Supporting Information

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SI Materials and Methods

Virus Design, Rescue, and Quantification. The modified nucleoprotein (NP) segment (A/PR/8/34) was generated by PCR and ligation. A SalI site was added into the NP 3′ UTR by site-directed mutagenesis to allow for duplication of the packaging sequence in a previously established influenza A virus (IAV) rescue vector (1). To replicate the 3′-NP packaging sequence the final 214 bp of NP were amplified using SalI containing primers. The SalI flanked PCR product was digested with SalI, cloned and sequence verified. The SalI insertion site was then used to ligate four copies of miR-142-3p targets, as previously described (2). These recombinant viruses with modified NP segments were rescued by using previously described reverse genetic techniques (3), plaque purified, and quantified by plaque assay.

Protein and RNA Analysis. Western blots were generated from total protein separated on a 15% (vol/vol) SDS/PAGE gel, transferred to nitrocellulose (Bio-Rad), blocked for 1 h with 5% (wt/vol) skim milk at 25 °C, and then incubated with the indicated antibody overnight at 4 °C. Actin (Abcam) and NP (kind gifts from P. Palese, Mount Sinai School of Medicine, New York, NY) at a concentrations of 1 μg/mL in 5% (wt/vol) skim milk. The 2′ mouse and rabbit antibodies (GE Healthcare) were used at a 1:5,000 dilution for 1 h at 25 °C. Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used as directed. All RNA samples were treated with DNase-I (Roche) according to the manufacturer's instructions and quantitative RT-PCR of cDNA samples was performed using KAPA SYBR FAST qPRC Master Mix (KAPA Biosystems) on a Mastercycler ep realplex (Eppendorf). The ΔΔCT values were calculated with replicates over the endogenous housekeeping gene, β-tubulin. Values represent fold-change over mock-infected samples.

Flow Cytometry and Antigen Presentation Assays. Lungs were processed into single cell suspension and incubated with: rat α-mouse CD8a (53-6.7), CD3e (145-2C11), CD45 (30-F11), and hamster α-mouse CD11c (HL3) purchased from Becton Dickinson. α-HA antibodies (PY102) were kindly provided by Peter Palese (Mount Sinai School of Medicine, New York, NY). Cells were fixed using FACS Lysing Solution (BD). For intracellular cytokine staining, cells were incubated with PA_{224} (SSLENFRAYV) or NP_{366} (ASNENMETM) (BioSynthesis) for 6 h with rIL-2 and BFA. Cells were then fixed, permeabilized in buffer containing 0.5% saponin (Acros Organics), and labeled with rat α-mouse IFN-γ (XMG1.2) (BD). All flow cytometry data were acquired on a BD FACS Calibur and analyzed using FlowJo software (TreeStar). Antigen presentation assays were performed as previously described (4). Briefly, 10^5 JAWS II antigen presenting cells were infected with either NPctrl or NP142t, incubated with UV inactivated IAV or pulsed with either 1 μM RSV $M2_{82}$ or IAV NP_{366} peptide. Twenty-four hours postinfection (hpi), cells were irradiated; 10^5 LacZ-inducible 53-A8.5 NP_{366}-specific CD8 T-cell hybridomas were added and cells were incubated overnight. Cells were fixed, incubated with X-Gal solution, and blue cells counted. JAWS II cells and 53-A8.5 hybridomas were kindly provided by David Woodland (Trudeau Institute, Saranac Lake, NY).

Generation of Bone Marrow Macrophages, Primary Lung Fibroblasts, and MDCK142 Cells. Femurs were removed from naive mice and bone marrow cells were cultured for a minimum of 10 d in RPMI containing 20% (vol/vol) FBS, L-glutamine, and 30% (vol/vol) conditioned media from L929 cells. Primary lung fibroblasts were generated by digesting naive mouse lungs with collagenase/liberase (Roche) for 1 h and cultured for a minimum of 7 d before use. Madin-Darby canine kidney cells engineered to express miR-142 (MDCK142) were generated by transfecting p142 expressing miR-142 in the lariat of a red fluorescent protein (RFP) and confers Neomycin resistance. Cells were cultured in standard medium supplemented with 1 mg/mL G418 (Sigma) for 4 wk and then sorted for fluorescence. Retinoic acid-inducible gene (RIG)-$I^{-/-}$ and RIG-$I^{+/+}$ mouse embryonic fibroblasts were kind gifts from Michael Gale Jr. (University of Washington, Seattle, WA).

