Supplementary Figure Legends

Supplementary Figure 1 (Related to Fgure 1)

(a) Membrane association of RIG-I in SenV-infected 293 cells. Mock-infected or SenV-infected 293 cell extracts were fractionated by sucrose gradient. Each fraction was immunoblotted for RIG-I and TRIM25 to assess their membrane associations. (b) Top: Relocalization of YFP-RIG-I (green) expressed in PH5CH8 cells during SenV infection. Bottom: Immunoblot assay of phospho-IRF-3 (p-IRF-3), total IRF-3 and IFIT1 protein level in PH5CH8 cells after 0 (mock-infected), 4, and 24 hr SenV infection. (c) Detection of *in vitro* transcribed 5'ppp Biotin-UTP labelled HCV poly-U/UC PAMP motif (PAMP) and X region motif (X) RNA. Left: input; X region motif RNA was slightly less labeled due to the nucleotide composition. Right: output product within membrane flotation gradient fractions 2, 4 or 6 recovered from transfected Huh7 cells shown in Figure 1e and 1f. RNA was detected by HRP-conjuaged anti-strepavidin antibody.

Supplementary Figure 2 (Related to Figure 2)

(a) Membrane association of TRIM25 in Huh7 and Huh7.5 cells. Mock-infected or SenV-infected Huh7 (top panels) and Huh7.5 (bottom panels) cell extracts were fractionated by sucrose gradient to determine TRIM25 membrane-association. (b) Loss of IRF-3 activation in TRIM25-knockdown (TRIM25 k/d) Huh7 cells during SenV infection. Mock-infected and SenV-infected Huh7 or TRIM25 k/d cell lysates were subject to immunoblot asasy for phospho-IRF-3 (p-IRF-3), total IRF-3, TRIM25. Tubulin levels were monitored as a loading control.

Supplementary Fig. 3 (Related to Figure 3)

(a) TRIM25 interacts with isoforms of 14-3-3 proteins. Panels show anti-Myc immunoblot of products recovered from anti-Flag immunoprecipitation of extracts from 293 cells expressing Flag-TRIM25/Myc-14-3-3ε, Flag-TRIM25/Myc-14-3-3η, or Flag-TRIM25/Myc-14-3-3σ after mock-infection of infection with SenV. Arrows indicate Myc-14-3-3ε . Lysate refers to

input protein. Tubulin levels were monitored for input control. (b) Immunoblot assay of phospho-IRF-3 and total IRF-3 protein levels in 14-3-3σ-knockdown, 14-3-3ε-knockdown, and NT control Huh7 cells after 0 (mock-infected), 4, and 24 hr SenV infection. (c) Loss of SenV-induced RIG-I translocation in 14-3-3ε-knockdown cells. Cellular distribution of YFP-RIG-I (green) and tubulin (blue) was monitored by fluorescence microscopy in NT control cells (see Fig. 2) and 14-3-3ε-knockdown cells (shown) that were mock-infected or infected with SenV for 4 hr. Graphs show the levels of YFP-RIG-I and tubulin present along the vector marked in each micrograph from nucleus to plasma membrane.

Supplementary Fig. 4 (Related to Figure 4)

(a) IFN-β promoter activity in 293 cells with transient expression of NT-control, 14-3-3σ, 14-3-3ε, or TRIM25shRNA. Luciferase activities were measured 24 hr after N-RIG (constitutively active) expression construct or vector control transfection. ε2 and ε3 denote shRNAs targeting different sequences of 14-3-3ε. Bottom panel shows immunoblot of protein levels in transfected 293 cells. (b-d) IFN-β promoter activity in NT control, MAVS-knockdown, 14-3-3σ-knockdown, 14-3-3ε-knockdown or TRIM25-knockdown stable Huh7 cells 24 hr after (b) N-RIG, (c) MAVS-SCR (scrambled at shRNA targeting sequence), or (d) IRF-3-5D, in comparison with the vector control. Error bars represent +/- standard deviation. *, p value <0.05; **, p value <0.01.

