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## Roles of Arenavirus Z Protein in Mediating Virion Budding, Viral Transcription-Inhibition and Interferon-beta Suppression

Junjie Shao, Yuying Liang, and Hinh Ly

Department of Veterinary and Biomedical Sciences, University of Minnesota, Twin Cities, 1988 Fitch Ave., 295 AS/VM Bldg., Saint Paul, MN 55108, USA

### Abstract

The smallest arenaviral protein is the zinc-finger protein (Z) that belongs to the RING finger protein family. Z serves as a main component required for virus budding from the membrane of the infected cells through self-oligomerization, a process that can be aided by the viral nucleoprotein (NP) to form the viral matrix of progeny virus particles. Z has also been shown to be essential for mediating viral transcriptional repression activity by locking the L polymerase onto the viral promoter in a catalytically inactive state, thus limiting viral replication. The Z protein has also recently been shown to inhibit the type I interferon-induction pathway by directly binding to the intracellular pathogen-sensor proteins RIG-I and MDA5, and thus inhibiting their normal functions. This chapter describes several assays used to examine the important roles of the arenaviral Z protein in mediating virus budding (i.e., either Z-self budding or NP-Z budding activities), viral transcriptional inhibition in a viral minigenome (MG) assay, and type I IFN suppression in an IFN $\beta$ -promoter mediated luciferase reporter assay.

### Keywords

arenavirus; Lassa virus; Pichinde virus; matrix protein; RING protein; virus budding; interferon suppression; transcription inhibition

## 1. Introduction

The *Arenaviridae* consists of a large group of bi-segmented single-stranded ambisense RNA viruses that can cause significant morbidity and mortality in humans. Lassa virus and Lujo virus (LUJV), found in Africa, and several other arenaviruses found in South America can cause severe hemorrhagic fever (HF). There are currently limited prevention and treatment modalities against these pathogenic arenaviruses. The only available vaccine (Candid #1) has been developed and used extensively to prevent Argentine hemorrhagic fever (AHF) caused by Junín virus (JUNV) (1). Ribavirin, a guanosine analog, has been used in treating arenaviral HF with mixed success and significant toxicity (2).

The arenaviral genome is composed of two segments: the L (large) segment encodes the Z matrix protein and the L polymerase protein, while the S (small) segment encodes the

glycoprotein (GPC) and nucleoprotein (NP). The smallest arenaviral protein of 90–99 amino acids in size, depending on the virus species, is the zinc-finger protein (Z) that belongs to the RING finger protein family (3). Like other known RING finger proteins (4), the arenavirus Z protein has been shown to interact with many cellular proteins, including but not necessarily limited to the promyelocytic leukemia protein (PML), eukaryotic elongation factor 4E (eIF4E) or components of the endosomal sorting complexes required for transport (ESCRT) (4, 5). In addition to interacting with cellular proteins, Z also interacts with the arenaviral L protein to latch the protein onto the genome in order to ensure that the L polymerase is incorporated into virion particles (6). The interaction between Z and the L polymerase of the Tacaribe virus (TCRV) appears to be essential for mediating viral transcriptional repression activity (7). Z appears to lock the L polymerase onto the viral promoter in a catalytically inactive state, thus limiting viral replication (6, 8, 9).

Z also serves as a main component required for viral budding by oligomerizing and forming the viral matrix of progeny virus particles (4). The Z homo-oligomerization process is likely important for its function in virion budding (10). A crystal structure of a dodecamer form of the LASV Z protein has recently been solved (11). It shows a ring-like structure of Z with highly basic residues, including a Lys-Trp-Lys triad in the center, as important for Z oligomerization. It is important to note that arenaviral NP protein appears to be required for efficient Z-mediated budding activity of the Tacaribe virus (TCRV) (12) and Pichinde virus (PICV) (13) possibly through specific NP-Z interactions (14, 15).

