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Short Communication

Expression of Raf kinase inhibitor protein is downregulated in response to Newcastle disease virus infection to promote viral replication

Renfu Yin,[1] Xinxin Liu,[2] Yuhai Bi,[3] Guangyao Xie,[1] Pingze Zhang,[1] Xin Meng,[1] Lili Ai,[1] Rongyi Xu,[1] Yuzhang Sun,[4] Tobias Stoeger[5] and Zhuang Ding[1]

Correspondence
Zhuang Ding
dingzhuang@jlu.edu.cn

[1]Department of Veterinary Preventive Medicine, College of Veterinary Medicine, Jilin University, Xi'an Road 5333, Changchun, Jilin 130062, PR China

[2]College of Quartermaster Technology Science, Jilin University, Xi'an Road 5333, Changchun, Jilin 130062, PR China

[3]CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, PR China

[4]China Animal Health and Epidemiology Center, Qingdao, Nanjing Road 369, Qingdao, Shandong 266032, PR China

[5]Comprehensive Pneumology Center, Institute of Lung Biology and Disease (iLBD), Helmholtz Zentrum München, Ingolstädter Landstraße 1, D-85764 Neuherberg/Munich, Germany

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Newcastle disease virus (NDV) causes a severe and economically significant disease affecting almost the entire poultry industry worldwide. However, factors that affect NDV replication in host cells are poorly understood. Raf kinase inhibitory protein (RKIP) is a physiological inhibitor of c-RAF kinase and NF-κB signalling, known for their functions in the control of immune response as well as tumour invasion and metastasis. In the present study, we investigated the consequences of overexpression of host RKIP during viral infection. We demonstrate that NDV infection represses RKIP expression thereby promoting virus replication. Experimental upregulation of RKIP in turn acts as a potential antiviral defence mechanism in host cells that restricts NDV replication by repressing the activation of Raf/MEK/ERK and IκBα/NF-κB signalling pathways. Our results not only extend the concept of linking NDV–host interactions, but also reveal RKIP as a new class of protein-kinase-inhibitor protein that affects NDV replication with therapeutic potential.

Newcastle disease virus (NDV), also designated avian paramyxovirus serotype 1 (AMPV-1), is a member of the genus *Avulavirus* in the family *Paramyxoviridae*. NDV infections cause a highly contagious and fatal viral disease affecting most species of birds that frequently leads to severe economic losses in the poultry industry across the globe (de Leeuw & Peeters, 1999; Swayne *et al.*, 2013). NDV is an enveloped virus containing a non-segmented, single-stranded, negative-sense RNA genome of approximately 15 186 nt that contains six genes encoding the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase protein (HN), large polymerase protein (L) and an additional protein, V, that is expressed by RNA editing of P mRNA (Swayne *et al.*, 2013). Strains of NDV can be classified as highly virulent (velogenic), intermediate (mesogenic) or non-virulent (lentogenic) on the basis of their pathogenicity for chickens (Swayne *et al.*, 2013). The clinical signs of a highly virulent NDV infection in susceptible birds can be extremely different depending on the strain of virus. Virulent strains that cause diarrhoea and frequently haemorrhagic intestinal lesions are called 'viscerotropic velogenic', and strains that cause respiratory and neurotropic signs are called 'neurotropic velogenic' (de Leeuw *et al.*, 2005; Dortmans *et al.*, 2011).

