Nucleolar localization of aprataxin is dependent on interaction with nucleolin and on active ribosomal DNA transcription

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The APTX gene, mutated in patients with the neurological disorder ataxia with oculomotor apraxia type 1 (AOA1), encodes a novel protein aprataxin. We describe here, the interaction and interdependence between aprataxin and several nucleolar proteins, including nucleolin, nucleophosmin and upstream binding factor-1 (UBF-1), involved in ribosomal RNA (rRNA) synthesis and cellular stress signalling. Interaction between aprataxin and nucleolin occurred through their respective N-terminal regions. In AOA1 cells lacking aprataxin, the stability of nucleolin was significantly reduced. On the other hand, down-regulation of nucleolin by RNA interference did not affect aprataxin protein levels but abolished its nucleolar localization suggesting that the interaction with nucleolin is involved in its nucleolar targeting. GFP-aprataxin fusion protein co-localized with nucleolin, nucleophosmin and UBF-1 in nucleoli and inhibition of ribosomal DNA transcription altered the distribution of aprataxin in the nucleolus, suggesting that the nature of the nucleolar localization of aprataxin is also dependent on ongoing rRNA synthesis. In vivo rRNA synthesis analysis showed only a minor decrease in AOA1 cells when compared with controls cells. These results demonstrate a cross-dependence between aprataxin and nucleolin in the nucleolus and while aprataxin does not appear to be directly involved in rRNA synthesis its nucleolar localization is dependent on this synthesis.

INTRODUCTION

Ataxia with oculomotor apraxia type 1 (AOA1) is a human autosomal recessive syndrome characterized by early-onset cerebellar ataxia and cerebellar atrophy (1–4). AOA1 resembles ataxia-telangiectasia (A-T) in its neurodegenerative phenotype, an autosomal recessive disorder characterized by neurodegeneration, but it lacks the extra-neurological features of A-T that include, radiosensitivity, immunodeficiency, hypogonadism and cancer susceptibility (5). Aprataxin, the product of the APTX gene, mutated in AOA1 (6,7) is a protein of 342 amino acids composed of three putative domains, a forkhed-associated (FHA) domain (8), a histidine-triad (HIT) protein domain and a DNA/RNA binding C₂H₂ zinc-finger domain (6,7). Although the cellular function of aprataxin is still elusive, recent reports have implicated aprataxin in DNA repair (9–13). Indeed, the marked sensitivity of AOA1 cells to DNA single-strand break-inducing agents such as methylmethane sulfonate and H₂O₂ (9–11), the increased chromosomal aberrations observed in response to DNA topoisomerase-I inhibitor camptothecin (CPT) (13) and its interaction with key players of the single-strand break repair machinery (9–12,14), suggest a role in DNA single-strand break repair.

Cellular localization of aprataxin determined by immunostaining and GFP–aprataxin expression revealed a nuclear localization distributed between the nucleoplasm and nucleoli (9). The nucleolus, a prominent organelle visible inside
the eukaryotic nucleus, is a dynamic nuclear structure that plays important roles in nuclear organization and function. The primary function of the nucleolus was established in the 1960s with the discovery of DNA sequences complementary to ribosomal RNA (rRNAs) and the presence of particles that corresponded to 18S and 28S rRNA (15–17). This organelle is organized into three distinct areas that reflect the vectorial process of ribosome biogenesis (18). Transcription of ribosomal DNA (rDNA) genes into precursor rRNAs (pre-rRNAs) by RNA polymerase I (RNA Pol I) occurs at the border between the fibrillar centres and the dense fibrillar component of the nucleolus. Maturation of the pre-rRNAs into rRNAs and assembly of the pre-ribosomes takes places in the surrounding granular components. In addition to its well-characterized function as a ‘ribosome factory’, many reports have implicated the nucleolus in other cellular processes such as maturation and export of mRNA, tRNA species and ribonucleoproteins, control of cellular proliferation, ageing and mitotic regulation (reviewed in 18,19).

Recent data suggest that the nucleolus acts as a cellular stress sensor (20–22). Given the role of aprataxin in genotoxic stress protection, its nucleolar localization and its interaction with DNA repair proteins (9–12,14), we attempted to identify novel aprataxin interacting proteins in order to understand better how aprataxin protects against cellular stress. We found several nucleolar partners, such as nucleolin (NCL/C23), nucleophosmin (NPM/B23) and upstream binding factor 1 (UBF-1/UBF) that are involved in rRNA transcription and maturation (19) and/or stress-response signalling (23,24). Although nucleolar localization of aprataxin was dependent upon ongoing rRNA transcription, no major defect in rRNA metabolism was observed in AOA1 cells.

