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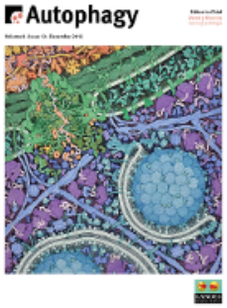

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Attenuation of TNFSF10/TRAIL-induced apoptosis by an autophagic survival pathway involving TRAF2- and RIPK1/RIP1-mediated MAPK8/JNK activation

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Keywords: autophagy, MAPK8/JNK, RIPK1/RIP1, TRAF2, TNFSF10/TRAIL, apoptosis

Abbreviations: 3MA, 3-methyladenine; CQ, chloroquine; DISC, death-inducing signaling complex; LC3, microtubule-associated protein 1 light chain 3; MAPK8/JNK, mitogen-activated protein kinase 8/ c-Jun N-terminal kinase; NFκB, nuclear factor-κB; TNFSF10/TRAIL, tumor necrosis factor (ligand) superfamily, member 10/tumor necrosis factor-related apoptosis-inducing ligand; TNFRSF10A/TRAIL-R1, tumor necrosis factor receptor superfamily member 10A/TNF-related apoptosis-inducing ligand receptor 1; TNFRSF10B/TRAIL-R2, tumor necrosis factor receptor superfamily member 10B/TNF-related apoptosis-inducing ligand receptor 2; TNFRSF10C/TRAIL-R3, tumor necrosis factor receptor superfamily member 10C/TNF-related apoptosis-inducing ligand receptor 3; TNFRSF10D/TRAIL-R4, tumor necrosis factor receptor superfamily member 10D/TNF-related apoptosis-inducing ligand receptor 4; TNFRSF11B/OPG, tumor necrosis factor receptor superfamily member 11B/osteoprotegerin; WTM, wortmannin

Although it is known that tumor necrosis factor-related apoptosis-inducing ligand (TNFSF10/TRAIL) induces autophagy, the mechanism by which autophagy is activated by TNFSF10 is still elusive. In this report, we show evidence that TRAF2- and RIPK1-mediated MAPK8/JNK activation is required for TNFSF10-induced cytoprotective autophagy. TNFSF10 activated autophagy rapidly in cancer cell lines derived from lung, bladder and prostate tumors. Blocking autophagy with either pharmacological inhibitors or siRNAs targeting the key autophagy factors BECN1/Beclin 1 or ATG7 effectively increased TNFSF10-induced apoptotic cytotoxicity, substantiating a cytoprotective role for TNFSF10-induced autophagy. Blocking MAPK8 but not NFκB effectively blocked autophagy, suggesting that MAPK8 is the main pathway for TNFSF10-induced autophagy. In addition, blocking MAPK8 effectively inhibited degradation of BCL2L1/Bcl-xL and reduction of the autophagy-suppressing BCL2L1–BECN1complex. Knockdown of TRAF2 or RIPK1 effectively suppressed TNFSF10-induced MAPK8 activation and autophagy. Furthermore, suppressing autophagy inhibited expression of antiapoptosis factors BIRC2/cIAP1, BIRC3/cIAP2, XIAP and CFLAR/c-FLIP and increased the formation of TNFSF10-induced death-inducing signaling complex (DISC). These results reveal a critical role for the MAPK8 activation pathway through TRAF2 and RIPK1 for TNFSF10-induced autophagy that blunts apoptosis in cancer cells. Thus, suppression of MAPK8-mediated autophagy could be utilized for sensitizing cancer cells to therapy with TNFSF10.

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/TNFSF10) is a promising anticancer agent that is undergoing preclinical and clinical studies for treating solid tumors including lung cancer.[1-4] TNFSF10 alone or in combination with other chemotherapeutics has shown promising anticancer activities through induction of apoptosis.[4] However, primary or acquired resistance to TNFSF10 in cancer cells has been documented and the molecular basis for TNFSF10 resistance is still elusive.[5-7] Therefore, understanding the underlying mechanisms for TNFSF10 resistance in cancer cells is important for improving the value of TNFSF10 as an anticancer therapeutic.

TNFSF10 induces apoptotic cell death in cancer by binding to its functional death receptors, death receptor (DR) 4 (TNFRSF10A/TRAIL-R1) and DR5 (TNFRSF10B/TRAIL-R2) to activate the extrinsic apoptosis pathway. TRAIL also activates c-Jun N-terminal kinase (MAPK8/JNK) and the transcription factor nuclear factor-κB (NFκB).[1] The ligation of TNFSF10 to TNFRSF10A or TNFRSF10B promotes

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the formation of the death-inducing signal complex (DISC), which consists of death receptors, FADD, CASP8/caspase-8 and CFLAR/c-FLIP. Through a self-cleavage process, CASP8 is activated in the DISC to trigger activation of downstream effector caspases such as CASP3 /caspase-3 and CASP7/caspase-7.[8] In cells with a weak CASP8-CASP3 signaling cascade, TNFSF10's apoptotic effect requires CASP8-mediated cleavage of the BH3-only BCL2/Bcl-2 family member BID to activate the intrinsic apoptosis pathway.[8] There are mechanisms that tightly control TNFSF10-induced apoptosis, which are utilized by cancer cells to counteract TNFSF10's cytotoxicity. These include NFκB activation to upregulate expression of antiapoptosis factors such as CFLAR and BIRC2,[9,10] and competitive inhibition of TNFRSF10A/B by decoy receptors (DcR) 1 (TNFRSF10C/TRAIL-R3) and DCR2 (TNFRSF10D/TRAIL-R4), and osteoprotegerin (TNFRSF11B/OPG) to suppress formation of a functional DISC that mediates apoptosis.[11] In addition, we have identified a cellular signaling pathway consisting of EGFR, CFLAR, MCL1, PTGS2/Cox-2 and TGM2 for acquired TNFSF10 resistance.[12-14]

Autophagy is an intracellular catabolic process involved in degradation and recycling of long-lived proteins and organelles, which is important for cell survival under nutrient-starvation conditions, and for housekeeping through removal of exhausted, redundant and unwanted cellular components.[15,16] However, in certain circumstances autophagy leads to cell death.[15,16] During autophagy, cytosolic elements are first sequestered by a double-membrane vesicle to create autophagic vacuoles called autophagosomes, which are subsequently fused to lysosomes to form autolysosomes, where sequestered components are degraded by lysosomal enzymes.[15,16] The autophagy process is regulated by autophagy factors such as ATG7, ATG5 and BECN1, which play critical roles at different stages.[15,16] The anti-apoptotic BCL2 family proteins such as BCL2 and BCL2L1/BCL-xL bind BECN1 to inhibit autophagy and dissociation of these BCL2 family proteins from BECN1 promotes autophagy.[17] In accordance with its contradictory roles in cell death control, the effects of autophagy in cancer cells' response to chemotherapy are also complex: either protecting against or promoting therapy-induced death.[18-20]

It has been reported that TNFSF10 is able to induce autophagy in certain cancer cells,[21] protecting them by blunting TNFSF10's cytotoxicity and possibly contributing to TNFSF10 resistance.[22] However, how TNFSF10 induces autophagy has not been elucidated. In this report, we investigated TNFSF10-induced cellular signaling pathways for autophagy induction and found that MAPK8 activation mediated by TRAF2 and RIPK1 is crucial for TNFSF10-induced authophagy. Further, we showed that inhibiting autophagy promoted TNFSF10 DISC formation, suppressed anti-apoptotic factors, and potentiated TNFSF10-induced apoptosis in cancer cells. Thus, suppression of MAPK8-mediated autophagy could be a useful approach for sensitizing cancer cells to therapy with TNFSF10.

