



# EIF4A2 interacts with the membrane protein of transmissible gastroenteritis coronavirus and plays a role in virus replication

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## ABSTRACT

Transmissible gastroenteritis coronavirus (TGEV) is enteropathogenic coronavirus that causes diarrhea in pigs, and is associated with high morbidity and mortality in sucking piglets. The TGEV membrane (M) protein is a decisive protein for the proliferation of viral proteins, and is associated with virus assembly and budding. To identify the cellular proteins that interact with the TGEV M protein, yeast two-hybrid screening was employed, and seven cellular proteins were identified M-binding partners. Using the GST pull-down approach and a CO-IP assay, the M protein was found to interact with porcine intestinal cells via eukaryotic translation initiation factor 4-alpha (EIF4A2), an essential component of the cellular translational machinery. Additionally, confocal microscopy revealed that EIF4A2 and M were colocalized in the cytoplasm. Furthermore, the function of EIF4A2 in intestinal cells during TGEV infection was examined. A knockdown of EIF4A2 by siRNA markedly decreased M protein proliferation and TGEV replication in target cells. Thus demonstrating that EIF4A2 plays a significant role in TGEV replication. The present study provides mechanistic insight into the interaction between the TGEV M protein and intestinal cells which contributes to the understanding of coronavirus replication and may be useful for the development of novel therapeutic strategies for TGEV infection.

## 1. Introduction

Porcine transmissible gastroenteritis (TGE) is a highly contagious disease caused by transmissible gastroenteritis virus (TGEV), particularly in the colder seasons of winter and early spring (Piñeyro et al., 2018). The clinical symptoms of TGEV include acute diarrhea, vomiting, and dehydration. Although all aged swines can be infected with this virus, suckling piglets are the most susceptible and have a mortality rate as high as 100% (Mullan et al., 1994).

TGEV is a positive single stranded RNA virus with a full length of 28.5 kb genome, which have a typical cap structure at 5' end and a poly (A) tail at the 3' end, and encodes four structural proteins including spike [S], membrane [M], nucleocapsid [N], and envelope [E], as well as other five non structural proteins (Lai and Cavanagh, 1997; Eleouet et al., 1995). The M and E proteins might be thought to be involved in the formation of virus-like particles (VLPs) (Baudoux et al., 1998). In addition, the gene of M protein is highly conserved and encoded a decisive protein for viral proliferation (ZHENHUI Song et al., 2015). As an important structural protein of TGEV particles, the M protein is

located in the lipid envelop and associates with the Golgi complex in the cell, suggesting that the M protein plays a pivotal role in virus assembly and budding. Additional evidence indicates that the M protein is an indispensable part for the replication of virus particles in host cells, which may also be used for virus-specific diagnostics and treatment (Cologna and Hogue, 1998; De Haan et al., 1998; Zou et al., 2013). However, there have been few reports regarding the interaction between the TGEV M protein and intestinal cells. The identification of the cellular ligands which interact with viral proteins is likely to provide a better understanding of the dynamics of viral replication, virus-mediated cellular modulation, and pathogenic mechanism.

In the present study, using yeast two-hybrid screening, seven cellular proteins, including eukaryotic translation initiation factor 4-alpha (EIF4A2), were identified to be M-ligands. The eukaryotic translation initiation factor, EIF4A, is a number of RNA helicase, which is required for the binding of mRNA to 40S ribosomal subunits with function to unwind RNA secondary structures in the 5'-UTR of mRNA. There are three closely related EIF4A proteins in, EIF4A1, EIF4A2, and EIF4A3, which not only participate in initiation and translation but also

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associated with multiple life processes such as embryogenesis (Lu et al., 2014). Although both EIF4A1 and EIF4A2 display high protein homology and assemble into the EIF4F complex required for microRNA-mediated gene regulation, evidence suggests that EIF4A2, but not EIF4A1, plays a key role in this process of formation of the EIF4F complex (Meijer, 2013). EIF4A3 is localized in the nucleus, where it plays a role as an mRNA clamp and is related to nonsense-mediated mRNA decay (Chan et al., 2004).

Moreover, previous reports have shown that EIF4A2 interacts with VP1 of infectious bursal disease virus (IBDV) and inhibits the RNA polymerase activity of IBDV to reduce its replication in host cells (Tacke et al., 2004; Gao et al., 2017). Therefore, we aimed to elucidate whether EIF4A2 interacts with the M protein of TGEV and impacts virus replication. In this study, we demonstrate that EIF4A2 in intestinal cells associates with the TGEV M protein and further examined the specific role of EIF4A2 on TGEV replication by knocking down EIF4A2 expression using small interfering RNA (siRNA).

## 2. Materials and methods

### 2.1. Cells and viruses

Porcine intestinal epithelial cells (pIECs) were developed at our institute. The cells were isolated from the jejunum of a newborn piglet, grown in Dulbecco's modified eagle's medium (DMEM)/F-12 medium (GIBCO) with 10% fetal bovine serum (FBS, GIBCO BRL), and maintained in maintenance medium (DMEM/F-12 supplemented with 2% FBS) in a 5% CO<sub>2</sub> incubator. The TGEV Chongqing (CQ) strain was isolated from sick piglets with symptoms of diarrhea by our lab (Zhenhui et al., 2015).

### 2.2. Antibodies, strains, and plasmids

C-Myc antibodies, GST-tag antibodies, and His-tag antibodies were purchased from Sangon Biotech, China. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from Proteintech (USA). FITC-conjugated Goat anti-rat IgG (H + L) was purchased from BBI Life Sciences (USA), and Cy3-conjugated goat anti-rabbit IgG (H + L) secondary antibody was purchased from BOSTER, China. The beta tubulin rabbit polyclonal antibody ( $\beta$ -tubulin) and EIF4A2 rabbit polyclonal antibody were purchased from Proteintech (USA). The mAb to the TGEV M protein was kindly donated by Yu Bai from Wenzhou college of sciences and agriculture. Y2HGold and Y187 were purchased from Clontech, Japan. The *Escherichia coli* Rosetta strain was provided by Prof. Ji Xiang Li. The plasmids applied in the yeast two-hybrid, pFastBac™-M was stored in the laboratory at  $-80^{\circ}\text{C}$ , and both pGBKT7 and pGBKT7-53 were purchased from Clontech, Japan. pMD19-T simple was purchased from TaKaRa, Japan.

