

# MicroRNA-based strategy to mitigate the risk of gain-of-function influenza studies

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**Recent gain-of-function studies in influenza A virus H5N1 strains revealed that as few as three-amino-acid changes in the hemagglutinin protein confer the capacity for viral transmission between ferrets<sup>1,2</sup>. As transmission between ferrets is considered a surrogate indicator of transmissibility between humans, these studies raised concerns about the risks of gain-of-function influenza A virus research. Here we present an approach to strengthen the biosafety of gain-of-function influenza experiments. We exploit species-specific endogenous small RNAs to restrict influenza A virus tropism. In particular, we found that the microRNA miR-192 was expressed in primary human respiratory tract epithelial cells as well as in mouse lungs but absent from the ferret respiratory tract. Incorporation of miR-192 target sites into influenza A virus did not prevent influenza replication and transmissibility in ferrets, but did attenuate influenza pathogenicity in mice. This molecular biocontainment approach should be applicable beyond influenza A virus to minimize the risk of experiments involving other pathogenic viruses.**

The seasonal pathogen influenza A virus has the potential to cause catastrophic pandemics. Owing to the segmented nature and replication dynamics of its RNA genome, influenza A virus can undergo gene reassortment and/or accumulate mutations that permit its persistence and evolution in nature as well as its adaptation to new hosts<sup>3</sup>. For example, influenza A virus circulates in birds as a pathogen of the digestive and respiratory tracts but, on occasion, gains the capacity to jump species and establish respiratory infections in mammals<sup>3</sup>. Although many strains of influenza A virus circulate in migratory bird species at any particular time, the H5N1 strain has caused several hundred human deaths since 1997, and public health authorities are concerned that it may evolve to become as deadly as the 1918 H1N1 (ref. 4). The recent emergence of H7N9 infections in humans in China is causing similar concerns<sup>5</sup>.

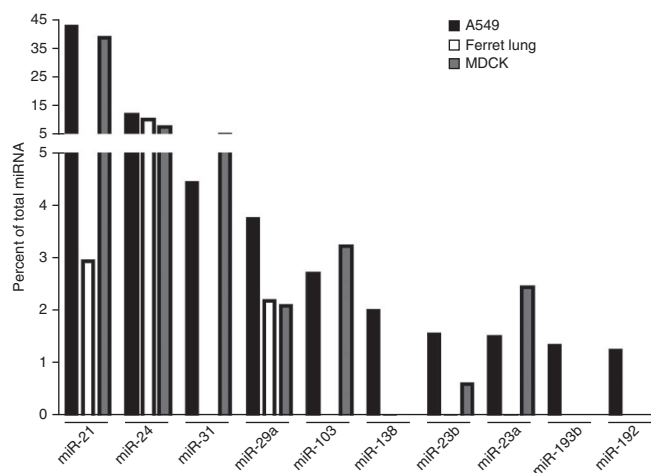
Although H5N1 is already capable of causing significant morbidity and mortality in humans, transmission thus far has been largely restricted to birds<sup>6,7</sup>. Humans infected with H5N1 seem to acquire

the virus from direct contact with birds. Human-to-human, ferret-to-ferret and pig-to-pig transmission of H7N9 viruses also appears to be inefficient<sup>8</sup>. However, as mutations may confer human transmissibility of H5N1 and H7N9 strains, defining the determinants of avian influenza virus transmission and using this information to monitor circulating avian strains regularly for transmission potential may improve global preparedness in the face of a novel pandemic. Recent findings suggest that three amino acid substitutions are sufficient to enable airborne transmission of some H5N1 strains between ferrets<sup>1,2,9</sup>; however, it should be noted that when introduced into currently circulating strains, these particular mutations are not sufficient to switch hemagglutinin binding preference from avian to human receptors<sup>10</sup>. In addition, natural recombinations between the seasonal H1N1 and avian H5N1 can confer transmissibility in guinea pigs<sup>11</sup>. These findings raised serious public concern about whether the scientific community should be generating mammalian transmissible influenza A viruses, even within the controlled setting of enhanced biosafety level 3 containment<sup>12</sup>. Here we present an approach to mitigate safety concerns associated with avian influenza A virus gain-of-function studies. Building on previous work showing that microRNA (miRNA)-binding sites engineered into the influenza A virus genome can suppress influenza A virus gene expression and infection<sup>13–15</sup>, we exploited endogenous miRNAs to limit viral tropism. Specifically, we exploit species-specific differences in miRNA expression to engineer a virus that is transmissible among animals of a desired experimental species but not among humans.

