G-Protein-Coupled Receptor and Ion Channel Genes Used by Influenza Virus for Replication

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ABSTRACT Influenza virus causes epidemics and sporadic pandemics resulting in morbidity, mortality, and economic losses. Influenza viruses require host genes to replicate. RNA interference (RNAi) screens can identify host genes coopted by influenza virus for replication. Targeting these proinfluenza genes can provide therapeutic strategies to reduce virus replication. Nineteen proinfluenza G-protein-coupled receptor (GPCR) and 13 proinfluenza ion channel genes were identified in human lung (A549) cells by use of small interfering RNAs (siRNAs). These proinfluenza genes were authenticated by testing in influenza virus A/WSN/33-, A/CA/04/09-, and B/Yamagata/16/1988-infected A549 cells, resulting in the validation of 16 proinfluenza GPCR and 5 proinfluenza ion channel genes. These findings showed that several GPCR and ion channel genes are needed for the production of infectious influenza virus. These data provide potential targets for the development of host-directed therapeutic strategies to impede the influenza virus productive cycle so as to limit infection.

IMPORTANCE Influenza epidemics result in morbidity and mortality each year. Vaccines are the most effective preventive measure but require annual reformulation, since a mismatch of vaccine strains can result in vaccine failure. Antiviral measures are desirable particularly when vaccines fail. In this study, we used RNAi screening to identify several GPCR and ion channel genes needed for influenza virus replication. Understanding the host genes usurped by influenza virus during viral replication can help identify host genes that can be targeted for drug repurposing or for the development of antiviral drugs. The targeting of host genes is refractory to drug resistance generated by viral mutations, as well as providing a platform for the development of broad-spectrum antiviral drugs.

KEYWORDS influenza, siRNA, virology, virus-host interactions
Viruses exploit host genes and their pathways to support entry, replication, and egress. Some of the most studied pathways exploited by influenza virus include the nuclear factor kappa B (NF-κB), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), protein kinase C/protein kinase R (PKC/PKR), toll-like receptor (TLR), and retinoic acid-inducible gene 1 (RIG-I) pathways (14–17). Anti-influenza drugs typically target viral proteins, but often these drugs can have reduced efficacy due to drug resistance acquired through antigenic shift and drift (18). For example, amantadine is no longer recommended for the treatment of influenza virus infection due to increased drug resistance, and the reduced efficacy observed for oseltamivir is linked to neuraminidase (NA) mutations (19), creating inconsistencies among therapies (20). In contrast, therapeutics targeting host genes necessary for virus replication could offer an approach refractory to drug resistance while providing broader-spectrum drug efficacy.

RNA interference (RNAi) is a conserved mechanism of posttranscriptional gene-specific regulation (21). RNAi can probe the virus-host interface to identify host genes necessary for virus replication (22–26). Genome-wide RNAi screening has uncovered key virus-host interactions, has helped identify drug targets for influenza viruses (27), and has been used to validate host genes important for virus replication (28–32). Small interfering RNAs (siRNAs) mediate posttranscriptional gene silencing via sequence-specific nucleolytic cleavage or translational inhibition upon interaction with their target mRNAs (29). siRNAs are rationally designed to be specific for one mRNA target (33).

G-protein-coupled receptors (GPCRs) are a family of seven-transmembrane cell surface receptor proteins that facilitate intracellular communication via activation of signal transduction pathways (34). Viruses use GPCRs to facilitate attachment, entry, replication, and egress. For example, HIV tropism is associated with the CXCR4/CCR5 coreceptor and GPCR15 (35–37). In addition, blocking of select GPCRs with drug antagonists obstructs Marburg virus and Ebola virus cell entry and replication (38). The overarching influence of GPCRs on the cell makes drugs that target GPCRs amenable to disease intervention. Similarly, ion channels (ICs) are assemblages of integral protein domains that allow transmembrane passage between the extracellular and intracellular components of the cell (39). ICs enable the influx/efflux of Na⁺, K⁺, Cl⁻, or Ca²⁺ ions, which regulate effector pathways. For example, inhibition of K⁺ channels at the early stages of Bunyamwera virus infection hinders virus replication postentry (40). In addition, Cl⁻ channels are important for herpes simplex virus 1 entry and virus-host fusion (41). Further, the Na⁺ channel opener SDZ-201106 can inhibit IAV replication via PKC pathway inhibition (42), and modulation of Cl⁻ or Na⁺ secretion/absorption in the respiratory tract contributes to the regulation of respiratory disease (43).

In this study, we used RNAi as a tool to survey the virus-host interface connected to GPCR and IC genes needed for influenza virus replication. Using siRNA pools to mediate RNAi, we examined GPCR and IC genes for their effects on influenza virus replication in A549 cells based on the following: (i) Z-score, (ii) Ingenuity Pathway Analysis software (2014) (IPA; Qiagen, Inc., Valencia, CA; Qiagen Knowledge Base; Qiagen.com; i.e., searching public databases and published texts), (iii) the availability of small-molecule inhibitors and antagonists, and (iv) targeting by microRNAs (miRs). The gene hits from the RNAi screen of A/WSN/33-infected A549 cells were validated following deconvolution using A/WSN/33. Confirmed hits were reexamined using A/CA/04/09- or B/Yamagata/16/1988-infected A549 cells. The findings from this study provide a better understanding of the virus-host interface and host genes needed for influenza virus replication and provide drug target information for the development of new drugs, or for the repurposing of existing FDA-approved drugs, to combat influenza.

RESULTS

An RNAi screen identifies GPCR genes. GPCR genes permit intracellular communication via signal transduction following activation (34) and are involved in virus replication (38, 44–46). We performed a genome-wide RNAi screen of GPCR genes required
for influenza virus replication in A549 cells. Briefly, A549 cells were reverse transfected with siRNA SMARTpools, and 48 h posttransfection, the cells were infected (multiplicity of infection [MOI], 0.001) with A/WSN/33. The levels of virus replication were determined, and a Z-score was applied that showed the number of standard deviations by which the gene knockdown event differed from the mean. A negative Z-score (≤−1.0) indicated decreased virus replication, while a positive Z-score (≥1.0) indicated increased viral replication. Our study focused on gene knockdown events that decreased influenza virus titers, since the goal was to determine strategies for host cell-targeted antiviral therapeutics.

We identified 185 GPCR genes whose knockdown resulted in Z-scores of ≤−1.0. Further evaluation of these genes with IPA and Gene Ontology (GO) analyses, as well as the implementation of selection criteria, identified 19 critical GPCR genes: ADGRF1, ADORA1, ADRB2, AGTR1, CSAR2, CCKBR, FFAR1, HCAR3, HCRTR2, HRH2, HTR1B, LGR4, LPAR3, MTNR1B, NMUR2, OXGR1, OXTR, P2RY12, and PRLHR (Table 1). GPCRs are grouped into six classes (A to F) based on sequence homology and functional similarity (34). Sixteen of 19 GPCR genes were identified as class A; ADGRF1 belongs to class B, C5AR2 is a nonclassical GPCR, and LGR4 is an orphan receptor. To limit off-target results, the 19 GPCR genes identified by SMARTpool screens were reexamined by deconvolution of the siRNA pools (24, 47). Here, A549 cells were transfected with individual ON TARGETplus (OTP)-modified siRNAs from the SMARTpool. OTP-siRNAs have improved gene targeting due to a dual-strand modification that provides increased interaction with the RNA-induced silencing complex (RISC), decreasing off-target effects by antisense strands (48).

