SUPPLEMENTAL DATA

EXPERIMENTAL PROCEDURES

Cell Culture. The mouse embryonic fibroblasts (*TRIM25*+/+, *TRIM25*-/+, and *TRIM25*-/- MEF) were described elsewhere (Gack et al., 2007). HEK293T, MEF, and L929 cells were propagated in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin-streptomycin, and 15 mM HEPES (pH 7.2-7.5). MDCK cells were propagated in Minimal Essential Medium supplemented with 10% fetal bovine serum, L-Glutamine, 1% penicillin-streptomycin, 15 mM HEPES (pH 7.2-7.5), 1 mM sodium pyruvate, 0.1 mM non-essential amino acid solution (Invitrogen Corp., Carlsbad, CA). Transient transfections were performed with calcium phosphate (Clontech) or FuGENE 6 (Roche) according to the manufacturer's instructions.

Plasmids. The pCAGGS NS1 expression vector encoding A/Brevig Mission/1/1918 NS1 was described previously (Basler et al., 2001). The pCAGGS NS1 expression vectors encoding the A/Puerto Rico/8/1934 NS1 wild-type, R38A/K41A mutant NS1, NS1 (aa 1-73), and NS1 (74-230) were described elsewhere (Basler et al., 2001; Mibayashi et al., 2007; Talon et al., 2000). cDNA encoding A/Puerto Rico/8/1934 E96A/E97A, A/Vietnam/1203/2004, A/Hong Kong/156/1997, and A/Swine/Texas/4199-2/98 NS1 were cloned into *EcoR*I and *Xho*I restriction enzyme sites in pCAGGS according to methodology detailed elsewhere (Talon et al., 2000).

All constructs for the transient expression of RIG-I and TRIM25 proteins in mammalian cells were derived from pEBG GST fusion vector or pEF-IRES-Puro expression vector. DNA fragments corresponding to the coding sequence of the RIG-I and TRIM25 genes were amplified from template DNA by PCR and subcloned into plasmid pEBG between *Kpn*I and *Not*I or into pEF-IRES-Puro between *Afl*II and *Not*I. V5- or Flag-tagged TRIM25 and RIG-I constructs were expressed from a modified pIRES-Puro encoding a C-terminal V5 or Flag tag, respectively. RIG-I mutants were generated by PCR using site-directed mutagenesis or overlapping PCR. Furthermore, Ebola virus VP35 (Zaire strain) and vaccinia virus E3L were cloned into pEF-IRES-Puro-V5. All constructs were sequenced using an ABI PRISM 377 automatic DNA sequencer to verify 100% conformance with the original sequence.

Luciferase Reporter Assay. HEK293T cells were seeded into 6-well plates. 24 h later, the cells were transfected with an IFN-β luciferase construct together with constitutive β-gal-expressing pGK-β-gal. 36 h post-transfection, WCLs were prepared and subjected to a luciferase assay (Promega). Luciferase values were normalized to beta-galactosidase to measure transfection efficiency.

Confocal Immunofluorescence Microscopy. Eighteen to twenty four hours after transfection, HeLa cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% (v/v) Triton X-100 for 15 min, blocked with 10% goat serum in PBS for 1 h and reacted with diluted primary antibody in PBS with 1% goat serum for up to 2 h at room temperature. After incubation, cells were washed extensively with PBS, incubated

with the appropriate secondary antibody diluted in PBS for 1 h at room temperature and washed three times with PBS. Confocal microscopy was performed using a Leica TCS SP laser-scanning microscope (Leica Microsystems) fitted with a 100× Leica objective (PL APO, 1.4NA) and Leica imaging software. Images were collected at 512 × 512–pixel resolution. The stained cells were optically sectioned in the *z* axis, and the images in the different channels (photo multiplier tubes) were collected simultaneously. The step size in the *z* axis varied from 0.2 to 0.8 μm to obtain 8 slices per imaged file. The images were transferred to a Macintosh G4 computer (Apple Computer, CA), and Photoshop (Adobe) was used to render the images.

Native PAGE. Native PAGE was performed using a 7.5 % acrylamide gel (Biorad). The gel was pre-run with 25 mM Tris and 192 mM glycine [pH 8.4] with or without 0.7% deoxycholate in the cathode and anode chamber, respectively, for 30 min at 30 mA. Samples in the native sample buffer (62.5 mM Tris-HCl [pH 6.8], 15 % glycerol) were applied on the gel and electrophoresed for 60-80 min at 10 mA followed by immunoblotting.

