Identification of Host Kinase Genes Required for Influenza Virus Replication and the Regulatory Role of MicroRNAs

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Abstract

Human protein kinases (HPKs) have profound effects on cellular responses. To better understand the role of HPKs and the signaling networks that influence influenza virus replication, a small interfering RNA (siRNA) screen of 720 HPKs was performed. From the screen, 17 HPKs (NPR2, MAP3K1, DYRK3, EPHA6, TPK1, PDK2, EXOSC10, NEK8, PLK4, SGK3, NEK3, PANK4, ITPKB, CDC2L5 (CDK13), CALM2, PKN3, and HK2) were validated as essential for A/WSN/33 influenza virus replication, and 6 HPKs (CDK13, HK2, NEK8, PANK4, PLK4 and SGK3) were identified as vital for both A/WSN/33 and A/New Caledonia/20/99 influenza virus replication. These HPKs were found to affect multiple host pathways and regulated by miRNAs induced during infection. Using a panel of miRNA agonists and antagonists, miR-149* was found to regulate NEK8 expression, miR-548d-3p was found to regulate MAPK1 transcript expression, and miRs -1228 and -138 to regulate CDK13 expression. Up-regulation of miR-34c induced PLK4 transcript and protein expression and enhanced influenza virus replication, while miR-34c inhibition reduced viral replication. These findings identify HPKs important for influenza viral replication and show the miRNAs that govern their expression.

Introduction

Influenza A viruses are ubiquitous, causing acute respiratory disease and substantial morbidity and mortality each year [1–3]. Although vaccination is central for controlling infection, treatment or prophylaxis with licensed antiviral drugs has been shown have >80% efficacy against the development of illness during inter-pandemic influenza periods [4]. Unfortunately, influenza has rapidly developed resistance to most antiviral drugs [5–8]. Given this and the difficulties with seasonal and pandemic influenza vaccine development [9,10], there is a need for new disease intervention strategies.

Influenza A viruses belong to the family *Orthomyxoviridae*, are enveloped, and have an eight segmented, negative-sense, single-stranded RNA genome that encodes up to 11 proteins [11]. The viral envelope contains the surface glycoproteins and antigenic determinants, hemagglutinin (HA) and neuraminidase (NA), as well as the membrane ion channel protein, M2. Within the virion, the matrix protein (M1) provides structure and secures the viral ribonucleoprotein (vRNP) complexes consisting of viral RNA coupled to nucleoprotein (NP) and the three polymerase proteins (PB1, PB2 and PA). The remaining viral proteins include the nonstructural proteins, NS1 and NS2, and the recently identified PB1-F2 protein found in some virus species. Alone, these 11 proteins are not sufficient to facilitate virus replication, and the

virus must infect a host cell to co-opt host proteins and pathways for the successful generation of progeny virus. Therefore, intracellular signaling events reflect the balance of virus replication and antiviral host responses. Understanding the virus-host interactions facilitating this balance could provide novel targets for disease intervention strategies.

RNA interference (RNAi) is an evolutionary conserved endogenous pathway to regulate gene expression in eukaryotes, and has been widely employed to study the impact of post-transcriptional gene silencing on biological processes. Meta-analysis of five recent RNAi-based studies that identified host genes important for influenza virus infection and replication [12–16], identified common pathways associated with steps in the virus life cycle [13,16–19]. Despite differences in methodology and reagents used in these studies, a level of accord was evident among the host cell pathways affected. Overlap was identified in pathways used for virus entry [16–19], fusion of the endosomal and viral membrane [13,17–19], transport of the viral components to the nucleus [14,15], as well as late events including export of the vRNP complex and RNA into the cytoplasm [12,13,16–18].

Viral infection triggers host responses that engage signaling networks which have a fundamental role in the anti-viral response. Previous studies have identified human protein kinases (HPKs) having key functions in influenza biology. By example these include protein kinase C (PKC) which is induced by viral binding

to cell surface [20,21], the extracellular signal-regulated kinase ERK [22,23] induced by accumulation of viral HA on the cell surface via PKC and regulating RNP export [24], and phosphatidylinositol-3 kinase (PI3K) [24]. The inhibition of this signaling network results in nuclear retention of the vRNP and decrease in influenza virus replication [22,25]. NF-κβ, a key mediator, is induced by accumulation of viral HA, NP and M1 proteins [25–34]. Additionally, the presence of viral dsRNA has been shown to activate signaling cascades involving IKK–NF-κβ, c-Jun N-terminal kinase (JNK), and P38 mitogen-activated protein kinase (MAPK) cascades all which regulate the expression of antiviral cytokines [35–37]. Together, these findings show the importance of HPKs in influenza virus replication making them targets for disease intervention strategies.

Post-transcriptional gene silencing by small non-coding micro-RNAs (miRNAs) has been recognized as an important mechanism of regulating host gene activity and is mediated via interaction between the miRNA seed site (nt 2–8 on the guide miRNA strand) and the 3′UTR of target gene(s) [38–40] to cause transcript decay or translational block. Owing to the short sequence complementarity between the miRNA "seed site" and the gene 3′UTR, >30κβhuman transcriptome is believed to be under miRNA regulation. It is thought that miRNAs have evolved to preferentially target and regulate key signaling nodes within a signal transduction network [41,42]. Though effects of miRNA per gene may be subtle, targeting multiple cellular pathways and targets lead to observable biological phenotypes. miRNAs can be considered molecular rheostats of gene function in contrast to siRNAs which catalyze significant reductions in gene mRNA levels. [39]. Antiviral signaling pathways, and the HPKs involved, are leading candidates for miRNA regulation due to dose-sensitivity and the fine-tuning nature of miRNAs [43]. MiRNA expression is modified in response to a variety of cell stimuli including virus infection, and studies have shown that viruses exploit miRNA deregulation for their own benefit. For example, miR-132 has been shown to be highly induced after herpes simplex virus-1 (HSV-1), and human cytomegalovirus (HCMV) infection, and to down-regulate the expression of interferon-stimulated genes thereby facilitating virus replication [44]. HSV-1 replication is suppressed when miRNA-101 (miR-101) targets a subunit of mitochondrial ATP synthase (ATP5B) [45]. Human immunodeficiency virus type 1 (HIV-1) down-regulates the expression of many cellular miRNAs [46], and for miR-17/92, miRNA suppression is required for efficient virus replication [47]. Influenza virus infection modulates multiple cellular miRNAs, and miR-323, miR-491, and miR-654 have been shown to inhibit viral replication by binding to the viral PB1 gene [48], while miR-507 and miR-136 have potential binding sites within the viral PB2 and HA genes [49]. Additionally, miR-26a and miR-939 regulate the replication of H1N1 influenza virus in MDCK cells [48]. These data show that miRNAs are important for governing aspects of the host response to influenza virus replication. While all these studies have identified patterns of miRNA deregulation following viral infection, pathways and genes regulated by these deregulated miRNAs remain largely unexplored.

The focus of this study was to identify kinases essential for influenza virus replication, and to determine miRNAs that regulate their expression. To this end, genetic screening was performed using siRNAs to identify and validate kinase genes required for infection or replication of A/WSN/33 influenza. Preliminary hits from the primary screen were independently validated using siRNAs targeting a novel seed site on the same gene, and multiple endpoint assays that included PCR to measure viral genome replication, TCID$_{50}$ to measure virus replication in

MDCK cells, and high-content analysis to quantitate intracellular levels and location of nucleoprotein, NP, staining. Eighteen HPKs were confirmed of which 3 were anti-viral and 15 pro-viral. Six of 18 HPKs modulated A/WSN/33 influenza virus replication as well as A/New Caledonia/20/99 virus as validated using the same endpoint assays. Computationally predicted miRNAs targeting the six HPKs were compared to miRNAs deregulated during influenza infection [50–54] to shortlist miRNAs for validation. Native miRNA activity of these miRNAs was inhibited or up-regulated using miRNA inhibitors or mimics, respectively, and their impact on gene expression and viral replication was analyzed using qRT-PCR and high throughput microscopy based screening. These studies showed that influenza deregulated multiple miRNAs involved in regulating HPKs found to be important for influenza replication. Together, these analyses identify novel miRNA-HPK interactions involved in influenza virus replication, and help to unravel principles of the virus-host interface.

Materials and Methods

Cell Culture and Viruses

To minimize biological variation, a single passage of A549 human lung epithelial cells (CCL-185, ATCC) and Madin–Darby canine kidney cells (MDCK, CCL-34, ATCC) were used for all assays from a frozen stock stored in 10% DMSO and 90% fetal bovine serum (FBS), in liquid nitrogen. The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, Thermo Scientific) supplemented with 5% heat-inactivated FBS (HyClone, Thermo Scientific) at 37°C and 5% CO$_2$. All cells were confirmed to be free of mycoplasma using a PlasmoTest kit (InvivoGen, San Diego, CA).

