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Supplementary Information for

Chimeric hemagglutinin vaccine elicits broadly protective CD4 and CD8 T-cell responses against multiple influenza strains and subtypes

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Supplementary Information Text

Materials and Methods

Vaccine and plasmid construction. All 102 full-length HA sequences from H1N1 viruses available in early 2009~2013 were downloaded from the NCBI database and aligned by the ClustalW algorithm from the BioEdit program. The most conserved amino acid at each position was chosen to create a consensus H1 sequence. The consensus hemagglutinin H5 (pCHA5-II) sequence was generated as described previously (1). The nucleotide sequences of consensus hemagglutinin H5 (pCHA5-II) and consensus H1 were cloned into the pcDNA expression vector, and the resulting plasmids were used as the templates for swap and chimeric HA construction. Swap H1/5 is composed of H1 as HA1 (amino acid 1~344) and H5 as HA2 (amino acid 345~520), giving H1 as globular head and H1+H5(HA2) stem. Swap H5/1 is composed of H5 as HA1 (amino acid 1~344) and H1 as HA2 (amino acid 345~520), giving H5 as globular head and H5+H1(HA2) stem. For chimeric H5/1 construct, the globular head domain is composed of the amino acid sequence between residues C52 and C277 (H3 numbering) and the stem region is comprised of portions of HA1 and HA2 subunits (amino acid 1~51 and 278~520). The oligonucleotide sequences of the primers for the constructs are listed in *SI Appendix,* Table S2. The transmembrane domain was replaced with the additional residues from the bacteriophage T4 fibritin foldon trimerization sequence (2), thrombin cleavage site and $(His)_6$-tag at the C-terminus of the HA. Both DNA sequences of consensus HA were optimized for expression by using human-preferred codons and various regions were amplified by PCR and subsequently cloned into the pcDNA vector for expression. Furthermore, the HA gene from influenza virus seasonal H1N1 Brisbane/59/2007, pandemic H1N1 California/07/2009, H3N2 Brisbane/10/2007, H7N9 A/Shanghai /2/2013 and avian flu H5N1 Vietnam/1194/2004 were also optimized, synthesized, and cloned into the pcDNA expression vector. The sequences were confirmed by DNA sequencing and prepared in high quality for protein expression and purification.

Expression of recombinant secreted HA from expressed cells. Human epithelial kidney (HEK) 293T and HEK293S cells were routinely maintained in DMEM (Gibco) supplemented with 10% Fetal bovine serum (Gibco). For transient transfection, 293T or 293S cells were seeded in a 10 cm dish (Nunc, Roskilde, Denmark) and all the procedures were performed according to the manufacture's protocol. Briefly, 293T or 293S cells at 80% confluency were transfected with Mirus TransIT®-LT1 (Mirus Bio) transfection reagent using a 3:1 ratio of reagent to plasmid DNA. TransIT®-LT1 reagents were diluted with Opti-MEM (Gibco) and the mixture was incubated for 5-20 minutes at room temperature. The solution was added with plasmid DNA and mixed completely followed by incubation for 15-30 minutes. Prior to transfection, cells were replaced with fresh DMEM (Gibco) medium supplemented with 10% Fetal bovine serum. The TransIT®-LT1 reagent/DNA complex was added to the cells and incubated for 48 h at 37 ºC. The expression of hemagglutinin was confirmed with immunoblots using anti-$(his)_6$ antibodies (Qiagen) or specific anti-hemagglutinin antibodies and the horseradish peroxidase (HRP)-conjugated secondary antibodies (PerkinElmer).

Purification of recombinant secreted hemagglutinins. For expression in human 293T cells, pcDNA carrying the gene of interest was prepared in high quality and transfected to

the cells with Mirus TransIT®-LT1 (Mirus Bio). After 48 h of transfection, the medium was collected and the cells were clarified by centrifugation at 1,000 × g for 10 mins. The supernatant was purified by Ni-NTA (nickel-nitrilotriacetic acid) affinity column (GE Healthcare). The supernatants were loaded onto Ni-NTA affinity column pre-equilibrated in 20 mM Tris-HCl pH 8.0 and 300 mM NaCl. The unbound proteins were washed out with imidazole gradient from 25 to 50 mM in 20 mM Tris-HCl pH 8.0 and 300 mM NaCl (Buffer A). Then the HA protein was eluted with 100 to 300 mM imidazole gradient in Buffer A. The purified HA proteins were concentrated by Amicon Ultrafiltration Unit (MW30K cutoff) (Millipore) in PBS, pH 7.4. The purity was monitored by using SDS-PAGE and the proteins were confirmed using Western blot with anti-$(his)_6$ antibodies (Qiagen) or specific anti-hemagglutinin antibodies and the horseradish peroxidase-conjugated secondary antibodies (PerkinElmer). Finally, the trimer form of HA proteins was obtained by using size-exclusion column, Superdex 200 Increase 10/300 GL gel filtration column (GE Healthcare).

Preparation of mono-glycosylated HA proteins. HEK293S cells, which are deficient in N-acetylglucosaminyltransferase I, was used to produce HA with high-mannose glycans (3). The purified HA protein from HEK293S cells was treated with Endo H (NEB) at 20 °C for overnight to produce the monoglycosylated HA_{mg}. The ratio of proteins to Endo H was 3 to 1 (w/v) for HA. Endo H and mono-glycosylated HA protein were then separated by Superdex 200 Increase 10/300 GL gel filtration column (GE Healthcare). The HA_{mg} proteins were concentrated by Amicon Ultrafiltration Unit (MW30K cutoff) (Millipore) in PBS, pH 7.4 and confirmed by SDS-PAGE and LC-MS/MS analysis.

