TOWSON UNIVERSITY
COLLEGE OF GRADUATE STUDIES AND RESEARCH

# KSHV VFLIP INTERACTS WITH THE ITCH/A20 UBIQUITIN-EDITING COMPLEX VIA A SUMO-DEPENDENT MECHANISM

by

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**THESIS APPROVAL PAGE**

This is to certify that the thesis prepared by Kevin Herold entitled KSHV vFLIP interacts with the Itch/A20 ubiquitin-editing complex via a SUMO-dependent mechanism, has been approved by his committee as satisfactory completion of the requirement for the degree of Master of Science.

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ABSTRACT

The Kaposi's Sarcoma Herpesvirus VFLIP Interacts With Itch/A20 Ubiquitin-Editing Complex Via a SUMO-Dependent Mechanism

Kevin G. Herold

KSHV is known to cause Kaposi Sarcoma (KS), and has been implicated in two B-cell lymphoproliferative disorders. We have previously demonstrated that expression of RTA, the lytic switch protein, results in the proteasomal degradation of vFLIP (Viral FLICE Inhibitory Protein) and down regulation of NF-κB associated gene expression early in lytic reactivation. We have also demonstrated that RTA interacts with the cellular Itch/A20 complex and that Itch and A20 are required to induce degradation of vFLIP. Here we provide evidence for vFLIP interaction with Itch and A20 via a SUMO-dependent mechanism. We have identified a SUMO interaction motif (SIM) in vFLIP through sequence analysis and demonstrate that a SIM deficient vFLIP cannot interact with SUMO. SIM deficient vFLIP is unable to activate NF-kB. Preliminary studies suggest that Itch is SUMOylated and cannot interact with SIM-deficient vFLIP. This suggests that the Itch/A20 complex may interact with vFLIP via a SUMO-dependent mechanism.

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# CHAPTER I:

# INTRODUCTION

Kaposi's sarcoma (KS) is a type of cancer that is most commonly characterized by the development of dark purple lesions on the skin. The disease has four distinct clinical variants that are based upon the level of immunosuppression and the severity of the infection, with the AIDS-related form of KS being particular severe[1]. In this form, the afflicted can experience disfigurement, hemorrhaging, gastrointestinal bleeding, and respiratory failure[2]. Though the contracting of AIDS results in an increased risk of KS, HIV infection alone does not result in the development of KS. For KS to occur, infection by the KS-associated herpes virus (KSHV) is necessary. KSHV is also associated with two B-cell lymphoproliferative disorders:Multicentric Castleman's Disease (MCD) and Primary Effusion Lymphoma (PEL)[3]. KSHV genomes have been detected in nearly all cases of HIV-seropositive PEL and MCD patients and in 50% of HIV-seronegative MCD cases.

KSHV is one of 130 herpesviruses that have been identified, and out of those 130, only eight are found to infect humans. These 130 herpesviruses are divided into 3 subfamilies: alpha, beta, and gamma. Each of the subfamily is classified according to their genome, biological characteristics and their structural organization. The gamma herpesviruses, of which KSHV is a member, are known for establishing periods of lasting latency in the host, which are punctuated by periods of short intermittent lytic replication[4]. In a KSHV-infected host, most cells are latently infected, with only a small population, about three percent, lytically infected[5]. The KSHV primarily infects lymphoid cells but has also been known to infect monocytes and endothelial cells[6].

After the release of the viral genome into the host cell, the KSHV establishes latency, which begins with the rapid circularization of the viral genome into an episome, a small circular piece of DNA anchored to a chromosome in the nucleus. Only a small subset of genes from the episome are expressed, whose purpose is to promote cell survival, persistence of the viral genome, and evasion of the host immune response. KSHV dedicates a large portion of its genome to evade detection by the host[7]. There are viral interferon (IFN) regulatory factors that downregulate IFN signaling in the host and thus prevent the activation of immune cells, anti-apoptotic proteins, and miRNAs that regulate innate antiviral immunity during latency. During lytic replication, all viral genes are expressed resulting in the regulation of the DNA damage response, angiogenesis, the promotion of cell proliferation, and the shutdown of host cell protein synthesis[2,7]. The expression of all viral genes will also lead to the assembly of the virus and the release from the infected cell. It is unclear, which stimuli result in the switch from latency to lytic replication, but it is suspected that hypoxia, reactive oxygen species and inflammatory cytokines are involved[8,9]. Chromatin regulatory factors appear to be involved that relax the compact chromatin structure of the episome, allowing for all viral genes to be expressed[10].

Nuclear factor-kappa B (NF-κB) signaling plays an integral part in promoting latency in KSHV. High levels of the transcription factor NF-κB result in the inhibition of gamma herpesvirus lytic promoter activation, lytic protein synthesis and viral replication. One of the proteins encoded by KSHV in latency is the viral FLICE inhibitory protein (vFLIP) which is a homolog of cellular FLIP[8]. The FLIP proteins are well known as inhibitors of death receptor-induced apoptosis, with apoptosis being a common response

to limit viral infection[11]. This inhibition is accomplished by binding to the adapter proteins TRADD and FADD, and preventing the recruitment and activation of caspase 8 whose activation cascade results in apoptosis[12]. It is known that vFLIP results in the activation of NF-κB and the expression of inflammatory cytokines such as interleukin 6 (IL-6) and genes that promotes KSHV latency[13].

The expression of KSHV vFLIP results in significant activation NF-κB in multiple cell types[14]. Chaudhary et al. (1999) suggested that vFLIP interacts with the IκB kinase (IKK) complex and that this interaction may be necessary for vFLIP to activate the NF-κB pathway. This kinase complex has been found to induce phosphorylation of IκB proteins, which are inhibitory proteins that bind to the NF-κB transcription factor and prevent it from localizing to the nucleus. When IκB is phosphorylated it is ubiquitinated and degraded by the proteasome, allowing NF-κB to activate transcription of NF-κB dependent genes[11]. In previous studies, KSHV infection led to the constitutive phosphorylation of IκB and vFLIP was found bound to the IKKy subunit of the activated IKK complex[11]. This suggests that vFLIP is activating the IKK complex, leading to the activation of NF-κB and the transcription of genes that promote KSHV latency.

Recent data gathered in the Ehrlich lab provide evidence that vFLIP may bind to the proteins A20 and Itch. Together, Itch and A20 form an ubiquitin-editing complex that modifies the ubiquitination of proteins essential to NF-κB activation such as Receptor-interacting protein 1 (RIP1). This complex is also necessary for the lytic activator protein, RTA, to induce degradation of vFLIP, enhancing lytic reactivation. Itch is a E3 ubiquitin ligase that is required for the deactivation of NF-κB signaling and is necessary for the recruitment of A20 to RIP1[15]. A20 is a zinc finger protein and E3 ligase that is expressed

with the activation of NF-κB[16]. A20 is known to downregulate NF-κB through two ubiquitin-editing domains. This downregulation occurs through the removal of K63-linked ubiquitin chains from RIP1 using the N-terminal domain of A20 and the conjugation of K48-linked ubiquitin chains to RIP1[17]. The K48-linked ubiquitin chains will signal for the degradation of RIP1 at the proteasome, which prevents further NF-κB from being activated.

