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RESEARCH ARTICLE

Replacement of huntingtin exon 1 by *trans*-splicing

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Abstract Huntington's disease (HD) is an autosomal-dominant neurodegenerative disorder caused by polyglutamine expansion in the amino-terminus of huntingtin (HTT). HD offers unique opportunities for promising RNA-based therapeutic approaches aimed at reducing mutant HTT expression, since the HD mutation is considered to be a "gain-of-function" mutation. Allele-specific strategies that preserve expression from the wild-type allele and reduce the levels of mutant protein would be of particular interest. Here, we have conducted proof-of-concept studies to demonstrate that spliceosome-mediated *trans*-splicing is a viable molecular strategy to specifically repair the *HTT* allele. We employed a dual plasmid transfection system consisting of a pre-mRNA *trans*-splicing module (PTM) containing *HTT* exon 1 and a *HTT* minigene to demonstrate that *HTT* exon 1 can be replaced in *trans*. We detected the presence of the *trans*-spliced RNA in which PTM exon 1 was correctly joined to minigene exons 2 and 3. Furthermore, exon 1 from the PTM was *trans*-spliced to the endogenous *HTT* pre-mRNA in cultured cells as well as disease-relevant models, including HD patient fibroblasts and primary neurons from a previously described HD mouse model. These results suggest that the repeat expansion of *HTT* can be repaired successfully not only in the context of synthetic minigenes but also within the context of HD neurons. Therefore, pre-mRNA *trans*-splicing may be a promising approach for the treatment of HD and other dominant genetic disorders.

Keywords Neurodegeneration · Huntington's disease · RNA-based therapeutics · Spliceosome-mediated *trans*-splicing

Introduction

Huntington's disease (HD) is an autosomal-dominant neurodegenerative disorder caused by polyglutamine expansion in the amino-terminus of huntingtin (HTT). It is characterized by irrepressible abnormal movements termed chorea which intensify progressively. Furthermore, cognitive function deteriorates and leads to dementia, and death usually occurs within 20 years of disease onset. The pathology of HD is marked by selective and progressive neuronal cell loss in the striatum, specifically caudate and putamen, which is often accompanied by loss of cells in the cerebral cortex and widespread brain atrophy [1].

HTT is a protein of approximately 350 kD which is encoded by 67 exons. It appears to be unique since it has no sequence homology with other proteins, and *HTT* knockout in mice produces a lethal phenotype as early as embryonic day 9, suggesting a lack of functional compensation by other proteins [2–4]. Except for the extreme amino-terminus, containing the polyglutamine region and proline-rich segments, the entire protein is predicted to be composed of

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36 α-helical HEAT repeats. These repeats fold into a spiral structure and may serve as docking sites for other proteins [5, 6]. HTT is expressed ubiquitously in humans and rodents with the highest levels found in CNS neurons and testes [7, 8]. Intracellularly, HTT is associated with various organelles, including the nucleus, endoplasmic reticulum and Golgi complex [9–11]. It is therefore likely that HTT performs multiple functions according to its subcellular context.

The genetic defect causing HD resides in exon 1 of the *HTT* gene. Exon 1 of the wild-type gene contains a polymorphic stretch of uninterrupted CAG trinucleotide repeats, which is translated into a series of consecutive glutamine residues, the polyglutamine tract. The normal repeat length ranges up to 35. Between 36 and 41 repeats, penetrance is variable. Above 41 repeats, penetrance is complete, and there is a strong inverse correlation between length and age of disease onset [12, 13]. The repeat expansion is generally believed to cause a toxic gain-of-function affecting multiple cellular functions including transcription [14, 15], apoptosis [15, 16], vesicular trafficking [17], cholesterol metabolism [18], and endoplasmic reticulum function [19]. Some experiments also demonstrate a possible loss-of-function due to decreased levels of wild-type protein [20, 21]. For example, wild-type HTT stimulates the production of brain-derived neurotrophic factor (BDNF), a neuronal survival factor, and its decrease in HD may be directly relevant to striatal neuron death [21]. Therefore, an ideal therapy would decrease the levels of mutant HTT while at the same time increasing the amount of wild-type protein.

Huntington's disease is caused by a defined, single mutation, i.e., the CAG repeat expansion. Thus, it is particularly well suited for gene therapy approaches. In particular, allele-specific strategies which preserve expression from the wild-type allele and reduce the levels of mutant protein would be especially advantageous. We therefore explored the possibility of replacing exon 1 of *HTT* with a corrected, non-pathogenic exon 1 sequence using spliceosome-mediated pre-mRNA *trans*-splicing. Most splicing reactions occur in *cis* where both 5′ and 3′ splice sites are located within one RNA molecule. *Trans*-splicing, however, occurs between two separate RNAs: the endogenous pre-mRNA and the "corrected" PTM RNA. The mature mRNA contains exons from both primary transcripts. Both *cis*- and *trans*-splicing follow similar mechanisms and are directed by the spliceosome complexes. Several forms of *trans*-splicing have been reported in different species from lower to higher eukaryotes including rodents and humans [22–31]. A recent study showed that *trans*-splicing occurs naturally in normal human cells where 5′ exons of the *JAZF1* pre-mRNA are joined to 3′ exons of the *JJAZ1/SUZ12* pre-mRNA. This chimeric RNA is translated into JAZF1-JJAZ1, a functional protein with anti-apoptotic activity [32]. Similarly, the ETS fusion protein SLC45A3-ELK4 which is prominent in a subset of prostate cancers commonly occurs in the absence of chromosomal rearrangements and has been ascribed to *trans*-splicing [33]. Thus, *trans*-splicing is a naturally occurring process used for the generation of alternative transcripts.

We present proof-of-principle experiments demonstrating that 5′ exon replacement of *HTT* by spliceosome-mediated pre-mRNA *trans*-splicing can be achieved in cultured cells using a binary transfection system consisting of a *HTT* minigene and a pre-mRNA *trans*-splicing module (PTM). Furthermore, we demonstrate the feasibility of *trans*-splicing exon 1 from the PTM to the endogenous *HTT* pre-mRNA. These results suggest that *trans*-splicing can be used to repair the pathological expansion of *HTT* exon 1 without impeding the function of the normal allele.

Materials and methods

Plasmid constructs

The *HTT* minigene contained exon 1 with 42 CAG repeats and exons 2 to 3 separated by intervening sequences. The sequences were based on Genbank accession number NT_006051. The two introns were shortened to 860 and 109 bp, respectively, to make the construct amenable to plasmid cloning. The PTM construct consisted of three portions: (1) the replacement exon 1 of *HTT* with 21 CAG repeats, (2) the splicing domain with an U1 snRNP binding site at the 3′ end of exon 1 and a triplet repeat of an intronic splice enhancer, and (3) the tether which binds to intron 1 by antisense base pairing. The constructs were generated by custom gene synthesis (Geneart). The minigene was subcloned into pCI-neo (Promega) where its expression was driven by the cytomegalovirus (CMV) promoter/enhancer. The PTM was inserted behind the CMV promoter into pMU1 [34]. pMU1 also contained an eGFP expression module expressed from a separate promoter. For viral delivery, the PTM was inserted into the lentiviral vector pSIN18.

