A peptidylc inhibitor-based therapeutic approach that simultaneously suppresses RNA- and protein-mediated toxicities in polyglutamine diseases

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Abstract

Polyglutamine (polyQ) diseases represent a group of progressive neurodegenerative disorders that are caused by abnormal expansion of CAG triplet nucleotides in disease genes. Recent evidence indicates that not only mutant polyQ protein, but also their corresponding mutant RNAs contribute to the pathogenesis of polyQ diseases. Here, we describe the identification of a 13-amino acid peptide, P3, which binds directly and preferentially to long CAG RNA within the pathogenic range. When administered to cell and animal disease models, as well as to patient-derived fibroblasts, P3 inhibited expanded CAG RNA-induced nucleolar stress and suppressed neurotoxicity. We further examined the combined therapeutic effect of P3 and polyQ-binding peptide 1 (QBP1), a well-characterized polyQ protein toxicity inhibitor, on neurodegeneration. When P3 and QBP1 were co-administered to disease models, both RNA and protein toxicities were effectively mitigated, resulting in a notable improvement of neurotoxicity suppression compared with the P3 and QBP1 single treatment controls. Our findings indicate that targeting toxic RNAs and/or simultaneous targeting of toxic RNAs and their corresponding proteins may open up a new therapeutic strategy for treating polyQ degeneration.
Introduction

Polyglutamine (PolyQ) diseases represent a group of dominantly inherited progressive neurodegenerative diseases (Orr and Zoghbi, 2007). These diseases are caused by genomic CAG trinucleotide repeat expansion in the coding region of the disease genes in which the CAG triplet repeats function as the codon for the amino acid glutamine. After gene transcription and protein translation, two primary toxic species - mRNA containing expanded CAG repeats and protein carrying an expanded polyQ domain - are produced in the neurons. These two mutant biomolecules induce neurotoxicity through multiple pathogenic pathways that lead to neurodegeneration (Fiszer and Krzyzosiak, 2013; Nalavade et al., 2013; Williams and Paulson, 2008). Recently, an additional RNA-dependent mechanism was reported by which toxic RNAs are translated into additional protein species with expanded homopolymeric amino acid tracts through the mechanism of repeat-associated non-ATG (RAN) translation initiation (Cleary and Ranum, 2014).

Ribosome biogenesis is essential for cellular protein synthesis. The ribosome is a ribonucleoprotein complex composing of ribosomal RNAs and ribosomal proteins. Failure in ribosomal RNA transcription induces nucleolar stress, and cells undergo apoptosis. Thus, nucleolar stress is a cellular response designed to eliminate cells that fail to carry out efficient protein synthesis due to ribosome biogenesis defects (Boulon et al., 2010). A reduction in ribosomal RNA transcription leads to an imbalance of cellular levels of ribosomal RNAs and ribosomal proteins, and this results in an increased level of unassembled free ribosomal proteins which are the proteinaceous components of the ribosome (Zhang and Lu, 2009). These free ribosomal proteins are targeted by the MDM2 E3 ubiquitin ligase for poly-ubiquitination and
subsequent proteasome degradation. The engagement of MDM2 with free ribosomal protein degradation causes a cellular buildup of the p53 protein, which is a physiological substrate of MDM2. The cellular accumulation of p53 triggers the activation of mitochondrial-mediated apoptosis (Wang et al., 2014). Nucleolar stress response has been implicated in the pathogenesis of various neurodegenerative diseases (Parlato and Kreiner, 2013), including Alzheimer’s and Parkinson’s Diseases, and Amyotrophic Lateral Sclerosis. Our laboratory was the first to provide evidence that nucleolar stress is involved in the pathogenesis of polyQ diseases, including Machado Joseph Disease (MJD/SCA3) and Huntington’s Disease (HD) (Chan, 2014; Kreiner et al., 2013; Lee et al., 2011; Tsoi and Chan, 2013; Tsoi and Chan, 2014; Tsoi et al., 2012). We showed that expanded CAG RNA interacts directly with the nucleolar protein nucleolin (NCL), and that this RNA-protein interaction prevents NCL from binding to the *upstream control element* (*UCE*) of the *ribosomal RNA* (*rRNA*) promoter. This then leads to *UCE* hypermethylation and down-regulation of *pre-45s rRNA* transcription, which eventually triggers nucleolar stress-induced apoptosis (Tsoi et al., 2012). We further showed that the overexpression of exogenous NCL protein inhibits *UCE* hypermethylation, restored *pre-45s rRNA* transcription and suppresses the nucleolar stress induced by expanded CAG RNA expression (Tsoi et al., 2012). These findings suggest that inhibition of the NCL-expanded CAG RNA interaction may offer a viable therapeutic strategy to suppress RNA toxicity in polyQ diseases. The human NCL protein carries four RNA recognition motifs (RRMs) (Ginisty et al., 1999), and our previous investigation pinpointed RRM2 and 3 as the interacting regions in NCL that mediate its binding to expanded CAG RNA (Tsoi et al., 2012).
Peptidylic inhibitors have been demonstrated to disrupt the RNA-protein interaction resulting in suppression of viral replication (Hamy et al., 1997). This prompted us to develop peptidylic inhibitors that could mitigate expanded CAG RNA toxicity. We scanned through a series of synthetic peptide sequences derived from RRM 2 and 3 of the NCL protein, and identified a 13-amino acid peptide, P3, which could bind directly and preferentially to expanded CAG RNA (calculated $K_D = 52.70 \pm 2.21 \mu M$). Next, we further demonstrated that P3 disrupted the interaction between endogenous NCL protein and the expanded CAG RNA. The introduction of the P3 peptide to cells expressing expanded CAG RNA resulted in the restoration of the interaction between NCL and $UCE$, and the level of pre-45s rRNA expression. We further showed that P3 suppressed cell death in both cell and $Drosophila$ disease models, and in MJD patient-derived fibroblasts. Our findings indicate that expanded CAG RNA toxicity can be targeted by peptidylic inhibitors. Finally, various peptidylic inhibitors (Arribat et al., 2013; Kazantsev et al., 2002; Mishra et al., 2012), including QBP1 (Nagai et al., 2000), have been reported to be capable of targeting polyQ protein toxicity by inhibiting misfolding and aggregation of expanded polyQ disease protein (Popiel et al., 2013). When P3 was co-administered with the polyQ protein toxicity inhibitor QBP1 (Nagai et al., 2000), the combined treatment of RNA- and protein-triggered toxicities lead to even greater suppression of neurodegeneration in vivo in $Drosophila$ model of MJD. Our findings indicate that targeting toxic RNAs alone may be sufficient to elicit a significant therapeutic benefit, while the simultaneous targeting of both toxic RNAs and their corresponding toxic proteins is desirable to treat polyQ disease more efficaciously.
Results

Identification of a peptide that prevents the binding of NCL to expanded CAG RNAs

We previously reported that expanded CAG RNA triggered nucleolar stress in polyQ diseases (Tsoi and Chan, 2013; Tsoi et al., 2012). We showed that the over-expression of full-length Nucleolin (NCL) protein restores rRNA transcription and suppresses the pro-apoptotic events triggered by expanded CAG RNA (Tsoi et al., 2012). This suggests that targeting the interaction between mutant RNA and NCL with inhibitors, such as peptides, represents a novel therapeutic direction. According to our previous observations (Tsoi and Chan, 2013; Tsoi et al., 2012), the information available on the structure of the RRMs of NCL protein (PDB ID=2KRR) (Arumugam et al., 2010), and the RRM/RNA binding interface (Daubner et al., 2013), we synthesized six peptides (P1-P6) that covered the NCL RRM2 and 3 regions (Fig. 1A). The ability of individual peptides to interrupt the RNA-protein interaction between the in vitro transcribed expanded CAG RNA, MJD_{CAG78}, and the purified GST-NCL protein was determined by a glutathione S-transferase pull-down assay (Tsoi et al., 2012). Two peptides, P3 and P5, were found to be capable of interfering with NCL binding to MJD_{CAG78} RNA (Fig. 1B). We focused our subsequent investigation on P3 as this peptide was derived from NCL RRM2, whose structure was elucidated previously (Arumugam et al., 2010).

