LETTERS

Drosophila RNAi screen identifies host genes important for influenza virus replication

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All viruses rely on host cell proteins and their associated mechanisms to complete the viral life cycle. Identifying the host molecules that participate in each step of virus replication could provide valuable new targets for antiviral therapy, but this goal may take several decades to achieve with conventional forward genetic screening methods and mammalian cell cultures. Here we describe a novel genome-wide RNA interference (RNAi) screen in *Drosophila*[1] that can be used to identify host genes important for influenza virus replication. After modifying influenza virus to allow infection of *Drosophila* cells and detection of influenza virus gene expression, we tested an RNAi library against 13,071 genes (90% of the *Drosophila* genome), identifying over 100 for which suppression in *Drosophila* cells significantly inhibited or stimulated reporter gene (*Renilla* luciferase) expression from an influenza-virus-derived vector. The relevance of these findings to influenza virus infection of mammalian cells is illustrated for a subset of the *Drosophila* genes identified; that is, for three implicated *Drosophila* genes, the corresponding human homologues *ATP6V0D1*, *COX6A1* and *NXF1* are shown to have key functions in the replication of H5N1 and H1N1 influenza A viruses, but not vesicular stomatitis virus or vaccinia virus, in human HEK 293 cells. Thus, we have demonstrated the feasibility of using genome-wide RNAi screens in *Drosophila* to identify previously unrecognized host proteins that are required for influenza virus replication. This could accelerate the development of new classes of antiviral drugs for chemoprophylaxis and treatment, which are urgently needed given the obstacles to rapid development of an effective vaccine against pandemic influenza and the probable emergence of strains resistant to available drugs.

Influenza, a highly contagious disease of birds and mammals, is caused by negative-strand RNA viruses of the family Orthomyxoviridae. Influenza outbreaks kill millions of people worldwide during pandemic years and hundreds of thousands during other years. Since their first lethal infection of humans in 1997, H5N1 influenza A viruses have spread throughout Asia and to Europe and Africa, posing a major risk for a new influenza pandemic[2]. To provide rational bases for improved treatment and control of influenza virus infection, we sought to advance understanding of viral infection mechanisms by elucidating previously unknown virus–host cell interactions. Many steps in the viral life cycle, including intracellular trafficking, gene expression, replication and virion assembly, depend on interactions with specific host cell gene products. Although most such host molecules remain elusive, emerging results indicate that their identification and characterization can provide new insights into the mechanisms by which viruses complete their life cycle, and hence illuminate potentially valuable targets for prophylactic and therapeutic intervention[3–5].

Systematic, genome-wide RNAi analysis offers an exciting tool to identify host genes that function in viral replication. Such analysis is facilitated by well-developed model systems such as *Drosophila*, the genome of which contains only ~14,000 genes, nearly all of which can be specifically targeted for high efficiency messenger RNA depletion by double-stranded RNA (dsRNA) libraries[1]. Because of its powerful genetics and conservation with vertebrates, *Drosophila* has been used to make numerous critical contributions to mammalian cell biology[6–9]. Thus, in principle, *Drosophila* RNAi studies could accelerate identification of host interactions essential for influenza virus replication.

Because *Drosophila* D-Mel2 cells do not express the human influenza virus receptor α2,6-linked sialic acid (Supplementary Fig. 1), we predicted that wild-type human influenza virus would not be able to infect them. Indeed, we did not detect viral protein expression by immunofluorescence assays in *Drosophila* D-Mel2 cells inoculated with influenza virus A/WSN/33 (WSN; H1N1) (data not shown). To bypass this block to wild-type influenza virus entry, we generated a genetically modified virus, Flu-VSV-G-GFP (FVG-G), in Madin-Darby canine kidney (MDCK) cells by replacing the receptor-binding haemagglutinin (*HA*) and neuraminidase (*NA*) genes with genes encoding vesicular stomatitis virus glycoprotein G (VSV-G) and enhanced green fluorescence protein (eGFP), respectively[10,11] (Fig. 1a). Because the envelopes of the resulting virions bear VSV-G, which mediates entry into mammalian, *Drosophila* and other cells[12], FVG-G virions should readily infect *Drosophila* cells. Twenty-four hours after infection, GFP fluorescence was detected in FVG-G-infected *Drosophila* D-Mel2 cells (Supplementary Fig. 2). We also confirmed by real-time polymerase chain reaction (PCR) that influenza virus RNA replication occurred in *Drosophila* cells (Supplementary Fig. 3). However, *Drosophila* cells infected with FVG-G did not release detectable virions into the medium, as assayed by infectivity tests on MDCK cells and by electron microscopy (data not shown). This was due, at least in part, to failure of *Drosophila* cells to express some viral proteins required for virion assembly and infectivity (Supplementary Fig. 4). Thus, *Drosophila* cells can support influenza virus replication from post-entry to at least the protein expression phase of the viral life cycle. This span encompasses multiple other steps in the life cycle, including cytoplasmic release of

