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Ubiquitin ligase SYVN1/HRD1 facilitates degradation of the SERPINA1 Z variant/α-1-antitrypsin Z variant via SQSTM1/p62-dependent selective autophagy

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ABSTRACT

SERPINA1/AAT/α-1-antitrypsin (serpin family A member 1) deficiency (SERPINA1/ AAT-D) is an autosomal recessive disorder characterized by the retention of misfolded SERPINA1/AAT in the endoplasmic reticulum (ER) of hepatocytes and a significant reduction of serum SERPINA1/AAT level. The Z variant of SERPINA1/AAT, containing a Glu342Lys (E342K) mutation (SERPINA1^{E342K}/ATZ), the most common form of SERPINA1/AAT-D, is prone to misfolding and polymerization, which retains it in the ER of hepatocytes and leads to liver injury. Both proteasome and macroautophagy/autophagy pathways are responsible for disposal of SERPINA1^{E342K}/ATZ after it accumulates in the ER. However, the mechanisms by which SERPINA1^{E342K}/ATZ is selectively degraded by autophagy remain unknown. Here, we showed that ER membrane-spanning ubiquitin ligase (E3) SYVN1/HRD1 enhances the degradation of SERPINA1^{E342K}/ATZ through the autophagy-lysosome pathway. We found that SYVN1 promoted SERPINA1^{E342K}/ATZ, especially Triton X 100-insoluble SERPINA1^{E342K}/ATZ clearance. However, the effect of SYVN1 in SERPINA1^{E342K}/ATZ clearance was impaired after autophagy inhibition, as well as in autophagy-related 5 (*atg5)* knockout cells. On the contrary, autophagy induction enhanced SYVN1-mediated SERPINA1^{E342K}/ATZ degradation. Further study showed that SYVN1 mediated SERPINA1^{E342K}/ATZ ubiquitination, which is required for autophagic degradation of SERPINA1^{E342K}/ATZ by promoting the interaction between SERPINA1^{E342K}/ATZ and SQSTM1/p62 for formation of the autophagy complex. Interestingly, SYVN1-mediated lysine 48 (K48)-linked polyubiquitin chains that conjugated onto SERPINA1^{E342K}/ATZ might predominantly bind to the ubiquitin-associated (UBA) domain of SQSTM1 and couple the ubiquitinated SERPINA1^{E342K}/ATZ to the lysosome for degradation. In addition, autophagy inhibition attenuated the suppressive effect of SYVN1 on SERPINA1^{E342K}/ATZ cytotoxicity, and the autophagy inducer rapamycin enhanced the suppressive effect of SYVN1 on SERPINA1^{E342K}/ATZ-induced cell apoptosis. Therefore, this study proved that SYVN1 enhances SERPINA1^{E342K}/ATZ degradation through SQSTM1-dependent autophagy and attenuates SERPINA1^{E342K}/ATZ cytotoxicity.

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Introduction

SERPINA1/AAT/α-1-antitrypsin (serpin family A member 1), a member of the serpin peptidase inhibitors (SERPIN) family, is the most abundant circulating protease inhibitor found in human plasma. It is a 52-kDa glycoprotein synthesized predominantly by hepatocytes and secreted into the circulation to control excessive proteolytic activity of neutrophil elastase in the lower respiratory tract.[1, 2] SERPINA1/AAT-deficiency (SERPINA1/AAT-D) is one of the most common serious hereditary disorders in the world. AAT-D is widely known in populations of European ancestry, with an estimated prevalence of one case per 3000 to 5000 persons in the United States. AAT-D is also affects individuals in all racial subgroups worldwide.[3] The original and most common cause of SERPINA1/AAT-D is the Z variant of SERPINA1/AAT (SERPINA1^{E342K}/ATZ), which contains a single amino acid substitution of lysine for glutamic acid at position 342, which causes a destabilization of the tertiary structure, resulting in abnormality in the folding and polymerization of SERPINA1^{E342K}/ATZ.[4, 5] Accumulation of SERPINA1^{E342K}/ATZ polymers and insoluble aggregates in the endoplasmic reticulum (ER) of hepatocytes is primarily responsible for liver injury by gain-of-function proteotoxicity, such as transient juvenile hepatitis, hepatic fibrosis, and increased susceptibility to hepatocellular carcinomas (HCC).[5, 6] In addition, the retention of SERPINA1^{E342K}/ATZ in the ER causes a significant decrease in serum SERPINA1^{E342K}/ATZ levels. Within the lung, the loss-of-function of SERPINA1/AAT leading to dysregulated elastase

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activity is the main driver of emphysema and chronic obstructive pulmonary disease (COPD).[7, 8] Although liver transplantation remains the only definitive therapy for SERPINA1/AAT-D-associated liver disease until now, recent studies have been searching for the potential targets for disposal of the toxic protein aggregates by harnessing a cellular metabolic homeostasis mechanism.[9, 10] Therefore, elucidating the mechanisms by which hepatocytes degrade SERPINA1^{E342K}/ATZ may lead to identification of novel therapeutic options for liver disease due to SERPINA1/AAT-D.

Several intracellular pathways are required for the disposal of SERPINA1^{E342K}/ATZ after it accumulates in the ER.[8, 9, 11-15] Early studies show that the proteasome participates in intracellular degradation of SERPINA1^{E342K}/ATZ.[11-14] Indeed mutant SERPINA1^{E342K}/ATZ is one of the first identified substrates for the pathway termed as ER-associated degradation (ERAD).[12, 16] Like other misfolded ER proteins, disposal of SERPINA1^{E342K}/ATZ in the ER includes recognition by chaperones, retrotranslocation into the cytosol and ubiquitin-proteasome-dependent degradation.[17, 18] More than a dozen E3 ubiquitin ligases function in ERAD.[16-19] SYVN1/HRD1 (synoviolin 1), a mammalian ortholog of *Saccharomyces cerevisiae* Hrd1/Der3, is an ER membrane-anchored RING finger-containing ubiquitin ligase. Many studies have proved that SYVN1 is the major mediator of substrate ubiquitination during ERAD.[20] SYVN1 is also involved in the degradation of nonERAD substrates such as TP53 (tumor protein p53), ERN1 (endoplasmic reticulum to nucleus signaling 1), NFE2L1 (nuclear factor, erythroid 2 like 1), PRDM1 (PR/SET domain 1), MAPT (microtubule-associated protein tau), and polyglutamine expanded HTT (huntingtin).[21-26] Furthermore, SYVN1 is highly expressed in the rheumatoid synovium and considered a novel pathogenic factor for rheumatoid arthritis.[22, 27] A recent report shows that SYVN1 is indispensable for the dislocation of the null Hong Kong variant of SERPINA1/ATT (NHK) from the ER.[28] Our previous study also confirms that SYVN1 interacts with SERPINA1^{E342K}/ATZ and targets it for degradation by the proteasome.[29]

Nevertheless, there is enough evidence to prove that the proteasomal pathway could not completely account for the removal of SERPINA1^{E342K}/ATZ. Studies in human cell line models of SERPINA1/AAT-D first led to the recognition that autophagy is activated by intracellular accumulation of SERPINA1^{E342K}/ATZ, which alleviates or reverses hepatic fibrosis in SERPINA1/AAT-D mouse models.[9, 15, 30] Therefore, autophagy appears to be particularly important in SERPINA1/AAT-D in that autophagy is specifically activated and plays a key role in the removal of SERPINA1^{E342K}/ATZ. Conventionally, autophagy commonly refers to macroautophagy, which requires membrane trafficking and remodeling to form autophagosomes and delivers its internal contents to the lysosome for degradation.[10, 31] Although autophagy has been regarded as a random cytoplasmic degradation process, the involvement of ubiquitin as a targeting signal for selective autophagy is rapidly emerging.[32-34] Meanwhile, the selectivity is mediated by autophagy receptors, such as SQSTM1/p62 (sequestosome 1) and NBR1 (NBR1, autophagy cargo receptor), which interact with both ubiquitin conjugated to the target proteins and autophagosome-specific MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3), and promote autophagy.[35, 36] Therefore, we wondered if the ubiquitin ligase SYVN1 targets SERPINA1^{E342K}/ATZ for selective autophagy degradation. Here, we revealed that SYVN1 predominantly mediated lysine 48 (K48)-linked polyubiquitination of SERPINA1^{E342K}/ATZ and promoted the polyubiquitinated SERPINA1^{E342K}/ATZ to interact with ubiquitin-associated (UBA) domain of SQSTM1 for further autophagy degradation. In addition, we found that autophagy inhibition limited the suppressive effect of SYVN1 on SERPINA1^{E342K}/ATZ-induced cytotoxicity, while autophagy activation enhanced the suppressive effect of SYVN1 on SERPINA1^{E342K}/ATZ-induced cell apoptosis.

Results

Autophagy is involved in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation

Our previous study has shown that the proteasome is involved in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation.[29] Here we wondered whether SYVN1 targets SERPINA1^{E342K}/ATZ for autophagic degradation, at least in part. To answer this question, HEK293T cells were transfected with the plasmids as indicated in Fig. 1A and treated with the lysosome inhibitor NH_4Cl. We found that SYVN1 significantly reduced the total level of SERPINA1^{E342K}/ATZ by 47% (Fig. 1A and B, lane 5 vs lane 3), but it exerted only a minor impact on wild-type SERPINA1/AAT (Fig. 1A and B, lanes 4 vs lane 2), which is consistent with our previous findings.[29] These results indicate that SYVN1 is more likely to target mutant SERPINA1^{E342K}/ATZ for degradation. More remarkably, NH_4Cl treatment effectively diminished the effect of SYVN1 on SERPINA1^{E342K}/ATZ levels (Fig. 1A and B, lane 10 vs lane 5). The autophagic inhibitor chloroquine (CQ) also attenuated the effects of SYVN1 on SERPINA1^{E342K}/ATZ levels (Fig. S5D, lane 6 vs lane 2). However, the autophagy inducers Torin1 and rapamycin (Rapa) further decreased SERPINA1^{E342K}/ATZ levels in the presence of SYVN1 (Fig. 1C and D, lane 4 vs lane 2, lane 6 vs lane 2). Interestingly, autophagy-enhancing drug carbamazepine (CBZ) seemed to have little impact on SERPINA1^{E342K}/ATZ levels (Fig. 1C and D, lane 8 vs lane 2). To know whether downregulation of SERPINA1^{E342K}/ATZ levels by SYVN1 is due to the increase of SERPINA1^{E342K}/ATZ degradation, cycloheximide (CHX, 100 μg/ml) was used to inhibit new protein synthesis. We found that bafilomycin A_1 (Baf A1) treatment attenuated SERPINA1^{E342K}/ATZ degradation mediated by SYVN1 (Fig. 1E and F, lane 4 vs lane 2). On the contrary, EBSS that induces autophagy due to severe nutrient starvation enhanced the effect of SYVN1 on SERPINA1^{E342K}/ATZ degradation (Fig. 1E and F, lane 6 vs lane 2). To confirm the role of SYVN1 in autophagic SERPINA1^{E342K}/ATZ degradation, 3 *SYVN1* siRNA specifically targeting different sequences of human *SYVN1* were transfected into human embryonic kidneys (HEK) 293T cell line (Fig. 1G and H), and NH_4Cl was then used to inhibit lysosome activity. We found that SERPINA1^{E342K}/ATZ is more stable in *SYVN1* knockdown cells (Fig. 1 I and J, lane 2 vs lane 1). After NH_4Cl treatment for 6 h, the SERPINA1^{E342K}/ATZ level was increased in *SYVN1* knockdown cells compared with that in NC-siRNA control cells (Fig. 1I and J, lane 4 vs lane 3). These results imply that SYVN1 enhances autophagic SERPINA1^{E342K}/ATZ clearance.

