**TRAF6 autophagic degradation by avibirnavirus VP3 inhibits antiviral innate immunity via blocking NFκB/NF-κB activation**

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**ABSTRACT**

Ubiquitination is an important reversible post-translational modification. Many viruses hijack the host ubiquitin system to enhance self-replication. In the present study, we found that *Avibirnavirus* VP3 protein was ubiquitinated during infection and supported virus replication by ubiquitination. Mass spectrometry and mutation analysis showed that VP3 was ubiquitinated at residues K73, K135, K158, K193, and K219. Virus rescue showed that ubiquitination at sites K73, K193, and K219 on VP3 could enhance the replication abilities of infectious bursal disease virus (IBDV), and that K135 was essential for virus survival. Binding of the zinc finger domain of TRAF6 (TNF receptor associated factor 6) to VP3 mediated K11- and K33-linked ubiquitination of VP3, which promoted its nuclear accumulation to facilitate virus replication. Additionally, VP3 could inhibit TRAF6-mediated NFKB/NF-κB (nuclear factor kappa B) activation and IFNB/IFN-β (interferon beta) production to evade host innate immunity by inducing TRAF6 autophagic degradation in an SQSTM1/p62 (sequestosome 1)-dependent manner. Our findings demonstrated a macroautophagic/autophagic mechanism by which *Avibirnavirus* protein VP3 blocked NFKB-mediated IFN production by targeting TRAF6 during virus infection, and provided a potential drug target for virus infection control.

**Abbreviations:** ATG: autophagy related; BafA1: bafilomycin A1; CALCOCO2/NDP52: calcium binding and coiled-coil domain 2; Cas9: CRISPR-associated protein 9; CHX: cycloheximide; Co-IP: co-immunoprecipitation; CRISPR: clustered regularly interspaced short palindromic repeats; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GST: glutathione S-transferase; IBDV: infectious bursal disease virus; IF: indirect immunofluorescence; IFNB/IFN-β: interferon beta; mAb: monoclonal antibody; MAP1LC3/LC3: microtubule associated protein 1 light chain 3; MOI: multiplicity of infection; MS: mass spectrometry; NBR1: NBR1 autophagy cargo receptor; NFKB/NF-κB: nuclear factor kappa B; NBL1: NBL1 autophagy cargo receptor; OPTN: optineurin; pAb: polyclonal antibody; PRRs: pattern recognition receptors; RNF125: ring finger protein 125; RNF135/Riplet: ring finger protein 135; SQSTM1/p62: sequestosome 1; TAX1BP1: tax1 binding protein1; TICD50: 50% tissue culture infective dose; TRAF3: TNF receptor associated factor 3; TRAF6: TNF receptor associated factor 6; TRIM25: tripartite motif containing 25; Ub: ubiquitin; Wort: wortmannin; WT: wild type.

**Introduction**

Ubiquitination, a reversible post-translational modification, participates in multiple biological processes, including substrate protein stability, subcellular transport, and signaling transduction [1–5]. Ubiquitin (Ub) is conjugated to lysine (K) residues of substrate proteins by an enzymatic cascade, including E1 activation enzymes, E2 conjugation enzymes, and E3 ubiquitin ligases [6,7]. Ub contains seven lysine residues K6, K11, K27, K29, K33, K48, and K63 and gives rise to multiple potential polyubiquitin signals [8]. Importantly, the type of polyubiquitination determines the different biological effects. K11-, K27-, and K63-linked polyubiquitination have been shown to be relevant to signaling transduction, protein stability, trafficking, and subcellular distribution [4–5,9–13], whereas, K48-linked polyubiquitination is commonly involved in ubiquitin-proteasome degradation [14]. Recently, many studies have reported that ubiquitination participates in the life cycle of diverse viruses, e.g., *Avibirnavirus* [15], influenza A virus [16–19], porcine circovirus type 2 [20], Ebola virus [21], and *Seneacavirus A* [22].

Infectious bursal disease virus (IBDV), the representative member of the genus *Avibirnavirus*, belongs to the *Birnaviridae* family [23]. The genome of IBDV is a double-strand RNA (dsRNA) comprising segment A and segment B [24]. Segment A encodes nonstructural protein VP5 and structural proteins VP2, VP3, and VP4 [25,26]. Segment B encodes the RNA-dependent RNA polymerase VP1 [27,28]. Many reports demonstrated that IBDV protein VP3 plays an important role in virus morphogenesis, the negative regulation of type I interferon (IFN) β, and cell autophagy [29–31]. The impact of VP3 ubiquitination on viral replication are...
unclear, although the roles of chemical modification of VP1 and VP4 have been reported [15,25,32,33].

An increasing number of E3 ubiquitin ligases are implicated to resist virus infection by mediating host antiviral immune responses [34]. However, many viruses have evolved elaborate mechanisms to hijack host E3 ubiquitin ligases to support self-replication [21–35]. TRAF6 (TNF receptor associated factor 6), an E3 ubiquitin ligase, plays an essential role in the NFκB/NF-xB (nuclear factor kappa B) signaling pathway and multiple pattern recognition receptor (PRR) signaling pathways [40]. In the NFκB signaling pathway, TRAF6 is auto-ubiquitinated through K63-linked ubiquitin chains [41], followed by activation of a complex comprising MAP3K7/TAK1 (mitogen-activated protein kinase kinase kinase 7), TAB2 (TGF-beta activated kinase 1 [MAP3K7] binding protein 2), and TAB3 (TGF-beta activated kinase 1 [MAP3K7] binding protein 3) [42–44]. This complex then induces phosphorylation of the IKK (inhibitor of IKKB/IκB) kinase complex (CHUK/IKKa, IKKB/IKKβ, and IKKBG/IKKy), resulting in activation of NFκB and IFNβ production [45]. In addition, TRAF6’s E3 ubiquitin ligase activity can also mediate host protein ubiquitination, and affects substrate protein stability and subcellular localization [3,4,10]. However, to the best of our knowledge, TRAF6-mediated viral protein ubiquitination has not been reported.

The objective of this study was to explore whether Avibirnavirus protein VP3 undergoes ubiquitination and, if so, how this might regulate its function. We demonstrated that VP3 was a ubiquitinated protein during infection and interacts with the zinc finger domain of TRAF6 to promote its ubiquitination, which facilitated VP3 nuclear accumulation and virus replication. Surprisingly, we found that VP3 induced TRAF6 autophagic degradation in an SQSTM1/p62-dependent manner, which inhibited TRAF6-dependent NFκB activation and IFNβ production to support viral replication.