RESULTS

Aprataxin interacts with nucleolin and other proteins

We have previously shown, by GST pull-down assays, that aprataxin is in a complex with nucleolin (9). In order to substantiate this and identify other interacting partners, we used a GST pull-down with the aprataxin–FHA domain followed by mass spectrometric analysis of bound proteins. Several proteins that bound to the FHA-domain of aprataxin were identified using MALDI-TOF (Fig. 1A). These included, XRCC1, PARP-1, nucleolar transcription factor 1 (UBF-1) and nucleolin. This method of analysis identified nine distinct peptides whose mass/charge corresponded to regions that covered most of nucleolin (Fig. 1B). Interaction between aprataxin and nucleolin was confirmed using co-immunoprecipitation (IP) with an antibody against aprataxin (Fig. 1C).

Nucleolin–aprataxin interaction does not require the presence of nucleic acids

Previous results have shown that both aprataxin and nucleolin bind to RNA and/or DNA (25–27). To examine whether the presence of nucleic acid was required for the complex formation between aprataxin and nucleolin, total cell extracts from control (C3ABR) cells were treated with either RNase A or DNase I before immunoprecipitating aprataxin. As shown in Figure 2A, both nucleolin and nucleophosmin, another nucleolar partner protein of nucleolin, co-immunoprecipitated with aprataxin regardless of pre-treatment with DNAse or RNase, suggesting that nucleic acid is not required for complex formation. However, there was an increase in the amount of both nucleolin and nucleophosmin associated with aprataxin in the DNase and RNase pre-treatments. This lack of dependence on nucleic acid was
further supported by GST pull-down assays using GST alone and aprataxin GST–FHA (Fig. 2B). In this experiment, pull-down was performed first, and then subjected to RNAse or DNAse digestion. Again, nucleolin bound to aprataxin–FHA domain regardless of RNAse or DNAse treatment, confirming that nucleic acids are not required for the interaction (Fig. 2B). As a positive control, pull-downs were also probed with XRCC1 (Fig. 2B), a DNA protein known to interact with aprataxin–FHA domain in a phosphorylation-dependent manner (10,11).

**Phosphorylation of nucleolin is required for binding with aprataxin**

Previous reports have shown that the FHA domain of aprataxin mediates the interaction with CKII-phosphorylated XRCC1 (10,11). As we had demonstrated that this domain also mediates binding to nucleolin, we determined whether phosphorylation might also be involved in the nucleolin–aprataxin interaction. Proteins were resolved by SDS–PAGE followed by staining with Pro-Q Diamond™ to detect phosphoproteins bound to GST–FHA. An intense staining band of ~110 kDa was identified as nucleolin using MALDI-TOF (Fig. 3A). The predicted molecular size of nucleolin is 77 kDa but the presence of highly acidic regions, interspersed with basic sequences, causes nucleolin to migrate at 100–110 kDa (28,29). In addition, nucleolin contains multiple phosphorylation sites that could alter gel mobility (reviewed in 30). To assess the importance of phosphorylated nucleolin in aprataxin binding, endogenous aprataxin was immunoprecipitated from cell extracts and immunoprecipitates were treated with lambda phosphatase (APPase) (Fig. 3B). APPase disrupted the complex between nucleolin and aprataxin thus confirming that nucleolin binds to aprataxin in a phosphorylation-dependent manner, most likely via the FHA domain of aprataxin.

**Mapping the interaction domains on aprataxin and nucleolin**

In order to map the interacting domains between aprataxin and nucleolin we used a series of GST fusion proteins covering the entire length of aprataxin (Fig. 4A). Nucleolin and nucleophosmin only interacted with the FHA domain of aprataxin (Fig. 4B). To define the region of interaction for aprataxin on nucleolin, we performed GST pull-down assays using a series of GST nucleolin constructs encompassing the various functional domains of nucleolin (Fig. 4C). Immunoblotting of the bound material revealed that aprataxin bound only the N-terminal GST-fusion product (amino acids 1–140) of nucleolin as shown in Figure 4D. However, it did not bind to a GST-fusion product (amino acids 1–55), suggesting that the region required for binding is located between the amino acids 55 and 140. It is evident that nucleophosmin also binds to this region of nucleolin (Fig. 4D).

