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CORRESPONDENCE

Antisense RNA foci are associated with nucleoli and TDP-43 mislocalization in C9orf72-ALS/FTD: a quantitative study

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Three main mechanisms are thought to contribute to neurodegeneration in C9ORF72 amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (C9-ALS/FTD): toxicity from transcribed expanded repeat RNAs, toxicity from RAN-translated dipeptide repeat proteins (DPRs), and loss of C9ORF72 protein function [4, 5, 9, 11, 12, 18]. Sense and antisense RNA foci have both been consistently observed in C9-ALS/FTD neuropathology [1, 3, 5, 8, 9, 18] and hypothesized to cause neurodegeneration by sequestering critical RNA-binding proteins [2, 6]. Antisense, but not sense RNA foci have been shown to correlate with mislocalization of TDP-43, a signature protein of ALS and frontal–temporal lobar degeneration (FTLD) [1]. A unique circumferential studding of nucleoli by antisense RNA foci was observed in a case report of two C9-FTLD cases [17] and recently re-discussed with two additional cases [16]. Understanding the relative contributions from sense and antisense strands to pathogenesis is critical, since antisense oligonucleotides (ASOs) are expected to ameliorate toxicity whether stemming from RNAs or DPRs.

We evaluated RNA foci in five C9-ALS cases with and without FTD in our short-postmortem interval ALS repository [13]. C9-ALS/FTD was confirmed by repeat-primed PCR and Southern blotting. Consistent with the literature, both sense and antisense RNA foci were widely distributed throughout the nervous systems [1, 3, 5, 9, 18]. They were not concentrated in the motor or frontal cortex, the anatomical regions of direct relevance to C9-ALS/FTD (Supplemental Fig. 1a and b). Overall, the mean prevalence of sense and antisense RNA foci over multiple CNS regions were 16% and 20% in neurons and 1% and 1% in glia, respectively (Supplemental Fig. 1c). Antisense RNA foci were significantly denser than sense RNA foci within the RNA foci-positive neurons ($p < 0.0001$; Supplemental Fig. 1d). As reported in C9-FTD and one case of C9-ALS, we also observed that in large neurons, RNA foci were frequently localized to nucleoli, identified as a prominent immunofluorescent density within the nucleus, often directly abutting their circumference (Fig. 1a). We confirmed nucleolar localization using the nucleolar marker nucleolin (Fig. 1b). We quantified the association and overall, on average, 27% of antisense RNA foci but only 9% of sense RNA foci were perinucleolar. We saw that perinucleolar antisense RNA foci were more frequent in disease-related areas than non-disease-related areas and were more likely than sense RNA foci to be perinucleolar in disease-related areas, but this did not reach significance in disease-unrelated areas: 35% of antisense RNA foci but only 13% of sense foci in disease-related areas were perinucleolar, compared to 17% and 4%, respectively, in disease-unrelated areas (Supplemental Fig. 1e and f). The preponderance of nucleolar antisense RNA foci, but not sense RNA foci tended to correlate with the burden of TDP-43 pathology in frontal cortex (Supplemental Table 1).

