African swine fever virus cysteine protease pS273R inhibits pyroptosis by noncanonically cleaving gasdermin D

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African swine fever virus (ASFV) is the only member of the family Asfarviridae, genus Asfivirus. The ASFV virion is a large, icosahedral structure of approximately 200 nm. The ASFV genome contains a linear double-stranded DNA (170–190 kbp) that encodes approximately 150 proteins (5). Similar to other nucleocytoplasmic large DNA viruses (NCLDVs), ASFV encodes many proteins involved in not only the viral life cycle, including viral entry, DNA replication and repair, viral assembly, and egress (6) but also the evasion of host defenses, including the inhibition of host innate immune responses (such as type 1-interferon production and inflammatory responses) and cell death pathways (7).

ASFV encodes two large polyprotein precursors, pp220 and pp62, which are proteolytically cleaved by ASFV pS273R to yield the structural proteins required for virus assembly (8, 9). The cysteine protease pS273R encoded by ASFV is synthesized at the late stages of viral infection and is localized within cytoplasmic viral factories (9). The overall structure of the ASFV pS273R is represented by two domains named the N-terminal “arm domain” and the “core domain” (10). The “arm domain” contains the residues from M1 to N83, which is unique to ASFV and plays an important role in maintaining the enzyme activity of ASFV pS273R (10). The “core domain” contains the residues from N84 to A273, which shares a high degree of structural similarity with chlamydial-deubiquitinating enzyme, sentrin-specific protease, and adenovirus protease.

Pyroptosis is a recently discovered form of programmed cell death that is activated in response to diverse microbial ligands, including bacterial flagellin, toxins, lipopolysaccharide, and DNA that gains access to the cell cytosol (11). Pyroptosis is characterized by the activation of inflammatory caspases (such as caspase-1 and caspase-4/5/11) and pore formation in the cellular plasma membrane, resulting in the release of a large number of proinflammatory cytokines (12). Recently, gasdermin family members, such as gasdermin D (GSDMD), were found to be cleaved by inflammatory-related caspases to execute pyroptosis. For example, GSDMD, a critical mediator of pyroptosis, can be cleaved within a linker between the N-terminal domain and the C-terminal domain by activated caspase-1 (13, 14), caspase-4/5/11 (15, 16), and caspase-8 (17, 18). Subsequently, the N-terminal domain of human GSDMD (GSDMD-N1–275) oligomerizes to be inserted into the plasma membrane, resulting in pore formation (19). It is
widely accepted that the pore formation by human GSDMD-N1–275 results in the loss of osmotic homeostasis, leading to cell swelling and death, which releases inflammatory factors to inhibit and clear intracellular pathogens (20, 21). Recently, the caspase-3-mediated cleavage of GSDME (22) and the granzyme A-mediated cleavage of GSDMB were also reported (23).

Here, we report that swine GSDMD is a novel binding partner of the ASFV-encoded pS273R. ASFV infection decreased the expression of GSDMD by cleavage of GSDMD at the G107-A108 pair to yield a nonfunctional GSDMD-N fragment consisting of amino acids (aa) 1 to 107 (GSDMD-N1–107). Mechanistically, unlike the GSDMD-N1–275 produced by caspase-1, the GSDMD-N1–107 produced by pS273R did not induce pyroptosis and inhibit ASFV replication. Our findings reveal a previously unrecognized novel mechanism by which ASFV evades the host antiviral innate immune responses.

Results

GSDMD is a novel target of the ASFV pS273R protease

To investigate the novel function of ASFV-encoded pS273R (ASFV pS273R) on the host cellular immune responses, we first screened and identified the host proteins that interact with ASFV pS273R by pull down-mass spectrometry. The cell lysates of porcine alveolar macrophages (PAMs) were incubated with His-pS273R protein bound to Ni Sepharose. ASFV pS273R-binding proteins were eluted and stained with Coomasie brilliant blue. We found that several specific bands were observed in the eluted proteins in the His-pS273R group (lane 3) compared with the control group (lane 1 and lane 2) (Fig. 1A). Then, the gels containing these specific bands (lane 3) were cut into three pieces as indicated and analyzed by mass spectrometry. As shown in Table 1, 12 pS273R-interacting proteins were identified in the sample 1# (sample 2# and 3# data not shown). Among them, GSDMD, a pyroptosis executor, was selected for subsequent studies due to its score and role in pyroptosis.

