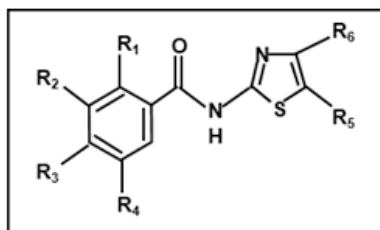


Supplementary Information

**Nitazoxanide inhibits paramyxovirus replication by
targeting the Fusion protein folding: role of glycoprotein-
specific thiol oxidoreductase ERp57**

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A**B**

		IC ₅₀ [μg/ml]	LD ₅₀ [μg/ml]
Nitazoxanide			
R ₁ =OCOCH ₃	R ₂ =H	0.30 ± 0.01	>50
R ₃ =H	R ₄ =H		
R ₅ =NO ₂	R ₆ =H		
Tizoxanide			
R ₁ =OH	R ₂ =H	0.50 ± 0.01	>50
R ₃ =H	R ₄ =H		
R ₅ =NO ₂	R ₆ =H		
RM4820			
R ₁ =OCOCH ₃	R ₂ =H	0.40 ± 0.02	>50
R ₃ =H	R ₄ =H		
R ₅ =Br	R ₆ =H		
RM4832			
R ₁ =OH	R ₂ =H	0.50 ± 0.04	>50
R ₃ =H	R ₄ =H		
R ₅ =Br	R ₆ =H		
RM4848			
R ₁ =OH	R ₂ =H	0.40 ± 0.03	>50
R ₃ =H	R ₄ =H		
R ₅ =Cl	R ₆ =H		
RM5038			
R ₁ =OCOCH ₃	R ₂ =H	0.50 ± 0.02	>50
R ₃ =H	R ₄ =H		
R ₅ =Cl	R ₆ =H		

Supplementary Table 1. Antiviral activity of thiazolides against Sendai virus. **A**, General structure of thiazolides. **B**, Evaluation of the antiviral activity of the second generation thiazolides RM4820, RM4832, RM4848, and RM5038 as compared to nitazoxanide (NTZ) and tizoxanide (TIZ). AGMK cells mock-infected or infected with SeV under single-step conditions (3 PFU/cell) were treated with different concentrations (0.01, 0.1, 1, 10, and 50 μg/ml) of the compounds immediately after the virus adsorption period. Cell viability and virus yield were determined at 24h p.i. by MTT assay and hemagglutination assay, respectively. Data, expressed as IC₅₀ (50% inhibitory concentration) and LD₅₀ (50% lethal dose), represent the means±S.D. of triplicate experiments. Lack of SD in the LD₅₀ column indicates that values were identical.

Supplementary Figure Legends

Supplementary Figure 1. Antiviral activity of tizoxanide in SeV-infected AGMK cells. **A,B**, AGMK cells mock-infected or infected with SeV under single-step (3 PFU/cell, *top panels*) and multistep (0.01 PFU/cell, *bottom panels*) conditions were treated with different concentrations of nitazoxanide (NTZ) (**A**), tizoxanide (TIZ) (**B**), or vehicle immediately after the adsorption period. Virus yield (○) was determined at 24h (single-step) or 48h (multistep) p.i. by hemagglutinin (HA) titration. Data, expressed as HAU/ml, represent the means±S.D. of quadruplicate samples. $*=P<0.05$; $**=P<0.01$. Cell viability (24h) of mock-infected cells (△) was determined by MTT assay (n=4). Absorbance (O.D.) of converted dye was measured at λ 570nm. **C**, AGMK cells were infected and treated as in (**A,B**). Virus yield was determined in quadruplicate samples at 24h (3 PFU/cell) or 48h (0.01 PFU/cell) p.i. by HA titration. TIZ and NTZ IC₅₀ (50% inhibitory concentration) was calculated using Prism 5.0 software. Selectivity Indexes (SI) are shown.

Supplementary Figure 2. Thiazolides inhibit SeV replication in human lung A549 cells.

A,B, Human alveolar type II-like epithelial A549 cells mock-infected or infected with SeV under single-step (3 PFU/cell, *top panels*) and multistep (0.01 PFU/cell, *bottom panels*) conditions were treated with different concentrations of nitazoxanide (NTZ) (**A**), tizoxanide (TIZ) (**B**) or vehicle immediately after the adsorption period. Virus yield (○) was determined at 24h (single-step) or 48h (multistep) p.i. by HA titration. Data, expressed as HAU/ml, represent the means±S.D. of quadruplicate samples. $*=P<0.05$; $**=P<0.01$. Cell viability (24h) of mock-infected cells (△) was determined by MTT assay (n=4). Absorbance (O.D.) of converted dye was measured at λ 570nm. **C**, Immunoblot analysis for SeV-F, ubiquitin and β -actin in whole-cell extracts of mock-infected and SeV-infected A549 cells treated for 24h with NTZ (5 μ g/ml), proteasome inhibitor bortezomib (BTZ) (25 nM), glycosylation inhibitor tunicamycin (TM) (5

µg/ml) or vehicle. A decrease in ubiquitinated protein levels is noted in bortezomib-treated cells in the presence of NTZ. Full-length blots/gels are presented in Supplementary Figure 15.

Supplementary Figure 3. Nitazoxanide inhibits SeV-F and RSV-F glycoprotein translocation to the cell surface. **A**, Levels of plasma membrane SeV-F glycoprotein (*red*) were detected at 24h p.i. by indirect immunofluorescence in SeV-infected A549 cells treated with 5 µg/ml NTZ or vehicle. Nuclei are stained with Hoechst (*blue*). Images were captured using an Olympus Fluoview FV1000 confocal laser scanning system. Merge and zoom images are shown. Scale bar, 20 µm (zoom, 7 µm). **B**, Confocal images of Flag-RSV-F (red) distribution on plasma membrane in HeLa cells transfected with RSV-F/ORF/C-Flag tag construct (RSV-F) or pcDNA3 empty vector (Control), and treated as in (**A**). Nuclei are stained with Hoechst (blue). Merge and zoom images are shown. Scale bar, 20 µm (zoom, 7 µm).

Supplementary Figure 4. Nitazoxanide alters ERp57 interaction with SeV fusion protein. SeV-F/ERp57 interactions (visualized as red spots) detected at 24h p.i. by PLA in SeV-infected AGMK (**A**) and A549 (**B**) cells treated with 5 µg/ml NTZ or vehicle. Nuclei are stained with DAPI (blue). Merge and zoom images are shown. Scale bar, 20 µm (zoom, 7 µm).

Supplementary Figure 5. Nitazoxanide alters ERp57/calnexin interactions in SeV-infected cells. ERp57/calnexin (CNX) interactions (visualized as red spots) detected at 16h p.i. by PLA in SeV-infected AGMK (**A**) and A549 (**B**) cells treated with 5 µg/ml NTZ or vehicle. Nuclei are stained with DAPI (blue). Merge and zoom images are shown. Scale bar, 20 µm (zoom, 7 µm).

Supplementary Figure 6. Reversal of nitazoxanide antiviral activity in SeV-infected cells. **A**, AGMK cells mock-infected or infected with SeV (3 PFU/cell) were treated with NTZ (10 µg/ml) or vehicle after virus adsorption. At 6h p.i. in 50% of the cultures the drug was removed,

