Replication of a low-pathogenic avian influenza virus is enhanced by chicken ubiquitin-specific protease 18

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Abstract

Previous research revealed the induction of chicken USP18 (chUSP18) in the lungs of chickens infected with highly pathogenic avian influenza viruses (HPAIVs). This activity was correlated with the degree of pathogenicity of the viruses to chickens. As mammalian ubiquitin-specific protease (USP18) is known to remove type I interferon (IFN I)-inducible ubiquitin-like molecules from conjugated proteins and block IFN I signalling, we explored the function of the chicken homologue of USP18 during avian influenza virus infection. With this aim, we cloned chUSP18 from cultured chicken cells and revealed that the putative chUSP18 ORF comprises 1137 bp. Comparative analysis of the predicted aa sequence of chUSP18 with those of human and mouse USP18 revealed relatively high sequence similarity among the sequences, including domains specific for the ubiquitin-specific processing protease family. Furthermore, we found that chUSP18 expression was induced by chicken IFN I, as observed for mammalian USP18. Experiments based on chUSP18 over-expression and depletion demonstrated that chUSP18 significantly enhanced the replication of a low-pathogenic avian influenza virus (LPAIV), but not an HPAIV. Our findings suggest that chUSP18, being similar to mammalian USP18, acts as a pro-viral factor during LPAIV replication in vitro.

INTRODUCTION

The innate immune system is the first immune barrier to invading pathogens. The type I interferon (IFN I) plays an important role by establishing an antiviral state in the host through the induction of IFN I-stimulated genes (ISGs), such as 2,5-oligoadenylate synthetase (OAS), double-stranded RNA-activated protein kinase and Mx [1]. Chicken IFN I (chIFN I) expression is strongly induced upon infection by avian influenza virus (AIV) [2, 3]. ChIFN I stimulation protects chicken cells against AIV infection [4, 5]. Chickens are vulnerable to highly pathogenic avian influenza viruses (HPAIVs), but the mechanism by which their innate immune response acts against HPAIV infection, including the involvement of chIFN I, is not well understood.

Previously, our microarray analysis revealed the induction of several immune-related genes in the lungs of chickens infected with HPAIVs to a degree that was related to the pathogenicity of the viruses to the chickens [6]. Among these, chicken ubiquitin-specific protease 18 (USP18; chUSP18), a homologue of human USP18 (huUSP18), which is involved in the ISG15 system, displayed 25-fold induction at 24 h after an HPAIV infection. Human ISG15 (huISG15) is a ubiquitin-like molecule that is up-regulated by IFNs and covalently conjugated to a target protein through sequential enzymatic reactions that are collectively termed ISGylation, which is governed by E1 activating enzyme (Ube1L), E2-conjugating enzyme (UbcH8) and E3 ligase (mainly Herc5), and induced by IFNs [7]. ISGylated proteins are subjected to further degradation by autophagy [8]. USP18 acts as a deISGylation enzyme that specifically removes ISG15 from conjugated proteins through its isopeptidase activity [9]. In influenza A virus-infected human cells, it was reported that huISG15 was specifically conjugated to the viral NS1 and M1 proteins, and through this ISGylation suppressed viral replication [10]. Therefore, it is conceivable that huUSP18 favoured influenza virus replication through removal of ISG15 from the targeted proteins. In accordance with this finding, ISG15-knockout mice exhibited increased susceptibility to influenza A virus.
infection, although viral replication in ISG15-knockout MEF cells was scarcely affected [11, 12]. Recently, it was reported that the isopeptidase activity-specific inactivation of mouse USP18 (mUSP18) enhanced ISGylation, thereby increasing resistance to influenza B virus infection [13].

In addition to its isopeptidase activity, USP18 also blocks IFN I signalling [14]. The signal from IFN I binding to the IFNAR1/IFNAR2 complex receptor on the cell surface is transmitted downstream through the Janus kinase and the signal transducer and activator of transcription (JAK-STAT) pathway [15, 16]. Once IFN I binds to the receptor, the cytoplasmic tails of IFNAR2 and IFNAR1 are phosphorylated by JAK1 and TYK2, respectively, which are constitutively associated with them. Their phosphorylation leads to the binding, phosphorylation and subsequent dimerization of STAT1 and STAT2. A complex of the dimer and IRF9 can translocate to the nucleus and induce the expression of ISGs. MUSP18 can interfere with IFN I signalling by competing with the JAK1 protein for binding to the IFNAR2 receptor. It was found that mUSP18 suppressed the replication of vesicular stomatitis virus in MEF cells independently of the ISG15 pathway [14].

