Development and characterization of monoclonal antibodies against p72 protein of African swine fever virus reveals a novel conserved B-cell epitope

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Highlights

- Discovery of a novel and conserved linear B-cell epitope (aa 130-152) on the major capsid protein p72 of ASFV.
- Structural insights confirm the epitope's surface accessibility on the p72 trimer, making it a prime target for antibodies.
- Validated utility for serodiagnosis through strong reactivity with sera from infected pigs.

Abstract

African swine fever (ASF), caused by African swine fever virus (ASFV), is a highly contagious disease that has spread globally, posing a significant threat to swine production and international trade. As rapid diagnosis is crucial for controlling ASF, its major capsid protein, p72, has become a key target for diagnostic and vaccine development. In this study, we generated five monoclonal antibodies (mAbs) against the p72 protein by immunizing mice with inactivated virus. Using phage display technology, we identified the epitope for one mAb as a novel linear B-cell epitope within amino acids 130-152 of the p72

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protein. Structural and homology analyses revealed that this epitope is highly conserved across diverse ASFV genotypes and is exposed on the surface of the p72 trimer. Importantly, the epitope showed strong reactivity with sera from ASFV-positive swine. These findings offer a foundation for creating improved serological diagnostics and designing epitope-based vaccines against ASFV.

Keywords: ASFV, monoclonal antibody, B-cell epitope, diagnostic development

1. Introduction

African swine fever (ASF) is a lethal disease of domestic pigs and wild boars caused by the African swine fever virus (ASFV) (Zhao *et al.* 2023). Since its identification in Kenya in 1921, ASF has spread to numerous countries, resulting in significant socioeconomic impacts (Eustace Montgomery 1921, Li and Zheng 2025, Xin *et al.* 2023, Zhao *et al.* 2023). In 2018, the first outbreak of ASF occurred in China, causing significant economic damage (Zhou *et al.* 2018). As the sole member of the *Asfarviridae* family, ASFV is one of the most complex DNA viral structures as known, featuring a multilayered architecture comprising an outer membrane, capsid, inner membrane, core shell, and genomic DNA (Liu *et al.* 2019, Ruedas-Torres *et al.* 2024, Wang *et al.* 2021b) . The viral genome ranges from 170-194 kb across different strains, containing 151-174 open reading frames (ORFs) with over 50% remaining functionally uncharacterized (Chandana *et al.* 2024).

The icosahedral capsid, forming the outermost virion layer, is predominantly assembled from p72 protein encoded by the *B646L* gene (Zhu *et al.* 2024a). The major capsid protein (MCP) p72 represents approximately 33% of the total virion mass and serves as the primary structural component (Alejo *et al.* 2018). Abnormal expression of the p72 protein leads to the accumulation of electron-dense membrane-like structures within the viral factory, which inhibits capsid formation and leads to failure of ASFV particle assembly (García-Escudero *et al.* 1998). Beyond its structural role, p72 mediates host-virus interactions critical for viral invasion and immune evasion (Orosco 2024, Song *et al.* 2025, Zhu *et al.* 2024b). Due to its abundance and immunogenicity, p72, along with p54 and p30 proteins, is a primary antigenic target recognized during natural infection and a cornerstone of diagnostic assays (Hu *et al.* 2023, Liberti *et al.* 2023, Miao *et al.* 2023, Petrovan *et al.* 2020).

While several p72-targeting antibodies exist, there remains a need for well-characterized monoclonal antibodies (mAbs) with defined epitopes for improved diagnostic applications and vaccine design. This study aims to generate and characterize novel p72-specific mAbs and map their binding epitopes to contribute to ASF control strategies. We generated and characterized five specific mAbs against the ASFV p72 protein. Through phage display technology, we identified a novel linear epitope (amino acids 130-152) of the p72 protein. This epitope is highly conserved across different ASFV strains and strongly reacts with ASF-positive sera. We also utilized our antibodies to track the subcellular localization of p72 during viral infection. The findings presented here deepen our understanding of p72 biology and provide a valuable resource for developing improved diagnostic tools for ASFV.

2. Materials and methods

2.1 Biosafety statement and facility

All infectious African swine fever virus (ASFV) experiments were conducted under Animal Biosafety Level 3 (ABSL-3) containment at Huazhong Agricultural University. Inactivated virus samples were handled in a Biosafety Level 2 (BSL-2) laboratory for subsequent analysis.

2.2 Cells, viruses and animals

Female BALB/c mice (6-8 weeks old) were procured from the Laboratory Animal Center of Huazhong Agricultural University.

SP2/0 murine myeloma cells (ATCC CRL-1581) were cultured in complete RPMI 1640 medium (Gibco) supplemented with 20% fetal bovine serum (FBS; ExCell Bio) and 1% penicillin-streptomycin (Invitrogen). Vero (ATCC CRL-1587), LLC-PK1 (ATCC CL-101), and HEK-293T (ATCC CRL-3216) were maintained in complete Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 1% penicillin-streptomycin. Primary porcine alveolar macrophages (PAMs) were isolated from 20-30-day-old piglets and cultured in RPMI 1640 medium with 10% FBS (ExCell Bio). All cell cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere.

The ASFV genotype II strain SXH1 was used in this study (Cao *et al.* 2024). The virus was inactivated with binary ethyleneimine (BEI) as previously described (Li *et al.* 2023) .

2.3 Reagents and antibodies

The ST-tag antibody, anti-p54 antibody, anti-p30 antibody and p30 protein were generated and maintained in our laboratory (Yu *et al.* 2021). The ASF-positive serum used in the experiment was also prepared and stored in our laboratory (Xu *et al.* 2024). Antibodies and reagents, including 4′,6-diamidino-2-phenylindole (DAPI), RIPA lysis buffer, 5× loading buffer (Beyotime, China), Peroxidase-AffiniPure Goat Anti-Human IgG (H+L) (Jackson ImmunoResearch Inc, USA), HRP Goat Anti-Mouse IgG (ABclone, China), Alexa Fluor® 488 Donkey anti Mouse IgG (antGene, China), HY-K0214 Protein G Agarose (MCE, China), Mouse Monoclonal Antibody Isotyping Elisa Kit (Proteintech, China), Thermo Scientific PageRuler Plus (Thermo Scientific, USA),QuickAntibody-Mouse 5W (Biodragon, China).

