Termination of NF-κB activity through a gammaherpesvirus protein that assembles an EC5S ubiquitin-ligase

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Host colonisation by lymphotropic gammaherpesviruses depends critically on the expansion of viral genomes in germinal centre (GC) B cells. Yet, host and virus molecular mechanisms involved in driving such proliferation remain largely unknown. Here, we show that the ORF73 protein encoded by the murid herpesvirus-4 (MuHV-4) inhibits host nuclear factor-kappa B (NF-κB) transcriptional activity through poly-ubiquitination and subsequent proteasomal-dependent nuclear degradation of the NF-κB family member p65/RelA. The mechanism involves the assembly of an ElonginC/Cullin5/SOCS (suppressors of cytokine signalling)–like complex, mediated by an unconventional viral SOCS-box motif present in ORF73. Functional deletion of this SOCS-box motif ablated NF-κB inhibitory effect of ORF73, suppressed MuHV-4 expansion in GC B cells and prevented MuHV-4 persistent infection in mice. These findings demonstrate that viral inhibition of NF-κB activity in latently infected GC centroblasts is critical for the establishment of a gammaherpesvirus persistent infection, underscoring the physiological importance of proteasomal degradation of RelA/NF-κB as a regulatory mechanism of this signalling pathway.

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Introduction

Being obligatory intracellular parasites, viruses have evolved a variety of mechanisms to modulate specific host signal-transduction pathways to favour their own replication. One of such mechanisms targets nuclear factor-kappa B (NF-κB), a family of ubiquitously expressed transcription factors that bind specific DNA sequences, κB sites, in the promoter region of a variety of genes, to modulate their rate of transcription/ expression. In mammalian cells, the NF-κB family comprises five members—p65/RelA, RelB, c-Rel, p105/p50 and p100/ p52 (Blanc et al, 1992)—that can form homo- or heterodimers (Saccani et al, 2003). Under homoeostasis, NF-κB dimers are sequestered in the cytoplasm by the inhibitory IκB proteins, which include IκBα, IκBβ and IκBε. These mask the nuclear localisation signal of the NF-κB dimers, impairing their nuclear translocation. Once exposed to pro-inflammatory stimuli, mammalian cells activate an IκB kinase (IKK) complex that phosphorylates IκB molecules leading to their poly-ubiquitination and proteasomal degradation. This, in turn, promotes nuclear translocation of NF-κB dimers, where they bind to eukaryomatised κB sites to activate target gene transcription (Karlin and Ben-Neriah, 2000).

Genes regulated through NF-κB are involved in critical biological functions, including inflammation and apoptosis as well as cell proliferation. One essential aspect of the NF-κB signal-transduction pathway is that it must be tightly regulated to afford a fast response to a given stimulus, terminating this response as soon as the stimulus is no longer present. Although mechanisms regulating NF-κB activation have been studied extensively, those regulating its termination are less well perceived. Termination of NF-κB activity was initially thought to rely exclusively on the de novo expression of IκB molecules. Once resynthesised, IκBε enters the nucleus where it dissociates NF-κB dimers from κB sites, shuttling NF-κB dimers back to the cytoplasm (Arenzana-Seisdedos et al, 1997). However, in recent years, it has become apparent that many other mechanisms control the extent of NF-κB activation, including direct poly-ubiquitination and subsequent proteasomal degradation of promoter-bound RelA (Saccani et al, 2004). This mechanism is regulated through the activation of multimeric E3 ubiquitin-ligases, which accept ubiquitin from E2 ubiquitin-conjugating enzymes, and transfer it to specific substrates promoting their degradation by the proteasome (Weissman, 2001). Two cellular proteins, PDLIM2 (Tanaka et al, 2007) and SOCS1 (suppressors of cytokine signalling 1; Ryo et al, 2003), have been identified as mediators of RelA poly-ubiquitination. In the case of SOCS1, this protein functions as the substrate recognition component of an ECS (ElonginC–Cullin2/5–SOCS) E3 ubiquitin-ligase (Ryo et al, 2003). SOCS proteins comprise several family members each of which share a C-terminal 40-amino-acid module that is known as the SOCS-box, which mediates the interaction with ElonginB/C and Cullin2/5 modules, bridging the substrate of ubiquitination to the E2
ubiquitin-conjugating enzyme (Yoshimura et al., 2007). By virtue of assembling an ECS\(^{\text{SOSC1}}\) ligase and interacting with NF-\(\kappa\)B subunits, SOCS1 directly ubiquitinates and proteasomal degradation of promoter-bound NF-\(\kappa\)B members and efficient termination of transcriptional responses (Ryo et al., 2003; Saccani et al., 2004).

Viruses have evolved several mechanisms to modulate NF-\(\kappa\)B activity (Hiscott et al., 2006). One example is the NS5B protein of hepatitis C virus that targets the IKK complex resulting in IKK inhibition and suppression of NF-\(\kappa\)B activity (Choi et al., 2006). Another strategy relies on the expression of viral I\(\kappa\)B-like proteins that stably interact with NF-\(\kappa\)B dimers, but lack the consensus serine residues phosphorylated by IKK. Thus, these viral inhibitors are not degraded in response to IKK activation, which prevents NF-\(\kappa\)B translocation to the nucleus and the subsequent NF-\(\kappa\)B-driven gene transcription. This is the case for the A238L I\(\kappa\)B-like protein of African swine fever virus (Powell et al., 1996; Revilla et al., 1998; Tait et al., 2000). In addition, host NF-\(\kappa\)B dimers can be targeted directly for degradation by viral proteins, as exemplified by the 3\(\epsilon\)C-encoded protease of poliovirus that cleaves the transcription-activation domain (TAD) of RelA to inhibit NF-\(\kappa\)B signalling (Neznanov et al., 2005).

