

## ***Abstract***

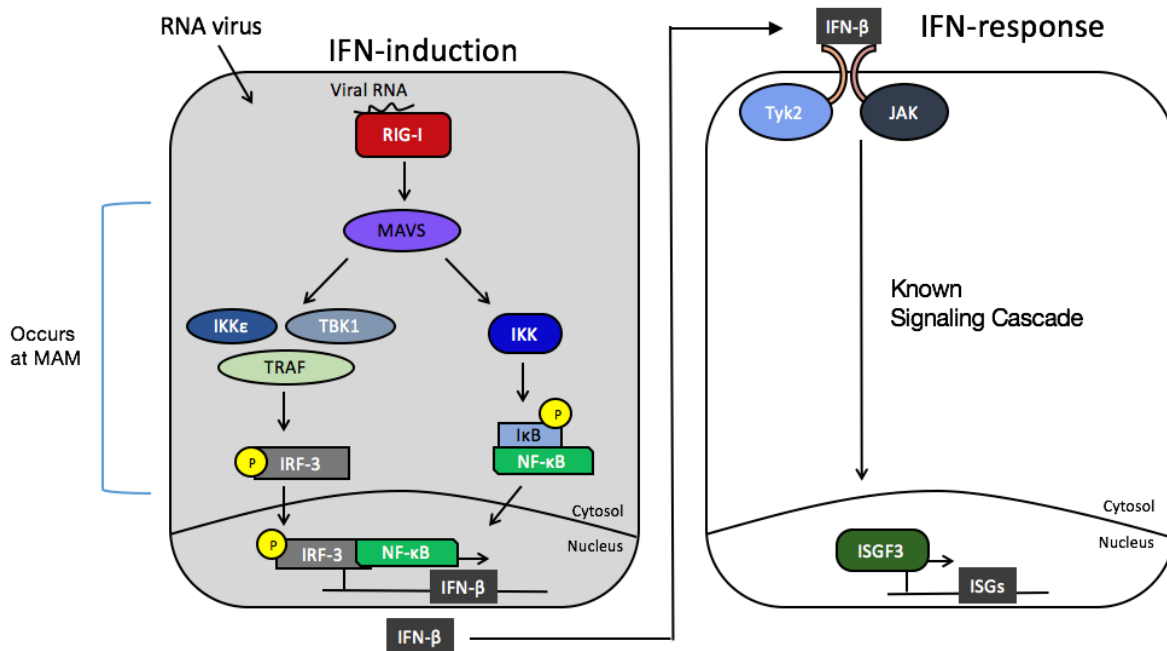
When viruses from the broad flaviviridae family of viruses including hepatitis C virus (HCV), yellow fever, and zika, infect a host cell, the host's pattern recognition receptors (PRR), such as the RNA virus sensor protein RIG-I, bind viral pathogen-associated molecular patterns (PAMPs), such as ssRNA. The activation of RIG-I leads to the downstream induction of the molecular message of viral attack to cells, cytokine interferon-beta (IFN- $\beta$ ), which leads to the further induction of several interferon-stimulated genes (ISGs), which limit viral replication. Proteins that are involved in the IFN- $\beta$  induction pathway are regulated by post-translational modifications. Here, I have identified that RAB1B, a GTPase protein involved in vesicle trafficking, positively regulates IFN- $\beta$  during viral infection. Additionally, we have determined that during viral infection, RAB1B is post-translationally modified in a RIG-I dependent fashion by ufmylation, a poorly understood conjugation of a protein's lysine (K) residue to the modification UFM1, which that has been implicated in ER homeostasis and stress response, inhibition of apoptosis, and protein stability. Further, I demonstrate that UFM1 and its E3 ligase UFL1, which conjugates UFM1 to a substrate protein, also both positively regulate IFN- $\beta$  during viral infection and that a putative, ufmylated lysine residue responsible for RAB1B function during innate immunity is K170. Consequently, I also constructed lysine to alanine substitution constructs of RAB1B at K170, K171, and K194 using site-directed mutagenesis that express in cell culture and eliminate the ability for the residue to be ufmylated for future experimentation. Altogether, we demonstrate a novel function for RAB1B and its ufmylation in regulating the innate immune system through induction of IFN- $\beta$ .

## ***Introduction***

Innate immunity is the body's non-specific response to foreign pathogens, activated by the chemical properties of a pathogen's antigens [1]. Innate immunity differs from adaptive immunity, in which the body processes pathogens, creates antibodies specific to the pathogen's antigens, and remembers individual pathogens to defend the body from future attacks [1]. In humans, innate immunity can be generally divided into three main events: 1) a host cell must first recognize that it is under pathogen attack 2) the cell must alert itself and other surrounding host cells of the pathogen infection and 3) the surrounding cells must fight the pathogen attack through an appropriate immune response. During a viral attack, all three of these events are facilitated by networks of antiviral signaling cascades. While these antiviral pathways have been well documented [2, 6, 7], there is still mounting evidence that there are other regulatory proteins and mechanisms involved in innate immunity that have yet to be implicated. This gap of knowledge is problematic because the innate immune system must be tightly regulated in order to clear viral infection without causing excessive inflammation or cellular damage. My research is focused on the first two events of the innate immune response during viral infection and seeks to answer what other cellular mechanisms and machineries are used by host cells to discriminate

between their own host features, or self-antigens, from viral, non-self-antigens [1, 2, 6, 7] and regulate the production of a molecular message of viral attack to other cells [1].

When a virus infects a host cell, unique viral antigens called pathogen-associated molecular patterns (PAMPs)—such as viral ssRNA or dsRNA—are detected by host pattern recognition receptors (PRRs) typically expressed in dendritic cells, macrophages, monocytes, neutrophils and epithelial cells [2, 6, 7, 12]. PRRs, which can exist as membrane-bound or intracellular cytoplasmic receptors, recognize conserved molecular motifs unique to each pathogen [6, 7, 12]. There are several types of PRRs used to detect viral PAMPs or infection in the host. One class of cytoplasmic PRRs is the RIG-I-like receptors (RLRs). There are three mammalian RLRs: RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation factor-5) and LGP-2 (laboratory of genetics and physiology-2). Additionally, RLRs all possess a DExD/H-box RNA helicase domain that allows for unwinding or disassociation of RNA-structures or RNA-protein complexes to aide in recognition of viral genetic material [12]. My lab is interested in the recognition of self from viral non-self-antigens during the innate immune response related to hepatitis C virus (HCV), in the hepacivirus genus and other viruses of the flavivirus genus such as yellow fever, zika, and dengue. The PAMPs of viruses within this broad family of flaviviridae are typically recognized by the RLR, RIG-I. As a result, we are primarily interested in RIG-I and how its activation and signaling leads to the downstream induction of the molecular message of viral attack to cells, cytokine interferon-beta (IFN- $\beta$ ), and the further induction of several interferon-stimulated genes (ISGs).



