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Host Src controls gallid alpha herpesvirus 1 intercellular spread in a cellular fatty acid metabolism-dependent manner

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ABSTRACT

Viral spread is considered a promising target for antiviral therapeutics, but the associated mechanisms remain unclear for gallid alpha herpesvirus 1 (ILTV). We previously identified proto-oncogene tyrosine-protein kinase Src (Src) as a crucial host determinant of ILTV infection. The present study revealed accelerated spread of ILTV upon Src inhibition. This phenomenon was independent of either viral replication or the proliferation of infected cells and could not be compromised by neutralizing antibody. Neither extracellular vesicles nor the direct cytosol-to-cytosol connections between adjacent cells contributed to the enhanced spread of ILTV upon Src inhibition. Further genome-wide transcriptional profile analyses in combination with functional validation identified fatty acid metabolism as an essential molecular event during modulation of the intercellular spread and subsequent cytopathic effect of ILTV by Src. Overall, these data suggest that Src controls the cell-to-cell spread of ILTV in a cellular fatty acid metabolism-dependent manner, which determines the virus's cytopathic effect.

1. Introduction

The spread of infectious viruses between host cells is important for viral replication, virulence, and pathogenesis (Flint et al., 2009). Two routes exist for viral spread: cell-free spread, which involves infection of new cells through extracellular virions or vesicles containing infectious materials released by infected cells, and cell-to-cell spread, which involves viral particle transmission between cells via cell-to-cell contacts or direct connections (Mothes et al., 2010; Sattentau, 2008). Cell-free spread is well established and important for distant viral dissemination, such as transmission between hosts and tissue-to-tissue spread within the host body through blood, lymph and cerebrospinal fluid. During distant spread, viruses must be released from host cells. However, once released into the extracellular environment, viruses are exposed to several barriers that block their subsequent cell-free spread, including biophysical barriers such as mucous membranes, cell intrinsic barriers associated with host immunosurveillance, and kinetic barriers due to the processes of random fluid-phase diffusion, attachment of a virus to the host cell membrane and the penetration of viruses bound to host cells. To achieve success in distant dissemination, all of these barriers must be overcome by viruses. Considering these disadvantages of cell-free spread, many viruses have evolved to exploit cell-to-cell spread as an alternative strategy to overcome the above barriers. Cell-to-cell spread has been proven to be important for infection with many clinically important viruses, such as herpesviruses, poxviruses, measles virus, hepatitis C virus, human immunodeficiency virus type 1, and rabies virus (Christian et al., 1971; Ritzenthaler, 2011; Sattentau, 2008, 2011; Wheeler, 1960).

For alphaherpesviruses, the main route of spread in tissues is cell-to-cell spread during primary infections in mucocutaneous tissue, establishment of latent infection in sensory neurons, and reactivation from latency, during which the virus spreads intercellularly between the mucocutaneous tissue and sensory neurons (Abaitua et al., 2013; Arvin et al., 2007). The cell-to-cell spread inhibitory activity of infection-elicited neutralizing antibodies has been found to be extremely limited in herpes simplex virus 1 (HSV-1)- and HSV-2-infected subjects

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(Criscuolo et al., 2019). Avian gallid alpha herpesvirus 1 (ILTV) belongs to the family *Herpesviridae* and the subfamily *Alphaherpesvirinae*, which continues to cause great economic losses in the poultry industry (Ou and Giambrone, 2012) despite successful protection by vaccination (Coppo et al., 2013). Similar to other alphaherpesviruses, ILTV establishes latency in the nervous system after acute infection of the upper respiratory tract and cannot be cleared from the host by any currently available therapeutic treatment; therefore, reactivation of ILTV from latent infection frequently occurs once the host is under stress or host immunity is compromised (Hughes et al., 1989, 1991; Ou and Giambrone, 2012). Thus, elucidation of the mechanisms responsible for cell-to-cell spread can facilitate the development of novel therapeutic strategies to control virus reactivation and the design of effective vaccines to combat ILTV.

Proto-oncogene tyrosine-protein kinase Src (Src) was originally isolated from a chicken sarcoma and was thought to contribute to oncogenesis (Vogt, 1971). Src has been shown to be an important factor for a wide range of biological processes, such as cell proliferation, adhesion, division, and death, angiogenesis, and organization of the cytoskeleton. Src has also been reported to be involved in infections with a wide range of viruses, including polyoma virus, dengue, HIV-1, coronaviruses, influenza virus, and herpesvirus (Bolen et al., 1984; Burkard et al., 2015; Cheshenko et al., 2005; Gaur et al., 2012; Krishnan et al., 2006; Kumar et al., 2016; McCarthy et al., 2016), suggesting that Src may be a new potential target for antiviral therapeutic agents and may have potential for repurposing existing FDA-approved Src inhibitors. However, no clinical applications of antiviral Src-targeting agents have been reported, which may be at least partially due to the complicated roles of Src in its regulation of the multiple biological processes in which it is involved. The biological effects of Src are complex and sometimes controversial. For example, some studies suggest that Src is essential in PDGF-induced DNA synthesis (Barone and Courtneidge, 1995; Twamley-Stein et al., 1993); however, other studies of PDGF-induced DNA synthesis indicate that Src is not absolutely required and even plays the opposite role (DeMali and Kazlauskas, 1998; Rosenkranz et al., 2000). In addition, Src is considered both a survival factor and a death factor depending on specific cellular conditions (Fan et al., 2007, 2013, 2014; Iqbal Hossain et al., 2015). Our previous study identified Src as the key host determinant of ILTV infection (Li et al., 2015). We found that activation of host Src upon ILTV infection raises the threshold of host cells for ILTV infection-induced cell death, thus maintaining the survival of infected cells and ensuring a high level of viral replication. However, therapies targeting Src not only repress viral replication but also exacerbate the pathological effects of ILTV and even increase the likelihood of death of the host. Because the underlying molecular mechanisms have not been sufficiently investigated, the development of Src-based therapeutics is limited.

Viral replication requires host cellular metabolism. While similarities exist, virus-specific metabolic requirements by different viruses have been identified. Many viruses have been shown to induce fatty acid synthesis in host cells, which provides a stable carbon source and sufficient energy for viral replication, viral spread and even the prolonged survival of infected cells (Rodríguez-Sánchez and Munger, 2019; Sanchez and Lagunoff, 2015; Sanchez et al., 2017). Although the mechanism remains unclear for ILTV, the utilization of host fatty acid metabolism has been found to be important for both latent and lytic infections with human gamma herpesvirus, Kaposi's sarcoma-associated herpesvirus (KSHV), and the survival of infected cells (Delgado et al., 2012; Sanchez et al., 2017). Thus, a better understanding of the involvement of fatty acid metabolism in viral infection may lead to the development of novel therapeutic approaches targeting specific cellular metabolic processes. In the present study, we observed accelerated cell-to-cell spread of ILTV with Src inhibition in a cellular fatty acid metabolism-dependent manner, which determines the cytopathic effect (CPE) of ILTV. Given the importance of cell-to-cell spread during ILTV infection as evidenced by the efficient protection against ILTV infection provided to a host by cell-mediated immunity rather than humoural immunity (Coppo et al., 2013), the mechanism that we revealed may be valuable for the future development of more rational anti-ILTV vaccination.

2. Materials and methods

2.1. Viral strain and cell line

The virulent ILTV-LJS09 strain (GenBank Accession No. JX458822) is stored at the Harbin Veterinary Research Institute of CAAS. This strain can be propagated in a chemically immortalized leghorn male hepatoma (LMH) cell line with clear CPEs observed (Kong et al., 2013; Zhao et al., 2013). ILTV-EGFP was constructed by replacing the *US9* gene of ILTV-LJS09 with the EGFP coding sequence as described previously (Li et al., 2018). LMH cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cell cultures were incubated at 37 °C in 5% CO_2.

2.2. Reagents

The Src inhibitors PP1 (2 μm) and PP2 (20 μm) were purchased from Sellckchem.com (Selleck chemicals LLC, Houston, TX). The 5-tetradecyloxy-2-furoic acid (TOFA, 60 μm), an allosteric inhibitor of acetyl Co-A carboxylase, and the C75 (8 μm), a synthetic fatty-acid synthase (FASN) inhibitor, were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO). Given that all inhibitors we used were dissolved in dimethyl sulfoxide (DMSO), cells treated with DMSO at the same volumes were used as chemical control. Dil and Calcein AM were purchased from Beyotime Biotechnology (Beyotime Biotech, Shanghai, China).

