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Friedreich's ataxia – a case of aberrant transcription termination?

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Reduced expression of the mitochondrial protein Frataxin (FXN) is the underlying cause of Friedreich's ataxia. We propose a model of premature termination of *FXN* transcription induced by pathogenic expanded GAA repeats that links R-loop structures, antisense transcription, and heterochromatin formation as a novel mechanism of transcriptional repression in Friedreich's ataxia.

Keywords: antisense transcription, Friedreich's ataxia, GAA repeats, R-loops, repeat expansion diseases, transcription termination

Abbreviations: FRDA, Friedreich's ataxia; FXN, Frataxin; H3K9me2/me3, histone H3 lysine 9 methylation; RNAP II, RNA polymerase II; dsRNA, double-stranded RNA; UTRs, untranslated regions; PAS, polyadenylation signal.

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Expanded GAA Repeats Induce an Altered Chromatin Environment at the *FXN* Locus in Friedreich's Ataxia

Tandem repeats make up ~3% of the human genome.[1] These sequences are often highly polymorphic and both their normal variability as well as pathological changes can drastically affect phenotype, predominantly via altering gene expression. Mutations in selected short tandem repeats composed of 3–6 nucleotide units are responsible for ~30 severe neurological and neuromuscular diseases, including fragile X syndrome, myotonic dystrophy, Huntington's disease, and Friedreich's ataxia (FRDA).[2] Friedreich's ataxia, the most common inherited form of ataxia, is a fatal neurodegenerative disease that results from large expansions of GAA trinucleotide repeat sequences in intron 1 of the *Frataxin* (*FXN*) gene, leading to transcriptional repression.[3] While unaffected individuals carry less than 40 repeats, patients have large GAA tracts reaching several hundreds of GAAs.[3]

In 2003, Saveliev et al.[4] demonstrated that insertion of these expanded polypurine-polypyrimidine repeats induce position effect variegation leading to transcriptional silencing of the nearby located transgenes. Detailed histone modification analyses conducted in various FRDA animal and cell line models, as well as in FRDA patient cells, readily confirmed the heterochromatin-like characteristics of this locus, featuring enriched histone H3 lysine 9 methylation (H3K9me2/me3) and decreased histone H3 and H4 acetylation.[5-7] These GAA-induced chromatin changes were localized in direct proximity of the GAA repeats and did not spread more than 2 kb from the expanded repeats.

Although significant downregulation of *FXN* expression (~75-95%) is undoubtedly the underlying cause of the FRDA, the molecular trigger as well as mechanisms of the transcriptional repression induced by the expanding intronic GAA repeat tract are unclear. Importantly, transcript stability and *FXN* pre-mRNA splicing are not affected by GAA expansion, nor has formation of GAA repeat-containing RNA foci been detected in FRDA samples.[8] A consensus in the field exists regarding significant changes in histone modification patterns induced by GAA-expansions. Treatment with particular histone deacetylase inhibitors results in the moderate elevation of frataxin mRNA levels and is accompanied by increased histone acetylation both in the promoter and vicinity of the GAAs.[8,9] On the contrary, treatment with the G9a histone methyltransferase inhibitor BIX-01294 decreases the level of H3K9me2 but fails to reactivate *FXN* expression.[10] Additional investigation into potential combinatorial approaches is necessary to fully elucidate the effects of histone modification-targeted therapies on *FXN* transcription.

Evidence has been presented indicating that transcription initiation at the *FXN*

promoter is affected by spreading of the heterochromatin-like environment toward the transcription initiation region.[11,12] Other studies indicated that transcription elongation rather than initiation is critically affected by expanded GAAs.[10,13,14] RNA polymerase II (RNAP II) profiling data (both total and serine 2 phosphorylated RNAP II) showed that RNAP II recruitment and transcription initiation proceed with similar efficiency in both control as well as in FRDA cells.[10] Notably, an antisense transcript termed *FAST1* has been identified in proximity to the GAA repeats.[15] Strand-specific RNAP II profiling experiments could determine whether transcription initiation and elongation of sense and antisense transcripts contribute to this conflicting data. Pre-mRNA quantitation analyses demonstrated a significant decrease of the primary transcript downstream of the expanded GAAs, indicating that elongation is the primary step affected by GAA expansion.[14] These conclusions are supported by RNA sequencing analyses of ribo(-) transcripts in control and FRDA cell lines showing a defect in transcription elongation rate (Li et al., unpublished).

