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Viral hijacking of the host ubiquitin system to evade interferon responses
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The post-translational attachment of ubiquitin or ubiquitin-like modifiers (ULMs) to proteins regulates many cellular processes including the generation of innate and adaptive immune responses to pathogens. Vice versa, pathogens counteract immune defense by inhibiting or redirecting the ubiquitination machinery of the host. A common immune evasion strategy is for viruses to target host immunoproteins for proteasomal or lysosomal degradation by employing viral or host ubiquitin ligases. By degrading key host adaptor and signaling molecules, viruses thus disable multiple immune response pathways including the production of and response to interferons as well as other innate host defense mechanisms. Recent work further revealed that viruses inhibit the ligation of ubiquitin or ULMs or remove ubiquitin from host cell proteins. Thus, viruses succeed in either stabilizing negative regulators of innate immune signaling or thwart host cell proteins that are activated by ubiquitin or ULM-modification.

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Essential role of the UPS for viral entry and replication
Viruses utilize the host ubiquitin pathway at each stage of their life cycle including entry, genome replication, and egress [3,4]. This is illustrated by recent reports that diverse viral families are unable to enter cells or replicate if the ubiquitin proteasome system is disabled by proteasome inhibitors, a treatment that also depletes free ubiquitin. Such treatment trapped viruses in the endosomes and dense lysosomes, but did not affect initial endocytosis [5]. By contrast, proteasomal inhibitors blocked endocytosis of influenza virus due to the blockade of ubiquitination of epsin 1, a cargo specific adaptor for clathrin [6]. For herpes simplex virus it was shown that UPS activity was required at a post-penetration step to transport the incoming capsid to the nucleus [7]. Thus, several unrelated viral families depend on the UPS system even before the onset of viral replication. In poxvirus-infected cells, two groups reported that inhibitors of the proteasome or of E1 enzymes delayed expression of early viral genes and blocks the formation of virus replication factories resulting in complete inhibition of intermediate and late gene expression [8,9]. The UPS system is also required for the replication of coxsackie virus 3B since proteasome inhibition, ubiquitin knockdown, or increasing deubiquitinase

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activities all prevented CV3B replication [10]. Similarly, replication of human respiratory syncitial virus was decreased in the presence of proteasome inhibitors [11]. Although it has been speculated that proteasome inhibitors in clinical use might have anti-viral activity [8], it has yet to be demonstrated that these compounds are able to inhibit viral replication in vivo. Taken together, these studies highlight the importance of the UPS for viral infection.

**Ubiquitin-mediated viral evasion of interferon-induction**

One reason the UPS is essential for viral replication is that many viruses use or inhibit the UPS to modulate the innate immune response, particularly the production and function of type I interferons (IFN), which include multiple IFNα subtypes and IFNβ. While the anti-viral activities of IFN have been known for over 50 years, the molecular details of how virus infection actually triggers IFN synthesis have only emerged in the past decade (for a recent review see [12]). As the roles and regulation of receptors, adaptors, signal transducers, and transcription factors in IFN induction come to light, corresponding viral counter-mechanisms are uncovered (reviewed in [13]) including many that involve the ubiquitin pathway [14]. The current model of virus-mediated induction of IFNβ and the IFN mediated responses with viral interventions is illustrated in Figure 1.

