Human RBMY regulates germline-specific splicing events by modulating the function of the serine/arginine-rich proteins 9G8 and Tra2-β

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Summary

RBMY is a male germline RNA binding protein and potential alternative splicing regulator, but the lack of a convenient biological system has made its cellular functions elusive. We found that human RBMY fused to green fluorescent protein was strictly nuclear in transfected cells, but spatially enriched in areas around nuclear speckles with some components of the exon junction complex (EJC). Human RBMY (hRBMY) and the EJC components Magoh and Y14 also physically interacted but, unlike these two proteins, hRBMY protein did not shuttle to the cytoplasm. In addition, it relocalised into nucleolar caps after inhibition of RNA polymerase II transcription. Protein interactions were also detected between RBMY and splicing factors 9G8 and transformer-2 protein homolog β (Tra2-β), mediated by multiple regions of the RBMY protein that contain serine/arginine-rich dipeptides, but not by the single region lacking such dipeptides. These interactions modulated the splicing of several pre-mRNAs regulated by 9G8 and Tra2-β. Importantly, ectopic expression of hRBMY stimulated the inclusion of a testis-enriched exon from the *Acinus* gene, whereas 9G8 and Tra2-β repressed this exon. We propose that hRBMY associates with regions of the nucleus enriched in nascent RNA and participates in the regulation of specific splicing events in the germline by modulating the activity of constitutively expressed splicing factors.

Key words: hRBMY, Alternative splicing, Testis, Exon-junction complex, SR proteins

Introduction

Alternative splicing (AS) is a major contributor to protein diversity. In human, 90% of genes are estimated to undergo AS (Wang et al., 2008) and up to 50% of disease-causing mutations might affect splicing (Wang and Cooper, 2007). The most striking example of AS is that of the *Dscam* gene in *Drosophila melanogaster*: the combination of four clusters of mutually exclusive exons and 20 constant exons can potentially give rise to over 30,000 different mRNA isoforms and as many different proteins (Schmucker, 2007). Important classes of AS trans-regulators have been described, such as the families of serine/arginine-rich (SR) proteins and heterogeneous ribonucleoprotein (hnRNP) proteins (Bourgeois et al., 2004; Long and Caceres, 2009; Martinez-Contreras et al., 2007). As a general rule (although there are a number of exceptions), SR and hnRNP proteins act in an antagonistic manner to promote or repress exon inclusion, respectively. Most of these splicing regulator proteins are ubiquitously expressed but at different anatomic levels. In addition to these well-known factors, only a few tissue-specific AS regulators have been identified, such as Nova in the brain and the recently characterised epithelial splicing regulatory proteins (Grabowski, 2000; Warzecha et al., 2009), which hence cannot explain the vast majority of the tissue-specific splicing events observed over a wide range of tissues. It is therefore important to identify more such tissue-specific splicing regulators and to investigate their putative role in the corresponding tissues.

Several large-scale computational analyses have recently highlighted differences in AS level across a number of different human tissues. The human brain displays the highest level of AS, followed by liver and testes (Yeo et al., 2004). Moreover, testes (and brain) display the highest number of skipped exons, and phylogenetically divergent AS is more frequent in this tissue (Kan et al., 2005; Xu et al., 2002). The differentiation of spermatogonial germ cells to produce spermatozoa is accompanied by drastic changes in gene expression and is tightly regulated, notably by AS of a large number of transcripts encoding proteins specifically involved in this process (Elliott and Grellscheid, 2006; Kimmins et al., 2004). However, how AS regulation is achieved in the germline is still poorly understood.

Some members of a family of trans-acting factors composed of RNA-binding motif protein Y-linked (RBMY) and of its paralogs hnRNP G and hnRNP G-T have been proposed to regulate splicing specifically in the germline (reviewed in Elliott, 2004). The prototypical member of this family, RBMY, contains an RNA recognition motif (RRM) in the N-terminal part and a central region (SRGY domain) formed by four repeats of a 37 amino acid peptide in human, including a total of five SRGY tetrapeptides (Fig. 1A). The linker region between these two domains also contains some SR dipeptides. The C-terminal region of RBMY has no well-identified protein motif. Several reports indicated that RBMY directly interacts with a variety of factors involved in splicing

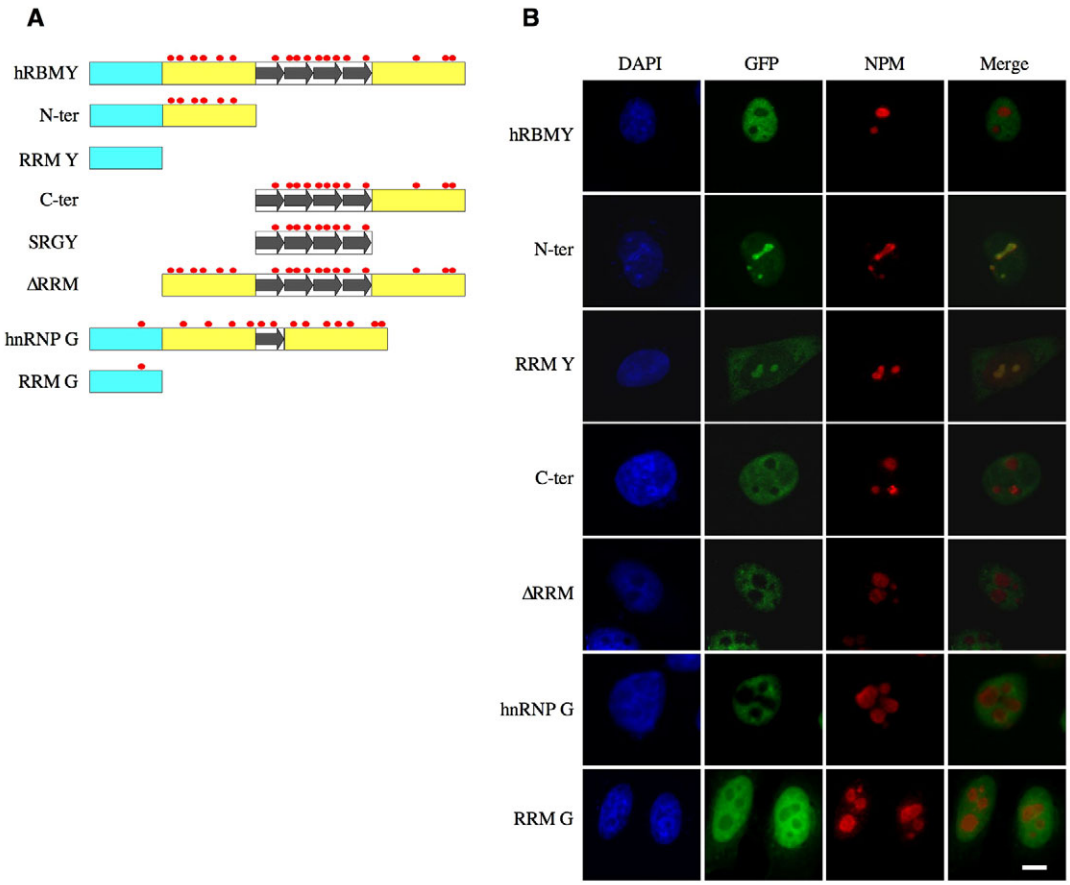

Fig. 1. In vivo subcellular localisation of hRBMY and its different domains. (**A**) Schematic structure of the different GFP constructs used in this study. hRBMY, its human paralog hnRNP G, and the different deletion constructs are represented. Each cDNA was fused downstream of the cDNA encoding GFP (not represented here). The RNA recognition motifs (RRM) and the SRGY boxes are represented by a blue box and arrows, respectively. The RS dipeptides are indicated as red dots above the constructs. (**B**) Subcellular localisation of hRBMY and its different domains. HeLa cells were transfected with the constructs depicted in A and the localisation of the GFP-tagged proteins was observed by direct fluorescence (green signal). hRBMY, hnRNP G and the constructs that do not contain the RRM domain are distributed in the nucleoplasm and excluded from the nucleoli. By contrast, hRBMY-derived constructs containing the RRM domain but no SRGY box are concentrated in the nucleoli, as shown by superposition of the GFP signal with a nucleolar marker (nucleophosmin, NPM, detected using a specific antibody). Scale bar: 5 µm. As shown in supplementary material Fig. S1, the concentration of GFP-RRM Y in nucleoli is not due to the overexpression of the mutant in transfected cells.

regulation, including some SR proteins, T-STAR and Sam68. Most of these protein interactions are mediated by the SRGY boxes (Elliott et al., 2000; Venables et al., 2000). RBMY can alter in vitro splicing of SR-dependent substrates (Elliott et al., 2000), or in cellulo splicing of various transformer-2 protein homolog β (Tra2-β)-dependent AS models (α-tropomyosin and SMN2 exon 7) (Hofmann and Wirth, 2002; Nasim et al., 2003). The overexpression of the RBMY paralog protein, hnRNP G, also regulates some AS events in cellulo (Hofmann and Wirth, 2002; Nasim et al., 2003).

