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Abstract of the Dissertation

Mechanisms of Endoplasmic Reticulum Stress-Induced Cell Death

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In cells, incorrectly folded proteins or compromised protein turnover induce cellular stress known as endoplasmic reticulum (ER) stress. Significantly, ER stress plays an important role in numerous physiological and pathological conditions, including degenerative diseases and cancer. In an attempt to resolve ER stress cells activate a response known as the unfolded protein response (UPR). While, initially a protective response, the UPR can induce cell death if ER stress is not overcome. As the precise molecular mechanisms that regulate ER homeostasis and UPR-related cell death remain elusive, I focused my initial studies on understanding these processes. In the course of this study it was discovered that autophagy, a process utilized by cells to degrade misfolded and/or damaged proteins, is important for restoring proper ER function. However, unabated autophagy can promote cell death. As autophagic protein degradation is ultimately carried out by lysosomal hydrolases, I also studied how perturbations within lysosomes affect ER function and cellular homeostasis. An endogenous inhibitor of lysosomal cathepsins known as squamous cell carcinoma antigen 1 (SCCA1), a member of the serine protease inhibitor (Serpin) family of proteins, was expressed in cells to modulate lysosomal function. Significantly these studies revealed that SCCA1 is able to protect cells against lysosomal injury resulting from DNA alkylating agents and hypoosmotic stress. This protection is due to SCCA1's ability to inhibit lysosomal rupture, thus preventing cytotoxic release of lysosomal hydrolases into the cytosol. Conversely, due to the inhibition of lysosomal protein degradation, SCCA1 promotes cell death in response to ER stress. This SCCA1-mediated cell death in response to ER stress is carried out by intracellular aggregation and subsequent activation of caspase-8. Hence, on one hand SCCA1 inhibits cell death induced by lysosomal damage, while on the other hand it sensitizes cells to ER stress by activating caspase-8. Our studies have thus uncovered a novel mechanism of cell death in response to ER stress. Given that SCCA1 expression is elevated in numerous cancers, these findings may offer insight into selective treatment strategies for SCCA1 expressing cancers.

Dedication Page

I would like to dedicate this dissertation to my family. To my wonderful husband Todd Pettigrew, whose constant love and support have helped push me through the many discouraging moments and failed experiments. To my beautiful daughter Makayla Pettigrew who is the light of my life; I hope I will inspire and instill the same work ethic as that imparted on me by my mother, Sharon Ullman, and father Raymond Ullman.

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List of Abbreviations

3-MA	3-methyladenine
APAF1	apoptotic peptidase activating factor 1
ARF	ADP reibosylation factor
ATF4	activating transcription factor-4
ATF6	activating transcription factor-6
Atg	autophagy related protein
BFA	brefeldin A
BH	Bcl-2 homolgy
BMK	baby mouse kidney
C/EBP	CCAAT-enhancer-binding protein
Ca^{2+}	calcium
Cath	cathepsin
Cb5	cytochrome b5
CBF	CCAAT binding factor
CHOP	C/EBP homologous protein
CHX	cycloheximide
COXIV	cytochrome C oxidase/ complex IV
CQ	chloroquine
Cyt. C	cytochrome C
DAPI	4',6-diamidino-2-phenylindole
DED	death effector domain
DISC	death inducing signaling complex
Dox	doxycycline
DUB	deubiquitinase
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
EDEM	ER degradation enhancing α-mannosidase-like protein
ER	Endoplamic Reticulum
ERAD	ER-associated degradation
ERSE	ER stress-response element
FACS	Fluorescence-activated cell sorting
FADD	Fas-Associated protein with Death Domain
FasL	Fas ligand
FITC	Fluorescein isothiocyanate
GADD	growth arrest and DNA-damage inducible gene
GFP	green fluorescent protein
GRP78/Bip	glucose-regulated proein 78
HMGB1	high mobility group box 1
HMW	high molecular weight
HSP	heat shock protein
IAP	inhibitor of apoptosis
IL	interleukin
IRE1	inositol requiring protein

JNK	c-Jun N-terminal kinase
LAMP	lysosomal associating membrane protein
LC3	microtubule-associated protein 1 light chain 3
LMP	lysosomal membrane permeabilization
MAF	moafosfamide
Mcl-1	myeloid cell leukemia sequence 1
MEF	mouse embryonic fibroblasts
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
MOMP	mitochondrial outer membrane permeabilization
Ni-NTA	nickel-nitriloacetic acid
PARP	Poly(ADP-ribose) polymerase
PDI	protein disulfide isomerase
PE	phosphatidylethanolamine
Pep-A	pepstatin A
PERK	PKR-like endoplasmic reticulum kinase
PI	propidium iodide
PKR	protein kinase activated by double stranded RNA
ROS	reactive oxygen species
RSL	reactive site loop
S1P	site-1 protease
S2P	site-2 protease
SCC	squamous cell carcinoma
SCCA	squamous cell carcinoma antigen
SEM	standard error of the mean
Serpin	serine protease inhibitor
shRNA	short hairpin RNA
siRNA	small interfering RNA
SQSTM1	sequestome 1
TEM	transmission electron microscopy
Tet	tetracycline
Thp	thapsigargin
TNF	tumor necrosis factor
Tom40	Transporter Outer Membrane Complex 40
TRAF2	TNF receptor-associated factor 2
Tuni	tunicamycin
Ub	ubiquitin
Untr	untreated
UPR	unfolded protein response
UV	ultraviolet
VEGF-A	vascular endothelial growth factor-A
Wt	wild-type
XBP1	X-box-binding protein 1

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Vita, Publications and/or Fields of Study

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I. Introduction

A eukaryotic cell is a highly organized unit that contains a number of subcellular organelles. These organelles function in an organized fashion to maintain intracellular homeostasis and unresolved damage to these organelles can lead to apoptosis. The endoplasmic reticulum (ER) is the primary site for protein synthesis, modification and folding within the cell. Additionally, the ER plays key roles in regulating Ca^{2+} release as well as in sterol and lipid synthesis. Being as it plays such vital roles in the cell, it is imperative that the ER function properly. Numerous stimuli can cause disturbances in ER homeostasis such as increased protein translation, defective modification of proteins, alterations within the ER microenvironment, including changes in redox state and calcium levels, as well as defects in protein turnover. In an attempt to overcome the stress the cell elicits a program known as the unfolded protein response (UPR), which involves the activation of three ER associated-stress transducers whose overall function is to decrease global protein translation while increasing the expression of chaperone proteins which aid in the proper folding of proteins. Additionally, protein degradation by both the proteasome as well as the lysosome is enhanced, so as to dispose of improperly folded proteins. If the cell is unable to restore proper protein folding the UPR triggers a cell death response.

Significantly many diseases are associated with Endoplasmic Reticulum stress including cancer, neurological disorders and diabetes. Some of the disease symptoms, such as in Alzheimer's and Parkinson's disease, are manifestations of an excessive amount of cell death due to unresolved ER stress. However, in cancer ER stress helps manipulate cell survival responses such that cell death is evaded leading to the proliferation and survival of damaged and altered cells. Thus it becomes apparent that understanding the mechanisms of ER stress induced cell death are important for both helping to prevent diseases resulting for a profusion of cell death as well as diseases where cell death needs to be restored. While apoptosis is thought to be the main mode of ER stress-induced cell death, autophagy and necrosis are other death pathways that should not be disregarded, as it is shown here that cells unable to undergo conventional apoptosis are still susceptible to cell death in response to prolonged ER stress. Uncovering the mechanisms of these alternative death pathways will provide valuable insight into possible treatment strategies for cells, such as cancer cells, that often times have defects in their apoptosis machinery.

1) Endoplasmic Reticulum Stress

A. Unfolded Protein Response (UPR)

Proteins that are folded in the ER are imported into the ER as they are translated on ER associated ribosomes. Upon the conclusion of translation the step-wise process of folding occurs which requires the assistance of numerous chaperone proteins. The environment of the ER lumen is very sensitive to imbalances. For example perturbations in calcium levels within the ER disrupt the proper function of calcium dependent ER chaperone proteins. Additional types of stresses, such as glucose depletion, which leads to improper glycosylation of proteins, as well as unfavorable oxidation status in the ER, which affects disulfide bond formation, can also induce protein misfolding and subsequent ER stress **(89, 134, 168)**. Inherently responsive to the presence of misfolded proteins within the ER lumen are three stress transducers which are ER-localized transmembrane proteins. Together these stress sensors activate a response known as the unfolded protein response (Fig. 1). This response is so essential that it has been found conserved among all mammalian species, as well as yeast, and worm organisms. Initially the UPR causes an overall decrease in new protein translation, while the translation of certain chaperone proteins, necessary for assisting in proper protein folding, is increased **(48, 172)**. Some proteins however are unable to attain proper folding conformation and thus it is imperative that they be disposed of. Two protein degradation pathways that are activated to dispose of accumulated misfolded proteins are the ER-associated protein degradation (ERAD) pathway and autophagy (Fig. 1) **(159)**. While ERAD involves the degradation of soluble proteins by the proteasome, autophagy is responsible for degrading not only soluble proteins but also protein aggregates that are unable to be degraded by the proteasome. If the ER is unable to overcome the stress prolonged induction of the UPR leads to increased transcription of pro-apoptotic factors, leading to cell death **(172) (133)**. Additionally, excessive autophagy due to prolonged ER stress can contribute to cell death.

Fig 1. ER Stress and the UPR. In response to various stimuli such as glucose deprivation, hypoxia, or Ca^{2+} imbalances, proteins misfold, leading to ER stress. To cope with the stress the ER activates the unfolded protein response (UPR), which involves activation of three stress transducers, PERK, ATF6, and IRE1, whose overall function is to decrease global protein synthesis while selectively increasing the synthesis of proteins that aid in protein refolding and degradation. In addition to the three stress transducers, protein degradation by the proteasome as well as the lysosome are also important processes in response to ER stress.

i. Signaling through ER membrane stress transducers

Upon accumulation of misfolded proteins within the ER three stress transducers become activated. While one of these stress transducers, protein kinase activated by double stranded RNA (PKR)-like endoplasmic reticulum kinase (PERK) is responsible for attenuating overall protein translation, the other two stress transducers, inositol requiring-1a (IRE1), and activating transcription factor-6 (ATF6) are responsible for inducing the transcription of chaperone proteins. These ER chaperone proteins are responsible for performing numerous duties such as post-translational modification, disulfide bond formation, folding, assembly and quality control of newly synthesized proteins **(143)**. Thus upon ER stress when there is an abundance of misfolded proteins it is imperative that the presence of these chaperone proteins be increased to facilitate the correct refolding and assembly of ER proteins as well as prevent their aggregation. Additionally, certain chaperones have the ability to attenuate apoptotic stimuli by performing such functions as regulating ER stress signals as well as protein degradation processes **(99)**. Among the ER chaperone proteins, perhaps the most notable and most widely studied is glucose-regulated protein 78 (GRP78/BiP). Under normal conditions BiP is bound to the three ER stress transducers, holding them in an inactive state, additionally BiP can bind to unfolded hydrophobic regions of proteins and assist in their proper folding. Upon ER stress the increased presence of malfolded proteins necessitates more BiP aiding in regaining proper protein conformation, thus BiP must release from stress transducers to help with protein folding, allowing the stress transducers to become activated and transmit signals across the ER to the cytosol and nucleus **(77, 81)**.

As mentioned above attenuation of overall protein translation is an important response to ER stress, and this response is carried out by the PERK signaling pathway. Upon ER stress BiP releases from the luminal side of PERK, allowing it to autophosphorylate and dimerize, thereby leading to its activation. PERK is then responsible for phosphorylating eIF2α, subsequently decreasing its ability to serve as an efficient initiator of global translation **(120)**. However there are a few exceptions, such as stress responsive genes, whose translation is now favored. One such exception is the transcription factor ATF4, that binds to genes involved in restoring ER homeostasis. One target of ATF4 is the phosphatase growth arrest and DNA-damage inducible gene 34 (GADD34), which is responsible for dephosphorylating eIF2α, which is necessary for restoring proper protein translation after recovery from ER stress. Another ATF4 target is CEBP-homologous protein (CHOP), which when transiently induced favors cell recovery; however prolonged induction of CHOP in a strong inducer of apoptosis **(128, 143, 165)**.

While PERK is responsible for attenuating overall protein translation the other two ER stress transducers IRE1 and ATF6 are necessary for inducing the expression of chaperone proteins that assist in protein folding and modification. IRE1 is activated via dimerization followed by transphosphorylation, which subsequently allows its C-terminal, endoribonuclease activity to cleave an intron from the mRNA of X-box-binding protein 1 (XBP1). This splicing event allows XBP1 to be translated into a transcription factor that goes on to upregulate transcription of genes containing the ER stress-response element (ERSE) promoter. Among the proteins that contain an ERSE promoter are the chaperone proteins BiP, GRP94 and calriticulin **(159)**. While the activation of ATF6 begins like the other stress transducers via release of BiP, the subsequent step is quite distinct in that ATF6 translocates to the golgi where it is proteolytically processed by two resident golgi proteases, site-1 protease (S1P) and site-2

protease (S2P), into a 50kDa transcription factor that translocates to the nucleus where it promotes the transcription of genes containing ER stress-responsive promoter element. Its ability to promote gene expression also requires binding of the heterotrimeric CCAAT binding factor (CBF) to a distinct site within the ERSE promoter. Among the genes that are upregulated by ATF6 are the ER-resident chaperone proteins, BiP, GRP94, protein disulfide isomerase (PDI), and proteins involved in ERAD **(31)**.

While the expression of chaperone proteins in the ER is increased in order to handle the elevated amount of unfolded proteins, not all proteins are able to reach their correct folding conformation. A buildup of misfolded proteins is very detrimental to the cell, possibly leading to proteotoxicity, and thus these proteins must be degraded. One mechanism that cells utilize to control elevated amounts of misfolded proteins is endoplasmic reticulum associated degradation (ERAD). While ERAD is important for degradation of soluble proteins it is unable to degraded non-soluble, aggregated proteins, which are instead degraded by autophagy.

ii. Endoplasmic Reticulum Associated Degradation

Proteins that are unable to reach their proper conformation are detected by the enzyme a1,2-mannosidase I, which cleaves a mannose group from an oligosaccharide group on the protein. Subsequently this protein is recognized by the ER degradation enhancing α–mannosidase-like protein (EDEM), which translocates the misfolded protein to the Sec61 translocation pore. The misfolded protein is then unfolded and retro-translocated through the pore into the cytoplasm where it becomes poly-ubiquitinated, an event that facilitates its recognition and subsequent degradation by the 26S proteasome. Conjugation of ubiquitin to proteins is a process that involves the action of three enzymes, an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2), and an ubiquitin ligase (E3). Polyubiquitinated proteins are then recognized by chaperone proteins such as CDC48/p97, and possibly sequestosome 1 (p62), which escort them to the 19S regulatory particle of the 26S proteasome. The 19S particle then presents the protein to the 20S particle of the proteasome which harbors enzymatic activity including chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing activities **(53)**. The importance of proteasomal clearance of proteins is evident by the finding that inhibition of proteasomal enzymatic activity, through treatment with drugs such as Velcade and MG132, is a strong inducer of cell death **(106, 169)**.

iii. Autophagy

In addition to degradation of proteins by ERAD, autophagy marks another pathway of degradation. The process of autophagy involves the sequestration of portions of the cytoplasm into intracellular membrane vesicles known as autophagosomes, subsequently autophagosomes fuse with lysosomes and their content is degraded. Initially autophagy was described as a process that occurs is response to starvation as a means to provide energy to the cell. However it is now recognized as a response to numerous types of stress such as ER stress, DNA damage and oxidative stress. The formation of autophagosomes occurs via the action of two ubiquitin-like conjugation systems that are orchestrated by a family of ATG proteins. The first conjugation system involves the conjugation of Atg12-Atg5 via the action of the E1-like enzyme Atg7, and the E2-like protein Atg10. The second system involves the conjugation of Atg8 (LC3) to phosphatidylethanolamine (PE), through the action of proteins Atg7 and Atg3.

This lipidated form of LC3 is known as LC3-II and because of its localization and accumulation on autophagosome membranes is widely used as a marker for autophagy **(69, 79, 88)**. While autophagy can randomly sequester portions of the cytosol, such may be the case when autophagy is induced in response to starvation, it can also be a selective process that can identify and degrade aggregated proteins and damaged organelles. The mechanism of selective autophagy is believed to involve the recognition of ubiquitinated aggregates by ubiquitin receptors at the autophagosome, namely sequestosome 1 (p62) and NBR1 **(68)**.

ER stress has been shown to induce the transcription of numerous genes, including those involved in autophagy such as ATG8 (LC3) and ATG14. Additionally the conversion of LC3-I to LC3-II has been observed to be increased when ER stress is induced, potentially through activation of the PERK-eIF2α pathway, which enhances transcription of proteins involved in LC3's conversion. While there are still questions that need to be addressed as to the main mediator of autophagy induced by ER stress, one supposed candidate is IRE1, which leads to the activation of c-Jun N-terminal kinase (JNK). Studies utilizing agents that block the IRE1-TRAF2-JNK pathway show that drug induced ER stress in this system is unable to activate autophagy **(54, 105, 167)**.

Initially, autophagy in response to ER stress acts as a survival response, functioning to degrade misfolded and aggregated proteins, as well as damaged organelles, such as portions of the ER **(80)**. However, like the ER stress response, while a limited amount of stress will induce a protective response, when the stress is more severe the response will trigger cell death. While excessive autophagy may trigger apoptosis it can also serve as a cell death program in and of itself, for example when apoptosis pathways are blocked **(150, 170)**.

B. ER stress response in Cancer

Due to the rapid proliferation of cancer cells, coupled to an insufficient blood supply, the environment of a cancer cell is often times hypoxic, nutrient depleted and low in glucose. These limiting environmental conditions thus result in a certain level of ER stress, which may actually contribute to tumor viability and resistance to treatment. Furthermore because many cancers have defects in their apoptosis machinery they are able to sustain an increased level of ER stress, and advantageously use features of the ER stress response, such as increased expression of chaperone proteins, to promote their growth. Currently, two approaches are being explored to exploit the elevated UPR response observed in cancer, one of which is to block critical mediators of the UPR to try and block pro-survival signals, while the other approach attempts to further enhance ER stress to shift in favor of a pro-death response **(50, 124)**.

i. Elevated ER stress in cancer

In the onset of malignancy cancer cells are faced with poor environmental conditions due to poor vascularization, which limits oxygen and nutrients as well as causes changes in environmental pH. Numerous responses are activated in response to these stresses including the unfolded protein response. Heightened ER stress leads to increased expression of chaperone proteins which is actually beneficial to cancer cells because they increase folding capacity which would be necessary for highly proliferative cells which have a higher output of protein production. For example, myeloma cells as well as other secretory cells, such as pancreatic tumors, require a heightened UPR response to meet the high demands of protein production. A

decreased UPR response in these cell types may result in cell death due to overwhelming stress from misfolded and aggregated proteins **(50)**. Additionally, many chemotherapeutics that are currently being used, such as etoposide and doxorubicin, have been shown to induce ER stress which could be good or bad for helping to destroy tumor cells **(40, 65, 118)**. If tumor cells are able to withstand a high degree of ER stress than using therapies that induce ER stress may be ineffective because tumor cells will be resistant. However, if tumor cells are already exposed to a high degree of ER stress than treating them with additional ER stress may shift in favor of a pro-death response. Importantly, while the UPR is known to function as both a pro-survival as well as a pro-death response, many times cancer cells are resistant to the pro-death consequences, possibly because they are able to suppress components in this pathway or because they have compromised apoptotic machinery, a common phenotype of cancer cells.

ii. Altered UPR in Cancer

As mentioned previously cancer cells often have elevated levels of chaperone proteins, one of the best characterized, GRP78/Bip, has been reported to be highly expressed in numerous cancers ranging for breast to colon cancer. Interestingly many of these studies have linked increased BiP levels to apoptosis resistance in cancer cells **(81, 100)**. For example one report presents data that dormant human epidermoid carcinoma cells are resistant to treatment with doxorubicin and etoposide due to increased PERK activation which leads to increased BiP levels, subsequently leading to decreased Bax activation and in turn decreased initiation of apoptosis **(118)**. Another possibility is that increased expression of BiP makes it more difficult for it to be released from the three ER stress sensors, thus allowing cancer cells to tolerate more protein misfolding and subsequent ER stress before transducing a stress response. Other proposed mechanisms for BiP's ability to inhibit apoptosis are through its inactivation of caspases 7 and 12 as well as through inactivation of Bik **(50)**. While PERK mediated BiP increases are linked to cancer cell survival, PERK has also been observed to have additional roles such as in promoting angiogenesis via expression of VEGF-A **(13)**. In addition to GRP78, the ER chaperone proteins GRP94 and calriticulin have been reported to contribute to cancer cell resistance to ER stress **(99)**.

Interestingly, it has been observed that most melanoma cell lines are insensitive to ER stress. One mechanism responsible for their resistance has been shown to be through increased expression of the anti-apoptosis Bcl-2 family protein, Mcl-1. The increased expression of Mcl-1 was attributed, in part, to increased transcription via IRE1α and ATF6 activation **(59)**. Indeed the Ire1/XBP1 pathway of the ER stress response is thought to be important for the growth of various types of cancers that are exposed to limiting environmental conditions. It has been observed that cancers of the breast as well as hepatocellular carcinomas and numerous other cancers have increased expression of XBP1 **(71)**. Additionally experiments conducted with MEF's or human fibrosarcoma tumor cells deficient in XBP1, were shown to be impaired in their ability to grow tumor xenografts **(127)**. Furthermore the Ire1 arm of the UPR is thought to be an essential mediator of angiogenesis that functions to upregulate VEGF-A expression, thus with impaired Ire1 cancer cells are unable to withstand hypoxic environmental conditions **(37)**.

iii. Treating cancers with ER stress

Two approaches are currently being investigated to try and harness the increased UPR in cancer cells to preferentially target these cells for death. While one school of thought believes taking advantage of cancer cells heightened ER stress and further promoting ER stress will drive cells towards cell death another thought is to inhibit ER stress. The idea behind inhibiting ER stress relies on the idea that cancer cells need a certain level of UPR in order to increase chaperone proteins and inhibit apoptosis. Additionally, most non-cancerous cells are not under chronic ER stress, thus using ER stress inhibitors would be non-toxic and have a minimal effect and consequences on normal cells. However a few cell types, such as B-cells that produce antibodies or insulin secreting pancreatic beta cells, require a functional UPR response to meet the high protein production demands, thus inhibiting the UPR in these cells could have detrimental consequences **(58, 104, 122)**.

Several drugs used clinically to treat various types of cancers have been reported to increase the unfolded protein response. Among these agents are proteasome inhibitors, which result in decreased protein degradation and a subsequent increase in poly-ubiquitinated proteins and aggregates **(38, 43-44, 52, 75, 78, 116, 138, 148)**. Inhibiting the proteasome inhibits ERAD a process that is vital for the UPR response to dispose of misfolded proteins. One such inhibitor, bortezomib (Velcade) has proven to be quite effective in treating multiple myeloma cells, which are secretory tumors that have high protein production. Treatment of these cells leads to induction of pro-apoptotic responses from the UPR such as activation of CHOP. Additionally, treatment of a human pancreatic cancer cell line L3.6pl, with bortezomib in combination with the anticancer drug cisplatin, was shown to be effective in treating cells, leading to an accumulation of ubiquitinated proteins and resulting in proteotoxicity and subsequent cell death **(98)**.

In addition to proteasome inhibitors other drugs targets that are being explored are the chaperone proteins, such as HSP90, which are critical in regulating folding and stability of numerous proteins involved in processes such as cell growth and differentiation. One such HSP90 inhibitor, 17-allylamino-17-demethoxygeldanamycin, was found to activate all three branches of the UPR, and subsequently result in cell death **(30)**. Another chaperone that has received a lot of attention as a possible drugable target is Hsp78/BiP. Several naturally occurring compounds such as epigallocatechin gallate (EGCG) found in green tea and salicyclic acid, have been shown to inhibit BiP, however it is likely that these compounds affect numerous other cellular functions. Specific GRP78 targeting peptides that were fused to the chemotherapeutic agent taxol, were shown to be effective in inducing apoptosis in cancer cells **(66)**. Additionally, there is a synthesized compound, versipelostatin, that inhibits Grp78 expression and in doing so represses the UPR, resulting in significant cell death in cells under chronic ER stress, such as cancer cells **(108-109)**.

In addition to the UPR targeting compounds mentioned above there are several other compounds that offer some promise in treating cancers. Brefeldin A, which inhibits the transport of proteins from the ER to the Golgi by inhibiting the ADP ribosylation factor (ARF), has been observed to induce cell death in numerous cancer cell lines such as, Jurkat, HeLa, leukemia, colon, prostate, and adenoid cystic sarcoma cells **(16, 22, 47, 129, 154)**. HIV protease inhibitors, such as nelfinavir and atazanavir, have also been reported to induce ER stress. These compounds cause the accumulation of polyubiquitinated proteins, leading to aggresome formation and subsequent cell death in malignant glioma cell lines **(116)**.

2) Cell Death Pathways

The foremost characterized cell death mechanism has been apoptosis, stretching back to its first description by Carl Vogt in 1842, while describing the maturation of a tadpole losing its tail. However it wasn't until 1965 that John Foxton Ross Kerr, with the help of the electron microscope, could define apoptosis morphologically as a programmed form of cell death. He recognized that apoptosis was a controlled process that a cell utilizes to eliminate cells that are no longer needed, have been damaged or are dangerous **(63)**. At this time it was distinguished from the other so called "spontaneous and traumatic" cell death known as necrosis. Since 1965 the topic of cell death has expanded tremendously so that now in addition to apoptosis and necrosis there is yet another form of cell death known as autophagy. Furthermore, the initial idea that necrosis is a spontaneous un-programmed event is now being called into question as it is now being shown that there are specific mediators of this process, whose inhibition protects cells. The importance of non-apoptotic cell death pathways is becoming more evident especially when considering cancer treatments. It is known that many times cancers cells have defects in their apoptotic machinery and yet these cells are still susceptible to death induced by chemotherapeutics.

With respect to cell death induced by endoplasmic reticulum stress, apoptosis is currently the main mode of cell death thought to be induced. However, it is also known that ER stress induces autophagy, which in the absence of apoptosis could potentially lead to cell death. Additionally, various responses to significant ER stress, such as dramatic increases in lysosomal activity and possible calcium perturbations within the cell can lead to circumstances that facilitate necrotic cell death through activation of lysosomal proteases. Thus with the idea that ER stress can be a target of cancer therapies, combined with the knowledge that most cancers are resistant to apoptosis it becomes apparent that further studies into alternative mechanisms of ER stress-induced cell death needs to be addressed.

A. Apoptosis

Referred to as "type 1 programmed cell death" apoptosis is a cell death process that is critical in organismal development as well as maintaining tissue homeostasis. Morphologically this process is characterized by nuclear condensation, nucleosomal fragmentation, and ordered degradation of cytoplasmic materials. Critical to the process of apoptosis are the Bcl-2 family of proteins whose members include both pro-apoptotic as well as anti-apoptotic proteins. Among the pro-apoptotic members are Bax and Bak, while anti-apoptotic members include proteins such as Bcl-2, Bcl-xL, and Mcl-1. Additionally important in regulating apoptosis are the BH3-only proteins, which function to regulate the activities of the pro-apoptotic and anti-apoptotic Bcl-2 proteins. Members of the BH3-only family of proteins include Bad, Bim, Bid, BNIP3, Blk, Hrk, Noxa, and Puma **(26, 29)**. The process of apoptosis can be separated into two pathways, the intrinsic or mitochondrial dependent pathway, and the extrinsic or mitochondrial independent pathway. While the intrinsic pathway is activated by internal cellular stresses such as DNA damage and ER stress, the extrinsic pathway is induced by extracellular stimuli such as the binding of death ligands, which belong to the tumor necrosis factor (TNF) superfamily, to cell surface death receptors.

i. Intrinsic Apoptosis

Intrinsic apoptosis begins with the activation of a BH3-only protein, which are strictly controlled both at the transcriptional as well as post-translational level. For example DNA damage induces p53 which goes on to increase the transcription of BH3 only proteins Puma and Noxa. On the other hand Bim has been shown to be controlled at the post-translational level, cellular stress can lead to a relocalization of Bim from the cytoskeleton, where it is bound to the dynein light chain of the microtubular dynein motor complex, to its presence at the mitochondria where it can bind to and inhibit the anti-apoptotic function of Bcl-2. Activation of BH3 only proteins, albeit through transcriptional upregulation or post-transcriptionally, leads to the binding of these proteins to anti-apoptotic Bcl-2 proteins, thus causing them to release there inhibitory hold on pro-apoptotic proteins Bax and Bak. Additionally BH3 only proteins bind to pro-apoptotic proteins causing a conformational change that allows them to oligomerize **(25, 29)**. For example, Bim can bind to Bax allowing a conformational change to occur that results in the relocalization of Bax from the cytosol to the mitochondrial membrane where it can now oligomerize with Bak. Thus upon stress stimuli and BH3 only activation, the pro-apoptotic proteins Bax and Bak oligomerize and insert into the outer mitochondrial membrane, facilitating the release of cytochrome C from the mitochondria. Release of cytochrome *c* from mitochondria induces the formation of the apoptosome, a multimeric complex formed by Apaf-1 and procaspase-9. Within this complex caspase-9 is activated resulting in the cleavage and activation of caspase-3 and caspase-7, which cleave key structural and regulatory proteins to bring about apoptotic cell death **(34, 119)**.

In addition to intrinsic apoptosis mediated by proteins functioning at the mitochondria, Bcl-2 proteins are also found to regulate cell death at the ER membrane **(2, 74)**. Upon ER stress the pro-apoptotic proteins Bax and Bak can, in addition to the mitochondria, form oligomers on the ER membrane, via ER stress-induced conformational changes that take place in these proteins. Their oligomerization compromises the ER membrane allowing the release of calcium. Additionally, caspase 12 activation was observed to occur and be dependent on Bax and Bak oligomerization on the ER membrane, which subsequently led to the activation of downstream effector caspases. Thus in addition to Bax and Bak initiating apoptosis from the mitochondria membrane they are additionally able to do so at the ER membrane in response to ER stress **(136, 175)**.

2) Extrinsic apoptosis

Extrinsic cell death begins with the binding of a trimeric pro-apoptotic ligand, such as FasL, to a cell-surface death receptor, such as Fas/CD95. The formation of this trimeric ligand-receptor complex leads to the recruitment of adaptor molecules, such as Fas-associated death domain (FADD), which subsequently recruits the initiator pro-caspase-8 via its death effector domain (DED). This complex, known as the death inducing signaling complex (DISC), facilitates the cleavage and self-processing of pro-caspase 8 into active caspase-8, which is responsible for going on to cleave and activate the executioner caspases 3 and 7. Often time the extrinsic pathway is not robust enough to ellicit cell death and requires further signal amplification through involvement of the intrinsic pathway. The two pathways merge through the BH3 only protein Bid, which is activated upon cleavage by caspase-8 into tBid. Bax undergoes a conformational change following interaction with tBid allowing it to go to the mitochondrial and facilitate intrinsic cell death **(4-5)**.

B. Autophagy

While autophagy, as mentioned above, is initially activated as a survival response, if it occurs in excess it can result in cellular demise **(79)**. Autophagic cell death, also known as "type II programmed cell death" is distinguished by the accumulation of double and multi-membraned electron dense vacuoles. However, it is still a topic of debate on whether autophagy in and of itself is a cell death mechanism or whether it occurs concurrently with cell death. As stated above, autophagy is induced by numerous stimuli including nutrient and energy stress, ER stress, hypoxia, redox stress, and mitochondrial damage. Autophagy is believed to function as a mechanism of cell death because when apoptosis is compromised it is observed that activation of autophagy can result in cell death **(170) (139)**. However, it is possible that the frequent presence of autophagosomes in dying cells is because cells upregulate autophagy in an attempt to dispose of harmful molecules, such as protein aggregates, or damaged organelles and that cells die from apoptosis or necrosis only once the autophagic system of defense has been overwhelmed. Additionally, several lines of evidence support autophagy having a role in regulating cell survival and tumor suppression. For example, monoallelic deletion of beclin 1 occurs in 40-75% of all human ovarian, breast, and prostate cancers **(1)**. Additionally, mice with a heterozygous loss of beclin 1 are observed to have an increased rate of spontaneous tumor development **(117) (171)**. However, the issue of autophagy as a tumor suppressor is a bit more complex, as it is also observed to have effects that support tumor growth, especially in tumor cells that are hypoxic and nutrient deprived. Furthermore, it has been reported that inhibition of autophagy enhances the efficacy of cancer chemotherapy agents, thus resulting in greater tumor regression **(3, 21)**. These conflicting reports make it difficult to definitively label autophagy as a mechanism of cell death.

C. Necrosis

Initially regarded as an uncontrolled, catastrophic process, necrosis, known as type III cell death, is defined as cell death that lacks the features of apoptosis or autophagy. However, necrosis is now distinguished from the other forms of cell death by morphological features such as early plasma membrane rupture and dilatation of cytoplasmic organelles, in particular mitochondria. Necrosis is regarded negatively because it is associated with traumatic, unnecessary cell death that often leads to inflammation, tissue damage, and an immune response. However, recent observations suggest that necrosis may not be as uncontrolled as originally thought. Indeed there are signal transduction pathways leading to necrosis that are believed to be at least partially responsible for maintaining the homeostasis of intestinal epithelial cells **(10)**.

The idea that necrosis may be a programmed event is supported by the finding that plasma membrane receptors, when bound by a specific ligand can lead to necrosis, which can many times be inhibited. For example, treatment of L929 mouse fibrosarcoma cells with TNFα has been shown to result in TNF receptor activation and subsequent necrotic cell death. In this scenario mitochondrial ROS is shown to play a role in promoting necrosis and thus its inhibition using antioxidants is observed to inhibit necrotic cell death **(131, 135)**. Additionally, it has been shown that following necrosis-inducing stimuli, certain controlled processes can be observed such as mitochondrial dysfunction, enhanced generation of reactive oxygen species, ATP depletion, proteolysis by calpains and cathepsins, and early plasma membrane rupture.

Furthermore it has been shown in *C. elegans* that treatment with lysosomal damaging agents, such as heat shock, hypotonic shock, oxidative stress and hypoxia results in necrotic cell death that requires caplain and lysosomal cathepsins and thus inhibition of these proteases by a serine protease inhibitor (serpin), SRP-6, is capable of inhibiting cell death **(86)**.

i. Lysosomal Membrane Permeabilization

In addition to the mitochondria, the lysosome is another organelle that is susceptible to damage by various types of stressing stimuli such as reactive oxygen species (ROS), DNA damage, photodamage, hypotonic shock, lysosomotrophic agents, and microtubule toxins **(17, 61, 86, 147)**. These agents can cause lysosomal membrane permeabilization, which allows the release of lysosomal hydrolases that can go on to damage critical cellular constituents indiscriminately. Additionally, if there is an overwhelming amount of lysosomal damage this can result in cellular acidification. While massive lysosomal damage results in necrotic cell death, it is believed that minimal damage, allowing the release of selective lysosomal hydrolases, can participate in activation of specific cellular proteins, such as caspases, and thus activate apoptotic cell death. The lysosomal cathepsins, B, L, and D have all been implicated in activating apoptosis when selectively released from the lysosome in response to certain stimuli. Interestingly, it has been proposed that lysosomal cathepsins are themselves inducers of LMP, functioning in a feedback loop whereby limited damage to the lysosomal membrane allows selective release of cathepsins, which can then cause further damage to the lysosome **(157)**. In addition, calpains have been shown to promote lysosomal release of cathepsins by compromising the integrity of the lysosomal membrane, thereby facilitating either apoptotic or necrotic cell death **(20, 161-162, 177)**. Calpains are activated by increased calcium concentrations within the cytosol, which could occur when the endoplasmic reticulum, a main storage site for calcium, becomes damaged. Thus, various stimuli can lead to lysosomal damage and subsequent leakage of acid hydrolases, such as cathepsins, and depending on the extent of the release of these hydrolases cell death can occur either through apoptosis or necrosis.

