Changes in intranuclear mobility of mature snRNPs provide a mechanism for splicing defects in spinal muscular atrophy

Allyson Kara Clelland, Alexandra Beatrice E. Bales and Judith Elizabeth Sleeman*

University of St Andrews, School of Biology, MBSB North Haugh, St Andrews, Fife KY16 9TF, UK

*Author for correspondence (jes14@st-andrews.ac.uk)

Accepted 8 February 2012
Journal of Cell Science 125, 2626–2637
© 2012. Published by The Company of Biologists Ltd
doi: 10.1242/jcs.096867

Summary

It is becoming increasingly clear that defects in RNA metabolism can lead to disease. Spinal muscular atrophy (SMA), a leading genetic cause of infant mortality, results from insufficient amounts of survival motor neuron (SMN) protein. SMN is required for the biogenesis of small nuclear ribonucleoproteins (snRNPs): essential components of the spliceosome. Splicing abnormalities have been detected in models of SMA but it is unclear how lowered SMN affects the fidelity of pre-mRNA splicing. We have examined the dynamics of mature snRNPs in cells depleted of SMN and demonstrated that SMN depletion increases the mobility of mature snRNPs within the nucleus. To dissect the molecular mechanism by which SMN deficiency affects intranuclear snRNP mobility, we employed a panel of inhibitors of different stages of pre-mRNA processing. This in vivo modelling demonstrates that snRNP mobility is altered directly as a result of impaired snRNP maturation. Current models of nuclear dynamics predict that subnuclear structures, including the spliceosome, form by self-organization mediated by stochastic interactions between their molecular components. Thus, alteration of the intranuclear mobility of snRNPs provides a molecular mechanism for splicing defects in SMA.

Key words: Nuclear dynamics, SnRNPs, Spinal muscular atrophy, Splicing speckles

Introduction

The spliceosome is a multi-component molecular machine responsible for the catalysis of pre-mRNA splicing. The organization of the spliceosome within mammalian cell nuclei is a complex and dynamic process. Splicing small nuclear ribonucleoproteins (snRNPs) form the core of the spliceosome. Each snRNP comprises a core of uridine-rich small nuclear RNA (U1, U2, U5, U4–U6, U11, U12) complexed with the seven Sm proteins (Sm B/B′, D1, D2, D3, E, F and G) and other proteins specific to each snRNP (reviewed by Will and Lührmann, 2001). The major spliceosome comprises U1, U2, U5, U4–U6 snRNPs, whereas in the minor spliceosome U1 and U2 are replaced by U11 and U12 (Tarn and Steitz, 1996). Splicing snRNPs have a complex pattern of localization within the cell, reflecting their complex biogenesis. With the exception of U6, whose biogenesis is thought to be entirely nuclear (reviewed by Patel and Bellini, 2008), snRNAs are transcribed by RNA PolII in the nucleus then exported to the cytoplasm where the Sm proteins are assembled into a ring structure around the snRNA (Kambach et al., 1999; Stark et al., 2001). This process is co-ordinated and controlled by the survival of motor neurons (SMN) complex (reviewed by Battle et al., 2006; Chari et al., 2009). Decreased amounts of SMN protein lead to the inherited neurodegenerative condition spinal muscular atrophy (SMA). Following the assembly of the Sm core, the 5′ cap of the U snRNA becomes hypermethylated and the 3′ end is trimmed before the partially mature snRNP can be reimported into the nucleus. On entry into the nucleus, snRNPs accumulate in Cajal bodies (Carvalho et al., 1999; Sleeman and Lamond, 1999) where their maturation continues (reviewed by Cioce and Lamond, 2005). In many, but not all, cells Cajal bodies colocalize with their twin structures, gems (Gemini of Cajal bodies) that contain the SMN protein (Liu and Dreyfuss, 1996). Cajal bodies are also implicated in the recycling of snRNPs after spliceosome disassembly (Schaffert et al., 2004; Sleeman, 2007; Stanek et al., 2008; Stanek et al., 2003). Mature splicing snRNPs localized to nuclear speckles (also termed interchromatin granules clusters; IGCS), which are thought to provide a reservoir of mature snRNPs for recruitment to active spliceosomes when needed (reviewed by Spector and Lamond, 2011). Various strategies for modelling the kinetic behaviour of snRNPs and other pre-mRNA splicing factors suggest that splicing factors are in constant dynamic exchange in and out of speckles and that the speckles form by self-organization that is dependent on the dynamic interactions of their components (reviewed by Iborra and Cook, 2002; Misteli, 2001a; Misteli, 2001b; Misteli, 2010). The active spliceosome itself is also proposed to be a self-organizing structure formed as a result of stochastic interactions between individual protein and RNA components (reviewed by Rino and Carro-Fonseca, 2009). Because the movement of splicing factors and other proteins within the nucleus occurs by anomalous diffusion, with their movement slowed by transient binding to specific sites of

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial Share Alike License (http://creativecommons.org/licenses/by-nc-sa/3.0/), which permits unrestricted non-commercial use, distribution and reproduction in any medium provided that the original work is properly cited and all further distributions of the work or adaptation are subject to the same Creative Commons License terms.
biological importance (Kruhlak et al., 2000; Phair and Misteli, 2000; Rino et al., 2007), the intranuclear dynamics of snRNPs reflect the balance of their activity in spliceosomes and storage in speckles.

