Senataxin resolves RNA:DNA hybrids forming at DNA double-strand breaks to prevent translocations

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Ataxia with oculomotor apraxia 2 (AOA-2) and amyotrophic lateral sclerosis (ALS4) are neurological disorders caused by mutations in the gene encoding for senataxin (SETX), a putative RNA:DNA helicase involved in transcription and in the maintenance of genome integrity. Here, using ChIP followed by high throughput sequencing (ChIP-seq), we report that senataxin is recruited at DNA double-strand breaks (DSBs) when they occur in transcriptionally active loci. Genome-wide mapping unveiled that RNA:DNA hybrids accumulate on DSB-flanking chromatin but display a narrow, DSB-induced, depletion near DNA ends coinciding with senataxin binding. Although neither required for resection nor for timely repair of DSBs, senataxin was found to promote Rad51 recruitment, to minimize illegitimate rejoining of distant DNA ends and to sustain cell viability following DSB production in active genes. Our data suggest that senataxin functions at DSBs in order to limit translocations and ensure cell viability, providing new insights on AOA2/ALS4 neuropathies.
Mutations in the SETX gene are responsible for the rare neurological disorders ALS4, a dominantly inherited form of amyotrophic lateral sclerosis and ataxia with oculomotor apraxia type 2 (AOA2) which are associated with an early onset neurons degeneration (for review see ref. 1). As a consequence, patients display strong ataxia as well as oculomotor troubles (AOA2) or muscle weakness (ALS4), generally occurring before their 30’s. SETX encodes a helicase, strongly conserved throughout evolution that has been implicated in a large variety of biological processes, from transcription termination, to meiosis completion and maintenance of genomic integrity1. At a molecular level, studies of the yeast senataxin homolog Sen1p established that this helicase displays an unwinding activity toward RNA:DNA hybrids2–4. Multiple genomic studies in both yeast and mammals recently unveiled that RNA:DNA hybrids mostly form as RNA polymerases progress throughout the genomes, by the re-hybridization of the nascent RNA to the template DNA strand, leading to triple-stranded structure called R-loops (reviewed in ref. 5). While R-loops display a strong ability to naturally form at GC-skewed promoters due to an enhanced thermodynamic stability of C-rich DNA: G-rich RNA duplexes5,7, multiple complexes regulate their occurrence throughout the genome, including RNA splicing/processing factors and specific helicases, such as senataxin6,9. R-loops formation and processing play a crucial role in terminating transcription at least in yeast (reviewed in ref. 8). In agreement, mutations in sen1 trigger defective transcription termination and increased transcriptional read-through, especially on short transcribed units such as tDNA, tRNA, and small non-coding or coding genes (for review see ref. 10). Such a function of R-loops processing and senataxin in transcriptional termination was also proposed to be conserved in higher eukaryotes, in a manner that would also involve dsRNA processing factors such as Drosha and DGC8 as well as components of the RNA exosome (for review ref. 11). However, beyond their role in regulating transcription, R-loops also represent a severe threat to genome integrity, proposed to arise beyond their role in regulating transcription, R-loops also represent a severe threat to genome integrity, proposed to arise due to both the susceptibility of the displaced single-stranded DNA to damaging agents as well as their potential to impede replication fork progression (reviewed in refs. 12–14). In agreement, increased damage occurrence and genome instability was observed in cells deficient for R-loops processing factors such as AQR15. Additionally in yeast, sen1 mutations are associated with an increased transcription-associated genome instability,16,17. On the other hand, few studies also suggested that senataxin may play a more direct role at damage sites. Senataxin/Sen1p interacts with repair proteins in yeast and mammals and localizes to the site of damage during replication18,19. Moreover, SETX mutant mice display defective meiosis and Spo11-mediated DSB persistence20. Finally, depletion of senataxin/Sen1p triggers sensitivity to some DNA damaging agents such as H2O2 and UV21–23, a feature also observed in AOA2 patient cell lines22,24,25. However, senataxin-depleted cells are not radiation sensitive26, suggesting that it may not function at sites of DNA double-strand break (DSB), a form of DNA damage largely induced upon irradiation.

Yet, recent studies have suggested that R-loops or/and RNA:DNA hybrids likely form at DSBs. An assay using a duplex specific nuclease detected RNA:DNA hybrids upstream the I-SceI site upon DSB formation26. Additionally a mutant form of RNAse H1, devoid of RNA exonuclease activity accumulates at the site of laser induced damages27. Finally, in Schizosaccharomyces pombe, RNA:DNA hybrids were shown to form during resection, regulating RPA filament formation28. The exact mechanism that leads to such R-loops or/and RNA:DNA hybrids formation remains unclear and may either relate to the transcriptional extinction observed at damaged sites or to de novo RNA PolII loading at DNA ends and subsequent RNA production at the break point, two features that have been previously proposed to occur in many organisms (for review see ref. 12).

In order to gain insights into R-loops biology at DSBs, here we set to assess a potential function of senataxin at sites of DNA DSBs. Using ChiP-seq and DRIP-seq, we uncovered that senataxin is recruited specifically at DSBs induced in transcriptionally active genes, which exhibit RNA:DNA hybrids accumulation following DSB induction. Senataxin distribution around DSBs coincided with a local decrease in R-loops and senataxin depletion triggered increased DSB-induced RNA:DNA hybrids formation, suggesting that senataxin processes DNA damage induced RNA:DNA hybrids. We found that senataxin is not required to sustain resection, nor rapid repair at these DSBs. Yet, it promotes Rad51 foci formation, counteracts translocations and sustains viability following DSB production in active genes, hence identifying a crucial and unanticipated role for senataxin in DSB repair.

