Supplementary Materials

Table S1. Primers used in this study. Related to STAR methods.

Figure S1. Stable expression of TRIM25 in CRFK cells exceeds endogenous levels, and TRIM25 restricts influenza viruses but not Sendai virus or retroviruses. Related to Figure 1. (A) Comparison of stably expressed TRIM25 protein levels in CRFK cells to endogenous levels of TRIM25 in A549 and 293T cells treated with 1000 U/mL IFN-α (PBL Assay Science #11100-1) for the indicated amount of time. Each lane contains 30 μg of protein and was probed using mouse anti-TRIM25 (BD Biosciences #610570) and mouse anti-βActin antibodies (Santa Cruz #sc-47778). The upper band corresponds to a ubiquitinated form of TRIM25 that is observed in cell lines that express high levels of TRIM25. This upper band happens to overlap with a non-specific band that appears in CRFK cells. **(B)** The CRFK cells from **Figure 1A** that stably express the indicated TRIM25 or TRIM5α were infected with 0.2 plaque-forming units (pfu)/cell of the 2009 pandemic influenza A virus strain A/California/09. Whole cell extracts were collected at 8 hours post infection (hpi) and subjected to immunoblotting using an anti-Udorn goat polyclonal antibody that recognizes HA, NP, and M1 (Chen et al., 2007). Actin was probed for a loading control. **(C)** Influenza B virus (B/Florida/4/2006 Yamagata lineage, obtained through BEI Resources, NIAID, NIH, NR-41795) infection of A549 RIG-I/TRIM25 KO cell lines (**Figure S4**) stably expressing control vector, Human (Hu), or Gibbon (Gi) FLAG-tagged TRIM25 (T25) constructs. Cells were infected with 0.002 pfu/cell under the same conditions as influenza A virus infection (see STAR methods) except that all virus incubation steps were carried out at 34°C. Whole cell extracts were collected at 24 hours post infection and probed with serum (1:10000) from ferrets subjected to influenza B virus infection, which was kindly provided by Jonathan McCullers (Huber et al., 2008). The major bands represent hemagluttinin (HA), nucleoprotein (NP), and non-structural protein 1 (NS1). Lysates were also probed with mouse

anti-TRIM25 and mouse anti-βActin for expression and loading controls, respectively. **(D)** Sendai virus (formerly parainfluenza virus 1, obtained through BEI resources, NIAID, NIH, NR-3227) infection of A549 RIG-I/TRIM25 KO cell lines (**Figure S4**) stably expressing control vector, Human (Hu), or Gibbon (Gi) FLAG-tagged TRIM25 (T25) constructs. Cells were infected with 20 hemagglutination units per sample under the same conditions as influenza A virus infections (see STAR methods). Whole cell extracts were collected at 24 hours post infection and probed with a guinea pig anti-Sendai virus antibody (1:10000, BEI Resources NR-3228) that recognizes the Sendai virus nucleoprotein (N). Lysates were also probed with mouse anti-TRIM25 and mouse anti-βActin for expression and loading controls, respectively. **(E)** Retroviral infection of CRFK cells stably expressing the indicated construct. Human and Rhesus TRIM5α are shown as controls. Cell lines were infected with single-cycle VSV-G pseudotyped HIV-1 (left), FIV (middle), or NB-MLV (right). Each virus carried a GFP reporter and infectivity is shown relative to the empty vector control (see STAR methods for virus production and experimental protocol). All experiments were carried out in triplicate and are representative of two experimental replicates.

Figure S2. Amino acid alignment of human and gibbon TRIM25. Related to Figure 1. RING, B-box 1, B-box 2, coiled-coil, and B30.2 domains are highlighted in gray boxes. Asterisks indicate conserved amino acid positions.

Figure S3. Human and gibbon TRIM25 bind NS1 equally. Related to Figure 1. (A) Co-immunoprecipitation assay using 293T whole cell extracts infected with Udorn virus at 2 pfu/cell. Cells were transfected with 2 micrograms of FLAG-tagged human TRIM25 (Hu T25), gibbon TRIM25 (Gi T25) or an empty vector control 48 hours prior to infection. Cells were collected at 9 hours post infection and whole cell extract was subjected to the co-immunoprecipitation protocol described in the STAR Methods, using 3xFLAG peptide for elution. Eluates were probed using

the indicated antibodies. **(B)** Co-immunopreciptation assay in 293T cells transfected with 2 micrograms of human (Hu) TRIM25, gibbon (Gi) TRIM25, or an empty vector control along with 2 micrograms of either wild-type (wt) PR8 NS1 or a TRIM25-binding mutant of PR8 (mut) containing R38A and K41A mutations (Gack et al., 2009). Cells were collected and lysed 48 hours post transfection. Co-immunoprecipitation of whole cell extracts and western blotting was performed as described for panel A. **(C)** Co-immunoprecipitation assay in 293T cells transfected with indicated amount of plasmids encoding TRIM25 coiled-coil linker 2 truncation (aa190-469) or vector control, along with PR8 NS1. Cells were collected and lysed 48 hours post transfection. Co-immunoprecipitation of whole cell extracts and western blotting was performed as described for panel A.