We screened small RNAs from human and ferret lung cells as well as Madin-Darby canine kidney cells (MDCK) by small RNA deep sequencing. As ferrets are routinely used in influenza A virus transmissibility experiments, and MDCK cells are often used to grow influenza A virus, we searched for miRNAs that are abundant in human A549 lung cells but absent in ferret lung cells and MDCK cells (the latter two species are both of the order *Carnivora*). Small RNAs from human lung A549 cells, MDCK and primary ferret lung were isolated, cloned and deep sequenced. Plotting specific reads over total reads allowed us to identify miRNAs that were abundant in A549 human lung cells (defined as >1% of total) but absent or nearly absent, in both ferret

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**Figure 1** Species-specific miRNA expression. Small RNAs were isolated from the human lung epithelial cell line A549, ferret lung and MDCK cells. Small RNAs were cloned and deep sequenced. Graph depicts percent of each miRNA among total miRNA, and displays the ten miRNAs most highly expressed in A549 cells.

lung and MDCK cells (Fig. 1 and Supplementary Table 1). Potential candidate miRNAs included miR-138, miR-193b and miR-192.

We analyzed by northern blot small RNAs isolated from human A549 cells and ferret lung to corroborate the miRNA expression pattern observed using sequencing (Supplementary Fig. 1a) and to rule out potential binding of other miRNAs to the northern blot probe, a labeled antisense oligonucleotide used to detect small RNA that can also act as a surrogate for miRNA target sites. In contrast to the sequencing data, northern blot analysis revealed substantial expression of miR-193b in ferret lung; northern blot analysis supported only miR-138 and miR-192 as miRNAs with the potential to facilitate ‘molecular biocontainment’. We next measured the abundance of miR-192 and miR-138 in murine lung; only miR-192 was detectable at levels sufficient to silence influenza A virus (Supplementary Fig. 1b). Furthermore, northern blot analyses of human lower respiratory tract bronchus and alveolar cell lines revealed robust miR-192 expression (Supplementary Fig. 1c). Additionally, quantitative PCR revealed that upper respiratory tract primary human nasal epithelial cells express approximately twofold more miR-192 than do alveolar cells (Supplementary Fig. 1d). These data are consistent with previous studies demonstrating miR-192 expression in primary human tracheal bronchial epithelial cells<sup>16</sup>. Given the disparate expression of miR-192 in ferret versus in human and murine respiratory tracts, we pursued miR-192 as an miRNA potentially capable of restricting influenza A virus replication in humans and mice but not in ferrets or MDCK cells.

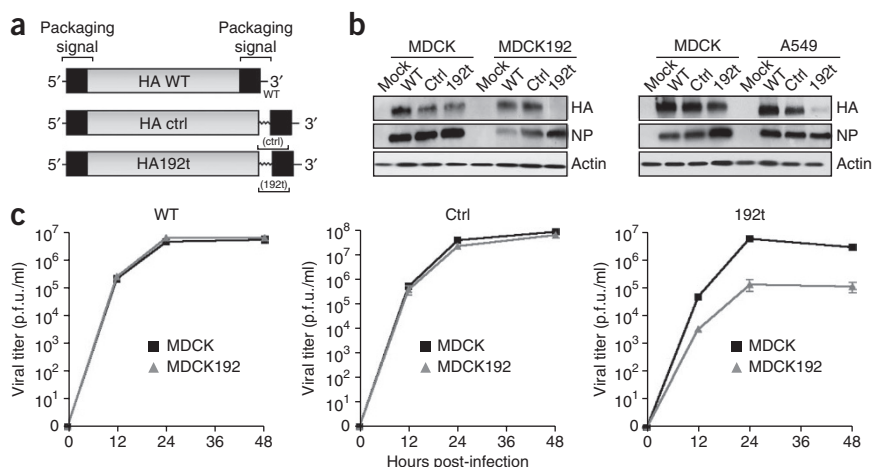
Past studies that engineered miRNA target sites into the influenza A virus RNA genome inserted the miRNA target site into the nucleoprotein segment because nucleoprotein is essential for virus replication and fitness<sup>13,14</sup>

