EXPLORING RBM6 OVEREXPRESSION: IMPACT ON CELL DIVISION AND GENE SPLICING IN HELA CELLS

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ACKNOWLEDGEMENT:

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This project has been a significant learning opportunity, and I am hopeful that my contributions have advanced our understanding of the intrinsic RBM6. I am grateful for this incredible experience and the opportunity to work alongside such dedicated individuals.

Thank you.

ABSTRACT:

The RNA-binding motif protein 6 (RBM6) serves as an alternative splicing factor and tumor suppressor gene initially identified as a gene mapped to a lung cancer tumor suppressor gene locus on chromosome 3p21. Sharing structural similarities with RBM5 and RBM10, such as their two RNA recognition motifs (RRM) domains involved in posttranscriptional gene expression, two zinc finger-like regions, a G-patch and an OCRE domain, as well as a YjbI homology domain not found in RBM5 or RBM10. RBM6 is a splicing factor or interacts with other splicing factors based on its localization to IGCs (interchromatin granule clusters), also known as nuclear speckles, nuclear domains enriched in pre-mRNA splicing factors located in the nucleoplasm of mammalian cells. Changes in RBM6 expression have been reported to alter alternative splicing, to alter the cloning efficiency of HeLa cells, and RBM6 was shown to bind preferentially to a consensus sequence. For example, one study showed that decreased expression of RBM6 caused increased exclusion of NUMB exon 9, the opposite of what is observed following depletion of RBM10. It is notable that these effects could only be observed when RBM6 was stably knocked down in cell lines. While changes in RBM6 expression impact pre-mRNA splicing and cell proliferation, its precise mechanism is unclear. We are creating RBM6 cell lines that overproduce the protein in HeLa cells, as well as studying the effect of transient expression of normal and mutated RBM6 proteins. We will determine if overexpression of RBM6 will promote, and whether this correlates with changes in the rate of cell division. Using mutations in the protein we will investigate how the overexpression of RBM6 promotes the exclusion of the gene NUMB exon 9 and alters the splicing of other pro-apoptotic genes.

KEY WORDS: RBM6, RBM, RNA, HeLa, Alternative Splicing, Cell Proliferation

INTRODUCTION:

RNA-Binding Motif (RBM) Proteins: Regulators of mRNA Processing and Translation

RNA-binding motif (RBM) proteins are a class protein that contain RNA-recognition motifs (RRMs), RNA-binding domains, and ribonucleoprotein motifs (Li et al. 2021). The RBM family proteins are a subgroup of the RNA-binding proteins (RBPs) which are commonly involved in RNA splicing, localization, translation, and transportation (Li et al. 2021). Like RBPs, the RBM proteins are also involved in biological activities, such as pre-mRNA splicing, RNA stability, mRNA translation and can therefore regulate alternative splicing by binding to the exon/intron region near the splice site of mRNA, in the case of RBM6, binding to the exon region near the splice site. (Li et al. 2021). RNA-binding motif (RBM) proteins make up a large family of RNA-binding proteins that all regulate mRNA processing and translation and have therefore been aberrantly expressed in cancer, and like RBM6, can serve as potential alternative splicing factors (Machour et al. 2020). The RNA-binding motif protein 6 (RBM6) is a known alternative splicing factor that is known to act as a tumor suppressor gene (Machour et al. 2020).

RBM6, was initially identified as a gene that mapped to a lung cancer tumor suppressor gene locus on chromosome 3p21 (Gure et al. 1998) and as one of two genes located at the 3p21.3 region frequently deleted in non-small cell lung cancer (the other being RBM5) (Timmer et al. 1999). As a nuclear protein that forms foci corresponding to splicing speckles (Machour et al. 2020), RBM6 is structurally very similar to RBM5 and RBM10, sharing 30-50% of amino acid sequences (Bechara et al., 2013) including two RRM domains, an OCRE domain and a G patch. RBM6 regulates gene expression and alternative splicing of genes involved in processes like tumorigenesis (Machour et al. 2020) and therefore, RBM6 is a splicing factor or interacts with other splicing factors based on its localization to IGCs (interchromatin granule clusters) (Heath et al. 2010).

Sequences of the N-terminal region of the protein was shown to bind to other RBM6 molecules while the C-terminal region containing the G patch domain was sufficient to localize the protein to speckles. (Heath et al 2010). Depletion of RBM6 altered splicing of numerous genes typically causing increased skipping of cassette exons (Bechara et al. 2013). Using CLIP, RBM6 was demonstrated to bind to a consensus sequence. Depletion of RBM6 had a similar effect in the splicing of specific proapoptotic genes as RBM5 and the opposite of RBM10 (Bechara et al. 2013) and is therefore presumed that increased expression of RBM6 caused increased inclusion of NUMB exon 9. RMB6 was identified as a regulator of homologous recombination (HR) repair of DNA double-stranded breaks (Machour et al. 2020). In an experiment conducted, it was found that RBM6 “regulates alternative splicing-coupled nonstop-decay of the positive HR regulator Fe65/APBB1” (Machour et al. 2020). A reduction in RMB6 thus leads to the reduction of Fe65 protein levels, damaging homologous recombination as well as DNA double-stranded breaks (Machour et al. 2020). While RBM6 is downregulated in laryngocarcinoma tissues and cell lines (Wang et al. 2019), RBM6 overexpression repressed the laryngocarcinoma tumor growth (Wang et al. 2019).

These previous studies analyzing RBM6 have provided some insight into its function but raise more questions than they have provided answers. RBM6 is widely believed to function as a tumor suppressor but prior experiments show that depletion of RBM6 in HeLa cells causes a decreased ability of the cells to proliferate (Bechara et al. 2013). RBM6 appears to regulate the same exons as other tumor suppressor genes while sometimes causing the same effect, and sometimes causing the opposite effect. Through our studies, we will continue to investigate RBM6 as an alternative splicing factor to better understand how its expression affects cellular proliferation in hopes of explaining the apparent inconsistencies in our current understanding. We hope this can lead to significant breakthroughs in hopes of curing some cancers related to RBM6. Creating RBM6 cell lines that overproduce the protein in HeLa cells, we therefore unveil the mystery if the overexpression of RBM6 promotes the exclusion of the gene NUMB exon 9 most commonly found in nucleosome speckles in hopes of uncovering the function of RBM6 in alternative splicing. We will do so through the use of cell culture experiments, protein analyses, and protein assays, a process similarly seen in studies that analyzed the effect of the depletion of RBM6 in the gene NUMB exon 9 (Bechara et al. 2013).

RBM6 Structure

RBM6, an RNA-binding-motif protein part of the RNA-binding-protein family, mapped to the 3p21.3 region contains two RNA recognition motifs (RRMs), one Zinc finger domain, a G-patch and OCRE domain, all shown to be involved in the regulation of alternative splicing (Machour et al. 2021) (Figure 1). In addition to having these domains and motifs, it has been shown that RBM6 is a nuclear protein that forms foci corresponding to splicing speckles, and therefore its regulation of gene expression and alternative splicing influence cellular processes like tumorigenesis (Machour et al. 2021). Typically mutated in human cancer, RBM6 has been identified as a novel regulator of homologous recombination (HR) repair of DNA double-strand breaks, regulating nonstop-decay of a positive HR regulator, Fe65/APBB1 (Machour et al. 2021). Studies therefore elucidated that RBM6 knockdown leads to a severe reduction in Fe65 protein levels and consequently impairs HR of DSBs (Machour et al. 2021). While RBM6 is one of the man RNA-binding proteins in the family, it also shares characteristics with other RBM proteins such as RBM5 and RBM10, all which also regulate alternative splicing and cell proliferation.

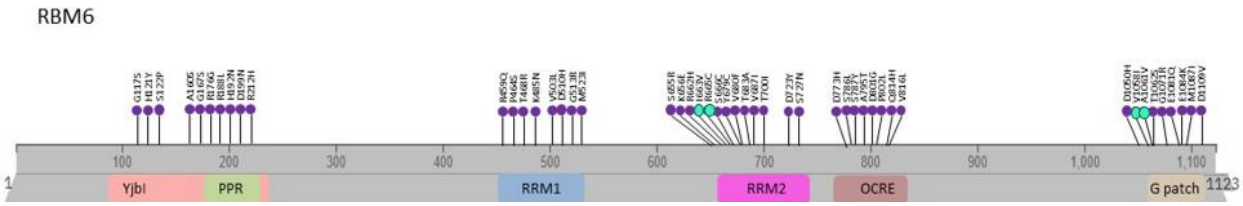

Figure 1: RBM6 Structure: This unique structure of RBM6 consists of an OCRE domain, a G patch domain, and RRM1 and RRM2 domain, a PPR domain, and a Yjbl domain distinct to only RBM6. Each lollipop reflects different mutations in RBM6 which the turquoise lollipops reflect the different mutations being studied in this paper (I663V, S665C, V1058I, A1061V).