No homologue of mammalian ISG15 has been identified in chickens; therefore, the function of chUSP18 in AIV infection, if any, has yet to be elucidated. In this study, to elucidate the function of chUSP18, we cloned the cDNA of chUSP18 transcripts derived from chUSP18 and identified ORFs as well as regulatory elements in a non-coding region. In addition, we assessed the effects of chUSP18 over-expression and depletion in chicken cells on viral replication, RNA transcription and protein expression, as well as those of host pro-viral proteins, to clarify whether the expression of chUSP18 favoured AIV infection in vitro.

RESULTS

Characteristics of chicken USP18

To molecularly characterize chicken chUSP18, we identified the transcriptional start and terminations sites of chUSP18 in cultured LMH cells using 5′- and 3′-RACE methods with chUSP18-specific primers, which were designed using the sequence of USP18 (XM_416398.2) in the NCBI database. The longest cDNA derived from the chUSP18 transcripts was 2633 bp in length, with a putative ORF of 1137 bp and 5′- and 3′-untranslated regions of 1469 bp (Fig. 1a). Using the NCBI database, we identified an ISRE motif, which has been reported to be an IFN-stimulated response element [17], upstream of the transcriptional start site of chUSP18 (Fig. 1b). The putative chUSP18 protein translated from this ORF consisted of 379 aa, producing a molecular weight of 43.8 kDa (Fig. 1c). We confirmed by PCR amplification that the cDNA sequence of chUSP18 from DF-1 cells was not different in the ORF region and identified a single-base substitution in both the 5′- and 3′-untranslated regions compared to two consensus sequences from DF-1 and LMH cell strains. Comparative analysis of the putative aa sequence with the human (NP_059110.2) and mouse sequences (CAJ18436.1) revealed that chUSP18 exhibited relatively high sequence identity with the homologues [chicken/human identity: 186/356 (52 %); chicken/mouse identity: 183/357 (51 %)], and we uncovered domains specific for the ubiquitin-specific processing protease family, such as a Cys box at aa 61–76, a QQDAQEF motif at aa 142–148, a LPQILVHLLRF consensus sequence at aa 253–264, and His boxes at aa 308–325, 337–345 and 352–355 (Fig. 1c), as previously reported [18]. Transcript variant analysis revealed at least three additional transcript variants of the chUSP18 ORF (bands b, c and d in Fig. 1a). In transcript b, in-frame deletions were found in the fourth and fifth exons, and the putative aa sequence from this transcript consisted of 323 aa and featured the deletion of 56 aa containing the QQDAQEF and LPQILVHLLRF motifs and the His boxes. Transcript c was poly-adenylated at an upper-stream putative poly (A) site in the untranslated region compared to the full-length transcript.

Next, chUSP18 protein expression in DF-1 cells was examined by transient transfection with a chUSP18-expressing vector containing the full-length ORF and the 5′-untranslated region, which was designated pCMV14-USP18. Twenty-four hours after transfection, chUSP18 proteins fused with FLAG tags at the C-terminus were detected by Western blotting with FLAG antibody. ChUSP18 proteins with at least three different molecular weights were observed (Fig. 1d, left). Because there are two ATG codons at 9 and 72 nucleotides downstream of the initial ATG codon (Fig. 1d, right) in the chUSP18 coding sequence, respectively, we performed site-directed mutagenesis of these ATG codons to determine which serves as the dominant translational start site. It was demonstrated that the third ATG codon is used as the dominant start site in DF-1 cells, and all three ATG codons were functional as the translational start site, as three different molecular sizes of chUSP18 were observed by Western blotting of the mutated constructs (Fig. 1d left).