SUPPLEMENTARY FIGURE LEGENDS

Suppl. Fig. 1. TRIM25-NS1 interaction. **(A)** At 48 h post-transfection with NS1 together with vector, TRIM25-V5 or ΔCCD TRIM25-V5, HEK293T WCLs were used for IP with α-V5 followed by IB with α-NS1 or α-V5. Expression of NS1 proteins in the WCLs was tested by α-NS1 immunoblotting. **(B)** After transfection of HEK293T with vector or TRIM25-V5 together with NS1 of the indicated Influenza A strains, WCLs were subjected to immunoprecipitation (IP) with α-V5 followed by IB with α-NS1 or α-V5. NS1 protein expression was determined in the WCLs by IB with α-NS1. **(C)** HEK293T cells were transfected with vector or TRIM25-V5 together with HA-tagged NS1, NS1 RNA-binding domain (RBD) or NS1 effector domain (ED). Whole cell lysates (WCLs) were subjected to IP with α-V5 followed by IB with α-HA or α-V5. WCLs were used for IB with α-HA to show the expression of NS1 proteins. Lc: light chain.

Suppl. Fig. 2. Precipitation of NS1 with TRIM25 or poly (I)-poly (C).

(A) The recombinant fusion proteins maltose-binding protein (MBP)-TRIM25-Flag, GST, and GST-WT NS1 were purified from bacteria. Purified MBP-TRIM25-Flag fusion protein was incubated with either purified GST or purified GST-WT NS1. Coomassie staining of anti-Flag M1 affinity gel precipitates resolved by SDS-PAGE revealed that MBP-TRIM25-Flag co-precipitated with GST-WT NS1. The arrows indicate positions of MBP-TRIM25-Flag (full-length and main degradation product), GST-WT NS1, and light chain from the anti-Flag M1 affinity gel. **(B)** WT and E96A/E97A NS1, but not R38A/K41A proteins are precipitated by Sepharose-poly (I)-poly (C) resin from total cell

lysates prepared from influenza virus infected A549 cells. Precipitated proteins were resolved by SDS-PAGE and NS1 proteins detected by IB with α-NS1.

Suppl. Fig. 3. E96A/E97A and R38A/K41A NS1 mutants do not co-localize with TRIM25. HeLa cells were transfected with NS1 E96A/E97A alone, NS1 R38A/K41A alone, NS1 E96A/E97A together with TRIM25-V5, or NS1 R38A/K41A together with TRIM25-V5. At 20 h post-transfection, cells were stained with α-NS1 (green), α-V5 (red) and Hoechst 33256 (nucleus, blue).

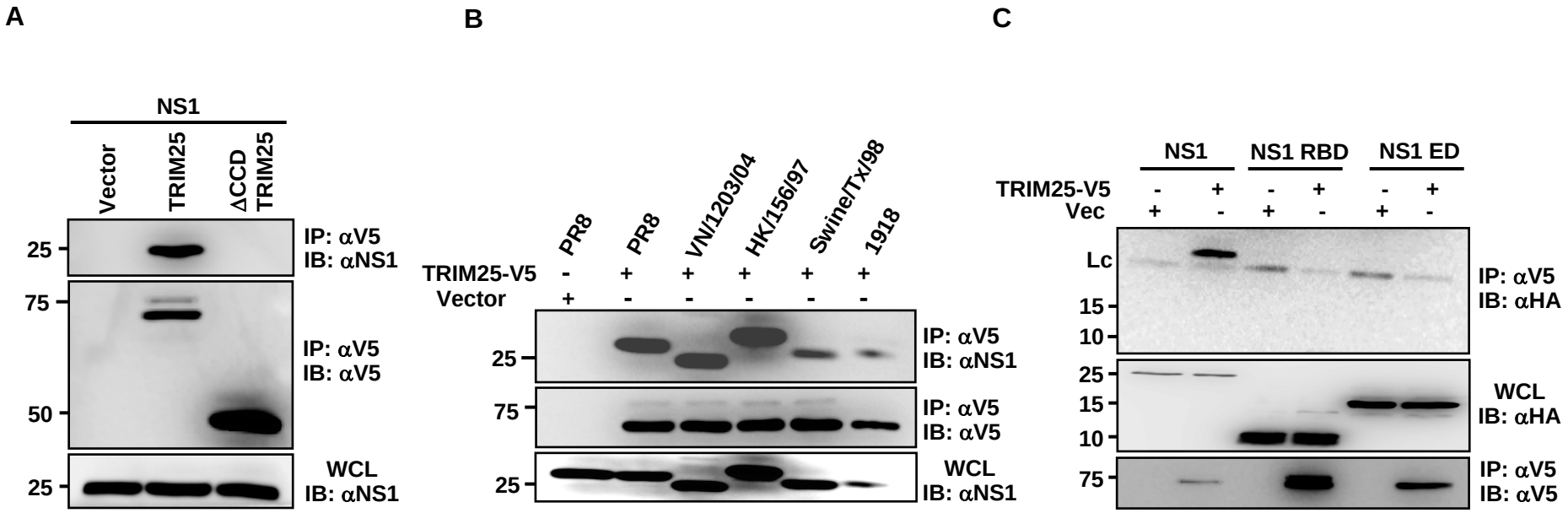
Suppl. Fig. 4. WT NS1, but not R38A/K41A and E96A/E97A NS1 mutants interact with RIG-I. HEK293T cells were transfected with empty vector or RIG-I-Flag together with NS1 WT, NS1 E96A/E97A or NS1 R38A/K41A. At 48 h posttransfection, WCLs were subjected to IP with α-Flag, followed by IB with α-NS1 or α-Flag. The expression of NS1 proteins was determined by IB with α-NS1.

