Regulating autophagy: a novel role for SETX (Senataxin)

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Mutations in the gene encoding SETX, also known as Senataxin, are mainly linked to two distinct neurodegenerative diseases, a cerebellar ataxia known as oculomotor apraxia type 2 (AOA2) and a form of juvenile amyotrophic lateral sclerosis, ALS4 (Chen et al., 2004; Moreira et al., 2004). SETX is an RNA/DNA helicase that functions in multiple events related to RNA metabolism and DNA maintenance, including transcriptional termination at certain genes and the DNA damage response at replication stress foci. A key role attributed to SETX in both of these functions is in the resolution of R loops, potentially deleterious DNA:RNA hybrid structures that form during transcription (Aguilera and Garcia-Muse, 2012). As abnormal levels of R loops are frequently observed in neurological disorders, this role has been proposed as a link between SETX dysfunction and neurodegeneration (Richard and Manley, 2016). In a recently published study, however, we demonstrated that SETX plays critical roles in the progression of autophagy, the process employed by cells to eliminate defective proteins and organelles, through its effects on expression of autophagy-related genes (Richard et al., 2020). A hallmark of neurodegenerative disease is the abnormal accumulation of protein aggregates that eventually lead to cellular dysfunction and degeneration of neuronal tissues (Kurtishi et al., 2019). Not surprisingly, defective autophagy is strongly implicated in the development of such disorders (Finkbeiner, 2020). As described in this perspective, we now suggest that this novel role for SETX provides an additional pathway by which transcriptional and gene expression defects caused by mutations in SETX can lead to neurological disease. Indeed, in preliminary analyses, we have detected perturbed autophagy in samples from AOA2 and ALS4 patients harboring SETX mutations.

Autophagy and neurological disease: Macroautophagy, hereafter called autophagy, is a process of self-digestion, a way for cells to not only eliminate unwanted and toxic material, but also to recycle metabolites, which helps maintain cell homeostasis in both normal and stressed conditions, including starvation (Yin et al., 2016). Typically, autophagy starts with the formation of a cup-shaped membrane called a phagophore that extends and encloses a portion of the cytoplasm, sometimes including organelles such as damaged mitochondria. The resulting autophagosome travels along microtubules and fuses with a lysosome, generating an autolysosome whose contents are digested by acidic hydrolases. In addition to clearing unneeded organelles, autophagy also eliminates harmful protein aggregates. These are targeted for removal by ubiquitination and are recognized by specific adaptors that interact with proteins of the LC3 (microtubule-associated protein 1 light chain 3) family at the autophagosome membrane, which promotes their engulfment into autophagosomes. Regardless of the cargo, the process of autophagy is highly regulated from its initiation to the degradation of the autolysosome through a cascade of phosphorylation events that trigger extensive transcriptional regulation of autophagy and lysosomal-related genes. Autophagy dysfunction has been linked to an increasing number of diseases, in particular neurological disorders in which the accumulation of protein aggregates becomes toxic since neurological tissues are post-mitotic and therefore unable to dilute harmful material through cell division.

AOA2, ALS4 and SETX: As described below, our recent study now demonstrates that AOA2 is among the disorders that present impaired autophagosomal and lysosomal homeostasis, which include ALS (also known as Lou Gehrig's disease), ALS-FTD (frontotemporal dementia), Parkinson's and Huntington's diseases. AOA2 is a rare autosomal recessive cerebellar ataxia with juvenile/adolescent-onset. It is characterized by axonal sensorimotor neuropathy and progressive cerebellar atrophy resulting in poor balance and coordination, ultimately leading to the need of a wheelchair. Since 2004, occurrence of AOA2 has been genetically linked to mutations in the gene encoding SETX. Because of its putative RNA/DNA helicase properties and its connection to an autosomal recessive cerebellar ataxia, SETX was suspected to be a DNA damage repair (DDR) protein. Indeed, SETX interacts with several components of the DDR machinery and with RNA polymerase II which is consistent with its proposed role in promoting genome stability by preventing DNA damage caused by the accumulation of transcription-generated R loops. Shortly after being linked to AOA2, dominant missense mutations in *SETX* were found associated with ALS4 (Chen et al., 2004). In contrast to the more common form of ALS, Lou Gehrig's disease but similar to AOA2, ALS4 is not fatal, but death of motor neurons in the central nervous system results in muscle weakness. While slowly progressive but highly debilitating, there is no disease-modifying treatment for either ALS4 or AOA2.