Cell culture and transfection

HEK293 cells, U2OS cells, and HD patient fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) containing high glucose and supplemented with 10 % fetal bovine serum (Hyclone) and 100 U penicillin/100 µg streptomycin (Invitrogen) per mL. DBTRG cells were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum and 100 U penicillin/100 µg streptomycin

per mL. Cells were transiently transfected when they had reached approximately 90 % confluency using PEI or lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Minigene and PTM plasmids were co-transfected at the ratios indicated in the text. Sonicated salmon sperm DNA was used to equalize the total amount of DNA input where necessary. Alternatively, the PTM plasmid was transfected by itself in experiments designed for *trans*-splicing of endogenous *HTT* pre-mRNA. Cells were harvested 24–48 h post-transfection.

Isolation of primary cortical neurons

All animal experiments were carried out in accordance with the University of Missouri Animal Care and Use Committee. Cerebral cortices from 17-day-old embryos of YAC128 HD transgenic mice (developed by M. Hayden, Jackson Labs stock number 4938) were removed, placed into cold DMEM, and the meninges were discarded. The tissue was suspended in 2 mL of 0.25 % (w/v) trypsin at 37 °C for 40 min. Tissues were washed three times in 10–15 mL DMEM containing 10 % (v/v) FBS, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 7.5 mg/mL fungizone. Tissues were triturated with a glass-fired Pasteur pipette 20 times or until homogeneous. The homogenate was diluted with complete culture medium to the desired concentration and seeded on amine coated six-well plates (Becton–Dickinson). Every 3 days thereafter, half the medium was replaced with B27-AO neurobasal medium [100 IU/mL penicillin, 100 mg/mL streptomycin, 7.5 mg/mL fungizone, 10 ml of B27-AO and neurobasal medium (Gibco-BRL) to 500 mL]. The neurons were used for experiments after 7 days in culture (DIV7).

Generation of PTM lentivirus and transduction of cultured cells

The PTM under the control of the CMV promoter was cloned into the unique EcoRV restriction site of pSIN18 [35], thereby retaining the *GFP* expression cassette of this vector. Virus was produced by triple transfection of HEK293 FT cells with pSIN18-PTM, the helper plasmid psPAX2 (originally developed by D. Trono and obtained from Addgene) and the envelope plasmid pVSV-G for pseudotyping. After 48 h, cell culture supernatant was collected and filtered through a 0.45-μm PES membrane, followed by centrifugation at 53,000*g* for 90 min to pellet viral particles. Pellets were resuspended in phosphate-buffered saline (PBS) and stored at 4 °C until use. HEK293 T cells, HD patient fibroblasts or primary HD neurons from YAC128 mice were transduced with varying amounts of virus preparations in the presence of 8 μg/ml polybrene. Cells were harvested 48–72 h later for analyses.

RNA isolation and RT-PCR

RNA was isolated using Tri-Reagent (Sigma) following the manufacturer's instructions. RNA was resuspended in 10 mM Tris–HCl pH 8.2, 1 mM EDTA, and concentrations were measured using a Nanodrop (Thermo Fisher). cDNA was synthesized using 1 μg of RNA and random primers following the SuperScript III protocol (Invitrogen). PCR was performed using two different procedures. For amplifications outside the CAG repeat and the adjacent GC-rich region, Pfu enzyme (prepared in-house) with Thermopol buffer (New England Biolabs) was used. This method was not successful for amplification across the CAG repeat, and we therefore used Taq PCRx with 2× enhancer solution (Invitrogen) for these reactions. PCR products were visualized by agarose gel electrophoresis. Selected PCR products were cloned into the TOPO pCR2.1 vector (Invitrogen) and sequenced by the University of Missouri DNA Core Facility. Primer sequences were: GAPDH fw 5′ TCCGCGCAGCCGAGCCA; GAPDH rev 5′ ACGCCAG TGGACTCCACG; F1 5′ GCAGAAGTTGGTCGTGAG GC; F2 5′ CCGGCCATCTAGGCCAAGC; R1 5′ CACAC GGTCTTTCTTGGTAGCTG; R2 5′ CTGACAGACTGT GCCACTATG; R3 5′ ACTCTGCGTCATCACTGCACA GC; R4 5′ AGGCATTCGTCAGCCACCATCC; R5 5′ GA TAACTTTGTTGAGGCATTCG; Ex1.1 5′ CTGCTGGA AGGACTTGAGGG; Ex1.2 5′ GGCGGCTGAGGAAGCT GAGGA; HD53 5′ GGTTCTGCTTTTACCTGCGGC. The efficiency of the *trans*-splicing reaction was determined by RT-PCR with primer pair HD53 and R1, in which HD53 was fluorescently labeled. The PCR product was purified by phenol/chloroform extraction and Sephadex gel filtration followed by restriction digestion with PstI. The products were separated on an 8 % polyacrylamide gel and visualized and quantitated (Typhoon FLA 9000).

Immunofluorescence

Cortical neurons were cultured in eight-chamber slides. Cells were fixed with methanol at 4 °C for 30 min and air dried. After blocking for 1 h in PBS with 5 % normal goat serum and 0.1 % Triton X-100, wells were washed 3 × 5 min with PBS followed by incubation with mouse anti-NeuN antibody (1:50 dilution; Chemicon) in PBS with 0.1 % Triton X-100 overnight at 4 °C. After three washes, secondary antibody (TRITC-conjugated goat anti-mouse IgG, 1:200 dilution; Jackson Immunoresearch) was added for 2 h at room temperature. After three washes, the specimens were coverslipped with Vectashield and DAPI

(Vector Laboratories) and examined using a Leica 5500 compound microscope.

Results

The possibility of correcting exon 1 of *HTT* was explored in cell culture using transient expression plasmids. To this end, we adopted a dual plasmid strategy consisting of a novel *HTT* minigene and a *trans*-splicing plasmid, the PTM. The splicing competent *HTT* minigene consisted of a subgenomic segment of the *HTT* gene and expresses exon 1, exon 2 and exon 3 of human *HTT* and shortened intervening sequences (Fig. 1). The expanded allele in humans has on average 42 CAG repeats. We therefore designed exon 1 of the minigene to serve as a molecular model of disease by expressing 42 consecutive CAG repeats. This is substantially longer than the 21 CAG repeats of the PTM (described below), making size discrimination feasible. Exon 2 and exon 3 of the minigene were identical to the human *HTT* sequence. Intron 1 and intron 2 of the human gene are both larger than 10 kb and therefore are not well suited for standard mammalian expression vectors. Therefore, the 5′ and 3′ regions of each intron were fused to generate shorter intervening sequences: 860 bp for intron 1 and 109 bp for intron 2. The construct is splicing competent and produces the expected fully spliced mRNA after transfection (data not shown). The SV40 polyadenylation signal was located at the 3′ end of the construct. Expression was driven by the CMV promoter/enhancer.

