SIMIAN VARICELLA VIRUS AND VARICELLA-ZOSTER VIRUS IMMUNE EVASION OF NFκB ACTIVATION

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List of Abbreviations

α: alpha

β: beta

DD: death domain

DRG: dorsal root ganglion

E: early

EBV: Epstein Barr Virus

eIF2a: eukaryotic translation initiation factor 2A

γ: gamma

HCMV: Human cytomegalovirus

HHV: Human herpesvirus

HSV: Herpes Simplex Virus

HZ: herpes zoster

IE: immediate early

IFN: interferon

IκBα: inhibitor of kappa B alpha

IKK: IκB kinase

IRF: Interferon Regulatory Factor

ISG: interferon stimulated genes

JAK: Janus kinase

κ: kappa

KS: Kaposi sarcoma

KSHV: Kaposi sarcoma associated herpesvirus

λ: lamda

L: late

LPS: lipopolysaccharide

MDA5: Melanoma Differentiation-Associated protein 5

MYD88: Myeloid differentiation primary response gene 88

NEMO: NFκB essential modulator

NES: nuclear export signal

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

NHP: non-human primate

NIK: NFκB-inducing kinase

NLR: NOD-like receptors

NLS: nuclear localization signal

ORF: open reading frame

PAMP: pathogen associated molecular patterns

PHN: postherpetic neuralgia

PKR: Protein kinase-R

PRR: pattern recognition receptors

RIGI: retinoic acid-inducible gene 1

RIP: Receptor-interacting protein

RLR: RIG-I-like receptors

RM: rhesus macaques

STAT: Signal Transducer and Activator of Transcription

SVV: Simian Varicella Virus

TIR: Toll/interleukin-1 receptor

TLR: Toll-like receptors

TRAF: TNF receptor associated factor

TRIF: TIR-domain-containing adapter-inducing interferon-β

VZV: Varicella Zoster Virus

Abstract

Varicella Zoster Virus (VZV) causes chickenpox upon primary infection and establishes latency in ganglia. Reactivation from latency causes herpes zoster, which may be complicated by post-herpetic neuralgia. Innate immunity mediated by interferon and pro-inflammatory cytokines represents the first line of immune defense upon infection and reactivation. VZV is known to interfere with multiple innate immune signaling pathways including the central transcription factor NFκB. However the role of these inhibitory mechanisms *in vivo* is unknown. Simian varicella virus (SVV)-infection of rhesus macaques recapitulates key aspects of VZV pathogenesis and this model thus permits examining the role of immune evasion mechanisms *in vivo*. Here we compare SVV and VZV with respect to interference of NFκB activation. We demonstrate that both viruses prevent ubiquitination of the NFκB inhibitor IκBα, whereas SVV additionally prevents IκBα phosphorylation. We show that the ORF61 proteins of VZV and SVV are sufficient to prevent IκBα ubiquitination upon ectopic expression. We further demonstrate that SVV ORF61 interacts with β-TrCP, a subunit of the SCF ubiquitin ligase complex that mediates the degradation of IκBα. This interaction seems to inactivate SCF-mediated protein degradation in general since the unrelated β-TrCP-target Snail is also stabilized by ORF61. In addition to ORF61, SVV seems to encode additional inhibitors of the NFκB pathway since ORF61-deleted SVV still prevented IκBα phosphorylation and degradation. Taken together, our data demonstrate that SVV interferes with TNFα-induced NFκB activation at multiple levels which is consistent with the importance of these counter mechanisms for Varicella Virus infection.

Introduction

1.1 Herpesviruses

Herpesviruses are double stranded DNA viruses that infect a large range of hosts. To date over 200 herpesviruses have been identified and categorized into three branches: *Herpesvirales* (mammals, birds, and reptiles), *Alloherpesviridae* (fish and amphibians), and *Malacoherpesviridae* (bivalves) [1]. There are eight herpesviruses that infect humans: Herpes Simplex virus (HSV)-1/2 or HHV1/2), Varicella Zoster virus (VZV or HHV3), Human cytomegalovirus (HCMV or HHV5), Epstein–Barr virus (EBV or HHV4), human herpesviruses 6 (HHV6), human herpesviruses 7(HHV7), and Kaposi's sarcoma herpesvirus (KSHV or HHV8). Herpesviruses have co-evolved with their respective hosts for long periods of time and have become well adapted for replication and persistence within their specific species. In fact one of the key properties of all herpesviruses is that they are never fully cleared from their host and can remain in a latent state. They can also reactivate from latency and become transmissible and cause disease again.

The herpesvirus genome is composed of linear double stranded DNA but quickly circularizes in infected cells [1]. Between specific herpesviruses the size and GC content of the genome can vary greatly. Of the human viruses, VZV has the smallest genome at ~125kb while HCMV has the largest at ~230kb [1]. There are between 70 and 200 genes expressed, depending on the virus, of which about 40 are functionally conserved between all herpesviruses. Gene expression, particularly during the acute phase, follows a temporal cascade of immediate-early (IE), early (E), and late (L) genes [1]. IE gene expression does not require prior protein synthesis and is activated upon initial viral

entry. Some functions of IE proteins include transactivation of other viral genes and immune evasion. E gene expression requires IE gene expression but not viral DNA synthesis. The most important function of E gene products is their involvement in genome replication. The last temporal class is that of the L genes. L genes typically require viral DNA synthesis and the proteins are involved in the assembly of new virions and their egress out of the cell [1].

Surrounding the DNA core is the 100 nm capsid (Fig 1: Top). The capsid of all herpesviruses is made of 12 pentons and 150 hexons with a triangulation number of T=16 [2]. There are four essential proteins that make up the capsid [1]. Capsid proteins are synthesized in the cytoplasm but migrate back to the nucleus for assembly. During the viral replication cycle, in the nucleus, once the viral DNA has been replicated it is inserted into the capsids. Outside the capsid is a structure called the tegument [3]. The tegument contains fully functional proteins that are important for the virus during the initial infection (IE genes). As virus particles move through the cytoplasm along the pathway to egress, accumulated proteins can be added as part of the tegument [4]. The last structural feature of the herpesvirus virion is the envelope. The envelope is made up of a modified host lipid membrane and contains the viral glycoproteins, which are involved in viral entry [1]. The model that best describes how the viral envelope is formed is the two-step envelopment model. This model proposes that the primary envelope is gained and lost during transport across the nucleus and the final envelope is acquired in the cytoplasm/golgi (Fig 1: Bottom) [2].

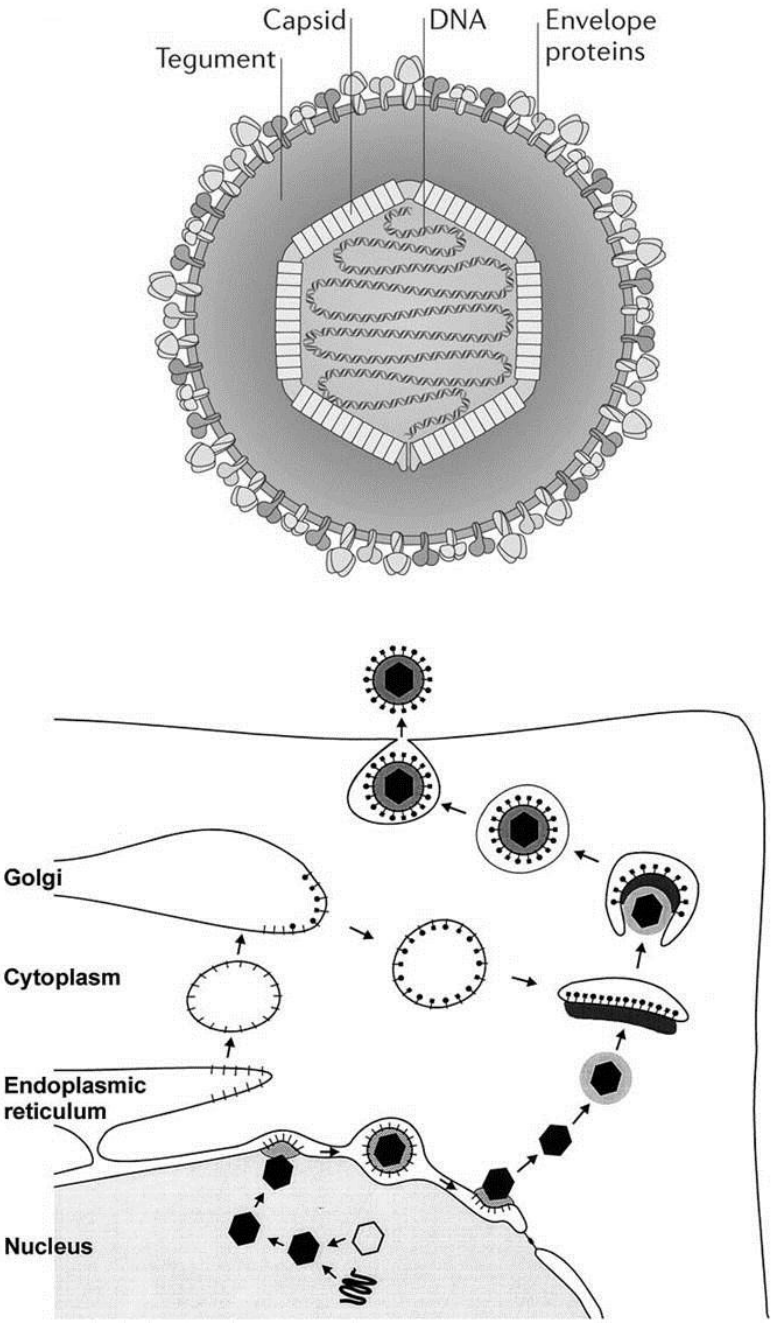

FIG 1. Herpesvirus structure and assembly pathway.
(Top) Herpesvirus architecture illustration. Authorized for reprint from Zerboni L, Sen N, Oliver SL, Arvin AM. Molecular mechanisms of varicella zoster virus pathogenesis. Nat Rev Microbiol. 2014;12(3):197-210.
(Bottom) Proposed pathway of herpesvirus egress. Authorized for reprint from Herpesvirus Assembly and Egress. Journal of Virology. 2002;76(4):1537

The *Herpesvirales* (formerly Herpesviridae) family has further been divided into three subfamilies: alpha, beta, and gamma. These subfamilies are divided based on biological properties such as replication time, tropism, and host range [1]. The human specific alpha-herpesviruses are HSV-1/2 and VZV. Herpes B-virus is a simian alpha-herpesvirus that has the ability to cause zoonotic infections in humans, which can be fatal [5]. Alpha-herpesviruses are characterized by rapid spread in culture and their propensity to establish latency in sensory ganglia. HSV-1/2 are the cause of herpes which typically presents as lesions at the site of infection, usually in or around the mouth and genitals [6]. The majority of HSV infections are asymptomatic, although virus can still be shed during this period [7, 8]. HSV can cause more serious injury when the infection occurs in the eye or brain causing conjunctivitis and HSV encephalitis, respectively [1]. After initial infection the virus establishes latency in neuronal cell bodies [9]. Reactivation seems to occur at rates that are proportional to viral load and inversely related to the amount of reactive CD8 T-cells surrounding the neurons [10]. The other human alpha-herpesvirus, VZV, is the focus of this dissertation and will be discussed in much greater detail (below), but briefly VZV is the causative agent of chickenpox and can reactivate decades later as herpes zoster (shingles) [11]. In addition to these two main diseases, VZV infection has also been associated with pneumonia, encephalitis, and acute retinal necrosis [12-15].

The beta-herpesviruses, which include HHV-6, HHV-7, and the largest herpesvirus HCMV, are characterized by long replication times and slow growth in culture. This group of viruses also tends to go latent in myeloid-derived cells [16, 17].

HHV-6 and HHV-7 are both ubiquitous viruses that typically result in asymptomatic infections [18]. Occasionally, infections with these viruses results in fever with roseola [19-21]. HCMV is also largely an asymptomatic ubiquitous virus [22]. However, HCMV infections are problematic when they occur during fetal development or in immunocompromised individuals. Congenital HCMV infection acts upon many organ systems during development and is a major cause of birth defects, the most common being sensorineural deafness [23-25]. HCMV has also been linked to many complications associated with organ transplant rejections, because these patients are under an immunosuppressive regimen [1, 26]. AIDS patients are another group at risk for HCMV related diseases [27, 28].

The third Herpesvirus subfamily is the gamma-herpesviruses, which include EBV and HHV-8 or Kaposi sarcoma herpesvirus (KSHV). EBV infection is associated with infectious mononucleosis [29]. Approximately half of the people that are infected with EBV will exhibit symptoms of mononucleosis including fever, pharyngitis, and fatigue, which can persist for weeks to months [30]. EBV is commonly spread via oral contact, through the sharing of food, utensils, or kissing; hence its colloquial name "the kissing disease" [31, 32]. EBV productively infects both B-cells and epithelial cells and can become latent in both [33]. Transformation of B-cells by EBV results in more serious cancers such as nasopharyngeal carcinoma, Burkitt, and Hodgkin lymphomas [34]. The other human gamma-herpesvirus is KSHV. KSHV is a human oncovirus that has been implicated in three different diseases, with the most noteworthy being Kaposi sarcoma (KS) [35]. KS is a tumor, originally described in 1872, that classically presents on the skin as red-purple nodules, which can further develop into plaques; this is predominantly

present in AIDS patients [36-38]. Other lymphoproliferative diseases KSHV has been linked to include primary effusion lymphoma and multicentric Castleman disease [39, 40].

1.2 The Immune System:

When a host encounters a virus or pathogen the response to the invader can be split into three main mechanisms: intrinsic, innate, and adaptive immune responses. The innate response to infection is not based on the specific individual pathogen, but is activated by a more general feature of the infection that creates an overall unfavorable environment for the pathogen to survive. In addition, the innate immune response induces and coordinates the adaptive response. The adaptive immune response is targeted to the individual pathogen by highly specific cells that have undergone germline rearrangements to produce receptors that identify specific components of the pathogen. All branches of the immune system are important for the clearance or control of pathogens, including VZV and other viruses, and therefore their induction and contributions to an overall anti-viral immune state within the host will be reviewed. For the focus and purpose of this document there will be a focus on the innate immune signaling of canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB).

1.2.1 Innate and Intrinsic Immune Induction and Response

The innate immune system is rapidly activated upon encounters with pathogens or other danger signals such as reactive oxygen intermediates or heat shock proteins and therefore becomes one of the first lines of defense against an infection[41]. The speed of

this response is critical due to the fast growth rates of bacteria and viruses. In fact, there are proteins and complexes already formed within a cell which do not require classical activation, which can act immediately and directly to limit viral replication. This form of immunity is called intrinsic immunity. Intrinsic immunity proteins are also known as restriction factors since they are typically constitutively expressed (although they are normally upregulated with classical activation of innate immune signaling) and can limit viral replication [42]. A few of the well-known restriction factors include members of the APOBEC and TRIM family of proteins [42, 43]. There are seven members of the APOBEC family which act as cytidine deaminases. These proteins act upon single stranded DNA and catalyze the change of cytidine to uridine via deamination [42]. APOBEC3G was identified for its antiviral effects against HIV-1 [44]. The result of APOBEC3G activity is that HIV-1 DNA becomes hypermutated (with G to A mutations) that end up limiting the stability of the viral genome [45]. In order to combat the effect of APOBEC3G HIV-1 Vif protein has evolved to direct APOBEC3G for ubiquitination and degradation [46]. The APOBEC family has also been implicated in the mutation of herpesviral DNA. Specifically, APOBEC3C has been demonstrated to hypermutate both HSV-1 and EBV genomes [47]. There have been at least 68 human genes identified that encode for TRIM proteins [48]. Two of the most notable TRIMs are TRIM5α and TRIM19/PML. TRIM5α is especially famous as it was found to be the protein responsible for restricting HIV-1 infection in old world monkeys [49]. TRIM5α mediates its antiviral effects by interacting with the viral capsid and promoting premature disassembly [50, 51]. TRIM5α also seems to be responsible for many of the species-specificities for retroviruses [52-54]. It is also partly responsible for limiting early stages

of HSV infection [55]. PML is present in the nucleus and is the major contributor to of the subnuclear ND10 protein complexes [56]. PML-ND10 bodies have been implicated as an antiviral restriction factor for many different viral families including Herpesviridae [57-59]. During VZV infection depletion of PML has been shown to enhance viral replication, which shows that PML-ND10 bodies are able to limit VZV replication to some degree [60]. PML interacts with the VZV capsid protein ORF23 sequestering viral capsids in the nucleus and preventing nascent virion formation [61]. This occurs across multiple cell types including neurons and epidermal cells [61]. Interestingly, VZV ORF61 and HSV-1 ICP0 have been implicated in disrupting ND10 bodies through the degradation of PML [62, 63]. This seems to be a critical function for VZV ORF61 during skin infection since disruption of ORF61 severely impaired VZV replication in skin xenografts [63].

Classically, activation of the innate immune system is based on the interaction between germline encoded pattern recognition receptors (PRRs) of the host with their cognate ligands; pathogen associated molecular patterns (PAMPs) which are produced by the foreign pathogen [41]. One major class of PRRs are the Toll-like receptors (TLRs). It has long been known that microbial and viral components can activate innate signaling pathways, but the discovery of the TLRs and other PRRs occurred relatively recently [64-66]. Humans have 10 functional TLRs with TLR 1,2,4,5, 6, and 10 located at the cell surface, which recognize extracellular PAMPs such as: lipopolysaccharide (LPS) by TLR4, flagellin by TLR5, and lipoproteins by TLR1/2 [67]. TLR 3, 7, 8, and 9 are found in endosomes which recognize intracellular PAMPs mainly consisting of foreign nucleic acids [67]. The TLRs themselves are type 1 membrane proteins with leucine rich

repeat ectodomains that recognize their ligands and an intracellular Toll/interleukin-1 receptor (TIR) domain that mediates downstream signaling [68]. The majority of downstream signaling is mediated by the TIR binding adaptor Myeloid differentiation primary response gene 88 (MYD88); TLR3 uses a different adaptor called TIR-domain-containing adapter-inducing interferon-β (TRIF), and TLR4 can use both [41].

Another class of PRR is the RIG-I-like receptors (RLR) which detect intracellular cytosolic forms of RNA, typically from viral infection. The two best described RLRs are retinoic acid-inducible gene 1(RIG-I) and Melanoma Differentiation-Associated protein 5 (MDA5). RIG-I detects short segments of dsRNA or uncapped RNA, while MDA5 detects longer dsRNA segments, both of which are typical intermediates in the replication cycle of RNA viruses [66, 69, 70]. It appears that RIG-I and MDA5 have a preference for the family of RNA viruses that they detect [69]. The signaling cascades for both of these RLRs end up promoting an antiviral state through NFκB activation and Type I IFN production [71]. Although it is not an RLR, Protein kinase-R (PKR) is another cytoplasmic RNA sensor that plays a key role in the antiviral immune state. When PKR senses foreign forms of RNA it undergoes conformational changes and phosphorylates eukaryotic translation initiation factor 2A (eIF2a) [72]. eIF2a is an important co-factor involved in normal protein synthesis, and when phosphorylated by PKR is inactive, halting protein production of both viral and host proteins [73].

Cytosolic DNA sensors are another group of PRRs. A few members of this group include IFI16, cGAS, zBP1, and STING. STING is an interesting protein because not only acts as a senor of DNA (cyclic dinucleotides) but also seems to mediate the signaling cascades for many of the other cytosolic DNA sensors [74, 75]. STING

activation (directly via sensing of DNA or indirectly via upstream sensors) results in activation of TBK1 which induces Type I interferon (IFNβ) production through Interferon Regulatory Factor 3 (IRF3) and NFκB activation by activating the IKK complex (details below) [76, 77]. This inevitably leads to the production of ISGs and proinflammatory cytokines resulting in an unfavorable environment for pathogen replication.

A fourth major class of PRR are the NOD-like receptors (NLR). NLRs have leucine rich repeat regions that function to sense microbial products and other danger signals. They evoke an innate immune response mainly through the induction of proinflammatory genes via NFκB or the activation of caspases [78]. One key innate immune protein complex that involves NLR family members is the induction and formation of the inflammasome [79]. There are various forms of the inflammasome, depending on what NLR is in the complex, but the well-known NLRP3 inflammasome complex forms to activate caspase-1 which is used to cleave IL-1β and IL-18 into their functional mature forms [79]. Both of these cytokines act to induce inflammatory genes, and can be used in conjunction with other cytokines to induce the production of IFNγ from NK and T-cells [80].

During infection, TLR2 and TLR9 appear to mediate the TLR of the responses for multiple different herpesviruses [81-84]. TLR2 likely recognizes a viral glycoprotein on the surface of the virion, although the specific agonist is unknown, while TLR9 senses viral DNA. VZV has specifically been shown to activate innate signaling pathways via TLR2 and TLR9 [85, 86]. A key difference between wild strains of VZV and the attenuated vaccine strain is due to the ability of the vaccine strain to signal through TLR2

and allow the development of a proper immune response [87]. Since TLR9 recognizes herpesviral DNA it stands to reason that the cytosolic DNA sensors also contribute to herpesvirus detection. In fact HSV-1 has been shown to mediate IFNβ production in a STING dependent manner via DDX41, IFI16, and cGAS [88-90]. It also seems that STING has a role in sensing VZV since knockdown of STING leads to increases in VZV replication [91]. Interestingly HSV-1 ICP0 degrades IFI16 therefore it could be that VZV ORF61 uses a similar mechanism to avoid detection from these types of sensors [89]. Regardless of the PRR activated the end result is normally activation of IRF3 and the IKK complex to promote Type I IFN expression and NFκB dependent transcription respectively.

1.2.1.1 Interferon Signaling

A major outcome of an activated innate immune response is the production of interferon (IFN) and activation of NFκB. IFNs were initially discovered as a result of their antiviral activity which was confirmed with IFN knockout mice [92, 93]. There are three classes of IFNs: Type I, II, or III. The chief Type I IFNs include IFNα and IFNβ [94]. The main Type II IFN is IFNγ [95]. Type III IFN such as IFNλ have just recently been classified and their functions during infection are under current research [96]. Type I and II IFNs have important roles in activating the immune response [95]. The expression of IFNβ is principally dependent on the phosphorylation of the transcription factor IRF3, which is induced upon PRR signaling (Fig 2: Left). Once the IFN is expressed it acts by binding to specific receptors that signal through Janus kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) pathways to induce a large

range of genes termed interferon stimulated genes (ISGs; Fig 2: Right) [97]. In most cell types IRF7 is an ISG that creates a positive feedback loop and is responsible for the increased expression of Type I IFN seen during infection [98-100]. Other notable ISGs that are important for their antiviral properties include PKR, 2-5A synthetase/RNase L, and Mx proteins, which control viral replication at the level of transcription and translation; although ISGs have been shown to target all aspects of the viral replication cycle [97]. It is well documented that IFN plays an important antiviral role against herpes viruses. Type I and II IFN synergistically block HSV-1 replication [101, 102]. The same is true for CMV infection, with Type I and II IFN regulating aspects of both acute and latent replication [103, 104]. Despite their relative novelty Type III interferons have been demonstrated to be highly antiviral in a number of respiratory and epithelial infections [105, 106]. Epithelial cells, one of the first cell types VZV would encounter in the lung (see below: VZV Tropism and Trafficking) are one of the few cell types that highly express the Type III IFN receptor [107]. IFNλ signaling results in JAK-STAT activation and the production of many ISGs that overlap with the genes expressed by Type I IFN signaling [108, 109]. Mordstein et al. used interferon receptor knockout mice (either single or double knockouts for Type I and or Type III) to examine the role IFNλ has in controlling many respiratory viral infections and their results show that IFNλ had an antiviral effect on influenza, RSV, HMPV, and SARS coronavirus [105, 110]. IFNλ has also been verified to be important in-vivo against HSV-2 at mucosal sites [111]. Nothing has been documented in regards to IFNλ and VZV in particular, but given the evidence of the role IFNλ has at mucosal sites and its ability to limit replication of other respiratory viruses it likely plays a role in the early stages of VZV pathogenesis. It

is equally likely since VZV can successfully replicate and move out of the lungs that VZV is able to interfere with IFNλ signaling. Since there is such an overlap between Type I and III signaling it is possible that the mechanisms that VZV employs to evade Type I IFN work double duty to evade Type III signaling as well. VZV replication is also very sensitive to the presence of interferon, both in vitro and in vivo, which is evidenced by the finding that interferon treatment of immunocompromised patients reduced the severity of varicella infection [112-115].

1.2.1.2 NFκB Signaling

The other major innate immune pathway that is activated by PRRs is the NFκB pathway [116]. NFκB is a group of transcription factors, which include RelA/p65, p50, p52, p105,p100, c-Rel, and RelB, that form hetero or homo dimers that bind specific DNA sequences to promote gene transcription [117]. NFκB factors are localized to the cytoplasm where they are maintained in a non-functional state by inhibitors of κB (IκB) [118]. Once signaling is initiated the IκBs are ubiquitinated and degraded, which allows the NFκB subunits to translocate to the nucleus and activate gene transcription [118]. The IFNβ promoter contains an NFκB binding site suggesting that NFκB is important for the interferon response [119, 120]. It was later determined in NFκB knockout mice that NFκB is not necessary for efficient IFNβ production, but instead plays a key role in the early stages of IFNβ transcription when IRF3 activation is low [121, 122]. This early IFNβ transcription has major acute antiviral properties, exemplified by the fact that RelA deficient fibroblasts are susceptible to multiple interferon sensitive RNA viruses, despite normal levels of IFNβ late in infection [121, 123]. Late in infection NFκB activation

leads to the induction of many key factors that are involved in different aspects of immunity, including proinflammatory cytokines (TNFα and IL-6), adhesion molecules (e-selectin), and chemokines (IL-8 and CCL5) [124]. These NFκB induced proteins aid in the recruitment of leukocytes, enhanced antigen presentation, and activation of the adaptive immune response. The proinflammatory cytokines also can exert antiviral properties. For example, TNFα has been demonstrated to have an antiviral effect that exceeded IFNα and IFNγ against multiple influenza viruses [125]. IL-1β, another NFκB driven gene, can block hepatitis C virus RNA replication, protein production, and works in conjunction with IFNα to enhance antiviral gene expression [126, 127]. NFκB also has anti-apoptotic and anti-necroptosis functions that allow an infected cell to prolong the proinflammatory gene expression, and thus prolong antiviral signaling [128, 129].

