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MicroRNA-mediated species-specific attenuation of influenza A virus

Jasmine T Perez[1], Alissa M Pham[1], Maria H Lorini[2], Mark A Chua[3], John Steel[2] & Benjamin R tenOever[1–3]

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Influenza A virus leads to yearly epidemics and sporadic pandemics. Present prophylactic strategies focus on egg-grown, live, attenuated influenza vaccines (LAIVs), in which attenuation is generated by conferring temperature sensitivity onto the virus. Here we describe an alternative approach to attenuating influenza A virus based on microRNA-mediated gene silencing. By incorporating nonavian microRNA response elements (MREs) into the open-reading frame of the viral nucleoprotein, we generate reassortant LAIVs for H1N1 and H5N1 that are attenuated in mice but not in eggs. MRE-based LAIVs show a greater than two-log reduction in mortality compared with control viruses lacking MREs and elicit a diverse antibody response. This approach might be combined with existing LAIVs to increase attenuation and improve vaccine safety.

Influenza A virus has the propensity to mutate and exchange segments, creating the need for annual vaccines that must be constantly updated with circulating strains identified by global monitoring[1]. Current LAIVs, such as FluMist, are temperature-sensitive reassortant viruses that contain segments derived from more than one strain[2–3]. Here we sought to develop a complementary attenuation strategy for influenza virus using microRNA (miRNA)-mediated gene silencing. miRNA-mediated viral attenuation, which relies on incorporation of miRNA target sequences into viral RNA, has recently been described for lentiviruses, picornaviruses and rhabdoviruses[4–7]. Transcript regulation by miRNAs occurs through direct binding, resulting in translational repression or cleavage[8]. miRNAs exhibit broad expression patterns, ranging from ubiquitous to tissue or lineage specific, and moderately affect global protein levels[9–11]. Although most miRNAs are evolutionarily conserved, a small percentage are species specific[12].

To determine the feasibility of miRNA-mediated attenuation of influenza A virus, we investigated whether infection affects pre-miRNA formation, maturation or post-transcriptional gene silencing. Tissue-specific and ubiquitous miRNAs, miR-124 and miR-93, respectively, were expressed exogenously in HEK293 cells in the context of virus infection (**Fig. 1a** and **Supplementary Fig. 1** online). Exogenous miR-93 and miR-124 were processed into pre-miRNA products and mature forms; this processing was unaffected by influenza A virus infection (**Fig. 1a**). Furthermore, endogenous miR-93 expression remained unchanged in both human and murine fibroblasts during the course of infection (**Fig. 1b**). Monitoring of miR-124–induced repression of a luciferase reporter containing known miR-124 target sequences[13] showed that miR-124 specifically suppressed 90% of luciferase activity; this inhibition was not disrupted by the presence of influenza A virus or NS1, the nonstructural RNA-binding protein responsible for disrupting many cellular processes (**Fig. 1c,d**)[14]. These data corroborate evidence[15] that influenza A virus and NS1 allow for proper miRNA biogenesis and gene silencing in mammalian cells and suggest that viral attenuation by MRE incorporation is feasible.

However, application of this strategy to influenza A virus is hindered by the fact that the viral mRNA terminates shortly downstream of the stop codon, and disruption of this region can result in packaging and replication defects[16]. We therefore incorporated MREs into the viral open reading frame. To design a virus that would be attenuated in humans and mice without reducing vaccine yield in eggs, we identified miRNA species that are not expressed in chicken but are ubiquitous in both murine and human lung tissue. Published deep sequencing results of miRNA profiles from *Gallus gallus*, *Mus musculus* and *Homo sapiens* identified miR-93 as an ideal candidate (**Supplementary Fig. 2a** online)[17–20]. The ubiquity of miR-93 in *Mus musculus* and *Homo sapiens* was corroborated by northern blot analysis, RT-PCR and RNA *in situ* data from *Mcm7*, which encodes miR-93 as part of an intron (**Supplementary Fig. 2b–d**).

To incorporate miR-93 target sites into influenza A virus, we identified regions in the viral genome that maintain high amino acid conservation between circulating strains but demonstrate plasticity at the RNA level. We chose the highly conserved segment five, which encodes nucleoprotein. We reasoned that this conservation would reduce the likelihood of escape mutants while allowing for DNA rescue of a diverse set of recombinant influenza A virus strains[2]. We identified two stretches of RNA that could be changed into miR-93 target sites without modifying the physical properties of the amino acids encoded at these sites (**Fig. 2a**). To ensure efficient and effective targeting, and to further decrease the possibility of revertants, we designed two near-perfect MREs at positions 225 (site 1, 93NP1) and 818 (site 2, 93NP2) of A/Puerto Rico/8/34 (A/PR/8/34), a laboratory H1N1 strain of influenza A virus that was adapted from a circulating 1934 strain[21] (**Fig. 2a**). Although codon usage at these sites is not conserved among circulating H1N1, H3N2 and H5N1 strains, these sites consistently maintain the same hierarchical class of amino acids,

[1]Microbiology Graduate School Training Program, [2]Department of Microbiology, [3]Global Health and Emerging Pathogens Institute, Mount Sinai School of Medicine, New York, New York, USA. Correspondence should be addressed to B.R.t. (benjamin.tenoever@mssm.edu).