To further characterize the interaction between ASFV pS273R and swine GSDMD, Flag-pS273R or HA-GSDMD alone or both were coexpressed in HEK293T cells, and the interaction and the subcellular colocalization of the two proteins were examined. As shown in Figure 1, B and C, ASFV pS273R interacted with and colocalized with GSDMD in the cytoplasm. In addition, we noticed that ASFV S273R interacted with the C-terminal of swine GSDMD and GSDMD in a low activity proteolysis. We also observed that the reduced abundance of full-length GSDMD (55 kDa) was accompanied by the appearance of an N-terminal fragment of GSDMD with apparent molecular masses of approximately 10 and 15 kDa (designated GSDMD-N), suggesting that ASFV pS273R may cleave swine GSDMD in vitro. To further confirm whether GSDMD is cleaved by ASFV pS273R, HEK293T cells were cotransfected with a plasmid encoding Flag-GSDMD and increasing amounts of a plasmid expressing ASFV pS273R. As shown in Figure 1D, the intensity of Flag-GSDMD diminished as the protein levels of ASFV pS273R were increased, and an N-terminal fragment of GSDMD began to appear, suggesting that GSDMD is cleaved by ASFV pS273R in a dose-dependent manner. To further confirm that ASFV pS273R directly cleaves GSDMD in vitro, purified ASFV pS273R (6 × His-pS273R) and GST-GSDMD were incubated, and the reaction products were detected with anti-His and anti-GSDMD antibodies. As shown in Figure 1E, we found that the purified ASFV pS273R cleaved GST-GSDMD and produced a GST-GSDMD-N fragment with a molecular mass of approximately 39 kDa. To further investigate whether GSDMD is specifically cleaved by ASFV pS273R, HEK293T cells were transfected with a plasmid expressing Flag-tagged GSDMA, GSDMB, GSDMC, GSDM, or GSDME in combination with an empty vector or a plasmid expressing HA-pS273R. As shown in Figure 1F, GSDMD, but none of the other GSDMDs chosen in this study, was cleaved by ASFV pS273R.

ASFV pS273R interacts with and cleaves GSDMD in ASFV-infected PAMs

To test whether ASFV pS273R interacts with endogenous GSDMD in PAMs upon ASFV infection. PAMs were infected with ASFV HLJ/18 and a Co-IP assay was performed. We found that ASFV pS273R interacted with endogenous GSDMD in PAMs infected with ASFV (Fig. 2A). Meanwhile, we also noticed that the ASFV pS273R colocalized with endogenous GSDMD in the cytoplasm in PAMs following ASFV infection (Fig. 2B). These data suggested that ASFV pS273R specifically interacts with GSDMD. To investigate the impact of ASFV infection on the integrity of GSDMD, we analyzed the expression of GSDMD in PAMs either mock-infected or infected with ASFV at different multiplicities of infection (MOIs). As shown in Figure 2C, upon viral infection with increasing doses of ASFV, the levels of endogenous GSDMD decreased in a dose-dependent manner, and only an approximately 40 kDa cleavage product (designated GSDMD-C) was recognized by the anti-GSDMD antibody against the C-terminus of GSDMD.

ASFV pS273R protease activity is required for GSDMD cleavage

To explore whether the protease activity of ASFV pS273R is required for the cleavage of GSDMD, we evaluated the impact of Z-VAD-FMK, a pan-caspase inhibitor, on GSDMD cleavage by pS273R. As shown in Figure 3, A and B, Z-VAD-FMK did not affect ASFV pS273R-mediated GSDMD cleavage although it inhibited caspase-1-mediated GSDMD cleavage, suggesting that ASFV pS273R-mediated GSDMD cleavage was not dependent on the activities of host caspases. His168 and Cys232 are key amino acids that form the two catalytic sites of ASFV pS273R (9). To further verify this issue, we constructed three plasmids expressing different mutant forms of ASFV pS273R, such as pS273R-H168R, C232S, and H168R/C232S (Fig. 3C), and used them to analyze GSDMD cleavage. As shown in Figure 3D, ASFV pS273R with H168R, C232S, or H168R/C232S (DM) substitutions within the active site disrupted its protease activity, resulting in loss of the cleavage of
GSDMD. Hence, these results demonstrate that the protease activity of ASFV pS273R is essential for GSDMD cleavage.

**GSDMD is cleaved at Gly-107 by ASFV pS273R**

Previous reports demonstrated that ASFV pS273R preferentially cleaves Gly-Gly (G-G) amino acid pairs within pp62 and pp220 (9). According to the features of the pS273R cleavage sites with pp62 and pp220 (Fig. 4A, above), we examined the amino acid sequence of the swine GSDMD for potential ASFV pS273R cleavage sites and found that four regions bearing several glycines (G) resemble the signature G-G sequences of the proteolytic sites of ASFV pS273R (Fig. 4A, bottom). Therefore, we inferred that at least four potential ASFV pS273R cleavage sites may exist in swine GSDMD. To define the putative cleavage site, we constructed a...
series of GSDMD mutants, in which the glycine was replaced with alanine (such as G78A, G107A, G320A, and G345A). GSDMD or its mutants were coexpressed with ASFV pS273R in HEK293T cells to identify the actual cleavage sites. As shown in Figure 4B, GSDMD-G107A was absolutely resistant to ASFV pS273R. However, other GSDMD mutants did not prevent ASFV pS273R cleavage.