Modified from Dr. Dia Beachboard

**Figure 1. Intracellular innate immune response to RNA viral infection.**

This diagram depicts the innate immune events upon RNA viral infection in the RIG-I pathway. Upon viral infection, ssRNA is detected by RIG-I. RIG-I interacts with MAVS at the mitochondrial associated ER membranes (MAM). This leads to the recruitment of several antiviral protein interactions at the MAM, which result in the downstream signaling to IFN-β. IFN-β is secreted in a paracrine or autocrine fashion, where cells receive the cytokine and initiate the Jak/Stat pathway to induce hundreds of ISGs (IFN stimulated genes). These ISGs limit viral replication and help clear the virus infection.

**Figure 1** summarizes our current understanding of how the RIG-I antiviral pathway regulates innate immunity. Upon binding to viral PAMPs such as ssRNA with a 5' triphosphate or short dsRNA in the cytoplasm, the RNA sensor protein RIG-I undergoes a conformational change that promotes its activation and oligomerization [2, 5, 6]. Other RIG-I activation steps include the removal of RIG-I inhibitory phosphorylation marks by protein phosphatases and further post-translational modifications from the proteins RIPLET, TRIM25, and 14-3-3ε [2, 6]. Then, RIG-I's N-terminal tandem caspase activation and recruitment domains (CARDs) interact with MAVS, the RIG-I signaling adaptor protein, and TRAF3, a downstream antiviral signaling protein, in the mitochondrial-associated ER membranes (MAM) [2, 5]. The MAM is a subdomain of the ER where this machinery that signals to protect against the viral RNA infection is organized. RIG-I and MAVS interactions result in a signaling cascade that further results in the activation of the protein kinases TBK1 and IKKε which, through phosphorylation, activate the IFN transcription factors, IRF3 and NF-KB [2, 5, 6, 7, 12]. These induced IFNs (IFN- β being a Type I IFN) are then secreted in an

autocrine or paracrine fashion to activate the Jak/Stat pathway, which signals the induction of hundreds of ISGs [2, 5, 6, 7, 12].

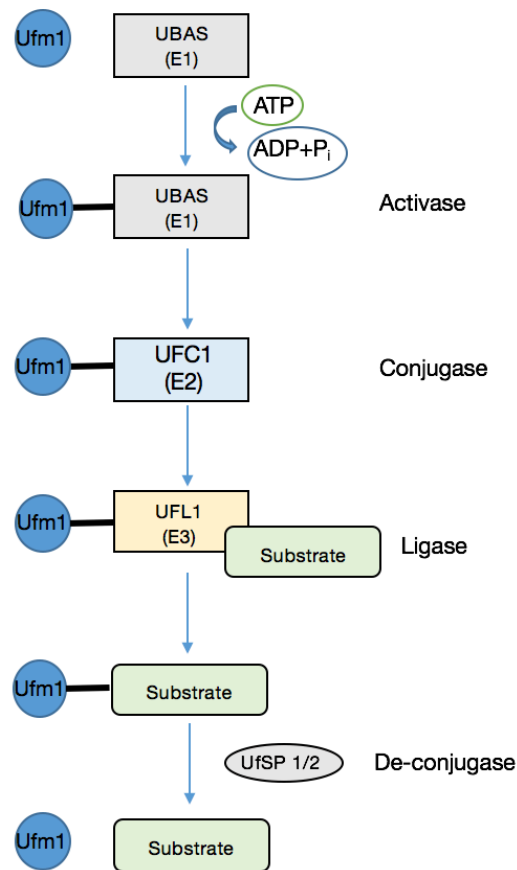
In order to understand which proteins or regulatory mechanisms could be potential candidates for innate immune regulation, we first needed to know which proteins may have a functional role at the MAM during the antiviral response. We suspected that those proteins that localized to the MAM upon activation of the RIG-I pathway were strong candidates. Interestingly, Horner et al. used a proteomic screen to implicate novel MAVS-interacting proteins organized in the MAM during virus infection that could be possible regulators of innate immune response in RNA virus infection [5]. Horner et al. examined the MAM proteome for proteins that had MAM-localization patterns during infection by two different RNA viruses that activate signaling of the RIG-I pathway: HCV and Sendai virus (SenV). Both types of RNA viral infections lead to different immune signaling responses within the host. During its infection, HCV evades the host's innate immune response by using its viral protease NS3/4A to cleave MAVS and thus inhibit downstream innate immune signaling [30]. On the other hand, SenV, does not have an inhibitory mechanism to evade host immune response and thus full RIG-I signaling downstream of MAVS is activated in SenV infection. Therefore, proteins with MAM-localization patterns during HCV infection were those at or leaving the MAM during infection. Proteins with SenV MAM-localization patterns entered the MAM during infection. The proteome consensus analysis found several proteins that commonly localized to the MAM during both types of RNA viral infection suggesting that these proteins may putatively play an integral role in virus innate immune regulation, including the protein RAB1B.

Interestingly, RAB1 has been shown to be crucial to the replication life cycle of several pathogens including legionella [17], chlamydia [3], and HCV [14]. During HCV infection, Sklan et al. determined that knocking down endogenous RAB1B (the most abundant Rab in liver cells—the target cell type of HCV) using siRNAs leads to a decrease in HCV RNA accumulation during HCV infection in the human liver hepatoma Huh7.5 cells [14]. Additionally, in non-infected cells, Rab1 depletion from cells has been demonstrated to disrupt the Golgi complex's structural integrity, the Golgi phenotype associated with HCV infection [18-20]. Similarly, the overexpression of the Rab1 GTPase-activating protein (GAP), TBC1D20 was also found to disrupt Golgi complex structure [14]. Sklan et al. suggested that during HCV infection, the combination of HCV protein NS5A and TBC1D20 might inactivate Rab1 GTPase activity, and using florescent microscopy, further assert that at sites of nascent viral replication, TBC1D20 inactivation of Rab1 GTPase activity might lead to the redirection of vesicle-trafficking towards ER-derived membranes containing viral proteins to promote virus replication. In this model, because depletion of RAB1B reduces HCV RNA accumulation, Sklan et al. hypothesizes that RAB1B, though GTPase function inactivated, is still necessary for formation of the HCV replication machinery. As discussed earlier, HCV typically evades host innate immune detection by inactivating proteins through protease cleavage or other means, and if Rab1 is a target of HCV innate immune evasion, this further implicates Rab1 as playing a crucial role during the antiviral response. We are

interested in confirming that RAB1B regulates the HCV antiviral response and if there are post-translational modifications that regulate RAB1B function during innate immunity.

