Cellular bases of the RNA metabolism dysfunction in motor neurons of a murine model of spinal muscular atrophy: Role of Cajal bodies and the nucleolus

Olga Tapia a, 1, Josep Oriol Narcis a, 1, Javier Riancho b, Olga Tarabal c, Lidia Piedrafita c, Jordi Calderó c, Maria T. Berciano a, Miguel Lafarga a, *

a *Department of Anatomy and Cell Biology and "Centro de Investigacion Biomedica en Red sobre Enfermedades Neurodegenerativas (CIBERNED)", University of Cantabria-IDIVAL, Santander, Spain*

b *Service of Neurology, University Hospital Marques de Valdecilla-IDIVAL-CIBERNED, Santander, Spain*

c *Department of Experimental Medicine, School of Medicine, University of Lleida and "Institut de Recerca Biomedica de Lleida" (IRBLLEIDA), Lleida, Spain*

ABSTRACT

Spinal muscular atrophy (SMA) is a severe motor neuron (MN) disease caused by the deletion or mutation of the *survival motor neuron 1* (*SMN1*) gene, which results in reduced levels of the SMN protein and the selective degeneration of lower MNs. The best-known function of SMN is the biogenesis of spliceosomal snRNPs, the major components of the premRNA splicing machinery. Therefore, SMN deficiency in SMA leads to widespread splicing abnormalities.

We used the SMNΔ7 mouse model of SMA to investigate the cellular reorganization of polyadenylated mRNAs associated with the splicing dysfunction in MNs. We demonstrate that SMN deficiency induced the abnormal accumulation of poly(A) RNAs in nuclear granules enriched in the splicing regulator Sam68. However, these granules lacked other RNA-binding proteins, such as TDP43, PABPN1, hnRNPA12B, REF and Y14, which are essential for mRNA processing and nuclear export. These effects were associated with changes in the alternative splicing of the Sam68-dependent *Bcl-x* and *Nrnx1* genes, as well as changes in the relative accumulation of the intron-containing *Chat*, *Chodl*, *Myh9* and *Myh14* mRNAs, which are all important for MN functions. Moreover, the massive accumulation of poly(A)

RNA granules in MNs was accompanied by the cytoplasmic depletion of polyadenylated mRNAs for translation.

We suggest that the nuclear accumulation of polyadenylated mRNAs in granules is an SMA hallmark in SMNΔ7 MNs. We also propose that the SMN-dependent abnormal accumulation of polyadenylated mRNAs and Sam68 in these granules reflects a severe dysfunction of mRNA metabolism in MNs and is an important contributor to SMA pathogenesis.

Keywords: Spinal muscular atrophy, motor neurons, poly(A) RNA retention, Sam68, nuclear speckles, poly(A) RNA granules

INTRODUCTION

The processing of mRNAs includes three essential modifications, capping, splicing and polyadenylation, which mainly occur at sites of transcription (co-transcriptional) (Girard et al., 2012; Moore and Proudfoot, 2009). An analysis of the human transcriptome revealed that >90% of protein-coding genes undergo alternative splicing, which generates multiple mRNA variants that encode the vast proteomic repertoire required for the protein interactome (Poulos et al., 2011). Another fundamental modification of most mRNAs is the addition of a polyadenosine tail at the 3' end. Polyadenylation is a modification necessary not only for the stability of mRNAs but also for their nuclear export (Kühn and Wahle, 2004). Poly(A)-binding protein nuclear 1 (PABPN1) plays a critical role in polyadenylation by strongly enhancing the processivity of poly(A) polymerase (Kühn and Wahle, 2004). Upon the completion of co-transcriptional processing, mRNAs are exported to the cytoplasm as parts of messenger ribonucleoprotein particles for translation and degradation. During their nuclear journey, mRNAs associate with several RNA-binding proteins that contribute to the regulation of their processing and export.

On their nuclear route, some normal and incompletely spliced mRNAs are trafficked and may be retained in nuclear speckles (Bahar Halpern et al., 2015; Girard et al., 2012; Johnson et al., 2000; Smith et al., 2007). These nuclear compartments store, assemble and recycle splicing factors and are also enriched in poly(A) RNAs and several mRNA processing factors, including PABPN1 (Calado and Carmo-Fonseca, 2000; Hall et al., 2006; Lamond and Spector, 2003; Tavanez et al., 2005).

Defects in premRNA splicing or polyadenylation often prevent the recruitment of mRNA export factors, resulting in the nuclear retention of immature or aberrant premRNAs (Schmid and Jensen, 2010). Thus, some abnormal mRNA transcripts may

accumulate in nuclear inclusions under pathological conditions, such as oculopharyngeal muscular dystrophy, myotonic dystrophy type 1 and fragile X-associated tremor/ataxia syndrome (Bengoechea et al., 2012; Klein et al., 2016; Qurashi et al., 2011; Smith et al., 2007). Moreover, we have previously reported that the dysfunction of nuclear RNA processing in the sensory ganglion upon proteasome inhibition induces the nuclear aggregation of polyadenylated mRNAs and the RNA-binding protein Sam68 (src-associated protein in mitosis of 68 kD) into a new nuclear structure called the "poly(A) RNA granule" (PARG) (Casafont et al., 2010; Palanca et al., 2014). The sequestration of crucial RNA-binding proteins in nuclear inclusions or granules may prevent their normal function and contribute to disease pathogenesis (Renoux and Todd, 2012).

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by the progressive degeneration and loss of spinal cord and brainstem motor neurons (MNs) (Soler-Botija et al., 2002; Talbot and Tizzano, 2017). SMA is caused by a homozygous deletion or mutation in the *survival of motor neuron 1* (*SMN1*) gene that results in decreased levels of the full-length SMN protein (Burghes and Beattie, 2009; Lefebvre et al., 1995). SMA patients carry a nearly identical *SMN1* gene paralogue named *SMN2*, which differs from *SMN1* by a C to T transition in exon 7 (Burghes and Beattie, 2009; Lefebvre et al., 1997). Although both the *SMN1* and *SMN2* genes encode the SMN protein, approximately 90% of the *SMN2* mRNA transcripts generate an alternatively spliced isoform that lacks exon 7 and encodes a truncated form of the SMN protein (SMNΔ7) that is rapidly degraded (Monani et al., 2000). Therefore, *SMN2* expression cannot fully compensate for the deficiency of the full-length SMN protein. The best-known function of SMN is the biogenesis of spliceosomal snRNPs (for a review, see (Cauchi, 2010; Matera and Wang, 2014)). Linked to this function, SMN

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deficiency in SMA produces alterations in this snRNP repertoire and causes widespread splicing defects that result in a severe dysregulation of mRNA metabolism in MNs (Bäumer et al., 2009; Doktor et al., 2017; Lotti et al., 2012; Tisdale and Pellizzoni, 2015; Zhang et al., 2008). Splicing alterations in the spinal cord in murine SMA models include widespread intron retention, particularly the retention of minor U12 introns, as well as a time-dependent differential expression of a number of exons associated with neurodevelopmental and cell stress pathways (Bäumer et al., 2009; Doktor et al., 2017; Jangi et al., 2017; Lotti et al., 2012; Zhang et al., 2008). In addition to the dysfunction of premRNA processing, as a pathogenic factor in SMA, recent studies identified ribosome biology and translation efficiency as key processes affected by SMN depletion, which may also contribute to MN degeneration and SMA pathogenesis (Bernabò et al., 2017; Tapia O et al., 2017).