Identification of N-linked glycosylation on HA proteins. Ten micrograms of protein were run on SDS-PAGE and were prepared for in-gel digestion. The desired proteins bands were excised with a sharp scalpel, diced into 1 mm pieces and placed into 1.3 ml eppendorf tubes. After washing twice with 500 µl of 25mM ammonium bicarbonate in 50% ACN (acetonitrile) for 3 min, the gel pieces were dried using a SpeedVac evaporator (Thermo). The dried samples were reduced by the addition of 100 µl of 50 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate (pH 8.5) at 37 °C for 1 h followed by centrifuge at 10,000 g for 1 min. The solution was removed and the gel samples were proceeded to an alkylation step by the addition of 100 µl of 100 mM iodoacetamide (IAA) in 25 mM ammonium bicarbonate (pH 8.5) and incubated in the dark at room temperature for 1 h. After washing with 500 µl of 50% acetonitrile in 25mM ammonium bicarbonate (pH 8.5) and 500 µl of 100% acetonitrile, the samples were centrifuged at 10,000 g for 1 min and the supernatant was removed completely. The gel samples were dried at a SpeedVac evaporator and redissolved with 200 µl of 25 mM ammonium bicarbonate (pH 8.5). Gel samples were then treated with 0.5 µg trypsin (Promega, Madison, WI, USA) and 1 µg chymotrypsin (Promega, Madison, WI, USA) for overnight. After an overnight digestion, the samples were added with 100 µl of 50% acetonitrile in 5 % TFA. The samples were sonicated for 10 sec, and then stopped for 10 sec. The processes were repeated 10 times. The supernatant containing peptide mixtures was removed from the sample tubes and transferred to new tubes. The procedure was repeated twice. The combined supernatants were dried in SpeedVac concentrator and processed for LC-MS/MS analysis.

Endotoxin measurement. Endotoxin levels were determined using the Pierce® LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific). Protein samples were diluted in 10, 20, 100, and 1000-fold, while endotoxin standards were prepared as 10, 5, 2.5, 1.25, 0.63, 0.31, 0.15, and 0 ng/ml. After the microplate was equilibrated in a heating block for 10 mins at 37 °C, protein samples or standards were mixed with Limulus Amebocye Lasate (LAL) Pyrochrome reagent (final volume 100 µl) (1:1) in endotoxin-free wells at 37 ºC for 10 minutes. One hundred µls of substrate solution were added to each well and the plates were incubated at 37 ºC for 6 mins. The reaction was stopped with the addition of 50 µl stop reagent (25 % acetic acid). The absorbance of wells was measured at 405 nm using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA). A standard curve was obtained by plotting the absorbance versus the corresponding concentrations of the standards. The standard curve was used to determine the endotoxin concentration of the samples. Endotoxin values of all purified proteins were < 0.5 ng/ml.

Mice vaccination. Adjuvant C34 was chemically synthesized as described (4) and dissolved in DMSO. Female 6- to 8-week-old BALB/c mice (n=10 per group) were immunized intramuscularly with 20 µg of purified chimeric HA_{fg} or HA_{mg} proteins in PBS, pH 7.4, and mixed with 50 µg of aluminum hydroxide (Alum; Sigma) or 2 µg of C34. Control mice were injected with phosphate buffer saline (PBS). Three vaccinations were given at two-week intervals. Blood was collected 14 days after the second or third immunization. The blood was incubated at 37 ºC for 30 minutes, and centrifuged at 1,2000 rpm for 10 mins to collect serum. The HA-specific antibodies in serum collected from vaccinated mice was assessed by enzyme-linked immunosorbent assay (ELISA) and neutralization assay.

Determination of HA-specific antibodies by ELISA. HA-specific antibody titers were detected by ELISA using H1N1 A/Brisbane/59/2007, H1N1 A/California/07/2009, H3N2 Brisbane/10/2007, H7N9 A/Shanghai/2/2013 and H5N1 Vietnam/1194/2004 HA proteins as the substrates. Ninety-six-well ELISA plate (Greiner bio-one, Frickenhausen, Germany) was coated with 100 µl of protein diluted in ELISA coating buffer, 100 mM sodium bicarbonate (pH 8.8), at a concentration of 5 µg/ml per well and covered with a plastic sealer at 4 °C for overnight. After the plates were blocked with 1% BSA in TBST (137 mM NaCl, 20 mM Tris-base, 0.05% Tween 20, pH 7.4) at 37 °C for 1 h and washed 3 times with TBST, the plates were incubated with 200 µl of mouse serum in 2-fold serial dilutions at 37 ºC for 2 h. After serum was moved and the plate was washed 6 times, HA-specific IgG was monitored by using 200 µl of secondary HRP-labeled anti-mouse antibody (1:8000) (PerkinElmer, Waltham, MA, USA). After 1 h of incubation at 37 ºC, the plates were washed 6 times with TBST and developed with 100 µl of the Super Aquablue ELISA substrate (eBioscience, San Diego, CA, USA) for 1 min. The reaction was stopped with the addition of 100 µl of 0.625 M oxalic acid. The absorbance of wells was measured at 405 nm using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA). The endpoint antibody titer was defined as the highest dilution of serum to produce an absorbance 2.5 times higher than the optical absorbance (OD) produced by the negative control (pre-immune serum). The background endpoint antibody titer was assigned as less than 1:50.

Harvest of bone marrow-derived dendritic cells. The GM-CSF–cultured bone marrow-derived dendritic cells (BMDCs) were prepared as described previously (5). Briefly, bone marrow single cell suspensions were subjected to RBC lysis to remove the red blood cells (RBCs). The remaining cells were cultured in 10 ml of RPMI 1640 supplemented with 20 ng/mL murine GM-CSF (eBioscience), 10 % FBS (BenchMark), 50 μM 2-ME, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were plated into each petridish to achieve the final cell density of 2×10^6 cells/petridish. The culture was replenished by adding 10 ml of fresh culture medium containing 20 ng/mL murine GM-CSF at day 3 and refreshed with one-half the volume of complete culture medium as described above at day 6. At day 8, immature BMDCs were harvested by collecting nonadherent cells by gently pipeting and re-plated the cells at a density of 10^6/ml. For CD8+ T cell assay, immature BMDCs were co-cultured with CD8+ T cell and chimeric HA proteins (0.1 mg/well in 100 μL) for 48 h. The number of granzyme B producing CD8+ T cell was determined by flow cytometric analysis after washing.

