

RESEARCH ARTICLE

# Structural prediction of porcine sialoadhesin V-set Ig-like domain sheds some light on its role in porcine reproductive and respiratory syndrome virus (PRRSV) infection

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**Abstract** Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failures in sows and respiratory diseases in pigs of all ages. PRRS virus (PRRSV) is its causative agent and has caused huge economic losses in the swine industry. Porcine sialoadhesin (pSn) is a putative receptor of PRRSV. Previous studies have shown that a pSn V-set Ig-like domain is significant in PRRSV infection. However, its structural details are not fully known, hindering our deep understanding of PRRSV infection. In this study, we successfully cloned, expressed and purified the pSn V-set Ig-like domain in *Drosophila* S2 cells. Then we tried to crystallize the target protein and predicted its structure. This will establish the foundation for the further structural study of pSn, deepen our understanding of the invasion mechanism of PRRSV, and support the structural information for the development of clinical drugs and vaccines against PRRSV.

**Keywords** PRRSV, porcine sialoadhesin, V-set Ig-like domain, *Drosophila* S2 cell, crystallization

virus, PRRS virus (PRRSV), was initially known as Lelystad virus in Europe<sup>[3]</sup> and when isolated in the USA, it was given the designation VR-2332<sup>[4]</sup>. PRRSV is an enveloped virus containing a single-stranded positive-sense RNA genome of about 15 kb in length and belongs to the family Arteriviridae along with equine arteritis virus, lactate dehydrogenase-elevating virus of mice, and simian hemorrhagic fever virus<sup>[5]</sup>.

PRRSV causes enormous economic losses in the swine industry every year<sup>[6]</sup> and consequently there are many studies focusing on its infection mechanism, including its host and receptors<sup>[7]</sup>. Swine are the only known natural host of PRRSV, with myeloid cells, particularly alveolar macrophages and dendritic cells, being the primary permissive cells. Virus invasion has been further shown to be mediated by the host cell surface receptors<sup>[8]</sup>. Currently, six putative receptors associated with PRRSV invasion have been identified, including heparin sulfate, sialoadhesin (Sn), and cluster of differentiation 163 (CD163)<sup>[9]</sup>.

Sn, also referred to CD169 or Siglec-1 (sialic acid-binding immunoglobulin-type lectin-1), is a macrophage-restricted lectin that binds to sialic acid. Sn belongs to the immunoglobulin (Ig) superfamily and is a type 1 transmembrane glycoprotein consisting of an extracellular Ig-like domain, a transmembrane domain and a short cytoplasmic tail<sup>[10]</sup>. The extracellular Ig-like domain is subdivided into 16 C2-set domains and a single N-terminal V-set Ig-like domain<sup>[11]</sup>. Sn participates in various physiological functions. It was first discovered as a sialic acid-dependent sheep erythrocyte receptor on bone marrow macrophages<sup>[12]</sup> and porcine Sn (pSn) was then assumed to be the receptor of PRRSV infection.

Duan et al. immunized mice against the membrane proteins from porcine alveolar macrophages, and then isolated a monoclonal antibody (mAb41D3). This anti-

## 1 Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first reported in the late 1980s in the USA. It has subsequently spread worldwide, and has become endemic in countries with a high level of swine rearing<sup>[1]</sup>. PRRS is characterized by reproductive failures in sows and respiratory symptoms in pigs of all ages<sup>[2]</sup>. Its causative

Received December 22, 2015; accepted January 26, 2016

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body was then demonstrated to abort PRRSV infection by neutralization of pSn<sup>[13]</sup>. Delputte et al. found the significant role of pSn in PRRSV infection and internalization into cells<sup>[14]</sup>. Van Breedam et al. further demonstrated that the major glycoprotein GP5 of PRRSV interacted with pSn *in vitro*<sup>[15]</sup>. They found that the sialic acid of GP5 was essential for PRRSV attachment to pSn and the N-terminal V-set Ig-like domain of pSn was responsible for the binding<sup>[16]</sup>. Despite considerable studies of pSn, the structural details of pSn, especially its N-terminal V-set Ig-like domain are still unknown, which hinders our deep understanding of pSn in PRRSV infection.