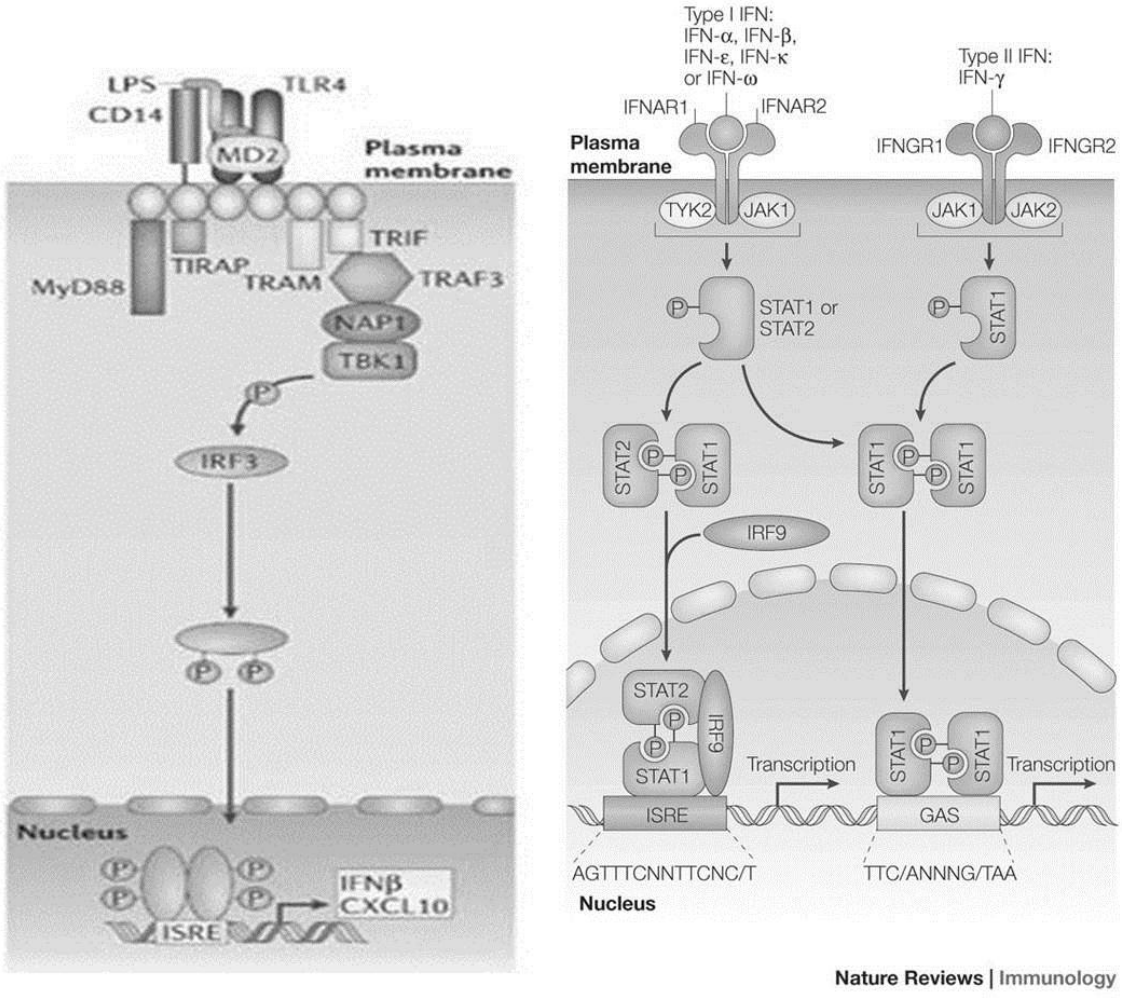

FIG 2. Interferon Induction and Signaling.

(Left) Type I interferon induction illustration. Authorized for reprint from Honda K, Taniguchi T. IRFs: master regulators of signaling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol. 2006;6(9):644-58.

(Right) Interferon signaling pathway. Authorized for reprint from Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol. 2005;5(5):375-86.

1.2.1.2.1 Non canonical NFκB Pathway

There are two distinct pathways of NFκB activation: the canonical and non-canonical. Unlike the rapid signaling of canonical NFκB the non-canonical pathway is relatively slow and is dependent upon de novo protein synthesis [130]. Also, it is only activated by a subset of TNFR signals whereas classical NFκB signaling is activated by a wide range of receptors [131]. The main dimer of non-canonical NFκB is composed of RelB complexed with p52 [131, 132]. This dimer depends on proteasomal processing of p100, the p52 precursor [131, 132]. The p100 precursor functions at two steps in the pathway: First, p100 mimics IκB by preventing RelB/p52 dimers from translocating to the nucleus, and second by being processed into the p52 subunits [133, 134]. The signaling steps that promote p100 processing are similar to those of the canonical system, involving phosphorylation, ubiquitination, and degradation; however the proteins involved are different. One of the principal proteins involved in the signaling cascade leading to p100 processing is NFκB-inducing kinase (NIK). Before the initial signaling step NIK is maintained in the cell at very low levels by interacting with an E3 ubiquitin ligase that contains TNF receptor associated factor (TRAF)3 and TRAF2 (Fig 3: Top). This association results in NIK's ubiquitination and subsequent degradation [135, 136]. When specific receptors are activated, TRAF2 and TRAF3 are degraded, which results in NIK stabilization [137, 138]. This stabilization coupled with continued synthesis allows NIK levels to accumulate. As levels of NIK increase, NIK becomes activated by autophosphorylation or though recruitment to receptor complexes [139, 140]. Once

activated, NIK phosphorylates p100, which becomes ubiquitinated and processed into p52 [141]. Although not well understood, NIK activation also leads to activation of the IκB kinase (IKK) alpha complex, which can in turn bind and phosphorylate p100 as well (Fig 3: Bottom) [132]. IKKα activation also acts as a potent negative feedback mechanism by phosphorylating NIK, leading to NIK degradation and the maintenance of NIK at a steady state level [142]. Once activated, non-canonical NFκB signaling leads the transcription of genes that aid in mature B-cell survival , stromal cell organization in secondary lymphoid organs, and T-cell differentiation [143-148].

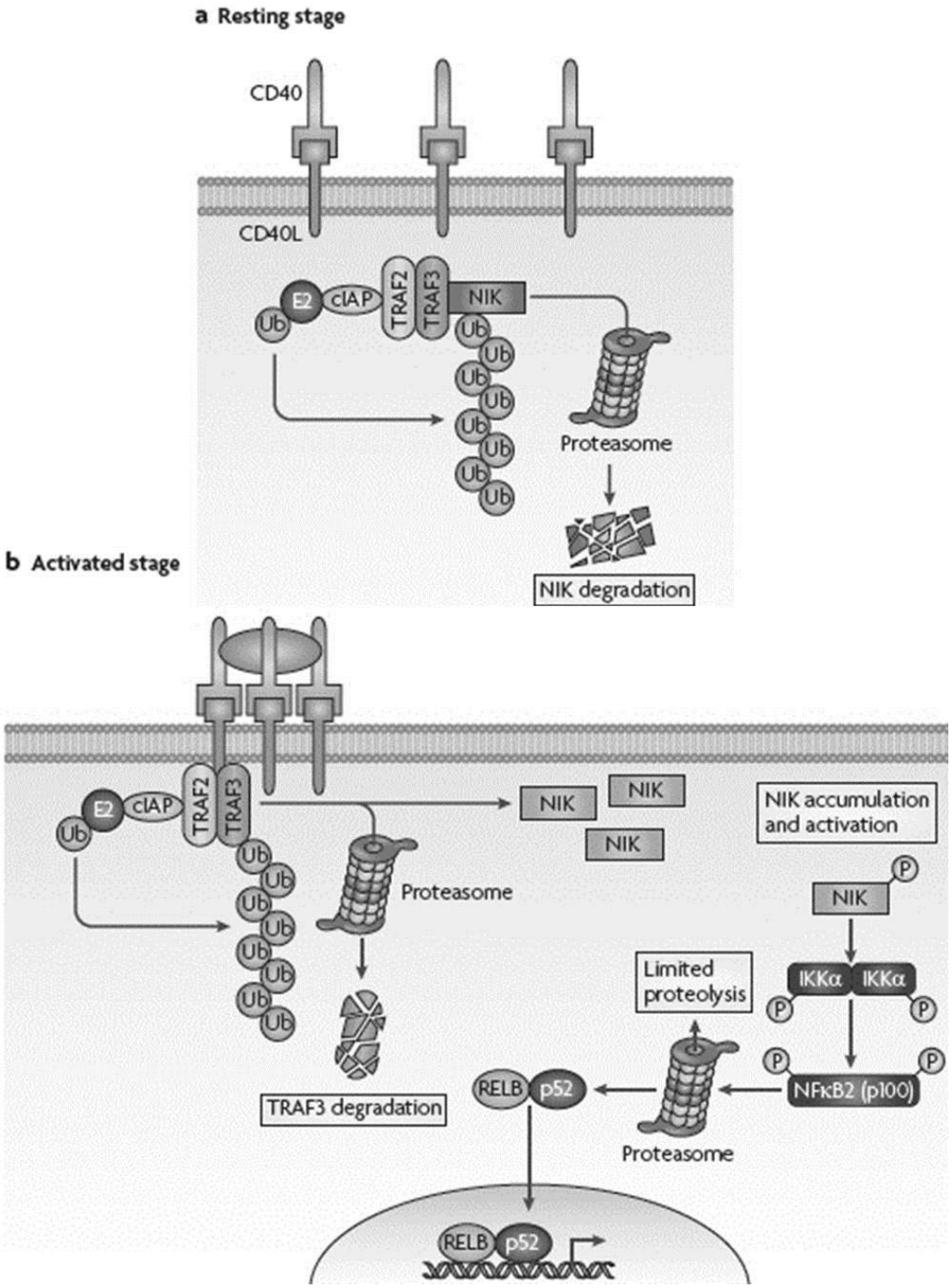

FIG 3. Non-canonical NFκB Signaling.

(Top) Non-canonical NFκB Signaling in resting cells.

(Bottom) Non-canonical NFκB Signaling in activated cells Interferon signaling pathway. Images authorized for reprint from Gyrd-hansen M, Meier P. IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nat Rev Cancer. 2010;10(8):561-74.

1.2.1.2.2 Canonical NFκB Pathway

The core of canonical NFκB signaling consists of a few stages. First various trigger events, including PRR and cytokine receptors binding their ligands, are able to begin the signaling cascade. In all cases the trigger events converge on the activation of the IKK complex [149]. Once the IKK complex is active it can phosphorylate the inhibitor of kappa B alpha (IκBα; prototypical IκB) which holds the NFκB subunits (typically p65:p50 dimer) primarily in the cytoplasm [149, 150]. Once phosphorylated, IκBα is ubiquitinated and degraded, which allows for NFκB translocation to the nucleus to bind specific DNA sequences [149]. This is a highly simplified version of what occurs during the NFκB activation, but highlights the two major regulatory aspects of the pathway: activation of IKK and the removal of inhibitory IκB which will be discussed in more detail below.

1.2.1.2.2.1 IKK Complex and Activation

The IKK complex is composed of three subunits: IKKα, IKKβ, and IKKγ or NFκB essential modulator (NEMO). The IKKα and IKKβ subunits are catalytically active while IKKγ acts as a regulatory subunit [151, 152]. Although IKKα and IKKβ can form homodimers, the complex as a whole is significantly more active when IKKα and IKKβ interact with each other [153, 154]. Also, IKKα homodimers seem to be more involved in non-canonical NFκB signaling [155]. The IKKγ subunit recruits the entire IKK complex to upstream activators, which in turn phosphorylate the activation loop of IKKβ [156, 157]. The phosphorylation of IκBα is predominantly due to the IKKβ

subunit since removal of serine residues in the activation loop of IKKα does not obstruct activation [156]. Also the presence of an intact IKKγ is necessary for IKK activation and NFκB signaling to occur; even IKKγ truncations that still allow IKKβ binding yield an inactive complex [158, 159]. Besides the phosphorylation of IKKβ, either by induced proximity oligomerization or by another kinase, the activation of the IKK complex also relies on the ubiquitination (K63 linked) of IKKγ [160-162]. In order to become active IKKγ interacts with multiple different proteins, but the main ones are TRAF family members, RIP kinases, and TAK1 (Fig 4) [149].

There are seven TRAF proteins in total and as we have seen (above: TRAF 3 in non-canonical NFκB signaling) they are key intermediates for the activation of NFκB. TRAF 2, 5, and 6 are well known activators of the canonical pathway [163-165]. As an intermediate the TRAF proteins link the IKK complex to upstream proteins that interact with the signaling receptors [166, 167]. For example, in TNFα signaling, TRAF2 bind the IKK complex and TNFR1 through the adaptor TRADD [168, 169]. Interestingly, the IKK/TRAF2 interaction seems to go through IKKα and IKKβ and not IKKγ [170]. The TRAF proteins are E3 ubiquitin ligases with N terminal RING domains and their K63 ubiquitination activity seems to play a role in the activation of IKK either directly or in conjunction with Receptor-interacting protein (RIP) kinases [163, 171].

RIP kinases seem to act in concert with the TRAF proteins in order to activate IKK (Fig 4). One of the best characterized RIPs is RIP1. RIP1 has a death domain (DD) allowing it to interact with many receptors and adaptor molecules, that also have a DD, and is essential for IKK activation from multiple receptors [172-174]. Besides the

interaction with receptors RIP1 can directly bind IKKγ aiding in the recruitment of the IKK complex [175]. In conjunction with TRAFs, RIP1 can become ubiquitinated by TRAFs which may aid in the stability of the IKKγ-RIP1 interaction due to a novel K63 ubiquitin binding domain in IKKγ (Fig 4: Top) [176-178]. The importance of the role that ubiquitination of RIP1 plays, at least for TNFα induced NFκB signaling, is contentious having been shown to be both necessary and dispensable [179, 180]. Although RIP1 is required for IKK activation it seems that its role is as an adaptor/scaffold and not a kinase since removal of its kinase domain still allows for normal IKK activation [181]. RIP2, behaves much like RIP: able to bind IKKγ, dispensable kinase function, and acceptor of TRAF ubiquitination, but instead of having a DD contains a caspase recruitment domain found in many NLRs [182, 183]. One model of IKK activation is the induced proximity model. This is based on the fact that oligomerization of IKK complex allow for self-activation [184, 185]. RIPs and TRAF proteins act as a scaffold to bridge IKK to signaling receptors and by bringing IKK complexes in close proximity to allow for their self-activation [186]. Alternatively, it has been shown that RIP2 and TRAFs can interact with TAK1, which may be an IKK kinase [171, 187].

TAK1 is a putative IKK kinase that may be required for IKKβ phosphorylation [188]. In many cases it seems to act in a RIP- or TRAF-dependent manner (Fig 4: Bottom) [171, 189, 190]. The requirement for TAK1 in IKK activation may be cell type- and or receptor-dependent since it is not necessary in all circumstances [191-194]. The general view is that RIP or TRAF K63 ubiquitination recruits not only the IKK complex (via IKKγ interaction) but also TAK1 containing complexes which then in turn phosphorylate IKKβ [195].

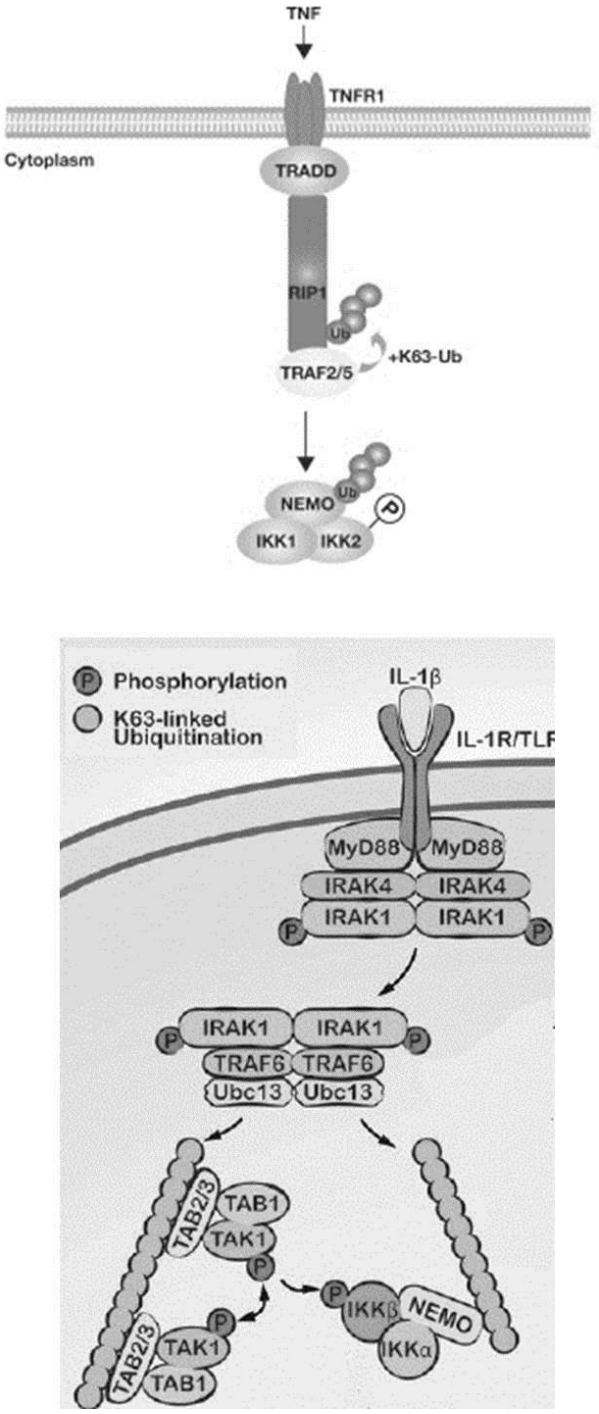

FIG 4. IKK Complex Activation.
(Top) IKK complex activation through TNFR1. Authorized for reprint from Jacque E, Ley SC. RNF11, a new piece in the A20 puzzle. EMBO J. 2009;28(5):455-6.
(Bottom) IKK complex activation through TNFR1 incorporating the use of TAK1. Authorized for reprint from Liu S, Chen ZJ. Expanding role of ubiquitination in NF-κB signaling. Cell Res. 2011;21(1):6-21.

1.2.1.2.2.2 IKK Deactivation

Once the IKK complex has been activated it needs to be tightly regulated. Although deactivation of the IKK complex is not well understood it does occur by multiple mechanisms. First, further auto phosphorylation of serine residues of IKKβ outside of its activation loop on the C-terminus are reported to have a negative effect on IKK activity [156]. Cellular phosphatases, such as protein phosphatase 1 target IKK for dephosphorylation [196]. Deubiquitinating enzyme CYLD and A20 have been shown to be strong negative feedback regulators of NFκB activity [197, 198]. After induction by NFκB, CYLD is involved with the deubiquitination of TRAF2, TRAF6, and IKKγ [199, 200]. A20 targets the K63 ubiquitin chains on RIP1 and TRAF6 for removal [201]. In addition to the removal of K63 from IKKγ, adaptor protein A20 can add K48 ubiquitin chains to RIP1 and target it for degradation [201]. Other negative regulators include SOCS-1 which seems to exert its effect through the ubiquitination and degradation of the p65 NFκB subunit [202, 203]. With multiple modes and levels of deactivation precise control of NFκB signaling is achieved.

1.2.1.2.2.3 Post IKK activation: IκBα

IκBα is the chief IκB that limits the p65:p50 NFκB dimer from transcriptional activity [204, 205]. IκBα is made up of three main regions: N-terminal, ankyrin repeat domain, and a C-terminal PEST domain (Fig 5: Top). The N-terminal region has two serine residues (S32/S36) contained within the degron motif (DSGXXS) of IκBα, which are phosphorylated as result of IKK activation [206-208]. Classically, it is understood that IκBα sequesters NFκB in the cytoplasm by virtue of masking the NLS on the NFκB

subunits, however the location and transport of IκBα:NFκB is a much more dynamic process [209]. IκBα contains a nuclear export signal (NES) which is required for the nuclear export of NFκB [210]. However, IκBα also contains a novel nuclear localization signal (NLS) in the second ankyrin repeat [211]. Crystal structures of the IκBα:NFκB complex reveal that IκBα only fully covers the p65 NLS of the p65:p50 dimer; thus leaving the p50 NLS exposed [212, 213]. This is exemplified in a homodimer of p50 which escapes IκBα cytoplasmic retention [214]. With an exposed p50 NLS and the novel NLS within the ankyrin repeat of IκBα, the IκBα:NFκB complex shuttles between the nucleus and cytoplasm; export being faster than import giving rise to the larger pool of the complex being found in the cytoplasm at steady state [215]. Although IκBα:NFκB complexes are found in the nucleus they are not active until IκBα has been degraded [215]. The first step in IκBα degradation involves the phosphorylation of the serines within the degron motif by IKK. Phosphorylation alone is not enough for IκBα to detach from NFκB [216]. However, once phosphorylation does occur the βTrCP–containing SCF complex, an E3 ubiquitin ligase, recognizes IκBα [217, 218]. The SCF complex is responsible for the addition of K48 linked ubiquitin chains on IκBα which relegates it for proteasomal degradation [218, 219]. The $SCF^{\beta TrCP}$ complex is also important for processing the precursors p100 and p105 into their active subunits p52 and p50 respectively [220]. Once IκBα has been degraded NFκB can translocate from the cytoplasm to the nucleus (Fig 5: Bottom). Since IκBα:NFκB complexes shuttle across the nucleus, the nuclear pool of IκBα:NFκB can be degraded to induce NFκB-dependent transcription very rapidly because these complexes are not subject to the translocation times and could be why there are temporal differences in NFκB-dependent gene

expression [221, 222]. Nuclear IκBα degradation is mediated through βTrCP ubiquitination and proteasomal degradation since these proteins are also located in the nucleus [207, 223-226]. Once active, NFκB promotes the transcription of more IκBα and p105 which creates another point of negative feedback where IκBα and p105 can bind NFκB dimers and promote their export from the nucleus [210, 227, 228].

Herpesviruses and NFκB have a very complex relationship. Some herpesviruses use NFκB to their advantage while others attempt to suppress NFκB signaling. EBV LMP1 protein is essential for the survival of the virus and immortalization of B-cells [229]. LMP1 functions by activating many signaling pathways including NFκB [230]. In addition to using NFκB for its anti-apoptotic effects LMP1 activation of NFκB minimizes TLR9 transcription and help EBV evade innate immune surveillance [231]. HCMV IE1 gene has NFκB-responsive portions in its promoter [232]. In addition to the upregulation of viral IE genes, IE1 also activates NFκB creating a positive feedback loop helping to maintain infection [233, 234]. HSV-1 has been shown to have both NFκB activating and inhibitory roles, demonstrating the tight control the virus requires over this pathway. In terms of pro-NFκB HSV-1 has been shown to have perpetual NFκB nuclear translocation and that inhibition of NFκB leads to lower viral yields [235-237]. HSV-1 also contains ICP0 which acts an anti-NFκB factor by degrading p50 and preventing TNFα induced-p65 nuclear translocation along with NFκB-dependent genes expression [238]. Unlike other herpesviruses VZV seems to only inhibit NFκB activity [239-242].

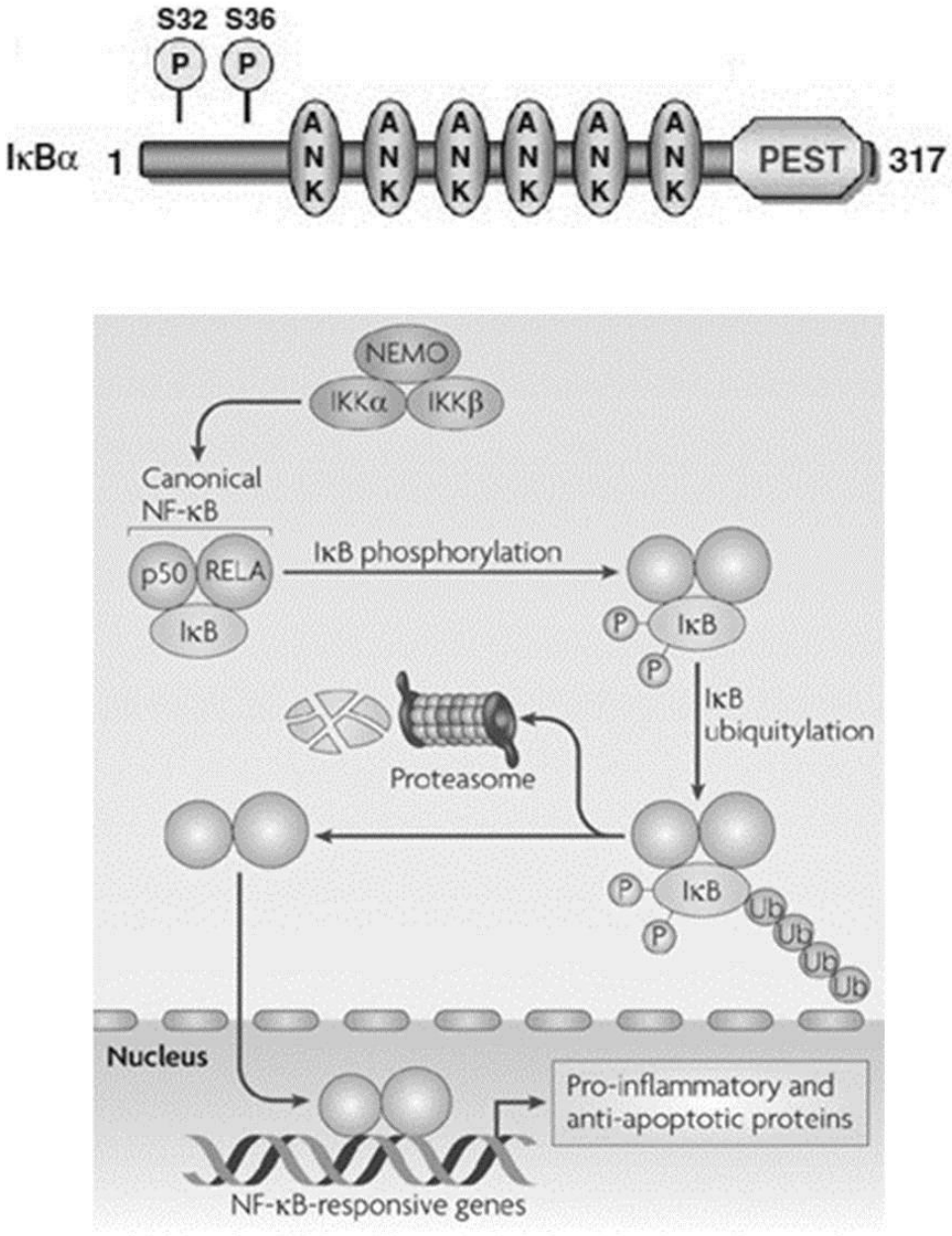

FIG 5. IκBα and its Role in Canonical NFκB signaling.
(Top) IκBα domains. Authorized for reprint from Perkins ND. Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. Oncogene. 2006;25(51):6717-30.
(Bottom) Canonical NFκB signaling downstream of IKK complex activation. Authorized for reprint from Pasparakis M. Regulation of tissue homeostasis by NF-kappaB signalling: implications for inflammatory diseases. Nat Rev Immunol. 2009;9(11):778-88.