The aim of this work is to analyze the subcellular reorganization of polyadenylated mRNAs and certain RNA-binding proteins induced by the SMN-dependent dysfunction of premRNA splicing in MNs from the SMNΔ7 mouse model of SMA. Our results indicate that the perturbation of premRNA splicing interferes with both the nuclear processing and export of polyadenylated mRNAs, resulting in their nuclear accumulation in PARGs. These granules are also enriched in the RNA-binding protein Sam68, which is an alternative splicing regulator of premRNAs (Iijima et al., 2011; Lukong and Richard, 2003; Richard, 2010). The formation of PARGs correlates with variations in the alternative splicing regulation of several mRNAs, including the Sam68-dependent *Bcl-x* and *Nrxn-1* mRNAs. Moreover, the massive accumulation of PARGs in MNs is associated with cytoplasmic depletion of polyadenylated mRNAs, supporting the existence of a severe dysfunction of both mRNA processing and translation, which may be an important pathogenic factor in SMA.

RESULTS

Distribution of polyadenylated RNAs in WT MN perikarya

With the exception of histone mRNAs, all mRNAs are polyadenylated and have a poly(A) tail that is essential for their export and stability (Kühn and Wahle, 2004; Moore and Proudfoot, 2009) (More and Prodfoot 2009; Kühn and Wahle, 2004). Therefore, *in situ* hybridization of poly(A) RNAs with an oligonucleotide poly(dT) probe, which recognizes the poly(A) tail, is a good approach for studying the perikaryal distribution of global mRNAs in MNs.

After staining with propidium iodide (PI), a cytochemical fluorescent stain for nucleic acid detection, dissociated MNs from wild-type (WT) mice exhibited a prominent nucleolus and large RNA-rich cytoplasmic areas of the Nissl substance, a classic name used to designate the neuronal protein synthesis machinery (for a review, see (Peters et al., 1970)) (Fig. 1A). Immunolabeling for the TMG-cap and coilin, which are markers of nuclear speckles and Cajal bodies, respectively, revealed the organization of these two nuclear compartments involved in mRNA processing. As shown in Fig. 1B and C, several nuclear speckles and at least one Cajal body were prominent nuclear structures in WT MNs. We next performed fluorescence *in situ* hybridization for the detection of poly(A) RNAs in dissociated WT MNs. Poly(A) RNAs, in addition to being diffusely distributed throughout the nucleus, excluding the nucleolus, were concentrated in nuclear speckles (Fig. 1D, E). As expected, in the cytoplasm, poly(A) RNAs were accumulated in the Nissl substance, the main site of mRNA translation (Fig. 1D, E). Triple labeling for poly(A) RNA in combination with the TMG-cap and PABPN1, two molecular markers of nuclear speckles, demonstrated

the accumulation of poly(A) RNAs in these nuclear compartments (Fig. 1E-H), as confirmed by plotting the fluorescence intensity profile across a line (Fig. 1I).

Polyadenylated mRNA relocalizes in nuclear poly(A) RNA granules in SMNΔ7 MNs

Next, we analyzed whether the dysfunction of RNA metabolism in SMNΔ7 MNs affects the nuclear and cytoplasmic organization of poly(A) RNAs. *In situ* hybridization revealed an important nuclear reorganization of poly(A) RNA in some SMNΔ7 MNs during both the late-presymptomatic (postnatal day [P]5) and symptomatic (P14) stages, compared with the staining pattern of WT MNs (Figs. 1E, 2A-F). There were notable variations in poly(A) RNA signal intensity in nuclear speckles and, remarkably, the *de novo* formation and accumulation of round and sharply defined bodies enriched in poly(A) RNA, which were identified as PARGs (Casafont et al., 2010) (Fig. 2B-F).

The PARGs, which ranged from 0.2 to 1.5 μm in diameter, were distributed throughout the nucleus, excluding the nucleolus. They frequently appeared in close proximity to the nucleolus and nuclear speckles (Fig. 2B, C, E). Two structural configurations of PARGs were found: compact nuclear bodies and ring-shaped structures with a poly(A) RNA-rich ring enclosing a hybridization signal-poor central region (Fig. 2C, F). Quantitative analysis showed that approximately 17% and 28% of MNs had PARGs at P5 and P14, respectively (Fig. 2G), ranging in number from very few to more than 50 PARGs per nucleus.

Interestingly, MNs harboring a large number of PARGs commonly exhibited a weaker poly(A) RNA hybridization signal in the cytoplasm (Fig. 2E, F). Moreover, the cytoplasmic accumulation of poly(A) RNAs in stress granules (Anderson and Kedersha, 2006; Hua and Zhou, 2004) was not observed in SMNΔ7 MNs. Changes in the relative concentration of poly(A) RNA in the cytoplasm were validated by a densitometric

analysis of the fluorescence hybridization signal intensities at P14. Multiple measurements of the poly(A) RNA signal intensities were performed in the peripheral cytoplasm, where the Nissl substance is largely distributed in MNs. A significant reduction of the relative poly(A) RNA concentration was detected in the Nissl substance of PARG-containing MNs, compared with PARG-free MNs, from both SMNΔ7 and WT mice (Fig. 2H).

Electron microscopy analysis of PARG-containing MNs revealed structural features of severe neuronal dysfunction, including a paucity of the protein synthesis machinery, nuclear shape aberrations with numerous nuclear envelope invaginations (Fig. 3A) and nucleolar segregation of the granular component (Fig. 3B, C), as we have recently reported (Tapia O et al., 2017). Ultrastructural analysis confirmed the presence of two categories of PARGs: rounded, compact electron-dense bodies (Fig. 3A) and larger spherical bodies composed of an electron-dense capsule enclosing an amorphous matrix of very low electron density (Fig. 3A, left inset). In addition, PARGs normally appeared to be closely surrounded by a layer of perichromatin granules (Fig. 3C, left inset), which are sites of storage for hnRNP complexes (Biggiogera and Fakan, 1998). Importantly, PARGs localized in euchromatin domains, wherein co-transcriptional premRNA processing occurs (Girard et al., 2012; Moore and Proudfoot, 2009), and they frequently appeared in close proximity to the nucleolus (Fig. 3B) and interchromatin granule clusters (Fig. 3A, right inset), the ultrastructural counterpart of nuclear speckles (Lamond and Spector, 2003).

The PARG is a distinct nuclear compartment

To establish the identity of the PARG as a distinct nuclear structure in SMA MNs, we performed double labeling for poly(A) RNA in combination with molecular markers of nuclear compartments, such as coilin (Cajal bodies), SMN (gems), the TMG-cap

(nuclear speckles) and the 20S proteasome (clastosomes). In a recent study, we demonstrated that the reduced levels of SMN in MNs from SMNΔ7 mice were associated with a severe depletion of Cajal bodies (Tapia O et al., 2017), nuclear structures involved in the biogenesis of both spliceosomal snRNPs and nucleolar snoRNPs (for a review, see (Lafarga et al., 2017; Machyna et al., 2013). Co-staining for poly(A) RNA and coilin confirmed the depletion of typical Cajal bodies in SMNΔ7 MNs. Nevertheless, small coilin-positive and poly(A) RNA-negative residual Cajal bodies were occasionally found adjacent to PARGs, but as two clearly distinct nuclear structures (Fig. 4A). Similarly, gems, SMN-positive and coilin-negative nuclear bodies (Lafarga et al., 2017; Liu and Dreyfuss, 1996), were never observed in SMNΔ7 MNs, and SMN was not concentrated in PARGs (Fig. 4B). Moreover, PARGs did not concentrate spliceosomal snRNPs, which typically appeared to be enriched in nuclear speckles immunolabeled for the TMG-cap of spliceosomal snRNAs (Fig. 4C). Finally, the catalytic 20S proteasome, a molecular marker of clastosomes, which are nuclear bodies enriched in ubiquitylated proteins and active 20S proteasomes (Lafarga et al., 2002), was not concentrated in PARGs (Fig. 4D).

