Senataxin, defective in ataxia oculomotor apraxia type 2, is involved in the defense against oxidative DNA damage

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A defective response to DNA damage is observed in several human autosomal recessive ataxias with oculomotor apraxia, including ataxia-telangiectasia. We report that senataxin, defective in ataxia oculomotor apraxia (AOA) type 2, is a nuclear protein involved in the DNA damage response. AOA2 cells are sensitive to H2O2, camptothecin, and mitomycin C, but not to ionizing radiation, and sensitivity was rescued with full-length SETX cDNA. AOA2 cells exhibited constitutive oxidative DNA damage and enhanced chromosomal instability in response to H2O2. Rejoining of H2O2-induced DNA double-strand breaks (DSBs) was significantly reduced in AOA2 cells compared to controls, and there was no evidence for a defect in DNA single-strand break repair. This defect in DSB repair was corrected by full-length SETX cDNA. These results provide evidence that an additional member of the autosomal recessive AOA is also characterized by a defective response to DNA damage, which may contribute to the neurodegeneration seen in this syndrome.

Introduction

Ataxia-telangiectasia (A-T) represents a paradigm for several autosomal recessive ataxias characterized by defects in the recognition and/or repair of DNA damage (Lavin and Shiloh, 1997). The protein defective in A-T, A-T mutated (ATM), recognizes and is activated by DNA double-strand breaks (DSBs) to signal this damage to the cell cycle checkpoints and the DNA repair machinery (Kurz and Lees-Miller, 2004). Loss of ATM function results in hypersensitivity to ionizing radiation (IR), cell cycle checkpoint defects, genome instability, increased cancer incidence, and neurodegeneration (Hernandez et al., 1993). A-T–like disorder (A-TLD), as a result of hypomorphic mutations in the MRE11 gene, most closely resembles A-T in its clinical phenotype (Taylor et al., 2004). Mre11 functions in a complex with Rad50 and Nbs1 (defective in Nijmegen breakage syndrome) to localize to sites of DNA DSB. This complex acts upstream of ATM in sensing DSB and ensures efficient activation of ATM (Uziel et al., 2003; Cerosaletti and Concannon, 2004; Lee and Paull, 2005). Once activated, ATM phosphorylates a series of substrates, including Nbs1, which acts as an adaptor molecule for control of the intra-S and G2/M cell cycle checkpoints.

AOA2 cells are sensitive to H2O2, camptothecin, and mitomycin C, but not to ionizing radiation, and sensitivity was rescued with full-length SETX cDNA. AOA2 cells exhibited constitutive oxidative DNA damage and enhanced chromosomal instability in response to H2O2. Rejoining of H2O2-induced DNA double-strand breaks (DSBs) was significantly reduced in AOA2 cells compared to controls, and there was no evidence for a defect in DNA single-strand break repair. This defect in DSB repair was corrected by full-length SETX cDNA. These results provide evidence that an additional member of the autosomal recessive AOA is also characterized by a defective response to DNA damage, which may contribute to the neurodegeneration seen in this syndrome.

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Abbreviations used in this paper: AOA, ataxia oculomotor apraxia; A-T, ataxia-telangiectasia; A-TLD, A-T–like disorder; ATM, A-T mutated; CPT, camptothecin; DSB, double-strand break; IR, ionizing radiation; MMC, mitomycin C; NFF, normal foreskin fibroblast; SSB, single-strand break.

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syndrome is characterized by cerebellar atrophy, oculomotor apraxia, peripheral neuropathy, and elevated serum α-fetoprotein in some cases (Le Ber et al., 2005; Criscuolo et al., 2006). The gene defective in AOA2, \textit{SETX}, was recently identified (Moreira et al., 2004). Mutations in \textit{SETX} are also associated with an autosomal dominant, juvenile onset form of ataxia with spastic paraplegia (Chen et al., 2004). Senataxin, the predicted protein encoded by \textit{SETX} is 2,677 amino acids in length and contains a seven-motif domain at its C terminus, typical of the superfamily I of DNA/RNA helicases (Moreira et al., 2004). Senataxin has extensive homology to the \textit{Schizosaccharomyces pombe} Sen1p proteins that possess helicase activity and are required for the processing of diverse RNA species that include transfer RNA, ribosomal RNA, small nuclear RNA, and small nucleolar RNA (Ursic et al., 1997). Sen1p proteins are also related to other DNA/RNA helicases, Upf1, involved in nonsense-mediated decay (Weng et al., 1996) and IGHMBP2, defective in a form of spinal muscular atrophy (Grohmann et al., 2001). Use of global and candidate-specific two-hybrid screens identified Rpo21p, a subunit of RNA polymerase II and Rnt1p, an endoribonuclease required for RNA maturation, as a Sen1p-interacting protein (Ursic et al., 2004), providing further support for a role in RNA processing. Recently, Steinmetz et al. (2006) showed that a single amino acid mutation that compromises Sen1 function in \textit{Saccharomyces cerevisiae} altered the genome-wide distribution of RNA polymerase II, providing evidence for a role in transcription regulation. Interestingly, Sen1p was also shown to interact with Rad2p, a DNase required for nucleotide excision repair after DNA damage (Ursic et al., 2004). These observations on yeast orthologues, together with an overlapping phenotype with other autosomal recessive ataxias with oculomotor apraxia, which are characterized by defective DNA repair, led us to investigate whether senataxin might also play a role in the DNA damage response. We show here that senataxin is primarily a nuclear protein and that AOA2 cells have increased sensitivity to H$_2$O$_2$, camptothecin (CPT), and mitomycin C (MMC), but a normal response to IR, compared with controls. To determine whether senataxin was responsible for this cellular phenotype, we cloned full-length \textit{SETX} cDNA and demonstrated complementation of agent sensitivity in stably transfected AOA2 cell lines. The increased sensitivity to H$_2$O$_2$ was associated with a defect in DNA DSB repair, but there was no defect in repair of SSBs or in DSBs produced by IR exposure. These results add further substance to the hypothesis that a defective DNA damage response contributes to the neurodegenerative phenotype in a subgroup of the autosomal recessive ataxias.