The innate immune response to NDV infection relies primarily on the induction of the IFN response and related pathways (Cheng *et al.*, 2014; Krishnamurthy *et al.*, 2006; Susta *et al.*, 2013). NDV also encounters a range of antiviral responses, which are proposed to depend on the protein kinase R (PKR)-induced PKR/eIF2α signalling cascade and sequestosome 1 (SQSTM1)-mediated selective degradation of mitochondria by autophagy, called 'mitophagy' (Meng *et al.*, 2014; Zhang *et al.*, 2014). Meanwhile, there

is increasing evidence suggesting that other classes of genes, such as the interferon-inducible protein ISG12a and IFN-gamma-signalling mediator Rac1, are also involved in virus–host interactions (Liu *et al.*, 2014; Puhlmann *et al.*, 2010). In addition to hosts, viruses, such as herpes simplex virus 1 (HSV-1) (Mostafa *et al.*, 2013), human cytomegalovirus (HCMV) (Cantrell & Bresnahan, 2005), influenza virus (IV) (Goodman *et al.*, 2011) and others (Saribas *et al.*, 2014; Stracker *et al.*, 2004; Trobaugh *et al.*, 2014), regulate host and viral genes to enhance viral replication.

Raf kinase inhibitor protein (RKIP), a member of the phosphatidylethanolamine-binding protein (PEBP) family, has been shown to be involved in numerous cellular processes, such as cell proliferation, differentiation and apoptosis (Johnson *et al.*, 2014; Lopez-Ilasaca *et al.*, 1997; Sisto *et al.*, 2014). Previously identified as a novel inhibitor of cancer metastasis, RKIP is a widely expressed protein that negatively regulates the Raf/MEK/ERK signalling pathway by binding to Raf and blocking Raf-mediated phosphorylation of MEK (Antoun *et al.*, 2012; Yeung *et al.*, 1999). RKIP also works as a scaffold protein that negatively regulates the assembly of the IKK complex leading to the degradation of IκB releasing the NF-κB complex for nuclear translocation (Beshir *et al.*, 2010; Tang *et al.*, 2010). Furthermore, activation of Raf/MEK/ERK and the NF-κB pathway are required for normal replication of certain viruses, such as IV (Pinto *et al.*, 2011), Kaposi's sarcoma-associated herpesvirus (KSHV) (Ford *et al.*, 2006), human immunodeficiency virus (HIV) (Zhu *et al.*, 2011) and others (Mathers *et al.*, 2014), to promote viral replication, prevent virus-induced apoptosis, and mediate the immune response to the invading pathogen (Hiscott *et al.*, 2001). However, there is little information available in the literature regarding RKIP inhibitory mechanisms in the context of NDV infection. Therefore, in the present study, the role of RKIP in NDV replication and the cellular response to NDV infection were elucidated.

An isolate of the NDV strain NA-1 from geese (velogenic, 10^6 $CCID_{50}$ per 0.1 ml) used in this study was replicated in the allantoic cavity of 9- to 10-day-old embryonated specific pathogen-free (SPF) chicken eggs (Merial, Beijing) and purified directly from the allantoic fluid as described in our previous study (Yin *et al.*, 2010). The primary chicken embryo fibroblast (CEF) and the DF-1 cell line were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FBS (Gibco, Life Technologies) and maintained with 5 % FBS. The virus titre was quantified by the micro-HA method as described previously (Yin *et al.*, 2010). Total RNA isolation, first strand cDNA synthesis, quantitative PCR (qPCR) performance with SYBR green and qPCR data analysis have been described previously (Yin *et al.*, 2011). Primers specific to RKIP (forward: GACATTGAGCCATCATCCAG, reverse: CTACGCAGCCACCTGATCT) were used for detection of mRNA levels. Anti-RKIP, anti-ACTB and anti-mouse IgG–HRP antibodies were all purchased from Santa Cruz Biotechnology. All experimental protocols used in this work were reviewed and approved by the Experimental Animal Council of Jilin University, China.

In line with the previous studies (Dortmans *et al.*, 2010; Yin *et al.*, 2010), the virus titres in supernatants significantly increased from 12 to 72 h post-infection (p.i.) in DF-1 cells after infection with NDV at an m.o.i. of 1. In detail, viral titres at 12 h p.i. only yielded in a 7.0-fold increase but further increased to 36.8-fold by 24 h p.i. and 111.4-fold by 48 h p.i., followed by a decrease back to 13.0-fold at 72 h p.i., as compared with the uninfected cells (Fig. 1a).