R-loop formation inhibits *FXN* transcription

The critical link between the DNA mutation and silencing of *FXN* is missing. Results of 2 recent studies point toward a novel molecular mechanism by which long GAAs can impede transcription. First, Groh et al.[13] uncovered that expanded GAAs form R-loop structures in both human FRDA cells and model cell lines, and elimination of these DNA-RNA hybrids by RNase H1 overexpression reactivated frataxin transcription in a luciferase reporter system. R-loop formation has been observed for other expanded repeats, including transcribed CTG, CGG, CCCCGG repeats,[16-19] as well as at GAAs in prokaryotic models.[20] The work by Groh et al. was the first report showing that these structures *per se* may be important not only for GAA instability but also for transcriptional silencing. Thus, these analyses identified a likely trigger for transcriptional inhibition. However, the exact mechanism linking R-loops with chromatin changes remained unknown.

R-loops have been implicated in a plethora of molecular processes including DNA rearrangements, repeat instability, recombination and silencing of centromeric chromatin (reviewed in[21]). Recent work by Skourti-Stathaki et al.[22] revealed a critical connection between R-loop formation, repressive chromatin marks and transcription terminators in mammalian genes. Initial studies by the same group demonstrated that R-loops facilitate RNAP II pausing before efficient termination in yeast.[23] Continuation of these studies in mammalian cells revealed that R-loops located nearby transcription termination regions represent RNAP II pause sites that induce antisense transcription leading to the recruitment of DICER, AGO1 and AGO2. Moreover, G9a histone methyltransferase recruited to these R-loop regions deposits H3K9me2 marks further stimulating the recruitment of HP1γ and reinforcing RNAP II pausing at the termination regions.[22] These findings were supported by genome-wide analyses indicating that the R-loop-mediated termination mechanism applies to a substantial subset of mammalian genes.[22]

Altogether, the formation of R-loops, increased levels of H3K9me2 and HP1γ binding, and the presence of the antisense *FAST1* transcript all in proximity to the repeats in FRDA patient cells strongly suggest that a similar, RNAi-based transcription termination mechanism may be responsible for aberrant *FXN* silencing (**Fig. 1**). *FAST1* expression is significantly higher in FRDA cells when compared to controls lacking expanded GAAs,[15] thus mimicking R-loop stimulated antisense transcription at the termination regions.[22] Moreover, the *FAST1* transcript was also demonstrated to form a double-stranded RNA (dsRNA) with the *FXN* sense transcript, and the RNA duplex interacted with argonaute proteins, Ago 1 and Ago 2.[24] However, the identified *FXN-FAST1* dsRNA aligns to the transcription initiation region and not to the GAAs. Results of a recent genome-wide screen aimed to uncover a modulator of GAA repeat stability and fragility in yeast demonstrated that expanded GAAs serve as promoters and recruit transcription initiation factors,[25] suggesting a potential mechanism to generate GAA•TTC dsRNA from the GAA repeat region. Therefore, either *FAST1* is a longer antisense transcript originating at the GAA region, or a second putative antisense transcript is initiated at the expanded GAA repeats (**Fig. 1B**).

Premature termination of *FXN* transcription is induced by the GAA repeat expansion in FRDA

A clear resemblance between R-loop mediated transcription termination at the 3′ untranslated regions (UTRs) and silencing of the mutated *FXN* gene suggests that transcriptional deficiency in FRDA may not be caused simply by defective initiation or elongation, but rather might involve recognition of aberrant transcription termination signals. Indeed, transcription through the expanded GAAs can be restored by overexpression of RNase H but not by inhibition of H3K9me2 methylation, similar to the 3'UTR R-loop associated termination.[10,13,22] Additionally, termination of transcription frequently occurs at multiple locations within a 3′ UTR, demonstrating that some termination signals can be bypassed by elongating RNAP II. In support, FRDA cells harboring expanded repeats express a small fraction, 5–25%, of *FXN* mRNA relative to control cells indicating that the GAA-mediated transcription impediment is not 100% efficient and can be overcome, allowing for some transcripts to escape premature termination.

RNAP II pausing and transcription termination occurs in the R-loop region downstream of the polyadenylation signal (PAS) in the subset of genes identified as containing pause-type termination regions.[22] Intron 1 of the *FXN* gene shares some characteristic sequence features of transcription termination regions. Notably, several canonical AAUAA signals are present in the vicinity of the GAA repeats followed by GU rich sequences resembling the downstream sequence element shown to facilitate 3′ end mRNA formation. Additionally, polymorphisms of that region, especially polyA stretches 5′ and 3′ of the GAAs, may facilitate transcription termination when R-loops are present. In fact, the GAA region in the *FXN* gene evolved from an Alu element,[26] and polyA sequences typical for Alu sequences have

been mutated to create canonical AAUAAA PAS motifs.[27]

Thus, R-loop formation, followed by antisense transcription and activation of the RNAi pathway could lead to dsRNA-induced local chromatin changes and aberrant transcription termination at the mutated *FXN* gene. This model, if validated in FRDA cells, could shift therapeutic efforts toward alleviating R-loop formation at the GAA repeats rather than targeting the silencing histone marks that appear to be a consequence of the DNA-RNA hybrid formation. Until recently R-loops have been considered a rare transcription nuisance that may affect genome stability. Discoveries of the past 2 years have demonstrated that these hybrid structures are rather frequent and are indispensable to gene expression regulatory processes.[21] Therefore, targeting pathogenic R-loops has to be conducted very precisely and specifically without affecting physiologically important DNA-RNA hybrids.

