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Research Article

# Naturally occurring PA$^{E206K}$ point mutation in 2009 H1N1 pandemic influenza viruses impairs viral replication at high temperatures

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ABSTRACT

The emergence of influenza virus A pandemic H1N1 in April 2009 marked the first pandemic of the 21st century. In this study, we observed significant differences in the polymerase activities of two clinical 2009 H1N1 influenza A virus isolates from Chinese and Japanese patients. Sequence comparison of the three main protein subunits (PB2, PB1, and PA) of the viral RNA-dependent RNA polymerase complex and subsequent mutational analysis revealed that a single amino acid substitution (E206K) was responsible for the observed impaired replication phenotype. Further *in vitro* experiments showed that presence of PA$^{E206K}$ decreased the replication of influenza A/WSN/33 virus in mammalian cells and a reduction in the virus's pathogenicity *in vivo*. Mechanistic studies revealed that PA$^{E206K}$ is a temperature-sensitive mutant associated with the inability to transport PB1–PA complex to the nucleus at high temperature (39.5 °C). Hence, this naturally occurring variant in the PA protein represents an ideal candidate mutation for the development of live attenuated influenza vaccines.

## 1. Introduction

The influenza A virus is an important human respiratory pathogen, responsible not only for seasonal epidemics resulting in significant morbidity and mortality annually, but also for influenza epidemics and pandemics worldwide, representing a significant threat to public health. In April 2009, a novel H1N1 influenza virus A of swine origin, named pdm09, emerged in Mexico and spread globally (Avit et al., 2009; Smith et al., 2009). Since then, the pandemic H1N1 has led to approximately 284,500 deaths, with more than 214 countries with confirmed H1N1 influenza A cases. Currently, A(H1N1)pdm09 is still circulating seasonally among humans (Mishra et al., 2010; Sherbany et al., 2014; Nogales et al., 2018; Palese and Shaw, 2013); thus, further characterisation of this virus is of great importance for controlling the pandemic influenza A virus.

The influenza A virus is a member of the *Orthomyxoviridae* family with a negative-sense, single-stranded, segmented RNA genome that consists of eight viral RNA (vRNA) segments that encode at least 10 major proteins (Wise et al., 2012; Krumbholz et al., 2011; Muramoto et al., 2013; Selman et al., 2012). Its viral ribonucleoprotein complex (vRNP) is made of an RNA-dependent RNA polymerase (RdRp) and nucleoproteins (NPs), and is the minimal functional unit for transcription (vRNA + mRNA) and replication (mRNA + cRNA + vRNA) to occur in the nucleus of infected cells. The RdRp of influenza A virus is a heterotrimeric complex that comprises the polymerase basic protein-1 (PB1), polymerase basic protein-2 (PB2), and polymerase acidic protein (PA). PB1 is the core of the RdRp of influenza, containing the enzyme motifs required for viral RNA polymerization activity; PB2 subunit is responsible for the recognition and binding to the host pre-mRNA cap structures; and the endonuclease activity of PA cleaves the cap structure to initiate capped RNA-primed transcription. vRNA replication is facilitated by a *trans*--acting polymerase, and the initiation of cRNA and vRNA synthesis is primer-independent. Thus, to fulfil its functions in the nucleus, the efficient assembly of RdRp from individually cytosolic expressed PB1, PB2, and PA subunits is a prerequisite. Sufficient evidence supports that PB1 and PA are assembled into a dimeric complex and transported into the nucleus where the PB1-PA dimer is assembled with individually transported PB2 into a trimeric RdRp complex, which will then interact with vRNA and NPs to form the vRNPs.

Crystallography studies revealed that RdRp is a compact particle with PB1 at its centre, capped on one face by PB2, and clamed between the two globular domains of PA. The PA subunit is 716 amino acids (aa) long and

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differs from the other subunits in that it has an overall negative charge. The PA protein is divided into two main domains: an endonuclease domain (1–197 aa) and a large C-terminal domain (257–716 aa). The N- and C-terminal domains of PA are connected by a PA linker (198–256 aa), which offers the PA protein a degree of conformational flexibility (Stevaert et al., 2016; Stubbs et al., 2014; Meyer et al., 2016). The PA subunit has been shown to possess protease activity that degrades both viral and host proteins, and regulates vRNA synthesis (Rodriguez-Frandsen et al., 2015; Hu et al., 2015; Fodor et al., 2013). Recently, PA has also been reported to be interacted with innate immunity. Influenza virus PA could block IRF3 activation and suppress IFN-β production (Yi et al., 2017). Avian influenza virus PA protein strongly suppresses host antiviral defense by interacting with and degrading Janus kinase 1 (JAK1) (Yang et al., 2023).

In this study, we compared the RdRp activities of two naturally derived 2009 H1N1 influenza A viruses isolated from Chinese and Japanese patients, and identified a single aa substitution (E206K) in the linker region of the PA protein that was significantly associated with RdRp activity in vRNP reconstitution systems. We investigated the regulatory mechanism of $PA^{K206}$ in the genetic background of the influenza A/WSN/33(H1N1) virus (WSN virus).

*In vitro* viral growth and mouse virulence were evaluated, revealing that $PA^{E206K}$ was associated with decreased viral growth in the early step of the virus replication cycle in mammalian cells, which consequently resulted in later death and less weight loss in mice compared with the wild-type virus. Furthermore, we found that $PA^{E206K}$ exhibited a temperature-sensitive phenotype *in vitro*. Overall, our findings suggest that 206 aa of PA protein plays a major role in the regulation of virus growth.

## 2. Materials and methods

### 2.1. Cells

Human lung carcinoma cell line A549, human embryonic kidney cell lines 293T and 293A, chicken fibroblast cell line DF-1, and Madin–Darby canine kidney cell line (MDCK) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with penicillin (100 U/mL), streptomycin (10 mg/mL), and 10% foetal bovine serum (Gibco) at 37 °C in a humidified incubator with 5% $CO_2$ in air and split 1:2 at confluence.

### 2.2. Plasmids and antibodies

pcDNA plasmids for the *in vitro* reconstitution of the ribonucleoprotein system of influenza A/Beijing/2009 (H1N1) and A/Kurume/2009 (H1N1) were designed based on the RNA sequences of the respective viruses. pcDNA plasmids for the reconstitution of the ribonucleoprotein system of A/WSN/33(H1N1) were kindly provided by Prof. Ervin Fodor (University of Oxford, UK). Rabbit anti-influenza HA antibody was purchased from Sino Biological (Beijing, China). The mouse anti-influenza NP antibody (MAB8251) was purchased from Merck Millipore (Burlington, MA, USA). Rabbit anti-actin antibody was purchased from Cell Signalling Technology (Danvers, MA, USA). Rabbit anti-PB1, anti-PA, and anti-PB2 antibodies were purchased from GeneTex Technology (Irvine, CA, USA).

### 2.3. Reverse genetics

Recombinant viruses (influenza A/WSN/33 virus and $PA^{E206K}$ mutant virus) were generated using the pHW2000 eight-plasmid system (Hoffmann et al., 2000). HEK293T cells ($5 \times 10^5$) and MDCK cells ($5 \times 10^5$) were transfected with pHW2000-PB2, pHW2000-PB1, pHW2000-PA (wild-type or $PA^{E206K}$), pHW2000-HA, pHW2000-NP, pHW2000-NA, pHW2000-M, and pHW2000-NS. At 24 h post-transfection, DMEM containing 10% FBS was replaced with DMEM containing 0.5% FBS. The virus supernatant was harvested at 48 h and stored at −80 °C.

### 2.4. Western blotting

Cells were collected and lysed with a cytobuster (Merck Millipore, Burlington, MA, USA) for 25 min at 25 °C, and debris were removed by centrifugation at 10,625 ×*g* for 15 min. The lysates were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and immunoblotted with the indicated antibodies and IRDye secondary antibodies (LI-COR Bioscience, Lincoln, NE, USA). Protein bands were detected using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA), and their relative levels were analysed using the integrated software of the Odyssey system.

