Specific splicing defects in *S. pombe* carrying a degtron allele of the Survival of Motor Neuron gene

Yannick Campion, Henry Neel, Thierry Gostan, Johann Soret and Rémy Bordonné*

Institut de Génétique Moléculaire de Montpellier (IGMM), CNRS UMR 5535/IFR122, Université Montpellier I and II, Montpellier Cedex 5, France

Spinal muscular atrophy results from deletions or mutations in the survival of motor neuron (*SMN1*) gene. The SMN protein has an essential role in the biogenesis of spliceosomal snRNPs, but the link between a defect in this process and specific splicing inhibition of pre-mRNAs has not been established. In this study, we report the construction of a temperature-degtron (*td*) allele of the *Schizosaccharomyces pombe* SMN protein and show that its depletion at 37°C affects splicing and formation of U1, U2, U4 and U5 snRNPs, but not of U6 and U3 ribonucleoproteins. The function of the *tdSMN* allele in snRNP assembly is already perturbed at 25°C, suggesting a deleterious effect of the tag at this temperature. Using a genome-wide approach, we report that introns react unequally to lower levels of snRNPs in *tdSMN* cells and that increasing the length of the polypyrimidine tract can improve the splicing efficiency of some, but not all, affected introns. Altogether, our results suggest that the defects observed in *tdSMN* fission yeast cells mimic splicing deficits observed in SMN-deficient metazoan cells.

The EMBO Journal (2010) 29, 1817–1829. doi:10.1038/emboj.2010.70; Published online 16 April 2010

Subject Categories: RNA

Keywords: fission yeast; pyrimidine tract; SMN; snRNP; splicing

Introduction

Spinal muscular atrophy (SMA) is a common human genetic disease in which degeneration of the motor neurons of the spinal cord results in subsequent muscular atrophy (Roberts *et al*, 1970). The disease is due to recessive mutations affecting the survival of motor neurons (*SMN1*) gene, which is homozygously deleted or mutated in SMA patients (Lefebvre *et al*, 1995). The SMN protein is part of a macromolecular complex, which contains at least eight additional proteins (Gemins 2–8 and unrip) and has an essential role in the biogenesis of spliceosomal U snRNPs (Paushkin *et al*, 2002; Neuenkirchen *et al*, 2008). The assembly process of

these U snRNPs follows an ordered pathway: after transcription, m⁷G-capped snRNAs U1, U2, U4 and U5 are exported to the cytoplasm, where they bind to the seven Sm proteins B/B', D1, D2, D3, E, F and G. The proper assembly of the Sm core domain is required for subsequent m³G cap formation of the U snRNA by Tgs1 methyltransferase and, following this step, the particle is actively transported to the nucleus (Will and Lührmann, 2001; Mouaikel *et al*, 2002).

The SMN complex is involved in the formation of the Sm protein heptameric ring and in its association with snRNAs (Fischer *et al*, 1997; Meister *et al*, 2001a). The SMN protein confers specificity during canonical Sm core assembly and prevents association of non-specific RNAs (Pellizzoni *et al*, 2002; Palfi *et al*, 2009). The function of the SMN machinery is regulated by the methylosome containing pICln, the methyltransferase PRMT5 and the WD45 protein (Friesen *et al*, 2001; Meister *et al*, 2001b). The snRNP core formation occurs by a two-step mechanism in which pICln first stabilizes intermediate subcomplexes of Sm proteins and, subsequently, the SMN complex functions as a catalyst, allowing ring closure of the Sm protein core complex on the snRNA (Chari *et al*, 2008).

In addition to the role of SMN in RNA metabolism, it has been proposed that the SMN protein possesses a motor neuron-specific function. Indeed, the SMN protein co-localizes with cytoskeletal proteins in dendrites and axons of spinal cord motor neurons *in vivo* and can be found in motile granules that are located in neurites and growth cones of cultured neurons, suggesting a function of SMN in nucleocytoplasmic, dendritic or axonal transport (Bechade *et al*, 1999; Cifuentes-Diaz *et al*, 2002; McWhorter *et al*, 2003; Winkler *et al*, 2005; Zhang *et al*, 2006).

With regard to the housekeeping role of SMN in snRNP biogenesis, recent studies show that a reduction of SMN levels decreases cell viability and alters the repertoire of snRNAs, the profile of which depends on the cell type and tissues of an SMA mouse model (Gabanella *et al*, 2007; Zhang *et al*, 2008). Using exon microarrays, different studies revealed specific alterations in splicing in various tissues from the SMA mouse and identified differences in pathways associated with neuronal development (Zhang *et al*, 2008; Bäumer *et al*, 2009). However, exon microarrays allow to distinguish between different isoforms of a gene and to identify alterations in exon usage, but they are not suitable to characterize splicing defects such as intron retention events, which could affect the coding capacity and/or stability of the resulting mRNA and have a role in pathogenesis. Moreover, although it is now clear that SMN depletion is responsible for splicing alterations, the mechanistic bases of these events remain to be determined.

To address these questions, we choose to analyse defects induced by SMN deficiency in fission yeast, which carries an SMN orthologue (Hannus *et al*, 2000; Owen *et al*, 2000; Paushkin *et al*, 2000). The *Schizosaccharomyces pombe* *smn1* + (*YAB8*; SPAC2G11.08c) gene is essential, but the precise role of the SMN protein in snRNP formation and the

*Corresponding author. Institut de Génétique Moléculaire de Montpellier, UMR 5535 CNRS, 1919 route de Mende, Montpellier 34293, France. Tel.: +33 4 67 61 36 47; Fax: +33 4 67 04 02 31; E-mail: remy.bordonne@igmm.cnrs.fr

Received: 16 October 2009; accepted: 24 March 2010; published online: 16 April 2010

effects of its depletion on splicing have not been assessed in this model organism. Fission yeast is of particular interest in the study of snRNP biogenesis and RNA processing, as numerous studies have demonstrated that *S. pombe* contains a splicing machinery more closely reflecting the archetype of a metazoan spliceosome: introns are found in 43% of genes, individual genes typically contain multiple introns and the fission yeast gene structure is more similar to that of vertebrate genes than to that of budding yeast (Käuffer and Potashkin, 2000; Wood *et al*, 2002). In addition to being an important organism in the study of various fundamental processes, *S. pombe* is also increasingly used for identifying the functions of genes related to human diseases (Wood *et al*, 2002).

To characterize splicing defects induced by SMN deficiency directly at the level of each intron, we constructed a strain carrying an *S. pombe* SMN temperature-degtron allele (tdSMN) and used tiling microarrays, which provide an unbiased tool to investigate gene expression on a genome-wide scope. In this report, we show that depletion of SMN at 37°C affects the formation of Sm-associated RNPs but not that of U6 and U3 ribonucleoproteins. Remarkably, at 25°C, cells carrying the tdSMN allele are viable but the U1, U2 and U5 snRNPs are differentially assembled and found at lower levels. Using a genome-wide approach, we further show that cells carrying the tdSMN allele exhibit variable splicing defects on different introns and that lengthening the polypyrimidine tract restores splicing of some, but not all, intron reporters. Altogether, our results demonstrate a direct link between SMN deficiency and splicing inhibition of only a subset of introns, supporting the view that similar defects might occur in SMN-deficient metazoan cells.