To determine whether the NDV infection was sufficient to regulate RKIP expression, RKIP protein and mRNA levels in NDV-infected DF-1 cells from 12 to 72 h p.i. were characterized. As depicted in Fig. 1(b, c), NDV infection dramatically downregulated RKIP expression in DF-1 cells, with minimum mRNA expression levels at 24 h p.i. (Fig. 1b), followed by undetectable protein levels at 48 h p.i. (Fig. 1c). However, mRNA and protein levels of RKIP were found to partly recover at 72 h p.i., matching decreasing virus titres at this time point. Taken together, the results indicate that NDV infection significantly downregulates RKIP expression, with NDV titres preceding the RKIP expression-repression.

To elucidate whether decreased expression of RKIP in NDV-infected cells is the consequence of NDV induction and is beneficial for the virus growth, the effect of RKIP knockdown on NDV replication was assessed. Firstly, two RKIP stable knockdown cell lines from the immortalized chicken fibroblast cell line DF-1, with two distinct small hairpin RNA (shRNA) sequences against RKIP (shRKIP-A: 261–CAGAGAATGGCATCACTTC–280; shRKIP-B: 654–GACACTGGTTTATAGCACT–673) were generated using the RNAi-Ready pSIREN-RetroQ Vector (Clontech, Takara) according to the manufacturer's instructions. As compared with the scramble control, no detection of RKIP protein and 2.6-fold knockdown of mRNA were found in both RKIP knockdown cell lines (Fig. 2a). Both knockdown cell lines demonstrated increased virus titres compared with the scramble control (Fig. 2b). To further validate these results, RKIP transient knockdowns in primary chicken fibroblast (CEF) cells were generated by siRNA (Sangon) at a 25 nM concentration, targeting the same genes as described before for the shRNA knockdown in the DF-1 cell line. CEF cells were treated with either siRKIP-A or siRKIP-B and infected with NDV (m.o.i.=1) 4 h post-transfection (p.t.) for different time points, and both siRNAs were maintained for at least 48 h p.t. and decreased (1.9-, 2.5-, 4.5-, 2.8- and 1.1-fold knockdown of RKIP mRNA at 4, 12, 24, 48 and 72 h p.t. as compared with the scramble control) target gene levels (Fig. 2c). Subsequently, supernatants were analysed for virus titres from 4 to 72 h p.i., and the results confirmed those obtained from the RKIP stable knockdown cell lines. In fact, both RKIP transient knockdown CEF cell preparations displayed an approximately 2.8-, 6.5- and 7.8-fold increase in NDV titre at 4, 12 and 24 h p.i., followed by a further increase