2.3. RNA interference and transfection

A short-interfering RNA (siRNA) pool that specifically recognizes different sequences of the *Src* mRNA and a control siRNA with no specific target site in chickens were used (Li et al., 2015). Transfection of siRNA was conducted using an N-TER nanoparticle siRNA transfection system (Sigma Aldrich) according to the manufacturer's instructions.

2.4. Viral quantitation

LMH cells were infected with ILTV at a multiplicity of infection (MOI) of 0.1. The indicated MOI was obtained according to the number of cells to be infected and the estimated number of infectious particles, based on plaque-forming units detected in LMH cells. Levels of virus replication were determined using plaque assays and ILTV-specific qPCR assays as previously described (Li et al., 2018). To determine the total level of viral replication, both cell-associated viruses and the viruses released into supernatant were collected for virus quantification. Cells were lysed via three rounds of freezing-thawing.

2.5. Flow cytometry and immunofluorescence

We conducted FACS analyses using a BD FACScan and CellQuest software version 4.0.2 (BD, Mountain View, CA). The activation of Src was assayed using an antibody against phosphorylated SRC at Y416 (EMD Millipore, Billerica, MA, USA), followed by a secondary goat anti-mouse antibody conjugated to APC (Jackson Laboratory, Bar Harbor, ME). Mouse IgG was used as an isotype control (Abcam Trading Company, Ltd., Shanghai, China). The proportion of ILTV-infected cells was determined via detection of EGFP-positive cells or by using a rabbit polyclonal antibody against ILTV glycoprotein I (Li et al., 2015), followed by a secondary goat anti-rabbit antibody conjugated to FITC (Jackson Laboratory). The background was determined by normal

rabbit control serum from non-immunized rabbits. Cell death was assayed by propidium iodide (PI) staining of living cells. For immunofluorescent examination, a fluorescent signal was detected with an EVOS FL fluorescence microscope (AMG, Bothell, WA). All cell nuclei were highlighted with Hoechst 33342 (Sigma Aldrich).

2.6. Time-lapse microscopy

ILTV-EGFP-infected cells and uninfected cells were co-cultured at a ratio of 1:50 in the absence or presence of Src inhibitors, PP1 and PP2, in 35-mm dishes. Dishes were placed on a heated chamber at 37 °C in an environmentally controlled mini-incubator maintained at 5% CO_2. Images were captured on an UltraView VoX Confocal Imaging System (PerkinElmer, Waltham, MA) in a Nikon Eclipse Ti stand (Nikon Instruments, Melville, NY) with a 20 × lens under the control of Volocity software (v3.3.0, PerkinElmer).

2.7. Extracellular vesicle preparation and examination

Extracellular vesicle (including exosomes and microvesicles) preparation and examination were performed according to previous description (Jung and Mun, 2018). LMH cells were cultured to near confluency in a 75 cm dish, and cells were mock infected or infected at a MOI of 1 in serum-free DMEM at 4 °C for 1 h. Then cells were washed once with PBS and cultured in extracellular vesicle-free full DMEM medium in the absence or in the present of PP1 or PP2. Medium was harvested after 24 hpi and extracellular vesicles were purified by differential centrifugation at 4 °C (300 × *g* for 10 min, 2000 × *g* for 20 min, 10000 × *g* for 40 min using a Beckman JA-25.15 fixed angle rotor, then ultracentrifuged 100000 × *g* for 90 min using a Beckman SW-41 swinging bucket rotor). The extracellular vesicle pellets generated were resuspended in serum-free DMEM, fixed and dried. Then samples were coated with gold/palladium alloy by sputter coating and examined under a Hitachi H-7650 transmission electron microscope (Hitachi High Technologies, Shanghai, China), and images were taken using an AMT CCD camera (Advanced Microscopy Techniques, Woburn, MA).

2.8. Protein extraction and western blotting

Western blotting was performed under reduced denaturing conditions according to previously described procedures (Li et al., 2014). Briefly, cells were washed with ice-cold PBS, and soluble proteins were extracted with cell lysis buffer (100 mM Tris-HCl pH = 8, 150 mM NaCl, 1% NP-40, phosphatase and protease inhibitor cocktail tablets (Abcam) according to the manufacturer's protocol). The protein concentration was determined using the Bio-Rad Bradford assay (Bio-Rad, Hercules, CA) and BSA standards (Sigma Aldrich). An equal amount of protein was separated by SDS-PAGE. Antibodies against Src (EMD Millipore) and actin (Sigma Aldrich) were used.

2.9. RNA sequencing

Genome-wide gene expression profiling of LMH cells was performed using RNA deep sequencing by Annoroad Gene Technology Co., Ltd, (Beijing, China). Library construction was performed following Illumina (San Diego, CA) manufacturer instructions. Samples were sequenced on an Illumina HiSeq 2500 instrument.

2.10. RT-qPCR

RNA was isolated from LMH cells using RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. RT-qPCR was performed using the SYBR PrimeScript™ Kit (TaKaRa Bio Inc, Tokyo, Japan) as described previously (Cong et al., 2013). Primer sequences are presented in Table 1. Data is calculated with $2^{-\Delta\Delta CT}$ method and results were presented as Log_2 fold change.

Table 1
List of RT-qPCR primers.

Primers (5′-3′)		Sequence
SRC	F	AGTACATGAGCAAGGGGAGC
	R	AGCCATATCGACGAGCTGTG
CCNB2	F	GATGGGAAGACGATCAATGGAC
	R	CTGGATGACTTTGTAAGAAGCGG
CD3E	F	AGACTACGAGCCCATCAG
	R	TAGCCAGAAGCATTCAGC
DDX47	F	TACGAGCTCTGAAGTTCCTAGTG
	R	CAGTATCTTATCCACCTCTGTCTC
FARSB	F	AACTTCCACTCAATAAGCTCACAG
	R	TTCCGTAGAACACAGGGCA
FST	F	CTTATCCGAGCGAGTGTG
	R	GGTCTTCGTTAATGGAGTTG
FSTL4	F	TCACATCCAAAGACTGGCCC
	R	CTGCTGCCCCTTCATACCTC
HSPA14	F	AGAACGAGGAGGTTGTGGG
	R	AGGATCTGCTTTACTTTCACCA
IFNGR2	F	AGACTGAGTGCAGTTTCCT
	R	GGCCCTATGGTAGTGTTTCTC
KIT	F	GGTTAAAGGAAATGCTCGTCTC
	R	ATTCCATAAGACCAGACATCAC
NEGR1	F	GACACATCTCTCCATCTG
	R	GGGCAAAGTTTACTGTTAC
RFLB	F	GTGCCGTCATCTTCACCT
	R	CTTGAGAAGAATCGGCCGC
SMAD9	F	GGTTCTGCCTGGGACTGC
	R	AGATGGACACCTTTTCCTATGTG
ST6GALNAC2	F	TAACACCTCCTCCAACAC
	R	CAGCCTGAAGACCAAATC
TNFRSF21	F	CGGCATCTCTTCCAAACAC
	R	AAGAGTTCAGACCTTTGGGA
TNFRSF8	F	ATCAGAGGCACTTCAGAGAC
	R	GTTCAACTTGGCACAGTCC

2.11. High-throughput data analysis

RNA sequencing data was analyzed with a web-based tool, Galaxy (Blankenberg et al., 2010). Pathway analysis was performed with DAVID (gene-enrichment analysis using EASE Score, a modified Fisher Exact *P*-Value, as threshold) (Huang et al., 2009). RNA sequencing raw data was uploaded to NCBI with accession number GSE98629.

2.12. Statistical analysis

The SPSS software package (SPSS for Windows version 13.0, SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Data obtained from several experiments are reported as the mean ± standard deviation (SD). The significance of differences between two groups was determined with Student's t-test. One-way or two-way analysis of variance with the Bonferroni correction was employed for multigroup comparisons. For all analyses, a probability (p) value of < 0.05 was considered statistically significant.

