**Supplementary Material**

**Senecavirus A induces mitophagy to promote self-replication through direct interaction of 2C protein with K27-linked ubiquitinated TUFM catalyzed by RNF185**

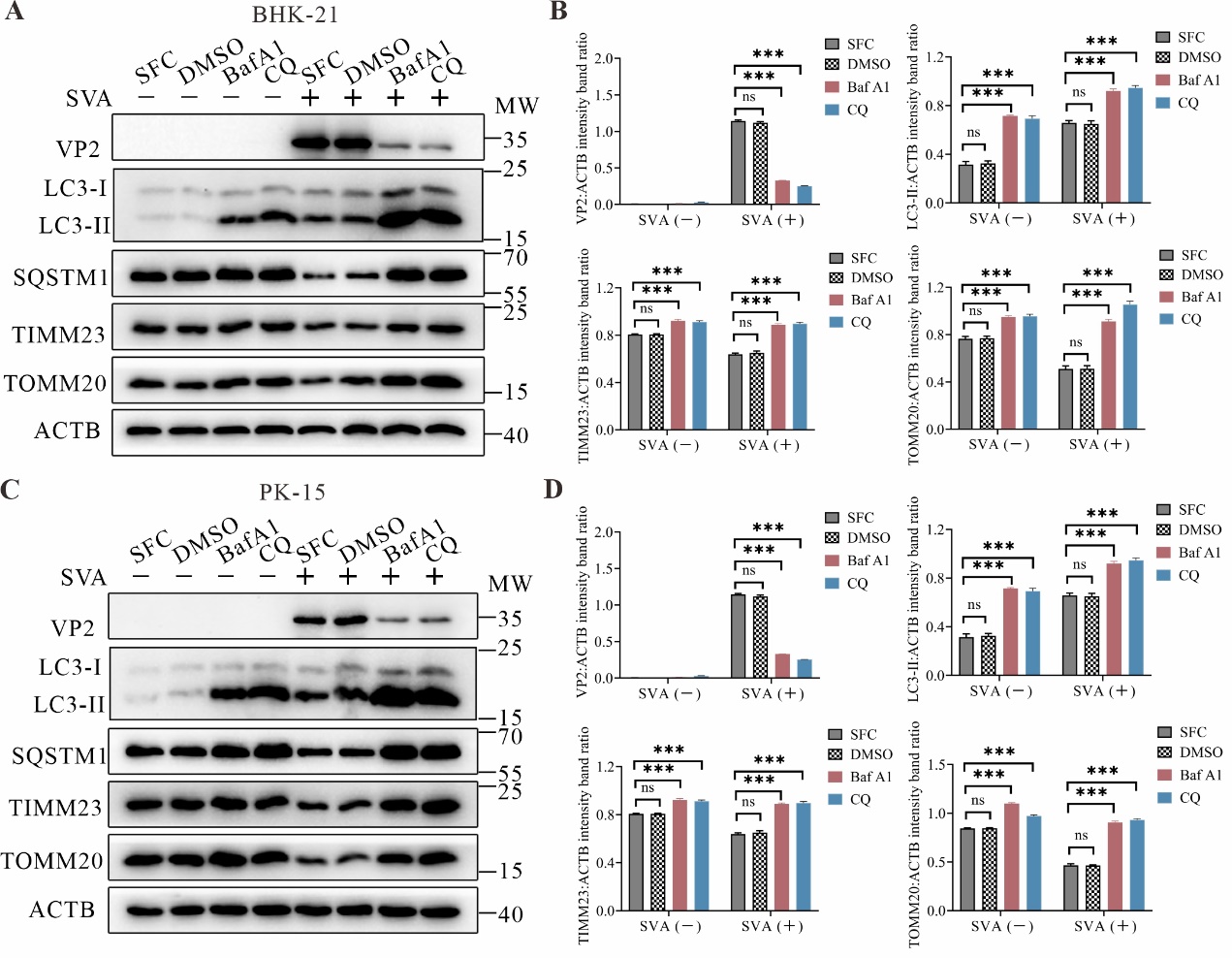
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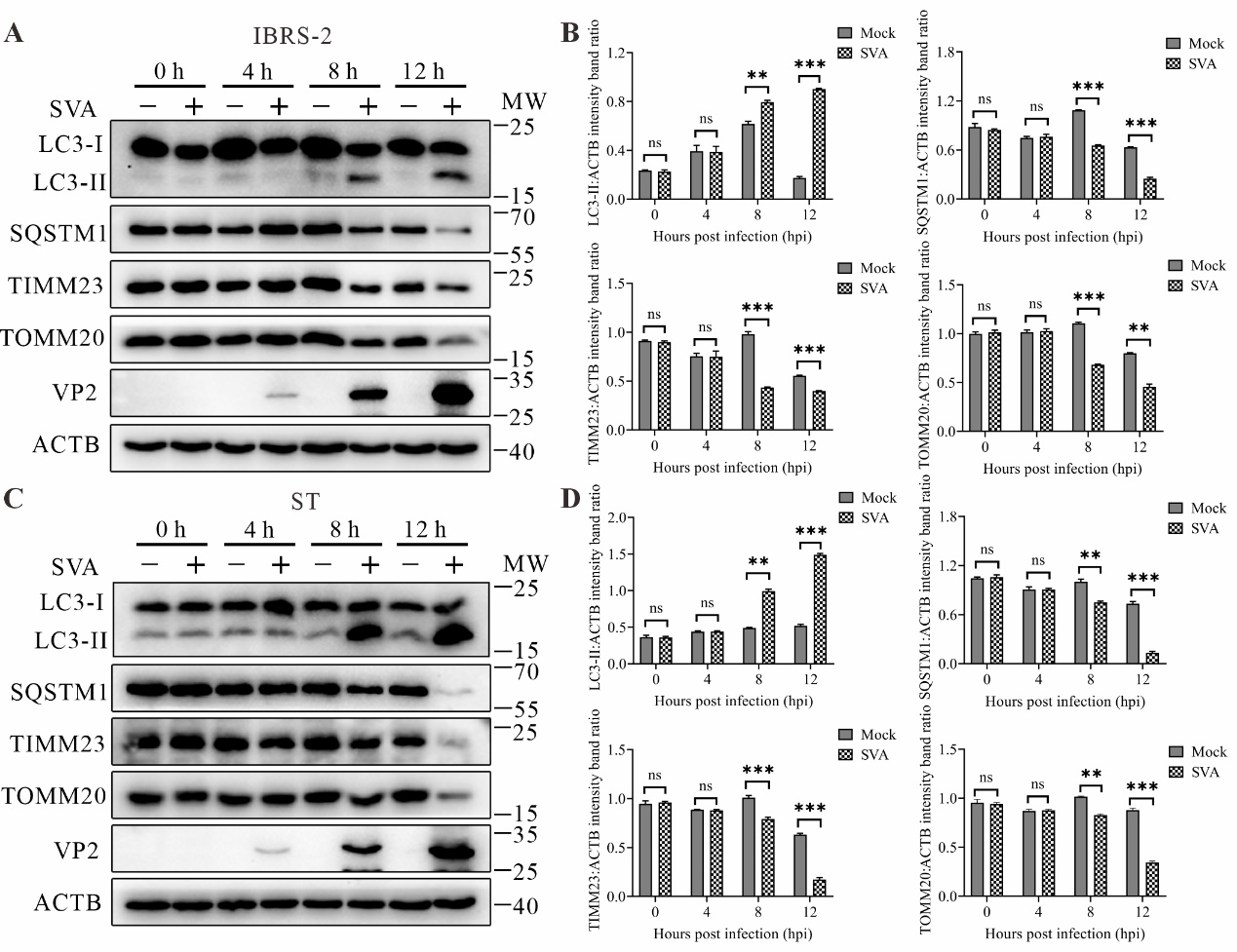
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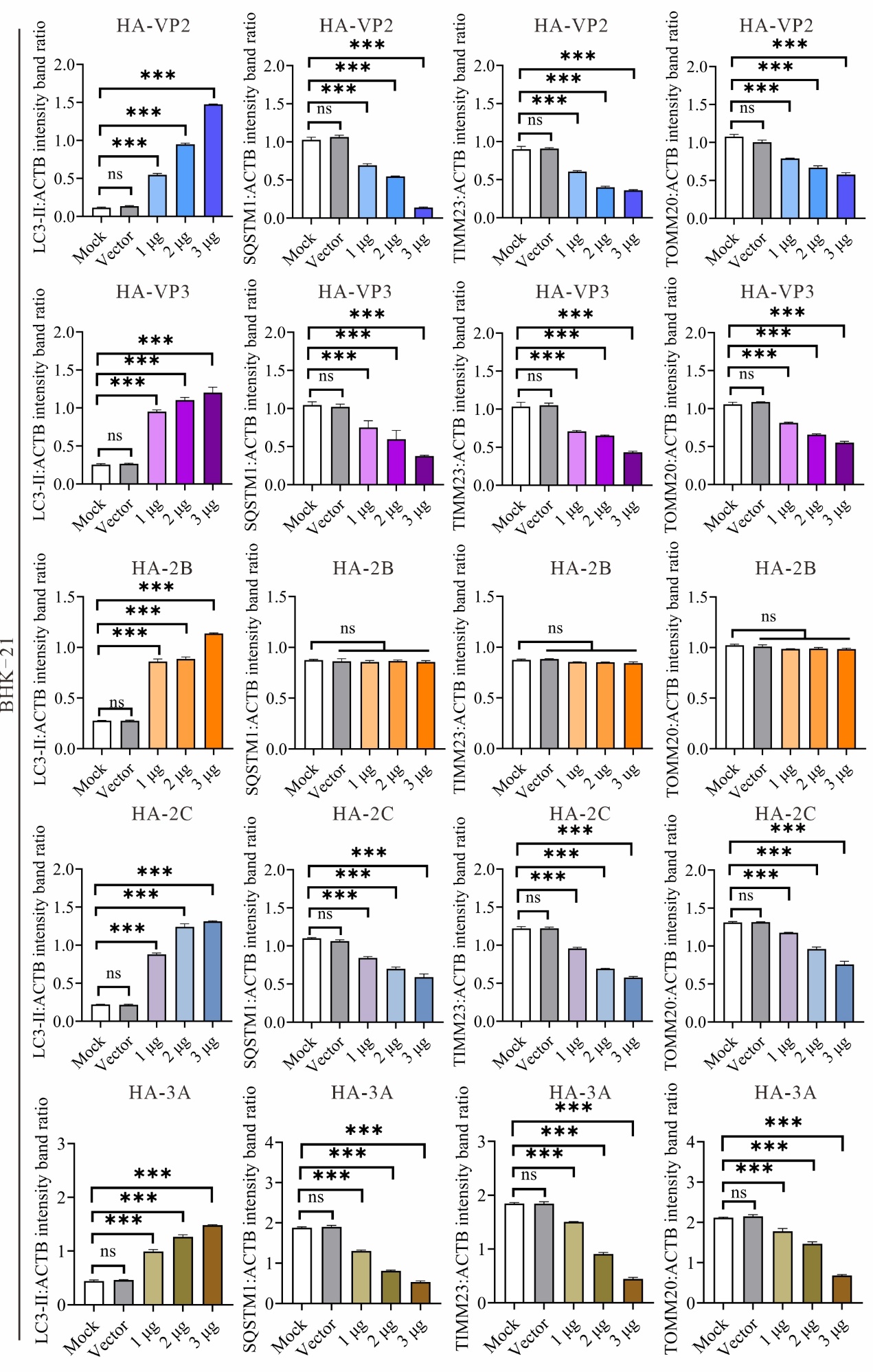
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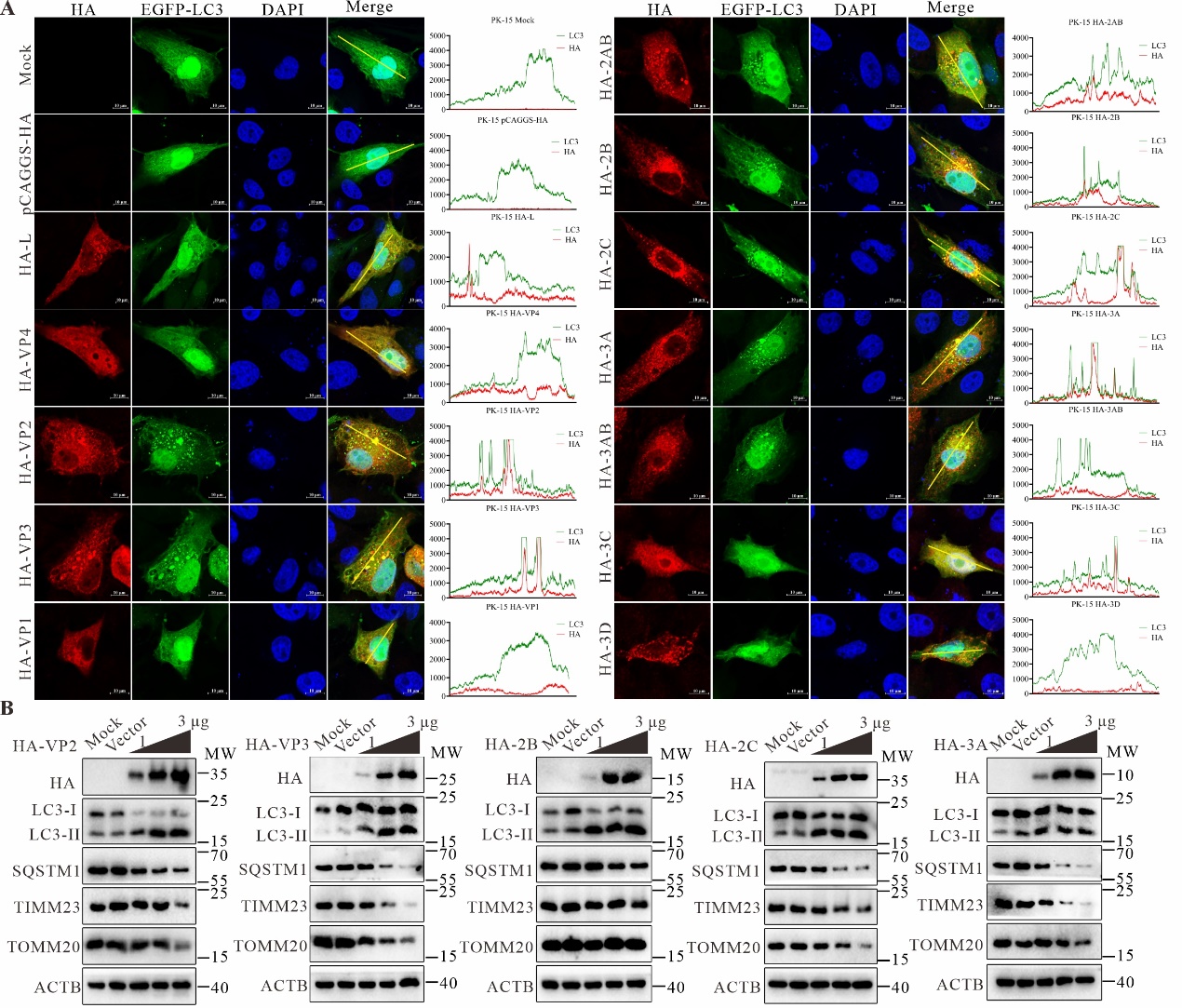
**Figure S1.** Pharmacological blockade of autophagy-lysosomal degradation activity can rescue the degradation of mitochondrial membrane