Since the function of Rab1 is primarily facilitating vesicle trafficking, we were curious if the literature has ever implicated vesicle trafficking in the innate immune response and if so, how Rab1 function could possibly play a role. Interestingly, vesicle trafficking from the ER to the Golgi has been directly implicated in antiviral innate immune signaling of the RIG-I pathway. van Zuylen et al. found that during the innate immune response, TRAF3, a cytoplasmic adaptor protein for the domains of multiple receptors, interacts with p115 and Sec16A, known factors involved in vesicle trafficking [15]. Upon infection, p115 and Sec16A help localize TRAF3 to the ER-to-Golgi compartments and their depletion was shown to decrease the production of IFNs. During infection, van Zuylen et al also found that at the ER-to-Golgi compartments, TRAF3 was positioned to interact with MAVS to signal the innate immune response. Other work has implicated Rab1 recruitment of p115 to ER exit sites (ERES) for ER-to-Golgi vesicle trafficking [21-22]. TRAF3 has also been known to recruit other MAVS-interacting proteins, such as TBK1, to the MAM during infection to stimulate IFN production. Kim et al. has shown that the TRAF3/TBK1 complex formation during infection was necessary for the positive regulation of IFN [31]. If RAB1B likely is a positive regulator of IFN production, RAB1B may be responsible for this TRAF3/TBK1 complex formation. As a result, we hypothesize that RAB1B may play a role in facilitating both the TRAF3 recruitment of TBK1 to the MAM and possibly the TRAF3/TBK1 complex interaction with MAVS during infection.

To date, the literature on RAB1B does not explicitly link RAB1B to the signaling of interferon in the RIG-I pathway. However, as will be discussed in more depth later, preliminary results show that RAB1B positively regulates IFN- $\beta$  induction during viral infection. Although we found that RAB1B does have an antiviral function, we were interested to learn how RAB1B itself could be regulated during infection. We found three pieces of evidence that guided us to hypothesize a regulatory role of RAB1B for the post-translational modification called Ufm1 during viral infection. Using a sensitive protein array profile of all the substrates that are modified by ubiquitin and other ubiquitin-like modifications, Merbi et al. found that RAB1B is ufmylated during yeast mitosis. [9]. However, Merbi et al. did not describe the mechanism of Ufm1 induction or function on RAB1B. Additionally, Zhang et al, found that cells under ER stress induced by thapsargin, which results in inhibited vesicle-trafficking, upregulate the post-translational modification and other components of the Ufm1 system [16]. Because viral infection poses cellular ER stress and since RAB1B is involved in the organization of antiviral machinery at the ER, we suspect that post-translational Ufm1-modifications on RAB1B may play a putative role in the innate immune response.



**Figure 2. Ufm1 ufmylation system.** Ufm1 (dark blue) is a ubiquitin-like modification. The E1 activase protein UBAS (grey rectangle) uses ATP to attach to Ufm1. UBAS transfers Ufm1 to the E2 conjugase UFC1 (light blue). UFC1 then transfers the modification to the E3 ligase UFL1 (yellow), which binds a substrate protein of interest (green) and attaches Ufm1 to the substrate. The de-conjugase enzymes UfSP1 and UfSP12 (grey oval) can deattach the Ufm1 modification from the substrate.

Interestingly, the Horner et al. MAM proteomics study also identified that the protein UFL1, a member of the Ufm1 system, is recruited to the MAM during both SenV and HCV infection [5]. UFL1 is an E3 ligase that adds the post-translational, ubiquitin-like modification Ufm1 onto proteins. The covalent addition of Ufm1 to proteins is termed ufmylation and occurs on the lysine residues of proteins [23]. Ufmylation requires three main enzymes: E1 activase protein (UBA5), E2 conjugase protein (UFC1), and an E3 ligase protein (UFL1). The protease UFPS2 cleaves a portion of UFM1 to activate it, promoting interactions with UBA5 [23]. UBA5 then transfers UFM1 to UFC1. UFL1 then covalently attaches UFM1 to the substrate protein [13, 16, 23]. UFM1 additionally has been known to target UFBP1, and ufmylated UFBP1 has been shown to increase UFL1 activity [23]. UFL1 has also been shown to post-translationally modify ribosomal proteins and may play a role in coordinating subunit joining and mRNA interactions

[13]. The covalent attachment between UFM1 and the substrate protein can be reversed by the additional de-conjugation function of UFPS2 [23] (Figure 2).

While the mechanism of Ufm1 addition has been described in the literature, the function of the ufmylation modification has not been well-characterized. Many other post-translational modifications such as phosphorylation, ubiquitination, and neddylation covalently bind to proteins and generally mature them to direct localization behaviors, influence protein-protein interactions, or modify protein catalytic properties [11]. The process of ufmylation has been implicated during the regulation of ER homeostasis and stress response [16], regulation of hematopoietic development, embryonic development, erythroid differentiation, inhibition of apoptosis, and protein stability [9, 16, 23]. However, the influence of the ufmylation modification on proteins during these processes is poorly understood.

In order to begin to understand how RAB1B and the ufmylation of RAB1B regulate the antiviral response during innate immunity, here, I first will demonstrate that RAB1B positively regulates IFN- $\beta$  production during viral infection as shown through luciferase assays and immunoblotting. Additionally, I will also demonstrate that when both expressed individually and together, the ufmylation modification UFM1 and the E3 ligase UFL1 also positively regulate IFN- $\beta$  production, as shown through luciferase assays. We further confirm that RAB1B is ufmylated by UFM1 during viral infection in a RIG-I dependent fashion only during viral infection, as realized through immunoprecipitation and immunoblotting. We have also found a putative lysine (K) residue K170 that is ufmylated only during infection using mass spectrometry and consequently have created mutant RAB1B constructs with lysine to alanine substitutions at K170 using site-directed mutagenesis (SDM) to assess in future experiments whether the K170 residue is crucial for as a possible UFM1 target for regulation of RAB1B antiviral function. Altogether our data demonstrates a novel function of RAB1B and ufmylation in the innate immune system by positively regulating IFN- $\beta$ .

## ***Materials and Methods***

### **Cell lines and Viruses**

Human hepatoma Huh7 cells and human embryonic kidneys (HEK) 293T cells were grown in complete Dulbecco's modification of Eagle's medium (cDMEM; Mediatech) supplemented with 10% fetal bovine serum (HyClone), 2.5 mM HEPES, and 1% penicillin and streptomycin.

The 293T RAB1B KO cell line was created using clustered regularly interspaced short palindromic repeats/Cas9. The guide RNA (gRNA) sequence targeting RAB1B exon 4 was cloned into the plasmid px330. Constructs together with blasticidin vector were transfected into 293T cells by FuGENE 6. Twenty-four hours after transfection, cells were selected with blasticidin (10  $\mu$ g/ml) for another 72 hours, and single colonies were picked and propagated. Clones were identified by immunoblotting with anti-RAB1B

antibody. Genomic editing was determined by TA-cloning of the RAB1B genomic amplicons and sanger sequencing.

	Forward	Reverse
sgRNA primers	CACCgGGGGGCTCATGGCATCATCG	AAACCGATGATGCCATGAGCCCCC <sub>c</sub>
Sequencing primers	CTCAGCTGACCTGCTCCTCT	GGTGCCCAGAAGGTCTACAA

The Huh 7 RIG-I knock-out (KO) cell line was generated by deleting RIG-I using clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9). The deletion of RIG-I in this cell line was verified using immunoblotting (Christine Vasquez, unpublished).