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genomic RNA-containing viral ribonucleoprotein complexes (vRNPs), vRNP import into the nucleus, mRNA synthesis from the negative-strand viral RNA genome, mRNA export to the cytoplasm and translation.

For high-throughput, functional genomics analysis of influenza virus replication in *Drosophila* cells, we engineered Flu-VSV-G-R.Luc (FVG-R), in which *VSV-G* and *Renilla* luciferase genes replaced the viral *HA* and *NA* open reading frames (Fig. 1b). FVG-R virions were then used with an RNAi library (Ambion) against 13,071 *Drosophila* genes (~90% of all genes) to identify host genes affecting influenza-virus-directed *Renilla* luciferase expression (Fig. 1c). Two independent tests of the entire library were performed (Supplementary Table 1). For 176 genes for which dsRNAs inhibited FVG-R-directed luciferase expression in both replicates, repeated secondary tests using alternate dsRNAs to control for possible off-target effects confirmed the effects of 110 genes (Supplementary Tables 2 and 3). This confirmation rate is comparable to that in a *Drosophila* screen with a natural *Drosophila*-infecting virus[5]. Cell viability testing identified six genes with potentially significant cytotoxic effects; these were excluded from further consideration (Supplementary Information and Supplementary Table 3). Secondary tests of candidate genes for which dsRNAs increased FVG-R-directed luciferase expression produced a much lower confirmation rate, suggesting a higher rate of off-target or other false-positive effects in this class (Supplementary Information and Supplementary Table 4).

