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RBM10 Modulates Apoptosis and Influences TNF- α Gene Expression

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Abstract: Recent evidence suggests that protein encoded by the RNA Binding Motif 10 (RBM10) gene has the ability to modulate apoptosis. The objective of this study was to test this hypothesis by manipulating RBM10 expression levels and examining the downstream consequences. The results showed that transient overexpression of RBM10 correlated with significantly elevated levels of tumour necrosis factor alpha (TNF- α) mRNA and soluble TNF- α (sTNF- α) protein, and increased apoptosis (phosphatidyl serine exposure on the outer cell membrane and nuclear condensation). Stable RNA interference-mediated RBM10 knockdown clones were less susceptible to TNF- α -mediated apoptosis, and had decreased sTNF- α protein levels. Elevated levels of TNF- α associated with RBM10 overexpression resulted from increased TNF- α transcription, not TNF- α mRNA stabilization. These results suggest that RBM10 has the ability to modulate apoptosis, and that it does so via a mechanism involving alterations to TNFR super family-mediated signaling. These data provide the first direct evidence that human RBM10 can function as an apoptosis modulator and cytokine expression regulator.

Keywords: RBM10, RBM5, TNF-alpha, apoptosis, cancer, transcription

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Introduction

The RNA Binding Motif 10 (RBM10) protein shares ~50% identity with RBM5 (Fig. 1), suggesting that the two genes encoding these proteins are paralogues.^{1,2} Paralogues typically have different but related functions, but while the function and mechanism of action of RBM5 are beginning to be elucidated, there is as yet limited data to connect the observations concerning RBM10 into a meaningful role.

Circumstantial evidence suggests that RBM10 does indeed have a related function to RBM5, and that like RBM5, RBM10 is a modulator of apoptosis as well as an RNA binding protein involved in the regulation of co-transcriptional modification of pre-mRNA. Firstly, expression of RBM10 and the pro-apoptotic factors BAX and caspase 3 were positively correlated in primary breast tumour specimens.^{3,4} Secondly, in primary chondrocytes induced to hypertrophy following exposure to glucocorticoid, an increase in RBM10 expression was associated with increased apoptosis and decreased proliferation.⁵ Thirdly, RBM10 has been purified from prespliceosomal complexes,⁶⁻⁸ and has a RanBP2 zinc finger that is predicted to interact with the sequence AGGUAA in 5' splice sites,⁹ data which suggest that RBM10 plays a related but different role to RBM5 in the regulation of alternative splicing.

In humans, RBM10 pre-mRNA can be alternatively spliced to produce at least three variants, RBM10v1, RBM10v2 and RBM10v3.¹⁰ RBM10v1 and RBM10v2 are widely expressed, differ only by the inclusion of one exon (exon 4) in variant 1, and encode proteins of approximately 100 and 95 kDa, respectively. Variant 3 (Genbank Accession Number AK024839) was identified in primary smooth muscle cells of the coronary artery, has an alternate start site and a 23 bp deletion in exon four which results in a frameshift and premature stop codon within exon 4.

Until recently, all expression and function studies related to RBM5 were carried out independently of any parallel analyses of RBM10. Recently, however, a significant relationship between RBM10 and RBM5 was demonstrated in HeLa cells: where as depletion of RBM5 alone was insufficient to induce an alteration in Fas receptor pre-mRNA splicing, depletion of RBM5, RBM10 and the structurally related factor RBM6 was associated with preferential exon 6 inclusion,²

suggesting a degree of functional synergy between at least two of the three RBM proteins. It is with this in mind that we set out to examine, directly, the involvement of RBM10 in apoptosis modulation. We accomplished this by experimentally manipulating RBM10 expression levels, examining the apoptotic response and identifying key downstream factors associated with altered function. For these analyses we used two cancer cell lines that are susceptible to TNFR super family-mediated apoptosis, and for which previous studies relating to RBM5 showed functional relevance, Jurkat and MCF-7¹¹⁻¹³ (MCF-7 cells using PBOX-6 and caspase 7 in place of caspase 8).¹⁴

Materials and Methods

Cell culture

MCF-7 human breast adenocarcinoma cells were obtained from David Seldon, Boston University, U.S.A., in 2003. JKM1 cells, a Fas-sensitive subclone of the Jurkat human T lymphoblastic leukemia cell line,¹⁵ were obtained from Gwyn Williams in 1998. Cells were grown as previously described.^{12,13}

Subcloning

RBM10v1 (KIAA0122, GenBank Accession Number X52328), in the Bluescript plasmid vector pBS.SK, was kindly provided by Takahiro Nagase at the Kasusa DNA Research Institute (Kisarazu, Chiba, Japan). To generate the pcDNA3.RBM10v1 subclone, RBM10v1 cDNA in the pBS vector was amplified using the HindIII-linked forward primer RBM10F.² HindIII (Table S1) and the EcoRI-linked reverse primer RBM10Rb'.EcoRI, and ligated to pcDNA3 (Invitrogen, Burlington, Canada) following digestion of both amplicon and receiving vector with HindIII and EcoRI (NEB, Pickering, Canada). To generate the pEGFP.RBM10v1 subclone, RBM10v1 cDNA in the pBS vector was amplified using RBM10Fe. EcoRI and RBM10Rb'.EcoRI, then ligated to an EcoRI digested and dephosphorylated pEGFP. Sense orientation constructs were identified following SacI digestions.

RBM10v2 (GenBank Accession Number NM_152856), in the cytomegalovirus based plasmid vector pCMV-XL6, was purchased from Origene Technologies (Cedarlane, Burlington, Canada). To generate the pcDNA3.RBM10v2 subclone, pCMV-



Figure 1. RBM5 and RBM10 have significant sequence homology. Amino acid sequences, Accession numbers NM_005676, NM_152856 and NM_005778 for RBM10v1, RBM10v2 and RBM5, respectively, were aligned using the multiple sequence alignment program CLUSTALW 1.18, with default settings. Consensus key: (*) fully conserved residue; (:) conservation of strong groups; (.) conservation of weak groups. Boxed sequences with solid lines represent the core RRM domains, boxed sequences with hatched lines represent zinc finger (ZF) sequences, the solid underlining delineates the nuclear localization sequence and the hatched underlining delineates the G-patch. Pair wise alignments found RBM10v1 and RBM10v2 to have 100% sequence homology, excluding exon 4 of RBM10v1; RBM10v2 and RBM5 to have 55% sequence homology, and; RBM10v1 and RBM5 to have 53% sequence homology.

XL6.RBM10v2 and pcDNA3 were digested with NotI (NEB, Pickering, Canada). pcDNA3.RBM10v2 constructs in the sense orientation were identified following XhoI digestions (NEB, Pickering, Canada).

To generate the pEGFP.RBM10v2 subclone, RBM10v2 cDNA in the pCMV-XL6 vector was amplified using the RBM10FeEcoRI and RBM10RbEcoRI primers, then ligated to EcoRI digested then dephosphorylated



pEGFP. Sense orientation constructs were identified following SacII digestion.

Transfections

For the PCR-Array studies, MCF-7 cells were transiently transfected using Lipofectamine Reagent and Lipofectamine PLUS (Invitrogen, Burlington, Canada), according to manufacturer's instructions. Briefly, 750 μ L Opti-MEM (Invitrogen, Burlington, Canada) containing 4 μ g of either pcDNA3.RBM5, pcDNA3.RBM10v1 or pcDNA3.RBM10v2 DNA were mixed with 20 μ L Lipofectamine PLUS, then 750 μ L Opti-MEM containing 30 μ L Lipofectamine Reagent was added. After 15 minutes, 5 mL of Opti-MEM were added to the transfection mixture. The diluted transfection mixture was placed on the adherent cells, following one rinse of the cells with Opti-MEM. Transfection medium was changed after four hours and replaced with D-MEM/F-12 containing 10% fetal bovine serum (Invitrogen, Burlington, Canada). The following morning, the medium was once again replaced with fresh serum containing medium.

