

# The Deubiquitylase USP5 Knockdown Reduces Semliki Forest Virus Replication in Hela Cells

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**ABSTRACT:** Semliki Forest Virus (SFV), a member of the *Alphavirus* genus in the *Togaviridae* family, is a small-enveloped, positive-sense single-stranded RNA (+ssRNA) virus. The virus is spread by mosquitos and can infect humans, resulting in mild febrile disease with symptoms that include fever, myalgia, arthralgia, persistent headaches and asthenia. Virulent strains of SFV in mice cause lethal encephalitis by infecting neurons in the central nervous system. In on-going experiments in the research group using a focused siRNA screen we have investigated the role of deubiquitylases (DUBs) during SFV infection (as a model alphavirus) and monitored the effect of DUB depletion on cell viability after infection. We identified a group of DUBs that have a pro-viral effect. The DUB, USP5, from this screen was validated to determine its effect upon viral replication. Here, we show that depleted USP5 in HeLa cells resulted in SFV RNA and viral yield at 8 h post-infection being significantly reduced. In the multi-step viral growth curve assay, in the absence of USP5, similar yields of SFV were determined at 2 and 4 h post-infection. However, a significant reduction in the infectious viral particles release at 6, 8, 10 and 12 h post-infection was observed and this could be reversed by direct constraining viral replication. These results raise the potential for USP5 to play a distinct role in the replication of SFV, suggesting that USP5 may be a possible anti-viral therapy for alphavirus infection.

**Keywords:** Alphavirus; RNA virus; SFV; DUB; USP5; siRNA.

حذف انزيم الديبوكيتيليز USP5 يختزل قابلية فيروس غابة سملكي على التكاثر في خلايا هيللا

عامر نوبگان

**المخلص:** فيروس غابة سملكي، ينتمي الى جنس فيروسات الفا عائلة توكافيريدي، محتواه الجينومي من نوع الحمض النووي الريبي، خيط منفرد و موجب الشحنة وجزئية هذا الفيروس محاطة بغلاف و حجمها صغير. ينتقل عن طريق البعوض و يسبب إصابة للانسان ينتج عنها مضاعفات مثل حمى خفيفة يرافقها ألم العضلات و المفاصل وصداع مستمر و إعياء عام. سلالات هذا الفيروس الضارية تسبب التهاب الدماغ القاتل للفئران عن طريق إصابة الخلايا العصبية في الجهاز العصبي المركزي. من خلال التجارب المختبرية المستمرة في مختبرنا و باستخدام تقنية حذف الجين بواسطة الية siRNA، تحققنا من دور انزيمات الديبوكيتيليز أثناء إصابة خلايا الإنسان المضيف بفيروس غابة سملكي (كأنموذج لفيروسات جنس ألفا) وذلك بحذف هذه الانزيمات من خلايا المضيف و مراقبة حيوية الخلايا بعد الإصابة. توصلنا الى تشخيص مجموعة من هذه الانزيمات التي تلعب دور مباشر في احداث إصابة فايروس غابة سملكي. انزيم ال USP5 واحد من هذه الانزيمات الذي ادى حذفه من خلايا هيللا الى احداث اختزال معنوي في مستوى الحمض النووي الريبي لفيروس غابة سملكي بعد 8 ساعات من الإصابة. بينت نتائج التحري عن عدد جزيئات الفيروس المتحررة من الخلايا المصابة خلال فترات زمنية متعددة، بأن عدد جزيئات الفيروس المتحررة لا تتأثر بعد 2 و 4 ساعات، بينما اختزلت إعداد جزيئات الفيروس المتحررة بشكل معنوي بعد 6، 8، 10 و 12 ساعة من وقت بدء الإصابة في الخلايا المحذوف منها انزيم ال USP5 مقارنة بالخلايا الطبيعية. أظهرت الدراسة الحالية بأن انزيم ال USP5 يلعب دور مميز في تكاثر واحداث الإصابة في خلايا الانسان و بالتالي قد يكون استهداف و حذف هذا الإنزيم من الخلية مضادا للحد من إصابات فيروسات نوع الفا.

**الكلمات المفتاحية:** فايروس نوع الفا، فايروس الحمض الريبي، فايروس غابة سملكي، انزيمات الديبوكيتيليز، انزيم USP5 تقنية حذف الجين siRNA .



