Functional role for senataxin, defective in ataxia oculomotor apraxia type 2, in transcriptional regulation

Amila Suraweera1, YiChieh Lim1,2, Rick Woods1, Geoff W. Birrell1, Talat Nasim3, Olivier J. Becherel1 and Martin F. Lavin1,2,*

1Radiation Biology and Oncology Laboratory, Queensland Institute of Medical Research, Brisbane, QLD 4029, Australia, 2Centre for Clinical Research, University of Queensland, Brisbane, QLD 4029, Australia and 3Department of Medical and Molecular Genetics, King’s College London, Guy’s Hospital, London SE1 9RT, UK

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Ataxia oculomotor apraxia type 2 (AOA2) is an autosomal recessive neurodegenerative disorder characterized by cerebellar ataxia and oculomotor apraxia. The gene mutated in AOA2, SETX, encodes senataxin, a putative DNA/RNA helicase which shares high homology to the yeast Sen1p protein and has been shown to play a role in the response to oxidative stress. To investigate further the function of senataxin, we identified novel senataxin-interacting proteins, the majority of which are involved in transcription and RNA processing, including RNA polymerase II. Binding of RNA polymerase II to candidate genes was significantly reduced in senataxin deficient cells and this was accompanied by decreased transcription of these genes, suggesting a role for senataxin in the regulation/modulation of transcription. RNA polymerase II-dependent transcription termination was defective in cells depleted of senataxin in keeping with the observed interaction of senataxin with poly(A) binding proteins 1 and 2. Splicing efficiency of specific mRNAs and alternate splice-site selection of both endogenous genes and artificial minigenes were altered in senataxin depleted cells. These data suggest that senataxin, similar to its yeast homolog Sen1p, plays a role in coordinating transcriptional events, in addition to its role in DNA repair.

INTRODUCTION

Neurodegenerative disorders are heterogeneous in nature and include autosomal recessive ataxias with oculomotor apraxia (AOA) that are characterized by cerebellar ataxia and a combination of different ophthalmological and neurological signs (1). AOA includes ataxia-telangiectasia (A-T), ataxia-telangiectasia like disorder (A-TLD), ataxia oculomotor apraxia type 1 (AOA1) and ataxia oculomotor apraxia type 2 (AOA2) (2). A related syndrome, spino cerebellar ataxia with axonal neuropathy is characterized by a defect in tyrosyl-DNA phosphodiesterase 1 (3). These syndromes have an overlapping clinical phenotype and are characterized by a defective response to DNA damage that appears to contribute to the neurodegenerative phenotype (2,4–6). The gene mutated in AAO2, SETX, consists of an open reading frame of 8031 bp, 26 exons and is predicted to code for a protein of 2677 amino acids (7). This protein was named senataxin due to its high homology to Saccharomyces Sen1p proteins (7). Mutations in SETX are also thought to be responsible for juvenile amyotrophic lateral sclerosis, an autosomal dominant disorder (8). We have previously shown that senataxin is a nuclear protein, involved in the response to oxidative stress (6). AOA2 cells are sensitive to the DNA damaging agents H2O2, camptothecin and mitomycin C, but display normal sensitivity to ionizing radiation. These cells also show evidence of constitutive oxidative DNA damage and the rejoining of H2O2-induced DNA double strand breaks is significantly reduced compared with controls (6).

The C terminus of senataxin contains a classical seven-motif domain found in the superfamily I of helicases, suggesting that it is a putative DNA/RNA helicase (7). Sen1p proteins possess

*To whom correspondence should be addressed at: Queensland Institute of Medical Research, PO Royal Brisbane Hospital, Brisbane, QLD 4029, Australia. Tel: +61 733620341; Fax: +61 733620106; Email: Martin.Lavin@qimr.edu.au

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assays using RNA helicase activity and are required for the processing of tRNA, rRNA, small nuclear and small nucleolar RNA (9). The C-terminal domain of senataxin also shares extensive homology with RENT1/Upf1 and IGHMB2, two members of the superfamily I of helicases (7). RENT1/Upf1 has been shown to be involved in nonsense-mediated RNA decay (10) and possesses both DNA and RNA helicase activity with a 5′ → 3′ polarity of unwinding (11). IGHMB2, dysfunctional in spinal muscular atrophy (SMA) with respiratory distress, has been described as a DNA binding protein with transcriptional transactivating properties (12,13). Sen1p interacts with Rpo21p (a subunit of RNA polymerase II) and Rnt1p (an endoribonuclease required for RNA maturation), suggesting a role for Sen1p in RNA processing, transcription and transcription-coupled DNA repair (14). A single amino acid substitution in the helicase domain of Sen1p, sen1-E1597K (a heat sensitive mutant allele of SEN1) that compromised the function of Sen1p, has been shown to alter the genome-wide distribution of RNA polymerase II in non-coding and protein-coding genes, suggesting a role for Sen1p in transcriptional regulation (15).