Like the mitochondria, which has Bcl-2 anti-apoptotic proteins as well as inhibitor of apoptosis (IAP) proteins to prevent unwarranted activation of apoptosis and caspases, the cell also contains proteins that can block the action of proteases that are aberrantly released from the lysosome. For example, Hsp70 has been shown to localize to lysosomal membranes and protect them from damage induced by stimuli such as etoposide, TNF-α and oxidative stress **(103)**. Additionally, there are several endogenous inhibitors of lysosomal hydrolases, such as cystatins and serine protease inhibitors (serpins). While cystatins are fairly unselective and inhibit a range of endopeptidases, serpins are more selective as to which proteases they inhibit. As their name implies, the vast majority of serpins inhibit serine proteases, however several serpins are capable of also inhibiting cysteine proteases, namely the cathepsins **(67)**.

3) Squamous Cell Carcinoma Antigen 1 (SCCA1)

One protein that has been proposed to play an important role in limiting injury caused by aberrant leakage of lysosomal proteases is SCCA1, a member of the serine protease inhibitor (serpin) family of proteins. Interestingly, SCCA was first identified as a serological marker for advanced squamous cell tumors in the cervix, and later was found to be associated with other types of cancer that were epithelial in origin such as lung cancer, head and neck cancer, and melanomas **(151)**. Evidence now also suggests that it may be useful as a marker for certain types of advanced breast cancers **(23)**. While the name serpin suggests inhibition of serine proteases, SCCA1 as well as it mouse paralog SerpinB3b, actually targets cysteine proteases such as cathepsin K, L, S, and V. It has been suggested that SCCA is found increased in certain cancers because the lysosomes within these cells are more vulnerable and thus must be protected from stimuli that could induced permeabilization and subsequent cell death **(140, 144)**.

A. Mechanism of inhibition

SCCA1 is a member of the clade B family of serpins, distinguishable from the other serpins by their cytosolic presence, in contrast to being secreted. All serpins adopt a metastable structure, consisting of three β sheets, nine α helices, and a reactive site loop (RSL). It is the reactive site loop, located at the C-terminus, which is responsible for acting as bait for the protease target. Within the RSL a single amino acid, which is designated the P1 residue, acts as the primary determinant for target specificity. Upon recognition of a serpin by the protease the two form non-covalent interactions. Peptide bond hydrolysis at the bond formed between the serpins P1 residue and the proteinase target leads to a dramatic conformational change within the serpin and proteinase, facilitating the formation of a very stable complex. This interaction distorts the active sites of both the serpin and the protease, thus rendering them both inactive **(57)**.

The SCCA locus in humans maps to locus 18q21, which contains 10 gene members of the clade B family of serpins. Corresponding mouse clade B serpin genes map to chromosome 1D. The mouse clade B serpin locus at 1D is expanded with respect to its human counterpart at 18q21, such that the human squamous cell carcinoma antigen (SCCA) locus encodes the two human clade B serpins *SERPINB3* and -*B4*, while in mice there are four genes, serpinb3a, -b3b, -b3c, and –b3d. While human SCCA1 and SCCA2 are nearly 91% identical at the amino acid level, their target proteinases are substantially different **(7-8)**. Human SCCA2 is responsible for inhibiting cysteine proteases Derp1 and Derf 1 as well as serine proteases such as human mast cell chymase and cathepsin G. SCCA1 on the other hand inhibits lysosomal cysteine proteases such as cathepsin K, L, S and V **(85)**. In mice serpinb3c and b3d have no known inhibitory activity, while serpinb3a has been shown to inhibit cysteine proteases cathepsin L and Derp1 as well as the serine proteases HMC and cathepsin G. Serpinb3b was shown to have inhibitory activity for serine proteases trypsin and plasmin as well as cysteine proteases cathepsin L and V **(7)**.

B. SCCA inhibition of cell death

While it is anticipated that SCCA1 has a role in preventing cellular demise due to activation of lysosomal proteases, the role of SCCA1 in limiting cell death extends beyond its

ability to block cathepsin activity. Indeed it has been observed that SCCA can prevent both necrotic as well as apoptotic cell death in response to numerous stimuli. Additionally, it has been reported that SCCA1's ability to inhibit certain types of apoptotic cell death is an RSL independent event.

As mentioned above SCCA has been found overexpressed in numerous types of cancers, however this protein has also been found to be expressed at low levels in normal epidermis. Interestingly, it was observed that when skin is exposed to UV irradiation SCCA1 is upregulated as well as translocated from its normal location in the cytosol to the nucleus. Additionally, following irradiation mRNA levels from cultured keratinocytes shows a marked elevation in SCCA1. Significantly increased SCCA1 expression is shown to protect cells from UV induced cell death via binding to phosphorylated JNK. Upon binding of SCCA1 to p-JNK the kinase activity of JNK is inhibited, thus preventing activation of downstream proteins such as c-Jun and subsequent cell death **(62)**. In addition to UV irradiation SCCA1 has also been shown to protect cells from apoptosis induced by TNF-α, IL-2 and the chemotherapeutic 7-ethyl-10-hydroxycamptothecin via an unknown mechanism that may involve inhibition of cytochrome C activity **(49, 142)**.

In addition to its ability to inhibit apoptotic cell death SCCA1 has also been shown to inhibit necrotic cell death. In C. elegans the SCCA1 homolog, SRP-6 was shown to protect animals from stimuli such as hypotonic shock, hypoxia and oxidative stress that induce a lysosomal dependent cell death. The mechanism of protection was through SCCA1 inhibition of calpain as well as lysosomal cathepsins. Inhibition of these enzymes prevented lysosomal permeabilization and subsequent necrotic cell death **(86)**.

C. SCCA1 in Cancer

It has been reported that often times cancer cells have alterations in their lysosomes, such as altered lysosomal trafficking, enhanced size, and increased expression of proteases. These changes may render cancer cells more susceptible to stimuli that can damage lysosomal membranes, which would allow the release of harmful proteases. To protect against lysosomal permeabilization cancer cells may increase the expression of lysosomal protease inhibitors, such as cystatin A and B as well as SCCA1. The finding that SCCA1 is present in the serum of patients with various types of epithelial cancers, such as lung cancer, head and neck cancer, melanomas, and hepatocellular carcinoma, suggests it plays a role in cancer **(153)**. Further studies have shown that indeed SCCA1 is a useful diagnostic/prognostic marker for certain squamous cell carcinomas, where its elevated expression is associated with a poor prognosis and response to treatment, allegedly due to increased resistance to cell death **(97)**. There have been several studies that have shown SCCA1 in cancer cells confers resistance to cell death **(49) (153)**.

One example of SCCA1 promoting tumor cell growth is in the cervical cancer cell line SKG IIIa, which normally produces a high level of SCCA1. When SCCA1's expression is inhibited using an antisense construct, in comparison to control cells, these cells are impaired in their ability to grow tumors in vivo. Attenuated tumor growth is shown to be the result of increased cell death, characterized as apoptosis, as well as increased infiltration of natural killer (NK) cells, which supported enhanced cell death of cancer cells **(141)**. This finding is further supported by data which utilized a mouse lung SCC cell line, KLN-205, that normally expresses a low level of SCCA. When this cell line was transduced with a SCCA1 plasmid, then

subsequently injected into nude mice, the cells with high levels of SCCA1 were able to grow substantially larger tumors. When tumors were sectioned and biopsied those from the SCCA1-high expressing cells had significantly lower levels of apoptotic cells **(142)**. Additional studies revealed that not only does elevated expression of SCCA1 promote tumor growth but it also protects cell from treatment with various types of damaging stimuli. For example when the SKG IIIa cells were treated in vitro with etoposide, control SKGIIIa cells were significantly more resistant than the cells containing antisense SCCA1 **(142)**. Furthermore, similar studies were performed in PCI-51 cells, a human SCC of the head and neck which expresses a very low level of SCCA1. When these cells were transduced with a retroviral SCCA1 construct they were observed to be more resistant to treatment with the chemotherapeutic SN-38 as well as TNF-α in comparison to control cells, which was attributed to a decrease in apoptosis **(142)**.

Additional studies have provided further evidence that SCCA1 is able to protect cells from TNFα treatment. When a human oral squamous cell carcinoma cell line was treated with TNFα, cells stably overexpressing SCCA1 were resistant to cell death, while control cells were observed to succumb to apoptosis via cytochrome C release followed by caspase activation. In this report the investigators claim that SCCA1 inhibits apoptosis via blocking cytochrome C release through a currently unknown mechanism **(49)**.

Cathepsin L (cath L) as stated above is one target of SCCA1. In a study looking at the effects of resveratrol (RSV), an antimicrobial compound found in various plant based foods such as nuts and grapes, on cervical cancer cells it was found to decrease cell growth and enhance cell death by promoting LMP. Additionally, RSV enhanced autophagy, indicated by the increase in LC3II as well as autophagosomes. Upon prolonged treatment with RSV, apoptotic cell death ensued via a cath L dependent pathway. Interestingly, in this study SCCA1 was found to protect cancer cells from RSV-induced cell death and when its expression was decreased using small interference RNA (siRNA), RSV cytotoxicity was dramatically enhanced. Thus this study came to the conclusion that SCCA1 was able to protect against LMP and subsequent cath L release into the cytosol, making cervical cancer cells resistant to RSV treatment **(55)**.

As more evidence arises in support of SCCA1 having a pro-cancerous and anti-apoptotic role, it becomes increasingly apparent that targeting SCCA1 may be therapeutically advantageous. Thus it is important to try and uncover how SCCA1 affects cell fate. While studies point toward a role for SCCA1 in preventing cell death it is currently still unknown how SCCA1 performs this role. Uncovering the mechanisms by which SCCA1 protects cells from death inducing stimuli may aid in the development of therapeutics that can more effectively target SCCA1 expressing cancers.

II. Results

1) Prolonged ER stress induces cell death in Bax/Bak doubly deficient cells

The Bcl-2 family of proteins are essential in coordinating the process of apoptosis. Within this family are both anti-apoptotic as well as pro-apoptotic members. Among the pro-apoptotic proteins are Bax and Bak, whose activation and subsequent oligomerization on the mitochondrial membrane lead to mitochondrial outer membrane permeability, which facilitates the release of cytochrome C, leading to caspase activation. Among the numerous stresses that have been shown to activate intrinsic apoptosis are those that induce ER stress, such as tunicamycin, an inhibitor of N-linked glycosylation; thapsigargin, an inhibitor of sarcoplasmic/endoplasmic Ca^{2+}-ATPases; and Brefeldin A, an inhibitor of ER-Golgi transport. While it has been previously shown that cells deficient in both Bax and Bak are resistant to apoptosis, we wanted to determine what the response would be to sustained ER stress, a physiologically relevant occurrence.

In order to observe the response to prolonged ER stress, mouse embryonic fibroblasts (MEFs) were treated with the ER stress inducers tunicamycin (0.5μg/ml), thapsigargin (0.1mM), and Brefeldin A (0.5 μg/ml). Propidium iodide (PI) exclusion was used to determine cell viability, as only cells with compromised plasma membranes will stain positive for PI. As previously reported we found the wild-type MEFs were mostly all dead after 1-2 days of treatment, while the Bax/Bak doubly deficient cells were protected (Fig. 1A). However, upon sustained treatment with ER stress the apoptosis deficient *bax*$^{-/-}$*bak*$^{-/-}$ MEFs gradually died (Fig. 1A). Thus it seems that ER stress can induced two stages of cell death, one that is dependent on Bax and Bak and one that is slower occurring and Bax/Bak independent. Additionally, in further support of this observation, MEF's over-expressing the anti-apoptotic protein Bcl-xL, while initially protected from cell death induced by tunicamycin, thapsigargin, and Brefeldin A, eventually died in response to long term treatment with these drugs (Fig. 1B).

2) Necrosis may be responsible for ER stress-induced cell death in *bax*$^{-/-}$*bak*$^{-/-}$ cells

Our results indicate that cell death in response to ER stress can occur independent of conventional apoptosis, thus we sought to determine what mechanism was responsible. While observing the cells under a phase-contrast microscope it was noted that the dead *bax*$^{-/-}$*bak*$^{-/-}$ MEFs displayed features, such as plasma membrane dilation and disruption, which are characteristic of necrotic cells (Fig. 2A). To further determine whether apoptosis was responsible or whether a different mode of cell death was induced in the *bax*$^{-/-}$*bak*$^{-/-}$ MEFs treated with ER stress, PARP cleavage was examined. Poly(ADP-ribose) polymerase (PARP) is a substrate for activated caspase 3, which cleaves the full length 114 kDa protein into an 89 kDa fragment. While PARP cleavage is observed in wild-type cells 1-2 days following treatment with brefeldin A or thapsigargin, the *bax*$^{-/-}$*bak*$^{-/-}$ MEFs did not display any PARP cleavage, even after 3-4 days of treatment, at which time cell death in these cells is comparable to wild-type cells at 1-2 days (Fig. 2B). Furthermore wild-type cells treated with tunicamycin for 48 h show nucleosomal fragmentation, whereas wild-type cells overexpressing Bcl-xL do not, thus further suggesting that cell death in apoptotic defective cells in response to prolonged ER stress is not due to apoptosis (Fig. 2C).

To further establish whether necrosis may be the mechanism responsible for cell death in the Bax/Bak cells treated with ER stress, the presence of high-mobility-group box protein 1 (HMGB1) in cell medium was examined. It has been reported that HMGB1, while normally

found tightly associated with the DNA of apoptotic cells, is released extracellularly from necrotic cells. Thus, we wanted to observe whether HMGB1 was found in the medium of ER stress treated *bax*$^{-/-}$*bak*$^{-/-}$ MEFs, thus indicating necrosis may be the mechanism of cell death. As expected, treatment of *bax*$^{-/-}$*bak*$^{-/-}$ cells with the DNA alkylating drug MNNG (0.5μM), which has been reported to induce necrosis, caused the extracellular release of HMGB1 within 24 hours of treatment, as noted by its presence in cell culture media. Significantly, HMGB1's presence in culture media was also observed in *bax*$^{-/-}$*bak*$^{-/-}$ cells after 4 days of treatment with thapsigargin, tunicamycin, and brefeldin A (Fig. 2D). These preliminary results suggest that prolonged treatment of apoptosis deficient cells with ER stress-inducers can lead to necrosis.

3) ER stress induces autophagy

Upon visualization of wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ MEFs treated with ER stress, it was observed that both cell lines had an increased presence of multi- or double layered membrane structures that enclosed electron dense material, which is characteristic of autophagosomes (Fig. 3A and B). Additionally, while 2 days of treatment with tunicamycin lead to chromatin condensation, a characteristic feature of apoptosis, in wild-type cells, this was not observed in *bax*$^{-/-}$*bak*$^{-/-}$ cells. While apoptotic features were not observed in the apoptosis deficient *bax*$^{-/-}$*bak*$^{-/-}$ cells, autophagosomes persisted (Fig. 3A and B). To further examine whether autophagy was induced by ER stress, the conversion of microtubule associated protein 1 (MAP1) light chain 3 (LC3) from its cytosolic (LC3-I) to membrane bound (LC3-II) form was visualized in cells expressing GFP-tagged LC3. It is observed that upon treatment with tunicamycin, thapsigargin, and brefeldin A, GFP-LC3 changes it pattern within the cell from a diffuse cytosolic presence to punctate (Fig. 4A and B). Furthermore, autophagy appears to be similarly induced in both wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ MEFs. Additionally, this process is inhibited by 3-methyladenine (3-MA), which blocks a Class III PI3K that is required for autophagosome formation (Fig. 4A and B). In contrast treatment with chloroquine, a chemical that increases lysosomal pH, thereby preventing lysosomal turnover, causes accumulation of autophagosomes (Fig. 4A and B).

4) ER stress-induced autophagy is not affected by the Bcl-2 family of proteins

The process of apoptosis is mediated by the Bcl-2 family of proteins. Interestingly, it has also been reported that members of this family can regulate autophagy. Beclin-1, first identified as a Bcl-2 interacting protein, is responsible for forming a complex with the lipid kinase protein Vps34, as well as Vps15, thereby forming the core complex necessary for autophagy induction. Beclin-1 has also been shown to interact with Bcl-2 family members such as Bcl-xL, Bcl-w, and MCL-1, and upon doing so inhibit autophagosome formation. It has been proposed that Beclin-1 interaction with Bcl-2 proteins prevents its interaction with Vps34, thus preventing autophagy induction. In light of these observations it is possible that the pro-apoptotic proteins Bax and Bak may affect autophagy via there interaction with Bcl-2. To determine whether Bax and Bak have an effect on autophagy, the formation of autophagosomes in response to ER stress treatment was quantitated in wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells. Similar levels of GFP-LC3 puncta were observed in wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells (Fig. 5A). In addition to looking at GFP-LC3 puncta as a readout for autophagy, immunoblotting for LC3 can also be used as a means to quantitate autophagy. Upon conversion of LC3-I to the membrane bound form LC3-II, a phosphatidylethanolamine (PE) group is added, causing the PE-conjugated LC3-II to migrate

faster than LC3-I on an SDS-PAGE gel. Indeed we found that treatment with ER stressors, thapsigargin, tunicamycin, and brefeldin A leads to LC3 conversion, apparent by the mobility shift of LC3I (18kD) to LC3II (16kD), in both wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells (Fig. 5B). Additionally, the conversion of LC3 appeared as early as 4 h after treatment and seemingly occurred at similar time points and levels in wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells, thus further supporting our above data that the autophagic response in *bax*$^{-/-}$*bak*$^{-/-}$ cells is similar to wild-type cells. The UPR responsive protein CHOP was also examined to determine whether the response to ER stress was similar in wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells. It was observed that induction of CHOP occurred as early as 4 h in both wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells and to similar levels, suggesting the UPR response is not altered by the absence of Bax and Bak (Fig. 5B). Furthermore, as in Fig. 4A and 4B, the conversion of LC3 was suppressed by 3-MA, while it was enhanced by treatment with chloroquine (Fig. 5C). Treatment with 3-MA or chloroquine did not affect CHOP induction. Additionally, the lysosomal protease inhibitors E-64-D and pepstatin A enhanced the accumulation of LC3-II to a similar degree in wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells, indicating autophagic turnover is also similar in wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells (Fig. 6A and B). These results indicate that deficiency in Bax and Bak has no significant effect on ER stress-induced autophagy. Altogether these results further suggest that ER-stress induced autophagy is not affected by the absence of the pro-apoptotic proteins Bax and Bak.

To further characterize the possible effect the pro-apoptotic Bcl-2 proteins Bax and Bak may have on ER stress-induced autophagy, overexpression of Bax and Bak wild-type and mutant proteins in *bax*$^{-/-}$*bak*$^{-/-}$ cells were studied. It is possible that due to their interaction with Bcl-2 or their possible effect on calcium homeostasis that Bax and/or Bak are capable of activating autophagy. Additionally, it has recently been shown that the ER-targeted Bcl-2/Bcl-xL is a more potent inhibitor of autophagy then wild-type Bcl-2, possibly through their interaction with Beclin 1 or through influencing ER Ca^{2+} flux **(28, 54, 110)**. Moreover, Bax and Bak have also been shown to perturb ER calcium stores **(101-102, 136, 175)**. Thus, it would be informative to observe whether ER targeted Bax or Bak have any effect on autophagy. To address this issue we utilized ER-targeted Bak, which unlike Bax whose membrane-targeting domain structure remains controversial, has a well defined carboxy-terminal hydrophobic transmembrane domain. This Bak mutant was created by replacing the C-terminal transmembrane domain of Bak with the ER targeting sequence of cytochrome b5 (cb5). This Bak-cb5 mutant has previously been shown to localize to the ER where it facilitates ER Ca^{2+} release and subsequent apoptosis **(175)**.

In an attempt to answer the above questions, DsRed-tagged Bak or Bax were co-transfected with GFP-LC3 into *bax*$^{-/-}$*bak*$^{-/-}$ MEFs. To verify that these proteins were functional, DsRed-tagged Bax, Bak and Bak-cb5 were left untreated or treated with BFA or tunicamycin for 24 h and cell death subsequently measured by PI exclusion. As expected in comparison to *bax*$^{-/-}$*bak*$^{-/-}$ MEFs expressing a vector control, cell death was enhanced in cells expressing Bax, Bak, or Bak-cb5 (Fig. 7A). Furthermore, it was observed that Bak was predominantly localized to mitochondria indicated by its punctate localization and colocalization with the mitochondrial marker COX IV (Fig. 7B). Expression of DsRed-Bak did not induce autophagy, nor did it affect tunicamycin-induced autophagy (Fig. 8B and 8C). Furthermore, the ER-targeted Bak mutant, Bak-cb5, also failed to affect autophagy induction (Fig. 7C). Bax was predominantly localized in the cytosol. In response to ER stress, it translocated from the cytosol to mitochondria. Similar to Bak, expression of DsRed-Bax failed to induce autophagy and did not alter ER stress-induced autophagy (Fig. 8A and 8C). Taken together, our findings indicate that unlike the antiapoptotic

Bcl-2 family proteins, the multi-domain proapoptotic Bcl-2 proteins Bax and Bak do not affect autophagy, nor do they affect Bcl-2's ability to regulate autophagy.

5) Autophagy promotes cell death in apoptosis deficient cells

Autophagy has been shown to promote either cell survival or death. Given that autophagy can be induced by ER stress, we evaluated whether autophagy plays a role in $bax^{-/-}bak^{-/-}$ cell death resulting from prolonged ER stress. Cells treated with ER stress in the presence of the autophagy inhibitor 3MA appeared to be more adherent to the culture plates, and maintained relatively normal fibroblast appearance (Fig. 9A). In addition, when measured by plasma membrane permeability, 3MA significantly enhanced the viability of $bax^{-/-}bak^{-/-}$ cells in response to ER stress (Fig. 9B). These results indicate that autophagy can enhance cell death in $bax^{-/-}bak^{-/-}$ cells. In contrast, 3MA enhanced ER stress-induced cell death in apoptosis-competent wild-type cells (Fig. 9C). These findings suggest that autophagy may have an opposite effect in determining cell fate in response to ER stress in apoptosis-competent and deficient cells. To determine more specifically whether autophagy plays a role in Bax/Bak-independent cell death in response to ER stress, the essential autophagy factor Atg5 was knocked down by short hairpin RNA in $bax^{-/-}bak^{-/-}$ MEFs. Similar to 3MA, inhibition of autophagy by Atg5 knockdown resulted in significant protection against thapsigargin and brefeldin A (Fig. 10A and 10B).

The above results suggest that autophagy can promote cell death in apoptosis-deficient cells. To determine whether this is a peculiar effect that can only be observed in $bax^{-/-}bak^{-/-}$ cells, Bcl-xL was ectopically expressed at similar levels in $atg5^{+/+}$ and $atg5^{-/-}$ cells to block apoptosis. When treated with ER stress, $atg5^{-/-}$ cells died faster than $atg5^{+/+}$ cells (Fig. 10C), consistent with recent reports that autophagy acts as a survival mechanism to suppress apoptosis in response to ER stress **(73, 105)**. As anticipated, Bcl-xL protected both $atg5^{+/+}$ and $atg5^{-/-}$ cells from acute apoptosis. In response to prolonged ER stress the Bcl-xL-overexpressing cells progressively died. Interestingly, Bcl-xL-overexpressing $atg5^{-/-}$ MEFs survived better than Bcl-xL-overexpressing $atg5^{+/+}$ MEFs (Fig. 10C). Taken together, our findings indicate that while autophagy serves as a survival mechanism in apoptosis-competent cells, those with defects in apoptosis can utilize autophagy as a means to promote non-apoptotic cell death in response to ER stress.

6) Necrosis may be induced as a cell death mechanism in apoptotic deficient cells treated with ER stress

While the above results support a role for autophagy in promoting ER-stress induced cell death in Bax and Bak deficient cells, it remains to be determined whether autophagy is the primary mediator of cell death in these cells. Indeed, it is observed that inhibition of autophagy in apoptosis deficient cells does not block cell death but rather delays it. Thus, it is possible that another mechanism of cell death, such as necrosis, may be activated by prolonged exposure to ER stress or prolonged autophagy. Several previous studies have reported that necrosis can be induced by ER stress, through several mechanisms such as calpain activation **(64)** and CHOP induction **(70, 90)**.

One of the proteins that is activated upon prolonged ER stress is C/EBP homologous protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153

(GADD153). While numerous studies have shown that CHOP plays an important role in mediating apoptosis when there is unresolved ER stress, **(94, 173)** a limited number of studies have also suggested a role for CHOP in mediating necrosis **(70, 90)**. Interestingly, CHOP induction occurs at similar times and levels in wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells treated with ER stress (Fig. 5B and 5C). It is foreseeable that prolonged induction of CHOP could influence the transcription of proteins that could promote necrosis, such as GADD34 and ERO1, two known downstream targets **(90)**. Furthermore, tunicamycin resistant clones were isolated from 10cm plates of *bax*$^{-/-}$*bak*$^{-/-}$ cells that were treated every other day for 14 days with tunicamycin. Interestingly, it was observed that in addition to having elevated Bip expression, these clones lacked CHOP induction (Fig. 11A and 11B). To further elucidate whether CHOP was responsible for inducing cell death in *bax*$^{-/-}$*bak*$^{-/-}$ cells, short hairpins targeting CHOP were transfected into *bax*$^{-/-}$*bak*$^{-/-}$ cells, and cells were subsequently treated with tunicamycin for the indicated amount of time (Fig. 11C and 11D). While CHOP knockdown may confer some protection from ER stress, it is unlikely that CHOP is the main mediator of cell death, as cells expressing little CHOP were not significantly protected in comparison to parental *bax*$^{-/-}$*bak*$^{-/-}$ cells (Fig. 11D). Additionally, a CHOP knockout cell line was used to study the effects of ER stress on apoptosis deficient cells. The anti-apoptotic protein Bcl-xL was overexpressed in these cells and cells were subsequently treated with tunicamycin. Treatment with tunicamycin in wild-type and *chop*$^{-/-}$ MEFs induced a similar amount of cell death. In comparison, cells expressing Bcl-xL were observed to be protected, however no significant difference was observed between wild-type cells and those lacking CHOP (Fig. 11E). It is probable that CHOP repression alone is not sufficient to confer protection and other changes, such as Bip overexpression, are necessary to confer protection from ER stress.

As mentioned above it has been suggested that calpains are able to promote necrosis. Calpains are calcium-dependent, non-lysosomal cysteine proteases that have been reported to be activated in response to ER stress, due to increases in cytosolic calcium **(132, 145-146)**. While reports suggest that calpain activation leads to caspase 12 activation and subsequent apoptotic cell death, it is possible in apoptosis deficient *bax*$^{-/-}$*bak*$^{-/-}$ cells that calpains may lead to necrotic cell death via lysosomal release of cathepsins into the cytosol. Indeed it has been observed that activated μ-calpain is found at the lysosomal membrane where it facilitates lysosomal membrane permeabilization, allowing release of cathepsins into the cytosol where they go on to degrade cellular proteins and induce necrotic cell death **(161-164)**.

To determine whether calpains are possibly facilitating cell death in *bax*$^{-/-}$*bak*$^{-/-}$ cells in response to ER stress, cells were either treated with the ER stress-inducing drug tunicamycin alone, or in combination with PD150606 ([3-(4-Iodophenyl)-2-mercapto-(Z)-2-propenoic acid], which is a cell permeable, selective calpain inhibitor that is directed towards the calcium binding site of calpain **(155)**. This inhibitor is able to inhibit both μ and m calpains. Additionally, to determine the role of caspases in ER stress-induced cell death, the broad caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone) was also added in combination with tunicamycin. In response to Z-VAD-FMK, wild-type cells showed moderate protection at 24 h, while combination treatment of tunicamycin and PD150606 showed no protection (Fig. 12A). Treatment of *bax*$^{-/-}$*bak*$^{-/-}$ cells with Z-VAD-FMK showed little protection, however unlike in wild-type cells, combined treatment with PD150606 showed substantial protection from cell death in response to prolonged tunicamycin treatment (Fig. 12B). This suggests that calpains play a role in mediating cell death in apoptosis deficient cells treated for a prolonged period of time with ER stress.

It is possible that calpains may facilitate cell death in apoptosis deficient cells by compromising the integrity of the lysosome, thereby allowing the leakage of lysosomal proteases which can go on to induce necrotic cell death, an event that has been previously reported to occur in response to various types of stimuli **(86)**. Indeed we have found that treatment of *bax*$^{-/-}$*bak*$^{-/-}$ cells with the lysosomal inhibitor chloroquine, in combination with ER stressors, enhances cell death (Fig. 12C). Chloroquine exerts its affect by lowering lysosomal pH and preventing lysosomal turnover, which can allow for lysosomal content to accumulate, leading to enlarged lysosomes, which may be more unstable and sensitive to rupturing. To determine whether lysosomal cathepsins were indeed responsible for mediating cell death in response to prolonged ER stress, we attempted to endogenously inhibit cathepsins and observe whether this prevented cell death.

7) SCCA1 inhibits cathepsin activity and lysosomal damage

Previous studies have indicated that several intracellular serpins are capable of inhibiting lysosomal cysteine peptidases such as cathepsin K, L, S and V **(140, 144)**. Srp-6, a SCCA1 homolog in *C. elegans*, protects cells from necrosis induced by lysosomal injury caused by hypotonic stress, hypoxia, heat shock, and oxidative stress **(86)**. Many of these stress conditions are often encountered by cancer cells during tumor development and anti-cancer therapy. We expressed Flag-tagged SCCA1 and its mouse homolog SerpinB3b in *bax*$^{-/-}$*bak*$^{-/-}$ baby mouse kidney (BMK) cells **(33)** to examine whether SCCA1 can block necrotic cell death induced by lysosomal injury resulting from DNA alkylating damage or hypotonic shock (Fig. 13A) **(46, 86, 174)**. The use of *bax*$^{-/-}$*bak*$^{-/-}$ cells facilitates the study of alternative death pathways in cells with compromised mitochondrial apoptotic machinery, which is a main feature of cancer cells **(26, 156, 176)**. During lysosomal injury, cathepsins are released into the cytosol and can be detected by a fluorogenic substrate specific for activated cathepsin L. While the DNA alkylating agent mafosfamide and hypotonic shock induced cytosolic cathepsin activity in parental cells, indicating lysosomal injury, SCCA1 and SerpinB3b blocked this cathepsin activation (Fig. 13B). The activation of cathepsin L correlated with a loss of lysosomal integrity in parental cells, indicated by the decrease in LysoTracker Red staining (Fig. 13C and 13D). However, expression of SCCA1 or SerpinB3b resulted in larger size lysosomes, whose integrity was preserved in response to mafosfamide treatment (Fig. 13C and 13D).

8) SCCA1 inhibits cell death caused by lysosomal damaging agents

The ability of SCCA1 and SerpinB3b to block lysosomal damage correlated with its ability to suppress cell death induced by mafosfamide, as well as another DNA alkylating agent MNNG, and hypotonic shock (Fig. 14A and 14B). This is consistent with our previous finding that necrosis can be induced by these types of cellular damage **(174)**, and the report showing that serpin can inhibit necrosis caused by lysosomal injury **(86)**. Furthermore, caspase cleavage in response to mafosfamide treatment was not observed, further supporting that apoptosis was not contributing to cell death (Fig. 14C). The ability of SCCA1 to block lysosomal injury and cell death was not restricted to apoptosis deficient cells, as the same effect was observed in apoptosis competent MCF10A or Hs578T cells (Fig. 15A-E). To verify that the protection against lysosomal injury is specifically conferred by SCCA1, *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells ectopically expressing SCCA1 were infected with lentiviral SCCA1 shRNA to knockdown its expression (Fig. 16A-D). Indeed knockdown of SCCA1 resensitized cells to lysosomal injury indicated by

decreased cell survival (Fig. 16B), increased cathepsin L activation (Fig. 16C), and decreased LysoTracker Red staining (Fig. 16D) in response to hypotonic shock. The resensitization to lysosomal injury was also observed in apoptosis-competent MCF10A cells using a tetracycline-inducible knockdown of ectopically expressed SCCA1 (Fig. 15A-C). Taken together, these results indicate that SCCA1 and SerpinB3b are able to protect cells from stimuli that induce lysosomal membrane permeabilization and necrosis.

9) SCCA1 enhances ER stress-induced cell death

In addition to hypotonic shock and DNA alkylating damage, *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells expressing either SCCA1 or SerpinB3b were treated with the ER stress inducers tunicamycin, an inhibitor of N-linked glycosylation, and thapsigargin, an inhibitor of the SERCA calcium pump. Surprisingly, unlike the protective effect of SCCA1 and SerpinB3b in cells treated with DNA alkylating damage and hypotonic shock, they markedly enhanced cell death induced by ER stress in *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells (Fig. 17A and 17B). The pro-death effect of SCCA1 and SerpinB3b is not limited to *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells, as it was also found to promote cell death in human breast cell lines in response to ER stress (Fig. 17C-E).

10) Lysosomal function is compromised in SCCA1 expressing cells

The enhanced cell death in serpin-expressing cells correlates with their inability to efficiently degrade lysosomal content, indicated by both the enlarged size of their lysosomes as well as their inhibition of cathepsin activity (Fig. 13B-D, 18A and 18B). Additionally, while ER stress markedly enhanced lysosomal presence in parental cells, this was not observed in SCCA1-expressing cells, further suggesting their ability to cope with ER stress is compromised (Fig. 18A and 18B). Consistent with the compromised lysosomal degradation, there was a higher steady-state level of UPR response as shown by elevated levels of GRP78/Bip in SCCA1 and SerpinB3b-expressing cells (Fig. 18C). Both GRP78/Bip and GADD153/CHOP inductions took place earlier in these cells (Fig. 18C), indicating that the cells may attempt to upregulate the chaperon/UPR machinery as SCCA1 inhibits lysosomal protein degradation. In addition to *bax*$^{-/-}$ *bak*$^{-/-}$ BMK cells, enhanced CHOP induction was also observed in Hs578T cells ectopically expressing SCCA1. Knockdown of SCCA1 in these cells restored the normal UPR response (Fig. 22E).

11) SCCA1's ability to enhance ER stress-induced cell death requires its protease inhibitory activity

Essential to serpins' protease inhibitory activity is their reactive site loop (RSL), which is the domain responsible for binding to their protease target. In order to determine whether the death-promoting effects of SCCA1 and SerpinB3b depend on their ability to inhibit proteases, mutants were constructed which harbored a point mutation within the RSL (Fig. 19A). The mutant SCCA1-F352A has been previously described to have impaired protease inhibitory activity **(85)**. Moreover, we generated two SerpinB3b mutants, SerpinB3b-E348A and SerpinB3b-S350G. These mutant proteins were expressed at similar levels as their wild-type counterparts (Fig. 19B). All three mutants had impaired ability to inhibit cathepsin L activity in response to hypotonic shock (Fig. 19C). We then used SCCA1-F352A and SerpinB3b-S350G

for subsequent experiments. Correlatively to their impaired ability to inhibit cathepsins, both mutants failed to prevent cell death as efficiently as the wild-type SCCA1 or SerpinB3b in response to mafosfamide and hypotonic shock (Fig. 19D and 19E). Conversely, these mutants failed to enhance cell death in response to ER stress (Fig. 19F and 19G and 17E). These results indicate that the protease inhibitory activity of serpins is essential for their ability to protect cells from lysosomal injury, and to enhance ER stress-induced cell death. The failure of SCCA1-F352A and SerpinB3b-S350G mutants to enhance ER stress-induced cell death also indicates that the enhanced death in cells expressing wild-type SCCA1 and SerpinB3b was not merely caused by protein overexpression.

