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THE A20/ITCH UBIQUITIN-EDITING COMPLEX IS REQUIRED FOR KSHV RTA-INDUCED DEGRADATION OF VFLIP

by

Jennifer Chmura

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Elana Ehrlich
Chair, Thesis Committee — Date: 4/4/14

Michelle Snyder
Committee Member — Date: 4/4/14

Petra A. Tsuji
Committee Member — Date: 4/4/14

Janet DeLany
Dean of Graduate Studies — Date: 4-29-14

ABSTRACT

The A20/Itch ubiquitin-editing complex is required for KSHV RTA-induced degradation of vFLIP

Jennifer C. Chmura

Kaposi's sarcoma-associated herpesvirus (KSHV) is a gamma-2 herpesvirus that causes lymphoid and endothelial tumors through establishing two replication states: latent and lytic. Latency is maintained in part through expression of viral FLICE inhibitory protein (vFLIP) which inhibits apoptosis and activates nuclear factor-kappaB (NF-κB) signaling. Lytic replication is dependent on the major transactivator of lytic gene expression, replication and transcription activator protein (RTA). vFLIP or NF-κB inhibition results in lytic reactivation, suggesting RTA may have a role in vFLIP stability. Data show RTA destabilizes vFLIP and inhibits vFLIP-induced NF-κB signaling by inducing ubiquitin-proteasome-mediated vFLIP degradation. This thesis provides evidence that the ubiquitin E3-ligase activities of Itch and A20, but not RTA, are required for vFLIP degradation. Immunoprecipitation evidence of A20, Itch, RTA, and vFLIP interaction suggests RTA interacts with the A20/Itch ubiquitin-editing complex to induce vFLIP degradation, inhibiting NF-κB signaling and allowing for lytic reactivation.

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CHAPTER I:

INTRODUCTION

Herpesviruses are enveloped viruses with a double-stranded DNA genome (Hayward et al., 1984). Over 130 herpesviruses have been identified, with eight known to infect humans, and they are classified into subfamilies: alpha, beta, and gamma (Damania, 2004). The herpesvirus subfamilies differ in their host range, target cell, and site of latency (Roizman and Baines, 1991). Kaposi's sarcoma-associated herpesvirus (KSHV) is an oncovirus in the gamma subfamily involved in the pathogenesis of Kaposi´s sarcoma (KS) and two B-cell lymphoproliferative diseases known as primary effusion lymphoma (PEL) and multicentric Castleman´s disease (MCDs) (Rivas et al., 2001; Izumiya et al., 2005), which are commonly found in HIV-infected and immunosuppressed individuals (Cai et al., 2010; Zhang et al., 2012).

While all viruses undergo lytic replication, unique to herpesviruses is the ability to maintain a latent (inactive) state for the life of the host while still retaining the ability to switch to a lytic replication cycle (Roizman and Baines, 1991; Black, 2008). KSHV samples have been found to be predominantly composed of latently infected cells with about three percent of cells lytically infected (Staskus et al., 1997) demonstrating the importance of both latent and lytic states in KSHV pathogenesis. Latency is essential as it enables KSHV to establish persistent infection by encoding a limited number of proteins important for immune evasion and tumorigenesis (Lee et al., 2010; Ye et al., 2011), while lytic replication is needed for KSHV to infect new target cells and activate cytokines favorable for tumorigenesis (Yang et al., 2008). The virus switches from the latent state

to an infectious lytic replication after exposure to a stimulus (Vandevenne et al., 2010). There are a number of stimuli suspected to trigger lytic reactivation, namely hypoxia, HIV, inflammatory cytokines, and reactive oxygen species (reviewed in Ye et al., 2011), and reactivation is most likely the result of a combination of these stimuli.

While lytic reactivation is triggered by different stimuli, it has been shown that a number of latency factors have inhibitory effects on lytic replication (Brown et al., 2003; Izumiya et al., 2003; Lan et al., 2004; Wang et al., 2005; Yang and Wood, 2007); therefore, it is logical that these latency factors may need to be turned off for lytic replication to occur. KSHV has been shown to use a major protein degradation pathway, the ubiquitin-proteasome pathway, to degrade lytic suppressers (Yu et al., 2005; Yang et al., 2008; Gould et al., 2009). The proteasome is a multi-component macromolecule that degrades proteins and, like the small protein ubiquitin, it is ubiquitous in eukaryotic cells (reviewed in Voges et al., 1999). Ubiquitin attached in the form of a polyubiquitin chain signals to send the target protein to the proteasome for degradation. Three components are required for conjugation of the ubiquitin linkage to the target protein: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and the ubiquitin-ligase (E3) (reviewed in Gao and Luo, 2006). The ubiquitin E3-ligase is important because it recognizes a specific target protein (reviewed in Lecker et al., 2006). The replication and transcription activator protein (RTA) is the transactivator of lytic gene expression and has been shown to use its own intrinsic ubiquitin E3-ligase activity to degrade repressors of lytic replication (Yu et al., 2005; Yang et al., 2008; Gould et al., 2009).

In order to examine the mechanisms of RTA's inhibition of lytic repressors, the factors required for the latent state should be explored. Nuclear factor-kappaB (NF-κB) is a transcription factor with a central role in the immune response (reviewed in Ghosh and Karin, 2002) making NF-κB signaling a target for manipulation by viruses (reviewed in Hiscott et al., 2006). When KSHV infected lymphocytes were treated with an NF-κB inhibitor they exhibited enhanced lytic gene expression (Brown et al., 2003), providing evidence that NF-κB has an inhibitory effect on lytic replication. From these data it was logical to assume that NF-κB must be turned off for lytic replication to occur. In order to turn off NF-κB signaling, it is plausible that activators of the NF-κB pathway may be targeted. The KSHV latent viral FLICE inhibitory protein (vFLIP) has been previously shown to activate NF-κB (Chaudhary et al., 1999; Liu et al., 2002). In addition to vFLIP's function in activating NF-κB, vFLIP is also an inhibitor of death receptor-induced apoptosis and inhibits procaspase-8 cleavage (Field et al., 2003; VanHoudt et al., 2007), and has been shown to be required for lymphoid cell survival (Guasparri et al., 2004). Based on this information, and since RTA is the switch that turns on lytic gene expression (reviewed in West and Wood, 2003), it was the logical next step to examine if RTA has an inhibitory effect on vFLIP.

Preliminary data gathered by Dr. Ehrlich provided evidence that RTA targets vFLIP for proteasome-mediated degradation resulting in the down-regulation of NF-κB responsive gene expression during lytic reactivation. Data from a reporter assay indicated that RTA expression inhibited vFLIP-induced NF-κB activation (Figure 1).

Additionally, the expression of RTA resulted in destabilization of vFLIP (Figure 2), and this effect was abrogated by treatment with the proteasome inhibitor MG132 (Figure 3).

From these preliminary data it is clear that RTA is responsible for vFLIP degradation because without RTA expression vFLIP is stable. However, the exact mechanism of RTA-induced vFLIP degradation is unknown. My thesis focused on identifying the ubiquitin E3-ligases that are responsible for RTA-induced vFLIP degradation; a degradation pathway that may contribute to the KSHV latent to lytic switch. Both latent and lytic states contribute to cancer formation and are needed for KSHV to maintain a successful infection (reviewed in Ye et al., 2011). Consequently, examining the mechanism of the latent to lytic switch will aid in our understanding of KSHV pathogenesis.

This thesis provides evidence that while RTA does not directly polyubiquitinate vFLIP, the lytic inducer does induce vFLIP degradation and down-regulation of vFLIP-induced NF-κB associated genes, specifically TNFα and ICAM1. Additionally, we demonstrate that amino acids 11-150 in RTA are required for the degradation of vFLIP. We found that RTA utilizes the ubiquitin E3-ligase activity of the Itch/A20 ubiquitin-editing complex. Our data confirm that the ubiquitin E3-ligase activity of Itch and A20 are required for vFLIP degradation. Lastly, we provide evidence of A20, Itch, RTA, and vFLIP interaction. Taken together, our data suggest that RTA utilizes the A20/Itch ubiquitin-editing complex to degrade vFLIP in order to inhibit NF-κB signaling and allow for lytic reactivation.

CHAPTER II:

MATERIALS AND METHODS

Cell Line Maintenance and Transfection

Human Embryonic Kidney 293T (HEK 293T) cells were cultured in DMEM medium 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) linear, MW~25,000 (Polysciences, Inc. Cat#23966) at a ratio of 1µg plasmid DNA:3µl PEI. For immunoprecipitations, cells were transfected using the Calcium Phosphate method. A total amount of 450µl of water and plasmid were added to 50µl of $CaCl_2$, and 500µl of 2xHBS were added drop wise while vortexing. After 5 min of incubation the mixture was added to the cells. 24h post transfection the media was changed and if appropriate, 2.5µM of MG132 was added.

Reagent, Plasmids, and Antibodies

The reagent MG132 (Boston Biochem) was used in this study. Plasmids pCMV-myc-RAUL-WT and the pCMV-myc-RAUL dominant-negative mutant (C1051A) were provided by Cecile Pickart (You et al., 2003). pSEW R01 (WT RTA), pSEW R03, pSEW R04, pSEW R06, pSEW R11, RTA C141S, and RTA H145L were described previously (Yu et al., 2005; Wang et al., 2003). Flag-A20, Flag-A20-C624A/C627A, Flag-Itch, Flag-Itch-C830A, GFP-RNF11, GFP-RNF11-C99A, and GFP-RNF11-Y40A were provided by Ed Harhaj (Shembade et al., 2008; Shembade et al., 2009; Shembade et al., 2010), and myc-vFLIP from Gary Hayward. The following primary antibodies were used: anti-RTA (G. Hayward), anti-cmyc (Millipore), anti-Itch (BD Transduction

Laboratories), anti-β-actin (Sigma-Aldrich), anti-tubulin (Sigma-Aldrich), anti-RAUL (Sun et al., 1999), anti-Flag (Sigma-Aldrich), anti-A20 H-100 (Santa Cruz Biotechnology, Inc.), and anti-GFP (Thermo Scientific). The secondary antibodies used were anti-mouse-HRP and anti-rabbit-HRP (Jackson ImmunoResearch) as well as anti-Chicken IgY (Thermo Scientific).