The role of Sen1p in different facets of RNA metabolism, together with a number of neurological disorders characterized by abnormal RNA transactions, led us to determine whether senataxin, in addition to its role in the response to oxidative stress and the neurodegeneration observed in this syndrome, is involved in transcriptional regulation and pre-mRNA processing. Misregulation of transcription and aberrant pre-mRNA processing in AOA2 may contribute to the oxidative stress and the neurodegeneration observed in this syndrome.

RESULTS

Senataxin interacts with proteins involved in transcription and pre-mRNA processing

In order to gain insight into the cellular function of senataxin, interacting proteins were co-immunoprecipitated from control lymphoblastoid cells (C2ABR and C3ABR) with a senataxin-specific antibody, resolved by SDS–PAGE and identified by Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Fig. 1A). Several protein bands (Fig. 1B, 1–3) were detected that were absent from the antibody only and control serum (Ig). These bands were excised from the gel, digested with trypsin and identified by mass spectrometry. In parallel, GST pull-downs using a series of GSTs that encompass the entire senataxin protein were also employed to identify interacting proteins (Fig. 1C and D). Bands absent from the GST only lane, indicating a specific binding to senataxin were excised, trypsin-digested and subjected to mass spectrometry for identification. In total, over 30 proteins were identified as senataxin-interacting proteins by mass spectrometry, the details for some of which appear in Supplementary Material, Tables S1 and S2. Only bands (1–13) that correspond to proteins that were identified as playing a role in RNA metabolism are shown (Fig. 1D). In this study, we primarily focused on proteins implicated in RNA metabolism (Table 1), given the known role of the senataxin yeast homolog Sen1p in RNA transactions (9,14). These proteins fall into three functional classes: pre-mRNA processing factors that include hnRNP1, C1, M and the spliceosomal protein 155 (SAP155/SF3b1), the RNA binding protein nucleolin and the transcription termination and polyadenylation factors (PABP1 and 2). These interactions were confirmed by co-immunoprecipitation of SAP155 and nucleolin using an anti-senataxin antibody [Ab-1, raised against the C-terminal region of the protein (6)] (Fig. 2A and B). These proteins were not co-immunoprecipitated from AOA2 cells lacking senataxin protein, suggesting a specific interaction. To further confirm the interaction between senataxin and nucleolin, nucleolin GST pull-downs were carried out using five overlapping nucleolin-GST fragments generated against full-length nucleolin (16) (Fig. 2C). Senataxin was found to bind specifically to the N-terminal region (N1 and N2) of nucleolin (Fig. 2C). The interaction between senataxin and PABP was also confirmed by co-immunoprecipitation of PABP using senataxin Ab-1 (Fig. 2D). PABP was not co-immunoprecipitated from AOA2 cells. The interaction between senataxin and RNA binding proteins prompted us to investigate whether senataxin, similar to Sen1p, would interact with RNA polymerase II. As shown in Fig. 2E, this is indeed the case and neither senataxin nor RNA polymerase II was detected in anti-senataxin immunoprecipitates from AOA2 cells. Furthermore, senataxin co-immunoprecipitated with RNA polymerase II (Fig. 2F). This interaction was not observed in AOA2 cells lacking senataxin, confirming a specific interaction between the two proteins. Having demonstrated an interaction between senataxin and several RNA processing factors, we determined whether this protein might also bind another key component necessary for RNA processing, survival motor neuron (SMN) protein. SMN protein, defective in the common human neurodegenerative disorder, SMA (17), is essential for the biogenesis of small nuclear RNA (snRNA)—ribonucleoproteins, the major components of the pre-mRNA splicing machinery (18). As shown in Fig. 2G, senataxin interacts with SMN providing additional support for a role for senataxin in RNA processing. Confirmation of direct protein–protein interactions between senataxin and these proteins was demonstrated by repeating the co-immunoprecipitations in the presence of Benzonase™, a potent nuclease that degrades both DNA and RNA (Supplementary Material, Fig. S1A–D). In addition, senataxin GST pull-downs confirmed the interaction between senataxin and nucleolin, SAP155, PABP, RNA polymerase II and SMN and mapped the interaction domain(s) on senataxin (Fig. 2H). Interactions with these proteins occurred at specific regions across the entire senataxin protein suggesting that other functional domains may reside within senataxin in addition to its conserved DNA/RNA helicase domain (7).

Senataxin is required for the transcriptional regulation of SOD1, IMPDH2, CYC and RPL36

In addition to being a helicase, Sen1p is also an RNA polymerase II termination factor required for transcriptional regulation of the yeast genes SOD1, NRD1, HRP1, CPR1, RPL36 and IMD2 (15,19). Since senataxin is the human homolog of
Sen1p, and since we showed that this protein interacted with RNA polymerase II (Fig. 2E and F), RNA polymerase II chromatin immunoprecipitation (ChIP) assays were carried out on control and AOA2 cells to determine whether senataxin, similar to Sen1p, had a role in transcriptional regulation.

$\text{Cu/Zn superoxide dismutase (SOD1)}$, IMP dehydrogenase 2 ($\text{IMPDH2}$), cytochrome c ($\text{CYC}$), cyclophilin A ($\text{CypA}$) and ribosomal protein L36 ($\text{RPL36}$) genes were chosen as they are the human homologs of the yeast genes regulated by Sen1p (15). Primer sets for these genes were chosen within the coding region of each gene to generate PCR products of $\sim$200–500 bp with both genomic DNA and mRNA templates.