or into NS1 (ref. 15). However, to prevent the possible reassortment of the hemagglutinin segment, here we chose to insert the miR-192 target site into segment four, which encodes hemagglutinin. To this end, we duplicated the 5′ packaging sequence of segment four viral RNA, thus creating an untranslated region (UTR) at the 3′ end of hemagglutinin mRNA (Fig. 2a). Without modifying the duplicated packaging sequence, we inserted scrambled noncoding RNA (control) or four fully complementary miR-192 target sites (192t) into this region (Fig. 2a). These modifications did not alter the amino acid composition of hemagglutinin or the biology of segment four, as the miRNA target sites are inserted downstream of the hemagglutinin stop codon but upstream of the duplicated packaging sequence.

For *in vitro* and *in vivo* studies, we used an H5 hemagglutinin in which the polybasic cleavage domain, a virulence motif that confers protease-independent virus replication and systemic spread<sup>17–19</sup>, is replaced with an avirulent H5 (termed HALo)<sup>20</sup>. The H5 HALo virus, in the influenza A/PR8 backbone, maintains high replicative capacity *in vitro* and in mice<sup>20</sup>. Engineered virus strains were rescued by plasmid-based transfection and amplified in MDCK cells, which do not express miR-192 (Supplementary Fig. 1e).

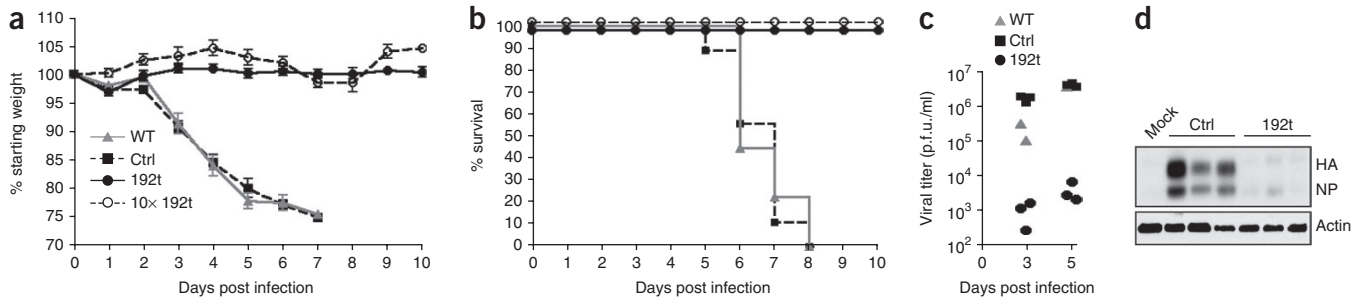
After a single cycle of infection, we constructed growth curves to measure the abundance of hemagglutinin and nucleoprotein in MDCK cells, MDCK cells engineered to express miR-192 (MDCK192) or A549 human lung epithelial cells that express endogenous miR-192. WT influenza A virus, influenza A virus engineered to express a scrambled miRNA target site (control) and influenza A virus engineered to express the miR-192 target site (192t) expressed similar amounts of hemagglutinin and nucleoprotein in MDCK cells. However, hemagglutinin expression from the 192t virus was ablated in MDCK192 or A549 cells, suggesting that the hemagglutinin segment was subject to post-transcriptional silencing in a sequence-specific manner (Fig. 2b).

To measure the effect of miR-192 targeting on influenza A virus replication, we constructed growth curves based on multiple cycles of infection. WT and control virus strains grew to similar amounts regardless of miR-192 expression (Fig. 2c and Supplementary Fig. 2).