2.4 Generation of monoclonal antibodies (mAbs)

Two healthy female BALB/c mice, aged 6–8 weeks, were immunized with inactivated virus, and serum antibody titers were analyzed by indirect Enzyme-linked immunosorbent assay (ELISA) and an immunofluorescence assay (IFA). After booster immunization, spleen cells from the immunized mice were fused with SP2/0 myeloma cells, following a previously established experimental protocol (Yu *et al.* 2021). Positive hybridoma cells were identified by IFA screening, and the positive hybridoma cell line was isolated through two rounds of subcloning. Hybridoma cells secreting monoclonal antibodies (mAbs) were then injected into mice to induce ascites formation to obtain high-titer monoclonal antibodies. These antibodies were then purified using a Protein G affinity column (MCE, China).

2.5 Identification of monoclonal antibodies

To screen mAbs against the p72 protein, we expressed the full-length protein by cloning the *B646L* gene

(ASFV reference: GenBank MK795937) into the pCAGGS plasmid vector. This was achieved using *Nhe*I and *Bam*HI restriction sites with primers detailed in **Appendix A.**, resulting in the successful construction of pCAGGS-p72-ST eukaryotic expression plasmids. The recombinant plasmids were transfected into HEK-293T cells for 28 h (h), followed by fixation with 4% paraformaldehyde (15 min, room temperature (RT)) for IFA or lysis with RIPA buffer (Beyotime, China) for Western blot analysis.

To evaluate the ability of the monoclonal antibodies to react with the p72 protein during viral infection, LLC-PK1 cells were infected with ASFV at a multiplicity of infection (MOI) of 0.5. At 36 hours post infection (hpi), the cells were fixed with 4% paraformaldehyde for 15 min at RT for IFA or lysed with RIPA lysis buffer for Western blotting analysis.

2.6 Immunofluorescence assay (IFA)

After 28–30 h of transfection or 24 h of infection, samples were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. The IFA was performed according to the previously described method (Cao *et al.* 2024).

2.7 Western blotting

Protein samples were prepared by lysing cells in RIPA buffer with 1 mmol L^{-1} PMSF, followed by denaturation in 5× SDS loading buffer at 95°C for 10 min. The Western blot analysis was then completed following the published method (Cao *et al.* 2024).

2.8 Enzyme-linked immunosorbent assay (ELISA)

Flat-bottom polystyrene plates were coated with p30 protein or inactivated virus using 0.05 mol L^{-1} carbonate buffer (pH 9.6) at 4°C overnight. After blocking with 5% skim milk (2 h, RT), plates were incubated with diluted serum or antibody samples (2 h, RT), followed by HRP-conjugated goat anti-mouse IgG (45 min, RT). Following washes with PBST (0.05% Tween-20 in PBS), 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added. The reaction was stopped after 10 min, and the absorbance at 450 nm was measured.

2.9 Identification of the antigenic epitopes

Viral peptides recognized by antibodies were identified using VirScan technology (Fig. 5A). Screened peptides (56-amino acids) were truncated and fused with glutathione S-transferase (GST) in the pGEX-6P-1 plasmid. Specific primers containing *BamHI*/*XhoI* restriction sites were synthesized (Appendix A). The recombinant plasmids were sequenced and transformed into *Escherichia coli* BL21. Protein expression was induced with 0.5 mmol L^{-1} isopropyl-β-d-thiogalactoside (IPTG; 6 h, 37 °C), and the products were analyzed by Western blotting.

2.10 Antigenic epitope analysis

The p72 protein sequences from 13 ASFV isolates (NCBI database) were aligned using ESPript 3.0 to assess epitope conservation. Epitope locations were visualized on p72 trimer structures using PyMOL.

2.11. Statistical analysis

Data are expressed as mean±SD and analyzed using GraphPad Prism 9.

3. Results

3.1 Generation of mAbs against ASFV

The antigenicity of the inactivated ASFV immunogen was confirmed by indirect ELISA, which showed strong reactivity with ASFV-positive swine serum, comparable to the p30 positive control (Fig. 1-A). The mice were immunized with the inactivated virus, and after three immunizations, the titers of antibodies against the ASFV protein in the sera of the two mice were measured using indirect ELISA and IFA. The antibody titer reached as high as 1:12,800 per mouse (Fig. 1-B and C). Subsequently, splenocytes from immunized mice were used to generate hybridomas, which were screened for the production of ASFV-specific antibodies.

3.2 Identification of mAbs against the p72 protein

Five hybridoma clones (11F6, 21B10, 28H1, 32G5, and 38C9) secreting mAbs against the p72 protein were identified by IFA. These mAbs were produced in ascites and purified using a Protein G affinity column, achieving over 95% purity as determined by SDS-PAGE (Fig. 2-A). Subtype analysis revealed that the heavy chain of the 32G5 antibody belonged to the IgG2a subtype, whereas the remaining four antibodies were of the IgG1 subtype, all possessing kappa light chains (Fig. 2-B). The IFA results demonstrated that all five monoclonal antibodies exhibited strong reactivity with the p72 protein expressed in HEK-293T cells, showing high concordance with the labeled antibodies, thereby validating their specific recognition (Fig. 2-C). Western blotting analysis further confirmed the specific binding of these five antibodies to the ASFV p72 protein, indicating that all five antibodies recognize linear epitopes of the p72 protein (Fig. 2-D). These results confirmed that all five antibodies successfully detected the full-length p72 protein expressed in cells.

3.3 Reactivity of mAbs to ASFV

To evaluate the reactivity between the mAbs and the p72 protein under viral infection conditions, LLC-PK1 cells were infected with ASFV at an MOI of 0.5. Samples were collected at 24 hpi for IFA and Western blotting analysis. The IFA results demonstrated strong reactivity of all the monoclonal antibodies with ASFV-infected cells, showing significant colocalization with the p30 protein (Fig. 3-A). The ability of the mAbs to recognize the viral p72 protein was further analyzed via Western blotting, with anti-p54 mAbs used as a control to confirm the successful infection and replication of ASFV. All five mAbs effectively recognized the ASFV p72 protein (Fig. 3-B), which was consistent with previous results.

