

1 **Supplementary Figure Legends**

2

3 **Supplementary Figure 1. NSP14 mediates NF- κ B activation.** (a) HEK293 cells were co-
4 transfected with 0.2 μ g of the indicated FLAG-tagged SARS-CoV-2 gene along with NF- κ B
5 reporter and pRL-SV40. After 48 hours, cells were harvested, and the relative reporter activity
6 was determined by calculating the ratio of firefly luciferase to *Renilla* luciferase. The *p*-value was
7 calculated by one-way ANOVA followed by Dunnett's multiple comparisons test. Lysates were
8 blotted with anti-FLAG and anti- α -tubulin antibodies. (b) HEK293 cells were co-transfected with
9 0.05 μ g, 0.1 μ g, and 0.15 μ g of FLAG-tagged SARS-CoV-2 NSP14 along with NF- κ B reporter and
10 pRL-SV40. After 48 hours, cells were harvested, and the relative reporter activity was determined
11 by calculating the ratio of firefly luciferase to *Renilla* luciferase. Lysates were blotted with anti-
12 FLAG and anti- α -tubulin antibodies. (c) 0.1 μ g of FLAG-tagged SARS-CoV-2 NSP14 and 0.1 μ g
13 of FLAG-tagged SARS-CoV-2 NSP10 were co-transfected with the NF- κ B reporter and pRL-SV40
14 into HEK293 cells. Western blotting demonstrates the expression levels of NSP14-FLAG and
15 NSP10-FLAG. The position of NSP14 and NSP10 is denoted. (d) FLAG-tagged SARS-CoV-2
16 NSP14 or the indicated zinc finger mutant was co-transfected with the NF- κ B reporter and pRL-
17 SV40 into HEK293 cells. After 48 hours, cells were harvested, and the relative reporter activity
18 was determined by calculating the ratio of firefly luciferase to *Renilla* luciferase. Lysates were
19 blotted with anti-FLAG and anti- α -tubulin antibodies. The *p*-value was calculated by one-way
20 ANOVA followed by Tukey's multiple comparisons test (b, c, d).

21

22 **Supplementary Figure 2. NSP14 activates NF- κ B via linear ubiquitination.** (a) HEK293 cells
23 were transfected with FLAG-tagged NSP14 genes derived from various coronaviruses. After 48
24 hours, cell lysates were subjected to immunoprecipitation and subsequent blotting using the
25 indicated antibodies. (b) Lysates of HEK293 cells and two HOIP knockout cell lines were blotted

26 as indicated. (c) Blotting analysis of lysates from HEK293 cells and two UBC13 knockout cell
27 lines. (d-e) Wild-type, HOIP knockout (d), and UBC13 (e) knockout HEK293 cells were stimulated
28 with 10 ng/mL TNF α for designated times. Subsequently, cell lysates were analyzed by Western
29 blotting. (f) Wild-type HEK293 cells, along with two UBC13 knockout and two HOIP knockout
30 HEK293 cell lines, were transfected with FLAG-tagged SARS-CoV-2 NSP14 along with NF- κ B
31 reporter and pRL-SV40. After 48 hours, cells were collected, and the relative reporter activity was
32 determined by calculating the ratio of firefly luciferase to *Renilla* luciferase. (g) Blotting analysis
33 of lysates from OTULIN wild-type and knockout HEK293 cells. (h) OTULIN wild-type and knockout
34 HEK293 cells were stimulated with 10 ng/mL TNF α for designated times. Cell lysates were blotted
35 using the indicated antibodies. (i) Wild-type and two OTULIN knockout HEK293 cell lines were
36 transfected with FLAG-tagged SARS-CoV-2 NSP14 with the NF- κ B reporter and pRL-SV40. The
37 *p*-value was calculated by two-way ANOVA followed by Sidak's multiple comparisons test (f, i).

38

39 **Supplementary Figure 3. NSP14 interacts with HOIP.** (a) NSP14-HA was co-transfected with
40 FLAG-tagged HOIP, HOIL-1, or SHARPIN into HEK293 cells. After 48 h, cell lysates were
41 subjected to immunoprecipitation with the anti-FLAG antibody and subsequent blotting with anti-
42 FLAG and anti-HA antibodies. (b) NSP14-FLAG or GFP-FLAG was transfected into HEK293
43 cells. After 48 h, cell lysates were immunoprecipitated with the anti-FLAG antibody and blotted
44 using the indicated antibodies. (c) Vector or NSP14-HA was transfected into A549 cells. After 48
45 h, cells were fixed and stained with anti-HOIP (red), anti-HA (green), and DAPI nuclear stain
46 (blue). Scale bar = 10 μ m. (d) Schematic of HOIP mutants. PUB: Peptide N-glycanase/UBA or
47 UBX-containing proteins; ZnF: Zinc finger; NZF: Npl4 zinc finger; UBA: Ubiquitin-associated;
48 RBR: RING between RING fingers. (e) FLAG-tagged HOIP or the indicated mutant was co-
49 transfected with Myc-tagged (NSP14-Myc) into HEK293 cells. After 48 h, cell lysates were
50 immunoprecipitated with the anti-FLAG antibody and blotted as indicated.

51

52 **Supplementary Figure 4. The IKK complex is required for NSP14-mediated NF- κ B**

53 **activation.** (a) Co-transfection of FLAG-tagged NSP14 with IKK α , IKK β , or p65, along with NF-
54 κ B reporter and pRL-SV40, into HEK293 cells. After 48 h, cells were collected, and the relative
55 reporter activity was determined by calculating the ratio of firefly luciferase to *Renilla* luciferase.
56 (b-d) Wild type, IKK α knockout (b), IKK β knockout (c), or NEMO knockout (d) HEK293 cells were
57 stimulated with 10 ng/mL TNF α for designated times. Cell lysates were blotted as indicated. (e-
58 g) Transfection of vector or FLAG-tagged NSP14 with NF- κ B reporter and pRL-SV40 into wild-
59 type, IKK α knockout (e), IKK β knockout (f), and NEMO knockout (g) HEK293 cells. After 48 h,
60 cells were collected, and the ratio of firefly luciferase to *Renilla* luciferase was calculated to
61 determine the relative reporter activity. (h) Vector or FLAG-tagged NSP14 was transfected with
62 the NF- κ B reporter and pRL-SV40 into wild-type and TBK1 knockout MEFs. After 48 hours, cells
63 were harvested, and the relative reporter activity was determined by calculating the ratio of firefly
64 luciferase to *Renilla* luciferase. (i) FLAG-tagged NSP14 or NSP14^{K/R} was transfected with Myc-
65 tagged IKK α or IKK β into HEK293 cells, along with NF- κ B reporter and pRL-SV40. After 48 hours,
66 cells were harvested, and the relative reporter activity was determined by calculating the ratio of
67 firefly luciferase to *Renilla* luciferase. Lysates were blotted with anti-FLAG, anti-Myc, and anti- α -
68 tubulin antibodies. The *p*-value was calculated by two-way ANOVA followed by Sidak's multiple
69 comparisons test (a, e, f, g, h) or one-way ANOVA followed by Tukey's multiple comparisons test
70 (i).