12) SCCA1 promotes apoptosis in response to ER stress independently of the mitochondrial apoptotic pathway

Cell death induced by ER stress is by and large attributed to mitochondria-dependent apoptosis **(51)**. As Bax and Bak play essential roles in mitochondrial apoptosis, our finding that SCCA1 and SerpinB3b promote cell death in *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells indicates a form of cell death that is independent of the mitochondrial apoptotic pathway. Indeed, while treatment of wild-type cells with tunicamycin led to cytochrome C release from mitochondria, SCCA1-expressing *bax*$^{-/-}$ *bak*$^{-/-}$ BMK cells failed to release cytochrome C as shown by both subcellular fractionation and immunofluorescence (Fig. 20A and 20B). However, DAPI staining in SCCA1 cells following tunicamycin treatment revealed an increase in apoptotic nuclear condensation and fragmentation, a process that occurs as a result of caspase activation, suggesting a caspase-mediated apoptosis in these *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells (Fig. 21A). We then examined caspase activation in *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells overexpressing SCCA1, using wild-type cells proficient in mitochondrial apoptosis as a control. As expected, apoptosis-competent wild-type cells treated with tunicamycin showed apoptotic cleavage of caspase-3, PARP, and caspase-8. While these apoptotic events were not observed in *bax*$^{-/-}$*bak*$^{-/-}$ BMK parental cells, SCCA1-expressing *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells were observed to have cleaved caspase-8 after 16 hours of treatment followed by both caspase-3 and PARP cleavage (Fig. 21B). A fluorometric caspase-8 activity assay revealed that while tunicamycin treatment induced little caspase-8 activation in the parental *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells, it greatly induced caspase-8 activation in SCCA1-expressing *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells after tunicamycin treatment for 16 hours (Fig. 21C). SCCA1's ability to promote caspase-8 cleavage was not a process confined to cells lacking Bax and Bak, as apoptosis competent MCF10A and Hs578T cells ectopically expressing SCCA1 were also observed to have an increased amount of cleaved caspase-8 compared to the parental cells (Fig. 22A, 22C and 22E). Knockdown of SCCA1 in SCCA1 expressing *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells, MCF10A, and Hs578T cells reduced cell death and caspase-8 cleavage induced by tunicamycin (Fig. 22A-E, 17C and 17D). These results indicate that in response to ER stress SCCA1 promotes cell death that may involve activation of caspase-8, and may be independent of mitochondrial apoptosis.

13) SCCA1 promotes caspase-8 oligomerization and activation

Caspase-8 activation has been associated with extrinsic apoptosis, which is stimulated by the engagement of the proapoptotic death ligands, such as tumor necrosis factor-α (TNF α) and Fas ligand (FasL), with their cell surface receptors. Binding of ligands to death receptors induces their oligomerization allowing the recruitment of adaptor proteins and subsequently pro-

caspase-8, thus forming the death inducing signaling complex (DISC). This complex brings pro-caspase-8 proteins into close proximity, which facilitates their activation and self-processing **(6)**.

In addition to caspase-8, other initiator caspases such as caspases-2 and 9 have been proposed to be activated through a model whereby their induced proximity facilitates their oligomerization and activation **(9, 24, 112, 123, 130)**. We then tested whether oligomerization of caspase-2, 8, and 9 was occurring in SCCA1-expressing cells upon ER stress. Size exclusion chromatography was performed and fractions assayed for caspase-8 activity. An increased amount of full-length (55/53 kDa) as well as cleaved (44/42 kDa) caspase-8 was found in high molecular weight fractions 3-7 in treated SCCA1-expressing cells as compared to parental cells (Fig. 23A). Correspondingly, these same fractions had the highest caspase-8 activity, suggesting that activation occurred as a result of caspase-8 oligomerization (Fig. 23B). In contrast, no obvious caspase-2 or caspase-9 high molecular weight aggregates were observed (Fig. 23A), indicating that caspase-8 is the major initiator caspase that forms oligomers in response to ER stress. The oligomerization of caspase-8 was further confirmed using the bimolecular fluorescence complementation (BiFC) assay. In this assay caspase-8 was fused to the N-terminal half and C-terminal half of Venus fluorescent protein, then subsequently transfected into cells. Only upon interaction of caspase-8 will these non-fluorescent fragments by brought into proximity allowing reconstitution of an intact fluorescent protein. In comparison to parental cells, SCCA1-expressing cells displayed a marked increase in fluorescence upon tunicamycin treatment, indicating that SCCA1 promotes proximity-driven oligomerization of caspase-8 in response to ER stress. Similar to parental cells, SCCA1-expressing *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells expressing shSCCA1 had very little fluorescence upon tunicamycin treatment, indicating SCCA1 is responsible for facilitating caspase-8 proximity-driven oligomerization (Fig. 23C). To confirm that caspase-8 was indeed involved in the pathway of cell death promoted by SCCA1, caspase-8 was knocked down by short hairpin RNA (shRNA). SCCA1 cells expressing the shRNA against caspase-8 were protected from ER stress (Fig. 24A). Importantly, although ER stress has been shown to induce TNFα expression **(56)**, the caspase-8-mediated apoptosis promoted by SCCA1 is unlikely through the death receptor as TNFα treatment did not show significant difference in cell death between parental and SCCA1-expressing cells (Fig. 24B). Together, these results indicate that ER stress can trigger caspase-8 oligomerization, activation, and subsequent apoptosis in the presence of SCCA1.

14) SCCA1 leads to decreased proteasome activity and increased protein poly-ubiquitination and aggregation

It has recently been shown that caspase-8 aggregation at the DISC is dependent on its ubiquitination **(60)**. While performing caspase-8 immunoblots it was observed that there was an increased amount of high molecular weight (HMW) species in SCCA1 and SerpinB3b-expressing cells, suggesting that SCCA1 and SerpinB3b may promote caspase-8 aggregation by enhancing its ubiquitination (Fig. 25A). To determine whether SCCA1 promotes ubiquitination of caspase-8, parental or SCCA1-expressing MCF10A cells were transfected with His-tagged ubiquitin and treated with tunicamycin. Cell lysates were precipitated with Ni-NTA-agarose. Upon immunoblotting with caspase-8 it was observed that SCCA1-expressing cells had an accumulation of ubiquitinated caspase-8, which was further enhanced by treatment with tunicamycin (Fig. 25B). Sequestosome 1/p62 is a cellular protein that has been shown to interact noncovalently with ubiquitin and localize to protein aggregates. Co-immunoprecipitation assay

showed that the amount of p62 associating with caspase-8 was significantly higher in tunicamycin-treated SCCA1-expressing Hs578T cells compared to parental cells and cells expressing the SCCA1-F352A mutant (Fig. 26A). Additionally, colocalization of caspase-8 and p62 was examined by immunofluorescence. Partial colocalization of caspase-8 and p62 was observed in both untreated and tunicamycin-treated parental cells. However in sharp contrast, the majority of p62 puncta were found to associate with caspase-8 in untreated SCCA1-expressing cells. The colocalization of p62 and caspase-8 was further enhanced by tunicamycin treatment (Fig. 26B).

15) SCCA1 expression represses global protein degradation by the proteasome

To address the question of whether caspase-8 ubiquitination is a specific event or whether global cellular ubiquitination is increased in SCCA1-expressing cells, a western blot looking at total cellular protein ubiquitination was performed. Ubiquitination in both SCCA1 and SerpinB3b-expressing cells was increased compared to parental cells (Fig. 27A). The increased global ubiquitination is dependent on serpin's protease inhibitory activity as both SerpinB3b-S350G and SCCA1-F352A mutants showed drastically decreased ubiquitination (Fig. 27A). Knockdown of SCCA1 in SCCA1-expressing cells also led to decreased ubiquitination (Fig. 27B). The increased protein ubiquitination correlated with decreased proteasomal degradation in SCCA1-expressing cells, as the half-life of an established proteasomal substrate, Mcl-1, was prolonged in SCCA1 cells (Fig. 27C). To further test the theory that the enhanced global ubiquitinated proteins is a result of impaired proteasomal degradation in SCCA1-expressing cells, a ubiquitin-luciferase reporter construct specifically degraded by the 26S proteasome was utilized **(87)**. This reporter construct is rapidly degraded by the 26S proteasome leading to low luciferase activity. Upon inhibition of proteasomal protein degradation, luciferase levels increase **(87)**. In comparison to parental cells, SCCA1-expressing cells displayed a significant decrease in proteasomal activity. The proteasomal inhibitory activity was impaired in SCCA1-F352A mutant cells (Fig. 27D). Additionally, inhibition of proteasomal function was also observed in *bax*$^{-/-}$*bak*$^{-/-}$ cells expressing SerpinB3b as well as in Hs578T cells expressing SCCA1 (Fig. 27E and 27F). Interestingly, combined treatment of tunicamycin and the proteasome inhibitor MG132 significantly enhanced cell death in parental *bax*$^{-/-}$*bak*$^{-/-}$ cells, whereas in SCCA1 and SerpinB3b expressing cells there was no significant difference (Fig 28A). Furthermore, cell death in parental cells treated with both tunicamycin and MG132 occurred at a similar rate as tunicamycin treated SCCA1 and SerpinB3b cells, suggesting that in these cells a synergistic affect is not observed because the proteasome is already inhibited by SCCA1 or SerpinB3b (Fig. 28A). Taken together, these results indicate that SCCA1 and SerpinB3b can inhibit global protein ubiquitination thereby promoting caspase-8 ubiquitination, which may facilitate its aggregation as previously reported **(60)**.

16) SCCA1 does not directly inhibit proteasomal enzymatic activity

One possible mechanism of SCCA1 and SerpinB3b inhibition of proteasomal degradation is through the direct inhibition of the proteasomal enzymatic activity. However, both the proteasomal chymotrypsin and trypsin activities were not inhibited in SCCA1-expressing cells, suggesting that decreased proteasomal degradation occurs at an early step or is an indirect consequence of SCCA1 expression (Fig. 29A and 29B). Additionally we attempted to determine

whether SCCA1 was able to inhibit deubiquitinase (DUB) activity, a reasonable hypothesis being that the majority of deubiquitinases (DUB's) are cysteine proteases. While poly-ubiquitin chains target proteins for proteasomal degradation, these chains must be removed prior to protein insertion into the catalytic core of the proteasome. Failure to remove ubiquitin chains results in impaired proteasomal degradation and thus can cause a buildup of ubiquitinated proteins within the cell. Using an assay to detect overall DUB activity within the cell it was observed that DUB activity was indeed significantly reduced in SCCA1 cells; however the decrease did not appear to be substantial enough to be the sole reason for the large increase in ubiquitinated proteins (Fig 28C).

17) SCCA1 blocks lysosomal turnover

Another possible mechanism for SCCA1 inhibition of proteasomal protein degradation could be an indirect consequence of impaired lysosomal degradation. Indeed it has been observed that inhibition of lysosomal degradation leads to a build-up of ubiquitinated proteins and a decreased flux of proteins through the proteasome at a stage upstream of proteasome catalytic activity **(72, 137)**. It is foreseeable that SCCA1's ability to inhibit lysosomal proteases may have an effect on lysosomal activity and protein turnover, and indirectly influencing proteasomal degradation. To determine whether lysosomal turnover is affected, the subcellular localization pattern of LC3, which undergoes lipidation and localizes to autophagasome membranes, and is consequently degraded in autolysosomes, was examined using GFP-conjugated LC3. SCCA1 cells showed a marked increase in the amount of GFP-LC3 puncta at the basal state (Fig. 30A). It is well documented that ER stress can induce autophagy flux and stimulates lysosomal turnover **(105, 152, 167)**. Tunicamycin treatment induced LC3 puncta formation in parental cells, suggesting an increase in autophago-lysosomal activity. However, this tunicamycin-induced LC3 puncta formation was not as drastic in SCCA1-expressing cells (Fig. 30A). Consistent with this, a long-lived protein degradation assay showed that while autophago-lysosomal protein degradation was stimulated by tunicamycin in parental cells, it was indeed suppressed in SCCA1-expressing cells (Fig. 30B). To further confirm the impaired lysosomal degradation upon SCCA1 expression, cells were either left untreated or treated with the lysosomal inhibitors E64D and pepstatin A (PepA). While treatment with E64D plus PepA caused the accumulation of LC3-II in parental and SCCA1-F352A mutant cells, SCCA1 cells had a higher basal level of LC3-II, which was not further enhanced by treatment with the lysosomal inhibitors (Fig. 30C-E). In agreement with above data, tunicamycin treatment led to an increase in LC3-II in parental and SCCA1-F352 cells, which was further increased in parental cells by the lysosomal inhibitors. In contrast, tunicamycin-induced LC3-II accumulation was not further enhanced by the lysosomal inhibitors in SCCA1-expressing cells (Fig. 30D). In contrast, SCCA1 knockdown in SCCA1-expressing cells restored the lysosomal degradation flux (Fig. 30D). In addition to *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells, similar findings were observed in MCF10A cells expressing SCCA1 (Fig. 30F). These findings suggest that in SCCA1-expressing cells, the ER stress-induced autophago-lysosomal turnover was blocked.

Interestingly, SCCA1 has been shown to suppress c-Jun NH2-terminal kinase-1 (JNK1) **(62)**. Additionally, it has been shown previously that the IRE1-JNK pathway is required for autophagy activation after ER stress **(105)**. While our data shows JNK phosphorylation is suppressed in SCCA1 expressing cells we find autophagy can still be induced in these cells by tunicamycin treatment (Fig. 31A and 30A). Furthermore using a JNK inhibitor in parental cells

to mimic SCCA1's inhibition of JNK, we find cell death is enhanced in response to ER stress (Fig. 31B). It is possible that the effects of JNK on cell survival and autophagy in apoptosis deficient cells is different than in apoptosis competent cells and will require a more in depth study to elucidate its role in these processes.

To further verify that lysosomal degradation is indeed affected by SCCA1, the mCherry-EGFP-LC3 construct was utilized **(107)**. This construct contains the monomeric red fluorescent protein mCherry, which can only be degraded when the pH is below 4.5, and an enhanced green fluorescent protein (EGFP), which is degraded at a pH below 6.0. While early and late endosomes have a pH of 6.1 and 5.5 respectively, lysosomes have a pH of 4.7, thus while EGFP is degraded by late endosomes and lysosomes mCherry is not **(107)**. In parental untreated cells there are very few puncta and mostly diffuse yellow staining was observed, due to the combined presence of mCherry and EGFP. Upon treatment with tunicamycin there was an increase in puncta, the majority of which are red, indicating that tunicamycin induces autophagy flux (Fig. 32A). SCCA1 cells however, had an increased basal level of puncta, which were almost all yellow, indicating a defect in lysosomal degradation. The size of the yellow puncta was further increased upon treatment with tunicamycin, indicating that while ER stress induces autophagy influx, SCCA1 blocks lysosomal degradation, hence facilitates protein aggregations at autolysosomes (Fig. 32A). Similar effects were observed in apoptosis-competent Hs578T cells expressing SCCA1 (Fig 32B). Taken together, these results demonstrate that SCCA1 can block lysosomal protein degradation, particularly when under ER stress.

18) SCCA1 promotes caspase-8 aggregation and activation on lysosomes

The above observations suggest that ER stress could possibly induce the aggregation of caspase-8, which normally would then be degraded by lysosomes. However in the presence of SCCA1, lysosomal degradation is blocked, which would facilitate the aggregation and subsequent activation of caspase-8. This theory would suggest that caspase-8 can be activated intracellularly on lysosomes. To address this, cells were stained with LysoTracker Red and immunofluorescence was performed using an antibody for caspase-8. As previously observed, in comparison to parental cells, SCCA1 cells have a higher basal level of lysosome staining (Fig. 13C, 13D, and 33A). While there is little association of caspase-8 with lysosomes in parental cells, this association is markedly enhanced in SCCA1 cells (Fig. 33A). In response to tunicamycin treatment the level of lysosomal staining dramatically increases in parental cells. Additionally, the pattern of caspase-8 staining appears more punctate than what was observed in untreated conditions. Nevertheless there appears to be only a few lysosomes that colocalize with caspase-8. In stark contrast, SCCA1 cells are observed to have large caspase-8 aggregates that colocalize with lysosomes (Fig. 33A). Additionally, to determine whether the aggregated caspase-8 that colocalizes with lysosomes is active, a FITC-conjugated caspase-8 substrate peptide, FITC-IETD-FMK, was incubated with tunicamycin treated parental and SCCA1 cells along with LysoTracker Red. Staining for activated caspase-8 in parental cells was low and was not observed to colocalize with lysosomes. In comparison, the FITC signal was stronger in SCCA1 cells and was found to form large aggregates that colocalized with lysosomes, indicating that the aggregated caspase-8 found at lysosomes is indeed active (Fig. 34B). These observations suggest that ER stress can lead to the accumulation of caspase-8 at lysosomes, allowing their association and activation which contributes to enhanced cell death in SCCA1

cells. Our results support the idea that SCCA1 indirectly leads to caspase-8 aggregation though blocking the degradation of caspase-8 aggregates, however we have not explored the possibility that SCCA1 may also play a more direct role in aggregate formation. Indeed we have found that SCCA1 can itself be ubiquitinated which may play some role in promoting aggregate formation (Fig. 29D). With regards to how exactly SCCA1 inhibits cathepsin activity and lysosomal turnover it is possible that SCCA1 inhibits cathepsins that are aberrantly released from partially damaged lysosomes, preventing them from doing further damage to lysosomes and other cellular membranes and proteins. To try and address these questions we performed cellular fractionation with trypsin digestion to distinquish between proteins in the cytosol, membrane heavy fraction (containing mitochondria, ER and lysosomes), and membrane light fraction (consisting almost exclusively of lysosomes). Trypsin digestion was performed to determine whether proteins were membrane bound or within the lumen of membrane structures. Immunoblotting with an antibody for cytochrome C oxidase (COXIV), a mitochondrial transmembrane protein located within the inner membrane, revealed that the heavy fraction contained mitochondria, whereas the light fraction did not. Furthermore, COXIV was present in both untreated and trypsin treated heavy fractions, while TOM40, an outer-mitochondrial membrane protein was degraded by trypsin digestion, suggesting that the trypsin digestion worked efficiently (Fig. 35A). LAMP1 was observed to be abundant in the heavy fraction, and was also present in the light fraction, treatment with trypsin lead to a slight decrease which is likely due to LAMP1's transmembrane and cytosolic domains which are sensitive to trypsin digestion. It is also observed that LAMP1 is stroner in the heavy fraction of SCCA1 cells which may be explained by the increased size of lysosomes within these cells. Interestingly, while caspase-8 can be found predominantly in the cytosol, it is also present in membrane bound fractions. Treatment with tunicamycin reduces the level of membrane bound caspase-8 in parental cells, possibly due to lysosomal degradation, while caspase-8 is increased in tunicamycin-treated SCCA1 cells (Fig 35A and 35B). Additionally cleaved caspase-8 is found to be higher in both the heavy and light fractions of SCCA1 treated cells in comparison to parental cells. Significantly we observed that while a large proportion of SCCA1 was located in the cytosol, it was also present in both heavy and light fractions. Furthermore, SCCA1 was partially resistant to trypsin digestion in the heavy fraction implying that it may gain access into the lumens of membrane structures (Fig 35A and 35B). These results may suggest that a proportion of SCCA1 is able to gain access to lysosomal lumens where it can inhibit cathepsins and prevent lysosomal degradation, however further experimentation is required to support this idea.

III. Discussion

Endoplasmic reticulum stress has been found to contribute to a variety of pathological disorders ranging from cardiovascular disease to neurodegenerative diseases and cancer. Thus studying the cellular responses to ER stress is important for understanding how these diseases transpire and even more importantly how they can be successfully treated. Being that the ER is the main site for protein folding and secretion it is inherently important that the ER performs these processes correctly in order to maintain the fidelity of all cellular processes. When normal ER function is compromised due to any number of stressful conditions, this leads to an accumulation of misfolded proteins within the ER lumen, a situation known as ER stress. Multiple signaling pathways, activated by ER stress, attempt to regain ER homeostasis through processes such as decreasing overall protein translation, while simultaneously up-regulating proteins involved in folding, quality control, trafficking, and ER-associated degradation (ERAD). The normal response to unresolved ER stress is activation of apoptotic cell death, a process controlled by the Bcl-2 family of proteins. However, we find that in the absence of apoptotic cell death other cell death processes, such as necrosis, may be activated.

1) Apoptosis deficient cells are susceptible to cell death in response to prolonged ER stress

With respect to apoptosis activated by intracellular stress, such as ER stress, the Bcl-2 family of proteins are key regulators. Included in this family are both pro-apoptotic and anti-apoptotic members. The function of these proteins is to antagonize each other thereby regulating MOMP, and the release of apoptogenic factors from mitochondria into the cytosol. In addition, these proteins play pivotal roles in regulating ER function **(18-19, 41)**. Our results show that both *bax*$^{-/-}$*bak*$^{-/-}$ and Bcl-xL-overexpressing cells are deficient in their ability to undergo apoptosis in response to ER stress. However, we also observe that while cell death is delayed it is not prevented in these cells when exposed to prolonged ER stress. Additionally, conventional apoptotic features, such as plasma membrane blebbing, nuclear condensation, and caspase activation, were not detected. Rather, features which are typically characteristic of necrotic cells were observed, such as plasma membrane dilation and release of intracellular content such as HMGB1 (Fig. 2).

Necrosis plays a significant role in multiple developmental, physiological, and pathological conditions **(42, 45)**. During necrosis cytoplasmic organelles dilate and the cell swells, the plasma membrane then ruptures releasing cellular content into the extracellular environment, which can mediate an inflammatory response. Apoptosis and necrosis can often be initiated in response to the same types of insults depending on the dose or duration. For example cytokines, ischemia, heat, irradiation, and pathogens can induce both apoptosis and necrosis in the same cell population **(93, 113, 177)**. In addition, inhibition of caspases required for apoptosis has been shown to change the morphological appearance of cell death from apoptosis to necrosis **(76, 84)**. Thus, under certain circumstances when apoptosis is blocked, necrosis can compensate. Necrosis is induced by depletion of ATP stores necessary for proper cellular functioning. Sustained UPR, in the absence of apoptosis, may deplete cellular energy stores in an attempt to resolve the ensuing stress and damage, thus leading to necrosis.

2) Autophagy is induced in response to ER stress regardless of the presence of pro-apoptotic Bcl-2 proteins Bax and Bak

When seeking the molecular mechanism for ER stress-induced Bax/Bak-independent cell death, we observed that autophagy is induced by ER stress. This observation is consistent with recent findings showing that autophagy is induced in both yeast and mammalian cells in response to ER stress **(11, 27, 35, 54, 73, 105, 167)**. In addition, recent studies have shown that anti-apoptotic Bcl-2 and Bcl-xL proteins can modulate autophagy induction, and that their ER localization appears to be important for this activity **(54, 110, 139)**. Although the exact role of Bcl-2/Bcl-xL in regulating autophagy remains elusive, one mechanism that has been proposed for how these anti-apoptotic proteins regulate autophagy is through their interaction with Beclin 1 **(110, 139)**. This interaction requires the BH1 and BH2 domains of Bcl-2 **(110)**. As the BH1 and BH2 domains of Bcl-2 are also required for Bax interaction **(166)**, there exists the possibility that the steady-state levels of Bax/Bak may have an effect on autophagy induction. Our results show that ER stress appears to induce similar amounts of autophagy in wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells. Overexpression of Bak or Bax does not induce autophagy (Fig. 8). Thus Bax and Bak do not affect autophagy, nor do they affect Bcl-2's ability to regulate autophagy.

Autophagy was originally described as a cell survival mechanism essential for coping with nutrient starvation conditions. It has been proposed that under certain circumstances autophagy can be a mechanism of cell death **(115, 139, 170)**. In light of these findings it is feasible that autophagy could function initially as a survival mechanism, however if the stress is not resolved accumulation of autophagosomes can interfere with normal membrane trafficking and ultimately lead to apoptosis **(80, 83)**. Consequently it is difficult to ascribe one specific function of autophagy pertaining to whether it serves a protective or damaging role and likely there are multiple possibilities. The physiological state of the cell and the nature of the insults may also determine whether autophagy will function mainly as a cell survival or death activating response **(32, 35)**. With respect to ER stress, it is suggested that autophagy helps the cell adapt to the stress, perhaps through aiding in the degradation of misfolded proteins and/or sequestration of portions of the ER, thereby averting apoptosis **(11, 73, 105)**. This would suggest that cells deficient in autophagy would die at a faster rate than normal cells when exposed to ER stress.

3) Autophagy promotes cell death in apoptosis deficient cells while it protects apoptosis competent cells

While we show that autophagy can be induced to similar levels in wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells in response to ER stress, the resulting outcome of this response appears to be much different. Inhibition of autophagy using 3MA or by Atg5 knockdown was able to delay cell death in *bax*$^{-/-}$*bak*$^{-/-}$ cells. In contrast, wild-type cells capable of undergoing apoptosis were more sensitive to ER stressors when autophagic machinery was impaired. Thus in wild-type cells, excessive ER stress causes apoptosis. Autophagy apparently confers protection from apoptosis, possibly by promoting protein degradation to resolve the stressing conditions. Similar protection was reported in polyglutamine 72 repeat treated cells **(73)**. The IRE-JNK pathway is required for autophagy induction and cytoprotection in response to ER stress **(105)**. In contrast, here we find in apoptosis-deficient cells prolonged autophagy provokes cell death, and its absence imparts protection. This may be because in these cells, excessive ER stress fails to induce apoptosis. The stressing situation keeps accumulating to a point where autophagy is massively induced subsequently leading to cell damage and necrosis. This is evidenced by our observation that two

days after ER stress treatment, the wild-type cells appeared apoptotic, whereas *bax*$^{-/-}$*bak*$^{-/-}$ cells still retain a large amount of autophagosomes (Fig. 3).

Prior studies performed using apoptotosis-inducing drugs, such as TNFα and etoposide, have attributed a role for autophagy in promoting a non-apoptotic form of cell death **(139, 170)**. We show here that ER stress can trigger Bax/Bak-independent cell death that is associated with autophagy. The question is then raised as to, in response to ER stress, whether autophagy is the responsible mechanism by which treated *bax*$^{-/-}$*bak*$^{-/-}$ cells are dying or whether the cell death is executed through other forms of cell death. Based on both morphological and biochemical criteria of dying cells it appears as though the type of cell death occurring in *bax*$^{-/-}$*bak*$^{-/-}$ cells in response to ER stress is necrotic. Inhibition of autophagy did not completely abrogate this Bax/Bak-independent cell death. This indicates that cell death in response to ER stress may involve complex cell death pathways. Consequently, autophagy, rather than acting as a distinguished form of cell death, instead contributes to the cell fate determination by differentially affecting apoptosis and necrosis. These findings may be pathologically relevant to cancer cells that have impaired apoptosis. In this case promoting autophagy by inducing ER stress may promote death by necrosis, which due to its proinflammatory nature may in itself promote cancer cell growth. On the other hand blocking autophagy in apoptotosis-deficient cells may enhance survival of damaged cells, thus also potentially contributing to tumorigenesis or resistance to therapy.

4) A programmed form of necrosis may be responsible for ER stress-induced cell death in cells lacking Bax and Bak

In an attempt to gain better insight into the possibility that continued ER stress could activate necrosis in apoptosis deficient cells we sought to identify mediators of this process. In addition to the ER's role in protein folding it is also an important Ca^{2+} storage site, and in fact contains the highest concentration of Ca^{2+} within the cell **(160)**. It is possible that Ca^{2+} released from damaged ER can lead to calpain activation, which has been previously shown to lead to caspase-12 activation and subsequently to mitochondrial dependent apoptosis **(95)**. It is also feasible that in the absence of apoptosis, such as in the case of cancer cells that often times have defects in apoptotic machinery, that activated calpains can compromise lysosomal membranes. Damaged lysosomal membranes can lead to cellular acidification as well as release of cathepsins that can subsequently go on to damage cellular proteins and membranes **(86, 92)**. Using a selective calpain inhibitor we found that while apoptosis competent cells were not protected from ER stress, apoptosis deficient cells were. Furthermore, treatment with ZVAD-FMK, a broad caspase inhibitor did not significantly protect apoptosis deficient cells from ER stress, suggesting that calpain is capable of activating non-apoptotic cell death in response to ER stress (Fig. 12). We next sought to determine whether calpain was promoting cell death by compromising lysosomal integrity, leading to cathepsin release, a process that has been previously observed in C.*elegans* in response to numerous types of stimuli **(86)**. In support of this possibility, we found that chloroquine, an inhibitor of lysosomal turnover, increased ER stress-induced cell death, possibly by causing lysosomes to become more sensitive to permeabilzation (Fig. 12).

The ability of ER stress to induce cell death in apoptosis deficient cells may be an especially important finding when considering cancer cells. Often times in order to cope with cellular changes such as elevated protein synthesis, energy consumption, and byproduct disposal, cancer cells acquire lysosomal changes such as elevated lysosome number, size, and expression

of lysosomal hydrolases **(39)**. These conditions can make cancer cells more susceptible to lysosomal injury, which depending on its extent and duration, may lead to both apoptotic and non-apoptotic cell death. Not surprisingly, cancer cells develop cytoprotective mechanisms, such as elevated levels of Hsp70, sphingomyelin, and serpins, to guard against this vulnerability **(111, 142)**.

5) SCCA1 protects cells from necrosis induced by stimuli that cause lysosomal permeabilization

To mimic a more innate occurance we decided to overexpress an endogenous cathepsin inhibitor to determine whether cell death in apoptosis deficient cells was proceeding through a pathway that involved cathepsin activation. One such family of proteins that is able to perform such a task is the serpins, specifically SERPINB3, which is known in humans as SCCA1. Indeed, in agreement with previous reports showing the protective role of serpins against lysosomal injury, we find here that SCCA1 protects against insults such as DNA alkylating damage and hypotonic shock by inhibiting lysosome hydrolases and preventing lysosomal permeabilization (Fig 13, 14A and 14B, 15, and 16). Additionally, we have observed that cancer cells naturally expressing a high level of SCCA1 are unable to survive and proliferate when SCCA1's expression is knocked down using a short hairpin, thus suggesting that this protein is imperative to cell survival of these cancer cells **(23)**.

6) SCCA1 enhances ER stress induced cell death through caspase-8 aggregation and activation

Interestingly, despite its protective role against lysosomal injury, we find here that SCCA1 promotes cell death induced by ER stress (Fig. 17). This cell death appears to be apoptotic, indicated by both the morphological features and the activation of caspases (Fig. 14C, 21, 22A, C, and E). ER stress-induced apoptosis has been previously reported to occur via caspase-12 in murine cells **(96)**, or through induction of the BH3-only proteins Bim **(114)** and Puma **(121)**. As apoptosis-mediated by caspase-12, Bim, and Puma requires Bax and Bak **(175-176)**, our finding that ER stress-induced apoptosis in SCCA1-expressing cells that lack Bak and Bax, indicates that this apoptosis is not mainly mediated by caspase-12 and the BH3-only proteins. This theory is further supported by the observation that cytochrome C release does not occur in SCCA1-expressing *bax*$^{-/-}$*bak*$^{-/-}$ cells upon ER stress treatment, albeit the apoptotic nature of the cell death (Fig. 20). Furthermore, while ER stress has been reported to upregulate the expression of death ligands TNFα, FasL, and TRAIL **(56)**, the death receptor apoptotic pathway is unlikely the cause for enhanced apoptosis rendered by SCCA1, as cell sensitivity to TNFα was not affected by the status of SCCA1 expression (Fig. 24B). Importantly, we show here that although the death receptor pathway is not involved, ER stress-mediated cell death in SCCA1-expressing cells is a result of caspase-8 activation.

Caspase-8 is an initiator caspase that has been well characterized to be activated through its aggregation and autocleavage at the DISC upon the interaction of death ligands and their cell surface receptors **(5, 14)**. In addition to DISC-mediated caspase-8 aggregation and activation, caspase-9 is activated via aggregation on apoptosomes **(82, 126)**, caspase-2 via PIDDosomes **(15, 149)**, and caspase-1 and 5 via inflammasomes **(91)**. A recent study shows that the aggregation of caspase-8 on the DISC is facilitated by its ubiquitination and interaction with the

ubiquitin-binding protein SQSTM1/p62 **(60)**. Here we show that intracellular caspase-8 ubiquitination and aggregation, induced by ER stress, can initiate an apoptotic response independently of the death receptor pathway in SCCA1 expressing cells and that this is a result of lysosomal inhibition causing inefficient degradation of caspase-8 aggregates (Fig 36). This, together with a recent finding in our laboratory that inhibition of proteasome degradation leads to enhanced caspase-8 proximity and activation in intracellular membranes (Pan et al. Submitted elsewhere), suggests a novel mechanism for caspase-8 activation in response to proteotoxic stress. While our results indicate that this cell death coincides with SCCA1's ability to increase global protein ubiquitination, including that of caspase-8, it remains to be determined whether ubiquitination is a prerequisite for caspase-8 intracellular aggregation and activation.

Our findings indicate that cells with high SCCA1 can be specifically targeted by ER stress to enhance cytotoxicity. Significantly, many cancers are under chronic ER stress, leading to a heightened UPR response. However, numerous studies have shown that the UPR response in cancer cells is altered such that pro-survival signals are favored while pro-death signals are inhibited **(59, 118)**. Currently two approaches are being explored to exploit the elevated UPR response observed in cancer, one of which is to block the pro-survival signals, while the other approach attempts to further enhance ER stress to shift in favor of a pro-death response **(36)**. Several drugs used clinically to treat various types of cancers have been reported to increase the unfolded protein response. Among these agents are proteasome inhibitors, which result in decreased protein degradation and a subsequent increase in poly-ubiquitinated proteins and aggregates **(38, 52, 138, 148)**. With regards to cancer cells expressing SCCA1, given that their UPR is already elevated, treating them therapeutically with agents that further enhance ER stress may shift the UPR in favor of a pro-death response, thus leading to a better outcome.

IV. Current and Future Perspectives

The aim of this study was to understand the mechanisms of ER stress-induced cell death to elucidate alternative cell death pathways that apoptosis resistant cells may be sensitive to. In the course of this study the protein SCCA1, which is expressed in various types of cancer, was found to sensitize cells to ER stress. The implications of this study may have a confound impact on the design of therapatuics aimed at targeting cancer cells expressing SCCA1. Additionally, our studies reveal that cancers under high levels of ER stress may be sensitive to agents that block protein degradation, such as proteasome inhibitors and lysosomal protease inhibitors.

Importantly, this study provides a mechanism for cell death induced by proteotoxicity and is the first study to show that caspase-8 can induce cell death through cytosolic aggregation, which is distinct from its known ability to be activated by membrane bound death receptors. It is well established that the majority of cancers are resistant to conventional apoptosis pathways, thus uncovering novel pathways to cell death may further aid in developing therapeutics that can induce cell death in these cancers.

While this study uncovers a novel mechanism for ER–stress induced cell death, it leaves several questions unanswered. One important consideration is whether these findings would hold true for an in vivo system. Our lab hopes to further explore this consideration by treating mice bearing tumors, which have either a high or low level of SCCA1 expression, with ER-stress inducers, such as tunicamycin or thapsigargin and subsequently look at tumor growth and regression. Additionally, we show that ER-stress induced cell death in Bax/Bak doubly deficient cells is strongly prevented by calpain inhibition; however we have not further pursued how exactly calpain activation promotes cell death. It would be interesting to try and elucidate the molecules downstream of calpain that are important mediators of what could be another potential novel cell death pathway. Regarding the ability of SCCA1 to block proteasome activity, further experimentation is required to determine whether this is solely due to SCCA1's ability to inhibit lysosomal turnover or whether it has a more direct action on proteasome activity. With respect to SCCA1's ability to block lysosomal turnover, while we have some data that suggests SCCA1 may gain access to lysosomal lumens where it can inhibit lysosomal cathepsins, further work is necessary to confirm this. In addition we show that SCCA1 promotes caspase-8 ubiquitination, aggregation, and association with p62, but whether or not ubiquitination and association of p62 are necessary for aggregate formation and wether other proteins are involved in formation of these aggregates, remains unknown. Lastly, we show that caspase-8 aggregates at lysosomes, where it subsequently becomes activated, raising the questions of whether this activated caspase-8 is on the outside or inside of lysosomes and if it is inside how is it activating cell death. While our last figure suggests that caspase-8 is on the outside of lysosomal membranes, further experiments are required to validate this idea.

