Nuclear retention of full-length HTT RNA is mediated by splicing factors MBNL1 and U2AF65

Xin Sun1,7, Pan P. Li1, Shanshan Zhu1, Rachael Cohen1, Leonard O. Marque1, Christopher A. Ross1,2,3,4, Stefan M. Pulst5, Ho Yin Edwin Chan6, Russell L. Margolis1,2,4 & Dobrila D. Rudnicki1,4

Huntington’s disease (HD) is caused by a CAG repeat expansion in the huntingtin (HTT) gene. Recent evidence suggests that HD is a consequence of multimodal, non-mutually exclusive mechanisms of pathogenesis that involve both HTT protein- and HTT RNA-triggered mechanisms. Here we provide further evidence for the role of expanded HTT (expHTT) RNA in HD by demonstrating that a fragment of expHTT is cytotoxic in the absence of any translation and that the extent of cytotoxicity is similar to the cytotoxicity of an expHTT protein fragment encoded by a transcript of similar length and with a similar repeat size. In addition, full-length (FL) expHTT is retained in the nucleus. Overexpression of the splicing factor muscleblind-like 1 (MBNL1) increases nuclear retention of expHTT and decreases the expression of expHTT protein in the cytosol. The splicing and nuclear export factor U2AF65 has the opposite effect, decreasing expHTT nuclear retention and increasing expression of expHTT protein. This suggests that MBNL1 and U2AF65 play a role in nuclear export of expHTT RNA.

Huntington’s disease (HD) is caused by CAG repeat expansions in the huntingtin (HTT) gene1. While there is undoubtedly support for the role of expHTT protein in HD pathogenesis2, efforts to ameliorate HTT protein–induced toxicity have not yet yielded an effective treatment. Based on evidence from other CAG/CTG repeat expansion diseases, including spinocerebellar ataxias type 3 and 8 (SCA3 and SCA8, respectively)3–5 and the HD genocopy Huntington’s disease-like 2 (HDL2)6,7, multimodal pathogenesis, including RNA toxicity, is likely to be involved in HD8,9. Indeed, at least three mechanisms of expHTT RNA neurotoxicity have been proposed. First, RNA with sufficiently long stretches of CUG or CAG triplets forms hairpin structures10 leading to the sequestration of RNA-binding proteins (RBPs), including the splicing factor MBNL17,9,11, with complications that include widespread splicing abnormalities. In support of this hypothesis, expanded HTT (expHTT) RNA in patient-derived HD fibroblasts aggregates into foci that co-localize with MBNL1, with the predicted consequence that at least some transcripts under MBNL1 control are misspliced11,12. Second, bidirectional HTT transcripts13 may provide a source of Dicer-generated CAG/CUG repeat siRNAs capable of targeting cellular transcripts containing complementary repeats, with potentially lethal consequences8,14. Third, repeat-associated non-ATG translation (RAN), an RNA-dependent mechanism triggered by structural abnormalities of

1Department of Psychiatry and Behavioral Sciences, Division of Neurobiology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. 2Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. 3Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. 4Program of Cellular and Molecular Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. 5Department of Neurology, University of Utah, Salt Lake City, Utah, USA. 6Laboratory of Drosophila Research, School of Life Sciences, Faculty of Science, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China. 7Guangdong-Hong Kong-Macau Institute of CNS Regeneration, Jinan University, Guangzhou, Guangdong, China. Correspondence and requests for materials should be addressed to D.D.R. (email: drudnic1@jhu.edu)
repeat expansion-containing transcripts, may lead to expression of proteins containing expanded tracts in HD. These three mechanisms are non-mutually exclusive and suggest that ignoring RNA-mediated pathogenic pathways in HD risks missing opportunities to develop novel therapies that will complement efforts aimed at reducing HTT protein-mediated neurotoxicity.

In the current study, we provide further evidence of the cytotoxicity of untranslated expHTT and describe how nuclear retention of expHTT is regulated, in opposite directions, by the splicing factors MBNL1 and U2 small nuclear ribonucleoprotein auxiliary factor 65 (U2AF65). We hypothesize that nuclear retention of expHTT is likely to lead to increased neurotoxicity, and therefore that pathways leading to this phenomenon may provide valuable therapeutic targets.

