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## Assessing activity of Hepatitis A virus 3C protease using a cyclized luciferase-based biosensor



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### ABSTRACT

Hepatitis A is an acute infection caused by Hepatitis A virus (HAV), which is widely distributed throughout the world. The HAV 3C cysteine protease (3C<sup>Pro</sup>), an important nonstructural protein, is responsible for most cleavage within the viral polyprotein and is critical for the processes of viral replication. Our group has previously demonstrated that HAV 3C<sup>Pro</sup> cleaves human NF-κB essential modulator (NEMO), a kinase required in interferon signaling. Based on this finding, we generated four luciferase-based biosensors containing the NEMO sequence (PVLKAQ↓ADIYKA) that is cleaved by HAV 3C<sup>Pro</sup> and/or the *Nostoc punctiforme* DnaE intein, to monitor the activity of HAV 3C<sup>Pro</sup> in human embryonic kidney cells (HEK-293T). Western blotting showed that HAV 3C<sup>Pro</sup> recognized and cleaved the NEMO cleavage sequence incorporated in the four biosensors, whereas only one cyclized luciferase-based biosensor (233-DnaE-HAV, 233DH) showed a measurable and reliable increase in firefly luciferase activity, with very low background, in the presence of HAV 3C<sup>Pro</sup>. With this biosensor (233DH), we monitored HAV 3C<sup>Pro</sup> activity in HEK-293T cells, and tested it against a catalytically deficient mutant HAV 3C<sup>Pro</sup> and other virus-encoded proteases. The results showed that the activity of this luciferase biosensor is specifically dependent on HAV 3C<sup>Pro</sup>. Collectively, our data demonstrate that the luciferase biosensor developed here might provide a rapid, sensitive, and efficient evaluation of HAV 3C<sup>Pro</sup> activity, and should extend our better understanding of the biological relevance of HAV 3C<sup>Pro</sup>.

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### 1. Introduction

Hepatitis A virus (HAV) is the commonest cause of clinically apparent viral hepatitis. Almost 1.4 million new cases of HAV infection are estimated to occur globally each year, with 11%–22% requiring hospitalization [1–3]. HAV is a small nonenveloped RNA virus with a single-stranded RNA genome of approximately 7500 nucleotides, and is a member of the family Picornaviridae. The single large open reading frame in the HAV genome is divided into three functional regions, designated P1, P2, and P3. The P1 region encodes the capsid polypeptides (VP1, VP2, and VP3), and the P2 and P3 regions encode the nonstructural polypeptides, which are

predicted to be processed into mature viral proteins by viral proteases [1,4–7]. The HAV 3C cysteine protease (3C<sup>Pro</sup>) is responsible for most cleavage within the viral polyprotein, supporting viral replication and proliferation [1,7,8].

Its pivotal role in viral replication and proliferation makes HAV 3C<sup>Pro</sup> one of the major targets in the design of anti-HAV drugs [6]. The traditional screening methods used to detect HAV 3C<sup>Pro</sup> activity are so slow and inefficient that they do not meet the requirements for high-throughput screening. Therefore, a simple, efficient, high-throughput method for the detection of HAV 3C<sup>Pro</sup> activity at the cell level, which fully reflects the characteristic biological activity of the protease, is urgently required. Compared with fluorescence-based assays, bioluminescence-based assays are markedly more sensitivity, with a wider dynamic range in detecting the activities of proteases [9–11]. Firefly luciferase is a 61-kDa monomeric enzyme that catalyzes the oxidation of firefly luciferin, which then emits a yellow-green light in the presence of ATP and oxygen [12,13]. As a

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reporter protein, firefly luciferase is widely used to detect apoptosis and enzyme activity, and to screen for antiapoptotic drugs and identify enzyme recognition sequences [14–16]. Previous research has shown that DNA polymerase III intein (DnaE) from *Nostoc punctiforme* (Npu) catalyzes the protein *trans*-splicing reaction at an extraordinarily high rate and is widely used in the cyclization of proteins without affecting their activities [17–19]. Therefore, DnaE was fused to a randomly mutated thermally stable firefly luciferase construct, modified at residue 358 or 233, to increase the sensitivity of the firefly luciferase assay of apoptosis. In a series of experiments, these recombinant luciferase reporters displayed increased sensitivity and yielded reproducible data with low background [16,20].