P3 preferentially modulated ribosomal RNA transcription in cells that expressed expanded CAG RNA

We next investigated whether P3 could mitigate expanded CAG RNA toxicity in cells. Following over-expression of CAG RNA - EGFP_{CAG78} – in HEK293 cells, a reduction in the level of pre-45S rRNA was observed compared to the cells over-expressed with EGFP_{CAG27} (Fig.
However, when P3 was co-expressed, the levels of pre-45s rRNA were restored to 70% of the $\text{EGFP}_{CAG27}$ control. The effect was not due to P3 affecting the levels of $\text{EGFP}_{CAG78}$ RNA (Fig. S1). Further, P3 expression had no effect on the level of pre-45s rRNA in cells expressing the control construct $\text{EGFP}_{CAG27}$ (Tsoi et al., 2012) (Fig. 1D). Our data thus highlight the specificity of P3 action towards expanded CAG RNA. As rRNA transcription is mediated by RNA polymerase I, we further examined whether P3 expression would affect the expression levels of genes that are transcribed by RNA polymerases II (GAPDH) and III ($U6$ and $tRNA^{met}$), and observed no change in RNA polymerase II- or III-mediated transcription in either $\text{EGFP}_{CAG27}$ or $\text{EGFP}_{CAG78}$ RNA-expressing cells (Fig. S2). This indicates that P3 expression does not affect cellular gene transcription in general.

**Structure-activity relationship of P3**

We next investigated the structure-activity relationship of P3. Since basic and aromatic side-chains usually play crucial roles in protein/peptide-nucleic acid interaction, we speculated that four residues in P3, namely Lys3, Lys5, Tyr9 and Phe12, are involved in RNA binding. These residues have previously been reported to play pivotal roles in RNA-protein (peptide) interactions (Iwakiri et al., 2012), including RRM-RNA interaction (Jenkins et al., 2011; Morozova et al., 2006). Hence, we generated five $P3$ point mutant constructs ($P3MT1-5$; Fig. 1C). The $P3MT1-4$ constructs each carry a single alanine substitution mutation at positions Lys3, Lys5, Tyr9 and Phe12, respectively, whereas $P3MT5$ carries a Tyr9Ala Phe12Ala double mutations. Expression of the $P3MT$ constructs was first confirmed by RT-PCR analysis, and their expression did not alter the levels of $\text{EGFP}_{CAG78}$ RNA (Fig. S1). Next, we examined the expression level of pre-45s rRNA in HEK293 cells co-transfected with both the $\text{EGFP}_{CAG}$
individual P3MT constructs. In contrast to the P3WT positive control, rRNA transcription could not be restored via the expression of any of the P3MT constructs in EGFP<sub>CAG78</sub> RNA-expressing cells (Fig. 1D). This indicates that Lys3, Lys5, Tyr9 and Phe12 are all essential for P3 functioning. P3MT5 was used as a negative control in our subsequent experiments.

**Mechanism of action of P3-mediated suppression of expanded CAG RNA toxicity**

Expanded CAG RNA-induced nucleolar stress is initiated by a reduction in the binding of NCL to the UCE of the rRNA promoter, which results in UCE hypermethylation (Tsoi and Chan, 2014). We next hypothesized that the expression of P3 prevents sequestration of NCL by expanded CAG RNA, allowing it to resume its normal cellular role in rRNA transcription regulation. To test this, HEK293 cells were co-transfected with EGFP<sub>CAG78</sub> and P3 constructs, and chromatin immunoprecipitation was performed to determine whether P3 restores the binding of endogenous NCL to UCE. Indeed, co-expression of P3 restored the interaction between endogenous NCL and UCE in cells expressing EGFP<sub>CAG78</sub> RNA (Fig. 1E). No such effect was observed in the negative control P3MT5. We further found that the expression of P3WT, but not P3MT5, suppressed UCE hypermethylation in EGFP<sub>CAG78</sub> RNA-expressing cells (Fig. 1F). Our findings indicate that P3 can effectively suppress expanded CAG RNA-induced nucleolar stress (Tsoi et al., 2012).

We previously demonstrated that expanded CAG RNA-induced apoptosis is mediated through the caspase pathway (Tsoi et al., 2012). We therefore examined the activity of distinct caspase pathways in cells that expressed EGFP<sub>CAG78</sub> RNA. The result showed that the activity of caspase 9, but not that of caspase 8, was elevated in HEK293 cells expressing expanded CAG RNA (Fig.
S3). When cells were co-transfected with P3 and EGFP<sub>CAG78</sub>, caspase 9 activity was significantly suppressed when compared with the EGFP<sub>CAG78</sub>-transfected cells (Fig. 1G). This supports the idea that the intrinsic apoptotic pathway is involved in expanded CAG RNA toxicity, and is in line with our previous observations that expanded CAG RNA induced mitochondrial cytochrome c release (Tsoi et al., 2012).

**P3 interacts directly with expanded CAG RNA**

We previously showed that NCL utilizes its RRM domains to interact with expanded CAG RNA (Tsoi et al., 2012). We next tested if P3, which is derived from NCL’s RRM2, interacts physically with expanded CAG RNA using isothermal titration calorimetry (iTC) (Li et al., 2009; Wong et al., 2014). We first showed that the synthetic wild-type P3 peptide associated with unexpanded MJD<sub>CAG27</sub> RNA with a $K_D$ value of 127.60±26.88 µM (Fig. 2A). When compared with its interaction with MJD<sub>CAG27</sub> RNA, P3 bound to expanded MJD<sub>CAG78</sub> RNA with a lower $K_D$ value (52.70±2.21 µM; Fig. 2B). This result indicates that P3 has a stronger interaction with RNA containing expanded CAG repeat RNA. In addition, we showed that P3 binding depends on the integrity of the CAG repeat, as the peptide interacted weakly with CAA interrupted-CAG repeat in the context of MJD transcript, MJD<sub>CAA/G78</sub> ($K_D$: 384.81±57.77 µM; Fig. 2C). Taken together, P3 peptide interacts preferentially with long continuous CAG triple repeat sequences. Our findings thus far demonstrate that P3 suppresses expanded CAG RNA toxicity (Fig. 1G) by binding directly to expanded CAG RNA (Fig. 2) leading to subsequent release of NCL (Fig. 1B) and restoration of <i>pre-45s rRNA</i> transcription (Fig. 1D-F).
Administration of synthetic P3 peptide suppressed expanded CAG RNA-induced cell death in vitro

Cell-penetrating peptides (CPPs) have been widely used as a vehicle to enhance delivery of therapeutics across the cell membrane (Koren and Torchilin, 2012), including the peptidylic inhibitors of polyQ protein toxicity QBP1 (Popiel et al., 2007; Popiel et al., 2009) and htt\textsuperscript{NT} (Mishra et al., 2012). The TAT peptide is a CPP derived from the HIV-1 virus transactivator of transcription protein, which has been reported to mediate the translocation of proteins across the cell membrane (Frankel and Pabo, 1988; Green and Loewenstein, 1988). We therefore synthesized TAT-P3 fusion peptides (TAT-P3WT and TAT-P3MT5) (Fawell et al., 1994; Vives et al., 1997) and tested the effect of the fusion peptides on expanded CAG RNA toxicity. We first examined whether TAT-P3 treatment was capable of neutralizing expanded CAG RNA-mediated cytotoxicity in HEK293 cells and observed a dose-dependent reduction of cytotoxicity (Fig. 3A), as detected by the lactate dehydrogenase (LDH) cytotoxicity assay (Banez-Coronel et al., 2012). The calculated maximal inhibitory concentration (IC\textsubscript{50}) value was 4.369±1.140 µM.

