Antiviral Research 170 (2019) 104571

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

# An endogenous retroviral element exerts an antiviral innate immune function via the derived lncRNA lnc-ALVE1-AS1

Shihao Chen[a,1], Xuming Hu[a,**,1], Isabelle H. Cui[b], Shaogen Wu[c], Chunfeng Dou[a], Yangyang Liu[a], Zhen Sun[a], Songlei Xue[a], Tuoyu Geng[a], Zongping Liu[d,e,f], Aijian Qin[d,e,f], Hengmi Cui[a,e,f,g,*]

[a] Institute of Epigenetics and Epigenomics and College of Animal Science and Technology, Yangzhou University, 48 East Wenhui Road, Yangzhou, Jiangsu, 225009, China
[b] Department of Pathology, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY, 10065, USA
[c] School of Medicine at Rutgers, The State University of New Jersey, 185 S Orange Ave, Newark, NJ, 07103, USA
[d] College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu, 225009, China
[e] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, 225009, China
[f] Institute of Comparative Medicine, Yangzhou University, Yangzhou, 225009, China
[g] Joint International Research Laboratory of Agricultural & Agri-Product Safety, The Ministry of Education of China, Yangzhou University, Yangzhou, Jiangsu, 225009, China

ARTICLE INFO

*Keywords:*
Endogenous retroviruses
Antisense lncRNAs
Innate immunity
Antiviral defence
Epigenetic regulation

ABSTRACT

Endogenous retroviruses (ERVs) constitute an important component of animal and human genomes and are usually silenced by epigenetic mechanisms in adult cells. Although ERVs were recently reported to be linked to early development, tumorigenesis and autoimmune disease, their impacts on antiviral innate immunity and the underlying mechanisms have not been elucidated. Here, we provide the first direct evidence of an endogenous retroviral element affecting antiviral innate immunity via its derived antisense long non-coding RNA (lncRNA). We found that an antisense lncRNA, which is called lnc-ALVE1-AS1 and is transcribed from the endogenous avian leukosis virus in chromosome 1 (ALVE1), distinctly inhibited the entry and replication of exogenous retroviruses in chicken embryonic fibroblasts (CEFs). This behaviour is at least in part attributed to the induction of an antiviral innate immune pathway by ALVE1 activation, suggesting that an activated endogenous retroviral element may induce antiviral defence responses via its derived antisense lncRNA. We also found that lnc-ALVE1-AS1 mediated these effects by activating the TLR3 signalling in the cytoplasm. Our results provide novel insights into the antiviral innate immune function of ERVs, suggesting that ERVs may play an important role in antiviral defences and provide new strategies for the development of new vaccines.

## 1. Introduction

Endogenous retroviruses (ERVs) are believed to be the remnants of ancient retroviral infections (Stoye, 2012) and constitute an important component of host viromes (Virgin, 2014), which make up about 8% of the human and 10% of mouse genomes, respectively (Stocking and Kozak, 2008). Due to their age, the majority of the ERVs contain numerous deletions and mutations that compromise or abrogate their protein-coding ability, and these sequences were ever presumed to be 'redundant junk' or 'useless' DNAs without any function. Recently, ERVs were shown to be intricately connected to human evolution (Hayward et al., 2015; Stoye, 2012), early development (Grow et al., 2015; Wang et al., 2014) and to tumourigenesis (Chiappinelli et al., 2015a; Roulois et al., 2015). In addition, accumulating evidence suggests that ERVs play important roles not only in pathogenesis of immune disorders, but also in proper functioning of the immune system (Volkman and Stetson, 2014).

Usually, ERVs are typically silenced in adult tissues by epigenetic mechanisms including, but not limited to, DNA methylation (Maksakova et al., 2008). An association between DNA

*Abbreviations:* ERV, endogenous retroviruses; 5-Aza-dC, 5-Aza-2′-deoxycytidine; ALVE1, endogenous avian leukosis virus in chromosome 1; lncRNA, long non-coding RNA; CEF, chicken embryonic fibroblast; ALVJ, avian leukosis virus subgroup J; dsRNA, double-stranded RNA; ISG, interferon stimulated gene

* Corresponding author. Institute of Epigenetics and Epigenomics and College of Animal Science and Technology, Yangzhou University, 48 East Wenhui Road, Yangzhou, Jiangsu, 225009, China.
** Corresponding author. Institute of Epigenetics and Epigenomics, Yangzhou University, 48 East Wenhui Road, Yangzhou, Jiangsu, 225009, China.
*E-mail addresses:* hxm@yzu.edu.cn (X. Hu), hmcui@yzu.edu.cn (H. Cui).
[1] Authors contributed equally.

https://doi.org/10.1016/j.antiviral.2019.104571
Received 17 April 2019; Received in revised form 27 July 2019; Accepted 29 July 2019
Available online 30 July 2019

hypomethylation and the induction of ERVs has recently been reported, and ERVs that were induced have also been proposed to participate in the induction of interferon signalling during this process. Recent studies have shown that the endogenous retroviruses that were reactivated by DNA methylation inhibitors, which were used for the treatment of colon and ovarian cancer cells, can induce a type I interferon response (Chiappinelli et al., 2015b; Roulois et al., 2015). Another study in zebrafish also showed that ERVs can be reactivated by the DNA hypomethylation that is caused by the mutation of *Dnmt1* or *Uhrf1*, which leads to a robust activation of type I interferon (Chernyavskaya et al., 2017). These reports also indicate that ERVs are involved in regulation and activation of the innate immune functions.

Bioinformatics profiling has suggested that activated ERVs are the source of long non-coding RNAs (lncRNAs) and that in this way, ERVs have assumed an important role in the regulatory network of development as they have evolved (Chuong et al., 2017; Kelley and Rinn, 2012). For example, the primate-specific ERV HERV-H has been identified as an RNA scaffold that recruits transcriptional activators to regulate stem cell pluripotency (Lu et al., 2014). Another ERV-lncRNA named HPAT5, which is derived from both a primate-specific HUERS-P1 ERV and an Alu element, was also discovered to promote pluripotency by functioning as a molecular sponge (Durruthy-Durruthy et al., 2016). These results indicate that ERV-derived lncRNAs are novel regulators of biological responses. However, it is still unclear whether ERV-derived lncRNAs can independently play a pivotal role in the antiviral innate immunity when the ERVs are reactivated?

In this study, we show that the treatment with the inhibitor 5-Aza-2′-deoxycytidine (5-Aza-dC) leads to the derepression of endogenous retroviruses, which can also produce lncRNAs that further give rise to activating the antiviral signalling in chicken embryonic fibroblast cells (CEFs). We found that an antisense lncRNA, lnc-ALVE1-AS1, derived from the chicken endogenous retrovirus ALVE1, plays an important role as a regulatory element in the activation of viral defence responses in CEFs. We also explored the mechanism though which the ERV-derived lnc-ALVE1-AS1 contributes to antiviral innate immunity, thereby providing an additional layer of activation in antiviral innate immunity. This study provides novel insights into the antiviral innate immune function of ERVs, which is obviously very important for understanding the mechanism of antiviral defence and for the developing new antiviral vaccines.

## 2. Materials and methods

### 2.1. Ethics statement

Animal work was performed in strict accordance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals of Yangzhou University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Yangzhou University (licence number: 06R015).

### 2.2. Cell culture

Primary chicken embryo fibroblast cells (CEFs) were prepared from 10-day-old specific pathogen-free (SPF) embryos obtained from Merial Vital (Laboratory Animal Technology Co., Ltd.). The cells were seeded into 6-well plates in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 5% foetal bovine serum (FBS) and cultured at 37 °C in 5% $CO_2$ and 95% humidity.

### 2.3. RNA-seq analysis

A library for lncRNA sequencing was prepared and sequenced at the Novogene Bioinformatics Institute on the Illumina HiSeq 2000 platform, and 100-bp paired-end reads were generated. Raw data (raw reads) in the FASTQ format were processed through in-house Perl scripts. The Q20, Q30 and GC contents of the clean data were then calculated. All downstream analyses were based on high-quality clean data.

Raw FASTQ files corresponding to DMSO- and 5-Aza-dC-treated samples (3 biological replicates each) were aligned to the chicken genome (gga4.0) using TopHat v2.0.9. The mapped reads for each sample were assembled by both Scripture (beta2) (Guttman et al., 2010) and Cufflinks (v2.1.1)(Trapnell et al., 2010) using a reference-based approach. The coding potential of the transcripts was predicted using four tools [the coding–non-coding index (CNCI), coding potential calculator (CPC), Pfam scan and phylogenetic codon substitution frequency (PhyloCSF)], and transcripts without coding potential comprised our candidate set of lncRNAs. Differential expression analysis of both lncRNAs and coding RNAs in each sample was performed using Cuffdiff v2.2.1 (Trapnell et al., 2010, 2012). Differences with a $P$ value $< 0.05$ indicated transcripts that were differentially expressed in biological replicate samples.

### 2.4. Rapid amplification of cDNA ends (RACE)

Both 5′- and 3′-RACE were performed with the SMARTer® RACE 5′/3′ Kit (cat # 634860, Takara) to obtain the ends of the ALVE1 transcripts. In brief, 1 μg of total RNA (with genomic DNA removed) was converted into RACE-Ready first-strand cDNA using the 5′- or 3′-CDS Primer A, which was provided by the kit. Then, the 5′- or 3′-RACE PCR was conducted using a Universal Primer (provided by the kit) and 5′ or 3′ gene-specific primers (see Supplementary Table 1) to generate 5′ and 3′ cDNA fragments. The RACE products were characterized using a 1.5% agarose gel and then cloned into a TA vector for sequencing.