Induction of chUSP18 expression in vitro upon infection by recombinant AIVs or IFN-β stimulation

To identify a stimulus that induces chUSP18 expression, we first measured chUSP18 mRNA levels in two cell lines, DF-1 and LMH, after infection with two recombinant influenza viruses previously generated by our group [6]. One is a reverse genetics-generated A/chicken/Yamaguchi/7/2004 strain (H5N1; rHP) that causes 100 % lethality when 10⁶ 50 % egg infectious dose (EID₅₀) viruses are inoculated intranasally into chickens. The other is a reverse genetics-generated reassortant virus, designated LP (W/PA), that does not cause lethal infection in chickens intranasally inoculated with 10⁶ EID₅₀ viruses, although it has the same HA and NA with the rHP. Our previous study demonstrated that chUSP18 mRNA expression was higher in the lungs of chickens infected with rHP at 24 h post-infection (p.i.)
Fig. 1. ChUSP18 transcripts and aa sequence. (a) ChUSP18 transcript variants are depicted by boxes indicating each exon with numbers, and grey boxes represent the coding regions. The arrow denotes the direction and binding site of primers specific for RACE analysis. Transcript a represents the longest transcript hypothesized from the RACE products from LMH cells. In DF-1 cells, the transcripts corresponding to a are denoted by a' and a". The three putative transcriptional variants are denoted by b, c and d. (b) Upstream sequence of chUSP18. The 5' end of the longest cDNA is underlined. The ISRE motif is boxed. (c) chUSP18 from LMH cells was aligned with human and mouse USP18 homologues. Identical and conserved residues are coloured black and grey, respectively. The conserved regions of the ubiquitin-specific processing protease family are boxed in reference to a previous report [18]. The grey
compared to those infected with LP (W/PA). In the in vitro experiment in this study (Fig. 2), the peak of chUSP18 mRNA expression was observed at 24 h p.i. in LP (W/PA)-infected DF-1 cells and at 48 h p.i. in rHP-infected cells, and it was also higher in the cells infected with rHP than in those with LP (W/PA). In the same experiments, rapid increases of IFN-α (chIFN-α) and IFN-β (chIFN-β) mRNA levels were observed at 12 h p.i. in LP (W/PA)-infected DF-1 cells and at 24 h p.i. in rHP-infected cells (Fig. 2). Similar phenomena were observed in LMH cells infected with these viruses. Thus, it was suggested that chUSP18 transcription was induced following the expression of IFN-1 genes.

We next determined whether stimulation by chIFN-1 induced the expression of chUSP18, as an ISRE motif was identified as described previously (Fig. 1b). As shown in Fig. 3, the chUSP18 mRNA levels increased significantly at 4 h after treatment with chIFN-β in DF-1 cells in the same manner as chicken MX1 (chMX1) and OAS-like genes (chOASL) (Fig. 3).

ChUSP18 expression enhanced LPAIV replication in DF-1 cells

To investigate the function of chUSP18 during virus infection, we examined viral replication in DF-1 cells that over-expressed chUSP18 from pCMV14-USP18. The replication of A/chicken/Yokohama/aq55/2001 (LP) was significantly enhanced, by almost eightfold, in the chUSP18-over-expressing cells compared to that in cells transfected with the control vector, whereas that of A/chicken/Yamaguchi/7/2004 (HP) was not significantly altered even at 12 h p.i. in addition to 24 h p.i. (Fig. 4a, b). ChIFN-β did not exert any effect on this phenomenon, as no effect on viral replication was observed in either LP- or HP-infected cells pre-treated with chIFN-β.

To ensure that the over-expression of chUSP18 indeed enhanced LP replication, we examined viral replication when chUSP18-specific siRNA was simultaneously transfected with pCMV14-USP18. Three siRNAs (si375, si401 and si883) that effectively reduced both chUSP18 mRNA and chUSP18 protein expressions in cells over-expressing chUSP18 compared to a negative control (NC) were used in the following experiments (Fig. 4c). Among the siRNAs, si401 and si883 also reduced LP replication with statistical significance (Fig. 4d).