PARGs concentrate the RNA-binding protein Sam68.

Since the RNA-binding protein Sam68 is a regulator of *SMN2* alternative splicing (Pedrotti et al., 2010), we investigated its nuclear reorganization in SMN-deficient MNs from SMNΔ7 mice. In WT MNs, co-staining for Sam68 and poly(A) RNA revealed a predominant nuclear localization of Sam68, which excluded the nucleolus and poly(A) RNA-positive nuclear speckles (Fig. 5A-C). The nuclear distribution was non-homogeneous, with extensive areas of diffuse staining, and a few irregular domains in which higher levels of Sam68 accumulation were observed (Fig. 5B). Although the basic nuclear pattern of Sam68 immunostaining was preserved in SMNΔ7 MNs, this

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splicing regulator was strongly concentrated in PARGs (Fig. 5D-F). Representing the fluorescence intensity profiles of poly(A) RNA and Sam68 across a line confirmed the colocalization of both signals in PARGs from SMNΔ7 MNs, as well as the absence of Sam68 in nuclear speckles (Fig. 5G, H). Similarly, immunogold electron microscopy for Sam68 showed that both the compact and the ring-shaped PARGs were decorated with numerous gold particles (Fig. 5I, J).

We next investigated whether the accumulation of Sam68 in PARGs was associated with changes in Sam68 mRNA and protein levels in tissue extracts from the spinal cord at P5 and P14. Although we observed a trend indicating the downregulation of Sam68 mRNA levels in SMNΔ7 mice, this decrease was not significant in the qRT-PCR validation (Fig. 5K). Similarly, western blotting analysis revealed no significant changes in Sam68 protein levels in samples from SMNΔ7 mice compared with those from WT littermates (Fig. 5L). However, as expected, a severe reduction of SMN protein levels was observed in SMNΔ7 mice (Fig. 5L).

Previous studies have demonstrated that Sam68 regulates the alternative splicing of two important genes for neuronal function, *Bcl-x* and *Nrxn1,* which encode an apoptotic regulatory factor and the presynaptic membrane protein neurexin, respectively (Iijima et al., 2011; Paronetto et al., 2007a). These studies also suggest that the relocation of Sam68 in nuclear foci affects the alternative splicing of its premRNA targets (Paronetto et al., 2007a). This finding prompted us to investigate whether the partial relocation of this splicing regulator in PARGs is associated with changes in the balance of *Bcl-x* and *Nrxn1* splicing isoforms. The *Bcl-x* transcript is alternatively spliced to generate the antiapoptotic *Bcl-x(L)* variant or the proapoptotic *Bcl-x(s)* variant (Boise et al., 1993). Real-time PCR quantification of these two variants in spinal cord extracts showed a significant decrease of the *Bcl-x(s)/Bcl-x(L)* ratio in SMNΔ7 mice

compared with WT mice (Fig. 5M). Similarly, we found a significant reduction of the *Nrxn1 4(-)/Nrxn1 4(+)* ratio in SMNΔ7 mice (Fig. 5M), which reflects a higher relative abundance of the isoform *4(+)*, which includes exon 20 in *Nrxn1* alternatively spliced segment 4 (AS4).

PARGs did not concentrate other RNA-binding proteins involved in mRNA processing and export

Having demonstrated the concentration of Sam68 in PARGs, we then proceeded to investigate the possible accumulation in these granules of other RNA-binding proteins involved in nuclear mRNA processing and export, such as PABPN1, TDP43 (TAR DNA-binding protein 43), hnRNP (heterogeneous nuclear ribonucleoprotein) A2/B1, hnRNP M3, Y14 and REF/Aly.

Since PABPN1 binds to the poly(A) tail of polyadenylated RNAs (Kühn and Wahle, 2004) and is required for efficient poly(A) RNA export from the nucleus (Apponi et al., 2010), we investigated changes in its nuclear pattern and protein levels in WT and SMNΔ7 mice. PABPN1 was concentrated in nuclear speckles in both WT and SMNΔ7 MNs (Figs. 6A, 7B-D), but not in PARGs (Fig. 7F), indicating that PABPN1 is not linked to the poly(A) RNAs within these granules. Moreover, western blotting analysis revealed significantly reduced PABPN1 levels in the spinal cord of SMNΔ7 mice compared with those of WT animals (Fig. 7E).

TDP43 plays a central role in the pathogenesis of MN diseases (Lee et al., 2012). This protein is a component of the hnRNP particles that regulate the splicing of a variety of premRNAs (Lee et al., 2012; Sephton et al., 2011). TDP43 immunolabeling showed a diffuse nuclear localization, excluding nuclear speckles and the nucleolus, in both WT and SMNΔ7 MNs, but the protein was not detectable in pathological PARGs (Figs. 6B, 7G). Similarly, immunostaining for two hnRNP family proteins, A2/B1 and

M3, which are involved in packing nascent premRNA and in alternative splicing regulation (Bekenstein and Soreq, 2013; Smith et al., 2014), revealed their diffuse nuclear distribution in both WT and SMNΔ7 MNs (Figs. 6C, D, 7H, I). No labeling of PARGs was detectable for the A2/B1 and M3 proteins (Fig. 7H, I).

To further investigate the possible concentration of post-spliced mRNA-binding proteins in PARGs, we focused on the detection of REF/Aly and Y14, two RNA-binding proteins that are directly implicated in the nuclear trafficking and export of mRNAs from the nucleus (Kim et al., 2001; Rodrigues et al., 2001). REF and Y14 showed a diffuse nuclear localization, which excluded the nucleolus, in addition to being concentrated in nuclear speckles in both WT and SMNΔ7 MNs (Figs. 6E, F, 7J, K). Interestingly, neither export factor was accumulated in pathological PARGs (Fig. 7J, K). The lack of colocalization of the REF/Aly and Y14 proteins with poly(A) RNA-positive PARGs was confirmed by the representation of their fluorescence intensity profiles across a line (Fig. 7F-K).

Accumulation of intron-containing premRNAs encoding essential proteins for MN function in SMNΔ7 mice.

Previous studies have demonstrated that SMA severity correlates with the decreased assembly of spliceosomal snRNP complexes, which leads to widespread defects in the splicing of genes expressed in MNs (Doktor et al., 2017; Lotti et al., 2012; Zhang et al., 2008). On this basis, we investigated whether the nuclear accumulation of polyadenylated mRNAs in PARGs was associated with the splicing dysfunction of four genes, *Chat, Chodl, Myh9* and *Myh14*, which are important for MN maturation and synapse development and function (Bäumer et al., 2009; Newell-Litwa et al., 2015). These genes encode choline acetyltransferase (*Chat*) and chondrolectin (*Chodl*), which are processed by the major U2-dependent spliceosome, and the non-muscle myosin II

isoforms IIA (*Myh9*) and IIC (*Myh14*), which are processed by the minor U12-dependent spliceosome. Splicing efficiency was analyzed by estimating the percentage of the unspliced (exon-intron sequence) forms of the *Chodl*, *Chat*, *Myh9* and *Myh14* premRNAs by qRT-PCR in spinal cord RNA extracts. Importantly, we found that in relation to WT samples, samples from P5 SMNΔ7 animals had a significant increase in the proportion of unspliced forms of the *Chat* and *Myh14* mRNAs, while the accumulation of the unspliced forms was extended to the four examined premRNAs, *Chodl*, *Chat*, *Myh9* and *Myh14*, during the late symptomatic stage (P14) (Fig. 8A, B). No significant changes in unspliced *Actb* (the beta-actin housekeeping gene) premRNAs were detected when samples from WT and SMNΔ7 mice were compared at P5 or P14 (Fig. 8A, B).