Next, we tested if the effect of the TAT-P3 peptide on cytotoxicity is due to the suppression of NCL-mediated nucleolar stress (Tsoi et al., 2012). We first performed real-time PCR analysis to confirm that TAT-P3 treatment did not affect the expression level of \textit{EGFP\textsubscript{CAG78}} RNA (Fig. S1). When compared with \textit{EGFP\textsubscript{CAG78}} RNA-expressing HEK293 cells, \textit{pEGFP\textsubscript{CAG78}}-transfected cells that were co-treated with the synthetic TAT-P3 peptide (12 µM) showed an increased levels of \textit{pre-45s rRNA} (Fig. 3B), \textit{18S rRNA} (Fig. 3C) and \textit{UCE/NCL} interaction (Fig. 3D), as well as a reduction in \textit{UCE} hypermethylation (Fig. 3E), p53 protein level (Fig. 3F), and caspase 9 activity (Fig. 3G). The above effects were not detected in cells co-treated with the TAT-P3MT5 negative
control peptide. Taken together, our results indicate that intracellular delivery of synthetic P3 peptide suppresses expanded CAG RNA-induced nucleolar stress and subsequently cell death. Although P3 physically interacts with cellular RNAs that carry short non-toxic CAG repeats \((MJD_{\text{CAG27}}} \text{RNA; Fig. 2A})\), it did not induce caspase activation in cells expressing normal length of CAG repeats (Fig. 1G). We performed a set of control experiments to show that P3 does not act on the polyQ protein and that its effect is specific for expanded CAG repeat-containing RNAs, but not RNAs containing other trinucleotide repeat expansions. First, we performed Western blot analysis to examine whether TAT-P3 treatment would alter protein translation of \(\text{ataxin 2 (ATXN2) CAG} \) mRNAs of different repeat lengths (22, 42, 55 and 72 CAGs). We observed no effect of TAT-P3 on the levels of ATXN2 proteins (Fig. S4A). Next, we found that the adult eclosion rate of wild-type \(\text{Drosophila} \) (Fig. S4B) and the viability of primary rat cortical neurons (Fig. S5) were not compromised when these models were treated with up to 1 mM and 25 µM of the TAT-P3 peptide respectively. Finally, we showed that TAT-P3 had no effect on staurosporine-induced cell death \(\text{in vitro} \) (Fig. S6A), nor \(\text{in vivo} \) toxicity induced by the expression of expanded \(\text{CUG} \) (Garcia-Lopez et al., 2008) and \(\text{CGG} \) (Jin et al., 2003) RNAs in \(\text{Drosophila} \) (Fig. S6B). Taken together, our findings demonstrate that the P3 peptide displays specificity for expanded CAG RNA-induced toxicity.

Simultaneous suppression of RNA- and protein-induced toxicities in polyglutamine neurodegeneration

Since both expanded CAG RNA and polyQ protein contribute to neurotoxicity in polyQ degeneration (Fiszer and Krzyzosiak, 2013; Nalavade et al., 2013), we next tested a combined therapeutic approach to concomitantly target RNA and protein toxicities. In addition to CPP (Fig.
3), we also tested whether the peptide transfection reagent DeliverX (DX) (Deshayes et al., 2004) could be used to deliver synthetic peptides to cells and mitigate expanded CAG RNA toxicity. Our results showed that DX-mediated delivery of 4 µM of synthetic P3 peptide could effectively restore both pre-45s rRNA level and NCL/UCE interaction, and suppress caspase 9 activity in our EGFP<sub>CAG78</sub> RNA toxicity-only cell model (Tsoi et al., 2012) (Fig. S7A-C). We further showed that DX-assisted intracellular delivery of P3 did not alter RNA polymerase II- and III-mediated gene expression (Fig. S8), indicating that the suppressive effect of the P3 peptide is specific for pre-45s rRNA transcription mediated by RNA polymerase I (Fig. S7A). After validating the DX-assisted peptide delivery protocol, we utilized the MJD<sub>CAG78</sub> cell model (Tsoi et al., 2011) in our subsequent analyses because this model exhibits both expanded CAG RNA and polyQ protein toxicities. When MJD<sub>CAG78</sub>-transfected cells were treated with the synthetic P3 peptide, the levels of rRNA was restored to the MJD<sub>CAG27</sub> control level (Fig. 4A, B). Taken together, our results indicate that DX-mediated intracellular peptide targeting is effective in neutralizing expanded CAG RNA toxicity (Fig. 4A, B).

The QBP1 peptide (Nagai et al., 2000) is one of the most studied polyQ protein toxicity peptidylic inhibitors which has been demonstrated to target disease protein misfolding and aggregation (Popiel et al., 2013). Hence, we used the QBP1 peptide as a model polyQ protein toxicity inhibitor in our subsequent investigations. We first made use of the rRNA as a readout to test whether the co-delivery of the P3 and QBP1 peptides would interfere with the suppression effect of P3 on RNA toxicity. Our results clearly showed that when P3 was co-delivered with QBP1 or its scrambled control peptide (QBP1 SCR) to MJD<sub>CAG78</sub>-transfected cells, the rRNA level was restored back to the control level (Fig. 4A, B). In contrast, both P3MT5-control peptide
treatment groups (P3MT5+QBP1 and P3MT5+QBP1 SCR) failed to rescue the rRNA defects (Fig. 4A, B). This indicates that QBP1 co-delivery has no effect on the efficacy of P3. A similar result was obtained from MJD patient-derived fibroblasts (Fig. 4C), further substantiating the application of peptide-based therapeutic interventions for expanded CAG RNA toxicity.

The binding immunoglobulin protein (BiP), also known as GRP-78 (Munro and Pelham, 1986), is a molecular chaperone responsible for protein refolding. Up-regulation of BiP has been reported in polyQ diseases (Duennwald and Lindquist, 2008; Kouroku et al., 2002; Leitman et al., 2013). X-box binding protein 1 (XBP1) is transcriptional factor that regulates chaperone gene expression, and its activation requires the excision of a 26-nucleotide fragment from the unspliced XBP1 transcript (XBP1U) to generate the active spliced XBP1S mRNA for subsequent production of the functional XBP1 protein (Yoshida et al., 2001). To monitor polyQ protein toxicity, we used both BiP gene induction and XBP1 splicing as readouts to monitor polyQ protein toxicity, and found that the expression of MJDQ78 protein induced BiP transcription (Fig. 4D) and XBP1S production (Fig. 4E) in our MJD CAG78 cell model. The DX-assisted delivery of QBP1, but not QBP1 SCR scramble control, reduced the cellular BiP expression and production of XBP1S (Fig. 4D, E; Fig. S9). This indicates that the QBP1 peptide suppresses polyQ protein toxicity and the suppression is not affected by the co-delivery of the P3 peptide. An effective suppression effect of QBP1 was also detected in MJD patient-derived fibroblasts (Fig. 4F).

When evaluating the overall inhibitory effects of the different treatment groups (Fig. 4A-F), the P3WT+QBP1 SCR group only conferred suppression on RNA toxicity, as shown by rRNA restoration, while the P3MT5+QBP1 group solely mitigated protein toxicity, as determined by a
reduction in BiP induction and XBP1S production (Fig. 4A-F). This indicates the respective suppression specificity of P3 and QBP1 in RNA and protein toxicities. In comparison to the P3WT+QBP1 SCR and P3MT5+QBP1 single treatment groups, the P3WT+QBP1 co-treatment group was found to yield the most marked suppression of both expanded CAG RNA and polyQ protein toxicities as evidenced by both the significant restoration of rRNA defects and the reduction of BiP mRNA induction/XBP1S production in the HEK293 (Fig. 4A, B, D and E), as well as the MJD patient-derived fibroblast (Fig. 4C, F) cells. As a step further, we evaluated the efficacy of P3+QBP1 co-treatment in suppressing cytotoxicity in our MJD_{CAG78} RNA/protein cell model. Based on the result of the LDH cytotoxicity assay, cells treated individually with either the functional P3WT (P3WT+QBP1 SCR) or QBP1 (P3MT5+QBP1) peptide yielded only a partial inhibition of cell death (Fig. 4G; Fig. S10). Intriguingly, the P3WT+QBP1 co-treatment group suppressed MJD_{CAG78} RNA/protein-induced cell death more effectively when compared with the single treatment groups (Fig. 4G). This demonstrates that P3WT+QBP1 co-treatment exerts an additive protective effect on MJD_{CAG78} cell death conferred by both RNA and protein toxicities.

