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R-loops highlight the nucleus in ALS

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Amyotrophic lateral sclerosis (ALS) is a severely debilitating neurodegenerative disease linked to mutations in various genes implicated in cytoplasmic RNA metabolism. Recent studies from genetic models have also helped reveal connections between various ALS-linked factors and RNA-DNA hybrid (R-loop) regulation. Here, we examine how such hybrid-regulatory processes are pointing to a key role for the nucleus in ALS. We also present a potential molecular mechanism in which hybrids may represent at least one of the long sought after missing links between different ALS genes. Our opinion is that RNA-DNA hybrids will play a key role in deciphering ALS and other human diseases.

Keywords: Amyotrophic lateral sclerosis (ALS), RNA-DNA hybrid, R-loop, genome instability, stress granules, C9ORF72, SOD1, Senataxin, Ataxin2, TDP43, FUS

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Beyond cytoplasmic RNA Dysregulation in ALS

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the loss of upper motor neurons in the cortex as well as lower motor neurons in the brainstem and spinal cord.[1] ALS cases are presented as 10% familial and approximately 90% sporadic in nature.[1] This relentlessly progressive disease is strongly linked to cytoplasmic proteinaceous aggregates associated with mutations in a number of genes including *SOD1* (superoxide dismutase), *TDP43* (TAR DNA-binding protein 43; a.k.a. *TARDP*), *FUS* (fused in sarcoma), *SETX* (senataxin), and *ATXN2* (ataxin-2), as well as repeat expansions at *C9ORF72* (chromosome 9 open reading frame 72) (**Table 1**).[2-8] Studies have pointed to the convergence of these seemingly disparate mutations onto RNA metabolism.[3-9] Specifically, a hallmark of ALS pathology is the accumulation of cytoplasmic aggregates containing translationally inert RNA and associated proteins, called stress granules.[9] Interestingly, the RNA-binding proteins TDP43, FUS, and ATXN2 strongly associate with these stress granules and are critical for their formation.[10,11] Moreover, mutations in these proteins as well as SOD1 and C9ORF72 are thought to induce the aberrant accumulation of stress granules and related RNA processing foci.[12,13] Thus, disruption of cytoplasmic RNA metabolism is closely associated with ALS.

However, it remains unclear if aberrant cytoplasmic RNA regulation is a driver or rather a bystander in ALS pathobiology. Could the dysregulation of cytoplasmic RNA metabolism be preventing RNA-binding proteins such as TDP-43, FUS, and/or ATXN2 from performing or promoting disease-suppressing nuclear functions? Could such nuclear roles help maintain overall genome structure and function? Recently uncovered connections between some ALS gene products and genome-destabilizing nucleic acid structures containing RNA-DNA hybrids (or R-loops) may help provide answers to these questions.[6,14-17] Here we explore how crosstalk between RNA-DNA hybrids and ribonucleoprotein bodies linked to RNA metabolism may lead to an overarching mechanism of ALS pathobiology. We also discuss how this mechanism could point to new connections between different genes commonly mutated in ALS patients. In addition, we highlight important future directions for research that promises to reveal even more intriguing links between RNA-DNA hybrid regulatory processes, neurodegenerative diseases in particular, and human health in general. We note that our overall goal

Table 1. ALS gene products proposed to play a role in an overarching pathobiological mechanism implicating nuclear RNA-DNA hybrids and cytoplasmic stress granules.*