Supplementary Experiment Procedures.

Plasmids and constructs. Flag-tagged RIG-I constructs have been described (Saito et al., 2007). pRK5-HA-ubiquitin constructs were obtained from Addgene (Addgene plasmid 17603, 17605, and17606). 14-3-3ε, 14-4-3σ, and TRIM25 expression constructs were generated by cloning specific PCR products produced from HEK293(293) cell cDNA into pCDNA3.1 or pEF-Tak(Saito et al., 2007). TRIM25-targeting shRNA constructs were

obtained from Addgene (Addgene Plasmid 12449 and 12450). shRNA construct targeting 14-3-3ε and 14-4-3σ were purchased from Sigma-Aldrich (SHCLNG-NM_006761, SHCLNG-NM_006142).

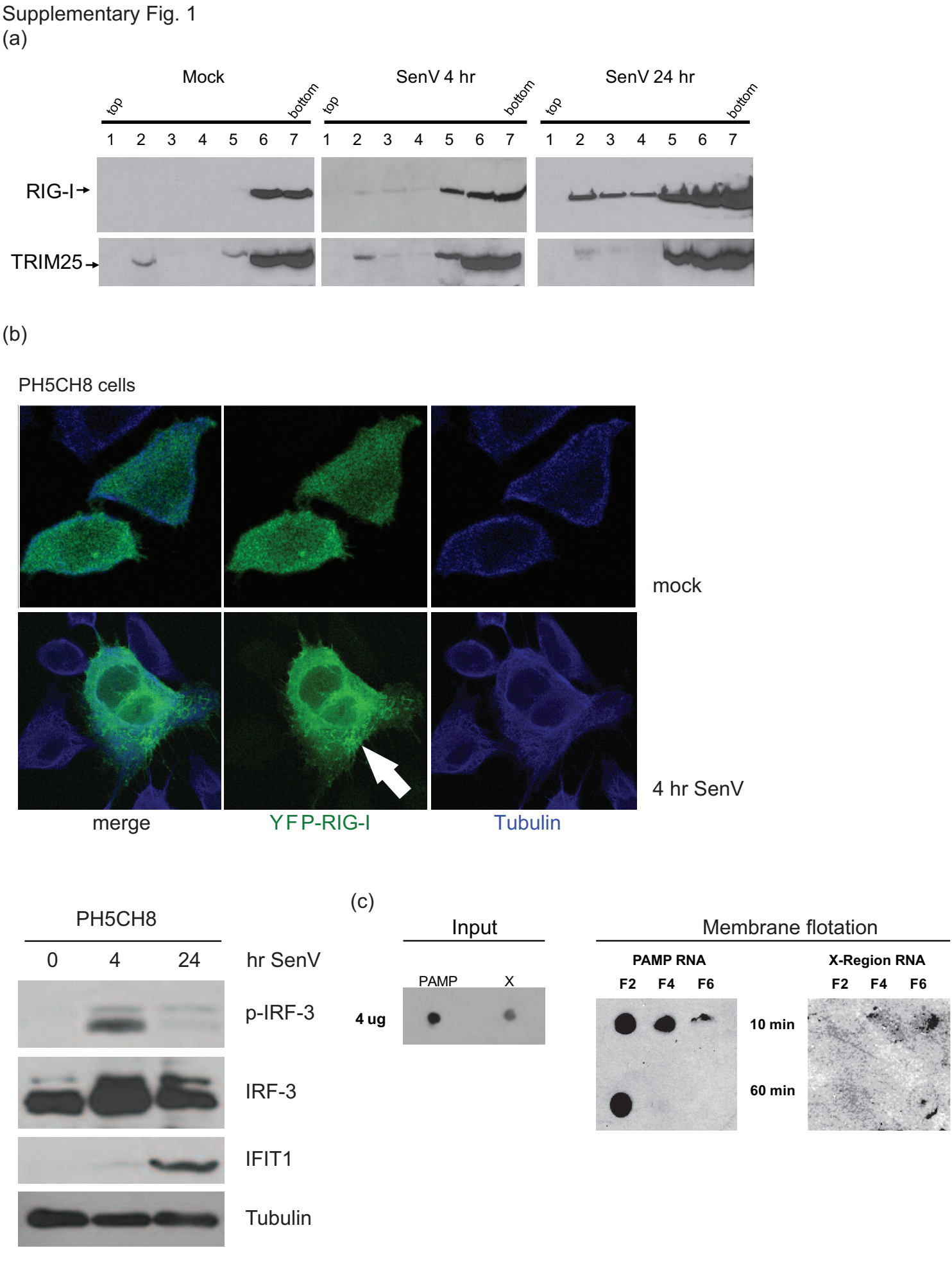
RIG-I intracellular distribution. Huh7 cells were transfected with YFP-RIG-I for 24 hr and then infected with SenV for 2 to 24 hours. Cells were incubated in complete media containing 1 μg/mL CM-DiI for plasma membrane staining and then fixed in 4% formaldehyde in PBS. In some experiments, cells were immunostained with anti-tubulin. Cells with comparable level of YFP-RIG-I expression were selected for fluorescence intensity quantification of YFP-RIG-I and CM-DiI staining or tubulin staining using Nikon NIS-Elements software. Relocalization index was calculated by the ratio of YFP fluorescence intensity in the perinuclear region to the non-perinuclear region. The vector was drawn from the nuclear membrane to the plasma membrane. Perinuclear region and non-perinuclear region were determined from the mid-point of the vector to the nuclear membrane or to the plasma membrane, respectively.