**Nucleolin is less stable in AOA1 cells lacking aprataxin**

As aprataxin and nucleolin are present in a complex in vivo, the absence of one of these components could influence the stability of the complex and perhaps the stability of the other protein. To investigate this, total cell extracts from control (C2ABR and C3ABR) and AOA1 (L939, L938) cells were examined by western blotting using nucleolin and aprataxin antibodies. As shown in Figure 5A, no aprataxin protein was detected in AOA1 cells confirming previous observations that AOA1 cells lack this protein (9,10). Surprisingly, nucleolin appeared to be more degraded in AOA1 cells when
compared with controls as detected by loss in the primary band and appearance of a lower molecular size bands (Fig. 5A). To verify this, cells were treated with cycloheximide (CHX) to inhibit de novo protein synthesis and determine the stability of the protein. Under these conditions, nucleolin was rapidly turned over and degraded in AOA1 (L939) cells, whereas there was little evidence of degradation in control (C3ABR) cells (Fig. 5B). Another protein, β-actin, was equally stable in both cell types under these conditions (Fig. 5B). These results suggest that the lack of aprataxin protein affects nucleolin stability. Down-regulation of aprataxin by short interfering RNA (siRNA) has also been shown to affect the steady-state levels of XRCC1 (11). We also investigated whether lack of aprataxin would affect the nucleolar localization of nucleolin in AOA1 cells. Immunostaining of normal foreskin fibroblasts (NFF) and primary skin fibroblasts derived from an AOA1 patient (FD105), carrying the APTX truncating mutation W279X, confirmed previous observations that aprataxin is distributed between the nucleoplasm and the nucleolus in normal cells and undetectable in AOA1 fibroblasts (Fig. 5C) (9,10). It is also evident that a proportion of aprataxin is distributed around the periphery of the nucleolus (Fig. 5C). Interestingly, a significant decrease in the nucleolar staining of nucleolin was observed in AOA1 fibroblasts that may result from the release of degraded nucleolin into the nucleoplasm (Fig. 5C). As lack of aprataxin destabilized nucleolin, we then tested...
whether over-expression of aprataxin would affect nucleolin protein levels. Total cell extracts from HeLa cells expressing GFP–aprataxin were analysed by immunoblotting with aprataxin antibody and showed increased nucleolin protein levels (Fig. 5D).

**Down-regulation of nucleolin by siRNA abolishes aprataxin nucleolar localization**

As we demonstrated that lack of aprataxin destabilized nucleolin, it was therefore possible that a lack of nucleolin could also affect aprataxin steady-state levels. To address this, we transfected HeLa cells with nucleolin-specific siRNA (siNCL) to down-regulate nucleolin expression and then assayed cell lysates by immunoblotting (Fig. 6A). Although an efficient and specific down-regulation of nucleolin was achieved, no changes were observed in nucleophosmin and β-tubulin protein levels and the amount of aprataxin protein was unaffected (Fig. 6A). We extended this approach to a HeLa cell line expressing GFP–aprataxin and determined whether the nucleolar localization of aprataxin was dependent on its interaction with nucleolin. Down-regulation of nucleolin dramatically reduced the nucleolar localization of GFP–aprataxin (Fig. 6B). The integrity of the nucleoli was not affected in cells where nucleolin was down-regulated, when compared with cells having normal levels of nucleolin, as demonstrated by the merge of the phase contrast and nucleolin immunostaining images (Fig. 6C). As previously observed, this did not reduce aprataxin protein levels in this system (Fig. 6D), suggesting that the interaction with nucleolin is responsible for the targeting and/or maintaining aprataxin in the nucleolus.