ALS-linked gene	Suspected function/impact within ALS	Estimated % of FALS and SALS	Refs
C9ORF72 (Chromosome 9 open reading frame 72)	Codes for protein of unknown function. Intronic HRE generates G4DNA leading to shortened nucleolar-disrupting transcripts.	40% and 7%	7, 16, 41, 43
SOD1 (Superoxide dismutase 1)	Converts free radicals to hydrogen peroxide. Mutations may cause various cellular defects including genome and RNA metabolism-destabilizing free radical build-up.	20% and 3%	2
ATXN2 (Ataxin-2; PBP1 in yeast)	RNA binding protein that interacts with polyA-binding factors. Important for stress granule formation in yeast and human. Yeast protein key to RNA-DNA hybrid and R-loop suppression. Mutations may increase ALS risk by promoting hybrid accumulation at various genomic locations, which may also lead to aberrant transcript accumulations and RNA metabolism stress.	<1% and 5%	6, 15
TDP43 (TAR DNA-binding protein 43)	RNA binding protein involved in multiple mRNA processing activities. Mutations deplete protein from the nucleus and engage it in cytoplasmic ribonucleoprotein aggregates such as stress granules.	5% and 1%	3, 64, 68
FUS (Fused in sarcoma)	RNA binding protein with proposed roles in RNA metabolism. Mutations mimic and lead to TDP43 pathological features.	4% and <1%	5, 63
SETX (Senataxin; Sen1 in yeast)	Resolves RNA-DNA hybrids in both yeast and human. Mutations may increase ALS risk by promoting hybrid accumulation at various genomic locations, which may also lead to aberrant transcript accumulations and RNA metabolism stress.	Unknown	47

*For a full list of ALS genes, please see reference 37.

here is to present a broad and thought-provoking hypothesis that may connect seemingly unrelated processes without conducting a detailed review of the field.

RNA-DNA hybrids, R-loops, and Their Regulatory Processes

The ability of newly generated transcripts to ultimately leave their site of transcription is critical to overall cell function. However, it is becoming increasingly clear that newly synthesized RNA can invade the DNA duplex behind an advancing RNA polymerase.[12,13,17] This invasion generally occurs before the emerging transcript can be assembled into competing RNA-protein complexes.[18,19] Importantly, cells do have an arsenal of molecules that help prevent or eliminate such events.

Invasion of a DNA duplex by an emerging transcript creates a so-called R-loop structure, which harbours an RNA-DNA hybrid opposite a single-stranded DNA region (**Fig. 1A**). The ability of RNA to reinvade the DNA duplex is favored by the presence of guanine clusters (G-clusters) especially near the 5′ end of the emerging transcript.[20] In addition, such R-loop structures may be further stabilized if the displaced single-stranded DNA is prone to form a type of looped 3-dimensional structure known as G-quadruplex-DNA (G4DNA).[21] G4DNA sequences are abundant across the eukaryotic genome and are especially enriched within rDNA repeats and telomeres.[22,23] Furthermore, G4DNA-containing regions can generate G4RNA-harbouring transcripts, which could further compromise downstream RNA-regulatory processes.[24]

Although R-loops can provide an additional level of regulation of gene expression and possibly other chromosomal processes, aberrant R-loop accumulation is a major threat to genome stability.[17,25,26] Therefore, cells have evolved mechanisms to control R-loop levels across the genome (**Fig. 1**).[17] First, the suppression of transcription, rapid degradation, and/or export of hybrid-prone transcripts greatly limit excessive R-loop accumulation.[12,14,18,27,28] Second, conserved RNaseH enzymes can degrade the RNA component of formed hybrids.[29] Third, conserved enzymes including the Pif1 helicase can limit R-loops by unwinding G4DNA and/or resolving RNA-DNA hybrids.[30-32]

