Reactivation of nonsense-mediated mRNA decay protects against C9orf72 dipeptide-repeat neurotoxicity

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Amyotrophic lateral sclerosis is a deleterious neurodegenerative disease without effective treatment options. Recent studies have indicated the involvement of the dysregulation of RNA metabolism in the pathogenesis of amyotrophic lateral sclerosis. Among the various RNA regulatory machineries, nonsense-mediated mRNA decay (NMD) is a stress responsive cellular surveillance system that degrades selected mRNA substrates to prevent the translation of defective or harmful proteins. Whether this pathway is affected in neurodegenerative diseases is unclear. Here we report the inhibition of NMD by arginine-rich dipeptide repeats derived from C9orf72 hexanucleotide repeat expansion, the most common cause of familial amyotrophic lateral sclerosis. Bioinformatic analysis of multiple transcriptome profiles revealed significant overlap of upregulated genes in NMD-defective cells with those in the brain tissues, micro-dissected motor neurons, or induced pluripotent stem cell-derived motor neurons specifically from amyotrophic lateral sclerosis patients carrying C9orf72 hexanucleotide repeat expansion, suggesting the suppression of NMD pathway in these patients. Using Drosophila as a model, we have validated that the C9orf72 hexanucleotide repeat expansion products could lead to the accumulation of the NMD substrates and identified arginine-rich dipeptide repeats, including poly glycine-arginine and poly proline-arginine, as the main culprits of NMD inhibition. Furthermore, in human SH-SY5Y neuroblastoma cells and in mouse brains, expression of glycine-arginine with 36 repeats (GR36) was sufficient to cause NMD inhibition. In cells expressing GR36, stress granule accumulation was accompanied by decreased processing body formation, which contributed to the inhibition of NMD. Remarkably, expression of UPF1, a core gene in the NMD pathway, efficiently blocked neurotoxicity caused by arginine-rich dipeptide repeats in both cellular and Drosophila models. Although not as effective as UPF1, expression of another NMD gene UPF2 also ameliorated the degenerative phenotypes in dipeptide repeat-expressing flies, indicating that genetically reactivating the NMD pathway could suppress dipeptide repeat toxicity. Finally, after validating tranilast as an NMD-activating drug, we demonstrated the therapeutic potential of this asthma drug in cellular and Drosophila models of C9orf72 dipeptide repeat neurotoxicity. Therefore, our study has revealed a cellular mechanism whereby arginine-rich C9orf72 dipeptide repeats could inhibit NMD activities by reducing the abundance of processing bodies. Furthermore, our results suggested that activation of the NMD pathway could be a potential therapeutic strategy for amyotrophic lateral sclerosis with defective RNA metabolism.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fast progressive, debilitating motor neuron disease with limited treatment options. There is no cure for the disease at present, and most commonly prescribed ALS drug—riluzole—can only prolong survival for a few months (Bensimon et al., 1994). In 2017, a free-radical scavenger, edaravone, became the second FDA-approved ALS drug in more than 20 years after showing its benefits in slowing down disease progression (Abe et al., 2017), even though the mode of action was not clear. Thus, there is a pressing need for disease-modifying therapeutic agents for ALS.

Although 90% of ALS cases are sporadic, advances in genetics have provided more clues for understanding the pathogenesis and offered new hope for treatments. The C9orf72 hexanucleotide repeat expansion (HRE) is the most common cause of familial ALS and frontotemporal dementia (FTD), and accounts for >10% of sporadic ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Taylor et al., 2016). Once the repeats reach more than 30–60, HRE becomes pathogenic (Gijselink et al., 2018). Current evidence suggests that three mechanisms may contribute to the development of C9orf72 HRE-related ALS and FTD: dipeptide repeats (DPRs), abnormal RNA species, or reduced C9orf72 gene expression (Gendron and Petrucelli, 2018). While the exact roles of HRE-derived RNA foci in disease progression remain to be clarified, accumulating evidence has suggested that DPRs are the more toxic products (Mizielska et al., 2014; Tao et al., 2015; Tran et al., 2015). The HRE produces five forms of DPRs via repeat-associated non-ATG translation (Ash et al., 2013; Mori et al., 2013; Schludi et al., 2015), and protein inclusions containing the DPRs are observed in the cerebellum, cortex, and hippocampus regions in C9orf72 ALS patients (Mori et al., 2013; Schludi et al., 2015). Among these DPRs, the arginine-rich DPRs [poly proline-arginine (PR) and glycine-arginine (GR)] are important contributing factors of neurotoxicity (Kwon et al., 2014; Mizielska et al., 2014; Tran et al., 2015; Kanekura et al., 2016; Zhang et al., 2016). Besides arginine-rich DPRs, poly glycine-alanine (GA) may cause toxicity by modulating protein aggregation (May et al., 2014; Zhang et al., 2014, 2016; Lee et al., 2017; Schludi et al., 2017).

Recent evidence has shown that arginine-rich DPRs could also affect the formation of stress granules rich in RNA and RNA-binding proteins (RBPs) (Lee et al., 2016; Boeynaems et al., 2017; Green et al., 2017; Li et al., 2017; Cheng et al., 2018; Zhang et al., 2018a, b).Remarkably, several RBPs, including TDP-43, FUS, TAF15, EWSR1 and TIA1, have been genetically linked to ALS/FTD (Ling et al., 2013; Mackenzie et al., 2017). Furthermore, these proteins are also found in stress granules (Taylor et al., 2016). Mutations in genes encoding these RBPs likely affect their functions and could cause dysregulation of RNA production, processing, transport, degradation and translation (Ling et al., 2013; Taylor et al., 2016; Mackenzie et al., 2017). Therefore, dysfunction in RNA metabolism could be a common disease-causing mechanism in ALS/FTD (Ling et al., 2013; Renton et al., 2014; Taylor et al., 2016). One of the pathways regulating mRNA abundance is nonsense-mediated mRNA decay (NMD), which was originally described as a quality control mechanism in eukaryotes to degrade defective mRNA with premature stop codon (He and Jacobson, 2015). Subsequently, NMD was found to regulate gene expression, especially of those genes with long 3’ untranslated regions (Lykke-Andersen and Jensen, 2015). The activation of the NMD pathway requires three conserved essential regulatory up-frame shift proteins (UPF1, UPF2, and UPF3) and the formation of a large protein complex to facilitate the cleavage of mRNA by endonucleases and exonucleases (Kervestin and Jacobson, 2012; He and Jacobson, 2015). Those key components of NMD machinery are found in processing bodies (P-bodies), which are membranless ribonucleoprotein granules, rich in proteins, involved in RNA degradation (Sheth and Parker, 2006; Durand et al., 2007; Parker and Sheth, 2007; Shukla and Parker, 2016; Hubstenberger et al., 2017). Thus, P-bodies were considered as reservoirs for untranslated RNA and proteins regulating RNA decay, and could also be the site for NMD (Sheth and Parker, 2006; Durand et al., 2007; Protter and Parker, 2016). Whether the NMD pathway is disturbed in ALS has never been carefully evaluated.