Results

Senataxin is recruited at DSBs produced in active genes. To assess senataxin recruitment at sites of DSBs, we used the DSB inducible via AsiSI (DIVA) cell line, which allows to induce clean DSBs throughout the genome29,30. In this cell line, 4 hydroxytamoxifen (4OHT) treatment induces the relocalisation of a stably expressed restriction enzyme (AsiSI) that in turn triggers the production of multiple DSBs at annotated positions across the genome, in a homogeneous manner in the cell population hence allowing the use of chromatin immunoprecipitation (ChiP)30. To obtain a quantitative and genome-wide assessment of senataxin binding and distribution at DSBs, we performed ChiP followed by high throughput sequencing (ChiP-seq) against senataxin before and after DSB induction in DIVA cells (respectively −4OHT and +4OHT). We observed an accumulation of senataxin in a 1–2 kb window surrounding AsiSI sites following 4OHT treatment (see examples in Fig. 1a). In vivo, AsiSI does not produce DSB at each of its 1211 annotated recognition sites, likely due to both DNA methylation and chromatin compaction30. Our recent studies allowed us to characterize AsiSI sites cleavage efficiency, using BLESS (direct in situ breaks labeling, enrichment on streptavidin and next generation sequencing) throughout the genome and to identify a set of 80 DSBs robustly induced following 4OHT treatment31 (Clouaire, T. et al., manuscript submitted). Senataxin binding was significantly enriched following 4OHT on the AsiSI sites population that exhibits cleavage compared to the uncut AsiSI recognition sites (Fig. 1b). Heatmaps revealed that senataxin recruitment did not necessarily correlate with the cleavage efficiency, indicating that genomic and/or epi-genomic features influence senataxin binding at DSBs (Fig. 1c).

Importantly, while AsiSI-induced DSBs mostly lie within promoters or gene bodies of active genes, some do reside in intergenic regions or in genes exhibiting no or very low level of RNA PolII (refs. 29,31 and see examples later). We previously reported that preexisting transcriptional activity strongly influences both DSB repair and signaling events35–38. Hence, we further tested whether DSB-induced senataxin recruitment may vary depending on the transcriptional activity of the broken locus. For this, we performed ChiP-seq mapping of the elongating form of RNA polymerase II (RNA PolII-S2P), of the total RNA PolIII, as well as RNA-seq, prior DSB induction. DSBs were further sorted according to their transcriptional status prior to damage induction. Senataxin recruitment following DSB induction correlated with total RNA PolIII enrichment levels preceding damage (Fig. 1d) as well as with elongating RNA PolII and RNA levels (Supplementary Fig. 1A, B). Moreover, inspection of
Fig. 1 Senataxin is recruited at DSB induced in active loci. a Genome browser screenshots representing senataxin ChIP-Seq reads count before AsiSI activation (−4OHT) and after damage induction (+4OHT) at two individual AsiSI sites. The BLESS signal (indicative of cleavage efficiency (Clouaire, T. et al., manuscript submitted)) is also shown. Close up profiles are also shown below each screenshot. b Box plots representing senataxin ChIP-seq count before (−4OHT) and after (+4OHT) DSB induction at sites displaying AsiSI-induced cleavage (“cut”, 80 AsiSI sites) or not (“uncut”, 1139 AsiSI sites). Center line: median; box limits: 1st and 3rd quartiles; whiskers: maximum and minimum without outliers. P values are indicated (Wilcoxon Mann-Whitney test). c Heatmaps representing senataxin ChIP-seq count over a 10 kb window centered on the DSB before (−4OHT) and after (+4OHT) DSB induction. DSBs are sorted according to decreasing cleavage efficiency (based on BLESS data set (Clouaire, T. et al., manuscript submitted)). d Box plots representing senataxin ChIP-seq count before (−4OHT) and after (+4OHT) DSB induction at AsiSI “cut” sites sorted according to total RNA Polymerase II occupancy on a 10 kb window surrounding AsiSI sites (20 DSBs in each category). Center line: median; box limits: 1st and 3rd quartiles; whiskers: maximum and minimum without outliers. Points: outliers. e Genome browser screenshots representing senataxin ChIP-Seq reads count before AsiSI activation (−4OHT) and after damage induction (+4OHT) at four individual AsiSI sites, exhibiting either high (left) or low (right) transcriptional activity (indicated by RNA PolII S2P ChIP-seq mapping and RNA-seq −4OHT). The BLESS signal (Clouaire, T. et al., manuscript submitted) indicates that all sites display equivalent cleavage following 4OHT treatment.
individual sites indicated that senataxin did not accumulate at broken intergenic or silent regions, although they were robustly cleaved (see BLESS signal) and showing high level of XRCC4 recruitment (assessed by ChiP-seq)\(^26\) (Fig. 1e, Supplementary Fig. 1C). We previously demonstrated that transcriptionally active genes exhibit preferential binding of Rad51 and homologous recombination (HR) repair\(^29\). In agreement, senataxin displayed a significantly enhanced recruitment at DSB enriched in Rad51 compared to DSBs exhibiting low levels of Rad51 (Supplementary Fig. 1D). Altogether these data indicate that senataxin is recruited to damage sites with a strong preference for DSBs induced in transcriptionally active loci, preferentially repaired by HR.

**Senataxin removes DSB-induced RNA:DNA hybrids in active loci.** A well described function of senataxin is its ability to unwind RNA:DNA hybrids, hence regulating R-loops stability on the genome. Given senataxin recruitment at DSBs, we further investigated R-loops distribution at AsISI-induced DSBs. For this, we performed DRIP-seq using the S9.6 antibody that displays a strong specificity for RNA:DNA hybrids\(^32,33\) before and after DSB induction. As expected\(^6,7\), DRIP-seq signal was enriched on active genes, peaking at TSS and TTS, validating our sequencing results (Supplementary Fig. 2A, B). Notably, we could observe robust changes in R-loops distribution following damage, with an increase of RNA:DNA hybrids around the break site (Fig. 2a, Supplementary Fig. 2C). When taken collectively, following DSB induction, RNA:DNA hybrids were mildly but significantly enriched (7% increase) on a 10 kb window surrounding cut sites compared to uncut sites (Fig. 2b). Interestingly, while senataxin accumulation was strongly correlating with the transcriptional activity of the broken locus (Fig. 1 and Supplementary Fig. 1), this was less the case for RNA:DNA hybrids accumulation. Indeed, we could detect their formation upon damage at few (example Supplementary Fig. 2D, E)—but not all (example Supplementary Fig. 2F)—untranscribed loci (no detectable signal for RNA-seq or elongating RNA PolII). While total RNA PolII (hence likely not in an elongating form) was readily detectable prior DSB induction at some of these untranscribed loci (Supplementary Fig. 2D), others did not display any RNA PolII before damage induction (Supplementary Fig. 2E), suggesting that at least in a few instances RNA:DNA hybrids may also be able to form at untranscribed loci, devoid of RNA PolII (Discussion), albeit at lower levels.