V. Figures

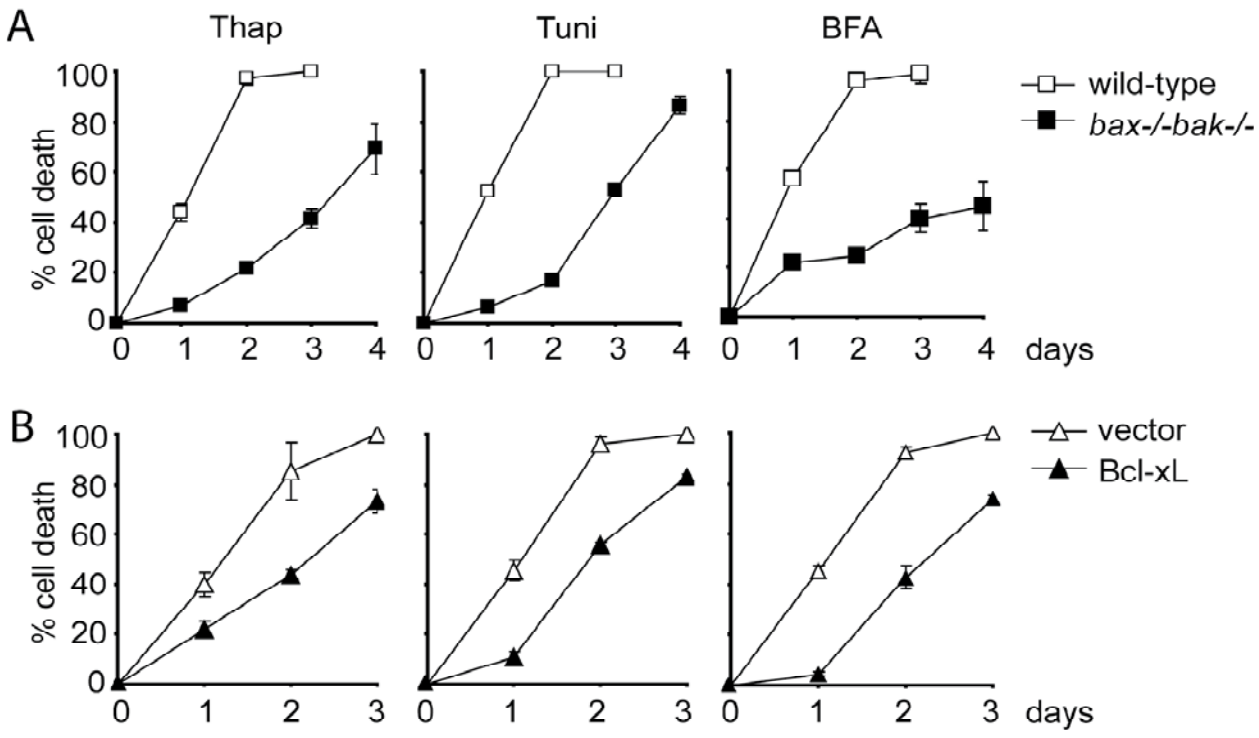


Figure 1. Prolonged ER stress induces cell death in Bax/Bak doubly deficient cells.

(A) ER stress can induce non-apoptotic cell death. Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were treated with thapsigargin (0.1 μM), tunicamycin (0.5 μg/ml) or brefeldin A (0.5 μg/ml). At the indicated time points cells were collected and cell death was measured by PI exclusion. Data are averages of triplicate treatments ± S.E.M. ($P < 0.01$ for all time points). (B) MEFs expressing Bcl-xL and the vector control were treated with ER stressors and cell death was measured as in (A).

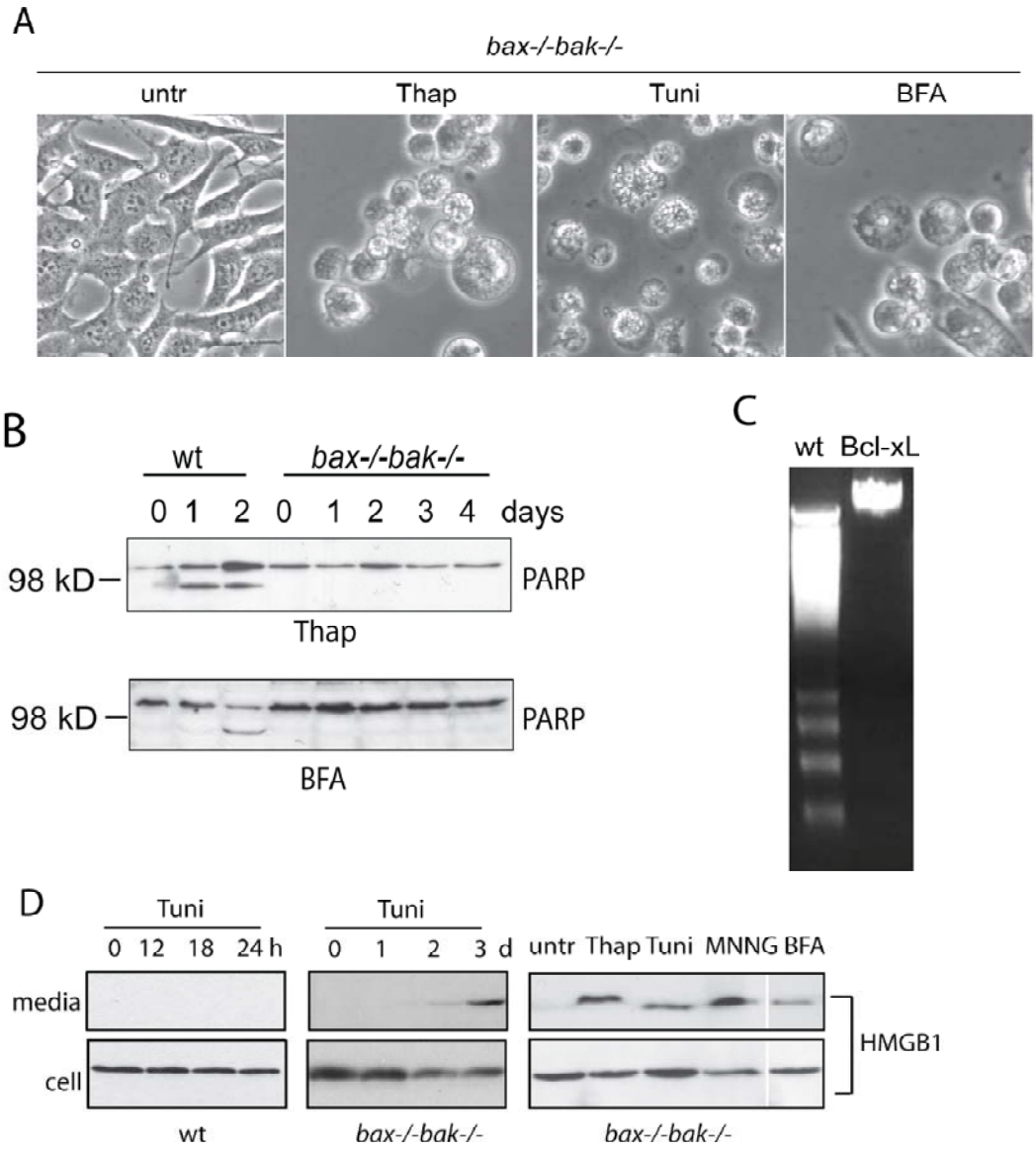


Figure 2. ER stress induces necrosis-like cell death.

(A) *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were treated with thapsigargin, tunicamycin or brefeldin A for 72 hours then photographed under a phase-contrast filter using a x32 objective. (B) Caspase3-dependent PARP cleavage does not occur in *bax*$^{-/-}$*bak*$^{-/-}$ MEFs. Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells were treated with either brefeldin A or thapsigargin for the indicated times, cell lysates were prepared followed by immunoblotting for PARP. (C) Wild-type cells treated with tunicamycin for 48 h show nucleosomal DNA laddering, while Bcl-xL expression blocks it. (D) *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were treated for 72 hours with thapsigargin (Thap), tunicamycin (Tuni), and brefeldin A (BFA). As a positive control for HMGB1 release, *bax*$^{-/-}$*bak*$^{-/-}$ cells were treated with MNNG (500 μM) for 30 min, then cultured in drug free media for 24 hours. Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells were treated with tunicamycin (0.5 μg/ml) for the indicated periods of time. Cell culture media and cell lysates were collected and probed for HMGB1 by immunoblotting.

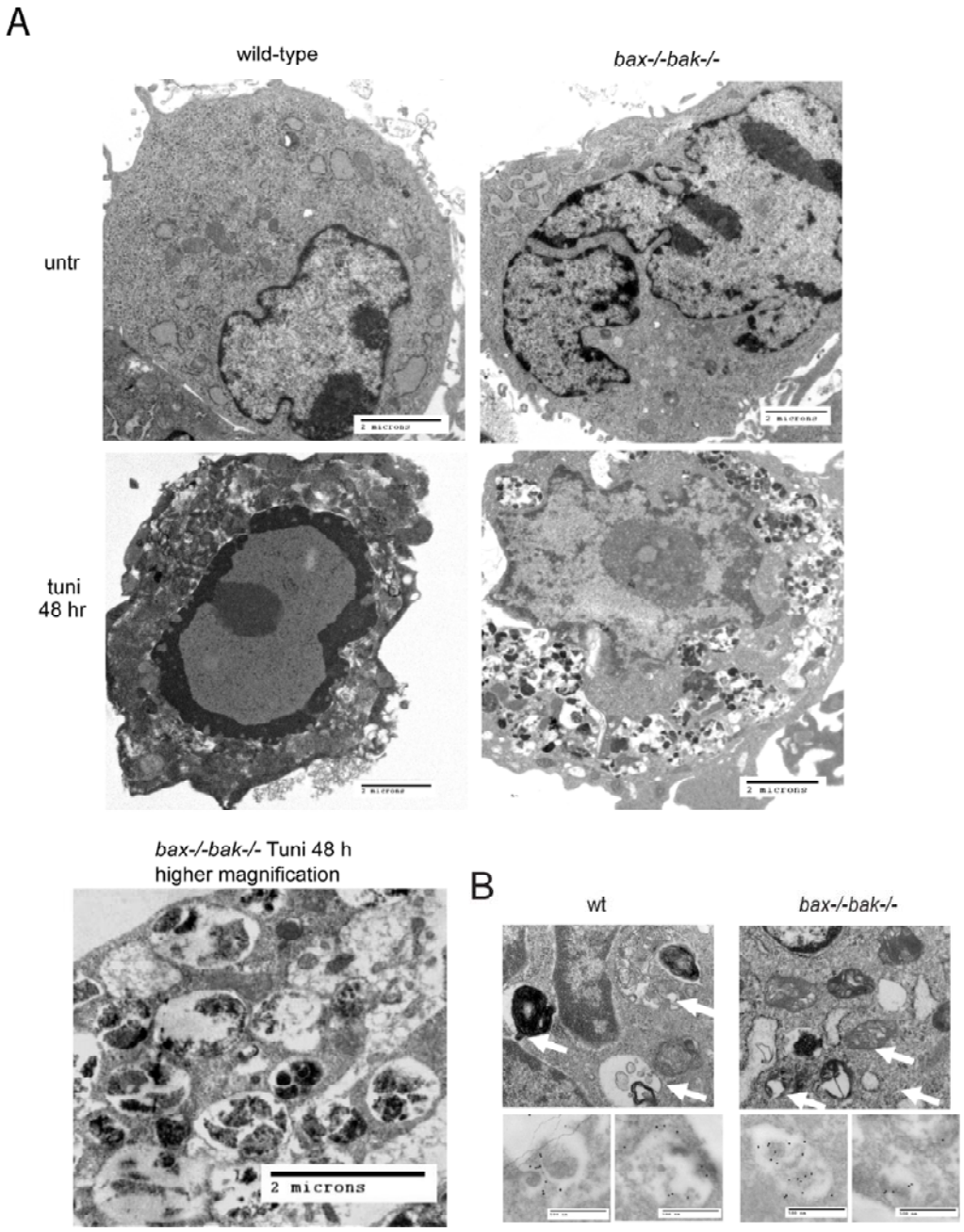


Figure 3. ER stress induces autophagosome formation.

(A) Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were treated with tunicamycin (0.5 μg/ml) for 48 hours. Cells were then fixed and observed with an electron microscope and representative images are shown. Note the chromatin condensation in wild-type cells treated for 48 hr. (B) Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ MEFs stably expressing GFP-LC3 were treated with tunicamycin (0.5 μg/ml) for 12 h. immunohistochemistry electron microscopy was then performed using an anti-GFP antibody. Note the presence of gold particles indicative of autophagosomes in both wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells. Bar=500 nm.

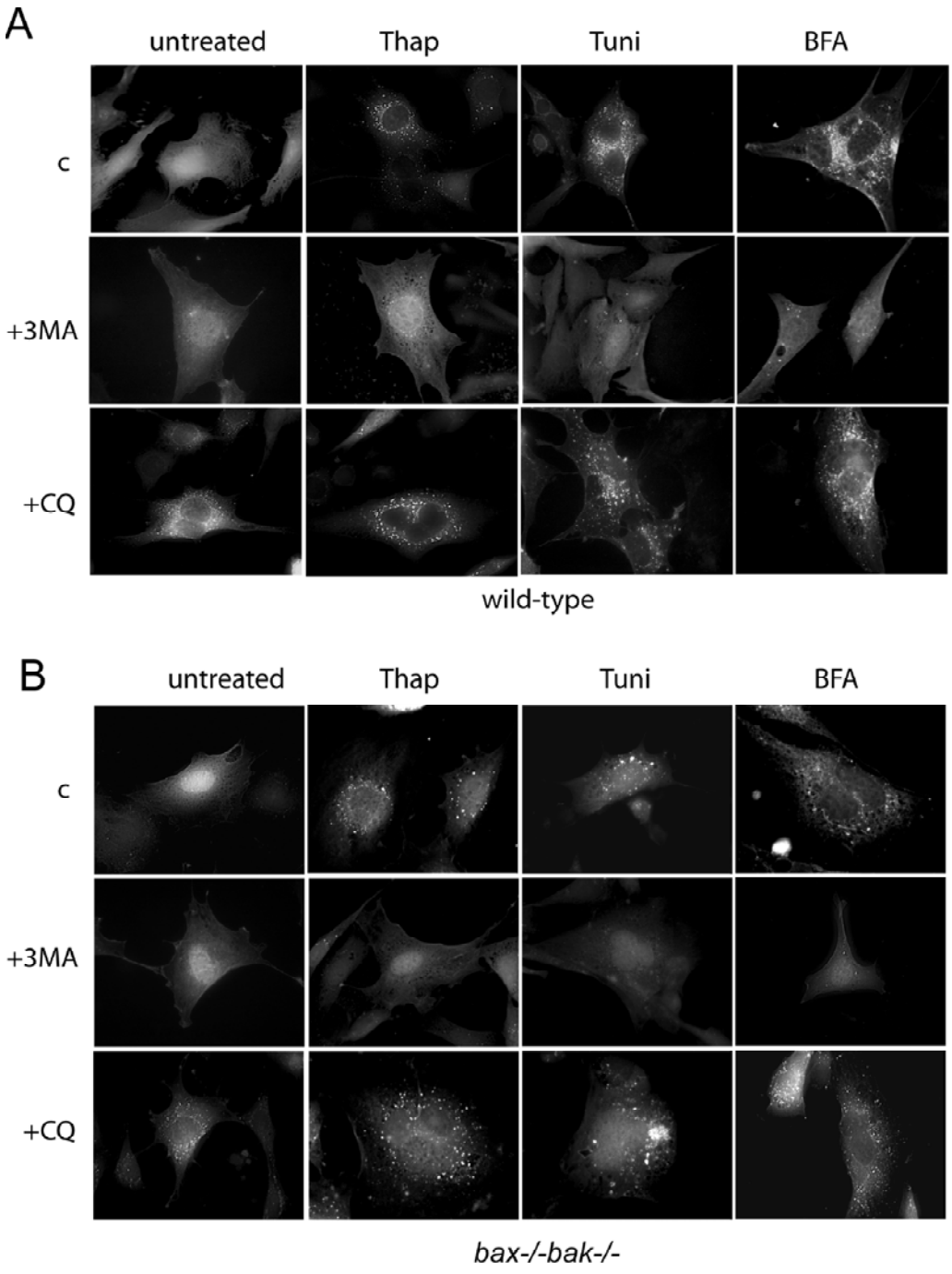


Figure 4. ER stress induces autophagy.

(A-B) Wild-type (A) or *bax*$^{-/-}$*bak*$^{-/}$ (B) cells stably expressing LC3-GFP were treated for 8 hours with the indicated ER stress alone or in combination with 3MA (5 mM) or chloroquine (CQ, 10 μM) and analyzed by fluorescence microscopy. Formation of LC3-GFP puncta was observed in cells exposed to ER stress and this effect was reduced upon combined treatment with 3MA, whereas it was enhanced upon co-treatment with CQ.

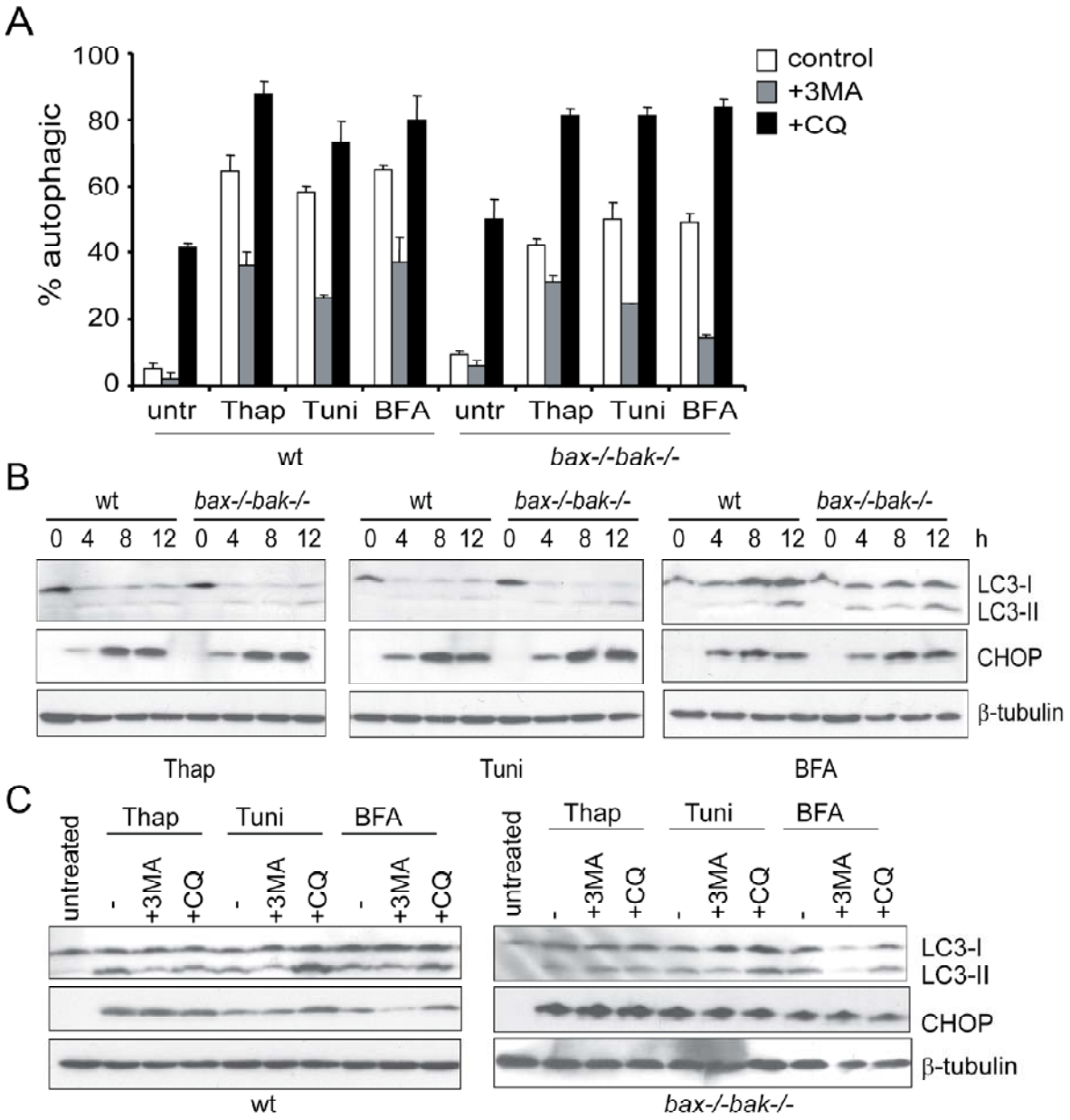


Figure 5. Autophagy is induced in *bax*$^{-/-}$*bak*$^{-/-}$ cells in response to ER stress.

(A) Quantification of LC3-GFP puncta positive cells in wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells. Percentage of cells showing punctated LC3-GFP was determined and the values represent the mean ± S.E.M. of 3 countings ($P < 0.01$ for all data points). (B) Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were treated for the indicated times with the indicated ER stress. Thirty micrograms of total cell lysates was subjected to immunoblotting assay using antibodies against LC3 and CHOP. β-tubulin was probed as a control for equal loading. (C) Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were treated for 16 hours with the indicated ER stress alone or in the presence of either 3MA or CQ. Total lysates were prepared and LC3-I to LC3-II conversion was determined by immunoblotting. Experiments were performed with assistance from Dr. Yongjun Fan.

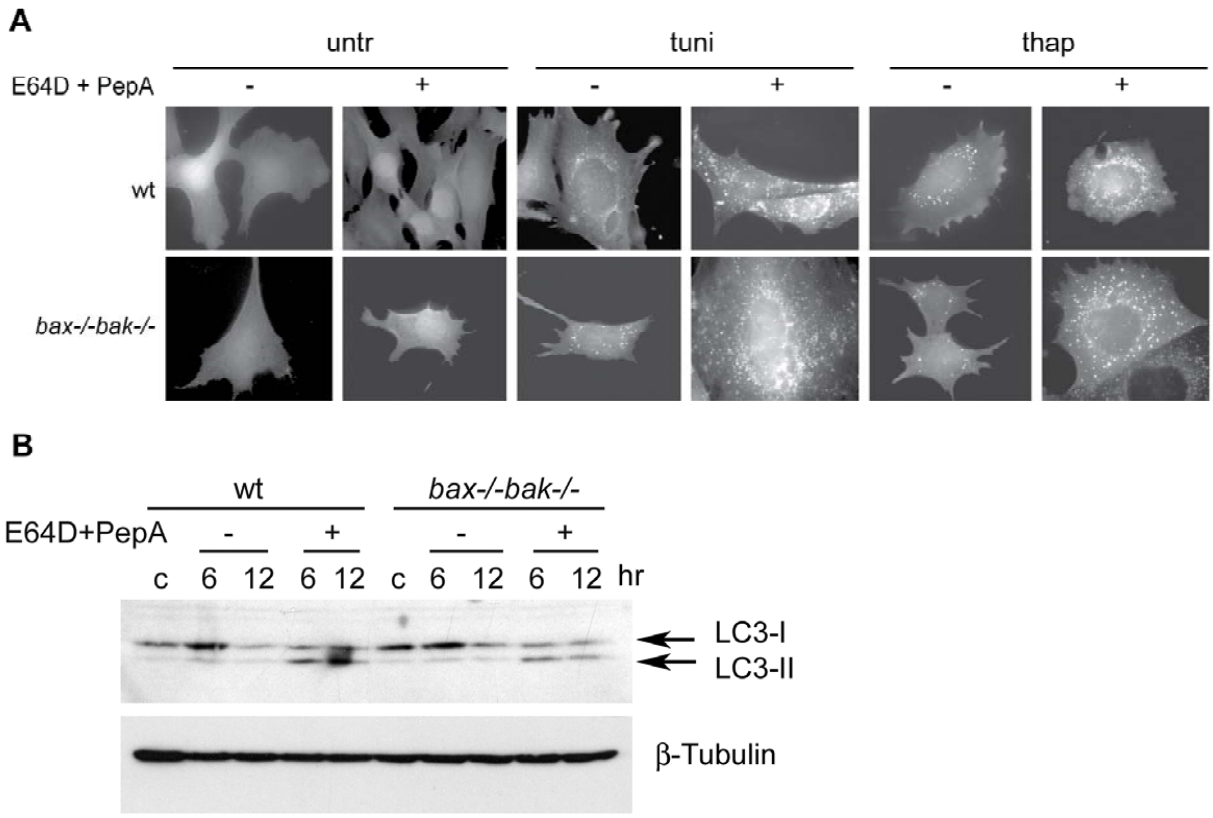


Figure 6. Lyosomal turnover is similar in wild-type and *bax-/-bak-/-* cells.

(A) Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells stably expressing LC3-GFP were treated with tunicamycin or thapsigargin in the absence or presence of the lysosomal protease inhibitors E-64-D (10 μg/ml) and pepstatin A (10 μg/ml). Twelve hours later, cells were fixed and observed under microscope. Note that the protease inhibitors enhanced the autophagosome formation induced by ER stress. (B) Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells were treated with tunicamycin for 6 or 12 hours in the absence or presence of E-64-D and pepstatin A. Thirty micrograms of cell lysates were probed with an anti-LC3 antibody. Note the similar accumulation of LC3-II in the presence of the lysosomal protease inhibitors.

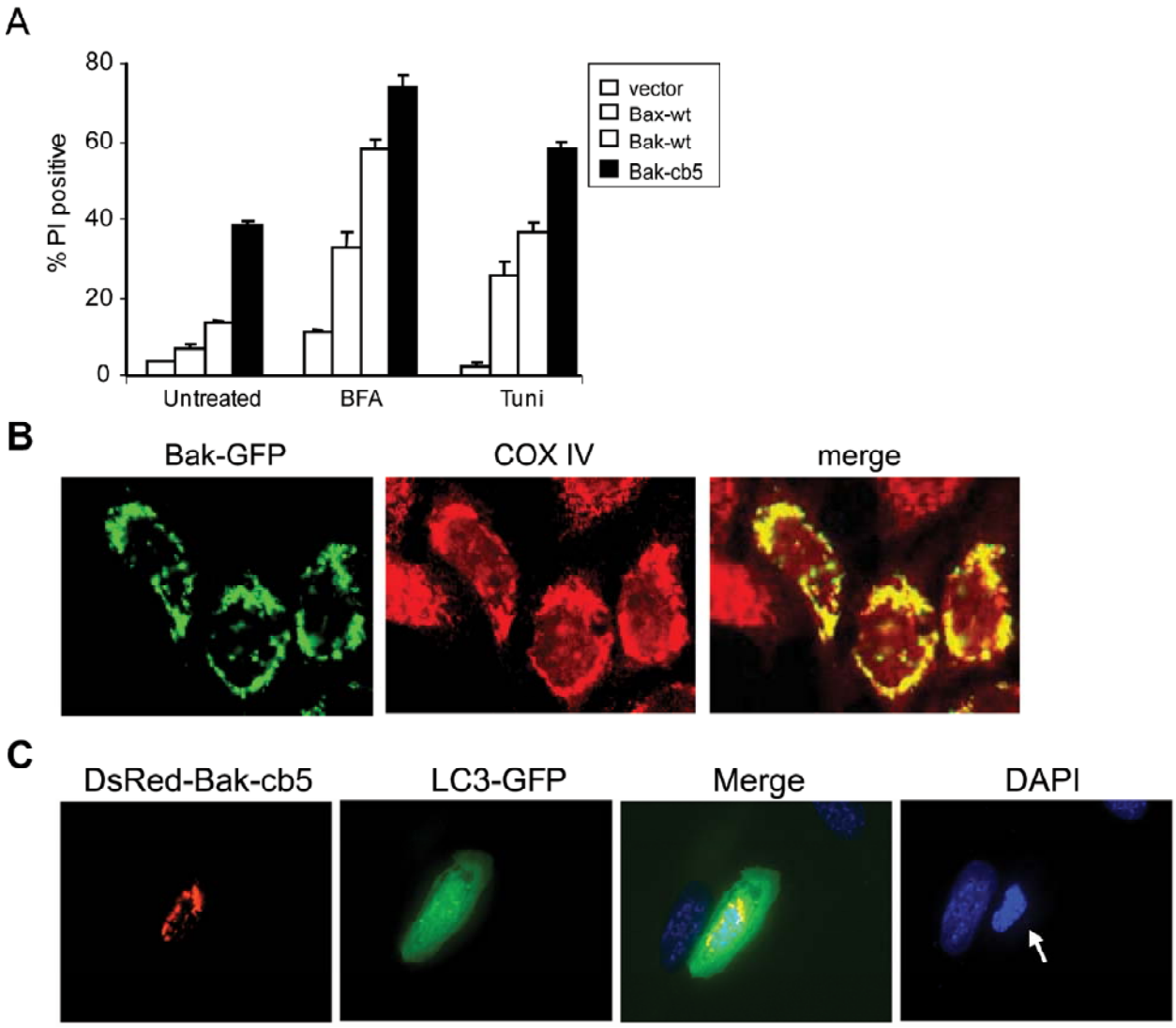


Figure 7. Multi-domain pro-apoptotic Bcl-2 proteins do not affect autophagy.

(A) *bax*$^{-/-}$*bak*$^{-/-}$ MEFs expressing Bax, Bak, or Bak-cb5 were left untreated or treated with BFA (0.5 μg/ml) or tunicamycin (0.5 μg/ml) for 24 hours. Cell death was determined by PI exclusion. GFP-tagged Bak, which localizes to mitochondria, was transfected into MCF7 cells for 24 h, cells were immunostained for COX IV, a mitochondrial marker, note the colocalization of Bak with COX IV. (C) DsRed-Bak-cb5, which targets the ER membrane, was transfected *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were fixed and stained with DAPI to visualize nuclei. Note that Bak-cb5 induced nuclear condensation (arrow), and failed to induce autophagy.

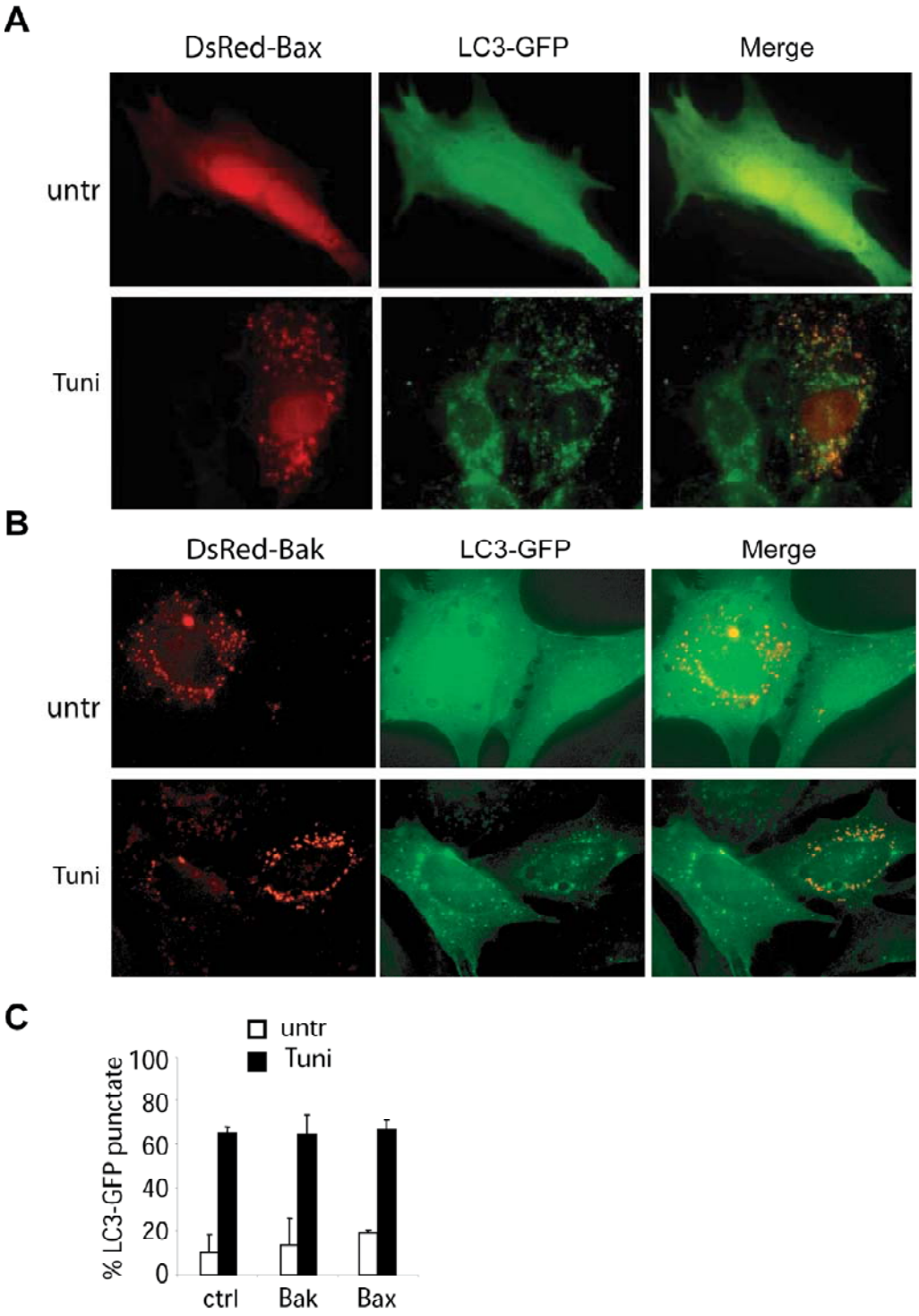


Figure 8. Multi-domain pro-apoptotic Bcl-2 proteins do not affect autophagy.

(A-C) *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were transfected with LC3-GFP together with DsRed-tagged Bax or Bak. (A) 24 h later cells were left untreated or treated with tunicamycin (0.5 μg/ml) for 8 h, fixed and then observed under a Zeiss Axiovert fluorescence microscope. Tunicamycin treatment induced Bax translocation to mitochondria, which did not affect the induction of autophagy.

