EXPERIMENTAL CELL RESEARCH 312 (2006) 3379–3388

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Research Article

WT1 interacts with the splicing protein RBM4 and regulates its ability to modulate alternative splicing in vivo

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ARTICLE INFORMATION

Article Chronology:
Received 1 March 2006
Revised version received
26 June 2006
Accepted 17 July 2006
Available online 25 July 2006

Keywords:
Alternative splicing
RNA binding proteins
Speckles
Wilm's tumor suppressor
Supraspliceosome

ABSTRACT

Wilm's tumor protein 1 (WT1), a protein implicated in various cancers and developmental disorders, consists of two major isoforms: WT1(–KTS), a transcription factor, and WT1 (+KTS), a post-transcriptional regulator that binds to RNA and can interact with splicing components. Here we show that WT1 interacts with the novel splicing regulator RBM4. Each protein was found to colocalize in nuclear speckles and to cosediment with supraspliceosomes in glycerol gradients. RBM4 conferred dose-dependent and cell-specific regulation of alternative splicing of pre-mRNAs transcribed from several reporter genes. We found that overexpressed WT1(+KTS) abrogated this effect of RBM4 on splice-site selection, whereas WT1(–KTS) did not. We conclude that the (+KTS) form of WT1 is able to inhibit the effect of RBM4 on alternative splicing.

Introduction

RNA-binding proteins (RBPs) are a large group of *trans*-acting factors [1] that influence post-transcriptional processes such as constitutive and alternative splicing, RNA stability, transport, capping and 3′ end formation [2,3]. They include SR proteins (serine–arginine domain-containing proteins) and hnRNPs (heterogeneous nuclear ribonucleoproteins) and contain at least one RNA-binding domain, such as an RNA recognition motif (RRM), KH domain and RGG box. Many have been categorized as spliceosomal components [4,5] and have been found to regulate splice-site selection [6]. A protein having structural features consistent with a possible role in RNA processing is the RBP RBM4.

RBM4 has been described recently as a novel splicing regulator, which shares a nuclear import pathway with SR proteins, being delivered to the nucleus by transportin-SR2 (TRN-SR2), an importin β-like nuclear transporter [7]. Using an adenovirus E1A reporter, Lai et al. showed that RBM4 is able to modulate alternative 5′ splice-site and exon selection in vivo [7]. Furthermore, RBM4 seems to directly influence the expression of the skeletal muscle-specific α-tropomyosin isoform [8].

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doi:10.1016/j.yexcr.2006.07.008

RBM4 contains two consensus RRMs and a retroviral-type zinc finger (RTZF). In this way, it resembles the SR protein 9G8. Unlike SR proteins, it does not contain an RS rich C-terminal domain, but several alanine-rich stretches, which Lai et al. termed CAD (C-terminal alanine-rich domain). Similar to RS domains, the RBM4 CAD region is important for protein–protein interaction as well as the splicing effect. We [9] and others [7] have also shown that RBM4, like many other splicing regulators, resides in speckles, subnuclear structures that are enriched in pre-messenger RNA splicing factors and are located in the interchromatin regions of the nucleoplasm [10].

Although it is clear that RBM4 is able to modulate splicing, it is not known yet whether RBM4 binds to other splicing factors or how RBM4-mediated splicing itself is regulated. We thus set out to determine further potential RBM4 interactors and their possible function. Here we show that RBM4 interacts with WT1 and that its ability to regulate alternative splicing is inhibited by one of the two isoforms of WT1.

Materials and methods

Plasmids

Full-length *RBM4* cDNA – purchased from the IMAGE consortium (BC000307) in pOTB (pOTB-RBM4) – was subcloned into pGEM T-Easy to create pGEM-RBM4. A full-length *RBM4* fragment from pGEM-RBM4 was ligated into pEGFP-C2 (Clontech) to create GFP (green fluorescence protein)-RBM4. Deletion-mutants were amplified from pOTB-RBM4 and cloned into pGEM T-Easy. Two-step PCR was carried out using PCR Supermix High Fidelity (GIBCO Invitrogen) and 35 cycles of 95°C for 20 s and 68°C for 3 min followed by 10 min at 68°C. pGEM-M7 was generated using primers 5′-accagagcggctactatcggtacgggaaagaggg-3′ and 5′-ccctctttcccgtaccgatagtagccgctctggt-3′ containing 2 nucleotide (nt) changes in *RBM4* at nt 540 (G→A) and nt 549 (G→A), which convert the first two Cys residues of the RTZF into Tyr. PCR for pGEM-M7 was as above except for use of Pfx (Invitrogen) for amplification, 5 min for annealing and post-PCR digestion with 10 U *DpnI* for 3 h.

pGBK-plasmids were generated by subcloning from pGEM-plasmids into pGBKT7 (Clontech). DNA-binding domain (pACT-BD) plasmids were kindly provided by N. Hastie, R. Davies and M. Niksic.

pCMV-RBM4-HA was cloned by inserting a full-length *RBM4* fragment from GFP-RBM4 into pCMV-HA (Clontech). pACT-WT1+KTS [11] contained the full-length WT1 variant, which includes sequences of the first alternative exon encoding 17 amino acids (aa), as well as the alternative 3 aa KTS between the 3rd and 4th zinc finger of WT1. pACT-WT1–KTS misses the 3 aa KTS. pCMV-WT1(+KTS)-*myc* was created by insertion of a full-length WT1 fragment from pACT-WT1 into pCMV-Myc (Clontech).

