Supplementary Information

A cell-based screening system for influenza A viral RNA transcription/replication inhibitors

Makoto Ozawa[1,2†], Masayuki Shimojima[3‡], Hideo Goto[3], Shinji Watanabe[2,4], Yasuko Hatta[2§], Maki Kiso[3], Yousuke Furuta[5], Taisuke Horimoto[3¶], Noel R. Peters[6], F. Michael Hoffmann[6], and Yoshihiro Kawaoka[1,2,3,4*]

[1]Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan
[2]Influenza Research Institute, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI 53711, USA
[3]Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan
[4]ERATO Infection-Induced Host Responses Project, Saitama 332-0012, Japan
[5]Toyama Chemical Co., Ltd., Toyama 930-8508, Japan
[6]UW Small Molecule Screening Facility and Medicinal Chemistry Core Facility, University of Wisconsin-Madison, Madison, WI 53792, USA

[†]Present address: Laboratory of Animal Hygiene, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan
[‡]Present address: Laboratory of Veterinary Microbiology, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan.
[§]Present address: FluGen, Inc. 545 Science Drive, Madison, WI 53711, USA
[¶]Present address: Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.

[*]Correspondence should be addressed to:
Yoshihiro Kawaoka, DVM, Ph.D.
Influenza Research Institute, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, 575 Science Drive, Madison, WI 53711, USA
Phone: +1-608-265-4925
Fax: +1-608-265-5622
E-mail: kawaokay@svm.vetmed.wisc.edu

Legends to Supplementary Figures

Supplementary Figure S1. Virus-like vRNA expression by transduction with an envelope-uncoated retroviral vector. 293 cells were transduced with the VSVG-coated (left panels) and -uncoated (right panels) retroviral vectors for the expression of the GFP-encoding virus-like RNA. Simultaneously, the cells were transfected with plasmids for the expression of the polymerase subunits and NP. Forty-eight hours later, GFP expression (green) and nuclei counter-stained by Hoechst dye (blue) were examined by fluorescence microscopy. Scale: 100 μm.

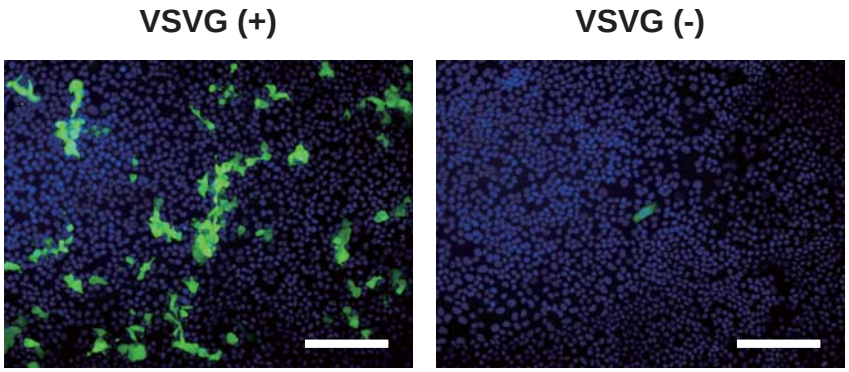
Supplementary Figure S2. Characterization of 293vRNP-Puro cells. (A) Detection of mRNAs for viral proteins in 293vRNP-Puro cells. Total RNAs extracted from 293 (lanes 1, 3, 6, 8, 11, 13, 16, 18, 21, and 23) and 293vRNP-Puro cells (lanes 2, 4, 7, 9, 12, 14, 17, 19, 22, and 24) were reverse transcribed using an oligo(dT)16 primer and amplified by PCR with specific primers for the 3' and 5' ends of the open reading frames of PB2 (lanes 1-4), PB1 (lanes 6-9), PA (lanes 11-14), and NP (lanes 16-19) and hGAPDH-specific primers (lane 21-24). The open reading frames of PB2, PB1, PA, and NP were 2280-, 2274-, 2151-, and 1497-nt in length, respectively. As positive controls, plasmids encoding the open reading frames of each protein were amplified by PCR with each specific primer set, respectively (lanes 5, 10, 15, and 20). The DNA sizes are shown on the left of panel. M, marker for DNA size; P, positive control; RT (-), omission of reverse transcription; RT (+), addition of reverse transcription. (B) Detection of virus-like RNA in 293vRNP-Puro cells. Total RNAs extracted from 293 (lanes 1 and 2) and 293vRNP-