1.2.2 Adaptive Response:

The adaptive branch of the immune system, unlike the innate, is specific for an individual antigen. The innate immune system helps drive antigen uptake and migration of APCs, like dendritic cells (DCs), to lymph nodes [243]. In the lymph nodes the DCs will present antigenic peptides on MHC molecules to activate CD4 and CD8 T-lymphocytes. The type of adaptive response depends on the type of infection and on whether the pathogen is intra- or extracellular. Regardless, CD4+ cells are important for both. Extracellular pathogens typically induce a Th2 "helper" T-cell profile which will aid in the activation of B-cells which produce an antibody response that will help with neutralization and engulfment of the foreign body. If the infection is intracellular, i.e. viral, the cellular immune response will be skewed towards a CD8+ T-cell mediated response. CD8+ T-cells, also known as killer or cytotoxic T-cells, have the ability to seek out infected cells (via MHC-I:antigen:TCR interaction) and kill them via a release of cytotoxins such as perforins and granzymes [244]. CD4+ T-cells in this case are important for the secondary expansion and maintenance of memory CD8+ T-cells [245-247]. A brief overview of normal adaptive responses to viral infection and how they relate to herpesvirus, including VZV, will be discussed.

All nucleated cells in the body express MHC-I. This allows CD8+ T-cells to scan the body for cells that are infected with intracellular pathogens (viruses) or that have become abnormal (cancer). In general, during protein degradation peptides are formed by the proteasome and get trafficked to the ER via the TAP transporter [248]. These peptides are self and/or pathogen derived. Peptides of 8-10 amino acids long can be loaded onto MHC-I molecules which promotes the stability of the MHC-I complex [248]. Once

MHC-I has bound peptide the stable complex is exported to the cell surface where it can interact with other cells. During a HSV-1 infection DCs are productively infected and show impaired presentation for many co-stimulatory molecules [249]. HSV-1 also induces apoptosis in infected DCs [250]. It is ensuing phagocytosis of non-infected DC that stimulates CD8+ T-cells through cross presentation [250]. Both CD8+ and CD4+ T-cell populations are important for controlling infection [251, 252]. During VZV infection it has been demonstrated that DCs are permissive to infection [253-255]. Immature DCs that have been infected with VZV are not able to upregulate MHC or co-stimulatory molecules, while VZV infection of mature DCs results in downregulation of these important immune markers [241, 255, 256]. In addition VZV infected DCs were reduced in their capacity to activate T-lymphocytes while at the same time being able to transmit the virus to T-cells for continued infection (see VZV tropism). The cell-mediated immune response is critical in controlling VZV. The possession of early VZV-specific T-cells resulted in a milder case of varicella, while the absence of T-cells resulted in persistent viremia [257]. Healthy adults have circulating T-cell populations, both CD4+ and CD8+, to a wide variety of VZV proteins [258-260]. In fact VZV-specific memory T-cells can be found in 1 out of 20,000-40,000 PBMCs [261]. A loss of cellular immunity shows a relationship with susceptibility and incidences of zoster [262, 263]. Upon reactivation of the virus T-cell numbers increase and play an important role in controlling VZV replication [263, 264]. In the SVV non-human primate (NHP) model CD4+ T-cells are necessary for control of SVV infection [265]. Haberthur et al. showed that depletion of CD4+ T-cells had a much larger impact on SVV infection than depletion of CD8+ T-cells or B-cells [265]. Along with increased numbers of T-cells VZV also

induces IgG, IgA, and IgM responses from B-cells [266, 267]. The role antibodies play in a VZV infection is indeterminate and minor at best. VZV IgG given as prophylaxis reduces varicella rate, however given after the onset of varicella it has no effect [268, 269] Also, children with T-cell immunodeficiencies still develop severe disease despite normal antibody responses, and B-cell depletion during SVV infection of NHP did not alter virus levels or disease severity [265, 270].

1.3 VZV

1.3.1 VZV Diseases:

VZV is a human neurotropic alpha herpesvirus which upon primary infection causes varicella (chickenpox) and is also the cause of herpes zoster (HZ,shingles) when the virus reactivates [11]. For many years VZV was considered a relatively benign virus and a childhood rite of passage [271]. However, with the introduction of chemotherapy to treat childhood cancers, two children were killed by varicella and the deadly nature of VZV was soon apparent [272]. With advancements in medical science, such as the use of chemotherapeutics to treat cancers and immunosuppressants for transplant recipients, there was a rise in the incidence of HZ and its related complications.

Each year there are approximately one million reported cases of HZ in the US, with over half of them being in persons age 50 or older [273]. It is believed that 1 out of 3 people will develop HZ in their lifetime and that 25% of those will be hospitalized for HZ complications [274]. HZ begins with the development of pain, itching, or burning sensations followed by a unilateral papular rash [275, 276]. The rash further develops vesicles (which can be transmitted to susceptible individuals) and typically begins to

crust by day 10 [275]. Although HZ itself is seldom life threatening there are many debilitating complications that arise from VZV infection and reactivation [277]. The most common complication associated with HZ is postherpetic neuralgia (PHN). PHN is characterized by constant severe pain that persists for months (even years) after the absence of the zoster rash [278, 279]. About 40% of people 60+ years old will develop PHN [277]. PHN is difficult to manage and typically antivirals are not beneficial, although there are cases where treatment with the herpes antiviral drug valacyclovir or famciclovir has been effective in reducing duration of the pain [277, 280, 281]. Other VZV complications include VZV vasculopathy, myelopathy, hearing loss, and retinal necrosis [277, 282-284].

1.3.2 VZV Vaccines:

There are two current FDA approved vaccines on the market for varicella and herpes zoster, Varivax and Zostavax respectively. They are both live attenuated virus vaccines based on the Oka strain of VZV. The Oka strain was attenuated by passaging the virus in guinea pig cells followed by approximately 30 passages in cell culture [285].Varivax was licensed for routine use (in the United States) in 1995 and has been extremely effective in reducing the number of varicella cases [286]. Initially, one dose of Varivax was recommended and protected about 85% of children [287]. However, there were cases of children developing varicella despite immunization that prompted the CDC to recommend a two dose strategy in 2006 [288, 289]. Since implementing the two dose regimen, protection from varicella has been increased to 98% [290-292]. Another key aspect to the usefulness of the varicella vaccine is that immunity does not wane over time, since contracting varicella as an adult is much more severe and can even be deadly

[293]. The data collected thus far shows that there is very little decrease of immunity to VZV up to 14 years post vaccination [294, 295]. The varicella vaccine also seems to be effective in reducing the rate of HZ when compared to those with a natural infection [296, 297].

The varicella vaccine has been largely successful; however the same cannot be said for the HZ vaccine. The HZ vaccine, Zostavax, was developed in 2005 and the only difference between it and the varicella vaccine is that Zostavax has a dose that is 14 times greater than that in Varivax [298, 299]. Zostavax has been shown to be mildly effective at reducing the incidence rate of HZ by 51% and PHN and other zoster related complications by 61% [299, 300]. In addition, unlike its varicella counterpart, Varivax, individuals vaccinated with Zostavax exhibit waning immunity (IgG titer) within a year and a return to pre-vaccination levels within three years [301]. It has also been shown that the vaccine efficacy is uncertain five years post vaccination [302]. Surprisingly, despite increases in varicella vaccination, there is a linear rate of increase of the incidence of HZ in the United States [274], a trend that has been occurring since the 1940s [303, 304]. One theory is that the increased incidence of HZ is due to adults no longer being exposed to children with varicella (due to the vaccine) which helped maintain and boost their immunity [305, 306]. This theory is unlikely because as mentioned above the linear trend of HZ started long before wide-spread vaccination was common, and because the rate of HZ has not increased at a quicker pace since vaccination became common [307, 308]. More likely explanations for the increase in HZ are the growing population of elderly and immunocompromised individuals in addition to the increased use of therapies that reduce the ability of the immune system to control and or fight infection. These, in conjunction

with improvements in diagnostics are the likely reasons for increases in HZ incidence [275].

1.3.3 VZV Genome:

The genome of VZV, like other herpes viruses, is comprised of a double stranded DNA organized into unique long (UL) and a unique short (US) regions flanked by inverted repeat regions TRL/IRL and IRS/TRS respectively [309, 310]. Typically the genome can be found as two isoforms with UL region the same in 95% of isolates [311]. The complete genome was sequenced in 1986 and found to have 124,884 base pairs [312]. Over the roughly 125kbp VZV encodes a minimum of 70 genes of which about 2/3 of them are necessary for in-vitro replication [313]. The gene expression follows a temporal sequence of immediate early (IE), followed by early, and then late genes. The IE genes (ORFs 4, 62, and 63) do not require de-novo synthesis and are typically involved host immune evasion and in the transactivation of other IE, E, and L genes. The early genes are mainly involved in DNA replication with ORF16 and ORF28 making up the DNA polymerase [309]. VZV ORF18 and ORF19 are involved in the conversion of ribonucleotides to dexoyribonucleotides to be used during replication [314]. Other genes involved in replication include ORF5, the origin of replication binding protein, ORF29, single stranded DNA binding protein, and ORF8, a dUTPase [310]. Late gene expression occurs after successful viral DNA replication and includes proteins that are structural in nature for the assembly, egress, and subsequent infection of newly made virions. Late genes include ORF40 and ORF54, which respectively make up the majority of the nucleocapsid and the entry site for viral DNA into the nucleocapsid [309]. Another group of genes important for the virus include the 9 glycoproteins (gB, gC, gE, gH, gI,

gK gL, gM, and gN). Notably, gB (ORF31) and gH/L (ORF37/ORF60) form the fusion complex allowing the virus to bind and enter cells [315].

1.3.4 VZV Tropism and Trafficking:

The spread of VZV is unique amongst herpesviruses in that infection is thought to be transmitted through inhalation [316-319]. The virus initially infects upper respiratory mucosal epithelial cells and then believed to spread to the tonsils and other lymphoid tissues where it infects T-cells [320-322]. While in T-cells two VZV kinases, ORF47 and ORF66, play a major role. ORF47 is responsible for the correct trafficking of gE and IE62, a necessary aspect of virion formation [323]. Initially the importance of ORF47 was not realized since it was not necessary for growth in tissue culture and ORF47 mutants have limited growth on skin xenografts [324, 325]. Despite these findings, ORF47 is indispensable for growth in T-cells [326, 327]. VZV ORF66 is a viral kinase responsible for the late stage cytoplasmic localization of IE62 and incorporation into the tegument [328-330]. Like ORF47 it was found to be unnecessary for growth in vitro or in skin and fibroblasts [331, 332]. Much like ORF47, ORF66 is necessary for in vivo growth in T-cells, despite normal localization of IE62 without its kinase function [332]. The infected T-cells can then disseminate throughout the body and deliver the virus to the skin where the characteristic rash will develop [310, 333]. Typically in the skin, due to the cell-associated nature of the virus, VZV will create polykaryocyte, multinucleated cells that have been fused together, to enhance spread, however it can still infect skin cells from release of mature virions [320, 334]. Besides the minimal fusion complex of gB and gH/gL, which are essential for skin infection, gE is also very important [335-337]. It has been demonstrated that a single amino acid mutation, serine 31 to an alanine

drastically impairs growth in skin [338]. Also, a naturally occurring VZV (VZV-MSP) with a different amino acid substitution in gE (aspartic acid to an asparagine at position 150) has an accelerated rate of spread in skin [339]. Post skin infection, the virus then infects and goes latent in neurons. VZV can infect neurons through two different methods. The first is by infecting the neuronal axon from innervated skin lesions where the virus uses retrograde transport up the axon to the cell body [275, 340]. The second is directly, during periods of viremia [320, 334, 341, 342].

1.3.5 VZV Evolution:

Despite the fact that VZV research has not been the highest priority among herpesviruses there is a surprisingly large amount of data concerning its phylogenetics and whole-genome sequences. Based on the amino acid sequence data of six conserved herpesviral genes (VZV ORFs 28/29/30/31/40/42) herpesviruses have been grouped into three categories: alpha, beta, and gamma [1]. VZV has been split into five clades based off of sequence data. The first strain of VZV to be sequenced was the Dumas strain [312]. The next was the Japanese Oka strain (parental and vaccine) sequenced in 2002 [343]. Over the course of the next few years 12 more VZV strains were fully sequenced [344-347]. During VZV SNP sequence analysis it became readily apparent groups of VZV genomes could be distinguished on a geographic basis [348-350]. Looking at the over 20 complete VZV sequences available, a 2008 consortium of scientists subgrouped the geographic VZV genotypes into the five clades [351]. Clade 1, 3, and 4 encompass Europe and North America. Clade 2 is Asian while clade 5 is Indian [352]. Isolates within an individual clade share more than 99.9% identity, while isolates between clades

share 99.8% identity; areas of large variability between clades occurs at the origin of replication [346].

Using this data it has been determined that the base of the phylogenetic tree for alphaherpesviridae is to have been around 400 million years ago [353]. In a geological time scale this is a notable time frame that coincides with the formation of the large landmass known as Pangea and shows that VZV most likely had an "Out of Africa" evolution along with humans [350]. As vertebrates continued to evolve so did the herpesviruses. The likely precursor to VZV was SVV. The genetic and immunological similarities between the two viruses are compelling and VZV can be used to immunize monkeys against SVV [354]. Prior to the formation of Pangea there were (and continue to be) herpesviruses that infect marine life such as the oyster and abalone [355]. The abalone herpesvirus is very fascinating since it was found to be neurotropic and create lesions in cerebral ganglia demonstrating that the neurotropism of herpesvirus has probably been around for an extremely long time [356].

Transmission of VZV is a very interesting, because it different from the other herpesviruses. The most common method of herpesvirus spread from one individual to another is via saliva; usually through kissing or though food sharing as is the case for HSV-1, CMV, and EBV [357-359]. Other methods include sexual transmission (HSV-2) and from mother to child through breast milk (CMV) [360, 361]. VZV on the other hand is the only herpesvirus to be spread primarily through aerosolization [316, 318, 319, 353, 362]. Also, since VZV asymptomatic shedding is rarely detected and reactivation usually only occurs once per individual, VZV uses reactivation, zoster, as its primary means of persistence within a population. Without reactivation later in life VZV would quickly die

out. For instance, when an infected individual (typically young) has primary varicella they will typically infect those around them that they come in contact with, usually a small group, especially if we consider early human life as small hunter-gather societies. After those people were infected and the virus went latent there would be no means of transmission to new individuals and the virus would not survive hence, reactivation late in life as zoster [363, 364]. Zoster is contagious, and therefore when we are much older, reactivation of the virus allows the transmission to new generation that has yet to come in contact with the virus [11, 353]. This is key since VZV does not reactivate as often as other alphaherpesvirus giving it less chance of transmission [365]. Both HSV-1/2 have been shown to be transmitted during asymptomatic periods [7, 8, 351, 366-368], whereas this would be very unlikely to occur for VZV [369]. However, infectious VZV has been recovered from the saliva of individuals during asymptomatic periods, but in this case the individuals were astronauts and represent a very specific and unique population [370, 371]. VZV has a novel mode of bi-generational transmission, with primary infection followed by reactivation late in life.

1.3.6 Animal Models for VZV:

A major issue hampering the study of VZV is its strict human specificity. Despite this much of what is known today about VZV has come from the studies involving animal models of infection. The animals used to study VZV include guinea pigs, rats, modified mice, and NHP. Each of these model systems has provided insight into the pathogenesis of the virus; however they all have critical shortcomings.

One of the earliest animal models used in the study of VZV is that of the guinea pig. It has been demonstrated that both Hartley and weanling guinea pigs can be infected

intranasally and subcutaneously and seroconversion is achieved [317, 362]. One major caveat of guinea pig infection by VZV is that it requires a guinea pig-adapted strain which is prepared by serial passage (10-30 times) through guinea pig cells and at times further passaged in human cells [285, 317, 362, 372, 373]. Although groups have achieved seroconversion with this model, human clinical symptoms, such as the development of a rash, are varying. Myers et al. found that 80% of animals infected IM developed exanthema, which could be reduced with the introduction of previously infected guinea pig serum [372]. However, in other studies from multiple groups exanthema was never observed in any animals [317, 374]. The guinea pig model has demonstrated support for transmission of the virus via aerosolized droplets through natural infection during co-habitation [317, 362]. The lack of immunological tools available for the guinea pig in conjunction with the variable reports of clinical symptoms and the fact that only an adapted strain of the virus can be used hinders the pervasive use of guinea pigs as a model to study VZV pathogenies.

In addition to guinea pigs other rodents, such as mice and rats have been used to study VZV. Like the guinea pig VZV infection of these animals results in seroconversion without any clinical manifestations [375-378]. Corneal inoculation of mice allows the virus to spread and establish a latent infection which was determined by PCR for VZV DNA [378]. This group found that the trigeminal ganglia were most frequently positive for VZV DNA 33 dpi [378]. However, this study was never able to detect viral antigens and has not been reproduced which could call into question the relevancy of the data to humans [378]. The greatest advantage of the rat model is the associated rat pain model. One of the largest complications with HZ is the associated pain known as PHN which can

last years after the HZ rash subsides, and is largely resistant to current therapies [379]. The rat model for VZV associated pain was developed by Fleetwood-walker and involves injection of cell-associated VZV subcutaneously into the footpad of winstar rats, and then monitoring paw withdrawal in response to mechanical and thermal stimuli up to 33 dpi [380]. These studies have been repeated and expanded upon by others [381, 382]. These studies show that footpad movement response times for allodynia and hyperalgesia occur only in the VZV infected rats and not in the controls [380]. It is very interesting that along with pain responses behavioral changes also occur in the rats [382]. Hasnie et al. found that along with lower footpad withdrawal thresholds the virus-infected animals had an anxiety-like ambulation pattern in open field paradigm studies. This is not unlike the depression and behavioral changes seen in patients with PHN [379]. The rat-pain model is a useful tool to test and develop novel PHN therapies.

VZV infection of NHP in most cases produces similar results to what has been documented during infection of immune-competent rodents. Monkey species are divided into New World (Platyrrhini) monkeys, which are found in Central and South America, and Old World (Catarrhines) monkeys, which inhabit Africa and Asia [383]. VZV infection of marmosets (*C. jacchus*; a New world monkey) via oral-nasal-conjunctival application or IV resulted in seroconversion without clinical rash, however they did develop morphological changes in lung tissue [384]. VZV infection of Old World monkeys has performed similarly. Felsenfeld et. al immunized patas monkeys (*E. patas*) with VZV and found that it provided protection against SVV challenge [354]. Although this study showed the antigenic relatedness of VZV and SVV, the monkeys inoculated with VZV failed to produce any clinical symptoms [354]. Recent experimental infection

of cynomologus macaques (*M. fascicularis*; an Old World monkey) with VZV was carried out as part of an experiment to create novel HIV/SIV vectors [385]. Much like previous reports they found seroconversion with a lack of clinical manifestations, however they also found VZV-specific T-cells [385]. There are also several reports of higher order apes naturally acquiring VZV [386, 387]. In 1984 the Cincinnati Zoo reported that a gorilla developed a self-limited varicella-like illness that generated VZV antibodies and was determined to be VZV by restriction digest of the isolate [388]. This prompted the idea that higher order hominoids could be a natural host for VZV and experiments with chimpanzees were initiated. Those studies indicated that chimpanzees inoculated subcutaneously with VZV developed a papular rash (only) near the injection site and VZV DNA was detected in PBMCs via PCR. Although in this study of limited sample size it seems VZV is able to infect non-human hominids there is extreme ethical, practical, and feasible concerns to using them for continued research.

One method to get around the strict human specificity of the virus was the use of humanized SCID (SCID-hu) mice for the study of VZV pathogenesis [389]. The SCID-hu mice are implanted with human fetal thymus and livers under the kidney capsule and are injected with VZV-infected cells [322]. This research has shown that VZV infects both CD4 and CD8 positive T-cells though the detection of cells positive for VZV DNA and the spread of infectious virus via transwell assays [322]. These in vivo studies correlate with other in vitro studies showing VZV infection of tonsillar CD4+ T-cells [321]. Ku et al. demonstrated that the population of tonsillar CD4+ T-cells most susceptible to VZV infection seemed to be activated (CD69+) memory (CD45RA-) cells that have skin homing markers such as CLA and CCR4 [321]. Human fetal skin has been

implanted subcutaneously into these mice as well, and inoculation of the skin graft with VZV leads to a characteristic varicella phenotype with vesicular lesions [322]. Together, these experiments have given us a major insight into how VZV traffics within the body. VZV-infected tonsil T-cells were given IV to SCID-hu mice with human skin implants which demonstrated the transfer of VZV from T-cells to the skin with recoverable VZV from the skin 7 dpi, epidermal thickening, and VZV lesion 10-21 dpi [333]. In addition to the in vivo trafficking data of VZV that the SCID-hu mouse model has provided, it has also been extremely useful for determining the role of viral proteins in vivo. Using this model it was determined that VZV ORF 47 was necessary for the successful infection of T-cells as well as skin implants [327]. It has also been shown that glycoprotein C (ORF14) is necessary for skin infection [327, 390]. In addition, using the SCID-hu mouse model it was determined that ORF66 is necessary for optimal T-cell infectivity [327]. These are all important findings since it had previously been determined that none of these viral proteins (ORF14, 47, and 66) were necessary for in vitro replication of the virus [325, 331, 390]. The humanized SCID mouse model has shown how important an in vivo system is in studying VZV.

1.4 SVV

1.4.1 SVV Genome

One of the earliest recorded reports of SVV came from the Liverpool School of Tropical Medicine in 1967 when 5 out of 17 vervet monkeys died with a papular rash and it was determined that the cause was a new member of the herpesvirus family [391]. Since this incident others episodes have occurred involving patas monkeys that develop

severe varicella-like symptoms (fever, full body rash, and mortality) [392-394]. In 1973 an outbreak occurred in macaque monkeys that closely mimicked the mild varicella that we see in most humans [395]. Since these outbreaks it has been determined that the causative agent is simian varicella virus, a member of the alphaherpesviruses, which has clinical, genetic, phylogenetic, and immunological similarities with VZV [354, 396, 397]. The genome is the smallest of the herpesviruses at 124.78 kb and is only slightly smaller than its VZV counterpart (124.88 kb) [398, 399]. SVV and VZV overall have a collinear genome and share between 70-75% DNA homology [400, 401]. SVV has 69 distinct ORFs and shares between 27-75% amino acid identity with VZV [402]. The major sequence differences between the two viruses occur at the 5' end of the genome. SVV contains a unique 665 bp section that is conserved (although varies in size between strains of SVV) that is not present in VZV [403]. SVV encodes ORFA, a 293 amino acid truncated version of SVV/VZV ORF4 [404]. SVV also lacks a homolog to VZV ORF2, which is a membrane phosphoprotein that was deemed dispensable for VZV replication in the cotton rat model [399, 405]. Another difference is that a latency associated transcript (LAT) has been described for SVV and not VZV. This LAT is antisense to SVV ORF61, the HSV-1 ICP0 homolog, and therefore in this regard closely resembles HSV-1and its ICP0 LAT [406-409]. Due to the high degree of homology which encompasses genetic, antigenic, and clinical aspects naturally occurring SVV infection of non-human primates is an advantageous alternative to study VZV pathogenesis.

1.4.2 SVV Animal Models

SVV infection of NHP is a tremendously advantageous tool to study VZV pathogenesis in a naturally occurring in vivo setting due to their high degree of genetic and antigenic

relatedness. However, not all monkey species are created equal, and there are differences between species during an SVV infection.

Patas monkeys were some of the first to be reported to have developed SVV infections and were used to demonstrate the antigenic relatedness between SVV and VZV in challenge studies [354, 392-394]. In these studies it was documented that Patas developed severe symptoms and suffered a large mortality rate (over 50%) [392, 394, 402]. Although this did not mimic what we expect during a VZV infection it did provide a useful model to test the ability of drugs and other therapeutic agents to prevent and limit illness. The ability of phosphonoacetic acid (PAA), a known inhibitor of herpesvirus DNA synthesis, to limit SVV replication was tested in patas monkeys [410]. In this study they gave PAA IM twice daily for 10 days starting 40 hours after infection with SVV [410]. The PAA treated animals developed SVV antibodies but never any clinical symptoms; in addition virus was not recovered from lymphocytes [410]. Acyclovir, a guanosine analog that limits viral DNA replication, blocked the occurrence of an SVV rash and improved overall health in patas but failed to have any effect on viremia [411]. Another known anti-herpesviral is adenine arabinoside 5' monophosphate (ara-AMP), which was found to inhibit SVV in-vitro as well [412, 413]. However, in an in vivo context, SVV infected patas given ara-AMP was completely ineffective as all monkeys (treated and control) developed a rash and viremia [413].

SVV infection of cynomolgus macaques has been used extensively to develop a working VZV model. Initially, there were two major outbreaks of SVV in cynomolgus monkey colonies: one at the Washington primate center and the other at the Tsukuba primate center [414]. The outbreak in Washington had a mortality rate of 2%, which was

much lower than what was seen with other species previously [395, 414]. Mahalingam et al. developed a natural infection model where SVV-seronegative monkeys were co-housed with monkeys that had previously been intratracheally inoculated with SVV [415]. Using this procedure with cynomolgus monkeys showed that they developed the characteristic varicella rash 10-14 days post exposure and then the virus went latent [416]. These studies were extended to examine reactivation though the addition of immunosuppressants and total body irradiation [416]. Mahalingam et al. reported the appearance of a zoster rash in 1 out of 4 animals [416]. In a second study Mahalingam et al. reported zoster rash in 3 out of 4 cynomolgus monkeys after treatment with immunosuppressants [417]. One caveat of the natural infection model is that the antibody titers in these monkeys are much lower (50-1000 times) than what we see with VZV infection in humans and direct infection of SVV in monkeys [416, 418-421]. This could be a possible reason that the Mahalingam group achieved reactivation so quickly (SVV DNA detected by day 7) [416, 422].