Figure 1. Identification of senataxin-interacting proteins by mass spectrometry. (A) Flow diagram showing the methodology used for identifying and confirming senataxin-interacting proteins. (B) Following SDS–PAGE on a 10% gel, senataxin IPs were silver stained and bands 1–3 were excised, trypsin-digested and analyzed by MS. Excised bands are indicated by the arrows. (C) Regions on senataxin for which nine overlapping GST fragments were generated. The helicase domain of senataxin is shown. (D) Following separation on a 12% SDS–PAGE, senataxin GST pull-downs were stained and multiple bands were excised, trypsin-digested and analyzed by mass spectrometry. Bands (1–13) correspond to proteins involved in RNA metabolism that were identified by mass spectrometry. C2ABR and C3ABR, control lymphoblastoid cells; Ig, pre-immune sheep sera (negative control); Ab-1, senataxin Ab-1.
Immunoblotting (Fig. 2E) and immunoprecipitation (Fig. 2F) revealed that there were similar levels of RNA polymerase II in control and AOA2 cells. Following immunoprecipitation, PCR was used to amplify and quantitate the extent of binding of RNA polymerase to SOD1, IMPDH2, CYC, CypA and RPL36 loci. CypA was used to normalize the binding levels of the other genes given that similar levels in both the INPUT (samples after crosslinking, prior to immunoprecipitation) and OUTPUT were observed in both control and AOA2 cells (Fig. 3A). There was significantly less RNA polymerase II binding to the SOD1 (68%), IMPDH2 (47%), CYC (59%) and RPL36 (70%) genomic regions in AOA2 compared with control cells (100%) (Fig. 3A and C). Differential binding of RNA polymerase II has previously been correlated to reduced transcription efficiency (20). Thus, in order to determine whether this is also the case in AOA2, endogenous mRNA transcript levels of SOD1, IMPDH2, CYC and RPL36 in AOA2 cells were compared with controls. The results in Figure 3B and C demonstrate a decrease in the endogenous mRNA transcript levels of SOD1, IMPDH2, CYC and RPL36 in AOA2 compared with control cells, in agreement with the reduced level of binding of RNA polymerase II to these genes observed in AOA2.

As a complementary approach to confirming the reduced binding of RNA polymerase to these genomic loci in senataxin deficient cells, small RNA interference (RNAi) was also employed. Using three distinct RNAi oligoribonucleotides targeted against the 5’ end of SETX (Fig. 3D, upper panel), senataxin expression was knocked-down by ~90%, whereas control RNAi did not affect senataxin expression levels (Fig. 3D, lower panel). Cells transfected with SETX RNAi mimicked AOA2 cells (Supplementary Material, Fig. S2). As expected, reduced interaction with RNA polymerase II, SAP155 and SMN were observed in cells transfected with SETX (Supplementary Material, Fig. S2A and B). Knockdown of senataxin also caused elevated levels of 8-oxo-dG, a characteristic of AOA2 cells (6) (Supplementary Material, Fig. S2C and D). Under these conditions, the expression of a number of the senataxin-interacting proteins remained unchanged (Fig. 3D, lower panel). Binding of RNA polymerase II to SOD1, CYC, IMPDH2 and CypA loci was subsequently determined in these cells by ChIP analysis. There was significantly less RNA polymerase II binding to SOD1 with SETX 1 (35%), SETX 2 (34%) and SETX 3 (34%), compared with cells treated with control RNAi (Fig. 3E and G).

For CYC, there was 39, 29 and 6% less binding of RNA polymerase II in cells transfected with SETX 1, SETX 2 and SETX 3, respectively, and for IMPDH2, 77, 86 and 64% less binding, respectively (Supplementary Material, Fig. S3A and B). CypA was used to normalize the binding levels. Endogenous mRNA transcript levels of SOD1, IMPDH2 and CYC in senataxin knocked-down cells were also determined. The results in Figure 3F and G and Supplementary Material, Fig. S3 demonstrate a decrease in the endogenous mRNA transcript of SOD1, CYC and IMPDH2 in agreement with those obtained with AOA2 cells.

### Table 1. Senataxin binding proteins

<table>
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<tr>
<th>Protein</th>
<th>Mascot score</th>
<th>No. peptides matched</th>
<th>Identified by MS</th>
<th>Identified by co-IP</th>
<th>Confirmed by GST pull-down</th>
<th>Function</th>
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<td>111</td>
<td>20</td>
<td>+</td>
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<td>hnRNP M</td>
<td>86</td>
<td>10</td>
<td>+</td>
<td></td>
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<td>Transcription and pre-mRNA processing</td>
</tr>
<tr>
<td>hnRNP C</td>
<td>68</td>
<td>6</td>
<td>+</td>
<td>−</td>
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<td>Transcription and pre-mRNA processing</td>
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<td>+</td>
<td>−</td>
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<td>14</td>
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<td>−</td>
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<td>Regulates translation and stability of mRNA</td>
</tr>
<tr>
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<td>Regulates translation and stability of mRNA</td>
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<td>22</td>
<td>+</td>
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Senataxin deficient cells exhibit abnormal transcription termination

