STEM-LOOP BINDING PROTEIN (SLBP) REGULATES THE EXPRESSION OF THE HIV-1 RESTRICTION FACTOR APOBEC3G

By

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Class of 2016

Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Bachelor of Science with Honors in Applied Mathematics-Biology

In the Department of Biology at Brown University

Submitted 24th April 2016

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ACKNOWLEDGEMENTS

I would like to thank Dr. Ming Li for the commitment and unrelenting support throughout my undergraduate research. Through Dr. Li, I have received mentorship and training that helped me to think logically and critically about scientific problems. I also extend my gratitude to Dr. Bharat Ramratnam for support in conducting experiments, and Prof. Basilis Gidas of the Department of Applied Mathematics for providing second advising for my honors thesis work. Lastly, I extend deep appreciation to The Karen T. Romer Undergraduate Teaching and Research Award (UTRA) committee for providing funding to support my research work on the Human Immunodeficiency Virus Type 1 (HIV-1).

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ABSTRACT

Lymphocytes possess innate mechanisms for regulating the HIV-1 viral cycle. Using proteomics and functional analysis, we recently identified Stem-Loop Binding Protein (SLBP) as a histone related HIV-1 host factor that restricts integration and transcription of HIV-1. Our findings also demonstrated that SLBP depletion leads to a decrease in the HIV-1 restriction factor APOBEC3G (apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G) in lymphocytic cells at both the RNA and protein levels. In this study, we use various techniques to further investigate the molecular mechanism for the novel relationship between SLBP and APOBEC3G.

Keywords: HIV-1, APOBEC3G, SLBP, Restriction factor

INTRODUCTION

Globally, HIV/AIDS is estimated to cause about 1.1 - 1.6 million deaths every year [1]. Despite the use of effective antiretroviral therapy (ART), HIV-1 infection remains incurable owing to the persistence of a viral reservoir that harbors replication-competent provirus within the host cellular DNA. HIV-1 relies on host cell machinery to integrate into the genome and replicate to produce infectious virions. The dependence of HIV-1 on host cell machinery has generated interest into innate mechanisms of controlling viral infection.

In the course of the viral cycle, HIV-1 interacts with cellular host proteins to facilitate successful replication. According to HIV-1 Human Interaction Database, around 3, 592 host proteins have been shown to interact with HIV-1. Only a few have been shown to significantly inhibit HIV-1 infection. These proteins, known as HIV restriction factors, block different processes of the HIV-1 life cycle including integration of viral DNA and release of newly synthesized virions. Some of the restriction factors identified in recent discoveries include APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G, A3G)[2-6], SAMHD1 (SAM domain and HD domain-containing protein 1)[7], tetherin/bst-2(bone stromal tumor protein 2)[8] and TRIM5α (Tripartite motif-containing protein 5 alpha)[9].

The restriction mechanisms of the host proteins have been shown to target different parts of the HIV-1 replication cycle. APOBEC3G, a 48 kDa protein of the APOBEC superfamily of proteins, is packaged into assembling HIV-1 virions, and transferred to target cells. In the cell, it has been shown to target viral transcription through association with the viral Reverse Transcriptase Complex (RTC) to deaminate cytidine residues in nascent single-stranded negative strand viral DNA [6]. Tetherin, a 30-36 kDa type II transmembrane glycoprotein, on the other hand, has

been shown to have both an N-terminal transmembrane domain and a C-terminal glycosyl-phosphatidylinositol (GPI) anchor. This has led to a model, in which BST-2, by means of its N-terminal transmembrane domain and its C-terminal GPI anchor, tethers otherwise fully detached whole virions to the producing cell[8].