We also investigated the role of *Atg5* (autophagy-related 5) in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation. We found SYVN1 significantly decreased SERPINA1^{E342K}/ATZ levels in

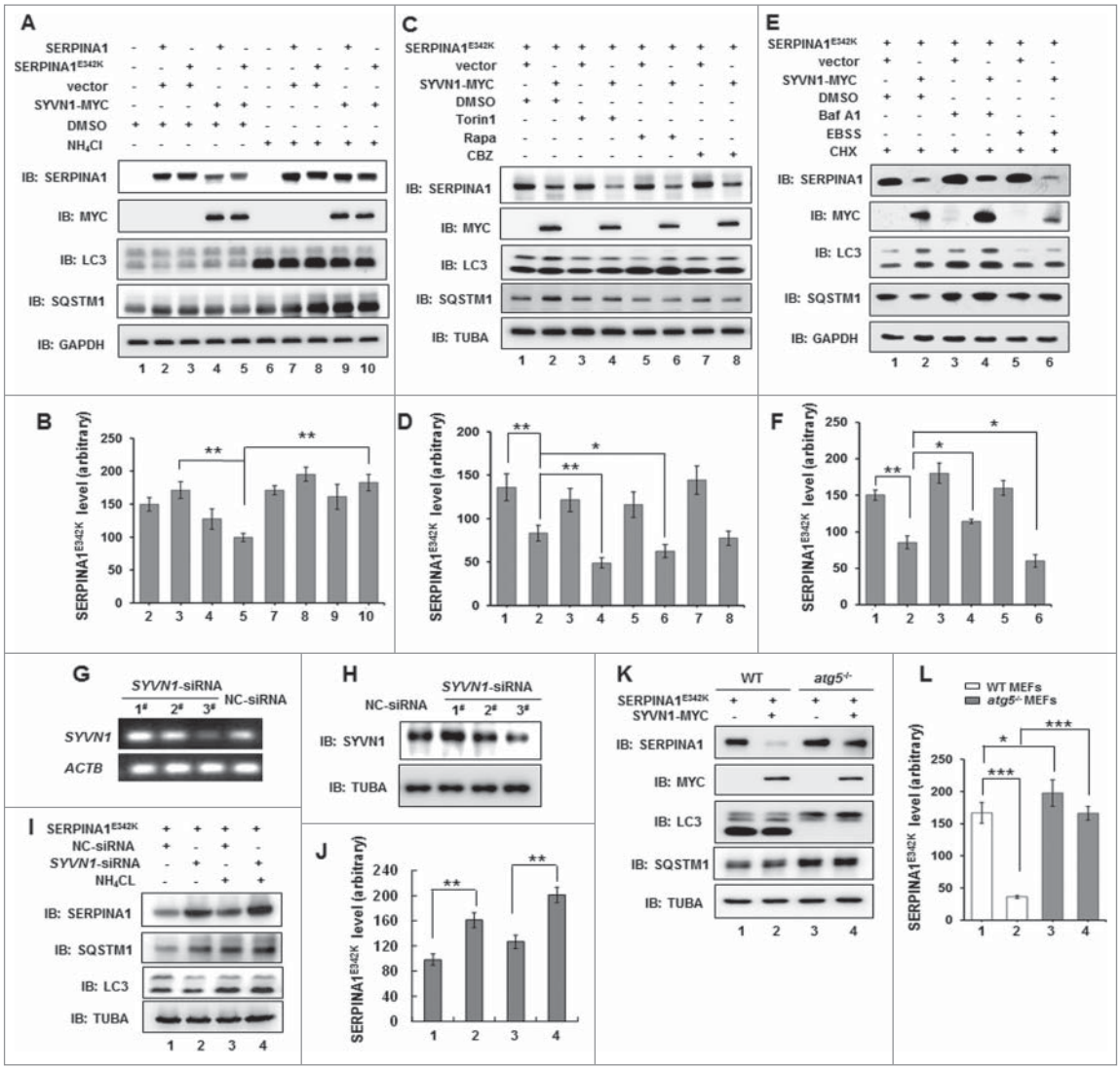

Figure 1. Autophagy is involved in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation. (A) Lysosome inhibitor NH_4Cl significantly inhibits the decrease of intracellular SERPINA1^{E342K}/ATZ induced by SYVN1. HEK 293T cells were transfected with the plasmids as indicated, and then treated with NH_4Cl (10 mg/ml) for 6 h. Cell lysates were analyzed by immunoblotting (IB) with the indicated antibodies. (B) Densitometric data of SERPINA1^{E342K}/ATZ and wild-type SERPINA1 levels in (A) were normalized to GAPDH. (C) Effects of autophagy inducers on SYVN1-mediated SERPINA1^{E342K}/ATZ degradation. HEK 293T cells were transfected with the plasmids as indicated, and then treated with Torin1 (20 nM), rapamycin (Rapa) (10 μM), or carbamazepine (CBZ) (100 μM) for 6 h. Cell lysates were analyzed by IB with the indicated antibodies. (D) Densitometric quantization of the SERPINA1^{E342K}/ATZ bands in (C). (E) Effects of autophagy inhibition and activation on SYVN1-mediated SERPINA1^{E342K}/ATZ degradation under the condition of SERPINA1^{E342K}/ATZ synthesis inhibition by cycloheximide (CHX). HEK 293T cells were transfected with the plasmids as indicated, and then incubated with CHX (100 μg/ml) and Earle balanced salt solution (EBSS) or bafilomycin A_1 (Baf A1) (20 nM) for 6 h. Cell lysates were analyzed by IB with the indicated antibodies. (F) Densitometric analysis of SERPINA1^{E342K}/ATZ levels in (E). (G and H) Identification of the effectiveness of *SYVN1* siRNAs. Endogenous SYVN1 expression was determined by RT-PCR (G) and IB (H) in HEK 293T cells at 48 h after transfection with 3 siRNAs targeting the *SYVN1* gene. (I) Lysosome inhibition causes SERPINA1^{E342K}/ATZ to be more stable in *SYVN1* knockdown cells. 293T cells were cotransfected with a SERPINA1^{E342K}/ATZ expression construct and *SYVN1*-siRNA, and then treated with NH_4Cl (10 mg/ml) for 6 h. The levels of SERPINA1^{E342K}/ATZ were analyzed by IB. (J) Quantitative data of (I). (K) *Atg5*-dependent autophagy promotes SYVN1-mediated SERPINA1^{E342K}/ATZ degradation. Wild-type (WT) and autophagy-deficient (*atg5*$^{-/-}$) MEFs were transfected with the indicated plasmids, and SERPINA1^{E342K}/ATZ levels were determined by IB. (L) Densitometric analysis of SERPINA1^{E342K}/ATZ levels in (K). All the quantitative data were presented as mean ± SD of at least 3 independent experiments. Band intensities were normalized to the corresponding loading controls. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. NC, negative control.

wild-type mouse embryonic fibroblasts (MEFs) (Fig. 1K and L, lane 2 vs lane 1), but this effect was mostly abolished in autophagy-deficient *atg5*$^{-/-}$ MEFs (Fig. 1K and L, lane 4 vs lane 2). All above results indicate that autophagy is involved in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation.

To directly observe autolysosomal localization of SERPINA1^{E342K}/ATZ facilitated by SYVN1, mCherry-GFP-SERPINA1^{E342K}/ATZ and SYVN1 plasmids were transfected into 293T cells, and the cells were treated with the vacuolar ATPase-inhibitor Baf A1 that blocks acidification of the lysosomes and thereby lysosomal degradation, but does not affect the fusion of autophagosomes with lysosomes. Because GFP is not stable in an acidic environment, mCherry-GFP-SERPINA1^{E342K}/ATZ in acidic vesicles displays red fluorescence only, and in neutral structures displays both green and red fluorescence (yellow puncta). By counting the fluorescent structures in cells, we found that red-only structures (mCherry-GFP-SERPINA1^{E342K}/ATZ in acidic vesicles) were about 38% in SYVN1-expressing cells, but only 11% left after the cells were treated with Baf A1 (Fig. S1A and B). Meanwhile, the yellow neutral structures were increased after treatment with Baf A1 (Fig. S1A and B). Consistently, after treatment with Baf A1 for 6 h, SYVN1 increased the colocalization between SERPINA1^{E342K}/

ATZ and LAMP1 (lysosomal-associated membrane protein 1), the major lysosomal membrane glycoprotein (Fig. S2A and B). These images strengthen our conclusion that SYVN1 facilitates SERPINA1^{E342K}/ATZ translocation to autolysosome.

SYVN1 is especially responsible for removal of insoluble SERPINA1^{E342K}/ATZ via the autophagy pathway

Our previous study shows that SYVN1 increases the solubility of SERPINA1^{E342K}/ATZ and mainly accelerates the detergent-insoluble SERPINA1^{E342K}/ATZ clearance.[29] To determine whether autophagy participates in SYVN1-mediated insoluble SERPINA1^{E342K}/ATZ clearance, we separated detergent-insoluble and -soluble fractions in the transfected cells as described previously.[29] It was shown that SERPINA1/AAT was only present in the soluble fraction whereas SERPINA1^{E342K}/ATZ was present in both the detergent-soluble and -insoluble fractions. SYVN1 significantly decreased the levels of insoluble SERPINA1^{E342K}/ATZ (Fig. 2A and B, lane 12 vs lane 10), but NH_4Cl treatment partially restored the decreased amount of insoluble SERPINA1^{E342K}/ATZ (Fig. 2A and B, lane 16 vs lane 12), even though NH_4Cl hardly affected soluble SERPINA1^{E342K}/ATZ levels (Fig. 2A and B, lane 8 vs lane 4). Another autophagy inhibitor, Baf A1, also exhibited a similar effect on insoluble SERPINA1^{E342K}/ATZ levels in SYVN1-expressing cells (Fig. 2C and D, lane 14 vs lane 13). More interestingly, the proteasome inhibitor MG132 also increased insoluble SERPINA1^{E342K}/ATZ levels in the presence of SYVN1, which further verified that the proteasome is also involved in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation (Fig. 2C and D, lane