Sen1p was identified as an RNA polymerase II termination factor for non-coding RNA genes and more recently was demonstrated to have a role in the transcriptional termination of several short protein-coding genes (15,19). On the basis of these observations together with the results described here, revealing differential binding of RNA polymerase II to several genes in senataxin deficient cells, we determined whether abnormalities might also exist in transcription termination. Termination of transcription was assayed using a reporter construct (CSAG-1) containing a sequence specific region of the chondrosarcoma associated gene 1 (CSAG1) mRNA and its poly(A) signal upstream of the EGFP gene. Read-through was measured by EGFP expression, located downstream of the poly(A) site (Fig. 4A). To investigate read-through in senataxin deficient cells, transfection efficiency of both control (C3ABR) and AOA2 (SETX-2RM) was determined using a GFP expression vector and flow cytometry (FACS) (Supplementary Material, Fig. S4). As shown in Supplementary Material, Figure S4A, a very low transfection efficiency was observed for AOA2 (SETX-2RM) lymphoblastoid cells using electroporation and Amaxa Nucleofection. Transient transfections of AOA2 lymphoblastoid cells with CSAG-1 read-through reporter construct failed to detect expression in these cells using RT–PCR but were readily detected in both C3ABR and HeLa cells (Supplementary Material, Fig. S4B). Hence, we employed the RNAi-based approach to knockdown senataxin expression levels in order to measure read-through in senataxin depleted cells. Read-through was determined in senataxin-depleted cells using RT–PCR with a specific primer pair that amplified transcripts downstream.
of the poly(A) signal in the reporter constructs (F1/R2). A primer set (F1/R1) was employed as an internal standard to amplify CSAG1 upstream of the poly(A) site (Fig. 4A) and β-2M was used as an additional control in RT–PCR. Read-through was detected with all three of the SETX RNAi knockdowns (Fig. 4B, upper panel) and there was no evidence of read-through with the control RNAi. Quantitation of the data showed significant levels (P < 0.05) of read-through for all three SETX RNAi (Fig. 4C). Given the role of the senataxin yeast homolog, Sen1p, in the termination of transcription of non-coding snRNA and snoRNA in yeast (15), we also assayed for read-through of the endogenous non-coding RNAs U3, U8, U13, U1, U6, U4A and HTR, by RT–PCR. In contrast to Sen1p, which is required for RNA polymerase II-dependent transcription termination of both protein-coding and non-coding RNA genes (15), senataxin does not appear to be required for transcriptional termination of non-coding RNA (data not shown).

**Senataxin modulates splicing efficiency**

All facets of transcription including initiation, elongation, RNA processing and chain termination are interconnected and coordinately regulated (21). Since we observed defects in RNA expression and transcription termination when senataxin was depleted or mutated, we determined the potential role of this protein in mRNA splicing. Using a double reporter assay where two in-frame reporters, β-galactosidase (β-gal) and luciferase are separated by an intron that contains termination codons interrupting the reading frame (22,23) (TN24 construct, Fig. 5A), we measured splicing efficiency in senataxin-depleted cells. β-Gal is expressed irrespective of splicing and acts as the internal standard, whereas luciferase expression is only observed after efficient splicing of the intron containing the termination codons. The ratio of luciferase to β-gal signal, therefore, provides an indication of the splicing efficiency. Similar to that observed with the
CSAG-1 read-through construct, TN24 expression was not detectable after transient transfection in AOA2 (SETX-2RM) lymphoblastoid cells compared with HeLa and control (C3ABR) cells, as shown by RT–PCR (Fig. 5B). Thus, splicing assays were also performed in HeLa SETX RNAi knocked-down cells. Depletion of senataxin using all three SETX RNAi reduced the splicing efficiency of the reporter construct by ~50–60% in HeLa cells ($P < 0.05$) compared with control RNAi (Fig. 5C). The splicing efficiency of the reporter construct with all three SETX RNAi sequences was also significantly decreased ($P < 0.05$) in two other cell lines, U-251 (a glioblastoma multiforme cell line of neuronal origin) and A549 (lung cancer cell line), when compared with the control RNAi (Fig. 5D and E).
on alternative splice-site selection, we employed another cell line, U-251. Similar to that with HeLa cells, senataxin expression was also depleted by RNAi in U-251 cells. A significant increase in exon 2 inclusion was observed for the Tra2β1 minigene in U-251 cells after depletion of senataxin with all three SETX RNAi (Fig. 6C).

