VIRULENCE
2022, VOL. 13, NO. 1, 1407–1422
https://doi.org/10.1080/21505594.2022.2112821

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SLC1A3 facilitates Newcastle disease virus replication by regulating glutamine catabolism

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ABSTRACT

As obligate intracellular parasites, viruses rely completely on host metabolic machinery and hijack host nutrients for viral replication. Newcastle disease virus (NDV) causes acute, highly contagious avian disease and functions as an oncolytic agent. NDV efficiently replicates in both chicken and tumour cells. However, how NDV reprograms host cellular metabolism for its efficient replication is still ill-defined. We previously identified a significantly upregulated glutamate transporter gene, solute carrier family 1 member 3 (*SLC1A3*), during NDV infection via transcriptome analysis. To investigate the potential role of *SLC1A3* during NDV infection, we first confirmed the marked upregulation of *SLC1A3* in NDV-infected DF-1 or A549 cells through p53 and NF-κB pathways. Knockdown of *SLC1A3* inhibited NDV infection. Western blot analysis further confirmed that glutamine, but not glutamate, asparagine, or aspartate, was required for NDV replication. Metabolic flux data showed that NDV promotes the decomposition of glutamine into the tricarboxylic acid cycle. Importantly, the level of glutamate and glutaminolysis were reduced by *SLC1A3* knockdown, indicating that *SLC1A3* propelled glutaminolysis for glutamate utilization and NDV replication in host cells. Taken together, our data identify that *SLC1A3* serves as an important regulator for glutamine metabolism and is hijacked by NDV for its efficient replication during NDV infection. These results improve our understanding of the interaction between NDV and host cellular metabolism and lay the foundation for further investigation of efficient vaccines.

ARTICLE HISTORY
Received 20 December 2021
Revised 2 August 2022
Accepted 9 August 2022

KEYWORDS
Infection; host; Newcastle disease virus; *SLC1A3*; glutaminolysis

Introduction

Newcastle disease virus (NDV), an enveloped single stranded negative sense RNA virus, is a member of the *Paramyxoviridae* family [1] and causes an acute, highly contagious disease. Animals susceptible to this disease include mainly chickens and turkeys. NDV infection leads to high morbidity in chickens and heavy losses to the poultry industry [2,3]. Glutamine is a nonessential amino acid, second only to glucose in terms of energy anaplerosis and biomass synthesis. The conversion of glutamine to α-ketoglutarate (α-KG) via glutamate serves as a route for entry of glutamine-derived carbons into the tricarboxylic acid (TCA) cycle [4]. During the glutamine catabolism, glutamine can be converted into α-KG, which participates as a supplementary substrate in the TCA cycle. In addition, α-KG and aspartate/alanine can be converted into glutamate and oxaloacetic acid/pyruvate by glutamic-oxaloacetic transaminase 1, 2 (*GOT1*, *GOT2*) and alanine aminotransferase (*ALT*) [5]. Glutamine can contribute nitrogen atoms; thus, it can also be used in the synthesis of glutathione, ammonia, and purines. In many cancer cells, glutamine is often utilized as a substrate to replenish the TCA cycle, given that the carbon source of glucose is used to produce lactic acid and fatty acids [6]. Some studies have confirmed that glutamine is required for viral replication. The production of poliovirus virion in HeLa cells decreased in a medium free of glucose and glutamine, and reverted to normal levels after the medium was supplemented with glutamine [7]. In 2008, Munger et al. have reported for the first time that glutamine uptake is

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/21505594.2022.2112821.

markedly upregulated after human cytomegalovirus (HCMV) infection [8]. Subsequently, in the absence of glutamine, ATP levels and viral production could be rescued after supplementation with TCA cycle [9]. Some studies reported that vaccinia virus (VACV) relies on glutamine extracellular uptake or catabolism to ensure its replication in host cells [10–12]. A global metabolomic screen of VACV-infected primary HFF cells showed that glutamate and glutamine levels increased. The lack of glutamine in growth media has significantly reduced the production of infectious virus particles [10]. These data suggest that when glucose is diverted to produce lactic acid, the virus upregulates glutamine catabolism in infected cells, which supports the survival and replication of the virus. Amino acids are the basic units for the synthesis of proteins and peptides. They play vital roles during the processes of cell biology and contribute to the production of bioactive molecules, which participate in the regulation of cell signalling transduction and metabolism [13–15]. Therefore, maintaining amino acid homoeostasis is essential for cell survival. There are three key factors which regulate homoeostasis in cells: (1) entry and exit through amino acid transporters (AATs); (2) biosynthesis of amino acids in cells; and (3) recycling of damaged organelles and protein degradation [16,17]. Solute carrier family 1 member 3 (*SLC1A3*), also known as EAAT1, a sodium-dependent glutamate/aspartate transporter 1, is a member of the high-affinity glutamate transporters [18]. Glutamate transporters are critical to the termination of excitatory neurotransmission and the supply of glutamate to cells for metabolic processes [18,19]. In addition to astrocytes, *SLC1A3* can be expressed in peripheral organs, indicating its metabolic significance beyond the central nervous system. *SLC1A3* mediates the cellular transport and regulation of glutamate/aspartate and can also remove extracellular glutamate [20]. Ralphe et al. reported that *SLC1A3* is also localized in the mitochondrial membrane, as a glutamate carrier, to facilitate the malate/aspartate shuttle [21,22].

Few studies have been reported on the host metabolism of NDV. Lipid metabolism was significantly affected in NDV-infected black-bone chickens. In the early stage of NDV infection, lipogenesis was inhibited, whereas lipolysis was strengthened [23]. Moreover, NDV replication can be inhibited by disturbing cholesterol homoeostasis [24]. Our previous results showed that NDV had elevated glucose uptake and glycolysis in host cells [25]. In addition, amino acid metabolism was significantly altered during NDV infection, but the detailed mechanism has not been elucidated [26]. Regarding the important role of AATs in the homoeostasis of amino acid metabolism, we speculated that they might be involved with NDV replication. In this study, we found that *SLC1A3* was required for effective infectious NDV production. *SLC1A3* also contributed to the production of glutamate and to the maintenance of the TCA cycle. Moreover, glutamine was necessary for NDV replication. Our findings will help to understand the demand for amino acids and how to regulate the host cell amino acid metabolism during NDV replication, which may provide novel clues and directions for future research.

Materials and methods

Cells and viruses

DF-1, 293T, and A549 cell lines were purchased from ATCC (USA). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) or F12K with 10% foetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO_2.

NDV strains Herts/33 (a standard NDV virulent strain) and LaSota (an attenuated strain) were obtained from the China Institute of Veterinary Drug Control (Beijing, China). NDV strain SH15 (a moderately virulent strain) was isolated and preserved by our laboratory in 2015 (GenBank accession number: KY247177). These viruses were proliferated in 10-day-old specific-pathogen free embryonic chicken eggs and viral titres were determined as 50% of the tissue culture infective dose ($TCID_{50}$), as described previously [27].

Antibodies and reagents

An antibody against NDV nucleoprotein (NP) was produced and preserved in our laboratory. Antibodies against β-actin, *p*-p53, p53, p21, *p*-IκBα, IκBα, LAMP1, Na,K-ATPase, and COX IV were purchased from Cell Signalling Technology (Danvers, MA, USA). Anti-SLC1A3 antibody (NB100–1869) was purchased from NOVUS Biotechnology (Centennial, CO, USA).

Lipofectamine 2000, CellMask™ Green Plasma Membrane Stain (C37608), MitoTracker™ Deep Red FM (M22426), and Lysosome Enrichment Kit (89839) were purchased from Invitrogen Thermo Fisher Scientific (Waltham, MA, USA). DAPI (C1002), Enhanced Cell Counting Kit-8 (C0042), and Cell Mitochondria Isolation Kit (C3601) were purchased from Beyotime Biotechnology (Shanghai, China). The siRNA was synthesized by GenePharma Co., Ltd (Shanghai, China). SYBR Green qPCR Mix (p2092)

was purchased from Dongsheng Biotech (Guangzhou, China). Enhanced chemiluminescence reagent kit was purchased from Share-Biotechnology (Shanghai, China). Plasma Membrane Protein Isolation and Fractionation Kit was purchased from Invent Biotechnology (Beijing, China). TFB-TBOA, an inhibitor of glutamate transporter, was purchased from Tocris Bioscience (Shanghai, China). The inhibitors, PFTα, BAY 11–7082, and chloroquine (CQ), were purchased from Selleck (Houston, TX, USA).

Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR was performed as described previously [28]. Total RNAs from cell samples were extracted and reverse transcribed to cDNA. The primers used in this study are listed in Table S1. All experiments were performed in triplicate. The data were analysed using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method [29].

Western blot and immunofluorescence

Western blot analysis was performed as described previously [30]. Cells were treated with 200 μL of lysis buffer for 15 min and denatured in a 100°C water-bath for 5 min. Samples were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Finally, the bands were visualized using an enhanced chemiluminescence substrate kit.

