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BRIEF REPORT

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STUB1 polyadenylation signal variant AACAAA does not affect polyadenylation but decreases *STUB1* translation causing SCAR16

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Abstract

We present three siblings afflicted with a disease characterized by cerebellar ataxia, cerebellar atrophy, pyramidal tract damage with increased lower limb tendon reflexes, and onset of 31 to 57 years, which is not typical for a known disease. In a region of shared homozygosity in patients, exome sequencing revealed novel homozygous c.*240T > C variant in the 3′UTR of *STUB1*, the gene responsible for autosomal recessive spinocerebellar ataxia 16 (SCAR16). In other genes, such an alteration of the evolutionarily highly conserved polyadenylation signal from AATAAA to AACAAA is known to highly impair polyadenylation. In contrast, RNA sequencing and quantification revealed that neither polyadenylation nor stability of *STUB1* mRNA is affected. In silico analysis predicted that the secondary structure of the mRNA is altered. We propose that this change underlies the extremely low amounts of the encoded protein in patient leukocytes.

KEYWORDS
3′UTR, cerebellar atrophy, polyadenylation, SCAR16, STUB1

Autosomal recessive cerebellar ataxias display clinical and genetic heterogeneity, and a rare form is spinocerebellar ataxia 16 (SCAR16) (Shi et al., 2013). SCAR16 is characterized by generally adolescent-onset ataxia with cerebellar atrophy, gait instability, and cognitive impairment (Depondt et al., 2014). The disease is caused by biallelic variants in *STUB1* (MIM# 607207), which encodes STIP1 homology and U-box containing protein 1. In total, 27 different homozygous or compound heterozygous variants have been reported, but none is a polyadenylation signal alteration (Supplementary Table S1). In several other genes many examples of such signal variants have been shown to impair polyadenylation, and thus eventually either the protein is synthesized at a very low level or its sequence is altered.

Polyadenylation signal AATAAA is the best known of all regulatory elements in the 3′UTR of mammalian mRNA and is required for efficient termination of transcription, cleavage at a specific site downstream of the signal, and addition of generally 150–250 adenosine nucleotides to the 3′ end (Bienroth, Keller, & Wahle, 1993; Guhaniyogi & Brewer, 2001). The poly(A) tail has a role in mRNA stability and translation efficiency (Faure, Ogurtsov, Shabalina, & Koonin, 2016; Yang et al., 2003). Polyadenylation signal sequence is highly conserved in human genes. For example, in the position of T, C has not been reported

in any gene but G has been reported in 2.99% of human genes (Tian, Hu, Zhang, & Lutz, 2005). In vitro studies have shown that replacing the T at this position by a C decreases mRNA polyadenylation to 4.0±2.0% of wild type in a simian virus 40 gene (Sheets, Ogg, & Wickens, 1990). T > C transition in the signal sequence of *HBB* in beta-thalassemia patients was shown to impair processing after the signal site (Orkin, Cheng, Antonarakis, & Kazazian, 1985). The same mutation in *BMP1* causes a bone fragility syndrome due to low levels of the transcript and thus decreased amounts of the encoded protein (Fahiminiya et al., 2015). We identified the same variant in *STUB1* and showed that the novel homozygous variant causes a drastic decrease in the amount of cellular STUB1 without impairing the polyadenylation or the stability of the mRNA.

Other sequences in the 3′UTR can play roles in various post-transcriptional regulation mechanisms including stability, nuclear transport, and subcellular localization (Kislauskis, Li, Singer, & Taneja, 1993). Some of those functions are hypothesized to mainly depend on the secondary structure of the molecule, for example, by rendering *cis* elements such as target sequences of miRNAs more exposed (Haas, Sczakiel, & Laufer, 2012) or impairing the rate of translation (Faure et al., 2016). Thus, sequence alterations in the 3′UTRs of many genes have been reported to lead to disease.

We performed clinical and genetic investigations on a family from Northern Cyprus. The three affected sibs had cerebellar ataxia, cerebellar atrophy, and pyramidal tract damage with increased lower limb tendon reflexes but no Babinski response, ankle clonus, lower limb spasticity, or hypogonadism, which is not typical for a known disease. They were born to first-cousin parents, and 16 of their unaffected relatives also participated in the study (Figure 1A). Written informed consent for the study was obtained from all participants according to the regulations of the Boğaziçi University Institutional Review Board for Research with Human Participants, which approved the study protocol.