In this study, we describe a novel viral mechanism to suppress NF-\(\kappa\)B activity. We provide conclusive evidence that the latency-associated protein ORF73 encoded by murid herpesvirus-4 (MuHV-4), a gammaherpesvirus genetically related to the human pathogens Epstein–Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV) (Simas and Efstratiou, 1998), targets nuclear RelA for proteasomal degradation. Gammaherpesviruses are among the most prevalent of human pathogens owing to their ability to cause persistent infections (Rickinson and Kieff, 2001). Persistent infection is associated with both lymphoid and epithelial tumours, which occur with increased incidence following immune suppression (Damania, 2004; Sunil-Chandra et al., 1994). Thus, the control of gammaherpesvirus infections represents a major clinical goal. A critical determinant of persistence is the ability to establish latency in memory B cells. Access to this cell type is gained by virus-driven lymphoproliferation of germinal centre (GC) B cells (Thorley-Lawson, 2001). During expansion of latency in B cells, viral genomes replicate in step with normal cell division. This process is mediated by viral episome maintenance proteins, which include Epstein–Barr nuclear antigen-1 (EBNA-1) of EBV (Yates et al., 1985) and ORF73 of gammaherpesviruses (Ballestas et al., 1999; Hall et al., 2000). ORF73 proteins have also been shown to function as nuclear regulators of transcription and to interact with several cellular proteins to modulate host functions, postulated to be involved in latency regulation (Verna et al., 2007). The new mechanism here described underlying ORF73-mediated RelA degradation, involves the assembly of an ECS\(^{\text{SOSC1}}\) complex mediated by an unconventional SOCS-box-like motif present in ORF73. We found that ECS\(^{\text{SOSC1}}\) mimics the host ECS\(^{\text{SOSC1}}\) ubiquitin-ligase inhibiting tumour necrosis factor (TNF)-induced NF-\(\kappa\)B activation. Infection with recombinant viruses, bearing disruptive mutations in the ORF73 SOCS-box-like motif, rendered these viruses incapable of inducing lymphoproliferation in GC B cells and prevented persistent infection in mice. This finding emphasises the physiological importance of proteasomal degradation of NF-\(\kappa\)B as a prompt terminator of this signalling pathway.

### Results

**Identification of ORF73 as an inhibitor of NF-\(\kappa\)B transcriptional activity**

Experiments were designed to investigate whether NF-\(\kappa\)B transcriptional activity was modulated in mammalian cells transiently expressing ORF73. Human HEK 293T cells were transiently co-transfected with a synthetic NF-\(\kappa\)B reporter containing three copies of \(\kappa\)B consensus sequences driving the expression of firefly luciferase (Winkler et al., 1996), with or without ORF73. TNF was used as a prototypical stimulus leading to NF-\(\kappa\)B activation. Control cells, which did not express ORF73, responded in a dose-dependent manner to TNF; that is, the higher the TNF concentration the higher the luciferase activity (Figure 1A, filled bars). In contrast, cells expressing ORF73 were unable to respond effectively to TNF, even at the highest concentration tested (50 ng/ml) (Figure 1A, open bars), an indication that the viral protein impairs TNF-driven NF-\(\kappa\)B activation.

We next assessed whether ORF73 would be modulating the NF-\(\kappa\)B inhibitor I\(\kappa\)Bz by interfering with its degradation and/or resynthesis kinetics. Control transfected or ORF73-expressing cells, exposed to TNF, were monitored by western blot for the expression of I\(\kappa\)Bz. The pattern of I\(\kappa\)Bz degradation was similar in control versus ORF73-expressing cells (Figure 1B, first panel, lanes T0 and T1), indicating that ORF73 does not modulate the signal-transduction pathway, driven by TNF receptor 1 (TNFR1), that leads to I\(\kappa\)Bz degradation. However, I\(\kappa\)Bz resynthesis was severely compromised in ORF73-expressing cells (Figure 1B, first panel, lanes T2–T4). These results are in good agreement with the inhibitory effect observed for the reporter assay, as I\(\kappa\)Bz is one of the primary targets of NF-\(\kappa\)B transcriptional activity.

**ORF73 directly targets the Rel homology domain of RelA**

Next, we hypothesised that ORF73 was directly targeting one or more members of the NF-\(\kappa\)B family. We performed reporter gene assays where the primary NF-\(\kappa\)B family member, RelA, was transiently overexpressed in different combinations with p50 and c-Rel, in the presence or absence of ORF73. Overexpression of these NF-\(\kappa\)B family members over-rides the sequestering effect of endogenous I\(\kappa\)B molecules (Anrather et al., 1999), as revealed by a significant fold induction of NF-\(\kappa\)B transcriptional activity (Figure 1C, filled bars). Co-expression of ORF73 resulted in the inhibition of the transcriptional activity in all NF-\(\kappa\)B combinations tested (Figure 1C, open bars), an indication that ORF73 directly targets NF-\(\kappa\)B proteins to inhibit their transcriptional activity.

The Rel homology domain (RHD) is a consensus sequence shared by all NF-\(\kappa\)B proteins: RelA, RelB, c-Rel, p105/p50 and p100/p52. RelA, RelB and c-Rel contain an additional TAD responsible for interaction with the basal transcriptional machinery (Chen and Greene, 2004). To investigate which functional RelA domain was targeted by ORF73, we used two artificial fusion proteins depicted in Figure 1D (Anrather et al., 1999). The RelA(RHD)–VP16 fusion protein is composed of the RHD of RelA (aa 2–320) in frame with the TAD derived from the herpes simplex virus VP16 protein. The construct Tet–RelA(TAD) has the DNA-binding domain from the bacterial tetracycline repressor fused to the C-terminal portion of RelA (aa 286–551), which includes its TAD. The transcrip-
The activity of NF-κB proteins was assessed on transient co-transfection with the NF-κB-responsive promoter (κB-luc; Figure 1E), or with a reporter containing seven tetracycline operons (TetO-luc; Figure 1F). ORF73 impaired the transcriptional activation of RelA(RHD)–VP16, as assayed with the κB-luc promoter (Figure 1E). In clear contrast, the transcriptional activity of Tet–RelA(TAD) was not inhibited by ORF73 expression, as assessed with the promoter TetO-luc (Figure 1F). In this case, a higher luciferase activity was observed, though this was not a specific effect of ORF73 on the TAD of RelA, as expression of the viral protein resulted in an analogous increase of Tet-VP16 transcriptional activity (data not shown). These results indicate that ORF73 inhibits the activity of NF-κB proteins by a mechanism that specifically targets their RHD domain.

