Appendix File Table of Contents

Appendix Figure S1 …… 2

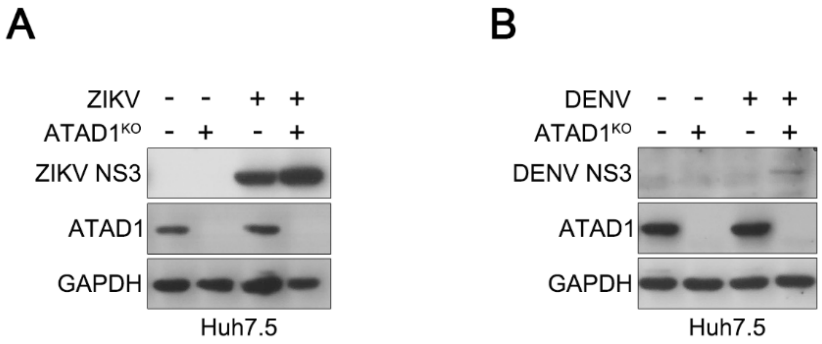
Appendix Figure S2…… 3

Appendix Figure S3…… 4

Appendix Figure S4…… 5

Appendix Figure S5…… 7

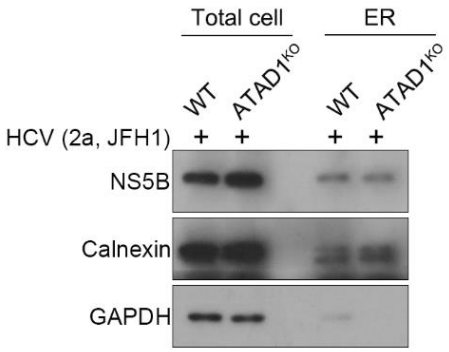
Appendix Figure S1



Appendix Figure S1. Knockout of ATAD1 slightly enhanced ZIKV and DENV infections in Huh7.5 cells.

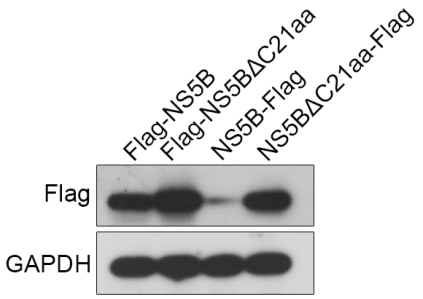
(A and **B)** WT and ATAD1^{KO} Huh7.5 cells were infected with ZIKV (A) or DENV (B) for 36 hours, and then the cells were harvested and analyzed by western blotting with anti-NS3 and anti-ATAD1 antibodies.