RIP1 is recruited to the tumor necrosis factor receptor 1 (TNFR1) with the binding of the ligand TNFα, and serves as a regulator of inflammation, necroptosis and apoptosis. When RIP1 is modified with K63-linked polyubiquitin, it acts as a docking site for NF-κB essential modulator (NEMO or IKKγ) which will lead to the activation of NF-κB. Though RIP1 exhibits kinase activity, this kinase activity is not required for the activation of the NF-κB transcription factor[18].

Data collected from the Ehrlich Lab had demonstrated that when A20 is immunoprecipitated, vFLIP is co-immunoprecipitated, which indicates that vFLIP is interacting with A20. Additionally, when vFLIP is immunoprecipitated, it results in the co-immunoprecipitation of Itch, suggesting that vFLIP interacts with Itch. These interactions were observed even in the absence of RTA. Additionally, vFLIP was found to be stable in the presence of RTA when Small Ubiquitin-related Modifier (SUMO) interaction motifs (SIMs) were mutated in vFLIP. Additionally, vFLIP SIM mutants were unable to activate the NF-κB pathway.

These preliminary data provide evidence for vFLIP manipulation of the NF-κB pathway in latency as vFLIP was found interacting with NF-κB proteins in the absence of RTA. Since Itch and A20 are known to remove K63-linked ubiquitin and add K48-linked

ubiquitin, it is possible that vFLIP may be interfering with that activity in order to keep the NF-kB pathway active. Also interaction with SUMOylated proteins may be an important component for vFLIP to keep the NF-κB pathway active.

SUMOylation involves the enzymatic conjugation of SUMO proteins to a substrate protein. These SUMO proteins are approximately 10kD in size, and there are four isoforms (SUMO 1-4) encoded by the human genome. SUMO modification can serve in the recruitment of proteins as part of a complex, prevent interaction with SUMOylated proteins, or result in a change in the conformation of the SUMOylated protein[19]. Though ubiquitination and SUMOylation utilize a cascade consisting of three classes of enzymes (E1 activating enzymes, E2 conjugating enzymes, and E3 ligases), these enzymes have no overlap in how they are utilized. There are three general consequences of SUMOylation: interference between a protein and its binding partner, inducing a conformational change in the target protein, and providing a binding site on a protein for another protein[20].

Because previous data collected in our lab demonstrated that A20 and Itch interact with vFLIP in the absence of RTA, and that vFLIP may have a SIM, I hypothesized that vFLIP interacts with the Itch/A20 ubiquitin-editing complex in a SUMO-dependent manner. My thesis is focused on determining whether the mechanism by which vFLIP interacts with Itch and A20 is one that is SUMO-dependent and what impact that interaction may have on the NF-κB pathway; a pathway that is essential for KSHV to maintain a state of latency in the host cell. My thesis consists of three aims, the first of which was to confirm that vFLIP has a SIM and that vFLIP can interact with SUMO proteins. For my second aim I wanted to determine whether Itch and A20 were

SUMOylated. Lastly, for my third aim I wanted to determine whether the vFLIP SIM was necessary for vFLIP interaction with Itch and A20. It is known that the KSHV genome includes viral oncogenes that have the potential to induce KS and that during latency genes are expressed that inactivate tumor suppressor pathways[2,3]. Thus by elucidating the interaction between vFLIP and the ubiquitin-editing complex, a better understanding of how KSHV contributes to carcinogenesis can be reached.

Here I provide evidence that vFLIP has a SIM site that is necessary for vFLIP to interact with SUMO 1 and SUMO 2/3. Additionally, I demonstrate that Itch and A20 are SUMOylated by SUMO 1 and SUMO 2/3, and interaction of vFLIP with A20 and Itch is SIM dependent. Taken together, these data suggest that vFLIP interacts with Itch and A20 via a SUMO-dependent mechanism. Lastly, I provide evidence that vFLIP affects the ubiquitination of RIP1, which could be an effect of vFLIP interacting with the Itch/A20 ubiquitin-editing complex.

# CHAPTER II:

# MATERIALS AND METHODS

## *Cell Line Maintenance and Transfection*

Human Embryonic Kidney 293T (HEK 293T) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and were grown at 5% $CO_2$ at 37°C. Cells were transfected at 60-70% confluency using 1µg/mL polyethyleneimine (PEI) at a ratio of 1µg plasmid DNA: 3µl PEI. After 5 min of incubation, the mixture was added to the cells. 24h post transfection the medium was changed and, if appropriate, 2.5µM of MG132 were added.

## *Reagent, Plasmids, and Antibodies*

The proteasome inhibitor MG132 (Boston Biochem) was used to prevent RIP1 degradation. FLAG-A20, was provided by Ed Harhaj[15], FLAG-Itch was provided by Annie Angers, and myc-vFLIP by Gary Hayward[21]. The following primary antibodies were used: anti-RTA (G. Hayward), anti-cmyc (Millipore), anti-Itch (BD Transduction Laboratories), anti-Flag (Sigma-Aldrich), anti-A20 (BD Transduction Laboratories), and anti-GFP (Thermo Scientific). The secondary antibodies used were anti-mouse-HRP, anti-rabbit-HRP, and anti-mouse-AP (Jackson ImmunoResearch).

## *Immunoblot Analysis*

Proteins were separated on 12%-PAGE or Any kD mini-PROTEAN Precast Gel (Biorad) with Tris-glycine running buffer. The proteins were then transferred to a PVDF membrane using a semi-dry transfer system at 20V for 20 minutes. The membranes were blocked in 5% non-fat dry milk for one hour. Primary antibodies were diluted in with 2.5% non-fat dry milk at 1:1000 and applied to the membranes. The membranes were

incubated on a shaker at 4°C overnight and were washed in PBS with 0.1% Tween the following day. Secondary antibodies were applied to the membranes in 2.5% non-fat dry milk at 1:1000. The membranes were incubated at room temperature on a shaker for one hour and afterward were washed with PBS and 0.1% Tween. Proteins were visualized with the addition of enhanced chemiluminescence (ECL) substrate and the detection of the luminescence on x-ray film or scanned by a Li-COR C-DiGit Blot Scanner.

*Immunoprecipitation*

Transfected cells with appropriate constructs were harvested 48 hours post-transfection with phosphate-buffered saline (PBS) and centrifuged at 1500 rpm for 10 minutes. The PBS was removed and 1mL of lysis buffer with 10µl of a protease inhibitor cocktail kit (Thermo Scientific) was added to each cell pellet. When appropriate 12.5µL of 5 mM NEM were added to each cell pellet. Cell lysates were centrifuged at 9,700 rpm for 5 minutes to remove cell debris. The resulting supernatant was precleared with protein A/G PLUS-agarose (Santa Cruz) for 30 min at 4°C. The lysates were transferred to a new 1.5mL tube and their protein concentrations were measured and normalized with a Pierce BCA protein assay kit. Approximately 50 µg of protein were transferred to new 1.5mL tube to serve as control lysate. One µg of the appropriate primary antibody was added to the remaining cell lysate and incubated on a rotator overnight at 4°C. 25µL of protein A/G-agarose were added the following day for 1 hour with four subsequent washings of RIPA lysis buffer. 50µl of 2X Laemmli Buffer were added and samples were boiled followed by incubation at 100°C for 10 minutes. Samples were visualized through immunoblot analysis as described above.