As a defense mechanism, HIV-1 has evolved to offer counter-restriction mechanisms against the restriction factors. HIV-1 encodes for viral accessory proteins that have been shown to target restriction factor pathways. In the case of APOBEC3G, the HIV-1 protein produces Vif (viral infectivity factor) that binds to APOBEC3G and recruits cellular ubiquitin ligase complex that comprises the cullin5 scaffold protein, elongins B and C, Rbx2, and an unidentified E2 conjugating enzyme [6]. This results in A3G polyubiquitination and proteasomal degradation, and therefore averts the encapsidation of A3G into nascent viral particles [3, 5, 10]. Other mechanisms employed by HIV-1 to inhibit HIV-1 restriction factors include the downregulation of BST-2 by the HIV-1 protein vpu (viral protein unique).

In recent studies, our experiments using Stable Isotope Labeling by Amino acids in Cell culture (SILAC) to characterize the peripheral blood cell proteomes of HIV-1 infected individuals with variable levels of plasma virus, identified histone proteins to be significantly decreased in individuals with high viral load. This differential expression was traced to a histone-RNA hairpin binding protein known as stem-loop binding protein (SLBP). SLBP is a 31-kDa protein that binds the 3' end of histone mRNA. SLBP is necessary for pre-mRNA processing and accompanies the histone mRNA to the cytoplasm for translation [11]. In terms of HIV-1 replication, cellular SLBP depletion rendered the chromatin structures of the viral LTR and host gene high mobility group protein A1 (HMGA1) more open for promoter engagement leading to higher levels of HIV-1

genomic integration and proviral transcription. Increased integration and transcription of the HIV-1 genome would likely result in elevated levels of the virus.

Preliminary investigations into SLBP suggested the existence of a previously unknown and uncharacterized relationship between SLBP and APOBEC3G. Based on our findings, SLBP depletion at both RNA and protein level resulted in a concomitant decrease in APOBEC3G. Reports suggest that immune responses associated with natural HIV infection could lead to expression of cytokines that induce APOBEC3G gene expression and APOBEC3G complex assembly [12]. In this study, we attempt to elucidate molecular mechanisms by which SLBP regulates the expression of APOBEC3G at both the transcriptional and translational level.

MATERIALS & METHODS

Cell culture and Transfection

CEM (ATCC® CCL-119) Cells were grown in RPMI-1640 cell growth medium. Two million cells were then dissolved in supplement solution from Cell Nucleofector™ Solutions before adding 3 µl of siRNA. The cells were then electroporated using 4D-Nucleofector™ System before adding growth media. After 48hrs, the cells were separated from the supernatant via centrifugation, before proceeding with RNA and protein extraction.

Western blotting

Protein was extracted from cell cultures using Mammalian Protein Extraction Reagent (MPER Thermo Scientific). The protein concentration was then determined by micro BCA assay, and run on an iBlot precast Gel (Life technologies). The proteins in the gel were then transferred onto a PVDF membrane. The membrane was then blocked for 1hr using blocking buffer (LI-COR), before adding primary antibodies anti-SLBP, anti-ACTB and anti-APOBEC3G for incubation at 4 °C overnight. Finally, the membrane was incubated with IRDye 680LT or 800CW conjugated secondary antibodies before analysis with the Odyssey CLx Infrared Imaging System (LI-COR).

RT-PCR

Total RNA was extracted from cultured cells using TRIzol® (Life technologies). The concentrations were then determined, before using equal amounts for cDNA synthesis and PCR using a thermocycler gradient (Eppendorf). Real-time PCR was performed on Mastercycler ep realplex (Eppendorf).

All reactions were performed in 96-well plates with the following reagents in a final volume of 20µl: 1µl of primers (10nM each for forward and reverse) and 2x Maxima® SYBR Green qPCR Master Mix. 10ng of cDNA was added to this mixture. Each sample was done in a triplicate form for both the target gene and housekeeping gene. Finally, a cycle threshold value (Ct) value was obtained for each sample, and used to calculate the relative expression of the target gene.

Promoter accessibility analysis by EpiQ®

Assay was performed according to protocols supplied by Bio-Rad's EpiQ® Chromatin Analysis Kit. Each promoter accessibility value was calculated by EpiQ Chromatin Kit Data Analysis Tool (Bio-Rad, version 1.1.18.0915). Triplicate values from both the control and the experimental were analyzed by Student's t-test. Results were considered to be statistically significant when $p \leq 0.05$ (two-sided). Heatmap Builder® (Version 1.0) was also employed to comparatively visualize promoter accessibility.

Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed according to protocols supplied by SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signaling). Anti-SP1 (Cell Signaling) was used as the capture antibody. Normal rabbit IgG (Cell Signaling) was used as negative control.

Dual-Glo Luciferase Assay

Promoter region of human APOBEC3G, spanning from -650 to +50 nucleotides to the translation starting site, was PCR-amplified and cloned into pGL3-Basic vector (Promega). Dual-Glo Luciferase Reagent (Promega) was used according to the manufacturer's instructions.

Luminescence was measured by using a TopCount NXT Microplate Scintillation and Luminescence Counter (PerkinElmer).

Interferon PCR Arrays

Interferon α, β Response RT² Profiler® PCR Arrays (Qiagen) were employed to quantify mRNA expression of 84 interferon response genes. Standard real-time PCR protocols were followed. Three replicate arrays were performed on both the control and the experimental samples. In order to determine significantly up- and down-regulated candidates, conservative cut-off values were calculated as follows: median and standard deviation (σ) of relative expression levels were calculated using log-transformed data of all quantified interferon response genes. The cut-off value (median±2σ) was calculated in log space that was then transformed back into linear space. Candidates which appeared in all three replicates would be prioritized for down-stream experiments.

RESULTS

SLBP /APOBEC3G depletion at the RNA level

To investigate the effect of SLBP depletion on APOBEC3G RNA and protein levels, we employed western blotting and quantitative PCR techniques. Two batches of CEM cells were cultured in media before siRNA transfection via electroporation. The CEM cells were transfected with siRNA control for one plate and siRNA SLBP for the other plate. RT-PCR employed to measure the RNA levels of control vs the SLBP knockdown demonstrated a decrease in levels of SLBP RNA in the mutant, and a corresponding decrease in levels of APOBEC3G RNA. For the Control, however, the RNA levels of SLBP and APOBEC3G remained relatively constant (Figure 1).

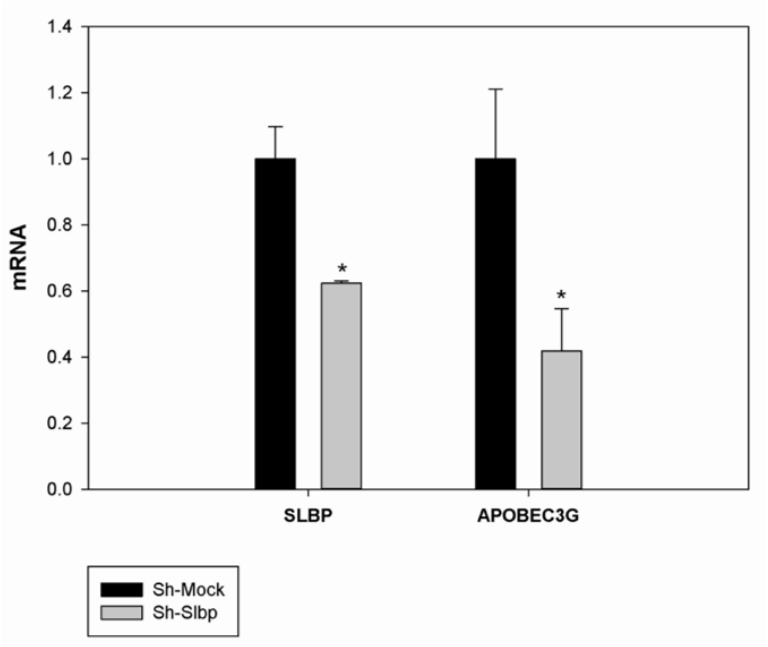

*Figure 1: APOBEC3G RNA level significantly decreased in the SLBP depleted cells (*P<0.05)*

SLBP /APOBEC3G depletion at the protein level

Next, we questioned whether the decrease in APOBEC3G RNA was reflected at the translational level. To test this, we utilized the western blotting technique to compare the protein levels of both SLBP and APOBEC3G. The western blots in Figure 2 show a significant decrease in SLBP protein in the knockdown cells, and a corresponding decrease in APOBEC3G protein levels. For the control however, both proteins demonstrate relatively high levels of expression.

