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Reduced autophagy upon C9ORF72 loss synergizes with dipeptide repeat protein toxicity in G4C2 repeat expansion disorders

Manon Boivin[1,2,3,4], Véronique Pfister[1,2,3,4], Angeline Gaucherot[1,2,3,4], Frank Ruffenach[1,2,3,4], Luc Negroni[1,2,3,4], Chantal Sellier[1,2,3,4,*] & Nicolas Charlet-Berguerand[1,2,3,4,**]

Abstract

Expansion of G4C2 repeats within the C9ORF72 gene is the most common cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Such repeats lead to decreased expression of the autophagy regulator C9ORF72 protein. Furthermore, sense and antisense repeats are translated into toxic dipeptide repeat (DPR) proteins. It is unclear how these repeats are translated, and in which way their translation and the reduced expression of C9ORF72 modulate repeat toxicity. Here, we found that sense and antisense repeats are translated upon initiation at canonical AUG or near-cognate start codons, resulting in polyGA-, polyPG-, and to a lesser degree polyGR-DPR proteins. However, accumulation of these proteins is prevented by autophagy. Importantly, reduced C9ORF72 levels lead to suboptimal autophagy, thereby impairing clearance of DPR proteins and causing their toxic accumulation, ultimately resulting in neuronal cell death. Of clinical importance, pharmacological compounds activating autophagy can prevent neuronal cell death caused by DPR proteins accumulation. These results suggest the existence of a double-hit pathogenic mechanism in ALS/FTD, whereby reduced expression of C9ORF72 synergizes with DPR protein accumulation and toxicity.

Keywords amyotrophic lateral sclerosis; autophagy; C9ORF72; neurodegeneration; RAN translation

Subject Categories Neuroscience; RNA Biology; Translation & Protein Quality

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Introduction

With one individual affected in ~50,000 people, amyotrophic lateral sclerosis (ALS) is the third most common neurodegenerative disease. ALS is characterized by degeneration of both upper and spinal motor neurons that leads to progressive skeletal muscle paralysis, ultimately resulting in death from respiratory failure generally 3–5 years after age of onset. ALS patients can present significant clinical, genetic, and histopathological overlaps with frontotemporal dementia (FTD), a presenile dementia affecting frontal and temporal brain regions (Lomen-Hoerth *et al*, 2002; Ringholz *et al*, 2005; Neumann *et al*, 2006). Consequently, ALS and FTD are now considered as two extremes of a same disease continuum. This notion is emphasized by the identification of an expansion of GGGGCC (G4C2) repeats within the first intron of the *C9ORF72* gene as the most common inherited cause for both ALS and FTD in Caucasian populations of northern Europe and North America (DeJesus-Hernandez *et al*, 2011; Renton *et al*, 2011; Gijselinck *et al*, 2012; Majounie *et al*, 2012).

Expanded G4C2 repeats are pathogenic through three main non-exclusive mechanisms. First, transcripts containing expanded repeats accumulate in RNA foci that recruit various RNA-binding proteins, potentially altering their localization and functions (Almeida *et al*, 2013; Donnelly *et al*, 2013; Lagier-Tourenne *et al*, 2013; Lee *et al*, 2013; Mizielinska *et al*, 2013; Cooper-Knock *et al*, 2014; Haeusler *et al*, 2014; Conlon *et al*, 2016, 2018). Second, sense G4C2 and antisense C4G2 expanded repeats are repeat-associated non-AUG (RAN) translated into dipeptide repeat (DPR)-containing proteins, which form inclusions throughout the brain of patients with C9-ALS/FTD (Ash *et al*, 2013; Gendron *et al*, 2013; Mori *et al*, 2013; Zu *et al*, 2013), as well as in mice expressing expanded G4C2 repeats (Chew *et al*, 2015; O'Rourke *et al*, 2015; Peters *et al*, 2015; Jiang *et al*, 2016; Liu *et al*, 2016). Overexpression of DPR proteins using artificial ATG codons for their translation initiation leads to neurodegeneration in cell and animal models, notably through

1 Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Illkirch, France
2 Université de Strasbourg, Illkirch, France
3 Centre National de la Recherche Scientifique, UMR7104, Illkirch, France
4 Institut National de la Santé et de la Recherche Médicale, U1258, Illkirch, France
*Corresponding author. Tel: +33 388 653 309; Fax: +33 388 653 201; E-mail: sellier@igbmc.fr
**Corresponding author. Tel: +33 388 653 309; Fax: +33 388 653 201; E-mail: ncharlet@igbmc.fr

alteration of mitochondria (Dafinca *et al*, 2016; Lopez-Gonzalez *et al*, 2016; Choi *et al*, 2019), DNA repair (Walker *et al*, 2017; Lopez-Gonzalez *et al*, 2019), nuclear and nucleolar organization (White *et al*, 2019; Zhang *et al*, 2019), and/or nucleocytoplasmic transport (Kwon *et al*, 2014; May *et al*, 2014; Mizielinska *et al*, 2014; Wen *et al*, 2014; Zhang *et al*, 2015, 2016; Freibaum *et al*, 2015; Jovičić *et al*, 2015; Tao *et al*, 2015; Boeynaems *et al*, 2016; Khosravi *et al*, 2017). Third, expanded G4C2 repeats promote DNA epigenetic changes that lead to decreased expression of C9ORF72 mRNA and protein levels in C9-ALS/FTD individuals (DeJesus-Hernandez *et al*, 2011; Gijselinck *et al*, 2012; Almeida *et al*, 2013; Waite *et al*, 2014; van Blitterswijk *et al*, 2015; Xiao *et al*, 2015; Saberi *et al*, 2017; Frick *et al*, 2018; Viodé *et al*, 2018). Interestingly, knockdown of C9ORF72 expression in zebrafish or in human motor neuron differentiated from iPS cells leads to some neuronal cell dysfunctions and cell death (Ciura *et al*, 2013; Shi *et al*, 2018). These results suggest that haploinsufficiency of C9ORF72 may play a role in the pathogenesis of ALS/FTD. However, the absence of neurodegeneration in mice knockout for C9orf72 argues against a sole loss of function of C9ORF72 as a cause of ALS/FTD (Lagier-Tourenne *et al*, 2013; Koppers *et al*, 2015; O'Rourke *et al*, 2016; Atanasio *et al*, 2016; Burberry *et al*, 2016; Jiang *et al*, 2016; Sudria-Lopez *et al*, 2016). Hence, the pathological consequences of C9ORF72 reduced expression in ALS/FTD remain to be clarified. Similarly, by which molecular mechanisms sense G4C2 and antisense C4G2 repeats are translated, and how such translation modulates repeat toxicity are key points that remain to be investigated.

Using constructs in which expanded repeats were embedded within the natural *C9ORF72* human sequence, we found that the G4C2 sense repeats are mostly translated into poly(glycine–alanine) (polyGA) and to a lesser extent into poly(glycine–arginine) (polyGR)-containing proteins through initiation to near-cognate start codons that are located upstream of the G4C2 repeats. Near-cognate initiation codons are codons differing from the AUG sequence by one base (CUG, GUG, UUG, ACG, AAG, etc.), but that can still initiate translation provided they are embedded in a correct Kozak consensus sequence (Kozak, 1989; Peabody, 1989). Moreover, we found that the antisense *C9ORF72* transcript containing expanded C4G2 repeats is mostly translated into a poly(proline–glycine) (polyPG)-containing protein using a canonical AUG start codon embedded in a poor Kozak consensus sequence. As a result of their non-optimal translation initiation, DPR proteins are expressed at low levels and consequently present limited toxicity in neuronal cells. Furthermore, accumulation of DPR proteins is prevented by their constant degradation through autophagy. As the C9ORF72 protein regulates autophagy (Amick *et al*, 2016; Sellier *et al*, 2016; Sullivan *et al*, 2016; Ugolino *et al*, 2016; Webster *et al*, 2016; Xiao *et al*, 2016; Yang *et al*, 2016; Jung *et al*, 2017; Chitiprolu *et al*, 2018; Ho *et al*, 2019), we tested whether C9ORF72 modulates DPR expression. Importantly, reduced expression of C9ORF72 increases the accumulation and toxicity of DPR proteins expressed under their natural sequences. Finally, we screened various pharmacological compounds known to activate autophagy and found that mTOR inhibitors and some phenothiazine derivatives, which are clinically approved antipsychotic drugs known to activate autophagy in neuronal cell culture (Tsvetkov *et al*, 2010), prevent the neuronal cell death caused by the synergic toxicity of DPR protein accumulation upon C9ORF72 reduced expression.