Results

Avibirnavirus VP3 protein undergoes ubiquitination during infection

To detect the chemical modification of Avibirnavirus VP3 protein, lysates from IBDV-infected DF-1 or 293 T cells were subjected to immunoblotting assays. The results showed that VP3, with an approximate molecular mass of 40–100 kDa, was detected (Figure 1A), which displayed a similar modified phenotype in avian DF-1 cells and human 293 T cells. To confirm that the protein bands with larger molecular weights were the ubiquitinated VP3, the lysates of IBDV-infected DF-1 cells and 293 T cells co-transfected with MYC-VP3 (VP3 overexpression) and HA-Ub (Ubiquitin overexpression) plasmids were subjected to immunoprecipitation with anti-VP3 or anti-MYC mouse monoclonal antibodies (mAbs), respectively. The results showed that VP3 was heavily modified by polyubiquitin chains (Figure 1B and 1C). However, ubiquitinated VP3 was not detected in purified IBDV particles (Figure 1D), suggesting that ubiquitination of VP3 was strictly controlled during the different stages of the viral life cycle. Taken together, these data suggested that VP3 was ubiquitinated during infection.

Avibirnavirus VP3 has multiple ubiquitination sites

To identify the ubiquitination sites on Avibirnavirus VP3, mass spectrometry (MS) assays were used. The results showed that VP3 contained five ubiquitination sites: K73, K135, K158, K193, and K219 (Figure 2A). To further verify these ubiquitination sites, we constructed different mutants of VP3 bearing single Lys (K)-to-Arg (R) substitutions and all five substitutions (5KR) and performed immunoprecipitation assays. The results revealed that polyubiquitin chain conjugation to the K73R, K135R, K158R, K193R, and K219R mutants were reduced in comparison with wild-type (WT) VP3 (Figure 2B) and the ubiquitination of 5KR mutant VP3 was almost completely abolished (Figure 2C), confirming that residues K73, K135, K158, K193 and K219 were the major ubiquitination sites on VP3.

VP3 directly binds to TRAF6 to block proteasome degradation and enhance nuclear localization

Ubiquitination affects protein stability [22,46]. To investigate the impact of ubiquitin on VP3 stability, 293 T cells were co-transfected with vectors expressing MYC-VP3 or MYC-VP3 ubiquitination site mutants along with HA-Ub, and then subjected to immunoblotting and reverse transcription PCR (RT-PCR) assays. The results showed that ubiquitin strongly increased the protein level of WT VP3, but not that of the VP3[5KR] mutant, although the levels of both WT and 5KR mutant VP3 mRNA did not change significantly (Figure 3A). Meanwhile, Ubiquitin (Ub) overexpression markedly increased the half-life of VP3 in cells treated with cycloheximide (CHX) to block de novo protein synthesis (Figure 3B). These findings demonstrated that ubiquitination of VP3 contributed to protein stability rather than degradation.

Ubiquitin E3 ligases are necessary for the ubiquitin system [47]. To determine the E3 ubiquitin ligase that modulated VP3, several host E3 ubiquitin ligases, TRAF3 (TNF receptor associated factor 3), TRAF6, RNF125 (ring finger protein 125), RNF135 (ring finger protein 135), TRIM25 (tripartite motif containing 25), and TAX1BP1 (Tax1 binding protein1), were selected. Among the selected E3 ligases, western blotting detected that only TRAF6 overexpression obviously enhanced VP3 protein stability (Fig. S1), indicating that TRAF6 was relevant to the stability of VP3. Subsequently, the interaction between TRAF6 and VP3 was detected. As shown in Figure 3C and 3D, co-immunoprecipitation (co-IP) assays showed that TRAF6 interacted with VP3 during co-transfection or IBVD infection. In addition, in vitro glutathione-S-transferase (GST) affinity-isolation assays further confirmed the direct association between TRAF6 and VP3 (Figure 3E). To determine whether TRAF6 served as an E3 ligase for VP3 ubiquitination, we detected the level of polyubiquitin linkages on VP3 in the presence or absence of TRAF6. As shown in Figures 3F and 3G, the level of VP3 ubiquitination was dramatically enhanced by TRAF6 overexpression. Considering that the type of polyubiquitin chains
conjugated to the lysine residues of the substrate protein provokes distinct biological effects \[^{[48-51]}\], we detected ubiquitin linkage types conjugated onto VP3 driven by TRAF6. The results showed that TRAF6 was the major E3 ubiquitin ligase that catalyzes K11- and K33-linked polyubiquitination of VP3 (Figure 3H). Subsequently, we assessed if Lys 73, 135, 158, 193, and 219 in VP3 were the major sites of TRAF6-mediated ubiquitination. As shown in Figure 3I, ubiquitin chain conjugation to the VP3 Lys 73, 135, 158, 193, and 219 Arg mutants was greatly reduced in TRAF6 expressing cells compared with those of WT VP3, and the promotion of TRAF6 on VP3 ubiquitination was almost completely abolished when the five lysines were substituted with arginine. Taken together, these data demonstrated that TRAF6 enhanced the K11- and K33-linked ubiquitination of VP3 at Lys 73, 135, 158, 193, and 219.

To investigate if TRAF6-mediated ubiquitination played a role in modulating the stability of VP3, we evaluated the levels of WT and 5KR mutant VP3 protein in 293 T cells overexpressing TRAF6. As shown in Figure 3J, overexpression of Ub and TRAF6 greatly enhanced the WT VP3 protein level, but not the level of VP3[5KR], and the level of VP3 mRNA showed no obvious change. However, TRAF6 still could upregulate the levels of the K73R, K135R, K158R, K193R, and K219R mutants dramatically (Fig. S2). These results demonstrated that TRAF6 might increase the stability of VP3 through inducing its ubiquitination. To further assessed the impact of TRAF6 on VP3 stability, cells expressing MYC-VP3 or co-expressing MYC-VP3 and FLAG-TRAF6 (TRAF6 overexpression) were treated with CHX or the ubiquitin proteasome inhibitor, MG132. We observed that VP3 levels were gradually reduced in CHX treated cells without FLAG-TRAF6 overexpression, but overexpressing TRAF6 prevented such reduction (Figure 3K). Meanwhile, VP3 gradually accumulated upon MG132 treatment in cells with or without TRAF6 overexpression, and was higher in