We showed recently that the RRM of human RBMY (hRBMY) interacts specifically with short stem-loop structures capped by a conserved CA/UCAA sequence (Skrisovska et al., 2007). However, this specificity of interaction, which is not conserved in the mouse RBMY protein, could not be correlated with any RRM-dependent effect of the protein in splicing regulation. RBMY has diverged strongly from other hnRNP G family proteins in humans and, more strikingly, RBMY protein sequence has also rapidly evolved between primates and rodents. Indeed, whereas mRBMY is similar to mouse and human hnRNP G and has only one 37-aa SRGY box (Elliott et al., 1996), 22 amino acids differ between the human and mouse RRM of RBMY (Skrisovska et al., 2007). Finally mRBMY and human and mouse hnRNP G proteins have nine RGG, RGR or RRG motifs, whereas hRBMY harbours only two such tripeptides. Therefore, it is possible that human RBMY might have acquired novel properties in primates relative to its mouse orthologue or to hnRNP G proteins.

Here, we sought to investigate the properties of the different domains of hRBMY in relation to their cellular and subnuclear localisation, as well as their ability to interact with various splicing regulatory proteins and to modulate AS in cellulo. Because there is no available cell line to study endogenously expressed RBMY, we transiently expressed GFP-tagged hRBMY and several mutant derivatives in human cell lines to carry out our investigation. We found that hRBMY is concentrated at the periphery of nuclear speckles and interacts with some SR or SR-like proteins, as well as with some components of the exon-junction complex (EJC). We also found that the interaction of hRBMY with SR proteins 9G8 (also known as splicing factor, arginine/serine-rich 7) and Tra2-β affects splicing of their specific target pre-mRNAs in cellulo. Together, our data suggest that hRBMY is located in sites of active

gene expression in the cell, and affects splicing in trans by modifying the activity of SR and SR-like proteins or by preventing their access to their target mRNAs.

Results

The SRGY boxes and the RRM domain have different subcellular localisation properties

We analysed the contribution of four different regions of hRBMY on the targeting of this protein within HeLa cells: the N-terminal RRM, the linker region, the SRGY domain and the C-terminal region (Fig. 1A). These different regions of the RBMY protein were fused downstream of the green fluorescent protein (GFP) (Fig. 1A) and the subcellular localisation of the resulting tagged proteins was determined by direct fluorescence microscopy. As shown in Fig. 1B, full-length hRBMY protein was dispersed throughout the nucleoplasm but did not have a speckled pattern like SR proteins in somatic cells, or a punctate localisation like endogenous hRBMY in spermatocytes. The hRBMY distribution in HeLa cells resembled most closely the endogenous RBMY localisation in spermatogonia and round spermatids (Elliott et al., 1998). A very similar distribution was observed with the tagged proteins containing SRGY boxes, i.e. the C-terminal and ΔRRM constructs (Fig. 1B). By contrast, the RRM-containing proteins (N-terminal or RRM) concentrated in the nucleoli, as shown by superposition with nucleophosmin (Fig. 1B; compare GFP to NPM and merged signal). The concentration of GFP-RRM Y in nucleoli is not due to the overexpression of the mutant in transfected cells (supplementary material Fig. S1). These observations indicated first that hRBMY contains at least two nuclear localisation signals (NLS). One is located in the N-terminal half of the protein (between the start codon and amino acid 220) and another in the C-terminal half (between amino acid 221 and the end). Indeed, all tagged proteins, except the RRM alone, had an exclusive nuclear distribution, and it is most likely that the RS dipeptides contribute to these two NLS. Secondly, these observations show that the RBMY RRM contains a nucleolar localisation motif, most probably because it interacts strongly with a nucleolar component. However, in the full-length RBMY protein, the localisation determinants in the C-terminal half are dominant, making the effect of this nucleolar localisation motif invisible.

We also tested the localisation of GFP-fused hnRNP G. The full-length hnRNP G displayed the same nucleoplasmic distribution as hRBMY, and was also excluded from the nucleolus (Fig. 1B). However, unlike the RRM of hRBMY and in line with the numerous amino acid changes observed between the RRM of both proteins, the RRM of hnRNP G did not concentrate in the nucleolus.

Human RBMY is enriched in perispeckle regions

We next used confocal microscopy to gain more information on the distribution of hRBMY in the nucleus relative to other nucleoplasmic components. Thin optical sections of transfected HeLa cells revealed a broad distribution of hRBMY in the nucleoplasm, with variations in certain nucleoplasmic regions (Fig. 2A). Overexpression of GFP-hRBMY did not disturb significantly the distribution of endogenous nuclear proteins (supplementary material Fig. S2). A comparison with the distribution of the endogenous SR protein 9G8, which accumulates in nuclear speckles, showed clearly that hRBMY is not concentrated in speckles but that it was often found enriched in the perispeckle region (Fig. 2B). The C-terminal and ΔRRM protein fusions, as well as GFP-hnRNP G, showed the same general distribution (Fig. 2A) but a much less obvious perispeckle distribution. These data suggest that all domains

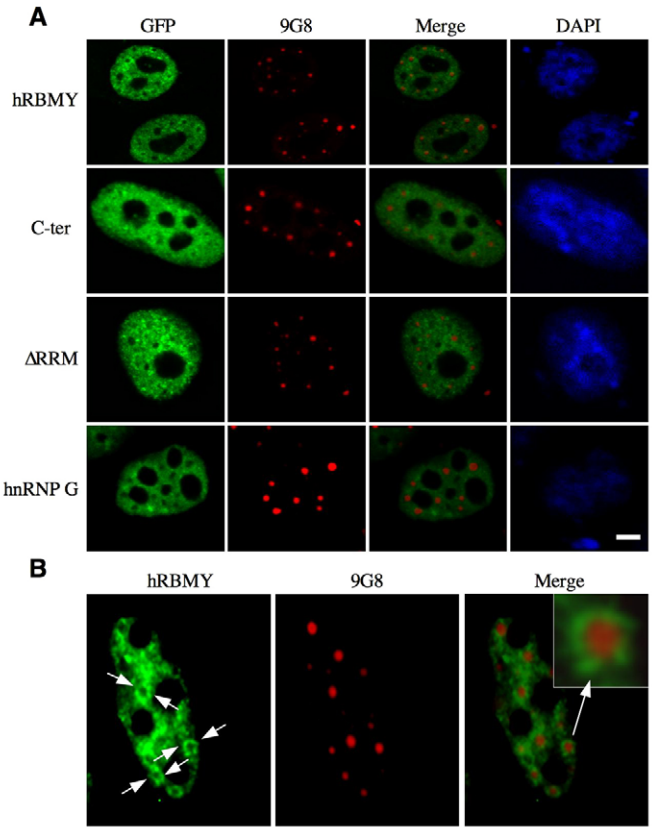

Fig. 2. hRBMY preferentially localises in the perispeckle region of the nucleus. (**A**) The localisation of hRBMY and 9G8 (which accumulates in speckles) was analysed by confocal microscopy with 0.5 μm optical sections. GFP-tagged hRBMY or mutants and hnRNP G were visualised by direct fluorescence and endogenous 9G8 was visualised by immunofluorescence. hRBMY is distributed in the nucleoplasm, as observed also with hnRNP G or with hRBMY mutants containing the SRGY boxes, but is concentrated around speckles. Scale bar: 8 μm. (**B**) Preferential localisation of hRBMY in the perispeckle regions. The experiment was carried out as in A, and a typical nucleus is shown. Some perispeckle regions enriched in hRBMY are shown by arrows on the left, and the inset (indicated by an arrow) shows a magnified view of a speckle (red) and the perispeckle localisation of hRBMY (green).

of RBMY contribute to its specific localisation. Interestingly, the perispeckle distribution of hRBMY was reminiscent of that observed for Magoh and MLN51, two components of the EJC that assembles on the mRNA during splicing (Degot et al., 2004). Therefore, our data strongly indicated that hRBMY is selectively enriched in a nuclear compartment dedicated to transcription, splicing of pre-mRNA and its subsequent transport (Hall et al., 2006; Mintz et al., 1999).

