Supplementary Table 1. X-ray data collection and refinement statistics.

	Mos99 NA	Bil69 NA	SD93 NA + Zanamivir
Data collection			
Wavelength (Å)	0.97856	0.97872	1.12723
Resolution (Å)	1.397	1.537	1.645
Resolution Range [a]	43.458-1.397 (1.401-1.397)	46.049-1.537 (1.563-1.540)	76.340-1.700 (1.650-1.645)
Space group	I 4 2 2	C 2	P 4 2_1 2
Cell dimensions			
a, b, c (Å)	136.15, 136.15, 150.77	116.34, 137.94, 138.25	107.95, 107.95, 78.62
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 92.16, 90.00	90.00, 90.00, 90.00
Total reflections	3,233,337	2,400,688	520,061
Unique reflections	138,645	322,494	54,981
Multiplicity [a]	23.0 (14.7)	7.5 (7.5)	9.5 (6.6)
Completeness (%) [a]	99.9 (99.2)	100.0 (100.0)	96.8 (97.3)
$<I/\sigma_I>$ [a]	20.2 (2.2)	12.1 (2.1)	11.5 (2.1)
R_{merge} (%) [a]	10.8 (126.0)	10.4 (98.9)	12.7 (77.4)
R_{meas} (%) [a]	11.3 (130.7)	11.1 (106.4)	13.8 (83.4)
$CC_{1/2}$ [a]	0.999 (0.674)	0.998 (0.778)	0.998 (0.895)
Refinement			
Resolution (Å)	43.46-1.40	25.00-1.54	25.00-1.65
No. reflections	138,634	304,302	51,828
R_{work}[c] / R_{free}[d]	0.151/0.164	0.166/0.180	0.188/0.215
No. atoms			
Protein	3,055	12,156	3,016
Water	583	2,004	317
Sugars/Inhibitor	124	408	149
B-factors			
Protein	14.2	14.9	18.0
Sugars/Inhibitor	25.1	25.5	33.1
Water	30.8	29.8	26.2
RMSD from ideal geometry			
Bond lengths (Å)	0.007	0.003	0.005
Bond angles (°)	1.12	0.995	1.210
Ramachandran statistics (%)			
Favored	99.7	95.7	96.1
Outliers	0.00	0.00	0.00
PDB code	Pending	Pending	Pending

[a] Numbers in parentheses refer to the highest resolution shell.

[b] $R_{merge} = \Sigma \, |I_i - <I_i>| \, / \, \Sigma \, I_i$ where I_i = the intensity of the ith reflection and $<I_i>$ = mean intensity.

[c] $R_{work} = \Sigma \, |F_o - F_c| \, / \, \Sigma \, |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

[d] R_{free} was calculated as for R_{work}, but on a test set comprising 5% of the data excluded from refinement.

Supplementary Table 2. Primers for SD93 combinatorial mutagenesis experiment.

Primer name	Sequence
SD93lib-N387K-VF	5'-CGT ACG TCT CAC TGG TCC AAA CCT AAA TCC AAA TTG CAG-3'
SD93lib-VR	5'-CGT ACG TCT CAA AGC ACT TCC ATC AGT CAT TAC TAC TGT-3'
SD93lib-E248G-R249K-F	5'-CGT ACG TCT CAG CTT CAG RAA RAG CTG ATA CTA AAA TAC TAT TCA TTG AGG AGG GGA AA-3'
SD93lib-I265T-F	5'-TAA AAT ACT ATT CAT TGA GGA GGG GAA AAT CGT TCA TAY TAG CCC ATT GTC AGG AAG TGC-3'
SD93lib-336-346-5mut-F	5'-YAT TGC CKG RAT CCT AAC AAT GAG RAA GGG RGT CAT GGA GTG AAA GGC TGG GCC-3'
SD93lib-336-346-5mut-R	5'-ACY CCC TTY CTC ATT GTT AGG ATY CMG GCA ATR GCT ACT GCT GGA GCT GTC GTT-3'
SD93lib-E369K-R	5'-ACT TTG AAG GTT TCA TAA CCT GAG CGT AAC TYC TCG CTG ATC GTT CTT CCC ATC CA-3'
SD93lib-G381E-R	5'-CGT ACG TCT CAC CAG CCT YCA ATG ACT TTG AAG GTT TCA TAA CCT GAG CGT-3'
SD93lib-recover-F	5'-CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT CTA GTA ATG ACT GAT GGA AGT GCT-3'
SD93lib-recover-R	5'-GAC TGG AGT TCA GAC GTG TGC TCT TCC GAT CTA CTT GCC TAT TTA TCT GCA ATT-3'