71

72 **Supplementary Figure 5. MAVS knockout impairs ISG but not proinflammatory factor**

73 **expression.** (a) MAVS wild-type and knockout A549 cells were infected with HCoV OC43 for the
74 designated times. Cell lysates were blotted as indicated, and band densitometry was calculated
75 using Image J. The ratio of phosphorylated IKK to total IKK in each lane was indicated. (b) HOIP

76 wild-type and knockout H1299 cells were infected with 1 MOI of HCoV OC43 for 16 h. Real-time
77 PCR was conducted to determine the relative mRNA levels of proinflammatory factors and ISGs.
78 (c) MAVS wild-type and knockout A549 cells were infected with 1 MOI of HCoV OC43 for 16 h.
79 Real-time PCR was conducted to determine the relative mRNA levels of proinflammatory factors
80 and ISGs. (d) MTT assays of A549 cells treated with DMSO, 30 μ M HOIPIN-8, or 10 μ M IKK-16
81 for 5 days. The *p*-value was calculated by two-way ANOVA followed by Sidak's multiple
82 comparisons test.

83

84 **Supplementary Figure 6. HOIPIN-8 inhibits HCoV OC43 viral replication.** (a) HOIP wild-type
85 and knockout A549 cells were infected with 0.01 MOI of HCoV OC43 for the designated days.
86 MTT assays were performed to determine cell viability. (b) HOIP wild-type and knockout H1299
87 cells were infected with 0.01 MOI of HCoV OC43 for the designated days. Lysates were blotted
88 as indicated. (c) A549 cells were treated with DMSO, HOIPIN-8, or IKK-16 for 2 h and then
89 infected with 0.01 MOI of HCoV OC43 for the designated days. TCID₅₀ of culture supernatants
90 containing HCoV OC43 were determined on Vero cells. The *p*-value was calculated by two-way
91 ANOVA followed by Sidak's multiple comparisons test. (d) A549 cells were treated with DMSO or
92 HOIPIN-8 for 2 h and then infected with 0.1 MOI HCoV OC43 for 24 h. Cells were stained with
93 anti-dsRNA (red) and DAPI (blue). The relative ratio of positively stained cells is summarized in
94 the graph. The *p*-value was calculated by *t*-test.

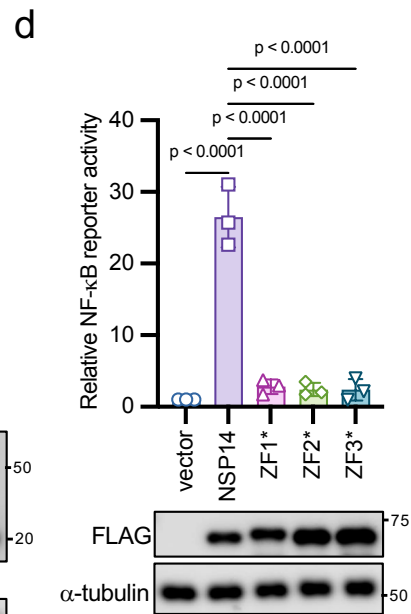
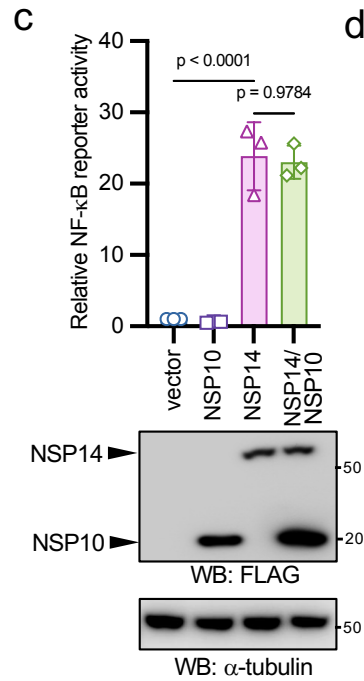
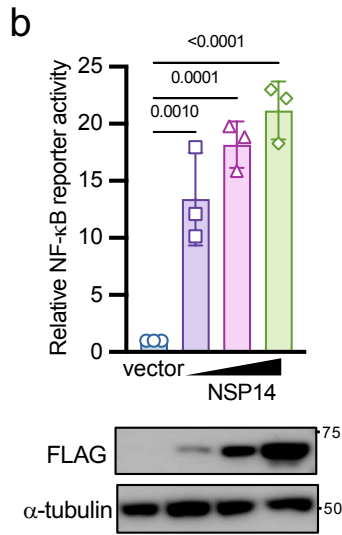
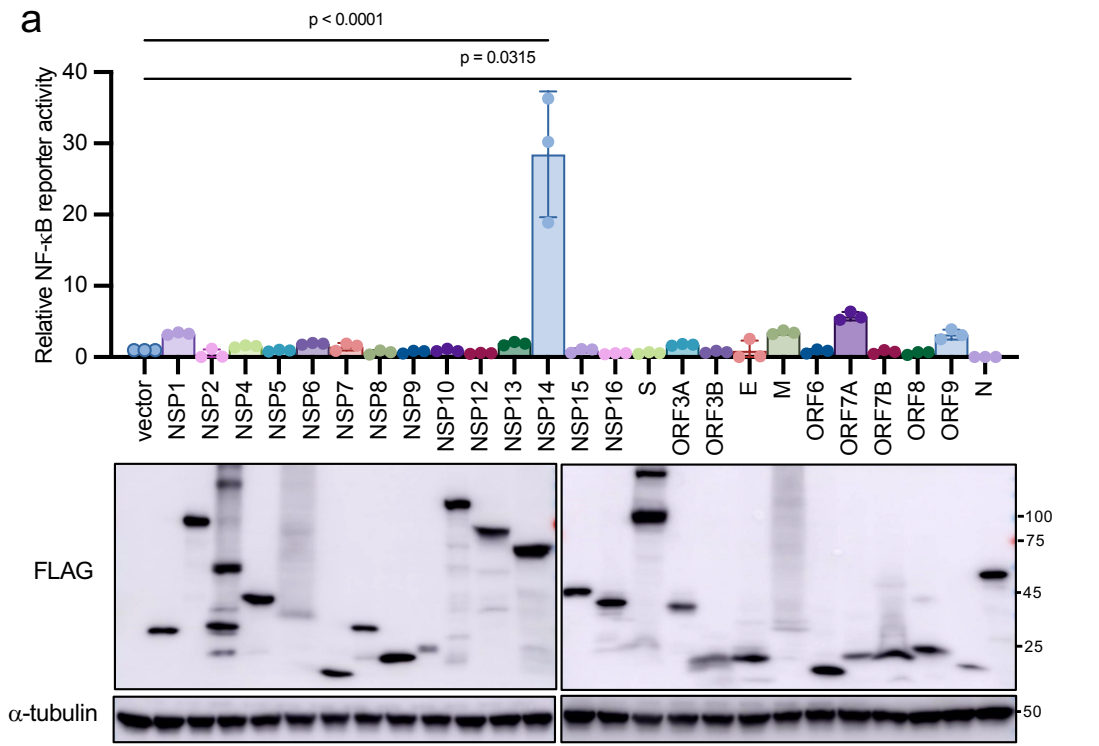