Recently, our group has previously shown that HAV 3C<sup>pro</sup> cleaves the glutamine residue at position 304 (Gln304) of human NF- $\kappa$ B essential modulator (NEMO) to inhibit the expression of type I interferon (IFN) [21]. Therefore, in this study, we combined the HAV 3C<sup>pro</sup> cleavage recognition sequence of NEMO with a simple reporter system involving firefly luciferase to produce a cell-based protease reporter system, generating the reporters 233-HAV (233H), 358-HAV (358H), 233-DnaE-HAV (233DH), and 358-DnaE-HAV (358DH). The cyclized firefly luciferase protein expressed by 233DH was successfully cleaved by HAV 3C<sup>pro</sup>, resulting in a significant increase in luciferase activity. The luciferase activity of 233DH was confirmed to be specifically dependent on HAV 3C<sup>pro</sup> activity, but not on the protease activities of other viruses, including the noroviruses (NoV), severe acute respiratory syndrome coronavirus (SARS coronavirus, SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), Human coronavirus 229E (HCoV-229E), porcine epidemic diarrhea virus (PEDV), and porcine deltacoronavirus (PDCoV), confirming the specificity of 233DH for HAV 3C<sup>pro</sup> activity. Taken together, we might have developed a rapid and sensitive method to detect HAV 3C<sup>pro</sup> activity in HEK-293T cells.

## 2. Materials and methods

### 2.1. Cells

Human embryonic kidney cells (HEK-293T) and human hepatoma cells (Huh-7), obtained from the China Center for Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C under 5% CO<sub>2</sub> in a humidified incubator.

### 2.2. Plasmids

The cDNA expression constructs encoding wild type HAV 3C<sup>pro</sup> and its catalytically inactive mutant (C172A) have been described previously [21]. The 3C-like protease (3CL<sup>pro</sup>) genes from NoV, SARS-CoV, MERS-CoV, HCoV-229E, PEDV, and PDCoV were also synthesized/amplified and cloned into pCAGGS-HA-C.

The DNA sequence encoding the conserved residues in DnaE [20] was synthesized and cloned into the pCAGGS-MCS vector to construct pCAGGS-DnaE. The sequences encoding the C-terminal fragment (amino acids 235–544) and the N-terminal fragment (amino acids 4–233) of firefly luciferase were amplified from the firefly luciferase reporter vector pGL4.21luc2P/Puro (Promega, USA), respectively. With two-step overlapping PCR, the sequences encoding amino acids 4–233 and 235–544 were cloned together into the pCAGGS-MCS vector, to generate the pCAGGS-233 vector. The sequences encoding other N-terminal (amino acids 4–354) and C-terminal fragments (amino acids 358–544) of firefly luciferase were also amplified and cloned to generate the pCAGGS-358 vector. Oligonucleotides corresponding to the amino acid sequence

PVLKAQ↓ADLYKA of NEMO (cleaved by HAV 3C<sup>pro</sup>) or ENLYFQ↓S (cleaved by Tobacco etch virus (TEV) 3C<sup>pro</sup>) [13,21] was also ligated into the pCAGGS-233 vector to construct the 233H reporter or the control 233 reporter, respectively. The sequence encoding amino acids 4–233 and 235–544 fused to the corresponding amino sequence PVLKAQ↓ADLYKA or ENLYFQ↓S was cloned into pCAGGS-DnaE, to generate the 233DH reporter or the control 233D reporter, respectively. The same construction strategy was used to generate the 358H reporter, the 358DH reporter, and their control reporters 358 and 358D, respectively. The sequences of biosensor expression plasmids are shown in Table S1.

### 2.3. Western blotting analysis

Briefly, HEK-293T cells cultured in 60 mm dishes were transfected with the various plasmids. After 30 h, the cells were harvested by the addition of lysis buffer, and the protein concentrations were measured in the whole-cell extracts. The proteins in the extracts were separated with 12% SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were incubated with antibodies and secondary antibodies. To confirm the expression levels of HA-tagged wild-type (WT) and mutant 3C<sup>pro</sup>, an anti-HA antibody (MBL, Japan) was used in immunoblotting analysis. An anti-goat monoclonal secondary antibody (Promega, USA) was used to detect the expression of each luciferase reporter. The expression of  $\beta$ -actin was monitored with an anti- $\beta$ -actin mouse monoclonal antibody (Beyotime, China) to confirm equal protein loading.

