

1 **Methods for supplementary Figure 4.**

2

3 **Determination of long-term stability of FlaB, dFlaB and FlaB<sup>ΔD2D3</sup> by measuring TLR5-**  
4 **dependent NF-κB activation.**

5 293T cells were seeded in 24-well plates at  $2 \times 10^5$ /well and incubated for 24h.  
6 Subsequently, cells were transfected with p3x Flag-hTLR5 (100 ng/well), the pNFκB-luc (100  
7 ng/well), and 50 ng/well of pCMV-β-Gal using 5 μl/well of Effectene (Qiagen, Hilden  
8 Germany). Twenty-four hours after transfection, the culture media were removed and replaced  
9 by serum-free Gibco® DMEM (Thermo Fischer Scientific Inc. Waltham, MA). The cells were  
10 treated with FlaB, dFlaB, or FlaB<sup>ΔD2D3</sup> and incubated for 24 h. The PBS-treated cells were used  
11 as the control group. The medium was removed and cells were treated with 1x lysis buffer 100  
12 μl/well (Promega, Madison, WI) and kept at room temperature for 1h. To determine NF-κB-  
13 activation, 30 μl of cell lysate was transferred to 96-well opaque plate for the measurement of  
14 luciferase activity while another 30 μl to of cell lysate was transferred to 96-well cell culture  
15 plate for β-Gal activity measurement. Luciferase activity was normalized to LacZ expression  
16 using the control expression plasmid pCMV-β-Gal (Clontech, Takara Bio, Kyoto, Japan). The  
17 luciferase activity was measured by a luminometer (MicroLumatPlus LB 96V; Berthold, Wilbad,  
18 Germany) while β-Gal activity was read by a microplate reader (Molecular Devices Corp.,  
19 Menlo Park, CA) at 420 nm.

20

21

## 22 **Supplementary information**

23

24 **Supplementary Figure 1. Monomeric (left) and oligomeric (right) structures of FlaB variants.**  
25 **(A) WT FlaB, (B) dFlaB and (C) FlaB<sup>AD2D3</sup>.** ND2-D3-CD2 domains (D2D3) are colored in red  
26 and the removed 19 residues are in blue. D2D3 are solvent exposed in both monomeric and  
27 oligomeric forms.

28

29 **Supplementary Figure 2. Development of recombinant dFlaB and FlaB<sup>AD2D3</sup>.** Recombinant  
30 plasmids were constructed as described in Materials and Methods. The vector maps and amino  
31 acid sequences of recombinant proteins are shown

32

33 **Supplementary Figure 3. MALDI-TOF analysis report.** Called up peptide sequences by  
34 MALDI-TOP analyses are shown in bold red.

35

36 **Supplementary Figure 4. Determination of TLR5-dependent NF- $\kappa$ B stimulating activity by**  
37 **FlaB, dFlaB and FlaB<sup>AD2D3</sup>.** The relative luciferase activities in the cell extracts were analyzed  
38 by a dual-luciferase reporter assay system and normalized using the pCMV- $\beta$ -Gal plasmid as a  
39 control. The same molar ratio of proteins was used, and PBS was used as a negative control.

40

41 **Supplementary Figure 5. sH3N2-specific antibody response after intranasal vaccination.**  
42 Groups of mice were intranasally vaccinated with PBS (PBS), 4  $\mu$ g FlaB (FLaB), 4  $\mu$ g dFlaB  
43 (dFlaB), 1.5  $\mu$ g H3N2 A/Switzerland/9715293/2013 NIB-88 split vaccine (sH3N2), 1.5  $\mu$ g sH3N2  
44 plus 4  $\mu$ g FlaB (sH3N2+FlaB), or 1.5  $\mu$ g sH3N2 plus 4  $\mu$ g dFlaB (sH3N2+dFlaB) three times at  
45 two-week intervals. Two weeks after the last immunization, serum was collected and sH3N2-  
46 specific serum IgG<sub>1</sub> or IgG<sub>2a</sub> titers were determined by ELISA.

47

48 **Supplementary Figure 6. sH1N1-specific antibody response after intranasal vaccination.**  
49 Groups of mice were intranasally immunized with PBS, FlaB, dFlaB, H1N1 A/Brisbane/59/07  
50 split vaccine (sH1N1), sH1N1 plus FlaB (sH1N1+FlaB), or sH1N1 plus dFlaB (sH1N1+dFlaB)  
51 three times at two-week intervals. Two weeks after the last immunization, serum was collected  
52 and sH1N1-specific serum IgG titer was determined by ELISA.

53

54 **Supplementary Figure 7. RMSF plots for FlaB and dFlaB.** Three molecular dynamics  
55 simulations were performed for each variant. Though overall RMSF patterns are similar each other,

56 notable changes are observed in the truncated region (shaded in blue). The high fluctuation due to  
57 the removal of 19 amino acids may have resulted in low antigenicity of dFlaB.