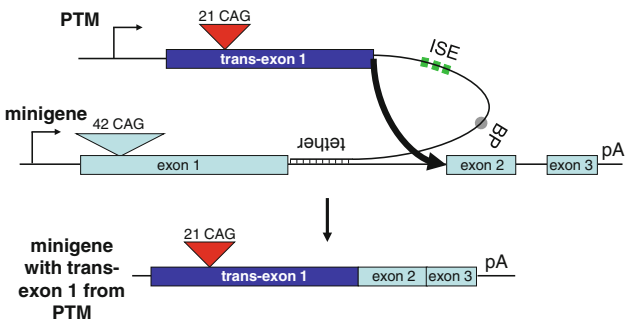

Fig. 1 Schematic of the minigene and the PTM *trans*-splicing constructs. The minigene contains exons 1 to 3 from the human *HTT* gene and shortened introns 1 and 2 as well as a polyadenylation signal (*pA*). Exon 1 was designed to harbor 42 consecutive CAG repeats. The *trans*-splicing construct (termed *PTM*) contains exon 1 of human *HTT* with 21 CAG repeats, followed by an engineered intron. Features include an optimized U1 snRNP binding site at the 5′ splice site, a triplet repeat of intronic splice enhancers (*ISE*), a branch point (*BP*), and a tether region complementary to the 5′ region of intron 1 which serves to bring both molecules into close proximity and facilitating the *trans*-splicing reaction (*thick arrow*). The resulting chimeric RNA contains *trans*-exon 1 spliced to *cis*-exons 2 and 3. The constructs are not drawn to scale

The *HTT trans*-splicing donor plasmid (termed PTM) was designed to contain the complete *HTT* exon 1 with 21 CAG repeats which represents a non-pathological number of triplets (Fig. 1). Directly downstream, we placed a U1 snRNP binding site, followed by a spacer and three repeats of the intronic splice enhancer (ISE) sequence, TGCATG, each separated by a short spacer sequence. A branch point (BP) sequence was inserted further downstream. Finally, 100 bp of sequence complementary to the 5′ end of *HTT* intron 1 was added as the "tether". This tether serves to bind the PTM RNA to the minigene pre-mRNA, thus bringing the two molecules into close proximity. The *trans*-splicing reaction is indicated by the thick arrow (Fig. 1).

In order to specifically detect and discriminate between the RNAs derived from the minigene, the PTM, and the expected *trans*-spliced product, we designed primers for RT-PCR. The specificity of these primers was determined in preliminary experiments which demonstrated that amplification of the individual cDNAs occurred only with the appropriate primer combinations (Fig. 2). Briefly, primers F1, and R1 to R2 are specific for the minigene, whereas F2 is specific for the PTM. Therefore, a PCR product obtained with forward primer F2 and the reverse primer R1 or R2 is indicative of *trans*-splicing between PTM exon 1 and minigene exon 2. Such a product was not obtained when amplifying from the minigene (Fig. 2a) or the PTM (Fig. 2b) alone, demonstrating the fidelity of detection. Amplification with primers Ex1.1 and Ex1.2 which anneal to exon 1, in conjunction with forward primer F2, resulted in the generation of the expected PCR products from the PTM (Fig. 2b).

To test the capacity of the PTM RNA to *trans*-splice to the minigene pre-mRNA, the two plasmids were co-transfected into HEK293 cells. Total RNA was isolated and RT-PCR was performed using the PTM-specific primer F2 and minigene-specific reverse primers in exon 2 (R1 and R2) and exon 3 (R3–R5). Specific bands of the expected sizes were observed, suggesting that *trans*-splicing occurred between the two RNAs (Fig. 3). The F2–R4 PCR product was cloned, and sequence analysis identified the correct exon junctions, demonstrating that *trans*-exon 1 was correctly spliced to exons 2–3 of the minigene (Suppl. Fig. 1). Taken together, the detection of products using F2 and reverse primers R1–R5 is indicative of the successful joining (*trans*-splicing) of PTM exon 1 to minigene exons 2 and 3.

Since both plasmids contain exon 1, i.e., a stretch of homologous sequence, it is possible that the two plasmids could recombine, thereby generating a DNA molecule containing PTM exon 1 and minigene exon 2 and exon 3. This recombined DNA could then give rise to a RNA species that is indistinguishable from the *trans*-spliced RNA species. To address this issue, the CMV promoter driving

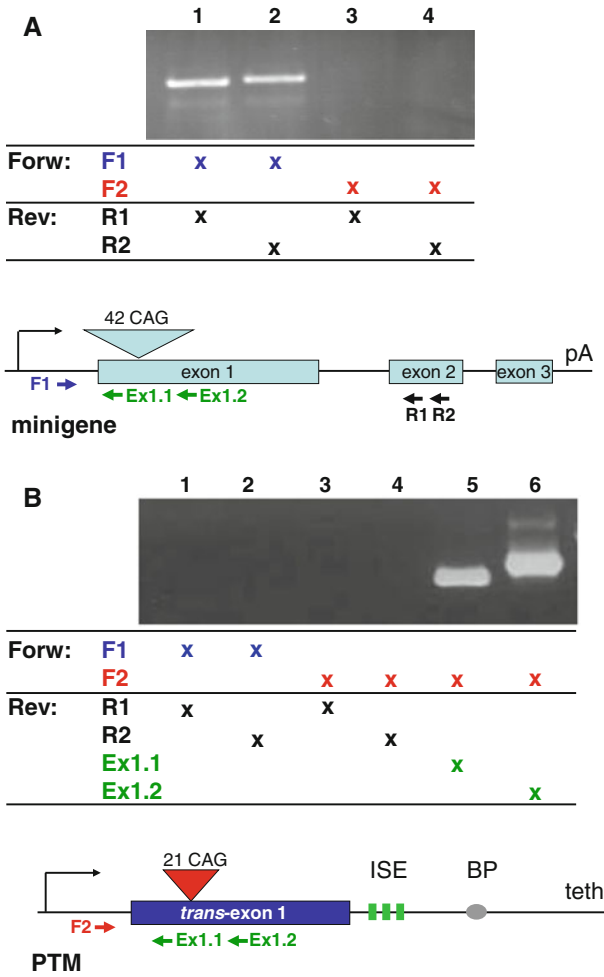

Fig. 2 Primer specificity. Primers were designed to specifically discriminate between the cDNAs generated from the minigene and PTM plasmids, as well as the presumptive *trans*-splicing product. To this end, we took advantage of the sequence divergence in the 5′-UTRs of the two cDNAs. Primer F1 binds uniquely to the minigene, but not to the PTM, whereas primer F2 specifically recognizes the PTM but not the minigene. Reverse primers R1 and R2 are located in exon 2 (minigene only), whereas reverse primers Ex1.1 and Ex1.2 are complementary to both constructs. **a** PCR with the minigene and F1 (*lanes 1* and *2*) or F2 (*lanes 3* and *4*) and the indicated reverse primer only generates a specific product with the F1 primer. **b** PCR with the PTM and F1 (*lanes 1* and *2*) or F2 (*lanes 3–6*) and the indicated reverse primer only generates a specific product with F2 and Ex1.1 or Ex1.2 (*lanes 5* and *6*). Identical specificities were observed when cDNA from HEK293 cells co-transfected with these two plasmids was used as PCR input (data not shown)