### 2.5. Strand-specific reverse transcription quantitative PCR

The strand-specific reverse transcription (RT) primers and gene-specific primers are described in Supplementary Table 1. The gDNA Eraser-treated RNA samples were reverse-transcribed with strand-specific RT primers at 42 °C for 15 min with the PrimeScript® Reverse Transcriptase (cat #RR047B, Takara).

Strand-specific quantitative PCR (qPCR) was performed with gene-specific primers and the SYBR Green Master Mix (cat # RR820B, Takara) on the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The *GAPDH* RNA levels were used as internal controls to normalise gene expression.

### 2.6. RNA FISH

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, permeabilised with 0.25% Triton X-100 for 5 min, washed with PBS, blocked with 2% BSA for 30 min and incubated with the anti-env mAb JE9 in PBS at room temperature for 45 min. The cells were then washed in PBS, incubated with goat anti-mouse IgG conjugated with the Alexa Fluor 488 dye (Sigma-Aldrich) and stained with DAPI dye (Sigma-Aldrich) at room temperature for an additional 10 min. Images were captured and merged with a Leica SP8 confocal microscope (20X). For FISH, a set of probes for lnc-ALVE1-AS1 were ordered from a commercial source (RiboBio Co., Ltd.). To achieve a sufficient signal-to-background ratio, we designed multiple probes to target the lnc-ALVE1-AS1 sequence. A set of 15–20 probes covering the entire length of the RNA molecule provided optimal signal strength. The pooled FISH probes were dissolved to a final concentration of 25 μM in RNase-free storage buffer and stored protected from light at −20 °C. Coverslips for each group were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min, permeabilised with 0.25% Triton X-100 for 5 min, washed with PBS, and incubated overnight with the lnc-ALVE1-AS1 probe mixture at 37 °C. For the co-localization studies, the cells were fixed in 4% paraformaldehyde for 5 min after RNA FISH and then subjected to

immunofluorescence staining. The pictures were captured and merged with a Leica SP8 confocal microscope.

### 2.7. Construction of the plasmid overexpressing lnc-ALVE1-AS1

lnc-ALVE1-AS1 was amplified from chicken CEF cDNA and then cloned into the pcDNA3.1 (+) vector (Invitrogen); the plasmid was verified using Sanger sequencing. The PCR primer pairs are listed in Supplementary Table 1.

### 2.8. Viral infection

Viral infection experiments were performed with the avian tumour virus ALVJ. CEFs were seeded into six-well plates and infected with the ALVJ virus at a multiplicity of infection (MOI) of 5 (unless otherwise indicated) for the indicated hours.

### 2.9. 5-Aza-dC treatments

Cells were seeded into six-well plates and cultured for 24 h before drug treatment. The demethylation agent 5-aza-2′-deoxycytidine (5-Aza-dC; cat #A3656, Sigma-Aldrich) was dissolved in DMSO. The 5-Aza-dC dose was selected based on previous study (Leonova et al., 2013) and our preliminary data, and the cells were treated with 5 μM 5-Aza-dC for 96 h. DMSO treatment was used as a control.

### 2.10. Western blotting analysis

Cell lysates were prepared with cell lysis buffer (cat #9803, Cell Signalling Technologies) and resuspended in 4 × Laemmli loading Buffer. Cell lysates were subjected to SDS-PAGE and Western blot analysis was performed with the appropriate antibodies. Antibodies against HA (cat #sc-7392) and GAPDH (cat #sc-166574) were from Santa Cruz Biotechnology. TLR3 antibody was purchased from Novus Biologicals (cat #NBP2-24565). Rabbit anti-STAT1 was purchased from ABclonal (cat # A0027). Rabbit anti-phospho-STAT1 (Tyr701) was from EMD Millipore (cat #07–307); and anti-β-actin was from Sigma-Aldrich (cat #A5441).

### 2.11. Northern blotting

Northern blotting assays were performed with the DIG Northern Starter Kit (cat #12039672910, Roche) according to manufacturer's instructions. Briefly, total RNA was extracted from 5-Aza-dC-treated CEFs using the TRIzol® Reagent, and a RT-PCR was performed with an oligo (dT) primer using the Expand Reverse Transcriptase System. PCR was then performed with the specially designed primers lnc-ALVE1-AS1-probe-F (5′-TAATACGACTCACTATAGGGACCATCTGAGTCCTTTGTG-3′, which contains the T7 RNA polymerase promoter sequence) and lnc-ALVE1-AS1-probe-R (5′-TGTTCCATTGTCATCGCTAA-3′) using the Expand High Fidelity PCR System. DIG-labelled RNA probes were generated according to the *in vitro* transcription labelling technique and used to hybridise to membrane-blotted nucleic acids according to standard methods. The hybridised probes were immunodetected with anti-digoxigenin-AP, and the fragments were visualized with the ready-to-use chemiluminescence substrate CDP-Star.

### 2.12. Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected, and the ALVJ p27 antigen was measured by ELISA (IDEXX) following the manufacturer's instructions.

### 2.13. Coding potential analysis of lnc-ALVE1-AS1

Full-length lnc-ALVE1-AS1 was cloned into the eukaryotic expression vector pcDNA3.1 with an N-terminal start codon (ATG) and a human influenza haemagglutinin (HA) tag in all three reading frames. The plasmids were then transfected into HEK293 cells. Immunoblotting was used to detect the HA tag after 48 h. Cells transfected with a plasmid containing GFP with an HA tag were used as a positive control. Data are representative of three independent experiments. Bioinformatics analysis confirmed that lnc-ALVE1-AS1 had no coding capability (http://cpc.cbi.pku.edu. cn/programs/run_cpc.jsp).

### 2.14. Gene knockdown by RNAi

Stealth RNAi™ siRNAs specific for lnc-ALVE1-AS1, chicken TLR3 and negative control Stealth RNAi™ siRNA were designed and synthesized by Invitrogen (Life Technologies, MD, USA). The sequences of the Stealth siRNAs were listed in Supplementary Table 1. For lnc-ALVE1-AS1 knockdown, cells were transfected with the control or *lnc-ALVE1-AS1* siRNA for 48 h and then collected for gene expression analysis. For TLR3 knockdown, cells were transfected with the control or TLR3 siRNA for 24 h and then transfected with the control or lnc-ALVE1-AS1 for another 36 h.

### 2.15. Analysis of coeffect of TLR3 and lnc-ALVE1-AS1 on innate immune-related genes

The cells were pretreated with 100 μM Amlexanox (InvivoGen) for 1 h and then transfected with the control or lnc-ALVE1-AS1 for another 36 h. Total RNA was extracted using the TRIzol reagent to analyse expression of innate immune-related genes.

### 2.16. Statistical analyses

The statistical analysis was performed with the Statistical Package for the Social Sciences (version 16.0) software. Statistical significance between groups was determined by two-tailed unpaired Student's t-test with a P value threshold of $< 0.05$.

## 3. Results

### 3.1. The DNA demethylation agent 5-Aza-dC inhibits ALVJ replication in vitro

We found that the DNA methylation inhibitor 5-Aza-dC functioned in a manner similar to antitumour therapies in humans when it was used for the treatment of avian leukosis virus subgroup J (ALVJ), which is a retrovirus that can cause haemangiomas and myeloid tumours in chickens (Justice et al., 2015). Using a virus-free chicken embryo fibroblast (CEF) that was infected with ALVJ for 24 h as a viral infection model, we treated the ALVJ-infected CEFs with 5 μM of 5-Aza-dC for 96 h. The results showed that the treatment of ALVJ-infected CEFs with 5 μM of 5-Aza-dC had little effect on cell growth and on the activity of CEFs (Fig. 1A) but significantly inhibited the envelope protein secreted by ALVJ compared to that of the controls (Fig. 1B), suggesting that 5-Aza-dC inhibited the proliferation of ALVJ in CEFs. We further confirmed that treatment of ALVJ-infected CEFs with 5 μM of 5-Aza-dC significantly inhibited the proliferation of ALVJ in CEFs compared to that of the controls by confocal immunofluorescence (Fig. 1C).