Figure S1

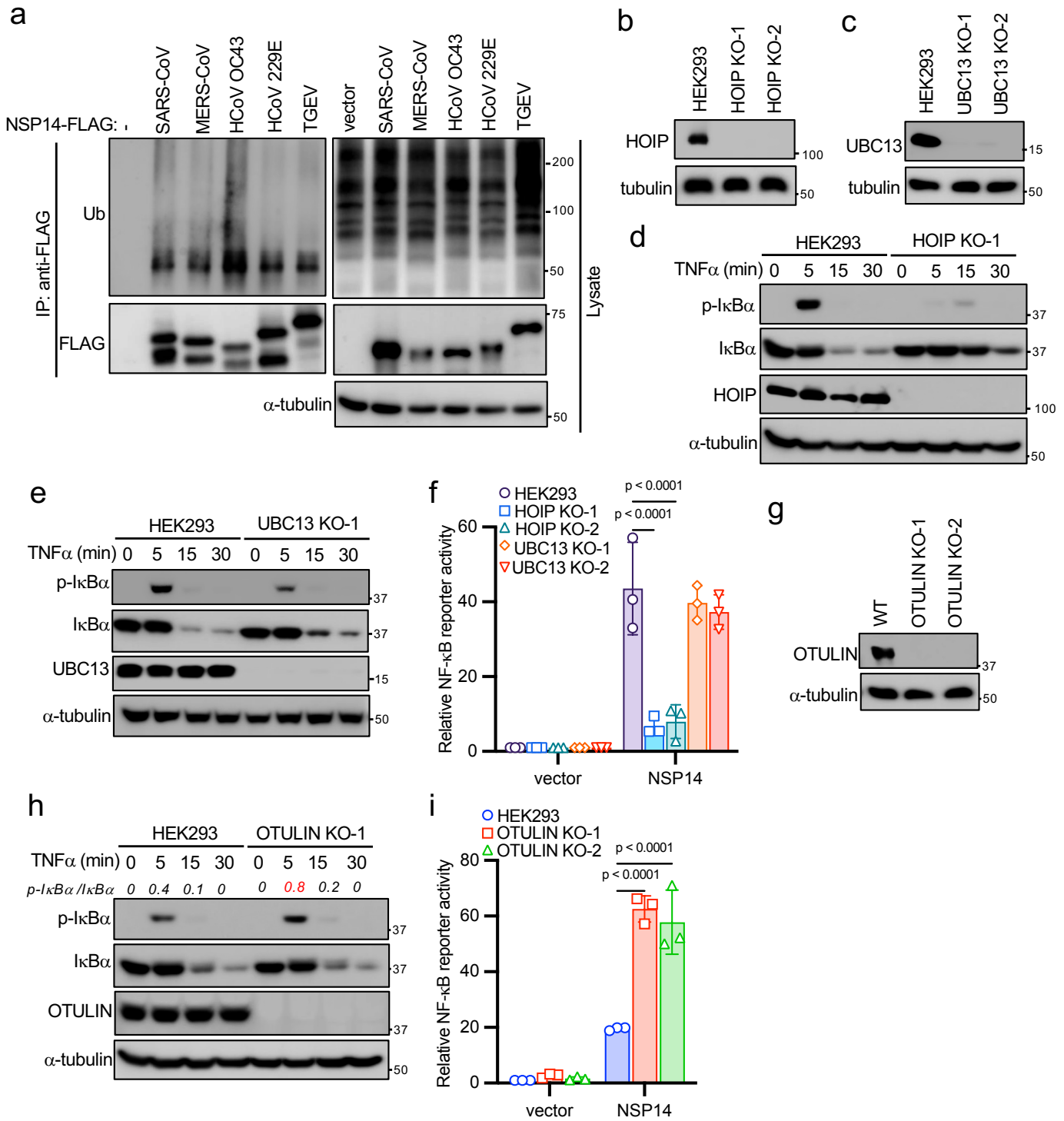


Figure S2

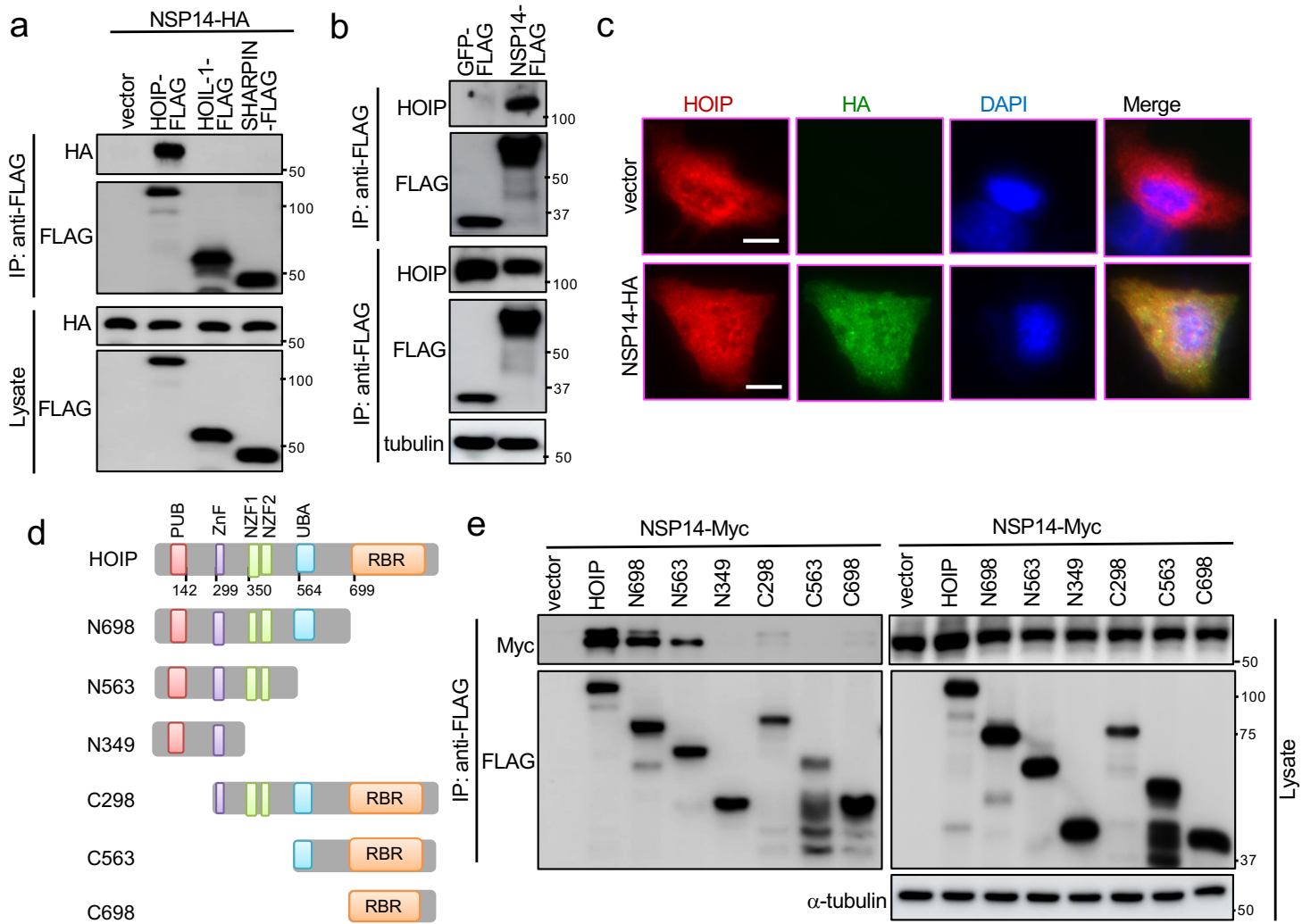


Figure S3