Compared with the human GSDMD sequence, we predicted that Asp279 (D279) is a site at which swine GSDMD is cleaved by caspase-1 (Fig. 4C). To further test whether G107 is indeed the cleavage site, we generated a GSDMD-G107A/D279A double mutant (GSDMD-DM). ASFV pS273R was coexpressed with GSDMD, GSDMD-G107A, GSDMD-D279A, and GSDMD-DM. As shown in Figure 4D, we found that GSDMD and GSDMD-D279A were still cleaved by ASFV pS273R. However, GSDMD-G107A and GSDMD-DM were completely resistant to ASFV pS273R cleavage. In contrast, GSDMD-D279A and GSDMD-DM were resistant to caspase-1 (Fig. 4E). Consistent with these results, we also noticed that GSDMD-C108–488 could be cleaved by caspase-1 (Fig. 4F). Taken together, these findings suggest that swine GSDMD is cleaved at G107 by ASFV pS273R and at D279 by caspase-1.

**Table 1**

Results of mass spectrometry analysis of the host proteins that interact with pS273R

| Sample name | Accession | Mass (Da) | Score | Matches | Sequences | emPAI | Protein description |
|-------------|-----------|-----------|-------|---------|-----------|-------|---------------------|
| 1           | GSDMD     | 51,941    | 613   | 30 (21) | 12 (10)   | 2.22  | Gasdermin D         |
| 2           | ARHGAP45  | 126,235   | 277   | 24 (11) | 20 (9)    | 0.36  | Minor histocompatibility protein HA-1 isoform X3 |
| 3           | NOP9      | 70,389    | 160   | 8 (3)   | 7 (2)     | 0.15  | Uncharacterized protein |
| 4           | WDR7      | 165,837   | 116   | 13 (4)  | 9 (4)     | 0.08  | WD repeat domain 7  |
| 5           | RUFY3     | 68,422    | 60    | 4 (2)   | 3 (1)     | 0.1   | Protein RUFY3 isoform X4 |
| 6           | CHERP     | 104,279   | 53    | 5 (1)   | 5 (1)     | 0.03  | Calcium homeostasis endoplasmic reticulum protein |
| 7           | ARCE1     | 68,254    | 39    | 7 (1)   | 6 (1)     | 0.05  | Uncharacterized protein |
| 8           | RPL4      | 48,287    | 39    | 3 (2)   | 2 (2)     | 0.14  | Ribosomal protein L4 |
| 9           | ECE2      | 172,699   | 35    | 3 (1)   | 3 (1)     | 0.04  | Uncharacterized protein |
| 10          | FLII      | 150,898   | 31    | 2 (0)   | 2 (0)     | 0.02  | FLII actin remodeling protein |
| 11          | CRIP1     | 89,296    | 21    | 1 (0)   | 1 (0)     | 0.39  | Cysteine-rich intestinal protein 1 |
| 12          | STAT3     | 81,896    | 20    | 7 (0)   | 5 (0)     | 0.04  | Signal transducer and activator of transcription |

**Figure 2.** ASFV-encoded pS273R targets endogenous GSDMD in ASFV-infected PAMs. A, PAMs were infected with ASFV at an MOI of 0.1 for 36 h, and then the cell lysates were coimmunoprecipitated with mouse IgG or anti-pS273R polyclonal antibody (pAb). The cell lysates and the immunoprecipitants were analyzed by Western blot with anti-GSDMD and anti-pS273R pAbs. B, PAMs were infected with ASFV at an MOI of 0.1 for 36 h. The cells were probed with rabbit anti-GSDMD monoclonal antibody (mAb) (green) and mouse anti-pS273R mAb (red). The cell nuclei (blue) were stained with DAPI. C, PAMs were mock-infected or infected with ASFV at MOIs of 0.1, 1, and 10. At the indicated time points, the cell lysates were analyzed by Western blot with antibodies against GSDMD, p72, pS273R, and GAPDH. ASFV, African swine fever virus; DAPI, 4,6-diamidino-2-phenylindole; GSDMD, gasdermin D; MOI, multiplicity of infection; PAMs, porcine alveolar macrophages.
The cleavage fragments of GSDMD by pS273R are unable to induce pyroptosis