### 3.2. Long noncoding RNA-associated chicken endogenous retroviruses could be induced by the DNA methylation inhibitor 5-Aza-dC

The mechanism of action of DNA methylation inhibitor in cancer therapy has recently been reported to induce the transcription of ERVs that activate the viral recognition and interferon response pathway (Chiappinelli et al., 2015b; Roulois et al., 2015). To investigate the mechanism of the antiviral activity of DNA methylation inhibitor 5-Aza-dC, we decided to focus on the actively expressed ERVs and their

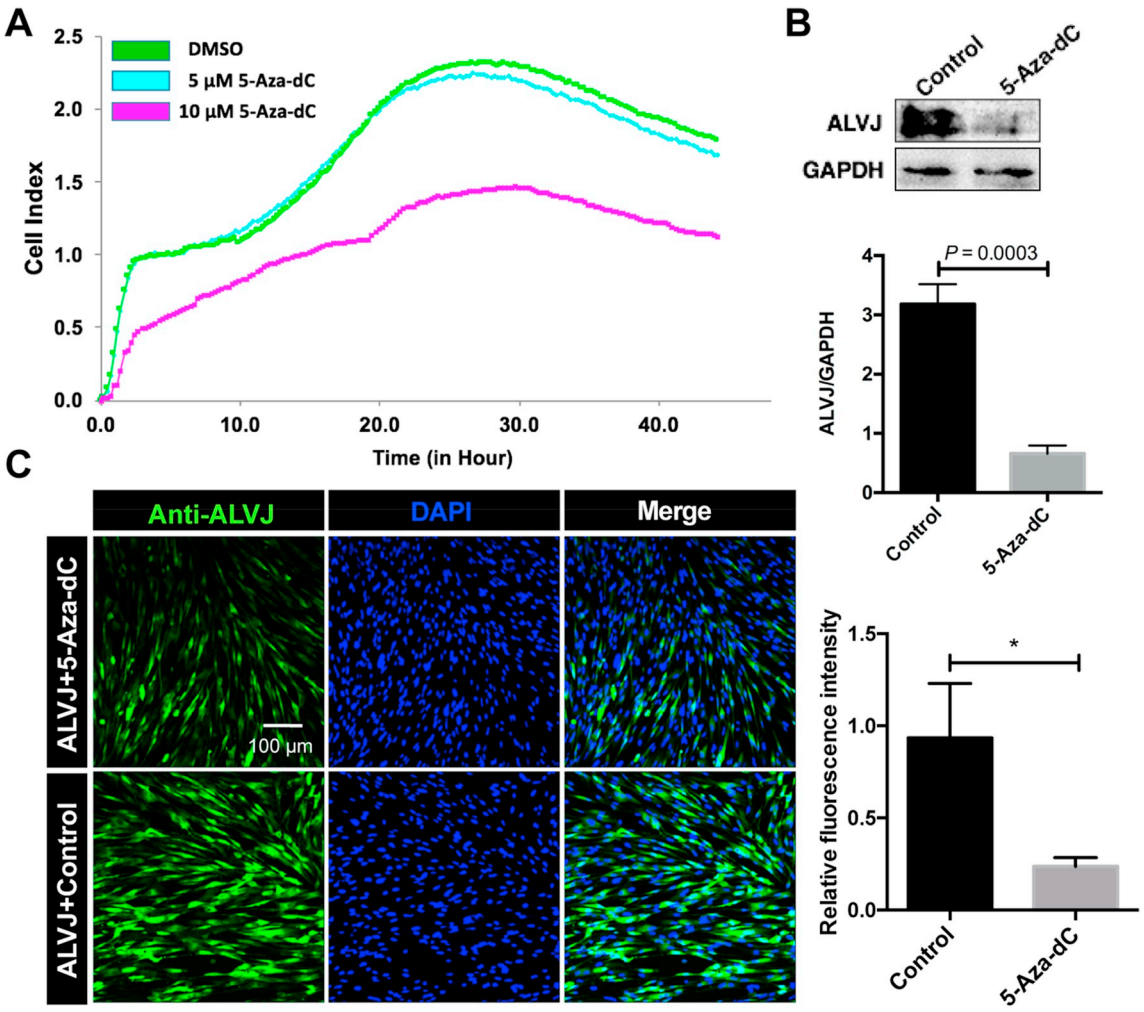


**Fig. 1. DNA demethylation agent 5-Aza-dC inhibits ALVJ replication in vitro.** (A) CEFs were treated with vehicle (DMSO) or with 5 μM or 10 μM 5-Aza-dC and were plated on E-plates. Cell index curves indicating the cell proliferation and activity were monitored using the xCELLigence system. (B) Western bolt analysis of ALVJ-env protein in CEFs that were infected with ALVJ for 24 h and were then treated with 5 μM 5-Aza-dC for 96 h. ALVJ was quantified by ImageJ software normalized to GAPDH. Data are presented as mean ± SD from three independent experiments. (C) CEFs were infected with ALVJ and treated 24 h later with 5 μM 5-Aza-dC. After 96 h of treatment, CEFs were then visualized with confocal microscopy to observe the ALVJ env protein. Images were acquired using a 20 × /NA 0.75 lens on a Leica SP8 confocal microscope running LAS AF Lite version 2.6.3 software. ALVJ env stained with Alexa-Fluor-488 (green) and nucleus stained with DAPI (blue) were excited at 488 nm by a white light laser and at 405 nm by a blue–violet laser diode, respectively. Scale bar, 100 μm. Images show a representative of three independent experiments. Relative fluorescence intensity of ALVJ and DAPI per group were quantified by ImageJ (normalized to the control group). Data are presented as mean ± SD from three independent experiments. Statistical significance was analyzed by two-tailed Student's t-test (*$P < 0.05$).

transcripts, long noncoding RNAs (lncRNAs). To further reveal whether lncRNAs derived from chicken endogenous retroviruses were activated, we employed high-throughput RNA sequencing (RNA-seq) to analyse and to identify lncRNAs derived from chicken endogenous retroviruses, ERV-lncRNAs. RNA-seq analysis revealed that compared to those of the controls, 320 lncRNAs were upregulated after 5-Aza-dC treatment, while only 85 were downregulated (Fig. 2A, Supplementary Table 2), indicating that these lncRNAs could be regulated by DNA methylation. Moreover, sequence alignment analysis revealed that a total of 59 of the 405 differentially expressed lncRNAs were derived from chicken endogenous retroviruses, suggesting that ERVs in the genome may be an important source of lncRNAs and that the expression of these lncRNAs could be activated by the DNA methylation inhibitor 5-Aza-dC. We also found that compared to those of the controls, 51 of the differentially expressed lncRNAs that were derived from chicken ERVs were significantly up-regulated, but only 8 lncRNAs derived from chicken ERVs were significantly downregulated (Fig. 2B, Supplementary Table 3), which suggests that the molecular mechanism thought which 5-Aza-dC inhibits the proliferation of ALVJ may be related not only to the activation of innate immune-related genes (Liu et al., 2016), but also to the abnormal activation of ERV-lncRNAs.

### 3.3. Identification of the chicken endogenous retrovirus-derived lnc-ALVE1-AS1

Therefore, we investigated whether these ERV-derived lncRNAs contributed to antiviral innate immunity. Of these 59 ERV-derived lncRNAs, the second-most upregulated transcript mapped to chicken endogenous retrovirus ALVE1 locus, attracted our attention. We named this transcript lnc-ALVE1-AS1, because it is derived from the antisense transcripts of ALVE1 (Supplementary Table 3). With 5′ and 3′ rapid amplification of cDNA ends (RACE) (Fig. 3A) and with strand specific RT-PCR, we discovered that the transcript of this lncRNA is 2136 nucleotides long without an intron, which was also be supported by northern blotting (Fig. 3B and C). To confirm that lnc-ALVE1-AS1 had no coding capability, full-length lnc-ALVE1-AS1 was cloned into the eukaryotic expression vector pcDNA3.1, with all three coding patterns shown in Fig. 3D, and the result suggested that lnc-ALVE1-AS1 is a long