Immunoblot Analysis

Transfected cells with appropriate constructs were harvested 48h post-transfection by direct addition of 2.5x Laemmli Buffer followed by incubation at 100°C for 10 min. Lysates were electrophoresed on a 10% SDS-PAGE or Any kD Mini-PROTEAN TGX Precast Gel (Biorad) and Tris-glycine running buffer. Proteins were transferred to a PVDF membrane using a semi-dry transfer system at 20V for 20min. Membranes were blocked in 5% non-fat dry milk in PBS for 1hr followed by incubation with primary antibody overnight at 4°C and secondary antibody for 1hr at room temperature. Proteins were visualized by addition of ECL substrate and detection of chemiluminescent signal on x-ray film.

Immunoprecipitation

Transfected cells with appropriate constructs were harvested 48h post-transfection with PBS and centrifuged at 1500 rpm for 5 min. 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. Cell lysates were centrifuged to remove cell debris and 50µl of each sample were saved as a control lysate. The remaining amount of the samples was precleared with protein A/G PLUS-agarose (Santa Cruz) for 30 min at 4°C. The lysate

was transferred to a new 1.5mL tube and 1µg of the appropriate primary antibody was added to the cell lysate and incubated on a rotator overnight at 4°C. 25µl of protein A/G-agarose were added the following day for 1hr with 4 subsequent washings of RIPA lysis buffer. 50µl of 2.5x Laemmli Buffer were added and samples were boiled followed by incubation at 100°C for 10 min. Samples were visualized through immunoblot analysis as described above.

Luciferase Reporter Assay

NF-κB activation was quantified using the Dual Luciferase reporter assay system (Promega). Briefly cells were transfected with indicated plasmids plus the two reporter plasmids pNF-κB-Luc (Stratagene) and pGL4.70[hRluc]. Cell lysates were prepared according to the manufacturer's protocol. Luciferase was measured on a GloMax-Multi Microplate Multimode Reader (Promega). Data were taken as a ratio of firefly/renilla luciferase.

RNA Extraction, Reverse Transcription, and qPCR

At 72h post-transfection, cells were harvested for RNA isolation. RNA was extracted using the Promega SV Total RNA Extraction Kit. DNase-treated RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions. The resulting cDNA was used for quantitative polymerase chain reaction (qPCR) performed on an ABI Prism 7000 Sequence Detection System using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Fermentas) as per manufacturer's specifications. vFLIP, ICAM1, TNFα, GAPDH, and β-actin were amplified using the following primers: β-actin F 5'-CAT GTA CGT TGC TAT CCA

GGC-3', R 5'-CTC CTT AAT GTCACG CAC GAT-3'; GAPDH F 5'-AAT CCC ATC ACC ATC TTC CAG-3', R 5'-AAA TGA GCC CCA GCC TTC-3'; ICAM1 F 5'-CAA TGT GCT ATT CAA ACT GCC C-3, R 5'-CAGCGTAGGGTAAGGTTCTTG-3'; TNFα F 5'-ACT TTG GAG TGA TCG GCC-3', R 5'-GCT TGA GGG TTT GCT ACA AC-3'; vFLIP F 5'- GGATGCCCTAATGTCAATGC-3',R 5'-GGCGATAGTGTTGGAGTGT-3'. Relative gene expression was calculated using the ΔΔCt method using the average of GAPDH and β-actin housekeeping genes for normalization, and reactions were performed in triplicates. Data were analyzed for statistical significance using a fixed-effects one-way ANOVA with alpha set at 0.05 using JMP (Version 10.0). Error bars represent the standard error.

CHAPTER III:

PRELIMINARY RESULTS

RTA inhibits vFLIP-induced NF-κB activation

It has been previously reported that NF-κB is required for the maintenance of latency (Brown et al., 2003; Grossman and Ganem, 2008) and that vFLIP induces NF-κB activation (Chaudhary et al., 1999; Liu et al., 2002; Field et al., 2003; Matta et al., 2007; Matta and Chaudhary, 2004). These data suggest that NF-κB associated gene expression might need to be down-regulated for the virus to enter the lytic cycle of replication. To determine whether RTA has an effect on NF-κB signaling, HEK 293T cells were transfected with vFLIP and/or RTA and NF-κB activity was assessed using a luciferase reporter containing five NF-κB response elements. vFLIP expression induced a 15-fold NF-κB activation as expected; however, co-transfection with RTA reduced the activation by three-fold (Figure 1, vFLIP alone compared to vFLIP + RTA). The role of RTA in blocking vFLIP-induced NFκB activation could be explained by one or more of the following mechanisms: RTA-induced degradation of vFLIP or RTA-induced down-regulation of components of the NFκB pathway. To evaluate whether RTA was targeting components of the NF-κB pathway, the effect of RTA on TNFα induced NF-κB activation was assessed. While co-transfection with RTA consistently resulted in a dose-dependent 2-3 fold decrease in vFLIP-induced NF-κB activation, RTA had no significant effect on NF-κB activation stimulated by TNFα, suggesting that RTA was targeting an aspect of vFLIP-induced NF-κB activation, rather than a component of the signal transduction pathway (data not shown). These data suggest that RTA expression

counteracts latency by inhibiting vFLIP-induced NF-κB activation by targeting vFLIP function.

vFLIP protein is decreased in the presence of RTA

To evaluate the role of RTA on vFLIP stability, HEK 293T cells were transfected with myc-vFLIP in the presence and absence of RTA (Figure 2, lanes 2 and 4). Co-transfection of RTA with vFLIP resulted in greatly decreased levels of vFLIP protein as detected by western blotting technique (Figure 2, lane 4).

RTA induces proteasome-mediated degradation of vFLIP

To evaluate whether vFLIP protein stability in the presence of RTA would be altered by the proteasome inhibitor MG132, HEK 293T cells were transfected with myc-vFLIP in the presence and absence of RTA and increasing doses of MG132. Even the smallest dose of MG132 in the presence of RTA resulted in vFLIP stability when compared to a lane without the addition of MG132 (Figure 3, lane 4 compared to lane 2), suggesting that RTA is inducing vFLIP degradation via the proteasome.

Figure 1: RTA inhibits vFLIP-induced NF-κB activation. HEK 293T cells were transfected with myc-vFLIP, RTA, and empty vector control 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 Promega luminometer.

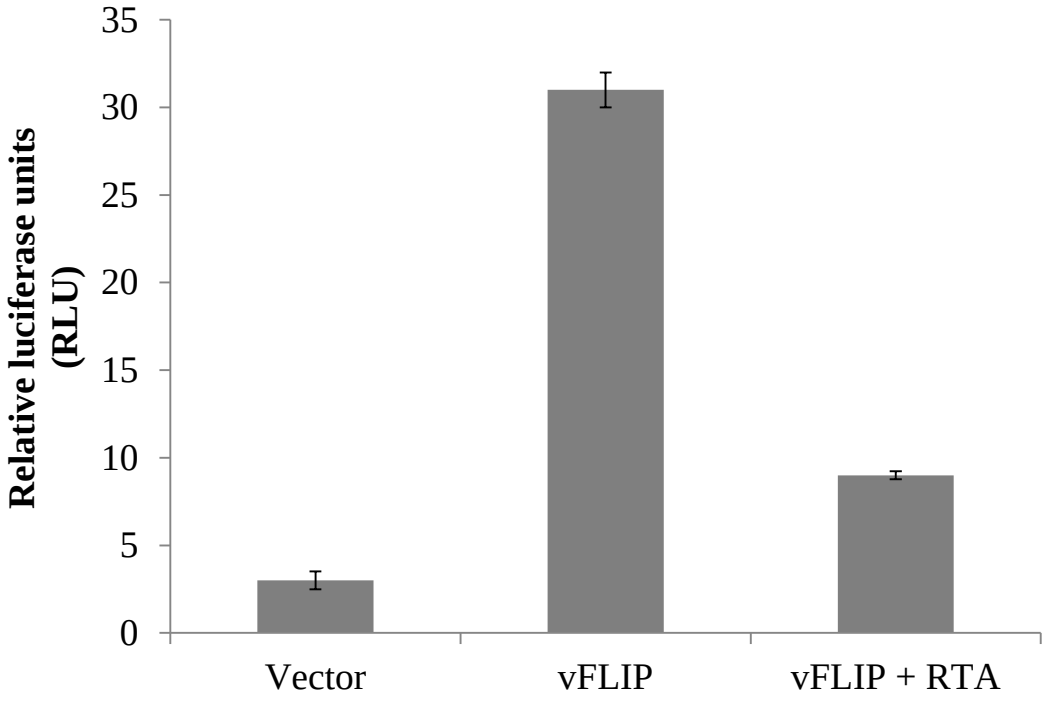

Relative luciferase units (RLU)
35
30
25
20
15
10
5
0
Vector
vFLIP
vFLIP + RTA

Figure 2: vFLIP protein is decreased in the presence of RTA. HEK 293T cells were transfected with myc-vFLIP, RTA, and empty vector control (pcDNA) where indicated. 48h post-transfection, cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against myc(vFLIP), RTA, and β-actin.