In this study, we investigated whether C9orf72 HRE products could affect the NMD pathway. By coupling bioinformatic analysis of various transcriptome studies with validation experiments in multiple models of C9orf72 HRE and DPRs, we found that C9orf72 arginine-rich DPRs caused accumulation of transcripts normally degraded by the NMD pathway in patients and various models, suggesting the inhibition of NMD. Our cell biology studies revealed that the NMD inhibition is likely caused by decreased formation of P-bodies in cells expressing arginine-rich DPRs. More importantly, we have demonstrated in cellular and Drosophila models that reactivation of the NMD pathway by genetic and pharmacological approaches could rescue C9orpha models that reactivation of the NMD pathway by genetic and pharmacological approaches could rescue C9orf72 arginine-rich DPR-induced neurotoxicity. Therefore, our results suggested that NMD pathway is a promising therapeutic target and an NMD-activating asthma drug—tranilast—could be a potential drug for ALS with dysfunctional RNA metabolism.

Keywords: ALS; nonsense-mediated mRNA decay; C9orf72; dipeptide repeats; tranilast

Abbreviations: ALS = amyotrophic lateral sclerosis; DPR = dipeptide repeat; FTD = frontotemporal dementia; HRE = hexanucleotide repeat expansion; NMD = nonsense-mediated mRNA decay; P-body = processing body
Materials and methods

Fly stocks

The UAS-DPRs transgenic flies were generous gifts of Dr Adrian Isaacs (Mizielska et al., 2014) (University College London). UAS-GFP-DPRs transgenic flies were described previously (Xu and Xu, 2018). \( W^{1118} \) and Elav\(^{C155} \), Gal4 were kind gifts from Dr Fude Huang (Shanghai Advanced Research Institute). \( w^* \); \( P\{\text{tubP-GAL80ts}\}20; \text{TM2/TM6B, Tb1, w*}\); \( P\{\text{UASp-Hsap\SNCA.A30P}\}40.1, \text{w1118}; P\{\text{UAS-HTT.128Q.FL}\}f27b, \) and \( \text{w} [^*\text{mW hs}]=\text{GawB} \) \( 10D42, \) \( \text{w*}\); \( P\{\text{UAS-GFP-DPRs}\}\) were obtained from the Department of Neurogenetics, Institute (w[^*]). \( P\{\text{w[ + mW hs]=GawB}\}10D42, \) \( \text{w*}\); \( P\{\text{UAS-GFP-DPRs}\}\) were kind gifts from Dr Fude Huang (Shanghai Advanced Research Institute). \( w^*; P\{\text{TRip.JF01974}\}
attP2 were obtained from the Department of Neurogenetics, Institute (w[^*]). \( P\{\text{w[ + mW hs]=GawB}\}10D42, \) \( \text{w*}\); \( P\{\text{UAS-GFP-DPRs}\}\) were kind gifts from Dr Fude Huang (Shanghai Advanced Research Institute). \( w^*; P\{\text{tubP-GAL80ts}\}20; \text{TM2/TM6B, Tb1, w*}; P\{\text{UASp-Hsap\SNCA.A30P}\}40.1, \text{w1118}; P\{\text{UAS-HTT.128Q.FL}\}f27b, \) \( \text{w*}; P\{\text{ubp-GAL80ts}\}20; TM2/TM6B, Tb1, w*; P\{\text{UaspgFp.Upf1}\}2, w*; P\{\text{UAS-Upf2.A}\}2, w*; P\{\text{UAS-Arc1.WT}\}3 \) and \( \text{y1 v1}; P\{\text{TRip.JF01974}\}
attP2 were obtained from Bloomington Drosophila Stock Center (BDSC). \( \text{y1 v1}; P\{\text{TRip.GL01485}\}
attP2 was the UAS-RNAi line used for knockdown of UPF1 under the control of Gal4. All flies were maintained at 24°C on a 12:12 h light:dark cycle at 60% humidity on a standard medium.

Plasmids

The G4C2 and DPR constructs were generous gifts of Dr Adrian Isaacs (Mizielska et al., 2014) (University College London). To generate GFP-tagged DPR constructs, untagged DPR constructs were subcloned into pEFGP-C1 vector. The flag-hUPF1 plasmid was generated by RT-PCR from the cDNA of SH-SY5Y cells and cloned into Flag-pcDNA3.1 vector. The coding sequence of DCP1 luciferase NMD reporter was constructed as described (Boelz et al., 2006; Keeling et al., 2013). Briefly, the in-frame \( \text{Renilla luciferase/}\)β-globin fusion gene containing a premature stop codon in exon 2 (N39X) was generated by PCR mutagenesis and cloned into the psiCHECK-2 vector (Promega).

Climbing assay

Negative geotaxis behaviour was used to examine the fly mobility. Briefly, 10 male flies (aged 10 days) were transferred to the testing vials. Flies were tapped down to the bottom of the vials, and their climbing behaviour was video recorded. The height of each fly after 5 s was determined (Liu et al., 2015). A total of three trials were performed for each vial, and the mean height for flies in each vial was calculated. At least three vials were used for each genotype.

Lifespan assay

For each group, 20 flies were collected 5 days after eclosion and transferred to a fresh vial. Every 5 days, the flies were moved to new vials with fresh food, and the number of dead flies was recorded. The survival rate for each group was calculated until the death of all flies.