In our study, we cloned, expressed and purified pSn V-set Ig-like domain in a *Drosophila* expression system. We then crystallized it and predicted its structure to determine the role of pSn in PRRSV infection. Our work provides the molecular basis for further structural study of pSn, supports its role in PRRSV infection, and may help in the elucidation of PRRSV invasion.

## 2 Materials and methods

### 2.1 Construction of pSn V-set Ig-like domain expression vector

The cDNA encoding the V-set Ig-like domain (UniProt entry A7LCJ3, residues Val23–Val135) was amplified from the full length pSn (DQ176853) by polymerase chain reaction (PCR) using the following primers: 5'-GAA-GATCTGTTTCCAGCCCCGAG AC-3' (sense primer, the underline indicates the location of a *Bgl*III site); 5'-CGACGCGTCACGGTGACAACCTGTGCCT-3' (anti-sense primer, the underlined nucleotides indicate the location of a *Mlu*I site). The amplified PCR product was

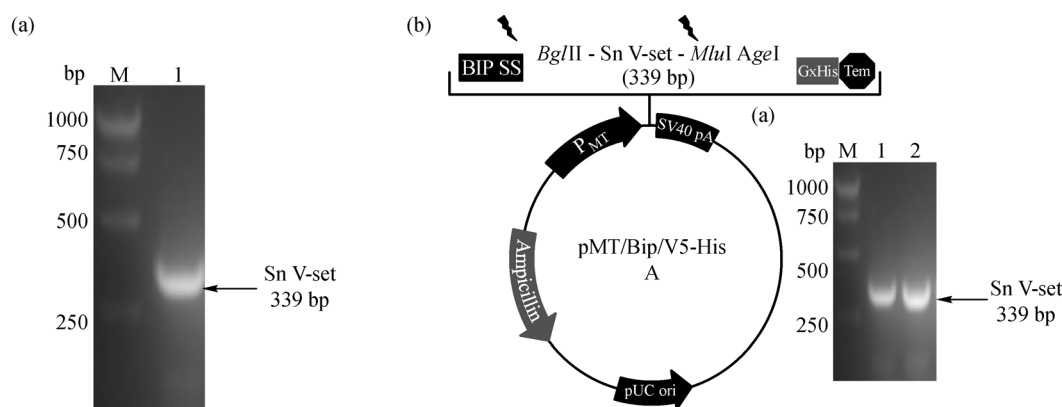
isolated and inserted into the expression vector pMT/BiP/Blast/V5-HisA of the *Drosophila* expression system between the *Bgl*III and *Mlu*I sites (Fig. 1). The construct was transformed into a competent *Escherichia coli* strain DH5 $\alpha$  (Takara Biotechnology Co. Ltd., Dalian, Liaoning, China) which was then screened on Luria-Bertani (LB) plates (0.5% yeast extract, 1% tryptone, 1% NaCl and 2% agar) containing 100  $\mu\text{g}\cdot\text{mL}^{-1}$  ampicillin (Beijing Solarbio Science and Technology Co. Ltd., Beijing, China) to select positive colonies. After colony PCR, the recombinant pSn V-set Ig-like domain expression vector was verified by sequencing by Shanghai Sangon Biotech Co. Ltd. (Shanghai, China).

