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Repository Citation

Romo LS, Mohn ES, Aronin N. (2018). A Fresh Look at Huntingtin mRNA Processing in Huntington's Disease. RNA Therapeutics Institute Publications. <https://doi.org/10.3233/JHD-180292>. Retrieved from https://escholarship.umassmed.edu/rti_pubs/37

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Review

A Fresh Look at Huntingtin mRNA Processing in Huntington's Disease

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Abstract. Huntington's disease (HD) is an inherited neurodegenerative disorder caused by a mutation that expands the polyglutamine (CAG) repeat in exon 1 of the huntingtin (*HTT*) gene. Wild-type HTT protein interacts with other proteins to protect cells against toxic stimuli, mediate vesicle transport and endocytosis, and modulate synaptic activity. Mutant HTT protein disrupts autophagy, vesicle transport, neurotransmitter signaling, and mitochondrial function. Although many of the activities of wild-type HTT protein and the toxicities of mutant HTT protein are characterized, less is known about the activities of *HTT* mRNA. Most putative HD therapies aim to target mutant *HTT* mRNA before it is translated into the protein. Therefore, it is imperative to learn as much as we can about how cells handle both wild-type and mutant *HTT* mRNA so that effective therapies can be designed. Here, we review the structure of wild-type and mutant *HTT* mRNA, with emphasis on their alternatively polyadenylated or spliced isoforms. We then consider the abundance of *HTT* mRNA isoforms in HD and discuss the potential implications of these findings. Evidence in the review should be used to guide future research aimed at developing mRNA-lowering therapies for HD.

Keywords: Huntington disease, mRNA, 3'UTR, alternative splicing, RNA 3' End Processing

***HTT* mRNA IS PROCESSED INTO SEVERAL ALTERNATIVELY POLYADENYLATED ISOFORMS THAT CHANGE THEIR ABUNDANCE IN HD**

Experimental Huntington's disease (HD) therapies target mutant *HTT* mRNA before it is processed into the toxic HTT protein [1–4]. Therefore characterization of *HTT* mRNA alternatively polyadenylated isoforms may inform therapeutic design. Cleavage and polyadenylation of nascent mRNAs at the end

of transcription is a critical step in mRNA maturation and is directed by nucleic acid motifs in the 3' untranslated region (UTR) of the transcript [5]. One of the motifs is a hexanucleotide sequence called a polyA signal. The canonical polyA signal is AAUAAA, but other nucleotide variations can elicit cleavage and polyadenylation at lower efficiency [6–8]. Most mammalian mRNAs possess multiple cleavage and polyadenylation sites that give rise to mRNA isoforms of varying 3'UTR lengths, a phenomenon termed alternative polyadenylation [9]. The choice of cleavage site depends on the transcription rate and the concentration of RNA binding proteins in the nucleus [10]. The length of the resulting 3'UTR ultimately impacts the metabolism of each respective mRNA isoform (for in-depth review, see reference 11) [11]. Briefly, regulatory factors bind *cis* elements in the 3'UTR to determine the localization and stability of

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the mature transcript [5, 11–13]. For example, brain-derived neurotrophic factor (*BDNF*) is transcribed into two predominant mRNA 3'UTR isoforms. The longer isoform is localized to dendrites, where it plays a role in long-term potentiation [14]. The shorter isoform stabilizes mRNA in cell bodies after depolarization [14, 15]. The stability of the mature transcript is dictated by the length of the 3'UTR via interactions with microRNA (miRNA) and RNA binding proteins [16, 17]. miRNAs target and destabilize mRNAs, and most miRNA sites are located in the 3'UTR after the first polyA site [16]. In T cells, mRNAs with extended 3'UTRs have twice as many miRNA target sites and produce less protein than mRNAs with shorter 3'UTRs [18]. Modulation of mRNA stability by RNA binding proteins also leads to changes in the amount of protein translated [19]. In yeast, deletions in many RNA binding proteins result in decreased mRNA steady-state levels [20]. Thus, alternative polyadenylation can affect mRNA activity, stability, and translation [21].