Neurologic examination for all three patients was indicative of cerebellar ataxia, and brain MRI results showed marked pure cerebellar atrophy (Figure 1B). A summary of the findings is presented in Supplementary Table S2. Age of onset was 57 years for male patient and 31 and 37 years for female patients (217 and 209, respectively). Patients had scanned speech and displayed wide based gait. Dysmetria, dysdiadochokinesia, and action tremor were found in the finger-to-nose and rapid alternating movements tests. Increased deep tendon reflexes without Babinski response or spasticity, indicative of pyramidal tract damage, was found, and nystagmus and cognitive dysfunction were observed in patient 217. Clinical course varied; patient 217 was the most severely affected of all, with earliest onset and fastest disease progression—loss of ambulation at 38 years of age, bedridden at 44 years, and death at 47 years due to the disease. The other 16 members of the family did not have any clinical phenotypes.

Genetic studies, RNA analysis and immunoblotting assay were performed to investigate the molecular etiology of the disease in the family (see, supplemental material and methods). We first used the dense SNP data of a mixture of patient DNA samples to identify regions of shared homozygosity in patients who were possibly identical by descent from a recent common ancestor. We identified five such regions >1 Mb that totaled to approximately 12.4 Mb (Supplementary Table S3) and together harbored 127 genes. To search for the putative causative mutation in the candidate regions, we evaluated novel and rare (<0.01 in all populations reported) variants in the exome file of a patient. We found three candidates among the exonic, splicing and UTR variants that were possibly damaging to the protein encoded (Supplementary Table S4). The two nonsynonymous variants were predicted as not damaging to protein by three or all four of the computational algorithms we used. The remaining variant disrupted the polyadenylation signal of a known SCA gene, namely, *STUB1*. Thus, we hypothesized that this latter variant underlies the disease in the patients: *STUB1* 3′UTR c.*240T > C (NM_005861.3) (Figure 1C), altering the polyadenylation signal AATAAA to AACAAA. Online tool DNA Poly(A) Signal Miner (https://dnafsminer.bic.nus.edu.sg/PolyA.html) predicted that the polyadenylation signal common to both transcript isoforms of the gene was most likely disrupted. The variant was found to segregate with the disease (Supplementary Figure S1A). It was not present in any database and is located in a highly conserved region of the gene (Supplementary Figure S1B). The predicted consequence of the disruption of the signal is that the new sequence is not recognized, and thus cleavage of the pre-mRNA at a downstream site and subsequent addition of a poly(A) tail would not occur. This prompted us to investigate whether polyadenylation of *STUB1* mRNA is impaired.

We sequenced *STUB1* cDNA generated from total RNA isolated from blood cells of patient 205 and noncarrier sibling 203 as control using a forward amplification primer specific to the junction of exons 6 and 7 and reverse primer R1 specific to the junction of encoded sequences and the poly(A) tail (left) or reverse primer R2 specific to genomic sequences downstream of the signal (right) (Supplementary Figure S2A). The sequences reflected the genotypes of the sampled individuals, with substituted C clearly present in the patient sample, indicating that normal polyadenylation had taken place (Supplementary Figure S2B). We then determined the relative levels of wild type and mutant *STUB1* transcripts to investigate whether the stability is altered. Real-time PCR using cDNA samples generated from total RNA from two patients (209 and 217), two heterozygotes for the variant (215 and 307), and two noncarriers (310 and 314) indicated that there was no remarkable difference in the results for cells with different genotypes (Supplementary Figure S2C and S2D).