## 1. Introduction

The alphaviruses are a group of small-enveloped viruses with positive-sense single-stranded RNA genomes; they belong to the *Alphavirus* genus of the *Togaviridae* family. Alphaviruses are known to cause a variety of arthritic diseases and encephalitis in humans and animals. They have been classified as Old World or New World viruses depending on their geographical distribution. Those classified as Old World viruses, include Semliki Forest virus (SFV), Chikungunya virus (CHIKV), Sindbis virus and Ross River Virus. These viruses typically cause fever, rash, and arthritic symptoms during infection. New World viruses include Venezuelan equine encephalitis virus and Eastern/Western equine encephalitis viruses [1]. Most members of the *Alphavirus* genus are arboviruses, which are transmitted to vertebrate hosts by arthropod vectors. The vectors include mosquitoes, ticks and lice, in which alphaviruses produce persistent, lifelong and asymptomatic infections [2]. SFV was identified in central, eastern and southern Africa, and was first isolated in 1942 from *Ae. abnormalis* mosquitoes in the Semliki Forest in Uganda [3,4]. Infection with SFV can result in a mild febrile illness with symptoms that include fever, myalgia, arthralgia, persistent headaches and asthenia during recovery. Only one fatal case of SFV infection has been documented in humans, which was in a laboratory worker in Germany, who was thought to be immunocompromised [5].

The basic life cycle of alphaviruses has been well studied [1,6]. The lifecycle starts with the attachment of the virus particle to the cellular receptor. E2 interacts with the cell surface receptor to initiate the endocytic internalization of the SFV virion, fusion of the viral envelope is triggered by low pH and the nucleocapsid is released into the cytoplasm. Uncoating of the nucleocapsid frees the 12kb positive-sense ssRNA genome, which is translated into four non-structural proteins (nsPs 1-4). The nsPs combine to generate a replicase complex, which generates dsRNA that is then used to produce genomic and subgenomic vRNAs.

These replication proteins enable the replication of the input genomic RNA and translation of the subgenomic mRNA into structural proteins (E1, E2, E3 and K6). Structural proteins are translocated across the endoplasmic reticulum, processed and transported through the Golgi to the plasma membrane. Cytoplasmic assembly of virus nucleocapsid, coat and genomic RNA produces the nucleocapsid core, which associates with processed glycoproteins at the plasma membrane, resulting in budding [2,7].

Ubiquitin is a 76-amino-acid polypeptide present in all eukaryotic cells [8]. All viruses rely extensively on host machinery to achieve successful infection. Accordingly, viruses have evolved to enhance or inhibit ubiquitylation of specific substrates to either enhance viral replication or inhibition specific cellular processes [9]. Ubiquitylation of protein is a reversible process, which facilitates the termination of ubiquitin-dependent signaling. The isopeptide that links ubiquitin and substrate and/or ubiquitin molecules in a polyubiquitin chain are cleaved by deubiquitylating enzymes (deubiquitylases or DUBs) [10]. The human genome encodes about 100 DUBs that are distinguished by their catalytic mechanisms and are grouped into six families according to their catalytic domains [11,12].

Ubiquitin-specific protease 5 (USP5), also known as isopeptidase T (isoT), disassembles an assortment of unanchored polyubiquitin chains (Lys 6, 11, 27, 29, 33, 48 and 63) to recycle free mono-ubiquitin [11,13,14]. USP5 is believed to play a role in preventing the accumulation of poly-ubiquitin chains, which would otherwise overwhelm the proteasome by competing with ubiquitinated proteins targeted for degradation [10,15,16]. Although DUBs regulators of many specific cellular pathways that play a role during different virus infections, a functional role for individual DUBs during alphavirus infection has not yet been established [17,18].

In on-going experiments in the research group, a screening of a DUB siRNA library identified a number of DUBs that play functional roles during alphavirus infection. Depletion of these hits in HeLa cells led to an increase in cell viability following SFV infection and subsequent experiments confirmed that depletion of these DUBs lead to decreased virus replication (predicted to be pro-viral) and thus could potentially be candidate anti-viral targets. The DUB, USP5, was further characterised and identified as being required for SFV replication.

DUBs have been closely studied as drug targets for cancer; and several DUB-targeting drugs are under investigation [19,20]. The overall goal of this type of work is to identify DUBs, such as USP5, that are required for efficient SFV replication, and so may lead to the identification of targets for anti-viral therapy to treat alphavirus infection.

## 2. Material and Methods

*Cell lines and Virus Stocks.* HeLa, Baby Hamster Kidney-21 (BHK-21) and Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10% (v/v) heated-inactivated foetal bovine serum (FBS), termed growth media. Maintenance medium Dulbecco's modified Eagle's Medium was supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 2.5% (v/v) FBS. All cell monolayers were grown under high humidity incubation at 37 °C and 5% CO<sub>2</sub>, were mycoplasma-free and cultured for limited passages. SFV clone 4 was used throughout this study [21]. SFV was amplified and stocks titrated in Vero cells.