OTP-siRNA-transfected A549 cells were infected (MOI, 0.01) with A/WSN/33, and after 48 h, the levels of infectious virus production were determined by a plaque assay. GPCR genes that were knocked down by OTP-siRNAs and had decreases in virus plaque titers for two or more individual OTP-siRNAs were further evaluated. For example, silencing of the MTNR1B gene by transfecting cells with siRNA 4 from the SMARTpool markedly reduced influenza virus titers, but transfection of siRNA 1, 2, or 3 had only a modest effect (Fig. 1A); thus, the MTNR1B gene was not considered further. Additionally, silencing of the NMUR2 or PRLHR gene had no substantial effect on viral titers (Fig. 1A). In contrast, OTP-siRNA knockdown of the ADGRF1, ADORA1, ADRB2, AGTR1, CSAR2, CCKBR, FFAR1, HCAR3, HCRTR2, HRH2, HTR1B, LGR4, LPAR3, OXGR1, OXTR, or P2RY12 gene resulted in decreased virus titers (≤−1.0) for two or

### Table 1: GPCR genes from a genome-wide RNAi screen

| Gene       | Function                                                                 | Z-score* |
|------------|--------------------------------------------------------------------------|----------|
| ADGRF1     | G-protein-coupled receptor 110                                            | −2.0     |
| ADORA1     | Adenosine A1 receptor                                                    | −2.1     |
| ADRB2      | Adrenoceptor beta 2, surface                                             | −1.8     |
| AGTR1      | Angiotensin II receptor, type 1                                          | −1.6     |
| CSAR2      | Complement component 5a receptor 2                                      | −1.9     |
| CCKBR      | Cholecystokinin B receptor                                               | −2.8     |
| FFAR1      | Free fatty acid receptor 1                                               | −2.1     |
| HCAR3      | Hydroxycarboxylic acid receptor 3                                       | −1.8     |
| HCRTR2     | Hypocretin (orexin) receptor 2                                           | −1.9     |
| HRH2       | Histamine receptor H2                                                   | −2.3     |
| HTR1B      | 5-Hydroxytryptamine (serotonin) receptor 1B, G protein coupled           | −1.5     |
| LGR4       | Leucine-rich repeat containing G-protein-coupled receptor 4              | −1.6     |
| LPAR3      | Lyosphosphatidic acid receptor 3                                         | −1.6     |
| MTNR1B     | Melatonin receptor 1B                                                    | −1.7     |
| NMUR2      | Neuromedin U receptor 2                                                  | −1.7     |
| OXGR1      | Oxoglutarate (alpha-ketoglutarate) receptor 1                           | −1.3     |
| OXTR       | Oxytocin receptor                                                       | −1.4     |
| P2RY12     | Purinergic receptor P2Y, G protein coupled, 12                           | −1.5     |
| PRLHR      | Prolactin-releasing hormone receptor                                     | −1.9     |

*A negative Z-score indicates a proinfluenza gene.*
more siRNAs (Fig. 1A and B), and knockdown of the C5AR2, CCKBR, OXTR, or P2RY12 gene gave the greatest reduction in virus titers for two or more siRNAs (Fig. 1B). Knockdown of the ADGRF1, ADRB2, CSAR2, CCKBR, HCRTR2, LPAR3, OXTR, or P2RY12 gene yielded a greater reduction in infectious viral titers than knockdown of the mitogen-activated protein kinase kinase (MAP2K) gene (−9.54-fold change), which is known to limit the replication of influenza virus and thus to reduce infectious viral titers.

**FIG 1** Deconvolution of siRNA pools. siRNA pools targeting GPCR (A, B) and IC (C) genes were deconvoluted and reverse transfected at a final concentration of 50 nM in A549 cells. At 48 h post-siRNA transfection, the A549 cells were infected (MOI, 0.001) with A/WSN/33; supernatants were collected, and virus titers were determined by an MDCK plaque assay. Experiments were performed in triplicate and assayed in duplicate. Results are presented as heat maps depicting fold changes in influenza virus titers (in PFU per milliliter) from titers with a nontargeting control siRNA (siNTC). A positive fold change equates to an increase in PFU per milliliter over the control. A negative fold change equates to a decrease in PFU per milliliter over the control. A zero fold change equates to no change in PFU per milliliter over the control. Asterisks indicate significant differences from the control by two-way mixed analysis of variance with Dunnett's multiple-comparison test (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001). siNTC results are corrected to zero to reflect the baseline change in replication (which is zero). Results are normalized to those for the siNTC control.
OTP-siRNAs were further evaluated. Silencing the plaque assay. IC genes that showed decreased plaque titers for two or more individual KCNA7 Z-scores of analyses, yielding 13 IC genes (WSN/33 after 48 h (24, 56). Levels of infectious in SMARTpools were deconvoluted (1 siRNA pool per treatment; 4 siRNAs per target) and into an acidi viruses attach to the cell membranes during infection and incorporate the membrane SCNN1D Glutamate receptor, ionotropic, delta 2 evaluated (58, 60). Thus, a total of seen. Following reverse transfection for 48 h, the A549 cells were infected with either SCNN1D and GRID2 Glutamate receptor, ionotropic, N-methyl-D-aspartate 3A. KCNA7 Potassium voltage-gated channel, shaker-related subfamily, member 7 KCNA8 Potassium voltage-gated channel, shaker-related subfamily, beta member 2 KCNIP2 Kv channel-interacting protein 2. MCOLN2 Mucolipin 2 SCN7A Sodium channel, non-voltage gated 1, delta subunit SCN11D Sodium channel, voltage gated, type VI, alpha subunit 4.3-fold change). Silencing 2.0, or 2.0) were further evaluated.

An RNAi screen identifies IC genes. Ion channels (ICs) are membrane-spanning proteins that allow for ion flux across cellular membranes (51), which affects signaling cascades and effector functions (52), as well as the activity and stability of viral proteins (53–55). Thus, ion channels affect influenza virus replication (40–42), since influenza viruses attach to the cell membranes during infection and incorporate the membrane into an acidic endosome, triggering conformational changes in HA (56, 57). We screened 352 IC genes for their importance in influenza virus replication and found Z-scores of ≤−1.0 for 173 IC genes. These proviral genes were analyzed by IPA and GO analyses, yielding 13 IC genes (ASIC1, CACNA1C, CHRNA1, GABRA3, GRID2, GRIN3A, KCNA7, KCNE2, KCNIP2, KCNMB2, MCOLN2, SCN7A, and SCN11D) (Table 2). OTP-siRNA SMARTpools were deconvoluted (1 siRNA pool per treatment; 4 siRNAs per target) and reverse transfected into A549 cells, and then the cells were infected (MOI, 0.01) with A/WSN/33 after 48 h (24, 56). Levels of infectious influenza virus were determined by a plaque assay. IC genes that showed decreased plaque titers for two or more individual OTP-siRNAs were further evaluated. Silencing the CACNA1C, CHRNA1, GRIN3A, KCNA7, KCNE2, KCNIP2, KCNMB2, or SCN7A gene did not detectably affect virus titers relative to those for non-targeting siRNA controls (siNTC) (Fig. 1C); however, silencing the ASIC1, GABRA3, GRID2, MCOLN2, or SCN11D gene resulted in a <−1.0-fold change. Silencing ASIC1 led to a greater reduction in influenza virus titers than silencing the MAP2K gene (−4.3-fold change). Silencing SCN11D resulted in a small decrease in viral titers; however, since SCN11D is targeted by the ion channel inhibitor triamterene, and thus, a potential repurposed drug, identified by IPA, was available, this gene was further evaluated (58, 60). Thus, a total of five ion channel genes—ASIC1, GABRA3, GRID2, MCOLN2, and SCN11D—were further evaluated.