**Viral inhibition of IRF3 activation**

Examples for ubiquitin-mediated degradation of components of the signaling pathway leading to IFN synthesis are pestiviruses such as Classical swine fever virus (CSFV) and Bovine viral diarrhea virus (BVDV) [15-17] as well as rotaviruses [18,19]. These viruses induce proteasome-dependent degradation of IRF3. HIV-1 also mediates IRF3 degradation via Vpr-directed and Vif-directed ubiquitination of the protein [20]. Similarly, Ebola virus VP35 protein has been shown to inhibit IFN synthesis in immune cells by promoting SUMOylation of IRF7 by the cellular E3 ligase TRIM25, an E3 ligase required for ubiquitin-dependent degradation of IRF3. HIV-I also mediates IRF3 degradation via PIAS1 [21]. IFN expression can also be impaired by blocking the signaling pathways leading from virus detection to transcription factor activation. This is well exemplified by the inhibition of RIG-I activity by the NS1 protein of influenza A virus. Gack et al. recently showed that this protein interacts with and blocks the activity of TRIM25, an E3 ligase required for ubiquitin-dependent interaction between RIG-I and IPS1 [22]. NS1 was shown to prevent TRIM25-dependent ubiquitination of RIG-I and thus activation of IRF3-dependent IFN secretion thereby allowing the virus to evade its anti-viral effects. Moreover, in the absence of this phenotype mutant virus was highly attenuated thereby demonstrating its importance for virus replication and transmission.

**Viral inhibition of NFκB activation**

To prevent NFκB activation viral proteins either directly bind to NFκB to inhibit its translocation to the nucleus or they mediate degradation of NFκB. The poxviral protein CP77 prevents NFκB activation by binding with its N-terminal six ankyrin repeat region to the p65 subunit of NFκB [23]. Another poxvirus protein that contains pyrin domain containing protein M013 also has been shown to bind to RelA/p65 and prevent localization of NFκB to the nucleus [24]. African swine fever virus even encodes a homolog of human IκB, A238L that is resistant to phosphorylation and thus irreversibly binds and inactivates NFκB [25]. A direct degradation of p65/RelA was reported for the murid herpesvirus-4 (MuHV-4) latency associated protein ORF73. MuHV-4 ORF73 has an unconventional suppressor of cytokine signaling (SOCS)-box motif that associates with the host ubiquitin-ligase complex ElonginC/Cullin5/SOCS to mediate poly-ubiquitination and subsequent proteasomal degradation of p65/RelA [26]. Functional deletion of SOCS-box motif in ORF73 ablated NFκB degradation and resulted in suppressed viral replication in germinal center B cells and prevented persistent infection in mice indicating that suppression of NFκB activity is essential for viral persistence. Viruses also utilize host machinery that negatively regulates NFκB activation to turn off the innate immune response. For instance, the host de-ubiquitin (DUB) enzyme A20 terminates TLR-dependent NFκB activation by removing ubiquitins from ATF6. A20 is prematurely upregulated by measles virus P protein thus preventing NFκB activation [27]. An unusual targeting of the ubiquitin complex that mediates IκB degradation was observed for the rotavirus NSP1 protein. NSP1 mediates the ubiquitination and degradation of the F-Box substrate recognition protein, β-transducin repeat containing protein (β-TrCP) that binds to and degrades IκB through the ubiquitin-ligase complex Skp-1/Cul1/F-Box (SCF) [28]. However, the same SCFβ-TrCP complex is utilized by HIV-1 Vpu to target host anti-viral effectors CD4 and bone marrow stromal antigen-2 (BST2). This demonstrates a case in which different viruses utilize the same host ubiquitin ligase in different contexts to target host proteins to their advantage.

