



REVIEW

Immunology

Viral deubiquitinases and innate antiviral immune response in livestock and poultry

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ABSTRACT. Among many of the pathogens, virus is the main cause of diseases in livestock and poultry. A host infected with the virus triggers a series of innate and adaptive immunity. The realization of innate immune responses involves the participation of a series of protein molecules in host cells, including receptors, signal molecules and antiviral molecules. Post-translational modification of cellular proteins by ubiquitin regulates numerous cellular processes, including innate immune responses. Ubiquitin-mediated control over these processes can be reversed by cellular or viral deubiquitinases (DUBs). DUBs have now been identified in diverse viral lineages, and their characterization is providing valuable insights into virus biology and the role of the ubiquitin system in host antiviral mechanisms. In this review, we briefly introduce the mechanisms of ubiquitination and deubiquitination, present antiviral innate immune response and its regulation by ubiquitin, and summarize the prevalence of DUBs encoded by viruses (*Arteriviridae*, *Asfarviridae*, *Nairoviridae*, *Coronaviridae*, *Herpesviridae*, and *Picornaviridae*) infecting domestic animals and poultry. It is found that these DUBs suppress the innate immune responses mainly by affecting the production of type I interferon (IFN), which causes immune evasion of the viruses and promotes their replication. These findings have important reference significance for understanding the virulence and immune evasion mechanisms of the relevant viruses, and thus for the development of more effective prevention and treatment measures.

KEY WORDS: action mode, innate antiviral immune response, livestock and poultry, prevalence, viral deubiquitinases

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The natural immune system of host cell is triggered when it is infected by pathogens such as parasites, bacteria and viruses. In the case of viruses, sensing viral proteins or specific forms of viral nucleic acid trigger an innate immune response that leads to the expression of antiviral molecules, including type I interferons (IFNs), proinflammatory cytokines, and chemokines [28]. In eukaryotes, the key mechanisms that trigger and modulate these immune responses are post-translational phosphorylation and ubiquitination of cellular immune factors that alter their interactions, localization, stability, or activity.

Ubiquitination of most proteins is the process by which the ubiquitin protein binds to its substrate protein. The ubiquitination pathway comprises E1, E2, and E3 enzymes, ultimately responsible for the conjugation of ubiquitin to protein substrates [36, 52]. The E3 ligase usually determines substrate specificity, although the E2-conjugating enzyme can also play a role in substrate selection. These three enzymes, in turn, catalyze the connection of C-terminus of ubiquitin to the ε-amino group of the target protein's lysine (Lys, K) to form an isopeptide bond and ubiquitinate the target protein. The ubiquitin molecule itself also has multiple Lys sites (such as K6, K11, K27, K29, K33, K48, and K63) that can be ubiquitinated to produce ubiquitin chains with different connections (such as the common K48 and K63 ubiquitin chains). Studies have shown that ubiquitination is involved in many important biological processes such as immune response, inflammatory response, cell cycle, cell connections, cell apoptosis, DNA repair, protein degradation, and tumor development [6].

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Some virus-encoded ubiquitins can directly or indirectly affect ubiquitin-regulation pathways in host cells. For example, both the infected-cell protein 0 (ICP0) protein encoded by herpesvirus-1 and the Epstein-Barr virus nucleäre antigen 1 (EBNA1) protein encoded by Epstein-Barr virus are E3 ubiquitin ligases with typical RING domains [39, 56]. ICP0 protein is an immediate early protein, and EBNA1 protein is an important regulator of Epstein-Barr virus genome replication and transcription. They can directly ubiquitinate p53 for degradation, and can also bind to the intracellular DUB ubiquitin-specific protease (USP) 7, which inhibits the deubiquitination of murine double minutes 2 (MDM2) by USP7 and stabilize and increase the content of MDM2 in cells. MDM2 is a p53-specific E3 ubiquitination ligase, which can ubiquitinate p53 leading to degradation by the proteasome, and resulting in inhibition of apoptosis mediated by p53 and proliferation of cells.

Some viruses encoding the E3 ubiquitin ligase that is integrated with two transmembrane domain structure type of membrane proteins, such as Kaposi sarcoma herpesvirus (KSHV) encoding ubiquitin ligase K3 and K5 proteins can induce degradation of major histocompatibility complex (MHC-I), thus affecting antigen presented [3]. KSHV also encodes another immediate early protein, an E3 ubiquitin ligase, which degrades the IFN regulatory factor, thereby blocking the production of IFN and destroying the antiviral response of the cells [33].

In addition to encoding for ubiquitination enzyme, viruses can also encode ligand proteins to interact with ubiquitination enzyme in cells. The high-risk human papillomavirus E6 protein encoded by HPV16 and HPV18 can form complex with intracellular E6AP (an E3 ubiquitin ligase) and ubiquitinate p53, Bak, MDM7 and E6TP1, causing degradation of these proteins and abnormal cell proliferation. E6/E6AP can ubiquitinate c-Myc protein and bind to a complex that acts on the telomerase-catalyzed subunit (hTERT) promoter to activate hTERT expression. E6/E6AP also induces ubiquitination of the telomerase inhibitor NFX1-91, which promotes the expression of hTERT and up-regulates telomerase activity in cells [43]. Another oncogenic protein of HPV, E7, recruits cellular-derived Cullin2, Elongin B/C and Rbx to form the E3 ubiquitin ligase complex, which ubiquitinates and degrades the cyclin-suppressor pRb, promoting cell proliferation [50]. The Vif protein encoded by retroviruses such as human immunodeficiency virus-1 (HIV-1) can also recruit intracellular Cullin5, Elongins B/C and Rbx1 to form the E3 ubiquitin ligase complex, causing non-permissive ubiquitination of the intracellular regulator APOBEC3G and inhibiting its entry into virions, thereby promoting viral infection and immunosuppression [29]. Vpu protein of HIV-1 and US11 and US2 of human cytomegalovirus can also recruit ubiquitin ligase in cells and catalyze the ubiquitination degradation of MHC-I and CD4 molecules, reducing antigen presented, resulting in immune escape, and promoting virus infection [57].