Comparative Analysis of RBM6 with RBM10 and RBM5

RBM6 is part of the subgroup family of RNA-binding proteins (RBPs) which also include the RNA-binding motif protein 5 (RBM5) and the RNA-binding motif protein 10 (RBM10). RBM6, RBM5 and RBM10, all share a similar domain organization and exhibit high sequence similarity with RBM6 (30% and 50%, respectively) (Soni et al. 2023). This is surprising, because RBM5 and RBM6 would be expected to be very similar, since they are located in tandem on chromosome 3 and are likely the result of a recent gene duplication. Studies have also shown that the chromosomal region (3p21.3) comprising the RBM6 and RBM5 genes is frequently deleted in genomes of heavy smokers and lung cancer patients (Soni et al. 2023). In addition to this founding, RBM6, RBM5, and RBM10 share an OCRE domain, a G patch domain, and RRM1 and RRM2 domain (Figure 2) and are all involved in alternative splicing of regulation of NUMB pre-mRNA. NUMB encodes an inhibitor of NOTCH pathway, which is hyper-activated in ≈40% of human lung cancers and linked to breast cancer, making inhibition of the NOTCH pathway a promising approach for cancer therapy (Soni et al. 2023). Altogether, analyzing the role of RBM6 overexpression in HeLa cell and its mutants can help elucidate the differences between RBM6, 5, and 10, despite their high similarities.

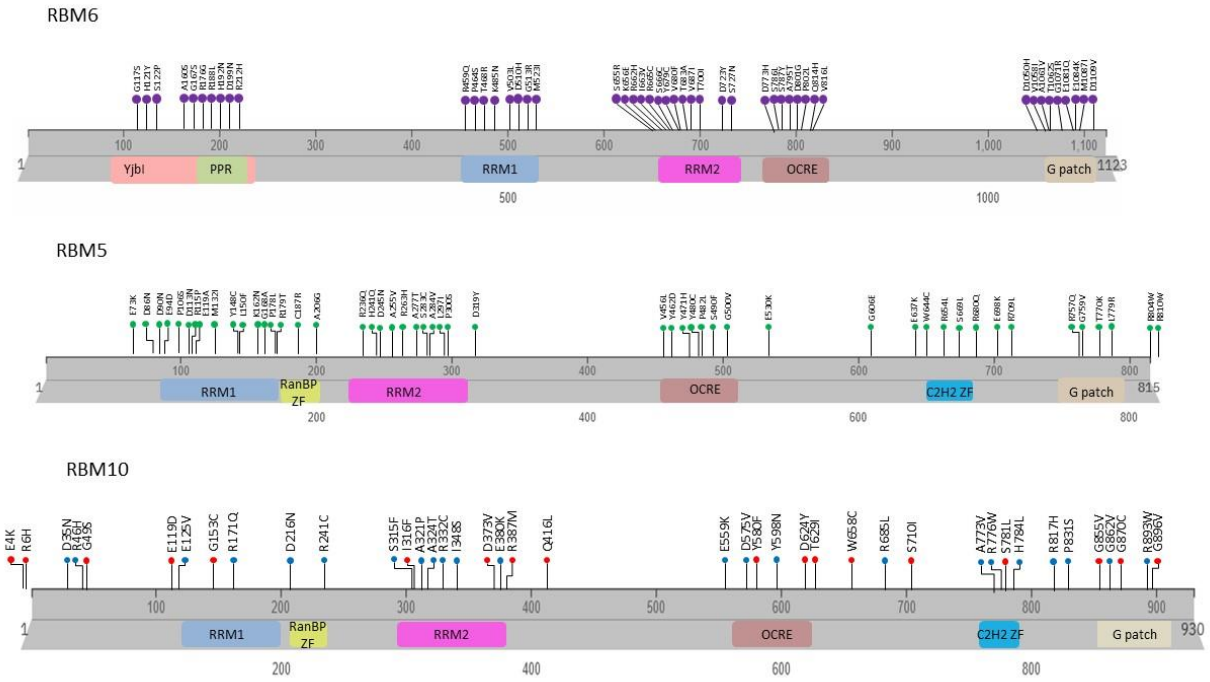

Figure 2: RBM6, RBM5, and RBM10 Structure: RBM6 , RBM5, and RBM10 together share an OCRE domain, a G patch domain, and RRM1 and RRM2 domain. The PPR domain, and a Yjbl domain are distinct to only RBM6 and RBM10 and RBM5 share a C2H2 ZF domain and RanBP ZF domain. Each lollipop for the plasmids RBM5, 6 , and 10 reflect different mutations being studies right now, highlighting the key differences and similarities within each RBM protein.

NUMB Exon 9 as an Endocytic Adaptor Protein and Tumor Suppressor

The endocytic adaptor protein NUMB acts as a tumor suppressor through downregulation of oncogenic pathways in multiple cancer types, and the identification of splicing alterations, giving rise to changes in NUMB protein isoform expression, indicating that NUMB may promote tumorigenesis (Zhang et al. 2022). Studies have shown that NUMB exon 9 inclusion, which results in production of a protein isoform with an additional 49 amino acids, NUMB isoform 10, is a feature of multiple cancer types, and therefore specific deletion of exon 9-included NUMB isoforms cell growth (Zhang et al. 2022). According to the studies by Bechara *et al.* while exon 9 skipping generates a repressor of the pathway and of cell proliferation, exon 9 inclusion correlates with reduced NUMB protein levels and activation of the NOTCH pathway, an important regulator of cell proliferation. This is why changes in NUMB exon 9 inclusion are among the most frequent tumor-associated alternative splicing changes observed in cancers like lung cancers (Bechara et al. 2013). This study further elucidated that increased levels of NUMB exon 9 inclusion were found to be frequent in non- small lung cancer tumors, correlating with higher NOTCH activity, suggesting that the modulation of NUMB alternative splicing could have therapeutic effects in cancer treatment and that knockdown of RBM6 leads to exclusion of exon 9, resulting in increased skipping of exon 9 of the NUMB gene (Figure 3), suggesting that

overexpression of RBM6 in HeLa cells would therefore lead to increased inclusion of NUMB exon 9 (Bechara et al. 2013)

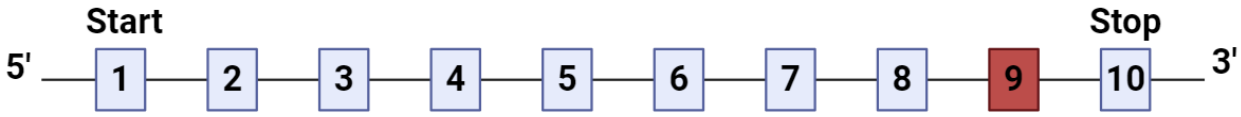

Figure 3: NUMB Construct: Construct of NUMB gene highlighting Exon 9

Investigating RBM6 Functionality: Insights from Bechara et al. 2013

According to Bechara, RBM6 depletion led to a decreased clonogenic capacity, meaning that cells were unable to form colonies, indicating that the protein RBM6 could play a role in promoting the ability of cell proliferation (Bechara et al. 2013). Through microassay analysis this study also highlights how the depletion of RBM6 promoted exclusion of cassette exons, promoting the inclusion of only 29% of its target cassette exons, and therefore may act as a splicing activator (Bechara et al. 2013). This result distinguished the splicing regulation mechanism for RBM6 to be different compared to RBM5 and 10, which were predicted to mainly have repressive activities on splicing, and therefore suggesting that RBM6 would promote the exclusion of the majority (71%) of its target cassette exons (Bechara et al. 2013). Lastly, to further investigate the involvement of RBM6 in NUMB alternative splicing regulation HeLa cells depleted of RBM6 were transfected expressing cDNAs encoding one of the two NUMB protein isoforms, NUMB-PRRS or NUMB-PRRL (Bechara et al. 2013), with isoform NUMB-PRRS generated upon exon 9 skipping and NUMB-PRRL being the isoform formed upon exon 9 inclusion (Bechara et al. 2013). The results of this study therefore showed that upon RBM6 depletion, the predominant isoform formed was NUMB-PRRL, suggesting the inclusion of exon 9, tied to increased cell proliferation, suggesting that the effects of RBM6 depletion on cell proliferation could be to altered expression of NUMB isoforms (Bechara et al. 2013).

