Inhibition of A20 deubiquitinase activity by KSHV vFLIP: A SUMO dependent mechanism?

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Abstract
KSHV vFLIP is a potent activator of NFκB signaling and an inhibitor of apoptosis and autophagy. Inhibition of vFLIP function and NFκB signaling promotes viral reactivation. Here we provide evidence for a novel function of vFLIP in promoting NFκB signaling through inhibition of the DUB activity of the negative regulator, A20. We demonstrate interaction of vFLIP with the Itch/A20 ubiquitin editing complex. We have identified a SUMO interaction motif in vFLIP that is required for NFκB activation. We observed a decrease in vFLIP induced NFκB when the SIM in vFLIP was mutated. Small molecule inhibition of SUMOylation resulted in a dose dependent increase lytic reactivation and virus production. Our results suggest a role for SUMO in mediating vFLIP function and provide evidence for vFLIP modulation of the negative regulation of NFκB signaling by A20. Our results provide further insight into the function of vFLIP and SUMO in the regulation of NFκB signaling and the latent lytic transition.
Introduction

Kaposi’s sarcoma herpesvirus is a member of the γ2 subfamily of herpesviruses and the causative agent of Kaposi’s sarcoma\textsuperscript{1,2}. The KSHV genome has been found in the cells of two B-cell lymphoproliferative diseases: primary effusion lymphoma (PEL) and Multicentric Castleman’s disease (MCD) and is associated with two inflammatory syndromes, immune reconstitution inflammatory syndrome-KS (IRIS-KS) and KSHV inflammatory cytokine syndrome (KICS)\textsuperscript{3–5}. KSHV has been classified as a Group 1 carcinogen by the International Agency for Research on Cancer and the National Toxicology Program 14\textsuperscript{th} Report on Carcinogens\textsuperscript{6}.

The KSHV genome contains several viral homologs of cellular genes, many of which promote immune evasion, cell survival and proliferation. KSHV exists mostly as a latent infection, where the viral genome is tethered to the host chromosome by LANA and infectious virions are not produced. Nascent virions are produced during periods of lytic replication induced by expression of the viral transactivator, RTA\textsuperscript{7}.

KSHV oncogenesis is, in part, attributed to genes expressed during latency. Viral FLICE inhibitory protein (vFLIP or K13), is a latently expressed gene that was originally identified as an inhibitor of apoptosis, due to the presence of tandem death effector domains\textsuperscript{8,9}. vFLIP is a potent activator of NFκB signaling and this activity is dependent on interaction with IKKγ\textsuperscript{10–12}. vFLIP has also been shown to promote NFκB signaling through upregulation of IKKε and CADM1 and inhibition of the SAP18/HDAC1 complex resulting in activation of NFκB via acetylation of p65\textsuperscript{13–15}. NFκB signaling is required for the virus to maintain latency, as chemical inhibition of this signaling pathway has been shown to promote lytic replication\textsuperscript{16,17}.

vFLIP plays a role in oncogenesis and genome instability. A transgenic mouse model of vFLIP expression displays persistent NFκB activation and an increased incidence of lymphoma as well as B cell abnormalities similar to those observed in MCD (reviewed in \textsuperscript{18}). More recently, vFLIP was shown to increase LINE-1 retrotransposition which may promote genome instability\textsuperscript{19}.

NF-κB signaling induces expression of negative regulators that limit the inflammatory response. A20 (TNFAIP3), one such negative regulator of NFκB, is induced by vFLIP. A20 is a ubiquitin editing protein with both C-terminal ubiquitin ligase activity and N-terminal deubiquitinase activity. In one well characterized mechanism, A20 forms a ubiquitin editing complex with Itch, RNF11, and TAX1BP1, and downmodulates NFκB signaling through removal of K63-linked
polyubiquitin chains from RIPK1 followed by addition of K48-linked polyubiquitin chains, resulting in degradation of RIPK1 via the proteasome. A20 is reported to deubiquitinate a number of signaling intermediates within the NFκB pathway in addition to RIPK1, including IKKγ, TRAF6, TRAF2 and MALT120–22.