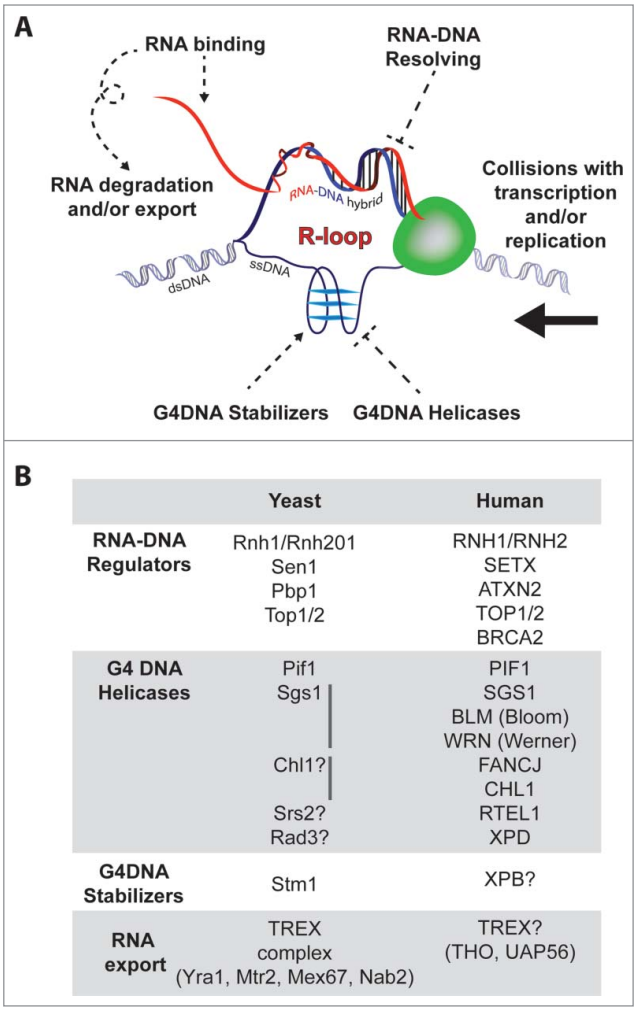
Factors linked to various diseases including premature aging and cancer have also been linked to R-loop regulation.[26,33-36] Intriguingly, a string of recent studies is revealing unexpected links between RNA-DNA hybrids and a number of factors that are typically mutated in ALS patients. Together, these studies raise the possibility that RNA-DNA hybrids could play a key role in ALS pathobiology.

RNA-DNA hybrids May Link *C9ORF72*, *SETX*, and *ATXN2*

Deciphering ALS has proven difficult as the disease is linked to mutations in an unexpectedly large number of genes (**Table 1**) (please also see ref. 37 for a full list of ALS genes). However, statistical data support the idea that a relatively small number of these genes such as *C9ORF72* are causal with the majority of other genes being disease modifiers (**Table 1**).[38-40] For

example, ALS modifier gene mutations may decrease the age of onset, hasten disease progression, or increase the severity of ALS pathobiology observed in patients carrying a causal mutation.[38,40] It remains difficult to completely eliminate the possibility that particularly severe mutations/disruptions of genes currently viewed as modifiers may actually be sufficient to trigger ALS.[38] Importantly, the fact that ALS is linked to many seemingly different genes has often hindered research aiming to decipher the pathobiological processes underlying the disease. However, recent studies are pointing to molecular connections between the different ALS genes. This may ultimately help decode the genetics of this devastating disease.

R-loops within C9ORF72 - Hexanucleotide (GGGGCC) repeat expansions (HRE) within the first introns of the *C9ORF72* locus constitute the most common genetic mutation in ALS patients, representing approximately 40% and 7% of familial and sporadic cases, respectively (**Table 1**).[7,8,16,41] Of note, these patients typically present with an overlapping disease termed frontotemporal dementia (FTD).[42] Although events leading to *C9ORF72* HRE remain unclear, *C9ORF72* HRE DNA sequences constitute G4DNA-containing R-loops that interfere with RNA Pol II transcriptional elongation and lead to the generation of truncated G4RNA-containing *C9ORF72* transcripts.[16,43] These truncated transcripts bind ribonucleoproteins including the nucleolar protein nucleolin, which in turn leads to the aggregation of aborted transcripts and associated factors within the nucleus and cytoplasm.[16,43] Consistent with this finding, C9ORF72 HRE ALS patient cortex tissues show evidence of nucleolar stress.[16] In addition, motor neurons derived from induced pluripotent stem cells of *C9ORF72* HRE patients exhibit nucleolin mislocalization.[16] Furthermore, the expression of $(GGGGCC)_{21}$ abortive transcripts mimics nucleolin pathological signatures observed in *C9ORF72* HRE ALS patient cells.[16] Considering the prevalence of *C9ORF72* HRE in ALS cases, these findings point to aberrantly formed/stabilized R-loops within *C9ORF72* HRE, as well as