3.4 Comparison of binding strength

To determine the affinity of the five mAbs for the p72 protein with its native folding structure under viral infection conditions, whole-cell lysates were collected from cells infected with the virus at 36 hpi. The supernatant of the lysate was incubated with 2 μg of each mAb at 4°C for 12 h, followed by enrichment with Protein A+G magnetic beads. The Western blotting results revealed that all five mAbs specifically reacted with the naturally folded p72 protein, with 21B10 exhibiting the highest binding strength for the p72 protein (Fig. 3-C).

3.5 Subcellular localization of the p72 protein during viral infection

To investigate the subcellular localization of the ASFV p72 protein during virus infection, Vero cells infected with ASFV at different time points were collected, and IFA was performed using the mAb 32G5. As shown in Fig. 4, the p72 protein was detected by IFA as early as 2 hpi in ASFV-infected Vero cells. This early detection is likely due to the p72 protein being located on the capsid of the virion, making it easily exposed to antibodies. At 4 and 6 hpi, the p72 protein was predominantly present in the cytoplasm and tended to move closer to the periphery of the nucleus. From 8 hpi to 18 hpi, the p72 protein was observed to accumulate primarily in the viral factory, as indicated by its colocalization with the p54 protein, a marker for the viral factory. Notably, at 10 hpi, a portion of the p72 signal began to diverge from the viral factory aggregation site toward the cell membrane, suggesting that virion assembly may have been completed and that the virions were subsequently transported to the plasma membrane.

3.6 Identification of p72 antigenic epitopes

The binding site of mAb 32G5 on the p72 protein was mapped using phage display technology (VirScan), which initially identified a 56-amino acid region spanning residues 112--168 (Fig. 5-A). Following the truncation method illustrated in Fig. 5-B, we subsequently truncated the peptide segment (112-168 aa), fused the truncated sequence with a GST tag, and expressed in *E. coli*. Western blotting analysis confirmed the successful expression of all the truncated proteins and demonstrated that the 32G5 antibody exhibited strong reactivity to the peptide segment spanning amino acids 122-158 (Fig. 5-C). This segment was further divided into five overlapping fragments, as shown in Fig. 5-D. The truncated proteins were similarly fused with the GST tag and expressed. The results indicated that the 32G5 antibody strongly recognized the peptide segment encompassing amino acids 130-152 (Fig. 5-E). Our experimental findings confirm that the sequence ^{130}AHGQLQTFPRNGYDWDNQTPLEG^{152} plays an essential role in conferring specific recognition when presented linearly.

To evaluate the reactivity of this antigenic epitope, we conducted prokaryotic expression of the 122-158 aa-GST fusion protein and purified it using glutathione resin (Fig. 5-F). The titer of the monoclonal antibody was evaluated by ELISA on the basis of the purified 122-158aa-GST protein (Fig. 5-G), which demonstrated that the titer of the 32G5 antibody was 1:3.2×10^5. The ELISA results in Fig. 5-G demonstrated that the other four antibodies do not bind to this epitope. The antigenicity of the identified epitope was subsequently assessed via ELISA using ASFV-positive serum, as illustrated in Fig. 5-H. This epitope is effectively recognized by ASFV-positive serum, further confirming that this region represents a naturally recognized antigenic region.

3.7 Conservation analysis of p72 B-cell epitopes

To understand the conservation of the B-cell epitope identified in this study across different strains, we performed multiple sequence alignments of representative strains from various regions. As shown in Fig. 6-A, among the 13 reference ASFV isolates, three belong to genotype II, whereas the remaining ten strains represent other genotypes. The amino acid sequence comparison conducted via ESPript 3 software revealed that the epitope spanning amino acids 130-152 is highly conserved among ASFV strains.

3.8 Spatial structure of the p72 B-cell epitope

To elucidate the spatial structure of the newly identified epitope, the B-cell epitope was visualized and analyzed via PyMOL software. The trimer structure of the p72 protein (PDB: 6KU9) was retrieved from the RCSB Protein Data Bank (PDB) database, and the spatial structure of the selected epitope region was displayed via PyMOL. The linear epitope spanning amino acids 130-152 is located on the surface of the p72 protein structure (Fig. 6-B).

4. Discussion

The ongoing global spread of African swine fever (ASF) highlights an urgent need for improved diagnostic tools and effective vaccines, both of which rely on a deep understanding of key viral antigens. The major capsid protein p72 is a central player in the ASFV life cycle and a primary target of the host immune response, making it a focal point for such research. This study successfully generated five new monoclonal antibodies (mAbs) against p72, providing valuable reagents for ASFV research.

The p72 protein serves as the primary antigen of ASFV and is a critical target for the development of ASF subunit vaccines and serological diagnostic techniques (Liao *et al.* 2024, Wang *et al.* 2024). As a key capsid protein of the viral particle, p72 not only plays a central role in ASFV serotyping but also represents an effective target for eliciting neutralizing antibodies. In this study, we used inactivated viruses to immunize mice, which likely preserved the native conformation of the protein in the virion (Fig. 1-B-D). Five monoclonal antibodies, namely, 11F6, 21B10, 28H1, 32G5, and 38C9, were successfully generated using the hybridoma technique. All five mAbs exhibited strong and specific reactivity with the recombinant p72 protein expressed in eukaryotic cells as well as in virus-infected cells (Figs. 2-C and 3-A). Subcellular localization analysis of the p72 protein at different stages of infection. At 2 hpi, p72 appeared as punctate structures within the cytoplasm. While by 12–18 hpi, in the later stage of infection, the p72 protein began to spread from the viral factory to the cell membrane as bright punctate structures (Fig. 4). This result indicates that the mAb (32G5) is a valuable tool for tracking the subcellular localization of p72 protein, and by extension, viral factories and assembled virions, during the infection cycle. This behavior may be attributed to the high abundance of the p72 protein in viral particles and its location in the capsid region, which facilitates recognition by antibodies. Furthermore, these findings confirm that the p72 protein is indeed an important target for detecting antigens in the blood.