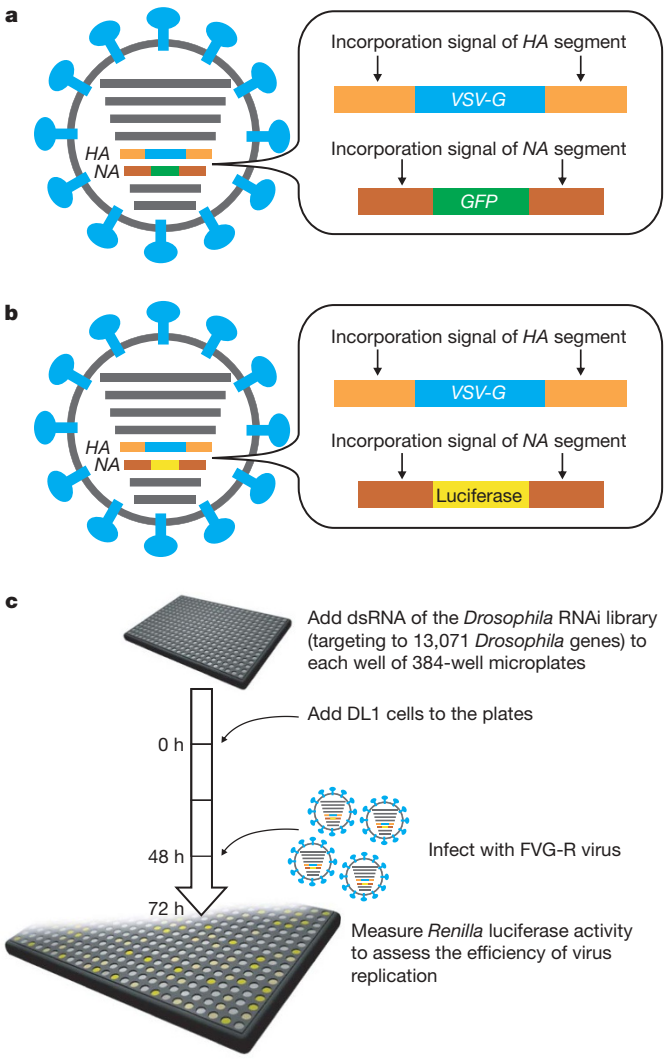

Figure 1 | Overview of genome-wide RNAi screen to identify host factors involved in influenza virus replication in *Drosophila* cells. **a**, **b**, Schematic diagrams showing recombinant influenza viruses. Shown are FVG-G, in which genes encoding the HA and NA proteins were replaced with the *VSV-G* and *eGFP* genes, respectively (**a**), and FVG-R, in which the genes encoding the HA and NA were replaced with the *VSV-G* and *Renilla* luciferase genes, respectively (**b**). **c**, Schematic diagram of the systematic analysis of host genes affecting influenza virus replication and gene expression in *Drosophila* cells. Experimental details are given in Methods.

Among the over 100 candidate genes found to be important for influenza virus replication in *Drosophila* cells, we selected the human homologues of several encoding components in host pathways/machineries that are known to be involved in the life cycle of influenza virus, for example, *ATP6V0D1* (endocytosis pathway), *COX6A1* (mitochondrial function) and *NXF1* (mRNA nuclear export machinery), for further analysis in mammalian cells to assess the relevance of our *Drosophila* results[13–17]. *ATP6V0D1* encodes subunit D of vacuolar (H^+)-ATPase (V-ATPase), a proton pump that functions in the endocytosis pathway (that is, the acidification and fusion of intracellular compartments[18]). *COX6A1* encodes a subunit of cytochrome *c* oxidase (COX), an enzyme of the mitochondrial electron transport chain that catalyses electron transfer from cytochrome *c* to oxygen[19]. *NXF1* encodes a nuclear export factor critical for exporting most cellular mRNAs containing exon–exon junctions[20,21].

As a first test for the possible contribution of these gene products to influenza virus replication in mammalian cells, we treated human HEK 293 cells twice at 24-h intervals with short interfering RNAs (siRNAs; siGENOME, Dharmacon) against the human homologue of each selected *Drosophilia* gene. Twenty-four hours after the second siRNA treatment, the cells were infected with FVG-R virus and, two days later, *Renilla* luciferase activity was measured to assess viral replication and gene expression. siRNA against *ATP6V0D1* or *COX6A1* markedly decreased *Renilla* luciferase activity (Fig. 2a), but not cell viability (Supplementary Fig. 5a), suggesting that these genes have important roles in influenza virus replication in mammalian cells, as in *Drosophila* cells. Inhibition was not caused by off-target effects because, for each gene, each of four distinct siRNAs inhibited FVG-R-directed expression of *Renilla* luciferase (Supplementary Table 6). Because *COX6A1* encodes a subunit of mitochondrial electron transport chain complex IV, COX, we used specific inhibitors to test whether in HEK 293 cells influenza virus also required other complexes in this chain (Fig. 2c). Inhibitors of complexes III, IV and V selectively inhibited FVG-R-directed *Renilla* luciferase expression by 50- to 100-fold, whereas complex I and II inhibitors had little or no effect. Thus, in mammalian cells, influenza virus depends on multiple late stages but not early stages in the mitochondrial electron transport chain.

Treatment for four days with siRNA against *NXF1* decreased mammalian cell viability (data not shown), as predicted by the critical role of *NXF1* in general host cell metabolism. Accordingly, the total incubation time with siRNA against *NXF1* was shortened to 36 h by transfecting cells with the siRNA twice at a 12-h interval, infecting with FVG-R virus 12 h later, and assaying for *Renilla* luciferase at 12-h post-infection. Under these conditions, cell viability was not detectably affected (Supplementary Fig. 5b) whereas *Renilla* luciferase activity was reduced by nearly fivefold (Fig. 2b). Whereas recent results indicated that influenza virus protein NS1 binds to *NXF1* to inhibit host mRNA export[17], these results imply that influenza virus RNAs and/or proteins are transported by an *NXF1*-dependent pathway (see also Supplementary Information).