3. Results

3.1. Inhibition of Src promotes ILTV spread

An ILTV-LSJ09 strain expressing enhanced green fluorescent protein (EGFP), which exhibits no significant difference from the wild type in either viral replication or the CPEs of infection as described previously (Li et al., 2018), was employed to monitor viral infection. With this EGFP strain, we investigated the effect of Src repression on the transmission of ILTV in leghorn male hepatoma (LMH) cells with a multiplicity of infection (MOI) of 0.1. We first performed Src depletion using pooled siRNAs targeting different sequences of *Src*. Src depletion was confirmed by western blotting (Fig. 1A). Upon ILTV infection, an EGFP signal was observed in approximately 10% of cells at 24 h post infection

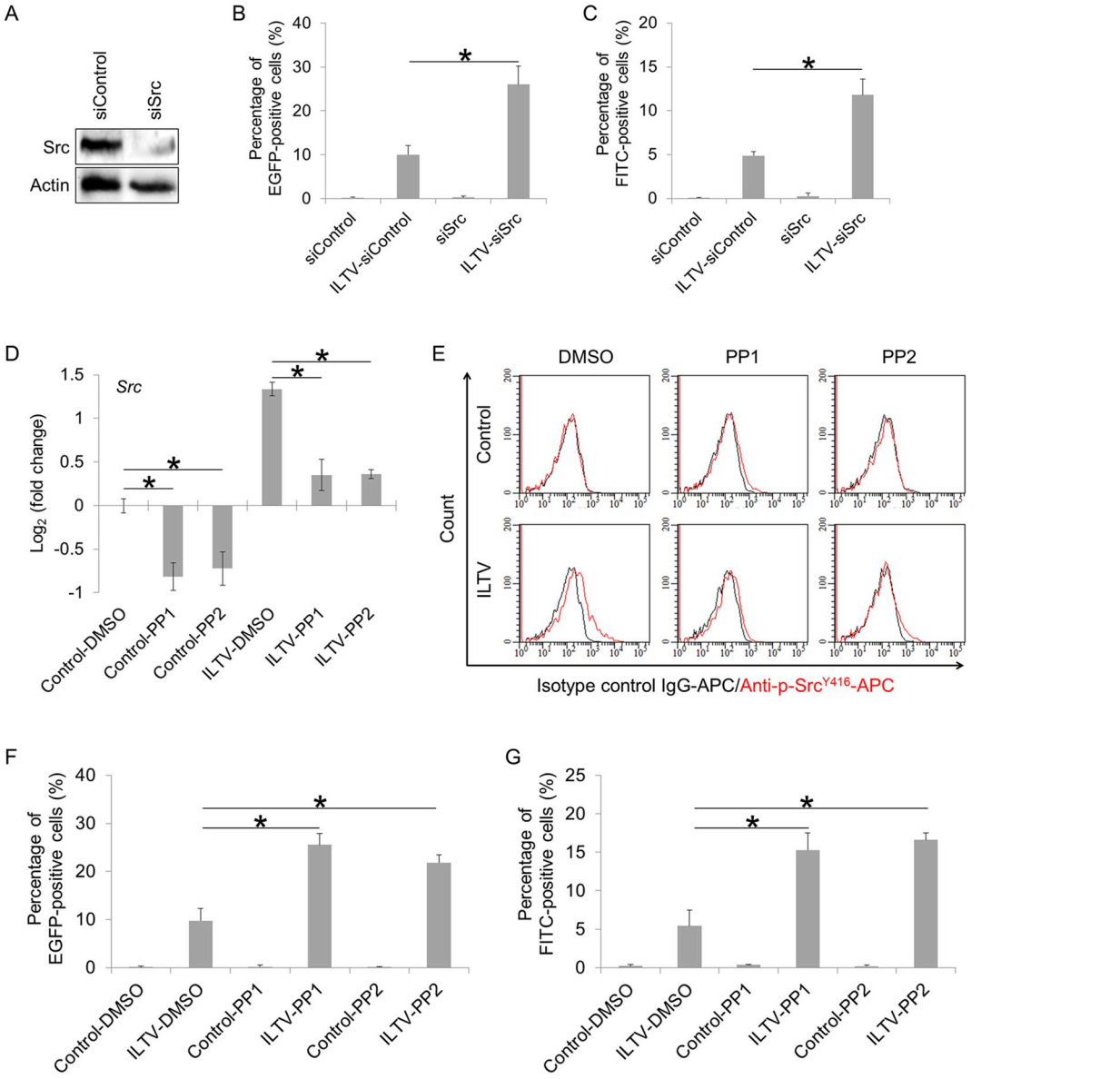


Fig. 1. Inhibition of Src promotes the spread of ILTV. (A) The depletion of Src in LMH cells was performed with a pool of two siRNAs targeting different sequences of *SRC,* and the protein levels of Src were assessed by immunoblotting. Actin was used as a loading control. (B and C) The percentage of infected cells at 24 h post infection upon depletion of Src was quantified by FACS via detecting EGFP-positive cells (B) or using a polyclonal antibody against glycoprotein I of ILTV produced in rabbits followed by an APC-conjugated anti-rabbit second antibody (C). The background was determined by normal rabbit serum. (D and E) Src inhibition by PP1 or PP2 was assayed by RT-qPCR (D) and FACS using an antibody specifically recognizing phosphorylated Src at Y-416 followed by an APC-conjugated anti-rabbit secondary antibody (E). The background was determined by a normal rabbit IgG control. (F and G) The percentage of infected cells at 24 h post infection upon inhibition of Src was quantified by FACS via detecting EGFP-positive cells (F) or using a polyclonal antibody against glycoprotein I of ILTV produced in rabbits followed by an APC-conjugated anti-rabbit second antibody (G). The background was determined by normal rabbit serum. The results in B, C, D, F and G are presented as the mean ± SD, n = 3. Asterisks indicate statistical difference ($p < 0.05$).

(hpi), which met the expected proportion at an MOI of 0.1 and was increased significantly by Src depletion as proven by the detection of EGFP-positive cells via fluorescence-activated cell sorting (FACS) (Fig. 1B). A similar conclusion was obtained using an antibody specifically targeting the gI protein of ILTV as assayed by FACS (Fig. 1C), suggesting that the proportion of EGFP-positive cells can be used to reflect ILTV spread in our model. Consistent with these findings, identical results were obtained for cells with repression of Src activity by one of two widely used Src inhibitors, PP1 or PP2, both of which repressed *Src* transcription and Src phosphorylation at Y416 in LMH cells (Fig. 1D and E) as assayed by FACS (Fig. 1F and G). The above findings demonstrate induced spread of ILTV with repression of Src activity in LMH cells.

3.2. Accelerated viral spread with Src inhibition is independent of viral replication

To address whether viral replication contributed to the accelerated ILTV dissemination by Src inhibition in LMH cells, we measured the total viral titre, including both cell-associated viruses and the viruses released into supernatant, using ILTV-specific qPCR (Fig. 2A) and plague formation assays (Fig. 2B). Consistent with our previous findings that Src is important for ILTV to maintain a high level of viral production (Li et al., 2015), the final production of viruses was significantly reduced by both Src inhibitors as assayed at 5 days post infection (dpi) for cells treated with PP1 or PP2 and at 6 dpi for cells treated with DMSO when all infected cells were dead (corresponding

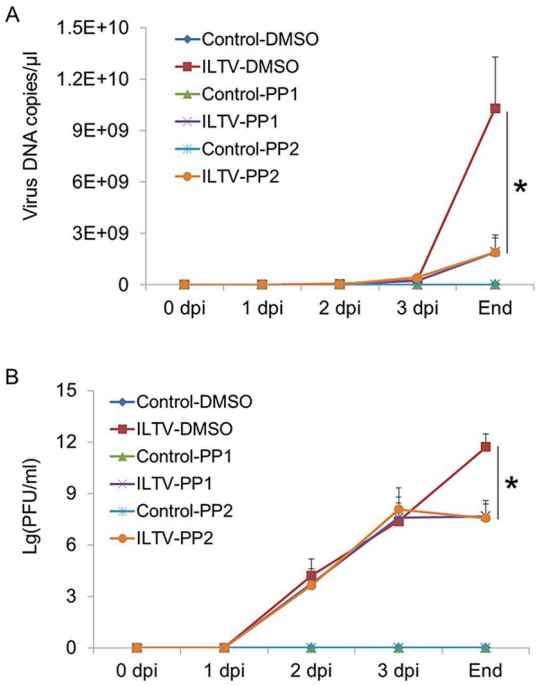


Fig. 2. The effects of Src inhibition on the replication of ILTV in LMH cells. (A and B) The replication of ILTV in LMH cells was determined by ILTV-specific qPCR (A) and plaque assays (B), respectively. The results are presented as the mean ± SD, n = 3. Asterisks indicate statistical difference ($p < 0.05$).

time points are indicated by "End" in Fig. 2A and B). However, neither method revealed any difference in viral titres between cells in the absence or presence of Src inhibitors within three dpi, and during this period, the viral genome copies continued to increase in all infected groups. The existence of virions was even undetectable at 1 dpi (Fig. 2B). The similar levels of viral replication within 3 dpi demonstrated that the accelerated viral spread by Src inhibition that we observed at 24 hpi is independent of viral replication.