Exploring RBM6 Mutations in HEK 293 Cells: Implications and Insights

Building on the insights from Bechara et al. (2013), which demonstrated RBM6's crucial role in promoting cell proliferation and regulating alternative splicing, particularly of the NUMB gene, our study further delves into the functional implications of RBM6 mutations. By exploring these mutations in the widely used HEK 293 cell line, we aim to elucidate how specific alterations in RBM6, such as the I663V, V1058I, and A1061V mutations, impact its function and potentially contribute to cancer development, as observed in somatic mutations reported in the COSMIC database (Tate et al. 2019). This approach allows us to deepen our understanding of RBM6's role in cellular processes and its potential significance in cancer biology.

The HEK293 human cell lineage is widely used in cell biology and biotechnology. The human embryonic kidney (HEK) 293 cell line is used in experiments such as transient transfections, and is broadly used for biomedical research for (Lin et al. 2014). First derived in 1973 from the kidney of an aborted human embryo of unknown parenthood by transformation with sheared Adenovirus 5 DNA (Graham et al 1977), the transfection of RBM6 mutations can reveal how the

function of the RBM6 protein is altered by the mutations. Mutations were selected using two criteria. First, mutations were selected from those identified as somatic mutations in cancerous tumors that had been reported to the COSMIC database (Tate et al 2019). Second, mutations were selected because the altered amino acids in conserved domains as discussed above. The I663V mutation is located in RRM2, while V1058I and A1061V are in the G patch domain.

Expressing these proteins (I663V, V1058I, A1061V) in HEK293 cells can provide information about how the overexpression of RBM6 alters functions related to cancer formation. Because RBM6 is expected to promote the exclusion of the gene NUMB exon 9 and alters the splicing of other pro-apoptotic genes. Through the expression of normal and mutated RBM6 (mutations include I663V, V1058I, A1061V, and R665C), we seek to determine if the mutations alter the ability of RBM6 to regulate NUMB splicing and uncover the mechanisms behind RBM6's role in alternative splicing. We can also determine if the mutations change the affect RBM6 has on cell division and apoptosis, potentially offering new insights into cancer treatment strategies. While the mutations R665C and I663V are found in the RRM2 domain of RBM6, A1061V and V1058I are found in the G-Patch domain of RBM6. Previous studies have shown that the RRM2 domain of RBM6 forms a hydrophobic binding pocket analogous to the UHM of RBM17, which leads to further investigation of the mutations in the RRM2 domain of RBM6 such as I663V and R665C to understand if RBM6 can act as a competitive inhibitor against RBM17 on SF3b155. The mutations V1058I and A1061V in the G-patch domain of RBM6 in unison with I663V and R665C can together be studied to understand if the overexpression of RBM6 promotes the exclusion of the gene NUMB exon 9, altering the splicing of other pro-apoptotic genes.

RESULTS:

Creating Expression Vectors for Genetic Engineering

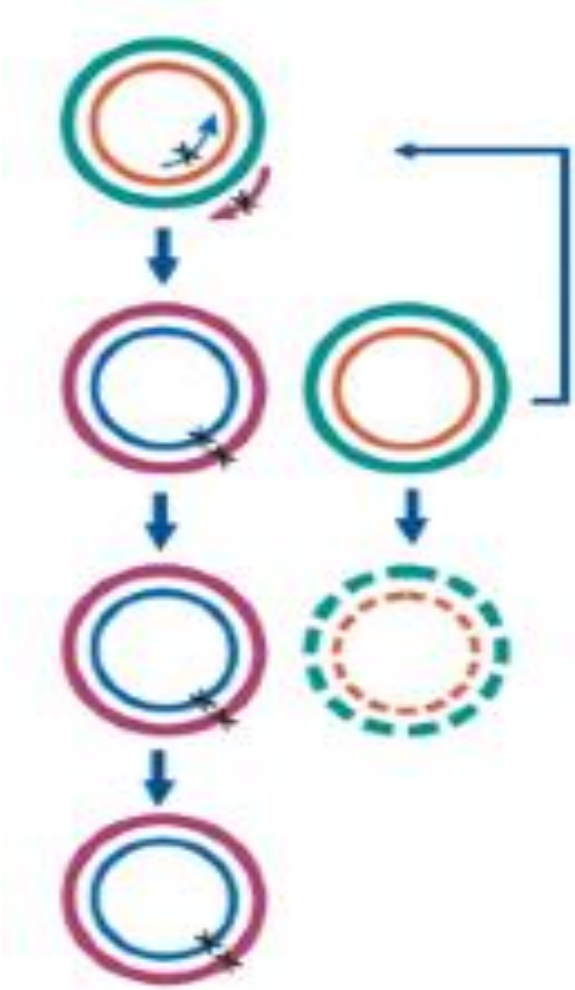

Figure 4B: Mutation Expression Vector: Figure demonstrating the insertion of mutations in plasmid. In this case, the RBM6 mutations I663V, V1058I, R665C, and A1061V were created using this process, inserting the mutations into the RBM6+ cDNA.

Expression vectors are used to study the expression of specific genes. The creation of expression vectors consists of cDNA being synthesized and cloned into a plasmid containing sequences needed to express an mRNA. Mutations may be introduced into the sequence (Figure 4B), and the use of PCR cloning fuses a tag to the C- or N-terminus of the protein and adds other sequences required for expression and selection. When the resulting plasmid is introduced to mammalian cells, a tagged protein will be produced. Using the tag fused to sequences of RBM6 and its mutations, we can isolate intact complexes that contain the fused protein to identify the protein and RNA components found in the complex and any changes caused by mutations. Figure 4A demonstrates the conceptual procedure of the creation of the RBM6 expression vector used to overexpress RBM6 in HeLa cells in order to determine its effect in cell proliferation and alternative splicing. The RBM6 expression vectors were built by using RT-PCR to amplify the RBM6 sequences (Figure 5A) and these were inserted into a plasmid with intron and poly(A) sequences. In the case of RBM6 mutations, the sequence was changed to contain the specific mutation. We used PCR based cloning to fuse a myc tag to the N-terminus of RBM6 along with an SV40 early promoter. (Figure 5B) The same process was used for the normal and mutated RBM6 proteins.

