Multiple pathways of toxicity induced by *C9orf72* dipeptide repeat aggregates and G$_4$C$_2$ RNA in a cellular model

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Abstract

The most frequent genetic cause of amyotrophic lateral sclerosis and frontotemporal dementia is a G_4C_2 repeat expansion in the *C9orf72* gene. This expansion gives rise to translation of aggregating dipeptide repeat (DPR) proteins, including poly-GA as the most abundant species. However, gain of toxic function effects have been attributed to either the DPRs or the pathological G_4C_2 RNA. Here we analyzed in a cellular model the relative toxicity of DPRs and RNA. Cytoplasmic poly-GA aggregates, generated in the absence of G_4C_2 RNA, interfered with nucleocytoplasmic protein transport, but had little effect on cell viability. In contrast, nuclear poly-GA was more toxic, impairing nucleolar protein quality control and protein biosynthesis. Production of the G_4C_2 RNA strongly reduced viability independent of DPR translation and caused pronounced inhibition of nuclear mRNA export and protein biogenesis. Thus, while the toxic effects of G_4C_2 RNA predominate, DPRs exert additive effects that may contribute to pathology.

Introduction

30

31 Expansion of a GGGGCC hexanucleotide repeat (hereafter G_4C_2) within the first intron of the

32 *C9orf72* gene is the most frequent genetic cause of amyotrophic lateral sclerosis (ALS) and

33 frontotemporal dementia (FTD) (Renton et al., 2011). Mutant *C9orf72* in patients suffering

34 from ALS/FTD can have more than a thousand G_4C_2 repeats, while healthy individuals

35 possess usually less than twenty repeats (Gijselinck et al., 2016; Nordin et al., 2015).

36 Transcripts with expanded G_4C_2 tracts are translated by repeat associated non-AUG (RAN)

37 translation in all reading frames and in both strands, resulting in the synthesis of five different

38 dipeptide repeat proteins (DPRs): poly-GA, poly-GR, poly-GP, poly-PR and poly-PA (Mori,

39 Arzberger, et al., 2013; Mori, Weng, et al., 2013; Zu et al., 2013), all of which have been

40 detected in patient brains (Mori, Arzberger, et al., 2013; Mori, Weng, et al., 2013; Zu et al.,

41 2013). Poly-GA is the most abundant of the DPRs, followed by the other sense strand-

42 encoded forms (poly-GP and poly-GR) (Mori, Weng, et al., 2013; Schludi et al., 2015). In

43 patient brain and cellular models, DPRs accumulate in deposits that can be found in the

44 nucleus and cytoplasm, including neurites (Ash et al., 2013; Gendron et al., 2013; Mori,

45 Arzberger, et al., 2013; Mori, Weng, et al., 2013; Schludi et al., 2015; Zu et al., 2013). Poly-

46 GA aggregates are localized mainly in the cytoplasm (Zhang et al., 2016), whereas arginine-

47 containing DPRs (R-DPRs; polyGR and polyPR) accumulate in the nucleus, specifically the

48 nucleoli (Kwon et al., 2014; May et al., 2014; Moens et al., 2019; Wen et al., 2014; White et

49 al., 2019; Yamakawa et al., 2015; Zhang et al., 2014). However, in patients, poly-GR and

50 poly-PR predominantly form cytoplasmic inclusions, with only a fraction of cells containing

51 para-nucleolar inclusions that co-localize with silent DNA (Schludi et al., 2015). Interestingly,

52 the less frequent intranuclear poly-GA inclusions in both cell models and patient brain are

53 excluded from the nucleoli (Schludi et al., 2015).

54   Both loss- and gain-of-function mechanisms have been suggested to contribute to

55   *C9orf72* pathology (reviewed in (Balendra & Isaacs, 2018)). Despite its location in a non-

56   coding part of the gene, the G_4C_2 expansion can alter the expression level of the C9ORF72

57   protein (Rizzu et al., 2016; Waite et al., 2014). However, *C9orf72* knockout mouse models

58   have failed to fully recapitulate ALS- or FTD-related neurodegenerative phenotypes,

59   suggesting that loss of C9ORF72 protein is not the only contributor to pathology (Burberry et

60   al., 2016; Burberry et al., 2020; Koppers et al., 2015; O'Rourke et al., 2016; Sudria-Lopez et

61   al., 2016; Zhu et al., 2020). Toxic functions induced by this expansion have been studied in

62   various cellular and animal models, and non-exclusive RNA- and protein-based mechanisms

63   of toxicity have been proposed. However, the main contributor to gain of function toxicity in

64   the disease remains to be defined.

65   mRNA containing the pathological G_4C_2 expansion has been shown to form stable G-

66   quadruplexes in the nucleus that retain RNA binding proteins and induce splicing defects

67   (Cooper-Knock et al., 2014; Donnelly et al., 2013; Gitler & Tsuiji, 2016; Haeusler et al.,

68   2014; Sareen et al., 2013; Simon-Sanchez et al., 2012; Xu et al., 2013). R-DPRs have been

69   additionally shown to interact with membrane-free phase separated compartments, such as the

70   nucleolus, causing nucleolar stress and dysfunction of nucleolar quality control, as well as

71   impairment of nucleocytoplasmic trafficking (Frottin et al., 2019; Hayes et al., 2020; Kwon et

72   al., 2014; Lee et al., 2016; Mizielinska et al., 2017; Shi et al., 2017; Tao et al., 2015; Vanneste

73   et al., 2019; White et al., 2019). Additionally, cytoplasmic poly-GA aggregates associate

74   extensively with proteasomes, interfere with proteasome activity (Guo et al., 2018), and have

75   been found to colocalize with p62, ubiquitin and several components of the proteasome

76   machinery in patient brain (Al-Sarraj et al., 2011; Guo et al., 2018; May et al., 2014; Mori,

77   Weng, et al., 2013). In mice expressing poly-GA, aggregates have been observed to sequester

78   nuclear pore components (Zhang et al., 2016), thereby interfering with nucleocytoplasmic

79   protein transport.

80   Differences between model systems as well as discrepancies in DNA constructs and

81   repeat lengths utilized in various studies have precluded a clear mechanistic understanding of

82   *C9orf72* toxicity pathways. Here we used G_4C_2 repeat-containing constructs as well as

83   synthetic constructs encoding DPR proteins of comparable lengths without generating G_4C_2

84   RNA to analyze the relative contributions of RNA and DPR species to cellular pathology.

85   Focusing on nuclear and cytoplasmic toxicity pathways, we found that cytoplasmic, but not

86   nuclear poly-GA aggregates impaired nucleocytoplasmic transport. However, nuclear poly-

87   GA was more toxic and interfered with nucleolar protein quality control and protein synthesis.

88   The G_4C_2-containing RNA species induced a strong accumulation of poly-adenylated mRNA

89   within the nucleus and dramatically inhibited protein biosynthesis. Thus, poly-GA protein and

90   G_4C_2 RNA interfere with multiple cellular key pathways, with the RNA component exerting

91   the major toxic effects limiting cell viability.

92

93   **Results**

94   **Nuclear and cytoplasmic poly-GA aggregates differ in toxicity**

95   Both G_4C_2 RNA and DPR proteins resulting from the pathological *C9orf72* expansion have

96   been suggested to mediate gain of toxic function effects in various models (reviewed in

97   (Balendra & Isaacs, 2018). To investigate the contribution of poly-GA proteins to cellular

98   pathology, we engineered ATG-driven synthetic constructs expressing 65 GA repeats fused

99   C-terminally to GFP (GA_{65}-GFP). Notably, these constructs do not contain any G_4C_2

100   hexanucleotide motifs (Figure 1A and Figure 1-figure supplement 1A).
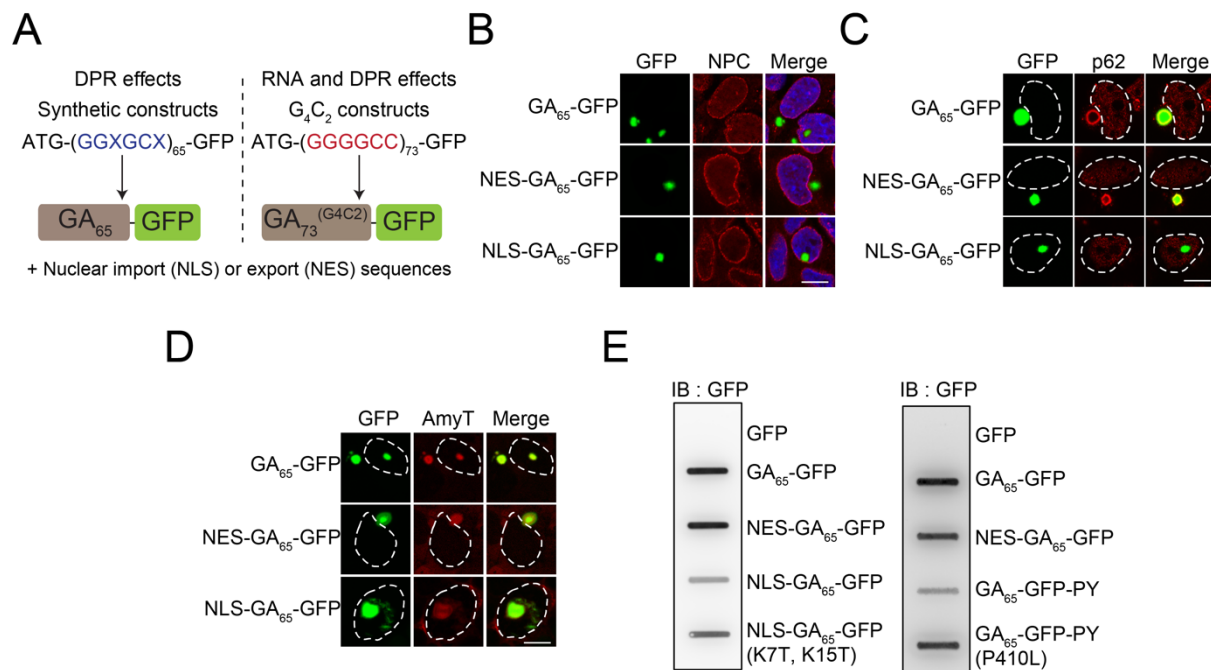
Figure 1 - Cytoplasmic and nuclear poly-GA aggregates are amyloid-like but differ in solubility. (**A**) Schematic representation of the poly-GA encoding constructs used. Two different classes of poly-GA encoding constructs were used to study dipeptide repeat protein (DPR) mediated toxicity in presence or absence of RNA repeat regions: synthetic constructs that do not contain G_4C_2 motifs and are translated into GA_{65} (left), and constructs containing 73 G_4C_2 repeats that encode GA_{73} (right). Both classes contained ATG start codons and were fused in frame to GFP. N-terminal nuclear import (NLS) or export sequences (NES) were present when indicated. (**B**) The indicated constructs were transfected into HEK293T cells. Antibodies against nuclear pore complexes (NPC, red) were used to detect the nuclear membrane, poly-GA was visualized by GFP fluorescence (green), nuclei were counterstained with DAPI. (**C**) NES-GA_{65}-GFP aggregates co-localize with p62. The indicated constructs were transfected into HEK293T cells. p62 (red) was detected by immunofluorescence and poly-GA was visualized by GFP fluorescence (green). White dashed lines delineate the nucleus based on DAPI staining. (**D**) Cytoplasmic and nuclear aggregates can be stained with an amyloid specific dye. The indicated constructs were transfected into HEK293T cells, and stained with AmyTracker (AmyT, red). Poly-GA DPRs were visualized by GFP fluorescence (green). White dashed lines delineate the nucleus based on DAPI staining. (**E**) Cytoplasmic GA aggregates are SDS insoluble. The indicated constructs were transfected into HEK293T cells. GFP was expressed as a soluble control protein. Cells were lysed and analyzed for SDS insoluble poly-GA aggregates by filter retardation assay. GFP antibody was used for detection. Scale bars represent 10 µm.