Influenza A/WSN/33 (H1N1) virus and A/New Caledonia/20/99 (H1N1) were grown in the allantoic cavities of 9-day-old embryonated chicken eggs as previously described [55]. Virus stocks were titrated in MDCK cells as previously described [56] and a 50% tissue culture infectious dose (TCID$_{50}$) was determined using the method described by Reed and Muench [57].

siRNAs and Transfection Assay

The siGENOME library is shipped as a series of 96 well plates with 0.5 nmol of lyophilized siRNA per well. The HPK library contains 9 master plates (a total of 720 gene targets). A siRNA arrayed library containing four pooled siRNAs per target gene for 720 different human protein kinase genes (Dharmacon siARRAY siRNA Library (G-003505 Human Protein Kinase Lot 08105), Thermo Scientific) was used for the primary siRNA screen. Controls included a siRNA targeting mitogen-activated protein kinase kinase 1 positive control (siMEK, 5′-GCACAUGGAUG-GAGGUUCU-3′, 5′-GCAGAGAGAGCAGAUUUGA-3′, 5′-GAGCAGAUUUGAAGCAACU-3′, 5′-CCAGAAAG-CUAAUUCAUCU-3′, siGENOME smartpool, Dharmacon M-003571-01), a negative non-targeting control siRNA (siNEG, 5′-UAGCGACUAAACACAUCAA-3′, siCONTROL Non-Targeting siRNA #1, Dharmacon D-001210-01-05), and a control for cellular cytotoxicity (TOX, Dharmacon D-001500-01-05). Each SMARTpool siRNA reagent used in the primary screen was validated for on-target activity by Dharmacon Thermo Fisher and consists of four rationally designed siRNAs targeting a distinct region of the target mRNA to achieve gene knockdown and reduce the incidence of off target effects (Table S1). To assure maximum silencing of target gene expression in our system, transfection and detection conditions were first optimized using the validated SMARTpool siMEK positive control (Figure S2A). The sequences for all siRNA duplexes provided with Dharmacon's

siGENOME libraries are proprietary and confidential and could not be listed. A list of the 720 HPK targeted by the library are listed in Table S1, as well as the siRNA sequences used for validation screen. All siRNAs were resuspended in Dharmacon siRNA buffer to a concentration of 1 µM and stored at −80°C.

All siRNAs were reverse transfected into A549 cells at a 50 nM final concentration using 0.4% Dharmafect 1 (Dharmacon) and incubated at 37°C and 5% CO_2 for 48 h. Cell cytotoxicity was evaluated in all siRNA transfected cells compared to the TOX positive control and non-target negative control number. A "percent cytotoxicity" of the controls is determined for each experimental siRNA and cytotoxicity was determined to be >20% based on the bioluminescent measurement of adenylate kinase (ToxiLight BioAssay Kit, Lonza) (Table S1). A SafireX$_2$ luminometer (Tecan U.S., Durham, NC) was used for the luminescence readout. All RNA interference (RNAi) experiments were carried out according to the Minimum Information for an RNAi Experiments (MIARE) guidelines [58].

Infection Assays

The infection assay involved 1 h incubation at 37°C with either A/WSN/33 or A/New Caledonia/20/99 at the indicated multiplicity of infection (MOI). After 1 h incubation at 37°C, cells were rinsed with PBS and replenished with fresh media without virus. Cells used to study A/WSN/33 were cultured and infected in DMEM supplemented with 5% FBS and cells used to study A/New Caledonia/20/99 in DMEM supplemented with 0.2% bovine serum albumin (Sigma Aldrich) and 1 µg/mL of TPCK trypsin (Worthington).

Supernatants were harvested 48 h after infection and the viral titers were determined by TCID$_{50}$ [59] or plaque assay [57] on MDCK cells. For TCID$_{50}$, 2×10^4 MDCK cells per well in 100 µl were plated in a 96-well flat-bottom cell culture plate. The virus samples were 1/10 serially diluted and each diluted sample was used to infect the replicate wells by incubating 50 µl of the sample on a confluent monolayer of MDCK cells at 37°C and 5% CO_2. At 72 h, the supernatants from all wells were transferred to a V-bottom plate. Chicken red blood cells (50 µl at 0.5%) were added and incubated for 1 h at 4°C. The agglutination wells were counted and used to determine TCID$_{50}$ values [57,60]. Plaque assays were performed with 1/10 serial dilutions of the virus samples on a confluent monolayer of MDCK cells, overlaid with Avicel containing either 5% FBS or 1 µg/ml TPCK-trypsin [56]. Cells were incubated for 72 h and then plaques were visualized by staining with 0.1% crystal violet.

Normalization and Z-score Analysis

The primary screen data was normalized by correcting the raw data for across plate variation. Specifically, the percent inhibition of infectious virus was calculated for each experimental siRNA such that the difference of the experimental HPK siRNA treated well (X$_{siHPK}$) was subtracted from the mean of the negative control well values ($_{siNEG}$) and then divided by the difference of the means of the negative control and the TOX control ($_{siTOX}$) for each plate:

$$\text{Percent Inhibition} = \frac{\overline{X}_{siNEG \; plate \; A} - X_{siHPK \; plate \; A}}{\overline{X}_{siNEG \; plate \; A} - \overline{X}_{siTOX \; plate \; A}}$$

The primary screen was performed in at least two independent experiments, and each experiment was performed in duplicate, yielding a dataset of ≥4 replicates for each X$_{siHPK}$. The mean of the replicates for each of the 720 X$_{siHPK}$ was calculated and the resulting dataset was standardized using Z-score analysis (Table S1), whereby the mean (µ) of the data becomes zero and the standard deviation (SD) becomes 1. A "positive hit" in the primary screen was considered to have a $\text{Z-score} \geq \mu \pm 2 \, \text{SD}$ using the formula below.

$$\text{Z-score} = \frac{(\text{Normalized score} - \text{mean of the population})}{\text{SD of the population}}$$

Quantitative Real-time PCR

The RNA from A549 cells was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity of total RNA was determined using a NanoDrop ND-1000 Fluorospectrometer (NanoDrop Technologies, Wilmington, DE). Equal amounts of RNA were then reverse transcribed to cDNA using random hexamers and MuLV reverse transcriptase (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems) in accordance to the manufacturer's protocol.

For quantification of influenza M gene expression, qPCR was performed using 200 nM internal probe (M +64, 5′-FAM-TCA GGC CCC CTC AAA GCC GA-BHQ-1-3′), 400 nM forward primer (M +25, 5′-AGATGAGTCTTCTAACCGAGGTCG-3′), and 400 nM reverse primer (M-124, 5′-TGCAAAAACATCTT-CAAGTCTCTG-3′) following a previously described TaqMan assay [61]. The cycling conditions for qPCR were 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 15 sec. The qPCR was carried out and analyzed with a Stratagene Mx3005P instrument and software (La Jolla, CA). Copy numbers were determined by generation of a standard curve using plasmid DNA encoding influenza M gene [61]. Results reported for these studies were the averages of at least three replicates.

To determine the gene silencing efficiency associated with siRNA treatment, qPCR was performed using QuantiTect SYBR Green PCR Master Mix (Qiagen) according to the manufacturer's instructions with the primer sequences described in Table S4. Relative expression level was calculated using the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Fold changes were calculated against the mean of negative control siRNA treated cells. Methodology and data analysis for all qPCR experiments were carried out according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [62].

Indirect Immunofluorescence to Detect Influenza NP

A549 cells were fixed with 3.7% formaldehyde and permeabilized with 0.5% Triton X-100 (Sigma), 10% FBS (Hyclone) in PBS. A549 cells were incubated with a primary antibody against the viral NP (25 µg/mL, H16-L10, HB65; ATCC) diluted in PBS with 10% FBS for 1 h at room temperature. Following this, the cells were incubated with Alexa-488 goat anti-mouse secondary antibody (A11001, Invitrogen) diluted 1:500 in PBS with 10% FBS for 1 h in the dark, and the cells were subsequently stained for 20 min with 1 µg/mL of 4′-6-Diamidino-2-phenylindole stain (DAPI, Invitrogen). The cells were imaged using the EVOS digital inverted fluorescent microscope (Advanced Microscopy Group, Bothell, WA) at two wavelengths, 488 nm to detect Influenza infected cells expressing NP, and 350 nm for nuclear DNA bound by DAPI. For quantification of NP immunofluorescence, cells were fixed, permeabilized and stained as above and 20× images were acquired and analyzed using Cellomics ArrayScan VTI High

Content Imager and Cellomics ArrayScan software (Thermo Fisher Scientific).

Indirect Immunofluorescence to Detect HPK Expression

A549 cells in 96 well plates were transfected with HPK specific miRNA inhibitors or mimics and incubated for 48 h. Cells were fixed with 4% formaldehyde in PBS and permeabilized with 0.5% TritonX-100 in PBS/20 min/RT. Cells were incubated with biotinylated antibodies against HPKs (10 ug/ml) diluted in PBS+5% BSA for 2 h at room temperature or 4°C overnight, washed with PBS (10 min×3) and incubated with Streptavidin-Alexa 488 conjugated secondary antibodies for 3 hrs. Excess secondary antibody was removed by washing with PBS thrice (10 min each wash) and nuclei were finally stained with 4′-6-Diamidino-2-phenylindole stain (DAPI, Invitrogen) for 10 min at RT. Plates were scanned with Cellomics ArrayScan VTI scanner using Target Acquisition protocol at 20× magnification. DAPI fluorescence was measured in channel 1 and Alexa 488 fluorescence was measured in channel 2. A minimum of 5000 valid channel 2 objects were counted per well in triplicate for each mimic/inhibitor treatment. Data was exported into csv files and statistically analyzed using GraphPad Prism version 5.0.

miRNA Target Prediction and Literature Analysis

miRNAs targeting the selected HPKs were mined using miRWalk [63]. Briefly, miRWalk tabulates the miRNAs targeting a gene of interest based on predictions from multiple algorithms and can be used to either find miRNAs targeting a gene(s) or vice versa. Lists were exported into spreadsheets containing data on miRNA deregulation during influenza infection and compared. Most predictions with significant scores have (1) a significant seed region match and (2) are evolutionarily conserved as per TargetScan [64].

miRNA Validation

A549 cells (2×10^4) were transfected with either a miRIDIAN miRNA antagonist (25 nM), a miRIDIAN miRNA agonist (12.5 nM) (Dharmacon), or a siRNA targeting the HPK under study using Lipofectamine 2000 (Invitrogen) per the manufacturer's protocol. miRNA let-7f (expressed at ∼750 copies in A549 cell [65]) was used to optimize inhibitor or mimic concentrations, and at 25 nM miRNA antagonist concentration, generally reduced miRNA expression by >85%, while transfection with 25 nM miRNA agonists led to an increase in miRNA levels (Figure S4). The miRIDIAN miRNA antagonists are chemically modified dsRNAs consisting of a central region complementary to the mature miRNA flanked by 0-16 nt long sequences with reverse complement to pri-miRNA to enhance specificity and reduce RISC incorporation [66–68]. Conversely, agonists are chemically modified dsRNAs that increased the guide strand concentration in RISC complex, thus increasing native miRNA mediated repression [68,69]. Cells treated with an equal concentration of a non-targeting control sequence (mock) were used to control for non-sequence-specific effects in miRNA experiments. After 48 h post-transfection, the cells were infected (MOI = 0.01) with A/WSN/33 for 48 h. Virus replication and HPK knockdown was assayed by high content microscopy and HPK expression was analyzed using qPCR (for transcript) and high content screening (for protein). For qPCR, 18S rRNA was used as housekeeping control. Fold changes were calculated against the mean of mock treated cells.