Three *HTT* mRNA 3'UTR isoforms are produced via alternative cleavage and polyadenylation at three different sites (Fig. 1A) [22, 23]. The longest mRNA isoform is 13.7 kilobases, the mid-length isoform is 12.5 kilobases, and the shortest isoform is 10.3 kilobases [22, 23]. All three isoforms are conserved in mice; conservation in other animals is unknown [23]. The long *HTT* mRNA isoform polyA signal, ATTA AAA, has higher *in vitro* cleavage and polyadenylation rates than the short (AGTAAA) and mid-length (AAUGAA) isoform signals [6]. However, the relative abundance of these mRNA isoforms differs across tissues [22, 23]. For example, the long isoform is more abundant in the brain while the short isoform predominates in peripheral tissues such as liver and muscle [22, 23].

The abundance of *HTT* mRNA 3'UTR isoforms changes in HD. Although all three isoforms are found in HD and control brains, the steady-state abundance of these mRNA isoforms differs in fibroblasts and brain tissue of HD patients compared to controls [23]. Isoform changes appear to be tissue-specific and arise from both *HTT* alleles [23]. An example of this isoform change in the human motor cortex is illustrated in Fig. 1B. The abundance of the *HTT* short and mid-length mRNA isoforms in HD motor cortex is approximately double that in control, although the isoforms remain of lower abundance (12% of total *HTT* mRNA) than the long isoform. It is possible that this observed shift in abundance is due to increased neuronal death in HD, which alters the cell population

in the brain. However, mRNA expression has been shown to be comparable in HD caudate tissue and HD caudate neurons, despite extensive neuronal loss [24]. Moreover, the aforementioned isoform study used motor cortex from grade 1 and 2 HD brains, in which neuronal loss is limited [23, 25–27].

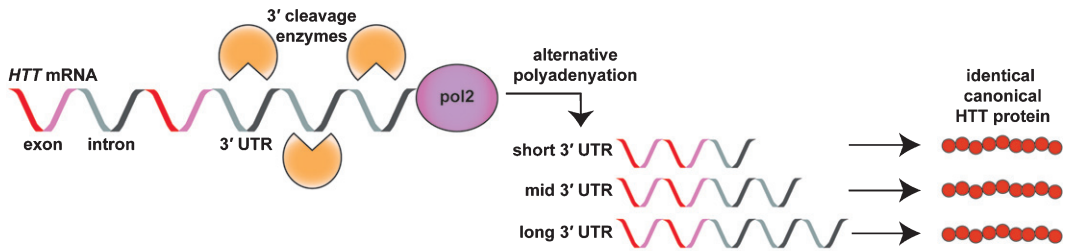
The mechanism underlying mRNA 3'UTR isoform alterations in HD is not established. However, one study determined that knockdown of an RNA binding protein, CCR4-NOT transcription complex subunit 6 (*CNOT6*), in human control fibroblasts resulted in *HTT* mRNA 3'UTR isoform amounts similar to those in HD fibroblasts [23]. Furthermore, *CNOT6* expression is decreased in HD patient motor cortex [23]. Taken together, these findings suggest that altered expression of certain RNA binding proteins in HD may contribute to mRNA isoform abundance changes. In addition to *CNOT6*, three 3' end processing factors are differentially expressed in human HD motor cortex: *CPSF2*, *PCBP2*, and *THOC5* [23]. These proteins should also be explored as potential causes of *HTT* 3'UTR isoform shifts in HD.