**Results**

An untranslatable expHTT RNA fragment is cytotoxic in the absence of both ATG- and non-ATG-initiated translation. An HTT exon 1 fragment encoding expHTT RNA with 80 CAG repeats is cytotoxic in SH-SY5Y cells. To test the toxicity of untranslated HTT transcripts with CAG repeats ranging in length from 23 triplets (normal) to 128 triplets (extreme juvenile onset), and including a length of 45 triplets (typical adult-onset HD), we overexpressed truncated HTT RNA in the SH-SY5Y and SK-N-MC neuroblastoma cell lines. To obtain the non-translatable N63QnHTT construct containing a sequence corresponding to the first 63 amino acids of HTT plus the repeat region by deleting the ATG 5′ to the repeat region and placing a STOP codon immediately 5′ to the repeat (Fig. 1A). Transfection of these untranslatable N63QnHTT constructs (with 23, 45, 66 or 128 CAG triplets), in a pcDNA3.1 vector, into SH-SY5Y cells resulted in repeat-dependent cytotoxicity, as measured by caspase-3/7 activity assay performed 72 hours after transfection (Fig. 1B). Recently, it was shown that, in addition to the toxic polyglutamine (polyGln), toxic polyalanine (polyAla) and polyserine (polySer) can be produced from expHTT by RAN translation in specific cell types. To confirm that in our experiments cytotoxicity is indeed caused by toxic HTT.
RNA itself, we cloned untranslatable N63(CAG)nHTT sequences into a vector in which each sense-strand reading frame is tagged (N63(CAG)nHTT-RAN plasmid) (Supplementary Fig. S1) and tested for the presence of RAN translation proteins in SH-SY5Y cells. With the exception of N63(CAG)150HTT RNA, which expresses a polyAla-containing RAN product, no evidence of RAN translation was observed in SH-SY5Y cells expressing expHTT (Supplementary Fig. S1). This confirms that expHTT RNA with a CAG repeat size within the range most frequently found in HD patients is cytotoxic in a neuroblastoma cell model.

Untranslated expHTT RNA is a significant contributor to cytotoxicity in HD. We next tested the cytotoxicity of expHTT RNA, using expanded expHTT protein as a point of reference, and whether RAN translation contributes to expHTT RNA toxicity. We first determined that, unlike SH-SY5Y cells (Fig. S1B), SK-N-MC cells are permissive of RAN translation. Transfection of non-translatable N63(CAG)nHTT plasmids with 66 and 150 CAG triplets into SK-N-MC cells resulted in high levels of RAN translation products encoding polyAla. Expression of polyGln and polySer was observed following overexpression of 66 CAG triplets (Supplementary Fig. S1C). We therefore transfected SH-SY5Y cells with either non-translatable N63(CAG)nHTT plasmids (with 23, 66 or 128 CAG triplets) expressing only HTT RNA, or, as an approximate reference point for neurotoxicity, unmodified, translatable N63QnHTT plasmids (with 16, 80 or 148 CAG triplets) expressing both HTT RNA and protein. We used truncated HTT constructs for these experiments, as these constructs enabled us to control for levels of transcription and to measure cytotoxicity in an unbiased manner using chemical readouts. Although repeat lengths were not precisely matched between the constructs expressing translatable and non-translatable transcripts due to slight shifts in repeat lengths during plasmid preparation, similar levels of RNA were expressed from all HTT constructs and triggered comparable levels of cytotoxicity at each repeat length (Fig. 1C). We next performed the same experiment in SK-N-MC cells to determine if RAN translation significantly affects toxicity in this model. Once again, constructs expressing only expHTT RNA and constructs expressing both expHTT RNA and protein triggered similar levels of cytotoxicity (Fig. 1D). Taken together, the data indicate that expHTT RNA significantly contributes to cytotoxicity and that RAN translation does not add to cytotoxicity in a neuroblastoma model of HD. Whether this is true in the context of FL HTT and in other cell types remains to be examined.

MBNL1 decreases foci formation of expanded full-length HTT RNA. Nuclear RNA foci, punctate aggregates of expanded RNA, are hallmarks of RNA-mediated toxicity. In HD, RNA foci have been detected in HD patient–derived fibroblasts expressing 44, 68, 69 or 151 CAG triplets. Using fluorescence in situ hybridization (FISH) with a 2′-O-methylated CUG riboprobe, we observed RNA foci in truncated N63HTT constructs expressed in SK-N-MC cells (Supplementary Fig. S2) and in the cortex of the N586–82Q transgenic mouse model of HD (Supplementary Fig. S2). Next we asked whether full-length (FL) expHTT RNA forms foci in neuronal cells, as it is possible that the additional sequence may influence the capacity of expHTT to form RNA foci. To test this, we overexpressed FL expHTT with 82 CAG triplets (FL-HTTQ82, Fig. 2A) in SK-N-MC cells. As predicted, FL expHTT RNA formed RNA foci (Fig. 2B) similar in appearance to those observed following expression of truncated expHTT, and resistant to DNase and sensitive to RNase treatment (Fig. 2C). Full-length normal HTT RNA with 82 CAG triplets (FL-HTTQ23) also formed foci in a small percentage of cells, presumably as a consequence of overexpression. These results demonstrate that both truncated and FL expHTT RNA form structures that facilitate RNA aggregation into RNA foci, and hence that foci formation is not an artifact of a short transcript.