Cell culture and transfection

Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Life Technologies) with 10% foetal bovine serum (FBS; Life Technologies) and antibiotics, at 37°C with 5% CO2. For quantitative PCR or NMD reporter analysis, cells were transfected with 3 μg GFP-GA36 or GFP-GR36 plasmids using Lipofectamine\(^\text{TM}\) 2000 reagent (Life Technologies) in 12-well plates. For immunostaining, cells were plated in 24-well plates and co-transfected with 0.5 μg GFP-GA36 or GFP-GR36 and 0.5 μg pcDNA3.1 or Flag-hUPF1 plasmids. The siRNA construct targeting UPF1 was transfected using Lipofectamine RNAiMAX (13778, Life Technologies). Cells were harvested 72 h after transfection for the subsequent studies. The sequence of siRNA was obtained from Mendell et al. (2004).

Stereotaxic injection of AAV-DPRs in mouse brain

Seven-week-old male wild-type C57BL/6 mice were purchased from SLAC Laboratory Animal Company. All animal experiments were approved and performed in accordance with the regulations of the Ethics and Animal Care and Use Committee of the Institute of Neuroscience, Shanghai Institutes for Biological Sciences. GFP-GA36 and GFP-GR36 were cloned into the adeno-associated virus (AAV) vector AOV-021 pAAV-CMV-MCS-3FLAG, and packaged by ObiO Inc with a titre of \( 6 \times 10^{12} \). Seven-week-old C57 mice were anaesthetized with pentobarbital sodium (80 mg/kg) and fixed on stereotaxic apparatus. AAV-GFP-GA36 and AAV-GFP-GR36 (1.5 μl) were injected into the hippocampus dentate gyrus (Bregma AP, –2.0 mm; ML, ±1.5 mm; DV, –1.9 mm) in the contralateral hemisphere. Mice were then maintained with proper food and water supply in a controlled environment. Five weeks post-injection, mice were either perfused intracardially with 4% paraformaldehyde for immune-fluorescent labelling or sacrificed for tissue collection and the subsequent quantitative PCR analysis.

Immunoprecipitation and immunoblotting

The procedures for immunoprecipitation and immunoblotting were described previously (Jiang et al., 2018). Briefly, transfected SH-SY5Y cells (15 cm dish, 48 h after transfection) were lysed in NP-40 buffer with protease inhibitors. After centrifugation at 12,000 rpm for 15 min, 1 mg of protein lysates were precleared with IgG for 1 h, followed by incubation with the control IgG or UPF1 antibody (1:50, ab109363, Abcam) overnight. After incubation with protein A/G sepharose beads (sc2003, Santa Cruz) for 1 h, the beads were washed and immunoprecipitated proteins were eluted by boiling in loading buffer, followed by western blot. Antibodies used in western blot included UPF1 antibody (1:3000, ab109363, Abcam), GFP antibody (1:3000, P30010, Abmart), DCPI\(^{\text{z}}\) antibody (1:1000, H00055808-M06, Abnova), β-tubulin antibody (1:3000, M20005S, Abmart), GAPDH antibody (1:10000, 60004–1, Proteintech) and HRP-conjugated secondary antibodies (Jackson lab).

Immunofluorescence

The mouse brains were dehydrated with 30% sucrose and embedded in optimum cutting temperature compound.
Frozen brains were then cryosectioned at a thickness of 40 μm. For SH-SY5Y cells, 48 h after transfection, the cells were fixed with 4% paraformaldehyde for 30 min. For flies, adult brains were dissected in phosphate-buffered saline (PBS) followed by 4% paraformaldehyde fixation for 30 min. The subsequent procedures were identical for all samples, with washes performed in PBST (0.3% Triton X-100 in PBS) followed by blocking and antibody incubation using PBST with 5% BSA as diluent. Primary antibodies used include: chicken anti-Flag (1:500, A10262, Thermo Fisher Scientific), mouse anti-NeuN, clone A60 (1:50, MAB377, Millipore), rabbit anti-Cleaved Caspase-3 (Asp175) (1:400, 9661 S, Cell Signaling Technology), mouse anti-LSM1 (1:50, TA503121, ORIGENE), mouse anti-DCP1α (1:50, H00055808-M06, Abnova), rabbit anti-PABP (1:200, ab21060, Abcam) and mouse anti-Flag (1:500, M20008L, AbMart). After primary antibody incubation at 4°C overnight, fluorescent labelled secondary antibodies (1:500, Thermo Fisher Scientific) were used for 2 h at room temperature. Finally, the slides were mounted in 70% glycerol. Images were acquired with Nikon A1 or Olympus FV10i confocal microscope and processed with Fiji software. Images were shown as maximum projection processing of multiple confocal scanning planes.

Quantitative PCR analysis

Fly heads, SH-SY5Y cells and mouse dentate gyrus were collected for various experiments and processed in TRIzol reagent (Thermo Fisher Scientific) for RNA extraction. For quantitative PCR, 1 μg RNA was reverse transcribed (Takara), followed by PCR amplification with SYBR® green (CFX-connector, Bio-Rad). Gene expression data were calculated by the delta-delta Ct method. Gadph was used as a reference control. The sequences of all the primers are available upon request.

Dual luciferase NMD reporter assays

GFP or GFP-tagged DPRs were co-transfected with the NMD reporter into SH-SY5Y cells for 48 h. Cells were collected and analysed following the instructions of dual-luciferase reporter assay system kit (E1910, Promega).

Drug treatment

Tranilast, niflumic acid, febuxostat and nitazoxanide were purchased from Sigma-Aldrich and dissolved in DMSO at a concentration of 10 mM as stock solution. For SH-SY5Y cells, 24 h after transfection, chemicals were added to cell medium to the working concentration and incubated for 24 h, followed by washing with PBS and subsequent experiments. For flies, tranilast was added to the Drosophila food at the final concentration of 10 μM. The parent flies were removed after laying eggs on food. Embryo development was then analysed until death.