These RIG-I KO and RAB1B KO cells were cultured as described earlier.

Sendai virus strain Cantell was obtained from Charles River Laboratory.

### Plasmids and Transfection

The following plasmids were used in this study: pEF-Tak-Flag-RAB1B and pEF-Tak-Flag-UFL1, generated by Dr. Dia Beachboard; pJLM1-Flag-UFM1, gift of Dr. Craig McCormick, Dalhousie University. The pEF-TAK-Flag-RAB1B (Horner et al., PLOS ONE, 2015) plasmid has been previously described and the pJLM1-flag-UFM1 was a gift from Craig McCormick (Dalhousie University). The plasmid encoding UFL1 was generated by cloning the PCR amplified product (UFL1, MGC cloneID- BC036379) into the pEF-Tak expression vector using NotI and PmeI (Saito et al., PNAS, 2007). All plasmids sequences we confirmed by sequencing.

The RAB1B K-to-A mutants K170A, K171A, and K194A in pEF-Tak-RAB1B were generated using site-directed mutagenesis (QuickChange Lightning kit; Stragene) using the primers in the table below:

### Primers:

	Sequence	TM
Ra1b K170A F	GCTGAAATCGCTAAGCGGATG	56.6
Ra1b K170A R	CATCCGCTTAGCGATTTTCAGC	
Ra1b K171A F	GAAATCAAAGCTCGGATGGGG	56.2
Ra1b K171A R	CCCCATCCGAGCTTTGATTTTC	
Ra1b K194A F	CCCCTGTAGCTCCGGCTGGC	65.4
Ra1b K194A R	GCCAGCCGGAGCTACAGGGG	

DNA transfections were done using FuGENE 6 (Roche).



### **IFN- $\beta$ promoter luciferase assays**

IFN- $\beta$  promoter luciferase assays were done as previously described, 18 hrs after either mock or SenV infection (1:20) in serum-free DMEM [26].

### **Immunoprecipitation**

Cell extracts were harvested in 1x ufmlyation RIPA lysis buffer (150mM NaCl, 0.5% sodium deoxycholate, 1% NP40, 50 mM Tris pH 8, H<sub>2</sub>O) supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail II (Millipore) and cleared by centrifugation. Protein samples were quantified by BioRad protein quantification assay.

Flag-tagged UFM1 samples (500ug) to be pulled down by anti-FLAG antibodies conjugated to magnetic beads (Sigma) were incubated overnight at 4°C with headover-tail rotation with the anti-FLAG beads and then washed three times in 1x ufmlyation RIPA lysis buffer and once in 1x tris-buffered saline (TBS). Following, samples were eluted by using purified FLAG peptide (100 ng/ul) and concentrated using Amicon concentration filters (3 kD cutoff) to 30 ul. Samples were analyzed by electrophoresis on 4-15% SDS-PAGE gels and Sypro-Ruby staining, and then resolved on imaging software.

### **Immunoblotting**

Cells were lysed using a modified RIPA buffer (10mM Tris [pH 7.5], 150mM NaCl, 0.5% sodium deoxycholate, and 1% Triton X-100) supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail II (Millipore). Protein sample were quantified by BioRad protein quantification assay and 10-15 ug of protein were loaded and run on a 4-15% SDS-PAGE gel and transferred to a nitrocellulose or PVDF membrane using TurboTransfer Buffer (BioRad). Membranes were blocked in StartingBlock (Thermo-Fisher) buffer and washed in PBS-T or TBS-T (for membranes probed with anti-P-IRF3). Then, membranes were incubated with primary antibodies, washed, and then incubated with species-specific horseradish peroxidase-conjugated antibodies (Jackson ImmunoResearch), treated with of ECL+ (GE Healthcare), and imaged on X-Ray film or LiCOR imaging.

### **Antibodies**

The following antibodies were used for immunoblot analysis or immunoprecipitation: anti-Flag-HRP (1:2000 dilution; Sigma), anti-IRF3 (1:1000; [24] ), anti-tubulin (1:5000; Sigma), anti-P-IRF3 (1:1000 dilution; Cell Signaling), anti-ISG-60 (1:1000; Santa Cruz), anti-Sendai Virus (1:1000; MBL), mouse-anti-HA (5 ug), anti-RAB1B (1:1000 dilution; Santa Cruz), anti-UFL1 (1:1000; [25]), anti-HA(1:1000; Abcam).

### **Mass Spectrometry (MS)**

Huh7 cells were transiently transfected with plasmids expressing Flag-RAB1B or empty vector, and then infected with SenV at 200 HAU. After 24 h, cells were lysed in IP lysis buffer (10mM Tris [pH 7.5], 150mM NaCl, 0.5% sodium deoxycholate, and 1% Triton X-100) and 500ug of lysate was immunoprecipitated with Flag-M2 beads (Sigma). Immunoprecipitated pellets were washed 3 times in IP lysis buffer before

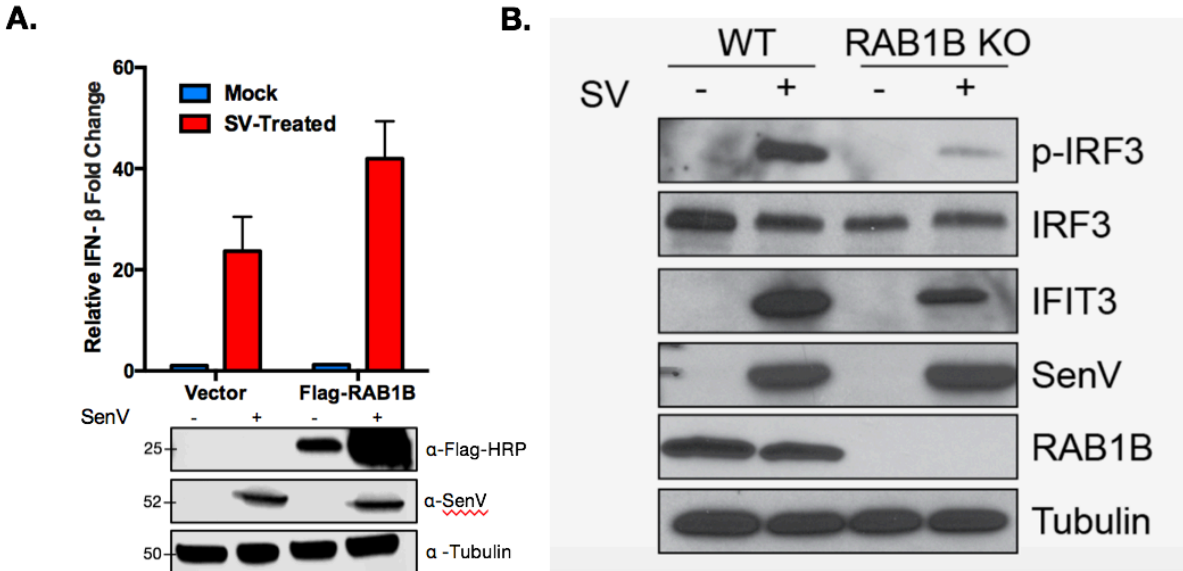
elution in Laemmli buffer for 10 min at 95 °C. Samples were then sent to the Duke Proteomics and Metabolomics Core for mass spectrometry analysis. The Core subjected Coomassie stained SDS-PAGE bands corresponding to RAB1B to standardized in-gel trypsin digestion. Extracted peptides were lyophilized to dryness and resuspended in 12 uL of 0.2% formic acid/2% acetonitrile. Each sample was subjected to chromatographic separation on a Waters NanoAquity UPLC equipped with a 1.7 µm BEH130 C18 75 µm I.D. X 250 mm reversed-phase column. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Following a 3 µL injection, peptides were trapped for 3 min on a 5 µm Symmetry C18 180 µm I.D. X 20 mm column at 5 µl/min in 99.9% A. The analytical column was then switched in-line and a linear elution gradient of 5% B to 40% B was performed over 90 min at 400 nL/min. The analytical column was connected to a fused silica PicoTip emitter (New Objective, Cambridge, MA) with a 10 µm tip orifice and coupled to a QExactive Plus mass spectrometer (Thermo) through an electrospray interface operating in a data-dependent mode of acquisition. The instrument was set to acquire a precursor MS scan from m/z 375-1675 with MS/MS spectra acquired for the ten most abundant precursor ions. For all experiments, HCD energy settings were 27v and a 120 s dynamic exclusion was employed for previously fragmented precursor ions.