In conclusion, these results suggest an alternative molecular mechanism to RAN translation that may explain production of DPR proteins from *C9ORF72* sense and antisense repeats. Furthermore, these data support a double-hit mechanism in ALS/FTD, where the reduced expression of C9ORF72 synergizes the accumulation and toxicity of DPR proteins.

Results

Expanded sense G4C2 repeats are translated into polyGA and polyGR

To assess translation of the sense GGGGCC repeats, we cloned 80 G4C2 repeats embedded within the natural human *C9ORF72* sequence and fused these repeats to the GFP sequence in all three possible frames (Fig EV1A). These frames were named according to the DPR protein potentially encoded by the expanded G4C2 repeats, namely glycine–alanine (GA), glycine–proline (GP), and glycine–arginine (GR). Cell transfection and immunoblotting against the GFP indicated that expanded G4C2 repeats embedded in the natural *C9ORF72* sequence are predominantly translated into the GA frame and with a much lower efficiency in the GR frame (Fig 1A). In contrast, we found no or very limited translation of the G4C2 repeats into the GP frame. Direct observation of the GFP fluorescence confirmed marginal levels of polyPG (Fig EV1B), excluding a potential bias of aggregation and insolubility that would impair detection of this protein by immunoblotting. Furthermore, RT–qPCR quantification indicates similar levels of RNA expression for polyGA, polyGP, and polyGR constructs, indicating that the absence of polyGP at the protein level is not caused by a lack of its expression at the RNA level (Fig EV1C). As a further control, cell transfections of GFP-tagged polyGA, polyGP, and polyGR expressed under artificial ATG start codons demonstrate expression of all three DPR proteins by fluorescence (Fig EV1D) and immunoblot analyses (Fig EV1E). Expression of polyGP into a stable protein when using an artificial ATG start codon suggests that its absence when using the natural *C9ORF72* sequence is not due to a bias of insolubility and/or rapid degradation of this protein.

Next, we characterized the translation initiation sites of the polyGA and polyGR proteins. Expanded G4C2 repeats embedded in their natural *C9ORF72* sequence were modified so that a lysine, target of the LysC enzyme, is present downstream of three G4C2 repeats in the GA frame. Immunoprecipitation of the polyGA DPR protein followed by LysC digestion and LC-MS/MS analysis revealed a translation initiation to a CUG near-cognate codon embedded in a correct Kozak consensus sequence and located 24 nucleotides upstream of the G4C2 repeats (Fig 1B). This CUG start site of polyGA is identical to the one found in three other independent studies (Green *et al*, 2017; Sonobe *et al*, 2018; Tabet *et al*, 2018). Mass spectrometry indicates that the initial amino acid of the polyGA DPR protein is a methionine, suggesting that the CUG codon is decoded by an initiator Met-tRNA despite imperfect match. Mutation of this CTG codon into CTT abolished polyGA DPR protein expression (Fig EV1F), confirming the importance of this near-cognate codon for translation of the G4C2 repeats in the GA frame. In contrast, mutation of the CTG codon into a canonical

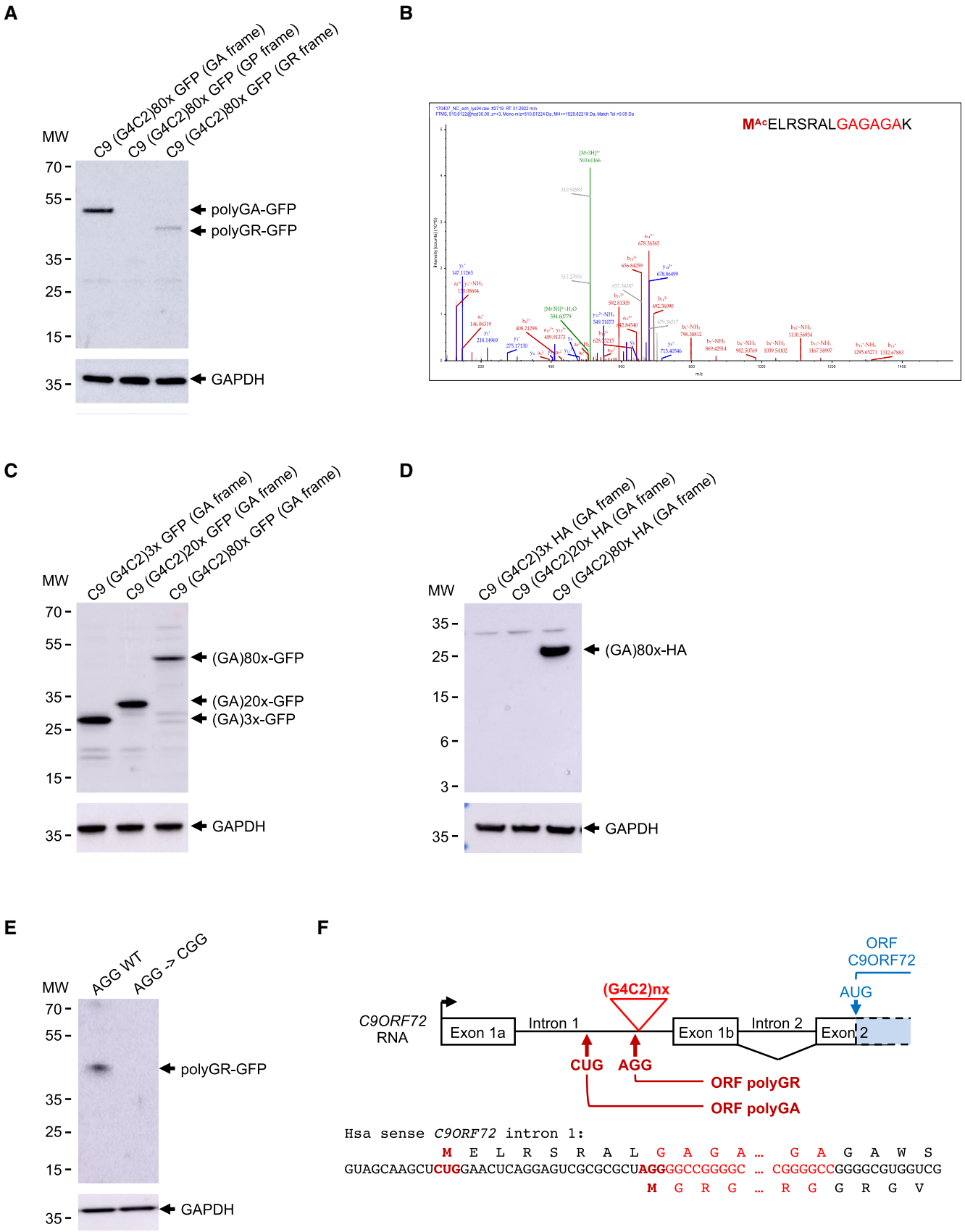

Figure 1.

Figure 1. Expanded G4C2 repeats are translated into polyGA and polyGR.

A Immunoblotting against the GFP or the GAPDH of proteins extracted from HEK293 cells transfected for 24 h with 80 G4C2 repeats embedded in the human sense *C9ORF72* and fused in all three possible frames with the GFP deleted of its ATG.

B LC-MS/MS spectra of the N-terminal part of the GFP-immunoprecipitated and LysC-digested polyGA protein expressed as in (A).

C, D Immunoblotting against the GFP- (C) or the HA-tag (D) or the GAPDH of proteins extracted from HEK293 cells transfected for 24 h with 3, 20 or 80 G4C2 repeats embedded in the human sense *C9ORF72* sequence fused to the GFP in the GA frame.