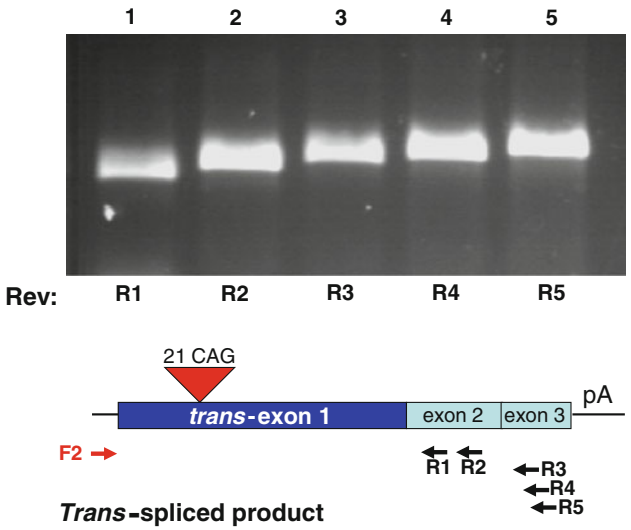

Fig. 3 Detection of *trans*-splicing of exon 1 of the PTM to exons 2 and 3 of the minigene. HEK293 cells were co-transfected with the minigene and PTM constructs and RNA was isolated 48 h later. RT-PCR was performed with PTM-specific F2 forward primer and one of the minigene-specific reverse primers, R1–R5. A specific *trans*-spliced product of the expected size was detected using reverse primers for exon 2 (R1, R2) and exon 3 (R3–R5)

the expression of the PTM was deleted; therefore, the *trans*-splicing could not occur. The minigene plasmid was co-transfected into HEK293 cells with identical amounts of PTM plasmid with or without the CMV promoter, RNA was isolated and RT-PCR was performed. Expression of the PTM was predictably essentially undetectable without the CMV promoter, and the putative *trans*-splicing product was absent (Fig. 4, left side). Recombination should have occurred at a similar rate irrespective of the presence of the promoter since identical amounts of DNA were used. Therefore, this strongly suggests that the *HTT* mRNA species are indeed products of *trans*-splicing. As an additional control, the tether within the PTM was made into the reverse complement. The tether consists of a sequence complementary to the coding strand. It allows for base-pairing with the pre-mRNA, or potentially with the coding strand of the gene. To further confirm that the observed PCR product originates from an interaction of RNAs, we tested a PTM in which the tether was made into the reverse complement (sense) sequence. The PTM with either the sense or the antisense tether was co-transfected with the minigene into HEK293 cells and RT-PCR was performed on isolated RNA to detect *trans*-splicing products. As expected, the efficacy of the sense tether was drastically reduced, suggesting again that the generation of the chimeric *HTT* mRNA species is a result of bona fide *trans*-splicing (Fig. 4, right side).

To determine whether *trans*-splicing was dose-dependent, a dose–response experiment was performed in which a constant level of minigene target was co-transfected into HEK293 cells with increasing quantities of the PTM plasmid (Fig. 5a). Results indicated that the amount of *trans*-spliced product increased with the level of PTM expression over nearly a log-fold change in the PTM concentration (Fig. 5a). As expected, *trans*-splicing was not detected in the absence of the PTM, whereas *trans*-splicing was detected even at the lowest concentration of the PTM tested. The PTM dose-dependent *trans*-splicing of the minigene occurred similarly in U2OS osteosarcoma

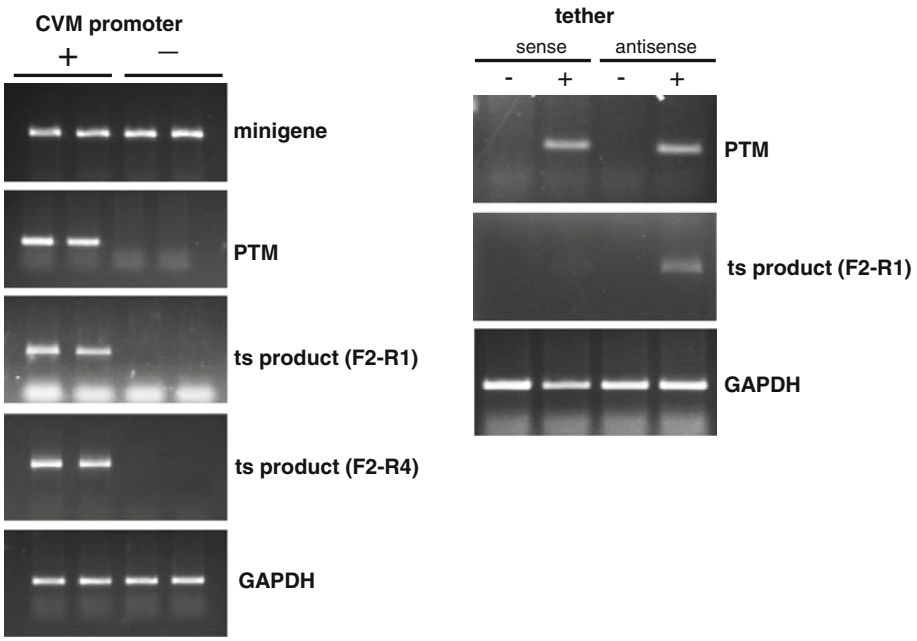

Fig. 4 The *trans*-splicing product is generated by interaction of RNA molecules, not via plasmid DNA recombination. **a** HEK293 cells were co-transfected with identical amounts of the minigene plasmid and a PTM construct either with (*left lanes*, labeled "+") or without (*right lanes*, labeled "−") the CMV promoter. After RNA isolation and RT-PCR using appropriate primer pairs (Fig. 3), mRNA from the minigene (*top panel*) or the PTM (*second panel*) was detected. As expected, minigene expression levels were comparable with or without the CMV promoter in the PTM construct. However, in the absence of the CMV promoter in the PTM, only a very small amount of PTM mRNA was detected. Residual levels of PTM RNA which can be detected after prolonged PCR (data not shown) are likely due to the presence of AAV ITRs in the PTM plasmid which have weak intrinsic promoter activity. However, no *trans*-splicing product was detected using reverse primers for either exon 2 (*third panel*, F2–R1) or exon 3 (*fourth panel*, F2–R4) when the CMV promoter was absent in the PTM. GAPDH was used as internal control. Duplicate reactions are shown. **b** A PTM plasmid in which the 100-bp tether domain sequence (antisense) was replaced by its reverse complement (sense) was co-transfected with the minigene construct into HEK293 cells. With this sense construct, the *trans*-splicing reaction was much less effective

and DBRTG glioblastoma cells (Fig. 5b, c). This suggests that *trans*-splicing parameters are amenable to optimization and that different cell types are capable of repairing *HTT* exon 1 via *trans*-splicing.

The minigene system is a straightforward means to initially investigate *trans*-splicing activity; however, the system is intrinsically artificial and the level of "target" transcript produced by the CMV promoter is exceptionally high. To begin to examine *HTT trans*-splicing in a more complex environment, we next sought to determine if the PTM is also suitable for *trans*-splicing of endogenous *HTT* pre-mRNA. HEK293 cells were transfected with the PTM plasmid alone. RT-PCR was performed with the primer combination F2–R1, and a product of the expected size was detected at all concentrations of the PTM plasmid (Fig. 6). This result indicates that exon 1 of endogenous *HTT* is amenable to replacement by *trans*-splicing.