Human RBMY is a non-shuttling protein

Some SR and hnRNP proteins have been shown to shuttle between the nucleus and the cytoplasm to take part in RNA biogenetic processes other than splicing. For example, 9G8 regulates mRNA export and translation in the cytoplasm (Huang et al., 2003; Huang and Steitz, 2001; Swartz et al., 2007). We performed heterokaryon experiments to determine whether also hRBMY shuttles between the nucleus and the cytoplasm (Fig. 3A). After 24 hours of transfection with various GFP-tagged proteins, HeLa cells were fused to NIH 3T3 cells and the GFP signal analysed 3 hours after

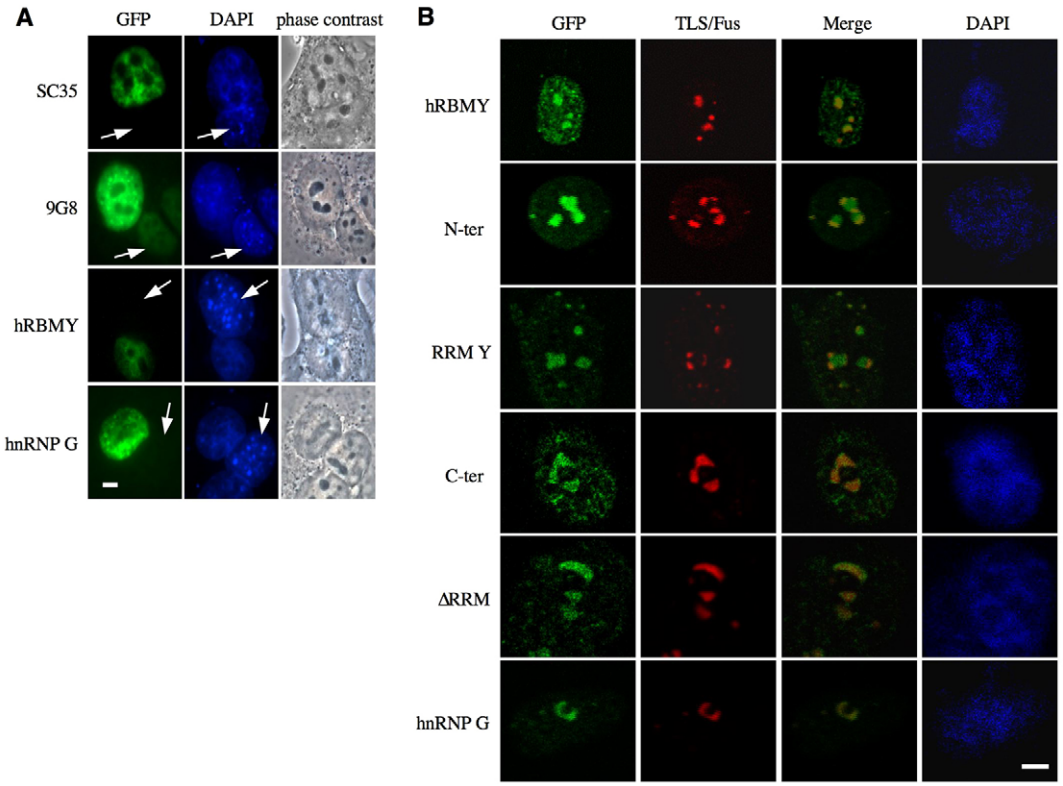

Fig. 3. Localisation of the non-shuttling hRBMY is dependent on ongoing transcription. (**A**) Analysis of nucleocytoplasmic shuttling by transient expression in interspecies heterokaryons. HeLa cells were transfected with the plasmids expressing the fusion proteins indicated. After 24 hours, cells were fused with mouse NIH 3T3 cells. The nucleus of NIH 3T3 cells is indicated by an arrowhead in the DAPI-stained panels. The localisation of the GFP fusion proteins was determined by direct fluorescence. Scale bar: 5 µm. (**B**) HeLa cells transfected with plasmids expressing various GFP fusion proteins, as indicated, were treated with actinomycin D before fluorescence analysis by confocal microscopy. Endogenous TLS/Fus, which was detected with a specific antibody (red), was used as marker of the nucleolar caps. Merged signals between GFP and TLS/Fus are given to indicate the colocalisation of both proteins. All the constructs, except the RRM and the N-terminal constructs, relocalised to nucleolar caps after inhibition of transcription. Scale bar: 8 µm.

cell fusion. In agreement with previous observations (Caceres et al., 1998), the 9G8-GFP protein was detected in the mouse nuclei, whereas SC35-GFP remained confined to the human nuclei (Fig. 3A). As their GFP signal also remained restricted to HeLa nuclei, we concluded that hRBMY and hnRNP G are non-shuttling proteins, strongly suggesting that they only have nuclear functions and that they do not intervene in cytoplasmic events.

The subnuclear distribution of RBMY is dependent on RNA and active transcription

To determine whether the observed subnuclear distribution of hRBMY or its deletion mutants is mediated through binding to nuclear RNA or RNA-protein complexes, we treated cells with RNase A (supplementary material Fig. S3). The signal of both the full-length protein and the C-terminal part of hRBMY strongly decreased in nuclei after RNase treatment, whereas the N-terminal region was only partially released from the nucleoli where it was concentrated. This suggested that hRBMY is associated or embedded into RNA-containing subnuclear complexes, and that normal subnuclear localisation relies on RNA integrity.

To further assess the link between the synthesis of nascent RNA transcripts and the nuclear distribution of hRBMY, transcription by RNA polymerases I and II was inhibited with 5 µg/ml actinomycin D. It has been shown that after inhibition of transcription a broad set of proteins, including some factors involved in pre-mRNA processing (PSF, TLS/FUS, hnRNP H/F as well as p68, p72 and RHAU helicases) relocalise to specific structures called nucleolar caps (Haaf and Ward, 1996; Iwamoto et al., 2008; Shav-Tal et al., 2005). As expected, inhibition of transcription in transfected HeLa cells resulted in the relocalisation of TLS/FUS in nucleolar caps (Fig. 3B). Interestingly, we observed that an important fraction of hRBMY colocalised with TLS/FUS in these structures. Identical results were obtained from each hRBMY construct harbouring the SRGY boxes but lacking the RRM, as well as with hnRNP G, which indicates that targeting of RBMY to nucleolar caps is RRM-independent. By contrast, the localisation of tagged proteins containing the RRM but no SRGY box (N-terminal and RRM) were not affected by transcription inhibition (Fig. 3B), although these already accumulated in nucleoli in normal conditions (Fig. 1B). No hRBMY relocalisation was observed at a concentration of actinomycin D (5 ng/ml), which alters only RNA polymerase I transcription (data not shown). Collectively, the results showed that hRBMY behaves in a dynamic and specific manner in the nucleus.

hRBMY interacts with specific SR or SR-like proteins and with some EJC components in cellulo