Figure 1. Avibirnavirus VP3 is ubiquitinated during infection. (A) Western blotting analysis of viral protein VP3. DF-1 and 293 T cells were infected with IBDV (MOI = 10) for 12 h. Cellular lysates were subjected to western blotting with anti-VP3 mouse mAbs. (B) Viral protein VP3 undergoes ubiquitination during infection. IBDV-infected DF-1 cells at an MOI of 10. At 12 h after infection, cellular lysates were used for VP3-immunoprecipitation and western blotting with anti-ubiquitin and anti-VP3 mouse mAbs. (C) VP3 is ubiquitinated during transfection. 293 T cells were co-transfected with vectors expressing MYC-VP3 and HA-Ub for 48 h. Cellular lysates were subjected to an immunoprecipitation assay with anti-VP3 mouse mAbs and western blotting with the indicated antibodies. (D) Ubiquitinated VP3 is not present in purified IBDV particles. IBDV virions were subjected to western blotting analysis using anti-ubiquitin and anti-VP3 mouse mAbs.
Figure 2. Identification of ubiquitination sites on VP3. (A) Mass spectrometry identification of ubiquitinated VP3 revealed multiple ubiquitination sites. (B and C) Confirmation of the ubiquitination sites on VP3 using an immunoprecipitation assay. Vectors expressing MYC-VP3 and its mutants, together with HA-Ub, were separately co-transfected into 293 T cells for 48 h. The lysates were subjected to VP3-immunoprecipitation and western blotting with the indicated antibodies.
Figure 3. Viral protein VP3 targeting to TRAF6 enhances its stability via blocking proteasome-mediated degradation. (A) Ubiquitin overexpression increases the stability of the WT VP3 protein but not the 5KR mutant VP3. 293 T cells were co-transfected with vectors expressing MYC-VP3 or MYC-VP3[5KR] and HA-Ub for 24 h. Western blotting and RT-PCR assays were used separately to assess the expression levels of VP3 protein and mRNA. (B) Ubiquitin prolongs the lifespan of viral protein VP3. Vectors expressing MYC-VP3 and HA-Ub or empty vector were co-transfected into 293 T cells for 24 h, followed by treatment with CHX (100 μg/ml). (C and D) TRAF6 interaction with viral protein VP3. Cellular lysates from 293 T cells transfected with vectors expressing FLAG-TRAF6 and MYC-VP3 (C) or DF-1 cells infected with IBDV (D) were subjected to co-IP and western blotting assays using the indicated antibodies. (E) TRAF6 interaction with viral protein VP3 by GST affinity-isolation
TRAF6-overexpressed cells (Figure 3L). Consistently, in TRAF6 deficient 293 T cells constructed using the clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9) method (Fig. S3), as shown in Figures 3M and 3N, VP3 had shorter life span and lower accumulation in TRAF6 deficient 293 T cells treated with CHX and MG132, respectively.

In addition, TRAF6-mediated ubiquitination was reported to promote the nuclear trafficking of substrates to enhance protein stability [4,10]. Thus, to determine if TRAF6-mediated ubiquitination of VP3 involves modulation of VP3 nuclear transport, VP3 was analyzed by an immunoblotting assay in the cytoplasm and nucleus of 293 T cells co-transfected with MYC-VP3, HA-Ub, and FLAG-TRAF6. As shown in Figure 3O, the accumulation of VP3 increased significantly in the nuclear fraction of cells overexpressing TRAF6. These findings indicated that VP3 directly bound to TRAF6 to enhance its nuclear accumulation by blocking proteasome-dependent degradation of VP3.

The zinc finger domain of TRAF6 is crucial to enhance the stability and nuclear accumulation of viral protein VP3

To explore if the increase of VP3 stability induced by TRAF6 depended on the RING domain with E3 ubiquitin ligase activity, we constructed a TRAF6 mutant possessing a cysteine (C)-70-to-alanine (A) substitution in the RING domain and found that overexpression of WT, and the C70A mutant TRAF6, greatly enhanced the concentration of VP3 (Figure 4A), suggesting that TRAF6-mediated VP3 stability was RING domain-independent. Thus, we hypothesized that TRAF6 involved in the stability of VP3 functions through other domains. Subsequently, we constructed different TRAF6-truncation mutants, named as TRAF6ΔRING, TRAF6ΔZN, TRAF6ΔZinc and TRAF6ΔTRAF-C mutants (Figure 4B), and detected their interaction with VP3. In western blotting and co-IP assays, we observed that WT TRAF6, TRAF6ΔRING and TRAF6ΔTRAF-C, but not TRAF6ΔZN and TRAF6ΔZinc, interacted with VP3 and increased its stability (Figure 4C). To eliminate the impact of high expression, we only assessed the association of TRAF6ΔZm and TRAF6ΔZnc and VP3, and discovered that the zinc finger domain deletion of TRAF6 abolished the interaction with VP3 (Figure 4D).

Similarly, to investigate whether the ubiquitination sites on VP3 affected the binding to TRAF6, the interaction of different VP3 mutants with TRAF6 were detected using co-IP and western blotting. The results exhibited that 5KR mutant VP3 completely lost the ability to interact with TRAF6 and the interaction of VP3 K135R mutant with TRAF6 also was reduced significantly. However, the TRAF6 association with VP3 mutants K73R, K158R, K193R or K219R hardly changed compared with that of WT VP3 (Figures 4E and 4F). Subsequently, we further revealed that the zinc finger domain was essential for TRAF6-mediated VP3 ubiquitination (Figure 4G). Notably, a recent study revealed that zinc region might have E3 ubiquitin ligase activity [52]. Thus, we speculated that TRAF6-mediated ubiquitination of VP3 might be zinc finger domain-dependent rather than RING domain. Subsequently, to investigate whether the mutant TRAF6C70A retained a capacity to mediate VP3 ubiquitination, we constructed different E2 conjugation enzymes and determined that UBE2D1 (ubiquitin conjugating enzyme E2 D1) was co-binding partner of TRAF6 and viral protein VP3 (Fig. S4A and S4B). Moreover, in vitro ubiquitination assay, we verified that the VP3 ubiquitination driven by TRAF6-UBE2D1 complex was irrelevant to RING domain activity of TRAF6 (Figure 4H). Additionally, we observed that TRAF6-mediated VP3 nuclear accumulation also depended on its zinc finger domain (Figure 4I). Altogether, these data demonstrated that VP3 interacted with the zinc finger domain on TRAF6, which was crucial to enhance the stability and nuclear accumulation of VP3.

The zinc finger domain of TRAF6 mediates VP3 ubiquitination and is crucial for sustaining virus replication

To evaluate the impact of ubiquitination of VP3 at K73, K135, K158, K193, or K219 on IBDV replication, T7 RNA polymerase promoter rescue plasmid, T7-A, possessing VP3K73R, VP3K135R, VP3K158R, VP3K193R, VP3K219R, or VP3[5KR] were generated by mutation using the wild type T7-A plasmid as the template. These mutants and wild-type T7-A along with T7-B were separately co-transfected into BSR77 cells, followed by infection of fresh DF1 cells. As shown in Figure 5A, except for the recombinant mutants rK135R and r5KR, the recombinant viruses rWT, rK73R, rK158R, rK193R, and rK219R successfully rescued IBDV replication, indicating