Results

TNFSF10 induced autophagy in different human cancer cells. We first investigated if TNFSF10 was able to induce autophagy in cancer cells derived from different human tumors. The conversion of MAP1LC3B-I (LC3B-I) to MAP1LC3B-II (LC3B-II), a hallmark of autophagy, was readily detected in the bladder cancer cell line UM-UC3, lung cancer cell line A549 and prostate cancer cell line PC3 as early as 1 h after TNFSF10 treatment (**Fig. 1A and B**; **Fig. S1A**). Consistent with autophagy induction, the expression level of the autophagic protein SQSTM1/p62 was gradually reduced in TNFSF10-treated cells (**Fig. 1A and B**; **Fig. S1A**). To further confirm that the observed changes of MAP1LC3B and SQSTM1 were specifically due to autophagy, a MAP1LC3B and SQSTM1 turnover assay to detect autophagy flux was conducted. In this experiment, the lysosome inhibitor chloroquine (CQ) was used to determine whether TNFSF10 increased MAP1LC3B-II production and did not simply suppress MAP1LC3B-II clearance. In all three cell lines, while either TNFSF10 or chloroquine alone caused moderate increases of MAP1LC3B-II, the incubation of cells with both TNFSF10 and chloroquine further elevated MAP1LC3B-II expression (**Fig. 1C and D**; **Fig. S1B**). Similarly, TNFSF10-induced reduction of SQSTM1 was also effectively blocked by CQ (**Fig. 1C and D**). These autophagy flux results strongly suggested that TNFSF10 induces autophagy in cancer cells.[23] Furthermore, with transfection of a GFP-LC3 plasmid, we examined MAP1LC3B puncta formation, another hallmark of autophagy, after TNFSF10 treatment in A549 cells. Indeed, TNFSF10 strongly augmented the number of cells with increased MAP1LC3B puncta and MAP1LC3B puncta formation within the cell (**Fig. 1E and F**).[24] Taken together, these results strongly suggested that TNFSF10 induces autophagy in different types of human cancer cells.

Autophagy protected cancer cells against TNFSF10-induced cytotoxicity. To determine the role of autophagy in TNFSF10-induced cancer cell cytotoxicity, we first employed different autophagy inhibitors, wortmannin (WTM), 3-methyladenine (3MA) and CQ, for blocking autophagy at different steps of the autophagic pathway.[23] While the inhibitors by themselves had little cytotoxicity, they remarkably increased TNFSF10-induced cell death in all the three cancer cell lines (**Fig. 2A and B**; **Fig. S1C**). Both 3MA and wortmannin blocked TNFSF10-induced autophagy, which was shown by reduced basal MAP1LC3B-II and autophagic flux (**Fig. 2C**; **Fig. S2**). To further confirm these observations, siRNA targeting *ATG7* or *BECN1* was used to block autophagy. Knockdown of either ATG7 or BECN1, which was confirmed by western blot (**Fig. 2D**), effectively potentiated TNFSF10-induced cytotoxicity (**Fig. 2D**). The *BECN1* and *ATG7* siRNAs effectively blocked TNFSF10-induced increase of MAP1LC3B-II (**Fig. 2E**). These results were in agreement with previous reports that TNFSF10-induced autophagy is a cytoprotective signal against TNFSF10-induced cell death.[22,25,26]

Autophagy suppressed TNFSF10-induced apoptotic signaling. We then examined if blocking autophagy affects TNFSF10-induced activation of the extrinsic apoptosis pathway. When the UM-UC-3 and A549 cells were pretreated with wortmannin, TNFSF10-induced activation of CASP8 and CASP3 was strongly potentiated. Consistent with caspase activation, the cleavage of PARP1, a biomarker of apoptosis, was also substantially enhanced (**Fig. 3A and C**). Using GST-TNFSF10 as a

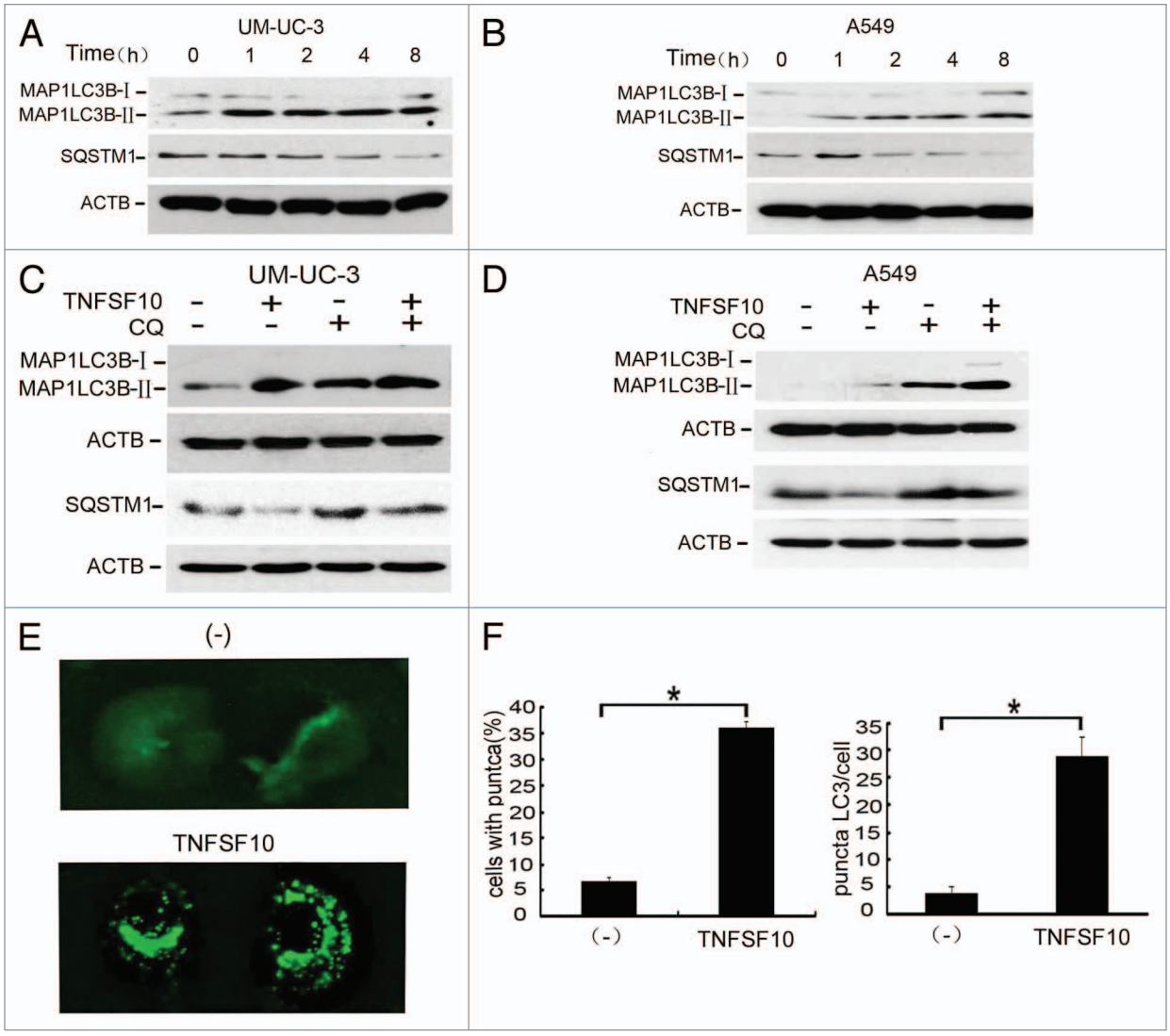

Figure 1. TNFSF10 induced autophagy in cancer cells. (**A and B**) The cells were treated with TNFSF10 (60 ng/ml for UM-UC-3, 200 ng/ml for A549 and 150 ng/ml for PC-3) for indicated times. The indicated proteins were detected by western blot. ACTB was detected as a loading control. (**C and D**) The cells were pretreated with chloroquine (CQ, 20 μM) for 30 min and then treated with TNFSF10 (60 ng/ml for UM-UC-3 and 200 ng/ml for A549) for an additional 2 h (for MAP1LC3B) and 8 h (for SQSTM1). The indicated proteins were detected by western blot. ACTB was detected as a loading control. (**E**) A549 stably transfected with GFP- LC3 were treated with TNFSF10 (200 ng/ml) for 1 h or remained untreated. Photographs were taken under a fluorescence microscope. (**F**) Quantification of cell numbers with GFP-LC3 puncta (left) and number of puncta per positive cell. Data shown are the mean ± SD *$p < 0.01$.