RelA nuclear levels are diminished in ORF73-expressing cells

We proceeded to investigate whether ORF73 would be functioning at the nuclear level by impairing the binding of NF-κB to DNA κB sites. We performed electromobility shift assays (EMSA) in the presence of oligonucleotides bearing the consensus κB site from the immunoglobulin promoter region. Cells were transiently transfected with ORF73, or control transfected, and NF-κB activation was induced by TNF. As illustrated in Figure 2A, NF-κB DNA-binding levels associated with each sample were assayed by western blotting.
reduced in ORF73-expressing cells. This decrease did not reflect ORF73 interference with NF-κB DNA binding to κB sites but rather correlated with diminished levels of nuclear RelA. HEK 293T cells were transiently transfected with an ORF73 expression plasmid, or control transfected. After 24 h of culture, cells were stimulated with TNF for 40 min, or left unstimulated. Nuclear extracts were prepared and subjected to electromobility shift assays (EMSAs) using the NF-κB consensus oligonucleotide from the immunoglobulin promoter region. The percentage of NF-κB binding present in each condition is shown. Nuclear extracts were analysed by immunoblotting to determine the input of RelA and ORF73 present in each binding reaction. Densitometry analysis of RelA nuclear levels present in each experimental condition, normalised to the nuclear protein LaminB, is shown. (B) ORF73 protein does not bind to κB sequences. Nuclear extracts from HEK 293T cells expressing ORF73 were subjected to a supershift assay using the specified antibodies. (C) Immunofluorescence analysis of ORF73-expressing cells. HEK 293T cells were stimulated with 50 ng/ml of TNF for the times indicated and then subjected to immunostaining with anti-RelA and anti-ORF73 antibodies. –, without; +, with; α, anti; WB, western blotting.

Figure 2 RelA nuclear levels are diminished in ORF73-expressing cells. (A) Reduction of NF-κB binding to κB sequences in ORF73-expressing cells correlates with diminished levels of nuclear RelA. HEK 293T cells were transiently transfected with an ORF73 expression plasmid, or control transfected. After 24 h of culture, cells were stimulated with TNF for 40 min, or left unstimulated. Nuclear extracts were prepared and subjected to electromobility shift assays (EMSAs) using the NF-κB consensus oligonucleotide from the immunoglobulin promoter region. The percentage of NF-κB binding present in each condition is shown. Nuclear extracts were analysed by immunoblotting to determine the input of RelA and ORF73 present in each binding reaction. Densitometry analysis of RelA nuclear levels present in each experimental condition, normalised to the nuclear protein LaminB, is shown. (B) ORF73 protein does not bind to κB sequences. Nuclear extracts from HEK 293T cells expressing ORF73 were subjected to a supershift assay using the specified antibodies. (C) Immunofluorescence analysis of ORF73-expressing cells. HEK 293T cells were stimulated with 50 ng/ml of TNF for the times indicated and then subjected to immunostaining with anti-RelA and anti-ORF73 antibodies. –, without; +, with; α, anti; WB, western blotting.

When transiently expressed in resting HEK 293T cells, ORF73 localised primarily to the nucleus, as assessed by immunofluorescence (Figure 2C, panel e). TNF treatment did not affect ORF73 nuclear localisation (Figure 2C, panels f and h), whereas it caused prompt translocation of RelA from the cytoplasm to the nucleus (Figure 2C, panels a and b). However, in cells expressing ORF73, there was a significant decrease in the levels of nuclear RelA after TNF stimulation, without concomitant accumulation of this protein in the cytoplasm (Figure 2C, panels d and h). These results are in
good agreement with the approximately 75% decrease in NF-κB DNA-binding activity in ORF73-expressing cells observed in Figure 2A, which likely reflect the reduced overall levels of nuclear RelA (approximately 70% decrease). Taken together, these data suggest that ORF73 targets nuclear RelA in a manner that decreases its level of nuclear expression after TNF stimulation.

**ORF73 triggers poly-ubiquitination and degradation of nuclear RelA**

As RelA nuclear degradation through poly-ubiquitination is one of the mechanisms through which NF-κB activity can be downmodulated (Saccani et al., 2004), we investigated whether ORF73 was targeting nuclear RelA for poly-ubiquitination/degradation. By performing a nickel-nitrolotriacetic acid (Ni-NTA) pull-down, in the presence of histidine-tagged ubiquitin, we observed that ORF73 expression significantly enhanced RelA poly-ubiquitination similarly to SOCS1 overexpression (Figure 3A, compare lanes 5 and 6), an effect enhanced by the proteasome inhibitor MG132 (Figure 3B, lane 4), indicating that ORF73-mediated RelA poly-ubiquitination targets RelA to proteasome degradation. To further confirm this activity in a more relevant biological context, we tested the ability of ORF73 to mediate the poly-ubiquitination of endogenous RelA following stimulation with TNF. Under the influence of the proteasomal inhibitor MG132, in comparison with control transfected cells, higher levels of poly-ubiquitinated RelA were detected in the presence of ORF73 (Figure 3C, compare lanes 3 and 6). These data suggest that ORF73 promotes the ubiquitination of nuclear RelA and its subsequent proteasomal-dependent degradation.

**ORF73 immunoprecipitates exhibit E3 ubiquitin-ligase activity**

As ORF73 does not possess known catalytic domains that could justify poly-ubiquitination activity, we investigated whether ORF73 could be part of a cellular E3 ubiquitin-ligase. To that end, ORF73 was immunoprecipitated from control transfected, or ORF73-expressing cells, and the respective immunoprecipitates were subjected to an in vitro ubiquitination reaction in the presence of exogenous ubiquitin-activating (E1) and conjugating enzyme (E2) UbcH5a, together with GST–RelA as a substrate. The presence of ligase activity directed towards RelA was analysed by immunoblot with an anti-GST serum. As shown in Figure 3D, in the presence of ATP, ORF73 immunoprecipitates specifically catalysed the poly-ubiquitination of RelA, indicating that ORF73 is a component of a cellular E3 ubiquitin-ligase with substrate specificity towards RelA.

**ORF73 interacts with ElonginC and Cullin5 reconstituting an E3 ubiquitin-ligase**

Recently, LANA encoded by ORF73 from KSHV was also shown to possess E3 ubiquitin-ligase activity, acting as a SOCS protein responsible for substrate recognition and specificity (Cai et al., 2006). As E3 ubiquitin-ligases must interact physically with their targets to exert their function, we investigated whether ORF73 was associated with RelA in vivo. In cells overexpressing RelA and co-expressing ORF73, the latter protein was able to efficiently co-immunoprecipitate RelA (Figure 4A).