Previous reports showed that the cleavage product (GSDMD-N1–107) of human GSDMD by active caspase-1 induces pyroptosis by forming a large pore in the cell plasma membrane (9, 19). We constructed two plasmids expressing swine GSDMD-N1–107 and GSDMD-C108–488 that mimic cleavage products generated by ASFV pS273R (Fig. 5A). We also constructed two other plasmids expressing swine GSDMD-N1–279 and GSDMD-C280–488 that mimic the cleavage products generated by active caspase-1. The expression levels of these proteins were verified (Fig. 5B). To visualize the cellular distribution of transiently expressed WT GSDMD and the cleavage products of GSDMD, HEK293T cells were transfected with a plasmid expressing GSDMD-WT and its cleaved fragments, including GSDMD-N1–107, GSDMD-C108–488, GSDMD-C280–488, GSDMD-C108–279, and GSDMD-N1–279, respectively. Alexa Fluor 594-conjugated wheat germ agglutinin was used to stain the plasma membrane. We found that GSDMD-WT and GSDMD-N1–107 diffusely localized in the cytosol and nuclear, GSDMD-C108–488, GSDMD-C280–488, and GSDMD-C108–279 localized in the cytosol whereas only GSDMD-N1–279 localized in the cell membrane (Fig. 5C). Consistent with these results, GSDMD-N1–279 but not GSDMD and other cleaved fragments, induces cell death with typical pyroptosis morphological features (Fig. 5D). Furthermore, we found that only GSDMD-N1–279 induced LDH release (Fig. 5E) and decreased cell viability (Fig. 5F). Taken together, these results indicate that pS273R may cleave GSDMD to produce GSDMD-N1–107 to lose its function.

The effect of GSDMD mutations on ASFV replication

Previous studies reported that several viral proteases can cleave GSDMD to affect pyroptosis, resulting in the regulation of viral replication (24, 25). Therefore, we first tested whether the swine GSDMD cleavage products generated by ASFV pS273R and caspase-1 are involved in ASFV replication. The plasmids expressing GSDMD, GSDMD-N1–107, GSDMD-C108–488, GSDMD-N1–279, and GSDMD-C280–488, GSDMD-C108–279 were ectopically transfected into MA-104 cells (Fig. 6A), a suitable cell line for ASFV growth (26). Meanwhile, the cells were infected with ASFV for 24 h, and ASFV genomic DNA was extracted for quantitative PCR analysis. As shown in Figure 6C, ASFV replication significantly decreased in the cells expressing GSDMD-N1–107. However, other cleavage products of GSDMD had no inhibitory effect on ASFV replication. Based on the above results, we further tested whether the cleavage of GSDMD by endogenous pS273R disrupts the GSDMD-N1–279-mediated...
pyroptosis and further affects ASFV replication. MA-104 cells transfected with plasmids coexpressing GSDMD and caspase-1 were chosen as positive control, which induces pyroptosis. MA-104 cells were transfected with a plasmid coexpressing GSDMD or its mutants, together with an empty vector or a plasmid expressing caspase-1 as indicated, and then infected with ASFV. Compared with the cells coexpressing GSDMD and caspase-1, ASFV replication has a slight decrease (Fig. 6E). Meanwhile, the LDH release significantly increased in the cell supernatants of the cells coexpressing GSDMD-G107A and caspase-1, ASFV replication has a slight decrease (Fig. 6E). Meanwhile, the LDH release significantly increased in the cell supernatants of the cells coexpressing GSDMD-G107A and caspase-1, which inhibits GSDMD-N1–279-mediated pyroptosis, resulting in the inhibition of the host antiviral innate immune responses and the enhancement of ASFV replication.

GSDMD is cleaved by several RNA viral proteases

The RNA virus genome can be translated into a polyprotein, which can be processed into mature functional proteins by viral protease(s) (27, 28). It is well known that several viral proteases can cleave host proteins to regulate host innate immune responses (29). To test whether swine GSDMD is cleaved by the indicated viral proteases, GSDMD was coexpressed with some major proteases encoded by several viruses belonging to the Coronaviridae, Arteriviridae, and Picornaviridae. As shown in Figure 7, A–C, we found that GSDMD was cleaved to produce a 25-KD fragment by porcine epidemic diarrhea virus (PEDV) Nsp4, porcine transmissible gastroenteritis virus (TGEV) Nsp4, and enterovirus type 71 (EV71) 3C protease (Fig. 7B), but not by foot-and-mouth disease virus (FMDV) 3C protease (Fig. 7C). These results suggest that GSDMD is also a
new target of some RNA viral proteases, which may be required for these RNA viruses infection-mediated pyroptosis.