Figure S4. Analysis of A549 RIG-I/TRIM25 knockout (KO) cell lines. Related to Figure 2. DNA sequencing analysis of **(A)** A549 RIG-I KO cell line and **(B,C)** A549 RIG-I/TRIM25 double KO cell line. A region that encompases the first exon of RIG-I or TRIM25 was amplified using PCR primers NRM923/NRM924 (RIG-I) or NRM925/926 (TRIM25) (**Table S1**). PCR products were TA-cloned into pCR4 (Invitrogen #K457502) and at least ten individual clones were sequenced using the M13R sequencing primer. PAM sequences used for Cas9 recognition are boxed. Resulting protein sequences are shown below each DNA alignment. **(D)** $1X10^5$ cells were boiled with 100 µl of SDS-PAGE loading buffer, 20 µl each were loaded onto an SDS-PAGE gel and blotted with anti-RIG-I, anti-TRIM25 and anti-βActin antibodies. See STAR Methods for experimental details regarding the generation of these cell lines.

Figure S5. TRIM25 RING mutations (C13/16A) inactivate K63-linked ubiqtuitin ligase activity, and analysis of RIG-I knockout 293T cells. Related to Figure 3. (A,B) Ubiquitination assay using TRIM25 RING mutants and either a wild-type ubiquitin construct **(A)** or a ubiquitin construct that has lysine encoded only at position 63 **(B)**. This mutant is used because the

ubiquitination of RIG-I by TRIM25 is mediated by the formation of K63-linked ubiquitin chains (Gack et al., 2007; Zeng et al., 2010). 293T cells were transfected with the indicated combination of TRIM25 and ubiquitin. RING domains were mutated by introducing alanines (C13A/C16A) into the first zinc-coordinating motif. Whole cell extracts were collected at 48 hours post transfection and probed using the indicated antibodes. **(C)** DNA sequencing analysis of 293T RIG-I KO cell lines. A region that encompases the first exon was amplified using PCR primers NRM923 and NRM924 (**Table S1**). PCR products were cloned into pGEM-T (Promega #A1360) and individual clones were sequenced using NRM924. Cas9 guide sequence is shown along with the PAM sequence. Resulting protein sequence is shown below. Amino acids that are known to be critical for RIG-I CARD domain function are in red text (Peisley et al., 2014). **(D)** $1X10^5$ cells were boiled with 100 µl of SDS-PAGE loading buffer, 20 µl each were loaded onto an SDS-PAGE gel and blotted with anti-RIG-I and anti-βActin antibodies. See STAR Methods for experimental details regarding the generation of these cell lines.

Figure S6. Purification of influenza vRNPs from virus particles, and purification of TRIM25 from insect cells. Related to Figure 4. (A) Influenza virus particles pelleted from media of infected cells were treated with detergent then loaded on a glycerol gradient. After ultracentrifugation, fractions were taken from the top to the bottom of the gradients. Samples of each fraction were subjected to SDS-PAGE and stained with Coomassie blue. Fractions 7 and 8 were pooled for further experiments. Molecular weight marker was loaded on the left. **(B)** Sf9 insect cells were infected with baculoviruses encoding his-tagged TRIM25 genes. Cells were scraped from the plates 48 hours post-infection. His-tagged TRIM25 proteins were purified from cell lysates with Ni-NTA affinity column and size-exclusion chromatography (see STAR Methods for experimental details). Fractions containing the peaks of purified proteins were pooled, quantified, resolved on an SDS-PAGE gel and stained with Coomassie blue. Left panel,

molecular weight marker and indicated amount of BSA were used as control. Right panel, duplicates of 5 µl of TRIM25 proteins at 0.2 µg/µl concentration were loaded.

Figure S7. Co-immunoprecipitation assays of FLAG-TRIM25 with individual polymerase subunits or the nucleocapsid protein (NP) of Udorn virus, and His-tagged human and gibbon TRIM25 bind to vRNA or cRNA with similar affinity. Related to Figures 4 and 5. (A) 293T cells were co-transfected with plasmids expressing FLAG-TRIM25 (gibbon TRIM25 with RING inactivating mutations) and GFP-fused PB2, PB1, PA, NP. All influenza proteins were cloned from the Udorn strain. 48 hours after transfection, cell lysates were incubated with mouse control antibody (Ctr) or FLAG antibody. Cellular lysis and immunoprecipitation was carried out as described for **Figure S3**. Whole cell extracts and immunoprecipitation eluates were probed with mouse anti-FLAG antibody and mouse anti-GFP antibody (Pierce #MA5-15256, 1:1000 dilution). **(B)** The pcDNA3 plasmid containing full length NS segment was linearized by NotI and used as a template for T7 polymerase to synthesize vRNA or cRNA in vitro. The cRNA was purified by gel extraction, then mixed with the indicated concentrations of His-TRIM25 proteins, and set on ice for 30 minutes in a total volume of 20 µl reaction buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 5% glycerol). Protein-RNA mixture was then loaded onto a 1.2% agarose gel in pre-cooled 0.5X TBE buffer and run at 100V in a cold room. Gel was stained with SYBR-gold (Thermo #S11494) and scanned with a Typhoon FLA9500 scanner (GE).