We previously reported that RTA induces the degradation of vFLIP early in lytic reactivation resulting in the termination of NFκB signaling, presumably to promote transition from latency to lytic replication23. RTA induced degradation of vFLIP is dependent on the activity of the Itch ubiquitin ligase24. We identified mutants of vFLIP that are unable to interact with Itch and cannot activate NFκB. Here we report that vFLIP interacts with the A20/Itch ubiquitin editing complex and this interaction occurs independently of RTA. We propose that vFLIP inhibits A20 DUB activity thereby modulating NFκB signaling through interference with negative regulation. We demonstrate reduced A20 activity and increased levels of RIPK1K ubiquitin conjugates following stimulation with TNFA in the presence of vFLIP.

Small Ubiquitin-like Modifier (SUMO) proteins, when covalently conjugated to substrate proteins, can modulate the stability, interaction and activity of proteins. SUMO is a ubiquitin-like protein that, like ubiquitin, has diverse and wide-ranging effects on cellular processes. We have identified a SUMO interacting motif (SIM) in vFLIP. vFLIP SIM mutants exhibited reduced SUMO1 and SUMO 2/3 binding and were unable to activate NFκB signaling. Small molecule inhibition of SUMO conjugation resulted in increased virus production, suggesting a role for SUMO the latent to lytic transition.

Taken together, our findings suggest a novel role for vFLIP in activation of NFκB signaling via inhibition of the DUB activity of A20. This interaction may occur via a SUMO dependent mechanism, as we have previously reported that a SIM mutant of vFLIP cannot interact with Itch, a member of the A20 containing ubiquitin editing complex. These data increase our understanding of how vFLIP maintains latency.

Results and Discussion

vFLIP interacts with Itch and A20 in the absence of RTA

We previously reported that RTA induces the degradation of vFLIP via the cellular ubiquitin ligase, Itch. We hypothesized that RTA was recruiting Itch to vFLIP to promote ubiquitination
and degradation of the viral protein. Upon further characterization of the interactions between vFLIP and Itch as part of the Itch/A20 ubiquitin editing complex, we observed interaction between vFLIP, Itch and A20, even in the absence of RTA (Fig 1a-b). Based on this data we hypothesized that vFLIP interacts with the Itch/A20 ubiquitin editing complex in latency and RTA expression, occurring early in lytic reactivation, results in activation of Itch and/or A20 ubiquitin ligase activity (either directly or indirectly), resulting in the subsequent ubiquitination and degradation of vFLIP. If this model were correct, we would expect to see degradation of additional targets of Itch and/or A20 in the presence of RTA. In fact, we detected a modest decrease in the levels of A20, a known target of Itch, in cells transfected with RTA compared to empty vector control (Fig. 2a). We evaluated additional Itch and/or A20 substrates, RIPK1 and c-Jun, by immunoblot and observed a modest decrease in protein levels compared to the internal tubulin control (Fig. 2b).

To further examine the effect of RTA on the ubiquitination of Itch and A20 substrates, we conducted an analysis of the ubiquitinated proteome in SILAC labeled RTA transfected 293T cells using the anti-diglycine remnant (K-ε-GG) antibody. We identified 193 ubiquitination sites with differential ubiquitination in 146 proteins (Fig. 2c). We detected two known Itch or A20 substrates that exhibited a significant increase in ubiquitination in the presence of RTA. We observed a 1.5-fold increase in the ubiquitination of BRAT1, a known Itch substrate, and a nearly 3-fold increase in ubiquitinated UBE2N, a known A20 substrate, in RTA transfected cells compared to empty vector transfected controls (Fig. 2d). Taken together, this data suggests that while RTA may play a role in the alteration of the ubiquitome, and perhaps activation of the Itch ubiquitin ligase or Itch/A20 ubiquitin editing complex, the exact mechanism remains unclear.