Statistical Analysis

Statistical analysis for validation screen, pathway analysis, and miRNA studies were performed using Student's t test with the GraphPad Prism 5 software. Statistical significance (p<0.05) is indicated by a single asterisks or double asterisks if highly significant (p<0.001).

Results

RNAi Screen Identifies Host Kinase Genes Important for Influenza Virus Replication

HPKs are key mediators of signaling events in a cell and inhibition of HPKs has been shown to reduce progeny virus production both *in vitro* and *in vivo* [70]. To better understand the contribution of HPKs during influenza virus replication, an RNAi-based genetic screen was performed and the hits validated (Figure S1). siRNA SMARTpools individually targeting 720 different HPKs were transfected into A549 cells followed by influenza A/WSN/33 infection. Levels of infectious virus were determined by $TCID_{50}$ and the effects of siRNA treatment quantified using a z-score method where a positive z-score (>2.0) indicates HPK knockdown resulting in an increase in viral replication and a negative z score (<−2.0) value indicates a resulting decrease in virus replication. Using the $|z|\pm>2.0$ identified 22 of the 720 HPK genes as modulators of influenza A/WSN/33 replication (Table 1; Figure S2A+B) of which 3 (NPR, MAP3K1, DYRK3) increased influenza virus replication and were designated as anti-viral HPKs, while silencing 19/22 genes (EPHA6, TPK1, PDK2, C9ORF96, EXOSC10, NEK8, PLK4, SGK3, NEK3, PANK4, ITPKB, CDC2L5/CDK13, CDK3, CALM2, PRKAG3, ERBB4, ADK, PKN3, HK2) decreased influenza virus replication compared to siNEG transfected cells and were designated pro-viral HPKs (Table 1, Figure 1A, Figure S2B). Cells transfected with the positive control, i.e. siMEK, had consistently lower influenza virus titers compared to siNEG transfected cells (Table S1).

To rule out false positive/negative hits due to potential siRNA off-target effects [71], primary hits were retested using a novel synthetic siRNA targeting the same gene but at a different seed site (Table S2), and HPKs that exhibited identical phenotypes as the primary screen were thus validated. Transfection of siRNAs targeting NPR2, **MAP3K1, DYRK3,** EPHA6, TPK1, PDK2, EXOSC10, **NEK8, PLK4, SGK3,** NEK3, PANK4, ITPKB, **CDK13,** CALM2, ADK, PKN3, and HK2 followed by gene specific qPCR demonstrated >80% silencing of the target mRNA (Figure S3). The novel siRNAs targeting genes CDK3, PRKAG3, ERBB4, and C9ORF96 were unable to knockdown mRNA expression and were excluded from the validation screen. Transfection of A549 cells with the novel siRNAs for the 18 HPKs followed by A/WSN/33 influenza infection modulated influenza replication significantly (p<0.01) similar to those obtained in the primary screen confirming the relevance of the HPK genes (Figure 1A). These findings were substantiated with additional endpoint assays that included measurement of viral genome replication by qPCR (Figure 1B), and influenza nucleo-protein determined by high content analysis (Figure 1C). Thus, 17 of 18 HPK hits repeated the original screen phenotype, i.e. increased or decreased virus replication. Identifying 22 hits out of 720 in the primary screen yielded a positive hit rate of 3.1% which is consistent with other related screens [12–16]. The majority of the hits were novel; however, PANK4, NEK8, ITPKB, CALM2 and HK2 have been identified in other influenza screens (Table 1; Table S3) [12–16]. A meta-analysis of the validated HPK hits combined with those previously identified revealed that although specific genes are not consistently identified, many genes are in
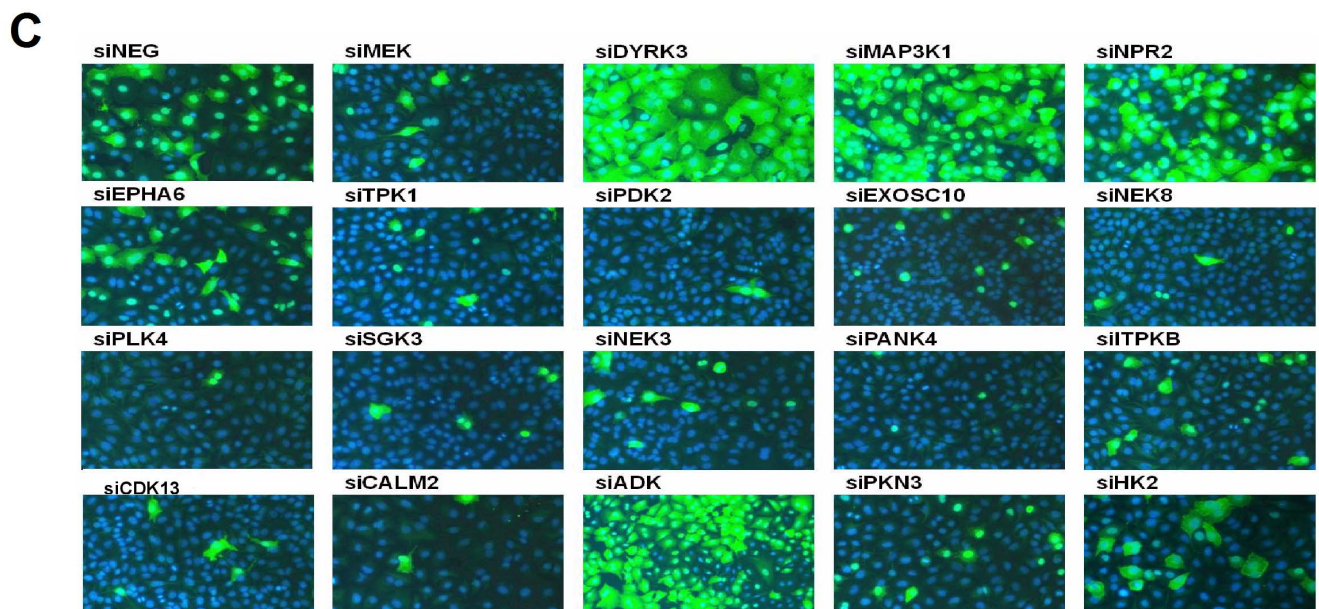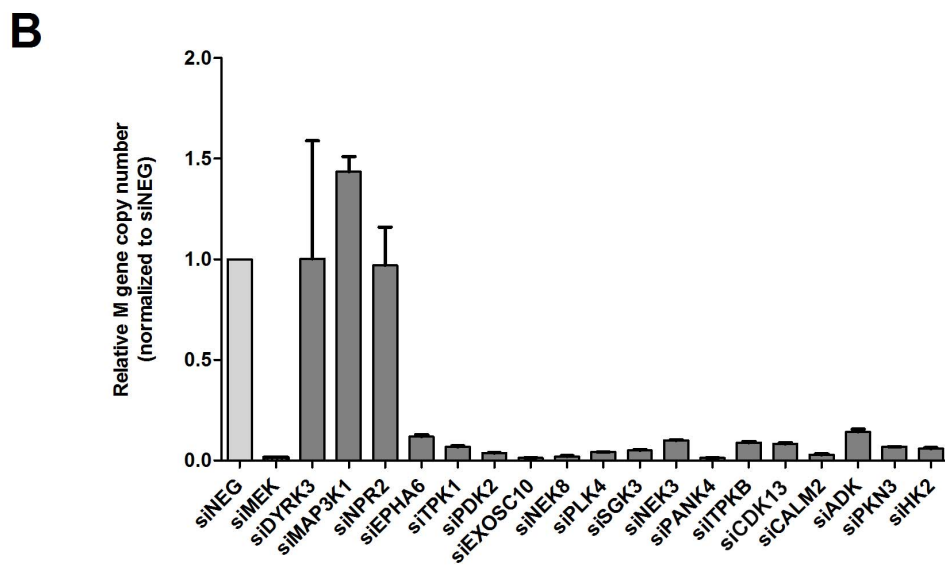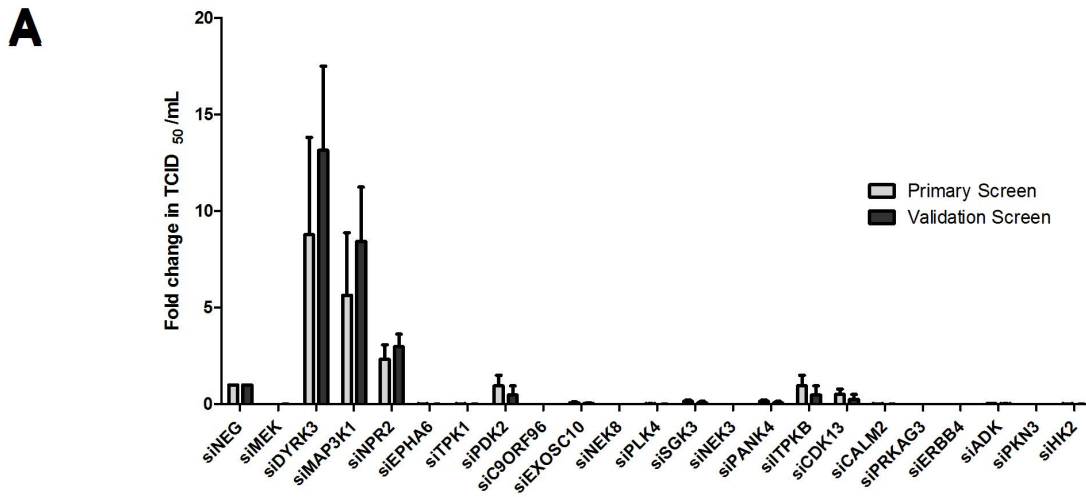
A