58

59 **Supplementary Video 1. Representative molecular dynamics simulations of (A) WT FlaB and**  
60 **(B) dFlaB. ND2-D3-CD2 domains (D2D3) are colored in red and the removed 19 residues are in**  
61 **blue.**

62

63 **Supplementary Data 1. BepiPred-2.0, FoldX alanine scanning and the Parker**  
64 **hydrophobicity scales of FlaB.**

65 **Supplementary Table 1. PCR primer used in this study**

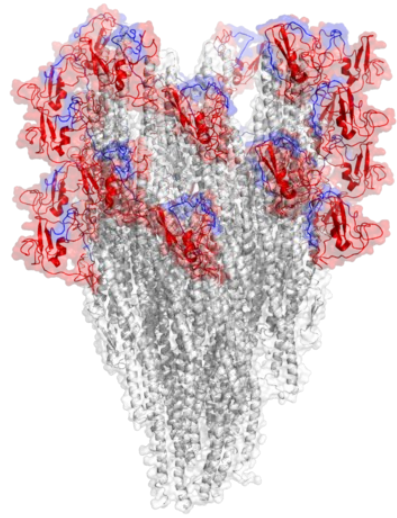
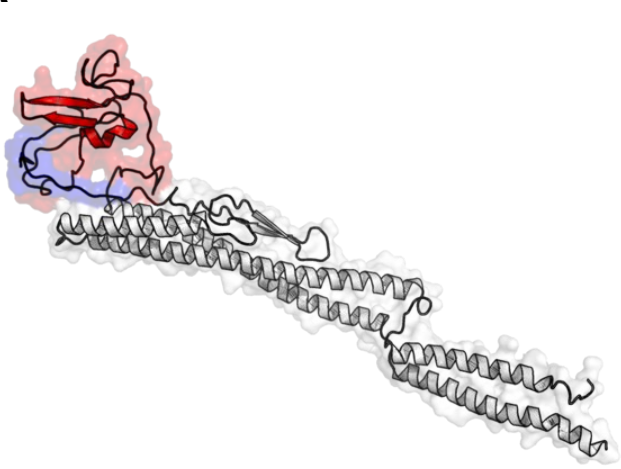
<b>Gene</b>	<b>Direction</b>	<b>Sequence</b>
dFlaB	Up/Forward	5' GGAATTC <u>CATATG</u> ATGGCAGTGAATGTAAATAC 3'
	Up/Reverse	5' TGCAATCGTCAAGTC CACGCCGCCCATCAT 3'
	Down/Forward	5' ATGATGGGCGGCGTG GACTTGACGATTGCA 3'
	Down/Reverse	5' CGG <u>CTCGAG</u> GCCTAGTAGACTTAGCGCTG 3'
FlaB <sup>AD2D3</sup>	Up/Forward	5' GGAATTC <u>CATATG</u> ATGGCAGTGAATGTAAATAC 3'
	Up/Reverse	5' CTCTTGCGCACCTTG CACGTTGTCAGAGCG 3'
	Down/Forward	5' CGCTCTGACAACGTG CAAGGTGCGCAAGAG 3'
	Down/Reverse	5' CGG <u>CTCGAG</u> GCCTAGTAGACTTAGCGCTG 3'

66 Underlined sequences indicate the restriction enzyme recognition sites.

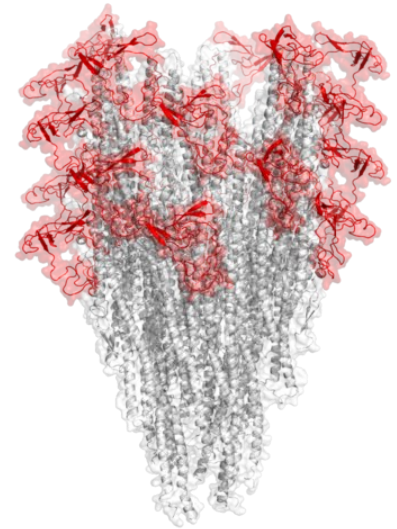
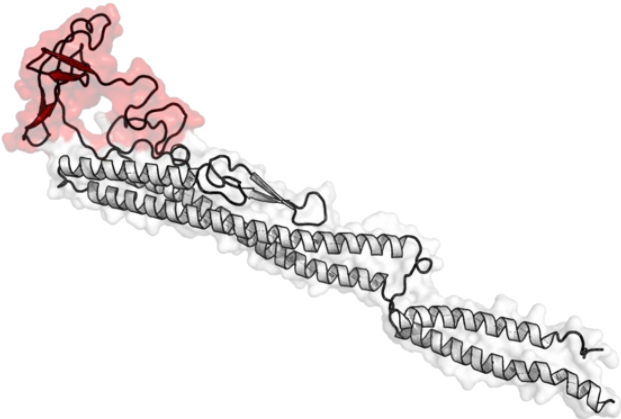
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# Supplementary Figure 1