Interestingly, at active genes, beyond the accumulation surrounding the DSBs, we could also detect a decrease in R-loops across damaged genes bodies and termination sites (Fig. 2a for examples, Supplementary Fig. 2G for averaged profiles). Moreover, careful examination of our high-resolution data revealed that despite an increase of RNA:DNA hybrids formation on ~10 kb window around DSBs, at active genes we could also observe a sharp 1–2 kb decrease of RNA:DNA hybrids at the exact sites of senataxin accumulation (Fig. 2c, d). Depletion of senataxin using a siRNA (Supplementary Fig. 3A) triggered an increase in DSB-dependent RNA:DNA hybrids accumulation proximally to a DSB (Fig. 2e). Thus, our high resolution data reveal that the pattern of R-loops shows complex alterations upon DSB induction. Altogether our data suggests that RNA:DNA hybrids form at DSB flanking chromatin and that, at active genes senataxin recruitment contribute to their removal in the immediate vicinity of DSBs.

**Senataxin promotes cell survival upon active genes breakage.** To further assess SETX function in DSB repair, we used our improved version of the DIVA cell line, whereby AsISI-ER is also fused to an auxin inducible degron (AID). In this cell line, auxin addition triggers the degradation of the enzyme and hence repair of AsISI-induced DSBs\(^39\). We first assessed the survival of AID DIVA cells following break induction and repair, in both control and senataxin-depleted cells (Supplementary Fig. 3A). Clonogenic assays revealed that depletion of senataxin using siRNA does not trigger cell death in absence of exogenous damage (Fig. 3a, –4OHT). DSB induction for 4 h, followed by auxin addition (+4OHT + auxin) only led to a mild survival defect in control cells (Fig. 3a) indicating that these cells recover well from the induction of DSBs by AsISI, as previously reported\(^34\). In contrast, senataxin depleted cells exhibited a strong sensitivity to AsISI-induced DSBs (Fig. 3a, Supplementary Fig. 3B).

Since AsISI-induced DSBs exhibit clean DNA ends and undergo repeated cycles of cleavage, we further tested the effect of senataxin depletion at DSBs produced by other means. Etoposide is an inhibitor of Topoisomerase II (TOP II) and multiples studies have revealed that TOP II exerts a critical function at active genes in order to unwind supercoils and release topological constraints that form at transcriptionally active regions\(^35\). Hence, etoposide-induced DSBs exhibit a biased distribution throughout the genome, being preferentially located in active promoters (~30%) and genes bodies (~40%)\(^36\). Interestingly, senataxin depletion also triggered enhanced sensitivity to etoposide (Supplementary Fig. 3C). On the other hand, ionizing radiation (IR) induces DSBs randomly across the genome, hence being mainly located in intergenic loci that represent over 95% of higher eukaryotes genomes. Notably, and in agreement with a previous report\(^22\), senataxin depletion did not trigger enhanced sensitivity to irradiation (Supplementary Fig. 3D). Our data therefore suggest that senataxin exerts an important function in cell survival specifically following break induction in active loci.

**Senataxin regulates γH2AX accumulation.** To further decipher the function of senataxin in DSB repair and to understand the lethality observed following DSB induction in senataxin deficient cells, we analyzed the effect of senataxin depletion on γH2AX foci formation following DSB induction. We found that senataxin depletion did not abolish foci formation and rather triggered enhanced γH2AX signal in 4OHT-treated cells (Fig. 3b). Interestingly, senataxin depletion also increased γH2AX signaling following etoposide treatment (Supplementary Fig. 3E) but not following IR (Supplementary Fig. 3F), suggesting a function of senataxin in regulating γH2AX foci formation at DSBs induced in active genes. To further strengthen these data, we tested whether a short global transcription extinction preceding break induction was able to rescue the increased γH2AX signaling observed in senataxin-depleted cells. A pretreatment of DIVA cells with cytosine, a well characterized transcription inhibitor, partially reduced γH2AX in damaged DIVA cells following senataxin depletion (Fig. 3c). Altogether, our data suggest that senataxin is involved in regulating γH2AX establishment at DSBs induced in transcriptionally active loci.

We next investigated the consequences of senataxin depletion on DSB repair kinetics. Immunofluorescence performed at different time points after auxin addition revealed that γH2AX foci disappeared with the same kinetics in both control and SETX siRNA-treated cells (Fig. 4a, b). To refine this analysis, experiments were also performed using a high content microscope which allows to sort G1 versus G2 cells based on their DNA content\(^34\). Similarly we could not detect any delay of γH2AX foci clearance following auxin addition in SETX-deleted G1 and G2 cells (Supplementary Fig. 4A). Additionally, AID DIVA cells allow to assay repair kinetics at specific DSBs using a protocol based on
the ligation of a biotinylated oligonucleotide followed by streptavidin purification and quantitative PCR measurement of purified DNA.30,37 Once more, senataxin depletion did not trigger repair delay at two DSBs found to be enriched in senataxin following 4OHT treatment (Fig. 4c). In addition, this held also true in G1- and G2-arrested cells following treatment with lovastatin and RO-3306 respectively (Supplementary Fig. 4B).