**rDNA transcription is required for normal localization of aprataxin in the nucleolus**

As aprataxin is targeted to the nucleolus by its interaction with nucleolin, a multifunctional phosphoprotein involved in rRNA synthesis and processing (30,31), we investigated whether the nucleolar localization of aprataxin might be related to active rDNA transcription. HeLa cells expressing GFP–aprataxin were either left untreated or treated with RNA synthesis inhibitors. Treatment with α-amanitin (50 μg/ml), a transcription inhibitor specific for both RNA polymerases II and III (32), did not affect GFP–aprataxin localization in unfixed cells (Fig. 7A). On the other hand, low concentrations of actinomycin D (50 ng/ml), that specifically inhibit RNA Pol I and consequently rRNA synthesis (33), produced a re-localization of GFP–aprataxin into peri-nucleolar caps and into the nucleoplasm (Fig. 7A). To confirm that endogenous aprataxin is localized in the same way as the GFP–aprataxin fusion protein, we used aprataxin immunostaining. The results in Supplementary Material, Fig. S1A demonstrate a similar pattern to GFP fluorescence and aprataxin immunostaining. Exposure of the cells to actinomycin D (50 ng/ml) revealed peri-nucleolar localization with aprataxin antibody (Supplementary Material, Fig. S1B) similar to that observed with the GFP construct (Figs 7 and 8). This is in agreement with previous observations that actinomycin D also causes a redistribution of the rDNA that become concentrated in clusters at the periphery of the nucleolus (34,35). To rule out the possibility that transcription inhibitors could trigger aprataxin degradation resulting in disappearance from the nucleolus, immunoblotting was performed on total cell extracts treated with a range of transcription inhibitors. Neither aprataxin nor nucleolin protein levels were affected by transcription inhibition confirming a genuine nuclear re-localization of aprataxin (Fig. 7B).

Other transcription inhibitors were also employed to check for effects on localization of aprataxin. Treatment with CPT, a specific inhibitor of DNA topoisomerase I, and RNA Pol I at the concentration used (36), led to the redistribution...
of aprataxin within the nucleolus (Fig. 8A). 5,6-dichlorobenzamidazole riboside (DRB), an adenosine analogue that unravels and disperses the nucleolar transcription units within the nucleus, without abolishing their transcription (37), caused dispersion of GFP–aprataxin signal throughout the nucleolus (Fig. 8A). This re-localization, resembles a ‘necklace-like’ pattern (37), and is transient since replacement of the DRB-containing media with fresh media restored the normal nucleolar localization of aprataxin after incubation for 2 h (data not shown). A ‘necklace-like’ pattern was also observed for nucleolin after DRB and this coincided with that of aprataxin (Fig. 8A). As observed previously for aprataxin (Fig. 7A) treatment with actinomycin D caused a re-localization of nucleolin to peri-nucleolar caps and it co-localized with aprataxin (Fig. 8A). α-amanitin failed to alter the pattern of nucleolin staining.

As nucleolar localization of aprataxin depends on ongoing rRNA synthesis, we performed co-localization experiments with the transcription factor UBF-1, a marker of rRNA synthesis that is required for RNA Pol I transcription initiation (19,38). As shown in Figure 8B, untreated cells demonstrate some co-localization between UBF-1 and aprataxin within the nucleolus, suggesting that they may interact. Indeed, as observed in Figure 1, UBF-1 was identified by mass spectrometry as an aprataxin-binding protein (see also Supplementary Material, Fig. S2A). The interaction between aprataxin and UBF-1 was confirmed using co-immunoprecipitation and GST pull-downs, and shown to be through the aprataxin–FHA domain (Supplementary Material, Fig. S2B and S2C). Interaction of UBF-1 with nucleolin was also demonstrated using GST pull-downs and this was through the N-terminal region of nucleolin, a region that also binds aprataxin and nucleophosmin (Supplementary Material, Fig. S2D). When rRNA synthesis was inhibited with actinomycin D,
UBF-1 and aprataxin re-localized to the same peri-nucleolar caps (Fig. 8B). A similar redistribution of the major components of the rRNA transcription machinery in response to actinomycin D has been reported previously (34,39).

**AOA1 cells do not have a major rRNA synthesis defect**

As aprataxin, nucleolin and UBF-1 co-localized in the nucleolus and de-localized after RNA Pol I inhibition, we investigated whether aprataxin was required for efficient rRNA synthesis. In vivo rRNA synthesis was compared in control and AOA1 cells lacking aprataxin. Total RNA from control and AOA1 cells was extracted and subjected to RT–PCR analysis to determine the relative proportion of the pre-rRNA transcripts (47S/45S) and 28S rRNA using a recently described method (40). An outline of the various rRNA transcripts species is depicted in Figure 9A. While slightly reduced levels of 47S/45S pre-RNAs were observed in AOA1 cells when compared with control cells, only a very minor difference was noticed regarding the 28S rRNA region (Fig. 9B), suggesting that AOA1 cells do not exhibit a major defect in rDNA transcription.