For the overexpression studies depicted in Figure 3, the overexpression-associated ELISA assays (Table 2), the generation of RBM10 KD clones and the transcription and stabilization studies, cells were transiently transfected using Lipofectamine 2000TM (Invitrogen, Burlington, Canada), according to manufacturer's instructions. 16 μ g of DNA and 48 μ L of Lipofectamine 2000 in 1 mL of Opti-MEM were added to cells 24 hours after seeding at a density of 15×10^4 cells mL⁻¹ in a total volume of 10 mL.

RNA extraction

Total RNA was isolated from transfected MCF-7 cells using an RNease[®] Kit (Qiagen, Mississauga, Canada), according to manufacturer's instructions. RNA quantity and quality were evaluated using a bioanalyzer (Agilent Technologies, Mississauga, Canada). RIN cutoff value was 8.6. For RNA expression level analyses of transfected genes, first strand cDNA was generated using Superscript II reverse transcriptase (Invitrogen, Burlington, Canada). For PCR-Array analyses, first strand cDNA was generated using the Reaction Ready First Strand cDNA Synthesis Kit recommended by the PCR-Array manufacturer (SABiosciences/Cedarlane, Burlington, Canada).

PCR amplification

The following primers were used to confirm overexpression of transfected cDNAs: LU15(2) and LU15(3),¹³ RBM10F, RBM10Rs, RBM10e4R and RBM10v1/v2R (Table S1). Primers RBM10F and RBM10Rs were used to determine the level of RBM10v1 and RBM10v2 overexpression, prior to performing the PCR-Array. Primers RBM10F, RBM10e4R and RBM10v1/v2R were used to determine the level of RBM10v1 and RBM10v2 overexpression, respectively, prior to performing the ELISA assay. The PCR program was 95 °C for 5 minutes, followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 45 seconds, followed by 72 °C for 10 minutes. Densitometric analysis of the electrophoresed amplicons was carried out using AlphaEase FC software (Alpha Innotech).

PCR-Array analysis

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using the apoptosis-specific RT² ProfilerTM PCR-Array (Cat.# APHS-012A, SABiosciences, Frederick, MA) and an ABI PRISM[®]7900HT sequence detection system (Applied Biosystems, Streetsville, Canada). For cDNA synthesis, 2 μ g of RNA was used. The PCR program was performed at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Each experiment was performed in triplicate. The Array results were analyzed using SABiosciences software. Relative expression was calculated using the comparative Ct method. Array genes were normalized to actin.

Validation primers for RT-qPCR verification of the PCR-Array results were generated using ABI Express 2.1 software (Applied Biosystems, Streetsville, Canada), following SABiosciences disclosure of the accession numbers of the gene sequences corresponding to the Array oligonucleotide sequences (TNF- α : NM_000594; TRAIL: NM_003810; TNF- β : NM_000595). For cDNA synthesis, 2 μ g of RNA was used. For primer sequences see Table S1. Template RNA was identical to that used in the PCR-Array experiments. The PCR verification program was 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Relative expression was calculated using the standard curve method.

Enzyme-linked immunosorbent assay (ELISA)

Microtiter ELISA plates with anti-TNF- α monoclonal antibody (R&D Systems/Cedarlane, Burlington, Canada) were used to estimate levels of ligand in the cell culture medium. Medium was concentrated using centriprep Ultracel[®] YM-10 columns (Millipore/Billerica, MA). Standards and samples were plated in duplicate or triplicate. Optical density was determined using a plate reader set to 450 nm with a correction at 540 nm. The data presented in Table 2 are from two independent RBM10v1 transfections, analysed in triplicate, and one RBM10v2 or RBM5 transfection, analyzed in duplicate.

Luciferase assay

Construct pGL3-TNFPro-UTR contains 1300 bp of promoter and 800 bp of 3'-UTR of the TNF- α gene from the mouse macrophage cell line RAW 264.7.^{16,17} pGL3-TNFPro-UTR was co-transfected with the GFP-based constructs into MCF-7 and Jurkat cells. Both adherent and floating cells were collected for expression analysis. Luciferase activity was monitored using the Luciferase Assay System (Promega/Fisher Scientific, Nepean, Canada) and the luminescence protocol of the Multimode Detection Software version 3.0.0.5 of the Beckman Coulter DTX880 Multimode Detector. GFP levels were measured using the fluorescence protocol.

Flow cytometry

For the overexpression studies, Jurkat cells were transiently transfected with pEGFP, pEGFP.RBM10v1 or pEGFP.RBM10v2 and harvested 48 hours after transfection. Cells were exposed to propidium iodide (PI) (Sigma-Aldrich, Oakville, Canada) and phycoerythrin (PE) conjugated Annexin-V (Biovision/Cedarlane, Burlington, Canada) for 15 minutes. Using a Beckman Coulter Cytomics FC500, ten thousand events were collected, gating only the transfected population. Fluorescence was measured using FL1 for EGFP (Ex: 488 nm and Em: 507 nm), FL2 for Annexin-V-PE (Ex: 488 nm and Em: 578 nm) and FL4 for PI (Ex: 505 nm and Em: 615 nm). For the knockdown studies, cells were exposed to PI and fluorescein isothiocyanate (FITC) conjugated Annexin-V (Biovision/Cedarlane, Burlington, Canada) for five

minutes. Ten thousand ungated events were collected. Fluorescence was measured using FL1 for FITC (Ex: 490 nm and Em: 525 nm) and FL4 for PI.

Apoptosis assays

For the nuclear condensation studies (Fig. 3), 72 hours post transfection cells were treated with 10 μ g/mL Hoechst 33258 (Ex: 346 nm, Em: 460 nm) (kindly provided by Dr. Appana, Laurentian University) using standard procedures. For the PS flip studies (Fig. S2), cells were stained with an Alexa Fluor 647-conjugated Annexin-V (Ex: 647 nm, Em: 665 nm) (Invitrogen, Burlington, Canada), according to the manufacturer's instructions. Images were captured using a Zeiss inverted Axiovert 200 M microscope and LSM 5 software, Version 3.2.

shRNA constructs

Two HuSH 29-mer shRNA constructs against RBM10 (TI308329, TI308330) (OriGene Technologies/Cedarlane, Burlington, Canada) were used to target locations unique to the RBM10 transcript. Constructs "29" and "30" target exon 6 and are not variant specific. Target sequences are listed in Table S1. Two negative control shRNA constructs were TR20003 (pRS plasmid) and TR30003 (pRS non-effective GFP plasmid).

Western blotting

Western blotting was performed as previously described.¹³ 50 μ g of extracted protein were electrophoresed through SDS-PAGE gels and transferred to PVDF membrane (Whatman/VWR, Mississauga, Canada) and specific proteins detected by ECL chemiluminescence using Hyper film ECL (GE Healthcare, Mississauga, Canada). The primary antibodies used were rabbit anti-RBM10 (1:5000, A301-006A, Bethyl Laboratories Inc/Cedarlane, Burlington, Canada), rabbit anti-RBM5¹³ (1:2500), mouse anti-TNF- α (1:200, sc-52746, Santa Cruz Biotechnologies Inc, Santa Cruz, CA) and mouse anti- α tubulin (1:10000, sc-8035, Santa Cruz Biotechnologies Inc., Santa Cruz, CA). Secondary antibodies used were the HRP-conjugated goat anti-rabbit (1:10000, sc-2004, Santa Cruz Biotechnologies Inc., Santa Cruz, CA) and goat anti-mouse (1:10000, sc-2005, Santa Cruz Biotechnologies Inc.,



Santa Cruz, CA). Densitometry was performed using AlphaEaseFC 4.0 software.

Transcription and stabilization studies

RBM10v1 or a vector control were transfected into Jurkat cells, and 48 hours after transfection the polymerase II inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole riboside (DRB) (Sigma-Aldrich, Oakville, Canada), at 50 μ M, was added.¹⁸ Dimethylsulphoxide (DMSO) was used as vehicle. Levels of endogenous TNF- α mRNA were measured using RT-qPCR and confirmation of RBM10v1 overexpression was carried out using RT-PCR.

