A SUMO-dependent interaction between Senataxin and the exosome, disrupted in the neurodegenerative disease AOA2, targets the exosome to sites of transcription-induced DNA damage

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Senataxin (SETX) is an RNA/DNA helicase implicated in transcription termination and the DNA damage response and is mutated in two distinct neurological disorders: AOA2 (ataxia oculomotor apraxia 2) and ALS4 (amyotrophic lateral sclerosis 4). Here we provide evidence that Rrp45, a subunit of the exosome, associates with SETX in a manner dependent on SETX sumoylation. We show that the interaction and SETX sumoylation are disrupted by SETX mutations associated with AOA2 but not ALS4. Furthermore, Rrp45 colocalizes with SETX in distinct foci upon induction of transcription-related DNA damage. Our results thus provide evidence for a SUMO-dependent interaction between SETX and the exosome, disrupted in AOA2, that targets the exosome to sites of DNA damage.

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Senataxin (SETX) is the human homolog of the yeast superfamily I RNA/DNA helicase Sen1 [Kim et al. 1999]. Sen1 is a component of the Nrd1 complex, which is involved in RNA polymerase II (RNAP II) transcription termination and processing of many noncoding RNAs as well as termination on some protein-coding genes [Ursic et al. 1997; Kim et al. 2006; Steinmetz et al. 2006; for review, see Richard and Manley 2009]. Interest in SETX increased when it was found that mutations in SETX can lead to two distinct neurological disorders. Moreira et al. (2004) identified mutations, all recessive, in patients with an autosomal ataxia, AOA2 [ataxia oculomotor apraxia 2], while Chen et al. (2004) showed that distinct mutations in SETX—in this case, all dominant—were linked to a juvenile form of ALS (amyotrophic lateral sclerosis or Lou Gehrig’s disease), ALS4.

As a putative RNA/DNA helicase and Sen1 homolog, SETX has been suspected to play an important role in termination/RNA processing. This is consistent with its role in neurological disorders, which have also increasingly been found to involve defects in RNA metabolism [Strong 2010]. SETX has been shown to function in RNAP II transcription termination by resolving R-loop formation at G-rich pause sites located downstream from some polyadenylation signals, thereby allowing degradation of the downstream cleaved RNA by the 5′-to-3′ exoribonuclease Xrn2 [Skourtí-Stathákí et al. 2011]. Sen1 was also shown to function more generally in R-loop resolution during transcription, potentially helping to prevent genomic instability [Mischo et al. 2011]. Indeed, Sen1 is located at replication forks and displaces R loops to allow fork progression across RNAP II transcription units [Alzu et al. 2012]. Likewise, a recent study suggests that SETX also resolves R-loop structures formed at sites of collision between the transcription and replication machineries, in conjunction with DNA repair factors [Yuce and West 2013]. Consistent with this, disruption of SETX in mice revealed an accumulation of R loops and double-strand breaks [DSBs] in germ cells [Becherel et al. 2013]. It is also known that SETX plays a role in the DNA damage response after oxidative stress [Suraweera et al. 2007].

Even though recent work has provided insight into the function of SETX and highlighted the importance of the protein in neurodegenerative disease, how disease mutations affect SETX function is unknown. To date, >80 mutations linked to AOA2 have been described, scattered throughout the SETX ORF. These mutations include ~40 missense mutations clustered within the N terminus and helicase domains of SETX as well as many nonsense mutations, indicating that SETX loss of function is likely responsible for AOA2 [Moreira et al. 2004]. Eight SETX missense mutations associated with ALS4 have been identified that appear to be dominant, gain of function [Chen et al. 2004; Arning et al. 2013]. Here we describe an interaction between SETX and Rrp45, a component of the exosome complex known to function in RNA turnover and quality control. Strikingly, we show that this interaction is dependent on modification of SETX by sumoylation and that both sumoylation and the interaction are disrupted by AOA2, but not ALS4, mutations. Finally, we show that SETX and Rrp45 colocalize in nuclear foci following the induction of transcription-related DNA damage, suggesting a role for the exosome in the response to DNA damage and providing insight into the SETX function relevant to AOA2 disease.