Yeast two-hybrid (Y2H) assay

The Matchmaker Two-hybrid System 3 (Clontech) was used according to the manufacturer's directions. Briefly, budding *Saccharomyces cerevisiae* strains AH109 or Y187 were cultured in YPAD (1% yeast extract, 2% peptone, 0.01% adenine, 2% dextrose) and cotransformed, using the lithium acetate method, with 0.1 μg each of pGBK-plasmid and pACT-plasmid for interaction testing. The yeast were then plated on synthetic dropout medium deficient in leucine, tryptophan, histidine and adenine (SD(–L/–W/–A/–H)) or deficient in leucine, tryptophan (SD(–L/–W)), depending on the strain used, and incubated at 30°C for 1 week. The transformants were analyzed for β-galactosidase (β-gal) expression by filter assay or liquid culture assay using Galacton Star chemiluminescent reagent according to the Clontech manual.

Cell culture and transient transfection

HEK293 cells (ATCC, CRL-1573) and HeLa cells (ATCC, CCL-2) were grown in Dulbecco's Minimal Essential medium containing sodium pyruvate (GIBCO Invitrogen), supplemented with 10% FBS (GIBCO Invitrogen) and 5 U/ml penicillin/streptomycin. All cells were maintained at 37°C in an atmosphere of 5% CO_2. Prior to transient transfections, cells were grown to 60–80% confluency and then transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 24–48 h after transfection, the cells were fixed for immunostaining. Cells for splicing assays were transfected by the calcium chloride method or with Lipofectamine 2000 and RNA was extracted 18–48 h post transfection.

Antibodies

Polyclonal antibody (produced to order by Alpha Diagnostics) was generated in rabbits by inoculating peptides corresponding to aa 41–59 (N-terminus) of human RBM4. Mouse or rabbit anti-WT1 were from DAKO (6F-H2) or Santa Cruz Biotechnologies (C-19), respectively. Mouse anti-c-*myc* and rabbit anti-HA-Tag were purchased from Clontech and Sigma, respectively. Secondary antibodies were: Alexa Fluor 488 goat anti-rabbit or anti-mouse IgG, Alexa Fluor 594 goat anti-mouse or anti-rabbit IgG, Alexa Fluor 594 donkey anti-goat (all from Invitrogen), as well as sheep anti-mouse horseradish peroxidase (HRP)-linked and donkey anti-rabbit HRP-linked (Amersham Pharmacia). Rabbit polyclonal antibody against CBP80 [12] was kindly provided by Iain Mattaj (EMBL).

Western blotting and immunoprecipitation

Nuclear extracts from 10^7 HEK293 were prepared using a NuCLEAR Extraction kit (Sigma). For immunoprecipitation, 20 μl of nuclear extracts (~100 μg) was pre-cleared with 20 μl Protein A–agarose (Roche) before addition of 3–5 μg antibody cross-linked to 20 μl Protein A–agarose. Immunocomplexes were incubated for 6 h at 4°C with frequent mixing, washed 5 times with PBS and resolved by 12% SDS-PAGE. Proteins were electroblotted onto a nitrocellulose membrane (Amersham Pharmacia). Membranes were blocked for 1 h in 5% blocking agent (Amersham Pharmacia) then incubated for 1 h with primary antibody followed by 1 h with secondary antibody before detection by ECL-plus (Amersham Pharmacia) according to the manufacturer's instructions. CBP80 was probed with rabbit polyclonal antibodies [12] diluted 1:500 or 1:1000 in NET buffer with 1 M NaCl and visualized with goat anti-rabbit IgG horseradish peroxidase conjugate, diluted 1:10,000.

In vitro protein–protein interaction magnetocapture assay

In vitro protein interaction assay was performed using the EasyXpress Protein Synthesis kit and Ni-NTA magnetic agarose beads (both from QIAGEN), according to the manufacturer. Briefly, two-step PCR products were generated for in vitro translation of full-length RBM4 and WT1(+KTS) proteins. RBM4-specific primers were 5′-agaaggagataaacaatggtgaagctgttcatcgg-3′ and 5′-tggtgatggtggtgacccaaaaggctgagtaccgc-3′ and resulted in a first-round product containing a C-terminal 6× His tag. WT1 first-round primers were 5′agaaggagataaacaatgtccgacgtgcgggac-3′ and 5′ggatgagaccaggcagaaagcgccagctggagttt-3′. Second-round PCR was then performed using adapter primers supplied by the manufacturer. The second PCR product was then used as a template for in vitro translation of the 2 proteins, using *E. coli* extracts. Translated proteins were incubated together for 1 h and then purified using Ni-NTA magnetic agarose beads (QIAGEN) and a magnetic separator. Purified proteins were run on 10% SDS-PAGE, blotted onto PVDF membrane and detected with RBM4- and WT1-specific antibodies (as described above).

Intracellular localization studies

For visualization of fluorescence, cells were grown on Lab-Tek chamber slides (Nunc) and fixed with ice-cold methanol for 2 min. Following 1 h of blocking with 10% goat serum (Sigma), the cells were incubated sequentially with primary and secondary antibody for 1 h each. Finally, the cells were stained with 300 nM DAPI (Molecular Probes) and mounted with glycerol/gelatin/PBS (Sigma). Two- and three-channel fluorescent images were acquired on a Zeiss Axioplan 2 imaging microscope with an Axiocam HRm camera.

Splicing assays

These were performed essentially as described [13]. Briefly, a splicing reporter minigene was cotransfected with an increasing amount of RBM4 expression construct (GFP-RBM4 or pCMV-RBM4-HA) into HEK293. One microgram of WT1 (+KTS)-myc or WT1(–KTS)-myc was added to the transfection where indicated. Empty parental vector was added to ensure that the same amounts of DNA were transfected, then 18 h after transfection, RNA was isolated using QIAGEN RNAeasy columns. RT-PCR was performed using the primers pclforward, 5′-ggtgtccactcccagttcaa-3′, and SMNex8rev, 5′-gcctcaccaccgtgctgg-3′ for SMN2 involving 30 cycles of 94°C for 30 s, 58°C for 1 min and 72°C for 1 min. For RT-PCR of minigene SRp20, primers were T7, 5′-taatacgactcactataggg-3′, and X16R, 5′-cctggtcgacactctagatttcctttcatttgacc-3′, and conditions were 20 cycles of 94°C for 50 s, 55°C for 50 s and 72°C for 1 min. The resultant splicing band pattern was quantified using the Totallab System (Phoretix, Nonlinear Dynamics, UK) or a 2100 Bioanalyzer (Agilent Technologies).