Deep Sequencing. Deep sequencing was performed on A549 cells untreated or infected with influenza A/PR/8/34 at a multiplicity of infection (MOI) of 1.0 for 12 h and has been described previously (5). Briefly, RNA adaptors were ligated to small RNA fractions, reverse-transcribed, and treated with RNase H. The cDNA library was amplified by PCR using 18 cycles and size-selected for ∼105–130 bp products on a 6% PAGE gel. The resulting libraries were quantified on a Flashgel (Lonza) using a Quant Ladder (Lonza) and assayed for quality on a Bioanalyzer (Agilent). The libraries were amplified onto SOLiD sequencing beads by emulsion PCR using the SOLiD ePCR Kit (Applied Biosystems). The templated beads were isolated, enriched, and were deposited into an eighth of a SOLiD sequencing slide (Applied Biosystems) using the SOLiD Bead Deposition Kit (Applied Biosystems). The slides were sequenced on a SOLiD Version 3 (Applied Biosystems). Color-space base calling and quality value assignment were performed on the SOLiD on-instrument cluster. The resulting reads were processed using the SOLiD Small RNA Analysis Tool (Applied Biosystems). Base-space reads were generated from color-space reads using the SOLiD System GFF Conversion Tool. The sequences were translated from color-space to sequence space and mapped to annotated miRNAs deposited on mirbase (http://www.mirbase.org).

Small RNA Northern Blot Analysis. Small RNA Northern blots and probe labeling were performed as previously described (5). Probes used include: anti–miR-142: 5′-TCCATAAAGTAGGAAACACTACA-3′ and anti-U6: 5′-GCCATGCTAATCTTCTCTGTATC-3′.

Primer Extension. Primer extensions were performed as previously described (6). Briefly, RNA from infected cells was reverse transcribed using primers specific for the positive strand of the NP RNA 5′–TGATTTCAGTGGCATTCTGG-3′. cDNA was resolved on a 6% denaturing gel, transferred to hybond membrane (GE Healthcare), and visualized by autoradiogram.

MHC I Tetramers. Tetramers HA_{533} (H-2K(d)/IYATVAGSL), and NP_{147} (H-2K(d)/TYQRTRALV) were obtained from National Institute of Allergy and Infectious Disease MHC Tetramer Core Facility (Atlanta, GA).

IAV NP 3′ UTR Cloning and Sequencing. RNA from whole lungs of three mice infected with NPctrl or NP142t 4 d postinfection (dpi) was extracted and reverse-transcribed using a primer specific for the 3′ UTR duplication. The 3′ UTR was then PCR-amplified with Econotaq PLUS green (Lucigen) using primers specific for the 3′ UTR of NP. Forward: TGACATGAGGACCGAAATCA and reverse: AGTAGAAACAAGGGTATTTTTCTT. Bands were gel purified cloned into pCR-TOPO 2.1 (Invitrogen) according manufacturer's protocols. Nine clones from NPctrl and NP142t were sequenced using the M13R primer. Sequences were then aligned to miR-142 target site or control sequence.

1. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc Natl Acad Sci USA* 97:6108–6113.
2. Brown BD, et al. (2007) Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nat Biotechnol* 25:1457–1467.
3. Perez JT, et al. (2009) MicroRNA-mediated species-specific attenuation of influenza A virus. *Nat Biotechnol* 27:572–576.
4. Crowe SR, et al. (2003) Differential antigen presentation regulates the changing patterns of CD8+ T cell immunodominance in primary and secondary influenza virus infections. *J Exp Med* 198:399–410.
5. Perez JT, et al. (2010) Influenza A virus-generated small RNAs regulate the switch from transcription to replication. *Proc Natl Acad Sci USA* 107:11525–11530.
6. Vreede FT, Brownlee GG (2007) Influenza virion-derived viral ribonucleoproteins synthesize both mRNA and cRNA in vitro. *J Virol* 81:2196–2204.