Raw LC-MS/MS data files were processed in Proteome Discoverer (Thermo Scientific) and then submitted to independent Mascot searches (Matrix Science) against an SwissProt database (Human taxonomy) containing both forward and reverse entries of each protein (20,322 forward entries). Search tolerances were 5 ppm for precursor ions and 0.02 Da for product ions using trypsin specificity with up to two missed cleavages. Carbamidomethylation (+57.0214 Da on Cysteine residues) was set as a fixed modification, whereas oxidation (+15.9949 Da on Methionine residues) and ufmylation (+156.0899 Da on Lysine residues) were considered dynamic mass modifications. All searched spectra were imported into Scaffold (v4.3, Proteome Software) and scoring thresholds were set to achieve a peptide false discovery rate of 1% using the PeptideProphet algorithm.

## **Results**

### **RAB1B positively regulates IFN-β**

To determine whether RAB1B could be a regulator of IFN- β signaling, I over-expressed FLAG-RAB1B in 293T cells and subjected samples to either mock or SenV infection and used a luciferase assay to measure the IFN- β promoter activity through the proxy of IFN- β promoter luciferase reporter gene expression. I found that 18 hours post-infection, SenV infected samples of RAB1B had an approximate two-fold increase in IFN- β promoter activity as compared to vector infected samples (Figure 3). This suggests that during SenV infection, RAB1B is a positive regulator of IFN-β signaling.



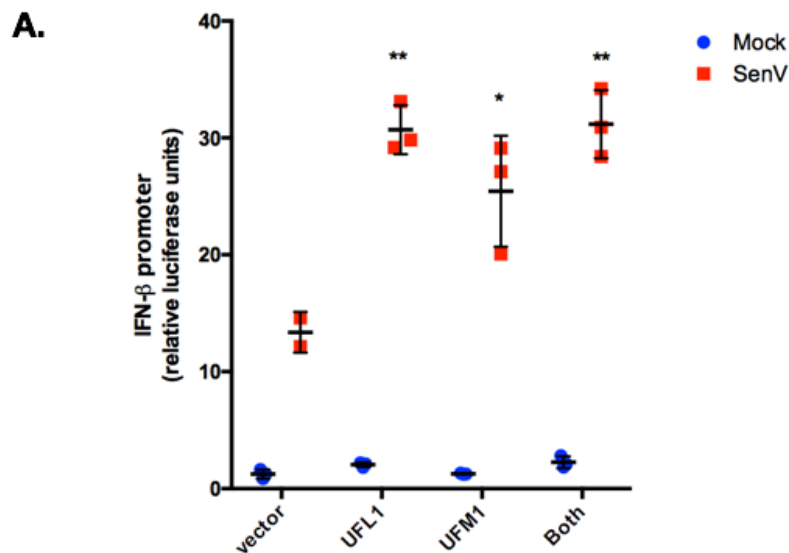
**Figure 3. RAB1B positively regulates IFN-β induction.**

A. (Above) Luciferase assay measures IFN-β promoter activity in 293T cells mock or SenV infected, 18 hours post infection. (Below) α-Flag-HRP immunoblotting detects the vector and FLAG-RAB1B plasmid expression. B. Lysates from WT and RAB1B knock-out (KO) 293T cells, mock or SenV infected, collected 18 hours post-infection were immunoblotted for p-IRF3, IRF3, IFIT3, SenV, RAB1B, and tubulin.

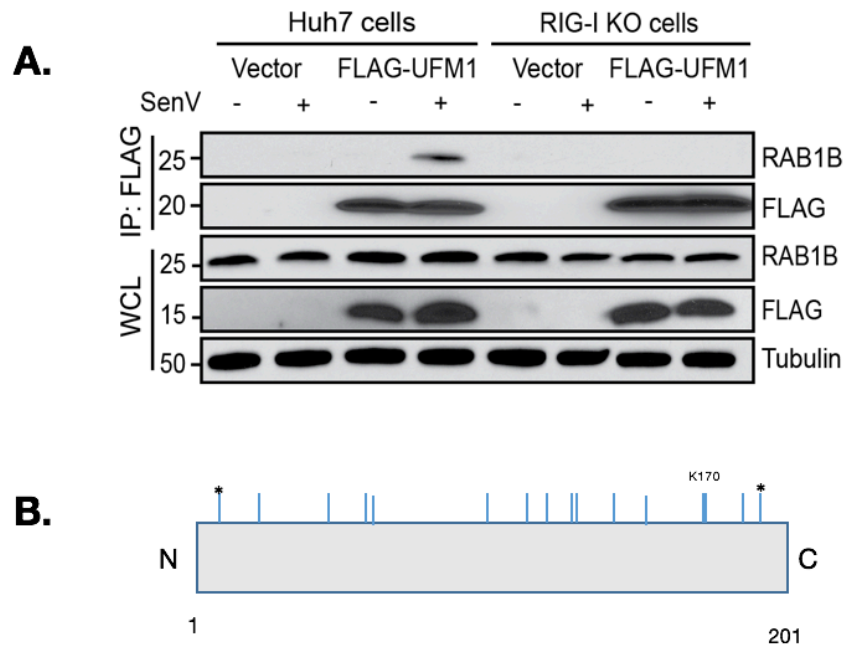
Since RAB1B was found to be a positive regulator of IFN-β signaling, I next wanted to determine how loss of RAB1B in cells affected IFN-β signaling. I subjected both WT 293T cells and RAB1B KO 293T cells to either mock or SenV infection and harvested lysates at 18 hours post infection. I then immunoblotted their lysates for RAB1B and other proteins in the RIG-I pathway: the unphosphorylated and inactive IFN-B transcription factor IRF3; the activated, phosphorylated IRF3; the interferon stimulated gene IFIT3 (known to be signaled downstream of IFN-B) (Figure 3B). Immunoblotting verifies the loss of RAB1B expression in the KO cells. Further, the results indicate that in cells that lack expression of RAB1B, there is less expression of the ISG IFIT3 and less phosphorylation of the transcription factor IRF3. Interestingly, when I blot for IFIT3, the RAB1B KO SV infected samples show an increase in SV protein production compared to WT SV (Figure 3D) and a decrease in the interferon induced IFIT3 as compared to WT SV infected samples. This suggests that RAB1B is working upstream of the ISG production and is necessary for mitigating the production of SV viral proteins. Interestingly, there is a significant decrease in the presence of IFN-B transcription factor p-IRF3 in infected RAB1B KO cells as compared to infected WT cells, while the amount of IRF3 remains qualitatively constant across samples. Because RAB1B deletion reduces production of p-IRF3 and IFIT3, this also suggests that RAB1B reduces IFN-β induction during SenV infection and demonstrates that RAB1B may regulate IFN-β induction somewhere upstream of p-IRF3 in the RIG-I pathway. Taken together, RAB1B is necessary for the positive regulation of the RIG-I pathway during SenV infection.