Supplementary Table 3. Primers for Bil69 combinatorial mutagenesis experiment.

Primer name	Sequence
Bil69lib-VR	5'-CGT ACG TCT CAA CTC CCA TCA GTC ATT ACT ACT GT-3'
Bil69lib-VF	5'-CGT ACG TCT CAG CTC AGG TTA TGA AAC TTT CAA AG-3'
Bil69lib-R249K-F	5'-CGT ACG TCT CAG AGT GCT TCA GGG ARA GCC GAT ACT AGA ATA CTA TTC ATT-3'
Bil69lib-D286G-R	5'-CCT ATT AGA GCC TTT CCA GTT GTC TCT GCA GAT ACA TCT GAC GYC AGG ATA TCG AGG ATA-3'
Bil69lib-I302V-M307V-R	5'-CTA TAA TCT TTC AYA TTT ATG TCT ACG AYG GGC CTA TTA GAG CCT TTC AG TTG TCT CTG-3'
Bil69lib-I302V-M307V-F	5'-CCC RTC GTA GAC ATA AAT RTG AAA GAT TAT AGC ATT GAT-3'
Bil69lib-D329N-K334S-1-R	5'-CTG CAA TGG CTC TTG CTA GAT CTG TCG TYG TTT CTA GGT GTG TCG CCA AC-3'
Bil69lib-D329N-K334S-2-R	5'-CTG CAA TGG CTA CTG CTA GAT CTG TCG TYG TTT CTA GGT GTG TCG CCA AC-3'
Bil69lib-D329N-K334S-1-F	5'-RAC GAC AGA TCT AGC AAG AGC CAT TGC AGG AAT CCT AAC AAT GAG AGA GG-3'
Bil69lib-D329N-K334S-2-F	5'-RAC GAC AGA TCT AGC AGT AGC CAT TGC AGG AAT CCT AAC AAT GAG AGA GG-3'
Bil69lib-N356D-R	5'-TTG CTG ATC GTT CTT CCC ATC CAC ACG TCA TTT CCA TYG TCA AAG GCC CAG CCT-3'
Bil69lib-L370S-R	5'-CGT ACG TCT CAG AGC GTR AAT CCT TGC TGA TCG TTC TTC CCA TCC ACA CGT-3'
Bil69lib-recover-F	5'-CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT CTA CAG TAG TAA TGA CTG ATG GGA GT-3'
Bil69lib-recover-R	5'-GAC TGG AGT CGA CGT GTG TCT TCC GAT CTC TTT GAA AGT TTC ATA ACC TGA GC-3'

A

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HK68   MNPNQKIITIGSVSLTIATVCFLMQIAILVTTVTLHFKQYECDSPASNQVMPCEPIIIERNITEIVYLNNTTIEKEICPK 80
Vic11  MNPNQKIITIGSVSLTISTICFFMQIAILITTVTLHFKQYEFNSPPNNQVMLCEPTIIERNITEIVYLTNTTIEKEICPK 80

HK68   VVEYRNWSKPQCQITGFAPFSKDNSIRLSAGGDIWVTREPYVSCDHGKCYQFALGQGTTLDNKHSNDTIHDRIPHRTLLM 160
Vic11  PAEYRNWSKPQCGITGFAPFSKDNSIRLSAGGDIWVTREPYVSCDPDKCYQFALGQGTTLNNVHSNNTVRDRTPYRTLLM 160