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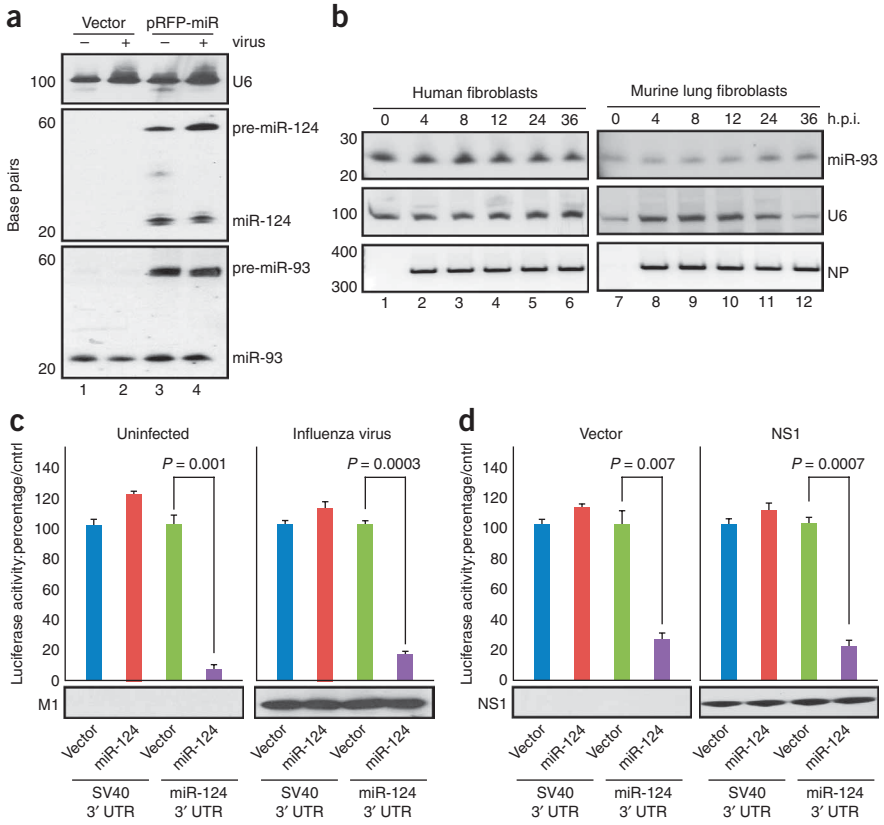

Figure 1 Influenza A virus infection does not disrupt cellular miRNA function. (**a**) Northern blot of exogenous miR-93, miR-124 and U6 small nuclear (sn)RNA after mock or influenza A virus infection (multiplicity of infection (MOI) = 10) of HEK293 cells. (**b**) Northern blot of endogenous miR-93 and U6 snRNA after influenza A virus infection (MOI = 3) for hours indicated in human and murine fibroblasts. NP, nucleoprotein. h.p.i., hours post infection. (**c**) HEK293 cells expressing either vector or miR-124 and luciferase reporter constructs containing control SV40 or miR-124 MRE-containing 3′ UTRs. Cells were infected with influenza A virus (MOI = 1) and subsequently measured for luciferase activity. (**d**) HEK293 cells expressing influenza A virus NS1, miR-124 and luciferase reporter constructs as described in **c**. For **c** and **d**, firefly luciferase activity was normalized to *Renilla* luciferase; data are the means of three independent experiments; error bars represent ± s.d. Western blots beneath each graph depict the expression of matrix 1 (M1) and NS1 proteins.

that is, the side chain at these positions maintains its hydrophobicity, polarity and/or charge (**Supplementary Fig. 3** online).

Modifying the coding region of segment five at sites one and two generated high-affinity miR-93 binding sites that exceed the complementarity of canonical MREs[8] (**Fig. 2a**). However, these modifications resulted in three amino acid substitutions, I63L, S262T and I265L, all of which remained within their hierarchical order and thus still resembled the natural variation observed at these loci.

As three amino acid substitutions were required to generate MRE-containing nucleoprotein, we designed a control virus, called parental (PRNTL), that contained these three amino acid substitutions and included additional mutations that disrupt miR-93 binding (**Fig. 2a**). PRNTL allowed us to distinguish phenotypic differences due to miRNA processing from indirect effects mediated by changes in nucleoprotein structure.