Enzyme-linked immunospot (ELISpot) assay. ELISPOT plates were coated with anti-mouse IFN-γ, IL-4 (Mabtech AB, Stockholm, Sweden) or granzyme B (R&D Systems) according to the manufacturer's instructions. The plates were washed four times and incubated for 30 min with RPMI-1640 supplemented with 10 % Fetal bovine serum (Gibco). For the detection of IFN-γ, IL-4 and granzyme B-secreting cells from chimera-immunized mice, splenocytes were collected and cultured at 5 X 10^5 per well at 37 °C in 5% CO_2 for 24 h with specific peptides from HA for restimulation. The cells were removed and incubated with biotinylated anti-mouse IFN-γ, IL-4 (Mabtech AB) or granzyme B (R&D Systems) specific antibody. The plates were washed five times before the addition of streptavidin-ALP conjugate and developed with ready-to-use BCIP/NPT substrate. Following drying, the number of resulting spots was analyzed with an Immune Spot Reader (Cellular Technology Ltd.). Data were obtained from triplicate wells.

Neutralization assay. The culture supernatant containing 100 $TCID_{50}$ of virus was mixed with equal volume of two-fold serially diluted serum and incubated at 37 °C for 1 h. The mixtures were then added to MDCK cells in each well of a 96-well plate and incubated at 37 °C for 3 days. The cells were added 30 μl CellTiter-Glo (Promega) to determine the number of viable cells based on quantitation of the ATP present. The neutralizing activity of serum was determined as the maximal dilution fold that significantly protected cells from virus-induced death.

Microneutralization assay. An infection medium (DMEM supplemented with 0.3 %BSA, 2 μg/ml TPCK-Trypsin) containing virus at 100 $TCID_{50}$ was mixed in equal volume with two-fold serial dilutions of serum and incubated at 37 °C for 1 h. The mixture was then added to MDCK cells (1.5×10^4 cells per well) in each well of a 96-well plate and incubate at 37 °C for 16-20 h. The cells were washed with PBS, fixed in acetone/ methanol solution (vol/vol 1:1), and blocked with 5% skim milk. After 1 h of incubation at 37 °C, the wells were washed 6 times with PBST, and the virus titer was monitored by using 100 μl of mAb against influenza A NP (1:2500). After 1 h of incubation at 37 °C, the wells were washed 6 times with PBST and added 100 μl of secondary HRP-labeled anti-rabbit antibody (1:5000) (PerkinElmer, Waltham, MA, USA). After 1 h of incubation at 37 °C, the wells

were washed 6 times with PBST again and developed with 50 µl of the 1-Step Ultra TMB substrate (Thermo) for 1 min. The reaction was stopped with the addition of 50 µl of 1 M H_2SO_4. The absorbance of wells was measured at 450 nm using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA).

Antibody dependent cell mediated cytotoxicity reporter assay. MDCK cells (1×10^4 cells per well) in each well of a 96-well flat-bottom plate were incubated at 37 °C for 24 h. The next day, 1×10^4 MDCK cells were infected with influenza viruses at multiplicity of infection (MOI) of 1 for 24 h. The medium was then replaced with Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 4% Low IgG Serum followed by addition of serial dilutions of antisera from chimeric HA protein-vaccinated mice and incubated at 37 °C for 30 min. Jurkat effector cells expressing mouse FcγRIII (Promega) were suspended in RPMI 1640 medium containing 4% low IgG FBS and the target cells: effector cells ratio of 1:5 were added to the infected MDCK cells. After incubation at 37 °C for 6 h, assay plates were removed from the 37 °C incubator and equilibrated for 15 min at ambient temperature before Bio-Glo™ Luciferase Assay Buffer (Promega) was added in a 1:1 ratio. Luminescence was measured on a CLARIOstar plate-reader.

Virus challenge experiments. Two weeks after three vaccinations at two week intervals, the immunized mice were challenged intranasally with 10 LD_{50} (the virus doses leading to 50% of the death of mice) of H1N1 California/07/2009, H1N1 A/New Caledonia/1999, H1N1 A/WSN/1933, H1N1 A/Solomon Islands/03/2006 and a reassortant H5N1 virus A/Vietnam/ 1194/2004/NIBRG14 and H5N1 A/Turkey/1/2005/NIBRG23. After infection, the mice were observed daily for 14 days, and survival and body weight were recorded. The percentage of body weight was calculated for each individual animal per group by comparing the daily weight to the pre-challenge weight, and mice losing more than 25% of their initial weight were sacrificed and scored as dead. Mouse studies were approved by the Institutional Animal Care and Use Committee of Academia Sinica. All animal experiments were performed under biosafety level-3 enhancement conditions.

Expression and purification of recombinant F10 antibodies. The plasmid that encodes the F10 antibody was transfected into serum-free adapted FreeStyleTM 293F cells by using polyethyleneimine and was cultured in FreeStyleTM 293 Expression Medium (Gibco) in 125 ml sterile Erlenmeyer flasks, rotating at 135 rpm on an orbital shaker platform. The supernatant was collected 72 h after transfection and the cells were clarified by centrifugation at 1,000 × g for 10 mins. The supernatants were loaded onto Protein-A column (GE Healthcare) that was pre-equilibrated in 5 Column Volumes (CV) of phosphate buffered saline (PBS) washing buffer (pH 7.0), followed by 5 CV of washing buffer. The F10 antibody was eluted with 0.2 M glycine buffer (pH 2.5) and the fractions were collected into tubes containing 0.5 mL 1 M Tris–HCl pH 9.0 for neutralization. The purity was monitored by using SDS-PAGE.

Statistical analysis. The animal experiments used for evaluation of immune responses were repeated at least three times (n =5 per group), and the virus challenge studies were done at least twice (n = 10 per group). The response of each mouse was counted as an individual data point for statistical analysis. Data obtained from animal studies were

examined by using two-way ANOVA from Prism; data were presented as mean ± SEM and differences were considered significant at *P < 0.05; **P <0.01; ***P < 0.001.