*USP5 siRNA knockdown.* All individual USP5 siRNAs were obtained from Qiagen as follows: siRNAs 1-4 were SI00052416, SI00052423, SI00052430 and SI03076717 respectively. The All-Stars positive control (Hs Death; SI04381048) and All-Stars negative non-targeting control siRNA (siControl or siC; SI03650318) siRNAs were purchased from Qiagen. For deconvolution of the USP5 siRNA pool, siRNA knockdown was carried out in an opaque-

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walled and clear-bottomed 96-well plate format. HeLa cells in a duplicate 96-well plate were reverse-transfected in triplicate with USP5 siRNAs, corresponding to the original pool used in the preliminary screen (P), along with each individual siRNA (siRNAs 1-4) at a final concentration of 20 nM using lipofectamine RNAiMax (Invitrogen). At 72 h post-transfection (monitoring by microscope every 24 h), one plate was infected with SFV at a MOI of 2 and the second plate was mock infected. At 16 h post-infection, cell viability was measured using the CellTiter-Glo assay (Promega) in accordance with the manufacturer's protocol. Both a negative non-targeting control siRNA control (siC) and positive control (Hs Death) were included.

For USP5 knockdown in 6-well plate format, HeLa cells were reverse-transfected with individual siRNAs at a final concentration of 20 nM. 72 h post-transfection (monitoring by microscope every 24 h) the cells were either infected with SFV at 2 MOI or mock infected. The cells were incubated at 37 °C and 5% CO<sub>2</sub> for a further 8 h, before being used to monitor the efficiency of knockdown (USP5 mRNA level) and viral infection (SFV RNA level). Supernatants were saved at 8 h post-infection to determine the levels of SFV virus. SFV yields were titrated by plaque assay.

For siRNA knockdown in 10-cm tissue culture format, cells were reverse-transfected with individual siRNAs at a final concentration of 20 nM. Cells were incubated at 37°C and 5% CO<sub>2</sub>, the following day, 6 ml of fresh media was added to each dish. After 48 h, cells were harvested and reseeded at 1x10<sup>6</sup> cell/well into four wells of a 6-well plate. Plates were incubated at 37 °C and 5% CO<sub>2</sub> for a further 24 h. 72 h post-transfection (monitoring by microscope every 24 h) the cells were either infected with SFV at 2 MOI or mock infected. The cells were monitored for efficiency of knockdown (USP5 protein level) and supernatants were saved at different time points post-infection to determine the multi-step viral growth curves.

*RNA Extraction and qPCR.* Cell lysis and extraction of total RNA was performed using RNeasy Plus Mini Kit (Qiagen) in accordance with the manufacturer's instructions. RNA concentration and purity of each sample was assayed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Massachusetts, USA), and RNA integrity was assessed by gel electrophoresis. Using oligo-d(T) primer (Promega) and RevertAid H-minus M-MuLV reverse transcriptase (Thermo Scientific), 1 µg of RNA was reverse transcribed to cDNA. Quantitative PCR (qPCR) was performed (in triplicate) using the iTaq™ Universal SYBR® Green Supermix (BioRad 172-5121, USA), and a Rotor-Gene Q real-time PCR cycler (Qiagen). The following PCR primers were used in this study: ACTB 5'-CACCTTCTACAATGAGCTGCGTGTG-3' (Forward) and 5'-ATAGCACAGCCTGGATAGCAACGTAC-3' (Reverse) [22]; USP5 5'-CGGGACCAGGCCTTGAA -3' (Forward) and 5'-TCGTCAATGTGACTGAAGATCCA -3' (Reverse); SFV 5'-CGCATCACCTTCTTTTGTG-3' (Forward) and 5'-CCAGACCACCCGAGATTTT-3' (Reverse) [23].

A two-step qPCR cycling profile was performed for all primer pairs. An initial enzyme activation/denaturation step at 95 °C for 3 mins was followed by 40 cycles of 94 °C/15 s, 55 °C or 60 °C/30 s. Melt curves were read at 0.5 °C intervals from 55 °C to 95 °C. qPCR data were analysed using Rotor-Gene software. Cycle threshold (Ct) values were determined for each sample and compared to beta-actin (ACTB), using the 2<sup>-ΔΔCt</sup> method [24].

*Immunoblotting.* Protein lysates were generated from whole cell extracts by lysing cell pellets with Laemmli buffer followed by incubation for 10 mins at 90 °C-100 °C, then centrifuged. The 1x Laemmli buffer was prepared as follows: [50 mM Tris-Cl pH 6.8, 2% sodium dodecyl sulphate (SDS) and 10% glycerol]. Protein samples for loading were adjusted to a final loading volume using 1x Laemmli buffer with 10% bromophenol blue (BPB) (w/v) and 1 M dithiothreitol (DTT); samples were heated at 90 °C-100 °C for 5 mins prior to analysis. Equal amounts of each sample were loaded and run alongside the ColourPlus™ protein ladder (broad range, 11-245 KDa).