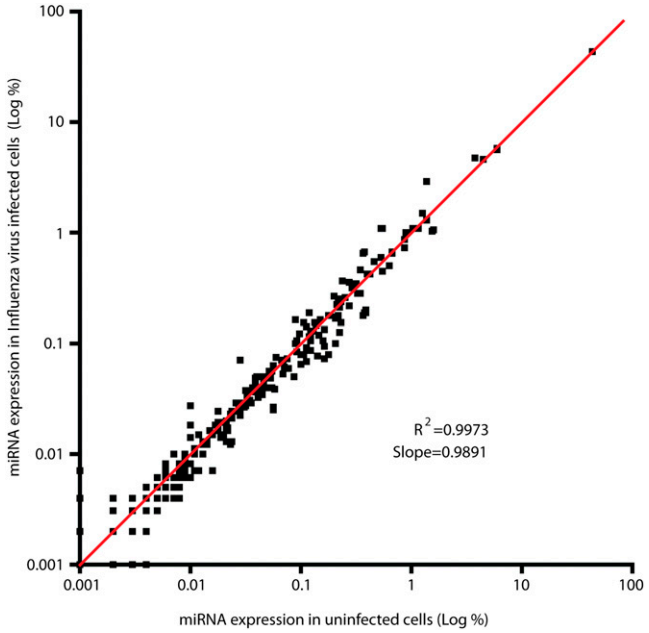

Fig. S1. The cellular small RNA profile is not altered during IAV infection. A549 cells were either uninfected or infected with influenza A/PR8. At 16 hpi, RNA was harvested and analyzed by 454 sequencing platform. The percent expression of each annotated miRNA in uninfected (*x* axis) and IAV-infected (*y* axis) is depicted.

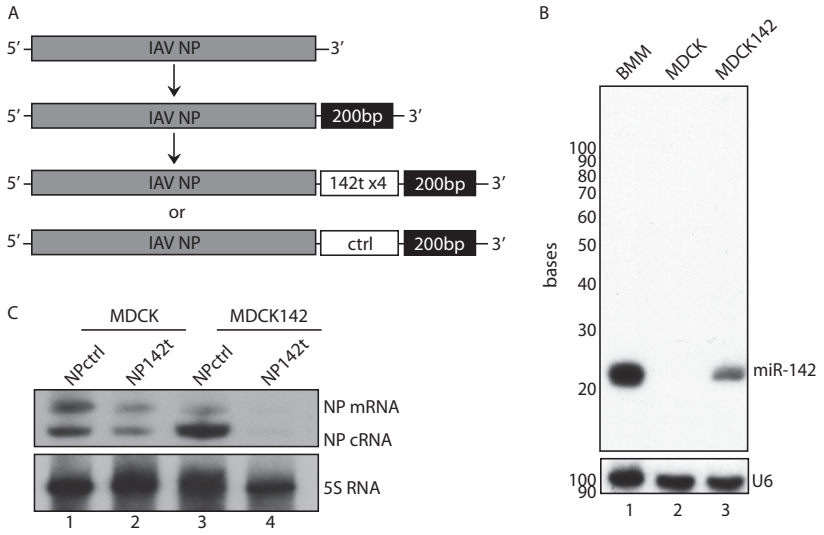

Fig. S2. Generation of miR-142 targeted IAV. (*A*) Model depicting duplication of the packaging sequence of the NP mRNA and the insertion of four perfect miR-142 target sites upstream of the duplicated packaging sequence. (*B*) Northern blot analysis for miR-142 (*Upper*) and U6 (*Lower*) of RNA from bone marrow macrophages (BMM), MDCK, and MDCK142. (*C*) MDCK or MDCK142 were infected with either NPctrl or NP142t at an MOI of 2. At 18 hpi, RNA was reverse-transcribed with primers specific for the positive strand of the NP gene (*Upper*) or 5S RNA as a loading control (*Lower*).

	control		miR-142t	# of conserved
	UUUCCAGGGGCGGGGAGUCUUCG		UCCAUAAAGUAGGAAACACUACA	miR-142t sites
1	••••••••••••••••••••••••	10	••••••••••••••••••••••••	4/4
2	••••••••••••••••••••••••	11	••••••••••••••••••••••••	4/4
3	••••••••••••••••A•••••••	12	••••••••••••••••••••••••	4/4
4	••••••••••••••••••••••••	13	••••••••••••••••••••••••	4/4
5	••••••••••••••••••••••••	14	••••••••••••••••••••••••	4/4
6	••••••••••••••••••••••••	15	•••••G••••••••••••••••••	3/4
7	••••••••••••••••A•••••••	16	••••••••••••••••••••••••	4/4
8	••••••••••••••••••••••••	17	••••••••••••••••••••••••	4/4
9	••••••••••••••••••••••••	18	••••••••••••••••••••••••	4/4