Table S1: Primers used in this study

Primer Name	Sequence (5'-3')	Orientation	Purpose
NRM257	GGTGCTGGTGCTGGTGCTATGGCAGAG CTGTGCCCC	Forward	Amplify TRIM25 from Human, Orangutan, or Gibbon cDNA. Includes N-term $(Gly\text{-}Ala)_3$ linker
NRM025	CTACTTGGGGGAGCAGATGG	Reverse	
NRM284	TCGCCACCATGGACTACAAGGACGACG ACGACAAGGGTGCTGGTGCTGGTGCT	Forward	Add 1xFLAG and Kozak sequence
NRM285	GGTGCTGGTGCTGGTGCTATGGCACAG CTGTGCCCC	Forward	Amplify TRIM25 from Talapoin cDNA. Use NRM025 as reverse primer
NRM534	GTCTCCTGCGACTTTAACAG	Forward	cat GAPDH, qPCR primers
NRM535	GTAGCCAAATTCATTGTCATACC	Reverse	
NRM537	CTATCATTCCCTCCCAACCA	Forward	Udorn H3N2 HA segment, qPCR primers
NRM538	TAAGCAAAACACCCTGAAGTTG	Reverse	
NRM540	CCCTTGGAGATACTTTGGTC	Forward	Udorn H3N2 NP segment, qPCR primers
NRM541	ATCCAGCACACAAGAGTCAG	Reverse	
NRM543	GTTCTTATCCATGATTGCTTGG	Forward	Udorn H3N2 NS1 segment, qPCR primers
NRM544	TGTCAAGGGACTGGTTCATG	Reverse	
NRM420	TAACCATGAAGACTATCATT	Reverse	Udorn HA vRNA amplification for RT step
NRM421	ACTGAGTGACATCAAAATCA	Reverse	Udorn NP vRNA amplification for RT step
NRM423	ACAAAGACATAATGGATTCC	Reverse	Udorn NS1 vRNA amplification for RT step
NRM749	AGTCCGATCTCTGAAGAAGTTTC	Forward	cat MxA, qPCR primers
NRM750	GCCGTACACTTGCAGGAT	Reverse	
NRM751	AGCTCAAGCAGCAGATATG	Forward	cat ISG-15, qPCR primers
NRM752	CACTGGGGTGTGAGTTCATA	Reverse	
NRM753	ATCCTGGAGGAAATCATGGA	Forward	cat IFN-b, qPCR primers
NRM754	CACAGACGCTGTACTCCTT	Reverse	
NRM874	GCCGAGGAGCTGTCGGCCTCCATCGCC CTGGAGCCCTTCAAGGAGCCGGTC	Forward	Site-directed mutagenesis primers for C13/16A RING mutations. Can be used for both Human and Gibbon TRIM25
NRM875	CTTGAAGGGCTCCAGGGCGATGGAGGC CGACAGCTCCTCGGCCAGGGGGCAC	Reverse	
NRM694	GGTGCTGGTGCTGGTGCTATGGCTTCT GGAATCCTGGTTAATG	Forward	Amplify Human TRIM5a from cDNA
NRM695	TCAAGAGCTTGGTGAGCACAGAG	Reverse	
NRM909	GATCGGCTTCGCGCAGATCAGGCATCC CTAAGAGGAAGGGGCAGC	Forward	Site-directed mutagenesis primers for R38/41A TRIM25 binding mutations in NS1 from influenza PR8 strain
NRM910	TCCTCTTAGGGATGCCTGATCTGCGCG AAGCCGATCAAGGAATGG	Reverse	
NRM951	GGTGCTGGTGCTGGTGCTTCCCTGAGC CAGGCCAGC	Forward	Amplify TRIM25 coiled-coil+Linker2 domain (aa190-469) from human (NRM951/952) or gibbon (NRM951/953) TRIM25 pLPCX plasmids
NRM952	CTATTTGTTGTGGGCGGTGTTGTAGTC	Reverse	
NRM953	CTATTTATTGTGGGCGGTGTTGTAGTC C	Reverse	
NRM923	TCCCTGCTTTCCCCGCTC	Forward	RIG-I exon 1 amplification. Primers flank the exon.
NRM924	CACCCTTAAGCAAGTCACTTCACC	Reverse	
NRM911	CACCGTTGCAGGCTGCGTCGCTGCT	Forward	These primers were annealed and cloned into pLentiCRISPRv2. Used to disrupt exon 1 of RIG-I in A549 RIG-I/TRIM25 double KO cells
NRM912	AAACAGCAGCGACGCAGCCTGCAAC	Reverse	

NRM913	CACCGGGATTATATCCGGAAGACCC	Forward	These primers were annealed and cloned into pLentiCRISPRv2. Used to disrupt exon 1 of RIG-I in 293T RIG-I KO cells and A549 RIG-I KO cells.
NRM914	AAACGGGTCTTCCGGATATAATCCC	Reverse	
NRM925	CCTGACGGCTTCAGGGACT	Forward	TRIM25 exon 1 amplificaton. Primers flank exon.
NRM926	CCCCTTTCTACTCTGACATTGGAG	Reverse	
NRM915	CACCGGCACGACAGCTCCTCGGCCA	Forward	These primers were annealed and cloned into pLentiCRISPRv2. Used to disrupt exon 1 of TRIM25 in A549 RIG-I/TRIM25 double KO cells
NRM916	AAACTGGCCGAGGAGCTGTCGTGCC	Reverse	
as717	ACCGTCAAGGCTGAGAACGG	Forward	human GAPDH, qPCR primers
as718	GTGGTGAAGACGCCAGTGGA	Reverse	
NRM772	GCCACCATGGCAGAGCTGTGCCCC	Forward	Amplify human/gibbon TRIM25 with N-term Kozak sequence and no stop codon
NRM773	CTTGGGGGAGCAGATGGAGA	Reverse	
NRM784	TTATCATTACTTGTACAGCTCGTCCATGC	Reverse	Used with NRM772 to amplify TRIM25-gfp constructs