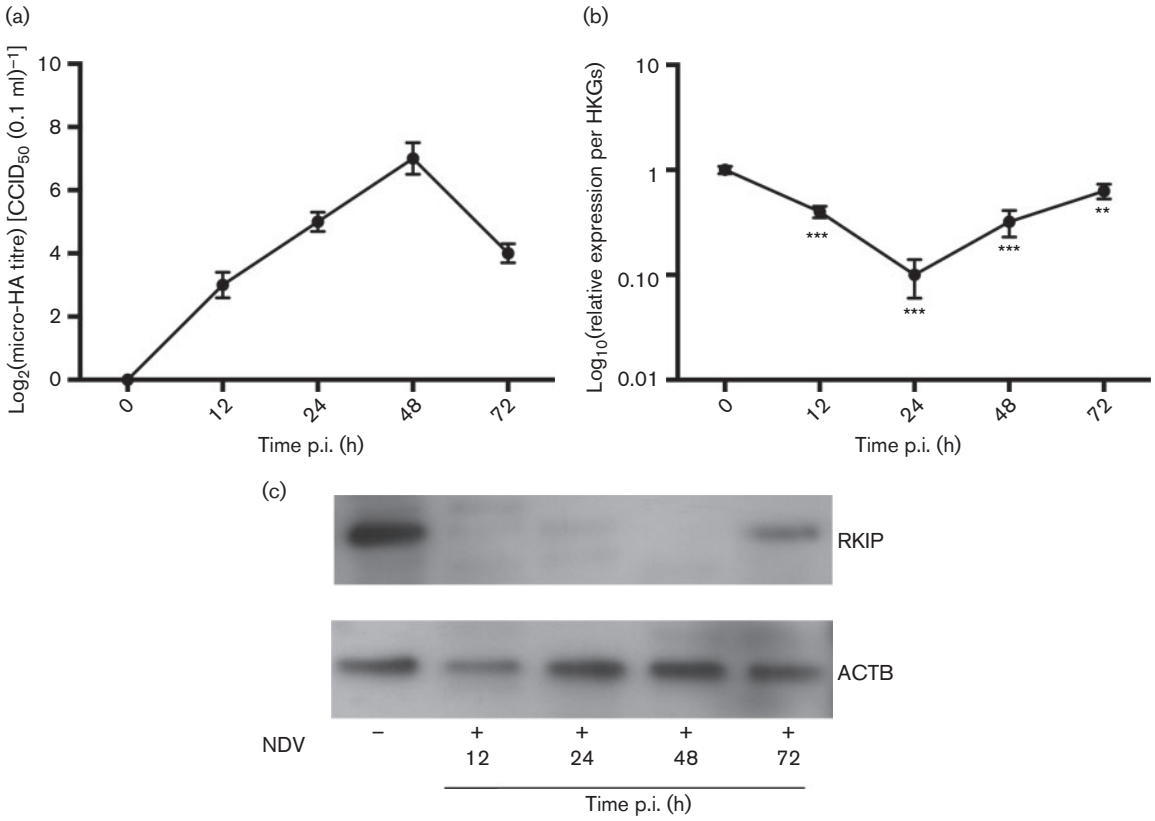

Fig. 1. Time-course dynamic of viral replication (a) and RKIP expression (b, c) in DF-1 infected with NDV NA-1 strain at an m.o.i. of 1.qPCR data were normalized to the geometric mean of three different housekeeping genes (ACTB, SHDA and HMBS) and calculated using the $2^{-\Delta\Delta C_t}$ method. Twenty micrograms total proteins were used for Western blot with ACTB considered as a loading control. All data are derived from two independent experiments. Values are shown as mean ± SEM ($n = 5-6$) and differences were considered significant if $^*P<0.05$, $^{**}P<0.01$ or $^{***}P<0.001$ as compared with the control group.

of 13.0-fold at 48 h p.i., compared with those detected in the scramble control, respectively. However, by 72 h p.i., similar levels of virus titres (1.1-fold) were exhibited for the scramble and RKIP transient knockdown cells (Fig. 2d), which were not observed for shRNA before. Taken together, these results show that reducing RKIP expression and activity might regulate host response in a way that promotes NDV replication.

After we demonstrated that inhibition of RKIP expression not only increases virus titres in immortalized cell lines, but at the same time increases the amount of virus in CEF cells we aimed to determine whether RKIP might be a potential antiviral target to counteract NDV infections. Therefore, we constructed an RKIP overexpression plasmid based on the CMV-inducible eukaryotic expression vector pCMV-BD (Promega) and thereby generated a DF-1 cell line stably overexpressing RKIP (ovRKIP), according to the manufacturer's instructions. As intended, a significantly higher RKIP expression could be observed in stable ovRKIP transfected cells, over either empty expression plasmid (eep)- or un-transfected (ut) cells (Fig. 2e). Thereafter, ovRKIP cells were infected with NDV (m.o.i.=1) for 12, 24, 48 and 72 h p.i. and supernatants were analysed for virus titres. Upon NDV infection, as compared with the eep control, virus titres in ovRKIP cells decreased by 31.1, 68.4, 83.6 and 70.9 % at 12, 24, 48 and 72 h p.i., respectively (Fig. 2f). Therefore, our data demonstrate that overexpression of RKIP is sufficient to inhibit NDV growth.