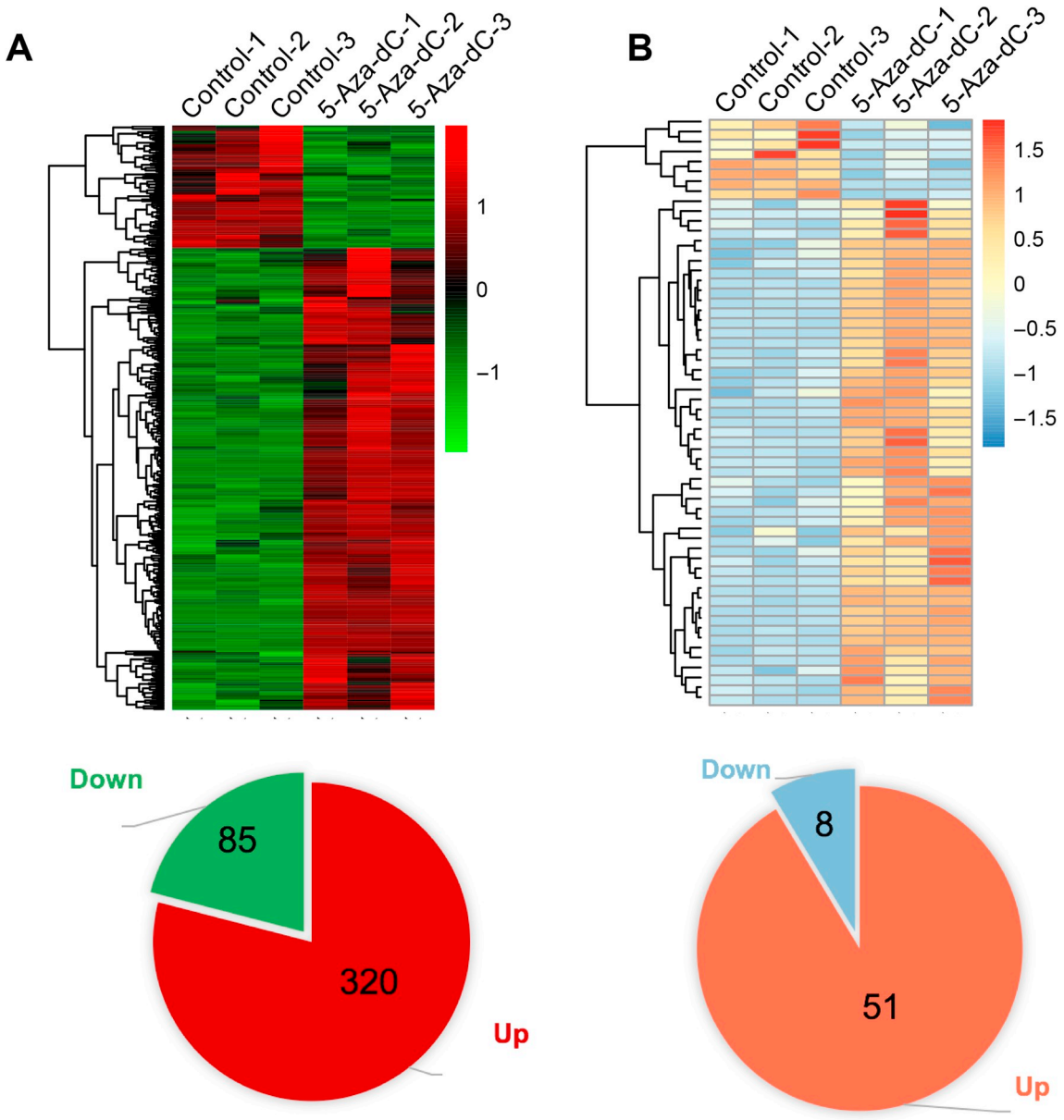


**Fig. 2. LncRNA-associated chicken endogenous retroviruses could be induced by the DNA methylation inhibitor 5-Aza-dC.** (A) Heatmap representation of the differentially expressed lncRNAs in embryo fibroblast cells that were treated with 5-Aza-dC or with DMSO (control) for 96 h. The threshold value was defined by at least a 2-fold down- or upregulation compared to results for the CEFs that were treated with DMSO (top). Pie chart showing the number of up-regulated and down-regulated lncRNAs (bottom) (B) Heatmap representation of the differentially expressed lncRNAs derived from chicken endogenous retroviruses (top). Pie chart showing the number of up-regulated and down-regulated ERV-lncRNAs (bottom).

noncoding RNA. Subsequently, Stellaris FISH probes were designed to visualize the locations and expressions of lnc-ALVE1-AS1, and the results showed that the lnc-ALVE1-AS1 was predominantly localized in the cytoplasm, with little expression in the nucleus (Fig. 3E). In addition, lnc-ALVE1-AS1 was expressed at higher levels in the tissues of the immune system, including in the bursa, thymus, and spleen, at 5 days of age than at 35 days of age (Fig. 3F), suggesting that lnc-ALVE1-AS1 could be involved in the early immune system in chickens.

### *3.4. lnc-ALVE1-AS1 triggered antiviral innate immunity by activating innate immune-related genes*

Evidence is accumulating that endogenous retroviruses can cause a type I interferon response via viral mimicry in cancer cells and may play a role in antitumour and antiviral signalling (Chernyavskaya et al., 2017; Chiappinelli et al., 2015b; Roulois et al., 2015). To investigate whether the ERV derived lnc-ALVE1-AS1 is also involved in the type I IFN signalling pathway, we transfected CEFs with a lnc-ALVE1-AS1 expression plasmid. As exogenous nucleic acids can also evoke some degree of antiviral response, we designed the plasmids expressing antisense lnc-ALVE1-AS1 or GFP as controls for the experiment. The results showed that transiently expressed lnc-ALVE1-AS1 could trigger the expression of multiple genes that are responsible for the interferon response, including the interferon beta gene (IFN-β) and a panel of interferon-stimulated genes (ISGs) (IFI27-L2, IFIT5, ISG12-2, MX1, OASL, and RASD2) (Fig. 4A). We also observed that key upstream genes in the type I interferon pathway (IFN-β, IRF7, and STAT1) were upregulated by lnc-ALVE1-AS1 RNA in CEFs, a kind of non-immune cells, compared to those of the controls (Fig. 4A). These data suggested that the expression of lnc-ALVE1-AS1 significantly activated the interferon response compared to that in the control groups, excluding any possibility of an exogenous nucleic acid-evoked antiviral response.

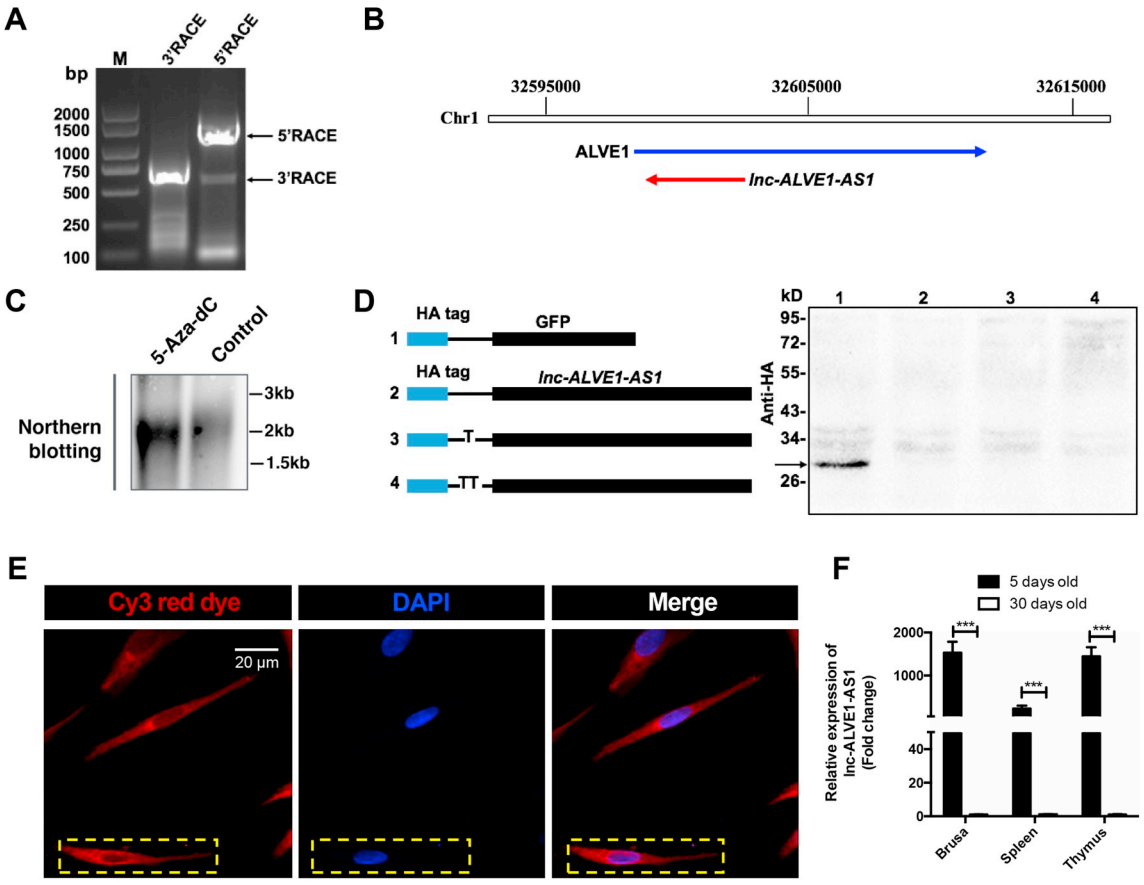


**Fig. 3. Identification and expression analysis of the ALVE1-derived lnc-ALVE1-AS1.** (A) Transcriptional start site and end site were separately identified with a 5′ cap adapter and a 3′ poly(A) adapter using special primers in a RACE assay that was performed with CEFs treated with 5-Aza-dC (B) Schematic overview of lnc-ALVE1-AS1 from the avian endogenous retroviral element ALVE1 in the chicken genome. (C) Northern blotting analysis of lnc-ALVE1-AS1 expression in CEFs treated with 5-Aza-dC or with DMSO (control). (D) Coding potential analysis of lnc-ALVE1-AS1. Full-length lnc-ALVE1-AS1 was cloned into the eukaryotic expression vector pcDNA3.1 with an N-terminal start codon (ATG) and an HA-tag in all three reading frames, and the plasmids were subsequently transfected into HEK293 cells. Immunoblotting was used to detect the HA tag after 48 h. Cells transfected with a plasmid containing GFP with an HA tag were used as a positive control. (E) Confocal microscopy images of lnc-ALVE1-AS1 stained with a Stellaris FISH probe in CEFs. Images were acquired using a 100 × /NA 1.4 lens on a Leica SP8 confocal microscope running LAS AF Lite version 2.6.3 software. Lnc-ALVE1-AS1 stained with Cy3 red dye probe (red) and nucleus stained with DAPI (blue) were excited at 552 nm by a white light laser and at 405 nm by a blue–violet laser diode, respectively. Scale bar, 20 μm. (F) Q-PCR analysis of the ALVE1-derived lnc-ALVE1-AS1 in the chicken bursa, spleen and thymus at 5 and 30 days of age. The relative expression of lnc-ALVE1-AS1 was normalized to GAPDH in each sample. Data are presented as mean ± SD from three independent experiments. Statistical significance was analyzed by two-tailed Student's t-test (***$P < 0.001$).