RNA foci are thought to be one of the major sites of interaction between RNA with repeat expansions and RBPs, including the splicing factor MBNL1. Sequestration of MBNL1 by expanded CUG repeat-containing RNA foci depletes functional MBNL1 in the nucleus and induces dysregulation of alternative splicing in myotonic dystrophy type 1 (DM1). Similar co-localization of MBNL1 with expanded CUG RNA foci has been detected in SCA8 and HDL2. CAG foci sequestering MBNL1 have been detected in HD patient–derived fibroblasts and a Drosophila model of SCA3. To further examine the relationship between MBNL1 and HTT RNA foci in neuronal-like cells, we co-transfected plasmids encoding FL-HTTQ82 and either GFP or GFP-MBNL1 (Fig. 3A) into SK-N-MC cells. While multiple isoforms of MBNL1 exist, we focused on MBNL1 isoform C, also known as the 42-kD isoform, containing 388 amino acids. We examined the quantitative effect of MBNL1 on foci formation of FL-HTT RNA. As shown in Fig. 3B, 48 hours after co-expression of a GFP control plasmid and expanded FL-HTTQ82, RNA foci were detected in ∼12% of nuclei (FL-HTTQ82 + GFP). Presumably as a consequence of overexpression, normal FL-HTTQ23 RNA also formed RNA foci, though in a much smaller subset of cells (Fig. 3B, FL-HTTQ23 + GFP). Overexpression of GFP-MBNL1 had no significant effect on the overall percentage of cells containing RNA foci generated by expression of either FL-HTTQ23 or FL-HTTQ82 (Fig. 3B, FL-HTT + GFP-MBNL1). However, GFP-MBNL1 overexpression decreased the mean number of nuclear FL-HTTQ82 RNA foci, but not of FL-HTTQ23 RNA foci (Fig. 3C, FL-HTTQ82 + GFP versus FL-HTTQ82 + GFP-MBNL1). Representative images of RNA foci used in these quantitative analyses are shown in Supplementary Figure S3. Therefore, while overexpression of MBNL1 does not alter the total number of cells containing expHTT foci, it markedly decreases the number of expHTT foci in each cell in which foci are detectable. No significant changes in the size
of the foci were observed. This led us to hypothesize that MBNL1 changes the pool of expHTT available for foci formation.

**MBNL1 increases nuclear retention of FL expHTT RNA and decreases the expression of FL expHTT protein.** If foci formation results in sequestration of RNA with a consequent increase in total nuclear expHTT RNA, then a MBNL1-induced decrease in foci would be predicted to reduce total nuclear expHTT RNA. However, unexpectedly, we determined that FL-HTTQ82 RNA is increased in the nuclear fractions of SK-N-MC cells relative to FL-HTTQ23, and that expression of MBNL1 further increased nuclear retention of FL-HTTQ82 RNA by ~170% and decreased cytoplasmic FL-HTTQ82 RNA to ~40% of control (Fig. 4A, FL-HTTQ82 + GFP-MBNL1). Knock-down of endogenous MBNL1 by siRNA decreased nuclear levels of FL-HTTQ82 RNA by ~1.3 fold (Fig. 4B, FL-HTTQ82 + MBNL1 siRNA). Neither MBNL1 overexpression nor MBNL1 knock-down changed the total cellular level of FL HTT RNA (Fig. 4C,D). Together, these data suggest that nuclear retention of expHTT RNA is not
simply a function of the formation of RNA foci, but involves other processes that are in part mediated by MBNL1.

If MBNL1 increases nuclear retention of expHTT RNA, one consequence should be a decrease in the levels of the expHTT protein. We therefore assessed the levels of FL HTT protein following overexpression of MBNL1 in SK-N-MC cells. As predicted, overexpression of MBNL1 reduced the levels of FL-HTTQ82 to ~40% of control (Fig. 5A) but had no significant effect on the levels of FL-HTTQ23 (Fig. 5A). While FL HTT does not form significant protein aggregates in the SK-N-MC cell model, we observed that MBNL1 reduces both soluble and insoluble N63Q148, which readily forms protein aggregates in SK-N-MC cells (data not included). Conversely, knock-down of endogenous MBNL1 increased the levels of FL-HTTQ82 protein by ~1.5 fold, but did not have a significant effect on FL-HTTQ23 protein (Fig. 5B). We did observe variability in FL-HTTQ23 experiments, perhaps reflecting a small and inconsistent effect of MBNL1 on shorter repeats.

To further confirm that the effect of MBNL1 on nuclear retention of expHTT is primarily dependent on the expanded CAG repeat, we examined the effect of MBNL1 on levels of endogenous HTT, ATXN2 and ATXN3 proteins with normal repeat sizes, as well as on the expression of an exogenously expressed protein without a repeat (mRFP). MBNL1 overexpression had no significant effect on the expression of any of these proteins (Supplementary Fig. S4). Consistent with this set of observations, knock-down of MBNL1 did not change the levels of endogenous proteins with normal length repeats.

Figure 3. MBNL1 decreases the number of FL-HTTQ82 RNA foci. (A) Schematic representation of GFP-MBNL1 plasmids. ZFs, zinc finger motifs. SK-N-MC cells were co-transfected with FL-HTT and GFP-MBNL1 plasmids for 48 hours and subjected to FISH. GFP plasmid was used as a control. Foci analysis was performed by Nikon Eclipse E400 microscopy. In each treatment, numbers of GFP-positive cells and foci-containing GFP-positive cells were counted. (B) Percentage of cells containing RNA foci. (C) Average foci number. Both experiments, two-way ANOVA, n = 3 biological replicates; *P < 0.05, **P < 0.01, ns = no significance.
and only minimally increased mRFP expression (Supplementary Fig. S4). These data indicate that the MBNL1-associated decrease in expHTT protein expression is primarily dependent on the presence of an expanded repeat.