Results

Construction of an *S. pombe* td SMN strain

It has been reported that the *S. pombe* SMN protein shares structural and functional similarities with the human SMN

protein (Hannus *et al*, 2000; Owen *et al*, 2000; Paushkin *et al*, 2000). We therefore used the *S. pombe* system to investigate the function of SMN. We explored the use of a temperature-sensitive degtron (td) SMN allele to obtain a homogeneous population of cells depleted of SMN in a short period of time. We constructed a strain in which the N-terminus of SMN is fused to the DHFR degtron (Dohmen and Varshavsky, 2005) and expressed from a moderate *nmt41* promoter (Supplementary Figure 1A). Correct integration of the N-degtron tag at the *smn1* + chromosomal locus was confirmed by PCR amplification with different pairs of primers (Supplementary Figure 1B). The tdSMN strain is viable (Figure 1A) but grew slower at 25°C, with tdSMN cells growing at a rate that was two-fold lower than that of wild type (Figure 1B). When transferred to 37°C, the growth rate of the tdSMN strain decreased rapidly in liquid cultures (Figure 1B) and cells failed to grow on plates, compared with wild-type cells (Figure 1A). This phenotype was complemented by transformation of the tdSMN strain with a plasmid carrying the wild-type SMN gene under the *nmt41* promoter but not with the empty vector (Figure 1C). As monitored by western blotting, the tdSMN protein is correctly expressed at 25°C and its degradation clearly occurs after a shift to 37°C (Figure 1D).

The *S. pombe* SMN protein is required for efficient splicing and snRNP formation

Splicing inhibition was readily visible in the tdSMN strain after the shift to non-permissive temperature (Supplementary Figure 2A and B), confirming that SMN is essential for splicing (Hannus *et al*, 2000), and demonstrating further that the N-degtron tool can be used to efficiently monitor the function of SMN in this process.

In mammals, it has been shown that the SMN complex has a crucial role in the formation of the Sm core heptameric ring and in the association of this complex to spliceosomal snRNAs. Thus, we tested whether SMN is required for

Figure 1 Properties of tdSMN cells. (A) Serial dilutions of wild-type and tdSMN cells were spotted onto rich media and grown at the indicated temperature. (B) Growth curves of wild-type and tdSMN cells in rich media at 25 or 37°C. Cell growth was followed by measuring the optical density at 600 nm at the indicated times. (C) Serial dilutions of tdSMN cells transformed with the indicated plasmids were grown on plates at 25 and 37°C for 5 days. Plasmid pREP-SMN contains the *S. pombe* SMN gene, whereas pREP is the empty vector. (D) The tdSMN protein is degraded at non-permissive temperature. Equivalent amounts of cell extracts, prepared from wild-type (wt) and tdSMN (td) strains grown at 25°C or after a 2 h shift at 37°C, were fractionated on SDS-PAGE and immunoblotted with anti-HA antibodies. The predicted molecular mass of the tdSMN protein is 35 kDa. The level of a cross-reacting band (arrow) shows that similar amounts of extracts were loaded into each lane.

Figure 2 SMN depletion in *S. pombe* gives rise to a decrease in snRNA levels. (A) Northern blot analysis of RNA isolated from wild-type and tdSMN cells before and after an 8 h shift to 37°C. The RNA was separated on 6% polyacrylamide/8 M urea gel, subjected to northern blot analysis and hybridized with oligonucleotide probes for the indicated RNA species. The tRNA^{Ser} was used as loading control and for quantification analyses. (B) Quantification of the northern blot analysis shown in (A) by scanning densitometry of the snRNAs present in tdSMN cells after an 8-h shift to 37°C, compared with cells grown at 25°C (time 0). (C) Quantification of the northern blot shown in (A) by scanning densitometry of the snRNAs present in tdSMN and wild-type cells when grown at 25°C.

stability of these snRNAs. This was carried out by northern blot analysis of RNA extracted from wild-type and tdSMN cells after shift to 37°C for 8 h. The U1, U2, U4 and U5 snRNA levels decrease in the tdSMN mutant after an 8-h shift at 37°C, whereas similar amounts are found in the wild-type strain (Figure 2A). Quantification analysis using tRNA^{Ser} as internal control showed that U1, U2 and U5 were more sensitive to SMN depletion (50–70% decrease) than U4 snRNA (35% decrease; Figure 2B) and this might be because of the protective association of U4 in the U4/U6 di-snRNP.

Given that SMN was also proposed to be required for the formation of the Lsm heptameric ring and for the assembly of snoRNPs in mammals (Terns and Terns, 2001), we also analysed the levels of the *S. pombe* U6 snRNA and U3 snoRNA and found that they are unchanged after the shift of tdSMN cells at 37°C (Figure 2A and B). Taken together, these results demonstrate unambiguously that in *S. pombe* the SMN protein is required for the accumulation of the spliceosomal U1, U2, U4 and U5 snRNAs but not for the U6 and U3 RNA species.

Interestingly, an examination of the blots in Figure 2A indicated differences in the levels of individual snRNAs in the tdSMN mutant cells grown at permissive temperature when compared with wild type (compare lanes 3 and 1). Quantification of the blot showed that the amounts of U1, U2 and U5 were lower in tdSMN cells by about 50%, whereas levels of U4 decreased by only 20%, the U3 snoRNA and U6 snRNA being only moderately reduced (Figure 2C). Together with the slow growth phenotype of tdSMN cells observed at permissive temperature, these observations suggest that the function of the tdSMN allele in snRNP assembly might be already affected at 25°C. This might be due to the incorrect folding of the fusion protein or to steric hindrance of the td tag (see Discussion section).

Determination of snRNP assembly status by glycerol gradient sedimentation

To determine the status of snRNPs under physiological conditions, we analysed snRNP complexes by glycerol gradient sedimentation, followed by northern blot analysis, an approach previously used to characterize the snRNP distribution in *Saccharomyces cerevisiae* and in *S. pombe* (Bordonné *et al*, 1990; Huang *et al*, 2002). As shown in Figure 3A, individual mono-snRNPs (U1, U2, U5; fractions 3–19) and di-snRNP U4/U6 (fractions 11–17) are found in the top fractions of the gradient and a multi-U2/U5/U6 tri-snRNP migrated near the bottom of the gradient in fractions 27–29 in extracts prepared from wild-type and tdSMN cells grown at 25°C. It has been previously reported that *S. pombe* contains almost undetectable amounts of U4/U5/U6 tri-snRNP, and instead contains high amounts of spliceosomal-like U2/U5/U6 particles, in contrast to *S. cerevisiae* and metazoan systems (Huang *et al*, 2002; Ohi *et al*, 2007).

A difference between wild-type and tdSMN patterns relates to a U5 form that can be observed easily in fractions 3–7 in the wild-type cells, and the amount of which decreases in tdSMN cells grown at 25°C (Figure 3A). This form likely corresponds to free U5 snRNP and a decrease in its amount can also be observed on native gel analysis (see below, Figure 4B). Finally, in both strains, a higher-order U1 snRNP complex was also found to sediment at the bottom of the gradient (fractions 25–29) as previously reported (Newo *et al*, 2007).

We next tested the effect of SMN depletion on snRNP stability by analysing the profile of snRNPs in cells shifted to 37°C for 4 h, an intermediate time at which snRNA levels are almost unchanged in tdSMN cells. When compared with cells grown at 25°C, a similar snRNP distribution was observed in extracts prepared from wild-type cells, although a

Figure 3 Analysis of the snRNP profiles by glycerol gradient sedimentation. Splicing extracts prepared from the indicated strains were subjected to glycerol gradient sedimentation. The RNAs were extracted from the indicated fractions, separated under denaturing conditions and transferred to a nylon membrane for northern blot analysis using probes specific for the indicated snRNAs. The fraction number of each gradient and the positions of the different snRNP complexes are shown at the bottom. Extracts corresponding to wild-type (WT; upper panels) or tdSMN cells (td; lower panels) grown at 25°C are shown in (A) and extracts obtained after a 4 h shift at 37°C are shown in (B).

lower amount of heavy sedimenting U1 particles is found in fractions 27–29 (Figure 3B, upper panel). Upon SMN depletion in tdSMN cells, U2/U5/U6 snRNP was no longer found in extracts prepared from cells grown for 4 h at 37°C (Figure 3B, fractions 27–29 in the lower panel) and U1 snRNP was also not present in high sedimenting particles. Altogether, these experiments show that depletion of the SMN protein alters the snRNP profile, thereby demonstrating an essential role for the SMN protein in snRNP formation and maintenance.