IMPLICATIONS FOR ALTERED ABUNDANCE OF *HTT* mRNA 3'UTR ISOFORMS IN HD

HTT mRNA 3'UTR isoforms are translated into identical proteins. However, the different *HTT* 3'UTR isoforms have distinct localizations, stabilities, polyA tail lengths, and different sites for RNA binding proteins and microRNAs [23]. In wild-type and HD fibroblasts, the mid-length mRNA isoform is more abundant in the cytoplasm whereas the short and long isoforms are more abundant in the nucleus [23]. The short isoform has the longest half-life, followed by the mid-length, then long isoform [23]. This may be because the polyA tail length of the short isoform is 50 nucleotides longer than that of the mid-length and long isoforms, both of which have more microRNA binding sites than the short isoform [23]. Further work should investigate whether rates of translation significantly differ among the mRNA isoforms. Taken together, these findings suggest *HTT* 3'UTR shifts in HD impact *HTT* mRNA and protein load.

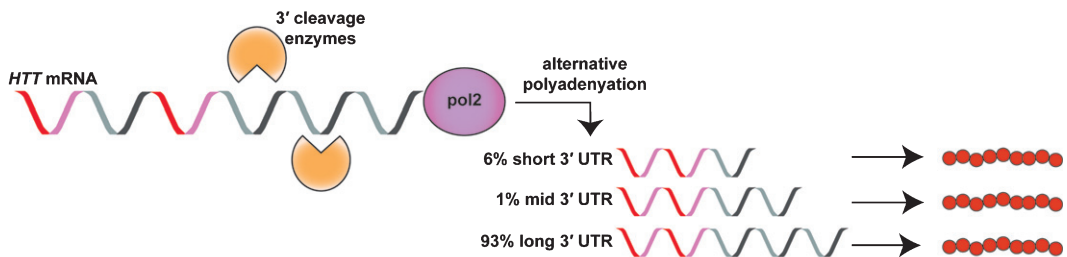
The impact of altering mRNA and protein load due to 3'UTR isoform shifts has been documented in several other diseases. In patients with non-anterior uveitis, a single nucleotide polymorphism in the interferon regulatory factor 5 (*IRF5*) mRNA creates a polyadenylation site, resulting in a short mRNA that is associated with the development of macular

A An overview of *HTT* mRNA 3' end processing



B *HTT* mRNA 3' end processing in normal versus HD motor cortex

Normal



HD

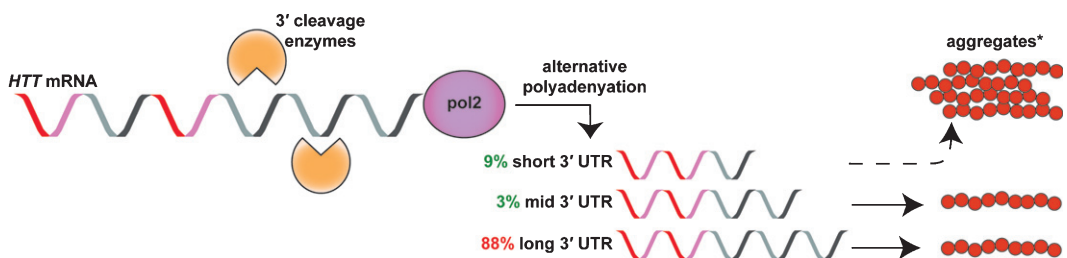


Fig. 1. *HTT* mRNA is processed into several alternatively polyadenylated isoforms that change their abundance in HD versus normal human motor cortex. A) The *HTT* gene is transcribed into mRNA by RNA polymerase 2 (pol2). During transcription, nascent *HTT* mRNA can be alternatively cleaved and polyadenylated at three putative polyA sites in its 3' UTR producing a 10.3 kb (short), 12.5 kb (mid), or 13.7 kb (long) transcript [22, 23]. These alternatively polyadenylated isoforms are translated into the canonical *HTT* protein. B) In HD patient motor cortex, the amount of the short and mid 3' UTR isoforms increases relative to the long isoform [23]. *The short 3' UTR isoform forms more aggregates than the long isoform *in vitro* [30], suggesting altered isoform abundance may have an impact on the formation of abnormal protein-protein interactions.