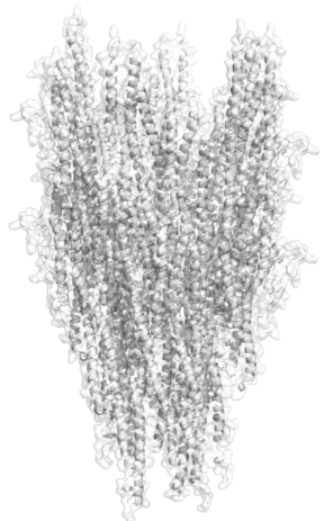
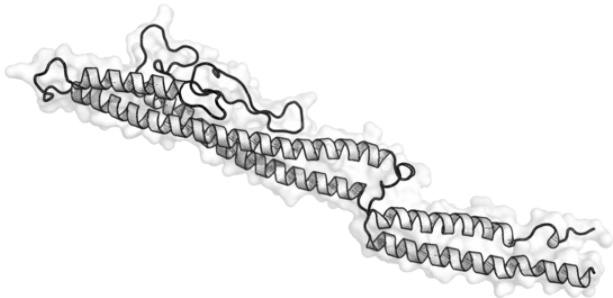
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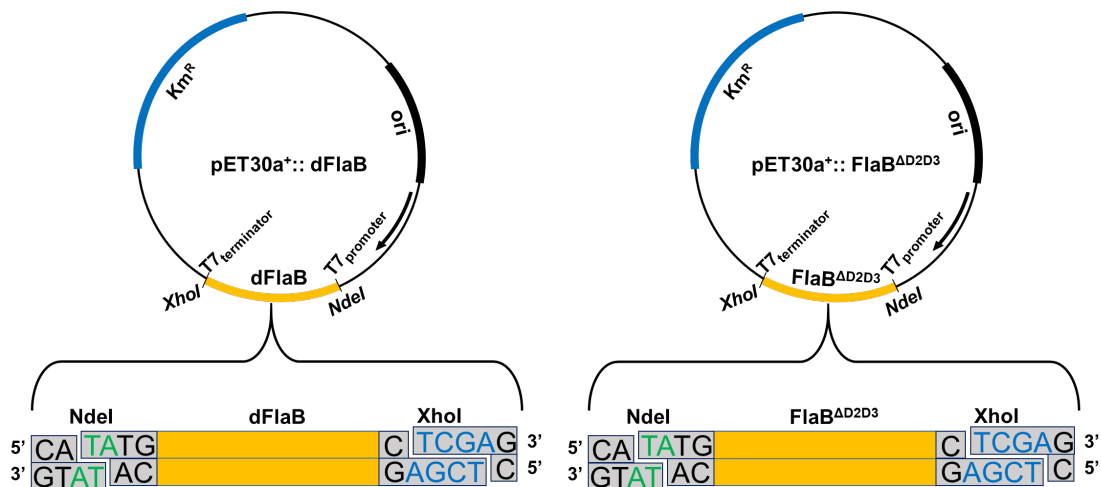
b



c



# Supplementary Figure 2



## dFlaB

HMMAVNVNTNVAAMTAQRYLNNANSAQQTSMERLSSGFKINSAKDDAAGLQISNRLNVQSRGLDVAVR  
NANDGISIAQTAEGAMNETTNI LQRMRDLSLQSANGSNSKSERVAIQEEVTALNDELNRIAETTSFGGNK  
LLNGTYGTKAMQIGADNGEAVMLSLKDMRSDNVMMGGVSYQAEEGKDKNWNVAAGDNDDLTIALTDSF  
GNEQEIEINAKAGDDIEELATYINGQTDLVKASVGE~~GG~~KLQIFAGNNKVQGEIAFSGSLAGELGLGEGKN  
VTVDTIDVTTVQGAQESVAIVDAALKYVDSHRAELGAFQNRFNHAISNLDNINENVNASKSRIKDTDFAK  
ETTQLTKTQILSQASSILAQAKQAPNSALSLLGLEHHHHHH

## FlaB<sup>ΔD2D3</sup>

HMMAVNVNTNVAAMTAQRYLNNANSAQQTSMERLSSGFKINSAKDDAAGLQISNRLNVQSRGLDVAVR  
NANDGISIAQTAEGAMNETTNI LQRMRDLSLQSANGSNSKSERVAIQEEVTALNDELNRIAETTSFGGNK  
LLNGTYGTKAMQIGADNGEAVMLSLKDMRSDNVMMGGVSYQAEEGKDKNWNVAAGDNDDLTIALTDSF  
GNEQEIEINAKAGDDIEELATYINGQTDLVKASVGE~~GG~~KLQIFAGNNKVQGEIAFSGSLAGELGLGEGKN  
VTVDTIDVTTVQGAQESVAIVDAALKYVDSHRAELGAFQNRFNHAISNLDNINENVNASKSRIKDTDFAK  
ETTQLTKTQILSQASSILAQAKQAPNSALSLLGLEHHHHHH