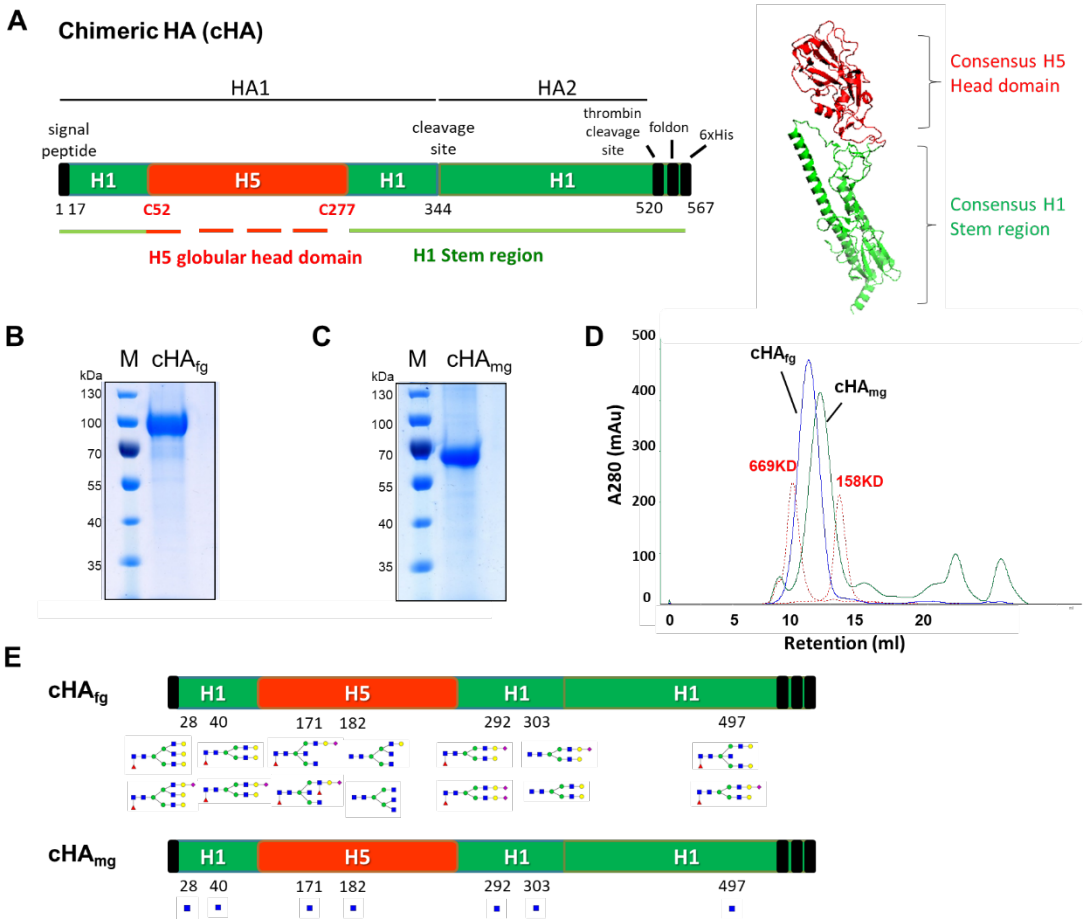


Fig. S1. Design and preparation of chimeric HA proteins. (A) The designed influenza HA sequences were constructed using the consensus H1N1 sequence and the consensus H5N1 sequence pCHA5-II to generate the chimeric HA. The globular head domain is composed of the amino acid sequence between residues C52 and C277 (H3 numbering). The stem region is composed of portions of HA1 and HA2 subunits. The protein structures were downloaded from the Protein Data Bank ID code 2IBX (VN1194 H5 HA) and 3LZG (A/California/04/2009). Final images were generated with PyMol. Because no structure of a consensus HA has been published, the image of the head domain of the avian flu H5 (Vietnam/1194/2004) and the stem region of the pandemic H1N1 (California/07/2009) are used for the chimeric HA construct. (B-D) Purification of chimeric HA protein and gel-filtration chromatography analysis. (B) The purified HA proteins were analyzed by SDS/PAGE. M: molecular weight marker. left: cHA_{fg}, the fully glycosylated cHA directly purified from HEK293T cells; (C) cHA_{mg}, the monoglycosyalted cHA purified from HEK293S cells and digested with endoglycosidase H. (D) Gel filtration analysis of purified secreted HA proteins. The fully glycosylated cHA from HEK293T cells (blue line) and the monoglycosyalted cHA (green line) existed as a trimer (>200 kDa) as shown in chromatograph. The figure represents superimposed elution profiles of HEK293T cell-expressed cHA proteins overlaid with calibration standards (dotted red line). (E) A schematic figure to mark the main glycans on the glycosites of cHA_{fg} and cHA_{mg} determined by LC-MS/MS. The general glycan symbols were followed.

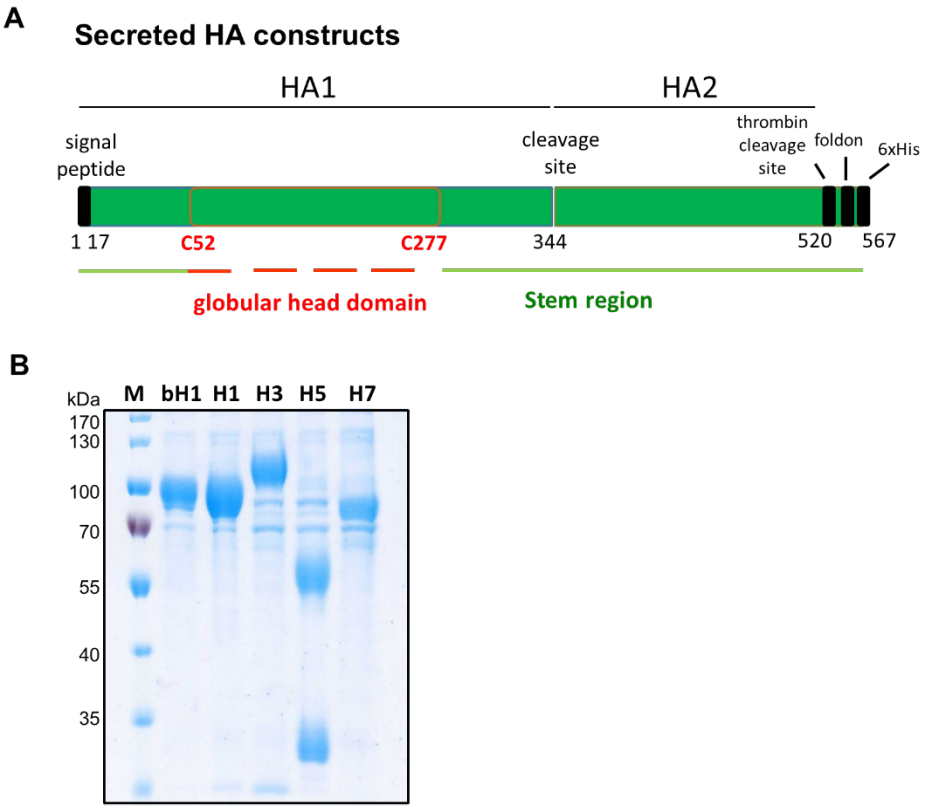


Fig. S2. The constructs of secreted HA and purification. (A) The sequence encoding the ectodomain of HA was prepared in the expression vector pcDNA and transfected to HEK293T cells. The protein was engineered to contain a stabilization/trimerization signal, foldon, as well as a C-terminal $(His)_6$ tag for purification. (B) The purified HA proteins were analyzed by SDS/PAGE. M: molecular weight marker. Lane 1: H1N1 (A/Brisbane/59/2007) HA protein; lane 2: H1N1 (A/California/07/2009) HA protein; lane 3: H3N2 (Brisbane/10/2007) HA protein; lane 4: H5N1 (Vietnam/1194/2004) HA protein; lane 5: H7N9 (A/Shanghai/2/2013) HA protein.