ligand, a GST pull-down experiment was conducted to examine the formation of TNFSF10 DISC. As shown in **Figure 3B and D**, blocking autophagy with chloroquine significantly increased the recruitment of FADD and CASP8 to form DISC. These results suggested that autophagy attenuates TNFSF10's cytotoxicity, at least in part, through suppressing TNFSF10-induced apoptotic signaling.

Inhibiting MAPK8 activation suppressed TNFSF10-induced autophagy and potentiated TNFSF10's cytotoxicity. To determine the signaling pathways that are involved in TNFSF10-induced autophagy, we used specific inhibitors for MAPK8 and NFκB to interfere with autophagy induction. The MAPK8 inhibitor completely abolished TNFSF10-induced autophagy (**Fig. 4A and B**; **Fig. S3**). In contrast, the NFκB blocking agent IKBKB inhibitor had little effect on TNFSF10-induced autophagy (**Fig. 4A and B**). These inhibitors were effective in suppressing their respective pathways (**Fig. 4A and B**).[14] Consistent with the cytoprotective role of autuphagy, blocking MAPK8 also substantially potentiated TNFSF10-induced cell death (**Fig. 4C and D**). Furthermore, blockage of MAPK8 potentiated TNFSF10-induced apoptosis, which was shown by enhancement of CASP8 activation and PARP1 cleavage (**Fig. 4E**). These results suggested that MAPK8 was the main pathway for TNFSF10 to induce cytoprotective autophagy.

MAPK8 mediated autophagy though degradation of BCL2L1 and reduction of the BCL2L1–BECN1 complex. In starvation-induced autophagy, MAPK8 phosphorylates the prosurvival BCL2 family of proteins to disrupt the complex of

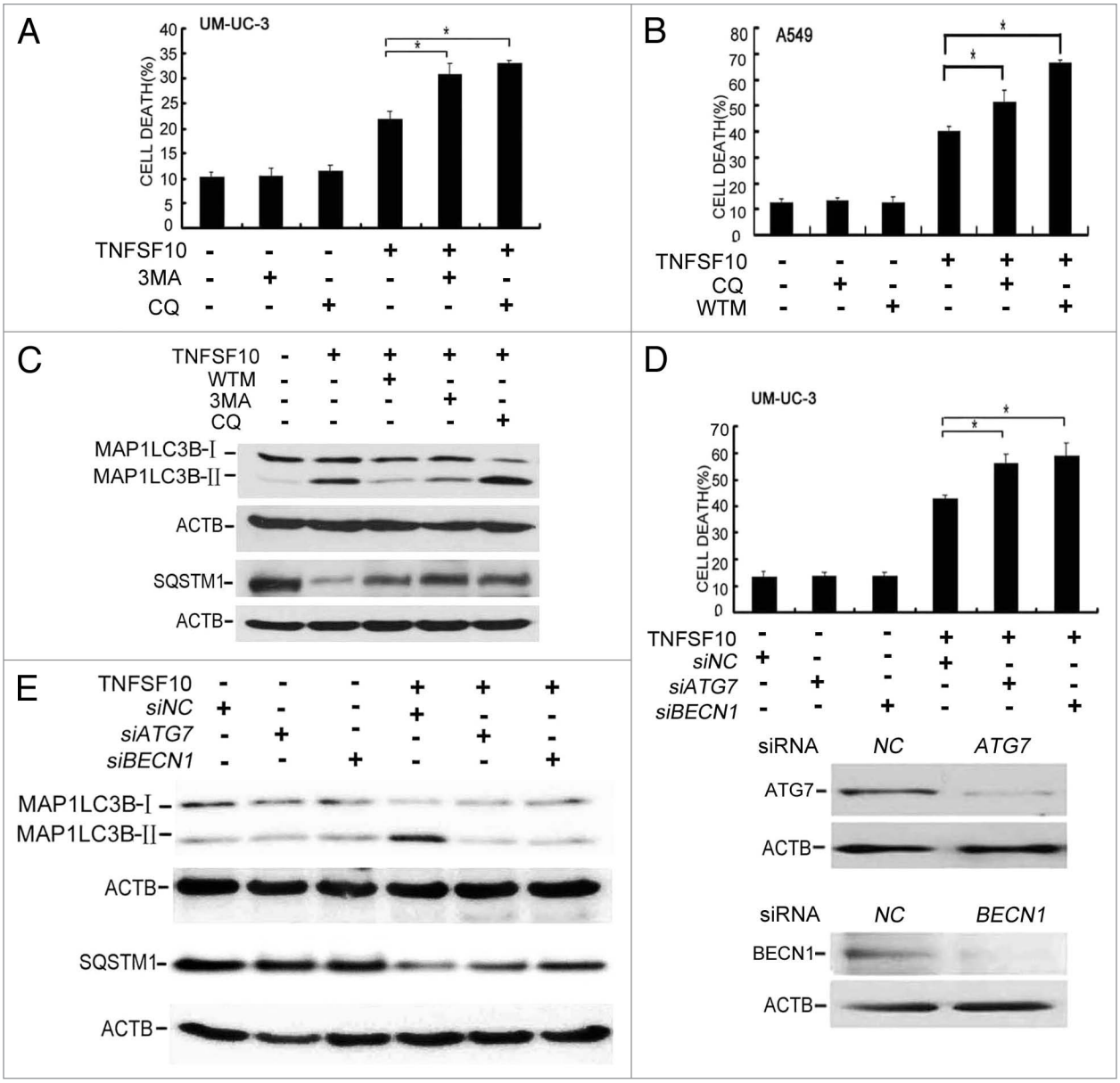

Figure 2. Autophagy protected cancer cells against TNFSF10-induced cytotoxicity. (**A and B**) The cells were pretreated with 3MA (10 mM), CQ (20 μM), or wortmannin (1 μM) for 30 min and then treated with TNFSF10 (60 ng/ml for UM-UC-3 and 200 ng/ml for A549) for 24 h. Cell death was measured by LDH release assay. (**C**) UM-UC-3 cells were pretreated with different inhibitors (CQ, 20 μM; wortmannin, 1 μM; 3MA, 10 mM) for 30 min and then treated with TNFSF10 (60 ng/ml) for an additional 2 h (for MAP1LC3B) and 8 h (for SQSTM1), respectively. The indicated proteins were detected by western blot. ACTB was detected as a loading control. (**D**) Upper, UM-UC-3 cells were transfected with the indicated siRNAs (10 nM) for 24 h and then treated with TNFSF10 (60 ng/ml) for an additional 24 h. Cytotoxicity was detected by LDH release assay. Data shown are the mean ± SD *$p < 0.01$. Lower, UM-UC-3 cells were transfected with the indicated siRNAs (10 nM) for 24 h. The indicated proteins were detected by western blot. (**E**) UM-UC-3 cells were transfected with the indicated siRNAs (10 nM) for 24 h, and then treated with TNFSF10 (60 ng/ml) for an additional 2 h (for MAP1LC3B) and 8 h (for SQSTM1). The indicated proteins were detected by western blot. ACTB was detected as a loading control.