Epitope identification has contributed to the development of diagnostic tools and vaccines (Wang *et al.* 2021a). Given that p72 exists as a trimer within viral particles, identifying epitopes on trimeric spikes, composed of three p72 molecules, is essential for studying the antigenic characteristics of p72. The most exposed top of the screw propeller-like cap, which consists of the tips of the DEN, HIN, and DEC loops, may harbor neutralizing antigenic epitopes and can guide the design of ASFV vaccines (Liu *et al.* 2019, Yu *et al.* 2024). In this study, we employed phage display technology (VirScan) (Shrewsbury *et al.* 2024, Shrock *et al.* 2020), which enables high-throughput screening of antigenic epitopes. Through this technology, we determined that the 32G5 antigenic epitope was located in the 130-152 amino acid region at the tips of the DEN (DEN β1-4) (Fig. 6-B). Notably, this sequence is highly conserved across strains of

different genotypes and shows strong reactivity with ASF-positive serum (Figs. 6-A and 5-H). These findings will facilitate the establishment of serological tests for ASFV.

5. Conclusion

In this study, we developed and characterized five novel monoclonal antibodies specific to the ASFV p72 protein. Using these reagents, we identified and precisely mapped a previously unknown B-cell epitope at amino acids 130-152. This epitope is highly conserved across ASFV genotypes, is structurally exposed on the virion surface, and is recognized during natural infection. The mAbs generated are valuable tools for future research into ASFV pathogenesis, while the well-characterized epitope represents a prime candidate for the development of next-generation peptide-based diagnostics and broadly protective epitope-focused vaccines. These findings provide a tangible step toward creating improved tools to control the global spread of ASF.

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Declaration of competing interest

The authors report that no conflicts of interest are associated with this work.

Ethical approval

All animal procedures complied with institutional ethical standards and were approved by Huazhong Agriculture University's Animal Ethics Committee (Protocol No. HZAUMO-2025-0020).

References

Alejo A, Matamoros T, Guerra M, Andrés G. 2018. A Proteomic Atlas of the African Swine Fever Virus Particle. *J Virol,* **92**.

Cao H, Zhang M, Liao Z, Li D, He X, Ma H, Li P, Yu X, Peng G, Xie S, He Q, Li W. 2024. A porcine kidney-derived clonal cell line with clear genetic annotation is highly susceptible to African swine fever virus. *Vet Res,* **55**, 42.

Chandana M S, Nair S S, Chaturvedi V K, Abhishek, Pal S, Charan M S S, Balaji S, Saini S, Vasavi K, Deepa P. 2024. Recent progress and major gaps in the vaccine development for African swine fever. *Braz J Microbiol,* **55**, 997-1010.

Eustace Montgomery R. 1921. On A Form of Swine Fever Occurring in British East Africa (Kenya Colony). *Journal of comparative pathology and therapeutics,* **34**, 159-191.

García-Escudero R, Andrés G, Almazán F, Viñuela E. 1998. Inducible gene expression from African swine fever virus recombinants: analysis of the major capsid protein p72. *J Virol,* **72**, 3185-3195.

Hu Y, Wang A, Yan W, Li J, Meng X, Chen L, Li S, Tong W, Kong N, Yu L, Yu H, Shan T, Xu J, Tong G, Zheng H. 2023. Identification of Linear Epitopes in the C-Terminal Region of ASFV p72 Protein. *Microorganisms,* **11**.

Li M, Pan Y, Xi Y, Wang M, Zeng Q. 2023. Insights and progress on epidemic characteristics, genotyping, and preventive measures of PEDV in China: A review. *Microbial Pathogenesis,* **181**, 106185.

Li M, Zheng H. 2025. Insights and progress on epidemic characteristics, pathogenesis, and preventive measures of African swine fever virus: A review. *Virulence,* **16**, 2457949.

Liao H C, Shi Z W, Zhou G J, Luo J C, Wang W Y, Feng L, Zhang F, Shi X T, Tian H, Zheng H X. 2024. Epitope mapping and establishment of a blocking ELISA for mAb targeting the p72 protein of African swine fever virus. *Appl Microbiol Biotechnol,* **108**, 350.

Liberti R, Colabella C, Anzalone L, Severi G, Di Paolo A, Casciari C, Casano A B, Giammarioli M, Cagiola M, Feliziani F, De Giuseppe A. 2023. Expression of a recombinant ASFV P30 protein and production of monoclonal antibodies. *Open Vet J,* **13**, 358-364.

Liu Q, Ma B, Qian N, Zhang F, Tan X, Lei J, Xiang Y. 2019. Structure of the African swine fever virus major capsid protein p72. *Cell Res,* **29**, 953-955.

Miao C, Yang S, Shao J, Zhou G, Ma Y, Wen S, Hou Z, Peng D, Guo H, Liu W, Chang H. 2023. Identification of p72 epitopes of African swine fever virus and preliminary application. *Front Microbiol,* **14**, 1126794.

Orosco F L. 2024. African swine fever virus proteins against host antiviral innate immunity and their implications for vaccine development. *Open Vet J,* **14**, 941-951.

Petrovan V, Murgia M V, Wu P, Lowe A D, Jia W, Rowland R R R. 2020. Epitope mapping of African swine fever virus (ASFV) structural protein, p54. *Virus Res,* **279**, 197871.

Ruedas-Torres I, Thi To Nga B, Salguero F J. 2024. Pathogenicity and virulence of African swine fever virus. *Virulence,* **15**, 2375550.

Shrewsbury J V, Vitus E S, Koziol A L, Nenarokova A, Jess T, Elmahdi R. 2024. Comprehensive phage display viral antibody profiling using VirScan: potential applications in chronic immune-mediated disease. *J Virol,* **98**, e0110224.

Shrock E, Fujimura E, Kula T, Timms R T, Lee I H, Leng Y, Robinson M L, Sie B M, Li M Z, Chen Y, Logue J, Zuiani A, McCulloch D, Lelis F J N, Henson S, Monaco D R, Travers M, Habibi S, Clarke W A, Caturegli P, Laeyendecker O, Piechocka-Trocha A, Li J Z, Khatri A, Chu H Y, Collection M C-, Processing T, Villani A C, Kays K, Goldberg M B, Hacohen N, Filbin M R, Yu X G, Walker B D, Wesemann D R, Larman H B, Lederer J A, Elledge S J. 2020. Viral epitope profiling of COVID-19 patients reveals cross-reactivity and correlates of severity. *Science,* **370**.