Protein concentration was assessed using a bicinchoninic acid (BCA) protein assay kit, (Pierce, UK) in accordance with the manufacturer's instructions. A final volume of 30 µg containing 25 µl of proteins was resolved on 10% SDS-PAGE gels, which were then transferred onto nitrocellulose membranes. Membranes were then blocked on a rocker for 2 h in BLOTTO [5% (w/v) skimmed milk powder in PBS plus 0.05% (v/v) Tween 20] and prepped with appropriate primary antibodies. Membranes were incubated overnight at 4°C. Appropriate secondary antibodies labelled with HRP were used and signals detected by enhanced chemiluminescence (ECL) (Pierce Bio-Technology) in accordance with the manufacturer's protocol. Quantification of bands (densitometry) was performed using ImageJ. Actin, ACTB, (Abcam ab6276) and USP5 (Proteintech 10473-1-AP) antibodies were used throughout this study. Anti-rabbit and anti-mouse HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology.

*Multi-step viral growth curves.* HeLa cells in 10 cm tissue culture dishes were transfected with either USP5 siRNAs (1 and 4) or siC for 48 h before being seeded into three wells of a 6-well format. At 72 h post-transfection, cells at a confluence of 80%-90% were either infected with SFV at 2 MOI or mock infected. After 1 h incubation at 37 °C, viral inoculum was aspirated and cells were fed and kept with 1 ml pre-warmed maintenance medium for 2, 4, 6, 8, 10 and 12 h. At each time point, 200 µl of supernatant was collected and replaced with 200 µl of fresh, pre-warmed maintenance medium at set time points, then immediately frozen at -80 °C. To assess the SFV titre, the frozen time point supernatants were defrosted and titrated by plaque assay in triplicate on BHK-21 cells twice, to give a total of six observations. The log<sub>10</sub> values of the final SFV titres were confirmed and the means of two independent experiments were compared against siC values.

*Plaque assays formation.* SFV stocks were titrated using BHK-21 as follows: cells were cultured in a 6-well plate until around 80%-90% confluence. Cells were then infected with 10-fold dilutions of SFV for 1 h. After 1 h adsorption at 37 °C, viral inoculum was aspirated and 2x DMEM (supplemented with 5% FCS) and 2% low melting point agarose

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overlay were added. Plaques were visualised at 72 h post-infection by staining with crystal violet. Plaques were counted, and virus titres calculated as plaque forming units per ml (pfu/ml), taking into account the original dilutions made.

### 3. Results

#### *Deconvolution of USP5 siRNA pool*

In previous results from our research group, a loss-of-function siRNA screen was undertaken to identify DUBs that may play a role during alphavirus replication (data are in the process of publication). A custom designed QIAGEN siRNA library targeting 92 known or predicted DUBs, in pools of 4 individual siRNAs specific for each DUB, was used to knockdown expression in HeLa cells in duplicate, prior to infecting one plate with virus and monitoring cell viability. We predicted that depletion of DUBs could lead to either an increase or decrease in cytopathic effect (CPE) after SFV infection, as a result of decreased or increased replication respectively, reflecting pro- or anti-viral DUB functions. To monitor this, the cell viability ratio for infected versus uninfected siRNA depleted cells was determined, with the overall goal of identifying DUBs reflecting pro-viral functions that may be potential therapeutic targets. The DUB USP5 was further characterised and was identified as being (a pro-viral) required for SFV infection, acting at the role of viral replication in the original screen.

Using a deconvolution approach, DUB was deemed to play a role in SFV infection if the pool of four siRNAs and two or more of the individual siRNAs resulted in an increase in cell viability (decrease in CPE), based on the assumption that it is unlikely that more than one siRNA within a pool would have the same off-target effect. Deconvolution of the USP5 siRNA pool is shown in Figure 1A. Knockdown of USP5 in HeLa cells with the siRNA pool again led to a 45% increase in cell viability after SFV infection. Two individual USP5 siRNAs, siRNAs 1 and 4, also resulted in respective increases in cell viability of 53% and 47%. However, treatment of HeLa cells with USP5 siRNAs 2 and 3 resulted in respective decreases in cell viability of 74% and 47% Figure 1A. As USP5 was deconvoluted successfully, with pool and two individual siRNAs giving positive results, it was decided that this DUB would be followed up to investigate further its role during SFV infection.