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Figure 4. Viral protein VP3 binding to the zinc finger domain of TRAF6 mediated its ubiquitination. (A) The RING domain of TRAF6 with E3 ubiquitin activity had no impact on VP3 stability. 293 T cells were co-transfected with vectors expressing WT or C70A mutant TRAF6 and MYC-VP3 for 24 h. Cellular lysates were subjected western blotting assays with the indicated antibodies. (B) Schematic representation of TRAF6 and its truncated mutants. (C and D) The zinc finger domain of TRAF6 was required for the interaction with VP3. MYC-VP3 and FLAG-TRAF6 or TRAF6 deletion mutants were co-transfected into 293 T cells. Cellular lysates were subjected to FLAG-immunoprecipitation and western blotting using the indicated antibodies. (E and F) Ubiquitination sites on VP3 were required for binding to TRAF6. 293 T cells were co-transfected with vectors expressing MYC-VP3 or VP3 mutants and FLAG-TRAF6 for 48 h. Cellular lysates were subjected to immunoprecipitation with anti-FLAG mouse mAbs and western blotting assays using the indicated antibodies. (G) The zinc finger domain of TRAF6-mediated ubiquitination of VP3. Cellular lysates from 293 T cells overexpressing MYC-VP3, HA-Ub, and FLAG-TRAF6 or TRAF6 deletion mutants were subjected to an immunoprecipitation assay with anti-VP3 mouse mAbs and a western blotting assay using the indicated antibodies. (H) In vitro ubiquitination assays showed that the RING domain activity was unnecessary for TRAF6-mediated ubiquitination of VP3. IPTG-inducible HIS-tagged recombinant proteins E1(UBA1), UBE2D1, ubiquitin, TRAF6, TRAF6C70A and GST-VP3 were expressed.
that the ubiquitination of VP3 at K135 was essential for virus survival. Subsequently, TCID 50 detection exhibited that in vitro the replication abilities of the viruses rK73R, rK193R, and rK219R were markedly decreased in comparison with the rWT virus, and that the rK158R mutation was insignificant (Figure 5B). The consistent data was also obtained in the rK158R virus but not rK73R, rK193R virus infected bursal tissues of the specific pathogen free (SPF) chickens by RT-qPCR assay (Figure 5C). However, the histopathological analysis showed that rK158R and rK193R recombinant viruses, but not rK73R and rK219R viruses, induced more obvious lesions in the bursae of SPF chickens in comparison with rWT, rK73R and rK219R (Figure 5D), suggesting that the substitution of K158R and K193R in VP3 could strengthen the pathogenicity of IBDV. Collectively, these data demonstrated that the ubiquitination of VP3 supported viral replication.

Given that the zinc finger domain of TRAF6 was critical for VP3 binding and mediating its ubiquitination, we investigated the impact of overexpression or knockdown of TRAF6 on IBDV replication by detecting virus replication. TCID 50 detection showed that the virus titer increased significantly in TRAF6 overexpressing DF-1 cells infected with IBDV and decreased significantly in cells expressing a short hairpin RNA (shRNA) targeting TRAF6 compared with that in control cells infected with IBDV (Figure 5E-G). Meanwhile, we observed that WT TRAF6, but not TRAF6ΔZinc, dramatically promoted IBDV replication (Figure 5H). Considering that chicken is the natural host of IBDV infection, we further investigated the relationship of chicken TRAF6 and viral protein VP3. The results showed that chicken TRAF6 associated with VP3 and enhanced VP3 stability and IBDV proliferation by its zinc finger domain (Fig. S5A-D). Collectively, these results indicated that the ubiquitination sites of VP3 binding to the zinc finger domain of TRAF6 was necessary to ensure IBDV replication.

**VP3 facilitates TRAF6 autophagic degradation**

Considering that TRAF6 interacted with VP3, and IBDV infection induced an increase of LC3B expression and a decrease of TRAF6 and SQSTM1 expression in vitro and in vivo (Figure 6A), we hypothesized that VP3 might participate TRAF6 degradation in autophagosome-dependent manner. Therefore, we conducted a western blotting experiment for VP3 and TRAF6. The results revealed that VP3 significantly decreased the level of TRAF6 in a dose-dependent manner (Figure 6B). However, there was no significant change in the level of the TRAF6 transcript (Figure 6C), which suggested that VP3 affected the stability of TRAF6.

Next, we assessed the impact of VP3 on the half-life of TRAF6 using CHX treatment. The results demonstrated that the half-life of exogenous TRAF6 was significantly shortened in WT and 5KR mutant VP3 overexpressing cells compared with that of the control (Figures 6D and 6E). However, in comparison with WT VP3, TRAF6 was quickly degraded in the VP3[5KR] mutant overexpressing cells (Figure 6E). Moreover, the loss of ubiquitination at any ubiquitin sites of VP3 accelerated the degradation of TRAF6 by VP3 (Fig. S6).

Autophagy and the ubiquitin-proteasome system are two major intracellular degradative pathways. To determine if VP3 facilitated TRAF6 degradation by autophagy, an experiment using autophagy inhibitors, wortmannin (Wort) and bafilomycin A1 (BafA1) was conducted. As shown in Figure 6F, VP3-mediated TRAF6 degradation was almost blocked using the autophagy inhibitors Wort and BafA1, but not by MG132. To further confirm the role of autophagosomal in VP3-induced TRAF6 degradation, we constructed autophagy-related genes ATG7 and ATG5 knockout 293 T cells (Fig. S7A and S7B). ATG7 knockout partially inhibited TRAF6 degradation induced by VP3 (Figure 6H). Collectively, our data indicated that VP3-mediated degradation of TRAF6 was an autophagosome-dependent event.

**VP3 blocks NFKB activation by promoting autophagic degradation of TRAF6 in SQSTM1/p62-dependent manner**

To identify the proteins responsible for the autophagic degradation of TRAF6, we investigated the role of autophagy receptors, such as SQSTM1, OPTN (optineurin), CALCOCO2/NDP52 (calcium binding and coiled-coil domain 2), NBR1/NBR1 autophagy cargo receptor) and TAX1BP1 [53]. Of the five receptors, only SQSTM1 interacted with TRAF6 and strongly promoted TRAF6 degradation (Fig. S8A and S8B). To further identify the domain of SQSTM1 responsible for mediating the degradation of TRAF6, we constructed a series of SQSTM1-truncation mutants, deleting the ubiquitin-binding domain (UAB) responsible for binding to ubiquitinated substrates [54], Phox1 and Bem1p (PBP1) required for activating autophagy [55], TRAF-binding domain (TBS), and the LC3-interacting region (LIR) that is critical for recruiting substrates into autophagosomes for lysosomal degradation [56]. The PB1, TBS, and LIR-deleted SQSTM1 mutants obviously decreased TRAF6 degradation in comparison with WT SQSTM1 and the UAB-deleted SQSTM1 mutant (Fig. S8C), suggesting that SQSTM1 mediated TRAF6 autophagic degradation.