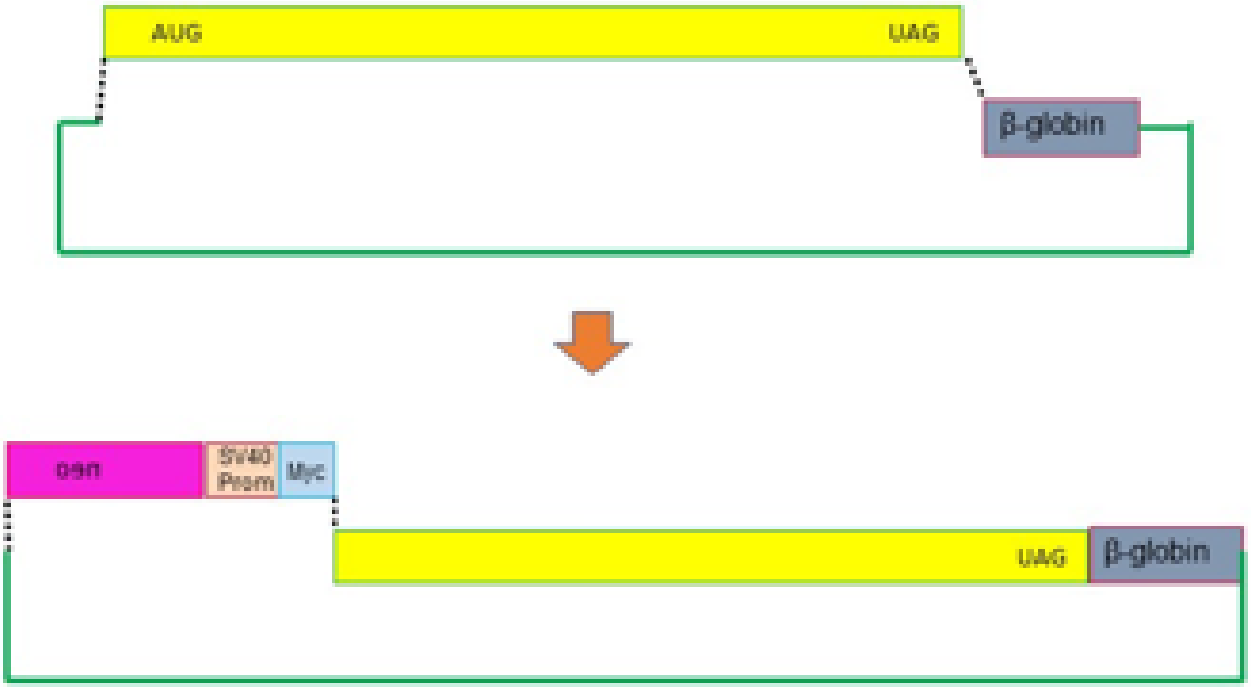

Figure 4A: RBM6 Expression Vector: RBM6 expression vector includes the addition of the neo-SV40 promoter and Myc-tag at N-terminus and addition of β-globin at C-terminus. RBM6 tagged vector is then used to transfect plasmid into HeLa cells.

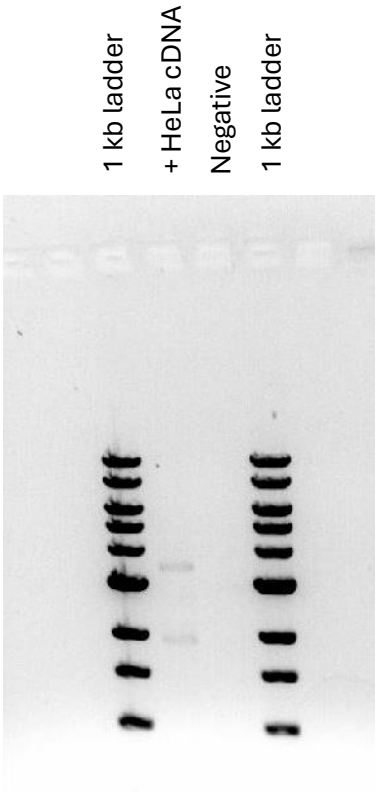

Figure 5A: RT-PCR of Human RBM6 protein. RT-PCR was performed using HeLa cell cDNA with Q5 DNA Polymerase in standard conditions. The size of the products is consistent with the size predicted for RBM6 (3436 bp). The product also yielded a smaller nonspecific product near the 2000 bp band. Samples were run on 0.7% Agarose gel using 1kb ladders.

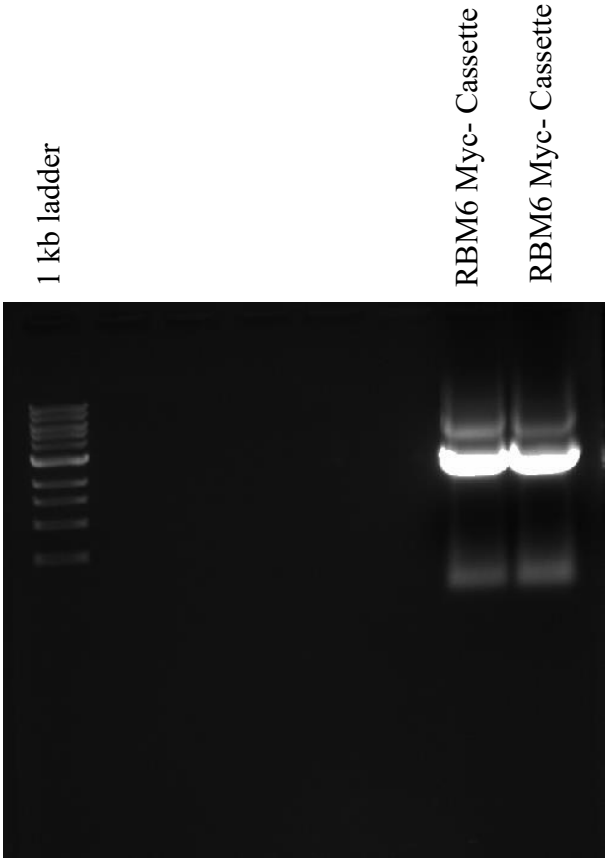

Figure 5B: PCR with neo-SV40 Pr-Myc: Figure 5A reflects the analytical gel from a neo-SV40 Pr-Myc PCR of the protein RBM6. The N-terminal Myc tag fused properly with RBM6 and a bright band with a high concentration is seen near the 3.4 kbp region. Gel will then be purified from Agarose gel to be used in megaprimer PCR.

Studying the Function of RBM6 in Human Hela Cell Lines

The HeLa cell line, first described in 1952 (Gey et al 1952), are the single most utilized cell line for the study of mammalian cell and molecular biology (reviewed in Landry et al 2013) HeLa cell lines were made by transfection to establish stable cell lines followed by G418 selection for the tagged-RBM6 expression vector. To create an expression vector of RBM6 with the neomycin resistance gene (neo) , creation of RBM6 cDNA using neo-SV40 promoter gene is used with the SV40 promoter driving the expression of RBM6, while the neo gene would confer resistance to the antibiotic G418 (geneticin). Upon purifying plasmid DNA for transfection with Midi Kit, ethanol precipitation of plasmid was done to prepare for co-transfection of RBM6+. When transfecting cells with this vector, along with a plasmid containing a gene of interest, the use of G418 selection would allow for the isolation of cells that have successfully taken up and expressed the RBM6 expression vector. G418 is an aminoglycoside antibiotic that kills cells that do not express the neo gene, providing a means to select for cells that have incorporated the vector and are expressing RBM6. This selection process ensures that only cells expressing the RBM6 gene survive, allowing for the study of RBM6's effects in a controlled cellular environment.

Four clones of growing cells were obtained and three cell lines (HeLa RBM6-1, HeLa RBM6-3, and HeLa RBM6-4) continued to grow and were used to determine if stable RBM6 protein expression was obtained. Cells were grown and were harvested and because unequal numbers of cells were used, a Bradford protein assay was ran to determine the concentration of protein in each sample, so when a western blot was run we would have with equal amounts of cells from each cell line. The Bradford assay's sensitivity allows for the detection of even low concentrations of protein, which is important for analyzing proteins that may be expressed at varying levels in the RBM6+ HeLa Cell Lines, ensuring accurate protein quantification of RBM6 protein in each sample, crucial for loading equal amount of protein onto a gel, enabling precise Western Blot analysis to compare RBM6 expression levels between different samples (Figure 6A) The amount of protein in each sample was determined by comparison of the value determined from the assay to the corresponding value from a known BSA standard curve. (Figure 6B).

SAMPLE ID	ng/uL	1 (ABS)
I	25	0.003
H	125	0.007
G	250	0.018
F	500	0.021
E	750	0.023
D	1000	0.026
C	1500	0.036
B	2000	0.041
A	5000	0.101
HELA RBM6 1	1715	0.042
HELA RBM6 3	3915	0.086
HELA RBM6 4	3315	0.074
HELA RBM6 +	3265	0.073

Figure 6A: Bradford Assay Table: Bradford protein assay using Bradford Reagent was used and absorption of samples A-I were measured before determining absorption of harvest cell lines (HeLa RBM6-1, HeLa RBM6-3, HeLa RBM6-4, and HeLa RBM6 +). Concentration in ng/µL) was calculated using regression line equation.