Not finding any difference in polyadenylation or the amounts of mRNA in cells with different genotypes prompted us to investigate for any differences in the secondary structures of the allelic *STUB1* mRNA molecules. Computational RNA secondary structure predictions using RNAfold web server indicated that the 3′UTR sequences of the reference and variant alleles were similar in two regions (nucleotides c.*1–237 and c.*252–278) but not within the region in between (nucleotides c.*238–251) that contains the signal (c.*238–243). Reference T was predicted to locate in an unpaired hairpin loop whereas variant C was in a paired structure of the junction of two loops. Additionally, free energies of the thermodynamic ensemble (ΔG) of the secondary structures were predicted by Gibbs free energy minimization. The reference transcript was found to have a slightly lower ΔG (−104.84 kcal/mol) than the variant transcript (−105.88 kcal/mol), indicating that the former was somewhat more stable (Supplementary Figure S3). mRNA

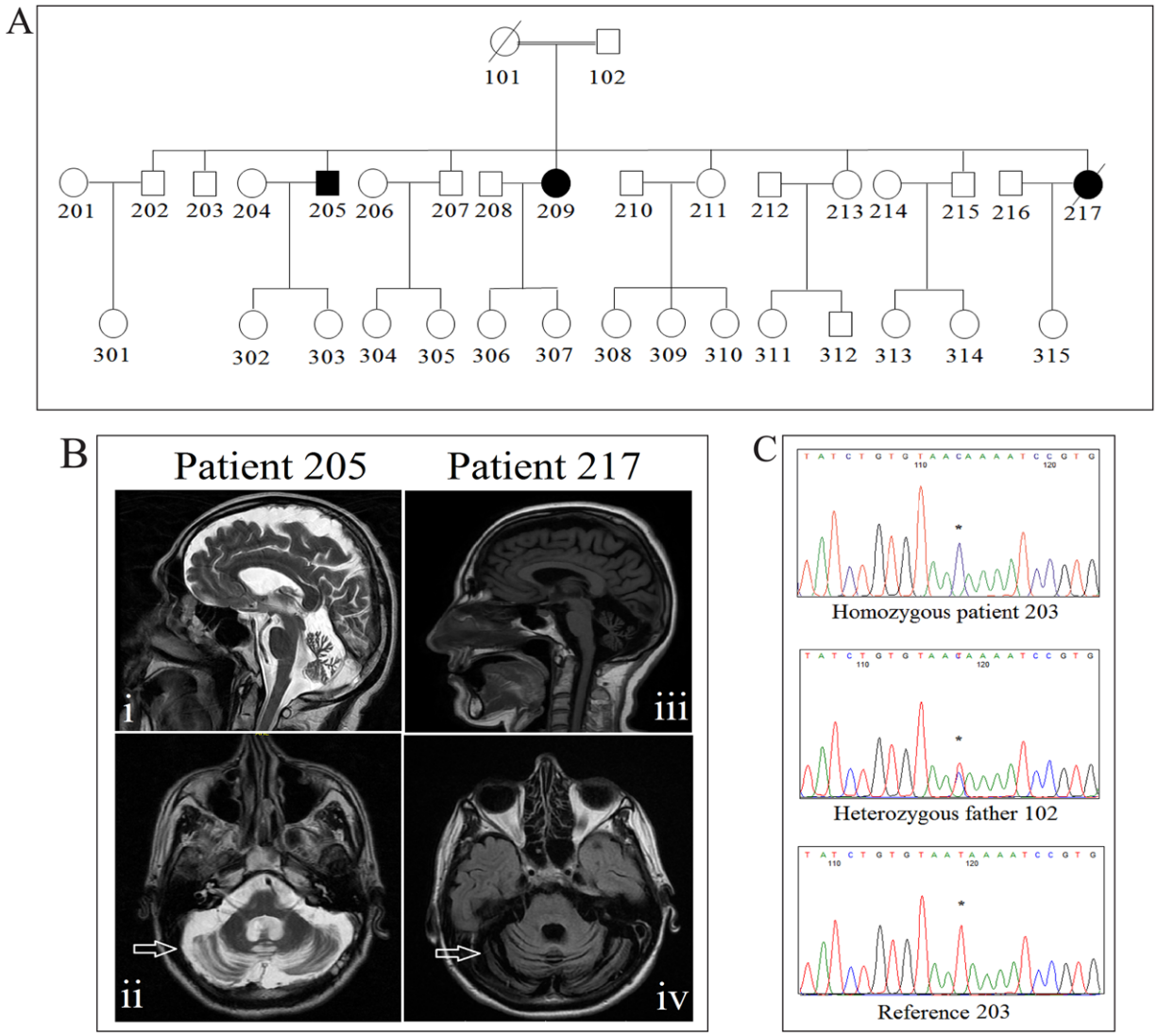

FIGURE 1 Patients and mutation. A: Pedigree of the family. Parents are first cousins. Shading indicates affected status. B: Noncontrast brain MRI scans for T2 sagittal sequence **(i)**, Axial sequence **(ii)**, T1 sagittal sequence **(iii)** and FLAIR sequence **(iv)**. Severe cerebellar atrophy (arrows) without any signal change in white and grey matters is evident. C: Electropherograms for *STUB1* c.*240T > C

secondary structures of reference and variant alleles were compared also by RNAstructure web server, and similar results were obtained. In total, 20 different mRNA structure models were identified for each allele. Again, T was predominantly located in an unpaired hairpin loop structure whereas 18 out of the 20 predicted structures (90%) of variant C were intramolecularly paired in conjunction of two loops. Comparing structures of the same window size for the transcripts indicated that in all cases the free energy value for the T transcript was lower than for the C transcript, suggesting that the T transcript is more stable (data not shown).