B

	Yeast	Human
RNA-DNA Regulators	Rnh1/Rnh201 Sen1 Pbp1 Top1/2	RNH1/RNH2 SETX ATXN2 TOP1/2 BRCA2
G4 DNA Helicases	Pif1 Sgs1 Chl1? Srs2? Rad3?	PIF1 SGS1 BLM (Bloom) WRN (Werner) FANCJ CHL1 RTEL1 XPD
G4DNA Stabilizers	Stm1	XPB?
RNA export	TREX complex (Yra1, Mtr2, Mex67, Nab2)	TREX? (THO, UAP56)

Figure 1. R-loop regulatory factors. (**A**) RNA modulatory processes involving RNA binding, degradation, and export cooperate with RNA-DNA hybrid resolving/suppressing factors and G4DNA helicases to suppress R-loop accumulation. On the other hand, G4DNA stabilizing factors can promote R-loop accumulation. R-loop accumulation can lead to genome-destabilizing collisions with transcription and/or replication machinery. (**B**) Examples of yeast and human R-loop regulators that may or may not be linked to ALS pathobiology.

subsequent downstream deleterious effects of truncated G4RNA transcripts including nucleolar disruption, as a possible key player in ALS pathobiology.

SETX resolves RNA-DNA hybrids - Additional links between R-loop formation and ALS originated from the study of the yeast protein Sen1. Through its RNA-DNA resolving ability, Sen1 suppresses R-loop accumulation and related transcription-induced recombination events.[18] Of note, the ability of Sen1 to resolve RNA-DNA hybrids may also underlie the ability of this protein to mediate transcriptional termination by eliminating hybrids within the 3′UTR-coding region of genes.[44,45] *SETX* is the human ortholog of the yeast *SEN1* gene.[46] *SETX* mutations are linked to juvenile ALS (**Table 1**).[47] Of note, *SETX* mutations are also linked to another neurodegenerative disease called AOA2 (ataxia with occulomotor apraxia type 2), providing additional support for this gene having a critical role in neural function.[46] Importantly, similar to yeast Sen1, the human SETX protein unwinds RNA-DNA hybrids and prevents collisions between the transcription and replication machinery at RNA Pol II-transcribed genes across the genome.[14,48] Although it remains to be determined if the R-loop accumulation associated with

SETX mutations directly leads to neurodegeneration, it is clear that SETX-dependent suppression of RNA-DNA hybrids is important for the maintenance of genome stability.[14,48] Therefore, while *C9ORF72* HRE links R-loops and possible subsequent nuclear defects to ALS, SETX may point to a broader role for R-loop accumulation across the genome in ALS pathobiology.