### 2.4. Luciferase reporter gene assays

The luciferase reporter constructs 233H, 358H, 233DH, 358DH and their controls (233, 358, 233D, 358D respectively) were used to detect HAV 3C<sup>pro</sup> activity. HEK-293T cells or Huh-7 cells plated in 48-well plates were transfected with various 3C<sup>pro</sup> and 3CL<sup>pro</sup> expression plasmids or the empty control plasmid, together with the luciferase reporter plasmid and pRL-TK (Promega) (used as an internal control to normalize transfection efficiency). The cells were lysed 36 h later, and the firefly luciferase and Renilla luciferase activities were determined with a luciferase reporter assay system (Promega, USA), according to the manufacturer's protocol. The data represent the relative firefly luciferase activities normalized to the corresponding Renilla luciferase activities.

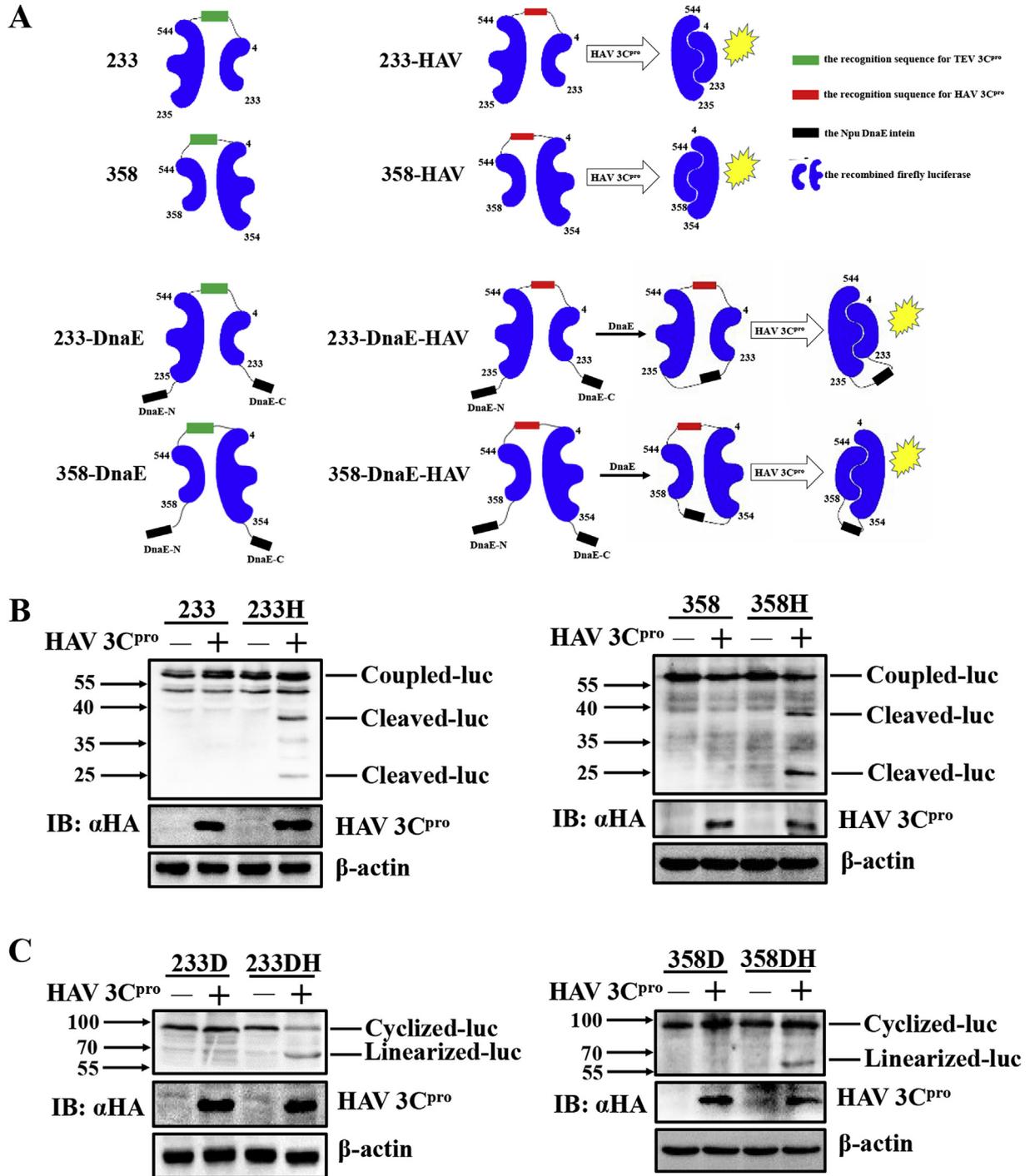
### 2.5. Statistical analysis

The results are presented as the means  $\pm$  standard deviations (SD) of at least three experiments. Significant differences were detected with Student's *t*-test.  $P < 0.05$  was considered statistically significant (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ ; ns, not significant).