To ascertain whether nucleoprotein function was compromised by the I63L, S262T and I265L substitutions, we used an antisense reporter construct to determine polymerase function[22]. Exogenous expression of the three main polymerase components PB1, PB2, PA and either the wild-type or the PRNTL nucleoprotein was used to measure RNA-dependent RNA-polymerase (RdRp)-driven luciferase expression. The data showed that the three conservative substitutions made to nucleoprotein did not substantially affect its function, but did result in 20% reduction of RdRp activity (**Fig. 2b**). To determine the effects of these amino acid substitutions in the context of virus infection, we compared an H1N1 strain (A/PR/8/34) encoding PRNTL nucleoprotein to wild type (WT) A/PR/8/34. PRNTL and WT virus infection were similar in cell culture; however, *in vivo* infections indicated that PRTNL was attenuated (**Fig. 2c**). This attenuation may reflect the decreased RdRp activity of PRNTL, which would be more apparent under the selective pressure of an *in vivo* infection than in immortalized cell culture.

To determine how MRE-containing viruses compare to the PRNTL virus *in vivo*, we infected mice with increasing titers of either PRNTL or 93NP1/2 recombinants. Despite lethality in both cohorts at titers $>1 \times 10^5$ plaque forming units (PFU), mortality was restricted to infections with the PRNTL strain at intranasal inocula of 10^4 and 10^3 PFU, generating a greater than two-log difference in lethal dose between PRNTL and the MRE-containing 93NP1/2 (**Fig. 2d**); median lethal dose (LD_{50}) of 1.7×10^3 and 2.15×10^5 for PRTNL and 93NP1/2, respectively[23]).

Next, we determined whether MRE-containing viruses are attenuated *in ovo*. We inoculated 10-d-old embryonated chicken eggs with 100 PFU of wild-type A/PR/8/34/H1N1 (WT), PRNTL, 93NP1, 93NP2 or 93NP1/2 (**Fig. 2e**). Two days after infection, allantoic fluid was harvested and titers of $\sim 10^8$ PFU/ml were observed for each of the five strains, suggesting no attenuation *in ovo* (**Fig. 2e**). Furthermore, to illustrate the versatility of this technology, we used the MRE-containing nucleoprotein segments to rescue H5N1 6:2 reassortants, generating viruses antigenically recognized as A/Vietnam/1203/04/H5N1 via modified hemagglutinin and neuraminadase gene expression[24]. As with the H1N1 strains, the reassortant viruses demonstrated no attenuation in eggs, growing to titers of $\sim 10^8$ PFU/ml (**Fig. 2f**).

To determine whether species-specific attenuation was a result of miRNA-mediated gene silencing, we infected *Dicer*$^{-/-}$ murine fibroblasts, which do not process miRNAs (**Supplementary Fig. 2b**), and wild-type murine fibroblasts with A/PR/8/34-based PRNTL, 93NP1, 93NP2 or 93NP1/2 strains (**Fig. 3a** and **Supplementary Fig. 4** online). In wild-type fibroblasts, PRNTL virus produced abundant levels of hemagglutinin, 93NP1 and 93NP2 showed mild attenuation of hemagglutinin production, and 93NP1/2 produced no hemagglutinin (**Fig. 3a**). Attenuation was attributed to miRNA targeting as these same viral strains replicated to high, similar levels in the absence of *Dicer* (**Fig. 3a**). To ensure that this attenuation was miR-93 specific, we pretreated cells with Mercury locked nucleic acid (LNA) anti-miR-93 or scrambled RNA and subsequently infected cells with PRNTL, 93NP1 or 93NP1/2 (**Fig. 3b**). In the presence of anti-miR-93, 93NP1/2 levels were greater than twice that observed with scrambled