**Results**

**Detection and subcellular localization of senataxin**

Cell lines were established from two patients with AOA2, fibroblasts (SETX-1RM) and a lymphoblastoid cell line (SETX-2RM).

from 50 measurements in each case. \( P < 0.001 \) (t test). [F] Confirmation of nuclear localization of senataxin using competition with antigen (senataxin GST-1), recognized by Ab-1, to inhibit binding in NFF cells. Bars, 20 μm.
Identification of the mutations in SETX was performed by PCR followed by DNA sequencing. The results in Fig. S1 A (available at http://www.jcb.org/cgi/content/full/jcb.200701042/DC1) show that for SETX-1RM, a homozygous deletion of 1 kb occurred at the cDNA level as a result of a large deletion in genomic DNA, which resulted in the deletion of exons 14–21 of SETX. Mutation in SETX was also homozygous for SETX-2RM, involving exon 23 skipping because of a missense mutation at the splice site (IVS 23 + 5 G > A; Fig. S1 B).

To detect the presence of senataxin protein and investigate its subcellular localization, we produced two polyclonal antibodies against the C-terminal (Ab-1/Ab-3) and another against the N-terminal (Ab-2) regions of the protein (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200701042/DC1). Specificity of the antibodies was determined by dot blot analysis (Fig. S2, B–E). The two C-terminal antibodies reacted only with a GST fusion protein corresponding to this C-terminal region of the molecule (senataxin GST-1), whereas the N-terminal antibody specifically recognized this region of the molecule (senataxin GST-2). Preimmune serum failed to detect senataxin GST. The results in Fig. 1 A show that Ab-1 detected the presence of a single prominent band at ~300 kD for two control cell lines (C2ABR and C3ABR), two AOA1 cell lines (L990 and L938), an unclassified AOA cell line (ATL2ABR) but was absent in an AOA2 cell line (SETX-2RM), as predicted from the exon-skipping mutation. The same pattern was observed with all three antibodies (unpublished data). Immunoprecipitation with Ab-1 followed by immunoblotting with the same antibody also detected a single band of the same size, which was again absent in the AOA2 cells (Fig. 1 B). Non-specific antisera failed to immunoprecipitate senataxin. It was not possible to detect senataxin in primary fibroblasts with up to twofold increased protein loading by immunoblotting on total cell extracts (unpublished data). However, immunoprecipitation with Ab-1 followed by immunoblotting with two senataxin antibodies (Ab-1 and Ab-2) revealed the presence of senataxin in normal foreskin fibroblasts (NFFs; Fig. 1 C). This might be explained by a lesser amount present in the fibroblasts or a reduced relative amount of nuclear protein in fibroblasts compared with lymphoblasts. A slightly lower molecular size band for senataxin was detectable in an AOA2 fibroblast (SETX-1RM), consistent with the deletion of exons 14–21, which would maintain the reading frame but would lead to the loss of 322 amino acids (Fig. 1 C). Fractionation of cell extracts followed by immunoblotting with senataxin antibodies revealed that this protein was primarily a nuclear protein (Fig. 1 D). Senataxin was present in the cytoplasm but markedly reduced compared with that in the nucleus. It is notable that the amount of senataxin varied with cell type being highest in lymphoblastoid cells. Comparison of AOA2 (SETX-1RM) fibroblasts with NFFs (controls) using immunofluorescence demonstrated reduced nuclear staining in the AOA2 cells (Fig. 1 E). To confirm that the nuclear staining was specific, we performed competition experiments with antigen (senataxin GST-1) corresponding to a C-terminal peptide (Fig. 1 F). The results in Fig. 2 A confirm that senataxin, detected by both N- and C-terminal antibodies, is predominantly a nuclear protein and appears to be excluded from the nucleolus, as shown.