proteins in SVA-infected host cells. (**A**) BHK-21 cells were mock infected or infected with SVA (MOI=0.1). The cells were further cultured in fresh medium supplemented with 100 μM of CQ, 100 nM of Baf A1, DMSO solvent control, or solvent-free control (SFC), respectively. At 24 hpi, the cells were analyzed by western blot. Representative results are shown and similar results were obtained in three independent experiments. (**B**) Densitometry of the target protein bands shown in (A) and calculation of their relative densitometric ratios to ACTB/β-actin. Data are shown as mean ± SD of three independent experiments (two-way ANOVA; ns, no significance; \*\*\**P* < 0.001). (**C**) PK-15 cells were infected and analyzed as described in (A). (**D**) Densitometric analysis of the target protein bands shown in (C).



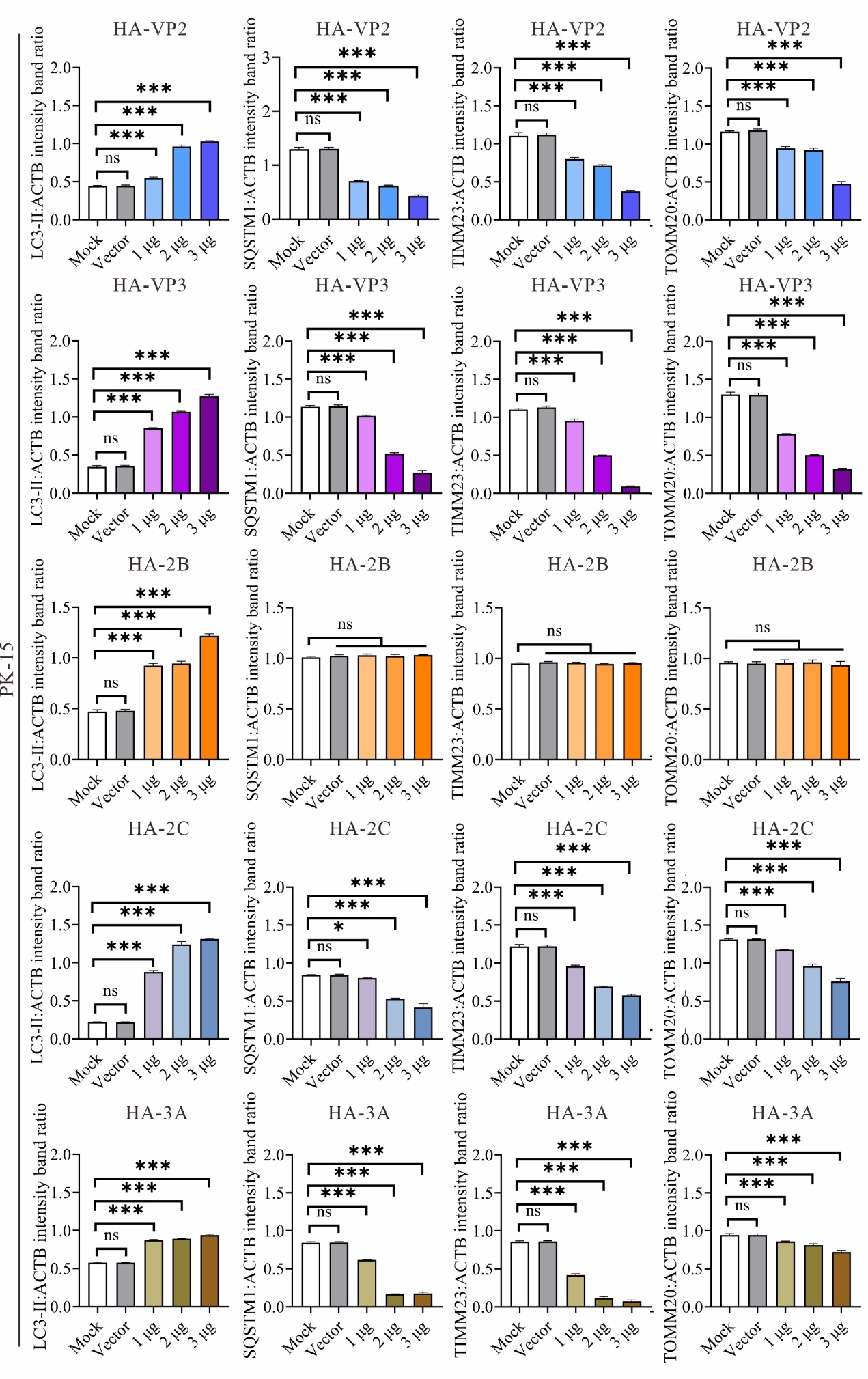
**Figure S2.** SVA infection promotes LC3 conversion and mitochondrial membrane protein degradation in IBRS-2 and ST cells. (**A**) IBRS-2 cells were mock infected or infected with SVA (MOI=0.1). At 0, 4, 8, and 12 hpi, the cells were analyzed by western blot. Representative results are shown and similar results were obtained in three independent experiments. (**B**) Densitometry of the target protein bands shown in (A) and