Arenaviral Z protein has also recently been shown to inhibit the type I interferon-induction pathway by directly binding to the N-terminal CARD domain of the intracellular pathogen-sensor proteins RIG-I and MDA5 (16). Z proteins from all known pathogenic arenaviruses [e.g., LASV, LUJV, JUNV, Machupo (MACV), Sabia (SABV), Chapare (CHPV), Guanarito (GTOV), and Dandenong (DANV)] as well as the relatively low pathogenic lymphocytic choriomeningitis virus (LCMV) are able to bind to RIG-I as well as MDA5 and thus, suppress the production of interferon-beta (IFN $\beta$ ). In contrast, Z proteins of many known non-pathogenic arenaviruses do not bind to RIG-I or MDA5 and fail to inhibit the IFN-induction pathway (16). The fact that this small Z protein plays multiple roles in arenaviral replication and in modulating host immune responses to viral infection highlights the importance of this viral protein as a potential target for antiviral drug development. This chapter describes several assays used to examine the important roles of the arenaviral Z protein in mediating virus budding (i.e., either Z-self budding or NP-Z budding activities), viral transcriptional inhibition in a viral minigenome (MG) assay, and type I IFN suppression in an IFN- $\beta$ -promoter-mediated luciferase reporter assay.

## 2. Materials

1. Cells: Human kidney epithelial 293T cells were grown in DMEM supplemented with 10% FBS and 50  $\mu$ g of penicillin-streptomycin/ml.
2. Plasmids: For PICV Z protein and NP expression constructs, genes was amplified from the respective plasmid of the full-length PICV L or S segment using primers containing hemagglutinin (HA) or Myc tag at the C terminus, and

cloned into the pCAGGS expression vector. IFN- $\beta$  promoter-directed LUC plasmid was used for Z induced interferon suppression assay. MGLF7-5Rluc3 plasmid was used for Mini-genome assay. pSV- $\beta$ -Galactosidase Control Vector was purchased from promega. Human RIG-I N terminal domain (RIG-iN) and LASV Z gene (with HA tag) were cloned into the pCAGGS vector as previously described (16).

3. Assay kits for Beta-galactosidase, Firefly luciferase, and Renilla luciferase.
4. Promega cell culture lysis 5X reagent
5. Lipofectamine 2000.
6. PCR thermal cycler.
7. Microcentrifuge.
8. Ultracentrifuge.
9. Waterbath.
10. Enhanced chemiluminescence (ECL) kit to detect bands in Western blots.
11. ECL detection device (Thermo).
12. Polyethylene glycol (PEG).
13. Plate reader.
14. 2X HBS: 50 mM HEPES, 280 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.10.
15. 2.5 M calcium chloride stock solution.
16. RIPA buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl.

### 3. Methods

#### 3.1. Z-self budding assay

1. 293T cells were seeded in 10 cm tissue culture dish at  $3 \times 10^6$ /dish with complete DMEM.
2. On the morning of the day of transfection, replace cell-culture media with an equal aliquot of fresh complete DMEM.
3. Prepare plasmids and calcium chloride mix fresh as follows:  
10  $\mu$ l pCAGGS-PICV-Z-HA plasmid (1  $\mu$ g/ $\mu$ l)  
50  $\mu$ l 2.5 M calcium chloride  
440  $\mu$ l water  
Add 500  $\mu$ l 2X HBS to above mixture while vortexing at the lowest speed setting for 10 s. The mixture was added dropwise using a pipetman into dish. Media were replaced with same amount of complete DMEM 6 h post-transfection.

4. 48 h post-transfection, supernatants were collected and centrifuged at  $15,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The budded VLP in the cleared supernatants were precipitated with 8% PEG 8000, 0.5 M sodium chloride, collected after centrifugation at  $15,000 \times g$  for 15 min, and lysed in RIPA buffer.
5. Cell lysates and the budded VLP were analyzed on a 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and Z proteins were detected by Western blotting with the anti-HA antibody.
6. Figure 1 shows results of the wildtype PICV Z-self budding as VLP.