We then examined the effect of chIFN-β-induced chUSP18 expression on LP replication in DF-1 cells transfected with siRNA. When the chUSP18-depleted DF-1 cells were treated with chIFN-β, all siRNAs significantly reduced LP replication (Fig. 4e). Conversely, no significant reduction of replication was observed in the absence of chIFN-β treatment, although significant chUSP18 depletion was evident (Fig. 4e). Taken together, these results clearly indicated that chUSP18 enhanced LP replication in those cells.

ChUSP18 expression affects LPAIV replication at the viral mRNA transcription and protein expression levels

To determine the point at which chUSP18 affects viral replication, we measured the mRNA levels of the viral nucleoprotein (NP) and matrix protein (M) genes in cells infected with LP virus. As shown in Fig. 5(a), when DF-1 cells were
treated with chIFN-β after the depletion of chUSP18 using si375, si401 or si883, significant reductions of NP and M mRNA expression were observed at 24 h p.i. for all siRNAs (NP: 39, 28 and 38 % of control, respectively; M: 26, 13 and 22 % of control, respectively), whereas among those treated without chIFN-β, chUSP18-depleted cells using si375 significantly increased NP and M mRNAs at 8 and 24 h p.i. and at 8 h p.i., respectively. In addition, a significant increase of NP mRNA levels was observed at both 8 and 24 h p.i. when DF-1 cells over-expressing chUSP18 were treated without chIFN-β (data not shown).

We also measured NP and M1 protein expression in cells infected with LP virus. As shown in Fig. 5(b), the total amount of NP protein in DF-1 cells transfected with si375, si401 or si883 was significantly reduced to 13, 4 and 14 % of control, respectively, when the cells were treated with chIFN-β, whereas among those not treated with chIFN-β, it was significantly reduced in chUSP18-depleted cells using si375. The reason for the distinct behaviour of viral mRNA and protein in si375-transfected cells was not the effect on viral mRNA and protein, but rather the fluctuation of actin mRNA and protein in the cells (data not shown), although the reason for this was not clear. In addition, under the same treatment, M1 protein expression was significantly increased by 22 % of control, respectively, whereas among those treated with chIFN-β, followed by LP infection, the ratio of NP-positive cells in chUSP18-depleted cells at 24 h p.i. was significantly lower than in the control (Fig. 6b), whereas there was no significant difference in relative fluorescence level among the NP-positive cells (Fig. 6c). These results indicate that the virus-infected chUSP18-depleted cells appeared to produce NP protein to a level that was equivalent to that of the control cells; however, later steps of virus maturation and/or a multi-step replication of the virus seemed impaired.

To further elucidate the mechanism by which chUSP18 affects virus replication in chIFN-β-treated cells, we next measured the mRNA expression levels of the MX1 and OASL genes, which are regulated by the JAK/STAT-mediated IFN signalling pathway in chUSP18-depleted cells treated with chIFN-β. The expression of both genes in chUSP18-depleted cells was sustained at 24 h after chIFN-β treatment, while it was reduced in NC (Fig. 7). It appeared that sustained anti-viral host proteins were able to maintain the anti-viral state in chIFN-β-treated chUSP18-depleted cells. Together, these results suggest that the pro-viral effect of chUSP18 may be governed, at least in part, by the interfering IFN signalling pathway that otherwise negatively feedbacks the IFN-induced anti-virus state of the cell.