calculation of their relative densitometric ratios to ACTB/β-actin. Data are shown as mean ± SD of three independent experiments (two-way ANOVA; ns, no significance; \*\**P* < 0.01; \*\*\**P* < 0.001). (**C**) ST cells were infected and analyzed as described in (A). (**D**) Densitometric analysis of the target protein bands shown in (C).



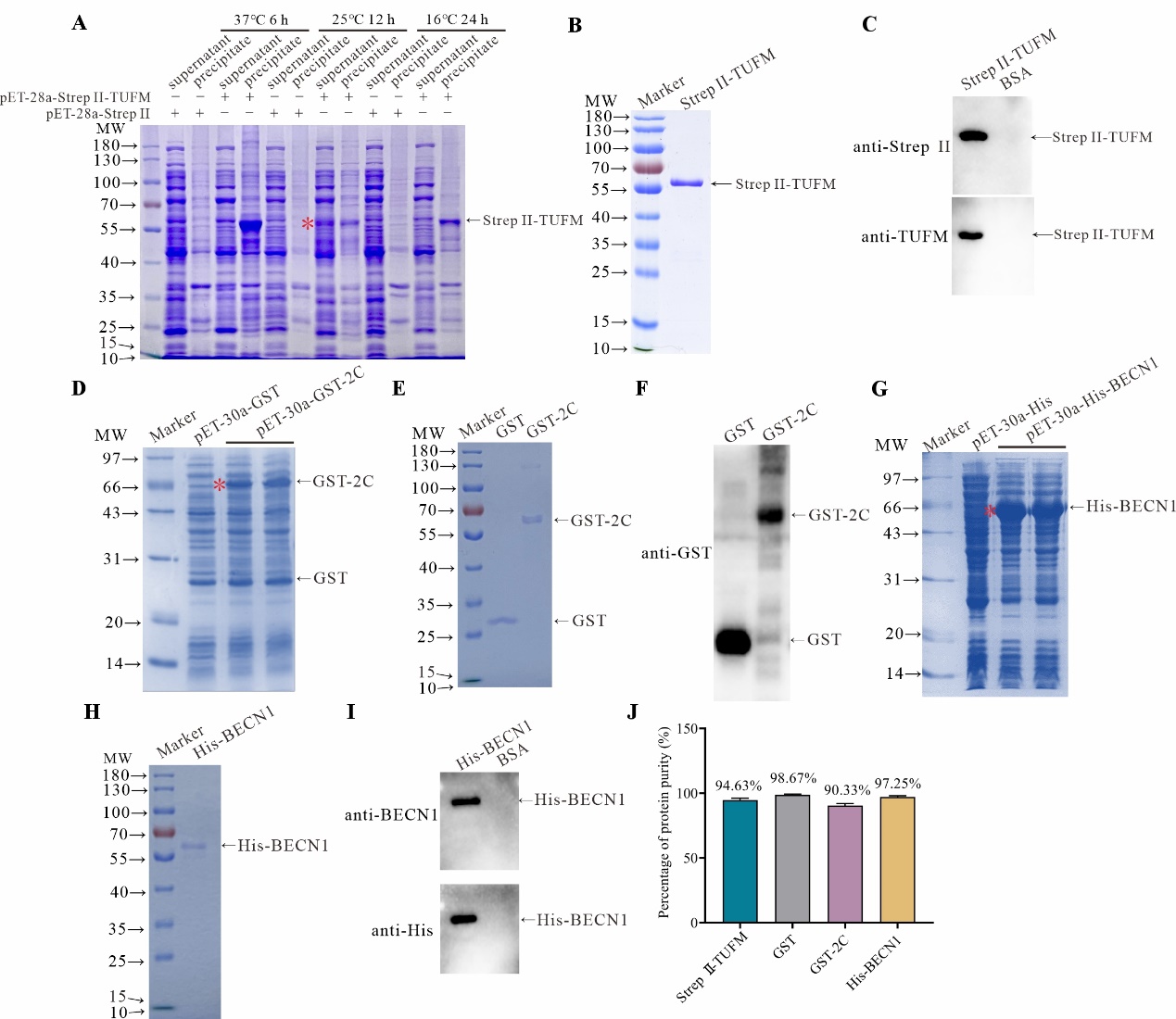
**Figure S3.** Densitometry of the target protein bands shown in Figure 6D and calculation of their relative densitometric ratios to ACTB/β-actin. Data are shown as mean ± SD of three independent experiments (two-way ANOVA; ns, no significance; \*\*\**P* < 0.001).