Splice-site selection was also determined using a second minigene, SRp20. The structure and splicing patterns for this gene appear in Fig. 6D, upper panel. RT–PCR analysis to distinguish the two alternate transcripts revealed a defect in exon 4 inclusion in cells treated with SETX 1 compared with control RNAi (Fig. 6D, lower panel). Again, transient transfection of AOA2 lymphoblastoid cells with the SRp20 minigene failed to detect expression in these cells, but was readily detected in HeLa cells (Supplementary Material, Fig. S5). Quantitation showed a marked reduction in exon 4 inclusion by ~80% (P < 0.05) (Fig. 6E). The splicing pattern of endogenous SRp20 was also determined by RT–PCR. Quantitation showed a 70% reduction of exon 4 inclusion of endogenous SRp20 in SETX RNAi treated cells compared with control (Fig. 6E). We also investigated exon 4 inclusion of the SRp20 minigene in U-251 cells depleted of senataxin. Similar to that with HeLa cells, a significant reduction of exon 4 inclusion was also observed in U-251 cells (P < 0.05) (Fig. 6F).

**DISCUSSION**

We showed here that senataxin, the protein defective in the neurological disorder AOA2 (7), interacts with several proteins involved in transcriptional regulation and pre-mRNA processing. Loss of senataxin by either mutation of SETX in AOA2 cells or depletion by RNAi revealed abnormalities in transcription termination and mRNA processing. This is reminiscent of the role of its yeast homolog Sen1p that has been shown to be involved in the processing of tRNA, rRNA and small nuclear and nucleolar RNAs (9). Sen1p is an RNA polymerase II termination factor required for transcriptional regulation of many yeast genes (15,19). Use of a ChIP–Chip analysis of RNA polymerase distribution, showed that a single amino acid substitution of Sen1p, within its helicase domain, altered the genome-wide distribution of RNA polymerase II in non-coding and protein-coding genes (15). Here we demonstrated that senataxin also interacts with RNA polymerase II and show that there is significantly less RNA polymerase II binding to the SOD1, IMPDH2, CYC and RPL36 genomic loci in both AOA2 and senataxin knocked-down cells compared with controls. This mirrors the differential binding of RNA polymerase II to these loci observed previously for Sen1p mutants (15). The differential binding of RNA polymerase II observed at these loci in senataxin deficient cells was also reflected at the level of mRNA expression of these genes, suggesting that senataxin similar to Sen1p has a role in regulating transcription.

For transcription termination by RNA polymerase II to occur, a functional polyadenylate signal and downstream sequence elements are required (25,26). The poly(A) signal consists of the highly conserved hexanucleotide AAUAAA, located 15–20 nucleotides upstream from the 3′ cleavage
Mutation of the poly(A) site has been shown to result in transcription beyond the transcription termination site (26). Together with Nrd1 and Nab3, Sen1p functions as an RNA polymerase II termination factor (19,28). Although no known human homologs of Nrd1 and Nab3 have been identified, we demonstrated here that senataxin, similar to its yeast homolog, is involved in transcription termination. Unlike Sen1p, which affects transcription termination of both protein-coding and non-coding RNA (15), senataxin loss only affected the termination of coding-RNA and not that of the non-coding RNAs investigated. Thus the neurodegenerative phenotype of AOA2 patients may not be as a consequence of the aberrant synthesis of regulatory non-coding RNAs, as previously suggested (15), but instead, due to the misregulation of transcription of coding-RNA.

Gene expression can be regulated at both the transcriptional and post-transcriptional levels by mRNA processing and splicing (29–32). Alternative splicing is a process which gives rise to functional complexity in the human genome as a result of a single pre-mRNA giving rise to multiple mRNAs and therefore functionally different protein products (33–36). For alternative splicing to occur, in addition to the spliceosome, regulatory factors that change the choice of splice sites by binding to pre-mRNA are also required (33,36). Using a double reporter assay to measure RNA splicing efficiency in cells depleted of senataxin, we observed a dramatic reduction in splicing efficiency in cells with reduced SETX expression levels.
reduction in splicing efficiency, indicating a pivotal role for senataxin in mRNA splicing. This is in agreement with the presence of a DNA/RNA helicase motif in senataxin and its predicted role as a helicase in the unwinding of nucleic acids during transcription and/or splicing (7). A measure of alternative splice-site selection using minigenes demonstrated that senataxin deficient cells had an altered splicing pattern compared with normal cells. Furthermore, senataxin was found to influence alternative splice-site selection of the endogenous counterparts of these minigenes. The reduced efficiency of splicing and alternative splice-site selection in senataxin depleted cells together with the capacity of senataxin to interact with several key proteins involved in RNA processing support an important role for this protein in RNA transactions. However, it is evident that the role of senataxin in transcription and RNA processing is related to efficacy and not to a major defect which would explain why AOA2 cells are viable and capable of normal proliferation. Since the phenotype in AOA2 patients appears to be confined largely to the nervous system, it is possible that the defect is more exacerbated in this cell type or that gene expression, more critical to that tissue, is affected (2). Alternatively, the loss of senataxin might have an indirect effect on RNA processing for example by inducing oxidative stress. There is evidence that AOA2 cells are in a constitutive state of oxidative stress (6), which is supported by results obtained here for cells depleted of senataxin. Oxidative stress-induced DNA damage has the potential to block RNA polymerase II-dependent transcription and disrupts pre-mRNA processing, leading to neuronal cell death (37,38). Furthermore, aberrant pre-mRNA splicing induced by oxidative stress may lead to increased production of toxic transcripts by altering the ratio