We proceeded to test whether ORF73 could associate with other cellular components known to interact with SOCS-box-containing proteins, namely ElonginC and Cullin5. Transiently expressed ORF73 was able to co-immunoprecipitate with endogenous levels of ElonginC and Cullin5 (Figure 4B). The ability of ORF73 to promote RelA poly-ubiquitination, combined with its ability to interact with ElonginC and Cullin5, suggested that the inhibitory effect of ORF73 on NF-κB activity resided on its ability to assemble an EC5S E3 ubiquitin-ligase complex.

To confirm that inhibition of NF-κB by ORF73 was dependent on ElonginC and Cullin5, we tested whether ORF73 would inhibit NF-κB activity in cells in which the expression of those E3 ubiquitin-ligase components was suppressed using small interfering RNAs (siRNAs). Specific targeting of ElonginC and Cullin5 by siRNA was confirmed by a decrease in the expression levels of these two proteins (Figure 4C). When endogenous ElonginC and Cullin5 expression was inhibited, the ability of ORF73 to promote RelA poly-ubiquitination was considerably reduced (Figure 4D), suggesting that the mechanism used by ORF73 to inhibit NF-κB is dependent on the recruitment of cellular proteins to reconstitute an E3 ubiquitin-ligase.

**Mutation of the SOCS-box-like motif in ORF73 abrogates its inhibitory effect on NF-κB transcriptional activity**

SOCS-box-containing proteins interact with ElonginB/C and Cullin5/Rbx2 modules through specific degenerated amino-acid sequences referred as BC and Cul boxes, respectively (Kamura et al., 2004). Analysis of MuHV-4 ORF73 primary structure and by comparison with the previously identified BC and Cul5 boxes from LANA of KSHV (Cai et al., 2006), ORF73 appears to lack an obvious BC box. However, close to the C-terminal end, ORF73 presents a sequence homologous to LANA Cul5 box: VSLPLPVPGTTQCVTY (Figure 5A). To determine whether this region was involved in ORF73 E3 ubiquitin-ligase functions, we substituted SOCS-box consensus amino acids (underlined) with alanines, and named this ORF73 mutant protein ORF73–SOCS. Immunofluorescence experiments were performed to analyse whether the introduced mutations affected the subcellular localisation of the protein. As observed in Figure 5B, ORF73–SOCS localises in the nucleus of transfected cells. Strikingly, in ORF73–SOCS-expressing cells, and upon TNF stimulation, RelA nuclear levels are normal and comparable to those observed in control transfected cells (Figure 5B, panels d and f). Moreover, in TNF-stimulated cells, RelA and ORF73–SOCS colocalised in the nucleus (Figure 5B, panel f), demonstrating that the introduced mutations did not affect ORF73 subcellular localisation, whereas they affected the ability of ORF73 to promote RelA nuclear degradation. Immunoprecipitation experiments revealed that ORF73–SOCS still interacted with RelA (Figure 5C), whereas lost the ability to recruit ElonginC (Figure 5D) and to a lower extent Cullin5 (Figure 5E). Taken together, these data revealed the existence of two distinct functional domains in ORF73: a domain involved in RelA interaction, which is distinct from the motif implicated in the recruitment of ElonginC and Cullin5. Although the latter can be attributed to the ORF73 SOCS-box, the former remains to be identified.

Further experiments showed that the ORF73–SOCS mutant was unable to potentiate RelA poly-ubiquitination.
confirming that the amino-acid substitutions introduced disrupted the ORF73 SOCS-box domain, and that the presence of this motif is essential for ORF73 to function as a mediator of RelA poly-ubiquitination. To investigate whether ORF73–SOCS failure to promote RelA poly-ubiquitination would be reflected by its inability to suppress NF-κB transcriptional activity, we performed gene reporter assays in ORF73–SOCS-expressing cells. The effect of the expression of ORF73–SOCS on NF-κB transcriptional activity, in conditions of TNF stimulation, was assessed by quantifying the luciferase activity present in each experimental condition (Figure 6B). We observed that disruption of the ORF73–SOCS–RelA poly-ubiquitination present in each condition was assayed as described in (A). (C) ORF73-mediated poly-ubiquitination of endogenous RelA. HEK 293T cells transiently expressing ORF73 or control cells were stimulated with TNF (50 ng/ml) for 3 h or left unstimulated with or without an additional hour of treatment with MG132 (10 μM). Total cell lysates were subjected to immunoprecipitation with an anti-RelA antibody, and the presence of ubiquitinated RelA was examined by immunoblotting using an anti-ubiquitin (Ubiq) antibody. (D) ORF73 immunoprecipitates exhibit E3 ubiquitin-ligase activity in vitro. HEK 293T cells were transfected with or without an ORF73-expressing plasmid, as indicated. After culture, cells were lysed and total cellular extracts were subjected to immunoprecipitation with anti-Myc. Immunoprecipitates were incubated with recombinant ubiquitin-activating enzyme (E1) and conjugating enzyme (E2) UbcH5a, together with ubiquitin and GST–RelA as substrates. The reactions were resolved by SDS–PAGE, and the presence of ubiquitinated RelA was analysed by immunoblotting with an anti-GST antibody. –, without; +, with; α, anti; IP, immunoprecipitation; PD, pull down; TCL, total cellular lysates; WB, western blotting.
SOCS-box motif impaired ORF73 ability to inhibit NF-κB-mediated signalling.

Taken together, the data obtained with the ORF73–SOCS mutant corroborate that ORF73 functions as a SOCS-box-containing protein to assemble an EC5SORF73 E3 ubiquitin-ligase that recognises RelA and promotes its poly-ubiquitination. This leads to the subsequent proteasomal-dependent degradation of RelA, resulting in a strong termination of RelA/NF-κB activity.