*GST-Pull-down Assay*

A 5mL culture of BL21 cells that could be induced to express Glutathione S-transferase (GST) tagged SUMO 1, SUMO 2/3, wildtype vFLIP or VE-vFLIP was used to inoculate 50mL of LB Broth. After 1.5-2 hours, when the optical density of the culture was around 0.4-0.7, expression was induced with 0.1mM IPTG. The samples were placed in 50mL conical tubes and pelleted at 3,100 rpm for 15 minutes. The pellet was placed in a -20°C freezer overnight. Each pellet was resuspended in 1mL of PBS in a new 1.5mL tube, 10µL of a protease inhibitor cocktail were added to each tube, and sonicated four times for 30 seconds. The samples were spun down at 9,700 rpm for 1 minute and the supernatant transferred to a new tube. The SUMO samples were added to glutathione-sepharose aliquots and incubated at room temperature for 1 hour. For the *in vitro* assay VE-vFLIP or wildtype vFLIP from BL21 cells was added to each of the 1.5mL tubes containing the SUMO and the glutathione-sepharose at room temperature for 1 hour. The samples were then pelleted at 2,500 rpm for 5 minutes and the lysate discarded. The pellets were washed 5 times with GST wash buffer and 50µL of 2X Laemmli sample buffer added to each pellet. Samples were analyzed using the described immunoblot analysis. Purified wildtype vFLIP and VE-VFLIP were also harvested from transfected 293T cells for use for the *in vitro* assay. Transfected cells with appropriate constructs were harvested 48 hours post-transfection and centrifuged at 1,500 rpm for 10 minutes. The PBS was removed and 1mL of lysis buffer with 10µl of a protease inhibitor cocktail kit (Thermo Scientific) were added to each cell pellet and the cells sonicated four times for 10 seconds.

The samples were pelleted at 9,700 rpm for 1 minute, the supernatant added to a new 1.5mL tube, and vFLIP samples were incubated with the SUMO and glutathione-sepharose.

# CHAPTER III:

# BACKGRAOUND DATA

*Itch and A20 are required for the RTA induced degradation of vFLIP*

In order for KSHV to remain latent in the host cell, NF-κB must be activated and vFLIP is known to induce the activation of NF-κB[22]. It has been previously demonstrated that RTA inhibits gene expression from NF-kB activation by targeting vFLIP for proteasomal degradation. In order for this to occur, RTA must recruit some ubiquitin E3 ligase to attach the ubiquitin proteins that will target vFLIP for proteasomal degradation[23]. Itch is an E3 ubiquitin ligase that is known to ubiquitinate cFLIP, of which vFLIP is a homolog, targeting it for degradation at the proteasome[24]. Thus this protein could be the E3 ligase co-opted by RTA to target vFLIP for degradation. To evaluate if this was the case, a dominant negative mutant of Itch (C830A) that lacked ubiquitin ligase activity was utilized. HEK293T cells were transfected with RTA, vFLIP and either wild-type or Itch mutant. vFLIP was stabilized in the presence of the Itch mutant and also was found to be stable in the presence of wild-type vFLIP (Figure 1a). It is possible that vFLIP was found to be stable in the presence of wild-type Itch because it is a dominant negative phenotype from the overexpression of Itch. Itch is part of a ubiquitin-editing complex with A20 and thus increased levels of Itch due to its overexpression could lead to the disruption of complex stoichiometry, which could inhibit its activity, resulting in the mutant phenotype[25]. A20 also has E3 ligase activity and thus could also be co-opted by RTA to ubiquitinate vFLIP[17]. A mutant form of A20 with a mutated E3 ligase domain (C624A/C627A) was utilized to evaluate our hypothesis. HEK293T cells were

transfected with vFLIP, RTA and either wild-type or mutant A20. Similar to the result with mutant Itch, vFLIP was found to be stable with the mutant with the wild-type forms of A20 in the presence of RTA (Figure 1b).

*vFLIP interacts with components of the components of the A20/Itch Ubiquitin-Editing Complex*

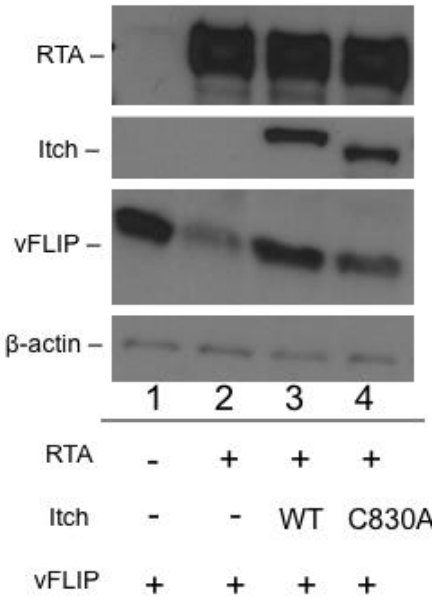
After determining that the E3 ligase activity of Itch and A20 were necessary for RTA to target vFLIP for degradation, it had to be determined whether these components interacted with vFLIP. HEK 293T cells were transfected with A20 and vFLIP in the presence and absence of RTA. When A20 was immunoprecipitated vFLIP was co-immunoprecipitated even in the absence of RTA (Figure 2a). In a similar experiment HEK 293T cells were transfected with Itch and vFLIP in the presence of RTA and when vFLIP was immunoprecipitated, Itch was co-immunoprecipitated (Figure 2b). These data suggest that Itch and A20 interact with vFLIP in the presence of RTA and that vFLIP is able to interact with A20 in the absence of RTA.

*vFLIP has a putative SIM that is required for NF-kB Activation and for RTA induced vFLIP degradation*

In previous experiments it has been observed that vFLIP localizes to promyelocytic leukemia (PML) nuclear bodies (NBs) in the nucleus which are most commonly known for being SUMOylated[26]. Because of the association of vFLIP with these PML NBs, it was hypothesized that vFLIP could have a SIM. vFLIP may have a putative SIM and SUMOylation site, as determined through sequence analysis (Figure 3a). Point mutations were made in this region to evaluate whether the vFLIP SIM was required for the activation of NF-kB. One vFLIP mutant had a valine mutated to a

glutamic acid in the SIM (VE-vFLIP). A second vFLIP mutant had a leucine mutated to a glutamic acid in the SIM (LE-VFLIP). A third vFLIP mutant was also examined with a lysine in a SUMOylation site mutated to an arginine. HEK293T cells were transfected with either an empty vector, wild-type vFLIP, KR-VFLIP, LE-vFLIP, or VE-vFLIP and a luciferase reporter and the relative luciferase calculated as a ratio of Luciferase: Renilla. With both the LE and VE vFLIP, we observed a decrease in the activation of NF-κB, highlighting the necessity of the vFLIP SIM for NF-κB activation (Figure 3b). The KR mutant resulted in an increase in NF-κB activation but the reason for this remains unclear and will not be the focus of this thesis (Figure 3b).

A

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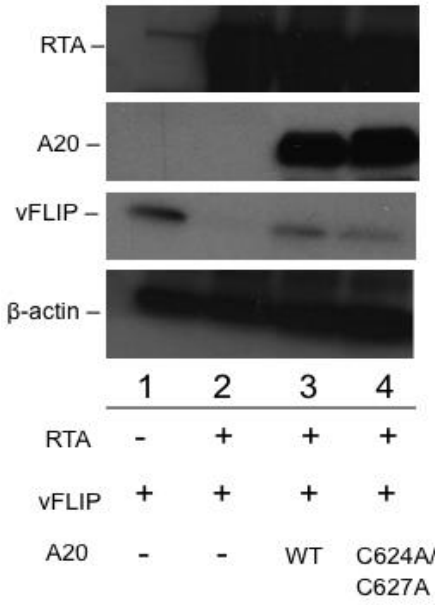


**Figure 1: Itch and A20 are required for RTA induced degradation of vFLIP.** HEK 293T cells were transfected with myc-vFLIP, Flag-Itch WT, Flag-Itch-C830A, and/or empty vector control where indicated (A) or myc-vFLIP, RTA, Flag-A20 WT, Flag-A20-C624/627A, and/or empty vector where indicated (B). The cells were harvested 48 hours after transfection and analyzed via western blotting.