E Immunoblotting against the GFP or the GAPDH of proteins extracted from HEK293 cells transfected with either a wild-type or a mutant (AGG into CGG) construct containing 80 G4C2 repeats embedded in sense *C9ORF72* fused to the GFP in the GR frame.

F Scheme of human *C9ORF72* sense transcript with intron 1 retained. Expanded G4C2 repeats, CUG and AGG near-cognate initiation codons, and polyGA and polyGR ORFs are indicated in red. C9ORF72 ORF is indicated in blue.

ATG start codon enhanced by fivefold to 10-fold expression of polyGA (Fig EV1G), highlighting the weak translation initiation efficiency of the natural *C9ORF72* sequence. As a control, RT–qPCR quantification shows that mutation of the CTG start codon does not modify polyGA expression at the RNA level (Fig EV1H), Next, cell transfection of various lengths of G4C2 repeats (3×, 20×, and 80×) fused to the GFP in the GA frame indicated that translation occurs with expanded G4C2 repeats of any lengths (Fig 1C). In contrast, fusion of G4C2 repeats to a smaller HA-tag (9 amino acids, ~1 kDa) in the GA frame resulted in detection of polyGA with expanded (80×) G4C2 repeats, but only trace of proteins with 3 or 20 G4C2 repeats (Fig 1D). This is likely caused by the rapid degradation of these small proteins, as inhibition of the proteasome and autophagy degradation pathways allowed detection of the polyGA protein with 20 G4C2 repeats (Fig EV1I). These results suggest that translation initiation occurs at a CUG near-cognate codon in the GA frame independently of the length of the G4C2 repeats. However, in absence of an expansion, the resulting protein is small and unstable, impairing its detection in normal conditions. In contrast, repeat expansion or fusion of the repeat to a large tag, such as the GFP protein, results in expression of a stable and detectable polyGA DPR protein.

Translation initiation of polyGA to a near-cognate start codon questions whether a similar mechanism may occur for polyGP and polyGR. However, in the GP frame a stop codon is located just before the G4C2 repeats, preventing expression of a polyGP protein from any upstream putative near-cognate initiation codons. Concerning the polyGR DPR protein, mass spectrometry failed to identify its N-terminus, but its expression is likely to be dependent of a near-cognate initiation mechanism as mutation of an AGG near-cognate codon located just upstream of the G4C2 repeats and in the GR frame impairs expression of this protein (Fig 1E). As a control, mutation of this AGG codon does not affect RNA expression (Fig EV1J). This AGG codon is located just before the repeats, leading to a methionine adjacent to the GR repeats, which may explain why we failed to identify it by mass spectrometry. Of interest, translation initiation at this AGG near-cognate codon is weak and results in limited expression of polyGR compared to polyGA (Fig 1A). To summarize this first part, translation initiation at either a CUG or an AGG near-cognate codon is predicted to result in a polyGA or a polyGR DPR protein, which is composed of a short N-terminus of either 8 or 1 amino acids, followed by a central repeated glycine–alanine or glycine–arginine stretch which length corresponds to the number of expanded G4C2 repeats and a C-terminus ending in either *C9ORF72* intron 2 or exon 2 according to splicing or retention of intron 2 (Fig 1F).

Expanded antisense C4G2 repeats are translated into polyPG

The *C9ORF72* gene is transcribed in both sense and antisense directions. To assess the translation of the antisense CCCCGG repeats, we cloned one hundred C4G2 repeats, embedded within the natural human *C9ORF72* antisense sequence, and fused to a HA-tag in all three possible frames, namely proline–alanine (PA), proline–glycine (PG), and proline–arginine (PR). Cell transfection and immunoblotting against the HA-tag indicated that expanded C4G2 repeats embedded in the antisense *C9ORF72* sequence are predominantly translated into the PG frame (Fig 2A). Fusion of the C4G2 repeats in all three frames to the GFP followed by cell transfection and direct observation of the GFP fluorescence confirmed that translation occurs mostly in the PG frame (Fig EV2A). As a control, RT–qPCR analysis indicates similar RNA levels of polyPA, polyPG, and polyPR (Fig EV2B). Furthermore, polyPA and polyPR proteins are correctly expressed when cloned downstream of artificial ATG start codons (Fig EV2C and D), excluding that the non-detection of these proteins is caused by a potential bias of protein insolubility and/or instability.

As C4G2 repeats appear to be translated mostly into polyPG, we next investigated the translation initiation mechanism of this protein. HA immunoprecipitation followed by LysC digestion and LC-MS/MS analysis revealed translation initiation to a canonical AUG start codon located 195 nucleotides upstream of the repeats (Fig 2B). Deletion of the *C9ORF72* antisense sequence containing this initiation codon abolished polyPG expression (Fig EV2E), demonstrating the importance of this initiation sequence for polyPG translation. As a control, RT–qPCR indicated that deletion of the sequence containing this ATG does not alter RNA expression (Fig EV2F). Of interest, this AUG start codon is located in a poor Kozak sequence and its mutation into a consensus Kozak sequence enhanced expression of the polyPG protein by threefold to fivefold (Fig EV2G). Consistent with translation starting before the repeats, expression of polyPG fused to the GFP is independent of the C4G2 expansion size (Fig 2C). In contrast, fusion of expanded (100×) or non-expanded (10×) C4G2 repeats to the small HA-tag resulted in a detectable polyPG protein only with 100 G4C2 repeats (Fig 2D). The absence of detection of the small polyPG protein with 10 C4G2 repeats fused to the HA-tag is likely due to its instability and rapid turnover as inhibition of the proteasome and autophagy degradation pathways allowed its accumulation and detection (Fig EV2H). These data indicate that the polyPG DPR protein is translated through initiation to a canonical AUG start codon, independently of the repeats. However, in the absence of a C4G2 expansion, the resulting polyPG protein is small and likely too unstable to be significantly observed in control conditions. In contrast, either expansion of the repeats or

