Figure S1

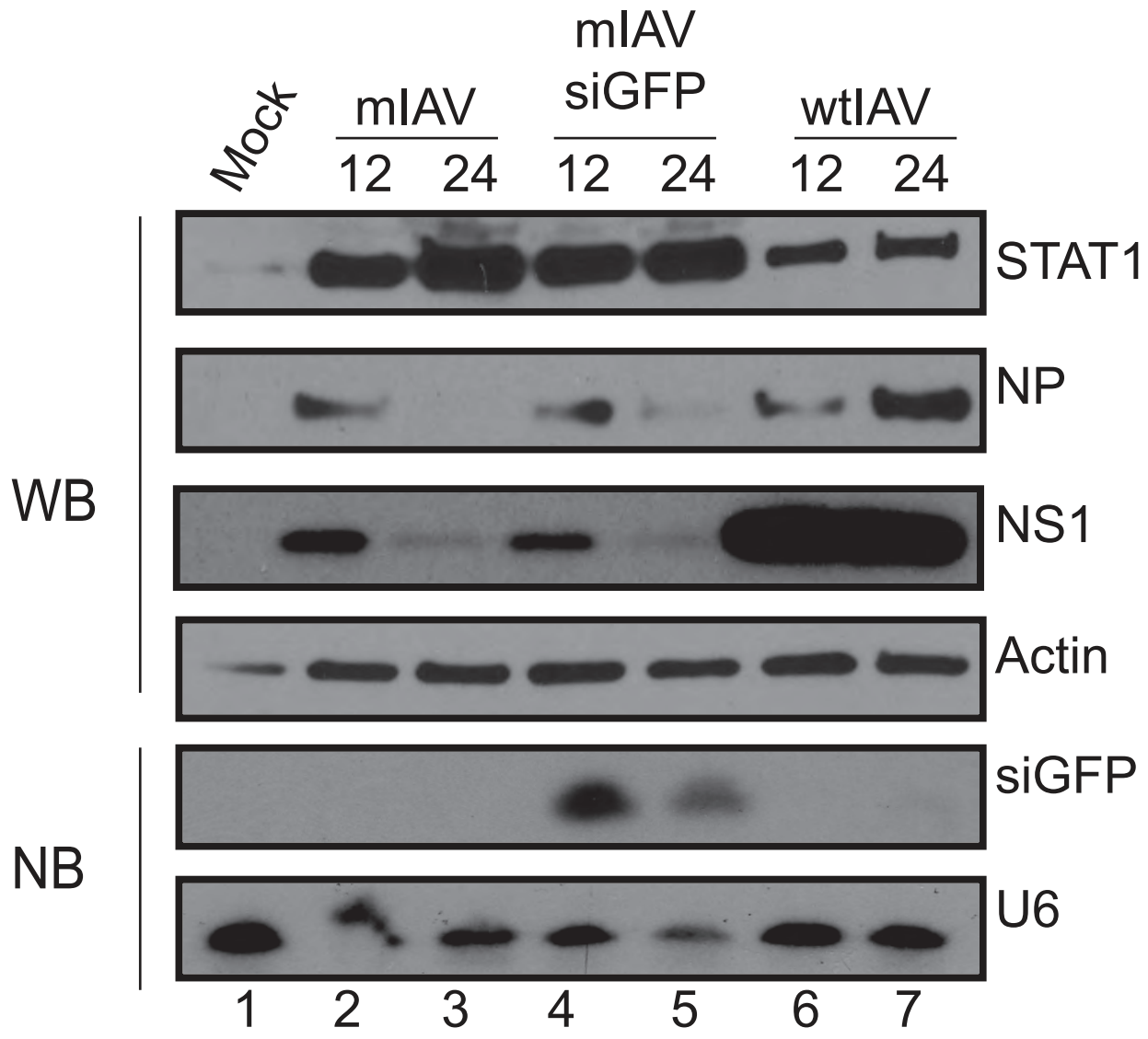

Mock
mIAV
12
24
mIAV
siGFP
12
24
wtIAV
12
24
STAT1
NP
NS1
Actin
siGFP
U6
WB
NB
1
2
3
4
5
6
7