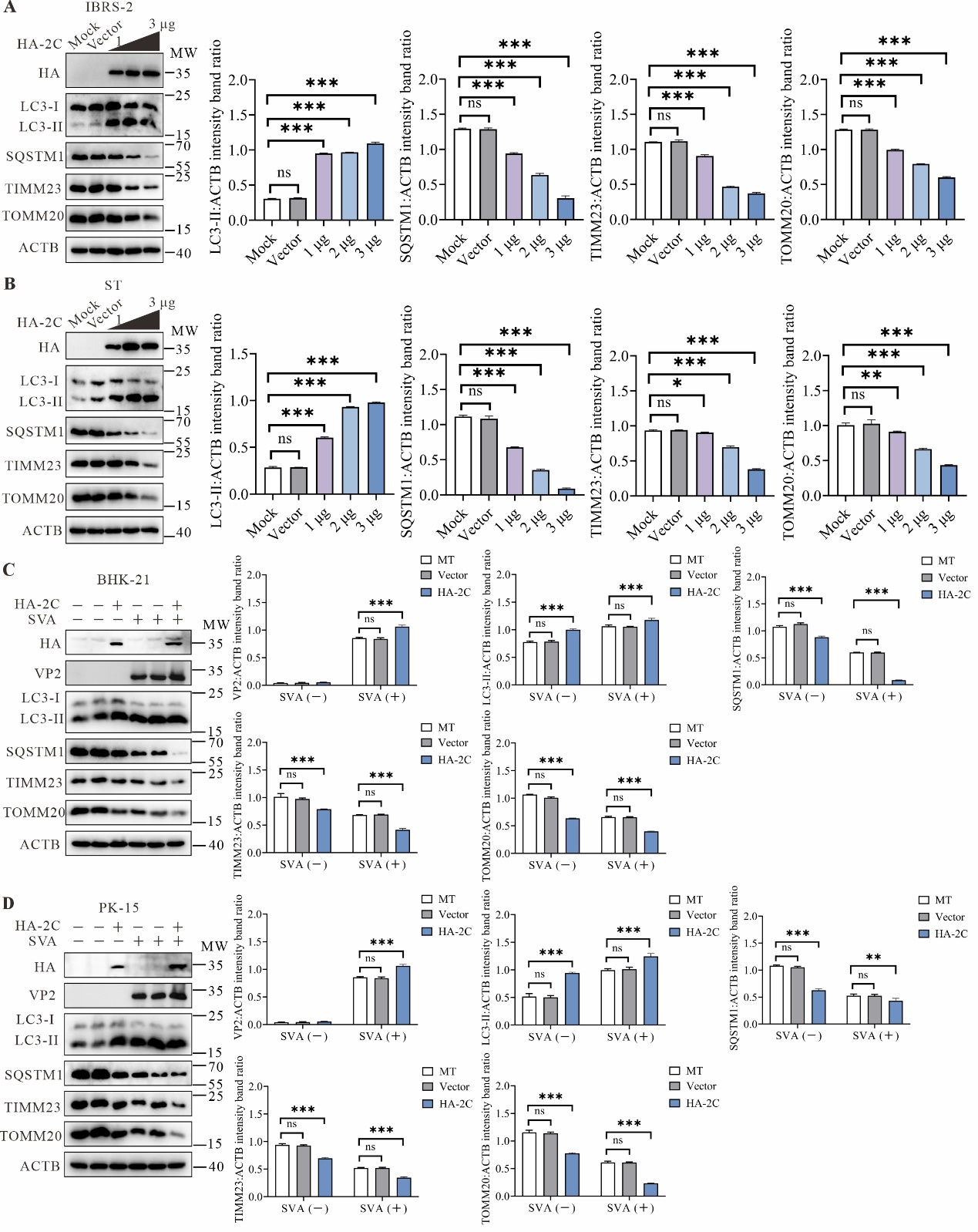
**Figure S4.** Single proteins of VP2, VP3, 2C, and 3A can independently induce mitophagy in PK-15 cells. (**A**) PK-15 cells were co-transfected with pEGFP-N2-LC3 and each of the recombinant pCAGGS-HA plasmids expressing HA-tagged SVA proteins. At 24 hpt, the cells were analyzed by confocal immunofluorescence analysis using anti-HA mAb and Alexa Fluor 568-conjugated goat anti-mouse IgG as the primary and secondary antibodies, respectively. Cell nuclei were counterstained with DAPI. The right panels show the fluorescence intensity profile of EGFP-LC3 (green) and HA-tagged SVA proteins (red) measured along the line drawn by ImageJ. Representative results are shown, and similar results were obtained in three independent experiments. Scale bar: 10 μm. (**B**) PK-15 cells were mock transfected or transfected with empty vector (pCAGGS-HA) or with 1, 2, and 3 µg/well of each of the recombinant plasmids pCAGGS-HA-VP2, pCAGGS-HA-VP3, pCAGGS-HA-2B, pCAGGS-HA-2C, and pCAGGS-HA-3A. At 36 hpt, the cells were analyzed by western blot. Representative results are shown and similar results were obtained in three independent experiments.