Distinctive GPCR and IC genes are utilized for the replication of influenza virus strains and subtypes. To better understand GPCR and IC genes that have influenza virus strain and type differences, the GPCR and IC genes were evaluated following A/CA/04/2009 or B/Yamagata/16/1988 infection of A549 cells using a plaque assay and a 50% tissue culture infective dose (TCID50) assay. Our initial RNAi screen investigated A/WSN/33 infection of A549 cells at a lower MOI (0.001). To corroborate earlier data, gene hits were confirmed using individual OTP-siRNAs and a higher MOI (0.01) of A/WSN/33. The higher MOI of 0.01 was repeated for RNAi silencing of GPCR and IC genes in A549 cells infected with A/CA/04/2009 or B/Yamagata/16/1988. Briefly, A549 cells were transfected with OTP-siRNAs (2 siRNAs per target, transfected individually) targeting a GPCR or ion channel gene selected from the A/WSN/33 deconvolution screen. Following reverse transfection for 48 h, the A549 cells were infected with either A/WSN/33 (MOI, 0.01), A/CA/04/2009 (MOI, 0.1), or B/Yamagata/16/1988 (MOI, 0.1).

### TABLE 2 IC genes from a genome-wide RNAi screen

| Gene   | Function                                      | Z-score* |
|--------|-----------------------------------------------|----------|
| ASIC1  | Acid-sensing (proton-gated) ion channel 1     | −1.8     |
| CACNA1C| Calcium channel, voltage dependent, L type, alpha 1C subunit | −2.2     |
| CHRNA1 | Cholinergic receptor, nicotinic, alpha 1 (muscle) | −1.5     |
| GABRA3 | Gamma-aminobutyric acid (GABA) A receptor, alpha 3 | −1.5     |
| GRID2  | Glutamate receptor, ionotropic, delta 2       | −1.8     |
| GRIN3A | Glutamate receptor, ionotropic, N-methyl-D-aspartate 3A | −1.5     |
| KCNA7  | Potassium voltage-gated channel, shaker-related subfamily, member 7 | −1.5     |
| KCNA8  | Potassium voltage-gated channel, shaker-related subfamily, beta member 2 | −1.7     |
| KCNE2  | Potassium voltage-gated channel, Isk-related family, member 2 | −1.4     |
| KCNIP2 | Kv channel-interacting protein 2              | −1.9     |
| MCOLN2 | Mucolipin 2                                   | −1.9     |
| SCN7A  | Sodium channel, non-voltage gated 1, delta subunit | −1.5     |
| SCN11D | Sodium channel, voltage gated, type VII, alpha subunit | −2.0     |

* A negative Z-score indicates a proinfluenza gene.
Forty-eight hours postinfection, the titer and 50% tissue culture infective dose were determined by a plaque assay and a TCID$_{50}$ HA assay, respectively. The results showed that silencing 16 GPCR and 5 IC proinfluenza genes individually was associated with a 2-fold decrease in influenza plaque formation in A549 cells infected with A/WSN/33. Supernatants were collected 48 h postinfection. Infectious viral titers (expressed as PFU per milliliter) and TCID$_{50}$ titers were determined by an MDCK plaque assay and sample titration on MDCK cells followed by an HA assay, respectively. Plaque assay data for GPCR (A) and ion channel (B) genes and TCID$_{50}$ data for GPCR (C) and ion channel (D) genes are presented as the inverse of the fold decrease from the level with nontargeting control siRNA (siNTC) for three independent experiments performed in triplicate. A positive increase in the fold change equates to a decrease in PFU per milliliter or TCID$_{50}$ per milliliter from that with siNTC. Data show means ± standard errors of the means for three independent experiments performed in triplicate. Asterisks indicate significant differences from the control by ordinary one-way analysis of variance with Dunnett’s multiple-comparison test (*, $P < 0.05$; **, $P < 0.001$; ***$ P < 0.0001$; ****, $P < 0.00001$). siNTC results are corrected to zero to reflect the baseline change in replication (which is zero). Results are normalized to those for siNTC. Numbers under graphs represent individual siRNAs from the SMARTpool (siRNA 1, 2, 3, or 4) targeting a particular gene.

FIG 2 Validation of host gene targets for A/WSN/33-infected A549 cells. A549 cells were reverse transected (50 nM) with OTP-modified siRNAs (2 siRNAs per gene target) from the deconvolution siRNA screen in triplicate and were incubated for 48 h. The A549 cells were infected (MOI, 0.01) with A/WSN/33. Supernatants were collected 48 h postinfection. Infectious viral titers (expressed as PFU per milliliter) and TCID$_{50}$ titers were determined by an MDCK plaque assay and sample titration on MDCK cells followed by an HA assay, respectively. Plaque assay data for GPCR (A) and ion channel (B) genes and TCID$_{50}$ data for GPCR (C) and ion channel (D) genes are presented as the inverse of the fold decrease from the level with nontargeting control siRNA (siNTC) for three independent experiments performed in triplicate. A positive increase in the fold change equates to a decrease in PFU per milliliter or TCID$_{50}$ per milliliter from that with siNTC. Data show means ± standard errors of the means for three independent experiments performed in triplicate. Asterisks indicate significant differences from the control by ordinary one-way analysis of variance with Dunnett’s multiple-comparison test (*, $P < 0.05$; **, $P < 0.001$; ***$ P < 0.0001$; ****, $P < 0.00001$). siNTC results are corrected to zero to reflect the baseline change in replication (which is zero). Results are normalized to those for siNTC. Numbers under graphs represent individual siRNAs from the SMARTpool (siRNA 1, 2, 3, or 4) targeting a particular gene.