Previous two-hybrid analyses or in vitro assays indicated that some SR or SR-like proteins (SRp30c, SRp20, Tra2-β), as well as T-STAR, efficiently interact with mouse or human RBMY, whereas Sam68, 9G8 and other SR proteins interact more weakly (Elliott et

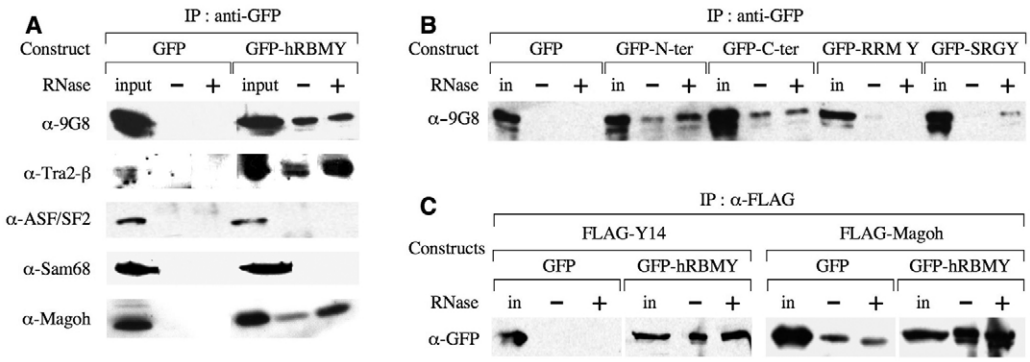

Fig. 4. hRBMY interacts with some SR and SR-like proteins and with components of the exon-junction complex in cellulo. (**A**) Co-immunoprecipitations were performed on HEK293-EBNA cells transfected with GFP-hRBMY or GFP as control, using the anti-GFP 2A3 antibody. The presence of putative hRBMY interactors was tested using antibodies directed against endogenous proteins, following or not a treatment of the pellets with RNase A, as described in Materials and Methods. hRBMY interactions are revealed with 9G8, Tra2-β and Magoh independently of RNA, but not with ASF/SF2 or Sam68. (**B**) Mapping of the domains of hRBMY required for its interaction with 9G8. Immunoprecipitations of GFP-tagged deletion variants of hRBMY were carried out as in A. The N-terminal and the C-terminal mutants displayed the strongest interaction for 9G8. (**C**) HEK293-EBNA cells were co-transfected first with GFP or GFP-hRBMY and, second, with FLAG-Y14 (left panel) or FLAG-Magoh (right panel). The FLAG-tagged proteins were immunoprecipitated and western blots were carried out using the anti-GFP antibody. hRBMY interacted with both components of the EJC in an RNA-independent way.

al., 2000; Venables et al., 2000). In cellulo, hnRNP G interacts more efficiently with Tra2-β than with RBMY (Hofmann and Wirth, 2002). To analyse the hRBMY partners in a human cell context, we transfected HEK293-EBNA cells with GFP-hRBMY and performed immunoprecipitation at high salt concentration (400 mM KCl) using an anti-GFP antibody. Consistent with previous data, we found that endogenous Tra2-β and 9G8 substantially co-immunoprecipitated with GFP-hRBMY (Fig. 4A), whereas SRp20 was detected in lower amounts (data not shown). These interactions were independent of RNA (Fig. 4A). By contrast, under identical salt conditions we did not detect any significant interaction of hRBMY with the other SR proteins ASF/SF2 (Fig. 4A) or SC35 (data not shown), or with Sam68 (Fig. 4A).

Published yeast two-hybrid experiments found that the SRGY boxes alone were sufficient to promote protein-protein interactions of RBMY with partner proteins (Venables et al., 2000). We directly mapped the domains of hRBMY through which it bound to 9G8 or Tra2-β (Fig. 4B and data not shown) and found that all constructs, except the GFP-RRM construct, interacted with both SR proteins. Our results indicated that hRBMY interacts with specific splicing regulators in a human cellular context and that this interaction is probably mediated by the RS dipeptides that are mainly located in the linker region and the SRGY boxes, but not in the RRM. Hence, although the SRGY region of RBMY is sufficient to promote interactions with partner proteins, such interactions are not limited to this region of the molecule.

The preferential colocalisation of hRBMY with EJC components at the periphery of speckles raised the possibility that RBMY could interact with some proteins of this complex. Like 9G8 and Tra2-β, endogenous Magoh, one protein of the core EJC, was detected in the GFP-hRBMY immunoprecipitated complex (Fig. 4A). However, we could not detect any interaction with the endogenous Y14 protein, which forms an heterodimer with Magoh, because the anti-Y14 antibody we used gave only poor signals (data not shown). To overcome this problem, we co-transfected HEK293-EBNA cells with constructs expressing FLAG-Y14 and GFP or GFP-hRBMY. The FLAG-antibody-immunoprecipitated proteins were probed with anti-GFP antibody. Consistent with a specific interaction between RBMY and the EJC, we observed that GFP-hRBMY, but not GFP, interacted with Y14 (Fig. 4C). We further validated our experiments using ectopically expressed proteins, and similarly found that GFP-hRBMY interacted with a coexpressed FLAG-Magoh more strongly than GFP (Fig. 4C). Finally, interactions between hRBMY and 9G8, Tra2-β or Magoh were also observed when a 3×FLAG tag was used for hRBMY instead of the GFP tag that we used in our study (supplementary material Fig. S4B).

Human RBMY affects splicing of Tra2-β- and 9G8-regulated exons in trans

Because the RBMY-interacting 9G8 and Tra2-β proteins are well-studied splicing regulators, we next analysed the role of hRBMY in terms of splicing control of pre-mRNA substrates regulated by these two partners. RBMY is known to affect the splicing activity of Tra2-β protein (Nasim et al., 2003), so if this is mediated by protein interactions a prediction of our protein interaction data described above is that multiple regions of the RBMY protein should independently be able to modulate splicing inclusion regulated by Tra2-β and 9G8. To test this, HeLa cells were transfected with hRBMY, its deletion mutants or hnRNP G, together with reporter pre-mRNAs that are specifically regulated by 9G8, Tra2-β or both proteins. To ensure the nuclear expression of each of the hRBMY fusion constructs (Fig. 1A), an additional NLS was inserted between the GFP tag and each hRBMY coding region. The NLS relocated the RRM exclusively into the nucleus without changing its nucleolar accumulation, but did not change the localisation of any other RBMY variants (supplementary material Fig. S5). The amount of each transfected plasmid construct was adjusted in order to obtain a similar expression level of the various GFP-hRBMY proteins in transfected cells, which was monitored by western blotting with an anti-GFP antibody (see Figs 5 and 6).

The first minigene we tested contained exons 3 to 4 of the *9G8* gene and the intervening intron, and was dependent on 9G8 protein for regulation. The intron 3 region is regulated by AS, with the production of at least three alternative isoforms by inclusion and/or exclusion of a variable internal exon that has two alternatively

