Nuclear accumulation of mRNAs underlies G4C2 repeat-induced translational repression in a cellular model of C9orf72 ALS

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ABSTRACT
A common feature of non-coding repeat expansion disorders is the accumulation of RNA repeats as RNA foci in the nucleus and/or cytoplasm of affected cells. These RNA foci can be toxic by sequestering RNA-binding proteins, thus affecting various steps of post-transcriptional gene regulation. However, the precise step that is affected by C9orf72 GGGGCC (G4C2) repeat expansion, the major genetic cause of Amyotrophic Lateral Sclerosis, is still poorly defined. In this work, we set out to characterise these mechanisms by identifying C9orf72 RNA binding proteins. Sequestration of some of these factors into RNA foci was observed when a (G4C2)31 repeat was expressed in NSC34 and HeLa cells. Most notably, (G4C2)31 repeats widely affected the distribution of Pur-alpha and its binding partner FMRP, which accumulate in intra-cytosolic granules that are positive for stress granules markers. Accordingly, translational repression is induced. Interestingly, this effect is associated to a marked accumulation of poly(A) mRNAs in cell nuclei. Thus, defective trafficking of mRNA, as a consequence of impaired nuclear mRNA export, might affect translation efficiency and contribute to the pathogenesis of C9orf72 ALS.
INTRODUCTION

During the last years, the discovery of mutations in the FUS/TLS, TARDBP and C9orf72 genes extended to Amyotrophic Lateral Sclerosis (ALS) the concept that alterations in RNA metabolism are a major determinant of motor neuron degeneration, similarly to what occurs in other neurodegenerative diseases (Walsh et al., 2015). Alterations in alternative splicing have been suggested to cause the disease, but other steps of post-transcriptional gene regulation could be equally involved (Achsel et al., 2013). C9orf72, the major genetic determinant of ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011), is a prime example of how little we understand: analogies with other repeat expansion diseases suggest three possible scenarios that could play a role in the pathogenesis of ALS linked to C9orf72 GGGGCC (G4C2) repeat expansions. One stems from reduced expression of the C9orf72 protein, the others from the accumulation of repeat-containing RNAs that may either trap specific RNA-binding proteins disrupting RNA processing, or undergo an unconventional mode of translation (repeat-associated non-ATG (RAN) translation), which results in the accumulation of toxic poly-dipeptides (Gendron et al., 2014). However, whether accumulation of toxic RNA species and/or haploinsufficiency of C9orf72 are the leading pathological mechanisms in ALS is unknown.

A common feature of non-coding repeat expansion disorders is the accumulation of RNA repeats as RNA foci in the nucleus and/or cytoplasm of affected cells. These foci are able to sequester RNA-binding proteins, which can affect various steps of post-transcriptional gene regulation, such as alternative mRNA splicing, translational regulation, mRNA transport, or mRNA decay (La Spada and Taylor, 2010). In samples from C9orf72 patients as well as patient-derived iPSC, RNA foci containing the sense as well the antisense RNA repeat sequence are likewise detected (DeJesus-Hernandez et al., 2011; Donnelly et al., 2013), indicating that the sequestration of RNA-binding proteins and hence a dysregulation in one of the steps of mRNA metabolism might well play a role in ALS. The precise step that is affected, however, remains ill defined, and our understanding of C9orf72 toxicity thus is still elusive. As a first hint to characterize these mechanisms, we used a biotinylated RNA containing (G4C2)31 repeats to identify C9orf72 RNA binding proteins. Based on the ability of (G4C2)31 repeats to bind proteins involved in translational control, we analysed whether protein translation could be affected in cultured cells expressing the same repeats. Indeed, we observed that the expression of (G4C2)31 is able to activate a stress response that leads to a general reduction of translation. In these conditions, C9orf72 repeats strikingly induce an abnormal nuclear accumulation of poly-adenylated mRNAs. Thus, nuclear retention of mRNAs, as a consequence of C9orf72 repeats ability to impair nuclear mRNA export, might contribute to ALS pathogenesis.
RESULTS

(G4C2)₃₁ repeats aggregate in RNA foci in mouse motoneuronal NSC34 cells

In order to produce GGGGCC repeat expansions with selected lengths, we synthesized two complementary oligonucleotides containing three G4C2 repeats and overhangs to allow head-to-tail ligation. Annealed oligos were ligated, fractionated on an agarose gel, and various repeat lengths were cloned in an expression vector (pcDNA5/FRT/TO). With this approach, we were able to select a repeat of 31 uninterrupted GGGGCC units (G4C2)₃₁ that was transiently transfected into mouse motoneuronal-like NSC34 cells, and compared to a plasmid containing 10 repeats (G4C2)₁₀. Expression of (G4C2)₃₁ is sufficient to induce the appearance of intense intranuclear RNA foci (Fig. 1A), as stained by fluorescence in situ hybridization (FISH) analysis using a Cy3-conjugated (C4G2)₄ RNA probe. Under the same conditions, 10 repeats, whose expression levels are comparable to the (G4C2)₃₁ (Fig. 1B), do not form intracellular foci (Fig. 1A). A cytoplasmic staining is also evident in cells transfected with the longer repeat, with cytoplasmic foci occasionally appearing (Fig. 1A). As expected from the nature of the probe target, treatment with RNase, but not DNase, completely abolishes the signal (Supplementary Fig. 1). Transfection of NSC34 cells with a plasmid bearing 270 repeats of the trimer TTC, that is expressed to similar levels as (G4C2) 10 and 31 (Fig 1B), does not induce the appearance of Cy3-(C4G2)₄ positive signals (Fig. 1A). Similarly, a CUG RNA expanded repeat, that is known to form RNA foci, is not recognised by the Cy3-(C4G2)₄ probe (Supplementary Fig. 1), further indicating the specificity of the experimental conditions used. The same (G4C2)₃₁ repeats were inserted upstream of a GFP coding sequence and transfected in NSC34 cells. As shown in Fig. 1C,D, about 30% of GFP-positive cells showed foci formation after 48 hrs of transfection, while in the same conditions of GFP expression, no foci are detectable in cells transfected with a GFP-(G4C2)₁₀ plasmid. Therefore, the accumulation into nuclear foci is a prevalent feature of (G4C2)₃₁ RNA repeat expression. Further, overexpression of 31 repeats is sufficient to reproduce a key pathological phenotype that characterizes C9orf72 patients carrying longer expansions.