Forty-eight hours postinfection, the titer and 50% tissue culture infective dose were determined by a plaque assay and a TCID$_{50}$ HA assay, respectively. The results showed that silencing 16 GPCR and 5 IC proinfluenza genes individually was associated with a 2-fold decrease in influenza plaque formation in A549 cells infected with A/WSN/33 (Fig. 2A and B), A/CA/04/2009 (Fig. 3A and B), or B/Yamagata/16/1988 (Fig. 4A and B). Notably, there was a >100-fold decrease in TCID$_{50}$ for A/WSN/33 (Fig. 2C and D), a >10-fold decrease in TCID$_{50}$ for CA/04/2009 (Fig. 3C and D), and a >10-fold decrease in TCID$_{50}$ for B/Yamagata/16/1988 (Fig. 4C and D). These differences in the fold change are likely related to the virus replication dynamics and growth kinetics. The A/WSN/33 and CA/04/2009 strains replicate at a higher tempo and to higher titers than B/Yamagata/16/1988 (52, 53). As shown in Fig. 2, siRNA silencing of the LGR4, LPAR3, OXGR1, ASIC1, GABRA3, or MCOLN2 gene markedly reduced A/WSN/33 virus titers from those with siNTC while also showing a reduction in virus titers from those with siMAP2K2 (4.4-fold decrease) (Fig. 2A and B). The effects of individually silencing the 16
GPCR and 5 IC genes on A/CA/04/2009 replication were also determined (Fig. 3). The results show that siRNAs targeting the \textit{AGTR1}, \textit{HCRTR2}, \textit{P2RY12}, or \textit{GRID2} gene substantially reduced A/CA/04/09 replication (Fig. 3A and B). Silencing \textit{P2RY12} also showed a considerable reduction in virus titers from those with siMAP2K (6.84-fold reduction) (Fig. 3A). The result of individually silencing 16 GPCR genes and 5 IC genes on B/Yamagata/16/1988 replication was also determined (Fig. 4). Importantly, silencing the \textit{HRH2} or \textit{GRID2} gene substantially reduced the B/Yamagata/16/1988 titer, and targeting \textit{HRH2} resulted in a reduction in the virus titer greater than that with siMAP2K (30-fold reduction) gene silencing (Fig. 4A and B). These results confirm earlier results from the A/WSN/33 screen and show that several GPCR and IC genes affect A/CA/04/09 and B/Yamagata/16/1988 replication.

\textbf{DISCUSSION}

RNAi screens have aided in the discovery of essential features of the host-virus...
interface, specifically the host pathways used to facilitate virus replication (23, 54), and have provided information used to develop disease intervention strategies (28, 29). GPCRs and ICs are implicated in the replication mechanisms of several RNA viruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Marburg virus, Ebola virus, and HIV, but have not been well described for influenza virus (38, 40, 41, 46, 55). In this study, we identified GPCR and IC genes used by influenza virus for replication and determined influenza virus strain and type differences. We screened 390 GPCR and 349 IC genes, of which 19 GPCR and 13 IC genes were selected for validation studies. Secondary validation by siRNA pool deconvolution yielded 16 confirmed GPCR genes (ADGRF1, ADORA1, ADRB2, AGTR1, CSAR2, CCKBR, FFAR1, HCAR3, HCRTR2, HRH2, HTR1B, LGR4, LPAR3, OXGR1, OXTR, and P2RY12) and 5 IC genes (ASIC1, GABRA3, GRID2, MCOLN2, and SCNN1D) (Fig. 1). The genes from the RNAi screen were validated by using two individual OTP-siRNAs and testing the effects on A/WSN/33

FIG 4 Validation of host gene targets for B/Yamagata/16/1988-infected A549 cells. A549 cells were reverse transfected (50 nM) with OTP-modified siRNAs (2 siRNAs per gene target) from the deconvolution siRNA screen in triplicate and were incubated for 48 h. The A549 cells were infected (MOI 0.01) with B/Yamagata/16/1988. Supernatants were collected 48 h postinfection. Infectious viral titers (PFU per milliliter) and TCID_{50} titters were determined by an MDCK plaque assay and sample titration on MDCK cells followed by an HA assay, respectively. Plaque assay data for GPCR (A) and ion channel (B) genes and TCID_{50} data for GPCR (C) and ion channel (D) genes are presented as the inverse of the fold decrease from the level with nontargeting control siRNA (siNTC) for three independent experiments performed in triplicate. A positive increase in the fold change equates to a decrease in PFU per milliliter or TCID_{50} per milliliter from that with siNTC. Data show means ± standard errors of the means for three independent experiments performed in triplicate. Asterisks indicate significant differences from the control by ordinary one-way analysis of variance with Dunnett’s multiple-comparison test (**, P < 0.05; ***, P < 0.001; ****, P < 0.0001). siNTC results are corrected to zero to reflect the baseline change in replication (which is zero). Results are normalized to those for siNTC. Numbers under graphs represent individual siRNAs from the SMARTpool (siRNA 1, 2, 3, or 4) targeting a particular gene.
replication using a higher MOI (0.01) to ensure robust infection. These studies used two endpoints to evaluate the effects of knockdown on influenza virus replication: infectious virus titers (expressed in PFU per milliliter), quantitated by plaque assays, and the amount of virus required to infect 50% of cells (50% tissue culture infective dose [TCID₅₀]), measured by HA assays (Fig. 2). siRNA silencing of GPCR genes LGR4, LPAR3, and OXGR1, and silencing of IC genes ASIC1, GABRA3, and MCOLN2, in A549 cells yielded substantial decreases in A/WSN/33 titers, showing that these genes are needed for A/WSN/33 replication. Of note, the decreases in virus plaque numbers were greater than those with the control siRNA siMAP2K (4-fold decrease), which targets mitogen-activated protein kinase, shown to be required for influenza virus replication (49, 50).

To examine influenza virus strain differences, siRNA-transfected A549 cells were infected with A/CA/04/2009, a representative circulating strain of human influenza A virus, and levels of virus replication were determined by quantitation of infectious virus (by plaque assay) and determination of the TCID₅₀ following transfection (Fig. 3). Silencing of GPCR and IC genes gave results similar to those for A/WSN/33-infected A549 cells, where influenza virus titers linked to the GPCR genes AGTR1, HCRTR2, and P2RY12 and the IC gene GRID2 were considerably reduced. Of note, silencing P2RY12 reduced virus titers 6-fold more than the siMAP2K control. We also examined the potential for influenza virus type differences linked to GPCR and IC genes in A549 cells by evaluating the replication of B/Yamagata/16/1988 after siRNA transfection (Fig. 4). siRNA silencing of GPCR and IC genes also yielded reduced B/Yamagata/16/1988 replication, but the reductions were statistically significant (P < 0.01) only for the HRH2 and GRID2 genes; targeting HRH2 yielded a reduction in virus titers greater than that with siMAP2K (30-fold change).