**Figure 2** Species-specific miRNAs can be exploited to control influenza A virus tropism.

(a) Schematic illustrating WT (unmanipulated) and engineered (control and 192t) hemagglutinin viral segments. For the engineered segments the complete packaging signal (black) was duplicated, allowing for insertion of the control or miR-192 target sequences. Gray area represents hemagglutinin open reading frame. (b) MDCK and MDCK192 cells (left panel) or A549 cells (right panel) were infected with WT, control or 192t H5 HALo virus. After a single cycle of infection, abundance of viral hemagglutinin (HA) and nucleoprotein (NP) was determined by western blot analysis. Actin, loading control. (c) Replication kinetics of viruses depicted in a in MDCK and MDCK192 cells. Data points indicate plaque quantifications performed in triplicate at indicated hours post-infection. Error bars, mean  $\pm$  s.e.m. Data are representative of two independent experiments.



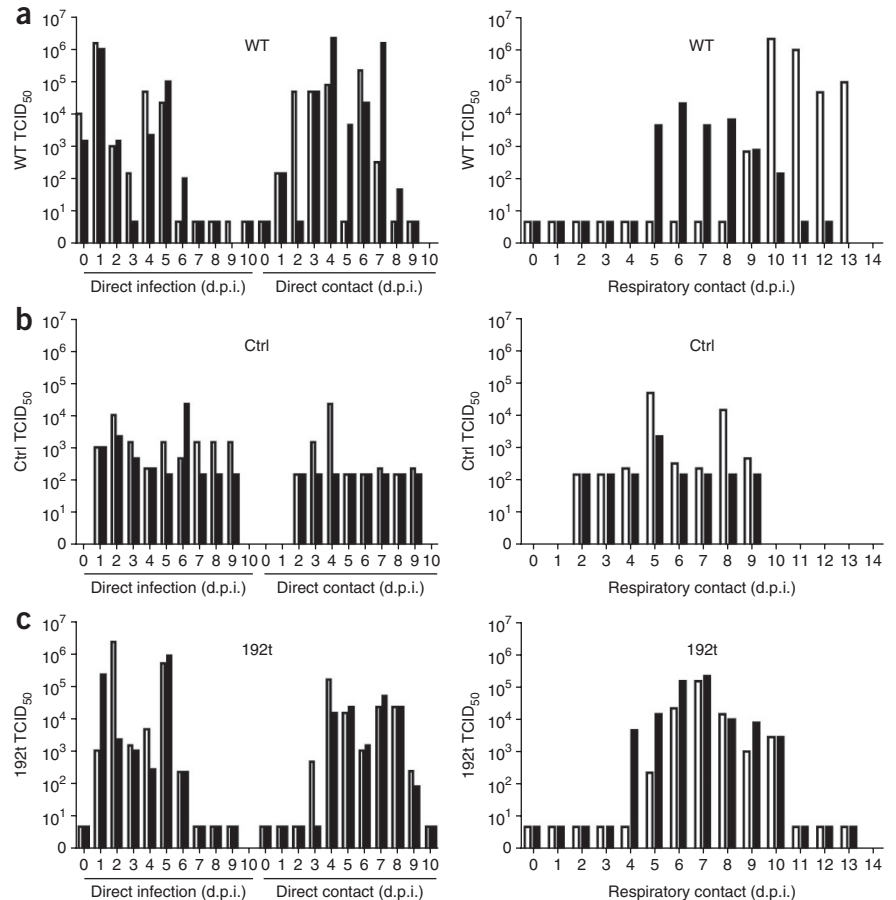
**Figure 3** Species-specific miR-192 targeting attenuates influenza A virus infection in mice. (a,b) Mice were infected with 100 plaque forming units (p.f.u.) of WT, control or 192t H5 HAlo virus (or with 1,000 p.f.u. of 192t virus, indicated as 10 $\times$ ) intranasally and weight loss **a**, and mortality **b**, were assessed daily ( $n = 9$  WT, 9 control, 10 192t and 5 10 $\times$ 192t mice per group). (c) Mice were infected as in **a** and virus titered from whole lung at 3 and 5 days post-infection ( $n = 3$  mice per group; each dot represents an individual mouse). (d) Mice were infected as in **a** and viral hemagglutinin and nucleoprotein protein expression from whole mouse lung were determined by western blot analysis 3 d after infection. Actin, loading control. Data are representative of two independent experiments. Morbidity and mortality data were pooled from two experiments with 4 or 5 mice per group in each experiment. Error bars, mean  $\pm$  s.e.m.