these proteins with BECN1, which promotes the initiation of autophagy.[27] Thus, we examined if TNFSF10-induced MAPK8 activation modulated the prosurvival BCL2 family of proteins. A decreased expression level of BCL2L1 was detected as early as 1 h post-TNFSF10 treatment. A slight decrease of MCL1 expression was also detected while BCL2 was largely unaffected (**Fig. 5A**; **Fig. S4A**). The degradation of BCL2L1 was suppressed by blocking lysosomal degradation with CQ but not by blocking caspases with z-VAD (**Fig. S4B**), suggesting that this degradation is unlikely the result of caspase-mediated cleavage.[28] Suppressing MAPK8 but not NFκB restored the BCL2L1 level, suggesting that MAPK8 mediated TNFSF10-induced BCL2L1 decrease (**Fig. 5B**). Suppression of either MAPK8 or NFκB had little effect on the expression level of MCL1 and BCL2 (**Fig. 5B**). Because MAPK8 dissociates the complex of BCL2L1 and BECN1 to trigger autophagy,[29,30] we examined

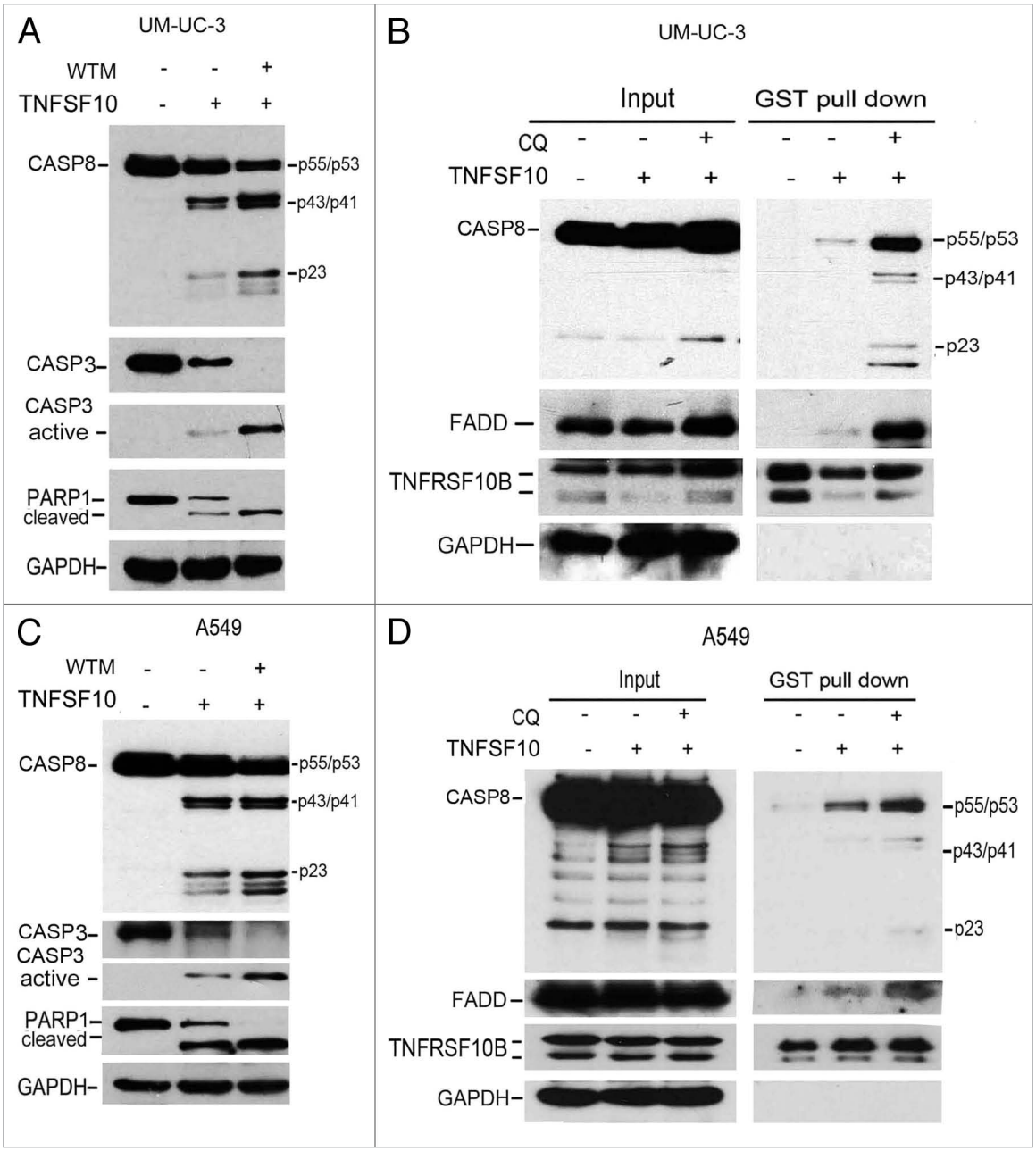

Figure 3. Autophagy suppressed TNFSF10-induced apoptotic signaling. (**A and B**) UM-UC-3 and A549 cells were pretreated with wortmannin (1 μM) for 30 min and then treated with TNFSF10 (60 ng/ml for UM-UC-3 and 200 ng/ml for A549) for an additional 4 h. CASP8, CASP3, and PARP1 were detected by western blot. GADPH was detected as a loading control. (**B and D**) DISC formation detected by GST pull-down assay. The cells were pretreated with CQ (20 μM) for 30 min, then treated with GST-TNFSF10 (60 ng/ml and 200 ng/ml for A549) for 15 min or left untreated. GST-TNFSF10 (60 ng/ml) was added to lysates from unstimulated cells to preciptate unstimulated TNFSF10 receptors. GST-TNFSF10-bound proteins were analyzed for the presence of TNFRSF10B, FADD, and CASP8 by western blot. GADPH was detected as loading and negative control.

if TNFSF10 induced reduction of the complex consisting of BCL2L1 and BECN1, by immunoprecipitation. The results showed that BECN1 was constitutively bound to BCL2L1, which was reduced after TNFSF10 treatment causing a decrease of BCL2L1 expression (**Fig. 5C**; **Fig. S4C**). Interestingly, the reduction of the BECN1–BCL2L1 interaction was restored when MAPK8 was blocked, which was associated with the restoration of BCL2L1 expression (**Fig. 5D**; **Fig. S4D**). Although a BECN1–BCL2 complex was also detected, TNFSF10 had no detectable effects on this complex (**Fig. 5C and D**; **Fig. S4C and S4D**). In contrast, no MCL1 was coimmunoprecipitated with BECN1, suggesting that the interaction of BECN1 and BCL2L1 was specifically involved in TNFSF10-induced autophagy regulation. These results suggested that TNFSF10-induced MAPK8 activated BECN1 for autophagy by alleviating the suppression of BCL2L1 on BECN1.