The results suggest that influenza virus strains and types coopt similar GPCR and IC genes as part of the replication process in A549 cells but have the ability to utilize different genes in similar pathways (54, 61). It has been reported that the tempo of signal transduction and host gene expression is associated with viral replication and virus production dynamics (61). It is possible that different host genes are used for influenza virus replication in other cell types, particularly since transformed cell lines can have distinct gene expression (62). This is a caveat with A549 cells, since some host genes identified as important may not translate to primary cell cultures. Additionally, the findings in this study were limited to 48 h postinfection (p.i.) due to the high-throughput screening procedure, and the later phases of virus replication were not evaluated. Additionally, GPCR signaling is a complex network; each GPCR complex may have a number of isoforms and splice variants that create hundreds of combinations of G proteins. Thus, differences in cell signaling associated with the kinetics of infection and/or GPCR isoforms/splice variants can go unnoticed (34). In addition, the configuration of the G protein affects not only which transmembrane receptor it can bind to but also which downstream target is affected (34, 63, 64). GPCR Gα subunits are grouped into four families (Gα₁, Gα₂, Gα₃, and Gα₁₂/13) based on sequence homology, consisting of approximately 20 distinct Gα subunit proteins due to splice variants (65). The host genes ADORA1, AGTR1, HTR1B, and P2RY12 are coupled to Gαi (Fig. 5), while the ADRB2, HCRTR2, and HRH2 genes are coupled to Gαs (Fig. 6). Gαi signaling inhibits adenyl cyclase, which decreases intracellular cAMP levels, while Gαs signaling stimulates adenyl cyclase, prompting the opposing effect. Modulation of cAMP levels regulates the duration and intensity of cAMP signaling via feedback mechanisms (66). G proteins have been implicated in late stages of influenza virus infection, specifically virus budding (67–69). The host genes AGTR1, CCKBR, FFAR1, HCRTR2, OXGR1, and OXTR were associated with Gαi signaling by IPA (Fig. 7). Gαs signaling is associated with multiple downstream pathways, but the best characterized are those associated with phospholipase Cβ (PLC) activation and phosphatidylinositol 3-kinase (PI3K) (70). Alteration of this pathway has been shown to play a regulatory role in the clathrin-mediated and clathrin-independent endocytosis pathways utilized by influenza virus at entry (71). The host genes ADGRF1 and LGR4 are orphan receptors, with no identified endogenous
ligand (72–74). C5AR2 is a nonclassical GPCR, and although it is a seven-transmembrane receptor, it does not couple to a G protein and instead binds β-arrestins (75, 76). In this study, we show that siRNA silencing of the GPCR genes AGTR1, CCKBR, FFAR1, HCRTR2, OXGR1, and OXTR inhibits A/WSN/33, A/CA/04/2009, and B/Yamagata/16/1988 replication in A549 cells.

IPA of the validated IC genes determined in this study suggested that several genes affected influenza virus replication. ASIC1 is an acid-sensing sodium channel gene whose regulation is controlled by activation of the PKC pathway (77); however, it remains unclear how ASIC1 is necessary for viral replication. Similarly, GRID2 (or GluRδ2) is an orphan glutamate receptor gene whose function is poorly understood (78). SCNN1D (the delta subunit of the epithelial sodium channel [δENaC]) is one of four subunits that compose the epithelial sodium channel located on the apical...
surfaces of polarized tissues, e.g., the lung. It is involved in Na\textsuperscript{+} transport across the transepithelial surface during Na\textsuperscript{+} reabsorption (60, 79). In this study, silencing of SCNN1D reduced virus replication, suggesting a novel role for this subunit compared to its \( \alpha \), \( \beta \), and \( \gamma \) counterparts (80). GABRA3 has been shown to be expressed in the

**FIG 6** Ga\textsubscript{s} signaling pathway generated by IPA. The ADRB2, HCAR3, and HRH2 genes were associated with Ga\textsubscript{s} signaling by IPA. AC, adenylly cyclase; cAMP, cyclic AMP; PKA, protein kinase A; RGS2, regulators of G protein signaling; RAP1A, Ras-related protein Rap-1A; RAPGEF 2, 3, and 4; Rap guanine nucleotide exchange factors 2, 3, and 4; SRC, Src protein kinase; B-RAF, RAF proto-oncogene serine/threonine-protein kinase; MEK 1/2, mitogen-activated kinases 1 and 2; ERK 1/2, extracellular signal-regulated kinases 1 and 2; CNG, cyclic-nucleotide-gated ion channel; HCK, tyrosine protein kinase; RYR, ryanodine receptor; ER, endoplasmic reticulum; CREB, cAMP response element-binding protein; Elk-1, ETS-like-1 protein.
lung (81), and its activation is linked to autophagy (81), a strategy used by influenza viruses to promote replication (82). We show that GABRA3 silencing reduces viral replication. It has been shown that MCOLN2 is associated with improved influenza virus, dengue virus, yellow fever virus, and equine arteritis virus infectivity (83), possibly by promoting virus trafficking between the early and late endosomes and releasing virus...
into the cytosol independently of interferon (IFN) signaling (83). Our findings concur, showing that siRNA silencing of MCOLN2 decreases influenza virus replication and that MCOLN2 is an important host factor not only for the replication of IAVs but also for that of IBVs, which was not previously known.

Understanding the host factors used by influenza virus during entry, replication, and egress can help identify targets for drug repurposing or for the development of novel antiviral drugs. Targeting of host factors is refractory to the development of drug resistance generated by viral mutations (18). Here, we identify several GPCR and ion channel genes that can be targeted by FDA-approved drug antagonists and/or inhibitors (Table 3). For example, P2RY12 (a GPCR gene) can be targeted by the drug clopidogrel bisulfate (Plavix), which is currently approved for the inhibition of platelet aggregation and the treatment of patients with acute coronary syndrome (84–86).

Interestingly, the AGTR1 gene (a GPCR gene) has been shown to be associated with the coronavirus infection pathway, which has a possible link between angiotensin-converting enzyme 2 (ACE2) and lung injury (127–129). AGTR1 can be targeted by angiotensin receptor blockers (ARBs), including candesartan, which has been suggested as a treatment for coronavirus disease 2019 (COVID-19) (87). ARBs have shown efficacy in decreasing lung injury in animal models of acute respiratory distress syndrome (ARDS), but not without potential side effects (88). Further studies are needed to determine the importance of this association with COVID-19. The ion channels ASIC1 and