To further detect whether VP3-induced autophagic degradation of TRAF6 was SQSTM1-dependent, we analyzed the relationship between SQSTM1 and VP3. Co-IP assays showed that VP3 only interacted significantly with SQSTM1 (Figures 7A and 7B). Subsequently, SQSTM1-deficient 293 T cells were generated (Figure 7C), and western blotting demonstrated that VP3-induced TRAF6 degradation was
The zinc finger domain of TRAF6 binding to VP3 is essential to support viral replication. (A) IFA analysis of recombinant viruses. DF-1 cells were infected with recombinant rWT or mutant virus for 18 h, followed by IFA assay with antibodies against viral protein VP2, as described in the Materials and methods. (B) The one step growth curve of rescued viruses. DF-1 cells were infected with recombinant viruses rWT, rK73R, rK158R, rK193R, and rK219R, respectively. At the indicated time after infection, cells were harvested and subjected to virus titer detection by the TCID\(_{50}\). (C) Copies of IBDV genome were detected by RT-qPCR in vivo. ns: \( p > 0.05 \) nonsignificant difference, *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \) significant difference. (D) Histopathological examination of bursae at 5 dpi. (E) chTRAF6 overexpression facilitates IBDV replication. DF-1 cells transfected with vectors expressing FLAG-chTRAF6 or the corresponding empty vector were infected with IBDV (MOI = 10) for the indicated times. Cellular lysates were used for virus titer detection. (F) Screening of an efficient shRNA against chTRAF6. Four shRNA against TRAF6 were cloned into vector pGreenPuro\textsuperscript{™} shRNA (Biovector Science Lab, Inc., SI505A-1). These constructs and a control (scramble) shRNA were transfected separately into DF-1 cells for 48 h. Cellular lysates were subjected to western blotting using anti-chTRAF6 antibody. ACTB levels served as the loading control. Oligonucleotide sequences for an shRNA against chicken TRAF6 are listed in Table S3. (G) ChTRAF6 knockdown suppresses IBDV replication. The two effective shchT6#3 and shchT6#4 and a negative control shCON were transfected into DF-1 cells for 24 h, respectively. The resultant cells were infected, and then the viral titers were detected as described in (B). (H) The zinc finger domain of TRAF6 interacting with VP3 is necessary to promote virus replication. DF-1 cells overexpressing WT or zinc finger domain deletion mutant TRAF6 were infected with IBDV (MOI = 10). At 12 and 24 h after infection, cells were harvested and subjected to virus titer detection according to the TCID\(_{50}\).
dramatically blocked in SQSTM1 knockout cells (Figures 7D and 7E), indicating that VP3-induced TRAF6 degradation was SQSTM1-dependent. To further analyze whether IBDV VP3 protein could drive receptor SQSTM1-mediated selective autophagy, we detected impacts of VP3 overexpression on the autophagy level in cells with or without SQSTM1. As shown in Figure 7F, the capacity of VP3 on the induction of autophagy was almost completely abolished in SQSTM1-deficient cells, which indicated that VP3 could facilitate selective autophagy via SQSTM1. In addition, given that TRAF6 interaction with SQSTM1 increased selective autophagy [57], we hypothesized that the association of TRAF6 and SQSTM1 could be elevated by IBDV or viral protein VP3. As shown in Figures 7G and 7H, the interaction between TRAF6 and SQSTM1 was significantly enhanced in the presence of IBDV, WT VP3, or VP3[5KR] mutants. In addition, we observed that WT VP3 and VP3[5KR] obviously accelerated SQSTM1-induced TRAF6 degradation (Figure 7I). These findings clearly indicated that IBDV VP3 facilitated selective autophagy degradation of TRAF6 through increasing the association of TRAF6 with SQSTM1.

Discussion

The host ubiquitin (Ub) system plays important roles against diverse virus infections. TRIM22 and TRIM32 induce K48-linked polyubiquitination of the nucleocapsid protein (NP) and polymerase basic protein 1 (PB1) of influenza A virus for degradation and to restrict viral replication, respectively [16,49]. The pRNFIN14 protein mediates K27-linked polyubiquitination of the NS4B protein of swine fever virus, leading to its degradation [51]. However, recently, many studies have revealed that Ub system can be hijacked by viruses to promote viral proliferation. E3 ligase ASB8 and E2 ubiquitin-conjugating enzyme UBE2L6 separately promote the ubiquitination of the Nsp1α protein of porcine reproductive and respiratory syndrome virus and RNA polymerase 3D of Senecavirus A to enhance stability and facilitate virus replication [22,50]. In the present study, our data demonstrated that the stability of Avibirnavirus VP3 could be enhanced via ubiquitination, leading to the promotion of viral replication (Fig. 3A, 3B, 5B and 5C). Additionally, we also revealed that ubiquitinated VP3 could be detected in IBDV-infected cells, but not in purified virions, which indicated that ubiquitination of VP3 was strictly controlled during infection and did not participate in the assembly of IBDV virions. Similarly, NP and PB1 proteins of influenza A virus can be efficiently conjugated to ubiquitin during replication and these ubiquitinated proteins are not incorporated into mature viral particles [16,49].

In this study, viral protein VP3 exhibited the K11- and K33-linked polyubiquitination by interacting directly with zinc finger domain of TRAF6, which increased VP3 nuclear accumulation (Fig. 3H and 4G and I). E3 ubiquitin ligases mediate the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to specific substrate proteins [47]. To mediate self-ubiquitination and regulate viral replication, host E3 ubiquitin ligases can be employed by viral proteins, e.g., Ebola virus protein VP35 [21], porcine circovirus type 2 Cap protein [20], the polymerase basic protein 1 and nucleocapsid protein of influenza A virus [16,17,49,58], and the Nsp1α protein of porcine reproductive and respiratory syndrome virus [50]. Previous studies demonstrated that TRAF6-mediated polyubiquitination modulates the subcellular trafficking of the substrate protein to enhance its stability [2,10]. Moreover, the RING and zinc finger domains on TRAF6 are essential for its ubiquitin E3 ligase activity [59]. Thereby, we concluded that during Avibirnavirus replication, VP3 hijacks TRAF6 for supporting viral proliferation. Additionally, our recent study reveals that Avibirnavirus VP3 in nucleus can target directly and de-SUMOylate API5 to repress IFNβ production [60], implying that TRAF6-mediated VP3 ubiquitination enhanced its nuclear accumulation to inhibit API5-mediated IFN expression (Figure 8).

TRAF6 is also an important adaptor of NFKB and diverse PRR pathways [40,61]. The activation of NFKB regulates the production of type I IFNs [62]. Many viruses can evade host antiviral response through affecting the expression or activity of TRAF6, such as human immunodeficiency virus-1 [63], human parainfluenza virus type 2 [64], human T-cell leukemia virus type 1 [3], and classical swine fever virus [65]. Additionally, IFNβ plays a critical role in the host response against IBDV infection [66]. Moreover, Avibirnavirus protein VP3 is inhibitor of IFIH1/MDA5-induced IFNβ production [30]. In present study, IBDV infection or VP3 overexpression induced decreased TRAF6 levels, and VP3 promoted the autophagic degradation of TRAF6 by targeting autophagic receptor SQSTM1, finally resulting in the inhibition of NFKB activation-dependent IFNβ production induced by TRAF6. Thus, we believe that Avibirnavirus protein VP3 inactivated TRAF6-dependent antiviral innate immunity to support viral replication.