Immunofluorescence assay was performed as described previously [31]. A549 cells were inoculated on coverslips and infected with NDV at 1 MOI for 18 h. A549 cells were fixed in 4% paraformaldehyde for 10 min, and incubated with the primary antibody for 1 h at 37°C. After washing with PBS, the cells were incubated with the secondary antibody for 45 min. The cell nuclei were stained with DAPI (Beyotime, Shanghai, China) for 10 min. Lastly, the coverslips were observed using an LSM 880 Zeiss confocal microscope.

siRNA transfection and construction of SLC1A3 knockdown cells

The siRNA targeting human *SLC1A3* (si-A3), and the nontargeting siRNA control (si-NC) were provided by GenePharma (Shanghai, China) and transfected as described previously [30]. The si-*SLC1A3* sense sequence was: 5′-GCUUGUGGGUGCUGUGAUATT-3′, and the si-*SLC1A3* antisense sequence was: 5′-UAUCACAGCAC CCACAAGCTT-3′. The si-NC sense sequence was: 5′-UUCUCCGAACGUGUCACGUTT-3′, and the si-NC antisense sequence was: 5′-ACGUGACACGUUCGGA GAATT. A short hairpin RNA targeting *SLC1A3* (sh*SLC1A3*, 5′-CGACAGTGAAACCAAGATGTA-3′) and a negative control (Control, 5′-TTCTCCGAACGT GTCACGT-3′) were synthesized by Sangon Biotech (Shanghai, China). To generate an *SLC1A3* stable knockdown cell line, A549 cells were infected with lentiviral shRNA for *SLC1A3* (sh*SLC1A3*) and selected using puromycin as described previously [32].

Amino acid quantitative analysis and targeted metabolomics analysis

NDV Herts/33 particles were obtained using a red blood cell absorption method as described previously [33] for amino acid profiling. The cells (A549/DF-1) were washed using precooled phosphate-buffered saline (PBS), and the cell pellets were collected by centrifugation (4°C, 12,000 g for 10 min). After freeze-drying, the cell samples were dissolved in sterile water and then stored at −80°C. The resulting supernatant was extracted with 900 μL of methanol/acetonitrile/water (2:2:1, v/v/v), followed by incubation at −20°C for 1 h and centrifugation (4°C, 12,000 g for 15 min) for liquid chromatography – mass spectrometry detection. Pingli He Laboratory of China Agricultural University (Beijing, China) assisted with the amino acid profile analysis. For metabolomic analysis, the cell samples were obtained as described previously [26]. Cells (A549/DF-1) were infected with NDV Herts/33 at 1 MOI. There were approximately 1×10^7 cells per sample. After freezing in liquid nitrogen, samples were stored at −80°C until metabolomic analysis.

Stable isotope tracing

A549 cells seeded in 100 mm dishes (5×10^6 cells/dish) were grown to 80% and were infected with NDV (Herts/33, MOI = 1). After washing with PBS, cells were incubated in DMEM with 10% dialysed FBS and [U-$^{13}C_5$]-glutamine. After 18 h of viral infection, cells were rinsed with 5% mannitol at room temperature. After adding ice-cold methanol (60%), the cells were frozen in liquid nitrogen for 30 s. The cell suspension was extracted using 900 μL of precooled methanol/acetonitrile/water (40:40:20, v/v/v), followed by incubation at −20°C for 1 h. Samples were then dried under vacuum and redissolved with 50 μL of acetonitrile/water (50:50, v/v) until LC-MS/MS analysis.

Nutrient deprivation studies

DMEM (Cat# D5030, Sigma-Aldrich, St. Louis, MO, USA) was used as the basic medium. The formula of 1 L complete medium was as follows: 8.3 g basic medium, 1.0 g glucose, 0.584 g L-glutamine, 3.7 g sodium

bicarbonate, 2 mM glutamine, 0.2 mM glutamate, 0.2 mM aspartate and 0.2 mM asparagine. An incomplete medium was prepared according to the need without the addition of the corresponding nutrients.

Statistical analysis

GraphPad Prism 7.0 software was used for the statistical analyses. Values of $p < 0.05$ were considered statistically significant. Data are presented as the mean ± standard error of the mean. Significance in all figures is denoted as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Results

NDV infection induced reprogramming of host amino acid anabolism

NDV infection induced changes in host metabolism [23,25], and altered multiple metabolite contents and metabolic pathways in host cells, and most of which are involved in the amino acid metabolism [26]. To confirm whether amino acid metabolism was affected in host cells after NDV infection, we quantitatively analysed amino acids in NDV-infected DF-1 and A549 cells. NDV infection altered the metabolism of 15 amino acids, among which 14 amino acids were upregulated and only L-Ala was downregulated, in DF-1 cells compared with mock-infection cells (Figure 1(a)). Under the same conditions, similar changes to the amino acid metabolism were observed in the NDV Herts/33-infected A549 cells (Figure 1(a)), demonstrating that mRNA levels of most amino acids, including aspartate and glutamate, were increased after NDV infection. Furthermore, the amino acid profile analysis indicated that proportions of glutamate and aspartate were higher than that of other amino acids in NDV-infected DF-1 and A549 cells (Figure 1(c) and (d)). Moreover, amino acid profile analysis of purified NDV Herts/33 particles indicated a similar composition and proportion of amino acids to NDV-infected DF-1/A549 cells, with the highest proportion of glutamic acid (Figure 1(e)). These results suggested that NDV infection induced reprogramming of host amino acid anabolism to benefit its own replication.

NDV infection induced significant upregulation of SLC1A3

AATs are membrane-bound transport proteins that participate in amino acid transport between cells and cellular organelles. AATs are divided into cationic, anionic, and neutral AATs according to different transport substrates and mechanisms [16,34]. Our previous results showed that NDV significantly upregulated of *SLC1A3* during infection by transcriptome (data not shown). To further investigate the potential role of AATs in amino acid homoeostasis of NDV-infected cells, we harvested cells at 6, 12, 18, and 24 h after NDV infection (Herts/33, LaSota and SH15 strains). The mRNA levels of various AATs (i.e. *SLC1A2*, *SLC1A3*, *SLC25A25*, *SLC7A14*, *SLC7A7*, *SLC6A19*, and *SLC38A2*) were analysed using qRT-PCR. *SLC1A3* was distinctly increased in Herts/33-infected A549 cells (Figure 2(a)). Furthermore, both LaSota and SH15 strains upregulated *SLC1A3* (Figure 2(b) and (c)). Solute carrier family 1 (SLC1) consists of five high-affinity glutamate transporters, namely *SLC1A1*, *SLC1A2*, *SLC1A3*, *SLC1A6*, and *SLC1A7*, as well as two neutral amino acid transporters, *SLC1A4* and *SLC1A5*. We further analysed whether these seven transporters were affected during NDV infection (Figure 2(d)). The results showed that the mRNA levels of *SLC1A3* were significantly enhanced after NDV infection. *SLC1A3* expression in A549 cells infected with NDV at 0.1, 0.5, 1 and 5 MOI was upregulated in a dose-dependent pattern (Figure 2(e)). The enhanced expression of *SLC1A3*, a glutamate and aspartate transporter, is essential in maintaining cell viability [4]. We considered the possibility that the transport of glutamate and aspartate by *SLC1A3* adapted to the stimulation induced by viral infection, which is beneficial to viral replication. With further verification, this result was confirmed in other cell lines, similar to H1299, HeLa, LoVo, HuH-7, HN13, CAL27, DF-1, and CEF samples harvested at different time points and analysed using qRT-PCR (Fig. S1). We further examined the protein levels of *SLC1A3* and NDV NP in A549 (Figure 2(g)) and DF-1 (Figure 2(h)) cells after Herts/33 infection. Clearly, the protein levels of *SLC1A3*, induced by NDV infection at various doses, displayed a similar pattern to the mRNA levels of *SLC1A3*. The expression levels of *SLC1A3* in mock-infected A549/DF-1 cells were considerably low or undetectable. These results demonstrated that NDV infection upregulated *SLC1A3* expression to enhance the transport of glutamate and aspartate, which may promote viral replication in host cells.