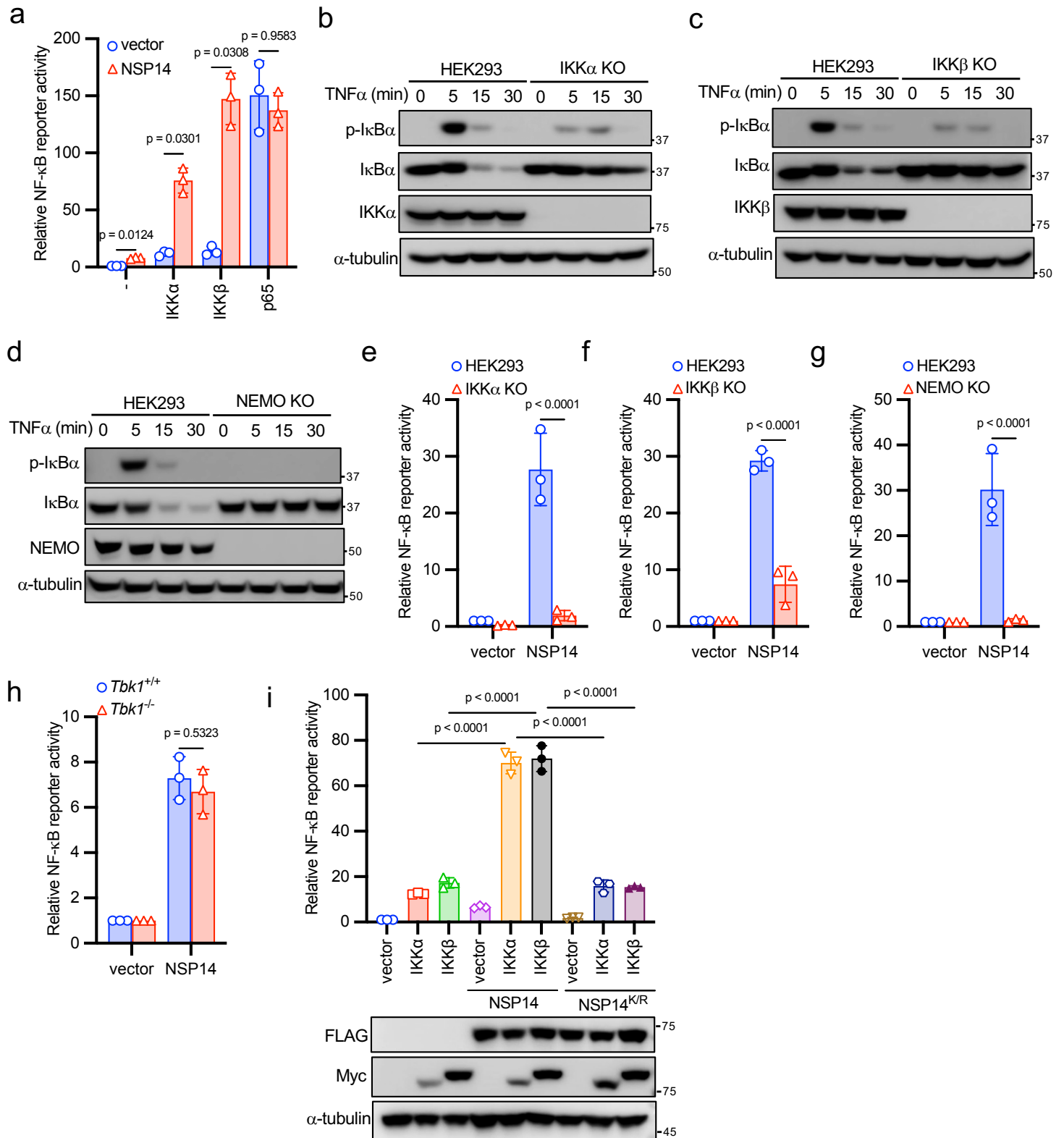


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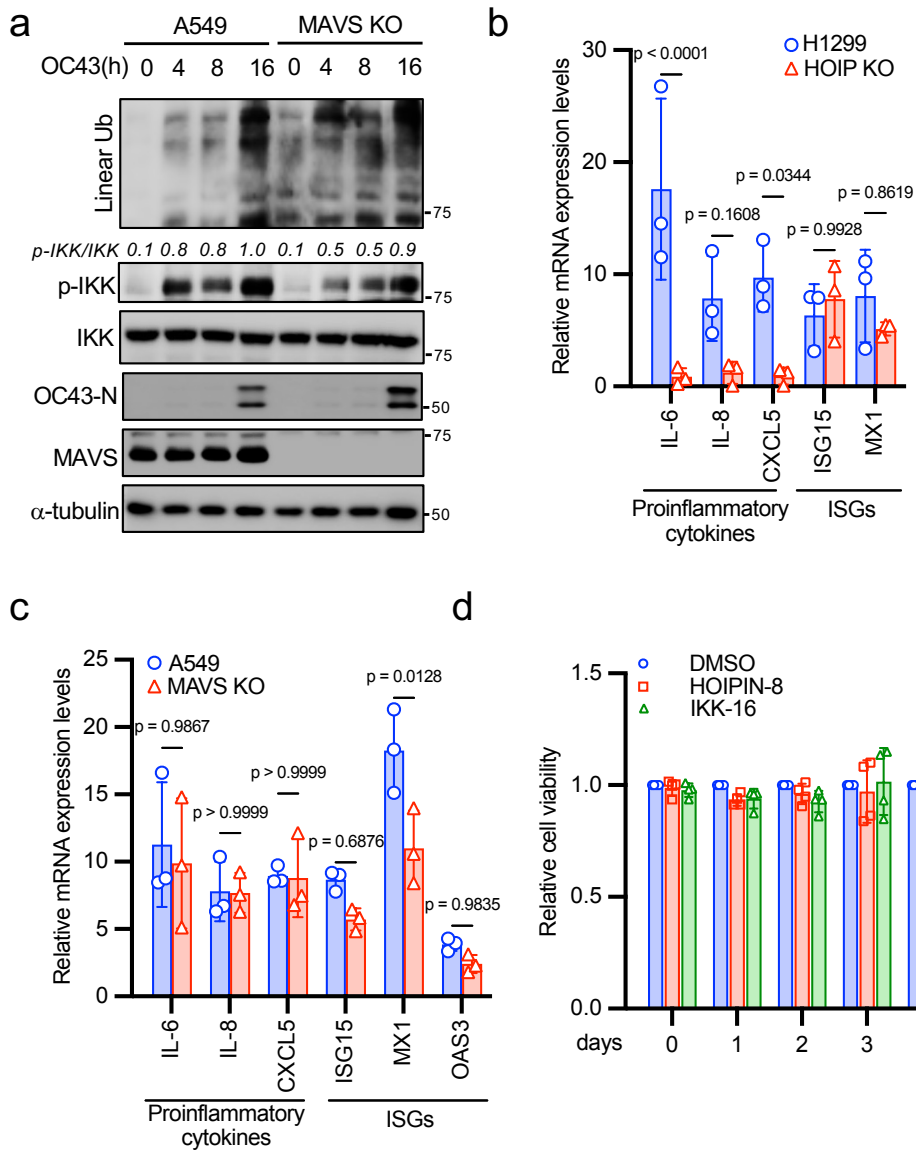