SETX is linked to autophagy: In addition to its role in the DDR, SETX is a master regulator of gene expression and has been found to control transcription initiation and termination at specific genes, as well as pre-mRNA splicing (Groh et al., 2017). To explore how defects in gene expression that result from impaired SETX function might lead to neurological disorders, we examined the transcriptome of U87 glioblastoma-astrocytoma cells after siRNA-mediated knockdown of SETX by microarray and deep sequencing analyses. Transcript levels of hundreds of genes were affected by depletion of SETX, most of which showed reduced abundance, and the knockdown had global effects on pre-mRNA processing. Specifically, SETX depletion resulted in a general lengthening of 3′ UTRs and increased levels of intron retention, which supports roles for SETX in multiple stages of mRNA metabolism, including the regulation of alternative polyadenylation and alternative splicing. Intriguingly, we noticed that among the genes whose transcript levels were significantly perturbed by SETX knockdown are several that are involved in lysosome organization and biogenesis and in the regulation of autophagy. This prompted us to explore whether SETX, by controlling the expression of autophagy-related genes, could control autophagy.

SETX depletion led to the upregulation of multiple genes involved in the stimulation of autophagy and, correspondingly, we detected increased levels of immature, precursor autophagosomes in both normally growing and starved cells. This suggests a role for SETX in restricting the early stages of autophagy under normal and autophagy-inducing conditions. However, by assaying autophagic flux, we found that SETX is also critical for efficient progression through the stages of autophagy. Specifically, by monitoring the lipidation status and localization of the autophagy marker protein LC3, we determined that SETX depletion impairs the development of mature autophagosomes, an early, post-initiation event in autophagy, which was an expected observation since expression of genes involved in this process was downregulated. Overall, our data indicate that, whereas dysregulation of autophagy-related genes by depletion of SETX can differentially affect multiple stages of autophagy, the net effect of SETX deficiency in U87 cells is impaired autophagy. Indeed, consistent with defective autophagic processes, SETX knockdown led to a striking increase in levels of ubiquitinated proteins, increased mitochondrial mass, and, in a protein aggregation assay, the knockdown led to accumulation of a huntingtin-based, polyglutamine-containing reporter protein. These data strongly point to essential roles for SETX as a regulator of genes involved in promoting the clearance of protein aggregates and mitochondria (**Figure 1**), defects of which are the very hallmarks of several neurological disorders.

Although SETX deficiency resulted in defective expression of genes not limited to those encoding autophagy regulators, we suggest that impaired autophagy specifically connects SETX to neurological disease. To examine this potential link further, we obtained patient-derived AOA2 fibroblasts, determined that they harbor novel mutations in the SETX gene, and then looked for signs of perturbed autophagy in motor neurons derived from the fibroblasts through reprogramming and differentiation. Strikingly, the AOA2 patient-derived tissues, but not tissues derived from unaffected family members, showed increased levels of BECN1 (beclin 1), a marker of autophagy induction. Although further tests for defects in autophagy produced varied results among the limited number of available patient samples, this consistent observation aligns with our finding that SETX functions in regulating early stages of autophagy. Interestingly, in a preliminary analysis of ALS4 fibroblasts, which harbor the dominant L389S mutation in SETX, we detected increased levels of proteins involved in autophagy initiation such as BECN1 as well as a decrease in lysosome formation visualized by immunofluorescence of the lysosomal membrane glycoprotein LAMP1 (lysosomal associated membrane protein 1) (unpublished data). In agreement with defects in autolysosome formation in the ALS4 cells, cytosolic protein aggregates of the DNA/RNA binding protein TARDBP/TDP-43 (TAR DNA-binding protein) have been detected in post-mortem lumbar spinal cords of ALS4 patients