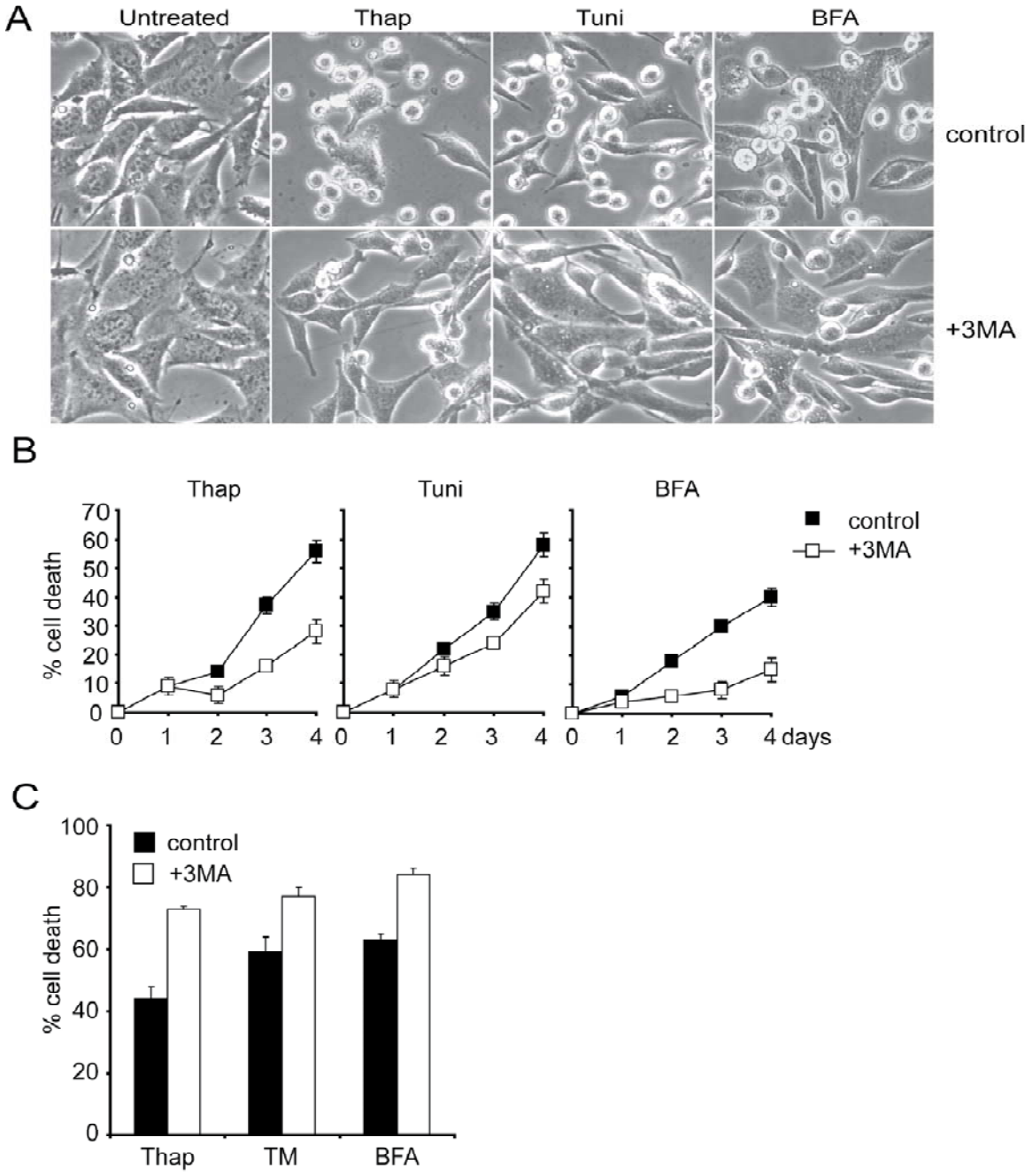


Figure 9. Apoptosis-deficient MEFs are protected from ER stress-mediated cell death by 3MA, while death is enhanced in apoptosis-competent cells.

(A) Cell morphology was assessed by phase-contrast microscopy in *bax*$^{-/-}$*bak*$^{-/-}$ MEFs after 3 days of treatment with ER stressors alone or in the presence of 3MA. (B) *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were treated for 1-4 days with the indicated ER stressors alone or in combination with 3MA. Cell death was determined by PI exclusion. (C) Wild-type MEFs were treated for 24 h with indicated ER stressors alone or in combination with 3MA. Cell death was determined by PI exclusion. Results shown are averages of triplicate samples +/- S.E.M. (P<0.01 for all treatments).

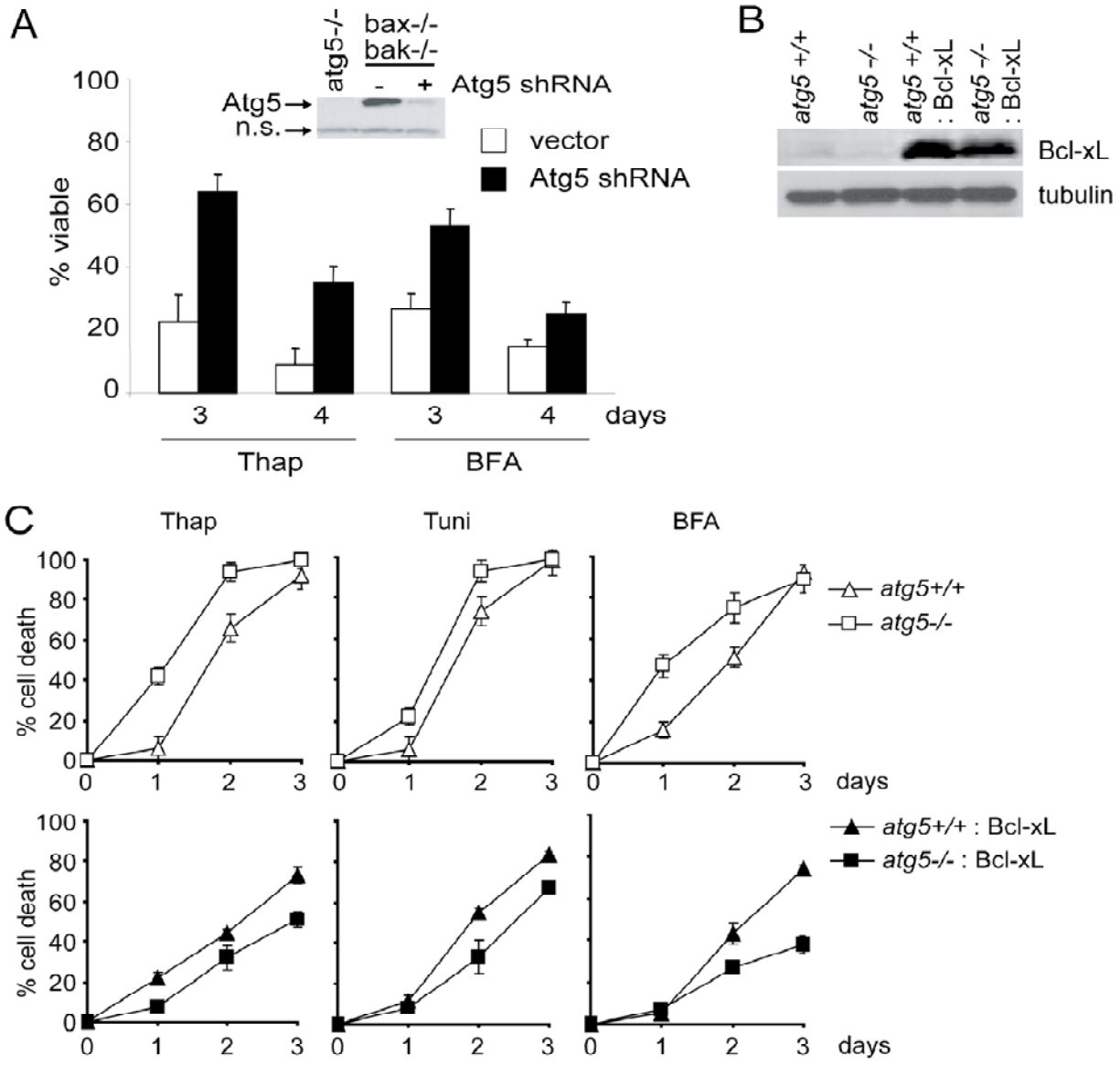


Figure 10. Atg5 knockdown delays cell death in *bax*$^{-/-}$*bak*$^{-/-}$ MEFs in response to ER stress.

(A) Atg5 was knocked down in *bax*$^{-/-}$*bak*$^{-/-}$ MEFs, cells were then treated for 3-4 days with either thapsigargin or brefeldin A. Percent cell survival was determined by trypan blue exclusion and taken as an average of triplicate samples ± S.E.M. ($P < 0.01$ for all time points). Immunoblotting to show decreased Atg5 expression in shAtg5 transfected *bax*$^{-/-}$*bak*$^{-/-}$ MEFs and *atg5*$^{-/-}$ MEFs, n.s. is a non-specific band used as a loading control. (B) *atg5*$^{-/-}$ and *atg5*$^{+/+}$ MEFs were infected with a retroviral construct expressing Bcl-xL, expression was visualized by immunoblotting. (C) Cells were treated for the indicated times with ER stressors and cell death was measured by PI exclusion and indicated as an average of triplicate samples ± S.E.M. ($P < 0.01$ for all time points). Experiments were performed with the assistance of Dr. Yongjun Fan.

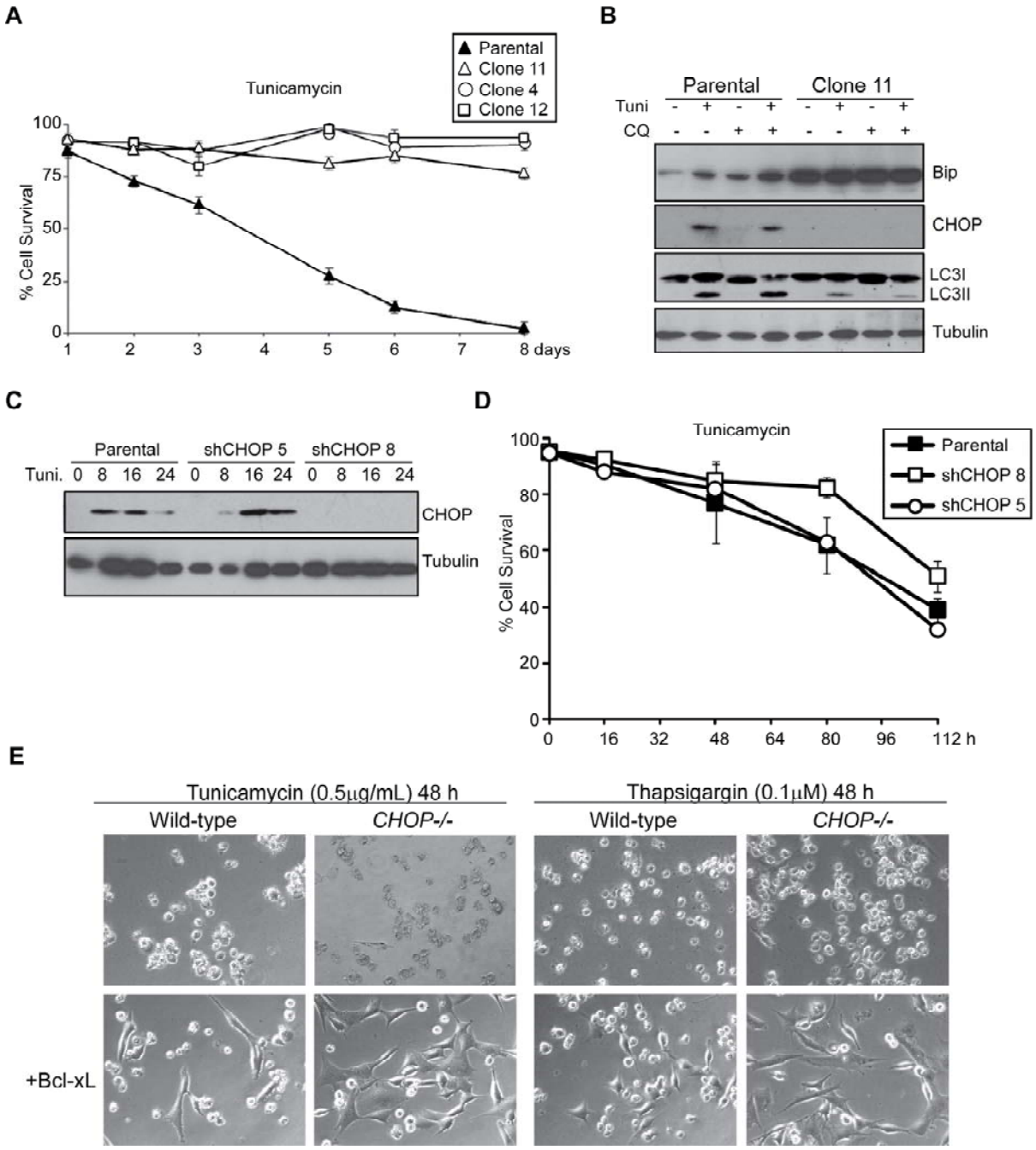


Figure 11. CHOP is not sufficient to induce cell death in apoptosis deficient cells.

(A-B) *bax-/-bak-/-* BMK cells were treated with tunicamycin (0.5 mg/ml) for 8 days and colonies that survived were isolated (A). Tunicamycin resistant clone 11 was probed for expression of Bip, CHOP and LC3, following treatment (B). (C) Short-hairpins against CHOP were expressed in *bax-/-bak-/-* BMK cells, lysates from tunicamycin treated cells were then immunoblotted to verify knock-down efficiency. (D) Cell death in response to tunicamycin treatment was analyzed using propidium iodide exclusion. (E) *CHOP-/-* cells were transfected with Bcl-xL and subsequently treated with tunicamycin, representation phase contrast images are shown.

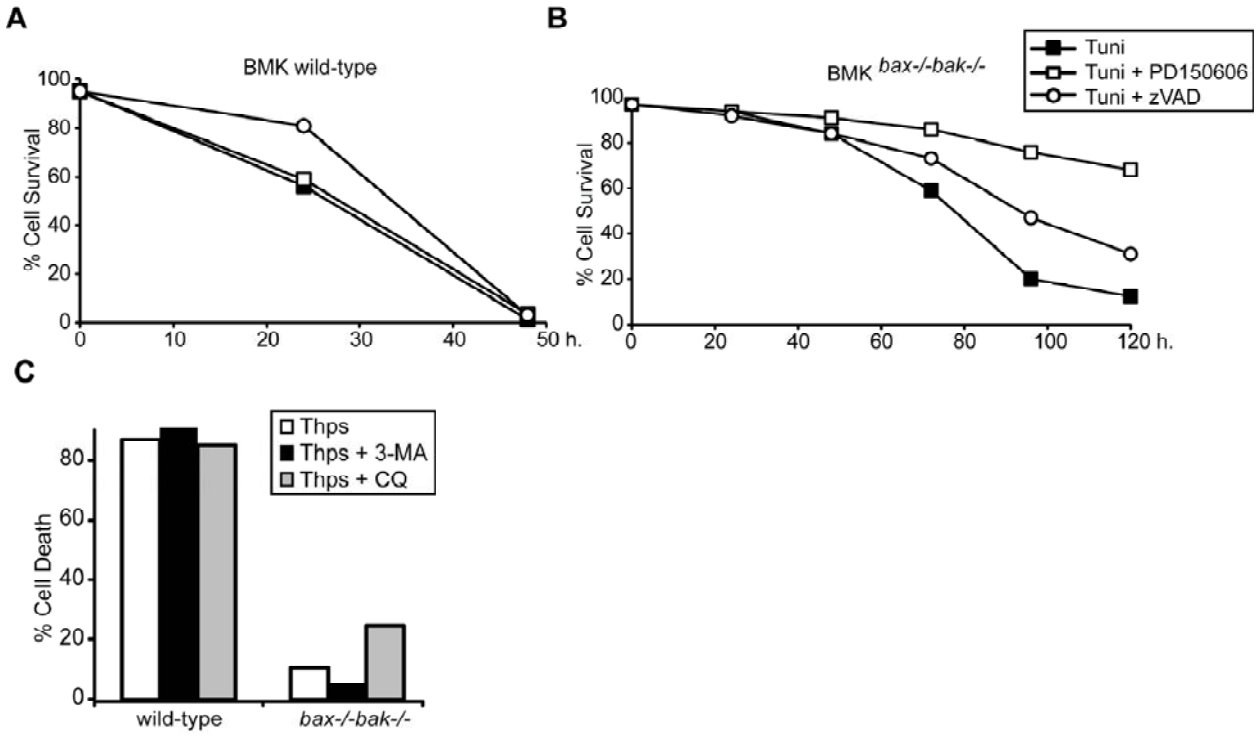


Figure 12. ER stress-induced cell death in apoptosis deficient cells may be through programmed necrosis.

(A-B) BMK wild-type (A) or *bax-/-bak-/-* (B) cells were treated with tunicamycin (0.5 μg/ml) alone or in combination with the broad caspase inhibitor zVAD-fmk,(20 μM) or the selective calpain inhibitor PD150606 (50 μM), cell death was then measured by PI exclusion. Note that while zVAD moderately protects apoptosis competent cells from tunicamycin treatment, PD150606 has no effect on wild-type cells but dramatically protects apoptosis deficient *bax-/-bak-/-* cells. (C) Lysosomal build-up promotes ER stress-induced cell death in *bax-/-bak-/-* cells. Wild-type or bax-/-bak-/- BMK cells were treated with thapsigargin alone, or in combination with either 3-methyladenine (5 mM), an inhibitor of autophagosome formation or chloroquine (10 μM), an inhibitor of autophago-lysosome turnover.

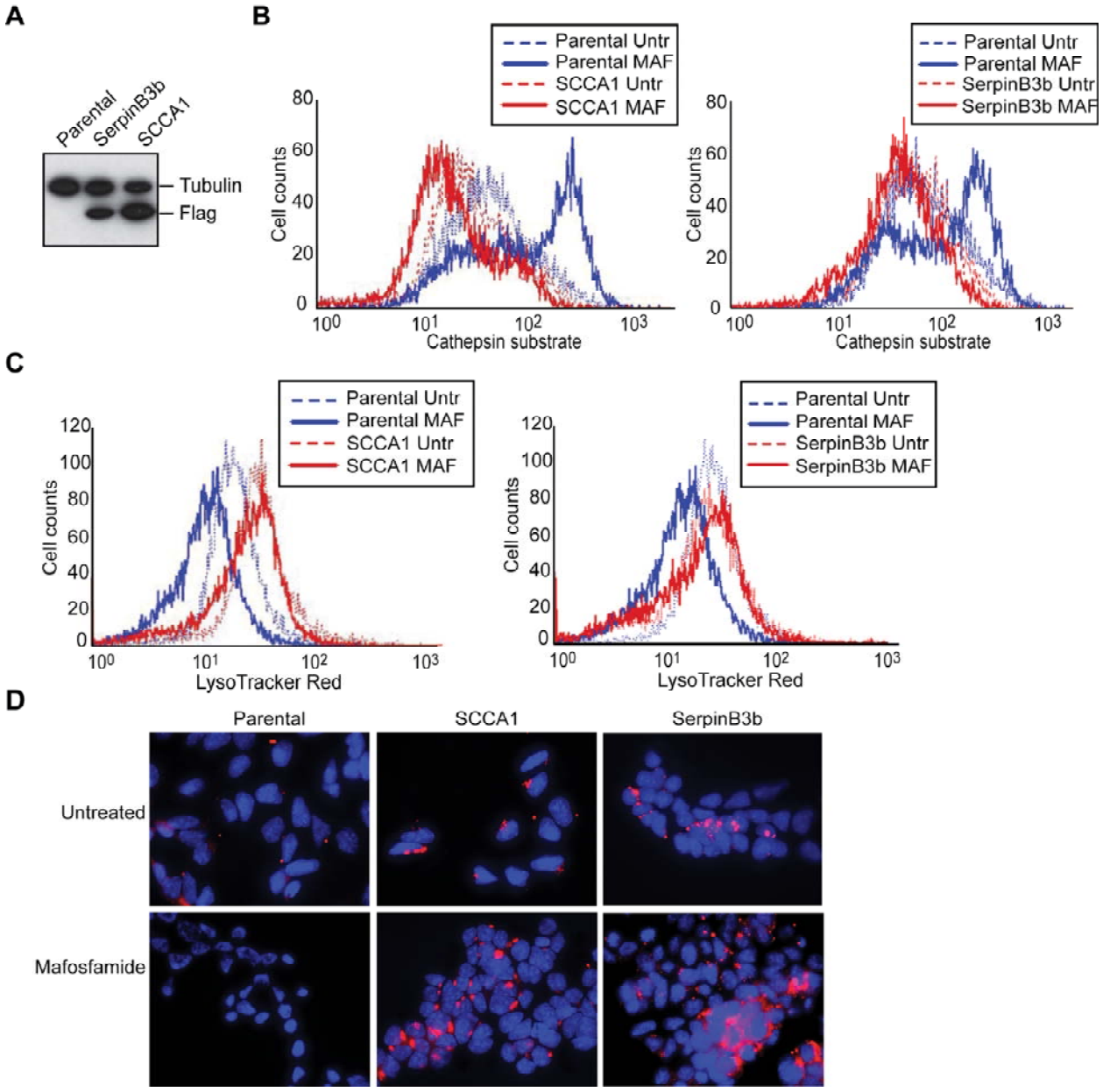


Figure 13. SCCA1 inhibits cathepsin activity and lysosomal damage.

(A) *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells were stably transfected with either Flag-tagged human SCCA1, or its mouse paralog SerpinB3b. Protein expression was confirmed by immunoblotting using an anti-Flag antibody. Tubulin was probed for equal loading. (B and C) Parental or Flag-SCCA1 expressing cells were treated for 6 h with mafosfamide (50 μg/ml), stained with either a flourogenic cathepsin L substrate (B), or with LysoTracker Red (C), then analyzed via flow cytometry. (D) Parental or Flag-SCCA1 expressing cells were left untreated or treated with mafosfamide for 16h. Cells were stained with LysoTracker Red and DAPI, and photographed.

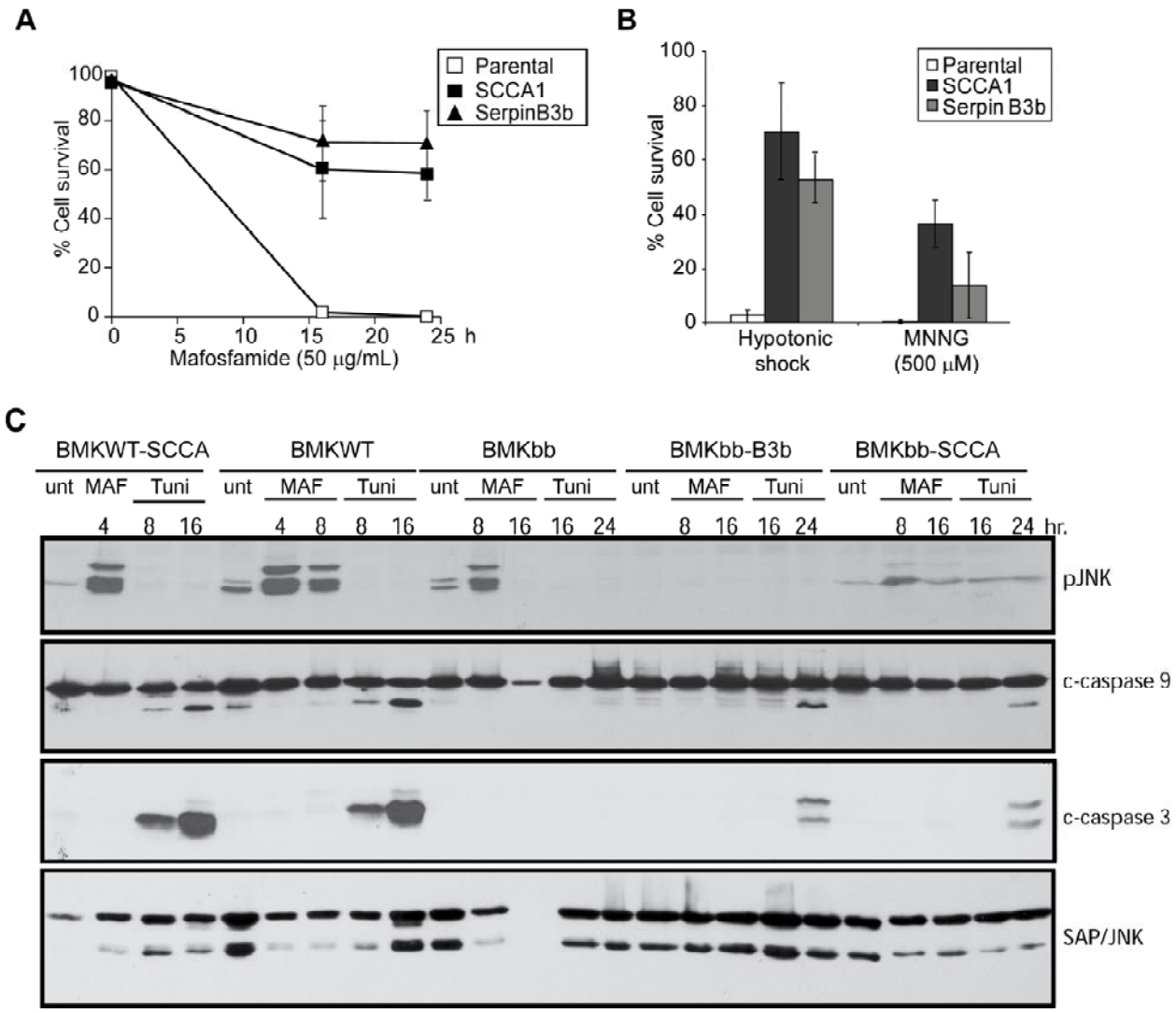


Figure 14. SCCA1 and SerpinB3b protect cells from lysosomal-mediated cell death that is not apoptosis.

(A and B) Parental cells or those expressing either Flag-SCCA1 or Flag-SerpinB3b were treated with MNNG (500 μM) or hypotonic shock for 16 h (A), or with mafosfamide (50 μg/ml) for indicated periods of time (B). Cell death was measured by PI exclusion using flow cytometry. (C) BMK wild-type, wild-type cells expressing SCCA1, as well as bax and bak deficient parental cells, or those expressing SCCA1 or SerpinB3b, were treated with either mafofsamide or tunicamycin for the indicated amount of time, lysates were then immunoblotted for caspase cleavage and phosphorylated JNK, total JNK was used as a loading control.

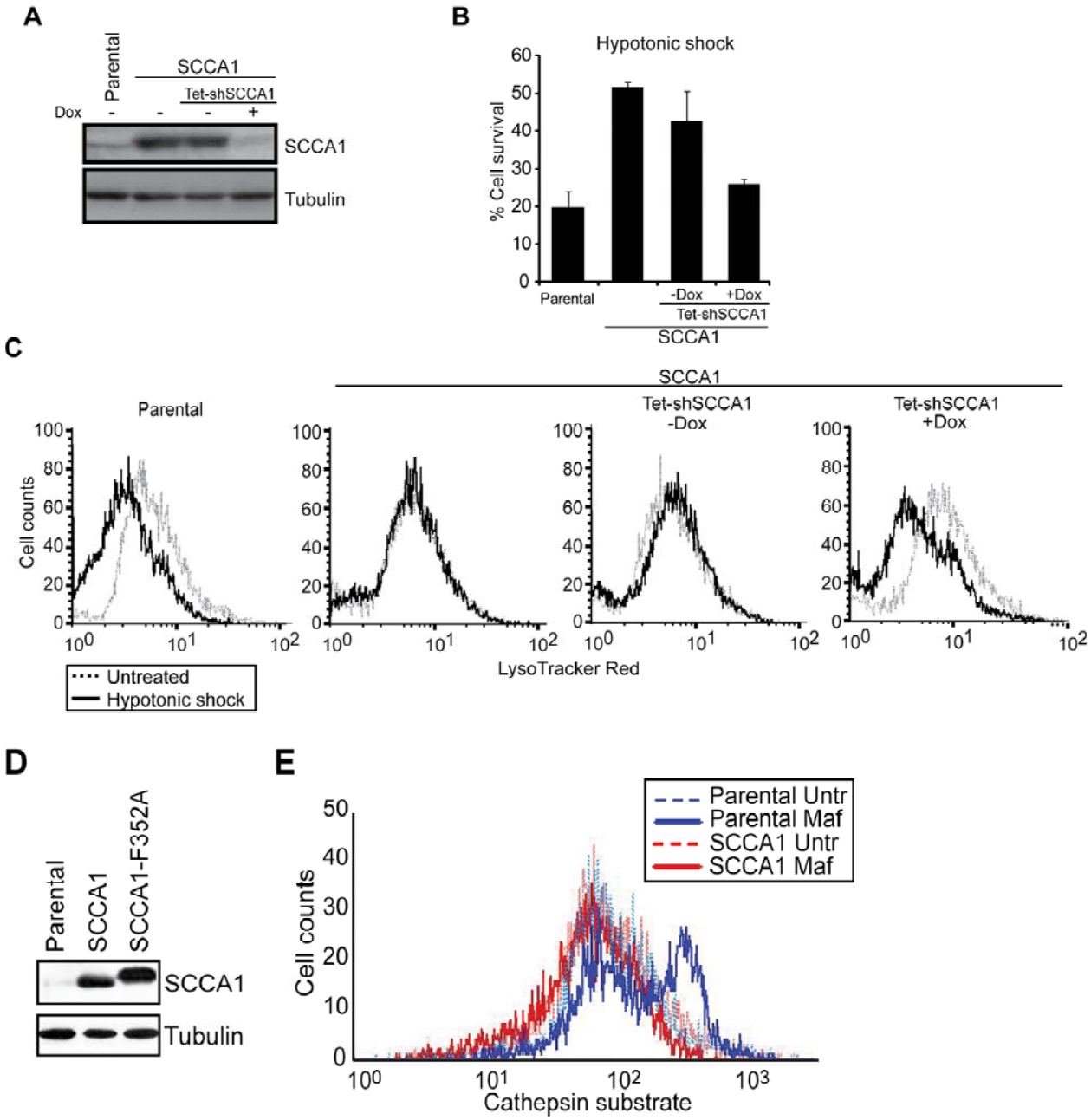


Figure 15. SCCA1's effect on cell death in response to lysosomal injury and ER stress in apoptosis competent MCF10A and Hs578T cells.

(A) MCF10A cells overexpressing SCCA1 were infected with a tet-inducible shSCCA1, addition of doxycycline for 3 days lead to decreased SCCA1 expression in these cells as indicated by immunoblotting. (B) Cells were then treated with hypotonic shock for 16 h and cell death measured by PI exclsion. (C) Lysosomal presence was determined by staining cells with LysoTracker Red following treatment with hypotonic shock for 4 h. (D) Wild-type SCCA1 or the inactive SCCA1-F352A mutant was expressed in Hs578T breast cancer cells. (E) Cells were treated for 6 h with mafosfamide (50 μg/ml), then stained with a fluorogenic cathepsin L substrate and analyzed by flow cytometry

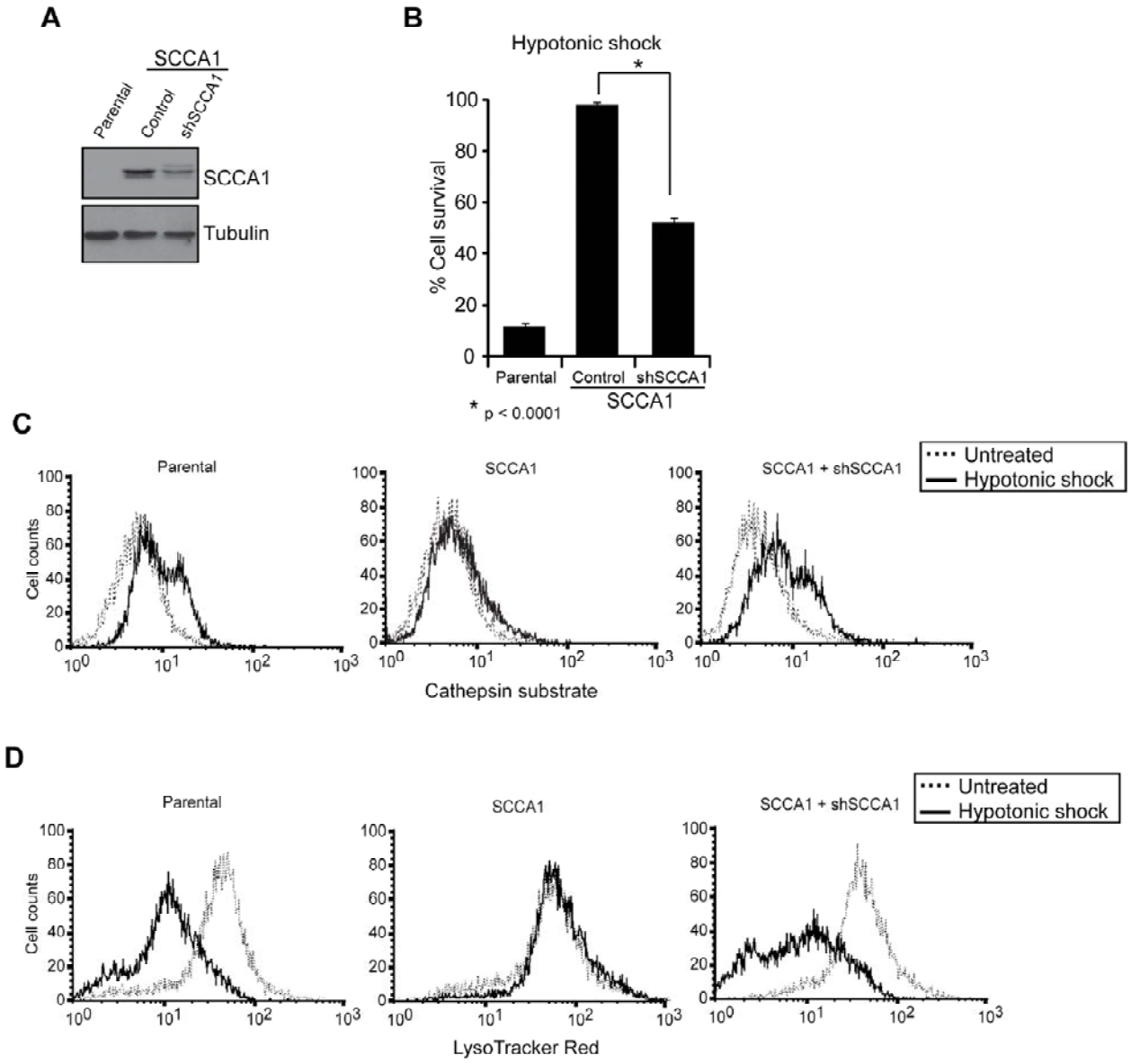


Figure 16. Protection from lysosomal damaging agents is specifically due to SCCA1 expression.

(A) Parental *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells expressing Flag-SCCA1 were infected with either a control lentiviral shRNA or one targeting SCCA1. SCCA1 knockdown was confirmed by immunoblotting. (B) Parental or Flag-SCCA1 cells expressing a control shRNA or shSCCA1 were treated with hypotonic shock for 16 h. Cell death was measured by PI exclusion. Cells were also analyzed for cathepsin activity (C) as well as lysosomal presence (D) following a 6 h treatment of hypotonic shock.