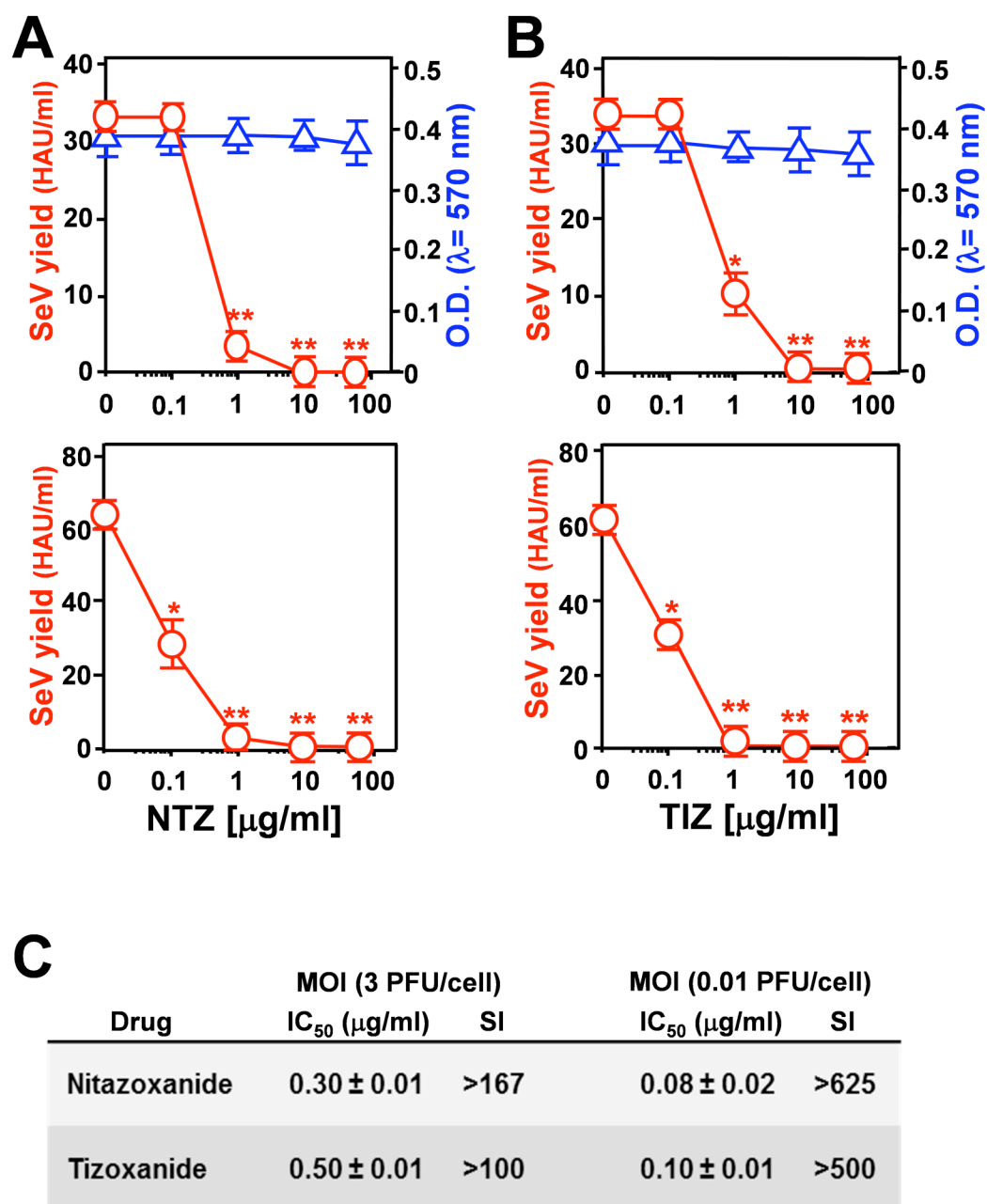
and cell monolayers were washed three times with phosphate-buffered saline and incubated in culture medium in the presence of vehicle; NTZ was kept in the remaining 50% of the cultures. At 24h p.i., whole-cell extracts were analyzed for SeV-F and β -actin levels by Western blot. **B**, In parallel samples, virus yield was determined at 24h p.i. by HA titration. Data, expressed as HAU/ml, represent the means $\pm$ S.D. of quadruplicate samples. $^*P<0.01$. Full-length blots/gels are presented in Supplementary Figure 15.

Supplementary Figure 7. Tizoxanide glucuronide does not affect SeV replication and ERp57 activity. **A**, Structure of tizoxanide glucuronide (TIZ-Glc). **B**, AGMK cells were mock-infected or infected with SeV (3 PFU/cell) and treated with different concentrations of TIZ (*top panel*), TIZ-Glc (*bottom panel*) or vehicle immediately after the adsorption period. Virus yield (**O**) was determined in quadruplicate samples at 24h p.i. by HA titration. Data, expressed as HAU/ml, represent the means $\pm$ S.D. of quadruplicate samples. $^*P<0.05$; $^{**}P<0.01$. Cell viability (24h) of mock-infected cells (**Δ**) was determined by MTT assay (n=4). Absorbance (O.D.) of converted dye was measured at λ 570nm. **C**, Kinetics of diethylenetriamine glutathione disulfide (Di-E-GSSG) reduction into fluorescent EGSH catalyzed by ERp57 (20 nM) in the presence of 30 μ M TIZ, 30 μ M TIZ-Glc or vehicle (C). Data are expressed as arbitrary fluorescence units (AFU).

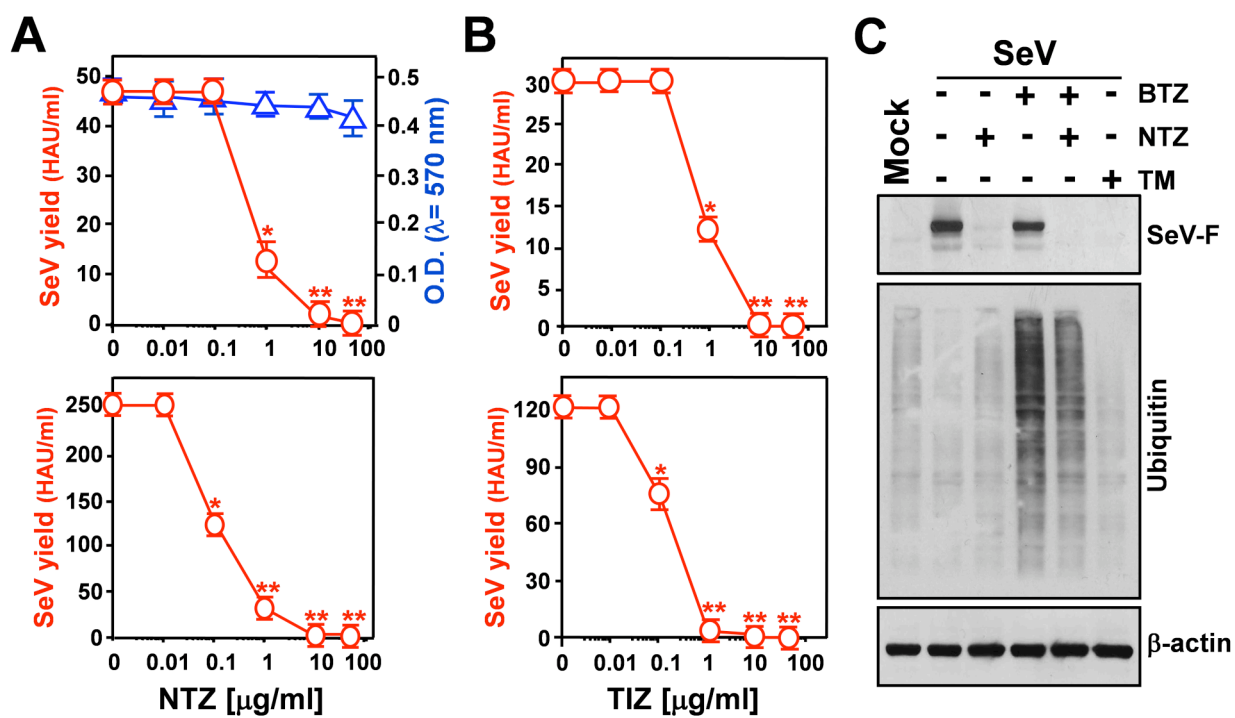
Supplementary Figure 8. Effect of ERp57 silencing on F-protein levels in thiazolide-treated SeV-infected cells. IB for SeV-F, ERp57 and β -actin in WCEs from AGMK cells transfected (48h) with scramble-RNA (scRNA) or ERp57-siRNA (siERp57), and mock-infected (M) or infected with SeV (3 PFU/cell) and treated with different concentrations of NTZ, 1 μ g/ml tizoxanide (TIZ) or vehicle (-) immediately after the adsorption period for 16h. Full-length blots/gels are presented in Supplementary Figure 16.

Supplementary Figure 9. Effect of nitazoxanide on the level of GRP78 and different ER proteins in SeV-infected cells. IB for GRP78 (anti-KDEL antibodies), ERp57, ERp72, calreticulin, XBP1, SeV-F and α -tubulin in WCEs from AGMK and A549 cells uninfected (**A**), or infected with SeV (3 PFU/cell) or mock-infected (**B**) and treated with 5 μ g/ml NTZ, 2.5 μ g/ml tunicamycin (TM) or vehicle (C) for different times. In SeV-infected cells treatment was started immediately after the adsorption period and continued for different times post infection (p.i.). Full-length blots/gels are presented in Supplementary Figures 17 to 19.