Vervet monkeys have also shown susceptibility to SVV infection and were among the earliest species reported to contract the virus [391]. Vervet monkeys belong to the genus *Chlorocebus* which includes the African green, grivet, tantalus, and malbrouck species. There have been multiple outbreaks within the *Chlorocebus* species that typically have a high (over 50%) fatality rate, such as in the UK when 9 out of 17 and in Louisiana when 5 out of 9 grivet monkeys died [423, 424]. Intratracheal inoculation of African green monkeys with SVV resulted in long-term acute replication and viremia [425]. Further, these monkeys maintained a status of persistent viremia with DNA found in CD4+ and CD8+ T-cells years after the initial inoculation [425]. Due to the large

mortality rate and other factors a natural infection model (mimicking the one used for cynomologus monkeys) was tested. It was determined that African green monkeys developed a mild rash which resolved and SVV DNA was found to be limited to ganglia [415]. Additional follow up studies revealed that the development of a rash is not necessary for ganglionic infection and that the hematogenous route of viral trafficking may play a larger role than epithelial retrograde transport in ganglionic infection [426]. Natural infection of African green monkeys followed by immunosuppressants have been used to study reactivation of SVV from latency, however, much like the parallel experiments done with cynomolgus monkeys, the low antibody titer achieved with the natural infection model may be the reason why reactivation was easily achievable [417].

SVV infection of rhesus macaques (RM) was originally reported to be mild in nature compared to other species when an outbreak occurred in the Washington primate research center in 1969 [395]. During this episode, none of the 19 RM that showed signs of infection died, whereas SVV infection of other old world monkeys has had high mortality rates [395]. SVV infection of RM has been shown to recapitulate many features of VZV pathogenesis and provides the first opportunity to natively address many host-virus interactions [407]. Intrabronchial infection of RM with SVV produces a varicella-like rash that resolves by day 21, viremia, adaptive immune responses, and the establishment of latency only in neuronal ganglia [407]. This model also produces the high antibody titers that humans have with VZV [418, 420, 421]. One of the hallmarks of latent SVV is the presence of a LAT antisense to ORF61 which was first described for vervet monkeys [409]. This was confirmed with SVV infection of RM as well [407, 408]. Interestingly, when RM were infected with mutant SVV lacking ORF61 only minor

differences (decreased viral loads and increased pDCs and IFNβ) compared to a wild-type infection occurred, and the virus was still able to establish latency [427]. A possible reason why this mutant virus behaved much like the wild-type is one of the outcomes of chapter 2 of this dissertation, but it may relate to the fact that SVV encodes multiple proteins that act in concert on immune signaling pathways [242]. Reactivation of SVV in RM was very recently archived though transport stress and or total body irradiation and treatment with tacrolimus and prednisone, a combination that had failed in the past [422, 428]. After reactivation, SVV antigens were found in multiple cell types including skin, lungs, gangli, macrophages, dendritic cells, and T-cells [428]. Zoster rash was also present [428]. With the recent ability to achieve reactivation along with all the other clinical and immunological parallels between SVV infection of RM and VZV in humans, SVV in RM has proven to be an important model for VZV and continued study with this model will further our understanding of both viruses.

1.5 Viral Immune Evasion:

In order to establish an efficient infection most viruses have developed multiple mechanisms to limit and avoid detection by our immune system. These mechanisms act to limit the intrinsic, innate, and adaptive branches of the immune systems. Critical pathways and components of anti-viral immunity can and will be targeted by several different viral proteins, within a single virus. Typically the number of proteins that a virus employs to block an immune pathway is proportional to the importance that pathway has in controlling viral infection. Herpesviruses are especially known to have multiple proteins targeting several immune pathways; this is probably due to the fact that they

establish persistent lifelong infections within their hosts. Below is a brief overview of strategies herpesviruses employ to modulate immune signaling with a focus on VZV

1.5.1 Interferon Evasion

Interferons are an important class of cytokines that act as part of the innate immune response to limit viral spread very early on in host defense. There are three groups of interferons, Type I (alpha and beta), Type II (gamma), and Type III (lamda). The expression of these proteins will induce a large number of genes termed interferon-stimulated genes (ISG) that act to limit viral replication. Some ISG proteins include the Mx proteins, PKR, and OAS/RNaseL which disrupt viral protein production and DNA replication [429]. To combat these effects viral proteins will block both the induction of interferons and their signaling pathways. CMV uses IE86 to block transcription of IFNβ through blocking NFκB activation, and IE1 to interfere with STAT2 affecting ISG induction [430, 431]. VZV is very susceptible to IFNs, especially Type I, demonstrated by an in vitro reduction in cytopathic effects [114, 115]. This is highlighted even more by the fact that treatment with IFNα reduces the severity of varicella in patients and that VZV infection of SCID-hu in mice that have been given Type I IFN neutralization antibody showed more widespread viral replication and skin lesion formation [112, 113, 333]. In order to combat IFN effects VZV ORF63 plays a major role. It has been demonstrated that ORF63 blocks the effects of IFNα by first limiting the expression of IFNα itself and by limiting the phosphorylation of eIF-2α which would reduce overall protein synthesis [333, 432]. ORF63 has also been implicated in blocking IFN signaling by disrupting the activation of JAK-STAT signaling by degrading IRF9 [433]. This same

study also reveals that VZV employs another unidentified protein that limits STAT2 phosphorylation [433]. VZV ORF61 has been shown to limit both IFNβ induction by targeting activated IRF3 for degradation and NFκB activation by blocking IκBα degradation [240-242, 434]. Along with ORF61 blocking IRF3 mediated cytokine induction, ORF62 and ORF47 interfere with IRF3 activation [435, 436]. Although it seems Type I IFN have a larger role in controlling VZV, VZV has mechanisms to limit Type II IFN signaling as well. VZV ORF66 has been shown to interfere with IFNγ signaling by downregulating STAT1 phosphorylation in T-cells [437]. The myriad number of VZV viral proteins that block induction or signaling of IFN shows how critical the elimination of IFN signaling is for proficient viral infection.

1.5.2 MHC Evasion

Another major mechanism of immune evasion is the downregulation of MHC class I molecules and preventing antigen processing and presentation to avoid CD8+ T-cell detection and killing. CMV is particularly good at manipulating the MHC-I antigen presentation pathway and targets the pathway at every step possible. HCMV first encodes US2 and US11which both relocate and degrade MHC-I heavy chain from the ER in an ubiquitin-proteasomal dependent manner; thus preventing the maturation of class I molecules from reaching the cell surface [438-441]. US3 blocks peptide loaded MHC-I from leaving the ER [442, 443]. US6 inhibits TAP by binding directly to TAP and limiting ATP binding, thus blocking peptide translocation into the ER and incorporation into MHC-I molecules [444-446]. HSV-1 ICP47 is also a TAP inhibitor, but it acts as a competitive inhibitor for TAP peptide binding [447-449]. Although VZV does not contain an HSV-1 ICP47 homolog, it does contain ORF9a a homolog to BHV UL49.5, a

TAP inhibitor similar to ICP47 [450, 451]. However, upon examination ORF9a does not seem to affect MHC-I downregulation [452]. The major modulator of MHC-I in VZV is ORF66, a kinase that limits MHC-I surface expression [452]. ORF66 impairs surface levels of MHC-I by disrupting its transport from the Golgi to the surface in both fibroblasts and T-cells [256, 452]. This facet of immune evasion is critical during the skin infection phase, allowing VZV to replicate while evading CD8+ T-cell surveillance. One major consequence of MHC-I downregulation is the increased activation and killing by NK cells due to "missing-self" [453]. NK cells are a type of lymphocyte that surveys the body for stressed or abnormal cells to kill through the production of cytokines based off a complex integration of both activating and inhibitory signals [454]. One of the main inhibitory signals is the recognition of surface MHC-I and proper antigen processing with peptide leader sequences presented on HLA-E [455, 456]. HCMV employs multiple mechanisms to evade NK cell activation while still disrupting antigen processing and presentation which include encoding its own MHC-1 homolog, downregulating activating NK cell ligands (MIC proteins), and containing its own signal peptide to be loaded on HLA-E independent of a functional TAP [457-461]. To date there have been no reports of any functional consequences, in regards to NK cells, due to MHC-I retention by VZV ORF66, although it is known that VZV infected cells are susceptible to NK cell killing [462-464].

MHC-II expression is conventionally restricted to a limited subset of cells: B cells, DC, monocytes, and CD4+ T-cells, however it has been shown to be upregulated on human fibroblasts upon exposure to IFNγ [465, 466]. This is important because activation of CD4+ T-cells can increase the anti-viral immune state by the production of IFNγ,

TNF, and IL-2 which aid the growth and survival of cytotoxic T-cells and enhance the inflammatory response [467]. It has also been shown that a population of CD4+ MHC-II restricted T-cells are able to aid in the lysis of target cells [468]. In order to counteract this host response herpesviruses have evolved methods to inhibit MHC-II signaling just like they have done for MHC-I signaling. HCMV has at least three proteins that block MHC-II signaling by aberrant localization and degradation [469-471]. HSV-1 has been shown to modulate surface MHC-II levels multiple ways: decreased expression of the host invariant chain and redistribution of DR and DM by an interaction with gB [473-475]. VZV is also able to modulate MHC-II. Abendroth found that VZV infection is able modulate IFNγ-induced expression of MHC-II at multiple signaling points. First, it was demonstrated that VZV downregulates the transcript levels of: MHC-II DRa, the MHC-II tranactivator CIITA, and IRF1 RNA in response to IFNγ [476]. Next, they verified that the IFNγ signaling pathway was disrupted by limited expression of STAT1 and JAK2 proteins [476]. Lastly, they saw that MHC-II downregulation was conserved in vivo by in situ hybridization of skin biopsies of people with active varicella or zoster rashes while in nearby uninfected cells MHC-II was expressed [476]. This inhibition of IFNγ-induced upregulation of MHC-II has been extended to human keratinocytes, another important cell type for VZV replication in the skin [477, 478].

1.5.3 NFκB Evasion

Another important cell type that is vulnerable to VZV infection is the DC population. Although it is not one of the major three cell types (see tropism) that VZV infects, the DC population is critical in inducing a proper immune response. DCs are the

chief APC in the periphery that capture, process, and present antigens to naïve CD4 cells, along with the necessary co-stimulatory ligands, to initiate the adaptive immune response [479]. VZV infection of DCs most likely occurs at the mucosal sites of initial infection, where the DC then travels to the nearest lymph node which results in VZV being transferred to T-cells and then throughout the body to the skin [253]. Abendroth was the first to show that VZV is able to have a productive infection in immature DC and transfer virus to fibroblasts and T-cells [253]. In the same study, despite the fact that the virus was replication-competent they found no downregulation of MHC (I and II) or other co-stimulatory markers [253]. Since DCs play such a large role in the immune response it would be advantageous for the virus to modulate it while in this cell type. The infection and transmission of VZV in the Abendroth et al. study was in an immature DC population. In order to be the potent immune activators that they are known to be, maturation, which increases the expression of MHC and co-stimulatory markers (CD80/86), needs to occur [480, 481]. VZV infection of mature DC behaved exactly like one would expect, from the viruses' standpoint, with downregulation of MHC-I, CD80/86, and CD83 [255]. Mature DC VZV infection also impaired the ability of DCs to activate allogeneic T-cells [255]. Although the proteins that are responsible for this phenotype are largely unknown it would seem like modulators of NFκB are being targeted since many of the cytokines and co-stimulatory markers are transcriptionally regulated by NFκB [482]. In fact VZV ORF61 has been shown to modulate NFκB activity in human DCs [241]. Further studies have been carried out on the various subsets of DCs that VZV infects [254]. One of the more interesting subsets is the pDC population for their ability to be recruited to sites of inflammation and produce large

amounts of Type I interferon [483]. Since Type I IFN is detrimental to VZV growth this population might be controlling VZV spread in the skin [484]. Note, VZV infection of pDCs does severely limit the amount of IFNα able to be produced [254]. Also, SVV infection of RM with a virus deficient for ORF61 showed a significant increase in the number of pDC present and IFNβ being produced [427].

1.6 ORF61

VZV ORF61 is a 62-65 kDa phosphoprotein and is the functional homolog to HSV-1 ICP0 [485, 486]. It is present within the first hour of infection and localizes to the nucleus with a diffuse fluorescence in a similar manner to what has been reported for ICP0 [486, 487]. VZV ORF61 and ICP0 also share transactivator functions [488-490]. However, unlike ICP0 which has only been shown to activate genes, ORF61 can activate as well as repress viral transcripts [491]. Another feature that ORF61 and ICP0 share is a zinc binding RING finger domain in their amino terminus. In both proteins this RING domain is critical for its transactivation ability [492, 493]. This domain is also responsible for the E3 ubiquitin ligase function of these proteins [494-496]. When ORF61 or ICP0 are knocked out from their respective viruses both yield virus that is able to replicate but has impaired growth kinetics and small plaque phenotype [497, 498]. Although they are functionally similar proteins, and VZV ORF61 can partially rescue an ICP0 null HSV-1 virus, it cannot replicate all of ICP0 functions. Therefore ORF61 has it has its own diverse and unique functions [499]. In order to study VZV ORF61 we looked at examining SVV ORF61 since VZV and SVV are highly similar both in terms of genetics and disease pathology (see above sections on SVV genome and RM animal

model). In addition, through studying SVV we have the ability to examine the effect of any in vitro findings in a natural in vivo setting.

Like ICP0, VZV ORF61 had been implicated in innate immune evasion of NFκB and Type I IFN pathways [238, 240, 241, 434, 500-502]. With specific regard to the role ORF61 played in NFκB modulation, we sought out to see if ORF61 (SVV/VZV) could inhibit NFκB signaling and if so to elucidate the mechanism of that inhibition.

Chapter 2: The ORF61 protein encoded by Simian Varicella Virus and Varicella Zoster Virus inhibits NFκB signaling by interfering with IκBα degradation

2.1 Introduction

Varicella Zoster Virus (VZV) is a member of the alphaherpesvirinae subfamily and is the causative agent of chickenpox and herpes zoster (HZ). Following primary infection, VZV establishes latency in ganglia. Reactivation from latency, which typically occurs later in life due to a weakened or compromised immune system, causes HZ or shingles. HZ is characterized by a painful itching rash that typically appears on the trunk of the body along a thoracic dermatome. The occurrence of HZ is associated with serious debilitating complications, which include post-herpetic neuralgia (PHN), blindness, paralysis, or hearing loss. PHN is characterized by pain or allodynia that remains after the HZ rash has subsided [379, 503, 504]. In vivo research on VZV has been constrained in the past due to the lack of an adequate animal model. Simian varicella virus (SVV) is organizationally and genetically similar to VZV sharing about 75% DNA homology and a co-linear genome [505]. Recently, SVV infection of Rhesus macaques (RM) has been shown to recapitulate many features of VZV pathogenesis including a varicella-like rash that disappeared around 3 weeks post infection. Latency was confirmed by detection of viral DNA in neuronal ganglia months after primary infection [407]. Thus, SVV-infection of RM can be used as a model for VZV-infection.

Nuclear Factor kappa B (NFκB) signaling plays a critical role in the establishment of antiviral immune responses [506]. NFκB signaling drives the expression of many

proteins that aid to block viral replication and stimulate the development of specific adaptive immune responses. These factors include pro-inflammatory cytokines, regulators of apoptosis, and chemokines [118, 507-509]. NFκB signaling is initiated by pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) as well as cytokine receptors. These include Toll-like receptor (TLR) 4, TLR-3, IL-1R and TNFR1 that are activated by lipopolysaccharide (LPS), double-stranded RNA, IL-1 and TNFα, respectively [118, 510-512]. Signaling through PRRs leads to the phosphorylation of the inhibitor of NFκB kinase (IKK) complex that is composed of the subunits IKKα, IKKβ, and IKKγ or NEMO. The activated IKK complex phosphorylates the inhibitor of NFκB (IκBα), which keeps the NFκB subunits RelA or p65 and p50 inactive. Phosphorylation of IκBα leads to its rapid degradation and the release of the NFκB subunits, which will initiate expression of numerous genes [118, 513]. NFκB-IκBα subunits have been shown to shuttle between the nucleus and cytoplasm [215]. Degradation of IκBα and activation of NFκB occurs predominantly in the cytoplasm, but has been observed in the nucleus as well [207, 215]. IκBα degradation is mediated by an E3 ubiquitin ligase complex that consists of the F-box protein β-TrCP, Skp1, Cullin1, and the adaptor protein Roc1 or Rbx1 (the $SCF^{\beta TrCP}$ complex). Targets of this complex contain a phosphodegron domain (DSGΦXS, Φ indicates hydrophobic domain), which is recognized by β-TrCP upon phosphorylation of the two serines. This is followed by the ubiquitination of the target protein and subsequent degradation by the proteasome [206, 514].

Numerous viruses code for immune evasion mechanisms that target the NFκB signaling pathway, illustrating the prominent antiviral role of NFκB-mediated protein

expression [515]. For VZV it was shown that the virus interferes with TNFα-induced NFκB promotor activity by preventing the degradation of IκBα [240]. This interference seems to be at least partially mediated by ORF61 since Sloan et al. showed that ORF61 expression led to the stabilization of IκBα in TNFα-treated HEK 293T cells [241]. However, Zhu et al. reported that overexpression of ORF61 had only a minor effect on Sendai virus-induced NFκB-signaling indicating a pathway-specific counter mechanism [434]. Instead, they showed that ORF61 interacts with phosphorylated IRF3 and induces degradation of the protein thereby preventing the induction of IFNβ-expression [434]. VZV ORF61 is expressed with immediate early kinetics and is highly homologous to the herpes simplex virus 1 protein ICP0 [485] which has also been involved in preventing innate immune activation [516]. In addition, ORF61 was shown to trans-activate or -repress the transcription of other VZV proteins, including its own promotor [490, 491, 493, 517, 518]. The ORF61 protein encodes an N-terminal RING domain, typically found in ubiquitin ligases and known to be required for its gene regulatory functions [493] as well as the degradation of IRF3 [434]. The isolated RING domain displayed ubiquitin ligase activity *in vitro* [495] and VZV ORF61 was shown to regulate its own stability via autoubiquitination [496].

In the presented study we demonstrate that, similar to VZV, infection with SVV leads to the stabilization of IκBα [240, 241]. We further demonstrate that SVV ORF61, which shares 42.8% amino acid identity with the VZV ORF61 [505] and regulates viral gene expression [497], also prevents IκBα degradation. Interestingly, IκBα phosphorylation was allowed, but degradation was prevented in ORF61-expressing cells stimulated with TNFα, resulting in the accumulation of phosphorylated IκBα. Inhibition of degradation is

likely the consequence of SVV ORF61 forming a complex with the ubiquitin ligase subunit β-TrCP, thereby preventing TNFα-induced ubiquitination of IκBα by the $SCF^{\beta\text{-}TrCP}$ complex. This interaction seems to broadly interfere with $SCF^{\beta\text{-}TrCP}$ function since SVV ORF61 also affected the turnover of β-TrCP target Snail. Similarly, VZV ORF61 prevented β-TrCP-mediated IκBα ubiquitination indicating that this molecular mechanism is conserved between the two viruses. In addition to inhibiting IκBα ubiquitination, we show that SVV, but not VZV, prevents the phosphorylation of IκBα, suggesting that SVV codes for at least one additional protein that contributes to the evasion of NFκB signaling.

2.2 Results

2.2.1 SVV blocks NFκB activation at or downstream of IKK activation

Previous studies have shown that VZV infection inhibits TNFα-induced activation of the NFκB pathway [240, 241]. To determine whether SVV similarly interferes with NFκB signaling we studied TNFα-induced NFκB activation in SVV-infected telomerized rhesus fibroblasts (TRFs) that stably express firefly luciferase under control of an NFκB promoter and constitutively express *Renilla* luciferase (TRF NFκB). We infected TRF NFκB cells with SVV.eGFP by co-incubating uninfected and SVV-infected cells at a 5:1 ratio. After 42 hours, the cells were incubated with increasing concentrations of rhesus (Rh) or human (Hu) TNFα for 6 hours. Immunofluorescence microscopy for GFP confirmed that nearly all cells were infected (data not shown). NFκB activity was calculated as the ratio between induced firefly and constitutive *Renilla* luciferase expression. The latter was measured to control for cell death resulting from viral

infection. Mock-infected cells showed a dose dependent increase in both Rh- and HuTNFα-induced NFκB activation, whereas NFκB promoter activity was significantly reduced in SVV-infected cells (Fig 6A).

NFκB is activated by many different signaling pathways that are induced by PAMPs, such as microbial RNA or DNA, LPS, or by pro-inflammatory cytokines, such as TNFα and IL-1β. While NFκB-terminal signaling employs multiple alternative upstream factors, they all converge at the IKK complex [149]. To determine if SVV inhibits a common event in NFκB activation we stimulated mock- and SVV.eGFP-infected TRF NFκB cells with increasing concentrations of poly I:C (activator of the MDA5/RIG-I pathway [519]), phorbol 12-myristate 12-acetate (PMA; activates NFκB via protein kinase C [520]), LPS (activator of TRIF signaling [521]) and IL-1β for 6 hours. At 42 hours post infection (p.i.), mock-infected cells showed a dose-dependent increase in firefly luciferase expression for all stimuli, which is indicative of NFκB activation (Fig 6B). In contrast, NFκB activation by all stimuli was significantly reduced in SVV-infected cells (Fig 6B). These data show that SVV-induced inhibition is not limited to the TNFα-specific pathway and suggest that SVV targets a common event in the signaling cascade at the level of or downstream from IKK activation.

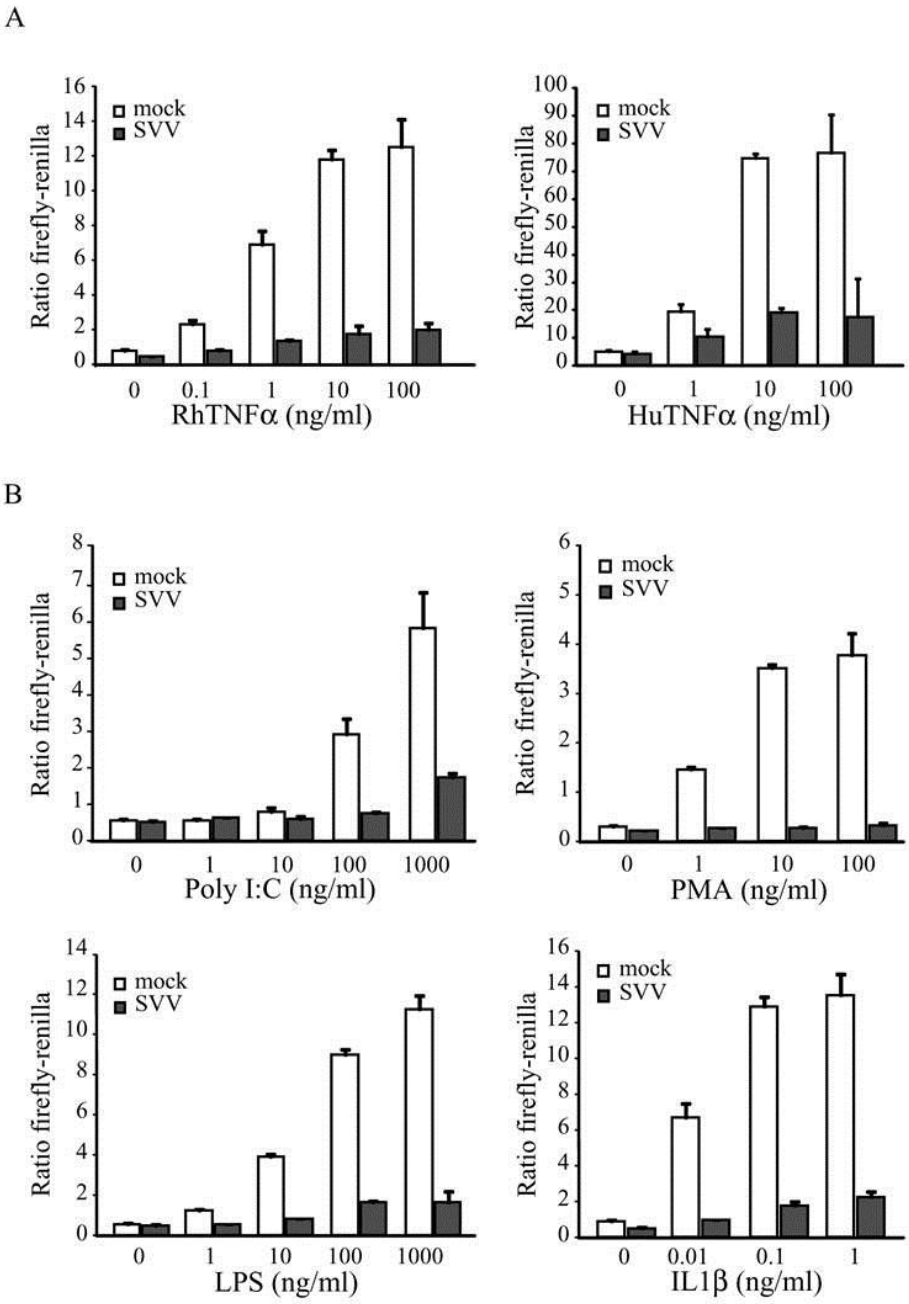

FIG 6 SVV inhibits NFκB **activation induced by various stimuli.** TRFs stably expressing firefly luciferase under an NFκB promoter and constitutively active Renilla luciferase (TRF-NFκB) were mock infected or infected with SVV.eGFP at a ratio of 5:1. At 42 h p.i., the cells were stimulated with the indicated increasing concentrations of RhTNFα and HuTNFα (A) or poly(I·C), PMA, LPS, and IL-1β (B) for 6 h. Firefly and Renilla luciferase expression was measured using a dual-luciferase reporter assay, and NFκB activity was determined by normalizing the firefly signal to the Renilla signal. The results from one out of three (TNFα) or two (other stimulants) independent experiments are shown. The error bars indicate standard deviations.