Then, we knocked down lnc-ALVE1-AS1 using RNA interference (RNAi) to further confirm the role of lnc-ALVE1-AS1 in antiviral innate immunity. The knockdown of lnc-ALVE1-AS1 led to a significant decrease in the expression of innate immune-related genes compared to that in the controls (Fig. 4B). These genes included a panel of interferon-stimulated genes (IFI27-L2, IFIT5, ISG12-2, MX1, OASL, and RASD2) (Fig. 4B). At the same time, the key upstream genes in the type I interferon pathway (IFN-β, IRF7, and STAT1) were also down-regulated in CEFs after the knockdown of lnc-ALVE1-AS1 compared to those in the controls (Fig. 4B). In CEFs transfected with lnc-ALVE1-AS1, western blot result shows that STAT1 and phosphorylation of STAT1 were considerably increased when compared to the CEFs transfected with the control RNA or GFP (Fig. 4C).

These data show that lnc-ALVE1-AS1 is involved in the induction of type I interferon genes, but we also wondered whether lnc-ALVE1-AS1 expression provides the cells with viral resistance. To address this question, we transfected the lnc-ALVE1-AS1 into CEFs that had been infected with avian leukosis virus subgroup J (ALVJ). CEFs were infected with ALVJ for 24 h and were then transfected with the lnc-ALVE1-AS1-expressing plasmid or the antisense sequence of lnc-ALVE1-AS1 expressing plasmid (mock) for 36 h. We observed that the exogenous virus ALVJ replication was significantly inhibited by lnc-ALVE1-AS1 compared to that in the control without the lnc-ALVE-AS1 transfection (Fig. 5A). The viral titres were also significantly lower in the lnc-ALVE1-AS1 group than in the control group (Fig. 5B). The inhibition of ALVJ by lnc-ALVE1-AS1 compared to that of the control was further confirmed by confocal immunofluorescence microscopy analysis (Fig. 5C). These findings, together with the above-mentioned results, raise the possibility that lnc-ALVE1-AS1 might function in antiviral innate immunity.

### 3.5. lnc-ALVE1-AS1 was involved in the activation of TLR3 signalling

We then elucidate how lnc-ALVE1-AS1 induces an antiviral innate immune response. Our results indicate that the overexpression of lnc-ALVE1-AS1 significantly induced the expression of the dsRNA sensors IFIH1 and TLR3 compared to that of the controls (Fig. 4A). Consistently, the key cytosolic sensor genes TLR3, IFIH1 and MD21D1 were down-regulated when lnc-ALVE1-AS1 expression was knocked down compared to that of the controls (Fig. 4B). This data helped us to infer that

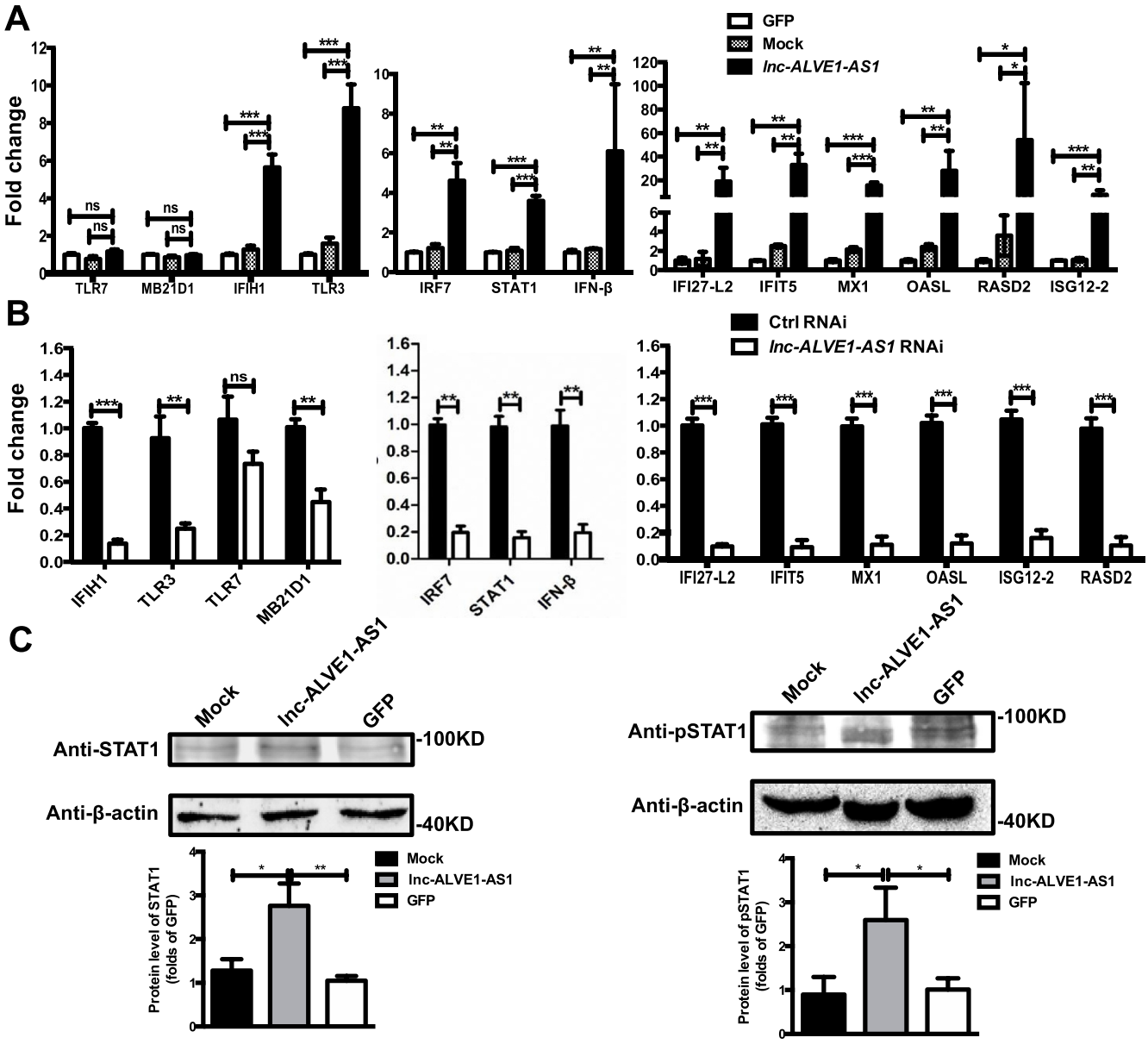


**Fig. 4. lnc-ALVE1-AS1 positively regulates the production of both IFN-β and ISGs.** (A) Q-PCR analysis of the pattern-recognition receptors including TLR7, MB21D1, IFIH1 and TLR3 (left panel); IRF7, STAT1 and IFNβ mRNA (middle panel); and the interferon-stimulated genes (right panel) in CEFs transfected with pcDNA3.1-GFP, -antisense RNA (Mock) or -lnc-ALVE1-AS1 for 36 h. Fold change (Y axis) represents relative expression of the genes normalized to GFP groups. Data are presented as mean ± SD from three independent experiments. (B) Relative expression analysis by Q-PCR of the pattern-recognition receptors including TLR7, MB21D1, IFIH1 and TLR3 (left panel); the IRF7, STAT1 and IFNβ mRNA (middle panel); and the interferon response genes (right panel) in CEFs that were transfected with the lnc-ALVE1-AS1 siRNA or with control siRNA (Ctrl RNAi) for 48 h. Fold change (Y axis) represents relative expression of the genes normalized to Ctrl RNAi between the lnc-ALVE1-AS1 RNAi and the Ctrl groups. Data are presented as mean ± SD from three independent experiments. (C) Western blot analysis of STAT1 and phospho-STAT1 expression in CEFs transfected with pcDNA3.1-antisense RNA (Mock), -lnc-ALVE1-AS1 or -GFP for 48 h. β-actin was used as the loading control. Levels of STAT1 or p-STAT1 were determined by ImageJ normalized to β-actin. STAT1 and p-STAT1 data was further adjusted by dividing with data from control GFP (GFP = 1). Data are presented as mean ± SD from three independent experiments. Statistical significance was analyzed by two-tailed Student's t-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, non-significant).

the molecular mechanisms through which the overexpression of lnc-ALVE1-AS1 induced an innate immune response may have been related to cytosolic nucleic acid sensors, including the dsRNA sensors TLR3 and IFIH1. Several recent studies in mammals have also reported that endogenous retroviral RNAs can trigger type I interferon signalling by the cytosolic pattern recognition receptors, such as IFIH1 and TLR3 (Chiappinelli et al., 2015a; Licht, 2015; Yu et al., 2012). However, to our knowledge, it has been well known that MDA5 (IFIH1) recognizes long-dsRNA (> 1–2 kb in length) (Rice et al., 2014; Roers et al., 2016). TLR3 recognizes not only perfect dsRNA larger than 40 bp (Roers et al., 2016), but also recognizes structured RNA that contains an incomplete stem with bulge and internal loops (Tatematsu et al., 2013, 2014). We thus speculate that TLR3 are centrally involved in lnc-ALVE1-AS1 triggering cytosolic sensor to induce an interferon response. Therefore, immunofluorescence and RNA FISH were performed to confirm whether TLR3 recognizes lnc-ALVE1-AS1 in vivo. We observed the co-localization of TLR3 and lnc-ALVE1-AS1 in CEFs with confocal microscopy (Fig. 6A), suggesting that TLR3 may recognizes lnc-ALVE1-AS1 in