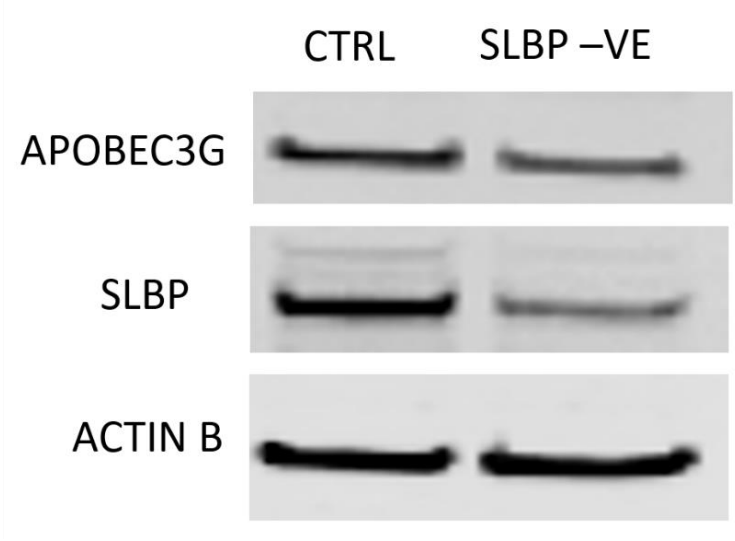

Figure 2: Western blot showing the effect of SLBP depletion on APOBEC3G in CEM cells.

SLBP depletion is associated with decreased promoter accessibility of APOBEC3G

SP1 has been previously identified as a promoter for APOBEC3G [13]. We decided to investigate whether SLBP depletion reduced accessibility of the SP1 promoter. To determine the effect of SLBP on the affinity of the transcriptional factors of APOBEC3G, a chromatin immunoprecipitation assay EpiQ® was used. The assay compared the promoter accessibility of APOBEC3G under different expressions of SLBP proteins-ranging from low concentrations to high concentrations, and using GAPDH as a control gene. Our results shown in Figures 3, 4, and

5, demonstrate that low concentrations of SLBP directly correlate with decreased accessibility of the APOBEC3G promoter SP, and an overall lower binding affinities in SLBP depletion conditions.

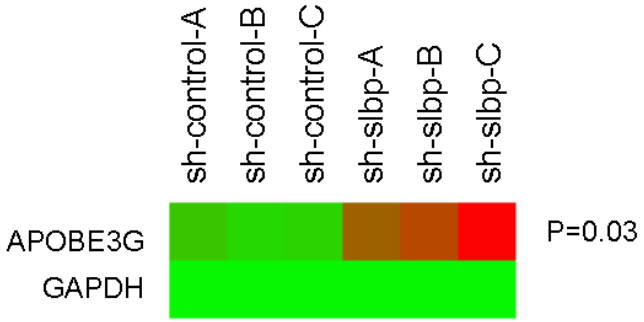

Figure 3: Heatmap of APOBEC3G promoter accessibility under mock and SLBP depletion conditions. Three replicates were performed by EpiQ® assay. GAPDH was used as the control gene.