Figure S2

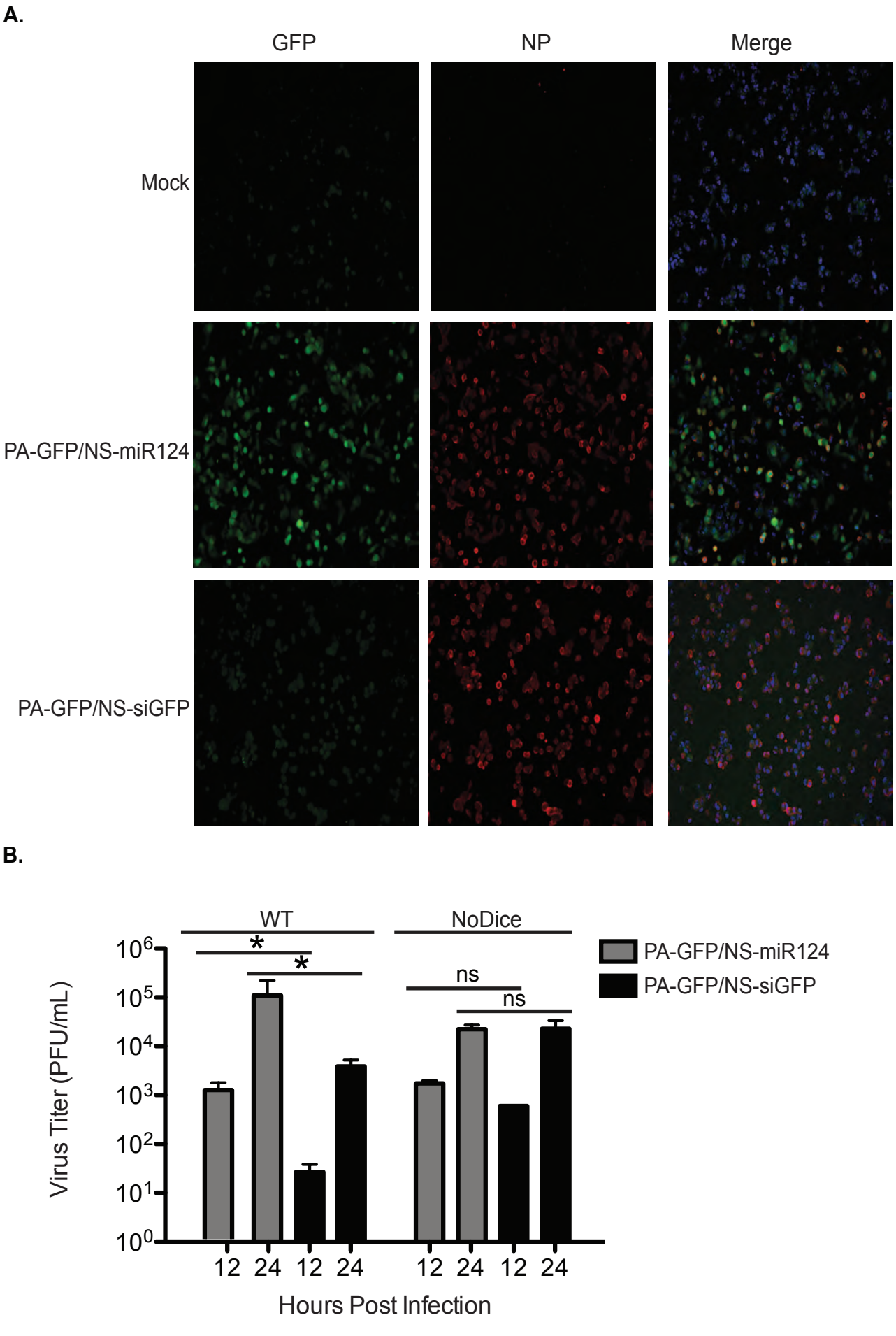

A.
GFP
NP
Merge
Mock
PA-GFP/NS-miR124
PA-GFP/NS-siGFP
B.
WT
NoDice
*
*
ns
ns
PA-GFP/NS-miR124
PA-GFP/NS-siGFP
Virus Titer (PFU/mL)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
12 24 12 24 12 24 12 24
Hours Post Infection