## UFL1 and UFM1 positively regulate IFN- $\beta$

The literature has shown that the Ufm1 system is upregulated during ER-stress conditions in the ER, and during the innate immune response, the E3 ligase UFL1 (which adds Ufm1 to substrate proteins) localizes to the MAM, where the antiviral machinery for innate immunity is organized [5,13]. To understand if the ubiquitin-like Ufm1 modification and UFL1 regulate IFN-  $\beta$  induction, in 293T cells, I overexpressed Ufm1 and UFL1 individually and also co-expressed Ufm1 and UFL1 in either mock or SenV infection conditions. Using the luciferase assay which measures the amount of IFN-  $\beta$  promoter activity, I saw an approximate 2.5 fold increase in SenV infected UFL1 samples and ~2.08x increase in SenV infected UFM1 samples as compared to the amount of IFN-  $\beta$  signaling in SenV infected vector samples (Figure 4). This demonstrates that both UFL1 and UFM1 individually are positive regulators of IFN-  $\beta$ . Interestingly, the co-expression of UFM1 and UFL1 resulted in a similar amount of signaling activity to IFN-  $\beta$  as either UFL1 or UFM1 alone, indicating that there might be a maximum upper limit to the amount of IFN-signaling that the Ufm1 system can induce. I will further discuss the implications of this max threshold of IFN-B signaling later in the discussion.



**Figure 4. UFL1 and UFM1 positively regulate IFN-  $\beta$ .** A. Luciferase assay measures IFN-  $\beta$  promoter activity in samples where vector, UFL1, UFM1, and UFL1+UFM1 were overexpressed in 293T cells and subjected to mock or SenV infection. (18 hours post infection). Individual data points are shown for each sample, as well as the mean and standard deviation. All statistics were done using ordinary one-way ANOVA using corrections for multiple comparisons. All experimental points were compared to vector SenV. (p-values: \* =  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*= $p < 0.001$ )



**Figure 5. RAB1B is ufmylated.** A. WT or RIG-I knock out (KO) Huh7 cells were transfected with vector or FLAG-UFM1 and mock or SenV treated. Lysates of these samples were harvested 18 hours post-infection and immunoprecipitated for FLAG and immunoblotted for RAB1B and FLAG. B. A linearized depiction of the RAB1B protein. The blue lines depict the location of a lysine (K) residue on RAB1B. K170 was characterized by mass spectrometry as conjugated to UFM1 during SenV infection 18 hours post-infection. Currently, the residues marked by \* (K10 and K194) have currently not been characterized for ufmylation during infection.

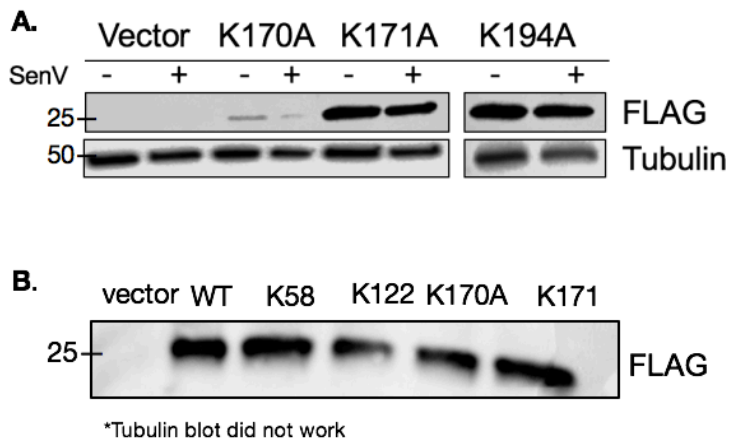
*Figure A. courtesy of Dr. Dia Beachboard and  
Figure B. modified from Dr. Dia Beachboard.*

## RAB1B is ufmylated during SenV infection

Since the literature has characterized RAB1B as being ufmylated with the modification Ufm1, and I have shown that Ufm1 and UFL1 both positively regulate IFN- $\beta$  induction, the lab tested whether RAB1B was ufmylated during SenV infection. FLAG-UFM1 or vector was overexpressed in either WT Huh 7 cells or Huh7 RIG-I KO cells, and both cell types were infected with either mock or SenV treatment. The proteins bearing the FLAG protein motif were pulled down using immunoprecipitation for each sample. When immunoblotting for anti-FLAG in the immunoprecipitation samples, we see that FLAG-UFM1 is found in the mock and SenV treated WT and RIG-I KO cells (Figure 5). However, when RAB1B is immunoblotted, we only see enrichment of RAB1B in SenV-treated WT sample and not in the SenV infected RIG-I KO sample. This suggests that RAB1B is ufmylated by Ufm1 only during SenV infection, and RAB1B ufmylation requires activation from RIG-I.

Because RAB1B has been shown to be ufmylated during SenV infection, we tried to determine on which lysine residues RAB1B is ufmylated. FLAG-RAB1B was overexpressed in Huh7 cells and infected with either mock and SenV. Using immunoprecipitation for FLAG, FLAG-RAB1B and their protein binding partners in both mock and SenV infection were purified using SDS-PAGE gel and visualized by coomassie staining of the gel (Figure 5). These samples were sent for mass spectrometry analysis to the Duke Proteomics and Metabolomics Core and based on the limited coverage of analysis, K170 was found to be ufmylated only during SenV infection. Because trypsin was used as the enzyme used to fragment RAB1B prior to mass spectrometry, there is one lysine each on the N and C terminus of RAB1B that remains uncharacterized for ufmylation. The experiment to characterize these remaining lysine residues is currently in progress, but the results of the mass spectrometry may not be received prior to the deadline of this thesis. Regardless, K170 is a putative residue that is involved in regulating the induction of IFN-  $\beta$  by RAB1B during infection.