**The MBNL1-associated increase in nuclear expHTT RNA depends on the RNA binding capacity and nuclear localization of MBNL1.** We next sought to confirm that the MBNL1-induced increase in the level of nuclear expHTT RNA is dependent on the capacity of MBNL1 to bind to RNA. MBNL1 has four CCCH-type zinc finger motifs, which are all necessary for MBNL1 to bind RNA. A C-terminal splicing domain includes a recently identified nuclear localization signal. We therefore made two different deletions of MBNL1: a deletion of the first zinc finger (MBNL1Δ12–46) and a deletion of the C-terminal splicing domain (MBNL1Δ251–388, Fig. 3A). MBNL1Δ12–46 showed an attenuated capacity to increase nuclear FL expHTT RNA (Figs 4A, 5C). Similarly, MBNL1Δ251–388, which, unlike MBNL1 and MBNL1Δ12–46, is localized to both the nucleus and cytoplasm (Supplementary Fig. S5), did not significantly increase nuclear levels of expHTT RNA (Figs 4A, 5C). These data indicate that the MBNL1-mediated increase of nuclear expHTT RNA requires MBNL1’s RNA-binding capacity and nuclear localization, and suggest that MBNL1 may reduce the nuclear export of expHTT.

**MBNL1 reduces non-ATG-dependent translation of an expHTT RNA fragment.** N63(CAG) 66HTT transcripts without an ATG codon produce non-ATG–translated polyAla-containing peptides in SK-N-MC cells (Supplementary Fig. S1). We next tested the effect of MBNL1 on the expression of the polyAla peptides produced by RAN translation. MBNL1 decreased the expression of polyAla peptides (Fig. 6), consistent with the MBNL1-induced increase in the nuclear/cytoplasmic ratio of expHTT.
Figure 5. MBNL1 decreases expression of expanded FL-HTT protein. (A) SK-N-MC cells were co-transfected with FL-HTT and GFP-MBNL1 plasmids, and levels of FL-HTT protein were assessed by western blot 72 hours post-transfection. GFP plasmid was used as a control. Overexpression of MBNL1 decreased levels of FL-HTT Q82. Student's t-test, n = 3 biological replicates. **P < 0.01, ns = no significance, versus GFP group. (B) SK-N-MC cells were first transfected with MBNL1 siRNA and FL-HTT plasmid. Levels of FL-HTT were assessed by western blot. Control siRNA was used as a control. Knock-down of endogenous MBNL1 increased expression of FL-HTT Q82. Student's t-test, n = 3 biological replicates. *P < 0.05, ns = no significance, versus control siRNA group. (C) SK-N-MC cells were co-transfected with FL-HTT and GFP-MBNL1 plasmids, and levels of FL-HTT protein were assessed by western blot 72 hours post-transfection. GFP plasmid was used as control. Overexpression of GFP-MBNL1 Δ12-46 (loss of first zinc finger) and GFP-MBNL1 Δ251-388 (loss of C-terminal splicing domain) abolished the effect of MBNL1 on FL-HTT Q82 levels. One-way ANOVA, n = 3 biological replicates. *P < 0.05, ns = no significance, versus GFP group.
Consistent with this result, expression of either MBNL1Δ12–46 or MBNL1Δ251–388 resulted in a significant increase in expression of the polyAla peptides (Fig. 6) indicating that MBNL1 that is not localized to the nucleus, or that cannot bind expHTT, facilitates the nuclear export and translation of expHTT, including RAN translation.

Effect of MBNL1 on expHTT RNA is not CAG repeat- or disease-specific. The capacity of MBNL1 to bind CAG repeats in vitro may depend on the RNA hairpin structure formed by strong C-G pairings. To indirectly test this possibility in our cell model, we modified HTT constructs N90Q45 and N90Q145 (each encoding the first 90 amino acids in HTT) by interrupting CAG triplets with CAA triplets (as depicted in Fig. 7A). Addition of MBNL1 resulted in decreased expression of these interrupted constructs (Fig. 7A), demonstrating that the effect of MBNL1 on expHTT is not dependent on a pure CAG repeat.

HD is one of nine autosomal dominant neurodegenerative diseases caused by CAG repeat expansions. A CAG/CTG repeat expansion in ATXN2 causes spinocerebellar ataxia type 2 (SCA2). To test if the effect of MBNL1 is disease-specific, we co-expressed MBNL1 with a FL GFP-tagged ataxin-2 (ATXN2) construct. GFP-MBNL1 overexpression significantly decreased the expression of expanded ATXN2Q58 and ATXN2Q104 protein to 50% and 20% of control, respectively (Fig. 7B). Conversely, MBNL1 knock-down increased expression of expanded ATXN2Q104 by ~2.5 fold (Fig. 7C). ATXN2Q22 was also up-regulated by MBNL1 knock-down (Fig. 7C). Interestingly, MBNL1 Δ12–46 had no significant effect on the level of expanded ATXN2Q104, while MBNL1 Δ251–328 significantly increased expanded ATXN2Q104 expression (Fig. 7D). We conclude that the effect of MBNL1 on protein expression of transcripts with CAG repeats, and particularly on expanded repeats, is not limited to expHTT. However, the degree to which MBNL1 binds to different CAG repeat–containing transcripts may be influenced by disease-specific sequences flanking the CAG repeats.