3.3. Inhibition of Src promotes ILTV spread in a cell-to-cell manner

Next, a detailed investigation of ILTV dissemination was carried out by monitoring the spread of EGFP using time-lapse microscopy. ILTV-EGFP-infected cells (used as donor cells) and uninfected cells (used as target cells) were co-cultured at a ratio of 1:50 in the absence or presence of a Src inhibitor. As shown by time-lapse microscopy, EGFP signals appeared between 3 and 6 hpi in all ILTV-infected groups. The transmission of the EGFP signal first occurred in cells treated with the Src inhibitor after 12 hpi but was rarely observed in cells in the absence of Src inhibition throughout the observation period (Fig. 3A). Interestingly, the EGFP signal mainly spread from infected cells to adjacent uninfected cells rather than uninfected cells without direct contact with infected cells. Therefore, we hypothesized that the distant transmission by extracellular ILTV particles or extracellular vesicles did not contribute to the enhanced ILTV spread that we observed. To address this hypothesis, a rabbit polyclonal antibody against ILTV glycoprotein I produced in our lab (Li et al., 2015) was utilized as a neutralizing antibody to block virions released into culture medium by infected cells. The neutralization of extracellular virions by the antibody was verified by the observation of EGFP-positive cells (Fig. 3B). However, the promotion of ILTV spread by Src inhibition was not compromised by the addition of this neutralizing antibody as assayed by FACS (Fig. 3C). This hypothesis was further confirmed by the fact that neither the amount nor the infectivity of extracellular vesicles (including both exosomes and microvesicles) secreted into culture medium was affected by Src inhibition in the presence of the neutralizing antibody (Fig. 3D). Taken together, the above findings suggest that Src inhibition promotes ILTV spread mainly in a cell-to-cell manner.

In addition to viral replication, the initial infection and the proliferation of infected cells may have also contributed to the enhanced viral spread by Src inhibition in our experimental system. Given that the initial infection was the same for all groups in our studies, we next addressed whether the accelerated ILTV transmission by Src inhibition was due to the proliferation of infected cells. To answer this question, ILTV-EGFP infected cells (used as donor cells) and uninfected cells labelled with Dil (used as target cells) were co-cultured at a ratio of 1:50 in the absence or presence of a Src inhibitor upon the addition of neutralizing antibodies, as indicated in Fig. 3E (left panel). Src inhibition by either inhibitor significantly increased the proportion of cells that were positive for both EGFP and Dil and the proportion of cells that were positive for EGFP but negative for Dil (Fig. 3E, right panel) as assayed by FACS, suggesting that both the proliferation of infected cells and the cell-to-cell spread of viruses contributed to the enhanced spread of ILTV by Src inhibition in our system. Src inhibition most likely promoted ILTV spread via the latter mechanism since the proportion of EGFP/Dil double-positive cells was approximately nine-times that of cells positive for EGFP only. Taken together, the above findings suggest that Src inhibition promotes ILTV transmission mainly in a cell-to-cell manner.

Inhibition of Src promotes cell-to-cell spread of ILTV independently of direct cytosol-to-cytosol connection.

Alphaherpesviruses can transmit intercellularly from infected cells to neighbouring cells by either passing through cytosol-to-cytosol connections directly or crossing the contacts between cells (Kadiu and Gendelman, 2011; Lucas, 2006). To determine the pathway by which Src inhibition promotes cell-to-cell spread of ILTV, the dissemination of Calcein AM, a dye that is soluble in cytoplasm and can freely pass through cytosol-to-cytosol connections, was assayed. ILTV-infected or uninfected cells stained with Calcein AM were used as donors, and cells labelled with Dil were used as targets. Given that the proportion of cells positive for both Calcein and Dil detected by FACS was too low to be analysed at ratio of 1:50 (lower than 1% in all groups), donors and targets were co-cultured at a ratio of 1:4 in the presence of a Src inhibitor or DMSO as indicated (Fig. 4A). As assayed by FACS, direct cytosol-to-cytosol connections occurred naturally between uninfected cells and were significantly promoted by ILTV infection (Fig. 4B). However, these connections were not affected by Src activity. Together with the low frequency at a ratio of 1:50, this finding excluded the possibility of the involvement of direct cytosol-to-cytosol connections in the enhanced intercellular spread of ILTV by Src inhibition.

Inhibition of Src promotes ILTV cell-to-cell spread in a cellular fatty acid metabolism-dependent manner.

To elucidate the molecular mechanism underlying the accelerated cell-to-cell spread of ILTV by Src inhibition, a genome-wide transcriptional profile analysis was conducted. RNA isolated from mock cells or ILTV-infected cells with distinct Src activities was subjected to RNA sequencing. Bioinformatics analyses identified 3599 genes differentially expressed among groups based on the following criteria: (i) a p-value < 0.01, (ii) a q-value < 0.001, and (iii) a fold change > 1.5 (Table S1). Hierarchical clustering analysis using these differentially expressed genes demonstrated efficient clustering of biological replicates for each group of cells (Fig. 5A). Among these groups, the genome-wide transcription profiles of uninfected cells and the genome-wide transcription profiles of infected cells were clustered separately. A total of 721 up-regulated genes and 1028 down-regulated genes were identified upon ILTV infection in the absence of Src inhibition (Fig. 5B). For validation purposes, the transcription levels of 15 randomly selected genes were examined using an RT-qPCR assay. The RT-qPCR results corresponded to those from the RNA-seq assays (Fig. 5C).

Among the 1749 genes significantly altered by ILTV infection in the absence of Src inhibition, the up-regulation of 309 genes and the down-regulation of 497 genes were halted by PP1 treatment, and the up-