To compare the abundance of STUB1 in individuals with homozygous, heterozygous, and wild type genotypes, total protein samples extracted from leukocytes from peripheral blood were resolved on SDS-PAGE and immunoblotted using STUB1 and ACTIN-specific antibodies. A semiquantitative protein expression assay revealed a drastically low amount (11%) of STUB1 in the leukocytes of the two surviving patients as compared to heterozygous cells (58%) and wild type cells (100%) (Figure 2).

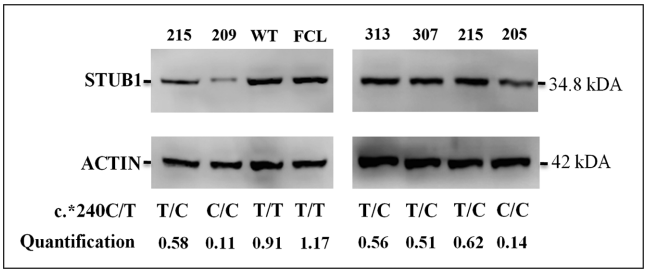

FIGURE 2 Protein quantification for cells with different genotypes. Comparison of STUB1 in leukocytes from heterozygous 215, patient 209 and wild type (WT), and commercial human fibroblast cell line (FCL) used as control (left). Similar assay for heterozygous 313, 307 and 215 and patient 205 (right). STUB1 was normalized to ACTIN, which was set to 1.00, and the ratio of intensity of STUB1 to ACTIN was calculated

Considering that the gene of the novel 3′UTR variant we identified is responsible for SCAR16, the variant is in homozygous state in

patients and not in any other family member, and the amount of the encoded protein is drastically low in patients, we hypothesize that the variant underlies the disease in the family, even though the disease is not typical SCAR16. All patients have the clinical signs of pyramidal tract damage with increased lower limb tendon reflexes commonly observed in SCAR16 but no Babinski response, ankle clonus, lower limb spasticity, or hypogonadism, which also have been reported in SCAR16. Clinical phenotype of STUB1 deficiency is generally variable (MIM# 615768). Also, onset of the ataxia in our patients is considered late (between 31 and 57 years of age), as to date only two patients have been reported with late ataxia onset; one was a 33-year-old woman with hypogonodotropic hypogonadism and the other was a 49-year-old man with lower limb spasticity (Heimdal et al., 2014; Synofzik et al., 2014). Onset of the ataxia in SCAR16 is variable and can be as early as infancy but is generally mid-twenties. Perhaps the late onset in our patients is due to the synthesis of some wild type protein, as observed in Figure 2.