Results

Overexpression of RBM10 correlates with TNF- α mRNA upregulation

To begin to investigate the role of RBM10 as a modulator of apoptosis we used an RT-qPCR-based pathway-focused gene expression profiling system in which we could simultaneously examine the expression of 84 apoptosis-associated genes. The MCF-7 cell line was chosen for these analyses since (a) it had previously been used in RBM5 apoptosis function studies,¹² and (b) transient expression is generally better than in Jurkat cells, the other cell line in which the majority of the RBM5 apoptosis function studies were carried out.^{11,13,19,20}

Cells were transiently transfected with cDNA encoding the two most prevalent RBM10 isoforms, RBM10v1 and RBM10v2,¹⁰ or RBM5. Experiments in which RNA overexpression was roughly equal were used for statistical comparisons. RBM5 was

~3–5-fold overexpressed, RBM10v1 was ~5–9-fold overexpressed and RBM10v2 was ~3–6-fold overexpressed (data not shown). The results of individual transfections are presented as Table S2. Of the 84 apoptosis-associated genes represented on the Array, including seven receptor ligands, only two receptor ligands (TNF- α and TRAIL) showed significant expression changes. TNF- α expression levels were significantly upregulated 2-fold or greater in 6/8 transfections, and approached significance in 1/8. Likewise, TRAIL expression levels were significantly upregulated 2-fold or greater in 6/8 transfections, with notably higher upregulation occurring in the RBM10v2 overexpressors. Grouped data per gene showed the upregulation of caspase 4 (CASP 4) was significant in the cells overexpressing RBM10v2 and RBM5. Significant upregulation of TNFSF7 and TNFRSF9 was observed in the RBM10v1 overexpressors (Table 1 and Table S3).

TNF- α and TRAIL expression was re-examined in repeat RT-qPCR reactions, and confirmed to be upregulated (Fig. S1).

Overexpression of RBM10 correlates with sTNF- α protein upregulation

We next examined ligand expression at the protein level, concentrating on TNF- α , which was upregulated in cells overexpressing both RBM10 variants (and RBM5). Additional transient transfections in MCF-7 cells were carried out, using GFP-tagged RBM constructs, overexpression was confirmed by RT-qPCR and soluble, excreted TNF- α (sTNF- α) protein levels were examined by ELISA.

Table 1. Genes, represented on the PCR-Array, with the most significant expression changes associated with RBM10 or RBM5 overexpression. Information extracted from Table S3.

Gene	RBM10v1		RBM10v2		RBM5	
	Fold-change	P-value	Fold-change	P-value	Fold-change	P-value
Ligands						
TNF-alpha	3.26	0.0054	3.75	0.0057	3.71	0.0001
TRAIL/TNFS F10	2.19	0.0847	8.13	0.0001	2.24	0.0038
TNF-beta/LTA	2.92	0.2472	133	0.0670	2.20	0.0052
TNFSF7	2.89	0.0005	168	0.1668	127	0.4270
Receptor						
TNFRSF9	2.60	0.0028	126	0.1074	183	0.0451
Other						
CASP4	2.23	0.0762	3.46	0.0230	2.29	0.0236

Note: Significances were calculated using a Student's unpaired *t*-test.

Table 2. Summarized TNF- α expression level data from MCF-7 transfectants.

	RBM over expression	Fold-change + SD ligand RNA	P-value	Fold-change + SD soluble ligand protein	P-value
RBM10v1	22-fold	2.32 \pm 0.27	0.0001	2.87 \pm 0.33	0.0002
RBM10v2	10-fold	3.60 \pm 0.15	0.0010	2.00 \pm 0.00	0.0000
RBM5	2.8-fold	2.08 \pm 0.11	0.0059	2.67 \pm 0.47	0.0299

Note: Significances were calculated using a Student's unpaired *t*-test.

The results, presented in Table 2, demonstrate that both TNF- α mRNA and sTNF- α protein were significantly elevated in the RBM transfected cell populations compared to the GFP control transfected population.

Confirmation of TNF- α mRNA upregulation by RBM10 using a luciferase reporter construct

Upregulation of TNF- α by RBM10 in MCF-7 cells was confirmed using a TNF- α /luciferase promoter/reporter construct. In addition, upregulation of TNF- α by RBM10 was examined in Jurkat T cells, since Jurkat cells are not only also sensitized to apoptosis by overexpressed RBM5,¹¹ but highly responsive to TNF- α , suggesting that they might be more responsive to RBM10 overexpression than the MCF-7 breast cancer cells. The results (Fig. 2) showed that luciferase activity was increased following transfection with both RBM10v1 or RBM10v2, in both

cell lines. Of three different transfection experiments into Jurkat cells, two demonstrated 18 to 24-fold higher expression of luciferase in the RBM10 transfectants compared to the controls. Thus, while there was considerable variability in luciferase expression in the Jurkat cells, the results did suggest that these cells were more responsive to RBM10 overexpression than the MCF-7 cells. Based on these results, Jurkat cells were used for the majority of the functional assays.

Overexpression of RBM10 is pro-apoptotic

Previous studies demonstrated that overexpression of RBM5 increased the sensitivity of Jurkat and CEM-C7 T cells to TNF- α -mediated apoptosis,^{11,21} a phenomenon we now attribute to elevated levels of endogenously expressed sTNF- α . Since overexpression of RBM10 is also associated with elevated levels of endogenously expressed sTNF- α , we postulated

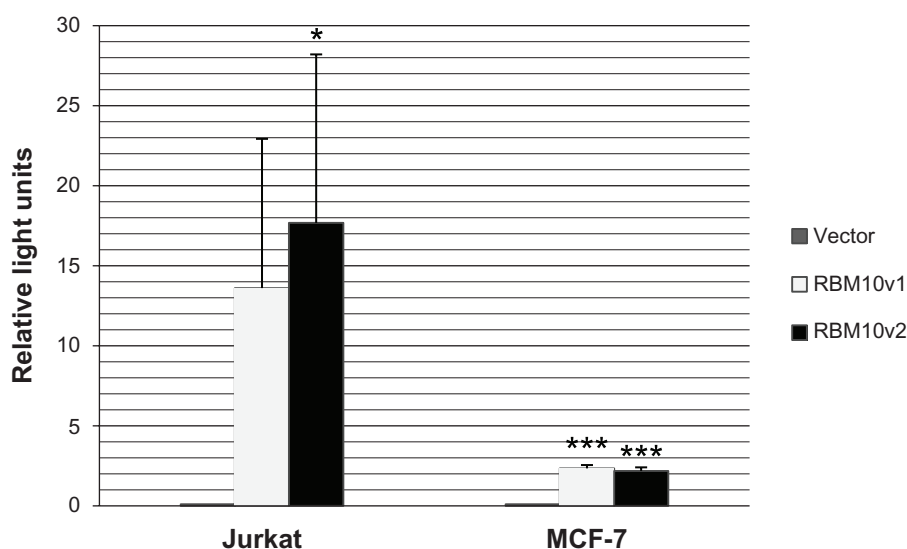


Figure 2. Luciferase activity measured in Jurkat and MCF-7 cells, 48 hrs after co-transfection of pGL3-TNFPro-UTR with pEGFP, pEGFP.RBM10v1 or pEGFP.RBM10v2. Differing transfection efficiencies were accounted for by normalizing the luciferase levels to the level of GFP. Data were from three transfection experiments per cell line.

Notes: Significance was measured using an unpaired Student's *t*-test, where **P* = 0.04 and ****P* \leq 0.0001.