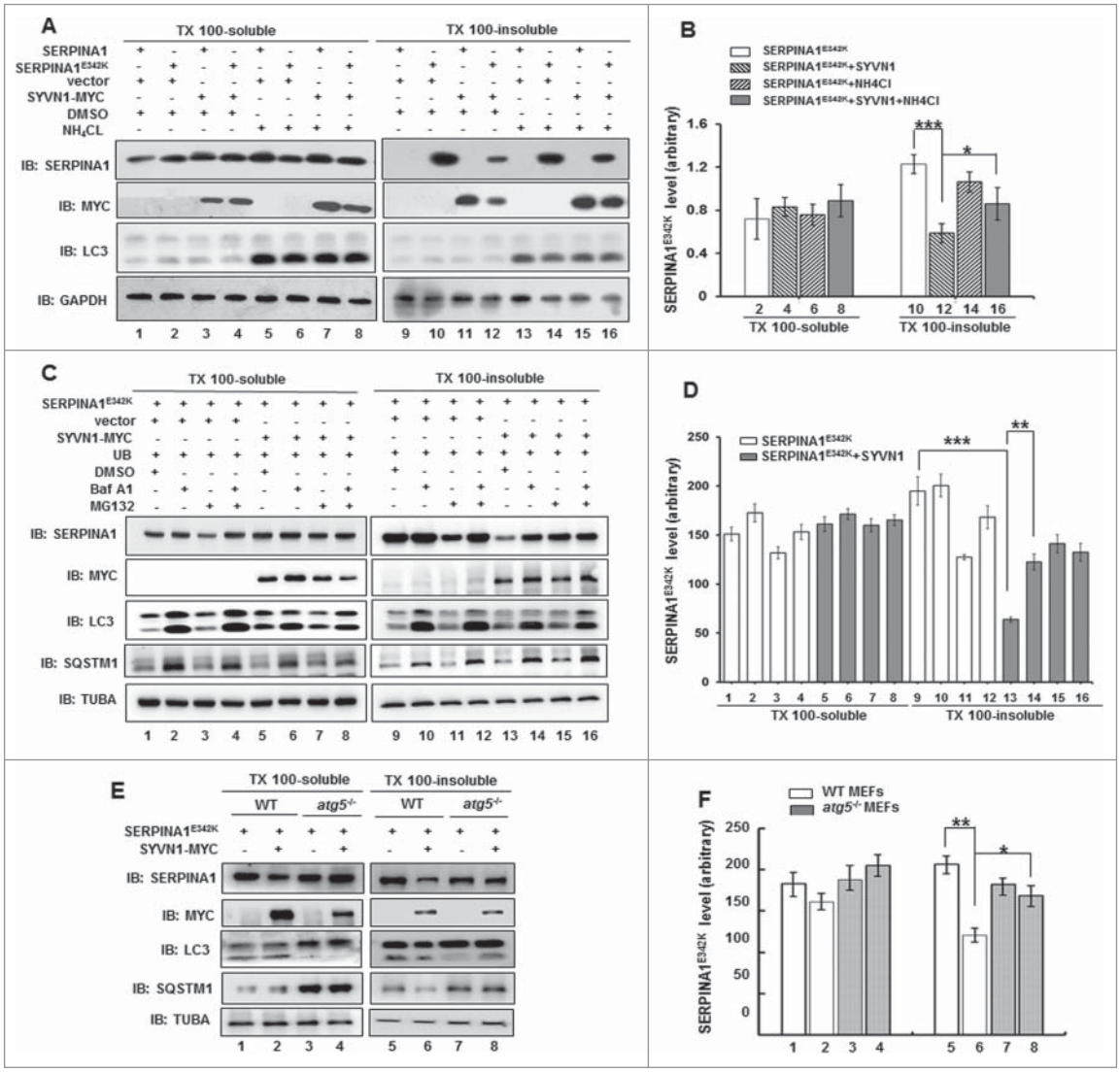

Figure 2. SYVN1 is especially responsible for removal of insoluble SERPINA1^{E342K}/ATZ via the autophagy pathway. (A) Autophagy inhibition restored the decrease of Triton X-100-insoluble SERPINA1^{E342K}/ATZ mediated by SYVN1. The transfected cells were lysed in RIPA buffer and the whole cell lysates were separated into the supernatant and pellet fractions. The levels of Triton X-100-soluble or -insoluble SERPINA1^{E342K}/ATZ were analyzed by IB. (B) The quantitative analysis of the SERPINA1^{E342K}/ATZ amount in (A). (C) Effects of lysosome and proteasome on SYVN1-mediated Triton X-100-insoluble SERPINA1^{E342K}/ATZ clearance. HEK 293T cells were transfected with the indicated plasmids, and then treated with Baf A1 (20 nM) and MG132 (20 μM) for 6 h. The levels of Triton X-100-soluble or -insoluble SERPINA1^{E342K}/ATZ were analyzed by IB with anti-SERPINA1/AAT antibody. (D) Quantitative data of SERPINA1^{E342K}/ATZ in (C). (E) Autophagy deficiency attenuates the effect of SYVN1 on insoluble SERPINA1^{E342K}/ATZ clearance. WT and *atg5*$^{-/-}$ MEFs were transfected with the indicated plasmids. The levels of Triton X-100-soluble or -insoluble SERPINA1^{E342K}/ATZ were analyzed by IB. (F) The quantitative analysis of the SERPINA1^{E342K}/ATZ amount in (E). All the quantitative data were presented as mean ± SD of at least 3 independent experiments. Band intensities were normalized to the loading control. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. TX 100, Triton X-100.

15 vs lane 13). *atg5*$^{-/-}$ MEFs were also used to confirm the effect of SYVN1 on insoluble SERPINA1^{E342K}/ATZ clearance. As shown in Fig. 2E and F, SYVN1 dramatically decreased insoluble SERPINA1^{E342K}/ATZ levels in WT MEFs (lane 5 vs lane 6), but SYVN1 hardly affected insoluble SERPINA1^{E342K}/ATZ levels in *atg5* knockout MEFs (lane 8 vs lane 7). Therefore, SYVN1 is mainly responsible for disposal of insoluble SERPINA1^{E342K}/ATZ via the autophagy pathway.

SYVN1-mediated SERPINA1^{E342K}/ATZ polyubiquitination is required for SERPINA1^{E342K}/ATZ degradation through the autophagy pathway

Our previous work demonstrates that SYVN1 acts as an E3 ubiquitin ligase that modifies SERPINA1^{E342K}/ATZ and enhances polyubiquitinated SERPINA1^{E342K}/ATZ degradation by the proteasome.[29] Moreover, protein aggregates tagged with ubiquitin are usually believed to be targeted selectively for autophagic degradation.[37] We are particularly interested in the issue of whether SYVN1-mediated SERPINA1^{E342K}/ATZ polyubiquitination is required for SERPINA1^{E342K}/ATZ degradation through the autophagy pathway. To clarify this issue, the E3 activity-deficient mutant SYVN1C1A plasmid (a mutation in the first 2 zinc-coordinating cysteine residues in the RING finger domain) was used for transfection into 293T cells. As predicted, Baf A1 inhibited the reduction of SERPINA1^{E342K}/ATZ mediated by SYVN1 (Fig. 3A and B, lane 5 vs lane 2), but it did not affect SERPINA1^{E342K}/ATZ levels in the cells transfected with SYVN1C1A (Fig. 3A and B, lane 6 vs lane 3), implying SYVN1 E3 activity is required for SERPINA1^{E342K}/ATZ degradation. To further explore the potential role of deubiquitination in SERPINA1^{E342K}/ATZ degradation through the autophagy pathway, the deubiquitinating enzyme ubiquitin specific peptidase 2, splice variant A (USP2A) was used to remove polyubiquitin chains from SERPINA1^{E342K}/ATZ. As shown in Fig. 3C and D, USP2A dramatically removed polyubiquitin chains (lanes 3 and 4) and also inhibited the reduction of SERPINA1^{E342K}/ATZ induced by SYVN1 (lane 4 vs lane 2), suggesting that ubiquitination of SERPINA1^{E342K}/ATZ is required for its subsequent degradation. These results provide a strong evidence for functional E3 activity of SYVN1 that is a prerequisite for SERPINA1^{E342K}/ATZ degradation through the autophagy pathway.

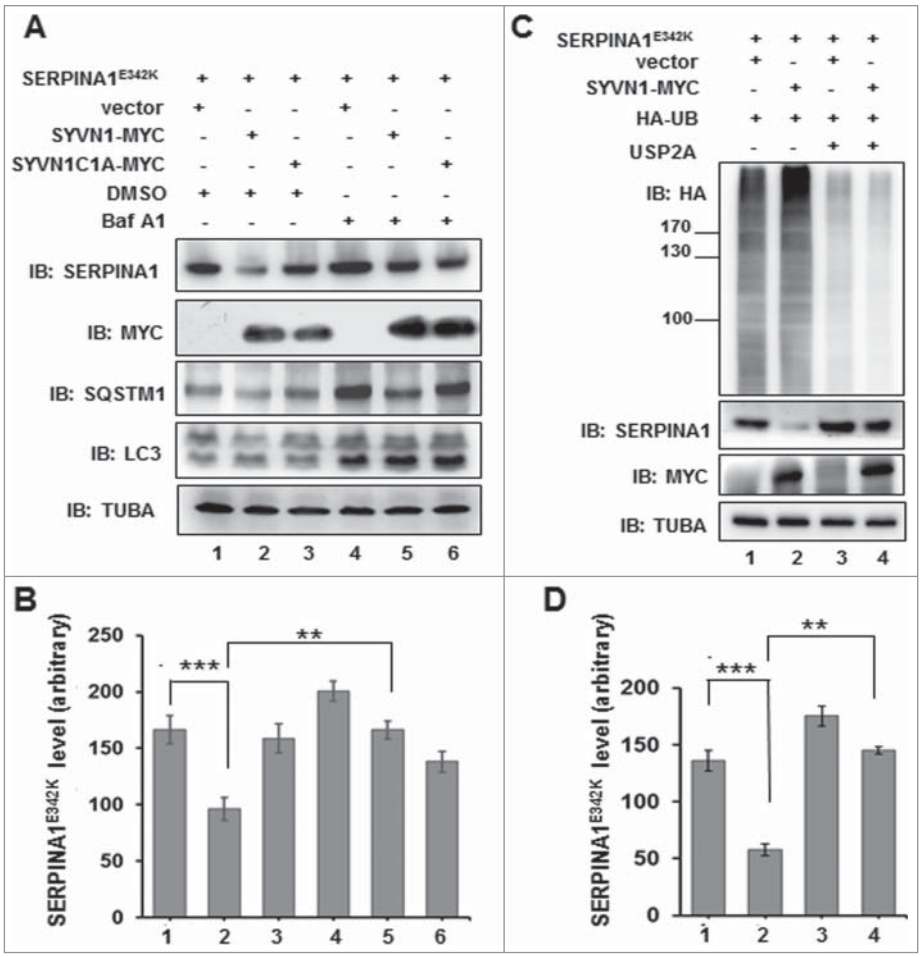

Figure 3. SYVN1-mediated SERPINA1^{E342K}/ATZ polyubiquitination is required for SERPINA1^{E342K}/ATZ degradation through the autophagy pathway. (A) SYVN1 E3 activity is required for lysosome targeting SERPINA1^{E342K}/ATZ degradation. The transfected cells were treated with Baf A1 (20 nM) for 6 h before lysis and the whole lysates were analyzed by IB with the indicated antibodies. (B) Densitometric analysis of SERPINA1^{E342K}/ATZ levels in (A). (C) SERPINA1^{E342K}/ATZ deubiquitination almost abolishes SYVN1-mediated SERPINA1^{E342K}/ATZ degradation. HEK 293T cells were transfected with SERPINA1^{E342K}/ATZ, SYVN1-MYC, HA-ubiquitin, and FLAG-USP2A plasmids. SERPINA1^{E342K}/ATZ and ubiquitin levels were determined by IB with anti-SERPINA1/AAT and anti-HA antibodies, respectively. (D) Quantitative data of (C). All the quantitative data were expressed as mean ± SD from at least 3 independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. UB, ubiquitin.