Expression of GA_{65}-GFP in HEK293T cells resulted in the formation of bright fluorescent inclusions in most transfected cells. The inclusions were mostly cytoplasmic, except for a small fraction of cells with nuclear foci (Figure 1B). This phenotype is in agreement with reports on the localization of poly-GA aggregates in patient brain, where poly-GA aggregates are also observed in the neuronal cytoplasm and nucleus (Mori, Weng, et al., 2013; Schludi et al., 2015). Using engineered β-sheet proteins, we have previously

127   reported that otherwise identical aggregation-prone proteins display distinct toxic properties

128   when targeted to different cellular compartments (Frottin et al., 2019; Vincenz-Donnelly et

129   al., 2018; Woerner et al., 2016). To test whether this is also the case for poly-GA, we

130   generated compartment-specific variants of the poly-GA proteins. We restricted the

131   expression of poly-GA to the cytoplasm by adding a nuclear export signal (NES-GA$_{65}$-GFP)

132   or targeted the protein to the nucleus using a double SV40 nuclear localization signal (NLS-

133   GA$_{65}$-GFP). NES-GA$_{65}$-GFP accumulated in the cytoplasm and formed inclusions similar to

134   those of GA$_{65}$-GFP (Figure 1B). Directing the protein to the nucleus resulted in an increased

135   number of cells with nuclear aggregates (Figure 1B). However, a number of NLS-GA$_{65}$-GFP

136   expressing cells also contained cytoplasmic inclusions, suggesting that aggregate formation in

137   these cells occurred before the transport of the poly-GA proteins into the nucleus.

138   Poly-GA forms p62 positive inclusions in the cytoplasm of neurons (Guo et al., 2018).

139   We were able to replicate this phenotype in our cellular system and observed p62-positive

140   poly-GA inclusions of GA$_{65}$-GFP and NES-GA$_{65}$-GFP (Figure 1C) (May et al., 2014; Mori,

141   Weng, et al., 2013). Both nuclear and cytoplasmic poly-GA inclusions were stained

142   throughout with AmyT, a small amyloid-specific dye (Figure 1D). The aggregates were also

143   recognized by the anti-amyloid antibody (OC) (Figure 1-figure supplement 1B), which

144   recognizes generic epitopes common to amyloid fibrils and fibrillar oligomers (Kayed et al.,

145   2007). However, while the nuclear aggregates stained homogenously with OC, cytoplasmic

146   GA$_{65}$-GFP inclusions showed only a peripheral reaction (Figure 1-figure supplement 1B). The

147   differential antibody accessibility of the inclusion core suggests that the nuclear and

148   cytoplasmic DPR aggregates, though both amyloidogenic, differ in structural properties such

149   as packing density.

150   Analysis of the solubility of the nuclear and cytoplasmic GA$_{65}$-GFP aggregates

151   supported this interpretation. NES-GA$_{65}$-GFP was retained in a filter retardation assay in the

152   presence of SDS, while most NLS-GA$_{65}$-GFP passed through the filter (Figure 1E). The

153   difference in detergent solubility was not due to the presence of the NLS sequence, since

154   similar results were obtained using the FUS-derived C-terminal nuclear localization signal

155   (PY) (Gal et al., 2011) (Figure 1E). Moreover, disabling of the nuclear targeting sequences by

156   point mutations resulted in the reappearance of SDS insoluble aggregates (Figure 1E). Thus,

157   despite being amyloid-like, nuclear and cytoplasmic poly-GA aggregates have different

158   physico-chemical properties.

159   The granular component (GC) of the nucleolus has recently been shown to function as

160   a protein quality control compartment (Frottin et al., 2019). Interestingly, the nuclear

161   aggregates of NLS-GA$_{65}$-GFP altered the localization of the GC marker protein

162   nucleophosmin (NPM1), while cytosolic NES-GA$_{65}$-GFP had no such effect (Figure 2A).

163   Nuclear GA$_{65}$-GFP aggregates induced the dislocation of NPM1 from the GC phase of the

164   nucleolus, with accumulation of NPM1 at the aggregate periphery (Figure 2A). However, the

165   nuclear GA$_{65}$-GFP deposits did not alter the distribution of the RNA polymerase I subunit

166   RPA40, a marker of the fibrillar center of the nucleolus, the site of rRNA synthesis

167   (Figure 2B). The NPM1-containing GC phase is responsible for pre-ribosome particle

168   assembly and has been shown to accommodate proteins that have misfolded upon stress.

169   These proteins enter the GC phase and are maintained in a state competent for refolding and

170   repartitioning to the nucleoplasm upon recovery from stress (Frottin et al., 2019). In contrast

171   to poly-GA, R-DPRs enter the GC phase of the nucleolus and convert it from liquid-like to a

172   more hardened state, thereby impairing its quality control function (Frottin et al., 2019; Lee et

173   al., 2016). To test whether nuclear poly-GA aggregates also affect nucleolar quality control,

174   we expressed the synthetic poly-GA constructs in HEK293T cells together with nuclear

175   firefly luciferase fused to the red fluorescent protein mScarlet (NLS-LS), a metastable protein

176   that enters the GC phase upon stress-induced misfolding (Frottin et al., 2019). In control cells,

177   NLS-LS accumulated in the nucleolus upon heat stress and largely repartitioned to the

178   nucleoplasm within 2 h of recovery (Figure 2C, Figure 2-figure supplement 1A). A similar

179   result was obtained upon expression of cytoplasmic NES-GA$_{65}$-GFP. However, in the

180   presence of aggregates of NLS-GA$_{65}$-GFP, NLS-LS failed to efficiently repartition to the

181   nucleoplasm (Figure 2C, Figure 2-figure supplement 1A), indicating that the nuclear poly-GA

182   aggregates compromise nucleolar protein quality control.

183   We next measured the viability of HEK293T cells expressing poly-GA in cytosol or

184   nucleus using the MTT assay. While the expression of NES-GA$_{65}$-GFP did not cause toxicity,

185   cell viability was significantly reduced upon expression of NLS-GA$_{65}$-GFP (Figure 2D). This

186   effect was reproduced in cells transfected with a plasmid coding for GA$_{65}$ from an alternative

187   degenerated and G$_4$C$_2$-free DNA sequence (GA$_{65}$(2)) (Figure 1-figure supplement 1A, Figure

188   2-figure supplement 1B), further excluding G$_4$C$_2$ mRNA as a cause of toxicity. Decreased

189   viability was also observed when GA$_{65}$-GFP was targeted to the nucleus via the alternative

190   PY localization signal (Figure 2-figure supplement 1C). Importantly, both NES-GA$_{65}$-GFP

191   and NLS-GA$_{65}$-GFP were expressed at levels comparable to GA$_{65}$-GFP (Figure 2-figure

192   supplement 1D). Point mutations in the NLS or PY targeting sequence prevented

193   accumulation of GA$_{65}$-GFP in the nucleus and restored viability (Figure 2-figure supplement

194   1C). Together these results indicate that the difference in toxicity between nuclear and

195   cytoplasmic poly-GA is caused by compartment-specific properties of the aggregates

196   independent of their targeting sequences and mRNA.
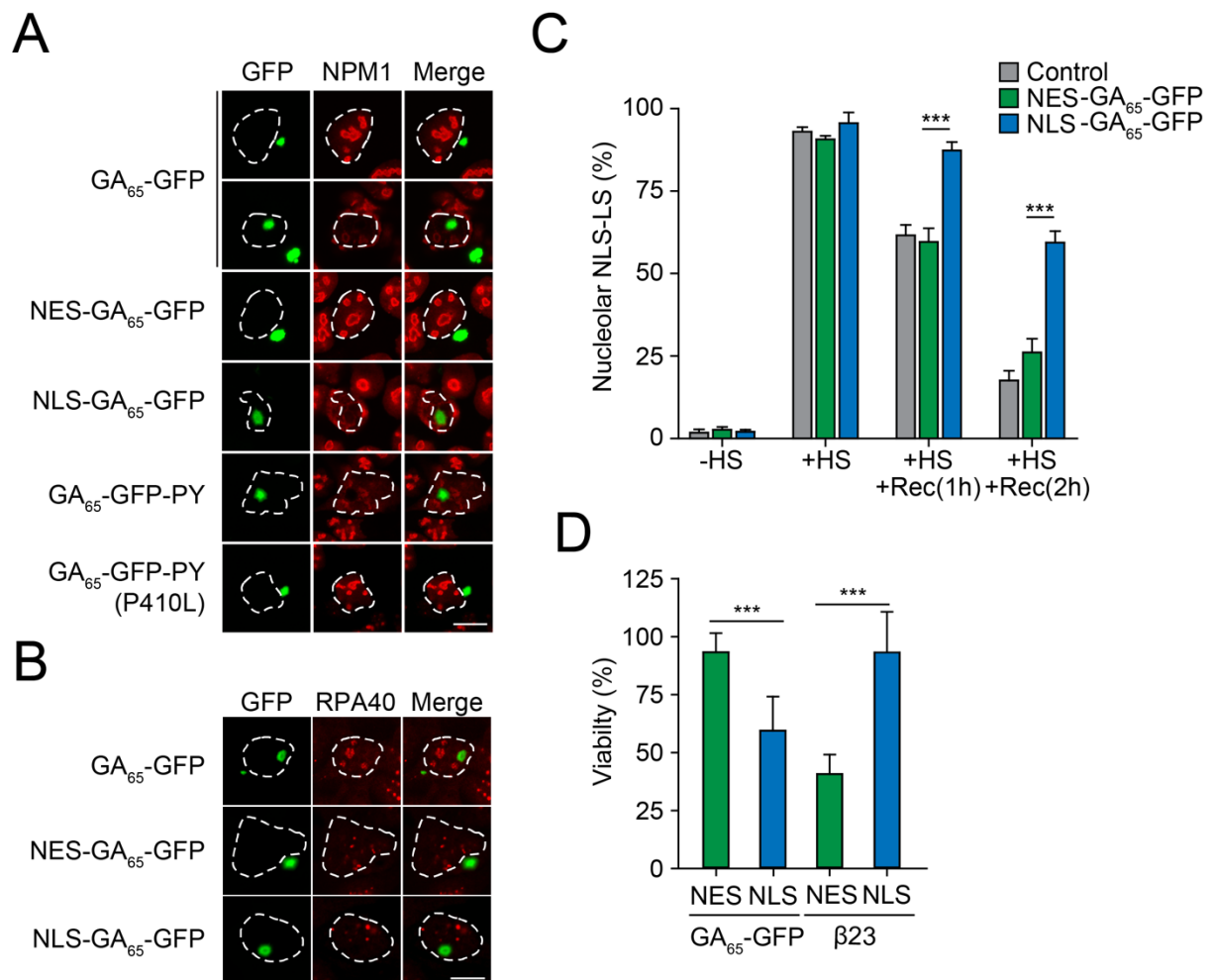
Figure 2 - **Nuclear poly-GA aggregates compromise nucleolar integrity and impair cell viability.** (**A**) Nuclear poly-GA aggregates alter nucleophosmin (NPM1) localization. The indicated constructs were transfected into HEK293T cells. Cells were fixed and stained with anti- NPM1 antibodies (red). Poly-GA was visualized by GFP fluorescence (green). (**B**) Nuclear poly-GA aggregates are not nucleolar. The indicated constructs were transfected into HEK293T cells, followed by staining with antibodies against DNA-directed RNA polymerases I and III subunit RPAC1 (RPA40) (red). Poly-GA was visualized by GFP fluorescence (green). (**C**) Nuclear poly-GA aggregates disrupt nucleolar protein quality control. HEK293T cells were co-transfected with NLS-firefly luciferase fused to mScarlet (NLS-LS) and the indicated poly-GA constructs or GFP as a control. Cells were maintained at 37°C (–HS) or subjected to heat stress 43°C (+HS) for 2 h of heat stress and recovery (+HS +Rec) for 1 h and 2 h. Cells with nucleolar NLS-LS were counted and the results plotted as percentage of transfected cells. Data are shown as mean + SD (n = 3). p-Value of two-sided t-test is displayed (*** $p \leq 0.001$). Representative immunofluorescence images are shown in Figure 2–figure supplement 1A. (**D**) Nuclear poly-GA is toxic. HEK293T cells were transfected with the indicated constructs and MTT cell viability assays were performed 4 days after transfection. Data were normalized to cells transfected with empty vector. Data are shown as means + SD (n ≥ 3); p-Values of two-sided t-test are shown (*** $p \leq 0.001$). White dashed lines delineate the nucleus based on DAPI staining. Scale bars represent 10 μm.