edema [28]. Similarly, single nucleotide polymorphisms associated with Parkinson's disease promote formation of the longest mRNA isoform of alpha synuclein [29]. This results in accumulation of the protein and localization to mitochondria, both *in vitro* and in Parkinson's patient brains, potentially causing pathology [29]. In HD, the increase in the *HTT* short mRNA isoform in patient motor cortex may contribute to toxicity. *In vitro* experiments have shown that cells transfected with the more-stable short *HTT* mRNA isoform have more aggregates than those transfected with the long *HTT* isoform, suggesting that *HTT* 3' UTR isoforms differentially affect the formation of abnormal protein-protein interactions [30]. Further studies should investigate whether this affects

HD pathogenesis. Finding one mRNA isoform to be more pathogenic than the others may provide new therapeutic strategies for *HTT* RNA silencing. However, the exact role of *HTT* 3' UTR isoform changes in HD remains to be established.

***HTT* mRNA IS PROCESSED INTO SEVERAL SPLICE ISOFORMS THAT MAY CHANGE THEIR ABUNDANCE IN HD**

mRNA splicing occurs concurrently with transcription and polyadenylation. During this process, splicing proteins interact with *cis*-factors to exclude introns and join exons. Like polyadenylation, the

splicing process is influenced by several factors, including RNA binding protein concentration, RNA-RNA base-pairing, and the chromatin environment, to produce different transcripts from the same gene [31]. In fact, alternative splicing and polyadenylation can be co-regulated by certain RNA binding proteins [32]. Heterogeneous ribonucleoprotein H (HNRNPH1), a mediator of mRNA alternative splicing, also suppresses cryptic polyA sites, and two 3' end formation proteins—cleavage and polyadenylation specificity factor (CPSF) and Symplekin (SYMPK)—also regulate splicing of internal exons [33, 34]. However, unlike alternative polyadenylation, alternative splicing results in mRNA isoforms that are translated into different protein products, which may have unique functions or post-translational modifications [31]. For example, upon neuronal activation some *BDNF* splice variants are localized to dendrites in rat hippocampus whereas others are restricted to the soma, allowing for targeted expression of the BDNF protein [35].

HTT nascent mRNA undergoes alternative splicing. The resulting splice isoforms are of low abundance compared to the most common (canonical) splice isoform, and the translation and function of most *HTT* splice variants has not been established (Fig. 2A) [36]. In wild-type mouse brains, *HTT* can be alternatively spliced to lack either exon 28, exon 29 or exons 34–44, or spliced to retain a portion of intron 28; the mRNA isoforms lacking exon 28/29 are also present in human brains [37, 38]. Isoforms lacking exon 28/29 or retaining intron 28 are most abundant in cerebellum and least abundant in the striatum [37]. The mRNA isoform lacking exon 29 is expressed at lower levels in HD Q150 knock-in mice compared to wild-type mice (Fig. 2B, ~20% lower) [37].

Another study identified five more *HTT* mRNA splice isoforms in human embryonic stem cell lines differentiated into neural precursors [39]. The expression of the mRNA isoforms ranged from 0.8% to 13.6% of the most common transcript. Only one of these mRNA splice isoforms has also been detected in human tissues. This isoform is hominid-specific and contains a previously undiscovered exon between exons 41 and 42, dubbed 41b [39]. Eighteen additional alternatively spliced isoforms with abundances ranging from 1% to 7% of the canonical transcript have been identified using high-throughput sequencing of control and HD human brain tissue [36]. However, this study did not attempt to compare HD to control brains, so it is unclear if the abundance of these *HTT* splice variants changes in HD

patients. Finally, PCR and sequencing of *HTT* cDNA from human brain identified twenty-two mRNA splice variants, including 41b and a transcript lacking exons 4–6 [40]. However, the study found no difference between the abundance of these mRNA splice isoforms in HD and control human brains [40].