The consensus polyadenylation signal hexamer is highly conserved across mammals. In vitro nucleotide substitutions within a simian virus 40 polyadenylation signal sequence indicated that alterations in the sequence decrease polyadenylation to different degrees (Sheets et al., 1990). Also, alterations in polyadenylation signals have been reported in some disease genes, mostly in *HEMOGLOBIN-BETA LOCUS (HBB)* and *HEMOGLOBIN-ALPHA LOCUS 2 (HBA2)*. The same T > C transition we identified in *STUB1* (AATAAA > AACAAA) was reported in *HBB* in several homozygotes with intermediate or severe beta-thalassemia (Haddad et al., 2017; Italia et al., 2012; Losekoot et al., 1991; Serjeant et al., 2011). The variant drastically impairs RNA cleavage and polyadenylation at the regular site, and polyadenylation occurs instead at an alternative signal 900 bases downstream of the altered signal (Orkin et al., 1985). Variant AACAAA has been reported also for *BMP1*, also in homozygous state, in three unrelated children with a bone fragility syndrome, and low levels of the transcript were found in skin fibroblasts (Fahiminiya et al., 2015). Our findings contrast with both of those reports, as we found that mRNA was polyadenylated at the appropriate site and its level was normal, suggesting that instead transport of the mRNA to cytoplasm or its translation could have been impaired, or even the protein could be misfolded due to a decrease in the rate of translation.

In some genes sequence alterations in 3′UTR sequences outside of the signal of many genes have been reported to lead to disease, as some such sequences play roles in various posttranscriptional regulatory mechanisms including stability, nuclear transport, and subcellular localization of mRNA (Hesketh, 2004; Kislauskis et al., 1993). Some of those functions are hypothesized to mainly depend on the secondary structure of the molecule, for example, by rendering *cis* elements such as target sequences of miRNAs more exposed (Haas et al., 2012). Another mechanism is that altered mRNA secondary structure affects the ribosomal speed, which in turn causes the nascent peptide to fold differently (Faure et al., 2016; Kaiser, Goldman, Chodera, Tinoco, & Bustamante, 2011). Misfolded proteins are usually degraded. As the variant in our patients does not affect polyadenylation or stability of the transcript, the drastic decrease in the cellular amounts of STUB1 is likely a consequence of the change in the 3D structure the transcript through one of these mechanisms. Our findings are unique in the sense that signal variant AACAAA in our patients does not impair processing at the mRNA 3′UTR indicating that other elements are important in this process. Further studies could unravel new *cis* element(s) that causes the polyadenylation machinery to recognize this signal variant in the *STUB1* transcript despite the alteration.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

Bienroth, S., Keller, W., & Wahle, E. (1993). Assembly of a processive messenger RNA polyadenylation complex. *EMBO J*, *12*(2), 585–594.

Depondt, C., Donatello, S., Simonis, N., Rai, M., van Heurck, R., Abramowicz, M., … Pandolfo, M. (2014). Autosomal recessive cerebellar ataxia of adult onset due to STUB1 mutations. *Neurology*, *82*(19), 1749–1750.https://doi.org/10.1212/WNL.0000000000000416

Fahiminiya, S., Al-Jallad, H., Majewski, J., Palomo, T., Moffatt, P., Roschger, P., … Rauch, F. (2015). A polyadenylation site variant causes transcript-specific BMP1 deficiency and frequent fractures in children. *Human Molecular Genetics*, *24*(2), 516–524. https://doi.org/10.1093/hmg/ddu471

Faure, G., Ogurtsov, A. Y., Shabalina, S. A., & Koonin, E. V. (2016). Role of mRNA structure in the control of protein folding. *Nucleic Acids Research*, *44*(22), 10898–10911.

Guhaniyogi, J., & Brewer, G. (2001). Regulation of mRNA stability in mammalian cells. *Gene*, *265*(1-2), 11–23.

Haas, U., Sczakiel, G., & Laufer, S. D. (2012). MicroRNA-mediated regulation of gene expression is affected by disease-associated SNPs within the 3′-UTR via altered RNA structure. *RNA Biology*, *9*(6), 924–937. https://doi.org/10.4161/rna.20497

Haddad, F., Trabelsi, N., Chaouch, L., Darragi, I., Ouslati, M., Boudriga, I., … Ouragini, H. (2017). Homozygous mutation on the β-globin polyadenylation signal in a Tunisian patient with β-thalassemia intermedia and coinheritance of Gilbert's syndrome. *Hemoglobin*, *41*(2), 147–150. https://doi.org/10.1080/03630269.2017.1322610