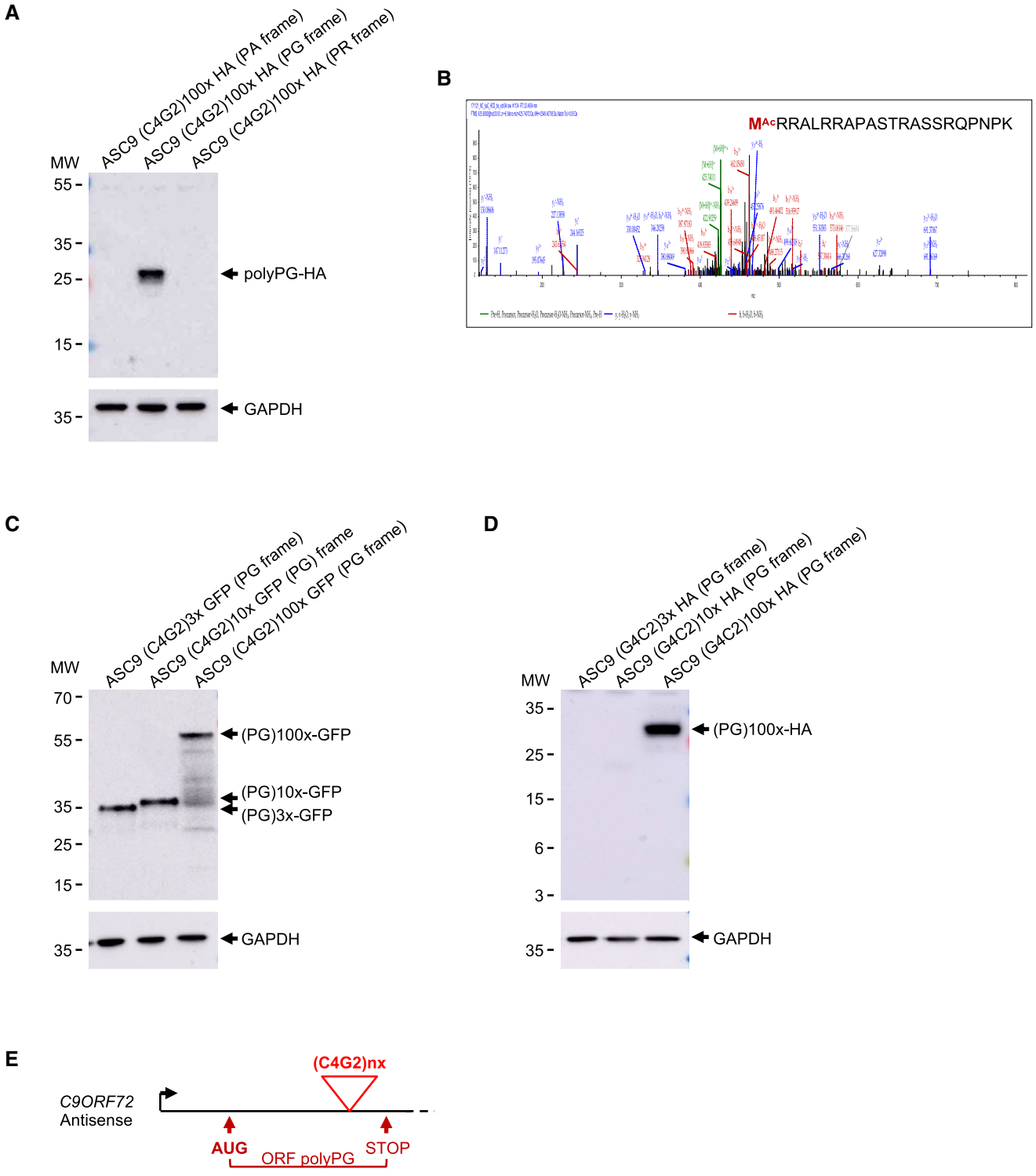
A
ASC9 (C4G2)100x HA (PA frame)
ASC9 (C4G2)100x HA (PG frame)
ASC9 (C4G2)100x HA (PR frame)
MW
55
35
25
15
35
polyPG-HA
GAPDH
B
MAcRRALRRAPASTRASSRQPNPK
C
ASC9 (C4G2)3x GFP (PG frame)
ASC9 (C4G2)10x GFP (PG) frame
ASC9 (C4G2)100x GFP (PG frame)
MW
70
55
35
25
15
35
(PG)100x-GFP
(PG)10x-GFP
(PG)3x-GFP
GAPDH
D
ASC9 (G4C2)3x HA (PG frame)
ASC9 (G4C2)10x HA (PG frame)
ASC9 (G4C2)100x HA (PG frame)
MW
35
25
15
6
3
35
(PG)100x-HA
GAPDH
E
(C4G2)nx
C9ORF72
Antisense
AUG
STOP
ORF polyPG
Hsa antisense C9ORF72:
AAAGUAAAAAUGCGUCGAGCUCUGAGGAGAGCCCCCGCUUCUACCCGCGCCUCUUCCCGGCAGCCGAACCCCAAACAGCCACCCGCCAGGAUGCCGCCU
M R R A L R R A P A S T R A S S R Q P N P K Q P P A R M P P
CCUCACUCACCCACUCGCCACCGCCUGCGCCTCCGCCGCCGCGGGCGCAGGCACCGCAACCGCAGCCCCGCCCCGGGCCCGCCCCCGGGCCCGCCCCGA
P H S P T R H R L R L R R R G R R H R N R S P A P G P P P G P P R
CCACGCCCCGGCCCCGGC … CCCCGGCCCCUAGCGCGCGACUCCUGA…
P R P G P G … P G P .

Figure 2.

Figure 2. Expanded C4G2 repeats are translated into polyPG.

A Immunoblotting against the HA-tag or the GAPDH of proteins extracted from HEK293 cells transfected for 24 h with 100 C4G2 repeats embedded in the human antisense *C9ORF72* and fused in all three possible frames with a HA-tag.

B LC-MS/MS spectra of the N-terminal part of the HA-immunoprecipitated and LysC-digested polyPG protein expressed as in (A).

C, D Immunoblotting against the GFP- (C) or the HA-tag (D) or the GAPDH of proteins extracted from HEK293 cells transfected for 24 h with 3, 10, or 100 C4G2 repeats embedded in the human antisense *C9ORF72* sequence fused to the GFP in the PG frame.

E Scheme of human antisense *C9ORF72* transcript. Expanded C4G2 repeats, AUG initiation codon, and polyPG ORF are indicated in red.

fusion to the GFP protein increases the size and the stability of this protein, enabling its detection. In summary, antisense C4G2 repeats are translated into a polyPG protein, which is predicted to be composed of a N-terminus of 65 amino acids rich in proline and arginine, a central repeated proline–glycine stretch which length corresponds to the number of expanded C4G2 repeats and a stop codon located just after the repeats (Fig 2E).

Decreased expression of C9ORF72 synergizes DPR protein toxicity

Next, we tested the toxicity of the polyGA, polyPG, and polyGR DPR proteins expressed under their natural sense or antisense *C9ORF72* human sequences. Consistent with their low expression, their transfection into neuronal cells resulted in low toxicity with only 10–20% of neuronal cell death (Fig 3A). This toxicity is likely due to translation of the repeats into DPR proteins and not caused by G4C2 or C2G4 RNA toxicity, as mutation or deletion of DPR protein initiation codons (CTG for polyGA, AGG for polyGR, and ATG for polyPG) resulted in negligible cell death (Fig 3A). As positive controls, transfection of polyGA, polyPG, and polyGR cloned downstream of canonical ATG start codons embedded in consensus Kozak sequences (gccATGg) resulted in higher DPR protein expression (Figs EV1C and EV2C) and consequently in higher neuronal cell toxicities, especially for polyGR (Fig 3A). These results are similar to the toxicity reported for DPR proteins driven by artificial ATG start codons and where polyGR are more toxic than polyGA or polyGP (May *et al*, 2014; Mizielinska *et al*, 2014; Yamakawa *et al*, 2015; Lopez-Gonzalez *et al*, 2016, 2019; Schludi *et al*, 2017; Zhang *et al*, 2018; Choi *et al*, 2019). DPR proteins form protein aggregates that are degraded by autophagy (Cristofani *et al*, 2018). Indeed, bafilomycin A1-mediated inhibition of autophagy enhances the accumulation of DPR proteins (Figs 3C and D, and EV3A and B). As the C9ORF72 protein regulates autophagy and as C9ORF72 protein expression is reduced in neuronal tissue from C9-positive ALS/FTD individuals, we tested whether decreased expression of C9ORF72 may modulate the expression and toxicity of DPR proteins. Immunoblotting indicated that siRNA-mediated depletion of C9ORF72 expression leads to an accumulation of polyGA, polyPG, and polyGR DPR proteins expressed under their natural start codons (Fig 3B–D). However, DPR accumulation upon partial decreased expression of C9ORF72 was milder compared to the complete block of autophagy induced by bafilomycin treatment (Fig 3C and D). These results are consistent with C9ORF72 decreased expression causing only a partial alteration of autophagy (Sellier *et al*, 2016). In that aspect, siRNA-mediated depletion of C9ORF72 impaired the recruitment of the P62/SQSTM1 autophagy adaptor protein to polyGA aggregates (Fig EV3C). As a control, decreased expression of C9ORF72 does not modify co-localization of ubiquitin with polyGA inclusions (Fig EV3D). These data suggest that C9ORF72 plays an early role in autophagy at the step of autophagosome formation. Furthermore, siRNA-mediated depletion of C9ORF72 leads to increased lysosomal labeling (Fig EV3E). As a control, endosome labeling is not altered by decreased expression of C9ORF72 (Fig EV3F). These observations are consistent with recent reports showing a role of the C9ORF72/SMCR8 complex at lysosomes (Amick *et al*, 2016; Sullivan *et al*, 2016; Corrionero & Horvitz, 2018; Lan *et al*, 2019), and suggest a complex function of C9ORF72 both at early and at late steps of the autophagic process. As decreased expression of C9ORF72 promotes DPR protein accumulation, we then tested whether C9ORF72 may modulate DPR toxicity. Importantly, reduced expression of C9ORF72 substantially increased the neuronal cell death induced by polyGA, polyPG, and polyGR DPR proteins expressed under their natural start codons (Fig 3E). Of technical interest, toxicity induced by expression of polyGA, polyPG, and polyGR proteins expressed under artificial ATG start codons was less sensitive to C9ORF72 loss, probably due to the already high expression and toxicity of these ATG-driven DPR proteins (Fig 3E). As negative controls, no toxicity was observed when the number of G4C2 repeats was reduced to control size or when DPR protein start codons (CUG for polyGA, AGG for polyGR, or AUG for polyPG) were deleted (Fig 3E). These controls indicate that G4C2 and C4G2 expanded repeats are likely not overly toxic at the RNA level. Furthermore, these results suggest a double-hit mechanism where the decreased expression of C9ORF72 synergizes the toxicity of DPR proteins expressed under their natural start codons.