Notably, Avibirnavirus-encoded VP4 had been identified as a serine protease and a phosphoprotein, which contributes the cleavage of viral polyprotein precursor (NH2-pVP2-pVP4-pVP3-COOH) [25,67,68]. In recent years, increasing evidences shows that some viral proteinases, i.e. aichi virus 3C protease [69], coxsackievirus B3 (CVB3) viral proteinase 2A [70] and Seneca Valley virus (SVV) 3Cpro [71], can target selective
Figure 6. VP3 facilitates TRAF6 autophagic degradation. (A) IBDV infection induced autophagy and decrease of TRAF6 expression in vitro and in vivo. DF-1 cells were infected with IBDV strain CT (MOI = 10) for indicated time. Two-week-old SPF chickens were infected with IBDV strain CT (10^6.5 TCID50/0.2 ml) for 120 h and chicken's bursa was collected. (B) VP3 decreases TRAF6 levels in a dose-dependent manner. Vectors expressing FLAG-TRAF6 and MYC-VP3 or the corresponding empty vector were co-transfected into 293 T cells for 24 h. Cellular lysates were subjected to RT-PCR as described in the Materials and methods. (D) VP3 overexpression shortens the half-life of TRAF6. Vectors expressing FLAG-TRAF6 and MYC-VP3 or corresponding empty vector were co-transfected into 293 T cells for 24 h. The resultant cells were treated with cycloheximide (CHX, 100 μg/ml). (E) The effects of WT and 5KR mutant VP3 on TRAF6 stability. 293 T cells were co-transfected with vectors expressing FLAG-TRAF6 (500 ng) and MYC-VP3 (500 ng), its mutant 5KR (1 μg) or control vector MYC-GFP for 24 h, and then treated with CHX for the indicated time. (F) VP3 facilitates TRAF6 autophagic degradation. Vectors expressing FLAG-TRAF6 and MYC-VP3 or empty vector were co-transfected into 293 T cells for 36 h, followed by treatment for 12 h with autophagosomal inhibitors, bafilomycin A1 (100 nM) or wortmannin (10 μM) and ubiquitin proteasome inhibitor, MG132 (10 μM). (G and H) Immunoblotting analysis of FLAG-TRAF6 in cellular lysates from WT, ATG7 (G) or ATG5 (H) knockout cells transfected with vectors expressing MYC-VP3. WT, ATG7 or ATG5 knockout 293 T cells were co-transfected with vectors expressing FLAG-TRAF6 and different doses of MYC-VP3 for 36 h. Cellular lysates of samples from (A), (B) and (D-H) were subjected to western blotting using the indicated antibodies. ACTB levels served as a loading control.
autophagy receptor SQSTM1 to downregulate its expression for ensuring viral propagation. In present study, Avibirnavirus infection induced decreased TRAF6 levels by VP3 targeting autophagic adaptor SQSTM1. Considering these data, whether viral protein VP4 also binds SQSTM1 to induce TRAF6-autophagic degradation during Avibirnavirus infection deserves further investigation.

In summary, our findings demonstrated that Avibirnavirus VP3 could be ubiquitinated at the Lys residues 73, 135, 158, 193, and 219. Ubiquitin chains conjugated to the Lys residues 73, 135, 193, and 219 on VP3 played an essential role in ensuring IBDV replication. In addition, the zinc finger domain of TRAF6 interacted with VP3 and mediated K11- and K33-linked ubiquitination of VP3 to enhance its nuclear accumulation, resulting in the inhibition of SUMOylated API5-mediated IFNβ production and the increase of viral replication. Meanwhile, VP3 induced TRAF6 selective autophagy degradation by enhancing the recognition of TRAF6 by SQSTM1, leading to the inhibition of TRAF6-mediated NFκB activation and IFNβ production (Figure 8).

Materials and methods

Cells and viruses

DF-1 (ATCC, CRL-12203) and HEK293T (ATCC, CRL-11268) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, 12,100–088) containing 10% fetal bovine serum (FBS). BSRT cells stably expressing MYC-SQSTM1 and FLAG-tagged autophagy receptors were stored with a multiplicity of infection (MOI) of 10. At different time points, cells were harvested and lysed in Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, P0013F), ATP (D7378-1 ML) and the dual luciferase reporter gene assay kit (Rg028) were purchased from Beyotime Biotechnology. Phenylmethylsulfonyl fluoride (PMSF; P8340) and 1,4-dithio-DL-threitol (DTT; 3483–12–3) were purchased from Solarbio. G418 (ant-gn-1), poly (I:C) (tlrl-pciv), and puromycin (58–58–2) were purchased from InvivoGen. The RevertAid RT reverse transcription kit (K1622) was obtained from Thermo Fisher Scientific. Biobest transfection reagents (BB0002) were provided by BioBEST Biotechnology. Protein A/G PLUS-Agarose (sc-203) was purchased from Santa Cruz Biotechnology. RNase-Free DNase (M6101) was purchased from Promega. The PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (RR047A) and TB Green’ Premix Ex Taq’ (Tli RNaseH Plus) (RR420A) were purchased from Takara.

Constructs

Dual luciferase reporter plasmid IFNB was generously provided by Prof. Jihui Ping from the Nanjing Agricultural University. Reporter plasmid NFKB (D2206) was purchased from Beyotime Biotechnology. TRAF6 ORFs amplified from DF-1 and 293 T cells were separately inserted into vector pCMV-FLAG-N vector (Clontech, 635,688) or pCMV-MYC-N vector (Clontech, 635,689) designed as FLAG-chTRAF6, FLAG-TRAF6 and MYC-TRAF6, respectively. Vectors expressing MYC-SQSTM1 and FLAG-tagged autophagy receptors (SQSTM1, OPTN, CALCCO2/NDP52, NBR1, and TAX1BP1), MYC-VP3, rescued plasmid IBDV strain CT T7-A and T7-B, HA-Ub, HA-UbK48, and HA-UbK63 were stored in our laboratory. MYC-SQSTM1-(T2, T3, T4) and FLAG-TRAF6, pET-32A-TRAF6 and FLAG-chTRAF6 mutants were constructed by mutation using the WT plasmid as the template. MYC-SQSTM1-T1 or T5 and FLAG-tagged TRAF3, RNF125, RNF135, TRIM25, UBE2D2 and UBE2D3 were generated by standard molecular biology techniques. Except for UBE2D2 and UBE2D3, the expression plasmids of human FLAG-tagged E2 conjugated enzymes were constructed by ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co., Ltd, C113-02). Several mutants of MYC-VP3, rescued plasmid T7-A, and HA-Ub were constructed by site-specific mutation experiments. All primers used are summarized in Table S1.