Figure S1

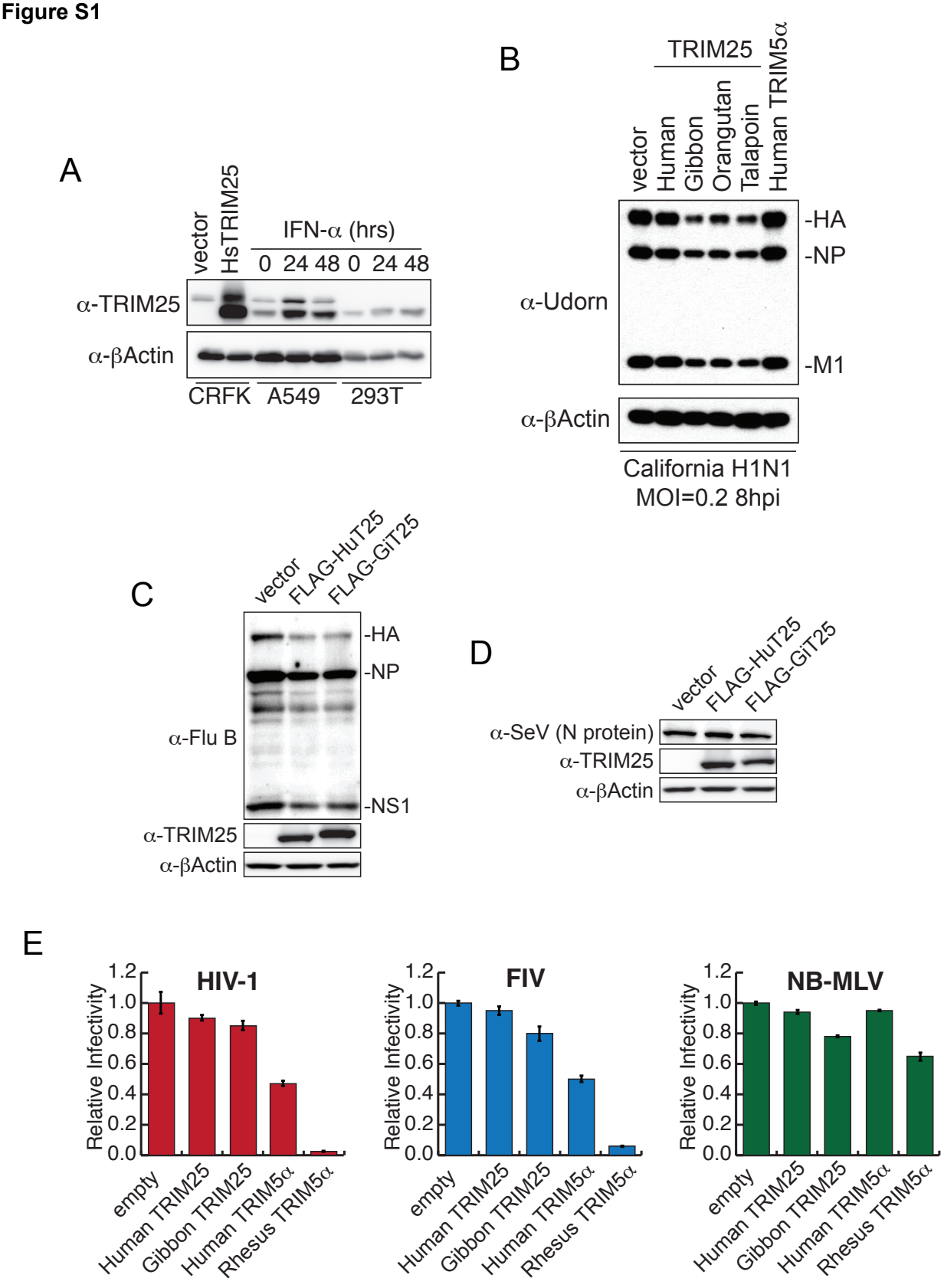

A
vector
HsTRIM25
IFN-α (hrs)
0 24 48 0 24 48
α-TRIM25
α-βActin
CRFK A549 293T
B
TRIM25
vector
Human
Gibbon
Orangutan
Talapoin
Human TRIM5α
-HA
-NP
-M1
α-Udorn
α-βActin
California H1N1
MOI=0.2 8hpi
C
vector
FLAG-HuT25
FLAG-GiT25
-HA
-NP
α-Flu B
-NS1
α-TRIM25
α-βActin
D
vector
FLAG-HuT25
FLAG-GiT25
α-SeV (N protein)
α-TRIM25
α-βActin
E
HIV-1
FIV
NB-MLV
Relative Infectivity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
empty
Human TRIM25
Gibbon TRIM25
Human TRIM5α
Rhesus TRIM5α