Figure 2. **Senataxin is a nucleoplasmic protein.** [A] Immunostaining for senataxin was performed in NFFs using both Ab-2 and Ab-3. RNA polymerase II identifies the nucleoplasm, and DAPI identifies the nucleus. [B] Senataxin does not localize to the nucleolus as shown by nonoverlapping staining pattern for senataxin and nucleolin, a specific marker of the nucleolus in NFFs, SETX-1RM, and HeLa. Bars, 20 μm. [C] Subcellular fractionation of control cells (C2ABR) was used to detect senataxin by immunoblotting using Ab-1. Cyto, cytoplasmatic fraction; Np, nucleoplasmatic fraction; No, nucleolar fraction. Detection of nucleolin and RNA polymerase II was used to determine the purity of the fractions. Total cell extract from C2ABR and SETX-2RM cells are also shown.

A staining pattern similar to that of RNA polymerase II, a nucleoplasmic protein. Further support for this is provided by the failure of senataxin to colocalize with nucleolin in the nucleolus of NFFs or HeLa cells (Fig. 2 B). This was also the case for SETX-1RM fibroblasts, which contain a truncated form of senataxin. Finally, evidence that senataxin is predominantly a nucleoplasmic protein was provided by subcellular fractionation and immunoblotting (Fig. 2 C). Under these conditions, a prominent senataxin band was detected in the nucleoplasm but not in the nucleolus or cytoplasm. Immunoblotting for RNA polymerase II and nucleolin was performed to demonstrate the integrity of the cellular fractions (Fig. 2 C).

**Sensitivity of AOA2 cells to DNA damaging agents and complementation of the defect**

Because it has been demonstrated that cell lines from patients with other autosomal recessive ataxias, which overlap in their clinical phenotype with AOA2, are sensitive to DNA damaging
agents, we determined whether this might also be the case for AOA2. The results in Fig. 3 A reveal that AOA2 lymphoblastoid cells (SETX-2RM) show a normal pattern of sensitivity to IR but have increased sensitivity to H2O2, CPT, and MMC, compared with controls. AOA1 cells are also sensitive to H2O2, as we have previously shown (Gueven et al., 2004). A-T cells showed normal sensitivity to these DNA damaging agents but hypersensitivity to IR. Increased sensitivity was also observed with H2O2, MMC, and CPT in AOA2 fibroblasts (Fig. 3 B). Although these agents give rise to either cross-links or SSBs and DSBs in DNA, there is also evidence that their toxicity is associated with redox-related pathways (Pagano et al., 2005). AOA1 fibroblasts were also sensitive to H2O2 (Fig. 3 B). As an additional measure of sensitivity, we compared the levels of H2O2-induced chromosome aberrations. The results in Fig. 3 C reveal approximately two induced chromosome aberrations per metaphase in H2O2-treated control cells, whereas a further twofold increase was observed in AOA2. To demonstrate that the increased sensitivity to H2O2 was due to loss of senataxin, we cloned full-length \( \text{SETX} \) cDNA into the Epstein-Barr virus–based expression vector pMEP4, containing a FLAG tag sequence, and established stable cell lines as previously described (Zhang et al., 1997). DNA sequencing detected five polymorphic changes in \( \text{SETX} \) and three nonconserved amino acid changes compared with the sequence in the database (available from GenBank/EMBL/DDBJ under accession no. AB014525). This construct, pSETX1, contains a metallothionein promoter, which allows inducible expression of senataxin by CdCl2. Induction of senataxin was detected by immunoprecipitation with anti-senataxin and anti-FLAG antibodies followed by immunoblotting with the respective antibodies (Fig. 4 A, right). Immunoblotting on total cell extracts confirmed that AOA2 (SETX-2RM) parental cells lack senataxin as compared with control cells (Fig. 4 A, left). Exposure of \( \text{SETX} \)-transfected AOA2 cells to H2O2 after induction of senataxin corrected the H2O2 hypersensitivity in these cells (Fig. 4 B). On the other hand, AOA2 cells transfected with empty vector remained hypersensitive to H2O2. \( \text{SETX} \) cDNA also corrected the H2O2-induced hypersensitivity to H2O2.
Chromosome aberrations in AOA2 cells (Fig. 4 C), whereas vector alone did not change the number of aberrations.