\*Underlined Red: deleted amino acid

# Supplementary Figure 3

## Protein: FlaB

**Enzyme:** Trypsin: cuts C-term side of KR unless next residue is P.

**Fixed modifications:** Carbamidomethyl (C)

**Variable modifications:** Oxidation (M)

**Protein sequence coverage: 70%**

Matched peptides shown in **bold red**.

```
1 MAVNVNTNVA AMTAQRYLYLN ANSAQQTSME RLSSGFKINS AKDDAAGLQI
51 SNRLNVQSRG LDVAVRNAND GISIAQTAEG AMNETTNILO RMRDLSLQSA
101 NGSNSKSERV AIQEEVTALN DELNRIAETT SFGGNKLLNG TYGTKAMQIG
151 ADNGEAVMLS LKDMRSDNVM MGGVSYQAEE GKDKNWNVAA GDNDLTIALT
201 DSGNEQEIE INAKAGDDIE ELATYINGQT DLVKASVGEG GKLQIFAGNN
251 KVQGEIAFSG SLAGELGLGE GKNVTVDTID VTTVQGAQES VAIVDAALKY
301 VDSHRAELGA FQNRFNHAIS NLDNINENVN ASKSRIKDTD FAKETTQLTK
351 TQILSQASS ILAQAKQAPN SALSLLG
```

Unformatted sequence string: 377 residues (for pasting into other applications).

## Protein: dFlaB

**Enzyme:** Trypsin: cuts C-term side of KR unless next residue is P.

**Fixed modifications:** Carbamidomethyl (C)

**Variable modifications:** Oxidation (M)

**Protein sequence coverage: 61%**

Matched peptides shown in **bold red**.

```
1 MAVNVNTNVA AMTAQRYLYLN ANSAQQTSME RLSSGFKINS AKDDAAGLQI
51 SNRLNVQSRG LDVAVRNAND GISIAQTAEG AMNETTNILO RMRDLSLQSA
101 NGSNSKSERV AIQEEVTALN DELNRIAETT SFGGNKLLNG TYGTKAMQIG
151 ADNGEAVMLS LKDMRSDNVM MGGVSYQAEE GKDKNWNVAA GDNDLTIALT
201 DSGNEQEIE INAKAGDDIE ELATYINGQT DLVKASVGEG GKLQIFAGNN
251 KVQGEIAFSG SLAGELGLGE GKNVTVDTID VTTVQGAQES VAIVDAALKY
301 VDSHRAELGA FQNRFNHAIS NLDNINENVN ASKSRIKDTD FAKETTQLTK
351 TQILSQASS ILAQAKQAPN SALSLLG
```

Unformatted sequence string: 377 residues (for pasting into other applications).

## Protein: FlaBΔD2D3

**Enzyme:** Trypsin: cuts C-term side of KR unless next residue is P.

**Fixed modifications:** Carbamidomethyl (C)

**Variable modifications:** Oxidation (M)

**Protein sequence coverage: 40%**

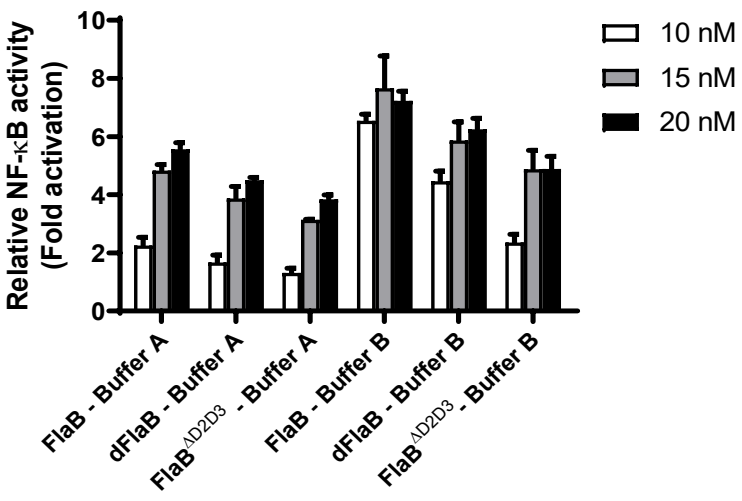
Matched peptides shown in **bold red**.

```
1 MAVNVNTNVA AMTAQRYLYLN ANSAQQTSME RLSSGFKINS AKDDAAGLQI
51 SNRLNVQSRG LDVAVRNAND GISIAQTAEG AMNETTNILO RMRDLSLQSA
101 NGSNSKSERV AIQEEVTALN DELNRIAETT SFGGNKLLNG TYGTKAMQIG
151 ADNGEAVMLS LKDMRSDNVM MGGVSYQAEE GKDKNWNVAA GDNDLTIALT
201 DSGNEQEIE INAKAGDDIE ELATYINGQT DLVKASVGEG GKLQIFAGNN
251 KVQGEIAFSG SLAGELGLGE GKNVTVDTID VTTVQGAQES VAIVDAALKY
301 VDSHRAELGA FQNRFNHAIS NLDNINENVN ASKSRIKDTD FAKETTQLTK
351 TQILSQASS ILAQAKQAPN SALSLLG
```