SLC1A3 was involved in NDV replication

To further study the role of *SLC1A3* during NDV replication in host cells, we effectively knocked down *SLC1A3* expression using siRNA. *SLC1A3* levels and NDV replication were detected by qRT-PCR and western blot, respectively. As expected, the mRNA and

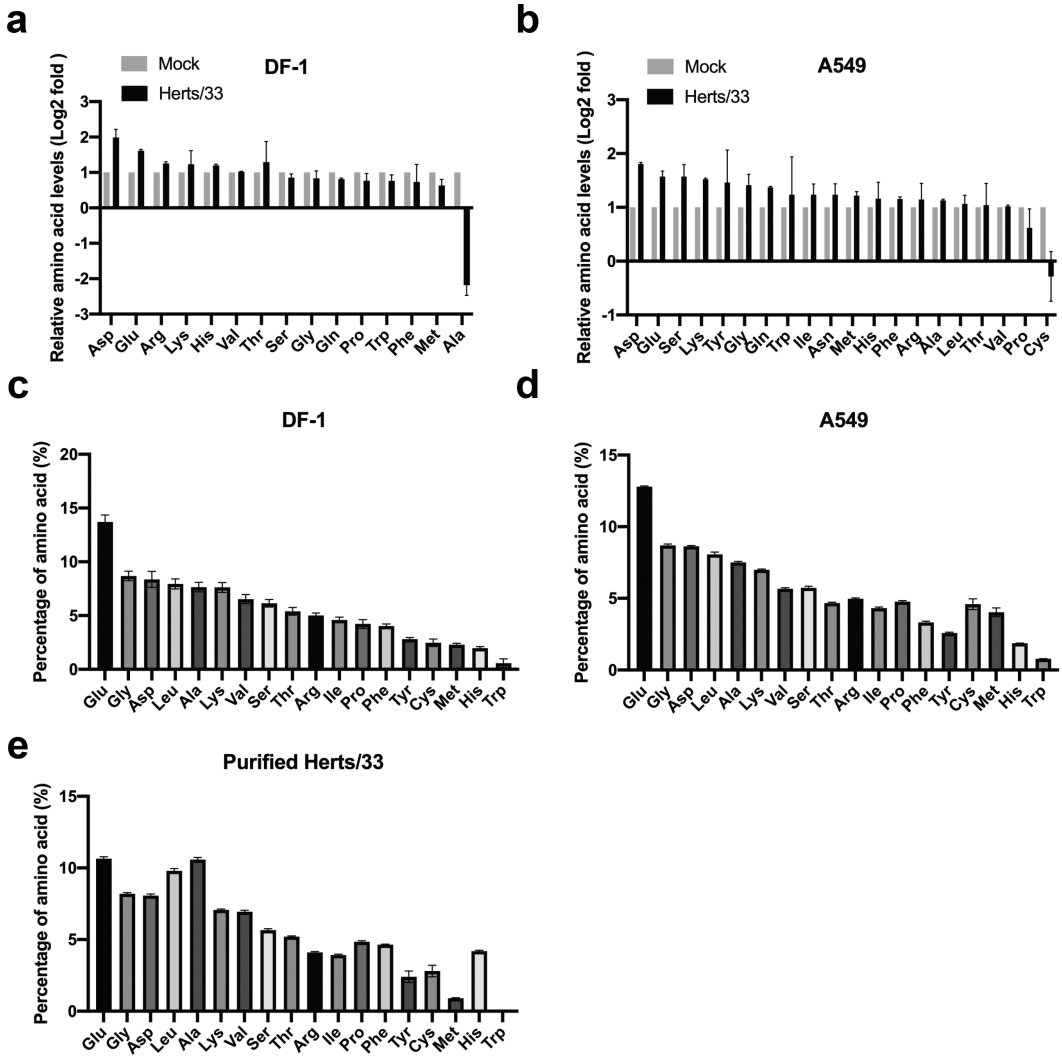

Figure 1. NDV infection induced amino acid metabolic reprogramming in host cells to match composition of NDV particles. (a-b) Changes to amino acid metabolism in NDV-infected DF-1 cells (a) and A549 cells (b). DF-1/A549 cells were infected with NDV Herts/33 at 1 MOI, then harvested at 12 h.p.i. For metabolomic analyses. (c-e) Amino acid profile analysis of DF-1 cells (c), A549 cells (d), and NDV Herts/33 particles (e). DF-1/A549 cells were washed with ice-cold PBS, and the supernatant was centrifugated. After freeze-drying, DF-1/A549 cell samples were dissolved in sterile water. NDV Herts/33 particles were prepared using a red blood cell absorption method as described previously [33]. These samples were then subjected to amino acid profile analysis.

protein expression levels of *SLC1A3* in si-A3 cells were markedly inhibited compared with the si-NC control cells (Figure 3(a) and (c)). By silencing *SLC1A3*, expression of NDV NP in the cells, and the titres of NDV in the cell supernatant were both decreased (Figure 3(b) and (c)). *SLC1A3* knockdown A549 cells (sh*SLC1A3*) were successfully constructed by using the shRNA and lentivirus packaging system. Western blot analysis and qRT-PCR confirmed the reduction in sh*SLC1A3* cells. We then evaluated whether *SLC1A3* knockdown affected NDV replication. In sh*SLC1A3* cells, the NP protein level and the viral titres in the cell supernatant were significantly decreased at 12 hours post infection (h.p.i.) (Figure 3(d) and (e)). In addition, *SLC1A3* could transport glutamate and aspartate, which may be closely related to cell metabolism. We also investigated the cell proliferation activity of control and sh*SLC1A3* cells. Interestingly, we observed that *SLC1A3* knockdown significantly decreased the proliferative activity of the host cell after NDV infection. However, no significant difference was observed between knockdown and uninfected cells (Figure 3(f) and (g)). Subsequently, to further validate the significance of *SLC1A3* in NDV replication, we distinguished the replication curve of Herts/33 in the control and sh*SLC1A3* cells (Figure 3(h)). The results showed that the knockdown of *SLC1A3* notably impaired viral infection, and the difference was significant at 12, 18, and 30 h.p.i., indicating the complex role of *SLC1A3* during NDV infection. Moreover, after treatment with TFB-TBOA,

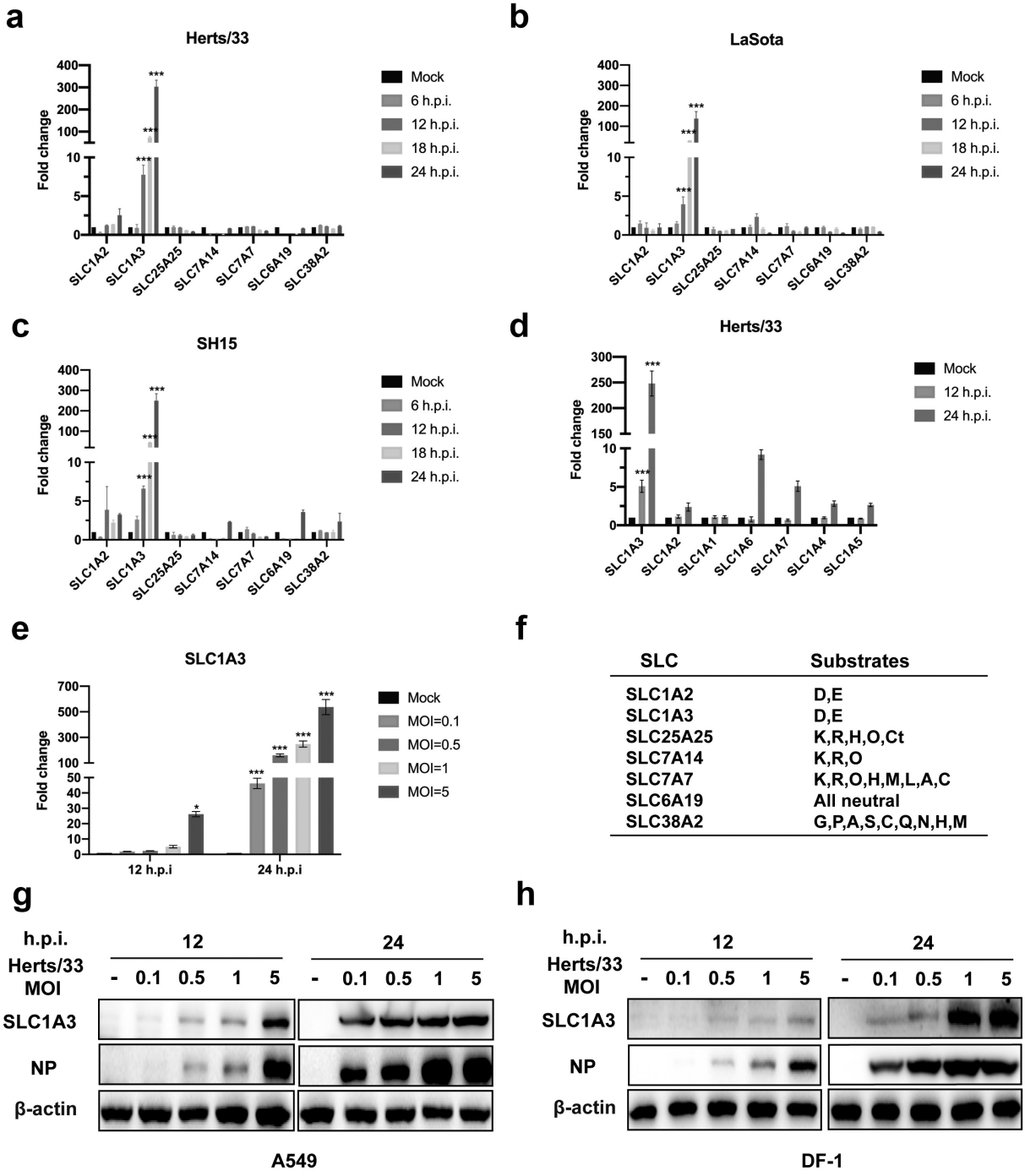

Figure 2. NDV infection induced significant *SLC1A3* upregulation. (a-c) A549 cells were harvested at 6, 12, 18 and 24 h.p.i. after NDV infection (Herts/33 or LaSota or SH15), and non-infected cells were used as the control. The mRNA levels of different amino acid transporters (*SLC1A2*, *SLC1A3*, *SLC25A25*, *SLC7A14*, *SLC7A7*, *SLC6A19*, *SLC38A2*) were determined using qRT-PCR. β-actin was used as an internal reference gene. (d) A549 cells were harvested at 12 and 24 h.p.i. after NDV infection (Herts/33, MOI=1). The mRNA levels of the SLC1 family were detected after NDV infection in A549 cells. (e) A549 cells were harvested after NDV infection (Herts/33, MOI=0.1, 0.5, 1, 5) and were used to detect the mRNA levels of *SLC1A3*. (f) Amino acid transporters and their substrates. (g-h) the expression levels of *SLC1A3* were detected in A549/DF-1 cells post-NDV infection.

a glutamate transporter inhibitor, the expression levels of *SLC1A3* and viral NP, were decreased (Figure 3(i)). Taken together, these results suggested that NDV-mediated *SLC1A3* upregulation benefited NDV replication in A549 cells.