HK68   NELGVPFHLGTRQVCIAWSSSSCHDGKAWLHVCITGDDKNATASFIYDGRLVDSIGSWSQNILRTQESECVCINGTCTVV 240
Vic11  NELGVPFHLGTKQVCIAWSSSSCHDGKAWLHVCITGDDKNATASFIYNGRLVDSVVSWSKEILRTQESECVCINGTCTVV 240

HK68   MTDGSASGRADTRILFIEEGKIVHISPLSGSAQHVEECSCYPRYPGVRCICRDNWKGSNRPVVDINMEDYSIDSSYVCSG 320
Vic11  MTDGSASGKADTKILFIKEGKIVHTSTLSGSAQHVEECSCYPRYPGVRCVCRDNWKGSNRPIVDINIKDHSIVSSYVCSG 320

                             336                                          387
HK68   LVGDTPRNDDRSSNSNCRNPNNERGNQGVKGWAFDNGDDVWMGRTISKDLRSGYETFKVIGGWSTPNSKSQINRQVIVDS 400
Vic11  LVGDTPRKTDSSSSSHCLDPNNEEGGHGVKGWAFDDGNDVWMGRTINETSRLGYETFKVIEGWSNPKSKLQINRQVIVDR 400

HK68   DNRSGYSGIFSVEGKSCINRCFYVELIRGRKQETRVWWTSNSIVVFCGTSGTYGTGSWPDGANINFMPI 469
Vic11  GDRSGYSGIFSVEGKSCINRCFYVELIRGRKEETEVLWTSNSIVVFCGTSGTYGTGSWPDGADLNLMPI 469
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B

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Mos99  MNPNQKIITIGSVSLTIATICFLMQIAILVTTVTLHFKQYECNSPPNNQVMLCEPTIIERNITEIVYLTNTTIEKEICPK 80
Wy03   MNPNQKIITIGSVSLTISTICFFMQIAILITTVTLHFKQYEFNSPPNNQVMLCEPTIIERNITEIVYLTNTTIEKEICPK 80

Mos99  LAEYRNWSKPQCNITGFAPFSKDNSIRLSAGGDIWVTREPYVSCDPDKCYQFALGQGGTTLNNGHSNDTVHDRTPYRTLLM 160
Wy03   LAEYRNWSKPQCNITGFAPFSKDNSIRLSAGGDIWVTREPYVSCDPDKCYQFALGQGGTTLNNVHSNDTVHDRTPYRTLLM 160

Mos99  NELGVPFHLGTKQVCIAWSSSSCHDGKAWLHVCVTGDDENATASFIYNGRLVDSIGSWSKKILRTQESECVCINGTCTVV 240
Wy03   NELGVPFHLGTKQVCIAWSSSSCHDGKAWLHVCVTGDDENATASFIYNGRLVDSIVSWSKKILRTQESECVCINGTCTVV 240

Mos99  MTDGSASGKADTKILFIEEGKIVHTSPLSGSAQHVEECSCYPRYPGVRCVCRDNWKGSNRPIVDINYKDYSIVSSYVCSG 320
Wy03   MTDGSASGKADTKILFIEEGKIVHTSTLSGSAQHVEECSCYPRYPGVRCVCRDNWKGSNRPIVDINIKDYSIVSSYVCSG 320

                                                                       385
Mos99  LVGDTPRKNDSSSSSHCLDPNNEEGGHGVKGWAFDDGNDVWMGRTISEKLRSGYETFKVIEGWSKPNSKLQINRQVIVDR 400
Wy03   LVGDTPRKNDSSSSSHCLDPNNEEGGHGVKGWAFDDGNDVWMGRTISEKLRSGYETFKVIEGWSNPNSKLQINRQVIVDR 400

Mos99  GNRSGYSGIFSVEGKSCINRCFYVELIRGRKQETEVLWTSNSIVVFCGTSGTYGTGSWPDGADINLMPI 469
Wy03   GNRSGYSGIFSVEGKSCINRCFYVELIRGRKQETEVLWTSNSIVVFCGTSGTYGTGSWPDGADINLMPI 469
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C