### 3.2. NP-Z budding assay

1. 293T cells were seeded in a 10 cm tissue culture dish at  $3 \times 10^6$ /dish with complete DMEM.
2. On the morning of the day of transfection, replace culture media with an equal aliquot of fresh complete DMEM.
3. Prepare plasmids and calcium chloride mix fresh as follows:  
10  $\mu\text{l}$  pCAGGS-PICV-NP plasmid (1  $\mu\text{g}/\mu\text{l}$ )  
10  $\mu\text{l}$  pCAGGS-PICV-Z-HA plasmid (1  $\mu\text{g}/\mu\text{l}$ )  
50  $\mu\text{l}$  2.5 M calcium chloride  
440  $\mu\text{l}$  water  
Add 500  $\mu\text{l}$  2X HBS into above mixture by vortexing using the lowest speed setting for 10 s. The mixture was added dropwise using a pipetman into dish. Media were replaced with same amount of complete DMEM 6 h post transfection.
4. 48 h post-transfection, supernatants were collected and centrifuged at  $15,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The budded VLP in the cleared supernatants were precipitated with 8% PEG-8000, 0.5 M sodium chloride, collected after centrifugation at  $15,000 \times g$  for 15 min, and lysed by RIPA buffer. Cell lysates and the budded VLP were analyzed on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel and detected for Z and NP proteins by Western blotting with the anti-HA antibody and rabbit anti-PICV NP sera, respectively (*see Note 1*).
5. Figure 2 shows results of NP incorporation into the Z-mediated VLP.

### 3.3. IFN $\beta$ promoter-driven luciferase reporter assay

1. 293T cells were seeded into 12 well-plate at  $3 \times 10^5$ /well with complete DMEM.
2. In the morning of the day of transfection, culture media were replaced by an equal aliquot of fresh media.

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<sup>1</sup>-Western blot analyses were conducted using rabbit sera raised against specific short peptides within the NP protein as previously described (17). Peptide sequences and limited aliquots of the rabbit sera are available upon request.

3. Prepare DNA and calcium phosphate mix as follow:
  - 1  $\mu$ l pSV- $\beta$ -Galactosidase plasmid (50 ng/ $\mu$ l)
  - 1  $\mu$ l IFN $\beta$  promoter reporter plasmid (100 ng/ $\mu$ l)
  - 1  $\mu$ l pCAGGS-PICV-Z-HA or pCAGGS-LASV-Z-HA plasmid (1  $\mu$ g/ $\mu$ l)
  - 1  $\mu$ l pCAGGS-RIG-iN plasmid (50 ng/ $\mu$ l)
  - 5  $\mu$ l 2.5M calcium chloride
  - 47  $\mu$ l waterTo prepare triplicates for each test condition, mix as follows:
  - 3  $\mu$ l pSV- $\beta$ -Galactosidase plasmid (50 ng/ $\mu$ l)
  - 3  $\mu$ l IFN  $\beta$  promoter reporter plasmid (100 ng/ $\mu$ l)
  - 3  $\mu$ l pCAGGS-PICV-Z-HA plasmid or pCAGGS-LASV-Z-HA plasmid (1  $\mu$ g/ $\mu$ l)
  - 15  $\mu$ l 2.5M calcium chloride
  - 141  $\mu$ l waterInto each tube of DNA/calcium phosphate mixture, add 75  $\mu$ l 2xHBS, vortex using very low speed to mix, and add the mixture to 3 wells of cells (98  $\mu$ l per well)
4. 24 h post transfection, the supernatants were aspirated and cells were lysed by 100  $\mu$ l of Luciferase Cell Culture Lysis 1X Reagent. 20  $\mu$ l of cell lysate was used for Firefly luciferase assay and another 50  $\mu$ l of cell lysate was used for  $\beta$ -gal assay according to manufacturer's instructions.
5. The Firefly luciferase activity is normalized by dividing the Firefly luciferase reading by the  $\beta$ -gal reading. The IFN $\beta$  promoter activity is represented by the folds of the normalized Firefly luciferase activity of the test to that the control (see Note 2).
6. Figure 3 shows results of the LASV Z-mediated suppression of the IFN $\beta$  promoter activity.