Discussion

The ASFV genome contains 150 to 167 ORFs encoding more than 150 viral proteins (30). ASFV-encoded proteins execute different functions, including virus entry, viral gene transcription and replication, genome integrity maintenance, viral assembly, and egress. Similar to other viruses, ASFV infection induces apoptosis (31, 32), the Endoplasmic reticulum (ER) stress response (33), and the inflammatory responses (34) to antagonize viral infection. In addition, previous studies have reported that ASFV has evolved series of mechanisms to

Figure 5. The cleavage fragments of GSDMD by ASFV pS273R are unable to induce pyroptosis. A, schematic diagrams of GSDMD and its cleavage fragments produced by caspase-1, ASFV pS273R protease alone, or both. B, detection of the expression of GSDMD and its deletion mutants in HEK293T cells. C, HeLa cells were transfected with a plasmid encoding HA-tagged GSDMD or its cleaved fragments, including GSDMD-N1-107, GSDMD-C108-488, GSDMD-N1-279, GSDMD-C280-488, and GSDMD-C108-279. Alexa Fluor 594-conjugated wheat germ agglutinin (WGA) was added at 37 °C for 10 min to stain the plasma membrane. The colocalization of HA-GSDMD (green) and its cleavage fragments with the cell plasma membrane (red) and cell nuclei (blue) stained with DAPI was observed by confocal microscopy, and GSDMD-N1-279 was used as the positive control. D, HEK293T cells were transfected with HA-tagged GSDMD or its cleaved fragments. The representative views of pyroptosis morphology are shown in (D). E and F, detection of LDH release and cell viability of HEK293T cells expressing HA-tagged GSDMD and its cleaved fragments in pyroptosis. LDH release and ATP-based cell viability are expressed as means ± SD from three technical replicates. All the data shown are representative of three independent experiments. **p < 0.01, ***p < 0.001. ASFV, African swine fever virus; DAPI, 4,6-diamidino-2-phenylindole; GSDMD, gasdermin D.
evade host antiviral responses. For example, it has been experimentally shown that several immunoregulatory proteins suppress the host antiviral immune responses by reducing interferon production (35–37), activating NF-κB (38), and inhibiting apoptosis in ASFV-infected macrophages (39, 40). In this study, we found that GSDMD, an executor of pyroptosis, is a new target of ASFV pS273R (Fig. 2). ASFV pS273R interacted with and cleaved GSDMD at the G107 site to produce GSDMD-N1–107, which may disrupt the pyroptosis induced by GSDMD-N1–279 to benefit ASFV replication.

Pyroptosis has been defined as an inflammatory cell death process that is characterized by lytic forms of death resulting in the release of cytokines and other cellular factors to drive inflammation and alert immune cells to a pathogenic or sterile insult. As one of the six gasdermin family members (GSDMs), GSDMD has been identified as a substrate of several inflammatory caspases (41). For example, GSDMD can be cleaved by active caspases to produce the GSDMD-N1–275 and C-terminal repressor domain (GSDMD-N276–484) (13, 14). GSDMD-N1–275 oligomerizes to form large pores in the cell membrane,
which drives cell swelling and membrane rupture to release inflammatory cytokines (19, 41). Recently, other GSDMD family members, such as GSDME, have also been shown to be cleaved and form pores, resulting in pyroptosis (22).

ASFV pS273R belongs to the SUMO-1-specific protease family (9). It has been reported that ASFV pS273R is involved in the cleavage and maturation of pp220 and pp62 polyprotein precursors into core-shell proteins (9). However, whether ASFV pS273R participates in the regulatory function of the host immune responses is still unknown. In this study, GSDMD was found to be an ASFV pS273R-binding partner. Subsequently, we found that pS273R cleaved GSDMD in a dose-dependent manner when the two proteins were expressed in mammalian cells (Fig. 1). We tested all the members of the gasdermin family in swine and found that only GSDMD could be specifically cleaved by pS273R (Fig. 1F). Consistently, we found that the cleavage of GSDMD by pS273R was dependent on its protease activity but not host caspases activity (Fig. 3). Our findings indicated that pS273R may have a specific role in the regulation of ASFV infection-mediated pyroptosis.