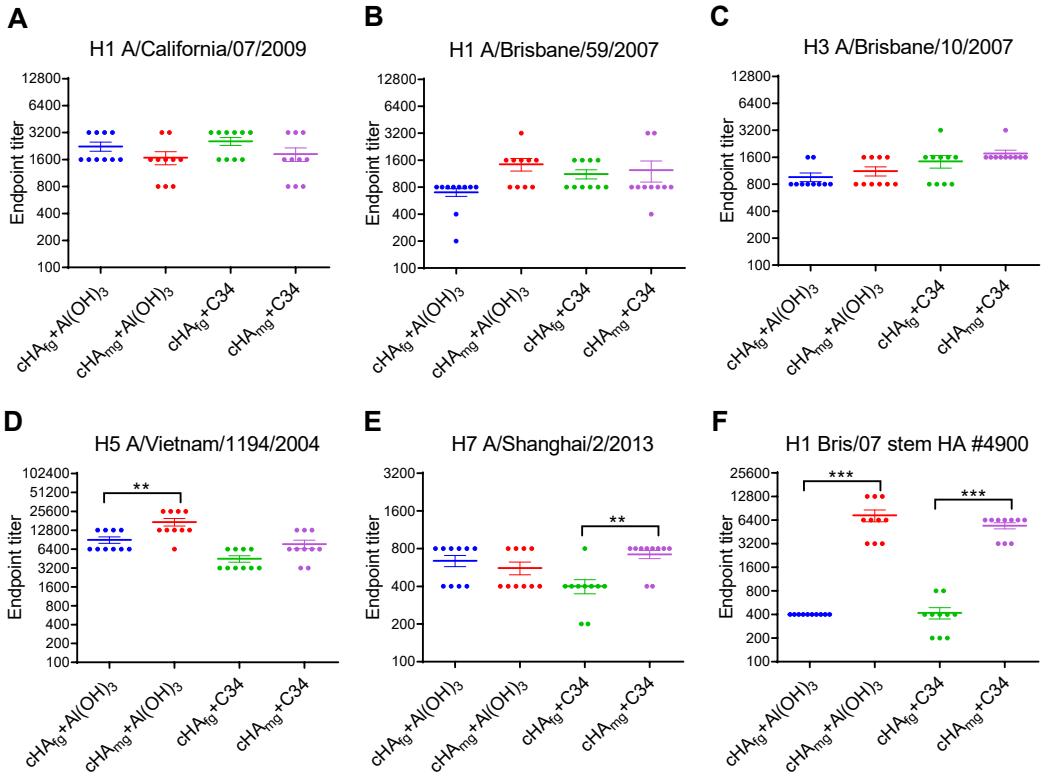


Fig. S3. HA binding activities of antisera from mice vaccinated with cHA_{fg} and cHA_{mg}. BALB/c mice (n=10 per group) were immunized at two-week intervals with cHA_{fg} or cHA_{mg} adjuvanted with $Al(OH)_3$ or C34. The antibody titers from the mice vaccinated with $Al(OH)_3$-adjuvanted cHA_{fg} (blue) and cHA_{mg} (red) vs. C34-adjuvanted cHA_{fg} (green) and cHA_{mg} (purple) were measured on day 28 by ELISA with the A/California/07/2009 H1N1 HA protein (A), A/Brisbane/59/2007 H1N1 HA protein (B), A/Brisbane/10/2007 H3N2 HA protein (C), A/Vietnam/1194/2004 H5N1 HA protein (D), A/Shanghai/2/2013 H7N9 HA protein (E) and the A/Brisbane/59/2007 (Bris/07) stem HA (#4900) protein (F) as the coating antigen. The endpoint antibody titer was defined as the highest dilution of serum to produce an absorbance 2.5 times higher than the optical absorbance (OD) produced by the negative control (pre-immune serum). Data were examined by using two-way ANOVA from Prism; differences were considered statistically significant at $^{**}P < 0.01$; $^{***}P < 0.001$. Data represents the mean ± SEM.