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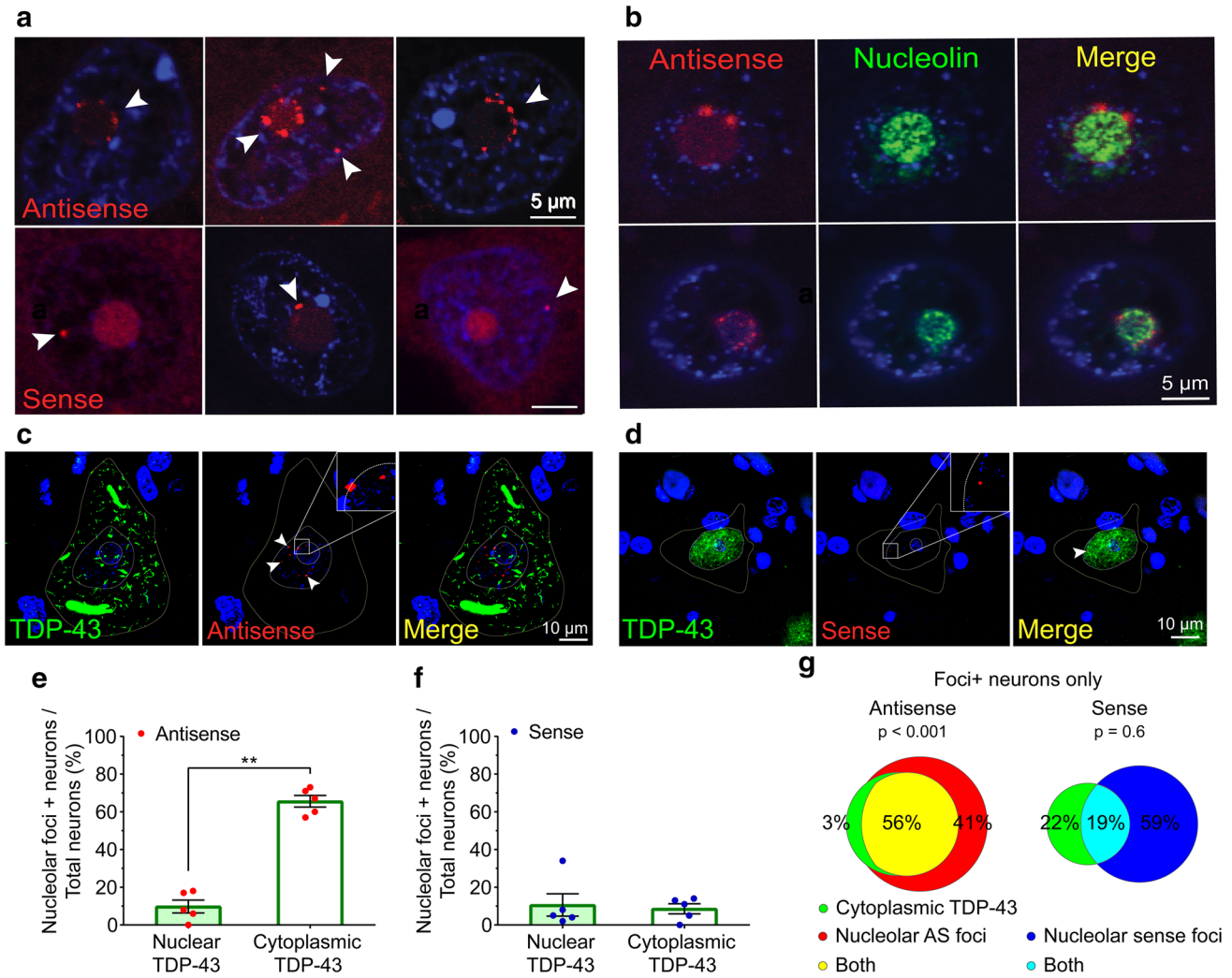

Fig. 1 Antisense RNA foci have unique neuropathological features compared to sense RNA foci. **a** Sense and antisense RNA foci (arrowheads) are observed in the nucleoplasm or on the periphery of nucleoli of neurons (outer, middle and innermost dashed lines mark the cytoplasm, nucleoplasm and nucleolus, respectively). **b** Nucleolar localization is confirmed by nucleolar marker nucleolin. **c**, **d** Neurons containing antisense RNA foci often have cytoplasmic aggregation of TDP-43 and neurons with sense RNA foci often have preserved nuclear TDP-43 (inset scale, 500 nm). **e** The percentage of neurons with nucleolar antisense RNA foci was significantly increased in neurons with cytoplasmic aggregation of TDP-43 compared to preserved nuclear TDP-43 ($p = 0.008$). **f** The percentage of neurons with nucleolar sense RNA foci was not significantly different in neurons with cytoplasmic aggregation of TDP-43 compared to preserved nuclear TDP-43 ($p = 0.7$, Mann–Whitney test). **g** The co-occurrence of nucleolar antisense RNA foci and cytoplasmic aggregation of TDP-43 is significantly greater than expected, while the co-occurrence of nucleolar sense RNA foci and cytoplasmic aggregation of TDP-43 is not. Area proportional Venn diagrams represent Fisher's exact test. Data are from the motor neurons within the anterior horn of the spinal cord