Toward a function for the conserved ATXN2 in hybrid suppression - Further support for links between ALS and the nucleus in general, as well as ALS and R-loops in particular, has recently been provided by a study in which we focused on the yeast RNA-binding protein Pbp1 (Pab1-binding protein 1). Eukaryotic rRNA genes (rRNA genes or rDNA) are arranged as tandem repeats on one or more chromosomes and provide the foundation for the nucleolar compartment.[49] The high copy number helps sustain ribosomal biogenesis and general protein synthesis. Unequal DNA recombination between rDNA units within the repeats allows cells to increase or decrease the number of rDNA units in response to stress conditions.[49] However, deregulated recombination within rDNA repeats can lead to chromosome instability and shorten cellular lifespan.[50-52] Importantly, non-coding transcripts are generated from bidirectional promoters within intergenic spacers located between rDNA units.[53-55] The disruption of ncRNA-suppressing processes such as silent chromatin assembly, early transcriptional termination, or exosome-mediated degradation leads to the accumulation of these ncRNAs but to varying degrees of rDNA instability.[53-55] This suggested that additional mechanisms might be preventing accumulating ncRNAs from destabilizing rDNA repeats. Indeed, we found that Pbp1, which harbours a Like SM (LSM) RNA-binding domain, binds to these intergenic ncRNAs and prevents them from engaging in non-coding RNA-DNA hybrids (ncRNA-DNA).[15] Importantly, substituting full length Pbp1 with a Pbp1 mutant that is lacking the Pab1-binding domain but not the LSM RNA-binding domain did not compromise ncRNA-DNA hybrid suppression.[15] Moreover, we found that a G4DNA-stabilizing protein called Stm1 further stabilizes R-loops formed in Pbp1-deficient cells. Intriguingly, caloric restriction, a dietary regimen in which the amount of glucose available to cells is decreased, essentially abolished hybrid accumulation in Pbp1-deficient cells.[15] This ability of caloric restriction is somehow dependent on the yeast RNaseH enzymes Rnh1 and Rnh201 as well as the G4DNA helicase Pif1, which may also have RNA-DNA hybrid resolving activity.[15,32] Of note, Pbp1 also suppresses hybrids at non-rDNA G4DNA sites near telomeres as well as within a couple of tested open reading frames pointing to a potentially broader role in R-loop suppression across the genome.[15] Furthermore, we observed that the R-loop suppressing ability of Pbp1 maintains cellular lifespan.

Extreme expansion of a polyglutamine (polyQ) site within human ATXN2, the ortholog of yeast Pbp1, was initially identified in spinal cerebellar ataxia type 2.[56-58] More recently, intermediate ATXN2 polyQ expansions were identified as a major risk factor for ALS.[6] An intriguing possibility is that *ATXN2* polyQ expansions may lead to R-loop accumulation within key coding or non-coding DNA sequences within loci with a central role in neural function. That various functions of Pbp1 and ATXN2 are likely conserved is supported by the fact that both proteins have LSM domains and play a role in the establishment of stress granules under stress, a point that we will further explore below.[6,59,60]

How R-loops may link C9ORF72, SETX, and ATXN2 - The disruption of an RNA-DNA hybrid-suppressing role for *ATXN2* and/or *SETX* could lead to R-loop accumulation at the G4DNA-containing *C9ORF72* HRE. This may further promote HRE expansion and its downstream effects on truncated G4RNA-containing transcripts as well as the sequestration of nucleolin. Consistent with this rationale, a recent study identified intermediate length polyQ expansion of *ATXN2* as a critical disease modifier in *C9ORF72* HRE patients.[40] Alternatively, *C9ORF72* HRE may be operating upstream of wild-type ATXN2. In this case, it is possible that accumulating truncated G4RNA-containing *C9ORF72* HRE transcripts may sequester the RNA-binding ATXN2 along with nucleolin within ribonucleoprotein aggregates.[16] This may in turn further promote *C9ORF72* HRE and help establish a vicious cycle. Through these connections, it is possible that ALS-linked genetic defects in *C9ORF72*, *SETX*, and *ATXN2* all lead to broader hybrid accumulations throughout the genome leading to chromosomal features that would be particularly destabilizing to the transcriptionally active neurons.