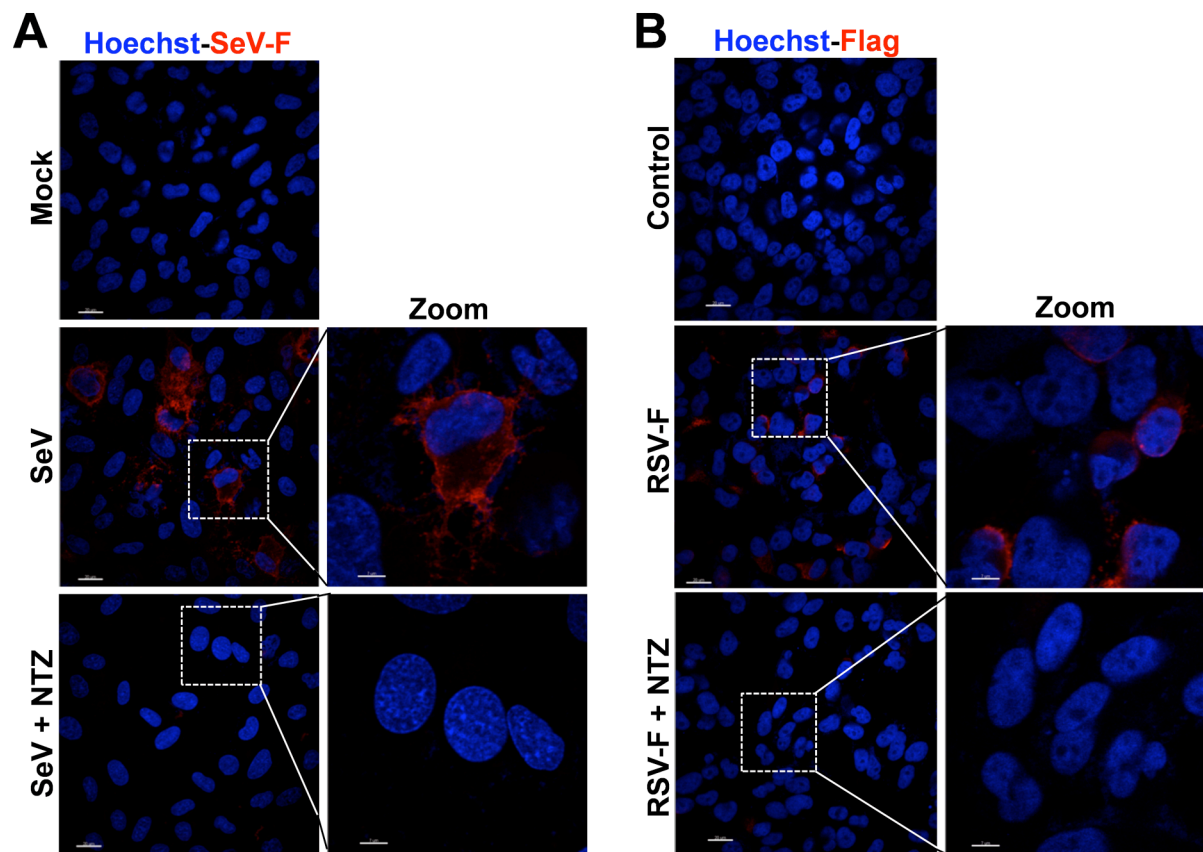
Supplementary Figures 10-19. Full-length blots/gels of data presented in Figure 2, Figure 3, Figure 8, and in Supplementary Figures 2, 6, 8 and 9.



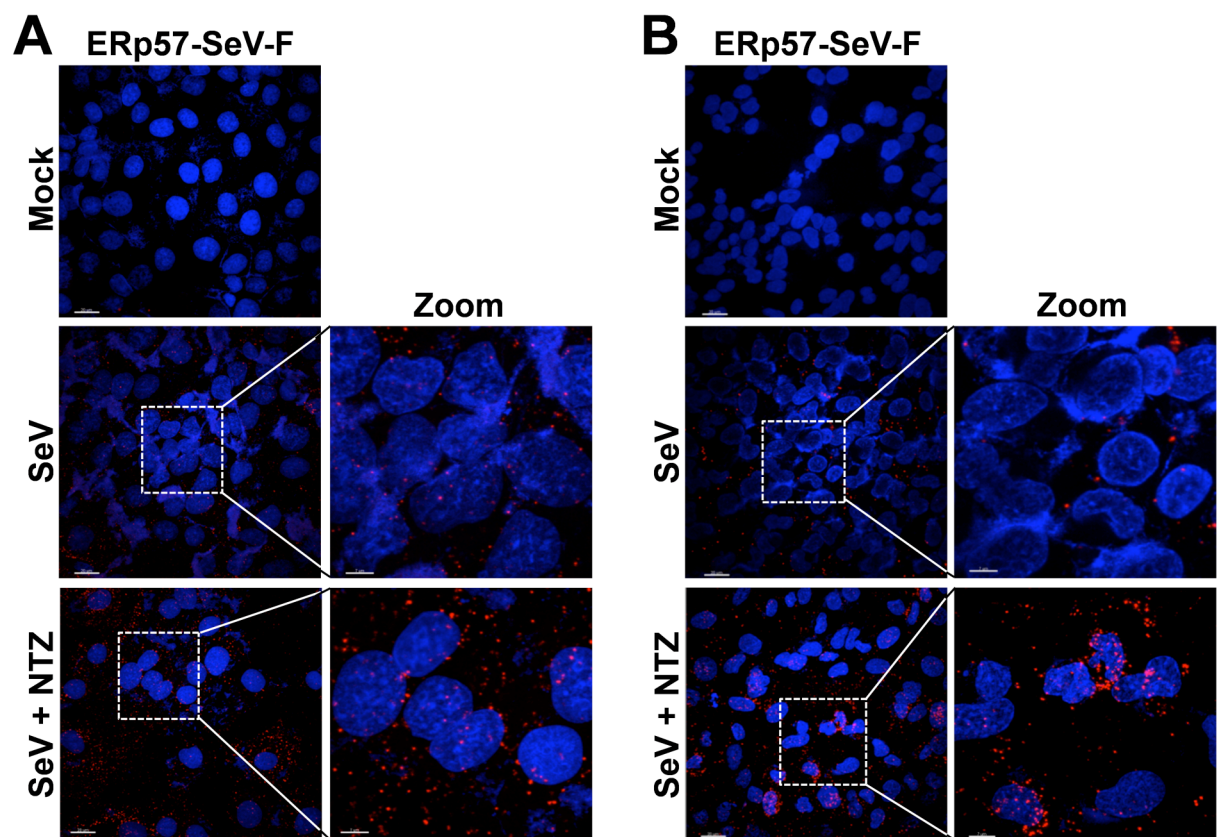
Supplementary Figure 1



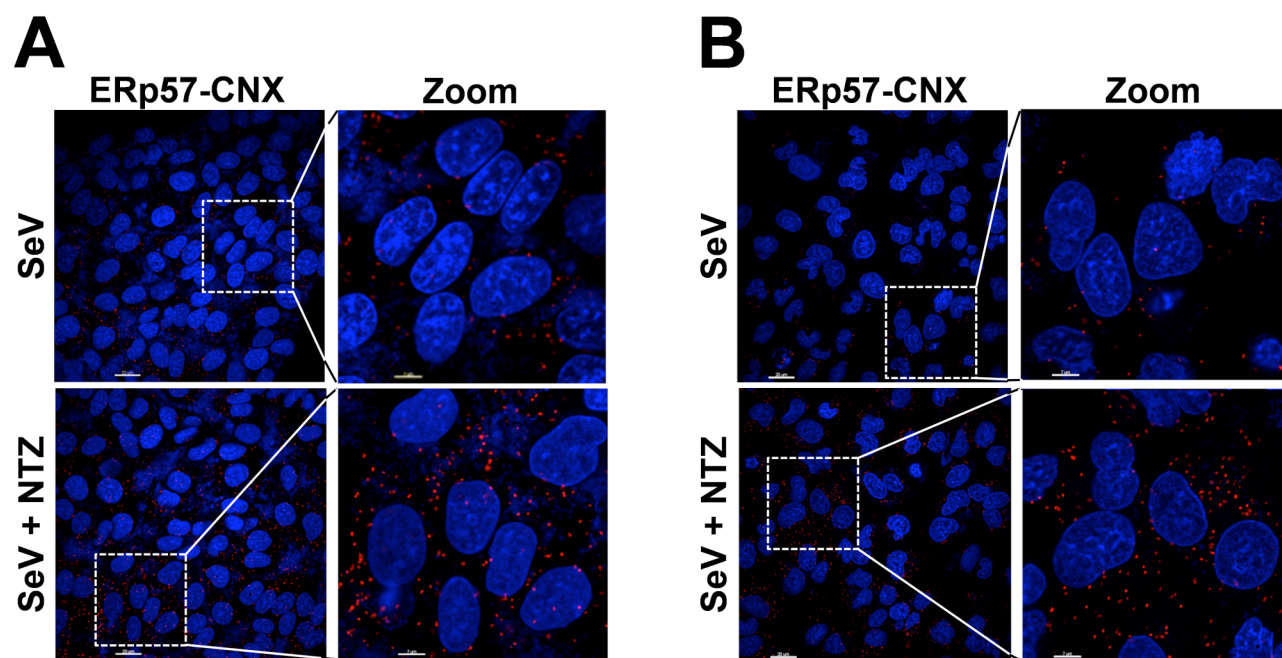
Supplementary Figure 2



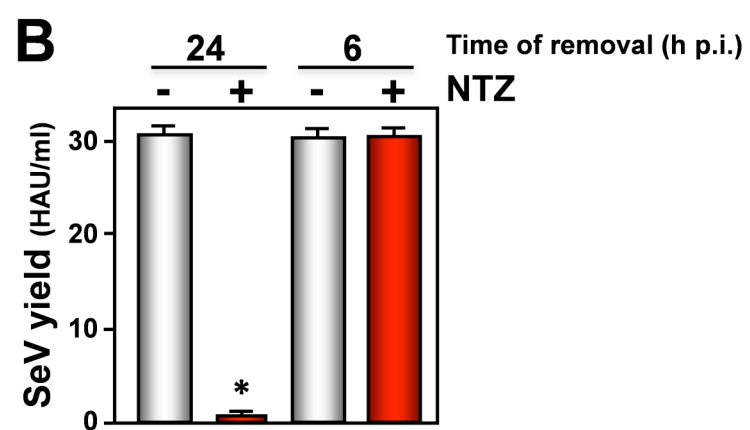
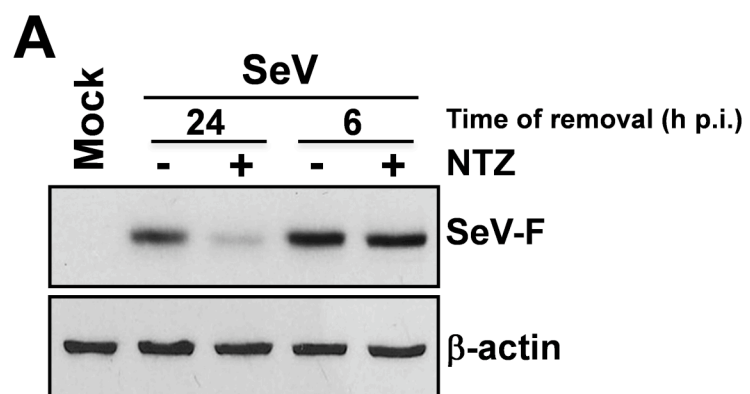
Supplementary Figure 3