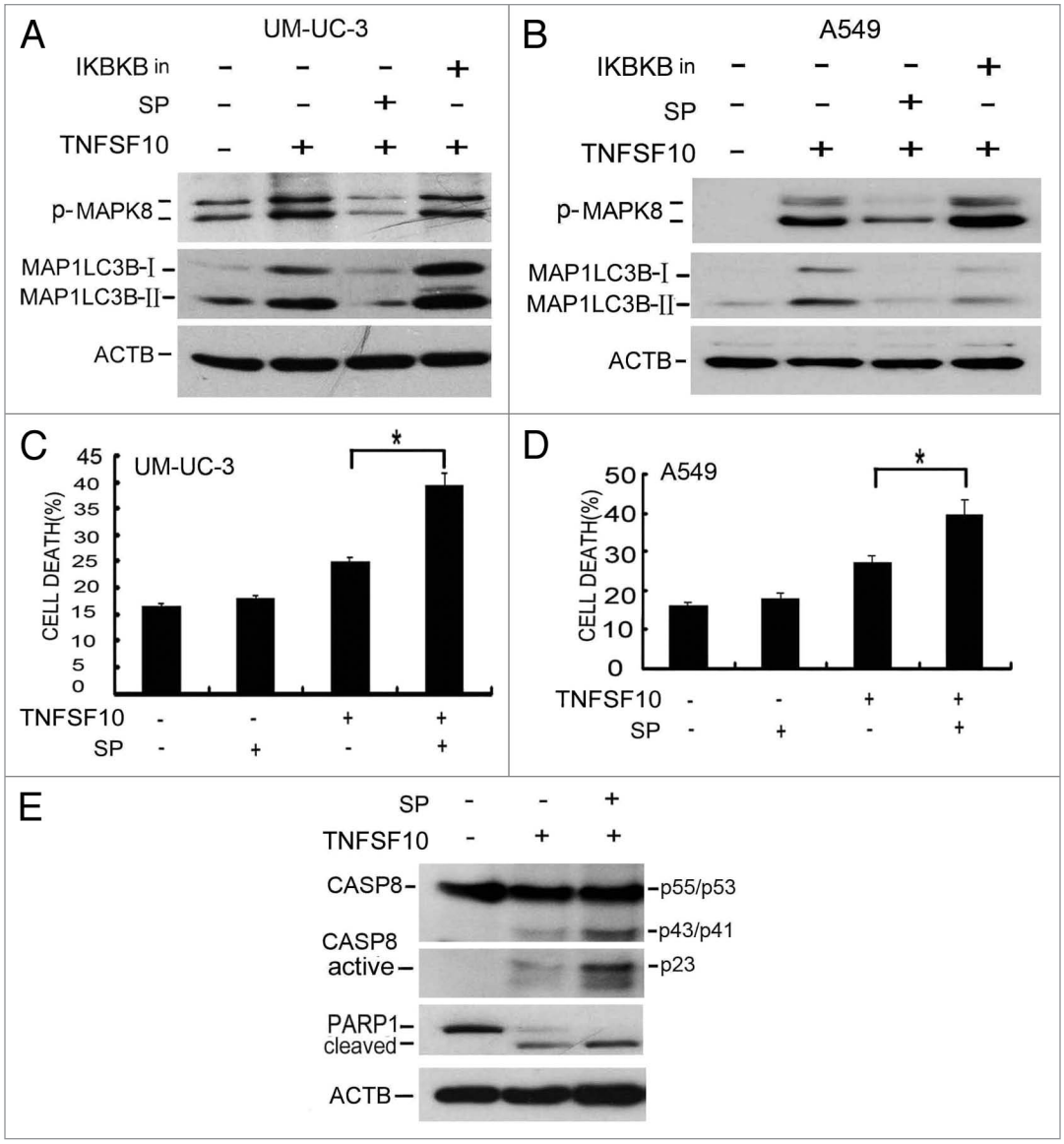

Figure 4. Inhibiting MAPK8 activation suppressed TNFSF10-induced autophagy and potentiated TNFSF10's cytotoxicity. (**A and B**) UM-UC-3 and A549 cells were pretreated with the indicated inhibitors (SP600125, 10 μM; IKBKB inhibitor, 50 μM) for 30 min and then treated with TNFSF10 (60 ng/ml for UM-UC-3 and 200 ng/ml for A549) for an additional 1 h. phospho-MAPK8 and MAP1LC3B were detected by western blot. ACTB was detected as a loading control. (**C and D**) UM-UC-3 and A549 cells were pretreated with SP600125 (10 μM) for 30 min and then treated with TNFSF10 (60 ng/ml for UM-UC-3 and 200 ng/ml for A549) for an additional 24 h. Cytotoxicity was detected by LDH release assay. Data shown are the mean ± SD *$p < 0.01$. (**E**) UM-UC-3 cells were pretreated with SP600125 (10 μM) for 30 min and then treated with TNFSF10 (60 ng/ml) for an additional 4 h. CASP8 and PARP1 were detected by western blot. ACTB was detected as a loading control.

Autophagy suppressed TNFSF10-induced degradation of anti-apoptotic proteins. We further investigated whether autophagy regulated anti-apoptotic factors. TNFSF10 treatment caused a slight decrease of BIRC2, BIRC3, XIAP and CFLAR. When the cells were cotreated with TNFSF10 and wortmannin, a dramatic decrease of these proteins was observed (**Fig. 6A and B**). Inhibition of autophagy through knockdown of either ATG7 or BECN1 also effectively potentiated TNFSF10-induced degradation of these anti-apoptotic proteins (**Fig. S5**). A similar effect of MAPK8 inhibition on these proteins was also detected (**Fig. 6A and B**). These results suggested that MAPK8-mediated autophagy protected these anti-apoptotic proteins from TNFSF10-induced reduction of these proteins.

Because anti-apoptotic proteins are regulated by degradation through the proteasome and lysosome crosstalk,[31] we further investigated the mechanisms of downregulation of these factors during autophagy blockage. The proteasome inhibitor MG-132 effectively restored the expression of BIRC2 in TNFSF10-treated cells (**Fig. 6C**). The cathepsin B inhibitor, which inhibits a subgroup of lysosomal proteases, and CQ, which prevents acidification required for lysosomal enzymes to function, also slightly increased BIRC2 expression without affecting the BIRC2 modification. These results suggested that