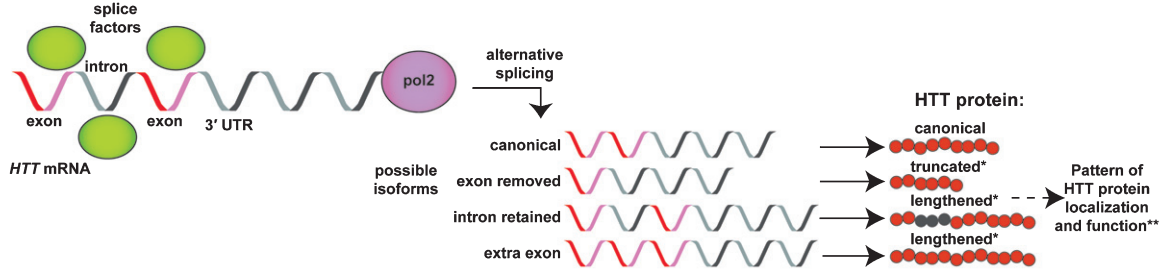
IMPLICATIONS FOR *HTT* mRNA SPLICE ISOFORMS IN HD

Many genes alter their splicing in HD patient brains, potentially due to sequestration of the splicing factor MBNL1 by mutant *HTT* mRNA, or to altered expression of other RNA binding proteins in HD [41, 42]. The abundance of *HTT* mRNA splice isoforms may also change in HD. If the isoform lacking exon 29 is found to be of lower abundance in HD patients as it is in HD mice, it may have functional consequences. Exon 29 contains a putative binding site for the Tap protein, which is involved in nuclear export of RNAs [37]. Loss of this binding site could result in nuclear retention of the *HTT* transcripts. However, this mRNA isoform is of such low abundance in human brain that it could only be detected after several rounds of amplification and could not be quantified via PCR [37]. Another *HTT* splice variant of interest is the isoform lacking exons 4–6 identified in human brain. Exons 4–6 contain a nuclear localization signal, two post-translational modification sites, and a predicted binding site for a nuclear localization protein [40]. Thus, a *HTT* protein lacking these domains may be localized differently than canonical *HTT* protein, which could affect pathology [43]. Evidence for a role of alternative splicing in disease pathology has been observed in tauopathies. Alternative splicing of the microtubule associated protein tau gene results in six isoforms; shifting the ratio of two of these isoforms leads to neurodegeneration in frontotemporal dementia [44]. For HD, it has not been established whether *HTT* mRNA splice isoforms are translated or whether they impact HD pathology. Future studies should investigate whether *HTT* mRNA splice variants, particularly the isoforms lacking either exon 28 or exons 4–6, play a role in HD.

AN ABERRANT *HTT* SPLICE ISOFORM FOUND IN HD MAY BE PATHOGENIC

Aberrant splicing refers to an abnormal variation in the splicing process and has been shown to contribute

A An overview of *HTT* mRNA alternative splicing



B Some splice variants change abundance in cerebellum of Q150 HD knock-in mouse model