Results and Discussion

To begin to investigate how disease mutations affect SETX function, we set out to identify SETX-interacting proteins using a yeast two-hybrid screen. Since SETX is mutated in two neurodegenerative diseases, we used a human brain cDNA library as prey and the N-terminal region of SETX [Nter-SETX: 1–665 amino acids] as bait. We chose this region because the corresponding region of Sen1 constitutes a protein–protein interaction domain.

[Keywords: exosome; Senataxin; sumoylation]

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(Ursic et al. 2004) and because a number of AOA2 and two ALS4 mutations lie in this region [Chen et al. 2006]. Among the cDNAs isolated (see also below) were several encoding the C terminus of Rrp45, a core component of the exosome [Housley et al. 2006]. One of these, Rrp45-Cter (206–439 amino acids) was analyzed further and shown to interact with Nter-SETX on selective medium lacking histidine and containing a high concentration (50 mM) of the histidine-competitive inhibitor 3-amino-1,2,4-triazole (3-AT), indicating a stringent interaction between the two proteins (Fig. 1A).

Given our interest in identifying SETX-interacting proteins relevant to AOA2 or ALS4, we next asked whether disease-related mutations affect the interaction, again using the yeast two-hybrid assay. Specifically, we tested the effects of three different N-terminal AOA2 missense mutations [E65K [EK] [Duquette et al. 2005], W305C [WC], and P413L [PL] [Moreira et al. 2004]] and two N-terminal ALS4 mutations [T3I [TI] and L389S [LS] [Chen et al. 2004]] on the interaction (Fig. 1B,C). Strikingly, the AOA2 mutant EK lost interaction with Rrp45-Cter on selective medium lacking histidine, and all three AOA2 mutant proteins lost interaction on medium supplemented with 3-AT. In contrast, the TI and LS ALS4 mutations did not affect the interaction (Fig. 1C). Western blot showed that expression levels of all of the SETX derivatives were similar (Supplemental Fig. 1A).

We next wished to gain more insight into the SETX–Rrp45 interaction. Efforts to show a direct interaction with purified recombinant proteins were unsuccessful [data not shown]. One explanation for this was that post-translational modifications might be required for interaction. In fact, our yeast two-hybrid screen also identified two components of the sumoylation machinery as Nter-SETX-interacting proteins: the SUMO-conjugating enzyme Ubc9 and the E3 SUMO ligase PIAS1 [see below; data not shown]. Additionally, Rrp45 was previously shown to interact with SUMO1 in a yeast two-hybrid screen and to contain a SIM [SUMO-interacting motif] in its unstructured C-terminal region [Minty et al. 2000]. We therefore set out to determine whether the interaction with SETX is SUMO-dependent. We first examined the ability of additional Rrp45 derivatives to interact with Nter-SETX in the yeast two-hybrid assay. These included full-length Rrp45 (FL), a shorter C-terminal derivative [residues 257–439, del1], and versions of these two deleted of the SIM [residues 388–403] (Fig. 1D). While both FL and del1 Rrp45 interacted with Nter-SETX on selective medium, in both cases, deletion of the SIM disrupted the interaction (Fig. 1E). Western blot confirmed comparable levels of protein expression [Supplemental Fig. 1B].

To confirm and characterize further the interaction between Rrp45 SIM and Nter-SETX, we tested several mutations of the SIM in the del1 mutant in the yeast two-hybrid assay. The Rrp45 SIM consists of a doublet of serines separated by one amino acid and surrounded by four hydrophobic residues on the N-terminal side [PIIL] and four acidic residues on the C-terminal side [EEEE] (Fig. 1D; Minty et al. 2000). We tested mutants [protein expression levels shown in Supplemental Fig. 1C] that include single-point mutations that had been previously to inhibit interaction with SUMO1 [S392A, S394A, E395A, and E397A] [Minty et al. 2000] and deletions or substitutions of two or four amino-acids [388–391-PIIL → EECP, Δ388–391, Δ390–391, Δ395–398-EEEE → AAAA, Δ395–398, and Δ395–396] within the hydrophobic and acidic residues of the SIM (Fig. 1F). While none of the single-point mutations important for SUMO1 binding affected the interaction with SETX, all of the other mutations [with one exception: Δ395–396] disrupted the interaction. These data strongly suggest that Rrp45 interacts with sumoylated Nter-SETX via its SIM in yeast cells.