### 2.3. Construction and identification of a bait vector with the TGEV M protein

The TGEV M protein was amplified from pFastBac™-M by PCR that added the EcoR I and Sal I restriction enzyme sites, and was then cloned into a pMD19-T simple vector. After verification by PCR, it underwent directional cloning into the bait vector, pGBKT7, of the yeast two-hybrid system and was identified by PCR. Y2HGold yeast were transformed with either pGBKT7-M or pGBKT7 (control). The expression product of Y2HGold (pGBKT7-M) was tested by Western blot using the c-Myc tag antibody (1:2000) as the primary antibody and HRP-conjugated goat anti-rabbit IgG (1:5000) as the secondary antibody. The yeast cultures were diluted 1/10 and 1/100, plated onto SD/–Trp plates, and incubated at  $30^{\circ}\text{C}$  for three to five days. The size and number of yeast colonies treated with pGBKT7-M were compared to that of the control to test the bait for toxicity. The yeast cultures were diluted at 1/10 and 1/100 and 100  $\mu\text{L}$  and spread onto SD/–Trp, SD/–

**Table 1**  
Primers for QRT-PCR.

Gene name	Primer sequence
EIF4A2	F: 5'–GAGTTCAAGGAGACCCCAAGC-3' R: 5'–AGCTTCAGCCTGCAACTTCT-3'
TGEV M	F: 5'–TGCTACTCTAACTTTGCTTTCAGG-3' R: 5'–CGCACTACTTGTCTCAACTTC-3'
$\beta$ -action	F: 5'–CTCTTCCAGCCCTCCTCC-3' R: 5'–GGTCTTGGCGATGTCC-3'

Trp/X- $\alpha$ -Gal, and SD/–Trp/X- $\alpha$ -Gal/AbA (Aureobasidin A) to test whether the bait induced autoactivation.

### 2.4. Yeast two hybrid screening

A yeast two-hybrid assay was performed according to the instructions of the Matchmaker® Gold two-hybrid system (catalog no.630489; Clontech). Briefly, the bait strain Y2HGold (pGBKT7-M) and Y187 (pIECs cDNA) were mixed and incubated at  $30^{\circ}\text{C}$  for 24 h until a three-lobed structure similar to a “Mickey Mouse” face was visible at 40 $\times$  magnification under a microscope.

The culture mixture was diluted at 1/10, 1/100, 1/1000, and 1/10000, and 100  $\mu\text{L}$  was spread onto SD/–Trp/–Leu/–Ade. All monoclonies that grew were plated out onto higher stringency.

SD/–Trp/–Leu/–Ade/–His. Finally, all of the white colonies from the second screening that grew on SD/–Trp/–Leu/–Ade/–His/X- $\alpha$ -Gal/AbA. The positive colonies were selected and inoculated into SD/–Trp/–Leu/–Ade/–His liquid medium. The positive prey plasmids were rescued and their cDNA inserts were sequenced to identify the candidate proteins.

### 2.5. GST pull-down assay

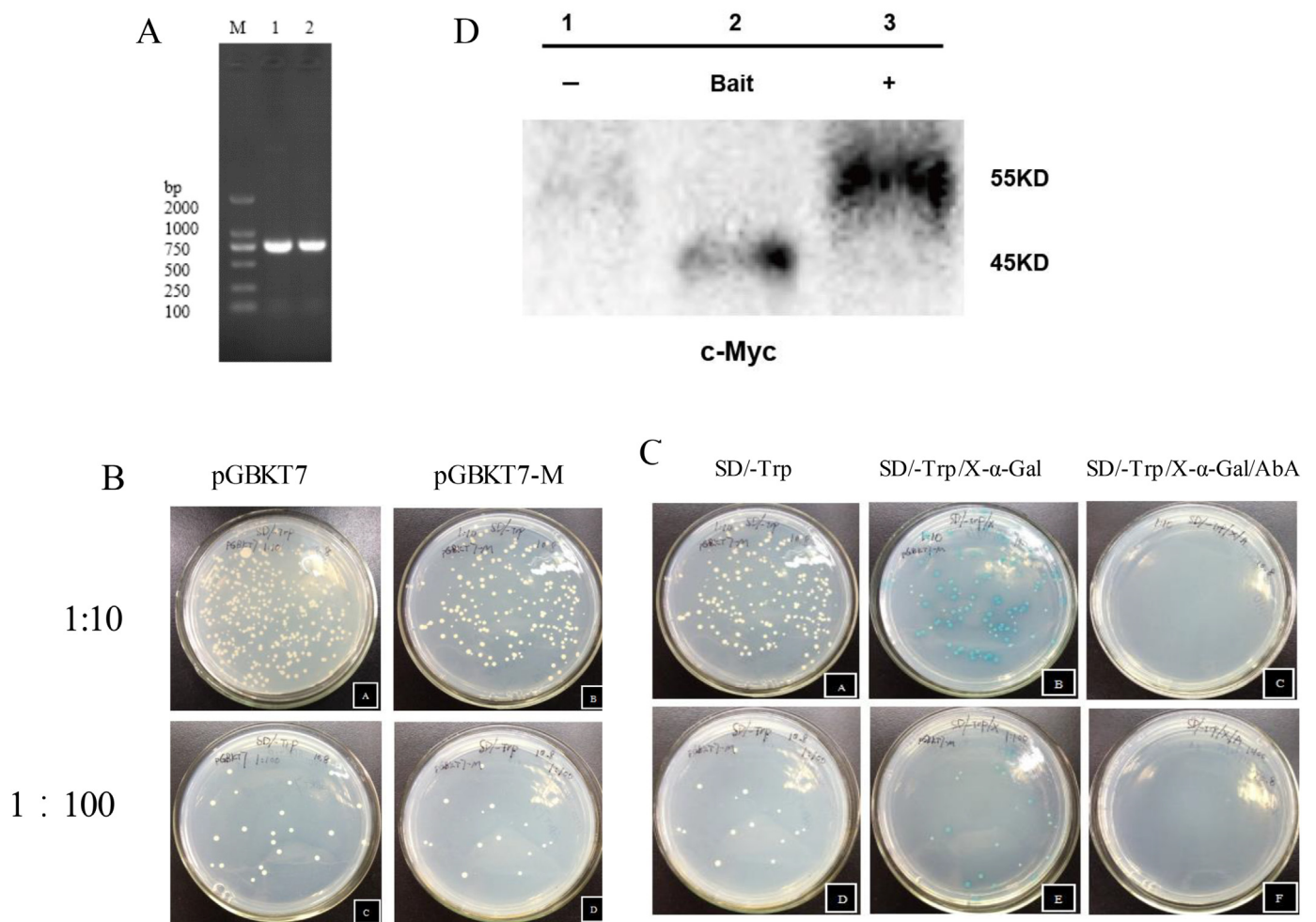
EIF4A2 appeared at a high frequency among the candidate proteins, which could be connected with virus proliferation and synthesis of viral proteins (JK Ndzinu et al., 2018; Gao et al., 2017). Therefore, the candidate protein, EIF4A2, was selected as a protein of interest for further verification of its interaction with the TGEV M protein. The GST-M fusion protein was expressed in *E. coli* Rosetta under induction by 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG), then immobilized on glutathione-sepharose 4B serving as a bait protein at  $4^{\circ}\text{C}$  for 6 h, followed by the addition of purified His-EIF4A2 protein at  $4^{\circ}\text{C}$  for 8 h. The isolated pull-down proteins were then analyzed by 12% SDS-PAGE analysis and Western blot. GST protein expression was used as a control.