Song J, Li J, Li S, Zhao G, Li T, Chen X, Hu B, Liu J, Lai X, Liu S, Zhou Q, Huang L, Weng C. 2025. Autophagy promotes p72 degradation and capsid disassembly during the early phase of African swine fever virus infection. *J Virol,* **99**, e0170124.

Wang A, Jiang M, Liu H, Liu Y, Zhou J, Chen Y, Ding P, Wang Y, Pang W, Qi Y, Zhang G. 2021a. Development and characterization of monoclonal antibodies against the N-terminal domain of African swine fever virus structural protein, p54. *Int J Biol Macromol,* **180**, 203-211.

Wang G, Xie M, Wu W, Chen Z. 2021b. Structures and Functional Diversities of ASFV Proteins. *Viruses,* **13**.

Wang L, Li D, Zeng D, Wang S, Wu J, Liu Y, Peng G, Xu Z, Jia H, Song C. 2024. Development of a fully automated chemiluminescent immunoassay for the quantitative and qualitative detection of antibodies against African swine fever virus p72. *Microbiol Spectr,* **12**, e0080924.

Xin G, Kuang Q, Le S, Wu W, Gao Q, Gao H, Xu Z, Zheng Z, Lu G, Gong L, Wang H, Zhang G, Shi M, Sun Y. 2023. Origin, genomic diversity and evolution of African swine fever virus in East Asia. *Virus Evol,* **9**, vead060.

Xu Q, Li D, Chen X, Liu X, Cao H, Wang H, Wu H, Cheng T, Ren W, Xu F, He Q, Yu X, Li W. 2024. In Vivo Study of Inoculation Approaches and Pathogenicity in African Swine Fever. *Vet Sci,* **11**.

Yu Q, Liang D, Fu W, Zhang L, Wang J, Zhang Z, Sun Y, Zhu D, Zheng B, Zhu L, Xiang Y, Zhao D, Wang X. 2024. p72 antigenic mapping reveals a potential supersite of vulnerability for African swine fever virus. *Cell Discovery,* **10**, 80.

Yu X, Zhu X, Chen X, Li D, Xu Q, Yao L, Sun Q, Ghonaim A H, Ku X, Fan S, Yang H, He Q. 2021. Establishment of a Blocking ELISA Detection Method for Against African Swine Fever Virus p30 Antibody. *Front Vet Sci,* **8**, 781373.

Zhao D, Sun E, Huang L, Ding L, Zhu Y, Zhang J, Shen D, Zhang X, Zhang Z, Ren T, Wang W, Li F, He X, Bu Z. 2023. Highly lethal genotype I and II recombinant African swine fever viruses detected in pigs. *Nat Commun,* **14**, 3096.

Zhou X, Li N, Luo Y, Liu Y, Miao F, Chen T, Zhang S, Cao P, Li X, Tian K, Qiu H J, Hu R. 2018. Emergence of African Swine Fever in China, 2018. *Transbound Emerg Dis,* **65**, 1482-1484.

Zhu J, Liu Q, Li L, Zhang R, Chang Y, Zhao J, Liu S, Zhao X, Chen X, Sun Y, Zhao Q. 2024a. Nanobodies against African swine fever virus p72 and CD2v proteins as reagents for developing two cELISAs to detect viral antibodies. *Virol Sin,* **39**, 478-489.

Zhu X, Li F, Fan B, Zhao Y, Zhou J, Wang D, Liu R, Zhao D, Fan H, Li B. 2024b. TRIM28 regulates the coagulation cascade inhibited by p72 of African swine fever virus. *Vet Res,* **55**, 149.

Figure captions

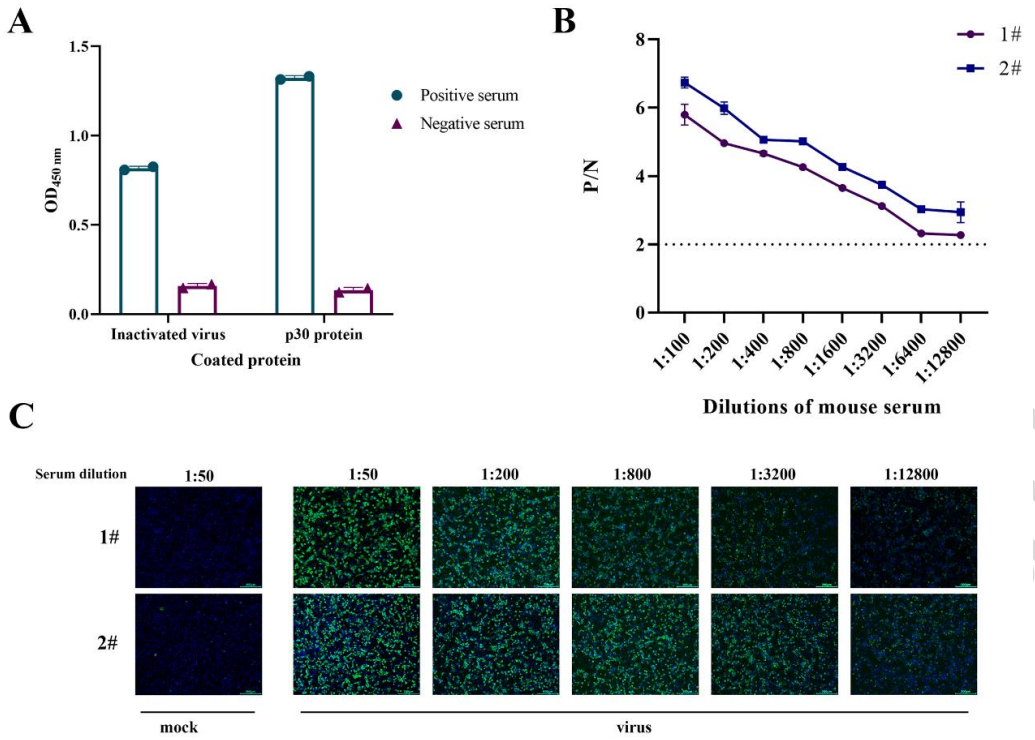

Fig. 1 Preparation of monoclonal antibodies. (A) Reactivity of the inactivated ASFV antigen with ASF-positive serum, as determined by direct ELISA. (B) Serum antibody titers in immunized mice were determined by indirect ELISA. Titers are presented as P/N values, calculated as $OD_{450\ nm}$ (sample)/$OD_{450\ nm}$ (negative control). (C) Serum antibody titers in immunized mice were detected by IFA. Scale bars, 200 μm.