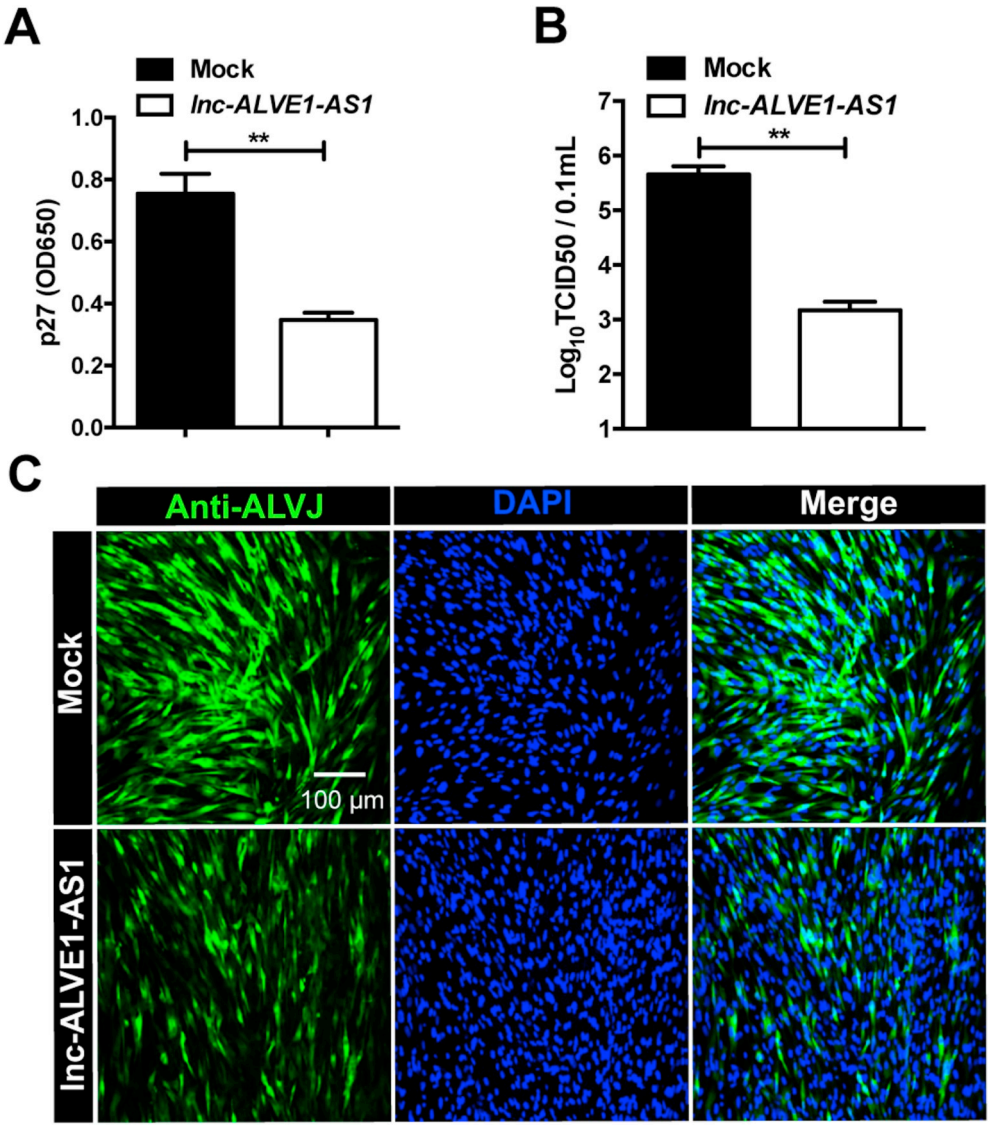


**Fig. 5. lnc-ALVE1-AS1 mediates the defense against the replication of ALVJ.** (A) ELISA of the p27 that was secreted by ALVJ in the supernatants of CEF cells that were infected with ALVJ for 24 h and were then transfected with pcDNA3.1-lnc-ALVE1-AS1 or with pcDNA3.1-antisense RNA (Mock) for 36 h. The value of OD650 were determined by the ELISA reader. Data are presented as mean ± SD from three independent experiments. (B) ALVJ titres based on a $TCID_{50}$ assay in the supernatants of CEF cells that were infected with ALVJ for 24 h and were then transfected with pcDNA3.1-lnc-ALVE1-AS1 or with pcDNA3.1-antisense RNA (Mock) for 36 h. The viral loads in the cell cultures per group were determined by $TCID_{50}$ using 96-well plates. Data are presented as mean ± SD from three independent experiments. (C) CEFs were infected with ALVJ for 24 h and were then transfected with a lnc-ALVE1-AS1-expressing plasmid or with an antisense sequence of lnc-ALVE1-AS1-expressing plasmid (Mock) for 36 h. The cells were then visualized with a Leica SP8 confocal microscope (20 × /NA 0.75 lens). ALVJ env stained with Alexa-Fluor-488 (green) and nucleus stained with DAPI (blue) were excited at 488 nm by a white light laser and at 405 nm by a blue–violet laser diode, respectively. Scale bar, 100 μm. Images show a representative example of three independent experiments. Statistical significance was analyzed by two-tailed Student's *t*-test (***$P < 0.001$).

this case. Western blotting was performed to further evaluate the effect of lnc-ALVE1-AS1 on the protein level of the dsRNA sensor TLR3 in CEFs (Fig. 6B). The TLR3 protein level was significantly increased by lnc-ALVE1-AS1 overexpression but was decreased when lnc-ALVE1-AS1 was knocked down in CEFs (Fig. 6B). These results suggest that lnc-ALVE1-AS1 RNA not only is associated with TLR3 in antiviral innate immunity but also may be involved in the regulation of TLR3 expression. Additionally, knocking down the dsRNA sensor TLR3 significantly blunted the lnc-ALVE1-AS1-induced innate immune response compared to that of the controls (Fig. 6C). Furthermore, the inhibition of the TLR3-type-I interferon signalling pathway with a Tbk1/Ikkε inhibitor (amlexanox) also reduced the gene responses that were induced by lnc-ALVE1-AS1 compared to those of the controls (Fig. 6D). These data suggested that lnc-ALVE1-AS1 induced an antiviral innate immune response via a TLR3-dependent pathway.

## 4. Discussion

ERVs have shaped the evolution of a transcriptional network underlying the IFN response, which is a major branch of innate immunity (Chuong et al., 2016). Moreover, lineage-specific ERVs have independently dispersed numerous IFN-inducible enhancers independently in diverse mammalian genomes (Chuong et al., 2016; Manghera and Douville, 2013; Nissen et al., 2013). Notably, immune reactivity to ERVs has been frequently observed in humans during infection, inflammation, autoimmunity and cancer. Recent evidence suggests that ERVs, an “enemy within”, may also play beneficial roles in tuning host immune reactivity (Kassiotis and Stoye, 2016) and in the regulatory evolution of innate immunity in combination with ERVs (Chuong et al., 2016). These observations suggest that ERV-derived transcripts may be involved in the innate immune system and may play a crucial role in protecting hosts against exogenous viral infection.

Although the activation of ERVs has been noted in various cancers and in autoimmunity, whether their activation is a consequence or a cause of disease remains poorly elucidated (Hayward and Katzourakis, 2015). In this study, we found that the loss of DNA methylation by the inhibitor 5-Aza-dC leads to the derepression of endogenous retroviruses, which can produce lncRNAs that lead to the induction of the antiviral signalling in chicken embryonic fibroblasts (CEFs). The identification of these new functional ERV-lncRNAs will be key to deciphering the mechanisms behind the 5-Aza-dC-mediated antiviral response and to providing an additional layer of activation in antiviral innate immunity. The antisense lncRNA lnc-ALVE1-AS1that is transcribed from the ERV ALVE1 was demonstrated to have antiviral