Suppl. Fig. 5. TRIM25 overexpression overcomes RIG-I inhibition by NS1. HEK293T cells were transfected with GST, GST-RIG-I 2CARD, or GST-RIG-I 2CARD and NS1 together with increasing amounts of TRIM25-V5. In addition, IFN-β-luciferase and pGK-β-gal were transfected. Luciferase and β-galactosidase values were determined as previously described (Gack et al., 2007). Data represent the mean $\pm$ SD (n=3). The expression of TRIM25 and NS1 was further determined by IB with α-V5 or α-NS1. Anti-actin immunoblotting was used as loading control.

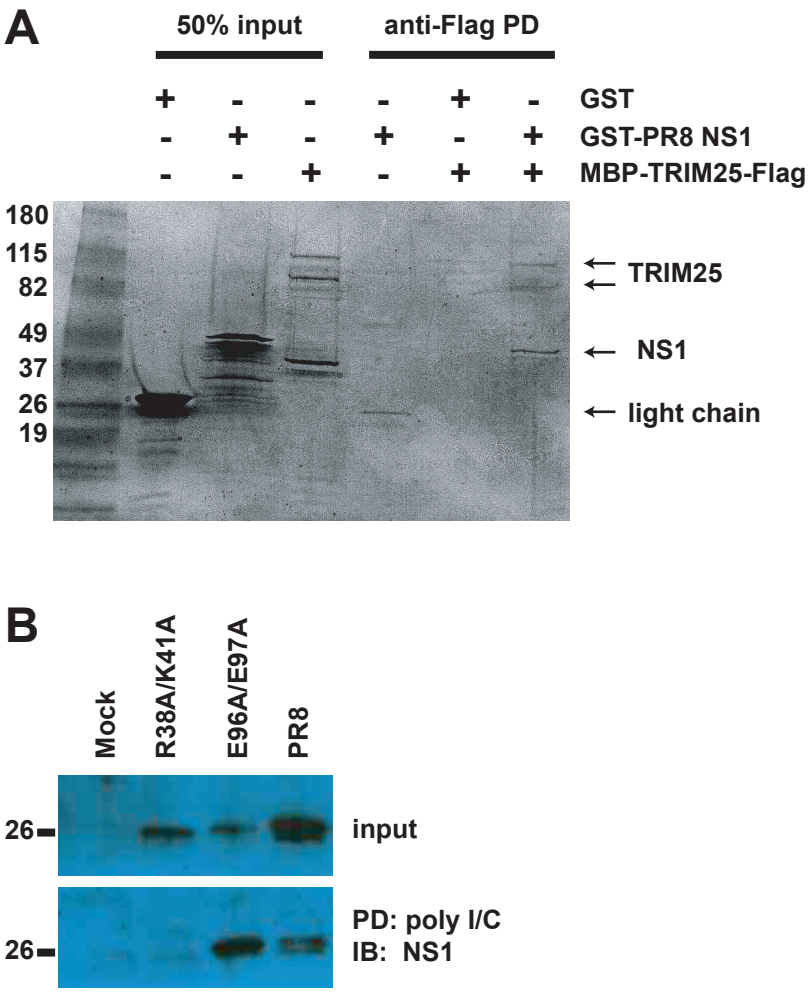
Suppl. Fig. 6. CCD-dependent TRIM25 multimerization is critical for its ubiquitin E3 ligase activity. (A) After transfection of HEK293T cells with TRIM25-Flag together with vector or the indicated V5-tagged TRIM25 constructs, WCLs were subjected to IP with α-V5 followed by IB with α-Flag or α-V5. TRIM25-Flag expression was determined in the WCLs by IB with α-Flag. **(B)** HEK293T were transfected with GST or GST-RIG-I 2CARD together with vector, TRIM25-V5 or ΔCCD-TRIM25-V5. WCLs were subjected to GST-PD followed by IB with α-GST. Expression of TRIM25 proteins in the WCLs was determined by IB with α-V5. Arrows indicate the ubiquitinated bands.