### 2.5. Immunofluorescence assay

293T cells were transfected with PB1-GFP and pcDNA-PA (wild-type) or pcDNA-$PA^{E206K}$. After 24 h, the cells were fixed with 4% paraformaldehyde and permeabilised with 0.2% Triton X-100 for 20 min. The cells were then blocked with 0.5% bovine serum albumin for 2 h at room temperature, incubated with the primary antibody overnight, and further incubated with the secondary antibody Alexa Fluor 594 goat anti-rabbit IgG (Zhongshan Jinqiao Biotech Co., Beijing, China) for 1 h at room temperature. The nuclei of the cells were labelled with 4', 6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO, USA). The cells were examined using a TCS SP5 laser scanning confocal microscope (Leica, Wetzlar, Germany).

### 2.6. Primer extension analysis

Primer extension analysis was performed as previously described (Fodor et al., 2002). The primers used were the following: 5′–TGGACTAGTGGGAGCATCAT–3′ to detect vRNA, 5′–TCCAGTATGGTTTTGATTTCCG–3′ to detect mRNA and cRNA, and 5′–TCCCAGGCGGTCTCCCATCC–3′ to detect *5S* RNA. Transcription products were resolved on 6% polyacrylamide gels containing 7 mol/L urea and visualised by autoradiography.

### 2.7. Plaque assay

MDCK cells ($2.5 \times 10^5$) were seeded on 12-well plates and incubated at 37 °C for 24 h. Recombinant wild-type and mutant viruses were serially diluted from $10^{-1}$ to $10^{-6}$ before infecting the cells and incubated at room temperature for 1 h. After deserting the viruses, 1% agarose/DMEM media containing 0.5 μg/mL TPCK-trypsin was added to the wells and incubated for 72 h at 37 °C. The cells were fixed with 4% formalin (Sigma-Aldrich) and stained with 1% crystal violet (Merck KGaA, Darmstadt, Germany). The number of plaque-forming units (PFU) was calculated based on the dilution factors.

### 2.8. Median tissue culture infectious dose ($TCID_{50}$) assay

For H1N1(A/California/04/2009) and H1N1(A/California/04/2009) $PA^{E206K}$, quantification of viral progenies was performed by the $TCID_{50}$ method. MDCK ($1.5 \times 10^4$) cells were seeded on 96-well plates and incubated at 37 °C for 24 h. Recombinant viruses were serially diluted from $10^{-1}$ to $10^{-6}$ before infecting the cells, and incubated at room temperature for 1 h. At 72 h post-infection (hpi), infected cells were scored for cytopathic effects under a microscope. The $TCID_{50}$ titres were calculated using the Reed-Muench method.

### 2.9. Mouse pathogenicity experiments

To determine the survival of infected mice, 6-week-old BALB/c female mice (Weitong Lihua Laboratory Animal Technology Co., Beijing, China) were anaesthetised with pentobarbital and infected intranasally with wild-type and $PA^{E206K}$ virus at $10^4$ PFU in 30 μL. Body weight was monitored daily for 15 days and the mice were observed for morbidity. For histopathological analysis, the lungs were removed on days 2 and 4 post-infection, fixed with 4% paraformaldehyde overnight, and dehydrated in 30% sucrose. The samples were frozen, cut into 10 μm-thick sections, and stained with haematoxylin and eosin.

### 2.10. Statistical analysis

Two-tailed independent Student's *t*-test was used to determine the significance of two-group comparisons. *P*-values $<0.05$ were considered statistically significant.

## 3. Results

### 3.1. Two clinical 2009 H1N1 influenza virus isolates have different RdRp activities

Since the RdRp of the influenza A virus is a key determinant of pathogenicity and host range, we compared the RdRp activities of two clinical 2009 H1N1 influenza virus isolates: A/Beijing/2009 isolated from a patient from Beijing (China) and A/Kurume/2009 isolated from an outpatient at Kurume University Hospital (Japan). The RdRp activities of the viruses were compared using an *in vitro* RNP reconstitution system in mammalian cells. Briefly, 293T cells were transfected with pcDNA plasmids (pcDNA-PB1, pcDNA-PB2, pcDNA-PA, pcDNA-NP) expressing PB2, PB1, PA, and NP proteins derived from A/Beijing/2009 or A/Kurume/2009, along with an RNA-expressing plasmid (pPOLI-HA-RT) encoding the viral HA segment of H1N1 viruses. At 24 h post-transfection, the HA protein levels, which acts as a reporter for the polymerase activity, were measured by Western blotting and the steady-state levels of the three viral RNA species (mRNA, vRNA, and cRNA) of the HA segment were measured by primer extension analysis. Overall, the RdRp activity was found to be markedly different between the two viruses (Fig. 1A), with A/Beijing/2009 showing a lower polymerase activity than A/Kurume/2009. Despite PB1, PB2, PA, and NP levels were comparable between the two vRNP reconstitution systems, the levels of HA derived from A/Beijing/2009 were significantly lower than those of HA from A/Kurume/2009. Next, we examined the synthesis of the three RNAs in the vRNP reconstitution system derived from each virus. Therefore, we performed primer extension to examine the levels of the three RNA sequences in the vRNP system, in which we replaced the pPOLI-HA-RT plasmid with pPOLI-NA-RT for convenience of detection. The levels of vRNA, cRNA, and mRNA in A/Beijing/2009 were generally lower than those in A/Kurume/2009 (Fig. 1B); thus, we then excluded the possibility that NP was responsible for the reduced polymerase activity of A/Beijing/2009. Substitution of A/Beijing/2009 NP protein with A/Kurume/2009 NP protein or vice-versa in the vRNP reconstitution system further showed that the substituted NP protein did not change the difference between the two vRNP systems (Supplementary Fig. S1), suggesting that the NP protein is not responsible for the reduced polymerase activity of the A/Beijing/2009 virus.

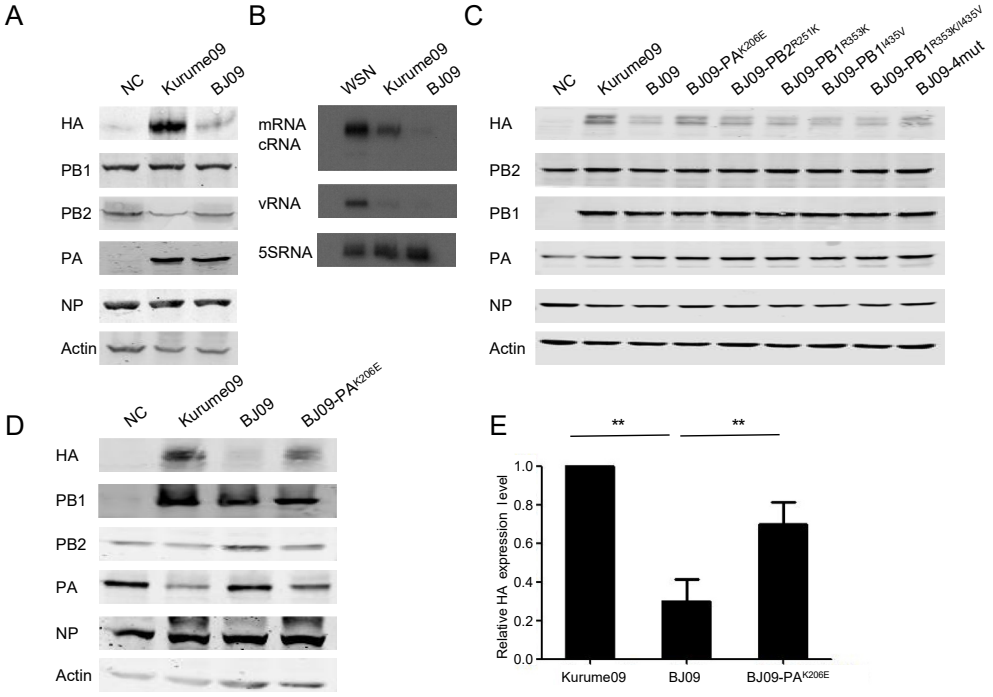


**Fig. 1.** Polymerase activities of influenza A/Beijing/2009 and A/Kurume/2009 viruses *in vitro*. **A** 293T cells were transfected with PB1, PB2, PA, and NP protein-expressing plasmids of A/Kurume/2009 or A/Beijing/2009, together with pPOLI-HA-RT (WSN virus). The transfected cells were incubated at 37 °C for 24 h and then lysed. The lysates were analysed by Western blotting. **B** 293T cells were transfected with PB1, PB2, PA, and NP protein-expressing plasmids of A/Kurume/2009 or A/Beijing/2009, together with pPOLI-NA-RT (WSN virus). Total RNAs were extracted and the levels of the three RNA species were evaluated by primer extension analysis. **C** Viral polymerase activity in 293T cells were compared among wild-type and variant viruses harbouring $PA^{K206E}$, $PB2^{R521K}$, or $PB1^{R353K/I435V}$. **D** Viral polymerase activity in 293T cells was compared among wild-type and variant viruses harbouring $PA^{K206E}$ of A/Beijing/2009. **E** Statistical analysis of HA levels in (**D**). HA values were standardized to the actin levels and normalized to those of HA in cells transfected with PB1, PB2, PA, and NP protein-expressing plasmids of A/Kurume/2009. Data are shown as mean ± standard error of the mean of three independent experiments.