### 3.4. Z-mediated viral mini-genome (MG) replication and transcription assay

#### 3.4.1. *In vitro* RNA transcription

1. The plasmid MGLF7-5Rluc3 was linearized by digestion with Nhe I restriction enzyme.
2. DNA was extracted by standard phenol/chloroform and isopropanol precipitation.

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<sup>2</sup>-When performing the IFN $\beta$  promoter assay, the amount of RIG-iN and Z plasmid is critical. Low Z and RIG-iN protein expression will lead to unreliable IFN $\beta$  readings.

3. Transcription reaction was assembled at room temperature according to Ambion MEGAscript® Kit manual. Calculate the volume of nuclease-free water to bring the total volume to 20  $\mu\text{L}$ , and to this amount add 2  $\mu\text{L}$  ATP, 2  $\mu\text{L}$  CTP, 2  $\mu\text{L}$  GTP, 2  $\mu\text{L}$  UTP, 2  $\mu\text{L}$  10X reaction buffer, 1  $\mu\text{g}$  linearized template DNA, and 2  $\mu\text{L}$  enzyme mix.
4. Sample was incubated at 37°C for 4 h.
5. 115  $\mu\text{L}$  nuclease-free water and 15  $\mu\text{L}$  ammonium acetate stop solution were added.
6. RNA was extracted by phenol/chloroform and isopropanol precipitated using the standard technique.
7. Carefully discard the precipitating solution and the RNA was resuspended in 80  $\mu\text{L}$  of DEPC-treated water.
8. RNA was quantified and adjusted to a concentration of 1  $\mu\text{g}/\mu\text{L}$  (*see Note 3*)

#### 3.4.2. Plasmid transfection:

1. 293T cells were seeded into 24-well plates at  $1 \times 10^5$  cells/well that contains 0.5 ml of DMEM with 10% FBS and 50  $\mu\text{g}/\text{ml}$  of penicillin and streptomycin.
2. On the morning of the day of transfection, a fresh aliquot of the cell media was added to the 293T cell culture.
3. DNA and calcium phosphate mixture was prepared at room temperature as follows:

Each of the 25- $\mu\text{L}$  of DNA mix (for one 24-well transfection) contains:

- 1  $\mu\text{L}$  Beta-gal reporter plasmid (50  $\text{ng}/\mu\text{L}$ )
- 1  $\mu\text{L}$  pCAGGS-PICV-Z-HA plasmid (1  $\mu\text{g}/\mu\text{L}$ )
- 1  $\mu\text{L}$  pCAGGS-NP plasmid (250  $\text{ng}/\mu\text{L}$ )
- 1  $\mu\text{L}$  pCAGGS-L plasmid (500  $\text{ng}/\mu\text{L}$ )
- 2.5  $\mu\text{L}$  2.5 M calcium chloride
- 18.5  $\mu\text{L}$  water

To prepare triplicates for each test condition, make a mixture as follows:

- 3  $\mu\text{L}$  Beta-gal reporter plasmid (50  $\text{ng}/\mu\text{L}$ )
- 3  $\mu\text{L}$  pCAGGS-PICV-Z-HA plasmid (1  $\mu\text{g}/\mu\text{L}$ )
- 3  $\mu\text{L}$  pCAGGS-NP plasmid (250  $\text{ng}/\mu\text{L}$ )
- 3  $\mu\text{L}$  pCAGGS-L plasmid (500  $\text{ng}/\mu\text{L}$ )
- 7.5  $\mu\text{L}$  2.5 M calcium chloride

<sup>3</sup>The integrity of the RNA needs to be validated by agarose gel electrophoresis prior to use in the transfection reaction.

55.5 µl water

Into each tube of DNA/calcium phosphate mixture ( $25 \times 3 = 75 \mu\text{l}$ ), add 75 µl of 2xHBS. Sample was gently mixed by vortexing using the lowest speed setting. The mixture was added dropwise using a pipetman into each well of cells (48µl of sample into each well of the 24-well plate).