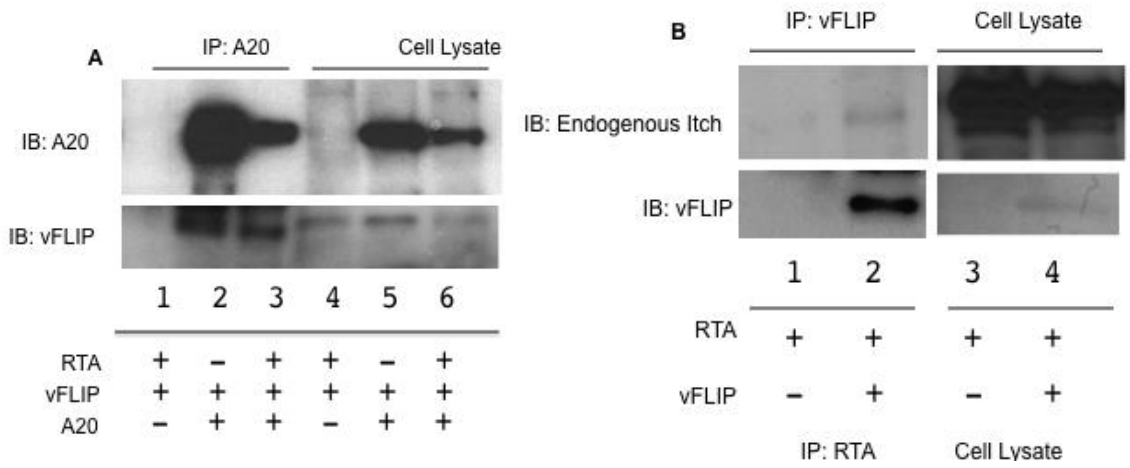


**Figure 2: vFLIP interacts with components of the A20/Itch ubiquitin-editing complex.** HEK 293T cells were transfected with myc-vFLIP, RTA, FLAG-A20 and empty vector control (A) or myc-vFLIP, RTA, and/or empty vector (B). 48 hours post-transfection, cells were harvested and processed for immunoprecipitation. Clarified lysates were incubated with anti-FLAG (A) or myc (B) antibody and protein A/G agarose to immunoprecipitate A20 (A) or vFLIP (B). Immunoprecipitates were analyzed via SDS-PAGE followed by western blotting.

A

| | |
|---|---|
| **WT-vFLIP** | 20 V**V**LF**L**L-X18-LKEE 48 |
| **VE-vFLIP** | 20 V**e**LFLL-X18-LKEE 48 |
| **LE-vFLIP** | 20 VVLF**e**L-X18-LKEE 48 |

B

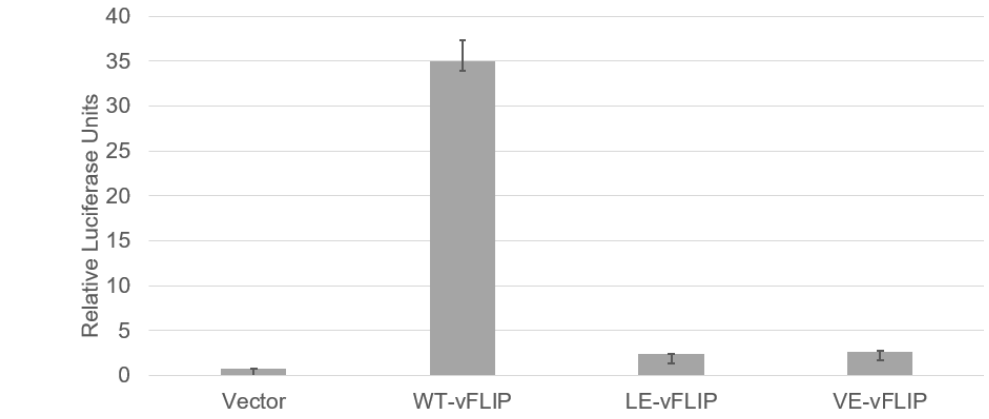


**Figure 3: vFLIP has a putative SIM that is required for NF-κB activation**. (A) Mutated residues are indicated in bold. the WT vFLIP sequenced. The VE and LE mutants have a mutated SUMO interaction motif (SIM). The KR mutant has a mutated SUMOylation site. The VE and LE mutants have a valine mutated to a glutamate. The KR mutant has a lysine mutated to an arginine. (B) HEK 293T cells were transfected with WT-vFLIP, LE-vFLIP, VE-VFLIP or empty vector where indicated, along with a luciferase reporter under control of an enhancer with five NF-κB binding sites and a Renilla control plasmid. NF-κB activity was detected as a ratio of Luciferase: Renilla using a luminometer.

# CHAPTER IV:

# RESULTS

*AIM 1: Does vFLIP interacts with SUMO 1 and SUMO 2/3?*

Because we had previously seen reduced activation of NF-κB with the SIM-deficient vFLIP mutant (Figure 3), we speculated that SUMOylation may be involved in the manipulation of the NF-κB pathway by vFLIP. Thus we confirmed that the putative vFLIP SIM is indeed a SIM. To determine whether the SIM was necessary for vFLIP to interact with SUMO 1 and/or SUMO 2/3, we carried out an *in vitro* pull-down assay. GST-tagged SUMO 1 or SUMO 2/3, and V5-HIS tagged wildtype vFLIP or SIM deficient mutant vFLIP (VE-vFLIP) were all expressed in *E.coli* (Figure 4a). The GST SUMO was bound to glutathione-sepharose and incubated with the wildtype or mutant vFLIP lysate. The samples were then analyzed by immunoblotting. In this assay, wildtype vFLIP was found to bind to both SUMO 1 and SUMO 2/3 but reduced binding to both SUMO proteins was observed when the vFLIP SIM mutant was incubated with SUMO 1 or SUMO 2/3 (Figure 4a). An *in vitro* pull-down assay was also carried out utilizing wildtype and VE vFLIP expressed in HEK293T cells to ensure proper folding of vFLIP and to confirm the results of the previous pull-down. SUMO 1 and SUMO 2/3 were both expressed in *E. coli* and bound to glutathione sepharose. The wildtype vFLIP in this pull-down assay was found bound to both SUMO 1 and SUMO 2/3, while binding of VE-vFLIP to both SUMO 1 and SUMO 2/3 was reduced compared to wildtype vFLIP (Figure 4b).