SLC1A3 was located on cell membranes, mitochondria, and lysosomes

Previous studies have reported that AATs can be found in intracellular compartments (vesicular, lysosome,

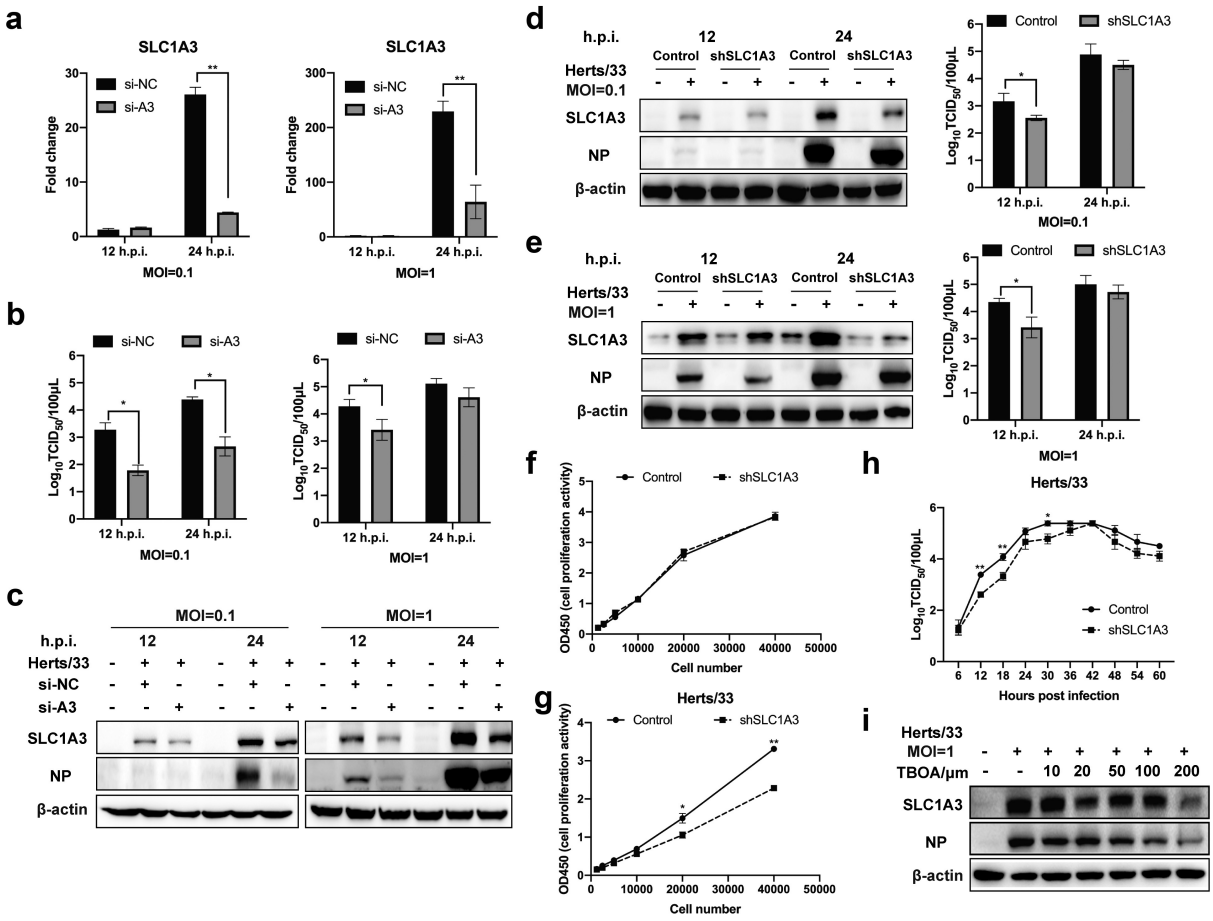

Figure 3. NDV replication level was decreased after knocking down *SLC1A3*. (a) A549 cells were transfected with siRNA (si-A3 and si-NC) followed by NDV (Herts/33, MOI= 0.1/1) infection, and cells were harvested to determine the effect of *SLC1A3* siRNA using qRT-PCR. (b, c) After treatment with siRNA, NDV-infected A549 cell samples and supernatant were harvested for $TCID_{50}$ measurement of NDV titers, and the cells were subjected to western blot analysis of the *SLC1A3* and NP expression. (d, e) A549 cells (Control and sh*SLC1A3*) were infected with NDV (Herts/33, MOI=0.1/1), and cell samples and supernatant were harvested for western blot and $TCID_{50}$ detection. (f, g) Cell proliferation activity of A549 cells (Control and sh*SLC1A3*) (F) and cells infected with NDV Herts/33 (MOI=0.1) (g) were measured using the Enhanced Cell Counting Kit-8. (H) the growth curve of NDV in A549 cells (Control and sh*SLC1A3*). Cells were infected with NDV Herts/33 (MOI=0.1). Supernatants were harvested at the indicated times and were subjected to the $TCID_{50}$ assay. (I) After treatment with TFB-TBOA, a glutamate transporter inhibitor, cells infected with NDV (Herts/33, MOI=1) were harvested at 12 h for western blot detection.

mitochondria, etc.) or the plasma membrane [16,21]. Therefore, they can promote the absorption, excretion, and exchange of amino acids. Considering the low or undetectable level of *SLC1A3* in uninfected A549 cells, localization of *SLC1A3* was identified following NDV infection. Colocalization of *SLC1A3* on the cell membrane was confirmed using immunofluorescence in infected cells (Figure 4(a)). Furthermore, *SLC1A3* was also specifically located on the mitochondria and lysosomes (Figure 4(b) and (c)). The fluorescence of *SLC1A3* in infected A549 cells was greater than that in uninfected cells. To further verify the location of *SLC1A3*, A549 cells underwent plasma membrane protein isolation, lysosome enrichment, and cell mitochondria isolation followed by NDV infection. The proteins of Na, K-ATPase, COX IV, and LAMP1 proteins were selected as markers for western blot analysis, respectively. As shown in Figure 4, the signals of these three marker proteins were strong, indicating that the extraction effect was good, and the target and cytoplasmic components were clearly distinguished. As expected, *SLC1A3* was detected in the components of membrane proteins, mitochondria, and lysosomes, but not in the cytoplasmic components (Figure 4(d) –(f)). In NDV-infected samples, the expression levels of *SLC1A3* were noticeably higher, and *SLC1A3* also gradually increased over time. Using immunofluorescence and western blot analysis, these results indicated that *SLC1A3* was located on the cell membrane, mitochondria, and lysosomes, which contribute to glutamate uptake from