Altogether, these data indicate that while SETX deficiency triggers enhanced γH2AX signaling, it is not associated with delayed DSB repair, neither in G1 nor in G2.

Senataxin regulates repair pathway choice. Given that senataxin was found to be recruited at DSBs induced in transcriptionally...
active loci, which are prone to undergo HR repair, we further investigated the function of senataxin on HR. Senataxin depletion impaired Rad51 foci formation, while increasing 53BP1 accumulation following DSB induction by AsiSI (Fig. 5a, b). Notably, the enhanced accumulation of 53BP1 observed in senataxin depleted cells was partially reversed by pretreating the cells with transcription inhibitors (cordycepin and 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB)) (Supplementary Fig. 5A, B).

Next, to further investigate the function of senataxin in repair pathway choice, we used the reporter constructs previously developed to quantitatively measure HR, single strand annealing (SSA), and NHEJ, using flow cytometry following I-SceI transfection. Senataxin depletion triggered a mild decrease of HR and SSA associated with a similarly mild increase of NHEJ (Fig. 5c). Importantly, western blot against I-SceI (myc tagged) indicated that this was not due to changes in I-SceI expression.

Because generation of ssDNA is the initial step for HR repair, we further examined the effect of senataxin depletion on DSB end...
SETX depletion enhances translocations. Given the strong requirement of SETX for survival upon DSB induction in active genes (Fig. 3), despite no clear delay in repair kinetics (Fig. 4), we next set to assess whether SETX could influence the quality of the repair reaction, more specifically the frequency of illegitimate rejoicing between distant DSBs, involved in the generation of translocations. Using high resolution Capture Hi-C, we recently demonstrated that DSBs can cluster when induced on active genes and identified the molecular identity of AsiSI-induced DSBs brought into spatial proximity within nuclear foci31. Hence, based on this knowledge we developed an assay to accurately measure the illegitimate rejoicing of closely clustered DSBs. We could detect rejoicing between DSBs induced on the same chromosome (between MIS12 and TRIM37 as well as in LINC0072 and LYRM2) (Fig. 6a), but also between DSBs induced on different chromosomes (MIS12::LYRM2 and TRIM37::RBMXL1) (Supplementary Fig. 6A). Importantly, SETX depletion led to a highly reproducible increase of all four translocations events compared to control cells (Fig. 6b, Supplementary Fig. 6B, C). Notably, this increase of translocations observed in senataxin depleted cells was partially rescued by a pretreatment with DRB (Fig. 6c) or upon overexpression of RNAseH1, which degrades RNA:DNA hybrids (Supplementary Fig. 6C). These data indicate that senataxin plays a key role in counteracting illegitimate rejoicing of DSBs induced in loci ongoing active transcription.

Discussion
In this study we set to better understand the formation of RNA:DNA hybrids at DSBs as well as the potential function of RNA:DNA hybrids removal factors in DSB repair, focusing on senataxin, a well characterized R-loops helicase. We discovered that senataxin is specifically recruited at DSBs induced in active loci, where it removes RNA:DNA hybrids forming in cis to broken loci. Senataxin is further required to regulate γH2AX signaling, to promote Rad51 loading and to minimize abnormal rejoicing of distant DNA ends (Fig. 6d).

Our genome-wide mapping indicates that RNA:DNA hybrids accumulate in cis to DSBs, as previously proposed26–28. However, it seems that proximal DSB-induced RNA:DNA hybrids may form differently depending on the transcriptional status of the damaged locus. At active genes, this RNA:DNA hybrids accumulation around DSBs is associated with an otherwise R-loops decrease across the entire damaged gene body. Several studies...
have reported that transcription is downregulated at the damaged gene, as well as on chromatin proximally flanking DSBs, although being globally maintained in the γH2AX domain at distance from the break (reviewed in refs. 12,42). The exact mechanism leading to transcriptional repression in cis to DSBs is not yet clear but involves the recruitment of chromatin modifying complexes 43–47, as well as ATM-induced modifications of transcription elongation factors45,47 yielding to a reduction in the elongating form of RNA PolII across the gene body48. Multiple studies have now established that R-loops accumulate at sites of paused or slowly elongating RNA PolII7,49. Hence, at active genes, DSB-induced RNA PolII stalling may contribute to the strong RNA:DNA hybrids and/or R-loops formation in cis to the break.

Notably, although it was not a general feature (Supplementary Fig. 2F), we could also identify few seemingly transcriptionally silent sites that displayed low, but detectable levels of RNA:DNA hybrids following breakage (Supplementary Fig. 2D, E), even when total RNA PolII was not present prior damage (Supplementary Fig. 2E). Hence de novo RNA PolII recruitment at DNA ends (a feature recently observed28,50) may, at least in some
instance, produce RNA:DNA hybrids in cis to DSBs. More dedicated systems that allow to induce DSBs at a larger number of transcriptionally silent loci (using multiple guides RNAs for Cas9 induced breaks for example) should help to understand the occurrence of such RNA:DNA hybrids as well as the mechanisms driving their formation. Additionally, it is important to note here that our study does not allow to determine whether the S9.6 signal detected at DSBs represent single stranded structures (R-loops) or double-stranded RNA:DNA hybrids. Indeed, in S. pombe, such hybrids were proposed to form as resection progresses, by the hybridization of a RNA to the resected single strand DNA. Strand-specific mapping of the RNA engaged in the hybrids detected at DSB, by DRIP-c-seq, should help to determine whether these resection-dependent RNA:DNA hybrids are conserved in mammalian cells and whether the increased S9.6 signal at DSBs observed in this study represent R-loops or RNA:DNA hybrids.