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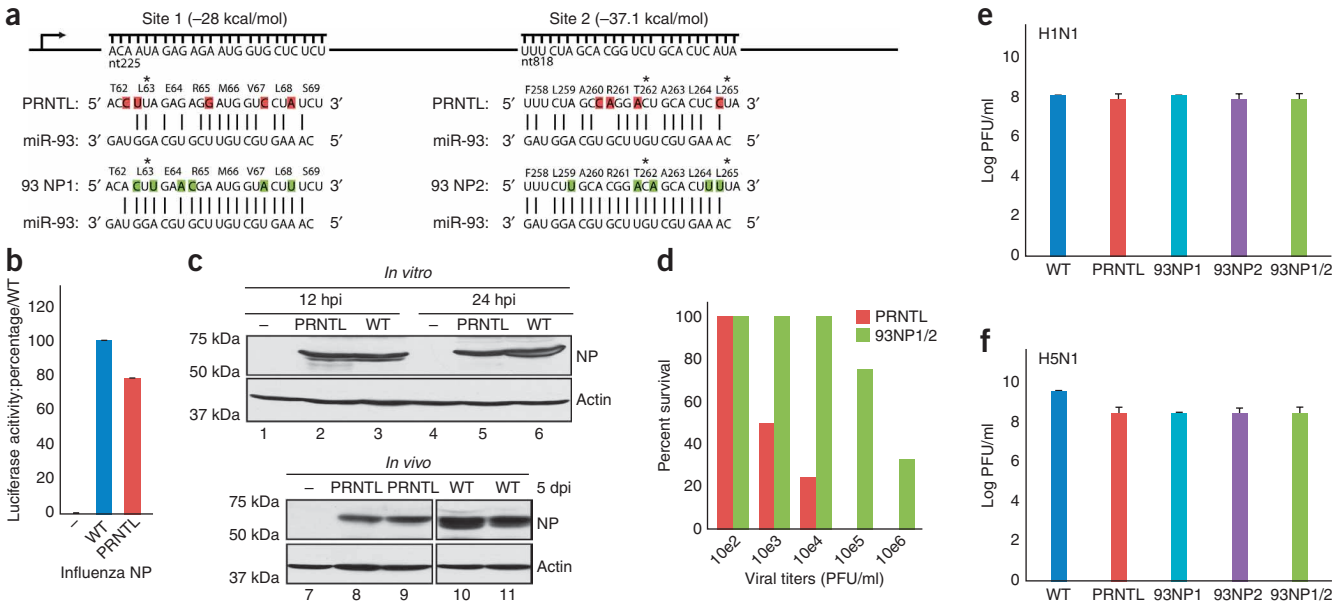

Figure 2 Species-specific attenuation of H1N1 and H5N1 influenza A viruses. (**a**) Schematic of RNA base substitutions generated to transform site 1 and site 2 in parental (PRNTL) or miR-93–targeted (93NP1/2) sites. *, denotes amino acid substitutions. Colored blocks denote nucleotide substitutions. Mean free energy is included as kcal/mol for each respective optimized site. (**b**) Influenza A virus polymerase-based firefly luciferase reporter assay in the context of no nucleoprotein (NP), wild-type (WT) NP or PRNTL NP. Data are the means of three independent experiments, normalized to *Renilla* luciferase; error bars represent ± s.d. (**c**) Western blot analysis of HEK293 cells or lung extract from BALB/c mice treated with WT influenza A/PR/8/34 or PRNTL viruses. Immunoblots of nucleoprotein and actin are shown. (**d**) Intranasal *in vivo* toxicity of PRNTL and 93NP1/2 viruses in BALB/c mice ($n = 4$/dose/strain). Inoculating titers given in PFU/ml. (**e**) Viral titers from PRNTL and MRE-containing H1N1 influenza A virus infections of 10-d-old embryonated chicken eggs. Titers were determined by hemagglutination and plaque assay from allantoic fluid and expressed as PFU/ml. Data are the means of four independent experiments. (**f**) Same as in e for H5N1 reassortant viruses.

control, indicating that attenuation of recombinant virus was a result of miR-93–specific suppression of nucleoprotein.

To further characterize the attenuation mechanism, we performed *in vitro* and *in vivo* kinetic, quantitative experiments comparing the levels of nucleoprotein and mRNA (**Fig. 3c,d**). As nucleoprotein is critical for copying template RNAs to form both mRNA and viral (v)RNA[25], we infected HEK293 cells and extracted total protein and RNA at 12 and 24 h after infection to determine whether decreases in nucleoprotein levels preceded the loss of mRNA. In cells infected with 93NP1/2, levels of nucleoprotein mRNA were elevated whereas protein levels were very low. This pattern of high nucleoprotein mRNA and low nucleoprotein was also observed *in vivo* 48 h after infection (**Supplementary Fig. 5** online). These results suggest that, despite the extensive complementarity, miR-93–mediated attenuation of influenza A virus was the result of translational repression. The overproduction of nucleoprotein mRNA may reflect the role of unbound nucleoprotein in the elongation of vRNA chains, loss of which biases the switch from transcription to replication[25].