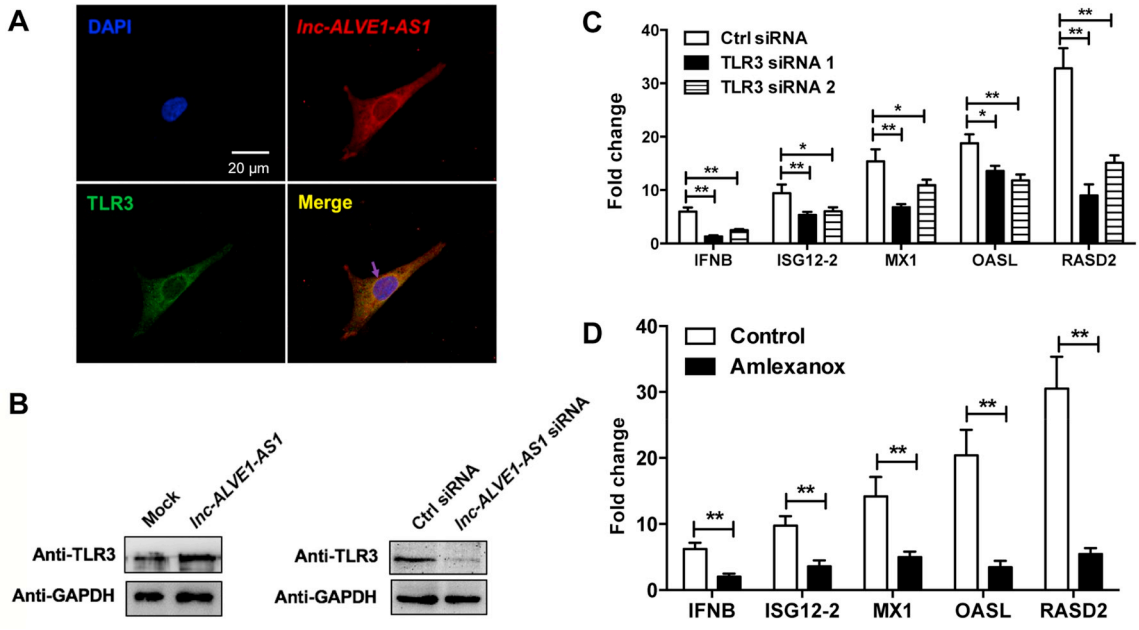


**Fig. 6. lnc-ALVE1-AS1 is involved in the activation of TLR3 signalling.** (A) Colocalization analysis of lnc-ALVE1-AS1, which was stained with a Stellaris FISH probe (red), combined with the immunofluorescence analysis of endogenous TLR3 (green) in CEFs that were transfected with lnc-ALVE1-AS1 for 36 h. Images were acquired using a 100 × /NA 1.4 lens on a Leica SP8 confocal microscope running LAS AF Lite version 2.6.3 software. Lnc-ALVE1-AS1 stained with Cy3 red dye probe (red) and TLR3 stained with Alexa-Fluor-488 (green) were excited at 552 nm and 488 nm respectively, and DAPI (blue) were excited at 405 nm. Scale bar, 20 μm. Images show a representative example. (B) Western blot analysis of the dsRNA recognition receptor TLR3 in CEFs that were transfected with lnc-ALVE1-AS1, antisense or GFP for 36 h (left) or that were transfected with lnc-ALVE1-AS1 siRNA or with control siRNA for 48 h (right). (C) Relative expression analysis of ISGs in CEFs that were transfected with TLR3 siRNA or with control siRNA for 24 h and were then transfected with lnc-ALVE1-AS1 or with antisense RNA (mock) for 36 h. Fold change (Y axis) represents relative expression of the indicated ISGs in the TLR3-knockdown CEFs or control transfected with or without lnc-ALVE1-AS1. (D) Relative expression analysis of ISGs in CEFs treated with the TLR3 signalling inhibitor amlexanox for 1 h and were then transfected with lnc-ALVE1-AS1 or with antisense RNA (mock) for 36 h. Fold change (Y axis) represents relative expression of the indicated ISGs in Amlexanox treatment or the control CEFs transfected with or without lnc-ALVE1-AS1. Data are presented as mean ± SD from three independent experiments. Statistical significance was analyzed by two-tailed Student's t-test (*$P < 0.05$ and **$P < 0.01$).

effects. This finding was obviously attributed, at least in part, to the induction of antiviral innate immunity. The activation of antiviral innate immunity by an ERV-derived lncRNA may be an important defence mechanism of the host during viral infection.

An important finding is that lnc-ALVE1-AS1, which is transcribed from the chicken ERV ALVE1, displayed obvious antiviral effects by activating the interferon (IFN) response in this study. Our results indicated that these effects were mediated by activating or binding to the Toll like receptor 3 (TLR3). Recent studies have shown that the antitumour effect of 5-Aza-dC is actually mediated by the dsRNA induction of endogenous retroviruses (Chiappinelli et al., 2015a; Licht, 2015; Roulois et al., 2015). Studies have also shown that TLR3 recognizes not only dsRNA, but also the incomplete stem structures of viral or host-derived RNA (Tatematsu et al., 2013, 2014) (Fig. 7). However, the mechanism by which lnc-ALVE1-AS1 regulates TLR3 is unknown yet. Based on our current results, we envision the following model of chicken ERV ALVE1 derived lnc-ALVE1-AS1 mediated antiviral innate immune signalling (Fig. 7). The transcript of the chicken endogenous retrovirus ALVE1, lnc-ALVE1-AS1, could be exported from the nucleus into the cytoplasm, where it activates the TLR3 pathway. The activation of TLR3 signalling results in the induction of IFN-β, which is critical to antiviral defence (McNab et al., 2015), and the subsequent the upregulation of ISGs, maintaining the antiviral state in CEFs.

Although we have shown that endogenous retroviral sequences play a functional role in activating innate immunity in CEFs, further studies are required to extend our findings to other species, such as humans and mice. We hypothesized that the existence of ERVs in the host genome may not be a coincidence but may reflect ancient retroviral adaptations to evolution and the plasticity of the host genome by positive selection. These endogenous viruses that “invaded” the human and animal genomes millions of years ago might be important components of the innate immune system. They could be exploited to combat of host infection by exogenous relatives (Best et al., 1997). Indeed, the innate immune system can mount resistance to re-infection, and this phenomenon, termed innate immune memory, may be exerted through endogenous retroviral elements (Netea et al., 2016).

In summary, our findings collectively show that an endogenous retroviral element exerts antiviral innate immunity via its derived antisense lncRNA, suggesting that ERV elements in the genome may play distinct antiviral protective roles. These findings may provide new insights into the function of ERVs in innate immunity and in antiviral defense in humans and in animals and may offer new strategies for antiviral treatment in the future.

## Authors’ contributions

S.C. and X.H. performed the experiment; S.C., X.H., C.D., Y.L., Z.S. and S.X. analyzed data; S.C., X.H., I.H.C., S.W., T.G. and H.C. wrote and edited the manuscript; Z.L. and A.J. provided viruses and other materials. H.C. supervised the research project. All the authors discussed the results and commented on the manuscript.

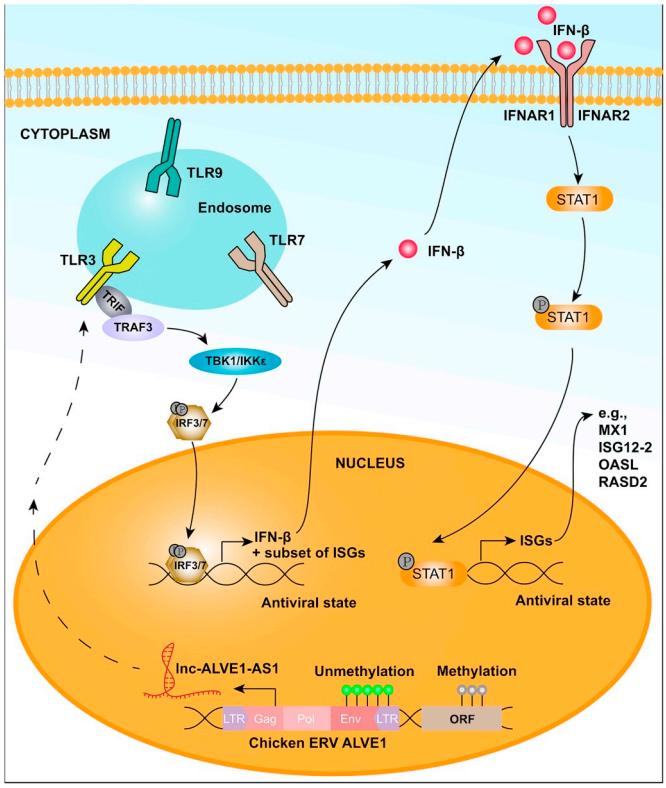


**Fig. 7. A model of the chicken ERV ALVE1-derived lnc-ALVE1-AS1-mediated innate immune signalling.** The transcript of the chicken endogenous retrovirus ALVE1, lnc-ALVE1-AS1, could be exported from the nucleus into the cytoplasm and activate the TLR3 pathway, the activation of which results in the induction of IFN-β and the subsequent upregulation of ISGs, thus maintaining the antiviral state in CEFs.

## Disclosures

The authors declare that there are no conflicts of interest.

## Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (91540117, 81773013 and 31602032), the National Key Research and Development Program of China (2016YFC1303604), the Priority Academic Program Development of Jiangsu Higher Education Institutions (Animal Science and Veterinary Medicine).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104571.