The inherited neurodegenerative condition SMA, one of the leading genetic causes of infant mortality, is caused by lowered levels of the SMN protein, with the degree of reduction of SMN showing some correlation with the severity of symptoms (Coovert et al., 1997; Lefebvre et al., 1997). Despite the well-defined role for SMN in early cytoplastmic stages of splicing snRNP assembly, the mechanism by which SMN reduction leads to symptoms of motor neuron loss and subsequent muscle degeneration is unknown. It is unclear whether motor neurons are particularly sensitive to defects in splicing snRNP maturation or whether SMN has an additional motor-neuron-specific function. A comparison of mouse models of SMA exhibiting differing phenotypic severities revealed a correlation between the degree of impairment of snRNP assembly and the severity of symptoms (Gabanella et al., 2007). Cell-type-specific and snRNP-specific alterations in the repertoire of snRNAs have been detected in severe SMA mice and in HeLa cells with 15% or less of normal SMN levels (Gabanella et al., 2007; Zhang et al., 2008). Widespread defects in splicing were also detected in severe SMA mice leading to the proposal that changes in the stoichiometry of snRNPs might result in splicing abnormalities by affecting the efficiency, rate and fidelity of spliceosome assembly on different introns (Zhang et al., 2008), with important motor neuron-specific transcripts preferentially affected. Defects in splicing events mediated by both the minor and the major spliceosomes have been identified in cells from SMA patients with 40–50% of normal SMN levels (Boulisfane et al., 2011; Fox-Walsh and Hertel, 2009; Wan et al., 2005), and SMN-deficient fission yeast also show splicing defects consistent with inefficient spliceosome formation (Campion et al., 2010). To date, however, no key neural transcripts affected by SMN-depletion in mammals have been identified and the molecular mechanism linking an altered snRNP repertoire to the varied and widespread splicing defects seen is not clear. We have examined the effect of SMN reduction on the localization and dynamics of splicing snRNPs in a human neural cell line and in fibroblasts from an SMA patient. Our data reveal significant alterations in the rates of movement of snRNPs within the nuclei of SMN-depleted cells that provide a possible mechanistic link between defects in ribonucleoprotein assembly and defects in splicing.

**Results**

**SMN can be reduced effectively by RNAi in HeLa and SH-SY5Y cells**

It is well established that lowered levels of SMN result in the loss of SMN from nuclear gems and defects in splicing snRNP maturation (Clelland et al., 2009; Girard et al., 2006; Gonsalvez et al., 2007; Lemm et al., 2006; Shargel and Matera, 2005). In order to investigate the effect of decreased levels of SMN on the structure of splicing factor speckles and on the dynamics of mature splicing snRNPs, we identified short interfering RNA (siRNA) sequences capable of reducing SMN levels in HeLa cells and in the neuroblastoma cell line SH-SY5Y. Transient transfection of HeLa cells with two independent siRNA duplexes to SMN (SMN 01 and SMN 05) led to a dramatic reduction in the number of SMN-positive nuclear gems after 24 hours compared with cells transfected with non-targeting duplexes or control duplexes targeting lamin A/C (Fig. 1A,B). Transfection of SH-SY5Y cells with siRNA duplexes to SMN resulted in a reduction to 48% of normal SMN protein levels after 24 hours and 32% of normal levels after 48 hours (Fig. 1C,D) compared with transfection with non-targeting duplexes or control duplexes targeting peptidyl-prolyl cis-trans isomerase B (PPIB).

**SMN reduction can be achieved using expression of shRNAs**

We have previously demonstrated that the neuroblatoma cell line, SH-SY5Y, has relatively low endogenous levels of SMN with a small overexpression of SMN able to increase the rate of accumulation of newly assembled snRNPs in nuclear speckles (Clelland et al., 2009). This cell line is an ideal model in which to study the effect of SMN reduction on mature snRNPs because it is the level of SMN that limits the splicing snRNP assembly pathway in SH-SY5Y cells. In order to investigate the dynamics of mature snRNPs in SMN-depleted SH-SY5Y cells, it was essential to unequivocally identify living cells with lowered levels of SMN. To achieve this, we used the sequence that we identified as capable of reducing SMN levels to generate plasmids to express small hairpin RNAs (shRNAs) from an H1 promoter with GFP expressed from a PGK promoter on the same plasmid (pSuperGFP). SH-SY5Y cells were transfected with these vectors and assayed for SMN expression after 24, 48 and 72 hours by indirect immunofluorescence using antibodies to SMN. Transfected SH-SY5Y cells, identified by expression of GFP, showed reduced SMN levels after 48 hours of shRNA expression (Fig. 2A–C). This reduction was also evident at 72 hours. Expression of a control plasmid expressing shRNAs to target PPIB had no effect on SMN levels or distribution (Fig. 2D). Quantification of the SMN fluorescence signal 48 hours after transfection with plasmids targeting SMN reveals a reduction to an average of 46% of normal SMN levels in whole cells and to an average of 56% of normal SMN in nuclei (Fig. 2E). These data demonstrate that shRNA expression can efficiently reduce SMN in SH-SY5Y cells to levels comparable with those seen in cells from SMA patients, but that longer incubation is required than for direct transfection of siRNA duplexes. Furthermore, the loss of SMN appears to be more pronounced in the cytoplasm than in the nucleus.

**Reduction of SMN does not disrupt the structure of splicing speckles**

Depletion of SMN has been demonstrated to lead to inefficient snRNP assembly (reviewed by Pellizzoni, 2007) and to a delay in newly assembled snRNPs reaching splicing factor speckles (Girard et al., 2006). However, the effect of SMN depletion on nuclear speckles containing mature splicing factors has not been examined in detail. Although the level of SMN available is the limiting factor in the rate of snRNP accumulation in speckles in SH-SY5Y neuroblastoma cells (Clelland et al., 2009), reduction of SMN levels using shRNA expression does not cause an appreciable defect in the localization of steady-state splicing factors in speckles (Fig. 3). The serine/arginine-rich (SR) domain splicing factor SC-35, core snRNP Sm proteins, the U1 snRNP-specific protein U1A and snRNAs (Fig. 3A–C, respectively), detected using an antibody to their tri-methyl guanosine (TMG) cap (Fig. 3D), all show normal localization in SH-SY5Y cells depleted of SMN for 48 hours (compare transfected cells, green on overlay, with control cells in the same panel). It is notable,
however, that SH-SY5Y cells show accumulations of TMG-capped snRNAs in their cytoplasm (arrowheads in Fig. 3D), suggesting that snRNP assembly is less efficient in these cells than in HeLa cells where such accumulations are not seen (Carvalho et al., 1999; Sleeman et al., 2003).

Mature snRNPs show alterations in their dynamics in cells depleted of SMN

Because the spliceosome is believed to assemble by self-organization, it is important to address the intranuclear dynamics of splicing factors in addition to their steady-state distribution. In order to study the dynamics of splicing snRNPs in SMN-depleted cells, we first established cell lines constitutively expressing mCherry-tagged SmB. mCherry–SmB is efficiently incorporated into snRNPs (Clelland et al., 2009) and colocalizes with endogenous Sm proteins within the nucleolus (Fig. 4A).