### 2.6. Coimmunoprecipitation Assay

The Co-IP assay was carried out using IP kit KIP-1(Protientech, China). pIECs infected with TGEV (MOI = 0.1) and control cells were harvested at 24 h and washed three times with cold-PBS. IP lysis buffer containing 1 mM protease inhibitor was added for 30 min on ice. After centrifugation at 12,000  $\times g$  for 15 min, the lysate supernatant which containing 1–2 mg of total protein was incubated with rabbit pAb to EIF4A2 (2  $\mu\text{g}$ ) overnight at  $4^{\circ}\text{C}$ . Then, 50  $\mu\text{L}$  resuspended Protein A/G-Agarose was added to this mixture and subjected to rotated incubation at  $4^{\circ}\text{C}$  for 4 h. After washing five times with washing buffer containing 1 mM protease inhibitor and centrifugation at 18  $\times g$  for 30 s, 40  $\mu\text{L}$  elution buffer was added to the elute immune complex. The isolated bound proteins were analyzed by Western blot. The TGEV mock-infected pIECs lysate was used as a control.

### 2.7. Transfection with siRNA against EIF4A2

pIECs were cultured in six-well plates, and the cell monolayer was



**Fig. 1.** Testing bait protein expression, autoactivation, and toxicity. (A) Yeast colony identification by PCR. M: DNA Marker DL2000; 1–2: PCR of colonies transformed with pGBKT7-M; (B) Identification of toxic of pGBKT7-M bait plasmid. Yeast harboring pGBKT7-M and control pGBKT7 were spread at two dilutions (1:10 and 1:100) on to SD/Trp plates, and the size and number colonies were compared to evaluate the toxicity of the bait plasmid; (C) Identification of autoactivation of the pGBKT7-M bait plasmid. pGBKT7-M were spread at 1/10 and 1/100 dilutions on SD/–Trp, SD/–Trp/X-α-Gal, and SD/–Trp/X-α-Gal/AbA for autoactivation testing; (D) Expression of M in yeast by Western blot. 1: pGBKT7 protein negative control; 2: pGBKT7-M protein; and 3: pGBKT7-53 protein positive control.

grown to a confluence of 70%–80%. Transfection with the siRNAs (three siRNAs targeting EIF4A2 produced by RIBO BIO, China) was performed with lipofecatmine<sup>®</sup>3000 in accordance with the manufacturer's instructions. The three siRNAs (20 nM) were complexed with lipofecatmine<sup>®</sup> 3000 by incubating them together at room temperature for 5 min. After removing the cell culture supernatant, the complex was added and incubated for 24 h prior to analysis.

**2.8. Virus titration**

PIECs were transfected with the best siRNA targeting (siRNA2, 5'-GGAGACTATATGGGAGCAA-3') against EIF4A2 for 24 h, then infected with TGEV (MOI = 0.1). The virus suspensions were collected at 0 h, 12 h, 24 h, and 48 h, serially diluted from 10<sup>-1</sup> to 10<sup>-7</sup>, and added to ST cells in 96-well culture plates. Each dilution was added to eight wells. The TCID<sub>50</sub> was calculated using the Reed and Muench method. The mock-treated cells and cells treated with TGEV at each time point served as controls.

**2.9. Immunofluorescence assay**

PIECs were infected with TGEV for 48 h. The cells were washed three times with PBS, and fixed in paraformaldehyde (4%) for 15 min at room temperature and permeabilized with 0.3% Triton X-100 for 10 min.