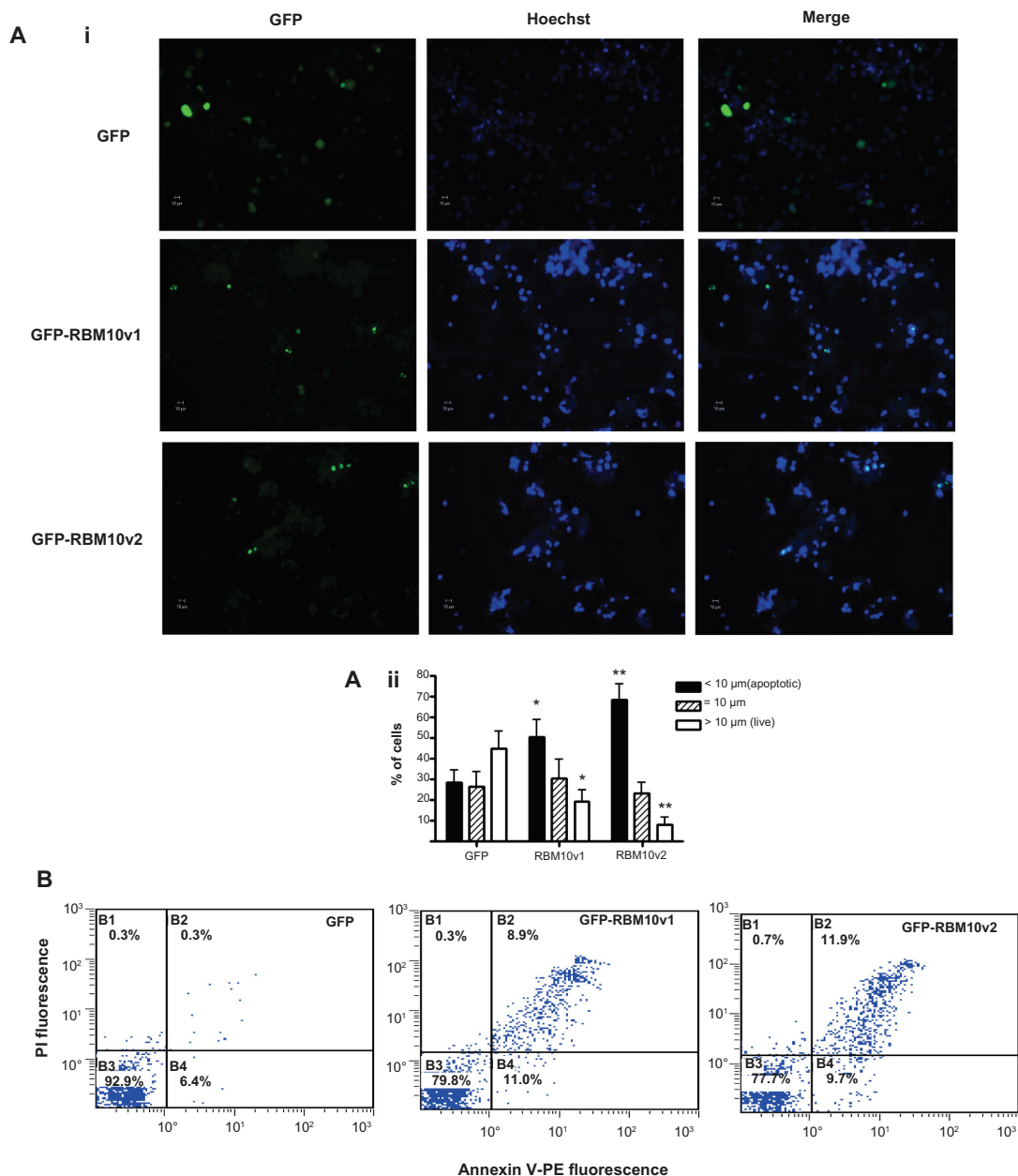


Figure 3. Apoptosis is observed in RBM10 overexpressing Jurkat cells. Cells were transiently transfected with pEGFP, pEGFP.RBM10v1 or pEGFP.RBM10v2. **(Ai)** Nuclear condensation/fragmentation. Cells were harvested 72 hours following transfection, incubated with 10 μg/mL of Hoechst 33258 (which binds to DNA and causes cells with more compacted DNA to fluoresce more brightly), and visualized using fluorescence microscopy. Green = GFP protein, blue = Hoechst staining of nuclei. **(Aii)** Five fluorescence images were taken for each construct and intact nuclei were measured and scored. The total number of intact nuclei used for each transfected population was 68, 39 and 34, for empty vector, RBM10v1 and RBM10v2, respectively. **(B)** Phosphatidyl serine exposure. Cells were harvested 48 hours following transfection. PS flip to the outer nuclear membrane was monitored by staining the cells with PE-conjugated Annexin-V, which binds to PS, and propidium iodide, which is only able to enter membrane damaged cells, thereby enabling a distinction between early apoptosis and secondary apoptosis/necrosis. Results were gated to the GFP-expressing cells.

Notes: Significance was measured using an unpaired Student's *t*-test whereby * and ** equal $P < 0.05$ and $P < 0.01$, respectively. Data are presented as a flow cytometer dot plot. Quadrant B1, lysed cells and debris; B2, late apoptotic/necrotic cells; B3, live cells; B4, early apoptotic cells. Results are from one assay, but are representative of a minimum of six assays.

that overexpression of RBM10 would also sensitize Jurkat cells to TNF- α -mediated apoptosis.

cDNA was transiently overexpressed in Jurkat cells and apoptosis (in the absence of an externally administered apoptogenic stimulus) was measured

using a variety of methods. Firstly, when the number of live cells were counted, the RBM10 transfected populations showed decreased cell numbers compared to the empty vector controls (data not shown). Secondly, the overexpression of both RBM10 variants

was associated with phosphatidyl serine (PS) on the outer cell membrane of intact GFP-expressing cells (Fig. 3B). Thirdly, nuclear condensation/fragmentation was examined. The microscopic images of Hoechst stained cells (Fig. 3Ai) clearly showed smaller, more intensely blue nuclei in the RBM10 transfected, than in the GFP transfected, populations (where GFP appears as small specs as a result of its nuclear localization as an RBM10-fusion protein).²² The results revealed a significantly reduced number of cells in the RBM10v1 and RBM10v2 transfected populations with diameters greater than 10 microns (live cells), and a significantly increased number of cells in the RBM10v1 and RBM10v2 transfected populations with diameters less than 10 microns (apoptotic cells), compared to the number in the GFP control transfected population (Fig. 3Aii).

Apoptosis was also examined in MCF-7 cells. As seen in Figure S2, the RBM10—in particular RBM10v1—transfected MCF-7 populations demonstrated more Annexin-V fluorescence than the GFP transfected MCF-7 population, although fluorescence was not necessarily coincident with GFP expression. Taken together, these results demonstrated that over-expressed RBM10 was pro-apoptotic in both Jurkat and MCF-7 cells.

Inhibition of RBM10 inhibits TNF-mediated apoptosis

Since the RBM10 overexpression levels associated with definitive apoptotic morphological changes may or may not reflect conditional physiological levels, we decided to restrict endogenous RBM10 expression, to obtain a potentially more physiological assessment of the ability of RBM10 to modulate apoptosis. Endogenous RBM10 expression was restricted using small interfering RNA. Stable knockdown (KD) clones were generated in order to prevent transient transfection-associated cell death from interfering with apoptosis analyses.

Using siRNAs that targeted both RBM10v1 and RBM10v2, four Jurkat clonal populations with a greater than 70% reduction in RBM10 expression were generated (designated J30.1, J30.2, J30.8 and J30.16) (Fig. 4A), and one MCF-7 clonal population (designated M29/30.2) (Fig. S3A). Because of the high degree of homology between RBM10 and RBM5, the specificity of the RBM10 KD effect was

verified by examining RBM5 protein expression levels in a subset of the RBM10 KD clones, and as seen in Fig. S3B, RBM5 protein expression levels were not affected by RBM10 KD.

To examine the effect of RBM10 inhibition on an apoptotic process, cells were exposed to TNF- α , which had previously been shown to trigger an apoptotic response in Jurkat and MCF-7 cells.^{11,12} In functional analyses, RBM10v1/v2 KD significantly reduced TNF- α -mediated cell death compared to the control, as seen by examining PS flip in Jurkat cells (Fig. 4B) and cellular morphology in the MCF-7 cells (Fig. S3C, D). While all the Jurkat RBM10 KDs showed reduced Annexin V staining (data not shown), the most dramatic results were seen in clones J30.8 and J30.16 (Fig. 4B).

Inhibition of RBM10 correlates with TNF- α down regulation

The robustness of our observed RBM10/TNF- α association was investigated by examining TNF- α expression in the RBM10 KD environment. We focused on the Jurkat cells, where expression level changes were greatest and for which any potential change in expression would be more easily measured. It was anticipated that, in apposition to the overexpression studies, RBM10 KD would be associated with decreased TNF- α expression. For this study, the RBM10v1/v2 KD clone with the most dramatic anti-apoptotic effect (J30.16) was used. By ELISA, the results demonstrated that sTNF- α protein levels were significantly reduced in the Jurkat RBM10 KD cells ($P = 0.02$) (Fig. 4C).