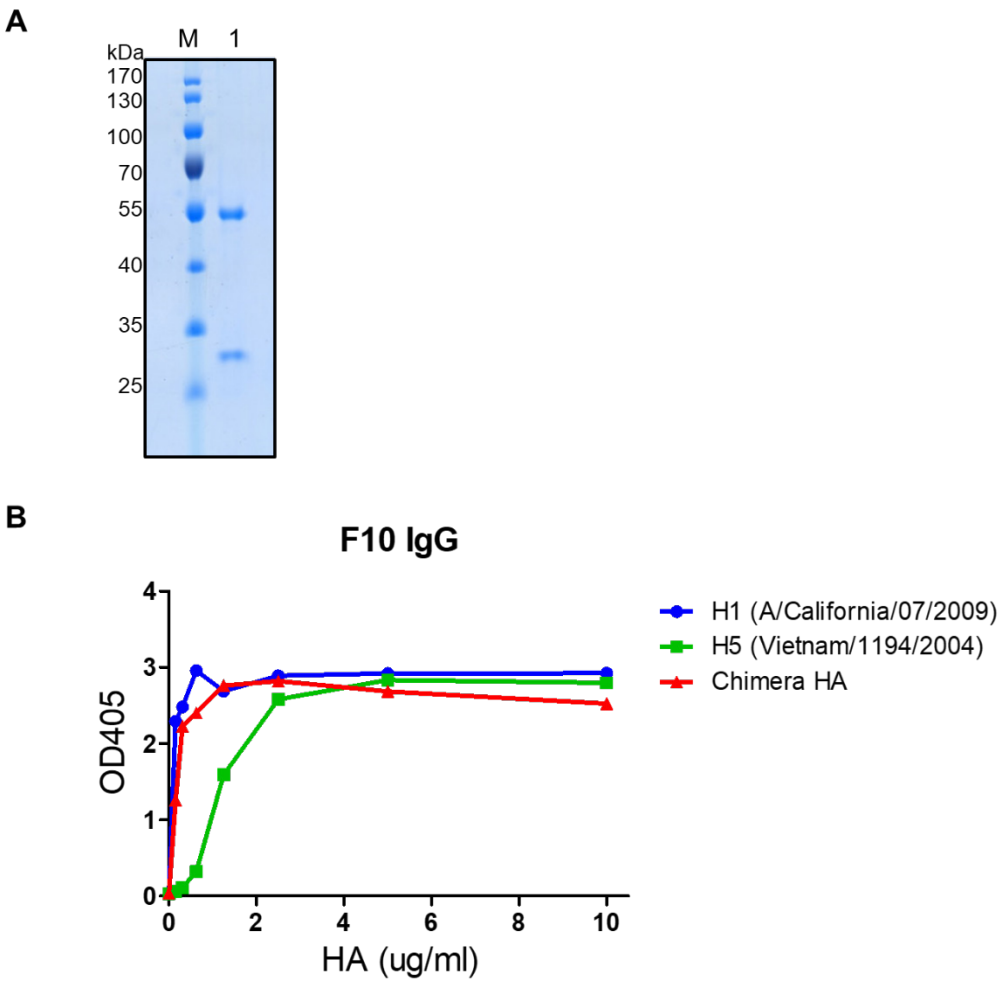


Fig S4. Binding of stalk-reactive antibodies (F10 IgG) to recombinant H1, H5 and cHAs. (A) The purified F10 was analyzed by SDS/PAGE. M: molecular weight marker. Lane 1: F10 antibody. (B) The binding affinities of F10 IgG and various HA were measured by using ELISA. The x-axis shows the concentration of various HA proteins and the y-axis shows the absorbance value at OD405 nm.

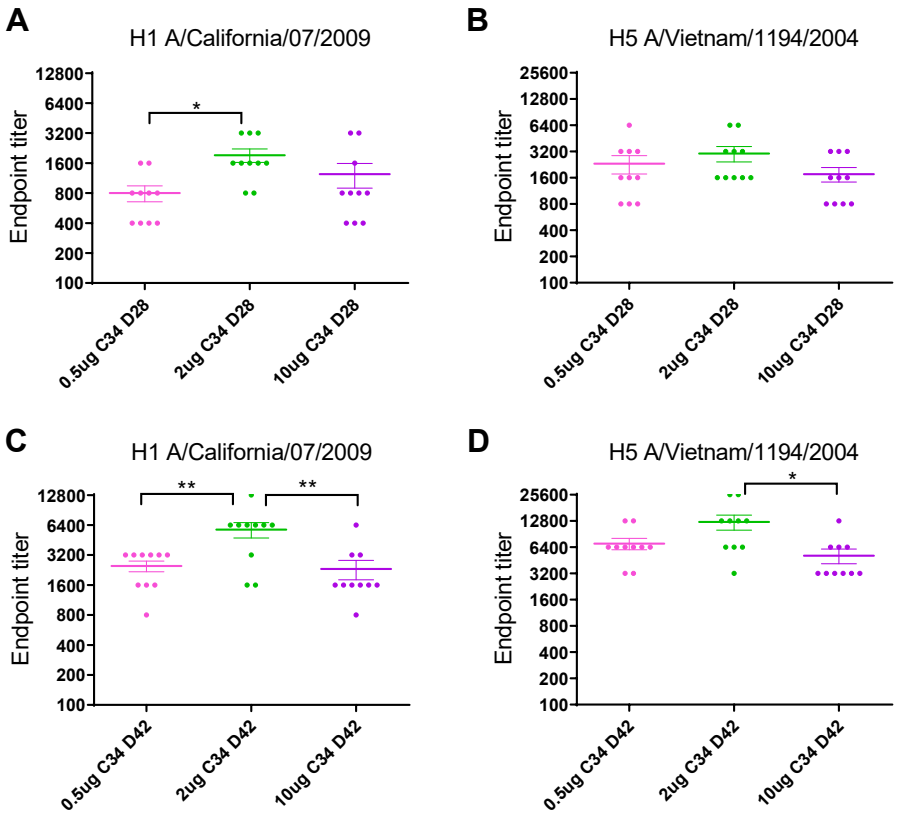


Fig S5. Dose-dependent effects of C34 on antibody titers. BALB/c mice (n=10 per group) were injected at two-week intervals with 20 μg cHA adjuvanted with 0.5 μg (Pink), 2 μg (Green) or 10 μg (Purple) of C34. Mice sera were collected two weeks after the second (D28) and third (D42) immunizations. The antibodies titers were measured by using ELISA with HA proteins of H1N1 A/California/07/2009 (A and C) and H5N1 Vietnam/1194/2004 (B and D). The P value of antibody titers was calculated by using two-way ANOVA from Prism; differences were considered statistically significant at *P <0.05; **P <0.01. Data represents the mean ± SEM.