### 2.2 Transfection and expression of pSn V-set Ig-like domain expression vector

*Drosophila* S2 cells were transfected with pMT/BiP/Blast/pSn V-set Ig-like domain-His expression vector by Cellfectin II according to the manufacturer's instructions (Invitrogen, Carlsbad, California, USA). Stably transfected S2 cells were selected in Schneider's Insect Medium (Sigma Aldrich Co. Ltd., St. Louis, Missouri, USA) supplemented with 10% fetal bovine serum and 25  $\mu\text{g}\cdot\text{mL}^{-1}$  blasticidin (Invitrogen, Carlsbad, California, USA). Target protein expression was induced by the addition of 0.75  $\text{mmol}\cdot\text{L}^{-1}$  CuSO<sub>4</sub> (final concentration) when the cell density reached  $4 \times 10^6$  cells per milliliter, and the culture supernatant was harvested by centrifugation 120 h after induction.

### 2.3 Characterization of recombinant pSn V-set Ig-like domain

The protein from the supernatant was harvested, applied to 15% SDS-PAGE and subjected to Western blotting. For



**Fig. 1** Cloning and expression vector construction of pSn V-set Ig-like domain. (a) PCR amplification of pSn V-set Ig-like domain. The arrow indicates the target gene of pSn V-set Ig-like domain (339 bp); M, DL2000 Marker; 1, the PCR product of pSn V-set Ig-like domain; (b) construction of pSn V-set Ig-like domain expression vector (left) and the colony PCR identification of the recombinant expression vector (right). M, DL2000 Marker; 1–2, colony PCR verification of pSn V-set Ig-like domain in the vector; the arrow indicates the target gene of pSn V-set Ig-like domain (339 bp).

Western blotting analysis, detection of target protein was performed with the mouse anti-His-Tag monoclonal antibody and horseradish peroxidase-labeled polyclonal goat anti-mouse antibody (Huamei Biotechnology Co. Ltd., Luoyang, Henan, China) followed by visualization using enhanced chemiluminescence (ECL). Moreover, samples of interest were sent to Shanghai Sangon Biotech Co. Ltd. (Shanghai, China) for mass spectrometry assay.

#### 2.4 Purification, crystallization and structural prediction of recombinant pSn V-set Ig-like domain

The recombinant protein was purified by Ni-NTA column (Merck, Kenilworth, New Jersey, USA) with elution at  $20 \text{ mmol} \cdot \text{L}^{-1}$  Tris-HCl, pH 8.0,  $150 \text{ mmol} \cdot \text{L}^{-1}$  NaCl and  $150 \text{ mmol} \cdot \text{L}^{-1}$  imidazole. Subsequently, the elution solution containing the target protein was dialyzed against the buffer  $20 \text{ mmol} \cdot \text{L}^{-1}$  Tris-HCl, pH 8.0,  $20 \text{ mmol} \cdot \text{L}^{-1}$  NaCl and concentrated by a Millipore ultra-centrifugation tube (Merck Millipore Co. Ltd., Carrigtwohill, Cork, Ireland). The purified protein was then applied to a 15% SDS-PAGE under reducing and non-reducing conditions. The protein was also treated with an amidase, PNGase F (Peptide-N-Glycosidase F) according to the manufacturer's instructions (New England BioLabs Inc., Ipswich, Massachusetts, USA) and applied to a 15% SDS-PAGE. Crystallization of pSn V-set Ig-like domain was done at room temperature by sitting drop vapor diffusion with an equal volume of recombinant protein at  $10 \text{ mg} \cdot \text{mL}^{-1}$  and crystallization reagents from crystallization screening kits (Hampton Research, Aliso Viejo, California, USA). Next, the protein structure prediction server (<http://swissmodel.expasy.org>) was used to predict and analyze its structure based on the crystal structure of murine Sn V-set Ig-like domain (PDB code 1QFP). The analysis results were displayed with PyMOL software<sup>[17]</sup>.