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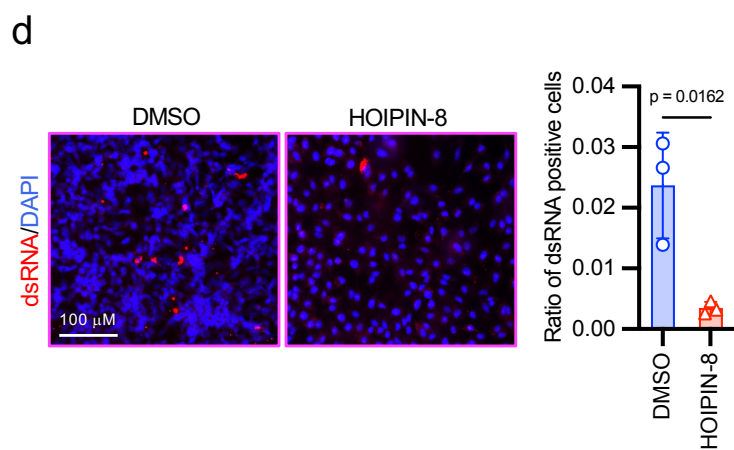
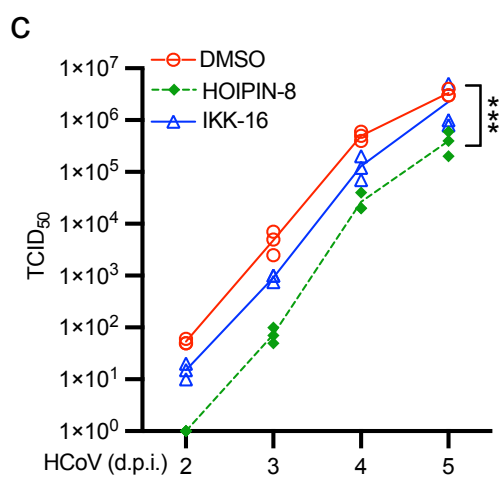
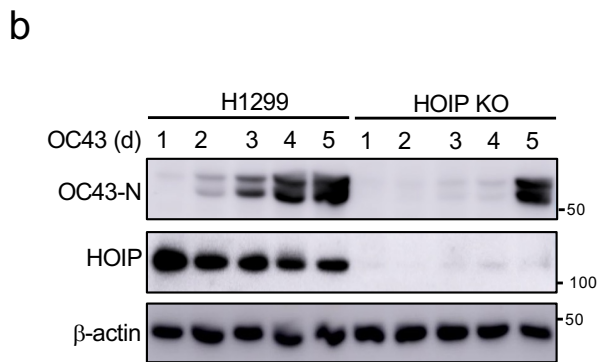
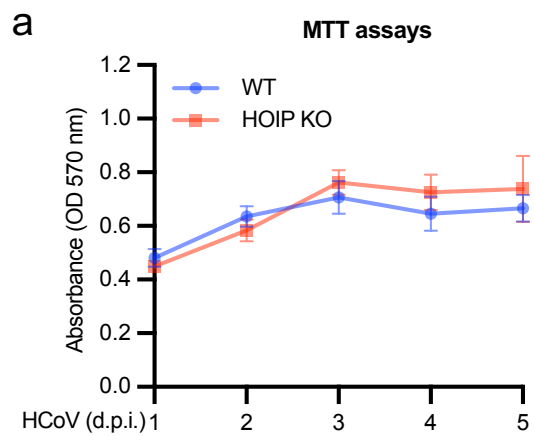


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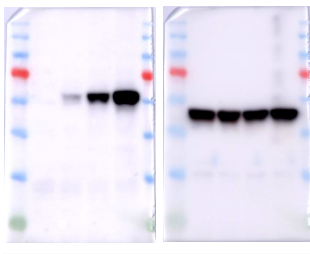
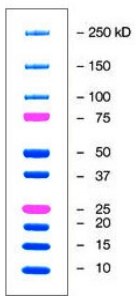


Fig. 1a

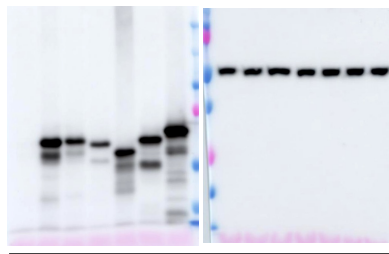


Fig. 1b

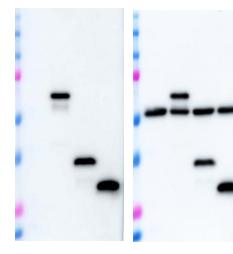


Fig. 1d

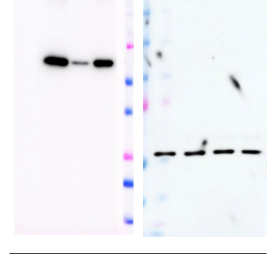


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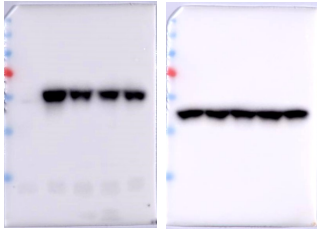


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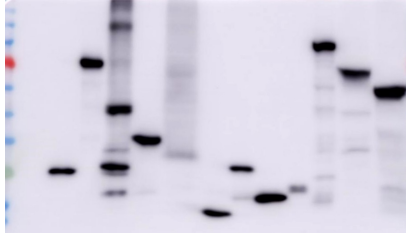


Fig. S1a

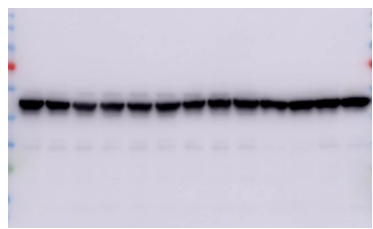
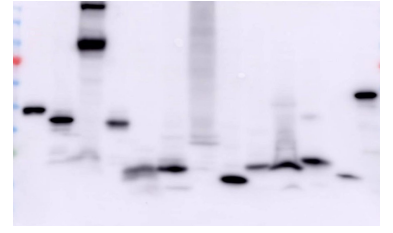
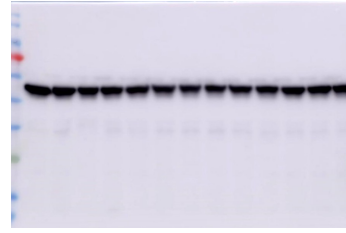


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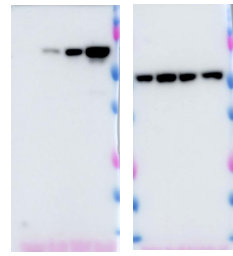


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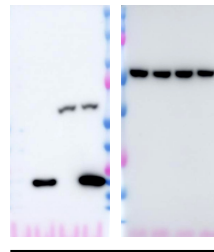


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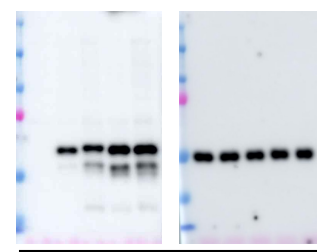


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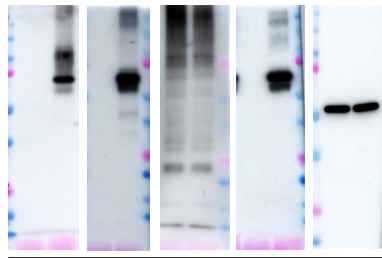


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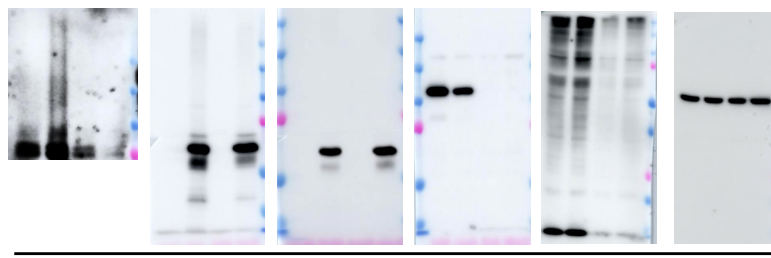


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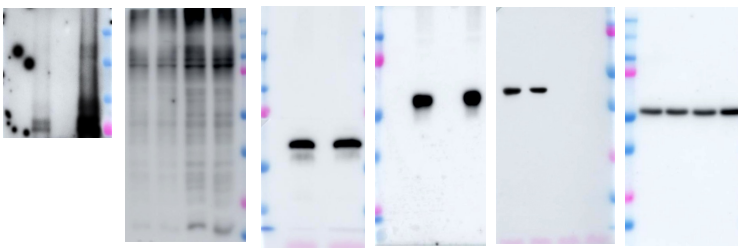