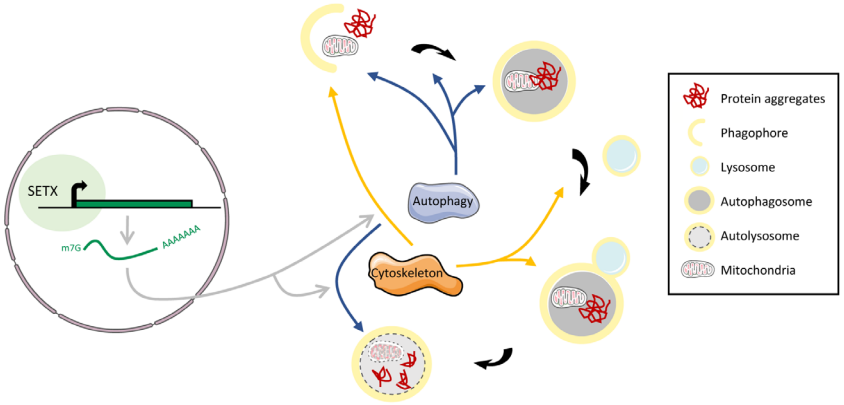

Figure 1 | **Potential mechanisms for the regulation of autophagy by SETX.**
SETX regulates transcription of genes that play roles at multiple steps in autophagy including initiation, elongation of the phagophore, formation of mature autophagosomes, and the degradation process (blue arrows). SETX also regulates expression and alternative splicing of several genes that encode actin cytoskeletal proteins (possibly through R-loop regulation), including some that function in the biogenesis of autophagosomes and in the transport and fusion of autophagosomes with lysosomes (orange arrows).

(Bennett et al., 2018). Our data implicating SETX dysfunction with defective autophagy, therefore, is well-supported by the observation of autophagy-related defects in neurological diseases in which SETX function is perturbed.

SETX and R loops: One surprising observation from our study is that depletion of SETX did not lead to a general increase in the number of R loops in U87 cells, as detected by immunoprecipitation of DNA:RNA structures followed by next generation sequencing (DRIP-seq). In fact, we detected reduced R-loop content, and perturbed expression levels, at many genes linked to cytoskeletal organization, which might indirectly affect autophagy since its progression depends on actin dynamics and microtubule scaffolding (see **Figure 1**). However, whereas our finding that SETX depletion did not generally elevate R-loop content indicates that SETX regulates autophagy and lysosomal gene expression independently of its putative role in R-loop resolution, it contrasts with the findings of others. R-loop accumulation has been reported after SETX knockdown, in SETX knockout mice, and in AOA2 induced pluripotent stem cells (iPSCs) and neuronal progenitors (Becherel et al., 2015), and it has become widely accepted that SETX functions in the resolution of R loops. The reason for the discrepancy is not clear. However, it is worth noting that, while many studies assessed R-loop accumulation by immunofluorescence using the RNA:DNA hybrid-specific S9.6 antibody, it has been shown that this antibody also has affinity for double-stranded RNAs, suggesting that many foci detected in these assays may derive from non-R-loop structures. Treating samples with RNaseH1, which degrades RNA:DNA hybrids specifically, may be a crucial control to include in such studies in order to validate the presence of R loops rather than other entities. Furthermore, R-loop signals observed by immunofluorescence show high intensity in the nucleolus and cytoplasm, suggesting that changes detected in overall R-loop cellular content do not necessarily reflect events associated with nucleoplasmic DNA. In agreement with our findings that SETX deficiency does not automatically correlate with increased R loops, SETX knockout mice show no detectable increase in the number of R loops in brain tissues, whereas proliferative tissues did show increased R-loop content (Yeo et al., 2014). This illustrates well the fact that cell type, context and species may drive the specific effects of SETX deficiency and that the link between SETX and R-loop resolution is more complex than anticipated and will require further study.

With a molecular mass of ~300 kDa, SETX acts as a large platform that orchestrates various functions through its many protein-interacting partners (see (Groh et al., 2017) for a recent and comprehensive review). While we unveiled a novel role for SETX in autophagy, which we believe may in part explain how SETX-associated mutations are specifically damaging to the brain, the precise molecular mechanisms involved in such regulation will necessitate further investigations. Specifically, future work should examine how defects in autophagy linked to SETX deficiency particularly affect the cerebellum, which is atrophied in most AOA2 cases. Future work is also needed to understand how both loss- and gain-of-function SETX mutations (as in AOA2 and ALS4, respectively), can result in impaired autophagy. As current animal models of neurodegeneration do not accurately recapitulate neurological disorders, iPSCs and neuron-derived iPSCs (e.g., iPSC-derived cerebellar Purkinje cells) may be reasonable alternatives for dissecting the molecular mechanisms leading to disease. Transcriptome combined with autophagy analysis after SETX deletion in multiple such systems will be important for confirming SETX-dependent regulation of autophagy in neurological contexts. In the case of rare diseases such as AOA2 or ALS4, this approach is somewhat more challenging due to the limited availability of samples, while conclusive results will only be achievable through studies that include samples from a large number of patients as well as their healthy or asymptomatic relatives. An already growing collaborative effort between the scientific community and patients will certainly accelerate our understanding of rare diseases, and uncover unexpected connections, such as the link between SETX and autophagy, that are crucial for developing disease-modifying compounds.

The authors declare no conflicts of interest.

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