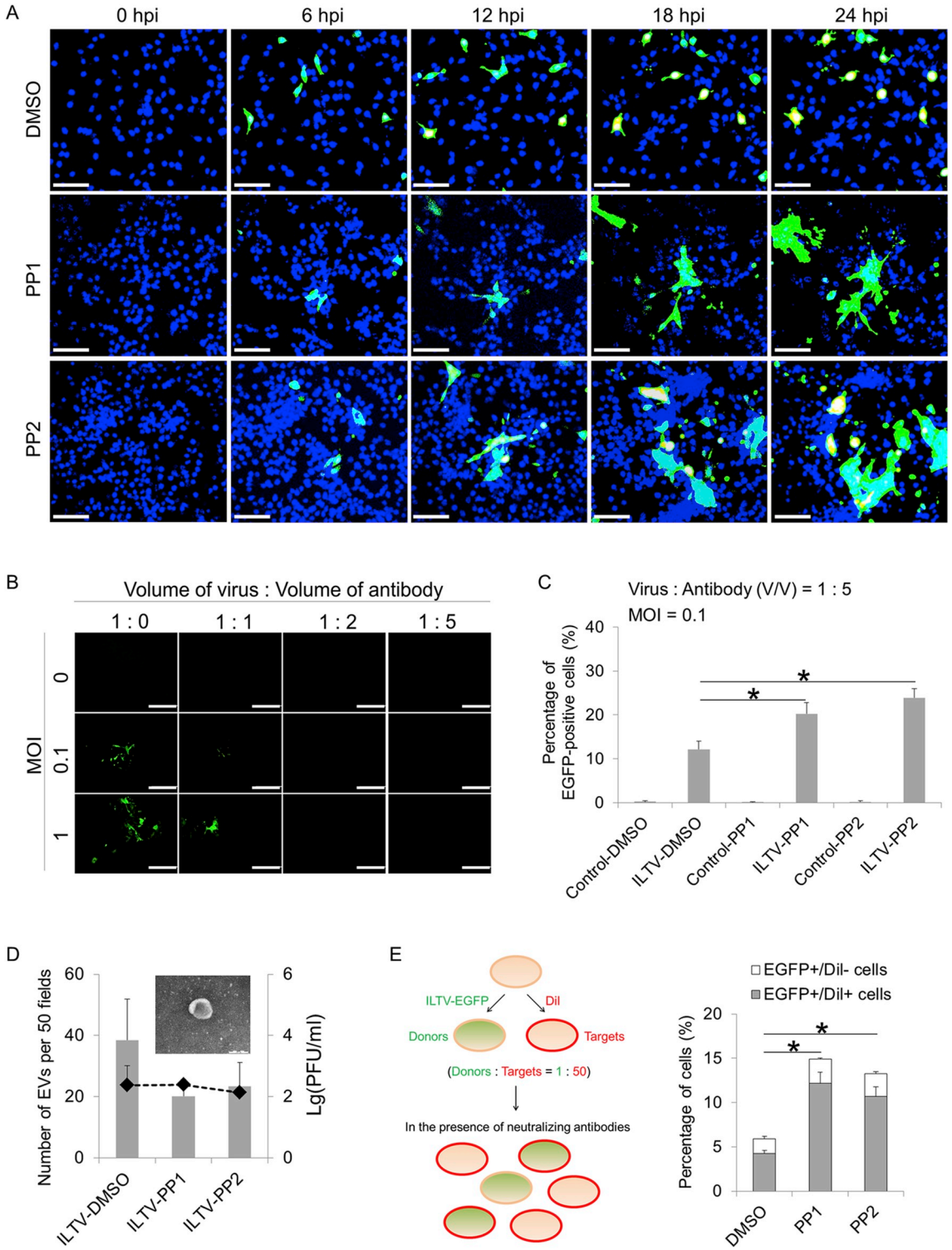


Fig. 3. Inhibition of Src promotes the cell-to-cell spread of ILTV. (A) ILTV-EGFP transmission was detected by time-lapse fluorescence microscopy via tracing the EGFP signal. ILTV-EGFP-infected cells (used as donor cells) and uninfected cells (used as target cells) were co-cultured at a ratio of 1:50 in the absence or presence of a Src inhibitor. Representative figures of ILTV-infected cells in the absence or presence of the Src inhibitor PP1 or PP2 at the indicated time points are shown. Cell nuclei were highlighted with Hoechst 33342 (blue). The scale bar indicates 70 μm. (B) Neutralization of ILTV by antibody. A polyclonal antibody against glycoprotein I of ILTV produced in rabbits was used as a neutralizing antibody and pre-incubated with different amounts of ILTV-EGFP at the indicated ratio (V/V). Normal rabbit serum was used as an isotype control. The expression of EGFP was observed by fluorescence microscopy at 24 h post infection. The scale bar indicates 400 μm. (C) The percentage of EGFP-positive cells in the presence of a neutralizing antibody at 24 h post infection was quantified by FACS. (D) The number and infectivity of extracellular vesicles secreted by ILTV-EGFP-infected cells in the absence or presence of a Src inhibitor were examined by electron microscopy and plaque assays. The representative transmission electron microscopy image showing the ultrastructure of extracellular vesicles we purified is provided. The scale bar indicates 200 nm. (E) Contribution of the proliferation of infected cells to the enhanced ILTV transmission by Src inhibition. Schematic presentation showing the design of the co-culture system. ILTV-EGFP-infected cells were used as donor cells. Target cells were labelled with the cell membrane dye Dil (Red). The percentages of cells positive for both EGFP and Dil and cells positive for only EGFP were assayed by FACS. The results in C, D and E are presented as the mean ± SD, n = 3. Asterisks indicate statistical difference ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

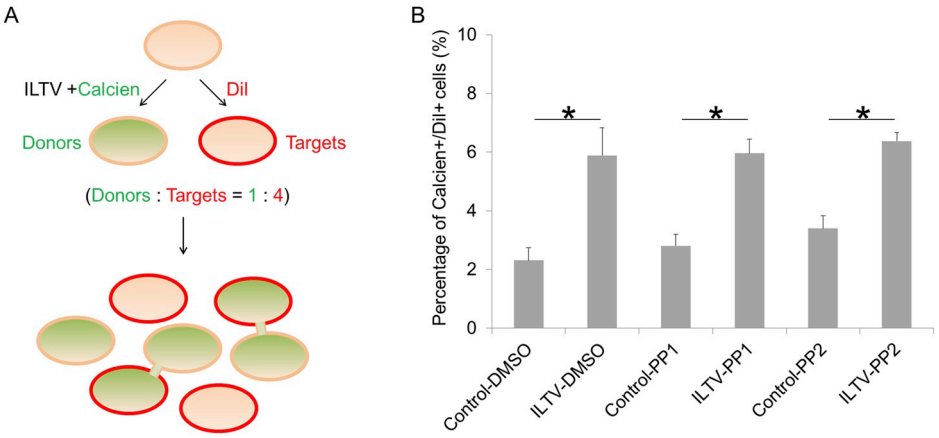


Fig. 4. Contribution of the direct connection between adjacent cells to the enhanced ILTV spread by Src inhibition. (A) Schematic presentation showing the design of the co-culture system. Donor cells were labelled with Calcein (Green). Target cells were labelled with the cell membrane dye Dil (Red). (B) The communication via direct cytosol-to-cytosol connection was examined by FACS via counting the proportion of cells positive for both Calcein and Dil 24 h post infection. The results are presented as the mean ± SD, n = 3. Asterisks indicate statistical difference ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

regulation of 341 genes and the down-regulation of 449 genes were attenuated by PP2 treatment (Fig. 5B). Accordingly, 234 up-regulated genes and 331 down-regulated genes were commonly regulated by PP1 and PP2 treatments (Fig. 6A, left panel) and named 'genes uniquely up-regulated/down-regulated by ILTV infection'. Among the genes unaffected by ILTV infection, 167 genes were up-regulated and 270 genes were down-regulated by PP1 treatment, and 301 genes were up-regulated and 459 genes were down-regulated by PP2 treatment upon ILTV infection (Fig. 5B), with 83 up-regulated genes and 128 down-regulated genes in common (Fig. 6A, left panel), which were named 'genes uniquely altered by Src inhibitors'. Considering the difference in transcriptional profiles between cells treated with PP1 or PP2, to avoid off-target effects of the inhibitors, only these gene subsets commonly altered by PP1 and PP2 treatments in ILTV-infected cells were included for further analyses. Pathway analysis using DAVID functional annotation with a p-value < 0.05 revealed pathways involved in fatty acid metabolic processes that were significantly enriched by 'genes uniquely down-regulated by ILTV infection' (Fig. 6A, right panel, Table S2) but did not uncover any pathway enriched by either 'genes uniquely altered by Src inhibitors' or 'genes uniquely up-regulated by ILTV infection'. Further DAVID functional annotation clustering analysis (enrichment score > 1.5) using 'genes uniquely down-regulated by ILTV infection' identified fatty acid metabolism-related biological processes as the cluster of the biological processes with the highest enrichment score (Fig. 6B).

To verify the role of fatty acid metabolism in the accelerated intercellular spread of ILTV by Src inhibition, two widely used chemicals that specifically inhibit fatty acid synthesis, namely, the allosteric inhibitor of acetyl Co-A carboxylase TOFA and the synthetic fatty-acid synthase inhibitor C75, were employed with or without the addition of palmitic acid (PAL), as shown in Fig. 7A. Both inhibitors did not affect the replication of viral DNA but significantly repressed virion production ($p < 0.05$) as assayed by ILTV-specific qPCR (Fig. 7B) and plague formation assays (Fig. 7C), suggesting that fatty acid metabolism is not required for viral genome replication but is essential for virion production. Considering the potential influence of the effects of TOFA and C75 on ILTV replication, neutralizing antibodies were added while investigating the role of fatty acid metabolism in the promotion of viral spread and cell death by Src repression via FACS (Fig. 7D and E). Both TOFA and C75 compromised the promoting effect of Src inhibition on viral spread (Fig. D). The inhibitory effects of TOFA and C75 on virion production and viral cell-to-cell spread in LMH cells upon Src inhibition were rescued by the addition of PAL (Fig. C and D). The enhanced death of infected cells by Src inhibition was also reduced by TOFA and C75 (Fig. E). No significant effect on cell death was observed in cells treated with only PP1, PP2, TOFA, or C75 (Fig. E), suggesting that these chemicals are not cytotoxic to LMH cells. Thus, our data demonstrate that fatty acid metabolism is essential for the regulation of the cell-to-cell spread and cytopathogenicity of ILTV by Src.