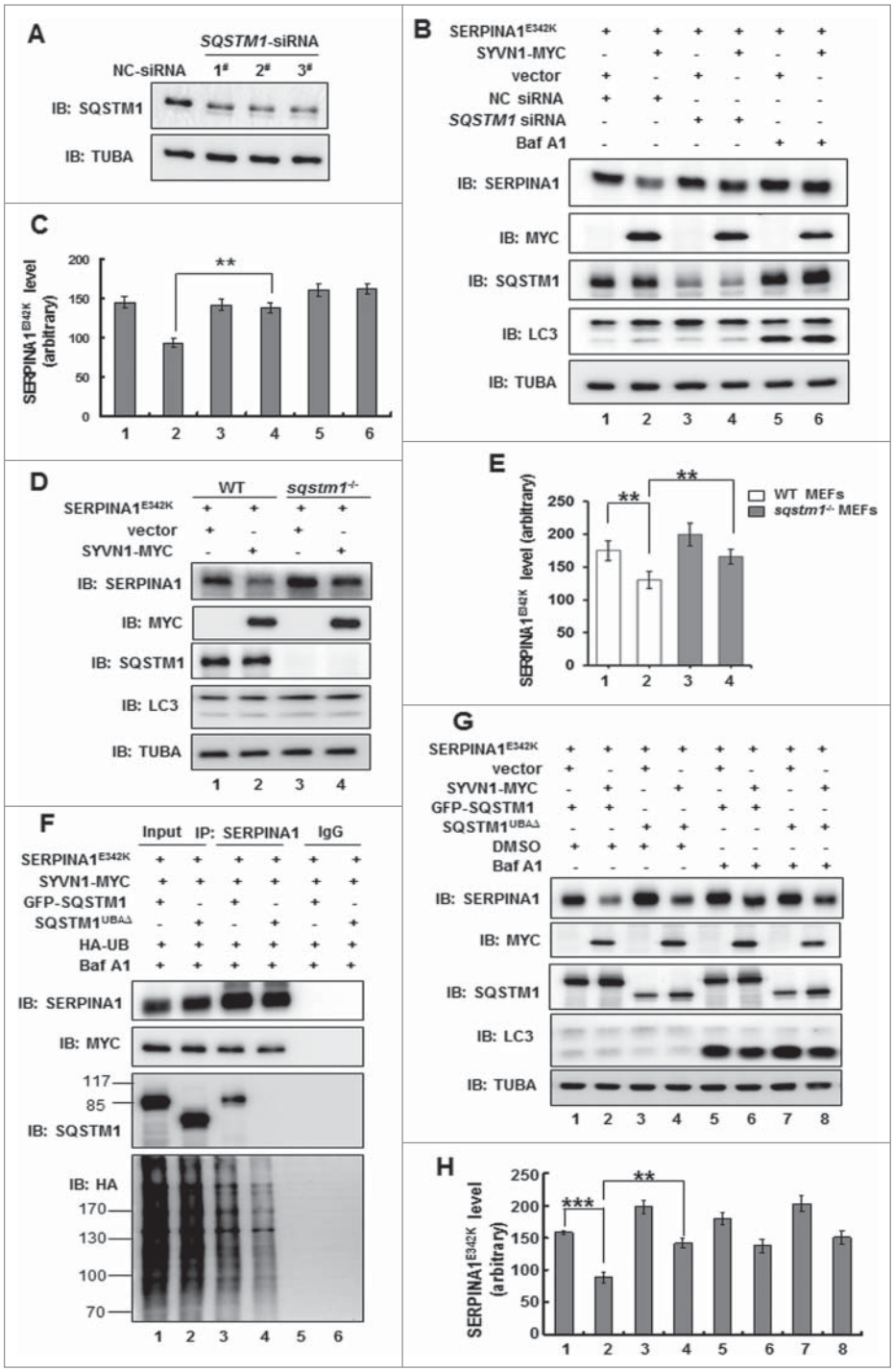

Figure 4. SQSTM1 is involved in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation by lysosomes. (A) Identifying the effectiveness of siRNAs for *SQSTM1* knockdown. HEK 293T cells were harvested at 48 h post transfection with 3 *SQSTM1* siRNAs and subjected to IB with anti-SQSTM1 antibody. (B) *SQSTM1* knockdown stabilizes SERPINA1^{E342K}/ATZ levels. HEK 293T cells were transfected with the indicated plasmids and *SQSTM1* siRNA and then treated with Baf A1 (20 nM) for 6 h. The proteins were detected by IB with the corresponding antibodies. (C) Band densitometry of SERPINA1^{E342K}/ATZ levels in (B) was quantified. (D) *sqstm1* knockout attenuated SYVN1-mediated SERPINA1^{E342K}/ATZ degradation. WT and *sqstm1* knockout MEFs were transfected with the indicated plasmids, and the levels of SERPINA1^{E342K}/ATZ were determined by IB. (E) Densitometric analysis of SERPINA1^{E342K}/ATZ levels in (D). (F) The UBA domain of SQSTM1 is required for the interaction between SQSTM1 and ubiquitinated SERPINA1^{E342K}/ATZ modified by SYVN1. HEK 293T cells were transfected with the plasmids expressing the full-length SQSTM1, or the SQSTM1$^{UBA\Delta}$ mutant, SYVN1-MYC, and HA-ubiquitin. The interaction between polyubiquitinated SERPINA1^{E342K}/ATZ and SQSTM1 was detected by coIP with anti-SERPINA1/AAT antibody followed by IB with anti-SQSTM1 and anti-HA antibody. (G) The UBA domain of SQSTM1 is involved in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation by the lysosome. The transfected cells were treated with Baf A1 (20 nM) for 6 h and then subjected to IB with anti-SERPINA1/AAT antibody. (H) Quantitative analysis of the SERPINA1^{E342K}/ATZ levels in (G). All the quantitative data were represented as mean ± SD of at least 3 independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. NC, negative control.

SQSTM1 is involved in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation via the autophagy pathway

Since autophagy receptors play an essential role in selective autophagy as scaffolding proteins for recognizing ubiquitinated substrates, we want to determine whether the classical autophagy receptor SQSTM1 is involved in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation. To answer this question, we first designed siRNAs to knockdown endogenous *SQSTM1* and observed whether SYVN1-mediated SERPINA1^{E342K}/ATZ degradation was affected by the absence of SQSTM1. As shown in Fig. 4A, endogenous SQSTM1 expression was effectively inhibited with *SQSTM1*-siRNAs. *SQSTM1* knockdown significantly reversed the decrease of SERPINA1^{E342K}/ATZ induced by SYVN1 (Fig. 4B and C, lane 4 vs lane 2). Similar results were observed in *sqstm1*knockout MEFs. *sqstm1* knockout reduced the effect of SYVN1 on SERPINA1^{E342K}/ATZ degradation compared with WT MEFs (Fig. 4D and E, lane 4 vs lane 2). The above results suggest that SQSTM1 is involved in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation. To figure out the functional domains of SQSTM1 involved in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation, we used a GFP-tagged SQSTM1$^{UBA\Delta}$ plasmid, a SQSTM1 mutant with the deletion of the C-terminal ubiquitin-associated (UBA) domain that can bind noncovalently to ubiquitin and recruit ubiquitinated proteins. We found that the full-length SQSTM1 interacted with ubiquitinated-SERPINA1^{E342K}/ATZ (Fig. 4F, lane 3), while SQSTM1$^{UBA\Delta}$ lost the ability to form a complex with ubiquitinated-SERPINA1^{E342K}/ATZ (Fig. 4F, lane 4). In addition, the SERPINA1^{E342K}/ATZ level was increased in the cells transfected with SQSTM1$^{UBA\Delta}$ compared with WT SQSTM1 plasmid (Fig. 4 G and H, lane 4 vs lane 2). These results suggested that the UBA domain of SQSTM1 is required for SYVN1-mediated SERPINA1^{E342K}/ATZ autophagic degradation.

SYVN1 promotes the interaction between SERPINA1^{E342K}/ATZ and SQSTM1

As mentioned above, the UBA domain of SQSTM1 is required for the interaction between SERPINA1^{E342K}/ATZ and SQSTM1, we wondered whether SYVN1 has some effects on the interaction between SERPINA1^{E342K}/ATZ and SQSTM1. As shown in Fig. 5A, SYVN1 increased the interaction between Lys48-linked ubiquitinated SERPINA1^{E342K}/ATZ and SQSTM1 after autophagy inhibition with Baf A1 (lane 6 vs lane 5). However, we did not find that the interaction between SERPINA1^{E342K}/ATZ and SQSTM1 was enhanced by SYVN1, although SYVN1 also increased Lys48-linked ubiquitination of SERPINA1^{E342K}/ATZ after proteasome inhibition with MG132 (Fig. S3, lane 6 vs lane 5). To further confirm the effect of SYVN1 on the interaction of SQSTM1 and SERPINA1^{E342K}/ATZ, SQSTM1 was immunoprecipitated with anti-SQSTM1 antibody and the binding of SERPINA1^{E342K}/ATZ was detected. As predicted, SYVN1 also increased the interaction between SERPINA1^{E342K}/ATZ and SQSTM1 (Fig. 5B, lane 6 vs lane 5). More interestingly, SYVN1 was pulled down with anti-SERPINA1/AAT antibody (Fig. 5A, lane 6), but not with anti-SQSTM1 antibody (Fig. 5B, lane 6), suggesting there is no direct interaction between SYVN1 and SQSTM1. Consistent with this result, the intensive colocalization of SERPINA1^{E342K}/ATZ and SQSTM1 was observed in the SYVN1-expressing cells after treatment with Baf A1 (Fig. 5C and D, indicated by white arrows), but few in the cells expressing only SERPINA1^{E342K}/ATZ. Similarly, we observed the colocalization of SERPINA1^{E342K}/ATZ and SYVN1 (Fig. 5C and D, indicated by yellow arrows), but there is no colocalization of SYVN1 and SQSTM1. These results indicate that SYVN1 promotes the interaction between SERPINA1^{E342K}/ATZ and SQSTM1 by interacting with SERPINA1^{E342K}/ATZ, but not directly interacting with SQSTM1.

K48-linked polyubiquitination mediated by SYVN1 promotes the interaction between SERPINA1^{E342K}/ATZ and SQSTM1

To examine whether SYVN1-mediated polyubiquitination is required for the interaction between SERPINA1^{E342K}/ATZ and SQSTM1, the deubiquitinating enzyme USP2A was used to remove polyubiquitin chains from SERPINA1^{E342K}/ATZ. It was found that USP2A completely blocked the SERPINA1^{E342K}/ATZ-SQSTM1 interaction, but it did not affect the SERPINA1^{E342K}/ATZ-SYVN1 interaction (Fig. 6A, lane 4), suggesting that ubiquitin-conjugated SERPINA1^{E342K}/ATZ modified by SYVN1 is critical for the interaction between SERPINA1^{E342K}/ATZ and SQSTM1. We further want to investigate which type of polyubiquitin chains linked to SERPINA1^{E342K}/ATZ is involved in SYVN1-mediated SERPINA1^{E342K}/ATZ-SQSTM1 interaction. To answer this question, the WT ubiquitin and the mutants K48 (containing only one lysine on lysine 48), K63 (containing only one lysine on lysine 63), K48R (substitution of lysine with arginine on lysine 48), and K63R (substitution of lysine with arginine on lysine 63) expression constructs were used (Fig. 6B). As shown in Fig. 6C, SYVN1 predominantly mediated formation of K48-linked SERPINA1^{E342K}/ATZ polyubiquitin chains under autophagy inhibition (lane 3), which is consistent with the results in Fig. 5A. More importantly, overexpressing the K63 mutant, but not the K48 mutant, abolished the interaction between SERPINA1^{E342K}/ATZ and SQSTM1 (Fig. 6C, lane 4). Similarly, substitution of lysine with arginine on lysine 48 (K48R mutation, which prevents the formation of K48-linked polyubiquitin chains) totally abolished SERPINA1^{E342K}/ATZ-SQSTM1 interaction (Fig. 6C, lane 5). However, the interaction of SERPINA1^{E342K}/ATZ-SQSTM1 was still observed using the K63R mutant containing a functional K48 residue (Fig. 6C, lane 6). Consistent with these results, the intensive colocalization of SERPINA1^{E342K}/ATZ aggregates and Lys48-linked polyubiquitin, not Lys63-linked polyubiquitin, was observed in SYVN1-expressing cells after treatment with Baf A1 (Fig. 6D, Fig S4). These results suggest that SYVN1-mediated K48-linked polyubiquitin chains are required for the SERPINA1^{E342K}/ATZ-SQSTM1 interaction.