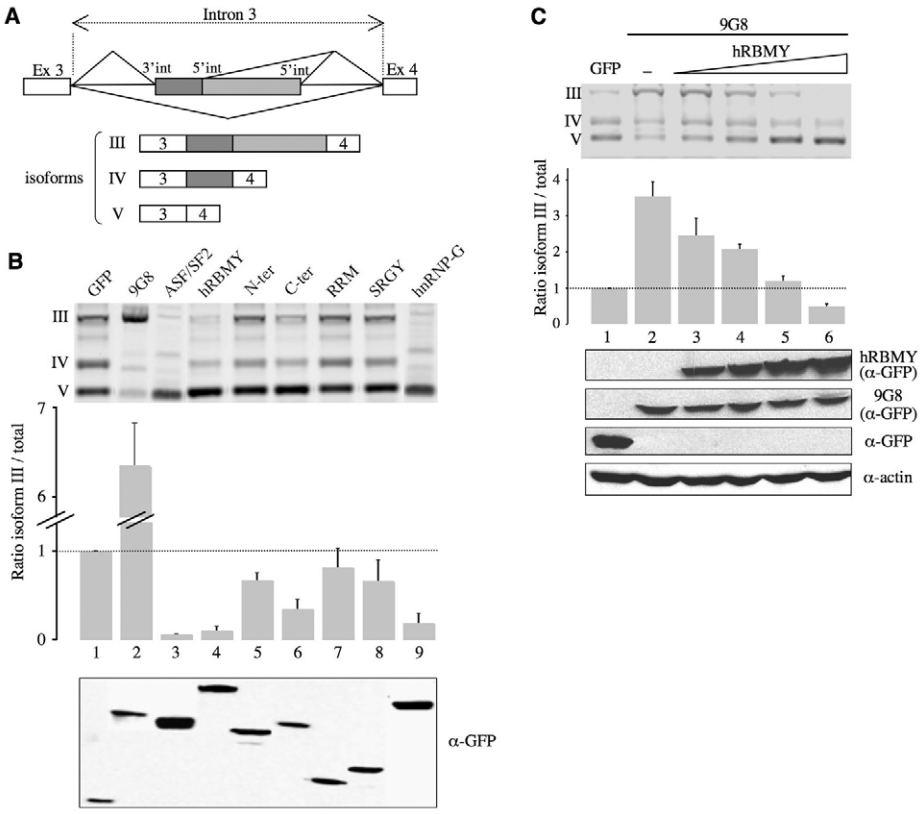

Fig. 5. hRBMY affects splicing of model minigenes in trans. (**A**) Alternative splicing of the region encompassing exons 3 to 4 of 9G8 mRNA produces at least three isoforms. Isoforms III and IV contain a large or small internal exon originating from intron 3, whereas isoform V corresponds to the classic mRNA and produces the full-length 9G8 protein. (**B**) The *9G8* minigene was co-transfected for 48 hours in HeLa cells with plasmids expressing various proteins or mutants, as indicated. The level of expression level of each protein (monitored by western blotting using an anti-GFP antibody) is shown at the bottom of the figure. The splicing pattern was analysed by RT-PCR and a representative ethidium-bromide-stained gel is shown. Three independent experiments were used for the quantitative analysis. (**C**) The *9G8* minigene was co-transfected with a constant amount of the plasmid expressing GFP-9G8 (50 ng) and increasing amounts (100-1000 ng) of GFP-hRBMY plasmid. Analysis of the splicing pattern was performed as described in B. In B and C, western blot analysis, using an anti-GFP antibody, shows the expression of the different recombinant proteins in the lower panel. Actin levels were monitored by western blot and used as a loading control in C.

spliced 5′ splice sites, as described in Fig. 5A (Lareau et al., 2007) (F.L. and J.S., unpublished results). In vivo, the AS pathway of this pre-mRNA is dependent on the balance between the concentration of 9G8 and that of other SR proteins, including ASF/SF2. In standard conditions, the three alternatively spliced isoforms were produced from the minigene in a roughly equivalent proportion in HeLa cells (Fig. 5B, lane 1), but when 9G8 was overexpressed, the large internal exon was strongly recognised, leading to the predominant synthesis of isoform III (Fig. 5B, lane 2). By contrast, when intracellular 9G8 protein was titrated by an excess of 9G8-high affinity RNA (not shown) or when the effect of 9G8 was counteracted by the overexpression of ASF/SF2 (Fig. 5B, lane 3), the internal exon was skipped and the isoform V was produced. In vivo, this AS allows a negative regulation of 9G8 expression because the inclusion of internal exons leads to the synthesis of mRNAs containing premature stop codons which are degraded by nonsense-mediated mRNA decay (Lareau et al., 2007) (F.L. and J.S., unpublished results).

Consistent with a negative regulation of endogenous 9G8-mediated splicing, coexpression of hRBMY with the *9G8* minigene favoured the production of isoform V, in a similar manner to ASF/SF2 (Fig. 5B, lanes 3 and 4). We also observed the same strong splicing effect with hnRNP G (Fig. 5B, lane 9) and with the hRBMY C-terminal construct (Fig. 5B, lane 6), whereas N-terminal and SRGY regions had only a moderate splicing effect (Fig. 5B, lanes 5 and 8), and the RRM alone had no effect at all (Fig. 5B, lane 7). This is strongly consistent with the interaction data described above (Fig. 4), in which the SRGY and RRM regions interacted respectively less strongly, or not at all with 9G8.

In order to see whether this protein-interaction-based model of splicing regulation extended to Tra2-β, we next sought to determine whether RBMY and its different domains could each modulate Tra2-β-dependent splicing. It has been shown that Tra2-β regulates splicing of its own transcripts at the level of exon 2 (Stoilov et al., 2004). Using a minigene encompassing exons 1 to 4 (Fig. 6A), we observed that inclusion of exon 2 was indeed dependent on Tra2-β concentration (Fig. 6B, lanes 1-2). Interestingly, coexpression of hRBMY had the opposite effect (Fig. 6B, lanes 3 and 2). The effect was also strong with the C-terminal part of hRBMY and hnRNP G, as well as with the SRGY boxes (Fig. 6B, lanes 5, 7-8), but again only weak with the N-terminal or the RRM constructs (Fig. 6B, lanes 4 and 6).

The results shown in Figs 5 and 6 showed that the RRM of hRBMY is not required for splicing modulation of the tested pre-mRNAs, but that there is a good correlation between the binding of hRBMY protein and its derivatives to 9G8 or Tra2-β and their effect on splicing of 9G8 and Tra2-β-dependent minigenes. Therefore, the simplest interpretation is that hRBMY, through its direct interaction with endogenous 9G8 or Tra2-β, titrated these proteins within the nucleus and thereby modulated splicing. To test this hypothesis, we next asked whether hRBMY was able to competitively counteract the effects of 9G8 and Tra2-β proteins. We analysed the effect of adding increasing amounts of GFP-hRBMY in the presence of a constant amount of 9G8- or Tra2-β-expressing vector, using the *9G8* and *Tra2-β* minigenes, respectively (Fig. 5C and Fig. 6C). In both cases, we observed a progressive and proportional antagonistic effect of hRBMY relative to the basal effect of 9G8 or Tra2-β, strongly suggesting that both proteins are directly targeted by hRBMY to repress their splicing activity. Finally, we also checked that a 3xFLAG-hRBMY has the same properties in splicing as the standard GFP-hRBMY used in Figs 5 and 6 (supplementary material Fig. S4C).