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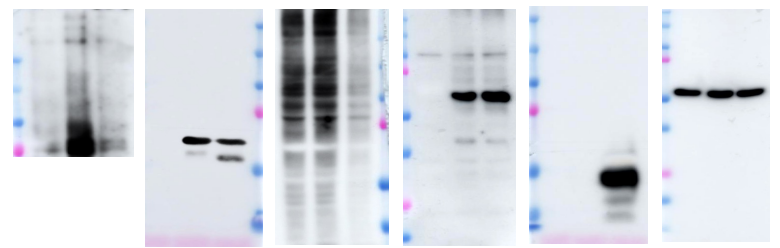


Fig. 2g

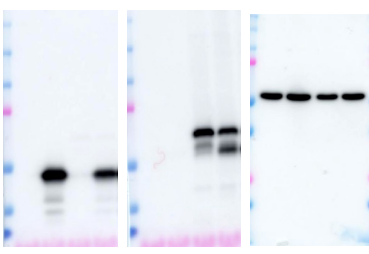


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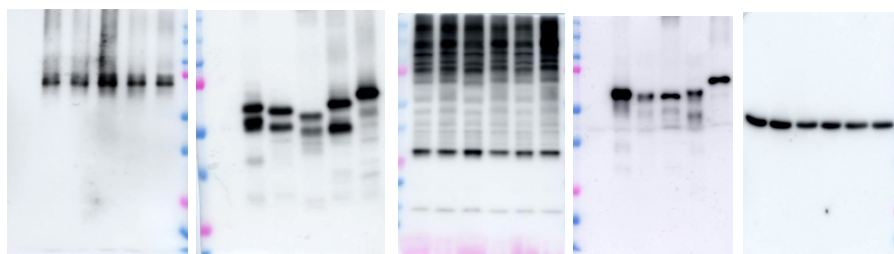


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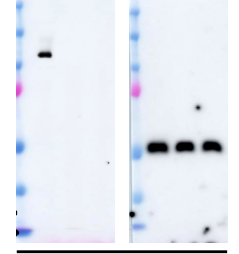


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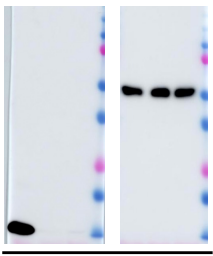


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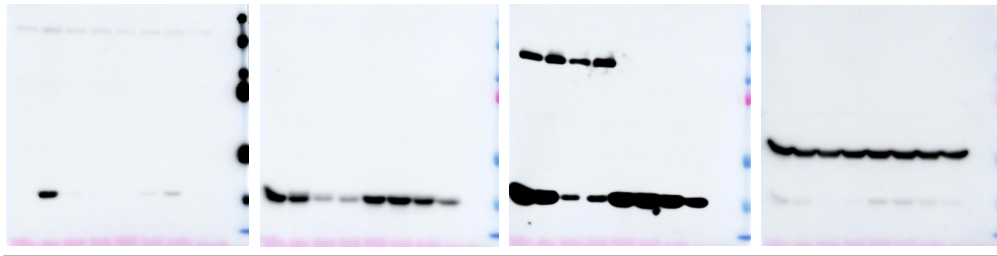


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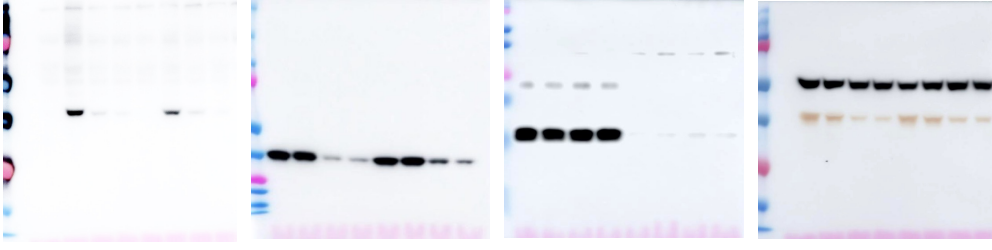


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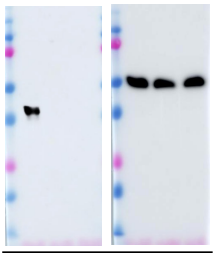


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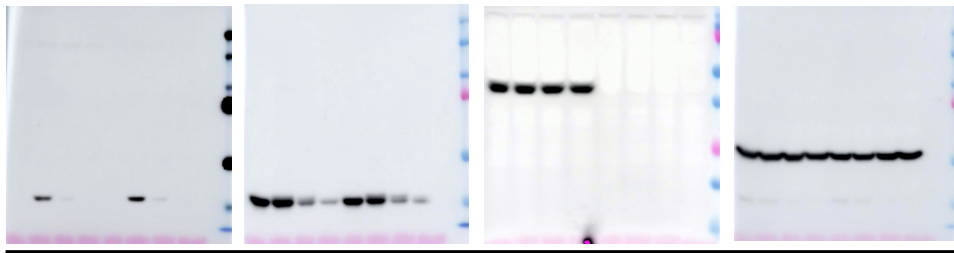


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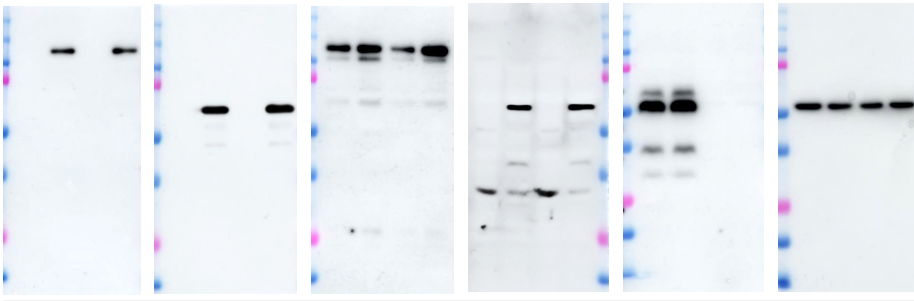


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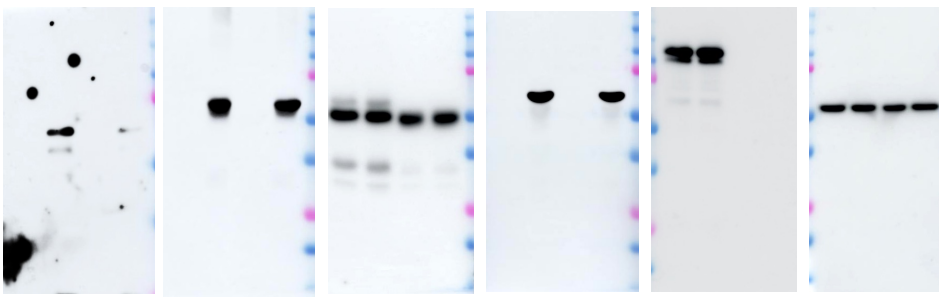


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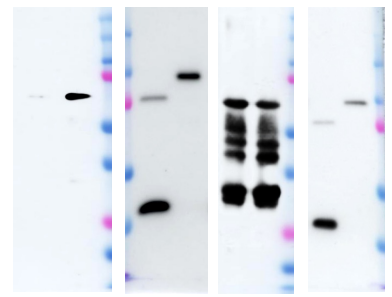