RNA sequencing

Four groups of flies [control PA36, (G4C2)_36, PR36, UPF1 RNAi] were used for RNA-Seq. Each genotype contains two replicates with a total of 40 female fly heads. RNA was extracted by TRIzol® reagent (Thermo Fisher Scientific). Whole transcriptome libraries preparation and sequencing were performed by Annoroad Gene Technology Corporation. Whole transcriptome libraries were constructed by using NEB Next Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs) followed the manufacturer’s instructions. The quality of the libraries was examined and quantified using the BioAnalyzer 2100 system (Agilent) and quantitative PCR (Kapa Biosystems). The resulting libraries were initially sequenced on an HiSeq X instrument (Illumina) and paired-end, 150-nucleotide reads were generated. Base calling was carried out using Illumina’s real-time analysis, and the raw sequence reads were exported in FASTQ format. The quality of the sequencing data was assessed by FastQC.

The FASTQ files containing raw sequence reads were aligned to fruit fly genome (BDGP6) by HISAT2 program (Version 2.1.0, default parameters). The number of reads mapped to each transcript were also calculated by HISAT2 (default parameters) based on the gene annotations from Ensembl Release 93 (www.ensembl.org). The read numbers were transcripts per kilobase million (TPM) from the HISAT2 result file. Compared with the control PA36 group, genes with P-value < 0.05 and log2-transformed fold change > 1 were considered as differentially expressed.

Biocomputational analysis

The raw reads count of transcriptome profiling in C9orf72-ALS and sporadic ALS were downloaded from the GEO database (GSE67196). DESeq (Anders and Huber, 2010) was applied to normalize the data and perform differential analysis. Using DESeq P-value < 0.05 and log2-transformed fold change > 1 as the cut-off, we identified 586 upregulated and 41 downregulated genes in the cerebellum, 389 upregulated and 44 downregulated genes in the frontal cortex.

The differentially expressed genes in UPF1/2/3-deficient cells were directly extracted from the supplementary tables of the corresponding papers (Mendell et al., 2006; Nguyen et al., 2012).

Statistical analysis

Data analysis and statistics were carried out using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA) and are detailed in the figure legends. P < 0.05 was considered as a significant change.

Data availability

Differentially expressed genes are listed in Supplementary Tables 4–6. The raw RNA-Seq data have been uploaded to GEO (GSE123172). All the other materials are available upon request.

Results

Inhibition of nonsense-mediated mRNA decay pathway in C9orf72 patients

To evaluate whether the NMD pathway could be disturbed in patients with C9orf72 HRE, we first conducted unbiased
bioinformatics analysis to compare published transcriptome datasets from various C9orf72 ALS-related sources with those from human cells with defective NMD pathways. C9orf72 ALS-related samples included brain tissues from ALS patients (n = 8 C9orf72 ALS and n = 10 sporadic ALS) (Prudencio et al., 2015), motor neurons collected from eight C9orf72 ALS patients using laser-guided microdissection (Cooper-Knock et al., 2015) and induced pluripotent stem cell (iPSC)-derived motor neurons from two C9orf72 ALS patients (Shi et al., 2018). The available NMD-defective transcriptome datasets were from studies using cells deficient of the essential components of the NMD pathway, including lymphocytes from five patients with UPF3B/NMD deficiency (Nguyen et al., 2012) and HeLa cells with depleted UPF1 or UPF2 (Mendell et al., 2004; Wittmann et al., 2006). It is worth noting that UPF3B/NMD-deficient patients exhibited a variety of neuropsychiatric defects, including intellectual disability (Nguyen et al., 2012). We found that there were a significant number of overlapping genes between the C9orf72 ALS cerebellum tissues and each human cell type with defective NMD functions (Fig. 1A–C and Supplementary Table 1), especially the primary lymphocytes from UPF3B−/− patients. Gene ontology analysis showed that stress response and immune system-related genes were significantly enriched among the overlapping genes (Supplementary Table 2). Similarly, we have observed a significant number of overlapping genes between NMD-defective cells and C9orf72-patient-derived iPSC motor neurons (Fig. 1D–F) or microdissected motor neurons from C9orf72 patient spinal cord (Supplementary Fig. 1A). In every pair of compared groups, we observed essentially no overlapping downregulated genes, and none of the downregulated genes in C9orf72 ALS brains are NMD substrate genes. Since even UPF1/UPF2/UPF3-deficient cells only showed a few (<6) commonly upregulated genes in pair-wise comparison (Wittmann et al., 2006; Nguyen et al., 2012), our results strongly suggested that C9orf72 HRE products could cause the accumulation of mRNA species regulated by NMD in C9orf72 patients. We also compared the transcriptome of the sporadic ALS brain tissues with that of UPF3B/NMD lymphocytes and UPF1/2-deficient cells and found a much lower number of overlapped upregulated genes (Supplementary Fig. 2), suggesting that the accumulation of NMD-regulated mRNA was an effect specifically related to C9orf72 products.

Arginine-containing C9orf72 dipeptide repeats inhibit the nonsense-mediated mRNA decay pathway