Preparation of supraspliceosomes

Supraspliceosomes were prepared from HEK293 cells using a protocol described previously [14,15]. Briefly, nuclear supernatants enriched for supraspliceosomes were prepared from purified nuclei of HEK293 cells by micro-sonication of the nuclei and precipitation of the chromatin in the presence of tRNA. The nuclear supernatants were fractionated in 10–45% glycerol gradients in 100 mM NaCl, 10 mM Tris·HCl, pH 8.0, 2 mM $MgCl_2$ and 2 mM vanadyl ribonucleoside complex. Centrifugations were carried out at 4°C in a SW41 rotor run at 41,000 rpm for 90 min (or an equivalent $\omega^2 t$).

Results

RBM4 interacts with WT1

On the basis of its striking similarities with other RBPs and its ability to modulate splicing, we used Y2H to test for interaction of RBM4 with a battery of proteins involved in splicing: $U2AF^{35}$, $U2AF^{65}$, U1-70K, ASF/SF2, SC35, SRp20, WT1 (+KTS) (isoform containing both exon 5 and exon 9) and the Cajal-body marker protein p80-coilin. SNF4, the noncatalytic effector subunit of kinase SNF1, was used as negative control. Only WT1(+KTS) interacted with RBM4, as determined by growth on SD(–L/–W/–A/–H) plates, followed by β-gal filter assay (Fig. 1A and data not shown). We then tested the other major isoform of WT1, WT1(–KTS), for interaction with RBM4. In 2 independent assays on SD(–L/–W/–A/–H) plates and on SD(–L/–W), only a few very small colonies grew, suggesting a possible weak interaction of WT1(–KTS) with RBM4. However, when we measured these colonies in liquid culture β-gal assays, the relative light units were below the negative control (data not shown) and, since they were negative on β-gal filter assays (Fig. 1A), were thus considered background.

To determine which domain of RBM4 interacts with WT1, we generated several mutated RBM4 DNA-binding constructs (Fig. 1A, left) and cotransformed each with pACT-WT1(+KTS). Both pGBK-M4 (both RRMs deleted) and pGBK-M7 (zinc finger domain mutated) were still able to bind WT1, while pGBK-M1 (C-terminal region deleted) did not bind. Moreover, a mutant construct containing only the C-terminal region of RBM4 (pGBK-M8) bound WT1. Thus, the C-terminal region, but not the RRM domains, nor the zinc finger of RBM4 is necessary and sufficient for interaction with WT1. The RBM4–WT1(+KTS) interaction was confirmed by coimmunoprecipitation from HEK293 cells cotransfected with HA-tagged full-length RBM4 (pCMV-RBM4-HA) and c-*myc*-tagged full-length WT1 (pCMV-WT1(+KTS)-myc). WT1(+KTS) was present in RBM4-HA immunoprecipitates (Fig. 1B).

We further confirmed the interaction between RBM4 and WT1(+KTS) by using in vitro-translated proteins. RBM4 and WT1(+KTS) were in vitro-translated from specific PCR products, incorporating a C-terminal 6× His tag for RBM4. In vitro-translated RBM4 and WT1 crude lysates were incubated together and then purified using Ni-NTA magnetic agarose beads, which bind to 6× His tags. Crude lysates and purified proteins (eluates) were then subjected to Western blotting, detected with RBM4 antibodies and reprobed with WT1-specific antibodies. We could show that, in assays containing both RBM4 and WT1 proteins, a band at about 94 kDa could be detected with WT1 antibodies, suggesting that WT1 and RBM4 were pulled down by the beads as a complex (Fig. 1C).