Regardless of whether they arise from an unscheduled activity of RNA Polymerase II blocked by the lesion, or by de novo transcription from DNA-ends, DSB-induced RNA:DNA hybrids may exert some important function in the DDR. First, these RNA:DNA hybrids could contribute to setup an adequate chromatin landscape. Indeed, R-loops at genes have been proposed to modify chromatin structure. For instance, R-loops trigger H3–S10 phosphorylation linked to chromatin condensation. Notably, the Tip60 complex has repeatedly been complexed at break site, where it mediates H4 and H2A acetylation and subsequent recruitment of repair proteins. Hence one can hypothesize that R-loops and/or RNA:DNA hybrids will contribute to set up the proper chromatin landscape required at DSBs to ensure accurate and timely repair. Second, RNA:DNA hybrids may contribute in promoting premature transcription termination of broken genes. Indeed, a large amount of studies established a function for R-loops, as well as for senataxin, in terminating transcription including for promoter-associated bidirectional non-coding RNA (for instance reviewed in refs. 1,11). In this regard it is interesting that dsRNA processing factors such as Drosha and Dicer contribute to this process.

Hence, at damaged genes, R-loops and senataxin may promote premature termination in order to allow either RNA PolII clearance from the damaged region to favor accessibility of repair proteins, or efficient recycling of RNA PolII to resume transcription after repair. It is tempting to speculate that the reported function of Dicer and Drosha in DDR may at least in part, relate to the transcriptional termination at genes experiencing a DSB.

Interestingly we found that, although senataxin depletion did not delay DSB repair, it impaired Rad51 foci formation, and conversely increased accrual of 53BP1. Decreased HR and increased NHEJ were also observed following senataxin depletion using HR and NHEJ reporter constructs although to a milder extent, which may be due to low R-loops formation on these substrates. Notably, senataxin depletion did not impede resection, suggesting that senataxin and/or RNA:DNA hybrids removal are critical at a step subsequent to ssDNA generation. However, resection was only monitored up to 1.6 kb from the DSB, so we cannot exclude a function of senataxin in regulating more long range resection events. Notably, we also found that senataxin regulates γH2AX establishment and counteracts the illegitimate rejoining of distant DNA ends, suggesting that R-loops removal is required to minimize translocations and maintain genome integrity following production of DSB in active genes. The mechanism by which senataxin impacts on γH2AX is currently unknown but may involve the regulation of ATM recruitment or activity. Alternatively, senataxin and/or R-loops may regulate the DSB-flanking chromatin structure, modifying its ability to undergo H2AX phosphorylation. Equally, how senataxin counteracts translocations needs to be investigated. We recently proposed that γH2AX spreading on entire topologically associated domains (TAD) likely modifies the properties of the chromatin fiber which could translate into changes in chromatin mobility within the nucleus. Given that DSBs induced in active genes were found to display enhanced clustering ability, we can speculate that the increased aberrant joining of distant DSBs observed in senataxin depleted cells arise from an increased DNA ends mobility triggered by the enhanced γH2AX establishment.

Importantly, SETX is a gene mutated in two severe neurological diseases, AOA2 and ALS4, associated with progressive neurodegeneration. In this regard it is interesting that DSBs have been shown to be produced as a consequence of neuronal activity and further genomic studies suggested they likely arise in active genes. Given that senataxin exerts its anti-translocation and survival-promoting functions only for DSBs induced in active genes, we propose that this “Transcription Coupled DSB repair” function of senataxin may contribute to neuron loss in AOA2/ALS4 patients.

**Methods**

**Cell culture.** U2OS were retrieved from ATCC and modified with a plasmid encoding for the restriction enzyme (pBabe-AssiER and pAID-AssiER). U2OS, DIVa (AssiER-UR-U2OS), and AID-DIVA (AID-AssiER-UR-U2OS) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with antibiotics, 10% FCS (InVitrogen) and either 1 µg/ml puromycin (DIVa cells) or 800 µg/ml G418 (AID-DIVA cells) at 37 °C under a humidified atmosphere with 5% CO2. The cell lines were regularly checked for mycoplasma contamination. For AssiER-dependent DSB induction, cells were treated with 300 nM 4OHT (Sigma, H7904) for 4 h. When indicated, 4OHT-treated cells were washed three times in

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**Fig. 5** Senataxin depletion decreases HR but does not impede resection. a Rad51 staining performed in untreated or 4OHT-treated DIVa cells (4 h), after transfection with control or SETX siRNA as indicated. Scale bar: 10 µm. Right panel shows the quantification of the Rad51 nuclear signal within foci (>100 nuclei) from a representative experiment. Center line: median; box limits: 1st and 3rd quartiles; whiskers: maximum and minimum without outliers. P values are indicated (unpaired t-test). b 53BP1 staining performed in untreated or 4OHT-treated DIVa cells (4 h), after transfection with control or SETX siRNA as indicated. Scale bar: 10 µm. Right panel shows the quantification of the 53BP1 nuclear signal within foci (>100 nuclei) from a representative experiment. Center line: median; box limits: 1st and 3rd quartiles; whiskers: maximum and minimum without outliers. P values are indicated (unpaired t-test). c HR (top panel), SSA (middle panel), and NHEJ (bottom panel) usage was measured using cell lines harboring specific reporter constructs in control or senataxin-deficient (siRNA-transfected) dedicated cells. Myc-I-Scel expression was controlled by western blot in each condition. Mean and s.e.m. of at least three biological replicates are shown (as indicated). P values are indicated (paired t-test). d The site specific resection assay has been described earlier. Briefly, DNA purified from damaged or undamaged cells is digested by dedicated restriction enzymes (as indicated) and digestion-resistant DNA (single stranded DNA) is measured by qPCR, using primers pairs apart from the restriction sites. Here, we optimized this assay at two AssiER-induced DSBs that were shown to undergo HR. e Resection assay at the two DSBs in control or SETX-siRNA transfected cells. Values were normalized against the % of ssDNA detected in control cells before 4OHT treatment. Avera and s.e.m. of four biological replicates are shown. P values are indicated (paired t-test).
pre-warmed PBS and further incubated with 500 µg/mL auxin (IAA) (Sigma; I5148) for the indicated time. For transcriptional inhibition, DRB (Sigma, 100 µM) or cordycepin (Sigma, 50 µM) was added to the medium 1 h prior to 4OHT (4 h) and auxin (2 h) treatments. Cells were arrested in G1 using a 48 h treatment with 40 µM lovastatin (Mevinolin from LKT Laboratories) and in G2 with a 24 h treatment with 40 µM Ro-3306 (CDK1 inhibitor, Calbiochem). For clonogenic assays in U2OS cells, DSBs were induced either by increasing doses of etoposide (Sigma) for 16 h as indicated or by irradiation with a Cs137 source (Biobeam 8000).