| Target  | Drug name | PubChem ID | CAS no. | Action     | Chemical formula | Reference(s) |
|---------|-----------|------------|---------|------------|------------------|--------------|
| ADORA1  | Aminophylline | 9433 | 317-34-0 | Antagonist  | C_{18}H_{24}NO_{10} | 98           |
|         | Dyphylline   | 3182 | 479-18-5 | Antagonist  | C_{18}H_{24}NO_{10} | 98           |
|         | Istradeylline| 5311037 | 155270-99-8 | Antagonist  | C_{18}H_{24}NO_{10} | 98           |
|         | Pentoxifylline | 4740 | 6493-05-6 | Unknown    | C_{18}H_{24}NO_{10} | 99, 100      |
|         | Theophylline  | 2153 | 58-55-9 | Antagonist  | C_{18}H_{24}NO_{10} | 98           |
| AGTR1   | Azilsartan  | 135415867 | 147403-03-0 | Antagonist  | C_{25}H_{20}N_{6}O_{5} | 101, 102     |
|         | Candesartan  | 2541 | 139481-59-7 | Antagonist  | C_{25}H_{20}N_{6}O_{5} | 87, 102, 103 |
|         | Eprosartan   | 5281037 | 133040-01-4 | Antagonist  | C_{25}H_{20}N_{6}O_{5} | 102          |
|         | Losartan     | 3961 | 114798-26-4 | Antagonist  | C_{25}H_{20}N_{6}O_{5} | 102, 104, 105 |
|         | Valsartan    | 60846 | 137862-53-4 | Antagonist  | C_{25}H_{20}N_{6}O_{5} | 102, 106     |
| HTR1B   | Asenapine   | 3036780 | 65576-45-6 | Antagonist  | C_{18}H_{16}CINO | 107          |
| P2RY12  | Cangrelor   | 9854012 | 163706-06-7 | Inhibitor   | C_{18}H_{16}Cl_{2}F_{2}N_{2}O_{2}P_{2}S_{2} | 108          |
|         | Clopidogrel  | 60006 | 113665-84-2 | Antagonist  | C_{18}H_{16}CINO | 84–86, 102, 109–111 |
|         | Prasugrel   | 6918456 | 150322-43-3 | Antagonist  | C_{18}H_{16}CINO | 102, 109, 112 |
|         | Ticagrelor  | 9871419 | 274693-27-5 | Inhibitor   | C_{18}H_{16}CINO | 113          |
|         | Ticlopidine  | 5472 | 55142-85-3 | Antagonist  | C_{18}H_{16}CINO | 86, 102, 109, 110 |
| ADRB2   | Carteolol HCl | 40127 | 51781-21-6 | Antagonist  | C_{18}H_{16}CINO | 114          |
|         | Labetalol   | 3869 | 36694-69-6 | Antagonist  | C_{18}H_{16}CINO | 102          |
|         | Levobunolol | 39468 | 47141-42-4 | Antagonist  | C_{18}H_{16}CINO | 102, 115     |
|         | Metipranolol | 31477 | 22664-55-7 | Antagonist  | C_{18}H_{16}CINO | 58, 116      |
|         | Sotalol     | 5253 | 39390-20-9 | Antagonist  | C_{18}H_{16}CINO | 117          |
|         | Timolol     | 33624 | 26839-75-8 | Antagonist  | C_{18}H_{16}CINO | 118          |
| HRH2    | Asenapine   | 3036780 | 65576-45-6 | Antagonist  | C_{18}H_{16}CINO | 107          |
|         | Famotidine  | 5702160 | 76824-35-6 | Antagonist  | C_{18}H_{16}CINO | 119          |
|         | Lafutidine  | 5282136 | 118288-08-7 | Antagonist  | C_{18}H_{16}CINO | 120          |
| ASIC1   | Amiloride   | 16231 | 2609-46-3 | Inhibitor   | C_{18}H_{16}CINO | 90           |
|         | Diclofenac  | 3033 | 15307-86-5 | Inhibitor   | C_{18}H_{16}CINO | 121          |
| GABRA3  | Bicuculline | 10237 | 485-49-4 | Antagonist  | C_{18}H_{16}CINO | 122          |
| OXTR    | Atosiban    | 5311010 | 90779-69-4 | Antagonist  | C_{18}H_{16}CINO | 123, 124     |
| SCNN1D  | Amiloride   | 16231 | 2609-46-3 | Inhibitor   | C_{18}H_{16}CINO | 125          |
|         | Triamterene | 5546 | 396-01-0 | Inhibitor   | C_{18}H_{16}CINO | 58, 116, 126 |
SCNN1D can be inhibited by amiloride, which has been shown to suppress the replication of coxsackievirus B3 (CVB3) and foot-and-mouth disease virus (FMDV) (89, 90). These examples show the therapeutic potential of drug repurposing to target host factors needed for virus replication.

To summarize, this study identified and evaluated GPCR and IC genes coopted by influenza viruses (A/WSN/33, CA/04/2009, B/Yamagata/16/1988) for replication and identified strain and type differences. Collectively, the identification of these GPCR and IC genes provides the opportunity to develop host-directed virus control strategies to limit influenza virus replication and disease using drug repurposing or the development of novel antivirals.

MATERIALS AND METHODS

Cells and viruses. Type II human lung epithelial (A549) cells (ATCC CCL-185) were propagated in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT) supplemented with 5% heat-inactivated fetal bovine serum (HI-FBS; Atlas Biologics Inc., Fort Collins, CO). Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) were propagated in DMEM supplemented with 5% HI-FBS. All experiments were performed using log-phase A549 or MDCK cells.

A/WSN/33 (H1N1; ATCC VR-825), which is lab adapted and trypsin independent (38, 39), A/CA/04/2009 (H1N1; BEI Resources), and B/Yamagata/16/1988 (BEI Resources) were grown in 9-day-old embryonated chicken eggs as described previously (91). The A/WSN/33 and A/CA/04/2009 viruses used in siRNA validation experiments were propagated in MDCK cells (91). Viral titers were determined by plaque assays and were calculated using the Reed and Muench method (92–94).

siGENOME screen. siGENOME plates received from Dharmacon/Horizon Discovery were preloaded with 0.5 nmol of pooled, lyophilized siRNAs targeting 390 GPCR or 349 IC genes. siRNAs were designed to ensure ≥85% knockdown of target gene expression, and optimal antisense-strand RISC loading is guaranteed (95). siRNA pools were resuspended in siRNA resuspension buffer to a concentration of 1 μM, aliquoted, and stored at −80°C until use. For the screen, A549 cells were reverse transfected with siRNA SMARTpools or siRNA controls (50 nM) and were incubated at 37°C under 5% CO₂ for 48 h to allow for gene silencing prior to infection as described previously (24, 30). Briefly, transfections were performed in a 96-well plate format in triplicate. The siRNA SMARTpools were diluted in Hanks’ balanced salt solution (HBSS; GIBCO), added to the plate, and incubated at room temperature (RT) for 5 min. Following incubation, 0.4 μl of DharmaFECT 1 transfection reagent (Horizon Discovery) and 9.6 μl of HBSS per well were added, and the mixture was incubated for 20 min at RT. Lastly, 80 μl containing 1.5 × 10⁴ A549 cells in DMEM supplemented with 5% HI-FBS was added to each well, and the mixture was incubated at 37°C under 5% CO₂ for 48 h. After transfection, the cells were washed twice with phosphate-buffered saline (PBS), infected with A/WSN/33 at an MOI of 0.001 to reduce defective interfering particles, and incubated at 37°C under 5% CO₂ for 48 h. After infection, the supernatant was collected and analyzed by a TCID₅₀ assay for virus replication by HA titers as described previously (24). HA titer results were normalized to those with siNTC. A primary screen was performed twice in two independent experiments. Results were pooled and analyzed. All RNA interference (RNAi) experiments were completed according to the Minimum Information about an RNAi Experiment (MIARE) guidelines (96).

Host genes with a Z-score of ≥−1.0 were considered proinfluenza because siRNA silencing reduced virus replication from that with nontargeting controls. A total of 185 GPCR genes and 173 IC genes were proinfluenza genes (94). These genes were evaluated by Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc., Redwood City, CA) and Gene Ontology (GO) analysis. Comprehensive gene interaction networks were determined by combining IPA and GO analysis to identify relationships, functions, mechanisms, and pathways. Following IPA and GO analysis, the GPCR and IC host genes identified were evaluated for their abilities to be targeted by miRs. These data were used to select 19 proinfluenza GPCR and 13 proinfluenza ion channel candidates for further examination.

siRNA pool deconvolution and validation. The four siRNAs per SMARTpool were individually examined in a deconvolution assay to eliminate false-positive results and to determine the most effective siRNAs for reducing influenza virus replication. Plates containing 0.5 nmol of individual lyophilized ON-TARGETplus (OTP) siRNAs (Horizon Discovery) against a single host gene target were tested. OTP-modified siRNAs contained a modification within seed regions to reduce off-target effects and to increase selectivity and effectiveness. siRNAs were suspended in siRNA buffer according to the manufacturer’s recommendations to a concentration of 1 μM, aliquoted, and stored at −80°C until use.