Earlier findings showed NDV infection induced phosphorylation of IκBα, a crucial player in the activation of the IκBα/NF-κB signalling axis, and ERK1/2, a kinase for the activation of Raf/MEK/ERK signalling (Ng *et al.*, 2013; Paulmann *et al.*, 2014). To get insight into the pathway of RKIP-related inhibition of virus replication, we set out to confirm whether NDV infection-induced phosphorylation of IκBα and ERK1/2 also applied under the conditions used here. Phosphorylated IκBα levels were measured, applying the FunctionELISA IκBα kit (Active Motif) and the ratio of phosphorylated ERK1/2 levels to ERK levels was measured with the phospho-ERK ELISA kit (Thermo Fisher Scientific, Life Technologies) according

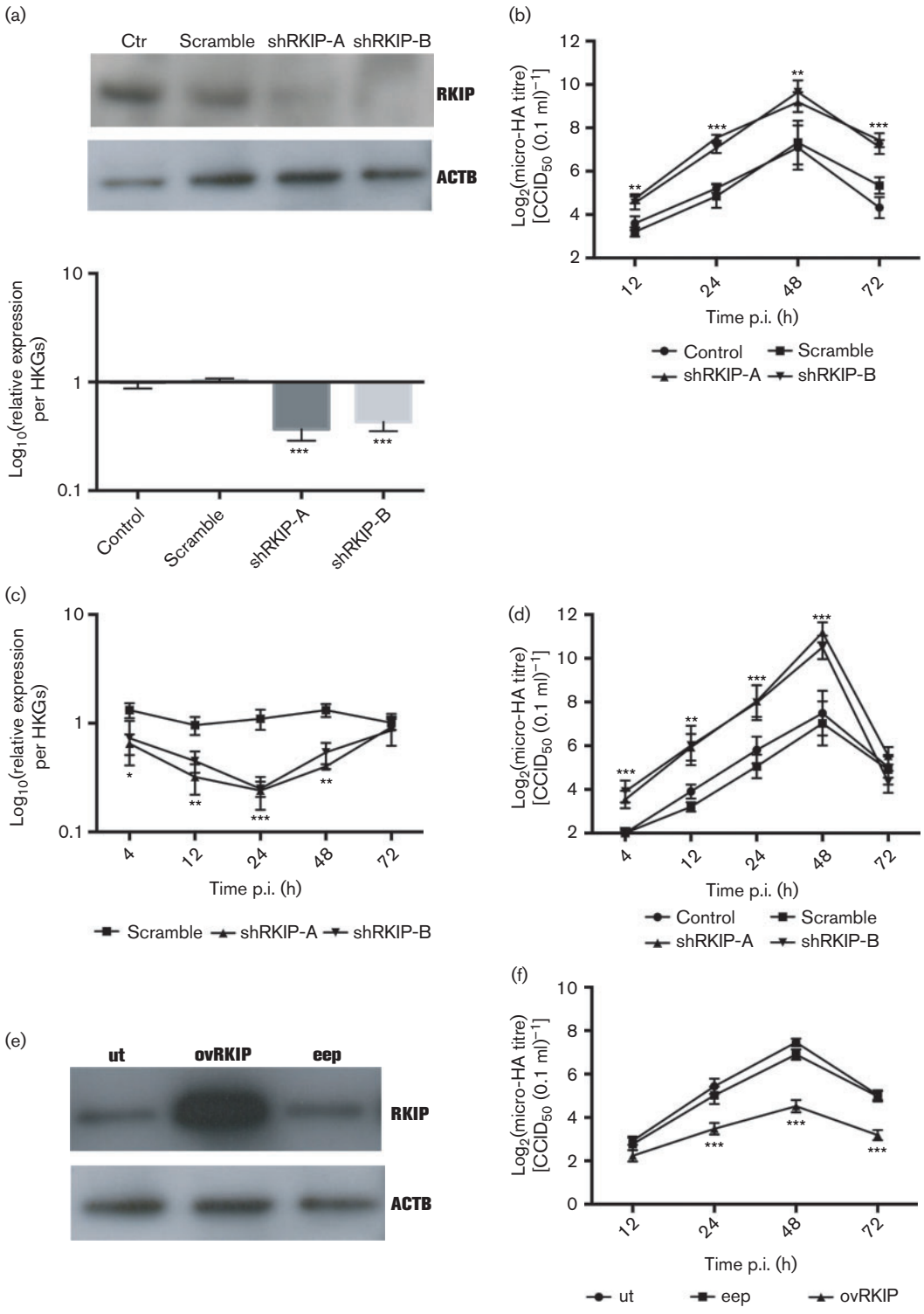

Fig. 2. RKIP modulates NDV replication *in vitro*. (a) DF-1 stably knocked down with shRNA against RKIP was generated by retro viral infection. Knockdown was confirmed by Western blot (upper) and qPCR (lower). (b) Virus titres were measured in NDV-infected control, scramble, shRKIP-A and shRKIP-B stable knocked down DF-1 cells by the micro-HA method. (c) CEF cells transiently knocked down with siRNA against RKIP were generated by transfection. Knockdown was confirmed by qPCR. (d) Virus titres were measured in transient knockdown CEF cells infected with NDV. (e) Expression levels of RKIP in un-transfected (ut), empty expression plasmid (eep) and RKIP stable overexpressed (ovRKIP) DF-1 cells were measured with Western blot. (f) Virus titres were measured in ut, eep and ovRKIP at 12, 24, 48 and 72 h p.i. of NDV (m.o.i.=1) infection. All data are derived from two independent experiments. Values are shown as mean±SEM (n=5–6) and differences were considered significant if *P<0.05, **P<0.01 or ***P<0.001 as compared with the control or scramble group.