We next sought to determine if the effect of MBNL1 on translation is CAG repeat-specific. CUG repeat expansion in JPH3 causes HDL2, in part via RNA-mediated neurotoxicity. Alternative splicing of JPH3 results in transcript variants in which the CUG repeat resides in the 3′ UTR or within open reading frames translated into polyAla or polyleucine. We expressed a JPH3 construct in which the repeat is in-frame for translation into polyAla (JPH3Ala55) with and without MBNL1 and observed that MBNL1 decreased the expression of JPH3Ala55 protein (Fig. 7E). This experiment suggests that the
Figure 7. Effect of MBNL1 on expHTT RNA is not CAG repeat- or disease-specific. (A) SK-N-MC cells were co-transfected with N90QnHTT plasmid and GFP-MBNL1 plasmid and levels of N90HTT protein were assessed by western blot. GFP plasmid was used as a control. MBNL1 still decreased levels of expanded N90QnHTT encoded by CAA-interrupted CAG repeats (shown by underlines). N90Q145 HTT plasmid, like N90Q45 HTT plasmid, has heavily CAA-interrupted CAG repeats. Both experiments, Student’s t-test, \( n = 3 \) biological replicates. ***P < 0.001, versus GFP group. (B) SK-N-MC cells were co-transfected with GFP-ATXN2 and GFP-MBNL1 plasmids and levels of GFP-ATXN2 protein were assessed by western blot. GFP plasmid was used as control. Overexpression of MBNL1 decreased levels of expanded GFP-ATXN2. All experiments, student’s t-test, \( n = 3 \) biological replicates. *P < 0.05, **P < 0.01, ns = no significance, versus GFP group. (C) SK-N-MC cells were first transfected with MBNL1 siRNA and GFP-ATXN2 plasmid. Levels of GFP-ATXN2 were assessed by western blot. Control siRNA was used as a control. Knock-down of endogenous MBNL1 increased expression of GFP-ATXN2. Student’s t-test, \( n = 3 \) biological replicates. *P < 0.05, versus control siRNA group. (D) SK-N-MC cells were co-transfected with GFP-ATXN2 and GFP-MBNL1 plasmids, and levels of GFP-ATXN2 protein were assessed by western blot 72 hours post-transfection. GFP plasmid was used as a control. Overexpression of MBNL1 \( \Delta 12-46 \) (loss of first zinc finger) and MBNL1 \( \Delta 251-388 \) (loss of C-terminal splicing domain) abolished the effect of MBNL1 on GFP-ATXN2Q104 levels. One-way ANOVA, \( n = 3 \) biological replicates. *P < 0.05, ns = no significance, versus GFP group. (E) SK-N-MC cells were co-transfected with JPH3Ala55 and GFP-MBNL1 plasmids and levels of JPH3Ala55 protein were assessed by western blot 72 hours post-transfection. GFP plasmid was used as a control. Overexpression of MBNL1 decreased the levels of JPH3Ala55, encoded by expanded CUG repeats. Student’s t-test, \( n = 3 \) biological replicates. ***P < 0.001, versus GFP group.
effect of MBNL1 on the expression of proteins derived from transcripts with expanded repeats is not CAG repeat-specific.

**U2AF65 stimulates nucleocytoplasmic export of FL expHTT RNA.** Our data so far support the idea that MBNL1 increases nuclear retention of expHTT RNA and other transcripts with expanded repeats by interfering with nuclear export processes. The nuclear export of mRNA is mainly mediated by the nuclear export factor 1 (NXF1) receptor pathway\(^3\), which was recently implicated in the nuclear retention of expanded ATXN3 RNA in a fly model of SCA3\(^3\). In this model the protein U2AF65 interacts with expanded CAG repeat-containing RNA and serves as an adaptor to link the transcript to NXF1. The same export mechanism may be disrupted in HD\(^3\). Interestingly, U2AF65 and MBNL1 were previously identified as splicing factors that compete with each other at a cardiac troponin T (cTNT) splicing site\(^3\). To determine if the NXF1 pathway is involved in the nuclear export of expHTT, we overexpressed U2AF65 with FL expHTT. In SK-N-MC cells, overexpression of U2AF65 increases the levels of expHTT protein (Fig. 8B), opposite to the effect observed with MBNL1. We therefore speculated that nuclear retention of expHTT RNA in HD may be triggered by an aberrant interaction of expHTT with MBNL1, with a consequent loss of U2AF65 binding and a disruption of NXF1 pathway-mediated export of expHTT RNA. Consistent with this speculation, co-expression of MBNL1 blocked the effect of U2AF65 on FL-HTTQ82 expression (Fig. 8C). The effect is unlikely to derive from an artifact of construct expression levels, as U2AF65 and MBNL1 do not appear to influence the endogenous expression of each other (Fig. 8D).