4. Cells were incubated in a 37°C CO<sub>2</sub> incubator for 24 hours.

#### 3.4.3. RNA Transfection at 24 h after plasmids transfection:

1. 4 h before transfection, an aliquot of fresh antibiotics-free DMEM was used to replace the overnight cell culture media.
2. For each well, 1 µg of *in vitro*-transcribed RNA (*see* Note 3) was diluted into 25 µl OptiMEM® I Medium.
3. For each well, 1 µl of Lipofectamine™ 2000 was diluted into 25 µl OptiMEM® I Medium and incubated for 5 min at room temperature.
4. The diluted RNA was added to diluted Lipofectamine™ and incubated at room temperature for no more than 10 min.
5. RNA-Lipofectamine™ 2000 complexes were directly added to each well containing cells and mixed gently by rocking the plate back and forth. The cells were incubated at 37°C in a CO<sub>2</sub> incubator.
6. The cell medium was replaced by fresh DMEM medium (10% FBS) at 6 h post transfection.
7. 24 h post RNA transfection, cells were washed once by PBS. Then cells were lysed by lysis buffer for Renilla luciferase assay.

#### 3.4.4. Renilla luciferase assay:

1. 20 µl of cell lysate was used for Renilla luciferase assay and another 50 µl of cell lysate was used for β-gal assay according to manufacturer's instructions.
2. Renilla luciferase activity is normalized by dividing the Renilla luciferase reading by the β-gal reading. The minigenome replication activity is represented by the folds of the normalized Renilla luciferase activity of the test to that of the control.
3. Figure 4 shows results of Z-mediated viral mini-genome inhibition.