**siRNA and plasmid transfection.** siRNA transfections were performed with a Cell Line Nucleofector kit V (Program X-001, Amaxa) according to the manufacturer’s instructions and cells were assayed 48 h after siRNA nucleofection. The following siRNA transfections were performed with a Cell Line Nucleofector kit V (Program X-001, Amaxa) according to the manufacturer’s instructions and cells were assayed 48 h after siRNA nucleofection. The following

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**Fig. 6** Senataxin counteracts the formation of translocations. **a** Rejoining of distant DSBs were detected by PCR, following DSB induction and repair (+4OHT + IAA 2 h) at breaks recently shown to undergo clustering[31]. DNA sequencing confirmed the nature of the amplified products. **b** MIS12::TRIM37 and LINC00271::LYRM2 rejoining frequencies were analyzed before or after 4OHT + IAA treatment, by quantitative PCR in AID DIVA cells transfected with control or SETX directed siRNA. Mean and s.e.m. of five biological replicates are shown. P values are indicated (one sample t-test). **c** MIS12::TRIM37 rejoining frequency was analyzed in control or SETX-depleted AID DIVA cells pretreated or not with DRB prior to 4OHT addition as indicated. Mean and s.e.m. of three biological replicates are shown. P value is indicated (paired t-test). **d** Model: R-loops form as the RNA Polymerase II progresses across the gene. The induction of a DSB elicits ATM activity which triggers RNA polymerase II stalling at the vicinity of the DSB, hence decreasing R-loops across the gene body. On another hand, R-loops and/or RNA:DNA hybrids accumulate in cis to the DSB, due to stalled RNA PolII generating short, abortive, RNAs which thread back in the DNA duplex, or/and potentially de novo PolII transcription from DNA end. Senataxin is further recruited to remove RNA:DNA hybrids at the vicinity of the break induced in active loci. Senataxin and/or R-loop removal, regulate γH2AX establishment, promote Rad51 loading and minimize the occurrence of translocation by a mechanism that still need to be investigated.
ChIP followed by high throughput sequencing and RNA-seq. Cells were crosslinked with formaldehyde (1%) and blocked with 500 µl of TE buffer (Tris 10 mM pH 8, EDTA 0.5 mM pH 8) with 30 µg of proteinase K for 30 min at 37 °C. Crosslink was reversed in the presence of 0.5% SDS at 70 °C overnight with 100 µl of Proteinase K in 100 mM NaCl. After 2 h, proteins were removed by phenol/chloroform and pre-washed three times with 700 µl of phenol/chloroform to eliminate phenol. DNA fragments were cleaned by phenol-chloroform extraction and ethanol precipitation. 

To classify DSBs based on transcriptional activity, RNA PolII-S2P ChIP-seq, RNA-seq, and DRIP-seq data sets were analysed. SETX ChIP-seq, RNA-seq, and DRIP-seq samples were sequenced using Illumina NextSeq 500 (single-end, 80 bp reads for SETX ChIP-seq; paired-end, 75 bp reads for RNA-seq and single-end, 85 bp reads for DRIP-seq) at EMBL Genomics core facilities (Heidelberg, Germany). The quality of each raw sequencing file (fastq) was verified with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). ChIP-seq and DRIP-seq files were aligned to the reference human genome (hg19) and processed using a classical ChIP-seq pipeline: bwa (http://bio-bwa.sourceforge.net/) for mapping and samtools (http://www.htslib.org/) for duplicate removal (rmdup), sorting (sort), and indexing (index). RNA-seq was mapped to a custom human genome (hg19 merge with ERCC92 sequences) to avoid mapping ERCC sequences on the human genome and processed as the same way as ChIP-seq, except for the alignment in paired-end mode with STAR and without remove potential duplicate.

Coverage for each aligned ChIP-seq data set (bam) were computed with the tracklayer R package and normalized using total read count for each sample. Coverage data was exported as bwig (bigwig format) for further processing.

Averaged ChIP-seq, RNA-seq, and DRIP-seq profiles were generated using the R package sgdplot2. For profile visualization, the transcriptional position relative to Assi site and the y-axis represents the mean coverage at each bp (Fig. 2d). For metagene profiles, the mean coverage was computed in two parts: first, mean coverage was computed in 200 bp intervals 3 kb upstream TSS and downstream TSS. Second, gene bodies were divided into 100 equally sized bins, so average profiles could be computed as a percent of entire gene length. Profiles were computed for all genes (Supplementary Fig. 2B) or for genes either directly or located near a DSB (Supplementary Fig. 2C).

Clonogenic assays. After siRNA transfection, AID-DIvA and U2OS cells were seeded at a clonal density in 10 cm diameter dishes. After 48 h, U2OS cells were treated with 400 µl of IP buffer for 10 min at room temperature. After the final wash, beads were resuspended in 250 µl of 1× IP buffer and incubated with 60 µg of Proteinase K for 45 min at 55 °C. Digested DRIP samples were then cleaned with phenol-chloroform extraction and ethanol precipitation. Air-dried DRIP pellets were resuspended in 45 µl of 10 mM Tris-HCl pH 8. DRIP experiment was assayed by qPCR using primers located at SNRNP (negative control), RPLA13 (positive control) and RBXmL1 (Assi site): SNRNP_FWD: GgCACAATGAGTGGAGTGG; SNRNP_REV: TTAATATGGCACCAGggT; RPLA13_FWD: FWAATGTTGGCATTCTTCTCG; RPLA13_REV: CCAATTCCCGCAAGACTCTCA. RBXmL1_FWD: GATTTGCATTTGGGTGAGAC; RBXmL1_REV: CATCCCTGCAAACAGTCTG.