vFLIP inhibits the deubiquitinase activity of A20

A20 is a well characterized negative regulator of NFκB signaling. Following stimulation of NFκB via the TNF receptor (TNFR), A20 downregulates signaling through removal of K63 linked polyubiquitin chains from RIPK1 and in concert with Itch, adds K48 linked polyubiquitin, resulting in RIPK1 degradation via the proteasome. It was previously reported that vFLIP induces the expression of A20. It has been proposed that A20 expression, in the context of latent KSHV infection, is necessary to limit the inflammatory phenotype induced by persistent NFκB signaling. We hypothesized that A20 activity needs to be tightly regulated, as excessive activity has the potential interfere with latency and cell survival, and inhibition of NFκB signaling has been shown to promote apoptosis and lytic reactivation. To this end, we assessed the impact of vFLIP on A20 DUB activity. Using purified K63-linked tetraubiquitin, A20 and vFLIP, we
evaluated A20 DUB activity via \textit{in vitro} assay. Addition of purified A20 alone to tetraubiquitin, resulted in cleavage of tetraubiquitin to faster migrating mono and polyubiquitin species (Ub-3, Ub-2, Ub-1), however addition of recombinant vFLIP resulted in a dose dependent decrease in DUB activity (Fig. 3a).

A well characterized target of A20 DUB activity is RIPK1, following TNFR stimulation. Within 30 minutes of TNFR stimulation, transient K63-polyubiquitin conjugates of RIPK1 can be detected via western blot. By 2hrs post stimulation, K63 ubiquitin conjugates are removed by A20. To determine whether vFLIP inhibits DUB activity in the context of NF\(\kappa\)B signaling, we evaluated K63 linked RIPK1 ubiquitin conjugates following stimulation with TNFA. Cells were transfected with wild-type or K63-only ubiquitin and vFLIP where indicated. Endogenous RIPK1 was purified and immunoprecipitates were probed for HA-tagged wild type or K63 only ubiquitin. Control cells, lacking vFLIP, displayed the characteristic increase in RIPK1 ubiquitin conjugates after 30 minutes of TNFA treatment, followed by deubiquitination 2h post treatment (Fig. 3b). Addition of vFLIP, however, resulted in detection of sustained RIPK1 ubiquitin conjugates, which was accentuated in cells transfected with K63-only ubiquitin (Fig. 3b). These data, taken together, suggest that vFLIP has an inhibitory effect on A20 DUB activity.

\textit{vFLIP has a SUMO interaction motif (SIM)}

PML nuclear bodies have been implicated in antiviral defense, apoptosis, and the DNA damage response as well as other cellular processes. They are known to be highly modified by the small ubiquitin-like modifier SUMO. In our initial vFLIP studies, we observed vFLIP colocalization with PML via immunofluorescence assay, prompting the question as to whether vFLIP has a SIM (unpublished observation). Analysis of the vFLIP protein sequence resulted in the identification of a putative tandem SIM spanning amino acids V21-VLFLLN-V28 based on the following consensus motif (V/L/I, X, V/L/I, V/L/I) or (V/L/I, V/L/I, X, V/L/I) (Fig. 4a). Indeed, wild-type vFLIP was able to bind recombinant SUMO 1 and SUMO 2/3, albeit to a lesser extent, while mutant vFLIP V22E, was no longer able to bind to either SUMO 1 or 2/3 (Fig. 4b).

We previously reported that this vFLIP mutant was resistant to RTA induced degradation and was unable to activate NF\(\kappa\)B, suggesting that this SIM is important for interaction with components of the NF\(\kappa\)B signaling pathway and RTA\(^{23,24}\). We previously reported that vFLIP V22E is unable to interact with the Itch ubiquitin ligase\(^{24}\). To determine whether Itch and A20 are SUMOylated, we immunoprecipitated FLAG tagged Itch or A20 and probed the 500mM NaCl washed immunoprecipitates with antibody against endogenous SUMO 1 or SUMO 2/3. We observed corresponding SUMO 1 and 2/3 bands in the Itch immunoprecipitates (Fig. 4c).
together, these results suggest that vFLIP may interact with the Itch/A20 ubiquitin editing complex via a SUMO dependent mechanism as the SIM mutant vFLIP V22E cannot interact with Itch nor activate NFκB and Itch is modified by SUMO 1 and 2/3.