A549 cells were reverse transfected with one of four OTP-siRNAs as described elsewhere (44). Briefly, siRNAs targeting a given GPCR or IC gene (Table 4), nontargeting control siRNA (siNTC), siMAP2K (siRNA targeting the mitogen-activated protein kinase 1 gene), or the RNAi transfection control siTOX was used at a final concentration of 50 nM, and transfected cells were incubated at 37°C under 5% CO₂ for 48 h to allow for gene silencing prior to infection. Transfections were performed in a 96-well plate in triplicate. Briefly, siRNA reverse transfection was done using 0.4% DharmaFECT 1 transfection reagent, where siRNA was preincubated with DharmaFECT 1 in serum-free DMEM at RT for 20 min. A549 cells were suspended in DMEM supplemented with 5% HI-FBS, and 1.5 × 10⁴ cells were added to each well. Transfection plates were incubated at 37°C under 5% CO₂ for 48 h. After transfection, the medium was decanted, and the cells were washed twice with PBS and then infected with A/WSN/33 (MOI, 0.001) diluted in infection medium (MEM plus 0.3% bovine serum albumin [BSA] plus 1 μg/ml l-(tosylamido-2-
| siRNA no. | Gene designation | Gene ID | GenBank accession no. | Target sequence |
|----------|------------------|---------|-----------------------|-----------------|
| si1      | ADGRF1           | 266977  | NM_025048             | CACAUGGGCUAAUAGAAU |
| si2      | ADORA1           | 134     | NM_000674             | AGAGAGGCCAUGACCAAG |
| si3      | ADRB2            | 154     | NM_000024             | UGAUAGUGUCACGACGA |
| si4      | AGTR1            | 185     | NM_032049             | UGGAAGGCAGAAUACAA |
| si5      | CSAR2            | 2702    | NM_018485             | CCAUCACCGCGCAAGC |
| si6      | CCKBR            | 887     | NM_176875             | GUGAAGGCUUGACGAC |
| si7      | FFAR1            | 2864    | NM_005303             | CGCUACAACGCGGCA |
| si8      | HCAR3            | 8843    | NM_006018             | UCAAAUACAAUACAA |
| si9      | HCRTR2           | 3062    | NM_001526             | GAGGACCGGACGCA |
| si10     | HRH2             | 3274    | NM_022304             | CGAAAGGACCAUCACA |
| si11     | HTR1B            | 3351    | NM_000863             | GGAACCGGACAGUAC |
| si12     | LGR4             | 55366   | NM_018490             | AGAAUACUGUACGUA |
| si13     | LPAR3            | 23566   | NM_012152             | UUAACAGCGGACGUA |
| si14     | MTNR1B           | 4544    | NM_005959             | GCACACACCAUACAG |
| si15     | NMUR2            | 56923   | NM_020167             | CCAUGGGACUGAAC |

(Continued on next page)
| siRNA no. | Gene designation | Gene ID | GenBank accession no. | Target sequence |
|----------|------------------|---------|-----------------------|-----------------|
| si1      | OXGR1            | 27199   | NM_080818             | CGGAUGAACUCAAUCAUA |
| si2      | CAUCGUUCUCAUCAUA |         |                       |                 |
| si3      | CCGAUGACCUCAACUA |         |                       |                 |
| si4      | CCACUAGCAUUUAGC   |         |                       |                 |
| si1      | OXTR             | 5021    | NM_000916             | GGAUCACGUACGUACUA |
| si2      | UGGCAGAACUCGCGCA |         |                       |                 |
| si3      | GGCGUAGCUACUCCUA |         |                       |                 |
| si4      | GAGCAACUCUCAUGCU   |         |                       |                 |
| si1      | P2RY12           | 64805   | NM_176876             | GGUCAUGUCCGCAUGAAAA |
| si2      | GUACCGGUCAUACGUAGA |        |                       |                 |
| si3      | CAAGUUAACCUCCGAUA |        |                       |                 |
| si4      | CAAGUCAUUCUAGCUAA |        |                       |                 |
| si1      | PRLHR            | 2834    | NM_004248             | CAUCCAGCCUACGUCAU |
| si2      | GGAUCACGAGGCAUAAC |       |                       |                 |
| si3      | CAGGGGUUUCUGACUAUAU |      |                       |                 |
| si4      | GCAAAACUGUUGGGCUGU |       |                       |                 |
| si1      | ASIC1            | 41      | NM_0001095            | GGAAGUGCUACAGCUACA |
| si2      | CUUCGAAGCCAGCUAACA |       |                       |                 |
| si3      | CAAAACAGGGCAUGAGAA |       |                       |                 |
| si4      | UCAAAACAGCUAGCCUA |       |                       |                 |
| si1      | CACNA1C          | 775     | NM_000719             | GAGGGGACACAUCAUA |
| si2      | GGAUGUACGUACGUAA |         |                       |                 |
| si3      | GGUGAGCUACGUAAUA |         |                       |                 |
| si4      | GAAGAGACUCGUACUGG |         |                       |                 |
| si1      | CHRNA1           | 1134    | NM_000079             | GCCCAAGCUUGUCUCA | |
| si2      | UAACUGGCCUGUACUA |         |                       |                 |
| si3      | GACCAGGUGUCAACAG |         |                       |                 |
| si4      | UAAAUCAGUCCUGACGAC |      |                       |                 |
| si1      | GABRA3           | 2556    | NM_0000808            | GAAGAUGGCUUACAUAC |
| si2      | ACAUGAGGGGUCAACUAC |        |                       |                 |
| si3      | CGACUAGGACAAGACUA |        |                       |                 |
| si4      | ACAAGUCAGUCUUAACU |        |                       |                 |
| si1      | GRID2            | 2895    | NM_001510             | GAGCGAUCUCUGUUAAUG | |
| si2      | GGAAGAGCAUGACUGA |         |                       |                 |
| si3      | GGACUCAACCGGACAA |         |                       |                 |
| si4      | UCCUGAGACUCUUGCU |         |                       |                 |
| si1      | GRIN3A           | 116443  | NM_133445             | CGACGGAUUACAUCUUA | |
| si2      | CAGCUUACCCUAAGGA |         |                       |                 |
| si3      | CAAACUAAUCAGCUGAA |        |                       |                 |
| si4      | GAAAGAGCUUUGUUGUUG |       |                       |                 |
| si1      | KCNA7            | 3743    | NM_031886             | GCGAGAGGCGGUUGGU | |
| si2      | GAGGGCGUGUUGAUUGU |        |                       |                 |
| si3      | GAAACACUUGGGCAGGA |        |                       |                 |
| si4      | CACUGUGGGGCGCAGGAA |       |                       |                 |
| si1      | KCNE2            | 9992    | NM_172201             | GAAUCCUACUAUCUAC | |
| si2      | GAGCGGAACACUCCAUG |        |                       |                 |
| si3      | CGAGGGCCACACUCAAUGA |      |                       |                 |
| si4      | ACAACACAGCUAGCAAG |        |                       |                 |
| si1      | KCNIP2           | 30819   | NM_173197             | GAAUGUCAACCGGAAUUG | |
| si2      | CAGCGUGGACGAUUAUU |        |                       |                 |
| si3      | AAACAAUUCACGGCGCA |        |                       |                 |
| si4      | GAAAUUAUCUAGUGCUUGU |       |                       |                 |
| si1      | KCNMB2           | 10242   | NM_005832             | CCAACGUGCUUCAUCUAC | |
| si2      | UCCACGGAUAUAGAAUA |        |                       |                 |
| si3      | UCAACUUGGGCGUCAUA |        |                       |                 |
| si4      | GUACCUCUCCCUAUCAUGU |       |                       |                 |
| si1      | MCOLN2           | 255231  | NM_153259             | GCUCUAGAGGUAACGGGAAGA | |
| si2      | GACCAUAUCAUGAACAGA |        |                       |                 |
| si3      | UCAGAUACUUGGUUAUU |        |                       |                 |
| si4      | UCAGUGCUUCGUUAUUUAAU |     |                       |                 |
TABLE 4 (Continued)