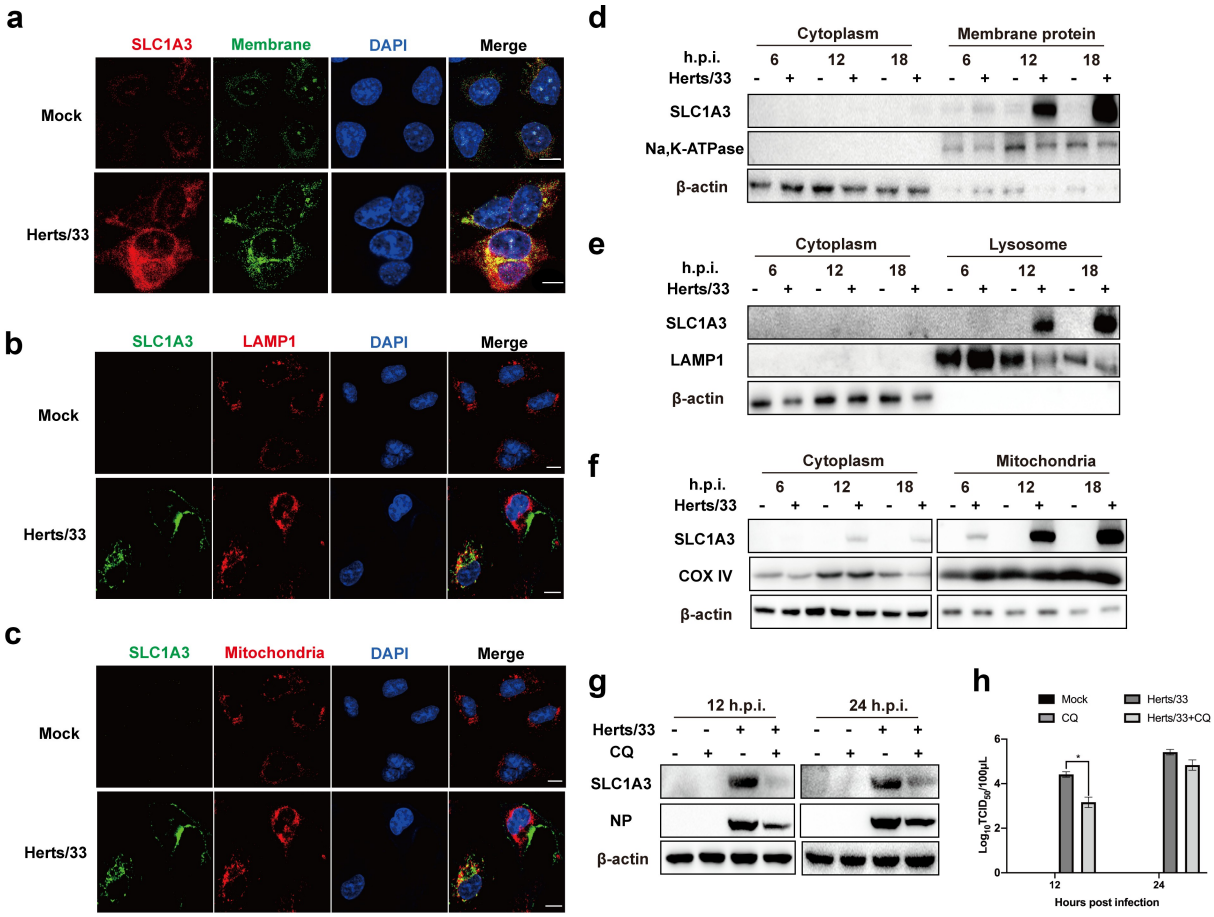

Figure 4. *SLC1A3* was located on the cell membranes, mitochondria, and lysosomes. (a-c) A549 cells were infected with NDV (Herts/33, MOI=1) for 18 h, followed by western blot analysis. SLC1A3 was detected using anti-SLC1A3 antibody. Cell plasma membranes were stained green. Lysosomes were detected using anti-LAMP1 antibody and stained red. Mitochondria were stained red. Cell nuclei were stained blue. Scale bars: 10 μm. (d-f) NDV-infected A549 cell samples were harvested at 6, 12, and 18 h.p.i., then underwent plasma membrane protein isolation, lysosome enrichment, and cell mitochondria isolation. Expressions of Na, K-ATPase (d), LAMP1(e), and COX IV (f) in isolated samples were analyzed using western blot and antibodies against Na, K-ATPase, LAMP1 and COX IV. Expression levels of SLC1A3 and β-actin were also detected. (g, h) Treatment with CQ, a lysosome inhibitor, inhibited SLC1A3 and NDV NP expression (g) and decreased virus titers (H), as detected using western blot and $TCID_{50}$ assay at 12 and 24 h.p.i.

media and cytoplasm. In addition, CQ treatment inhibited the expression levels of *SLC1A3* and NP in NDV-infected A549 cells (Figure 4(g)). Viral titres in the cell supernatant were significantly decreased at 12 h.p.i after CQ treatment (Figure 4(h)). These findings suggest that *SLC1A3* located on lysosomes was favourable for glutamate circulation and viral replication.

Glutamine is essential for NDV replication

Given the important role of AATs in amino acid homoeostasis, some evidence showed that glutamine transporter SLC1A5 was increased in latent KSHV-infected endothelial cells and included increased glutamine uptake for glutaminolysis [35]. We previously showed that *SLC1A3* was involved in NDV replication, and aimed to determine if *SLC1A3* affects the replication of NDV by regulating the transport of glutamate and aspartate. To determine if exogenous glutamate, aspartate, glutamine, or other nutritional components were required for A549 cells during NDV infection, we prepared alternative culture media for the experiment as follows: a complete culture medium (NC); media that only lacked glutamine, glutamate, asparagine, aspartate, or glucose; and media that only contained glutamine, glutamate, asparagine, or aspartate. We observed that cells in media containing no glucose could not grow or had died, thereby hindering NDV replication. However, cells grew well in other incomplete culture media (Figure 5(a)). Therefore, glucose was added to all culture media for all subsequent experiments. We then used western blotting to analyse the replication levels of NDV in cells grown in media

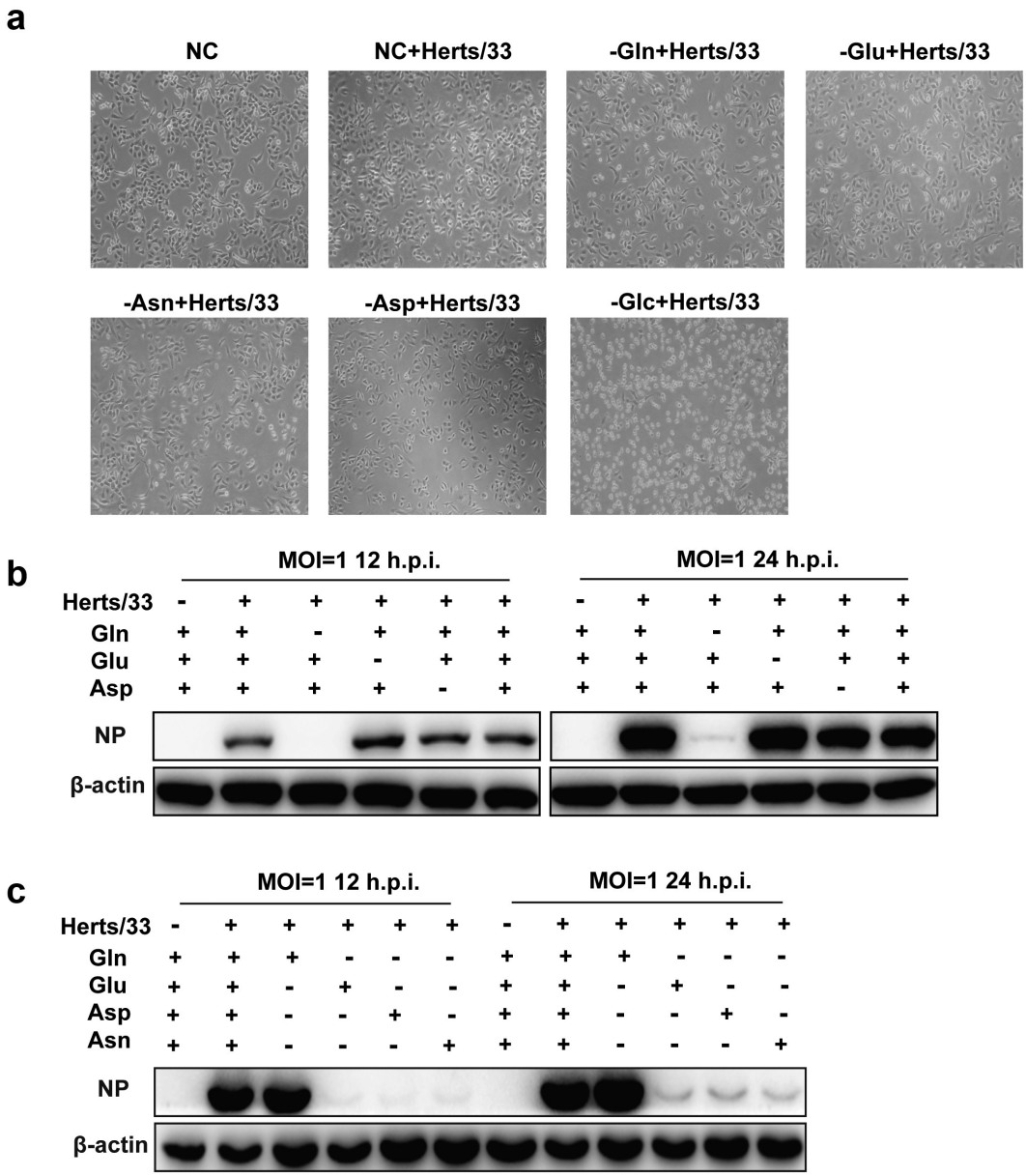

Figure 5. Detection of NDV replication levels after treatment with different culture media. (a) Microscopic observation of NDV-infected A549 cells in different culture media at 18 h.p.i. Cells were infected with NDV (Herts/33, MOI=1) and incubated at 37°C with 5% CO_2 for 1 h. Thereafter, the virus inoculum was removed by washing with PBS and were exposed to various culture media. Culture media were prepared as follows: complete culture medium (NC), and media that only lacked glutamine (−gln), glutamate (−glu), asparagine (−asn), aspartate (−asp), or glucose (−glc). (b) the replication level of NDV in different culture media. A549 cells were infected with NDV Herts/33 at 1 MOI in the absence of a certain nutrient component and cell samples were harvested at 12 and 24 h.p.i. For western blot analysis. (c) the NDV replication level in culture media containing only one nutrient (glutamine, glutamate, asparagine, or aspartate). A549 cells were infected with NDV Herts/33 at 1 MOI and cell samples were harvested at 12 and 24 h.p.i. For western blot analysis.

lacking a certain nutrient component. The results showed that NDV replication was inhibited only when the culture medium lacked glutamine, whereas no significant effect was observed when other nutrients were absent compared with that in the NC (Figure 5(b)). To verify this result, we used the incomplete culture medium containing only a single nutrient component for cells infected with NDV. We found that NDV successfully replicated only in the culture medium with glutamine (Figure 5(c)). These results indicated that glutamine contributed to NDV replication. Besides, the culture media with different concentrations of glutamine were prepared for the next experiment. Following NDV infection, the supernatant and cell

samples were harvested for $TCID_{50}$ and western blot analysis. The results showed that there was in a dose-dependent manner between NDV replication and glutamine concentration within the limits, as shown in Figures 6(a) and (b). Under glutamine starvation, the NDV replication level could not be rescued after supplementation with high concentrations of glutamate, aspartate, and asparagine (Figure 6(c) –(e)). These results showed that glutamine was essential and irreplaceable for successful NDV replication.