For DRIP-seq, samples from three DRIP experiments were pooled and sequenced using a 3 kb library (Diagenode) for 20 cycles of 30 s, 30 s off, high setting. Immunoprecipitated DNA was subjected to library preparation and single-end sequencing on a NextSeq 500 at EMBL GeneCore (Heidelberg, Germany).

**RNA extraction and RNA-seq library preparation.** For RNA-seq, Dlva cells (transfected with the control siRNA) were lysed using TRI reagent (SIGMA) and spiked-in with ERCC RNA Spike-In Mix (Thermo Fisher Scientific). Total RNA were recovered by chloroform extraction followed by isopropanol precipitation. Samples were treated with RNase-free DNase (Promega) for 1 h at 37 °C and purified by phenol/chloroform extraction followed by ethanol precipitation. Ribosomal RNA depletion and RNA-seq library preparation were performed at EMBL Genomics core facilities (Heidelberg, Germany) using TrueSeq Stranded Total RNA (Illumina).

**DNA-RNA immunoprecipitation.** DRIP assay was carried out according to the protocol described in ref. 12. Dlva cells were treated with 300 µM 4OHT (for 4hr, trypan blue was added at low speed and washed with DPBS (Life technology). Total nucleic acids were extracted with 0.5% SDS /Proteinase K (Thermo Fisher Scientific, Waltham, MA) treatment at 37 °C overnight and recovered by phenol-chloroform extraction and ethanol precipitation. DNA was digested by a restriction enzyme cocktail (20 units each of EcoRI, HindIII, BglIII, XbaI) (New England BioLabs) in 20 µg of DNA without RNase H treatment and 10 µg/mL of proteinase K for 1 h at 37 °C. Fragmented DNA was cleaned by phenol-chloroform extraction and ethanol precipitation followed by two washes with 70% ethanol. Air-dried pellets were resuspended in 10 µM Tris-HCl pH 7.5, 1 mM EDTA (TE). In total, 4 µg of digests was diluted in 450 µl of TE and 10 µl was reserved as input for qPCR. 50 µl of 10X IP buffer was added (final buffer concentration of 10 mM sodium phosphate, 140 mM sodium chloride, 0.05% Triton X-100) and 10 µl of 59.6 antibody (1 mg/ml, kind gift from E. Chedin, UC Davis). Samples were incubated with the antibody at 4 °C for 2 h on a wheel. 50 µl of Protein A/G Agarose (Pierce), previously washed twice with 700 µl of IP buffer for 5 min at room temperature, were added and samples were incubated for 2 h at 4 °C on a wheel. Each DRIP was then washed three times with 700 µl × 1 IP buffer for 10 min at room temperature. After the final wash, beads were resuspended in 250 µl of 1× IP buffer and incubated with 60 µg of Proteinase K for 45 min at 55 °C. Digested DRIP samples were then cleaned with phenol-chloroform extraction and ethanol precipitation. Air-dried DRIP pellets were resuspended in 45 µl of 10 mM Tris-HCl pH 8. DRIP experiment was assayed by qPCR using primers located at SNRNP (negative control), RPLA13 (positive control) and RBXmL1 (Assi site): SNRNP_FWD: GgCACAATGAGTGGAGTGG; SNRNP_REV: TTAATATGGCACCAGggT; RPLA13_FWD: FWAATGTTGGCATTCTTCTCG; RPLA13_REV: CCAATTCCCGCAAGACTCTCA. RBXmL1_FWD: GATTTGCATTTGGGTGAGAC; RBXmL1_REV: CATCCCTGCAAACAGTCTG.

For DRIP-seq, samples from three DRIP experiments were pooled and sequenced using a 3 kb library (Diagenode) for 20 cycles of 30 s, 30 s off, high setting. Immunoprecipitated DNA was subjected to library preparation and single-end sequencing on a NextSeq 500 at EMBL GeneCore (Heidelberg, Germany).

**Drpase assay.** The Drpase assay was performed as described in ref. 12. Drpase reactions were incubated at 37 °C for 30 min in a final volume of 20 µl containing: 1 µM of protein, 0.5 µM of DNA substrate and 50 mM Tris-HCl pH 7.5, 1 mM MgCl2. Reactions were stopped by 50 mM EDTA (PharmaCielo, Toronto, Canada), boiled at 95 °C for 10 min, and run on a 3% agarose gel.
either exposed at increasing doses of IR or treated with increasing doses of etoposide, as indicated. AID-DIvA cells were treated with 300 nM 4OH for 4 h and, when indicated, washed three times in pre-warmed PBS and further incubated with 500 µg/ml auxin for another 4 h. After three washes in pre-warmed PBS, complete medium was added to each AID-DIvA cells dish. After 10 days, U2OS cells and AID-DIvA cells were stained with crystal violet (Sigma) and counted. Only colonies containing more than 50 cells were scored.

**Immunofluorescence.** Cells were plated in glass coverslips, fixed with 4% paraformaldehyde for 15 min, permeabilized with Triton X 0.5%, and blocked with PBS-BSA 3% for 30 min at room temperature. Cells were then incubated with antibody against γH2AX (IBW301, 0.5-636, Millipore, 1:1000) and 53BP1 (Novus Biological, NB-300-104, 1:500) overnight at 4 °C. Cells were washed three times in PBS-BSA 3% and incubated with secondary antibody for 1 h. After three washes (one PBS-BSA 3% and two PBS 3% washes), cells were stained with Hoechst 33342 (Sigma). Staining against Rad51 (Santa cruz sc8349, 1:200) was performed using the Operetta automated high-content screening microscope (PerkinElmer). For quantitative image analysis, multiple fields were selected according to the B method, and appropriate parameters, such as the size parameters to ensure best foci detection: detection sensitivity 0.5 –0.9. G1 and G2 nuclei were selected on the basis of the Hoechst intensity, after visualization of the Hoechst distribution in all cells. Box plots represent the total nuclear signal intensity detected in foci.