### 3.2. Identification of polymerase activity determinants of 2009 H1N1 viruses in human cells

Nucleotide sequence analysis revealed that A/Beijing/2009 and A/Kurume/2009 are highly homogeneous. Thus, to identify which of the polymerase subunits was responsible for the reduced polymerase activity of the A/Beijing/2009 virus, we compared the sequences of PB1, PB2, and PA of A/Beijing/2009 and A/Kurume/2009. Sequence alignment revealed a total of four mutations between A/Beijing/2009 and A/Kurume/2009 (Table 1): two PB1 mutations (R353K and I435V), one PB2 mutation (R251K), and one PA mutation (K206E).

To identify the aa residues in the A/Beijing/2009 and A/Kurume/2009 RdRp proteins responsible for the difference in polymerase activity, we individually introduced A/Kurume/2009 residues into the A/Beijing/2009 background at the four identified sites and then compared the polymerase activity of the mutants and wild-type viruses in 293T cells. Most of the point mutations and the double mutants did not enhanced the A/Beijing/2009 polymerase activity (Fig. 1C), suggesting that PB1$^{R353K}$, PB1$^{I435V}$, and PB2$^{R251K}$ were not crucial for the reduced A/Beijing/2009 polymerase activity. In contrast, PA$^{K206E}$ led to 3-fold increased polymerase activity compared with the wild-type virus, indicating that the PA$^{K206}$ residue is the main contributor to A/Beijing/2009 polymerase activity regulation (Fig. 1D and E). Noteworthily, the effects of these mutations was not due changes on PB1, PB2, or PA levels (Fig. 1C).

Furthermore, we compared the aa sequences of the human H1N1, avian H1N1, and avian H5N1 (Table 2). After alignment of more than 2,000 unique PA protein sequences from the Influenza Virus Database of the National Center for Biotechnology Information from different years, we found that the 206 residue in PA is highly conserved in all human and avian influenza A viruses, with 99.8% human H1N1 strains and 100% avian H5N1 strains showing Glu (E) at point 206 of the PA protein, consistent with A/Kurume/2009. Interestingly, we also found a Lys (K) at position 206 of PA in most influenza B and C viruses. These findings implied that PA 206 residue may play a key role in regulating influenza virus characteristics.

### 3.3. Effect of PA$^{E206K}$ on the replication of WSN virus

Minireplicon system data indicated that a single aa mutation altered the reporter gene (HA) levels, which were driven by polymerase activity. To determine whether this alteration could influence viral replication, recombinant viruses were generated using reverse genetics. To ensure laboratory safety, we introduced PA$^{E206K}$ into the influenza A/WSN/33(H1N1) virus (WSN virus). In agreement with our previous results with A/Beijing/2009, introduction of PA$^{E206K}$ led to reduced polymerase activity (by 1.9-fold) of WSN virus (Fig. 2A and B). Next, we examined whether PA$^{E206K}$ could impact on the viral RNA synthesis machinery of WSN virus. Primer extension showed that the levels of vRNA, cRNA, and mRNA of PA$^{E206K}$ viruses were generally lower than those of the wild-type WSN in the vRNP reconstitution system (Fig. 2C and D). These results indicate that the presence of 206K in PA protein significantly reduces its ability to support viral polymerase activity.

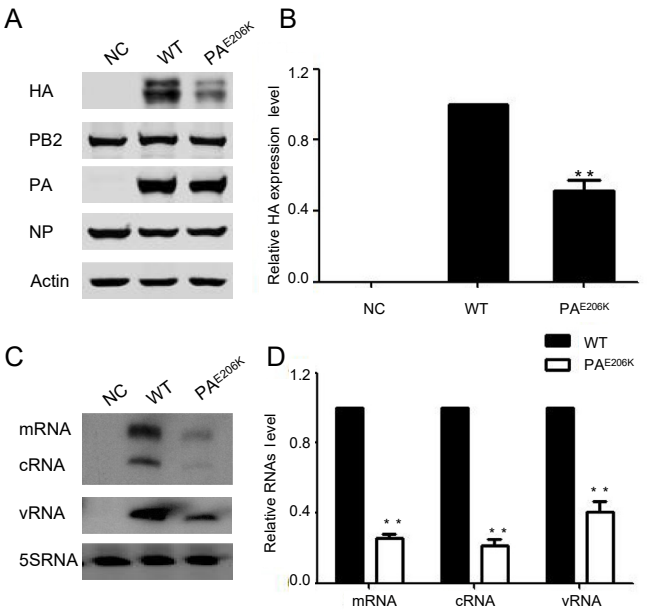


**Fig. 2.** PA$^{E206K}$ reduces polymerase activity of influenza A virus *in vitro*. **A** 293T cells were transfected with A/WSN/1933 virus RNP reconstitution system plasmids (pcDNA-PB1, pcDNA-PB2, pcDNA-NP, and pPOLI-HA-RT), along with pcDNA-PA or pcDNA-PA$^{E206K}$. HA levels were analysed by Western blotting. **B** Statistical analysis of HA levels in **A**. HA values were standardized to the actin levels and normalized to those of HA in cells transfected with control vector. **C** 293T cells were transfected with A/WSN/1933 virus RNP reconstitution system plasmids (pcDNA-PB1, pcDNA-PB2, pcDNA-NP, and pPOLI-NA-RT), along with pcDNA-PA or pcDNA-PA$^{E206K}$. Total RNAs were extracted and the levels of the three RNA species were analysed by primer extension analysis. **D** Statistical analysis of viral RNAs in (**C**). Values of the viral RNAs were standardized to the levels of 5S rRNA and normalized to those of viral RNAs in cells transfected with control vector. Data are represented as mean ± standard error of the mean of three independent experiments.

### 3.4. Effect of PA$^{E206K}$ on influenza A virus growth in vitro

To understand the role of PA$^{E206K}$ in influenza virus replication in cultured cells, a PA$^{E206K}$ mutant virus was generated using the WSN background. To compare the replication of wild-type and PA$^{E206K}$ WSN viruses, their single-step replication kinetics were examined at 4, 6, and 8 h in A549 cells infected at a multiplicity of infection (MOI) of 1. It showed that wild-type WSN grew more rapidly and up to a higher titre than PA$^{E206K}$ WSN (Fig. 3A). Similarly, multistep replication kinetics analysis of the two viruses in MDCK cells infected at an MOI of 0.001 also showed that the wild-type virus achieved a significantly higher titre than the PA$^{E206K}$ WSN virus at 12 and 24 hpi (Supplementary Fig. S2), but

**Table 1**
Amino acid mutations identified from BJ09 and Kurume 09.

| Segment | Site | BJ09 | Kurume09 |
|---|---|---|---|
| PA | 206 | K | E |
| PB1 | 353 | R | K |
| | 435 | I | V |
| PB2 | 251 | R | K |

**Table 2**
Amino acid frequencies at position 206 in PA of human H1N1, avian H1N1 and avian H5N1.

| PA amino acid at position 206 by virus | | | | | |
|---|---|---|---|---|---|
| Human H1N1 (n = 1740) | | Avian H1N1 (n = 395) | | Avian H5N1 (n = 122) | |
| Residue | Frequency (%[n]) | Residue | Frequency (%[n]) | Residue | Frequency (%[n]) |
| E | 99.8% (1737) | E | 100%(395) | E | 100%(122) |
| K | 0.1% (2) | K | 0 | K | 0 |
| V | 0.1% (1) | V | 0 | V | 0 |