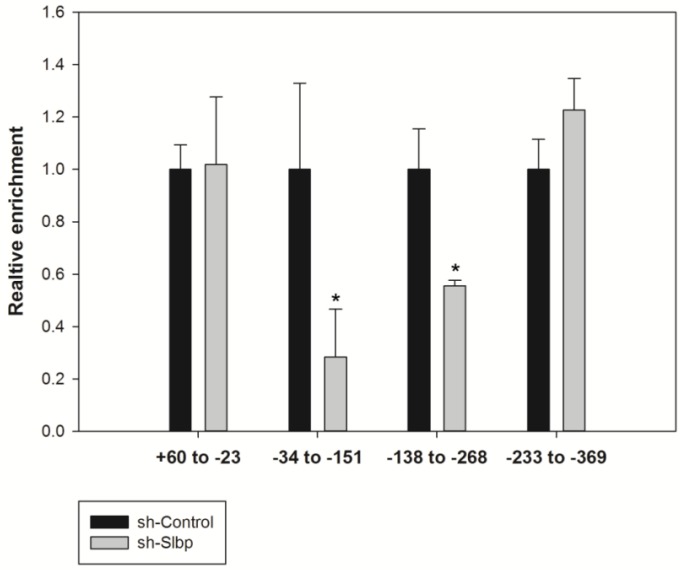

Figure 4: CHIP assay on SP1/APOBEC3G when SLBP is depleted. Relative reduction in nucleotides -368 to -34 with respect to the APOBEC3G transcription start site. All values are relative to non-immune IgG. (, p≤0.05)*

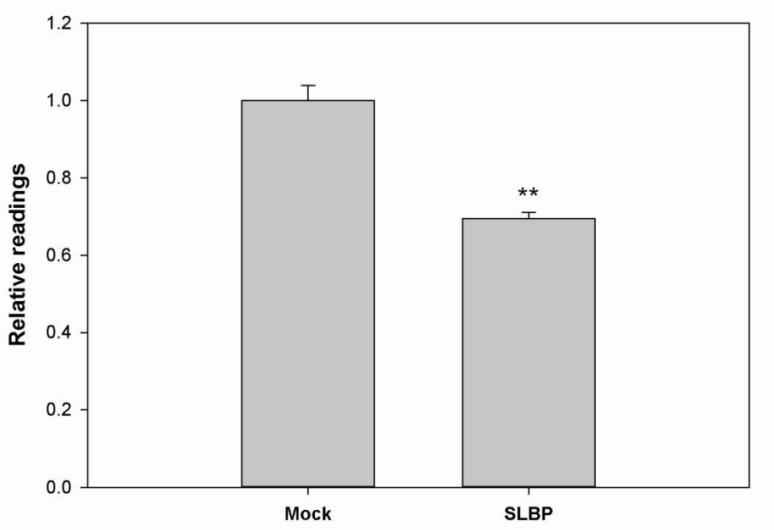

Figure 5: The APOBEC3G promoter region corresponding to nucleotides -650 to +50 was also investigated by use of a luciferase reporter assay. Experiments were performed in triplicate, and luciferase activity is expressed relative to that obtained in experiments. Lower luciferase reading on APOBEC3G promoter is associated with SLBP depletion.

SLBP regulates expression of APOBEC3G-associated interferon genes

An interferon PCR array on 84 interferon response genes revealed that several genes were differentially expressed in SLBP depleted conditions. Using the histogram of interferon mRNAs (Figure 6), we identified significantly downregulated and upregulated interferon genes. Interestingly, 3 of the genes CD70 (downregulated), IL15 (downregulated), and SOCS1 (upregulated) had been previously linked to APOBEC3G regulation (Table 1) [12].

A separate RT-PCR was then done to confirm the expression of the three interferon genes CD70, IL15 and SOCS1 in SLBP depleted conditions. As shown in Figure 7, SLBP depletion is associated with downregulation of both CD70 and IL15 and upregulation of SOCS1. The specific

functions of the three interferon genes are summarized in Table 2.