Two of these lines, mCherrySmBSHY03 and mCherrySmBSHY12, were used for fluorescence recovery after photobleaching (FRAP) analyses following transfection with a plasmid to reduce SMN levels or with control plasmids (targeting expression of PPIB or non-targeting). Transfected cells were identified by their expression of GFP. Using a Texas Red filter set to visualize mCherry, nuclear speckles were bleached using a fixed laser pulse of 1 second at 532 nm, and short adaptive time-lapse sequences were taken to determine the recovery kinetics (Fig. 4B,C). Comparison of these kinetics in SMN-depleted cells with control cells revealed a significant and substantial decrease in the half-time of recovery of mCherry–SmB-tagged snRNPs to splicing speckles in cells with lowered levels of SMN (Fig. 4D). The mobile fraction of mCherry–SmB was approximately 40%, was unaltered by depletion of SMN. The immobile fraction of mCherry–SmB was larger than that seen for many non-snRNP splicing factors, in agreement with previous data (Sleeman, 2007; Rino et al., 2007; Huranova et al., 2010). The nature of this immobile fraction is not known, but it is unlikely to represent snRNPs actively involved in pre-mRNA splicing as splicing occurs co-transcriptionally and not within speckles.

We have previously observed that the kinetics of exchange of photoactivatable GFP–SmB-containing snRNPs in Cajal bodies are best modelled using a two phase, rather than a one phase, exponential decay curve (Sleeman, 2007) with a slow moving and a rapidly moving pool of signal present. It is difficult to
Values are means ± s.e.m.

measurements were made from deconvolved z-stacks of a total of 40 cells, to 46% of control in whole cells and to 56% of control in nuclei. transfected and untransfected cells and nuclei demonstrate reduction of SMN expression at all three time points (D and other data not shown). In both transfected and untransfected cells, the decrease in SMN protein (A, arrowhead shows an SMN-positive nuclear speckle) is consistent with the presence of SMN-depleted HeLa cells and severe SMA mice, with the suggestion that the levels of minor snRNPs are preferentially affected by SMN depletion. However, these changes were different in different tissues and it is unclear whether all snRNPs or predominantly the minor U11–U12 snRNPs are affected. To address this, we extended our FRAP analyses to look independently at the dynamics of the U1 snRNP and the U11–U12 snRNPs. SH-SY5Y cell lines constitutively expressing the U1-snRNP-specific protein mCherry–U170K [(Ellis et al., 2008) a gift from David Lleres and Angus Lamond, University of Dundee, UK] were established and FRAP experiments performed as described above. SMN depletion resulted in a significant decrease (P<0.05) in half-time of recovery of mCherry–U170K to speckles (Fig. 5A,B). As with mCherry–SmB-labelled snRNPs, the change only affected the slower moving fraction of the signal, with the relative amounts of slow and fast signalunaltered. This is indicative of increased mobility of mature, fully assembled U1 snRNPs. We next investigated the mobility of the U11–U12 snRNPs. SH-SY5Y cell lines express the U11–U12-specific SNRNP35 protein was more dramatic than that seen for the U1-snRNP-specific U170K protein, suggesting that both the abundance and the mobility of the minor snRNPs are preferentially affected by SMN depletion.

Both the major spliceosomal U1 snRNP and the minor spliceosomal U11–U12 snRNPs have altered mobility in cells depleted of SMN

SmB is present in snRNPs from both the major (U1, U2) and minor (U11–U12) spliceosomes. Alterations in the repertoire of snRNPs have been implicated in the splicing defects seen in SMN-depleted HeLa cells and severe SMA mice, with the suggestion that the levels of minor snRNPs are preferentially affected by SMN depletion. However, these changes were different in different tissues and it is unclear whether all snRNPs or predominantly the minor U11–U12 snRNPs are affected. To address this, we extended our FRAP analyses to look independently at the dynamics of the U1 snRNP and the U11–U12 snRNPs. SH-SY5Y cell lines constitutively expressing the U1-snRNP-specific protein mCherry–U170K [(Ellis et al., 2008) a gift from David Lleres and Angus Lamond, University of Dundee, UK] were established and FRAP experiments performed as described above. SMN depletion resulted in a significant decrease (P<0.05) in half-time of recovery of mCherry–U170K to speckles (Fig. 5A,B). As with mCherry–SmB-labelled snRNPs, the change only affected the slower moving fraction of the signal, with the relative amounts of slow and fast signalunaltered. This is indicative of increased mobility of mature, fully assembled U1 snRNPs. We next investigated the mobility of the U11–U12 snRNP-specific protein SNRNP35, which shows substantial homology to U170K (Will et al., 1999) (a gift from Reinhard Lührmann, Max-Planck Institute for Biophysical Chemistry). SH-SY5Y cell lines were established constitutively expressing mCherry–SNRNP35. The localization of mCherry–SNRNP35 was clearly nuclear with a slight accumulation in nuclear speckles (Fig. 5C) as previously reported for the equivalent protein in Arabidopsis (Lorkovic et al., 2005). FRAP experiments again demonstrated a significant reduction (P<0.01) in the half-time of recovery of the slower moving fraction (Fig. 5D,E). Interestingly, the reduction seen for the U11–U12-specific SNRNP35 protein was more dramatic than that seen for the U1-snRNP-specific U170K protein, suggesting that both the abundance and the mobility of the minor snRNPs are preferentially affected by SMN depletion.
Increased snRNP mobility is seen in fibroblasts from a spinal muscular atrophy type 1 patient

To ascertain the relevance of changes in intranuclear snRNP mobility to SMA, we investigated the mobility of mCherry–U170K in fibroblasts from an SMA type 1 patient and his unaffected mother (Coriell cell repository lines GM03813 and GM03814, respectively). Again, no gross structural abnormalities of nuclear speckles were detected in the SMA patient fibroblasts (Fig. 6A,B). FRAP analyses of these fibroblasts expressing mCherry–U170K for 72 hours revealed a similar decrease in the half-time of recovery of this protein to speckles as was observed in SH-SY5Y cells depleted of SMN experimentally (Fig. 5A,B). This clearly demonstrates that defects in splicing snRNP mobility are associated with lowered levels of SMN in patients as well as in our cell culture models of SMA.