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SD93   MNPNQKIITIGSVTLTIATICFLMQIAILVTTVTLHFKQYECNSPPNNQVMLCEPTIIERNITEIVYLTNTTIEKEICPK 80
Mos99  MNPNQKIITIGSVSLTIATICFLMQIAILVTTVTLHFKQYECNSPPNNQVMLCEPTIIERNITEIVYLTNTTIEKEICPK 80

SD93   LAEYRNWSKPQCKITGFAPFSKDNSIRLSAGGDIWVTREPYVSCDPGKCYQFALGQGGTTLNNRHSNDTVHDRTPYRTLLM 160
Mos99  LAEYRNWSKPQCNITGFAPFSKDNSIRLSAGGDIWVTREPYVSCDPDKCYQFALGQGGTTLNNGHSNDTVHDRTPYRTLLM 160

SD93   NELGVPFHLGTKQVCIAWSSSSCHDGKAWLHVCVTGHDENATASFIYDGRLVDSIGSWSKNILRTQESECVCINGTCTVV 240
Mos99  NELGVPFHLGTKQVCIAWSSSSCHDGKAWLHVCVTGDDENATASFIYNGRLVDSIGSWSKKILRTQESECVCINGTCTVV 240

             248&249                265
SD93   MTDGSASERADTKILFIEEGKIVHISPLSGSAQHVEECSCYPRYPGVRCVCRDNWKGSNRPIVDINVKDYSIVSSYVCSG 320
Mos99  MTDGSASGKADTKILFIEEGKIVHISPLSGSAQHVEECSCYPRYPGVRCVCRDNWKGSNRPIVDINVKDYSIVSSYVCSG 320

             336 338&339 344 346                       369            381
SD93   LVGDTPRKNDSSSSSYCRNPNNEKGSHGVKGWAFDDGNDVWMGRTISEELRSGYETFKVIGGWSKPNSKLQINRQVIVDR 400
Mos99  LVGDTPRKNDSSSSSHCLDPNNEEGGHGVKGWAFDDGNDVWMGRTISEKLRSGYETFKVIEGWSKPNSKLQINRQVIVDR 400

SD93   GNRSGYSGIFSVEGKSCINRCFYVELIRGRKQETEVWWTSNSIVVFCGTSGTYGTGSWPDGGDINLMPI 469
Mos99  GNRSGYSGIFSVEGKSCINRCFYVELIRGRKQETEVLWTSNSIVVFCGTSGTYGTGSWPDGADINLMPI 469
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D

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Bil69  MNPNQKIITIGSVSLIIATVCFLMQIAILVTTVTLHFKQHECDSPSSNQVMLCEPIIIERNITEIVYLNNTTIEKETCPK 80
Bil71  MNPNQKIITIGSISLTIATICFLMQIAILVTTVTLHFKQYECDSPANNQVMPCEPIIIERNITEIVYLTNTTIEKEICPK 80

Bil69  LVEYRNWSKPQCKITGFAPFSKDNSIRLSAGGDIWVTREPYVSCDPGKCYQFALGQGGTTLDNKHSNDTIHDRIPHRTLLM 160
Bil71  LVEYRNWSKPQCKITGFAPFSKDNSIRLSAGGDIWVTREPYVSCDPGKCYQFALGQGGTTLDNKHSNDTIHDRIPHRTLLM 160

Bil69  NELGVPFHLGTRQVCIAWSSSSCHDGKAWLHVCVTGDDKNATASFIYDGRLVDSIGSWSQNILRTQESECVCINGTCTVV 240
Bil71  NELGVPFHLGTRQVCIAWSSSSCHDGKAWLHVCVTGDDKNATASFIYDGRLVDSIGSWSQNILRTQESECVCINGTCTVV 240

             249                     286    302 307
Bil69  MTDGSASGRADTRILFIEEGKIVHISPLSGSAQHVEECSCYPRYPGVRCICRDNWKGSNRPIVDINMKDYSIDSSYVCSG 320
Bil71  MTDGSASGKADTRILFIEEGKIVHISPLSGSAQHVEECSCYPRYPGVRCICRDNWKGSNRPVVDINVKDYSIDSSYVCSG 320