B

C

Figure 1. Validation of human protein kinase genes affecting influenza virus replication. A549 cells were reverse transfected with 50 nM of a non-target negative control siRNA (siNEG) or with siHPK and infected at a MOI = 0.01 with A/WSN/33. (A) 72 hours after infection, supernatants were harvested and the viral titers were determined by $TCID_{50}$ assay on MDCK cells (B) After a 48 hour infection, the effect of siRNA silencing on influenza virus replication was measured by quantifying the levels of influenza M gene expression. The RNA from siRNA-transfected and WSN-infected A549s was isolated and used for quantification with an influenza M-specific primer/probe set. Light gray bars indicate controls. Data show mean±SEM from 3 independent experiments. (C) After a 48 hour infection, cells were fixed, permeabilized and incubated with an anti-influenza virus NP monoclonal antibody and subsequently with an Alexa-488 goat anti-mouse secondary antibody and DAPI. The intracellular distribution of the viral RNPs (NP, green) and cellular nuclei (DAPI, blue) are shown.
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shared cell pathways important for influenza including PI3K/AKT signaling, NFKB, PKC/CA++ signaling, and p53/DNA damage pathways [72].

For the hit selection, it is important to identify false-negatives in which siRNAs with larger effects are not selected, and false-positives in which siRNAs with negligible effects are selected as hits. Assessment of the false-negative rate in these studies using a z-score $\geq \mu \pm 2$ SD revealed a rate of 87.5%. If the potential hits are expanded to those fitting a z-score $\geq \mu \pm 1SD$, the false-negative rate decreases to 23% because 37 HPK genes can be identified that overlap with HPK genes discovered in related published screens [12–16]. Since only the ADK gene was not validated, the false-positive rate in the primary screen was 4.5% (1 out of 22). This good false-positive rate is linked to the assay conditions, i.e. using pooled siRNA duplexes reported to reduce the rate of false-positives [73].

Validated HPK Genes Affect Replication of Different Influenza Strains

Influenza virus A/WSN/33 was chosen for the primary and validation screens based on its ability to replicate without exogenous trypsin. However, because it is a lab-adapted strain, another influenza virus strain was tested to validate the HPK hits important for influenza virus replication. Therefore, A549 cells were transfected with siRNAs targeting the hit HPKs and subsequently infected with influenza A/New Caledonia/20/99 (Figure 2). Of the 17 HPK genes identified important for A/WSN/33 replication, six (CDK13, HK2, NEK8, PANK4, PLK4, SGK3) emulated the phenotype following A/New Caledonia/20/99 infection, i.e. increased or decreased virus replication as measured by influenza NP localization (Figure 2A) and influenza M gene levels (Figure 2B). In addition, four of the six (HK2, NEK8, PANK4, PLK4) have also been identified important for influenza virus replication in other influenza-genome screens (Table 1; Table S3) [13,15,16].

Table 1. Human kinase genes identified important for influenza virus replication in primary screen.

Symbol	Name	Z-score	Log_{10} $TCID_{50}$/mL
NPR2	natriuretic peptide receptor B/guanylate cyclase B	2.64	6.5
MAP3K1	mitogen-activated protein kinase kinase kinase 1	2.55	5.7
DYRK3	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3	2.23	5.9
EPHA6	EPH receptor A6	−2.01	3.0
TPK1	thiamin pyrophosphokinase 1	−2.01	3.0
PDK2	pyruvate dehydrogenase kinase, isozyme 2	−2.03	2.7
C9ORF96	chromosome 9 open reading frame 96	−2.16	2.7
EXOSC10	exosome component 10	−2.16	2.7
NEK8*	never in mitosis gene a- related kinase 8	−2.18	2.7
PLK4*	polo-like kinase 4	−2.18	1.7
SGK3	serum/glucocorticoid regulated kinase family, member 3	−2.19	2.7
NEK3	never in mitosis gene a-related kinase 3	−2.22	2.7
PANK4*	pantothenate kinase 4	−2.35	1.7
ITPKB*	inositol 1,4,5-trisphosphate 3-kinase B	−2.35	1.7
CDC2L5	cell division cycle 2-like 5	−2.38	1.7
CDK3	cyclin-dependent kinase 3	−2.40	1.7
CALM2*	calmodulin 2	−2.59	n.v.
PRKAG3	protein kinase, AMP-activated, gamma 3 non-catalytic subunit	−2.59	n.v.
ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4	−2.59	n.v.
ADK	adenosine kinase	−2.59	n.v.
PKN3	protein kinase N3	−2.59	n.v.
HK2*	hexokinase 2	−2.59	n.v.

*Genes identified important for influenza in a previous screen; n.v., no detectable virus.
List of HPKs with Z scores ±>2.0 identified in primary screen. Positive z-scores indicated anti-viral HPKs while negative z-scores indicate pro-viral HPKs.
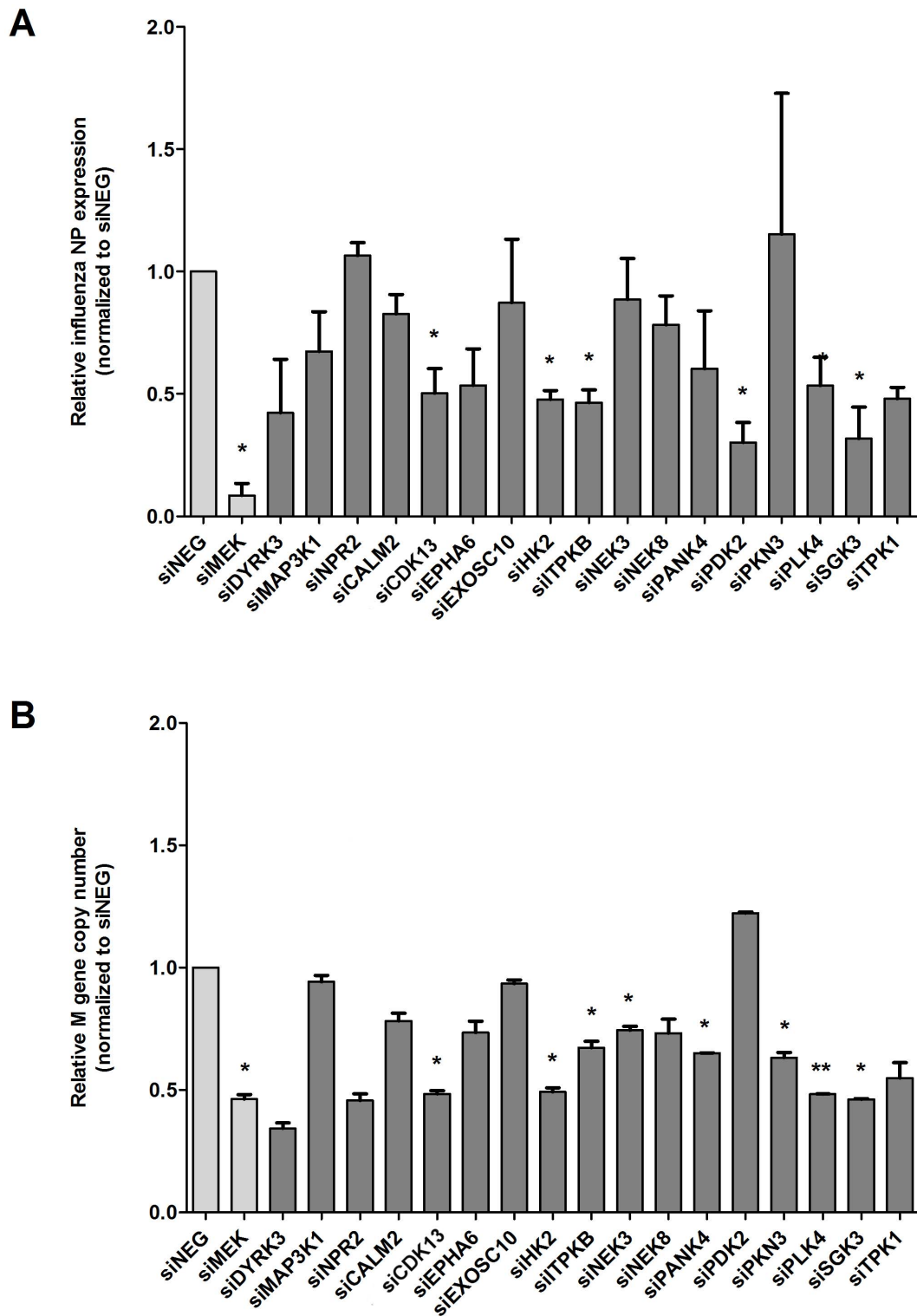doi:10.1371/journal.pone.0066796.t001