## References

Best, S., Le Tissier, P.R., Stoye, J.P., 1997. Endogenous retroviruses and the evolution of resistance to retroviral infection. Trends Microbiol. 5, 313–318.
Chernyavskaya, Y., Mudbhary, R., Zhang, C., Tokarz, D., Jacob, V., Gopinath, S., Sun, X., Wang, S., Magnani, E., Madakashira, B.P., Yoder, J.A., Hoshida, Y., Sadler, K.C., 2017. Loss of DNA methylation in zebrafish embryos activates retrotransposons to trigger antiviral signaling. Development 144, 2925–2939.
Chiappinelli, K.B., Strissel, P.L., Desrichard, A., Li, H., Henke, C., Akman, B., Hein, A., Rote, N.S., Cope, L.M., Snyder, A., Makarov, V., Budhu, S., Slamon, D.J., Wolchok, J.D., Pardoll, D.M., Beckmann, M.W., Zahnow, C.A., Merghoub, T., Chan, T.A., Baylin, S.B., Strick, R., 2015a. Inhibiting DNA methylation causes an interferon response in cancer via dsRNA including endogenous retroviruses. Cell 162, 974–986.
Chiappinelli, K.B., Strissel, P.L., Desrichard, A., Li, H., Henke, C., Akman, B., Hein, A., Rote, N.S., Cope, L.M., Snyder, A., Makarov, V., Buhu, S., Slamon, D.J., Wolchok, J.D., Pardoll, D.M., Beckmann, M.W., Zahnow, C.A., Mergoub, T., Chan, T.A., Baylin, S.B., Strick, R., 2015b. Inhibiting DNA methylation causes an interferon response in cancer via dsRNA including endogenous retroviruses. Cell 162, 974–986.
Chuong, E.B., Elde, N.C., Feschotte, C., 2016. Regulatory evolution of innate immunity through co-option of endogenous retroviruses. Science 351, 1083–1087.
Chuong, E.B., Elde, N.C., Feschotte, C., 2017. Regulatory activities of transposable elements: from conflicts to benefits. Nat. Rev. Genet. 18, 71–86.
Durruthy-Durruthy, J., Sebastiano, V., Wossidlo, M., Cepeda, D., Cui, J., Grow, E.J., Davila, J., Mall, M., Wong, W.H., Wysocka, J., Au, K.F., Reijo Pera, R.A., 2016. The primate-specific noncoding RNA HPAT5 regulates pluripotency during human preimplantation development and nuclear reprogramming. Nat. Genet. 48, 44–52.
Grow, E.J., Flynn, R.A., Chavez, S.L., Bayless, N.L., Wossidlo, M., Wesche, D.J., Martin, L., Ware, C.B., Blish, C.A., Chang, H.Y., Pera, R.A., Wysocka, J., 2015. Intrinsic retroviral reactivation in human preimplantation embryos and pluripotent cells. Nature 522, 221–225.
Guttman, M., Garber, M., Levin, J.Z., Donaghey, J., Robinson, J., Adiconis, X., Fan, L., Koziol, M.J., Gnirke, A., Nusbaum, C., Rinn, J.L., Lander, E.S., Regev, A., 2010. Ab initio reconstruction of cell type-specific transcriptomes in mouse reveals the conserved multi-exonic structure of lincRNAs. Nat. Biotechnol. 28, 503–510.
Hayward, A., Cornwallis, C.K., Jern, P., 2015. Pan-vertebrate comparative genomics unmasks retrovirus macroevolution. Proc. Natl. Acad. Sci. U. S. A. 112, 464–469.
Hayward, A., Katzourakis, A., 2015. Endogenous retroviruses. Curr. Biol. : CB 25, R644–R646.
Justice, J.t., Malhotra, S., Ruano, M., Li, Y., Zavala, G., Lee, N., Morgan, R., Beemon, K., 2015. The MET gene is a common integration target in avian leukosis virus subgroup J-induced chicken hemangiomas. J. Virol. 89, 4712–4719.
Kassiotis, G., Stoye, J.P., 2016. Immune responses to endogenous retroelements: taking the bad with the good. Nat. Rev. Immunol. 16, 207–219.
Kelley, D., Rinn, J., 2012. Transposable elements reveal a stem cell-specific class of long noncoding RNAs. Genome Biol. 13, R107.
Leonova, K.I., Brodsky, L., Lipchick, B., Pal, M., Novototskaya, L., Chenchik, A.A., Sen, G.C., Komarova, E.A., Gudkov, A.V., 2013. p53 cooperates with DNA methylation and a suicidal interferon response to maintain epigenetic silencing of repeats and noncoding RNAs. Proc. Natl. Acad. Sci. U. S. A. 110, E89–E98.
Licht, J.D., 2015. DNA methylation inhibitors in cancer therapy: the immunity dimension. Cell 162, 938–939.
Liu, M., Ohtani, H., Zhou, W., Orskov, A.D., Charlet, J., Zhang, Y.W., Shen, H., Baylin, S.B., Liang, G., Gronbaek, K., Jones, P.A., 2016. Vitamin C increases viral mimicry induced by 5-aza-2'-deoxycytidine. Proc. Natl. Acad. Sci. U. S. A. 113, 10238–10244.
Lu, X., Sachs, F., Ramsay, L., Jacques, P.E., Goke, J., Bourque, G., Ng, H.H., 2014. The retrovirus HERVH is a long noncoding RNA required for human embryonic stem cell identity. Nat. Struct. Mol. Biol. 21, 423–425.
Maksakova, I.A., Mager, D.L., Reiss, D., 2008. Keeping active endogenous retroviral-like elements in check: the epigenetic perspective. Cell. Mol. Life Sci. 65, 3329–3347.
Manghera, M., Douville, R.N., 2013. Endogenous retrovirus-K promoter: a landing strip for inflammatory transcription factors? Retrovirology 10, 16.
McNab, F., Mayer-Barber, K., Sher, A., Wack, A., O'Garra, A., 2015. Type I interferons in infectious disease. Nat. Rev. Immunol. 15, 87–103.
Netea, M.G., Joosten, L.A., Latz, E., Mills, K.H., Natoli, G., Stunnenberg, H.G., O'Neill, L.A., Xavier, R.J., 2016. Trained immunity: a program of innate immune memory in health and disease. Science 352, aaf1098.
Nissen, K.K., Laska, M.J., Hansen, B., Terkelsen, T., Villesen, P., Bahrami, S., Petersen, T., Pedersen, F.S., Nexo, B.A., 2013. Endogenous retroviruses and multiple sclerosis-new pieces to the puzzle. BMC Neurol. 13, 111.
Rice, G.I., Del Toro Duany, Y., Jenkinson, E.M., Forte, G.M., Anderson, B.H., Ariaudo, G., Bader-Meunier, B., Baildam, E.M., Battini, R., Beresford, M.W., Casarano, M., Chouchane, M., Cimaz, R., Collins, A.E., Cordeiro, N.J., Dale, R.C., Davidson, J.E., De Waele, L., Desguerre, I., Faivre, L., Fazzi, E., Isidor, B., Lagae, L., Latchman, A.R., Lebon, P., Li, C., Livingston, J.H., Lourenco, C.M., Mancardi, M.M., Masurel-Paulet, A., McInnes, I.B., Menezes, M.P., Mignot, C., O'Sullivan, J., Orcesi, S., Picco, P.P., Riva, E., Robinson, R.A., Rodriguez, D., Salvatici, E., Scott, C., Szybowska, M., Tolmie, J.L., Vanderver, A., Vanhulle, C., Vieira, J.P., Webb, K., Whitney, R.N., Williams, S.G., Wolfe, L.A., Zuberi, S.M., Hur, S., Crow, Y.J., 2014. Gain-of-function mutations in IFIH1 cause a spectrum of human disease phenotypes associated with upregulated type I interferon signaling. Nat. Genet. 46, 503–509.
Roers, A., Hiller, B., Hornung, V., 2016. Recognition of endogenous nucleic acids by the innate immune system. Immunity 44, 739–754.
Roulois, D., Loo Yau, H., Singhania, R., Wang, Y., Danesh, A., Shen, S.Y., Han, H., Liang, G., Jones, P.A., Pugh, T.J., O'Brien, C., De Carvalho, D.D., 2015. DNA-demethylating agents target colorectal cancer cells by inducing viral mimicry by endogenous transcripts. Cell 162, 961–973.
Stocking, C., Kozak, C.A., 2008. Murine endogenous retroviruses. Cell. Mol. Life Sci. 65, 3383–3398.
Stoye, J.P., 2012. Studies of endogenous retroviruses reveal a continuing evolutionary saga. Nat. Rev. Microbiol. 10, 395–406.
Tatematsu, M., Nishikawa, F., Seya, T., Matsumoto, M., 2013. Toll-like receptor 3 recognizes incomplete stem structures in single-stranded viral RNA. Nat. Commun. 4, 1833.
Tatematsu, M., Seya, T., Matsumoto, M., 2014. Beyond dsRNA: Toll-like receptor 3 signalling in RNA-induced immune responses. Biochem. J. 458, 195–201.
Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., Pachter, L., 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7, 562–578.
Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., Pachter, L., 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell

differentiation. Nat. Biotechnol. 28, 511–515.
Virgin, H.W., 2014. The virome in mammalian physiology and disease. Cell 157, 142–150.
Volkman, H.E., Stetson, D.B., 2014. The enemy within: endogenous retroelements and autoimmune disease. Nat. Immunol. 15, 415–422.
Wang, J., Xie, G., Singh, M., Ghanbarian, A.T., Rasko, T., Szvetnik, A., Cai, H., Besser, D., Prigione, A., Fuchs, N.V., Schumann, G.G., Chen, W., Lorincz, M.C., Ivics, Z., Hurst, L.D., Izsvak, Z., 2014. Primate-specific endogenous retrovirus-driven transcription defines naive-like stem cells. Nature 516, 405–409.
Yu, P., Lubben, W., Slomka, H., Gebler, J., Konert, M., Cai, C., Neubrandt, L., Prazeres da Costa, O., Paul, S., Dehnert, S., Dohne, K., Thanisch, M., Storsberg, S., Wiegand, L., Kaufmann, A., Nain, M., Quintanilla-Martinez, L., Bettio, S., Schnierle, B., Kolesnikova, L., Becker, S., Schnare, M., Bauer, S., 2012. Nucleic acid-sensing Toll-like receptors are essential for the control of endogenous retrovirus viremia and ERV-induced tumors. Immunity 37, 867–879.