**Viral use of the ubiquitin system to inhibit IFN-dependent signal transduction**

In addition to preventing the induction of IFN, viruses also inhibit the signal transduction pathway triggered upon binding of IFN to its receptor [13]. A key event in this signal cascade is the activation and nuclear translocation of STAT proteins that are frequent targets of viral counter-mechanisms. For example, parainfluenza simian virus 5 is known to degrade STAT1 via a proteasome-dependent mechanism [29] that involves co-opting a host cell E3 ligase [30]. Respiratory syncytial virus was found to degrade STAT2 through formation of an E3 ligase complex that includes both host proteins (Cul2, Rbx) as well as the viral NS1 protein [31]. More recently, Ashour et al. have shown that the Dengue virus NS5 protein mediates ubiquitination and proteasome-dependent degradation of STAT2
although the host cell molecules required for this are unknown [32]. Interestingly, this effect is exhibited only when cells sense the presence of pathogens via pattern-recognition receptors (PRRs) such as extracellular toll-like receptors (TLRs), or intracellular RIG-I like helicases (RLHs) or DNA binding proteins. PRRs recognize molecular signatures of pathogens, the single or double stranded RNA or DNA. TLR3 activates Toll-interleukin (IL)-1 resistance (TIR) domain-containing adaptor inducing IFN-b (TRIF), whereas TLR7 and 9 activate myeloid differentiation factor 88 (MyD88). Melanoma differentiation-associated gene (mda-5) and retinoic acid-inducible gene-1 (RIG-I) activate tumor necrosis factor (TNF) receptor associated factor 3 (TRAF3) via Cardif/MAVS/IP-10. Activated TRAF3 interacts with TRAF family member associated NF-kB activator (TANK), TANK binding kinase 1 (TBK-1), and the related IxB Kinase (IKK) to phosphorylate and translocate IRFs in the nucleus. In a parallel pathway, NF-kB activation is initiated by poly-ubiquitination of TRAF6 and receptor interacting protein-1 (RIP1). These adaptors recruit transforming growth factor (TGF) β—activating enzyme 1 (TAK1), NF-kB essential modifier (NEMO), and IxB Kinases (IKK) into a multi protein complex that phosphorylates inhibitor of NF-kB (I-kB) resulting in its ubiquitination and degradation by proteasomes. The degradation of I-kB reveals the nuclear localization signal of NF-kB that then translocates to the nucleus. In the nucleus, activated IRFs and NF-kB assemble at the IFN stimulated response element (ISRE) to induce IFN stimulated genes (ISGs). Viral intervention of these pathway proteins are marked in the figure as numbers and listed below in the virus (protein)[ref] format:

1. Influenza A virus (NS1) [22]; 2. Measles virus (P) [27]; 3. African swine fever virus (A238L) [25], Rotavirus (NSP1) [28]; 4. Murid herpesvirus-4 (ORF73) [26], Poxvirus (CP77) [23]; 5. classical swine fever virus [15], Bovine viral diarrhea virus [16], Rotavirus [18, 19], HIV-1 (Vpr, Vif) [20]; 6. Ebola virus (VP35) [21]; 7. Para influenza simian virus 5 [29, 30]; 8. Respiratory syncytial virus (NS1) [31]; 9. Hepatitis C virus [70]; 10. Human cytomegalovirus (US2, US11) [3], Kaposi’s Sarcoma associated herpesvirus (KSHV) (K3, K5) [1, 2]; 11. Influenza virus (NS1A) [42, 46–49], vaccinia virus (E3) [51]; 12. Coronavirus (PLP) [53]; 13. HIV-1 (E5D) [57, 61, 64–66], KSHV (K5) [63].