**DISCUSSION**

Aprataxin is a nuclear protein present in both the nucleoplasm and the nucleolus that interacts with proteins involved in DNA strand break repair (9–12,14). Although recent evidence implicates aprataxin in DNA repair processes (9–13), the details of its nucleolar function remain elusive. Using a combination of GST pull-downs followed by mass spectrometry analysis and IP, we found that the FHA domain of aprataxin binds with the nucleolar proteins nucleolin, nucleophosmin and UBF-1 in good agreement with its nucleolar localization (9) and suggests a potential function for aprataxin in the nucleolus.

Nucleolin is a ubiquitous, non-histone, nucleolar RNA-binding phosphoprotein found in abundance in the nucleolus, the most prominent structure in the nucleus (30,41). Numerous reports have implicated the involvement of nucleolin either directly or indirectly in the regulation of cell proliferation and growth (30,41), chromatin decondensation (42), cytoplasmic nucleolar transport of ribosomal components, pre-ribosomal particles assembly (43) and replication and nucleogenesis (44). Nucleolin is a modular phosphoprotein that contains a highly negatively charged N-terminal domain, multiple phosphorylation sites for casein kinase II (CKII), cdc2 and protein kinase C-ζ domain, which contains a highly negatively charged N-terminal nucleogenesis (44). Nucleolin is a modular phosphoprotein found in abundance in the nucleolus, the most prominent structure in the nucleus (30,41). Numerous reports have implicated the involvement of nucleolin either directly or indirectly in the regulation of cell proliferation and growth (30,41), chromatin decondensation (42), cytoplasmic nucleolar transport of ribosomal components (43) and replication and nucleogenesis (44). Nucleolin is a modular phosphoprotein that contains a highly negatively charged N-terminal domain, multiple phosphorylation sites for casein kinase II (CKII), cdc2 and protein kinase C-ζ, four RNA recognition motifs (RRM) and a glycine-arginine rich (GAR) C-terminus (30). In the context of DNA damage recognition, it is of interest that Mre11 and 53BP1 also contain GAR motifs (45,46).

We demonstrated that the FHA domain of aprataxin binds phosphorylated nucleolin in good agreement with previous findings that demonstrated that FHA domains are phosphopeptide-interaction motifs (47–49). Clements et al. (10) have shown that aprataxin binds XRCC1 and XRCC4 in a phosphorylation-dependent manner most likely through its FHA domain. In those experiments, XRCC1 and XRCC4 were phosphorylated in vitro by CKII which enhanced binding to aprataxin for XRCC1, but was absolutely required for XRCC4. Phosphorylation-dependent binding between XRCC1 and aprataxin was also demonstrated by Luo et al. (11). They showed that XRCC1 is phosphorylated in vivo and in vitro by CKII and that CKII phosphorylation on Ser518, Thr 519 and Thr 523 largely determines aprataxin binding to XRCC1 through its FHA domain. It seems unlikely that CKII is responsible for the phosphorylation on nucleolin that determines its binding to aprataxin, as the specific CKII inhibitor (6,7-tetrabromobenzotriazole) did not interfere with this binding (unpublished data). There are several potential phosphorylation sites within the acidic domain of nucleolin, the region through which it interacts with aprataxin. The identity of the protein kinase involved remains to be determined.

It seems likely that the interaction between aprataxin and nucleolin is of physiological significance, as we have demonstrated a requirement for nucleolin to retain aprataxin in the nucleolus and a need for aprataxin to stabilize nucleolin in that organelle. AOA1 cells lacking aprataxin show evidence of nucleolin degradation, more diffuse and less intense nucleolar staining and instability of nucleolin protein. Furthermore, over-expression of GFP–aprataxin increases the steady-state levels of nucleolin protein confirming that aprataxin influences its stability. It is quite common that a particular component of a multi-protein complex affects the stability of the other members of the complex. For example, in A-T-like disorder, cells lacking full-length Mre11 protein, a component of the Mre11-Rad40-Nbs1 complex involved in sensing and signaling DNA double-strand breaks (50), both Nbs1 and Rad50 are destabilized in the absence of Mre11 (51).
Although nucleolin is found almost exclusively within the nucleolus, assays based on interspecies heterokaryons have shown that nucleolin shuttles between the nucleus and cytoplasm (43,52). Furthermore, fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) analyses demonstrate that factors involved in rRNA transcription (UBF-1), rRNA processing (nucleolin, fibrillarin, and others), and ribosome assembly (nucleophosmin) exchange rapidly between the nucleoplasm and nucleolus (53). FLIP experiments for GFP–aprataxin also demonstrate rapid movement of this protein within the nucleoplasm and nucleolus (Supplementary Material, Fig. S3). When a sub-nuclear region (position 1) was bleached, the fluorescence in nearby regions (positions 2–4) declined at a $\tau$ value of approximately 9s (Supplementary Material, Fig. S3C). This value is in agreement with those reported for both nucleolin and UBF-1, which were also 9s when measured by FRAP (53). Thus all of these three nucleolar proteins that interact together in the nucleolus have comparable mobilities.