#### *Quality control of deconvolution for USP5 siRNA pool*

Poor transfection efficiency, poor knockdown efficiency of the target transcript and siRNA-treatment toxicity, due to either depletion of the specific DUB or off-target effects, are important variables that could potentially influence the data from these deconvolution experiments. To monitor transfection efficiency and toxicity, issues steps were included in this protocol. However, it was not possible to monitor the target knockdown efficiency in this protocol; this is addressed in the next section.

Transfection efficiency was monitored by using the ALL-Stars Hs Death siRNA, which induces a high degree of cell death by targeting and depleting essential human survival genes. Hs Death siRNA induced cell death was monitored visually by light microscopy and cell viability was measured using CellTiter-Glo assay (Cell Viability Luciferase Assay). The CellTiter-Glo assay demonstrates an increase in cell viability based on ATP levels. ATP is an indicator of metabolically active cells, thus the number of viable cells can be assessed based on the amount of ATP available. The ATP Cell Viability Luciferase Assay offers a highly sensitive homogenous assay for quantifying ATP in cell cultures. Triplicate wells of the 96-well plate were treated with Hs Death siRNA. Cells were assessed at 72 h post-transfection; successful transfection of the HeLa cells should result in a high level of cell death. In all experiments, Hs Death transfected cells showed an estimated 85%-95% reduction in confluence, which was supported by monitoring the cell viability in the transfected, but uninfected plate which increased numbers of floating cells (died cells) were observed.

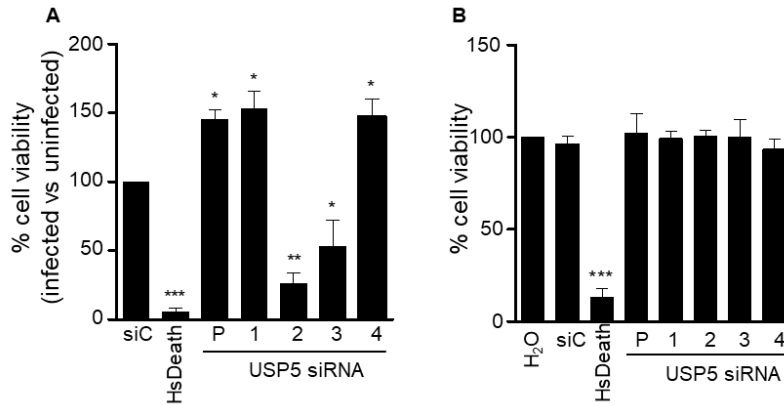
Monitoring of USP5 siRNAs induced toxicity was carried out two ways. Firstly, examination of cells by light microscopy was performed at 72 h post-transfection. The majority of the DUB siRNAs were associated with little visible direct toxicity. This was evidenced by an estimated confluency of cells of greater than 90%, with few floating cells in the media and healthy looking cells.

These microscopy observations were supported by monitoring the cell viability in the transfected but uninfected plate. This was determined as part of the overall assay (to determine the ratio of cell viability of infected vs uninfected), but here it is analysed on its own, as a percentage relative to untransfected cells (H<sub>2</sub>O control wells were transfection without siRNA to control the potential effect of transfection reagents on the cell) to assess siRNA induced toxicity (after 88 h transfection).

As predicted by the visual/cell viability evaluation at 72 h, transfection with Hs Death resulted in an 87% reduction in cell viability compared to untransfected cells in H<sub>2</sub>O control wells (Figure 1B).

A non-targeting control siRNA (siC) had marginal effect on the cell viability ratio post-transfection, with changes of 4% observed. Depletion of USP5 by either the pool of the four siRNAs or individual siRNAs (1 and 3) had no effect on cell viability respectively. However, individual siRNAs (2 and 4) had a marginal reduction in cell viability of 1% and 6% respectively, (Figure 1B).

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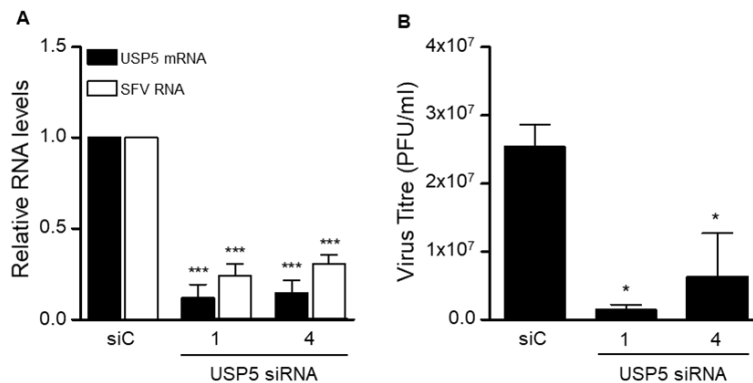
**Figure 1.** Deconvolution and quality control for USP5 siRNA pool.