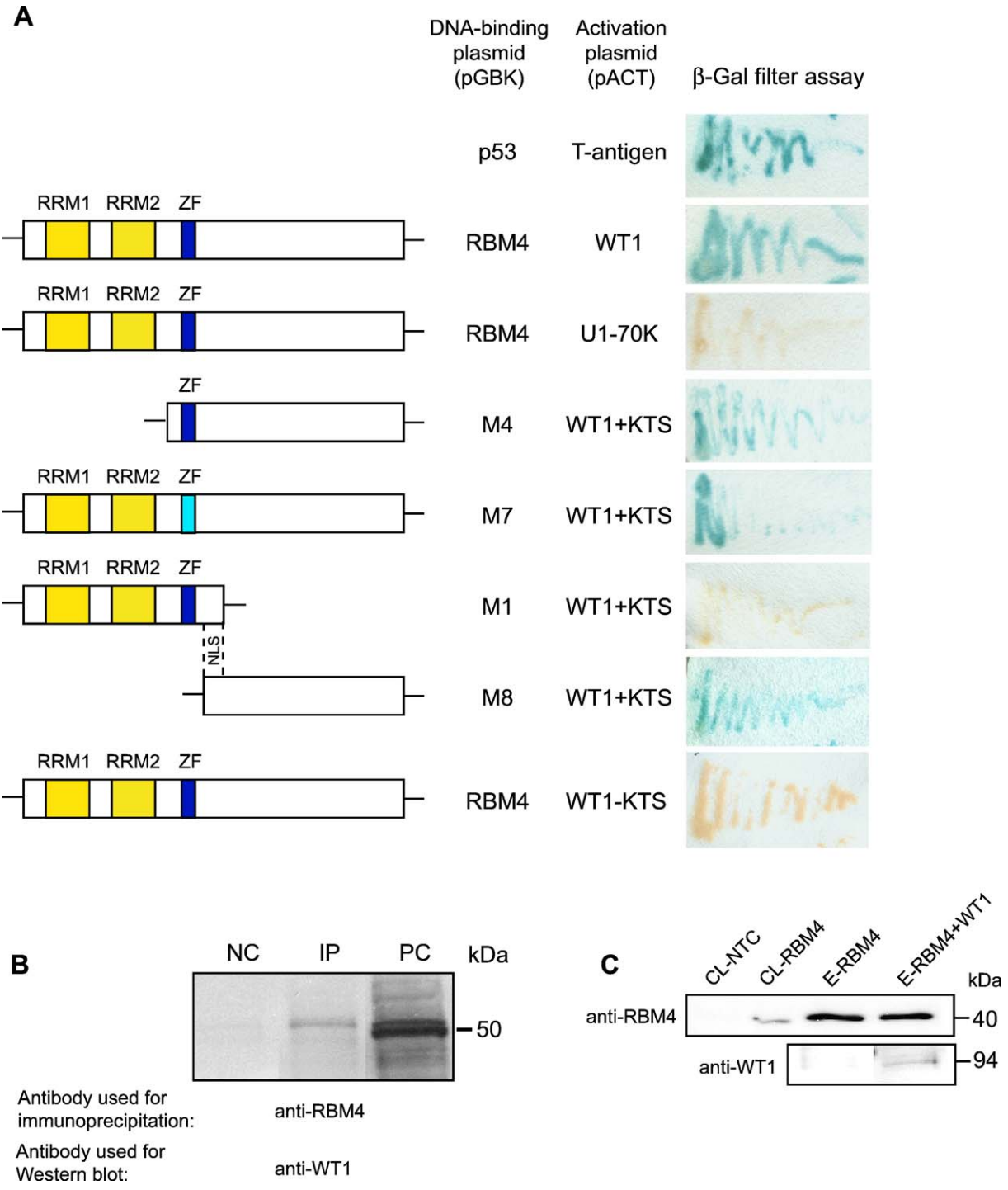

Fig. 1 – Interaction of RBM4 with WT1. Panel A shows yeast two-hybrid analysis. Yeast were cotransformed with DNA-binding plasmids (vector pGBK) and activation-plasmids (vector pACT) encoding the proteins indicated. Colonies were grown on the appropriate dropout plates prior to β-gal assay. p53 and T-antigen interaction was used as a positive control. pGBK-constructs of wild-type RBM4 and different mutations are shown on the left. RRM, RNA recognition motif; ZF, zinc finger; NLS, putative nuclear localization signal. Panel B shows coimmunoprecipitation of RBM4 with WT1+KTS in HEK293 cells transfected with RBM4-HA and WT1(+KTS)-myc. Nuclear extracts (100 μg) were immunoprecipitated with 5 μg antibodies (IP) or control IgG (NC) using Protein A–agarose. Ten microliters of the complexes was resolved on an SDS-PAGE gel, blotted and the presence of WT1 was confirmed with anti-WT1 antibody. Positive control (PC) was nuclear extract alone. (C) In vitro protein–protein interaction of RBM4 and WT1+KTS. In vitro-translated proteins were incubated together, purified by magnetocapture using Ni-NTA beads and subjected to Western blotting. CL=crude lysate, E=eluate.

In the control eluate (RBM4 proteins only), no such complex could be detected (Fig. 1C).

Together, these data suggest that RBM4 interacts with WT1 (+KTS) through its C-terminal region.

RBM4 colocalizes with WT1

In contrast to the RBM4 data of the Tarn group [7], we found that not only the C-terminal region of GFP-RBM4, but also full-length GFP-RBM4, was expressed in speckles (data not shown). WT1 is known to localize within the nucleus in speckles, defined compartments or in a diffuse manner, depending on the protein isoform [16]. The speckled entities correspond to substructures believed to be storage or assembly sites of splicing factors [17,18]. To determine whether RBM4 colocalizes with WT1, we transiently transfected HEK293 and HeLa cells with pCMV-RBM4-HA and pCMV-WT1(+KTS)-myc to overexpress HA-tagged RBM4 and c-*myc*-tagged WT1(+KTS). Immunofluorescence was performed with anti-HA and anti-c-*myc* antibodies. In each cell line, RBM4 and WT1 showed a speckled pattern and we saw extensive colocalization between the proteins, as exemplified for HeLa cells in Fig. 2A. Endogenous RBM4 and WT1 also colocalize in the nucleus, with most of the speckles overlapping or being in very close proximity (Fig. 2B).

RBM4 and WT1 are found in large nuclear complexes cosedimenting with supraspliceosomes

We then tested whether RBM4 was part of the supraspliceosome [19], in which endogenous pre-mRNAs are packaged with all five spliceosomal U snRNPs, together with other splicing factors and additional pre-mRNA processing activities [20]. To this end, nuclear supernatants enriched with supraspliceosomes were prepared from HEK293 cells and were fractionated on a glycerol gradient. Supraspliceosomes sedimented in fractions 8–14 (Fig. 3) and peaked in fractions 10–11, as confirmed by EM visualization and sedimentation of the cap binding protein CBP80 [20]. We have previously shown that CBP80 is associated with supraspliceosomes and represents a good marker for these large nuclear ribonucleoprotein (lnRNP) complexes. The distribution of RBM4 and WT1 across the gradient was analyzed by Western blotting using our polyclonal anti-RBM4 antibody and anti-WT1 (C-19) antibodies, respectively. Both RBM4 and WT1 cosediment with supraspliceosomes in glycerol gradients and appear in many of the same fractions (Fig. 3). The distribution pattern of both was not, however, identical, RBM4 appearing a little earlier than WT1 and peaking in fractions 11–12, while WT1 peaked in fractions 13–14.