*AIM 2: Are TAX1BP1, A20 and Itch are SUMOylated?*

Since we have confirmed that vFLIP has a SIM, which is required for NF-κB activation, we hypothesized that vFLIP may be interacting with a SUMOylated protein that is involved in the NF-κB pathway. In previous experiments we have seen vFLIP interacting with Itch and A20 (Figure 2), members of a ubiquitin-editing complex involved in the shutoff of the NF-κB pathway[17]. We hypothesized that SUMOylation of Itch and/or A20 might be required for interaction with vFLIP. In order to determine whether A20 was SUMOylated, HEK293T cells were transfected with A20 or an empty vector and were immunoprecipitated using anti-FLAG antibody. To ensure that only the SUMOylation ofA20 was examined and not SUMOylated proteins found in complex, a 500mM NaCl RIPA wash was utilized. In this experiment A20 was found to be SUMOylated by SUMO 2/3 (Figure 5a). In the same experiment, the SUMOylation state of TAX1BP1, was also examined as it has a role in the assembly of the Itch and A20 complex[27]. TAX1BP1 was found to be SUMOylated by SUMO 2/3, as determined by immunoblotting (Figure 5a).

We then examined whether Itch was SUMOylated by SUMO 2/3 by transfecting HEK293T cells with Itch, A20 or an empty vector, immunoprecipitating the FLAG-tagged proteins and blotting for SUM0 2/3. We observed SUMO 2/3 modification of Itch but not A20 in this experiment (Figure 5b).

In order to determine whether Itch and A20 were SUMOylated by SUMO 1, and whether that SUMOylation was influenced by vFLIP, we transfected HEK293T cells with FLAG-tagged Itch or A20, myc-vFLIP, or an empty vector where indicated. We then immunoprecipitated the FLAG-tagged proteins and immunoblotted for SUMO 1 to

determine the SUMOylation state of those proteins. Itch and A20 were found to be modified by endogenous SUMO 1, but there was no detectable difference in the level of SUMOylation in the presence of vFLIP (Figure 5c).

*AIM 3: Is the vFLIP SIM is necessary for vFLIP to interact with Itch and A20*

After confirming that Itch and A20 are indeed SUMOylated, we next had to determine whether vFLIP interacts with Itch and A20 through a SUMO-dependent mechanism. We transfected HEK293T cells with FLAG tagged Itch, an empty vector, and myc tagged wildtype vFLIP, or SIM-deficient VE-vFLIP. We then immunoprecipitated the myc tagged vFLIP proteins and examined vFLIP interaction with Itch through immunoblotting. Itch was co-immunoprecipitated with wildtype vFLIP, but there was reduced to no binding to the vFLIP SIM mutant thus demonstrating that the vFLIP SIM mediates the interaction with Itch (Figure 6a). We then examined whether vFLIP interaction with A20 was SIM-dependent. We transfected HEK293T cells with FLAG-tagged A20, an empty vector, and myc tagged vFLIP and VE-vFLIP and immunoprecipitated the FLAG-tagged proteins. Less VE-vFLIP was co-immunoprecipitated with A20 compared to wildtype vFLIP which suggests that the interaction between vFLIP and the Itch/A20 ubiquitin-editing complex is dependent on SUMO (Figure 6b).

*vFLIP alters the ubiquitination of RIP1*

The previous data provide evidence that vFLIP interacts with Itch and A20 through a SUMO dependent mechanism but the purpose of this interaction remained unclear. This interaction is observed in the absence of RTA, and may serve as a means to promote NF-κB signaling by inhibiting the function of the Itch/A20 ubiquitin-editing

complex. To evaluate whether this inhibition is taking place, we examined the formation of RIP1 ubiquitin conjugates in the presence and absence of vFLIP. HEK293T cells were transfected with either vFLIP or an empty vector and treated with tumor necrosis factor alpha (TNFα) prior to their harvesting to activate the NF-κB pathway. The cells were also treated with 1μM MG132, a proteasome inhibitor, to prevent degradation of RIP1 at the proteasome. Approximately 2 hours after treatment with TNFα, there was a noticeable increase in the quantity of RIP1 ubiquitin conjugates that was not present at the same timepoint in the presence of vFLIP (Figure 7). This suggests that vFLIP does have an effect on the formation of RIP1 ubiquitin conjugates, and thus may be interfering with the activity of the Itch/A20 ubiquitin-editing complex.

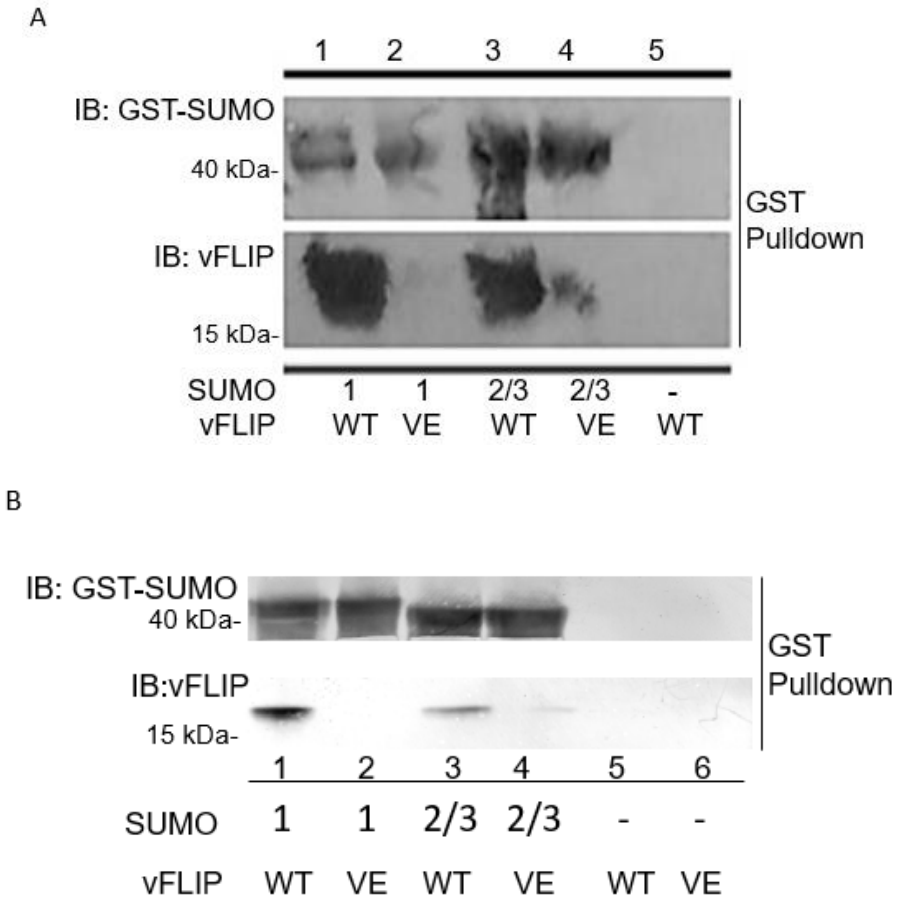


**Figure 4. vFLIP interacts with SUMO 1 and SUMO 2/3 *in vitro.*** (A) Recombinant GST-SUMO and wild type or SIM-deficient V5-HIS vFLIP were expressed in *E. coli* and HEK 293T cells were transfected with myc-vFLIP. V5-HIS tagged vFLIP was incubated with GST tagged SUMO bound to glutathione-sepharose. (B) Recombinant GST-SUMO was expressed in *E. coli*, and HEK 293T cells were transfected with myc-vFLIP. Myc tagged vFLIP was incubated with GST tagged SUMO bound to glutathione-sepharose. Purified proteins were analyzed via immunoblotting