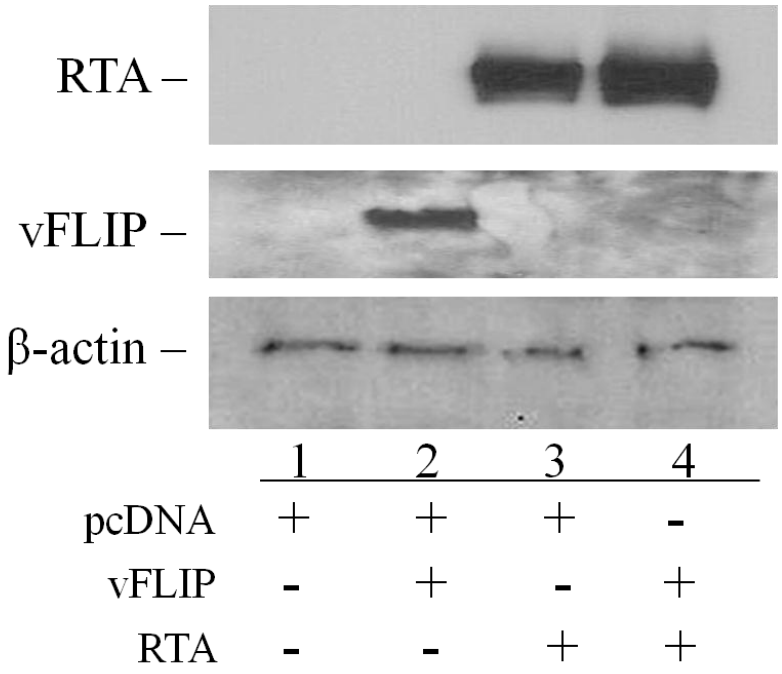
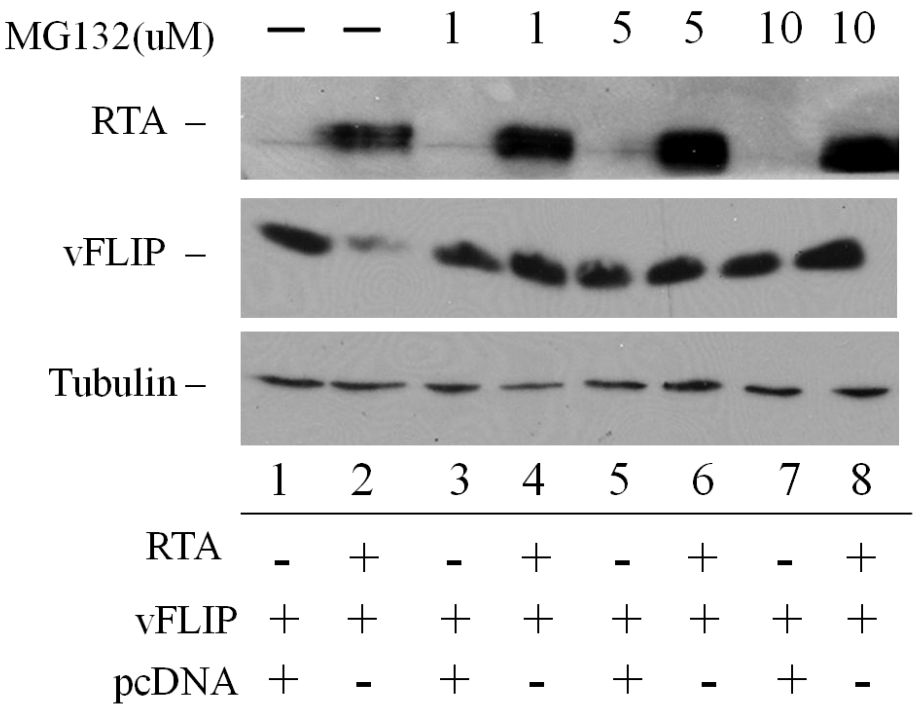

RTA –
vFLIP –
β-actin –
1 2 3 4
pcDNA + + + -
vFLIP - + - +
RTA - - + +

Figure 3: RTA induces proteasome-mediated degradation of vFLIP. HEK 293T cells were transfected with myc-vFLIP and RTA or empty vector control (pcDNA) where indicated. The proteasome inhibitor MG132 was added 12h post transfection at the indicated concentrations. Dimethyl sulfoxide (DMSO), the solvent for MG132, was added in the absence of MG132 in lanes 1 and 2. 48h post transfection, cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against myc(vFLIP), RTA, and tubulin.


MG132(uM) – – 1 1 5 5 10 10
RTA –
vFLIP –
Tubulin –
1 2 3 4 5 6 7 8
RTA - + - + - + - +
vFLIP + + + + + + + +
pcDNA + - + - + - + -

CHAPTER IV:

RESULTS

RTA inhibits vFLIP-induced expression of TNFα and ICAM1 in HEK 293T cells

To further examine the role of RTA on vFLIP function, the effect of RTA on vFLIP induction of two NF-κB responsive genes was examined. Dr. Ehrlich previously used an NF-κB signaling pathway qPCR Array to identify genes whose transcripts were up-regulated by vFLIP in HEK 293T cells. We chose to validate vFLIP-induced expression of TNFα and ICAM1 mRNA in the presence and absence of RTA in transfected HEK 293T cells. While both ICAM1 and TNFα expression were negligible in the control cells transfected with an empty vector, transfection of myc-vFLIP resulted in 24-and 103-fold increases in TNFα and ICAM1 expression respectively (Figure 4A and B, lane 2 with vFLIP alone). However, co-transfection of RTA with myc-vFLIP resulted in an 11-and 13-fold reduction in TNFα and ICAM1 expression, respectively, bringing gene expression down to background levels (Figure 2A and B, lane 4 with vFLIP + RTA). RTA alone had no effect on TNFα and ICAM1 transcript levels (Figure 4A and B, lane 3 with RTA alone).

RTA induces vFLIP degradation through recruitment of a novel cellular ubiquitin ligase

RTA has been reported to participate both directly and indirectly in the degradation of several inhibitors of lytic replication (Yu et al., 2005; Yang et al., 2008; Gould et al, 2009; Yu and Hayward, 2010). To evaluate whether RTA is directly promoting the degradation of vFLIP, two mutants in the ubiquitin E3-ligase domain of RTA, RTA C141S and RTA H145L, were used. These mutants cannot degrade IRF7, and

RTA H145L is also defective for degradation of Hey-1 and K-RBP (Yu et al., 2005; Yang et al., 2008; Gould et al, 2009). In HEK 293T cells co-transfected with vFLIP and either RTA mutant (C141S and H145L), vFLIP protein levels were just as efficiently reduced as with wild-type (WT) RTA (Figure 5, lanes 2, 3 and 4), which indicates that while RTA is promoting vFLIP degradation, the degradation does not require the intrinsic ubiquitin E3-ligase activity of RTA.

Amino acids 11-150 in RTA are required for vFLIP degradation

Because RTA is not using its intrinsic ubiquitin E3-ligase activity to destabilize vFLIP, it is likely that RTA is recruiting another cellular ubiquitin ligase. Therefore, to determine whether a specific domain of RTA is required for RTA-induced degradation of vFLIP, RTA truncation mutants (Figure 6A) were used and their effects on vFLIP stability were assessed (Figure 6B). The results with RTA mutants R03(1-377), R04(1-273), R06(151-548), and R11(**Δ**11-272) are compared with WT RTA(1-691) (Figure 6B, lanes 5, 6, 7, and 8 compared to lane 4). All of these mutants were previously shown to still bind to C/EBPα and/or DNA, suggesting that despite their truncation they are still folding well enough to maintain some of their functional domains (Wang et al., 2003). Co-transfection of R06 RTA(151-548) and R11(**Δ**11-272) with vFLIP resulted in a moderate defect in vFLIP degradation (Figure 6B, compare lanes 4 to 7 and 8). The R03(1-377) and R04(1- 273) proteins were not detected on western blots because they are missing the epitope recognized by our RTA antibody (located at amino acids 527-539). It is likely that expression of RTA was sufficient because vFLIP was completely degraded (Figure 6B, lanes 5 and 6). These data suggest that a region within amino acids

11-150 is likely to be critical for forming a stable interaction with either vFLIP or a cellular protein required for vFLIP destabilization or both. This region contains the ubiquitin ligase domain but is distinct from the region reported to bind IRF7.

RTA-induced vFLIP-degradation does not occur via a RAUL-dependent mechanism

While RTA has intrinsic ubiquitin E3-ligase activity targeting IRF7, RTA was also previously shown to recruit and stabilize the cellular HECT ubiquitin E3-ligase RTA-Associated Ubiquitin Ligase (RAUL) leading to degradation of both cellular IRF3 and IRF7 (Yu and Hayward, 2010). To evaluate the possible role of RAUL in RTA-induced degradation of vFLIP, we used a dominant-negative mutant of RAUL that has a C1051A mutation at the catalytic site in its HECT domain (You et al., 2003). In the presence of RTA and endogenous RAUL, vFLIP is efficiently degraded (Figure 7, lane 4), however upon addition of the mutant myc-tagged RAUL ubiquitin E3-ligase in the presence of RTA, there was no significant increase in vFLIP levels (Figure 7, lane 5). These data suggest that RTA-induced vFLIP degradation does not occur via a RAUL-dependent mechanism.