Differential snRNP assembly in tdSMN cells

We also used native gel electrophoresis to analyse the snRNP composition of the different extracts after a shift to 37°C. This approach has previously been used to study snRNP complexes in *S. pombe* (Huang *et al*, 2002) and allows the separation of RNA–protein complexes from free snRNA (Supplementary Figure 2C). The U1, U2, U4/U6 and U5 snRNPs can be found in the wild-type extract after a shift to 37°C (Figure 4A). In addition, a slowly migrating complex containing U2, U5 and U6 snRNPs corresponding to the tri-snRNP was also visible under similar conditions when probed with the corresponding oligonucleotides (Figure 4A). In contrast, with the exception of the U3 particle and, to a lesser extent, the U4/U6 snRNP, all snRNPs are strongly destabilized in extracts from tdSMN cells after 8-h incubation at 37°C, demonstrating that the SMN protein has an essential role in the stability of the spliceosomal snRNPs *in vivo*. It should also be noted that in wild-type extracts, U2 and U5 snRNPs are found in additional complexes (indicated by an asterisk in Figure 4A). These complexes correspond to the U2 and U5 forms found in fractions 13–19 in the glycerol gradient analyses (Figure 3) and likely represent splicing complex intermediates (Huang *et al*, 2002).

Given the reduction in the levels of individual snRNAs in the tdSMN mutant cells grown at permissive temperature detected by northern blot analysis (Figure 2A), we further investigated the snRNP content in tdSMN and wild-type cells

grown at 25°C by native gel electrophoresis. Although both cell types contained equivalent amounts of U3 snoRNP, the tdSMN extract clearly contained less U1, U2 and U5 snRNPs (Figure 4B). Quantification of the gel after normalization using U3 as standard indicated that snRNP levels present in tdSMN cells represented approximately 50% of the wild-type levels for U1, 40% for U2, 80% for U4, 30% for U5 and 90% for U6. As expected from lower amounts of U2 and U5 snRNPs, the U2/U5/U6 snRNP level in tdSMN cells was also decreased and represented 45% of that found in wild-type cells (Figure 4C). In summary, these results show that tdSMN cells contain lower levels of U1, U2 and U5 mono-snRNPs, as well as decreased amounts of the U2/U5/U6 post-splicing particle, suggesting that splicing efficiency is already affected at 25°C in tdSMN cells.

The tdSMN mutant exhibits differential pre-mRNA splicing defects at 25°C

To test whether the reduced levels of snRNPs affect splicing in tdSMN cells at 25°C, we monitored the potential accumulation of introns in pre-mRNAs using tiling microarrays, which have previously been used to detect intron-containing RNAs in yeast (Zhang *et al*, 2007; Dutrow *et al*, 2008; Sayani *et al*, 2008). Three independent cultures were used for wild-type and tdSMN mutant, and probes prepared from the corresponding RNAs were used to hybridize Affymetrix tiling arrays. Using the Tiling Array Software (Affymetrix), diagrams were generated in which bars represent the differences between tdSMN and wild-type intensities (Figure 5A, panels a–d). Visual inspection of these tiling array profiles revealed a subpopulation of genes showing an increase in intronic signals in the tdSMN mutant compared with wild type. These higher intronic signals can occur with a significant decrease of signal in adjacent exonic sequences, as it is the case for the SPAC9G1.03c intron 1, the SPCC18B5.10c intron 2 and SPCC24B10.17 intron 2 (Figure 5A, panels a–c, respectively). In another subpopulation, no major change was

Figure 4 Analysis of snRNPs in tdSMN and wild-type cells by native gel electrophoresis. (A) Extracts were prepared from cells grown after an 8-h shift at 37°C and aliquots (20 µg) were separated on 4% native gels. The RNA was subjected to northern blot analysis and hybridized with probes for the indicated RNAs. Similar amounts of U3 snRNP are found for both extracts, showing that equivalent amounts of extracts were loaded into each lane. (B) Extracts were prepared from the indicated strains grown at 25°C, separated on native gels and treated as in (A). In both panels, asterisks point to intermediate splicing complexes observed for U2 and U5 snRNPs (see text for details). (C) Quantification of the northern blot analysis shown in (B) by scanning densitometry of the RNAs found in tdSMN compared with wild-type cells after growth at 25°C.

observed in intronic signals, whereas those corresponding to adjacent exonic sequences were strongly decreased (SPAC13A11.03 in Figure 5A, panel d), thereby indicating that intron retention is associated with an efficient degradation of the corresponding transcripts. These tiling array profiles were confirmed by semi-quantitative RT-PCR experiments showing that, for the above mentioned genes, the pre-mRNAs accumulate in tdSMN cells at the expense of the fully mature transcript (Figure 5A, right panels).

Interestingly, an inspection of the diagrams also revealed that introns from multi-intronic genes are not retained to the same extent in the tdSMN strain (see, for example, SPCC18B5.10c introns 1, 3, 4, 5 and 6; Figure 5A, panel b), suggesting that SMN deficiency does not result in a general splicing inhibition. In agreement with this, analysis of the tiling array profiles led to the identification of introns and exons showing no major changes in signals in the tdSMN strain as compared with the wild type (Supplementary Figure 3). It is noteworthy that RT-PCR analyses indicated that splicing of numerous pre-mRNAs is either very efficient (Figure 5B and Supplementary Figure 3, panels a and b) or affected at similar levels (Figure 5C and Supplementary Figure 3, panels c and d) in tdSMN and wild-type strains. Taken together, these observations established that SMN loss of function impairs splicing of a specific subset of introns independently of whether they are efficiently spliced in the wild-type strain.

Following bioinformatic analysis (described in the Materials and methods section), the difference between the mean signal intensities for each intron and that of the two adjacent exons was calculated to evaluate their retention index (Supplementary Table I). The names and properties of the top 20 introns showing the highest retention index are displayed in Table I and the primary sequences of the top 100 retained introns are shown in the Supplementary Figure 4.

Characterization of features common to retained introns

To find common features in the retained introns, we used the non-parametric Wilcoxon test (see Materials and methods and Supplementary methods). When the distribution of sequences corresponding to the 5' splice donor site, to the branch point (BP) or to the acceptor site in a given group was systematically compared with that of the rest of the 4614 fission yeast introns, no significant differences (P -value < 0.05) were observed between the two groups (data not shown). This suggests that splicing defects in tdSMN cells do not primarily rely on the presence of less consensual subsets of donor, BP or acceptor sequences in the affected introns.

In addition to the consensus splice sites, it has also been shown that, similar to their metazoan counterparts, fission yeast introns have polypyrimidine tracts (PPTs) that contribute to splicing. These PPTs are not only found between the BP and the 3' acceptor splice sites but also between the 5' splice site and the BP region, where they can participate in U2AF recruitment and 3' splice site identification (Romfo and Wise, 1997; Käufer and Potashkin, 2000; Kupfer *et al*, 2004; Sridharan and Singh, 2007). Although analysis of the position and size of PPTs located downstream of the BP adenosine did not define a group significantly different from the rest of the 4614 introns (Figure 6A), a comparison of the position and size of PPTs located upstream of the BP adenosine led to the identification of a first group of introns (1–455, Figure 6B) significantly distinct from the remaining 4159 (P -value: 0.008). As shown in Figure 6C, the main differences between the two groups are a threefold reduction of the frequency of PPTs located five nucleotides upstream of the BP adenosine (position –5) and a twofold increase in the frequency of PPTs located much far upstream (position –20). Comparison of the position and size of the upstream PPTs also led us to identify a much larger group (1–1827) that was also significantly