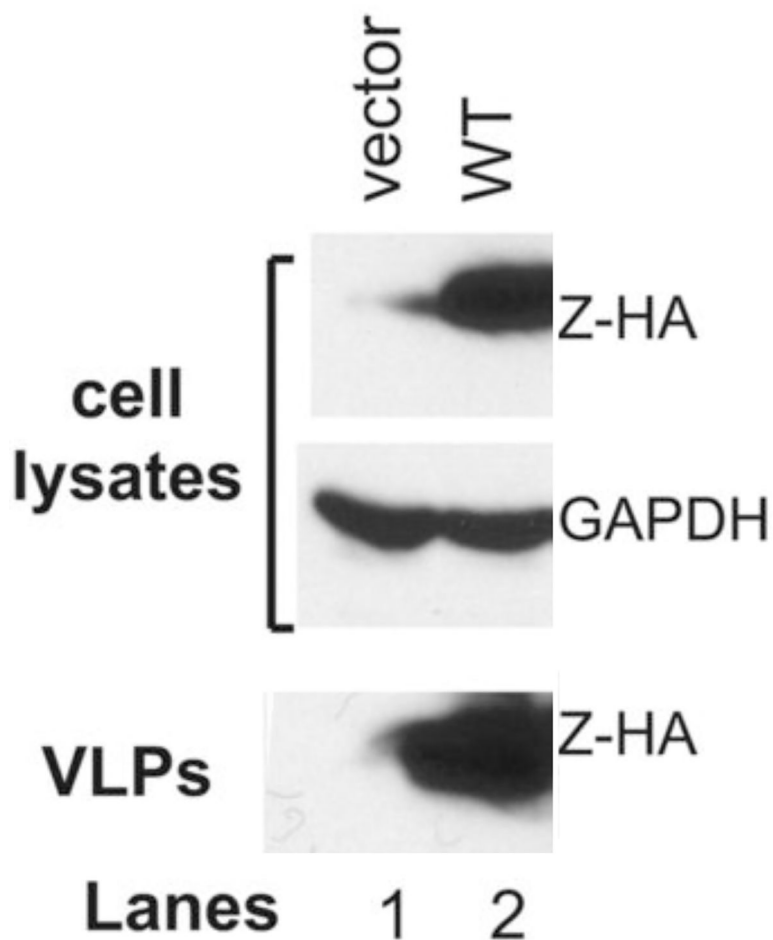
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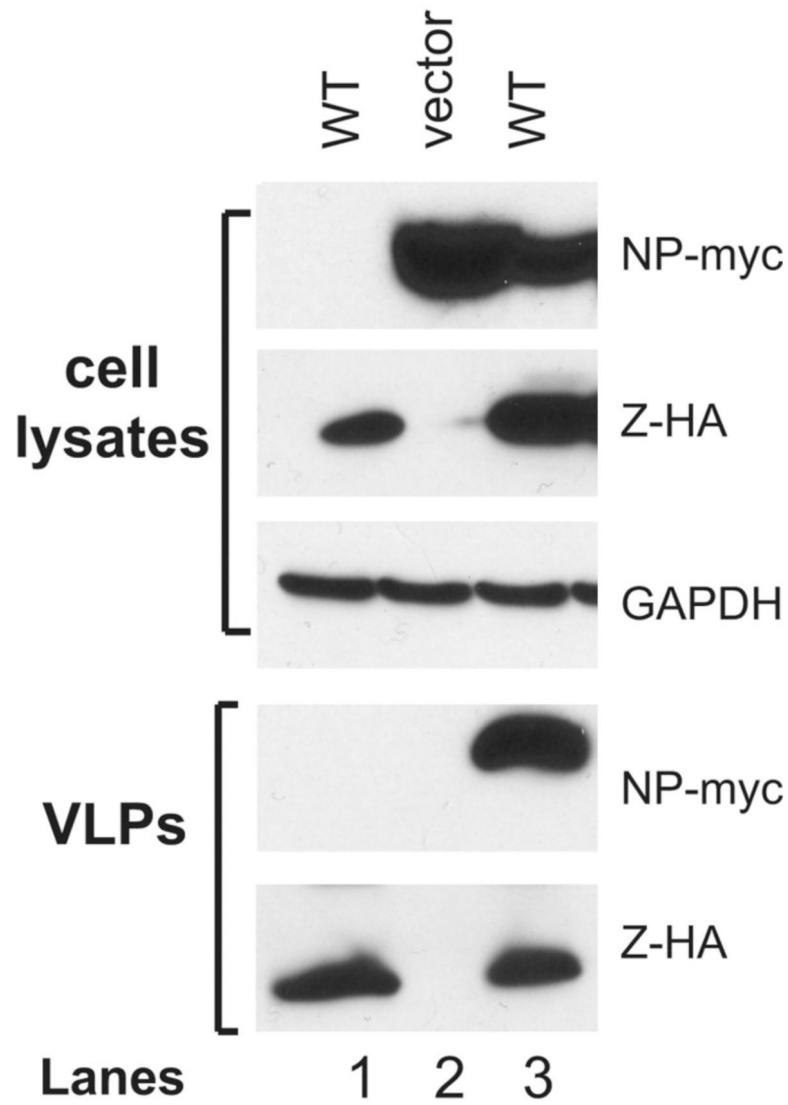
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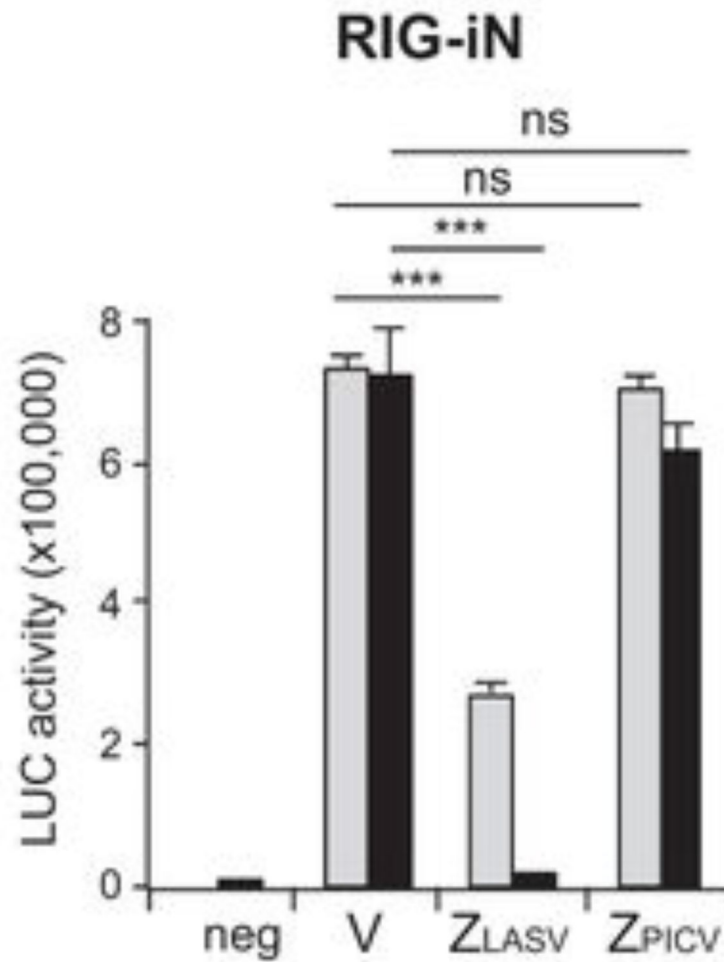