**DISCUSSION**

In this study, we demonstrated that chUSP18 expression increased the replication of an LP virus *in vitro*. Given the reported functions of mammalian USP18, two plausible mechanisms of chUSP18-mediated viral enhancement could be considered. First, chUSP18 may function as a deISGylase, as reported for huUSP18 [10]. However, no gene homologous to mammalian ISG15 has been found in the chicken genome, although other homologue genes involved in the ISGylation system, such as *Ube1L*, *UbCH8*, *HERC5* and...
Fig. 4. ChUSP18 expression enhances LP replication in DF-1 cells. (a, b) Virus titre in DF-1 cells electroporated with pCMV14 or pCMV14-chUSP18, incubated with growth medium with (+) or without (−) chIFN-β and infected with LP (a) or HP (b) at an m.o.i. of 0.1. (c) chUSP18-silencing efficiency in DF-1 cells electroporated simultaneously with pCMV14-USP18 and negative control (NC), si375, si401 or si883 is shown at mRNA (left) and FLAG protein levels (right). (d) Virus titre in DF-1 cells simultaneously electroporated with pCMV14-USP18 and NC, si375, si401 or si883, incubated with growth medium without chIFN-β and infected with LP at an m.o.i. of 0.1. (e) Virus titre in DF-1 cells electroporated with NC, si375, si401 or si883, incubated with growth medium with or without chIFN-β and infected with LP at an m.o.i. of 0.1. Virus titres and relative chUSP18 expression mRNA levels at 24 h post-infection (p.i.) are shown on the left and right, respectively. The chUSP18 expression level in cells treated with NC in growth medium without chIFN-β at 24 h p.i.
USP18, have been identified in the chicken genome database [19]. Some immune-related gene homologues, such as human IRF3, IRF9, TLR8 and TLR9, have not been found in the chicken genome [3, 19–21]. The absence of TLR9 from the chicken genome has been reported to be compensated for by chicken TLR21 [22]. Our previous study suggested that the function of RIG-I, another molecule missing from the chicken genome, was in part compensated by chicken MDA5 [23]. Given the evolutionary distance between mammals and galliform birds such as chickens and quails, the lack of ISG15 in chickens might be overcome by an as-yet unidentified ubiquitin-like molecule in the ISGylation system of chickens. It might also be possible that although the chicken genome encodes ISG15, insufficient readings of the chicken genome have not permitted its identification. In that case, further scrutiny of the chicken genome, along with the involvement of the catalytic activity of chUSP18 as isopeptidase, is needed.

Another mechanism of chUSP18-mediated viral replication may be that chUSP18 interferes with IFN I signalling to suppress the expression of genes involved in the chicken innate immune system. It has been reported that mUSP18 interfered with IFN I signalling by competing with JAK1 was set at 1.0 as a reference. Each bar represents the mean±SD from three independent experiments; *P<0.05 versus cells treated with growth medium without chIFN-β, #P< 0.05 versus pCMV14 or NC, Steel–Dwass test.

**Fig. 5.** ChUSP18 expression in cells infected with the LP virus affects both viral RNA transcription and protein expression. (a, b) Viral nucleoprotein (NP) and matrix (M) mRNA (a) and NP and M1 protein (b) levels in DF-1 cells electroporated with negative control (NC), si375, si401 or si883, incubated with growth medium with (+) or without (−) chIFN-β and infected with LP at an m.o.i. of 0.1. The expression level in cells treated with NC in growth medium without chIFN-β at 8 or 24 h post-infection (p.i.) for NP mRNA or protein, respectively, was set at 1.0 as a reference. (c) NP and M1 protein levels in DF-1 cells electroporated with pCMV14 or pCMV14-chUSP18, incubated with growth medium without chIFN-β and infected with LP at an m.o.i. of 0.1. The expression level in cells treated with pCMV14 in growth medium without chIFN-β at 24 h p.i. was set at 1.0 as a reference. Each bar represents the mean±SD from three independent experiments, *P<0.05 versus each siRNA in cells incubated with growth medium without chIFN-β, #P< 0.05 versus NC, Steel–Dwass test; ‡P< 0.01 versus pCMV14, Welch’s t-test.
protein for binding to the IFNAR2 receptor [14]. In this study, it was shown that the chUSP18 depletion resulted in the sustained expression of anti-viral protein mRNAs, such as MX1 and OASL, in chIFN-b-treated cells. A similar observation was made with human USP18, indicating that USP18 negatively regulated MX1 and IFIT1 expression through interfering with the type-I IFN-stimulated JAK/STAT pathway [24]. A contradictory result was also obtained in our study; when chUSP18–over-expressing cells were treated with chIFN-b followed by virus infection, enhancement of viral replication was not observed (Fig. 4a). However, it may be conceivable that chUSP18 was sufficiently expressed after chIFN-b treatment to interfere with IFN I signalling even in the control cells and no additive effect was seen from plasmid-driven chUSP18 enhancement. Therefore, it is possible that competition with JAK1 for IFNAR2 binding that otherwise leads to negative feedback on anti-viral host proteins in IFN-treated cells could be one of the pro-viral mechanisms of chUSP18.