To test the effects of these genes on authentic influenza viruses, we infected siRNA-treated HEK 293 cells with WSN virus or H5N1 influenza A/Indonesia/7/05 (Indonesia 7; isolated from a patient) or with VSV or vaccinia virus as controls. Progeny viruses were collected from the medium at 24 h (Indonesia 7, VSV or vaccinia virus) or 48 h (WSN) post-infection and were titrated. Depleting *ATP6V0D1* and *COX6A1* did not affect VSV or vaccinia virus replication, but decreased the WSN and Indonesia 7 virus yields by ~10-fold or more (Fig. 3a). Thus, *ATP6V0D1* and *COX6A1* are required for replication

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of influenza viruses but not VSV and vaccinia virus. Depleting the *NXF1* nuclear export factor, again using an accelerated 36-h time line, reduced Indonesia 7 virus titres by 20-fold (Fig. 3b), indicating that *NXF1* has a critical role in H5N1 influenza virus replication. Unfortunately, because WSN grows in HEK 293 cells more slowly than does Indonesia 7 virus, the WSN virus yield at 12-h post-infection was insufficient to test for an effect of *NXF1* depletion (data not shown). In comparison to Indonesia 7 virus, *NXF1* depletion had no effect on VSV or vaccinia virus yields (Fig. 3b). Moreover, siRNA against *NXF1* inhibited FVG-G, but not adenovirus, which, like influenza virus, depends on nuclear steps for genome transcription and replication (Supplementary Fig. 6). Thus, multiple genes identified in *Drosophila* cells correspond to important, selective host factors for influenza virus replication in mammalian cells.

The method that we have established, using systematic analysis of a *Drosophila* RNAi library with confirmation in mammalian cells, can be used to identify host gene products that affect influenza virus replication. This utility was demonstrated in experiments with authentic influenza viruses, including an H5N1 virus isolated from a human patient. One of the candidate genes tested in mammalian cells, *COX6A1*, which encodes a subunit of COX, acts as a critical enzyme in cytochrome-*c*-dependent electron transport in mitochondria. Influenza virus PB2 polymerase has a mitochondrial targeting signal[14], and PB1-F2, the second protein encoded by the influenza *PB1* gene, was reported to localize to inner and outer mitochondrial membranes and to delay influenza virus clearance by host antiviral responses[15]. Thus, *COX6A1* may be involved in PB2- and/or PB1-F2-mediated functions in mitochondria. Additionally, influenza vRNP

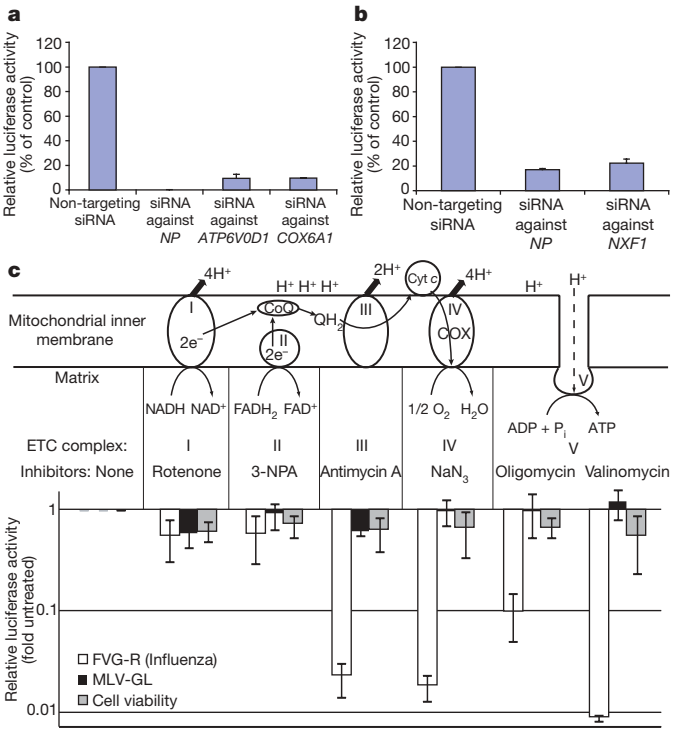

Figure 2 | Effect of selected siRNAs and inhibitors on *Renilla* luciferase expression in FVG-R-infected human cells. a–c, *Renilla* luciferase activity was measured in FVG-R-infected HEK 293 cells treated with siRNAs against *ATP6V0D1* and *COX6A1* (**a**), *NXF1* (**b**) or the indicated mitochondrial electron transport chain inhibitors (**c**). Inhibitors of complexes III, IV and V inhibited FVG-R-directed *Renilla* luciferase expression significantly, whereas complex I and II inhibitors had little or no effect. In contrast, the inhibitors had no significant effects on cell viability and *Gaussia* luciferase expression of a murine leukaemia virus derivative (MLV-GL) that, like FVG-R, depended on VSV G-mediated entry. All experiments were conducted three times in duplicate, with the results reported as mean ± s.d. 3-NPA, 3-nitroproprionic acid.