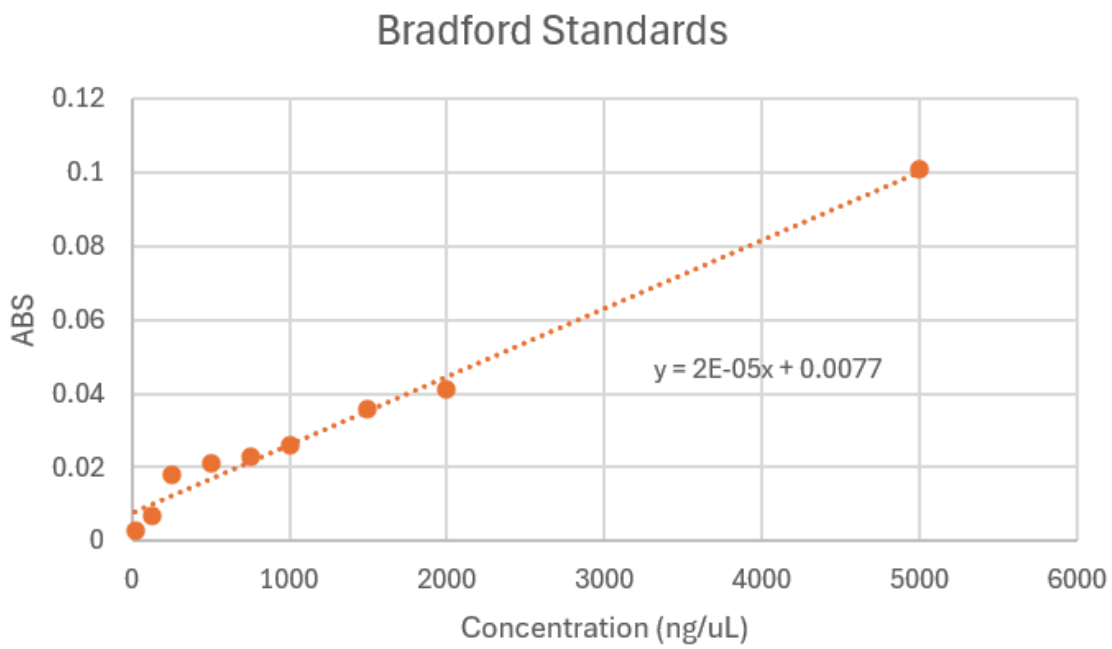

Figure 6B: Bradford Assay Regression Line: Bradford Protein Assay using Bradford Reagent showing the absorption of samples A-I as well as harvested cell lines (HeLa RBM6-1, HeLa RBM6-3, HeLa RBM6-4, and HeLa RBM6 +) was plotted into regression line where the equation y=2E-05x + 0.0077 was determined to calculate the concentration in ng/µL) for the harvested samples.

Prior to transfection, purified plasmid RBM6+ was digested with the restriction enzyme PvuI-HF in order to linearize the DNA without digesting any of the sequences needed to make G-418 or RBM6. After the enzyme was inactivated the DNA was prepared for transfection by precipitating it with ethanol. 10µg of fusion plasmid DNA was transfected into HeLa cells after resuspending the DNA in 450µl water, adding 50µl of freshly dissolved 2.5M CaCl2 for each plate and mixed with 500µl of 2xHBS at a pH = 7.05 per plate, letting mixture stand for 30-45 minutes before plating. After 24 hours the media was changed to contain G-418. This media was changed regularly until colonies of resistant cells could be seen. Clones were picked and grown on new plates until enough material was obtained to allow for the cells to be preserved and for protein to be harvested from the cells for study.

Upon 48 hours, the cells were harvested by aspirating the media from the plates, adding 5 ml of PBS and aspirating the plates from PBS as well and then using 1ml of RIPA-protease inhibitor mixture (990 µl RIPA and 10 µl of protease inhibitor) before transferring to a 1.5 ml microfuge tube, placed on the nutator at 4°C for 30 minutes, centrifuged for 20 min max speed at 4°C and finally the supernatant was transferred to a new 1.5 ml microfuge tube and stored at -80°C until Western Blot was conducted.

As stated in the methodology section, Western Blot under standard conditions was conducted for the harvested plasmid cell lines RBM6-1, RBM6-3, RBM6-4, and the negative control (Figure 7).

Figure 7 demonstrates the unsuccessful expression of harvested plasmid cell lines RBM6-1, RBM6-3, RBM6-4 in HeLa cells with residual bands showing on RBM6-1. Because the use of G418 selection process ensures that only cells expressing the RBM6 gene survive, allowing for the study of RBM6's effects in a controlled cellular environment, the presence of cells in each cell line (RBM6-1, RBM6-3, RBM6-4) demonstrates that RBM6 is expressed and therefore expression of cell lines in Western Blot should reflect a band at about ~200 kDa. Unfortunately, this is not what is shown. Western Blot of harvested plasmid cell lines RBM6-1, RBM6-3, RBM6-4 in HeLa cells will be redone in hopes of expression verification occurring. Success of Western Blot of harvested plasmid cell lines RBM6-1, RBM6-3, RBM6-4 in HeLa cells will therefore allow the continuous investigation of the overexpression of RBM6 and its correlation with changes in the rate of cell division in order to determine how this alters the splicing of other pro-apoptotic genes.

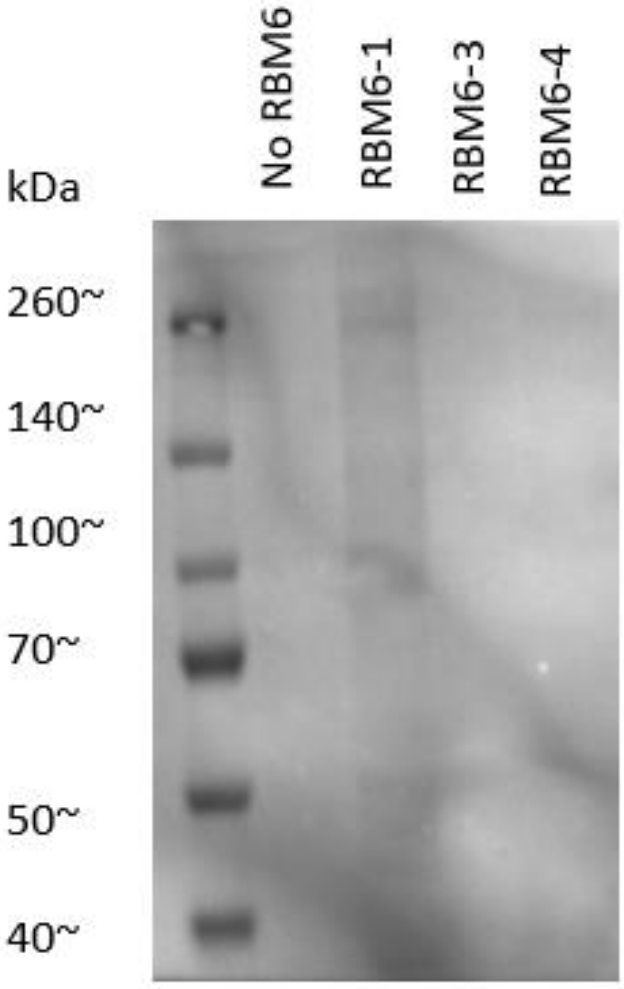

Figure 7: RBM6 HeLa Western Blot: Western Blot of harvested plasmid cell lines RBM6-1, RBM6-3, RBM6-4 in HeLa cells. Verification of the Co-transfected RBM6+ G148 selected HeLa cells show inconclusive results and failed to determine the expression of stable cell lines through the analysis of Western Blot.

Although all harvested cell lines showed resistance to G418 selection, only the RBM6-1 cell line exhibited unexpected "bands" that are not fully understood and do not yield definitive results. Further investigation and analysis, as well as completion of Western Blotting, are necessary to determine the expression of RBM6+ G418 in HeLa cells.