Virus infection and western blotting

DF-1 cells were mock-inoculated or infected with IBDV at a multiplicity of infection (MOI) of 10. At different time points, cells were harvested and lysed in Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, P0013C). Protein samples were prepared and separated by SDS-PAGE, followed by transfer to nitrocellulose blotting membranes (GE Healthcare Life Science, 10,600,001). After blocking with 5% skim milk containing 0.1% Tween 20 (Amresco, 0777–1 L) for 1 h at room temperature, the membranes were washed three times with phosphate-buffered saline (PBS; 137 mM NaCl [Sinopharm Chemical Reagent Co., Ltd, 10,019,318], 10 mM Na2HPO4 [Sinopharm Chemical Reagent Co., Ltd, 10,020,318], 1.8 mM NaH2PO4 [Sinopharm Chemical Reagent Co., Ltd, 10,019,318]).
Figure 7. VP3 blocks TRAF6-mediated NFKB activation by SQSTM1-targeted TRAF6 autophagic degradation. (A and B) VP3 interacts strongly with SQSTM1, but not with OPTN, CALCOCO2, NBR1, and TAX1BP1. Vectors expressing MYC-VP3 and different FLAG-tagged autophagy receptors were co-transfected into 293 T cells for 48 h, respectively (A). 293 T cells were transfected with MYC-VP3 for 36 h (B). Cellular lysate samples from (A) and (B) were subjected to an immunoprecipitation assay using anti-FLAG mouse mAbs or anti-VP3 mouse mAbs, followed by an immunoblotting assay using the indicated antibodies. (C) Generation of SQSTM1 knockout 293 T cells. The genomic DNA and cellular lysates from WT or SQSTM1 knockout 293 T cells were subjected to T7 endonuclease I (upper) and western blotting assays with anti-SQSTM1 rabbit pAbs (bottom). (D and E) VP3 promotes TRAF6 degradation in autophagic receptor SQSTM1-dependent manner. WT or SQSTM1 knockout 293 T cells were transfected with vectors expressing FLAG-TRAF6 and MYC-VP3 (D) or MYC-VP3 alone (E) for 36 h. Cellular lysates were subjected to western blotting analysis using the indicated antibodies. (F) IBDV VP3 induced receptor SQSTM1-mediated selective autophagy. WT and SQSTM1 knockout cells were separately transfected with MYC-VP3 or corresponding empty vector for 42 h, the lysates were subjected to western blotting assays with indicated antibodies. GAPDH served as loading control. (G) 293 T cells were co-transfected with vectors expressing FLAG-TRAF6 and MYC-SQSTM1 for 30 h, followed by IBDV infection and autophagic inhibitor BafA1 (200 nM) treatment for 12 h. The lysates were immunoprecipitated using anti-FLAG mouse mAbs and immunoblotted using the indicated antibodies.
**IBDV virion purification**

Sucrose (Sinopharm Chemical Reagent Co., Ltd, 10,021,418) density gradient centrifugation was performed. DF-1 cells were infected with IBDV (MOI = 0.01) and harvested when the cytopathic effect was obviously displayed. After freeze-thawing three times, the cellular lysates were collected and centrifuged at 6,000 × g for 10 min. The supernatants were then ultra-centrifuged at 160,000 × g for 1.5 h at 4°C using an Optima L-100XP ultracentrifuge (Beckman Coulter). The pellets were resuspended and the viral suspension after centrifugation at 12,000 × g for 10 min at 4°C was transferred to Ultra-Clear centrifuge tubes (Beckman Coulter, 344,057). Sucrose solutions at various concentrations of 30% to 60% were prepared. Then, 1 ML of 30%, 40%, 50%, and 60% sucrose working solution were added sequentially to the bottom of the tube to form a density gradient. Samples were added on the top of the gradient, and then centrifuged at 133,900 × g for 3.5 h at 4°C. Fractions at different sucrose concentration interfaces were collected. Finally, purified IBDV particles were subjected to 12% SDS-PAGE followed by Coomassie Brilliant Blue R250 staining.

**Protein expression and purification**

The plasmids pGEX-4 T-1-VP3, pET-28A-TRAF6, pET-32A-TRAF6, pET-32A-TRAF6C70A, pET-32A-UBA1 and pET-32A-UBE2D1 were transformed into Escherichia coli BL21 (pLysS; Sangon Biotechnology, B528415-0010) competent cells, respectively, and then induced with 1 mM IPTG (Sangon Biotechnology, A100399-0005). Mc-lysates were lysed by binding buffer by sonication. After centrifugation at 12,000 × g for 10 min at 4°C, HIS-tagged recombinant proteins, GST, and GST-VP3 in the supernatants were purified using Ni-NTA agarose (QIAGEN, 30,210) and Glutathione (GST) Resin (GenScript, L00206) according to manufacturer’s instructions and eluted in elution buffer. For HIS-tagged recombinant proteins, the binding buffer was 50 mM Tri-HCl, pH 8.0, 10 mM imidazole; and the elution buffer was binding buffer containing 80 mM imidazole. For GST and GST-VP3, the binding buffer was 50 mM Tri-HCl, pH 8.0, 150 mM NaCl; and the elution buffer was binding buffer containing 10 mg/5 ml reduced glutathione (Sangon Biotechnology, A100399-0005).

**Co-immunoprecipitation and GST affinity-isolation assays**

For the co-IP assay, cells were lysed in NP-40 lysis buffer containing PMSF for 4 h. After centrifugation at 12,000 × g for 10 min at 4°C, the supernatants were incubated with the indicated antibodies and protein A/G PLUS-agarose for 4 h at 4°C. For the GST affinity-isolation assay, purified GST (10 μg) and GST-VP3 (10 μg) recombinant proteins were separately mixed with purified HIS-TRAF6 (20 μg) in 500 μL NP-40 lysis buffer for 4 h at 4°C, followed by adding the GST resin. After centrifugation at 1,000 × g for 5 min at 4°C, the pellets were washed with NP-40 lysis buffer and then lysed in RIPA lysis buffer (Beyotime Biotechnology, P0013B) for immunoblotting analysis.

**Mass spectrometry (MS)**

To determine the ubiquitinated sites on viral protein VP3, MYC-VP3 was transfected into 293 T cells. At 48 h after transfection, the cells were harvested and lysed in NP-40 lysis buffer containing PMSF and N-ethylmaleimide (NEM) for 4 h at 4°C. The supernatants were subjected to an immunoprecipitation assay using anti-MYC mouse mAbs. The immunoprecipitation complexes were subjected to SDS-PAGE and Coomassie Brilliant Blue R250 staining. Subsequently, the regions of the staining gels beyond 25 kDa were mixed together and subjected to Liquid Chromatography Mass Spectrometry (LC-MS) analysis in APTBio (Shanghai, China) to identify the specific ubiquitinated sites of VP3.

**Cellular fractionation**

The process has been described previously [75].