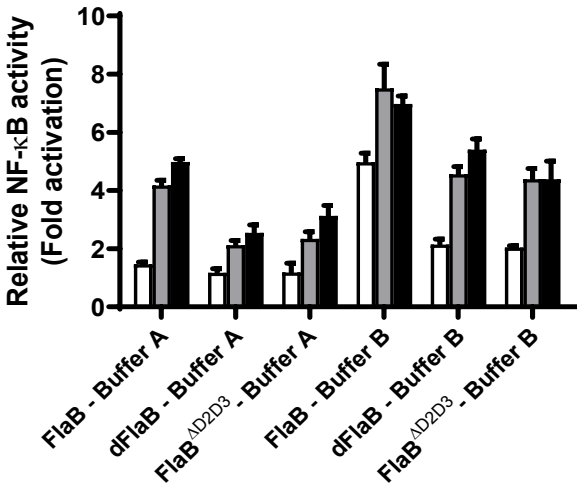
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# Supplementary Figure 4

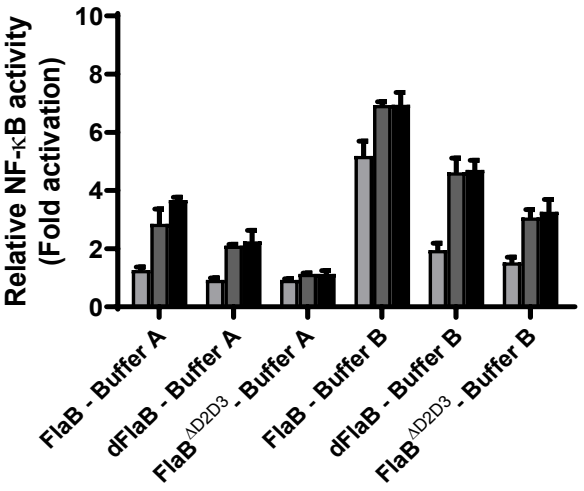
## Fresh proteins



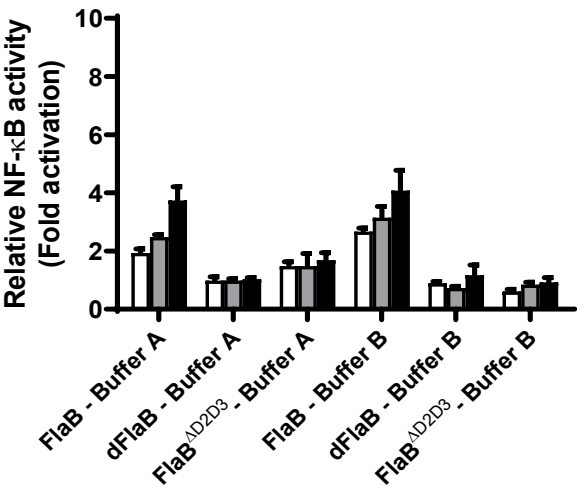
## 1 month (4 °C)



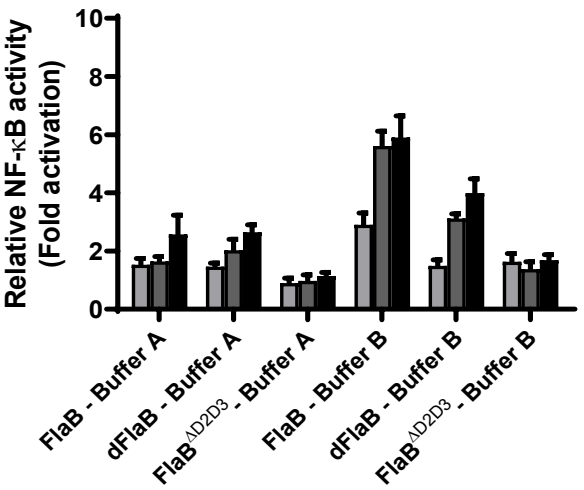
## 1 month (-20 °C)