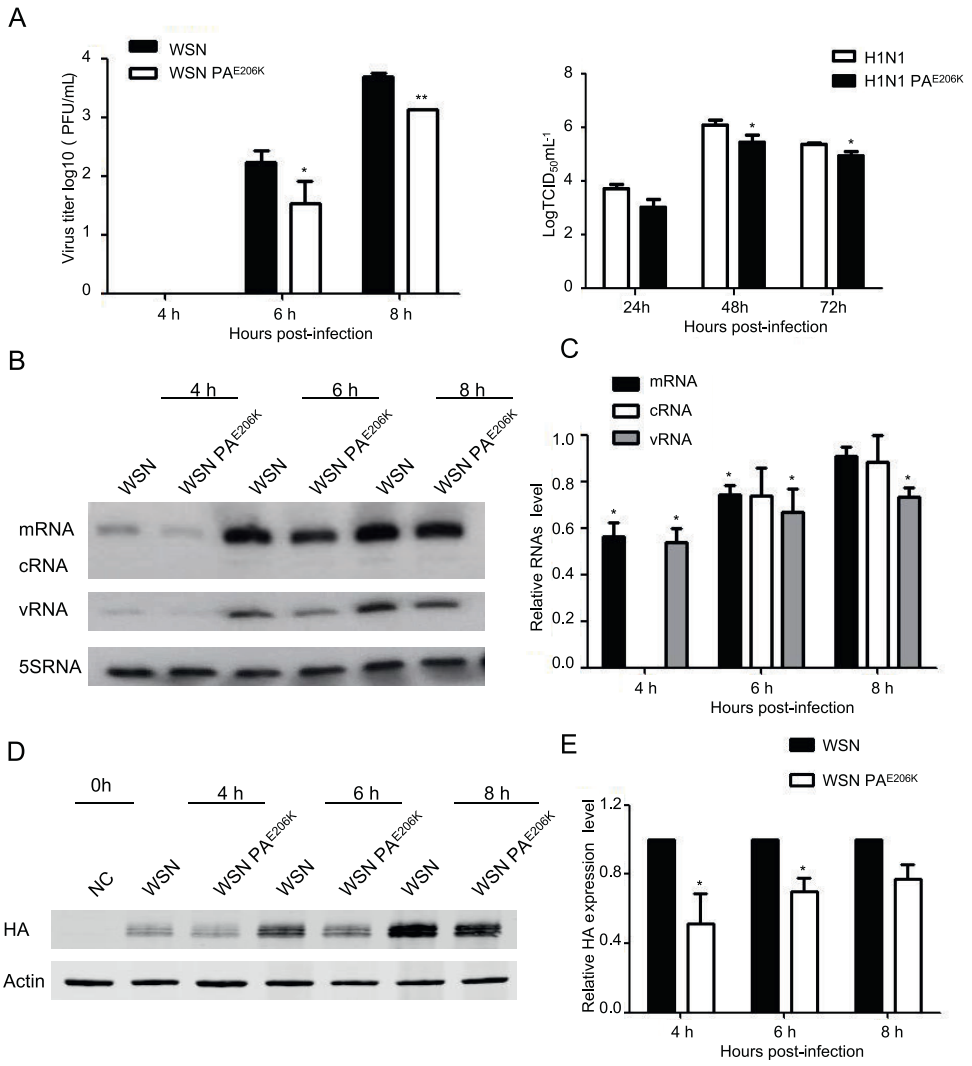


**Fig. 3.** PA$^{E206K}$ impairs replication of influenza A virus *in vitro*. **A** A549 (left) or MDCK (right) cells were infected with wild-type or PA$^{E206K}$ WSN ( H1N1 PA$^{E206K}$) viruses at an MOI of 1 (left) or 0.01 (right) and cultured at 37 °C. Viral titres were measured by plaque assays at the indicated time points. Error bars represent the standard deviations of the results of three independent experiments. **B** A549 cells were infected with wild-type or PA$^{E206K}$ WSN viruses (MOI=1). At the indicated time post-infection, total RNAs were extracted and the levels of the three RNA species were analysed by primer extension analysis. **C** Statistical analysis of viral RNAs in (**B**). (**D**) A549 cells were infected with wild-type or PA$^{E206K}$ WSN viruses (MOI=1). At the indicated time post-infection, cells were harvested and lysed for Western blotting analysis. **E** Values of HA were standardized to the actin levels and normalized to those of HA in cells infected with wild-type virus. Data are represented as mean ± standard error of the mean from three independent experiments.

similar virus titres were observed between the two viruses at 36, 48, 60, and 72 hpi (Supplementary Fig. S2A). Collectively, these results suggest that the E206K substitution in PA protein impairs the viral growth capacity in human and mammalian cells.

We then conducted assays to evaluate the performance of vRNP polymerase in viral transcription and replication of wild-type and PA$^{E206K}$ WSN viruses (MOI = 1.0) in A549 cells. Primer extension assays showed that mRNAs, cRNAs, and vRNAs levels were significantly lower in A549 cells infected with WSN PA$^{E206K}$ than in those with wild-type WSN at 4 and 6 hpi (Fig. 3B and C). These data demonstrated that PA$^{E206K}$ reduces viral transcription and replication in human A549 cells. Moreover, Western blot analysis indicated that WSN PA$^{E206K}$ produced significantly lower levels of HA proteins at 4 and 6 hpi (Fig. 3D and E). Similarly, MDCK infected with H1N1 (A/California/04/2009) viruses harbouring PA$^{E206K}$ showed lower growth rates than wild-type H1N1 (Fig. 3A), as determined by the TCID$_{50}$ method. Moreover, Western blot analysis showed that the levels of viral NP and PB1 proteins were lower in H1N1 PA$^{E206K}$ infected cells than in wild-type H1N1 infected cells at 6 hpi (Supplementary Fig. S2B). Taken together, these data showed that PA$^{E206K}$ impairs viral replication in MDCK and A549 cells, particularly during the initial 12 h of infection.

### 3.5. *Effect of PA$^{E206K}$ on the pathogenicity of influenza A virus in vivo*

Next, we examined the growth and pathogenicity of PA$^{E206K}$ in mice. Six-week-old female BALB/c mice were intranasally infected with $10^2$, $10^3$, $10^4$, or $10^5$ PFU of wild-type or PA$^{E206K}$ WSN. A mortality rate of 100% and fatality rate of 83.3% were observed in mice infected with $10^5$ and $10^4$ PFU of wild-type WSN virus, whereas 100% mortality and a 50% fatality rate were observed in mice infected with $10^5$ and $10^4$ PFU of WSN PA$^{E206K}$ (Supplementary Fig. S3). Mouse median lethal dose values for wild-type and mutant WSN were $10^{2.40}$ and $10^{3.40}$ PFU, respectively. Thus, 11 mice were intranasally infected with $10^4$ PFU of either virus, and survival rates and body weight were monitored daily for 15 days. Overall, mice infected with wild-type WSN exhibited more weight loss and a higher fatality rate than mice infected with WSN PA$^{E206K}$ virus