             329    334 336                    356        370
Bil69  LVGDTPRNDDRSSKSNCRNPNNERGNHGVKGWAFDNGDDVWMGRTISKDLRSGYETFKVIGGWSTPNSKSQINRQVIVDS 400
Bil71  LVGDTPRNNDRSSSSYCRNPNNERGNHGVKGWAFDDGNDVWMGRTISKDSRSGYETFKVIGGWSTPNSKSQINRQVIVDS 400

Bil69  DNRSGYSGIFSVEGKSCINRCFYVELIRGREQETRVWWTSNSIVVFCGTSGTYGTGSWPDGANINFMPI 469
Bil71  DNRSGYSGIFSVEGKSCINRCFYVELIRGREQETRVWWTSNSIVVFCGTSGTYGTGSWPDGADINLMPI 469
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Supplementary Figure 1. Sequence alignment of influenza virus H3N2 neuraminidase (NA).

Alignments of NA amino acid sequences between **(A)** HK68 and Vic11, **(B)** Mos99 and Wy03 **(C)** SD93 and Mos99, and **(D)** Bil69 and Bil71. Residues that are not conserved between sequences are highlighted in pink. Mutations of interest are labeled in red. NA head domain is from residues 82 to 469.
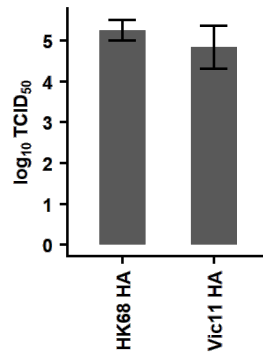
Supplementary Figure 2. Pairing HK68 NA with Vic11 HA does not affect virus replication fitness. The replication fitness of viruses that carry HK68 NA but different HAs (HK68 HA and Vic11 HA) was examined by a virus rescue experiment. Error bars indicate the standard deviation of three independent experiments. Virus titer was measured by $TCID_{50}$.