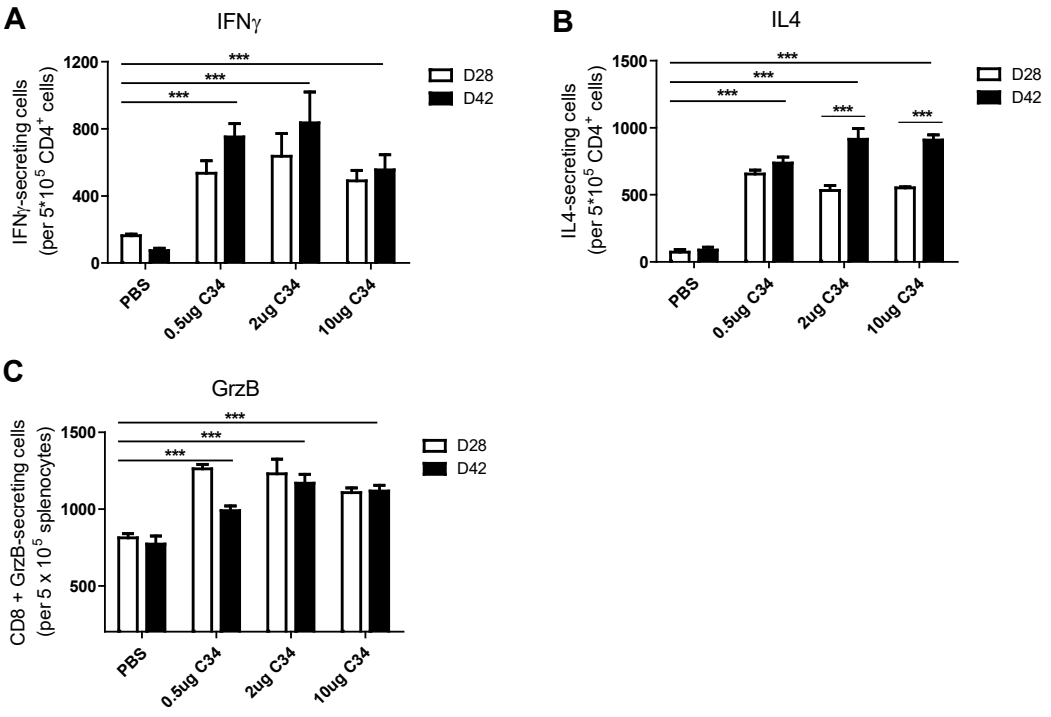


Fig S6. Dose-dependent effects of C34 on antigen-specific cytokine-secreting cells. BALB/c mice (n=5 per group) were injected at two-week intervals with 20 µg of purified cHA adjuvanted with three different doses of C34 at 0.5, 2 and 10 µg. The splenocytes of cHA immunized mice were obtained after the second (D28) and third (D42) immunizations. (A) IFN-γ and (B) IL4-secreting cells were assessed by Elispot analysis. (C) The number of granzyme B producing CD8+ T cell in splenocytes was determined by Elispot analysis using specific peptides. ***P < 0.001. The P value were calculated with Prism software using two-way ANOVAs.

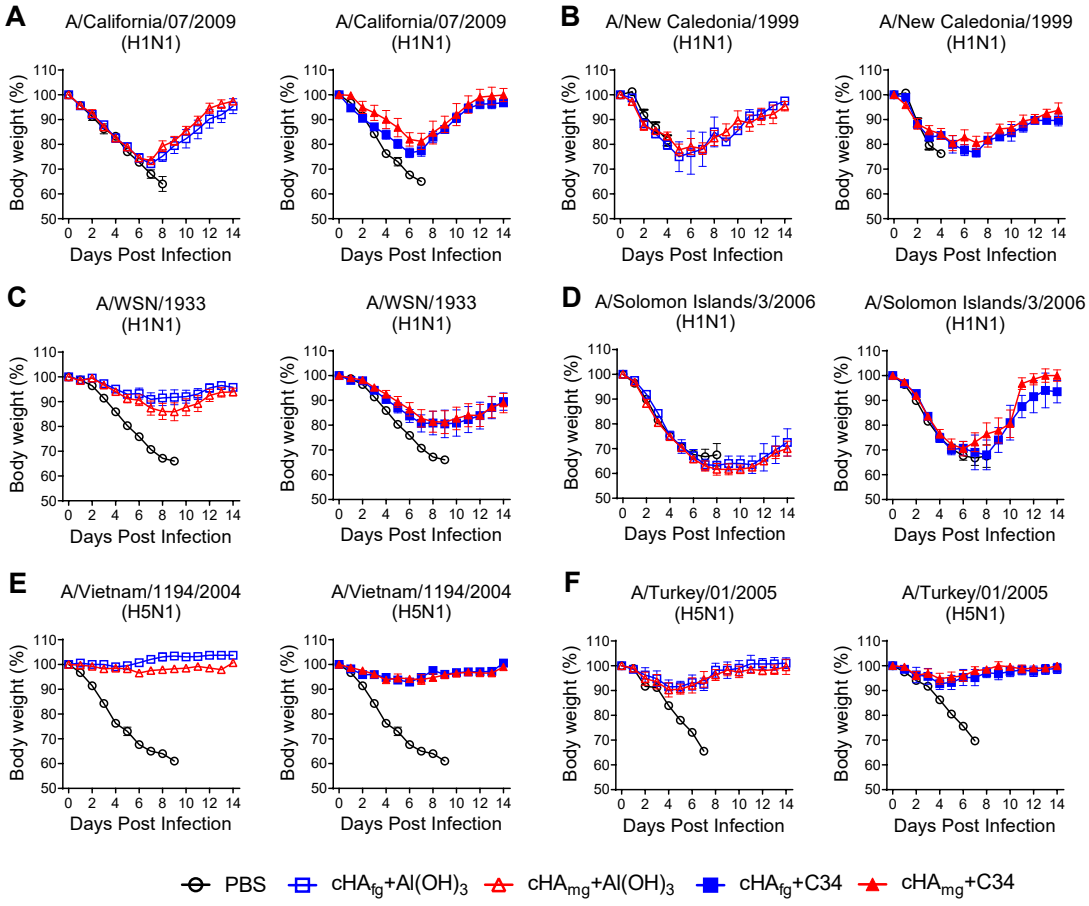


Fig S7. The body weight of cHA$_{fg}$ or cHA$_{mg}$ vaccinated mice challenged by H1N1 and H5N1 viruses at lethal dose. Body-weight changes of immunized mice challenged with H1N1 A/California/07/2009 (A), H1N1 A/New Caledonia/1999 (B), H1N1 A/WSN/1933 (C), H1N1 A/Solomon Islands/03/2006 (D), H5N1 A/Vietnam/1194/2004 (E), or H5N1 A/Turkey/1/2005 (F) viruse were monitored for 14 days after infection. Body-weight change is presented as mean ± SEM.

Table S1. The N-linked glycan structures of cHA in fully-glycosylated and mono-glycosylated proteins analyzed by LC–MS/MS.