Itch and A20 ubiquitin E3-ligase activity is required for vFLIP degradation in the presence of RTA

The intrinsic ubiquitin E3-ligase activity of both RTA and RAUL, the ubiquitin E3-ligase RTA has been shown to recruit and stabilize, were not required for RTA-induced vFLIP degradation, thus, we hypothesized that RTA was likely recruiting another cellular ubiquitin E3-ligase for vFLIP degradation. In order to identify which cellular ligase may be recruited by RTA, a literature search was performed to locate ideal

targets. Itch is a cellular ubiquitin E3-ligase that has been reported to target the cellular FLICE inhibitory protein (cFLIP) for proteasome-mediated degradation (Chang et al., 2006). vFLIP is a truncated homolog of cFLIP (Thome et al., 1997); consequently, we hypothesized that it is possible that vFLIP could be targeted for degradation by Itch.

To determine if the ubiquitin E3-ligase activity of Itch was required for vFLIP degradation in the presence of RTA, an Itch mutant with impaired E3-ligase activity, Itch-C830A, was used. The catalytically inactive mutant, Itch-C830A, presents with a dominant-negative effect due to the ability to compete with endogenous Itch (Omerovic et al., 2007). In the presence of RTA and Itch-C830A, vFLIP was stabilized, compared to the rapid degradation of vFLIP induced by RTA in the absence of the Itch dominant-negative mutant (Figure 8A, compare lane 4 to lane 2). This suggests that the ubiquitin E3-ligase activity of Itch is required for vFLIP degradation. Evidence also indicates that vFLIP stability in the presence of Itch-C830A is dose-dependent; as vFLIP stabilization correlates with increasing amounts of Itch-C830A (Figure 8B). However, vFLIP was also stabilized when WT Itch was co-transfected with RTA (Figure 8A, lane 3), suggesting that Itch may be part of a complex. If Itch is part of a complex, then increased levels of WT Itch due to overexpression will affect the stoichiometry of the complex resulting in a dominant-negative phenotype.

Another literature search was performed to identify Itch complexes of interest. Tax is a regulatory protein of human T cell leukemia virus type 1 (HTLV-1) and is similar to KSHV's vFLIP in that it causes proliferation through its activation of NF-κB (reviewed in Pilotti, et al. 2013). NF-κB is activated by many viruses to increase viral

replication and evade cell apoptosis and it is a common method by viruses to interfere with host cell signaling by targeting components that inhibit NF-κB (Reviewed in Lisowski & Witkowski, 2003).

In 2008, a ubiquitin-editing complex that reduced inflammatory signaling through the inactivation of signaling proteins such as RIP1, an intermediate of the cytokine tumor necrosis factor (TNF) was described (Shembade et al., 2008). The ubiquitin-editing complex consisted of three main components: TAX1BP1, a cellular protein that interacts with Tax, Itch, and A20. A20 is a ubiquitin-editing enzyme that has been shown to terminate NF-κB signaling through inhibiting signaling intermediates like RIP1; in the absence of A20, persistent NF-κB signaling occurs (Shembade et al., 2008). When A20 forms the ubiquitin-editing complex with Itch described above, the complex works to shut off NF-κB signaling through altering the ubiquitination profile of NF-κB signaling intermediates. Thus, viruses may attempt to impede the ability of A20 and Itch or the ubiquitin-editing complex as a whole to shut off NF-κB signaling.

The authors found that TAX1BP1 recruited Itch to A20 through two 'PPXY' motifs resulting in RIP1 inactivation. They also found that A20 cleaved K63-linked polyubiquitin chains of RIP1, and that both A20 and Itch promoted K48-linked polyubiquitination, leading to the proteasome-mediated degradation of RIP1. However, Tax inactivated this complex by disrupting the interaction between TAX1BP1, A20, and Itch. Subsequently, the ring finger protein 11 (RNF11) was shown to be a critical component of this ubiquitin-editing complex and is required for the interaction of A20 with RIP1 (Shembade et al., 2009).

Due to the similarities between HTLV-1's Tax and KSHV's vFLIP, we examined whether the ubiquitin-editing complex Shembade et al. described could be involved in vFLIP degradation. Specifically, we were interested in examining the components of that ubiquitin-editing complex that had ubiquitin E3-ligase activity, and these components were Itch and A20. Since we previously presented evidence that the ubiquitin E3-ligase activity of Itch is required for vFLIP degradation in the presence of RTA, the ubiquitin E3-ligase activity of A20 was examined.

An A20 dominant-negative mutant, A20-C624A/C627A, that is unable to polyubiquitinate its substrates, was used to determine if the ubiquitin E3-ligase activity of A20 was required for vFLIP degradation in the presence of RTA. In the presence of RTA and A20-C624A/C627A, vFLIP was stabilized compared to the degradation of vFLIP seen in the absence of A20-C624A/C627A (Figure 8C, lane 4 compared to lane 2) indicating that the ubiquitin E3-ligase activity of A20 is required for vFLIP degradation. vFLIP was also stabilized in the presence of WT A20 and RTA. This is likely due to a dominant-negative effect produced by the overexpression of one component of a multiprotein complex (Figure 8C, lane 3). vFLIP stability in the presence of A20-C624A/C627A was also found to be dose-dependent, with noticeably increasing amounts of vFLIP stabilization coinciding with increasing amounts of A20-C624A/C627A (Figure 8D).

RNF11 expression is not required for RTA-induced vFLIP degradation

Since the ability of A20 to interact with and degrade RIP1 is dependent on the expression of RNF11 (Shembade et al., 2009), we wanted to investigate whether RNF11

was needed for the A20/Itch ubiquitin-editing complex to degrade vFLIP. Specifically, we wanted to know if the ubiquitin E3-ligase activity of RNF11 was needed for vFLIP degradation or if RNF11 was required for the formation or function of the A20/Itch ubiquitin-editing complex. Two RNF11 mutants were used: RNF11-C99A and RNF11-Y40A. The point mutation of RNF11-C99A occurs in the RING domain, eliminating the ability of RNF11 to act as a ubiquitin E3-ligase. RNF11-Y40A has a mutated 'PPXY' motif which can interfere with the ability of RNF11 to associate with other proteins, specifically ubiquitin E3-ligases, such as A20 (Shembade et al., 2008). In the presence of RTA and each RNF11 mutant transfected separately or together, vFLIP was degraded as efficiently as in the presence of RTA alone (Figure 9, lanes 4, 5, and 6 compared to lane 2). This indicates that RNF11 expression is not important for RTA-induced vFLIP expression, and is further supported by evidence that vFLIP is also efficiently degraded in the presence of RTA and RNF11-WT (Figure 9, lane 3), suggesting that RNF11 is not included in the A20/Itch ubiquitin-editing complex we describe for vFLIP degradation, since we would expect vFLIP to be stabilized due to a disruption of the ubiquitin-editing complex stoichiometry.

RTA and vFLIP interact with components of the A20/Itch ubiquitin-editing complex

We demonstrated that the ubiquitin E3-ligase activities of Itch and A20 are required for vFLIP destabilization in the presence of RTA. The next step was to look for interaction between these components to further characterize the mechanism of RTA-induced vFLIP degradation. We transfected HEK 293T cells with WT A20, vFLIP, and RTA as indicated (Figure 10A). Immunoprecipitation of A20 resulted in co-

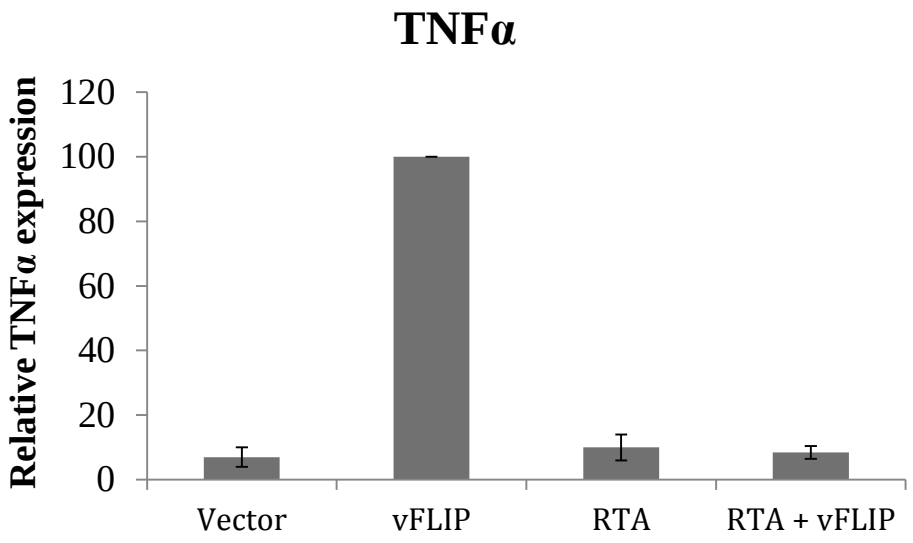
immunoprecipitation of vFLIP (Figure 10A, lane 2 and lane 3), indicating that vFLIP interacts with A20. In another experiment, HEK 293T cells were transfected with RTA with and without vFLIP as indicated (Figure 10B). Immunoprecipitation of vFLIP result in co-immunoprecipitation of endogenous Itch (Figure 10B, lane 2). This suggests that Itch and vFLIP interact.

We also immunoprecipitated RTA. HEK 293T cells were transfected with vFLIP, RTA, WT-Itch, and WT-A20 as indicated (Figure 10C). Immunoprecipitation of RTA resulted in co-immunoprecipitation of WT-A20 and vFLIP (Figure 10C, lane 2). A second RTA immunoprecipitation was performed; HEK 293T cells were transfected with vFLIP and RTA as indicated (Figure 10D). Immunoprecipitation of RTA resulted in co-immunoprecipitation of endogenous Itch and vFLIP (Figure 10D, lane 2).