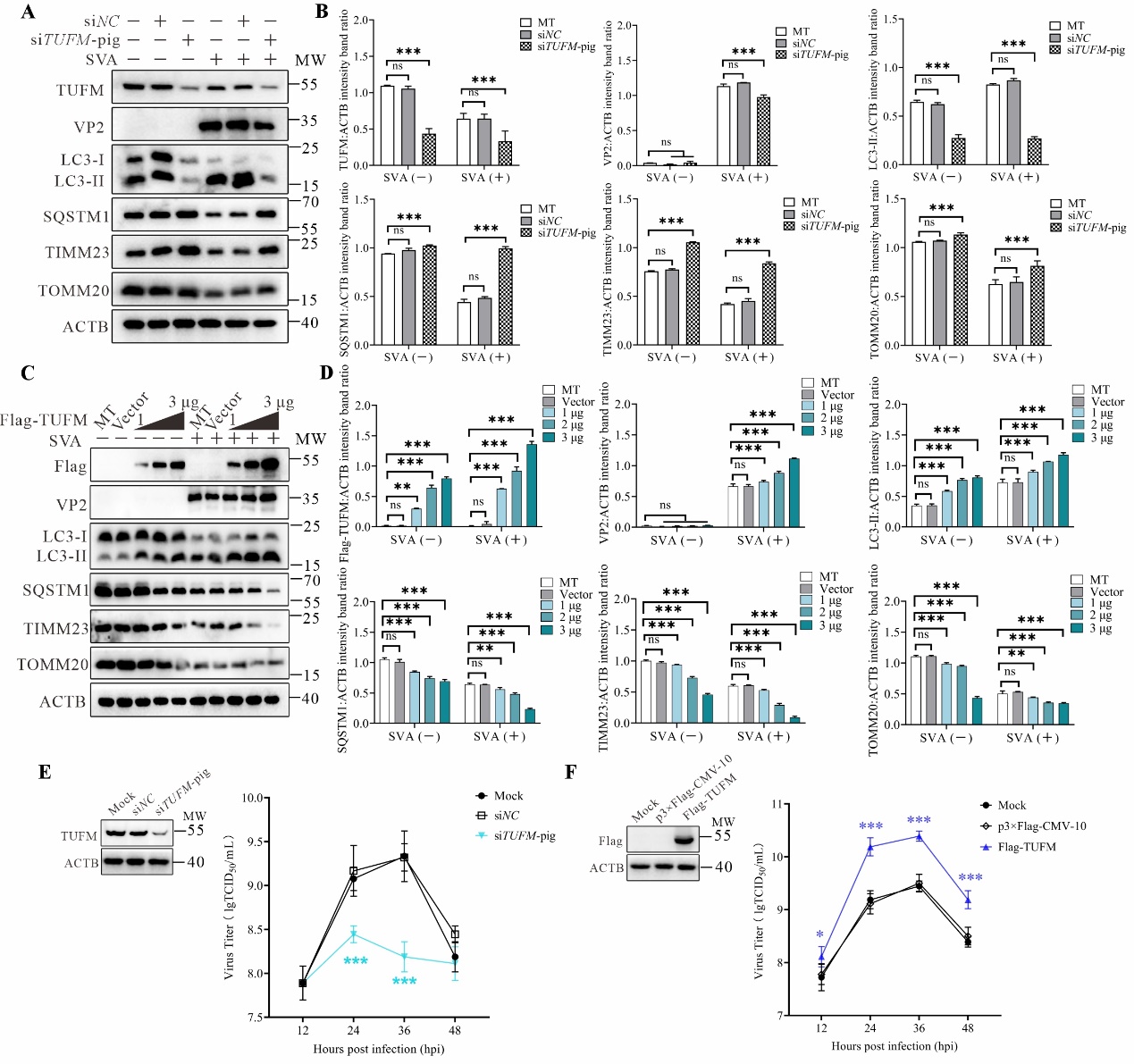
**Figure S5.** Densitometry of the target protein bands shown in Fig. S4B and calculation of their relative densitometric ratios to ACTB/β-actin. Data are shown as mean ± SD of three independent experiments (two-way ANOVA; ns, no significance; \**P* < 0.05; \*\*\**P* < 0.001).



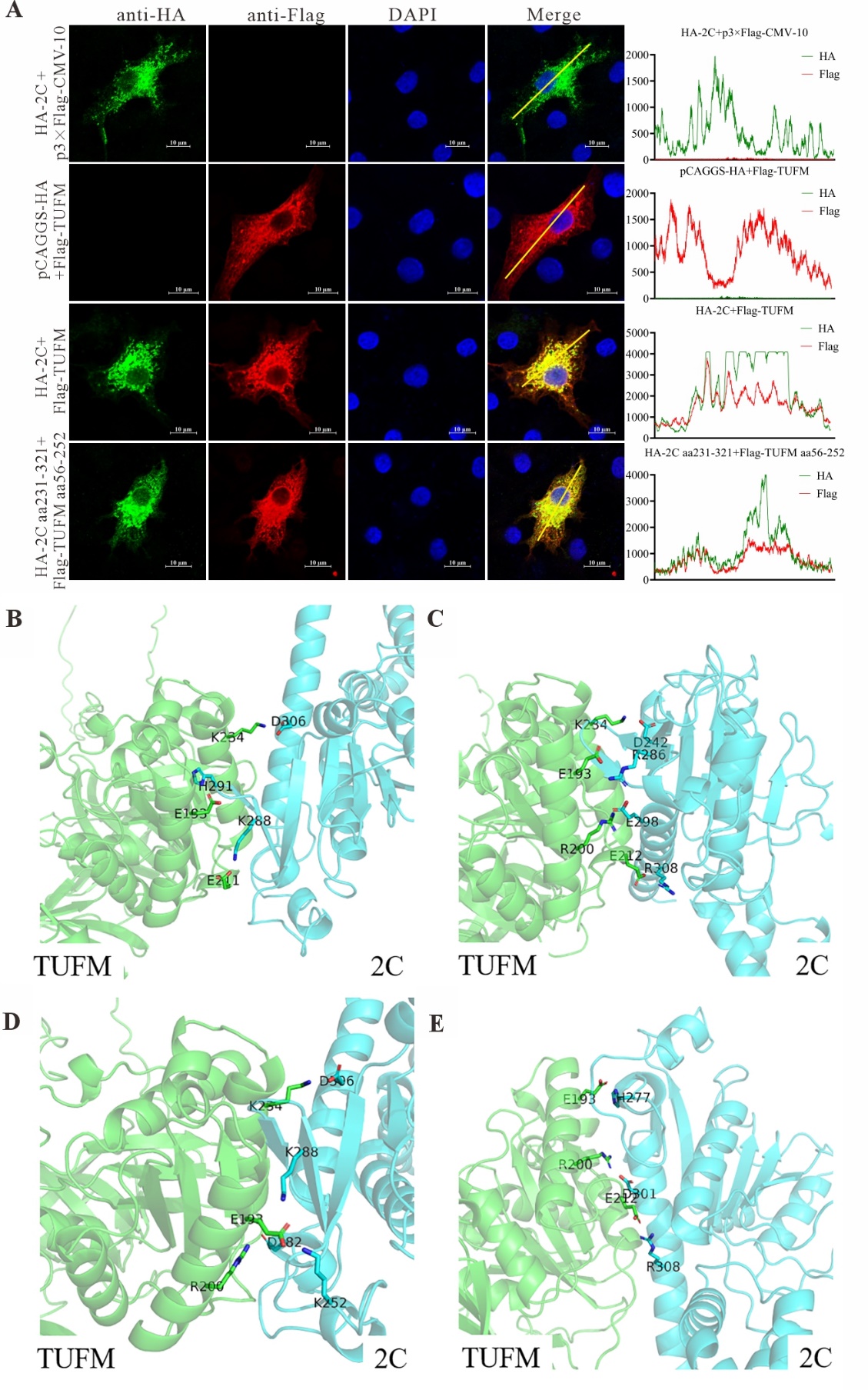
**Figure S6.** Prokaryotic expression, purification, and identification of TUFM, 2C, and Beclin1 proteins. (**A**) pET-28a-Strep II-TUFM and pET-28a-Strep II were individually transformed into *E. coli* BL21 competent cells, which were then induced with 0.5 mM IPTG for 6 h at 37°C, 12 h at 25°C, and 24 h at 16°C. Both supernatant and precipitate of the bacterial lysates were analyzed by SDS-PAGE. The red asterisk indicates the highest expression level of soluble Strep II-tagged TUFM protein. (**B**) Strep II-TUFM fusion protein was purified from the supernatant of bacterial lysates using Streptactin Beads 4FF and then analyzed by SDS-PAGE. (**C**) Western blot analysis of the purified Strep Ⅱ-TUFM protein using primary antibodies against Strep II and TUFM, along with bovine serum albumin (BSA) as an unrelated protein control. (**D**) pET-30a-GST-2C and pET-30a-GST were individually transformed into *E. coli* BL21 competent cells, which were then induced with 0.5 mM IPTG for 12 h at 25°C. The whole-bacterial lysates were analyzed by SDS-PAGE. The red asterisk indicates the target protein of GST-2C. (**E**) GST-2C fusion protein was purified from the precipitate of bacterial lysates using Glutathione-Sepharose 4B bead column and then analyzed by SDS-PAGE. (**F**) Western blot analysis of the purified GST-2C protein using anti-GST primary antibody. (**G**) pET-30a-His-BECN1 and pET-30a-His were individually transformed into *E. coli* BL21 competent cells, which