**Constitutive oxidative DNA damage in AOA2 cells**

Because AOA2 cells were sensitive to H$_2$O$_2$, CPT, and MMC, all of which are capable of generating oxidative stress (Pagano et al., 2005), we investigated whether there might be an inherent defect in coping with oxidative damage in these cells. The presence of 8-oxo-deoxyguanosine (8-oxo-dG) was determined as a marker of reactive oxygen species–mediated DNA damage (Cooke et al., 2000). The results in Fig. 5 A reveal a high basal level of 8-oxo-dG in AOA2 fibroblasts (SETX-1RM), but not in controls (NFFs). Quantitation of 8-oxo-dG fluorescence intensity reveals an approximately twofold increase in AOA2 cells compared with controls (Fig. 5 B). Exposure of cells to H$_2$O$_2$ caused an increase in 8-oxo-dG in both controls and AOA2, but the extent of staining remained higher in the AOA2 cells (Fig. 5 A and B).

To determine whether this represented more general oxidative damage in AOA2 cells or a defect at the level of DNA, we also assayed for protein nitrotyrosination and lipid peroxidation. The results showed background levels for both 3-nitrotyrosine and 4HNE-Michael adducts in AOA2 cells similar to those observed in normal fibroblasts, suggesting that these cells were not undergoing generalized oxidative stress (Fig. 5, C and D).

**Repair of oxidative damage to DNA**

Given that AOA2 cells were sensitive to agents that cause oxidative stress, and because these cells had evidence of constitutive oxidative DNA damage, we investigated their DNA repair capacity. For DNA SSB repair, we used alkaline elution analysis. Using this assay, we failed to observe any difference between control and AOA2 cells over a time course of 1-h repair (Fig. 6 A). These results were confirmed by determining intracellular NAD(P)H levels, which represents a reliable method to monitor imbalance in break repair in cells (Nakamura et al., 2003). Again, in this case, there was no evidence for a defect in the extent or duration of DNA single-strand breakage (Fig. 6 B).

H$_2$O$_2$ is also capable of inducing DSBs in DNA (Dahm-Daphi et al., 2000). To detect DSBs arising in DNA as a result of oxidative damage and eliminate those that occur as a consequence of DNA replication fork movement across a site of damage, cells were grown to confluence. DNA DSBs were detected by measuring H2AX phosphorylation visualized as foci at sites of DNA damage, a quantitative assessment for the appearance and repair of DNA DSBs (Rothkamm and Lobrich, 2003). Approximately equal numbers of γH2AX foci were detected in control and AOA2 cells between 10 and 50 min after treatment with H$_2$O$_2$ (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200701042/DC1), and in both cases, these foci coincided with MDC1 foci, further supporting that they were sites of DNA DSB (Fig. 7 A). However, the extent of disappearance of these foci appeared to be slower in AOA2 cells at 8 h after treatment than in controls (Fig. 7 A). Quantitation of these results showed that there was a significant difference between AOA2 and controls at 4, 6, and 8 h after treatment. By 8 h after treatment, 10% of the foci remained in control nuclei compared with
Figure 6. DNA strand break repair in AOA2 cells. (A) SSB repair of control (C3ABR) and AOA2 (SETX-2RM) cells in response to H$_2$O$_2$-induced DNA damage measured by alkaline elution at the indicated times after treatment. SSBs were induced by treating the cells with 20 μM H$_2$O$_2$ for 15 min at 37°C. (B) Measure of intracellular NAD(P)H depletion in response to H$_2$O$_2$ and methyl methanesulfonate (MMS) treatment was also used to determine appearance of SSBs in DNA. Error bars indicate SD.

39% in AOA2 cells, indicating a defect in repair of DSBs (Fig. 8 A). To determine whether this was a general defect in repair of DSBs, cells were exposed to IR, and the rate of disappearance of γH2AX foci was measured (Fig. 7 B). No difference in the rate of DSB repair after IR was observed (Fig. 8 B). The reduced capacity of AOA2 cells to repair the DSB induced by H$_2$O$_2$ does not appear to be explained by a trivial reason, such as widespread oxidative damage to proteins after incubation with H$_2$O$_2$ in the form of nitrotyrosinated proteins, as there was no evidence of gross damage (Fig. 5 C). To investigate further whether senataxin played a direct role in the DNA damage response, we performed transient transfections of AOA2 cells with SETX-GFP cDNA and assayed for correction of the DNA DSB defect after H$_2$O$_2$ treatment. Use of the SETX-GFP construct allowed us to differentiate between transfected and untransfected cells, providing an internal control for scoring of γH2AX foci. The results in Fig. S4 A reveal that the number of γH2AX foci are comparable in unlabeled and GFP-labeled cells. However, by 8 h after treatment, the number of γH2AX foci in GFP-labeled cells is significantly lower than that of γH2AX foci in nontransfected cells (Fig. S4 B). Similar experiments in transfected control fibroblasts revealed that the number of γH2AX foci was the same in unlabeled and GFP-labeled cells at 30 min after treatment with H$_2$O$_2$, and both cell types efficiently repaired the DNA DSBs by 8 h (Fig. S4, C and D). Foci were counted in at least 50 GFP-transfected and unlabeled cells and quantitated to reveal that SETX-cDNA corrected the DSB defect in AOA2 cells.