Ubiquitination modification of proteins can be removed by cellular DUBs, which can reverse ubiquitination by hydrolyzing polyubiquitin (polyUb) chain and plays an important regulatory role in ubiquitin-mediated signaling pathway. There are currently seven known classes of DUBs that act to reverse ubiquitin and ubiquitin-like modifications: (1) USP, (2) autophagin (ATG), (3) ubiquitin C-terminal hydrolase (UCH), (4) ovarian tumor (OTU) domain proteins, (5) Josephin-domain (JD) or Machado-Joseph disease (MJD) proteins, (6) ubiquitin-like protein-specific protease (ULP), and (7) JAMM (JAB1/MPN domain-associated metalloisopeptidase) domain proteins [49, 64].

Cellular ubiquitinase and DUB regulate the immune system by regulating the levels of nuclear factor kappa-B (NF- κ B). Several proteins, such as CYLD, A20, Cezanne, USP11, USP15, and USP17, were found to stabilize various inhibitors of NF- κ B by deubiquitination, down-regulate the amount of active NF- κ B, and thereby regulate cell immune response [10, 20].

Viruses with the encoding DUBs can simulate host's DUBs, actively destroy the cellular ubiquitin-dependent process, inhibit innate antiviral immune response, and thus promote virus replication [25]. Virus can coevolve with host to acquire a similar mechanism to trick immune system and establish infection.

A well-known example of a viral DUB is UL36^{USP} of Herpes simplex virus 1 (HSV-1). UL36^{USP} was found to be active toward both Lys48- and Lys63-linked polyUb chains. Wang *et al.* demonstrated that the ectopic expression of UL36^{USP} decreased Sendai virus-mediated production of IFN- β , indicating that UL36^{USP} is involved in the modulation of cellular innate immune signaling pathways. To probe the role of UL36^{USP} during an infection, the authors used an HSV-1 bacterial artificial chromosome system to generate recombinant HSV-1 with a catalytically inactive UL36^{USP}(Cys40Ala). Infections with the UL36^{USP} knockout virus resulted in increased production of IFN- β , while virus containing wild-type UL36^{USP} impaired cellular IFN- β production, demonstrating that UL36^{USP} is an IFN antagonist [34].

Corona virus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is in the midst of a global pandemic and has become a major threat to human life and health. Virus-encoded DUB have also been found in previous studies of coronaviruses. The *in vitro* DUB and deISGylating activities of papain-like protease (PLP or PLP^{pro}) of SARS-CoV were confirmed in parallel by independent groups, demonstrating activity toward Lys48-linked ubiquitin chains [2, 42]. This DUB activity has been implicated in the downregulation of cellular innate immune responses [17], consistent with the observation that SARS-CoV infection prevents IFN- β induction in infected cells [63].

ANTIVIRAL INNATE IMMUNE RESPONSE AND ITS REGULATION BY UBIQUITIN

The first step of the innate immune response is the detection of pathogen-associated molecular patterns by a large repertoire of pattern-recognition receptors (PRRs) present on both immune and non-immune cells. Intracytosolic sensors for the detection of viral nucleic acids in virtually all cells are retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), NOD-like receptors, and viral DNA sensors [cyclic-GMP-AMP (cGAMP) synthase (cGAS)] and interferon-gamma-inducible protein (IFI16) are the key sensors [46, 62]. RIG-I-like receptors include RIG-I and melanoma differentiation-associated factor 5 (MDA5), which bind different forms of viral RNA leading to their activation and oligomerization that will induce the interaction with mitochondrial

antiviral signaling protein (MAVs). MAVs recruits signaling molecules such as E3 ligases of the tumor necrosis factor (TNF) receptor-associated factor (TRAF) protein family to activate kinase complexes.

TRAF6 will recruit the transforming growth factor- β -activated protein kinase (TAK) complex that will phosphorylate the inhibitor of NF- κ B kinase γ (IKK γ) [also known as NF- κ B essential modulator (NEMO)], which in turn will form a complex with IKK α/β . This complex will initiate the degradation of the NF- κ B inhibitor of NF- κ B α (I κ B α), which frees NF- κ B to promote transcription of pro-inflammatory cytokines. Another protein recruited to MAVs, TRAF3, activates a complex comprising of TRAF family member-associated NF- κ B activator (TANK)-binding kinase 1 (TBK1)/ inhibitor of NF- κ B kinase ϵ (IKK ϵ) to phosphorylate IFN regulatory factor (IRF) 3 and IRF7. Phosphorylation enables the IRF transcription factors to dimerize and induce the expression of type I IFNs in concert with NF- κ B [11, 28]. Secreted IFN- α/β will bind to IFN receptors and activate the Janus kinase–signal transducer and activator of transcription pathway, resulting in the transcription of interferon-stimulated genes (ISGs). ISG-encoded proteins, including ISG15, have antiviral activity by interfering with processes like viral replication or translation [59].

In DNA virus detection, cytoplasmic DNA of virus origin binds to sensor cGAS to stimulate synthesis of cGAMP [9]. cGAMP then activates the stimulator of interferon genes (STING), a membrane-bound protein on the endoplasmic reticulum, which recruits TBK1 and initiates IRF3-mediated transcription of type I IFNs and proinflammatory cytokines.

Activation of innate immune signaling is regulated, besides by phosphorylation, through ubiquitination performed by specialized E3 ligases such as the members of the family of tripartite motif-containing (TRIM) E3 ligases [53]. These and several other E3s conjugate Lys63-linked and Lys48-linked ubiquitin chains to RIG-I, MAVs, TBK1, and STING, as well as myeloid differentiation primary response (MyD) 88, TIR-domain-containing adaptor-inducing IFN- β (TRIF), interleukin-1 receptor-associated kinase 1 (IRAK1), and receptor-interacting protein 1 (RIP1), while TRAF3 and 6 induce their Lys63-linked auto-ubiquitination, which triggers activation [11, 15, 24]. I κ B α is degraded by the proteasome after Lys48-linked ubiquitin chains are conjugated, which enables downstream signaling [45].