## 3. Results

### 3.1. Generation of four biosensors to evaluate HAV 3C<sup>pro</sup> activity

As described in our previous study, HAV 3C<sup>pro</sup> recognizes and cleaves human NEMO at the Gln304 residue [21]. Thus, we combined the HAV 3C<sup>pro</sup> cleavage recognition sequence (PVLKAQ↓ADLYKA) of NEMO with a firefly luciferase reporter system to monitor the activity of HAV 3C<sup>pro</sup> in HEK-293T cells. To this end, pCAGGS-233 and pCAGGS-358 were fused to the cleavage sequence of NEMO recognized by HAV 3C<sup>pro</sup> to generate 233H and 358H, respectively (Fig. 1A). As described previously, DnaE is widely used in the cyclization of proteins because this cyclization does not



**Fig. 1.** Generation of four biosensor assays to evaluate HAV 3C<sup>pro</sup> activity. (A) Diagram of the generation of 233H, 358H, 233DH, and 358DH and their controls (233, 358, 233D, 358D respectively). The blue structure represents the peptide sequences of the recombinant firefly luciferase. Black rectangle represents the peptide sequence of DnaE used to cyclize the protein. Red rectangle represents the protease recognition site PVLKAQ↓ADIYKA used to assess HAV 3C<sup>pro</sup> activity, and the green rectangle represents the protease recognition site ENLYFQ↓S for TEV 3C<sup>pro</sup>, used as the control. (B) HEK-293T cells were transfected with 233H, 358H, or the corresponding controls (233, 358, respectively) and a plasmid encoding HAV 3C<sup>pro</sup>. Cell lysates were prepared at 30 h posttransfection and analyzed with western blotting. (C) HEK-293T cells were transfected with 233DH, 358DH, or the corresponding controls (233D, 358D, respectively) and the plasmid encoding HAV 3C<sup>pro</sup>. Cell lysates were prepared 30 h posttransfection and analyzed with western blotting. The lane with protein markers (thermo scientific, catalog no. 26616) includes 100-, 70-, 55-, 40-, 35-, 25-kDa molecular mass bands. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

affect their activities, and increases the sensitivity of firefly luciferase assays [13,16–20]. Therefore, 233-DnaE (233D) and 358-DnaE (358D), fused to split DnaE, were also fused to the cleavage sequence of NEMO to generate 233DH and 358DH, respectively (Fig. S1A). As shown in Fig. 1A, 233H, 358H, 233DH and 358DH

expressed the divided N-terminal (amino acids 2–232 or 2–354) and C-terminal fragments (amino acids 235–544 or 358–544) of firefly luciferase in the inactive state. In the presence of DnaE, the expressed N- and C-terminal fragments 233DH and 358DH were cyclized to restrict the movement of the two domains, locking the

enzyme into the more inactive form. If the recognition peptide inserted between the N-terminal fragment and C-terminal fragment of firefly luciferase was cleaved by HAV 3C<sup>pro</sup>, the two domains of firefly luciferase could theoretically interact freely and the enzyme would become fully active. In Fig. 1A, to detect any nonspecific cleavage by HAV 3C<sup>pro</sup>, the 233, 358, 233D, and 358D reporters were fused to the ENLYFQ↓S sequence, recognized by TEV 3C<sup>pro</sup>, and used as the controls for the corresponding proteins [13].

To test whether the four recombinant firefly luciferases fused to the cleavage sequence of NEMO were successfully recognized and cleaved by HAV 3C<sup>pro</sup>, HEK-293T cells were transfected with the HAV 3C<sup>pro</sup> expression plasmid, together with the reporter 233H, 358H, 233DH, or 358DH, or the corresponding control. A western blotting analysis showed that the protein bands from the cells cotransfected with HAV 3C<sup>pro</sup> and 233H, 358H, 233DH, or 358DH migrated fastest (Fig. 1B and C). The HAV-3C<sup>pro</sup>-induced cleavage of 233H and 358H produced two new cleaved bands (Fig. 1B). However, because of the cyclization conferred by DnaE, only 233DH and 358DH were linearized, displaying greater mobility after cleavage, and resulting in a slightly shorter product than the full-length protein on the western blot (Fig. 1C). No cleavage phenomenon was observed in the cells transfected with the 233, 358, 233D, or 358D reporter fused to the recognition sequence of TEV 3C<sup>pro</sup> (Fig. 1B and C). These results confirm that the four recombinant luciferase constructs fused to the cleavage sequence of NEMO are specifically recognized and cleaved by HAV 3C<sup>pro</sup>, suggesting their potential utility in assessing the HAV 3C<sup>pro</sup> activity in HEK-293T cells.