4. Discussion

Cell-to-cell spread of intracellular pathogens is one of the mechanisms that emerge during the co-evolution of a virus and its host, which is important not only for the pathogenesis of viral infection but also anti-microbial therapies. Currently, no antiviral drug targeting the latent infection of alphaherpesvirus is available, which can lead to outbreaks when the host is under stress or the host's immune system is compromised. During both the establishment of latent infection in sensory neurons and reactivation from latency, the virus spreads intercellularly between the mucocutaneous tissue and sensory neurons (Arvin et al., 2007; Abaitua et al., 2013). Thus, elucidation of the underlying mechanisms modulating the cell-to-cell spread of alphaherpesviruses may provide promising targets for the development of novel therapeutic strategies and for refining the rational design of effective vaccines to combat alphaherpesviruses. Despite extensive research efforts, the precise intercellular dissemination mechanisms remain largely unclear. ILTV shares common characteristics with other alphaherpesviruses, including its resistance to host humoural immune responses (Coppo et al., 2013), indicating the importance of intercellular virus spread during ILTV infection. Our previous studies identified Src as a key host determinant of ILTV infection. In the present study, we found that Src acts as the key orchestrator between ILTV and its host during infection through its control of viral cell-to-cell transmission and subsequent cell death in a cellular fatty acid metabolism-dependent manner. Our findings not only provide new insights regarding the mechanisms by which host Src regulates ILTV infection but may also help further reveal the mechanism of the cell-to-cell spread of other alphaherpesviruses.

Numerous efforts have been made to elucidate the mechanisms involved in the intercellular spread of viruses. The simplest and most widely used model of cell-to-cell spread is plaque formation in cell cultures with or without the addition of a neutralizing antibody, which reflects the cumulative processes of the host-virus interaction, including viral binding, penetration and replication, reorganization of cell-to-cell junctions, viral intercellular transport, and restriction mechanisms used by the host to resist infection, such as antiviral immune responses and factors (Dingwell et al., 1994; Johnson et al., 2001; Mettenleiter et al., 2009). Such models have been frequently used to investigate the processes of cell-to-cell spread and the essential viral and host factors for these processes (Farnsworth and Johnson, 2006; Krummenacher et al., 2003; Sourvinos and Everett, 2002). Recent studies utilizing advanced techniques and equipment to visualize viral infections in living cells, such as time-lapse microscopy, have revealed some novel features of viral cell-to-cell spread that had previously been unexpected and unrecognized. For instance, many viruses have been observed to spread

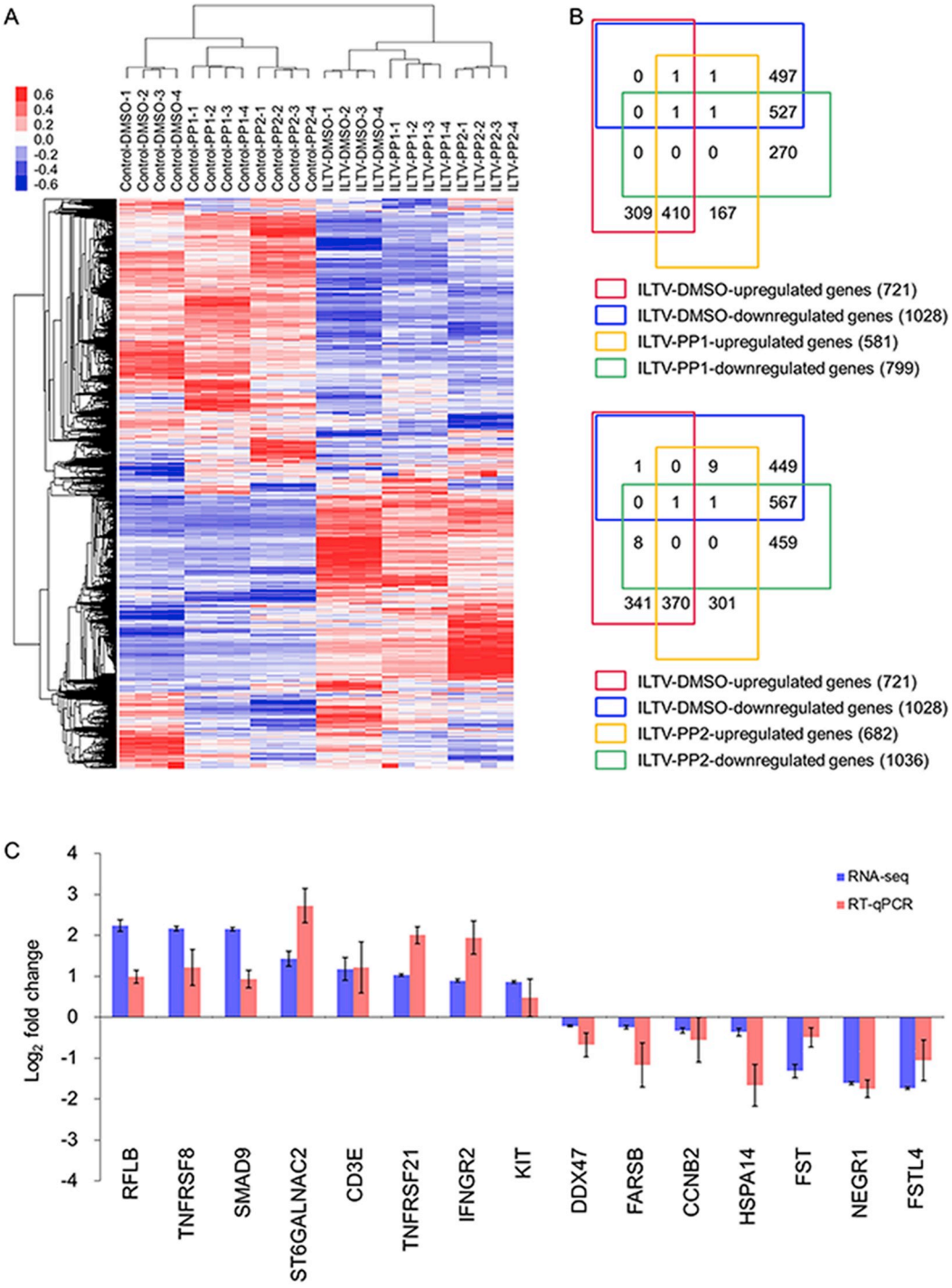
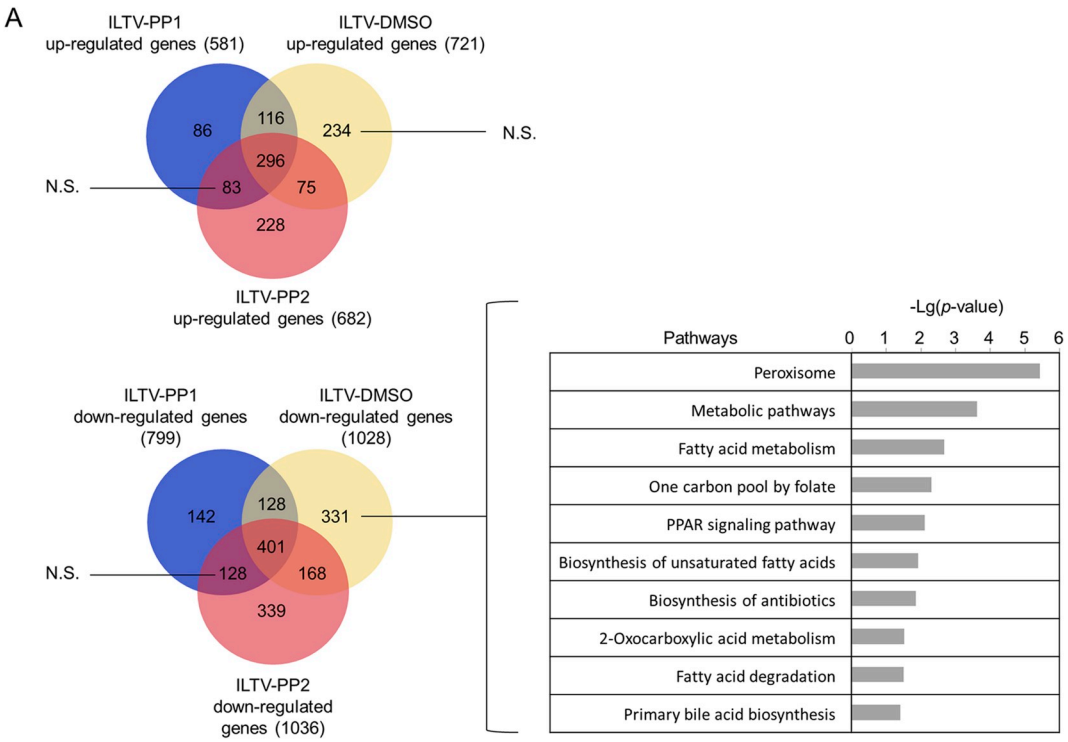