**Indirect immunofluorescence assay (IFA)**

Mock-infected or IBDV-infected DF-1 cells were fixed with an equal volume of methanol and acetone for 30 min at 0°C, and then treated with BafA1 (200 nM) for 12 h. The cellular lysates were used for immunoprecipitation and immunoblotting assays with the indicated antibodies. (H) Vectors expressing FLAG-TRAF6, MYC-SQSTM1, and MYC-VP3 or its mutant SKR were co-transfected into 293 T cells. At 30 h after transfection, the cells were treated with IFNB or NFκB reporter plasmids along with FLAG-TRAF6 in the presence or absence of the plasmid encoding VP3.
20°C, and then were incubated with anti-VP2 mouse mAbs overnight at 4°C. After washing three times with PBS (137 mM NaCl [Sinopharm Chemical Reagent Co., Ltd, 10,019,318], 2.7 mM KCl [Sinopharm Chemical Reagent Co., Ltd, 10,016,318], 10 mM Na2HPO4 [Sinopharm Chemical Reagent Co., Ltd, 10,020,318], 1.8 mM KH2PO4 [Sinopharm Chemical Reagent Co., Ltd, 10,017,618], pH 7.4), the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Inc., 172–1806) for 1 h at room temperature. After washing five times with PBS, the fluorescence signal was scanned under a fluorescence microscope (OLYMPUS, U-RFL-T).

**Virus rescue**

Recombinant IBDV strain CT possessing wild-type (WT) or K73R, K158R, K193R, or K219R mutant VP3 (designated as rWT, rK73R, rK158R, rK193R, and rK219R) were generated by reverse genetics, as described previously [15]. Briefly, WT or several mutants T7-A clones together with T7-B were co-transfected separately into BSRT7 cells for 72 h, with T7-A alone as a negative control. After freeze-thawing, cellular lysates after were centrifugated at 6, 000 × g for 10 min, and the supernatants were transferred into fresh DF-1 cells. At 3 h after infection, the supernatants were removed, followed by adding fresh DMEM containing 2% FBS for continuous culture for another 48 h.

**Viral titer detection**

DF-1 cells were infected with IBDV (MOI = 10). At different time points after infection, cells were harvested and freeze-thawed three times. After centrifugation at 12,000 × g for 10 min at 4°C, the supernatants were subjected to TCID50 (50% tissue culture infective dose) detection. Briefly, the viral suspension was diluted 10-fold using DMEM containing 2% FBS, and then added to fresh DF-1 cells. Eight repeats of each diluted sample were tested. At 48 h after infection, the cells were subjected to an IFA assay with anti-VP2 mouse mAbs. Virus titers were determined by observing the infected cells under a fluorescence microscope (OLYMPUS, U-RFL-T) and calculating the TCID50 per 0.1 ML.

**Reverse transcription PCR (RT-PCR) and quantitative real time reverse transcription PCR (qRT-PCR)**

For RT-PCR, total RNA was extracted and 1 μg of RNA treated by RNase-Free Dnase was reverse-transcribed into cDNA using a RevertAid RT reverse transcription kit (Thermo Fisher Scientific, K1622). Subsequently, VP3 and GAPDH mRNAs were amplified using 2 × Taq Master Mix (Vazyme Biotech Co., Ltd, P114-01). The PCR products were separated on 1.5% nucleic acid agarose gels, and the images were scanned using a Gel Documentation system (GenoSens 1880). For qRT-PCR, total RNA was extracted and reverse-transcribed into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser according to the manufacturer’s protocols. The cDNA was subjected to qPCR using TB Green® Premix Ex Taq™. The expression of ACTB/β-actin was used to normalize the relative abundance of indicated gene mRNA. The primers used are listed in Table S1.

**CRISPR-Cas9 knockout**

The TRAF6 gene target sequence (5’-TACTATACTCATCCAGAGAAC-3’), ATF5 gene target sequence (5’-AAATGTACTGTGTCTTCCA-3’), ATG7 gene target sequence (5’-AGAAGAAGCTGAAAGT-3’), and SQSTM1 gene target sequence (5’-AGGGCTTCTGCACAGCCGC-3’) were inserted separately into the guide RNA expression plasmid PX459 (Addgene, 62,988; deposited by Feng Zhang). The recombinant constructs were transfected separately into 293 T cells. At 36 h after transfection, the cells were selected using puromycin (10 μg/ml) for 48 h. Finally, the monoclonal cells were obtained using the limiting dilution method. T7 Endonuclease (Vazyme Biotech Co., Ltd, EN303-01) and immunoblotting assays were used to identify...
monoclonal cell lines. The primers used for PCR amplification are listed Table S2.

**Luciferase activity assay**

293 T cells were transfected with luciferase reporter plasmids (NFKB and IFNB), pRL-TK, and the indicated plasmids. At 36 h after transfection, the cells were harvested, and luciferase activity was detected using a dual luciferase reporter kit according to the manufacturer’s instructions. All experiments were repeated at least three times.

**In vitro ubiquitination assay**

HIS-tagged E1(UBA1), UBE2D1, ubiquitin, TRAF6, TRAF6	extsuperscript{C70A} and GST-VP3 proteins were purified from *E. coli*. Subsequently, *in vitro* ubiquitination assay was conducted as described previously [76]. Briefly, 50 μL of ubiquitination buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl	extsubscript{2}, 2 mM DTT, supplemented with 2 mM ATP) was mixed with ubiquitin (50 μg), UBA1 (2 μg), UBE2D1 (3 μg), TRAF6 or TRAF6	extsuperscript{C70A} (8 μg) and GST-VP3 (20 μg). The mixture was incubated at 37°C for 2 h, and followed by GST affinity-isolation assay. Finally, the sample was washed using 1 M urea for 60 min to exclude potential binding of unanchored polyubiquitin, then the sample was placed in SDS-loading buffer and boiled for 10 min. Samples were fractionated on 8% SDS-PAGE followed by western blotting with anti-ubiquitin mAb.

**Animal experiments**

Eighteen 2-week-old SPF chickens were randomly divided into six groups and infected with viruses at a dose of 0.2 ml (10	extsuperscript{6.5} TCID	extsubscript{50}/0.2 ml), respectively, to detect the replication characteristics of the rWT and different mutant viruses *in vivo*. The inoculated chickens were bled and sacrificed at 5 days post-infection (dpi). Bursae of chickens were collected and weighed. Viral loads in bursal tissues were detected using qPCR as described previously [77]. Simultaneously, bursae were collected for pathological examination.

**Acknowledgments**

This study is supported by grants from National Natural Science Foundation of China (Grant No.31630077) and China Agriculture Research System (Grant No. CARS-40-K13).

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

This work was supported by the Agriculture Research System of China [CARS-40-K13]; National Natural Science Foundation of China [31630077].

**Statistical analysis**

Statistical differences were assessed using one-way ANOVAs using IBM SPSS Statistics 20 (IBM Corp., Armonk, NY, USA). All data are presented as the mean ± SD of three independent experiments. For all experiments, *p* < 0.05 was considered statistically significant. In the figures *p* < 0.05, **p** < 0.01, ***p*** < 0.001, not significant (ns): *p* > 0.05.

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