ChUSP18 over-expression in DF-1 cells increased the replication of the LP virus but not the HP virus used in this study. This phenomena may be explained by differences in the viral proteins of the two strains in this study and ISGylation. It was reported that huISG15 attaches to lysine residues in the NS1 and M1 proteins of influenza A virus [10]. Comparing the LP and the HP viruses used in this study, there are differences in the aa sequences of their NS1 and

![Fig. 6. ChUSP18 depletion inhibits multiplication of LP virus. DF-1 cells electroporated with negative control (NC), si375, si401 or si883, incubated with growth medium with (+) or without (−) chIFN-β and infected with LP at an m.o.i. of 0.1. (a) Phase-contrast and fluorescence images of the cells stained with antibody against NP at 24 h post-infection (p.i.) are shown. Scale bar represents 100 µm. (b) Ratio of fluorescent cells to total cells in the images. #P<0.05, ##P<0.01 and ###P<0.0000001 versus NC, chi-square for independence test. (c) The fluorescence intensity of each cell was quantified and displayed as a box plot. The fluorescence intensity level in cells treated with NC in growth medium without chIFN-β was set at 1.0 as a reference.](image-url)
M1 proteins. Lysine residues at positions 172 and 217 in the NS1 protein of the LP virus are replaced by glutamic acid and asparagine, respectively, in that of the HP virus. In M1, position 230 in the LP virus is lysine, compared to arginine in HP. These lysine residues may be targeted by an unidentified ISG15-like molecule in chickens. Recently, it was reported that in influenza B virus huISG15 also attaches to NP in addition to NS1 and M1, and the ISGylated NP acts as an inhibitor of viral RNA synthesis [25]. Although it was demonstrated in the report that the lysine residues at position 156, 245, 279 and 478 in NP were ISGylated, there was no difference in the NP lysine residues between the LP and HP viruses. It may be that NP protein is not targeted by an ISG15-like molecule in chickens because there is no significant difference in the relative fluorescence level between the control cells and chUSP18-depleted cells, as shown in Fig. 6. It is also possible that other viral protein targeted by ISG15-like molecules might affect virus replication significantly. The mechanism by which chUSP18 is involved in the replication of HP needs to be further investigated.

Induction of chUSP18 by chIFN I in virus-infected cells enhances the replication of the LP virus, whereas most other IFN I-inducible factors, such as OAS and MX1, are inhibitory. It is possible that chUSP18 may have a role through negative feedback of the IFN I inducible system, including ISGylation. Viruses may take over the system to proliferate effectively in the host. Another possibility is that pathogens are allowed to replicate to some extent to induce an effective adaptive immune response. MUSP18 expression in metallophilic macrophages interferes with IFN I signalling, thereby increasing local replication of vesicular stomatitis virus [26] and allowing substantial antigen production in the metallophilic macrophage to induce an appropriate adaptive immune response by the host. ChUSP18 may play a similar role against LP virus. In this study, we demonstrated for the first time that chicken homologues of USP18 enhanced LPAIV replication in vitro, and further study is needed to elucidate the mechanism by which chUSP18 functions during avian influenza A virus replication and whether this protein acts in favour of the pathogen or infected host in vivo.

METHODS

Viruses and cells

The viruses used in this study were low-pathogenic avian influenza virus (LPAIV) of the H9N2 subtype, A/chicken/
mRNA-tag were used for viral NP vRNA and mRNA, The primer pairs vRNA-tag/NP-752R and NP-1466F/
quantitate chicken endogenous RT-R, chIFN-
57, 52, 56, 59, 62 or 60
chUSP18-RT-F/chUSP18-RT-R, chIFN-
a genes. The primer pairs ch