Given the lead from the bioinformatic analysis, we used the Drosophila model to assess whether C9orf72 GGGGCC (G4C2) HRE products could affect the expression of NMD-regulated genes *in vivo* by RNA-seq analysis. As multiple studies have shown that the arginine-rich DPRs generated from the HRE are the more toxic species (Mizielska et al., 2014; Tao et al., 2015; Tran et al., 2015), we examined the effects of both G4C2 HRE and proline-arginine dipeptide with 36 repeats (PR36) on NMD-regulated gene expression in *Drosophila*. We chose flies with pan-neuronal expression of PR36 because we have found previously that flies expressing glycine-arginine (GR36) could not survive beyond the pupa stage (Xu and Xu, 2018). We compared the brain transcriptome data from 5-day-old flies expressing (G4C2)36 (Supplementary Table 4) or PR36 (Supplementary Table 5) with those from flies with defective NMD pathways caused by deficient UPF1 (Supplementary Table 6) or defective UPF2. UPF1 deficiency was achieved by pan-neuronal expression of the UAS-UPF1 RNAi, and the RNA-seq data from UPF2-haploinsufficiency flies were retrieved from a previous study (Chapin et al., 2014). Flies expressing non-toxic proline-alanine 36 (PA36) (Xu and Xu, 2018) were used as control. In (G4C2)36-expressing flies, the expression of 337 genes showed increase (fold change > 2, \( P < 0.05 \)). Among those upregulated genes, 66 genes were also found to be accumulated in UPF1-knockdown flies (Fig. 1G) (\( P = 3.19 \times 10^{-35} \)), suggesting that the accumulation of NMD-regulated genes observed in human tissues (Fig. 1A–F) could also be recapitulated in the Drosophila model. Interestingly, no statistically significant overlap was found between (G4C2)36-expressing flies and UPF2 mutant flies (\( P = 0.6705 \)) (Supplementary Fig. 1B), suggesting that the UPF1 function could be more vulnerable to C9orf72 HRE toxicity than UPF2 function in flies. We then assessed the effects of PR36 and found that 67 of the 275 upregulated genes caused by PR36 expression were also accumulated in flies with UPF1 deficiency (\( P = 3.63 \times 10^{-42} \)) (Fig. 1H). Furthermore, 19 common genes were identified between PR36-expressing flies and UPF2 mutant flies (\( P = 0.0182 \)) (Supplementary Fig. 1C). Notably, among the two groups of genes commonly accumulated in UPF1-deficient flies and (G4C2)36- or PR36-expressing flies, 36% of them are identical (Fig. 1I) (\( P = 4.55 \times 10^{-16} \)), indicating that arginine-rich DPRs, such as PR, could effectively affect the NMD pathway and could be the main contributors to NMD inhibition by C9orf72 HRE. Because of this discovery, we focused on the inhibition of NMD mainly by DPRs in our subsequent studies. To validate the observations from transcriptomic studies, we examined the expression of four NMD substrates (Sin3A, Gadd45, Xrp1 and Arc1) (Giorgi et al., 2007; Bramham et al., 2008; Chapin et al., 2014; Nelson et al., 2016) in PR36-expressing flies and UPF1-deficient flies, as well as in flies expressing GFP-tagged GR36, which has lower toxicity than untagged GR36, but comparable toxicity to PR36 (Xu and Xu, 2018) (Fig. 1J). Those four selected genes were among the seven validated NMD substrates that were upregulated in both UPF1-deficient and UPF2 mutant flies (Supplementary Table 3), and have well-characterized functions such as stress response...
(Fornace et al., 1992) and synaptic regulation (Bramham et al., 2008). Consistent with the RNA-seq results, those four genes were upregulated in both PR36- and GFP-GR36-expressing flies, indicating inhibition of the NMD pathway as a common feature for arginine-rich DPRs (Fig. 1J). In contrast, other known neurotoxic proteins, such as Parkinson’s disease-causing A30P α-synuclein, Huntington’s disease-causing huntingtin poly-glutamine with 128 repeats (128Q), and another ALS/FTD-associated protein FUS, did not significantly affect the expression of these genes (Fig. 1K), suggesting that the upregulation of NMD targets Sin3A, Gadd45, Xrp1 and Arc1 is likely to be specific for C9orf72 arginine-rich DPRs. Taken together, the expression of C9orf72 arginine-rich DPRs likely causes the suppression of NMD in Drosophila.

To directly assess the effects of DPRs on NMD activity, we made a luciferase reporter gene fused to the β-globin gene with a premature stop codon, a standard paradigm for
Evaluating NMD activity (Boelz et al., 2006; Keeling et al., 2013) (Fig. 2A). The validity of the reporter was confirmed by increased reporter activity in cells with silenced UPF1 expression (Supplementary Fig. 3A and B). We found that GFP-GR36 expression elevated the NMD reporter gene expression by 5-fold in the human SH-SY5Y cell line (Fig. 2B). In contrast, control GFP or GFP-GA36 expression led to very little change in NMD reporter expression (Fig. 2B).

We have shown that arginine-rich DPRs were the main contributors to NMD inhibition by G4C2 repeats in Drosophila (Fig. 1G–I). To validate this conclusion in mammalian cells, we selected a group of overlapping genes from C9orf72-ALS brain with G4C2 repeats and NMD-deficient cells (Fig. 1A–F) and tested their expression in a human neuroblastoma cell line or in mouse brain expressing only the DPRs. Those selected genes also met our criteria to be expressed in both cerebellum and frontal cortex (Supplementary Table 1). In addition, the mammalian Arc1 homologue Arc and Gadd45, a stress-related gene, was also included as it was upregulated by poly PR/GR in Drosophila. The expression of most of those genes was significantly higher in human SH-SY5Y cells expressing GFP-GR36 than in cells expressing GFP-GA36 (Fig. 2C), and were further validated as NMD-regulated genes in cells with UPF1 silencing.

Figure 2 NMD pathway is inhibited in GFP-GR36-expressing human cells and mouse brains. (A) Schematic representation of the dual luciferase NMD reporter. The firefly luciferase was used as transfection efficiency control, whereas the renilla luciferase fused to a human β-globin with a premature stop codon mutation (N39X) was used to monitor the NMD pathway activity. (B) GFP-GR36 impaired the NMD function and led to the accumulation of an NMD reporter. Indicated constructs were co-transfected with the NMD reporter into SH-SY5Y cells. Mean ± SEM, n = 3 independent experiments, one-way ANOVA followed by Tukey’s multiple comparison tests. (C) The mRNA levels of common NMD targets upregulated in C9orf72-ALS patients’ cerebellum and frontal cortex (highlighted in Supplementary Table 1) were also increased in the human SH-SY5Y cells transfected with GFP-GR36, n = 3 independent experiments. Mean ± SEM, two-tailed Student’s t-test. TNFRSF11B was not detected (N.D.) due to its low expression in SH-SY5Y cells. (D) Schematic depiction of the experimental design. AAV expressing GFP-GA36 or GFP-GR36 was separately injected to the dentate gyrus (DG) in the contralateral hemispheres, and the tissues were collected 5 weeks after injection for mRNA analysis. (E) Representative images showing the robust expression of GFP-GA36 or GFP-GR36 in dentate gyrus. (F) Relative mRNA levels of the same genes as in C in AAV-GFP-GA36/GR36-injected animals. n = 3 male mice. Mean ± SEM, two-tailed Student’s t-test.
Using the NMD reporter construct, we found that DCP1 decapping enzyme DCP1 loss of P-bodies. Furthermore, we observed that arginine-rich DPRs could simultaneously contribute to the suppression of NMD activity by GFP-GA36 (Fig. 2F). These results demonstrate that the suppression of NMD by arginine-rich DPRs was conserved across species.