For the delivery and long-term expression of therapeutic candidates in the central nervous system, viral agents are frequently employed, and the feasibility and efficacy of delivering regulatory RNAs has been shown using several HD mouse models [36–41]. Therefore, we transferred the CMV-driven PTM into the pSIN18 lentivirus vector [35] and pseudotyped the virus with VSV-G, which confers a broad tropism and allows entry into a large number of cells, including neuronal cells. To initially test the effectiveness of the PTM lentivirus, HEK293 cells were transiently transfected with the 42 CAG minigene, followed by transduction with the PTM lentivirus. The specific *trans*-spliced mRNA was detected, suggesting that lentiviral vectors can be used successfully (Fig. 7a). Furthermore, we wished to explore if the virally encoded PTM could direct the exchange of endogenous *HTT* exon 1. To this end, HEK293 cells were transduced in the absence of the minigene, and the *trans*-spliced product was detected by RT-PCR (Fig. 7b). Decreasing viral multiplicity of infection correlated with reduced synthesis of PTM RNA and diminished abundance of the *trans*-spliced endogenous *HTT* pre-mRNA.

In order to determine whether the exon 1 replacement strategy was applicable to a more disease-specific context, we examined *trans*-splicing in primary fibroblasts from HD patients. First, fibroblasts were transiently transfected with the minigene target, followed by transduction with the PTM lentivirus. Cells were harvested 48–72 h after transduction. As observed before in the other cell types, the

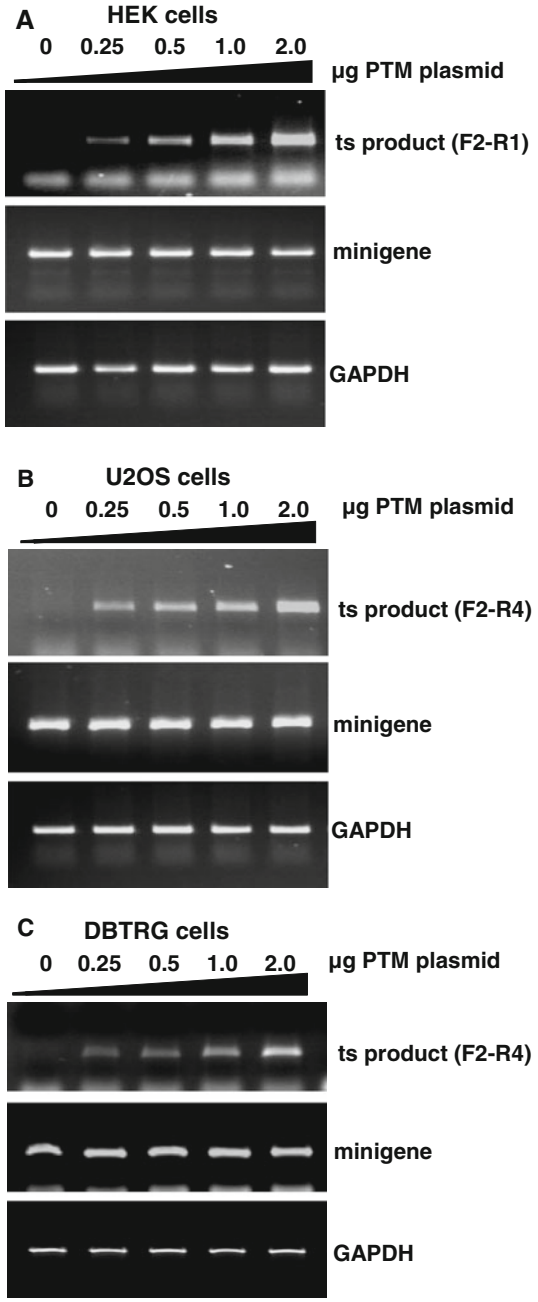

Fig. 5 The *trans*-splicing product titrates with PTM input in different cell lines. **a** HEK293 cells were co-transfected with a constant amount of minigene plasmid and increasing amounts of the PTM construct. After RNA isolation, expression levels of the *trans*-splicing product were determined using the F2 and R1 primer pair. The quality of the RNAs was tested by amplification of *GAPDH* with or without RT reaction (data not shown). **b**, **c** Expression levels of the *trans*-splicing product in U2OS and DBTRG cells was determined with the F2 and R4 primer pair and also correlated with the amount of PTM input

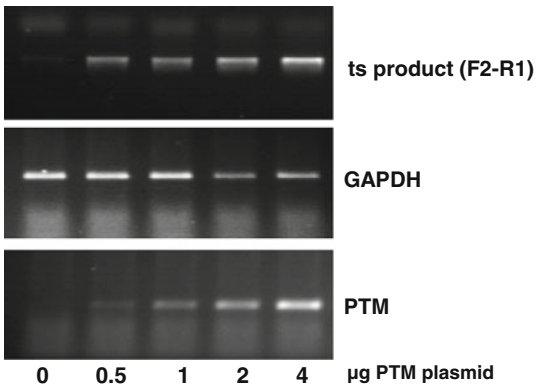

Fig. 6 *Trans*-splicing of endogenous *HTT* pre-mRNA. HEK293 cells were transfected with PTM plasmid alone. RT-PCR was performed using the *trans*-splicing-specific primer pair F2–R1. The expression levels of the *trans*-spliced product titrated with the amount of PTM plasmid input

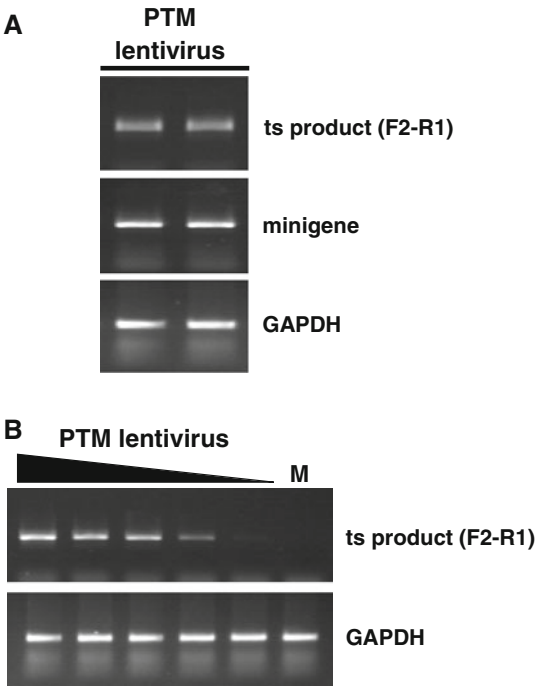

Fig. 7 Viral delivery of the PTM. **a** HEK293 cells were transiently transfected with the minigene plasmid, followed by transduction with a lentivirus harboring the PTM and an eGFP module (MOI: 25). Identical amounts of virus were administered to all wells. After 48 h, the cells were harvested and RNA was isolated for RT-PCR. Specific primer pairs were used to amplify GAPDH, minigene, and *trans*-spliced product. Duplicate reactions are shown. **b** Viral transduction (MOI: 50, 25, 12.5, 6, 2.5) was performed in the absence of the minigene. *Trans*-splicing to the endogenous *HTT* pre-mRNA of HEK293 cells was proportional to the amount of virus added. *M* mock transduction (no virus added)

amount of *trans*-spliced product titrated with the quantity of virus added to the fibroblasts cultures using constant minigene input (Fig. 8a). Importantly, *trans*-splicing was not restricted to the minigene as robust *trans*-splicing of endogenous *HTT* by the PTM lentivirus in the absence of minigene was detected in fibroblasts from three