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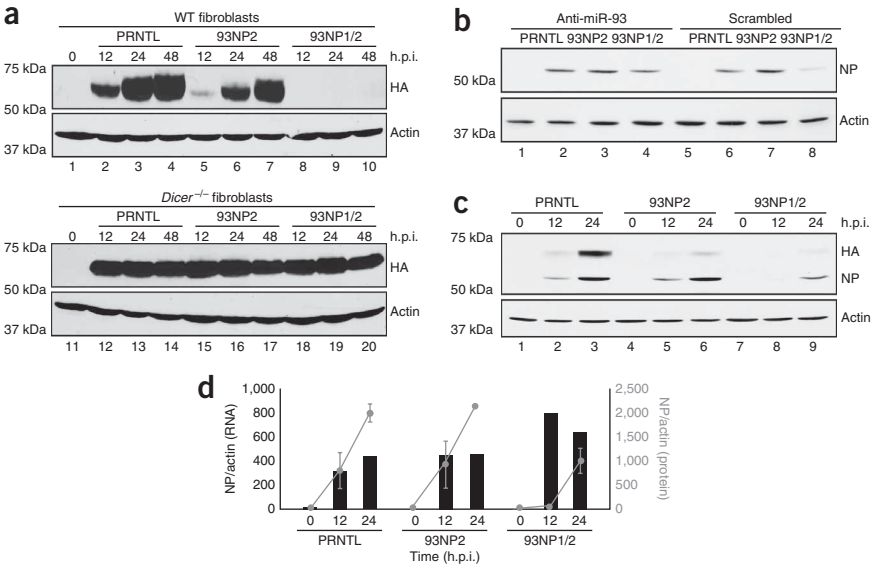

Figure 3 Characterization of miRNA-mediated attenuation of influenza A virus. (**a**) Western blot analysis of A/PR/8/34-based PRNTL, 93NP2 and 93NP1/2 infections harvested at hours indicated in wild-type (WT) and *Dicer*$^{-/-}$ murine fibroblasts. Immunoblots of hemagglutinin and actin protein levels are shown. h.p.i., hours post infection. (**b**) Western blot analysis of HEK293 cells treated with Mercury LNA anti-miR-93 or scrambled RNA oligonucleotides and infected as in **a**. Immunoblots of nucleoprotein and actin are depicted. (**c**) Western blot analysis of HEK293 cells infected as described in **a**. Immunoblots for hemagglutinin and nucleoprotein are shown. (**d**) Left-hand vertical axis: RNA levels determined by quantitative RT-PCR, standardized to actin and represented as copy number; error bars represent ± s.d. Right-hand vertical axis: quantification of protein levels by densitometry; nucleoprotein levels standardized to actin and represented in arbitrary units.