were then induced with 0.5 mM IPTG for 12 h at 25°C. The whole-bacterial lysates were analyzed by SDS-PAGE. The red asterisk indicates the target protein of His-BECN1. (**H**) His-BECN1 fusion protein was purified from the precipitate of bacterial lysates using Ni-NTA agarose beads and then analyzed by SDS-PAGE. (**I**) Western blot analysis of the purified His-BECN1 protein using primary antibodies against His and BECN1. (**J**) Determination of protein purity for the purified Strep II-TUFM, GST, GST-2C, and His-BECN1 by scanning the optical density values of all protein bands in each lane of SDS-PAGE using ImageJ. Results are representative of three independent experiments. Data are shown as mean ± SD of three independent experiments.



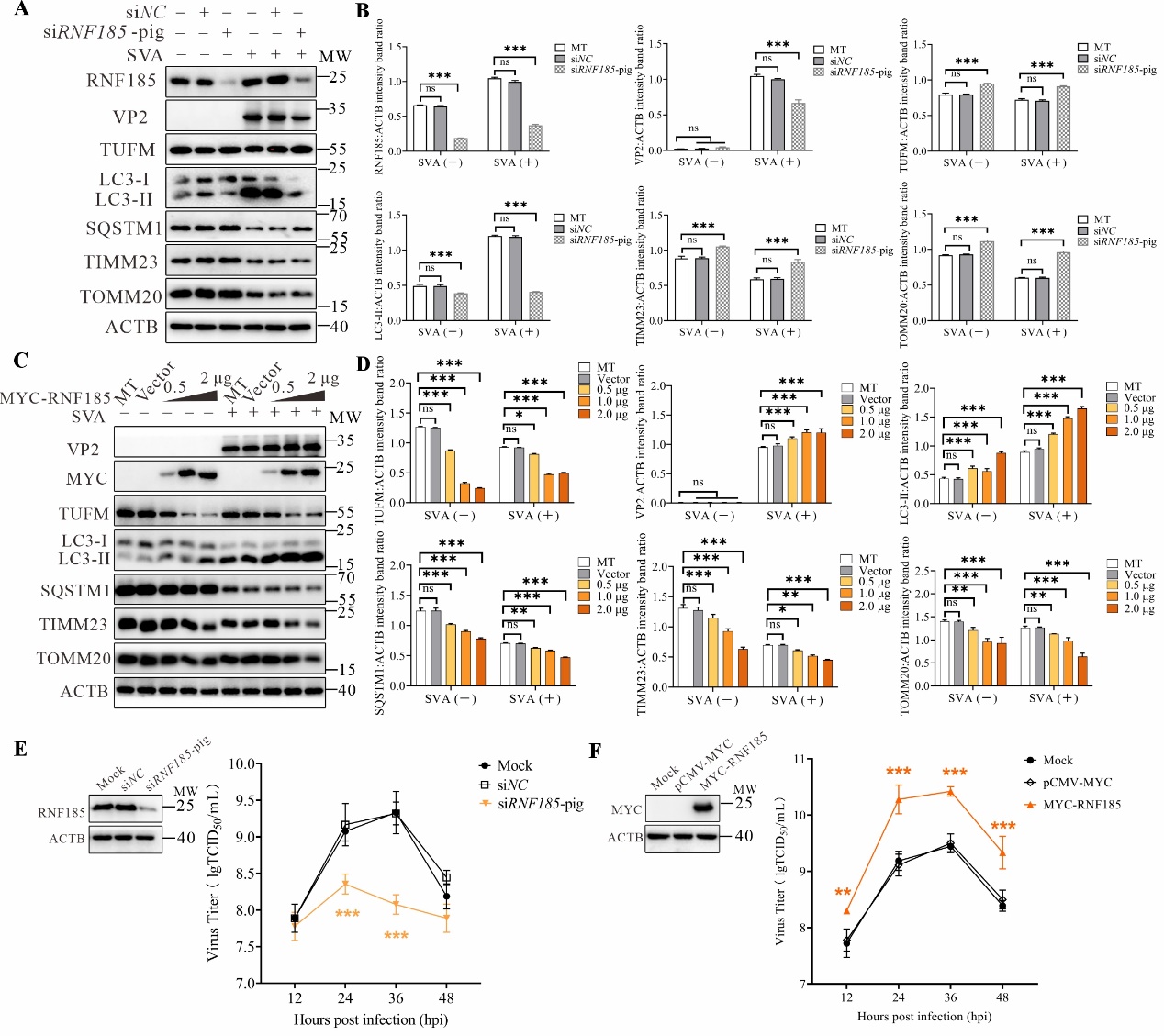
**Figure S7.** The 2C protein of SVA can independently induce mitophagy in host cells. (**A**) IBRS-2 cells were mock transfected or transfected with empty vector (pCAGGS-HA) or with 1, 2, and 3 µg/well of pCAGGS-HA-2C. At 36 hpt, the cells were analyzed by western blot. The right panels show the relative densitometric analysis of the target protein bands. (**B**) ST cells were treated and analyzed as described in (A). (**C**) BHK-21 cells were mock transfected (MT) or transfected with pCAGGS-HA or pCAGGS-HA-2C. At 24 hpt, the cells were mock infected or infected with SVA (MOI=0.1) for 24 h, and then analyzed by western blot analyses. The right panels show the relative densitometric analysis of the target protein bands. (**D**) PK-15 cells were treated and analyzed as described in (C). All experiments related to this figure were conducted in three independent biological replicates. Data are shown as mean ± SD of three independent experiments (two-way ANOVA; ns, no significance; \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001).