Upregulation of TNF- α by RBM10 occurs via a transcriptional mechanism rather than message stabilization

The first RBM10 mRNA target was recently identified: the rat RBM10 orthologue, S1-1, bound to the 3'-untranslated region (UTR) of the message encoding the angiotensin II receptor type 1 (AT1), in primary rat vascular smooth muscle cells.²³ This S1-1 binding to AT1 mRNA resulted in message stabilization. We therefore set out to determine if the elevated TNF- α expression associated with RBM10 overexpression resulted from TNF- α mRNA stabilization.

Jurkat cells transiently overexpressing RBM10v1 were exposed to the polymerase II inhibitor DRB,

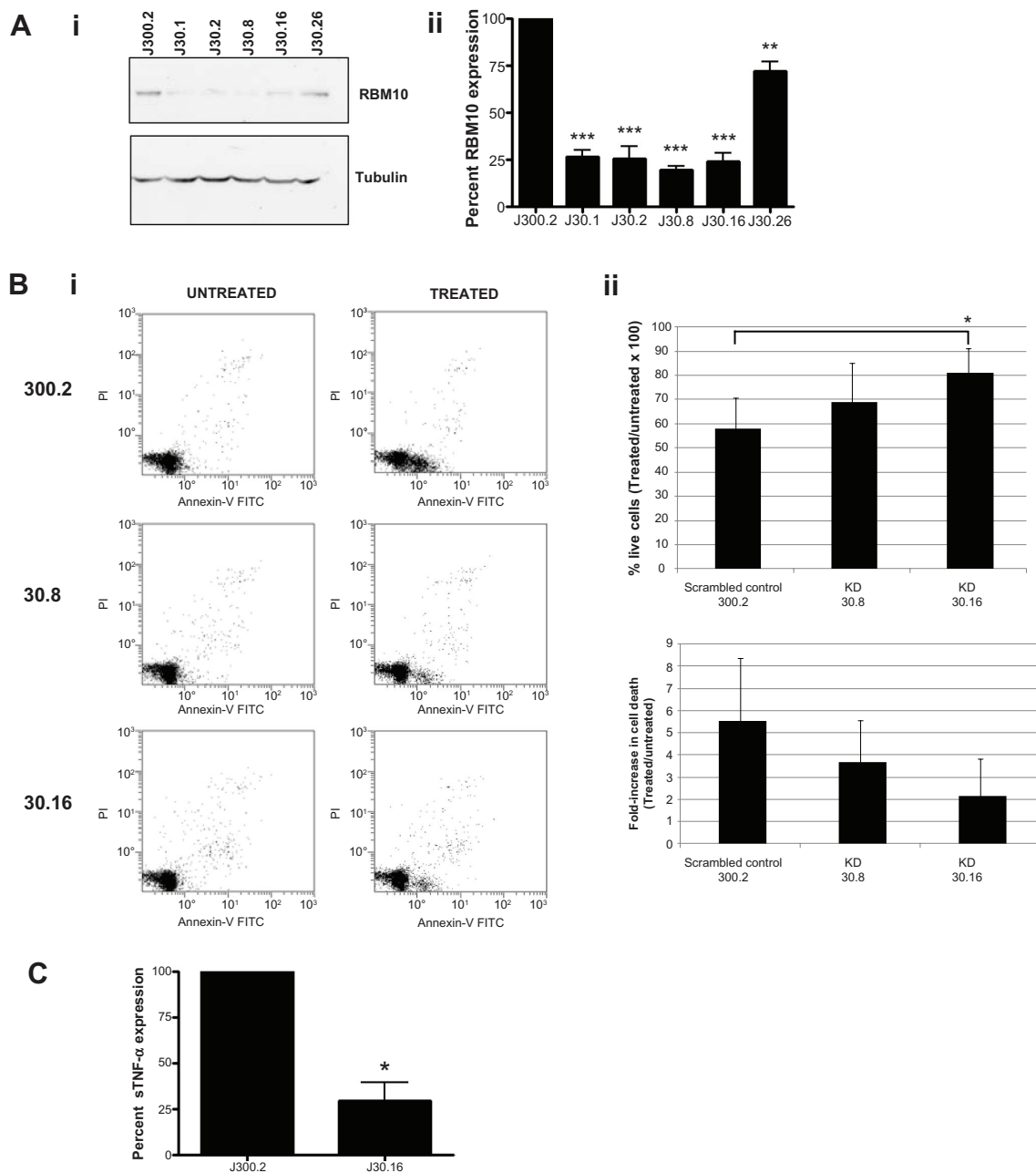


Figure 4. TNF- α -mediated apoptosis is inhibited in RBM10v1/v2 KD Jurkat cells. **(Ai)** Western blot data showing the level of RBM10 expression in various stable clonal populations. Western blots are representative of results from three separate lysates, each obtained from cell populations from three consecutive weeks of growth. **(Aii)** Graphed data summarizes the densitometric data obtained from the three separate Western blots for each clone. Error bars represent the standard error of the mean. **(B)** Flow cytometry data. Jurkat RBM10 KD clones J30.8, J30.16 and the control clone J300.2 were treated with 10 ng/mL TNF- α and 10 μ g/mL cycloheximide. Following treatment, cells were stained with FITC-conjugated Annexin-V and propidium iodide, and examined by flow cytometry. **(i)** Dot blot of one representative experiment, where cells were treated for 4 hrs. **(ii)** Graphed, summarized dot blot data from 4 biological replicates, two with 4 hr treatments and two with 16 hr treatments. **(C)** ELISA data of sTNF- α protein expression in RBM10 KD Jurkat clone J30.16 (fold-expression changes were calculated from two independent experiments, performed as technical triplicates). The supernatant was collected 48 hours after routine passage.

Notes: Significances were calculated using a Student's unpaired *t*-test. **P* < 0.05, ***P* < 0.005, ****P* < 0.0001.

and the levels of TNF- α RNA were measured. Since TNF- α message has a half-life of 2.5 hours,²⁴ and the cells were exposed to DRB for 4.5 hours, if the TNF- α transcripts were not being stabilized by RBM10 then it would be anticipated that by 4.5 hours of DRB

treatment the TNF- α transcript level would be almost one quarter of the minus treatment level.

The results showed that while overexpression of RBM10v1 was associated with significantly elevated levels of TNF- α mRNA compared to the

vector control in both untreated ($P = 0.01$) and DRB treated ($P < 0.05$) cells, there was approximately one quarter of the level of TNF- α in the treated as compared to the untreated samples (a significant decrease in both the vector ($P = 0.01$) and the RBM10v1 ($P < 0.01$) DRB treated samples) (Fig. 5). These results suggest that the increased levels of TNF- α observed in cells overexpressing RBM10v1 were not due to TNF- α mRNA stabilization but to increased transcription.

Discussion

RNA binding proteins are classified according to the structure of their consensus binding motif. "RBM" designated proteins contain at least one RNA recognition motif (RRM)¹⁰ but not all RBM-containing proteins have the RBM designation. Presently, fifty

proteins, many of which have no known function, are so classified in the HUGO Gene Nomenclature Committee Database. The most widely studied of these "RBM" proteins, including RBM4, RBM5, RBM9 and RBM17, are involved in RNA processing—more specifically in RNA splicing regulation^{2,25–28}

Our work with RBM10, a paralogue of RBM5, began with the hypothesis that, because of its high degree of homology with RBM5, which modulates apoptosis by regulating the alternative splicing of transcripts associated with death receptor signaling, RBM10 functions in a similar manner to RBM5. We began by determining if overexpression of RBM10 impacted on apoptosis-associated gene expression. To accomplish this we used an apoptosis-focused PCR-Array that incorporated oligonucleotides representing 84 genes known to play a role in the