2.2.2 SVV inhibits NFκB-signaling by preventing IκBα phosphorylation and degradation

To confirm that SVV inhibits NFκB-driven cytokine expression, we studied RANTES mRNA induction by qPCR in TRFs that were mock- or SVV.eGFP-infected for 48 hours and stimulated with TNFα during the final 1, 3 or 6 hours of infection. The mock-infected cells showed a time-dependent increase in RANTES mRNA expression, which was remarkably diminished in SVV-infected cells (Fig 7A). To determine the signal transduction step at which SVV interferes with NFκB activation we studied the nuclear localization of the NFκB complex in mock- and SVV-infected cells. TRFs were infected with SVV.eGFP for 48 hours and stimulated with TNFα for 45 minutes to activate NFκB. TNFα-treatment resulted in nuclear localization of the NFκB subunit p65 in uninfected cells, but not in SVV-infected cells (green/eGFP) (Fig 7B). Since the translocation of the p50/p65 heterodimer from the cytoplasm into the nucleus is dependent on IκBα degradation, which is preceded by IKK-mediated phosphorylation, we analyzed the phosphorylation and degradation of IκBα in SVV-infected cells. TRFs were mock- or SVV.eGFP-infected for 48 hours and subsequently stimulated with TNFα for up to 60 minutes. In mock-infected cells phosphorylated IκBα appears after 5 minutes of TNFα-stimulation and reduced IκBα levels are observed as early as 15 minutes after cytokine addition. After 60 minutes of stimulation, IκBα reappeared in mock-infected cells as a result of new synthesis (Fig 7C). In contrast, in SVV-infected cells, only very low levels of IκBα phosphorylation were observed regardless of TNFα-stimulation (Fig 7C, long exposure). Furthermore, IκBα was not degraded in SVV-infected cells and was detected at similar levels at all time points of TNFα stimulation (Fig 7C). These data

suggest that SVV interferes with IκBα phosphorylation, thereby stabilizing IκBα in TNFα-treated cells.

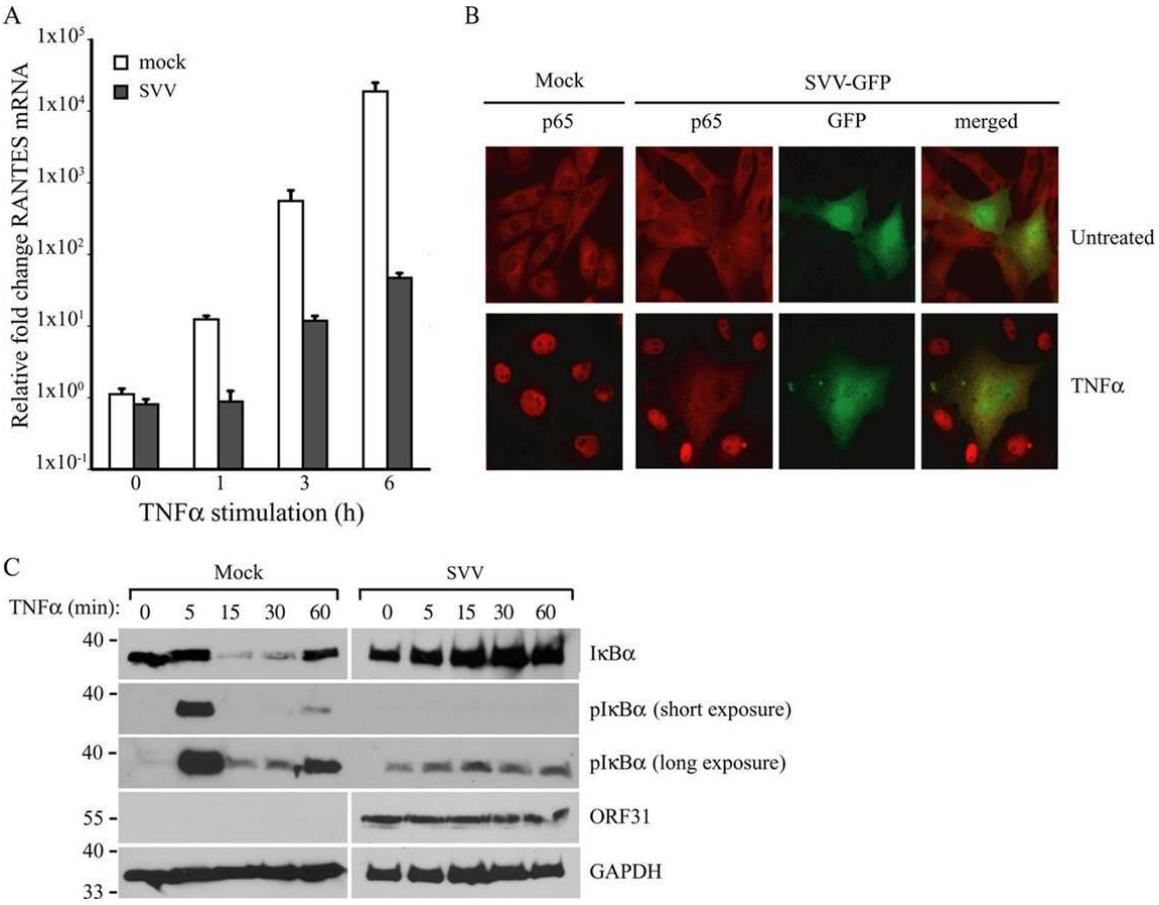

FIG 7 SVV inhibits NFκB**-induced cytokine production by preventing IκBα activation**. (A) TRFs were mock or SVV.eGFP infected (5:1 ratio) and stimulated with 100 ng/ml RhTNFα at 42 h p.i. for the indicated times. TNFα-induced RANTES mRNA expression was measured by qPCR using specific primers. The data were normalized to GAPDH mRNA expression in each sample and are shown as relative fold changes. Shown are the means and standard deviations of the results of two independent experiments with three replicates per sample in each experiment. (B) TRFs were mock or SVV.eGFP infected (10:1 ratio) for 48 h and stimulated with 100 ng/ml RhTNFα for 45 min. Nuclear localization of the NFκB subunit p65 was analyzed by immunofluorescence microscopy using a specific antibody (red). SVV.eGFP-infected cells appear green. (C) TRFs were mock or SVV.eGFP infected (5:1 ratio) for 48 h and stimulated with 100 ng/ml RhTNFα for the indicated times. Lysates of the cells were analyzed for IκBα and phosphorylated IκBα by SDS-PAGE and Western blotting using specific antibodies. ORF31 expression was analyzed to confirm SVV infection, and GAPDH was used as a protein-loading control. The results of one representative experiment out of three independent experiments are shown.

2.2.3 SVV ORF61 inhibits NFκB activation by preventing the degradation but not phosphorylation of IκBα

The inhibition of NFκB activation observed in VZV-infected cells has been attributed to the ORF61 protein [241]. VZV and SVV ORF61 share 42.8% overall amino acid identity [505], and both proteins are implicated in the transactivation of expression of other viral genes [497, 517]. To assess whether the ORF61 protein was responsible for the inhibition of NFκB signaling in SVV-infected cells we used recombinant adenovectors to ectopically express the protein. TRFs were transduced with an adenovector encoding SVV ORF61 (AdORF61 or AdFL-ORF61 in which ORF61 is N-terminally tagged with FLAG). Expression of the gene is dependent on the tetracycline-regulated transactivator, which is provided by co-transducing with AdTA. As a control we used TRFs transduced with AdORF61 or AdFL-ORF61 only. At 48 hours p.i. we studied TNFα-induced RANTES expression by qPCR. In the control cells we observed a time dependent increase in RANTES expression, but when ORF61 was expressed this response was strongly reduced (Fig 8A). Similar results were obtained for TRFs expressing the FLAG-tagged ORF61. Thus, SVV ORF61 inhibits TNFα-induced NFκB-signaling. Next, we determined whether ORF61 inhibits the nuclear translocation of NFκB as observed in SVV-infected cells. In TRFs transduced with AdFL-ORF61 alone, treatment with TNFα for 45 minutes resulted in the nuclear import of the NFκB subunit p65 (Fig 8B). In contrast, in TRFs that expressed ORF61 through co-transduction with AdTA, nuclear accumulation of p65 was not observed (Fig 8B). FLAG-ORF61 expression was confirmed by staining with a FLAG-specific antibody (Fig 8B). To study if this block in nuclear translocation of NFκB resulted from inhibited IκBα activation, we

stimulated FLAG-ORF61-expressing cells with TNFα for the indicated time points. In the absence of ORF61 expression, IκBα was phosphorylated after 5 minutes, degraded after 15 minutes and reappeared after 60 minutes of treatment with TNFα (Fig 8C). Conversely, IκBα degradation was not induced in the presence of ORF61 (Fig 8C) suggesting that ORF61 inhibited NFκB by preventing IκBα degradation. Interestingly, ORF61 did not affect cytokine-induced phosphorylation of IκBα (Fig 8C). Thus ORF61 appears not to be responsible for the inhibition of IκBα-phosphorylation observed in SVV-infected cells but rather inhibits IκBα degradation at a step that follows phosphorylation.

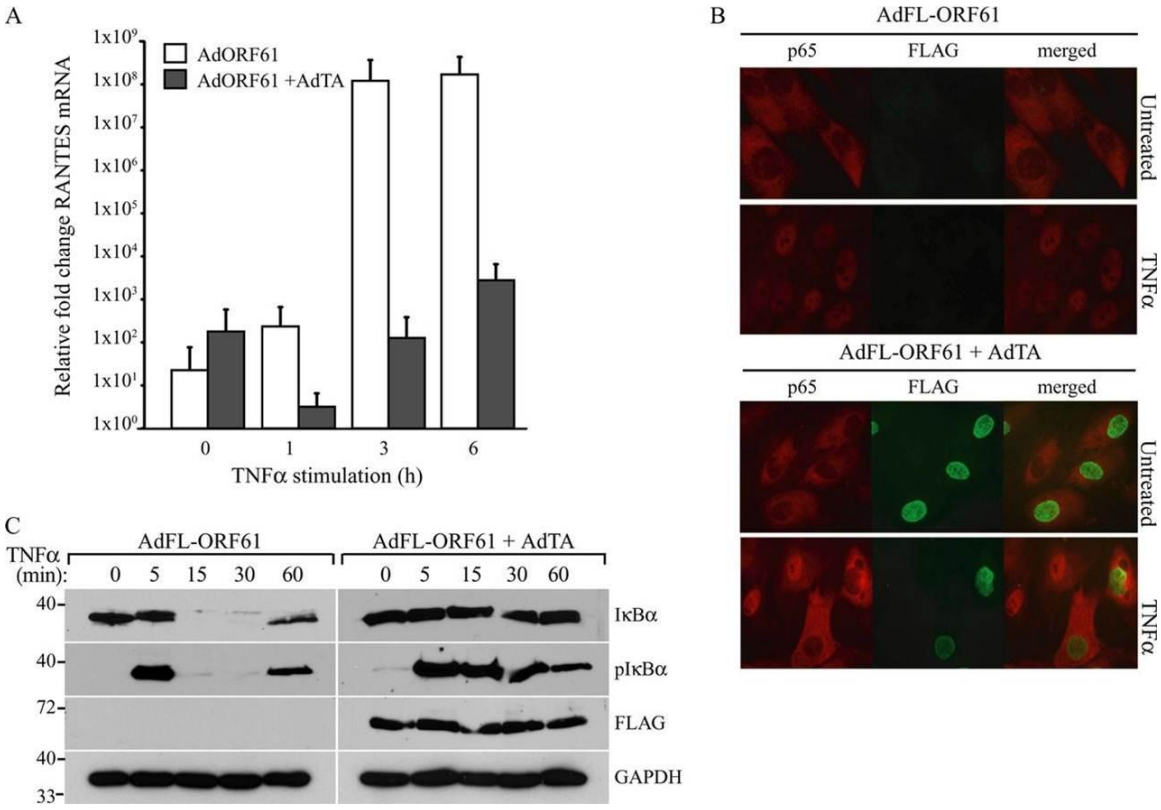

FIG 8 SVV ORF61 inhibits NFκB**-induced cytokine production by preventing IκBα degradation.** TRFs were coinfected with a recombinant adenovirus expressing SVV ORF61 (AdORF61) or FLAG-tagged ORF61 (AdFL-ORF61) at an MOI of 15 and an adenovirus expressing the tetracycline transactivator (AdTA) at an MOI of 7. TRFs infected with AdORF61 or AdFL-ORF61 only were used as a control. (A) TRFs were infected with AdORF61 only or with AdORF61 and AdTA and stimulated with 100 ng/ml RhTNFα for the indicated times at 48 h p.i. TNFα-induced RANTES mRNA expression was measured by reverse transcription-PCR and qPCR using specific primers. The data were normalized to the level of GAPDH mRNA expression measured in each sample and are shown as the relative fold change. Shown are the means and standard deviations of the results of two independent experiments with three replicates per sample in each experiment. (B) TRFs infected with AdFL-ORF61 (MOI, 15) only or AdFL-ORF61 (MOI, 15) and AdTA (MOI, 7) for 48 h were incubated with 100 ng/ml RhTNFα for 45 min, after which nuclear localization of the NFκB subunit p65 was analyzed by immunofluorescence microscopy using a specific antibody (red). The cells were stained with a FLAG-specific antibody to visualize FLAG-ORF61 expression (green). (C) TRFs infected with AdFL-ORF61 (MOI, 15) only or AdFL-ORF61 (MOI, 15) and AdTA (MOI, 7) for 48 h were incubated with 100 ng/ml RhTNFα for the indicated times. Lysates of the cells were analyzed for IκBα and phosphorylated IκBα by SDS-PAGE and Western blotting using the indicated antibodies. ORF61 expression was confirmed using a FLAG-specific antibody, and GAPDH was used as a protein-loading control. The results from one out of three independent experiments are shown.

2.2.4 SVV ORF61 inhibits TNFα-induced ubiquitination of IκBα by targeting β-TrCP

Upon being phosphorylated, IκBα is recognized by the F-box protein β-TrCP, which is associated with the proteins Skp1, Cul1, and the Ring protein Roc1/Rbx1, together forming the E3 ligase complex $SCF^{\beta\text{-}TrCP}$. $SCF^{\beta\text{-}TrCP}$ adds polyubiquitin chains to IκBα, which leads to the proteasomal degradation of IκBα and the subsequent release exposure of NLS of the NFκB subunits to the nucleus [206]. The results described above suggest that ORF61 inhibits NFκB signaling by preventing IκBα degradation post-phosphorylation. Conceivably, ORF61 could either interfere with the ubiquitination of IκBα or prevent the degradation of the ubiquitinated protein by the proteasome. To determine if IκBα ubiquitination is affected in ORF61-expressing cells, we used agarose-conjugated Tandem-repeated Ubiquitin Binding Entities (TUBEs) to isolate poly-ubiquitinated forms of IκBα [522]. TRFs were co-transduced with AdORF61 and AdTA and at 44 hours p.i. the cells were treated with MG132 for 3 hours to block the proteasome. The cells were subsequently stimulated with TNFα for 1 hour to initiate NFκB activation. Lysates of the cells were incubated with TUBEs and the resulting complexes were analyzed for the presence of IκBα by Western blot. In stimulated control cells (AdORF61 only) higher molecular weight forms of IκBα were detected corresponding to the poly-ubiquitinated form of the protein (Fig. 9A, lane 2). In contrast, ubiquitinated forms of IκBα were almost undetectable in ORF61-expressing cells treated with TNFα (Fig. 9A, lane 4). These results imply that ORF61 blocks the $SCF^{\beta\text{-}TrCP}$-mediated addition of ubiquitin chains to IκBα, thereby interfering with the degradation of the protein.

To determine whether ORF61 generally interferes with $SCF^{\beta\text{-}TrCP}$ function we examined the turnover of Snail, a well-known target of this ubiquitin ligase complex. Snail is phosphorylated by glycogen synthase kinase-3β (GSK-3β), creating the recognition motif or degron for β-TrCP [523]. GSK-3β is constitutively active in resting cells, therefore Snail is continuously ubiquitinated by $SCF^{\beta\text{-}TrCP}$ and subsequently degraded by the proteasome, resulting in a very short half-life [523]. TRFs were transduced with AdFL-ORF61 in the absence or presence of AdTA. As an additional control we used cells co-transduced with an adenovirus expressing GFP in a transactivator-dependent manner (AdGFP) and AdTA. At 48 hours p.i. low levels of Snail were detected in TRFs infected with AdFL-ORF61 only or with AdGFP/AdTA (Fig. 9B). In contrast, when FLAG-ORF61 was expressed we observed an accumulation of Snail, indicating that degradation of the protein by the $SCF^{\beta\text{-}TrCP}$ complex was inhibited by ORF61 (Fig. 9B). Therefore we conclude that ORF61 affects multiple $SCF^{\beta\text{-}TrCP}$ target proteins in addition to IκBα.

To assess whether inhibition of SCF-mediated ubiquitin-ligation involved a direct interaction between β-TrCP and ORF61 we performed co-immunoprecipitation experiments. As a viral control protein known to interact with β-TrCP we included the vaccinia virus (VACV) protein A49 previously reported to block the $SCF^{\beta\text{-}TrCP}$-mediated degradation of IκBα by interacting with β-TrCP [524]. We co-transfected HEK 293T cells with expression plasmids encoding HA-tagged β-TrCP and FLAG-tagged VACV A49 (FL-A49), FLAG-ORF61, or FLAG-tagged SVV ORF63 (FL-ORF63) as a negative control. Cells were harvested 48 hours post-transfection and the viral proteins were isolated by immunoprecipitation with a FLAG-specific antibody. β-TrCP was detected by

Western blot using an HA-antibody in immunoprecipitates of FL-A49 and FL-ORF61, but not FL-ORF63 (Fig. 9C). Therefore we conclude that, similar to the poxvirus protein A49, ORF61 specifically forms a complex with β-TrCP thereby preventing $SCF^{\beta\text{-}TrCP}$-mediated ubiquitination of target proteins such as IκBα and Snail.

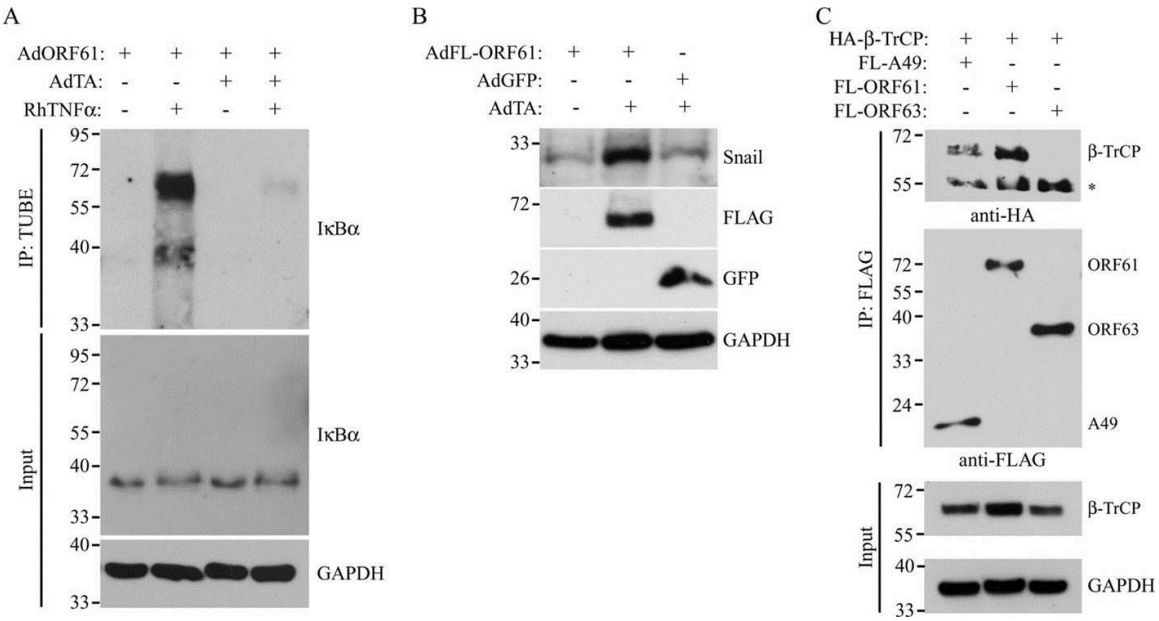

FIG 9 SVV ORF61 interferes with ubiquitination by SCF$^{\beta TrCP}$. (A) TRFs were infected with AdORF61 (MOI, 15) only or with AdORF61 (MOI, 15) and AdTA (MOI, 7). At 44 h p.i., the TRFs were incubated with 50 μM MG132 for 3 h, followed by stimulation with 100 ng/ml RhTNFα for 1 h. Agarose-conjugated TUBEs were used for the immunoprecipitation (IP) of ubiquitinated IκBα. Whole lysates and the immunoprecipitated complexes were then analyzed by SDS-PAGE and Western blotting using an IκBα-specific antibody. Input lysates were analyzed for GAPDH as a protein-loading control. ORF61 expression was confirmed using reverse transcription-PCR and PCR with ORF61-specific primers (data not shown). (B) TRFs were infected with AdORF61 (MOI, 15) only, with AdORF61 (MOI, 15) and AdTA (MOI, 7), or with AdGFP (MOI, 15) and AdTA (MOI, 7). At 48 h p.i., whole-cell lysates were analyzed for Snail, FLAG, and GFP expression by SDS-PAGE and Western blotting using specific antibodies. GAPDH was used as a protein-loading control. (C) HEK 293T cells were cotransfected with HA-tagged βTrCP and FL-A49, FL-ORF61, or FL-ORF63. At 48 posttransfection, the cells were lysed, and viral proteins were immunoprecipitated using a FLAG-specific antibody. The input lysates and immunoprecipitated complexes were analyzed for the presence of HA–βTrCP by SDS-PAGE and Western blotting using a specific antibody. Viral protein expression was confirmed in the input lysates using the FLAG-specific antibody, and GAPDH was used as a loading control. Shown are the results of one representative experiment out of three independent experiments.

2.2.5 The RING domain of SVV ORF61 is necessary for IκBα inhibition

The RING domain of VZV ORF61 has E3 ubiquitin ligase activity *in vitro* [495, 496]. This domain was found to be essential for the degradation of phosphorylated IRF3 [434] and for the inhibition of TNFα-induced NFκB-signaling [241]. Mutation of the cysteine at position 19 to a glycine residue (C19G) was shown to disrupt the RING domain of ORF61 and the protein's E3 ubiquitin ligase activity [493, 496]. We introduced this mutation in the SVV ORF61-expressing adenovirus (AdFL-ORF61 C19G) and studied whether this protein was still able to inhibit NFκB signaling. TRFs were co-transduced with AdTA alone, AdTA and AdGFP, AdFL-ORF61, or AdFL-ORF61 C19G for 48 hours followed by stimulation with TNFα for 6 hours to induce cytokine expression. Cells transduced with AdTA alone or AdTA/AdGFP showed RANTES expression upon TNFα stimulation, which was inhibited in AdTA/AdFL-ORF61-infected cells (Fig. 10A). In contrast, RANTES induction was not inhibited in ORF61 C19G-expressing cells (Fig. 10A) despite comparable expression levels of ORF61 and ORF61 C19G (Fig. 10B). We also analyzed IκBα degradation in these cells using Western blot. As shown in Fig. 10B, GFP- expressing cells displayed diminished IκBα levels after 20 minutes and restored IκBα levels after 60 minutes of treatment with TNFα. As observed in Fig. 8C, ORF61 expression stabilized IκBα, but this was not observed for ORF61 C19G (Fig. 10B). These data indicate that the inhibition of the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase complex by SVV ORF61 requires an intact RING domain and thus a functional ubiquitin ligase activity of ORF61.

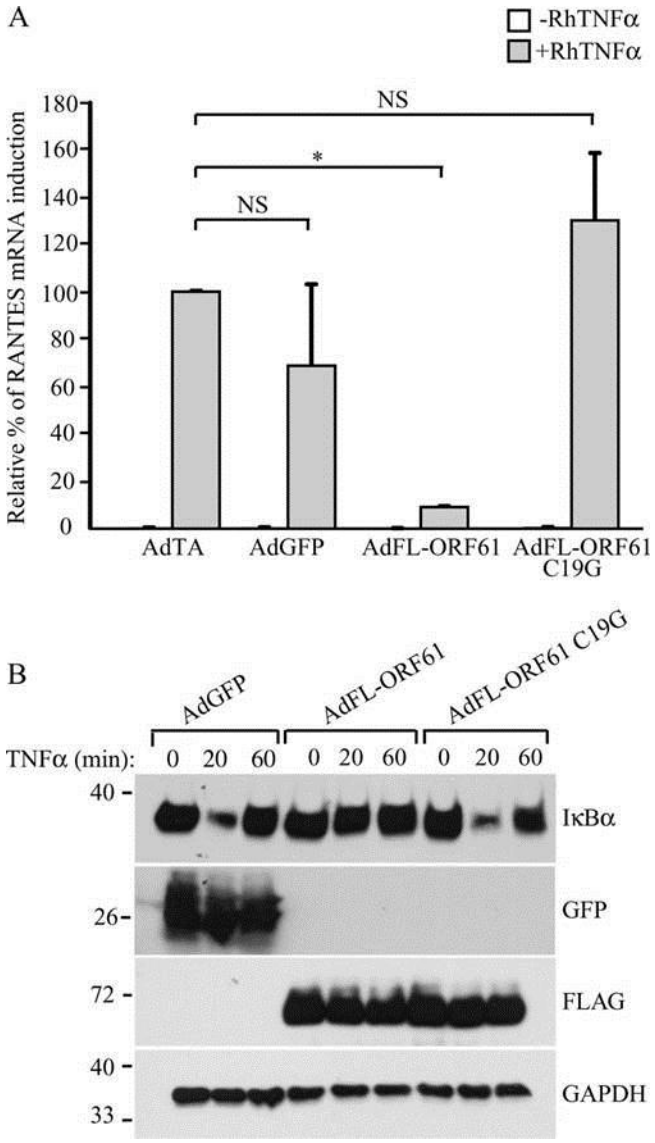

FIG 10 Inhibition of NFκB **signaling by SVV ORF61 is RING domain dependent**.
TRFs were infected with AdTA (MOI, 7) or coinfected with AdTA and AdGFP, AdFL-ORF61, or AdFL-ORF61 C19G (MOI, 15) for 48 h. (A) At 42 h p.i., the cells were stimulated with 100 ng/ml RhTNFα for 6 h. RANTES mRNA expression was measured by reverse transcription-PCR and qPCR using specific primers. The data were normalized to the level of GAPDH mRNA expression measured in each sample, and the relative fold change of RANTES was determined. The graph shows relative fold changes normalized to the induction in the control cells (AdTA only). Shown are the means and standard deviations of the results of two independent experiments with three replicates per sample in each experiment. NS, not significant; *, $P<0.05$. (B) At 48 h p.i.,the TRFs were incubated with 100 ng/ml RhTNFα for the indicated times.
Whole lysates of the cells were analyzed for IκBα, ORF61, and GFP expression by SDS-PAGE and Western blotting using specific antibodies. GAPDH was used as a protein-loading control. The results from one out of three independent experiments are shown.