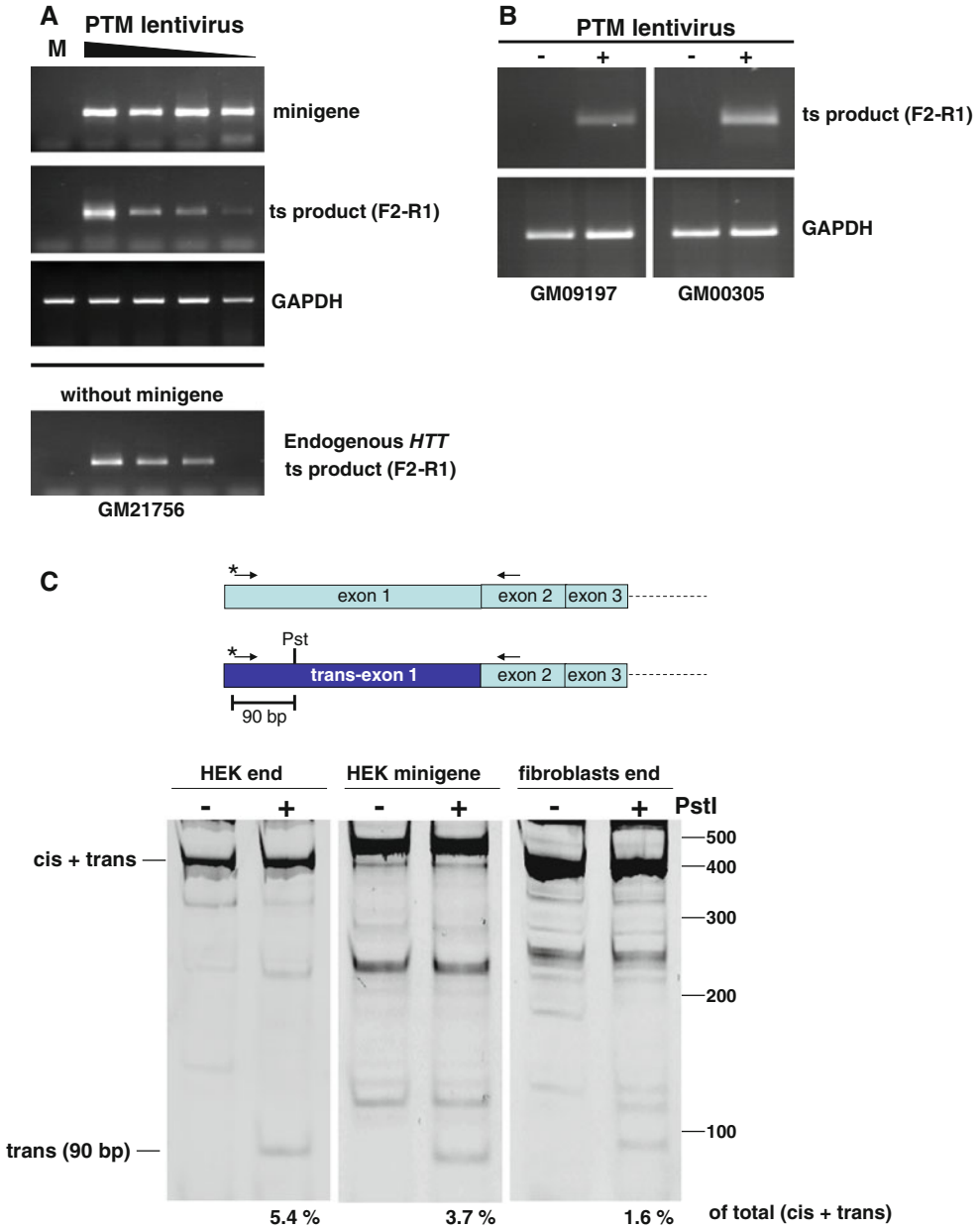

Fig. 8 **a** Primary fibroblasts from HD patients were transfected with identical amounts of minigene plasmid followed by transduction with varying amounts of the PTM lentivirus (*top panels*) (MOI: 126, 63, 31.5, 15). After 72 h, cells were harvested, RNA was isolated and RT-PCR was performed using the specific primer pair F2–R1. *Bottom panel* HD patient fibroblasts were transduced with the PTM lentivirus in the absence of minigene and endogenous *trans*-splicing was detected using the specific primer pair F2–R1. **b** Two additional HD patient fibroblast lines with different CAG repeat lengths (GM09197 and GM00305) were transduced with the PTM lentivirus (MOI 126 and 31.5, respectively) and *trans*-splicing to endogenous *HTT* pre-mRNA was detected. **c** The relative efficiency of the *trans*-splicing reaction was determined by performing PCR with a primer pair common to both the *cis*- and *trans*-spliced RNAs. The 5′ primer was labeled fluorescently (*asterisk*), and the products of the PstI digestion were separated on a 8 % non-denaturing polyacrylamide gel. The band intensities were quantified using a Typhoon FLA 9000, and the relative fraction of the 90-bp band is indicated below the scan. The (*cis* + *trans*) band varies in size between cell lines due to different CAG tract lengths. HEK293 have 16 and 17 CAG repeats (data not shown), the normal allele of the patient fibroblasts has 17 repeats (Corriell data), and the minigene has 42 repeats

independent HD patients (Fig. 8a, bottom panel, and b). These results suggest that the *trans*-splicing approach is successful in an important disease-appropriate context, primary HD cells.

The *trans*-spliced product is detected using specific primer pairs that differ from those used to detect the minigene mRNA. To quantify the relative amount of *trans*-spliced product generated, i.e., the efficiency of the *trans*-

splicing versus the *cis*-splicing reaction, a unique PstI restriction site was introduced in the 5′-UTR of exon 1 of the PTM (Fig. 8c). A common primer pair was then used to amplify both *cis*- and *trans*-spliced RNAs. The 5′ primer was labeled fluorescently to allow quantitation. After PstI digestion of the PCR product, the intensity of the 90-bp band is indicative of the relative fraction of *trans*-spliced RNA. In HEK293 cells, approximately 3 and 5 % *trans*-splicing was achieved when using endogenous *HTT* pre-mRNA, or the minigene as target (Fig. 8c). Similarly, patient fibroblasts transduced with the PTM lentivirus showed a slightly lower 1.6 % *trans*-splicing efficiency.