**Reduced processing body formation in C9orf72 dipeptide repeat expressing cells contributes to NMD suppression**

We then attempted to investigate the potential mechanism that could account for the NMD inhibition by C9orf72 DPRs. We excluded the possibility that arginine-rich DPRs could form a complex with UPF1 (Supplementary Fig. 4A) or affect the expression of UPF genes (Supplementary Fig. 4B and C). As P-bodies are ribonucleoprotein granules harbouring proteins participating in NMD, and could be the sites for NMD (Sheth and Parker, 2006; Durand et al., 2007; Protter and Parker, 2016; Hubstenberger et al., 2017), we examined the abundance of P-bodies by immunofluorescence labelling of endogenous specific P-body markers DCP1α (Fig. 3A) or LSM1 (Supplementary Fig. 5A) in cells transfected with GFP-GR36 or control GFP or GFP-GA36. The number of DCP1α or LSM1-positive P-bodies was significantly reduced in cells expressing GFP-GR36 (Fig. 3B and Supplementary Fig. 5B), and this effect was not due to decreased expression of the marker protein DCP1α (Supplementary Fig. 5C and D).

Given that stress granules and P-bodies are related ribonucleoprotein granules with some shared components (Kedersha et al., 2005; Durand et al., 2007; Protter and Parker, 2016; Youn et al., 2018), we assessed whether the decreased abundance of P-bodies was related to increased stress granules formed in cells expressing GFP-GR36 (Fig. 3C and Supplementary Fig. 5E). We examined the DCP1α-positive P-bodies in GFP-GR36-expressing cells with (SG+) or without (SG−) stress granules (Fig. 3C) and found that the abundance of P-bodies was significantly lower in cells with stress granules (Fig. 3D). As P-bodies and stress granules are dynamically linked (Kedersha et al., 2005), our results suggested that increased stress granule formation in cells expressing arginine-rich DPRs could simultaneously contribute to the loss of P-bodies.

We found overexpression of a specific P-body component decapping enzyme DCP1α could lead to increased abundance of P-bodies as determined by labelling endogenous LSM1 (Fig. 3E). Therefore, we assessed whether the inhibition of NMD could be rescued by overexpressing DCP1α. Using the NMD reporter construct, we found that DCP1α could alleviate the suppression of NMD activity by GFP-GR36 (Fig. 3F). Taken together, C9orf72 arginine-rich DPRs could suppress NMD by decreasing the abundance of P-body formation, which is likely influenced by concurrent increase of stress granules.

**Activation of NMD protects against C9orf72 dipeptide repeat neurotoxicity**

We then tested whether the activation of NMD may prevent C9orf72 DPR-induced toxicity. As UPF1 is a key protein initiating NMD, we co-transfected UPF1 with control GFP, GFP-GA36 or GFP-GR36 in human SH-SY5Y cells and assessed the number of cells with apoptosis marker activated caspase-3. UPF1 expression did not affect the abundance of co-transfected GFP-GR36 (Supplementary Fig. 6). While GFP-GR36 led to a great increase of activated caspase-3, co-expression of UPF1 completely prevented the apoptotic cell death (Fig. 4). Similarly, UPF1 effectively protected against (G4C2)36-induced apoptosis (Supplementary Fig. 7).

The protective role of UPF1 was also evident in Drosophila. First, knockdown of UPF1 with siRNA led to impaired motor function (Supplementary Fig. 8). Second, UPF1 was able to reverse the build-up of four NMD substrates (Fig. 1J and Supplementary Fig. 9A) and effectively rescued the loss of motor neurons and axon projection by PR36 (Fig. 5A–C and Supplementary Fig. 10). Finally, overexpression of UPF1 or UPF2 also rescued GFP-GR36-induced motor deficits and shortened lifespan (Fig. 5D and E). It is worth noting that UPF1 exhibited a much stronger rescue effect than UPF2, consistent with our earlier observation that UPF1 could be more susceptible to DPR toxicity (Fig. 1H and Supplementary Fig. 2C). One caveat in the lifespan study is that the UPF1 and UPF2 flies were not backcrossed for more than six generations. Regardless, our data strongly suggest that the restoration of the NMD activity by increasing the expression of core genes in the NMD pathway could alleviate neurotoxicity induced by arginine-rich DPRs.

Next, we aimed to test whether compounds activating NMD could be effective therapeutics in C9orf72 ALS models. In a previous study investigating the effects of NMD inhibitors, a short list of compounds that could activate NMD were identified using a dual-colour, bioluminescence-based screening assay (Nickless et al., 2014). We acquired four of the compounds (tranilast, nifulumic acid, febuxostat and nitazoxamide), and carried out validation assays to further confirm their NMD-activating effects using our β-globin-based NMD reporter (Fig. 2A) in human SH-SY5Y cells expressing C9orf72 DPRs. Surprisingly, we found that only tranilast demonstrated the activity consistent with the features of an NMD activator, exhibiting dose-dependent degradation of the NMD reporter (Fig. 6A), while not affecting PR expression (Supplementary Fig. 11). In contrast, nifulumic acid had...
no effect, whereas febuxostat and nitazoxanide exhibited inhibition of NMD in our reporter system (Supplementary Fig. 12). Based on these results, we chose tranilast for our subsequent studies to evaluate its therapeutic potential.

In human SH-SY5Y cells, tranilast could efficiently reactivate NMD activity as demonstrated by reduced expression of NMD reporter at a dose as low as 2.5 \( \mu \text{M} \) (Fig. 6A), and it could effectively suppress GFP-GR36 or GFP-GA36 expression (Fig. 3A). Tranilast also reduced the abundance of DCP1α-positive P-bodies per GFP + cell (Fig. 3B), indicating its ability to activate NMD. Furthermore, tranilast could suppress the formation of stress granules in GFP-GR36-expressing cells, as shown in Figure 3C.