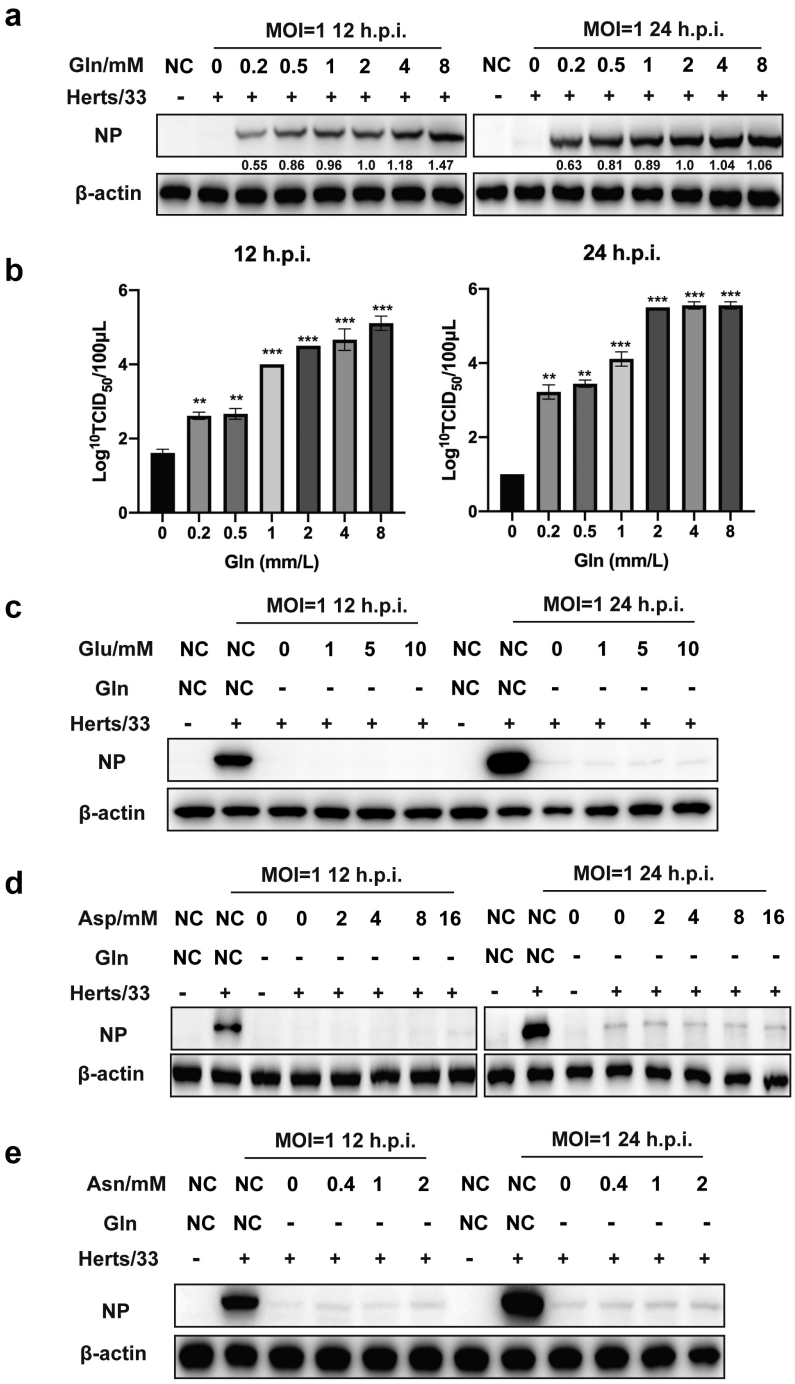

Figure 6. NDV replication relied on glutamine in a dose-dependent manner. (a, b) A549 cells were infected with NDV (Herts/33, MOI=1) for 1 h, and the supernatant was removed by washing with PBS. Thereafter, cells were exposed to culture media containing various glutamine concentrations at 1 h post infection, followed by western blot analysis and $TCID_{50}$ assay. (c-e) A549 cells were infected with NDV (Herts/33, MOI=1) for 1 h and then were exposed to complete culture medium (NC), glutamine-free medium (−gln), or glutamine-free medium supplemented with various concentrations of glutamate (Glu), aspartate (Asp), and asparagine (Asn). Cells were then harvested for western blot analysis.

SLC1A3 was involved in the regulation of glutamine catabolism during NDV infection

Our results showed that both *SLC1A3* and glutamine played important roles in NDV replication. To investigate a possible synergistic effect between *SLC1A3* and glutamine in NDV replication, we investigated NDV metabolism of glutamine by NDV and performed a metabolic flow analysis on U-$^{13}C_5$-labelled-glutamine cultured cells (Figure 7(a)). The labelled cells promoted the uptake of glutamine and catabolism to produce glutamate isotopologue [M + 5] under NDV infection (Figure 7(b)). Glutamine is known to serve as a crucial carbon source to replenish the TCA cycle and as a source of reduced nitrogen for some biological reactions [36–38]. Metabolic flux data showed that NDV promotes the decomposition of glutamine into the TCA cycle, and the intermediate metabolites, including the isotopologues of citrate[M+4], fumarate [M+4], malate [M+4], and aspartate [M+4], were markedly higher than those in mock cells (Figure 7(c)). Glutamine is converted to glutamate after catalysis by glutaminase (*GLS*), and the derived α-KG can then be converted and catalysed by *GLS*. Thereafter, the derived α-KG can be converted to isocitrate and provide acetyl-CoA for lipid synthesis by NADP+/NADPH-dependent isocitrate dehydrogenase 1 in the cytoplasm. In the reductive pathway, the intermediate metabolites including citrate [M + 5] were noticeably higher than those in the mock group, which indicated that NDV infection also activated the reductive pathway (Figure 7(d)). These results showed that NDV infection disrupted glutamine metabolism and increased glutamine catabolism by activating both pathways. To further investigate the effect of *SLC1A3* on glutamine metabolism regarding viral infection, we selected control and sh*SLC1A3* cells and evaluated the metabolic flow of U-^{13}C5-labelled glutamine. Compared with control cells, sh*SLC1A3* cells displayed lower intracellular glutamate levels, suggesting that the *SLC1A3* knockdown markedly reduced the ability of cells to generate glutamate (Figure 7(e)). A similar pattern was consistently