Suppl. Fig. 7. (A) Influenza A NS1 inhibits TRIM25 oligomerization. After transfection with vector or V5-TRIM25 B-boxes/CCD together with increasing amounts of NS1, HEK293T WCLs were used for IP with α-V5 followed by IB with α-TRIM25 or α-V5. WCLs were subjected to IB with α-NS1 or α-TRIM25 to determine NS1 and endogenous TRIM25 protein. **(B) Influenza A NS1 does not affect TRIM25 expression.** WCLs of HEK293T cells transfected with TRIM25-V5 and WT NS1 were subjected to IB with α-V5 or α-NS1. Anti-actin immunoblotting was used as loading control.

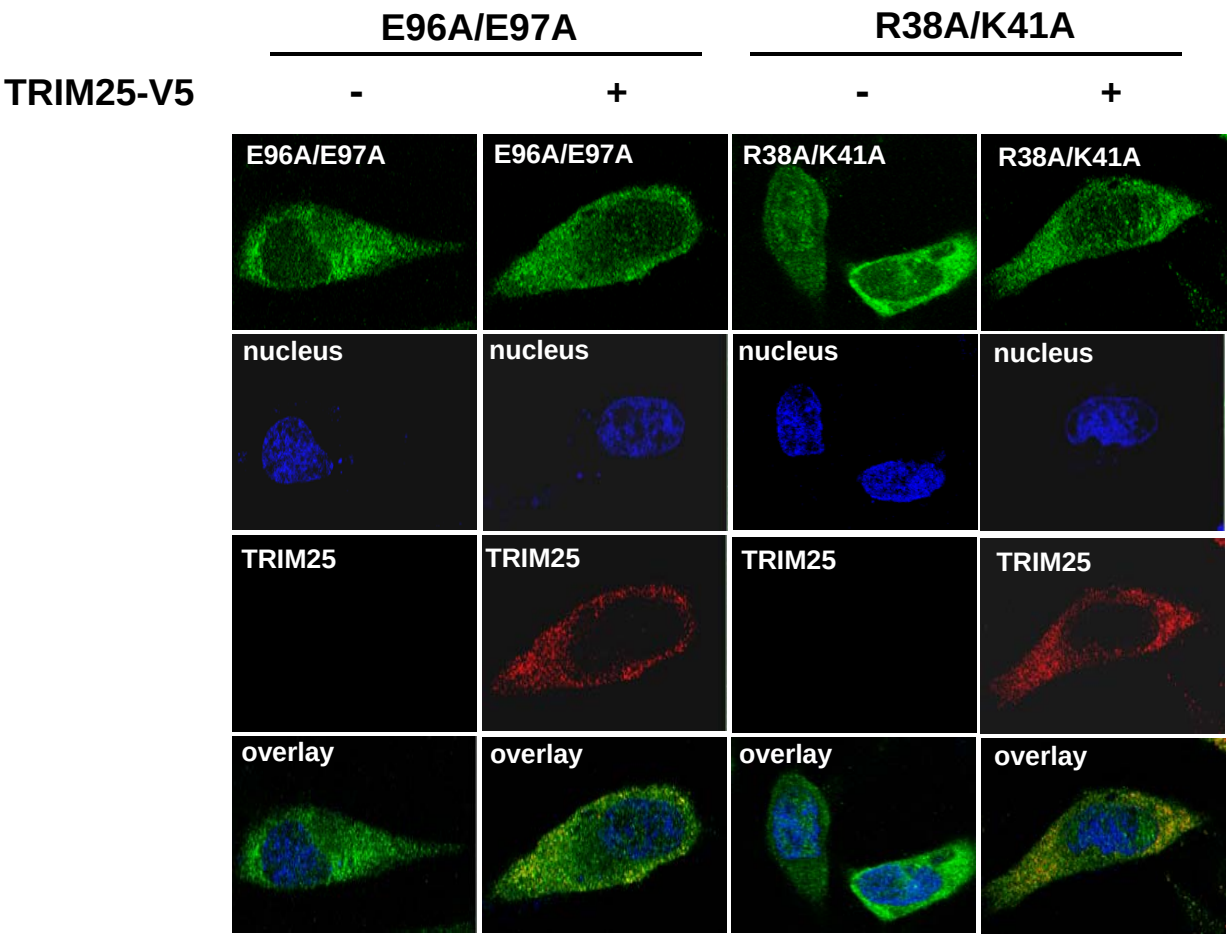
Suppl. Fig. 8. Interferon production from influenza-infected A549 cells by bioassay. Supernatants from A549 cells infected with the indicated influenza viruses were exposed to UV-light to inactivate infectious virus. Vero cells were incubated with 10-fold dilutions of the A549 supernatants prior to infection with NDV-GFP. The fluorescence micrographs depicted GFP expression resulting from NDV-GFP replication in the treated Vero cells.