(A) HeLa cells were reverse-transfected in duplicate 96 well plate with USP5 siRNAs corresponding to the original pool used in the preliminary screen (P) and each siRNA individually (siRNAs 1-4) along with the non-targeting control (siC). At 72 h post-transfection, one plate was infected with SFV at 2 moi, the second plate was mock infected. At 16 h post-infection cell viability was measured using the CellTiter-Glo assay. The percentage change in cell viability of infected vs. uninfected cells, relative to the siC treated control is shown. Hs Death was included as an additional control. The data shown are the mean of 3 independent experiments (+/- SEM). Data were analysed against siC by one way ANOVA with Tukey-one-way test (\* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ ). (B) Data representing the cell viability of USP5 and control siRNA treated cells 88 h post-transfection. The data shown are the mean of 3 independent experiments (+/- SEM). Data were analysed against H<sub>2</sub>O control by one way ANOVA with Tukey-one-way test (\* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ ).

### *Depletion of USP5 is reduced SFV replication*

USP5 was successfully validated by deconvolution, meeting the criteria that the original pool of four siRNAs and at least two of the individual siRNAs (1 and 4) repeated the effects observed in the original screen. Thus, USP5 siRNAs 1 and 4 were chosen to use for the follow up studies to investigate the role of USP5 during SFV infection. To verify the validity of the USP5 effect, several experiments were undertaken to determine if this was reflected at the level of virus RNA.

HeLa cells were reverse-transfected with USP5 siRNAs (1 and 4) for 72 h before being infected with 2 MOI of SFV and RNA extracted at 8 h post-infection. SFV RNA and USP5 mRNA levels were then measured by qPCR. USP5 siRNAs (1 and 4) led to USP5 transcripts being depleted by 88% and 85% respectively, which is statistically significant ( $p < 0.05$ ). For SFV RNA levels, depletion of USP5 with siRNAs 1 and 4 led to significant reductions in SFV RNA of 76% and 69% respectively (Figure 2A). The data were analysed and compared to siC using Tukey-one-way ANOVA. This showed that the differences were statistically significant (siRNA 1  $p \leq 0.001$  and siRNA 3  $p \leq 0.001$ ) (Figure 2A).



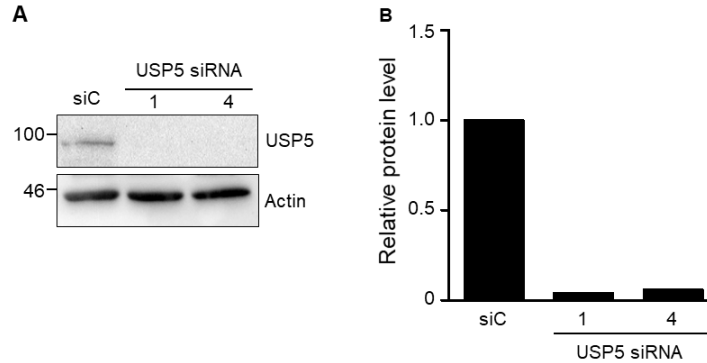
**Figure 2.** USP5 depletion leads to a decrease in SFV genomic RNA levels and SFV titre after infection.

(A) HeLa cells were transfected with USP5 siRNAs 1 or 4, or siC, incubated for 72 h before infecting with SFV at 2 moi. Total RNA was extracted at 8 h post-infection and analysed by qPCR for levels of SFV genomic RNA and USP5 mRNA. Total RNA was extracted 8 h post-infection, converted to cDNA using oligo d(T) before being analysed by qPCR for levels of SFV genomic RNA and USP5 mRNA. Data were normalised to actin and expressed as  $2^{-\Delta\Delta Ct}$  relative to the siC control. (B) SFV present in the supernatants from these experiments were titrated on monolayers of

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BHK-21 cells. The data show are the mean of 3 independent experiments ( $\pm$  SEM). Data were analysed against siC by one way ANOVA with Tukey-one-way test ( $*p \leq 0.05$ ,  $**p \leq 0.01$ ,  $***p \leq 0.001$ ).

It was also possible to determine the levels of SFV released from cells in these experiments. Supernatants were saved at 8 h post-infection and titrated on BHK-21 cells. For the supernatant for cells treated with USP5 siRNAs 1 and 4, the mean pfu/ml was  $1.5 \times 10^6$  pfu /ml and  $1.6 \times 10^6$  pfu /ml respectively. In comparison, supernatant from siC treated cells was  $2.5 \times 10^7$  pfu /ml. Data were analysed using Tukey one-way ANOVA as described previously. The results were statistically significant, with siRNA 1  $p \leq 0.05$  and siRNA 3  $p \leq 0.05$  (Figure 2B). Immunoblot analysis also confirmed siRNAs 1 and 4 led to a reduction in USP5 protein levels (Figures 3A and B).