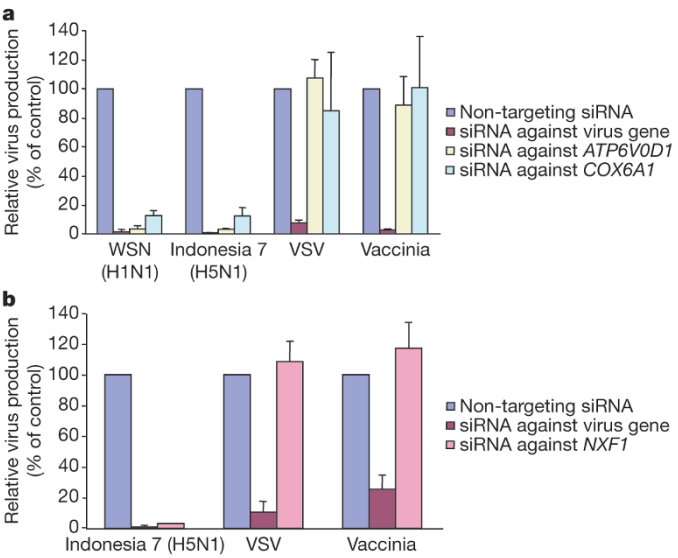

Figure 3 | Effect of siRNAs against selected genes on the replication of influenza viruses, VSV or vaccinia virus in human HEK 293 cells. a, **b**, The titres of influenza viruses (WSN and Indonesia 7), VSV and vaccinia virus in HEK 293 cells treated with siRNA against *ATP6V0D1* and *COX6A1* (**a**) or against *NXF1* (**b**) are shown. Experimental details are given in Methods and Supplementary Methods. All experiments were conducted three times, with the results reported as mean ± s.d.

export from the nucleus requires caspase 3 activation[22], which can be induced by cytochrome *c* release from mitochondria. Moreover, human BCL2 inhibits both cytochrome *c* release and influenza vRNP export[23], and COX function and changes in mitochondrial membrane potential have been linked to caspase activation[24]. These pathways may underlie our further findings (Fig. 2c) that influenza virus replication and expression in mammalian cells are strongly and selectively modulated by compounds that inhibit COX, cytochrome *c*-linked electron transport chain complex III, and normal ion transport across mitochondrial membranes (oligomycin and valinomycin). Potential roles of the other two host genes confirmed here in mammalian cells, *ATP6V0D1* and *NXF1*, in influenza virus replication are discussed further in the Supplementary Information.

The above results with multiple, diverse genes, including *ATP6V0D1*, *NXF1* and *COX6A1* as well as mitochondrial electron transport complexes III and V, demonstrate the feasibility and value of using *Drosophila* RNAi screening to identify previously unknown host factors with important and potentially unsuspected roles in influenza virus replication. Simultaneously, the genome-wide results from our *Drosophila* RNAi screen provide more than 100 additional candidate genes (many with unknown functions) to be tested in mammalian cells. We suggest that the same strategy could be applied to identify previously unknown host factors involved in the replication of other viruses, whenever at least a portion of their replication cycle is supported by *Drosophila* cells.

METHODS SUMMARY

Cells and viruses. MDCK cells, HEK 293 cells, baby hamster kidney (BHK) cells, D-Mel2 cells and DL1 cells were maintained as described in Methods. WSN, FVG-G, FVG-R and Indonesia 7 viruses were generated as described previously[10] and propagated in MDCK cells. VSV and vaccinia virus were grown in BHK and CV-1 cells, respectively. *Gaussia*-luciferase-expressing murine leukaemia virus (MLV-GL) and the GFP-expressing adenovirus were provided by J. Bruce and R. Kalejta[25], respectively.

***Drosophila* RNAi library analysis.** Double-stranded RNA of the *Drosophila* RNAi library (targeting 13,071 *Drosophila* genes) and DL1 cells were added to each well of 384-well microplates. After two days of incubation, cells were infected with the FVG-R virus. At one day post-infection, *Renilla* luciferase activity was measured as described below. Two independent analyses of the entire library were performed.