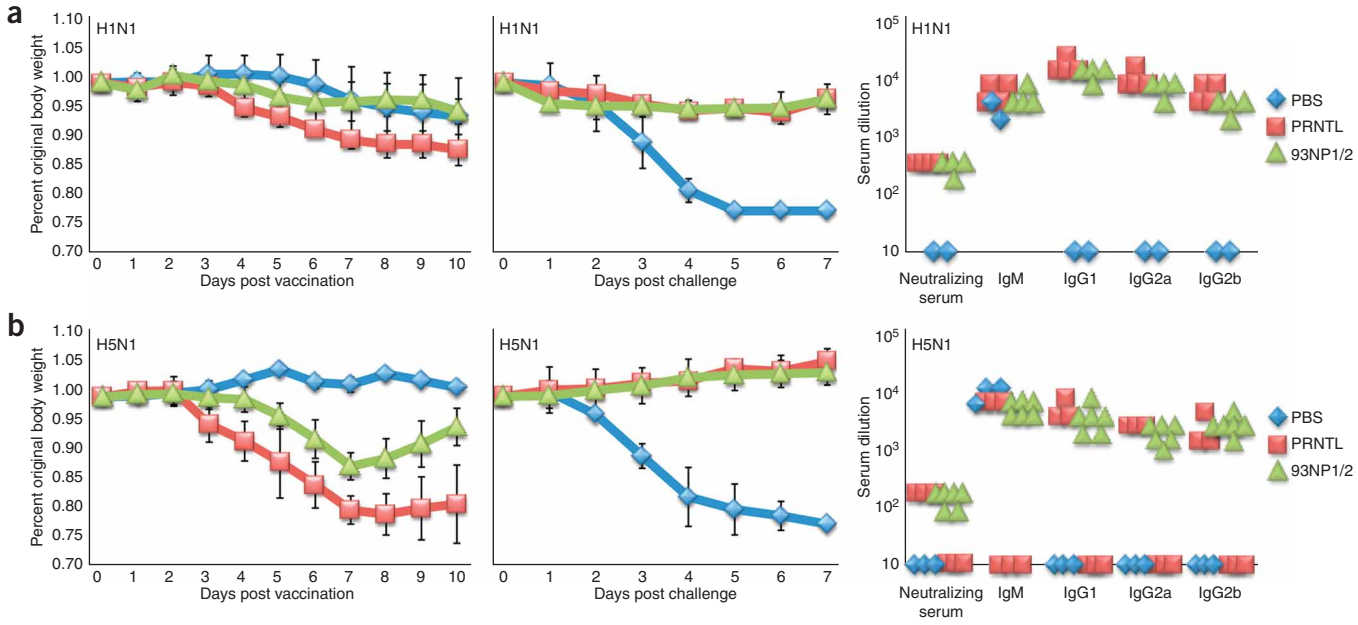

Figure 4 MRE-containing influenza A viruses as live attenuated vaccines. (**a**) Left: percent original body weight for mice vaccinated intranasally with 10e3 PFU of PRNTL or 93NP1/2 H1N1 viruses. Middle: percent original body weight after lethal challenge. Data represent the mean of each cohort ($n = 4$), errors bars are ± s.d. Right: antibody response after challenge, as determined by lowest serum dilution of positive hemagglutinin inhibition and immunoglobulin response as measured by enzyme-linked immunosorbent assay. (**b**) Same as in **a**, with H5N1 reassortant PRNTL or 93NP1/2 vaccinations.

Additional *in vivo* characterization of the MRE-containing influenza viral strains confirmed no changes in viral tropism and a normal cellular response to infection, demonstrating robust cytokine and transcriptional profiles (**Supplementary Fig. 6** online). To evaluate the possible generation of escape mutants, we analyzed RNA sequences from both *in vitro* and *in vivo* infections. Notably, we were unable to isolate escape mutants, suggesting that the flexible nature of miRNA targeting combined with the opposing rigid conservation of nucleoprotein may prevent reversion and add to the safety of this LAIV strategy.

To ascertain whether miR-93–seeded strains serve as effective LAIVs, we performed studies in mice with the A/PR/8/34 H1N1 PRNTL and 93NP1/2 recombinants (**Fig. 4a**). Inoculation of the PRNTL strain resulted in >10% weight loss compared with administration of 93NP1/2 or PBS. Furthermore, lethal challenge of these mice, 21 d after 93NP1/2 vaccination, resulted in 100% survival and induction of a robust repertoire of antibodies, including IgM, IgG1, IgG2a and IgG2b (**Fig. 4a**).

To further test the versatility of this MRE-based vaccine strategy, we inoculated mice intranasally with the miR-93–seeded H5N1 reassortants, which demonstrated no attenuation *in ovo* (**Fig. 2e**). Unlike the H1N1 vaccinations, H5N1 PRNTL resulted in 50% mortality and an average 20% loss in body weight (**Fig. 4b**). In contrast, all mice vaccinated with MRE-containing H5N1 survived, with only a small loss in body weight (**Fig. 4b**). Twenty-one days after vaccination, mice were challenged with a lethal dose of H5N1. Mock-vaccinated animals showed rapid weight loss and 100% mortality. In contrast, mice vaccinated with MRE-containing H5N1 displayed no signs of morbidity, indicating complete protection (**Fig. 4b**). Furthermore, serum samples from these mice were positive for neutralizing activity against wild-type H5N1 virus and, as in the case of H1N1 vaccinations, had high levels of IgM, IgG1, IgG2a and IgG2b (**Fig. 4b**).

In this report, we describe the effectiveness of harnessing the endogenous miRNA silencing machinery to achieve species-specific attenuation of influenza A virus. The MRE-containing LAIVs are attenuated in mammals while maintaining wild-type characteristics *in ovo*. Although the H1N1 reassortant MRE-containing vaccine described here does not protect against any influenza A strains currently in circulation, the H5N1 reassortant should be an effective vaccine for current H5N1 strains. This technology could be applied to any other influenza A strain. An advantage of MRE-based attenuation is that the degree of attenuation can be modulated by varying the number of MREs and/or the miRNA(s). This approach can also easily be adapted to tissue culture through the exploitation of miRNAs that are absent in select cell lines. Lastly, as the mechanism is distinct from temperature-based attenuation, this technology could be used in concert with FluMist as a means of increasing vaccine safety and extending the target demographic to include those presently excluded on the basis of their age.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturebiotechnology/.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

J.T.P. and B.R.t. designed and wrote the manuscript. Animal studies were conducted by A.M.P. and M.A.C. Experiments were done by J.T.P., A.M.P. and M.H.L. Cloning and rescue of H5N1 reassortants was done by J.S.

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ONLINE METHODS

Generation of recombinant virus. Human embryonic kidney cells (HEK293) were transfected with parental or mutant RNA-polymerase I–dependent nucleoprotein (pPol-I-NP) constructs, which produce vRNA transcripts, along with a Pol II–dependent nucleoprotein construct (pCAGGs-NP) and seven pDZ constructs that encode bidirectional vRNA and mRNA, as previously described[26–28]. Cells were harvested 24 h after transfection and injected into the allantoic fluid of 10-d-old embryonated chicken eggs. Live virus was isolated 48 h after infection and quantified by hemagglutinin assay using chicken red blood cells in Alsevers (CBT Farms) and plaque assay in Madin-Darby canine kidney (MDCK) cells. Plaque assays were limited to parental and wild-type virus as MDCK cells express significant levels of miR-93 (**Supplementary Fig. 2b**). H5N1 recombinant influenza A viruses were generated using internal components of H1N1 (A/PR/8/34) and the hemagglutinin and neuraminadase segments of H5N1 (A/Vietnam/1203/04). The hemagglutinin segment was modified by the removal of the polybasic cleavage site, as previously described[28].