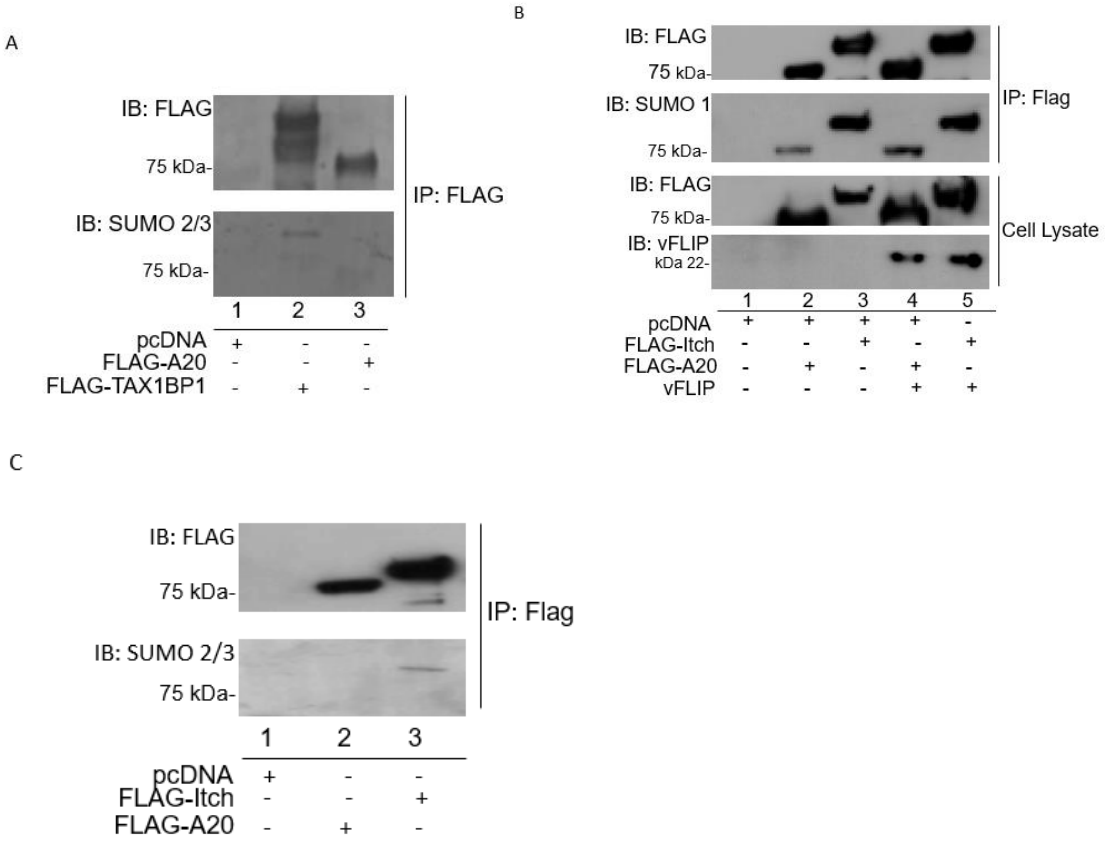


**Figure 5. TAX1BP1, Itch and A20 are SUMOylated.** HEK293T cells were transfected with (A) Flag-TAX1BP1, Flag-A20 or empty vector, or (B) FLAG-Itch, Flag-A20 or empty vector, or (C) Flag-Itch, Flag-A20 or empty vector, where indicated. (B) Cell lysates were processed for immunoprecipitation using anti-FLAG antibody. Immunoprecipitates were washed with 500mM NaCl. Immunoprecipitates were analyzed via SDS-PAGE followed by immunoblotting against Flag or endogenous SUMO 1 (B) or SUMO 2/3 (A, C).

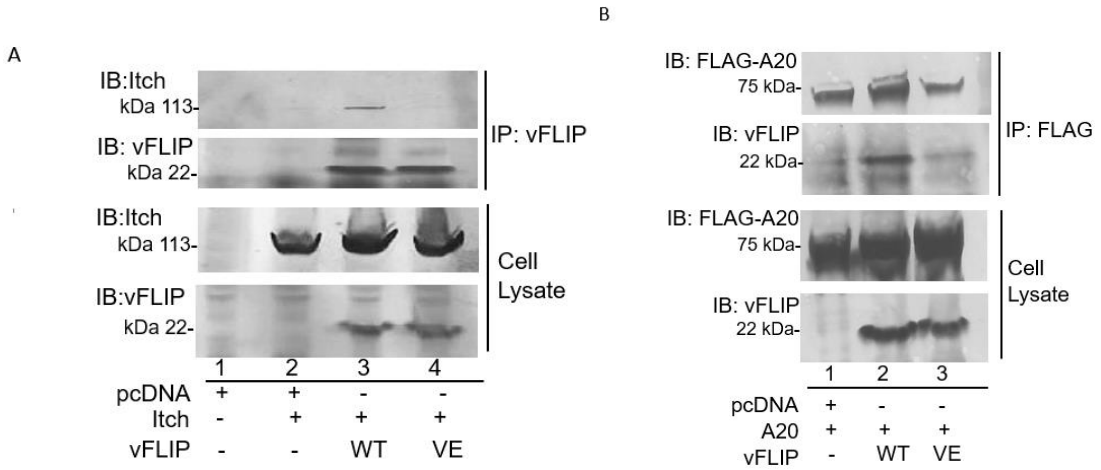


**Figure 6. vFLIP requires a SIM in order to interact with Itch and A20**. HEK 293T cells were transfected with (A) Flag-Itch or (B) A20, myc-tagged wildtype or VE SIM-deficient vFLIP, or an empty vector where indicated. Cell lysates were processed for immunoprecipitation using anti-FLAG antibody. Immunoprecipitates were analyzed by immunoblot as previously described.

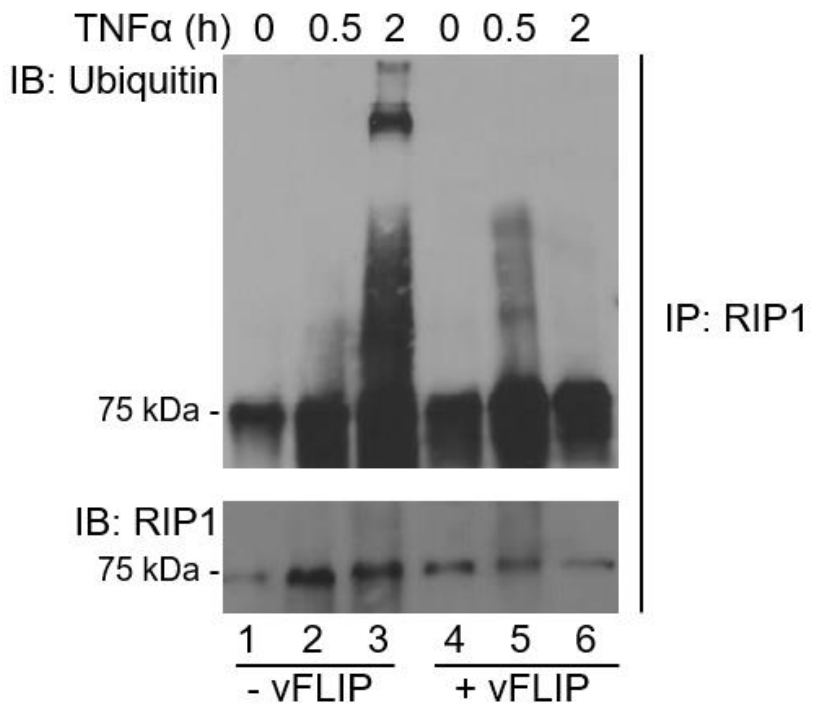


**Figure 7. vFLIP expression alters formation of RIP1 ubiquitin conjugates.** HEK293T cells were transfected with either WT myc-vFLIP or empty vector, then treated with TNFα at 0, 0.5, and 2 hours prior to harvesting, and analyzed via immunoprecipitation with anti-RIP1. Cells were also treated with 1μM MG132 16 hours before harvesting. A 50mM NaCl RIPA wash was used to prevent non-covalent interactions. Following immunoprecipitation, lysates were analyzed by immunoblotting with anti-Ub and anti-RIP1.