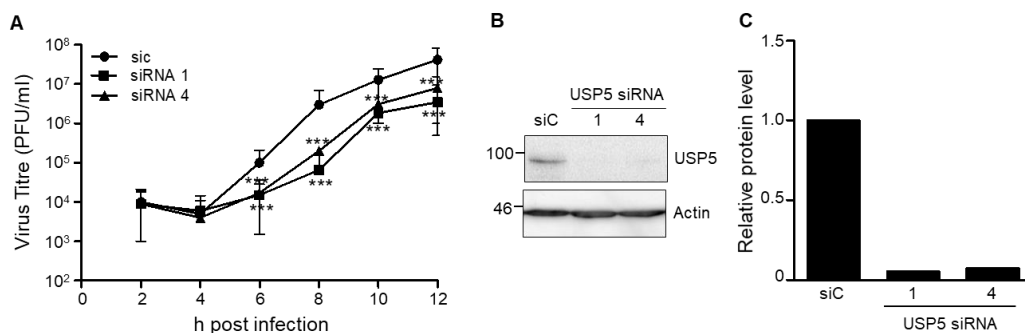


**Figure 3.** Efficiency of siRNA depletion of USP5 protein.

HeLa cells were transfected with the Qiagen USP5 siRNAs 1 or 4 or siC. Following 72 h incubation, the cells were lysed in Laemmli buffer. 30  $\mu$ g were then resolved by SDS-PAGE and immunoblotted for USP5 and actin. (A) Proteins were visualised using ECL detection. (B) Quantification of USP5 protein level was determined by quantitating relative to actin, and then compared to siC via Image J analysis. Data from one independent experiment.

### Multi-step viral growth curves

The preliminary screen and deconvolution assay reported the effect of USP5 depletion on virus infection by monitoring cell viability after 16 h post-infection. Subsequent experiments confirmed the effects of depletion upon the production of viral RNA and viral yield at 8 h post-infection. To test whether the absence of USP5 causes a defect in viral replication at different time points, HeLa cells were inoculated at a MOI of 2, and comparisons were made between the depletion of USP5 by siRNAs (1 and 4) treatment and siC treatment over the course of 12 h (Figure 4A). No differences in SFV replication were observed between siC and the depletion of USP5 by siRNAs (1 and 4) at 2 h and 4 h post-infection; siC produced less virus at 4 h post-infection compared to siRNA 1 and 4 (Figure 4A). In contrast, the SFV titre detected in siRNAs 1 and 4 was significantly reduced after 6-12 h post-infection compared to siC (Figure 4A). Plaque sizes and phenotypic homogeneity following SFV infection were assessed. Compared to siC, no differences in plaque size were detected with the depletion of USP5 by siRNAs 1 or 4. Immunoblot analyses of the efficiency of USP5 depletion mediated by siRNAs 1 and 4 at 72 h post-transfection are shown in Figures 4B and C.



**Figure 4.** Depletion of USP5 leads to a decrease in SFV infectious particle release in multi-step growth curves.

HeLa cells were transfected in a 10 cm dish with either USP5 siRNAs 1 or 4, or siC. At 48 h cells were reseeded into duplicate 6 well plates. After a further 24 h, (A) one plate was infected with SFV at 2 moi. Aliquots of the cultured supernatants were collected at the indicated time points (2, 4, 6, 8, 10 and 12 h) post-infection and SFV titres were quantified in triplicate on monolayers of BHK-21 cells. The data show are the mean of 3 independent experiments ( $\pm$  SEM).

SEM). Data were analysed against siC by one way ANOVA with Tukey-one-way test (\* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ ). (B) Representative immunoblot analysis of USP5 and Actin in lysates from siRNA treated cells. (C) Quantification of USP5 protein level was determined by quantitating relative to actin and then compared to siC via Image J analysis. Data from one independent experiment.

#### 4. Discussion

All viruses rely extensively on host machinery to achieve successful viral replication. Accordingly, viruses have evolved to enhance or inhibit ubiquitylation of specific substrates to either enhance viral replication or inhibition specific cellular processes. The reverse reaction is undertaken by a large family of enzymes, termed deubiquitylases, or DUBs. Many of these are crucial, not only to virus replication, but also in the regulation of the immune system against infection and vesicle trafficking. However, the specific role of DUBs in alphavirus infection had not been studied previously. Several studies have been carried out using multiple genome-wide screens to illustrate the mechanisms of viral infection, including influenza virus, Sindbis virus, West Nile Virus and HIV, and the host cell requirements to support or control infection [25–29].