Discussion

Antibodies directed against both extremities of senataxin detected a protein of ~300 kD, the predicted size from the reported open reading frame (Moreira et al., 2004). This protein was not detected in AOA2 lymphoblastoid cells with a mutation in SETX predicted to give rise to a prematurely truncated protein, but was detected as a lower molecular size form in fibroblasts (SETX-IRM) where an in-frame deletion was observed. We also provided firm evidence that senataxin is a nuclear protein with only minimal amounts in the cytoplasm. This protein is diffusely distributed throughout the nucleus with no evidence of localization to any subnuclear compartments. This is in contrast to a recent report demonstrating that senataxin was diffusely present in the cytoplasm and in the nucleolus in cultured cells (Chen et al., 2006). These data suggested that senataxin was expressed strongly in the cytoplasm in primate deep cerebellar nucleus but dull and diffuse in the nucleus. Using three different antibodies directed against both termini of senataxin, we showed diffuse nuclear labeling by immunofluorescence, which is supported by immunoblotting, revealing predominantly nuclear localization of the protein. We did not detect any senataxin in the nucleolus using either immunofluorescence or subcellular fractionation. It is of interest that Chen et al. (2006), using an N-terminal FLAG-tagged SETX construct, demonstrated only strong immunoreactivity throughout the nucleoplasm with an anti-FLAG antibody, in contrast to the results obtained with their anti-senataxin antibody. The authors interpreted this to mean that FLAG-senataxin is mislocalized. On the contrary, our data suggest that this is the correct localization.

Similar to that observed in A-T, A-TLD, and AOA1 cells, AOA2 cells are also characterized by sensitivity to DNA damaging agents. A-T cells are hypersensitive to IR and radiomimetic agents that give rise to DSBs in DNA (Taylor et al., 1975; Chen et al., 1978). A-TLD cells are also sensitive to these agents but not to the same extent as A-T (Stewart et al., 1999). The pattern of sensitivity described for AOA1 cells overlaps with that reported here for AOA2 (Gueven et al., 2004; Mosesso et al., 2005). Both cell types show increased sensitivity to H$_2$O$_2$, CPT, and MMC, but are not sensitive to IR. All three agents to which AOA2 cells are sensitive can give rise to DNA SSBs, but we found no evidence for a defect in SSB repair in AOA2. On the other hand, there is some evidence that AOA1 cells have a defect in SSB repair (Mosesso et al., 2005; Gueven et al., 2007), which is compatible with a role for aprataxin in resolving abortive DNA ligation intermediates (Ahel et al., 2006). Thus, the basis for the sensitivity to these agents appears to differ in AOA1 and AOA2 cells. The toxicity of these agents (MMC, CPT, and H$_2$O$_2$) is also associated with redox-related pathways (Pagano et al., 2005). A common observation in neurodegenerative disorders associated with DNA repair/signaling defects is an increase of spontaneous oxidative damages (Hayashi et al., 2001; Barzilai et al., 2002). Investigation of oxidative stress markers in AOA2 cells revealed higher basal levels of oxidative DNA damage...
(8-oxo-dG) compared with controls, suggesting a reduced capacity to repair this type of DNA lesion (Hoffmann et al., 2004). This is further supported by a failure to observe more general oxidative damage in these cells.