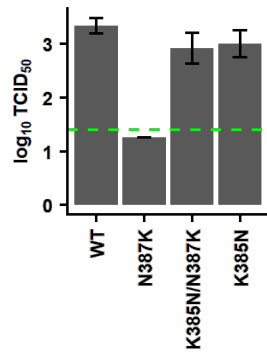
Supplementary Figure 3. K385N of Mos99 NA does not alter virus replication fitness. The replication fitness of different Mos99 NA mutants was examined by a virus rescue experiment. Error bars indicate the standard deviation of three independent experiments. Virus titer was measured by $TCID_{50}$. The green dashed line represents the lower detection limit.
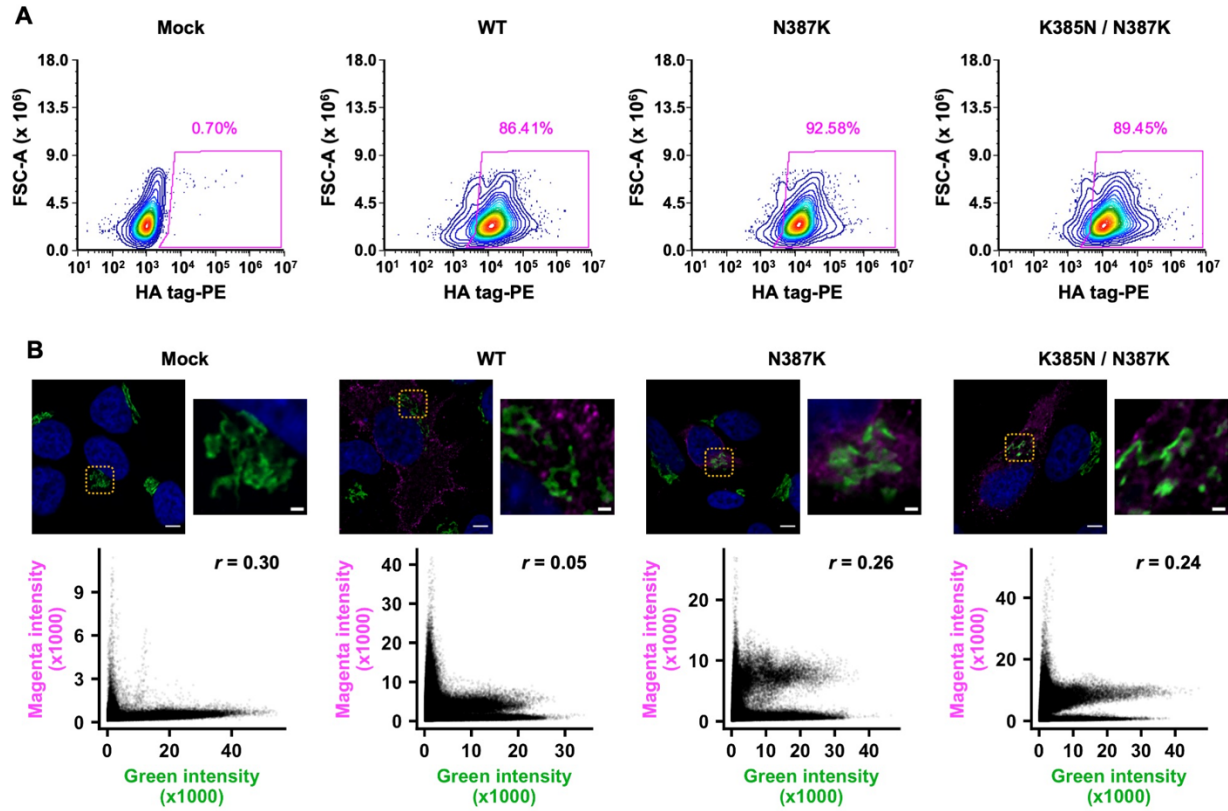
Supplementary Figure 4. Cellular expression and localization of Mos99 NA mutants. (A) Flow cytometry analysis of 293T cells that transiently expressed HA-tagged Mos99 NA mutants. **(B)** Confocal microscopy analysis of HA-tagged Mos99 NA mutants. Blue (DAPI), Green (GM130, Golgi), Magenta (NA). The orange box highlights the zoomed-in region, which is shown on the left. Scale bar for large image is 5 μm and scale bar for zoomed-in image is 2 μm. Below each micrograph is a cytofluorogram along with the Pearson correlation coefficient.
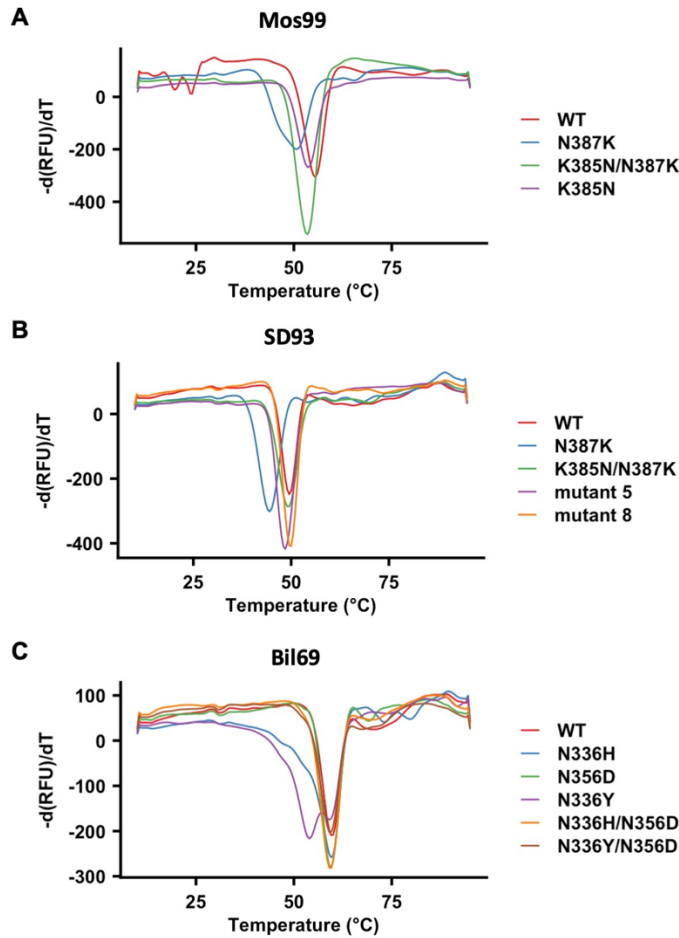