The alteration in splicing snRNP dynamics is a direct consequence of defective snRNP biogenesis in SMN-depleted cells

Splicing defects have been reported in several models of SMA including SMN-depleted cells, and early studies of SMN function also suggested an additional direct role in splicing (Pellizzoni et al., 1998). Because increased mobility of splicing factors can be caused by inhibition of transcription or splicing (Kruhlak et al., 2000; Phair and Misteli, 2000; Rino et al., 2007), it was important to determine whether the alteration of snRNP dynamics caused by SMN depletion occurs as a result of defects in splicing or is a potential mechanism for the defects. To address this, we analysed in detail the kinetic changes seen in cells treated to inhibit different stages of mRNA production. Cell lines stably expressing mCherry–SmB were treated with 5,6-dichloro-1-β-D-ribobenzimidazole (DRB) to inhibit transcription, spliceostatin A (SSA; a gift from Minoru Yoshida, RIKEN, Japan) (Kaida et al., 2007) to inhibit pre-mRNA splicing directly and leptomycin B (LMB) (Fornerod et al., 1997) to inhibit the export of nascent snRNAs and, hence, to indirectly inhibit snRNP assembly. In agreement with previous reports studying a number of different splicing factors (Rino et al., 2007), DRB treatment resulted in significantly faster (P<0.01) kinetics of exchange of snRNPs within speckles, again affecting preferentially the slower moving fraction (Fig. 7). However, in contrast to our results from SMN-depleted cells, DRB treatment also resulted in a significant increase (P<0.01) in the mobile fraction of rapidly moving signal and in noticeably altered speckle morphology (Fig. 7). Direct inhibition of splicing with SSA resulted in alterations in speckle morphology indistinguishable from those produced by inhibition of transcription by DRB (Fig. 7). However, this was associated with an increase in the mobile fraction of the slower moving component but no significant alteration in either τ1/2. We have previously reported that inhibition of snRNP assembly with LMB for 3 hours does not affect the interaction of transiently expressed PA–GFP–SmB-tagged snRNPs with speckles (Sleeman, 2007). This remains the case in cells stably expressing mCherry–SmB. However, treatment with LMB for 16 hours or more can successfully replicate the changes in dynamics seen in SMN-depleted cells (Fig. 7) with no discernible alterations to speckle morphology. This in vitro modeling indicates that the changes in snRNP dynamics seen in SMN-depleted cells do not result from indirect or direct inhibition of pre-mRNA splicing, but are a direct consequence of inefficient splicing snRNP maturation (Fig. 8).

Discussion

SMN depletion does not alter splicing speckle morphology but leads to significant changes in splicing snRNP dynamics

The role of SMN in splicing snRNP biogenesis is well established. Despite evidence that SMN depletion has a profound effect on the formation of new snRNPs (reviewed by Chari et al., 2009), no obvious alteration in speckle morphology is seen in SMN-deficient cells using antibodies to snRNP proteins, snRNAs or non-snRNP splicing factors (Figs 3, 6). Analysis of the exchange of snRNPs within splicing speckles, however, reveals a significant increase in the intranuclear mobility of mature snRNPs in SMN-depleted cells and in cells from an SMA patient. Increased mobility of snRNPs is indicative of their reduced interaction with binding sites within the nucleus. This observation emphasizes the importance of examining the flux of factors, rather than just their steady state localization, when investigating dynamic nuclear compartments.

Dynamics of both the major spliceosomal U1 snRNP and the minor spliceosomal U11–U12 snRNPs are affected by SMN depletion

Although there are now a number of reports of splicing defects associated with lowered levels of SMN, including both changes to alternative splicing and inaccurate or defective splicing, the mechanism for these defects is unclear. Alterations in the repertoire of snRNPs have been noted in HeLa cells, MN1 cells and mouse models of SMA (Gabanella et al., 2007; Zhang et al., 2008) with the minor U11 and U12 snRNPs preferentially affected. However, splicing defects seen in mouse models...
(Bäumer et al., 2009; Zhang et al., 2008) and higher rates of errors in splice site pairing seen in SMA patient fibroblasts (Fox-Walsh and Hertel, 2009) do not appear to preferentially affect transcripts spliced by the minor spliceosome. Other studies have not detected significant alterations in the amounts of steady-state snRNPs (Boulisfane et al., 2011; Girard et al., 2006; Lemm et al., 2006). Defects in assembly of the minor spliceosome tri-snRNP have been implicated in altered splicing of minor introns in SMA patient lymphoblasts (Boulisfane et al., 2011). We have identified alterations in the dynamics of bulk splicing snRNPs using mCherry-tagged core Sm proteins in cells expressing shRNAs targeting SMN. More detailed analyses revealed that these alterations affect both the major spliceosomal U1 snRNP (tagged with mCherry–U170K) and the minor spliceosomal U11–U12 snRNP.

Fig. 4. See next page for legend.
snRNP (tagged with mCherry–SNRNP35). Although the alterations in dynamics of mCherry–SmB-tagged snRNPs were unlikely to exclusively reflect defects in the mobility of the minor spliceosomal snRNPs, present at 100 times lower amounts that the major snRNPs, the equivalent alterations seen using mCherry–U170K as a tag confirm that the major U1 snRNP, and not just the minor U11–U12 snRNP, shows increased mobility. The alteration in U11–U12 snRNP dynamics was, however, greater than that in U1 snRNP dynamics, in broad agreement with the body of literature demonstrating more profound changes in the amounts of minor spliceosomal snRNPs in SMA models. Our data link SMN depletion to changes in the dynamic behaviour of snRNPs of the major and the minor spliceosomes.