Compared with the specific cleavage sites of pS273R in pp220 and pp62, we noticed that the cleavage sites in pp220 and pp62 have a highly conserved Gly-Gly signature: Gly-Gly-Xaa (9). Therefore, we speculated that the cleavage sites on GSDMD may have the same characteristics. Consistent with our speculation, mutation analysis showed that the cleavage of GSDMD by S273R occurred at the site G107-A108 (Fig. 4). The results suggest that once GSDMD is cleaved by caspase-1 to produce GSDMD-N1–279 and GSDMD-C280–488, GSDMD-N1–279 may be cleaved by pS273R to produce GSDMD-N1–107 and GSDMD-C108–279 (Fig. 4F). Subsequently, our findings revealed that over-expression of the three GSDMD truncated fragments (GSDMD-N1–107, GSDMD-C108–279, and GSDMD-C280–488) did not cause pyroptosis (Fig. 5), suggesting S273R may inhibit GSDMD-N1–279-induced pyroptosis by the cleavage of GSDMD-N1–279. Previous results revealed that several viral proteases can convert pyroptosis by the cleavage of GSDMD-N1–275. For example, enterovirus 71 (EV71) protease 3C directly cleaves human GSDMD to produce a shorter N-terminal fragment (1–193) of GSDMD, which results in disruption of the pore formation, thereby avoiding pyroptosis and ensuring viral persistence (25). Our results showed that the swine GSDMD-N1–279 significantly promoted cell death and affected the ASFV replication (Fig. 6), whereas the GSDMD-N1–107 and GSDMD-C108–279 fragments produced by ASFV S273R did not affect cell death.

It has been well documented that virus infection can activate pyroptosis-related cell death, which contributes to viral replication and transmission. For instance, SARS-CoV-2 infection activates the NLRP3 inflammasome to induce pyroptosis, which leads to GSDMD cleavage and inflammatory cytokines release to generate a cytokine storm (42, 43), suggesting that GSDMD is a promising target for the treatment of severe coronavirus-related diseases (44). Recently, several viral proteases have been found to cleave host GSDMD. For example, Zika virus (ZIKV) NS2B3 directly cleaves GSDMD to produce the N-terminus of GSDMD-N1–249, which promotes virus release through ZIKV-induced pyroptosis (24). In this study,

Figure 7. GSDMD is cleaved by several RNA viral proteases. A–C, HEK293T cells were transfected with a plasmid encoding Flag-tagged GSDMD together with a plasmid expressing viral proteases encoded by Arteriviridae and Coronaviridae (A) and Picornaviridae (B and C). The cell lysates were subjected to Western blot analysis with anti-HA, Flag, and GAPDH antibodies. GSDMD, gasdermin D.
ASFV ps273R inhibits pyroptosis by cleavage of GSDMD

we also noticed that the proteases from several other RNA viruses, including the EAV NSP4, PEDV 3C, and TGEV 3C proteases, but not the FMDV 3C protease, could also cleave GSDMD (Fig. 7). Taken together, different viruses may use their specific proteases to cleave GSDMD to regulate pyroptosis to affect viral replication, which may be likely to occur widely in the process of host cell innate immunity.

In this study, we found and verified the cleavage of swine GSDMD by ASFV ps273R, which is a novel mechanism to disrupt the pyroptosis mediated by GSDMD-N1-279 (Fig. 8). Interestingly, we also noticed that ps273R inhibited the type I interferon signaling pathway to inhibit interferon-stimulated genes production, suggesting that ps273R might interact with and cleave the core components in the interferon signaling pathway to subvert interferon responses (data not shown). Therefore, it is necessary to continue to screen and identify ps273R binding partners and then elucidate their roles in ASFV replication and pathogenesis.

Experimental procedures
Cell lines and viruses

PAMs were isolated from the lung lavage fluid from 4-week-old healthy specific pathogen-free piglets, as previously described (45) and maintained in RPMI-1640 medium containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin, 50 mg/ml streptomycin, and nonessential amino acids (Gibco). HEK293T cells and HeLa cells obtained from ATCC were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. All the cells were maintained at 37 °C with 5% CO2. The ASFV HLI/18 strain (GenBank accession number: MK333180.1) was isolated from the pigs, as previously described (46). A HAD assay was performed, as described previously (47). Briefly, primary peripheral blood mononuclear cells were seeded in 96-well plates. The samples were then added to the plates and titrated in triplicate using 10× dilutions. The quantity of ASFV was determined by the identification of characteristic rosette formation, which represents the hemadsorption of erythrocytes around the infected cells. HAD was observed for 7 days, and 50% HAD doses (HAD50) were calculated by using the method of Reed and Muench (48).