Drugs activating autophagy reduce DPR protein accumulation and toxicity

Autophagy is a highly regulated cellular process amendable to drug treatment (Rubinsztein *et al*, 2012; Galluzzi *et al*, 2017). Thus, we tested whether drugs known to activate autophagy may bypass C9ORF72 decreased expression and correct DPR protein aggregation and toxicity. The compounds tested included Torin1, KU0063794, and rapamycin, which are well-characterized mTOR inhibitors and thus activators of autophagy, and various phenothiazine derivatives, which are clinically approved antipsychotic drugs known to activate autophagy in neuronal cell culture (Tsvetkov *et al*, 2010). Interestingly, addition of not only Torin1, but also to a lesser extent either promethazine, chlorpromazine, or fluphenazine, decreases the accumulation of polyGA and polyPG DPR proteins expressed under their natural start codons (Fig 4A and B). These compounds can also decrease accumulation of a polyGA-GFP-tagged protein expressed under an artificial ATG start codon (Fig EV4A and B). Direct observation of the GFP fluorescence confirmed that these compounds decrease the accumulation of polyGA protein aggregates (Fig EV4C). Next, we tested

whether boosting autophagy may correct the toxicity of DPR protein expression. As inhibiting mTOR has many adverse consequences in animals (Zhang *et al*, 2011), we focused on phenothiazine derivatives, which are clinically approved drugs. Importantly, addition of promethazine alleviated neuronal cell death caused by the synergic toxicity caused by DPR protein accumulation upon C9ORF72 reduced expression (Fig 4C). Moreover, promethazine was also able to reduce cell death caused by expression of polyGA under an artificial ATG start codon (Fig EV4D). Overall, these results suggest that modulating autophagy could be of therapeutic interest in ALS/FTD to prevent the toxic accumulation of DPR proteins.

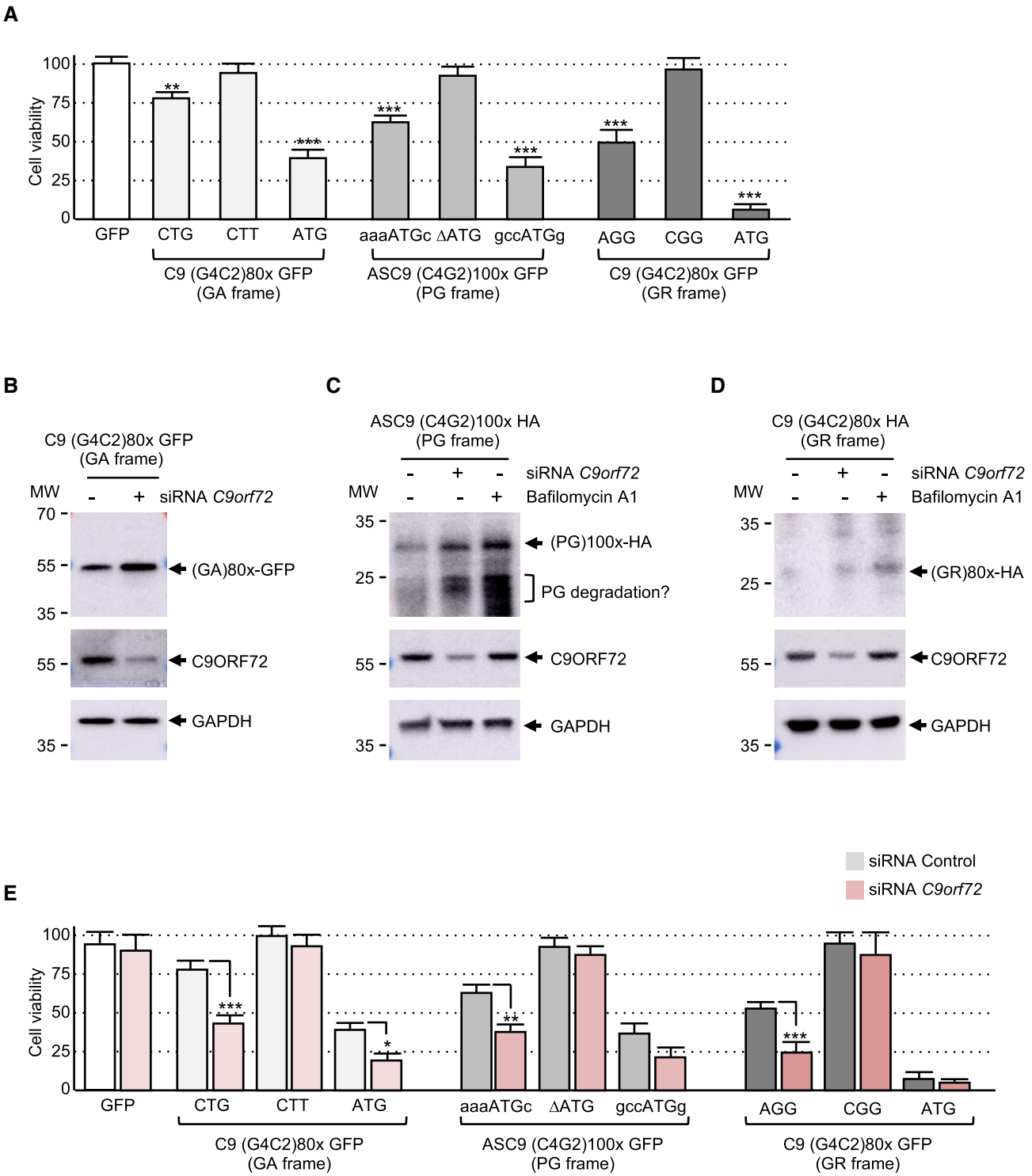

Figure 3.

Figure 3. Decreased expression of C9ORF72 synergizes DPR protein toxicity.

A Cell viability (TO-PRO-3 FACS staining) of GT1-7 neuronal cells transfected for 24 h with either wild-type or indicated mutant constructs containing either 80 G4C2 repeats or 100 C4G2 repeats embedded in sense or antisense *C9ORF72* fused to the GFP in the GA, PG, or GR frame.

B Immunoblotting against the GFP, endogenous C9ORF72, or the GAPDH of proteins extracted from Neuro2A cells transfected for 24 h with 80 G4C2 repeats embedded in the human sense *C9ORF72* sequence fused to the GFP in the GA frame and with either a control siRNA or a siRNA targeting *C9orf72* mRNA.

C Immunoblotting against the HA-tag, endogenous C9ORF72, or GAPDH of proteins extracted from Neuro2A cells transfected for 24 h with 100 C4G2 repeats embedded in the human antisense *C9ORF72* sequence fused to a HA-tag in the PG frame and with either a control siRNA or a siRNA targeting *C9orf72* mRNA or treated with bafilomycin A1 for 15 h.

D Immunoblotting against the HA-tag, endogenous C9ORF72, or GAPDH of proteins extracted from Neuro2A cells transfected for 24 h with 80 G4C2 repeats embedded in the human sense *C9ORF72* sequence fused to a HA-tag in the GR frame and with either a control siRNA or a siRNA targeting *C9orf72* mRNA or treated with bafilomycin A1 for 15 h.