| siRNA no. | Gene designation | Gene ID | GenBank accession no. | Target sequence |
|-----------|------------------|---------|-----------------------|------------------|
| si1       | SCNN1D           | 6339    | NM_001130413.4        | GCAUCAGGGUCAUGGUUCA |
| si2       |                  |         |                       | GCUCACUCCUCACCUCUCU |
| si3       |                  |         |                       | GAGAAUGGAAAGCAGCCACA |
| si4       |                  |         |                       | CUACACAAACCCUCUCA |

*A genome-wide RNAi screen was performed with siRNA SMARTpools to determine GPCR and IC gene hits for A/WSN/33-infected A549 cells. Hits were validated by deconvolution of the SMARTpools by testing each siRNA individually at a 50 nM final concentration. The table includes four siRNAs from each pool as well as relative gene sequence and target information. Gene hits were considered validated when two or more siRNAs yielded reduced viral replication when transfected individually.

Phenyl-ethyl chloromethyl ketone (TPCK)-trypsin (Worthington, Columbus, OH). Infected cultures were incubated for 48 h at 37°C in 5% CO2, and included siNTC and a siTOX siRNA control. siNTC (5′-UAGGCACUAACACAACUCA-3′) targets no known sequence; siMAP2K (5′-PAGAACCUCACAUUGGCUU-3′), 5′-PUCAAACUGCCUCUCUGCUU-3′, 5′-PAGUUGCUCCAAUCUGCCUCU-3′, 5′-PAGUGAAUUGCCUUUGGUU-3′, targeting MAP2K, which is required for influenza virus replication, was used as a positive control, i.e., for host-targeted decrease of influenza virus replication (45, 46); and siTOX was used to confirm siRNA transfection under transfection conditions. Following incubation, supernatants were collected and stored at −80°C until they were tested by plaque assays. For the selected gene targets, the two siRNAs that gave the greatest reduction in virus titers were used for all remaining studies.

**Validated hits.** A549 cells were transfected with individual OTP-siRNAs (2 siRNAs/gene target) from the deconvolution screen or with a control siRNA (siNTC, siMAP2K, or siTOX) at a final concentration of 50 nM in triplicate. Following transfection, the cells were infected with either A/WSN/33 (MOI, 0.01), A/CA/04/2009 (MOI, 0.1), or B/Yamagata/16/1988 (MOI, 0.1). The MOIs mediated low or no cytopathic effect (CPE). Following incubation, supernatants were removed and stored at −80°C until they were tested by plaque assays and TCID50 assays. Two independent experiments were performed.

**Cytotoxicity assay.** Any cytotoxic effects associated with siRNA silencing were determined using a ToxiLight BioAssay kit (Lonza, Rockland, ME). Results were normalized to those with the siTOX transfection control, which results in complete cell death 48 h posttransfection. SMARTpools were considered toxic if transfection resulted in luminescence equivalent to ≥20% of the luminescence of the siTOX control.

**Plaque assay.** Infectious virus titers were determined by plaque assays as described elsewhere (47, 48, 91). Briefly, supernatants were serially diluted 10-fold in MEM with 1 μg/ml TPCK-trypsin and were inoculated onto 90% confluent MDCK cell monolayers in 12-well tissue culture plates (Corning Costar, Cambridge, MA). The virus was adsorbed for 1 h at 37°C under 5% CO2 before the addition of 3 ml of overlay. The overlay medium contained 1 part liquid medium containing 10 × MEM supplemented with 200 ml L-glutamine (Gibco), HEPES solution (Gibco), 7.5% NaCHO3 (Gibco), penicillin-streptomycin-ampithycin B solution (Gibco), and 1 part 2.4% Avicel (FMC BioPolymer, Philadelphia, PA) in water or 1 part 1% agarose in water. Samples from A/WSN/33 or A/CA/04/2009 wells were incubated at 37°C under 5% CO2 for 5 days to allow for better plaque formation. The overlays were removed, the plates were washed twice with PBS, and the cell monolayers were fixed with acetone-methanol (80:20) for 20 min at RT. Following fixation, the plates were stained with crystal violet as described previously, and viral titers were determined (92, 93).

**TCID50 assay.** Endpoint titers were determined by a TCID50 assay as described previously (22, 25, 92). Briefly, supernatants collected from influenza virus-infected A549 cells were serially diluted 10-fold in triplicate on MDCK cells in 96-well plates. Influenza virus-infected MDCK plates were incubated 5 days using cell culture conditions described elsewhere (22, 25). Following incubation, an HA test was performed using 50 μl of supernatant from infected MDCK cells and 50 μl of 1% turkey red blood cells (RBC) for a final concentration of 0.5% in a round-bottom plate (97). The TCID50 titers were calculated using the Reed and Muench method (92).

**Hemagglutination assay.** Hemagglutination was used for viral diagnosis of influenza viruses (92, 97). Briefly, 2-fold serial dilutions of virus in PBS were dispensed into individual wells of a 96-well round-bottom microtiter plate (Corning Costar, Cambridge, MA). Then aliquots of turkey RBC were added to each well to 0.5% of final volume. The highest dilution at which clumping was observed was regarded as the HA titer of the sample.

**Statistics.** HA assay results were normalized to results for siNTC-transfected controls. The nontargeting control was set to an arbitrary value of 1. Genes were specified a Z-score, calculated as $Z = (x - \mu) / (\sigma / \sqrt{n})$, where $x$ is equal to the average HA value of each gene, $\mu$ is equal to the population mean of the HA, $s$ is equal to the standard deviation of each gene across the two independent experiments, and $n$ is equal to the number of genes within the populations (16). Genes in the primary screen that were $<1.5$ standard deviations from the plate mean in both duplicates were considered primary hits.

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