The synthesized cDNAs were used as templates for quantitative real-time PCR (qRT-PCR). The cDNA was mixed with primers specific for the target genes and SYBR Premix Ex Taq (TaKaRa), and subjected to qRT-PCR using an Applied Biosystems 7500 system (Applied Biosystems). PCR was performed under the following conditions: 1 cycle of 95 °C for 30 s and 40 cycles of 95 °C for 5 s, and 57, 52, 56, 59, 62 or 60 °C for chicken USP18, IFN-α, IFN-β, MX1, OASL or viral RNA, respectively, for 34 s. Chicken β-actin was used as an endogenous control to normalize quantification of the mRNA expression of the targeted genes. The primer pairs chβ-actin-RT-F/chβ-actin-RT-R, chUSP18-RT-F/chUSP18-RT-R, chIFN-α-RT-F/chIFN-α-RT-R, chIFN-β-RT-F/chIFN-β-RT-R, chMX1-RT-F/chMX1-RT-R and chOASL-RT-F/chOASL-RT-R were used to quantitate chicken endogenous β-actin, USP18, IFN-α, IFN-β, MX1 and OASL expression, respectively (Table 1). The primer pairs vRNA-tag-NP-752R and NP-1466F/
mRNA-tag were used for viral NP vRNA and mRNA, respectively (Table 1). The primer pairs M-906F/mRNA-
tag were used for viral M mRNA (Table 1). The relative expression levels of the target genes were analysed using the ΔΔCt method [29].

Sequence of full-length cDNA and alignment analysis
The cDNAs of the 5′- and 3′-untranslated regions of chUSP18 were synthesized using the SMARTer RACE cDNA amplification kit (TaKaRa). Subsequently, these regions were amplified by nested PCR with primers (Table 1) constructed on the basis of the USP18 DNA sequence (XM_416398.2) from the NCBI database. The primer pairs used were chUSP18-seq-R2/ UPM for the first PCR amplification and chUSP18-seq-R1/UPM for the second PCR amplification for 5′-untranslated regions, and chUSP18-seq-F1/UPM for the first PCR amplification and chUSP18-seq-F2/UPM for the second amplification for 3′-untranslated regions. Total RNA extracted from LMH or DF-1 cells infected with rHP was used as a template. The determined chUSP18 sequence from LMH cells has been submitted to DDBJ (accession no. LC036549). In transcript variant analysis, the 5′ and 3′ regions of chUSP18 were amplified by nested PCR using cDNA from DF-1 cells infected with LP and the following primer pairs: chUSP18-seq-R3/UPM (first PCR) and chUSP18-seq-R2/Universal Primer Short (second PCR) for the 5′ region, and chUSP18-seq-F3/UPM (first PCR) and chUSP18-seq-F1/Universal Primer Short (second PCR) for the 3′ region. Comparative analysis of the putative aa sequence with those of hUSP18 and mUSP18 was performed using CLUSTAL X 2.0 [30], and the alignment was depicted using GeneDoc 2.7 [31].

Construction of expression plasmids and site-directed mutagenesis
chUSP18 was amplified by PCR using cDNA from LMH cells and the primer pair chUSP18-F/chUSP18-R, and cloned using the Zero Blunt TOPO PCR cloning kit (Invitrogen). Subsequently, the chUSP18 fragment digested with HindIII/BamHI was inserted into the p3xFLAG-CMV-14 (pCMV14) expression vector (Sigma-Aldrich) to generate pCMV14-USP18. To mutate the ATG codons of interest, site-directed mutagenesis was performed using pCMV14-USP18 and a PrimeSTAR mutagenesis basal kit (TaKaRa) according to the manufacturer’s instructions. To identify the translational start codon, DF-1 cells were transfected with pCMV14-USP18 or its derivatives using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 h, whole proteins were collected using EzApply (ATTO) and subjected to Western blot analysis using anti-FLAG and anti-beta actin antibodies.