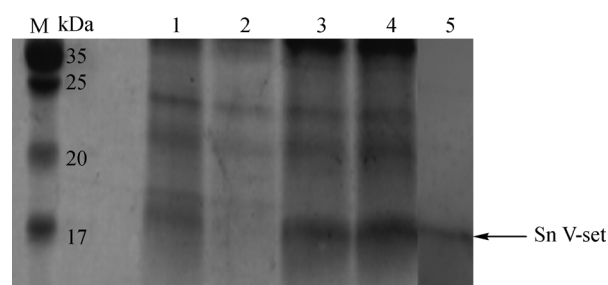
### 3 Results

#### 3.1 Construction of pSn V-set Ig-like domain expression vector

The target gene fragment of the V-set Ig-like domain was amplified from the full-length pSn cDNA by PCR. The amplified products were analyzed by 1% agarose gel electrophoresis. A 339 bp band was obtained, which indicated the target gene (Fig. 1a). We then constructed the pMT/BiP/Blast/pSn V-set Ig-like domain-His recombinant vector, transformed it into *Escherichia coli* DH5 $\alpha$  cells, and screened out positive clones by colony PCR. The results showed that the target gene fragment had been successfully inserted into the expression vector (Fig. 1b). Subsequently, the inserted target gene fragment was verified by the sequencing company (data not shown).

#### 3.2 Characterization of recombinant pSn V-set Ig-like domain

In Fig. 2, the Lanes 3 and 4 of SDS-PAGE show an apparent protein band of about 15 kDa from the supernatant of recombinant vector-stably transfected cells compared to that of blank S2 cells (Lane 1) and the empty vector-stably transfected cells (Lane 2). The relative molecular mass of this band was consistent with the theoretical mass value of pSn V-set Ig-like domain with enzyme sites and His-tag, which suggests it might be the target protein. The Western blotting assay against His-Tag showed that the recombinant vector-stably transfected cells expressed the recombinant pSn V-set Ig-like domain for the target protein was fused with His-tag (Lane 5, Fig. 2). The mass spectrometry further confirmed that the protein of interest matched with the target protein (Fig. 3). These results together demonstrated that recombinant vector-stably transfected cells expressed the pSn V-set Ig-like domain.



**Fig. 2** 15% SDS-PAGE and Western blotting identification of pSn V-set Ig-like domain. The arrow indicates the target protein of pSn V-set Ig-like domain. M, Protein marker; 1, inducible expression of blank S2 cells as a negative control; 2, inducible expression of empty vector-stably transfected S2 cells as a negative control; 3–4, inducible expression of recombinant pSn V-set Ig-like domain expression vector-stably transfected S2 cells; 5, anti-His tag Western blotting of inducible expression of recombinant pSn V-set Ig-like domain expression vector-stably transfected S2 cells.

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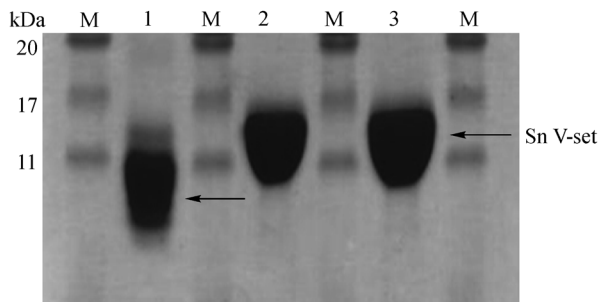
1 NPKVVENHVFQGRALLLGQVEQRTCSLLKDLQPQDSGSYNFRFEI
2 VVENHVFQGRALLLGQVEQR DLQPQDSGSYNFR

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**Fig. 3** Sequence alignment of mass spectrometry and target pSn V-set Ig-like domain. 1, Sequence of the target protein; 2, mass spectrometry result with the expressed protein of interest. The aligned sequences are colored in red.