Figure S3

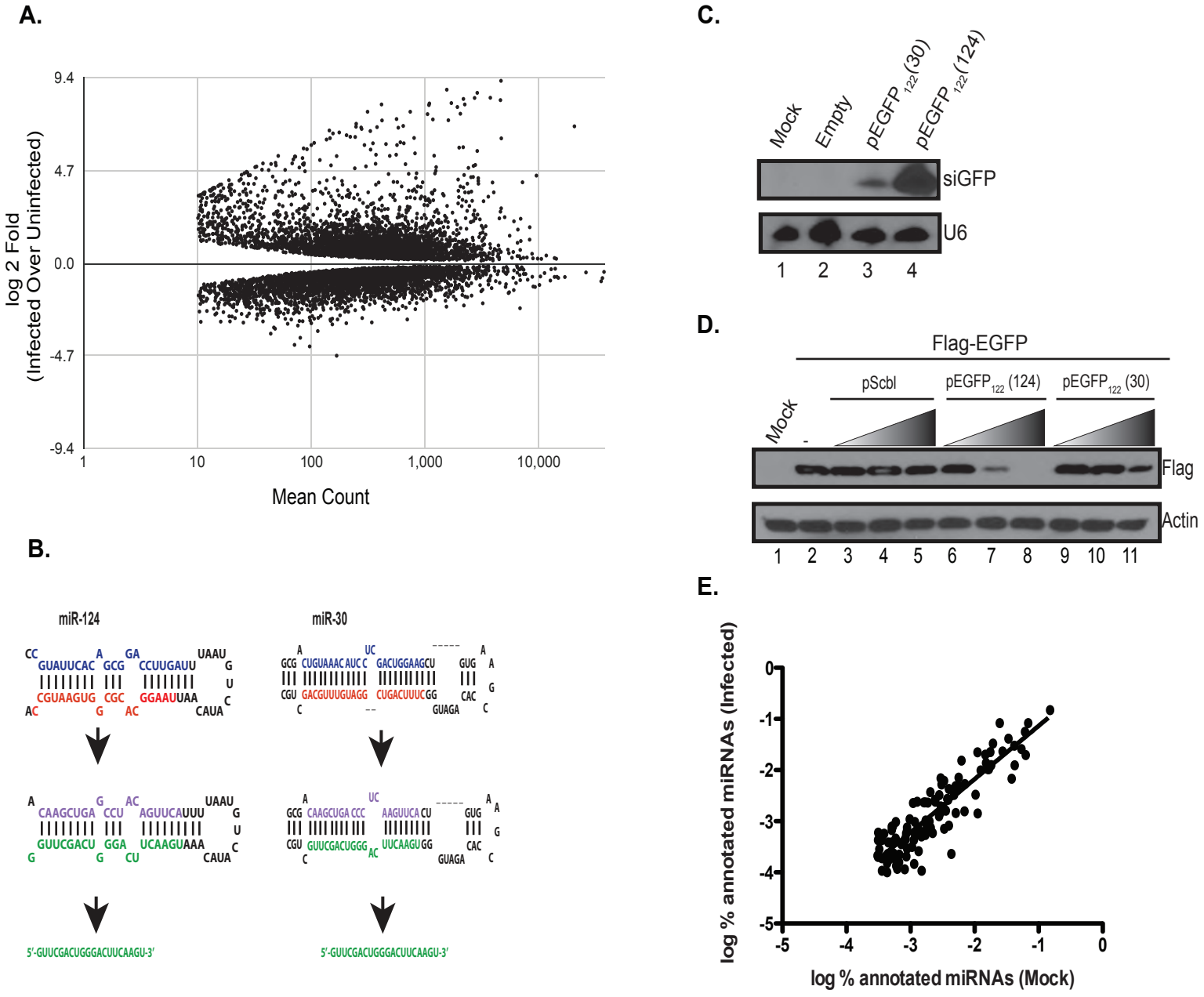

A.
9.4
4.7
0.0
-4.7
-9.4
log 2 Fold
(Infected Over Uninfected)
1
10
100
1,000
10,000
Mean Count
B.
miR-124
miR-30
5'-GUUCGACUGGGACUUCAAGU-3'
5'-GUUCGACUGGGACUUCAAGU-3'
C.
Mock
Empty
pEGFP 122 (30)
pEGFP 122 (124)
siGFP
U6
1 2 3 4
D.
Flag-EGFP
pScbl
pEGFP122 (124)
pEGFP122 (30)
Mock
Flag
Actin
1 2 3 4 5 6 7 8 9 10 11
E.
log % annotated miRNAs (Infected)
log % annotated miRNAs (Mock)
0
-1
-2
-3
-4
-5

Figure S4

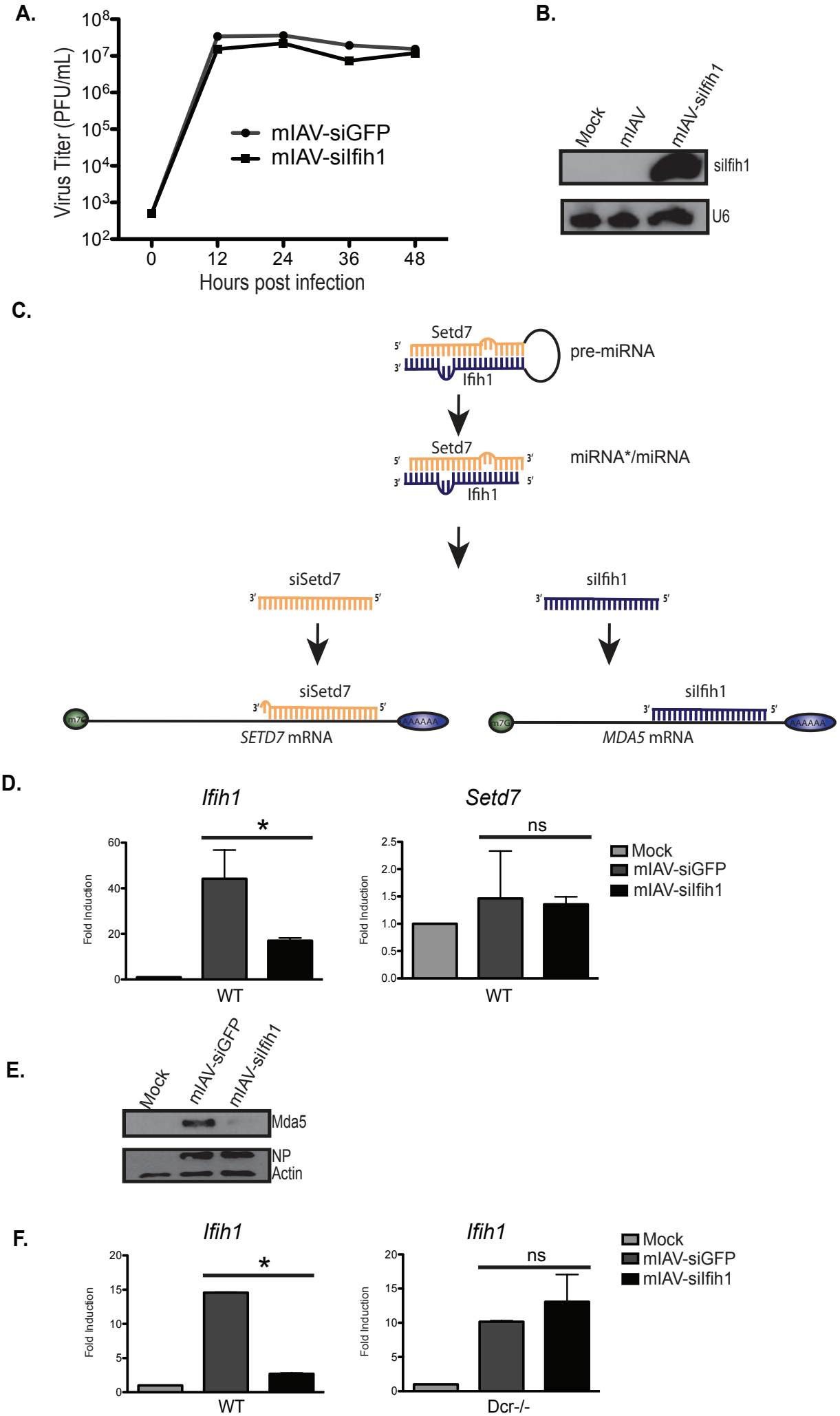

A.
Virus Titer (PFU/mL)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
mIAV-siGFP
mIAV-siIfih1
0
12
24
36
48
Hours post infection
B.
Mock
mIAV
mIAV-siIfih1
siIfih1
U6
C.
Setd7
Ifih1
pre-miRNA
miRNA*/miRNA
siSetd7
siIfih1
SETD7 mRNA
MDA5 mRNA
m7G
AAAAAA
D.
Ifih1
Setd7
*
ns
Fold Induction
WT
Mock
mIAV-siGFP
mIAV-siIfih1
E.
Mock
mIAV-siGFP
mIAV-siIfih1
Mda5
NP
Actin
F.
Ifih1
Ifih1
*
ns
Fold Induction
WT
Dcr-/-
Mock
mIAV-siGFP
mIAV-siIfih1