## 7 months (4 °C)

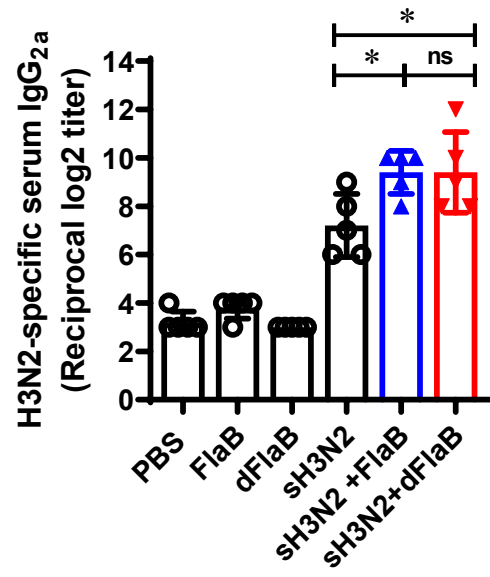
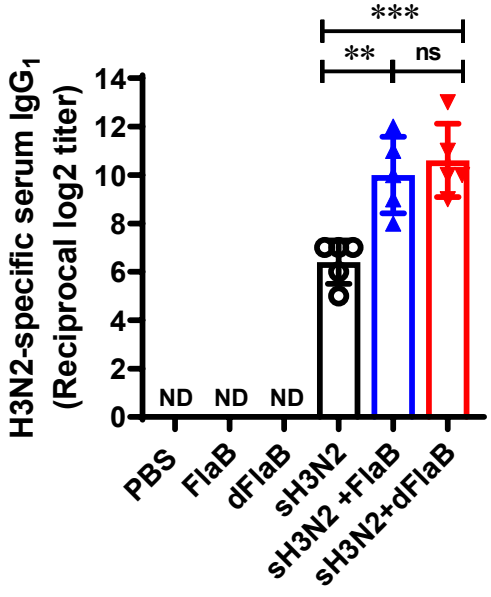


## 7 months (-20 °C)

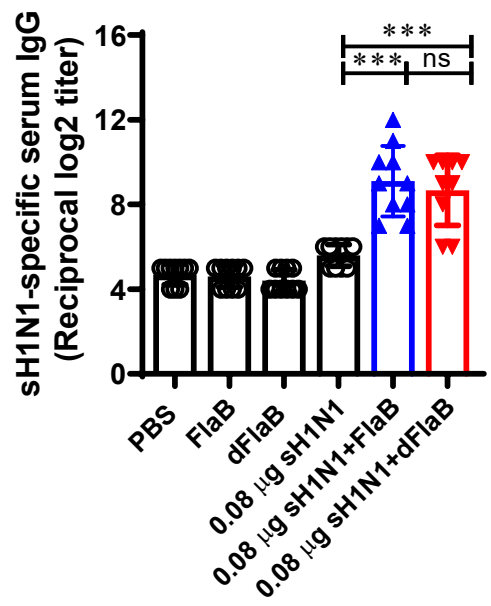
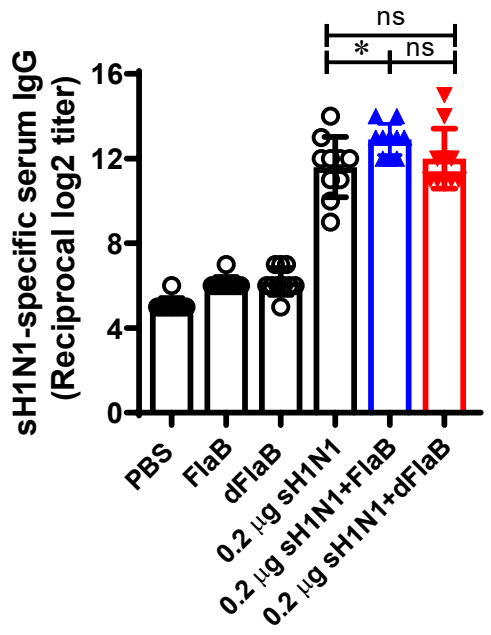