### 3.2. Cyclized luciferase-based biosensor (233DH) reliably detects HAV 3C<sup>pro</sup> activity in cells

To evaluate the reporters' functions in a luciferase activity assay, cells were cotransfected with each of the four reporters or their respective controls, pRL-TK, and the HAV 3C<sup>pro</sup> expression plasmid. At 36 h posttransfection, a dual-luciferase assay was performed. The results shown in Fig. 2A indicate that the activity of 233DH was significantly induced by HAV 3C<sup>pro</sup>, whereas that of the control reporter 233D remained low. However, although the activity of the 233H, 358H, and 358DH reporters also increased to some extent, the activities of the control reporters also increased as the expression of HAV 3C<sup>pro</sup> increased (Fig. 2A), suggesting that the activities of these reporters might be nonspecific. These results indicate that the 233DH reporter is the most sensitive and reliable of the four constructed. To further confirm the effect of 233DH in the luciferase activity assay, HEK-293T cells or Huh-7 cells were transfected with different amounts of the HAV 3C<sup>pro</sup> expression plasmid and the 233D or 233DH reporter. The results shown in Fig. 2B and C demonstrate that the luciferase activity of the 233DH reporter was effectively and dose-dependently induced by HAV 3C<sup>pro</sup> in both the HEK-293T and Huh-7 cells. The control reporter activity was not induced. Western blotting also showed that HAV 3C<sup>pro</sup> dose-dependently cleaved the recombinant firefly luciferase in the cyclized form, 233DH, producing a faster-migrating protein band, but did not cleave the control reporter (Fig. 2D). The cleavage shown in Fig. 2D is consistent with the fold induction in the luciferase activity shown in Fig. 2B and C, confirming the correlation between the luciferase activity assay and the cleavage of the reporter construct by HAV 3C<sup>pro</sup>.

### 3.3. Luciferase activity of 233DH is specifically dependent on HAV 3C<sup>pro</sup> activity

According to a previous study, Cysteine 172 (Cys172) of HAV 3C<sup>pro</sup> is part of the catalytic dyad and is essential to its protease

activity [22,23]. Comparison of the protein sequences and three-dimensional structures of HAV 3C<sup>pro</sup>, Foot-and-mouth disease virus (FMDV) 3C<sup>pro</sup>, enterovirus 71 (EV71) 3C<sup>pro</sup>, and human rhinovirus (HRV) 3C<sup>pro</sup> showed that the region around the Cys172 residue is conserved (data not show). Therefore, a mutant 3C<sup>pro</sup> at the Cys172 residue (3C<sup>pro</sup>-C172A) was constructed. To explore whether the luciferase activity of the 233DH reporter relies on the protease activity of 3C<sup>pro</sup>, HEK-293T cells were transfected with different amounts of plasmid encoding 3C<sup>pro</sup>-C172A or WT HAV 3C<sup>pro</sup>, together with the 233DH reporter and pRL-TK. Unlike the dose-dependent activation of luciferase activity induced by HAV 3C<sup>pro</sup>, the mutant 3C<sup>pro</sup>-C172A was incapable of activating the 233DH reporter (Fig. 3A). To explore the mechanism underlying the failure of overexpressed 3C<sup>pro</sup>-C172A to induce the reporter luciferase activity, WT HAV 3C<sup>pro</sup> or 3C<sup>pro</sup>-C172A was overexpressed in the presence of the recombinant firefly luciferase 233DH reporter. As shown in Fig. 3B, the cyclized form of the recombinant firefly luciferase was cleaved normally and dose-dependently by WT HAV 3C<sup>pro</sup>, generating a faster-migrating protein band. However, no cleavage product was observed when HAV 3C<sup>pro</sup>-C172A was overexpressed, confirming that the activation of this luciferase reporter system is specifically dependent on the protease activity of HAV 3C<sup>pro</sup>.