Cytoplasmic GA$_{65}$-GFP aggregates interfere with nuclear transport

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217   We have previously shown that artificial β-sheet proteins, when aggregating in the cytoplasm,

218   sequester nuclear transport factors and thereby interfere with transport of proteins and mRNA

219   across the nuclear envelope (Woerner et al., 2016). Similar observations were made for the

220   aggregates of various disease proteins including poly-GA and R-DPRs (Boeynaems et al.,

221   2016; Chou et al., 2018; Eftekharzadeh et al., 2018; Freibaum et al., 2015; Gasset-Rosa et al.,

222   2017; Grima et al., 2017; Jovicic et al., 2015; Khosravi et al., 2017; Kramer et al., 2018;

223   Solomon et al., 2018; Zhang et al., 2015; Zhang et al., 2016). To test which role the

224   localization of the poly-GA proteins plays in this process, we expressed NES-GA$_{65}$-GFP or

225   NLS-GA$_{65}$-GFP together with the reporter protein shuttle-mApple (S-mApple). This reporter

226   protein contains both nuclear import and export signals and consequently shuttles between

227   nucleus and cytoplasm. At steady state, S-mApple localized mainly to the cytoplasm, but

228   accumulated within minutes in the nucleus upon inhibition of nuclear export with leptomycin

229   B (LMB) (Wolff et al., 1997) (Figure 3).

230   As previously described, S-mApple was retained within the nucleus upon expression

231   of the cytoplasmic β-sheet protein NES-β17 (Figure 3), indicative of inhibition of nuclear

232   protein export (Woerner et al., 2016). Expression of NES-GA$_{65}$-GFP also impaired S-mApple

233   export from the nucleus (-LMB), but to a lesser extent than NES-β17 (Figure 3). A mild

234   impairment of nuclear protein import by NES-GA$_{65}$-GFP was also observed, as measured

235   upon inhibition of export with LMB (Figure 3). As for NLS-β17, nuclear poly-GA aggregates

236   had only a weak effect on protein export (Figure 3). Similarly, cells containing nuclear

237   aggregates did not show a significant change in S-mApple import into the nucleus (Figure 3).

238   These findings were replicated in cells displaying cytoplasmic or nuclear poly-GA aggregates

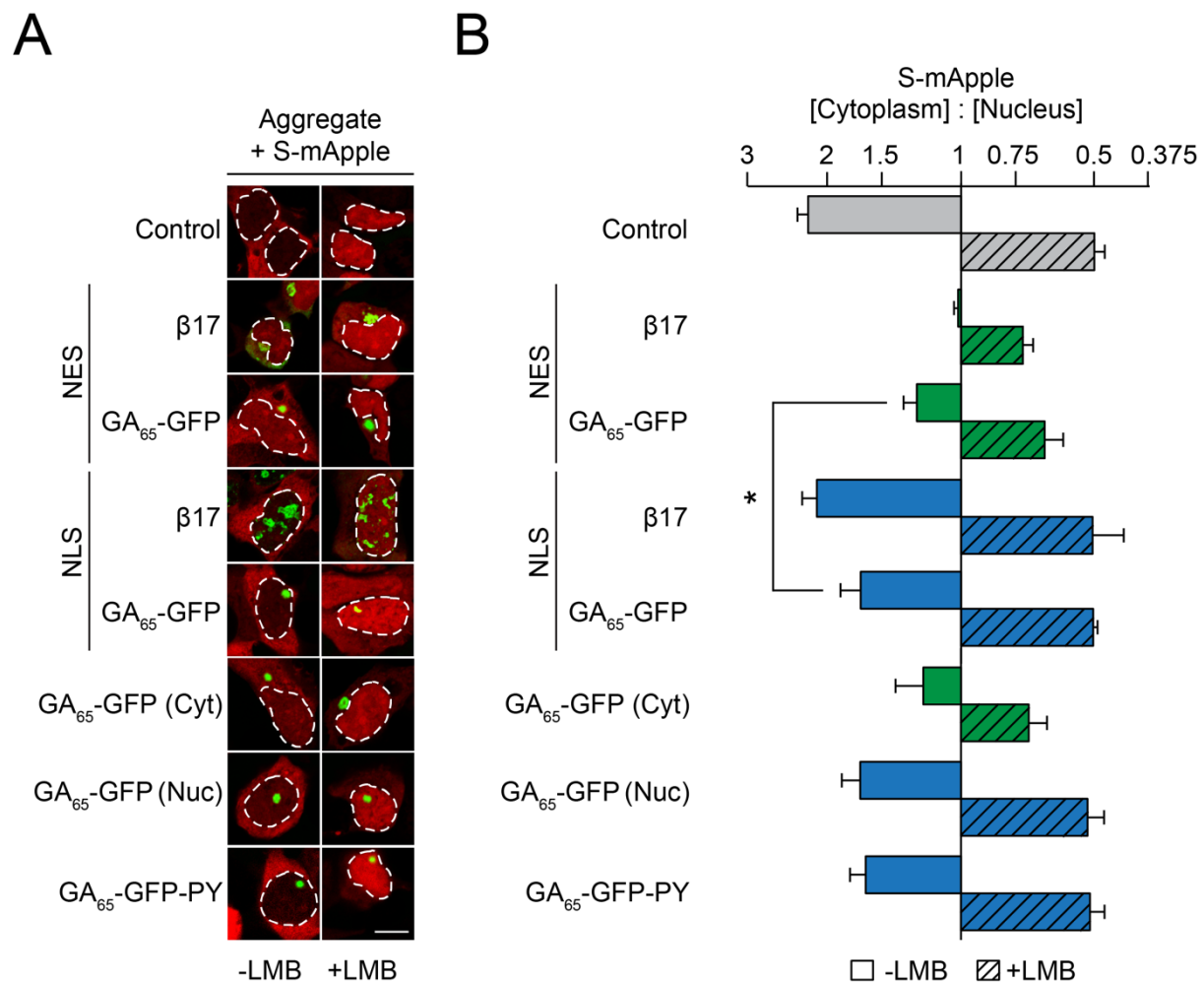239   of untargeted GA$_{65}$-GFP (Figure 3).

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Figure 3 - Cytoplasmic poly-GA aggregates impair nucleocytoplasmic protein transport. (**A**) Cytoplasmic poly-GA aggregates alter nuclear transport of a shuttling reporter protein. HEK293T cells were co-transfected with S-mApple (red) and either empty vector (Control), NES-β17, NES-GA$_{65}$-GFP, NLS-β17, NLS-GA$_{65}$-GFP, GA$_{65}$-GFP or GA$_{65}$-GFP-PY (green). Leptomycin B (LMB; 10 ng/ml) was added for 15 min when indicated. White dashed lines delineate nuclei based on DAPI staining. Scale bar represents 10 μm. (**B**) Quantification of S-mApple distribution from data in (A). The x-axis shows the enrichment of S-mApple concentration in the cytoplasm relative to the nucleus. Cells transfected with GA$_{65}$-GFP were further analyzed and divided into cells with cytoplasmic (Cyt) or nuclear (Nuc) aggregates. Data are means + SD, n = 3 independent experiments, >70 cells were analzyed per condition. * $p \leq 0.05$ from two-sided t-test.

We next monitored the nuclear translocation of p65, a subunit of the NF-κB complex, upon stimulation by the cytokine TNFα. In control cells, p65 is largely cytoplasmic and enters the nucleus upon treatment with TNFα (Figure 4A and B). Cells containing NES-GA$_{65}$-GFP aggregates displayed a potent inhibition of p65 translocation (Figure 4A and B). A similar effect was seen with cytoplasmic aggregates of polyQ-expanded Huntingtin-exon 1 (Htt96Q) as a positive control (Figure 4A and B) (Woerner et al., 2016). Cells containing cytoplasmic

12

258   aggregates of untargeted GA$_{65}$-GFP also displayed reduced p65 translocation (Figure 4A and

259   B). The observed translocation impairment was independent of an alteration of p65

260   phosphorylation and degradation of the inhibitor of nuclear factor κB (IκB) (Figure 4C). In

261   contrast, nuclear poly-GA aggregates showed only a limited effect on p65 translocation
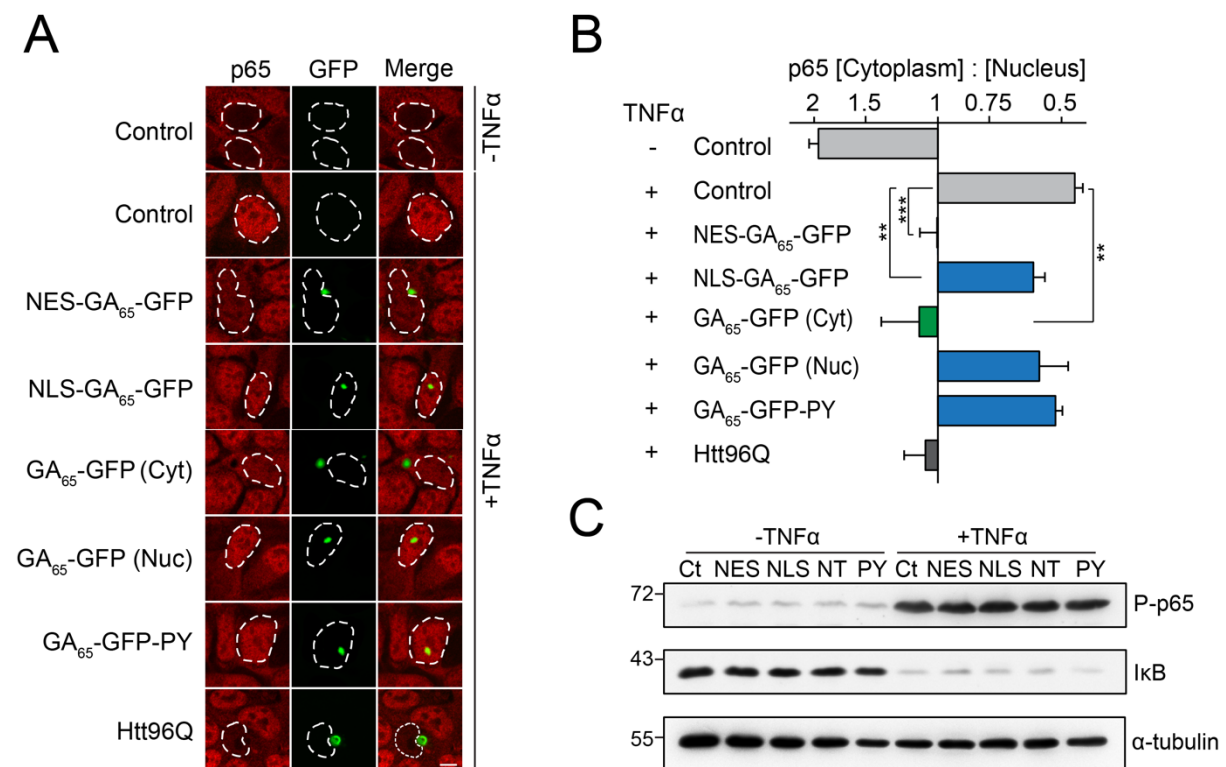
262   (Figure 4A and B).