Puro cells (lanes 3 and 4) were reverse transcribed using a random hexamer primer and amplified by PCR with primers specific for the 3' and 5' noncoding ends of HA vRNA (upper panel) and hGAPDH-specific primers (lower panel). The HA vRNA encoding the puromycin-resistance gene was 677-nt in length. As a positive control, pPolI/HA(0)Puro(0) was amplified by PCR (lane 5). The DNA sizes are shown on the left of each panel. M, marker for DNA size; P, positive control; RT (-), omission of reverse transcription; RT (+), addition of reverse transcription. (C) Functional expression of viral proteins required for vRNA transcription in individual 293vRNP-Puro cells. 293vRNP-Puro cells were mock-transduced (left panels) or transduced with a virus-like RNA-expressing adenovirus vector AdV/PolI-GFP [14] at a multiplicity of infection of 10 (right panels). Twenty-four hours later, GFP expression was examined by fluorescence microscopy (lower panels): upper panels show corresponding bright-field images.

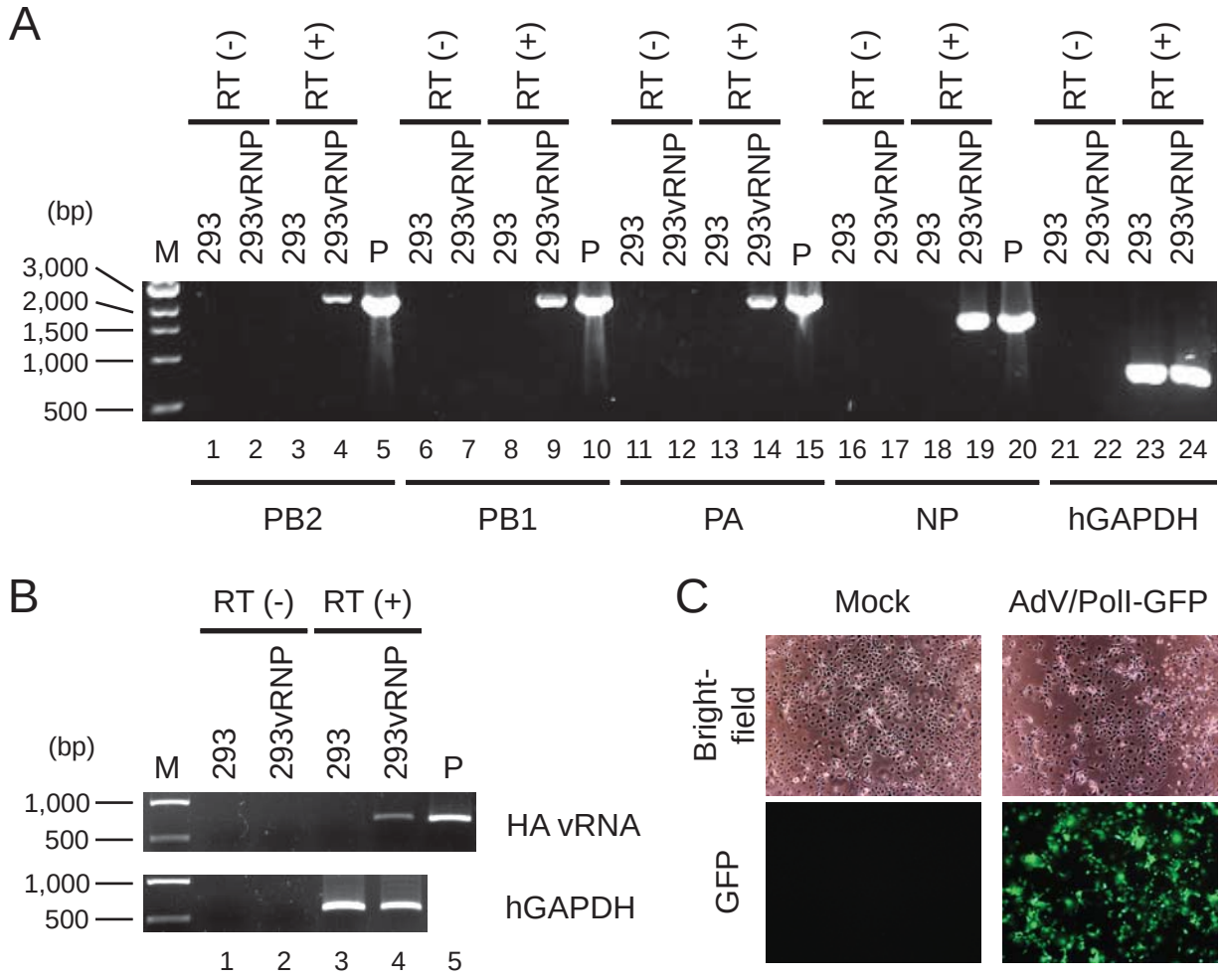
Supplementary Figure S3. Effect of favipiravir on influenza vRNA transcription/replication. (A) Effect of favipiravir on influenza viral RNA transcription/replication in a minigenome assay. 293 cells were transfected with plasmids for the expression of a virus-like RNA encoding the firefly luciferase gene together with expression plasmids for PB2, PB1, PA, NP, and *Renilla* luciferase, which served as an internal control. Simultaneously, the indicated concentration of favipiravir dissolved in DMSO was added to the cells. Twenty-four hours later, luciferase activity was measured by use of the Dual-Luciferase® Reporter Assay System (Promega). Error bars indicate standard deviations of three independent experiments. (B) Effect of favipiravir on 293vRNP-Puro cell viability. 293vRNP-Puro cells were cultured with the indicated