We noticed that different interactions were occurring. For instance, two RTA immunoprecipitations were performed, and each resulted in different co-immunoprecipitations: A20 and vFLIP (Figure 10C) compared to Itch and vFLIP (Figure 10D). Due to the interaction evidence presented, we hypothesized that there are different pools of interaction occurring between these components. vFLIP could be interacting with A20 and Itch individually or interacting with them both simultaneously in order to interfere with the A20/Itch ubiquitin-editing complex and allow for NF-κB signaling. The presence of RTA could affect the interaction between vFLIP, A20, and Itch, to allow the A20/Itch ubiquitin-editing complex to function and degrade vFLIP, subsequently inhibiting NF-κB signaling. Overall, these data supported our hypothesis that RTA is co-opting the Itch/A20 ubiquitin-editing complex to target vFLIP for degradation.

Figure 4: RTA inhibits vFLIP-induced expression of TNFα and ICAM1 in HEK 293T cells. HEK 293T cells were transfected with myc-vFLIP, RTA, and empty vector control. At 72h post-transfection, cells were harvested for RNA isolation. For qPCR total RNA were isolated, reverse transcribed and quantified on an ABI7000 with using primers for TNFα and ICAM1 and Sybr green. The housekeeping genes used in the analysis were β-actin and GAPDH. Data were analyzed using the ΔΔCt method and presented as relative to vFLIP. **A.** vFLIP-induced expression of TNFα, p= 0.0001 and **B.** ICAM1, p= 0.0001 in the presence or absence of RTA calculated relative to vFLIP alone. Error bars represent standard error.

A


TNFα
Relative TNFα expression
120
100
80
60
40
20
0
Vector
vFLIP
RTA
RTA + vFLIP

B

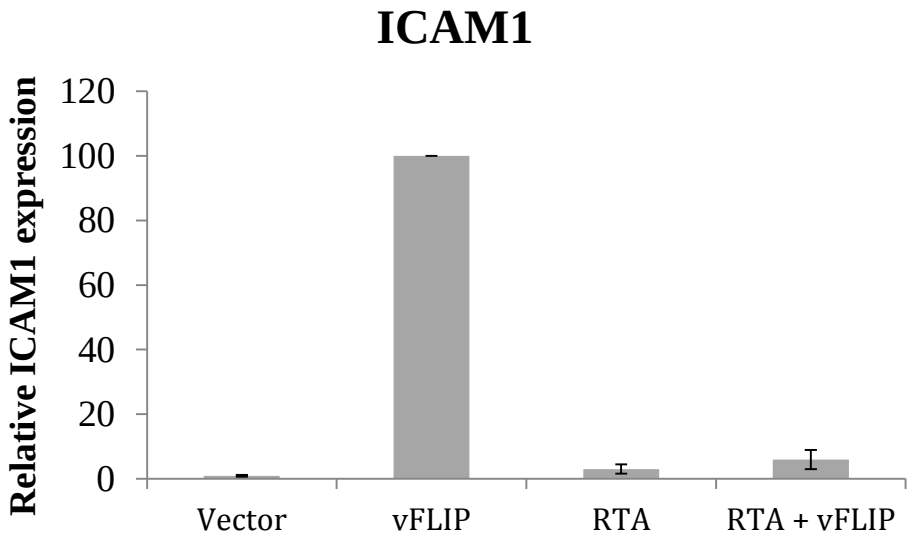
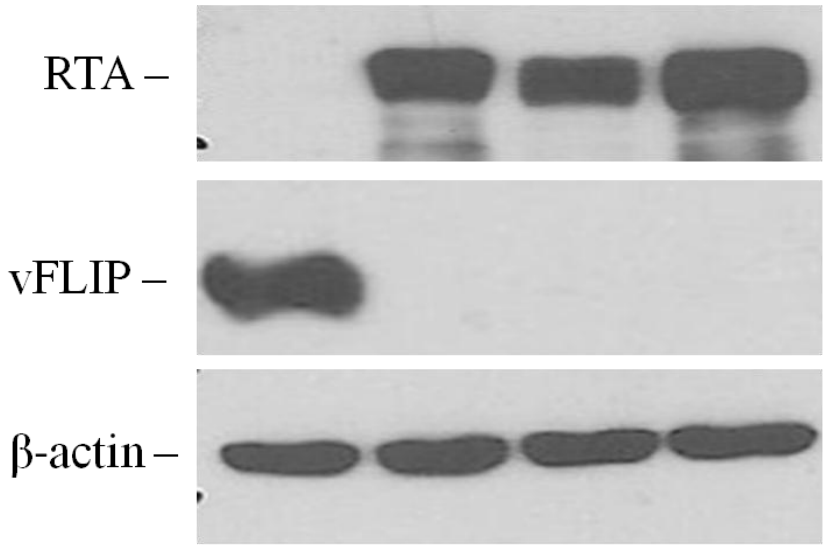
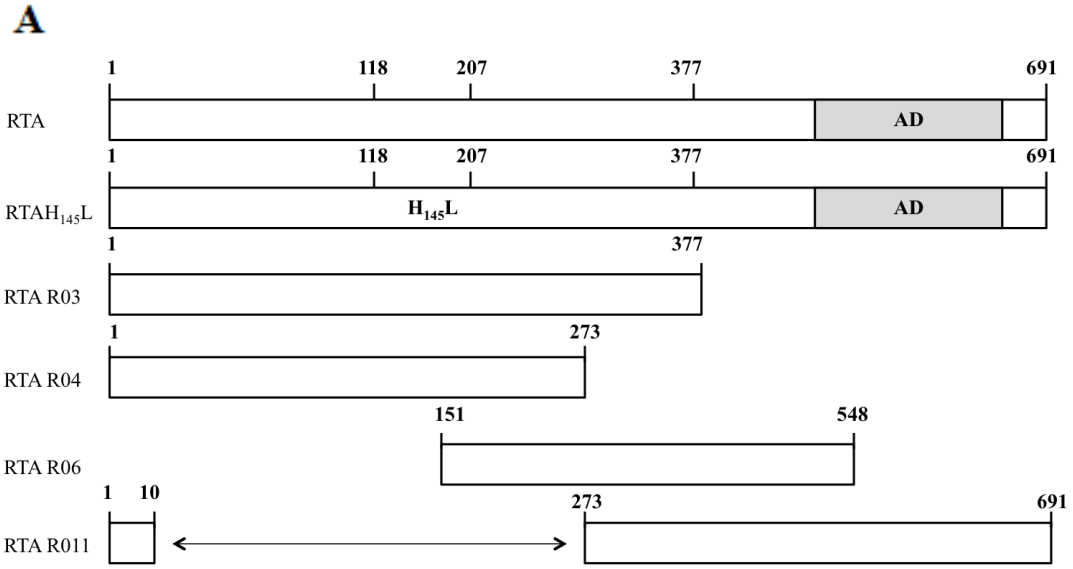

ICAM1
Relative ICAM1 expression
120
100
80
60
40
20
0
Vector
vFLIP
RTA
RTA + vFLIP

Figure 5: RTA induces vFLIP degradation through recruitment of a novel cellular ubiquitin ligase. HEK 293T cells were transfected with myc-vFLIP, WT or mutant (C141S and H145L) RTAs, and empty vector control (pcDNA). 48h post transfection, cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against RTA, myc(vFLIP) and β-actin.



	1	2	3	4
RTA	-	WT	C141S	H145L
vFLIP	+	+	+	+
pcDNA	+	-	-	-

Figure 6: Amino acids 11-150 in RTA are required for vFLIP degradation. A. Illustration depicting the RTA truncation mutants used in this study. Note, that the rabbit RTA antibody recognizes amino acids 527-539, so not all mutants were detected by western blot. **B.** HEK 293T cells were transfected with myc-vFLIP, wild type or mutant RTA, and empty vector control (pcDNA). 48h post-transfection cells were harvested and analyzed by SDSPAGE followed by western blot with antibodies against RTA, myc(vFLIP), and β-actin.