### RAB1B K to A mutants express



#### Figure 6. FLAG-RAB1B K to A mutants express.

FLAG-RAB1B lysine (K) to alanine (A) mutants were created using site-directed mutagenesis (SDM) and immunoblotting for  $\alpha$ -Flag-HRP determines these mutant constructs express in 293T cells as shown in A and B.

As a result of knowing that at least K170 on RAB1B is ufmylated during SenV, I used site-directed mutagenesis to change the K170 of RAB1B into an alanine (A). The choice to mutate the lysine to an alanine was made because the R-group of lysine is a long hydrocarbon tail that possess a positively charged, terminal  $\text{NH}_3^+$  group. The alanine R-group is simply a methyl group to eliminate both normal properties of lysine residue due to its the stereochemistry and charged nature. Additionally, mutation of K170 is

not one of the known mutations of RAB1B (S22N, Q67L, N121I) noted in the literature that effectively impedes the domains that allow RAB1B to function as GTPase, vesicle trafficking protein [28, 29]. Additional RAB1B mutant constructs for FLAG-K194A and FLAG-K171A were created on similar logic for possible future experimentation to also be discussed later.

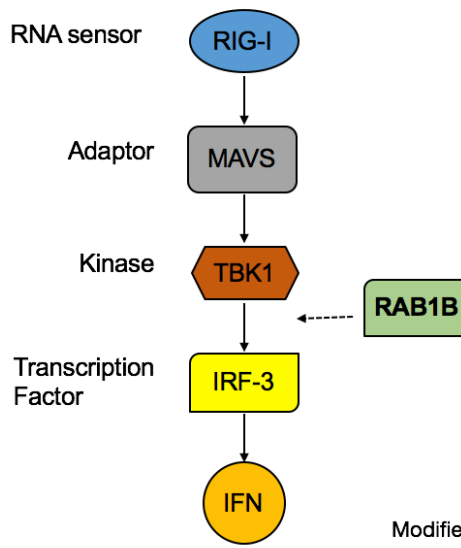
To clone these K to A mutant RAB1B constructs, I performed PCR on pEF-Tak-RAB1B plasmids using primers (see Methods) that created amplified sequences of the K to A substitution on the codon for residue 170 of RAB1B. These PCR products were incubated with DpnI at 37°C for 5 minutes and then transformed into ultracompetent bacterial cells by first incubating on ice for 30 minutes and then heat shocking at 42 °C for 30 seconds. Following an hour long incubation at 37 °C in SOC media, the bacterial cells were plated on LB plates with an ampicillin selection factor. Putative clones from the growing colonies were grown in 3 ml LB and ampicillin media culture, mini-prepped, and sequence verified. These mutants were overexpressed in 293T cells and harvested lysates were immunoblotted for anti-FLAG. The presence of FLAG indicates that these mutant RAB1B constructs express and all possess the same molecular weight as WT at 25 kDa (Figure 6). In Figure 6A, K170A appears to express less than the other mutants during mock and SenV infection conditions, but this expression was done with an old dilution of the plasmid midiprep that has been freeze thawed several time for other experimentation. However, in Figure 6B, the expression immunoblot done immediately after cloning shows that K170A expresses in 293T cells similarly to other K to A mutants and WT RAB1B. (Note the tubulin blot for Figure 6B did not work.)

## ***Discussion***

Although RAB1B and members of the Ufm1 system have previously been reported to be recruited to the MAM during viral infection, RAB1B and the post-translational ufmylation modification UFM1 have never directly been shown to regulate the innate immune system. In my study, I demonstrate that RAB1B and ufmylation both individually play a regulatory role in activation of the innate immune system.

**Figure 7. Overview of Innate Immune RIG-I Pathway**

This diagram illustrates the RIG-I pathway. The solid arrows indicate known steps of the pathway and the dashed line indicates where it is hypothesized RAB1B acts during the pathway.



Modified from Dr. Stacy Horner

I first show that RAB1B positively regulates IFN- $\beta$  during SenV infection (Figure 1), and while I have identified that RAB1B regulates IFN- $\beta$  signaling upstream of p-IRF3 in the RIG-I pathway, we also want to determine at which regulatory step and with which proteins RAB1B interacts to regulate IFN- $\beta$  induction. One step of interest is during MAVS interactions with kinase TBK1 (Figure 7). During infection, TBK1 is activated when adaptor proteins like MAVS recruit TRAF E3 ubiquitin ligases to activate IFN-specific TBK1 functions through polyubiquitination [31]. Activated TBK1 then phosphorylates other serine and threonine clusters domains of upstream adaptor proteins like MAVS, which recruit IRF3 to the MAM innate immune complex by binding to its positively charged surfaces [31]. TBK1 then activates IRF3 by phosphorylation to be an IFN transcription factor. Since RAB1B has been known to interact with TRAF-associated proteins during cellular conditions similar to infection, we hypothesize that RAB1B could possibly regulate IFN- $\beta$  by being responsible for interacting with TRAF in some way to promote the TRAF-dependent TBK1/MAVS interactions during antiviral signaling. Based on the literature, RAB1B regulation of TRAF-TBK1 interactions seem promising because p115 and Sec16A localize TRAF3 to the ER to Golgi compartments during infection [15]. Since p115 is known to be recruited to the ER-to-Golgi sites by RAB1 [21,22], it is likely that RAB1B indirectly, through p115, helps recruit TRAF to the MAM which facilitates downstream TBK1/MAVS interactions for the innate immune response. To test these hypotheses, in the future, we could immunoblot lysates from WT and RAB1B KO cells with and without SenV infection for p-TBK1. If p-TBK1 is found in RAB1B KO SenV infected samples, RAB1B does not influence the autophosphorylation of TBK1 and likely is involved in TRAF3-related organization of antiviral machinery at the MAM. Additional experiments to assess the function of RAB1B in the TRAF-dependent TBK1-MAVS interactions could also include using confocal microscopy in WT and RAB1B KO cells to immunostain TRAF3, TBK1, and RAB1B (only in WT) and visualize the co-localization of these proteins during SenV



infection. We would expect in RAB1B KO cells, TRAF3 would not be recruited to the ER-to-Golgi compartments. Further, these interactions can be resolved biochemically, using immunoprecipitation to see if TRAF3 still interacts with TBK1 in RAB1B KO cells during infection. If all of these methods determine that RAB1B is not involved with TRAF-TBK1 interactions (a phenotype of continued TRAF-TBK1 interactions in RAB1B KO infected samples), the lab will use immunoprecipitation to determine if RAB1B is involved in TBK1-MAVS interactions or TBK1-IRF3 interactions.