Plasmids and reagents

To construct plasmids expressing HA-tagged and Flaggagged ps273R, cDNA corresponding to the ASFV S273R gene was cloned into the pCAGGS-HA (pHA) and pCAGGS-Flag (pFlag) vectors. Three plasmids expressing ASFV S273R and its mutants (S273R-H168R, C232S, and H168R/C232S (DM)) were constructed based on a plasmid expressing ASFV S273R by site-directed mutagenesis. The cDNAs corresponding to the swine GSDMA, GSDMB, GSDMC, GSDMD, and GSDME proteins were synthesized by GenScript and then cloned into the pHA and pFlag vectors. The cDNAs corresponding to the point mutants of swine GSDMD (G78A, G107A, G320A, G345A, D279A, and G107A/D279A (DM)) and the deleted mutants of GSDMD, including 1 to 107 amino acid (aa), 108 to 488 aa, 1 to 279 aa, and 280 to 488 aa of GSDMD, were cloned into the pHA vector. The pET-22b-S273R plasmid was a gift from Prof. Yu Guo of Nankai University, and the cDNA of GSDMD was cloned into the pGEX-6P1 vector to express and purify the recombinant GST-GSDMD fusion protein. The sequences of the primers used in this study are available upon request. All the constructs were validated by DNA sequencing. Alexa Fluor 594-conjugated wheat germ agglutinin (W11262) was purchased from Thermo Fisher Scientific. The pan-caspase inhibitor Z-VAD-FMK was purchased fromSigma-Aldrich.

Antibodies

Anti-Flag (14793S) and anti-HA (3724S) were purchased from Cell Signaling Technology. Anti-GAPDH (10494-1-AP) was purchased from Proteintech. The rabbit anti-GSDMDC1 polyclonal antibody (NBP2-33422) was purchased from Novus. IRDye 800CW goat anti-mouse IgG (H + L) (926-32210) was purchased from Sera Care, and IRDye 800CW goat anti-rabbit IgG (H + L) (925-32211) was purchased from LI-COR. Polyclonal antibodies against ASFV ps273R, p72, and p30 were prepared by immunizing mice using the purified recombinant ASFV ps273R, p72, and p30 proteins as immunogens. All animal procedures were approved by Harbin Veterinary Research Institute Animal Ethics Committee of CAAS.

Pull-down assay

PAMs (1 × 10⁶) were washed three times with PBS and lysed with cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM MgCl₂, 1 mM EDTA, and 10% glycerol) containing 1 mM PMSF and 1× protease inhibitor mixture (Roche Diagnostics). A Ni Sepharose column was washed three times with PBS. Ni Sepharose bound with His-ps273R or Ni Sepharose were incubated with the cell lysates of PAMs at 4 °C overnight. The resin was washed five times with wash buffer (20 mM Tris, 300 mM NaCl, and 20 mM
imidazole (pH 8.0)), and the candidate proteins were eluted with elution buffer (20 mM Tris, 300 mM NaCl, and 500 mM imidazole (pH 8.0)). Then, the eluted samples were resolved by SDS-PAGE and stained with Coomassie brilliant blue.

**MS analysis**

The gels containing His-pS273R-binding proteins were cut and named 1#, 2#, and 3# and then processed for LC-MS/MS to identify pS273R binding host proteins (45). Briefly, the samples were mixed with trypsin and digested at 37 °C overnight. LC-MS/MS was implemented by using a Dionex ultimate 3000 nano-LC system (Dionex) coupled with a Q Exactive mass spectrometer (Thermo Fisher Scientific) at Beijing Protein Innovation in China. The MS/MS signals were then processed against the uni_sus_9822 9822 database (122,086 sequences; 73,310,586 residues) using the Mascot software (Version 2.3.01, Matrix Science) with the following parameters: Fixed modification: Carbamidomethyl (C); Variable modification: Oxidation (M), Gln→Pyro-Glu (N-term Q); and maximum missed cleavages, 2; filter by score ≥31.

**Coimmunoprecipitation and Western blot analysis**

Co-IP and Western blot analysis were performed, as described previously (49). At 36 h posttransfection (hpt), the cells transfected with the different plasmids were lysed with the cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM MgCl2, 1 mM EDTA, and 10% glycerol) containing 1 mM PMSF and 1× protease inhibitor mixture (Roche Diagnostics). The cell lysates were incubated with anti-Flag agarose beads (A2220-5ML, Sigma) overnight at 4 °C on a roller. The immunoprecipitants were subjected to electrophoresis. To identify the interactions between endogenous proteins, PAMS were either mock-infected or infected with ASFV (1 MOI) for 36 h. The cell lysates then were incubated with anti-S273R monoclonal antibody or IgG for 8 h at 4 °C, and S273R complexes were captured using protein A+G-Sepharose (Pierce Protein A/G Plus Agarose, Thermo). The equal amounts of cell lysates and immunoprecipitants were resolved by 12% SDS-PAGE and then transferred to polyvinylidine difluoride membranes (ISEQ00010, Merck-Millipore). After incubation with primary and secondary antibodies as indicated, the membranes were visualized by an Odyssey two-color infrared fluorescence imaging system (LI-COR).