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265

266   **Figure 4** - **Cytoplasmic poly-GA aggregates inhibit nuclear import of p65.** (**A**) Cytoplasmic poly-GA
267   aggregates inhibit p65 nuclear translocation. HEK293T cells were transfected with empty vector (Control), NES-
268   GA$_{65}$-GFP, NLS-GA$_{65}$-GFP, GA$_{65}$-GFP, GA65-GFP-PY or Htt96Q-GFP (Htt96Q) (green) and analyzed for NF-
269   κB p65 localization (red) with and without TNFα treatment (30 min). White dashed lines delineate nuclei based
270   on DAPI staining. Scale bar represents 10 μm. (**B**) Quantification of NF-κB p65 distribution from data in (A). The
271   x-axis shows the enrichment of p65 in the cytoplasm relative to the nucleus. Data are means + SD (n = 3), >100
272   cells were analyzed per condition. ** $p \leq 0.01$, *** $p \leq 0.001$ from two-sided t-test. (**C**) Expression of poly-GA
273   does not alter the degradation of IκB and phosphorylation of p65. HEK293T cells were transfected with the
274   indicated constructs (Ct, Control; NES, NES-GA$_{65}$-GFP; NLS, NLS-GA$_{65}$-GFP; NT, GA$_{65}$-GFP; PY, GA$_{65}$-GFP-
275   PY) and treated as described in (A). Levels of IκB and phosphorylated NF-κB p65 (P-p65) were analyzed by
276   immunoblotting. α-tubulin served as loading control.

277 Cytoplasmic aggregates of β-sheet protein and other disease-linked proteins cause

278 mislocalization and sequester nuclear pore complexes and importins (Woerner et al., 2016).

279 Particularly, R-DPRs have also been shown to directly bind and interfere with cargo loading

280 onto importin at the nuclear pore (Hayes et al., 2020). While cytoplasmic poly-GA aggregates

281 had no apparent effect on the localization of the nuclear pore complex (Figure 1B), we found

282 that cells with cytoplasmic GA_{65}-GFP aggregates also frequently contained aggregate foci of

283 importins α1 (KPNA2), α3 (KPNA4) and β1 (KPNB1), not co-localizing with the poly-GA

284 inclusions (Figure 4-figure supplement 1). Additionally, importins accumulated at the

285 periphery of poly-GA inclusions (Figure 4-figure supplement 1). Nuclear poly-GA aggregates

286 had no effect on the distribution of these importins (Figure 4-figure supplement 1). Thus,

287 similar to the artificial β-sheet proteins, poly-GA aggregates induce compartment-specific

288 cellular defects and impair nucleocytoplasmic protein transport.

289

290 **G_4C_2 repeat mRNA causes nuclear mRNA retention and pronounced toxicity**

291 An aberrant distribution of mRNA has been observed in mouse motor neuron-like cells

292 expressing expanded G_4C_2 repeats and in *C9orf72* patient cortical neurons (Freibaum et al.,

293 2015; Rossi et al., 2015). We used an oligo-dT probe to test whether cytoplasmic poly-GA

294 aggregates, generated from constructs lacking G_4C_2, affect the cellular distribution of total

295 mRNA. In control cells, mRNA was present throughout the cytoplasm and in small nuclear

296 ribonucleic particles (Figure 5A) (Carter et al., 1991). Expression of NES-GA_{65}-GFP had only

297 a minor effect on cellular mRNA distribution (Figure 5A, B), in contrast to the expression of

298 cytoplasmic β-protein (NES-β17), which resulted in pronounced nuclear mRNA retention

299 (Woerner et al., 2016). Likewise, NLS-GA_{65}-GFP only caused limited mRNA retention in the

300 nucleus (Figure 5A, B). Thus, poly-GA aggregates interfere with the function of only a subset
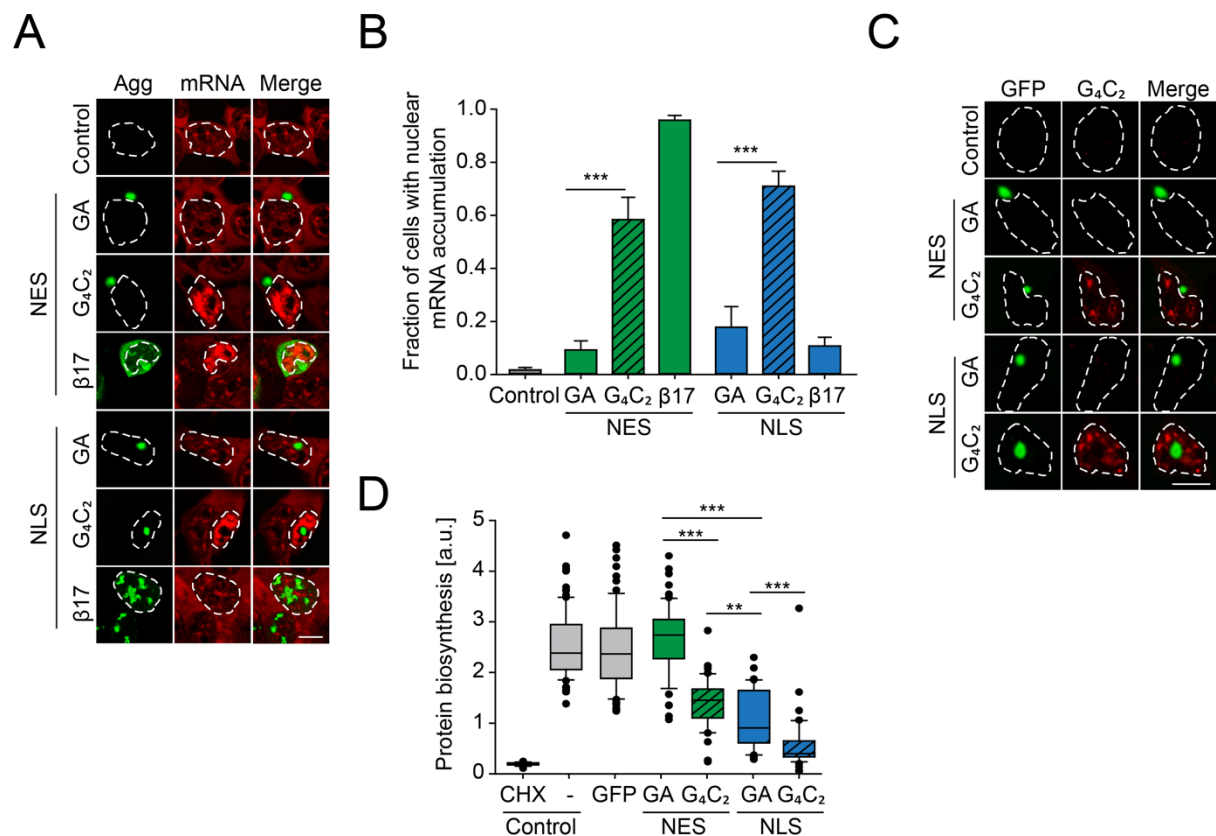
301 of nuclear transport factors.

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Figure 5 - **Protein biosynthesis defects correlate with retention of mRNA in the nucleus induced by G_4C_2 mRNA as well as presence of nuclear poly-GA aggregates.** (**A**) G_4C_2 containing constructs induce strong nuclear mRNA accumulation. HEK293T cells were transfected with the indicated constructs: empty vector (Control); NES-GA_{65}-GFP (NES-GA); NES-G_4C_2-GFP (NES-G_4C_2); NES-β17; NLS-GA_{65}-GFP (NLS-GA); NLS-G_4C_2-GFP (NLS-G_4C_2); NLS-β17. PolyA-RNA was detected by fluorescence *in situ* hybridization using a poly-dT probe (red); protein aggregates (Agg) green. (**B**) Quantification of data in (A). The graph shows the fraction of cells with nuclear mRNA accumulation. Data are means + SD (n=3), *** $p \leq 0.001$ from two-sided t-test. (**C**) G_4C_2 containing constructs induce the formation of G_4C_2 RNA foci. HEK293T cells were transfected with the indicated constructs:empty vector (Control); NES-GA_{65}-GFP (NES-GA); NES-G_4C_2-GFP (NES-G_4C_2); NLS-GA_{65}-GFP (NLS-GA); NLS-G_4C_2-GFP (NLS-G_4C_2). Cells were analyzed for GFP fluorescence (green) and C_4G_2 fluorescence by *in situ* hybridization (red). (**D**) Decreased protein biosynthesis caused by nuclear poly-GA and G_4C_2 mRNA. Protein biosynthesis was assayed in HEK293T cells transfected with the indicated constructs. Control cells were transfected with empty vector and treated with the translation inhibitor cycloheximide (CHX) when indicated. Boxplot of a representative experiment is shown. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to the 10th and 90th percentiles, outliers are plotted as circles. Welch's t-test was used to assess statistical significance (** $p \leq 0.01$; *** $p \leq 0.001$). The white dashed lines delineate the nucleus based on DAPI staining and the scale bars represent 10 μm.

Since the expression of poly-GA protein alone did not recapitulate the alterations of mRNA localization observed in *C9orf72* patient brain (Freibaum et al., 2015; Rossi et al., 2015), we next analyzed the effect of an ATG-driven poly-GA construct of 73 GA repeats encoded entirely by G_4C_2 motifs $(G_4C_2)_{73}$ (Figure 1A and Figure 1-figure supplement 1A).