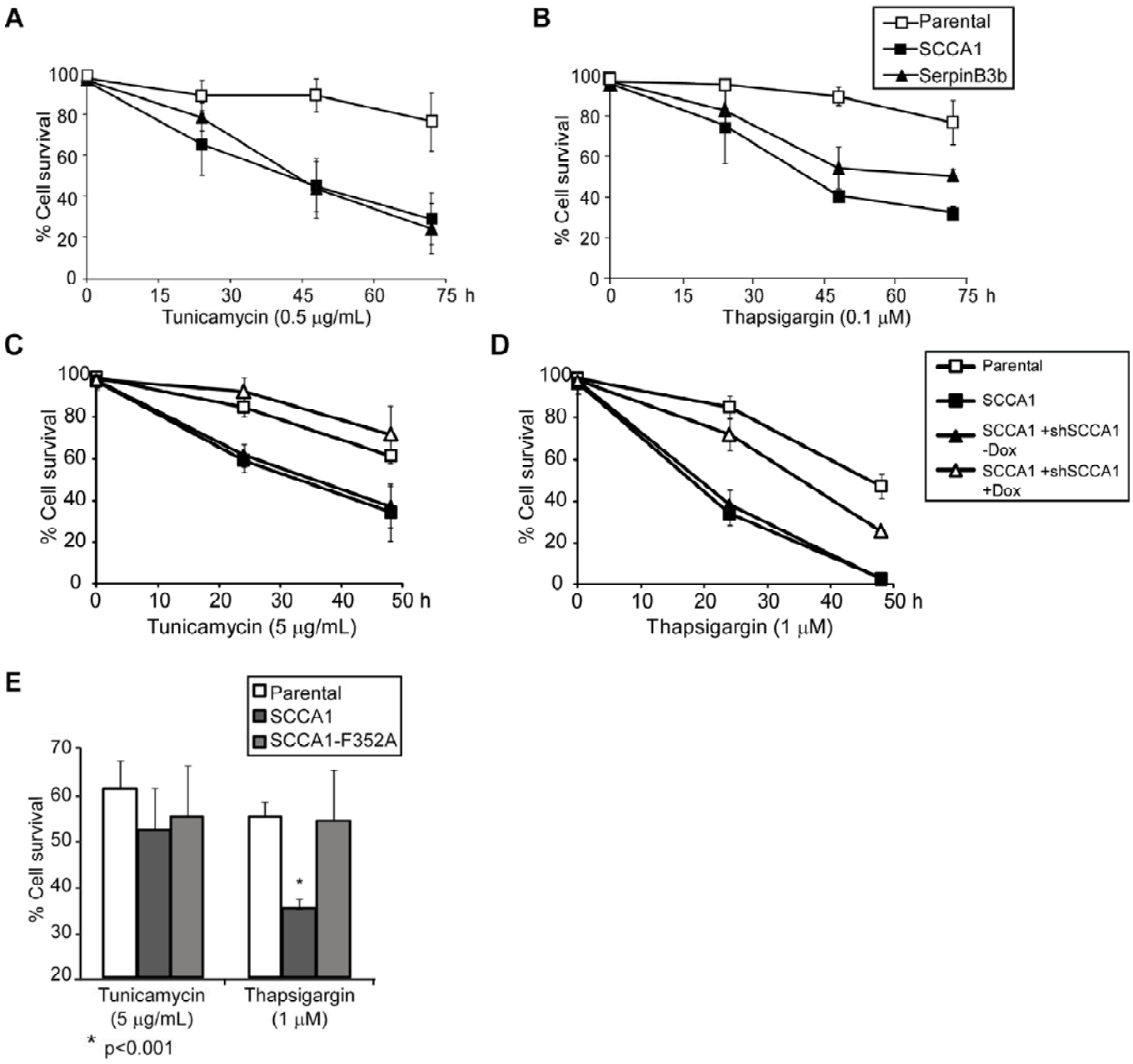


Figure 17. SCCA1 enhances ER stress-induced cell death.

(A and B) Parental *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells or those expressing Flag-SCCA1 or Flag-SerpinB3b were treated with tunicamycin (0.5 μg/ml) (A) or thapsigargin (0.1 μM) (B). Cell death was measured by PI exclusion. (C-D) MCF10A parental cells, SCCA1-expressing cells, and SCCA1-expressing cells containing a tet-inducible shSCCA1 either left untreated or treated with doxycycline for 3 days were treated with either tunicamycin (5.0 μg/ml) (C) or thapsigargin (1.0 μM) (D) for the indicated amount of time. Cell death was measured by PI exclusion. (E) Wild-type SCCA1 or the SCCA1-F352A mutant was expressed in Hs578T breast cancer cells that were subsequently treated with either tunicamycin (5.0 μg/ml) or thapsigargin (1.0 μM) for 48 h and cell death was measured by PI exclusion using flow cytometry.

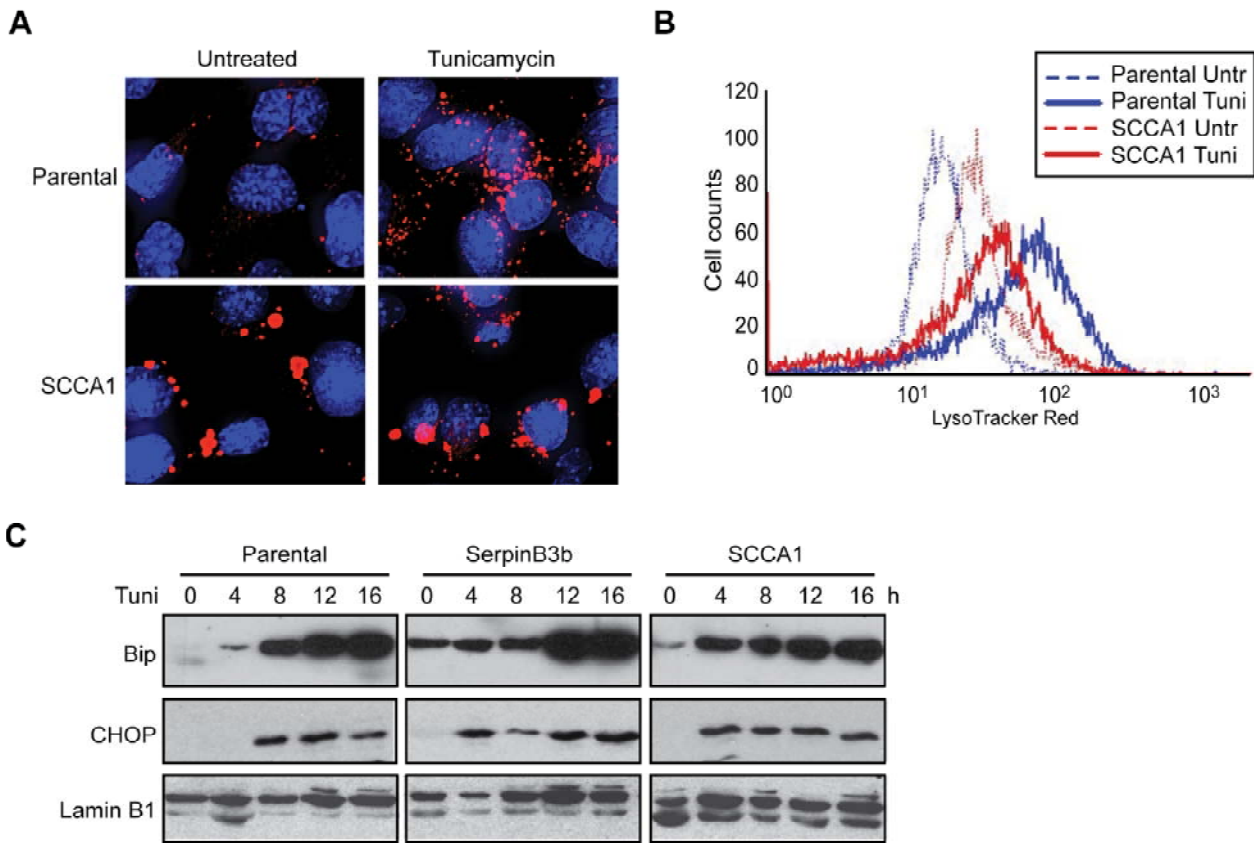


Figure 18. Lysosomal function is compromised in SCCA1 expressing cells.

(A and B) Cells were left untreated or treated with tunicamycin (0.5 µg/ml) for 16 h. Cells were stained with LysoTracker Red and DAPI. Cells were photographed and representative images are shown (A), or subjected to flow cytometry (B). (C) Cells were treated with tunicamycin for indicated times. The ER stress response was examined by immunoblotting with indicated antibodies.

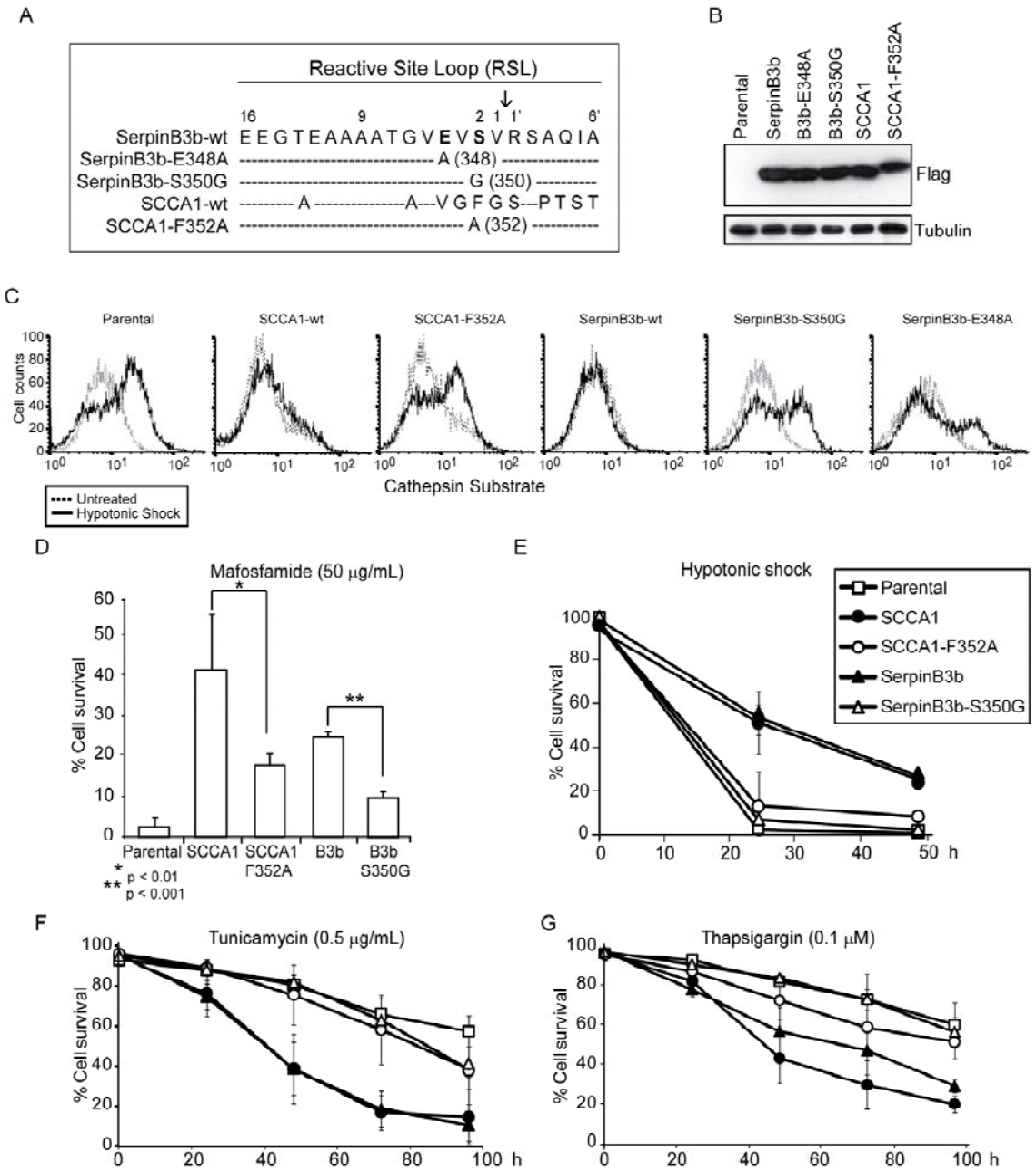


Figure 19. SCCA1's protease inhibitory activity is required to enhance cell death.

(A) Diagram of point mutations made within the RSL of SCCA1 and SerpinB3b. The numbering indicates the position of residues within the RSL and the arrow indicates the protease cleavage site. (B) Immunoblot of *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells stably expressing Flag-tagged wild-type and mutant SCCA1 and SerpinB3b. (C) Indicated parental, serpin-wt, and serpin-mutant cells were treated with hypotonic shock for 6 h, then incubated with a fluorogenic cathepsin L substrate, activity was determined by flow cytometry. (D-G) Parental, serpin-wt, and serpin-mutant cells were treated with mafosfamide (D), hypotonic shock (E), tunicamycin (F), or thapsigargin (G) for the indicated amount of time. Cell death was measured by PI exclusion.

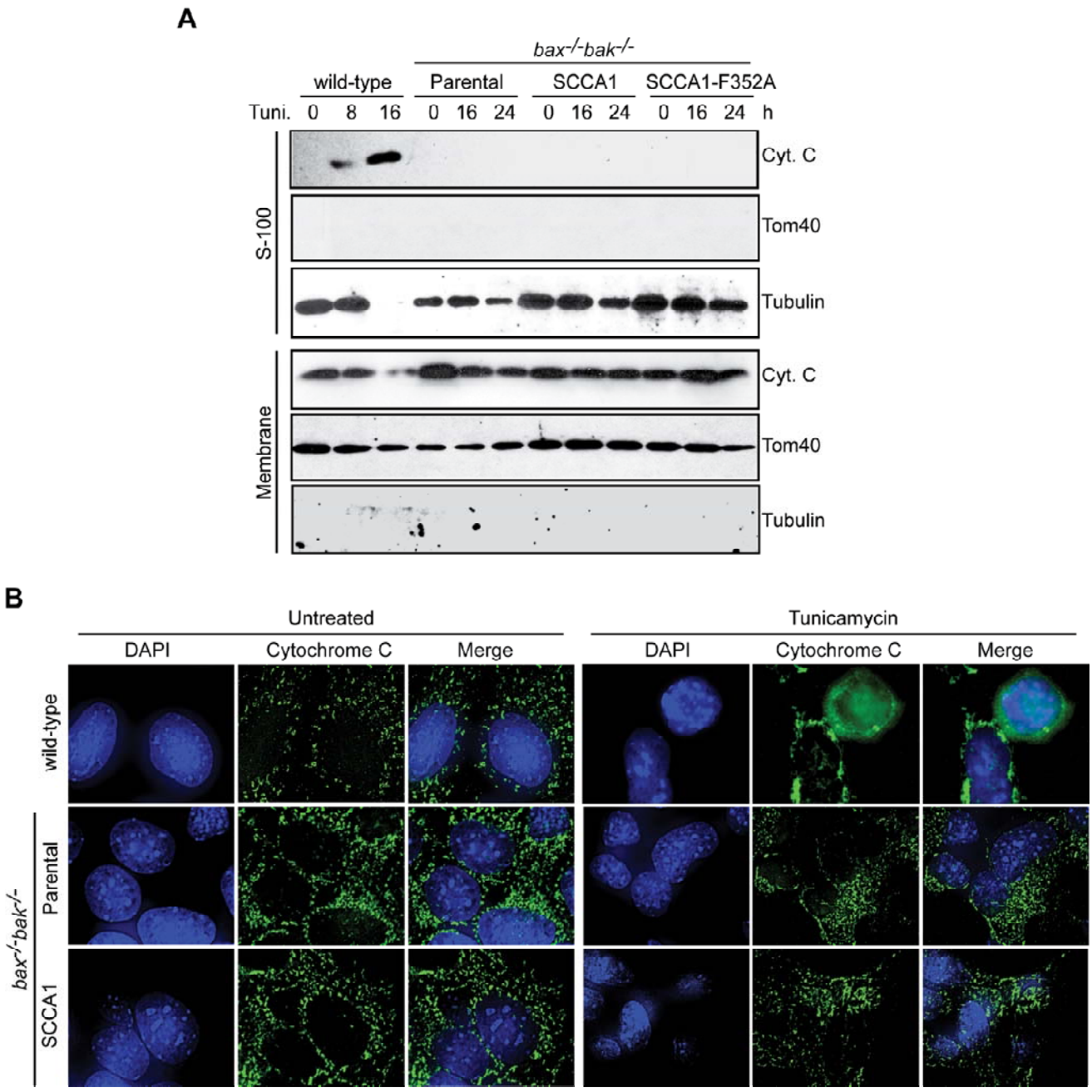


Figure 20. SCCA1 promotes mitochondrial independent cell death in response to ER stress.

(A) BMK wild-type, as well as *bax*$^{-/-}$*bak*$^{-/-}$ parental or those expressing SCCA1 or SCCA1-F352A were treated for the indicated amount of time with tunicamycin (0.5 μg/mL). Subcellular fractionation was performed, and the fractions probed with indicated antibodies. (B) BMK wild-type, as well as *bax*$^{-/-}$*bak*$^{-/-}$ parental and SCCA1-expressing cells were treated with tunicamycin for 24 h. Subcellular localization of cytochrome C (green) was visualized by immunofluorescence. DAPI (blue) was used to stain the nucleus.

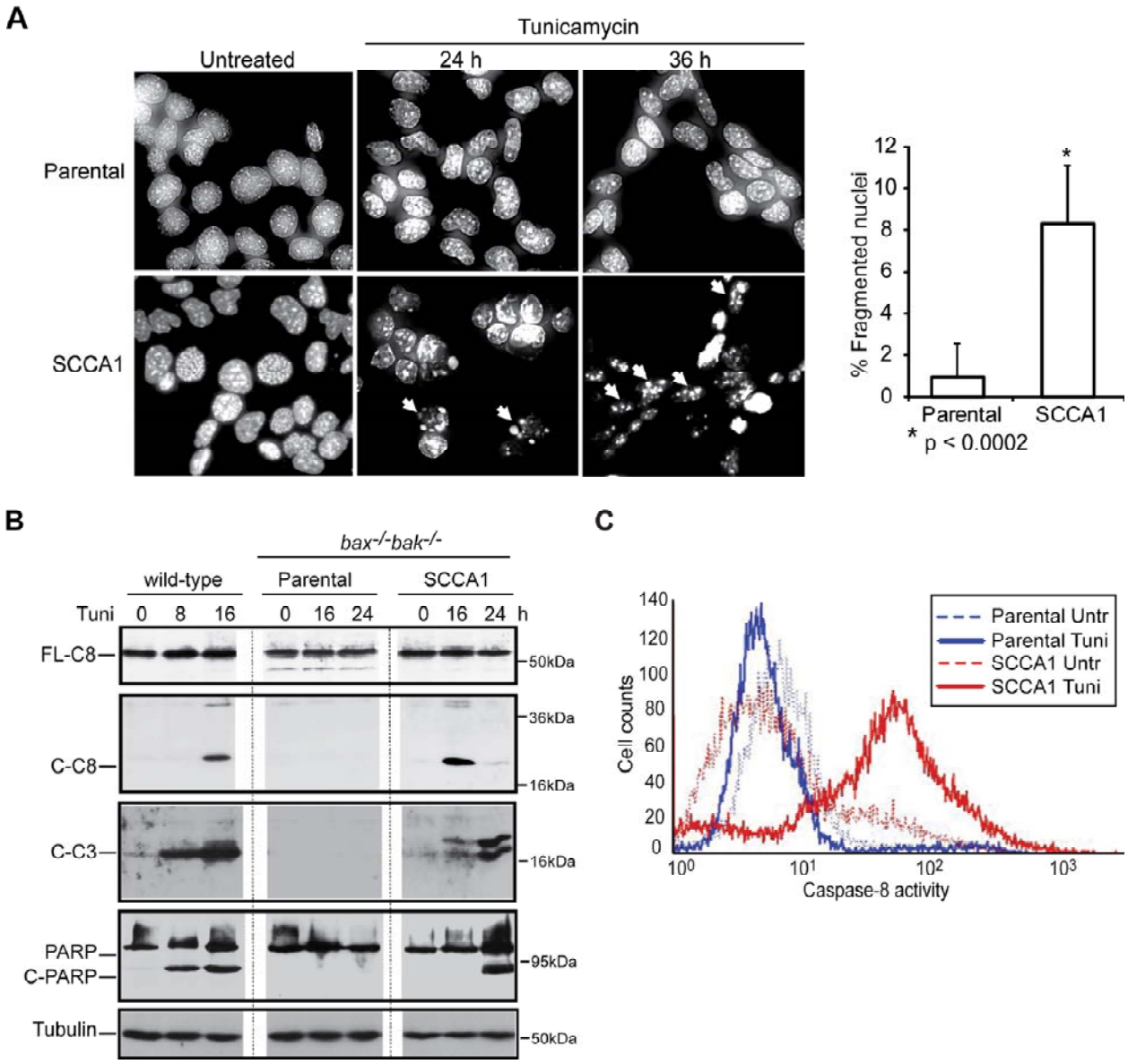


Figure 21. SCCA1 promotes caspase activation in response to ER stress.

(A) bax$^{-/-}$*bak*$^{-/-}$BMK parental and SCCA1-expressing cells were treated with tunicamycin (0.5 μg/ml) for 24 or 36 h, subsequently apoptotic nuclear morphology was visualized by DAPI staining. Quantification of fragmented nuclei was determined by performing 3 independent countings of 50 cells, the mean ± S.E.M. is shown. (B) Lysates from *bax*$^{-/-}$*bak*$^{-/-}$BMK parental and SCCA1-expressing cells, treated with tunicamycin (0.5 μg/ml) for the indicated amount of time, were probed with indicated antibodies. Wild-type BMK cells were used as a positive control for apoptosis induction. (C) *bax*$^{-/-}$*bak*$^{-/-}$ BMK parental and SCCA1-expressing cells were treated with tunicamycin (0.5 μg/ml) for 16 h. Caspase-8 activity was detected using the fluorogenic caspase-8 substrate FITC-IETD-FMK.

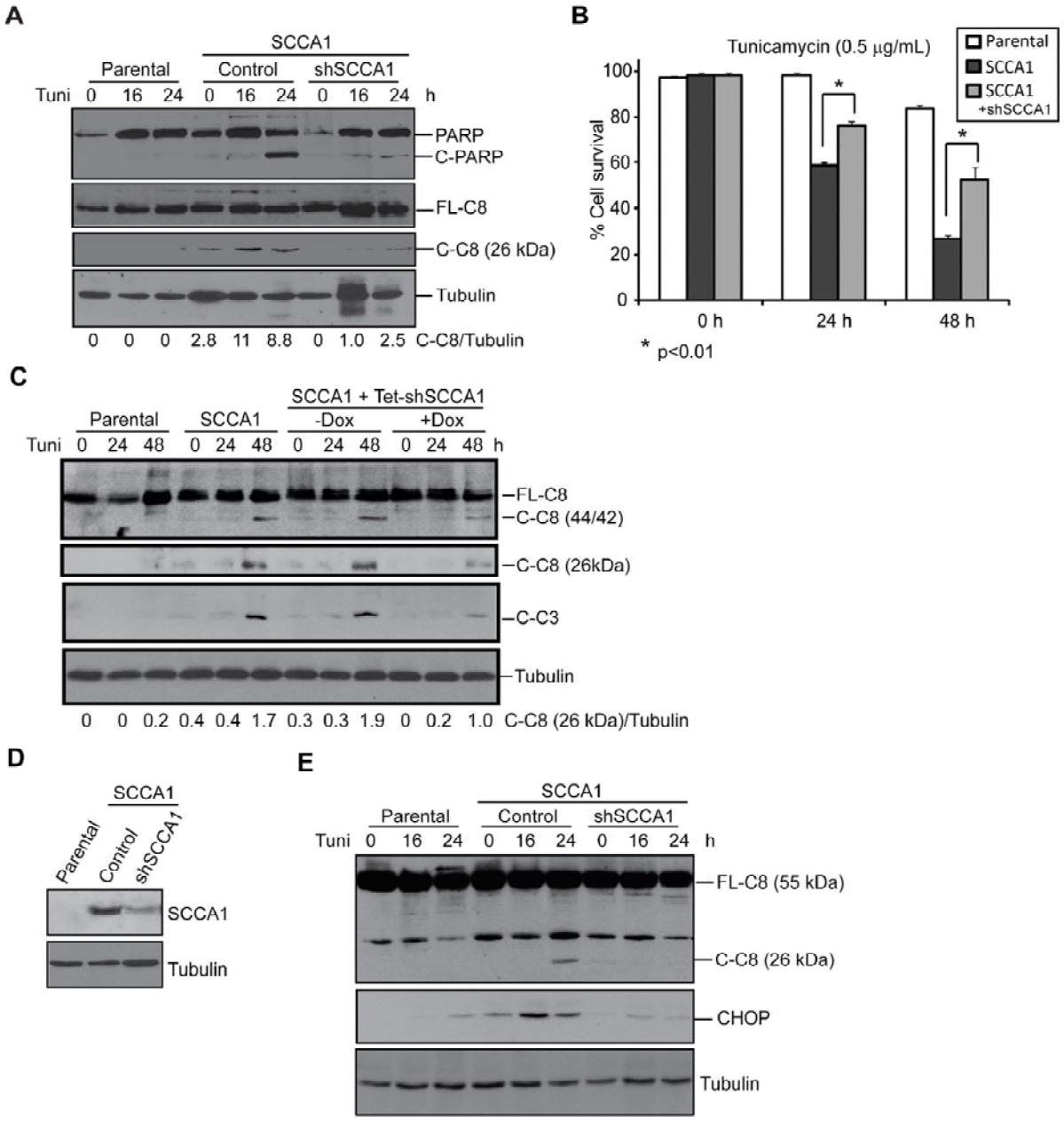


Figure 22. SCCA1 promotes caspase-8 cleavage in apoptosis competent and deficient cells.

(A-B) Parental and SCCA1-expressing BMK *bax-/-bak-/-* cells containing either a shControl or shSCCA1 were treated with tunicamycin (0.5 μg/ml) for the indicated amount of time. Cell death was measured by PI exclusion (B) and immunoblotting was performed to observe caspase-8 and PARP cleavage (A). (C) MCF10A parental cells, SCCA1-expressing cells, and SCCA1-expressing cells containing a tet-inducible shSCCA1 were treated with tunicamycin (5.0 μg/ml), subsequently caspase-8 and caspase-3 cleavage was observed. The amount of cleaved caspase-8 was determined by densitometric analysis of cleaved caspase-8 against tubulin. (D) shControl or shSCCA1 were introduced into SCCA1-expressing Hs578T cells. (E) Cells were treated for the indicated amount of time with tunicamycin (5.0 μg/ml), then probed for caspase-8 and CHOP.

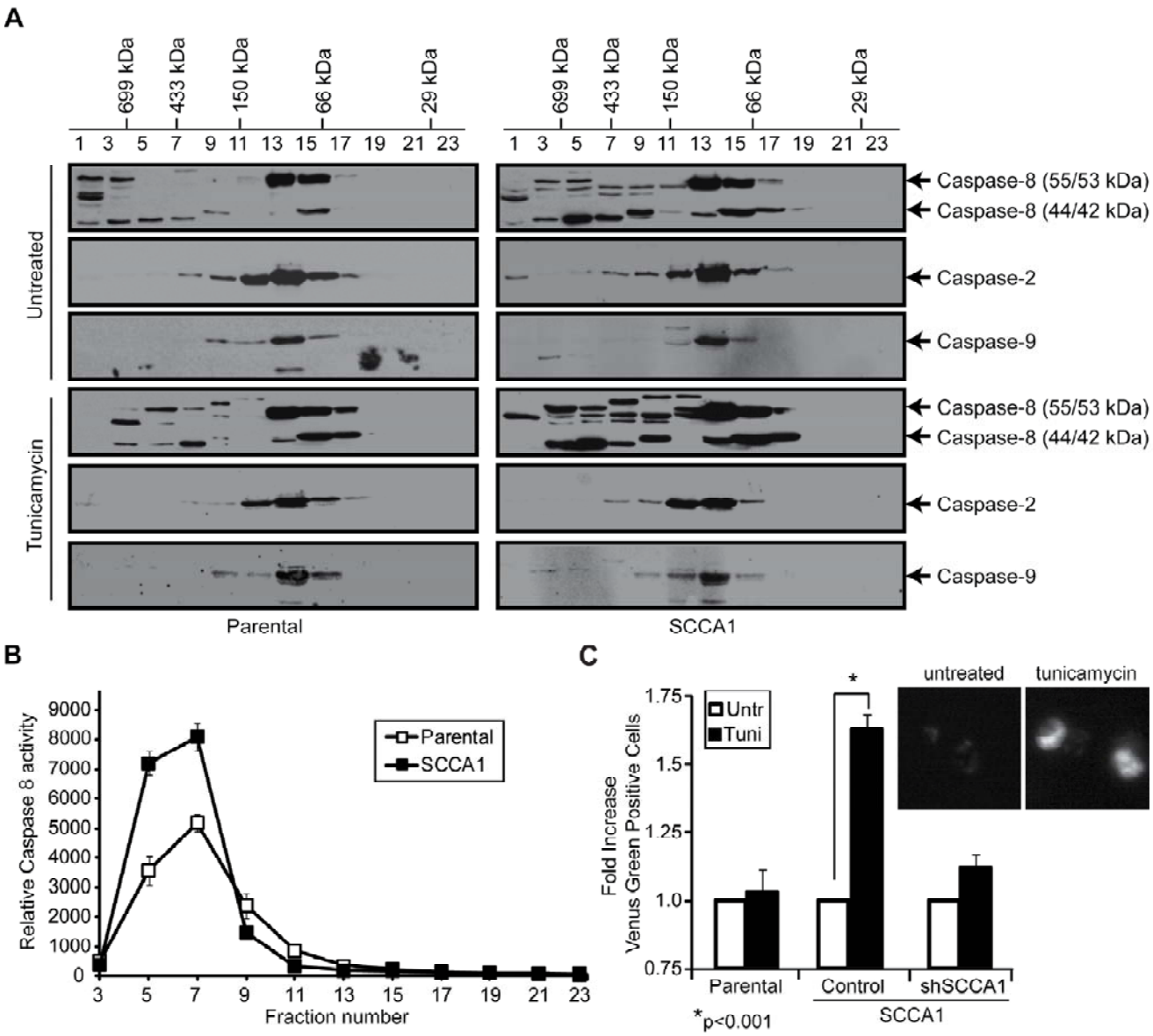


Figure 23. SCCA1 enhances caspase-8 oligomerization in response to ER stress.

(A) *bax*$^{-/-}$*bak*$^{-/-}$ BMK parental and SCCA1-expressing cells were treated with tunicamycin (0.5 μg/ml) for 16 h and size-exclusion chromatography was subsequently performed. Eluted fractions were probed for caspase-8, caspase-2, and caspase-9. Molecular weight of the fractions is indicated at the top. (B) Caspase-8 activity in tunicamycin-treated fractions was determined using a luminogenic caspase-8 substrate ITED. (C) Parental and SCCA1 cells expressing either a shControl or shSCCA1, were transfected with Venus-tagged caspase-8, which fluoresces only when there is interaction between caspase-8 molecules. Cells were left untreated or treated for 16 h with tunicamycin (0.5 μg/mL) and then analyzed by flow cytometry. Shown is the fold increase in fluorescent cells in tunicamycin treated cells compared to untreated cells. Representative images of untreated and tunicamycin treated SCCA1 cells transfected with Venus-tagged caspase-8 are shown. Experiments were performed with help from Dr. Ji-An Pan.

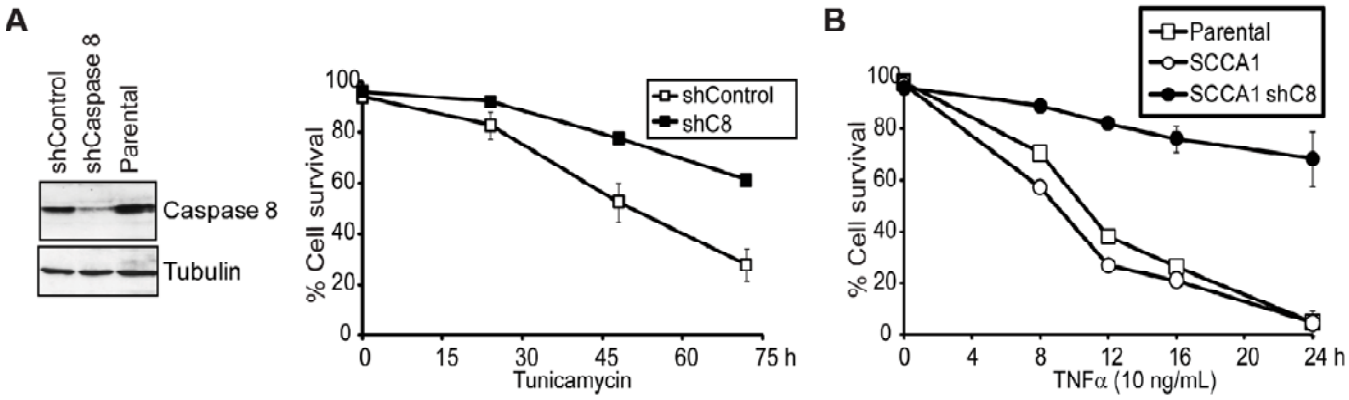


Figure 24. Caspase-8 knockdown protects SCCA1-expressing cells from ER stress-induced apoptosis.

(A) SCCA1-expressing *bax*$^{-/-}$*bak*$^{-/-}$ cells were infected with lentiviral shCaspase-8 or control shRNA. Caspase-8 knockdown was verified by immunoblotting. The cells were treated with tunicamycin and cell death measured by PI exclusion. (B) Parental and SCCA1-expressing cells as well as SCCA1-expressing cells containing shCaspase-8, were treated with TNFα (10 ng/mL) and cycloheximide (10 μg/mL) for the indicated amount of time and cell death measured by PI exclusion.

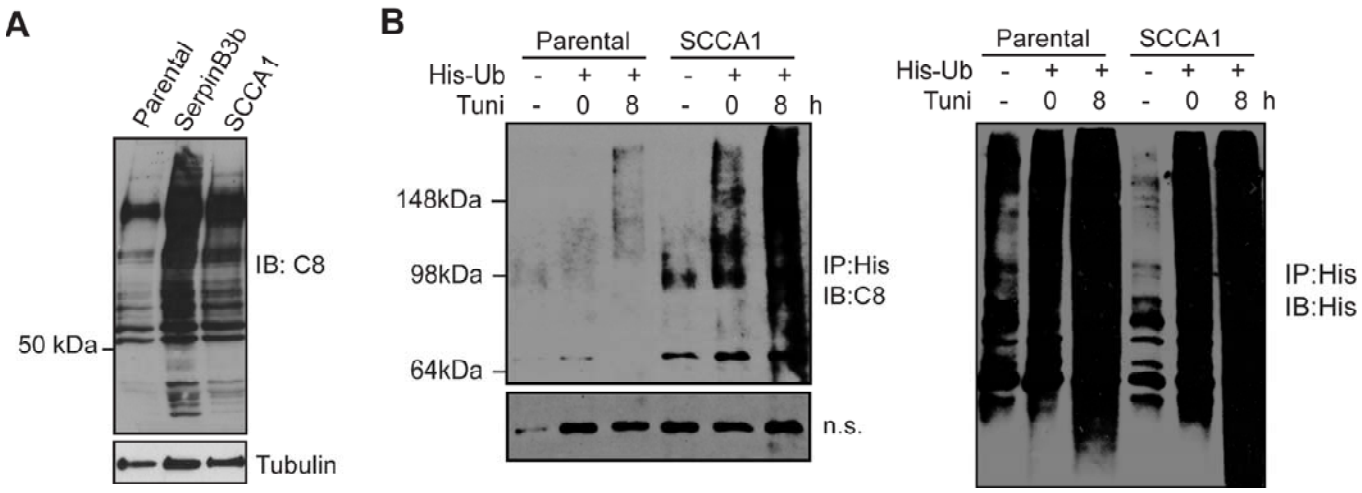


Figure 25. SCCA1 leads to increased caspase-8 ubiquitination

(A) bax$^{-/-}$*bak*$^{-/-}$ BMK parental, SCCA1-expressing, and SerpinB3b-expressing cells were immunoblotted for caspase-8. Note the increase in higher molecular weight protein bands in SCCA1 or SerpinB3b-expressing cells, suggestive of polyubiquitination. (B) MCF-10A parental and SCCA1-expressing cells were transfected with a polyhistidine-tagged Ub (His-Ub) and subsequently treated with tunicamycin (5 μg/ml) for 8h. Lysates were immunoprecipitated with a His antibody, and blotted for caspase-8 or His tag. A non-specific band (n.s.) was used to indicate equal loading. Experiments were performed with help from Dr. Ji-An Pan.