Glycan name		N28		N40		N171		N182		N292		N303		N497	
		cHA_{fg}	cHA_{mg}	cHA_{fg}	cHA_{mg}	cHA_{fg}	cHA_{mg}	cHA_{fg}	cHA_{mg}	cHA_{fg}	cHA_{mg}	cHA_{fg}	cHA_{mg}	cHA_{fg}	cHA_{mg}
None		0%	0%	3%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%
Deamidated		0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	7%	0%
N		0%	100%	0%	100%	0%	95%	0%	100%	0%	100%	1%	99%	0%	100%
NF		0%	0%	0%	0%	6%	0%	0%	0%	2%	0%	0%	0%	3%	0%
Man5		3%	0%	3%	0%	0%	0%	0%	0%	0%	0%	6%	0%	0%	0%
Man5NHS		0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	6%	0%	0%	0%
N-N3H5S0F0		0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	10%	0%	0%	0%
N-N3H5S1F0		0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	10%	0%	0%	0%
N-N3H5S0F1		1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
N-N3H5S1F1		0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	3%	0%	0%	0%
N-N3H4S0F0		0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	2%	0%	0%	0%
NNH3NN		0%	0%	0%	0%	0%	0%	2%	0%	0%	0%	0%	0%	0%	0%
H4NN		0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%	0%
NNH3NNF1-G0		0%	0%	0%	0%	0%	0%	1%	0%	0%	0%	0%	0%	0%	0%
Man6N1		1%	0%	5%	0%	0%	0%	0%	0%	0%	0%	9%	0%	0%	0%
BiF1-H		0%	0%	0%	0%	0%	0%	0%	0%	2%	0%	1%	0%	2%	0%
BiS1		0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	13%	0%	0%	0%
BiS1-H		0%	0%	0%	0%	0%	0%	10%	0%	0%	0%	0%	0%	0%	0%
BiS1F1-H		0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%	0%	0%	0%
BiS2		0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%	0%
BiS2-H		0%	0%	0%	0%	0%	0%	3%	0%	0%	0%	0%	0%	0%	0%
BiS2N1		0%	0%	0%	0%	0%	0%	2%	0%	0%	0%	0%	0%	0%	0%
Bi		0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	13%	0%	0%	0%
N-N5H4S0F0		0%	0%	0%	0%	0%	0%	21%	0%	0%	0%	0%	0%	0%	0%
N-N5H3S0F0		0%	0%	0%	0%	0%	0%	38%	0%	0%	0%	0%	0%	0%	0%
N-N5H3S0F1		0%	0%	0%	0%	0%	0%	10%	0%	0%	0%	0%	0%	0%	0%

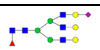
BiF1		6%	0%	12%	0%	0%	0%	0%	0%	7%	0%	8%	0%	11%	0%
Bi-H		0%	0%	0%	0%	0%	0%	5%	0%	0%	0%	0%	0%	0%	0%
BiN1		0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%	0%
BiN1F1-H		0%	0%	2%	0%	8%	0%	0%	0%	1%	0%	3%	0%	7%	0%
BiN1F2-H		0%	0%	0%	0%	2%	0%	0%	0%	0%	0%	0%	0%	0%	0%
BiN1F1		1%	0%	14%	0%	9%	0%	0%	0%	9%	0%	0%	0%	31%	0%
BiS1F1		3%	0%	25%	0%	6%	0%	0%	0%	34%	0%	9%	0%	38%	0%
BiS2F1		0%	0%	0%	0%	7%	0%	0%	0%	21%	0%	0%	0%	0%	0%
N-N5H4S1F1		0%	0%	0%	0%	23%	0%	0%	0%	0%	0%	0%	0%	0%	0%
BiN1S1F1		0%	0%	0%	0%	0%	0%	0%	0%	10%	0%	0%	0%	0%	0%
BiN1S1-H		0%	0%	0%	0%	0%	0%	2%	0%	0%	0%	0%	0%	0%	0%
N-N5H4S1F2		0%	0%	0%	0%	16%	0%	0%	0%	0%	0%	0%	0%	0%	0%
BiN1S1F1		0%	0%	1%	0%	6%	0%	0%	0%	0%	0%	0%	0%	0%	0%
N-N5H4S2F1		0%	0%	0%	0%	15%	0%	0%	0%	0%	0%	0%	0%	0%	0%
TriF1		43%	0%	2%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
TriS1F1		38%	0%	11%	0%	0%	0%	0%	0%	3%	0%	0%	0%	1%	0%
TriN1F1		3%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
BiF1N1S2		0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%	0%	0%	0%
TriS2F1		0%	0%	0%	0%	0%	0%	0%	0%	4%	0%	0%	0%	0%	0%
TetraF1		0%	0%	2%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
TetraS1F1		1%	0%	7%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
TetraS2F1		0%	0%	11%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
TetraH1S2		0%	0%	0%	0%	2%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Others		0%	0%	2%	0%	0%	5%	6%	0%	5%	0%	3%	0%	0%	0%

Table S2. The oligonucleotide sequences of the primers for the expression constructs

Target gene	Sequences
Swap H1/5	1F: AAGCTGGCTAGCATGAAGGCCATCCTGGTTGTGCTG
	2R: GGCGCCGAACAGGCCTCTGCTCTGGATGCTGGGAACG
	3F: AGCATCCAGAGCAGAGGCCTGTTCGGCGCCATCGCCG
	4R: TTAAACGCTAGCCACGCCGCTGATCTCCTCTCTC
Swap H5/1	1F: AAGCTGGCTAGCATGGAGAAGATCGTGCTGCTGCTC
	2R: GGCGCCAAACAGGCCTCTCTTCTTTCTTCTTCTCTCT
	3F: AGAAGAAAGAAGAGAGGCCTGTTTGGCGCCATTGCCG
	4R: TTAAACGCTAGCCTGGTAGATCCGGGTGCTTTCCAGC
Chimeric H5/1	1F: AAGCTGGCTAGCATGAAGGCCATCCTGGTTGTGCTG
	2R: GCCGTCCAGGTCGCACAGCTTGCCGTTGTGCTTATCT
	3F: CACAACGGCAAGCTGTGCGACCTGGACGGCGTGAAGC
	4R: CTGACAGGTGGTGTTGCAGTTGCCGTACTCCAGCTCG
	5F: GAGTACGGCAACTGCAACACCACCTGTCAGACCCCCA
	6R: TTAAACGCTAGCCTGGTAGATCCGGGTGCTTTCCAGC

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