Examples of viral protein mediated intervention of host pathogen stimulated IFN stimulation pathways using UPS. Host anti-viral signals are initiated when cells sense the presence of pathogens via pattern-recognition receptors (PRRs) such as extracellular toll-like receptors (TLRs), or intracellular RIG-I like helicases (RLHs) or DNA binding proteins. PRRs recognize molecular signatures of pathogens, the single or double stranded RNA or DNA. TLR3 activates Toll-interleukin (IL)-1 resistance (TIR) domain-containing adaptor inducing IFN-b (TRIF), whereas TLR7 and 9 activate myeloid differentiation factor 88 (MyD88). Melanoma differentiation-associated gene (mda-5) and retinoic acid-inducible gene-1 (RIG-I) activate tumor necrosis factor (TNF) receptor associated factor 3 (TRAF3) via Cardif/MAVS/IP-10. Activated TRAF3 interacts with TRAF family member associated NF-kB activator (TANK), TANK binding kinase 1 (TBK-1), and the related IxB Kinase (IKK) to phosphorylate and translocate IRFs in the nucleus. In a parallel pathway, NF-kB activation is initiated by poly-ubiquitination of TRAF6 and receptor interacting protein-1 (RIP1). These adaptors recruit transforming growth factor (TGF) β—activating enzyme 1 (TAK1), NF-kB essential modifier (NEMO), and IxB Kinases (IKK) into a multi protein complex that phosphorylates inhibitor of NF-kB (I-kB) resulting in its ubiquitination and degradation by proteasomes. The degradation of I-kB reveals the nuclear localization signal of NF-kB that then translocates to the nucleus. In the nucleus, activated IRFs and NF-kB assemble at the IFN stimulated response element (ISRE) to induce IFN stimulated genes (ISGs). Viral intervention of these pathway proteins are marked in the figure as numbers and listed below in the virus (protein)[ref] format: 1. Influenza A virus (NS1) [22]; 2. Measles virus (P) [27]; 3. African swine fever virus (A238L) [25], Rotavirus (NSP1) [28]; 4. Murid herpesvirus-4 (ORF73) [26], Poxvirus (CP77) [23]; 5. classical swine fever virus [15], Bovine viral diarrhea virus [16], Rotavirus [18, 19], HIV-1 (Vpr, Vif) [20]; 6. Ebola virus (VP35) [21]; 7. Para influenza simian virus 5 [29, 30]; 8. Respiratory syncytial virus (NS1) [31]; 9. Hepatitis C virus [70]; 10. Human cytomegalovirus (US2, US11) [3], Kaposi’s Sarcoma associated herpesvirus (KSHV) (K3, K5) [1, 2]; 11. Influenza virus (NS1A) [42, 46–49], vaccinia virus (E3) [51]; 12. Coronavirus (PLP) [53]; 13. HIV-1 (E5D) [57, 61, 64–66], KSHV (K5) [63].
Viral use of the ubiquitin system to prevent the function of IFN-induced genes

In the event that viruses fail to completely shut-down the induction of IFN or IFN-dependent signaling they face a multitude of anti-viral IFN-induced proteins (ISGs) [34]. Well-studied anti-viral ISGs include PKR, OAS, RNaseL, and Mx. While the pre-treatment of cells with IFN generally renders them resistant to viral infection, important ISGs that are induced during viral infection can be successfully counteracted by viruses. Here, we will focus on two ISGs whose function and viral counter-mechanisms are currently being elucidated: ISG15 and BST2/Tetherin.

ISG15

ISG15 is a ubiquitin-like modifier that is one of the most highly induced ISGs. This di-ubiquitin-like protein is conjugated to proteins (ISGylation), a process that utilizes UbE1L as E1 enzyme, UbcH6, UbcH8 or UbcM8 as the E2 enzyme and Here5, HHARI, and Efp as E3 ubiquitin ligases that are also IFN-induced [35,36]. This process is reversible via the action of the ubiquitin-specific protease UBP43. Although many ISGylated targets have been identified [37], the impact of ISGylation has yet to be established in individual cases. Recent examples include demonstration that IRF3 is positively regulated by ISG15 [38,39] whereas RIG-I is negatively regulated [40]. However, a broadly anti-viral function of ISG15 has been established in ISG15 and UbE1L null mice that exhibit increased susceptibility to influenza A and B, HSV-1, MHV68, and Sindbis virus [41,42]. How ISG15 mediates anti-viral functions requires elucidation. Given the plethora of ISGylated proteins it is possible that anti-viral effects are virus-specific. One ISGylation anti-viral mechanism is inhibition of ubiquitin-ligase activity of Nedd4, which is required for budding of Ebola, vesicular stomatitis, and rabies viruses [43]. ISG15 and UbE1L overexpression also inhibits ubiquitination of Tsg101 and Gag, a process essential for HIV budding [44].