Atypical ubiquitin chains also play a role in the activation of the innate immune response since NF- κ B essential modulator (NEMO) is a substrate for conjugation by Lys27, Lys29, and linear polyUb chains, and STING can be modified with Lys11 and Lys27 polyUb chains [44, 74]. Besides conjugated ubiquitin, unanchored ubiquitin chains (Lys63- or Lys48-linked) also seem to play a role in the regulation of innate immune signaling by providing a multimeric scaffold for the activation of RIG-I and MDA5 complexes [8, 32, 55]. In addition, ubiquitination of IRF3 has been observed and implicated in the induction of cellular proapoptotic pathways and in the negative regulation of IFN- β signaling [7, 80]. Negative regulation of the innate immune response is achieved by conjugation of Lys48-linked polyUb to degrade signaling factors and thereby dampen the expression of type I IFNs and pro-inflammatory cytokines. Additionally, actions of E3 ligases that attach different ubiquitin linkage chains can be counteracted by several specific cellular DUBs [15, 82]. CYLD (USP) is able to cleave linear and Lys63-linked ubiquitin chains, and it deubiquitinates RIG-I, TRAFs, and TBK1 [22].

PREVALENCE OF DUBS IN LIVESTOCK AND POULTRY VIRESES

Numerous studies have shown that in addition to viruses that infect humans, many viruses that infect livestock and poultry also have genes that encode DUBs. The constant interaction between the host and the pathogen leads to the formation of a cellular antiviral system to effectively destroy the invading pathogen, while the pathogen continuously adapts to the cellular antiviral system and evolves toward more effective genome replication machineries [66]. In this review, we focus on the DUBs found in livestock and poultry viruses, and emphatically introduce the mode of action of viral DUBs and their effects on innate immune response.

Arteriviridae

The family *Arteriviridae* is one of four virus families in the order Nidovirales. Arteriviruses are enveloped viruses with unsegmented plus-strand RNA genomes. At 12.7–15.7 kb, their genomes are considerably smaller than the coronaviruses' but they do share many characteristics, which include overall genome organization and use of discontinuous transcription to synthesize subgenomic RNAs. They lack the notable spike proteins of the coronaviruses. Members of the family include equine arteritis virus, porcine reproductive and respiratory syndrome virus, lactate dehydrogenase elevating virus of mice, and simian hemorrhagic fever virus. Arterivirus genomes are organized in the same overall manner as other virus families in the order Nidovirales. The ~13–16 kb positive-strand RNA genome contains 10–13 open-reading frames (ORFs). Approximately three-fourths of the genome encodes the nonstructural proteins (nsp) required in transcription and genome replication.

Equine arteritis virus (EAV): EAV is responsible for a common infection of horses worldwide. Many infections are asymptomatic or cause a mild upper respiratory tract disease; however, some of the infections might be more severe. An infrequent outcome of the infection is abortion in pregnant mares. Some infected stallions become persistent shedders of the virus but it can be cured by castration. This indicates a hormonal link (testosterone) to the viral persistence although the molecular events associated with persistence have not yet been determined. During acute infection, the virus is transmitted via aerosols, and the primary sites of replication are in the epithelial cells of the respiratory tract. The virus also infects macrophages and some lymphocytes thereby moving to regional lymph nodes. The infected cells then disseminate EAV throughout the horse. The virus is present in feces, urine, vaginal secretions, and semen.

Snijder *et al.* (1995) found that the replicase ORF1a polyprotein of EVA could be hydrolyzed at least into 6 nsp, among which the nsp2 cysteine protease had similar sequence with viral PLP, and the development of protease activity requires the appearance

of conserved Cys and His residues [61]. In 2007, Frias-Staheli and colleagues identified the OTU domain-containing proteases from nairoviruses and arteriviruses, two unrelated groups of RNA viruses, which hydrolyze ubiquitination and ISG15 from cell target proteins. They also showed that viral OTU domain-containing proteases inhibit NF- κ B-dependent signaling. Their study showed that the EAV protease has the property of DUB that helps the virus evade innate immune responses based on ubiquitination and ISG15 [21]. By using both *in vitro* and cell-based assays, van Kasteren and colleagues showed that PLP2 DUB activity is conserved in all members of the arterivirus family and that both arterivirus and nairovirus DUBs inhibit RIG-I-mediated innate immune signaling when overexpressed. They also found that both arteri- and nairovirus DUBs inhibit RIG-I ubiquitination upon overexpression, suggesting that both MDA5 (melanoma differentiation-associated factor 5) and RIG-I have a role in countering infection by arteriviruses [69]. van Kasteren and colleagues further confirmed the DUB function of arterivirus PLP2, which suppresses the innate immune response in infected host cells [71].

Porcine reproductive and respiratory syndrome virus (PRRSV): PRRSV is an economically important virus, which are found in both farm-raised and wild pigs. The virus most often spreads through direct contact but can also be spread by aerosol route. The virus can be introduced into a herd via infected animals, semen, or contaminated fomites. PRRS has two distinct clinic presentations: reproductive failure and postweaning respiratory disease. Reproductive disease causes increased numbers of stillborn piglets, mummified fetuses, premature births, and weak piglets.

The nsp2 of PRRS virus contains a cysteine protease domain at its N-terminal, belonging to the OTU protease family. Sun and colleagues demonstrated that the PRRSV nsp2 OTU domain antagonizes type I IFN interfering with the NF- κ B signaling pathway. Further analysis revealed that the nsp2 OTU domain has Ub-deconjugating activity. This domain inhibits the activation of NF- κ B by interfering with the polyUb of I κ B α , thereby preventing the degradation of I κ B α . The results of the OTU domain mutation experiment indicated that certain mutations that had a destructive effect on viral replication can impair the ability of nsp2 to inhibit NF- κ B activation [65]. The selection of PRRS OTUs for its host ubiquitin and ubiquitin like substrates, such as ISG15, may vary greatly and is considered as a potential virulence factor. The virulence differences are dependent of the different PRRSV strains. Deaton *et al.* (2014) determined the specificity of OTUs of two PRRSV strains with different virulence. Although the OTUs of both of the strains showed deubiquitinating activity and significantly low deISGylating activity, the highly pathogenic PRRSV showed a strong preference for lysine 63-linked polyUb, which is related to the regulation of innate immune response. The pathogenicity of the strains may be related to the increased immunosuppression and virulence caused by OTU [16].

Asfarviridae

The viruses of *Asfarviridae* are large, enveloped double-stranded DNA viruses. African swine fever virus is the only known DNA arbovirus and is transmitted by soft ticks of the genus *Ornithodoros*. Virus strains are distinguished by their virulence to swine, which ranges from highly lethal to subclinical infection.

African swine fever virus (ASFV): ASFV is a severe disease of domestic swine with mortality rates approaching up to 100%. No vaccine is currently available. Quarantine and slaughter are the only effective control measures [67]. ASFV is a large, icosahedral virus that contains a linear double-stranded DNA genome (170 to 190 kbp) encoding approximately 165 genes [77].