Since the primary defect in HD manifests itself in the brain, we were interested in examining the possibility of *HTT* exon 1 replacement in neurons. To this end, primary cortical neuron cultures were prepared from embryonic day 16.5–17.5 brains of YAC128 transgenic mice. Importantly, these mice carry a complete human *HTT* gene with 128 CAG repeats in exon 1, including the intronic sequence which is targeted by the PTM's tether [42]. To ascertain the purity of the neuronal cultures, cells were plated in eight-well chamber slides and processed for immunohistochemistry. Staining with an antibody against NeuN, a neuron-specific marker, showed that the vast majority of the cultured cells were neurons (Fig. 9, top panels). We then transduced the neurons with the PTM lentivirus and isolated RNA after 72 h. As expected, PCR products were not generated when we used wild-type neurons from mice not expressing the human *HTT* gene (data not shown). In contrast, the specific *trans*-splicing product was successfully detected after RT-PCR with the specific primer set in HD neurons from YAC128 mice expressing the human *HTT* gene (Fig. 9, bottom panel), demonstrating that the pathogenic expanded exon 1 region can be successfully replaced by spliceosome-mediated pre-mRNA *trans*-splicing in this important disease context.

Discussion

Targeting RNA for the treatment of inherited disorders is an alternative to conventional gene replacement therapy. RNA-based approaches have a number of potential advantages [43]. For example, since targeted sequences are generally relatively short, they can easily be corrected with current delivery systems, such as adeno-associated virus. In addition, insertional mutagenesis and adverse effects due to

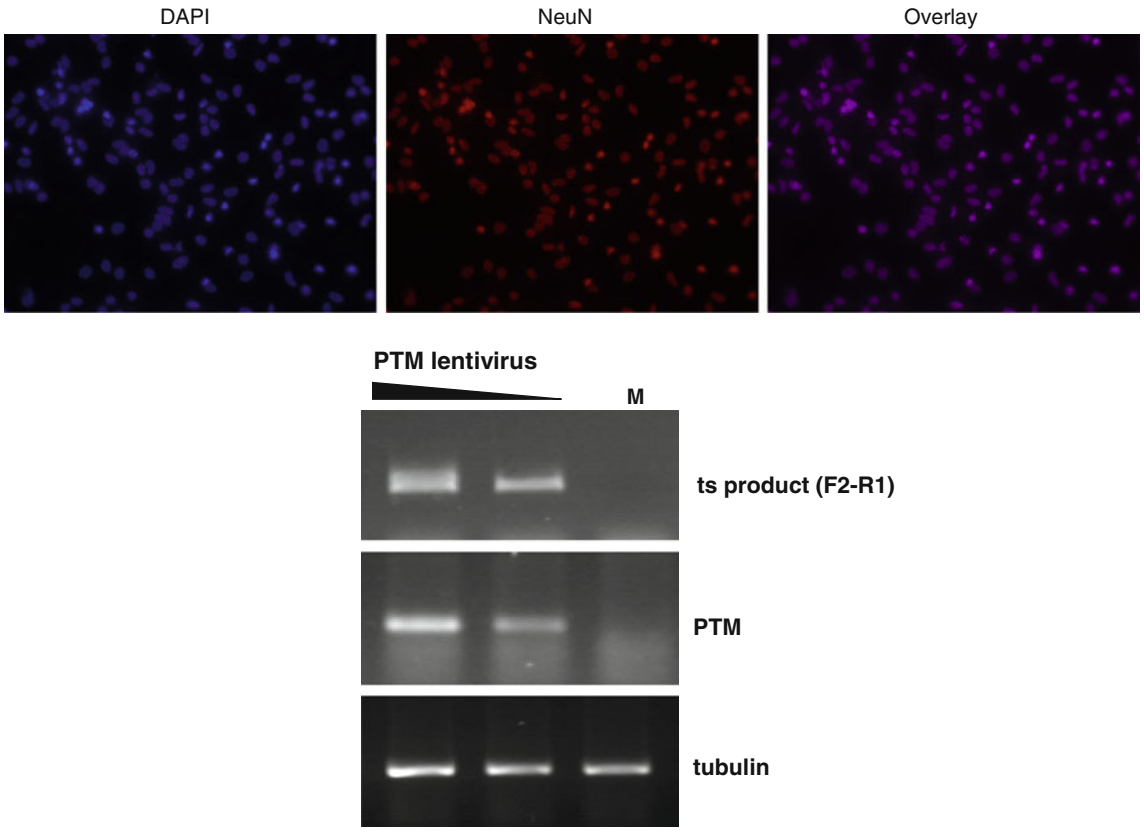

Fig. 9 Primary cortical neurons were isolated from YAC128 HD transgenic mice and cultured until DIV7. *Top panels* Cells were fixed and stained with the neuronal nuclear marker, NeuN, and counter-stained with the nuclear stain DAPI to assess the relative purity of the preparations. *Bottom panel* Cells were transduced with the PTM lentivirus and harvested 72 h later. After RNA isolation and RT-PCR, the *trans*-splicing product of transgenic *HTT* pre-mRNA was detected using the specific primer pair F2–R1

genomic integration can be avoided. Importantly, the modification of the disease-related RNA takes place within the framework of a normal regulatory environment where the spatial and temporal expression of the underlying gene is controlled by its intrinsic regulatory mechanisms, and optimal expression is ultimately controlled via the endogenous promoter.

Dominantly inherited disorders, such as HD, are particularly well suited for correction by RNA targeting because the alternative therapeutic introduction of a functional gene does not eliminate the underlying toxic gain-of-function caused by expression of the mutant allele. In this case, reducing or inhibiting the expression of the mutant allele would be advantageous. A promising approach to the suppression of *HTT* expression is the use of small RNAs. Antisense oligonucleotides have been used successfully to reduce *HTT* mRNA expression in vitro and in cell culture [44, 45]. These approaches targeted regions common to wild-type and mutant *HTT* and demonstrated the feasibility of suppressing *HTT* expression. A recent study using peptide nucleic acid and locked nucleic acid chemistry revealed that it is possible to target the CAG repeat region of *HTT* and *ATXN3* [46]. Depending on the specific sequence of the oligonucleotide and the concentration used, mutant protein levels were reduced while the amount of wild-type protein was not strongly affected. It is unclear how much loss of wild-type HTT can be reasonably tolerated in a therapeutic approach. Non-allele-specific knockdowns of HTT suggest that wild-type HTT can be reduced below 50 % of normal levels, at least in the short term, although this was shown to be accompanied by significant changes in gene expression profiles whose consequences need to be explored [38, 47]. On the other hand, *HTT* gene knockout in the mouse results in embryonic lethality [2–4], and reduced levels lead to developmental brain defects and perinatal lethality [48]. In addition, lack of HTT in the adult mammalian brain has adverse effects [20, 49]. This suggests that a certain level of wild-type HTT is required for appropriate brain function, making allele-selective approaches highly attractive. Significant discrimination between the expanded and the wild-type allele has been successfully demonstrated by targeting SNPs in the mutant allele using RNAi in vitro [50–53]. HD-associated SNPs have been described [54], and a survey of 225 human samples found that about 75 % of expanded alleles were associated with three specific SNPs that were successfully targeted with a cocktail of five siRNAs, suggesting that the majority of HD patients would be amenable to such a therapeutic regimen [53]. Similarly, Hayden and colleagues have recently developed SNP-based antisense oligonucleotides that reduce mutant HTT expression in an allele-selective fashion [55]. This is potentially very powerful approach to the reduction of mutant HTT in the brain, with the possible drawback that the patient population is not homogeneous in the occurrence of SNPs, and different antisense oligonucleotides may have to be tailored towards specific individuals. Importantly, McBride and colleagues demonstrated that inhibitory RNAs incorporated into artificial miRNA vector scaffolds supported efficient expression and silencing while at the same time exhibiting low levels of toxicity in mouse brain [37], and that reduction of HTT expression levels in rhesus monkeys is overall well tolerated [56].