Figure 5 Differential splicing defects in tdSMN cells revealed by microarrays analyses. **(A)** Gene models are annotated with signals derived from the tiling arrays corresponding to the difference between the intensities found in tdSMN and wild type. Scale on the y-axis represents the log2 fold change between the treatment (tdSMN) and control (WT) group signals. The panels on the right correspond to RT-PCR validations performed on total RNA isolated from the wild-type (WT) and tdSMN (td) cells grown at 25°C. The indicated introns were PCR amplified with exon-specific primers and PCR products were separated on a 1.5 or 2.5% agarose gel and visualized by ethidium bromide staining. **(B)** Validation tests were performed for introns that do not appear at the top of the list, as well as for a non-intronic gene (*SPAC3C7.09*), demonstrating similar RNA content in the samples. **(C)** RT-PCR analyses were also performed on pre-mRNAs that are not efficiently spliced in wild-type or tdSMN cells.

different from the remaining 2787 introns (Figure 6B; *P*-value 0.006), showing no clear differences, except for position -7, in which a twofold increase in the frequency of tracts consisting of five pyrimidines, at the expense of tracks

containing 6, 7, 8, 10 and more pyrimidines, was observed (Figure 6D). No difference was detected between the 455 and 1827 intron groups and the corresponding remaining *S. pombe* introns with regard to the frequencies of splicing

Table I Names and properties of the top 20 inefficiently spliced introns in tdSMN cells

Accession number	Gene symbol/Description	Biol. process/Mol. function	Intron	Intron mean intensity	Exon mean intensity	Retention index
SPCC18B5.10c	TREX complex subunit Tex1	mRNA export from nucleus	2	4.413	−0.359	4.773
SPAC9.07c	GTPase Rbg1	GTPase activity	2	3.064	−0.599	3.664
SPAC9G1.03c	Ribosomal protein L30	Translation	1	2.743	−0.308	3.051
SPBC17D11.08	WD repeat protein	Golgi complex	3	3.453	0.454	2.999
SPBC1711.04	Methylenetetrahydrofolate reductase	Folate biosynthesis	1	2.044	−0.666	2.710
SPCC970.05	Ribosomal protein L36	Translation	1	2.270	−0.263	2.533
SPBC1685.09	Ribosomal protein S29	Translation	2	2.920	0.396	2.523
SPAP27G11.03	D123 family	Mitotic cell cycle control	1	2.211	−0.189	2.400
SPAPB18E9.01	tRNA methyltransferase Trm5	tRNA methylation	1	1.569	−0.813	2.383
SPBC13E7.09	Verprolin	Actin nucleation	1	3.269	0.891	2.377
SPBC106.18	Ribosomal protein L25	Translation	1	2.070	−0.255	2.325
SPAC17A5.07c	Cysteine peptidase Ulp2	Protein desumoylation	3	2.510	0.219	2.290
SPBC11G11.02c	Actin cortical patch component End3	Actin filament organization	1	2.073	−0.140	2.214
SPBC646.15c	Pex16 family	Peroxisome membrane protein import	1	2.072	−0.135	2.208
SPCC285.14	TRAPP complex subunit Trs130	ER to Golgi transport	4	2.254	0.055	2.198
SPAC4A8.07c	Sphingoid long chain base kinase	PKC activation	2	2.251	0.056	2.194
SPBC29A10.06c	Conserved fungal protein	Unknown	1	1.282	−0.908	2.190
SPBC336.08	Spindle pole body protein Spc24	Monopolar attachment	1	2.550	0.434	2.116
SPAC3A11.06	Sorting nexin Mvp1	Protein vacuolar targeting	7	2.073	−0.041	2.115
SPBC83.01	UBA/EH/EF hand domain protein Ucp8	Actin cortical patch assembly	2	1.744	−0.316	2.061

signals and the location of the PPT (upstream and/or downstream of the BP) when size and position were not taken into account (Supplementary Figure 5A and B).

Given that the strength of optimal PPTs can be modulated by suboptimal donor, acceptor and/or BP sequences, it is likely that introns carrying such sites introduce a bias in our statistical studies on the PPT. Thus, we also performed the analysis of PPT distribution in the subset of *S. pombe* introns containing the strongest consensus 5' splice site (GTA^A/TGT), 3'splice site (TAG) and BP sequence (CTAAC). Among the 622 introns fulfilling these criteria (Supplementary Table II), a group of 140 were found to be significantly different (*P*-value: 0.029) from the remaining 482 (Figure 7A) when the distribution of upstream PPTs was considered, whereas no groups could be defined on analysis of the positions of PPTs located downstream of the BP adenosine (Figure 7B). As shown in Figure 7C, the most notable differences between both groups are a fivefold reduction in the frequency of PPTs located six nucleotides upstream of the BP adenosine and a threefold increase in the frequency of PPTs located much far upstream (position −20) in the group of 140 introns. Altogether, our statistical analyses support the idea that the size and position of the PPT located upstream of the BP constitute, for some introns, important determinants involved in the differential splicing defects observed in tdSMN cells.

Mutations increasing the length of the polypyrimidine tract enhance splicing efficiency of several pre-mRNA reporters in tdSMN cells

To determine whether the polypyrimidine tract constitutes a determinant accounting for efficient splicing in the tdSMN background, we constructed splicing reporters carrying genes containing retained introns (Figure 8 and Supplementary Table I) in which we introduced mutations generating

11–22-nucleotide-long PPTs (see Supplementary Figure 6 for sequences) immediately upstream of the BP adenosine. These constructs were transformed in both wild-type and tdSMN cells, and splicing of the corresponding transcripts was analysed by semi-quantitative RT-PCR experiments. As shown in Figure 8, in wild-type cells, most of the RNAs expressed from plasmids were not as efficiently spliced as endogenous transcripts. This result is in agreement with a previous report showing that the relative yield of fully spliced mRNA is higher for transcripts expressed from a single-copy chromosomal locus (Romfo *et al*, 2000). Conversely, precursors are far more abundant in RNA prepared from cells carrying reporter genes on plasmids, even in wild-type cells (Romfo *et al*, 2000). For four reporter genes (*SPBC106.18*, *SPBC947.14c*, *SPAC4F10.14c* and *SPBC1703.10*; Figure 8A and Supplementary Figure 7), mutations generating a long PPT increased the splicing efficiency of the corresponding mutated intron in tdSMN cells, whereas splicing of the same intron was not significantly modified in wild-type cells. It is noteworthy that splicing efficiency of the *SPBC106.18*- and *SPBC1703.10*-mutated introns in tdSMN cells was restored at nearly the same level compared with that of wild-type introns in wild-type cells. For the *SPAC630.03* reporter, splicing efficiency of the mutated intron was increased in tdSMN cells at a level similar to that observed in wild-type cells (Figure 8B). For the two other splicing reporters tested, (*SPCC24B10.17* and *SPAC9G1.03c*, Figure 8C), no significant changes in the splicing efficiency of mutated introns, either in wild-type or in tdSMN cells, could be observed. Altogether, these results indicate that splicing of some retained introns can be restored in tdSMN cells by increasing the length of the polypyrimidine tract immediately upstream of the BP sequence. As splicing efficiency could be restored only partially or not at all for several other mutated introns, these results also demonstrate that, aside from the lack of an upstream

Figure 6 Characterization of determinants common to retained introns by statistical analyses. **(A)** Wilcoxon *P*-values for the downstream pyrimidine tract position for the entire list of introns sorted by decreasing retention indexes. Whatever the group size, no *P*-value is below the significance limit (≤ 0.05). Moreover, all values fluctuate around 0.6, indicating an absence of correlation between downstream PPT position and retention indexes. **(B)** Wilcoxon *P*-values for the upstream pyrimidine tract position for the entire intron list sorted by decreasing retention indexes. Most of the *P*-values calculated up to a First Group Size (FGS) of ~ 2000 introns are localized below the significance limit (≤ 0.05), indicating a correlation between upstream PPT positions and retention indexes. Two specific group pairs are defined by the *P*-value variation curve. In the first one (introns 1–455 and the 4159 others), the two distributions defined are extremely different (*P*-value = 0.008). In the second one (introns 1–1827 and the 2787 others), the two distributions are even more different (*P*-value = 0.006). **(C)** Histograms depicting the frequency of PPTs of five or more pyrimidines at the indicated position upstream of the branch point adenosine. The size of the two groups being compared and the Wilcoxon *P*-value are indicated. **(D)** Histograms depicting the frequency of PPTs of the indicated size at position –7 upstream of the branch point adenosine. The size of the two groups being compared and the Wilcoxon *P*-value are indicated.