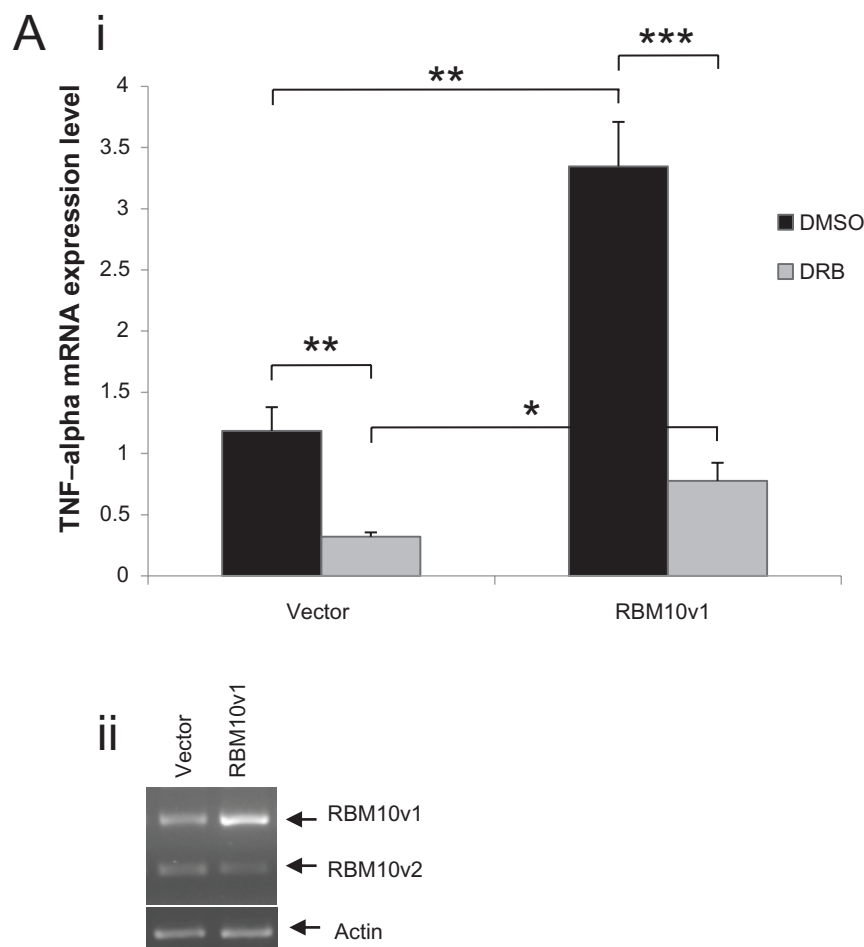


Figure 5. RBM10v1 increases TNF- α transcription. **(Ai)** TNF- α mRNA expression levels in Jurkat cells, measured using RT-qPCR and normalized to S28, 48 hrs after transfection with pEGFP or pEGFP.RBM10v1 and 4.5 hrs after treatment with 50 μ M DRB or the equivalent volume of DMSO vehicle. Data are from one transfection analyzed in duplicate. **(Aii)** Verification of RBM10v1 overexpression in the transfectants, measured following RT-PCR in the DMSO control samples.

Notes: Significances were calculated using a Student's unpaired t -test. * $P < 0.05$, ** $P = 0.01$, *** $P < 0.01$.



regulation of apoptosis. Included on the Array were sequences corresponding to a subset of the death receptor, death receptor ligand, Bcl-2 and caspase family members.

Overexpression of RBM10v1, RBM10v2 or RBM5 was shown to correlate with changes in the expression levels of a number of transcripts, but the only transcript whose expression was significantly altered following overexpression in each instance was TNF- α . Additional significant expression changes were observed, but appeared to be gene-specific. For instance, expression of TNF- β /LTA was significantly changed only in the RBM5 overexpressing cells, expression of the receptor and ligand TNFRSF9 and TNFSF7 was significantly changed only in the RBM10v1 overexpressing cells, and expression of caspase 4 and TRAIL was significantly changed in the RBM10v2 and the RBM5 overexpressing cells (Table 1). Taken together, these results suggested that, like RBM5, RBM10v1 and RBM10v2 were capable of modulating apoptosis via TNF- α -associated regulatory mechanisms.

TNF- α signaling is complex, and involves the regulation of expression and/or activity of proliferation and apoptosis factors, with much cross-talk between various pathways. Outcome (proliferation versus apoptosis) is influenced by a number of factors, including which intracellular complexes are formed following cytokine crosslinking,²⁹ and which form of the cytokine cross links with which receptor, eg, membrane bound TNF- α has high affinity for both TNFR1 and TNFR2, whereas sTNF- α has a much higher affinity for the death domain-containing TNFR1.³⁰ We investigated the relationship between RBM10 overexpression and TNF- α protein expression by ELISA, and found a positive correlation between both RBM10v1 and RBM10v2 protein expression and the pro-apoptosis-associated sTNF- α protein (Table 2).

Expression of TNF- α is regulated at various levels, including transcription, mRNA half-life, translation and protein cleavage. Transcription is regulated by a number of factors, including NF- κ B, NFAT family members, AP-1, AP-2, Ets-1, C/EBP β and ATF-2. Transcript half-life is regulated by RNA binding proteins, including AUF-1, HuR and ZFP36/Tis11/Nup475/Gos24, which bind to the adenine and

uridine-rich elements (AREs) in the TNF- α 3'-UTR and increase message turnover by destabilizing the mRNA.³¹ Binding of RNA binding proteins, such as TIAR, within the TNF- α 3'-UTR also influences translation.³² The metalloproteinase ADAM17/TACE is responsible for proteolytic modification of mTNF- α to sTNF- α .^{33,34} Using a reporter gene construct, we demonstrated that RBM10v1 upregulation correlated with increased transcription, but not transcript half-life, of TNF- α (Fig. 5). Whether or not RBM10 interacts directly or indirectly with elements in the 1300 bp upstream region of the TNF- α DNA used in this study remains to be established. Current evidence suggests that RBM10 functions in a similar manner to RBM5, as a regulator of alternative splicing. As mentioned in the introduction, a study by Valcárcel and colleagues suggests that RBM10 plays an integral role in regulating the alternative splicing of FasR.² In addition, we have data demonstrating that inhibition of RBM10 in multiple cancer cell lines correlates with significant alterations in the splicing of a number of transcripts (Sutherland and Chabot, manuscript in preparation). Although to our knowledge no RNA binding proteins have been shown to interact directly with the promoter region of TNF- α , a number of RRM, KH and RGG RNA binding domain containing proteins are capable of binding directly to DNA.³⁵⁻³⁷ For instance, TIAR (through its RRM1 domain) binds with high affinity to single-stranded thymidine-rich DNA within the VEGF gene;³⁸ the TIAR homologue, TCBP, binds to double-stranded DNA and is thought to function as a transcriptional regulator,³⁹ and; two KH-domain containing RNA binding proteins, HnRNP K and FBP, bind to far upstream sequences within the c-myc promoter and function as transcriptional enhancers.^{40,41} Determining whether RBM10 regulates TNF- α transcription directly (by binding to the TNF-promoter) or indirectly (for instance, by altering the splicing of transcription factors) will form part of our future research efforts.

Interestingly, no matter what the level of RBM10 overexpression in the MCF-7 cells (which ranged from 2.5 to 22-fold), TNF- α was never upregulated more than approximately 4-fold, demonstrating a "threshold effect". This was not observed in the Jurkat cells. We speculate that the reason MCF-7 cells experienced less dramatic changes in TNF- α expression levels

than Jurkat cells (as shown in Fig. 2) relates to the fact that apoptosis is a particularly important process to immune cell function, immune cell apoptosis is highly regulated by TNF- α signaling and MCF-7 cells lack certain regulatory cofactors or steps that function to regulate TNF- α production in Jurkat cells.