Immunoprecipitation. Cells were lysed in ice-cold RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 5mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of Protease Inhibitor Cocktail (Roche) for 10 min. Lysates were clarified by centrifugation and incubated with 2ug of antibodies for 16 hours followed by Protein A/G agarose for 1 hr at 4° C. The immunocomplexes were washed 3-times with cold RIPA buffer and resuspended in 15ul of 2X SDS sample buffer for SDS-PAGE.

Luciferase reporter assay. Dual luciferase assays to measure Interferon β promoter activity were conducted as described (Saito et al., 2008) .

Statistical analysis. Data were compared using the Student's t-test.

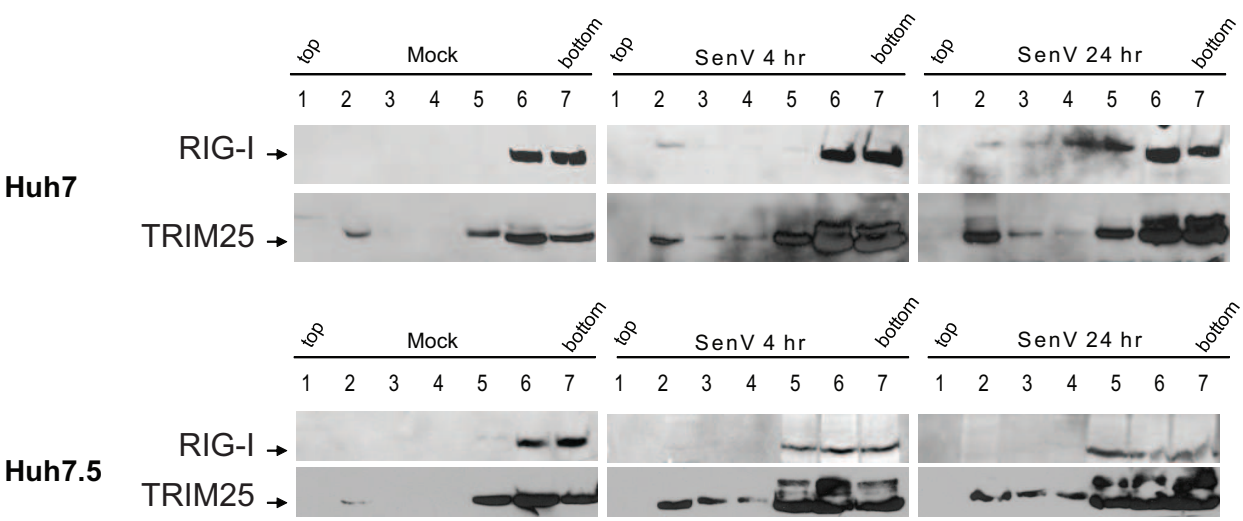
Supplementary Figure 1



Supplementary Figure 2

Supplementary Fig.2

(a)