Figure S5

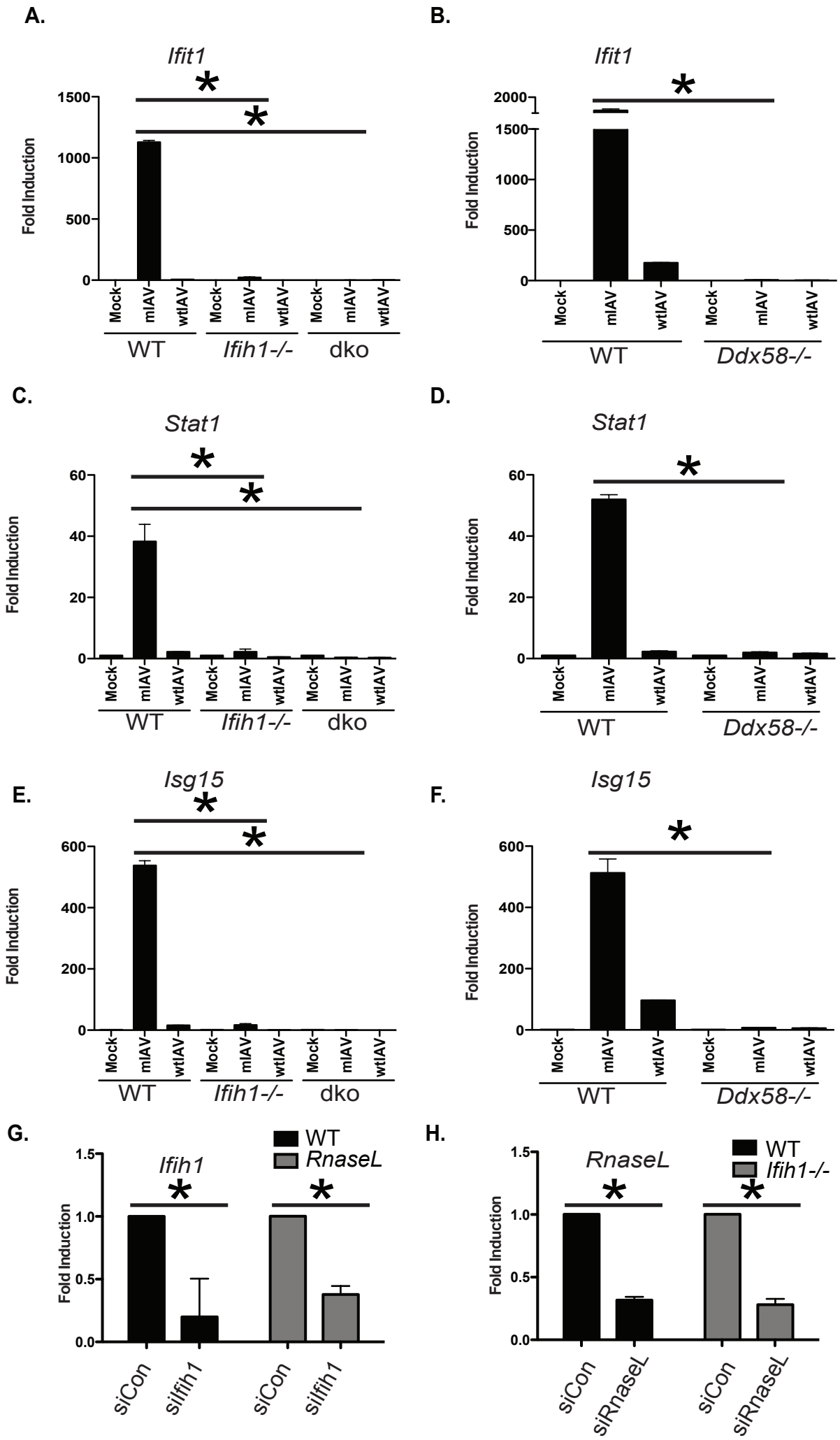

A.
Ifit1
Fold Induction
1500
1000
500
0
Mock
mIAV
wtIAV
WT
Ifih1-/-
dko
B.
Ifit1
2000
1500
1000
500
0
WT
Ddx58-/-
C.
Stat1
60
40
20
0
D.
Stat1
E.
Isg15
600
400
200
0
F.
Isg15
G.
Ifih1
WT
RnaseL
1.5
1.0
0.5
0.0
siCon
siIfih1
H.
RnaseL
WT
Ifih1-/-
siRnaseL