PPT, additional determinants are responsible for the splicing changes observed on SMN deficiency.

Discussion

In this study, we used the *S. pombe* model organism and a genome-wide approach to analyse the function of SMN in snRNP biogenesis *in vivo* and to characterize pre-mRNA splicing defects associated with SMN deficiency. Cells carrying a *td* allele of SMN display alterations in the assembly of snRNPs and exhibit splicing defects affecting a subset of

introns. Interestingly, we found a moderate, although statistically significant, change in the size and position of upstream PPTs in the retained introns, suggesting that these elements represent influential, but not unique, determinants for efficient splicing of some introns in tdSMN cells.

The SMN protein is required *in vivo* for optimal snRNP assembly and function of the spliceosome

Consistent with the role of the SMN complex in the formation of the heptameric Sm ring and in its transfer to snRNA, we found that depletion of the *S. pombe* SMN protein generates

Figure 7 The PPTs locate more upstream to the branch point in the retained introns displaying strong consensus sites. (A) Wilcoxon P -values for the upstream PPT position for the consensus introns list (composed of 622 introns) sorted by decreasing retention indexes. Most of the P -values calculated up to a First Group Size (FGS) of 140 introns are localized below the significance limit (≤ 0.05), indicating a correlation between upstream PPT positions and retention indexes. This ranked position (FGS = 140) defines two significantly different distributions (P -value = 0.029). (B) Wilcoxon P -values for the downstream PPT position for the consensus introns list sorted by decreasing retention indexes. Whatever the group size, no P -value is below the significance limit (≤ 0.05). Moreover, all the values are fluctuating around 0.6, indicating no correlation between downstream PPT position and retention indexes. (C) Histograms depicting the frequency of PPTs of five or more pyrimidines at the indicated position upstream of the branch point adenosine. The size of the two groups being compared and the Wilcoxon P -value are indicated.

instability of the spliceosomal U1, U2, U4 and U5 snRNPs *in vivo*. Such instability is consistent with studies in human cells and in *S. cerevisiae*, showing that snRNAs not assembled with Sm core proteins become degraded (Sauterer *et al*, 1988; Bordonné and Tarassov, 1996). Our results also demonstrate that the U6 snRNA and U3 snoRNA remain stable on depletion of SMN in *S. pombe*, even after a prolonged shift at a non-permissive temperature, suggesting that their assembly does not depend on the SMN complex. This is in contrast to the situation in mammals in which SMN has been suggested (but never shown directly) to be involved in the formation of various macromolecular complexes including U3 and U6 RNPs. Thus, in *S. pombe*, defects resulting from SMN depletion might be restricted to components associated with the Sm core complex. In this regard, our RT-PCR experiments have revealed that TER1, the RNA component of the fission yeast telomerase, which is capped with m_3G and contains an Sm-binding site (Leonardi *et al*, 2008; Webb and Zakian, 2008), is unstable in tdSMN cells on SMN depletion, indicating that TER1 is bound by an Sm core complex (data not shown).

We also found that the td tag already hinders the function of the SMN protein at permissive temperature. Because of steric hindrance of the tag or incorrect folding of the fusion protein, it is possible that the loading of Sm proteins onto the SMN complex, or association of another factor to this complex, is hampered. Remarkably, our native gel analysis shows that snRNPs are differentially affected, with U1, U2 and U5 levels being the most reduced at 25°C when compared with wild type. Although it was shown that SMN-deficient cells contain altered levels of snRNAs (Gabanella *et al*, 2007; Zhang *et al*, 2008), our report is the first demonstrating directly that snRNPs are differentially assembled *in vivo* upon SMN deficiency. Moreover, our data also provide evidence for a direct link between snRNP levels and the function of the splicing machinery. Indeed, in addition to a change in the relative proportions of individual snRNPs, we found that tdSMN cells contain lower amounts of U2/U5/U6 tri-snRNP compared with wild-type cells. Given that U2/U5/U6 tri-snRNP contains RNA splicing intermediates and represents post-splicing particles or stable multi-snRNP complexes in the late stages of the splicing reaction (Huang *et al*, 2002;

Figure 8 Splicing efficiency of some retained introns is improved in tdSMN cells by a polypyrimidine tract located immediately upstream of the branch point. Semi-quantitative RT-PCR experiments were performed using RNA extracted from wild-type and tdSMN cells transformed with the indicated splicing reporters and grown at 25°C. The precursor and mature forms of the amplified region are schematized on the right. The fraction of spliced product [mature/(mature + precursor)] was determined using the Syngene Genetools software. The fold change in splicing efficiency observed with the mutated splicing reporters (mean of triplicate experiments ± s.d.) is indicated. (A) Reporters showing increased splicing of the mutated intron in tdSMN cells but not in wild-type cells. (B) Reporter showing increased splicing in both wild-type and tdSMN cells. (C) Reporters showing no increase in splicing efficiency. White asterisks in the RPL25A, YPT1 and EMP24 panels represent PCR heteroduplexes that were described in Eckhart *et al* (1999) and that were not considered in the quantification analyses.

Ohi *et al*, 2007), our results indicate that splicing capabilities or kinetics are lowered in tdSMN cells.

Low levels of snRNPs inhibit splicing of introns to varying degrees

Our genome-wide approach allowed us to directly monitor the effect of an SMN deficiency on splicing of the full fission yeast 'intronome' at 25°C. We found that defects in snRNP assembly in tdSMN cells do not modify the processing of transcripts similarly but generate differential splicing defects. Our results demonstrate directly that pre-mRNAs react differently to lower levels of snRNPs and that the activity of the spliceosome is selectively perturbed when snRNP assembly is altered. We were able to detect a retention of intronic sequences in RNAs of many genes irrespective of intron number, which, in contrast, was the sole described common feature of the aberrantly spliced transcripts in the SMA mouse model (Zhang *et al*, 2008). The fact that introns from a given gene are not affected similarly also demonstrates that the observed alterations in intron splicing in tdSMN cells are not correlated with differences in the expression levels of the transcript. Such a coordinated mechanism, coupling increased splicing efficiency with increased transcription, was indeed observed for many introns in *S. pombe* (Wilhelm *et al*, 2008).

Previous studies in budding yeast using splicing-specific microarrays already showed that introns are not all affected in the same way in response to environmental stress or in strains carrying mutated splicing factors (Clark *et al*, 2002; Burckin *et al*, 2005; Pleiss *et al*, 2007a, b), raising the question of how the spliceosome discriminates between introns. In *S. cerevisiae*, it has been proposed that optimal splicing efficiency occurs on co-transcriptional U1 recruitment, which is independent of exon length. In contrast, U2 and U5 recruitment is dependent on exon length and post-transcriptional splicing occurs more efficiently for genes with short second exons (Moore *et al*, 2006; Tardiff *et al*, 2006). This model does not account for the observed splicing deficiency in tdSMN cells, as no correlation between the length of the downstream exons and retention of the upstream introns could be observed.

Our studies show that both the size and position of the PPT might account for the retention of some introns in tdSMN cells. This is in agreement with previous studies showing that PPTs are important for efficient splicing in fission yeast (Romfo and Wise, 1997; Sridharan and Singh, 2007). Because of the short size of pyrimidine tracts or because of their position relative to the BP, lower amounts of U2 snRNP in tdSMN cells might give rise to inefficient spliceosome formation and splicing of introns. This is supported by

studies showing that splicing of introns lacking PPTs is more dependent on U2AF (and consequently on U2 snRNP recruitment) than introns with long PPTs located upstream of the BP (Huang *et al*, 2002; Sridharan and Singh, 2007).