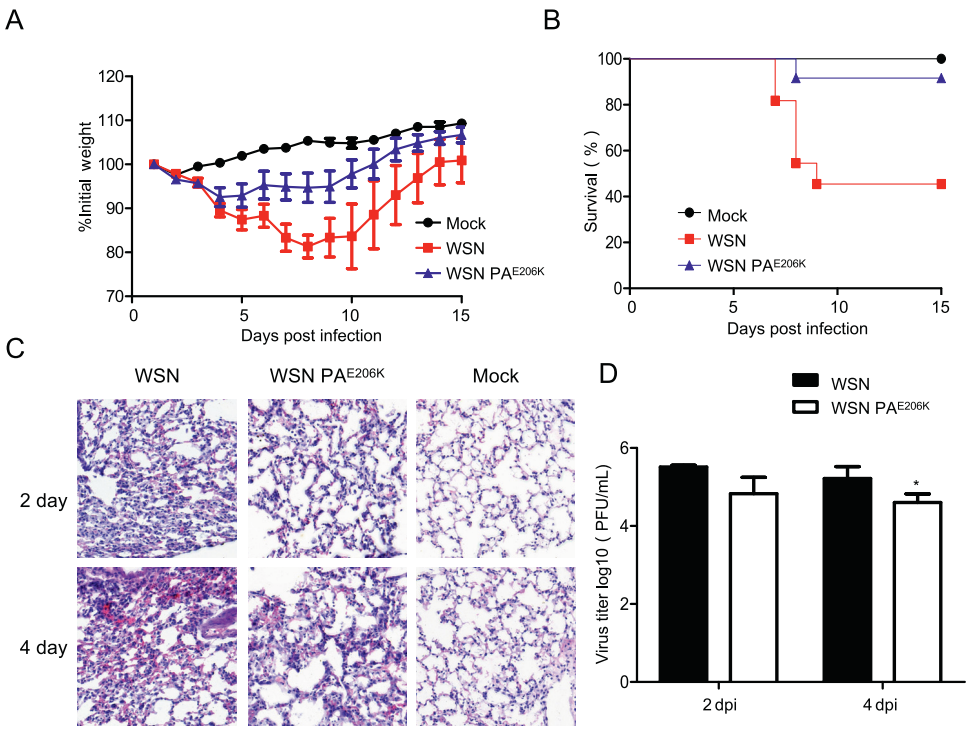


**Fig. 4.** Pathogenicity of wild-type and $PA^{E206K}$ WSN *in vivo*. BALB/c mice were intranasally inoculated with 30 μL PBS (Mock) or $10^4$ PFU (in 30 μL) of wild-type or $PA^{E206K}$ viruses (n = 11 per group). Mouse body weights (**A**) were monitored daily for 15 days and the survival rates (**B**) were calculated. **C** Lung sections were stained with haematoxylin and eosin. Magnification: ×100. **D** Viral titres from mice lung on days 2 and 4 post-infection. Data are shown as mean ± standard error of the mean (n = 3).

(Fig. 4A and B). Histological analyses further showed that the wild-type virus caused more severe pathology than the mutated virus, with more severe inflammatory cell infiltration, alveolar injury, and other pathological changes on days 2 and 4 post-infection (Fig. 4C). Consistently, virus titres in the lungs of WSN $PA^{E206K}$-infected mice were decreased by 0.7 and 0.6 log on days 2 and 4 post-infection compared with those infected with wild-type WSN (Fig. 4D). These data suggest that $PA^{E206K}$ is less pathogenic than the wild-type virus.

### 3.6. $PA^{E206K}$ is a temperature-sensitive mutant

It has been shown that the PA protein contains two domains connected by a 58-residue linker (198 to 256 aa). Moreover, the PA linker region was shown to have an identical structure in influenza A and B virus polymerases (Fan et al., 2019; Lukarska et al., 2017), and several positions in this domain have been confirmed to display a temperature-sensitive phenotype (Da Costa et al., 2015; Meyer et al., 2016). To test whether the herein identified $PA^{E206K}$ variant displayed a temperature-sensitive phenotype in mammalian cells, we measured the polymerase activity in two RNP reconstitution systems at different temperatures. Upon co-transfection of 293T cells with pPLOI-HA and pcDNA-PB1, pcDNA-PB2, pcDNA-PA (wild-type)/pcDNA-$PA^{E206K}$, and pcDNA-NP at 33, 37, and 39.5 °C, the HA levels were measured as a reflection of the overall polymerase activity. $PA^{E206K}$ showed to promote a temperature-sensitive phenotype in the RNP reconstitution system, with a level of polymerase activity of 80%, 55%, and 30% of that of the wild-type activity at 33, 37, and 39.5 °C, respectively (Fig. 5A and B). Next, analysis of the replicative abilities of the recombinant mutant viruses in MDCK cells (MOI = 0.001 PFU/mL) also showed that the growth of the WSN $PA^{E206K}$ virus was markedly delayed at 39.5 °C, with the virus titres at 24 hpi being of 0 PFU/mL, in contrast to $10^3$ PFU/mL for wild-type WSN (Fig. 5C). At 37 and 33 °C, the mutants replicated less efficiently than the wild-type virus and displayed similar multiplication kinetics in MDCK cells (Supplementary Fig. S4). These results suggest that the mutant $PA^{E206K}$ exhibits a temperature-sensitive phenotype.

### 3.7. $PA^{E206K}$ disrupts the cellular distribution of PB1 at high temperatures

The transcription and replication of the influenza virus occurs in the nucleus, which differs from those of other RNA viruses. Recent studies have indicated that efficient polymerase assembly and import to the nucleus are limiting factors for the viability of reassortant viruses (Watanabe et al., 2010; Hutchinson et al., 2012). To determine whether the $PA^{E206K}$ mutant could interfere with the nuclear import of the PA protein or the PA–PB1 complex, we co-transfected PB1-GFP and wild-type PA- or $PA^{E206K}$-expressing plasmids into 293A cells. Notably, PB1 localised to the nucleus when wild-type or mutated PA was co-expressed with PB1 at 37 °C. In contrast, PB1 and $PA^{E206K}$ were found mainly in the cytoplasm and were absent in the nucleus at 39.5 °C (Fig. 6A). Our findings suggest that the temperature sensitivity of $PA^{E206K}$ is associated with the inability of PA subunits to transport PB1 to the nucleus. Further analysis of the interaction between PA and PB1 at 37 and 39.5 °C showed that $PA^{E206K}$ was weakly associated with PB1 at 39.5 °C, but was similar to that of wild-type PA and PB1 at 37.5 °C (Fig. 6B). Hence, $PA^{E206K}$ can disturb the interaction between PA and PB1 at high temperatures.

### 3.8. $PA^{E206K}$ also affects influenza virus polymerase activity in DF-1 cells

The source of the 2009 H1N1 pandemic comprised genes derived from avian (PB2 and PA), human H3N2 (PB1), and classical swine (HA, NP, and NS) lineages (Smith et al., 2009), highlighting that adaptation of viral polymerase is necessary for efficient viral replication in new host species. Our results show that $PA^{E206K}$ reduced the viral polymerase activity of H1N1 in a vRNP reconstitution system in 293T cells. In agreement with this observation, $PA^{E206K}$ also significantly impaired the viral

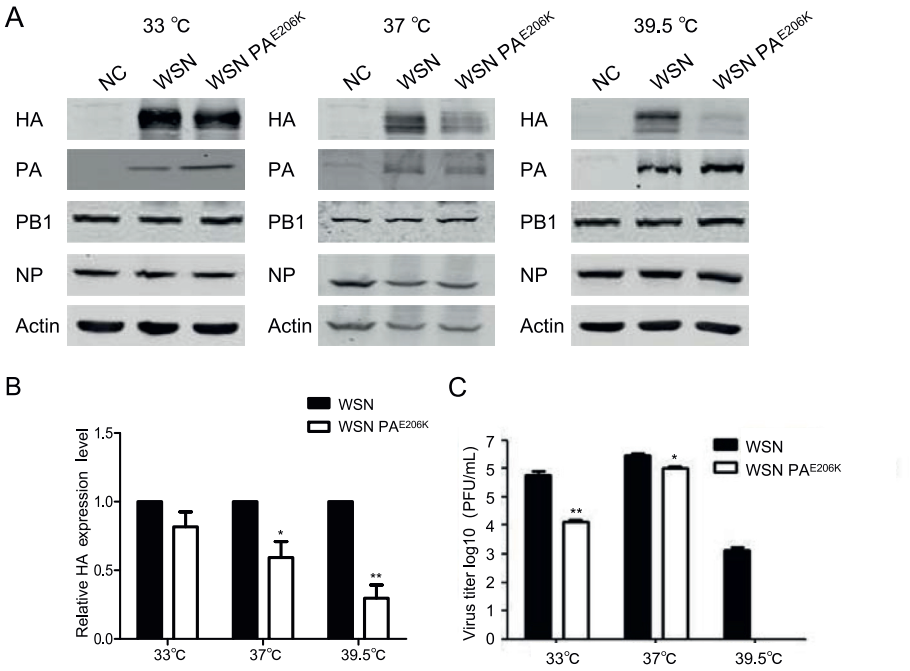


**Fig. 5.** Replication Kinetics of wild-type and $PA^{E206K}$ WSN viruses at different temperatures. **A** Viral polymerase activity was determined in a vRNP reconstitution system in 293T cells at different temperatures (33, 37, and 39.5 °C). **B** Statistical analysis of the HA levels in (**A**). Values of HA were standardized to the actin levels and normalized to those of HA in cells transfected with wild-type vector. **C** MDCK cells were infected with wild-type or $PA^{E206K}$ WSN at a multiplicity of infection of 0.001 at different temperatures (33, 37, and 39.5 °C) in A549 cells. Virus titres were determined via plaque assays at 24 h post-infection. Data represent the mean ± standard error of the mean of three independent experiments.

polymerase activity in DF-1 cells compared with the wild-type polymerase complex (Supplementary Figs. S5A and B). Furthermore, we introduced the mutation $PA^{E206K}$ in the context of A/Quail/Hong Kong/G1/97 (H9N2) reconstitution system and then compared the polymerase activities between $PA^{E206K}$ and wild-type polymerase (Supplementary Fig. S5C). Similarly, we also found that the presence of the introduced mutation $PA^{E206K}$ [A/Quail/Hong Kong/G1/97 (H9N2)] could reduce the activity of polymerase in vRNP reconstitution system in 293T cells. Hence, $PA^{K206}$ does not contribute to viral adaptation in new host species.