Repeat-binding proteins are involved in pre-mRNA splicing and mRNA translation

To get insights into the mechanisms whereby C9orf72 might induce cell toxicity, we used an in vitro-transcribed biotinylated RNA containing the (G4C2)₃₁ repeats to affinity purify proteins able to bind it. The major repeat-binding proteins from mouse brain and spinal cord extracts were visualised by Coomassie staining of SDS-polyacrylamide gels, excised, and identified by mass spectrometry (Supplementary Table 1 and Supplementary Fig. 2). Among the interactors, we found...
different factors involved in post-transcriptional gene regulation. Indeed, G4C2 repeat-binding proteins are enriched in members of the hnRNP family (hnRNP H, hnRNP U, hnRNP Q), that are known key regulators of alternative splicing (Chen and Manley, 2009). However, translational regulators also bind (G4C2)$_{31}$: these include initiation and elongation factors (EF1 alpha, eIF2 alpha, beta, gamma), and also Pur-alpha (Pura), Pur-beta and other translation regulatory proteins (ILF2, ILF3, RAX/PACT). To validate the mass spec data, a subset of the identified proteins was analysed by Western blot on proteins pulled-down by (G4C2)$_{31}$ RNA repeat from mouse brain and spinal cord (not shown), as well as mouse (NSC34) and human (SH-SY5Y) cultured neuronal cells. As shown in Fig. 2A, all the proteins tested could be detected, with different binding affinity depending on the stringency conditions. When the same proteins were analysed in extracts pulled-down with a biotinylated (TTC)$_{270}$ RNA repeat, only hnRNP H, eIF2a, eIF2b and RAX/PACT displayed a significant specificity for the (G4C2)$_{31}$ RNA repeat, while the others precipitate to the same or even higher degrees (Fig. 2B,C). On the contrary, none of the proteins tested was detected in control precipitates. Overall, these data suggest that G4C2 binding to Pura, hnRNP U, ILF2 and ILF3 might not be required for C9orf72 toxicity. Based on their described ability to bind the C9orf72 repeats (Lee et al., 2013; Mori et al., 2013; Reddy et al., 2013), we also checked for the presence into repeat binding partners of FUS, TDP43, hnRNP A2/B1, and SRSF1(Fig. 2D). Again, binding of these proteins to (G4C2)$_{31}$ was observed. Importantly, MBNL1, whose sequestration by CUG RNA repeats has a prominent role in DM1 pathogenesis, was in no instance precipitated by C9orf72 repeats (Fig. 2A), indicating that (G4C2)$_{31}$ repeat is selective for the binding to a subset of RNA binding proteins.

(G4C2)$_{31}$ repeats sequester RBPs into RNA foci and disturb the intracellular localization of Pura and FMRP

Sequestration of (G4C2)$_{31}$ binding proteins into RNA foci has been suggested to cause a decreased availability of those proteins, thus affecting their function and eventually leading to cell death. We therefore looked for the presence of the above identified proteins in RNA foci formed by (G4C2)$_{31}$ repeats in NSC34 cells. Among the proteins tested, hnRNP H, eIF2a, FUS, and ILF3 show a clear colocalization with RNA foci (Fig. 3A), while others do not significantly colocalise (not shown). To assess whether the accumulation of (G4C2)$_{31}$ binding proteins into RNA foci could be dependent on the cellular context, we also performed a similar analysis in human HeLa cells, obtaining comparable results (Fig. 3B). In both cellular types, however, the number of cells where RNA foci colocalise with those proteins is extremely low (less than 3%). Thus, while the presence of the proteins into RNA foci indicates that their interaction with G4C2 repeats also occurs in vivo, these
results suggest that the formation of RNA foci by the (G4C2)$_{31}$ repeats does not normally affect the overall subcellular distribution of the partners analysed. Pura represents a striking exception. Indeed, while this protein only occasionally shows a clear colocalization with RNA foci, in almost all the cells displaying (G4C2)$_{31}$ RNA foci formation the intracellular distribution of Pura is profoundly affected, with an evenly diffused localization in the cytosol of untransfected cells, while coalescing into cytosolic and nuclear granules in cells with RNA foci. A similar result was obtained with FMRP, a known binding partner of Pura (Fig. 4A). In cells transfected with (G4C2)$_{31}$ repeats, FMRP accumulates into cytosolic and, to a lesser extent, nuclear granules. The same pattern of Pura and FMRP delocalization is induced by (G4C2)$_{31}$ expression in HeLa cells (Fig. 4B). Interestingly, FMRP is also pulled down by (G4C2)$_{31}$ RNA (Supplementary Fig. 3), but differently from other partners analysed, this interaction is completely lost when the NaCl concentration is raised to 300 mM, suggesting that the binding of FMRP is not direct, and might be mediated by Pura itself. This conclusion is further supported by the observation that Pura and FMRP colocalise in cells expressing the (G4C2)$_{31}$ repeats, but not the (G4C2)$_{10}$ nor the (TTC)$_{270}$ repeats (Supplementary Fig. 3).

**G(G4C2)$_{31}$ repeats induce translational arrest**

The ability of (G4C2)$_{31}$ repeats to bind eIF2a, a central controller of protein translation, as well as the effects of repeat expression on the subcellular distribution of Pura and FMRP, whose functions in protein translation are well documented (Bagni and Oostra, 2013; White et al., 2009), prompted us to analyse whether (G4C2)$_{31}$ is able to affect protein translation. HeLa cells, a well established model to study stress response, were therefore transiently transfected with (G4C2)$_{31}$ repeats, and the presence of stress granules, where non-functional translation initiation complexes accumulate in response to stress, was analysed. Indeed, stress granules are formed in the vast majority of cells expressing the (G4C2)$_{31}$ repeats, but not in cells expressing similar amounts of (G4C2)$_{10}$ or (TTC)$_{270}$ RNAs, as measured by the presence of intra-cytoplasmic granules that are positive for TIAR, a known stress granule marker (Fig. 5A-D). Importantly, Pura granules (Fig. 5A), as well as FMRP granules (not shown), are positive for TIAR. Thus, C9orf72 repeat is able to activate in cells a stress response that induces stress granules formation.