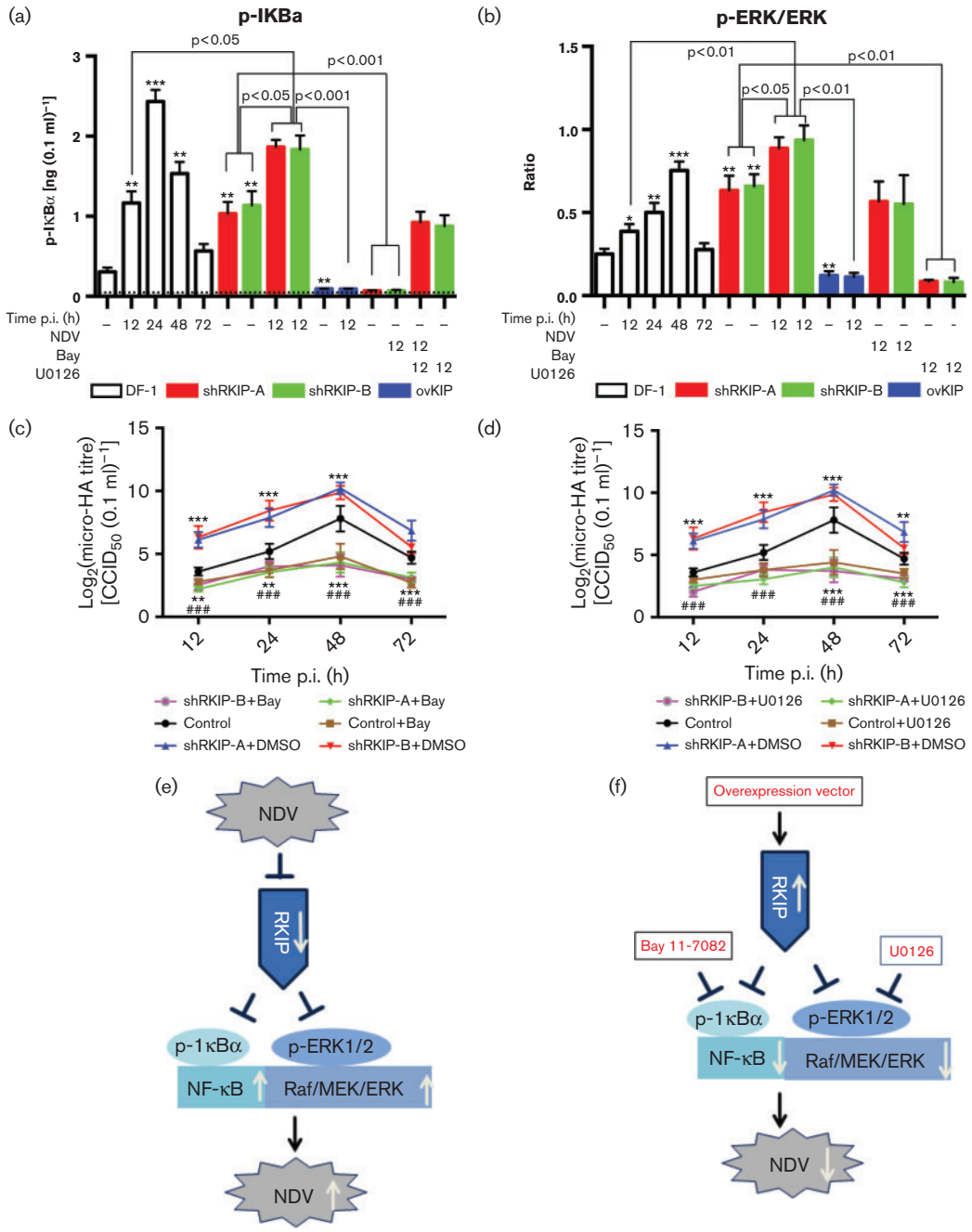

Fig. 3. NDV silences RKIP expression which promotes viral replication through enhanced Raf/MEK/ERK signalling and NF-κB pathway activation. The levels of phosphorylated IkBα (a) and ERK1/2 (b) were measured in DF-1, ovRKIP and shRKIP cells treated with different conditions. The limit of detection of p-IκBα level is 0.015 ng per 0.1 ml (dotted line). Virus titres in NDV-infected control DF-1 and shRKIP DF-1 treated with either Bay 11-7082 (c) or U0126 (d). (e) Schemata for interaction between host RKIP and NDV replication. (f) Anti-NDV strategies for RKIP mediated pathway. All data are derived from two independent experiments. Values are shown as mean ± SEM (n=5–6) and differences were considered significant if *P<0.05, **P<0.01 or ***P<0.001 as compared with the control group; ###P<0.001 as compared with the respective non-inhibitor treatment.

to the manufacturer's instructions. In fact we also observed significantly higher phosphorylation of both IκBα and ERK1/2 in shRKIP cells indicating that the Raf/MEK/ERK and IκBα/NF-κB pathways became activated in cells after RKIP knockdown and further depleted upon stable overexpression of RKIP in the respective transfected cell lines (Fig. 3a, b). Moreover, NDV infection induced higher phosphorylation of both IκBα and ERK1/2 in shRKIP