Figure 2. Human protein kinase genes affect H1N1 A/New Caledonia/20/99 virus replication. A549 cells were reverse transfected with 50 nM of siRNA specific for validated HPK genes and after 48 h the cells were infected with A/New Caledonia/20/99 at an MOI of 0.01 in the presence of 1 µg/ml TPCK-trypsin. After 48 h of infection, RNA was extracted and the effect of siRNA silencing of HPK genes on viral genome replication was measured by quantifying (A) influenza NP expression and (B) the level of influenza M gene. Data show mean±SEM of 3 independent experiments. *p<0.05 and **p<0.001 compared to control.
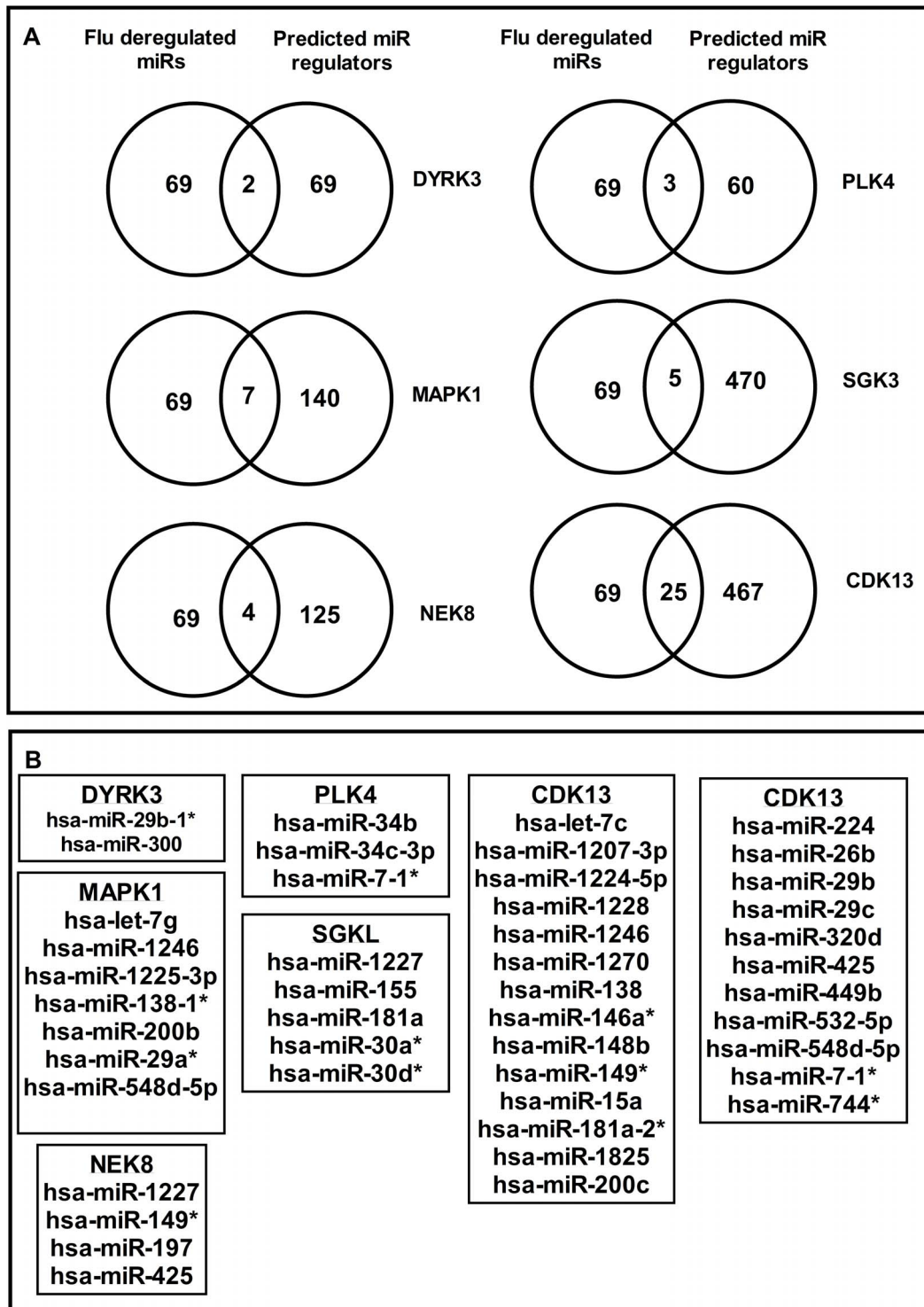doi:10.1371/journal.pone.0066796.g002

Figure 3. Identifying miRNA regulators of HPKs important for influenza replication. (A) Venn diagrams showing miRNAs common to computationally predicted miRNA regulators and influenza deregulated miRNAs. (B) miRNAs that are shared between computationally predicted HPK regulators and miRNAs deregulated during influenza infection.
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Systematic Analysis of miRNAs Predicted to Govern HPK Genes

Deregulation of cell cycle pathways is a hallmark of influenza virus infection and replication [74–76]. Meta-analysis of micro-array data from individuals with mild or severe influenza infections identified cell cycle control and apoptosis as the major pathways kinked to severity and outcome of influenza infection [75]. Four of the 6 validated HPKs, i.e. CDK13, NEK8, PLK4 and SGK3, have roles in cell cycle regulation. Similarly, two of 3 anti-viral
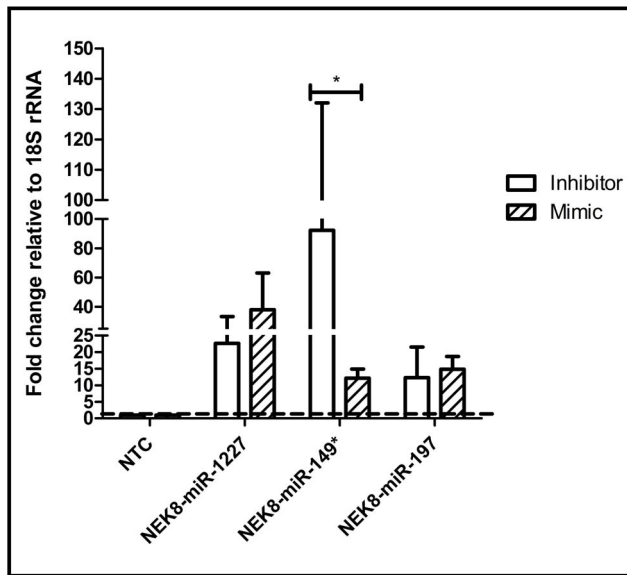
Figure 4. miRNA regulators of NEK8. A549 cells were transfected with 25 nM of miR-1227, -149* and -197 inhibitor/mimic for 48 hrs followed by RNA extraction and RT-qPCR with NEK8 specific primers. Expression data was normalized to 18S rRNA expression and shown as mean±SEM of independent experiments. *p<0.05 compared to control.

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HPKs (MAP3K1, DYRK3) are also implicated in regulation of cell cycle. Specifically, CDK13 has been shown to interact with L-type cyclins and regulate alternative splicing [77], as well as increase HIV mRNA splicing, and upon silencing leads to increased HIV replication via phosphorylation of serine/arginine-rich splicing factor 1 (SRSF1/ASF/SF2) [78]. NEK8 phosphorylates bicaudal D (Bicd2) [79], binds to centrosomes and regulates ciliogenesis [80]. PLK4 also regulates centriole duplication during cell cycle [81–83]. Repressing the autoregulation of PLK4 leads to centrosome amplification and increased p53 activity [84]. PLK4 activity is crucial for organization of the centrosome, the main microtubule organizing center (MTOC) in the cell that regulates not only cell division, but also movement of intracellular organelles such as late endosomes and lysosomes. Organization of the MTOC is important for influenza virus infection and replication since RNAi depletion of genes such as Histone deacetylase 8 (HDAC8) that are involved in MTOC organization led to reduced motility of early endosomes and lysosomes and misdistribution of intracellular vesicles and organelles [85]. SGK3 belongs to the three member family of serum glucocorticoid kinases (SGK1, 2 and 3), and has been shown to regulate influenza vRNP nuclear export into the cytoplasm [86]. SGK3 has been implicated in regulating cell survival [87]. MAP3K1 is a multifunctional protein and important for induction of IFN-β induction in response to poly I: C challenge via IRF-3 activation [88]. MAP3K1 also inhibits expansion of virus specific CD8+ T cells [89]. DYRK3 belongs to a family of dual specificity tyrosine kinases that activate by auto phosphorylation and catalyze phosphorylation of histone H3 and H2B. DYRK3 phosphorylates and activates sirtuin 1 (SIRT1) turnover, causes deacetylation of p53 and increased apoptosis [90]. Influenza virus infection upregulates mTORC1 signaling pathway [91] and inhibition of mTORC1 can significantly delay mortality due lethal challenge of influenza virus in mice [92]. DYRK3 has been shown to stabilize P-granule like structures and the mTORC1 pathway during

cellular stress. Inactivation of DYRK3 traps mTORC1 inside cytosolic stress granules while activation of DYRK3 promotes dissolution of stress granules and release of mTORC1 [93]. These observations show that the validated HPKs affect critical pathways during influenza infection and replication, thus miRNA regulation of these genes were examined during influenza infection.