At a higher concentration, H2O2-induced DSBs are also detected in DNA, as determined by the formation of γH2AX foci (Chen et al., 2005). They described the appearance of γH2AX foci in H2O2-treated cells after 2 h, which gradually decreased over 24 h. Coincidence was demonstrated between γH2AX and 53BP1 foci, providing additional evidence that these were DNA DSBs. In this study, we revealed a coincidence between γH2AX and MDC1 foci, a γH2AX-interacting protein that bridges the binding of the DNA damage response machinery to sites of DNA breaks (Stucki and Jackson, 2006). In the present study, these foci occurred to the same extent in both control and AOA2 cells in response to H2O2 treatment. However, the rate of loss of γH2AX foci was significantly reduced in AOA2 cells compared with controls from 4–8 h after treatment. The presence of high constitutive levels of 8-oxo-dG in AOA2 cells did not affect the number of breaks introduced into DNA by H2O2, but it is possible that these lesions might interfere with the rate of repair of the DSBs. An alternative but less likely explanation for the persistence of γH2AX foci in AOA2 cells is that these foci are indicative of aberrant chromatin structure by inappropriate rejoining of DNA breaks. Suzuki et al. (2006) provided this as an explanation to account for the persistence of γH2AX foci at a time when all the breaks were repaired on mitotic chromosomes. The defect in repair to DSBs was not a general one because AOA2 cells were as efficient as controls in repairing breaks induced by IR. These results suggest that the nature of the breaks is different after H2O2 and IR exposure. DNA damage caused by oxidation is complex and is deposited along the DNA molecule as single alterations or in clusters termed multiple DNA damage sites (Letavayova et al., 2006). This damage is primarily oxidized base damage and, when present at close proximity on opposite strands, can give rise to abortive base excision repair that leads to the formation of DNA DSBs (Wallace, 2002).
What distinguishes a DSB resulting from IR or H$_2$O$_2$ treatment? There is no easy answer to this, but it is evident that a DNA DSB arising from IR is efficient in attracting the Mre11 complex, activating ATM and the components of DNA repair (Bakkenist and Kastan, 2003; Kozlov et al., 2006). On the other hand, H$_2$O$_2$ is less efficient in activating ATM (Isai et al., 2005; unpublished data). Furthermore, PDGFβ receptor transactivation acts as an upstream mediator of ATM kinase stimulation in response to H$_2$O$_2$ and, under these conditions, p53 is phosphorylated only on Ser15 (Chen et al., 2003). These data suggest that the mechanism of activation of ATM by IR and H$_2$O$_2$ are different and provide support for a difference in the nature of the DNA DSB generated in each case. Further support for a difference in the nature of the damage inflicted by H$_2$O$_2$ and IR is demonstrated by a lack of correlation in sensitivity to these agents in different cell lines (Cantoni et al., 1989; Bouzyk et al., 2000; Gueven et al., 2004) and difference in mutagenesis capacity (Gustafson et al., 2000) and in their effects on cell cycle progression (Wharton, 1995). Thus, it is not inconceivable that senataxin plays a specific role only in the processing of breaks generated by oxidative DNA damage.

It appears from the shape of the DNA repair curve that the initial rate of removal of breaks is comparable in control and AOA2 and that the defect is in a slower component of repair. This could be explained by a defect in repairing a subgroup of DNA breaks arising as a consequence of oxidative damage. 8 h after treatment with H$_2$O$_2$, 10% of breaks remained unrepaired in control cells, whereas almost four times as many (39%) were still detected in AOA2 cells. The subgroup of breaks may relate to double-strand ends with damaged termini. Although it is likely that •OH generated from H$_2$O$_2$ is responsible for much of the damage, there is also evidence for metal-mediated DNA damage, which is not protected against with hydroxyl radical scavengers (Li et al., 1999). Although Artemis-dependent processing of a subgroup of DNA breaks in response to radiation damage has been reported (Riballo et al., 2004), it is unlikely that the involvement of senataxin is in the ATM–Artemis pathway because DSBs induced by IR are normally repaired in AOA2. The predicted protein sequence of senataxin contains a seven-helicase motif near its C terminus related to that present in the helicase superfamilies I and II (Gorbalenya et al., 1989), which play essential roles in maintaining genome integrity through their involvement in DNA replication, transcription, recombination, and repair (Hickson, 2003). Two members of the RecQ helicase family, WRN and RECQL4, mutated in Werner syndrome and Rothmund-Thompson syndrome, respectively, also protect against oxidative DNA damage (Szekely et al., 2005; Werner et al., 2006). The increased sensitivity to agents that cause oxidative stress and the reduced DSB repair in response to H$_2$O$_2$ exposure in AOA2 cells might also be explained by a defect in a helicase. However, although senataxin is an orthologue of yeast DNA/RNA helicases, no such activity has yet been demonstrated for this protein. A recent report by Steinmetz et al. (2006) showed that the yeast Sen1 helicase controlled the genome-wide distribution of RNA polymerase II, and a mutation that affected the function of this helicase led to profound changes in polymerase II distribution over noncoding and protein-coding genes, suggesting that Sen1 has an important role in control of gene expression. Given the similarity between the yeast and human Sen1 proteins, they suggested that mutations in senataxin may also lead to misregulation of transcription and in turn account for the progressive neurological defect in this syndrome. If it were to emerge that senataxin plays a similar role to yeast Sen1 in mammalian cells, it is not immediately evident how the transcriptional misregulation might account for some of the cellular characteristics that are described here for AOA2. Mouse cells lacking the WRN helicase exhibit altered expression of genes responding to oxidative stress (Deschenes et al., 2005). Furthermore, combining the defect with abrogation of the DNA repair gene poly (ADP-ribose) polymerase-1 (PARP-1) increased the extent of misregulation of gene expression. Aberrant transcription, chronic cellular stress, and apoptosis have also been suggested to contribute to the phenotype in Alzheimer’s disease (Toiber and Soreq, 2005). The appearance of oxidative stress in AOA2 as described here could thus be explained by misregulation of transcription. Failure to respond normally to DNA damage and repair DSBs arising as a result of oxidative DNA damage might also be explained by the down-regulation of specific genes that are important for this process. Alternatively, as senataxin is a large protein characterized by only one putative helicase domain, it is possible that it has other activities or controls other proteins through interaction. Being able to reconcile the AOA2 cellular characteristics described here with a possible defect in regulation of gene expression requires a greater understanding of the functioning of this protein.