2.2.6 ORF61 deletion does not restore NFκB-signaling in SVV-infected cells

To assess the contribution of ORF61 to the inhibition of NFκB-signaling by SVV we deleted ORF61 by BAC mutagenesis and SVVΔ61 was recovered. PCR analysis of DNA isolated from TRFs that were either mock-infected or infected with wild type (wt) SVV or SVVΔ61 confirmed deletion of the ORF61 gene (Fig. 11A). To address if phosphorylation and turnover of IκBα was differentially affected in the absence of ORF61, we stimulated the mock-, wt-, and SVVΔ61-infected cells with TNFα for the indicated times and monitored IκBα and pIκBα levels by Western blot. Mock-infected cells displayed diminished levels of IκBα after 20 minutes of TNFα stimulation and newly synthesized IκBα appeared after 60 minutes (Fig. 11B). In contrast, IκBα was not degraded in both wt- and SVVΔ61-infected cells and phosphorylation of IκBα was only observed in mock-infected cells (Fig. 11B). Since phosphorylation of IκBα was not affected by ORF61 this result is consistent with a separate viral inhibitory mechanism acting upstream of ORF61 to prevent IκBα degradation by reducing phosphorylation. Thus, SVV seems to encode multiple mechanisms to inhibit NFκB activation and deletion of ORF61 alone does not restore IκBα degradation and release of NFκB.

Since the targeting of β-TrCP by ORF61 results in the accumulation of Snail we also compared Snail levels in cells infected with wt or ORF61-deleted SVV by Western blot. However, compared to mock-infected cells, Snail accumulated both in wt and SVVΔ61-infected cells (Fig. 11C). These data indicate that SVV interference with the degradation of Snail by the $SCF^{\beta\text{-}TrCP}$ complex is not limited to ORF61.

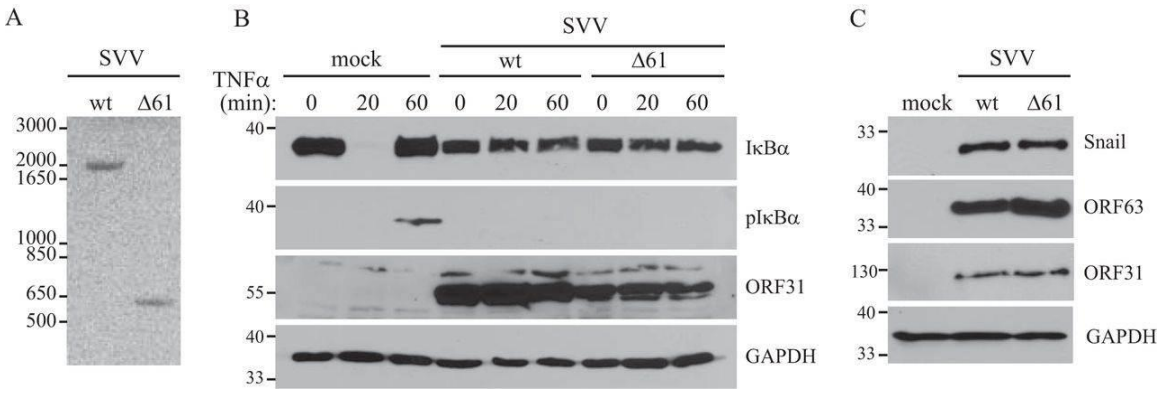

FIG 11 SVV ORF61 is not required for the inhibition of NFκB **signaling and Snail accumulation**. TRFs were mock infected or infected with SVV wt or an ORF61 deletion mutant (61) at a 5:1 ratio for 48 h. (A) PCR was performed on DNA extracted from the infected cells, and the presence of the ORF61 gene was studied using flanking primers. (B) At 48 h p.i., the cells were incubated with 100 ng/ml RhTNFα for the indicated times. Lysates of the cells were analyzed for IκBα and phosphorylated IκBα by SDS-PAGE and Western blotting using specific antibodies. Samples were stained for ORF31 to confirm SVV infection, and GAPDH was used as a protein-loading control. (C) Lysates of the infected cells were analyzed for Snail expression by SDS-PAGE and Western blotting using a specific antibody. Infection was confirmed using antibodies for ORF63 and ORF31, and GAPDH was used as a protein-loading control. The results of one representative experiment out of three independent experiments are shown.

2.2.7 VZV ORF61 blocks the ubiquitination of IκBα

Next we determined whether VZV blocks IκBα-activation by the same mechanism as SVV. MRC5 cells were either mock- or VZV.eGFP-infected and 48 hours p.i. the cells were stimulated with TNFα for the indicated times. IκBα was stabilized in VZV.eGFP-infected cells compared to mock-infected cells, but different from SVV, we did not observe an inhibition in IκBα-phosphorylation (Fig. 12A). To determine whether VZV ORF61 was able to inhibit IκBα degradation we stably expressed VZV ORF61 under the control of a tetracycline-inducible promotor in telomerized human fibroblasts (THFs). These fibroblasts express the reverse tetracycline Transcriptional Activator protein (rtTA) and incubation with doxycycline (Dox) induced ORF61 expression (Fig. 12B and C). The THF rtTA ORF61^{VZV} cells were incubated with 1 µg/ml Dox and after 48 hours the cells were treated with TNFα for the indicated times. In the absence of Dox, IκBα was degraded after 20 minutes of cytokine treatment, while Dox-dependent induction of ORF61 prevented IκBα degradation (Fig. 12B). To control for the effects of Dox-induced protein overexpression we included a THF rtTA cell line that stably expressed the GAG protein of Simian Immunodeficiency (THF rtTA GAG). We did not observe increased IκBα-stability in these cells, indicating that this was specifically induced by ORF61. Using the VZV ORF61expressing cells we further examined whether ORF61 inhibited ubiquitination of IκBα. THF rtTA ORF61^{VZV} cells were incubated with Dox for 48 hours and during the last 4 hours MG132 was added to the cultures to inhibit the proteasome. Upon stimulation of control cells with TNFα for 1 hour, poly-ubiquitinated IκBα was captured by TUBEs and visualized by Western blot (Fig. 12C). In contrast, poly-ubiquitinated IκBα was not detected in ORF61-expressing cells. Together,

these data indicate that, similar to SVV ORF61, VZV ORF61 inhibits ubiquitination of IκBα.

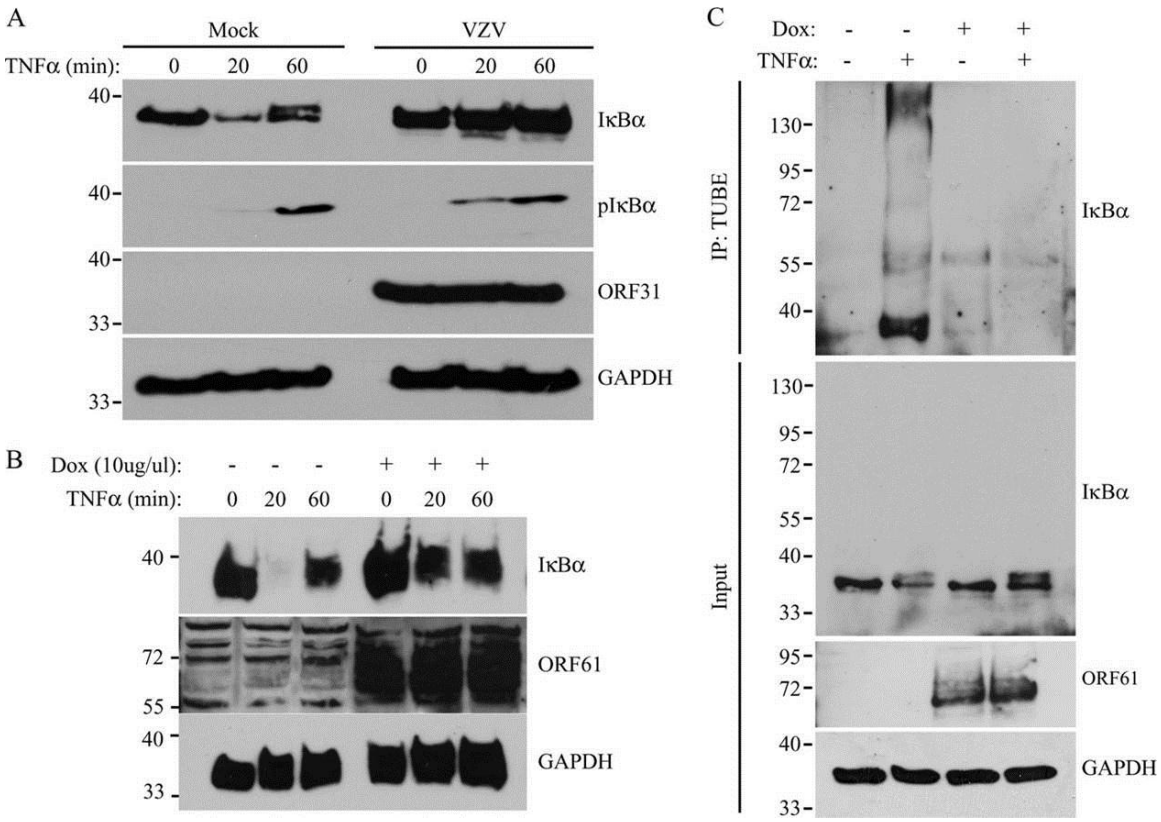

FIG 12 VZV ORF61 inhibits the ubiquitination of IκBα. (A) MRC5 cells were mock or VZV infected for 48 h at a 5:1 ratio and stimulated with 100 ng/ml HuTNFα at the indicated times. Lysates of the cells were analyzed for (phosphorylated) IκBα expression by SDS-PAGE and Western blotting using specific antibodies. The lysates were stained for ORF31 expression to control for infection, and GAPDH was used as a loading control. (B) THF rtTA ORF61VZV were incubated with 1 μg/ml Dox for 48 h, after which they were stimulated with 100 ng/ml HuTNFα to activate NFκB signaling. Lysates from the cells were stained for IκBα, ORF61, and GAPDH using specific antibodies in SDS-PAGE and Western blotting. (C) THF rtTA ORF61VZV were incubated with 1 μg/ml Dox for 44h and incubated in 50 μM MG132 for 3 h, followed by stimulation with 100 ng/ml RhTNFα for 1 h. Polyubiquitinated IκBα was immunoprecipitated from the lysates using TUBEs. Whole lysates and immunoprecipitated complexes were analyzed by SDS-PAGE and Western blotting using an IκBα-specific antibody. Input lysates were analyzed for ORF61 expression using a specific antibody, and GAPDH was used as a protein-loading control. Shown are the results of one representative experiment out of three independent experiments.

2.3 Discussion

The results presented in this study suggest that SVV inhibits NFκB activation by sequentially inhibiting phosphorylation and degradation of IκBα thus retaining the NFκB subunits p50 and p65 in the cytosol preventing transcription of antiviral genes. Our data indicate that SVV ORF61 is responsible for the stabilization of IκBα downstream of phosphorylation. The viral protein interacts with β-TrCP and this interaction likely prevents the ubiquitination and subsequent degradation of IκBα by the $SCF^{\beta\text{-TrCP}}$ complex. β-TrCP facilitates the ubiquitination of several target genes, including Snail. We found increased levels of Snail in SVV ORF61 expressing cells, indicating that ORF61 broadly impedes protein degradation mediated by β-TrCP. We further show that the RING domain of SVV ORF61 is critical for its ability to prevent IκBα degradation suggesting that the ubiquitin ligase activity of ORF61 might be involved in inhibiting ubiquitination of IκBα. Similar to SVV ORF61, expression of VZV ORF61 inhibited ubiquitination of IκBα indicating that the ORF61 proteins of the two viruses prevent NFκB activation by a similar mechanism.

Deletion of ORF61 from SVV did not restore NFκB signaling consistent with SVV encoding at least one additional protein that inhibits NFκB-activation upstream of ORF61 most likely at the level of IκBα phosphorylation. Phosphorylation of IκBα by the IKK-complex is required for its ubiquitination and degradation by the $SCF^{\beta\text{-TrCP}}$ complex [206, 514]. Infection with SVV resulted in low levels of phosphorylated IκBα and treatment with TNFα did not increase phosphorylation. Since SVV ORF61 alone did not block the phosphorylation of IκBα and phosphorylation was still observed in SVVΔ61 infected cells we concluded that an unknown SVV protein interferes with

phosphorylation followed by ORF61 preventing ubiquitination of IκBα in a sequential process. The sequential inhibition of IκBα degradation thus underscores the importance of NFκB-mediated innate immunity in controlling varicella viruses.

VZV-infection also leads to the stabilization of IκBα (Fig. 12A), confirming previous observations in VZV-infected dendritic cells [241] and fibroblasts [240]. Moreover, we demonstrate that isolated expression of VZV ORF61 prevents ubiquitination of IκBα similar to SVV ORF61. However, unlike SVV, we observed that TNFα-induced phosphorylation of IκBα was not inhibited in VZV-infected cells. This observation is consistent with a publication by Jones et al. that reported IκBα phosphorylation in VZV-infected cells [240]. These results suggest both overlapping and divergent mechanisms by which SVV and VZV target the NFκB pathway, a somewhat unexpected finding given the high homology between SVV and VZV [399].

At present the mechanism by which SVV blocks IκBα phosphorylation is unknown. We have not yet explored whether only TNFα-induced IκBα phosphorylation is inhibited or whether SVV prevents IκBα phosphorylation regardless of the stimulus. One possibility is that SVV targets the IKK complex to inhibit NFκB signaling, a strategy commonly used by viruses. For example, the vaccinia virus (VACV) protein B14 and the hepatitis C virus protein NS5B inhibit IKK-mediated phosphorylation of IκBα by directly interacting with IKKβ or IKKα, respectively [525-527]. In addition, the adenovirus protein E1A was shown to prevent UV-initiated IKKβ activation, but did not affect expression levels of the protein [528, 529]. In contrast, the herpes simplex virus 1 protein ICP27 prevents IκBα phosphorylation by binding to the protein IκBα itself [530]. The homolog of ICP27 in VZV is the ORF4 protein [531, 532]. SVV has an ORF4 homolog

[505] and, in addition, encodes ORFA, which is a 293 amino acid long truncated version of ORF4 that is not found in VZV [404, 533]. It is therefore possible that ORFA is involved in the inhibition of IκBα phosphorylation that was uniquely observed in SVV-infected cells.

SVV ORF61 was shown to prevent the TNFα-induced ubiquitination of IκBα. Typically, IκBα is phosphorylated by the activated IKK complex, creating a docking motif for β-TrCP to interact with IκBα and catalyze its ubiquitination [206, 514]. We demonstrate that SVV ORF61 interacts with β-TrCP. In VZV-infected cells ORF61 localizes predominantly to the nucleus [534]. Fractionation studies revealed that SVV ORF61 in AdFL-ORF61-transduced cells is also mostly nuclear, though small amounts of protein were detected in the cytoplasm (data not shown). This raises the question of how a nuclear protein can affect a mostly cytosolic pathway. NFκB-IκBα complexes shuttle have been shown to shuttle between the nucleus and the cytoplasm [215]. The nuclear complexes can be activated by TNFα resulting in the degradation of IκBα by proteasomes residing in the nucleus [207, 215]. Correspondingly, β-TrCP has been reported to localize to the nucleus through an interaction with heterogeneous nuclear ribonucleoprotein hnRNP-U [223]. We speculate that ORF61 interacts with nuclear β-TrCP, thereby preventing both nuclear IκBα degradation and preventing β-TrCP from translocating to the cytosol to act on cytoplasmic IκBα. Additionally, there is a small cytoplasmic fraction of ORF61 that could target cytoplasmic β-TrCP directly.

Amino acids 366-371 of ORF61 represent a motif (LSGPIKS) that is highly similar to the phosphodegron motifs found in β-TrCP substrates (DSGΦXS, Φ indicates hydrophobic amino acid) [206, 514]. Comparable phosphodegron-like (PDL) motifs were

found in viral proteins that target the NFκB pathway by interfering with the β-TrCP function. The Epstein-Barr virus (EBV) latent membrane protein (LMP) 1 and the VACV A49 protein were shown to block the degradation of IκBα by binding to β-TrCP [524, 535]. Mutagenesis analysis demonstrated that the PDL motifs both proteins were required for the inhibition in NFκB activation [524, 535]. Additionally, the NSP1 protein of the porcine rotavirus (RV) strain OSU was demonstrated to stabilize activated IκBα by binding to and degrading β-TrCP [536]. Morelli et al. showed that the N-terminal RING domain and the C-terminal PDL motif of OSU NSP1 were required for the inhibition of NFκB signaling [537]. We show that ORF61-mediated inhibition of NFκB activation requires an intact RING domain (Fig. 10). However, SVV ORF61 did not reduce the levels of β-TrCP expressed in HEK 293T cells (Fig. 9C) suggesting that the ubiquitin ligase function of ORF61 is not required to mediate degradation of β-TrCP. Interestingly, a comparison of many different human and porcine RV strains with respect to their capacity to inhibit and degrade β-TrCP revealed that while all NSP1 homologs that encode a PDL motif were able to inhibit NFκB activation, not all affected the expression levels of β-TrCP [537]. This indicates that β-TrCP degradation was not required for RV NSP1 to inhibit the SCF complex, suggesting that it is the interaction between NSP1 and β-TrCP that disrupts the proteins ability to ubiquitinate IκBα. Similarly, the RING domain of ORF61 might be required for a stable interaction. Alternatively, SVV ORF61 might mediate the degradation of other members of the $SCF^{\beta\text{-}TrCP}$ complex.

Our data demonstrate that VZV ORF61 also interferes with the ubiquitination of IκBα, thereby stabilizing the protein. In contrast to SVV ORF61 however, the VZV protein does not have an obvious PDL motif. Thus, it is possible that VZV ORF61

prevents IκBα ubiquitination and degradation via a different strategy than SVV ORF61. Alternatively, VZV ORF61 might associate with the $SCF^{\beta\text{-}TrCP}$ complex via a different motif.

The $SCF^{\beta\text{-}TrCP}$ complex is involved in the degradation of multiple host proteins, including β-Catenin, Snail [514] and p105 [538]. Since ectopic expression of SVV ORF61 resulted in the stabilization of Snail (Fig. 9B) we concluded that the inhibition of β-TrCP affects other $SCF^{\beta\text{-}TrCP}$-substrates as well. The human immunodeficiency virus (HIV) 1 Vpu also interacts with β-TrCP via its PDL motif [539]. This interaction prevents TNFα- and virus-induced degradation of IκBα [540]. Like SVV ORF61, Vpu was shown to affect other β-TrCP targets as well [541]. In addition, Vpu utilizes the E3 ubiquitin ligase activity of the $SCF^{\beta\text{-}TrCP}$ complex to degrade the anti-viral factor tetherin and CD4 [539, 542]. Mutagenesis studies showed that tetherin degradation is dependent on the interaction between Vpu and β-TrCP [539]. It would be interesting to study whether ORF61 redirects the $SCF^{\beta\text{-}TrCP}$ complex and regulates the stability of other endogenous or viral proteins.

Unexpectedly, deletion of ORF61 from the viral genome did not result in restored degradation of Snail (Fig. 11C). $SCF^{\beta\text{-}TrCP}$ complex-mediated degradation of Snail is dependent on phosphorylation of its phosphodegron motif by glycogen synthase kinase (GSK) 3β [543]. Interestingly, GSK-3β is also involved in TNFα-, LPS-, or IL1-β-induced NFκB-activation [544] and Takada et al. showed that treatment of $GSK\text{-}3\beta^{-/-}$ mouse fibroblasts with TNFα did not result in IKK-activation [544]. Since we observed that SVV inhibits IκBα phosphorylation independent of ORF61 it is conceivable that SVV interferes with GSK-3β function, thereby preventing IKK-activation and Snail

degradation, even in the absence of ORF61, which would explain Snail accumulation in SVVΔ61-infected cells. Interestingly, Liu et al. showed that VZV ORF12 drives the activation of GSK-3β by the phosphatidylinositol 3-kinase/Akt pathway in infected cells [545]. The authors speculate that this process is required for entry of the VZV into the cells and for protection from apoptosis since ORF12 is a tegument protein. It is thus possible that SVV activates GSK-3β via ORF12 immediately following infection to aid efficient replication and subsequently inhibits phosphorylation of GSK-3β through the unidentified protein to prevent NFkB-activation in the infected cells.

In conclusion, we have shown that SVV inhibits NFκB-driven protein expression via at least two sequentially operating strategies (Fig 13). We have established that SVV ORF61 interferes with the ubiquitination of IκBα by binding to β-TrCP. This interaction is likely dependent on the PDL motif that is present in the SVV ORF61 sequence. SVV also prevents the phosphorylation of IκBα, although the mechanism of this inhibition is presently unknown. It is common for viruses to inhibit signaling pathways at multiple levels. For example, IRF3-driven cytokine expression is inhibited by at least three different VZV proteins, including ORF61 [434], ORF47 [436] and IE62 [435]. Such sequential inhibitory mechanisms along a signal transduction pathway likely serve as fail-safe strategies to efficiently block an immune response. The viral resources devoted to inhibiting a given pathway are likely directly proportional to the antiviral impact of the respective immune response pathway. Thus, ORF61 might be responsible for eliminating residual phosphorylated IκBα that escaped the upstream inhibitory mechanism. Deletion of ORF61 alone thus does not restore NFκB-mediated innate immunity which might explain why SVV lacking ORF61 was still able to establish primary and latent infection

in rhesus macaques as reported by Meyer et al. [427]. However, ORF61-deleted SVV displayed reduced viral gene expression *in vivo*, which could either be related to the transactivator function of ORF61 or due to increased expression of antiviral genes that suppress viral gene expression. In addition, there was an increased frequency of plasmacytoid dendritic cells in the BAL and an increase in IFNβ gene expression in SVVΔORF61-infected animals [427]. Enhanced recruitment of dendritic cells and enhanced cytokine expression could be the direct result of increased NFκB-activity by infected cells due to lack of ORF61 inhibition or lack of ORF61 could indirectly affect innate immune responses by reduced transactivation of other viral genes that interfere with innate signaling pathways, such as IE62 [435] and ORF63 [432, 433]. If deletion of ORF61 together with the yet to be identified protein that inhibits IκBα phosphorylation restore NFκB activation it is to be expected that SVV infection will be severely attenuated by innate immunity while possibly maintaining or even improving the induction of SVV-specific adaptive immunity. Further delineation of NFκB-inhibitory pathways by SVV and VZV might thus lead to improved vaccine design.

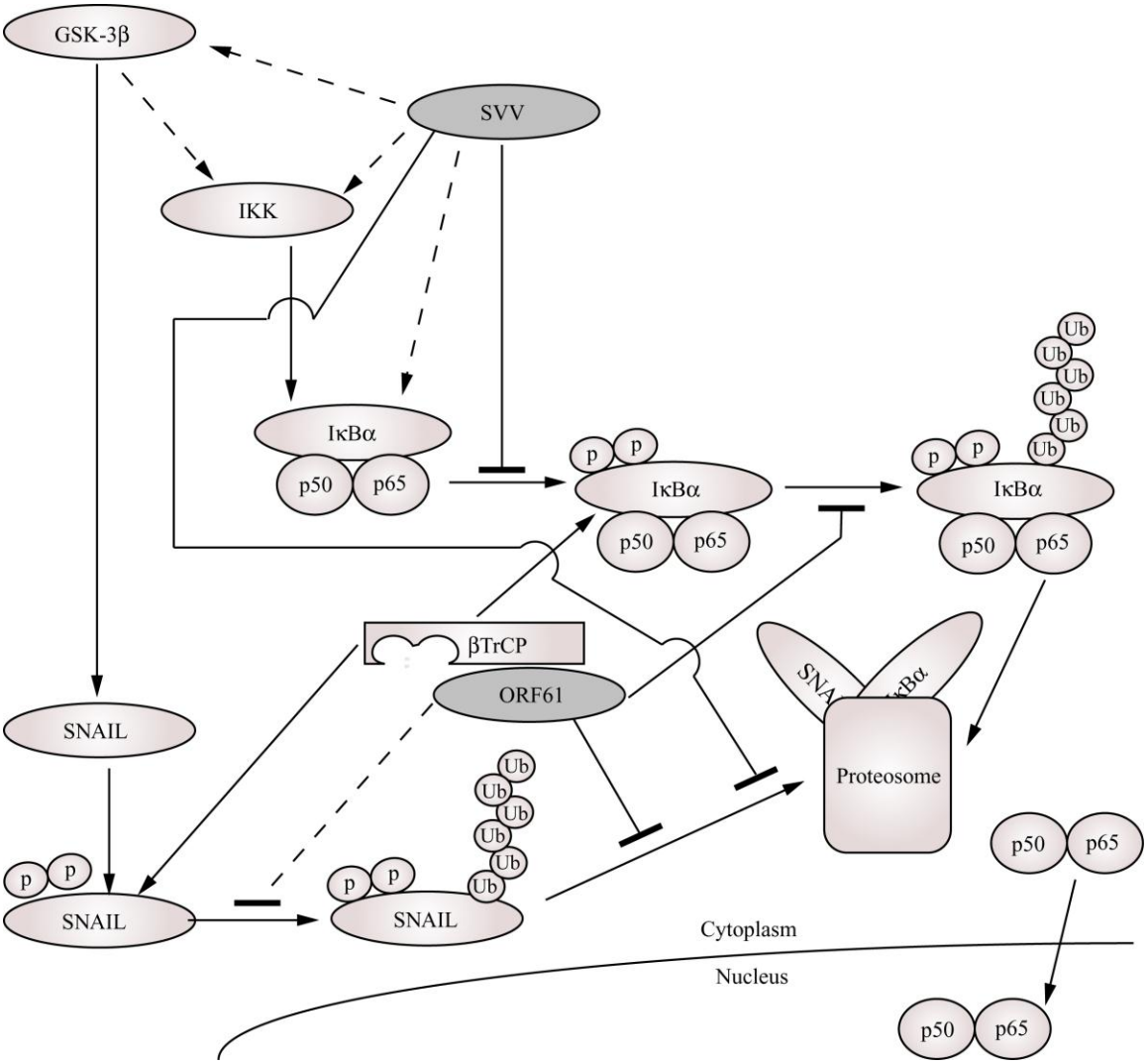

FIG 13. Proposed Model of SVV inhibition of NFκB activation. Key features are that SVV inhibits IκBα phosphorylation through a yet unknown process. SVV and VZV ORF61 limit the ubiquitination of IκBα post phosphorylation. SVV ORF61 was shown to interact with βTrCP and limit the degradation of Snail, another SCF-βTrCP substrate. Dashed lines represent unknown but possible points of interaction between the two proteins.