15

326   Comparable to the synthetic GA_{65}-GFP sequences, these constructs also generated poly-GA

327   protein aggregates in the cytoplasm or nucleus, respectively (Figure 5A, C). However, both

328   NES-$(G_4C_2)_{73}$-GFP and NLS-$(G_4C_2)_{73}$-GFP additionally generated G_4C_2 positive RNA foci in

329   the nucleus, as observed by fluorescent in situ hybridization (FISH) using a C_4G_2 probe

330   (Figure 5C). Cells expressing GA_{65}-GFP or $(G_4C_2)_{73}$-GFP without targeting sequence were

331   analyzed as well, but only the G_4C_2 constructs showed mRNA accumulation in the nucleus in

332   the majority of cells (Figure 5-figure supplement 1A). Furthermore, simultaneous

333   visualization of G_4C_2 RNA puncta with the C_4G_2 probe, total mRNA using an oligo-dT probe

334   and GA-GFP revealed that the presence of G_4C_2 RNA foci is associated with nuclear mRNA

335   accumulation, independent of the presence of visible poly-GA protein aggregates (Figure 5-

336   figure supplement 1B). Together these results indicate that G_4C_2 RNA, not poly-GA protein,

337   mediates retention of mRNA in the nucleus.

338   The nuclear mRNA retention in cells expressing G_4C_2 constructs was accompanied by

339   a marked reduction of protein synthesis as measured by the incorporation of a puromycin

340   derivative into newly translated proteins (Slomnicki et al., 2016) (Figure 5D, Figure 5-figure

341   supplement 1C). Interestingly, NLS-GA_{65}-GFP expressing cells also displayed reduced

342   protein synthesis, independently of G_4C_2 RNA. In contrast, NES-GA_{65}-GFP had no inhibitory

343   effect on protein synthesis (Figure 5D, Figure 5-figure supplement 1C). Notably, cells

344   expressing NLS-$(G_4C_2)_{73}$-GFP, accumulating both nuclear poly-GA protein and G_4C_2 RNA,

345   were almost completely translation inactive, similar to control cells treated with the

346   translation inhibitor cycloheximide (Figure 5D and Figure 5-figure supplement 1C). Thus,

347   both nuclear poly-GA protein and G_4C_2 RNA appear to have additive inhibitory effects on

348   protein biosynthesis. We next measured the viability of cells transiently transfected with

349   either synthetic or G_4C_2-containing constructs. Independent of the presence of a NES or NLS

350   targeting sequence, all G_4C_2 constructs markedly decreased cellular viability (Figure 6A),

351 indicating that toxicity was mediated by the expanded G_4C_2 RNA. Moreover, expression of

352 NLS-$(G_4C_2)_{73}$-GFP was more toxic than NLS-GA$_{65}$-GFP (Figure 6A), although the poly-GA

353 levels of the constructs were comparable (Figure 6-figure supplement 1A).

354   Long sequences of repeated G_4C_2 motifs can produce a series of different DPRs by

355 RAN translation (Ash et al., 2013; Gendron et al., 2013; Mori, Arzberger, et al., 2013; Mori,

356 Weng, et al., 2013; Zu et al., 2013). Since the R-DPRs (poly-PR and poly-GR) have been

357 reported to be toxic in cellular models (Boeynaems et al., 2016; Freibaum et al., 2015; Lee et

358 al., 2016; Shi et al., 2017; Tao et al., 2015; Zu et al., 2013), we investigated whether the G_4C_2

359 constructs produced R-DPRs at levels sufficient to explain the observed toxicity

360 independently of G_4C_2 RNA effects. To this end we engineered synthetic sequences resulting

361 in the ATG-driven synthesis of 73 GR or 73 PR repeats (GR$_{73}$-GFP and PR$_{73}$-GFP,

362 respectively) without G_4C_2 repeats. GR$_{73}$-GFP was located predominantly within the

363 cytoplasm and the nucleolus of HEK293T cells, while PR$_{73}$-GFP accumulated within the

364 nucleus and nucleolus (Figure 6-figure supplement 1B), as reported previously (Frottin et al.,

365 2019; Lee et al., 2016; May et al., 2014; White et al., 2019). While the expression of $(G_4C_2)_{73}$-

366 GFP dramatically reduced cellular viability, the R-DPRs were not measurably cytotoxic

367 (Figure 6A), consistent with an earlier report for constructs with longer repeat lengths (May et

368 al., 2014). We used specific anti-GR and anti-PR antibodies to determine the relative

369 accumulation of the R-DPRs in $(G_4C_2)_{73}$-GFP expressing cells in comparison to cells

370 expressing GR$_{73}$-GFP and PR$_{73}$-GFP. The antibodies recognized specific signals in cells

371 transfected with the respective R-DPR constructs, but failed to detect R-DPR protein in cells

372 expressing $(G_4C_2)_{73}$-GFP (Figure 6B), indicating that production of R-DPR protein from the

373 G_4C_2 constructs was very inefficient. Given that PR$_{73}$-GFP and GR$_{73}$-GFP were produced in

374 detectable quantities from the synthetic constructs without inducing toxicity, we conclude that

375 the pronounced toxicity observed upon expression of $(G_4C_2)_{73}$-GFP cannot be explained by

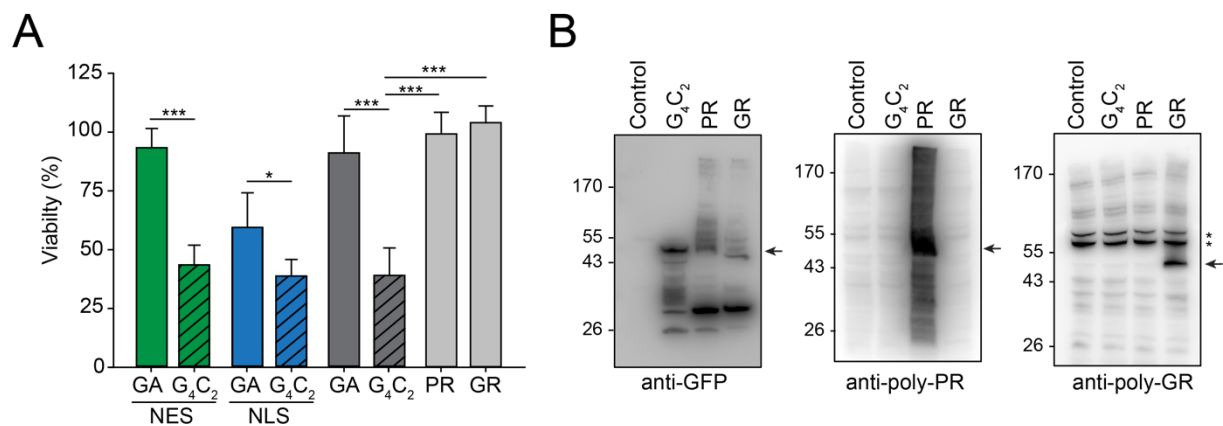376   RAN translation of R-DPR but is due to nuclear mRNA retention mediated by the G_4C_2 repeat

377   RNA.

378

379

380   **Figure 6 - Production of G_4C_2 mRNA strongly decreases cellular viability.** (A) G_4C_2 mRNA induces strong
381   toxicity. HEK293T cells were transfected with the indicated constructs: G_4C_2-GFP (G_4C_2), NES-G_4C_2-GFP (NES-
382   G_4C_2), NLS-G_4C_2-GFP (NLS-G_4C_2), GA_{65}-GFP (GA), NES-GA_{65}-GFP (NES-GA), NLS-GA_{65}-GFP (NLS-GA),
383   PR_{73}-GFP (PR), or GR_{73}-GFP (GR). MTT cell viability assays were performed 4 days after transfection. Data were
384   normalized to cells transfected with empty vector. Data are means + SD ($n \geq 3$). Part of this data is also shown in
385   Figure 2D. * $p \leq 0.05$; *** $p \leq 0.001$ from two-sided t-test. (B) $(G_4C_2)_{73}$-GFP does not produce detectable amounts
386   of arginine containing DPRs. HEK293T cells were transfected with the indicated constructs: empty vector
387   (control), $(G_4C_2)_{73}$-GFP (G_4C_2), PR_{73}-GFP (PR), GR_{73}-GFP (GR). Immunoblot analysis was then performed
388   against GFP (left), poly-PR (center), and poly-GR (right). A representative result of three biological repeats is
389   shown. The arrows indicate the main band of the respective DPRs and * indicate non-specific bands recognized
390   by the anti-GR antibody.

391

Discussion

392

393   We have employed a cellular model to differentiate possible mechanisms of toxicity

394   exerted by expansion of the G_4C_2 hexanucleotide tract within the *C9orf72* locus, the most

395   frequent genetic cause of ALS and FTD. Our results demonstrated that the G_4C_2 expansion

396   causes cellular toxicity in a manner dependent on both aggregates of G_4C_2-encoded DPR

397   proteins and the G_4C_2 repeat mRNA. These findings suggest a multiple hit model of additive,

398   but mechanistically independent, effects of proteotoxicity and RNA-mediated toxicity, with

399   the latter being the predominant toxic agent in the model system investigated.

400   Both DPRs and repeat mRNA interfered with different aspects of nucleocytoplasmic

401   transport. The G_4C_2 RNA inhibited the export of mRNA from the nucleus, consistent with a

18

402   dramatic impairment of protein synthesis that would be associated with strong neuronal

403   toxicity. Notably, this effect was independent of the presence of DPR protein aggregates.

404   Additionally, as has been reported for artificial β-sheet proteins and other disease-associated

405   aggregation-prone proteins such as Tau, Huntingtin, FUS and TDP-43 (Chou et al., 2018;

406   Dormann et al., 2010; Eftekharzadeh et al., 2018; Gasset-Rosa et al., 2017; Grima et al., 2017;

407   Woerner et al., 2016), cytoplasmic poly-GA aggregates partially inhibited the transport of

408   proteins across the nuclear pore. This observation is in agreement with the previously reported

409   sequestration of transport factors by poly-GA (Khosravi et al., 2017; Zhang et al., 2016).

410   Interestingly, other DPRs have also been reported to inhibit additional aspects of

411   nucleocytoplasmic transport. Poly-PR can directly obstruct the central channel of the nuclear

412   pore by binding to the FG domains of nuclear pore proteins (Shi et al., 2017), and R-DPRs

413   can disrupt cargo loading onto karyopherins (Hayes et al., 2020).

414   While the effects of cytoplasmic poly-GA (NES-poly-GA) aggregates on nuclear

415   transport were relatively well tolerated in our cell culture model, poly-GA aggregates within

416   the nucleus (NLS-poly-GA) were associated with substantial proteotoxicity. Nuclear poly-GA

417   formed aggregates at sites that were distinct from nucleoli, similar to the localization of poly-

418   GA aggregates observed in patient brain (Schludi et al., 2015). However, formation of these

419   inclusions altered the shape of nucleoli as a result of mislocalization and partial sequestration

420   of the abundant GC protein NPM1. Nuclear poly-GA aggregates interfered with the recently

421   described protein quality control function of the GC phase of the nucleolus (Frottin et al.,

422   2019), as demonstrated using the metastable firefly luciferase as a model substrate. In cells

423   containing nuclear poly-GA aggregates, misfolded luciferase failed to repartition from the

424   nucleolus to the nucleoplasm during recovery from stress. This inhibitory effect was

425   comparable to that previously observed for positively charged DPRs, such as poly-PR, which

426   accumulate directly within the GC phase of the nucleolus (Frottin et al., 2019; Mizielinska et

19

427 al., 2017; Tao et al., 2015). However, R-DPRs apparently do not accumulate in the nucleolus

428 in patient brain (Schludi et al., 2015), and thus may rather interfere with nucleolar quality

429 control by the mechanism described here for nuclear poly-GA (Frottin et al., 2019; Kwon et

430 al., 2014; White et al., 2019).