Figure 6. Loss of senataxin affects alternative splice-site selection. (A) Structure of the Tra2β1 minigene (24). Dotted lines indicate alternative splicing patterns. RT–PCR run on an agarose gel with the Tra2β1 minigene with control and SETX 1 on HeLa cells is also shown. (B) Quantitation of the percentage of exon 2 inclusion with the reporter and endogenous Tra2β1 in control and senataxin-depleted cells. (C) Quantitation of percentage of exon 2 inclusion with Tra2β1 minigene in U-251 cells transfected with control/SETX RNAi. (D) Structure of SRp20 minigene (24). Dotted lines indicate alternative splicing patterns. Lower panel: RT–PCR run on an agarose gel with the SRp20 minigene with control and SETX 1 on HeLa cells. (E) Quantitation of percentage of exon 4 inclusion with reporter and endogenous SRp20. (F) Quantitation of percentage of exon 4 inclusion with SRp20 minigene in U-251 cells transfected with control/SETX RNAi. Error bars indicate SD for three independent experiments.
of splice variants (38). Genes expressed in the nervous system are more frequently spliced than genes expressed elsewhere (39,40). Since alternative splicing is required for neuronal development (41), it is not surprising that defects in alternative splicing as those observed here in senataxin deficient cells result in human neurodegenerative disorders.

The potential role described here for senataxin in RNA metabolism appears to be very different to our previous report on a role for senataxin in the response to oxidative stress (6). However, similar observations have been made for its yeast homolog Sen1p, which is required for RNA processing but also binds Rad2p, implicating it in nucleotide excision repair (14). A resolution of these apparently disparate roles for senataxin may be provided in its interaction with RNA polymerase II. In this report, we revealed less binding of RNA polymerase II to a number of genes including SOD1. We also showed that this was reflected in reduced expression of these genes. SOD1 codes for the protein SOD1, a key factor involved in the resistance to oxidative stress, that converts superoxide radicals into less reactive oxygen and H2O2, the latter being subsequently converted into water and oxygen by glutathione peroxidase and catalase (42). Thus, abnormalities in transcription of SOD1 in AOA2 cells may result in increased levels of H2O2, which might in turn contribute to the hypersensitivity to H2O2 and the constitutive oxidative DNA damage observed in these cells (6).

Another intriguing observation that links RNA polymerase II to the DNA damage response is the report that the RNA polymerase II cofactor, PC4, is a suppressor of oxidative mutagenesis in both bacteria and yeast (43). A more direct involvement for PC4 in DNA repair has been demonstrated recently in mammalian cells (44). A combination of live cell microscopy, microirradiation and fluorescence recovery was employed to demonstrate an accumulation of PC4 at DNA damaged sites, suggesting that PC4 plays a role in the early response to DNA damage by recognizing single-strand DNA to initiate subsequent steps in DNA repair (44). It is tempting to suggest that senataxin and PC4, both of which bind RNA polymerase II, have roles in transcriptional regulation and protect against oxidative damage from H2O2 and may have a common function in the response to oxidative damage in DNA. PC4 has been shown to bind single-stranded DNA at sites of damage (43) and senataxin, being a putative helicase (7), may play a role in unwinding structures at the site of DNA damage.

In summary, we have demonstrated that senataxin interacts with a series of proteins involved in different aspects of transcription and mRNA processing. It seems likely that these interactions are of functional significance, since AOA2 cells with mutations in the SETX gene or cells depleted of senataxin show a series of abnormalities in transcription termination and splicing. Thus, senataxin shows similarity in function to its yeast homolog that has been shown to participate in processing of different RNA species (9). Although senataxin possesses a putative DNA/RNA helicase domain, no activity has yet been ascribed to the protein. The potential role of senataxin as a RNA helicase together with its capacity to bind to RNA polymerase II makes it a good candidate for playing a broad role in RNA processing and protection against oxidative stress (Fig. 7). In this model, senataxin would play multiple roles in regulating gene expression at different levels to protect neuronal tissue. This type of regulation also impacts on protecting against or carrying out repair of oxidative damage. Defects in this regulation could account for the neurodegenerative phenotype in AOA2 patients. Elucidation of the molecular mechanisms leading to neurodegeneration in AOA2 awaits the generation of an animal model for this syndrome.

MATERIALS AND METHODS

Immunoprecipitation

Senataxin immunoprecipitations (IPs) were carried out as previously described (6). Following IP, samples were either untreated, DNase treated (Promega), RNase A treated (Roche) or nuclease treated (Benzonase™, Novagen) according to the manufacturer’s instructions. Bound proteins were separated by SDS–PAGE and either silver stained as described (45) or immunoblotted with relevant antibodies.