DISCUSSION

The present study demonstrates that the SMN deficiency in SMNΔ7 MNs affects the nuclear distribution of polyadenylated mRNAs, resulting in their progressive accumulation in Sam68-positive PARGs. During the symptomatic stage, this response is associated with an increased proportion of intron-containing *Chat, Chodl, Myh9* and *Myh14* mRNAs, suggesting that the formation of PARGs correlates with a splicing perturbation in SMNΔ7 MNs. The accumulation of poly(A) RNA in PARGs appears to be a clear and early (P5) cellular indicator of the RNA metabolism dysfunction in SMA MNs. Indeed, MNs that contain numerous PARGs frequently exhibit other signs of neuronal dysfunction. These signs include the nucleolar segregation and cytoplasmic reduction of polyadenylated mRNAs, which support defective ribosome biogenesis and translation, according to previous studies in SMA mice (Bernabò et al., 2017; Tapia O et al., 2017). Collectively, our results suggest that splicing alterations in SMA MNs lead

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to a nuclear accumulation in PARGS of incompletely processed polyadenylated mRNAs, which could contribute to MN degeneration and SMA pathogenesis.

A poorly understood aspect of SMA is why MNs that carry the same deletion or mutation of the *SMN1* gene are affected differently. Our results confirm this heterogeneity, showing that at both P5 and P14, affected MNs carrying PARGs coexist with other MNs that have an apparently normal pattern of poly(A) RNA distribution. In this regard, differences in the pathogenic vulnerability of MNs have recently been correlated with individual differences in SMA protein levels in MNs from a particular SMA mouse (Rodriguez-Muela et al., 2017).

Although we have previously reported the presence of PARGs in sensory ganglion neurons upon proteasome inhibition (Casafont et al., 2010; Palanca et al., 2014), the present work provides the first demonstration that these structures are present in SMA MNs. The PARG represents a distinct nuclear entity that is clearly distinguishable by its structure and molecular composition from other nuclear compartments, such as nuclear speckles (Lamond and Spector, 2003), Cajal bodies (Cioce and Lamond, 2005; Gall, 2000; Lafarga et al., 2016), gems (Liu and Dreyfuss, 1996) and clastosomes (Lafarga et al., 2002). PARGs share with nuclear speckles the presence of poly(A) RNAs; however, PARGs lack spliceosomal snRNPs and display an ultrastructural configuration as sharply defined nuclear bodies that is clearly different from the interchromatin granule clusters of nuclear speckles (Lamond and Spector, 2003; Tapia O et al., 2017). Cajal bodies, the nuclear structures involved in spliceosomal snRNP biogenesis (Machyna et al., 2013), are absent in SMNΔ7 MNs (Tapia O et al., 2017); additionally, the Cajal body marker coilin was not detected in PARGs. Similarly, gems, SMN-positive and coilin-negative nuclear bodies of unknown function (Lafarga et al., 2017; Liu and Dreyfuss, 1996), have been reported in fetal MNs

(Young et al., 2001), but they are absent in postnatal and mature MNs from both WT and SMNΔ7 mice (Tapia O et al., 2017). Moreover, we did not detect SMN in PARGs. Finally, these RNA granules also lack the 20S proteasome, a molecular marker of clastosomes, which are nuclear proteolytic factories enriched in catalytic proteasomes, ubiquitylated proteins and proteasome substrates (Carmo-Fonseca et al., 2010; Lafarga et al., 2002).

PARG-containing MNs were already observed at P5, but they increased in number during the late symptomatic stage (P14), presumably reflecting the well-established asynchrony at the beginning of MN degeneration in SMNΔ7 mice (Tarabal et al., 2014). We propose that the formation of PARGs in SMNΔ7 MNs reflects a stress-related dysfunction of RNA metabolism, essentially in premRNA splicing. Consistent with this view, premRNA splicing has emerged as a well-known and important target of several stressing agents, resulting in alternative splicing dysregulation and splicing inhibition (Biamonti and Caceres, 2009a). In the case of SMA, several studies demonstrated that the hyperactivation of the endoplasmic reticulum stress pathway and widespread defects in splicing underlie the neurodegeneration observed in SMA MNs (Doktor et al., 2017; Jangi et al., 2017; Lotti et al., 2012; Ng et al., 2015; Zhang et al., 2008). Moreover, we have previously reported the formation of PARGs following proteasome inhibition-induced proteotoxic stress in rat sensory ganglion neurons, an experimental condition that produces a dysfunction of RNA metabolism and a disruption of the protein synthesis machinery (Casafont et al., 2010; Palanca et al., 2014).

An important finding in this study is the accumulation of Sam68 in PARGs. Sam68 is a member of the STAR (signal transducer and activator of RNA) family of RNA-binding proteins that bind both RNA and DNA and are involved in signal

transduction, transcription and alternative splicing regulation (Hartmann et al., 1999; Matter et al., 2002; Rajan et al., 2008; Richard, 2010). As a splicing regulator, Sam68 may promote exon inclusion or exclusion in certain neural premRNAs, including *SMN2*, *Bcl-x* and *Nrxn 1* (Iijima et al., 2011; Paronetto et al., 2007b; Richard, 2010). Regarding *SMN* genes, Sam68 is a physiological regulator of *SMN2,* but not of *SMN1,* splicing. Thus, Sam68 directly binds to the *SMN2* premRNA and acts as a splicing repressor of exon 7 inclusion in *SMN2* transcripts (Pagliarini et al., 2015; Pedrotti et al., 2010). In the present study, we detected no significant changes in the mRNA and protein levels of Sam68 in the spinal cords of SMNΔ7 mice compared with WT animals. Although the accumulation of Sam68 in PARGs should reduce its nucleoplasmic levels, potentially facilitating the inclusion of exon 7 in *SMN2* transcripts (Pagliarini et al., 2015; Pedrotti et al., 2010), its partial relocation in PARGs does not correct the defective *SMN2* splicing in SMNΔ7 mice. In fact, SMN protein levels are dramatically reduced in the spinal cords of SMNΔ7 mice during the symptomatic stages (Tapia O et al., 2017). However, our results show changes in the alternative splicing regulation of the Sam68-dependent *Bcl-x* and *Nrxn-1* mRNAs in the spinal cords of SMNΔ7 mice. It is well-established that Sam68 binds *Bcl-x* mRNA, and its intracellular levels regulate the balance of alternative splicing to produce pro-apoptotic *Bcl-x(s)* transcripts or anti-apoptotic *Bcl-x(L)* transcripts (Boise et al., 1993). In particular, whereas the upregulation of Sam68 enhances *Bcl-x(s)* splicing, its downregulation promotes the production of *Bcl-x(L)* transcripts (Paronetto et al., 2007a). In this context, our finding of a reduction of the *Bcl-x(s)/Bcl-x(L)* ratio in SMNΔ7 mice, compared with WT littermates, is consistent with a downregulation of Sam68. Regarding *Nrxn1* transcripts*,* changes in alternative splicing regulation result in a decreased *Nrxn1 4(-)/Nrxn1 4(+)* ratio, with a relative increase in the isoform that includes exon 20 in *Nrxn1* alternatively

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spliced segment 4 (AS4). Importantly, the neurexin protein 4(+) and 4(-) variants show different interactomes with proteins that are key mediators of synaptic formation and maintenance, including neuroligins (for a review, see (Iijima et al., 2011)). Thus, previous studies have demonstrated that neurexin 4(+) exhibits weak binding with the neuroligin-1B postsynaptic receptor, which affects synaptic adhesion (Chih et al., 2006; Dean et al., 2003). In this context, the Sam68-dependent relative abundance of neurexin 4(+) in SMNΔ7 mice might contribute to the synaptic dysfunction observed in SMA MNs. Collectively, our results suggest that the recruitment of Sam68 into PARGs impacts its normal function in splicing regulation.