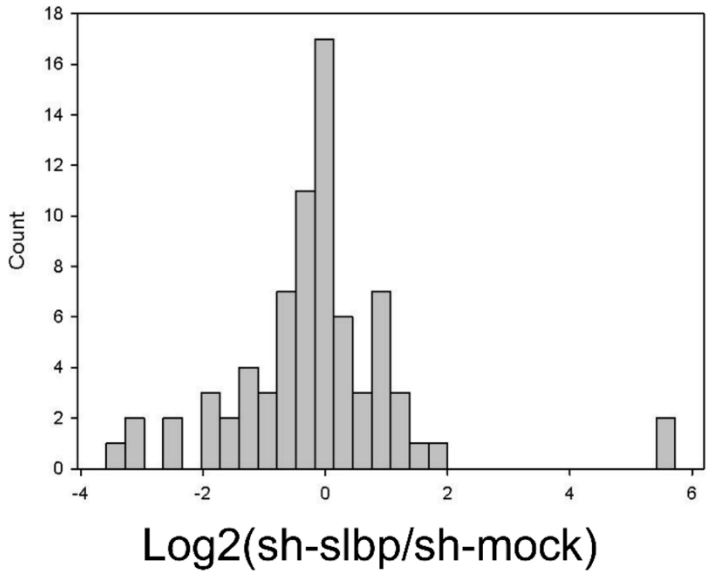

Figure 6: Histogram of mRNA ratios revealed symmetrical distribution along the ratio=1 (log2=0) trend line. The log2 transformed ratios were grouped into ratio bins, and the y-axis shows the relative number of detected ratios per bin. The analysis encompasses 84 interferon response genes.

Array replicate 1		*Array replicate 2*		*Array replicate 3*	
Name of interferons	Fold change	Name of interferons	Fold change	Name of interferons	Fold change
CD70	0.02	CAV1	0.18	CCL5	12.69
CXCL10	8.13	CCL5	12.47	CD70	0.03
IL15	0.02	CD70	0.05	IFNW1	13.60
IL6	9.41	IFIT3	0.15	IL15	0.09
PRKCZ	6.00	IFNE	10.20	IL6	0.24
SOCS1	5.22	IFNW1	38.07	MAL	5.34
VEGFA	11.99	IL15	0.08	PRKCZ	9.35
		MET	7.52	SOCS1	3.88
		MNDA	6.78	TLR3	0.23
		SOCS1	5.98	VEGFA	11.52
		TLR7	18.26		

Table 1: Significantly up- & downregulated interferon response genes found in SLBP depleted CCRF-CEM cells. The name and corresponding fold change (Shslbp/Shsmock) are given for each

candidate. Three array replicates were performed. Each array contains 84 interferon response genes plus positive and negative controls.

Name of interferons	SLBP dep.	Functions
CD70	↓	Induces the proliferation of costimulated T-cells and enhances the generation of cytolytic T-cells.
IL15	↓	Stimulates the proliferation of T-lymphocytes.
SOCS1	↑	Form part of a classical negative feedback system that regulates cytokine signal transduction.

Figure 7: Candidate interferons and their reported functions.

DISCUSSION

From our studies, it is clear that SLBP uses multiple possible pathways to regulate the expression of APOBEC3G. SLBP can regulate the expression of APOBEC3G both directly and indirectly: directly by reducing APOBEC3G promoter accessibility and indirectly by modulating the interferon pathway and perhaps upregulating expression of anti-APOBEC3G viral proteins (Illustrated in Figure 9). Although the APOBEC3G promoter SP1 had previously been identified as a promoter for APOBEC3G, our results demonstrated that SLBP can effect direct regulation of APOBEC3G by reducing accessibility and affinity of the APOBEC3G promoter SP1. More indirectly, however, SLBP effected differential expression of 3 interferon genes IL15, SOCS1, and CD70 which have been identified to be crucial in APOBEC3G regulation.

A third possible pathway is that SLBP depletion could upregulate the expression of the HIV-1 protein Vif, an APOBEC3G antagonist, previously discussed. In previous studies, we demonstrated the bifocal regulation of HIV by the histone binding protein SLBP. In one aspect, we examined that SLBP enhances integration and transcription of the HIV. In the second aspect, we demonstrate that this enhanced integration and transcription has a downstream effect of leading to higher Vif protein levels which act to protect the viral replication cycle. This interaction is however still under investigation.

Further downstream experiments to elucidate certain aspects of the SLBP/APOBEC3G relationship can be done. For the SLBP-Vif – APOBEC3G pathway in particular, we propose experiments to verify the effect of SLBP on the Vif protein. Furthermore, we ask whether SLBP overexpression is sufficient to restore APOBEC3G expression perhaps by overcoming Vif-induced degradation of APOBEC3G.