Mass spectrometry

Excised gel bands were destained (46) and trypsin digested (45) as described. Mass spectrometry was carried out as previously described (47,48). Briefly, MALDI-TOF mass spectrometry was carried out using a Microflex MALDI-TOF-PSD (Bruker Daltonics), operated in a positive ion reflectron mode. MS data were acquired using 350 shots of a nitrogen laser at 355 nm with a 20 Hz repetition rate and varying intensity. MS data were calibrated via close external calibration using the peptide standards (New England Biolabs), containing

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**Figure 7.** Neurodegeneration and the novel roles of senataxin. Lack of senataxin in both AOA2 and senataxin-depleted cells influences the binding of RNA polymerase II to specific gene loci and subsequently affects mRNA transcript levels, suggesting a regulatory role for senataxin during transcription initiation and/or elongation. Furthermore, a reduction in splicing and alternative splicing efficiency and increased transcriptional read-through was also observed. It is not surprising that multiple defects are observed in several aspects of RNA transactions given that both transcription and mRNA processing are intimately coupled and co-regulated (21). Thus, sensitivity of AOA2 cells to H2O2, elevated levels of oxidative DNA damage in senataxin deficient cells and the protective function of senataxin in the repair of H2O2-induced DNA double strand breaks, combined with the defects in transcription and mRNA processing observed in senataxin deficient cells may all act as compounding factors for the neurodegeneration observed in AOA2.
Angiotensin I (MH + 1296.69), Neurotensin (MH + 1672.92), ACTH (1–17 clip, MH + 2093.09), ACTH (18–39 clip, MH + 2465.20), ACTH (7–38 clip, MH + 3657.93). Protein identification was obtained using Mascot search engine (Version 1.9) and the NCBI database. Mass tolerance was set at 150 p.p.m. The search took into account carbomidomethylated cysteine and oxidized methionine and no other post-translational modifications were included.

Senataxin fragments for GST pull-downs

Nine overlapping fragments (S₁–S₅) covering full-length SETX were PCR amplified, cloned into a GST bacterial expression vector and purified (Supplementary Material and Methods). For GST pull-downs, GST and GST-S₁ to GST-S₅ (1 µg) were incubated overnight at 4°C with 1 mg Benzonase-treated control LCL (C3ABR) whole cell extract (6). Pull-downs were washed three times with lysis buffer (6) and resuspended in 20 µl sample loading buffer. Proteins were separated by SDS–PAGE and either silver stained or immunoblotted with senataxin Ab-1 (6), nucleolin (Medical and Biological Laboratories, #M019-3), SAP155 (Medical and Biological Laboratories, #D221-3), PABP (Santa Cruz Biotechnology Inc., #sc-28834), SMN #M019-3), and GST-N1 to GST-N₅ (1 µg) to GST-N₅ (1 µg) were incubated with 1 mg Benzonase-treated C3ABR whole cell extract (6). Pull-downs were washed three times with lysis buffer (6) and resuspended in 20 µl sample loading buffer. Proteins were separated by SDS–PAGE and immunoblotted with senataxin Ab-1 (6), nucleolin (Medical and Biological Laboratories, #M019-3), SAP155 (Medical and Biological Laboratories, #D221-3), PABP (Santa Cruz Biotechnology Inc., #sc-28834), RNA polymerase II CTD (Abcam, #ab5408), RNA polymerase II N-ter (Santa Cruz Biotechnology Inc., #sc-899) and SMN (BD Biosciences, #610646) antibodies. GST loading was determined by Coomassie staining.

Nucleolin GST pull-down assays

GSTs for nucleolin were prepared as previously described (16). Briefly, five overlapping fragments (N₁–N₅) covering full-length nucleolin were cloned into a GST bacterial expression vector and purified. For GST pull-downs, GST and GST-N₁ to GST-N₅ (1 µg) were incubated with 1 mg Benzonase-treated control LCL (C3ABR) whole cell extract (6). Pull-downs were washed three times with lysis buffer (6) and resuspended in 20 µl sample loading buffer. Proteins were separated by SDS–PAGE and immunoblotted with senataxin Ab-1. GST loading was determined by Coomassie staining.

Transfection with stealth RNAi

HeLa, U-251 and A549 cells were transfected with Stealth™RNAi Negative Control LO GC (#12935200) (Invitrogen) and Stealth™RNAi specific for the knock-down of SETX (Invitrogen): SETX 1 (CCACUAAACUCUGUACACUUGCU), SETX 2 (CGACUGGUGAAAGCCGUGUCAAAU) and SETX 3 (CCCAUUGCUCCUUACAGGGUGUGUA). Approximately 2.5–10×10⁵ cells were transiently transfected in a six-well plate with control/SETX RNAi oligoribonucleotide (5 µl of a 20 µM stock) using Lipofectamine™2000 (Invitrogen) as described by the manufacturer. Knockdown efficiency of SETX with RNAi was determined 48 h post-transfection by immunoblotting of whole cell extracts using senataxin Ab-1 (6). Rad50 protein levels (Upstate, #05-525) were used as the loading control. Relative expression of RNA polymerase II, SAP155, nucleolin and SMN were also determined in these extracts. Further experiments with SETX RNAi transfected cells were only performed if more than 80% knock-down of senataxin was observed.