The nuclear retention of immature or aberrant premRNAs can be triggered by a wide range of errors in mRNA processing, which prevent the recruitment of mRNA export factors (Schmid and Jensen, 2010). We believe that PARGs may contain incorrectly processed polyadenylated transcripts bound to Sam68, but lack other RNA-binding proteins involved in packing nascent mRNA (hnRNPA2/B1), splicing regulation (hnRNPM3 and TDP43) and polyadenylation (PABPN1). Consistent with the nuclear accumulation of poly(A) RNA in PARGs, REF and Y14, two RNA-binding proteins that link premRNA splicing to nuclear export, (Dreyfuss et al., 2002; Zhou et al., 2000) are not detectable in these granules. Moreover, we observed a reduction of the levels of PABPN1, a protein that, in addition to polyadenylation, is also involved in mRNA export from the nucleus (Apponi et al., 2010). Therefore, a deficiency of this export factor in SMNΔ7 MNs could also contribute to the accumulation of poly(A) RNA in PARGs.

Alternative splicing defects account for nearly 50% of human inherited diseases (Cáceres and Kornblihtt, 2002), and mis-splicing events are particularly prominent in neurodegenerative diseases (Dredge et al., 2001; Ibrahim et al., 2012). Previous studies

in mouse models of SMA have demonstrated SMN-dependent downregulated spliceosomal snRNPs and altered alternative splicing, preferentially in U12-dependent introns (Bäumer et al., 2009; Doktor et al., 2017; Lotti et al., 2012; Zhang et al., 2008). Our results extend the splicing defects in SMA MNs. Thus, we demonstrate here the accumulation of incompletely spliced (intron-containing) *Chat, Chodl, Myh9* and *Myh14* pre-mRNAs during the late symptomatic stage (P14), when a higher incidence of PARG-containing MNs occurs. Interestingly, the accumulation of intron-containing mRNAs impacts the transcripts processed by both the minor (*Myh9* and *Myh14)* and major (*Chat* and *Chodl)* spliceosomes, suggesting a global splicing dysfunction. Importantly, reduced *Chodl* expression has been demonstrated in SMA mouse MNs and linked to MN outgrowth defects (Bäumer et al., 2009; Sleigh et al., 2014).

Although MN functions, such as axonal RNA transport and translation control at the neuromuscular junction, are altered in SMA (Rossoll et al., 2003), spliceosome dysfunction plays a critical role in SMA pathogenesis (for a review, see (Tisdale and Pellizzoni, 2015)). We propose that the abnormal accumulation of polyadenylated RNAs in PARGs reported here, together with the previously demonstrated depletion of canonical (coilin-, SMN- and snRNP-positive) Cajal bodies (Sapaly et al., 2018; Tapia et al., 2012, 2017), are two cellular hallmarks of the global splicing pathology in SMA MNs. Notably, an enhancement of the nuclear poly(A) RNA signal, which is associated with the formation of cytoplasmic stress granules, has been reported in a cellular model of *C9orf72* amyotrophic lateral sclerosis(Rossi et al., 2015), suggesting that the nuclear accumulation of polyadenylated mRNAs may be a more general mechanism in the pathogenesis of MN diseases.

The reduction of the cytoplasmic poly(A) RNA signal in MNs containing a large number of PARGs supports a decrease in translation efficiency, with reduced

availability of polyadenylated mRNAs for protein synthesis. Consistent with this notion, after ultrastructural and immunocytochemical analyses, we have previously reported nucleolar alterations and a severe disruption of the protein synthesis machinery in MNs from SMNΔ7 mice (Tapia et al., 2017). Furthermore, a recent study on the transcriptome and translatome in SMA mice, by means of next-generation sequencing, provides an important catalogue of the mRNAs with altered translation efficiency (Bernabò et al., 2017). The negative impact of the reduced levels of cytoplasmic poly(A) RNAs on translation might be reinforced by the failure of SMNΔ7 MNs to recruit these RNAs into cytoplasmic stress granules, which is a protective cellular mechanism for the transient storage and stabilization of mRNAs during the stress-induced inhibition of translation (Anderson and Kedersha, 2006).

In conclusion, SMN deficiency in SMNΔ7 MNs causes a severe mRNA metabolism dysfunction, resulting in the abnormal nuclear accumulation of polyadenylated RNAs in PARGs, as well as the cytoplasmic depletion of these RNAs. We propose that the accumulation of incompletely processed polyadenylated mRNAs interferes with their export from the nucleus and affects their translation efficiency. The present study provides additional support for the hypothesis that the dysfunction of nuclear mRNA metabolism plays a critical role in MN degeneration and consequently in SMA pathogenesis.

MATERIAL AND METHODS

Animals

The $Smn^{+/-}$, $SMN2^{+/+}$, $SMN\Delta7^{+/+}$ heterozygous knockouts for mouse Smn (FVB.Cg-Tg[SMN2*delta7]4299Ahmb Tg[SMN2]89Ahmb Smn1tm1Msd/J, stock number 005025), which were purchased from The Jackson Laboratory (Sacramento, USA), were crossed to generate $Smn^{-/-}$, $SMN2^{+/+}$, $SMN\Delta7^{+/+}$ (hereafter referred to as SMNΔ7)

mice and *Smn*^{+/+}, *SMN2*^{+/+}, *SMNΔ7*[+/+] mice that were wild type for *Smn* (hereafter referred to as WT). SMNΔ7 mice exhibit a severe postnatal SMA phenotype with a mean lifespan of approximately two weeks (Le et al., 2005; Tarabal et al., 2014). Animal care and handling were performed in accordance with Spanish legislation (Spanish Royal Decree 53/2013 BOE) and the guidelines of the European Commission for the Accommodation and Care of Laboratory Animals (revised in Appendix A of the Council Directive 2010/63/UE). The experimental plan was examined and approved by the Ethics Committee of the University of Cantabria and the Committee for Animal Care and Use of the University of Lleida. On postnatal day (P)0, the identification of WT and SMNΔ7 mice was carried out by genotyping with PCR. DNA was extracted from the tail, as previously described (Tarabal et al., 2014). Age-matched WT littermates of mutant animals were used as controls.

Immunofluorescence and confocal microscopy

For immunofluorescence, four mice per group (WT and SMNΔ7) at P5 and P14 were perfused, under deep anesthesia with pentobarbital (50 mg/kg), with 3.7% paraformaldehyde (freshly prepared) in phosphate-buffered saline (PBS). The spinal cords were rapidly dissected, removed, post-fixed for 6 hours and washed in PBS. Transverse sections (160 μm thick) of the spinal cord were obtained with a vibratome, and small tissue fragments from the anterior horn were dissected out. The samples were transferred to a drop of PBS on a positively charged slide (Superfrost Plus, Thermo Scientific, Germany), and squash preparations of dissociated MNs were generated following the previously reported procedure (Pena et al., 2001). The samples were sequentially treated with 0.5% Triton X-100 in PBS for 45 minutes, 0.1 M glycine in PBS containing 1% bovine serum albumin (BSA) for 30 minutes and 0.05% Tween 20 in PBS for 5 minutes. Then, the samples were incubated for 3 hours with the primary

antibody containing 1% BSA at room temperature, washed with 0.05% Tween 20 in PBS, incubated for 45 minutes in the specific secondary antibody conjugated to FITC, TexasRed or Cy3, or Cy5 (Jackson, USA) and counterstained with PI for the detection of nucleic acids. The slides were then washed in PBS and mounted with the ProLong Anti-Fading Medium (Invitrogen).