ASFV multigene family consists 360 and 530 (MGF360/530) genes that affect viral growth in macrophage cell cultures and virulence in pigs. To define MGF360/530 gene function, Afonso *et al.* (2004) infected pig macrophages with parental ASFV (Pr4) virus and mgf360/530 deletion mutant virus (Pr4 Δ 35) and compared their transcriptional profiles. A swine cDNA microarray containing 7,712 macrophage cDNA clones was used to compare the transcriptional profiles of postinfected swine macrophages [1]. The study found that most Pr4 Δ 35 up-regulated genes were part of a type I IFN response or were genes that are normally induced by double-stranded RNA and/or viral infection. These include monocyte chemoattractant protein, transmembrane protein 3, tetratricopeptide repeat protein 1, a ubiquitin-like 17-kDa protein, ubiquitin-specific protease ISG43, an RNA helicase DEAD box protein, GTP-binding MX protein, the cytokine IP-10, and the PKR activator PACT. The absence of IFN- α in Pr4-infected macrophages suggests that MGF360/530 genes either directly or indirectly suppress the type I IFN response. In Pr4 Δ 35-infected macrophages, however, the virus is unable to inhibit the host type I IFN response, possibly because of defective growth or attenuated toxicity. The inhibition of type I IFN response induced by ASFV may be related to the Ub-specific protein ISG43.

Nairoviridae

Members of the family *Nairoviridae* produce enveloped virions with three single-stranded RNA segments comprising 17.1 to 22.8 kb in total. These viruses are maintained in arthropods and transmitted by ticks to mammals or birds. Crimean-Congo hemorrhagic fever virus is tick-borne and is endemic in most of Asia, Africa, Southern and Eastern Europe whereas Nairobi sheep disease virus (NSDV), which is also tick-borne, causes lethal haemorrhagic gastroenteritis in small ruminants in Africa and India.

NSDV: NSDV was first isolated from sheep blood in Kenya in 1910 and is known to be associated with disease of small ruminants, specifically sheep and goats, in a narrow band straddling the equator from Kenya in the east to Congo in the west. The virus can be transmitted transstadially by a range of ixodid ticks, including *Amblyomma variegatum*, but the endemic vector appears to be *Rhipicephalus appendiculatus*, in which transovarial transmission occurs. The disease in sheep and goats is characterized by fever, haemorrhagic gastroenteritis and abortion in pregnant animals. High mortality occurs when susceptible sheep or goats are introduced into an endemic area, but within such areas young animals appear to undergo benign infection as maternal immunity wanes, and there is a high prevalence of immunity in adult animals.

Honig *et al.* used CCHF (Crimean–Congo hemorrhagic fever) or NSD (Nairobi sheep disease) virus to infect Vero E6 cells to study the L RNA genome fragments using RT-PCR and nucleic acid sequencing techniques, and the analysis showed that they

were about twice the size of other bunya viruses [26]. The L segment and encoded proteins of CCHF virus (12,164 nucleotides and 3,944 amino acids, respectively) are similar in size and sequence to those of nairovirus Dugbe virus (12,255/62% and 4,036/62% in nucleotide and amino acid length/identity, respectively). An OTU-like protease was identified at the amino terminus of nairoviruses Dugbe, CCHF, and NSD L proteins, indicating that these proteins are members of the recently described OTU-like protease family, suggesting that these macromolecules may be polyproteins involved in autoproteolytically cleavage or associated with DUB.

Coronaviridae

Members of the family *Coronaviridae* are large, enveloped, single-stranded RNA viruses. These are the largest RNA viruses identified up to date, with genomes ranging from 25 to 32 kb and dimensions of virion between 118–136 nm in diameter. The 25–32 kb positive-strand RNA genome contains 7–10 ORFs. Almost two-thirds of the genome encode nsp that are required for transcription and genome replication. The virions are roughly spherical and are notable for large spike glycoprotein that extends 16–21 nm from the virus envelope. Coronaviruses have a wide range of hosts, including rodents, cats, dogs, chickens, turkeys, ducks, pigs, horses, and cattle. Over 60 coronaviruses (CoVs) have been isolated from bats (BtCoV) and most of these belong to the genus betacoronavirus. Bats serve as large (and highly mobile) CoV reservoirs; many bat species have their own unique BtCoV, suggesting a very long history of coevolution. Several important animal diseases are caused by CoVs, including avian infectious bronchitis, porcine epidemic diarrhea, porcine transmissible gastroenteritis, and mouse hepatitis. Until 2002, CoVs were only considered as minor pathogens of humans. However, several outbreaks of human infectious diseases caused by coronavirus since 2002 have increased serious global public health concerns, which include the outbreak of severe acute respiratory syndrome (SARS) in 2002, the outbreak of Middle East respiratory syndrome in 2014, and the most recent outbreak of Coronavirus disease 2019 (COVID-19) in 2019 [72].

Infectious bronchitis virus (IBV): IBV causes bronchitis in chicken that results in huge economic losses every year in the poultry industry globally. PLP of coronaviruses (CoVs) carries out proteolytic maturation of non-structural proteins that play a role in replication of the virus and performs DUB of host cell factors to scuttle antiviral responses.

To explain the substrate specificities of PLP, Kong *et al.* (2015) solved the crystal structure of PLP from IBV at 2.15-Å resolution. The overall structure is reminiscent of the structure of severe acute respiratory syndrome CoV PLP. However, unlike the SARS CoV PLP that lacks blocking loop 1 of DUBs, the IBV PLP has a short blocking loop 1-like loop. Access to a conserved catalytic triad consisting of Cys101, His264, and Asp275, is regulated by a flexible blocking loop 2. A model of Ub-bound IBV CoV PLP brings out key differences in substrate binding sites of PLPs. In particular, P3 and P4 subsites as well as residues interacting with the β -barrel of Ub are different, suggesting different catalytic efficiencies and substrate specificities. Results of the study show that IBV PLP cleaves peptide substrates KKAG-7-amino-4-methylcoumarin and LRGG-7-amino-4-methylcoumarin with different catalytic efficiencies. These results demonstrate that substrate specificities of IBV PLP are different from other PLP and that IBV PLP might target different ubiquitinated host factors to aid the propagation of the virus [38]. In another study, Yu *et al.* characterized the DUB activity of IBV PLP in the DF-1 chicken fibroblast cell line and the core domain of the DUB activity. They showed that IBV has DUB activity and confirmed that PLP-TM (PLP transmembrane domain) was not only a classic PLP encoded by IBV but was also a multifunctional protein that plays important roles in the regulation of interactions between IBV and host antiviral innate immune response proteins. Ub modifications play key regulatory roles in protein degradation and in innate and adaptive immunity signaling pathways. IBV PLP-TM may prevent the activation of host antiviral signaling pathways by degrading polyUb chains associated with ubiquitin proteins. Their result showed that IBV PLP can degrade Lys48- and Lys63-linked polyUb chains to monoubiquitin but not linear polyUb [79].