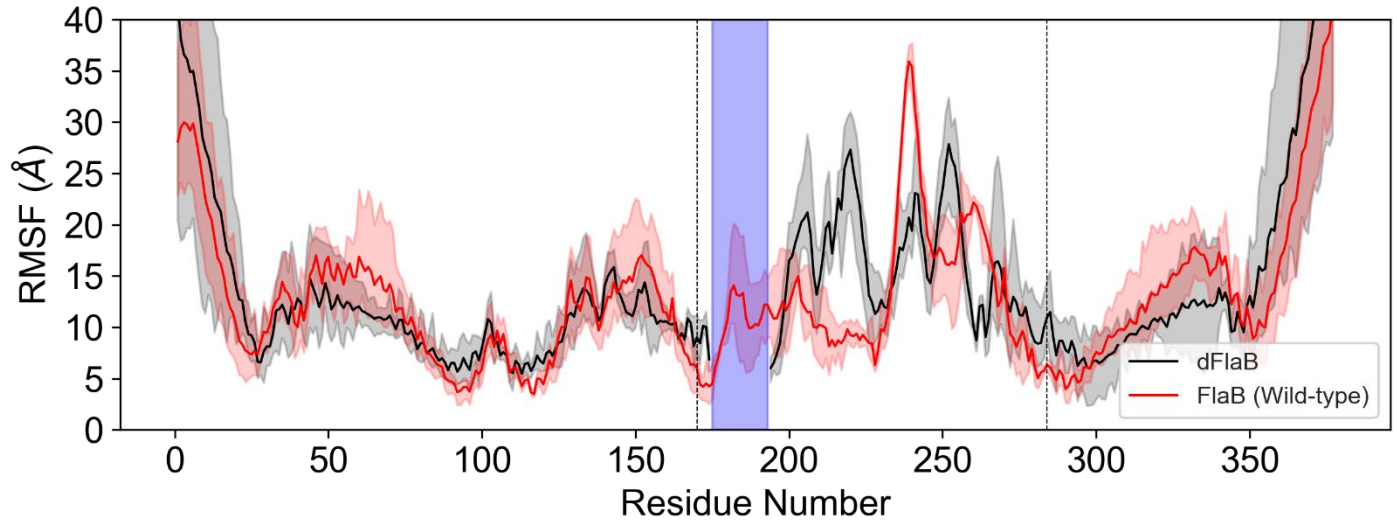
# Supplementary Figure 5



# Supplementary Figure 6



# Supplementary Figure 7



# Figure 2a: Unprocessed W blot

Figure. 2a Left

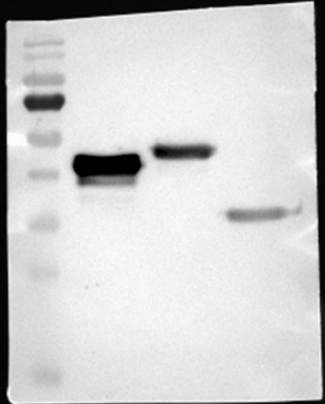