Figure S6

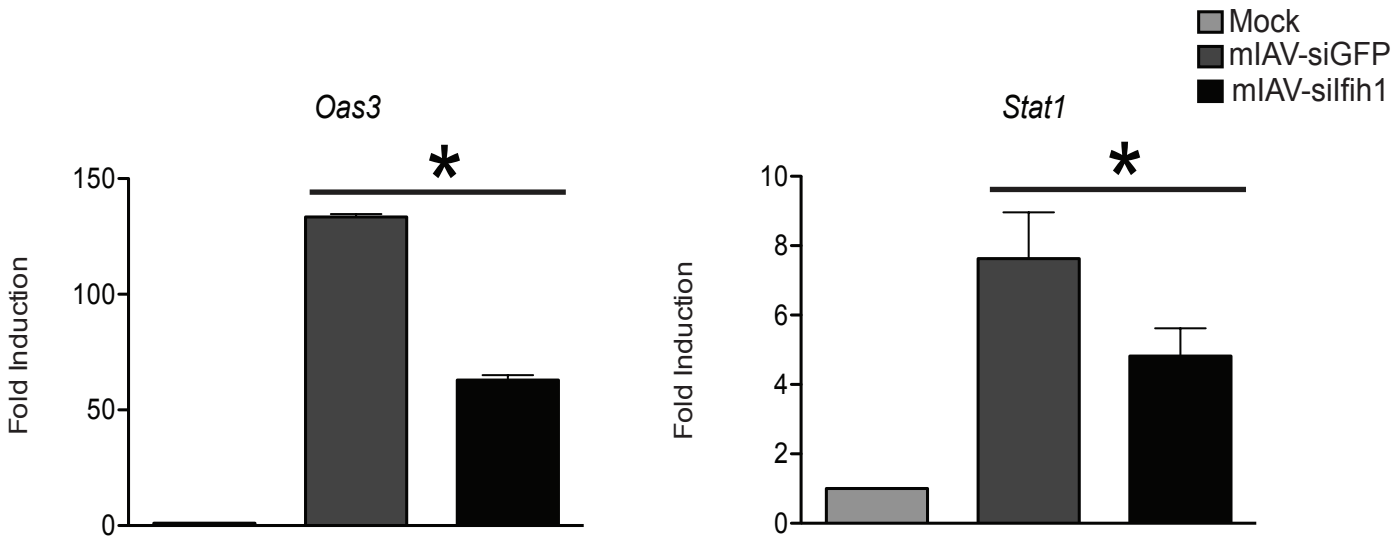
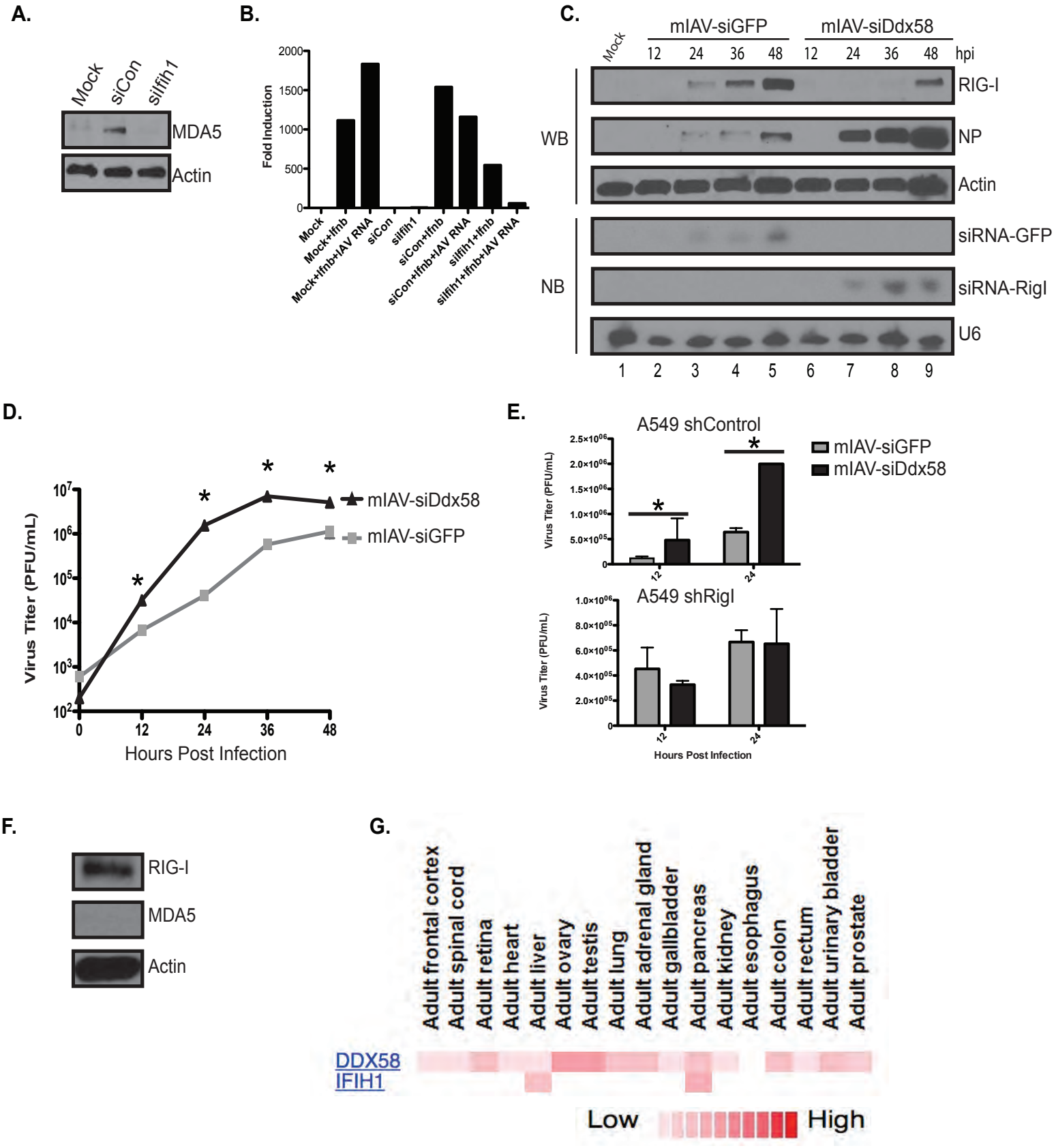

Mock
mIAV-siGFP
mIAV-silfih1
Oas3
Fold Induction
150
100
50
0
*
Stat1
Fold Induction
10
8
6
4
2
0
*

