Virology 531 (2019) 69–78

Contents lists available at ScienceDirect

Virology

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

Latency-associated nuclear antigen inhibits lytic replication of Kaposi's sarcoma-associated herpesvirus by regulating let-7a/RBPJ signaling

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ARTICLE INFO

Keywords:
Kaposi's sarcoma-associated herpesvirus
Latency-associated nuclear antigen
Let-7a
RBPJ
Latent

ABSTRACT

Latency-associated nuclear antigen (LANA) is the key factor in the establishment and maintenance of latency of Kaposi's sarcoma-associated herpesvirus (KSHV). A cellular protein, recombination signal binding protein for immunoglobulin kappa J region (RBPJ), is essential for the lytic reactivation of KSHV. However, whether RBPJ expression is regulated by KSHV is not clear. Here, we show that LANA upregulates let-7a and its primary transcripts in parallel with its reduction of RBPJ expression. An increase in notch intracellular domain (NICD) and the downregulation of NF-κB and LIN28B contribute to the upregulation of let-7a by LANA. Let-7a represses RBPJ expression by directly binding the 3′ untranslated region of RBPJ. Let-7a overexpression or RBPJ knockdown led to a dose- and time-dependent inhibition of lytic reactivation of KSHV. Collectively, these findings support a model wherein LANA inhibits the lytic replication of KSHV by regulating let-7a/RBPJ signaling.

1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as *Human herpesvirus 8* (HHV8), is a gamma herpesvirus associated with Kaposi's sarcoma (KS), primary effusion lymphoma (Cesarman et al., 1995), and multicentric Castleman's disease (Soulier et al., 1995). Like other herpesviruses, KSHV has a dual-phase life cycle, which includes latent and lytic modes, during its infection of endothelial cells and B lymphocytes (Chang et al., 1994). The virus remains in the latent state in most KSHV-infected host cells, from where it is occasionally reactivated to the lytic state, facilitating its cell-to-cell spread (Sun et al., 2017; Wu et al., 2006). Therefore, the latent and lytic replication of KSHV and the continuous infection of new cells are essential for persistent KSHV infection and its associated pathogenesis (Bottero et al., 2013; Chang and Kung, 2014).

During latency, the circular KSHV episome is retained in the nucleus at a low copy number and expresses only a few genes. Latency-associated nuclear antigen (LANA), encoded by the open reading frame 73 (ORF 73), is among the small number of KSHV genes expressed in all KSHV-infected cells during latency (Sun et al., 2014). LANA is a multifunctional protein that regulates both transcription and cell growth (Gjyshi et al., 2015; Lu et al., 2014; Wang et al., 2014). The replication and transcription activator (RTA) encoded by ORF50 controls the switch from latency to lytic replication (Deng et al., 2007; Papugani et al., 2008; Zhao et al., 2015). RTA initiates the expression of the lytic genes in an ordered cascade and is both necessary and sufficient to trigger the lytic switch (Gradoville et al., 2000; Lukac et al., 1999). Recombinant viruses that lack RTA establish latent infections but are unable to reactivate (Xu et al., 2005). RTA binds directly to RTA-responsive elements on the promoters of the target genes or uses cellular DNA-binding factors as adaptors to bind DNA, such as AP-1, C/EBP-a, and OCT-1, or recombination signal binding protein for the immunoglobulin kappa J region (RBPJ), (Papugani et al., 2008; Persson and Wilson, 2010).

RBPJ is a highly conserved, ubiquitously expressed protein and the major effector of Notch signaling (Wang et al., 2013). It can act as an adaptor for transactivators, but can also recruit corepressor complexes, thereby silencing gene expression. RTA binds to RBPJ, contributing to the activation of RTA itself, which initiates a positive feedback loop driving lytic reactivation (Carroll et al., 2007; Guito and Lukac, 2012; Liang and Ganem, 2004; Lu et al., 2012; Palmeri et al., 2011; Wang and Yuan, 2007). When RTA binds to RBPJ, it also induces the expression of

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https://doi.org/10.1016/j.virol.2019.02.019
Received 24 October 2018; Received in revised form 27 February 2019; Accepted 27 February 2019
Available online 27 February 2019
0042-6822/

many lytic genes, such as ORF6, ORF8, ORF19, ORF29a, ORF47, ORF50, ORF57, ORF59, ORF61, ORF65, ORF70, K2, K5, K6, K8, K14, and PAN, as well as the latency transcript cluster that includes LANA (Guito and Lukac, 2012; Izumiya et al., 2009; Lu et al., 2011; Persson and Wilson, 2010; Wang et al., 2010a). LANA induced by RTA also binds to RBPJ, which in turn inhibits RTA expression, and this negative feedback contributes to the establishment and maintenance of the viral latency (Lan et al., 2005a, 2005b, 2006; Lu et al., 2011). Thus, RBPJ serves as a mediator of the positive and negative feedback, functioning as a balancer to regulate KSHV lytic and latent replication. Although the binding of RBPJ to LANA contributes to the repression of RTA expression, RBPJ is only essential for KSHV lytic infection, but not its latent infection (Liang and Ganem, 2003; Scholz et al., 2013). RBPJ is almost undetectable on viral and cellular promoters in KSHV latent infected cells, but it is significantly enriched on these promoters after RTA expression during virus reactivation (Carroll et al., 2006). Besides that RBPJ is relocated to the promoters of these genes, the results also indicate that KSHV-latently-infected cells may have relatively low levels of RBPJ. These findings suggest that the expression of RBPJ is regulated by KSHV. Therefore, we tested the hypothesis that during latent KSHV infection, RBPJ expression is repressed by LANA or other latency-associated proteins.

In previous studies, we demonstrated that the cellular microRNA let-7a is downregulated in KS tumor tissues compared with the adjacent normal skin tissues and that let-7a represses KSHV reactivation by inhibiting the expression of its target gene *MAP4K4* (Tan et al., 2015). The 3′ untranslated region (UTR) of RBPJ also contains a conserved let-7a-binding site, indicating that RBPJ is directly targeted by let-7a. In the present study, we confirmed that LANA represses the lytic replication of KSHV via let-7a/RBPJ signaling. LANA upregulates let-7a in parallel with its downregulation of RBPJ expression. LANA induces let-7a transcription by increasing cellular notch intracellular domain (NICD) and promoting let-7a maturation by downregulating NF-κB and Lin28B. Let-7a represses RBPJ expression by directly binding to the 3′-UTR of its mRNA. RBPJ knockdown led to a dose- and time-dependent inhibition of lytic reactivation of KSHV. Taken together, our results indicate that the upregulation of let-7a and downregulation RBPJ by LANA contributes to the establishment and maintenance of latent KSHV infection in the host cell.

2. Materials and methods

2.1. Cell lines

293 T cells were cultured in Dulbecco modified Eagle medium (DMEM) medium supplemented with 10% (v/v) of FBS, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C under 5% CO_2. The KSHV positive cells, iSLK.219, were kindly gifted from Prof Ke Lan, were maintained in DMEM containing G418 100 μg/ml, puro 4 μg/ml, hygromycin 100 μg/ml, other condition is the same as 293 T cells. iSLK.219 cells contain recombinant KSHV.219 (rKSHV.219) (Vieira and O'Hearn, 2004)and express RTA under the control of a doxycycline (DOX)-responsive promoter. These cells constructively express GFP protein and express RFP in lytic replication cells.