Confocal images were obtained with an LSM510 (Zeiss, Germany) laser scanning microscope using a 63x oil (1.4 NA) objective. To avoid overlapping signals, images were obtained by sequential excitation at 488 nm, 543 nm and 633 nm to detect FITC, TexasRed or Cy3, and Cy5, respectively. Fluorescence profiles of confocal intensity signals across a line were generated to analyze the spatial association between poly(A) RNAs and the 2,2,7-trimethylguanosine Cap (TMG-Cap), poly(A)-binding protein nuclear 1 (PABPN1), Src-associated protein in mitosis of 68 kD (Sam68), TAR DNA-binding protein 43 (TDP43), heterogeneous nuclear ribonucleoproteins A2 and B1 (hnRNP A2/B1), heterogeneous nuclear ribonucleoproteins M3 and M4 (hnRNP M3/M4), the RNA export factor (REF) or the RNA-binding protein Y14 (Y14). Images were processed using Photoshop software.

The following primary antibodies were used for immunofluorescence: the mouse monoclonal antibodies anti-TMG-cap (NA02A, Oncogene), anti-REF (ab6141, Abcam), anti-Y14 (ab5828, Abcam) and anti-hnRNPM3/M4 (ab9548, Abcam), the rabbit polyclonal antibodies anti-PABPN1 (sc-67017, Santa Cruz Biotechnology), anti-Sam68 (sc-333, Santa Cruz Biotechnology), anti-coilin 204/10 (204.3 serum, (Bohmann et al., 1995)) and anti-TDP43 (10782-2-AP, Proteintech Inc. Group), and the goat polyclonal antibody anti-hnRNPA2/B1 (sc-10035, Santa Cruz Biotechnology).

In situ hybridization and quantification

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Tissue fragments from the ventral horn of the spinal cord were fixed with 3.7% paraformaldehyde and processed for fluorescence *in situ* hybridization (FISH). The tissue samples were then dissociated as described above. Preparations of MNs were permeabilized with TBS-E-SDS for 15 minutes at 37ºC, washed three times in 6x SSPE-0.1% Tween 20 for 15 minutes, and incubated with the probe containing tRNA for 3 hours at 42ºC in a humidified chamber. An oligo dT $_{(50)}$-mer, 5'-end labeled with biotin (MWG-Biotech, Germany) was used as a probe for fluorescence *in situ* hybridization (FISH) to poly(A) RNA. The hybridization mixture contained 80 ng of oligo dT(50), 2x SSC, 1 mg/ml tRNA, 10% dextran sulfate and 25% formamide. After hybridization, the MNs were washed in 6x SSC for 15 minutes and then washed in 4x SSC-0.1% Tween 20 for 15 minutes at room temperature. The hybridization signal was detected with FITC-avidin for 30 minutes. For the amplification of the hybridization signal, neuronal samples were incubated with avidin-biotin for 30 minutes, washed in 4x SSC-0.1% Tween 20 for 15 minutes and then incubated with FITC-avidin for 30 minutes. All samples were mounted with Vectashield (Vector, USA). Some samples were also processed for double- or triple-labeling experiments combining poly(A) RNA detection with immunofluorescence for TMG-cap, PABPN1, Sam68, TDP43, hnRNP A2/B1, hnRNPM3/M4, REF or Y14.

The quantitative analysis of the proportion of SMNΔ7 MNs containing PARGs was performed in squash preparations processed for FISH with the poly(dT) probe. The proportion of neurons containing these granules was estimated by direct examination of the different focal planes throughout neuronal nuclei, using a 40x objective. Quantification was performed on at least 100 MNs from three WT and SMNΔ7 mice.

The fluorescence intensity of poly(A) RNAs in the Nissl substance was quantified using the confocal Zeiss LSM 5 image analysis system. At least 30 confocal

images of MNs per animal from WT (n=3) and SMNΔ7 (n=3) mice were recorded using a 63x oil objective. For each MN, four measurements of poly(A) RNA signal intensity in the peripheral cytoplasm, where the RNA-rich Nissl substance is largely distributed, were made. The values were corrected for background staining by subtraction of a blank measurement taken outside the cell.

Electron microscopy

For conventional ultrastructural examination of MNs, WT and SMNΔ7 mice (n=3 per group) were perfused under deep anesthesia with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Transverse sections (500 μm thick) were obtained with a vibratome, and anterior horn tissue fragments were dissected out, rinsed in 0.1 M phosphate buffer, postfixed in 2% osmium tetroxide, dehydrated in acetone and embedded in araldite (Durcupan, Fluka, Switzerland). Ultrathin sections stained with uranyl acetate and lead citrate were examined with a JEOL 201 electron microscope.

For immunoelectron microscopy of Sam68, WT and SMNΔ7 mice were perfused with 3.7% paraformaldehyde in 0.1 M phosphate buffer. Tissue fragments of the ventral horn were washed in 0.1 M cacodylate buffer, dehydrated in increasing concentrations of methanol at −20°C, embedded in Lowicryl K4M at −20°C and polymerized with ultraviolet irradiation. Ultrathin sections were mounted on nickel grids and sequentially incubated with 0.1 M glycine in PBS for 15 min, 5% BSA in PBS for 30 min and the primary antibody for 2 h at 37°C. After washing, the sections were incubated with the specific secondary antibodies coupled to either 10 nm or 15 nm gold particles (BioCell, UK; diluted 1:50 in PBS containing 1% BSA). Following immunogold labeling, the grids were stained with lead citrate and uranyl acetate and examined with a JEOL 201 electron microscope. As controls, ultrathin sections were treated as described above without the primary antibody.

Real-time quantitative PCR (qRT-PCR) for relative gene expression analysis

Five SMNΔ7 (n=5) and 3 WT (n=3) mice were used for qRT-PCR studies. The mice were decapitated after being anesthetized, and the lumbar spinal cord was quickly removed and frozen in liquid nitrogen. RNA was isolated with Trizol following the manufacturer's instructions (Invitrogen, Carlsbad) and purified with the RNeasy kit (Qiagen, Hilden, Germany).

One microgram of RNA was reverse-transcribed to first-strand cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies) with random hexamers as primers. The cDNA concentration was measured in a spectrophotometer (Nanodrop Technologies ND-1000) and adjusted to 0.5 μg/μl. The expression of the mRNA candidates Src-associated protein in mitosis of 68 kD (*Sam68*), ß-actin (*Actb*), choline O-acetyltransferase (*Chat*), chondrolectin (*Chodl*), Myosin-9 (*Myh9*) and Myosin-14 (*Myh14*) was determined by qRT-PCR using gene-specific SYBR Green-based primers (Takara). RT-qPCR was performed with three biological replicates and technical triplicates/duplicates of each cDNA sample. The threshold cycle (Ct) for each well was determined. The results were normalized to the 45S ribosomal gene, which has no significant variation in expression across the sample sets and was therefore chosen as a normalizer in our experiments. Relative gene expression was calculated according to the 2-(ΔΔCt) equation (Livak and Schmittgen, 2001). Each value in this work represents the mean ± SD of independent samples obtained under the same conditions and compared to two replicated qRT-PCR analyses. The SYBR Green-based specific primers for murine RNAs were as follows: for the *Sam68* mRNA, 5´-CTCCAGCTAGGCCAGTGAA-3´ and 5´-TTGTGGGTAAAGCAACAGGA-3´; for the unspliced premRNA of *Actb* containing intron 1, 5´-GACTCCCAGCACACTGAACT-3´ and 5´-CTCAGGGCAGGTGAAACTGT-3; for