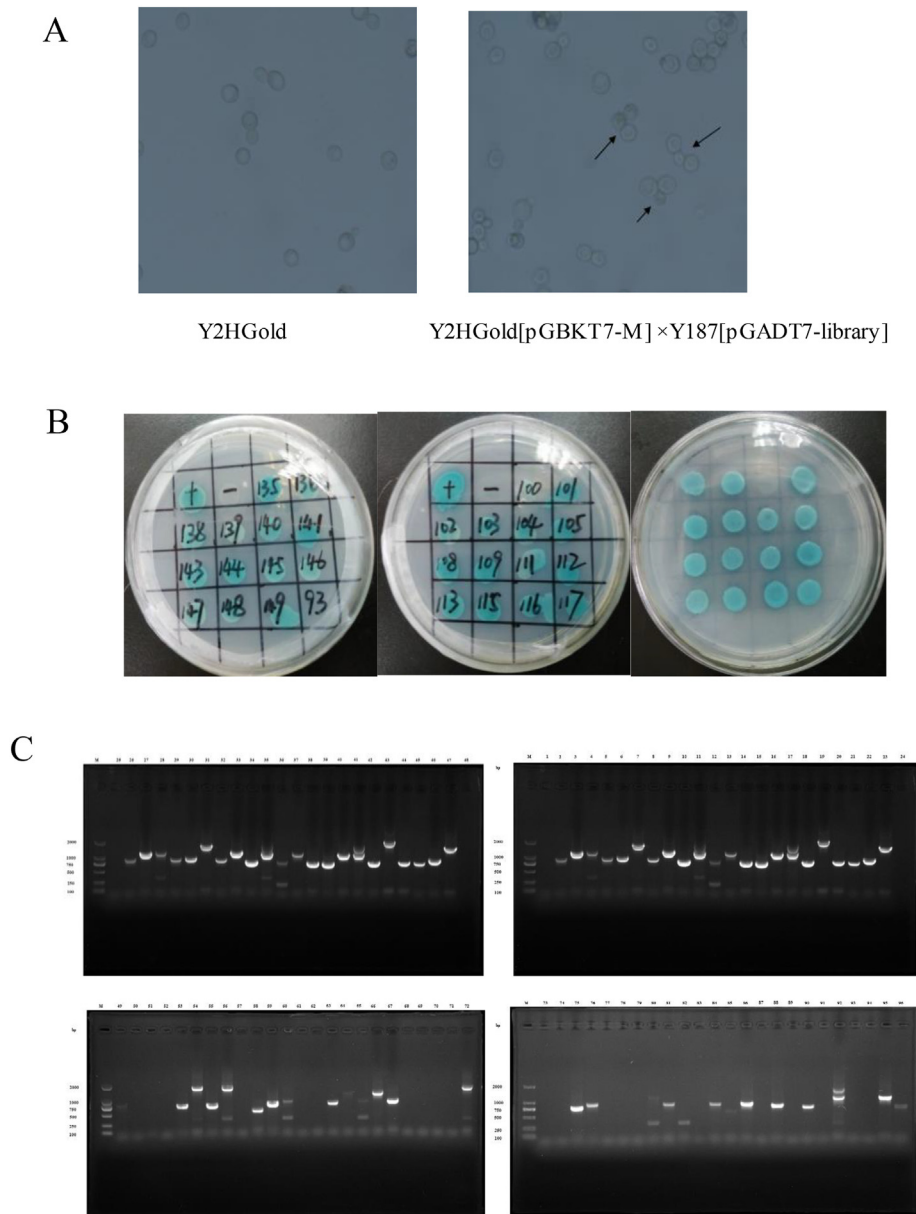
After blotting with 5% BSA (bovine serum albumin, BBI), the cells were incubated with TGEV M protein (1:100) mAb and rabbit pAb to EIF4A2 (1:50) overnight at 4 °C. The cells were then incubated with FITC-labeled goat anti-rat IgG (1:100) and Cy3-labeled goat anti-rabbit IgG (1:100). Subsequently, the cells were stained with 4,6-diamidino-2-phenylindole (DAPI) for 5 min and examined under a ZEISS LSM 800 with Airyscan.

**2.10. RNA extraction and quantitative real-time PCR (qRT-PCR)**

PIECs were transfected with siRNA2 against EIF4A2 for 24 h and then inoculated with TGEV for 48 h. Total RNA was extracted using the RNAiso plus (Invitrogen, USA) reagent and subjected to reverse transcription with PrimerScript<sup>™</sup> RT Master Mix (TaKaRa, Japan) according to the manufacturer's instructions. Quantitative PCR analysis was performed to amplify the membrane protein (M) gene and EIF4A2 using the cDNA as the template and the β-actin gene as the internal standard. Data analysis was based on the measurement of the cycle threshold (Ct). The relative level of M gene and EIF4A2 expression was calculated using the 2<sup>-ΔΔCt</sup> method. The primers are presented in Table 1.

**2.11. Western blotting**

PIECs were transfected with siRNA2 and infected with TGEV for



**Fig. 2.** A yeast two-hybrid system was used to screen for M-interacting proteins. (A) Diploid after yeast two-hybrid screening. The zygotes typically displayed a three-lobed structure similar to a “Mickey Mouse” face at 40 × magnification under a microscope; (B) The third screening using SD/–Trp/–Leu/–Ade/–His/X-α-Gal/AbA selective medium. A total of 108 blue clones were visible and identified by PCR (D) M: DL2000 DNA Marker; 1–96: PCR amplification products of the positive clones. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 2**  
TGEV M protein-associated cellular proteins identified by Yeast two-hybrid system.