Porcine epidemic diarrhea virus (PEDV): PEDV is the cause of an economically important swine disease. To study the mechanisms by which viruses evade or block innate immune responses, Xing *et al.* (2013) investigated the activation of IFN- β in PEDV-infected cells [76]. In their study, Vero E6 cells were co-transfected with IFN- β -Luc and pRL-TK reporter plasmids and then infected with PEDV. Their research suggested that PEDV PLP2 is not only a classical PLP encoded by a CoV genome, but also a multifunctional protein that plays important roles in regulation of the interactions of PEDV–host antiviral innate immune response. The study indicated that PEDV PLP2 is an IFN antagonist and its IFN antagonistic activity depends on the intact catalytic sites of C1729, H1888 and D1901. The cleaving target of PEDV PLP2 DUB is the ubiquitin chains from RIG-I and STING, thereby inhibiting the activation of type I IFN signaling.

Porcine transmissible gastroenteritis virus (PTGV): Wojdyla *et al.* (2010) reported the first structural and biochemical characterization of a purified coronavirus PLP1 domain, that of PTGV. Its tertiary structure was compared with SARS coronavirus PLP2, a downstream paralog that is conserved in the nsp3's of all coronaviruses. They identified both conserved and unique structural features that likely control the interaction of PL1^{PRO} with cofactors and substrates, including the tentative mapping of substrate pocket residues. The purified recombinant PTGV PLP1 was shown to cleave a peptide mimicking the cognate nsp2/nsp3 cleavage site. Like its PLP2 paralogs from several coronaviruses, PTGV PLP1 was also found to have deubiquitinating activity in an *in vitro* cleavage assay, implicating it in counteracting Ub-regulated host cell pathways, likely including innate immune responses [75].

Herpesviridae

Herpesviruses are large DNA viruses, with double-stranded DNA genomes ranging from 124 to 295 kb encoding 70–200 proteins. The *Herpesviridae* family consists of a subset of viruses within the order Herpesvirales that infect mammals, birds, and reptiles. The herpesvirus virion structure is composed of an icosahedral nucleocapsid hosting the viral genome, which is surrounded

by a viral matrix, or tegument, and contained within the viral envelope. All herpesviruses can establish lifelong infections in their respective hosts, remaining latent until periods of reactivation.

Marek's disease virus (MDV): MDV causes Marek's disease, which is a highly infectious, and highly tumorigenic lymphoproliferative disease of chicken, accompanied by high mortality. The genome of MDV is a double-stranded DNA. There is a tegument structure, which is divided into two layers: inner, capsid-associated part and outer, envelope-juxtaposed portion. UL36 is one of the components of inner part [4].

The herpesvirus USP family, whose founding member was discovered as a protease domain embedded in the large tegument protein of HSV-1, is conserved across all members of the *Herpesviridae*. UL36 protein (pUL36) is a large tegument proteins encoded by the UL36 gene of HSV and MDV viruses, approximately composed of more than 3000 amino acids, in which the N terminus has DUB activity (UL36^{USP}). Although the homology of UL36 amino acid sequences in EBV, murine cytomegalovirus, HSV and MDV is very low, these sequences contain similar USP active sites, that is, they commonly possess N-terminal Cys and C-terminal His and Asp sites [48].

Jarosinski *et al.* (2007) reported that USP activity is conserved in MDV [30]. A single amino acid substitution that abolishes the USP activity of the MDV large tegument protein diminishes MDV replication *in vivo*, which consequently limits the oncogenic potential of the virus. Expression of the USP transcripts in MDV-transformed cell lines further substantiates this hypothesis. The herpesvirus USP thus appears to be required not only to maintain a foothold in the immunocompetent host, but also to contribute to malignant outgrowths.

Several studies suggested that MDV encodes an USP within its UL36 gene, and the USP is highly conserved among herpesviruses and important for MDV replication and pathogenesis in MDV's natural host, the chicken. To further investigate the role of MDV USP, several recombinant MDV (rMDVs) were generated and their *in vitro* phenotypes were evaluated using plaque size and growth kinetics assays [68]. These studies also discovered that the N-terminus of pUL36 is essential for MDV replication and could not be complemented by ectopic expression of MDV USP. In addition, they demonstrated that the region located between the conserved glutamine (Q85) and leucine (L106) residues comprising the active site cysteine (C98) is also essential for MDV replication. Based on the analyzes of the rMDVs generated, it was concluded that MDV USP likely contributes to the structure and/or stability of pUL36 and affects replication and oncogenesis of MDV beyond its enzymatic activity.

Up to date, more than 100 kinds of DUBs have been discovered from human genome, among which USPs play an important role chromatin regulation, virus infection, tumorigenicity, and immune regulation [78]. Furthermore, it has been found that when combined with human USP associated factor 1 (hUAF1), the deubiquitination activity of hUSP1 can be increased by 35 times [13]. Zheng *et al.* (2017) identified a chicken USP1 homologue (chUSP1) from MDV [81]. They expressed and purified both chUAF1 and chUSP1 with or without putative catalytic core mutations using a Bac-to-Bac system before investigating their deubiquitination activity and kinetics using various substrates. The chUSP1 was shown to interact with chUAF1 both in cellular assays, in which the two proteins were co-expressed, and in *in vitro* assays using purified proteins. Heterodimerization with chUAF1 increased the deubiquitination activity of chUSP1 up to 54-fold compared with chUSP1 alone. The chUSP1/chUAF1 complex was found to have distinct substrate preferences; and efficient hydrolysis of ubiquitin dimers with K11-, K48-, and K63-linkages was observed.