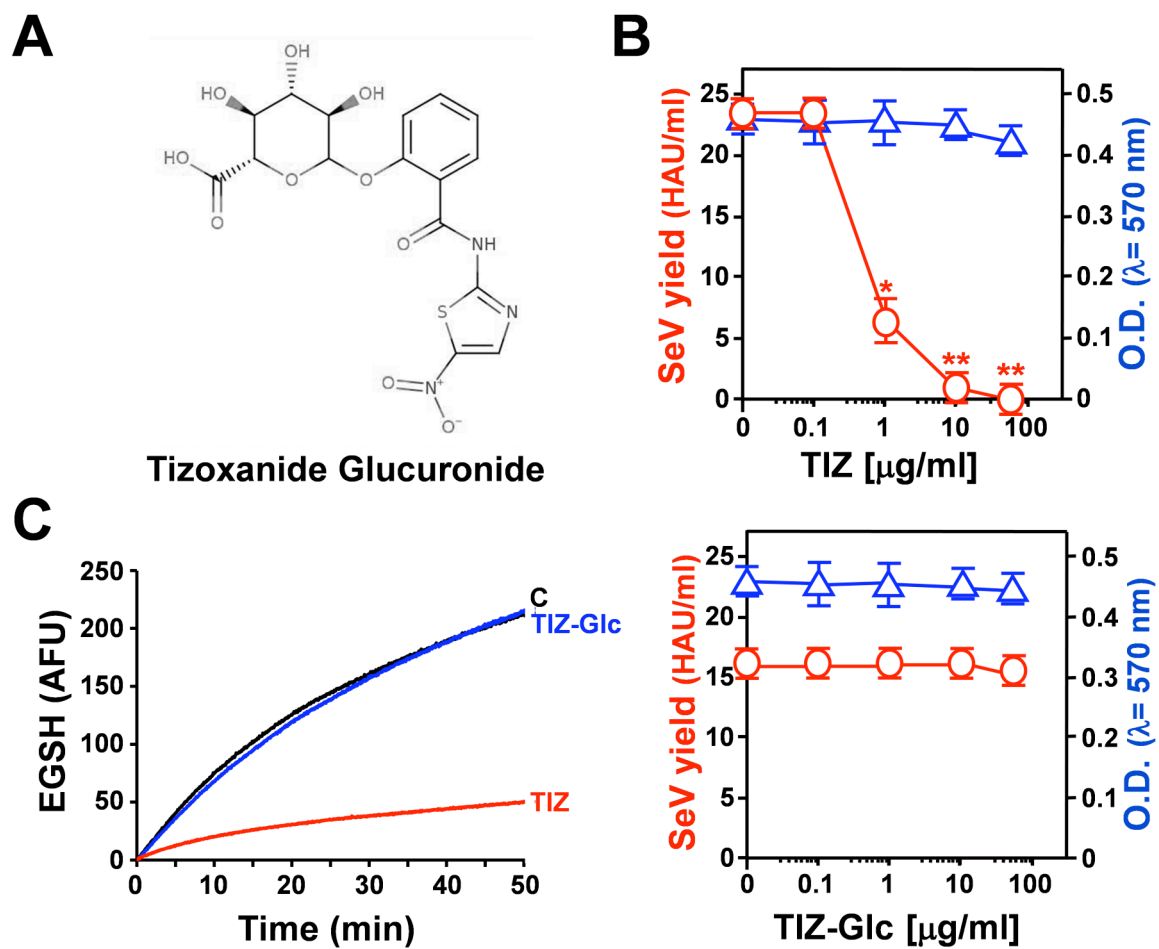
Supplementary Figure 4



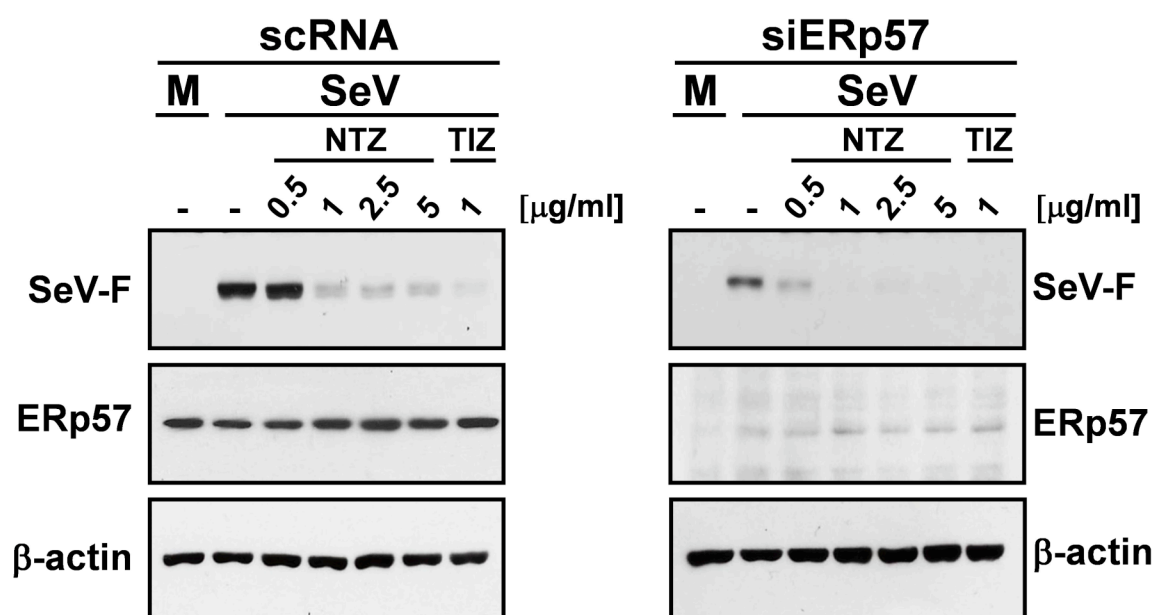
Supplementary Figure 5



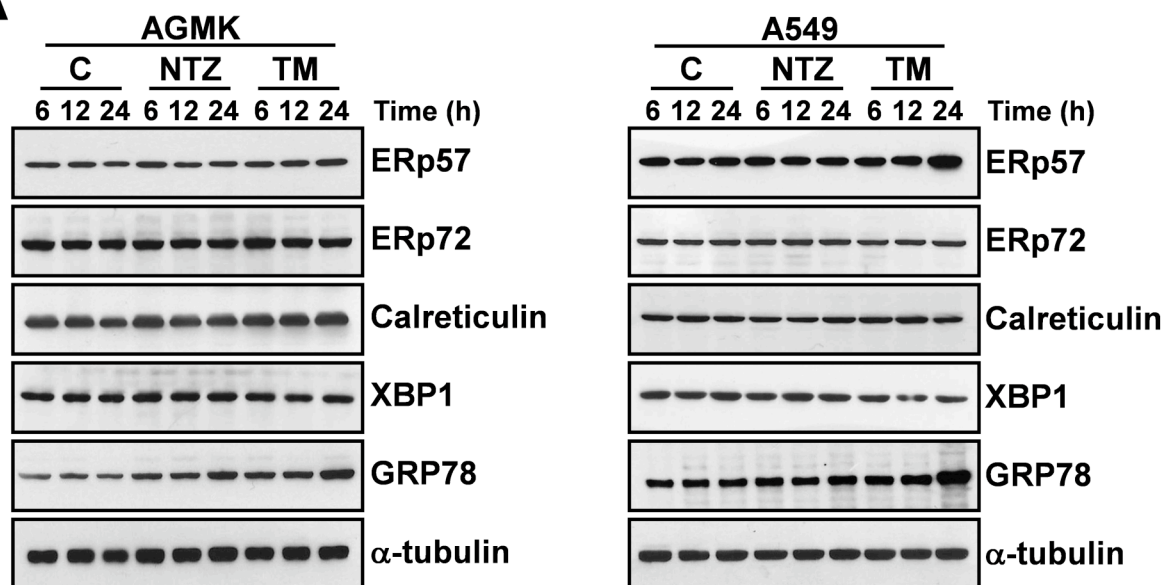
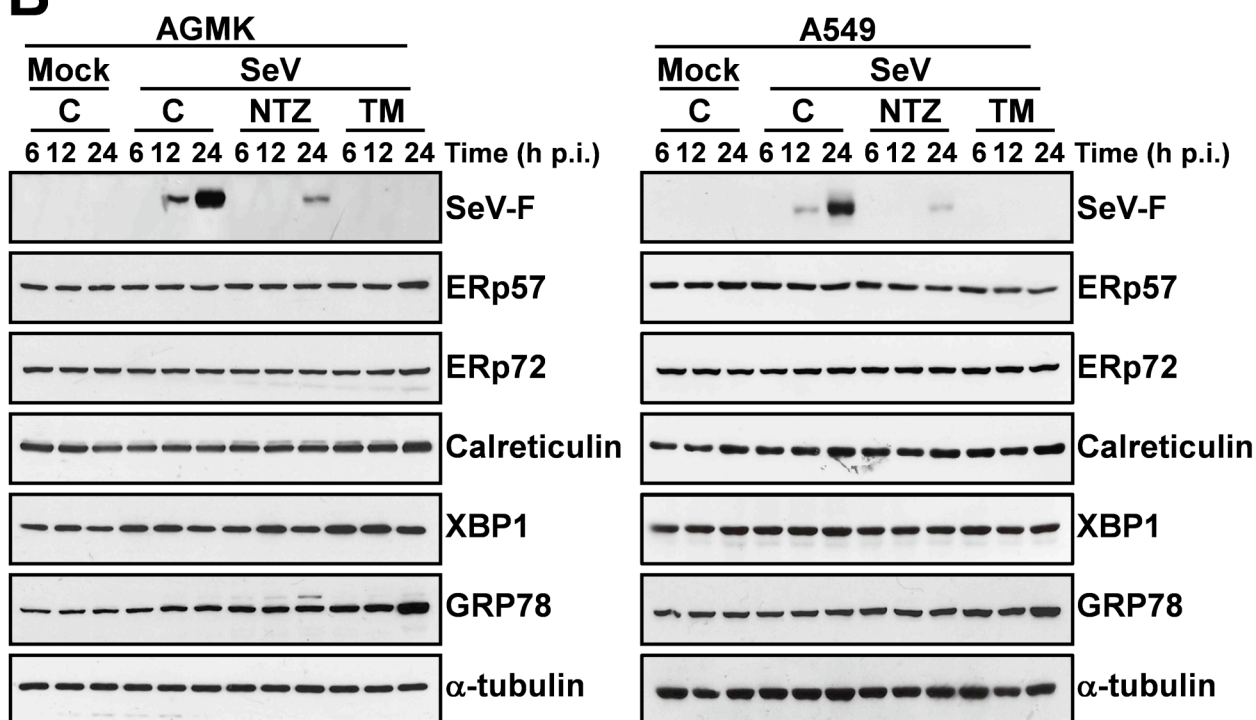
Supplementary Figure 6