the spliced mRNA of *Actb*, 5´-CAGCCTTCCTTCTTGGGTATG-3´ and 5´-GGCATAGAGGTCTTTACGGATG-3´; for the unspliced premRNA of *Chat* containing intron 3, 5´-CTTGGGGCCAGTCTGATAGC-3´ and 5´-GGACACATGGCTAGAAGGGG-3´; for the spliced mRNA of *Chat,* 5´-CTGGCTTACTACAGGCTTTACC-3´ and 5´-GTGGCCGATCTGATGTTGT-3´; for the unspliced premRNA of *Chodl* containing intron 3, 5´-GCTGTTGTCTCCCGCATCTT-3´ and 5´-AAGTGGAAGCGTTTGGGATT-3´; for the spliced mRNA of *Chodl,* 5´-GAAGCAGGCATAATTCCCAATC-3´ and 5´-TTCCCAAAGCAACCAGTATCA-3; for the unspliced premRNA of *Myh9* containing intron 6, 5´-CACTTGTGTGGCATTGGGAC-3´ and 5´-AAGAAGGACCTCTCCTCCGA-3´; for the spliced mRNA of *Myh9,* 5´-TGGTGCCAACATTGAGACTTAT-3´ and 5´-CCAGACAGCAGGTAGTAGAAGA-3´; for the unspliced premRNA of *Myh14* containing intron 5, 5´-TCACCCTGAAAAGATGCCCC-3´ and 5´-TCGAAGGTCCAGACAGGCAT-3´; and for the spliced mRNA of *Myh14,* 5´-GGAGCAAACATCGAGACCTATC-3´ and 5´-GGTAGAAGATATGGAAGCTGCATT-3´.

SDS-PAGE and immunoblotting

Spinal cords from WT (n=3) and SMNΔ7 mice (n=5) were lysed at 4°C in a buffer containing 50 mM Tris (pH 8), 150 mM NaCl, 2% Nonidet P-40, 1 mM MgCl$_2$, 1 mM dithiothreitol, and 10% glycerol and supplemented with EDTA-free complete protease inhibitor cocktail and PhosSTOP (Roche). The spinal cord samples were sonicated for 5 cycles of 30 seconds ON/OFF at 4°C using a Bioruptor Plus (Diadode) and incubated on ice for 20 min. The samples were then cleared by centrifugation at 14,000 rpm for 10 min at 4°C. The proteins were separated on 4-20% NuPage TG SDS–PAGE gels (Invitrogen) and transferred to nitrocellulose membranes using standard procedures.

Mouse monoclonal anti-ßIII-Tubulin (Sigma T8660) and rabbit polyclonal anti-PABPN1 and anti-Sam68 were used. Protein bands were detected with an Odyssey^{TM} Infrared-Imaging System (Li-Cor Biosciences) according to the Odyssey^{TM} Western-Blotting Protocol. Immunoblots were developed with anti-mouse IRDye800DX or anti-rabbit IRDye680DX (Rockland Immunochemicals, USA) secondary antibodies. For the quantitative analysis of the blots, ImageJ software was used (U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij).

Statistical analysis

For comparisons between WT and SMNΔ7 samples, data were analyzed using the GraphPad Prism 7 software and an unpaired Student's *t*-test. Significance was established at p≤ 0.05.

Author contributions

M.L., M.T.B., O. Tapia and J.C. conceived and designed the experiments. O.N., O. Tapia, O. Tarabal, L.P., J.C., M.T.B. and M.L. performed the experiments and analyzed the data. M. L., M.T.B. and J.C. wrote the paper.

FIGURE LEGENDS

Figure 1. Dissociated spinal cord MNs from WT mice at P14. (A) Propidium iodide (PI) staining of the nucleolus and Nissl substance. (B) Immunostaining for snRNP splicing factors with the anti-TMG-cap antibody shows the typical concentration of splicing factors in nuclear speckles. The samples were counterstained with PI. (C) Immunostaining for coilin illustrates a typical Cajal body free in the nucleoplasm. The samples were counterstained with PI. (D) FISH for poly(A) RNAs shows a diffuse nuclear pattern, which excludes the negative nucleolus, in addition to concentration in nuclear speckles. In the cytoplasm, poly(A) RNAs accumulate in irregular areas corresponding to the Nissl substance. (E-I) Triple labeling for poly(A) RNAs, the TMG-cap and PABPN1, demonstrating the colocalization of these three fluorescent signals in nuclear speckles, as revealed by the plot of fluorescence intensity profiles across a line. Scale bar: 5 μm.

Figure 2. FISH for poly(A) RNAs in WT and SMNΔ7 MNs at P5 and P14. (A) Typical distribution of Poly(A) RNA concentrated in nuclear speckles and the Nissl substance and diffuse in the nucleoplasm in a WT MN at P5. (B and C) Formation of PARGs in SMNΔ7 MNs at P5. Note the association of PARGs with the nucleolus and nuclear speckles (B and C) and the reduced hybridization signal in the Nissl substance in panel C. (D-F) Distribution of poly(A) RNAs in SMNΔ7 MNs at P14, showing three progressive stages of PARG accumulation in neuronal perikarya. Note the presence of two categories of PARGs, compact (D and E) and ring-shaped (F and inset), and the cytoplasmic depletion of poly(A) RNAs in MNs containing a large number of PARGs (E, F). Scale bar: 4 μm. (G) Proportion of MNs containing PARGs at P5 and P14. PARGs are already visible at P5, but the percentage of MNs containing PARGs notably increases during the late symptomatic stage (P14). ***p<0.0005 by Student's *t*-test. (H)

Scatter plot of the fluorescence intensity signals of poly(A) RNAs in the Nissl substance of WT and SMNΔ7 MNs at P14. Each dot represents the mean value of the four measurements of poly(A) RNA signal intensity performed for each MN. A significant reduction of the relative concentration of poly(A) RNAs is observed in PARG-containing SMNΔ7 MNs (n=93) compared with both WT and SMNΔ7 MNs without PARGs (n=112 and n=90, respectively). ***p<0.0005 by Student's t-test.

Figure 3. Electron micrograph of a PARG-containing MN during the end stage of disease (P14). (A) The cytoplasm is poor in rough endoplasmic reticulum cisterns; note the abnormal nuclear morphology, with numerous invaginations of the nuclear envelope. The nucleus exhibits two compact PARGs (arrows) and two interchromatin granule clusters (IGCs). Left inset: detail of a ring-shaped PARG surrounded by perichromatin granules (arrows). Right inset: interchromatin granule cluster with an associated PARG (arrow). Scale bar: 2 µm. (B, C) Nucleoli of SMNΔ7 MNs at P14, showing the segregation of large masses of the granular component (GC), the presence of a nucleolus-attached PARG (arrow in panel B) and a ring-shaped PARG free in the nucleoplasm and surrounded by perichromatin granules (arrow in panel C). Scale bar: 1 µm.

Figure 4. (A-D) Double labeling for poly(A) RNAs in combination with molecular markers of Cajal bodies (coilin, A), nuclear speckles of splicing factors (TMG-cap, B), gems (SMN, C) and clastosomes (20S proteasome, D) demonstrates that the PARG is a distinct nuclear structure. The plot of fluorescence intensity profiles across a line clearly demonstrates a lack of concentration of these molecular markers in PARGs. Scale bar: 4 µm.