RBM4 can influence alternative splice-site selection

Several reporters, containing an alternative exon flanked by constitutive exons (minigenes) (Fig. 4A) [21], were transfected into HEK293 cells along with increasing amounts of RBM4 plasmids, and the mRNA isoforms were detected by RT-PCR using primers directed at sequences located in the flanking constitutive exons. Fig. 4B shows results of representative agarose gels for minigenes SMN2 and SRp20. For SMN2, both GFP- and HA-tagged-RBM4 expression clones promoted exon

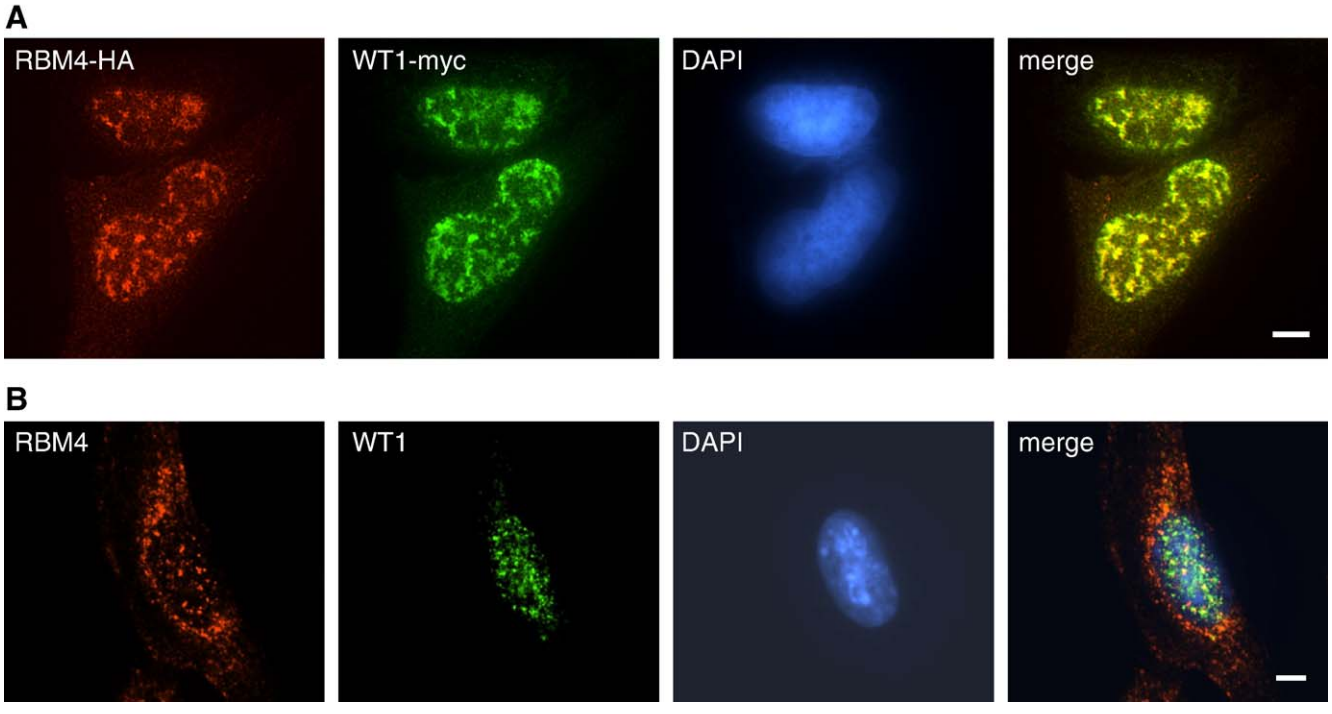

Fig. 2 – Colocalization of RBM4 and WT1 in the nucleus. (A) HeLa cells were cotransfected with pCMV-RBM4-HA and pCMV-WT1 (+KTS)-myc, fixed and immunodetection carried out using an anti-HA and an anti-myc antibody. (B) Endogenous expression of RBM4 and WT1. The nucleus was stained with DAPI. Merged image (yellow) indicates colocalization of RBM4 and WT1 in speckled domains in the nucleus. Scale bars: 5 μm.

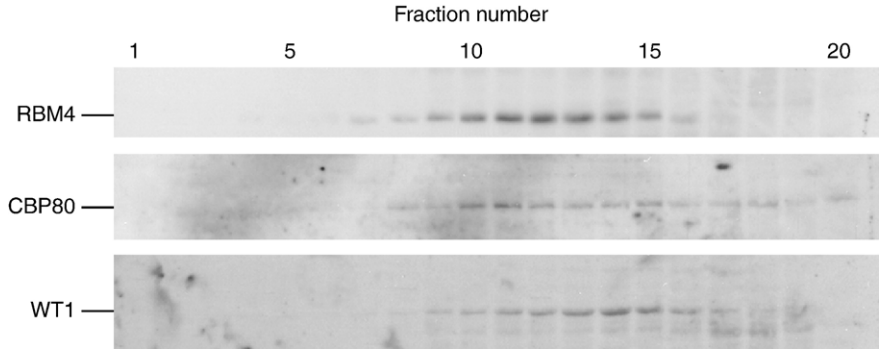

Fig. 3 – RBM4 and WT1 are present in supraspliceosomes. Nuclear supernatants enriched in supraspliceosomes prepared from HEK293 cells were fractionated in a 10–45% glycerol (v/v) gradient and collected (bottom to top) in 20 fractions. Aliquots from each fraction were analyzed by Western blotting using anti-RBM4 (upper panel), anti-CBP80 (middle panel) and anti WT1 (C-19) (lower panel) antibodies. TMV (tobacco mosaic virus) that sediments at 200S peaks at tubes 10–11 (data not shown).