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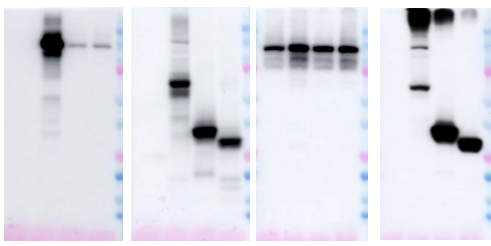


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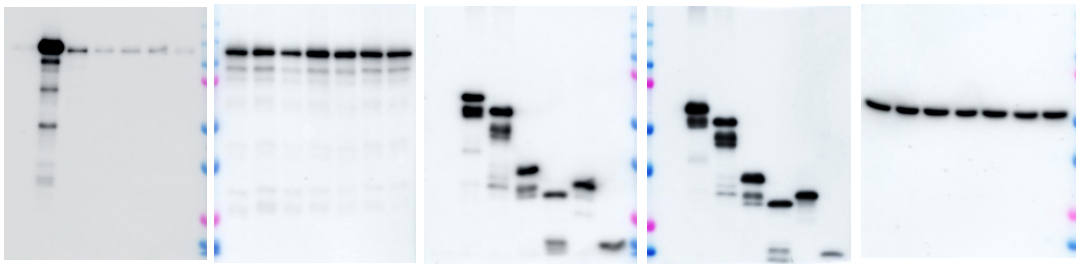


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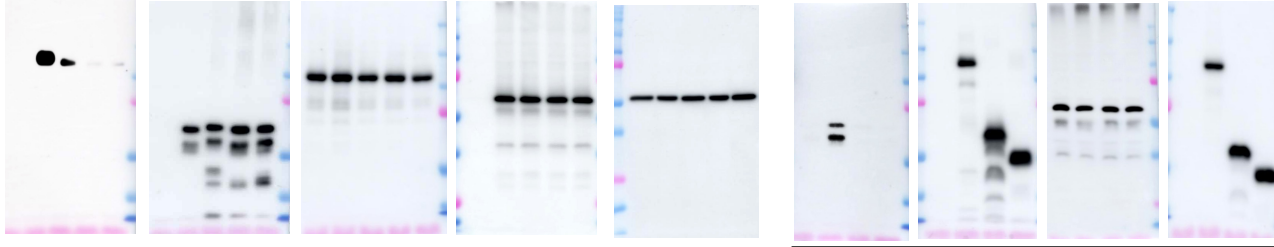


Fig. 3g

Fig. S3a

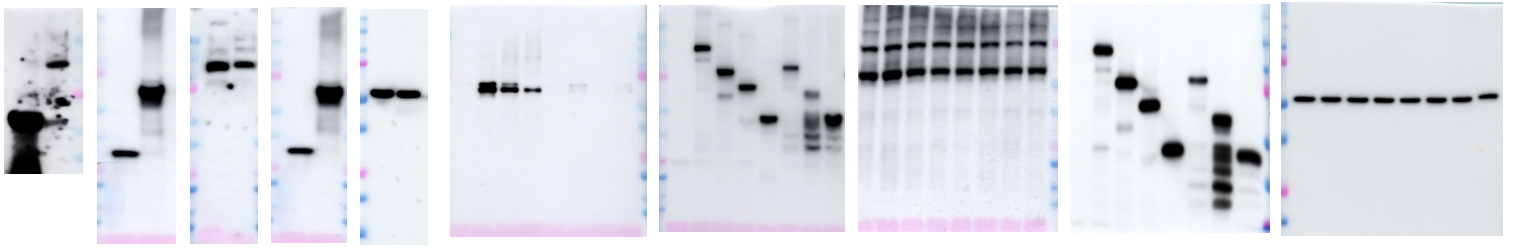


Fig. S3b

Fig. S3e

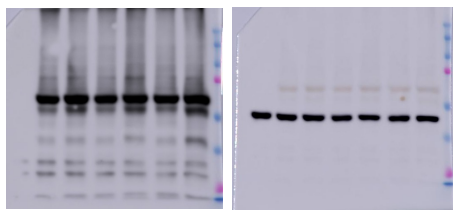


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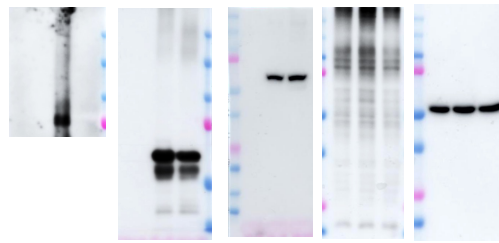


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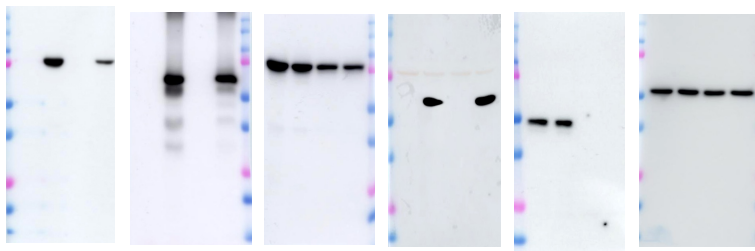


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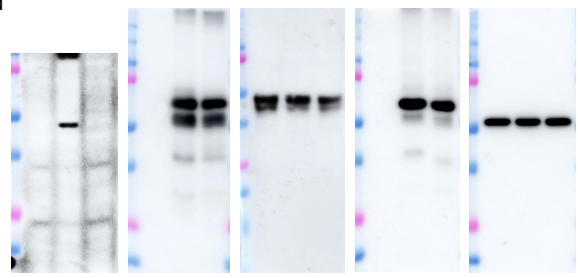


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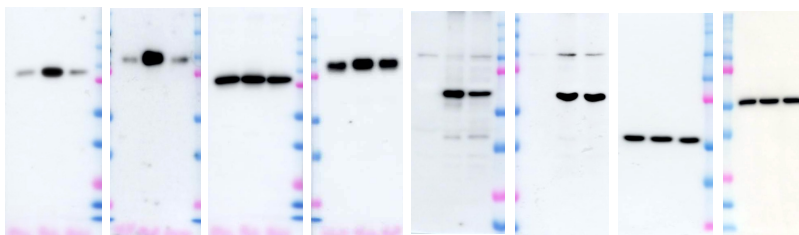


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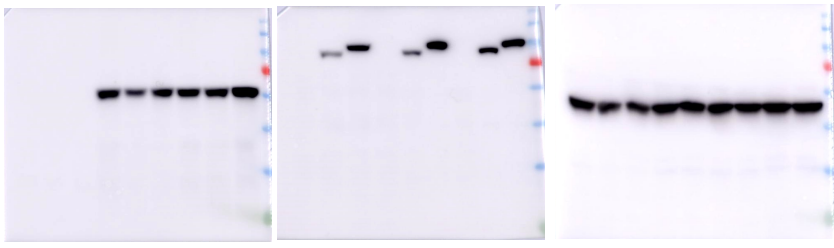


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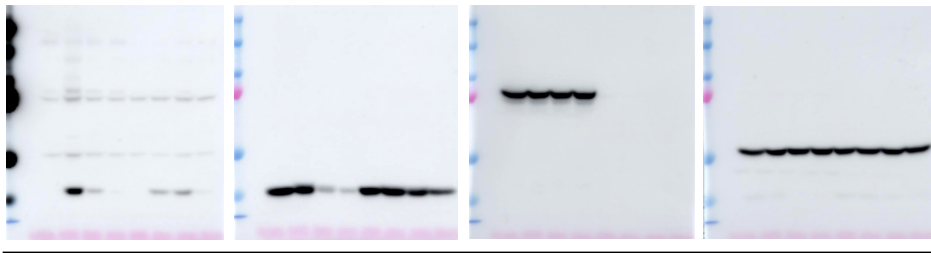