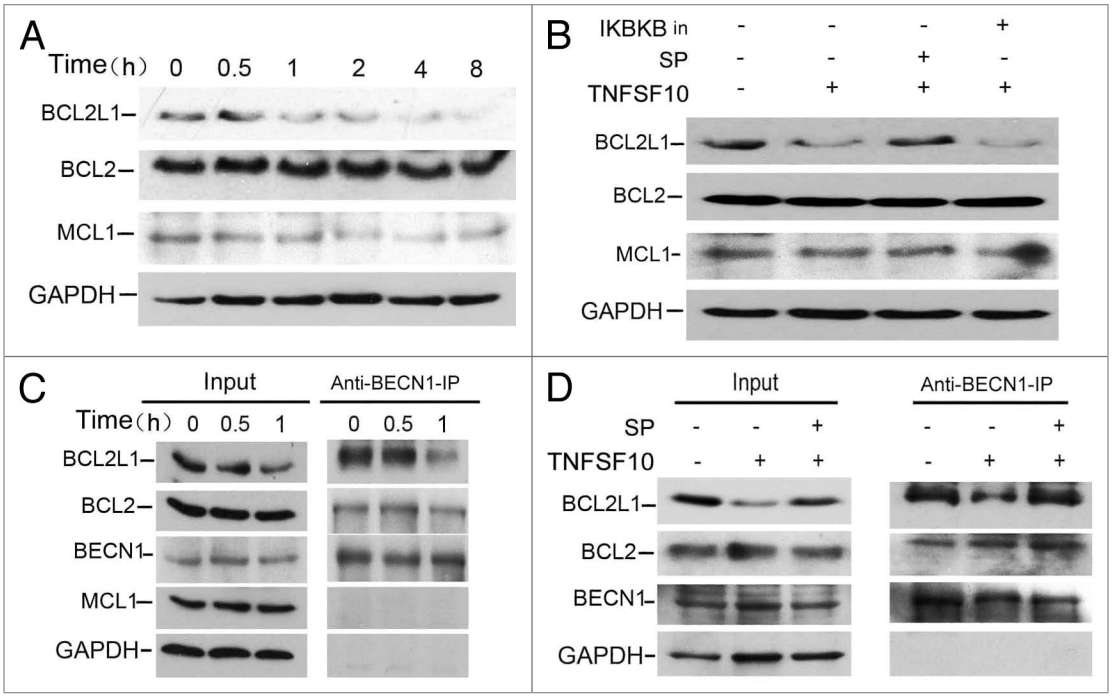

Figure 5. MAPK8 mediated autophagy through degradation of BCL2L1 and dissociation of the BCL2L1–BECN1 complex. (**A**) A549 cells were treated with TNFSF10 (200 ng/ml) for indicated times. The expression of proteins was detected by western blot. GADPH was detected as a loading control. (**B**) A549 cells were pretreated with the indicated inhibitors (SP600125, 10 μM; IKBKB inhibitor, 50 μM) for 30 min and then treated with TNFSF10 (200 ng/ml) for an additional 1 h. BCL2L1 and BCL2 were detected by western blot. GADPH was detected as a loading control. (**C**) A549 cells were treated with TNFSF10 (200 ng/ml) for indicated times (h). The proteins were detected by western blot after co-immunoprecipitation with a BECN1 antibody. (**D**) A549 cells were pretreated with the SP600125 (10 μM) for 30 min or untreated, then treated with TNFSF10 (200 ng/ml) for 1 h. The indicated proteins were detected by western blot after co-immunoprecipitation with a BECN1 antibody. GAPDH was detected as a loading control.

both proteasome and lysosome were involved in TNFSF10-induced and autophagy-mediated BIRC2 degradation. In contrast, the cathepsin B inhibitor, but not MG132 protected from TNFSF10-induced BIRC3, XIAP and CFLAR reduction, suggesting that the lysosomal but not the proteasomal pathway was involved in degradation of these proteins (**Fig. 6C**). Importantly, CQ suppressed SP600125-triggered BIRC2, BIRC3, XIAP and CFLAR decreases to different extents (**Fig. 6C**), suggesting that MAPK8-mediated protection of these anti-apoptotic proteins involved lysosomal degradation. This observation was consistent with the report that autophagy inhibition compromises the ubiquitin-proteasome pathway.[32] In addition, the pan-caspase inhibitor z-VAD also protected BIRC2 but had little effect on BIRC3, XIAP and CFLAR (**Fig. 6C**). Whether apoptosis or caspase-like protease activity in the proteasome mediates BIRC2 degradation needs further investigation. Nevertheless, these results suggested that the degradation of anti-apoptotic proteins involved different mechanisms, and both the proteasome and lysosome were involved during TNFSF10-induced autophagy.

RIPK1 and TRAF2 modulated TNFSF10-induced and MAPK8-mediated autophagy. We next investigated factors in the TNFSF10 receptor signaling cascade for TNFSF10-induced MAPK8 activation and autophagy. Knocking down either RIPK1 or TRAF2 partially inhibited TNFSF10-induced MAPK8 activation, suggesting that TNFSF10-induced MAPK8 activation required both RIPK1 and TRAF2 (**Fig. 7A**), consistent with our previous finding in mouse embryonic fibroblasts.[9] Consistent with the role of MAPK8 in autophagy induction, suppressing RIPK1 or TRAF2 inhibited TNFSF10-induced autophagy (**Fig. 7A and B**). Further, knockdown of RIPK1 or TRAF2 effectively potentiated TNFSF10-induced cytotoxicity (**Fig. 7C and D**). Knockdown of expression of RIPK1 and TRAF2 was confirmed by western blot (**Fig. 7E and F**). Although RIPK1 and TRAF2 may protect cells against apoptosis through other mechanisms such as NFκB, these results suggested that mediation of MAPK8-associated autophagy also contributed to the cytoprotective activities of RIPK1 and TRAF2.

Discussion

In this study, we first confirmed that TNFSF10 induced protective autophagy in different cancer cell lines. By dissecting the TNFSF10-induced signaling pathways, we then investigated the mechanism by which TNFSF10 activated autophagy. The results showed that the MAPK8 activation, mediated by RIPK1 and TRAF2, played a pivotal role in TNFSF10-induced autophagy. Furthermore, we found that degradation of the anti-apoptotic factors BIRC2, BIRC3, XIAP and CFLAR and enhancement of TNFSF10 DISC formation were involved in the promotion of TNFSF10-induced cytotoxicity by inhibiting autophagy. Therefore, inhibiting MAPK8 pathway-mediated autophagy

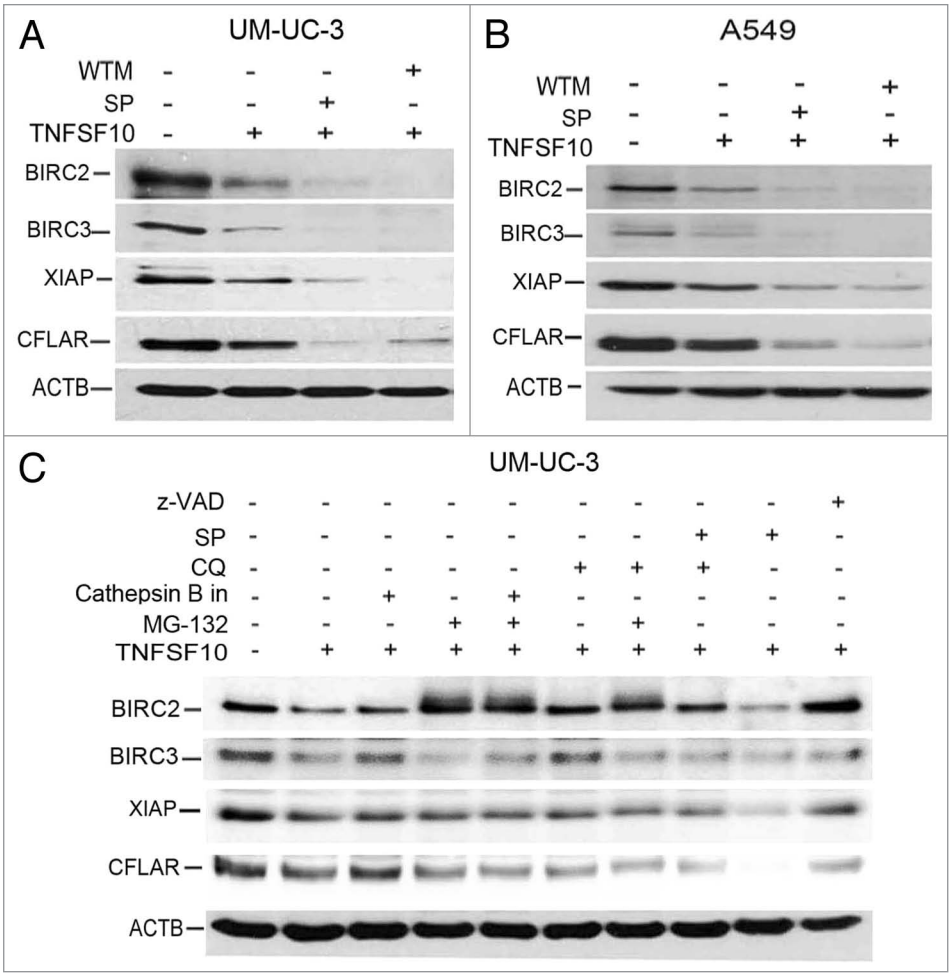

Figure 6. MAPK8 mediated autophagy-associated degradation of anti-apoptotic proteins. (**A–C**) UM-UC-3 and A549 cells were pretreated with the indicated inhibitors (SP600125, 10 μM; wortmannin, 1 μM; MG132, 10 μM; cathepsin B inhibitor, 10 μM; CQ, 20 μM; z-VAD, 10 μM) for 30 min and then treated with TNFSF10 (60 ng/ml for UM-UC-3 and 200 ng/ml for A549) for an additional 4 h. The indicated proteins were detected by western blot. ACTB was detected as a loading control.

will increase TNFSF10's anticancer activity and circumvent TNFSF10 resistance in cancer cells.