Figure S7


A.
Mock
siCon
silfih1
MDA5
Actin
B.
Fold Induction
2000
1500
1000
500
0
Mock
Mock+Ifnb
Mock+Ifnb+IAV RNA
siCon
silfih1
siCon+Ifnb
siCon+Ifnb+IAV RNA
silfih1+Ifnb
silfih1+Ifnb+IAV RNA
C.
mIAV-siGFP
mIAV-siDdx58
Mock
12
24
36
48
12
24
36
48
hpi
WB
NB
RIG-I
NP
Actin
siRNA-GFP
siRNA-RigI
U6
1
2
3
4
5
6
7
8
9
D.
Virus Titer (PFU/mL)
Hours Post Infection
mIAV-siDdx58
mIAV-siGFP
E.
A549 shControl
A549 shRigI
Virus Titer (PFU/mL)
mIAV-siGFP
mIAV-siDdx58
Hours Post Infection
F.
RIG-I
MDA5
Actin
G.
Adult frontal cortex
Adult spinal cord
Adult retina
Adult heart
Adult liver
Adult ovary
Adult testis
Adult lung
Adult adrenal gland
Adult gallbladder
Adult pancreas
Adult kidney
Adult esophagus
Adult colon
Adult rectum
Adult urinary bladder
Adult prostate
DDX58
IFIH1
Low
High

SUPPLEMENTAL DATA

Figure S1. Virus-encoded miRNAs do not increase ISG production over mIAV levels. Related to Figure 1.

Top three panels depict western blots of whole cell extract derived from A549 cells either mock treated or infected with mIAV, mIAV-siGFP, or wtIAV. Immunoblots were probed for STAT1, IAV nucleocapsid protein (NP), IAV non-structural protein 1 (NS1), and Beta-actin, which was used as a loading control. Bottom two panels depict northern blots of total RNA derived from A549s. Northern blots were probed for small RNA expression of the GFP targeting siRNA (siGFP) and the host splicing RNA, U6, which was used as a loading control.

Figure S2. siRNA-mediated self targeting of IAV. Related to Figure 2.

(A) Immunofluoresence of A549 cells either mock treated or infected (MOI=1) with PA-GFP/NS-siGFP or PA-GFP/NS-miR124. Panels demonstrate GFP, NP, and merged images with DAPI from left to right, respectively.

(B) Multi-cycle growth as described in Figure 2D. Mean ± SEM; *$p<0.05$ as assessed by Student's t test.

Figure S3. Validation of miR-124 as a scaffold to generate siRNAs. Related to Figure 3.

(A) RNA expression profile of mock treated vs. mIAV-siGFP infected (MOI=2) murine fibroblasts. Graph depicts the mean log2 fold change (y-axis) of gene expression in cells

infected over mock-treated conditions. Each dot depicts a single gene with the x-axis representing the mean read count. See Table S1 for complete list of genes.

(B) Schematic of mmu-miR-124-2 and hsa-miR-30a and the changes made to generate artificial siRNAs targeting GFP.

(C) Northern blot of total RNA from 293T cells mock treated, transfected with empty plasmid, or transfected with plasmids expressing the artificial miRNAs described in (B). Northern blots were probed form siGFP and U6.

(D) Western blot of whole cell extract derived from 293T cells mock treated or co-transfected with epitope tagged (Flag) GFP and increasing amounts of the plasmids described in (B). Immunblots were probed for Flag and Beta-actin.

(E) Small RNA profiling of A549 cells infected with the IAV library profiling as determined by small RNAseq. Shown are the log percentages of annotated miRNAs in infected and mock-treated cells.

Figure S4. Evaluation of Setd7 targeting of the Ifih1 hairpin. Related to Figure 4.

(A) Multi-cycle growth curves in MDCK cells infected (MOI=0.01) with mIAV-siGFP or -siIfih1. Error bars reflect the mean ± SEM. *$p<0.05$ as assessed by Student's t test.

(B) Small RNA northern blot of total RNA derived from murine fibroblasts infected (MOI=1) with mIAV or mIAV-siIfih1. Panels depict the siRNA against Ifih1, and the loading control U6.

(C) Schematic representation depicting the processing and subsequent targeting of the cognate mRNA of the 5p and 3p sides of the Ifih1-2 hairpin.

(D) qPCR of total RNA derived from murine fibroblasts were infected (MOI=2) with mIAV-siGFP or -siIfih1 using primers specific for *Ifih1* and *Setd7*. Mean ± SEM; *p<0.05 as assessed by Student's t test.

(E) Western blot of whole cell extract from mouse embryonic fibroblasts either mock treated or infected with mIAV-siGFP or miIAV-siIfih1 (MOI=5) for 24 hours. Immunoblots were probed for MDA5, NP and Beta-actin.

(F) Conditions as described in (D) but performed in wild type or Dicer-deficient ($Dcr^{-/-}$) fibroblasts.

Figure S5. Understanding the function of RIG-I and MDA5 in response to mIAV. Related to Figure 5.

(A) qPCR of *Ifit1* derived from wild-type, $Ifih1^{-/-}$, or $Ddx58/Ifih1^{-/-}$ (dko) murine fibroblast cells either mock treated or infected with mIAV or wtIAV (MOI=2). Error bars correspond to the mean ± SEM. *p<0.05 as assessed by Student's t test.

(B) qPCR of *Ifit1* derived from wild-type, or $Ddx58^{-/-}$ murine fibroblast cells either mock treated or infected with mIAV or wtIAV (MOI=2). Error bars correspond to the mean ± SEM. *p<0.05 as assessed by Student's t test.

(C) qPCR as described in (A) for *Stat1*.

(D) qPCR as described in (B) for *Stat1*.

(E) qPCR as described in (A) for *Isg15*.

(F) qPCR as described in (B) for *Isg15*.

(G) qPCR of *Oas2* derived from wild-type, or *RnaseL*$^{-/-}$ murine fibroblast cells either mock treated or infected with mIAV (MOI=2). Error bars correspond to the mean ± SEM. *p<0.05 as assessed by Student's t test.

(H) qPCR of *Oas2* derived from wild-type, or *Ifih1*$^{-/-}$ murine fibroblast cells either mock treated or infected with mIAV (MOI=2). Error bars correspond to the mean ± SEM. *p<0.05 as assessed by Student's t test.