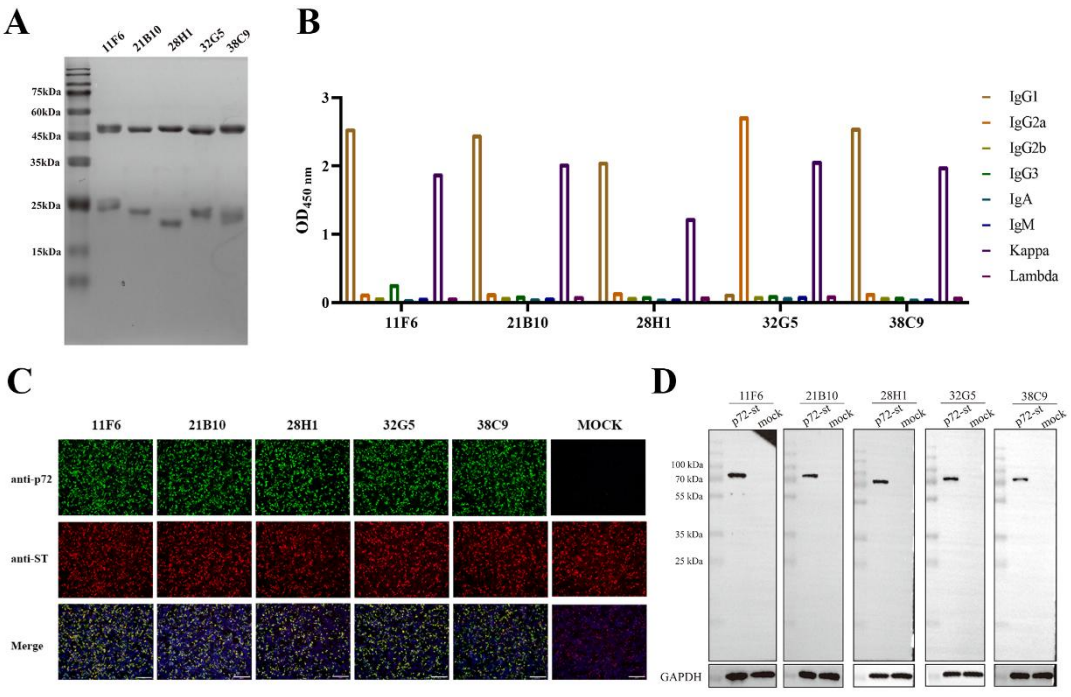

Fig. 2 Identification of candidate anti-p72 monoclonal antibodies. (A) Purification analysis of mAb ascites by SDS-PAGE under non-reducing conditions. The intact immunoglobulin bands are indicated. (B) Identification of the subtypes of all mAbs. (C) Analysis of monoclonal antibody reactivity by IFA. Cells were transfected with a pCAGGS-p72-ST expression plasmid. All five mAbs (green) show specific staining of p72. Successful transfection and protein expression were confirmed using an anti-ST-tag antibody (red). Nuclei were stained with DAPI (blue). Scale bars=200 μm. (D) Analysis of monoclonal antibody reactivity via Western blotting. All five mAbs specifically recognize a protein band at the expected molecular weight of approximately 72 kDa.