Phosphorylation of eIF2a is the leading mechanism that causes the formation of non-functional translation initiation complexes and their accumulation into stress granules, and the phosphorylation status of eIF2a is thus an indication of translational inhibition (Spriggs et al., 2010). P-eIF2a levels were therefore monitored with a phospho-specific antibody in HeLa cells transfected with (G4C2)$_{31}$ repeats. As shown in Fig. 6A, no significant increase in eIF2a phosphorylation was detectable in
cells expressing the \((G4C2)_{31}\) repeats, compared to cells expressing shorter expansions (10 repeats) or to mock transfected cells. Conversely, in the same cells treated with thapsigargin, a well-known inducer of ER stress through the activation of PERK kinase, a significant increase in P-eIF2a was detected. Thus, eIF2a phosphorylation does not seem to be a critical step in the stress response induced by \((G4C2)_{31}\).

To check whether this response is nonetheless sufficient to cause translation inhibition, cells were analysed for their ability to incorporate puromycin into nascent polypeptides as a measure of the rate of mRNA translation (Schmidt et al., 2009). Both Western blot analysis (Fig. 6B) and immunofluorescence staining (Fig. 6C,D) of transfected cells clearly shows that puromycin incorporation is considerably decreased in HeLa and NSC34 cells expressing \((G4C2)_{31}\), but not \((G4C2)_{10}\) nor \((TTC)_{270}\) repeats. As expected, pre-treating of cells with cycloheximide completely abolishes puromycin incorporation.

**mRNAs accumulate in nuclei of \((G4C2)_{31}\) expressing cells**

Previous data indicate that a significant inhibition of global mRNA translation is achieved in cells expressing \(C9orf72\) repeats independently from the phosphorylation of eIF2a, thus implying alternative mechanisms whereby translational inhibition is carried out by G4C2 expansions. One obvious possibility was that G4C2 RNA repeats might cause a failure in mRNA export from nuclei, eventually leading to the accumulation of poly(A) RNA into them. To investigate this possibility, cells were left untransfected or were transfected with \((G4C2)_{10}\) or \((G4C2)_{31}\) repeats, and poly(A) RNAs were visualized by oligo(dT) FISH. To exclude that the probe might recognize the poly(A) tail of the overexpressed \((G4C2)_{31}\) RNAs that indeed accumulates in nuclei, the repeat sequence was cloned under the control of an RNA polymerase III promoter, and a small poly(T) stretch was inserted immediately after the repeat sequence as a transcription termination signal. As shown in Fig. 7A,B,C, an enhancement of poly(A) RNAs signal in the nucleus was clearly detectable in cells displaying G4C2 RNA foci, compared to untransfected or \((G4C2)_{10}\) transfected cells (Supplementary Fig. 4A). Importantly, poly(A) RNAs accumulate as nuclear dots that only partially co-localise with \((G4C2)_{31}\) RNA foci, thus excluding a direct sequestration of poly(A) mRNAs by the repeat sequence. When stress granule formation was induced by sodium arsenite, poly(A) mRNAs aggregate in TIAR-positive stress granules, as expected (Fig. 7D). In this condition, however, no mRNA accumulation in cell nuclei was observed, suggesting that nuclear mRNA retention by \((G4C2)_{31}\) repeat is not a mere consequence of the formation of cytoplasmic stress granules. This conclusion is further supported by the subcellular distribution of the cytoplasmic poly(A) binding protein (PABPc) in cells expressing \((G4C2)_{31}\) repeats. It has been recently shown,
in fact, that nuclear accumulation of poly(A) RNAs is associated to re-localization in cell nuclei of PABPc, a protein with an established, fundamental role in cytosolic mRNA translation and stability (Burgess et al., 2011; Kumar and Glaunsinger, 2010), which is known to localise into stress granules upon stress (Kedersha et al., 2000). We therefore analysed PABPc distribution in cells expressing the (G4C2)$_{31}$ repeats. Immunofluorescence staining of cells shows that, as expected, PABPc has a prevalent cytoplasmic steady-state localization in a majority of untransfected cells. However, in a significant fraction of (G4C2)$_{31}$ transfected cells (10± 2%), an evident nuclear PABPc signal appears (Fig. 8A), while the cytosolic distribution of PABPc in cells expressing (G4C2)$_{10}$ or (TTC)$_{270}$ is unaffected (Fig. 8B) A similar result was obtained when cells were transfected with repeat-GFP plasmids (Supplementary Fig. 4B), or when nuclear and cytoplasmic fractions of cells were analysed by Western blot (Fig. 8C). Interestingly, a considerable number of transfected cells displays a clear accumulation of PABPc into RNA foci (Fig. 8A), and PABPc is pulled down by (G4C2)$_{31}$ RNA (Fig. 8D), but not by (TTC)$_{270}$ RNA. Importantly, PABPc only rarely colocalises with Pura-containing cytosolic stress granules that are induced by (G4C2)$_{31}$ repeats, differently from what is observed in cells treated with arsenite, where PABPc and Pura fully colocalise (Fig. 8E). Thus, a nuclear relocalization of PABPc is specifically induced by (G4C2)$_{31}$, and this is associated to nuclear retention of poly(A) mRNAs.