Small molecule inhibition of SUMOylation increases production of infectious virus
Our observations suggest that SUMOylation plays a role in regulation of NFκB signaling induced by vFLIP. Previous reports demonstrated the importance of vFLIP induced NFκB signaling for maintaining viral latency and cell survival, as inhibition with Bay 11-7082 and transfection with an IKBA dominant negative mutant resulted in increased apoptosis and lytic reactivation. We hypothesized that if SUMOylation was required for maintaining latency, we would see a dose dependent increase in lytic reactivation upon SUMOylation inhibition. To evaluate this model, we utilized Vero rKSHV.294 cells that contain a reporter gene that expresses secreted placental alkaline phosphatase (SeAP) from a tetracycline responsive promoter. SeAP is secreted into the media only after successful infection of 293 MSR Tet OFF cells with virus. Vero rKSHV.294 cells were treated with increasing concentrations of 2-D08. After 48h incubation, virus containing media was collected and added to 293 MSR Tet OFF cells. Media was assayed for SeAP activity following 72h incubation. We observed a significant dose dependent increase (p<0.0001) in SeAP activity at concentrations of 30 μM and higher (Fig. 4d). This data suggests that SUMOylation is important for maintaining latency in Vero rKSHV.294 cells.

Discussion
We have presented multiple observations supporting a novel mechanism by which vFLIP promotes NFκB signaling and latency. vFLIP is an established activator of NFκB signaling and this activity is associated with viral latency. However, activation of NFκB results in the expression of several negative regulators of the signaling pathway. Expression of one such negative regulator, A20, was shown to be induced by vFLIP. While NFκB signaling is important for maintaining latency, prolonged NFκB activation could contribute to an inflammatory phenotype. In fact, this is what occurs when negative regulators of NFκB are either naturally or experimentally defective. Deficiencies in Itch ubiquitin ligase expression or function are associated with immune deficiencies and the Itch -/- knock out mouse displays an “itchy” phenotype for which this gene is named. A20 -/- mice also display a phenotype associated with inflammation and autoimmunity, exhibiting hypersensitivity to TNF and premature death. To establish and maintain a latent infection, vFLIP must activate NFκB and signaling must be
sustained without killing the host and to accomplish this, the virus must control negative regulators of NFκB.

We observed interaction of vFLIP with the Itch and A20 ubiquitin editing complex. We previously reported that in the presence of RTA, Itch targets vFLIP for degradation. These observations suggest that vFLIP may be interacting with the Itch/A20 ubiquitin editing complex in latency and reactivation along with expression of RTA may be modulating the activity of this complex. We evaluated multiple known substrates of Itch via western blot and proteomic analysis and observed modest decreases in protein levels when RTA was expressed suggesting that RTA is altering Itch substrate stability. Our proteomics data revealed identification of 146 proteins with RTA dependent alterations in ubiquitination, however only two were known Itch or A20 substrates, suggesting that while RTA has a demonstrated effect on the cellular ubiquitome, the mechanism(s) governing this observation remains unclear. We reasoned that vFLIP interaction with the Itch A20 ubiquitin editing complex may function to promote NFκB signaling and expression of RTA abrogates signaling by inducing the degradation of vFLIP as well as other members of the complex.

vFLIP had no effect on Itch/A20 complex assembly, suggesting that vFLIP was not inhibiting protein complex formation as had been described with HTLV Tax. We observed, through in vitro assay and through immunoprecipitation of RIPK1 conjugates, inhibition of A20 DUB activity by vFLIP. Detection of sustained K63 ubiquitinated RIP1 in the presence of vFLIP, suggests that A20 DUB activity is limited thereby allowing for sustained NFκB signaling.