Figure S2

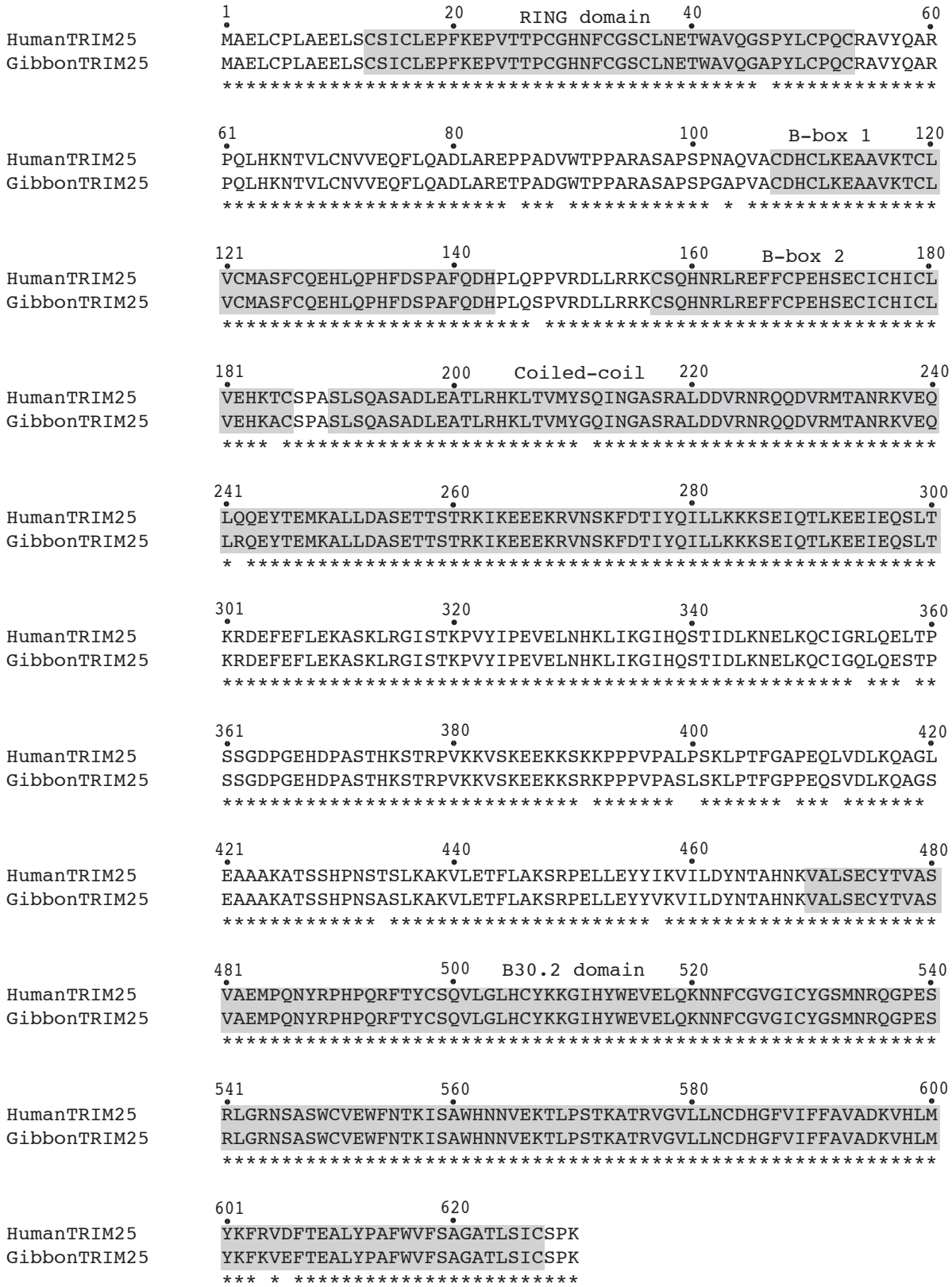

RING domain
B-box 1
B-box 2
Coiled-coil
B30.2 domain
HumanTRIM25
GibbonTRIM25

Figure S3

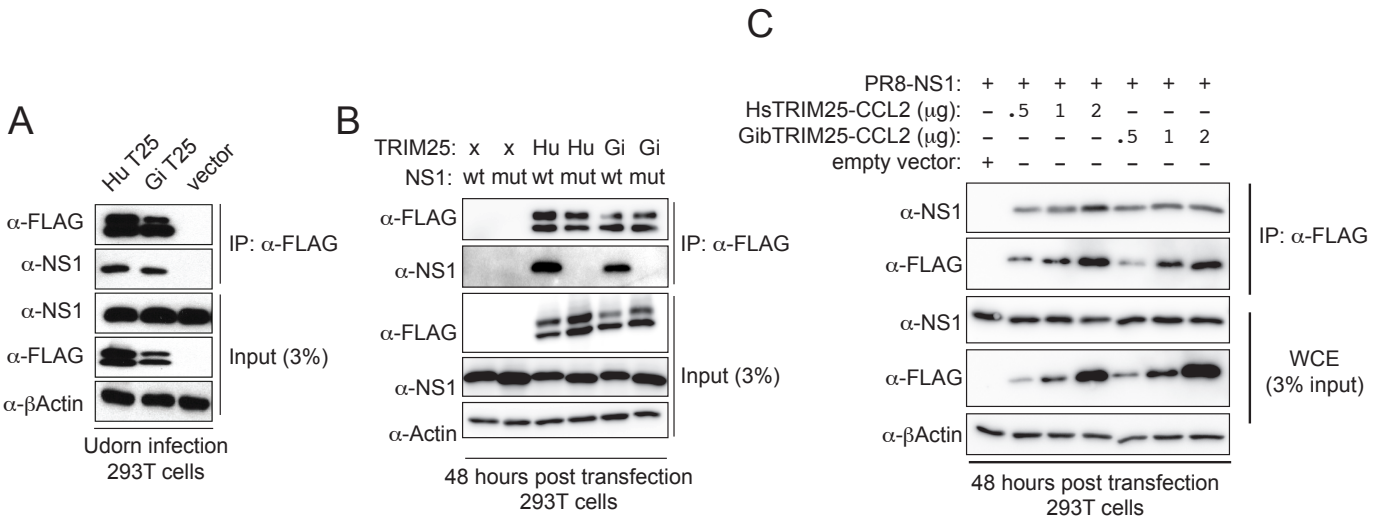

A
Hu T25
Gi T25
vector
α-FLAG
α-NS1
IP: α-FLAG
α-NS1
α-FLAG
α-βActin
Input (3%)
Udorn infection
293T cells
B
TRIM25: x x Hu Hu Gi Gi
NS1: wt mut wt mut wt mut
α-FLAG
α-NS1
IP: α-FLAG
α-FLAG
α-NS1
α-Actin
Input (3%)
48 hours post transfection
293T cells
C
PR8-NS1: + + + + + + +
HsTRIM25-CCL2 (μg): – .5 1 2 – – –
GibTRIM25-CCL2 (μg): – – – – .5 1 2
empty vector: + – – – – – –
α-NS1
α-FLAG
IP: α-FLAG
α-NS1
α-FLAG
α-βActin
WCE
(3% input)
48 hours post transfection
293T cells

Figure S4

A

A549 RIG-I KO, RIG-I Exon 1

DNA analysis

```
CopyA          ATGACCACCGTGCAGCGACGCAGCCTGCAAGCCTTCCAGGATTATATCCGG----CCCTGGACCCTACCTA...
CopyB          ATGACCACCGAGCAGCGACGCAGCCTGCAAGCCTTCCAGGATTATATCCGGAA--CCCTGGACCCTACCTA...
RIG-I exon 1   ATGACCACCGAGCAGCGACGCAGCCTGCAAGCCTTCCAGGATTATATCCGGAAGACCCTGGACCCTACCTA...
               ********** ****************************************    ***************