DISCUSSION

The presence of C9orf72 RNA inclusions in tissues from ALS/FTD patients, as well as the formation of similar inclusions in cellular systems expressing the repeats, including iPSC-derived motor neurons, provided an early support for the hypothesis that sequestration of RNA binding proteins by C9orf72 repeat might have a role in disease pathogenesis (Walsh et al., 2015). Evidence from histopathological analysis of RNA foci in tissues from ALS/FTD patients has shown a positive correlation between the number of repeat-containing foci and disease presentation or severity (Cooper-Knock et al., 2014; Mizielinska et al., 2013), further indicating that accumulation of C9orf72-derived G4C2 RNA repeats are primary involved in the neurodegenerative process. However, the nature of the mechanisms affected by this accumulation is still unknown. To get insights into this process, we have characterized the major binding partners of a (G4C2)$_{31}$ repeat. Our analysis showed that a substantial number of binding partners are RNA regulatory factors, as expected by the nature of the repeat sequence and in line with previous analyses showing that splicing and translation regulatory factors are enriched within the binding partners of C9orf72 (Cooper-Knock et al., 2014; Donnelly et al., 2013; Haeusler et al., 2014; Mori et al., 2013; Xu et al., 2013). In our study, translational regulators are particularly represented: these include initiation and
elongation factors (EF1 alpha, eIF2 alpha, beta, gamma), but also Pur-alpha (Pura), Pur-beta and other translation regulatory proteins (ILF2, ILF3, RAX/PACT). Trying to figure out which of these interactors might be more directly involved in C9orf72 repeat toxicity, we therefore reasoned that the amount of sequestration into RNA foci might represent a discriminating factor. Surprisingly, at least among the binding partners tested, only a limited number was found in association with RNA foci that are formed in cultured cells by the expression of 31 repeats, and even in these cases, the amount of co-localization was really low (Supplementary Table 2). Clearly, we cannot exclude that the length of the repeats, as well as the duration of the process, can influence the appearance of such a phenotype. Indeed, the observations that in brain tissues from C9orf72 ALS and FTD patients, who usually bear longer expansions that accumulate over a large period of time, a significant number of G4C2 RNA foci colocalizes with hnRNPH (Lee et al., 2013), and to a lower, but still significant extent, with SRSF2 and ALYREF (Cooper-Knock et al., 2014) argues in favour of such a conclusion. However, numerous inconsistencies emerge from studies where tissues from ALS/FTD patients have been surveyed for proteins that co-aggregate with RNA foci (Stepto et al., 2014), suggesting that a dynamic interaction between accumulated RNA repeats, either aggregated in foci or not, and selected targets, rather than an irreversible sequestration, might better describe the pathogenic mechanism (Cooper-Knock et al., 2014). Our results support this idea, and suggest that RNA repeats might affect the proper localization and/or function of a distinguished set of RNA binding proteins independently from their sequestration into nuclear foci, a conclusion that is also consistent with the presence of RNA repeats in the cytosol of cells, where they do not necessarily form RNA inclusions containing co-aggregated proteins. Among these RNA binding proteins, hnRNPH, eIF2a, eIF2b, and RAX might represent relevant targets of C9orf72 expansion, as their interaction with the (G4C2)31 repeat proved to be specific when compared to the binding to an unrelated, TTC repeat expansion, that is not sufficient to induce cellular phenotypes when expressed in NSC34 or HeLa cells. Yet, Pura, that has been implicated in G4C2-induced neurodegeneration (Xu et al., 2013), binds the two repeats with the same affinity. Thus, the nature of the particular RNA process affected by C9orf72 could not be easily anticipated by a colocalization analysis or simply argued by the types of binding partners found to be associated to the repeats, and a functional analysis is needed to draw any conclusion.

Functionally, the most striking effect of C9orf72 expression is the induction of translational arrest. Indeed, in this study we show for the first time that stress granules are formed in cells where C9orf72 RNA repeats accumulate into nuclear foci, and this phenotype well correlates with a strong reduction in the rate of global translation. Mounting evidence indicates that stress granule-associated translational repression might have a role in ALS pathogenesis. FUS and TDP-43, two
major ALS factors, are known to accumulate into stress granules upon stress, and ALS-linked mutations clearly affect stress granule formation, eventually impairing a neuroprotective effect of this process or generating a persistent presence of stress that might be harmful to neurons (Bentmann et al., 2013). Moreover, stress granule genes are potent modifiers of TDP-43 toxicity in Drosophila, and decreased translational repression has a protective effect (Kim et al., 2014). Thus, our observations extend to C9orf72-ALS this scenario. In the cell lines tested, however, phosphorylation of eIF2a, which results in decreased production of the ternary complex needed for translation initiation, does not seem the major mechanism whereby translational inhibition is achieved. Based on the interaction between the G4C2 RNA repeat and eIF2a, that we found to occur both in vitro and in cell culture, it could be assumed that eIF2a function might be impaired independently from its phosphorylation, and that this might in turn be responsible for the observed assembly of stress granules. Indeed, stress granules are formed when the availability of eIF2a, as well as other translational factors, is decreased by siRNA (Mokas et al., 2009), a situation that might be mimicked by RNA repeat sequestration. As an alternative, decreased mRNA export from cell nuclei, as suggested by the clear accumulation of poly(A) RNA in the nuclei of cells expressing (G4C2)_31 repeats, might explain, at least in part, this phenomenon. Which is the factor that provokes nuclear accumulation of poly-adenylated mRNAs in the presence of G4C2 RNA expression? One interesting possibility is that nuclear accumulation of PABPc might be the principal driver of mRNA nuclear retention. The cytoplasmic form of PABP (PABPc) is a nucleo-cytosol shuttling protein with a steady state expression in the cytoplasm, where it enhances translational efficiency by bridging the poly(A) tail to the cap-binding complex via the eIF4G protein (Smith et al., 2014). Further, PABPc is found into stress granules upon cell stress (Kedersha et al., 2000). However, PABPc is also known to relocalise in the nuclei of cells when accumulation of mRNAs in cell nuclei as well as translational inhibition are induced by a subset of viral proteins (Smith and Gray, 2010). Interestingly, the obligatory expression of a nuclear targeted PABPc protein is sufficient to cause a dramatic increase in poly(A) mRNAs in cell nuclei (Kumar and Glaunsinger, 2010). Thus, PABPc localization appears as an important determinant of mRNA fate. We observed in this work that C9orf72 G4C2 repeats bind to PABPc, sequester it into nuclear foci, and induce its nuclear accumulation. For these reasons, it is tempting to speculate that nuclear retention of PABPc by G4C2 might represent a crucial point in the pathogenic cascade that originates from RNA repeat expression, and could be in accordance with those evidence showing that PABPc is found in TDP43-containing inclusions in ALS patients (McGurk et al., 2014), thus proposing PABPc as a possible important factor in the pathology of ALS. Yet, nuclear accumulation of PABPc might be one required component of the observed phenomena, but other factors might be necessary as well.
Among the \textit{C9orf72} binding partners, the mRNA export adaptor ALYREF has been recently described to be highly represented in RNA foci in cerebellar granule cells and in motor neurons from \textit{C9orf72} ALS patients (Cooper-Knock et al., 2014). This observation suggests that accumulated RNA repeats might interfere with the proper delivery of mRNAs from nuclei to cytosol, eventually affecting gene expression. As a matter of fact, Fragile X premutated rCGG repeats, that are responsible for Fragile X-associate tremor/ataxia syndrome (FXTAS), induce nuclear accumulation of mRNAs, including mRNAs involved in stress response, in a \textit{Drosophila} model (Qurashi et al., 2011). Notably, this process is mediated by the interaction of the \textit{Drosophila} homologue of p68 RNA helicase, Rm62, with Pura, that we show in this work to be profoundly affected in its localization by \textit{C9orf72} expression, and that has been suggested to play a pivotal role in \textit{C9orf72}-mediated neurodegeneration in \textit{Drosophila} (Xu et al., 2013). Whether mRNA export defects represent a disease relevant mechanism in ALS cases due to \textit{C9orf72}, and how this process might influence the formation of RAN peptides from \textit{C9orf72} translation, an important contributing factor to \textit{C9orf72} toxicity, obviously needs further investigations. Yet, motor neurons seem to be particularly sensitive to alterations in this process, as mutations in the human \textit{GLE1} gene, that has a central function in nuclear mRNA export, are responsible for the autosomal recessive lethal congenital contracture syndrome-1 (LCCS1), a disease characterised by lack of anterior horn motor neurons and severe atrophy of the ventral spinal cord (Folkmann et al., 2013).