In our hands, RBM5 overexpression has more often been associated with increasing a cell's sensitivity to an exogenously administered apoptogenic stimulus than an outright pro-apoptotic response, whereas RBM10 overexpression has always been associated with a pro-apoptotic response. Just like the effect of RBM10 overexpression on TNF- α upregulation, this pro-apoptotic response shows a cell type-specificity. For instance, whereas in Jurkat cells apoptosis obviously occurred in the actual GFP-RBM10 fusion protein overexpressing population (since a gated GFP population was examined by flow cytometry) (Fig. 3), in MCF-7 cells many non-RBM10-overexpressing cells in the transfectant population were apoptotic (Fig. S2). The fact that many non-obviously transfected MCF-7 cells were undergoing apoptosis suggested that RBM10 overexpression was associated with the release of a soluble factor that promoted apoptosis within the population. The fact that some RBM10 overexpressing cells were also undergoing apoptosis suggested that this soluble factor could act in both an autocrine and a paracrine manner. Since sTNF- α upregulation was associated with RBM10 overexpression and sTNF- α is known to have the ability to induce apoptosis following binding to TNFR1, our experiments suggested that sTNF- α was at least one of the factors through which RBM10 functioned to regulate apoptosis. Indeed, downregulation of RBM10 not only inhibited TNF- α -mediated apoptosis in both Jurkat and MCF-7 cells, but also reduced expression of sTNF- α in the Jurkat cells (Figs 4 and S3). Our model is that cells that normally express a non-cytotoxic amount (pg/mL) of sTNF- α , can be induced—via upregulation of RBM10—to secrete greater quantities of sTNF- α , resulting in apoptosis in at least a subset of cells. The corollary is that inhibition of RBM10 decreases susceptibility to TNF- α -mediated apoptosis because the cells with reduced RBM10 also have less endogenous sTNF- α in their environment.

At this stage, our observations concerning RBM10, TNF and apoptosis are correlative, but they do demonstrate that RBM10 is involved in apoptosis regulation and that changes in RBM10 expression levels influence TNF- α expression levels. Since sTNF- α has a known pro-apoptotic capability, our study suggests that TNF- α is at least one mediator of RBM10-directed apoptosis. Current efforts in our lab are aimed at further defining the mechanistic relationship between RBM10 and TNF.

Since beginning the studies described in this manuscript, the first mRNA target for the rat orthologue of RBM10, S1-1, was identified—AT1, a G protein-coupled receptor involved in mediating smooth muscle cell proliferation. By binding to the 3'-UTR of AT1 mRNA, S1-1 served in a dual capacity to both stabilize the message and reduce the rate of transcription.²³ Interestingly, the downstream affect of this interaction was reduced proliferation and increased apoptosis.²³ Our work, described herein, is the first to show a functional role for human RBM10 in apoptosis modulation. RBM10 overexpression was associated with increased TNF- α mRNA and soluble protein expression, PS exposure on the outer cell membrane and decreased nuclear size, while RBM10 inhibition was associated with decreased TNF- α soluble protein expression and decreased PS exposure. It also demonstrated that, unlike S1-1 and AT1 in the smooth muscle cells, RBM10 can modulate apoptosis by a mechanism that involves increasing, as opposed to decreasing, transcription (of TNF- α), with no coordinated involvement of message stabilization. These results suggest that RBM10 uses different mechanisms to regulate the expression levels of different genes.

The involvement of the RBM10/RBM5 family of RNA binding proteins in multiple aspects of tumour necrosis factor signaling, including upregulation of TNF- α mRNA with an associated increase in sTNF- α protein (RBM10 and RBM5) (reported herein), splicing of caspase-2 pre-mRNA to generate the pro-apoptotic isoform (RBM5),⁴² regulation of FasR pre-mRNA splicing to generate the soluble anti-apoptotic isoform (RBM10? and RBM5)² and either blockage of TNF- α -mediated apoptosis in RBM5-null cells¹² or inhibition of TNF- α -mediated apoptosis in RBM10 KD cells (reported herein),



suggests that RBM10 and RBM5 are important players. Gaining a better understanding of the role played by both RBM10 and RBM5 in the regulation of these responses may contribute to the production of more targeted and efficacious TNF-based therapies for a wide spectrum of diseases, including cancer, diabetes and various autoimmune diseases.

Author Contributions

Conceived and designed the experiments: L.C.S. Analysed the data: K.W., M.L.B., L.C.S. Wrote the first draft of the manuscript: L.C.S. Made the critical revisions and approved the final version: L.C.S. All authors reviewed and approved of the final manuscript.

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Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this

section. The external blind peer reviewers report no conflicts of interest.

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Supplementary Materials

Table S1. Sequence information for PCR primers and RBM10 knockdown targets.

	Sequence 5'-3'
Subcloning primers	
RBM10F ² .HindIII	CCCAAGCTTTGGCTGGGAAGTGAAACGGA
RBM10Rb ¹ .EcoRI	GGAATTCGCCATCCAACACTCTGTCCT
RBM10Fe.EcoRI	GAATTCGGGTGGCTGGGAAGTGAAACGGA
RBM10R ² .HindIII	GCCGCAAGCTTCTGGGCCTCGTTGAAGCG
Overexpression confirmation primers	
RBM10F	TGGCTGGGAAGTGAAACGGA
RBM10Rs	GGATGTTGAGGGAGTGCTGA
RBM10e4R	CAGCATCCTCAGCATGACGA
RBM10v1/v2R	GTGTAGCGTCCTGCAAGTGA
Array validation primers	
qTNFf2	GGAGAAGGGTGACCGACTCA
qTNFr2	TGCCCAGACTCGGCAAAG
qLTAf	TGTTGGCCTCACACCTTCAG
qLTAr	TGCTGTGGGCAAGATGCAT
qTRAILf	AATCATCAAGGAGTGGGCATTG
qTRAILr	ATGACCAGTTCACCATTCTCAA
KD target sequence ID	
29	GCCTTCGTGCGAGTTTAGTCACTTGCAGGA
30	AGTCACTTGCAGGACGCTACACGATGGAT

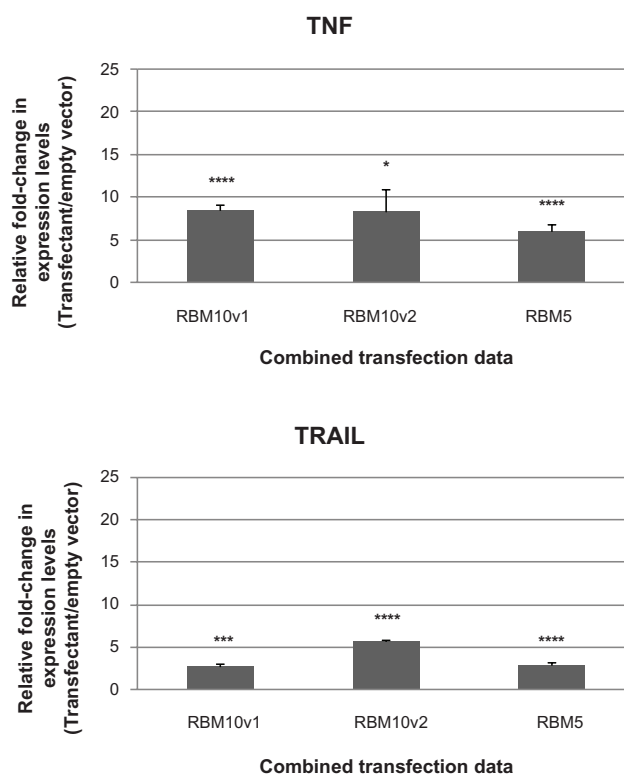


Figure S1. Validation of the PCR Array expression profiles in RBM5 or RBM10 overexpressing MCF-7 cells. The same mRNA from each of the transfections used in the PCR Array studies was re-examined by real-time qPCR. Relative expression was determined by extrapolation of the Ct value (the number of cycles required to reach a relative threshold level) for the cytokine or the control ribosomal protein S28 in pcDNA3 or RBM5 or RBM10 from either the cytokine or S28 standard curve, respectively. Cytokine expression was normalized to S28 expression. Data are presented as the relative fold-change in expression levels, transfectant to empty vector, combining transfections for each RBM. Error bars represent standard error of the mean for six (RBM10v1 or RBM10v2) or nine (RBM5) replicates.

Notes: Significances were calculated using a Student's unpaired *t*-test. **P* < 0.05, ****P* ≤ 0.001, *****P* ≤ 0.0001.