2.2. Plasmids

The 303 bp fragment of wild-type RBPJ 3'UTR containing the let-7 binding site, or mutated RBPJ 3'UTR, was synthesized and cloned into the PRL-TK dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA) to get the wt-PRL-TK-RBPJ 3'UTR or mt-PRL-TK-RBPJ 3'UTR. The target site for microRNA let-7a (5'-**C**TA**CC**T**C**T-3') in wt-PRL-TK-RBPJ 3'UTR was mutated to 5'-**A**TA**AC**A**C**T-3 in mt-PRL-TK-RBPJ 3'UTR. The RBPJ ORF were amplified used the primers, RBPJ-F: TCTCGAGCTCAAGCTTC***GAATT***CGCCACCATGGCGCCTGTTGTGAC AGG and RBPJ-R: GGTACCGTCGACTGCA***GAATT***CCCACTGTGGCTGT AGATGATGTG. The NICD expression plasmids were amplified used the primers, NICD-F: TCTCGAGCTCAAGCTTC**GAATT**CGC CACCATGCCTG AGGGCTTCAAAGTGTCTG and NICD-R: GGTACCGTCGACT GCA***GAATT***CCTTGAAGGCCTCCGGAATGCG. The bald italic letter show the *Eco*RI enzyme recognize site) and inserted into the pEGFP-N2 expression plasmid. The let-7a and let-7 sponge expression plasmids were constructed as previously described (Tan et al., 2015). Briefly, the let-7a encoding fragment was amplified by PCR using DNA extracted from 293 T cells with specific primers and cloned into pSilencer 4.1 (Invitrogen, Carlsbad, CA, USA). The let-7 sponge fragment, containing the complementary sequence of let-7, was synthesized and cloned into the pEGFP-C2. The ORF73 (LANA) expression plasmid pCAGGS-LANA was kindly gifted from Prof. Ke Lan. The plasmids were transfected into 293 T or iSLK.219 cells using lipofectamine (Invitrogen, Carlsbad, CA, USA).

2.3. Luciferase activity assay

Doses of pSilencer™ 4.1-Let-7a and wt-PRL-TK-RBPJ 3′UTR or mt-PRL-TK-RBPJ 3′UTR were co-transfected into 293 T cells using TurboFect Transfection Reagent (Thermo, Carlsbad, CA, USA). Cells were cultured in DMEM for 24 h, then collected and analyzed for luciferase activity. Luciferase activity was measured using the dual-luciferase reporter assay kit (Promega, Madison, WI, USA) and normalized to renilla luciferase activity and total protein level.

2.4. Isolation and quantification of intracellular and extracellular KSHV genomes

For determination of intracellular KSHV DNA copy numbers, 1×10^6 cells were harvested, washed in PBS, then the DNA was purified by phenol-chloroform extraction. Extracellular virion-associated KSHV genomes in culture supernatants of lytic induced cells were isolated as described (Lu et al., 2004). Briefly, the supernatant was centrifuged at 5000 g for 5 min, followed by addition of DNase I (Invitrogen, Carlsbad, CA, USA) at a concentration of 100 U/ml for 1 h to digest unprotected DNA. After heat inactivation of DNase I at 65 °C for 30 min in the presence of 10 mM EDTA, DNA was purified by phenol-chloroform extraction. Intracellular and extracellular viral DNA was analyzed by real-time PCR using ORF50 primers (ORF50F 5′-CGCAAT GCGTTACGTTGT TG-3′ and ORF50R 5′-GCCCGGACTGTTGAATCG-3′).

2.4.1. RNA inference assay

The commercial synthesized siRNAs specific target RBPJ (si-RBPJ: 5′-GGCTCTGTTTAATCGACTA-3′), LANA (si-LANA: 5′-GCATTTGTGTC TAGTCCTA-3′), si-LIN28B (si-LIN28B: 5′-CCAGTGGAATTCACAT TTA-3′) and the non-specific control siRNAs (Riobo, Guangzhou, China) were transfected into 293 T cells or iSLK.219 cells using riboFECT™ CP reagent (Riobo, Guangzhou, China). The knock-down efficiency was confirmed by detecting the targeted genes' mRNA and protein levels.

2.5. Quantitative real-time PCR

Total RNA from cells was reverse transcribed using the Thermoscript™ RT-PCR System (Invitrogen, Carlsbad, CA, USA). The viral DNA was extracted from iSLK.219 cells as described above. The quantity of RBPJ and LANA mRNA, and RTA DNA was assayed by real-time PCR performed by the methods described previously (Takara Bio Inc. Otsu, Japan) with specific primers. Real-time PCR data were presented using $2^{-\Delta\Delta Ct}$ method with β-actin as the internal control.

To measure the production of let-7 miRNA, cells were harvested and miRNAs were isolated using miRcute miRNA Isolation Kit (Tiangen Biotech, Beijing, China). The let-7 miRNAs were quantified using the methods as described previously (Takara Bio Inc. Otsu, Japan) with commercial primers (Riobo, Guangzhou, China). Data were analyzed by

Table 1
Primer sequences for quantitative real-time PCR.

Primer	Sequence (5′-3′)
LIN28B-F	CATCTCCATGATAAACCGAGAGG
LIN28B-R	GTTACCCGTATTGACTCAAGGC
P65-F	CACAAGGCAGCAAATAGACG
P65-R	GAGTTAGCAGTGAGGCACCA
RBPJ-F	GACTCAGACAAGCGAAAGCA
RBPJ-R	TTTGGAAGGTTTGGAGATGAC
pri-let-7afd-F	GAAACCTTTTGCTTCTTGTCT
pri-let-7afd-R	CCTCACTCTGATAGAGCAAT
pri-let-7a-2-F	ATACTGAATCCCTCAAAGCC
pri-let-7a-2-R	GAAAGGTAAGATTGGGTACGA
ORF73-F	GCCTACATCTCCCATCTCCA
ORF73-R	ATCCTCCTCGTCATCCTCCT
ORF50-F	CGCAATGCGTTACGTTGTTG
ORF50-R	GCCCGGACTGTTGAATCG
Actin-F	GAGCGGGAAATCGTCCGTGACATT
Actin-R	GATGGAGTTGAAGGTAGTTTCGTG
let-7a-F	GCGCCTGAGGTAGTAGGTTG
let-7a-R	CAGTGCAGGGTCCGAGGT
U6-F	CTCGCTTCGGCAGCACATATACT
U6-R	ACGCTTCACGAATTTGCGTGTC

$2^{-\Delta\Delta Ct}$ method with U6 as the internal control (Dittmer, 2003). The primer sequences used for quantitative PCR were listed in Table 1.