Chapter 3: Discussion and Future Directions:

3.1 ORF61 with Regards to Latency and Reactivation:

In addition to the multiple functions that ORF61 plays during acute infection it may also have an important role in the reactivation of the virus from latency. Reactivation of VZV, and of herpesviruses in general, is not a well-understood process. This stems from the fact that a robust latency/reactivation system has yet to be established for VZV due to the strict species specificity of the virus.

It was previously shown that corneal inoculation of mice with VZV led to spread of the virus to the nervous system where a non-productive latent infection was established and VZV mRNA could be detected up to 33 dpi [378]. It has also been documented that VZV infection of cotton rats, which have been used to study other human viruses that replicate poorly in other rodents, resulted in the detection of latent VZV DNA [546-548]. More recently, a mouse model that involves the implantation of fetal human dorsal root ganglion (DRG) xenografts into SCID mice was shown to be permissive for VZV infection [342]. This humanized mouse model showed robust replication of the virus in the human DRG during the first 14 days, which then resolved by 55 dpi, at which time infectious virus was no longer recoverable [549]. After 56 dpi neurons that persisted in the DRG showed signs of latent infection by of the presence of VZV genomes and limited viral transcripts [549]. Also no viral proteins were detected at this time [549]. However a major limitation of all these studies is that DRG reactivation of the virus was not documented. Also, the rodent models failed to produce clinical signs of VZV infection [378, 548]. The large cost, limited supply of fetal tissue, and technical

labor associated with the human DRG xenograft SCID mouse model should also be taken into consideration when deciding on the appropriate animal model.

The guinea pig has been used to examine VZV pathogenesis and recent reports have extended this model for the study of VZV latency [550, 551]. Ex-vivo, Chen et al. (2003) demonstrated that cell-free VZV was able to infect isolated enteric neurons from guinea pigs and the virus went latent expressing the same proteins seen in human ganglia latently infected with VZV (ORFs 4/21/29/40/62/63; [550, 552, 553]). In vivo, Gan et al. (2014) showed that IV injection of VZV infected T-cells lead to a latent infection of the enteric neurons [551]. However, like the mouse and rat models, natural reactivation was not documented. Also, the guinea pigs failed to develop any clinical signs or symptoms of VZV and it is not clear how well this model matches that of human VZV latency. However, it is also interesting that ex vivo reactivation of guinea pig enteric neurons was achieved through the ectopic expression of VZV ORF61 or HSV-1 ICP0 delivered via adenoviral vectors [554].

SVV infection of NHP has proven to be an invaluable resource to model VZV pathogenesis. Examining latent SVV infection has revealed key differences that distinguish SVV from VZV. To date no latency associated transcript (LAT) has been found for VZV and the major protein associated with latent VZV is ORF63 [552, 555]. In latent SVV infection of vervet monkeys, a LAT antisense to ORF61 is the predominant transcript along with expression of sense ORF61 and ORF21 [409]. SVV infection of RM led to the establishment of latent virus and revealed that again the most abundant transcript was that of a LAT antisense to ORF61 [408]. In this study the authors failed to find ORF21 but claimed that it could be due to the difference in monkey species used.

Reactivation of SVV in Cynomologus and African Green NHP has been shown following immunosuppression with tacrolimus, irradiation, or both, but a similar immunosuppressive regimen failed to reactivate SVV-infected RM [416, 417, 422]. This again may be due to the differences between NHP species or methods of initial SVV infection (natural vs intrabroncial). Reactivation of RM infected intrabroncially was recently achieved through stress with or without irradiation and an immunosuppression regimen [428]. SVV DNA was found in multiple cell types and monkeys developed a zoster-like rash mainly on the abdomen and axilla [428]. Abundant SVV DNA was detected in the lymph nodes primarily in activated macrophages and dendritic cells, and less so in CD3+ T-cells [428]. Traina-dorge et al. also demonstrated that in the absence of viremia, zoster skin lesions were still present suggesting anterograde transport from the axon to the skin [428]. One major difference between this study and the previous one that might explain the discrepancy in reactivation was the timing between the viral challenge and the immunosuppressive treatment. Traina-dorge et al.waited 5 months before starting immunosuppression whereas Meyer et al. waited 30 days [422, 428]. This new SVV RM reactivation model is an exciting break-through that, with future development and study, promises to yield valuable insight into herpesvirus latency. It would be of great interest to determine whether the SVVΔ61 virus is able to reactivate in this model.

Although a suitable model for SVV latency and reactivation is still in developmental stages it is clear that there are key differences in the transcriptional profiles between SVV and VZV during latency. The exact extent of VZV transcription and translation during latency is unknown. Examination of VZV latency transcriptome is

achieved through post-mortem obtained human ganglia. A few issues have arisen from the analysis of data collected in this manner. First, the tissue undergoes physiological changes (hypoxia) after death that may not recapitulate what is seen in an in vivo setting [556]. These postmortem changes might also influence the transcripts that are present. Second, the postmortem time it takes to obtain human ganglia seems to be a critical factor. A study demonstrated that there was a wide variability to the number and abundance of VZV transcripts detectable given longer times postmortem [557]. Therefore, the VZV latency transcriptome that has been described to date may be a result of postmortem events rather than what is seen during true in vivo latency. Another confounding factor is that the individuals that the tissue is derived from usually have died from other confounding factors (brain tumor, suicide, etc) which may or may not have some impact on VZV latency. Lastly, depending on the method of analysis different VZV transcripts have been found. cDNA sequence analysis has shown VZV ORFs 21, 29, 62, 63, and 66 to be present in infected human ganglia [558-560]. In situ hybridization has detected ORFs 4, 18, 21, 29, 40, 62, and 63 [552, 561, 562]. Multiplex RT-PCR of human ganglia (within 24 hours postmortem) detected ORFs 4, 11, 29, 40, 41, 43, 57, 62, 63, and 68 [563]. These different methods and the primer affinity for each VZV ORF vary and likely contribute to the differences between analyses. Analysis of the SVV transcriptome in latently infected RM mitigates many of the issues that are involved with the collection of human samples [564]. Latent SVV transcriptome analysis has revealed that SVV ORFs A, B, 4, 10, 61, 63, 64, 65, 66 and 68 can be detected during latency [408]. As mentioned before the most abundant transcript seems to be an antisense ORF61 LAT. Again, to date no VZV LAT has been described but the data

regarding the transcriptome of latent VZV is suggestive at best due to the inherent problems described above of examining postmortem human ganglia. In fact, Ouwendijk et al. did not find any VZV transcripts with postmortem times of less than 9 hours [557]. If a VZV LAT was present during these early times postmortem it could have easily been missed since Ouwendijk enriched for poly(A) RNA and examined VZV ORFs with multiplex primers which are not as specific for an individual ORF as individual primers are. Also, the HSV-1 LAT is not polyadenylated, and if that holds true for VZV then that it would have been excluded from their search [406]. It is possible that VZV differs from SVV and other neurotropic alpha-herpesviruses and does not contain LAT. It may regulate latency through an epigenetic strategy. Chromatin immunoprecipitation (ChIP) of latently infected MeWo revealed that the promoters of VZV ORF 62 and 63 were maintained in a euchromatic (H3K9Ac) state [565]. Euchromatic histone modifications also occur at the promoter region for the HSV-1 LAT [566, 567]. Histone modifications are also used by another human herpesvirus, EBV, to switch between different latency states [568-570]. Another major function of the HSV-1 LAT is to limit apoptosis in neuronal cells [571, 572]. Many of the properties of the HSV-1 LAT are also conserved in the BHV-1 LR gene. The BHV-1 LR gene is antisense to its ICP0 homolog and amply expressed during latency [573, 574]. BHV-1 LR also has anti-apoptotic functions [575]. It is possible that the anti-apoptotic property of the LAT is the more important function (rather than antisense regulation of viral ICP0 and ICP0 homologs). If this is true then it would match up well with that fact that the most prevalent VZV transcript found in latently infected human ganglia is to ORF63 which also has neuronal anti-apoptotic functions [558, 559, 563, 576, 577].

Despite what appears to be transcriptional differences, ORF61 may play a key role in both SVV and VZV. The major transcript of latent SVV is an antisense transcript of ORF61, which is similar to other neurotropic herpesviruses that express a LAT antisense transcript to their respective ICP0 homologs [578-580]. However, RM infected with SVV lacking ORF61 is still able to establish latency in sensory ganglia [427]. This has also been shown for VZV lacking ORF61 and for HSV-1 lacking ICP0 in a rodent model [548, 581]. These data suggest ORF61 is not necessary for the establishment of latency. Instead, ORF61 may be more important for virus reactivation. Expression of VZV ORF61 was sufficient to switch from a latent to a lytic replication cycle in guinea pigs [551]. Similarly, ICP0 expression from HSV-1 is necessary to induce efficient reactivation of HSV-1 from mouse derived models. [581, 582]. Another function of VZV ORF61 is to control the subcellular location of VZV ORF63, which is the major latent VZV protein. In latently infected cells, VZV ORF63 is sequestered in the cytoplasm, whereas during a lytic infection it can be found in the nucleus and cytoplasm [583]. The location of VZV ORF63 is important for viral replication since it modulates both viral and cellular promoters [583]. The expression of ORF61 allows for the nuclear location of ORF63 during reactivation [583]. These data suggest that ORF61 expression may be necessary for complete reactivation of VZV from latency. Recently, an in vitro VZV reactivation model based on low MOI VZV infection of human embryonic stem cell-derived neuron cells was published [340]. In this model, reactivation is achieved through the inhibition of PI3K by LY294002, a drug known to reactivate HSV-1 [340, 584]. It would be interesting to determine whether reactivation could be achieved in this model with a mutant VZV lacking ORF61. If ORF61 was indeed required for reactivation

it would be interesting to understand if the IFN and NFκB modulatory functions of ORF61 that we and others have demonstrated (which occur during acute replication) are also required for reactivation to occur [241, 242, 434]. Future work (detailed below) would be aimed to separate out ORF61's immune modulatory functions from its transactivation function to determine if one or both of these drives a successful reactivation event.

3.2 ORF61 Future Directions:

It is clear that many questions remain to be answered regarding the mechanisms used by varicella viruses to modulate NFκB signaling. One avenue for future work that builds on the data presented in this dissertation is further characterization of ORF61. It would be useful to determine the regions and key residues within ORF61 that allow interaction with other proteins, such as IκBα and βTrCP. We show in chapter 2 that SVV ORF61 binds to βTrCP (Fig 9C) and suggest that this interaction blocks βTrCP's ability to ubiquitinate its substrates. We propose that βTrCP interacts with ORF61 through a phosphodegron-like (PDL) motif (LSGPIKS) which is highly similar to the cognate DSGXXS motif which βTrCP normally binds. To investigate this we performed site-directed mutagenesis on ORF61 substituting each serine in the PDL motif for an alanine; individually or together. Using HEK 293T cells that stably express firefly luciferase under the control of an NFκB promoter and constitutively express *Renilla* luciferase (293 NFκB), we transfected wild-type ORF61, each of the single PDL mutants (S367A or S372A), or the double PDL mutant (S367A/S371A). After 42 hours, the cells were incubated with or without HuTNFα for 6 hours. NFκB activity was calculated as the ratio between induced firefly and constitutive *Renilla* luciferase expression. The latter was measured to control for cell death. Mock transfected cells show a clear increase in NFκB activation, while wt ORF61 actively blocked NFκB activation as expected (Fig 14). Much like wt ORF61 each of the PDL mutants (single and double) blocked NFκB activation as well (Fig 14). These preliminary data show that the PDL motif in ORF61 is not important for NFκB inhibition. Further characterization of these mutants need to be performed to determine if they retain the ability to interact with βTrCP. The results of

those experiments would be very interesting because if they fail to interact with βTrCP it would confirm that the PDL motif is the binding site for βTrCP. However, at the same time it would force us to reconsider our proposed mechanism for ORF61 NFκB inhibition since the PDL mutants are still able to inhibit NFκB activation without interaction with βTrCP. If the mutants are able to bind to βTrCP then it would suggest that ORF61 has a novel uncharacterized βTrCP binding site. To identify this site, truncation mutants would need to be made and tested for βTrCP interaction. Since the PDL mutants failed to restore NFκB activity it would also be informative to test the C19G RING mutant for βTrCP interactions since we have shown that this mutant restores NFκB activity. A new βTrCP interaction motif in ORF61 could have major implications and give insights into other unknown viral or cellular targets of βTrCP.

Multiple signaling pathways are controlled by the $SCF^{\beta TrCP}$ complex, and therefore the possibility remains that one of these other pathways, and not NFκB, is the true target of ORF61. This idea is supported by our data that other βTrCP targets (Snail) are also modulated in the presence of SVV ORF61. Since we have already shown Snail, a member of the Wnt signaling pathway, is affected it should be one of the first alternative pathways to be examined. The canonical Wnt/β-catenin pathway is critical for embryonic development and establishing tissue polarity [585]. Activation of the pathway leads to an increase in β-catenin which then translocates to the nucleus and will bind T-cell specific factor (TCF)/lymphoid enhancer binding factor 1 (LEF-1) to transactivate many genes involved in cell survival, maintenance, trafficking, and proliferation [586, 587]. Many of these functions would be important for a SVV/VZV infected cell. Interestingly the Wnt pathway is crucial for differentiation and development of neurons

and seems to provide an anti-apoptotic neuroprotective state [588-591]. This aspect of Wnt signaling would be extremely important for the maintenance of VZV infected neurons. βTrCP has also been implicated in the down regulation of the discs large tumor suppressor (hDlg) which plays a large role in the formation of epithelial cell-cell junctions [592, 593]. In isolated cells hDlg is rapidly degraded by the proteasome mediated through the $SCF^{\beta TrCP}$ complex [594, 595]. SVV/VZV may act to upregulate the amount of hDlg in order to promote polykaryocyte formation during the skin stage of infection. βTrCP has also been demonstrated to ubiquitinate and degrade the IFNAR1 subunit of the IFN receptor. ORF61 may interact with βTrCP to facilitate the degradation of the IFNR (analogous to HIV Vpu targeting tetherin for degradation) as yet another method to limit the effects of IFN [539, 596]. It would be very difficult to tease out the specific role NFκB has in controlling VZV infection. Mutation of the ORF61 amino acids that allow for the βTrCP interaction would have an effect on all βTrCP substrates and not just IκBα. Overexpression of βTrCP to out compete ORF61 would also have an effect on all βTrCP substrates. We could generate a cell line that expresses constitutively active forms of p50 and p65, however it has been documented that pretreatment of lung fibroblasts with TNFα (NFκB activator and target gene) blocks the replication of VZV, suggesting that NFκB (or another TNFα dependent pathway) is important for controlling viral replication [597, 598].

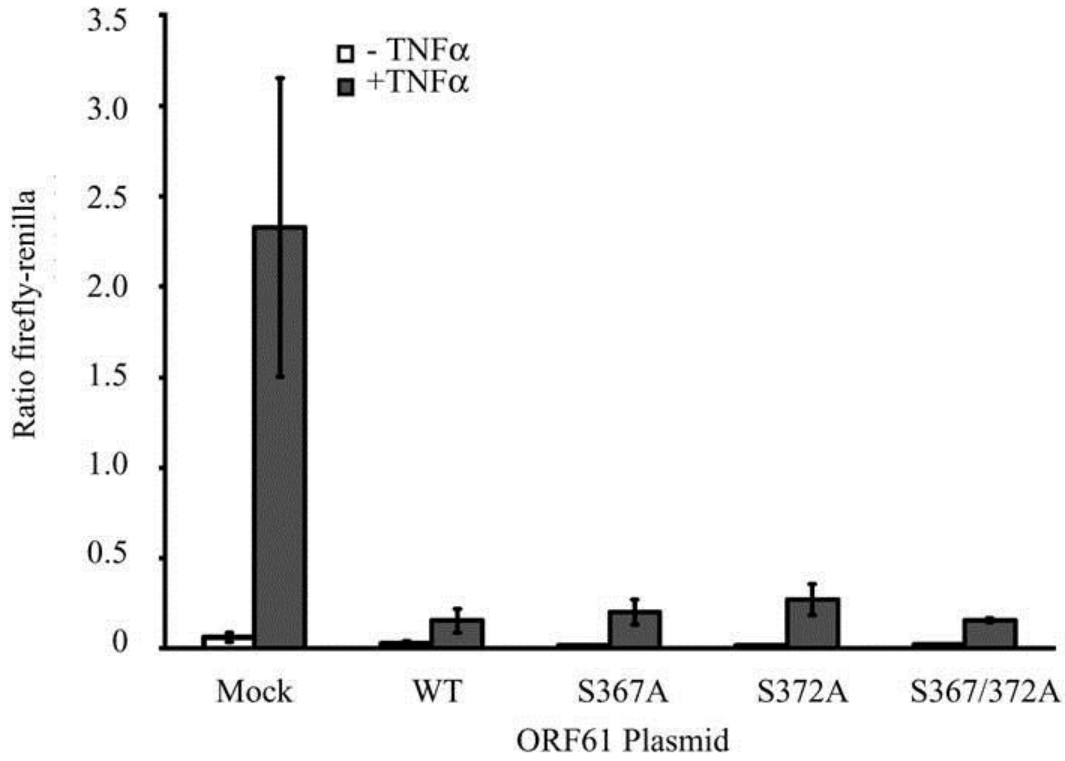

FIG 14 SVV ORF61 mutants inhibit NFκB **activation induced TNFa.** HEK 293T stably expressing firefly luciferase under an NFκB promoter and constitutively active Renilla luciferase were transfected with mock (empty plasmid) or the stated SVV ORF 61 pladmid. At 42 h p.i., the cells were stimulated with the indicated increasing concentrations of HuTNFα . Firefly and Renilla luciferase expression was measured using a dual-luciferase reporter assay, and NFκB activity was determined by normalizing the firefly signal to the Renilla signal. The error bars indicate standard deviations.

In addition to mapping the βTrCP-binding domain of ORF61, the IκBα-binding domain also needs to be determined. To establish this interaction, IκBα was immunopreciptitated (IP) from TRF cells infected with AdFL-ORF61 only, AdFL-ORF61 and AdTA, or with a tetracycline inducible adenovirus expressing FLAG-tagged Rhesus Cytomegalovirus protein UL159 (AdUL159-FL) in the presence of AdTA as a negative control for 48 h. The precipitated complexes were analyzed for the presence of IκBα and FLAG by SDS-PAGE and western blot. Only expressed ORF61 was able to be Co-IP from IκBα (Fig 15). Since βTrCP and IκBα are known to interact it could be possible that that we are immunopreciptitating a complex of ORF61, IκBα, and βTrCP in the two separate IκBα or βTrCP pull-downs. Note, βTrCP and IκBα should only be interacting upon IκBα phosphorylation which only occurs if the pathway is stimulated, which it has not been in these two experiments. Therefore, it seems that ORF61 has an IκBα interaction domain that is separate from its βTrCP interaction domain, and may act as a scaffold if future Co-IP experiments reveal that we are indeed co-immunopreciptitating a complex of IκBα, βTrCP, and ORF61. To investigate which portion of ORF61 is necessary for IκBα interaction we could use the same truncation mutants that we would make for the βTrCP interaction, but test for IκBα interaction instead. It seems redundant that ORF61 would need to bind both βTrCP and IκBα in order to block NFκB activation. Once both interacting domains have been determined we could test to see if one or both of them are responsible for the inhibition of NFκB activity. If it turns out that only the IκBα interaction is necessary for the NFκB phenotype we observe it would open up another avenue of inquiry into what other signaling pathway are targeted by the virus that are mediated in part by βTrCP.

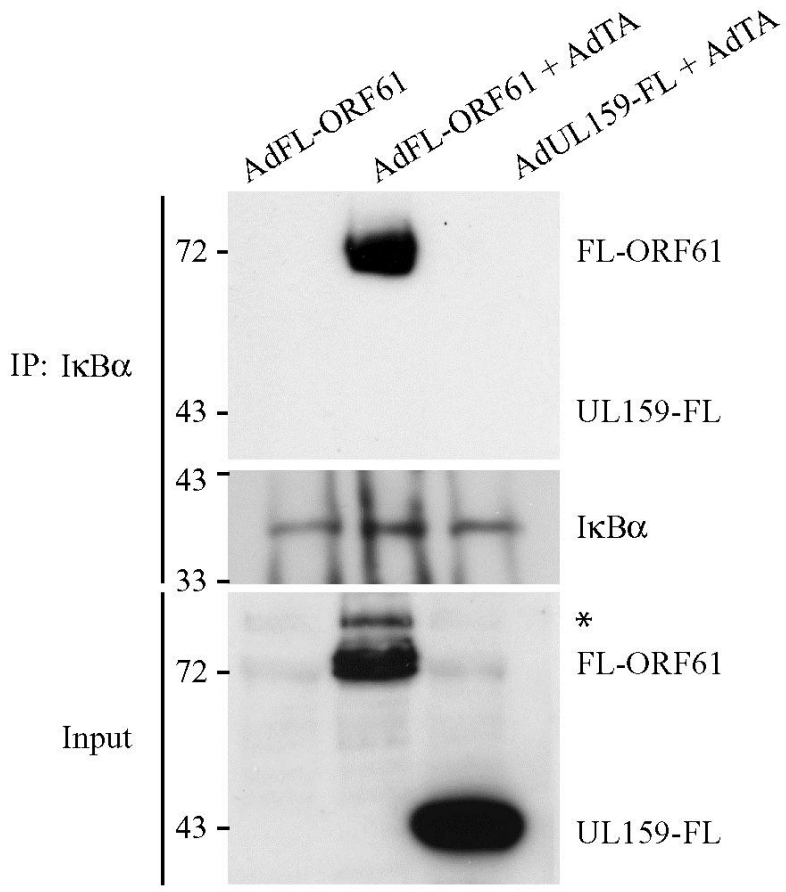

FIG 15. SVV ORF61 interacts with IκBα. IκBα was immunopreciptitated (IP) from TRF cells infected with AdFL-ORF61 only, AdFL-ORF61 and AdTA, or with a tetracycline inducible adenovirus expressing FLAG-tagged Rhesus Cytomegalovirus protein UL159 (AdFL-RhCMV159) in the presence of AdTA for 48 h. The precipitated complexes were analyzed for the presence of IκBα and FLAG by SDS-PAGE and western blot (upper panels). FLAG expression in the input lysate is shown (lower panel).

Another recent finding regarding SVV ORF61 that needs future exploration is that ORF61 appears to actively sequester IκBα in the cytoplasm (Fig 16). We observed this during a fractionation experiment to show that ORF61, although primarily in the nucleus, is also in the cytoplasm (Fig 16). It has been established that IκBα shuttles in and out of the nucleus and that there remains a pool of nuclear IκBα:NFκB complex to act immediately upon activation. It is plausible that in order to minimize this pool of "fast-acting" NFκB ORF61 removes it from the nucleus in addition to minimizing IκBα ubiquitination. Future studies would focus on how ORF61 mechanistically removes IκBα from the nucleus: active export, import block, or both. Since IκBα shuttles between the cytoplasm and nucleus my hypothesis is that ORF61 is carried out to the cytoplasm by IκBα, through its interaction with IκBα (see above), where it then can no longer re-enter the nucleus. Future experiments should look at the location (cytoplasmic or nuclear) of IκBα after stimulation of the pathway with TNFα. We know from previous data (chapter 2 Fig 8C) that ORF61 allows for the phosphorylation of IκBα; therefore it would be interesting to see if phosphorylated IκBα also remained sequestered in the cytoplasm.

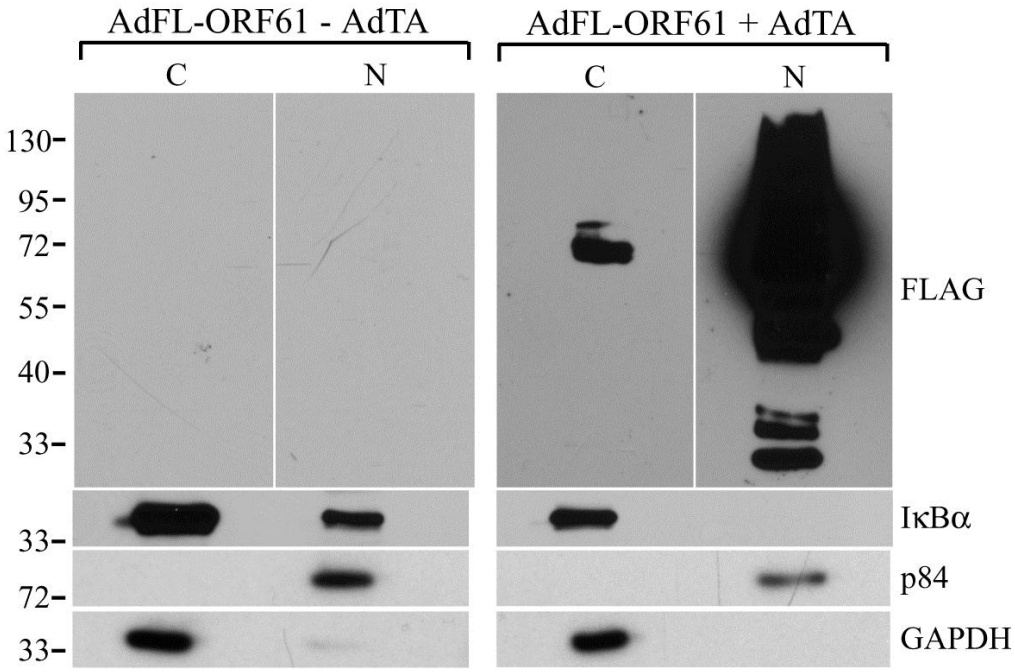

FIG 16. SVV ORF61 modifies the subcellular localization of IκBα. TRFs infected with AdFL-ORF61 (MOI, 15) only or AdFL-ORF61 (MOI, 15) and AdTA (MOI, 7) for 48 h. Samples were split into cytoplasmic or nuclear fractions. Lysates of the fractions were analyzed for IκBα and ORF61 by SDS-PAGE and Western blotting using the indicated antibodies. ORF61 expression was confirmed using a FLAG-specific antibody. P84 and GAPDH were used as fraction specific controls.

