV-like nuclear export signal (Chennathukuzhi et al., 2001), Translin was located in the cytoplasm in all but around 6% of cells where a nuclear/cytoplasmic expression pattern was observed, and treatment of transfected COS 7 cells with etoposide or γ-irradiation did not induce translocation of Translin into the nucleus. Why around 6% of cells exhibit a different localization for Translin is unclear, but these cells may represent those that have recently undergone mitosis, a point in the cell cycle when Translin would have access to DNA by virtue of nuclear envelope breakdown. Following the completion of mitosis, the nuclear envelope would reform, causing the Translin that was retained in the nucleus either being exported to the cytoplasm or degraded.

In contrast to Translin, and to a previous report indicating that TRAX is a predominantly cytoplasmic protein (Chennathukuzhi et al., 2001), our results show TRAX located in the nucleus in all cells examined (COS7, MCF7 or 3T3 cell lines), consistent with TRAX containing a nuclear localization signal (Aoki et al., 1997). However, co-expression of TRAX and Translin resulted in a different pattern of localization of both proteins – an increase in the number of cells with a nuclear Translin and an increase in cells expressing cytoplasmic TRAX. The changes in localization occurring when TRAX and Translin are co-expressed are consistent with an interaction between the two proteins affecting their ability to traffic between the nucleus and cytoplasm, and they also indicate that the relative expression levels of TRAX and Translin are likely to be important in determining their subcellular localization. Indeed, differences in the levels of endogenous or ectopically expressed TRAX and Translin in different cell types may explain why the reported subcellular localization of ectopically expressed Translin or TRAX may differ.

Because, in our experimental sytems, γ-irradiation did not have a significant effect in the subcellular localizations of TRAX, Translin or C1D, the finding that the SDS-resistant homodimers of C1D and TRAX interact only after γ-irradiation may indicate that a post-translational modification is required for the interaction. Considering that C1D is phosphorylated by DNA-PK, it is possible that upon γ-irradiation DNA-PK is activated and phosphorylates C1D, which in turn interacts with TRAX. DNA-PK was not found to immunoprecipitate with TRAX from mammalian cells, nor was it able to phosphorylate recombinant TRAX in vitro (data not shown). However, this does not exclude the possibility that TRAX could be phosphorylated by DNA-PK or a related kinase in vivo in response to DNA damage. Indeed, mouse TRAX has been found to be phosphorylated in testis germ cell nuclear extracts where many DNA DSBs are found due to ongoing recombination events (Chennathukuzhi et al., 2001).

One biological consequence of C1D/TRAX interaction could be to regulate the Translin/TRAX interaction. In this regard, we note that TRAX has been shown to enhance the DNA binding capacity of TB-RBP (Translin), while decreasing its RNA-binding ability (Chennathukuzhi et al., 2001). Because interaction with either C1D or Translin appears to require the putative LZ motif of TRAX, the increased expression of C1D that occurs in response to γ-irradiation (Yavuzer et al., 1998) (a signal that also triggers the stable interaction between C1D and TRAX in mammalian cells) could result in C1D masking the Translin docking site on TRAX and thereby preventing the Translin/TRAX complex formation and binding to DNA. Thus, increasing the C1D/TRAX interaction on γ-irradiation might represent a mechanism to inhibit any unwanted Translin-mediated recombination events before the damaged DNA is repaired. Indeed, in our in vitro competition experiments, we showed that binding of C1D and Translin to TRAX is mutually exclusive. Importantly, however, if TRAX binds to Translin first, then it is no longer available to bind to C1D. Therefore, C1D may act as a regulator of TRAX/Translin complex formation, rather than regulate the association of any

preformed TRAX-Translin complex. This is an important point. Our studies using fluorescently tagged proteins also failed to identify any dramatic change in subcellular localization of TRAX, Translin or C1D when expressed in any combination in response to γ-irradiation. This is in agreement with others (Chennathukuzhi et al., 2001) who also found no difference in the subcellular localization of Translin in response to DNA-damaging agents. We suspect that the results may reflect the relative timing of expression of the proteins in transfected cells. Thus, in the absence of γ-irradiation, C1D will not interact stably with TRAX and so no significant effect of C1D on the subcellular localization of TRAX and Translin would be expected. On γ-irradiation C1D would interact with TRAX, but this association would only inhibit the formation of new TRAX-Translin complexes. Against the high background of preformed TRAX-Translin complexes it would be difficult to detect any change in overall localization of the fluorescent proteins before the overexpression of C1D eventually results in apoptosis. Nevertheless, we suspect that the interaction between C1D and TRAX will represent an important mechanism regulating the association of TRAX and Translin and leading to alterations in the relative amounts of TRAX/Translin targeted to the nucleus. Further studies dissecting the interaction between C1D, TRAX and Translin and their requirements for DNA binding should help to address some of these issues.

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