2.6. Northern-blots

The let-7a miRNA expression levels were validated by northern blot hybridization using a High Sensitive miRNA Northern Blot Assay Kit (NB-1001, Signosis, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. The miRNAs isolated using miRcute miRNA Isolation Kit (Tiangen Biotech, Beijing, China), 45 μg miRNA was electrophoresed in 15% denaturing PAGE and transferred to a Hybond-NX nylon membrane, followed by UV cross-linking. Membranes were hybridized with oligonucleotide probes of let-7a (MP-0511, Signosis, Inc., Santa Clara, CA, USA). We hybridized blots with an oligonucleotide probe complementary to RNU48 (MP-0001, Signosis, Inc.) as a control. Digital images were acquired in the linear range of the scanner Fluor-S MultiImager (Bio-Rad). Intensities of band signals were quantified using the densitometric software Quantity One (Bio-Rad). miRNA amount was normalized with the corresponding RNU48 RNA in each sample.

2.7. Western blotting

All western blots were probed with specific antibodies directed against RBPJ or LIN28B (Abcam, Cambridge, MA, USA), LANA (Novus, Littleton CO, USA), p65 or β-actin (Santa Cruz Biotechnology, Santa Clara, CA, USA). β-Actin was used as the internal loading control.

3. Results

3.1. LANA increases let-7a and pri-let-7afd levels while reducing RBPJ levels

We asked whether LANA contributes to the regulation of let-7a and RBPJ expression. Therefore, we investigated the association between the expression levels of LANA, let-7a, and RBPJ. The cellular miRNA let-7a is derived from primary transcripts expressed from three different chromosomal locations, *pri-let-7afd* on chromosome 9, *pri-let-7a-2* on chromosome 11, and *pri-let-7a-3* on chromosome 22.

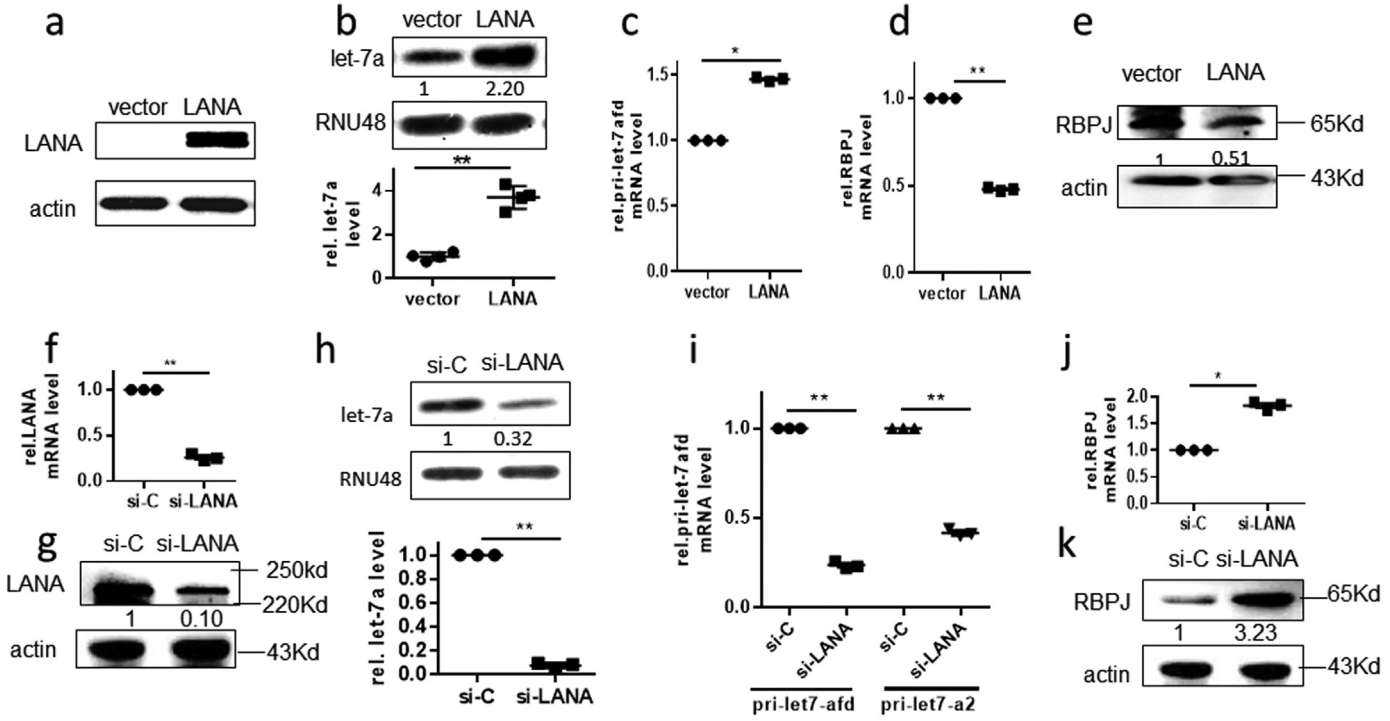
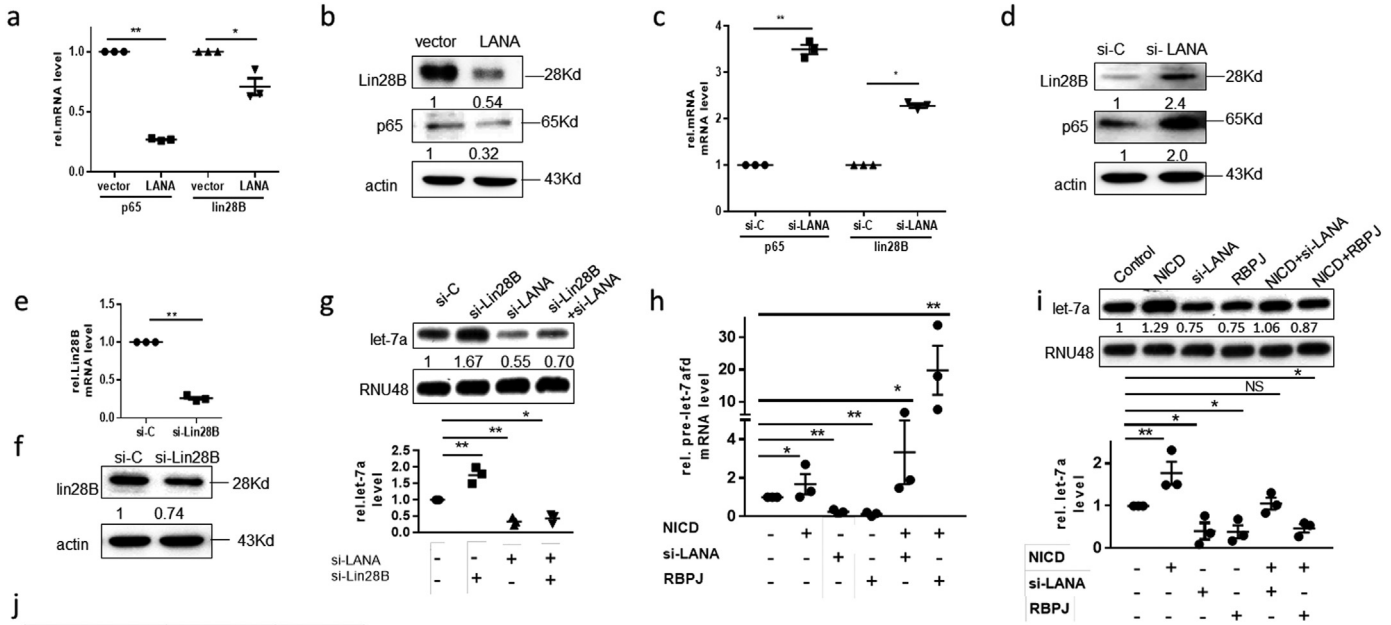