# CHAPTER V:

# DISCUSSION

In order to deal with host immune systems, viruses developed mechanisms that allow them to control the cell and facilitate their proliferation and survival. One of these mechanisms involves modulation of the NF-κB pathway which can promote cell proliferation and suppress apoptosis[28]. Previous studies have indicated that the activation of NF-κB by KSHV vFLIP is essential for the survival of KSHV infected primary effusion lymphoma cells[29]. It is also known that NF-κB activation by vFLIP suppresses lytic replication and thus keeps the virus in latency[8].

The data presented here demonstrate that vFLIP interacts with components of the Itch/A20 ubiquitin-editing pathway of the NF-κB pathway via a SUMO-dependent mechanism. We have demonstrated that vFLIP interacts with SUMO 1 and SUMO 2/3 and that the ability of vFLIP to interact with these proteins is disrupted when that SIM is mutated (Figure 4). Previous studies in our lab provided evidence for the interaction of vFLIP with Itch and A20 in the absence of RTA (Figure 2), so we hypothesized that the mechanism by which vFLIP is interacting with these proteins could involve SUMOylation, stemming from the data that vFLIP has a functional SIM. Itch, A20 and TAX1BP1 were found to be SUMOylated by SUMO 1 and SUMO 2/3 (Figure 5), and that if the vFLIP SIM was mutated, vFLIP interaction with Itch and A20 is greatly reduced (Figure 6). To confirm that Itch and A20 indeed interact with vFLIP via a SUMO-dependent mechanism the SUMOylation sites of Itch and A20 could be mutated and vFLIP interaction with those proteins assessed. TAX1BP1 was found to be

SUMOylated along with Itch and A20, but the role of this SUMOylation warrants further investigation, because TAX1BP1 has a role in the interaction between A20 and RIP1[27].

These data suggest a mechanism by which vFLIP interacts with Itch, and A20 and we speculated that because Itch and A20 form a ubiquitin-editing complex that is responsible for the inactivation and degradation of RIP1, vFLIP might be playing a role in the interfering with the of the activity of this complex in some way. A20 is known to be a strong inhibitor of NF-κB utilizing its de-ubiquitin and ubiquitin ligase domains to downregulate the transcription factor. A20 accomplishes this through the removal K63-linked ubiquitin chains from RIP1 and ubiquitin it with K48-linked ubiquitin, which targets the RIP1 for degradation at the proteasome[17]. Itch may also ubiquitinate RIP1 and has a role in the recruitment of A20 to RIP1. Together Itch and A20 form an ubiquitin-editing complex that inactivates NF-κB signaling[15]. When we examined the effect vFLIP on the formation of RIP1 ubiquitin conjugates, we found reduced levels of RIP1 ubiquitination (Figure 7). This suggests that vFLIP may be interfering with the ubiquitin ligase activity of the Itch/A20 ubiquitin-editing complex. Interference with this complex could prevent the inactivation of RIP1, resulting in more NF-κB activation downstream of the protein, thus promoting KSHV latency. The effect of vFLIP needs to be further characterized, as the ubiquitin antibody utilized cannot distinguish between K63 or K48-polyubiquinated proteins, so it is not known which function of the Itch/A20 ubiquitin-editing complex vFLIP is potentially affecting. Additionally, if vFLIP is preventing the degradation of RIP1 at the proteasome and this interaction is dependent on vFLIP having a functional SIM, then one would see more RIP1 degradation in the presence of the VE-vFLIP SIM mutant which requires further experimentation.

KSHV is known to cause three different cancers: Kaposi's sarcoma, endothelial tumors and primary effusion lymphoma. One factor several of the proteins expressed in latency have been implicated in deregulating the DNA damage response, transcription factor activities, and cell proliferation, which could lead to oncogenesis[1]. Since latent virus is found in KSHV-associated cancers and many current antiviral drugs target lytic replication, targeting the virus in latency could be potential therapy for KSHV-associated cancers[30]. Drugs that reactivate latent virus have been investigated, especially since most current antiviral drugs are ineffective against the latent virus. A greater understanding of the mechanisms involved in maintaining KSHV latency, such as how vFLIP manipulates Itch and A20, is necessary for the development of such therapeutics.

## LITERATURE CITED


1. Jha, H., Banerjee, S. & Robertson, E. The role of gammaherpesviruses in cancer pathogenesis. *Pathogens* **5,** 18 (2016).
2. Ganem, D. KSHV infection and the pathogenesis of Kaposi's sarcoma. *Annu. Rev. Pathol. Mech. Dis.* **1,** 273–296 (2006).
3. Cavallin, L. E., Goldschmidt-Clermont, P. & Mesri, E. Molecular and cellular mechanisms of KSHV oncogenesis of Kaposi's sarcoma associated with HIV/AIDS. *PLoS Pathog.* **10,** e1004154 (2014).
4. Damania, B. Oncogenic gamma-herpesviruses: comparison of viral proteins involved in tumorigenesis. *Nat. Rev. Microbiol.* **2,** 656–68 (2004).
5. Staskus, K. A. *et al.* Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. *J. Virol.* **71,** 715–9 (1997).
6. Paoli, P. D. E. & Carbone, A. Kaposi ' s Sarcoma Herpesvirus : twenty years after its discovery. 1288–1294 (2016).
7. Lee, H., Lee, S., Chaudhary, P. M., Gill, P. & Jung, J. U. Immune evasion by Kaposi's sarcoma-associated herpesvirus. *Future Microbiol.* **5,** 1349–1365 (2011).
8. Ye, F.-C. *et al.* Kaposi's sarcoma-associated herpesvirus latent gene vFLIP inhibits viral lytic replication through NF-κB-mediated suppression of the AP-1 pathway: a novel mechanism of virus control of latency. *J. Virol.* **82,** 4235–4249 (2008).
9. Vandevenne, P., Sadzot-Delvaux, C. & Piette, J. Innate immune response and viral interference strategies developed by human herpesviruses. *Biochem. Pharmacol.* **80,** 1955–72 (2010).

10. Purushothaman, P., Dabral, P., Gupta, N., Sarkar, R. & Verma, S. C. KSHV Genome Replication and Maintenance. *Front. Microbiol.* **7,** 1–14 (2016).

11. Liu, L. *et al.* The human herpes virus 8-encoded viral FLICE inhibitory protein physically associates with and persistently activates the Iκ B kinase complex. *J. Biol. Chem.* **277,** 13745–51 (2002).