TNFSF10 induces autophagy in leukemia (Jurkat), breast cancer (MCF7) and colon cancer (HCT 116) cell lines, and autophagy is protective against TNFSF10's cytotoxicity in these cancer cells.[22,25,26] However, the role of autophagy in a cell's fate can be cell type-specific.[15,16] Furthermore, how TNFSF10 induces autophagy in cancer cells is still elusive. Therefore, in this study we first confirmed that TNFSF10 was able to induce protective autophagy in lung, prostate and bladder cancer cell lines and then went on to investigate the signaling pathways required for TNFSF10-induced autophagy. The results identified the MAPK8 pathway as the main pathway for TNFSF10-induced autophagy. Blocking MAPK8 effectively suppressed TNFSF10-induced autophagy. As a kinase with multiple cellular functions, MAPK8 can be either pro-apoptotic or anti-apoptotic: transient MAPK8 activation is prosurvival while sustained MAPK8 activation results in cell death.[33-35] Although it is reported that MAPK8 contributes to TNFSF10-induced cell death, we clearly showed that in the tested cells suppression of MAPK8 promoted TNFSF10's cytotoxicity. These observations reflected the complexity of MAPK8 in cell-death regulation, which may involve cell type specificity and alternative functions of MAPK8 isoforms.[36] Consistent with a previous report for starvation-induced autophagy,[27] TNFSF10-induced MAPK8 activation caused dissociation of the BCL2L1–BECN1 complex. The released BECN1 triggers initiation of autophagy.[27] However, we were unable to detect TNFSF10-induced BCL2 phosphorylation. Instead, we found that TNFSF10 caused BCL2L1 degradation and suppressed the BCL2L1–BECN1 complex through MAPK8 activation. The degradation of BCL2L1 was suppressed by blocking lysosomal degradation with CQ but not by blocking apoptosis with z-VAD. The exact mechanism by which MAPK8 mediates BCL2L1 degradation for autophagy certainly deserves further study. Among the anti-apoptotic members of the BCL2 family, we found BCL2L1 and BCL2 but not MCL1 could bind to BECN1, and only the BCL2L1–BECN1 complex was suppressed by TNFSF10. This suggested that different anti-apoptotic members of the BCL2 family may be differently involved in autophagy, which is shown in a recent report.[37]

With a siRNA approach, we found that both RIPK1 and TRAF2 were required for TNFSF10-induced MAPK8 activation in cancer cells, which was consistent with our previous finding in mouse embryonic fibroblasts.[9] More importantly, knockdown of RIPK1 or TRAF2 effectively suppressed autophagy and potentiated TNFSF10-induced cell death. These results established a pathway consisting of RIPK1, TRAF2 and MAPK8 for TNFSF10-induced protective autophagy.

How autophagy inhibits TNFSF10-induced apoptotic cell death is not fully understood. We clearly showed that suppression of autophagy enhanced TNFSF10 DISC formation. TNFSF10-mediated autophagy counter-balances apoptosis by sequestering the large CASP8 subunit in autophagosomes for lysosomal degradation.[25] In agreement with this report, we found that suppressing autophagy substantially increased the large CASP8 subunit (p43). In addition, we found that autophagy also inhibited basal and TNFSF10-induced degradation of anti-apoptotic factors including IAPs and CFLAR that are involved in TNFSF10 resistance.[12] Thus, autophagy seems to suppress TNFSF10-induced cancer cell death in two ways: (1) by blocking apoptotic signaling

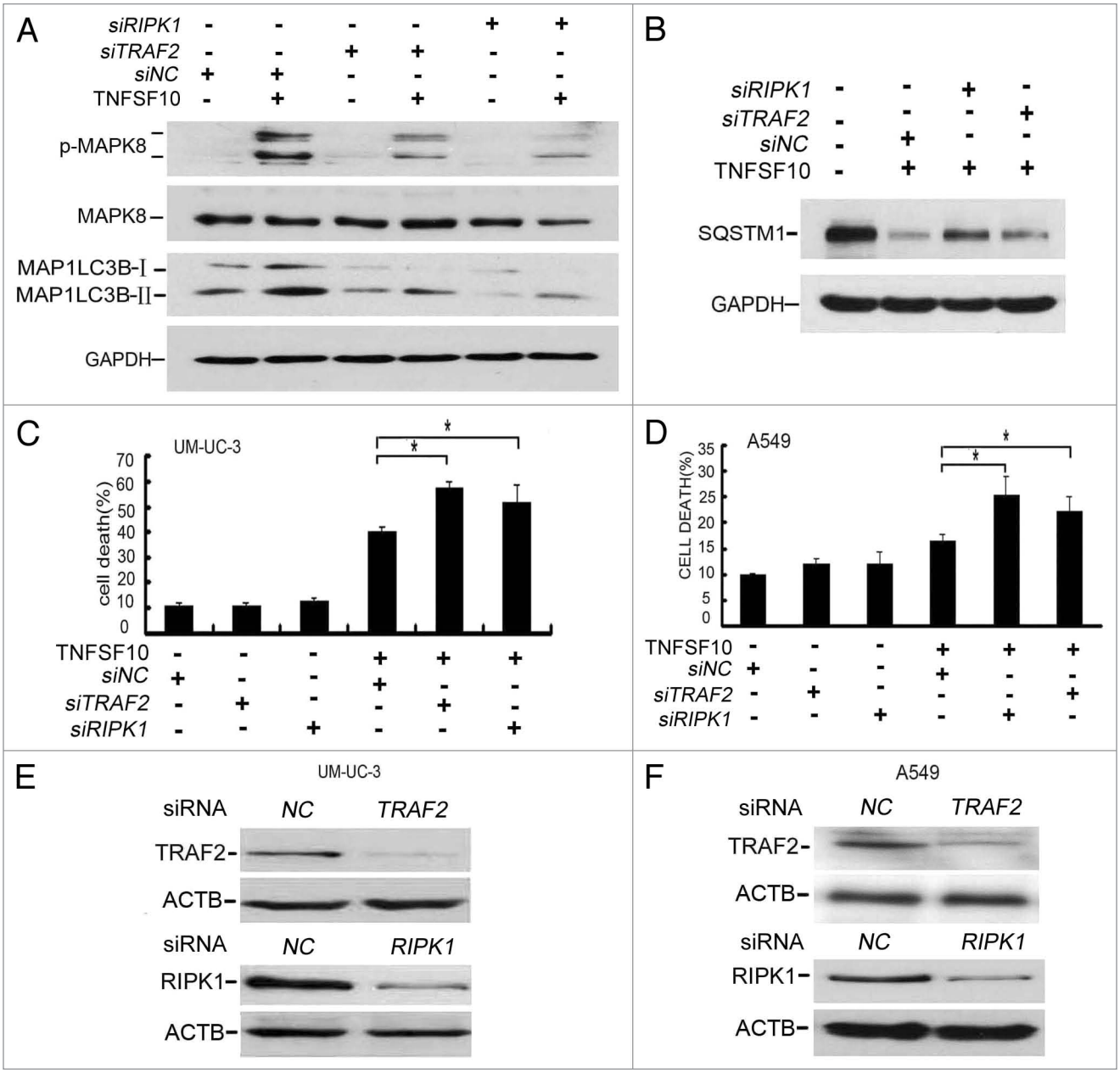

Figure 7. RIPK1 and TRAF2 modulated TNFSF10-induced and MAPK8-mediated autophagy. (**A and B**) UM-UC-3 cells were transfected with negative control or siRNAs against *RIPK1* or *TRAF2*. Twenty-four h post-transfection, the cells were treated with TNFSF10 (60 ng/ml) for for an additional 2 h (for MAP1LC3B) and 8 h (for SQSTM1). The indicated proteins were detected by western blot. GAPDH was detected as a loading control. (**C and D**) UM-UC-3 and A549 cells were transfected with the indicated siRNAs for 24 h and then treated with TNFSF10 (60 ng/ml for UM-UC-3 and 200 ng/ml for A549) for an additional 24 h. Cytotoxicity was detected by LDH release assay. Data shown are the mean ± SD *$p < 0.01$. (**E and F**) UM-UC-3 and A549 cells were transfected with the indicated siRNAs (10 nM) for 24 h. The indicated proteins were detected by western blot. ACTB was detected as a loading control.