Fig. 1. LANA increases let-7a and pri-let-7afd levels while reducing RBPJ levels. (a–e) 293 T cells were transfected with pCAGGS-LANA or empty vector for 48 h. (a) Protein levels of LANA were determined with western blotting. β-Actin was the loading control. (b) miRNAs were extracted and the let-7a levels were determined with northern blotting (upper) with RNU48 as the internal control and qPCR (lower) with U6 as the internal control. (c, d) Total RNA was extracted, and the levels of pri-let-7afd and RBPJ mRNA were determined with qPCR assays. β-Actin was the internal control. (e) Levels of RBPJ protein were determined with a western blotting assay. (f-k) iSLK.219 cells were transfected with si-RNA directed against LANA or nonspecific siRNA (as the control) for 48 h. (f) LANA mRNA levels were determined with a qPCR assay. (g) Levels of LANA protein were determined with a western blotting assay. (h) let-7a miRNA levels were detected with northern blotting (upper) and qPCR (lower). (i) RBPJ mRNA levels were determined with a qPCR assay. (j-k) RBPJ mRNA and protein levels were determined with a western blotting assay. Results shown are means ± SEM. *P* values were determined with Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.



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	Pri-let-7a	Let-7a
NICD	↑	↑
Si-LANA	↓	↓
RBPJ	↓	↓
NICD+si-LANA	↑	NS
NICD+RBPJ	↑	↓

Fig. 2. LANA increases let-7a expression by downregulating NF-κB and LIN28B and increasing NICD. (a, b) 293 T cells were transfected with a plasmid expressing LANA. (a) p65 and LIN28B mRNAs were quantified with qPCR, and (b) the levels of LANA, p65 and LIN28B proteins were quantified with western blotting. (c, d) iSLK.219 cells were transfected with si-LANA, and (c) the p65 and LIN28B mRNAs were quantified with qPCR, and (d) the LANA, p65 and LIN28B protein levels with western blotting. iSLK.219 cells were transfected with si-LIN28B. LIN28B mRNA and protein levels were quantified with qPCR and western blotting, respectively (e, f). (g) iSLK.219 cells were transfected with si-LANA and/or si-LIN28B, and the let-7a levels were determined with northern blotting (upper) and qPCR (lower). (h-j) iSLK.219 cells were cotransfected with plasmids expressing NICD, si-LANA, and RBPJ as indicated and harvested after 48 h. miRNAs and total RNA were extracted. (h) Levels of pri-let-7afd were quantified with qPCR, and (i) let-7a expression was quantified with northern blotting (upper) and qPCR (lower). (j) Changes in let-7a and pri-let-7afd induced by the indicated treatment are summarized. Results shown are means ± SEM. *P* values were determined with Student's *t*-test. $*P < 0.05$, $**P < 0.01$.

293 T cells were transfected with the plasmid pCAGGS-LANA or the empty vector pCAGGS. The expression of LANA protein in the 293 T cells was confirmed with western blotting (Fig. 1a). qPCR and northern blotting assays showed that LANA increased the expression of let-7a (Fig. 1b). LANA increased the levels of pri-let-7afd (Fig. 1c), whereas pri-let-7a-2 and pri-let-7a-3 were undetectable (data not shown), and reduced the RBPJ mRNA and protein levels in the 293 T cells (Fig. 1d and e). To confirm the effect of LANA on the expression of let-7a and RBPJ, iSLK.219 cells were transfected with a siRNA targeting LANA, and the expression of let-7a, the let-7a primary transcripts, and RBPJ was detected. The LANA mRNA (Fig. 1f) and protein (Fig. 1g) levels were knocked down by the siRNA. The knockdown of LANA in iSLK.219 cells reduced the expression of let-7a (Fig. 1h), pri-let-7afd, and pri-let-7a-2 (Fig. 1i), and pri-let-7a-3 was undetectable, whereas it increased RBPJ mRNA and protein levels (Fig. 1j and k). These results indicate that LANA increases let-7a and induces its primary transcription, but simultaneously reduces the expression of RBPJ.

3.2. LANA increases let-7a by downregulating NF-κB and LIN28B

let-7a is a tumor suppressor miRNA whose expression is regulated by several signaling molecules, and the NF-κB/LIN28B signaling pathway is one of the most important pathways involved (Joshi et al., 2016; Roos et al., 2015). A recent study shown that p65 protein and its phosphorylation levels were reduced by LANA (Mariggio et al., 2017). NF-κB induces LIN28B, and the RNA-binding protein LIN28 reduces let-7a by blocking the processing of the primary let-7a transcripts by Drosha and Dicer and/or inducing the degradation of these primary transcripts (Sangiao-Alvarellos et al., 2015). Therefore, we investigated whether NF-κB/LIN28B signaling contributes to the LANA-induced upregulation of let-7a. LANA reduced the expression of p65 and LIN28B in 293 T cells (Fig. 2a and b). In iSLK.219 cells, the knockdown of LANA increased the levels of p65 and LIN28 (Fig. 2c and d). In iSLK.219 cells, silencing Lin28B increased let-7a and si-LANA decreased let-7a, but when we silenced both Lin28 and LANA, let-7a decreased (Fig. 2e–g). Taken together, these results indicate that LANA increases let-7a levels by downregulating NF-κB/LIN28B signaling.

3.3. LANA induces pri-let-7afd transcription by increasing NICD

Several studies have shown that the notch intracellular domain (NICD), the active form of the notch receptor, increases let-7a expression (Patterson et al., 2014; Solomon et al., 2008). LANA activates notch signaling and increases cellular NICD (Lan et al., 2007). In this study, we investigated whether NICD is involved in the LANA-induced increase in let-7a transcripts. Our findings were as follows: (1) pri-let-7afd was increased in iSLK.219 cells transfected with NICD, with or without the cotransfection of si-LANA. The exogenous coexpression of NICD and RBPJ dramatically increased pri-let-7afd levels, although RBPJ alone reduced pri-let-7afd levels (Fig. 2h). These results indicate