Fig. 5. Genome-wide transcriptome analyses. (A) Hierarchical clustering analysis of 3599 genes differentially expressed in LMH cells at $p < 0.01$, $q < 0.001$, fold change > 1.5. Columns indicate arrays, and rows indicate genes. Values are normalized by row. Blue indicates repression, and red indicates promotion. (B) Venn diagram showing the intersections of genes significantly regulated by ILTV infection in mock LMH cells and LMH cells treated with PP1 (upper panel) or PP2 (lower panel). (C) The transcriptional level of 15 selected genes was examined by RT-qPCR and compared with RNA-seq data for validation. The results are presented as the mean ± SD, n = 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

faster than the limits imposed by their replication kinetics. For poxviruses, through the repulsion of superinfecting virions by infected cells mediated by viral proteins A33, A36, A56, K2, and B5, the speed of viral spread is four-times faster than that expected according to their replication kinetics (Doceul et al., 2010, 2012). Rapid viral cell-to-cell spread has also been observed for HIV and herpesviruses, and paracrine-dependent migration of uninfected cells to the site of infection (Abaitua et al., 2013; Igakura et al., 2003; Jolly and Sattentau, 2004) as well as intercellular spread via nanotubes (Eugenin et al., 2009) were thought to contribute to this phenomenon. Cytoplasmic connections can promote the spread of viruses such as HIV-1 and herpesviruses by transporting viral genomes, viral capsids or vesicles containing viral particles between adjacent cells (Digel et al., 2006; Fan et al., 2014; Lucas, 2006). The present study suggests that despite being enhanced by ILTV infection, direct cytosol-to-cytosol connections do not contribute to the promoted intercellular spread of ILTV by Src inhibition



B

Annotation Cluster 1 (Enrichment Score: 1.772)			
Term	**Count**	**P Value**	**Fold Enrichment**
GO:0006631~fatty acid metabolic process	9	0.024	2.560
GO:0032787~monocarboxylic acid metabolic process	12	0.023	2.159
GO:0006082~organic acid metabolic process	19	0.012	1.874
GO:0044281~small molecule metabolic process	39	0.000	1.869
GO:0019752~carboxylic acid metabolic process	17	0.020	1.860
GO:0043436~oxoacid metabolic process	17	0.021	1.846
GO:0044255~cellular lipid metabolic process	18	0.027	1.752
GO:0006629~lipid metabolic process	22	0.032	1.603
Annotation Cluster 2 (Enrichment Score: 1.647)			
Term	**Count**	**PValue**	**Fold Enrichment**
GO:0035058~nonmotile primary cilium assembly	5	0.002	9.584
GO:0042384~cilium assembly	11	0.001	3.759
GO:0060271~cilium morphogenesis	12	0.000	3.599
GO:0044782~cilium organization	11	0.001	3.489
GO:0010927~cellular component assembly involved in morphogenesis	12	0.003	2.829
GO:0030031~cell projection assembly	14	0.002	2.743
GO:0070925~organelle assembly	16	0.022	1.891

Fig. 6. The molecular events related to host fatty acid metabolism are commonly altered by Src inhibitors in ILTV-infected cells. (A) Venn diagram showing the intersections of up-regulated genes (upper panel) and down-regulated genes (lower panel) that were significantly regulated among subgroups in LMH cells. Pathway analysis with genes uniquely altered by both Src inhibitors and genes uniquely up-regulated or down-regulated by ILTV infection was performed with a *p*-value < 0.05. (B) DAVID's functional annotation clustering analysis of the biological processes enriched by 'genes uniquely down-regulated by ILTV infection' (Enrichment score > 1.5).

(Fig. 4). In addition, herpesviruses can travel from infected cells to neighbouring cells via budding at the basolateral intercellular junction or by crossing the tight junctions between cells (Cocchi et al., 2000; Dingwell et al., 1994; York and Johnson, 1993). Whether these mechanisms are responsible for the accelerated spread of ILTV that we observed remains unclear. Further studies utilizing transmission electron microscopy are needed to investigate the exact mechanism of the enhanced cell-to-cell spread of ILTV by Src inhibition.

Our previous and present studies suggest that Src plays a very complex role during ILTV infection. On one hand, Src activation raises the threshold of infected host cells for infection-induced host cell death, which prolongs the duration of ILTV replication in each infected cell and allows the infiltrated virus to achieve optimal replication. Meanwhile, along with Src activation, ILTV infection also tends to accelerate viral cell-to-cell spread. Unexpectedly, according to the findings of the present study, this process is restricted by the activation of host Src triggered by the ILTV infection itself. In sum, Src activation by ILTV not only ensures the ultimate replication of viruses in infected cells but also limits viral dissemination. Why does ILTV activate host Src to achieve these opposite functions? As we showed in previous