In contrast, 192t virus replication was blunted in MDCK192 and A549 cells compared to MDCK cells (Fig. 2c and Supplementary Fig. 2).

To determine the targeting potential of miR-192 within the dynamics of an intact pulmonary system, we infected C57BL/6 mice intranasally with WT, control or 192t virus. Consistent with miR-192 expression and *in vitro* virus growth (Fig. 2b,c), mice infected with 192t virus showed no signs of morbidity and did not succumb to the infection (Fig. 3a,b). In contrast, infection with WT or control virus resulted in weight loss and all animals died within 8 d after infection. Strikingly, even when the dose of 192t virus was increased by one order of magnitude, the mice showed no signs of morbidity or mortality. These results illustrate that species-restricted miRNA targeting of hemagglutinin can provide robust protection even when the lethal dose is exceeded by at least tenfold. In results consistent with the weight loss and survival data, we detected a reduction in pulmonary titers of 192t compared to WT or control viruses at 3 and 5 d after infection; we also noted a reduction in the amount of 192t hemagglutinin and nucleoprotein (Fig. 3c,d and Supplementary Fig. 3). To determine if the reduction in virus replication in the 192t-infected animals is a result of virus escape from miRNA targeting, either through mutation or complete excision, we plaque-purified 192t virus from the lungs of mice 5 d after infection and sequenced all isolates. All 17 of the 192t virus plaques isolated contained perfect miR-192 target sites (data not shown). These data are consistent with previous studies describing miRNA-mediated influenza A virus targeting, where

no evidence of virus escape *in vivo* was detected; these results demonstrate the safety of this molecular biocontainment platform<sup>13,14</sup>. Taken together these data demonstrate that species-specific miRNAs expressed in the lung are capable of blunting *in vivo* influenza A virus infections and preventing virus-induced disease.

To be useful in transmission studies, inserted miRNA target sites must not alter virus biology within the experimental animal model of interest. To determine if our engineering of miRNA target sites in influenza A viruses alters virus biology in ferrets, and to extend our results to a second viral strain, we used the seasonal H3N2 Wyoming strain, for which aerosol transmission in ferrets has been demonstrated<sup>7</sup>. Similar to how we engineered the H5 HAlo virus, we duplicated the



**Figure 4** Species-specific miRNA targeting of influenza A virus does not affect virus capacity to infect, replicate or transmit between ferrets. (a–c) Ferrets were directly infected with WT (a), control (b) or 192t (c) H3N2 viruses and monitored by nasal wash on indicated days post-infection (d.p.i.). Infections induced by direct contact (left panels) or respiratory contact (right panels) were determined by nasal wash of naive animals co-housed with directly infected animals. Open and closed bars represent individual ferrets.

5' packaging sequence of hemagglutinin viral RNA thus creating a 3' UTR in H3 mRNA, which allowed insertion of four perfect miR-192 target sites or a scrambled control site. To confirm miR-192-mediated attenuation of H3N2 virus, we measured growth kinetics in MDCK and A549 cells. We detected an ~100-fold reduction in growth of 192t compared to control virus in A549 cells (**Supplementary Fig. 4a,b**), suggesting that miR-192 can attenuate this H3N2 virus.

To assess the effect of miR-192 target-site engineering on the dynamics of virus spread, we performed ferret transmission studies. We directly compared the transmission of unmanipulated WT, control and 192t H3N2 viruses (**Fig. 4**)<sup>21</sup>. Naive animals were exposed to infected ferrets by direct contact (housed within the same cage) or by respiratory contact (separated, allowing only for aerosol transmission) and virus levels were monitored by nasal wash. Ferrets who were inoculated intranasally with WT, control or 192t had nasal washes with high viral titers as early as day one, which slowly dissipated over the 10-d infection (**Fig. 4a–c**). For WT, control and 192t H3N2 infections, infection was spread by direct and respiratory contact with ferrets inoculated intranasally (**Fig. 4a–c**). Consistent with the absence of miR-192 expression in ferret lungs, incorporation of miR-192 target sites did not prevent transmission in these animals. To ensure genetic stability of the recombinant hemagglutinin segment in ferrets, we isolated and sequenced virus from ferrets at day seven (**Supplementary Figs. 1–4** and **Supplementary Table 1**) after aerosol transmission; we detected no evidence of excision events (data not shown). Taken together these data suggest that the inserted miRNA target sites are stable *in vivo* and do not prevent transmission in this experimental animal model.