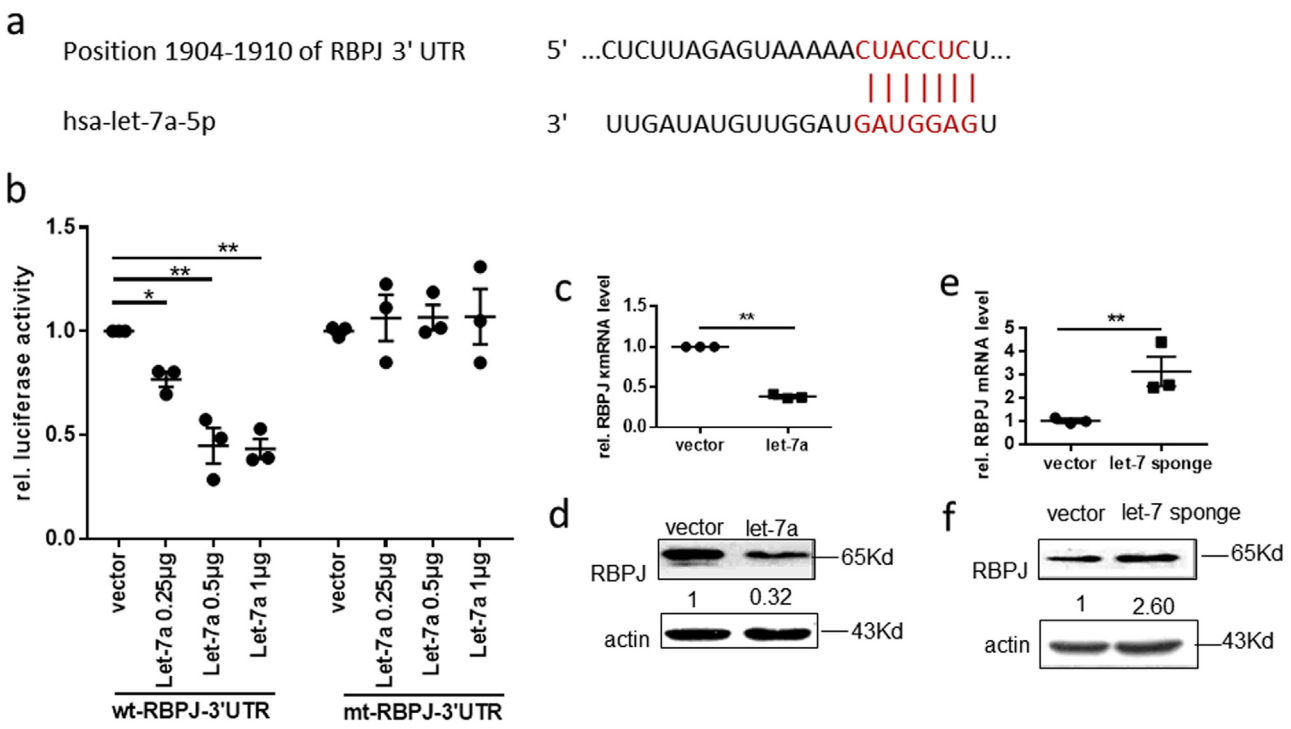

Fig. 3. RBPJ expression is directly inhibited by let-7a. (A) The 3′UTR sequence of RBPJ binding sites to let-7a predicted by targetscan (www.targetsacn.org). (B) 293 T cells were cotransfected with increasing doses of plasmid expressing let-7a or empty pSilencer4.1 and wt-RBPJ 3′-UTR or mt-RBPJ 3′-UTR. After 72 h, a luciferase activity was performed. (C) 293 T cells were transfected with plasmid expressing let-7a or empty pSilencer 4.1, and the RBPJ mRNA levels were determined with a qPCR assay, (D) and the RBPJ protein levels with western blotting. (E) 293 T cells were transfected with a let-7 sponge or empty vector, and the RBPJ mRNA levels were determined with a qPCR assay, and (F) the RBPJ protein levels with a western blotting assay. Results shown are means ± SEM. *P* values were determined with Student's *t*-test. $*P < 0.05$, $**P < 0.01$.

that NICD or activated notch signaling induces let-7a transcription (2). The overexpression of NICD increased let-7a and pri-let-7afd, but the exogenous coexpression of NICD and si-LANA only increased pri-let-7afd, and let-7a expression was unchanged (Fig. 2i). These results indicate NICD mainly induce let-7a transcription, while LANA increases let-7a through promoting both its transcription and maturation. The effects of NICD, si-LANA, and RBPJ on the expression of let-7a and pri-let-7afd are shown in Fig. 2j. Taken together, these results suggest that LANA increases the primary transcripts of let-7a by activating Notch signaling.

3.4. RBPJ expression is directly inhibited by let-7a

The parallel increase in let-7a and decline in RBPJ during the exogenous expression of LANA in 293 T cells and the binding site for let-7a on the 3′-UTR of RBPJ predicted with TargetScan 7.0 (www.targetscan.org) indicate that RBPJ is directly targeted by let-7a (Fig. 3a). We used a luciferase assay to explore the association between let-7a and its potential binding site in the RBPJ 3′-UTR. let-7a suppressed the luciferase activity from the wild-type RBPJ 3′-UTR in a dose-dependent manner but had no effect on the mutated RBPJ 3′-UTR (Fig. 3b). To further investigate the effects of let-7a on RBPJ expression, the mRNA and protein expression levels of RBPJ were detected in 293 T cells overexpressing or knocking down let-7a. qPCR and western blotting assays showed that let-7a inhibits the expression of RBPJ at the mRNA and protein levels (Fig. 3c and d). We also silenced let-7 in 293 T cells by transfecting let-7 sponge, to evaluate the effect of let-7a on RBPJ. let-7 knockdown increased the RBPJ mRNA and protein levels (Fig. 3e and f). All these results indicate that let-7a inhibits RBPJ expression by directly binding to its 3′-UTR.

3.5. Overexpression let-7a dose- and time-dependently inhibits the lytic reactivation of KSHV

In our previous study, we found knockdown of let-7a reactivated KSHV lytic replication in BCBL-1 cells (Tan et al., 2015). Here, we further investigate the effect of overexpression let-7a on KSHV lytic replication and production of infectious virus in iSLK.219 cells. The endothelial iSLK.219 cell line expresses a doxycycline (Dox)-inducible RTA gene incorporated into the cellular genome. The iSLK.219 cells were transfected with a series of concentrations (2, 4, 8, or 16 μg) of let-7a expression plasmids or empty vectors as the control for 24 h. The expression levels of let-7a were detected with qPCR assay. Overexpression let-7a decreases RBPJ protein levels in dose-dependent manner (Fig. 4a). Then the cells were treated with 500 ng/ml doxycycline (DOX) for another 36 h to induce the reactivation of KSHV. The green fluorescent protein (GFP)- and red fluorescent protein (RFP)-positive cells were observed with an inverted fluorescence microscope (Fig. 4b). The intracellular and extracellular KSHV genome copy numbers were determined with qPCR assays, and the results show that overexpression of let-7a reduced the intracellular and extracellular KSHV genome copy numbers in a dose-dependent manner (Fig. 4c and d). In the present study, we show let-7a downregulates RBPJ expression, attenuates KSHV reactivation, and decreases extracellular KSHV genome copies significantly.