In summary, we have shown here that accumulation of \textit{C9orf72} RNA repeats inside cells is associated to formation of stress granules, a marked translational repression and a striking accumulation of poly(A) mRNAs in cell nuclei. Our data therefore extend to \textit{C9orf72}-dependent ALS the emerging concept that stress granules-associated translational repression has a prominent role in neurodegenerative disorders, including Amyotrophic Lateral Sclerosis, and raise the intriguing possibility that the hexanucleotide expansion in \textit{C9orf72} may interfere with the cellular trafficking of poly(A) RNAs, thus increasing our current understanding of the molecular mechanisms underlying the pathogenesis of familial ALS.

**MATERIALS AND METHODS**

**DNA synthesis and plasmid construction**

CC(GGGGCC)\textsubscript{2}GGGG and CCGG(CCCCGG)\textsubscript{2}CC DNA fragments were synthesized with a 5’ phosphate (SIGMA). Complementary DNA strands were incubated at 95°C for 5 min in the presence of 100 mM potassium acetate, 2 mM magnesium acetate and 30 mM Hepes-KOH pH 7.4, then slowly cooled to 65°C. After 30 min, the reaction was further cooled to 4°C. 3 μg of annealed
oligos were ligated at room temperature for 24 hours by T4 DNA ligase (Promega) and then separated on 1% agarose gel. DNA fragments with different lengths were extracted and cloned into the XmnI site of pPCR-Script AmpSK(+) vector (Agilent Technologies). Plasmids containing 10 and 31 repeats were obtained and verified by automated sequencing. The hexanucleotide repeats were subcloned into the EcoRV/NotI sites of pcDNA5/FRT/TO expression vector (Invitrogen) and into BgIII/HindIII sites of pEGFP vector (Clontech). For subcellular distribution of poly(A) mRNAs, the repeats were inserted into NotI/BglII sites of pSUPER.retro.puro (Oligoengine), that contains an RNA polymerase III promoter, and previously modified for the presence of a transcription termination (6 thymidine residues immediately downstream the cloning site).

For (TTC)\textsubscript{270} cloning, a 1300bp DNA fragment containing 270 Friedreich's ataxia-associated repeats of a GAA sequence was PCR-amplified from genomic DNA of a human carrier and cloned into pGEM-T Easy Vector (Promega). NotI fragments were then inserted into pcDNA5/FRT/TO vector. Sequence orientation was controlled by restriction analysis of each vector and verified by automated sequencing, and the reverse orientation containing a 5'-(TTC)\textsubscript{270}-3' repeat was used for the experiments.

**Cell culture and transfection**

Mouse motor neuron-like NSC34 cells were grown in Dulbecco's modified Eagle's/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (tetracycline free, Lonza) at 37°C in an atmosphere of 5% CO\textsubscript{2} in air. Human neuroblastoma SH-SY5Y cells and cervical carcinoma HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in an atmosphere of 5% CO\textsubscript{2} in air. For transient expression of hexanucleotide repeats, NSC34 and HeLa cells were transfected with pcDNA5-(G4C2)\textsubscript{10}, pcDNA5-(G4C2)\textsubscript{31}, and pcDNA5-(TTC)\textsubscript{270} using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instruction.

**qRT-PCR analysis**

For quantitative RT-PCR (qRT-PCR), RNAs were isolated using TRIzol (Invitrogen) and treated with DNase. RNAs were quantified and reverse-transcribed with random primers according to a ImProm II reverse transcriptase kit (Promega). qRT-PCR was performed with LightCycler 480 SYBR Green I Master Mix (Roche). A 113bp sequence of pcDNA5/FRT/TO upstream the repeat cloning sites was amplified using the following primers: forward 5'-CGCAAATGGGCGGTAGGCGTG-3'; reverse 5'-CACTAAACGAGCTCGTCGACG-3'. Actin was also measured as an housekeeping gene.
RNA transcription *in vitro* and pull down assay

pPCR-Script-(G4C2)$_{31}$ and pGEM-(TTC)$_{270}$ vectors were linearized with *SacI* or *SalI*, respectively, and used as template for *in vitro* RNA transcription, that was performed with RNAMaxx High Yield Transcription Kit (Agilent Technologies) in the absence or presence of biotin-14-CTP (Invitrogen) in a one-to-one ratio of normal to modified cytosine, following the manufacturer's instructions. After treatment with RNAse-free DNase (Promega), RNA transcripts were purified with Phenol:Chloroform:Isoamyl alcohol 25:24:1 (Invitrogen). RNA was denatured at 90°C for 2 min, and incubated for 1 h at RT in the presence of 100 mM KCl, 10 mM MgCl$_2$ and 10 mM Tris-HCl, pH 7.4.

For pull down assay, mouse brain and spinal cords were homogenised using a Teflon homogenizer for 30 sec on ice in lysis buffer (250 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.5% Nonidet-P40, 0.5 mM DTT, 1 mM PMSF, protease inhibitor cocktail from Sigma-Aldrich). NSC34 and SH-SY5Y cell lysis was performed in the same buffer. A clear supernatant was obtained by centrifugation of cell and tissue lysates at 20,000xg for 5 min twice. Protein content was determined using Bradford protein assay (Bio-Rad). 1 mg of total protein extract was diluted in 0.5 ml of lysis buffer to a final NaCl concentration of 150 mM, and then pre-cleared with 60 µl of Dynabeads® M-280 Streptavidin (Invitrogen) for 30 min. The pre-cleared protein extract was incubated for 1 h with 5 µg of biotinylated (G4C2)$_{31}$ RNA, and then with 60 µl of Dynabeads® M-280 Streptavidin for 1 h. After five washes in lysis buffer, RNA-protein complexes were resuspended in 2x Laemmli sample buffer, boiled for 5 min and subjected to 4–12% NuPAGE gel electrophoresis (Invitrogen). A non-biotinylated (G4C2)$_{31}$ RNA was also pulled-down and analysed in the same conditions to check for unspecific binding of proteins. Gels were stained with Coomassie Blue for the following mass spectrometry analysis or subjected to Western blot analysis.