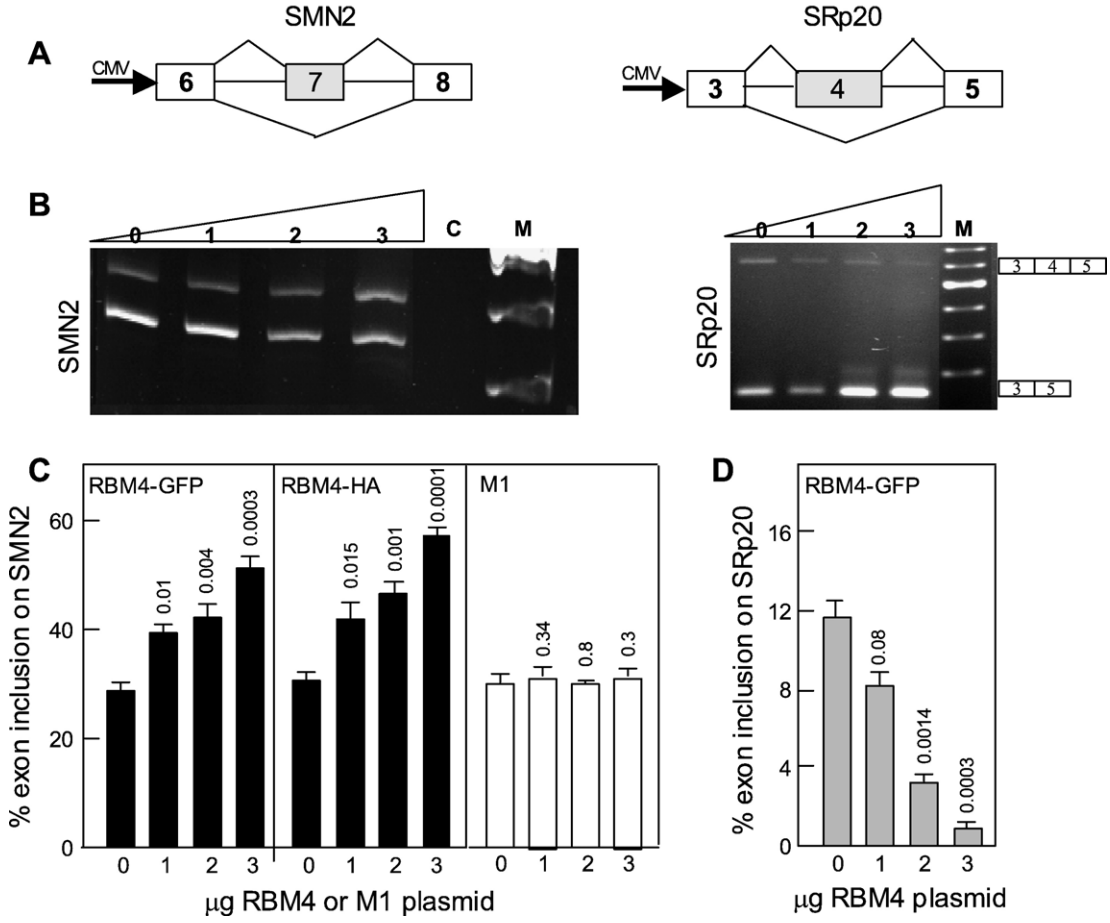

Fig. 4 – RBM4 influences alternative splice-site selection in HEK293 cells in vivo. Panel A shows structure of the SMN2 and SRp20 minigenes. Indicated are: alternative exon (gray), intron (horizontal line) and promoter (arrow). Lines connecting the exons indicate the two splicing patterns, either including or omitting the alternative exon. Panel B shows that RBM4 promotes exon 7 inclusion on minigene SMN2 and exon 4 exclusion on SRp20. An increasing amount of GFP-RBM4 or RBM4-HA (0–3 μg) was cotransfected with 1 μg of the minigene. Parental vector was added to ensure that similar amounts of cDNAs were transfected. The ethidium bromide stained gels are from representative experiments. Panels C and D show splicing results of 4 independent experiments using GFP-RBM4, HA-tagged RBM4 or mutant M1 (C-terminus deleted) with minigene SMN2 (C), as well as GFP-RBM4 with minigene SRp20 (D). Error bars indicate ± SE. All *p* values are from *t* test vs 0 μg RBM4 plasmid.

inclusion in a concentration-dependent manner (Fig. 4C). From a baseline of approximately 30%, such exon inclusion was elevated to 60%, which is comparable to the effect of tra2-β1 or SRp30c on pre-mRNA from the same minigene [22,23]. Deletion-mutant M1 lacking the C-terminal region of RBM4 was unable to alter splice-site selection of SMN2, indicating that the carboxyl terminal region of RBM4 is important for regulation of splice-site selection (Fig. 4C). RBM4 had the opposite effect on minigene SRp20 in that it stimulated the skipping of exon 4 (Fig. 4D). A weaker effect was observed on tra2-β1 minigene, with a reduction in exon inclusion from 30% down to 21% (data not shown). These results accord with findings by the Tarn group [7,8] that RBM4 modulates alternative 5′ site selection and is able to promote or prevent exon inclusion, depending on the minigene or pre-mRNA used.

WT1 suppresses the effect of RBM4 on alternative splicing

We next examined the functional significance of the binding of RBM4 to WT1 and tested the ability of WT1 to modulate the influence of RBM4 on splice-site selection. An increasing