We explored the feasibility of a novel RNA-based approach for the allele-specific suppression of mutant *HTT*, i.e., spliceosome-mediated pre-mRNA *trans*-splicing. In general, *trans*-splicing offers several advantages. Spatial and temporal expression patterns of HTT should remain unchanged, since the gene is driven by its intrinsic regulatory elements. In addition, exon replacement occurs only in cells expressing HTT, and adverse effects due to expression of the PTM in cells that do not express HTT should therefore be minimal.

An important part of Huntington pathology is the accumulation of specific protein aggregates. The expanded HTT protein forms high molecular weight, β-sheet-rich amyloid-like aggregates similar to those seen in other neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease. Similar protein aggregation structures are observed in prion diseases, such as Creutzfeld–Jakob disease, where these amyloid fibrils are responsible for the infection of healthy neurons. This raises the question whether expanded polyQ amyloids may also play a role in the propagation of the pathology from initially localized foci [57, 58]. Importantly, the aggregates also sequester other proteins, and it is possible that misfolded expanded HTT protein also recruits the normal, non-expanded HTT protein, analogous to the situation in prion disease. Support for this notion comes from work by Ren and coworkers [59], who showed that fibrillar polyQ aggregates can enter cells from the extracellular space. Furthermore, employing cellular reporters (HTT–gfp fusion proteins), these internalized polyQ fibrils recruit soluble, wild-type-length polyQ proteins and induce them to aggregate [59]. Since *trans*-splicing converts the mutant allele to wild-type, it presumably not only prevents expression of the mutant allele but also increases the level of the wild-type form. The excision of an exon 1 with expanded polyQ tract results in a truncated RNA species lacking a polyadenylation signal. Consequently, this fragment will be susceptible to degradation in the nucleus and is unlikely to be exported and processed by the ribosome. Therefore, the load of mutant protein will be reduced, and the associated cellular pathology, such as protein aggregation and recruitment of wild-type protein, should be reduced. Conceptually, the repair process is applicable to

varying repeat lengths, which could potentially allow the development of a single therapeutic molecule for all HD patients. Furthermore, *trans*-splicing confers essentially functional allele specificity. While the *trans*-exon 1 may integrate into the pre-mRNA derived from either allele, only the repair of the expanded molecule will have a (positive) functional effect, and the wild-type mRNA will remain normal. A potential limitation of this technique is the relatively low efficiency of *trans*-splicing and consequently the necessity of optimizing the PTM either empirically or by screening procedures [60].

To begin to address this issue, we performed proof-of-principle experiments to explore whether *trans*-splicing can be used for the replacement of the 5′ exon of *HTT*. We generated a prototype PTM that contained exon 1 of *HTT* with 21 CAG repeats and several engineered features to enhance its activity, including intronic splice enhancers and a U1 snRNP binding sequence at the 3′ end of exon 1. A tether of 100-bp complementary sequence was used to direct the PTM construct to the start of intron 1. The tether forms a double strand at the very 5′ end of intron 1 and masks the U1 snRNP binding site on the minigene pre-mRNA, thus favoring the strong intron 1 5′ splice site complex on the PTM RNA. A polyadenylation signal was omitted to avoid nuclear export and translation of the PTM RNA. When co-transfected with a splice-competent minigene, the PTM RNA was able to productively interact with minigene pre-mRNA, resulting in the generation of a chimeric mRNA, which we detected by RT-PCR using specific primers. This *trans*-splicing reaction occurred in all three cell lines tested, HEK293, U2OS, and DBTRG, as well as HD patient-derived fibroblasts and cultured neurons from YAC128 transgenic mice expressing human HTT with 128 CAG repeats, suggesting that *trans*-splicing is applicable to a range of cell types. Endogenous *HTT* is expressed ubiquitously. Although brain lesions are prominent in HD, many peripheral tissues are also affected [61], and targeting additional cell types should be a consideration in the development of therapeutic regimens.

The co-transfection system allowed us to demonstrate that 5′ exon replacement of *HTT* is feasible in principle. The *HTT* minigene target contained the complete exons 1 through 3 while the intervening introns were shortened to allow handling in a plasmid vector. The endogenous intron 1 of human *HTT* is over 11 kb long. RNA polymerase II interactions with subunits of the splicing machinery are thought to sequester exons near the polymerase, and very large introns may be looped away from this complex [62, 63]. It is conceivable that the functionality or the spatial organization of such a long intron is different from that of the shortened, 0.86-kb minigene intron 1, which may affect interactions of the PTM RNA with the *HTT* pre-mRNA. Therefore, we administered the PTM without the minigene to investigate *trans*-splicing of endogenous *HTT* pre-mRNA. Using RT-PCR, we were able to detect specifically the *trans*-spliced mRNA species whose abundance titrated with the amount of PTM plasmid transfected. This demonstrates that, using the prototype PTM, the endogenous *HTT* pre-mRNA can be repaired successfully. Furthermore, expression of the PTM via a lentiviral system resulted in successful *trans*-splicing of both the minigene and the endogenous *HTT* pre-mRNA, suggesting that this strategy is amenable to *HTT trans*-splicing in future in vivo studies.

The efficiency of the *trans*-splicing reaction was approximately 1–5 % using the prototype PTM in this study. It is not clear at present how much reduction of mutant *HTT* is necessary to achieve a long-term clinical benefit, and whether the level of *trans*-splicing observed here would be sufficient to prolong the time to disease onset or ameliorate the disease phenotype in an animal model of HD. Nevertheless, this study is a proof-of-principle that measurable levels of *trans*-splicing can be achieved in disease-relevant cell types, and future work will be directed towards optimization of this process.

Functional correction using *trans*-splicing has been reported in several models of human disease, including cystic fibrosis, hemophilia A, X-linked immunodeficiency, and various cancers [64–69]. For example, Liu et al. repaired the gene defect Δ508 in *CFTR*, which encodes the cystic fibrosis transmembrane conductance regulator, in explants of human cystic fibrosis airway epithelia in a xenograft model [64, 65]. They achieved a partial restoration of conductance in airway epithelial cells, thereby demonstrating functional improvements in a disease-relevant cell type. We developed the first *trans*-splicing strategy to increase the expression of full-length mRNA and functional SMN protein from the *SMN2* gene in the context of spinal muscular atrophy [34]. The first in vivo RNA repair by *trans*-splicing was performed in factor VIII hemophilia A-knockout mice, demonstrating the feasibility of transferring this methodology into an animal model [66]. A recent study by Wally et al. [70] demonstrated the potential power of *trans*-splicing for the allele-specific correction of a dominant-negative mutation in the *plectin* gene. A mutation in exon 9 of *plectin* causes increased protein aggregation and degradation, leading to the blistering skin disease epidermolysis bullosa. Combining an exon replacement strategy with viral delivery, the level of full-length *plectin* protein was increased >50 % in patient fibroblasts. These examples show that meaningful levels of repair can be achieved, suggesting that *trans*-splicing might be a promising new tool for the treatment of autosomal-dominant genetic disorders.

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