**Protein identification by mass spectrometry analysis**

Bands from gels were processed via tryptic proteolysis, after reduction and alkylation steps. The peptide mixtures were analysed by MALDI-ToF mass spectrometry (AutoFlexII, BrukerDaltonics) and the resulting peptide mass fingerprints used to identify proteins by Mascot search engine (Palermo et al., 2012).

All peptide mixtures were also analysed by a nano LCLTQ-Orbitrap mass spectrometer platform. After desalting (Rappsilber et al., 2007) and resuspension in 0.1% of formic acid, samples were loaded from an autosampler onto a 10 cm long silica capillary packed with C18 reverse phase resin,
using the Dionex Ultimate 3000 system (LC Packings, Dionex, Amsterdam, The Netherlands). The elution of peptides was monitored by a linear ion trap-orbitrap hybrid mass spectrometer (LTQ-Orbitrap Discovery, Thermo Fisher Scientific GmbH) equipped with a nanoelectrospray ion source (Thermo Fisher Scientific), operating in the positive ionization mode with a spray voltage of 1.9 kV. In data-dependent MS/MS scans, the five most abundant ions were fragmented in CID and analyzed in the linear trap. Data acquisition was controlled by Xcalibur 2.1. The MS/MS spectra were searched by MaxQuant (v. 1.4.1), against mouse UniProtKB FASTA database. The identifications with only one unique peptide were accepted.

Fluorescence *in situ* hybridization and immunofluorescence analysis

Cell cultures were grown on poly-L-lysine-coated glass coverslip, washed in PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Cells were then washed twice with 70% ethanol and stored in 70% ethanol at 4°C. Cells were rehydrated with 5 mM MgCl₂ in PBS for 30 min and then pre-hybridized in 35% formamide, 10 mM sodium phosphate, pH 7.0, and 2x SSC (300 mM NaCl, 30 mM sodium citrate) for 30 min at RT. For the probe hybridization, cells were incubated with 250 ng/ml of Cy3 labelled (C4G2)₄ (SIGMA) and/or Cy5-conjugated oligo(dT) in 30% formamide, 10% dextrane sulphate, 2x SSC, 0.2% BSA, 10 mM sodium phosphate, pH 7.0, and 0.5 mg/ml each of *E. coli* tRNA and sonicated salmon sperm DNA, at 37°C overnight in a humidified chamber. After hybridization, cells were washed twice with 35% formamide, 10 mM sodium phosphate, pH 7.0, and 2x SSC for 30 min each at 37°C; twice with 2x SSC, 0.1% Triton-X100, 15 min each at RT; twice with 0.2x SSC, 0.1% Triton-X100, 15 min each at RT. After washing, coverslips were mounted on the slides, or processed for immunofluorescence analysis as follows.

Cells were blocked for 30 min in PBS, 1% BSA and incubated for 1 h at 37°C with primary antibodies diluted in the same buffer. Cells were washed in PBS and incubated for 45 min with fluorophore-conjugated secondary antibodies diluted in PBS, 1% BSA. After rinsing in PBS, cells were stained with 1 μg/ml DAPI (SIGMA) and examined with a Zeiss LSM 510 Confocal Laser Scanning Microscope equipped with a 63× objective. Fluorescence images were processed using ZEN 2009 (Carl Zeiss) and Adobe Photoshop software.

**Protein extraction**

After rinsing with ice-cold PBS, cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.25% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 5 mM MgCl₂) containing a protease inhibitor cocktail (SIGMA). A clear supernatant was obtained by
centrifugation of lysates at 17,000xg for 10 min. Protein content was determined using Bradford protein assay (Bio-Rad).

For eIF2a phosphorylation measurement, cells were treated with 1 mM sodium orthovanadate for 30 min, then cell lysis was performed in RIPA buffer containing a protease inhibitor cocktail (SIGMA), 1 mM sodium orthovanadate, 50 mM sodium fluoride.

For translation rate measurement, prior of cell lysis, cell cultures were treated with 10 µg/mL puromycin for 10 min. Where specified, cells were previously treated with 1 mM cycloheximide for 10 min.

**Nuclear-cytosol fractionation**

After 24 hrs from transfection, HeLa cells were harvested, washed in ice-cold PBS and lysed in low-salt buffer (10 mM Hepes, pH 7.4, 42 mM KCl, 5 mM MgCl₂, 0.5% CHAPS, 1 mM DTT, 1 mM PMSF, 1 µg/ml leupeptin). After 10 min on ice, lysates were centrifuged at 2,000xg for 10 min. Supernatants were collected as cytosolic fractions, while pellets were resuspended in high-salt buffer (50 mM Tris–HCl, pH 7.4, 400 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet-P40, 10% glycerol, 2 mM DTT, 1 mM PMSF, protease inhibitor cocktail from Sigma-Aldrich). After 30 min on ice, lysates were centrifuged at 20,000xg for 15 min and supernatants were collected as nuclear fractions.

**Electrophoresis and western blot**

Protein samples were separated by SDS–PAGE and transferred to nitrocellulose membranes (Amersham). Membranes were blocked for 1 h in Tris-buffered saline solution, 0.1% Tween-20 (TBS-T) containing 5% non-fat dry milk, and then incubated for 2 hrs at room temperature or overnight at 4°C with indicated primary antibodies, diluted in TBS-T containing 2% non-fat dry milk. After rinsing with TBS-T solution, membranes were incubated for 1 h with the appropriated peroxidase-conjugated secondary antibody diluted in TBS-T containing 1% non-fat dry milk, then washed and developed using the ECL chemiluminescent detection system (Roche). Densitometric analyses were performed using ImageJ software program (National Institutes of Health).