These data demonstrate that species-specific miRNAs can be exploited to mitigate the potential risks associated with transmission studies of influenza A virus. Adding this proposed molecular biocontainment measure to existing physical safety precautions, such as the use of enhanced biosafety level 3 facilities and protocols, would greatly reduce the possibility of viral escape. One potential concern regarding this approach is the possibility of escape mutants. However, the absence of escape mutants in this study and in studies of comparable miRNA-targeting strategies suggest that escape is unlikely<sup>13,14</sup>. No alleviation of miRNA-mediated silencing was observed in viruses engineered to express more than one miRNA target site, even in viruses with polymerases of lower fidelity<sup>22</sup>. In events where escape does occur, the entire targeted sequence is excised<sup>23,24</sup>; we did not detect such an event in either of our *in vivo* systems. In any case, additional measures could be employed to further enhance safety including the targeting of multiple segments with either the same or unique species-specific miRNAs. Compounded targeting would not only minimize the possibility of escape but also further blunt replication in desired cells and hosts.

Lastly, it should be noted that this strategy is not necessarily restricted to influenza A virus. It may also be used to minimize risks associated with potential gain-of-function experiments of other highly pathogenic viruses including Ebola, SARS coronavirus and henipaviruses, among others. The only requirements for this approach are miRNAs that are absent in cells of the model system where replication is meant to occur but present in human cells, and a genetic system to permit the insertion of miRNA target sites into the genome.

## METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the [online version of the paper](#).

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## AUTHOR CONTRIBUTIONS

R.A.L. and R.A.A. designed and conducted the research and wrote the manuscript. B.K., T.S., C.F., A.S.G.-R., M.A. and K.X. were responsible for the transmission experiments. J.S.S. performed and analyzed deep sequencing experiments. M.A.C. provided technical support. D.P., A.G.-S. and B.R.T. designed research, oversaw the project and wrote the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## ONLINE METHODS

**Deep sequencing, northern blot and qPCR analysis.** Purification of total RNA, isolation, cloning of the small RNA fraction, sequencing and analysis on the Illumina platform, and northern blot analyses were done as previously described<sup>13</sup>. Northern blot probes: miR-192: 5'-GGCTGCAATTCATAGGT CAG-3', miR-193b: 5'-AGCGGGACTTTGTGGGCCAGTT-3', miR-138: 5'-CGGCCTGATTACAACACCAGCT-3' and U6: 5'-GCCATGCTAAT CTTCTCTGTATC-3'. Quantitative PCR for the detection of miR-192 was performed on 10 ng of RNA using the TaqMan (Applied Biosystems) reverse transcription kit according to the manufacturer's instructions. cDNA was then analyzed using TaqMan probes specific for mature miR-192 and miR-20a on a realplex thermocycler (Eppendorf). miR-20a was used for normalization as levels are similar between human A549 and ferret lung samples.

**Cell lines.** Calu3 (human bronchial epithelia) cells were purchased from American Type Culture Collection (ATCC) (HT-B55) and were confirmed to be free of mycoplasma. A549 (human small airway) cells were purchased from ATCC (CCL-185). Human nasal epithelial cells suspended in RNAlater were purchased from Promocell (C-14062). F56 (ferret fibroblasts) cells were derived directly from naive ferret lung parenchyma. MDCK cells were kindly provided by Peter Palese (MSSM). MDCK cells expressing full-length primary miR-192 (MDCK192) were generated in a similar manner to MDCK cells expressing miR-142 (ref. 13). These A549, MDCK and MDCK192 cells were not tested for mycoplasma contamination.