E Cell viability (TO-PRO-3 FACS staining) of GT1-7 neuronal cells co-transfected for 24 h with either a control siRNA or a siRNA targeting *C9orf72* mRNA and wild-type or mutant constructs containing either 80 G4C2 repeats or 100 C4G2 repeats embedded in sense or antisense *C9ORF72* fused to the GFP in the GA, PG, or GR frame.

Data information: Error bars indicate s.e.m. Student's *t*-test, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. $n = 5$ independent transfection.

Discussion

An expansion of G4C2 repeats within the first intron of the *C9ORF72* gene is the main genetic cause of ALS/FTD (DeJesus-Hernandez *et al*, 2011; Renton *et al*, 2011). Sense and antisense transcripts containing these repeats are RAN-translated into toxic DPR proteins (Ash *et al*, 2013; Gendron *et al*, 2013; Mori *et al*, 2013; Zu *et al*, 2013), which is a major pathogenic event in cell and animal models of ALS/FTD (Tran *et al*, 2015; Jiang *et al*, 2016; Liu *et al*, 2016; Moens *et al*, 2018). However, the molecular mechanisms by which expanded sense G4C2 and antisense C4G2 repeats are translated remain to be fully characterized.

Using repeats embedded in their natural human sequences, we found that expanded antisense C4G2 repeats are mostly translated into polyPG through initiation to a canonical AUG start codon located upstream of the repeats. In contrast, sense G4C2 repeats are translated mostly into polyGA and to a lesser extent into polyGR, through initiation to a CUG and an AGG near-cognate codon, respectively. In support of these results, translation initiation of polyGA from the exact same CUG near-cognate codon was independently identified in three other studies (Green *et al*, 2017; Sonobe *et al*, 2018; Tabet *et al*, 2018), and deletion of the sequence containing this CUG codon abolishes polyGA expression in neurons differentiated from C9-iPS cells with ~1,000 G4C2 repeats (Almeida *et al*, 2019). Of interest, expanded CGG repeats in the neurodegenerative disease fragile X-associated tremor/ataxia syndrome (FXTAS) are also translated into a toxic protein (FMRpolyGlycine) through initiation to a near-cognate start codon located upstream of the repeats (Todd *et al*, 2013; Kearse *et al*, 2016; Sellier *et al*, 2017). Near-cognate initiation codons are codons differing from the AUG sequence by one base, but that can still initiate translation provided they are embedded in a correct Kozak sequence (Kozak, 1989; Peabody, 1989). Ribosome profiling demonstrated that initiation to near-cognate codons is more common than previously thought and may occur in up to ~30% of mammalian genes (Ingolia *et al*, 2011; Fritsch *et al*, 2012). These data suggest that translation initiation at near-cognate codons located upstream of the repeats could be a common pathogenic mechanism in microsatellite expansion diseases, a hypothesis that may warrant further investigations (Gao *et al*, 2017). However, translation initiation at near-cognate codons should occur independently of the presence of downstream repeats, but DPR proteins are observed only in carriers of G4C2 repeat expansions and not in control individuals. Of interest, we noted that in the absence of an expansion, translation does indeed occur, but the resulting proteins are small and unstable and consequently hardly detectable. In contrast, repeat expansion increases the size and the stability of these DPR proteins, enabling their detection. As a note of caution, our experiments are based on cell transfection with constructs containing a limited number of repeats. Thus, it remains to test whether larger expansions would employ a different mechanism of translation, notably RAN translation initiating directly within the repeats and/or translation frameshifting. In that aspect, G4C2 repeat translation frameshift from the GA frame, initiated at the CUG near-cognate codon, to the GP and GR frames was recently reported in cell transfection assays (Tabet *et al*, 2018). However, deletion of this CUG codon abolishes polyGA translation, but does not affect polyGP and polyGR expression in C9-iPS cells (Almeida *et al*, 2019). Similarly, we found only trace amounts of polyGP expressed from the sense G4C2 repeats, suggesting a very low rate of frameshifting from the GA frame to the GP frame. However, this could be due to the lower sensitivity of our assay (immunoblotting) compared to the more sensitive nanoluciferase and *in vitro* translation assays used in Tabet *et al* In summary, we found that DPR proteins are produced through a classic mechanism of translation initiation at canonical AUG or near-cognate codons located upstream of the sense and antisense repeats, leading to expression of polyPG, polyGA, and, to a lesser degree, polyGR DPR proteins. Importantly, this expression pattern is similar to the one observed in patient tissues where inclusions of polyGA and polyGP/PG proteins are commonly observed, while fewer aggregates of polyGR are detected (Mackenzie *et al*, 2015; Davidson *et al*, 2016; Lee *et al*, 2017; Saberi *et al*, 2017).

A second important point of the present work is the synergic toxicity between C9ORF72 reduced expression and DPR protein expression (model presented in Fig 5). Indeed, we found that DPR proteins expressed under their natural sequences caused only limited toxicity, which is consistent with their limited expression. Mutation of their near-cognate codons into AUG canonical start codons enhanced both DPR protein expression and toxicity, especially for the polyGR DPR protein, as consistently reported in various recent studies (Lopez-Gonzalez *et al*, 2016, 2019; Zhang *et al*, 2018; Choi *et al*, 2019). Conversely, deletions of these DPR initiation codons abolished neuronal cell death, suggesting that expanded repeats cause little toxicity at the RNA level and that RAN translation initiating within the repeats is not sufficiently efficient to drive toxicity, at least with the limited number of repeats used in the