Supplementary Figure 7



Supplementary Figure 8

A**B**

Supplementary Figure 9

Fig. 2-B

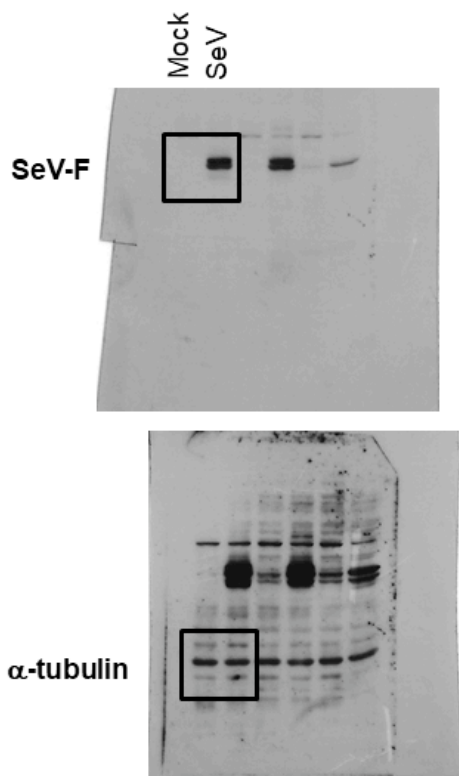
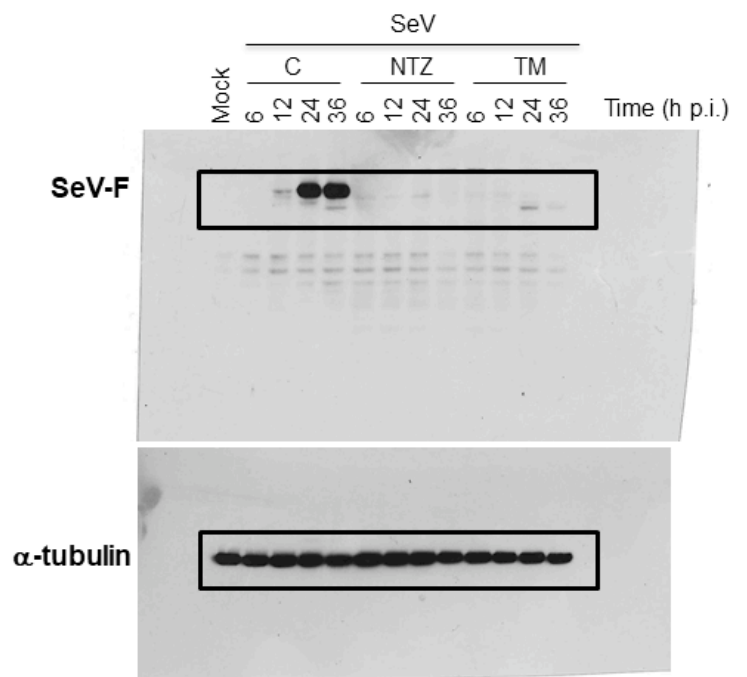
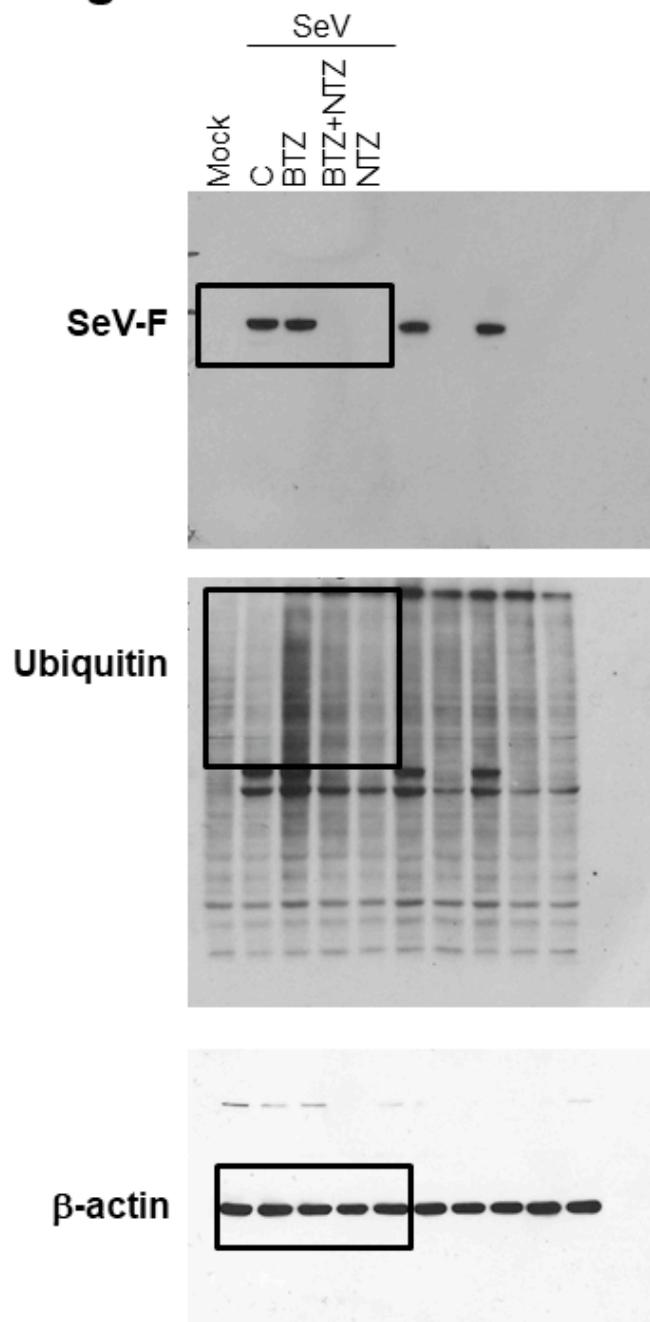