**Fluorescence microscopy**

To test the cellular localization of GSDMD and S273R, HEK293T cells were transfected with a plasmid expressing HA-GSDMD or Flag-S273R alone or both plasmids for 24 h. To test the cellular localization of endogenous GSDMD and S273R, PAM cells were infected with ASFV at an MOI of 0.1 for another 36 h. To test the cellular localization GSDMD or its deletion mutants, HeLa cells were transfected with a plasmid expressing HA-GSDMD or its deletion mutants. At 24 h posttransfection, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and then probed with primary antibodies as indicated and 4,6-diamidino-2-phenylindole. The samples were visualized with a Zeiss LSM-800 laser scanning fluorescence microscope (Carl Zeiss AG).

**Expression of recombinant proteins and cleavage assay in vitro**

The purification of recombinant proteins and the cleavage assay were performed in vitro, as described previously (14, 50). In brief, His-pS273R protein was purified from clarified bacterial lysates, as described previously (10). The GST-GSDMD protein was purified on a glutathione-agarose column. The two proteins were dialyzed and stored at −80 °C. To examine GSDMD cleavage in vitro, the aliquots of recombinant ASFV pS273R (His-pS273R) and GST-GSDMD were incubated in Buffer A (50 mM Hepes (pH 7.5), 3 mM EDTA, 150 mM NaCl, 0.005% (vol/vol) Tween-20, and 10 mM DTT). The reaction was incubated for 2 h at 37 °C, and the reactions were terminated by adding 1× loading buffer and then subjected to Western blot analysis.

**Cell viability and lactate dehydrogenase assay**

HEK293T cells (1.5 × 10⁴) grown in 96-well plates were transfected with the indicated plasmids using Lipofectamine 3000 (Thermo Fisher Scientific). After 36 hpt, cell viability was determined by the CellTiter-Glo 2.0 Assay (Promega, G9242). Cell death was measured by LDH assay using a CytoTox 96 NonRadioactive Cytotoxicity Assay kit (Promega, G1780).

MA-104 cells (1 × 10⁵) grown in 24-well plates were transfected with the indicated plasmids for 8 h after ASFV infection with 5 MOI. At 18 hpi, the supernatants were harvested, and the cell death was measured by LDH assay.

**Quantitative PCR**

To test the GSDMD cleavage fragments for ASFV replication, MA-104 cells (1 × 10⁵) were seeded on 24-well plates and cultured for 8 h after transfection with indicated plasmids (1 µg per well) for 24 h and then followed ASFV infection with 5 MOI. At 4 hpi, the cells were harvested. To test the endogenous S273R to cleaving GSDMD and GSDMD mutant (GSDMD-G107A) for ASFV replication, MA-104 cells (1 × 10⁵) were seeded on 24-well plates and cultured for 12 h after transfection with indicated plasmids (1 µg per well) for 8 h and then followed ASFV infection with 5 MOI. At 18 hpi, the cells were harvested. ASFV genomic DNA was extracted from MA-104 cells using a Qiagen DNA Mini Kit (Qiagen). Quantitative PCR was carried out on a QuantStudio5 system (Applied Biosystems) according to the OIE-recommended procedure.

**Flow cytometry**

MA-104 cells (2 × 10⁵) were seeded on 24-well plates and cultured for 12 h after transfection with indicated plasmids (0.5 µg per well) for 24 h and then followed ASFV infection with 1 MOI. At 36 hpi, the cells were harvested and then fixed...
ASFV pS273R inhibits pyroptosis by cleavage of GSDMD

with 4% paraformaldehyde and were analyzed by a Cytomics FC 500 flow cytometer (Beckman).

Statistics

All the statistical analyses were performed using one-way ANOVA and Student’s t test using GraphPad Prism 8 software (GraphPad Software Inc). The data are presented as the mean ± SD. The data are presented as the mean ± SD, * indicates a significant difference (p < 0.05), ** indicates a highly significant difference (p < 0.01), and *** indicates an extremely significant difference (p < 0.001).

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository (51) with the dataset identifier PXD028692.

Supporting information—This article contains supporting information. All primers used in this study are listed in Table S1.

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Abbreviations—The abbreviations used are: ASF, African swine fever; ASFV, African swine fever virus; Co-IP, coimmunoprecipitation; GSDMD, gasdermin D; hpt, hours posttransfection; MOI, multiplicity of infection; PAMs, porcine alveolar macrophages.

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