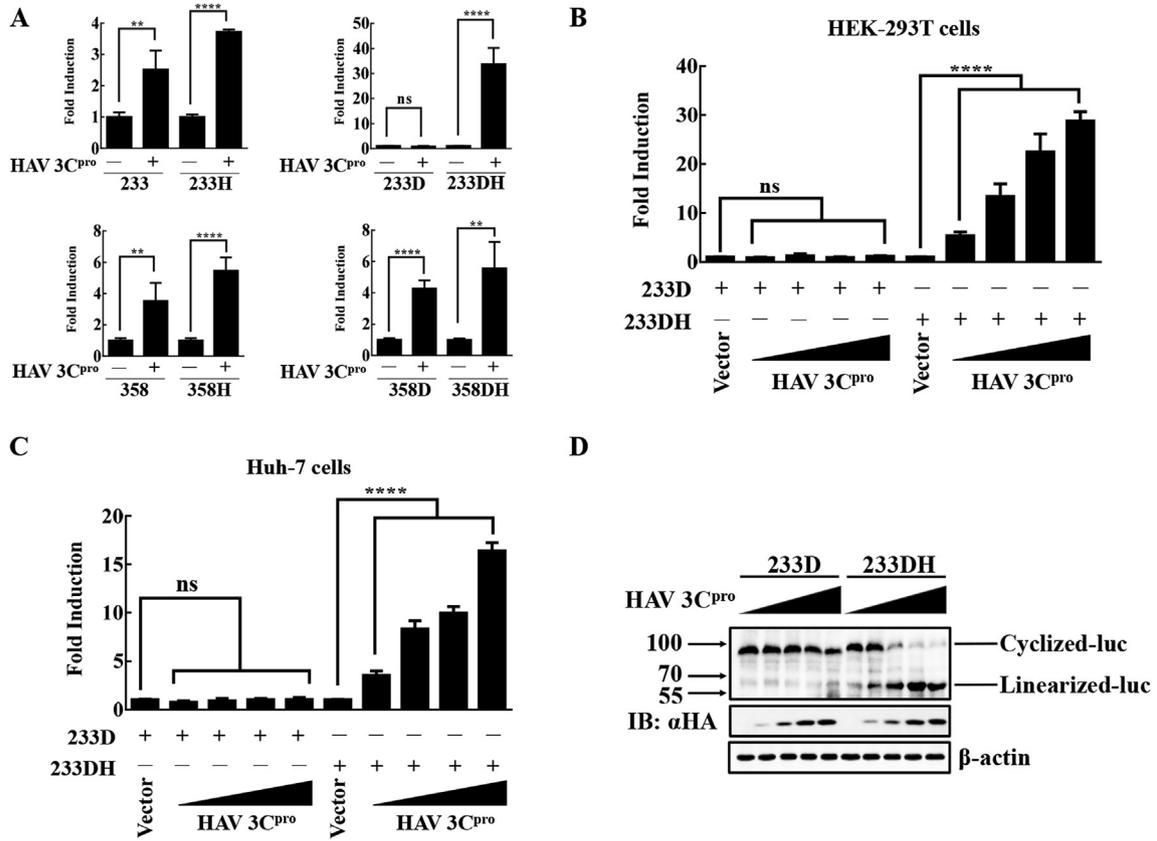
### 3.4. Luciferase reporter 233DH specifically reflects the protease activity of HAV 3C<sup>pro</sup>

To confirm the specificity of 233DH for HAV 3C<sup>pro</sup>, we used the 3C<sup>pro</sup> proteins of typical human viruses NoV, SARS-CoV, MERS-CoV, and HCoV 229E and animal coronaviruses PEDV and PDCoV as the negative controls. 3C<sup>pro</sup> of PEDV and PDCoV are both reported to cleave NEMO at Gln231, which differs from the peptide sequence of NEMO inserted into the 233DH reporter [24,25]. To analyze the luciferase activity, HEK-293T cells were transfected with plasmids expressing HAV 3C<sup>pro</sup> or 3C<sup>pro</sup> of NoV, SARS-CoV, MERS-CoV, HCoV 229E, PEDV, or PDCoV, together with the 233DH reporter and pRL-TK. As shown in Fig. 4A, HAV 3C<sup>pro</sup> successfully induced luciferase activity, whereas the other viral 3C<sup>pro</sup> proteases failed to activate the 233DH reporter. The western blot shown in Fig. 4B shows that, unlike HAV 3C<sup>pro</sup>, the other viral 3C<sup>pro</sup> proteases were unable to cleave the cyclized recombinant firefly luciferase, which is consistent with the result of the luciferase activity analysis. These data show that only the 233DH reporter fused to the peptide PVLKAQ↓ADIIYKA was specifically recognized and cleaved by HAV 3C<sup>pro</sup>, demonstrating the excellent potential utility of the 233DH reporter in screening for HAV 3C<sup>pro</sup>-specific inhibitors.