431 Expression of poly-GA from G_4C_2 containing constructs resulted in substantially

432 greater toxicity than expression from synthetic constructs, when similar poly-GA lengths and

433 amounts were compared. The G_4C_2 hexanucleotide repeat is thought to exert toxic effects in

434 part by forming higher order RNA assemblies in the nucleus which sequester RNA-binding

435 proteins (Almeida et al., 2013; Cooper-Knock et al., 2015; Cooper-Knock et al., 2014;

436 Donnelly et al., 2013; Haeusler et al., 2014; Mori, Lammich, et al., 2013; Reddy et al., 2013;

437 Rossi et al., 2015; Sareen et al., 2013; Xu et al., 2013). We found that G_4C_2 constructs always

438 resulted in strong inhibition of protein synthesis, even when coding for cytoplasmic poly-GA

439 (NES-GA_{65}), which itself alone did not impair protein biogenesis when produced from a

440 synthetic construct. However, because expression constructs based on G_4C_2 repeats also

441 generate the different RAN translation DPR products, a clear distinction between RNA and

442 DPR toxicity in previous studies had been difficult. We therefore compared not only the

443 relative toxicity of poly-GA and G_4C_2 constructs, but also measured the toxicity of poly-PR

444 and poly-GR constructs in the absence of G_4C_2 repeat RNA. Notably, expression of these

445 protein-only constructs did not induce overt toxicity, even when expressed at levels much

446 higher than those generated by RAN translation of the G_4C_2 repeat. These findings allowed us

447 to unequivocally attribute the major component of toxicity associated with G_4C_2 constructs to

448 the production of the pathological G_4C_2 RNA sequence. Indeed, DPRs may be undetectable in

449 the brain regions most affected by neurodegeneration in *C9orf72* patients (Schludi et al.,

450 2015). We note however, that nuclear poly-GA also interfered with protein biosynthesis,

451   presumably by impairing the nucleolar function in ribosome biogenesis, and thus could

452   enhance the toxic effects of G_4C_2 repeat RNA.

453   In summary, our results indicate that the G_4C_2 expansion in *C9orf72* interferes with

454   multiple nuclear functions, culminating in an inhibition of protein biogenesis, an outcome that

455   would be especially harmful to neuronal cells. These dominant toxic effects might be further

456   aggravated by a loss of function of the endogenous C9ORF72 protein, which is thought to

457   play a role in cellular quality control (Boivin et al., 2020; Sellier et al., 2016; Sullivan et al.,

458   2016; Yang et al., 2016; Zhu et al., 2020). Further research on the relative contribution of the

459   different toxic mechanisms will be important in developing therapeutic strategies.

Materials and Methods

Cell culture, transfection, and cell treatments

Human embryonic kidney 293T (HEK293T) cells were obtained from ATCC and maintained

in Dulbecco's modified Eagle's medium (DMEM) (Biochrom KG) supplemented with 10%

fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (Gibco),

2 mM L-glutamine (Gibco) and 5 μg/ml Plasmocin (InvivoGen). For heat stress (HS) and

recovery experiments, cells were either maintained at 37 °C (-HS), or placed in a 43 °C (+HS)

incubator for the indicated duration, or subjected to heat stress and then transferred back to

37 °C for recovery (+HS +Rec). Transient transfections were performed by electroporation

with the GenePulser XCell System (Bio-Rad) or with Lipofectamine 2000 (Invitrogen)

according to the manufacturer's instructions. For assessing nuclear import of p65, transfected

cells were treated with 20 ng/ml recombinant human TNFα (Jena Biosciences) for 30 min.

For translation inhibition, cycloheximide (CHX, Sigma-Aldrich) was dissolved in phosphate

buffered saline (PBS) and applied at a final concentration of 1 mM.

Plasmids

Degenerated sequences encoding 65 GA^{ggxgcx} repeats preceded by a start codon (ATG) and

flanked by NheI and BamHI restriction sites were chemically synthesized by GeneArt Gene

Synthesis (Invitrogen). The GC content of sequence 1 is 77.2%, while sequence 2 contains

81.5% GC (Figure 1-figure supplement 1A). Both GA$_{65}$ sequences were cloned into

pcDNA3.1-myc/His A plasmids in frame with GFP at the C-terminus. An N-terminal nuclear

export signal (NES) or an N-terminal double SV40 nuclear localization signal (NLS) were

inserted by site-directed mutagenesis. The alternative FUS-derived nuclear localization signal

(PY) was inserted C-terminally. Point mutations were introduced by site-directed

mutagenesis. Similarly, degenerated sequences encoding PR$_{73}$ and GR$_{73}$ were generated by

485 GeneArt Gene Synthesis (Invitrogen) and fused to the N-terminus of GFP. All sequences

486 contained an ATG start codon. Sequences encoding $(G_4C_2)_{73}$ repeats preceded by a start

487 codon (ATG) were generated as previously described by primer hybridization (Guo et al.,

488 2018). Similarly, the $(G_4C_2)_{73}$ construct was subcloned in place of the degenerated GA_{65}

489 sequence in the same NES/NLS-tagged GFP-containing vector. A GFP-only construct was

490 generated by deletion of the GA from the same vector. NLS-LS has been previously described

491 (Frottin et al., 2019). The plasmid encoding for S-mApple was generated from mApple-N1

492 (Addgene plasmid # 54567) a kind gift from Michael Davidson (Shaner et al., 2008). mApple

493 was then cloned between BamHI and XbaI to replace GFP in a previously described plasmid

494 encoding shuttle GFP (Woerner et al., 2016). c-myc-NES-β17, c-myc-NLS-β17 and Htt96Q

495 plasmids have been previously described (Woerner et al., 2016). All relevant plasmid regions

496 were verified by sequencing.

497

498 **Antibodies and dyes**

499 The following primary antibodies were used in this study: nuclear pore complex proteins

500 (Mab414), Abcam (24609); NPM1, Invitrogen (32-5200); NF-κB p65 (D14E12), Cell

501 Signaling Technology (#8242); c-Myc-Cy3 (9E10), Sigma (C6594); amyloid fibrils, OC,

502 Millipore (AB2286); GFP, Roche (11814460001); α-tubulin, Sigma Aldrich (T6199);

503 RPA40, Santa Cruz (sc-374443); IκBα (L35A5), Cell Signaling Technology (#4814);

504 phospho-NF-κB p65 (Ser536) (93H1), Cell Signaling Technology (#3033); KPNA2, Abcam

505 (ab70160); KPNA4, Abcam (ab84735); KPNB1, Abcam (ab2811); GAPDH, Millipore

506 (MAB374); poly-PR, Proteintech (23979-1-AP); poly-GR (5A2), Millipore (MABN778).

507 The following secondary antibodies were used: Mouse IgG-Alexa488, Cell signaling

508 Technology (#4408); Mouse IgG-Alexa555, Cell signaling Technology (#4408); Rabbit

509 IgG-Alexa555, Cell signaling Technology (#4408); Mouse IgG-Alexa488, Invitrogen

510   (21200); Mouse IgG-Alexa 633, Invitrogen (A21053); anti-mouse IgG-Peroxidase, Sigma

511   Aldrich (A4416); anti-rabbit IgG-Peroxidase, Sigma Aldrich (A9169). The amyloid dye

512   AmyTracker 680 (AmyT; Ebba Biotech AB) was used as previously described (Frottin et al.,

513   2019). Briefly, cells were fixed in 4% paraformaldehyde in PBS (GIBCO) for 20 min, washed

514   with PBS, permeabilized with Triton X-100 0.1 % for 5 min. AmyT was used at 1:500

515   dilution and incubated with the samples for 1 h at room temperature. Nuclei were

516   counterstained with 4', 6-Diamidine-2'-phenylindole dihydrochloride (DAPI, Molecular

517   Probes).

518

519   **Immunofluorescence and image acquisition**

520   Cells were grown on poly-L-lysine coated coverslips (Neuvitro). Cells were fixed with 4%

521   paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% bovine

522   serum albumin in PBS. Primary antibodies were applied in blocking buffer supplemented with

523   0.1% Triton X-100 and incubated overnight at 4°C. Appropriate fluorescent secondary

524   antibodies at a dilution of 1:500 were applied for 60 min at room temperature. Nuclei were

525   counterstained with DAPI before mounting samples with fluorescence-compatible mounting

526   medium (DAKO).

527   Confocal microscopy was performed at MPIB Imaging Facility (Martinsried,

528   Germany) on a ZEISS (Jena, Germany) LSM780 confocal laser scanning microscope

529   equipped with a ZEISS Plan-APO 63x/NA1.46 oil immersion objective. In case of multi-

530   fluorescence samples, a single-stained control sample was used to adjust emission and

531   detection configuration to minimize spectral bleed-through. Images of cells with inclusions

532   for co-localization studies were subjected to linear unmixing with spectra obtained from the

533   single-stained samples using ZEN software. When fluorescence intensities were directly

534   compared, acquisition settings and processing were kept identical. Images were analyzed with

535 ImageJ (Rasband, W.S., National Institutes of Health, USA) and assembled in Adobe

536 Photoshop CC (Adobe Systems Incorporated, Release 19.1.5).

537

538 **Cell viability assay**

539 HEK293T cells were transfected by electroporation as previously described (Woerner et al.,

540 2016). In brief, cells were electroporated with 20 µg of plasmid in 0.4 cm-gap electroporation

541 cuvettes (Bio-Rad). Cells were electroporated at 225 V, ∞ Ω, 950 µF exponential wave in a

542 GenePulser XCell System (Bio-Rad). After electroporation, cells were plated in a 24-well

543 plate in triplicates. MTT assays were performed 3 days after transfectrion. The growth

544 medium was replaced with fresh medium containing 5 µg/ml thiazolyl blue tetrazolium

545 bromide (Sigma) for 1 h. Formazan crystals were solubilized by addition of stop solution,

546 containing 40% N,N-dimethylformamide (Sigma-Aldrich), 16% SDS (Sigma-Aldrich) and

547 2% (v/v) acetic acid (Sigma-Aldrich). Absorbance at 570 nm and 630 nm was then recorded.

548 Alternatively, viability was measured using the CellTiter-Glo 2.0 Cell Viability Assay kit

549 (Promega) in the same conditions.

550

551 **Determination of nuclear import/export with S-mApple**

552 Cells were co-transfected with the indicated constructs and the reporter S-mApple. After 48 h,

553 cells were treated with 10 ng/ml of the CRM1 inhibitor Leptomycin B (LMB, Sigma Aldrich)

554 in DMSO for 15 min before cell fixation. Control cells received DMSO. The relative

555 concentration of S-mApple in the cytoplasm and nucleus was quantified by measuring the

556 fluorescence intensity ratio in cells from 3 independent experiments. Fluorescence intensities

557 were determined using ImageJ.

558

25

Protein biosynthesis assay by click-chemistry

559

560 Protein biosynthesis assays were carried out using the click-it plus O-propargyl-puromycin

561 (OPP) protein synthesis assay (ThermoFisher Scientific) according to the manufacturer's

562 instructions. Cells were transfected for 24 h with the indicated construct before metabolic

563 labelling and incubated with the OPP reagent for 30 min in normal growth conditions. As

564 control, protein translation was inhibited with cycloheximide (CHX, Sigma-Aldrich)

565 dissolved in phosphate buffer saline (PBS) and applied at a final concentration of 1 mM.