Inhibition of NF-κB signalling is essential for MuHV-4 latency

To directly investigate the biological relevance of inhibition of NF-κB signalling in gammaherpesvirus pathogenesis, we generated a recombinant MuHV-4 in which the ORF73 gene was modified to recapitulate the amino-acid substitutions of the ORF73 SOCS-box mutant, designated vSOCS. To assure that any phenotypic alteration in vSOCS was due to the engineered mutations in the SOCS-box and not from any spurious mutation introduced during mutagenesis, a second independent recombinant virus was engineered (vSOCSi). To characterise the role of the introduced mutations in a natural context of infection, we started to compare the kinetics of viral replication in vitro and during the acute phase of infection in lungs of Balb/c mice following intranasal inoculation. For comparative purposes, the viruses analysed included the vSOCS mutants alongside wild-type MuHV-4 (vWT) and a previously described (Fowler et al., 2003)
ORF73 frameshift mutant (v73FS) that encodes only the 163 amino acids of the N-terminus of the protein. All these viruses were analysed for genome integrity (Supplementary Figure S1) and showed identical in vitro growth (Supplementary Figure S2), as well as normal replication in acutely infected lungs (Figure 7A). Next, we proceeded to investigate the role of the introduced mutations for the ability of MuHV-4 to induce the expansion of latency in GC B cells.

To this end, we used three independent, but complementary, experimental assays: ex vivo explant co-culture assays to measure latent infection in total splenocytes, flow cytometry coupled to limiting dilution and real-time PCR to quantify the frequency of viral DNA-positive GC B cells, and in situ hybridisation analysis to identify virally infected cells within the spleen, as described earlier (Pires de Miranda et al., 2008). All three assays used were concurrent in that disruption of the SOCS-box motif in ORF73 leads to a severe latency deficit, characterised by its inability to induce the expansion of latent infection in GC B cells and persistence in the host (Figure 7B–D). This phenotype was comparable to the previously reported phenotype (Fowler et al., 2003) of a MuHV-4 lacking a functional ORF73 (v73FS in Figure 7B–D). Thus, at day 14 post-infection, the levels of the explant co-culture assay (Figure 7B) and the frequencies of infection in GC B cells (Figure 7C) obtained for the SOCS-box mutants were identical to those obtained for v73FS and significantly lower.
when compared with vWT. In good agreement, in situ hybridisation analysis of spleen sections from vSOSCs- or v73FS-infected mice exhibited a complete lack of expansion of latently infected cells (Figure 7D, panels b and c). This was in clear contrast with vWT where large clusters of latently infected cells were observed within GCs (Figure 7D, panel a).

Discussion

In this study, we provide compelling evidence that the ORF73 protein from the lymphotropic gammaherpesvirus MuHV-4 is a strong terminator of NF-κB-dependent transcription. Several viral proteins have been shown to interfere with the NF-κB pathway (Hiscott et al., 2006). Here, we report the first example of a viral protein modulating NF-κB signalling/activity in the nucleus by mimicking a physiological regula-

tory pathway of NF-κB response termination. We show that the mechanism involves the assembly by ORF73 of an E2S E3 ubiquitin-ligase. This complex targets nuclear-activated RelA for poly-ubiquitination and subsequent proteasomal degradation. The recruitment of ElonginB/C and Cullin5 is directed through an unconventional SOCS-box present in ORF73, whereas RelA recognition is mediated through an independent, as yet, unidentified structural motif. Thus, MuHV-4 has evolved to encode a protein that, in a manner equivalent to cellular SOCS1 (Ryo et al., 2003), efficiently terminates the NF-κB response. Taking into account that herpesviruses have co-evolved with their hosts, this finding emphasises the physiological relevance of the recently described regulation of the NF-κB pathway through poly-ubiquitination and proteasomal degradation of promoter-bound RelA. This regulatory mechanism has been proposed to function in synergy with resynthesised IkBz to efficiently terminate NF-κB responses (Saccani et al, 2004). The advantage to the virus is obvious. Independently of the nature of the stimulus, MuHV-4 directly targets activated NF-κB dimers bound to κB sites efficiently shutting down transcription of specific genes. This specificity is unique to MuHV-4 and contrasts with other viral mechanisms of NF-κB inhibition that modulate upstream signalling events, such as the IKK complex, thus affecting collateral cellular signalling pathways. However, this property of a viral protein assembling an E2S E3 ubiquitin-ligase is not exclusive to ORF73 of MuHV-4. The Vif protein encoded by human immunodeficiency virus-1 (Mehle et al., 2004), the E4ORF6 from adenovirus (Querido et al, 2001) and the ORF73 homologue LANA from KSHV (Cai et al., 2006), have recently been shown to assemble E2S E3 ubiquitin-ligase complexes through unconventional SOCS-box motifs. In the case of LANA, poly-ubiquitination is directed towards the cellular proteins p53 and VHL. The authors suggested that manipulation of these tumour suppressors by LANA could potentially create a propitious environment for the maintenance of latent infection and progression of KSHV-associated tumours. Owing to the absence of an amenable animal model of infection, the direct in vivo role of this LANA function was not possible to assess.

Unlike KSHV, the biological significance of the inhibition of NF-κB signalling by ORF73 of MuHV-4 for the pathogenesis of gammaherpesvirus infections can be directly addressed due to the availability of a murine animal model of infection (Simas and Efstatiou, 1998). Here, we show that a MuHV-4 recombinant virus with a disrupted SOCS-box motif abrogated the ability of the virus to expand in GC B cells and persist in the host. Although we cannot formally exclude that mutating four amino-acid residues in the SOCS-box motif in ORF73 of MuHV-4 is compromising its putative role as a viral episme maintenance protein, the findings presented here sustain the interpretation that inhibition of NF-κB activation is critical for amplification of latent virus in GC B cells and for persistence in the host. Notably, it has been recently shown that blocking the inhibition of NK-κB signalling mediated by EBV in latently infected cell lines results in the loss of virus genome copy number (Lu et al., 2008), providing evidence of a link between viral genome maintenance and NK-κB inhibition.