Supplementary Fig. 1



Supplementary Fig. 2



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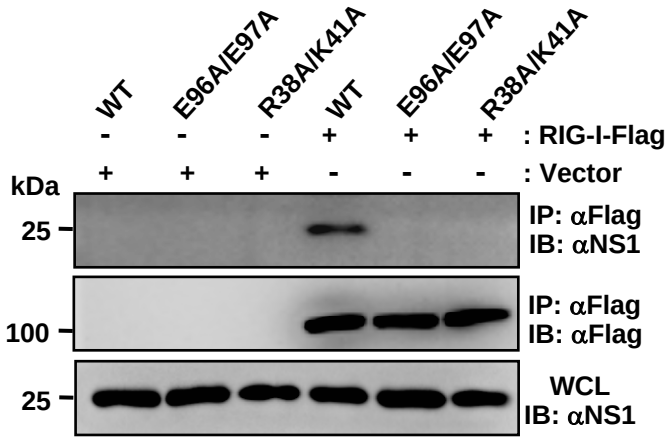

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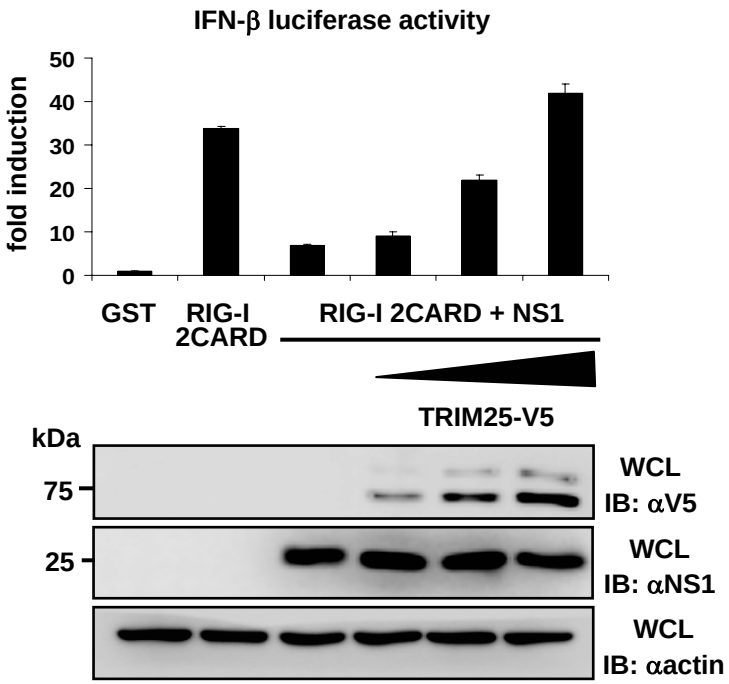
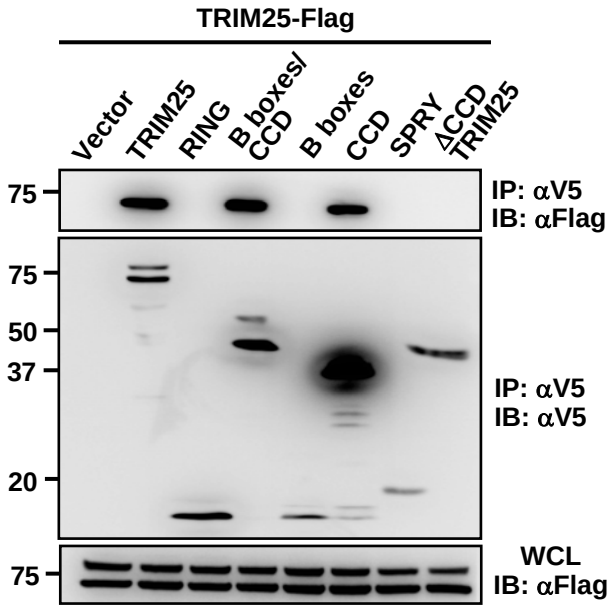