Supplementary Figure 5. Measuring the thermal stability of different NA mutants using SYPRO orange dye-based thermal shift assay. The first differential curves for the relative fluorescence unit (RFU) with respect to temperature were shown for **(A)** Mos99 NA WT and mutants, **(B)** SD93 NA WT and mutants, and **(C)** Bil69 NA WT and mutants. For SD93, mutant 5 represents E248G/R249K/Y336N/K344E/E369K/N387K, whereas mutant 8 represents E248G/R249K/Y336N/K344E/E369K/K385N/N387K.
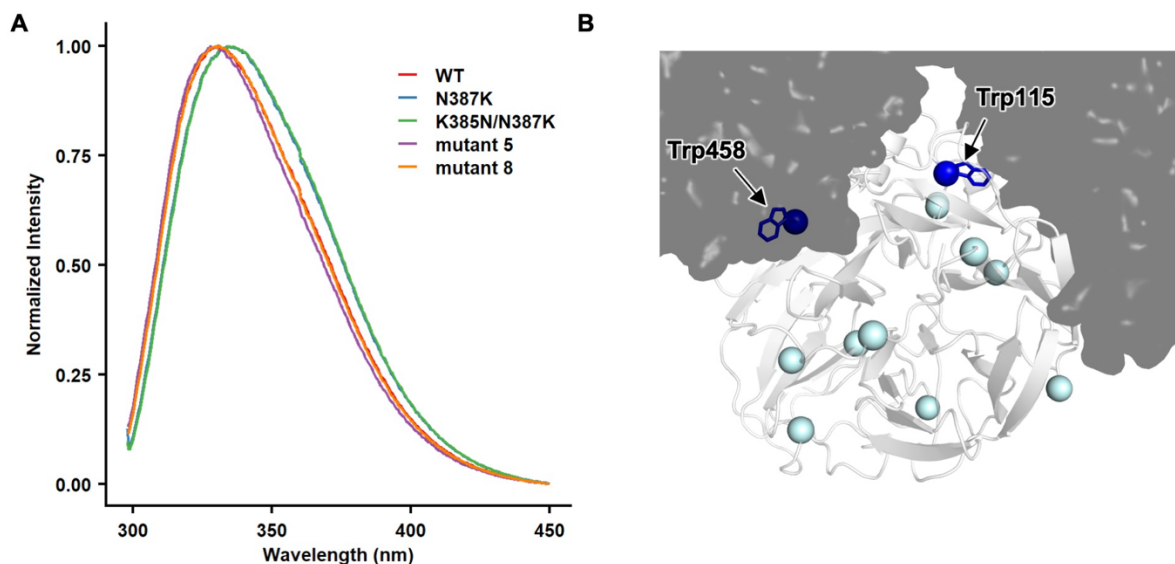
Supplementary Figure 6. Tryptophan emission spectrum of SD93 NA WT and mutants. (A) Normalized steady-state emission spectrum of SD93 NA WT and mutants, using λ_{exc} = 295 nm. Of note, the blue (N387K) and green (K385N/N387K) curves almost completely overlap. Mutant 5 represents E248G/R249K/Y336N/K344E/E369K/N387K, whereas mutant 8 represents E248G/R249K/Y336N/K344E/E369K/K385N/N387K. **(B)** Two out of 12 tryptophans in the head domain of SD93 NA are located at the protomer-protomer interface. Tryptophans are shown as spheres on one protomer that is in white cartoon representation, while the other three protomers are show as semitransparent black surface. The two tryptophans at the protomer-protomer interface, namely Trp115 and Trp458, are shown in blue. Other tryptophans are in cyan.