In this study, siRNA treatment of HeLa cells indicated a pro-viral role for USP5 during SFV infection. The long-term aim of the study was to identify potential targets for therapeutic intervention. Several factors may explain the lack of overlap between the results of the preliminary screen and the deconvolution. It is possible that there may have been technical issues from generating the pools for use in this study, as the original screen pools purchased from Qiagen were no longer available. Furthermore, as revealed in a study by Bushman and colleagues, data analysed from a screen performed in duplicate under identical experimental conditions, the yields of data sets only overlapped by an estimated 50% [30]. Three important variables that could potentially influence the data from these deconvolution experiments are poor transfection efficiency, poor knockdown efficiency of the target transcript and toxicity due to siRNA treatment, either due to depletion of the specific DUB or off-target effects [31,32]. The approach used in these validation experiments was unable to monitor the target knockdown efficiency. This is addressed in the section investigating the effect of USP5 depletion on SFV replication by monitoring the levels of USP5 transcripts and protein. However, steps were included in the protocol to monitor both transfection efficiency and toxicity issues (Figure 1B). Transfection efficiency was monitored by using the ALLStars Hs Death siRNA, which induces a high degree of cell death by targeting and depleting essential human survival genes [33].

In this study we propose that USP5 is essential for efficient SFV infection and replication. In its absence, induced by siRNA depletion in HeLa cells, SFV replication and the release of infectious viral particles during infection were significantly reduced. qPCR and plaque assay were used to demonstrate the effect upon SFV RNA levels and SFV titres at 8 h post-infection. In the multi steps viral growth curve assay, for the early hours of SFV infection, 2 and 4 h post-infection, similar SFV yields were determined among the siC and USP5 knockdown cells, suggesting that siRNAs 1 and 4 against USP5 had no effect on SFV uptake. However, SFV showed a lower titre at 6, 8, 10 and 12 h post-infection, indicating that USP5 may play a role within the cell only and late in infection. Taken together, these data implied that in HeLa cells, USP5 is required for efficient SFV replication.

It has been reported that the depletion of USP5 in mammalian cells, and in yeast, leads to the accumulation of unanchored ubiquitin chains; depletion of USP5 may also inhibit the function of proteasomes [13,14]. Many studies have shown that USP5 targets numerous cytosolic proteins and influences a broad range of cellular processes including DNA repair, immune responses and tumour cell proliferation [35–37]. However, the mechanisms by which USP5 exerts this strong pro-viral effect are not obvious.

Previous studies indicate that DUBs play a role as negative regulators of IFN and the production of pro-inflammatory cytokines such as MYSM1, A20 and CYLD [38–41]. Panda and colleagues reported that the absence of MYSM1 in murine macrophages led to the reduced replication of vesicular stomatitis virus as a consequence of increased type 1 IFN and pro-inflammatory cytokine production [41]. Alphaviruses are very sensitive to IFNs and pre-treating cells with IFN inhibits viral replication [42]. Findings by Reynaud and colleagues suggest that IFN $\beta$  pre-treatment could block alphavirus infection before RNA begins to replicate, but that it did not affect the process of virus entry and virion disassembly [43]. Ubiquitination and deubiquitination processes have been demonstrated to play regulatory roles, controlling various aspects of cellular pathways, including immunity [44–47]. Normally, E3 ligases activate TRAF3/6 by adding K63-linked polyubiquitin chains, leading to the production of IRF3/7 and NF- $\kappa$ B. This subsequently causes the induction of pro-inflammatory cytokines and type 1 IFNs [47,48]. Thus, in the absence of USP5 there may be overproduction of cytokines such as IFN $\beta$ , which induces a strong anti-viral environment. This would make USP5 attractive to explore in basic experiments to characterise its role during alphavirus infection; in particular, it would be useful to identify those proteins with which USP5 may interact.

#### 5. Conclusion

The overall goal of this type of work is to try to identify druggable proteins that may be possible to target during alphavirus infection. DUBs are commonly studied as drug targets in cancer, and a number of drugs are under investigation. From the outcome of our investigation, it is possible to conclude that USP5 may be a target anti-viral therapy for alphavirus infection. There are a number of areas that could be followed up from this work, in addition to

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the many unanswered questions regarding the role of USP5. This makes the continued characterisation of the role of DUBs during alphavirus infection a worthwhile objective for further research.

### Conflict of interest

The author declares no conflict of interest.

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