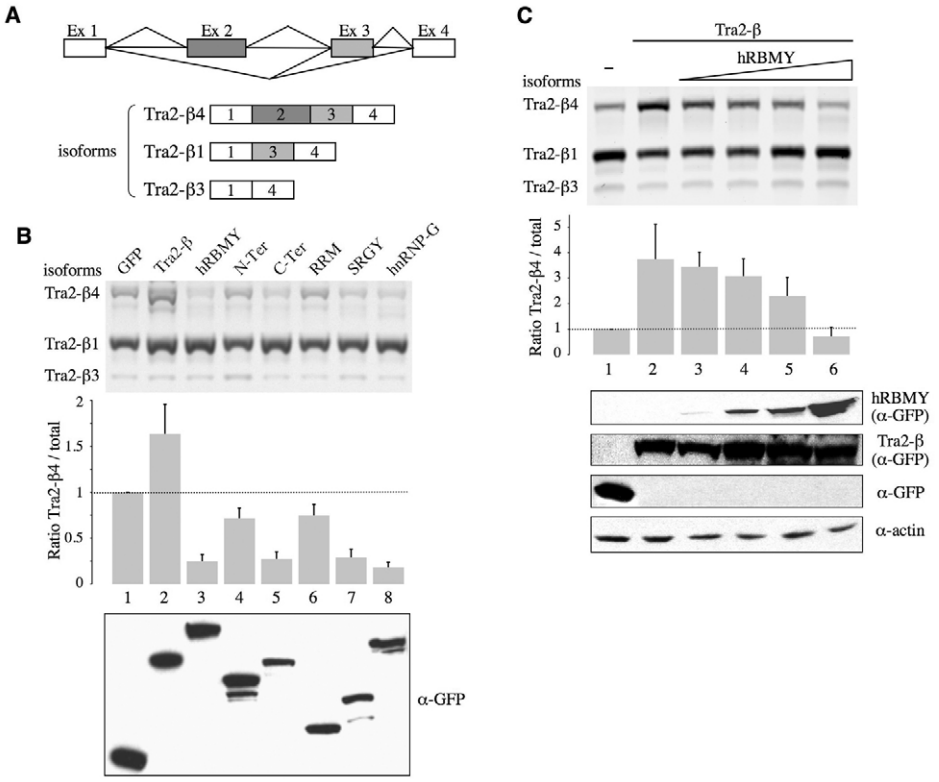

Fig. 6. Effect of hRBMY on a *Tra2-β* minigene. (**A**) The minigene consists of exons 1 to 4 of the *Tra2-β* gene. Exons 2 and 3 are alternatively spliced, depending on the amount of Tra2-β protein available in the cell. (**B**) The *Tra2-β* minigene was co-transfected in HEK293-EBNA cells with plasmids expressing various proteins. The splicing pattern was analysed as in Fig. 5B. (**C**) The *Tra2-β* minigene was co-transfected with a constant amount of GFP-Tra2-β (100 ng) and increasing amounts of GFP-hRBMY (100 to 1000 ng). The splicing pattern was analysed as in B. In B and C, the expression levels of recombinant proteins were analysed in the lower panel.

If RBMY functions as a splicing coregulator in the germline through interactions with Tra2-β and 9G8, a prediction is that there should be germ cell exons that are also antagonistically regulated by Tra2-β and 9G8. Therefore, we set out to identify alternative exons that could be regulated in this way and were specific to the germline. We first identified the *CREB* gene, in which exon D is alternatively spliced in various mouse and human tissues, but significantly enriched in brain and testis compared to other tissues (Berkowitz and Gilman, 1990; Ruppert et al., 1992). We identified exon D as a target for Tra2-β-mediated splicing repression (Y.L. and D.J.E., unpublished observations). A *CREB* minigene was constructed by inserting the exon D region in a *β-globin* gene context (pXJ41) (Fig. 7A). Transfection of the *CREB* minigene in HeLa cells resulted in a significant inclusion of exon D (Fig. 7B, lane 1), but inclusion was strongly inhibited by 9G8 or Tra2-β overexpression (Fig. 7B, lanes 2 and 3). Consistent with antagonistic regulatory activity rather than with a general inactivation of splicing, hRBMY affected splicing in an opposite way to 9G8 or Tra2-β (Fig. 7B, compare lane 4 to lanes 2 and 3), and increased exon D inclusion into the mature mRNA. HnRNP G and the C-terminal part of hRBMY also modulated splicing in a positive way (Fig. 7B, lanes 6-7).

Because of its testis-specific expression and ability to activate the splicing of *CREB* exon D, we postulated that RBMY should be able to activate the splicing of other testis-specific exons. We identified another exon, in the *Acin1* gene (exon 4), which was preferentially included in the testis of mouse compared to other tissues (Fig. 7C). A minigene containing the region of exons 3 and 4 of *Acin1* gene was constructed (sequence of exon 4 region is 92% identical in human and mouse) and the effect of 9G8 and Tra2-β on exon 4 splicing was tested (Fig. 7D,E). As for the *CREB* gene described above, we showed that 9G8 and Tra2-β efficiently repressed exon 4 inclusion (Fig. 7E, lanes 2-3). Although SR or SR-like proteins have often a positive effect on AS, a negative effect has also been described in a few examples, for instance in RON or CFTR pre-mRNA (Buratti et al., 2007; Ghigna et al., 2005). Very similarly to their effect on the *CREB* minigene, both hRBMY and hnRNP G significantly stimulated the inclusion of exon 4 from the *Acin1* minigene (Fig. 7E, compare lanes 4 and 7 to lane 1). This effect was RRM-independent because the N-terminal construct did not have any effect, whereas the C-terminal construct had strong splicing activity (Fig. 7E, lanes 5 and 6). Taken together, these observations strongly supported an action of hRBMY that leads to exon skipping or exon inclusion, depending on the substrate and on the mode of action of 9G8 and Tra2-β. The finding that a testis-activated exon in the *Acin1* pre-mRNA is included more efficiently when hRBMY is present might be the basis of its splicing regulation in the testis, where hRBMY is specifically expressed.

Discussion

Microdeletions of the Y chromosome are a frequent cause of male infertility. Human *RBMY* was identified as a potential candidate gene for causing this infertility, and its putative function in spermatogenesis was therefore investigated (Elliott et al., 1997; Ma et al., 1993). The RNA-binding capacity of hRBMY and its partial colocalisation with splicing factors in the early stages of spermatogenesis led to the hypothesis of possible functions in RNA processing during this process (Elliott et al., 1998). High-affinity target sequences recognised by the RRM domain of hRBMY were recently identified by SELEX (Skrisovska et al., 2007). To follow up this study and identify potential target mRNAs of RBMY, we performed in silico analyses (Sushma Grellscheid and D.E., unpublished results). However, so far we have been unable to associate the presence of a CA/UCAA-capped stem-loop (the

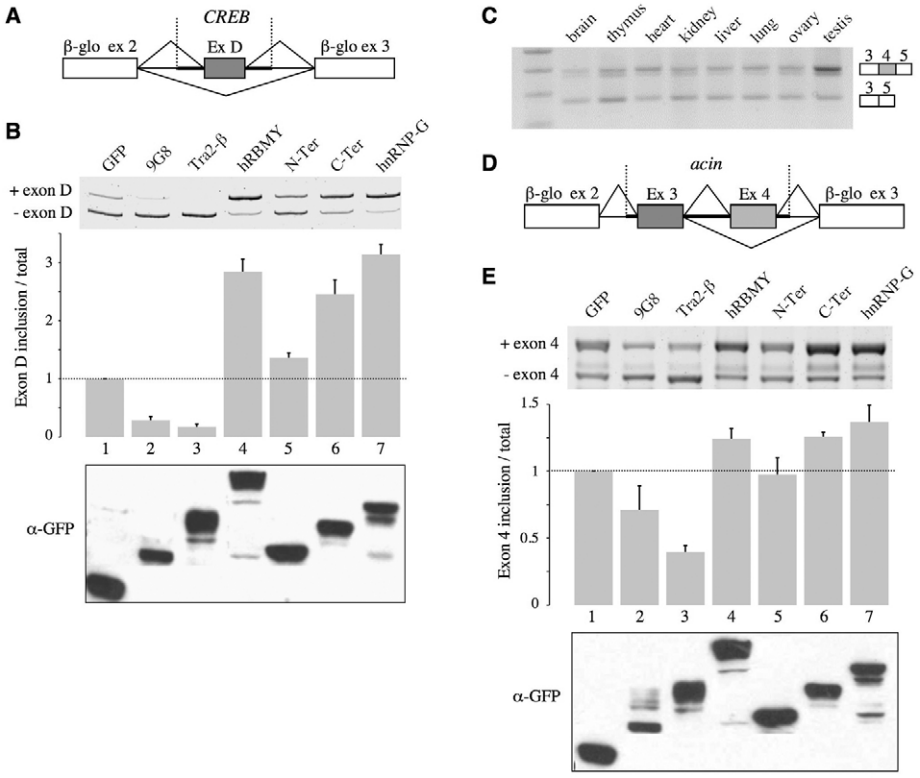

Fig. 7. hRBMY increases the inclusion of testis-specific exons. (**A**) Schematic organisation of the *CREB* minigene. Exon D and its flanking intronic sequences were inserted between the two *β-globin* exons of pXJ41. (**B**) This *CREB* minigene was co-transfected in HEK293-EBNA cells with plasmids expressing various proteins and the splicing pattern analysed as in Fig. 5B. (**C**) RT-PCR analysis showing the differential inclusion of exon 4 in the mRNA isoforms of the *Acin1* gene in various mouse tissues, as indicated. (**D**) Schematic organisation of the *Acin1* minigene. Exons 3 and 4 and flanking intronic sequences of mouse *Acin1* gene were cloned between the β-globin exons of pXJ41. (**E**) The *Acin1* minigene was co-transfected in HEK293-EBNA cells with plasmids expressing various proteins and the splicing pattern analysed as in Fig. 5B. In B and E, the expression levels of recombinant proteins were analysed in the lower panel.

hRBMY-specific RNA signature) in a given mRNA to a direct regulation of splicing after addition of hRBMY (N.D., C.F.B. and J.S., unpublished observations).