Initiation of a GC reaction is reliant not only on specific antigen stimulation through the immunoglobulin B-cell receptor but also on the interaction between the TNFR family
member CD40, expressed in B cells, and its ligand CD154, expressed by helper T cells. This molecular event triggers the activation of NF-κB transcription factors, the main downstream effectors of CD40 signalling. However, in the GC the gene expression profile of B cells changes markedly. In clear contrast with what is observed in naïve B cells, GC B cells fail to express most CD40 and NF-κB target genes (Basso et al., 2004; Klein and Dalla-Favera, 2008; Shaffer et al., 2001). Moreover, in GC B cells, the proteins c-Rel and RelA are localised in the cytoplasm, an indication of NF-κB inactivity and absence of CD40 signalling (Basso et al., 2004). It is noted that within the light zone of GCs and in memory B cells NF-κB members are again localised in the nucleus (Basso et al., 2004), which is indicative that activation of these transcriptional factors is needed for exiting from the proliferative GC stage promoting further B-cell differentiation. Taken together, these data suggest that during B-cell responses, NF-κB signalling needs to be transiently switched off during GC proliferation. Putting our present data into this context, we propose that inhibition of NF-κB by ORF73 is essential to promote the proliferation of latently infected B cells within GCs. According to our premise, ORF73 function relies on prior NF-κB activation and translocation of transcriptionally active dimers to the nucleus, which is critical for B-cell activation and initiation of GCs. This interpretation concurs with a previous study showing that constitutive inhibition of NF-κB from the initial stages of MuHV-4 infection impairs the establishment of latency (Krug et al., 2007). Hence, MuHV-4 modulation of NF-κB signalling must be tightly regulated to support GC formation, which needs transcriptionally active NF-κB, whereas progression into, and maintenance of a proliferative GC reaction requires the

**Figure 7** ORF73–SOCS virus shows a strong latency deficit. (A) ORF73–SOCS recombinant virus exhibits normal replication in the lung. Wild-type BALB/c mice were intranasally infected with 10⁴ p.f.u. of the indicated viruses. At the indicated days post-infection, lungs were removed and infectious viruses were titrated by plaque assay. (B) BALB/c mice were intranasally infected with 10⁴ p.f.u. of the indicated viruses. At day 14 post-infection, latent viruses in spleens were titrated by infectious centre assay. Each point represents the titre of an individual mouse. The dashed line represents the limit of detection of the assay. (C) BALB/c mice were intranasally infected with 10⁴ p.f.u. of the indicated viruses. At 14 days post-infection, reciprocal frequencies of viral infection in purified GC B cells (B220⁺/PNAhigh) were determined by limiting dilution and real-time PCR. Data were obtained from pools of five spleens per group. Bars represent the frequency of viral DNA-positive cells with 95% confidence intervals. (D) BALB/c mice were intranasally infected with 10⁴ p.f.u. of the indicated viruses. At day 14 post-infection, spleens were removed and processed for *in situ* hybridisation using probes derived from viral miRNAs 1–6. Panels (a–c) show representative spleen sections from each group of viruses. All sections are magnified × 200.
prompt termination of the NF-κB response. Thus, while ORF73 promotes the proliferation of MuHV-4-infected cells preventing premature differentiation, another yet unidentified signal must exist, viral or foreign, supporting further differentiation of latently infected centroblasts into long-lived memory B cells. Such timely regulation is consistent with the pattern of ORF73 transcription that we have shown before to be restricted to GC B cells but not to newly formed or follicular B cells (Marques et al., 2003).

Effective colonisation of the host by gammaherpesviruses requires the proliferation of latently infected B cells (Stevenson, 2004). Herein, we provide evidence for a novel ORF73 function, as a mediator of NF-κB activity termination through the assembly of an Elongin/Cullin/SOCS complex (E3) ubiquitin-ligase that targets the NF-κB subunit RelA. By mimicking GC physiological inhibition of NF-κB, ORF73 promotes the development of MuHV-4-driven GC-like reactions to expand the host pool of latently infected cells. Given the intimate association of lymphoproliferative disease with gammaherpesvirus persistent infection, this study reinforces NF-κB as a putative target for therapeutic intervention in such virus-driven malignancies.

Materials and methods

Plasmids

ORF73 expression plasmid was amplified by PCR from MuHV-4 genome, and the respective PCR product was cloned into pCMV-Myc (Clontech). pCMV-Myc encoding ORF73–SOCS was generated by site-directed mutagenesis using QuickChange kit (Stratagene). Myc-tagged versions of RelA, p50 and c-Rel; RelA chimaeras; and p45 (κB-luc) or p52 (TetO-luc) were described earlier (Winkler et al., 1996; Anrather et al., 1999). Histidine-tagged ubiquitin plasmid was kindly offered by Dr D Bohmann. Flag-tagged ElonginC and SOCS1 expression plasmids were provided by Dr E Burstein. Dr X-F Yu provided the Myc-tagged version of Cullin5.

Immunological reagents

ORF73 antiserum was generated by immunisation of New Zealand white rabbits (Abcam) with purified GST–ORF73 protein. Anti-RelA, anti-κBz, anti-Cul5, anti-ElonginC, anti-ubiquitin and anti-LaminB antibodies were purchased from Santa Cruz Biotechnology. Antibodies directed to c-Myc and Flag epitopes were from Clontech and Sigma, respectively. Actin was detected with a rabbit anti-actin polyclonal antibody (Sigma). Anti-GST and horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences. Fluorochrome-labelled secondary antibodies were from Jackson Immunoresearch.

Tissue culture, DNA/siRNA transfection

HEK 293T and NIH-3T3-CRE cells were cultured in DMEM plus 10% FCS, 2 mM glutamine, and 100 U/ml of penicillin–streptomycin. BHK-21 cells were cultured in GMEM supplemented as above plus 10% tryptose phosphate broth. Plasmid DNA and siRNAs were transfected with 300 ng of reporter vectors, 900 ng of each NF-κB member and 900 ng of ORF73/ORF73–SOCS expression plasmids.

In all transfections, a β-galactosidase expression plasmid (300 ng) was used to normalise luciferase values. After 48 h in culture, cells were left unstimulated or stimulated with appropriate TNF concentrations for 7 h. Cells were washed in PBS and lysed in 120 μl of reporter assay lysis buffer (Promega). Luciferase and β-galactosidase activities were assayed using Luciferin (Promega) and Gâlacron (Tropix), respectively. Light emission in each sample was quantified in a luminometer. Results are shown as the fold induction relative to luciferase activity measured in unstimulated or control transfected cells.

EMSA

EMSA were performed using the Lightshift Chemiluminescent EMSA kit (Pierce) for the NF-κB consensus oligonucleotide probe (5’-AGTTAGGAGGACTTCCCCGTC-3’, from the immunoglobulin promoter) 5’ end-labelled with biotin (Thermo Scientific Biopolymers). Binding reactions were made in a total volume of 20 μl by adding 5 μg of nuclear extracts to 20 fmol of probe in binding buffer (20 mM HEPES (pH 8.0); 50 mM NaCl; 1 mM EDTA; 5% glycerol; 0.05 μg/μl poly [dI-dC] and 0.5 mM DTT). After incubation at room temperature (r.t.) for 30 min, the electromobility of the probe was analysed in 6% native PAGE. For the supershift assays, antibodies recognising RelA, ORF73 or Actin were added to nuclear extracts at a final concentration of 0.05 μg/ml, and incubated at r.t. for 1 h, prior to the addition of the probe 5’ end-labelled with [32P]ATP (Amersham Life Science).