cells, not ovRKIP cells at 12 h p.i. Therefore, we thought to test further whether RKIP actually regulates the NDV replication through Raf/MEK/ERK signalling and the IκBα/NF-κB pathway. To test this we used the pharmacological inhibitors Bay 11-7082 and U0126 (Calbiochem, Merck Millipore), which were both dissolved in DMSO at a stock concentration of 100 mM. Bay 11-7082 has previously been shown to specifically inhibit the activation of IκBα/NF-κB and subsequent DNA binding by preventing phosphorylation of the inhibitor of κB (IκB) (Kim *et al.*, 2010). U0126, chemically known as 1,4-diamino-2, 3-dicyano-1,4-bis(2-aminophenylthio)butadiene, is a small molecule inhibitor of Raf/MEK/ERK signalling by selectively inhibiting phosphorylation of MEK1 and MEK2, family members of the mitogen-activated protein kinase kinases (MAPKKs) (Ong *et al.*, 2015). Firstly we analysed the effect of the inhibitor treatment for either Raf/MEK/ERK signalling or the IκBα/NF-κB pathway on the virus titres in RKIP stable knockdown DF-1 cells. To this point, we determined virus titres in shRKIP DF-1 cells infected with NDV (m.o.i.=1), with or without inhibitor treatment. As depicted in Fig. 3(c), shRKIP cells treated with medium conditioned with 20 μM Bay 11-7082 exhibited a decline of virus titres by 93.0, 95.2, 98.4 and 87.7 % at 12, 24, 48 and 72 h p.i. as compared with the non-treated cells. U0126, given at 20 μM in parallel, effectively reduced virus titres by 94.1, 96.5, 98.8 and 86.8 % at 12, 24, 48 and 72 h p.i., respectively (Fig. 3d). Furthermore, treatment of control DF-1 cells with medium conditioned with either Bay 11-7082 or U0126 caused significant reductions in viral titres as compared with cells without treatment (Fig. 3c, d). Our data thus clearly demonstrate that IκBα/NF-κB signalling as well as the Raf/MEK/ERK pathway are important factors required for NDV propagation and their inhibition limits virus replication. Upon infection, NDV silences RKIP expression, which promotes viral replication through enhanced Raf/MEK/ERK signalling and IκBα/NF-κB pathway activation.

In line with others reports, our data show that IκBα/NF-κB signalling is essential for efficient virus propagation, as inhibition of IκBα phosphorylation by IKK inhibition via Bay 11-7082 results in reduced virus titres, such as Venezuelan equine encephalitis virus (Amaya *et al.*, 2014), IV (Mazur *et al.*, 2007; Pinto *et al.*, 2011) and coxsackievirus (Sobotta *et al.*, 2012). Previous studies showed that NDV replication does not require NF-κB p50 and cRel and, accordingly, embryonic fibroblasts (MEF) derived from $p50^{-/-}$ and $cRel^{-/-}$ mice revealed no defect during the early time of infection. Increased NDV replication was, however, observed in MEF lacking NF-κB RelA (p65) subunit, since RelA is a specific requirement in early virus-induced IFN-β expression, although RelA, p50 and cRel play a relatively minor role in overall IFN-β production upon NDV infection (Wang *et al.*, 2007, 2010). But in the present study, we found that blocking phosphorylation of the IκBα by the IKK-specific inhibitor Bay 11-7082 impaired NDV propagation.

Our conclusion is that the drop in NDV titres at 72 h p.i. was a result of the recurrence of RKIP expression because the RKIP gene expression increase from 24 to 72 h p.i. preceded the drop of NDV titres from 48 to 72 h p.i. Since NDV infection obviously regulates PKIP expression, virus titres will need to precede RKIP gene expression if upstream of them. Also Fig. 2(c, d) shows that the effect, as transient siRKIP knockdown, had disappeared at 72 h p.t., and no difference in viral titre was observed at this time point, when comparing siRNAs, scramble and control CEF.

In summary, we show that NDV infection represses host RKIP expression, which subsequently results in enhanced Raf/MEK/ERK signalling and IκBα/NF-κB pathway activation, since this pathway is usually inhibited by RKIP activity (Fig. 3e). NDV replication, however, requires Raf/MEK/ERK and IκBα/NF-κB signalling, and virus propagation is, therefore, less efficient if these pathways are obstructed (Fig. 3f). Targeted overexpression of RKIP, therefore, can be exploited as an antiviral defence mechanism, and the results of this study could not only help to improve our understanding of NDV host interactions but also suggest a new avenue for the development of novel antiviral strategies.

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