Appendix Figure S2



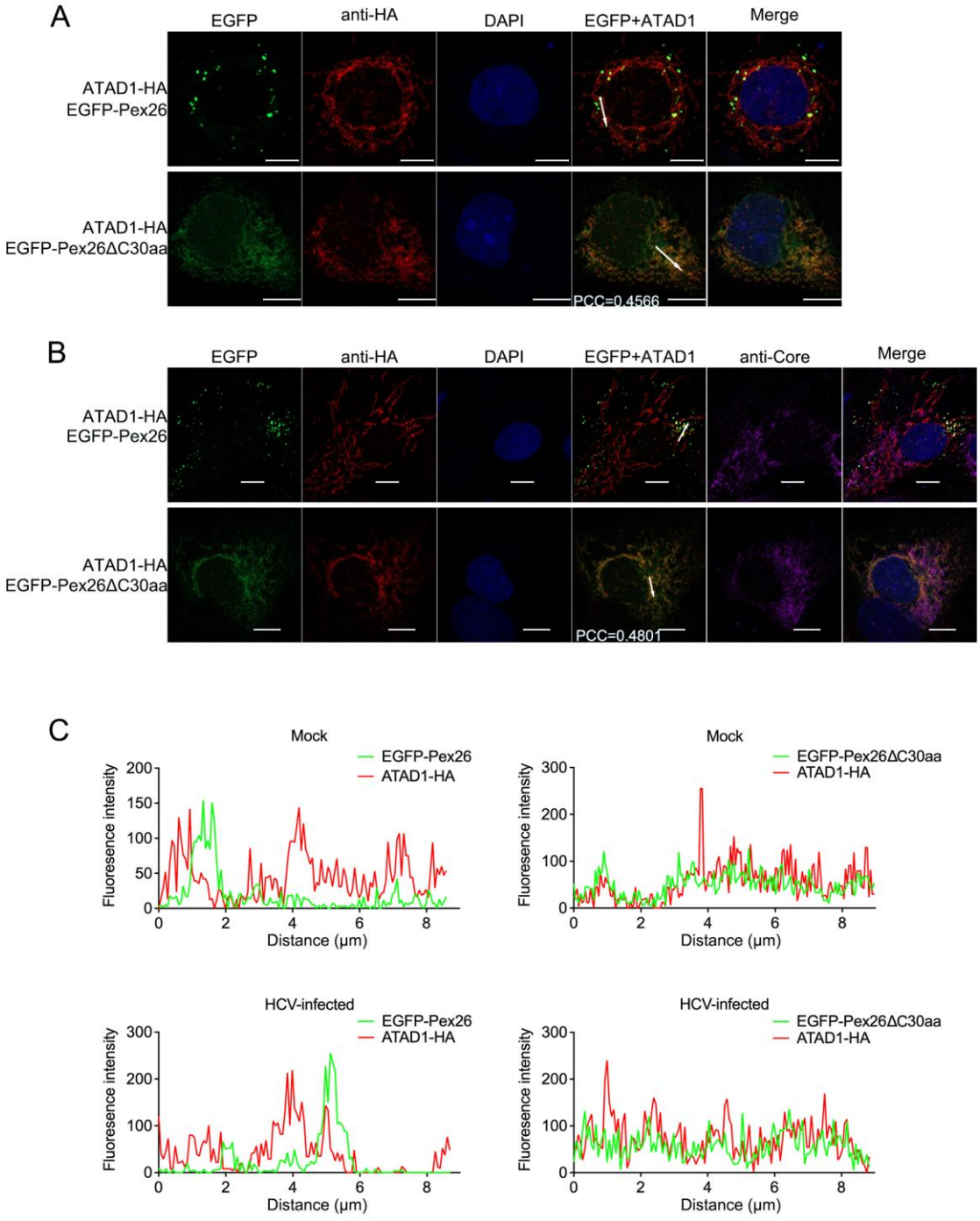
Appendix Figure S2. The co-localization of NS5B with ER in HCV-infected cells.
WT and ATAD1^{KO} cells were infected with JFH1 for 72 hours, and then the cells were harvested and isolated for ER using the Minute^{TM} ER enrichment Kit (Invent Biotechnologies, ER-036). Equal amounts of protein (2.7 μg) were analyzed by western blotting with anti-NS5B and anti-Calnexin antibodies.

Appendix Figure S3



Appendix Figure S3. The mobility on SDS-PAGE of full length NS5B and NS5BΔC21aa. 293T cells were transfected with plasmids expressing NS5B and NS5BΔC21aa, with Flag-tag at the N-terminus (Flag-NS5B and Flag-NS5BΔC21aa) or at the C-terminus (NS5B-Flag and NS5BΔC21aa-Flag) for 24 hours, and then the cells were harvested and analyzed by western blotting with anti-Flag antibody.

Appendix Figure S4



Appendix Figure S4. HCV infection did not apparently affect the function of ATAD1 in terms of its interaction with the mitochondria-targeting TA-protein Pex26 mutant.