miRNAs regulate multiple aspects of the host response to infection. While *in vivo* deregulation of host miRNA expression associated with influenza infection has been established, the pathways by which cellular miRNAs modulate host gene expression during influenza virus infection remain largely unexplored [94,95]. Analysis of miRNA regulation of the HPKs was performed using existing data on miRNA and mRNA expression during influenza virus infection. qPCR assays for expression profiling of the HPKs (Figure S5) and analysis of gene expression omnibus (GEO) datasets GDS3919 [75], GDS3919 [75], GDS3595 [96] and GDS2762 [97] indicated that HPKs could be shortlisted to a variable extent based on those that are expressed in vitro and in vivo in mice during influenza infection and replication. To identify miRNAs that regulate these HPKs, a list of miRNAs deregulated during influenza infection (Table S5) was compared to computational predictions for NEK8, PLK4, SGK3 and CDK13, MAP3K1 and DYRK3 genes [98] (Figure 3A) providing a shortlist of miRNAs for experimental validation (Figure 3B). Details of miRNA seed match with target gene 3′UTR are given in Table S6. A panel of miRNA inhibitors and mimics that have been shown to consistently prevent or increase the incorporation of miRNA guide strand into the RISC complex [68] were used to modulate native miRNA activity. Previous studies have established that 25 nM of miRNA inhibitor reduces native miRNAs ≥85% in 24 h and is not cytotoxic (Figure S4) [99]. Thus, a miRNA concentration of 25 nM was used in all transfection assays. An important caveat of this assay is that while miRNA inhibitors are miRNA-specific and able to distinguish between different members of the same miRNA family, miRNA mimics can affect native levels of all members of a miRNA family especially when the seed sites are conserved. Based on the dogma of miRNA action and our own prior studies [99,100], we expected a small but significant increase in target gene transcript/protein expression upon miRNA inhibition, and an opposite phenotype upon mimic supplementation. A549 cells were transfected with miRNA inhibitors or mimics, the cells assayed for cytotoxicity, and subsequently processed for HPK-specific qPCR to evaluate HPK gene expression, as well as gene-specific protein levels by anti-HPK antibodies. In parallel, similarly transfected A549 cells were infected with A/WSN/33 (MOI = 0.001) for 48 hrs, fixed and stained for influenza NP protein using an Alexa-488 coupled anti-NP antibody and analyzed using a high throughput Cellomics ArrayScan VTI microscope (Thermo Fisher). Data represent means of ≥ 5000 cells counted from at least 20 fields of triplicate wells for each treatment. Though all predicted miRNA HPK pairs were analyzed (Figure 3A and B), only data on miRNAs which impact HPK and/or virus replication is discussed hereafter. miRNAs targeting DYRK3, CDK13 and SGK3 did not alter HPK expression or viral replication and are not discussed further. Inhibition of miR-149* led to ~10 fold NEK8 induction of transcript, but not protein, while miR-149* mimic transfection reduced NEK8 transcript expression below the level of the control (Figure 4). Though NEK8 transcript is significantly induced by 24 hrs post A/WSN/33 infection (Figure S5). NEK8 protein expression was not readily detected as the native level of NEK8 has low level expression even in mock and NTC transfected cells. This is due to rapid proteasome mediated degradation of NEK8 protein [80]. No effect of mR-149* modulation on influenza NP
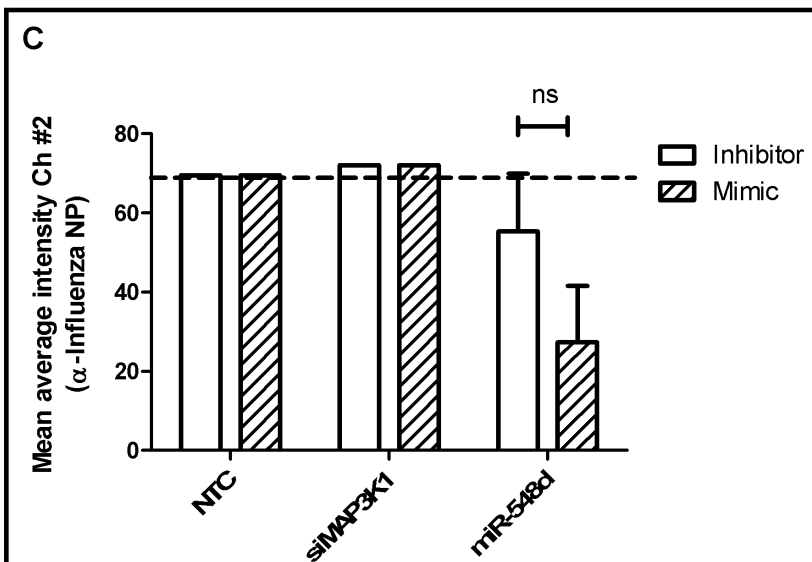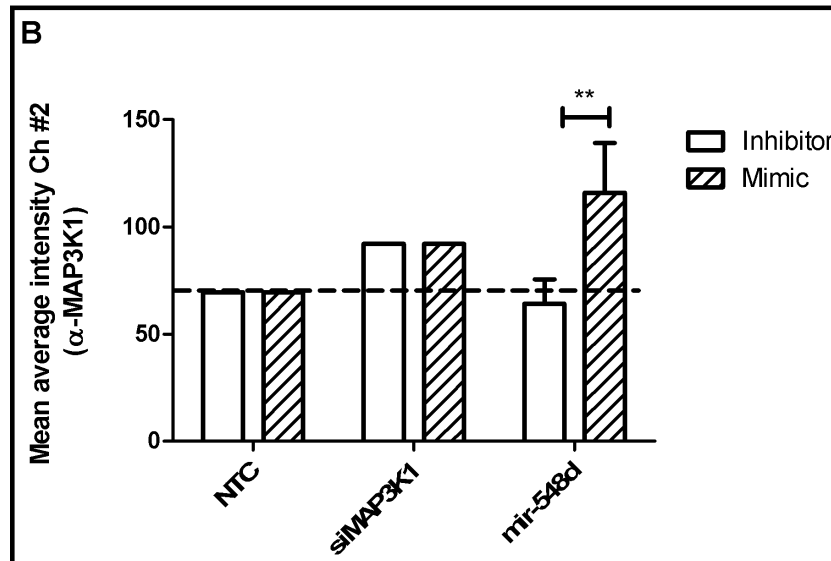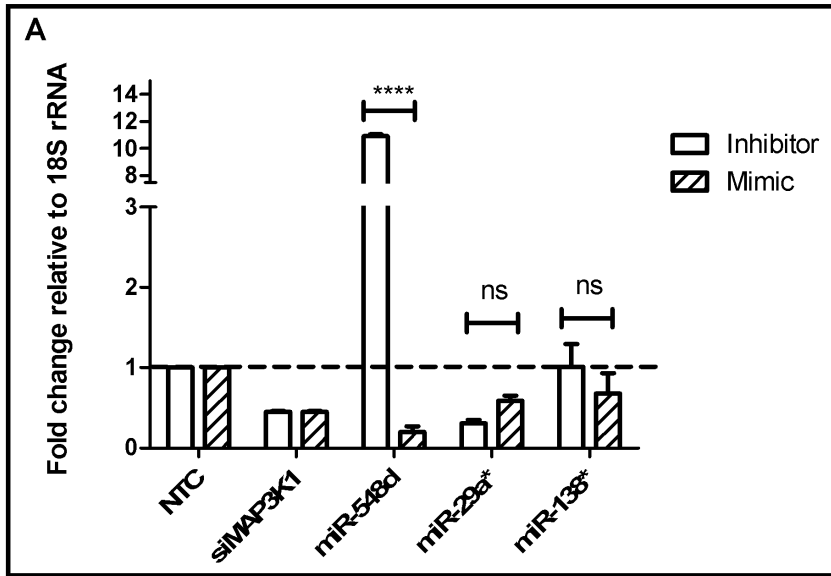
Figure 5. miRNA regulators of MAP3K1. (A) A549 cells were transfected with 25 nM of miR-548d, -29a and -138 inhibitor/mimic for 48 hrs followed by RNA extraction and RT-qPCR with MAP3K1 specific primers. Expression data was normalized to 18S rRNA expression and shown as mean±SEM of independent experiments. *p<0.05 compared to control. (B) A549 cells mock/transfected with miR-548d inhibitor/mimic for 48 hrs were fixed with 4% formaldehyde in PBS and stained for MAPK1 protein using biotinylated rabbit anti-MAP3K1 antibody (Abcam ab69533) and detected with Streptavidin-Alexa488. Cells were analyzed by Arrayscan Cellomics VTI scanner and data analyzed by GraphPad Prism. (C) A549 cells mock/transfected with miR-548d inhibitor/mimic for 48 hrs were infected with A/WSN/33 (MOI=0.001) for 48 hrs and stained for influenza NP protein using mouse-anti-NP coupled to Alexa-488 and analyzed as above in (B). Data show mean±SEM of two independent experiments. *p<0.05.
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staining was evident suggesting that under the conditions of assay, NEK 8 transcript modulation by miR-149* did not have any effect on viral replication. Indeed, miR-149* induction during influenza infection has been reported to occur only post 72 hrs (Table A1 from [50]) and this could explain the lack of effect of miR-149* inhibition on NEK8 protein. MAP3K1 transcript expression was significantly up-regulated by miR-548d inhibitor treatment while the mimic down-regulated MAP3K1 transcript expression (Figure 5A). miR-29a or miR-138* treatments did not have any appreciable effect on MAP3K1 expression (data not shown). miR-548d inhibitor/mimic treatments did not alter MAP3K1 protein expression (Figure 5B). This could be attributable to off-targeting given the modest expression of miR-548d during H1N1 infection (Table SA5 from [50]), and the significant "seed identity" between the 68 members of the miR-548 family (Text S1). Alternatively, seed shifting [101] and/or the indirect effects of miR-548d on MAP3K1 via miR-548d regulation of IFN-λ1 may have contributed to the outcome [102]. Similar to the NEK8 findings, MAP3K1 transcript modulation by miR-548d did not alter viral replication.