P3/QBP1 peptide co-treatment effectively suppressed polyglutamine neurodegeneration in vivo

Drosophila has been used as in vivo models to investigate peptidylic inhibitors of polyQ protein toxicity (Arribat et al., 2013; Kazantsev et al., 2002; Nagai et al., 2003). Peptide feeding was previously reported to be an effective way to deliver CPP-fusion QBP1 peptide to flies to mitigate polyQ protein toxicity, and it was demonstrated that 200 µM of QBP1 was capable of suppressing polyQ toxicity in vivo (Popiel et al., 2007). We utilized the full-length MJD_{CAG} fly
model, \textit{flMJD}_{CAG27/84}, (Warrick et al., 2005) to investigate the combined suppression effect of P3 and QBP1. The expression of expanded \textit{flMJD}_{CAG84} RNA and \textit{flMJDQ84} protein caused severe retinal degeneration, which can be quantified by the pseudopupil assay (Chan et al., 2011) (3.06±0.10; Fig. 5A, B). We observed a mild but significant suppression of neurotoxicity in \textit{flMJD}_{CAG84} flies after they were treated with either the functional P3 (the TAT-P3WT+TAT-QBP1 SCR group; 3.93±0.13) or QBP1 (the TAT-P3MT5+TAT-QBP1 group; 3.91±0.01) peptidylic inhibitor (Fig. 5A, B). We also expressed the \textit{flMJD}_{CAG84} transgene using the pan-neural \textit{Elav-GAL4} driver to test whether P3 could modulate expanded CAG RNA toxicity in nervous tissues other than the eye. Pan-neural expression of the \textit{flMJD}_{CAG84} transgene caused adult lethality, and TAT-P3 treatment partially but significantly delayed \textit{flMJD}_{CAG84}–induced lethality in flies (Fig. S11). This indicates the \textit{in vivo} suppression effect of P3 does not simply confine to the photoreceptor neurons in the eye, but can further be extended to other nervous tissues.

We next determined whether a concurrent inhibition of both RNA and protein toxicities would yield an additive effect on the rescue of neurodegeneration \textit{in vivo}. As expected, when \textit{flMJD}_{CAG84} flies were simultaneously treated with TAT-P3 and TAT-QBP1 peptides (200 µM each), a marked preservation of retinal integrity was observed as evidenced by a significant increase in the pseudopupil score (4.93±0.14) when compared with the single treatment groups (Fig. 5A, B). More importantly, no deleterious effect was observed when TAT-P3 and TAT-QBP1 were co-administered \textit{in vivo}, as indicated by the retinal integrity of the \textit{flMJD}_{CAG27} control flies (Fig. 5A, B). This finding is consistent with our cell-based toxicity (Fig. S10) and animal lethality (Fig. S4) investigations, in which P3 and QBP1 peptidylic inhibitors did not
elicit any dominant toxicity effect under our experimental conditions. We further found that treating flies with both non-functional peptidylic inhibitors, TAT-P3MT5 and TAT-QBP1 SCR, did not cause any suppression of neurodegeneration (2.95±0.09; Fig. 5A, B). This demonstrates that the TAT CPP component of the peptides did not contribute to the phenotypic suppression.

To further confirm that P3+QBP1 co-treatment mitigate both expanded CAG RNA and polyQ protein toxicities, we examined the expression levels of rRNA (Larson et al., 2012) (Fig. 5C, D), BiP (Chow et al., 2015) (Fig. 5E) and Xbp1S (Ryoo et al., 2007) (Fig. 5F) in flMJD CAG flies treated with TAT-P3 and/or TAT-QBP1. Real-time PCR analysis demonstrated a marked restoration of rRNA levels (Fig. 5C, D) in animals treated with TAT-P3 in combination with either TAT-QBP1 or TAT-QBP1 SCR (Fig. 5C, D). This confirms the suppression effect of the P3 peptide on expanded CAG RNA-mediated nucleolar stress induction in vivo. We next investigated the rescue effect of QBP1 on polyQ protein toxicity (Popiel et al., 2007) in our fly model. We first demonstrated that the expression level of the protein misfolding biomarkers BiP (Fig. 5E) and Xbp1S (Fig. 5F) were induced in the flMJD CAG84 flies when compared to that of the flMJD CAG27 control. This confirms that BiP and Xbp1S are reliable markers for monitoring protein toxicity in polyQ degeneration in vivo. When flMJD CAG84 flies were treated with TAT-QBP1 peptide either in combination with TAT-P3WT or TAT-P3MT5, we observed suppression of BiP induction (Fig. 5E) and Xbp1 splicing (Fig. 5F). We further showed that TAT-P3 and TAT-QBP1 treatment did not affect the protein expression of the unexpanded polyQ MJD disease protein (Fig. S12). Upon, TAT-QBP1 administration, we also observed that the stacking gel-residing SDS-insoluble expanded polyQ MJD protein was partially diminished (Fig. S12). Further, our findings illustrate that the mitigating effects of P3WT (Fig. 5C, D) and QBP1 (Fig.
5E, F; Fig. S12) on RNA and protein toxicity respectively were not influenced by the other co-administered peptide. Taken together, our data suggest that the simultaneous targeting of RNA and protein cellular toxicities using peptide agents is a viable approach for developing effective treatments for polyQ diseases.
**Discussion**

There are growing evidence that both mutant polyQ proteins (Williams and Paulson, 2008) and transcripts that encode the proteins (Fiszer and Krzyzosiak, 2013; Nalavade et al., 2013) contribute to the pathogenesis of polyQ diseases. Over the past decade, several peptidylic inhibitors have been developed to target polyQ protein toxicity, many of which have demonstrated promising therapeutic potential (Arribat et al., 2013; Kazantsev et al., 2002; Mishra et al., 2012; Nagai et al., 2000). However, the development of inhibitors for expanded CAG RNA-mediated neurotoxicity has been lagged behind. Peptidylic and small molecule inhibitors represent the two major groups of therapeutics for combating polyQ neurotoxicity, and both demonstrate significant therapeutic potential (Bauer and Nukina, 2009; Shao and Diamond, 2007). Of the two, peptidylic inhibitors are generally considered more selective (Cirillo et al., 2011). This property is particularly important in polyQ disease as a successful treatment requires an agent that can discriminate between mutant RNA/protein species and their wild-type counterparts.

Our previous investigation of RNA toxicity in polyQ diseases (Tsoi et al., 2012) led us to identify P3, a 13-amino acid peptide derived from NCL (Fig. 1), which is capable of neutralizing the nucleolar stress induced by expanded CAG RNA *in vitro* (Figs. 1, 3 and 4) and *in vivo* (Fig. 5). The P3 peptide preferentially binds to CAG RNA within the pathogenic repeat range, and diminishes the interaction between NCL and the mutant RNA (Fig. 6A). This leads to the reduction of UCE hypermethylation, restoration of pre-45s rRNA transcription, and blockade of nucleolar stress induction (Fig. 6A). We demonstrated that P3 is effective in suppressing RNA
toxicity in both an artificial expanded CAG RNA (Figs. 1, 3) and specific polyQ disease (Figs. 4, 5) models. This suggests that P3 is a generic peptidylic inhibitor against CAG RNA toxicity.