Heimdal, K., Sanchez-Guixé, M., Aukrust, I., Bollerslev, J., Bruland, O., Jablonski, G. E., … Johansson, S. (2014). STUB1 mutations in autosomal recessive ataxias - evidence for mutation-specific clinical heterogeneity. *Orphanet Journal of Rare Diseases*, *9*, 146.https://doi.org/10.1186/s13023-014-0146-0

Hesketh, J. (2004). 3′-Untranslated regions are important in mRNA localization and translation: Lessons from selenium and metallothionein. *Biochemical Society Transactions*, *32*(Pt 6), 990–993. https://doi.org/10.1042/BST0320990

Italia, K., Sawant, P., Surve, R., Wadia, M., Nadkarni, A., Ghosh, K., & Colah, R. (2012). Variable haematological and clinical presentation of b-thalassemia carriers and homozygotes with the Poly A (T→C) mutation in the Indian population. *European Journal of Haematology*, *89*(2), 160–164. https://doi.org/10.1111/j.1600-0609.2012.01810.x

Kaiser, C. M., Goldman, D. H., Chodera, J. D., Tinoco, I., Jr., & Bustamante, C. (2011). The ribosome modulates nascent protein folding. *Science*, *334*(6063), 1723–1727. https://doi.org/10.1126/science.1209740

Kislauskis, E. H., Li, Z., Singer, R. H., & Taneja, K. L. (1993). Isoform-specific 3'-untranslated sequences sort alpha-cardiac and beta-cytoplasmic actin messenger RNAs to different cytoplasmic compartments. *The Journal of Cell Biology*, *123*(1), 165–172.

Losekoot, M., Fodde, R., Harteveld, C. L., van Heeren, H., Giordano, P. C., Went, L. N., & Bernini, L. F. (1991). Homozygous beta+ thalassaemia owing to a mutation in the cleavage-polyadenylation sequence of the human beta globin gene. *Journal of Medical Genetics*, *28*(4), 252–255.

Orkin, S. H., Cheng, T. C., Antonarakis, S. E., & Kazazian, H. H., Jr. (1985). Thalassemia due to a mutation in the cleavage-polyadenylation signal of the human beta-globin gene. *The EMBO Journal*, *4*(2), 453–456.

Serjeant, G. R., Serjeant, B. E., Fraser, R. A., Hambleton, I. R., Higgs, D. R., Kulozik, A. E., & Donaldson, A. (2011). Hb s-β-thalassemia: Molecular, hematological and clinical comparisons. *Hemoglobin*, *35*(1), 1–12. https://doi.org/10.3109/03630269.2010.546306

Sheets, M. D., Ogg, S. C., & Wickens, M. P. (1990). Point mutations in AAUAAA and the poly (A) addition site: Effects on the accuracy and efficiency of cleavage and polyadenylation in vitro. *Nucleic Acids Research*, *18*(19), 5799–5805.

Shi, Y., Wang, J., Li, J. D., Ren, H., Guan, W., He, M., … Tang, B. (2013). Identification of CHIP as a novel causative gene for autosomal recessive cerebellar ataxia. *Plos One*, *8*(12), e81884. https://doi.org/10.1371/journal.pone.0081884

Synofzik, M., Schüle, R., Schulze, M., Gburek-Augustat, J., Schweizer, R., Schirmacher, A., … Xu, Y. M. (2014). Ataxia and hypogonadism caused by the loss of ubiquitin ligase activity of the U box protein CHIP. *Human Molecular Genetics*, *23*(4), 1013–1024. https://doi.org/10.1093/hmg/ddt497

Tian, B., Hu, J., Zhang, H., & Lutz, C. S. (2005). A large-scale analysis of mRNA polyadenylation of human and mouse genes. *Nucleic Acids Research*, *33*, 201–212. https://doi.org/10.1093/nar/gki158

Yang, E., van Nimwegen, E., Zavolan, M., Rajewsky, N., Schroeder, M., Magnasco, M., & Darnell, J. E., Jr. (2003). Decay rates of human mRNAs: Correlation with functional characteristics and sequence attributes. *Genome Research*, *13*(8), 1863–1872. https://doi.org/10.1101/gr.1272403

SUPPORTING INFORMATION

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