```

NCC PAM sequence

Amino acid analysis

```
CopyA   MTTEQRRSLQAFQDYIR-PWTLPTS*ATWPPGLGR
CopyB   MTTEQRRSLQAFQDYIR-NPGPYLHPELHGPLV*G
RIG-I   MTTEQRRSLQAFQDYIRKTLDPTYILSYMAPWFRE
        *****************             *
```

B

A549 RIG-I/TRIM25 KO, RIG-I Exon 1

DNA analysis

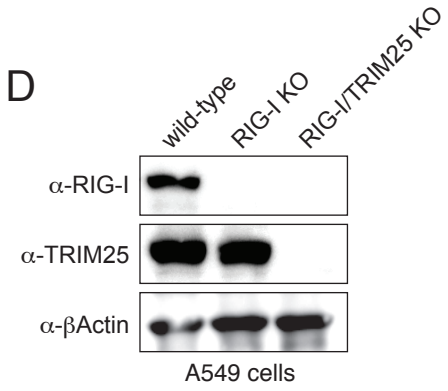
```
CopyA          ----------GCGACGCAG...
CopyB          ATGACCACCGAGCAAGCGACGCAG...
RIG-I exon 1   ATGACCACCGAGCA-GCGACGCAG...
                         *********
```

CCN PAM sequence

Amino acid analysis

```
CopyA   ---------------------------------- (no start codon)
CopyB   MTTEQATQPASLPGLYPEDPGPYLHPELHGPLV*G
RIG-I   MTTEQRRSLQAFQDYIRKTLDPTYILSYMAPWFRE
        *****                *
```

D



C

A549 RIG-I/TRIM25 KO, TRIM25 Exon 1

DNA analysis

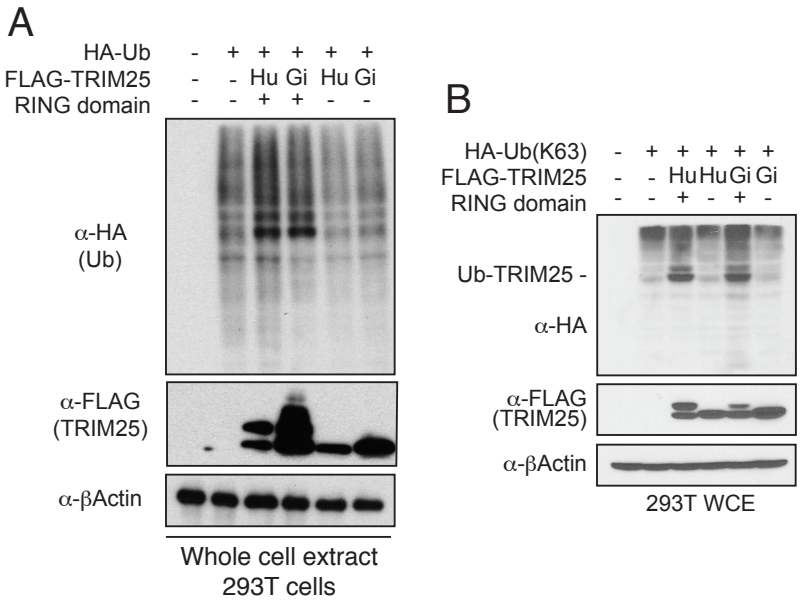
```
CopyA          ATGGCAGAGCTGTGCCCCCTGG-CGAGGAGCT...
CopyB          ATGGCAGAGCTGTGCCCCCTGG--GAGGAGCT...
TRIM25 exon 1  ATGGCAGAGCTGTGCCCCCTGGCCGAGGAGCT...
               ********************  ********
```