Materials and methods

Cell lines, survival, and induced chromosome aberrations

Lymphoblastoid cell lines (LCLs) from control (C3ABR and C2ABR), AOA1 (L938 and L939), Friedrich’s ataxia (FRA1A), and AOA2 (SETX-2RM) patients were cultured in RPMI 1640 medium (Invitrogen) containing 10% FCS ( JR Biosciences), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin (Invitrogen), and 100 U/ml streptomycin (Invitrogen), and maintained in a humidified incubator at 37°C/5% CO$_2$. Fibroblasts from an AOA2 patient (SETX-1RM), NFFs [a gift from P. Parsons, Queensland Institute of Medical Research, Queensland, Australia], and Hela were cultured in DMEM medium (Invitrogen) containing 10% FCS. LCLs were used at a density of $1 \times 10^6$ cells/ml, and adherent cells were used at 75% confluency except in the case of γH2AX foci experiments, where confluent cells were used. MMC, CPT, and H$_2$O$_2$ were purchased from Sigma-Aldrich. Irradiations were performed at room temperature using a $^{60}$Co source (Gammacell 40 Exactor [MDS Nordion]; dose rate 1.1 Gy/min). Lymphoblastoid cell viability (triplicate wells for each drug concentration) was measured by adding 0.1 ml of 0.4% trypan blue to 0.5 ml of cell suspension. The number of viable cells was counted, and viabilities were expressed as the number of cells in drug-treated wells relative to cells in untreated wells (percentage of viable cells), as previously described (Chen et al., 1978). The cells were incubated with the genotoxic agents for 1 h (MMC), 3 h (CPT), and 30 min (H$_2$O$_2$) before washing twice with PBS and suspension in culture medium. The number of viable cells was counted daily up to 4 d after treatment, and viability was calculated as described. The conditions were the same for fibroblasts, but survival was determined by colony formation. Cells were left for 2–3 wk to form colonies before staining with methylene blue and counting. Chromosomal aberrations were determined by treating cells with 2 mM H$_2$O$_2$ for 30 min in media under aerobic conditions. Colcemid (final concentration 0.1 µg/ml) was added immediately after treatment, 2 h before harvesting. The cells were treated for 15 min in 0.0075 M KCl and fixed in methanol ± glacial acetic acid (3:1), and the fixed cells were spread onto glass slides, air-dried, and stained with Giemsa. 50 metaphases were analyzed for each sample.
Cloning and expression of senataxin antigens

To produce senataxin antibodies, two regions of SETX were PCR amplified and cloned into bacterial expression vectors. In brief, a region of 450 bp of human SETX cDNA was PCR amplified from the 3' end of senataxin using Ab-1F/Ab-1R primer pair. This SETX fragment was subsequently cloned into Nhel and Noti sites of pTYB1 plasmid (New England Biolabs, Inc.), containing a C-terminal chitin binding domain. A second region of 1.1 kb of SETX was PCR amplified from the 5' end of senataxin using Ab-2F/Ab-2R primer pair. The 5' SETX fragment was cloned into EcoRI and NotI sites of pGEX-5X-1 plasmid (GE Healthcare), containing an N-terminal GST tag. Both of these constructs were transformed into Escherichia coli BL21 (DE3) plysE cells, and overexpression was induced using 0.3 mM IPTG.

Production and purification of anti-senataxin antibodies

To produce senataxin antibodies, two regions of SETX corresponding to the N and C termini of the protein were PCR amplified, cloned into bacterial expression vectors, and transformed into E. coli (BL21 [DE3] plysE). N- and C-terminal fusion proteins were affinity purified using chitin beads and glutathione–Sepharose resin, respectively, using the manufacturer's protocol. Sheep or rabbits were inoculated with senataxin antigens, and polyclonal antibodies were generated against the C (Ab-1/Ab-3) and N termini (Ab-2) of human senataxin using methods described previously (Bor-Peled and Raikher, 1996). Senataxin antibodies were affinity purified using a series of GST-only and GST-N/ter GST-C/ter columns.