Figure S6. Characterizing the changes in gene expression in the absence of MDA5. Related to Figure 6.

qPCR analysis on samples processed as in (Figure 6A) for *Oas3* and *Stat1*. Mean ± SEM; *p<0.05 as assessed by Student's t test.

Figure S7. Virus selection for MDA- and RIG-I-based silencing.

Related to Figure 7.

(A) Western blot of whole cell extract from A549 cells treated with control siRNA (siCON) or an siRNA targeting MDA5 (siMDA5). Immunoblots were probed for MDA5, and Beta-actin.

(B) Quantification of Figure 7D. Bands were first normalized to the internal control Actin and then to the mock sample using ImageJ.

(C) Upper panel shows Western blot of whole cell extract from A549 cells either mock treated or infected with mIAV-siGFP or mIAV-siDdx58 (MOI=0.01) and harvested at the indicated time points. Immunoblots were probed for RIG-I, NP and Actin. Lower panel depicts a Northern blot from the same conditions used in the Western blot. Northern blots

were probed for small RNA expression of siGFP, siDdx58 and the host splicing RNA, U6, which was used as a loading control.

(D) Multi-cycle growth curves in A549s infected (MOI=0.01) with mIAV-siGFP, or mIAV-siDdx58. Error bars reflect mean ± SEM; *p<0.05 as assessed by Student's t test.

(E) Virus levels in A549s either expressing a control shRNA (shControl) or a shRNA targeting *RIG-I* (shRigI) infected (MOI=0.01) with mIAV-siGFP, or mIAV-siDdx58. Error bars reflect mean ± SEM; *p<0.05 as assessed by Student's t test.

(F) Western blot of mouse lung depicting the endogenous levels of Rig-I and Mda5. Actin was used as a loading control.

(G) Heat map depicting the endogenous levels of RIG-I and MDA5 in human lungs. The heat map was generated using the publicly available Human Proteome Map software (http://www.humanproteomemap.org/index.php).

Table S1. Transcriptome profiling of mIAV infected fibroblasts. Related to Figure 3.

Log2 Fold induction of mRNA transcripts derived from murine fibroblasts as determined by mRNAseq. Cells were mock treated or infected with mIAV-siGFP for 12hrs (MOI=2) and sequenced as biological duplicates. P-values are denoted for each individual transcript mapped to the ENSEMBL annotated mouse genome.

Table S2. Artificial hairpin design. Related to Figure 3.

Differential expression of genes implicated in Table S1 for which artificial miRNAs were designed. Column A depicts the gene name, Column B the 3p siRNA, and Column C is the complete sequence assembly of the hairpin.

Table S3. Quantitative values for *in vivo* virus selection. Related to Figure 4.

Table depicts mIAV siRNA library strains, the siRNA sequence which defines them, and their relative proportion in the population.

Table S4. Quantitative values for *in vivo* virus selection of Sublibraries A, B, and C. Related to Figure 4.

Table depicts the targets of libraries A, B, and C and the normalized read counts for both replicates administered.

Table S5. Plaque-purified genotypes of mIAV-siGFP and –Ifih1. Related to Figure 4.

Table includes the assembled consensus sequence for each of the eight segments derived from single plaques of mIAV-siGFP and –siIFih1 as determined by mRNAseq.

Table S6. Antiviral genes impacted by mIAV-siIfih1. Related to Figure 6.

Table depicts mRNAseq expression data for cell mock treated or infected with mIAV-siGFP or –siIFih1.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

Mouse embryonic fibroblasts (both wild type and lacking Dicer), Madin-Darbin canine kidney cells (MDCK), human embryonic kidney cells (HEK293), 293T NoDice cells, and human alveolar epithelial cells (A549) were cultured at 37^{o}C in DMEM media (Gibco) supplemented with 10% FBS (Atlanta Biologicals), and penicillin/streptomycin (P/S, Corning), unless otherwise indicated. Infections were performed with media containing phosphate-buffered saline (PBS) supplemented with 3% BSA (MP Biomedicals) and P/S.

Virus Rescue

HEK293 cells were transfected with plasmids expressing bidirectional vRNA and mRNA as previously described (Fodor et al., 1999). The modified segment 8, which also included the split open reading frame and miRNA, also encoded for a mutant non-structural protein (NS1), all of which have been previously described (Donelan et al., 2003; Varble et al., 2010). Cells were collected 48 hours post transfection and injected into the allantoic fluid of 9-day old embryonated eggs. Rescued virus was extracted 48 hours post injection and quantified by hemagglutinin assay using chicken red blood cells (Lampire Biological Pharmaceuticals) in Alsevers and tittered by plaque assay on MDCK cells.

***in vitro* infections**

For multicicle growth curves, cells were administered virus in infection media for 1 hour and incubated at 37^{o}C. Following the 1 hr incubation, the infection media was removed and cells were washed three times with PBS. For multi-cycle growth curves in A549 or MDCK, the media was changed to DMEM containing P/S and trypsin (Sigma) at concentrations of 0.25 ug/mL or 1.0 ug/mL respectively. For single cycle infections, cells were incubated with complete DMEM supplemented with 10% FBS and P/S.

Animal Infections

C57BL/6 6-week old mice were purchased from Taconic. Mice were anesthetized with a mixture of ketamine, xylazine, and H_20 (1:1:4.6) via intraperitoneal injection. The mice were infected with $1x10^6$ pfu, unless otherwise indicated. Lungs were harvested at the indicated times and homogenized in 500 uL of PBS for plaquing on MDCK cells. All the

experiments performed were in accordance with the Icahn School of Medicine or University of Pennsylvania Institutions of Animal Care and Use Committee.