Yet not all viruses are affected by ISGylation [45] and several viruses have developed countermeasures against ISGylation. The NS1 protein of influenza B virus binds to ISG15 in a species-specific manner [46–48] and thus inhibits protein ISGylation. By contrast, the NS1A protein of influenza A is ISGylated that disrupts its nuclear localization [49]. Sumoylation of NS1 was also reported [50]. Inhibition of ISGylation has also been reported for Vaccinia Virus (VACV) [51]. Interestingly, this was dependent on the VACV E3L protein that was known previously as an inhibitor of PKR. E3L-deleted virus was growth-restricted in ISG15−, but not in ISG15+ cells and mice. Viruses are also known to deconjugate ISG15. Nairoviruses and arteriviruses encode OTU domain proteases that hydrolyze both ubiquitin and ISG15 from cellular target proteins [52]. Similarly, the coronavirus PLP protein acts as a de-ubiquitinating and de-ISGylating enzyme [53]. These viral counterstrategies probably pinpoint those ISGylation events most detrimental to a given viral species.

BST2/Tetherin

Bone marrow stromal antigen-2 (BST2/Tetherin) is an IFN-induced [54], glycosylated type II transmembrane protein with a unique topology since it also contains a glycosylphosphatidylinisotol (GPI) anchor at the C-terminus thus localizing it to the periphery of lipid rafts, forming a fence like structure [55]. We initially identified this protein in a proteomics screen as a new target of the viral E3 ligase K5 of Kaposi’s sarcoma virus [56] and, more recently, in a similar screen as dominant target for the HIV Vpu protein [57]. KSHV-K5 is a viral homolog of the membrane-associated RING-CH (MARCH) family of transmembrane ubiquitin ligases [58] that share the ability to ubiquitinate the intracellular domains of selected target proteins [59]. By comparison, HIV-1 Vpu does not possess ubiquitin-ligase activity on its own but interacts with the cellular F-Box protein β-TrCP, a subunit of the cellular SCF-complex. The finding that BST2/Tetherin is targeted by different viral immune evasion proteins raised this protein from obscurity, particularly since recent work revealed that BST-2 is responsible for tethering mature HIV-1 particles to the cell membrane in the absence of Vpu [60,61]. As recently reviewed elsewhere [62], this seminal work stimulated extensive research by a number of groups revealing that this IFN-induced protein prevents the egress of several unrelated viral families, including gamma-2 herpesviruses (KSHV), lentiviruses (EIAV, FIV) retroviruses (SIV, RSV, MPMV, HTLV-1, PFV), Filoviruses (Ebola, MV), and arena viruses (Lassa). While the exact details of this retention require identification, it seems that unlike other ISGs, BST2/Tetherin acts at a very late stage of infection. Many viruses counteract BST-2 function using different molecular mechanisms. In addition to KSHV-K5 and HIV-1 Vpu, Ebola virus envelope protein, and the nef protein of SIV and HIV-2 have been implicated in inhibiting BST2/Tetherin [62]. Since these evasion mechanisms are often highly species-specific, they probably contribute to host restriction. The molecular mechanism of BST2 downregulation by most viral proteins is not known, but some details emerged for KSHV-K5 and HIV-1 Vpu. In the case of KSHV-K5, cytosolic lysines of BST2 are directly ubiquitinated and this is required for lysosomal targeting [63]. By contrast, lysine-deleted BST2 is still targeted to lysosomes by HIV-1 Vpu in a β-TrCP-dependent manner, suggesting that ubiquitination is indirectly involved in this process [57,64]. In addition, Vpu can initiate the proteasomal destruction of BST2, particularly in situations when BST2 resides in the ER owing to overexpression [65,66]. Thus, ubiquitination is central to counteracting the anti-viral activity of BST2.

Conclusions

Ubiquitin-enabled interference of viruses with the innate immune response emerges as a central immune evasion...
mechanism in almost every viral species studied. Since viruses specifically target those innate immune responses that are particularly detrimental to a given viral species, studying the manipulation of ubiquitination has revealed novel host defense mechanisms. The identification of novel targets for viral ubiquitin ligases has been accelerated by proteomics approaches suggesting that it will be possible to use viral ubiquitin ligases to identify novel important key elements of host defense pathways [56, 67, 68, 69]. Studying ubiquitination events in virally infected cells thus holds great promise to unravel important modulators of the intricate relationship between host and pathogen.

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