We also confirmed that overexpression let-7a inhibited KSHV reactivation in a time-dependent manner. iSLK.219 cells were transfected with let-7a (4 μg) or empty vector for 24 h before 500 ng/ml DOX was added. The cells were harvested at 12, 24, 36, 48, and 60 h after DOX treatment. The GFP- and RFP-positive cells were observed with an inverted fluorescence microscope. Overexpression let-7a reduced the numbers of RFP-positive cells at 24, 36, 48 and 60 h after DOX treatment (Fig. 4e). The intracellular and extracellular KSHV genome copy numbers were determined with qPCR. The intracellular KSHV copies

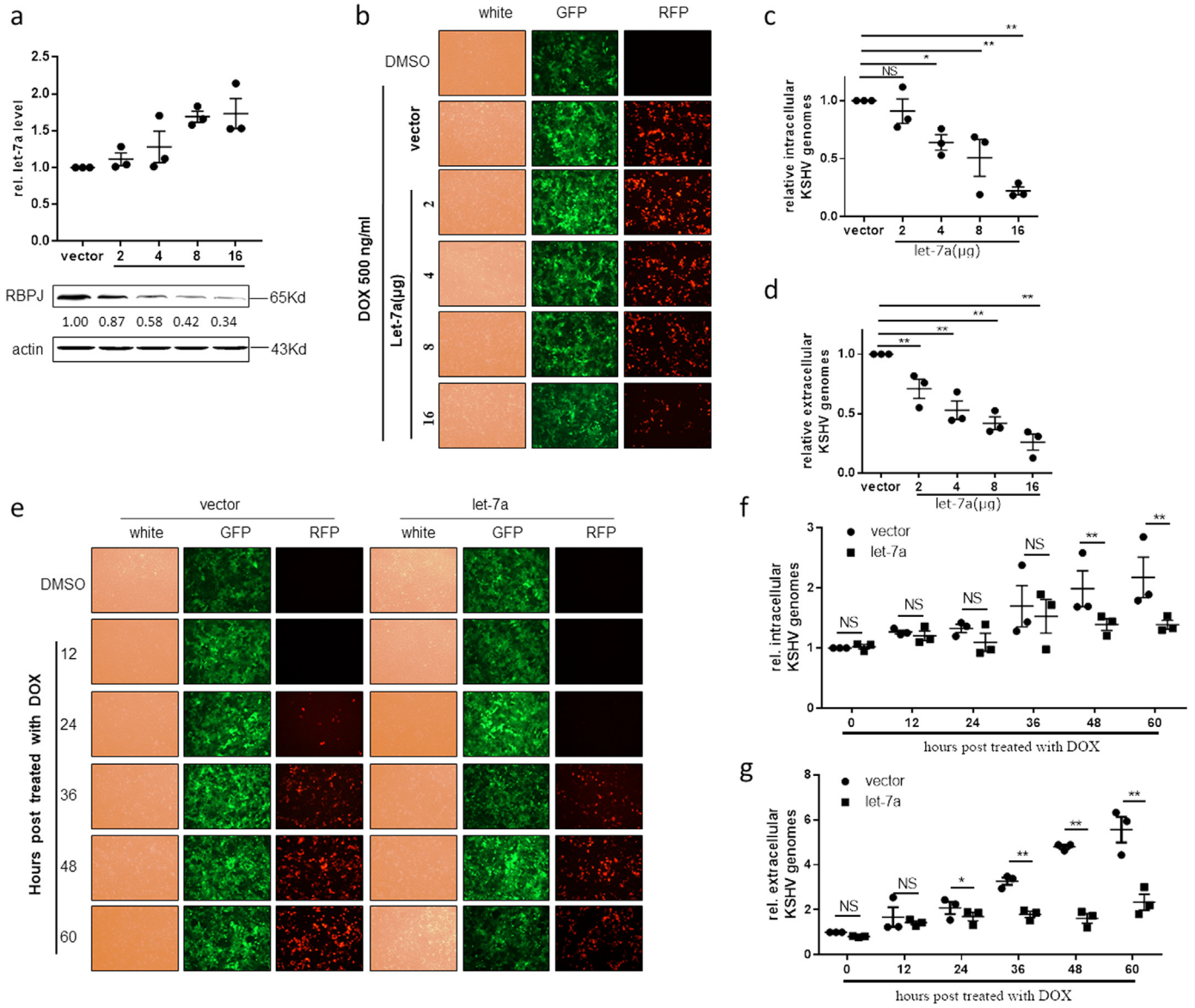

Fig. 4. Overexpression let-7a inhibits KSHV reactivation and replication. (a-d) iSLK.219 cells were transfected with the indicated doses of let-7a expression plasmid or empty pSilencer 4.1 vectors for 24 h. (a) the let-7a expression levels were determined by qPCR assay (upper) and the RBPJ protein levels were determined by Western-blot assay (lower). (b)The cells were then treated with doxycycline (500 ng/ml) for another 36 h. Cellular expression GFP and RFP was observed with inverted fluorescence microscopy. (c) The cells were harvested to extract the DNA, the relative numbers of intracellular KSHV genome copies were determined with qPCR. (d) The supernatants were harvested and treated with DNase for 30 min, after inactivating the DNase, the DNA be extracted and the relative numbers of extracellular KSHV virions were determined with qPCR. (e) iSLK.219 cells were treated with 4 μg let-7a or empty vectors for 24 h and then treated with doxycycline (500 ng/ml) for 12, 24, 36, 48, or 60 h. Cellular expression GFP and RFP was observed with inverted fluorescence microscopy. (f, g) The relative numbers of intracellular and extracellular KSHV genome copies were determined with a qPCR assay. Results shown are means ± SEM. *P* values were determined with Student's *t*-test. $*P < 0.05$, $**P < 0.01$. NS: not significantly different.

were modestly decreased in let-7a overexpression cell (Fig. 4f). The extracellular KSHV copies were significantly reduced by let-7a in 24 h till 60 h treated with DOX (Fig. 4g). These results demonstrate that overexpression let-7a delays the reactivation of KSHV.

3.6. RBPJ knockdown dose- and time-dependently inhibits the lytic reactivation of KSHV

RBPJ is one of the rate-limiting factors in the reactivation of KSHV mainly by binding RTA. The RBPJ deficient human B cell lines can be infected with recombinant KSHV, maintain the viral genome and re-activate but does not produce or produce only an amount of infectious virus (Scholz et al., 2013). Therefore, we hypothesized that lower RBPJ attenuates KSHV lytic replication. In this study, we speculated that reducing RBPJ inhibits KSHV reactivation. The RBPJ levels in iSLK.219 cells were knocked down with a series of concentrations (12.5, 25, 50, or 100 nM) of siRNA directed against RBPJ or a nonspecific siRNA (as a control), and the knockdown efficiency of RBPJ mRNA and protein was confirmed with qPCR and western blotting assay, respectively (Figs. 5a and 5b). iSLK.219 cells were transfected with si-RBPJ for 24 h before treatment with 500 ng/ml doxycycline (DOX). The GFP- and RFP-positive cells were observed with an inverted fluorescence microscope (Fig. 5c). The intracellular and extracellular KSHV genome copy numbers were determined with qPCR assays, and the results showed that si-RBPJ reduced the intracellular and extracellular KSHV genome copy numbers in a dose-dependent manner (Fig. 5d and e).