**Discussion**

There is growing evidence that mutant RNA contributes to the pathogenesis of multiple repeat expansion diseases\(^3\). Our data provide further support for a role of expHTT RNA in HD pathogenesis by demonstrating the cytotoxicity of both truncated non-translatable and RAN-translated expHTT RNA fragments containing expanded CAG repeats of different lengths (Fig. 1). Future model systems that use FL constructs and automated longitudinal monitoring of toxic damage to individual cells as developed by Finkbeiner and colleagues\(^3\), will help refine the quantitative and temporal relationship of RNA and protein toxicity in specific cell types.

Several HD mouse models have been used to demonstrate that alterations in protein sequence markedly suppress the phenotype of HD, including the YAC128 transgenic mice “short stop” (a mutation prevents expression of full length protein), the YAC128 line in which the caspase -6 cleavage site has been eliminated, and the BAC transgenic mice expressing full-length mutant huntingtin with serines 13 and 16 mutated to aspartate\(^3\). The lack of phenotype in these mice supports the argument that mutant protein, and not expHTT RNA, is essential to disease pathogenesis. In addition, the BACHD mouse model\(^3\), in which the expanded repeat is interrupted ([CAACAGCAGCAACAGCAA]n), reproduces many of the features of HD, leading to the speculation that the interruptions in the repeat prevent the formation of abnormal RNA structures, hence eliminating a contribution of RNA toxicity. However, it is difficult to reconcile this conclusion with the increasingly compelling data, primarily from cell models (including human HD cells), that RNA is also important in disease pathogenesis. Part of the problem is that mouse models of repeat disease are inherently imperfect—each defers from other mouse models, from cell models, and from human disease in the degree to which they recapitulate the mechanistic complexity of repeat diseases, including variables such as repeat length and integrity, transcript and protein expression levels, localization and aggregation of protein and RNA, and the presence of RAN translation\(^3\). It is not possible to evaluate the role of RNA toxicity in cell or mouse models without data on expression levels, sub-cellular distribution, and aggregation status of expHTT RNA. Finally, it is quite possible that longer repeats shift the relevant importance of RNA and protein toxicity, so that the long repeats used in most mouse models may obscure the role of RNA toxicity.

In further support for the role of RNA in HD, we show that truncated HTT RNA aggregates into RNA foci in neuronal-like cells and in neurons of an HD transgenic mouse model (Supplementary Fig. S2). We also show for the first time that FL expHTT RNA readily forms RNA foci in neuronal-like cells (Fig. 2). In addition, we show that co-expression of MBNL1 decreases the number of RNA foci formed from exogenous expHTT RNA (Fig. 3). The effect of MBNL1 on the expHTT RNA foci formation is opposite to recent data suggesting that an exon 1 expHTT fragment forms RNA foci only when co-expressed with MBNL1\(^2\). We posit that this discrepancy is primarily due to the sensitivity of our assay in detecting RNA foci formed by both truncated and FL expHTT transcripts; a difference in cell types may also contribute to the different findings.

Our data demonstrate that FL expHTT RNA is retained in the nucleus of neuronal cells, in agreement with evidence of nuclear retention of HTT RNA in the R6/2 mouse model of HD\(^4\) and our own data, and those of others, showing that nuclear retention of expHTT fragments is increased by MBNL1\(^2\). Counterintuitively, we determined that the retention is not due to expHTT RNA aggregation into RNA foci, but involves aberrant interaction between FL expHTT RNA and MBNL1 (Fig. 4).

Our evidence that neither knock-down nor overexpression of MBNL1 has a significant effect on the total levels of FL HTT RNA (Fig. 4) differs from *Drosophila* models of SCA3\(^3\) and myotonic dystrophy 2 (DM2)\(^3\). While the MBNL1 effect on total RNA levels may be specific for the fly models, it is also...
possible that the effect of MBNL1 on localization and/or levels of expRNA is dependent on the sequence flanking the repeat. Overexpression of MBNL1 decreases non-ATG-initiated translation, while both MBNL1 Δ12–46 and MBNL1 Δ251–388 significantly facilitate this translational mechanism (Fig. 6). One possible explanation is that MBNL1 Δ12–46 inefficiently binds to expHTT RNA. While this is not sufficient to trigger
nuclear retention, the imposed structural changes on the expHTT RNA may facilitate RAN translation of expHTT RNA. On the other hand, MBNL1 Δ251–388, which has full RNA-binding ability, may be insufficiently retained in the nucleus to induce nuclear retention of expHTT RNA (Supplementary Fig. S5), while in the cytoplasm it may bind to expHTT RNA and facilitate non-ATG–dependent translation. These effects of MBNL1 variants on RAN translation may provide useful clues to understanding RAN translation.