To date, multiple parallel pathogenic mechanisms have been reported to contribute to expanded CAG RNA toxicity (Evers et al., 2014; Marti and Estivill, 2013; Nalavade et al., 2013; Tsoi and Chan, 2014). In our study, we determined the empirical IC$_{50}$ value of our expanded CAG RNA toxicity peptidylic inhibitor P3 based on cell death inhibition in $EGFP_{CAG78}$ RNA-expressing cells (~4 µM; Fig. 3A). Recently, a small molecule compound, D6, was identified that is capable of correcting the pre-mRNA splicing in a HD patient-derived cell model (Kumar et al., 2012). Both D6 and P3 are capable of inhibiting particular RNA toxicity-associated molecular pathogenic mechanisms, namely RNA mis-splicing for D6 (Kumar et al., 2012) and nucleolar stress for P3 (Figs. 1, 3). More importantly, both studies unequivocally demonstrate that expanded CAG RNA toxicity can be targeted therapeutically. It would be of interest to further determine whether P3 and D6 suppress RNA toxicity through targeting the same set of cellular pathogenic events, or whether each of which has its own distinct set of suppression mechanism.

While our study describes the identification of the first peptidylic inhibitor that targets expanded CAG RNA toxicity, the prototypic P3 sequence may be further subjected to peptide engineering modifications (Ramos-Martín et al., 2014), such as N- and C-terminal truncation (Tomita et al., 2009), to improve its potency.

The QBP1 peptide is a well-characterized peptidylic inhibitors of polyQ protein toxicity (Nagai et al., 2000). Beta-sheet conformation transition of polyQ protein has been shown to be responsible for triggering protein toxicity in polyQ degeneration (Nagai et al., 2007), and QBP1
was reported to suppress protein toxicity by attenuating polyQ β-sheet conformation transition (Hervas et al., 2012; Nagai et al., 2007). In addition, QBP1 was found to be capable of inhibiting polyQ protein aggregation (Nagai et al., 2000). Both β-sheet conformation transition and aggregation of polyQ protein intimately associate with protein misfolding, and molecular chaperones are a class of cellular proteins responsible for promoting proper protein folding. Several previous observations have demonstrated that the expression level of multiple heat shock protein (hsp) genes are up-regulated in polyQ diseases (Huen and Chan, 2005; Huen et al., 2007; Tagawa et al., 2007), and such gene induction events are considered to be a cellular protective mechanism aiming to neutralize protein toxicity through promoting refolding of the polyQ disease protein. As one of the members of the molecular chaperone family, BiP protein levels were previously reported to be upregulated in polyQ disease (Duennwald and Lindquist, 2008; Kouroku et al., 2002; Leitman et al., 2013). In this study, we further showed that BiP gene expression as well as the spliced form of XBP1 mRNA, XBP1S, were induced in both in vitro and in vivo (Figs. 4, 5) conditions. This and previous findings emphasize a global activation of molecular chaperone machinery, including hsp such as BiP, to combat toxicity that associates with polyQ protein misfolding. Although QBP1 single treatment already resulted in a notable attenuation of cell death (Fig. 4E) and neurodegeneration (Fig. 5B), a P3/QBP1 co-treatment clearly led to a more complete suppression.

One challenging issue in therapeutic intervention of polyQ diseases is the delivery of inhibitors to the cellular targets, neurons in the central nervous system. Increasing number of peptide therapeutics enter clinical trial phases in recent years (Kaspar and Reichert, 2013), one of the reasons could be due to the development of newly emerging peptide drug technologies such as
cell-penetrating peptides (Fosgerau and Hoffmann, 2015). GRN1005 is a peptide-drug conjugate for treating advanced brain tumors, and it was found that intravenous administration of GRN1005 to patients resulted in the shrinkage of brain metastases (Kurzrock et al., 2012). This suggests intravenous delivery as a possible route for the delivery of P3 and QBP1 to the central nervous system (CNS) in polyQ patients. In addition, a short peptide sequence derived from the rabies virus glycoprotein was reported to be able to deliver proteins to the CNS (Fu et al., 2012). In our study, we showed that the attachment of the TAT cell-penetrating peptide (Frankel and Pabo, 1988; Green and Loewenstein, 1988) to both P3 and QBP1 did not alter their therapeutic properties (Figs. 3, 5). This opens up the possibility of further modifying the cell-penetrating peptide moiety of P3 and QBP1 for achieving CNS-targeting. However, the distinctive pathophysiology of the degenerating neurons in polyQ patients may make the outcome of the peptide delivery strategies less predictable. Nevertheless, our results indicate that an effective treatment strategy for polyQ disease may require simultaneous targeting of toxic RNA and protein species.
Materials and methods

Construction of plasmids

The \textit{pcDNA3.1-MJD\textsubscript{CAG27}}, \textit{pcDNA3.1-MJD\textsubscript{CAG78}}, \textit{pcDNA3.1-MJD\textsubscript{CAA/G78}}, \textit{pEGFP\textsubscript{CAG27}} and \textit{pEGFP\textsubscript{CAG78}} constructs were reported previously (Li et al., 2008; Tsoi et al., 2012). To generate the \textit{pcDNA3.1-\textit{myc-ATXN2}\textsubscript{CAG22/42/55/72}} constructs, \textit{ATXN2} DNA fragments containing 21 bp upstream and 105 bp downstream of the CAG repeats were PCR amplified from patient brain samples and cloned into \textit{pcDNA3.1(-)myc-His A} vector using \textit{EcoRV} enzyme. To generate peptide expression constructs, oligonucleotide His linkers were employed. All DNA oligos were ordered from Life Technologies Limited. The \textit{P3WT} linker was generated by annealing the following oligos: \textit{P3WTF} 5’-AAT TCA TGG ATG GTA AGT CAA AGG GTA TCG CTT ACA TCG AGT TCA AGT AAC-3’ and \textit{P3WTR} 5’-CG ATG TAC TTG AAC TCG ATG TAA GCG ATA CCC TTT GAC TTA CCA TCC ATG-3’. The \textit{P3MT1} linker was generated by annealing the following oligos: \textit{P3MT1F} 5’-AAT TCA TGG ATG GTG CTT CAA AGG GTA TCG CTT ACA TCG AGT TCA AGT AAC-3’ and \textit{P3MT1R} 5’-CG ATG TAC TTG AAC TCG ATG TAA GCG ATA CCC TTT GAC TTA CCA TCC ATG-3’. The \textit{P3MT2} linker was generated by annealing the following oligos: \textit{P3MT2F} 5’-AAT TCA TGG ATG GTA AGT CAA AGG GTA TCG CTT ACA TCG AGT TCA AGT AAC-3’ and \textit{P3MT2R} 5’-CG ATG TAC TTG AAC TCG ATG TAA GCG ATA CCC TTT GAC TTA CCA TCC ATG-3’. The \textit{P3MT3} linker was generated by annealing the following oligos: \textit{P3MT3F} 5’-AAT TCA TGG ATG GTA AGT CAA AGG GTA TCG CTT ACA TCG AGT TCA AGT AAC-3’ and \textit{P3MT3R} 5’-CG ATG TAC TTG AAC TCG ATG TAA GCG ATA CCC TTT GAC TTA CCA TCC ATG-3’. The \textit{P3MT4} linker was generated by annealing the following oligos: \textit{P3MT4F} 5’-AAT TCA TGG ATG GTA AGT CAA AGG GTA TCG CTT ACA TCG AGT TCA AGT AAC-3’
and P3MT4R 5’-TCG AGT TAC TTA GCC TCG ATG TAA GCG ATA CCC TTT GAC TTA CCA TCC ATG-3’. The P3MT5 linker was generated by annealing the following oligos: P3MT5F 5’-AAT TCA TGG ATG GTA AGT CAA AGG GTA TCG CTG CTA TCG AGG CTA AGT AAC-3’ and P3MT5R 5’-TCG AGT TAC TTA GCC TCG ATA GCA GCG ATA CCC TTT GAC TTA CCA TCC ATG-3’. The annealed linkers were ligated to pcDNA3.1 vector digested with EcoRI and XhoI.