Infections in mice. Animal infections were performed in 5-week-old BALB/c mice (Taconic). Mice were put under general anesthetic and treated intranasally. Pathogenic studies were performed on cohorts of 3–4 mice/inoculating dose. Mice were weighed daily and euthanized if they lost >25% of original body mass. Vaccinations were performed using 1 × 10e3 PFU of MRE-containing H5N1 or H1N1. Three weeks after infection, mice were rechallenged intranasally with either 10 LD_{50} of H5N1 6:2 recombinant or 100 LD_{50} of H1N1 (A/PR/8/34). Mice were monitored daily for signs of morbidity and mortality.

ELISA and HI assay. Serum was obtained post mortem through intraocular bleeding and treated with cholera filtrate (Sigma) overnight at 37 °C. ELISA and hemagglutinin assays were performed as previously described[29].

miRNA expression and targeted luciferase vectors. The red fluorescent protein minigene (pRFP) expressing miR-124 has been described elsewhere[13]. For generation of pRFP-miR-93, a 500-bp genomic fragment containing the pri-miR-93 locus was isolated from mouse genomic DNA by PCR amplification with High Fidelity PCR Master kit (Roche) per the provided protocol, using forward 5′-TAGTGGTCCTCTCTGTGCTACCG-3′ and reverse 5′-ATTGAACA AAAATGGGGACTCCT-3′ primers. The resulting PCR product was subcloned into PCR2.1-TOPO (Invitrogen) according to the manufacturer's instructions, and subsequently cloned into the pRFP minigene via PmeI-SpeI sites. Firefly luciferase constructs containing miR-124 MREs and control SV40 3′ UTRs were a kind gift from E. Makeyev (Nanyang Technological University, Singapore)[13].

Passaging of virus and sequencing of nucleoprotein. Human lung epithelial cells (A549) were infected with PRNTL or MRE-containing H1N1 (MOI = 0.01) in the presence of TPCK trypsin. Cells were washed 2 h after infection in PBS and then replaced with complete media. One hundred microliters of replacement media was removed from the infected plates 15 min after changing the media and tested by plaque assay to ensure absence of virus from the original inoculum, and 24 h after infection, 10 µl of supernatant (from a total volume of 1.5 ml) was serially transferred to naive cells for a total of 10 passages. Ten days after infection, RT-PCR was performed on total RNA and nucleoprotein PCR products were cloned and sequenced. For *in vivo* studies, 5-week-old BALB/c mice were treated with virus as above. Five days after infection, total RNA was harvested, and used to clone nucleoprotein for sequencing. Depicted sequences represent >25 individual colonies per cohort.

Tissue culture and *in vitro* infections. HEK293, A549, murine lung fibroblasts and MDCK cells were grown in DMEM (Mediatech), supplemented with 10% FBS (JM Bioscience) and 1% penicillin/streptomycin (P/S, Mediatech), unless otherwise indicated. Human astrocytoma U373 cells were supplemented with an additional 10 mM HEPES (GIBCO). Jurkat cells were grown in RPMI (Mediatech), supplemented with 10% FBS, and 1% P/S. Primary human dendritic cell RNA was kindly provided to us by A. Fernandez-Sesma (MSSM, NYC). *Dicer*−/− murine fibroblasts were a kind gift from A. Tarakhovsky (Rockefeller University, NYC) and Donal O'Carroll (EMBL, Monterotondo), and were grown in DMEM supplemented with 15% FBS, 1% nonessential amino acids (GIBCO), and 1% P/S. *In vitro* infections were performed in serum-free medium for 1 h; inoculum was washed and replaced with fresh complete medium without trypsin for indicated times. pRFP expressing HEK293 cells were infected with WT influenza A/PR/8/34 (MOI = 10) for 24 h, and subsequently harvested for total RNA. Human and murine lung fibroblasts were infected with WT A/PR/8/34 (MOI = 3) and harvested at the times indicated. Murine wild-type and *Dicer*−/− fibroblasts were infected with PRNTL or MRE-containing A/PR/8/34 viruses (MOI = 1). For anti-miR-93 experiments, HEK293 cells were transfected with 20 nM of Mercury LNA oligonucleotides (Exiqon) specific for human miR-93 or scrambled control for 24 h preceding infection. Subsequently, cells were infected with PRNTL or MRE-containing A/PR/8/34 viruses (MOI = 0.1) and harvested 24 h post infection. Comparison of nucleoprotein and RNA levels after infection was performed in HEK293 cells (MOI = 0.1).