U2AF65, a splicing factor and a component of the NXF1 receptor export pathway, stimulates nuclear export and increases the translation of expHTT RNA (Fig. 8), opposite to the effect of MBNL1. A potential explanation is that increased binding of MBNL1 to expHTT RNA also results in sequestration of U2AF65, disrupting the assembly of the NXF1 complex and leading to nuclear retention of expHTT RNA. Alternatively, and with more specificity, MBNL1 may interfere with the action of U2AF65 on expHTT, altering the entry of expHTT into the export pathway. While increasing U2AF65 levels is unlikely to decrease the toxicity of expHTT (Supplementary Fig. S6), further characterization of the expHTT RNA export pathway may identify novel targets with therapeutic potential. Intriguingly, the NXF1 receptor pathway was recently linked to DM1 by the finding that Aly/REF is associated with nuclear accumulation of expanded DMPK transcripts. Insertion of a WPRE (woodchuck post-transcriptional regulatory element) at the 3'-end of the expDMPK 3'-UTR stimulated the export of expanded DMPK RNA and rescued muscle cell differentiation. In addition, increasing Staufen1, a double-stranded RNA (dsRNA)-binding protein implicated in multiple post-transcriptional gene-regulatory processes, and thereby increasing nuclear export, rescued three hallmarks of DM1 pathology (aberrant splicing, nuclear export, and translation of expCUG RNA). A concern is that increasing nuclear export could elevate the levels of expHTT protein and increase neurotoxicity. However, if RNA toxicity is reduced, cells may more efficiently degrade or otherwise protect themselves from expHTT protein, consistent with the recognition that expHTT protein turnover is 3-fold more important for neuronal survival than is protein level. The net effect, we speculate, would be a decrease in neuronal toxicity. Targeting the interactions of MBNL1 and U2AF65 with expHTT with antisense oligonucleotides or small molecules may therefore have substantial therapeutic benefits.

Analysis of the effect of MBNL1 on the effect of expHTT toxicity is complicated by the finding that with or without the N-terminal zinc finger domain or NLS signal, MBNL1 itself increases cytotoxicity (Supplementary Fig. S6), and MBNL1 knockdown decreases SK-N-MC cell proliferation (Supplementary Fig. S6). Similarly, overexpression of U2AF65 reduced proliferation of SK-N-MC cells (Supplementary Fig. S6), complicating the interpretation of the apparent effect of U2AF65 on reducing expHTT toxicity. This is not unexpected as both MBNL1 and U2AF65 have multiple functions and any changes in the levels of RBPs are likely to disrupt multiple cellular pathways. Indeed, the effects of MBNL1 overexpression on models of repeat expansion disease appear to vary as a function of the model organism, the repeat expansion under exploration, and levels of transcript expression. We speculate that the effects of MBNL1 on missplicing and nuclear retention exist in homeostatic tension, and that disruption of the homeostasis, whether through overexpression or knockdown, can result in neurotoxicity.

Taken together, our data provide mechanistic support for the hypothesis that expHTT transcripts contribute to the pathogenesis of HD and support an examination of nuclear export pathways as a source of novel therapeutic targets for HD.

Materials and Methods

Reagents, cells and mice. Control siRNA (sc-37007) and human MBNL1 siRNA (sc-60988) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies used for immunoblott- ing were as follows: anti-huntingtin (MAB2166, 1:1000), anti-polylactamidine, 1C2 (MAB1574, 1:2000), anti-ATXN3 (MAB5360, 1:500) from EMD Millipore (Billerica, MA); anti-ATXN2 (611378, 1:500) from BD Transduction Laboratories (San Jose, CA); anti-beta-actin (ab8224, 1:5000) from Abcam (Cambridge, MA); anti-GFP (G10362, 1:2000), anti-myc (AHO0062, 1:2000), anti-HA (32–6700, 1:1000), -actin (ab8224, 1:5000) from Abcam (Cambridge, MA); anti-GFP (G10362, 1:2000), anti-myc (AHO0062, 1:2000), anti-HA (32–6700, 1:1000) from Life Technologies (Grand Island, NY); anti-MBNL1 (A2764, 1:1000) from Dr. Charles A. Thornton (University of Rochester, Rochester, NY); anti-mRFP (5F8, 1:1000) from Chromotek (Martinsried, Germany); anti-N-terminus of huntingtin (sc-8767, 1:200) from Santa Cruz Biotechnology; anti-FLAG (4C5, 1:1000) from Origene (Rockville, MD).

Neuroblastoma cell lines SK-N-MC (HTB-10; ATCC) and SH-SY5Y (CRL-2266; ATCC) were cultured in Dulbecco’s Modified Eagle Medium with 4.5g/L glucose and supplemented with 10% fetal bovine serum. Nine-month-old C57BL/6j wild type mice were from The Jackson Laboratory (Bar Harbor, ME), and N586-82Q transgenic HD mice were previously described. The breeding and subsequent use of mice were approved by the ACUC of Johns Hopkins University, Baltimore, MD. Mice were housed at the East Baltimore campus rodent vivarium and maintained on a standard circadian cycle with free access to water and standard chow.