CCN PAM sequence

Amino acid analysis

```
CopyA   MAELCPLA-RSCRAPSAWSPSRSRSPLRAATTSAGRA*M
CopyB   MAELCPL-GGAVVLHLPGALQGAGHHSVRPQLLRVVPE*
TRIM25  MAELCPLAEELSCSICLEPFKEPVTTPCGHNFCGSCLNE
        *******
```

Figure S5



C

293T RIG-I KO, RIG-I Exon 1

DNA analysis

```
CopyA          ATGACCACCGTGCAGCGACGCAGCCTGCAAGCCTTCC----------------------TGGACCCTACCTACATCCTG
CopyB          ATGACCACCGAGCAGCGACGCAGCCTGCAAGCCTTCCAGGATTATATCCGGAAGA----------------------TCCTG
RIG-I exon 1   ATGACCACCGAGCAGCGACGCAGCCTGCAAGCCTTCCAGGATTATATCCGGAAGACCCTGGACCCTACCTACATCCTG
               ********** ************************                                          *****
```

NGG PAM sequence

Amino acid analysis

```
Copy A MTTVQRRSLQAF-------LDPTYILSYMAPWFRE
Copy B MTTEQRRSLQAFQDYIRK------ILSYMAPWFRE
RIG-I  MTTEQRRSLQAFQDYIRKTLDPTYILSYMAPWFRE
       *** ********           ***********
```