**Figure S8.** TUFM functions as a positive regulator of SVA-induced mitophagy in PK-15 cells. (**A**) PK-15 cells were mock transfected (MT) or transfected with 40 pmol/well of si*TUFM*-pig or si*NC* for 24 h. Then, the cells were mock infected or infected with SVA (MOI=0.1) for an additional 24 h. The cells were analyzed by western blot analyses. Representative results are shown and similar results were obtained in three independent experiments. (**B**) Relative densitometric analysis of the target protein bands shown in (A). Data are shown as mean ± SD of three independent experiments (two-way ANOVA; ns, no significance; \*\*\**P* < 0.001). (**C**) PK-15 cells were mock transfected (MT) or transfected with p3×Flag-CMV-10 or 1, 2, or 3 μg/well of p3×Flag-CMV-TUFM. At 24 hpt, the cells were mock infected or infected with SVA (MOI= 0.1) for 24 h. The cells were analyzed by western blot analyses. Representative results are shown and similar results were obtained in three independent experiments. (**D**) Densitometric analysis of the target proteins shown in (C). (**E**) PK-15 cells were transfected and then infected as described in (A). The silencing of TUFM was confirmed by western blot prior to virus titration. At 12, 24, 36, and 48 hpi, viral samples were harvested, and viral titers were detected by TCID50 assay on BHK-21 cells. Data are shown as mean ± SD of three independent experiments (two-way ANOVA; \**P* < 0.05; \*\*\**P* < 0.001). (**F**) PK-15 cells were transfected and then infected as described in (C). Viral titrations were performed and analyzed as described in (E).



**Figure S9.** Validation of interactions between full-length TUFM and 2C proteins, as well as aa56–252 of TUFM and aa231–321 of 2C. (**A**) BHK-21 cells were co-transfected with pCAGGS-HA-2C and p3×Flag-CMV-TUFM, or pCAGGS-HA-2C(aa231–321) and p3×Flag-CMV-TUFM(aa56–252), alongside with their corresponding empty vector controls (pCAGGS-HA and p3×Flag-CMV-10). At 24 hpt, the cells were analyzed by confocal immunofluorescence assays using anti-HA, or -Flag mAbs and Alexa Fluor 488-, or 568-conjugated goat anti-mouse IgGs as the primary and secondary antibodies, respectively. Cell nuclei were counterstained with DAPI. The right panels show the fluorescence intensity profile of HA-2C (green), HA-2C(aa231–321) (green), Flag-TUFM (red), and Flag-TUFM (aa56–252) (red) measured along the line drawn by ImageJ. Representative results are shown and similar results were obtained in three independent experiments. Scale bar: 10 μm. (**B-E**) The other four predicted models of the TUFM-2C interaction complex were obtained using the Alphafold2 online server. The TUFM (left) and 2C (right) and proteins are marked in green and pink, respectively. The amino acid residues predicted to be possible interaction sites are labeled with one-letter type name in stick representation. These potential key amino acid residues of D306, H291 and K288 in B, D242, R286, E298 and R308 in C, D306, K288, D282 and K252 in D, and H277, D301 and R308 in E were selected for construction of single-site mutants.



**Figure S10.** RNF185 mediates TUFM degradation and functions as a positive regulator of SVA-induced mitophagy in PK-15 cells. (**A**) PK-15 cells were mock transfected (MT) or transfected with 40 pmol/well of si*RNF185*-pig or si*NC* for 24 h. Then the cells were mock infected or infected with SVA (MOI=0.1) for an additional 24 h. The cells were analyzed by western blot analyses. (**B**) Densitometry of the target protein bands shown in (A) and calculation of their relative densitometric ratios to ACTB/β-actin. (**C**) PK-15 cells were transfected with pCMV-MYC or 0.5, 1, or 2 μg/well of pCMV-MYC-RNF185 for 24 h, and then mock infected or infected with SVA at an MOI of 0.1 for additional 24 h. The cells were analyzed by western blot analyses. (**D**) Densitometric analysis of the target proteins shown in (C). (**E**) PK-15 cells were transfected and then infected as described in (A). The silencing of TUFM was confirmed by western blot prior to virus titration. At 12, 24, 36, and 48 hpi, viral samples were harvested, and viral titers were detected by TCID50 assay on BHK-21 cells. (**F**) PK-15 cells were transfected and then infected as described in (C). Viral titrations were performed and analyzed as described in (E). All experiments related to this figure were conducted in three independent biological replicates. Data are shown as mean ± SD of three independent experiments (two-way ANOVA; ns, no significance; \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001).