Transfection

Plasmid transfections were carried out using OptiMem and Lipofectamine 2000 (Invitrogen). Cells were transfected in suspension and the OptiMem media was changed to complete DMEM 4 hrs post transfection. siRNA transfections were performed with OptiMem and Lipofectamine RNAiMax (Invitrogen). Cells were also transfected in suspension with 6 pmol of the siRNA and the media was changed 4 hrs post transfection. The following siRNAs were used: Mda5 (Santa Cruz Biotechnologies), RnaseL (Santa Cruz Biotechnologes), and control siRNA (Santa Cruz Biotechnologies).

Northern Blotting

Small RNA northern blotting was performed as previously described (Pall and Hamilton, 2008). Probes include: GFP122: 5'-GCAAGCTGACCCTGAAGTTCA-3'; miR-124: 5'-TGGCATTCACCGCGTGCCTTAA-3'; anti-Ifih1: 5'-TCCAGAAGTTGTCAAATCTTA-3'; anti-U6: 5'-GCCATGCTAATCTTCTCTGTATC-3'.

qRT-PCR

Total RNA was extracted using TriZol (Invitrogen). The RNA was then subjected to reverse transcription using SuperScript II (Invitrogen) to generate cDNA. qPCR was performed using the KAPA SYBR FAST qPCR Master Mix (KAPA Biosystems) and

the Mastercycler e realplex (Eppendorf). The ddCT was calculated using tubulin as a housekeeping gene where mock treatments were used as a baseline in each respective experiment. Values represent the fold induction over mock-treated and the error bars depict +/- SEM of triplicates. Primers were designed using NCBI Primer Design or from previous publications (Broquet et al., 2011; Kim et al., 2014) and can be below.

Primer Name	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
mTubulin	TGCCTTTGTGCACTGGTATG	CTGGAGCAGTTTGACGACAC
mIfnb	AGATGTCCTCAACTGCTCTC	AGATTCACTACCAGTCCCAG
mIrf7	GGGCTGCAGTGGCTGAACGA	GCAGGTTAACTCCACTAGGT
mOasL	CACCGTTGTGCCCGCCTACA	CGTCGGCCGATGCTTCACGAA
mCxcl10	GCCTATCCTGCCCACGTGTTGA	GGCTTCACTCCAGTTAAGGAGCCC
mIfit3	TTTCTGAACTGCTCAGCCCACACC	GAAGGATCGCTTCCAGAGATTCCCG
mIsg15	CAGCAGTGGTTCCCTAGTTTAG	GGCAGTAGTCAGCCCTGTTAAT
mIfih1	CGATCCGAATGATTGATGCA	AGTTGGTCATTGCAACTGCT
mCxcl1	GCACCCAAACCGAAGTCATA	CTTGGGGACACCTTTTAGCA
mOas2	GGAAAGTGCCAGTAATGCAG	AGTCTTTGCCAGATCACTCCA
mOas3	TTCGGAAAGCCAGGCCTCTA	GAAGGCAGACTTGTGACCCA
mStat1	CGTGCAGTGATCGTTTCAGC	TGCAGGTTCGGGATTCAACA
hIfit1	TCGGAGAAAGGCATTAGATC	GACCTTGTCTCACAGAGTTC
hIfit2	CGTGGGAACCTGGTGACTAA	TCGTTCCAAGCATACCGTGA
hIfnb	GTCAGAGTGGAAATCCTAAG	ACAGCATCTGCTGGTTGAAG
hTubulin	GCCTGGACCACAAGTTTGAC	TGAAATTCTGGGAGCATGAC
hIfih1	CGGATATAAAGAATGTAACAT	ATGAGCATACTCCTCTGGTTTCA
hStat1	CTTCTCTGGCGACAGTTTTC	CCTTTCAATTTTACCTTCAG

Immunofluorescence

A549 cells were plated on cover slips and infected with the corresponding viruses at the indicated MOI. Cells were fixed in 4% formaldehyde/PBS 24 hours post infection, permeabilized with 0.5% Nonidet P-40 lysis buffer/PBS, and blocked with 2% BSA/PBS. Following blocking, the cells were incubated with the primary antibody (anti-NP; BEI

Resources Cat. No NR4544) 1% BSA/PBS overnight at 4^{o}C. Cells were washed with PBS and incubated with the secondary antibody for 1 hour at room temperature, and counterstained with DAPI (Life Technologies, Cat. No D1306). The cells were washed again three times with PBS before being mounted on slides and sealed. The cells were imaged using Zeiss Axioplan2.

Immunoblotting

Whole cell extracts were collected using Nonidet P-40 lysis buffer. Cells were incubated in buffer for 10 minutes and the soluble fractions were measured by standard Bradford assay after a 10 minute spin at 12, 000 rpm. 10 ug of total cell extract was subjected to SDS/PAGE on 4-15% polyacrylamide gels (BioRad). Following electrophoresis, protein was transferred to Hybond-c nitrocellulose membrane (Amersham) for two hours. The membranes were blocked in 5% milk for one hour and incubated with the primary antibody overnight at 4^{o}C. The membrane was washed 3 times with PBS with for 15 minutes and incubated with the secondary antibody for one hour at room temperature. Following incubation with the secondary antibody, the membrane was visualized with ECL as per the manufacture's instructions. Primary antibodies were used at 1 mg/mL and secondary antibodies were used at a dilution of 1: 5000. The following antibodies were used: anti-NP (BEI), anti-STAT1 (Santa Cruz), anti-RIGI (gift from A. Garcia-Sastre), anti-MDA5 (Enzo) anti-EGFP (Abcam), anti-Actin (NeoMarkers), ECL-linked anti-mouse and ECL-linked anti-rabbit (GE Healthcare).

Viral Genome Sequencing

The sequence of each segment of H1N1 (A/Puerto Rico/8/34) recombinant strains was generated by aligning overlapping reads from mRNA HiSeq data to create a consensus genome from a single plaque purification. Consensus genomes can be found in Table S5.

Statistical Analyses

Means and standard deviations were computed from biological triplicates. Results are represented as mean + the standard deviation (*,<0.05).