D

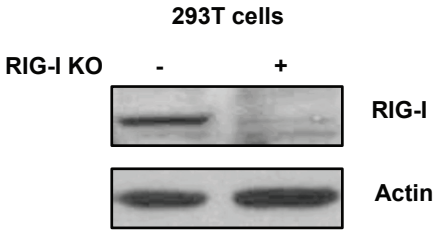


Figure S6

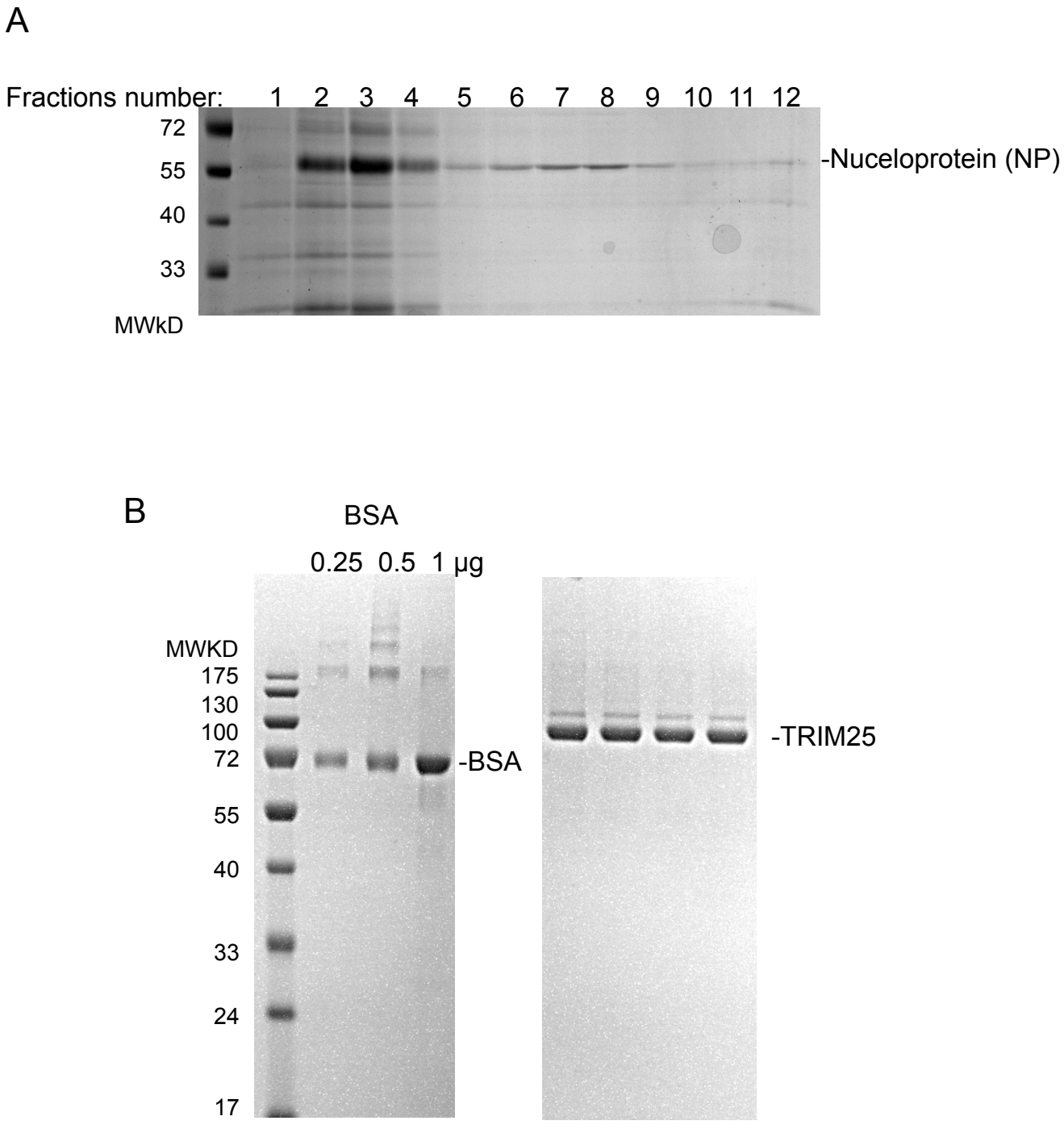
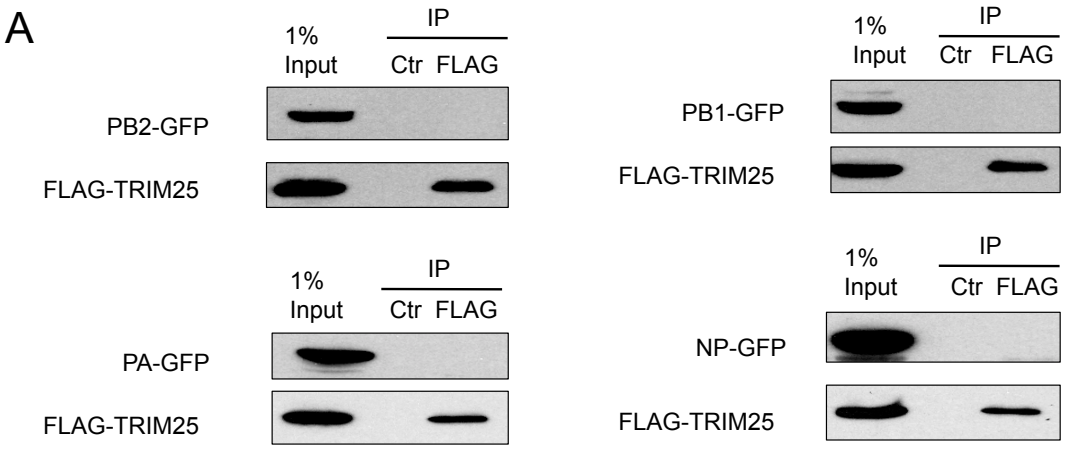
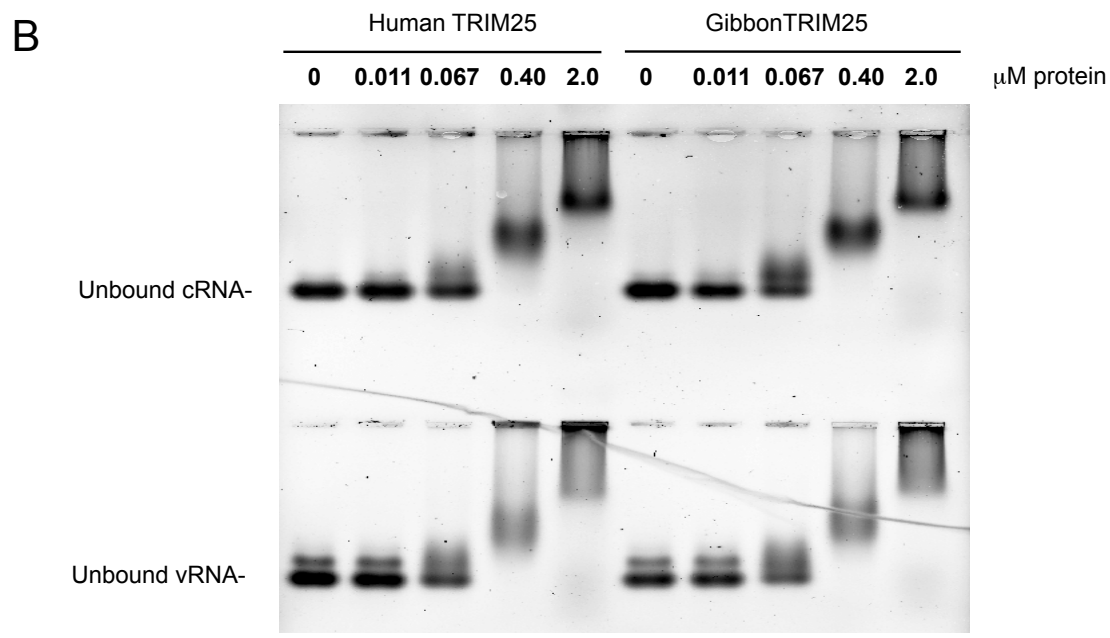

A
Fractions number: 1 2 3 4 5 6 7 8 9 10 11 12
72
55
40
33
MWkD
-Nucleoprotein (NP)
B
BSA
0.25 0.5 1 µg
MWKD
175
130
100
72
55
40
33
24
17
-BSA
-TRIM25

Figure S7





vRNA and cRNA: 50ng/20μl=9nM