Fig. S3. Lack of miR-142 targeted escape mutants during in vivo infections. Mice were infected with 40 pfu of IAV NPctrl or NP142t and on 4 dpi lungs were removed and the 3′ UTR of the NP gene was sequenced. Consensus sequence displayed on top with nucleotide changes annotated below for each clone. Only sequences for one of four miR-142 target sites shown.

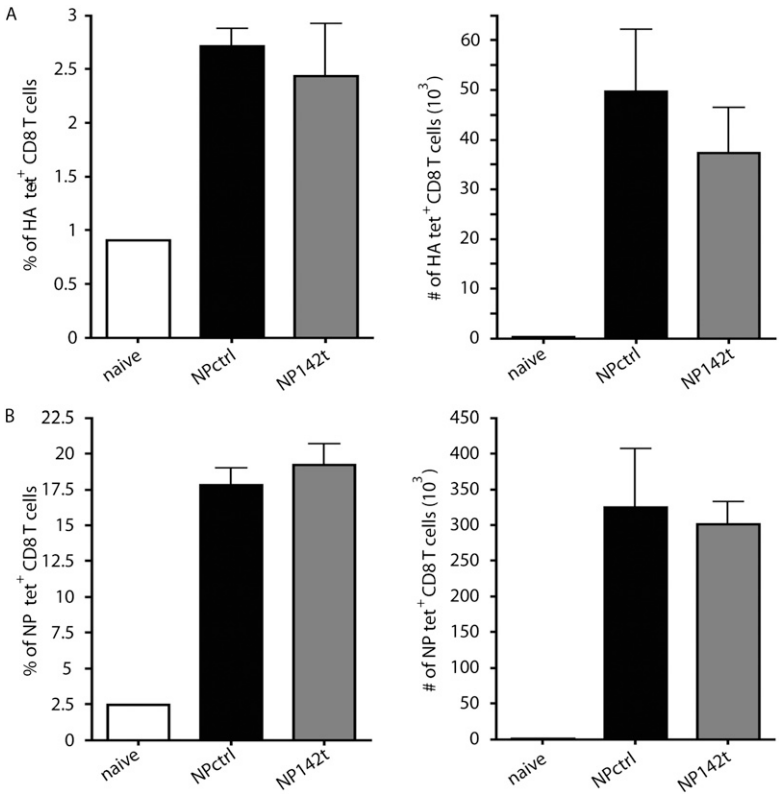

Fig. S4. Loss of virus amplification within hematopoietic cells does not alter generation of the antigen-specific adaptive immune response. BALB/c mice were infected with 40 pfu of IAV NPctrl or NP142t and 10 dpi the frequency (*A*) and total number (*B*) of IAV HA_{529} (*Upper*) and NP_{147} (*Lower*) tetramer$^+$ CD8 T cells was determined.

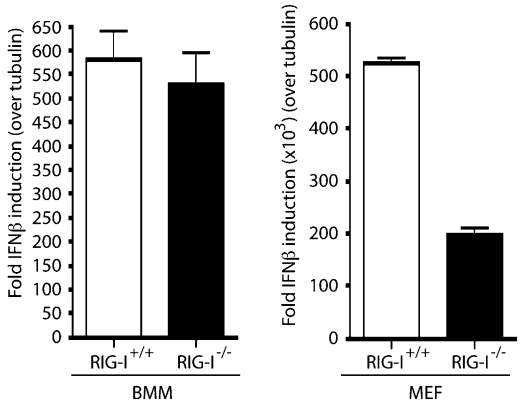

Fig. S5. RIG-I–deficient BMMs and fibroblasts can induce IFN-I following dsRNA stimulation. BMMs (*A*) or mouse embryonic fibroblasts (MEF) (*B*) from RIG-I$^{+/+}$ or RIG-I$^{-/-}$ littermate controls were transfected with dsRNA and IFN-β mRNA levels determined by quantitative RT-PCR at 12 hpi.