Although the cellular function(s) of aprataxin is not yet understood, the interaction with nucleolin, nucleophosmin, UBF-1 and the nucleolar localization of aprataxin suggests a possible role in transcription of rDNA and/or pre-rRNA processing. UBF-1 interacts with the RNA Pol I complex (54) and phosphorylation of UBF-1 is required for the initiation of rDNA transcription (55). As we identified UBF-1 as an aprataxin–FHA binding protein, it is very likely that aprataxin interacts with a phosphorylated form of UBF-1, and therefore might be part of the RNA Pol I transcription initiation complex. In support of this, we observed co-localization between aprataxin, nucleolin and UBF-1 in asynchronous cells, suggesting that aprataxin localizes to sites of rRNA synthesis. Measurement of rRNA expression in vivo in control and AOA1 cells that lack aprataxin protein revealed that only a small reduction in 47S/45S rRNA and 28S rRNA species was observed in AOA1 cells when compared with control cells suggesting that aprataxin does not play a major role in rRNA transcription and/or maturation. Inhibition of RNA Pol I transcription by low doses of actinomycin D induced a translocation of both aprataxin and UBF-1 to sub-structures surrounding the nucleolus that have been previously referred to as ‘peri-nucleolar caps’ (34). These caps contain nucleolar proteins, PML or Cajal body proteins and RNAs (34). It seems likely that the relocation and nucleolar capping of nucleolar proteins is not just a byproduct of transcriptional arrest, but rather is an active process that reshapes the nuclear compartment (34). Although a fraction of nucleolin re-localized to these peri-nucleolar caps, the majority of the protein was released into the nucleoplasm confirming previous reports (56). In addition, inhibition of RNA polymerase II transcription by α-amanitin did not affect aprataxin nucleolar localization.

Although aprataxin may not be an active player of rRNA synthesis, its interaction with DNA repair proteins (9–12,14) raises the possibility that aprataxin might play a role in the repair of DNA damage in the nucleolus. The nucleolus is a site of intense transcriptional activity, as it generates approximately $4 \times 10^6$ transcripts/22 h cell cycle to maintain the steady-state level of $3.5 \times 10^6$ ribosomes (57). The presence of DNA strand breaks in rDNA would be detrimental to the cells, as it would block the transcription machinery, leading to the potential accumulation of DNA single-strand breaks and thus impact on cell growth and proliferation and ultimately lead to cell death. It has been shown that AOA1 cells have increased numbers of chromosome aberrations after treatment with CPT, a DNA topoisomerase I inhibitor that induces single-strand breaks in DNA (13). Similar to the results obtained here for aprataxin, PARP-1, PARP-2 and DNA topoisomerase I were also shown to localize to nucleoli and to delocalize upon RNA Pol I transcription inhibition by low doses of actinomycin D and CPT (36,58). The interaction of aprataxin with DNA single-strand break repair proteins PARP-1, XRCC1 and DNA ligase III could facilitate the repair of such DNA damage and contribute to maintaining the integrity of nucleolar functions to ensure cell proliferation and cell growth. These peri-nucleolar caps may represent sites of repair similar to foci observed associated with nuclear chromatin in general in response to different DNA damaging agents. Using its DNA/RNA binding activity (27), aprataxin could scan the rDNA for DNA lesions and therefore recruit the DNA repair machinery to these sites via its interaction with nucleolin and components of the RNA Pol I machinery (UBF-1). The nucleotide hydrolase activity of aprataxin, albeit weak, would also support a role in DNA repair (27,59). The challenge ahead is to define further the role of aprataxin in the nucleolus including its possible involvement in DNA repair in that organelle.