Fig. 2-C



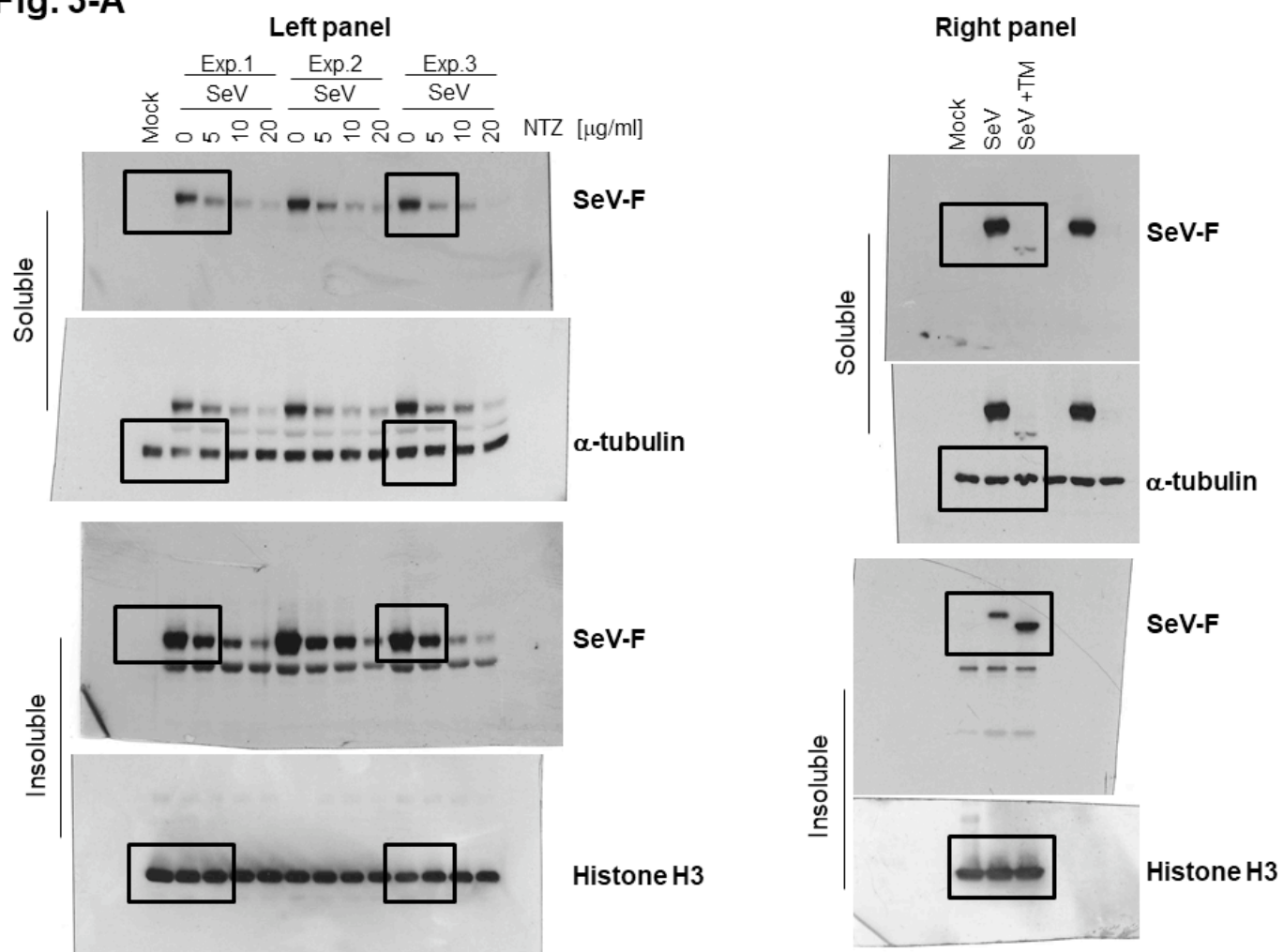
Supplementary Figure 10

Fig. 2-D



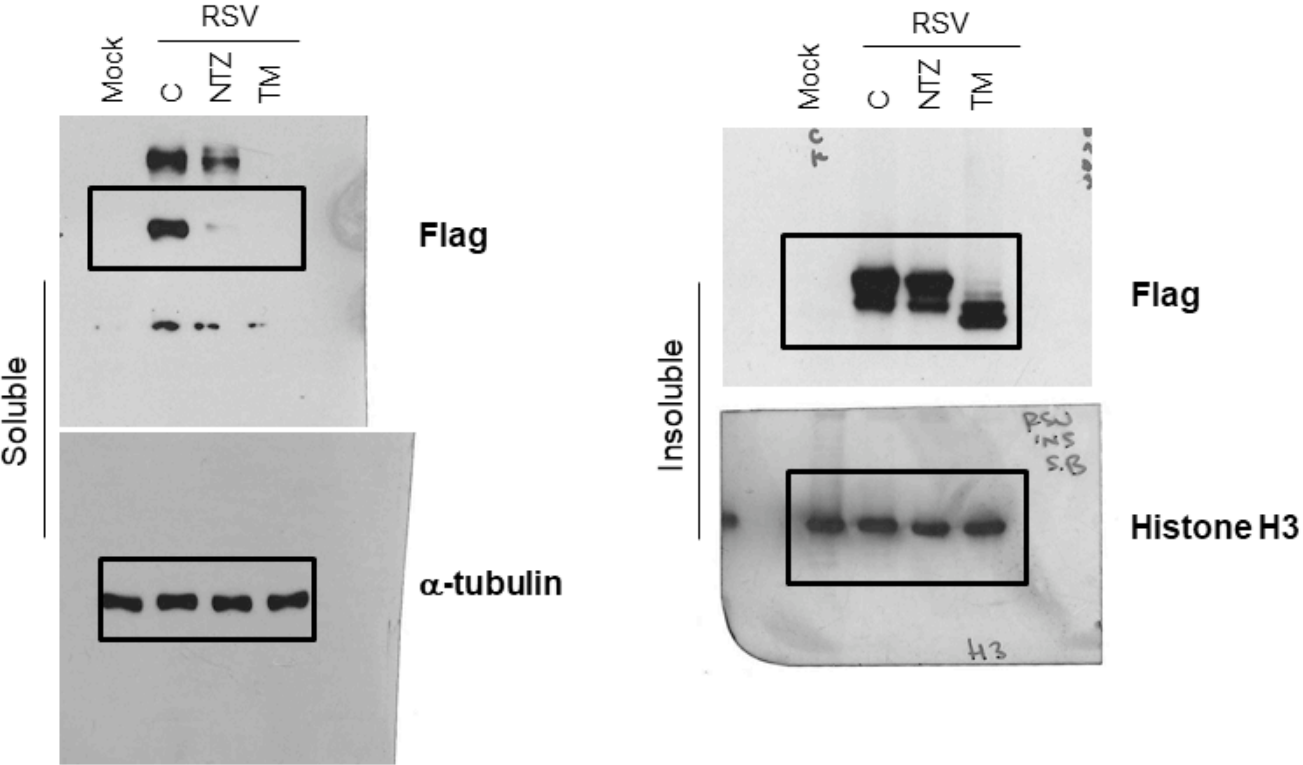
Supplementary Figure 11

Fig. 3-A



Supplementary Figure 12

Fig. 3-B



Supplementary Figure 13

Fig. 8-A

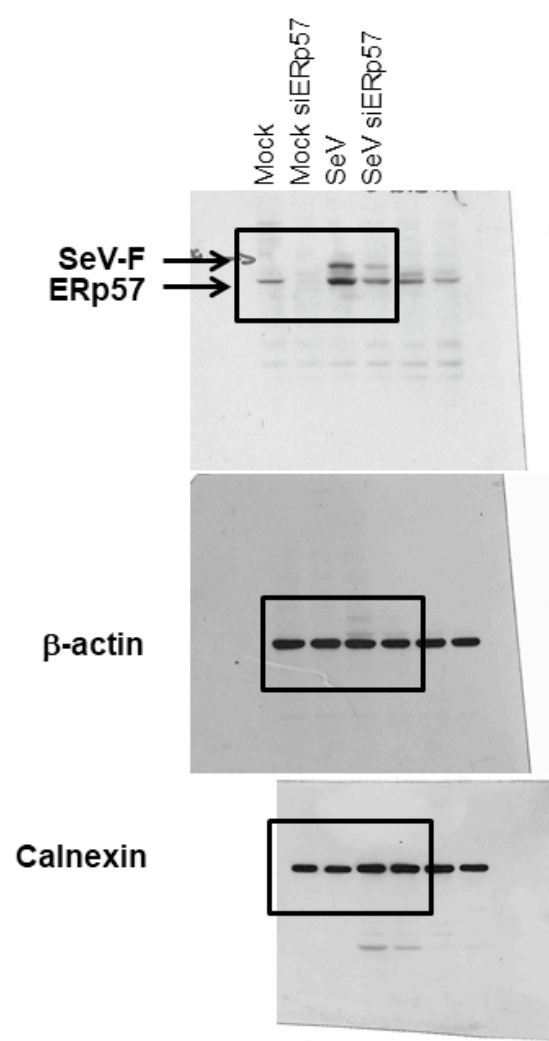
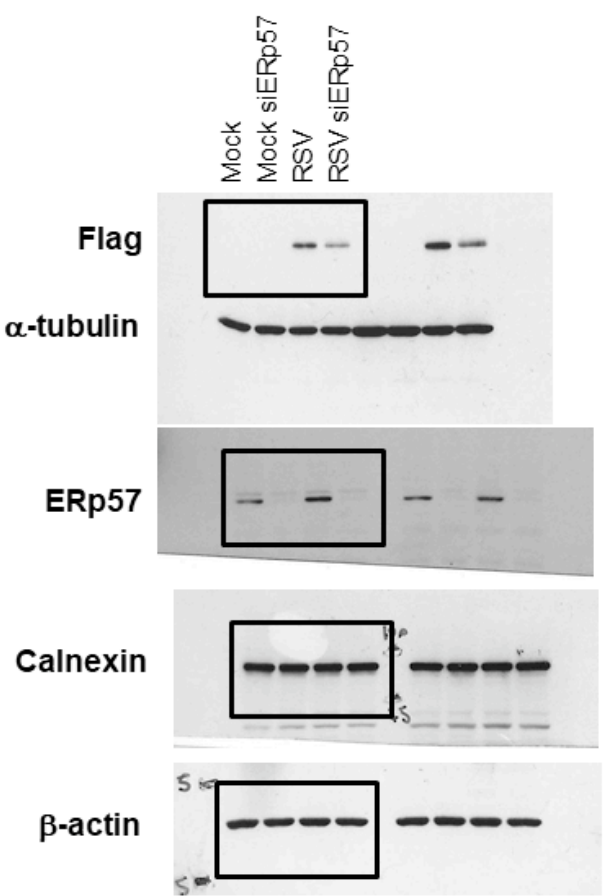
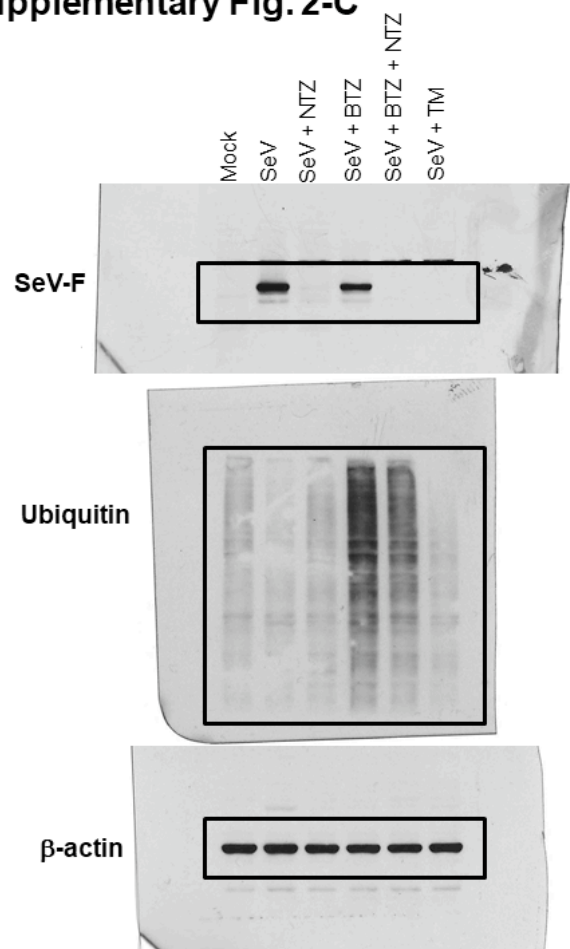