**Virus design, rescue and growth assays.** 181 and 154 nucleotides of the 3' end of H3/Wyoming and H5/Vietnam HALo mRNA were duplicated, respectively, and inserted immediately upstream of their cognate stop codons by site-directed mutagenesis. This recreated the endogenous open reading frame for hemagglutinin with a nonoverlapping packing sequence separated by a unique ClaI site. The ClaI site, located immediately 3' to the hemagglutinin mRNA stop codon, was used for insertion of miR-192 (5'-GGCTGTCAATTCATAGGTCAG-3') or scrambled (5'-ATCGATAGGAATTTACCAAAT-3') targeting sequences. Viruses were rescued by standard reverse genetics and plaque-purified, their sequences were confirmed and they were quantified by plaque assay. Multicycle growth curves were performed in MDCK, MDCK192 and A549 cells in the presence of TPCK trypsin. Supernatant was harvested at the indicated time points and titers assessed by plaque assay. The limit of detection for all virus titration experiments was 20 p.f.u.

**Western blot analysis.** Single-cycle infections were performed at a multiplicity of infection of 1 and whole protein was extracted and separated on a 7.5–10% SDS/PAGE gel and transferred to nitrocellulose (Bio-Rad). Membrane was blocked for 1 h with 5% skim milk at 25 °C, and then incubated with the indicated primary antibody overnight at 4 °C. Antibodies specific for actin

(PA5-17568 ThermoScientific), H5 HALo (DPJY02 NR-19870 biodefense and emerging infections (bei) Resources), and nucleoprotein (NR-4544 bei Resources) were added at a concentration of 1 µg/ml in 5% skim milk. Serum from H5 HALo infected mice was used at a 1:5,000 dilution. Secondary mouse antibodies (NA931V GE Healthcare) were used at a 1:5,000 dilution for 1 h at 25 °C. Protein was detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore) as directed by the manufacturer.

**Mouse infections.** All animal experiments were done in accordance with Mount Sinai School of Medicine Institution of Animal Care and Use Committee. C57BL/6 8-week-old female mice were purchased from Taconic. For these experiments mice were not randomized and investigators were not blinded. Mice were anesthetized with a ketamine, xylazine, H<sub>2</sub>O mixture then infected intranasally with 100 p.f.u. of WT, control or 192t H5 HALo virus. Where indicated mice were infected with 1,000 p.f.u. of 192t H5 HALo virus. Lungs were harvested at 3 and 5 d after infection and homogenized in PBS either for direct titering or protein analysis.

**Transmission studies.** Animal studies were done as described previously with minor modifications<sup>1</sup>. Briefly, transmission studies were performed in a BSL2<sup>+</sup> facility at the Department of Veterinary Medicine, University of Maryland. Animal studies described were carried out according to the guidelines of the Animal Care and Use Committee of the University of Maryland (protocol RO-09-93). For these studies ferrets were not randomized and investigators were not blinded. Prior to inoculation, 23-week-old female ferrets (Sayre, PA) were housed in holding BSL2<sup>+</sup> for 1.5 weeks to establish a baseline for temperature, weight and general health. Serum from each ferret was assayed by nucleoprotein-specific enzyme-linked immunosorbent assay to rule out prior influenza A virus exposure. Transponders were implanted subcutaneously to provide a means of identification and temperature determination. Ferrets were inoculated with 10<sup>5</sup> tissue culture infectious dose 50 (TCID<sub>50</sub>), the amount of virus required to infect 50% of infected cell cultures, diluted in PBS (0.5 ml total volume, 0.25 ml/nostril was administered intranasally). Directly infected ferrets were inoculated 1 d before the introduction of the naive direct contact (housed within the same cage) and respiratory contact ferrets (separated by wire mesh allowing for only aerosol transmission). Ferrets were given nasal washes (0.25 ml PBS/nostril) every day after the administration of anesthesia and assayed by FluDetect (Synbiotics Corp.). Nasal washes continued until animals tested negative for two consecutive days. To prevent cross contamination, ferrets were handled in the following order: respiratory contact, direct contact, direct infected. Gloves were changed between every virus group. Once RC ferrets tested positive, they became the last to be given nasal washes. All viruses, including nasal wash samples and virus stocks, were titrated in MDCK cells by TCID<sub>50</sub>. Titters were read by hemagglutination assay using 0.5% Turkey red blood cells 3 d after infection.