A
1
118
207
377
691
RTA
AD
RTAH145L
H145L
RTA R03
RTA R04
273
RTA R06
151
548
RTA R011
10
273
691

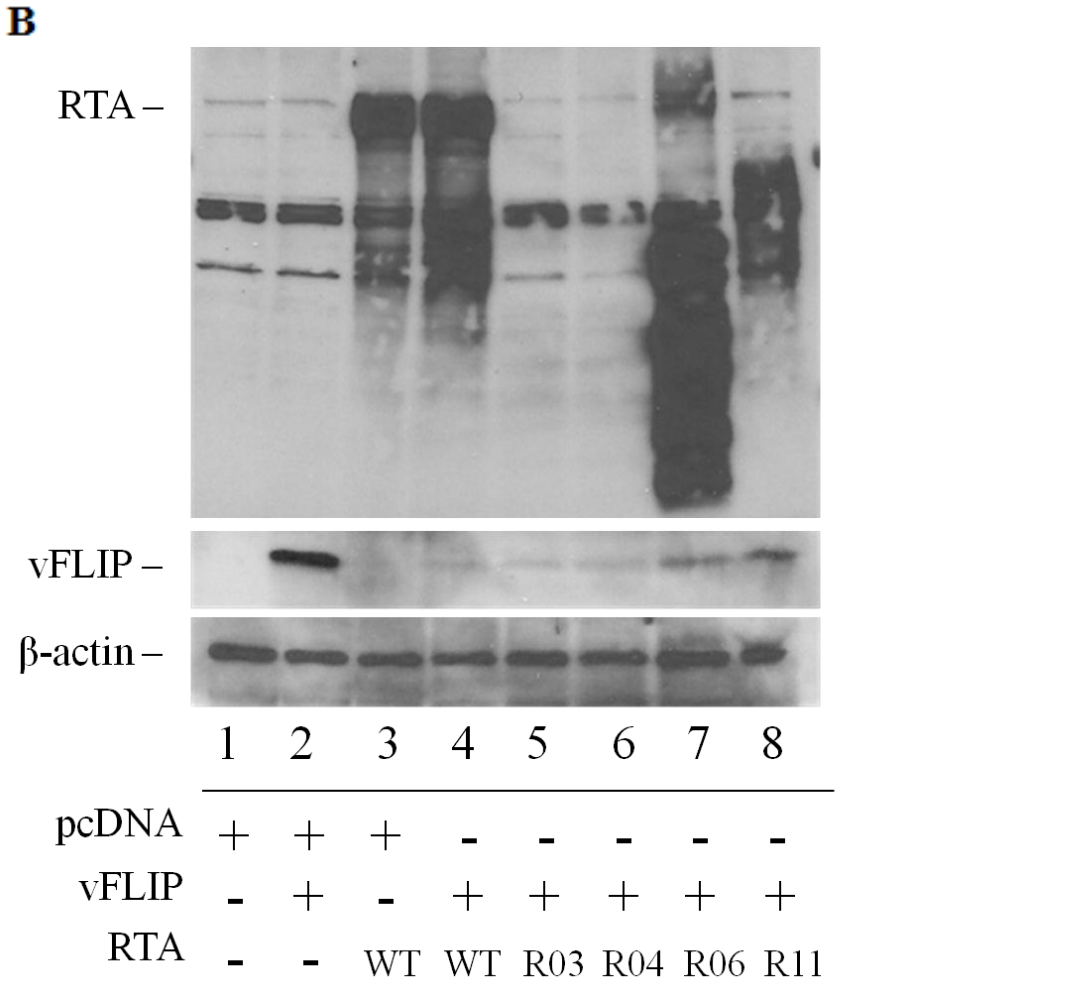

B
RTA –
vFLIP –
β-actin –
1 2 3 4 5 6 7 8
pcDNA + + + - - - - -
vFLIP - + - + + + + +
RTA - - WT WT R03 R04 R06 R11

Figure 7: RTA-induced vFLIP degradation does not occur via a RAUL-dependent mechanism. HEK 293T cells were transfected with myc-vFLIP, RTA, RAUL, myc-RAUL- mutant C1051A, and empty vector control (pcDNA) where indicated. 48h post transfection, cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against RTA, RAUL, myc(vFLIP and RAUL-C1051A), and β-actin.

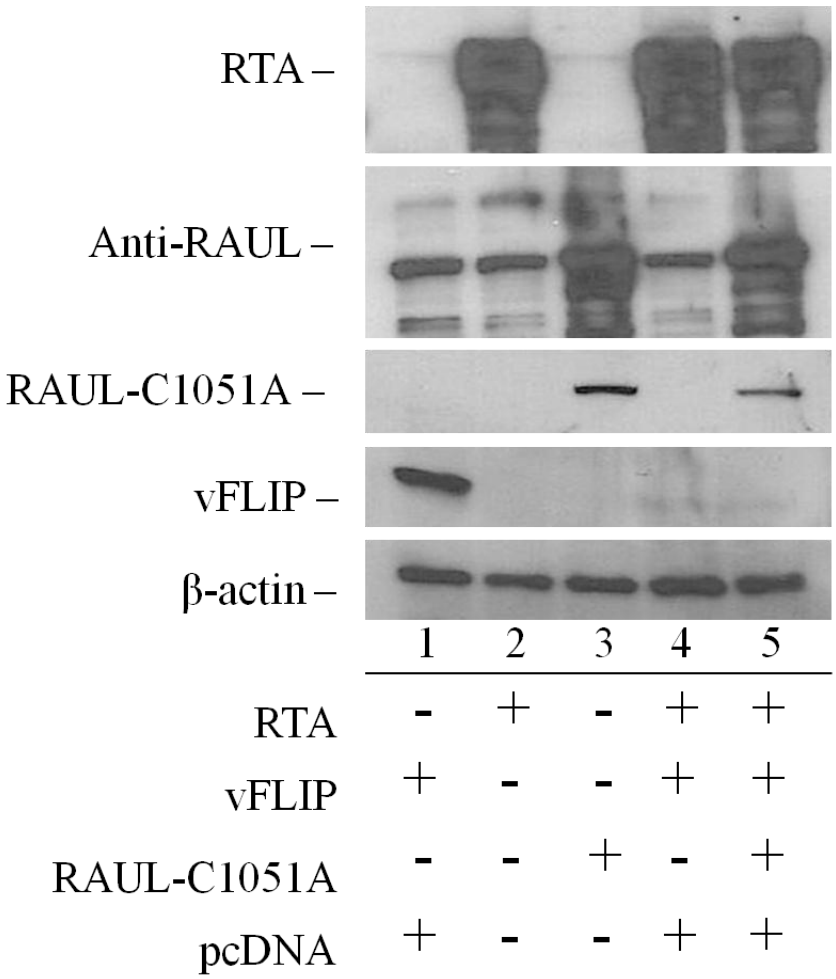

RTA –
Anti-RAUL –
RAUL-C1051A –
vFLIP –
β-actin –
1 2 3 4 5
RTA - + - + +
vFLIP + - - + +
RAUL-C1051A - - + - +
pcDNA + - - + +

Figure 8: Itch and A20 ubiquitin E3-ligase activity is required for vFLIP degradation in the presence of RTA. A. vFLIP was stabilized in the presence of WT Itch and Itch-C830A. HEK 293T cells were transfected with myc-vFLIP, RTA, Flag-Itch WT, Flag-Itch-C830A, and empty vector control (pcDNA). 48h post-transfection, cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against myc(vFLIP), RTA, β-actin, and Flag(Itch and Itch-C830A). **B.** vFLIP stability in the presence of Itch-C830A is dose dependent. HEK 293T cells were transfected with myc-vFLIP, RTA, and increasing levels of Flag-Itch-C830A and empty vector control (pcDNA). 48h post-transfection, cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against myc(vFLIP), RTA, β-actin, and Flag(Itch-C830A). **C.** vFLIP is stabilized in the presence of WT A20 and A20-C624/627A. HEK 293T cells were transfected with myc-vFLIP, RTA, Flag-A20 WT, Flag-A20-C624/627A, and empty vector control (pcDNA). 48h post-transfection, cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against myc(vFLIP), RTA, β-actin, and Flag(A20 WT and A20-C624/627A). **D.** vFLIP stability in the presence of A20-C624/627A is dose dependent. HEK 293T cells were transfected with myc-vFLIP, RTA, and increasing levels of Flag-A20-C624/627A and empty vector control (pcDNA). 48h post-transfection, cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against myc(vFLIP), RTA, β-actin, and Flag(A20-C624/627A).