**Repair kinetics at AsiSI sites**

High-throughput microscopy. AID-DIvA and U2OS cells were plated in 96-well plates until confluence. After fixation, permeabilization and saturation steps, γH2AX was stained overnight with γH2AX (IBW301, 0.5-636, Millipore) and the secondary antibody anti-mouse Alexa 647 (A21235, Molecular Probes). Nuclei were labeled with Hoechst 33342 (Sigma) at a final concentration of 1 µg/ml for 5 min. γ-H2AX foci were further analyzed with an Operetta automated high-content screening microscope (PerkinElmer). For quantitative image analysis, statistical analysis of multiple fields per well was acquired using x40 or x100 objective. 

**Data availability.** High throughput sequencing data have been deposited to Array Express under accession number E-MTAB-6318. Other data and source codes are available upon request.

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**Resection assay.** Measure of resection was performed as described in ref. 41 with the following modifications. DNA was extracted from fresh cells using the DNAeasy kit (Qiagen). In total, 400 ng of DNA were digested overnight at 37 °C using the Ban I restriction enzyme (16U per samples) that cuts at ~200 bp and 1626 bp from the DBS-KDEL3 and at 740 bp and 2000 bp for DBS-ASXL1. Digested and undigested samples were also treated with RNase H (Promega). Ban I was heat inactivated 20 min at 65 °C. Digested and undigested DNA were analyzed by qPCR using the following primers:

**RBMXL1-REV CATCCTTGCAAACCAGTCCT.**

**DSB-ASXL1_2000_REV: TGGAGCCCAATTCCTGAAG.**

ssDNA% was calculated with the following equation: ssDNA% = 1/(2^Ct digested – Ct undigested) + 0.5)*100.

**HR, NHEJ, and SSA repair assays.** The GCSS and RG37 cell lines have been derived from SV40 T-transformed human fibroblasts (GM6398 39,40. The U2OS SSA has been derived from the osteosarcoma cell line U2OS41. For siRNA transfection, 1 × 105 cells were transfected using Interferin (Ozyme, France) with 10 nM of siRNA according to the manufacturer’s instructions. Plasmid coding for I-SceI expression was transfected using JetPei (Ozyme, France) according to the manufacturer’s instructions. Twenty four hours after siRNA transfection, cells were washed and transfected with the I-SceI coding plasmid (1 µg). After 72 h, cells were collected after trypsin treatment and analyzed by flow cytometry (BD FacsCalibur) to detect and count GFP-positive cells in each condition. Percentage of GFP-positive cells was calculated on 25,000 sorted events.

**Translocation assay.** AID-DIvA cells were treated as indicated and then DNA was extracted from fresh cells using the DNAeasy kit (Qiagen). Illegitimate rejoining frequencies between MIS12 and TRIM37 (chr17_5390209 and chr17_57184285), LINC00217 and LRMY2 (chr17_5390209 and chr16_9304817), and TRIM37 and RBMXL1 (chr17_57184285 and chr19_8433139) were assessed by qPCR using the following primers: 

**MIC12_Fw: GACTGGCATAAGCGTCTTCG**

TRIM37_Rev: GCAATGAGTTCCTGATGTCC

Results were normalized using two control regions, both far from any AsiSI sites and γH2AX domain using the following primers:

**Ctrl_chr1_82844750_Fw: AGACATGGATGTTGCCAG**

**Ctrl_chr1_82844992_Rev: TCTCCCTCTTGTGTCACCA**

**Ctrl_chr17_9784962_Fw: ACAGTGGGACAGAAGACG**

**Ctrl_chr17_9785135_Rev: CTCCATCATGCAGCCTTGG**

Normalized translocation frequencies were calculated using the DeltaDeltaCt method from Bio-Rad CFX Manager 3.1 software42.

**Repair kinetics at AsiSI sites**

**High-throughput microscopy:** AID-DIvA and U2OS cells were plated in 96-well plates until confluence. After fixation, permeabilization and saturation steps, γH2AX was stained overnight with γH2AX (IBW301, 0.5-636, Millipore) and the secondary antibody anti-mouse Alexa 647 (A21235, Molecular Probes). Nuclei were labeled with Hoechst 33342 (Sigma) at a final concentration of 1 µg/ml for 5 min. γ-H2AX foci were further analyzed with an Operetta automated high-content screening microscope (PerkinElmer). For quantitative image analysis, statistical analysis of multiple fields per well was acquired using x40 or x100 objective to visualize ~2000 cells per well in triplicate.

**γ-H2AX, 53BP1, and Rad51 foci intensity quantification.** All quantification was performed using Columbus, the integrated software to the Operetta automated high-content screening microscope (PerkinElmer). DAPI or Hoechst nuclei were selected according to the B method, and appropriate parameters, such as the size and intensity of fluorescent objects, were applied to eliminate false-positive. Then γ-H2AX, Rad51 and 53BP1 foci were detected with the DAP method with adjusted parameters to ensure best foci detection: detection sensitivity 0.5–1; splitting coefficient, 0.5–1; background correction, >0.5–0.9. G1 and G2 nuclei were selected on the basis of the Hoechst intensity, after visualization of the Hoechst distribution in all cells. Box plots represent the total nuclear signal intensity detected in foci.

**Data availability.** High throughput sequencing data have been deposited to Array Express under accession number E-MTAB-6318. Other data and source codes are available upon request.

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Author contributions
S.C., N.P., T.C., and Y.C. performed experiments. V.R., M.A., and T.C. performed bioinformatic analyses of ChIP and DRIP data sets. Y.-L.L. and P.P. provided technical and conceptual assistance for DRIP experiments. G.L. conceived, supervised, analyzed experiments and wrote the manuscript. All authors commented and edited the manuscript.

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