**Figure 3** Decreased P-body formation in GFP-GR36-expressing cells leads to NMD inhibition. (A) Representative images showing the DCP1α-positive P-bodies in GFP-GA36 or GFP-GR36-expressing cells. Arrows indicate GFP + cells. (B) Quantification of the abundance of DCP1α-positive P-bodies per GFP + cell. Mean ± SEM, one-way ANOVA followed by Tukey’s multiple comparison tests. \( n = 9-10 \) fields, each with at least 15 GFP + cells. (C) Representative images showing the DCP1α-positive P-bodies and stress granules in GFP-GR36-expressing cells. Arrows indicate GFP-GR36 + cells containing stress granules. (D) Quantification of the abundance of DCP1α-positive P-bodies in GFP-GR36-expressing cell with (SG +) or without (SG –) stress granules. Mean ± SEM, two-tailed Student’s t-test. Each dot represents the average number of P-bodies in one field, \( n = 118 \) SG – cells and \( n = 56 \) SG + cells were scored. (E) Representative pictures showing DCP1α overexpression increasing LSM1 + P-bodies. The P-bodies in cells with or without mCherry-DCP1α expression were indicated by arrows or arrowhead, respectively. (F) NMD reporter activity in cells with indicated transfection condition. Mean ± SEM, one-way ANOVA followed by Tukey’s multiple comparison tests, \( n = 4-5 \) biological replicates.
(G4C2)_36-induced apoptosis (Fig. 6B and C and Supplementary Figs 13 and 14). In vivo, tranilast feeding starting at the larval stage led to suppressed expression of NMD substrates Arc1, Gadd45 and Xrp1, which were significantly induced in flies expressing PR36 (Fig. 6D). While GR36 expression in Drosophila caused a reduction in the number of pupae formed and significantly decreased pupa-to-adult viability, treatment with 10 μM tranilast improved the survival of GR36-expressing flies (Table 1). The female pupa-to-adult viability in the tranilast-treated GR36 group rose from 10–15% to >40%. In flies expressing the less toxic GFP-GR36, tranilast treatment led to a significant increase in the number of pupae formed (from mid-20 s to 56). Furthermore, a small number of male flies expressing GFP-GR36 could survive to the adult stage with tranilast feeding (Table 1). Finally, tranilast significantly improved the adult survival rate (Fig. 6E) and motor function (Fig. 6F) in flies expressing either PR36 or GFP-GR36.
Thus, NMD-activating compound tranilast could protect against the neurotoxicity induced by C9orf72 DPRs in human cells and Drosophila.

**Discussion**

By combining transcriptome analysis of tissues and cells from C9orf72 patients with experimental validation in various models, we have found that C9orf72 HRE products, especially the arginine-rich DPRs could inhibit the NMD pathway, potentially by suppressing the formation of P-bodies. Remarkably, reactivation of the NMD pathway could protect against the neurotoxicity caused by G4C2 expansion repeat products and therefore could be a promising therapeutic approach for ALS. Furthermore, we have suggested tranilast, an NMD-activating drug with good safety record, as a potential therapeutic agent for ALS.

Proper surveillance of various RNA species to remove faulty and excess RNA in the cells is vital for the survival and thriving of the organism. A major surveillance mechanism is the NMD pathway, which selectively targets mRNAs with premature termination codon or long 3’ untranslated region for degradation (He and Jacobson, 2015; Lykke-Andersen and Jensen, 2015). Because of its importance, the roles of NMD in human diseases, and even ageing and longevity, have been increasingly recognized (Bhuvanagiri et al., 2010; Son et al., 2017; Tabrez et al., 2017; Popp and Maquat, 2018). Interestingly, Connolly (2005) proposed a hypothesis that the translation of normally untranslated nonsense RNA due to a faulty surveillance system may contribute to the accumulation of misfolded protein and lead to neurodegeneration. However, there has been no direct evidence to connect the suppression of the NMD pathway to neurodegenerative diseases. For FTD-associated progranulin, mutations that cause a frameshift and premature termination codon actually stimulated the degradation of progranulin mRNA via NMD (Skoglund et al., 2009; Nguyen et al., 2018). Interestingly, Connolly (2005) proposed a hypothesis that the translation of normally untranslated nonsense RNA due to a faulty surveillance system may contribute to the accumulation of misfolded protein and lead to neurodegeneration. However, there has been no direct evidence to connect the suppression of the NMD pathway to neurodegenerative diseases. For FTD-associated progranulin, mutations that cause a frameshift and premature termination codon actually stimulated the degradation of progranulin mRNA via NMD (Skoglund et al., 2009; Nguyen et al., 2018). Our current study revealed that the inhibition of NMD could be one of the major consequences in C9orf72 ALS. Since ~40% of alternative spliced transcripts could generate premature stop codons and NMD is coupled to remove those defective mRNAs (Lewis et al., 2003; Tabrez et al., 2017), the suppression of NMD could have likely contributed to the extensive alternative splicing and polyadenylation defects seen in the C9orf72 brains (Prudencio et al., 2015). Besides the build-up of aberrantly spliced transcripts,
Figure 6  Tranilast, an NMD activator, ameliorates GR/PR toxicity in SH-SY5Y cells and flies. (A) The dose effect of tranilast on an NMD reporter in GFP-GA36 or GFP-GR36-transfected cells. Mean ± SEM, n = 3 independent experiments. Asterisks or hash symbols represents the statistically significant differences of luciferase activities between cells treated with indicated dosage and untreated control in GFP-GR36 group or GFP-GA36 group. One-way ANOVA followed by Tukey’s multiple comparison tests, ****, #### P < 0.0001. (B and C) Representative images showing the protective effects of 2.5 µM of tranilast on GFP-GR36-induced caspase3 cleavage. Quantification was shown in C. Mean ± SEM, two-way ANOVA, ***P < 0.005, n = 3–6 fields, with at least 100 cells per field. The protective effects of tranilast at the dosage of 2.5 and 7.5 µM were confirmed in two more replicate experiments. (D) The mRNA abundance of Arc1, Gadd45 and Xrp1 in the brains of 5-day-old female flies treated with DMSO or tranilast. Mean ± SEM, n = 3 independent experiments, one-way ANOVA followed by Tukey’s multiple comparison tests. *P < 0.05, **P < 0.01. (E) Feeding flies with 10 µM tranilast from larval stage prolonged the lifespan in GFP-GR36 or PR36-expressing flies. Log-rank test, each genotype includes n = 80 flies in four vials. As ElavC155 > GFP-GR36 expression in male is lethal, female flies were used for lifespan assay. (F) Tranilast treatment improved the mobility in PR36-expressing flies. The expression of PR36 was not affected by tranilast (data not shown). Male, mean ± SEM, n = 3 tubes, each dot represents the result from one vial of flies, two-tailed Student’s t-test. CTRL genotype = ElavC155/+; ♀ = female; ♂ = male.
accretion of some key NMD substrates could also be detrimental. Two of the well known NMD targets identified in GR/PR-expressing flies, Arc1 and Gadd45 (Fig. 1J), could contribute to DPR-induced neurotoxicity. Gadd45 has been shown to cause neurodegeneration in flies, and its degradation via NMD is essential for the viability (Nelson et al., 2016). In our study, we found that Arc1 partially mediated DPR neurotoxicity in flies (Supplementary Fig. 15). Therefore, the suppression of the NMD pathway would cause a broad impact in neuronal survival by increasing the expression of a variety of target genes.