#### 3.3 Purification and crystallization of pSn V-set Ig-like domain

After purification by Ni-NTA column, the target protein pSn V-set Ig-like domain was easily separated from other proteins with a high purity of 99% (Fig. 4, Lane 2). As



**Fig. 4** Purification, and testing for the presence of disulfide bonds and glycosylation of pSn V-set Ig-like domain. The arrow indicates the target protein of pSn V-set Ig-like domain. PNGase F (about 36 kDa) is not shown in the current figure. M, Protein marker; 1, the target protein under non-reducing condition; 2, the target protein under reducing condition without deglycosylation; 3, the PNGase F-treated target protein.

shown in Fig. 4 (Lanes 1 and 2), there was a difference between the bands of the purified protein under reducing and non-reducing conditions, which implies disulfide bonds may be present. The result (Fig. 4, Lanes 2 and 3) further shows there is no difference between the bands of the PNGase F-treated target protein and the untreated one, implying there may be no N-glycosylation. Crystallization was then attempted by sitting drop vapor diffusion, but no crystals formed.

### 3.4 Structural prediction of pSn V-set Ig-like domain

Since the primary crystal screening of the pSn V-set Ig-like domain was not achieved, we used the protein structure

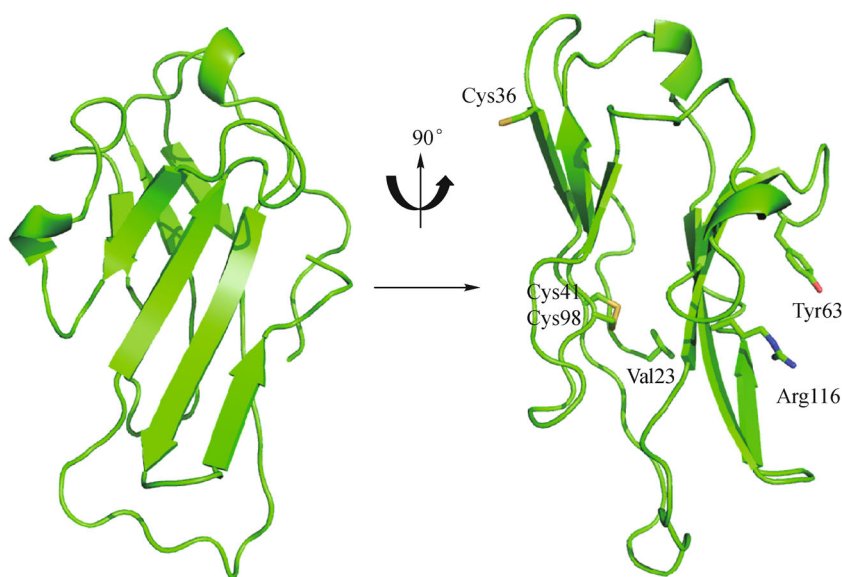
prediction server to predict and analyze its structure (Figs. 5–7).

As shown in Fig. 5, pSn V-set Ig-like domain is made up of seven  $\beta$ -strands and two helices. This domain contains 108 amino acids, from the N-terminal Val23 to C-terminal Gly130, including a free cysteine (Cys36) and a disulfide bond formed by Cys41 and Cys98. The predicted disulfide bond is similar to the one in its homolog, murine Sn (UniProt entry Q62230, with a identity of 67%) and consistent with our result from the 15% SDS-PAGE. In addition, there is no predicted N-glycosylation in the pSn V-set Ig-like domain, which is similar to that in its homolog, murine Sn (UniProt entry Q62230, with a identity of 67%) and consistent with the 15% SDS-PAGE result.