**Sensitivity to SMN depletion varies in different cell types**

The SH-SY5Y neuroblastoma cell lines analysed in this study are viable for at least 4 days with levels of SMN approximately 50% of wild-type, low enough to cause defects in snRNP mobility. However, we have so far been unable to establish stable SH-SY5Y cell lines with levels of SMN reduced by more than 10%. Experiments on HeLa cells (Zhang et al., 2008) demonstrated no loss of viability or changes in snRNP repertoire in cells expressing only ~20% of normal SMN levels, whereas the reduction to ~5% that is required to see changes in splicing also resulted in cell death over 2–3 days. However, SMA mice (Zhang et al., 2008) survive with levels of SMN low enough to result in splicing defects, and SMA patient fibroblasts are fully viable despite showing defects in snRNP mobility similar to those seen in SH-SY5Y cells with levels of SMN that are only achievable for short periods. Comparison of these data emphasizes the different sensitivities of different cell types to the depletion of SMN. Because SMA preferentially affects spinal motor neurons, despite the need for snRNP assembly and accurate splicing in all cell types, this is highly relevant to the pathology of SMA.

**Increased mobility of snRNPs suggests a mechanism for inefficient spliceosome formation resulting from defective snRNP biogenesis**

The mobility of proteins and RNAs within the nucleus occurs by anomalous diffusion, with reduced mobility resulting from interactions with specific binding sites. In the case of splicing snRNPs, the key interaction expected to retard their mobility is their incorporation into active spliceosomes (Rino et al., 2007). Increased mobility of splicing snRNPs in SMN-depleted cells is, therefore, indicative of reduced binding of snRNPs within active spliceosomes. Pre-mRNA splicing is a highly dynamic process, with spliceosome assembly in vivo proposed to occur by self-organization. Splice site selection is complex and determined by the balance of many positive and negative regulators (reviewed by Wahl et al., 2009). However, in the major spliceosome, the binding of U1 snRNP to the 5' splice site is a key requirement for the formation of the spliceosomal E complex during the earliest stages of spliceosome assembly. The widespread defects in pre-mRNA splicing seen by Zhang et al. appeared to preferentially involve genes with high numbers of introns and exons (Zhang et al., 2008). They included not only altered selection of alternative splice sites but also defective splicing, suggesting a general lack of fidelity in the splicing process. To investigate the cause of such splicing defects, it is important to determine whether the changes in snRNP dynamics we see are a consequence of defective splicing resulting from SMN depletion by a mechanism unrelated to snRNP biogenesis or represent a potential link between defective snRNP biogenesis and altered splicing.

Our in vivo modelling using inhibitors of different stages of pre-mRNA processing was designed to address a specific question: do the changes in snRNP mobility result from splicing defects or do they have the potential to cause them? The results clearly demonstrate that the changes seen in snRNP dynamics in SMN-depleted cells can only be replicated by reducing the snRNP assembly pathway. LMB is known to inhibit snRNP maturation at the point of snRNA export into the cytoplasm by binding directly to the export receptor CRM1 (Kudo et al., 1998). This step closely precedes the addition of Sm proteins to the snRNA, which is mediated by the SMN complex and impaired in SMN-depleted cells (Shpargel and Matera, 2005; Lemm et al., 2006). The inhibitor of transcriptional elongation, DRB, has traditionally been used as an indirect inhibitor of splicing. More recently SSA has been identified as a direct inhibitor of pre-mRNA splicing, believed to act through the formation of an arrested spliceosome containing U1 and U2 snRNPs (Roybal and Jurica, 2010) and impeding the transition from the A complex to the B complex. DRB, but not SSA, results in a significantly decreased half-time of recovery for the slower moving fraction of snRNPs, suggesting lower affinity interactions with spliceosomes in DRB-treated cells. However, both inhibitors also result in alteration of speckle morphology and a significantly higher mobile fraction of snRNPs, neither of which are seen in SMN-depleted cells. Mathematical modelling has previously demonstrated that decreasing the number...
of nucleoplasmic binding sites is sufficient to reproduce the effects on splicing factor kinetics and nuclear speckle morphology observed experimentally by inhibiting transcription using DRB, or splicing using a dominant-negative variant of the snRNP nuclear import adaptor snurportin 1 (Rino et al., 2007). Decreased pre-mRNA transcription (caused by DRB) or the presence of stable arrested spliceosomes (caused by SSA) would both be predicted to reduce the number of nucleoplasmic splice sites available to bind snRNPs. Inhibition of splicing snRNP biogenesis using LMB, however, accurately replicates the changes seen in snRNP dynamics following SMN depletion (Fig. 7B,C). The half-time of recovery of snRNPs is decreased indicating lowered affinity of interaction with spliceosomes while the mobile fraction remains the same, suggesting that the number of binding events remains constant. In addition to implicating the alterations in snRNP mobility as a cause, rather than a consequence, of inefficient splicing, this provides a direct molecular link between the known function of SMN in cytoplasmic stages of snRNP assembly and defects in splicing observed in numerous models, without needing to invoke other, as yet unidentified, functions for SMN. Although it is still unclear whether defective splicing of key motor neuron transcripts is at the root of the pathology of SMA, our data provide...
a mechanistic link between reduction of SMN, a key player in the generation of the cellular splicing machinery, and generalized defects in splicing.

There is increasing recognition that defects in RNA metabolism underlie many human diseases, particularly motor neuron diseases (reviewed by Cooper et al., 2009; Lemmens et al., 2009).
2010). The finely balanced patterns of splicing seen in mammalian cells allow for rapid and sensitive adjustments to protein expression during the development and maintenance of complex tissues and organs. However, their intricate nature is susceptible to perturbation, with defects in proteins involved in pre-mRNA splicing pathways implicated in degenerative conditions such as ALS and cancers, in addition to SMA. Recent research into the degenerative eye condition, retinitis pigmentosa (RP), in which heterozygous mutations in splicing factors associated with the U4/U6.U5 tri-snRNP are implicated, has drawn striking parallels with SMA. Both SMA and RP appear to be systemic splicing diseases with cell-type-specific symptoms (Linder et al., 2011; Tanackovic et al., 2011a; Tanackovic et al., 2011b). This implicates disrupted spliceosome assembly and dynamics as the cause of at least two degenerative conditions.