RNA polymerase II ChIP

For each precipitation, 0.1 volume of crosslinking mix (11% formaldehyde, 100 mM NaCl, 0.5 mM EGTA, 50 mM HEPES pH 8.0, supplemented with protease and phosphatase inhibitors) was added to ~2–5×10⁷ control (C3ABR), AOA2 (SETX-2RM) LCLs in culture and cells transfected with control and SETX RNAi. Crosslinking was stopped after 15 min with 0.1 volume of 1.25 M glycine, washed twice with PBS and lysed with ChIP lysis buffer (1% SDS, 10 mM EDTA pH 8.0, 50 mM Tris–HCl pH 8.0, supplemented with protease and phosphatase inhibitors) for 30 min at 4°C. DNA was sheared by sonication to an average size of 1000 bp and insoluble components removed by centrifugation. Two milligram of protein was diluted to 600 ml with dilution buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA pH 8.0, 20 mM Tris–HCl pH 8.0, supplemented with protease and phosphatase inhibitors) and pre-cleared with protein A-Sepharose/G-Sepharose beads (Amersham Biosciences) and 4 µg of salmon sperm DNA. RNA polymerase II was immunoprecipitated using 2 µg of RNA polymerase II CTD antibody overnight at 4°C. Immunocomplexes were recovered with protein A-Sepharose/G-Sepharose beads. Following extensive washing with wash buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA pH 8.0, 20 mM Tris–HCl pH 8.0), 500 mM salt buffer (same as wash buffer, but with 500 mM NaCl) and 1 mM salt buffer (same as wash buffer, but 1 mM NaCl), bound DNA fragments were eluted in elution buffer (1% SDS, 100 mM NaHCO₃, purified and analyzed by PCR with primer pairs SOD1F/SOD1R, IMPDH2F/IMPDH2R, CYCF/CYCR, RPL36F/RPL36R and CypAF/CypAR. Sequences of primers used for PCR are given in Supplementary Material and Methods). Twenty-four hour post-transfection of HeLa cells with control/SETX RNAi, cells were transfected with CSAG-1 using Lipofectamine 2000 (Invitrogen). Total RNA was extracted and cDNA synthesized 24 h post-transfection with CSAG-1. RT–PCR was carried with both EGFP and β-2M (housekeeping gene) using the primer pairs EGPF/EGPFR and β-2MF/β-2M (Supplementary Material and Methods). PCR products were quantitated using Image Quant 5.1 Molecular Dynamics Image or Quantity One 4.6.2 (Basic) Biorad software. Results for each experiment were calculated relative to the control sample and each experiment was carried out three independent times. Error bars show the standard deviation (SD). Significance was determined using a Student’s two-tailed t-test.

Transcription read-through with CSAG-1 reporter

A read-through reporter construct (CSAG-1) was generated by PCR amplification and cloning of the 3′-UTR of the CSAG1 gene into a mammalian expression vector (Supplementary Material and Methods). Twenty-four hour post-transfection of HeLa cells with control/SETX RNAi, cells were transfected with CSAG-1 using Lipofectamine 2000 (Invitrogen). Total RNA was extracted and cDNA synthesized 24 h post-transfection with CSAG-1. RT–PCR was carried with both EGFP and β-2M (housekeeping gene) using the primer pairs EGPF/EGPFR and β-2MF/β-2M (Supplementary Material and Methods), respectively. Following amplification, DNA was resolved on TAE agarose gels. Read-through was a measure of EGFP expression located downstream of the poly(A) site. The mRNA expression values
were quantitated as previously described. Read-through with EGFP was normalized to the expression of β-2M to obtain relative read-through.

**Luciferase double-reporter in vivo splicing assay**

The double-reporter plasmid (TN24) has been previously described (21,22). Twenty-four hour post-transfection of HeLa, U-251 and A549 cells with control/SETX RNAi, cells were transfected with TN24. Forty-eight hour post-transfection, cells were harvested and β-gal and firefly luciferase were measured using the β-gal Enzyme Assay System with Reporter Lysis Buffer (Promega) and the Luciferase Assay System with Reporter Buffer (Promega), respectively, using the manufacturer’s instructions. To determine transfection efficiency, total RNA was extracted and cDNA synthesized 48 h post-transfection with TN24. RT–PCR was carried with both TN24 and β-2M (housekeeping gene) using the primer pairs GalF 3301/LucR 3700 and β-2MF/β-2MR, respectively (Supplementary Material and Materials and Methods).

**Alternative splice-site selection with Tra2β1 and SRp20 minigenes**

HeLa and U-251 cells were transfected with control/SETX RNAi. Twenty-four hour post-transfection with control/SETX RNAi, cells were transfected with either the Tra2β1 or SRp20 minigenes (23,49) (kindly provided by Professor Stefan Stamm, Max-Planck Institute of Neurobiology, Germany). Total RNA was extracted and cDNA synthesized 24 h post-transfection with Tra2β1 and SRp20. RT–PCR was carried out with the primer pairs MGTraBam/MGTraRXho and T7/X16R (Supplementary Material and Materials and Methods), for the amplification of Tra2β1 and SRp20 minigenes, respectively. Alternative splice-site selection was measured as previously described (23,49). Alternative splice-site selection of endogenous Tra2β1 and SRp20 were measured in control and SETX RNAi cells using the primer pairs Traex1f/Tra2ex4r and SRp20f/SRp206r, respectively (Supplementary Material and Materials and Methods).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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