The most significant effects were observed for miR-34c and the PLK4 gene. While miR-34b and let-7i inhibitor/mimic treatments had no substantial effects on PLK4 transcript and protein expression (data not shown), miR-34c mimic considerably up-regulated PLK4 transcript (Figure 6A) and protein expression (Figure 6B), as well as influenza NP levels (Figure 6C). These observations are contrary to the dogma that miRNA inhibitor treatment should induce target gene expression, while mimic treatment should repress target gene expression. We believe these observations tie in well with known deregulation of cell cycle during influenza virus replication. Influenza infection has been shown to substantially alter the activity of p53, a master regulator of cell cycle via the NS1 protein [103–105]. Influenza NS1 associates with p53 [105] and RhoA protein [74] to regulate the host anti-viral response [103] causing cell cycle arrest in G0/G1 phase which is conducive to influenza replication. MiR-34c (and also miR-149*) expression is driven by p53 activation during influenza infection [104] to negatively regulate the activity of the transcription factor, Myc, which regulates S phase progression and DNA replication [106,107]. Inhibition of miR-34c accelerates S phase, promotes excessive DNA synthesis, and affects the Myc-regulated gene, Bcl2, which is important for cell cycle control. In contrast, the MiR-34c mimic affects Myc activity and arrests the cell cycle in S phase [106]. During cell cycle, the PLK4 transcript and active phosphorylated PLK4 kinase are detected at S phase, peak during M phase, and are subsequently degraded by proteasome-mediated protein decay [82,83]. miR-34c arrest of S phase cell cycle would prevent degradation of PLK4 transcript and protein, and thus help to explain the observation of increased PLK4 transcript and protein following miR-34c mimic treatment. Additionally, members of the miR-34 family, e.g. miR-34a/b-5, have been shown to enhance translation of neuronal tissue-specific polyadenylated transcript of β-actin [107]. Thus, a miR-34c mimic transfection can cause S phase cell cycle arrest and promote translation of target transcripts. Cells arrested in S phase would not be able to complete centriole duplication and accumulate

increased PLK4 transcript and protein as was observed in this study. Previous studies demonstrated that cells arrested in G0/G1 or S phase are more conducive to influenza virus replication relative to mock treated cells, or cells in G2/M phase and our observations thus provide mechanistic insight into these findings [76]. Thus, we hypothesize that during influenza virus infection, NS1 mediated p53 up-regulation triggers miR-34c activity to regulate cell cycle through Myc, PLK4 and NEK8 (Figure S6). While miR-34c is believed to primarily target c-Myc [108], and the findings from this study show that miR-34c positively regulates PLK4, other miR-34c targets cannot be discounted and warrant investigation. Interestingly, miR-34c-3p has been shown to be a major miRNA induced during infection by H1N1 and H5N1 influenza viruses [50], although its role in influenza biology is presently incompletely understood. Thus, these findings have helped to identify important miRNA regulated kinase pathways required for influenza infection and replication.

Discussion

The study of influenza virus biology has revealed complex mechanisms by which the influenza virus co-opts host cellular pathways to facilitate virus replication and evade the antiviral response. With the application of genome-wide screens, interactions between the virus and specific host cell components are now being identified and have led to new insights into viral and host interactions at different stages of the life cycle. A direct outcome of these studies can be the repurposing of old drugs for new conditions as was recently demonstrated for influenza [109].

A primary objective of this study was to identify human protein kinases that regulate influenza virus replication, and determine how miRNAs may govern their expression during influenza infection. A genome-wide siRNA screen of 720 HPK genes was evaluated. This screen identified 22 HPK that upon silencing led to increased or decreased influenza virus replication. Three HPKs (NPR, MAP3K1 and DYRK3) when silenced led to increased viral replication, suggesting that they have anti-viral activity. The remaining 19 HPKs when silenced decreased virus replication, suggesting that they are pro-viral and indispensable for viral replication. The preliminary hits identified from the primary screen were silenced using siRNAs targeting a novel seed site in the same gene and tested for impact on viral replication using multiple endpoint assays that evaluated viral genome, virus replication, and NP staining. A total of 18 HPKs (NPR2, MAP3K1, DYRK3, EPHA6, TPK1, PDK2, EXOSC10, NEK8, PLK4, SGK3, NEK3, PANK4, ITPKB, CDK13, CALM2, ADK, PKN3, and HK2) passed this validation and were tested with a seasonal strain of influenza virus where six HPKs that were identified as necessary for replication. It should be noted that other genes identified in the primary screen, i.e. CDK3, PRKAG3, ERBB4, and C9ORF96, were excluded from validation studies only because their expression was not silenced with the novel siRNA, and these HPKs may also have a role in virus replication. One primary screen hit, ADK, showed reduced M1 levels measured by qPCR, but did not validate in our NP localization analysis. Genomic changes would be expected to be subtle compared to changes in

stained for influenza NP protein using mouse-anti-NP coupled to Alexa-488 and analyzed as above in (B). Data show mean±SEM of two independent experiments. *p<0.05.
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NP localization and could account for these findings. To date, five independent genome-wide screens in mammalian cells have characterized host factors important for influenza virus replication [13,16–19]. A minimal overlap between screens was observed, but core pathways were found to be conserved. The minimal overlap between different virus strains is expected since the tempo of signal transduction and host gene expression is differentially induced by different virus strains which is linked to differences in replication dynamics and virus yield [110]. It is also likely that different influenza viruses may use alternate pathways for virus replication as host pathways were generally found to be conserved between screens [72]. This may be particularly relevant as A/WSN/33 which is a mouse-adapted virus and A/New Caledonia is a human virus.

The experimental approach used in this study to analyze influenza virus replication at 48 h post-siRNA transfection limits the findings to the later phases of the viral life cycle and may preclude HPKs that are important in the earliest phases of the viral life cycle. To avoid an extensive cross-talk due to cellular signaling post-infection, a very low MOI of infection was used to allow sufficient viral replication and avoid excessive cytopathic effect. Importantly, the screen in this study was validated using three endpoints to confirm the effect of host gene silencing on influenza virus replication. These endpoints included determining infectious virus titers as measured in MDCK cells, viral genome replication as determined by qPCR measurement of influenza M gene expression, and influenza NP as determined by high content analysis.

From the HPKs identified in the secondary validation screen, four pro-viral (NEK8, PLK4, SGK3 and CDK13) and two anti-viral (MAP3K1 and DYRK3) HPKs were assessed for miRNA regulation, and identified three HPKs (two pro-viral and one antiviral) (CDK13, MAP3K1 and PLK4) were found to have differential expression upon corresponding miRNA inhibitor/ mimic treatment suggesting that these miRNAs likely regulate these HPKs. Inhibitor/mimic treatments for two genes DYRK3 and SGK3 did not cause significant changes in transcript expression and hence were not pursued further. miRNAs-1227 and -149* inhibitor/mimic modulated NEK8 transcript expression though differences between inhibitor/mimic were significant only for miR-149*. miR-149* treatment did not affect NEK8 protein expression however and hence was not studied further. miR-149* is known to induce apoptosis by repressing Akt1 and E2F1 [111] and hence inhibition of miR-149* may explain increased NEK8 transcript levels. Contrary to current dogma that miRNA inhibition relieves native miRNA inhibition and causes increased expression of target genes, and vice versa, it was observed that miRNA inhibition reduced transcript levels for CDK13, MAP3K1 and PLK4 while mimic treatment induced protein expression. This may be either by off-targeting by miRNA inhibitor/mimics studies or by stabilizing target gene expression by poorly understood alternate mechanisms [98,112–114]. miR-548d inhibitor mediated MAPK1 transcript induction was not observed at the protein levels (Figure 5A–B). Expression of PLK4 transcript and protein were significantly modulated by miR-34c inhibitor/ mimic and these also had a significant impact on viral replication (Figure 6C) suggesting that PLK4 is an important miR-34c target during influenza replication. Analysis of existing literature suggests that miR-34c alters PLK4 activity by modulating the activity of either p53 or by stabilizing PLK4 translation. Influenza infection
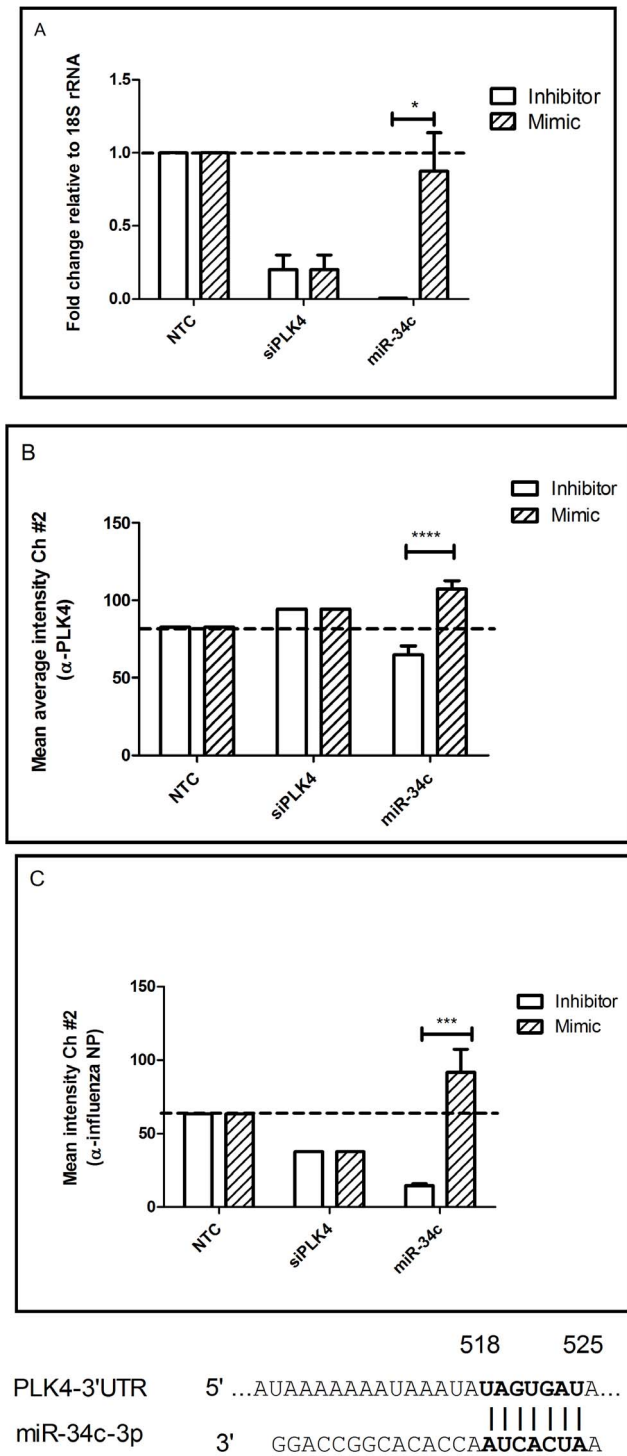
Figure 6. miRNA regulators of PLK4 (A) A549 cells were transfected with 25 nM of miR-34c inhibitor/mimic for 48 hrs followed by RNA extraction and RT-qPCR with PLK4 specific primers. Expression data was normalized to 18S rRNA expression and shown as mean±SEM of independent experiments. *p<0.05 compared to control. (B) A549 cells mock/transfected with miR-34c inhibitor/ mimic for 48 hrs were fixed with 4% formaldehyde in PBS and stained for PLK4 protein using biotinylated rabbit anti-PLK4 antibody (Abcam ab71394) and detected with Streptavidin-Alexa488. Cells were analyzed by Arrayscan Cellomics VTI scanner and data analyzed by GraphPad Prism. (C) A549 cells mock/transfected with miR-34c inhibitor/mimic for 48 hrs were infected with A/WSN/33 (MOI=0.001) for 48 hrs and