Electroporation of siRNA and expression plasmids
DF-1 cells for electroporation were washed with Opti-MEM (Gibco) and resuspended in Opti-MEM at a final concentration of 2.63 × 10⁶ cells ml⁻¹. Subsequently, 0.38 ml of the cells were mixed with 2000 pmol siRNA, electroporated in a 4 mm cuvette (Greiner bio-one) with a 200 V pulse at 960
microfarads using Gene Pulser Xcell (Bio-Rad) and then mixed with 0.5 ml of growth medium (Dulbecco’s modified Eagle’s medium with 10 % FCS). Depletion of endogenous or exogenous chUSP18 in DF-1 cells was achieved using Stealth RNAi (siRNA) specific for chUSP18 (Invitrogen), along with stealth RNAi negative control low GC (NC) (Invitrogen). Six siRNAs were designed using the BLOCKiT RNAi designer (Invitrogen), and three of them, si375, si401 and si883 (listed in Table 1), efficiently depleted chUSP18 expression. The silencing efficiency was determined by comparing the mRNA expression levels of chUSP18 between siRNA- and NC-transfected cells after incubation in growth medium for 4 h. Subsequently, the silencing efficiency for FLAG-USP18 expression between siRNA- and NC-transfected cells was simultaneously compared with mRNA expression in the aliquoted cells, as shown in Fig. 4 (c). For expression plasmids, DF-1 cells were electroporated at a final concentration of 70 pmol ml⁻¹ plasmid. The transfection efficiency of plasmid into DF-1 cells using electroporation was 84±2 %.

### Table 1. Sequences of siRNA and primers used in this study

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### Viral infection and titrations

DF-1 or LMH cells were seeded at 1 × 10⁶ cells well⁻¹ in six-well plates, and after 24 h the cells were washed twice with PBS and infected with LP (W/PA) or rHP in infection medium (minimum essential medium supplemented with 0.4 % bovine serum albumin, 1 % penicillin–streptomycin, 1 % fungizone and 3 % MEM vitamin solution) at 0.1 EID₅₀ cell⁻¹. Subsequently, the total cell RNA was collected at 12, 24 and 48 h.p.i and subjected to qRT-PCR analysis. Electroporated DF-1 cells were seeded in 12-well plates at a concentration of 0.2 × 10⁶ cells well⁻¹. After 24 h, the cells were incubated in growth medium with or without 1 µg ml⁻¹ chicken IFN-β (Abbexa) for 24 h and then infected with LP or HP in the infection medium with 1 µg ml⁻¹ acetylated trypsin from bovine pancreas (Sigma-Aldrich) at an m.o.i. of 0.1. At 24 h.p.i and 12 and 24 h.p.i for LP and HP, respectively, the culture supernatant was centrifuged, and the supernatant was subjected to qRT-PCR analysis for quantification of the viral vRNA. Total RNA or protein within the culture cells was collected at 8 and/or 24 h.p.i.
after washing the cells twice with PBS and subjected to qRT-PCR and Simple Western analysis, respectively.

**Western blotting, Simple Western and antibodies**

Extracts from transfected cells were separated on 5–20 % PAGE gels (ATTO) with SDS running buffer (EzRun, ATTO) and transferred to PVDF membranes (GE Healthcare). Detection of chemiluminescence was performed using ECL. Prime Western blotting detection reagent (GE Healthcare) and a ChemiDoc XRS Plus system (Bio-Rad) according to the manufacturer’s instructions. Simple Western was performed using Wes (ProteinSimple; Bio-Techne). Quantification to the manufacturer’s instructions. Simple Western was performed using Wes (ProteinSimple; Bio-Techne). Quantification of the viral protein expression was performed according to the manufacturer’s instructions. β-actin was used as an endogenous control to normalize protein expression. Anti-FLAG (F1804; Sigma-Aldrich), anti-β-actin [ab20272 (abcam) or A3853 (Sigma-Aldrich)], anti-influenza A virus NP (LS-C70554; LSBio) and anti-influenza A M1 antibodies (LS-C19375; LSBio) were used in the experiments.

**Immunofluorescence assay**

The electroporated cells were treated with or without chicken IFN-β for 24 h and then infected with LP at an m.o.i. of 0.1. At 24 h.p.i., the cells were washed once with PBS, fixed with 4 % paraformaldehyde for 15 min and then permeabilized with 0.5 % Triton X-100 for 10 min at room temperature. Virus NP was detected using anti-influenza A virus NP and Alexa Fluor 647 donkey anti-rabbit IgG (Invitrogen) for primary and secondary antibodies, respectively. Images were taken using an OLYMPUS IX73 phase contrast and fluorescence microscope and analysed by ImageJ image analysis software (version 1.47; NIH). To extract the fluorescent cells from the derived images, the fluorescent intensity of each cell was quantified, and cells that were lower than the highest value from non-infected cells were cut off. The extracted cells were subjected to subsequent analysis.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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