566 Samples were then fixed, permeabilized and the click reaction performed as recommended by

567 the provider. Samples were subsequently analyzed by confocal microscopy. The

568 concentration of labelled proteins was quantified by measuring the mean fluorescence

569 intensity in 100-250 cells using ImageJ. A representative experiment of three independent

570 experiments is shown.

571

Fluorescence in situ hybridization (FISH)

572

573 Visualization of mRNA and G_4C_2 RNA by FISH was carried out as previously described

574 (Woerner et al., 2016). HEK293T cells were fixed in 4% formaldehyde for 10 min at room

575 temperature and permeabilized with 0.1% Triton X-100, both in UltraPure SCC buffer

576 (Thermo Fisher Scientific). After washing with SSC buffer and with FISH buffer (10%

577 formamide in SCC buffer), the probes (either T30 or $(C_4G_2)_5$) were hybridized in FISH buffer

578 with 10% dextran sulphate (Sigma Aldrich) for 3 h at 42ºC, followed by additional washes in

579 FISH buffer. When required, immunostaining was performed in PBS-based buffers

580 afterwards. The fraction of transfected cells displaying abnormal accumulation of mRNA

581 (using poly-dT probe) within the nucleus was determined by confocal microscopy.

582

26

Filter retardation assay

For the filter retardation assay (Scherzinger et al., 1997; Wanker et al., 1999) cells were

harvested 24 h after transfection with the indicated plasmids, lysed in

radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific) and sonicated for 10 s.

After incubation for 30 min, protein concentration was measured by Bradford assay (Bio-

Rad), and equal amounts of lysates were filtered through a 0.2 µm pore size cellulose acetate

membrane (GE Healthcare) and washed with lysis buffer. The membrane was subsequently

immunoassayed with anti-GFP antibody. Antibody binding was detected using Luminata

Forte Western HRP substrate (Millipore) and pictures were acquired with a LAS-3000 camera

system (Fujifilm). AIDA (Raytest) software was used for analysis and quantitation.

Statistics

Significance of differences between samples was determined using unpaired Student's t test,

ubless stated otherwise. Significance levels: (*) for $p < 0.05$, (**) for $p < 0.01$, (***) for

$p < 0.001$.

Author contributions:

F.F. and M.P.B. designed and performed the experiments. F.F. supervised M.P.B..

F.F., M.S.H. and F.U.H. initiated and supervised the project, and wrote the paper.

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No competing interests declared.

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1025   **Supplementary Information**

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1028   **Multiple pathways of toxicity induced by *C9orf72* dipeptide repeat**

1029   **aggregates and G$_4$C$_2$ RNA in a cellular model**

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Degenerated sequence (1)

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GGAGCT GGTGCT GGTGCA GGCGCT GGGGCA GGGGCT GGCGCT GGTGCC GGTGCT GGTGCC GGCGCA
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GGCGCT GGTGCT GGCGCA GGGGCA GGCGCT GGGGCT GGTGCA GGCGCT GGTGCT GGCGCT GGTGCA
GGCGCT GGCGCT GGTGCT GGCGCT GGCGCA GGGGCA GGCGCA GGCGCA GGGGCT GGTGCT GGTGCC
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Degenerated sequence (2)

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G₄C₂ sequence

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GGGGCC GGGGCC GGGGCC GGGGCC GGGGCC GGGGCC GGGGCC GGGGCC GGGGCC GGGGCC GGGGCC
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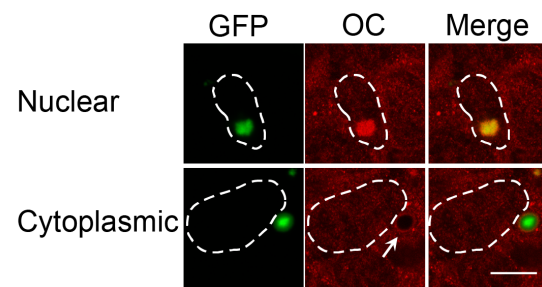
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Figure 1–figure supplement 1

Cytoplasmic and nuclear poly-GA aggregates differ in their staining properties. (**A**) Sequences of the GA expressing constructs used in this study. Sequences (1) and (2) are synthetic degenerated sequences that do not contain G_4C_2. A third construct only contains G_4C_2 repeats and is translated into GA_{73}. All three constructs contain an ATG start codon in frame with the GA coding sequence. (**B**) Cytoplasmic and nuclear aggregates can be stained with the OC antibody against amyloid fibrils. GA_{65}-GFP was expressed in HEK293T cells, and immunofluorescence analysis was performed against amyloid fibrils (OC, red). Poly-GA was visualized by GFP fluorescence (green). White dashed lines delineate the nucleus based on DAPI staining. Scale bar represents 10 µm.
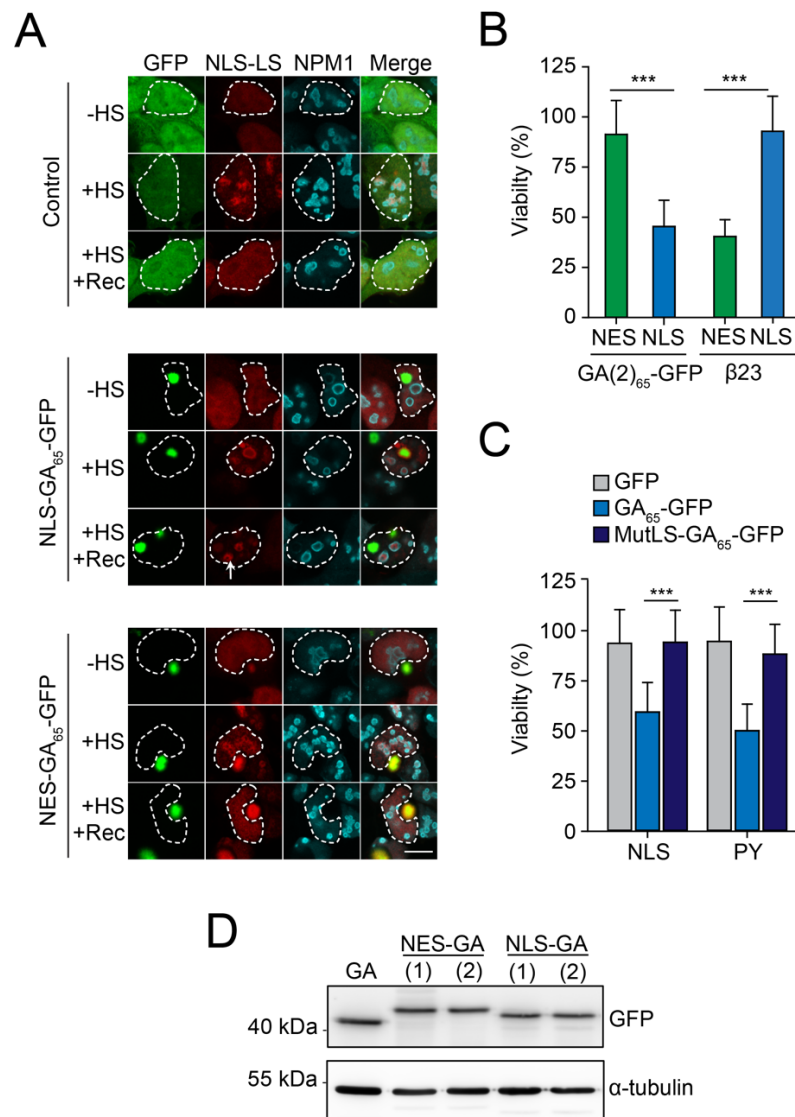
Figure 2–figure supplement 1