has been shown to up regulate expression of a large number of proteins [115–120], and the genes expressing these proteins are likely regulated at some level by miRNAs.

While the findings in this study show that HPKs are important for influenza replication, and key HPKs are regulated by miRNAs that are also deregulated during influenza infection, the number of miRNAs that were validated to be affected was limited. This could be either due to differential temporal expression profiles of the target genes and the miRNAs investigated, and/or affected by cell type-specific features, or the regulation small enough as not to lead to a detectable phenotype. While preliminary pathway analysis identified several key pathways regulated by the HPKs identified in this study, detailed analysis is needed but outside the scope of this study. Given the large number of potential miRNA targets, it is unlikely that one miRNA governing one HPK gene could sufficiently explain a phenotype, i.e. increased or decreased influenza virus replication. The genetic screen in this study yielded 17 candidate HPK genes based on a Z-score $\geq \mu \pm 2$ SD; however, lowering the threshold to a Z-score $\geq \mu \pm 1$ SD would have added an additional 37 HPK genes that overlapped with HPK genes discovered in related published screens [12–16]. Thus, it is likely that other HPK genes not identified in this study and the miRNAs governing their expression may be required or contribute to influenza virus replication.

There remains a gap in our understanding of the role of miRNA regulation of host genes, and how this interaction affects intracellular signaling pathways used during virus infection and replication. However, this study provides a framework for future studies, and contributes toward a better understanding of host-pathogen interactions which may help in accelerating the rational design of therapeutics aimed to control influenza infection and disease pathogenesis.

Supporting Information

Figure S1   RNA interference screen strategy for identi-fication of host factors affecting influenza infection. A) A549 cells were plated onto lyophilized siRNAs in 96-well flat-bottom plates and transiently transfected for 48 h with 50 nM siRNA. B) At 48 hours post-transfection, cells were infected with influenza virus A/WSN/33 (MOI 0.001). C) 48 hours post-infection, viral replication was assayed by titration of A549 cell supernatant on MDCK cells. Each siRNA given a score based on the number of wells with detectable virus, and primary hits determined using Z score analysis. D) Those hits were then validated using a novel siRNA to repeat the screen and E) phenotype was confirmed by influenza NP localization as well as assaying influenza viral genome replication via quantitative real time PCR detecting influenza M gene. F) Last, validated gene hits were associated with the cellular pathways they affect or intersect. MDCK, siNEG, non-target negative control siRNA; siMEK, mitogen-activated protein kinase kinase 2 siRNA positive control. (TIF)

Figure S2   (A) Optimization of siRNA knockdown using siMEK as a positive control. 48 hours after transfection with Dharmafect only (Mock) or Dharmafect+siMEK at 25 nM, 50 nM, and 100 nM concentrations in A549 cells, total RNA was extracted and used to quantify MEK-specific mRNA. The transcript copies divided by GAPDH of gene silenced cells normalized to the same values of non-target control siRNA transfected samples. (B) z-score plot for HPK hits from primary screen. Calculated Z scores of the human kinase library identified primary hits (z scores $\geq$ 2 and $\leq$ −2) whose silencing increased virus replication (positive Z score) and strongest

hits that decreased virus replication (negative Z score). The position of each cellular kinase gene identified important for virus replication in the primary screen are indicated. (TIF)

Figure S3   Novel siRNAs targeting primary screen hits validate HPKs. siRNAs targeting a novel site in the HPK hits from the primary screen (Table S2) were transfect-ed into A549 cells for 48 hrs followed by RNA isolation and RT-qPCR using gene specific primers. Fold changes were calculated relative to GAPDH as described in Materials and Methods previously. Values of control transfected cells are set as 0% silencing. The results are expressed as mean $\pm$ SD from a representative experiment performed in triplicate. (TIF)

Figure S4   Knockdown of miRNA by inhibitor treatment. We used the miRNA let-7f (expressed at 750 copies in A549 cell (PMID 17699775) as a candidate for optimizing inhibitor/mimic transfection. A549 cells (2×10^4 cells/well) were mock/transfected with 25 nM of let-7f inhibitor using Lipofectamine 2000 as per manufacturer's protocol for 24 hrs. RNA was extracted using Trizol and then used for qRT-PCR using let-7f specific forward oligo (5′-TGAGGTAGTAGATTGTATAGTTAAAAA-3′) and Universal reverse oligo as per Ncode miRNA qRT-PCR kit. Five tenfold serial dilutions of let-7f specific cDNA in triplicate from uninfected A549 cells were run in parallel to calculate copy numbers. As per MIQE guidelines only standards with RSq >0.99, PCR efficiency 90–110% and slope between -3.1 to -3.4 were used to calculate copy numbers. Data is represented as fold change relative to mock from two independent experiments. Error bars represent SEM. (TIF)

Figure S5   HPK expression in response to Influenza virus infection. A549 cells were infected with A/WSN/33 (MOI = 0.05) and RNA was isolated at 4 h, 12 h and 24 h post infection using Trizol as per manufacturer's instructions. HPK expression was analyzed using gene specific qPCR primers and plotted relative to 18S rRNA expression. Data represent means from two experiments. (TIF)

Figure S6   Predicted pathways of miRNA regulation of HPK expression during influenza infection. Schematic outline of cell cycle in mock infected cells. G1 to S phase transition is controlled by multiple factors of which Myc is a major regulator. Levels of PLK4 transcript and auto phosphorylated active PLK4 protein increase starting at S phase and peak at M phase. Active PLK4 targets its own degradation via proteasome mediated decay and is degraded.to complete cytokinesis and entry into G0/G1 phase. (B) Influenza (via NS1 protein) infection induces expression of p53 protein that induces miR-34c. miR-34c suppresses Myc mediated entry into S phase. Inhibition of miR-34c accelerates cell cycling and increased degradation of PLK4 protein and decreased influenza replication. miR-34c mimic arrests cells in G2/S phase by suppressing Myc leading to increased influenza replication. (TIF)

Table S1   Normalized scores, Z score analysis and cytotoxicity data for hits from host protein kinase screen. Table listing the location, siRNA target gene symbol and raw and normalized scores for all the kinases tested in this manuscript. Raw scores were normalized across the library to calculate mean score, standard deviation and Z-scores. Cytotox-icity data as measured by Toxilight assay is also shown. (DOCX)

Table S2 Sequences of siRNAs used in the study. Table shows accession numbers, gene symbol, gene id and siRNA sequences used for validation of hits from primary screen.
(DOCX)

Table S3 Summary of hits overlapping with other influenza whole genome siRNA screens. Data from other screens to identify host genes crucial for influenza virus replication were compared with the hits in our screen and are highlighted in red.
(DOCX)

Table S4 Oligonucleotide sequences of all primers used in the manuscript. Table lists the genes and the primers used for qPCR in this manuscript. Forward primers are appended with F and reverse primers are appended with R.
(DOCX)

Table S5 List of miRNAs differentially expressed during influenza infection. Table lists miRNAs shown to be differentially expressed during various influenza infections. Articles are referenced by PMIDs and miRNAs and targets if any are listed in adjoining columns.
(XLSX)

Table S6 Details of seed matches between miRNAs and 3′-UTRs. miRNA and 3′UTR alignment details were mined from **Targetscan** (www.targetscan.org). Details of seed match alignment, seed sequence etc are shown.
(XLSX)

Text S1 Alignment of miR-548 family miRNA sequences. Sequences of miR-548 family members were aligned using Clustal W application locally in BioEdit ver. 7.0 (Tom Hall). Alignment shows high degree of seed identity between multiple miR-548 members.
(RTF)

Author Contributions

Conceived and designed the experiments: LEA AB VM SMT RAT. Performed the experiments: LEA AB KC XY PB. Analyzed the data: LEA AB VM KC XY PB JC SMT RAT. Contributed reagents/materials/analysis tools: LEA AB VM KC XY PB JC SMT RAT. Wrote the paper: LEA AB SMT RAT.

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