Supplementary Fig. 5

A



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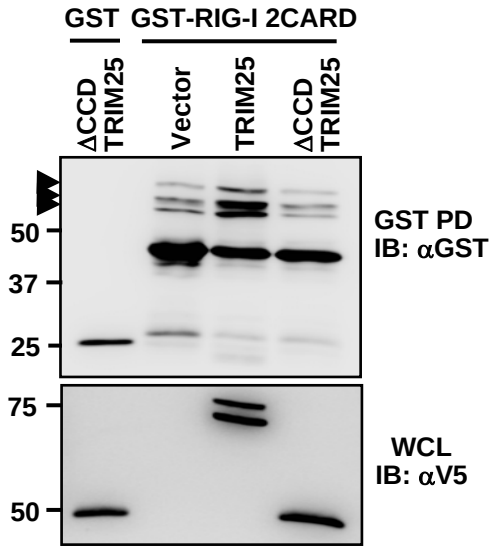

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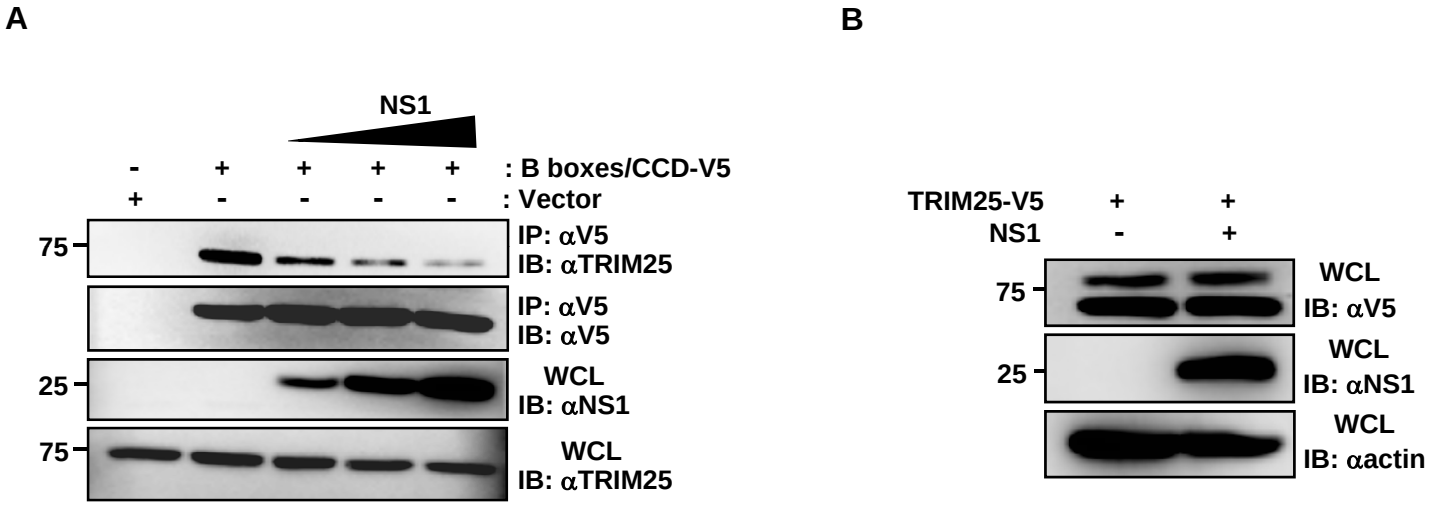