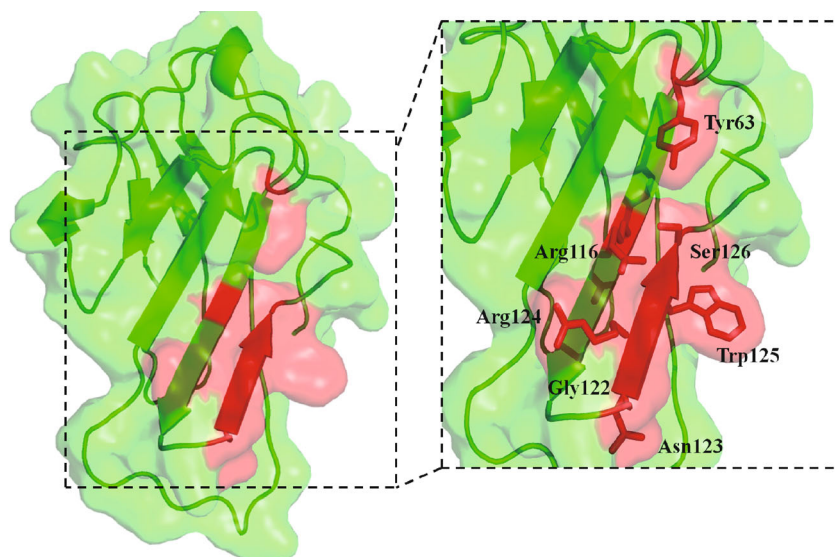
Tyr63 and Arg116 are reported to be the key binding sites for sialic acid<sup>[18]</sup>. In Fig. 6, Tyr63 and Arg116 protrude like a clamp, which may explain their binding to sialic acid in the structure. As shown in Fig. 6, all these residues form into an interface favored for the interaction with the sialic acid from the GP5 of PRRSV.

In Fig. 7, the sialic acid binding region is positively charged and matches with the sialic acid (negatively charged) from the GP5 of PRRSV.

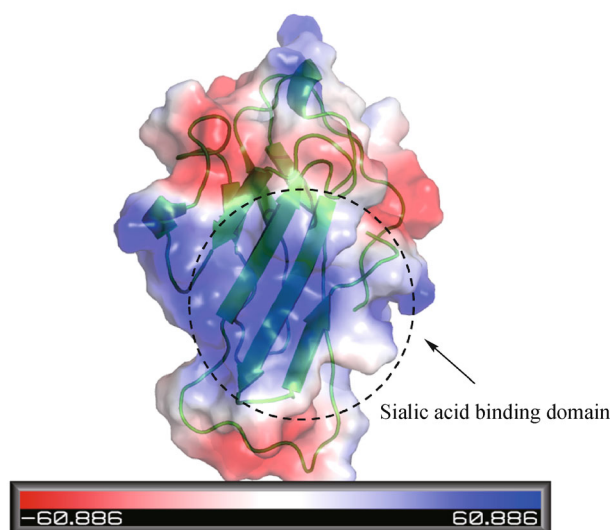
Altogether, our structural prediction and analysis support previous studies on the role of pSn in PRRSV infection and may contribute to the deep understanding of the PRRSV GP5 interaction with pSn V-set Ig-like domain.



**Fig. 5** Structural prediction of pSn V-set Ig-like domain. The right panel is yielded by anticlockwise rotation of the left panel along a longitudinal axis in the page-face (only the residues from Val23 to Gly130 are displayed). N-terminal Val23, the free cysteine Cys36, the disulfide bond formed by Cys41 and Cys98, and the key amino acids Tyr63 and Arg116 in the sialic acid-binding region are labeled.



**Fig. 6** The sialic acid-binding moiety of the pSn V-set Ig-like domain. The predicted structure of the pSn V-set Ig-like domain is shown in structural and surface representation, and colored in green (only the residues from Val23 to Gly130 are displayed). Residues from Gly122 to Ser126 are the sialic acid-binding region colored in red. Tyr63 and Arg116 are the key binding sites and also colored in red.



**Fig. 7** Surface charge analysis of the pSn V-set Ig-like domain. The predicted structure of the pSn V-set Ig-like domain was generated with vacuum electrostatics (only the residues from Val23 to Gly130 are displayed). The moieties with positive charge are colored in blue and those with negative charge in red. The sialic acid-binding region is circled by the dashed line and indicated by the arrow.