**Synthesis of peptides and CAG RNAs**

All peptides were purchased from GenScript USA Inc. The P3 peptide sequences are shown in Fig. 1a, c and the QBP1 sequence is shown as follows: QBP1, SNWKWWPGIFD. Amino acid sequence of the TAT cell penetrating peptide used in our study was YGRKKRRQRRR (Popiel et al., 2007). Sequences of the TAT-fusion peptides used in this study are shown as follows: TAT-QBP1, SNWKWWPGIFD-YGRKKRRQRRR; TAT-QBP1 SCR, WPIWSKGNDF-WYGRKKRRQRRR; TAT-P3WT, YGRKKRRQRRR-DGKSKGIAYIEFK and TAT-P3MT5, YGRKKRRQRRR-DGKSKGIAAIEAK. The purity of peptides used in cell experiments and in vitro binding was over 95%. Desalted peptides were used in Drosophila feeding assays. All RNAs were synthesized using the MEGAscript® kit (Ambion) as previously described (Tsoi et al., 2012), and the MJDCAG27, MJDCAG78 and MJDCAA/G78 RNAs were transcribed from linearized pcDNA3.1-MJDCAG constructs (Tsoi et al., 2011).

**Cell culture, plasmid transfection and peptide transfection**

Normal human fibroblasts (AG04351) and MJD patient-derived fibroblasts (GM06153) were obtained from the Coriell Institute for Medical Research (Camden, NJ, USA). Both HEK293
cells and fibroblasts were cultured at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Primary rat cortical neurons were isolated and cultured as previously described (Lau et al., 2008). Transient transfection of HEK293 cells was performed using Lipofectamine 2000 (Life Technologies). Peptides were delivered to HEK293 cells using the DeliverX (DX) Peptide Transfection Kit (Affymetrix) 4 h after DNA transfection. Four micromolar of peptides were used to transfect cells, except for the LDH cytotoxicity assay in which 2 μM of peptides were used. For the TAT-fusion peptide treatment, 12 μM of TAT-P3WT, TAT-P3MT5, TAT-QBP1 and TAT-QBP1 SCR peptides were added directly to the culture medium at the time of DNA transfection unless otherwise stated. At least two batches of independently synthesized peptides were used in the experiments.

**In vitro binding assay**

Purified nucleolin protein (GST-NCL) was purchased from Abnova (Taiwan), and the control GST protein was expressed and purified as mentioned in 5. One hundred micromolar of corresponding peptides were added to the CAG₇₈ RNA/GST-NCL mixture. The reaction mixture was incubated at 4°C with end-to-end rotation for 2 h. The beads were then washed 3 times with 1 ml of binding buffer. Each wash was conducted for 10 min at 4°C. After the washing steps, 100 μl of GST elution buffer (20 mM Tris-Cl, pH7.4, 20 mM glutathione) was used to elute the protein-RNA complex. RNA extraction was performed. Reverse transcription PCR was performed to amplify CAG amplicon with primers CAGF 5’-AAA AAC AGC AGC AAA AGC-3’ & CAGR 5’-TCT GTC CTG ATA GGT CC-3’. Band intensity was measured using Image J (Schneider et al., 2012). Each experiment was repeated for at least 3 times with consistent results obtained.
RNA extraction, reverse transcription-PCR and real-time PCR

RNA was extracted from cells or ten 6 day-old adult fly heads by Trizol reagent (Life Technologies), and 1 µg of purified RNA was then used for reverse-transcription using the ImPromII™ Reverse Transcription System (Promega). Random hexamer (Roche) was used as primers in reverse transcription. The amplicon of actin was amplified by primers actinF 5’-TGT GCA AGG CCG GTT TCG C-3’ & actinR 5’-CGA CAC GCA GCT CAT TGT AG-3’; the amplicons of P3WT and P3MT1-5 were amplified by primers P3F 5’-TAA TAC GAC TCA GTC CTG ATA GGT CC-3’ & P3R 5’-TAG AAG GCA CAG TCG AGG-3’; the amplicon of CAG78 RNA was amplified by primers CAGF 5’-AAA AAC AGC AGC AAA AGC-3’ & CAGR 5’-GTC CTG ATA GGT CC-3’; the amplicon of XBP1S for human was amplified by primers XBP1SF 5’-GGA GTT AAG ACA GCG CTT GG-3’ & XBP1SR 5’-ACT GGG TCC AAG TTG TCC AG-3’; and the amplicon of Xbp1S for Drosophila was amplified by primers Xbp1SF 5’-CAA CAG CAG CAC AAC ACC AG-3’ & Xbp1SR 5’-AGA CTT TCG GCC AGC TCT TC-3’.

Taqman gene expression assays were performed on an ABI 7500 Real-time PCR system and data were analyzed as previously described (Tsoi et al., 2012). The following probes were used: pre-45s rRNA (Assay ID: AILJIZM), pre-rRNA (Assay ID: AIMSG5U), 18S rRNA (Assay ID: Hs03928985_g1), human GAPDH (Assay ID: Hs99999905_m1), Drosophila GAPDH (Assay ID: Dm01841186), U6 (Assay ID: AI11MM6), tRNA^met (Assay ID: AIN1FB2), UCE (Assay ID: AIHSOGY) and actin (Assay ID: Hs99999903_m1), human BiP/GRP78 (Assay ID: Hs999999174_m1), Drosophila BiP/GRP78 (Assay ID: Dm01813415-g1), EGFP (Assay ID: Mr04097229_mr), and ATXN3 (Assay ID: Hs01026440_g1). Each experiment was repeated for at least 3 times.
**Western Blotting**

All protein samples were resolved on 12% SDS-PAGE, and detected using the following antibodies: 7F5 (Cell Signaling Technology; 1:1,000) for p53, 9B11 (Cell Signaling Technology; 1:2,000) for myc-tagged proteins. Tubulin was detected by E7 (Developmental Studies Hybridoma Bank; 1:5,000). Each experiment was repeated for at least three times, and comparable results were obtained.

**Chromatin immunoprecipitation and HpaII methylation assays**

Chromatin immunoprecipitation was performed according to (Tsoi et al., 2011; Tsoi et al., 2012). Antibody used was anti-nucleolin 3G4B2. To perform the HpaII methylation assay, genomic DNA was extracted from cells, followed by digestion with 2 units of HpaII or MspI (New England Biolabs) for 4 h at 37°C. The DNA products were incubated at 85°C for 15 min to heat-inactivate the restriction enzymes. The resulting DNA products were amplified by PCR. Amplicon of the human UCE was amplified by UCEF, 5’- CGT GTG TCC TTG GGT TGA CC-3’ and UCER, 5’- CGC GTC ACC GAC CAC GCC-3’. Each experiment was repeated for at least 3 times with consistent results obtained.

**Caspase activity assays**

Caspase activity was measured using the Caspase-Glo®8 and Caspase-Glo®9 assay systems (Promega) following manufacturer's instructions. TNF-related apoptosis-inducing ligand (TRAIL) served as positive control for caspase 8 activity assay, while staurosporine (STS) served as positive control for caspase activity 9 assay. EnVision® Multilabel Reader
(PerkinElmer) was used to measure the luminescence. Each sample was measured in duplicates, and each experiment was repeated for at least 3 times.