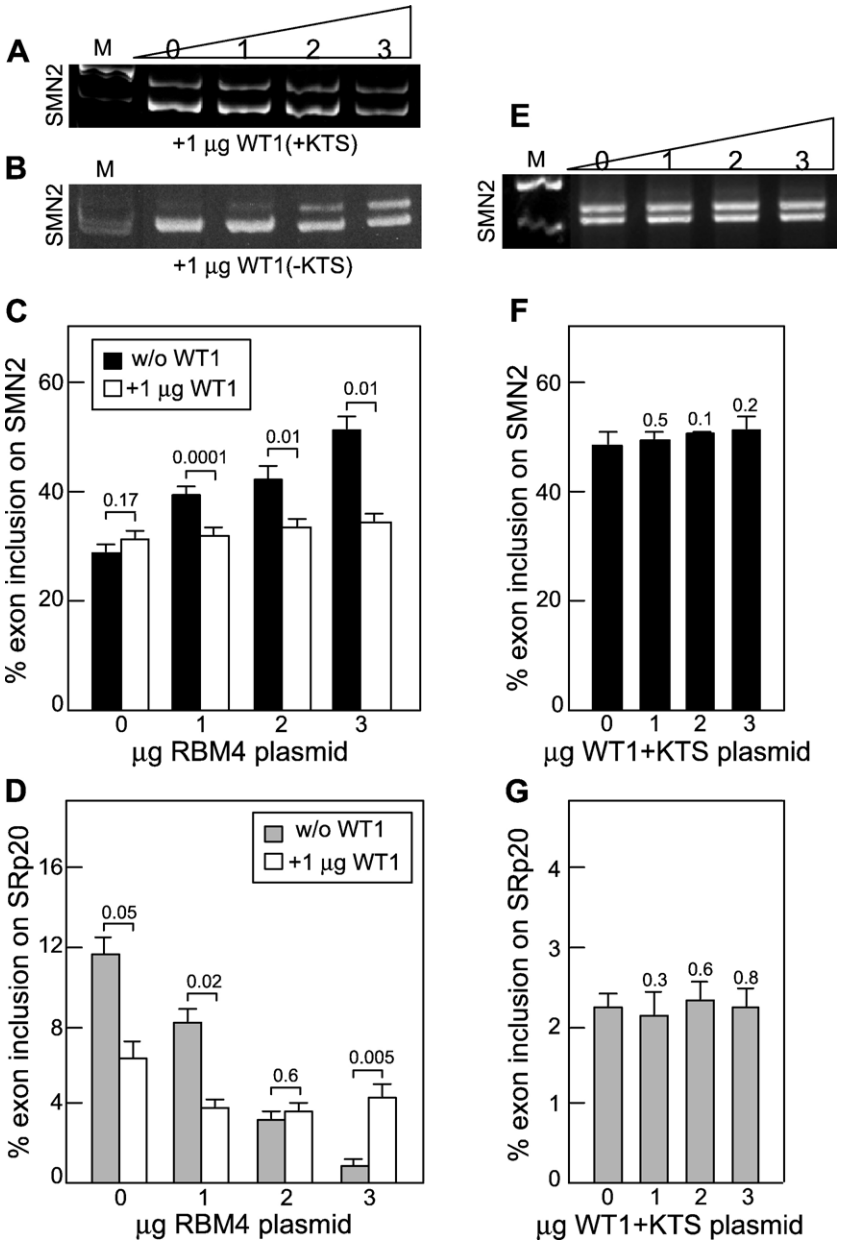

Fig. 5 – WT1 antagonizes the RBM4 splicing effect in HEK293 cells. Panels A and C show that WT1(+KTS) antagonizes the effect of RBM4 on SMN2. One microgram of WT1(+KTS)-myc was cotransfected with an increasing amount of GFP-RBM4 and the SMN2 minigene as indicated and compared with transfections without WT1 ('w/o WT1'). Panel C shows results from 3 independent experiments. Panel B shows that WT1(–KTS) is not able to inhibit the RBM4 splicing effect. Panel D shows that WT1(+KTS) suppresses the RBM4 splicing effect on SRp20 ($n=3$ independent experiments). (E–G) WT1 alone does not influence the splicing patterns of SMN2 and SRp20 minigenes.

amount of RBM4 (0, 1, 2, 3 μg) was cotransfected into HEK293 cells with a constant amount of WT1(+KTS) expression construct. As shown in Fig. 5, WT1(+KTS) abolished the influence of RBM4 on splice-site selection of SMN2 pre-mRNA (Figs. 5A and C) and significantly reduced the effect on SRp20 (Fig. 5D). This was most probably caused by sequestration of RBM4 by WT1(+KTS) and demonstrates that the interaction between RBM4 and WT1 occurs in vivo. A WT1(–KTS) plasmid, where 9 nucleotides at the end of exon 9 were deleted, was not, however, able to inhibit the RBM4 splicing effect (Fig. 5B), indicating that the KTS coding exon is necessary for the effect of WT1 on RBM4. To exclude the possibility that WT1 has an effect on splice-site choice of the minigenes tested, independently of RBM4, we transfected WT1(+KTS) plasmids alone and measured the effect in exon inclusion. We found that WT1 (+KTS) had no significant effect on splice-site choice with either SMN2 or SRp20 (Figs. 5E–G), suggesting that the inhibitory effect it has on the splice-site changes by RBM4 is mediated through its interaction with RBM4.

Discussion

The present study has shown that RBM4 binds to the tumor suppressor WT1, but not to any of the other splicing factors tested, including U2AF^{65}, which is known to interact with WT1 [11]. The possibility remains that RBM4 interacts with other splicing factors that we did not test. The interaction with WT1 is, however, of considerable interest. WT1 is involved in multiple molecular processes and has been implicated in 10–15% of Wilm's tumors, a pediatric renal malignancy [24,25] often seen with the congenital abnormalities WAGR (Wilms' tumor, aniridia, genitourinary abnormalities, mental retardation), Denys-Drash and Frasier syndromes [26–28]. In normal development, WT1 has a prominent role in the differentiation of the metanephric mesenchyme by mediating mesenchyme-to-epithelial transition (reviewed in [29,30]). Recently, we have shown that siRNA-mediated knockdown of WT1 ex vivo in developing mouse fetal kidneys results in a block of nephrogenesis, coupled with abnormal proliferation and apoptosis rates of WT1-expressing mesenchymal cells [31]. WT1 also plays a role in early hematopoiesis, and WT1 expression is associated with certain acute leukemias [32], as well as prostate and breast cancers [33–35].