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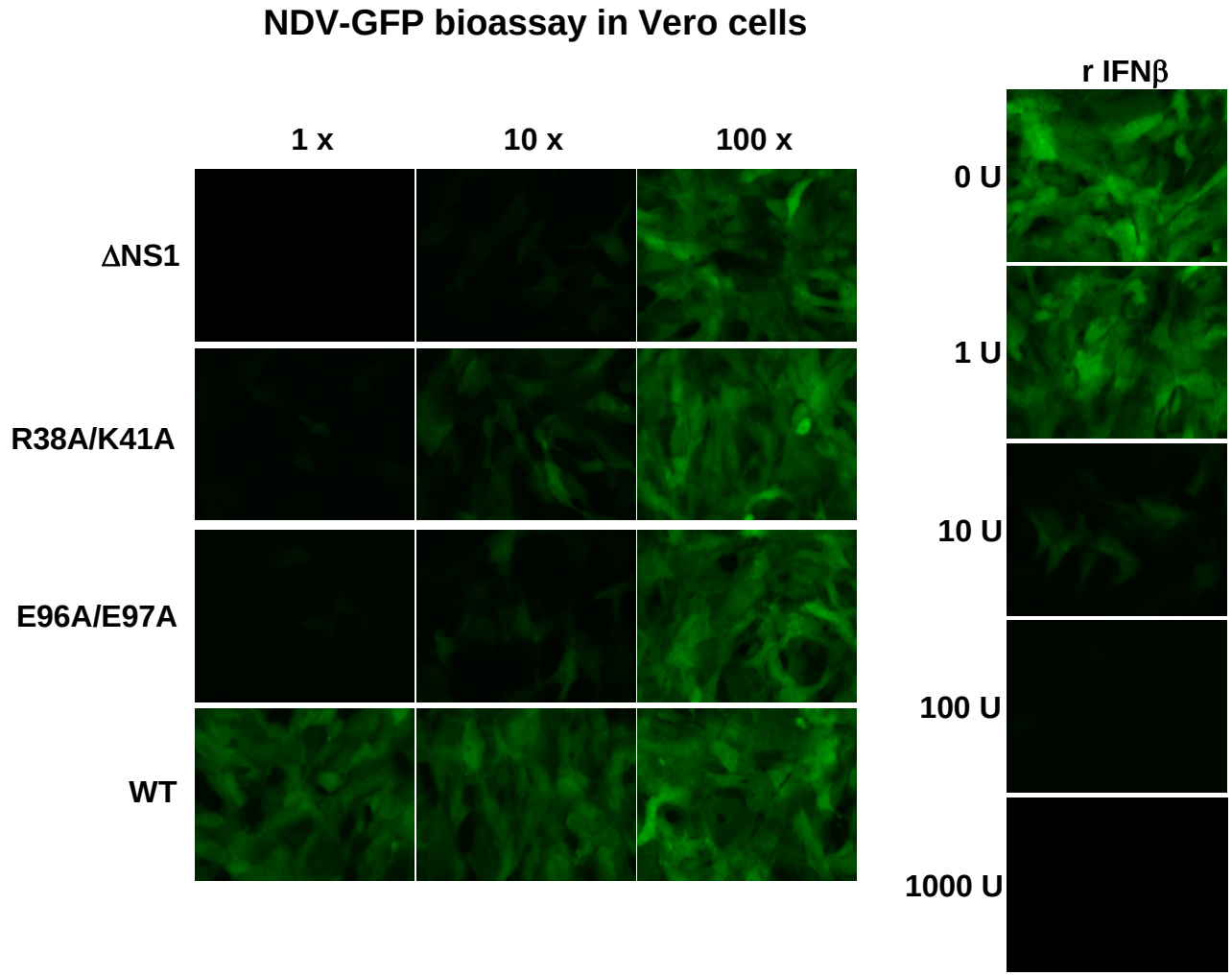

Supplementary Fig. 8