## 4. Discussion

The PA protein is one of the three subunits that consists of the heterotrimeric RNA-dependent RdRp of influenza viruses, and contributes to host adaptation and virulence of these viruses (Lee et al., 2017; Mehle et al., 2012; Bussey et al., 2011; Song et al., 2009). The 2009 pandemic H1N1 virus contains a unique combination of gene segments from swine, avian, and human viruses (Zhang et al., 2009; Trifonov et al., 2009); thus, similar to other influenza A viruses, this pandemic H1N1 may be associated with influenza A viruses co-infections. Therefore, it exhibits a change in replication and virulence. A previous study reported that the *PA* genes of the H1N1 pandemic virus increased vRNP activity and virulence of seasonal H1N1 when introduced into the genome of the seasonal virus, suggesting that PA may serve as a virulence factor of the H1N1 pandemic virus (Hsieh et al., 2011). In this study, we focused on the contribution of the $PA^{E206K}$ mutation in the 2009 pandemic H1N1 influenza virus.

Comparing the identity of PA sequences between two H1N1 pandemic influenza viruses (A/Beijing/2009 and A/Kurume/2009), the protein sequences of the two strains showed a high level of identity for the *PA* gene. Nevertheless, a novel PA substitution was identified (E206K), which reduces the polymerase activity and viral replication in mammalian cells. Most notably, few mutations that alter aa in polymerase complex subunits have been found to substantially affect polymerase activity, replication, virulence, or pathogenicity in mammals, as well as adaptation to new host species. Nevertheless, several studies have identified the aa positions in PA that affect replication and/or virulence. For example, $PA^{T162A}$ leads to a moderate decrease in protease activity and cRNA synthesis (Perales et al., 2000), and the $PA^{R638A}$ mutation destabilises the PA-RNA template interaction during elongation by promoting the synthesis of defective interfering RNAs, and causes severe attenuation of viral growth in cell culture. Moreover, the PA subunit has been shown to be involved in host adaptation; the avian H9N2 virus with $PA^{K356R}$ showed increased levels of viral transcription and virus production in human A549 cells; $PA^{K356R}$ along with $PB2^{E627K}$ may render avian H9N2 viruses adapted for human viruses (Xu et al., 2016); the $PA^{K185R}$ mutation of the H5N1 virus affects viral replication in both mammalian cells and mouse pathogenicity (Fan et al., 2014); and the aa substitution T157 in PA modulates polymerase activity for viral cRNA synthesis (Huarte et al., 2003).

The crystal structures of the RdRp revealed that the polymerase complex is formed by PB1 enclosed by the PA linker on one side and the N-terminal domain of PB2 on the other side (Fan et al., 2019; Lukarska et al., 2017; Hengrung et al., 2015). Using high-throughput mutagenesis and sequencing analysis, Chen et al. found that the PA linker is critical for polymerase activity and the formation of ionic interactions with the PA C-terminal channel. Furthermore, they indicated that the linker regions of PA do not tolerate insertional mutations, with deletions or insertions on the PA linker region impairing its binding with PB1, polymerase activity, and RNA synthesis. The PA linker plays a key role in the formation of a functional polymerase complex, which requires spatial arrangement (Wang et al., 2015). The aa at position 206 is located in the PA linker α7, α8, and α9 helices (201–257). Our data showed that mutation of Glu (E) to Lys (K) at position 206 in PA dramatically decreased polymerase activity and attenuated WSN virus growth in mammalian cells and mice. Two nuclear localization signals (NLSs) of the PA protein have been identified: NLS1 comprises the 124–139 aa and NLS2 comprises the

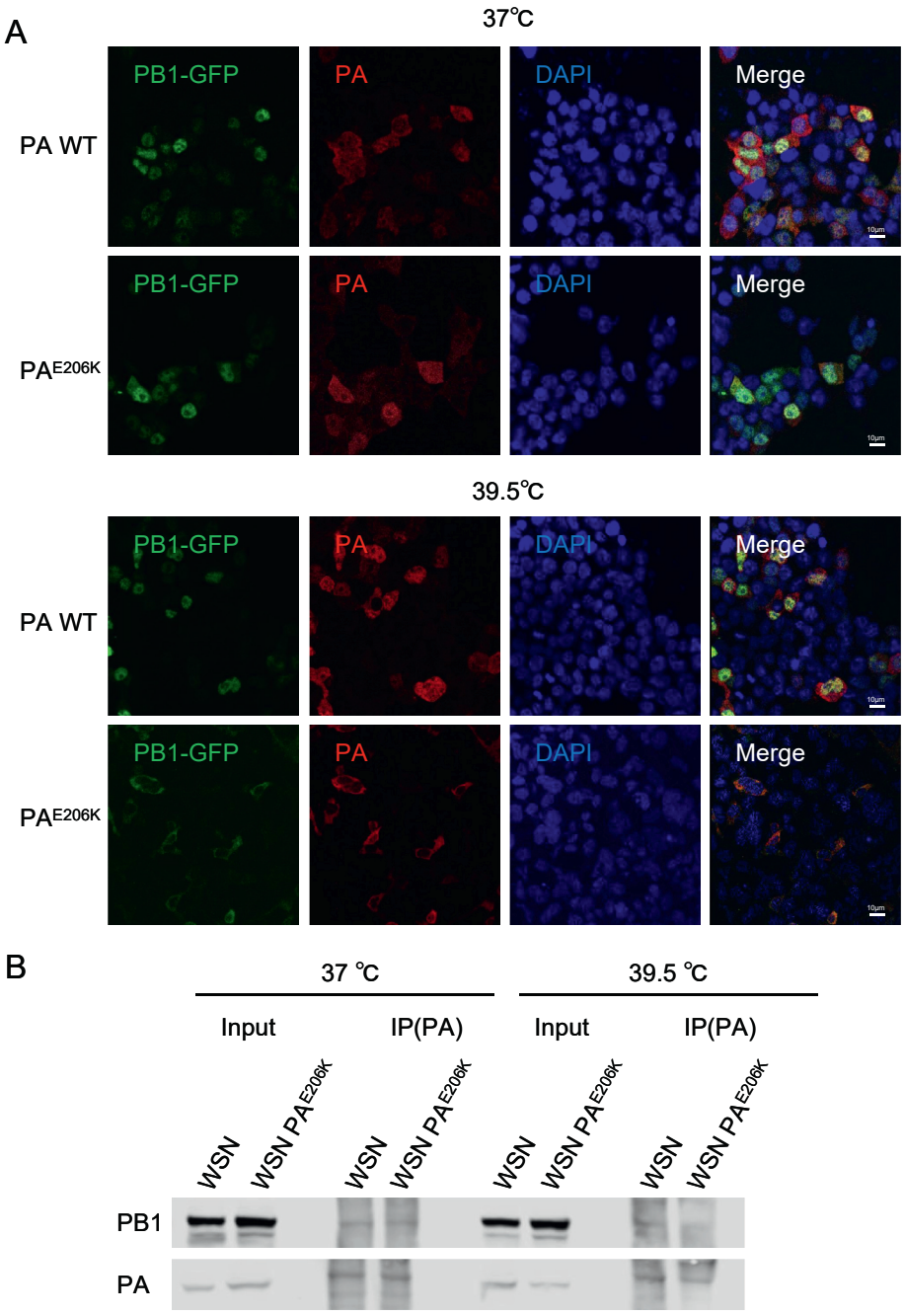


**Fig. 6.** Subcellular localization of PB1 and PA subunits (wild-type and PA$^{E206K}$) transiently expressed in 293T cells at 37 and 39.5 °C. **A** 293T cells were transfected with PB1-GFP and wild-type PA or PA$^{E206K}$ for 24 h. Cells were fixed and analysed by immunofluorescence. Scale bar = 10 μm. **B** 293T cells were transfected with PA or PA$^{E206K}$ with PB1. The transfected cells were incubated at 37 °C or 39.5 °C for 40h and then lysed with RIPA buffer. The lysates were analysed by co-immunoprecipitation with PA antibody.