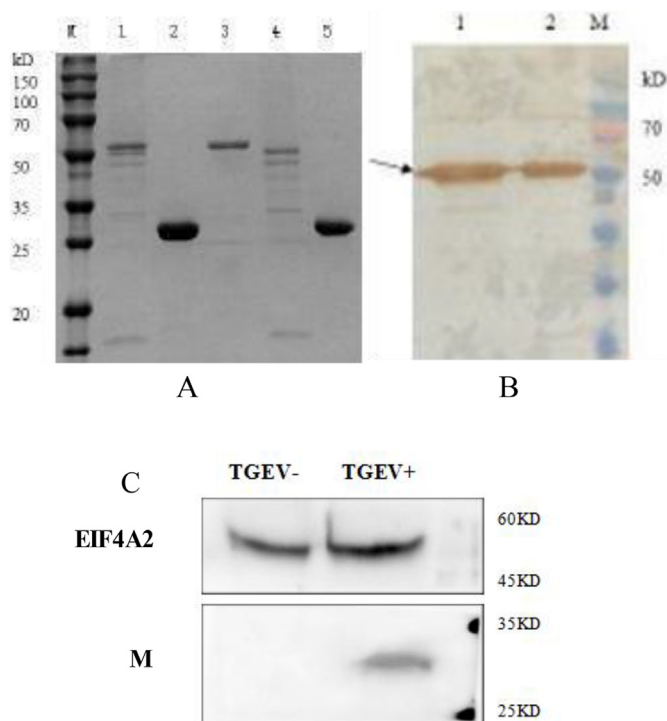
Clone	Protein name	NCBI number
14	multifunctional protein ADE2	XP_003129072
36	regulator of G protein signaling small	NP_001172113.1
57	nuclear ribonucleoprotein SmD3	NP_001230355.1
82	P2Xpurinergic receptor 7	XP_001926839.2
88	RNA polymerase II transcription subunit	XP_005653991.1
90	eukaryotic initiation factors 4A2	NP_001093665.1
94	Centromere-related proteins E	XP_013844806.1

48 h. pIECs were washed three times with cold-PBS and lysed in radioimmunoprecipitation assay (RIPA, 200 μL/well) buffer (Beyotime, China) containing the protease inhibitor, PMSF (0.2 μL). The protein concentrations of the resulting lysates was determined using a Pierce

BCA (Beyotime, China). After centrifugation at 11,000 × g for 12 min, proteins in the supernatant (40 μg protein) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gradient gels, and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 5% non-fat dry milk at room temperature, and incubated with the primary antibodies (1:600) at 4 °C overnight. After washing three times with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (Sangon Biotech) at 37 °C for 1 h and the proteins were visualized using 3,3'-diaminobenzidine and detected by enhanced chemiluminescence (ECL; Thermo Scientific) and autoradiography.

2.12. Statistical analysis

All calculations were performed using GraphPad Prism 7.0. All data



**Fig. 3.** GST pull-down and CO-IP were used to analyze the interaction between the M protein and candidate protein EIF4A2 by SDS-PAGE and Western blot. (A) The results of the GST pull-down by SDS-PAGE M: Prestained protein marker; 1: purified GST-M fusion protein + purified His-EIF4A2 fusion protein; 2: purified GST-tag protein + purified His-EIF4A2 fusion protein; 3: purified His-EIF4A2 fusion protein; 4: purified GST-M fusion protein; 5: purified GST-tag protein; (B) The results of the GST pull-down by Western blot M: Prestained protein marker; 1–2: His-EIF4A2 fusion protein. (C) pIECs infected with TGEV and control cells were harvested at 24 h, and the M protein was precipitated by an antibody in TGEV-infected group but not the mock group. TGEV + represent the pIECs infected with TGEV and TGEV- represent uninfected TGEV in pIECs.

are presented as the mean  $\pm$  SD or with the standard errors of the mean from three independent experiments. A one-way analysis of variance (ANOVA) and *t*-test were employed to determine the statistical differences between multiple groups. *P*-values < .05 were considered statistically significant (\**P*-value < .05; \*\**P*-value < .01; \*\*\**P*-value < .001).

### 3. Results

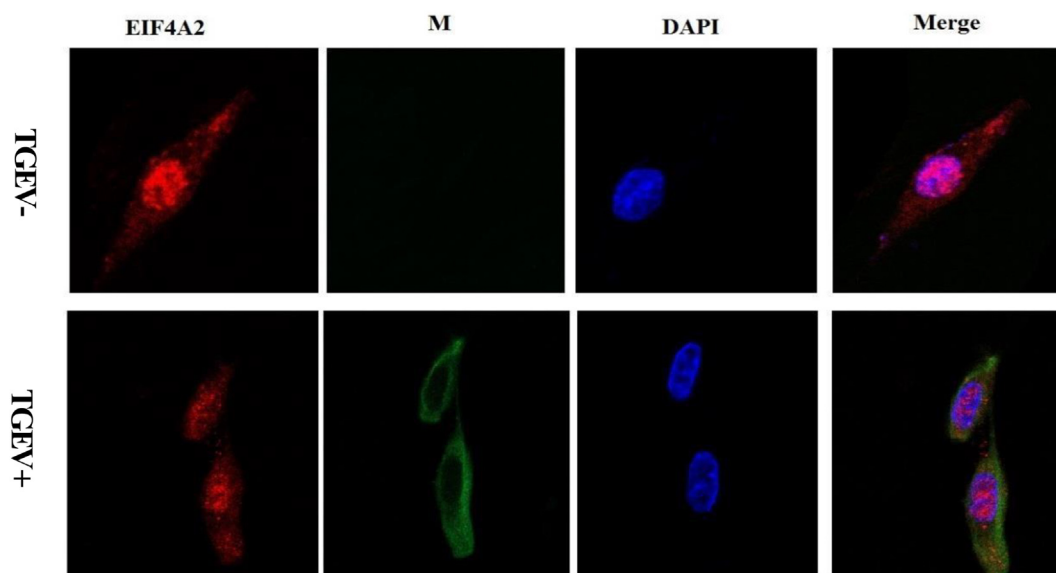
#### 3.1. Bait protein expression, autoactivation, and toxicity