We identified a SIM in vFLIP and observed interaction with SUMO-1 and 2/3. We previously reported that this motif is required for activation of NFκB, degradation of vFLIP by RTA and interaction with Itch. Taken together these data suggest that vFLIP interacts with the Itch/A20 complex via a SUMO dependent mechanism. We observed evidence of Itch SUMOylation and inhibition of global SUMOylation resulted in a dose dependent increase in infectious virus production.

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% CO₂ 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. 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. Vero rKSHV.294 cells containing a recombinant reporter KSHV clone were cultured in DMEM, 10% FBS, and 10 µg/ml G418 antibiotic. MSR-tet OFF 293T cells were cultured in DMEM and 10% FBS.

Reagents, Plasmids, and Antibodies

The proteasome inhibitor MG132 (Boston Biochem) was used in this study. FLAG-A20, was provided by Ed Harhaj, FLAG-Itch was provided by Annie Angers (REF), and myc-vFLIP by Gary Hayward. 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 run on 12% Tris-Glycine or Any kD mini-PROTEAN Precast Gel (Biorad) with Tris-glycine running buffer. The proteins were then transferred to a PVDF membrane using semi-dry transfer system at 20V for 20 minutes. The membranes were blocked in 5% non-fat dry milk in PBS for one hour. Primary antibodies were diluted in with 2.5% non-fat dry milk at 1uL antibody: 1000uL milk 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 1uL antibody: 1000uL milk. 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 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 48h post-transfection with PBS and centrifuged at 1500 rpm for 10 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. When appropriate 12.5µL of 5 mM NEM was added to each cell pellet. Cell lysates were centrifuged at 10,000 rpm for 5 minutes to remove cell debris. The resulting supematant 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 protein concentrations were measured and normalized with a Pierce BCA protein assay kit. Approximately 50 µg of protein was transferred to new 1.5mL tube to serve as control lysate. 1µg 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 1hr and washed 4x with RIPA lysis buffer. 50µl of 2X Laemmli Buffer were added and samples were boiled at 100°C for 10 min. Samples were visualized through immunoblot analysis as described above.

**GST-Pull-down Assay**

A 5mL culture of BL21 cells containing the indicated constructs (GST tagged SUMO 1, SUMO 2/3, wildtype vFLIP or VE-vFLIP) was used to inoculate 50 mL of LB Broth. After 1.5-2 hours, when the OD = 0.4-0.7, expression was induced with 0.1mM IPTG. Cells were spun down and stored at -20°C overnight. Pellets were resuspended in 1 mL of PBS with 10µL of a protease inhibitor cocktail and sonicated. The samples were spun down at 9700 RPM for 1 minute and the supernatant transferred to a new tube. GST-SUMO samples were added to glutathione-sepharose and incubated at room temperature for an hour. Purified wildtype vFLIP and VE-VFLIP was harvested from transfected 293T cells for use for the *in vitro* assay. Transfected cells with appropriate constructs were harvested 48h post-transfection with PBS and centrifuged at 1500 rpm for 10 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 and the cells sonicated 4 times for 10s. The samples were spun down at 9700 RPM for 1 minute and the supernatant added to a new 1.5mL tube and would be incubated with the SUMO and glutathione-sepharose.

**SEAP Assay**

Vero rKSHV.294 cells, containing a recombinant KSHV reporter and producing infectious virus, were plated in a six-well plate and treated with the indicated concentrations of the SUMO inhibitor. After one day, 293T MSR tet-OFF cells were plated. Twenty four hours later, media from the rKSHV.294 cells was transferred to the MSR tet-OFF plate. Two days later, 25 µl of media was harvested, and heat inactivated at 65°C for 30 minutes. The same samples were then transferred to a 96 well plate for SEAP assay using Great EscAPe SEAP Assay (Clontech) according to the manufacturer’s instructions. Results were read on a Molecular Devices Filtermax F5 platereader.