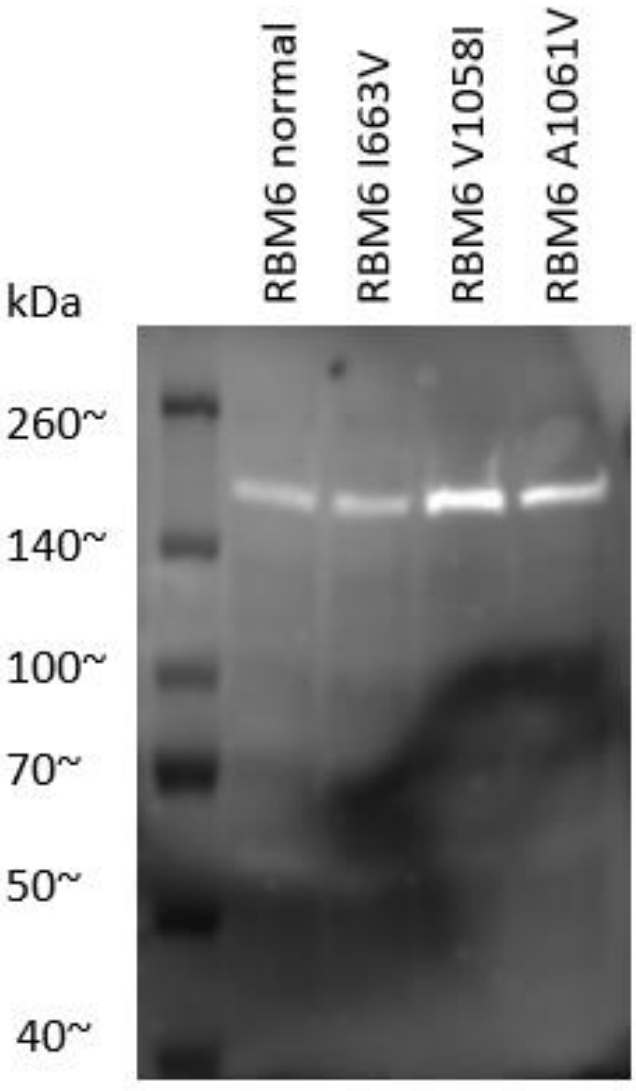

Figure 8: Western Blot of RBM6+ and Mutations: Western Blot of harvested RBM6+ and RBM6 mutations I663V, V1058I, and A1061V in HEK 293 cells. RBM6 V1058I shows strongest intensity of RBM6 plasmid compared to RBM6+, I663V, and A1061V with RBM6+ showing the least intensity. Expression of plasmids at the same molecular weight still supports the transfection of all plasmids into HEK 293 cells.

The first step in analyzing the effect of mutations is to determine if the mutated protein is stable when produced in 293 cells. RBM6+ and RBM6 mutations I663V, V1058I, and A1061V were transfected into HEK 293 cells through Expression of Transfected Gene Fusions using 10µl of fusion plasmid DNA, adding 50µl of freshly dissolved 2.5M CaCl2 for each plate and mixed with 500µl of 2xHBS at a pH = 7.05 per plate, letting mixture stand for 30-45 minutes before plating. Upon 48 hours, the cells were harvested by aspirating the media from the plates, adding 5 ml of PBS and aspirating the plates from PBS as well and then using 1ml of RIPA-protease inhibitor mixture (990 µl RIPA buffer and 10 µl of protease inhibitor cocktail) before transferring to a 1.5 ml microfuge tube, placed on the nutator at 4°C for 30 minutes, centrifuged for 20 min max speed at 4°C and finally the supernatant was transferred to a new 1.5 ml microfuge tube and stored at -80°C until Western Blot was conducted. As stated in the methodology section, Western Blot under standard conditions was conducted for the harvested plasmids RBM6+ and RBM6 mutations I663V, V1058I, and A1061V (Figure 8).

Successful Western Blot demonstrates successful expression of RBM6+ and RBM6 mutations I663V, V1058I, and A1061V about ~200 kDA. Expression of RBM6+ and RBM6 mutations I663V, V1058I, and A1061V allows possible co-transfection of RBM6+ and RBM6 mutations I663V, V1058I, and A1061V into NUMB-exon9 construct to investigate the effect of the overexpression of RBM6 and if it promotes the exclusion of the gene NUMB exon 9 and alters the splicing of other pro-apoptotic genes.

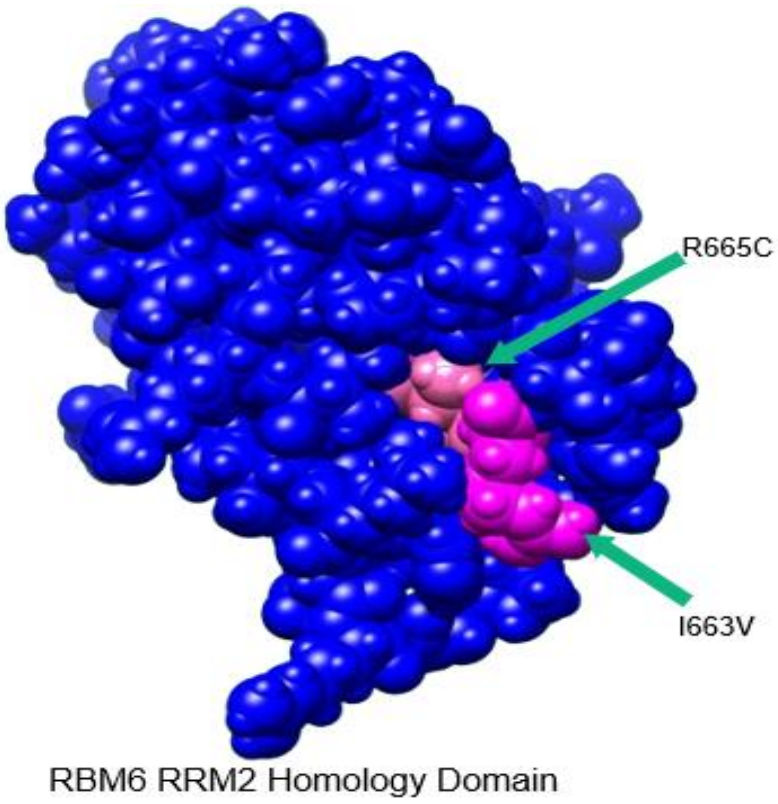

Figure 9: RRM2 Domain of RBM6: RRM2 homology domain of RBM6 depicting location of mutations R665C and I663V. The RRM2 domain of RBM6 is known to form a hydrophobic binding pocket analogous to the UHM of RBM17 and therefore mutations in the RRM2 domain of RBM6 such as I663V and R665C can be studied to understand if RBM6 can act as a competitive inhibitor against RBM17 on SF3b155.

Based on these successful expression of RBM6+ and RBM6 mutations I663V, V1058I, and A1061V, further investigation on the RRM domain of RBM6 and its mutations in this region such as the mutation I663V (Figure 9) can be investigated to see if when mutated, its RRM2 domain interacts and binds with SF3b155, a key subunit frequently mutated in hematologic malignancies and solid tumors that has also been shown to interact with the RRM2 domain found in RBM17. Because RBM6 and RBM17 share a conserved RRM2 domain that interacts with SF3b155, additional studies can be formulated to investigate how the RBM6 mutation 1663V found in the RRM2 domain of RBM6 effects the ability of RBM6 to act as a competitive inhibitor against RBM17 for SF3B1, influencing cellular proliferation.

METHODOLOGY:

Analytical Restriction Digestion of MiniPrep: Because the plasmids purified from bacteria are supercoiled, making them move abnormally in agarose gels, analytical restriction digest is used so that the DNA loaded onto gels is linear and well-behaved. Using 1µl of plasmid DNA from each miniprep, 1 µl 10X Buffer 2, 7.5 µl PCR water, and 0.5 µl restriction enzyme Pvu-1 HF (20000 U/ml) and place in 37°C water bath for 1 hour before storing or running an analytical gel at 90V for 30-45 minutes.

Chemical Transformation of E. coli XL 10-Gold Cells: The most basic method for introducing DNA into bacteria is through transformation, by incubating the bacteria in the presence of specific chemicals that induce the bacteria to take up DNA from their surroundings. Aliquots of ligation mixes were added to 200µl competent cells and incubated on ice for 30 minutes. The cells were heat shocked for 2-2½ minutes at 42°C. 1ml of SOC was added to each transformation and the cells were incubated 1 hour at 37°C to allow the expression of antibiotic resistance markers. Cells were plated on LB plates containing the appropriate antibiotics Ampicillin 100ng/µl and Kanamycin 50ng/µl. 50µl of aliquot-competent cells mixture was spread on 100mm plate, and the remaining mixture was vortexed, centrifuged and plated on another 100mm plate. Plates were incubated at 37°C.