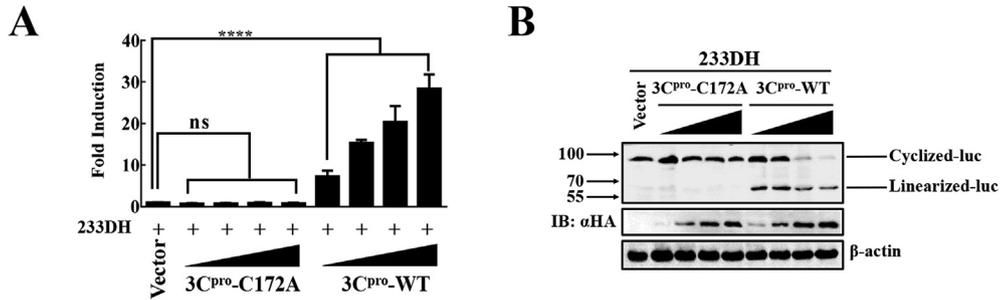
## 4. Discussion

In the development of anti-HAV drugs, HAV 3C<sup>pro</sup> is one of the major targets used to identify suitable compounds or small-molecule polypeptides that inhibit HAV. Therefore, the analysis of 3C<sup>pro</sup> activity in live cells with an efficient high-throughput strategy is critical [6,26,27]. Here, we developed the 233DH reporter system, based on cyclized recombinant firefly luciferase and the peptide substrate of HAV 3C<sup>pro</sup> in NEMO (PVLKAQ↓ADIIYKA) [21]. Our study demonstrates that the luciferase activity of the 233DH reporter is significantly activated by HAV 3C<sup>pro</sup>, confirming the excellent prospects of the 233DH reporter in the analysis of HAV 3C<sup>pro</sup> activity.

Because the rate of the protein *trans*-splicing reaction is high, DnaE is widely used to cyclize proteins without affecting their activities [17–19]. In this study, DnaE was used to cyclize the two domains of firefly luciferase to generate a cyclized recombinant firefly luciferase. The circularly permuted luciferase successfully



**Fig. 2.** Reporter 233DH reliably detects HAV 3C<sup>pro</sup> activity in cells. (A) HEK-293 cells were transfected with each of the four reporters or their corresponding controls, the pRL-TK plasmid, and the HAV 3C<sup>pro</sup> expression plasmid. Luciferase assays were performed 36 h after transfection. (B and C) HEK-293 cells or Huh-7 cells were transfected with 233DH, pRL-TK, and increasing quantities of plasmid encoding HAV 3C<sup>pro</sup>. The cells were harvested after 36 h and subjected to a dual-luciferase assay. (D) HEK-293T cells were cotransfected with 233DH and HAV 3C<sup>pro</sup> expression plasmid. Cell lysates were prepared 30 h posttransfection and analyzed with western blotting.