**Antibodies and reagents**

The following antibodies were used: anti-hnRNP H, anti-RAX, anti-laminB goat polyclonal (Santa Cruz); anti-hnRNP U (H-94) rabbit polyclonal (Santa Cruz); anti-ILF2 (G-3), anti-SF2/ASF (96), anti-PABP (10E10) mouse monoclonal (Santa Cruz); anti-eIF2a (D7D3), anti-phospho-eIF2a
(D9G8) rabbit monoclonal (Cell Signaling); anti-eIF2b rabbit polyclonal (GeneTex); anti-ILF3 (EPR3626) rabbit monoclonal (GeneTex); anti-FUS rabbit polyclonal (Bethyl Laboratories); anti-TDP43 rabbit polyclonal (ProteinTech); anti-β-actin mouse monoclonal (SIGMA); anti-Puromycin (12D10) mouse monoclonal (Millipore); anti-MBNL1 mouse monoclonal (P11) (a kind gift of Annalisa Botta); anti-hnRNP A2/B1 mouse monoclonal (GeneTex); anti-FMRP rabbit polyclonal (Ram2); anti-TIAR mouse monoclonal (BD Transduction Lab); anti-Pura mouse monoclonal (Abcam, ab77734); anti-Pura rabbit polyclonal (Abcam, ab79936). Anti-rabbit, anti-mouse and anti-goat peroxidase-conjugated secondary antibodies were from BioRad; Alexa Fluor-conjugated secondary antibodies were from Invitrogen. Anti-mouse Cy3 was from Jackson ImmunoResearch Laboratories. Poly-U, Poly-G, Sodium Arsenite, Thapsigargin, Puromycin, and Cycloheximide were from SIGMA. PERK inhibitor GSK2606414 was from Calbiochem. tRNA was from Roche, ssDNA from SIGMA.

Statistical analysis
Statistical analysis was performed using two-tailed unpaired Student’s t-test. Values significantly different from the relative control are indicated with asterisks. P-values of 0.05 or 0.01 were considered significant.

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COMPETING INTERESTS
The authors declare no competing interests.

AUTHOR CONTRIBUTIONS
S.R., A.S., V.G., F.B., M.N. and M.C. performed the experiments; A.G., L.D.F. and M.E.S. performed mass spec analysis and interpretation; S.R., T.A. and M.C. designed the experiments, interpreted the data, prepared and wrote the manuscript. G.C. and C.B. contributed to data
interpretation and provided key reagents. M.T.C. contributed to experimental design and to provide grant funding to support this study.

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**REFERENCES**


FIGURE LEGENDS

**Figure 1.** (G4C2)$_{31}$ repeats form intracellular RNA foci. (A) NSC34 cells were transiently transfected with plasmids coding for 31 (upper panel) or 10 (middle panel) G4C2 repeats, and for 270 TTC repeats (lower panel). After 48 hrs, cells were subjected to FISH analysis with a C4G2 RNA probe conjugated to Cy3 (red). Nuclei were stained with DAPI (blue). Scale bar: 10 µM. (B) Cells were transfection as in (A). After 48 hrs, expression of repeat-containing RNAs was analysed by RT-qPCR. Shown are the levels relative to the (G4C2)$_{10}$ repeat expressing cells. RNAs from untransfected cells, as well as input RNAs from (G4C2)$_{31}$-transfected cells were used as negative control. Mean ± SD is shown (n = 3). (C) NSC34 cells were transfected with plasmids coding for (G4C2)$_{31}$ or (G4C2)$_{10}$ repeats and GFP. After 48 hrs, cells were subjected to FISH analysis with a Cy3-C4G2 RNA probe. Nuclei were stained with DAPI (blue). Scale bar: 10 µm. (C) The percentage of GFP expressing cells that show RNA foci was scored (mean ± SD from n=3 independent experiments is shown).

**Figure 2.** (G4C2)$_{31}$ repeat RNA binds factors involved in pre-mRNA splicing and mRNA translation. (A) Lysates from NSC34 cells were incubated at the indicated NaCl concentrations with biotinylated (G4C2)$_{31}$ repeat. Pulled-down proteins were then analysed in Western blot with the indicated antibodies. 0.2% and 2% of inputs were analysed as a loading control. The two different ILF3 isoforms of 90 and 110 kDa are bound. (B) Lysates from NSC34 cells were incubated at 200 mM NaCl concentration in the absence (no RNA) or in the presence of biotinylated (G4C2)$_{31}$ or (TTC)$_{270}$ repeats, and analysed in Western blot as indicated. 2% of inputs were analysed as a loading control. (C) Quantification of the precipitation efficiency shown in (B). The amounts of the indicated proteins are expressed as mean±SD of arbitrary densitometric units (AU) relative to (G4C2)$_{31}$ precipitates. Values significantly different from relative controls are indicated with an asterisk when p<0.05 and two asterisks when p<0.01 (n=3). (D) Lysates from NSC34 or SH-SY5Y cells were incubated with biotinylated (G4C2)$_{31}$ RNA and pulled-down proteins were analysed in Western blot with indicated antibodies. Two different exposures of inputs and pull down from the same blot are shown.

**Figure 3.** (G4C2)$_{31}$ repeats sequester RNA binding protein into RNA foci. (A) NSC34 cells were transiently transfected with a plasmid expressing (G4C2)$_{31}$ repeats and analysed after 48 hrs by RNA FISH using a Cy3-(C4G2)$_{4}$ probe (red) and by immunofluorescence staining (green) with antibodies recognizing different RNA repeat binding proteins (eIF2a, FUS, hnRNP H, ILF3).
HeLa cells were transiently transfected with a plasmid expressing (G4C2)\textsubscript{31} repeats and analysed after 24 hrs as in (A). The overlay of the two colours is shown. Nuclei were stained with DAPI. Scale bar: 10 μm.

Figure 4. (G4C2)\textsubscript{31} repeats affect Pura and FMRP localization in NSC34 and HeLa cells. (A) NSC34 cells were transiently transfected with a (G4C2)\textsubscript{31}-expressing plasmid and analysed after 48 hrs by RNA FISH using a (C4G2)\textsubscript{4} probe conjugated to Cy3 (red) and by immunofluorescence staining (green) with antibodies anti-Pura (upper panels) and anti-FMRP (lower panels). In untrasfected cells, Pura and FMRP have a diffuse cytosolic localization, while in cells that express (G4C2)\textsubscript{31} repeats both proteins accumulate into cytosolic and nuclear granules. White arrowhead point to a nuclear granule where Pura and G4C2 repeat colocalise. Higher magnifications of the areas highlighted in the inset are shown together with DAPI staining. (B) HeLa cells were transiently transfected with (G4C2)\textsubscript{31} repeats and analysed after 24 hrs by RNA FISH as in (A). Scale bar: 20 μm.