186–247 aa (Yamaji et al., 2015). NLSs are recognised by host proteins, and allow viral and host protein complexes to pass through the nuclear pore complexes. As PA$^{E206K}$ belongs to NLS2, it may affect the efficient nuclear import of the viral genome in human cells. Interestingly, our data showed that PA$^{E206K}$ does not interfere with the PA–PB1 complex transport to the nucleus at 37 °C, whereas PB1 is mainly found in the cytoplasm when co-transfected with PA$^{E206K}$ at 39.5 °C. Moreover, our results also showed the PA$^{E206K}$ does not alter the interaction strength between PA and PB1 at 37 °C. Some host proteins that interact with PA affect the polymerase activity and viral replication (Hsu et al., 2013, Huarte et al., 2001). The majority of PA residues that affect polymerase activity in a host-specific manner are concentrated on the surface of the polymerase complex, and the linker region is also exposed to the surface. Thus, we speculate that the E206K substitution in the PA linker may interact with host factors, and that it is the strength of these interactions that affects viral polymerase activity.

Generation and characterisation of temperature-sensitive mutants are powerful tools for investigating essential steps in a virus life cycle. Recently, several temperature-sensitive mutations have been identified in PB1, PB2, and PA. The PA subunit is critical for modulating vRNP activity under thermal stress (Bussey et al., 2011; Zhang et al., 2012). In particular, a large series of PA linker mutants exhibit a temperature-sensitive phenotype, with nine aa positions (T201P, K213P, D216P, L219P, P221A, N222P, F223P, S225P, and L226P) in the PA linker being

identified as appropriate for engineering temperature-sensitive mutants. The temperature-sensitive phenotype of these mutants is associated with a defect in the transport of the PA–PB1 complex into the nucleus (Da Costa et al., 2015). Meyer et al. indicated that aa deletions at positions 207, 208, 209, and 210 in the PA linker result in temperature sensitivity and attenuated phenotypes (Meyer et al., 2016). Furthermore, they found that PB1 and PA Δ206 were mainly observed in the cytoplasm and were absent from the nucleus at 39.5 °C, where were transported into the nucleus at 33 °C. These findings suggest that $PA^{K206}$ may be unable to recruit PB1 to the nucleus at a higher temperature, which agrees with our results. In addition, they found that deletion of position 206 of PA does not allow virus recovery, suggesting that PA 206 residue may play a key role in the protein structure or function. Our results showed that $PA^{E206K}$ reduces viral replication in both mammalian cells and mice. Taken together, all these results suggest that this mutation can severely impair the PA structure and/or function.

It is well known that influenza vaccines can prevent influenza infection through immunisation. The global threat of pandemic influenza and H7N9 avian influenza highlighted the importance of vaccines, and promoted the development and evaluation of candidate vaccines. Currently, inactivated influenza vaccines (IIVs) and live attenuated vaccines (LAIVs) are the two main types of influenza virus vaccines available. Unlike IIV, which can induce a serum antibody response, seasonal LAIV induces serum and mucosal antibody responses, as well as T-cell responses. Cross-protection against antigenic drift variants of LAIV is attractive for controlling pandemic influenza (Subbarao et al., 2020). Most notably, some countries have continued to use LAIV as the vaccine of choice, based on these characteristics. Considering the different temperatures between the upper and lower respiratory tracts, temperature-sensitive viruses are a logical approach for vaccine development (Treanor et al., 2020; Weinberg et al., 2020; Lopez et al., 2020). Our results show that $PA^{E206K}$ displays a temperature-sensitive phenotype and attenuates influenza virus *in vitro* and *in vivo*, suggesting that the $PA^{E206K}$ variant can be used to design LAIVs.

## 5. Conclusions

In summary, in this study we identified a key mutation in PA (at position 206) that affects polymerase activity. This position lies in the PA linker that is responsible for binding PB1 and is involved in recruiting PB1 to the nucleus. Moreover, substitution of aa in this position results in temperature-sensitive phenotypes. Hence, our findings further our understanding on PA protein function.

## Data availability

All the data generated during the current study are included in the manuscript.

## Ethics statement

The animal study protocol was approved by the Ethics Committee of National Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College.

## Author contributions

Mengmeng Cao: conceptualization, data curation, formal analysis, validation, writing. Qiannan Jia: formal analysis, investigation, methodology. Jinghua Li: data curation, formal analysis, investigation, methodology. Lili Zhao: supervision, validation. Li Zhu: data curation, validation. Yufan Zhang: data curation, validation. Shan Li: data curation, validation. Tao Deng: conceptualization, project administration, supervision, writing.

## Conflict of interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2023.11.005.

## References

Avit, D.F.S., Jain, S., Finelli, L., Shaw, M.W., Lindstrom, S., Garten, R.J., Gubareva, L.V., Xu, X., Bridges, C.B., Uyeki, T.M., 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N. Engl. J. Med. 360, 2605–2615.

Bussey, K.A., Desmet, E.A., Mattiacio, J.L., Hamilton, A., Bradel-Tretheway, B., Bussey, H.E., Kim, B., Dewhurst, S., Takimoto, T., 2011. PA residues in the 2009 H1N1 pandemic influenza virus enhance avian influenza virus polymerase activity in mammalian cells. J. Virol. 85, 7020–7028.

Da Costa, B., Sausset, A., Munier, S., Ghounaris, A., Naffakh, N., Le Goffic, R., Delmas, B., 2015. Temperature-sensitive mutants in the influenza A virus RNA polymerase: alterations in the PA linker reduce nuclear targeting of the PB1-PA dimer and result in viral attenuation. J. Virol. 89, 6376–6390.

Fan, H., Walker, A.P., Carrique, L., Keown, J.R., Martin, I.S., Karia, D., Sharps, J., Hengrung, N., Pardon, E., Steyaert, J., Grimes, J.M., Fodor, E., 2019. Influenza A virus RNA polymerase structures provide insights into viral genome replication. Nature 573, 287–290.

Fan, S., Hatta, M., Kim, J.H., Le, M.Q., Neumann, G., Kawaoka, Y., 2014. Amino acid changes in the influenza A virus PA protein that attenuate avian H5N1 viruses in mammals. J. Virol. 88, 13737–13746.

Fodor, E., 2013. The RNA polymerase of influenza A virus: mechanisms of viral transcription and replication. Acta Virol. 57, 113–122.

Fodor, E., Crow, M., Mingay, L.J., Deng, T., Sharps, J., Fechter, P., Brownlee, G.G., 2002. A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase inhibits endonucleolytic cleavage of capped RNAs. J. Virol. 76, 8989–9001.

Hengrung, N., Omari, K.E., Martin, I.S., Vreede, F.T., Cusack, S., Rambo, R.P., Vonrhein, C., Bricogne, G., Stuart, D.I., Grimes, J.M., Fodor, E., 2015. Crystal structure of the RNA-dependent RNA polymerase from influenza C virus. Nature 527, 114–117.

Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G., Webster, R.G., 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. U.S.A. 97, 6108–6113.

Hsieh, E.F., Lin, S.J., Mok, C.K., Chen, G.W., Huang, C.H., Wang, Y.C., Chen, T.C., Chen, C.J., Ojcius, D.M., Shih, S.R., 2011. Altered pathogenicity for seasonal influenza virus by single reassortment of the RNP genes derived from the 2009 pandemic influenza virus. J. Infect. Dis. 204, 864–872.

Hu, J., Liu, X., 2015. Crucial role of PA in virus life cycle and host adaptation of influenza A virus. Med. Microbiol. Immunol. 204, 137–149.

Huarte, M., Sanz-Ezquerro, J.J., Roncal, F., Ortín, J., Nieto, A., 2001. PA subunit from influenza virus polymerase complex interacts with a cellular protein with homology to a family of transcriptional activators. J. Virol. 75, 8597–8604.

Huarte, M., Falcón, A., Nakaya, Y., Ortín, J., García-Sastre, A., Nieto, A., 2003. Threonine 157 of influenza virus PA polymerase subunit modulates RNA replication in infectious viruses. J. Virol. 77, 6007–6013.