**A20 purification**
A20-FLAG was transfected into 293T cells, and purified following 48 hours incubation. Nine 10 cm dishes of A20 transfected cells were harvested in ice cold PBS, centrifuged, and lysed in 1 mL 50 mM HEPES, pH 7.4, 100 mM KAc, 5 mM MgAc2, 100 μg/mL digitonin, 1 mM DTT, 1X EDTA-free Complete protease inhibitor cocktail (Thermo Scientific) for 20 min on ice. The lysate was centrifuged to remove cell debris. Supernatant was incubated with 100 μL of anti-Flag affinity resin (Sigma) for 1-1.5 hr at 4°C. The resin was washed three times in 1 mL lysis buffer, three times in 1 mL 50 mM HEPES, pH 7.4, 400 mM KAc, 5mM MgAc2, 100 μg/mL digitonin, 1 mM DTT buffer, and three times in 50 mM HEPES, pH 7.4, 100 mM KAc, 5 mM MgAc2 buffer. Elutions were carried out with one volume of 0.2 mg/ml 3X-Flag peptide in the final wash buffer at room temperature for 30 min. Two sequential elutions were combined to form the final fraction.

In vitro deubiquitination assay

V5-His tagged vFLIP was expressed in E. coli (BL21) and purified using Ni-NTA resin (ThermoFisher). A20-Flag was purified as previously described. Purified tetra-K63 ubiquitin was purchased from Boston Biochem. The following reagents were added to 20μl reactions where indicated: A20 (2μM), vFLIP (1μM, 5μM, 10μM), tetra-K63 Ub (500nM). Reactions were incubated at 37ºC for 2hrs followed by the addition of 4x Laemmli loading buffer. Reactions were analyzed by SDS PAGE followed by immunoblot.

Figure legends

Figure 1. vFLIP interacts with Itch and A20. A. vFLIP does not interfere with Itch/A20 interaction. 293T cells were transfected with Flag tagged Itch and/or myc tagged vFLIP where indicated. Itch was immunoprecipitated with Flag antibody and immunoprecipitates were analyzed by immunoblot against Flag, myc, and endogenous A20. B. vFLIP interaction with A20 occurs independent of RTA. 293T cells were transfected with Flag tagged A20, myc tagged vFLIP and/or RTA where indicated. Itch was immunoprecipitated with Flag antibody and immunoprecipitates were analyzed by immunoblot against Flag and myc.

Figure 2. RTA expression is associated with reduced stability of Itch substrates. A. A20, and B. cJun, and RIPK1 protein levels are reduced in the presence of RTA. 293T cells were transfected with Flag tagged A20, RTA or empty vector where indicated. Cell lysates were probed for Flag or endogenous cJun or RIPK. Actin or tubulin was used as a loading control. C.
RTA expression induces alterations in the ubiquitinated proteome. 193 sites in 146 proteins displayed differential ubiquitination in RTA expressing cells compared to vector transfected controls. SILAC labeled 293T cells were transfected with RTA or empty vector control. Lysates were processed for mass spectrometry and ubiquitinated peptides were enriched using the antidiglycine remnant (K-ε-GG) antibody. Samples were cleaned on C18 and analyzed by MS. MS spectra were processed and analyzed by Maxquant and Perseus. D. UBE2N and BRAT 1, known substrates of A20 and Itch displayed increased ubiquitination in RTA transfected cells.

Figure 3. vFLIP inhibits A20 deubiquitinase activity. A.vFLIP inhibits A20 deubiquitinase activity in vitro. In 20μl reactions the following reagents were added where indicated: tetra-K63 Ub (Boston Biochem) (500nM), V5-vFLIP (1,5,10μM), and A20 (2μM). Reactions were incubated at 37ºC for 2hours following addition of 4x Laemmli loading buffer. Samples were run on 15% SDS-PAGE gel and analyzed via immunoblot with antibody against ubiquitin. B. vFLIP abrogates the deubiquitination of RIPK1. HEK 293T cells were transfected with either empty vector control, HA-WT Ub, and or myc-vFLIP, then treated with TNF at 0, 0.5, and 2 hours after transfection and before harvesting, and analyzed via immunoprecipitation with anti-RIP1. Following immunoprecipitation, lysates were analyzed by immunoblotting with anti-HA and anti-RIP1.