The bait vector, pGBKT7, of the yeast two-hybrid system was constructed to screen the interactions between the TGEV M protein and host proteins, and the recombinant plasmid was successfully transformed into Y2HGold yeast cells (Fig. 1A). Yeast harboring pGBKT7-M and those harboring pGBKT7 (control) were spread at two dilutions onto SD/Trp plates, and the yeast size and number of colonies produced by pGBKT7-M were compared with that produced by the control pGBKT7. The results suggest that the bait vector displayed no toxicity toward the yeast (Fig. 1B). The yeast harboring pGBKT7-M were spread at two dilutions on three different selective media plates. No colony growth was observed on the SD/–Trp/X- $\alpha$ -Gal/AbA, indicating that there was no self-activation phenomenon to the reporter genes (Fig. 1C). A Western blot showed that pGBKT7-M produced an approximately 50 kDa fusion protein (Fig. 1D). These results indicate that the constructed pGBKT7-M bait vector could be used for screening host proteins that interact with the TGEV M protein using the yeast two-hybrid system.

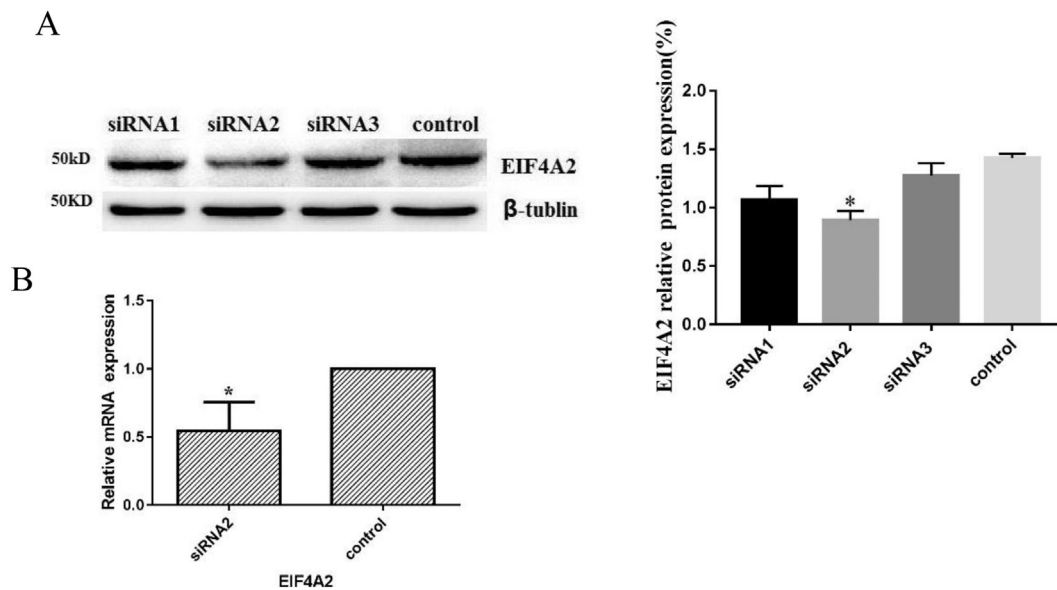
#### 3.2. Yeast two-hybrid system screening for M-interacting proteins

TGEV M protein-associated cellular proteins were identified using the yeast two-hybrid system. The zygotes typically displayed a three-lobed structure similar to a “Mickey Mouse” face at.

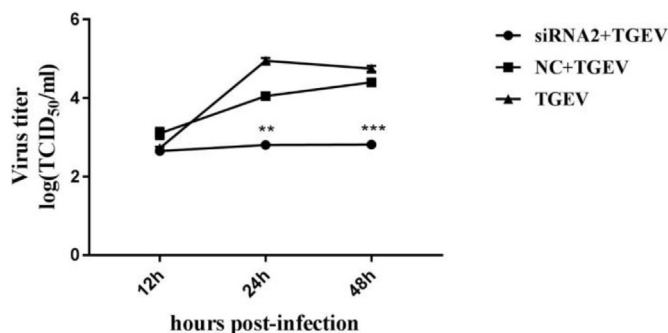
40 $\times$  magnification under the microscope (Fig. 2A) after the bait strain Y2Hgold (pGBKT7-M) and Y187 (pIECs cDNA) were mixed and incubated. After three rounds of screening on different nutritionally-deficient medium plates, a total of 108 blue clones that grew on the SD/–Trp/–Leu/–Ade/–His/X- $\alpha$ -Gal/AbA were visible and identified by PCR (Fig. 2B and C). Finally, a database search with sequence analysis



**Fig. 4.** Co-localization of EIF4A2 with the M protein. Determining the location of the M protein using FITC-conjugated goat anti-rat IgG (green) as the secondary antibody after pIECs were infected with TGEV. The red colour indicates the distribution domain of EIF4A2. The nucleus was stained with DAPI (blue). A ZEISS LSM 800 with Airyscan was used to examine the triple-stained location. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 5.** EIF4A2 gene silencing. pIECs were cultured in six-well culture plates and transfected with siRNAs (20 nM). The cell lysates were harvested for Western blotting at 48 h. The average densitometric intensity of EIF4A2 in the immunoblot analysis, with  $\beta$ -tubulin used as a control (A and B). The relative level of EIF4A2 mRNA expression was used to further evaluate the interference effect of siRNA2 by RT-qPCR at 36 h (C). \* $P < 0.05$ .



**Fig. 6.** Virus titration. EIF4A2-knockdown with siRNA2 cells and negative control knockdown cells were adsorbed with TGEV (MOI = 0.1) at 37 °C for 1 h. The culture supernatants of the cells and the virus-associated cells infected with TGEV were collected at different points. The viral titers are presented as the averages from three independent samples. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

resulted in the positive identification of seven different cellular proteins (Table 2). These proteins included the multifunctional protein, ADE2, regulator of G protein signaling, small nuclear ribonucleoprotein SmD3, P2X purinergic receptor 7, RNA polymerase II transcription subunit 1, eukaryotic initiation factor 4A2 (EIF4A2), and centromere-related protein E. Since it has been previously reported that the host translation initiation factor, EIF4A2, is involved in the promotion of virus proliferation and viral protein synthesis (Ndzinu et al., 2018; Li et al., 2016). EIF4A2 was used as the candidate protein for further research and validation.