Figure 5. (G4C2)\textsubscript{31} repeats induce stress granules formation. (A) HeLa cells were transiently transfected with a plasmid expressing (G4C2)\textsubscript{31} repeats. After 24 hrs, RNA foci were visualised by FISH analysis with a Cy3-(C4G2)\textsubscript{4} probe conjugated to Cy3 (red) together with immunofluorescence staining with antibodies anti-TIAR (green) and anti-Pura (blue). The overlay of the three colours (merge), and a higher magnification of the area highlighted in the inset are shown. In cells expressing (G4C2)\textsubscript{31} repeats, Pura accumulates in TIAR-positive stress granules. Nuclei were stained with DAPI (grey). Scale bar: 20 μm. (B) HeLa cells were transfected with (G4C2)\textsubscript{10} or (TTC)\textsubscript{270} plasmids as in (A). After 24 hrs, immunofluorescence staining with antibodies anti-TIAR (green) and anti-Pura (blue) was performed, together with DAPI staining (grey). (C) Cells were transfected as in (A) with the indicated plasmids. After 24 hrs, expression of repeat-containing RNAs was analysed by RT-qPCR. Shown are the levels relative to the (G4C2)\textsubscript{10} repeat expressing cells. RNAs from untransfected cells, as well as input RNAs from (G4C2)\textsubscript{31}-transfected cells were used as negative control. Mean ± SD is shown (n=3). (D) Cells transfected with the indicated plasmids and presenting stress granules were scored and plotted according to the presence (w/, red) or absence (w/o, blue) of RNA foci. Values are reported as mean percentage ± SD from three independent experiments. Values significantly different from relative controls are indicated with an asterisk when p<0.01.
Figure 6. (G4C2)31 repeats induce eIF2a phosphorylation-independent translational arrest.
(A) Untransfected HeLa cells or transiently transfected with mock, (G4C2)10 or (G4C2)31 plasmids, were grown in the absence or presence of the PERK inhibitor GSK2606414 (PI), at the indicated concentrations. After 24 hrs, where specified, cells were treated with 5 µM thapsigargin (TG) for 2 hrs, and cell lysates were analysed in Western blot with antibodies that specifically recognizes phosphorylated eIF2a (P-eIF2a) or total eIF2a. Lower panel shows the quantification of the bands, presented as the ratio of the phospho-eIF2a over the total eIF2a and expressed in arbitrary units (AU), and reported as mean ± SD; n = 4. Values significantly different from relative controls are indicated with an asterisk when p < 0.01. (B) HeLa cells were left untrasfected (NT), or were transiently transfected with an empty vector (mock), or vectors containing (G4C2)10, (G4C2)31 and (TTC)270 repeats. After 24 hrs, cells were treated with 10 µg/mL puromycin for 10 min and cell lysates were analysed in Western blot with an antibody anti-puromycin. β-actin was analysed as a standard for equal protein loading. Puromycin incorporation rate in untransfected cells pre-treated with 1 mM cycloheximide (CHX) for 10 min, was also analysed. Lower panel shows the quantification of the bands, expressed in arbitrary units (AU), relative to untransfected cells, and reported as mean ± SD; n = 3. Values significantly different from relative controls are indicated with an asterisk when p < 0.01. (C) HeLa cells were transiently transfected with a plasmid expressing (G4C2)31 repeats. After 24 hrs, cells were treated with puromycin, and then analysed by Cy3-(C4G2)4 RNA FISH (red) and by immunofluorescence staining with an anti-puromycin antibody (green). Nuclei were stained with DAPI (grey). (D) NSC34 cells were transiently transfected with (G4C2)31 repeats, and analysed as in (C) after 48 hrs.

Figure 7. mRNAs accumulate in the nuclei of (G4C2)31 repeats expressing cells. (A) NSC34 cells were transiently transfected with a plasmid (pSUPER.retro.puro) expressing (G4C2)31 repeats and analysed after 48 hrs by RNA FISH using a (C4G2)4 probe conjugated to Cy3 (red) and an oligo(dT) labelled with Cy5 (blue). Scale bar: 20 µm. (B) HeLa cells were transfected and analysed after 24 hrs as in (A). In cells expressing (G4C2)31 repeats mRNAs accumulate as nuclear dots, that partially colocalize with RNA foci. A higher magnification of the areas highlighted in the inset is shown, together with DAPI staining (grey). Scale bar: 10 µm. (C) Cells presenting RNA foci were scored and plotted according to the presence of mRNA accumulated in nuclei. At least 50 cells for each condition were counted. Values are reported as mean percentage ± SD from three independent experiments. (D) HeLa cells were treated with 1 mM NaAs for 30 min and analysed by RNA FISH using an oligo(dT) labelled with Cy5 (blue). Cells were also stained with an antibody anti-TIAR.

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(green). A higher magnification of the area highlighted in the inset is shown, together with DAPI staining (grey).

**Figure 8.** (G4C2)$_{31}$ repeats induce the re-localization of cytoplasmic PABP in cell nuclei. (A) HeLa cells were transiently transfected with (G4C2)$_{31}$ repeats and analysed after 24 hrs by RNA FISH (red) and by immunofluorescence staining with antibodies anti-PABPc (green) and anti-Pura (blue). Nuclei were stained with DAPI (grey). Scale bar: 20 μm. (B) HeLa cells were transfected with (G4C2)$_{10}$ or (TTC)$_{270}$ plasmids and stained after 24 hrs with anti-PABPc (green) and anti-Pura (blue) antibodies. (C) HeLa cells were transiently transfected with (G4C2)$_{31}$ repeats or left untransfected (NT). After 24 hrs, nuclear and cytosolic fractions from cells were isolated and analysed by Western blot using an antibody anti-PABPc. Pura and the nuclear protein lamin B were also examined as standards for equal protein loading in the two fractions. (D) Lysates from HeLa cells were incubated at 200 mM NaCl concentration in the absence (no RNA) or in the presence of biotinylated (G4C2)$_{31}$ or (TTC)$_{270}$ repeats, and analysed in Western blot with an anti-PABPc antibody. 0.5% input was analysed as a loading control. (E) HeLa cells were treated with 1 mM NaAs for 30 min and analyzed by immunofluorescence staining with anti-PAPBc (green) and anti-Pura (blue) antibodies. A higher magnification of the highlighted area is shown.
Figure 2

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