Alternatively, studies on cell cycle alteration indicate that TNF-alpha can induce SLBP depletion in hepatocytes [14]. Further experiments can therefore be done to investigate whether TNF-alpha can induce SLBP depletion in lymphocytic cells, and lead to a corresponding APOBEC3G downregulation. These particular experiments on immune signal regulation would be particularly relevant, as they mimic the natural environment of lymphocytic cells.

Our findings highlight the clinical relevance of the ability to harness the host innate immunity against viral infection. For one, we demonstrate that the expression of the HIV inhibitory protein APOBEC3G can be regulated at multiple levels including the basic transcriptional level. Secondly, we demonstrate that this regulation can be induced by immunoregulatory factors, which would typically be found in normal physiological environments in a cell. As such, these findings could inform the design of host factor targeted viral therapies that would less likely be toxic to lymphocytic cells.

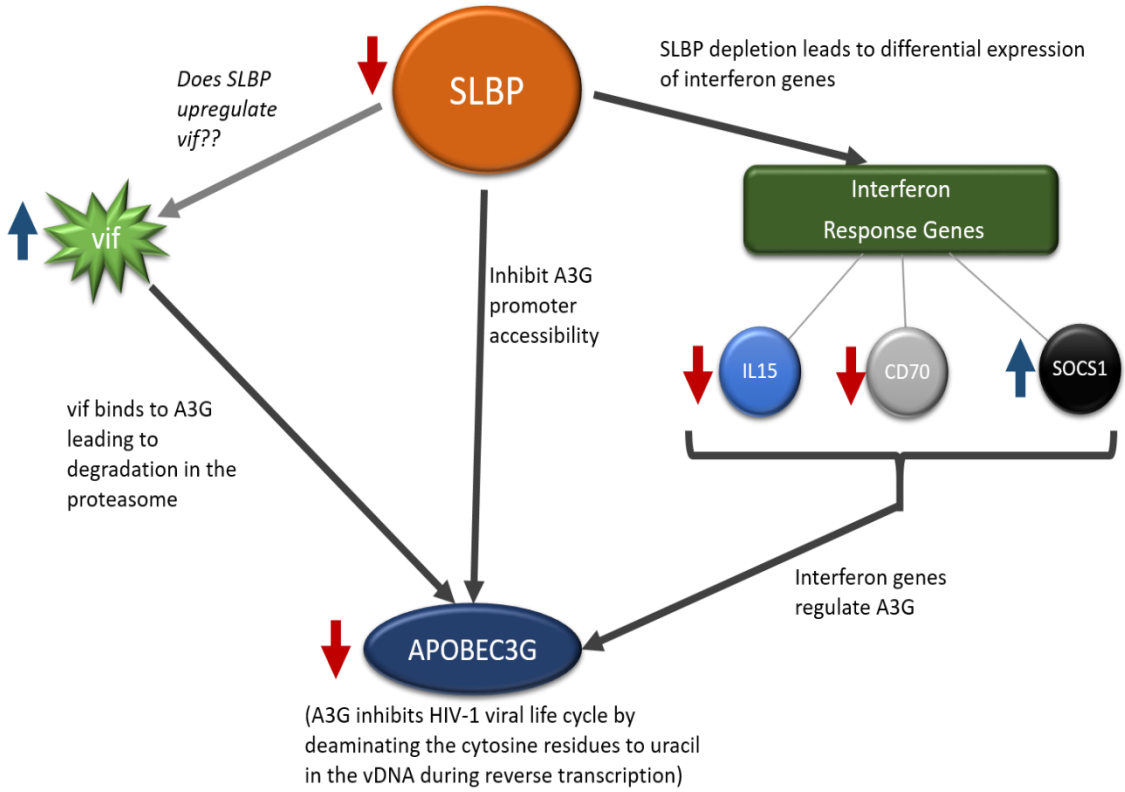

Figure 8: Direct and Indirect regulation of APOBEC3G by SLBP

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