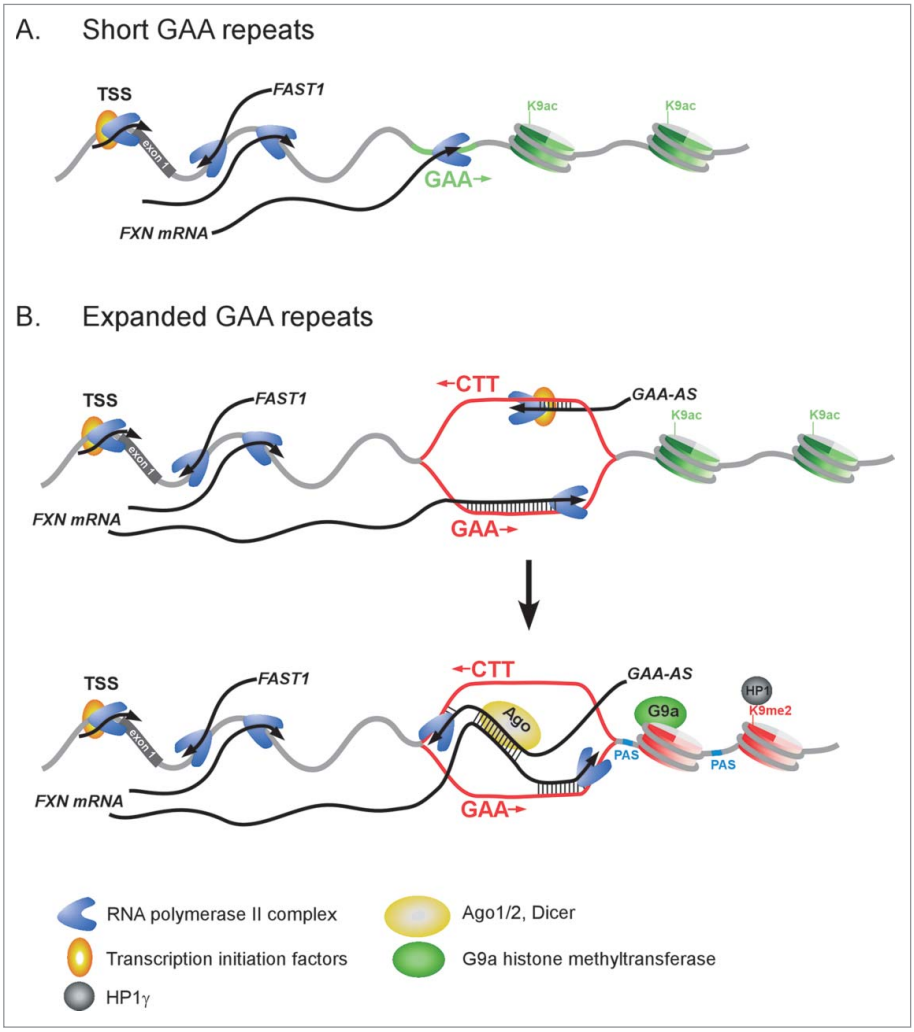

Figure 1. *Proposed model of R-loop induced aberrant transcription termination of the FXN gene at the expanded GAA repeats.* (**A**) Transcription of *FXN* harboring short GAAs (depicted as a green line) in unaffected cells. (**B**) Transcriptional termination at the expanded GAA region (depicted as a red line) is initiated by formation of R-loops between the *FXN* mRNA and DNA template strand. R-loops can stimulate antisense transcription (either *FAST1* or putative *GAA-AS*) at the GAA repeats. Recruitment of transcription factors to the GAAs can initiate synthesis of the antisense transcript at the repeat region. Bidirectional transcription of the expanded GAA•TTC sequences facilitates formation of a dsRNA between coding and non-coding strands, recruiting Ago/Dicer and histone methyltransferase (G9a), and leading to histone H3K9 methylation and HP1γ binding. Heterochromatin formation and appropriate sequence context (multiple PAS) result in recurrent, aberrant termination of RNA synthesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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