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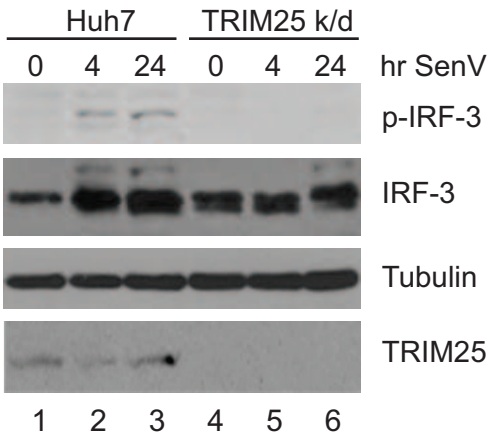
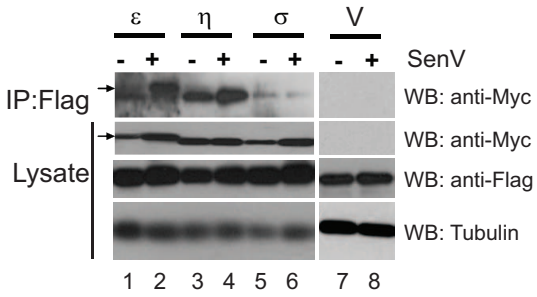


Supplementary Figure 3

Supplementary Fig. 3

(a)