**MATERIAL AND METHODS**

**Cell lines and antibodies**

Lymphoblastoid cell lines (LCL) from controls (C2ABR, C3ABR), AOA1 patients (L938, L939), NFF, fibroblasts from an AOA1 patient (FD105) carrying the mutation W279X and cervical adenocarcinoma cells (HeLa) were cultured in RPMI 1640 medium (Gibco BRL) containing 10% fetal calf serum (FCS) (JRH Biosciences), 2 mm L-glutamine (Life Technologies), 100 U/ml penicillin (Gibco BRL), 100 U/ml streptomycin (Gibco BRL) and maintained in a humidified incubator at 37°C/5% CO₂. Aprataxin antibodies have been previously described (9). A rabbit polyclonal aprataxin antibody was also produced against purified full-length recombinant human aprataxin protein. Antibodies used are the following, nucleolin (M019-3, MBL), nucleophosmin (#3542, 1/1000, Cell Signalling Technology), XRCC1 (AHP832, 1/5000, Serotec), UBF-1 (F-9, 1/1000, Santa Cruz), β-actin (AC-40, 1/1000, Sigma) and β-tubulin (2-28-33, 1/1000, Sigma).

**Mass spectrometry**

Gel slices containing protein bands of interest stained with Coomassie blue R250 were excised and processed as previously described (60). Peptides mixture resulting from trypsin digestion was analysed by MALDI-TOF (Applied Biosystems Voyager DE-STR). Mass data acquisitions were piloted by Data Explorer® MS processing software.
Aprataxin–GST and nucleolin–GST pull-down assays

Aprataxin–GST fusion proteins have been previously described (9). Briefly, for pull-down assays, the GST, GST–FHA, GST–NL, GST–HIT and GST–ZF fusion proteins were bound to glutathione Sepharose beads (Amersham). Total cell extracts were prepared by lysing 1 × 10^7 control cells (C3ABR) in 1 ml of lysis buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM ethylene diamine tetra-acetic (EDTA), 1% Igepal (NP-40), 5 mM dithiothreitol (DTT) supplemented with protease and phosphatase inhibitors]. Clear lysate corresponding to approximately 1–2 mg of total protein was mixed with 50 μL of each GST fusion containing beads and GST pull-downs were performed in a batch mode for 2 h at 4°C on a rotating wheel. GST pull-downs were subsequently washed three times with lysis buffer and/or high salt buffer [50 mM Tris–HCl pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Igepal (NP-40), 5 mM DTT supplemented with protease and phosphatase inhibitors] and the beads were resuspended in 20 μL of sample loading buffer. Proteins were separated on 10% SDS–PAGE, transferred onto nitrocellulose membranes (Pall Life Sciences) and detected using rabbit polyclonal aprataxin antibody. Proteins were separated on 10% SDS–PAGE, transferred onto nitrocellulose membranes (Pall Life Sciences) and detected using rabbit polyclonal aprataxin antibody.

rRNA expression analysis

Total cellular RNA was extracted using Tri-Reagent solution as described in the manufacturer’s protocol (Invitrogen). Residual DNA was digested by RNase-free DNase RQ01 (Promega). RNA was reverse-transcribed with Superscript III reverse transcriptase (Promega) for 60 min at 55°C. The resultant cDNA was amplified by PCR for 15–40 cycles of 30 s at 96°C, 30 s at 55°C for 28S and 47S/45S rRNA or 65°C for GAPDH and 30 s at 72°C. PCR primer sets used for 47S/45S rRNA were forward primer: 5′-CGAAGAAGGCTGC CGGGTCT-3′, reverse primer: 5′-CAGGCCCTCACTGCACGTG-3′, reverse primer: 5′-CCCAAATGAGAACACCAGGAG CGCACG-3′ and primer sets for 28S rRNA were forward primer: 5′-CGAAGCCAAGTCGAGCGGGTCT-3′, reverse primer: 5′-CTTAACGGTTCACGGCCCTC-3′. GAPDH was used as an internal standard with the following primer set, forward primer: 5′-GCGACCTCAGATCAGACGTG-3′, reverse primer: 5′-GCCGACCTCAGATCAGACGTG-3′, reverse primer: 5′-CTTAACGGTTCACGGCCCTC-3′ and reverse primer: 5′-GAGATGATGACCCTTCTTGG CTC-3′.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. The authors declared that they have no conflicts of interest.

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