## 4 Discussion

Previous studies have confirmed that pSn is an indispensable receptor in PRRSV infection. However, subsequent research discovered that PRRSV also infects African green monkey kidney epithelial cell line MA-104 and its derivative, MARC-145 cell line, which possess no Sn. In

addition, CD163 alone has been demonstrated to mediate PRRSV internalization<sup>[19]</sup>. Also, a recent study showed that Sn gene-knockout pigs could also be infected by PRRSV, which demonstrated that pSn was not required for infection of PRRSV and the presence of the pSn gene did not contribute to acute PRRS and PRRSV pathogenesis<sup>[20]</sup>. Therefore, the function of pSn in PRRSV invasion and whether it is an indispensable receptor for PRRSV remain controversial. Moreover, even if pSn is essential for PRRSV invasion, there is still a lack of the structural details of pSn V-set Ig-like domain and its interaction with PRRSV GP5. As a result, the structural study of pSn is of great importance.

In this study, we cloned, expressed, and purified the pSn V-set Ig-like domain in *Drosophila* S2 cells to obtain the target protein. The *Drosophila* Expression System is an insect expression systems that overcomes the shortcomings of the prokaryotic expression system as used in previous studies<sup>[21,22]</sup>. The exogenous protein can be post-translationally modified, including correct folding, glycosylation and disulfide bond formation. The target protein can be secreted into the culture medium and thus the processes of separation and purification are relatively easy. Meanwhile, the insect cell expression system has the advantage of high expression level compared with mammalian cell expression systems. Moreover, the characteristics of S2 cells makes them easy to culture in suspension without serum and CO<sub>2</sub>. Our research results have confirmed these advantages. The target protein is properly expressed, which is identified by immunology and mass spectrometry, and the purity of target protein can reach 99% after purification. All these results provide the molecular basis for further structural study of pSn.

Our predicted structure for pSn V-set Ig-like domain supports its role in PRRSV infection as well as explores its key structural domains and interaction sites with the virus. Notably, our predicted structure is significantly different from other predicted structures<sup>[22]</sup>. The differences are probably due to that previous predicted structures of pSn V-set Ig-like domain comprise more residues (aa1–150) than ours (UniProt entry A7LCJ3, aa23–135). The inclusion of more residues in the other studies may generate additional  $\beta$ -strands (12  $\beta$ -strands). Also, our predicted structure shows some minor differences from that of the murine Sn V-set Ig-like domain. The crystal structure of murine Sn V-set Ig-like domain contains one additional  $\beta$ -strand, which is not found in pSn at aa107–110. These differences may result from species variation in amino acid sequences. Since the structural prediction may not represent the genuine structure of the target protein, we are carrying out further crystallization of the pSn V-set Ig-like domain to elucidate its exact structural information. Crystallization would contribute to understanding whether the putative receptor Sn is indispensable in PRRSV invasion, deepen our understanding of the invasion mechanism of PRRSV, and finally support the structural information for the development of clinical drugs and vaccines.

## 5 Conclusions

The innovation of the present study was to use the *Drosophila* S2 cells to express the pSn V-set Ig-like domain, crystallize it, and predict its structure. The results have provided a molecular basis to determine the genuine structure of pSn V-set Ig-like domain, explored the key structural domains and interaction sites of the virus, promoted our understanding of its function in PRRSV infection, and laid the foundation for the deep exploration of the molecular mechanism of PRRSV infection.

**Acknowledgements** This study was supported by the National Natural Science Foundation of China (31490601), the National Program on the Key Basic Research Project (2014CB542702), and the Basic and Advanced Technology Research Program of Henan Province (162300410252). The *Drosophila* Expression System was kindly provided by Professor Mingdong Huang (Fujian Institute of Research on the Structure of Matter, Chinese Academy of Sciences, China).

**Compliance with ethics guidelines** Jie Hou, Rui Li, Hongfang Ma, Songlin Qiao, and Gaiping Zhang declare that they have no conflict of interest or financial conflicts to disclose.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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