**Isothermal titration calorimetry binding assay**

Experiments were carried out using a MicroCal iTC200 isothermal titration calorimeter (GE Healthcare) at 25°C. Data were analyzed using the Origin® scientific plotting software version 7 (Microcal Software Inc.). All RNAs and peptides were dissolved in binding buffer (20 mM MOPS, pH 7.0; 300 mM NaCl). The concentration of RNA was estimated with appropriate extinction coefficients at 260 nm on a Nanodrop 2000 (Thermo Scientific). A reference power of 8 μcal/s was used with an initial 0.5 μl of injection of peptide followed by 2.5 μl for all subsequent titrations points with a 60 s initial equilibrium delay and 150 s pause between injections. The samples were stirred at a speed of 1,000 r.p.m. throughout the experiment. The thermal titration data were fitted to the 'one binding site model' to determine the dissociation constant (K_D). At least two batches of independently synthesized peptides were used in the experiments. Each experiment was repeated for at least 3 times with consistent results obtained.

**Lactate dehydrogenase (LDH) cytotoxicity assay and IC_{50} determination**

Human embryonic kidney 293 cells were seeded on a 24-well plate at a density of 0.5×10^5, and pcDNA3.1-MJD_{CAG27/78} or pEGFP_{CAG78} DNA construct was used to transfect the cells. Four hours after DNA transfection, peptide transfection was performed as follows: P3WT-QBP1, P3WT-QBP1 SCR, P3MT5-QBP1 and P3MT5-QBP1 SCR (2 μM each). For staurosporine (STS; (Feng and Kaplowitz, 2002)) treatment, cells were treated with 1 μM of STS in conjunction with 12 μM TAT-P3WT. Lactate dehydrogenase enzyme activity in the cell culture
medium was measured 24 h (for STS experiment) or 72 h (for \( pEGFP_{CAG78} \) transfection experiments) post-treatments using the Cytotox 96 non-radioactive cytotoxicity assay (Promega). Each experiment was repeated for at least 3 times with consistent results obtained.

To detect the effect of P3WT on inhibiting cell death in \( EGFP_{CAG78} \) RNA-expressing HEK293 cells, LDH assay was employed. A density of 0.5×10^5 HEK293 cells were transfected with \( pEGFP_{CAG78} \) and various amounts of the TAT-P3WT peptide, 0.1, 0.5, 1, 2, 4, 5, 10 and 25 μM, were then added to individual culture wells. Seventy two hours after treatment, LDH enzyme activity in the cell culture medium was measured as described before. Experimental groups were normalized to the untransfected control. After normalization, data were analyzed using the dose response-inhibition curve (nonlinear regression-variable slope) to determine the IC\(_{50}\) value (Prism6 software, GraphPad Software, Inc.).

**Drosophila genetics, peptide feeding and assays**

Flies were raised at 21.5°C or 25°C on cornmeal medium supplemented with dry yeast. Fly lines bearing \( UAS-flMJD_{CAG27} \) and \( UAS-flMJD_{CAG84} \) (Warrick et al., 2005) were gifts from Professor Nancy Bonini (University of Pennsylvania, USA). The \( UAS-EGFP-CGG_{90} \) (Jin et al., 2003) and \( UAS-(CTG)_{480} \) (Garcia-Lopez et al., 2008) fly lines were obtained from Professors Stephen Warren (Emory University, USA) and Rubén Artero Allepuz (Universitat de València, Estudi General, Spain) respectively. The \( gmr-GAL4 \), \( elav-GAL4 \) and \( Oregon \ R \) fly lines were obtained from Bloomington Drosophila Stock Center. For pseudopupil assay, third instar larvae were fed with 200 μM of respective peptides dissolved in 2% sucrose solution for 2 h and then continued to culture in standard fly food at 21.5°C (Chau et al., 2006). Pseudopupil assay was performed
on 6 day-old adult flies as mentioned previously (Wong et al., 2008). Images were captured by
SPOT Insight CCD camera controlled by the SPOT Advanced software (Diagnostic instruments
Inc.). Image processing was performed using the Adobe Photoshop CS software (Adobe). Each
experiment was repeated for at least 3 times (n=10 fly heads) and consistent results were
obtained. For lifespan analysis, third instar larvae were fed with 200 µM of TAT-P3WT or TAT-
P3MT5 (dissolved in 2% sucrose solution) for 2 h and then continued to culture in standard fly
food at 25°C. Two days after eclosion, 10-15 adult flies were allocated to individual fresh non-
drug-containing food vials. At least 120 flies were analyzed per treatment group. The flies were
transferred to fresh vials every 3 days during the whole course of the experiment, and the number
of survived flies was counted every 3 days. Survival rate was calculated as area under survival
curve followed by one-way ANOVA analysis. For wild-type adult eclosion test, Oregon R third
instar larvae were fed with 500 µM or 1 mM of TAT-P3WT peptide dissolved in 2% sucrose
solution for 2 h, and then continued to culture in standard fly food at 25°C. Adult eclosion rate
was calculated as the number of adult flies divided by the number of larvae examined. Each
experiment was repeated for three times (n=60 larvae). Two batches of independently
synthesized peptides were used in the experiments.

**Statistical analyses**

Data were analyzed by one-way ANOVA followed by post hoc Tukey test. “*”, “**” and “***”
represent P<0.05, P<0.01 and P< 0.001 respectively, which are considered statistically
significant.
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Conflict of interest: The authors declare no conflict of interest.
References