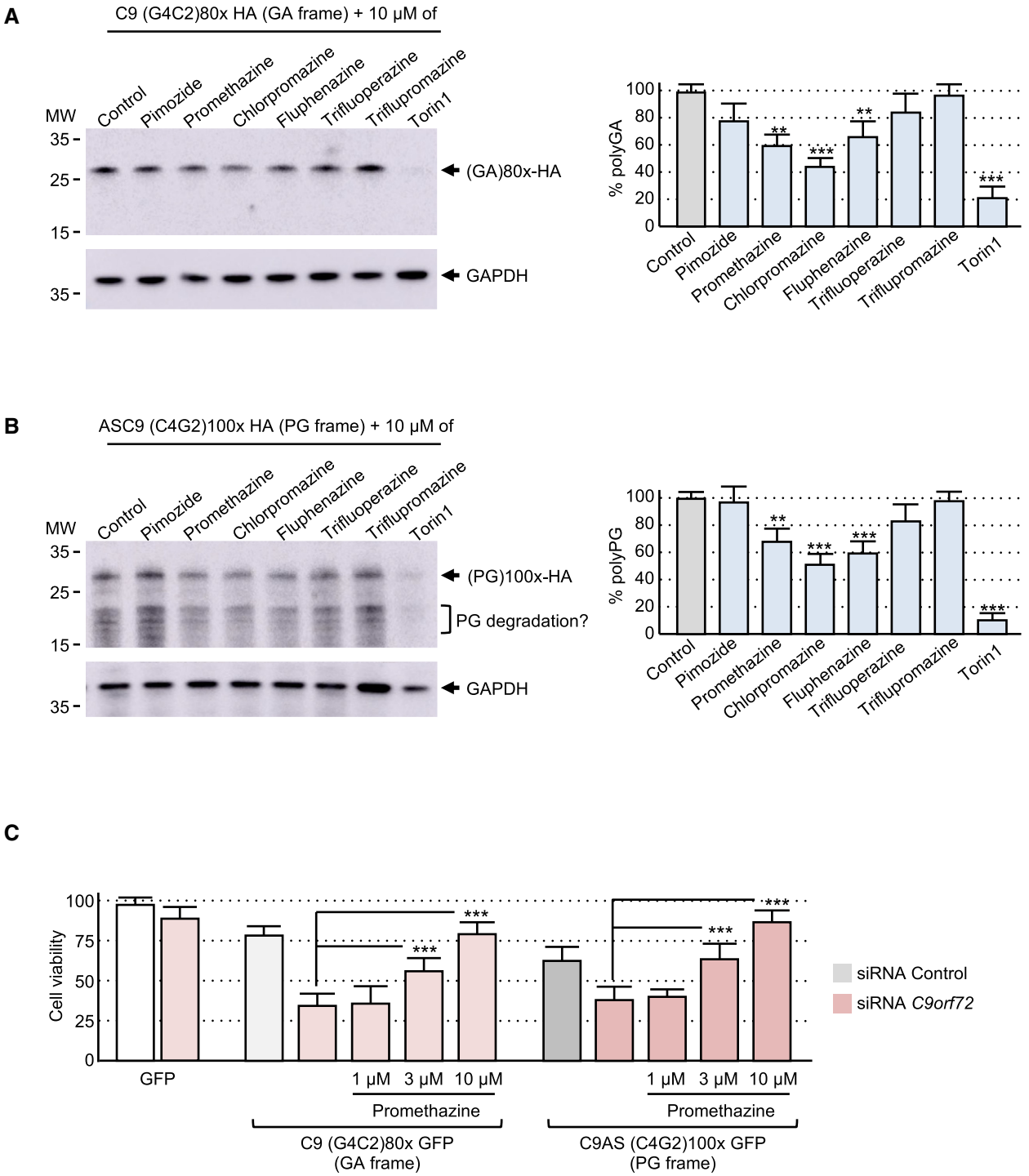

Figure 4. Promethazine reduces DPR protein accumulation and toxicity.

A Left panel, immunoblotting against the HA-tag or the GAPDH of proteins extracted from Neuro2A cells transfected for 24 h with a construct expressing 80 G4C2 repeats embedded in the human sense *C9ORF72* sequence fused to a HA in the GA frame and treated with 10 µM of the indicated compound for 15 h. Right panel, quantification of polyGA expression relative to the GAPDH.

B Left panel, immunoblotting against the HA-tag or the GAPDH of proteins extracted from Neuro2A cells transfected for 24 h with a construct expressing 100 C4G2 repeats embedded in the human antisense *C9ORF72* sequence fused to a HA-tag in the PG frame and treated with 10 µM of the indicated compound for 15 h. Right panel, quantification of polyPG expression relative to the GAPDH.

C Cell viability (TO-PRO-3 FACS staining) of GT1-7 neuronal cells treated with 1, 3, or 10 µM of promethazine and co-transfected for 24 h with either a control siRNA or a siRNA targeting *C9orf72* mRNA and a construct expressing either 80 G4C2 repeats or 100 C4G2 repeats embedded in sense or antisense *C9ORF72* fused to the GFP in the GA or PG frame.

Data information: Error bars indicate s.e.m. Student's *t*-test, $^{**}P < 0.01$, and $^{***}P < 0.001$. $n = 5$ independent transfection.

present study. Of interest, accumulation and thus toxicity of DPR proteins expressed under their weak natural initiation codons are prevented by their constant degradation, notably by autophagy, a pathway regulated by the C9ORF72 protein. As reduced expression of C9ORF72 leads to suboptimal autophagy and as expression of C9ORF72 is decreased in brain tissues of C9-positive ALS/FTD individuals, we tested whether reduced expression of C9ORF72 may modulate DPR protein accumulation and toxicity. Importantly, decreased expression of C9ORF72 increases the accumulation and thus the toxicity of polyGA, polyGR, and polyPG DPR proteins expressed under their native sequences. These data suggest that the sole reduction of C9ORF72 protein levels or the sole expression of DPR proteins under their native weak initiation codons may not be sufficient to drive pathogenicity in isolation. In contrast, expanded repeats in ALS/FTD may be pathogenic through a double-hit mechanism where the suboptimal autophagy due to C9ORF72 reduced expression may enhance the accumulation and toxicity of DPR proteins expressed under their natural sequences. These results are reminiscent of stress conditions that increase expression and toxicity of DPR proteins (Green *et al*, 2017; Cheng *et al*, 2018; Sonobe *et al*, 2018; Westergard *et al*, 2019). Our results are also consistent with the decreased expression of C9ORF72 that synergizes the toxicity of expanded G4C2 repeats in C9-BAC transgenic mice (Shao *et al*, 2019) or that enhances the toxicity of DPR proteins expressed under artificial ATG start codons in neurons differentiated from C9-iPS cells (Shi *et al*, 2018). Interestingly, a model where both loss of C9ORF72 and expression of DPR proteins are required to observe neuronal cell death is consistent with the absence of neurodegeneration in BAC transgenic mice expressing DPR proteins from their natural sequences but with normal levels of C9orf72 (O'Rourke *et al*, 2016; Peters *et al*, 2015), as well with the absence of a neuronal phenotype in mice knockout for C9orf72 but without DPR protein expression (Lagier-Tourenne *et al*, 2013; Koppers *et al*, 2015; O'Rourke *et al*, 2016; Atanasio *et al*, 2016; Burberry *et al*, 2016; Jiang *et al*, 2016; Sudria-Lopez *et al*, 2016). Furthermore, this double-hit mechanism is supported by the association of *C9ORF72* mRNA decreased levels with patient survival (van Blitterswijk *et al*, 2015), and by the absence of clinical symptoms in individuals with a limited (30 to 70 repeats) number of G4C2 repeats, who show inclusions of DPR proteins but normal levels of the C9ORF72 protein (Gami *et al*, 2015; McGoldrick *et al*, 2018).

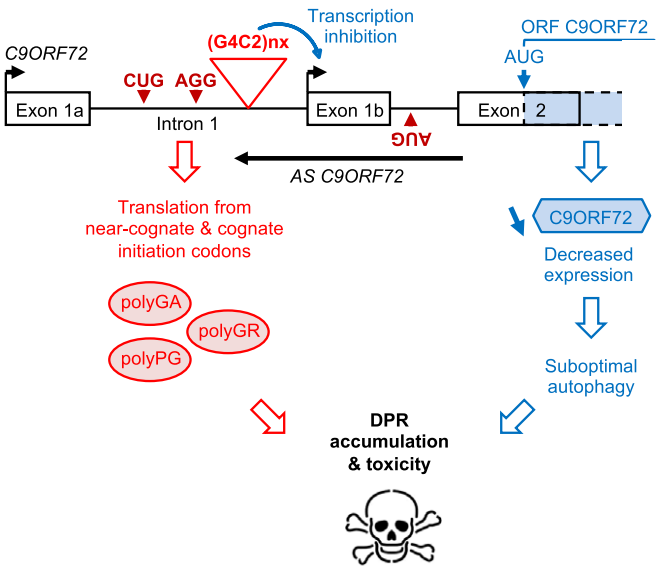

Figure 5. Model of C9ORF72 loss-of-function and DPR gain-of-function toxicity.

Expanded sense G4C2 and antisense C4G2 repeats are translated into polyGA, polyGR, and polyPG DPR proteins through initiation to near-cognate codons or a cognate ATG codon embedded in a poor Kozak sequence. Concomitantly, expanded G4C2 repeats promote epigenetic DNA changes that inhibit promoter 1b activity, ultimately resulting in decreased expression of the C9ORF72 protein. Reduced expression of C9ORF72 leads to suboptimal autophagy that promotes the toxic accumulation of polyGA, polyGR, and polyPG DPR proteins, ultimately resulting in neuronal cell death.