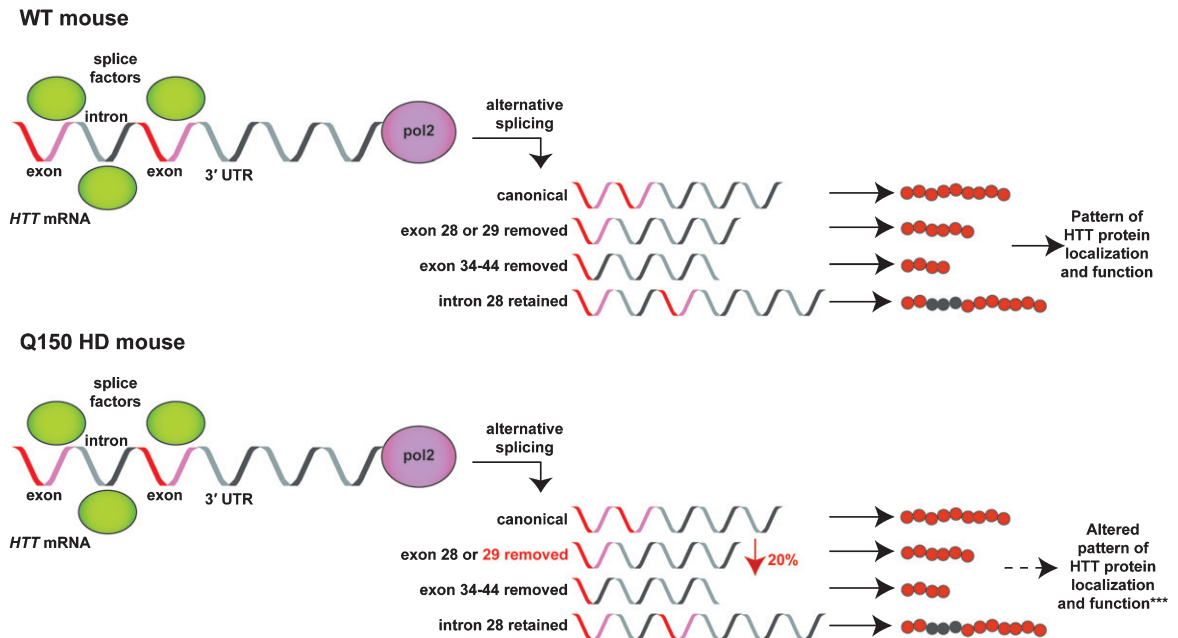


Fig. 2. *HTT* is transcribed into several splice isoforms in normal and HD tissues. A) In normal and HD cells, *HTT* mRNA can be alternatively spliced to lack an exon(s), producing a shorter mRNA and protein than the canonical transcript, or to include an extra exon or retain an intron, producing longer mRNA and protein [36–40]. *The translation of these isoforms has yet to be demonstrated. **However, if translated, splice variant protein products may have different localization and function. For example, a splice isoform of similar abundance in patient and control brains that excludes exons 4–6 lacks a nuclear localization signal [40]. B) In wild-type mice, *HTT* can be alternatively spliced to lack exon 28, 29, 24–44, or to retain intron 28 [37, 38]. These isoforms are of the same or unknown abundance in HD mouse models except the variant lacking exon 29, which is of decreased abundance in Q150 HD knock-in mice [37]. ***Decreased abundance may have an impact on *HTT* localization and function, as exon 29 has a binding site for a protein involved in RNA nuclear export (Tap) [37].

to disease [31]. In spinal motor atrophy, both copies of the survival motor neuron 1 (*SMN1*) gene are deleted, leaving the duplicate *SMN2* gene as the only source of the SMN protein [45, 46]. A frequent nucleotide variant in the *SMN2* gene at a splicing enhancement site leads to missplicing and an unstable truncated protein that exacerbates the disease [47]. Aberrant splicing may also occur in HD. An elegant study published by the Bates group characterized one truncated *HTT* mRNA splice isoform that may be pathogenic [48]. In HD patients and mouse models, but not controls, *HTT* is misspliced and polyadenylated at a cryptic

polyadenylation signal in intron 1, producing a truncated transcript [48] (Fig. 3). In mice, production of the aberrant transcript is correlated with the CAG repeat number. Although quantitation of this mRNA isoform is difficult because of its high GC-content and stable secondary structure, the truncated transcript has been detected via 3' RACE (rapid amplification of cDNA ends) in HD patient, but not control, fibroblasts and brain tissue [48–50]. This study also observed higher levels of *HTT* mRNA containing intron 1 in patient motor cortex and hippocampus compared to controls [50].