Immunofluorescence analysis

HEK 293T cells grown on poly-1-lysine-coated coverslips were transiently transfected with 1 μg of ORF73-expressing plasmid. After 24 h, cells were incubated with medium alone or stimulated with TNF (50 ng/ml) for 15 min. Cells were incubated in fixing solution (5% formaldehyde and 2% sucrose in PBS) for 15 min, and permeabilised (0.1% Triton X-100 in PBS) for 5 min. Immunostaining was performed with the appropriate antibodies diluted in PBS. Following staining, coverslips were washed and mounted onto microscope slides with Mowiol.

Immunoprecipitations

Transiently transfected HEK 293T cells with expression plasmids encoding RelA (3 μg), Cullin5 (4 μg), ElonginC (2 μg) and/or ORF73 (3 μg) were disrupted in ice-cold lysis buffer containing 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM NaF, 100 μM Na2VO4, and a cocktail of protease inhibitors (Complete; Roche). Cleared supernatants were processed for immunoprecipitation essentially as described (Pires de Miranda et al., 2008).

RelA ubiquitination in vivo

For analysis of endogenous RelA ubiquitination, total cellular lysates, prepared with lysis buffer as above, were immunoprecipitated with anti-RelA antibody and analysed by immunoblotting with anti-ubiquitin antibody. Levels of in vivo ubiquitinated over-expressed RelA were determined by pull-down of histidine-tagged ubiquitin (His6-ubiquitin) with Ni-NTA agarose beads. Cells were transfected with expression plasmids carrying His6-ubiquitin (4 μg), RelA (3 μg) and/or ORF73 (3 μg). When appropriate, cells were incubated in the presence of 10 μM of MG132 (Calbiochem). Transfected cells were lysed with ice-cold urea buffer containing 8 M urea, 50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 1% Triton X-100, 10 mM imidazole, 1 mM Na2VO4 and Complete. Cleared lysates were incubated for 3 h at 4°C with Ni-NTA beads. After incubation, beads were collected by centrifugation and washed three times with urea buffer. Proteins were eluted and denatured by boiling in Laemmli’s buffer and analysed by immunoblotting with anti-RelA antibodies.

RNA interference

All oligonucleotides were purchased from Ambion. Pre-designed siRNAs for human ElonginC or Cullin5 were transiently transfected into HEK 293T cells, at a final concentration of 30 nM each. Non-targeting siRNAs were used as controls. At 48 h post-transfection, cells were processed for Ni-NTA pull down.

In vitro ubiquitination assay

Cell lysates from HEK 293T cells transiently expressing ORF73, or control transfected, were subjected to immunoprecipitation with anti-Myc. Immunoprecipitates were resuspended in reaction buffer (40 mM HEPES (pH 7.4), 60 mM potassium acetate, 1 mM EDTA, 2 mM DTT, 5 mM MgCl2 and 10% glycerol) supplemented with recombinant ubiquitin (2.5 μg) (Biomol International), E1 (50 ng), UbH5a E2 (100 ng) (Calbiochem), GST–RelA (2.5 μg) and/or

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ATP-regenerating buffer (Biomol International). Reactions were incubated for 1 h at 30°C, resolved by SDS–PAGE and analysed by immunoblotting with anti-GST antibody.

**Generation of recombinant viruses**

MLV-Δ4 vSVGc/pSCCI recombinant viruses (with the amino-acid residues at positions 199, 202, 203 and 206 mutated to alanines) were independently generated by mutagenesis of the viral genome cloned as a bacterial artificial chromosome (BAC) (Adler et al., 2000). pCMVmyc-ORF73–SOCS was digested with HindIII and PciI to isolate the fragment harbouring the desired mutations, which was inserted into the BamHI-G genomic clone. Recombinant BamHI-G fragment was subcloned into the BamHI site of PST76K-SR shuttle plasmid. Shuttle plasmid was transformed into an Escherichia coli strain (DH10B) containing the wild-type MHV-68 BAC (pHA3). Following a multistep selection procedure, recombinant BAC clones were identified by the loss of the internal ORF73 KpnI restriction site. Both viruses were reconstituted as described (Pires de Miranda et al., 2008). v73F5 recombinant virus was reported earlier (Fowler et al., 2003).

**Analysis of recombinant viruses**

BALB/c mice (Instituto Gulbenkian de Ciência, Portugal) with 6–8 weeks of age were intranasally inoculated with 10^6 p.f.u. in 20 μl of PBS under halothane anaesthesia. At 3, 7, 10 and 14 days post-infection, lungs or spleens were removed and processed for subsequent analysis. Infectious virus titers in freeze-thawed lung homogenates were determined by suspension assay using BHK-21 cells. Latent viruses were examined using explant co-cultures of single-cell suspension splenocytes with BHK-21 cells. Plates were incubated for 4 (suspending assay) or 5 days (co-culture assay), fixed with 10% formal saline and counterstained with toluidine blue. Viral plaques were counted with a plate microscope. Frequencies of virus-genome-positive cells were determined by limiting dilution combined with real-time PCR, as described earlier (Marques et al., 2003). GC B-cell (B220^+^, PNA^hi^) populations were cytometry purified from pools of five spleens using a BD FACSAria Flow Cytometer (BD Biosciences). The purity of sorted populations was always >97%, as analysed by flow cytometry. Real-time PCR reactions were performed as reported (Pires de Miranda et al., 2008). In situ hybridisation was performed on formalin-fixed, paraffin-embedded spleen sections using digoxigenin-labelled riboprobes, generated by T7 transcription of pEHI.4 (Simas et al., 1998).