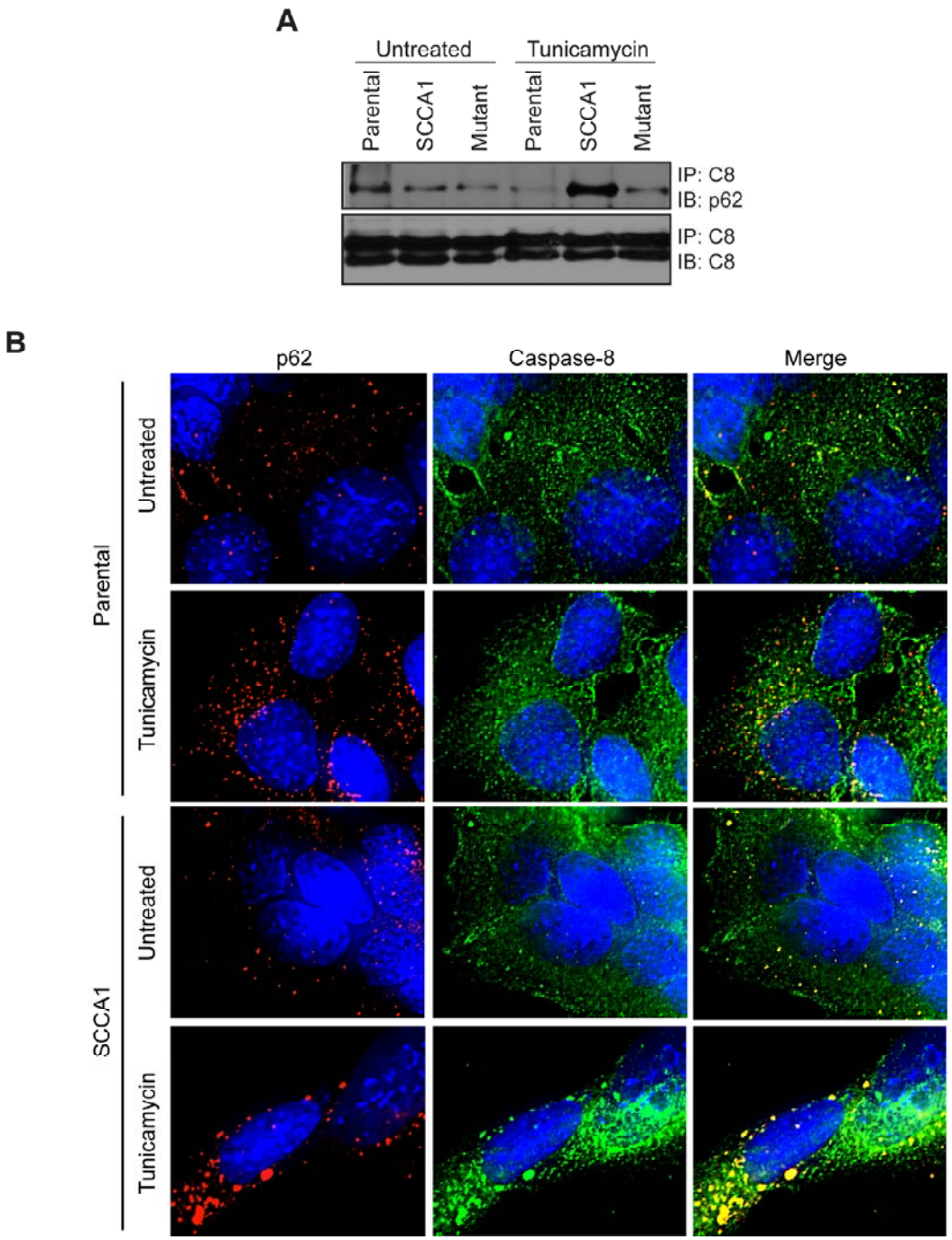


Figure 26. SCCA1 leads to increased caspase-8 interaction with SQSTM1/p62.

(A) Lysates from Hs578T parental or SCCA1-expressing cells, untreated or treated with tunicamycin (5 μg/ml) for 24 h, were immunoprecipitated with a caspase-8 antibody and blotted for p62 or caspase-8. (B) *bax*$^{-/-}$*bak*$^{-/-}$ BMK parental and SCCA1-expressing cells were left untreated or treated with tunicamycin (0.5 μg/ml) for 16 h. Immunofluorescence staining was performed using antibodies against p62 (red) or caspase-8 (green). Note increased co-localization of p62 and caspase-8 in SCCA1-expressing cells upon ER stress treatment.

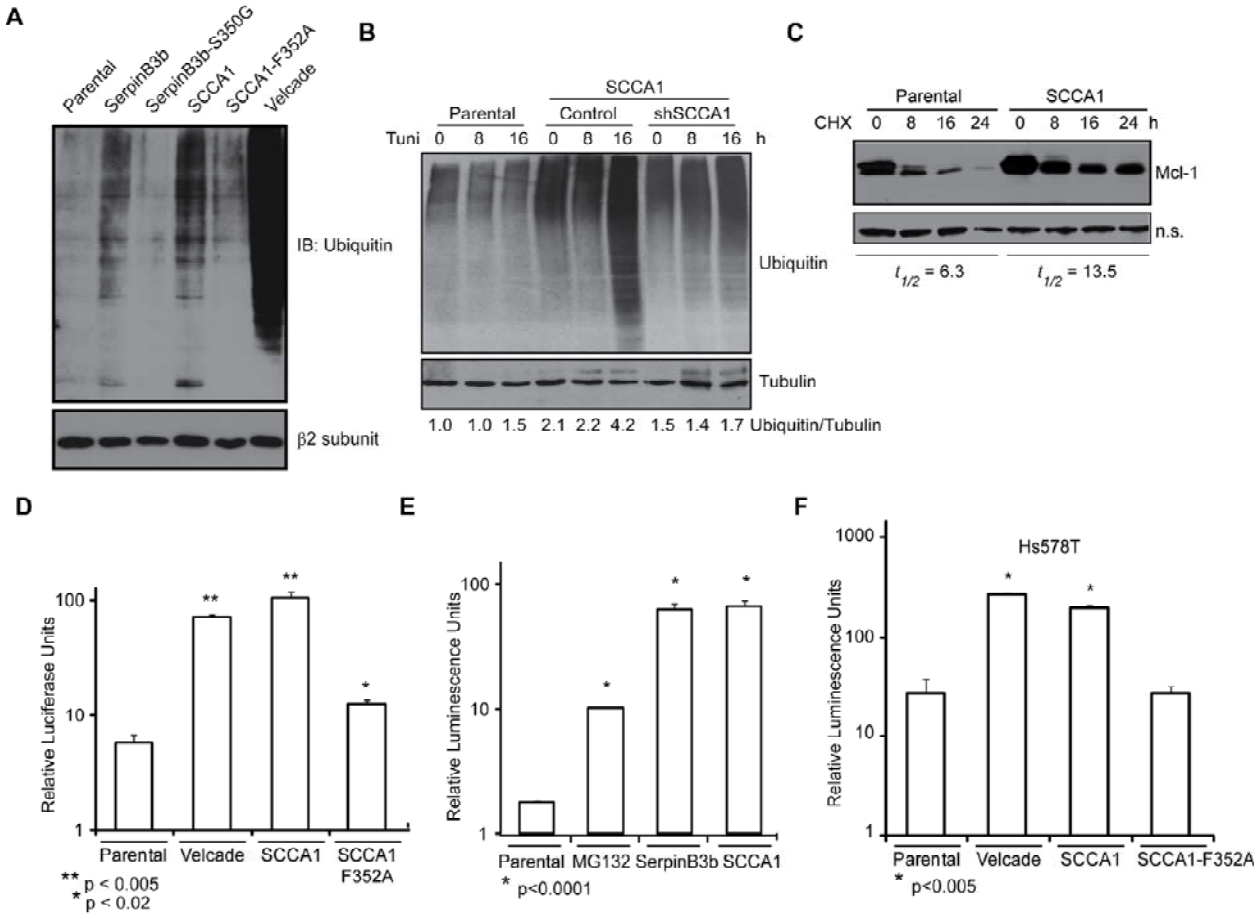


Figure 27. **SCCA1 expression represses global protein degradation by the proteasome.**

(A) Cell lysates from untreated BMK parental, Serpin wild-type, or Serpin mutant-expressing cells were examined for protein ubiquitination. Parental cells were treated with the proteasome inhibitor Velcade (2 μM) for 8 h, as a positive control. The proteasome β2 subunit was probed as a loading control. (B) Parental and SCCA1 cells expressing a shControl or shSCCA1 were treated with tunicamycin (0.5 μg/mL) for the indicated amount of time. Cell lysates were subsequently examined for protein ubiquitination. The amount of ubiquitination was determined by densitometric analysis of the intensity of ubiquitination standardizing against tubulin. (C) *bax*$^{-/-}$*bak*$^{-/-}$BMK parental and SCCA1-expressing cells were treated for the indicated amount of time with the protein translation inhibitor cycloheximide (10 μg/mL). Mcl-1, a protein degraded by the proteasome, was then probed for and the half-life ($t_{1/2}$) determined. (D-F) Parental, SCCA1, and SCCA1-F352A BMK *bax*$^{-/-}$*bak*$^{-/-}$ cells (D) or Hs578T cells (F), or SerpinB3b BMK *bax*$^{-/-}$*bak*$^{-/-}$ cells (E) were transfected with a ubiquitin-luciferase reporter construct to determine the efficiency of degradation of ubiquitinated proteins. Parental cells were treated with Velcade (2 μM) or MG132 (0.5 μM) for 8 h as a positive control. Luciferase activity in cell lysates was determined with a dual luciferase assay kit, which allows for standardizing transfection efficiencies based on Renilla luciferase activity.

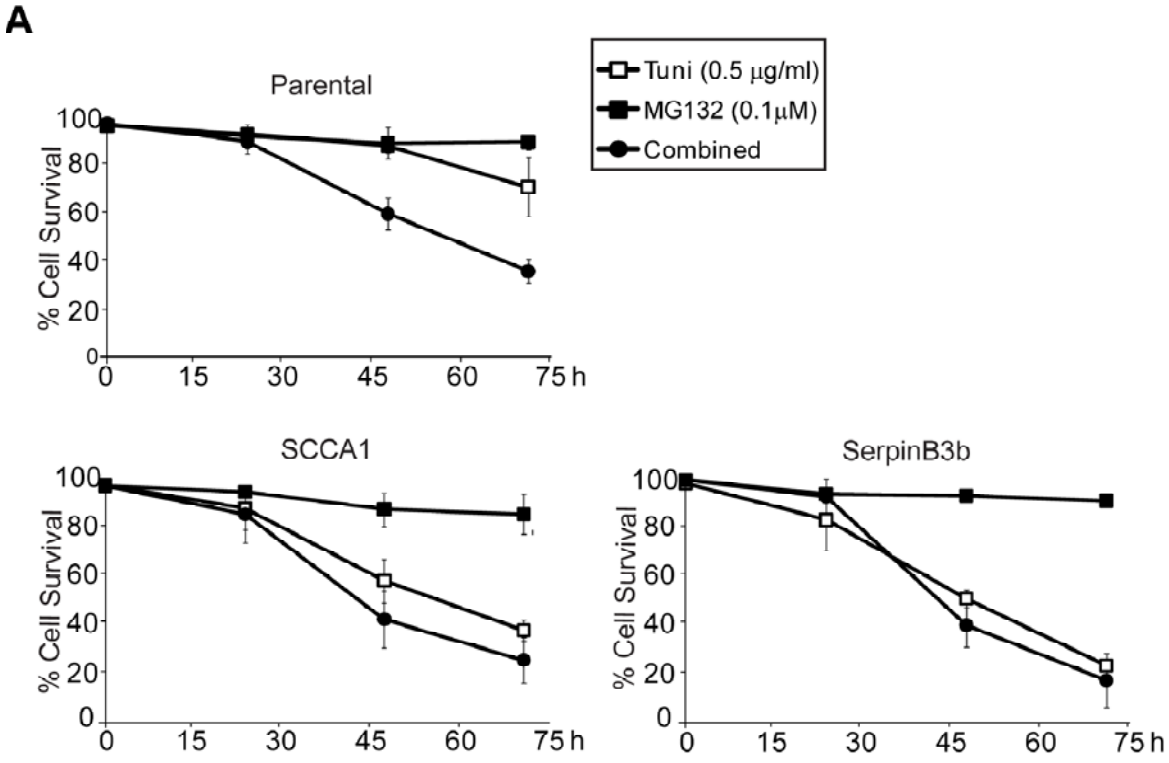


Figure 28. Inhibition of proteasome protein degradation enhances ER stress-induced cell death.

(A) Parental, SCCA1 expressing, or SERPINB3b expressing, BMK cells were treated for the indicated amount of time with either tunicamycin (0.5μg/ml) or MG132 (0.1μM), a proteasomal inhibitor, individually or simultaneously. Cell death was then measured by PI exclusion. Note that while the proteasomal inhibitor alone does not induce cell death it sensitizes cells to ER stress-induced cell death.

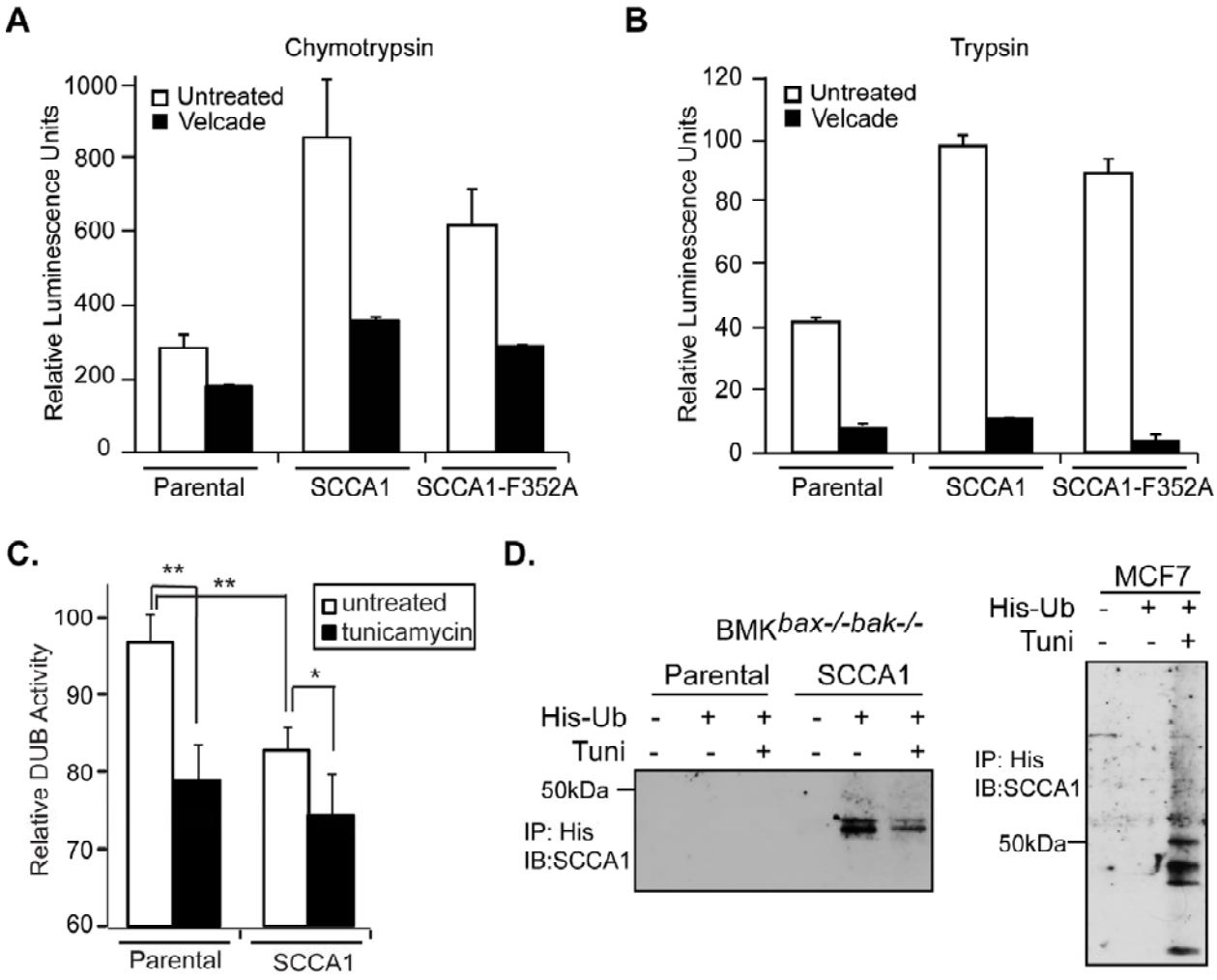


Figure 29. Proteasome Enzymatic Activity is not inhibited by SCCA1.

(A-B) Parental, SerpinB3b, and SCCA1-expressing *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells were left untreated or treated with Velcade (2 μM) for 8 h, proteasomal chymotrypsin-like (A) and trypsin-like (B) activities were assayed for using fluorogenic substrates. (C) Ubiquitin isopeptidase activity was measured in cell lysates of parental and SCCA1-expressing *bax*$^{-/-}$*bak*$^{-/-}$ BMK cells using the fluorogenic substrate ubiquitin-AMC. (D) Ubiquitination of SCCA1 was tested by transfecting indicated cells with His-tagged ubiquitin, performing an immunoprecipitation with a His antibody, then subsequently probing for SCCA1.

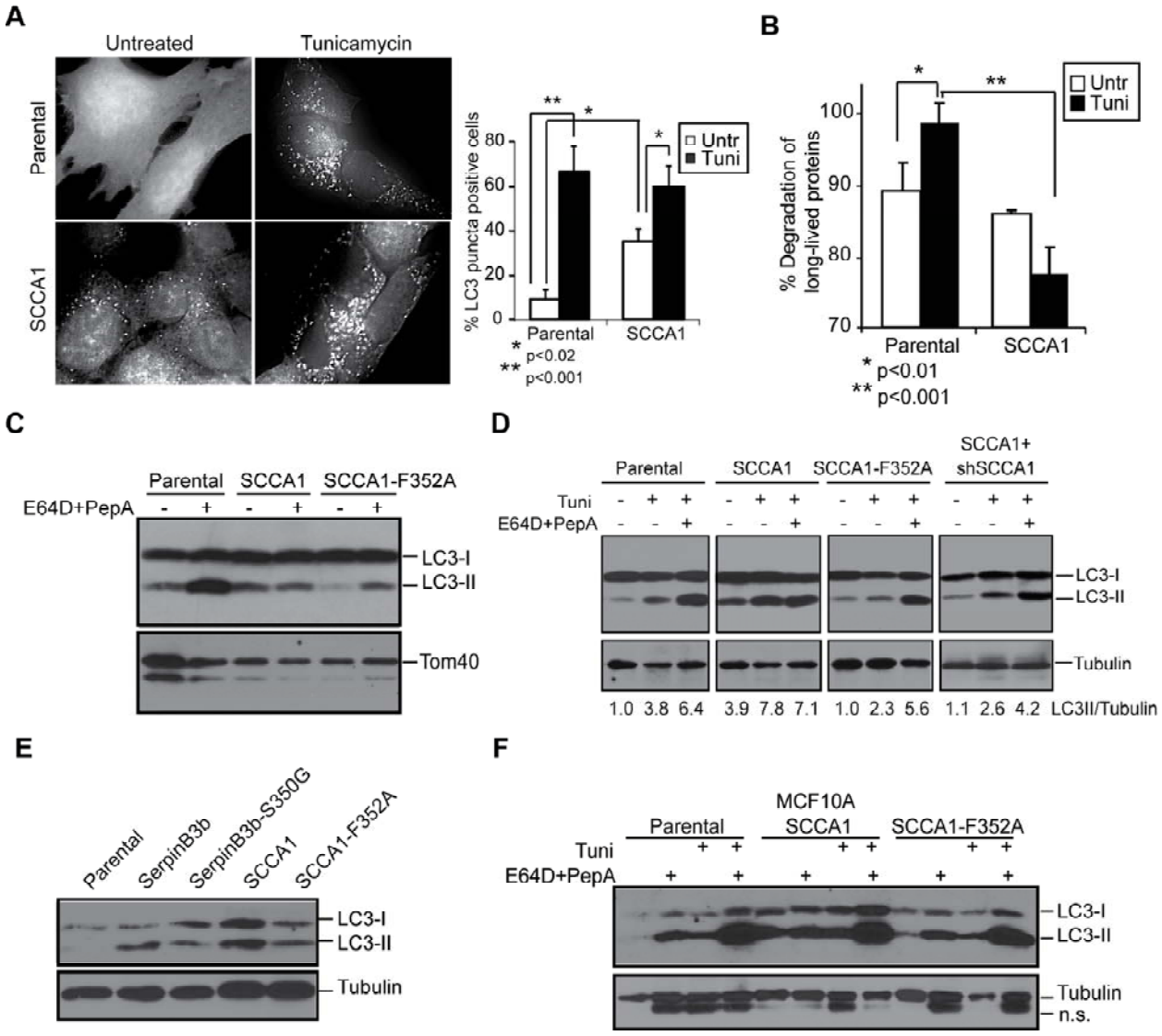


Figure 30. SCCA1 blocks lysosomal turnover.

(A) Parental or SCCA1 cells stably expressing GFP-LC3 were treated with tunicamycin for 8 h. Cells were observed under a deconvolution microscope. Percentage of cells showing punctated GFP-LC3 was determined for three independent countings of 50 cells, values represent the mean ± S.E.M. of these 3 countings. (B) BMK Parental and SCCA1 cells were labeled with [14C]-valine for 48 hr, then treated for 24 h with tunicamycin. Long-lived protein degradation was measured. (C) *bax*$^{-/-}$*bak*$^{-/-}$ BMK parental, SCCA1, or SCCA1-F352A expressing cells were left untreated or treated with E64D (10 μg/ml) and PepA (10 μg/ml) for 16 h. Cell lysates were probed for LC3. Tom40 was probed for equal loading. (D and F) Indicated BMK (D) or MCF10A (F) cells were treated with tunicamycin (0.5 μg/ml) alone or in combination with E64D (10 μg/ml) and PepA (10 μg/ml) for 16 h. Cell lysates were probed for LC3 and tubulin and the ratio of LC3II against tubulin was determined by Image J densitomerty. (E) Cell lysates from *bax*$^{-/-}$*bak*$^{-/-}$ BMK parental cells or those expressing wt or mutant serpins were probed for LC3.

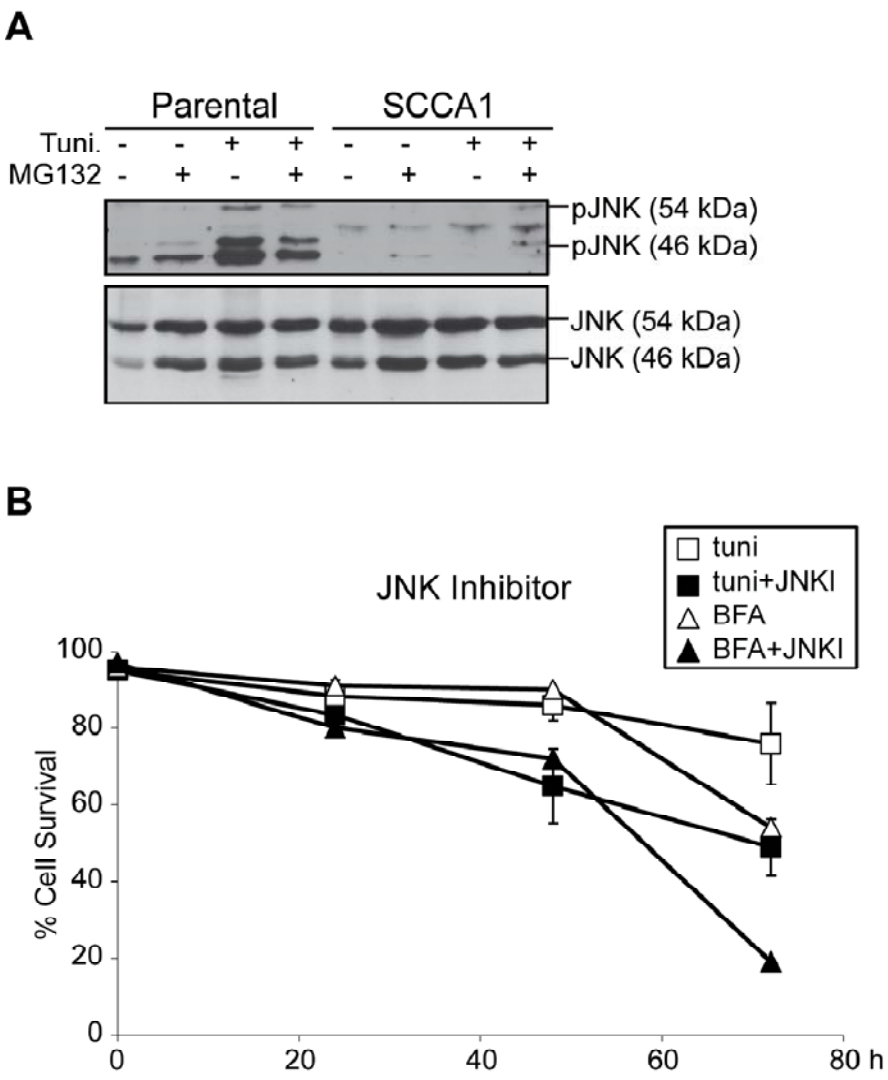
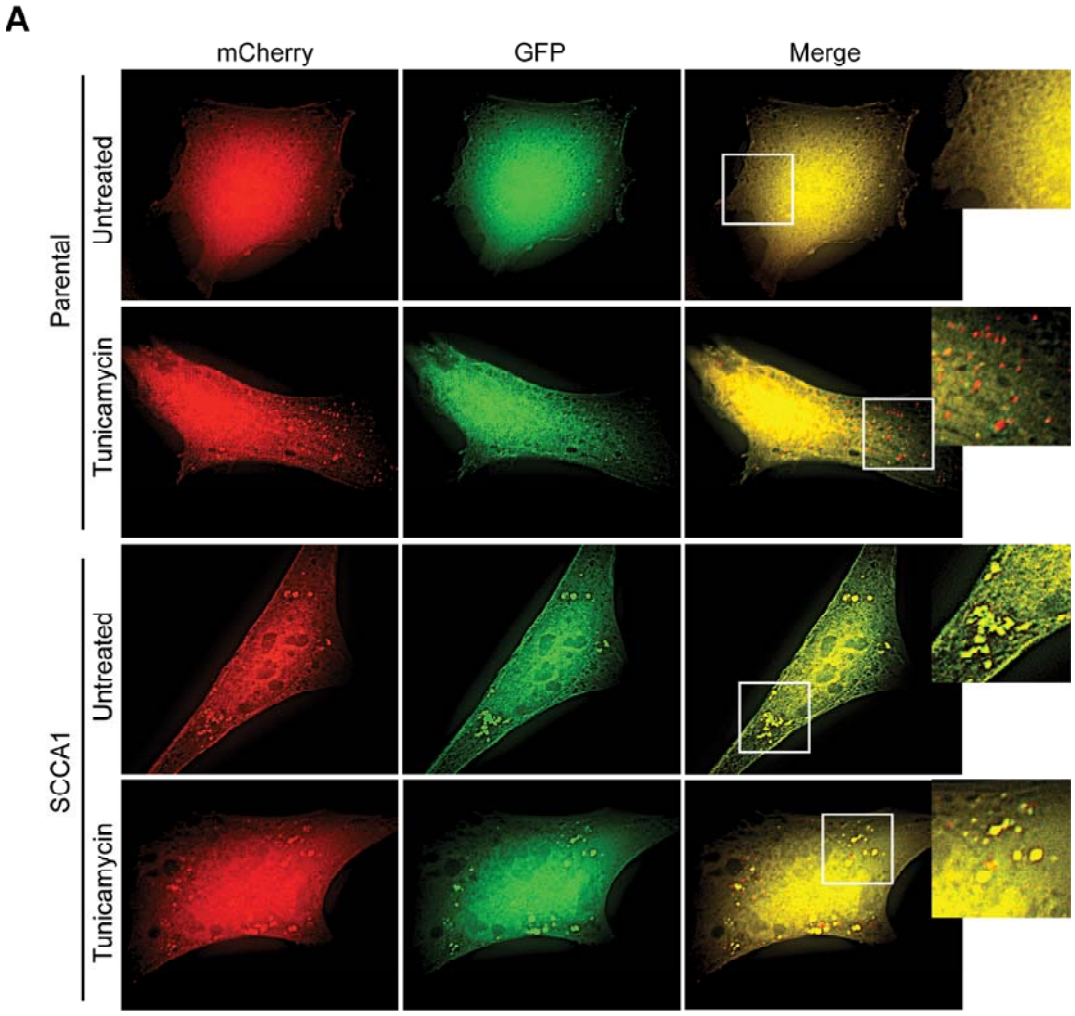


Figure 31. SCCA1 inhibition of JNK phosphorylation does not affect autophagy induction but may contribute to ER stress-induced cell death.

(A) Parental or SCCA1-expressing *bax-/-bak-/-* BMK cells were treated with tunicamycin (0.5 μg/ml) and MG132 (0.5 μM) alone or together for 16h, cell lysates were then immunoblotted for JNK phosphorylation. (B) Parental *bax-/-bak-/-* BMK cells were treated with ER stressor tunicamycin and brefeldinA alone, or incombination with a JNK inhibitor to mimic SCCA1's ability to inhibit JNK activity, cell death was then determined by propidium iodide exclusion.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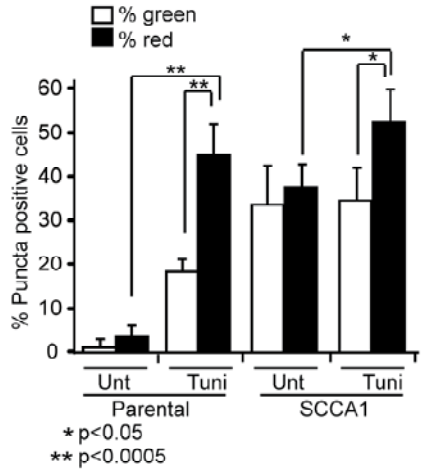
% green
% red
% Puncta positive cells
60
50
40
30
20
10
0
**
**
*
*
Unt
Tuni
Unt
Tuni
Parental
SCCA1
* p<0.05
** p<0.0005

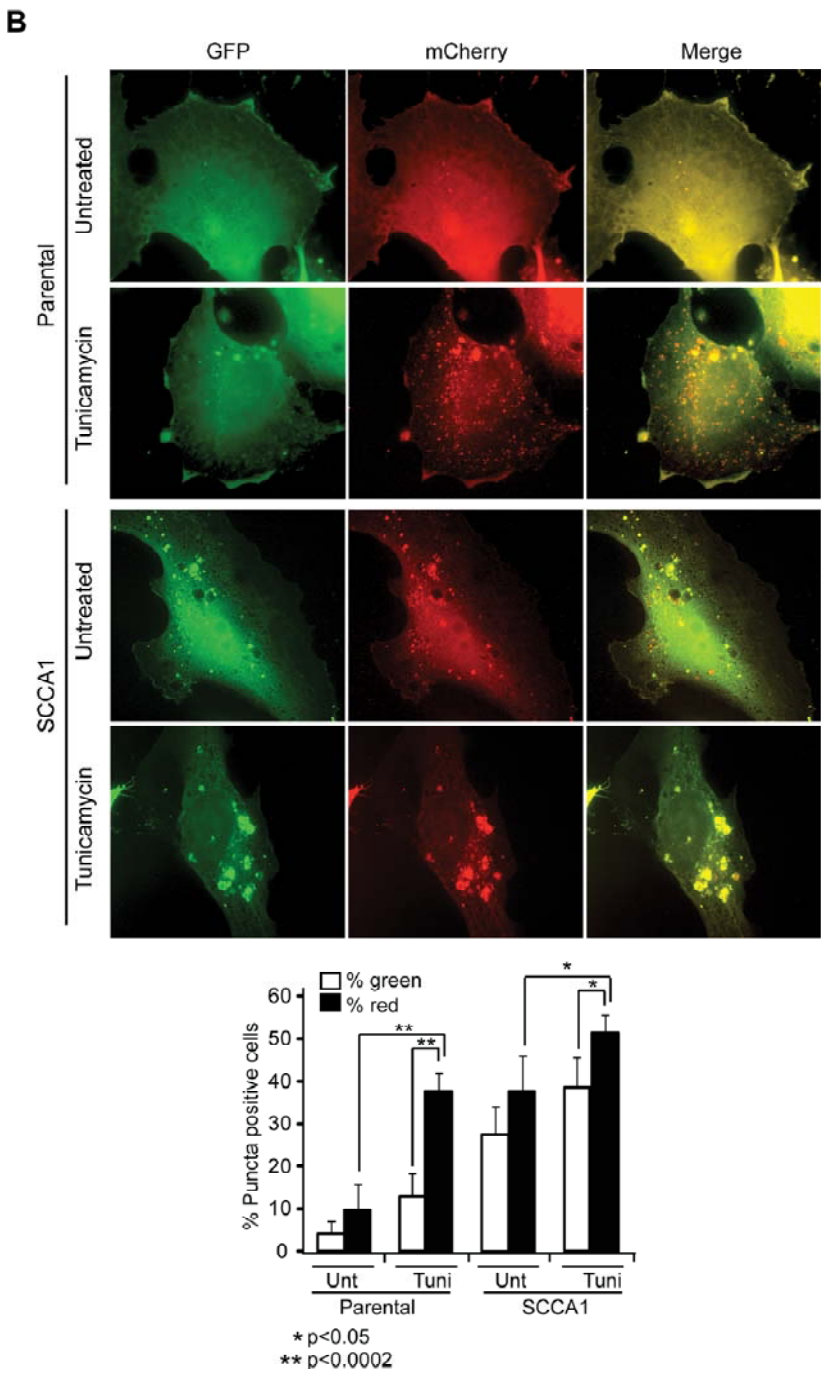

Figure 32. SCCA1 inhibits lysosomal degradation

Parental *bax*$^{-/-}$*bak*$^{-/-}$ BMK (A) or Hs578T (B) cells and those expressing SCCA1 were transfected with mCherry-EGFP-LC3, then treated with tunicamycin (0.5 μg/ml) for 16 h. EGFP can be degraded by endosomes and lysosomes, while mCherry is not, thus this construct can be used to measure lysosomal turnover. The amount of red and green puncta was quantitated and the percentage of cells showing green and red puncta was determined for three independent countings of 50 cells and the values represent the mean ± S.E.M. of these 3 countings.