### 3.3. EIF4A2 interacts with the TGEV M protein

Next, the interaction between the M protein and candidate protein EIF4A2 was verified using a GST pull-down assay and Co-IP. The His-EIF4A2 fusion protein solution was added to Glutathione<sup>™</sup>4B agarose beads adsorbed with the GST-M fusion protein at the same concentration. GST protein was used as a control to eliminate non-specific protein binding. As shown in Fig. 3A, SDS-PAGE electrophoresis and the Western blot results revealed that the EIF4A2 protein was present in the GST-M protein immobilized beads but not in the other gels and the Western blot indicated that the size of the His-EIF4A2 fusion protein

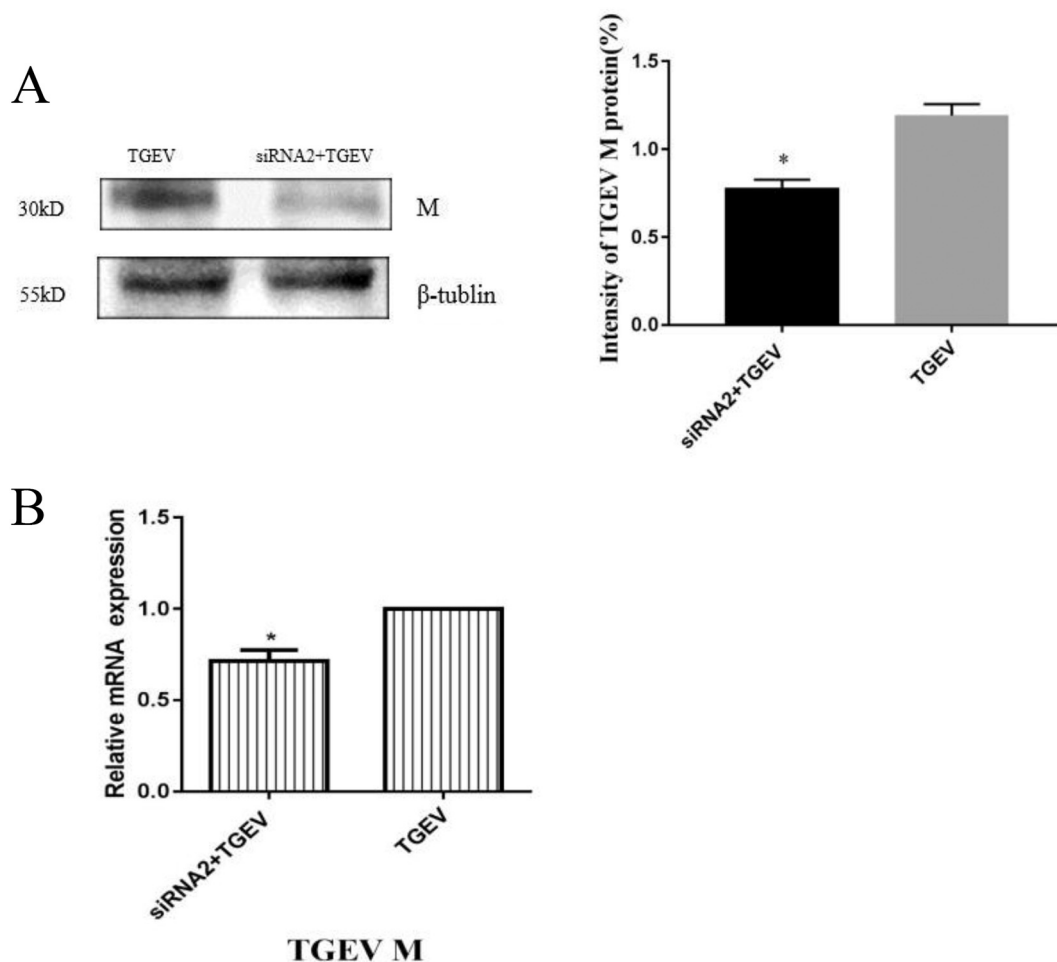
was approximately 50 kDa (Fig. 3B.). A Co-IP assay was performed to further verify the interaction between EIF4A2 and TGEV M in vitro. The Western blot presented in Fig. 3C shows that the M protein was precipitated by the antibody in the TGEV-infected group but not in the mock-treated group. These results confirm the interaction between EIF4A2 and the M protein both in vitro and in vivo.

### 3.4. Co-localization of EIF4A2 with the M protein by immunofluorescence

Indirect immunofluorescence was used to validate where EIF4A2 and M were localized in pIECs following TGEV infection. The results indicated that EIF4A2 was distributed in both the cytoplasm and nucleus. The green fluorescence of M protein and the red fluorescence of EIF4A2 were observed to be partially overlapping in the cytoplasm, indicating that EIF4A2 was co-localized with the M protein within pIECs during TGEV infection (Fig. 4).

### 3.5. Knockdown of EIF4A2 reduces TGEV replication in host cells

To further study the role of EIF4A2 in TGEV replication, the EIF4A2 protein of pIECs was inhibited using siRNA2. At 24 h post transfection, the cells were lysed with Trizol and the level of EIF4A2 mRNA expression was detected using RT-qPCR. At 48 h post transfection, the cells were lysed for EIF4A2 protein expression by Western blot. As shown in Fig. 5A, siRNA2 knocked down protein expression more efficiently than the other interference fragment, and quantitative analysis further confirmed a significant inhibitory effect of siRNA2 against EIF4A2 (Fig. 5B). To investigate the role of EIF4A2 on TGEV replication, EIF4A2 was knocked down in pIECs with siRNA2, which were subsequently infected with TGEV at an MOI of 0.1. The viral loads in the supernatants treated with siRNA2 were significantly lower compared to those of the negative siRNA- and mock-treated groups ( $P < 0.01$  and  $P < 0.001$ , respectively) (Fig. 6). Similarly, compared with the TGEV group, the level of M protein and mRNA expression decreased following the knockdown of EIF4A2 in pIECs and infected with TGEV for 48 h (Fig. 7A and B). Together, these results suggest that EIF4A2 interacts with the TGEV M protein and impairs TGEV replication.