Figure 4. vFLIP has a SIM. A. Sequence alignment illustrating SIM location and mutations generated B. SIM dependent interaction between vFLIP and SUMO 1 and SUMO 2/3. Recombinant GST-SUMO was expressed in E. coli and 93T cells were transfected with myc-vFLIP. Interaction was evaluated via GST pull-down assay. Cell lysates containing myc tagged vFLIP were incubated with GST tagged SUMO bound to glutathione-sepharose. Purified proteins were analyzed via western blot. C. Evidence for Itch SUMOylation. 293T cells were transfected with Flag-Itch, Flag-A20 or empty vector where indicated. Cell lysates were processed for immunoprecipitation using anti-FLAG antibody. Immunoprecipitates were washed with a 500mM NaCl followed by analysis via SDS-PAGE and western blot against Flag or endogenous SUMO 1 or SUMO 2/3. D. Small molecule SUMOylation inhibition results lytic reactivation. Infectious virus production was evaluated by treating Vero rKSHV.294 cells with SUMO inhibitor 2-D08 and quantifying secreted alkaline phosphatase following infection of 293T MSR tet-OFF cells.
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|        | A         | B         | C         | D         |
|--------|-----------|-----------|-----------|-----------|
|        | FLAG-A20  | RTA       | 193 sites in 146 proteins | Fold increased ubiquitination/control |
|        | RTA       | RIPK1     |          | UBE2N     |
|        | ACTIN     | TUBULIN   |          | BRAT1     |
| 1      |           |           |          |           |
| 2      |           |           |          |           |
| RTA    | -         | -         |          |           |
| A20    | +         | +         |          |           |
FIGURE 4

A

|   | WT  | V22E | L23E |
|---|-----|------|------|
|   | MATYEVLCVARKLGTDREVVLHNVFIPQPTLAQIGLHRAKKEEGRLFTPFLLAECL | MATYEVLCVARKLGTDREVVLHNVFIPQPTLAQIGLHRAKKEEGRLFTPFLLAECL | MATYEVLCVARKLGTDREVVLHNVFIPQPTLAQIGLHRAKKEEGRLFTPFLLAECL |
|   | FRAGRDDLRLDILHRPLERHLAGMSYFSPYTQLTVLHVDGELCARIDRSLFLISDS | FRAGRDDLRLDILHRPLERHLAGMSYFSPYTQLTVLHVDGELCARIDRSLFLISDS | FRAGRDDLRLDILHRPLERHLAGMSYFSPYTQLTVLHVDGELCARIDRSLFLISDS |
|   | IGRSTPQTFMLHVYCMENLDLLGPTVDALMSMLRSLSRVLQVRVQTLMLHLSFPS | IGRSTPQTFMLHVYCMENLDLLGPTVDALMSMLRSLSRVLQVRVQTLMLHLSFPS | IGRSTPQTFMLHVYCMENLDLLGPTVDALMSMLRSLSRVLQVRVQTLMLHLSFPS |
|   | SGHYRHTP | SGHYRHTP | SGHYRHTP |

B

IB: GST-SUMO 40 kDa
IB: vFLIP 15 kDa
SUMO 1 | 1 | 2/3 | 2/3 | - | -
vFLIP WT | VE | WT | VE | VE | VE

C

IB: FLAG
IB: SUMO1
IP: FLAG
IB: SUMO2/3
IB: vFLIP
IB: vFLIP
Input

1 | 2 | 3 | 4 | 5
FLAG-A20 | - | + | - | + | -
FLAG-Itch | - | - | + | - | +
vFLIP | - | - | + | + | +

D

p<0.0001

SeAP activity (fluorescence units)

SUMOylation inhibitor, 2-D08 (µM)

Control | 15 µM | 30 µM | 60 µM