Finally, our study provides an additional proof of concept that modulating autophagy may be of therapeutic interest in ALS/FTD. Indeed, previous studies have shown that stimulating autophagy is beneficial in SOD1, FUS, and TDP-43 cell or animal models of ALS (Hetz *et al*, 2009; Crippa *et al*, 2010; Wang *et al*, 2012; Castillo *et al*, 2013; Barmada *et al*, 2014; Perera *et al*, 2017; Marrone *et al*, 2018, 2019). In agreement with these works, we found that compounds known to induce autophagy, notably promethazine, are able to bypass the reduced expression of C9ORF72 and can promote the clearance of DPR proteins, thus reducing their toxicity. Promethazine is a sedative and antihistamine drug with antipsychotic effects that belongs to a class of phenothiazine derivatives known to activate autophagy in neuronal cell cultures (Tsvetkov *et al*, 2010). Interestingly, these compounds ameliorate neuronal cell dysfunctions and prevent neuronal cell death in SOD1, TDP-43, and FUS cellular and animal models of ALS (Barmada *et al*, 2014; Patten *et al*, 2017; Marrone *et al*, 2018, 2019). Furthermore, pimozide, a phenothiazine derivative related to promethazine, displayed some promising effects in a short randomized clinical trial of sporadic ALS (Patten *et al*, 2017). These results support the need for further investigations of autophagy activators in ALS/FTD.

In conclusion, these results suggest a model where the sole loss of C9ORF72 or the sole expression of DPR proteins under their native sense or antisense sequences is not sufficient to be pathogenic in isolation. In contrast, neuronal cell loss in ALS/FTD may result from a double-hit mechanism where the suboptimal autophagy due to the reduced expression of C9ORF72 may synergize toxicity of other stress, notably the accumulation of toxic DPR proteins translated from their weak natural initiation codons (Fig 5). Of clinical importance, this double-hit mechanism can be prevented by pharmaceutical compounds enhancing autophagy.

Materials and Methods

Constructions

Human sense *C9ORF72* sequence containing 80 G4C2 repeats interrupted by NheI, HindIII, BamHI, and EcoRI restrictions sites was cloned into pcDNA3.1 fused to a HA-tag or the GFP deleted of its ATG in all three frames. Human antisense *C9ORF72* sequence containing 360 nts upstream of 100 C4G2 repeats was cloned into pcDNA3.1 fused to a HA-tag or the GFP deleted of its ATG in all three frames. Mutations of size of the repeats, of initiation codons,

or of Kozak sequences were achieved by oligonucleotide ligations. To insure stability of the expanded repeats, all plasmids were transformed into STBL3 bacterial strain (Invitrogen) and grown at room temperature (22°C).

Immunofluorescence

Coverslip were incubated for 15 min in PBS with 4% paraformaldehyde, washed with PBS, and incubated in PBS plus 0.5% Triton X-100 during 10 min. The cells were washed three times with PBS, and the coverslips were incubated during 1 h with primary antibody against ubiquitin (ab134953, Abcam), EEA1 (ab109110, Abcam), M6PR (ab124767, Abcam), and P62/Sqstm1 (ab56416, Abcam). After washing with PBS, the coverslips were incubated with a goat anti-mouse or anti-rabbit secondary antibody conjugated with Alexa 488 or CY3 (Interchim SA) for 1 h, washed twice with PBS, and incubated for 3 min in PBS/DAPI (1/10,000 dilution). Coverslips were rinsed twice before mounting in Pro-Long media (Molecular Probes) and were examined using a Leica microscope.

Cell cultures, transfections, treatments, and viability assay

Neuronal GT1-7 cells were grown in DMEM 4.5 g/l glucose with 10% fetal calf serum, gentamicin, and penicillin at 37°C in 5% CO_2. HEK293 cells were growth in DMEM 1 g/l glucose with 10% FCS and gentamicin at 37°C in 5% CO_2. For transfection, cells were plated in DMEM and 0.1% fetal bovine serum and transfected for 24 h using Lipofectamine 2000 (Fisher Scientific) and/or RNAiMAX (Fisher Scientific) with either control siRNA or siRNA against C9orf72 (ON-TARGETplus, Dharmacon). After 1–3 days, neurons were analyzed by FACS analysis for viability or by immunofluorescence or Western blotting. Cells were treated with 100 nM of bafilomycin A1, 1 μM MG132, or the indicated concentration of drug (Sigma) during 15 h before analysis. For cell viability, cells were detached by scraping and resuspended in PBS. TO-PRO-3 iodide (Fisher Scientific, T-3605) was added at 20 nM to each sample and gently mixed just prior to analysis, and 30,000 cells were FACS-analyzed.

Western blotting

Proteins were denatured 3 min at 95°C, separated on 4–12% Bis-Tris Gel (NuPAGE), transferred on nitrocellulose membranes (Whatman Protran), blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) buffer, incubated with anti-GFP (ab290, Abcam), HA (ab130275, Abcam), C9ORF72 (3H10, homemade), and GAPDH (ab125247, Abcam) in TBS plus 5% non-fat dry milk, washed three times, and incubated with anti-rabbit or mouse peroxidase antibody (1:10,000, Cell Signaling) 1 h in TBS, followed by washing and ECL chemiluminescence revelation (Amersham ECL Prime).

Mass spectrometry analysis

HEK293 cells were transfected with HA- or GFP-tagged plasmid using Lipofectamine 2000 (Fisher Scientific) for 24 h. Proteins were purified by immunoprecipitation against the HA or GFP, separated on 4–12% Bis-Tris Gel (NuPAGE), and visualized by Coomassie blue staining. Gel bands were excised and subjected to in-gel reduction in 10 mM DTT in 100 mM NH_4HCO_3 (Sigma-Aldrich) for 1 h at 57°C, alkylated for 45 min in the dark with 55 mM iodoacetamide in 100 mM NH_4HCO_3 (Sigma-Aldrich), washed in 25 mM NH_4HCO_3, dehydrated with acetonitrile, and dried in SpeedVac 5301 Concentrator (Eppendorf). Then, the gel pieces were rehydrated with 12.5 ng/μl LysC solution (Promega) in 50 mM NH4HCO3 and incubated overnight at 37°C. The peptides were extracted twice with acetonitrile/water/formic acid—45/45/10, v/v/v followed by a final extraction with acetonitrile/formic acid (FA)—95/05, v/v. Extracted peptides were then analyzed using an Ultimate 3000 nano-RSLC (Thermo Scientific) coupled in line with an Orbitrap ELITE (Thermo Scientific). Peptides were separated on a C18 nanocolumn with a linear gradient of acetonitrile and analyzed within a Top 20 collision-induced dissociation data-dependent mass spectrometry with an inclusion list. Data were processed by database searching using SequestHT (Thermo Fisher Scientific) with Proteome Discoverer 1.4 software (Thermo Fisher Scientific) against a homemade database of all potential three frames translated proteins or peptides from the human *C9ORF72* sense and antisense sequences. Precursor and fragment mass tolerance were set at 7 ppm and 0.5 Da, respectively. Oxidation (M) and N-terminal acetylation were set as variable modification, and carbamidomethylation (C), as fixed modification. Peptides were filtered with the fixed value node of Proteome Discoverer 1.4.

RT–qPCR

RT–qPCR analyses were performed using the Transcriptor Reverse Transcriptase Kit (Roche) and the QuantiTect SYBR Green PCR Kit (Qiagen) in a LightCycler 480 (Roche) with 15 min at 94°C followed by 50 cycles of 15 s at 94°C, 20 s at 58°C, and 20 s at 72°C using aagttcatctgcaccaccg (fwd) and ttctgctggtagtggtcggcg (rev) primers to quantify the GFP expression, with *RPLP0* mRNA as standard (fwd: gaagtcactgtgccagccca, rev: gaaggtgtaatccgtctcca). Data were analyzed using LightCycler 480 analysis software and the $2\Delta C_t$ method.

Statistical analysis

All cell experiments are represented as average ± standard error of mean (s.e.m.) with significance determined using Student's *t*-test.

Data availability

The data that support the findings of this study are available within the article and its supplementary information files or from the corresponding authors on reasonable request.

Expanded View for this article is available online.

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"C9ORF72 ALS" (NCB); ANR-18-CE16-0019 "NewMutALS" (NCB); ANR-10-LABX-0030-INRT (IGBMC) and ANR-10-IDEX-0002-02 (IGBMC).

Author contributions

Experiments were performed by MB, VP, AG, FR, and CS. Data were collected and analyzed by LN, NC-B, and CS. The study was designed, coordinated, and written by CS and NC-B.

Conflict of interest

The authors declare that they have no conflict of interest.

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