**Fig. 7.** Western blotting and RT-qPCR analysis following siRNA2 interference and viral infection. At 24 h after interference, the cells were incubated with TGEV Miller. After 48 h, the cells were harvested and the expression of the TGEV M protein was detected by Western blot (A and B). At 36 h, real-time RT-PCR was performed to detect the level of TGEV M mRNA expression (C). \* $P < 0.05$ .

#### 4. Discussion

TGEV is a highly contagious disease in the *Coronavirus* that can cause acute diarrhea in piglets at 1–2 weeks of age, and causing severe economic losses (Duque and Ospina, 2017). Thus, identification of host proteins that interact with TGEV viral proteins is a vital for studying the mechanisms of viral pathogenesis. In this study, using a yeast two-hybrid system, we identified seven cellular proteins that can interact with the TGEV M protein. These proteins are primarily related to gene transcription, protein folding, protein degradation and metabolism. Of these, EIF4A2 was identified as a novel M ligand. However, EIF4A2 had not been identified in intestinal cells in previous study, likely due to the different cell types used to construct the cDNA library. In vivo, TGEV replicates in the intestinal epithelial cells of susceptible animals, inducing cell injury, villi atrophy, as well as reduced villous height and crypt depth (Haelterman, 1972; Schwegmann and Herrler, 2006; Xia et al., 2018). Therefore, it is important to identify host proteins in the natural target cells of TGEV to study viral replication and pathogenesis.

Previous studies have found that viral nucleic acids and structural proteins are assembled into viral particles in the region between the endoplasmic reticulum (ER) and Golgi, and that the particles are subsequently transported into the extracellular by budding of vesicles (Corse and Machamer, 2000; Kuo and Masters, 2010). Our previous research demonstrated that infectious virions entered the intestinal cells by membrane fusion and mature viruses budded into vacuoles, which were gradually to the cell membrane before being released (Song

et al., 2016). Moreover, research into TGEV has established that the M protein as a linker may contribute to initiate the viral particle assembly by interacting with genomic RNA and nucleoproteins in pre-Golgi compartments (Risco et al., 1998). Therefore, this study provides evidence that host cellular proteins interacting with the TGEV M protein will be serve to futher study of viral replication and pathogenesis. In the present study, EIF4A2 was identified to interact with the TGEV M protein using a GST pull-down assay and Co-IP. EIF4A2 is one of variants (EIF4A1 and EIF4A2) reported in mice and rabbits (Nielsen and Trachsel, 1988) and associates with EIF4A1 to form a complex (Marintchev et al., 2009). A previous study showed that EIF4A2 acts to limit IBDV replication in infected DF1 cells, which is consistent with our findings that knocking down EIF4A2 expression can inhibit the replication of TGEV in intestinal cells. Furthermore, we identified the mechanism by which EIF4A2 reduced TGEV replication in intestinal cells to be via the down-regulation of M expression.

Some studies have found that EIF4A2 plays a significant role in the virus infection cycle. For example, EIF4A2 reduces IBDV replication by inhibiting RNA polymerase activity in host cells (Li G et al., 2016). Similar findings have also been reported that human EIF4A2 was found to interact with the RdRp NS5B of hepatitis C virus by using a yeast two-hybrid system, which may facilitates the genome RNA synthesis of NS5B protein (Kyono et al., 2002). In the present study, our results suggest that EIF4A2 may be required for TGEV replication. Therefore, EIF4A2 may potentially serve as new therapeutic target for controlling TGEV infection.

Elucidating the interactions between host cell and viral components is essential for understanding coronavirus pathogenesis. In this study, the TGEV M protein was shown to interact with the eukaryotic translation initiation factor, EIF4A2. Moreover, the knockdown of EIF4A2 expression with small interfering RNA (siRNA) led to the inhibition of M protein synthesis and decreased viral copy numbers. Since such inhibition occurs during the later stages of the virus replication cycle, inhibition of M protein expression, which plays a key role in the morphological processes of coronaviruses, likely affects the assembly of viral particles, thereby leading to decreased TGEV titers released into the supernatant of pIEC. Therefore, EIF4A2 can play a dual role by either forming the EIF4F complex to activate translation or binding to the CCR4-NOT complex to facilitate miRNA-mediated transcription repression (Mathys et al., 2014). Furthermore, one study showed that cellular miRNAs can inhibit viral infection and that the virus mutates to escape suppression by cellular miRNAs (Zheng et al., 2013). Therefore, since the underlying mechanism by which EIF4A2/TGEV affects TGEV/EIF4A2 replication is extremely intricate and may involve cellular miRNAs, further research is required to confirm this mechanism.

## 5. Conclusions

In summary, this study is the first to identify proteins involved in the porcine intestinal epithelial interaction with the TGEV M protein using a yeast two-hybrid system. A total of seven cellular proteins were identified, which provided novel insight into the mechanism of coronavirus replication. The interaction between the cellular EIF4A2 and the TGEV M protein was confirmed in TGEV-infected pIECs. Moreover, the knockdown of EIF4A2 reduced TGEV replication in pIECs. Therefore, our results may be useful in understanding the mechanisms involved in TGEV replication and developing novel strategies for infection control.

## Competing interests

The authors declare that there are no conflicts of interest.

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