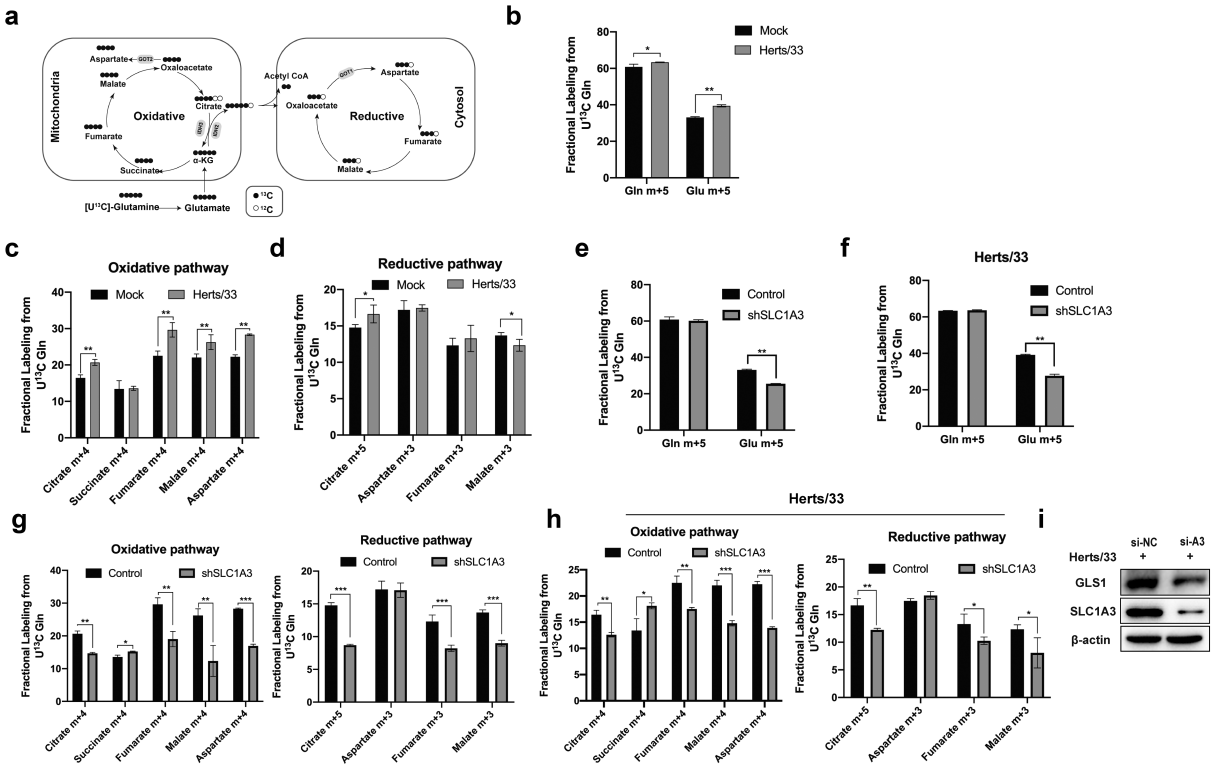

Figure 7. *SLC1A3* was involved in glutamine catabolism regulation during NDV infection. (a) Schematic diagram of the metabolic flow of labeled carbon atoms in the TCA cycle. Black dots denote 13C atoms originating from uniformly U-$^{13}C_5$-labeled glutamine. (b) A549 cells infected with NDV (Herts/33, MOI=1) received complete culture medium labeled with U-$^{13}C_5$-labeled glutamine. At 18 h.p.i., glutamine and glutamate were analyzed via metabolic analysis. (c, d) TCA cycle intermediates were analyzed using LC-MS. (E-H) A549 cells (Control and sh*SLC1A3*) were or were not infected with NDV (Herts/33, MOI=1) and received complete culture medium labeled with U-$^{13}C_5$-labeled glutamine. At 18 h.p.i., intracellular glutamine and glutamate were quantified by metabolic analysis (e, f). Stable isotopomer tracing analysis of U-$^{13}C_5$-labeled glutamine incorporation into reduction reactions and TCA cycle intermediates was conducted (G, H). (I) After treatment with siRNA, A549 cells infected with NDV (Herts/33, MOI=1) were harvested at 24 h.p.i. For western blot analysis. GLS1, SLC1A3, and β-actin were detected using corresponding antibodies.

observed after NDV infection (Figure 7(f)) in both control and knockdown cells. We also examined changes to TCA cycle intermediates and glutamine metabolism pathways. sh*SLC1A3* cells contained lower levels of citrate, fumarate, malate, and aspartate compared to control cells, suggesting that the oxidative reaction and reductive reactions were weakened (Figure 7(g)). The results in the NDV-infected groups were consistent with those in the mock groups (Figure 7(h)). By silencing *SLC1A3*, GLS was decreased (Figure 7(i)). *SLC1A3* was involved in the regulation of GLS, which may be used to promote glutaminolysis. Taken together, our data suggested that *SLC1A3* contributed to glutamate production and maintenance of the TCA cycle, in agreement with the previous work indicating that *SLC1A3* provides reducing equivalents to benefit the electron transport chain and TCA cycle [39].

The NF-κB pathway plays a positive role in regulating *SLC1A3* at the transcriptional level [40,41]. In addition, Tajan et al. demonstrated that *SLC1A3* expression was induced by p53 [38]. p53 increased the expression of *SLC1A3* to promote cancer cell survival under glutamine starvation. We validated the two pathways using inhibitor treatments (Figure 8). As shown in Figures 8(a) and (c), levels of *SLC1A3* were decreased slightly after treatment with inhibitors (PFTα and BAY11–7082) for 24 h. Additionally, the transcription levels of *SLC1A3* decreased significantly (Figure 8(b) and (d)). Therefore, activation of the p53 or NF-κB pathway was related to the increase of *SLC1A3* induced by NDV infection.

Discussion

Amino acid homoeostasis plays an important role during cell metabolism. Our results demonstrated that amino acid metabolism of DF-1 and A549 cells was reprogrammed after NDV infection. In this study, we determined that NDV-induced *SLC1A3* upregulation benefited viral replication. We also showed that glutamine was essential for viral replication in cells. Glutamine is a vital nutrient that enables cancer cells to survive and grow [42,43]. Glutamine provides carbon and nitrogen for biosynthesis, energy, and redox

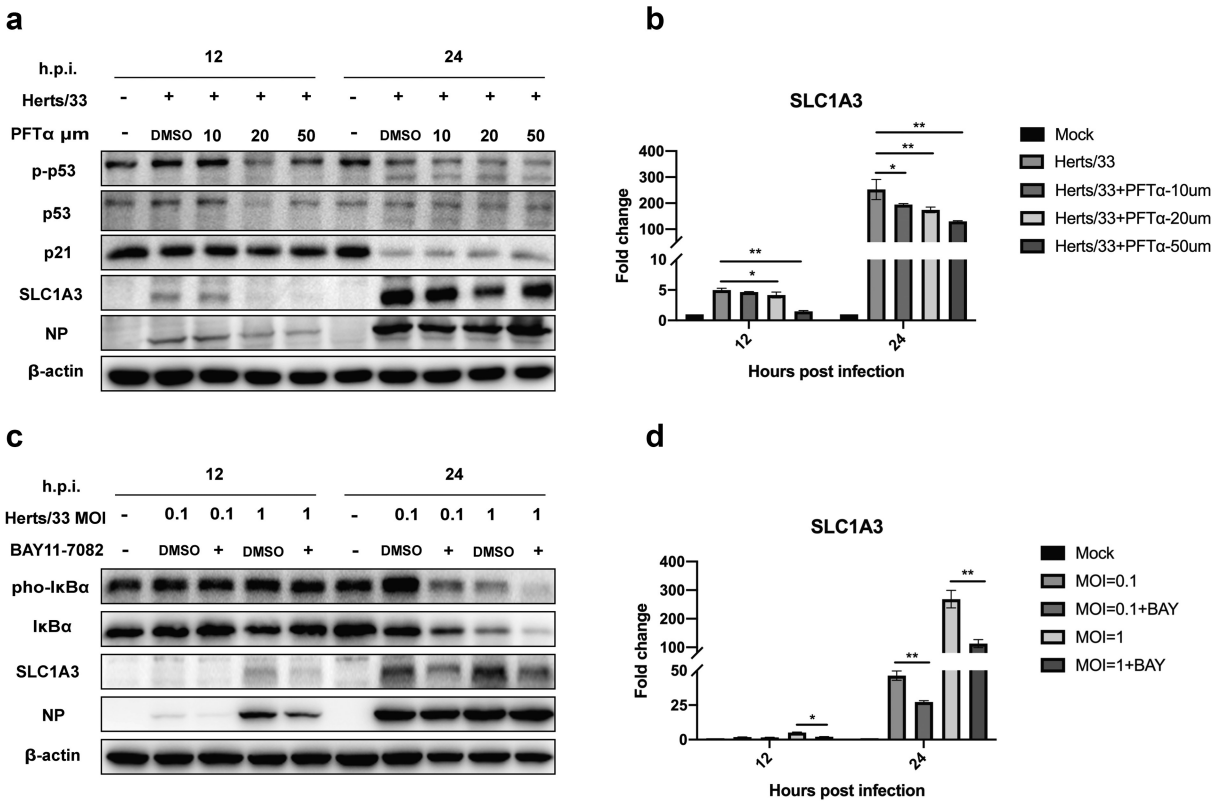

Figure 8. The expression levels of *SLC1A3* decreased after treatment with p53 and NF-κB pathway inhibitors. (a, b) in the presence of the p53 inhibitor PFTα (10, 20, or 50 μM), A549 cells infected with NDV (Herts/33, MOI=1) were harvested for western blot analysis and qRT-PCR. Antibodies against phopho-p53, total p53, p21, SLC1A3, NDV-NP, and β-actin were used. The mRNA levels of *SLC1A3* were analyzed using qRT-PCR. (c, d) Under BAY 11–7082 (10 μM) treatment, A549 cells infected with NDV (Herts/33, MOI=0.1, 1) were harvested for western blot and qRT-PCR analysis. Antibodies against phopho-IκBα, total IκBα, SLC1A3, NDV-NP, and β-actin were used. DMSO was used as a control. The mRNA levels of *SLC1A3* were analyzed using qRT-PCR.

homoeostasis [44]. Some evidence has recently proved that viruses modify specific metabolic pathways to meet the requirements for viral replication [8–10,45,46]. We found that NDV infection induced glutamine catabolism in A549 cells. Furthermore, increased glutamine catabolism and glutamate production were involved with *SLC1A3* upregulation. This work provided some new information about the demand for amino acids during NDV replication and the interaction between NDV and glutamine metabolism in the host cells.