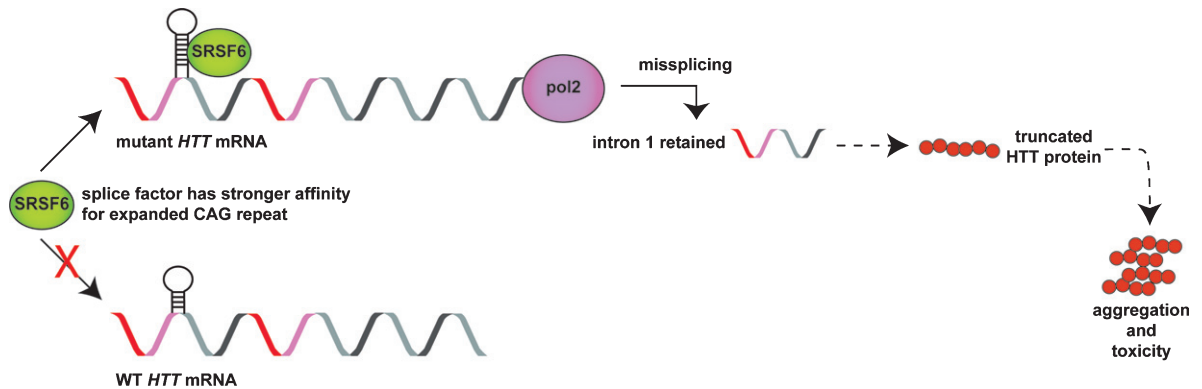


Fig. 3. *HTT* is alternatively spliced into a truncated isoform in HD cells. In HD fibroblasts and brain tissue, the splicing factor SRSF6 processes mutant *HTT* mRNA into a third alternatively polyadenylated splice isoform that terminates in intron 1. This isoform may be translated into the pathogenic N-terminal HTT protein prone to aggregation and toxicity [48–50].

Production of the transcript may be controlled by the serine/arginine-rich splicing factor 6 (SRSF6), which binds to expanded mutant *HTT* mRNA with a higher affinity than to wild-type mRNA (Fig. 3). A stop codon at the very beginning of intron 1 precludes the production of an intron-1 specific antibody, but immunoprecipitation of N-terminal HTT detected a protein of the appropriate size in HD mouse models that was not detected in controls [48]. The protein could not be immunoprecipitated from HD patient brains, possibly because it is highly prone to aggregation [50]. It has not been established if this protein represents translation of the truncated splice isoform or post-translational cleavage of the full-length protein. The association of this splice isoform with disease and the toxicity of N-terminal fragments of the HTT protein suggest that it could contribute to HD pathology. If the potential link between this splice isoform and disease can be established, intron 1 may be an exciting new therapeutic target.

CONCLUSIONS

For decades, HD research has focused on the toxic mutant HTT protein. However, recent studies suggest *HTT* mRNA may also influence cellular toxicity. There are multiple alternatively spliced or polyadenylated isoforms of mutant *HTT* mRNA. The studies detailed in this review suggest some isoforms of *HTT* mRNA may be more toxic than others, either independently or because of their protein products. However, research on *HTT* mRNA structure, activity, and toxicity is in its infancy with most of the studies described in this review being correlative. Differential handling and processing of *HTT* mRNA may be

a contributor to pathology, or it may be a result of pathology. Regardless, mutant *HTT* mRNA is an ideal target for HD therapeutics because it is translated into the pathogenic protein, and thus should continue to be rigorously investigated [4]. This will aid in the development of more efficient and effective HD therapies designed to target specific mutant *HTT* mRNA isoforms. For instance, if the truncated *HTT* mRNA isoform is found to be pathogenic, it could be targeted with interfering RNAs complementary to intron 1. If the short 3'UTR *HTT* isoform is found to be toxic, the proximal polyA site could be disrupted in the *HTT* gene. Moreover, knowledge of the differential pathogenicity and localization of *HTT* mRNA isoforms may suggest cellular compartments that could be targeted by RNA-lowering therapies. Thus, it is critical that future research address the current gaps in knowledge regarding differential toxicity of *HTT* mRNA isoforms in an effort to further characterize HD pathogenesis and guide recommendations for *HTT* mRNA-lowering therapies

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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