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siRNA treatment of mammalian cells. HEK 293 cells were transfected with siRNA by TransIT-TKO (Mirus). Cells were incubated for 12 h in experiments with *NXF1* or for 24 h with other genes, were transfected again under the same conditions, and inoculated with virus after 12 h for *NXF1* or after 24 h for other genes. After a further 12 h (for *NXF1*) or 48 h (other genes), the cells were harvested for the indicated analyses.

***Renilla* luciferase and cell viability assays.** *Renilla* luciferase activity and cell viability were measured with established *Renilla* luciferase and CellTiter-Glo assay systems (Promega) according to the manufacturer's instructions; signals were read with a GLOMAX 96 microplate luminometer.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions L.H., A.S., P.A. and Y.K. designed research; L.H., A.S., T.W. and C.A.N. performed research; L.H., A.S., T.W., E.S., M.A.N., P.A. and Y.K. analysed data; and L.H., A.S., T.W., P.A. and Y.K. wrote the manuscript.

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METHODS

Cells and viruses. MDCK cells, HEK 293 cells and BHK cells were maintained in minimum essential medium containing 5% fetal calf serum and antibiotics at 37 °C in 5% CO_2. D-Mel2 cells were maintained in *Drosophila*-SFM (GIBCO/Invitrogen) at 28 °C. DL1 cells were maintained in Schneider's *Drosophila* (SD) medium containing 10% FBS at 28 °C.

WSN, FVG-G, FVG-R and Indonesia 7 viruses were generated by a plasmid-based reverse genetics system[10] and were grown and titrated in MDCK cells. MLV-GL was produced by replacing the CD4 open reading frame in pCMMP-CD4-eGFP[26] with that of *Gaussia* luciferase (J. Bruce, unpublished observations). All experiments with Indonesia 7 virus were conducted in a biosafety level 3 containment laboratory approved for such use by the Centers for Disease Control and Prevention and the US Department of Agriculture.

***Drosophila* RNAi library analysis.** The *Drosophila* RNAi library (Ambion) contained 13,071 individual dsRNAs, each designed to specifically target a single *Drosophila* gene. Five microlitres of dsRNA (40 ng μl^{-1}) were added to each well of 384-well plates, after which 2×10^4 DL1 cells in 10 μl of SD medium were added to each well and incubated with the dsRNA at 28 °C for 60 min. Twenty microlitres of SD medium containing 20% FBS was then added to each well after incubation. Cells were treated with dsRNA for two days at 28 °C and were then inoculated with an amount of FVG-R virus corresponding to a multiplicity of infection of 10 for MDCK cells, and were then transferred to 33 °C. At one day post-infection, *Renilla* luciferase activity was measured as described below. Two independent analyses of the entire library were performed.

siRNA treatment of mammalian cells. The non-targeted siRNA used was siCONTROL 1 (Dharmacon). Duplex siRNAs against candidate genes were obtained from siGENOME (Dharmacon, see Supplementary Table 7 for sequences); the effects of siRNA were evaluated by reverse transcription followed by PCR (RT–PCR; Supplementary Fig. 7). The sequences of siRNA against the *NP* gene of influenza virus (GGAUCUUAUUUCCUUCGGAGUU)[27] and the *E3L* gene of vaccinia virus (AAUAUCGUCGGAGCUGUACAC)[28] were reported previously. The sequence of siRNA against the gene encoding the L protein of *VSV* (CGAGUUAUCCAGCAAUCAUUU) was designed using BLOCK-iT RNAi designer (Invitrogen). A HEK 293 cell suspension was seeded into the wells of a 24-well plate (2.0×10^4 cells per well), incubated for 1 h and transfected with siRNA (to a final concentration 10 nM; Dharmacon) using TransIT-TKO (Mirus), according to the manufacturer's instructions.

Inhibitor treatment of mammalian cells. Mitochondrial electron transport chain inhibitors were used at the following final concentrations: rotenone (0.5 μM), 3-nitroproprionic acid (1 mM), antimycin A (25 μM), NaN_3 (50 mM), oligomycin (25 μM) and valinomycin (0.9 μM). HEK 293 cells were treated with the inhibitors for 5 h before being infected with FVG-R or MLV-GL, incubated 18 h, and assayed for *Renilla* or *Guassia* luciferase, respectively. Cell viability was assayed in parallel by a firefly-luciferase-mediated ATP assay (CellTiter-Glo, Promega).

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