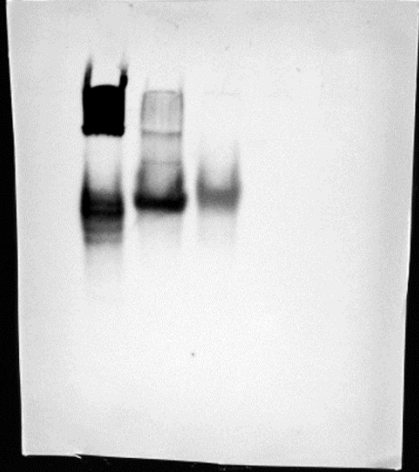
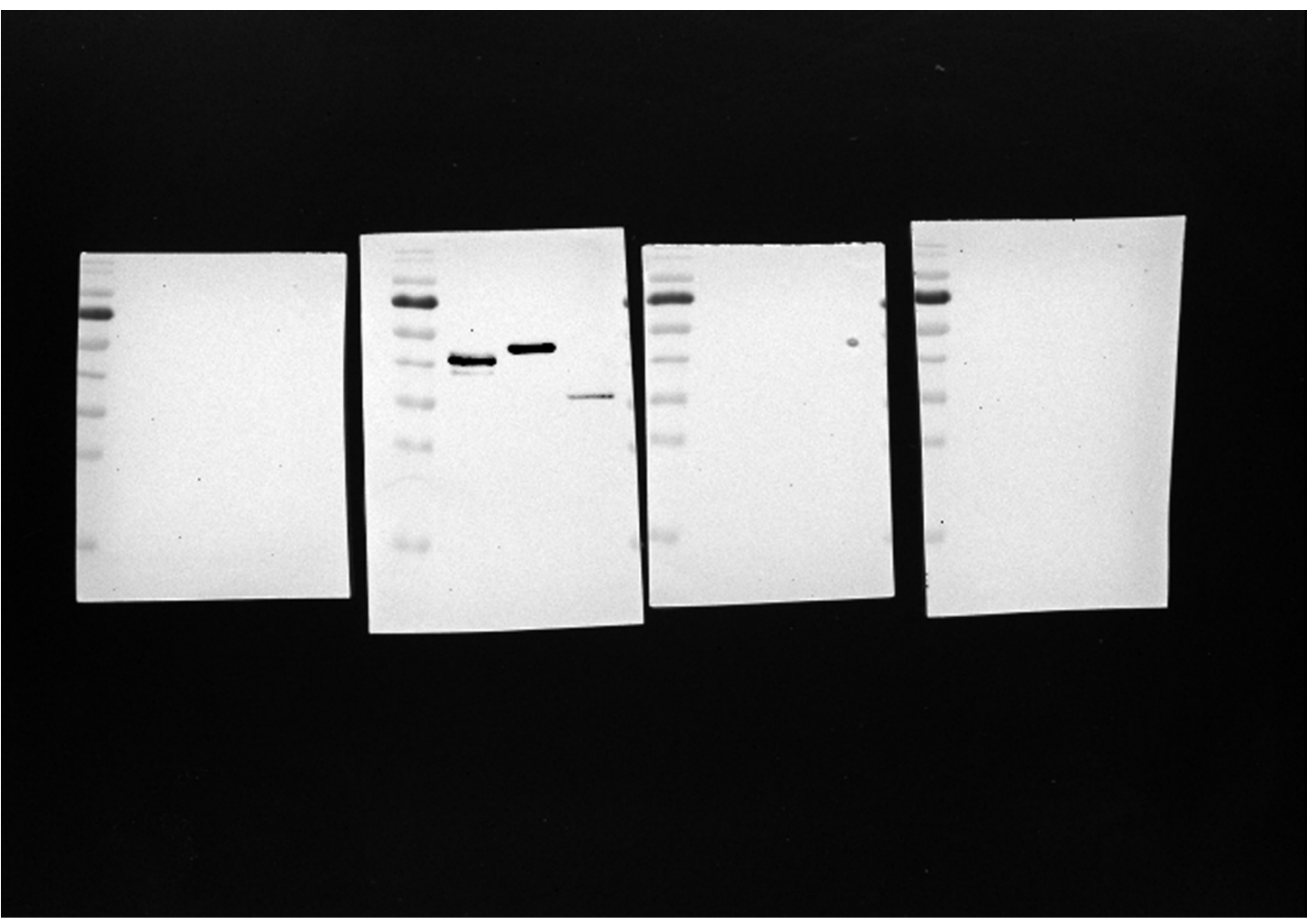
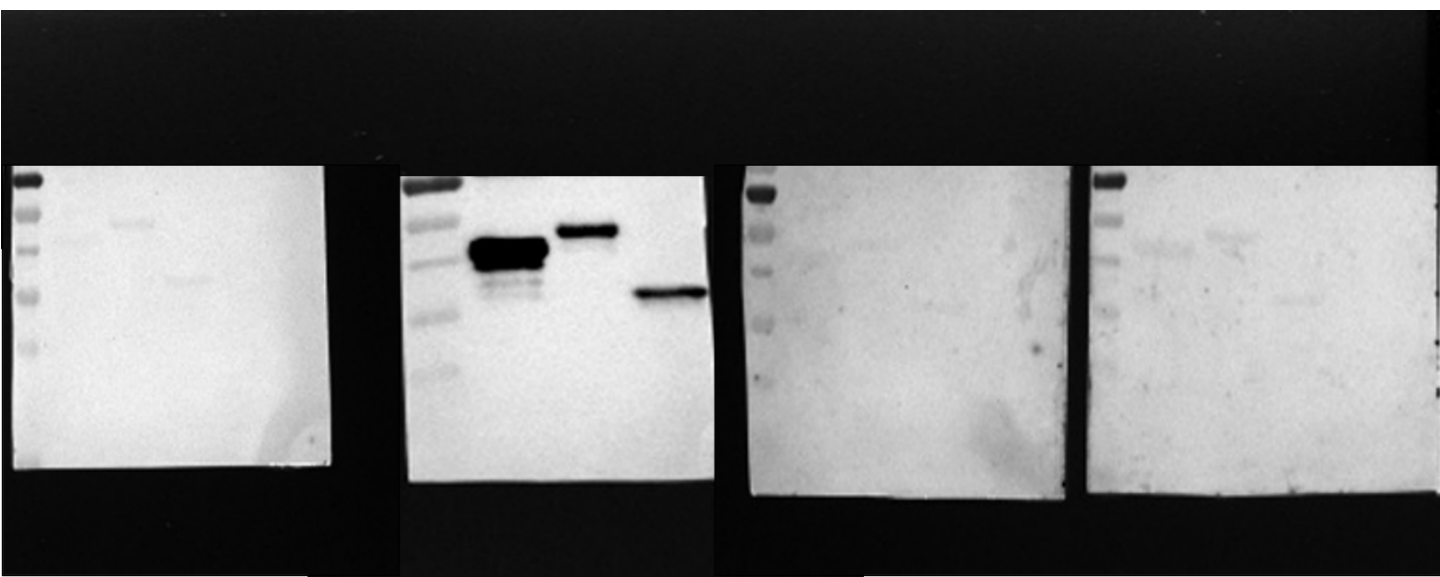


Figure. 2a Right



**Figure 3c: Unprocessed W blot**



**Figure 5b: Unprocessed W blot**