Autophagy inhibition reverses the suppressive effect of SYVN1 on SERPINA1^{E342K}/ATZ-induced cytotoxicity

As our previous study has indicated, SYVN1 plays a suppressive effect on SERPINA1^{E342K}/ATZ-induced cytotoxicity.[29]

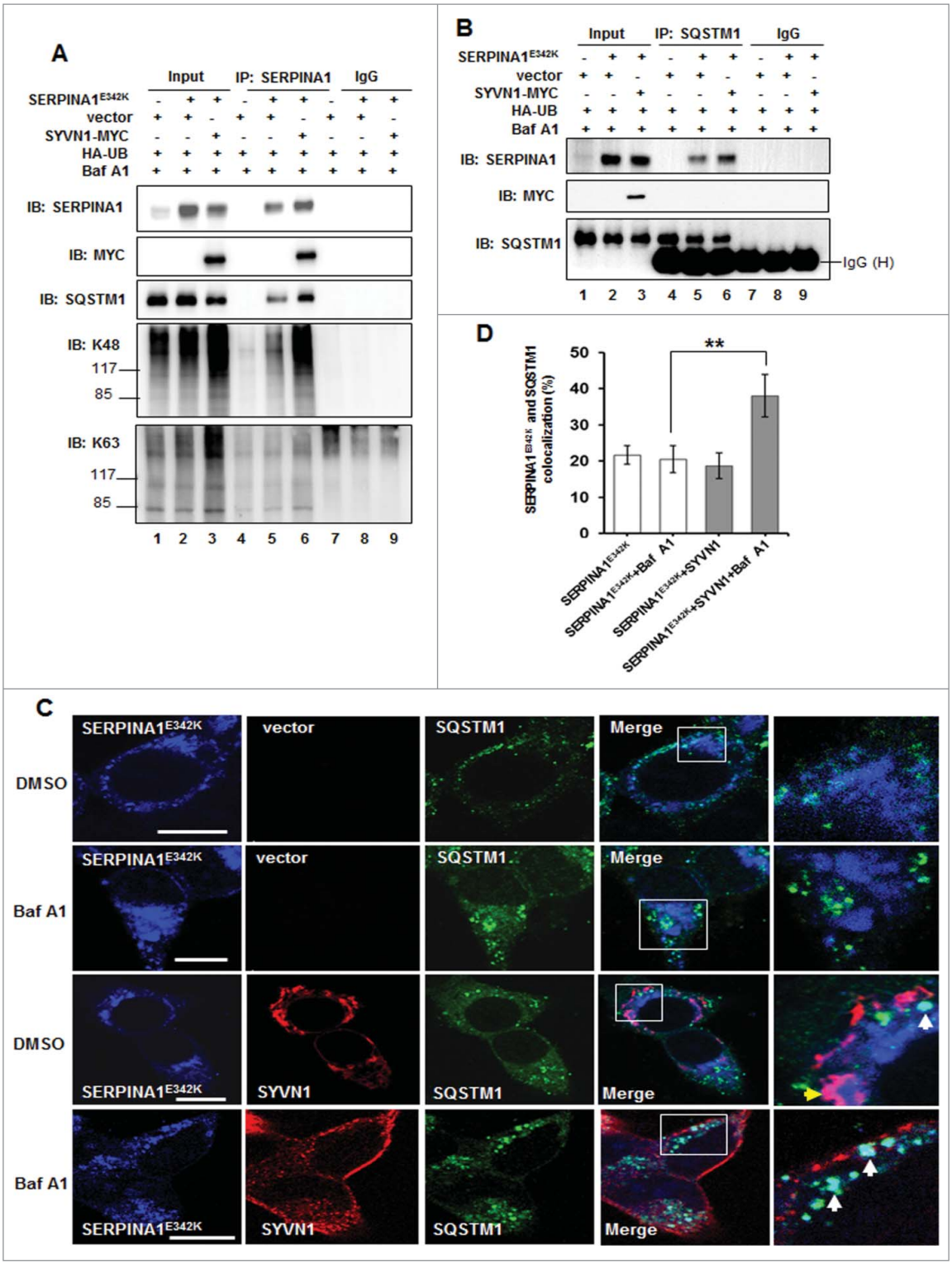

Figure 5. SYVN1 promotes the interaction between SERPINA1^{E342K}/ATZ and SQSTM1. (A) SYVN1 increases the interaction between polyubiquitinated SERPINA1^{E342K}/ATZ and SQSTM1. HEK 293T cells were transfected with the indicated plasmids and treated with Baf A1 (20 nM) for 6 h. The interaction between polyubiquitinated SERPINA1^{E342K}/ATZ and SQSTM1 was detected by coIP with anti-SERPINA1/AAT antibody followed by IB with anti-SQSTM1 and anti-K63 or -K48 ubiquitin antibodies. IgG served as a negative control. (B) Verifying the effect of SYVN1 on the interaction between SERPINA1^{E342K}/ATZ and SQSTM1 by using anti-SQSTM1 antibody. The transfected cells were treated with Baf A1 (20 nM) for 6 h. The interaction between SERPINA1^{E342K}/ATZ and SQSTM1 was detected by coIP with anti-SQSTM1 antibody followed by IB with anti-SERPINA1/AAT antibody. (C) SYVN1 increases the colocalization of SERPINA1^{E342K}/ATZ and SQSTM1. HEK 293T cells were transfected with SERPINA1^{E342K}/ATZ and SYVN1-MYC plasmids and treated with Baf A1 (20 nM) for 6 h. Immunofluorescent staining was performed using antibodies against MYC (red), SERPINA1/AAT (blue) and SQSTM1 (green). The images were collected by using confocal microscopy. The extensive colocalization of SERPINA1^{E342K}/ATZ and SQSTM1 was indicated by white arrows, while the colocalization of SERPINA1^{E342K}/ATZ and SYVN1 was indicated by yellow arrows. Scale bar: 20 μm. (D) Quantifying the colocalization between SQSTM1 and SERPINA1^{E342K}/ATZ in (C). SQSTM1 and SERPINA1^{E342K}/ATZ double-positive puncta in 15 to 30 cells per field were counted in a minimum of 5 different fields and analyzed with the "Colocalization Finder" plugin in ImageJ. The error bars represent standard division calculated for 5 fields. UB, ubiquitin.

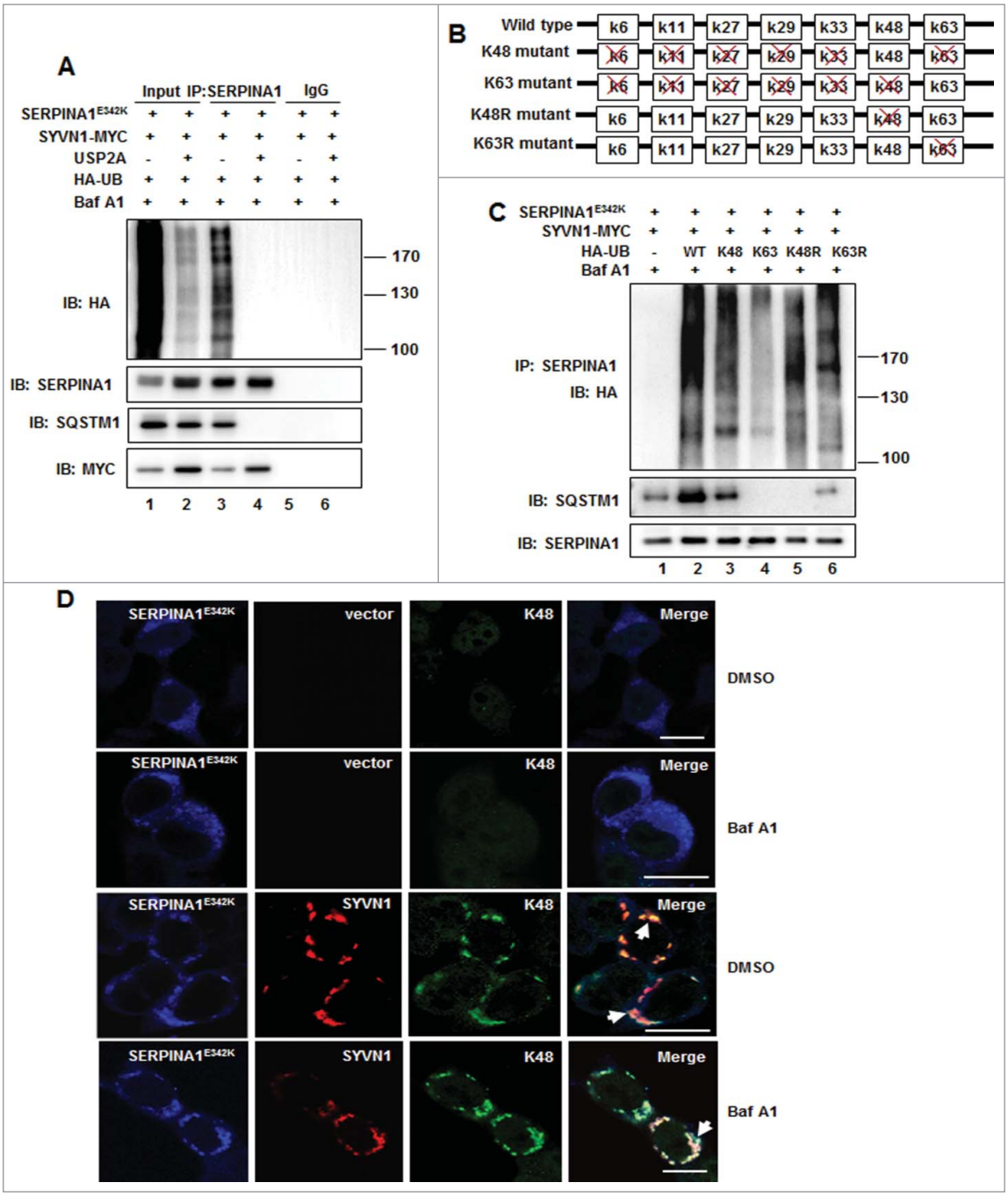

Figure 6. K48-linked polyubiquitination mediated by SYVN1 promotes the interaction between SERPINA1^{E342K}/ATZ and SQSTM1. (A) SQSTM1 interacts with SERPINA1^{E342K}/ATZ through polyubiquitin chains that were conjugated by SYVN1. HEK 293T cells were transfected as indicated and treated with Baf A1 (20 nM) for 6 h. The interaction between SERPINA1^{E342K}/ATZ and SQSTM1 was detected by coIP with anti-SERPINA1/AAT antibody followed by IB with anti-SQSTM1 antibody. (B) Schematic diagram of different ubiquitin mutants. (C) SYVN1-modified SERPINA1^{E342K}/ATZ with Lys48-linked polyubiquitin chains is required for the interaction between SERPINA1^{E342K}/ATZ and SQSTM1. HEK 293T cells were transfected with the plasmids that express SERPINA1^{E342K}/ATZ, SYVN1-MYC, and HA-ubiquitin (WT; K48, K63, K48R or K63R mutants) and treated with Baf A1 (20 nM) for 6 h. The interaction between ubiquitinated SERPINA1^{E342K}/ATZ and SQSTM1 was detected by coIP using anti-SERPINA1/AAT antibody followed by IB with anti-HA, anti-SERPINA1/AAT or anti-SQSTM1 antibodies. WT, wild type. (D) SYVN1 enhances the colocalization of K48-linked polyubiquitin chains and SERPINA1^{E342K}/ATZ. HEK 293T cells were transfected and treated as indicated. Immunofluorescent staining was performed using antibodies against K48-ubiquitin (green), MYC (red), and SERPINA1^{E342K}/ATZ (blue). The images were collected by using confocal microscopy. The extensive colocalization of SERPINA1^{E342K}/ATZ and K48-linked polyubiquitin chains were indicated by arrows. Scale bar: 20 μm.