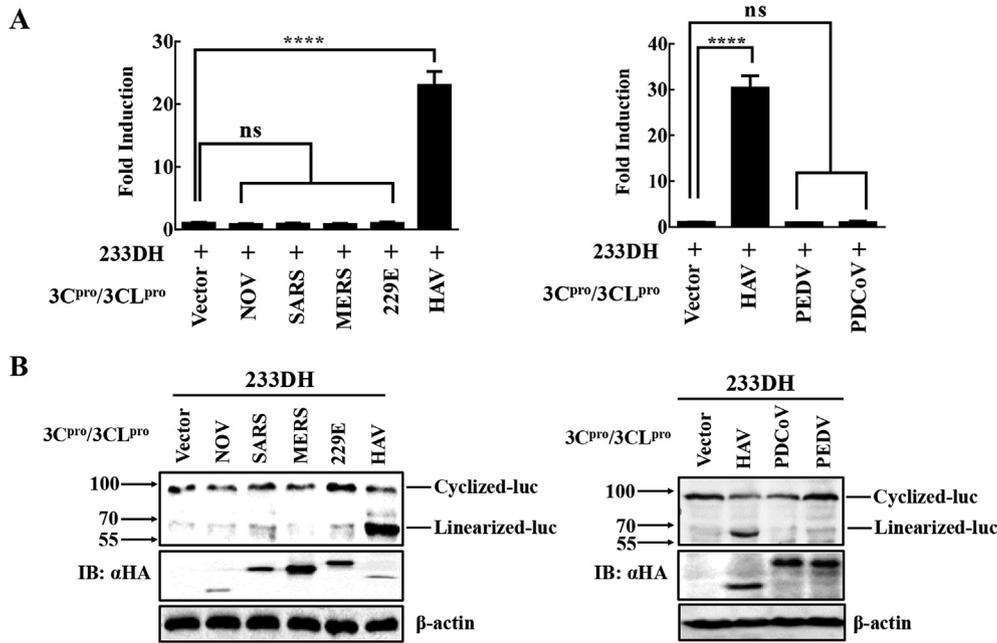


**Fig. 3.** Confirmation that 233DH is specifically dependent on the protease activity of HAV 3C<sup>pro</sup>. (A) HEK-293T cells were transfected with 233DH, pRL-TK, and increasing quantities of plasmid encoding mutant HAV 3C<sup>pro</sup>-C172A. Luciferase assays were performed 36 h after transfection. (B) HEK-293T cells were cotransfected with 233DH and the HAV 3C<sup>pro</sup>-C172A expression plasmid. Cell lysates were prepared 30 h posttransfection and analyzed with western blotting.

restricts the movement of the two domains of firefly luciferase and locks the enzyme in its less-active open form [13]. This underpins the high sensitivity and low background of the 233DH reporter system (Fig. 2A). However, the cyclized recombinant firefly luciferase in the 358DH reporter was not significantly activated by HAV 3C<sup>pro</sup>, and the 358D reporter fused to the sequence recognized by the TEV protease was slightly activated by HAV 3C<sup>pro</sup>. Moreover, the uncyclized firefly luciferase in 233H, 358H, and their control reporters could randomly form an active firefly luciferase, generating the high background observed in the luciferase activity analysis (Fig. S1B). We also found that the capacity of HAV 3C<sup>pro</sup> to activate 233DH depends on the residue (C172) involved in its protease activity (Fig. 3A). Therefore, this firefly luciferase system can also be

used to identify other potentially catalytic residues of HAV 3C<sup>pro</sup>. In addition, HAV 3C<sup>pro</sup> activated the 233DH reporter significantly more effectively than did the 3C<sup>pro</sup> proteases of NoV, SARS-CoV, MERS-CoV, HCoV 229E, PEDV, and PDCoV (Fig. 4A), revealing its potential utility in specifically detecting HAV 3C<sup>pro</sup> activity in HEK-293T cells. All these results confirm that the use of DnaE and the firefly luciferase fragments consisting of amino acids 4–233 and 235–544 significantly enhanced the sensitivity and specificity of the detection of HAV 3C<sup>pro</sup> activity. Therefore, the 233DH reporter could be selected to monitor HAV 3C<sup>pro</sup> activity in the future study.

In summary, using DnaE to cyclize firefly luciferase significantly enhanced the detection of HAV 3C<sup>pro</sup> activity in HEK-293T cells. The 233DH reporter system constructed represents an improved



**Fig. 4.** Luciferase reporter system specifically detects the protease activity of HAV 3C<sup>pro</sup>. (A) HEK-293T cells were transfected with 233DH, pRL-TK, and plasmid encoding 3CL<sup>pro</sup> of NoV, SARS-CoV, MERS-CoV, 229E, PEDV or PDCoV. Luciferase assays were performed 36 h after transfection. (B) HEK-293T cells were cotransfected with 233DH and plasmid expressing NoV, SARS-CoV, MERS-CoV, 229E, PEDV or PDCoV 3CL<sup>pro</sup>. Cell lysates were prepared 30 h posttransfection and analyzed with western blotting.

biosensor that is sensitive and yields reproducible data. It has potential utility in screening for HAV-3C-specific inhibitors or broad-spectrum inhibitors of the 3C proteases of viruses in the family Picornaviridae.

#### Conflict of interest

The authors declare that they have no conflicts of interest.

#### Acknowledgement

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2017.05.063>.

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