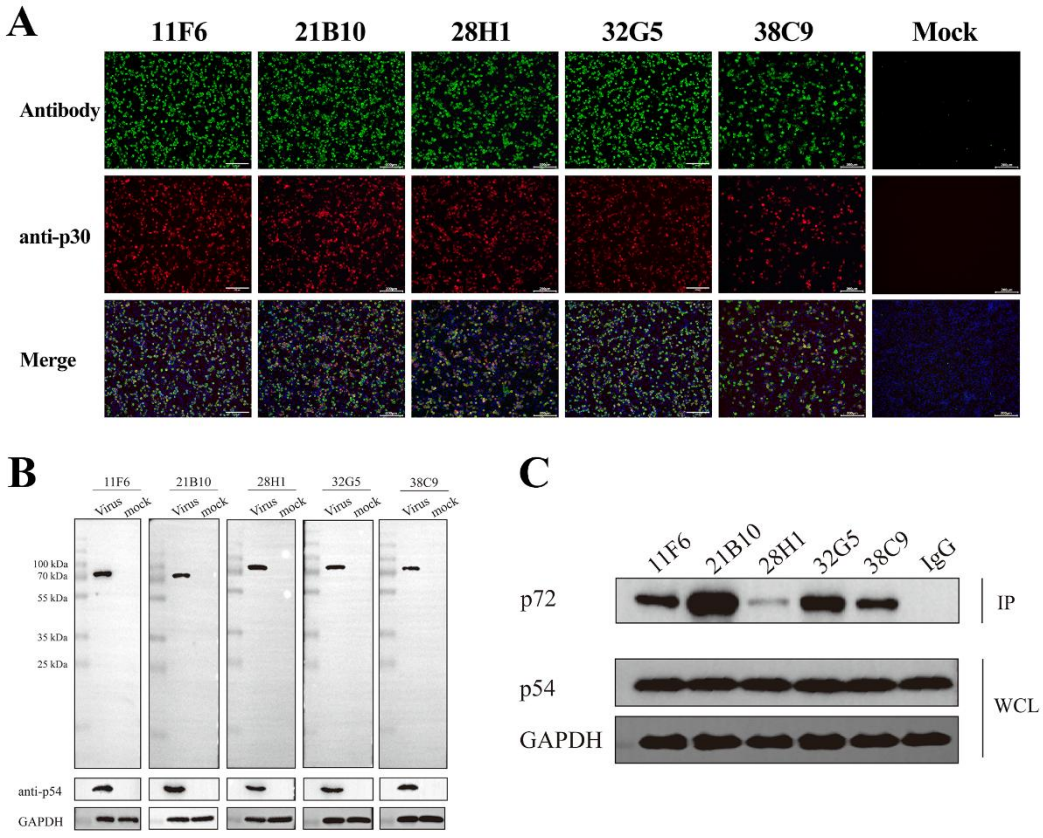

Fig. 3 Identification of the reactivity of monoclonal antibodies with ASFV. (A) IFA of monoclonal antibody reactivity in ASFV-infected LLC-PK1 cells at 24 hpi. Cells were labeled with mAbs (green, targeting p72) and the anti-p30 mAb (red) as an infection marker. Nuclei were stained with DAPI (blue). Scale bars, 200 μm. (B) Western blotting analysis of the p72 protein in porcine alveolar macrophages (PAMs) infected with ASFV. An anti-p54 mAb served as a marker for infection. (C) Analysis of mAbs binding by immunoprecipitation (IP).

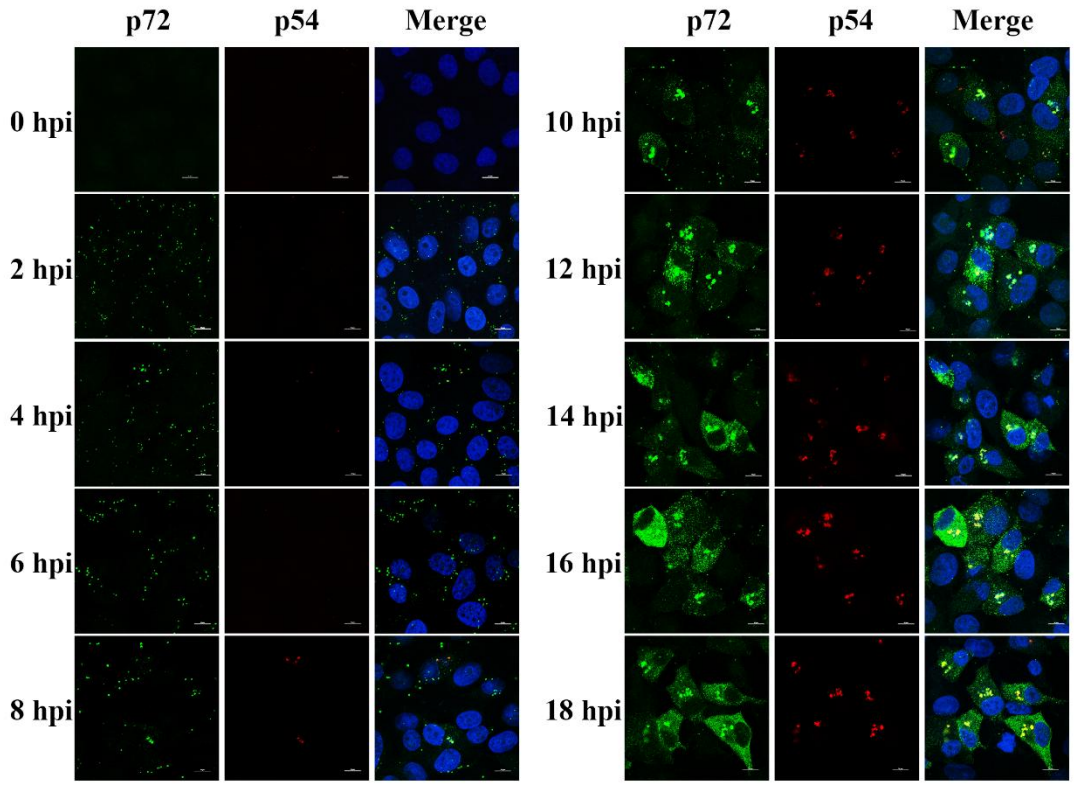

Fig. 4 Subcellular localization of the p72 protein in ASFV-infected cells at different times post infection. Vero cells were infected with ASFV (MOI = 0.5) and fixed at the indicated hours post-infection (hpi). The p72 protein was stained with mAb 32G5 (green), the viral factory was labeled with an anti-p54 antibody (red), and nuclei were stained with DAPI (blue). Scale bars=10 μm.