Therefore, we wondered whether autophagy inhibition affects the protective effect of SYVN1 on SERPINA1^{E342K}/ATZ cytotoxicity. To answer this question, HepG2 cells were cotransfected with SERPINA1^{E342K}/ATZ and SYVN1 and treated with Baf A1 for 6 h. As shown in Fig. 7**A**, the SERPINA1^{E342K}/ATZ-overexpressing cells showed relative abnormal morphologies, including round-shaped cell body, short and small processes and obvious aggregates in cytoplasm, some nuclei became smaller (Fig. 7 A1 to 3, indicated by arrows). On the contrary, SERPINA1^{E342K}/ATZ aggregates were significant reduced in cytoplasm and the nuclei became bigger and near to the normal after SYVN1 overexpression (Fig. 7 A7 to 9,

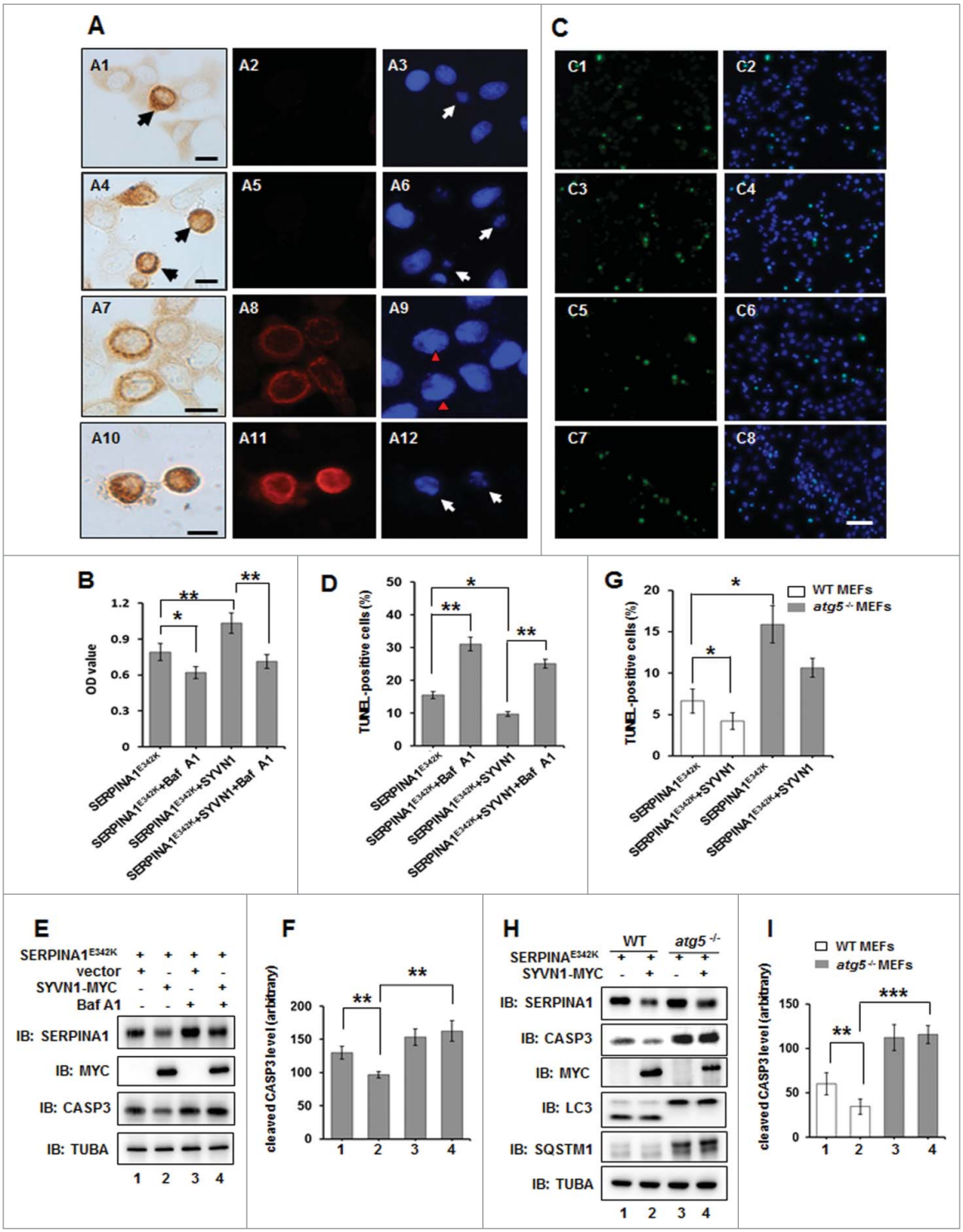

Figure 7. Autophagy inhibition reverses the suppressive effect of SYVN1 on SERPINA1^{E342K}/ATZ-induced cytotoxicity. (A) Autophagy inhibition attenuates the effect of SYVN1 on the morphology of HepG2 cells that overexpress SERPINA1^{E342K}/ATZ. HepG2 cells were transfected with SERPINA1^{E342K}/ATZ alone (A1 to A6) or cotransfected with SERPINA1^{E342K}/ATZ and SYVN1 (A7 to A12). Twenty-four h after transfection, the cells were treated with Baf A1 (20 nM) (A4 to A6, A10 to A12) or DMSO (A1 to A3, A7 to A9) for 6 h. Immunocytochemistry and immunofluscence were used to observe the morphology of cells and the expression of SERPINA1^{E342K}/ATZ (brown) and SYVN1 (red). DAPI (blue) staining was used to assess nucleus morphology. Scale bar: 20 μm. (B) Autophagy inhibition decreases the cell viability in SERPINA1^{E342K}/ATZ and SYVN1-expressing cells. HepG2 cells were transfected with the indicated plasmids. Twenty-four h after transfection, the cells were treated with Baf A1 (20 nM) for 12 h and processed for the MTT assay. (C) Autophagy inhibition facilitates apoptosis of the SERPINA1^{E342K}/ATZ and SYVN1-expressing cells. HepG2 cells were transfected with SERPINA1^{E342K}/ATZ alone (C1 and 2, C3 and 4), or cotransfected with SERPINA1^{E342K}/ATZ and SYVN1 expression constructs (C5 and 6, C7 and 8). Twenty-four h after transfection, the cells were treated with Baf A1 (20 nM) for 12 h (C3 and 4, C7 and 8) and processed for TUNEL assay. DAPI was used to determine the number of gross nuclei. Scale bar: 50 μm. (D) Quantitative analysis of the number of TUNEL-positive cells in (C). (E) Autophagy inhibition increases CASP3 activation in the SERPINA1^{E342K}/ATZ and SYVN1-expressing cells. HepG2 cells were transfected with SERPINA1^{E342K}/ATZ and SYVN1-MYC, and then treated with Baf A1 (20 nM) for 6 h. The cleaved CASP3 was examined by IB. (F) Quantitative data of cleaved CASP3 in (E). (G) Autophagy deficiency diminishes the suppressive effect of SYVN1 on cell apoptosis induced by SERPINA1^{E342K}/ATZ. WT and *atg5*$^{-/-}$ MEFs were transfected with SERPINA1^{E342K}/ATZ and SYVN1 plasmids, twenty-four h after transfection, the cells were processed for TUNEL assay. (H) Autophagy deficiency diminishes the suppressive effect of SYVN1 on CASP3 activation induced by SERPINA1^{E342K}/ATZ. WT and *atg5*$^{-/-}$ MEFs were transfected with SERPINA1^{E342K}/ATZ and SYVN1-MYC. The cleaved CASP3 was examined by IB. (I) The quantitative analysis of cleaved CASP3 amount in (H). All the quantitative data were presented as mean ± SD from at least 3 independent experiments. The TUNEL-positive cells were counted in 5 randomly selected fields from 4 sections of each group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

indicated by arrows). Baf A1 treatment enhanced SERPINA1^{E342K}/ATZ-induced cytotoxicity and reversed the protective effect of SYVN1. For example, the number of abnormal cells was significantly increased, the cells became small and round, processes disappeared, and many nuclei became pyknotic, even karyolysis (Fig. 7 A4 to 6, A10 to 12, indicated by arrows). These results suggest that autophagy inhibition abolishes the suppressive effect of SYVN1 on SERPINA1^{E342K}/ATZ-induced cytotoxicity. To confirm this finding, the MTT assay was used to determine the cells proliferation. It showed that SYVN1 coexpression significantly increased the viability of cells compared with the cells expressing SERPINA1^{E342K}/ATZ alone, whereas both Baf A1 and CQ treatment caused reduced cell viability (Fig. 7B, Fig. S5A). TUNEL staining showed that the number of apoptotic cells in SERPINA1^{E342K}/ATZ and SYVN1-coexpressing cells was lower than that in cells expressing only SERPINA1^{E342K}/ATZ. After treatment with Baf A1 or CQ for 12 h, the number of apoptotic cells was dramatically increased both in cells expressing only SERPINA1^{E342K}/ATZ and in SERPINA1^{E342K}/ATZ and SYVN1-coexpressing cells (Fig. 7C and D, Fig. S5B and C). Similar results were observed in *atg5*$^{-/-}$ MEFs (Fig. 7G). On the contrary, the autophagy inducer Rapa reduced apoptotic cells in the presence of SYVN1 (Fig. S5B and C). Meanwhile, we detected cleaved CASP3/caspase-3 levels to confirm the effects of autophagy and SYVN1 on cell apoptosis. As predicted, the cleaved CASP3 (~17 k_D) was downregulated by SYVN1 (Fig. 7E and F, lane 2 vs lane 1; Fig. S5D and E, lane 2 vs lane 1), but Baf A1 or CQ treatment increased the levels of cleaved CASP3 in SERPINA1^{E342K}/ATZ and SYVN1-coexpressing cells (Fig. 7E and F, lane 4 vs lane 2; Fig. S5D and E, lane 6 *vs* lane 2). Similarly, SYVN1 did not decrease the cleaved CASP3 level in *atg5*$^{-/-}$ MEFs (Fig. 7H and I, lane 4 vs lane 2). As expected, Rapa significantly decreased CASP3 activation in SERPINA1^{E342K}/ATZ-expressing cells (Fig. S5D and E, lane 4 vs lane 2). These results indicate that autophagy inhibition reverses the suppressive effect of SYVN1 on SERPINA1^{E342K}/ATZ-induced cytotoxicity.

Discussion

The substitution of Glu342Lys (E342K) in SERPINA1^{E342K}/ATZ is sufficient to inhibit its secretion and renders SERPINA1^{E342K}/ATZ prone to polymerization and aggregation. There are several mechanisms involved in SERPINA1^{E342K}/ATZ degradation, including ubiquitin-dependent and ubiquitin-independent proteasome pathways as well as nonproteasome-dependent pathways, such as autophagy.[12-15, 30, 38] The tendency for misfolded SERPINA1^{E342K}/ATZ to polymerize and aggregate may be especially important for determining which pathways are involved in degradation of SERPINA1^{E342K}/ATZ that retained in the ER. Our previous work has shown that SYVN1 decreases intracellular SERPINA1^{E342K}/ATZ levels, especially in the detergent-insoluble fraction, which in part can be attributed to SYVN1 increasing the solubility of SERPINA1^{E342K}/ATZ and facilitating SERPINA1^{E342K}/ATZ degradation by the proteasome.[29] Since the autophagy pathway is likely to decrease misfolded and aggregated proteins,[11, 39] we considered the possibility that SYVN1 selectively targets SERPINA1^{E342K}/ATZ to the lysosome for clearance. As predicted, autophagy inhibitors NH_4Cl, Baf A1, and CQ partially reversed the reduction of SERPINA1^{E342K}/ATZ induced by SYVN1. On the contrary, autophagy inducers Rapa, Torin1, and EBSS somehow facilitate SYVN1-mediated SERPINA1^{E342K}/ATZ degradation, which convincingly demonstrate that autophagy is involved in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation.