through inhibiting CASP8 and, (2) by enhancing anti-apoptotic pathways through stabilizing IAPs and CFLAR. Notably, although MCL1 was stabilized by MAPK8 similarly to BCL2L1, it seemed that MCL1 was not involved in autophagy regulation. Therefore, each prosurvival BCL2 protein may function differently in protecting cancer cells from TNFSF10-induced cytotoxicity.

The involvement of autophagy components such as BECN1 and SQSTM1 in apoptosis regulation, in an autophagy-dependent or -independent manner, is well documented. It has been reported that SQSTM1 binds and promotes full activation of CASP8 for enhancing cell death, suggesting a pro-apoptosis role of SQSTM1. Our results indicated that SQSTM1 had a cytoprotective role through mediating TNFSF10-induced autophagy. Although the role and mechanism of SQSTM1 in regulating CASP8 activation deserve further study, our results clearly suggested that SQSTM1-mediated autophagy protected cancer cells against TNFSF10-induced apoptosis.

The proteasome and lysosome impact one another and are involved in autophagy. Although autophagic protein degradation is lysosomal, autophagy also promotes ubiquitin-proteasomal protein degradation.[32] In our study, we found that both lysosomal and proteasomal degradation were involved in regulating anti-apoptotic proteins during TNFSF10-induced apoptosis. During TNFSF10-induced signaling, proteasome activity is required for BIRC2 degradation while the lysosome is involved in degradation of BIRC2/BIRC3 (c-IAPs) and CFLAR. Interestingly, both these protein degradation mechanisms are impacted by autophagy.

In summary, the results of this study identified a pathway consisting of TRAF2, RIPK1 and MAPK8 for TNFSF10-induced protective autophagy in cancer cells. Suppression of MAPK8-mediated autophagy through targeting the key components of this pathway could be useful for sensitizing cancer cells to therapy with TNFSF10.

Materials and Methods

Reagents and antibodies. Glutathione S-transferase (GST) TNFSF10 was prepared as described previously.[9,12] Antibodies against BCL2 (sc-7328), BECN1 (sc-48341), GAPDH (sc-32233) and TRAF2 (sc-877) were from Santa Cruz Biotechnology. Anti-phospho-MAPK8 (44682G) was from Invitrogen. Anti-FADD (556402), -BIRC2 (AF8181), -BIRC3 (552782), -CASP3 (559565), -TNFRSF10B (557868), -MAPK81 (554286), -SQSTM1 (610832), -RIPK1 (610458), -CASP8 (551242) and -poly(ADP-ribose) polymerase (PARP1, 611038) were from BD Biosciences. Anti-ACTB (A1978) and Anti-MAP1LC3B (L7543) were purchased from Sigma-Aldrich. Antibodies for BCL2L1 (2762) and XIAP (2042) were from Cell Signaling. Antibodies against CFLAR (AAP-440c) and MCL1 (3035-100) were from Alexis and BioVision, respectively. Anti-ATG7 (PA5–17216) was from Thermo Scientific. The MAPK8 inhibitor SP600125 (129-56-6), IKBKB/IKK inhibitor II (354812-17-2), MG132 (474790), cathepsin B inhibitor (219385) and wortmannin (19545-26-7) were from Calbiochem. Chloroquine (CQ, 50-63-5) was from Sigma-Aldrich. 3-Methyladenine (3MA, sc-205596) was from Santa Cruz Biotechnology. Short-interfering RNAs (*ATG7* siRNA, M020112 and *RIPK1* siRNA, M-00445-02-005) for selected genes and the nontargeting siRNA (Silencer® negative control #1 siRNA) were obtained from Dharmacom. *BECN1* siRNA (sc-29797) and *TRAF2* siRNA (sc-29509) were from Santa Cruz Biotechnology.

Cell culture. UM-UC-3, PC-3 and A549 cells were obtained from American Type Culture Collection and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Cytotoxicity assay. A cytotoxicity assay based on the release of lactate dehydrogenase (LDH) was conducted using a CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, G1782). Cells were seeded in 48-well plates at 70–80% confluence. After overnight culture, cells were treated as indicated in each figure legend. LDH release was determined as described previously.[38]

Western blotting and immunoprecipitation. Cell lysates were prepared by suspending cells in M2 buffer [20 mM TRIS-HCl (pH 7.6), 0.5% NP40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 1 mM sodium vanadate, and 1 μg/ml leupeptin]. Equal amounts of protein from each cell lysate were resolved by 12% or 15% SDS-PAGE and analyzed by western blot. The proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Millipore, WBKLS0500).[39] Each experiment was repeated at least three times and representative results are shown. For immunoprecipitation, cells were cultured in 60 mm dishes, treated as indicated in figure legends, and lysed in M2 buffer. Immunoprecipitation was performed as described previously.[40] Briefly, 20 μl Protein A agarose beads (50%) were coupled to 1 μg BECN1 antibody in PBS for 2 h at room temperature. Then 1 mg cell lysates were added and incubated with the beads by rotating overnight at 4°C. The beads were washed seven times with M2 buffer. The immunoprecipitants were eluted off the beads using electrophoresis sample buffer. The samples were boiled for 5 min and loaded on 12% SDS-PAGE gel. Proteins of interest were detected by western blot.

GST-pull-down and DISC analysis. UM-UC-3 cells were treated with 50 ng/ml of GST-TNFSF10 for 15 min, 30 min or left untreated. Cells were then washed twice with ice-cold PBS and lysed with 0.5 ml M2 buffer. The soluble fraction was incubated with 20 μl of (50%) glutathione-sepharose beads (Pharmacia,) for 3 h or overnight on a rotator at 4°C. GST-TNFSF10 (60 ng/ml) was added to the lysates prepared from nonstimulated cells to precipitate the nonstimulated receptors. After five washes with M2 buffer, the bound proteins were eluted by boiling for 3 min in SDS-PAGE loading buffer and resolved in 12% SDS/PAGE. The presence of TNFRSF10B, FADD, CASP8, and CFLAR was then determined by western blot.[12]

Knockdown protein expression by RNAi. UM-UC-3 and A549 cells were seeded in a 6-well plate and 48-well plate the day before transfection at 50% confluence. SiRNA was transfected with siRNA INTERFERin™ (Polyplus-transfection, 409-10). Twenty-four hours after transfection, the expression of proteins of interest was measured by western blot, and remaining cells were treated for cytotoxicity assays.

Fluorescence microscopy. A549 cells stably tranfected with GFP-MAP1LC3B were grown on glass coverslips, treated with TNFSF10 (200 ng/ml) for 1 h, then examined under a fluorescence microscope. Images shown are representative of three independent experiments. The percentage of punctate cells and average of puncta per positive cell were calculated.[24]

Statistical analysis. Data are expression as means ± SDs. Statistical significance was examined by one-way ANOVA.

In all analyses, $p < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/autophagy/article/22145

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