To characterize deubiquitinating activity and substrate specificity of UL36-DUB, the UL36 N-terminal fragments, UL36 (323), UL36 (480), and mutants were prepared using the Bac-to-Bac system. The deubiquitinating activity and substrate specificity of these recombinant UL36-DUBs were analyzed using various ubiquitin or ubiquitin-like (UbL) substrates and activity-based deubiquitinating enzyme probes [41]. The results indicated wild type UL36-DUBs presented the highest activity to K11, K48, and K63 linkage ubiquitin chains. The UL36 has higher cleavage efficiency for K48 and K63 polyUb than the linear ubiquitin chain (M1-Ub4), but no activity on various ubiquitin-like modifiers.

Pseudorabies virus (PrV): PrV is the causative agent of Aujeszky's disease, an infectious disease with a major economic impact in animal husbandry. PrV's natural hosts are porcines but it can infect nearly all mammals, excluding humans and other higher primates. In addition, PrV has been used increasingly as a model for elucidating the basic mechanisms of herpesvirus infection in cell culture and in the animals.

Herpesviruses specify a Ub-specific protease activity located within their largest tegument protein. Although its biological role is still largely unclear, a mutation within the active site abolished DUB activity and decreased virus replication *in vitro* and *in vivo*. To further elucidate the role of DUB activity for herpesvirus replication, the conserved active-site cysteine at amino acid position 26 within pUL36 of PrV (Suid herpesvirus 1), a neurotropic alphaherpesvirus, was mutated to serine [5]. Whereas one-step growth kinetics of the resulting mutant virus PrV-UL36 (C26S) was moderately reduced, in which the plaque size was decreased to 62% of that of the wild-type virus. Ultrastructural analysis revealed large accumulations of unenveloped nucleocapsids in the cytoplasm, but incorporation of the tegument protein pUL37 was not abolished. Mice post intranasal infection with PrV-UL36 (C26S) showed a survival time twice longer than those of mice infected with wild-type or rescued virus. Thus, the DUB activity is important for PrV replication *in vitro* and for neuroinvasion in mice.

Picornaviridae

Members of the family *Picornaviridae* are small RNA viruses. The Picornaviral genomes are single molecules of positive-sense RNAs with sizes ranging from 7,500 to 9,000 nt. Each of the Picornaviral genomes has a single open reading frame encoding a polyprotein of ~2,300 amino acids. To date there are 68 genera in the family *Picornaviridae*. Of importance to human health are several members of the genus *Enterovirus*. Foot and Mouth disease virus (FMDV) belong to infects hoof-stock such as cattle, sheep, pigs, and horses.

FMDV: FMDV infects a wide variety of cloven-hoofed domestic and wild animal species. Although the horse is refractory to infection, cattle, water buffalo, sheep, goats, llamas, camels, and swine are susceptible and develop clinical signs post infection. More than 70 species of wild mammals belonging to more than 20 families also are susceptible. Foot-and-mouth disease is one of the most devastating diseases of livestock due to its impact on the economy.

The leader proteinase (L^{pro}) of FMDV is a PLP that plays an important role in FMDV pathogenesis. Previously, it has been shown that L^{pro} is involved in the inhibition of the type I IFN response by FMDV. Wang *et al.* (2011) demonstrated that FMDV Lb^{pro} , a shorter form of L^{pro} , has deubiquitinating activity [73]. Sequence alignment and structural bioinformatics analyses revealed that the catalytic residues (Cys51 and His148) are highly conserved in FMDV Lb^{pro} of all seven serotypes and that the topology of FMDV Lb^{pro} is remarkably similar to that of USP14, a cellular DUB, and to that of SARS-CoV PLP, a coronaviral DUB. Both purified Lb^{pro} protein and *in vivo* ectopically expressed Lb^{pro} removed Ub moieties from cellular substrates, acting on both lysine-48- and lysine-63-linked polyUb chains. Furthermore, L^{pro} significantly inhibited UB of RIG-I, TBK1, TRAF6, and TRAF3, key signaling molecules in activation of type I IFN response. Collectively, these results demonstrate that FMDV Lb^{pro} possesses DUB activity in addition to serving as a viral proteinase and describe a novel mechanism evolved by FMDV to counteract host innate antiviral responses.

Taken together, it can be seen that different DUBs have been identified from several species of six virus families that infect livestock and poultry. These results are summarized in Table 1.

ACTION MODE OF DUBS

As mentioned above, a considerable number of viruses that infect livestock and poultry have DUBs, which are present in both DNA and RNA viruses. As shown in Table 1, the viral DUBs have PLP, USP, OTU and OTU-like protease. The widely distributed PLP belongs to the USP family of DUBs [42]. PLP is part of the replicase polyprotein and is involved in cleaving the nsp (nsps) to form the viral RNA replication complex. It might serve to protect viral replication complex from ubiquitination and degradation by proteasome. The second type of DUBs is OTU or OTU-like protease. These enzymes have similar structure and function to cellular DUBs. The third type of DUBs is pUL36, lacking obvious similarity to any known DUB, and representing a new family of DUBs [58].

According to the present study, there are two substrates of viral DUBs, namely ubiquitin and ISG15. They are more common for ubiquitin and less common for ISG15. The covalent attachment of ubiquitin to protein substrates, i.e., ubiquitination, plays a pivotal regulatory role in numerous cellular processes [12, 27, 31, 37, 51]. Ubiquitination can be reversed by DUBs and, not surprisingly, various virus groups encode such DUBs to influence ubiquitin-mediated host cell processes. The DUBs cleavage sites are concentrated at Lys48- and Lys63-linked polyUb chains [36, 42, 43, 58]. ISG15 is ubiquitin-like protein, and is a crucial regulator of the interferon signaling pathways. The expression of ISG15 is strongly induced by type I IFN. ISG15 is the first