**Figure 1. Analysis of the self-budding activity of the wildtype PICV Z protein.** 293T cell were transfected with an empty vector (lane 1) or the WT PICV Z expression vector (lane 2). Expressions of HA-tagged Z proteins (Z-HA) along with GAPDH as loading control in the cell lysates were analyzed by Western blot. Virus supernatants (VLP) were collected, precipitated by PEG-8000, separated on 15% SDS-PAGE, and analyzed by Western blot.



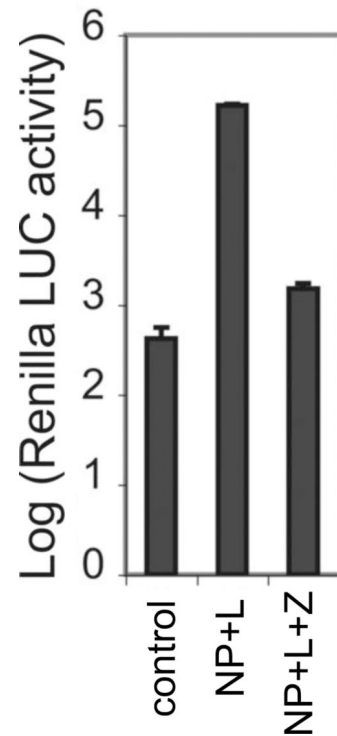
**Figure 2. NP incorporation into Z-mediated VLP formations.**

293T cells were transfected with WT PICV Z protein expression vector (lanes 1 and 3), empty vector (lane 2), and with the NP expression vector (lanes 2 and 3). The amounts of VLP released into the supernatants were analyzed by Western blot against Z and NP proteins on 15% SDS-PAGE along with GAPDH as loading control in the cell lysates.



**Figure 3. Z-mediated inhibition of viral RNA synthesis.**

PICV Z protein was examined for its ability to inhibit viral RNA transcription in the mini-genome assay. Control reaction contains the minigenomic RNA and the NP-expressing plasmid only, whereas other reactions also contain other protein-expressing plasmids (i.e., either L-expressing plasmid or L- and Z-expressing plasmids). Results shown are the average of at least three independent experiments with error bars representing standard deviations.



**Figure 4. Z-mediated suppression of the IFN $\beta$  promoter-driven luciferase reporter activity.** LASV Z, but not PICV Z, inhibits RIG-iN-induced IFN- $\beta$  activation in a luciferase (LUC)-based promoter assay. 293T cells were transfected with two different concentrations [100 ng (gray bars) and 1,000 ng black bars] of either an empty vector (V) or Z plasmids (LASV or PICV) together with IFN- $\beta$ -LUC,  $\beta$ -Gal, and RIG-iN plasmids. neg, negative control (no RIG-iN transfection).