Plasmid construction. Plasmids encoding truncated N-terminal HTT (N63QnHTT) and FL HTT (FL-HTTQn) were described by Cooper et al. and Ratovitski et al., respectively. N90QnHTT plasmids were obtained from Corell Institute for Medical Research (Camden, NJ). The translation start codon of N63QnHTT plasmids was deleted and a TAG stop codon was introduced immediately 5’ to the repeat to produce N63(CAG)nHTT plasmids. To examine RAN translation, N63(CAG)nHTT inserts...
were PCR-amplified and cloned into the Xhol and XbaI sites of the A8(*KKQ)3Tf1 vector to produce N63(CAG)nHTT-RAN plasmids. JPH3Ala55 plasmid expressing the JPH3 transcript in the polyalanine frame and JPH3-(CTG)55 plasmid expressing a non-translatable JPH3 transcript were described by Seixas et al. 32. GFP-MBNL1 plasmid expressing GFP-tagged human MBNL1 isoform C was previously described by Li et al. 33. GFP-MBNL1 Δ12–46 was constructed by deleting amino acids 12–46 of MBNL1 using primers 5′-CAC CAA TTC GGG ACA CAA ATG GAC GAG TAA TAC CAG GTC A-3′ (forward) and 5′-GGC ATT ACT CTT GGT TCC CGA ATT GGT GTG G-3′ (reverse) and the QuikChange II XL Site-Directed Mutagenesis Kit (Aglent Technologies, Santa Clara, CA). GFP-MBNL1 Δ251–388 was constructed by mutating a CAA codon to a TAA stop codon at amino acid 251 using primers 5′-ATG CTC GGC TAA TAC CAG GTC A-3′ (forward) and 5′-TGA CCT TGG ATG AGG CAG CCT CTA T-3′ (reverse) and the QuikChange II XL Site-Directed Mutagenesis Kit. pcDNA3.1 and pcDNA-mRFP plasmids were from Life Technologies. eEF1A1-Myc-FLAG and AKT-HA plasmids encoding myc- and FLAG-tagged eEF1A1 and HA-tagged AKT, respectively, were from Origene. U2AF65 and GFP-ATXN2Qn plasmids were previously described 30,34. All plasmids were confirmed by sequencing before use.

Fluorescence in situ hybridization and microscopy. SK-N-MC cells were transfected with plasmids expressing FL HTT transcripts and fixed in 4% paraformaldehyde 48 hours post-transfection. Frontal cortices from wild-type or N586-82Q HD mice were collected and frozen-sectioned. Fluorescence in situ hybridization (FISH) with or without DNase/RNase treatment was performed as previously described 7, 23. GFP-MBNL1 was previously described by Lin 35. SH-SY5Y and SK-N-MC cells were plated in 96-well plates (2,500 cells/well) and transfected with 0.2 μL of Lipofectamine 2000 (Life Technologies), and protein extracts were analyzed by immunoblotting 72 hours post-transfection. For knock-down of MBNL1, cells were first transfected with 200 pmol of siRNA. After 72 hours cells were further transfected with 100 pmol of siRNA plus 2 μg of plasmids and analyzed by immunoblotting 48 hours post-transfection. Cells were lysed with RIPA buffer (Sigma-Aldrich, St. Louis, MO), and the protein lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked, probed with primary antibody at 4°C overnight, washed, and incubated with HRP-conjugated secondary antibodies, and the proteins were then visualized using the ECL Prime Western Blotting System (GE Healthcare, UK) and Hyperfilm ECL (GE Healthcare). Western blots. SK-N-MC cells were plated in 6-well plates (200,000 cells/well), transfected with 4 μg of plasmids and 4 μL of Lipofectamine 2000 (Life Technologies), and protein extracts were analyzed by immunoblotting 72 hours post-transfection. For knock-down of MBNL1, cells were first transfected with 200 pmol of siRNA. After 72 hours cells were further transfected with 100 pmol of siRNA plus 2 μg of plasmids and analyzed by immunoblotting 48 hours post-transfection. Cells were lysed with RIPA buffer (Sigma-Aldrich, St. Louis, MO), and the protein lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked, probed with primary antibody at 4°C overnight, washed, and incubated with HRP-conjugated secondary antibodies, and the proteins were then visualized using the ECL Prime Western Blotting System (GE Healthcare, UK) and Hyperfilm ECL (GE Healthcare).

Caspase activity assay. SK-N-MC and SH-SY5Y cells were plated in 96-well plates (2,500 cells/well) and transfected with 0.2 μg of plasmids and 0.2 μL of Lipofectamine 2000. Cytotoxicity was assessed at
72 hours post-transfection by measuring caspase-3/7 activities using the Caspase-Glo 3/7 Assay system (Promega, Madison, WI).

**MTT proliferation assay.** SK-N-MC cells were plated in 96-well plate (5,000 cells/well) and transfected with 0.2 μg of plasmids and 0.2 μL of Lipofectamine 2000. Cell numbers before and after transfection were assessed 72 hours post-transfection using the Vybrant MTT Cell Proliferation Assay Kit (Life Technologies).

**Statistical analysis.** At least three biological replicates of each experiment were performed. Data were presented as mean ± SD. The results were analyzed using Student’s t-test, one-way analysis of variance (ANOVA) followed by Dunnett’s or Bonferroni’s post-hoc test, or two-way ANOVA followed by Bonferroni’s post-hoc test. Statistical significance was set at P value < 0.05.

**References(161,391),(843,996)
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Author Contributions
D.D.R. conceived and supervised the study; X.S., P.P.L., S.Z., R.C. and L.O.M. carried out the experiments and analyzed data; C.A.R., S.M.P., H.Y.E.C. and R.L.M. provided fundamental reagents and intellectual contribution; X.S. and D.D.R. were involved in writing the paper. All the authors had final approval of the submitted version.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

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