Our data showed that NDV infection caused reprogramming of amino acid metabolism in DF-1/A549 cells (Figure 1(a) and (b)), indicating that NDV could regulate the overall amino acid metabolism of infected cells to facilitate its own replication. The amino acids required for viral survival and replication are provided by infected cells; thus, the infected cells require reprogramming of amino acid metabolism to meet the needs of viral protein synthesis during viral replication. This concept was confirmed by the results of amino acid metabolism changes in NDV-infected cells. The amino acid profile in NDV-infected DF-1 and A549 cells showed high similarity to that of purified NDV particles, with glutamate and aspartate accounting for a larger proportion (Figure 1(c)–(e)).This suggests that NDV prefers glutamate and aspartate, and NDV infection reprogrammed cellular amino acid metabolism of the cells to benefit its own replication.

Starvation is one of several stress types occurring in all organisms. When the free amino acid pool is insufficient to support viral replication, it causes amino acid starvation in infected cells. All organisms have evolved various mechanisms to respond and adapt to starvation [47]. Cells respond to amino acid starvation by inducing autophagy, which captures and degrades proteins and damaged organelles [48,49]. Our previous study showed that autophagy is triggered by NDV infection in U251 glioma cells and is beneficial for efficient viral replication [50]. Recently, increasing evidence has shown that tumour cells need to upregulate certain AATs to meet their high demand for amino acids [51,52]. Therefore, we investigated the relationship between NDV infection and AATs. Our results revealed that AAT levels were upregulated in A549 cells infected with different strains of NDV, and *SLC1A3* was significantly increased ($p < 0.001$; undefined Figure 2(a)–(c)). Further experiments on five members of the SLC1 family showed that only *SLC1A3* was significantly upregulated after NDV infection, which occurred in a dose-dependent manner (Figure 2(d) and (e)). The upregulation of *SLC1A3* caused by NDV infection was confirmed in other cell lines, such as H1299 and HeLa cells, etc. (Fig. S1). However, it is unknown whether other viral infections may also lead to *SLC1A3* upregulation. After downregulating *SLC1A3*, the NDV replication level decreased (Figure 3). Consistently, *SLC1A3* knockdown significantly decreased the proliferative activity of host cells after NDV infection, whereas there was no significant difference between them in uninfected cells (Figure 3(f) and (g)). The results suggested that *SLC1A3* knockdown may meet the basic growth needs of A549 cells. However, additional cellular metabolic resources are required after NDV infection and replication; thus, *SLC1A3* may play a complex and important role in the preferential use of cellular metabolic resource for viral replication, resulting in the limited proliferation level of infected cells. These results suggested that *SLC1A3* may contribute to the survival and proliferation of NDV-infected cells.

SLC1A3 was detected in the components of membrane proteins, mitochondria, and lysosomes, and it was upregulated in NDV-infected cells. (Figure 4). However, we noticed that the LAMP1 levels were low at 12 and 18 h.p.i. in NDV-infected cells. We suggested that disrupted lysosome integrity was one of the reasons. When reactive oxygen species (ROS) levels are high, they can induce lysosomal membrane permeabilization, resulting in the translocation of cathepsins to the cytosol, inducing lysosomal-dependent cell death [53]. A previous study showed that NDV infection induces elevated mitochondrial ROS and mitochondrial damage [25], which may compromise lysosomal integrity.

Glutamine is a critical nutrient for cell metabolism, which viruses increase in hosts to support replication [8,45,54]. Viruses may upregulate glutamine metabolism for efficient replication. Our recent results confirmed that glutamine is essential for NDV replication and thus could not be replaced (Figure 5 and 6). Glutamine may provide carbon or nitrogen sources for the synthesis of viral proteins and nucleotides, which is necessary for viral replication. NDV infection may alter the metabolic pathway of glutamine, as well as increase of glutamine catabolism. Importantly, NDV replication supported by glutamine catabolism was regulated by *SLC1A3*, which contributed to the production of glutamate and maintenance of the TCA cycle (Figure 7). Previous study showed that NDV infection induced energy metabolism dysfunction and electron transport chain (ETC) dysfunction [25]. Therefore, increasing glutamine catabolism may promote ATP production and supply energy for viral replication. The mRNA levels of *GLS*, glutamine synthetase (*GLUL*), glutamate dehydrogenase (*GLUD2*), *GOT1*, *GOT2* and *ALT*, which are related to glutamine metabolism, were also detected (Fig. S2). The results showed that the mRNA level of *GLUL*

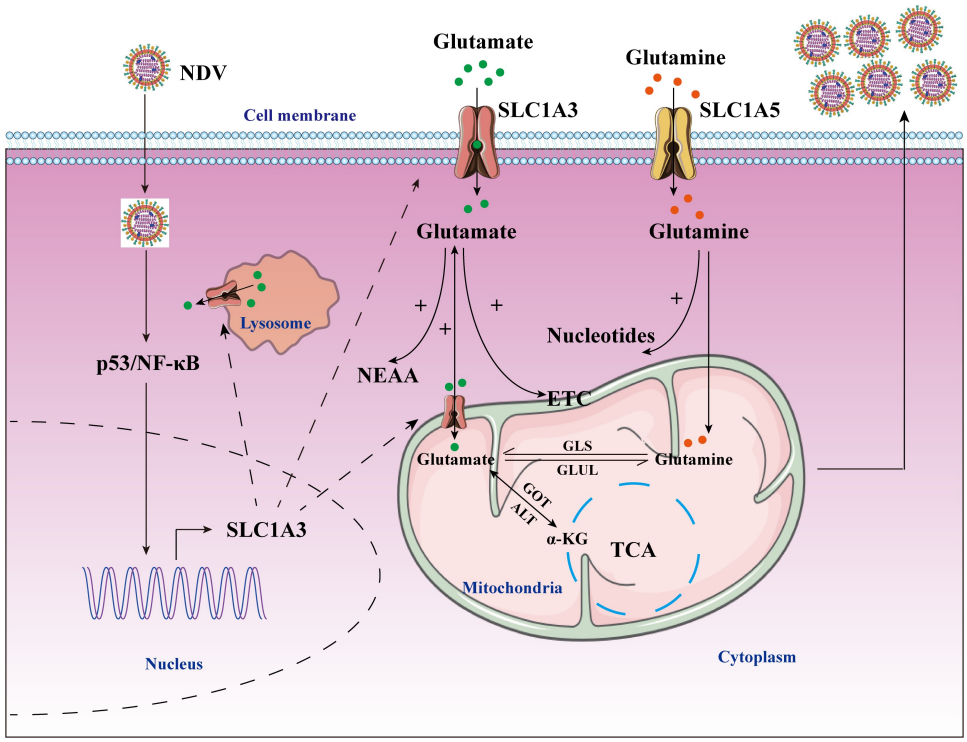

Figure 9. Schematic diagram of the molecular mechanism of NDV-mediated glutamine metabolism. NDV infection induced metabolic reprogramming in host cells to benefit viral protein synthesis and replication. NDV infection facilitated glutamate transport by increasing *SLC1A3* expression on cell membranes, lysosomes, and mitochondria in infected cells by activating p53 and NF-κB pathways. The transportation of glutamate contributes to maintaining functionality of the TCA cycle and the electron transport chain, as well as providing energy for viral replication. Glutamate can also produce some nonessential amino acids. In addition, glutamine can also be used for viral nucleotide synthesis. All these factors provide favorable conditions for viral replication during NDV infection.

initially decreased, then increased, whereas *GLS* increased after NDV infection, suggesting that glutamine anabolism decreased, and catabolism increased. Furthermore, the mRNA levels of *GLUD2*, *GOT1*, *GOT2* and *ALT* were also upregulated. These results confirmed that NDV infection significantly altered the glutamine metabolism in host cells.

Our results also indicated that the activation of p53 and NF-κB pathway activation was involved in increased *SLC1A3* induced by NDV (Figure 8). Kan et al. reported that NDV infection activated p53 in NDV-infected U251 cells and regulated glutathione metabolism [55]. Glutathione is composed of glutamate, cysteine, and glycine, and plays critical roles in protecting cells from oxidative damage and maintaining redox homoeostasis. These results collectively suggest that p53 activation plays an important role in NDV-infected cells and the relationship between different metabolic pathways is complex and interrelated.

In summary, our results revealed that NDV infection distinctly altered the amino acid metabolism of infected cells, and NDV showed a preference for glutamate and aspartate. For the first time, our findings revealed that NDV infection enhanced the expression and function of *SLC1A3*, a glutamate/aspartate transporter, to facilitate viral replication. *SLC1A3* upregulation increased glutamate uptake and transportation from media and cytoplasm to satisfy the needs of NDV replication, which may promote glutamine catabolism and glutamate production (Figure 9). These results will improve our understanding of the demand for amino acids, how to regulate the host cell amino acid metabolism during NDV replication, and the effect of amino acid metabolism on NDV replication.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was funded by grants 32030108 (to C. D.) and 91957120 (S.-H. L.) from the National Natural Science Foundation of China

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request (shoveldeen@shvri.ac.cn).

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