Materials and Methods

Cell culture and cell lines

Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 100 U/ml penicillin and streptomycin (Invitrogen). For immunofluorescence assays, cells were grown on coverslips (VWR International, Lutterworth, UK). For live-cell microscopy, cells were grown on 40 mm diameter coverslips (Intracel, Royston, UK). Stable cell lines were established using G418 selection of SH-SY5Y cells following transfection with plasmids pmCherry-SmB (Clelland et al., 2009) and pmCherry-U170K (Ellis et al., 2008) (a gift from David Lle´res and Angus I. Lamond, University of Dundee, UK) with Effectene transfection reagent (Qiagen) as described previously (Sleeman et al., 2001). snRNP35 (a gift from Cindy Will and Reinhard Lu¨hrmann, Max-Planck Institute for Biological Chemistry, Gottingen) was sub-cloned into mCherry-C1 (Clontech) by Dundee Cell Products, Dundee, UK. Stable cell lines were established as above. SMA patient and control fibroblasts were lines GM03813 and GM03814, respectively, from the Coriell Cell Repository (Coriell Planck Institute for Biological Chemistry, Gottingen) was sub-cloned into pSuper-GFP plasmids 48 hours or 72 hours before analysis. The coverslips were incubating at 50˚C for 30 minutes with 2% SDS, 100 mM b-mercaptoethanol and 62.5 mM Tris (pH 6.8).

Preparation of cell lysates and immunoblotting

Lysates were prepared as described previously (Sleeman et al., 2003). Cells were mounted in Prolong Gold (Invitrogen) and recorded using a DeltaVision Spectris deconvolution microscope with an Olympus 100x, 1.35 NA objective and a Coolsnap HQ camera (Photometrics). Optical sections, separated by 200 nm, were collected using a binning of 2x2. Images were restored using an iterative deconvolution algorithm using a calculated point-spread function (Volocity, Perkin Elmer). Images in Figs 1, 2 and 3 are maximum intensity projections of z-stacks covering the entire depth of the cells.

RNAi assays

ON-TARGET plus siRNA duplexes (Dharmacon) were introduced into HeLa cells and SH-SY5Y cells using RNAiFect (Qiagen) according to the manufacturer’s instructions. For shRNA expression, targeting sequences were cloned into pSuperGFP (Oligoengine, Seattle, WA) according to the manufacturer’s instructions, and the resulting plasmids transfected into cells. Sequences used were: lamin A/C: 5’-GGUGGUGAAGCAUCGUGGCU-3’; non-targeting (luciferase): 5’-UAAGGGCAUAUGAAGAGAUAC-3’; PPBP: 5’-GGUAAGACUGUCUCAAAAA-3’; SMN 01: 5’-CAGUGGAAAGUUGGGGACA-3’; SMN 05: 5’-UAUAUGGGGCUUCAGACAAA-3’. Lamin A/C was used as a positive control in immunofluorescence assays and PPIB in western blot assays because of the differences in performance of antibodies to the two proteins in the different assays.

Cell fixation, immunostaining and microscopy

HeLa cells grown on glass coverslips were fixed for 10 minutes at room temperature with 3.7% paraformaldehyde in PHEM buffer [60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2 (pH 6.9)]. Immunostaining was carried out as described previously (Sleeman et al., 2003). Cells were mounted in Prolong Gold medium (Invitrogen). Antibodies used were mouse monoclonal antibodies MANSMA1 anti-SMN (Young et al., 2000) (1:10), SC-35 (Sigma; dilution 1:2000), Y12 anti-Sm (Abcam; 1:100), anti-TMG (Calbiochem; 1:15) and rabbit 856 anti-U1A (Kambach and Mattaj, 1992) (1:500). Secondary antibodies were FITC- and TRITC-conjugated goat anti-mouse and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories; 1:250). Immunostained specimens were examined and recorded using a DeltaVision Spectris deconvolution microscope with an Olympus 100x, 1.35 NA objective and a Coolscope IQ camera (Photometrics).

Quantification of fluorescence signals

Quantification of the proportion of SMN signal in transfected and untransfected cells and nuclei was carried out using an intensity threshold on stacks of deconvolved z-sections. A segmentation algorithm was used to find ‘objects’ by applying a global threshold to each stack of z-sections. Objects were defined as sets of contiguous pixels above the specified threshold. To identify transfected cells, GFP signal was used to identify ‘objects’, to identify untransfected cells, SMN signal was used. To identify nuclei, DAPI staining was used for ‘object’ identification and the transfected cells identified manually by reference to the overlaid GFP signal. In each case, the total SMN signal contained in each cell or nucleus was normalized to the total SMN fluorescence of the brightest cell or nucleus in the field of view following a correction to allow for background fluorescence within the sample.

Live-cell microscopy and FRAP analyses

Cells were grown on 40 mm diameter glass coverslips and transfected with pSuper-GFP plasmids 48 hours or 72 hours before analysis. The coverslips were incubated at 50˚C for 30 minutes with 2% SDS, 100 mM b-mercaptoethanol and 62.5 mM Tris (pH 6.8).
transferred to an open chamber (Zeiss) within an environmental incubator (Solenent Scientific, Segensworth, UK) on an Olympus DeltaVision RT microscope (Applied Precision) with a quantifiable laser module including a 20 mW 532 nm laser, and maintained at 37°C with 5% CO₂. Transfected cells expressing GFP were identified using FITC filters. Nuclear speckles within selected cells were bleached using a 1 second 532 nm pulse at 100% laser power focused to a diffraction-limited spot of approximately 0.5 μm. The duration of bleaching achieved a reduction of fluorescence in the region of interest of approximately 60%. A single z-section of each cell was imaged using a Texas Red filter at three time points before the laser pulse and an adaptive time course of 32 timepoints after the laser pulse. For single-phase recovery analyses, images were normalized to mean intensity and models generated using the method of Axelrod (Axelrod et al., 1976) as implemented within SoftWoRx software. For comparison of one-phase and two-phase recovery, the intensities of the region of interest, corrected for photo-bleaching, were normalized against the initial pre-bleach fluorescence and plotted against time. One- and two-phase exponential association curves were fitted to each data set and an extra sum-of-squares F-test performed to determine which model gave the better fit (GraphPad Prism). In all cases, the two-phase curve gave the better fit (P<0.0001). Equations used were as follows:

\[ Y = Y_{max} \times \frac{[1 - \exp(-K \cdot X)]}{[1 - \exp(-2 \times K \cdot X)]} \]

Half-times of recovery and mobile fractions were compared using a one-way ANOVA test with a Tukey–Kramer post-test.