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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**References**

Adler H, Messerele M, Wagner M, Koszinowski UH (2000) Cloning and mutagenesis of the murine gammaherpesvirus 68 genome as an infectious bacterial artificial chromosome. J Virol 74: 6964–6974

Amrander J, Ciszmadia V, Soares MP, Winkler H (1999) Regulation of NF-kappaB RelA phosphorylation and transcriptional activity by p21(ras) and protein kinase Ceta in primary endothelial cells. J Biol Chem 274: 13594–13603

Arenzana-Seisdedos F, Turpin P, Rodriguez M, Thomas D, Hay RT, Virelizier JL, Dargentom C (1997) Nuclear localization of I kappa B alpha promotes active transport of NF-kappaB from the nucleus to the cytoplasm. J Cell Sci 110 (Part 3): 369–378

Ballestas ME, Kourilsky P, Israel A (1992) NF-kappa B and related mechanisms of assembly. Annu Rev Immunol 10: 641–644

Basso K, Klein U, Dalla-Favera R (2008) Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol 8: 22–33

Basso K, Klein U, Dalla-Favera R (2008) Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol 8: 22–33

Blank V, Kourilsky P, Israel A (1992) NF-kappaB and related proteins: Rel/dorsal homologies meet ankyrin-like repeats. Trends Biochem Sci 17: 135–140

Cai QL, Knight JS, Verma SC, Zald P, Robertson ES (2006) EC5S ubiquitin complex is recruited by KSHV latent antigen LANA for degradation of the VHL and p53 tumor suppressors. PLoS Pathog 2: e116

Chen LF, Greene WC (2004) Shaping the nuclear action of NF-kappaB. Nat Rev Mol Cell Biol 5: 392–401

Choi SH, Park KJ, Ahn BY, Jung G, Lai MM, Hwang SB (2006) Hepatitis C virus nonstructural 5B protein regulates tumor necrosis factor alpha signaling through effects on cellular IkappaB kinase. Mol Cell Biol 26: 3048–3059

Damania B (2004) Oncogenic gamma-herpesviruses: comparison of viral proteins involved in tumorigenesis. Nat Rev Microbiol 2: 655–668

Fowler P, Marques S, Simas JP, Estathiou S (2003) ORF73 of murine herpesvirus-68 is critical for the establishment and maintenance of latency. J Gen Virol 84: 3405–3416

Grundhoff A, Ganem D (2003) The latency-associated nuclear antigen of Kaposi’s sarcoma-associated herpesvirus permits replication of terminal repeat-containing plasmids. J Virol 77: 2779–2783

Hall KT, Giles MS, Goodwin DJ, Calderwood MA, Markham AF, Whitehouse A (2000) Characterization of the herpesvirus saimiri ORF73 gene product. J Gen Virol 81: 2653–2658

Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 25: 6844–6867

Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 25: 6844–6867

Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 25: 6844–6867

Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 25: 6844–6867

Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 25: 6844–6867

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Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 25: 6844–6867

Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 25: 6844–6867

Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 25: 6844–6867

Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 25: 6844–6867

Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 25: 6844–6867

Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 25: 6844–6867

Hiscott J, Nguyen TL, Argue...
Revilla Y, Callejo M, Rodriguez JM, Culebras E, Nogal ML, Salas ML, Vinuela E, Fresno M (1998) Inhibition of nuclear factor kappaB activation by a virus-encoded IkappaB-like protein. J Biol Chem 273: 5405–5411

Rickinson AB, Kieff E (2001) Epstein–Barr virus. In Field’s Virology, Fields BN, Knipe DM, Howley PM, Griffin DE (eds) Vol. 2, 4th edn, pp 2655–2670. Philadelphia: Lippincott Williams and Wilkins

Ryo A, Suizu F, Yoshida Y, Perrem K, Liou YC, Wulf G, Rottapel R, Yamaoa K, Lu KP (2003) Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. Mol Cell 12: 1413–1426

Saccani S, Marazzi I, Beg AA, Natoli G (2004) Degradation of promoter-bound p65/RelA is essential for the prompt termination of the nuclear factor kappaB response. J Exp Med 200: 107–113

Saccani S, Pantano S, Natoli G (2003) Modulation of NF-kappaB activity by exchange of dimers. Mol Cell 11: 1563–1574

Shaffer AL, Rosenwald A, Hurt EM, Giltinan JM, Lam LT, Pickeral OK, Staudt LM (2001) Signatures of the immune response. Immunity 15: 375–385

Simas JP, Bowden RJ, Paige V, Efstathiou S (1998) Four tRNA-like sequences and a serpin homologue encoded by murine gammaherpesvirus 68 are dispensable for lytic replication in vitro and latency in vivo. J Gen Virol 79 (Part 1): 149–153

Simas JP, Efstathiou S (1998) Murine gammaherpesvirus 68: a model for the study of gammaherpesvirus pathogenesis. Trends Microbiol 6: 276–282

Stevenson PG (2004) Immune evasion by gamma-herpesviruses. Curr Opin Immunol 16: 450–462

Sunil-Chandra NP, Arno J, Fazakerley J, Nash AA (1994) Lymphoproliferative disease in mice infected with murine gammaherpesvirus 68. Am J Pathol 145: 818–826

Tait SW, Reid EB, Greaves DR, Wileman TE, Powell PP (2000) Mechanism of inactivation of NF-kappa B by a viral homologue of I kappa b alpha. Signal-induced release of i kappa b alpha results in binding of the viral homologue to NF-kappa B. J Biol Chem 275: 34656–34664

Tanaka T, Grusby MJ, Kaisho T (2007) PDLIM2-mediated termination of transcription factor NF-kappaB activation by intranuclear sequestration and degradation of the p65 subunit. Nat Immunol 8: 584–591

Thorley-Lawson DA (2001) Epstein–Barr virus: exploiting the immune system. Nat Rev Immunol 1: 75–82

Verma SC, Lan K, Robertson E (2007) Structure and function of latency-associated nuclear antigen. Curr Top Microbiol Immunol 312: 101–136

Weissman AM (2001) Themes and variations on ubiquitylation. Nat Rev Mol Cell Biol 2: 169–178

Winkler H, Brostjan C, Csizmadia V, Natarajan G, Anrather J, Bach FH (1996) The intron-exon structure of the porcine E-selectin-encoding gene. Gene 176: 67–72

Yates JL, Warren N, Sugden B (1985) Stable replication of plasmids derived from Epstein–Barr virus in various mammalian cells. Nature 313: 812–815

Yoshimura A, Naka T, Kubo M (2007) SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol 7: 454–465

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