12. Thome, M. *et al.* Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* **386,** 517–521 (1997).

13. An, J., Sun, Y., Sun, R. & Rettig, M. B. Kaposi's sarcoma-associated herpesvirus encoded vFLIP induces cellular IL-6 expression: the role of the NF-κB and JNK/AP1 pathways. *Oncogene* **22,** 3371–3385 (2003).

14. Chaudhary, P. M., Jasmin, A., Eby, M. T. & Hood, L. Modulation of the NF-κB pathway by virally encoded Death Effector Domains-containing proteins. *Oncogene* 5738–5746 (1999).

15. Shembade, N. & Harhaj, N. The E3 ligase Itch negatively regulates inflammatory signaling pathways by controlling the function of the ubiquitin-editing enzyme A20. *Nat. Immunol.* **9,** 254–262 (2008).

16. Sakakibara, S. *et al.* A20/TNFAIP3 inhibits NF-κB activation induced by the Kaposi's sarcoma-associated herpesvirus vFLIP oncoprotein. *Oncogene* **32,** 1223–32 (2013).

17. Wertz, I., O'rourke, K., Zhou, H. & Eby, M. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF- κB signalling. *Nature* **430,** 2350–2354 (2004).

18. Ofengeim, D. & Yuan, J. Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. *Nat. Rev. Mol. Cell Biol.* **14,** 727–36 (2013).

19. Chang, P.-C. & Kung, H.-J. SUMO and KSHV Replication. *Cancers (Basel).* **6,** 1905–1924 (2014).

20. Geiss-Friedlander, R. & Melchior, F. Concepts in SUMOylation: a decade on. *Nat. Rev. Mol. Cell Biol.* **8,** 947–956 (2007).

21. Hayward, G. S., Ambinder, R., Ciufo, D., Hayward, S. D. & LaFemina, R. L. Structural organization of human herpesvirus DNA molecules. *J. Invest. Dermatol.* **83,** 29s–41s (1984).

22. Brown, H., Song, M., Deng, H. & Wu, T. NF-κB inhibits gammaherpesvirus lytic replication. *J. Virol.* **77,** (2003).

23. Ehrlich, E. S., Chmura, J. C., Smith, J. C., Kalu, N. N. & Hayward, G. S. KSHV RTA abolishes NF-κB responsive gene expression during lytic reactivation by targeting vFLIP for degradation via the proteasome. *PLoS One* **9,** (2014).

24. Chang, L. *et al.* The E3 ubiquitin ligase itch couples JNK activation to TNFα-induced cell death by inducing c-FLIP(L) turnover. *Cell* **124,** 601–13 (2006).

25. Prelich, G. Gene overexpression: Uses, mechanisms, and interpretation. *Genetics* **190,** 841–854 (2012).

26. Lallemand-Breitenbach, V. & de Thé, H. PML nuclear bodies. *Cold Spring Harb. Perspect. Biol.* **2,** 1–17 (2010).

27. Shembade, N., Harhaj, N. S., Liebl, D. J. & Harhaj, E. W. Essential role for TAX1BP1 in the termination of TNFα, IL-1- and LPS-mediated NF-κB and JNK signaling. *EMBO J.* **26,** 3910–3922 (2007).

28. Lisowska, K. & Witkowski, J. M. Viral strategies in modulation of NF-kappaB activity. *Arch. Immunol. Ther. Exp. (Warsz).* **51,** 367–375 (2003).

29. Guasparri, I., Keller, S. Cesarman, E. KSHV vFLIP is essential for the survival of infected lymphoma cells. *J. Exp. Med.* **199,** 993–1003 (2004).

30. Gorres, K. L., Daigle, D., Mohanram, S. & Miller, G. Activation and repression of Epstein-Barr Virus and Kaposi's sarcoma-associated herpesvirus lytic cycles by short- and medium-chain fatty acids. *J. Virol.* **88,** 8028–44 (2014).

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[redacted]

## EDUCATION

**Towson University.** Jan. 2014 - Present
M.S. in Biological Sciences
Expected Graduation Date: August 2016
GPA: 3.9
Coursework: Applied Biotechnology, Membrane Biology, Cellular Signaling, Gene Expression and Regulation, Microscopy and Microtechniques, Data Analysis and Interpretation, Virology

**University of Maryland, Baltimore County.** Aug. 2009 - May 2013
B.S. in Biological Sciences
Departmental Honors in Research
GPA: 3.5

## RESEARCH EXPERIENCE

**KSHV vFLIP Interaction with the A20/Itch Ubiquitin-Editing Complex.** Jan. 2014 - Present
*Towson University*
*Thesis Student*
*Principal Investigator: Elana Ehrlich, PhD*
For my master's thesis, I studied the role of the Kaposi's sarcoma herpesvirus protein vFLIP in maintaining latency in the infected cell. Specifically, I examined how vFLIP interacts with the A20/Itch Ubiquitin editing complex and the mechanism behind this interaction primarily In addition, I also examined the ubiquitination and SUMOylation of proteins involved in maintaining KSHV Latency.

**A Mathematical Model of Melanopsin Phototransduction.** Sept. 2011 - May 2013
*University of Maryland, Baltimore County*
*Undergraduate Researcher*
*Principal Investigators: Phyllis Robinson, PhD, Kathleen Hoffman, PhD*
As a part of the UBM program, I focused on developing a mathematical model of the phototransduction cascade of the photopigment melanopsin from existing electrophysiology data and from calcium imaging assays performed in the laboratory.

## TEACHING EXPERIENCE

**Graduate Teaching Assistant.** Jan. 2014 - May 2016
*Towson University*
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I have taught lab sections of BIOL 202L: Intro to Ecology and Genetics Lab, and lab sections of BIOL 120L: Principles of Biology. As a teaching assistant, I was responsible for preparing lab lectures and experiments, supervising students as they conducted these experiments, and grading lab reports.

## PROFESSIONAL PRESENTATIONS

*The Kaposi's Sarcoma Herpesvirus vFLIP Interacts With Itch/A20 Ubiquitin-Editing Complex Via a SUMO Dependent Mechanism.* Jun. 2016
Annual Meeting for the American Society for Virology
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*Kaposi's sarcoma herpesvirus manipulates the Itch/A20 ubiquitin-editing complex.* Nov. 2015
University of Maryland Virology Retreat.
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*Kaposi's sarcoma herpesvirus manipulates the Itch/A20 ubiquitin-editing complex.* Nov. 2014
University of Maryland Virology Retreat.
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*Kaposi's sarcoma herpesvirus manipulates the Itch/A20 ubiquitin-editing complex.* May 2014
American Society for Microbiology Annual Poster Presentation.
Baltimore, MD.

*Kaposi's sarcoma herpesvirus manipulates the Itch/A20 ubiquitin-editing complex.* April 2014
Undergraduate and Graduate Research and Performance Expo**.**
Towson, MD.

*A Mathematical Model of Melanopsin Activation*. May 2012
Undergraduate Research and Creative Achievement Day.
Baltimore MD.

*A Mathematical Model of Melanopsin Activation*. Nov. 2012
4th Annual Undergraduate Research Conference at the Interface of Biology and Mathematics.
Knoxville, TN.

*A Mathematical Model of Melanopsin Activation*. Oct. 2012
15th Annual Undergraduate Research Symposium in the Chemical and Biological Sciences.
Baltimore, MD.

*A Mathematical Model of Melanopsin Activation*. Aug. 2012
15th Annual CNMS Summer Undergraduate Research Fest.
Baltimore, MD