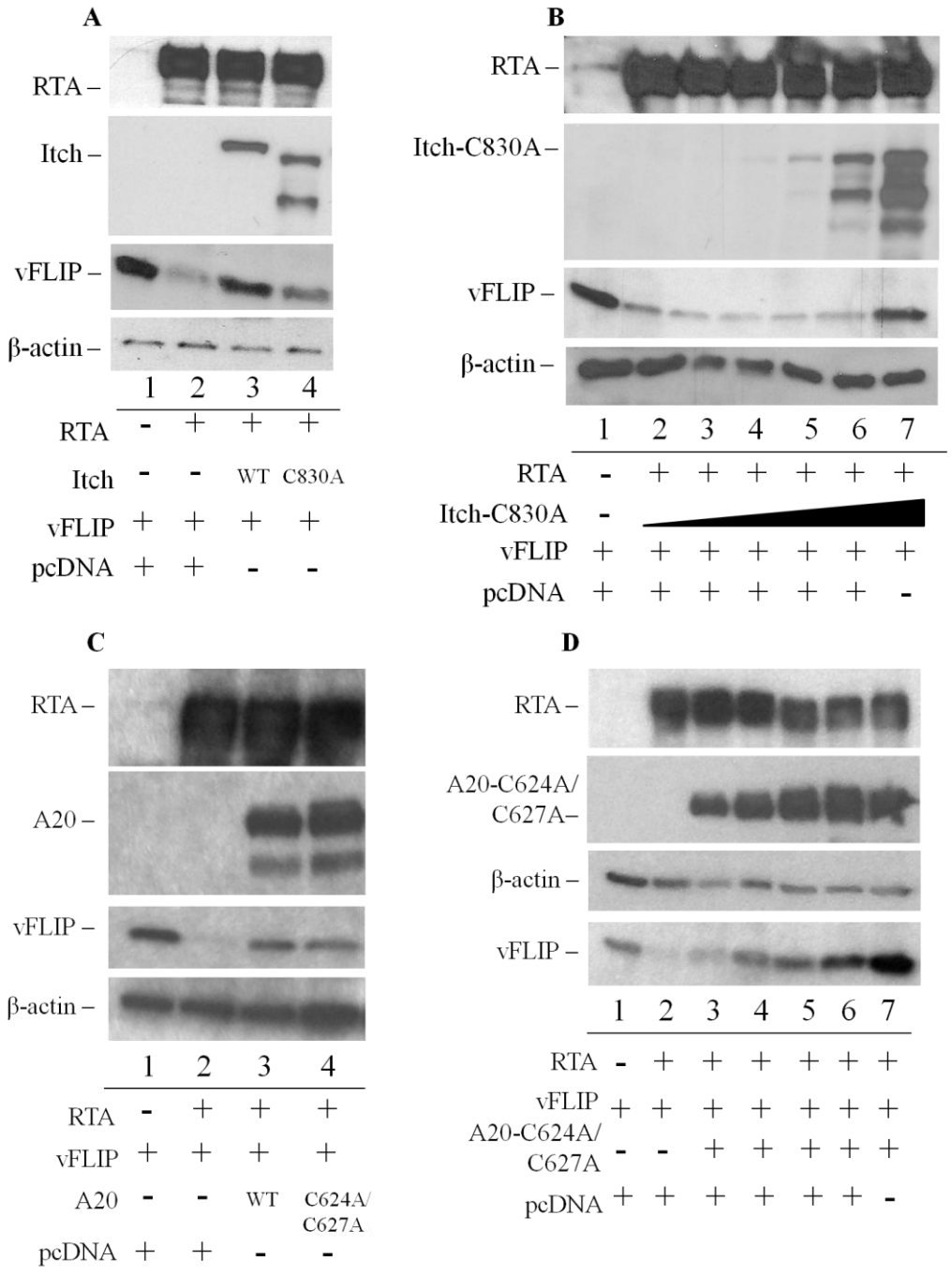

A
RTA –
Itch –
vFLIP –
β-actin –
1 2 3 4
RTA - + + +
Itch - - WT C830A
vFLIP + + + +
pcDNA + + - -
B
RTA –
Itch-C830A –
vFLIP –
β-actin –
1 2 3 4 5 6 7
RTA - + + + + + +
Itch-C830A -
vFLIP + + + + + + +
pcDNA + + + + + + -
C
RTA –
A20 –
vFLIP –
β-actin –
1 2 3 4
RTA - + + +
vFLIP + + + +
A20 - - WT C624A/ C627A
pcDNA + + - -
D
RTA –
A20-C624A/ C627A–
β-actin –
vFLIP –
1 2 3 4 5 6 7
RTA - + + + + + +
vFLIP + + + + + + +
A20-C624A/ C627A - - + + + + +
pcDNA + + + + + + -

Figure 9: RNF11 expression is not required for RTA-induced vFLIP degradation. vFLIP is degraded in the presence of RNF11 mutants. HEK 293T cells were transfected with myc-vFLIP, RTA, GFP-RNF11-WT, GFP-RNF11-C99A, GFP-RNF11-Y40A, and empty vector control (pcDNA). 48h post-transfection, cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against myc(vFLIP), RTA, β-actin, and GFP(RNF11, RNF11-C99A, and RNF11-Y40A).

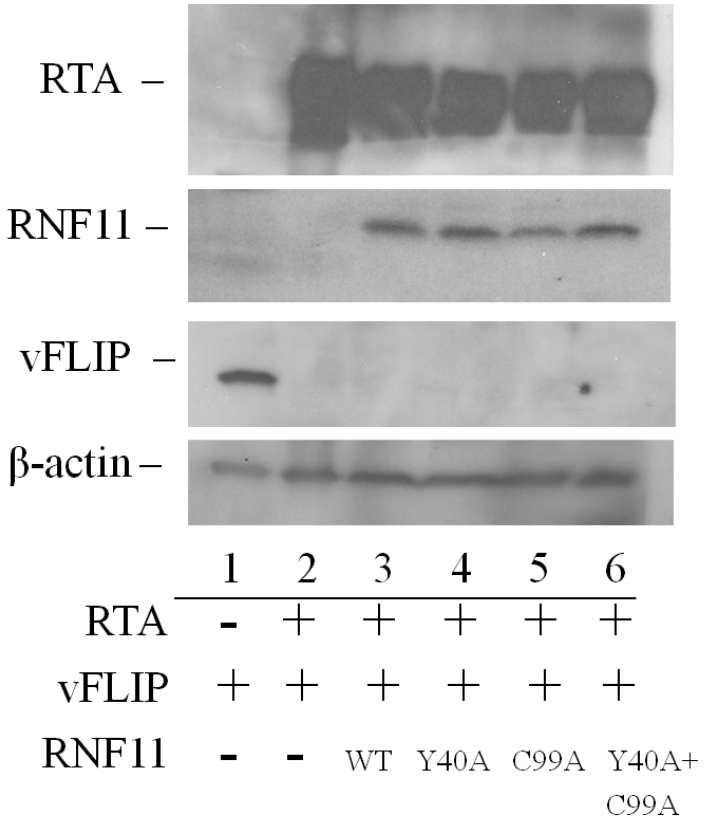
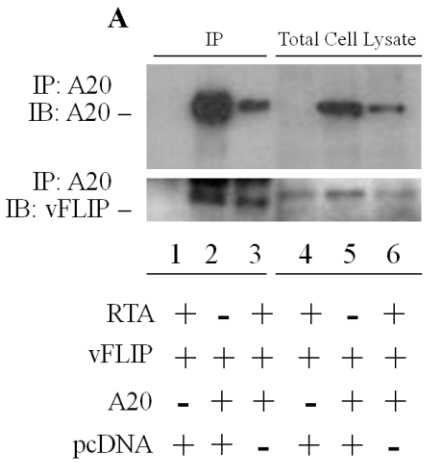
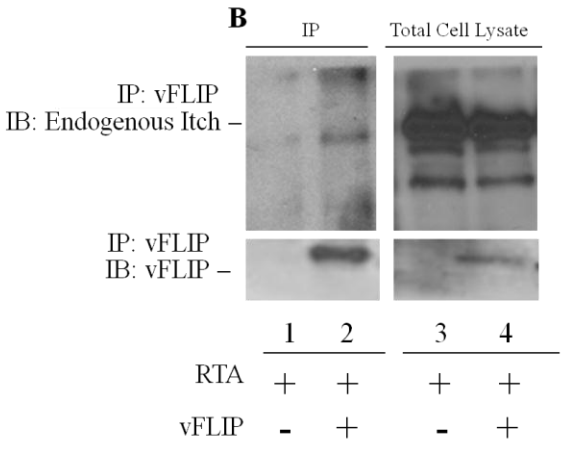
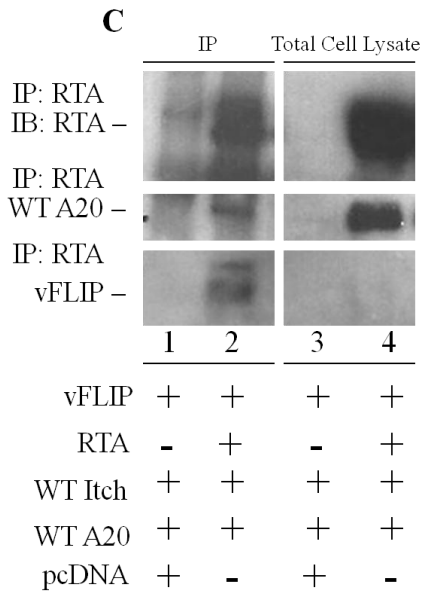

RTA –
RNF11 –
vFLIP –
β-actin –
1 2 3 4 5 6
RTA - + + + + +
vFLIP + + + + + +
RNF11 - - WT Y40A C99A Y40A+ C99A

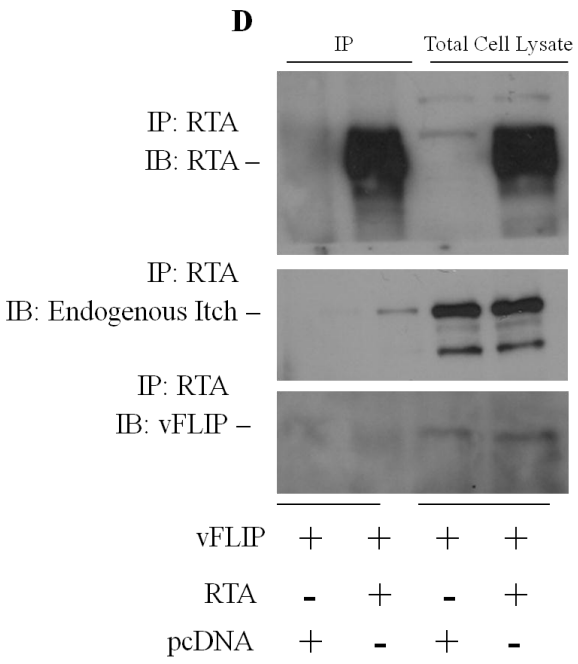
Figure 10: RTA and vFLIP interact with components of the A20/Itch ubiquitin-editing complex. A. Immunoprecipitation of A20 show interaction with vFLIP. HEK 293T cells were transfected with myc-vFLIP, RTA, Flag-A20-WT, and empty vector control (pcDNA). 24h post-transfection, the cell medium was changed and the cells were treated with 2.5μM of the proteasome inhibitor MG132. 48h post-transfection, cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against myc(vFLIP) and Flag(A20). **B.** Immunoprecipitation of vFLIP show interaction with Itch. HEK 293T cells were transfected using the calcium phosphate method with myc-vFLIP, RTA, and empty vector control (pcDNA). 24h post-transfection, the cell medium was changed and the cells were treated with 2.5μM of the proteasome inhibitor MG132. 48h post-transfection, cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against myc(vFLIP) and AIP4(endogenous Itch). **C.** Immunoprecipitation of RTA show interaction with A20 and vFLIP. HEK 293T cells were transfected using the calcium phosphate method with myc-vFLIP, RTA, Flag-A20-WT, Flag-Itch-WT, and empty vector control (pcDNA). 24h post-transfection, the cell medium was changed and the cells were treated with 2.5μM of the proteasome inhibitor MG132. 48h post-transfection, cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against RTA, myc(vFLIP), and Flag(A20). **D.** Immunoprecipitation of RTA show interaction with Itch and vFLIP. HEK 293T cells were transfected using the calcium phosphate method with myc-vFLIP, RTA, and empty vector control (pcDNA). 24h post-transfection, the cell medium was changed and the cells were treated with 2.5μM of the proteasome inhibitor MG132. 48h post-transfection,

cells were harvested and analyzed by SDS-PAGE followed by western blot with antibodies against RTA, myc(vFLIP), and AIP4(endogenous Itch).