Cloning of full-length SETX cDNA

Full-length SETX was PCR amplified and cloned into KpnI–Noti digested pMEP4 (see the supplemental text, available at http://www.jcb.org/cgi/content/full/jcb.200701042/DC1). For complementation studies, expression of senataxin was induced or mock-induced from pMEP4–transfected cells with 5 μM CdCl₂. SETX was also cloned into pEGFP-C2 by applying full-length SETX cDNA from pSPORT1_Si (RP2D Deutsches Ressourzenzentrum). SETX–GFP was used in transient transfection experiments.

PCR primers were designed to amplify two SETX fragments overlapping a unique Spel restriction site located at position 4432. Primer pairs HEF1/HER2 amplified a 5' region from 1 to 4375 bp and HEF3/HER3 amplified a 3' region from 4313 to 8038 bp. PCR amplification was performed with 100 ng C3ABR cDNA, 1 μg of each primer, 100 μM dNTP, 10 mM Tris-Cl pH 8.3, 50 mM KCl, 0.1% Tween 20, supplemented with protease and phosphatase inhibitors) for 1 h at 37°C. Insoluble components were removed by centrifugation at 16,000 g for 20 min at 4°C. For immunopurifications, 1 mg of total cell extract was precleared with 30 μl of protein G–Sepharose beads (GE Healthcare) for 3 h at 4°C. Senataxin was immunoprecipitated with 7.5 μg senataxin-Ab1 antibody overnight at 4°C. The following day, 40 μl of protein G–Sepharose beads was added for 1 h and incubated at 4°C. Immunoprecipitates were washed three times with lysis buffer and resuspended in 20 μl of sample loading buffer before separation of the proteins by SDS-PAGE. The proteins were then transferred onto a nitrocellulose membrane (Pall Life Sciences), and immunoblots were performed with the relevant antibodies.

Immunofluorescence

NF5s, AOA2 (SETX-1RM) fibroblasts, and HeLa were grown on glass coverslips for 48 h, washed with PBS, fixed in 2% paraformaldehyde/PBS/10 mM for 10 min, and processed for immunofluorescence as previously described (Becherel et al., 2006) using the relevant antibodies, senataxin Ab-3 (1:400) and Ab-2 (1:200), RNA polymerase II (1:400; ab5408 [Abcam]), neureilin (1:500; M0193 [MBL International Corporation]), 3-nitrotyrosine (1:100; MAB2945), and γH2AX (1:100; 393207 [Cellbiochem]). Fluorochromes conjugated to the relevant secondary antibodies were Alexa Fluor 488 and 594 (Invitrogen). Images were captured using a digital camera (AxioCam MRm; Carl Zeiss Microimaging, Inc.) attached to a fluorescent microscope (Axioscop2 mot plus; Carl Zeiss Microimaging, Inc.) using Plan Apochromat 1.4 oil differential interference contrast (DIC) ×63 magnification. Imaging medium was PBS, and acquisition was performed at ambient temperature (25°C). AxioVision LE 4.3 software was used to capture the individual images, which were assembled using Photoshop 7.0 (Adobe). Fluorescence intensity was quantitated on the raw images using the public domain software ImageJ version 1.34s (NIH) before their assembly in Photoshop 7.0. After assembly, contrast was enhanced on all images simultaneously in Photoshop 7.0 using the brightness and contrast tool. No further image processing (e.g., surface or volume rendering, γ adjustment) was performed.

Analysis of DNA SSB repair

To induce SSB, cells were exposed to 20 μM H₂O₂ in RPMI 1640 medium without supplements for 15 min at 37°C. To remove H₂O₂, 880 U/ml catalase was added. Cells were collected by centrifugation (1,500 U/min for 5 min) and washed with PBS/CMF (140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, and 1 mM KH₂PO₄). The numbers of SSBs were determined by an alkaline elution assay as previously described (Epe and Hegler, 1994). The numbers of SSBs in untreated control cells were subtracted in all cases. NAD(P)H depletion after H₂O₂ and MMS treatments was performed as previously described (Nakamura et al., 2003).

Repair kinetics of γ-H2AX foci

Cells were seeded onto coverslips, and experiments were performed on confluent fibroblasts. Cells were either irradiated or treated with H₂O₂ by adding 2 mM H₂O₂ into the growth medium for 10 or 30 min. Cells were washed with PBS and returned to fresh media. At the time points indicated, the cells were processed for immunofluorescence as described. Images were captured and assembled as described.

Online supplemental material

Fig S1 shows characterization of mutations in two AOA2 cell lines. Fig. S2 shows characterization of senataxin antibodies. Fig. S3 shows induction of γ-H2AX foci in control (NFF) and AOA2 (SETX-1RM) cells after exposure to 2 mM H₂O₂. Fig. S4 shows characterization of the DNA DSB repair defect in AOA2 cells. The supplemental text gives primers used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200701042/DC1.

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The authors declare that they have no conflict of interest.

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