We therefore decided to characterise each domain of hRBMY in order to better define the function of the whole protein. First, we observed that deletion mutants containing SRGY boxes, but no RRM, localised in the nucleus like the full-length protein, indicating that SRGY boxes have a dominant function over the RRM in controlling the normal spatial localisation of RBMY. SRGY-box-containing tagged proteins were nuclear but excluded from nucleoli whereas tagged proteins containing the RRM but no SRGY box concentrated into the nucleoli. The spatial localisation of the hRBMY RRM was very specific because the RRM of hnRNP G was not targeted to the nucleolus (Fig. 1B) and consistent with the different RNA binding properties of hRBMY and hnRNP G (Heinrich et al., 2009; Skrisovska et al., 2007). Second, we found that hRBMY displays a dynamic and specific nuclear localisation, which is associated with the transcription and maturation of pre-mRNAs. In physiological conditions, hRBMY does not concentrate in speckles, but localises preferentially in their periphery (Fig. 2B). Structurally, speckles are formed of a core [the interchromatin granule cluster (IGC)] and, at their surface, perichromatin fibrils (PF), which contain growing RNA transcripts (Hall et al., 2006; Lamond and Spector, 2003). Functionally, IGCs are thought to be compartments for storage, assembly and modification that supply splicing factors, notably SR and SR-like proteins, to surrounding active transcription sites. FRAP experiments indicated that the maximal residence time of ASF/SF2 in speckles is less than 1 minute (Phair and Misteli, 2000), suggesting that perispeckles are sites of very intensive exchanges between speckles and the surrounding areas. Although the perispeckle regions containing RBMY could not be easily visualised using SR-specific antibodies, they have been previously visualised by confocal microscopy studies that analysed the subnuclear localisation of other factors such as EJC components (Degot et al., 2004) or the transcription regulator CA150 (Sanchez-Alvarez et al., 2006). It has also been shown that components of the EJC are efficiently recruited at sites of abundant pre-mRNA synthesis and processing (Custodio et al., 2004). Therefore, perispeckle regions represent a crucial zone in the nucleus because they correspond to active gene transcription and processing sites.

The preferential localisation of hRBMY in the perispeckle region was confirmed by co-immunoprecipitation experiments (Fig. 4), which showed that hRBMY interacts, independently of RNA, with the EJC core components Magoh/Y14, as well as with some SR and SR-like proteins (9G8 and Tra2-β especially) that exchange rapidly between the speckle and perispeckle regions. Interactions between RBMY and Tra2-β had been revealed previously by yeast two-hybrid and in vitro studies (Venables et al., 2000) so that the interactions we identified in human cultured cells are most probably direct. By contrast, the interactions between RBMY and EJC core components have not been described before.

An interesting aspect of the interactions of hRBMY with its partners is that they seem to be transient and dynamic. Indeed, although 9G8 and Magoh/Y14 are shuttling proteins, RBMY is not (Fig. 3A), indicating that it is not continuously associated with its partners. This better supports a transient regulatory function rather than a constitutive function of RBMY in mRNA processing. In addition, whereas SR proteins concentrate in speckles of bigger size after inhibition of the transcription (Lamond and Spector, 2003). RBMY behaves very differently because it relocalised into nucleolar caps (Fig. 3B) along with a subset of 40 nuclear factors, including RNA binding proteins (Shav-Tal et al., 2005).

In agreement with previous work (Elliott et al., 2000; Venables et al., 2000), we showed that the SRGY boxes of hRBMY are

important for its interactions with SR or SR-like proteins. However we also observed that the N-terminal deletion mutant, which contains SR dipeptides but no SRGY tetrapeptide, also interacted strongly with the same factors (Fig. 4B), indicating that the numerous RS dipeptides that are scattered throughout the entire sequence of RBMY and other hnRNP G family proteins (except in their RRM) are important for protein-protein interactions. Furthermore, it has been shown that the phosphorylation of Tra2-β strengthens its interactions with RBMY, strongly suggesting that the RS domain of SR proteins are involved in these interactions (Venables et al., 2000). Therefore, it is possible that the phosphorylation of the SR dipeptides within SR proteins and RBMY regulates the interactions between these two groups of proteins. Importantly, our immunoprecipitation experiments indicate that interactions between hRBMY and SR proteins Tra2-β, 9G8 and SRp20 are very specific because under identical conditions hRBMY does not interact with ASF/SF2 or SC35. Although our experiments used transiently expressed RBMY, an immunoprecipitation performed on mouse testis showed that only a weak proportion of total endogenous RBMY could be recovered, making the detection of putative partners practically impossible. However, immunofluorescence of human testis sections shows that RBMY and Magoh are indeed present in the nuclei of the same cells (supplementary material Fig. S6), and that a partial subnuclear colocalisation might be detected in certain nuclei, notably when eIF4AIII, another component of the EJC core, was analysed (supplementary material Fig. S6B).

The fact that RBMY interacted with 9G8 and Tra2-β and that these interactions could occur preferentially in the perispeckle region prompted us to analyse the functional consequences of these interactions on the splicing activities of both SR proteins. By analysing the splicing of pre-mRNA substrates that are regulated by 9G8 or Tra2-β, we showed that the expression of hRBMY or of hnRNP G counteracted the activity of those SR proteins on splicing (Figs 5 and 6). There was a good correlation between the binding capacity of the different deletion mutants of hRBMY to interact with 9G8 and Tra2-β, and with their ability to modulate splicing activity of both proteins (Figs 5 and 6). Finally, we showed that hRBMY competed with the effect of 9G8 or Tra2-β on splicing in a concentration-dependent manner. The properties of hnRNP G are very similar to those of hRBMY, suggesting that in either case the RRM is not essential for splicing regulation of these pre-mRNAs. Taken together, these observations strongly suggested that both SR proteins are functionally targeted by RBMY or hnRNP G through the binding of the SR dipeptide-containing domains of hRBMY or hnRNP G.

This function of hRBMY, based on the model pre-mRNAs investigated here, brings additional support to previous proposals based on in vitro or in vivo studies using poorly specific pre-mRNA substrates (Elliott et al., 2000; Nasim et al., 2003; Venables et al., 2000), but interestingly differs from the mode of action proposed for hnRNP G in SMN2 exon 7 splicing (Hofmann and Wirth, 2002). In the model studied here, hnRNP G interacted with Tra2-β but both proteins cooperated to stimulate exon 7 splicing. This function of hnRNP G was also confirmed very recently by the group of Stefan Stamm (University of Kentucky, KY) using the same splicing model, but the effect of hnRNP G was independent of its RRM (Heinrich et al., 2009). It is not clear how the action of hnRNP G (and possibly RBMY) on the same protein (Tra2-β) might have two opposite effects on splicing. The same recent study also demonstrated that the RRM of hnRNP G recognises sequences containing CCC/A motifs, and microarray analysis showed that hnRNP G modulates splicing of a class of exons that are statistically enriched in such motifs relative to control exons (Heinrich et al., 2009). Therefore, in this situation, hnRNP G could regulate splicing through a standard mechanism involving the direct binding of the protein to specific motifs on the pre-mRNA sequence.

Interestingly, we found that hRBMY and hnRNP G are able to modulate splicing of two exons in *CREB* and *Acinus* genes, which are efficiently included in the testis. Both alternative exons are negatively regulated by 9G8 and Tra2-β and positively regulated by hRBMY or hnRNP G, a result that is consistent with what occurs in vivo in the testis (Fig. 7). Because the effect of hRBMY was independent of its RRM and opposite to that of SR proteins, it is probable that hRBMY and hnRNP G counteracted the activity of both SR proteins via the same mechanism as proposed above and acted as splicing co-activators. Significantly, similar effects were also observed when analysing a newly described testis-specific splicing event within the *transducing-like enhancer of split 4* (*TLE4*) gene (Liu et al., 2009).