DNA Sequencing: All DNA sequences were determined using GENEWIZ to verify that plasmids have the correct DNA sequence with minipreps who have a concentration more than 100ng/µl. Add a DNA volume equal to cDNA PCR + pBNG (3650 bp) and divide by ten. Using the concentrations from the nanodrop (ng/µl) calculate how many µl of the plasmid is equal to the number of ng and pipet the number of ng of the appropriate plasmid to the 1.5 ml tube, adding enough PCR water to make the volume in the tube 11.7 µl, adding the correct primer to each sample as *shown in the table below.*

Ethanol Precipitation: DNA was precipitated by addition of 2µl of yeast tRNA and NH_2OAc to a concentration of 2.5M followed by the addition of 3.5 volumes of 100% ethanol (based on the volume of the DNA-ytRNA mix). DNA was recovered by incubating for more than 2 hours at -20°C, followed by centrifugation at 4°C for 10 minutes.

Expression of Transfected Gene Fusions: 10µl of fusion plasmid DNA in a sterile snap cap tube to be transfected. PCR water was added to make the volume equal to 450µl for each plate. 50µl of freshly dissolved 2.5M $CaCl_2$ was added for each plate. In a separate tube, 500µl of 2xHBS at a pH = 7.1 was aliquoted per plate. A sterile plugged 1 ml serological pipet was inserted into the tube containing 2xHBS to generate a fine stream of bubbles to mis the solution. The mixture of DNA-PCR water and $CaCl_2$ was slowly added to this tube, and after the bubbling pipet was removed, the mix was allowed to stand for 30-45 minutes. 1 ml of precipitate was added dropwise to each plate of 293 cells. The plates were gently mixed and then returned to the incubator for 48 hours.

The cells were harvested by aspirating the media from the plates, adding 5 ml of PBS and aspirating the plates from PBS as well, taking care to remove as much liquid as possible. 1ml of RIPA-protease inhibitor mixture (990 µl RIPA and 10 µl of protease inhibitor) were added to each plate and the cells were scraped from the surface with a rubber policeman, and transferred to a 1.5 ml microfuge tube the cells were then placed on the nutator at 4°C for 30 minutes,

centrifuged for 20 min max speed at 4°C and the supernatant was transferred to a new 1.5 ml microfuge tube and stored at -80°C until further use.

Gel Electrophoresis: A 1/10 volume of 10x neutral gel loading dye was added to samples to be electrophoresed. DNA samples were electrophoresed on gels of 0.65-2.0% agarose (depending on whether gel is a preparative or analytical gel) in 89nM Tris-borate, pH 8.3, 2mM EDTA containing 5µg/ml ethidium bromide at 90 volts. For analytical digests of cloned DNAs, 0.5µg or less were loaded per lane. For preparative digests 5µg were loaded per lane.

Ligation of DNA Fragments into Digested Plasmids: Approximately 0.5-2 nmols of purified DNA fragment was mixed with 0.05-0.2µg of digested vector DNA in a total volume of 20µl 1xT4 ligase buffer. To "join" fragments with "sticky" ends, 1 unit of T4 DNA ligase was added. For blunt end ligations 3-5 units of ligase was added to each reaction. Ligation mixes were incubated to 12-14°C for 4-16 hours. Typically 1-2.5µl of ligation mix were used to transform E. coli.

Mega-Primer Cloning: Mega primer cloning, is a technique used to insert a desired mutation or sequence into a specific region of a DNA fragment or plasmid (RBM6 and mutations). The purpose of this method is to create a modified version of the original DNA sequence, either by introducing point mutations, deletions, insertions, or by fusing two separate DNA fragments together. *All Primers used as found in the table below.*

The process involves designing two long primers (mega primers) that contain the desired mutation or sequence at their 3' ends, as well as regions that are complementary to the target DNA. These mega primers are used in a PCR reaction along with the target DNA and a template plasmid. The PCR generates two DNA fragments with overlapping ends, which can then anneal to each other and be ligated together by DNA ligase. This results in a new DNA molecule containing the desired modification, which can be transformed into a host organism for further study or application. Mega primer cloning is particularly useful for site-directed mutagenesis and protein engineering studies. This is done through the use of restriction enzyme Dpn1 which recognizes and cuts DNA at the sequence "GATC" when the DNA is methylated on the "A" residue. Dpn1 is used to selectively digest DNA synthesized from E. Coli as the synthesize DNA is methylated on specific residues as part of the bacteria's defense mechanism against foreign DNA. Using plasmid + pBNG (3.65 kb), transfer 40 ng into PCR tubes and use 20 µl 5X Q5 polymerase buffer, 6 µl 25mM MgCl2, 10 µl 20X 5mM dNTPs, 2 µl BNG RBM6 20ng/µl, ~10 µl gel purified mega-primer 1250ng and run on PCR .

PCR of neo-SV40 Promotor Myc-Cassette To Prepare for Tagging via Mega Primer: PCR of the neo-SV40 promoter Myc-cassette serves to amplify a specific DNA sequence for subsequent use in tagging experiments via Mega Primer. It is done by mixing 28 µl PCR H20, 10 µl 20X dNTPs, 4 µl of 7.5 µM NSF-RR, 4 µl 7.5 µM RBM6-RR, 5ng/µl NS3M pBSR-neo-SV40 Pr-Myc plasmid template in one tube labeled "DNA mix" and 32.5 µl PCR H20, 10 µl 10X HF Buffer, 6 µl 25mM MgCl2 and 1.5 µl Expand DNA Polymerase (2.6 units) and transfer the 50 µl of DNA mix to enzyme mix before running on PCR. *All Primers used as found in the table below.*

Protein Assays: Conduct a Bradford assay by using 1000µl of Bradford reagent in 14 tubes labeled A-I, blank, plus the four samples being analyzed. Using thermos nanodrop-2000 UV-Vis at 590 λ, measuring the absorption of each sample, then conducting a regression line and best fit

line to determine the initial concentration of each sample to determine the µl needed to conduct a Western Blot analysis.

Purification of DNA from Agarose Gels: Purification of DNA from Agarose Gels is a critical method in molecular biology research, allowing for the isolation of target DNA fragments for subsequent applications such as cloning, sequencing, or gel electrophoresis analysis. This process involves excising the desired DNA band from the agarose gel that was obtained after running a preparative agarose and placing it in a 50°C heat block, ensuring that it is not above 0.35g, allowing it to melt, mixing every 2-3 minutes, followed by treatment with QG Buffer to equalize the volume of the sliced gel containing the target plasmid. Subsequently, purification is performed using a purification column, adding 100 µl QG buffer spinning in centrifuge for 1 min at max speed before discarding flow, adding 750 µl wash PE Buffer twice, spinning for 1 minute at maximum speed each time, adding and 30 µl EB Buffer in the column after transferring to new tube to remove impurities before using the Thermos nanodrop to determine the concentration before proceeding to the Mega-Primer PCR step. Ethanol precipitation may be necessary to further eliminate impurities from the purified DNA sample.

Purification of DNA Using the QIAprep Plasmid Midi Kit: The purpose of this procedure is to purify sufficient amounts of very pure plasmid DNA (≥100 µg) from bacterial colonies to allow for efficient transfection of animal cells. This is done by adding 1ml of LB with Ampicillin 100ng/µl and Kanamycin 50ng/µl, inoculating a colony in tube allowing it to shake in 37°C incubator for 6-8 hours @250-300 rpm before transferring to 250 ml sterilized flask and adding 50 ml of LB with Ampicillin 100ng/µl and Kanamycin 50ng/µl, allowing it to incubate again in in 37°C incubator for 16-24 hours @250-300 rpm. Purification of bacterial colonies includes the addition of 4 ml P1 buffer, 4 ml P2 buffer, 4ml P3 buffer, filtered through a purification column after being spun in Allegra centrifuge for 3000 rpm, 10 min at 4°C. This is followed by the addition of 3ml 100% Ethanol, 4ml Buffer QBT, 10ml Buffer QC, 5ml Buffer QF, 3.5 ml isopropanol before spinning again in Allegra centrifuge for 3000 rpm, 10 min at 4°C before pipetting 5ml cold 70% ethanol, decanting all ethanol and adding 400µl EB buffer before determining concentration with Thermos Nanodrop.