To explore whether silencing RBPJ attenuated KSHV reactivation in a time-dependent manner, iSLK.219 cells were transfected with siRNA

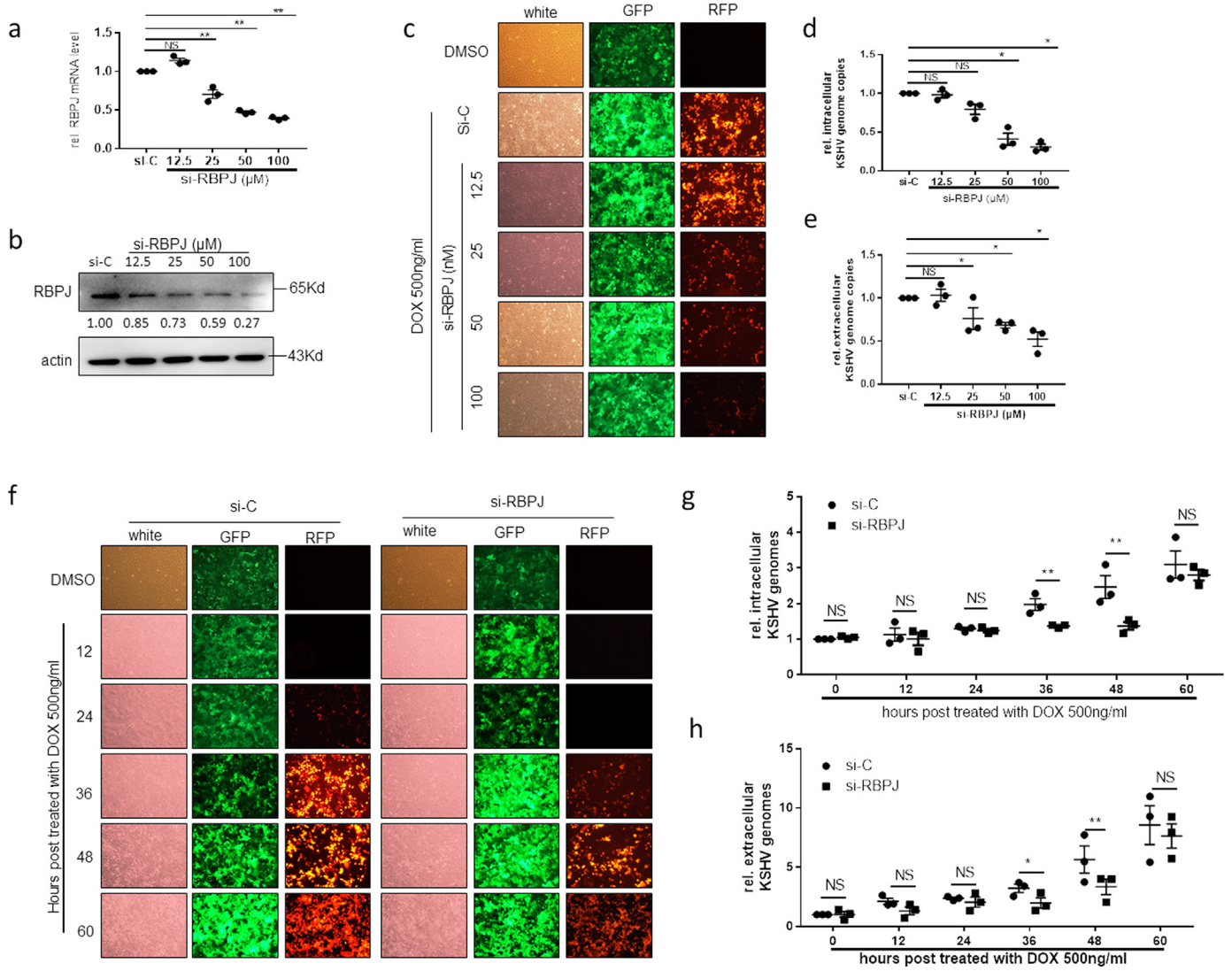

Fig. 5. RBPJ knockdown inhibits KSHV reactivation and replication. (a, b) iSLK.219 cells were transfected with the indicated doses of siRNA directed against RBPJ or a nonspecific control siRNA (as the control) for 24 h. (a) RBPJ mRNA levels were determined with qPCR, and (b) RBPJ protein levels with western blotting. The cells were then treated with doxycycline (500 ng/ml) for 36 h. (c) Cellular expression GFP and RFP was observed with inverted fluorescence microscopy. (d, e) The relative numbers of intracellular and extracellular KSHV genome copies were determined with a qPCR assay. (f) iSLK.219 cells were treated with 50 nM si-RBPJ or the si-control for 36 h, and then treated with doxycycline (500 ng/ml) for 12, 24, 36, 48, or 60 h. Cellular expression GFP and RFP was observed with inverted fluorescence microscopy. After treatment with DOX for 36 h, cells and supernatants were harvested, and (f, g) the relative numbers of intracellular and extracellular KSHV genome copies were determined with a qPCR assay. Results shown are means ± SEM. *P* values were determined with Student's *t*-test. $^*P < 0.05$. NS: not significantly different.

(50 nM) directed against RBPJ or nonspecific siRNA for 24 h, and then 500 ng/ml DOX was added to induce KSHV reactivation. The cells were harvested at 12, 24, 36, 48, and 60 h after DOX treatment. The GFP- and RFP-positive cells were observed with an inverted fluorescence microscope. Silencing RBPJ expression reduced the numbers of RFP-positive cells at 24, 36, and 48 h after DOX treatment (Fig. 5f). The intracellular and extracellular KSHV genome copy numbers were determined with qPCR 36 h after the DOX treatment and showed that si-RBPJ reduced the KSHV genome copy numbers (Fig. 5g and h). These results demonstrate that silencing RBPJ delays the reactivation of KSHV.

4. Discussion

LANA is the key viral protein in the maintenance of latent KSHV infection. The viral protein RTA switches KSHV infection from latent to lytic, and LANA represses RTA expression (Lan et al., 2005b). RBPJ is a cellular protein essential for the lytic reactivation of KSHV. RBPJ binds to RTA and induces many viral genes, including RTA itself, through the positive feedback loop to activate KSHV lytic replication. LANA expression also is promoted by RBPJ binding to RTA, and in turn, LANA binds to RBPJ and represses RTA expression (Lan et al., 2005a, 2005b). RBPJ is the mediator of the positive and negative feedback. RBPJ is essential for KSHV lytic replication, however, there is no evidence shown that RBPJ is essential for KSHV latent infection. The results showed that LANA down-regulated RBPJ was also helpful to maintain the latency status of KSHV. In this study, we found that LANA down-regulates RBPJ by increasing the cellular miRNA let-7a, and over-expression let-7a or knockdown RBPJ dose- and time-dependently inhibited the RTA-mediated lytic reactivation of KSHV. Collectively, these findings support a model wherein LANA inhibits the lytic replication of KSHV by regulating let-7a/RBPJ signaling.