Table 1. Deubiquitinases found in livestock and poultry viruses

Virus family/genome	Virus	Virus DUBs	Mode of action	References
<i>Arteriviridae</i> /RNA	EAV	PLP2	Hydrolyzing Ub and ISG15, acting targets MDA5 and RIG-I.	[21, 61, 69, 71]
	PRRSV	OTU	Inhibits the activation of NF- κ B by interfering with the polyUb of I κ B α . With deubiquitinating and deISGylating activity. Strong preference for lysine 63-linked polyUb.	[16, 65]
<i>Asfarviridae</i> /DNA	ASFV	USP (ISG43)	-	[1]
<i>Bunyaviridae</i> /RNA	NSDV	OTU-like protease	-	[26]
<i>Coronaviridae</i> /RNA	IBV	PLP	Degrading Lys48- and Lys63-linked polyUb chains to monoubiquitin but not linear polyUb.	[38, 79]
	PEDV	PLP2	Cleaving target is Ub chains from RIG-I and STING, thereby inhibiting the activation of type I IFN signaling.	[76]
	PTGV	PLP1	-	[75]
<i>Herpesviridae</i> /DNA	MDV	pUL36 or USP (within UL36 gene)	-	[30, 41, 68, 81]
	PrV	pUL36	-	[5]
<i>Picornaviridae</i> /RNA	FMDV	PLP (L^{pro})	Acting on both lysine-48- and lysine-63-linked polyUb chains. Inhibiting Ub of RIG-I, TBK1, TRAF6, and TRAF3.	[73]

EAV, equine arteritis virus; PRRSV, porcine reproductive and respiratory syndrome virus; ASFV, african swine fever virus; NSDV, nairobi sheep disease virus; IBV, infectious bronchitis virus; PEDV, porcine epidemic diarrhea virus; PTGV, porcine transmissible gastroenteritis virus; MDV, Marek's disease virus; PrV, pseudorabies virus; FMDV, foot-and-mouth disease virus; PLP, papain-like protease; OTU, ovarian tumor domain-containing protease; USP, ubiquitin-specific protease; pUL36, large tegument proteins encoded by the UL36 gene of *Herpesviridae*; L^{pro} , leader proteinase; ISG, interferon-stimulated gene; Ub, ubiquitin; MDA5, melanoma differentiation-associated factor 5; RIG-I, retinoic acid-inducible gene I; NF- κ B, nuclear factor-kappa B; polyUb, polyubiquitin; I κ B α , inhibitor of NF- κ B α ; STING, stimulator of interferon genes; IFN, interferon; TBK1, TANK (TRAF family member-associated NF- κ B activator)-binding kinase 1; TRAF, tumor necrosis factor receptor-associated factor; -, the mode of action remains unclear.

ubiquitin-like modifier to be identified. However, unlike ubiquitination, ISGylation known to promote degradation, but parallels ubiquitin's activating effects and plays an important role in the innate antiviral response although the detail mechanism is still unknown [47].

So far, the effects of viral DUBs on both viral infection and pathogenicity point mainly are known to impact innate immune responses of host, and are achieved by regulating signal transduction pathways to affect the expression of type I IFN. The immune system protects the organism against infections and any damage resulted from the infections. The first line of defense against pathogens is the innate immune response. In the case of a viral infection, it induces the type I IFN signaling cascade and eventually the expression of type I IFN, which then causes an antiviral state in the cells. However, many viruses have evolved with strategies to counteract this mechanism and to prevent the production of type I IFN. Type I IFNs belong to a family of cytokines, which have attracted much attention owing to their protective role against viral infection. IFNs are widely expressed cytokines that possess strong antiviral and immunomodulatory properties. IFN family is classified into three main types of cytokines, type I, type II and type III IFNs. IFN- α and IFN- β belongs to type I IFN family, while the type II IFN family includes only one cytokine: IFN- γ , which also exhibits antiviral activities [23]. The third type of IFNs is the IFN- λ family. In mammals, plasmacytoid dendritic cells (pDCs), monocytes, epithelial cells and fibroblasts are the main producers of type I IFNs [19], while type II IFNs are predominantly produced by NK cells and activated T cells. In spite of the fact that chicken type I IFNs are shown to inhibit viral infection both *in vivo* and *in vitro*, chicken pDCs have not been identified.

Although the effect of viral DUBs on the production of type I IFNs is not systematic enough, it can still be seen that these enzymes have negative regulatory effects on it through deubiquitination or de-ISGylation by targeting ubiquitinated signaling factors. The effect of the DUBs can be seen on multiple nodes of the type I IFNs signaling pathway, including MDA5, RIG-I, STING, TBK1, TRAF3, and TRAF6 in association with different viruses [21, 69, 71, 73, 76].

The role of viral DUBs in innate immune response of host cells can be described as follows (Fig. 1). PRRs, such as TLRs, NLRs, RIG-I, MDA-5, and cGAS, recognize the presence of invading pathogens by their PAMPs. The PRRs consider PAMPs to be an important component of the invading pathogen, which is crucial to its survival and has not been altered during evolution. When their ligands are present, the PRRs signals downstream, which initiates a complete response and activates the antiviral state of cell [35]. By contrast, the virus-encoded DUBs through deubiquitination and de-ISGylation regulate the production of type I IFNs in order to evade the innate antiviral immune response of cells or to promote viral infection.

DUBS INHIBITORS

Antiviral immunity is regulated by a variety of post-translational modifications, in which deubiquitination is also widely involved. The role of DUBs in the occurrence and development of virus infection is complex. Host DUBs can regulate its own innate immunity and the proliferation of viruses. The DUBs encoded by viruses can inhibit host antiviral immune response and promote the replication of the viruses. Due to the broad scope of DUB biology, they are emerging as a target class for inhibitor development. Through the intervention of DUBs with small molecule inhibitors, positive progress has been made in the study of tumor therapy and antiviral infection.

Currently, many DUBs inhibitors have been reported, which inhibit tumor development of humans and animals. Inhibitors b-AP15, azepan-4-ones, WPI 130, and tricyclic heterocyclics exert anti-tumor effects by inhibiting some UCH and USP family DUBs, respectively [14, 18].

Some DUBs' inhibitors are also used in the treatment of viral infections. P22077 and PR-619 are specific inhibitors of USP7 and USP47, which inhibit Gag processing and reduce the release of infectious virions in HIV-1 infection [60]. The DUBs' PL^{pro} encoded by SARS-CoV can cut ubiquitin and ISG15 binding, which helps SARS-CoV escape from human immune system. GRL0617 is a competitive PL^{pro}, exerting antiviral activity and inhibiting viral replication of SARS-CoV [54].