Nuclear poly-GA aggregates impair nucleolar protein quality control and cell viability. (**A**) Nuclear poly-GA aggregates disrupt nucleolar protein quality control. HEK293T cells were co-transfected with NLS-LS and the indicated poly-GA (NLS-GA$_{65}$-GFP or NES-GA$_{65}$-GFP) constructs or GFP as control. Cells were maintained at 37°C (–HS) or subjected to heat stress at 43°C for 2 h (+HS) and recovery (+HS +Rec) for 2 h. Samples were immunostained for NPM1 (cyan), NLS-LS and poly-GA proteins were visualized by mScarlet and GFP fluorescence, respectively. Merged panels are shown and a white dashed line delineates the nucleus based on DAPI staining. Scale bars represent 10 μm. Representative images of 3 independent experiments are shown. See Figure 2C for quantification. (**B**) NLS-GA$_{65}$-GFP produced from an alternative degenerated and G$_4$C$_2$-free DNA sequence also decreases cellular viability. HEK293T cells were transfected with the indicated constructs and MTT cell viability assays performed 4 days after transfection. Data were normalized to cells transfected with empty vector. Data are means + SD (n ≥ 3) and two-sided t-test is shown (*** $p ≤ 0.001$). β23 data are from Figure 2D and are shown as control. (**C**) Disabling nuclear targeting sequence functionality of GA$_{65}$-GFP rescues cellular viability. HEK293T cells were transfected with constructs encoding two different nuclear localization sequences (NLS or PY) fused to GFP, GA$_{65}$-GFP or GA$_{65}$-GFP with an inactivating mutation in the nuclear localization sequence (MutLS-GA$_{65}$-GFP), followed by MTT cell viability assay 4 days after transfection. Data were normalized to cells transfected with empty vector. Data are shown as means + SD (n ≥ 3). *** $p ≤ 0.001$ by two-sided t-test. Part of this data is also shown in Figure 2D. (**D**) HEK293T cells were transfected with the indicated constructs (GA$_{65}$-GFP; NES-GA$_{65}$-GFP; NES-GA$_{65}$(2)-GFP; NLS-GA$_{65}$-GFP; and NLS-GA$_{65}$(2)-GFP). Expression levels were determined by immunoblotting with anti-GFP antibodies, α-tubulin served as loading control.
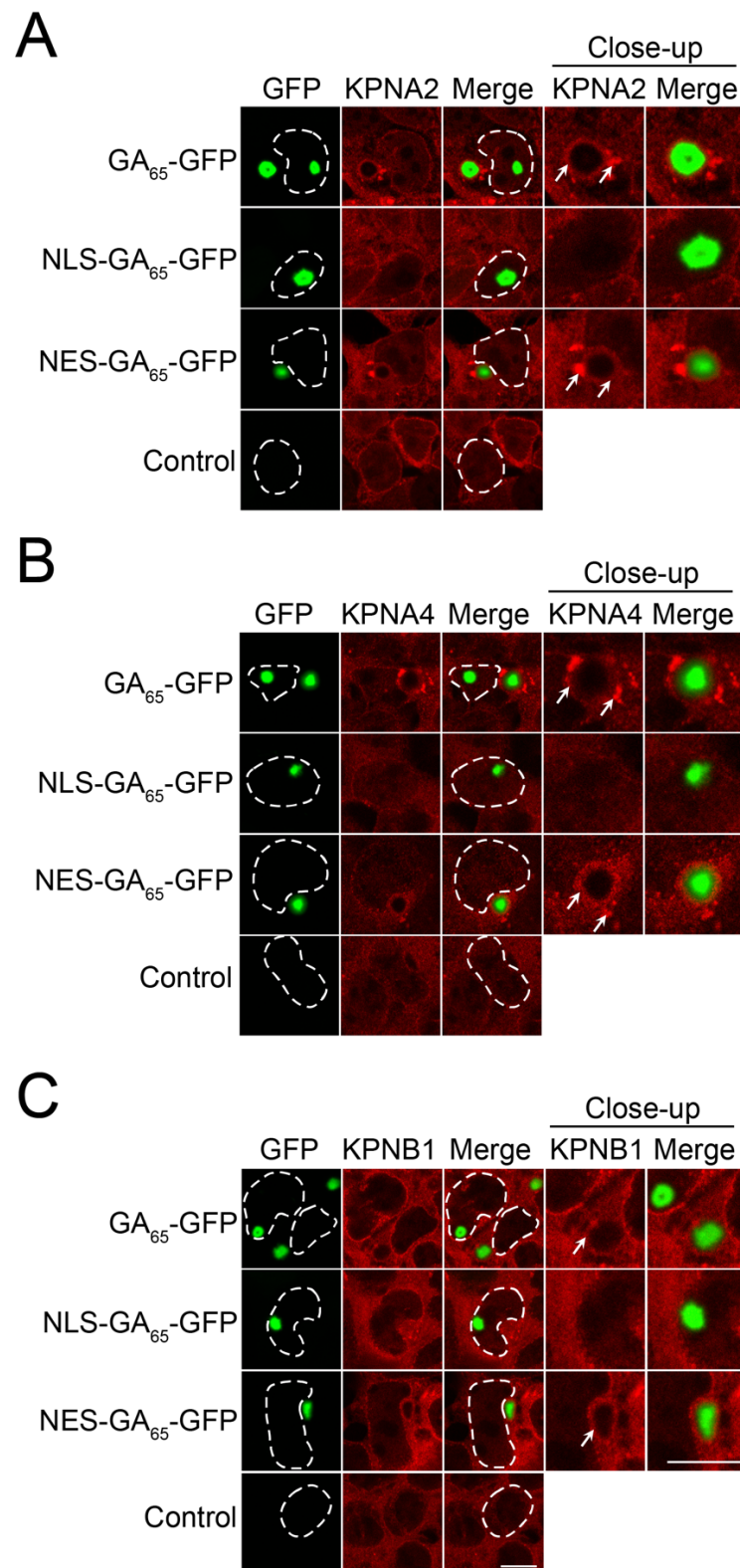
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1079 **Figure 4–figure supplement 1**
1080 **Importins form aggregates in cells containing cytoplasmic poly-GA aggregates and are partially**
1081 **sequestered.** HEK293T cells were left untreated (Control) or were transfected with GA$_{65}$-GFP, NES-GA$_{65}$-GFP
1082 or NLS-GA$_{65}$-GFP (green). Cells were then analyzed by immunofluorescence with antibodies against importin α-
1083 1 (KPNA2; (**A**)), importin α-3 (KPNA4; (**B**)) and importin β-1 (KPNB1; (**C**)) (red). Close-up views of inclusions
1084 are shown and arrows indicate importin aggregates and/or their sequestration at the periphery of the inclusion. The
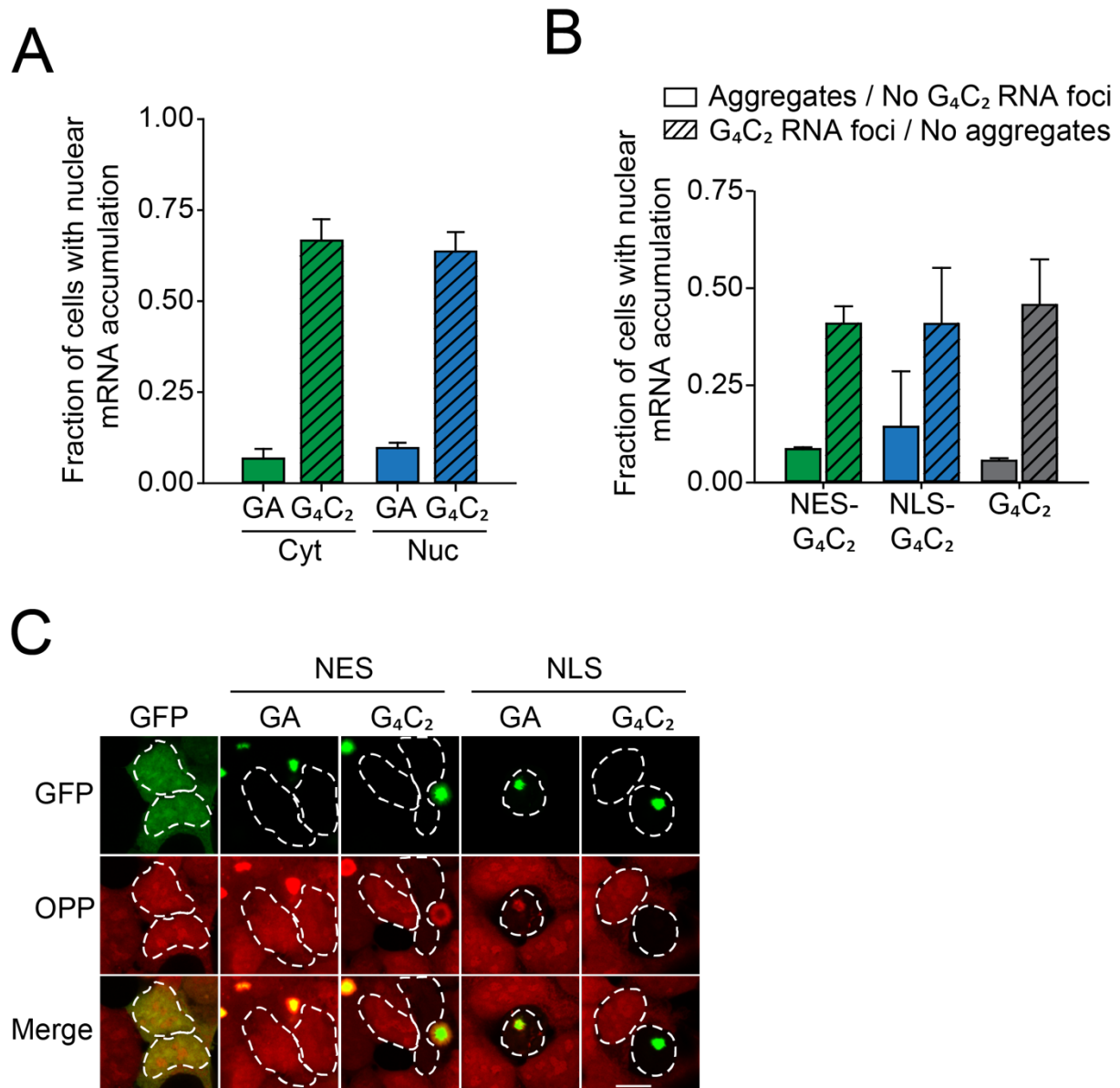1085 white dashed lines delineate nuclei based on DAPI staining. Scale bars are 10 μm.

Figure 5–figure supplement 1

Expression of G_4C_2 containing constructs induces mRNA retention in the nucleus. (**A**) Presence of G_4C_2 rather than aggregate localization causes nuclear mRNA retention. HEK293T cells were transfected with either GA_{65}-GFP (GA) or G_4C_2-GFP (G_4C_2) and analyzed for nuclear mRNA accumulation as in Figures 5A and B. Cells containing GA-GFP aggregates in the cytoplasm (Cyt) or nucleus (Nuc) were analyzed separately. Data are shown as means + SD (n = 3). (**B**) mRNA retention correlates with the presence of G_4C_2 positive nuclear foci. HEK293T cells were transfected with G_4C_2-GFP (G_4C_2), NES-G_4C_2-GFP (NES-G_4C_2) or NLS-G_4C_2-GFP (NLS-G_4C_2) and analyzed for mRNA nuclear accumulation as in Figure 5C. FISH was performed as in Figure 5C to detect G_4C_2 foci. Cells were categorized according to the presence of G_4C_2 positive foci as well as aggregates. Data are shown as means + SD (n = 3). (**C**) Decreased protein biosynthesis in presence of nuclear poly-GA and G_4C_2 mRNA. Newly synthesized proteins were labelled with O-propargyl-puromycin (OPP; red) in HEK293T cells transfected with the indicated constructs (green). Quantification of this experiment is shown in Figure 5D. The white dashed lines delineate the nucleus based on DAPI staining and the scale bar represents 10 µm.
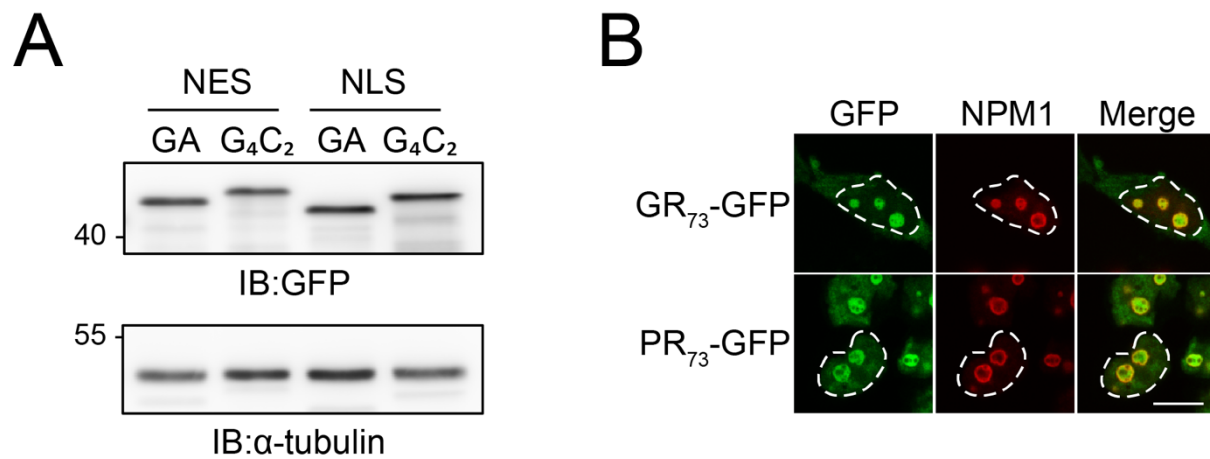
Figure 6–figure supplement 1

(**A**) Toxicity induced by G_4C_2 mRNA is not due to a higher poly-GA level. HEK293T cells were transfected with the indicated constructs: NES-GA$_{65}$-GFP (NES-GA); NES-G$_4$C$_2$-GFP (NES-G$_4$C$_2$); NLS-GA$_{65}$-GFP (NLS-GA); NLS-G$_4$C$_2$-GFP (NLS-G$_4$C$_2$). Poly-GA-GFP protein levels were analyzed by immunoblotting against GFP. α-tubulin served as loading control. A representative result of 3 independent experiments is shown. (**B**) Arginine containing DPRs accumulate in the nucleolus. HEK293T cells were transfected with the indicated constructs (GR$_{73}$-GFP (GR); PR$_{73}$-GFP (PR)). Cells were then stained with anti-nucleophosmin (NPM1, red). Expressed constructs are shown in green. The white dashed lines delineate the nucleus based on DAPI staining and the scale bar represents 10 μm.