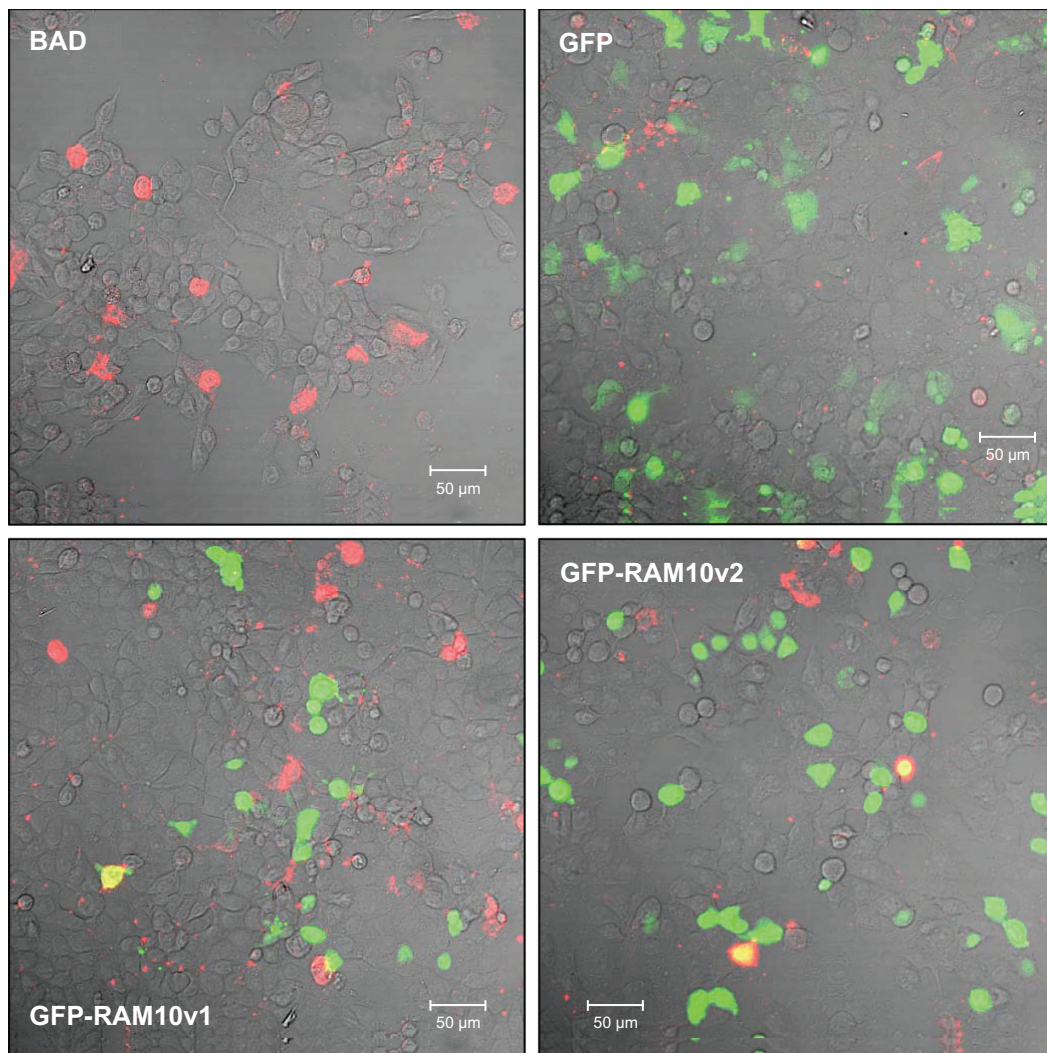


Figure S2. Phosphatidyl serine expression in MCF-7 cells. Cells were transiently transfected with an untagged BAD expression construct, pEGFP, or the GFP-tagged RBM10-fusion constructs, and 48 hours following transfection the cells were stained with Alexa Fluor 647-conjugated Annexin-V. Annexin-V bound PS was visualized using fluorescence microscopy. GFP transfected cells are green and the cells with exposed PS are red.

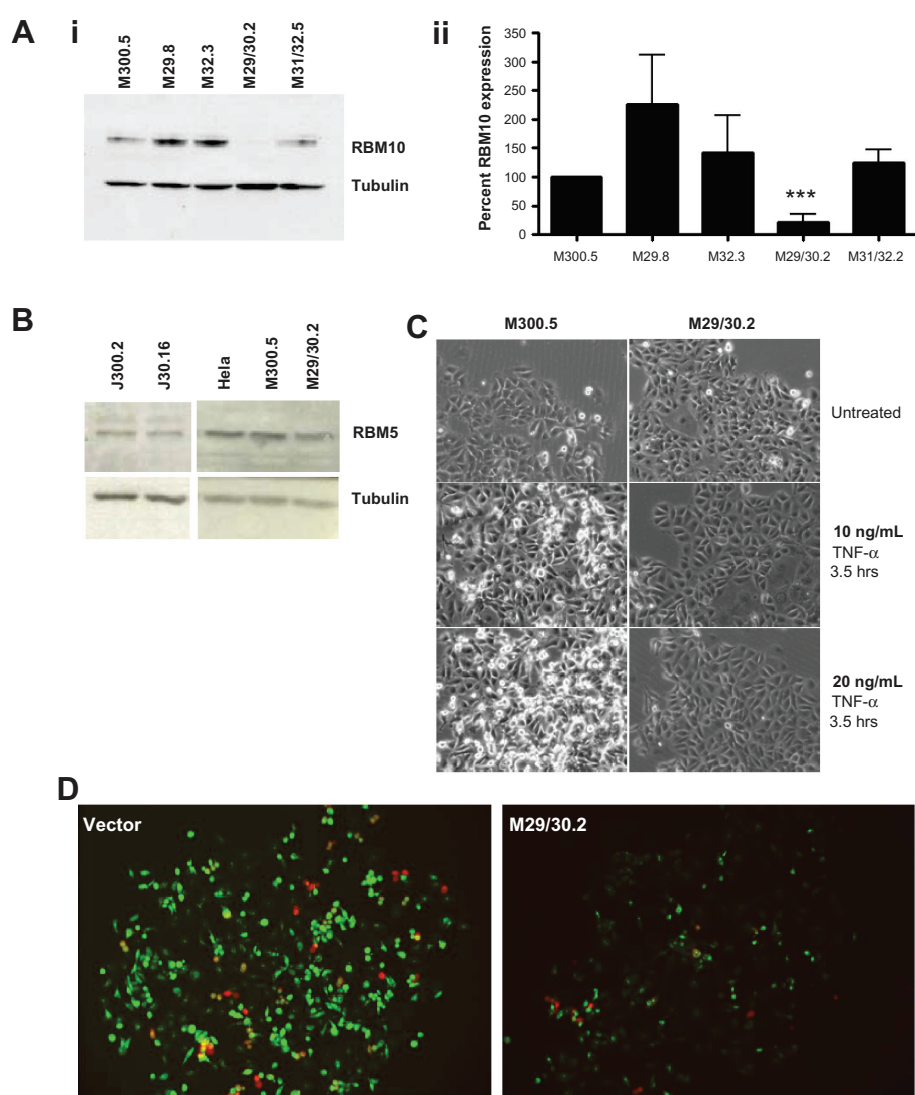


Figure S3. Inhibition of RBM10 expression in MCF-15 cells. **(Ai)** Western blot of RBM10 protein expression in four RBM10 KD clones, and **(Aii)** associated densitometric measurements, indicating that only clone M20/30.2 had significantly reduced RBM10 expression levels, compared to the non-effective GFP hairpin negative control. $***P = 0.004$ by a Student's unpaired *t*-test. **(B)** Western blot showing unaltered RBM5 expression by RBM10 KD clones, with HeLa cell extract as a positive control. **(C)** Phase contrast microscopy comparing MCF-7 morphology between control and RBM10 KD clones untreated, or treated with 10 or 20 ng/ml TNF- α and 10 μ g/ml cycloheximide, for the times indicated. Magnification 100 \times . **(D)** Fluorescence microscopy showing MCF-7 cells stained with 1 μ g/ml ethidium bromide and 0.1 μ g/ml acridine orange after treatment with 80 ng/mL TNF- α and 10 μ g/mL cycloheximide for 16 hours. Bright green cells are in early apoptosis whereas orange/red cells are in late apoptosis/necrosis. Magnification 100 \times .

Table S2. individual arrays.xlsx.

Table S3. grouped arrays.xlsx.



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