Acknowledgements
The authors would like to thank M. Yoshida (Riken, Japan) for providing splicingostatin A; D. Lleres and A. I. Lamond (University of Dundee, UK) for the plasmid to express mCherry–U170K; C. Will and R. Lührmann (Max-Planck Institute for Biological Chemistry, Göttingen) for the cDNA for SNRNP35; G. Morris (RJAH Orthopaedic Hospital, Oswestry, UK) for anti-SMN antibodies from the Wolfson Centre for Inherited Neuromuscular Disease antibody database; L. Trinkle-Mulcahy (University of Ottawa, Canada) for helpful discussions; and R. Elliott (University of St Andrews, UK) for critical reading of the manuscript.

Funding
This work was supported by the Wellcome Trust [grant number WT078810 to J.E.S.]; and the Royal Society [University Research Fellowship to J.E.S.]. Deposited in PMC for immediate release.

References
Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E. and Webb, W. W. (1976). Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. Biophys. J. 16, 1055-1069.

Battle, D. J., Kasim, M., Yong, J., Lotti, F., Lau, C. K., Mouaikel, J., Zhang, Z., Han, K., Wan, L. and Dreyfuss, G. (2008). The SMN complex: an assembly machine for RNP. Cold Spring Harb. Symp. Quant. Biol. 73, 313-320.

Bäumer, D., Lee, S., Nicholson, G., Davies, J. L., Parkinson, N. J., Murray, L. M., Gabilo, F., Butchbach, M. E., Saieva, L., Carissimi, C., Burghes, A. H. and Pellizzoni, L. (2007). Ribonucleoprotein assembly defects correlate with spinal muscular atrophy severity and preferentially affect a subset of spliceosomal snRNPs. PLoS ONE 2, e921.

Gudas, J., B., Tian, L., Ospina, J. K., Boisvert, F. M., Lamond, A. I., and Matera, A. G. (2002). Two distinct arginine methyltransferases are required for binding of Sm-class ribonucleoproteins. J. Cell Biol. 157, 733-740.

Huber, J., Dickmanns, A. and Lührmann, R. (2002). The importin-beta binding domain of snurportin 1 is responsible for the Ran- and energy-independent nuclear import of spliceosomal U snRNPs in vitro. J. Cell Biol. 156, 467-479.

Kambach, C. and Mattaj, I. W. (1999). Intracellular distribution of the U1A protein depends on transportable nuclear export signal. EMBO J. 18, 267-276.

Kambach, C., Ba¨umer, D., Lee, S., Nicholson, G., Davies, J. L., Parkinson, N. J., Murray, L. M., Chari, A., Paknia, E. and Fischer, U. (2009). The role of RNP biogenesis in spinal muscular atrophy. J. Cell Sci. 122, 3213-3222.

Linder, B., Dill, H., Hirmer, A., Brocher, J., Lee, G. P., Mathavan, S., Bolz, H. J., Gabanella, F., Butchbach, M. E., Saieva, L., Carissimi, C., Burghes, A. H., and Pellizzoni, L. (2007). Two distinct arginine methyltransferases are required for binding of Sm-class ribonucleoproteins. J. Cell Biol. 176, 265-269.

Liu, Q. and Dreyfuss, G. (1996). A novel nuclear structure containing the survival motor neuron protein. J. Biol. Chem. 271, 23221-23227.

Lemmens, R., Moore, M. J., Al-Chalabi, A., Brown, R. H., and Robberecht, W. (2010). RNA metabolism and the pathogenesis of motor neuron diseases. Trends Neurosci. 33, 249-258.

Linder, B., Dill, H., Hirmer, A., Brocher, J., Lee, G. P., Mathavan, S., Bolz, H. J., Gabanella, F., Butchbach, M. E., Saieva, L., Carissimi, C., Burghes, A. H., and Pellizzoni, L. (2007). Two distinct arginine methyltransferases are required for binding of Sm-class ribonucleoproteins. J. Cell Biol. 176, 265-269.

Mistelli, T. (2001a). The concept of self-organization in cellular architecture. J. Cell Biol. 155, 181-186.

Mistelli, T. (2001b). Protein dynamics: implications for nuclear architecture and gene expression. Science 291, 843-847.

Mistelli, T. (2010). Higher-order genome organization in human disease. Cold Spring Harb. Perspect. Biol. 3, 1-00077.

Mistelli, T. (2004). Coupled in vitro nuclear export and splicing events are a late feature of pathology in a mouse model of spinal muscular atrophy. J. Cell Biol. 165, 1355-1365.

Morris, G. (2002). The interdependence of nuclear structure and splicing defects in S. pombe carrying a degron allele of the Survival of Motor Neuron gene. EMBO J. 21, 75-86.

Pellizzoni, L., Z. J., Lehner. R., Forster, C., and Barta, A. (2005). Evolutionary conservation of minor U12-type spliceosomes between plants and humans. RNA 11, 1095-1107.

Pellizzoni, L. (2005). The survival motor neuron protein in spinal muscular atrophy. Hum. Mol. Genet. 6, 1205-1214.

Pellizzoni, L., Z. J., Lehner. R., and Matera, A. G. (2004). Coupled in vitro import of U snRNPs and SMN, the spinal muscular atrophy protein. Mol. Cell 16, 415-424.

Pellizzoni, L., Z. J., Gonsalvez, G. B., Bedenekko, J., Darzyynskiewicz, J., Gerace, L. and Matera, A. G. (2005). Cross-talk between snurportin1 subdomains. Mol. Biol. Cell. 16, 4660-4671.

Pellizzoni, L., Z. J., Gonsalvez, G. B., Bedenekko, J., Darzyynskiewicz, J., Gerace, L. and Matera, A. G. (2005). Cross-talk between snurportin1 subdomains. Mol. Biol. Cell. 16, 4660-4671.