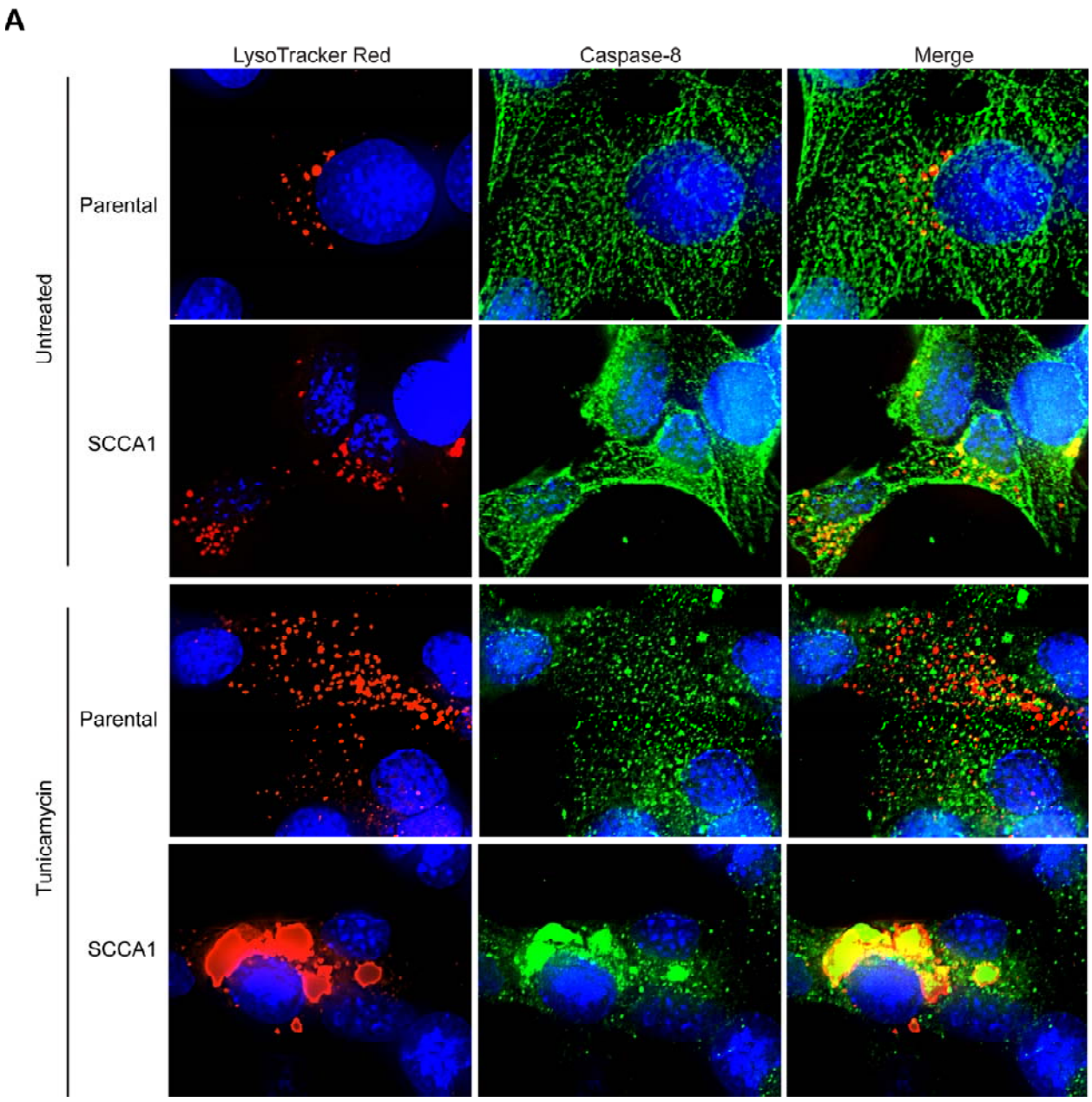

Figure 33. SCCA1 promotes caspase-8 aggregation on lysosomes.

(A) *bax*$^{-/-}$*bak*$^{-/-}$ BMK parental and SCCA1-expressing cells were left untreated or treated with tunicamycin (0.5 μg/ml) for 16 h. Immunofluorescence staining was performed using LysoTracker Red and an antibody against caspase-8 (green). Note increased co-localization of LysoTracker Red and caspase-8 in SCCA1-expressing cells upon ER stress treatment.

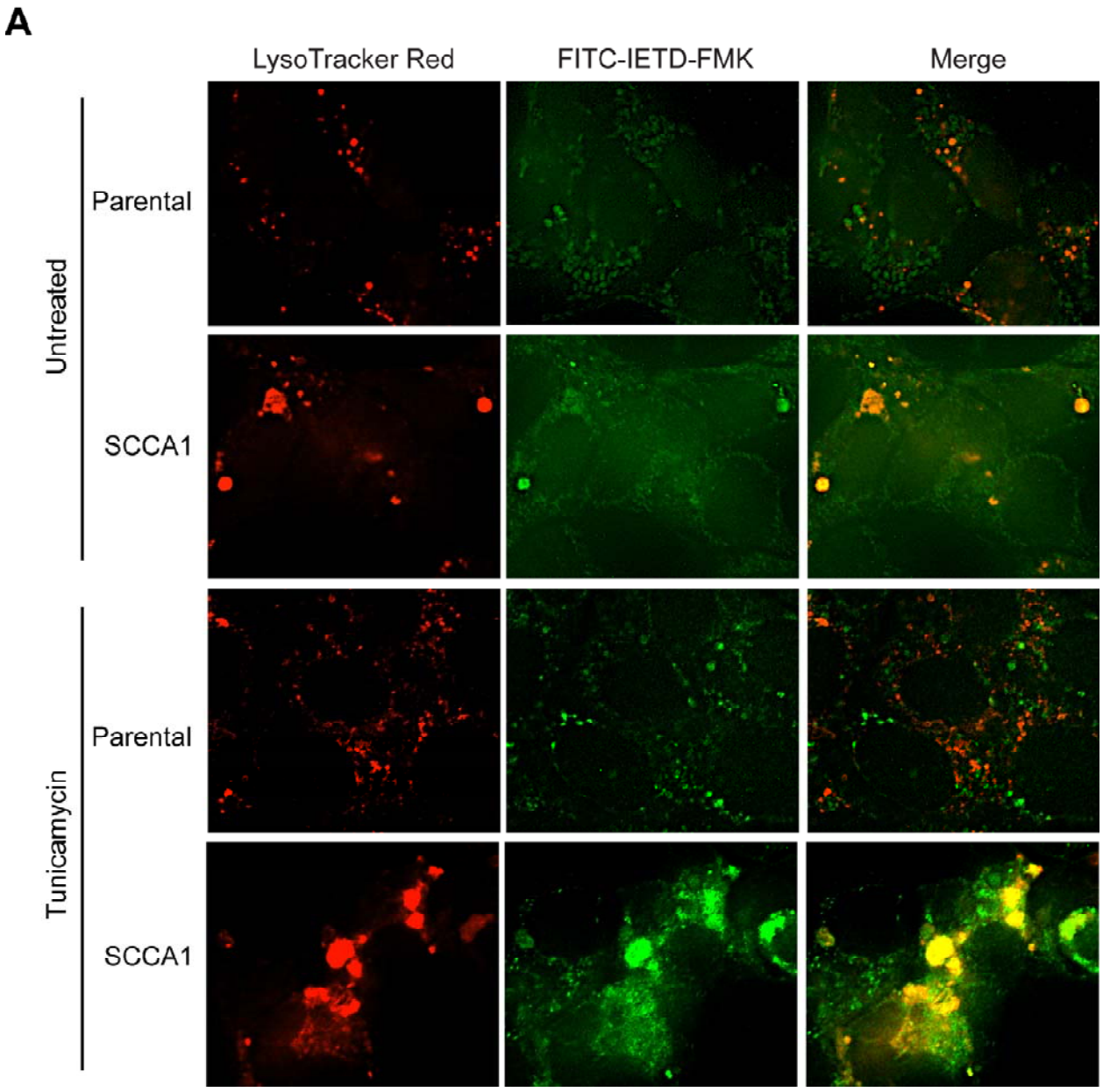

Figure 34. SCCA1 promotes caspase-8 activation on lysosomes.

(B) *bax*$^{-/-}$*bak*$^{-/-}$ BMK parental and SCCA1-expressing cells were left untreated or treated with tunicamycin (0.5 μg/ml) for 16 h. The substrate specific for activated caspase-8, FITC-IETD-FMK, was used to verify that the caspase-8 colocalizing with lysosomes was indeed active.

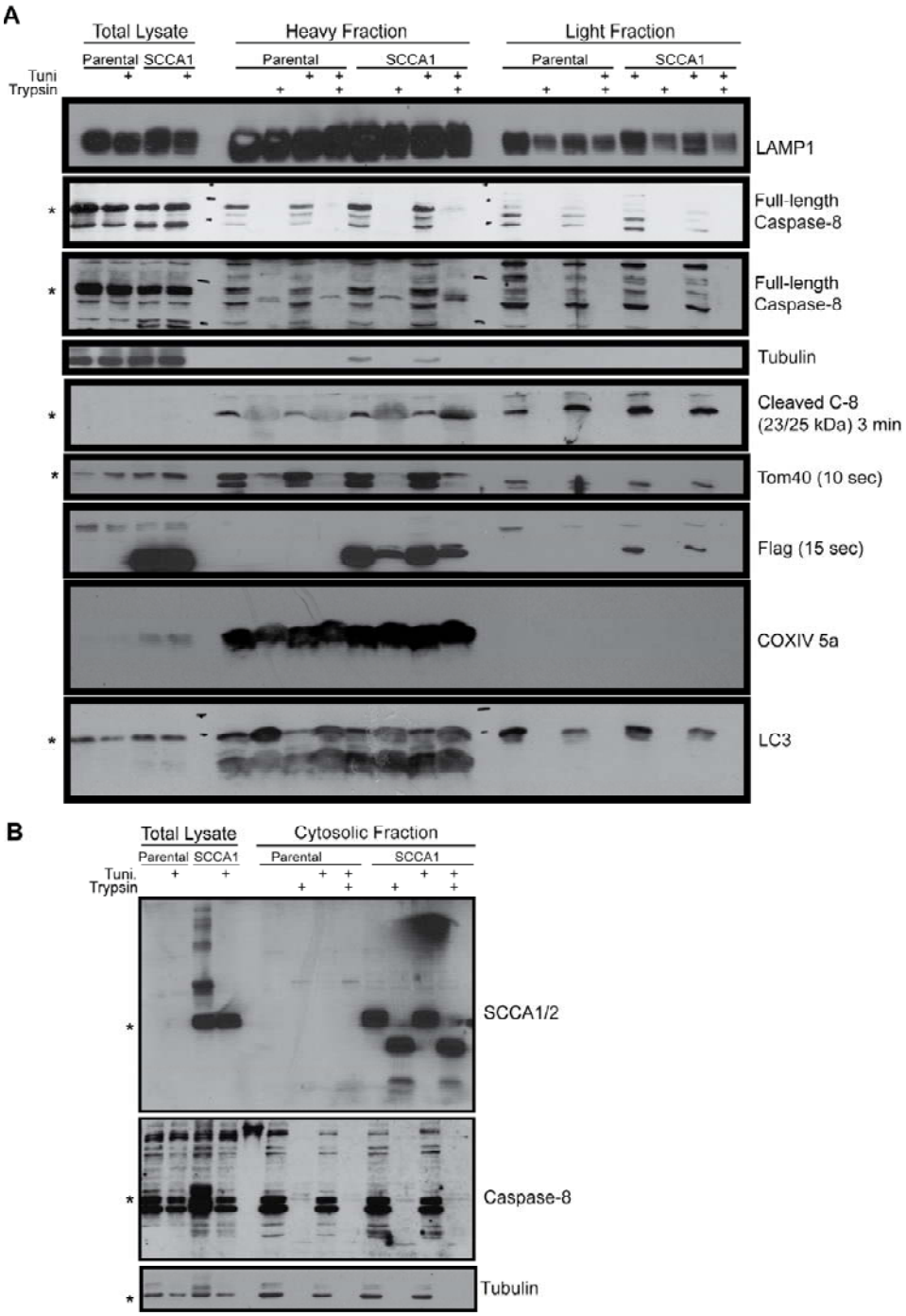

Figure 35. SCCA1 is not only cytosolic but also localizes to lysosomes. (A-B) Trypsin digestion was performed, to distinquish between luminal and cytosolic proteins, followed by subcellular fractionation on parental and SCCA1-expressing cells that were left untreated or treated for 16 h with tunicamycin. The heavy fraction contains mitochondria, ER, and lysosomes, while the light fraction consists exclusively of lysosomes. LAMP1 was used as a marker for lysosomal presence, while COXIV was used to identify mitochondria. (B) cytosolic fractions from subcellular fractionation were probed for SCCA1.

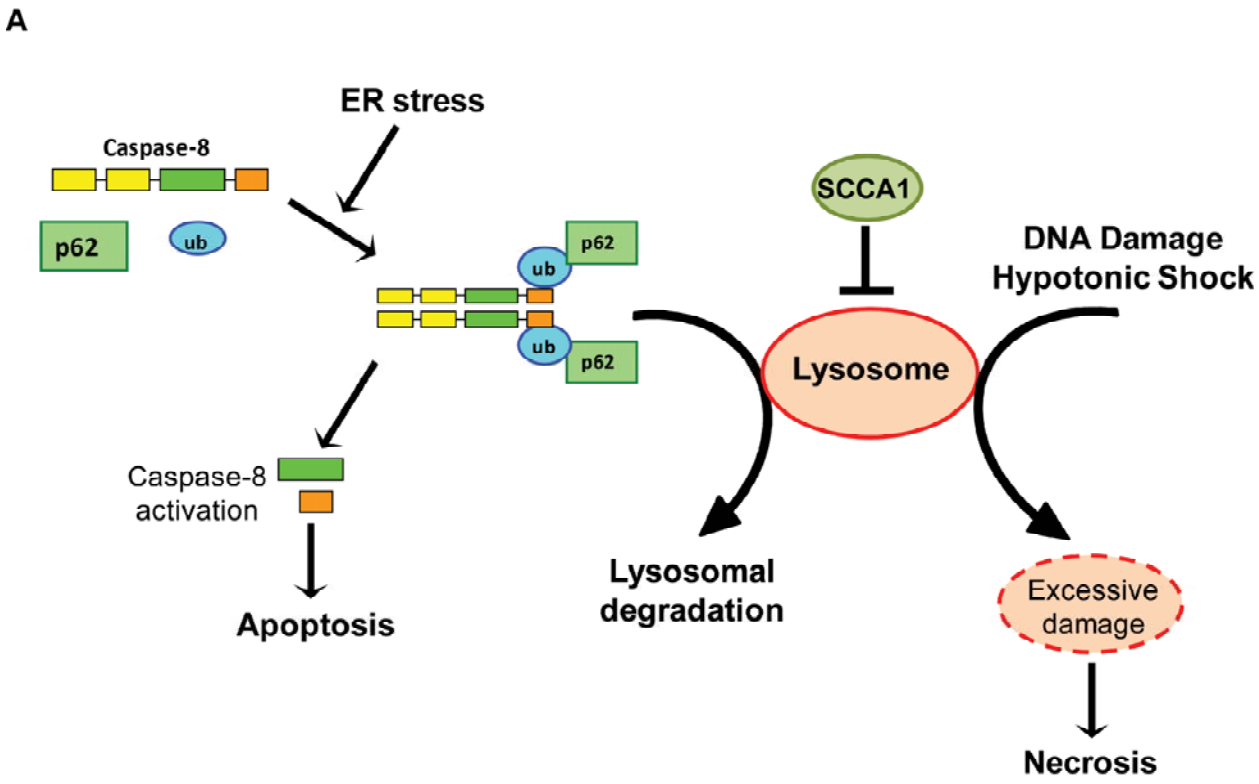

Figure 36. SCCA1 protects cells against lysosomal injury but enhances apoptosis in response to ER stress.

(A) On one hand, SCCA1 blocks necrosis induced by lysosomal injury in response to DNA alkylating damage and hypotonic shock. On the other hand, SCCA1 inhibits lysosomal protein degradation. Hence, in response to ER stress that leads to the accumulation of unfolded proteins, SCCA1 prevents lysosomal clearance of caspase-8/p62 aggregates, resulting in the subsequent activation of caspase-8 and apoptosis.

VI. Materials and Methods

1. Cell Lines, culture, and transfection

Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ murine embryonic fibroblasts (MEFs) were generated as previously described **(156)**. *atg5*$^{-/-}$ and *atg5*$^{+/+}$ cells were a kind gift from Dr. Noboru Mizushima at Tokyo Medical and Dental University. BMK, Hs578T, MEF, and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. MCF7 cells were cultured in RPMI1640 supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. MCF10A cells were cultured in DMEM/F12 supplemented with 5% donor horse serum (Invitrogen), 20 ng/ml epidermal growth factor (EGF) (Sigma), 10 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma), 100 units/ml penicillin and 100 μg/ml streptomycin. Transfection of BMK and Hs578T cells was performed using Lipofectamine 2000 (Invitrogen). Transfection of 293T and Phoenix cells was performed by calcium phosphate precipitation method.

2. Plasmids

The retroviral LPC-LC3-GFP was generated using the EGFP-tagged LC3 excised from pEGFP-C1-LC3 expression vector, a kind gift from Dr. Tamotsu Yoshimori at the National Institute of Genetics, Japan. 293T packaging cells were transfected with LPC-LC3-GFP using the calcium phosphate precipitation method. Twenty-four to seventy-two hours after transfection viral supernatant was collected, filtered through a 0.22-μm filter and subsequently used to infect MEFs. Cells were then selected for by treating with puromycin. Bcl-xL overexpression in Bax/Bak wild-type, *atg5*$^{-/-}$, and *atg5*$^{+/+}$ MEFs was performed in a similar manner using a retroviral plasmid expressing Bcl-xL. pcDNA3-DsRed-Bak, pcDNA3-DsRed-Bak-cb5, and pcDNA3-DsRed-Bak-ΔC (28) were transfected into 293T cells using Lipofectamine2000 (Invitrogen). Flag-SCCA1 plasmid was constructed by performing reverse transcription polymerase reaction (RT-PCR) from isolated RNA from MDA-MB-468 cells. The primers utilized in the PCR reaction contained a Flag tag at the N-terminus as well as a BamHI restriction site and a C-terminal XhoI restriction site (Forward primer, 5'-CGGGATCCATGGACTACAAGGACGACGATGACAAGACCATGAATTCACTCAGTGAA GCC -3'; Reverse primer, 5'- CCCTCGAGCATCTACGGGGATGAGAATCTGCCA -3'). The PCR product was ligated into pCR2.1-TOPO (Invitrogen). Flag-SCCA1 was then subcloned into the retroviral vector LPC between BamHI and XhoI sites. Flag-SerpinB3b was constructed in a similar manner using total RNA isolated from mouse embryonic fibroblasts (MEFs). The primers utilized contained an N-terminal Flag tag and BamHI restriction site as well as a C-terminal XhoI restriction site (Forward primer, 5'-CGGGATCCATGGACTACAAGGACGACGATGACAAGACCATGATTCGTTTTCATGCA GCT -3'; Reverse primer, 5'- CCCTCGAGGAAACATGTTTCCAGGCCTCAATT -3'). Site directed mutagenesis was utilized to generate point mutants for SCCA1 and SerpinB3b. All serpin constructs were expressed in cell lines using the technique mentioned above for LPC-LC3-GFP. The plasmid Ub-Fl utilized to monitor proteasomal protein degradation was a gift from Dr. David Piwnica-Worms **(87)**. mCherry-GFP-LC3 is a gift from Dr. Terje Johanson **(107)**. Lipofectamine reagent was used to transfect cells with Ub-Fl and mCherry-GFP-LC3 constructs.

3. Knockdown by shRNA

For the generation of Atg5 short hairpin (shAtg5), a PCR reaction was performed using a forward oligonucleotide with the sequence from an RNA polymerase III-specific U6 promoter (CAGTGGAAAGACGCGCAGGCA) in combination with reverse primer (AAAAAACAACTTGCTTTACTCTCTATCACCTCGAGCTGATAGAGAGTAAAGCAAGT TGGGTGTTTCGTCCTTTCCACAA) that contains a hairpin for *atg5* **(139)**, in addition to a sequence from the U6 promoter. This construct was subsequently cloned into a pBabe-puro-based retroviral vector. The retroviral vector was transfected into 293T packaging cells by calcium phosphate transfection. Virus-containing supernatant was collected 24-72 hours post transfection, and was used to infect MEFs. Stable shRNA knockdown of Atg5 was verified by immunoblotting. For knockdown of caspase 8, a lentiviral shRNA plasmid was purchased from Sigma (NM_001228.x-377s1c1). For knockdown of CHOP, lentiviral shRNA plasmids were purchased from Sigma; two constructs were observed to knockdown CHOP (TRCN0000103708 and TRCN0000103709). For knockdown of SCCA1, a lentiviral shRNA plasmid was purchased from Sigma (NM_006919.1-1113s1c1). For construction of the tetracyclin-inducible shSCCA1, the shRNA sequence from the lentiviral shRNA plasmid designed by sigma was ligated into the pLKO-Tet-On plasmid **(158)**. The lentiviral vectors were transfected into 293T packaging cells by lipofectamine transfection. Virus-containing supernatant was collected 24-72 hours post transfection, and was used to infect cells.

4. Retroviral and lentiviral infection

Retrovirus infection was performed as previously described **(152)**. 293T cells (used for murine cell infections) or Phoenix cells (for human cell infections) were plated into 6-well plates at a density of $4x10^5$. The following day cells were transfected with LPC-retroviral constructs, additionally helper virus was added to murine transfections. 24 h post transfection viral supernatant was collected from 293T or Phoenix cells, 10 μg/ml of polybrene (sigma) was added and subsequently the supernatant was filtered through a 0.45μM filter. The infection was performed three rounds at an 8 h interval. Three days after the initial infection, cells were selected with puromycin (InvivoGen). For lentiviral infections 293T cells were transfected with lentiviral constructs and packaging plasmids, and the infection performed similarly to retroviral infections. For expression of the lentiviral tetracycline-inducible shSCCA1, doxycycline (1 μg/ml) was added for seven days prior to treatment.

5. Reagents and antibodies

DMEM, valine-free DMEM, F-12, cathepsin L substrate (R6502) and LysoTracker Red were purchased from Invitrogen. Cholera toxin, human EGF, hydrocortisone, insulin, MG132, cycloheximide, MNNG, and pepstatin A (used at 10μg/ml) were purchased from Sigma, tunicamycin, E64D (10 μg/ml), caspase-8 detection Kit (FITC-IETD-FMK) and thapsigargin from Calbiochem, ^{14}C-valine from Amersham. The Dual Luciferase Reporter Assay System was purchased from Promega. Boc-Leu-Arg-Arg-AMC (Boc-LRR-AMC), for measuring proteasome trypsin-like activity and z-Gly-Gly-Leu-AMC (Z-GGL-AMC), for measuring chymotryptic-like activity were purchased from BioMol. Propidium iodide was purchased from

Molecular Probes. Mafosfamide (4-sulfoethylthio-cyclophosphamide L-lysine) was a gift from Dr. Myriam Malet-Martino at Université Paul Sabatier, France.

The following antibodies were used: HMGB1 (BD PharMingen), CHOP (Santa Cruz), Atg5 (ABGENT), and Bcl-xL (clone 13.6). SCCA1/2 (FL-390) (Santa Cruz, 1:1000), □-tubulin (Sigma, 1:2,000), Flag (M2, Sigma, 1:2,000), Bip/GRP78 (BD Transduction Laboratories, 1:2,000), CHOP/GADD153 (B3, Santa Cruz, 1:500), caspase-8 (R&D systems, 1:2,000 for WB; Alexis Biochemical, 1:100 for IF; Millipore for IP, 1 μg/mL), cytochrome C (Pharmingen International 65971A, 1:500 for IF; 65981A, 1:1,000 for WB), cleaved-caspase-3 (Cell Signaling, 1:1,000), Tom40 (H-300, Santa Cruz, 1:1,000), PARP (Cell Signaling, 1:2,000), p62/SQSTM1 (Abnova, 1:100,000 for WB and 1:1,000 for IF), LC3 (Cell Signaling Technology, 1:500; MBL, 1:1,000), ubiquitin (FK2H, Biomol, 1:10,000), Mcl-1 (Rockland Inc., 1:1,000), caspase 2 (MAB3507 Millipore, 1:500), lamin B1 (M-20, Santa Cruz, 1:1,000), peroxidase-conjugated secondary antibodies (Rockland, 1:2,000), rhodamine-conjugated secondary antibodies (Rockland, 1:500), fluorescein conjugated secondary antibodies (Rockland, 1:250).

6. Immunoblotting

Cells were lysed in RIPA buffer (1% sodium deoxycholine, 0.1% SDS, 1% Triton X-100, 10 mM Tris at pH 8.0, 0.14 M NaCl) with protease inhibitor cocktail (Roche). Thirty micrograms of protein was resolved on SDS-PAGE and transferred to nitrocellulose membranes. Densitometric analysis of the immunoblotting bands was performed using Image J.

7. Electron microscopy

Samples used for transmission electron microscopy (TEM) were processed using standard techniques. Briefly samples were collected and fixed with 2.0% paraformaldehyde/2.5% EM grade glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 37°C. After fixation, samples were placed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4), dehydrated in a graded series of ethyl alcohol and embedded in Durcupan resin. Ultrathin sections of 80 nm were cut with a Reichert-Jung UltracutE ultramicrotome and placed on formvar coated slot copper grids. Sections were then counterstained with uranyl acetate and lead citrate and viewed with a FEI Tecnai12 BioTwinG^2 electron microscope. Digital images were acquired with an AMT XR-60 CCD Digital Camera System.

8. Measurement of cell death

For analysis of cell death with MNNG treatment, cells were treated with MNNG for 30 min. Cells were washed and fed with fresh medium with no MNNG, and cultured for indicated periods of time. For hypotonic shock, mafosfamide, tunicamycin, and thapsigargin treatment the agents remained in the medium. For assessment of cell death, cells were harvested and resuspended in DMEM with propidium iodide (PI, 1μg/ml). Cell viability was determined by PI exclusion via flow cytometry using a FACs caliber. Alternatively trypan blue staining was also used as a measure of cell death. After treating cells for the indicated times cells were collected, harvested and stained with trypan blue (0.2%), and counted under a phase contrast light

microscope. Pictures were taken with a Zeiss AxioPlan 2 microscope using an x32 objective. Images were captured using a Spot camera (Diagnostic Instruments).

9. Observation and quantification of the LC3-GFP puncta formation

Cells were fixed in 4% paraformaldehyde for 10 min then washed with phosphate buffered saline (PBS). LC3-GFP transfectants were imaged and pictures taken with the Zeiss Axiovert Fluorescence microscope, using the x63 oil objective. To quantify the percent of autophagic cells, 100-200 cells were randomly selected and counted for autophagy induction, those containing more than 8 green puncta were considered autophagic and expressed as a percent of the total cells counted.

10. LysoTracker Red and cathepsin L activity

For measurement of LysoTracker red staining and cathepsin L activity, $5x10^4$ cells were plated in 12-well plates. Upon treatment, cell culture media containing floating cells were collected combined with the remaining adherent cells that were harvested by trypsination. Cells were pelleted and for LysoTracker red resuspended in 1ml of DMEM containing 50 nM LysoTracker red, for cathepsin L activity resuspended in 1ml of HBS containing 2mM EDTA and 10μM cathepsin L substrate. After 30 minute incubation cells were analyzed using a FACS caliber using the FL-3 channel for LysoTracker red and the FL-2 channel for cathepsin activity.

11. Subcellular fractionation

Subcellular fractionation was performed as previously described with modification **(175)**. Breifly, cells were collected via trypsinization, pelleted, and resuspended in hypotonic Buffer A (250 mM sucrose, 20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1× protease inhibitor cocktail) on ice for 30 min. Cells were disrupted by passing through 26-gauge needles 30 times, and then 30-gauge needles 20 times. Cell lysates were centrifuged at 750 *g* for 10 min at 4°C to get rid of unlysed cells and nuclei. The supernatant was centrifuged at 18,000 g for 1 h at 4°C. The supernatant was saved as the cytosolic and the pellet as the mitochondrial fractions. The mitochondrial fraction was lysed in RIPA buffer for Western blotting.

12. Immunofluorescence

Cells were plated onto glass coverslips in 24 well plates prior to treatment. After treatment cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. For LysoTracker red staining and caspase 8 activity assay, prior to fixing cells 0.1 μM LysoTracker red or 1 μl of FITC-IETD-FMK was added for 1 h, subsequently cells were washed three times with PBS. After fixation, cells were permeabilized in 0.1% Triton in PBS for 3 min, then blocked in 5% goat serum in PBS followed by incubation with primary antibody in blocking solution for 2 h at room temperature. Cells were then incubated with fluorophore-conjugated secondary antibodies in blocking solution for 1 h. 1μg/ml of DAPI was added to visualize the nucleus. Cells were mounted with IMMU-MOUNT (Thermo Scientific). Slides were observed and imaged using a Zeiss Axiovert 200M deconvolution fluorescence microscope, using the 63x oil objective.

13. Measurement of long-lived protein degradation

BMK cells ($5x10^4$) were plated into 6 well plates. After overnight recovery cells were labeled with 1.0 μCi/ml ^{14}C-L-valine in valine-free medium. 48 h post labeling, cells were washed three times with PBS and incubated in complete medium containing an excess of 10 mM unlabeled L-valine for 16 h to chase out short-lived proteins. Cells were then washed three times with PBS and cultured either in complete medium alone or in medium containing tunicamycin (0.5 μg/ml), both containing 10 mM unlabeled L-valine. The supernatant was removed 24 h later and precipitated with ice cold trichloroacetic acid (TCA) at a final concentration of 10%. Cells were washed 3 times with PBS and lysed in 0.05% SDS, then subsequently precipitated with TCA at a final concentration of 10%. TCA insoluble pellets were dissolved in 0.2 N NaOH. Radioactivity of both TCA soluble and insoluble samples was measured by liquid scintillation counter. The degradation of long-lived proteins was calculated by the radioactivity in TCA-soluble supernatant normalized against the total ^{14}C-radioactivity present in supernatants and cell pellets.

14. Measurement of proteasome activity

For measuring poteasome-mediated protein degradation BMK cells ($2.5x10^4$) or Hs578T cells ($5x10^3$) were plated into 24 well plates. After overnight recovery cells were transiently transfected, using Lipofectamine 2000, with a renilla control plasmid and the ubiquitin-luciferase bioluminescence imaging reporter (Ub-FL) **(87)**. After 5 h the transfection medium was changed to regular medium and 16 h later cells were left untreated or treated with the proteasome inhibitor Velcade (2 μM) or MG132 (0.5 μM) for 8 h. Cell lysates were made following the manufacturer's instructions for passive lysis (Dual-Luciferase Reporter Assay System, Promega), and luminescence measured in a 96 well white plate.

For measuring chymotrypsin and trypsin-like activity of the 26S proteasome, BMK cells ($5x10^6$) were plated into 10 cm plates and allowed to recover overnight. Cells were left untreated or treated with the proteasome inhibitor Velcade (2μM) for 8 h, subsequently media was removed from the cells and 1 ml of proteasome extraction/lysis buffer (10 mM Tris-HCl, pH 7.8; 5 mM ATP; 0.5 mM dithiothreitol; 5 mM $MgCl_2$) was added directly to 10 cm plates. The cell lysate was collected, incubated on ice for 15 min, sonicated for 15 sec, and centrifuged at 400 x g for 10 min at 4°C. 50 μg of the supernatant was incubated with 50 mM EDTA and 50 μM of fluorogenic substrate (Z-Gly-Gly-Leu-AMC, for chymotrypsin-like activity and Boc-Leu-Arg-Arg-AMC, for trypsin-like activity) and brought to a volume of 200 μl with the proteasome extraction/lysis buffer. The reaction was added to a 96 well black plate that was incubated at 37°C for 30 min and fluorescence measured at an excitation of 395 nm and emission of 460 nm, using a Spectra Max M5 plate reader (Molecular Devices). For measurement of ubiquitin isopeptidase activity (deubiquitinase activity) the fluorogenic substrate Ub-AMC was utilized and assays were performed as detailed in Berdtsson et al **(12)**. Briefly cells were left untreated or treated with tunicamycin (0.5 mg/ml) for 16 h, then lysates were made using an isopeptidase lysis buffer (25mM Hepes (pH 7.0), 5mM EDTA, 0.1% CHAPS, 5mM ATP, 10mM DTT), protein concentrations were standardized using BCA protein quantification and samples were adjusted to 25mg/ml. 1mM Ub-AMC was added to each sample and fluorescence was

quantitated using a 96 well white/opaque plate and an excitation of 380nm and emission of 460nm, using a Spectra Max M5 plate reader (Molecular Devices).

15. Measurement of caspase-8 activity

BMK cells (3 x 10^5) were plated ino 6 well plates and after overnight recovery were left untreated or treated for 24 h with tunicamycin. Floating and adherent cells were collected via trypsinization and centrifuged at 3,000 rpm for 5 min, cells were then resuspended in 1 ml of medium and 300 µl was subsequently removed and 1 µl of FITC-IETD-FMK (caspase-8 detection kit, Calbiochem) added. After 1 h incubation at 37°C cells were centrifuged at 3,000 rpm for 5 min. Supernatant was removed and cells washed twice with 0.5 ml of supplied wash buffer. Analysis was performed by flow cytometry.

16. Venus assay

Bimolecular fluorescence complementation (BiFC) constructs were made such that DNA sequences encoding amino acid residues 1–173 of mVenus (monomeric Venus Fluorescent Protein) **(125)** or DNA sequences encoding amino acid residues 155-239 of mVenus, were fused to the C-terminus of caspase-8. A linker GGSGSGSS was inserted between the Venus tag and caspase-8. Venus and linker sequences were provided kindly by Dr. Michael A. Frohman (Stony Brook University). This BiFC pair of caspase-8 constructs was subsequently transfected into BMK cells with Lipofectamine 2000. 24 h post transfection, cells ($1x10^5$) were plated into 6 well plates. After overnight recovery cells were treated for 16 h with tunicamycin, harvested, and the percentage of cells that fluoresced green was determined by flow cytometry.

17. Size-exclusion chromatography

BMK cells ($5x10^6$) were plated into six-10 cm plates. After overnight recovery, three plates were treated for 16 h with tunicamycin and three were left untreated. Cells were then harvested and lysed in IP lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1% triton X-100, 10 mM NaF, 100 µM orthovanadate, 200 µM PMSF) supplemented with protease inhibitor cocktail (Biosciences). Cell lysates were cleared by centrifugation at 4°C at top speed and filtered through 0.45 µm filters. 3 mg of the cleared lysate was loaded onto a superdex 200 10/300 GL column equilibrated with washing buffer (30 mM Tris, pH 7.5, 150 mM NaCl), subsequently eluted with washing buffer at a flow speed of 0.5 ml/min, and collected in 0.5 ml volumes, with the first sample being collected at 7.5 min.

18. Caspase-8 activity assay

30 µl of the eluted fractions from size exclusion chromatography were assayed for caspase 8 activity using a Caspase-Glo 8 assay kit (Promega), which uses a luminogenic caspase-8 substrate FITC-IETD-fmk. The luciferase activity values were read using a luminescence plate reader (Spectramax, Molecular Devices), according to manufacturer's instruction.

19. His-Ub assay for mammalian cells

3×10^6 cells were plated into three 10 cm culture dishes. After overnight recovery, the cells were transfected using Lipofectamine 2000, with 2 μg pMT107 plasmid, which expresses polyhistidine-tagged Ub (His-Ub) (a kind gift from Prof. Erich R. Mackow, Stony Brook University). 24 h post transfection all cells were harvested, mixed, and replated. After overnight recovery cells were treated with tunicamycin (5 μg/ml). After treatment, the cells were collected, washed twice with phosphate-buffered saline (PBS) and resuspended in 1 ml of Buffer A (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole, pH 8.0). After a 10 s sonication, each sample was incubated with 50 μl Ni-NTA-agarose (Qiagen) equilibrated with Buffer A and incubated for 3 h at room temperature with agitation. The agarose was precipitated by centrifugation and washed twice with Buffer B (10 mM Tris-Cl, pH 8.0, 8 M urea, 0.1 M NaH_2PO_4) and three times with 1:4 diluted Buffer B. The precipitates were resuspended in 100 μl of 2X Laemmli loading dye (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl pH 6.8) with 200 mM imidazole, boiled at 95°C for 10 min and subjected to western blotting.

20. Co-immunoprecipitation

HS578T and HS578T-SCCA cells (3×10^6) were plated into four 10 cm dishes, two for untreated and two for treated. After overnight recovery cells were treated for the indicated amount of time, harvested via trypsinization, pelleted, washed twice with PBS and lysed for 30 min in IP lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1% triton X-100, 10 mM NaF, 100 μM orthovanadate, 200 μM PMSF) supplemented with protease inhibitor cocktail (Biosciences). The cell lysates were cleared by centrifugation at 4°C. Subsequently, cell lysates were pre-cleaned with protein A/G agarose (Roche) beads and incubated with an anti-caspase 8 antibody and protein A/G overnight at 4°C, with rotation. The complexes were precipitated by a brief spin, washed three times with IP lysis buffer with 500 mM NaCl and twice with IP lysis buffer, and boiled in 2X SDS sample buffer at 95°C for 5 min.

21. Statistical Analysis

Data were expressed as average ± S.D. Student's t-test was used to compare the differences between two groups. For comparison between more than two groups, one-way ANOVA with Tukey's post-hoc test was used. Significance was judged when $p < 0.05$.

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