The let-7 miRNAs are tumor suppressor factors (Roush and Slack, 2008; Shimizu et al., 2010) and are also targeted by several viruses (Cheng et al., 2013; Edge et al., 2008; Wang et al., 2010b). The viral

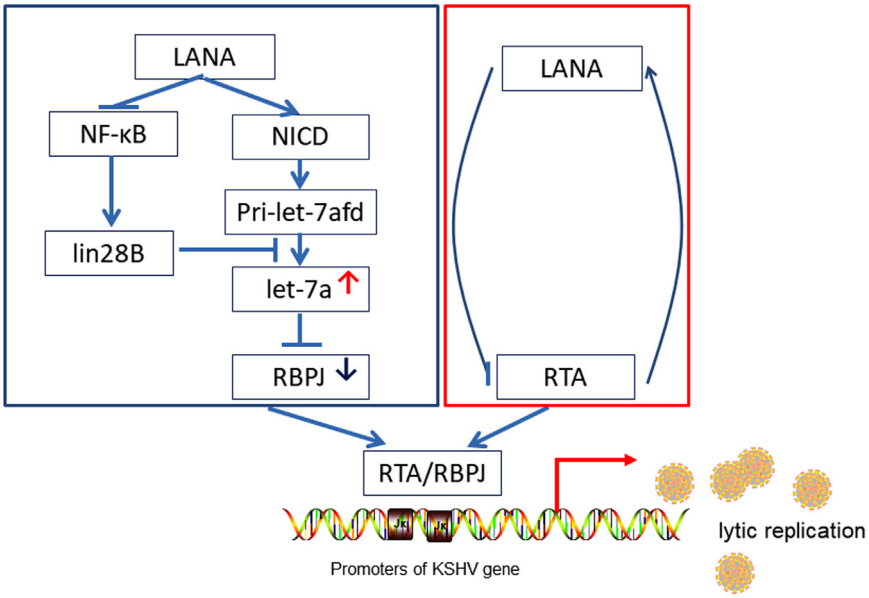

Fig. 6. Hypothetical model of the regulation of RBPJ by LANA through let-7a, which contributes to the inhibition of the lytic replication of KSHV.

protein EBNA1 encoded by Epstein-Barr virus also upregulates let-7a, and let-7a inhibits the lytic reactivation of EBV (Mansouri et al., 2014). In a previous study, we showed that let-7a also inhibits the lytic reactivation of KSHV (Tan et al., 2015). However, whether the expression of let-7a is regulated by KSHV and which viral genes are involved in this effect have been unclear. In this study, we confirmed that LANA upregulates the expression of let-7a, suggesting that the upregulation of let-7a contributes to the function of LANA in inhibiting the lytic reactivation of KSHV.

miRNAs are short single-stranded RNA molecules, approximately 22 nucleotides in length. They are involved in the posttranscriptional regulation of gene expression by binding to complementary sequences in the 3′-UTRs of their target mRNAs. miRNAs exist in three forms during their synthesis: primary miRNAs (pri-miRNAs), precursor miRNAs (pre-miRNAs), and mature miRNAs. LIN28B has been shown to act as a posttranscriptional repressor of let-7 biogenesis by binding to the loop portion of the pri-let-7 hairpin and the stem of pre-let-7 to inhibit the binding of Drosha or Dicer, thereby inhibiting their processing and reducing the levels of mature let-7. In the present study, we confirmed that LANA reduces the expression of p65 and LIN28B and that this repression of LIN28B contributes to the upregulation of let-7a by LANA.

Several studies have shown that the activation of Notch signaling upregulates the expression of let-7a (Patterson et al., 2014; Solomon et al., 2008). Ligand binding to the Notch receptor leads to the proteolytic release of NICD, which is translocated to the cell nucleus to activate the transcription of its target genes. LANA increases cellular NICD by reducing its ubiquitination and degradation (Lan et al., 2007). Therefore, we determined the effect of LANA on the primary transcripts of let-7a. As expected, LANA increased the primary transcripts of let-7a. NICD and LANA increased the levels of pri-let-7afd and let-7a, and si-LANA reduced them. However, the cotransfection of NICD and si-LANA in iSLK.219 cells only upregulated pri-let-7afd expression, whereas the levels of mature let-7a did not change. These results suggest that LANA increases let-7a both by inducing the expression of its primary transcripts and by promoting their processing. Li et al. reported that the inhibition of the Notch pathway upregulates the expression of lytic genes (Li et al., 2016), suggesting that an increase in cellular NICD and the upregulation let-7a contribute to the LANA-induced inhibition of the lytic replication of KSHV. Because LANA suppresses NF-κB/LIN28B signaling, we concluded that LANA induces the transcription of let-7a by increasing cellular NICD and promotes let-7a maturation by inhibiting NF-κB/LIN28B signaling.

The 3′-UTR of RBPJ contains a conserved let-7a-binding sequence, and RBPJ is essential for the lytic reactivation of KSHV. Our results show that RBPJ is directly inhibited by let-7a. Taking these results together, we identified the feedback loop: LANA increases let-7a, let-7a reduces RBPJ (Fig. 6). Through this feedback loop, RBPJ is maintained at a relatively low level in cells latently infected with KSHV.

In this study, we have shown that LANA reduces RBPJ by upregulating let-7a expression. RBPJ is essential for the lytic replication of KSHV by binding to RTA. We then investigated the relationship between let-7a or RBPJ levels and the lytic replication of KSHV. In iSLK.219 cells, overexpression let-7a or knockdown RBPJ inhibits the DOX-induced KSHV lytic reactivation in a dose- and time-dependently manner. Scholz et al. reported that RBPJ deficient human B cell lines infected with recombinant KSHV can maintain the viral genome, but fail to produce detectable levels of infectious virus (Scholz et al., 2013). Although both overexpression let-7a and RNA interference decrease RBPJ expression, it seems overexpression let-7a is more powerful effect on KSHV lytic replication, indicate that repressing other let-7a target genes such as MAP4K4 and Dicer (Mansouri et al., 2014; Tan et al., 2015) may also play a role.

In summary, LANA is the key regulator that maintains the latent infection of KSHV, but the mechanism is still not fully understood. Both the viral protein RTA and the cellular protein RBPJ are essential for the lytic replication of KSHV. The repression of RTA by LANA is well documented. Here, we show that reducing RBPJ through upregulating let-7a also contributes to the LANA-induced inhibition of KSHV lytic replication. LANA induces the expression of the primary transcripts of let-7a by increasing cellular NICD and promotes the processing of the let-7a primary transcripts by suppressing NF-κB/LIN28B signaling. let-7a then directly binds to the 3′-UTR of RBPJ and represses its expression. Therefore, we constructed a hypothetical model in which LANA downregulates RBPJ expression, contributing to the inhibition of the lytic replication of KSHV (Fig. 6). In conclusion, through a LANA/let-7a/RBPJ signaling, LANA controls let-7a and RBPJ levels to inhibit the lytic replication of KSHV.

Acknowledgments

We acknowledge Prof. Ke Lan for providing the LANA expression

plasmid and iSLK.219 cells. This work was supported by grants from the Natural Science Foundation of China (8177080336 and 81071636) and the Natural Science Foundation of Zhejiang Province (LQ18H190003 and LY12H16028).

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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