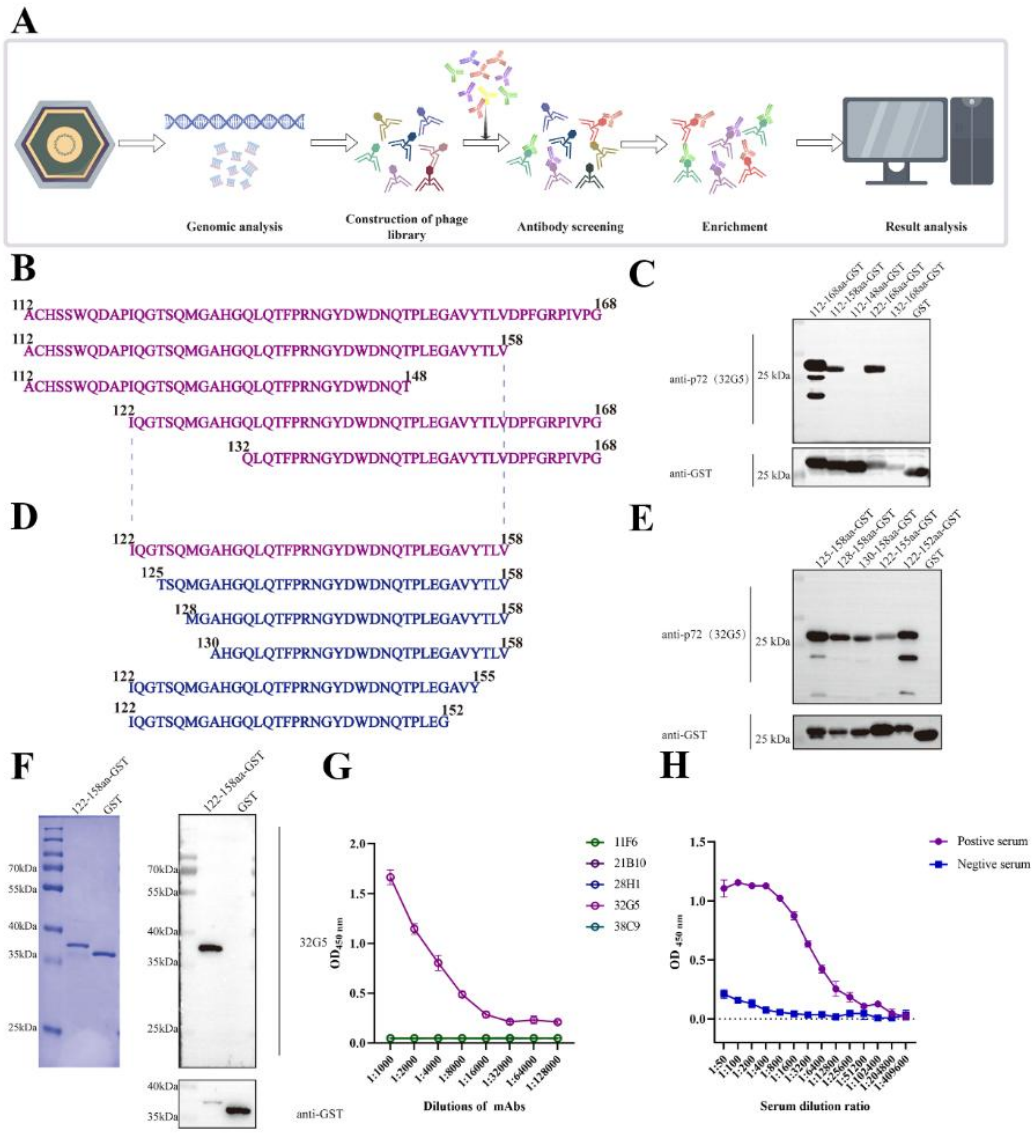

Figure 5. Identification of the antigenic epitope. (A) Schematic of the experimental strategy for identifying antigenic peptides using phage display library (VirScan) screening. (B) and (D) Schematic of the sequential truncation strategy applied to the initial 112-168 amino acid region to map the minimal epitope. (C) and (E) Western blot analysis of the expressed truncated GST-fusion proteins, probed with mAb 32G5 to identify reactive segments. The minimal epitope was localized to amino acids 130-152. (F) Coomassie-blue-stained SDS-PAGE and Western blot of the purified 122-158 amino acid-GST fusion protein (target protein) and GST tag alone (control). (G) Titration of mAb 32G5 by indirect ELISA using the 122-158 aa-GST protein as the coating antigen. (H) Reactivity of the identified epitope with ASFV-positive serum. ELISA was performed using the 122-158 aa-GST protein. The signal was calculated by subtracting the OD_{450} value of the GST-only control from the OD_{450} value of the experimental group.

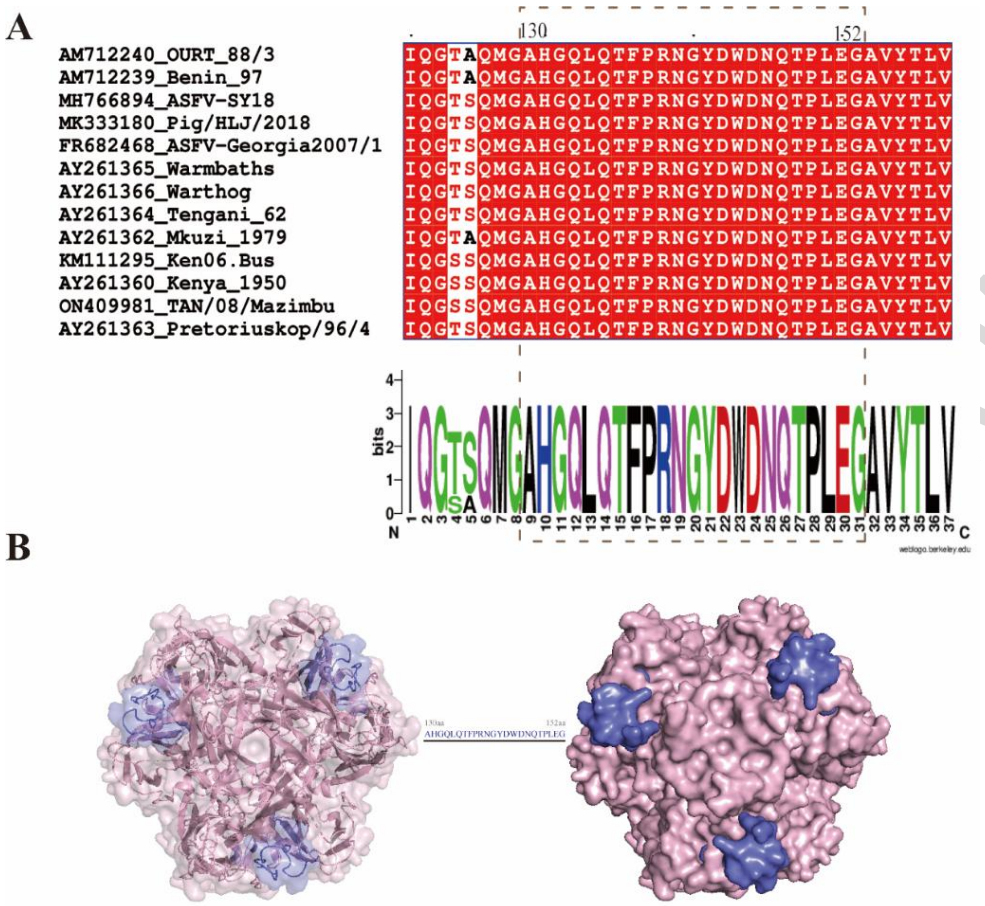

Fig. 6 Sequence analysis of the identified linear B-cell epitopes. (A) Conservation analysis of the p72 B-cell epitope. Multiple sequence alignment of the epitope region (amino acids 130–152) across p72 proteins from 13 representative ASFV strains shows complete conservation across all genotypes. (B) Spatial distribution of the epitopes recognized by the mAb 32G5. The epitope (amino acids 130-152, purple) is displayed on the surface of the p72 trimer structure (PDB: 6KU9). The structure was visualized and rendered using PyMOL.