Hence, using transiently transfected cells we have obtained convergent results showing that hRBMY concentrates in the perispeckle compartment of the nucleus where transcription and splicing take place, and that RBMY physically interacts with specific SR proteins and EJC components that are present or in close proximity to this compartment. Consistent with these observations, hRBMY is intrinsically capable of modulating alternative splicing of genes that are specifically regulated by its partners. The results strongly suggest that RBMY could function in germ cells as a co-regulator of a specific set of alternative splicing events.

Materials and Methods

Plasmids

All constructs expressing hRBMY deletion mutants were derived from the pGFP3-RBMY in which the hRBMY cDNA is flanked by *Eco*RI and *Not*I sites. A DNA sequence coding for SV40 NLS (amino acids PKKKRKV) was inserted in frame between GFP and RBMY coding sequences, using the *Eco*RI site and a newly created *Bsp*EI site. The different fragments of hRBMY cDNA were amplified by PCR from the pGFP3-RBMY plasmid, and inserted between *Bsp*EI and *Not*I sites, in place of the full-length cDNA. The fragments correspond to the following amino acids: 1-99 (RRM), 1-220 (N-terminal), 95-496 (ΔRRM), 220-372 (SRGY) and 220-496 (C-terminal). Equivalent constructs without NLS were also engineered by removing the *Eco*RI-*Bsp*EI fragment from each plasmid.

Different minigenes were used in our study. The *9G8* and *Tra2-β* minigenes are reported elsewhere (Lejeune et al., 2001; Stoilov et al., 2004). The *CREB* minigene was obtained by amplifying a 450-bp region encompassing exon D using 5′-TTTGGGTTTGATCCACAGGT-3′ and 5′-TGCTGGACTACCATTAGGAAGA-3′ and cloning the fragment into the *Mfe*I site of the *β-globin* intron of pXJ41 (Bourgeois et al., 1999). Similarly, the *Acin1* minigene was obtained by inserting a 968-bp fragment (amplified with 5′-AAAAAAAACAATTGGGACCCAGGCTTG-AGTCTTA-3′ and 5′-AAAAAAAACAATTGTGATGCCCTCTACTGGTGTG-3′) into the *Mfe*I site of pXJ41.

Cell culture, reagents and transfections

HeLa cells and HEK293-EBNA cells were grown in Dulbecco modified Eagle's medium (DMEM) supplemented respectively with 5% foetal calf serum (FCS) and antibiotics or 10% FCS, 1 mg/ml G418 and antibiotics. 24 hours before transfection, cells were plated into six-well plates at a density of 200,000 cells per well (HeLa cells), and into six-well plates or 6-cm dishes (HEK293-EBNA cells) at a density of 400,000 or 600,000 cells per dish, respectively. Transfections were performed with jetPEI (Polyplus transfection) according to the manufacturer's instructions. For transcription inhibition, 5 μg/ml of actinomycin D (Sigma) was added 2 hours before performing immunofluorescence.

For heterokaryon experiments, HeLa cells were used as donor cells and mouse NIH-3T3 cells were used as acceptor cells; the experiment was performed as described elsewhere (Caceres et al., 1998). Nuclei were counterstained with DAPI, and EGFP fusion proteins were visualised by fluorescence microscopy. For the analysis of localisation of RBMY after RNA hydrolysis, transfected HeLa cells were treated with 10 μg/ml of RNase (DNAse-free) in phosphate-buffered saline (PBS) for 15 minutes at room temperature, after permeabilisation. After several washes in PBS, cells were fixed and processed for immunofluorescence.

Immunofluorescence

HeLa cells were grown on coverslips and transfected for 24 hours with various constructs. After several washes in PBS, cells were fixed in 4% paraformaldehyde for 15 minutes and permeabilised in 0.5% Triton-X100 for 5 minutes. GFP signal was observed by direct fluorescence. Immunofluorescence experiments were performed as follows: fixed and permeabilised cells were incubated for 1 hour with the primary antibody diluted to 1:1000 in PBT (PBS pH 7.4, 0.15% BSA, 0.1% Tween-20). After three washes in PBS containing 0.1% Tween, the GAM-Cy3 antibody (Jackson ImmunoResearch) was added for 1 hour in the dark. Cells were mounted on microscope slides and examined using either a simple fluorescence microscope (Leica) or a Leica DM4000 B confocal microscope, equipped with a Leica 100× HCX Plan Apo CS 1.40 objective, in 0.5 µm optical sections.

Minigene analysis

The splicing minigene reporters were co-transfected with various hRBMY constructs for 48 hours. RNA was extracted with RNAsolv (Omega Bio-Tek) and treated with RNase-free DNAse I (Roche Diagnostics) for 30 minutes at 37°C. After an additional phenol-chloroform extraction, cDNA was produced from 2 µg RNA using Supercript II reverse transcriptase (Invitrogen) in 25 µl. PCR reactions were performed using Taq polymerase (Roche Diagnostics) according to the manufacturer's recommendations. After separation of the PCR products in an agarose gel, the bands were stained with ethidium bromide and quantified using a Typhoon 8600 imager (GE Healthcare). Intron 3 region of the *9G8* gene was amplified for 25 cycles under standard conditions in a final volume of 50 µl of PCR buffer containing 3 mM $MgCl_2$. The primers used are described elsewhere (Lejeune et al., 2001). The *Tra2-β* minigene was amplified as previously reported (Stoilov et al., 2004). The transcripts of *CREB* and *Acin1* minigenes were amplified using primers corresponding to sequences from the parental vector pXJ41/42: BBR81 (5′-ACGGTGCATTGGAACGGACC-3′) and XY90 (5′-AACCATTATAAGCTGCAAT-3′) for 25 cycles under standard conditions.

Immunoprecipitation

After 40 hours transfection, HEK293-EBNA cells were lysed in buffer P (20 mM Tris-HCl pH 7.4, 400 mM KCl, 1 mM DTT, 0.5% NP-40, 20% glycerol). Lysates were gently sonicated twice for 10 seconds and centrifuged at 16,000 ***g*** for 10 minutes at 4°C. Soluble fractions were used for immunoprecipitation (IP) after treatment or not with 10 µg/ml RNAse A for 15 minutes on ice. 1 µl of monoclonal anti-GFP antibody (2A3) was added to 250 µl of lysate and incubated for 2 hours at 4°C. 30 µl of protein G-Sepharose (GE HealthCare) was then added and the mixture incubated for 1 hour at 4°C under constant mixing. After three washes in buffer P, the bead pellet was resuspended in protein loading buffer (1×) and denatured for 5 minutes at 100°C. 10% of the input and 20-40% of the pelleted fraction were analysed by SDS-PAGE and western blotting.

The interaction with EJC components was tested using FLAG-tagged Y14 and FLAG-tagged Magoh (provided by Catherine Tomasetto, IGBMC, Illkirch, France). Cells were co-transfected with the FLAG-tagged cDNA and the GFP or the GFP-hRBMY constructs. Co-immunoprecipitations were performed using the anti-FLAG M2 affinity gel (Sigma-Aldrich) following the manufacturer's instructions.

Antibodies and western blotting

The following antibodies were used: affinity-purified rabbit anti-9G8 (C-terminal), anti-Sam68, monoclonal anti-GFP 2A5 (2A3 and 2A5 provided by Mustapha Oulad, IGBMC, Illkirch, France), affinity-purified rabbit anti-Tra2-β (N-terminal), monoclonal anti-Magoh 2D2 (provided by Catherine Tomasetto), and monoclonal anti-TLS/Fus (provided by Laszlo Tora, IGBMC, Illkirch, France). Cells were directly lysed in the loading buffer and proteins were resolved by 12% SDS-PAGE followed by transfer on nitrocellulose. Secondary antibodies conjugated to horseradish peroxidase were obtained from Jackson ImmunoResearch.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/1/40/DC1

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