Figure 5. Double labeling for poly(A) RNAs and Sam68 in WT (A-C) and SMNΔ7 (D-F) MNs at P14. (A-C) In the WT MNs, Sam68 exhibits a diffuse nuclear localization

with a few areas of higher intensity. (D-F) In the SMNΔ7 MNs, Sam68, in addition to being diffusely distributed throughout the nucleus, appears highly concentrated in two PARGs (F). Note the absence of Sam68 in nuclear speckles and the cytoplasmic depletion of poly(A) RNA. Scale bar: 3 μm. (G-H) The plot of the fluorescence intensity profiles of poly(A) RNAs and Sam68 across a line confirms the colocalization of both signals in a PARG and the concentration of poly(A) RNA, but not Sam68, in a nuclear speckle. Scale bar: 3 μm. (I, J) Immunoelectron microscopy localization of Sam68 in dense and ring-shaped PARGs. Scale bar: 200 nm. Insets, FISH detection of poly(A) RNAs in PARGs. (K) qRT-PCR of the relative levels of Sam68 mRNA in spinal cord extracts from WT (n=3) and SMNΔ7 mice (n=5). No significant differences (n.s.) were found when comparing WT and SMNΔ7 samples during both the P5 and P14 stages. (L) Representative western blot of Sam68 protein levels showing the dramatic reduction of SMN protein levels in spinal cord lysates from SMNΔ7 mice, compared with WT mice, and the absence of significant changes in Sam68 protein levels between WT and SMNΔ7 mice at the P14 stage. (M) qRT-PCR of the *Bcl-x(s)/Bclx-(L)* and *Nrxn14(-)/Nrxn1 4(+)* mRNA ratios in spinal cord extracts from WT and SMNΔ7 mice. A significant increase in the relative abundance of the antiapoptotic *Bclx-(L)* and *Nrxn 4(+),* which includes exon 20, splicing variants was detected in the SMNΔ7 mice at P14. **$p<0.005$ by Student's *t*-test.

Figure 6. (A-F) Double labeling for poly(A) RNAs and the RNA-binding proteins PABPN1 (A), TDP43 (B), hnRNP A2/B1 (C), hnRNP M3/M4 (D), REF (E) and Y14 (F) in WT MNs at the P14 stage. Whereas all proteins exhibit a nuclear localization, PABPN1 and REF appear concentrated in nuclear speckles. Scale bar: 4 μm.

Figure 7. (A-D) Double labeling for poly(A) RNA and PABPN1 shows the colocalization of both molecules in nuclear speckles in a PARG-free MN from an

SMNΔ7 mouse. Scale bar: 3 μm. (E) Representative western blotting analysis of PABPN1 levels in spinal cord lysates from WT and SMNΔ7 mice. Western blot bands for PABPN1 were normalized to Tubulin ß-III, which showed double immunoreactive bands at approximately 70 kDa and 55 kDa. We chose the 55 kDa band for normalization purposes since 50-55 kDa is the predicted and apparent molecular weight of Tubulin ß-III in WB analyses. The larger 70 kDa band observed could be due to cross-reactivity with a protein related to Tubulin ß-III or a post-translationally modified form of Tubulin ß-III. The bars represent a densitometric analysis of the WB bands for PABPN1 normalized to the 55 kDa Tubulin ß-III band and expressed as the mean ± SD of three independent experiments (WT (n=3) vs SMNΔ7 (n=5); **p < 0.005). (F-K) Double labeling for poly(A) RNAs and the RNA-binding proteins PABPN1 (F), TDP43 (G), hnRNPA2/B1 (H), hnRNPM3/M4 (I), REF (J) and Y14 (K) reveals an absence of the colocalization of poly(A) RNA with these RNA-binding proteins in PARGs, which was confirmed by plots of their respective fluorescence intensity profiles across a line. Scale bar: 4 μm.

Figure 8. (A, B) qRT-PCR determination of the intron-containing (unspliced) *Actb, Chat, Chodl, Myh9* and *Myh14* premRNAs reveals a significant increase of the relative abundance of their unspliced premRNAs in the spinal cords of SMNΔ7 mice compared with WT mice at P14 (B). During the late presymptomatic stage (P5), this increase was only detected in *Chodl* and *Myh9* premRNAs (A). The bars represent the mean ± SD of the relative fraction of the total RNA transcript that is unspliced premRNA from WT (n=3) and SMNΔ7 mice (n=5). n.s, not significant; **p<0.005 and ***p<0.0005 by Student's *t*-test. qRT-PCR analyses were always confirmed in triplicate.

The nuclear retention of immature or aberrant premRNAs can be triggered by a wide range of errors in mRNA processing that prevent the recruitment of mRNA export factors (Schmid and Jensen, 2010). In the SMNΔ7 mouse model of SMA, we have detected an abnormal accumulation of poly(A) RNAs in nuclear speckles in SMNΔ7 MNs during the symptomatic stage (P13-P14) of the disease. This poly(A) RNA retention appears to be transient and is followed by a progressive reduction of nuclear speckle concentration and PARG accumulation. The concentration of PABPN1 in the nuclear speckles of SMNΔ7 MNs suggests that it remains bound to poly(A) RNA, which occurs in WT MNs and non-neuronal cells (Calado and Carmo-Fonseca, 2000; Tavanez et al., 2005). Previous studies in cultured cells and mammalian neurons have shown that a considerable fraction of poly(A) RNAs is located in nuclear speckles under physiological conditions (Carter et al., 1991; Casafont et al., 2010; Palanca et al., 2014; Prasanth et al., 2005). Furthermore, the poly(A) RNA in nuclear speckles are proposed to constitute a new category of the nuclear regulatory RNA family, which is not transported to the cytoplasm (Huang et al., 1994; Prasanth et al., 2005). However, more recent studies using antibodies that specifically recognize active spliceosomes (phosphorylated SF3b155) have demonstrated that approximately 15 to 20% of premRNA splicing is post-transcriptional and occurs in nuclear speckles (Girard et al., 2012), clearly indicating the transient presence of polyadenylated mRNAs in nuclear speckles.

Consistent with this view, we have observed the retention of the intron-containing *Chat, Chodl, Myh9* and *Myh14* premRNAs, PARG formation and the cytoplasmic depletion of poly(A)RNA. Previous studies in HeLa cells indicated that the inhibition of splicing activity by the knockdown of CDC5l, an essential component for premRNA splicing, results in the accumulation in nuclear speckles of polyadenylated

premRNAs that have not completed splicing (Girard et al., 2012). The authors also showed that inhibiting the nuclear export pathway by knocking down the export factor REF/Aly causes poly(A) RNA to remain in nuclear speckles, even when splicing activity is preserved.

In this context, previous studies in human cells have reported the formation of Sam68-positive and poly(A) RNA-negative nuclear stress bodies (NSBs) in response to heat shock (Biamonti and Caceres, 2009b). However, PARGs represent a distinct nuclear compartment. Whereas PARGs appear as either electron-dense nuclear bodies or ring-shaped structures, NSBs are formed by clusters of densely packed perichromatin granules (Biggiogera and Fakan, 1998), never have a ring-shaped configuration and lack poly(A) RNA (Biamonti and Vourc'h, 2010). Sam68-positive nuclear foci have also been reported in HeLa and PC3 cells in response to DNA damage-induced genotoxic stress (Busà et al., 2010). These foci concentrate Sam68 together with several other RNA-binding proteins involved in alternative splicing and are active transcription sites, although the nature of the RNA generated in these foci has not been reported (Busà et al., 2010). Collectively, these results support the idea that the formation of distinct categories of Sam68-positive nuclear bodies/foci is part of the cellular stress response to severe RNA metabolism dysfunction.