However, alternative mechanisms of U2AF recruitment have to occur *in vivo* to accommodate the large diversity of intron architectures observed in the affected introns. Along this line, the retention of introns in tdSMN cells could be due to different competitive abilities when the level of snRNPs is limiting, each intron requiring particular combinations of *cis*-acting elements and *trans*-acting factors for efficient splicing. Consistent with this proposal, it was shown that introns differ in the extent and nature of their requirements for U1 snRNA *in vivo*, as out of three tested pre-mRNAs, only the second intron of the fission yeast *cdc2* pre-mRNA is spliced inefficiently because of the presence of a stable hairpin even in cells containing wild-type U1 snRNA (Alvarez *et al*, 1996). Thus, low levels of snRNPs in tdSMN cells might contribute to unmask the deleterious effects of suboptimal splice signals and/or abnormal structures in retained introns. In addition, recognition of splice sites in mammals often depends on their sequence context and on the presence of exonic and intronic splicing enhancer elements that bind to regulatory factors, such as members of the SR protein family, necessary to recruit the core splicing machinery (Long and Cáceres, 2009). In this regard, the presence of exonic splicing enhancers, which promote efficient splicing of reporter pre-mRNAs, has been characterized in fission yeast (Webb *et al*, 2005), thereby adding another level of complexity to the characterization of determinants responsible for intron retention in tdSMN cells. Altogether, our results strengthen the view of the cooperative nature and flexibility of spliceosome assembly.

Links between defects in snRNP assembly and SMA

Classification of the affected transcripts by gene ontology did not reveal a particular functional class of genes that would be more sensitive to lower snRNPs levels in tdSMN cells. Given that a broad range of transcripts is affected and that the splicing defects are not lethal, it is likely that various cellular processes might be less efficient but still functional in tdSMN cells, altering different metabolic activities and explaining the observed growth rate reduction. Our results showing that introns are removed differentially in tdSMN cells suggest that similar differential splicing defects could also occur in mammalian cells on SMN deficiency. Thus, splicing alterations of only a limited number of mRNAs coding for proteins having a crucial role in the function of motor neurons could be responsible for spinal muscular atrophy. The identification of these pre-mRNAs will certainly constitute a major challenge in the near future and enable the elucidation of the molecular basis of SMA.

Materials and methods

Strains and culture conditions

Strains SP200 (h⁺ leu1-32 ura4-D18 ade6-M210) and SP814 (h⁺ leu1-32 ura4-D18 ade6-M216) and derived diploid or haploid strains were used in this study. Standard methods were used for growth and genetic manipulation of *S. pombe* (Moreno *et al*, 1991). Cells were grown on YES or minimal EMM2 medium with adequate supplements.

Construction of the td-tagged fission yeast SMN gene

The td system has been useful for studying the functions of a number of proteins, both in *S. cerevisiae* (Dohmen and Varshavsky, 2005) and in *S. pombe* (Rajagopalan *et al*, 2004). The *tdSMN* degron allele was constructed using PCR primers with 80-nucleotide homologies to genomic DNA and 25-nucleotide homologies to the pSMRG2-nmt41-degronHA (Kana) plasmid (Gregan *et al*, 2003), which was kindly provided by S. Kearsey (University of Oxford). A DNA fragment was amplified and transformed into fission yeast cells as described earlier (Bähler *et al*, 1998). Correct homologous recombination of the tagged allele was checked by PCR amplification on genomic DNA.

Extract preparation, native gels and glycerol gradient sedimentation

Extracts were prepared as described previously (Huang *et al*, 2002). Briefly, after centrifugation, pellets were washed with water and cells were suspended in AGK400 buffer and resuspended to 1 g cells/ml in AGK400 buffer (10 mM HEPES-KOH (pH 7.9), 400 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 1 mM PMSF and 10% glycerol). Cell homogenates were frozen in liquid nitrogen and ground to a fine powder using a Freezer Mill 6770 grinder (Spex). The powder was then thawed on ice and centrifuged at 14 000 r.p.m. for 10 min at 4°C. The supernatant was recovered and spun at 55 000 r.p.m. for 30 min at 4°C in a TLA-100.3 rotor and dialysed for 2 h against buffer D (20 mM HEPES-KOH (pH 7.9), 0.2 mM EDTA, 100 mM KCl, 0.5 mM DTT, 1 mM PMSF, 20% glycerol). Aliquots of extracts were stored at -80°C.

For native gel analysis of snRNPs, 20 µg of extracts was loaded on 4% acrylamide gels (80:1) run in 25 mM Tris, 25 mM boric acid and 1 mM EDTA at 13 V for 16 h until bromophenol blue reached the bottom. The RNA was transferred onto a nylon membrane and subjected to northern blot analysis as described (Bordonné *et al*, 1990). Glycerol gradients were performed as previously described (Bordonné *et al*, 1990).

Reporter gene construction and RT-PCR analysis

Fission yeast genes were amplified from the Riken DNA Bank #6118 (Matsuyama *et al*, 2006) and cloned into the *S. pombe* plasmid pREP41 (Craven *et al*, 1998) using standard methods. Mutagenesis was performed by PCR using mutated primers.

For semi-quantitative RT-PCR analyses, total RNA was purified from exponentially growing cells with Tri-Reagent (Sigma) according to the manufacturer's procedure and treated with RQ1 RNase-free DNase (Promega). First-strand cDNAs were synthesized from 5 µg of total RNA and pd(N)6 random oligonucleotides with a First strand cDNA synthesis kit (Amersham). For RT-PCR analyses, one-tenth of the reaction was amplified with GoTaq polymerase (Promega) in a volume of 50 µl containing 20 pmol of primer pairs, 200 µM dNTPs and 1.5 mM MgCl₂ in 1 × GoTaq buffer and sequentially cycled 26 times. Linearity of amplification was observed for at least 27 cycles as tested by the addition of [α -³²P]-dCTP (3000 Ci/mmol) in the final cycles (See Supplementary Figure 7 for an example). Primer sequences and PCR regimes are available on request. The PCR products were separated on 1.5–2.5% agarose gels containing ethidium bromide and visualized under UV light. The gel images were digitally captured and analysed using Syngene Genetools software. The ³²P-labelled fragments were separated on non-denaturing 6% polyacrylamide gels and analysed using Typhoon 9200 scanner and Image Quant Software.

Microarray analysis

Cells were grown to exponential phase and total RNA was purified as mentioned above. Probe labelling was performed with a GeneChip WT Double-Stranded cDNA Synthesis Kit (Affymetrix) following the manufacturer's protocol. Labelled probes were hybridized to GeneChip *S. pombe* Tiling 1.0FR Arrays (Affymetrix) using the manufacturer's protocol. These arrays are comprised of over 1.2 million perfect match/mismatch probe pairs tiled through the complete *S. pombe* genome. Scanning and data collection were carried out by GeneChip Scanner 3000 7G (Affymetrix) and GeneChip Operating Software. Total RNA from three independent cultures of the td-SMN and wild-type strains was applied to the tiling arrays.

Raw tiling array data were analysed using the Tiling Analysis Software (TAS) package (Affymetrix). Data from triplicate arrays were combined in control and treatment groups, quantile

normalized and analysed by the two-samples normalization process. The Perfect Match/Mismatch (PM/MM) normalized intensities were scaled so that the median intensity value was equal to 100 in the signals Binary Analysis Result (BAR) files. A bandwidth of 40 nucleotides was chosen in agreement with the Affymetrix user guide to obtain a probe analysis window size of 81 bp, which corresponds to the average intron size in *S. pombe*. This TAS normalization process results in a BAR file containing the differences between control and treatment-normalized signal values (expressed in log2 scale). To analyse differences between exon and intron intensities, a specific R software algorithm was developed to calculate average signal intensities in all *S. pombe* exon and intron areas from TAS-normalized result files. First, all positions of genes containing at least one intron were extracted from the *S. pombe* (September, 2004) database of the Affymetrix DAS server to generate a sub-list made of 4614 discrete chromosomal areas. This step allowed us to define the peak positions usually identified by statistical tests and *P*-value analyses. These positions were then used to calculate the difference between the mean of signal intensities for each *S. pombe* intron and that of the two adjacent exons.