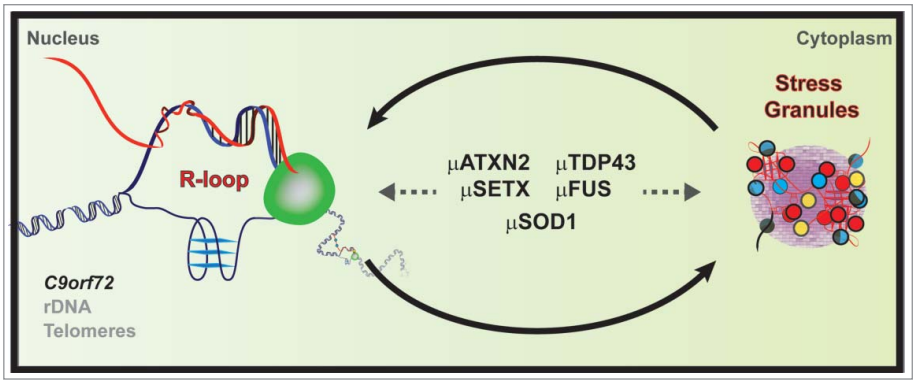


Figure 2. Putative feedback loop between R-loops and stress granules may connect various ALS genes at the molecular level. Mutations (μ) in ALS-linked factors can abrogate their ability to suppress R-loop formation and/or lead to their aberrant sequestration in RNA-processing foci such as cytoplasmic stress granules. Increased R-loop accumulation in turn leads to genomic instability and aberrant transcript accumulations, which in turn further promotes stress granule formation and the cycle continues. Motor neurons are predicted to be particularly sensitive to such a deleterious and vicious cycle.

Deleterious Feedback Loop Between RNA-DNA Hybrids and Stress Granules May Connect The Dots Between ALS Genes

While *C9ORF72*, SETX, and ATXN2 may control the formation and stability of RNA-DNA hybrids, cytoplasmic aggregation/retention of RNA-binding proteins, especially TDP43 and FUS, are a key hallmark of ALS pathology. One possibility is that nuclear R-loops and cytoplasmic ribonucleoprotein aggregates are part of a self-reinforcing loop that gains strength with disease progression (**Fig. 2**). Alternatively, it is possible that proteins such as TDP43 and FUS play unforeseen direct roles in RNA-DNA hybrid suppression.

Both TDP43 and FUS can bind DNA and RNA.[61-63] In addition, both proteins have been linked to various nuclear and cytoplasmic steps of mRNA processing and transport.[64-66] In contrast, ALS-linked mutant TDP43 is depleted from the nucleus, aggregates within the cytoplasm of affected neurons, and associates with stress granules.[67,68] Additionally, FUS accumulates at stress granules within the degenerating neurons of ALS patients.[5,11] Interestingly, ATXN2 associates with both TDP43 and FUS and is thought to alter TDP43 toxicity and FUS-related pathology.[6,11] Furthermore, ATXN2 is a component of stress granules in yeast and human cells.[59,69] Together, these findings point to a number of urgent questions. Are RNA-DNA hybrids accumulating excessively when TDP43 or FUS are aberrantly localized to stress granules? If R-loops are indeed forming, can this be attributed to an ability of mutant TDP43 or FUS to sequester ATXN2 into cytoplasmic stress granules? Alternatively, can nuclear TDP43 and FUS play a role in R-loop suppression? And if so, would that affect *C9ORF72* or intersect with the hybrid-suppressing ability of SETX and possibly ATXN2?

Concluding Remarks and Broad Questions

Overall, we have explored how excessive R-loop accumulation may connect the dots between different ALS genes and move us a step closer toward a putative unifying molecular model of ALS pathogenesis. We expect that future work by others and us will put this model to the test. It will also be critical to determine if excessive RNA-DNA hybrid levels are the underlying cause of motor neuron degeneration in ALS. Are hybrids accumulating in ALS patient-derived neurons? Can interventions aiming to alleviate hybrid accumulation result in reduced neurodegeneration? Do increased R-loop levels play a role somewhere along the pathway to degeneration? Does the loss of R-loop regulation act as a trigger that leads to toxic nuclear and cytoplasmic ribonucleoprotein aggregations? Importantly, R-loops are emerging as key players in a number of diseases including cancer and premature aging.[30,31,34] Thus, major efforts are needed to fully investigate connections between R-loops, ALS, and human health.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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