Quantitative-PCR and western blot. qPCR and analysis were performed by MSSM Quantitative Genomics core facility. RT-PCR and immunoblots were performed as recently described[29]. RT-PCR primers are listed below. Actin (Abcam), polyclonal A/PR/8/34 (a kind gift from A. Garcia-Sastre), monoclonal nucleoprotein (a kind gift from P. Palese, MSSM, NYC), IRF1 (Santa cruz, sc-640), STAT1 (Santa cruz, sc-417), and ISG54 (a kind gift from G. Sen) antibodies were all used at a concentration of 1 µg/ml and incubated overnight at 4 °C. Secondary mouse and rabbit antibodies (GE Healthcare) were used at a 1:5,000 dilution for 1 h at 25 °C. Full-length western blots are presented in **Supplementary Figure 7** online. miRNA RT-PCR primers include: Let7a: GTCCTGGCGCGGTGCTCT & TCTCTTGCTCCTTCCCT TGC; miR155: CATTTCAAGAACAACCTACCAGAGA & AAGTTTATCCAGC AGGGTGACTC; mIR16: TCTGATGTGAACACAAGGACATTCA & TTTCCAC CATCTTTACCCTGTTT3; miR93:GAAGCTCATGAGGCGTTACATAG & ATT GACCTGCCAGACATTGAG; miR181: CAACGGTTTCTGTCAGGATGAAT & AGGGGAACTGTGGTCACTATCAC; mIR21: TGCTTGGGAGGAAAATAAAC AAT & GACTCTAAGTGCCACCAGACAGA; tubulin: GCCTGGACCACAAGT TTGAC & TGAAATTCTGGGAGCATGAC.

pRFP and luciferase reporter transfections. Transfections for fluorescence confirmation of pRFP constructs were performed in HEK293s using 4 µg pRFP vector and Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Fluorescence was imaged 24 h after transfection. For subsequent infection with WT influenza A/PR/8/34, HEK293s were transfected as above, with 100 ng firefly luciferase 3′ UTR constructs, 10 ng constitutive *Renilla* luciferase, and 700 ng of miRNA pRFP construct and harvested 18 h after transfection and 12 h after infection (MOI = 1) and analyzed per the manufacturer's instructions (Dual-Luciferase Reporter Assay, Promega). For NS1 studies, HEK293 cells were cotransfected with pBluescript SK+ (Stratagene) or A/PR/8/34 NS1 (a kind gift from P. Palese, MSSM, NYC) in addition to 50 ng firefly luciferase 3′ UTR constructs, 10 ng constitutive *Renilla* luciferase, 350 ng miRNA pRFP constructs. Cells were harvested 24 h after transfection and analyzed as described above. RNA-dependent RNA polymerase activity of mutant nucleoprotein was accessed in HEK293 cells transfected with 250 ng of pDZ-NP-PRNTL1/2 or wild-type pDZ-NP and 100 ng pPol-I firefly luciferase and analyzed as previously described[22]. All firefly luciferase readings are expressed as a ratio to *Renilla* luciferase expression per sample, and averaged over three independent replicates.

Statistical analyses. Statistical analysis was performed using a two-tailed Student's *t*-test with an $n = 3$–8. $P < 0.05$ were considered significant, and error bars reflect ± s.d.

miRNA northern blot analysis. Northern blots in **Figure 1** were generated from total RNA and separated by PAGE (PAGE) with a 15% denaturing polyacrylamide gel containing 7.5 M urea and 1× TBE[14]. RNA was transferred to Hybond N+ membrane (Amersham) in 0.5× TBE, cross-linked and blocked overnight. Probes include: anti-miR-124: 5′-TGGCATTCACCGCGTGCCT TAA-3′, anti-miR-93: 5′-CTACCTGCACGAACAGCACTTTG-3′, and anti-U6: 5′-GCCATGCTAATCTTCTCTGTATC-3′. Probes were labeled using T4 polynucleotide kinase (Invitrogen) and [γ-^{32}-P]ATP (Perkin Elmer) and purified by Sephadex G-25 columns (GE Healthcare). Northern blots depicted in **Figure 2** were performed as previously described[30]. All northern blots portray a representative result that has been produced a minimum of three times.

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Incorporation of MREs into influenza A/PR/8/34 NP. Sites within influenza A/PR/8/34 nucleoprotein with partial complementarity to miR-93 were identified using Bibiserv's RNAhybrid algorithm. Nearly full complementarity was achieved with 3–5 steps of site-directed mutagenesis using the QuickChange kit and protocol (Stratagene) on the pPol-I driven nucleoprotein vector (a kind gift from P. Palese, MSSM, NYC).

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