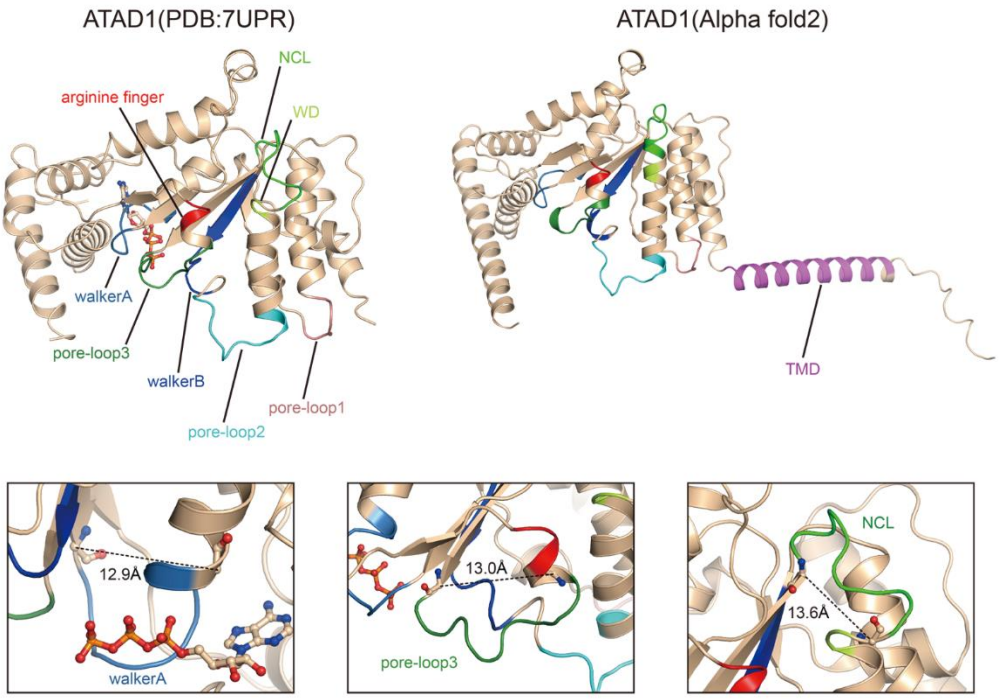
(A) Huh7.5 cells were co-transfected with EGFP-Pex26 or EGFP- Pex26ΔC30aa with ATAD1-HA for 24 hours. The cells were fixed and blotted with rabbit primary antibody anti-HA for 2 hours at room temperature, followed by incubation with Goat anti-Rabbit conjugated IgG (H+L) Highly

Cross-Adsorbed Secondary Antibody Alexa Fluor® 647 conjugate for 1 hour at room temperature. Immunostaining of ATAD1 was in red color, while nuclei were stained with Hoechst in blue color. Pex26 and Pex26ΔC30aa were visualized by EGFP (green). The PCC between Pex26ΔC30aa and ATAD1 were shown in the image using white color. The direction of arrow corresponds to the horizontal coordinate in ***panel C***. Scale bars, 10 μm.

(**B**) Huh7.5 cells were infected with JFH1 for 24 hours, and then co-transfected with EGFP-Pex26 or EGFP- Pex26ΔC30aa with ATAD1-HA for 24 hours. The cells were fixed and blotted with rabbit primary antibody anti-HA (red) and mouse primary antibody anti-Core (purple) for 2 hours at room temperature. Secondary antibody Goat anti-Rabbit conjugated IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor® 647 conjugate (HA, red) and Goat anti-Mouse conjugated IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor® 568 conjugate (Core, purple) were incubated for 1 hour at room temperature. Nuclei were stained with Hoechst (blue). Pex26 and Pex26ΔC30aa were visualized by EGFP (green). The PCC between Pex26ΔC30aa and ATAD1 were shown in the image using white color. The direction of arrow corresponds to the horizontal coordinate in ***panel C***. Scale bars, 10 μm.

(**C**) Distant colocalization analysis of Pex26 or Pex26ΔC30aa and ATAD1 in ***panels A*** and ***B*** were performed using Zen and processed by GraphPad Prism.

Appendix Figure S5



Appendix Figure S5. **The predicted structures of ATAD1 mutants.**
According to the parsed ATAD1 (PDB: 7UPR) and the TMD structure model predicted by Alpha Fold2, there are flexible linkers with sufficient flexibility located at either end of pore-loop 1, pore-loop 2, and TMD domains. Upon deletion of either pore-loop 1, pore-loop 2, or TMD domain, the linkers on both ends could come into contact without altering the secondary structure. However, in the case of walker A, walker B, pore-loop 3, WD motif, NCL, or arginine finger domain, which have distant or stable secondary structures at their respective ends, deletion would result in a change in spatial location leading to instability.