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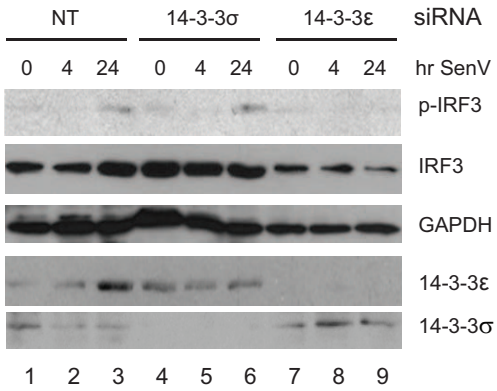
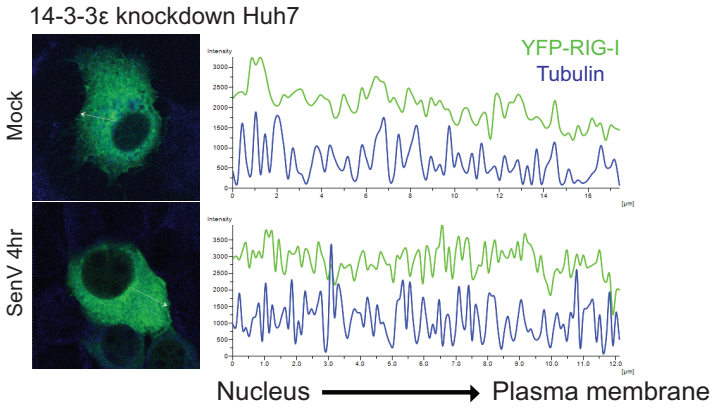
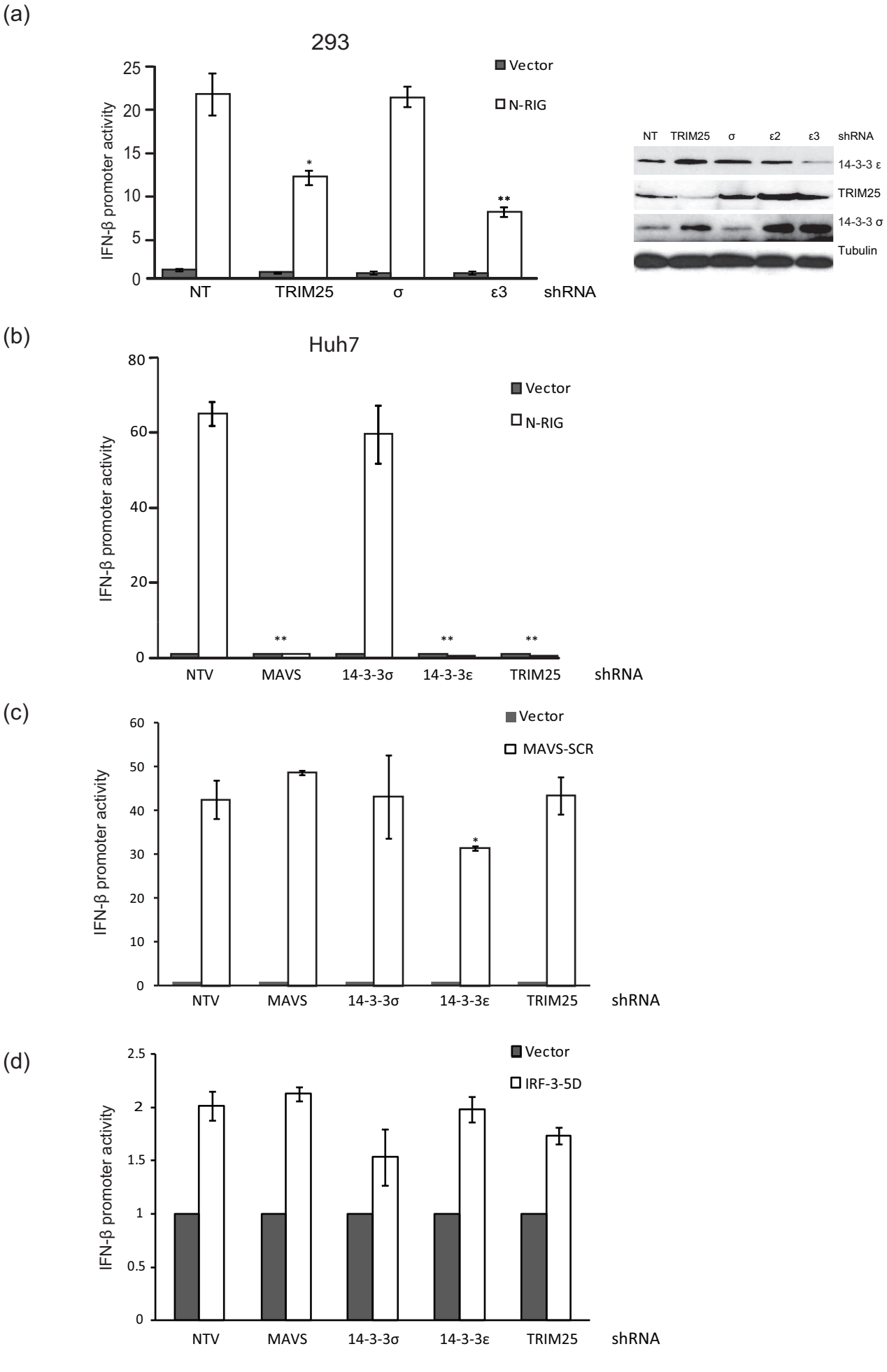


(c)



Supplementary Figure 4

Supplementary Fig. 4



Supplementary Table 1.
Sequences of 14-3-3 peptides identified by MS (IP with Flag-N-RIG):

Peptide sequence	Length (aa)	target
DSTLIMQLLR	10	Common to most 14-3-3 isoforms
AAFDDAIAELDTLSEESYK	19	14-3-3 epsilon
AASDIAMTELPPTHPIR	17	14-3-3 epsilon