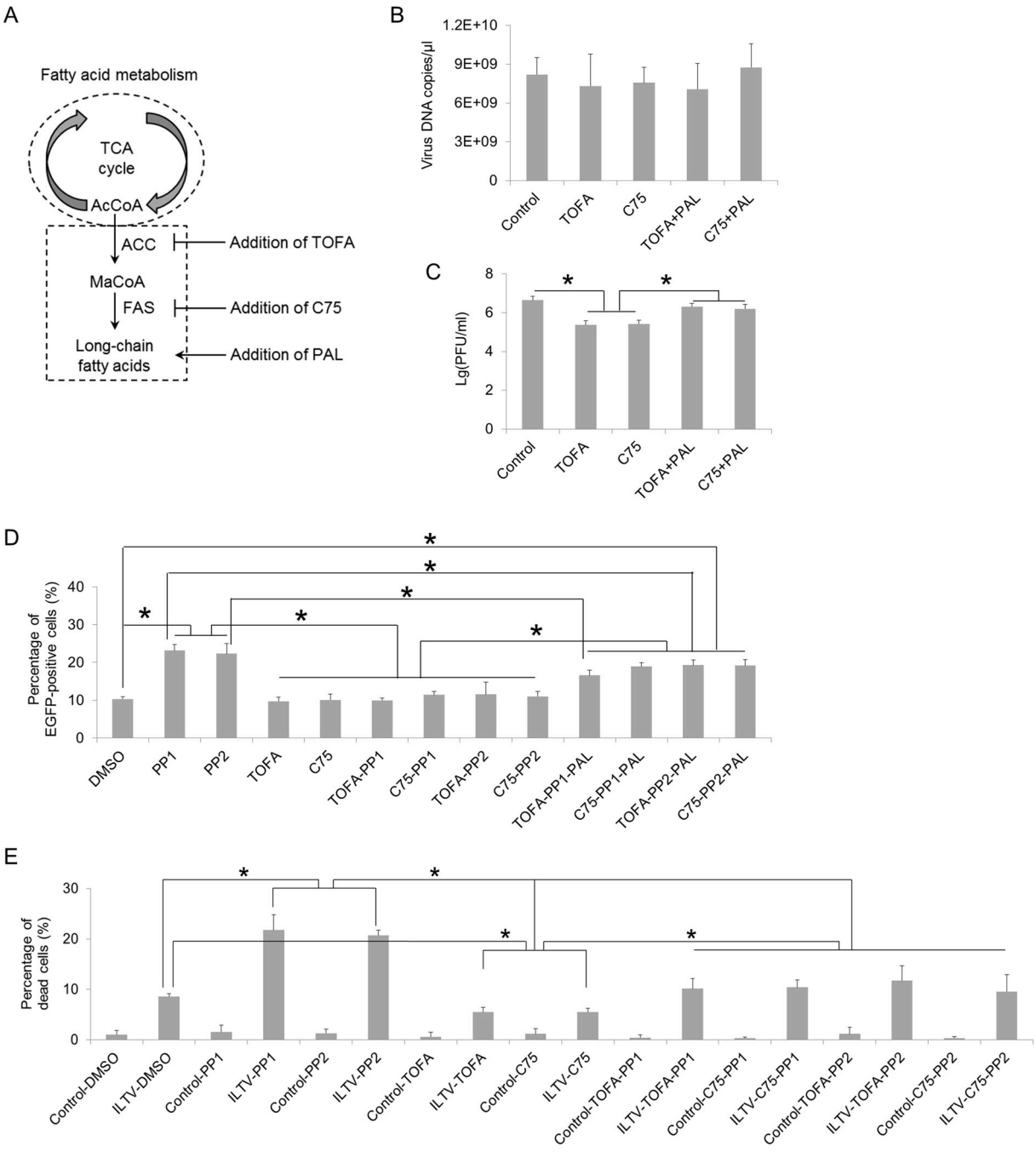


Fig. 7. Fatty acid metabolism is required for the promoted viral spread and cytopathogenicity by Src inhibition. (A) Schematic presentation showing the experimental design. (B and C) Statistic analyses of the effects of fatty acid synthesis on viral DNA replication and virion production were assayed by was determined by ILTV-specific qPCR (B) and plaque assays (C), respectively. (D) The effect of fatty acid synthesis on the accelerated viral spread by Src inhibition was quantified by FACS by detecting EGFP-positive cells at 24 h post infection. (E) The effect of fatty acid synthesis on the enhancement of ILTV infection-triggered cell death by Src inhibition was quantified by FACS via PI staining at 48 h post infection. The results in B to E are presented as the mean ± SD, n = 3. Asterisks indicate statistical difference ($p < 0.05$).

studies, Src inhibition not only represses ILTV replication but also reduces the threshold of infected cells for ILTV-triggered cell death and pathologic damage (Li et al., 2015). Considering the rapid establishment of latent infection during the initial infection with ILTV, the opposite effects of Src activation by ILTV infection most likely reflect a strategy that evolves during the co-evolution of ILTV and its host, which establishes a balance for the pathogen-host interaction.

Host metabolic requirements for maximal viral production have been examined in several human herpesviruses, including human cytomegalovirus (HCMV), herpes simplex virus 1 (HSV-1), and KSHV (Delgado et al., 2012; Munger et al., 2006, 2008; Sanchez et al., 2017; Vastag et al., 2011), and the results suggest that inhibition of specific cellular metabolic pathways can both eliminate latently infected cells and block lytic replication, thus providing opportunities for the development of novel therapeutic approaches for herpesviruses. Although similarities exist, significant differences in the utilization of host metabolic resources by these herpesviruses have been reported. For example, HCMV increased the levels of the tricarboxylic acid (TCA) metabolites fuelling fatty acid synthesis, while HSV-1 increased the level of flux to the TCA cycle to drive pyrimidine synthesis. The metabolic

signature of ILTV has not yet been addressed. Interestingly, our data demonstrate that Src inhibition promotes intercellular ILTV spread in a host cellular fatty acid metabolism-dependent manner (Fig. 7). Src is a myristoylated protein and can be found both within and outside of lipid rafts, as well as within endosomes, before translocating to the plasma membrane (Seong et al., 2009). Src can be activated by exogenous saturated fatty acids, such as palmitate and myristic acid, or the overexpression of cluster of differentiation 36 (CD36, also named fatty acid translocase), which is known to facilitate long-chain fatty acid uptake through the myristoylation of Src, leading to activation of its downstream signalling, such as JNK signalling (Holzer et al., 2011; Huang et al., 2017; Kim et al., 2017, 2019; Park et al., 2016). Nonetheless, given the increased fatty acid oxidation observed in mice with knockout of Fyn, another Src kinase family member (Bastie et al., 2007), Src activation by ILTV infection may limit viral intercellular spread by balancing host fatty acid metabolism, which can be negated by Src inhibition, resulting in accelerated viral spread and an exacerbated CPE of infection as observed in our model. This hypothesis is partially supported by our findings that inhibition of Src rescues the reduced transcription of genes involved in fatty acid metabolism (Fig. 6, Table S2), and that the promoting effects of Src inhibition on both intercellular viral spread and the cytopathogenicity of ILTV can be significantly attenuated by modulation of host fatty acid synthesis (Fig. 7). To exclude the potential off-target effect of Src inhibitor, two inhibitors were employed in our studies, and conclusion was made only when similar results were obtained using both inhibitors. The generation of LMH cell line and chicken embryo with Src knockout background using CRISPR/Cas9 system will be greatly helpful to further investigations of Src-mediated mechanisms in ILTV infection by excluding any cell type or chemical specific effect.

Considering the heterogeneous environment and the difficulty associated with controlling and monitoring the cell-to-cell spread of ILTV *in vivo*, *in vitro* studies were carried out to investigate the effect of Src on ILTV spread. Although primary cells isolated from chicken embryos can better mimic the *in vivo* process of ILTV infection, the instability of the biological characteristics of different isolates and the short lifespan of primary cells during *in vitro* culture limit their use in cellular and molecular mechanistic studies. LMH is the only cell line susceptible to ILTV infection *in vitro*. Our previous studies found that this cell line infected with ILTV can sufficiently mimic ILTV *in ovo* infection, and all Src-mediated mechanisms that we identified in LMH cells were validated *in ovo*, suggesting that LMH cells infected with ILTV are reliable *in vitro* models for studying ILTV infection (Li et al., 2015, 2018). In addition, we also analysed the only publicly available, genome-wide gene expression data of primary lung cells isolated from chicken embryos infected with a different virulent ILTV strain (Lee et al., 2010), which again revealed that host Src is the central regulator of the molecular network induced by ILTV infection in host cells (Li et al., 2015). All these results support the notion that the mechanism that we identified in LMH cells is universal in normal cells and tissues rather than a cell line-specific event. Thus, the LMH cell line was selected for the mechanistic study of Src regulation of ILTV infection in the present research. Further investigations using primary cultures of tissue samples or cells isolated from the mucosal epithelium of tracheal and conjunctival specimens infected with multiple ILTV strains are needed to determine the universality of the regulatory mechanism of Src on ILTV spread that we revealed in the present study.

5. Conclusion

ILTV causes an economically important chicken disease. Despite the extensive administration of live attenuated vaccines since the mid-twentieth century and the administration of recombinant vaccines in recent years, infectious laryngotracheitis outbreaks due to ILTV occur annually worldwide, and there is no cure for it. Similar to other alphaherpesviruses, ILTV establishes latency in the trigeminal ganglia after acute infection of the upper respiratory tract, and reactivation of viruses from latent infection occurs frequently once host immunity is compromised. No drug or efficient treatment targeting ILTV latent infection is currently available. We previously identified Src as a key host determinate of ILTV infection. However, therapies targeting Src not only repress viral replication but also exacerbate the pathological effects of ILTV. The underlying mechanisms of the side effects remain unknown. Our present study suggests that host Src controls the cell-to-cell spread of ILTV in a cellular fatty acid metabolism-dependent manner, which determines the CPE of ILTV. Given the importance of cell-to-cell spread during ILTV infection, our findings not only provide new insights regarding the mechanisms of ILTV infection but may also be valuable for the future development of novel therapeutic strategies and for refining the rational design of effective vaccines.

Author contributions

HL, SL and PW designed experiments and wrote the manuscript; ZW, BS, QG, YM, YL, ZC, HW, LC, and YS performed the experiments; ZW, BS, QG, HL SL and PW analyzed the data. All authors reviewed the results and approved the final version of the manuscript.

Conflicts of interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.08.011.

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