We evaluated the relationship between RNA foci and TDP-43 mislocalization using co-fluorescent in situ hybridization and immunofluorescence. We did this in neurons in the anterior horn of the spinal cord, where identification of motor neurons by location in the anterior horn is readily done (Fig. 1c, d). Nucleolar localization of RNA foci was confirmed by imaging through the *z*-plane. As previously reported [1], antisense RNA foci were associated with TDP-43 pathology and sense foci were not: antisense foci were seen in 67% of neurons with cytoplasmic aggregation of TDP-43 and 16% of neurons with preserved nuclear TDP-43 ($p = 0.008$; data not shown), compared to sense foci which were seen in 20% of neurons with cytoplasmic aggregation of TDP-43 and 18% of neurons with preserved nuclear TDP-43 ($p = 0.5$, data not shown). Importantly, the difference between antisense and sense foci held true for the perinucleolar RNA foci: perinucleolar antisense RNA foci were seen in 67% of neurons with cytoplasmic aggregation of TDP-43 and 20% of neurons with preserved nuclear TDP-43 ($p = 0.008$; Fig. 1e), compared to perinucleolar sense RNA foci, which were seen in 9% of neurons with cytoplasmic TDP-43 and 11% of

Table 1 Cytoplasmic aggregation of TDP-43 and nucleolar RNA foci in motor neurons within the anterior horn of the spinal cord by co-FISH-IF

	Nucleolar RNA foci				*p* value
	Cytoplasmic TDP-43		Nuclear TDP-43		
	Observed	Expected	Observed	Expected	
Antisense					
Foci positive	43	34	32	41	< 0.001
Foci negative	2	11	21	12	
Sense					
Foci positive	8	9	24	23	0.6
Foci negative	9	8	19	20	

neurons with preserved nuclear TDP-43 ($p = 0.7$; Fig. 1f and Table 1). Overall, the co-occurrence of TDP-43 abnormalities and perinucleolar antisense RNA foci was significantly greater than expected ($p < 0.001$; Fig. 1g), while the co-occurrence of TDP-43 abnormalities and perinucleolar sense RNA foci was as expected ($p = 0.6$; Fig. 1g).

The association of antisense RNA foci and nucleoli is important in light of recent discussions about nucleolar stress contributing to the pathogenesis of C9-ALS/FTD [6, 7, 10, 15]. The nucleolus is the site of many localized functions such as ribosomal biogenesis, and disruptions to nucleolar function can dramatically affect many functions such as rates of protein synthesis. Alterations in nucleolar morphology as well as altered rates of protein synthesis have been found in the C9-FTLD/ALS frontal cortex [10] and cellular model [6, 15], although not confirmed by all [14]. Thus far, sense RNA foci [6] and the arginine-containing DPRs, poly-GR and poly-PR [7, 15] have been implicated in causing, or at least to be associated with, nucleolar stress in C9-ALS/FTD pathogenesis. While antisense RNA foci were previously found to correlate with mislocalization of TDP-43 in C9-ALS [1] and antisense RNA foci studding of nucleoli have been observed in C9-FTLD [17], the co-occurrence of nucleolar antisense RNA foci with TDP-43 neuropathology has not been reported or quantitated. In our study, we found that nucleolar antisense RNA foci are significantly more associated with disease, especially with neuronal TDP-43 cytoplasmic aggregation and our findings bring the different aspects together. It has been suggested that nucleolar antisense RNA foci may form early in disease and precede TDP-43 aggregation [17].

In summary, antisense RNA foci have unique neuropathological features compared to sense RNA foci including nucleolar localization, anatomic distribution, and correlation with TDP-43 pathology. This reinforces that antisense RNA may play an important role in pathogenesis and be an important target for therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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