Pellizzoni, L., Z. J., Gonsalvez, G. B., Bedenekko, J., Darzyynskiewicz, J., Gerace, L. and Matera, A. G. (2005). Cross-talk between snurportin1 subdomains. Mol. Biol. Cell. 16, 4660-4671.

Pellizzoni, L., Z. J., Gonsalvez, G. B., Bedenekko, J., Darzyynskiewicz, J., Gerace, L. and Matera, A. G. (2005). Cross-talk between snurportin1 subdomains. Mol. Biol. Cell. 16, 4660-4671.

Pellizzoni, L., Z. J., Gonsalvez, G. B., Bedenekko, J., Darzyynskiewicz, J., Gerace, L. and Matera, A. G. (2005). Cross-talk between snurportin1 subdomains. Mol. Biol. Cell. 16, 4660-4671.

Pellizzoni, L., Z. J., Gonsalvez, G. B., Bedenekko, J., Darzyynskiewicz, J., Gerace, L. and Matera, A. G. (2005). Cross-talk between snurportin1 subdomains. Mol. Biol. Cell. 16, 4660-4671.
Altered snRNP dynamics in SMA

Phair, R. D. and Misteli, T. (2000). High mobility of proteins in the mammalian cell nucleus. Nature 404, 604-609.

Rino, J. and Carmo-Fonseca, M. (2009). The spliceosome: a self-organized macromolecular machine in the nucleus? Trends Cell Biol. 19, 375-384.

Rino, J., Carvalho, T., Braga, J., Desterro, J. M., Lührmann, R. and Carmo-Fonseca, M. (2007). A stochastic view of spliceosome assembly and recycling in the nucleus. PLOS Comput. Biol. 3, e201.

Rino, J., Carvalho, T., Braga, J., Desterro, J. M., Lührmann, R. and Carmo-Fonseca, M. (2007). A stochastic view of spliceosome assembly and recycling in the nucleus. PLOS Comput. Biol. 3, e201.

Shpargel, K. B. and Matera, A. G. (2005). Gemin proteins are required for efficient assembly of Sm-class ribonucleoproteins. Proc. Natl. Acad. Sci. USA 102, 17372-17377.

Sleeman, J. E. and Lamond, A. I. (1999). Newly assembled snRNPs associate with coiled bodies before speckles, suggesting a nuclear snRNP maturation pathway. Curr. Biol. 9, 1065-1074.

Sleeman, J. E., Ajuh, P., and Lamond, A. I. (2001). snRNP protein expression enhances the formation of Cajal bodies containing p80 coilin and SMN. J. Cell Sci. 114, 4407-4419.

Sleeman, J. E., Trinkle-Mulcahy, L., Prescott, A. R., Ogg, S. C. and Lamond, A. I. (2003). Cajal body proteins SMN and coilin show differential dynamic behaviour in vivo. J. Cell Sci. 116, 2039-2050.

Spector, D. L. and Lamond, A. I. (2001). Spliceosome U snRNP biogenesis, structure and function. Curr. Opin. Cell Biol. 13, 290-301.

Spector, D. L. and Lamond, A. I. (2011). Nuclear speckles. Cold Spring Harb. Perspect. Biol. 3, a000646.

Sprague, B. L. and McNally, J. G. (2005). FRAP analysis of binding: proper and fitting. Trends Cell Biol. 15, 84-91.

Stanek, D., Rader, S. D., Klingauf, M. and Neugebauer, K. M. (2003). Targeting of U4/U6 small nuclear RNP assembly factor SART3/p110 to Cajal bodies. J. Cell Biol. 160, 505-516.

Stanek, D., Pridalová-Hnilcová, J., Novotný, I., Huranová, M., Blazíková, M., Wen, X., Sapra, A. K. and Neugebauer, K. M. (2008). Spliceosomal small nuclear ribonucleoprotein particles repeatedly cycle through Cajal bodies. Mol. Biol. Cell 19, 2534-2543.

Stark, H., Dube, P., Lührmann, R. and Kastner, B. (2001). Arrangement of RNA and proteins in the spliceosomal U1 small nuclear ribonucleoprotein particle. Nature 409, 539-542.

Tanackovic, G., Ransijn, A., Ayuso, C., Harper, S., Berson, E. L. and Rivolta, C. (2011a). A missense mutation in PRPF6 causes impairment of pre-mRNA splicing and autosomal-dominant retinitis pigmentosa. Am. J. Hum. Genet. 88, 643-649.

Tanackovic, G., Ransijn, A., Thibault, P., Ahou Elela, S., Klinck, R., Berson, E. L., Chabot, B. and Rivolta, C. (2011b). PRPF mutations are associated with generalized defects in spliceosome formation and pre-mRNA splicing in patients with retinitis pigmentosa. Hum. Mol. Genet. 20, 2116-2130.

Tarn, W. Y. and Steitz, J. A. (1996). A novel spliceosome containing U11, U12, and U5 snRNPs excises a minor class (AT-AC) intron in vitro. Cell 84, 801-811.

Wahl, M. C., Will, C. L. and Lührmann, R. (2009). The spliceosome: design principles of a dynamic RNP machine. Cell 136, 701-718.

Wan, L., Battle, D. J., Yong, J., Gubitz, A. K., Kolb, S. J., Wang, J. and Dreyfuss, G. (2005). The survival of motor neurons protein determines the capacity for snRNP assembly: biochemical deficiency in spinal muscular atrophy. Mol. Cell. Biol. 25, 5543-5551.

Will, C. L. and Lührmann, R. (2001). Spliceosomal U snRNP biogenesis, structure and function. Curr. Opin. Cell Biol. 13, 290-301.

Will, C. L., Schneider, C., Reed, R. and Lührmann, R. (1999). Identification of both shared and distinct proteins in the major and minor spliceosomes. Science 284, 2003-2005.

Young, P. J., Le, T. T., thi Man, N., Burghes, A. H. and Morris, G. E. (2000). The relationship between SMN, the spinal muscular atrophy protein, and nuclear coiled bodies in differentiated tissues and cultured cells. Exp. Cell Res. 256, 365-374.

Zhang, Z., Lotti, F., Dittmar, K., Younis, I., Wan, L., Kasim, M. and Dreyfuss, G. (2008). SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. Cell 133, 585-600.