Molecules that inhibit DUBs have also been found in viral diseases of domestic animals and poultry. Li *et al.* infected HD11 cell lines with IBV, and found that the an overexpressed microRNA (miRNA), GGA-miR-30d, inhibited IBV replication, and the gene of DUBs, USP47, was the target gene of GGA-miR-30d, suggesting that miRNA could regulate IBV replication by regulating DUBs [40]. With development of related research, it is reasonable to believe that more DUBs' inhibitors will be discovered, and these inhibitors are promising for prevention and control of diseases caused by DUBs active viruses.

CONCLUSION

Taken together, the reported studies strongly suggest that viruses encode DUBs that act to inactivate cellular proteins involved in host innate immune signaling. These studies are of great significance in understanding virulence and infectivity of viruses and in exploring ways to control virus infections. However, the research on distribution of viral DUBs, as well as the properties, structures, action substrates and action mechanisms of enzymes is far from enough, which limits our comprehensive and scientific understanding of DUBs. Moreover, the role of viral DUBs is not limited to the innate immune response of host cells. It is likely that DUBs can modify a variety of metabolic processes of host cells and play a variety of biological roles, thus affecting their survival. Therefore, in-depth understanding of the effect and mechanism of DUBs will be of great significance for the development of DUBs inhibitors, the development of DUBs-based vaccines [70], and the provision of theoretical basis for the control of livestock and poultry viral diseases.

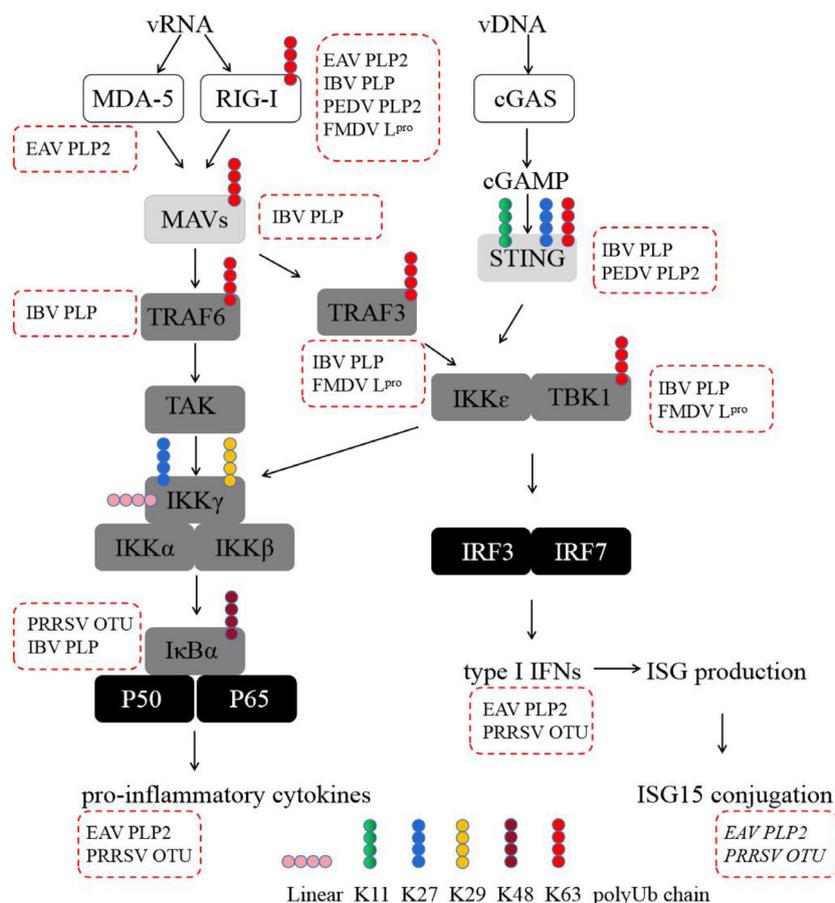


Fig. 1. Activation of the innate immune response and the effect of domestic and poultry viral deubiquitinases (DUBs) on it. White boxes represent the cytoplasmic receptors RIG-I, MDA5, and cGAS that can sense viral RNA or DNA via adaptor proteins MAVs or STING (light gray boxes), which in turn activate kinase complexes (dark gray boxes). Ultimately, transcription factors IRF3, IRF7, p50, and p65 (black boxes) are activated and translocate to the nucleus to induce the transcription of type I IFNs and pro-inflammatory cytokines. Differently linked polyUb chains involved in the activation of the innate immune response are represented by different colored chain balls. Dashed boxes placed next to innate immune signaling factors contain viral DUBs that remove ubiquitin chains from these specific targets. DUBs placed below the type I IFN or nuclear factor kappa-B (NF- κ B) pathway (inducing the expressing of pro-inflammatory cytokines) interfere with these pathways without knowing their exact substrate(s). Viral DUBs having deISGylating activity are shown in italic. The abbreviations used are: vRNA, viral RNA; vDNA, viral DNA; MDA5, melanoma differentiation-associated factor 5; RIG-I, retinoic acid-inducible gene I; MAVs, mitochondrial antiviral signaling protein; TRAF3, tumor necrosis factor receptor-associated factor 3; TRAF6, tumor necrosis factor receptor-associated factor 6; TAK, transforming growth factor- β -activated protein kinase; IKK α , inhibitor of nuclear factor-kappa B (NF- κ B) kinase α ; IKK β , inhibitor of nuclear factor-kappa B kinase β ; IKK γ , inhibitor of nuclear factor-kappa B kinase γ ; I κ B α , inhibitor of NF- κ B α ; P50, NF- κ B P50; P65, NF- κ B P65; cGAS, cyclic-GMP-AMP (cGAMP) synthase; cGAMP, cyclic guanosine monophosphate-adenosine monophosphate; STING, stimulator of interferon genes; IKK ϵ , inhibitor of nuclear factor-kappa B kinase ϵ ; TBK1, TRAF family member-associated NF- κ B activator-binding kinase 1; IRF3, interferon regulatory factor 3; IRF7, interferon regulatory factor 7; IFNs, interferons; ISG, interferon-stimulated gene; ISG15, interferon-stimulated gene 15; polyUb, polyubiquitin; EAV, equine arteritis virus; IBV, infectious bronchitis virus; PEDV, porcine epidemic diarrhea virus; FMDV, foot-and-mouth disease virus; PRRSV, porcine reproductive and respiratory syndrome virus; PLP, papain-like protease; L^{pro}, leader proteinase; OTU, ovarian tumor domain-containing protease.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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