The above model requires by definition that Nter-SETX is sumoylated in yeast. To test this, we performed immunoprecipitation-Western analysis from cells expressing either Gal4DBD-Nter-SETX or the Gal4DBD alone by immunoprecipitating with an anti-Gal4DBD antibody (Ab) and probing with an anti-SUMO Ab [anti-Smt3]. The results [Fig. 2A] revealed that Gal4DBD-Nter-SETX, but not Gal4DBD alone, was extensively sumoylated.
Bead purification under Nter-SETX is sumoylated in yeast and in HeLa cells by DELEDV VD 2229

remove any noncovalently hybrid screen. To extend these data, we tested the sumoylation (Myc-Ubc9) coimmunoprecipitated with Nter-SETX (Fig. 3A, WT + Ubc9 significantly increased sumoylation of Nter-SETX (Fig. 3A, lane). Indeed, overexpressed Ubc9 [Myc-Ubc9] communoprecipitated with Nter-SETX [Fig. 3A, IB: Ubc9], consistent with results from the yeast two-hybrid screen. To extend these data, we tested the sumoylation status of the other two AOA2 mutants (EK and WC) [Fig. 3B] and the TI ALS4 mutant [Fig. 3C]. Strikingly, the EK and WC mutations abolished Nter-SETX sumoylation in 6xHis-SUMO3 cells overexpressing Ubc9, while the TI mutant displayed a sumoylation pattern similar to Nter-SETX wild type. These data indicate that AOA2, but not ALS4, mutations negatively impact SETX sumoylation status in human cells and are consistent with the effects of these mutations on the SETX-Rrp45 interaction in yeast.

To confirm the above results, we subjected extracts from the 6xHis-SUMO3 cells cotransfected with Nter-SETX and Myc-Ubc9 to Ni²⁺ bead purification under denaturing conditions to remove any noncovalently associated proteins and analyzed the eluate by Flag Western. The results (Fig. 3D) indicate that Nter-SETX wild type and the TI ALS4 mutant were indeed extensively sumoylated, while the EK AOA2 mutant was not. We also examined whether the Nter-SETX/Ubc9 interaction in yeast (described above) was disrupted by any of the AOA2 mutations. To this end, we tested the interaction between Ubc9 and Nter-SETX wild type, the three AOA2 mutants, and the two ALS4 mutants by yeast two-hybrid [Fig. 3E; Supplemental Fig. 1D for protein expression levels]. Strikingly, the interaction between the EK mutant and Ubc9 was completely disrupted on selective medium with or without 3-AT, while WC and PL as well as the ALS4 mutations did not disrupt the interaction. The EK mutation therefore likely prevents sumoylation by interfering with Ubc9 binding, while the WC and PL mutations block sumoylation by another mechanism.

An important question is whether SETX and the exosome indeed interact in human cells and, if so, whether this interaction is SUMO-dependent. To address this, we performed coimmunoprecipitation experiments with HeLa nuclear extracts using anti-SETX or anti-Rrp45 Abs [Fig. 4A]. As anticipated from the above experiments, each Ab appeared to coimmunoprecipitate the other protein. Strikingly, though, the communoprecipitated proteins were detected primarily as lower-mobility isoforms. Thus, the SETX Ab detected predominantly a diffuse, higher-molecular-weight species in the anti-Rrp45 immunoprecipitation than was observed in the SETX immunoprecipitation [Fig. 4A, cf. Rrp45 IP and SETX IP lanes]. This is consistent with the communoprecipitated SETX consisting of a small fraction of the total SETX protein that was modified in some way and specifically is consistent with the idea that sumoylated SETX interacts preferentially with Rrp45. To address this possibility directly, we reprobed the blots with an anti-SUMO2 Ab, which indicated that the coimmunoprecipitating proteins were indeed sumoylated [Fig. 4A, right panel]. Our results also suggest that the fraction of Rrp45 interacting with SETX was also sumoylated [Fig. 4A, SETX IP and Rrp45 IP lanes]. Although we did not pursue this further, Rrp45 is indeed known to be sumoylated [Minty et al. 2000; Golebiowski et al. 2009]. Supporting the physiological significance of this interaction, siRNA-mediated knockdown of Rrp45 or the exosome-associated exonuclease Rrp6 in human U87 cells resulted in significant codelivery of SETX [Fig. 4B]. This decrease in SETX was not due to changes in mRNA levels [Supplemental Fig. 2A] and could be largely rescued by the proteasome inhibitor MG132 [Supplemental Fig. 2B].