A
IP
Total Cell Lysate
IP: A20
IB: A20 –
IP: A20
IB: vFLIP –
1 2 3 4 5 6
RTA + - + + - +
vFLIP + + + + + +
A20 - + + - + +
pcDNA + + - + + -


B
IP
Total Cell Lysate
IP: vFLIP
IB: Endogenous Itch –
IP: vFLIP
IB: vFLIP –
1 2 3 4
RTA + + + +
vFLIP - + - +


C
IP
Total Cell Lysate
IP: RTA
IB: RTA –
IP: RTA
WT A20 –
IP: RTA
vFLIP –
1 2 3 4
vFLIP + + + +
RTA - + - +
WT Itch + + + +
WT A20 + + + +
pcDNA + - + -


D
IP
Total Cell Lysate
IP: RTA
IB: RTA –
IP: RTA
IB: Endogenous Itch –
IP: RTA
IB: vFLIP –
vFLIP + + + +
RTA - + - +
pcDNA + - + -

CHAPTER V:

DISCUSSION

NF-κB modulation is an important strategy viruses use to control the host cell cycle and escape host defense mechanisms (Lisowska and Witkowski, 2003). In KSHV, NF-κB inhibition has been shown to promote lytic reactivation (Brown et al., 2003; Grossman and Ganem, 2008; Ye et al., 2008). A major strategy that the latently expressed KSHV vFLIP protein employs is to inhibit lytic reactivation through its activation of NF-κB (Sun et al., 1999). Due to its ability to modulate NF-κB, vFLIP has been shown to be essential for cell survival (Guasparri et al., 2004). RTA is the major transactivator of KSHV lytic gene expression and has been reported to target inhibitors of lytic reactivation (Yu et al., 2005; Yang et al., 2008; Gould et al., 2009). More recently RTA has been shown to have intrinsic ubiquitin E3-ligase activity (Yu et al., 2005), and the same group has provided evidence that RTA interacts with and stabilizes the cellular ubiquitin E3-ligase RAUL (Yu and Hayward, 2010).

The data presented here demonstrated that RTA induces ubiquitin-proteasome-mediated degradation of vFLIP and inhibits vFLIP-induced expression of the NF-κB associated genes, TNFα and ICAM1, in HEK 293T cells. We also presented data that RTA's intrinsic ubiquitin E3-ligase activity is not responsible for vFLIP degradation since vFLIP was destabilized in the presence of two RTA ubiquitin E3-ligase mutants (C141S and H145L). However, the region of amino acids 11-150 of RTA is important for vFLIP degradation, as vFLIP was stabilized with RTA truncation mutants lacking those regions. This evidence indicates that RTA may be interacting with a cellular

ubiquitin E3-ligase, likely in the region of amino acids 11-150, to promote vFLIP degradation.

As previously mentioned, RTA has been shown to interact with RAUL, and RAUL's role in RTA-induced degradation of vFLIP was evaluated. vFLIP was degraded in the presence of a dominant-negative RAUL ubiquitin E3-ligase mutant, indicating that the ubiquitin E3-ligase activity of RAUL may not be important for vFLIP degradation.

The ubiquitin E3-ligase activity of Itch was subsequently investigated due to the ability of Itch to target cFLIP for proteasome-mediated degradation. Using an Itch ubiquitin E3-ligase mutant, Itch-C830A, there was clear evidence that the ubiquitin E3-ligase activity of Itch was important for vFLIP degradation in the presence of RTA since vFLIP was stabilized when Itch-C830A was transfected in HEK 293T cells, in a dose-dependent manner. A striking observation was that vFLIP was stabilized with the transfection of WT Itch, suggesting that Itch is part of a larger complex. The overexpression of one component of a multiprotein complex can result in a dominant-negative effect that can affect the function of the complex, which could be the reason for vFLIP stabilization.

The ubiquitin-editing enzyme A20 has been shown to interact in a ubiquitin-editing complex composed of TAX1BP1, Itch, RNF11, and A20 working to terminate NF- κB signaling through inhibiting signaling intermediates such as RIP1 (Shembade et al., 2008; Shembade et al., 2010). This complex is disrupted by Tax, whose role in pathogenesis of HTLV-1 is similar to KSHV's vFLIP. Due to this similarity, the ubiquitin E3-ligase activity of A20 was assessed to determine if A20 played a role in

RTA-induced vFLIP degradation. Using a dominant-negative A20 mutant with impaired ubiquitin E3-ligase activity, A20-C624/627A, there was clear evidence that the ubiquitin E3-ligase activity of A20 is required for RTA-induced degradation of vFLIP. vFLIP was stabilized with the transfection of A20-C624/627A and this effect was dose-dependent. Additionally, WT A20 transfection stabilized vFLIP, supporting a dominant-negative phenotype. In the presence of either the A20 or Itch-defective ubiquitin E3-ligase mutants, vFLIP stability was not compromised; indicating that the ubiquitin E3-ligase activity of both A20 and Itch are required for RTA-induced vFLIP degradation.

The effect of RNF11 on the ability of the A20/Itch ubiquitin-editing complex to degrade vFLIP in the presence of RTA was assessed. Two RNF11 mutants that are both required A20 to interact with and degrade RIP1 were used, RNF11-C99A and RNF11-Y40A. Neither mutation restored vFLIP expression. Moreover, WT RNF11 did not result in vFLIP stabilization, suggesting that RNF11 is not part of the A20/Itch ubiquitin-editing complex.

Because the A20/Itch ubiquitin ligase complex is working to inactivate RIP1-signaling in the HTLV-1 model and A20 interacts with RIP1 in that model, it is possible that A20 may be interacting with vFLIP and/or RTA in order to prevent vFLIP from inducing NF-κB signaling. We presented immunoprecipitation data that vFLIP interacts with A20, Itch, and RTA, supporting this hypothesis. Immunoprecipitation also suggested that RTA interacts with both Itch and A20 and it is possible that there are different complexes forming. One may form during latent infection with vFLIP disrupting the A20/Itch ubiquitin-editing complex, much like Tax disrupting the

ubiquitin-editing complex described by Shembade et al. The second complex may form during the latent to lytic switch, with the presence of RTA altering the complex so that vFLIP can no longer disrupt the A20/Itch ubiquitin-editing complex and vFLIP can be targeted for degradation by the ubiquitin E3-ligase activity of A20 and Itch.

In summary, while RTA has its own intrinsic ubiquitin E3-ligase activity, that activity is not required for RTA-induced vFLIP degradation. A20 and Itch have been identified as the ubiquitin E3-ligases required for vFLIP ubiquitin-proteasome-mediated degradation in the presence of RTA. Our immunoprecipitation data support the hypothesis that RTA is co-opting this A20/Itch ubiquitin-editing complex to promote vFLIP degradation in order to down-regulate vFLIP-induced NF-κB gene expression and allow for the virus to enter the lytic replication cycle.

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JENNIFER CHMURA

[redacted]

EDUCATION

Master of Science, Molecular and Cellular Biology **May 2014**
Towson University
Thesis Advisor: Elana Ehrlich
Thesis: *The A20/Itch ubiquitin-editing complex is required for KSHV RTA-induced degradation of vFLIP*

- GPA: 4.0 / 4.0 Activities: Graduate Assistantship Advisory Council Member ♦ College Curriculum Committee Member

Bachelor of Science, Biology and Animal Science **May 2010**
University of Kentucky

REVELANT EXPERIENCE

Research Assistant **Summer 2012 – Present**
Towson University, Towson, MD

- Implemented sedimentation equilibrium technique which led to the discovery of a previously unidentified protein complex
- Created Standard Operating Procedures (SOPs) that resulted in above average output and shorter inventory turnaround time
- Nominated for 2013 Outstanding Graduate Student Award

Graduate Teaching Assistant **Fall 2012 – Present**
Towson University, Towson, MD

- Instruct three laboratory sections per semester for BIOL 120L: Principles of Biology
- Course management—prepare weekly lectures, manage student concerns, draft syllabus

Research Intern **Summer 2011**
Johns Hopkins Polycystic Kidney Disease Research Center

PUBLICATION AND PRESENTATIONS

- Ehrlich, E.S., Chmura, J.C., Smith, J.C., Kalu, N.K., and Hayward, G.S. (2014). KSHV RTA abolishes NFκB responsive gene expression during lytic reactivation by targeting vFLIP for degradation via the proteasome. *PLOS ONE* 9(3): e91359. doi:10.1371/journal.pone.0091359
- RTA induces vFLIP degradation. Poster presentation at the American Society for Virology Conference, University Park, PA, July 2013.
- Regulation of Polycystin 1 Cleavage. Poster presentation at Johns Hopkins University School of Medicine Summer Internship Program, Baltimore, MD, August 2011.