Purification of Plasmid DNA Using the QIAprep Miniprep Kit: The purpose of this procedure is to purify small amounts (2-20µg) of plasmid DNA from bacterial colonies. This is done by obtaining snap cap culture tubes per each culture and adding 3ml of LB with 3µl of Ampicillin 100ng/µl and Kanamycin 50ng/µl, inoculating a colony in tube allowing it to shake in 37°C incubator for 16-24 hours @250-300 rpm before obtaining 3 microcentrifuge (1.5ml) tubes per each inoculated liquid culture, pipetting 1.5ml of each culture into each of 2/3 tubes and centrifuge at 5000 x g (rcf) for 3 minutes to pellet the cells and pipet supernatant out. Add 250µl of P1 buffer into one of the tubes, resuspend, and transfer content to next tube, vortexing and adding 250µl P2 buffer, 350µl N3 buffer, mixing and spinning in microfuge max speed for 10 minutes before filtering and spinning supernatant through purification columns at 15000 rpm for 1 minute. Add 750µl PE buffer, spin 1 minute at 15000rpm twice before moving column to last tube, adding 50µl EB Buffer and spinning for 1 min at 15000 rpm before determining concentration with Thermos Nanodrop and storing at -20°C.

Restriction Digest: All restriction endonuclease digests were carried out in buffer and conditions recommended by the manufacturer. All digests contained DNA at a concentration of 100-200 µg/ml.

Test Amplification of RBM6 and RBM6 mutants using Q5 Polymerase: To test the amplification of RBM6 and RBM6 mutants, 0.5 µlQ5 Polymerase will be used in the polymerase chain reaction (PCR) methodology. Aliquots include the addition to 2.5 µl 20X dNTPs, 1µl HeLa cell cDNA, 5µl 5X Q5 DNA Polymerase Buffer, and 1µl of 7.5µM of the designated primers for each plasmid as shown in the table below.

Western Blot: To detect the expression of RBM6 and its mutants in 293 or HeLa Cells, Western blot analysis was performed according to standard protocols. This included making a 19/1-part buffer or 4x Sample buffer with 2-mercaptoethanol, adding 10-12.5 µl of sample buffer and 30-37.5 µl sample and heat at 95-100°C in heat block and load gel with 1X running buffer to run for 60 min @ 200V. After gel has been ran, transferring and incubation of antibody (Myc antibody) must be done with 800ml nanopure H20, 100ml methanol, 100ml 10X Tris Glycine (for transfer buffer), 10X TBS (24.22 g Tris base, 87.8 g NaCl and pH to 7.4 with HCl) , 1X TBST (500 µl TWEEN-20, 900 ml nanopure H20, and 100 ml 10X TBS), and blotto (5g nonfat dry milk diluted to 100 ml with 1X TBST) before development of blot.

SUPPLEMENTS:

Table S1

Primer	Sequence
RBM6-L	CTAGTCTAGATTACTTGTTGGGGCCCTCTTGAT
RBM6-R	AAGGAAAAAAGCGGCCGCGGAGGTTGTATCCCATGGAACTTG
hRBM6-RXXR	AGCAAACCTTTCTTCTTGGCTCCCACGAAAAGGTCCAGTTCTGTTAGCAGGTCGAGAATCCCCCCACCGTTTCAGGTCCTCCTCTGAGATCAGCTTC
NSF-RR	CTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAG CTGATACCGCTCGCCGCAAGGGTTCCGCAAGCTCTAGTCG
hRBM6 R665C sense	AAACTATCATGCTAAAGCGTATCTATTGTTCCACACCACCTGA
hRBM6 R665C AS	TCAGGTGGTGTGGAACAATAGATACGCTTTAGCATGATAGTTT
hRBM6 I663V sense	GAGCAAAACTATCATGCTAAAGCGTGTCTATCGTTCCACACCACCTG
hRBM6 I663V AS	CAGGTGGTGTGGAACGATAGACACGCTTTAGCATGATAGTTTTGCTC
hRBM6 V1058I sense	GACACTAGCAGCAAAGGAGGCTGTATCCAACAGGCTACT

hRBM6 V1058I AS	AGTAGCCTGTTGGATACAGCCTCCTTTGCTGCTAGTGTC
hRBM6 A1061V sense	GAGGCTGTGTCCAACAGGTTACTGGCTGGAGGAAAGG
hRBM6 A1061V AS	CCTTTCCTCCAGCCAGTAACCTGTTGGACACAGCCTC

DISCUSSION:

The goal of this study was to begin to resolve some unresolved issues about the function of RBM6 in splicing and cell division in cancer. In a prior study, Bechara demonstrated that RBM6 regulated alternative splicing of the NUMB gene and knocking down expression of the RBM6 protein promoted exon inclusion (Bechara et al. 2013). These experiments were done in stable cell lines where RBM6 was decreased. In these cells decreased RBM6 expression caused a decrease in cell proliferation compared to normal HeLa cells, the opposite of what would be expected for a tumor suppressor gene. They also showed that RBM6 specifically promotes the inclusion of a specific exon in the NUMB pre-mRNA, more specifically exon 9, and this leads to the production of the NUMB isoform previously shown to promote cell proliferation and tumorigenesis. (Bechara et a. 2013). In RBM6 knockdown cells, the splicing of many mRNAs was altered, but in many instances, knockdown promoted skipping of exons and while RBM6 often had the opposite effect on the splicing of particular exons that RBM10 had, RBM6 and RBM10 were frequently observed to have the same effect. And the effect of mutation in RBM6 had not been studied.

Through the creation of RBM6 cell lines that overproduce the protein in HeLa cells, as well as studying the effect of transient expression of normal and mutated RBM6 proteins, we are able to determine if the overexpression of RBM6 will promote cell division, and in hopes, also prove that if this overexpression correlates with changes in the rate of cell division. The use of RBM6 mutations in the protein enabled us to investigate if the overexpression of RBM6 promotes the exclusion of the gene NUMB exon 9 and alters the splicing of other pro-apoptotic genes.

Here, we re-investigate the work done by Bechara, through RBM6 cell lines that overproduce the protein in HeLa cells to determine if this overexpression will promote cell division. If shown that the overexpression of RBM6 in HeLa cells promotes cell division and proliferation, this research sheds light on how RBM6 and its downstream pathways could serve as potential therapeutic strategies for cancer treatment, as well as be used in target therapy and medicine where patients who have tumors exhibiting the dysregulation of RBM6 could potentially benefit from such personalized medicine in hopes of restoring normal splicing patterns.

In addition, the use of RBM6 mutations in the protein, which enabled us to investigate that the overexpression of RBM6 promotes the exclusion of the gene NUMB exon 9 and alters the splicing of other pro-apoptotic genes, could serve to better understand the role of NUMB isoforms in cancer, leading to further studies in cancer therapy medicine. While this study provides valuable insights into RBM6 and its role in cell proliferation, it is important to acknowledge several limitations that may impact the interpretation and generalizability of the findings.

While this study primarily relies on cell culture models, further studies using in vivo models or patient samples are needed to validate these findings in a more physiological context. Furthermore, additional research is needed to fully understand the broader implications of NUMB splicing in cancer biology. Based on these findings, further investigation on the RRM domain of RBM6 and its mutations in this region such as the mutation I663V can be investigated

to see if when mutated, its RRM2 domain interacts and binds with SF3b155, a key subunit frequently mutated in hematologic malignancies and solid tumors that has also been shown to interact with the RRM2 domain found in RBM17. Because RBM6 and RBM17 share a conserved RRM2 domain that interacts with SF3b155, additional studies can be formulated to investigate how the RBM6 mutation 1663V found in the RRM2 domain of RBM6 effects the ability of RBM6 to act as a competitive inhibitor against RBM17 for SF3B1, influencing cellular proliferation.

Through the creation of RBM6 cell lines that overproduce the protein in HeLa cells, as well as studying the effect of transient expression of normal and mutated RBM6 proteins, we are able to determine if the overexpression of RBM6 will promote cell division, and if this overexpression correlates with changes in the rate of cell division. The use of RBM6 mutations in the protein enabled us to investigate if the overexpression of RBM6 promotes the exclusion of the gene NUMB exon 9 and therefore alters the splicing of other pro-apoptotic genes. These findings will therefore allow us to compare the claims stated in *RBM5, 6, and 10 differentially regulate NUMB alternative splicing to control cancer cell proliferation* (Bechara et al. 2013) which demonstrated that knockdown versions of the protein RBM6 promoted exon inclusion, aiding in the role of RBM6 in regulating alternative splicing of the NUMB gene and therefore knockdown of RBM6 results in decreased expression of NUMB-10 and reduced cell proliferation (Bechara et al. 2013).

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