Hutchinson, E.C., Fodor, E., 2012. Nuclear import of the influenza A virus transcriptional machinery. Vaccine 30, 7353–7358.

Hsu, W.B., Shih, J.L., Shih, J.R., Du, J.L., Teng, S.C., Huang, L.M., Wang, W.B., 2013. Cellular protein HAX1 interacts with the influenza A virus PA polymerase subunit and impedes its nuclear translocation. J. Virol. 87, 110–123.

Krumbholz, A., Philipps, A., Oehring, H., Schwarzer, K., Eitner, A., Wutzler, P., Zell, R., 2011. Current knowledge on PB1-F2 of influenza A viruses. Med. Microbiol. Immunol. 200, 69–75.

Lee, I., Il Kim, J., Park, S., Bae, J.Y., Yoo, K., Yun, S.H., Lee, J.Y., Kim, K., Kang, C., Park, M.S., 2017. Single PA mutation as a high yield determinant of avian influenza vaccines. Sci. Rep. 7, 40675.

Lopez, C.E., Legge, K.L., 2020. Influenza A virus vaccination: immunity, protection, and recent advances toward A universal vaccine. Vaccines 8, 434.

Lukarska, M., Fournier, G., Pflug, A., Resa-Infante, P., Reich, S., Naffakh, N., Cusack, S., 2017. Structural basis of an essential interaction between influenza polymerase and Pol II CTD. Nature 541, 117–121.

Mehle, A., Dugan, V.G., Taubenberger, J.K., Doudna, J.A., 2012. Reassortment and mutation of the avian influenza virus polymerase PA subunit overcome species barriers. J. Virol. 86, 1750–1757.
Meyer, L., Sausset, A., Sedano, L., Da Costa, B., Le Goffic, R., Delmas, B., 2016. Codon deletions in the influenza A virus PA gene generate temperature-sensitive viruses. J. Virol. 90, 3684–3693.
Mishra, A.C., Chadha, M.S., Choudhary, M.L., Potdar, V.A., 2010. Pandemic influenza (H1N1) 2009 is associated with severe disease in India. PLoS One 5, e10540.
Muramoto, Y., Noda, T., Kawakami, E., Akkina, R., Kawaoka, Y., 2013. Identification of novel influenza A virus proteins translated from PA mRNA. J. Virol. 87, 2455–2462.
Nogales, A., Martinez-Sobrido, L., Chiem, K., Topham, D.J., DeDiego, M.L., 2018. Functional evolution of the 2009 pandemic H1N1 influenza virus NS1 and PA in humans. J. Virol. 92, e01206-e01218.
Palese, P., Shaw, M.L., 2013. Orthomyxoviridae. In: Knipe, D.M., Howley, P.M., Cohen, J.I., Griffifin, D.E., Lamb, R.A., Martin, M.A., Racaniello, V.R., Roizman, B. (Eds.), Fields Virology, sixth ed. Lippincott Williams and Wilkins, Philadelphia, pp. 1151–1185.
Perales, B., Sanz-Ezquerro, J.J., Gastaminza, P., Ortega, J., Santarén, J.F., Ortín, J., Nieto, A., 2000. The replication activity of influenza virus polymerase is linked to the capacity of the PA subunit to induce proteolysis. J. Virol. 74, 1307–1312.
Rodriguez-Frandsen, A., Alfonso, R., Nieto, A., 2015. Influenza virus polymerase: functions on host range, inhibition of cellular response to infection and pathogenicity. Virus Res. 209, 23–38.
Selman, M., Dankar, S.K., Forbes, N.E., Jia, J.J., Brown, E.G., 2012. Adaptive mutation in influenza A virus non-structural gene is linked to host switching and induces a novel protein by alternative splicing. Emerg. Microb. Infect. 1, e42.
Sherbany, H., McCauley, J., Meningher, T., Hindiyeh, M., Dichtiar, R., Markovich, M.P., Mendelson, E., Mandelboim, M., 2014. Return of pandemic H1N1 influenza virus. BMC Infect. Dis. 14, 710.
Smith, G.J., Vijaykrishna, D., Bahl, J., Lycett, S.J., Worobey, M., Pybus, O.G., Ma, S.K., Cheung, C.L., Raghwani, J., Bhatt, S., Peiris, J.S., Guan, Y., Rambaut, A., 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459, 1122–1125.
Song, M.S., Pascua, P.N., Lee, J.H., Baek, Y.H., Lee, O.J., Kim, C.J., Kim, H., Webby, R.J., Webster, R.G., Choi, Y.K., 2009. The polymerase acidic protein gene of influenza A virus contributes to pathogenicity in a mouse model. J. Virol. 83, 12325–12335.
Stevaert, A., Naesens, L., 2016. The influenza virus polymerase complex: an update on its structure, functions, and significance for antiviral drug design. Med. Res. Rev. 36, 1127–1173.
Stubbs, T.M., Te Velthuis, A.J., 2014. The RNA-dependent RNA polymerase of the influenza A virus. Future Virol. 9, 863–876.
Subbarao, K., 2020. Live attenuated influenza vaccines for pandemic preparedness. J. Ped. Inf. Dis. Soc. 9, S15–S18.
Treanor, J., 2020. History of live, attenuated influenza vaccine. J. Ped. Inf. Dis. Soc. 9, S3–S9.
Trifonov, V., Khiabanian, H., Rabadan, R., 2009. Geographic dependence, surveillance, and origins of the 2009 influenza A (H1N1) virus. N. Engl. J. Med. 361, 115–119.
Wang, L., Wu, A., Wang, Y.E., Quanquin, N., Li, C., Wang, J., Chen, H.W., Liu, S., Liu, P., Zhang, H., Qin, F.X.F., Jiang, T.J., Cheng, G.H., 2015. Functional genomics reveals linkers critical for influenza virus polymerase. J. Virol. 90, 2938–2947.
Watanabe, T., Watanabe, S., Kawaoka, Y., 2010. Cellular networks involved in the influenza virus life cycle. Cell Host Microbe 7, 427–439.
Weinberg, G.A., 2020. Live, attenuated influenza vaccine: present and future roles. J. Ped. Inf. Dis. Soc. 9, S1–S2.
Wise, H.M., Hutchinson, E.C., Jagger, B.W., Stuart, A.D., Kang, Z.H., Robb, N., Schwartzman, L.M., Kash, J.C., Fodor, E., Firth, A.E., Gog, J.R., Taubenberger, J.K., Digard, P., 2012. Identification of a novel splice variant form of the influenza A virus M2 ion channel with an antigenically distinct ectodomain. PLoS Pathog. 8, e1002998.
Xu, G., Zhang, X., Gao, W., Wang, C., Wang, J., Sun, H., Sun, Y., Guo, L., Zhang, R., Chang, K.C., Liu, J.H., Pu, J., 2016. Prevailing PA mutation K356R in avian influenza H9N2 virus increases mammalian replication and pathogenicity. J. Virol. 90, 8105–8114.
Yamaji, R., Yamada, S., Le, M.Q., Ito, M., Sakai-Tagawa, Y., Kawaoka, Y., 2015. Mammalian adaptive mutations of the PA protein of highly pathogenic avian H5N1 influenza virus. J. Virol. 89, 4117–4125.
Yang, H., Dong, Y.R., Bian, Y., Huo, C.Z., Zhu, C.C., Qin, T., Chen, S.J., Peng, D.X., Liu, X.F., 2023. The synergistic effect of residues 32T and 550L in the PA protein of H5 subtype avian influenza virus contributes to viral pathogenicity in mice. PLoS Pathog. 19, e1011489.
Yi, C.Y., Zhao, Z.Z., Wang, S.Y., Sun, X., Zhang, D., Sun, X.M., Zhang, A.D., Jin, M.D., 2017. Influenza A virus PA antagonizes interferon-βby interacting with interferon regulatory factor 3. Front. Immunol. 8, 1051–1059.
Zhang, H., Chen, L., 2009. Possible origin of current influenza A H1N1 viruses. Lancet Infect. Dis. 9, 456–457.
Zhang, W., Tu, J., Zhao, Z., Chen, H., Jin, M., 2012. The new temperature-sensitive mutation PA-F35S for developing recombinant avian live attenuated H5N1 influenza vaccine. Virol. J. 9, 97.