Figure 2. Nter-SETX is sumoylated in yeast and in HeLa cells by SUMO2/3. (A) Gal4DBD immunoprecipitation from extracts of H7c cells expressing HA-Rp45-Cter and Gal4DBD [lane a] or Gal4DBD-Nter-SETX wild type [lane b]. Nter-SETX expressed in yeast was detected by Western blot using an anti-Gal4DBD, and sumoylated Nter-SETX was detected using an anti-Smt3 antibody [Ab]. (B) Flag immunoprecipitation of Flag-tagged Nter-SETX expressed in HeLa cells. Nter-SETX isoforms were detected by Western blot using anti-Flag M2 Ab. (C) Flag immunoprecipitation of Flag-tagged Nter-SETX in HeLa cells stably expressing 6xHis-SUMO1 [lane 1], 6xHis-SUMO2 [lane 2], and 6xHis-SUMO3 [lane 3]. Nter-SETX isoforms were detected by Western blot using an M2-Flag Ab, and sumoylated Nter-SETX was detected with an anti-His Ab. [IB] Immunoblot; [I] input; [IP] immunoprecipitate.
indicating that decreased levels of the exosome result in enhanced proteasomal degradation of SETX.

Finally, we wished to obtain insight into the function of the SETX–exosome interaction. One possibility is suggested by two recent studies in humans and yeast revealing that SETX/Sen1 functions to resolve R loops when transcription and replication machineries collide (Alzu et al. 2012; Yuce and West 2013), which can otherwise result in DNA damage and genomic instability (Gan et al. 2011; Becherel et al. 2007). SETX has been implicated in this process by revealing that SETX/Sen1 functions to resolve R loops when the transcriptional machinery and lead to DNA damage which can result from collision of replication forks and transcription and replication machineries collide (Alzu et al. 2012; Yuce and West 2013). Indeed, evidence that AOA2 cells are defective in DNA repair underlie many neurological disorders (Krumova and Weishaupt 2013), to the best of our knowledge, this modification has not been shown previously to be affected directly by disease mutations, as we established for AOA2.

An important question is how the SETX–exosome interaction is relevant to AOA2 disease. Our data point to a role in the DNA damage response. It is well known that defects in DNA repair underlie many neurological diseases, including ataxias (Rass et al. 2007; McKinnon 2009). Indeed, evidence that AOA2 cells are defective in DNA repair, specifically of DSBs, has been presented (Suraweera et al. 2007). SETX has been implicated in this process by its ability to resolve cotranscriptional R-loop structures, which can result from collision of replication forks and the transcriptional machinery and lead to DNA damage and genomic instability (Gan et al. 2011; Becherel et al. 2013; Yuce and West 2013). While the neuronal cells affected in AOA2 are primarily post-mitotic, R loops leading to genomic instability can be created by multiple mechanisms (Aguilera and Garcia-Muse 2012), and SETX can function in resolving them.
Our data suggest that the exosome also functions in the process of DNA damage response. Although a possible role for the exosome in the AOA2 should be informative.