Fig. 8-B

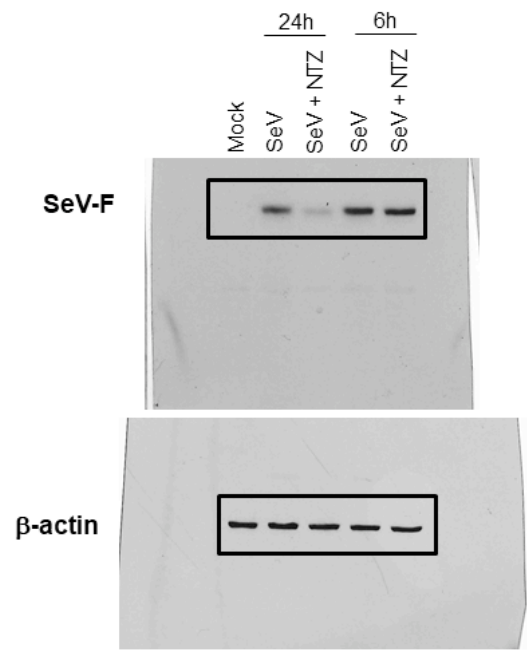


Supplementary Figure 14

Supplementary Fig. 2-C

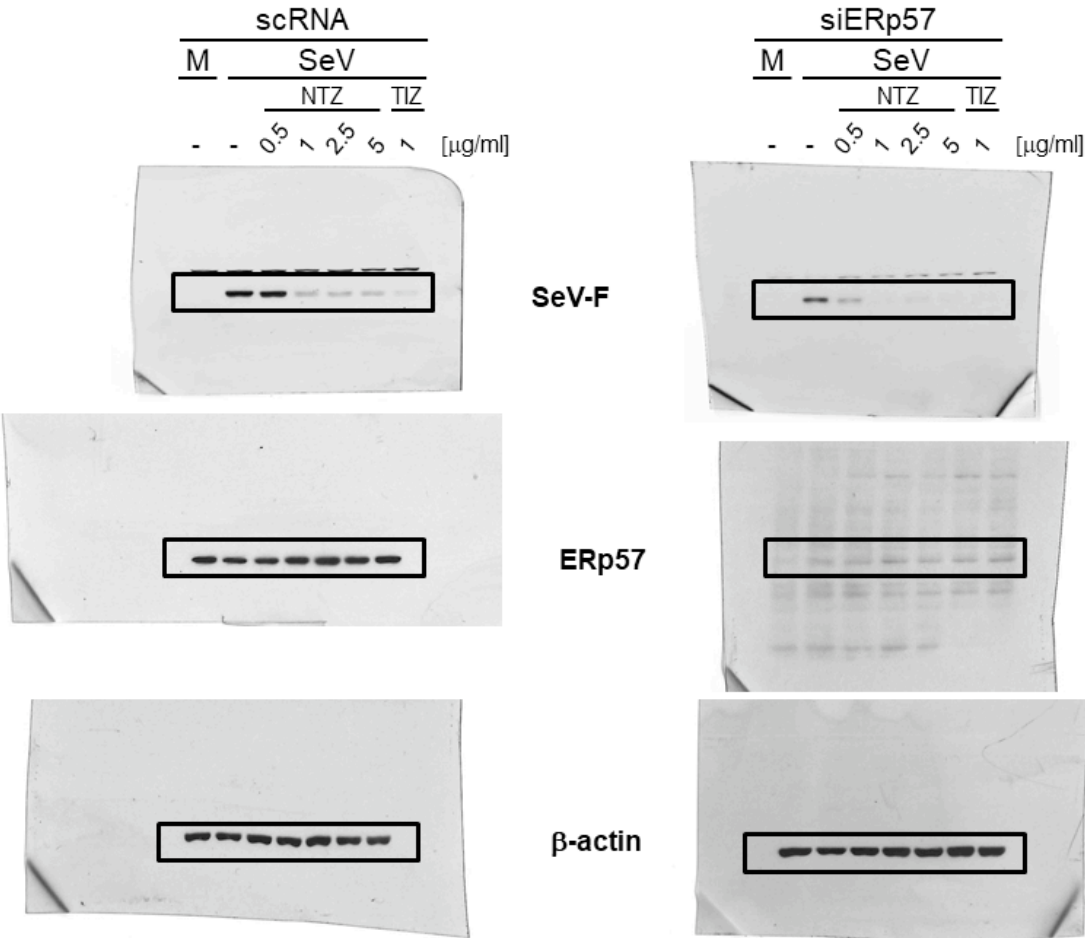


Supplementary Fig. 6-A



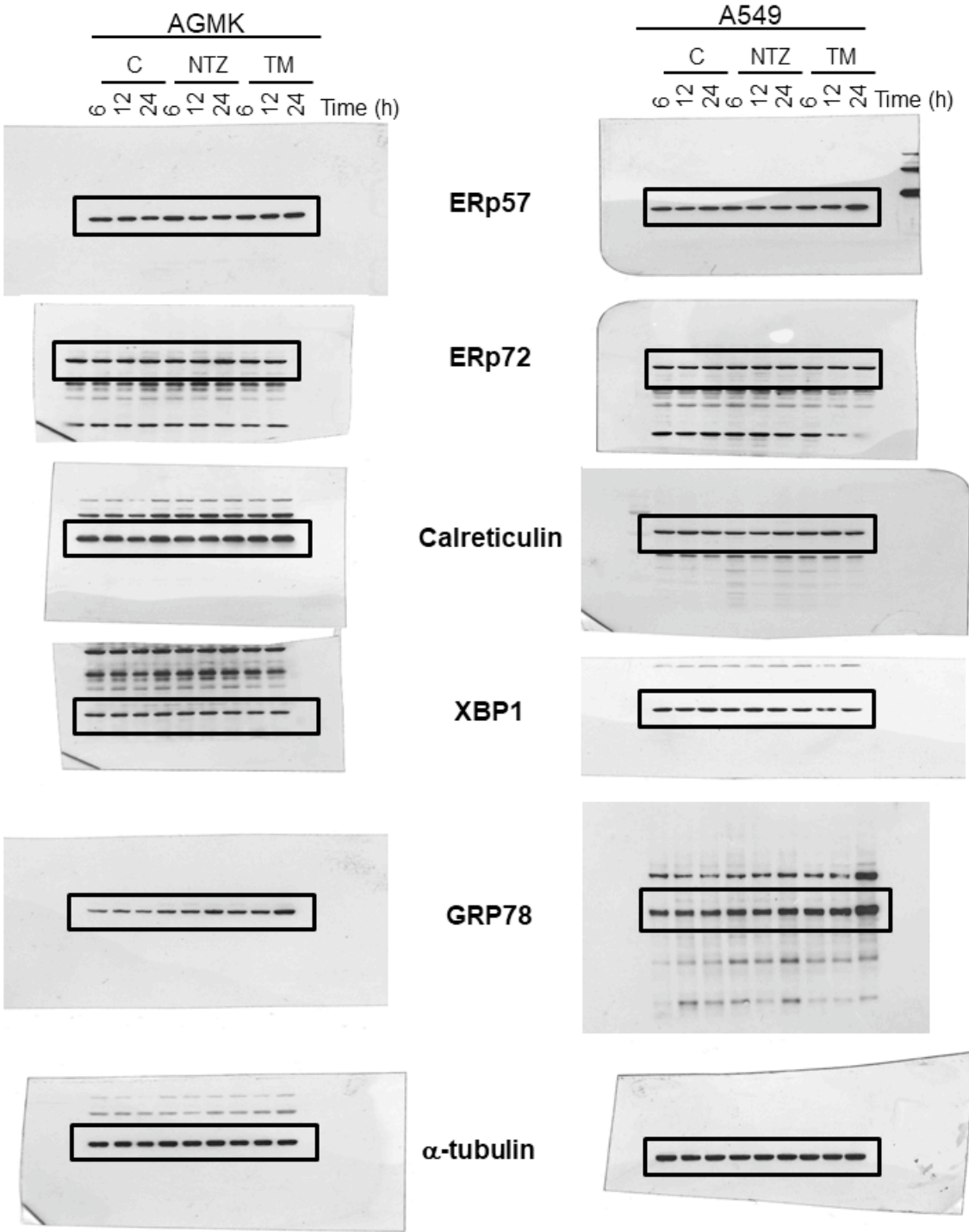
Supplementary Figure 15

Supplementary Fig. 8