We have found that the inhibition of NMD by the C9orf72 HRE products was mainly mediated by arginine-rich DPRs, which could increase cellular stress and promote the formation of stress granules while suppressing protein translation (Lee et al., 2016; Boeynaems et al., 2017; Green et al., 2017; Li et al., 2017; Cheng et al., 2018; Zhang et al., 2018a, b). In this report, we found that the formation of P-bodies was also suppressed by arginine-rich DPRs, thus contributing to the inhibition of NMD. This observation extends our understanding of the impact of DPRs on various ribonucleoprotein granules. Interestingly, the reduced abundance of P-bodies was accompanied by increased formation of stress granules in the same cell. As stress granules and P-bodies are dynamically linked (Kedersha et al., 2005; Proter and Parker, 2016), it is conceivable that dynamic stress granule formation may drain the components from P-bodies and lead to the disintegration of P-bodies. Given that poly GR/PR could interact with some RNA-binding proteins and affect the structures and dynamics of membraneless organelles (Lee et al., 2016; Lin et al., 2016), they could also directly affect the P-body formation while promoting stress granule formation in parallel. Unlike stress granules, P-bodies are much less dynamic structures, as determined by FRAP of fluorescent-tagged DCP1ζ (unpublished data). Therefore, the consequences of the disruption of P-bodies could be long-lasting. Although P-body formation is not entirely required for NMD (Eulalio et al., 2007), NMD activities would likely be affected by the displacement of various NMD-related proteins residing in P-bodies.

One key finding from our study is that the NMD pathway would be a promising downstream checkpoint for therapeutic intervention in cellular and in vivo models of C9orf72 ALS. This conclusion has provided strong evidence to support the emerging concept that NMD could be a therapeutic target for some ALS cases (Jaffrey and Wilkinson, 2018). Previous studies have shown beneficial effects of UPF1 in a TDP-43 rat model and FUS/TDP-43 cellular models (Barmada et al., 2015; Jackson et al., 2015), although the mechanisms were not clear. Our new findings strongly suggested that the NMD pathway could be a converging therapeutic target in ALS/FTD cases with a strong RNA dysregulation component, such as aberrant alternative splicing.

The beneficial effects of tranilast (N-[3,4-dimethoxycinnamoyl]-anthranilic acid; trade name: Rizaben) in our C9orf72 HRE models are encouraging. Tranilast has been used in Japan, South Korea and China to treat bronchial asthma since 1982 (Rogosnitzky et al., 2012; Darakhshan and Pour, 2015). It is a synthetic derivative of the tryptophan metabolite anthranilic acid, and has been shown to suppress autoimmunity in a mouse model of multiple sclerosis (Platten et al., 2005) and tested for the treatment of various proliferative disorders (Rogosnitzky et al., 2012). The exact molecular mechanism of tranilast action is unclear, but its current clinical applications could be due to its ability to suppress immune response, inflammation, histamine release and the TGF-β pathway (Darakhshan and Pour, 2015). Whether these effects are related to the ability of tranilast to activate NMD remains to be investigated. Tranilast-mediated immune suppression could be a consequence of NMD activation. We found that immune response-related NMD target genes are significantly enriched in the C9orf72 ALS brains, in which the NMD pathway is inhibited (Supplementary Table 2), thus further suggesting the potential beneficial effect of tranilast in C9orf72 ALS patients.

Overall, the long track record of tranilast as a well-tolerated, low toxicity drug would make it a highly promising choice for ALS therapy. A daily dose of 600 mg has been used in patients for tumour treatment, and orally-administered tranilast could pass the blood–brain barrier (Darakhshan and Pour, 2015). Therefore, it will be important to fully characterize the therapeutic effects of tranilast in other C9orf72 animal models, as well as in other ALS models. Tranilast clinical trials in ALS patients, particularly those carrying the C9orf72 HRE, could be attempted to meet the urgent need for new ALS therapies. With validation of its protective effect against C9orf72 HRE, tranilast may also be used in individuals carrying C9orf72 HRE at

### Table 1 Tranilast treatment from embryo stage partially rescues GR36- or GFP-GR36-caused developmental defects

| Treatment | Pupae formation | Pupae-to-adult viability (%) |
|-----------|----------------|-----------------------------|
| ElavC155-Gal4 × UAS-GR36 | CTRL 27 Male lethal | Female 3/27 (11.1) |
| 0.1% | Male lethal | Female 4/28 (14.3) |
| DMSO | Male lethal | Female 10/27 (37) |
| 10μM | Male lethal | Female 13/32 (40.6) |
| Tranilast | Female 23/56 (41.1) |
| ElavC155-Gal4 × UAS-GFP-GR36 | CTRL 27 Male leth | Female 10/23 (43.5) |
| 0.1% | Male lethal | Female 10/23 (43.5) |
| DMSO | Male lethal | Female 10/23 (43.5) |
| 10μM | Male lethal | Female 10/23 (43.5) |
| Tranilast | Female 23/56 (41.1) |

The numbers of pupae and viable adults with indicated genotype and treatment were listed. Bold indicates the beneficial effects. CTRL = control; DMSO = dimethyl sulfoxide.
pre-symptomatic stage to delay or prevent the disease onset.

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Competing interests

J.X and W.C.X. are co-inventors of patent application (CN201710646727) ‘The use of nonsense-mediated mRNA decay in the treatment and diagnosis of neurodegenerative diseases’.

Supplementary material

Supplementary material is available at Brain online.

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