Statistical analyses

For statistical analyses, we developed a specific function called *P-valueVariation* with the R statistical language. This function returns the Wilcoxon test *P*-value, allowing the comparison of non-parametric distributions between two groups defined by the function argument. The first group is defined from the beginning of the list to the argument (a ranked position from the entire intron list sorted by decreasing retention indexes). The second is set from the next argument position to the end of the list. The different *P*-values were obtained using the *P-valueVariation* function with all

the arguments chosen between the early beginning (Start position 30) to the close end (30 before ending) of the ranked list, with an increment of 1. The obtained *P*-values $((4614 - 30) - 30) = 4554$ values) have been plotted versus the argument to draw the *P-valueVariation* curves.

Accession numbers

Microarray data are accessible in the GEO database (accession number GSE17010).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank the members of the fission yeast community for sharing strains and plasmids. RDB6118 was provided by RIKEN BRC, which is participating in the National Bio-Resource Project of the MEXT (Japan). We thank members of the laboratory for stimulating discussions, E Schwob, E Bertrand and S Rader for critical comments on the manuscript and P Pasero for initial help in microarrays analysis. This study was supported by grants from the CNRS, the GIS 'Institut des Maladies Rares', AFM and ANR (ANR-06-MRAR-004-01) to RB.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Alvarez CJ, Romfo CM, Vanhoy R, Porter GL, Wise JA (1996) Mutational analysis of U1 function in *Schizosaccharomyces pombe*: pre-mRNAs differ in the extent and nature of their requirements for this snRNA *in vivo*. *RNA* **2**: 404–418
- Bähler J, Wu JQ, Longtine MS, Shah NG, McKenzie A, Steever AB, Wach A, Philippsen P, Pringle JR (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* **14**: 943–951
- Bäumer D, Lee S, Nicholson G, Davies JL, Parkinson NJ, Murray LM, Gillingwater TH, Ansorge O, Davies KE, Talbot K (2009) Alternative splicing events are a late feature of pathology in a mouse model of spinal muscular atrophy. *PLoS Genet* **5**: e1000773. doi:10.1371/journal.pgen.1000773
- Bechade C, Rostaing P, Cisterni C, Kalisch R, La Bella V, Pettmann B, Triller A (1999) Subcellular distribution of survival motor neuron (SMN) protein: possible involvement in nucleocytoplasmic and dendritic transport. *Eur J Neurosci* **11**: 293–304
- Bordonné R, Banroques J, Abelson J, Guthrie C (1990) Domains of yeast U4 spliceosomal RNA required for PRP4 protein binding, snRNP–snRNP interactions, and pre-mRNA splicing *in vivo*. *Genes Dev* **4**: 1185–1196
- Bordonné R, Tarassov I (1996) The yeast *SME1* gene encodes the homologue of the human E core protein. *Gene* **176**: 111–117
- Burckin T, Nagel R, Mandel-Gutfreund Y, Shiue L, Clark TA, Chang JL, Chang TH, Squazzo S, Hartzog G, Ares Jr M (2005) Exploring functional relationships between components of the gene expression machinery. *Nat Struct Mol Biol* **12**: 175–182
- Chari A, Golas MM, Klingenhäger M, Neuenkirchen N, Sander B, Engbrecht C, Sickmann A, Stark H, Fischer U (2008) An assembly chaperone collaborates with the SMN complex to generate spliceosomal snRNPs. *Cell* **135**: 497–509
- Cifuentes-Diaz C, Nicole S, Velasco ME, Borra-Cebrian C, Panozzo C, Frugier T, Millet G, Roblot N, Joshi V, Melki J (2002) Neurofilament accumulation at the motor endplate and lack of axonal sprouting in a spinal muscular atrophy mouse model. *Hum Mol Genet* **11**: 1439–1447
- Clark TA, Sugnet CW, Ares Jr M (2002) Genome wide analysis of mRNA processing in yeast using splicing-specific microarrays. *Science* **296**: 907–910
- Craven RA, Griffiths DJ, Sheldrick KS, Randall RE, Hagan IM, Carr AM (1998) Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe*. *Gene* **221**: 59–68
- Dohmen RJ, Varshavsky A (2005) Heat-inducible degron and the making of conditional mutants. *Methods Enzymol* **399**: 799–822
- Dutrow N, Nix DA, Holt D, Milash B, Dalley B, Westbrook E, Parnell TJ, Cairns BR (2008) Dynamic transcriptome of *Schizosaccharomyces pombe* shown by RNA–DNA hybrid mapping. *Nat Genet* **40**: 977–986
- Eckhart L, Ban J, Ballaun C, Weninger W, Tschachler E (1999) Reverse transcription-polymerase chain reaction products of alternatively spliced mRNAs form DNA heteroduplexes and heteroduplex complexes. *J Biol Chem* **274**: 2613–2615
- Fischer U, Liu Q, Dreyfuss G (1997) The SMN–SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell* **90**: 1023–1029
- Friesen WJ, Paushkin S, Wyce A, Massenet S, Pesiridis GS, Van Duyne G, Rappsilber J, Mann M, Dreyfuss G (2001) The methylosome, a 20S complex containing JBP1 and pICln, produces dimethylarginine-modified Sm proteins. *Mol Cell Biol* **21**: 8289–9300
- Gabanella F, Butchbach ME, Saieva L, Carissimi C, Burghes AH, Pellizzoni L (2007) Ribonucleoprotein assembly defects correlate with spinal muscular atrophy severity and preferentially affect a subset of spliceosomal snRNPs. *PLoS ONE* **2**: e921
- Gregan J, Lindner K, Brimage L, Franklin R, Namdar M, Hart EA, Aves SJ, Kearsley SE (2003) Fission yeast Cdc23/Mcm10 functions after pre-replicative complex formation to promote Cdc45 chromatin binding. *Mol Biol Cell* **14**: 3876–3887
- Hannus S, Bühler D, Romano M, Seraphin B, Fischer U (2000) The *Schizosaccharomyces pombe* protein Yab8p and a novel factor, Yip1p, share structural and functional similarity with the spinal muscular atrophy-associated proteins SMN and SIP1. *Hum Mol Genet* **9**: 663–674
- Huang T, Vilardell J, Query CC (2002) Pre-spliceosome formation in *S. pombe* requires a stable complex of SF1–U2AF(59)–U2AF(23). *EMBO J* **21**: 5516–5526
- Käufer NF, Potashkin J (2000) Analysis of the splicing machinery in fission yeast: a comparison with budding yeast and mammals. *Nucleic Acids Res* **28**: 3003–3010