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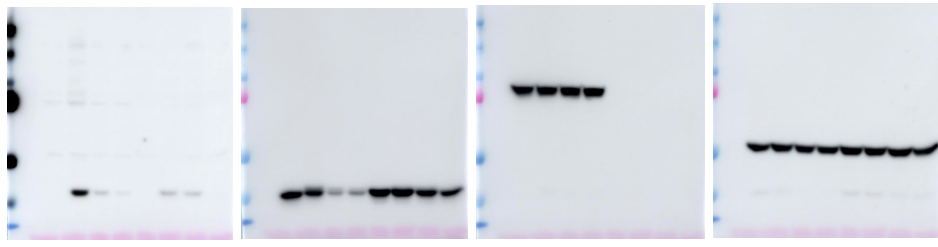


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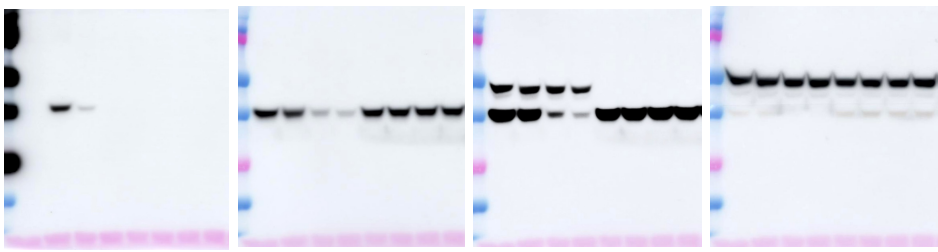


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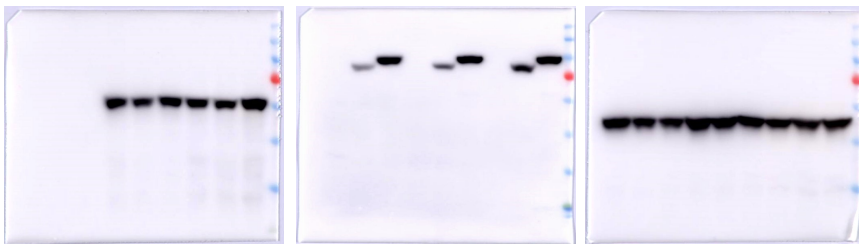


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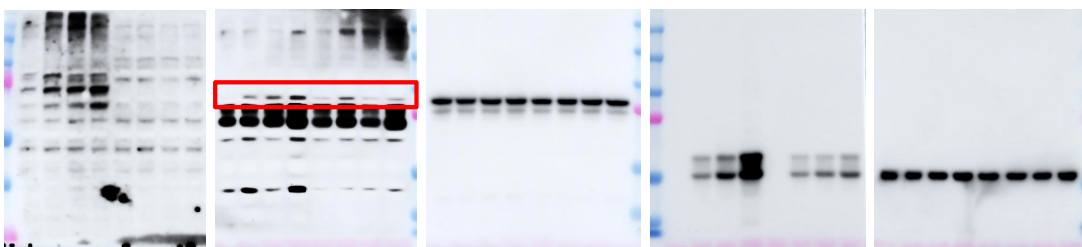


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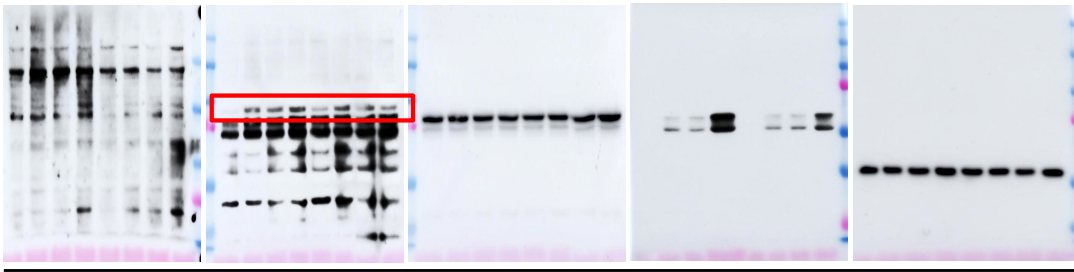


Fig. 6d

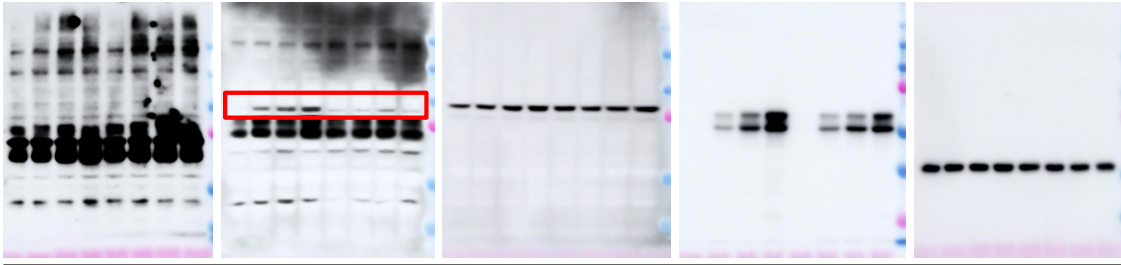


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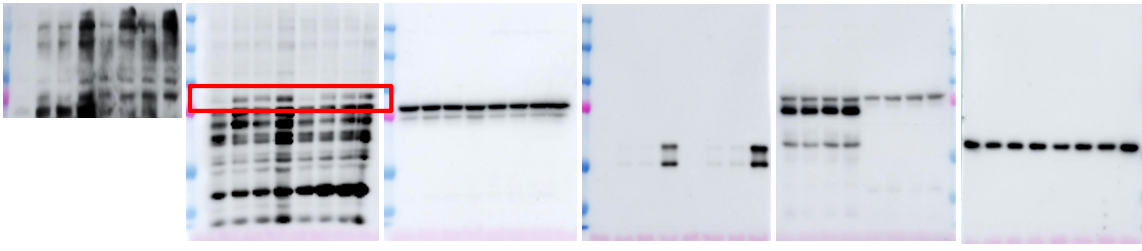


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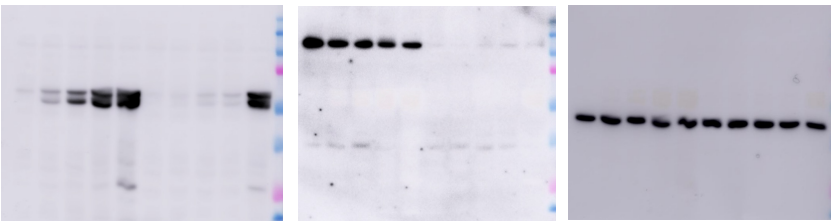


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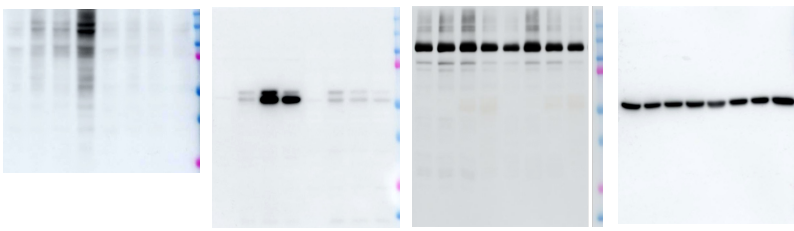


Fig. 7b