Fig. 1 Expression of P3 suppressed nucleolar stress in cells expressed with expanded CAG RNA. (A) Amino acid sequence of NCL peptides used in this study. (B) The P3 and P5 peptides...
disrupted the interaction between expanded CAG RNA and NCL. After in vitro binding of CAG\textsubscript{78} RNA and GST-NCL protein in the presence of NCL peptides, reverse transcription PCR was performed to detect the binding of CAG\textsubscript{78} RNA to GST-NCL. (C) Amino acid sequences of wild type (WT) and mutant (MT) P3 peptides. The mutated residues are underlined. (D) Expression of P3\textsubscript{WT} resumed the expression level of pre-45s rRNA in EGFP\textsubscript{CAG78} RNA-expressing HEK293 cells. Real-time PCR was performed to determine the expression level of pre-45s rRNA in cells co-transfected with EGFP\textsubscript{CAG} and P3 constructs. (E) Expression of P3\textsubscript{WT} resumed the physical interaction between nucleolin (NCL) and upstream control element (UCE) in EGFP\textsubscript{CAG78} RNA-expressing HEK293 cells. Chromatin immunoprecipitation was performed. Real-time PCR was performed to determine the amount of UCE in the immunoprecipitant. (F) Expression of P3\textsubscript{WT} resumed the DNA methylation status of UCE. “-” represents cells that were transfected with pcDNA3.1 empty vector. Genomic DNA was treated with either HpaII or MspI. HpaII is a methylation sensitive restriction enzyme while MspI is a methylation insensitive restriction enzyme. The enzyme-treated DNA was used in PCR. Amplicon UCE was amplified. MspI-treated samples were used as loading control. (G) Expression of P3\textsubscript{WT} suppressed caspase 9 activity in HEK293 cells expressing EGFP\textsubscript{CAG78} RNA. Experiments were repeated for at least 3 times and data are expressed as mean ± S.D.. *** indicates \( P<0.001 \).
Fig. 2 P3 directly interacted with CAG repeat-containing RNA. Isothermal titration calorimetry study of the binding of synthetic P3WT peptide (13 mM) to CAG RNA (10 μM) in vitro transcribed from (A) pcDNA3.1-MJD_{CAG27}, (B) pcDNA3.1-MJD_{CAG78} and (C) pcDNA3.1-MJD_{CAA/G78}. The top panel shows the raw thermogram and the bottom panel shows the binding isotherm fitted to a single-site model. The reported errors correspond to the S.D. of the fit. P3WT represents Peptide 3 wild type. Each experiment was repeated for at least 3 times with consistent results obtained, and only representative graphs are shown.
Fig. 3 P3 peptide treatment suppressed nucleolar stress in cell expressing expanded CAG RNA. (A) Dose-dependent effect of synthetic TAT-P3WT on the inhibition of cell death in
EGFP<sub>CAG78</sub> RNA-expressing HEK293 cells. A lactate dehydrogenase cytotoxicity assay was performed. The IC<sub>50</sub> value represents the concentration of TAT-P3WT that reduced LDH enzyme activity by 50% when compared with the no peptide treatment control group. Data are expressed as mean ± S.E.M. for at least 3 independent experiments. (B, C) Synthetic TAT-P3WT peptide (12 μM) treatment restored pre-45s rRNA (B) and 18S rRNA (C) levels in EGFP<sub>CAG78</sub> RNA-expressing HEK293 cells. Cells were treated with 12 μM of corresponding P3 peptides. Real-time PCR was performed to determine the level of pre-45s rRNA. “P3WT” represents synthetic P3 peptide without the TAT fusion. This serves as a control to demonstrate TAT-mediated intracellular delivery of P3 is crucial for its action. Experiments were repeated for at least 3 times and data are expressed as mean ± S.D.. (D) Synthetic TAT-P3WT treatment resumed the interaction between NCL and UCE in EGFP<sub>CAG78</sub> RNA-expressing HEK293 cells. Following chromatin immunoprecipitation, real-time PCR was performed to determine the amount of UCE in the immunoprecipitant. Experiments were repeated for at least 3 times and data are expressed as mean ± S.D.. (E) TAT-P3WT peptide treatment resumed the DNA methylation status of UCE in EGFP<sub>CAG78</sub> RNA-expressing HEK293 cells. “-” indicates cells were not treated with peptides. Genomic DNA was treated with either HpaII or MspI. HpaII is a methylation sensitive restriction enzyme while MspI is a methylation insensitive restriction enzyme. Digested DNA was used in PCR. Amplicon UCE was amplified. MspI-treated samples were used as loading control. Only representative gel photos are shown. (F) Synthetic TAT-P3WT peptide treatment inhibited p53 protein expression in EGFP<sub>CAG78</sub> RNA-expressing HEK293 cells. Western blotting was performed to determine the p53 expression level. Tubulin was used as loading control. The experiment was repeated for 3 times with consistent results obtained. Only representative blots are shown. (G) Synthetic TAT-P3WT peptide treatment
suppressed cell death in HEK293 cells expressing EGFP_{CAG78} RNA. Caspase 9 activity was determined. P3WT represents Peptide 3 wild type and P3MT5 represents P3 mutant 5. Experiments were repeated for at least 3 times and data are expressed as mean ± S.D.. * indicates \( P<0.05 \), ** indicates \( P<0.01 \) and *** indicates \( P<0.001 \).
Fig. 4 Cellular transfection of synthetic P3 and QBP1 peptides suppressed expanded CAG RNA-induced RNA toxicity and expanded polyQ protein-induced protein toxicity *in vitro.*
Expression analyses of RNA and protein toxicity biomarkers in HEK293 cells (A, B, D and E), and MJD patient-derived fibroblasts (GM06153) (C, F). Intracellular delivery P3WT peptide (4 μM) through peptide transfection restored expression level of rRNAs in MJD\textsubscript{CAG78}-transfected HEK293 cells (A, B) and MJD patient-derived fibroblasts (C). Delivery of QBP1 peptide (4 μM) reduced the induction level of BiP (D) and XBP1\textsubscript{S} (E) mRNAs in MJD\textsubscript{CAG78}-transfected HEK293 cells, and reduced BiP level MJD patient-derived fibroblasts (F). (G) Co-delivery of P3WT and QBP1 peptides (2 μM each) effectively inhibited cell death in MJD\textsubscript{CAG78}-transfected HEK293 cells. LDH activity was performed to measure cell death induced by expanded MJD\textsubscript{CAG78} RNA and MJDQ78 protein. A lactate dehydrogenase assay was performed to measure the cytotoxicity. P3WT represents wild type Peptide 3, P3MT5 represents P3 mutant 5, and QBP1 SCR represents scrambled control for QBP1. DX denotes DeliverX peptide transfection reagent while AG04351 denotes control human fibroblasts. For reverse transcription-PCR, only representative gels are shown and actin was used as loading control. Experiments were repeated for at least 3 times and data are expressed as mean ± S.D.. * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$. 
Fig. 5 P3/QBP1 co-treatment suppressed expanded CAG RNA-induced RNA toxicity and expanded polyQ protein-induced protein toxicity in vivo. (A) Co-delivery of P3/QBP1 effectively suppressed flMJDQ84 neurodegeneration in Drosophila. When compared with the control groups including blank, TAT-P3MT5/TAT-QBP1 SCR, TAT-P3WT/TAT-QBP1 SCR
and TAT-P3MT5/TAT-QBP1, transgenic *Drosophila flMJD<sub>CAG84</sub> disease model co-treated with TAT-P3WT and TAT-QBP1 peptides (200 μM each) more significantly suppressed neurodegeneration *in vivo*. Pseudopupil assay was performed on 6 day-old adult flies. (B) Statistical analysis of (A). Experiments were repeated for at least 3 times and data are expressed as mean ± S.D.. (C-E) Real-time PCR analyses of pre-rRNA, 18S rRNA and BiP mRNA levels *in vivo*. Treatment of *flMJDQ84* flies with TAT-P3WT in combination with other peptides (200 μM each) resumed pre-rRNA (C) and 18S rRNA (D) levels. Similarly, the TAT-QBP1 treatment in combination with other peptides (200 μM each) reduced BiP mRNA expression level (E). Data are presented as fold change of the relative pre-rRNA or BiP expression levels compared with the untreated samples. Experiments were repeated for at least 3 times and data are expressed as mean ± S.D.. * indicates *P*<0.05, ** indicates *P*<0.01, *** indicates *P*<0.001. (F) Reverse transcription-PCR analysis of Xbp1 expression *in vivo*. Treatment of *flMJDQ84* flies with TAT-QBP1 in combination with other peptides (200 μM each) reduced Xbp1S level. Experiments were repeated for at least 3 times, and only representative gels are shown. *actin* was used as loading control. The flies were of genotypes *w; gmr-GAL4 UAS-myc-flMJD<sub>CAG27</sub>/+; +/+* and *w; gmr-GAL4/+; UAS-myc-flMJD<sub>CAG84</sub>/+. 
Fig. 6 Schematic diagram illustrating mechanism of actions of P3 and QBP1 in suppressing RNA and protein toxicities of polyQ degeneration. **(A)** P3 suppressed expanded CAG RNA-induced nucleolar stress. **(B)** Suppression of RNA toxicity and protein toxicity utilizing the P3-QBP1 combination treatment strategy.
**Translational Impact**

**Clinical issue:**
Polyglutamine (polyQ) diseases, including Huntington’s disease, spinobulbar muscular atrophy, dentatorubral-pallidoluysian atrophy and several types of spinocerebellar ataxias, are caused by an abnormal expansion of CAG repeats in the respective disease genes. Increasing evidence indicates that both expanded CAG RNA and polyQ protein are the primary toxic species that trigger neuronal degeneration. Although inhibitors that target either RNA or protein toxicity have independently been discovered, it is not certain whether the simultaneous targeting of expanded CAG RNA and polyQ protein would cause a more effective suppression of degeneration.

**Results:**
Based on the Nucleolin protein sequence, we identified a 13-amino acid peptide, P3, that can suppress expanded CAG RNA toxicity. We co-treated animals with both P3 and a well-characterized protein toxicity peptide inhibitor QBP1. Our data clearly show that simultaneous P3/QBP1 co-treatment yielded a more significant suppression of neurodegeneration both *in vitro* and *in vivo* when compared with the single peptide treatment alone (P3 or QBP1).

**Implications and future directions:**
Expanded CAG RNA and polyQ protein represent two distinct toxic species which concomitantly contribute to polyQ degeneration. Our findings demonstrate that the simultaneous intervention of both RNA and protein toxicities could lead to a more complete suppression of polyQ degeneration. This study highlights the importance of targeting both toxic RNA and protein species in treating polyQ degeneration, and opens up a new direction of therapeutic treatment.