According to our previous results, SYVN1 remarkably decreases detergent-insoluble SERPINA1^{E342K}/ATZ levels, [29]

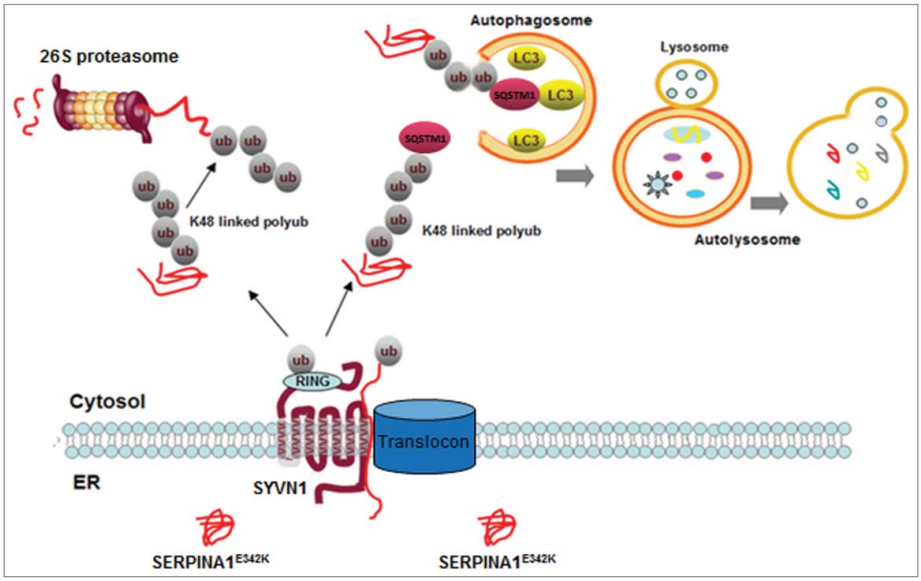

Figure 8. Schematic diagram of SYVN1 interacting with SERPINA1^{E342K}/ATZ and targeting it for degradation. SYVN1 mainly modifies SERPINA1^{E342K}/ATZ with K48-linked polyubiquitin chains and facilitates the interaction between ubiquitinated SERPINA1^{E342K}/ATZ and the UBA domain of SQSTM1, which leads to SERPINA1^{E342K}/ATZ subsequent degradation by the autophagy-lysosome pathway. On the other hand, SYVN1-mediated SERPINA1^{E342K}/ATZ ubiquitination with K48-linked polyubiquitin chains also targets SERPINA1^{E342K}/ATZ for proteasome degradation. These mechanisms may be helpful for understanding the clearance of SERPINA1^{E342K}/ATZ aggregates from the ER and subsequently relieving SERPINA1^{E342K}/ATZ cytotoxicity in hepatocytes.

we wondered whether autophagy modulation affects this process. For this purpose, SERPINA1^{E342K}/ATZ levels were analyzed both in detergent-soluble or -insoluble fractions of cell lysates. As we predicted, autophagy inhibitors NH_4Cl and Baf A1 partially reversed the decrease of insoluble SERPINA1^{E342K}/ATZ levels mediated by SYVN1, suggesting that insoluble SERPINA1^{E342K}/ATZ may be efficiently disposed by SYVN1 via the autophagy pathway. Similarly, MG132 treatment also partially reversed the decrease of insoluble SERPINA1^{E342K}/ATZ levels mediated by SYVN1. Our previous study found that SYVN1 still increased soluble SERPINA1^{E342K}/ATZ level in the supernatant after blocking SERPINA1^{E342K}/ATZ synthesis with CHX, suggesting the increased soluble SERPINA1^{E342K}/ATZ comes from the insoluble SERPINA1^{E342K}/ATZ. Based on this point, we speculated that SYVN1 directs the insoluble SERPINA1^{E342K}/ATZ into the soluble one and degrades soluble SERPINA1^{E342K}/ATZ through proteasome and lysosome pathways under physiological conditions. However, under autophagy inhibition, the balance between soluble and insoluble SERPINA1^{E342K}/ATZ was disrupted, more insoluble SERPINA1^{E342K}/ATZ aggregates were sequestered in ER or collected into intracellular inclusion bodies, which may explain why autophagy inhibitors more significantly increased the insoluble SERPINA1^{E342K}/ATZ levels, although SYVN1 was co-overexpressed with SERPINA1^{E342K}/ATZ.

The mCherry-GFP-SERPINA1^{E342K}/ATZ images and colocalization of SERPINA1^{E342K}/ATZ and LAMP1 further strengthen our conclusion that SYVN1 facilitates SERPINA1^{E342K}/ATZ translocation to autolysosome. However, another question is raised that the SERPINA1^{E342K}/ATZ that was retained in the ER may be directly degraded by the autophagosome together with the residual ER membrane, a process termed as reticulophagy or ER-phagy,[40] instead of ER-associated degradation (ERAD). It is well known that a crucial step for ERAD is the retrotranslocation from the ER lumen to cytosol. Generally, it has been believed that VCP (valosin-containing protein) activity is required for the retrotranslocation step. [41] Loss of VCP ATPase activity blocks proteasome degradation of several different ERAD substrates.[42] Therefore, we investigated the effect of VCP or its ATPase defective mutant construct VCP-QQ (deficient in AAA ATPase domains D1 and D2) on SERPINA1^{E342K}/ATZ degradation efficiency in the presence of SYVN1. We found that VCP-QQ mutant diminishes SERPINA1^{E342K}/ATZ degradation, especially the insoluble SERPINA1^{E342K}/ATZ degradation.[43] This result suggests that VCP is required for SYVN1-mediated SERPINA1^{E342K}/ATZ degradation. In other words, SYVN1 mediates SERPINA1^{E342K}/ATZ degradation at least partially through the ERAD pathway.

Although our data suggest that SYVN1 targets SERPINA1^{E342K}/ATZ degradation by the lysosome, the mechanisms by which SYVN1 specifically recognizes SERPINA1^{E342K}/ATZ and facilitates conversion of insoluble SERPINA1^{E342K}/ATZ to a soluble one are unknown. A recent study shows that ER-resident glycoprotein OS-9 (endoplasmic reticulum lectin) selectively binds to a misfolded mutant SERPINA1/AAT (NHK) through recognizing oligosaccharides on the NHK, then associates with the ER chaperone HSP90B1 (heat-shock protein 90 β family member 1), and interacts with SYVN1 through SEL1L (SEL1L ERAD E3 ligase adaptor subunit), which is required for the degradation of mutant SERPINA1/AAT.[16, 44] Both NHK and SERPINA1^{E342K}/ATZ belong to SERPINA1/AAT variants, they may share some similar mechanisms for SERPINA1^{E342K}/ATZ recognition in the ER. As we known, ubiquitin is a universal degradation signal used for protein disposal via the proteasome and autophagy.[45] Because ubiquitin possesses 7 internal lysine residues, polyubiquitin chains are formed as structurally distinct polymers via different linkages, leading to distinct functional outcomes.[36, 46] We have previously shown that SYVN1 is capable of mediating SERPINA1^{E342K}/ATZ ubiquitination,[29] but the type of polyubiquitin linkage with that SERPINA1^{E342K}/ATZ is modified is not clear. In fact, our results revealed that SERPINA1^{E342K}/ATZ with Lys48-linked polyubiquitin chains might be predominantly targeted for autophagic degradation. These observations depart from the generally accepted opinion that K48-linked polyubiquitin exclusively targets protein to the proteasome-dependent degradation,[47-49] whereas K63-linked polyubiquitin is involved in lysosomal degradation.[50] Notably, it has been reported that K48-linked ubiquitin-conjugated proteins accumulate when autophagy is impaired.[51, 52] Although the UBA domain of SQSTM1 is more likely to bind K63-linked polyubiquitin chains, it also binds to K48-linked polyubiquitin chains.[50, 53, 54] More interesting, SYVN1-mediated SERPINA1^{E342K}/ATZ ubiquitination with K48-linked polyubiquitin chains also targets SERPINA1^{E342K}/ATZ for proteasome-dependent degradation. Therefore, K48-linked polyubiquitinated SERPINA1^{E342K}/ATZ may be degraded both through lysosome and proteasome pathways. The precise selectivity and underlying mechanisms for the ubiquitinated SERPINA1^{E342K}/ATZ degradation is worth further exploring in detail.

Autophagy receptors are known to play pivotal roles in selective autophagy through recruiting and delivering cargo to the phagophore, the precursor to the autophagosome.[33, 55] Currently known autophagy receptor proteins include SQSTM1, NBR1, CBL (Cbl proto-oncogene), BNIP3L (BCL2 interacting protein 3 like), STBD1 (starch binding domain 1), OPTN (optineurin), and CALCOCO2 (calcium binding and coiled-coil domain 2).[33, 56-60] Among them, SQSTM1 simultaneously binds to polyubiquitinated proteins via its UBA domain and to the phagophore membrane component MAP1LC3B via its LC3-interacting region (LIR).[61, 62] In particular, our findings have strongly suggested that SERPINA1^{E342K}/ATZ and SQSTM1 indeed form a complex, which largely depends on SYVN1-mediated SERPINA1^{E342K}/ATZ ubiquitination mainly through K48-linked polyubiquitin chains. In addition, we found that the UBA domain of SQSTM1 interacted with the K48-linked polyubiquitin chains of SERPINA1^{E342K}/ATZ. These findings support that SQSTM1 functions as a cargo receptor to recruit SYVN1-mediated K48-linked polyubiquitination of SERPINA1^{E342K}/ATZ to autophagosomes. In light of the central role of SQSTM1 in organizing the autophagosome followed by autophagic degradation, it is conceivable that the SERPINA1^{E342K}/ATZ-SQSTM1 interaction could prominently augment the recruitment of SERPINA1^{E342K}/ATZ to the autophagy receptors before targeting to autophagosome in vivo. However, whether and how other autophagy receptors actually form complexes and are involved in this process is worth further study.

Although our results indicate that SYVN1 plays a role in autophagic degradation of SERPINA1^{E342K}/ATZ, it is noteworthy that SERPINA1^{E342K}/ATZ degradation does not rely only on SYVN1, because it was found that NH_4Cl treatment further increased the levels of SERPINA1^{E342K}/ATZ compared with DMSO controls in *SYVN1* knockdown cells in Fig. 1I. However, we can presume that autophagy is a predominant regulator in SYVN1-mediated SERPINA1^{E342K}/ATZ degradation from the result that SERPINA1^{E342K}/ATZ protein is more stable in *atg*5-knockout cells as compared with WT cells, although SYVN1 was overexpressed at the same time.

Consistent with our previous findings, SYVN1 helped to maintain the normal morphology of SERPINA1^{E342K}/ATZ-overexpressing cells and relieved SERPINA1^{E342K}/ATZ cytotoxicity.[29] While autophagy inhibition increases SERPINA1^{E342K}/ATZ cytotoxicity and counteracts the protective effect of SYVN1. Therefore, in our experimental system, accumulation of SERPINA1^{E342K}/ATZ will result in hepatic cell injury, and SYVN1 provides protection probably by increasing the clearance of SERPINA1^{E342K}/ATZ by lysosomes.

In summary, as shown in Fig. 8, we reported here that SYVN1 interacts with SERPINA1^{E342K}/ATZ and predominantly mediates SERPINA1^{E342K}/ATZ polyubiquitination with K48-linkage, which binds to the UBA domain of SQSTM1 to form a SERPINA1^{E342K}/ATZ-SQSTM1 complex, and the complex directs the ubiquitinated SERPINA1^{E342K}/ATZ to the lysosome for degradation. Given the fact that SERPINA1^{E342K}/ATZ is critically implicated in SERPINA1/AAT-D, targeting E3 SYVN1 may represent a new avenue for therapeutic intervention.

Materials and methods

Plasmids, antibodies, and reagents

pcDNA3.1/myc-His-SYVN1 (wild type), pcDNA3.1/myc-His-SYVN1C1A, pCIneo-SYVN1-FLAG, pcDNA3.1/Zeo(+)-SERPINA1^{E342K}/ATZ and pcDNA3.1/Zeo(+)-SERPINA1 plasmids have been described previously.[26, 29] Plasmids pRK5-HA-ubiquitin-WT, pRK5-HA-ubiquitin-K48, pRK5-HA-ubiquitin-K63, pRK5-HA-ubiquitin-K48R, pRK5-HA-ubiquitin-K63R, and pRK5-FLAG-USP2A were kindly provided by Professor Ronggui Hu at the Chinese Academy of Sciences[52] and Professor Hongbin Shu at Wuhan University (China).[63, 64] The pEGFP-MAP1LC3B plasmid was a kind gift from Professor Tamotsu Yoshimori's group at Okazaki University (Japan).[65] The pEGFP-SQSTM1 (wild type) and pEGFP-SQSTM1$^{UBA\Delta}$ plasmids were kindly provided by Professor Wei Ding at Capital Medical University of China.[66] The RFP-LAMP1 plasmid was provided by Professor Shengyun Fang at University of Maryland (USA). Plasmid pDest-mCherry-EGFP-vector was kindly provided by Professor Wanglai Hu at Anhui Medical University (China). To construct plasmid pDest-mCherry-EGFP-SERPINA1^{E342K}/ATZ, PCR was performed on pcDNA3.1/Zeo(+)-SERPINA1^{E342K}/ATZ using the primers 5′-GGAATTC-GATGATGCCGTCTTCTGTCTC-3′ and 5′-GGAATTCT-TATTTTTGGGTGGGATTCACC-3′. The PCR product of 1257 bp was digested with the mono-restriction endonuclease enzyme EcoRI (TaKaRa, 1040S), and the target fragment was inserted into pDest-mCherry-EGFP vector. The construct was verified via restriction enzyme digestion and DNA sequencing (Shanghai Sangon Biotech Co., Ltd.).