While understanding how RAB1B functions during infection will give insight into how the antiviral machinery organizes itself at the MAM to signal to IFN- $\beta$ , we also wanted to determine what regulates RAB1B function during viral infection and the antiviral response. We found that ufmylation has a novel, positive regulatory function during IFN- $\beta$  induction (Figure 4), and knowing that RAB1B has been previously reported to be ufmylated and that UFL1 localizes to the MAM during SenV infection, this supports the idea that RAB1B ufmylation could be responsible for RAB1B regulation of IFN- $\beta$ . The lab has additionally found that RIG-I signaling is necessary for RAB1B ufmylation during infection (Figure 5). I have also found that both the ubiquitin-like modification UFM1 and the E3 ligase UFL1, which adds UFM1 to a substrate protein, positively regulate IFN- $\beta$  production during SenV (Figure 4). These together suggest that ufmylation of RAB1B likely regulates the innate immune function of RAB1B. In the context of UFM1 system, I hypothesize UFL1 likely ufmylates RAB1B during infection, which could be further confirmed by using siRNAs to knock down UFL1 in SenV infected cell lysates that overexpress FLAG-UFM1, immunoprecipitating FLAG, and then immunoblotting for endogenous RAB1B and FLAG. If UFL1 ufmylates RAB1B during SenV infection, I expect that FLAG-UFM1 and RAB1B will not co-immunoprecipitate in infected samples treated with UFL1 siRNA. Additionally, we could use a luciferase assay to measure signaling to IFN in SenV infected cells with UFL1 knocked down using siRNAs. If UFL1 is necessary for regulating IFN-induction through modification of RAB1B, we would expect to see decreased levels of IFN-signaling. If we overexpress UFL1 in SenV infected RAB1B KO cells, using a luciferase assay, we would additionally expect decreased signaling to IFN- $\beta$  as compared to UFL1 overexpression in WT cells.

Interestingly, the co-expression of UFL1 and UFM1 does not lead to increased levels of IFN- $\beta$  signaling than either UFL1 or UFM1 alone (Figure 4). This suggests that there is a threshold level of IFN- $\beta$  signaling that can be induced by UFM1 and UFL1 and this signaling could be regulated by a negative-feedback loop by the UFM1 deconjugase UFPS2, which removes the UFM1 modification from an ufmylated substrate. Negative-feedback loops involving ubiquitination are well reported in the literature [27]. For the UFM1 system, which adds an ubiquitin-like modification, there is a likelihood that the deconjugase UFPS2 might cleave the portion of UFM1 conjugated to the protein of attachment. I hypothesize that there must be some increase of a protein or complex that is detected by the cell and switches the function of UFPS2 or that UFPS2 conjugase and deconjugase activity are both present and the sheer increase in UFM1-protein concentration allows for UFPS2 deconjugase activity. Further, if UFPS2 was

knocked down or the region of UFM1 that interacts with UFPS2 was deleted from the protein, we would see expect to see uninhibited signaling to IFN- $\beta$  that is greater than levels seen in the overexpression of UFL1 or UFM1 alone.

Additionally, as the mass spectrometry results reveal that at least K170 of RAB1B is uniquely ufmylated during SenV infection, I also want to determine whether K170 and other possible sites of RAB1B ufmylation individually positively or negatively enhance RAB1B function in the innate immune pathway. Ubiquitination in the RIG-I pathway is well-known to increase/decrease protein function in the IFN- $\beta$  cascade. For example, linear polyubiquitination of RIG-I has been shown to decrease enhancement of RIG-I innate immune function and that TRIM25 ubiquitination of RIG-I on K172 is necessary for MAVS interactions and downstream signaling in the IFN- $\beta$  cascade [27]. Consequently, it is unknown whether each UFM1 addition to RAB1B acts as a positive regulator of RAB1B induction of INF- $\beta$ . We also still are not aware how UFM1 modifications regulate RAB1B function in the innate immune system whether it enhances antiviral function, inhibits antiviral function, or the combination of both. Therefore, our current understanding of how ufmylation positively regulates IFN- $\beta$  signaling, simply refers to the net effect of ufmylation on innate immune signaling.

In preparation for knowing which lysine residues on RAB1B are ufmylated, I created individual lysine to alanine mutations of RAB1B at K residues 170, 171, and 194 using SDM. The selection of these specific residues for mutation was framed to test the function of RAB1B ufmylation on K170. K170A is a mutation that should inhibit ufmylation at that residue. The K194A mutation controls for the effect of any mutation on RAB1B antiviral function; and this mutation is not known to affect RAB1B function. K171A controls for fact that K170A is the specific ufmylated lysine necessary for antiviral function. Importantly, each of these mutant constructs (K170A, K171A, K194A) expresses and is soluble (Figure 6). In the future, to determine the functionality of the mutant constructs, whether the K170A mutation of RAB1B increases or decreases signaling to IFN- $\beta$ , I will set up a luciferase assay to measure IFN- $\beta$  promoter activity for cells overexpressing K170A, K171A, K194A in mock/SenV infection conditions. Because RAB1B deletion leads to similar Golgi phenotype exhibited during HCV infection [14]; RAB1B appears to be positively enhancing signaling to IFN- $\beta$  during infection; and ufmylation appears to have a net positive regulation of IFN- $\beta$  signaling, I hypothesize that ufmylation of RAB1B matures RAB1B for innate immune specific function. Further, based on preliminary data from the lab, I expect that K170A will decrease IFN- $\beta$  signaling and that RAB1B ufmylation promotes IFN- $\beta$  signaling.

For future directions, we hope to determine how exactly ufmylation regulates RAB1B function in signaling IFN- $\beta$  during the antiviral response. As discussed previously, post-translational modifications can change protein-protein interactions, protein localization, and protein catalytic properties, among several changes to protein function. Because I hypothesize that RAB1B facilitates TRAF-dependent TBK1/MAVS interactions during infection, ufmylation of RAB1B likely positively promotes p115 trafficking of TRAF to

the MAM and relocalizes RAB1B to the MAM. Assuming that our experiments show that RAB1B is necessary for TRAF trafficking to the MAM during infection, to test this, we could first do confocal microscopy in WT and cells expressing RAB1B with all ufmylation residues mutated and immunostain for RAB1B, UFM1, and TRAF and visualize the co-localization of these proteins during SenV infection. We would expect that in cells expressing the RAB1B mutant construct, TRAF3 and RAB1B would not be recruited to the ER-to-Golgi compartments. Additionally, If we determine that TRAF binds to TBK1 during infection due to RAB1B, to test whether ufmylation is necessary for TRAF-TBK1 interactions, we could use immunoprecipitation to see if TRAF still interacts with TBK1 in cells expressing RAB1B with mutations at all ufmylation sites. We would expect to see TBK1 to not co-immunoprecipitate with TRAF expressing the mutant RAB1B construct. These results together would hopefully demonstrate that ufmylation of RAB1B is necessary for TRAF-TBK1 interactions.

Overall, I have demonstrated a novel function of RAB1B in the innate immune system by positively regulating IFN- $\beta$ . I have also identified a novel post-translational modification UFM1 that conjugates to RAB1B during infection and have proposed consequential experiments to test for how UFM1 and ufmylation regulates RAB1B function in the antiviral RIG-I pathway.

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