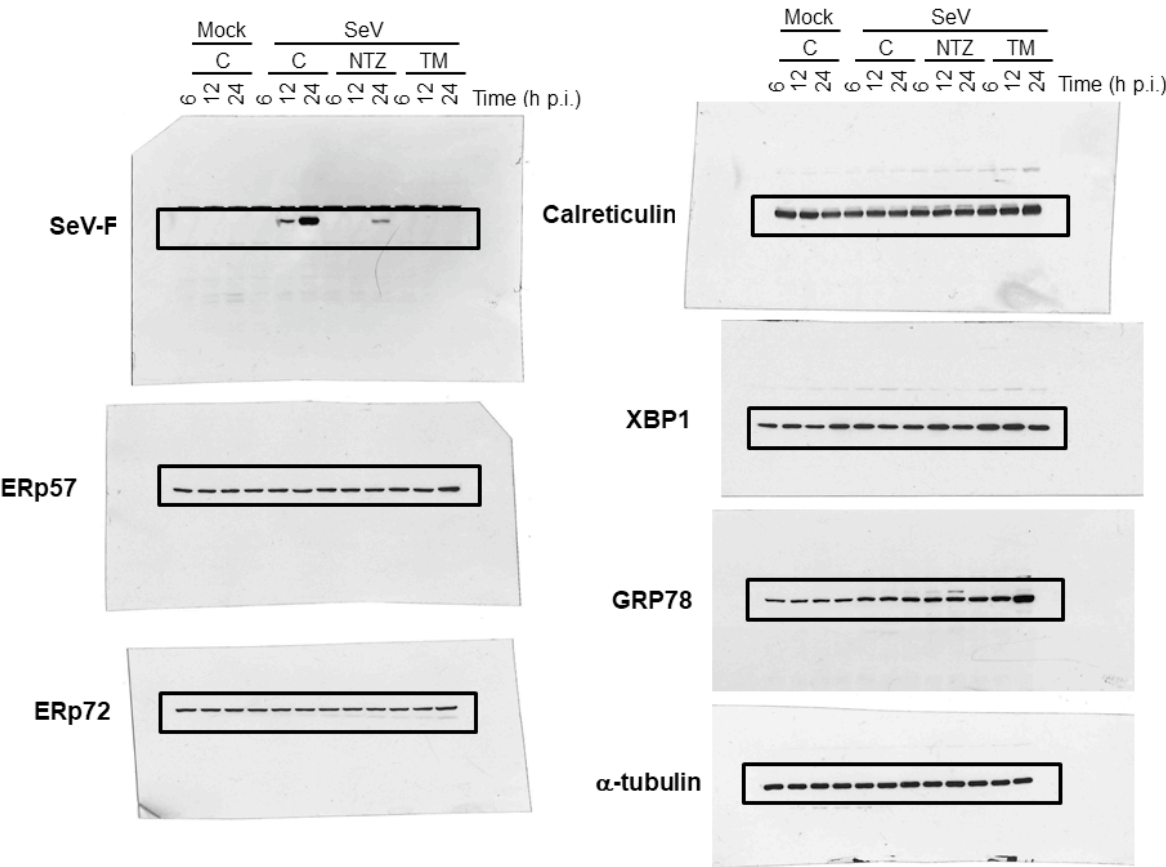
Supplementary Figure 16

Supplementary Fig. 9-A



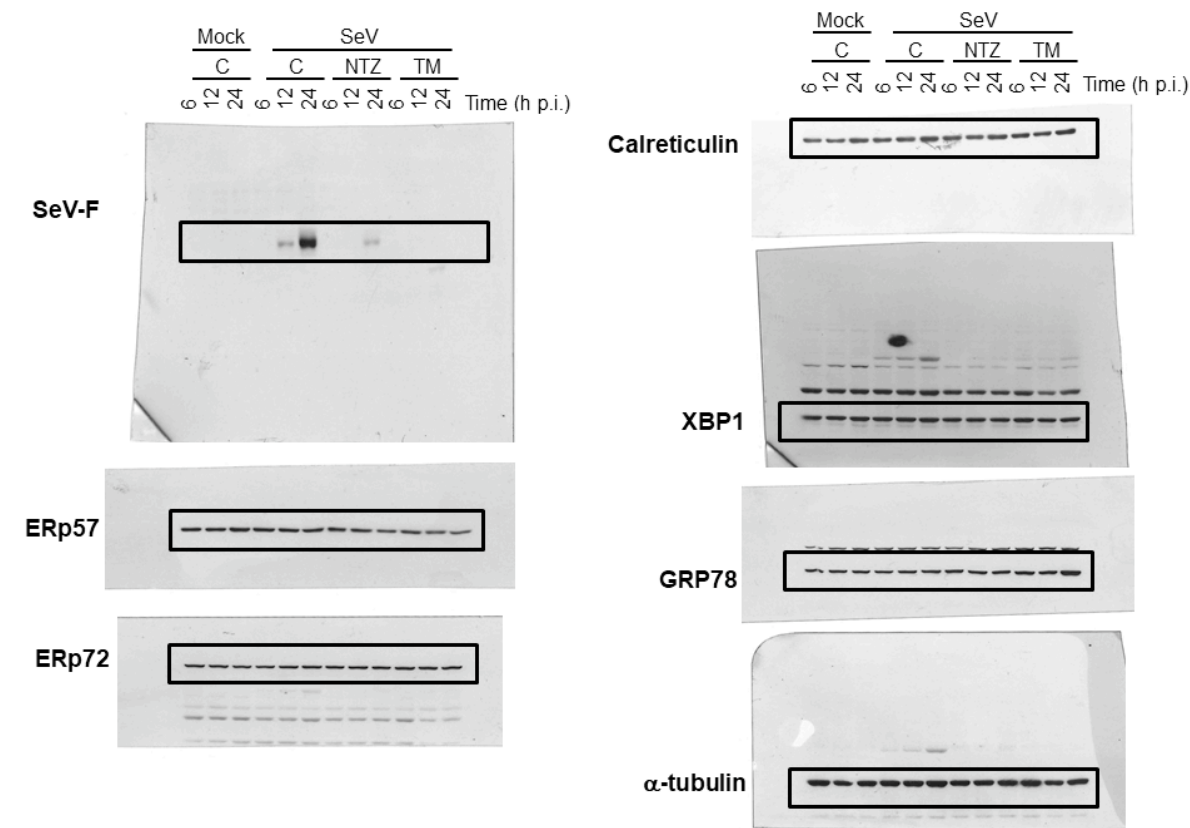
Supplementary Figure 17

Supplementary Fig. 9-B/AGMK



Supplementary Figure 18

Supplementary Fig. 9-B/A549



Supplementary Figure 19