The following antibodies were used: goat anti-SERPINA1/AAT (Bethyl Laboratories, A80–122A), rabbit anti-SYVN1 (Abcam, ab170901), rabbit anti-SQSTM1 (Sigma, P0067), rabbit anti-MAP1LC3B (Sigma, L7543), mouse anti-MYC (Sigma, A5963), rabbit anti-FLAG (Sigma, F3165), rabbit anti-HA (Sigma, h6908), rabbit anti-Ub (K48-specific) and anti-Ub (K63-specific) (Millipore, 05–1307, 05–1313), rabbit anti-cleaved-CASP3/caspase-3 antibody (CST, 9654s), mouse anti-TUBA/α tubulin (Sigma, t6199) and HRP-conjugated, mouse anti-GAPDH antibody (Proteintech, 10494–1-AP). Alexa Fluor 488-labeled anti-mouse IgG, Alexa Fluor 568-labeled anti-rabbit IgG, and Alexa Fluor 350-labeled anti-goat IgG (Invitrogen, A11029, A11036, and A21081). HRP-conjugated, anti-mouse secondary antibody (Sigma, GENA931), HRP-conjugated, anti-rabbit secondary antibody (Sigma, GENA934), HRP-conjugated, anti-goat secondary antibody (Sigma, A5420). The following reagents were used: autophagy inhibitors NH_4Cl (Sigma, A9434), Baf A1 (Abcam, AB120497), and CQ (Sigma, C6628); autophagy inducers EBSS (Sigma, E2888), Torin1 (Selleckchem, S2827), CBZ (Sigma, C4024), and rapamycin (Santa Cruz Biotechnology, sc-3504); proteasome inhibitor MG132 (Tocris, 1748); cycloheximide (CHX) (Sigma, R750107). Protein A/G plus agarose beads were from Pierce (20423, 15918–014).

Cell culture and transfection

Wild-type MEFs and *atg*5 gene knockout (*atg*5$^{-/-}$) MEFs were kindly gifted by Professor Hong Zhang (Institue of Biophysics, Chinese Academy of Sciences). *sqstm1* gene knockout (*sqstm1*$^{-/-}$) MEFs were kindly provided by Professor Yushan Zhu (Nankai University, China). Human embryonic kidney (HEK) 293T cell line, human HepG2, and SMMC-7721 hepatoma cell lines were obtained from Cobioer Biosciences (Nanjing, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, 1710802) supplemented with 10% fetal bovine serum (FBS) and transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen, 11668–019). When necessary, the empty vectors were used to bring the total DNA to equal amount for each transfection group.

RNA interference

293T cells were transfected with 50 to 100 nM siRNA using Lipofectamine2000 reagent. The mRNA and protein expression of knockdown gene were examined by RT-PCR and immunoblot respectively. The following siRNA were used: for *SYVN1*, siRNA 1 [CUGUGGUUACCUGACCAATT (sense)], siRNA 2 [GGCUGCAGAGCCUGCGUAATT (sense)], siRNA 3 [GGCCUGCCACUUCAGUCAATT (sense)] and the negative control siRNA (Shanghai GenePharma Co., Ltd). For *SQSTM1*, siRNA 1 [GUGACGAGGAAU UGACAAUTT (sense)], siRNA 2 [GGAGUCGGAUAACUGUUCATT (sense)], siRNA 3 [CCUGACAUUUAGUUGAUUATT (sense)] and the negative control siRNA (Shanghai GenePharma Co., Ltd).

Immunostaining

Transfected cells grown on coverslips were washed in PBS and fixed at room temperature in 4% paraformaldehyde for 20 min. Double immunofluorescent labeling was performed with according primary antibodies and fluorescence labeled second antibodies, respectively, using a previously published method.[26] Confocal images were captured using a confocal microscope (LSM710, Carl Zeiss, Tokyo, Japan) with ZEISS ZEN Imaging Software. The images of mCherry-EGFP-SERPINA1^{E342K}/ATZ were acquired randomly with confocal microscopy. Cells were outlined manually, and each channel was independently autothresholded using the same settings across all images from each channel. mCherry and GFP puncta were quantified using the "Analyze Particles" plugin in ImageJ. Double-positive puncta were identified and counted using the "Colocalization and Analyze Particles" plugin in ImageJ.[67] Quantitative measurement of protein-protein immunofluorescent colocalization was performed in ImageJ with "Colocalization Finder" plugin.

For cell morphology observation, double labeling of immunofluorescent SYVN1 and immunohistochemically-detected SERPINA1^{E342K}/ATZ was combined. Briefly, HepG2 cells grown on coverslips were transfected with SERPINA1^{E342K}/ATZ and SYVN1 plasmids and treated with 10 mg/ml NH_4Cl for 12 h. The cells were incubated with anti-SERPINA1/AAT antibody overnight at 4°C. After washing in PBS, the cells were incubated with the appropriate biotinylated secondary antibody at 37°C for 30 min. This was followed by further incubation with horseradish peroxidase-conjugated streptavidin (HRP-SA) at 37°C for 15 min. Immunostaining was developed with 3, 3-diaminobenzidine tetrahydrochloride (DAB) for about 1 min under light microscopy. After washing with PBS, the coverslips were incubated with mouse anti-MYC primary antibody for 2 h, followed by incubation with Alexa Fluor 568-labeled anti-mouse IgG for 1 h. Cell nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI; Sigma, D8417). Images were collected under a fluorescence microscope (Olympus, IX73, Tokyo, Japan).

Preparation of fractionated lysates into cytoplasmic soluble proteins and aggregates

293T cells were collected 24 h after transfection and lysed in lysis buffer (25 mM HEPES, pH 7.5, 0.3 M NaCl, 1.5 mM $MgCl_2$, 20 mM β-glycerol phosphate, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 1% Triton X-100 [Sigma, T8787], 10% glycerol, 5 μg/mlaprotinin [Sigma, 10236624001], 10 μg/ml leupeptin [Sigma, L2884], 1 mM PMSF [Bio BASIC PB0425], 1 mM Na3VO4 [Sigma, 450243], and 50 mM NaF [Sigma, 71519]). After centrifugation at 14,000 g for 10 min at 4°C, the supernatant was saved as the cytoplasmic soluble fraction and the pellet was resuspended in lysis buffer containing an additional 1% SDS (SINOPHARM, 30166428). The cell suspension was sonicated for 5 cycles at high intensity (30 s on/30 s off intervals) at 4°C. The supernatant was collected after centrifugation at 20,000 g for 30 min at 4°C and designated as the aggregate fraction. Then the SERPINA1^{E342K}/ATZ protein levels in cytoplasmic soluble fractions and aggregate fractions were examined by immunoblot.

Coimmunoprecipitation (coIP) and immunoblot (IB)

Protein lysates used for coIP were prepared from 293T cells. Cells were harvested and lysed in IP buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 1×protease inhibitor cocktail (Sigma, 4693159001) and then centrifuged at 12,000 g at 4°C for 15 min. The supernatant was collected and incubated with corresponding primary antibodies and protein A-agarose beads overnight at 4°C. The beads were washed for 5 times with IP buffer before processing for SDS-PAGE gel and then subjected to IB analysis.

Protein lysates used for IB were harvested and lysed in sample buffer containing 2% SDS followed by SDS-PAGE. Then the proteins were transferred to PVDF membranes and probed with the indicated antibodies, which were detected using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences 34095)

Cycloheximide chase analysis

Cells were transfected with the indicated plasmids and grown for 24 h. CHX (100 μg/ml) was used to inhibit protein synthesis. Equal number of cells was collected at the indicated time points. Levels of SERPINA1^{E342K}/ATZ in the samples were determined by IB as described above.

RT-PCR

Total RNA was extracted from 293T cells with Trizol reagent (Invitrogen, 15596018) according to the manufacturer's protocol. Reverse transcription was performed using PrimeScriptTM RT reagent Kit (Takara, DRR036A) at 37°C for 30 min. The primers used are listed as follows: *SYVN1*, forward 5′-CTCCTCCCTGGATGGGTATG-3′, reverse 5′-CCTCTGAGCTAGGGATGCTG-3′; *ACTB*/β-*actin*, forward 5′-TCACCAACTGGGACGACAT-3′ and reverse 5′-GCACAGCCTGGATAGCAAC-3′; The PCR apparatus (Thermo, PIKOREAL96, Finland) is used to amplify segments of DNA. Expression values were normalized with the *ACTB* mRNA level, which was quantified by Bandscan software.

MTT assay

The transfected cells were seeded into 96-well plates (3×10^3 cells/well). After treatment with the autophagy inhibitor Baf A1 or CQ for 12 h, cell proliferation was evaluated by the MTT assay as described previously.[68] In brief, MTT (500 μl; 5 mg/ml) was added to each well and incubated at 37°C for 4 h, and 100 μl DMSO was added to each well and vortexed vigorously to solubilize the colored crystals produced within the living cells. The absorbance was measured at 570 nm using a microplate reader (Thermo, Varioskan Flash, Finland).

TUNEL assay

HepG2, SMMC-7721 and *atg5*$^{-/-}$ MEFs were transfected with the indicated plasmids and processed for apoptosis assay using in situ cell death detection kit (Roche, 11684795910). Before staining, HepG2 and SMMC-7721 cells were treated with Baf

A1 or CQ or Rapa for 12 h. DAPI was used to stain the nuclei. Fluorescence images of cells were obtained with constant parameters of acquisition. The numbers of TUNEL-positive cells (green) and the total number of nuclei (blue) were counted in 5 randomly selected fields from 4 sections of each group. The histogram shows the proportion of TUNEL-positive cells in relation to the total cell number.

Statistical analysis

The data are presented as mean ± SD of at least 3 independent experiments. Data were analyzed with GraphPad Prism 5.01 (GraphPad software, San Diego, CA, USA). Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by the Dunnett test. A value of P less than 0.05 was considered to be statistically significant.

Abbreviations

ANOVA	one-way analysis of variance
ATG	autophagy related
Baf A1	bafilomycin A_1
CHX	cycloheximide
COPD	chronic obstructive pulmonary disease
CQ	chloroquine
DAB	3, 3-diaminobenzidine tetrahydrochloride
DAPI	4, 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
EBSS	Earle's balanced salt solution
ECL	enhanced chemiluminescence
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
HCC	hepatocellular carcinomas
HRP	horseradish peroxidase
IB	immunoblot
IP	immunoprecipitation
LIR	LC3-interacting region
MAP1LC3/LC3	microtubule-associated protein 1 light chain 3
3-MA	3-methyladenine
MEF	mouse embryonic fibroblast
NBR1	NBR1, autophagy cargo receptor
NH_4Cl	ammonium chloride
NHK	Hong Kong variant of SERPINA1/α-1-antitrypsin
PBS	phosphate-buffered saline
Rapa	rapamycin
RFP	red fluorescent protein
RT-PCR	reverse transcription-polymerase chain reaction
SERPIN	family acronym for "serpin peptidase inhibitors"
SERPINA1/AAT/α-1-antitrypsin	serpin family A member 1
SERPINA1/AAT-D	serpin family A member 1/α-1-antitrypsin deficiency
$SERPINA1^{E342K}$/ATZ	Z variant of SERPINA1
siRNA	small interfering RNA
SQSTM1	sequestosome 1
SYVN1	synoviolin 1
Ub	ubiquitin
UBA	ubiquitin-associated
USP2	ubiquitin specific peptidase 2
USP2A	splice variant "A" of USP2
WT	wild type

Disclosure of potential conflicts of interest

There is no potential conflict of interest that needs to be disclosed.

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