- Kupfer DM, Drabenstot SD, Buchanan KL, Lai H, Zhu H, Dyer DW, Roe BA, Murphy JW (2004) Introns and splicing elements of five diverse fungi. *Eukaryot Cell* **3**: 1088–1100
- Lefebvre S, Bürglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C, Millasseau P, Zeviani M, Le Paslier D, Frézal J, Cohen D, Weissenbach J, Munnich A, Melki J (1995) Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* **80**: 155–165
- Leonardi J, Box JA, Bunch JT, Baumann P (2008) TER1, the RNA subunit of fission yeast telomerase. *Nat Struct Mol Biol* **15**: 26–33
- Long JC, Caceres JF (2009) The SR protein family of splicing factors: master regulators of gene expression. *Biochem J* **417**: 15–27
- Matsuyama A, Arai R, Yashiroda Y, Shirai A, Kamata A, Sekido S, Kobayashi Y, Hashimoto A, Hamamoto M, Hiraoka Y, Horinouchi S, Yoshida M (2006) ORFeome cloning and global analysis of protein localization in the fission yeast *Schizosaccharomyces pombe*. *Nat Biotechnol* **24**: 841–847
- McWhorter ML, Monani UR, Burghes AH, Beattie CE (2003) Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding. *J Cell Biol* **162**: 919–931
- Meister G, Bühler D, Pillai R, Lottspeich F, Fischer U (2001a) A multiprotein complex mediates the ATP-dependent assembly of spliceosomal U snRNPs. *Nat Cell Biol* **3**: 945–949
- Meister G, Eggert C, Bühler D, Brahms H, Kambach C, Fischer U (2001b) Methylation of Sm proteins by a complex containing PRMT5 and the putative U snRNP assembly factor pICln. *Curr Biol* **11**: 1990–1994
- Moore MJ, Schwartzfarb EM, Silver PA, Yu MC (2006) Differential recruitment of the splicing machinery during transcription predicts genome-wide patterns of mRNA splicing. *Mol Cell* **24**: 903–915
- Moreno S, Klar A, Nurse P (1991) Molecular genetic analysis of the fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol* **194**: 795–823
- Mouaikel J, Verheggen C, Bertrand E, Tazi J, Bordonné R (2002) Hypermethylation of the cap structure of both yeast snRNAs and snoRNAs requires a conserved methyltransferase that is localized to the nucleolus. *Mol Cell* **9**: 891–901
- Neuenkirchen N, Chari A, Fischer U (2008) Deciphering the assembly pathway of Sm-class U snRNPs. *FEBS Lett* **582**: 1997–2003
- Newo AN, Lützelberger M, Bottner CA, Wehland J, Wissing J, Jänsch L, Käufer NF (2007) Proteomic analysis of the U1 snRNP of *Schizosaccharomyces pombe* reveals three essential organism-specific proteins. *Nucleic Acids Res* **35**: 1391–1401
- Ohi MD, Ren L, Wall JS, Gould KL, Walz T (2007) Structural characterization of the fission yeast U5.U2/U6 spliceosome complex. *Proc Natl Acad Sci USA* **104**: 3195–3200
- Owen N, Doe CL, Mellor J, Davies KE (2000) Characterization of the *Schizosaccharomyces pombe* orthologue of the human survival motor neuron (SMN) protein. *Hum Mol Genet* **9**: 675–684
- Palfi Z, Jaé N, Preusser C, Kaminska KH, Bujnicki JM, Lee JH, Günzl A, Kambach C, Urlaub H, Bindereif A (2009) SMN-assisted assembly of snRNP-specific Sm cores in trypanosomes. *Genes Dev* **23**: 1650–1664
- Paushkin S, Charroux B, Abel L, Perkinson RA, Pellizzoni L, Dreyfuss G (2000) The survival motor neuron protein of *Schizosaccharomyces pombe*. Conservation of survival motor neuron interaction domains in divergent organisms. *J Biol Chem* **275**: 23841–23846
- Paushkin S, Gubitz AK, Massenet S, Dreyfuss G (2002) The SMN complex, an assemblysome of ribonucleoproteins. *Curr Opin Cell Biol* **14**: 305–312
- Pellizzoni L, Yong J, Dreyfuss G (2002) Essential role for the SMN complex in the specificity of snRNP assembly. *Science* **298**: 1775–1779
- Pleiss JA, Whitworth GB, Bergkessel M, Guthrie C (2007a) Rapid, transcript-specific changes in splicing in response to environmental stress. *Mol Cell* **27**: 928–937
- Pleiss JA, Whitworth GB, Bergkessel M, Guthrie C (2007b) Transcript specificity in yeast pre-mRNA splicing revealed by mutation in core spliceosomal components. *Plos Biol* **5**: 745–747
- Rajagopalan S, Liling Z, Liu J, Balasubramanian M (2004) The N-degron approach to create temperature-sensitive mutants in *Schizosaccharomyces pombe*. *Methods* **33**: 206–212
- Roberts DG, Chavez J, Court SDM (1970) The genetic component in child mortality. *Arch Dis Child* **45**: 33–38
- Romfo CM, Alvarez CJ, van Heeckeren WJ, Webb CJ, Wise JA (2000) Evidence for splice site pairing via intron definition in *Schizosaccharomyces pombe*. *Mol Cell Biol* **20**: 7955–7970
- Romfo CM, Wise JA (1997) Both the polypyrimidine tract and the 3' splice site function prior to the first step of splicing in fission yeast. *Nucleic Acids Res* **25**: 4658–4665
- Sauterer RA, Feeney RJ, Zieve GW (1988) Cytoplasmic assembly of snRNP particles from stored proteins and newly transcribed snRNA's in L929 mouse fibroblasts. *Exp Cell Res* **176**: 344–359
- Sayani S, Janis M, Lee CY, Tosca I, Chanfreau GF (2008) Widespread impact of nonsense-mediated mRNA decay on the yeast intronome. *Mol Cell* **31**: 360–370
- Sridharan V, Singh R (2007) A conditional role of U2AF in splicing of introns with unconventional polypyrimidine tracts. *Mol Cell Biol* **27**: 7334–7344
- Tardiff DF, Lacadie SA, Rosbash M (2006) A genome-wide analysis indicates that yeast pre-mRNA splicing is predominantly post-transcriptional. *Mol Cell* **24**: 917–929
- Terns MP, Terns RM (2001) Macromolecular complexes: SMN-the master assembler. *Curr Biol* **11**: R862–R864
- Webb CJ, Romfo CM, van Heeckeren WJ, Wise JA (2005) Exonic splicing enhancers in fission yeast: functional conservation demonstrates an early evolutionary origin. *Genes Dev* **19**: 242–254
- Webb CJ, Zakian VA (2008) Identification and characterization of the *Schizosaccharomyces pombe* TER1 telomerase RNA. *Nat Struct Mol Biol* **15**: 34–42
- Wilhelm BT, Marguerat S, Watt S, Schubert F, Wood V, Goodhead I, Penkett CJ, Rogers J, Bähler J (2008) Dynamic repertoire of a eukaryotic transcriptome surveyed at single-nucleotide resolution. *Nature* **453**: 1239–1243
- Will CL, Lüthmann R (2001) Spliceosomal UsnRNP biogenesis, structure and function. *Curr Opin Cell Biol* **13**: 290–301
- Winkler C, Eggert C, Gradl D, Meister G, Giegerich M, Wedlich D, Lagerbauer B, Fischer U (2005) Reduced U snRNP assembly causes motor axon degeneration in an animal model for spinal muscular atrophy. *Genes Dev* **19**: 2320–2330
- Wood V, Gwilliam R, Rajandream MA, Lyne M, Lyne R, Stewart A, Sgouros J, Peat N, Hayles J, Baker S, Basham D, Bowman S, Brooks K, Brown D, Brown S, Chillingworth T, Churcher C, Collins M, Connor R, Cronin A *et al* (2002) The genome sequence of *Schizosaccharomyces pombe*. *Nature* **415**: 871–880
- Zhang H, Xing L, Rossoll W, Wichterle H, Singer RH, Bassell GJ (2006) Multiprotein complexes of the survival of motor neuron protein SMN with Gemins traffic to neuronal processes and growth cones of motor neurons. *J Neurosci* **26**: 8622–8632
- Zhang Z, Hesselberth JR, Fields S (2007) Genome-wide identification of spliced introns using a tiling microarray. *Genome Res* **17**: 503–509
- Zhang Z, Lotti F, Dittmar K, Younis I, Wan L, Kasim M, Dreyfuss G (2008) SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. *Cell* **133**: 585–600