concentration of favipiravir dissolved in DMSO in the presence (black circle) and absence (white circle) of puromycin for 96 h. Cell viability was measured by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Error bars indicate standard deviations of three independent experiments.

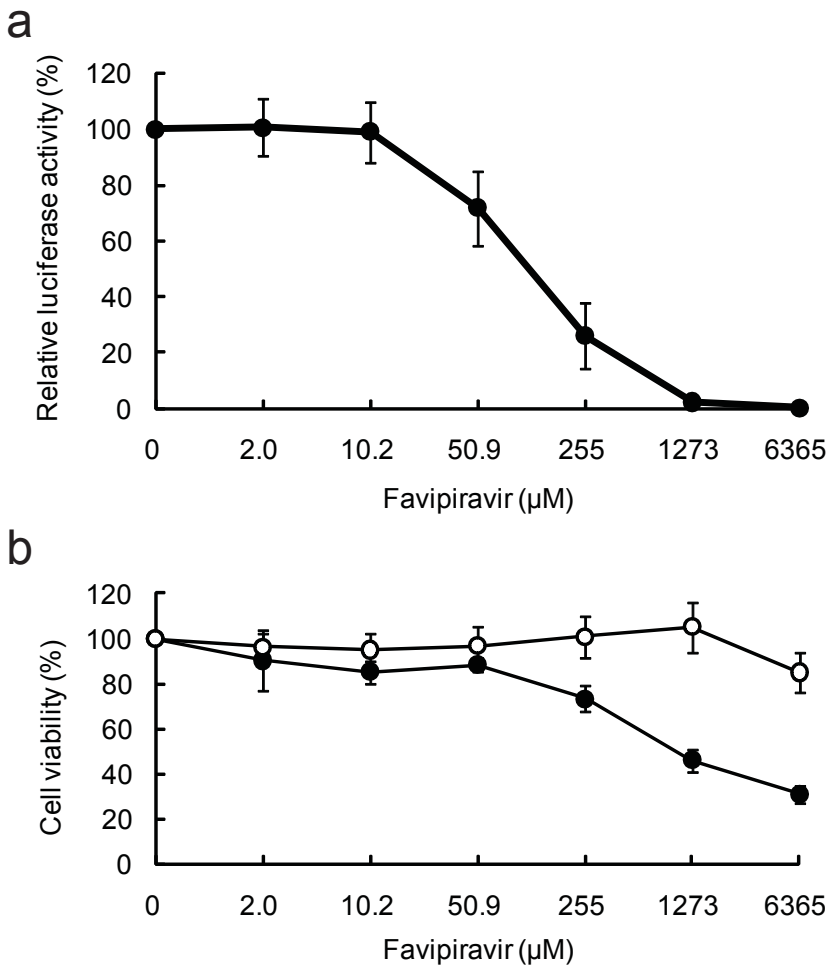
Supplementary Figure S4. Identification of influenza vRNA transcription/replication inhibitor candidates through 293vRNP-Puro cell-based screening. (A) Scheme of 293vRNP-Puro cell-based screening system for vRNA transcription/replication inhibitors. (B) Effect of identified compounds on 293vRNP-Puro cell viability. 293vRNP-Puro cells were cultured with the indicated compound (10 μM each) or DMSO (Ctrl) in the presence (black bar, from three independent experiments) and absence (gray bar, from two independent experiments) of puromycin for 96 h. Cell viability was measured by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Error bars indicate standard deviations.



Supplementary Fig. S1. Ozawa et al.



Supplementary Fig. S2. Ozawa et al.



Supplementary Fig. S3. Ozawa et al.

a

Procedures:

<Day 0> 293vRNP-Puro cells were seeded on 384-well plates (1,000 cells/well) in normal or puromycin-containing medium.

<Day 1> Compounds (10 μM) were added to the cells.

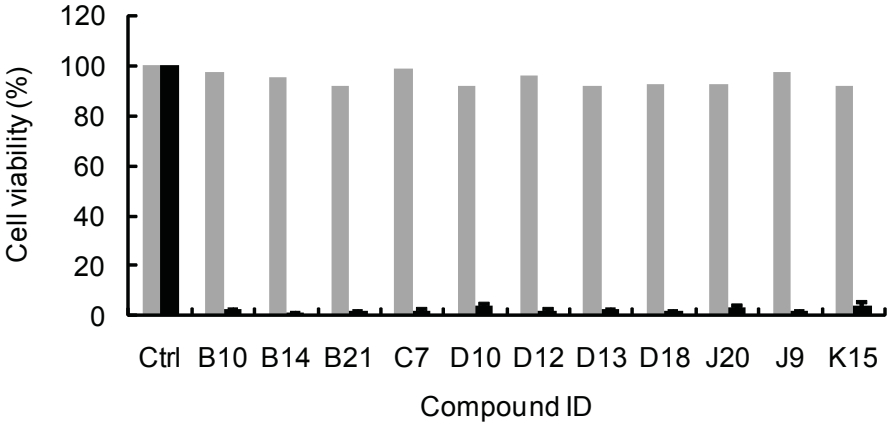
<Day 5> Cell viability was measured by CellTiter-Glo® Luminescent Cell Viability Assay.

Criteria:

<1st round> >50% and <20% cell viability in normal and puromycin-containing medium, respectively, were considered as positive.

<2nd round> >90% and <5% cell viability in normal and puromycin-containing medium, respectively, were considered as positive.

b



Supplementary Fig. S4. Ozawa et al.