Materials and methods

Yeast two-hybrid screen

We performed a yeast two-hybrid screen using a Matchmaker pretransformed human brain cDNA library in yeast strain Y187 (Clontech), which contains a LacZ reporter gene. The cDNA library was cloned into the pACTII vector (expressing Gal4-DNA-activating domain in fusion with a HA epitope), while the N-terminal domain of SETX (1–665 amino acids) was cloned in the pAS2AΔ vector (in fusion with a Gal4-DNA-binding domain, Gal4DBD) and transformed into yeast strain CG1945, which includes a HIS3 reporter gene. After mating, interactions were tested in a –Leu-Tep-His medium and by filter lift X-Gal assay.

Immunoprecipitations, His purification, and Western blots

For flag immunoprecipitation, 6×His-SUMO3 HeLa cells were transiently transfected with Flag-Nter-SETX wild type (WT) and mutants. Cells were washed twice with cold PBS and lysed with lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Na3HPO4, supplemented with a protease inhibitor cocktail [0.2 mM pepstatin A, 72 μM leupeptin, 26 μM aprotinin], 1 mM NaVO4, 50 mM NEM, and 1 mM PMSF for 20 min at 4°C). The lysates were centrifuged at 14,000 rpm in an Eppendorf centrifuge 5424 for 20 min at 4°C, and the supernatant was used for immunoprecipitation with 2 μg of M2 Flag Ab [Sigma, no. F1804] and protein G sepharose [Roche] for 1 h at 4°C. Immunoprecipitations were washed three times with 1 mL of lysis buffer. Protein samples were separated by 6% SDS-PAGE. The following Abs were used for immunoblotting: anti-Flag M2 [Sigma, no. F1804] and anti-His [Clontech, no. 631212].

Purification of 6×His-SUMO3 under denaturing conditions was performed as described in Tatham et al. (2009). SETX and Rrp45 immunoprecipitations were performed with HeLa nuclear extract. Cells were washed twice with cold PBS and lysed after 15 min of incubation on ice in lysis buffer (10 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate 1 mM EDTA, 1 mM DTT) supplemented with 1 mM PMSF and 200 mM iodoacetamide (Sigma) followed by 10 passages through a 21G1 needle. After centrifugation at 14,000 rpm for 15 min, the supernatant was collected and complemented with 1 vol of hypotonic buffer (10 mM Tris-HCl at pH 7.4, 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT) containing PMSF and iodoacetamide.

Proteins were immunoprecipitated with 2 μg of SETX Ab and 1 μg of Rrp45 for 4 h at 4°C. Immunoprecipitates were washed three times with 1 mL of lysis buffer. Protein samples were separated by 6% SDS-PAGE. Abs used for immunoprecipitations and/or Western blot were SETX [NBPI-94712 and NB100-57542 for immunoprecipitation], Rrp45 [NBPI-71702, Rrp6 [NBPI-32870], and SUMO2 [NBPI-95473] from Novus Biologicals, Ubc9 [H-81: sc-10759] from Santa Cruz Biotechnology, and GAPDH from Sigma. Western blot quantifications were performed using ImageJ.

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Figure 4. SETX and Rrp45 associate and colocalize in stress-induced nuclear foci. (A) Western blots of SETX and Rrp45 immunoprecipitations from HeLa nuclear extract. Blots were probing with anti-SETX and anti-Rrp45 [left panel] or anti-SUMO2 [right panel]. Arrows indicate unmodified SETX and Rrp45. (B) Western blot analysis [left panel] and quantification [right panel] of U87 whole-cell extracts after Rrp6 and Rrp45 knockdown. Proteins levels were normalized to GAPDH (n = 5, mean and SE are shown). [C] Immunofluorescence imaging of SETX and Rrp45 in HeLa cells after aphidicolin [APH] or control [DMSO] treatment for 24 h. Colocalizing foci in a typical cell [Merge] are highlighted with arrows. Bar, 10 μm. [D] Quantification of SETX foci colocalizing with Rrp45 foci after DMSO and APH treatment [n = 50 cells]. Similar results were observed in three independent experiments. [E] Three-dimensional immunofluorescence imaging of SETX and transient expression of Rrp45 in HeLa cells after aphidicolin [APH] or control [DMSO] treatment for 24 h. Colocalizing foci in a typical cell [Merge] are highlighted with arrows. Bar, 10 μm.
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