Alternative splicing, RNA editing and alternative translation initiation sites generate up to 24 WT1 isoforms, which seem to have overlapping but also distinct functions during embryonic development and the maintenance of organ function. In particular, the insertion or deletion at the end of exon 9 of only 9 nucleotides, which code for the three amino acids Lys–Thr–Ser (KTS), results in very different functions for the two isoforms [36]. While WT1(–KTS) has strong DNA binding capacities and acts as a transcriptional regulator [30], WT1(+KTS) has been found to be active post-transcriptionally, having an ability to bind RNA [37,38] and be incorporated into spliceosomes [11]. Furthermore, WT1(+KTS) binds to U2AF^{65} and colocalizes with splicing factors in speckles [16], compartments which represent storage sites for both constitutive and alternative splicing factors and which can be recruited to sites of active splicing [39–41]. The fact that RBM4 bound to and colocalized with WT1(+KTS) in speckles supports a role for this isoform in post-transcriptional regulation.

Similar to earlier findings [7], the C-terminal region of RBM4 (aa 176–364) was necessary for the effect of RBM4 on splicing and contained the protein binding site (mutant M8 was sufficient for interaction with WT1). It is thus possible that RBM4 modulates splicing by interacting with other splicing factors such as WT1. No known domains reside, however, in the C-terminal region of RBM4, and it remains to be determined which specific sites bind to WT1 and possibly other splicing factors.

We found that the ability of RBM4 to alter the splicing pattern of SMN2 and SRp20 pre-mRNAs was suppressed by WT1(+KTS). These results demonstrate that the interaction between RBM4 and WT1 is functional in vivo and that WT1 (+KTS) is critical for RBM4 splicing. They also support the notion that changes in the ratio of splicing regulators can affect alternative splicing of pre-mRNAs in vivo [42]. Furthermore, it is most important to note that only WT1(+KTS) but not WT1(–KTS) was able to inhibit the effect of RBM4 on splicing. This ties in with other data that point to the +KTS isoform as being an RNA processing factor. WT1(+KTS) may inhibit RBM4 by binding to the C-terminal region of RBM4 and sequestering it. The exact mechanism of inhibition will, however, need to be determined.

Splicing in nuclei of living cells occurs while the pre-mRNA is being packaged in supraspliceosomes [15,19]. These 21 MDa macromolecular complexes contain all five spliceosomal U snRNPs, together with other splicing factors [19,43], and package the entire repertoire of nuclear pre-mRNAs, independent of their length or number of introns [14]. Supraspliceosomes have been shown to be active in splicing and are composed of four functional native spliceosomes interconnected by the pre-mRNA [44]. This structure should enable efficient communication between the native spliceosomes, which is important to allow the non-sequential removal of introns and for splicing regulation and alternative splicing. Our observation that RBM4 and WT1 cosediment with supraspliceosomes is not only consistent with their effect on alternative splicing, but also supports their interaction and their possible cooperation in RNA processing.

A further interesting observation we made (data not shown) and which was made by others before [7,45] was that RBM4 also resides in nucleoli. Other splicing proteins also localize in nucleoli [45], and, interestingly, one of them is U2AF^{65}, which, like RBM4, interacts with WT1 [11]. WT1 itself, when expressed in *Xenopus* oocytes, accumulates in the RNA-rich granular component of nucleoli [37]. Although the role of RBM4 in nucleoli is yet to be determined, we have found an interaction with RNA-polymerase (pol) I in a preliminary yeast two-hybrid library screen (H. Mangs, M.A. Markus, and B.J. Morris, unpublished data), suggesting a role for RBM4 in rRNA processing.

There is compelling evidence that both pre-mRNA splicing and nucleolar events are linked with the export machinery and RNA localization. Both spliceosomal RBPs such as SR proteins or hnRNPs (for reviews see [46,47]), as well as nucleolar RBPs [48,49], are able to shuttle rapidly between the nucleus and the cytoplasm and associate with export components [50]. Recent findings also suggest that a subset of

mRNAs is exported from the nucleus through transient association with the nucleolus [51]. In this light, it is important to note that both RBM4 and WT1 are capable of nucleoplasmic shuttling [7,52,53]. A potential role for each in mRNA export thus merits investigation.

WT1 has also been implicated in sex determination. In mice lacking the WT1(+KTS) isoform, gonads develop as ovaries in both XX and XY animals owing to reduced expression of *Sry*, a gene essential for the initiation of male development [54]. Furthermore, WT1 also interacts with WTAP [55], the human homolog of *Drosophila* fl(2)d (female lethal (2)d), which is required throughout development and the adult life of *Drosophila*, and which is important for regulation of splicing of the sex determining genes SXL (sex-lethal) and tra (transformer) [56]. WT1(+KTS) could regulate the expression of critical testis differentiation genes at the post-transcriptional level (with SRY downstream of WT1), and WTAP could function as a cofactor for alternative splicing events regulated by WT1 [57]. Interestingly, RBM4 also interacts with cyclin A1 [58], which is essential for spermatocyte passage into the first meiotic division in mice [59]. WT1 (+KTS) is also required for normal development of the olfactory system [60], highlighting the importance of this isoform in development. A role for RBM4 in these events should also be investigated.

In conclusion, our findings show that the WT1 isoform containing amino acids KTS in exon 9 (WT1+KTS) is a direct regulator of the splicing factor RBM4. This adds to the importance of WT1 in post-transcriptional processing. Our results also invite further research on the role of RBM4 in Wilm's tumor, Denys-Drash and Frasier syndromes, as well as leukemia, breast and prostate cancers, and sex determination. Finding the direct targets of RBM4 could help in understanding the mechanisms of these conditions.

Acknowledgments

This work was supported by a grant from the Australian Research Council (to B.J.M.), The University of Sydney Cancer Research Fund (to M.A.M.), the Families of Spinal Muscular Atrophy (to S.S.), and the Association for International Cancer Research, U.K. (to M.L.). We thank Profs. N. Hastie, R. Davies and M. Niksic, MRC, Edinburgh, U.K. for providing us with pACT-plasmids.

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