The key irreversible step in NFκB activation is the proteolysis of IκBα, therefore to completely inhibit NFκB activation, varicella virus would need to block the degradation of IκBα in both the nuclear and cytoplasmic compartments. The recent data presented above and the data presented in chapter 2 of this project suggest that ORF61 accomplishes this through two interactions, one with IκBα and the other with βTrCP. Our current model regarding ORF61 function hypothesizes that initially the ORF61 NLS directs the protein primarily to the nucleus where it can interact with βTrCP to prevent the ubiquitination of IκBα:NFκB complexes. Consequently, this interaction blocks the nuclear pool of NFκB from becoming active. Once inside the nucleus, ORF61 can now bind IκBα, which allows translocation back to the cytoplasm as a complex with IκBα during IκBα:NFκB shuttling events. Once in the cytoplasm ORF61 prevents the re-entry of IκBα:NFκB and any further ubiquitination by cytoplasmic βTrCP.

3.3 SVV NFκB evasion Future Directions:

Another major area of research will be to determine the mechanism by which SVV blocks IκBα phosphorylation. We showed in chapter 2 Fig 11 that SVV lacking ORF61 was still able to inhibit NFκB activation by preventing IκBα phosphorylation and subsequent degradation. In addition, we showed that SVV is able to prevent NFκB activation from various stimuli (chapter 2 Fig 6), which suggests that SVV is blocking NFκB activation at the convergence point of IKK activation. IκBα is phosphorylated by the IKKβ subunit of the IKK complex, therefore future studies should include determining whether SVV can modulate the protein levels of IKK. In addition, during NFκB activation, the IKK complex is also phosphorylated and ubiquitinated. Additional

experiments could be performed to determine if any of these IKK activation events are inhibited by SVV infection, and if so which corresponding proteins are being affected.

Although it is reasonable to expect that SVV might affect the IKK complex to prevent the phosphorylation of IκBα, it is not the only potential target to investigate. SVV lacking ORF61 can still affect the GSK-3β-Snail pathway (chapter 2 Fig 11C). Snail activation occurs through the PI3K GSK-3β pathway. Briefly, GSK-3β is active in resting cells and phosphorylates Snail leading to the constitutive turnover of Snail [523]. Activation of certain signaling cascades such as Wnt or the PI3K/AKT pathway leads to the inactivation of GSK-3β and a subsequent rise in Snail [599, 600]. There have been conflicting reports on the role GSK-3β plays in the activation of the IKK complex [544, 601]. However, if GSK-3β is involved in IKK activation then by targeting this single protein, SVV could efficiently modulate both NFκB activation as well as Snail degradation. Follow-up work will be required to determine whether GSK-3β is directly targeted by SVV. It is possible that SVV inhibits both the NFkB and Snail pathways individually, or that inhibition of one pathway might impact the other depending on where the block occurs. Future work will be necessary to resolve these issues.

In addition to understanding how SVV modulates IκBα phosphorylation and Snail degradation, determining the responsible viral ORF(s) would be another important line of inquiry. One approach to discover which other ORFs inhibit NFκB activity would be to first create a series of SVV ORF expression constructs and then transfect into an NFκB reporter cell line to determine which gene(s) caused a reduction in luciferase output. Once the ORFs responsible for downregulating NFκB activity are identified, they could be further evaluated for their possible involvement in the observed inhibition of IκBα

phosphorylation. SVV ORFs that may potentially modulate NFκB activity are ORFA and ORF21. The data suggesting a role for SVV ORF21 is derived from an unpublished yeast-two-hybrid screen of all the VZV ORFs (personal communication). It was determined that VZV ORF21 had potential interactions with the IKK complex. Not a lot of information is known about VZV ORF21. VZV ORF21 is the HSV-1 UL37 homolog [602]. During HSV infection UL37 protein is a phosphorylated tegument protein that is involved in the maturation of the virus [603-605]. UL37 has also been demonstrated to bind DNA in complexes with ICP8 [606]. VZV ORF21 has been shown to localize in both the cytoplasm and nucleus and is a member of maturing nucleocapsids [607]. Unlike UL37, VZV ORF21 is not phosphorylated and does not bind DNA in complex with VZV ORF29, the HSV-1 ICP8 homolog [602]. In a subset of latency studies, VZV and SVV, ORF21 transcript was detectable [398, 409, 552, 558]. Although it may be transcribed in latently infected cells it seems that ORF21 is dispensable for the establishment of latency [608]. However, since the lack of IκBα phosphorylation appears to be unique to SVV it is likely that the responsible protein is present only in SVV but not VZV. ORFA is one of two SVV proteins that are not found in VZV. ORFA is a 293 amino acid long truncated version of ORF4 [404, 533]. ORF4 happens to be the VZV homolog of HSV-1 ICP27, which has been shown to block NFκB activation through a mechanism that prevents IκBα phosphorylation by binding to IκBα [530]. It is therefore possible that ORFA is involved in the inhibition of IκBα phosphorylation that was uniquely observed in SVV-infected cells. Once the responsible SVV ORF has been determined, follow-up studies to find the functional domains would be possible.

The data from these proposed experiments would be extremely useful for future in vivo work. We have already discovered that SVV, unlike VZV, is able to inhibit IκBα phosphorylation. Once the SVV ORF responsible for this inhibition has been identified the in vivo importance of NFκB signaling can be determined. Previous studies have evaluated SVV lacking ORF61 in the RM model, however at the time it was not known that there was a second viral protein that affected NFκB activation. The results from these studies indicated that there were no major differences in the immune responses elicited by wild type or ORF61-deleted SVV. Our in vitro VZV data indicate that during an infection VZV does not block IκBα phosphorylation. Therefore, infection of RM with SVV lacking both ORF61 and this unknown viral protein would further enhance our understanding of what would immunologically occur during a VZV lacking ORF61 infection in humans. I speculate that the SVV ORF61/ORF? double knockout virus would be attenuated and that RM infected with this virus would produce a much more robust immune response. If this were true it would provide evidence that a VZV ORF61 deletion virus might be a potential candidate for a new and possibly more effective vaccine.

Conclusions:

In this dissertation, I have described for the first time the underlying mechanism of how varicella virus ORF61 protein inhibits NFκB activation. We have identified binding partners for SVV ORF61and have begun to characterize the functional domains of the protein. In the process we have uncovered an interesting functional difference in the way SVV and VZV actively block NFκB signaling. The results from these studies are highlighted in the following conclusions:

1. SVV blocks NFκB activation from multiple different stimuli
2. SVV inhibits NFκB activation by preventing the phosphorylation and subsequent degradation of IκBα
3. SVV ORF61 is sufficient, but not necessary to block NFκB activation
4. SVV ORF61 prevents IκBα degradation by inhibiting its ubiquitination
5. SVV ORF61 interacts with the host SCF E3 ubiquitin ligase through βTrCP
6. SVV ORF61 is able to modulate the degradation of other substrates of the $SCF^{\beta TrCP}$ complex
7. An intact RING finger domain of SVV ORF61 is critical for ORF61 to inhibit IκBα degradation
8. The putative degron motif LSGPIKS in SVV ORF61 is not necessary for inhibiting NFκB activation
9. SVV ORF61 interacts with IκBα
10. The majority of SVV ORF61 is located in the nucleus, but a small fraction is in the cytoplasm

11. SVV ORF61 prevents IκBα from localizing to the nucleus (or SVV ORF61 sequesters IκBα in the cytoplasm)
12. VZV inhibits NFκB activation at a point downstream of IκBα phosphorylation
13. VZV ORF61 is sufficient to block IκBα degradation by inhibiting its ubiquitination.

Further characterization of ORF61 and its interacting proteins will provide new insights into how varicella virus evades the host immune response. In addition, these results and future studies to elucidate other SVV proteins involved in NFκB regulation might improve the in vivo SVV RM model to better mimic what we know to occur during a VZV infection. Deletion of multiple NFκB modulatory genes might prove to be the basis for a more attenuated virus that has to the potential to illicit a much stronger immune response, which in turn could lead to an improved vaccine.

Materials and Methods

Cell lines and recombinant viruses

Rhesus fibroblasts were life-extended through stable transduction of constitutively expressed human telomerase reverse transcriptase encoded in the lentivector pBABE and selected using 400μg/mL G418 generally as described [609]. The telomerized Rhesus fibroblasts (TRFs) were then stably transduced with replication-incompetent lentiviruses containing luciferase coding sequences from the firefly *Photinus pyralis* as well as *Renilla reniformis* that were, respectively, inserted downstream of an NFκB-dependent promoter or the CMV promoter (SA Biosciences). Transduced cells were selected using 3μg/mL puromycin. The reverse Tet-transactivator (rtTA) was stably introduced into THF by inserting the coding region into the retrovector pCFG5-IEGZ and transducing cells with derivative replication-incompetent virus as described [610]. THF rtTA cells stably expressing inducible VZV ORF61 and SIV GAG were generated using the pLVX lentivector system (Clontech) by cotransfecting pLVX along with vectors encoding vesicular stomatitis virus G (pMD2.G; VSV-G, Addgene #12259), and Gag/Pol (psPAX2; Addgene #12260) into HEK 293T cells using Lipofectamine LTX (Life Technologies) according to the manufacturer's protocol. Supernatant containing lentivirus was harvested from the transfected cells 48 hours post transfection, passed through 0.45 μM filters, and used to transduce THF rtTA cells in the presence of 5 μg/ml Polybrene (Hexadimethrine bromide; Sigma-Aldrich). This process was repeated 24 hours later and the resulting cell lines were grown in the presence of 3 μg/ml puromycin to select for cells that expressed the viral genes. TRFs, TRF-NFκB, THF rtTA, human embryonic kidney (HEK) 293T cells (ATCC) and the human fibroblast cell line MRC-5 (ATCC) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 140 IU of penicillin and 140 μg of streptomycin per ml of culture media.

TRFs cells were infected with SVV Delta strain that expresses eGFP (SVV.eGFP), which was inserted between US2 and US3 using homologous recombination [611] or SVVΔ61 (see

below). A monolayer of TRFs was infected by co-cultivation with previously infected cells at the described ratios in DMEM supplemented with 2% FBS. Complete infection with SVV.eGFP was verified by fluorescence microscopy. VZV infections with the recombinant VZV Oka strain in which eGFP was fused to the N-terminus of ORF66 (VZV.eGFP) (generously provided by P.R. Kinchington, University of Pittsburgh, Pennsylvania) [452] in MRC5 cells were performed in the same way as SVV-infections.

Reagents and antibodies

Rhesus and Human TNFα and IL-1β were obtained from R&D systems. Phorbol 12-myristate 13-acetate (PMA) (Enzo Life Sciences) was dissolved in DMSO and used at the indicated concentrations. Poly(I:C) and LPS were acquired from Sigma-Aldrich and used at the indicated concentrations. MG132 (Fisher Scientific) was dissolved in DMSO and used at 50 μM for 3 hours. For detection of cellular and viral proteins in Western blot we used the following antibodies: anti-IκBα sc-203 (Santa Cruz Biotechnology), anti-IκBα 10B (kindly provided by R.T. Hay, University of Dundee, Scotland) [612], anti-phospho-IκBα Ser32/36 (Cell Signaling Technology), anti-FLAG M2 (-Peroxidase) (Sigma-Aldrich), anti-Snail C15D3 (Cell Signaling Technology), anti-β-TrCP sc-33213 (Santa Cruz Biotechnology), and anti-HA HA-7 (Sigma-Aldrich). The monoclonal antibodies specific for SVV and VZV ORF31 (clone 31C_8) and ORF63 (clone 63_6) have been described previously [613]. VZV ORF61 was detected using a rabbit polyclonal that was described before and has been kindly provided by P.R. Kinchington, (University of Pittsburgh, Pennsylvania) [531]. Primary antibody binding was visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies specific for mouse (Santa Cruz) or rabbit (Thermo Scientific) IgG. The anti-FLAG M2 and an anti-NFκB p65 sc-372 (Santa Cruz Biotechnology) were used in immunofluorescence microscopy (IFA). Secondary antibodies used for IFA were Alexa Fluor 488 Goat anti-Mouse IgG and Alexa Fluor 594 Goat anti-Rabbit IgG (Life Technologies).

Plasmids

The HA-β-TrCP and FL-A49 expression plasmids were kindly provided by G.L. Smith (University of Cambridge, Cambridge UK) and have been previously described [524]. For the recombinant adenoviruses we cloned SVV ORF61 from DNA isolated from TRFs infected with SVV.eGFP using the DNeasy Blood and Tissue kit (Qiagen) with the following primers: 5'-CACCGAATTCACCATGAACCCCCCGGCGTATACC-3' (ORF61 FW) or 5'-CACCGAATTCACCATGGACTACAAGGATGACGACGATAAGAACCCCCCGGCGTATAC-3' (ORF61 FW-FLAG) and 5'-AATAAAGGATCCTTATTTTCTCCGTACCTTTTTAGTTAACATTTCAATGCG-3' (ORF61 Rev). Resulting PCR products were inserted in the adenovirus shuttle vector using EcoRI/BamHI sites. The RING mutant of ORF61 was generated using the shuttle vector with FLAG-ORF61 as a template and using the primers: 5'- CCACCGGGAACTCCGCTATATGCATGAGC-3' (QC FW) and 5'-GCTCATGCATATAGCGGAGTTCCCGGTGG-3' (QC Rev).

For the transfection vectors we used the same DNA as a template for PCR amplification of SVV ORF63. PCR was performed using the following primers: 5'-AATAAAGAATTCGCCACCATGGACTACAAGGATGACGACGATAAGCAGGCGCCCCGAG-3' (ORF63 FW-FLAG) and 5'-AATAAAGGATCCTTATGTATTGTGTACAGACTCTCGTAACTCCGTG-3' (ORF63 Rev). SVV ORF61 was amplified from the adenovirus shuttle vectors using the primers: 5'-GCCACCATGGACTACAAGGATGACGACGATAAG-3' (FW-FLAG) and 5'-TTATTTTCTCCGTACCTTTTTAGTTAACATTTCAATGCG-3' (ORF61 Rev). These PCR-generated products were inserted into the pcDNA3-IRES-nlsGFP, creating pcDNA3 FL-ORF63 and pcDNA3 FL-ORF61. VZV DNA was purified from MRC5 cells infected with VZV.eGFP and used as a template for PCR amplification of VZV ORF61. The primers used were 5'-AATAAAGAATTCGCCACCATGGACTACAAGGATGACGACGATAAGGATACCATATTAGCGGGCGGTAGC-3' ($ORF61^{VZV}$ FW-FLAG) and 5'-

AATAAAGCGGCCGCCTAGGACTTCTTCATCTTGTTTGGAATACC-3' (ORF61^{VZV} Rev). The PCR-generated product was inserted into the pLVX-Tight-Puro vector (Clonetech Laboratories). pLVX SIV GAG was created using DNA isolated from TRFs infected with a recombinant rhesus cytomegalovirus that expresses the protein [614] using the primers 5'-CACCGAATTCACCATGGGCGTGAGAAACTCCGTCTTG-3' (GAG FW), 5'-AATAAAGGATCCCTACTGGTCTCCTCCAAAGAGAGAATTGAG-3' (GAG Rev).

All PCRs were performed with either Expand High Fidelity PCR system (Roche) or AccuPrime Taq DNA polymerase High Fidelity (Life Technologies) and all sequences were verified.

Site-directed mutagenesis

Site-directed mutagenesis was carried out using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) in accordance with the supplied protocol. pcDNA3 FL-ORF61 was used as the original template. The primers used to create the S367A and S372A mutants were: 5'-CCAATTAGATTGCTAGCCGGTCCAATAAAATCGCCG-3' (S367A FW), 5'- CGGCGA TTTTATTGGACCGGCTAGCAATCTAATTGG-3' (S367A Rev) and 5'-CTATCCGGTCCAATAAAAGCGCCGGATGGCGGTTCAAC-3' (S372A FW), 5'-GTTGAACCGCCATCCGGCGCTTTTATTGGACCGGATAG-3' (S372A Rev). To create the double mutant S367/372A the single S367A mutant was used as the template with the primers 5'-CTAGCCGGTCCAATAAAAGCGCCGGATGGCGGTTCAAC-3' (S367/372A FW), 5'-GTTGAACCGCCATCCGGCGCTTTTATTGGACCGGCTAG-3' (S367/372A Rev). All sequences were verified.

Fractionation Assay

TRF cells were infected were infected with the indicated viruses. 48 hours p.i. the cells were harvested and resuspended in dounce buffer containing 100 mM KCL, 20 mM Hepes (pH 7.4), 0.1 mM EDTA, 0.2 mM sucrose (3%) and Halt protease inhibitor cocktail and Halt phosphatase inhibitor cocktail (Thermo Scientific). After 15 minutes on ice 10 μL 10% NP-40 was added.

Samples were then centrifuged and the supernatant was saved as the cytoplasmic fraction. The pellet was washed with cold PBS and subsequently lysed in RIPA buffer containing 1% NP-40, 10 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% trition X-100, 1% sodium deoxycholate acid, 0.1% SDS, Halt protease inhibitor cocktail, and Halt phosphatase inhibitor cocktail (Thermo Scientific). Laemmli sample buffer was added to all samples followed by western blot analysis.

Luciferase reporter assay

TRF-NFκB cells infected with SVV.eGFP were seeded in a black 96 well plate (Corning Incorporated) at 24 hours p.i. At 42 hours p.i. the cells were incubated with the indicated NFkB activators for 6 hours to induce expression of NFκB-driven firefly luciferase. Firefly and *Renilla* luciferase was measured using the Dual-Glo luciferase assay system (Promega) and luminescence was measured on a Veritas microplate luminometer (Promega). Data are presented as the ratio of firefly luciferase expression to *Renilla* luciferase expression.

Semiquantitative PCR

Total cellular RNA was harvested using the NucleoSpin RNA isolation kit (Machery Nagel) in accordance with the supplied protocol. Collected RNA concentration was measured with the NanoDrop 1000 Spectrophotometer (Thermo Scientific) and single-stranded cDNA was made using Maxima Reverse Transcriptase (Thermo Scientific) using the manufacturers protocol and random hexamers (TaKaRa). RANTES mRNA induction following TNFα-treatment was analyzed by semiquantitative real-time PCR (qPCR) using SYBR green PCR core reagents and Platinum Taq DNA Polymerase (Invitrogen). Reactions were performed using the Applied Biosystems® StepOnePlus™ Real-Time PCR System (Life Technologies). The primers that were used are RANTES Fw: 5'-CCTCGCTGTCATCCTCG-3' and RANTES Rev: 5'-GCACACACTTGGCGATC-3'. GAPDH was used as a housekeeping gene (GAPDH Fw: 5'-GCACCACCAACTGCTTAGCAC-3' and GAPDH Rev: 5'-TCTTCTGGGTGGCAGTGATG-3'). Relative expression of RANTES was calculated using the method described by Livak and Schmittgen [615].

Immunofluorescence microscopy

TRFs were seeded onto glass cover slips and infected the following day with the indicated viruses. Following infection and cytokine treatment the cells were washed twice with PBS and fixed with 3.7% formaldehyde (Fisher Scientific) in PBS for 40 minutes at room temperature (RT). The cells were washed again in PBS and incubated with 50 mM Ammonium Chloride (NH_4Cl) for 10 minutes to reduce nonspecific background. The cells were then permeabilized with 0.1% Triton X-100 for 4 minutes at RT and washed/blocked with 2% bovine serum albumin (Fisher Scienntific) in PBS (PBA). Fixed cells were incubated for 1 hour at 37°C with primary antibody diluted in PBA, washed in PBA and incubated with secondary antibodies diluted in PBA for 1 hour at 37°C. Cells were washed with PBA, followed by a PBS rinse and coverslips were then mounted on slides using ProLong® Gold Anti-fade Reagent with DAPI (Cell Signaling). Images were captured with an Axioskop 2 Plus fluorescence microscope and AxioVision v4.6 softaware (Zeiss).

Immunoprecipitations, TUBE pull down and Western blotting

For immunoprecipitation studies HEK 293T cells were transfected with 3 µg of the indicated plasmids using the Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's protocol. 48 hours post transfection the cells were lysed in a buffer containing 0.5% Nonidet P-40 (NP40), 50 mM Tris-HCL (pH 7.5), and 5 mM $MgCl_2$. The lysates were pre-cleared for 1 hour using Protein A/G PLUS-Agarose beads and normal mouse serum sc-45051 (Santa Cruz Biotechnology). The lysates were transferred to a new tube with an anti-FLAG antibody and Protein A/G PLUS-Agarose beads and incubated overnight at 4°C. The beads were washed with ice-cold Tris-buffered saline (20 mM Tris-HCl [pH8.0], 150 mM NaCl, and 0.1% Tween-20) and precipitated immune complexes were eluted from the beads by resuspending in Laemmli sample buffer (100 mM Tris-HCL [pH 8.0], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, Bromophenol blue) and boiling the samples for 5 minutes at 95°C.

For the ubiquitination experiment cells were washed once with ice-cold PBS and were subsequently lysed in a lysis buffer containing 1% NP-40, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 10% glycerol, Halt protease inhibitor cocktail, Halt phosphatase inhibitor cocktail (Thermo Scientific), and 15 μM N-Ethylmaleimide. Lysates were incubated with Protein A/G PLUS-Agarose beads for 30 minutes at 4°C to remove any nonspecific protein binding. Lysates were transferred to new tubes and incubated with Agarose-TUBE 2 (Lifesensors) overnight at 4°C. After 3 washes with ice-cold Tris-buffered saline (20 mM Tris-HCl [pH8.0], 150 mM NaCl, and 0.1% Tween-20) proteins were eluted with Laemmli sample buffer.

For all other Western blot analysis cell were directly lysed in Laemmli sample buffer. Immunoprecipitated complexes and lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Thermo Scientific). Membranes were incubated with primary antibodies followed by HRP-conjugated secondary antibodies. Bound HRP-labeled antibodies were visualized using either SuperSignal West Pico Chemiluminescent Substrate, Pico substrate mixed with Western Blot Signal Enhancer, SuperSignal West Femto Maximum Sensitivity Substrate or ECL2 Western Blotting Substrate (Thermo Scientific).

Recombinant adenovirus production and infection

The generation of infectious recombinant adenovirus expressing SVV ORF61, SVV FL-ORF61, SVV FL-ORF61 C19G, and GFP was previously described [616, 617]. The vectors contain a tetracycline-responsive promoter and require the addition of a tetracycline-regulated transactivator (tTA) [618], which was provided by co-transducing with AdTA. TRFs were transduced in six-well clusters with the purified adenoviruses and AdTA at the indicated MOI in 0.5 ml of serum-free DMEM. After 2 hours of rocking at 37°C, 1.5 ml of DMEM supplemented with 10% FBS was added and incubation continued for a total of 48 hours.

Statistical analysis

P-values were determined using an unpaired Student’s t-test.

SVV BAC mutagenesis

The SVV BAC pSVV-FrDX in E.Coli strain DH10B was kindly provided by W.L. Gray (University of Arkansas for Medical Sciences, Little Rock) [619]. To create a ΔORF61 deletion mutant based on this wild type (wt) by homologous recombination, primers containing 50 base pair homology to regions flanking ORF61 (Fw 5′-AAAACAGTATTACAGTAAAATAACATGTAACTATGTGAATGTACATTGCT-3′ and Rev 5′-CTAACGGCATATTTGGGCCTGGTTTTTGGGGACATCTATCTTCCACAGTC-3′) were used to amplify a kanamycin (Kan) resistance cassette from plasmid pCP015 [620]. The pCP015 forward primer binding site (5′-GTAAAACGACGGCCAGT) and reverse primer binding site (5′-GAAACAGCTATGACCATG) were added to the 3′ end of the mutagenesis primers. Purified SVV BAC DNA was transformed into the E.coli strain SW105 that has heat-inducible λ-recombination genes and an arabinose-inducible FLP recombinase [621]. Bacterial cultures were grown in LB-medium at 30°C until an OD of 0.6 at 600 nm was reached, and the λ-recombinant genes were heat-induced by shaking at 42°C in a water bath for 15 minutes. The bacteria were subsequently chilled on ice for 10 minutes and made electrocompetent by washing 4 times with cold, deionized water. For recombination, the generated competent E.coli were electroporated with the PCR product using a MicroPulser (Bio-Rad) and selected for Kan and chloramphenicol (Cm) resistance at 30°C on LB agar for 36 hours. Cm/Kan resistant colonies were grown in LB-medium and BAC DNA was isolated from the bacteria. Restriction digest was performed using EcoRI and the resulting DNA fragments were separated by electrophoresis on a 0.75% agarose gel in 0.5xTBE-buffer. Restriction patterns of the generated clones were compared to the parental wt and to an *in silico* restriction analysis.

To induce the FLP recombinase excising the Kan^R cassette, clones were grown in LB with Cm until they reached an OD of 0.5 at 600 nm and incubated with 1 mg/ml arabinose for 1 hour. The bacteria were streaked out on an LB plate with Cm selection using an inoculation loop and incubated overnight at 30°C. After colonies were visible, clones were replica plated first on Cm/Kan LB agar followed by Cm LB agar, and colonies were selected that had lost Kan^R and characterized by restriction digest and partial sequencing. To reconstitute the virus, BAC DNA was prepared from the bacteria, and transfected into Vero cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Viral plaques appeared approximately 7 days post-transfection.

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