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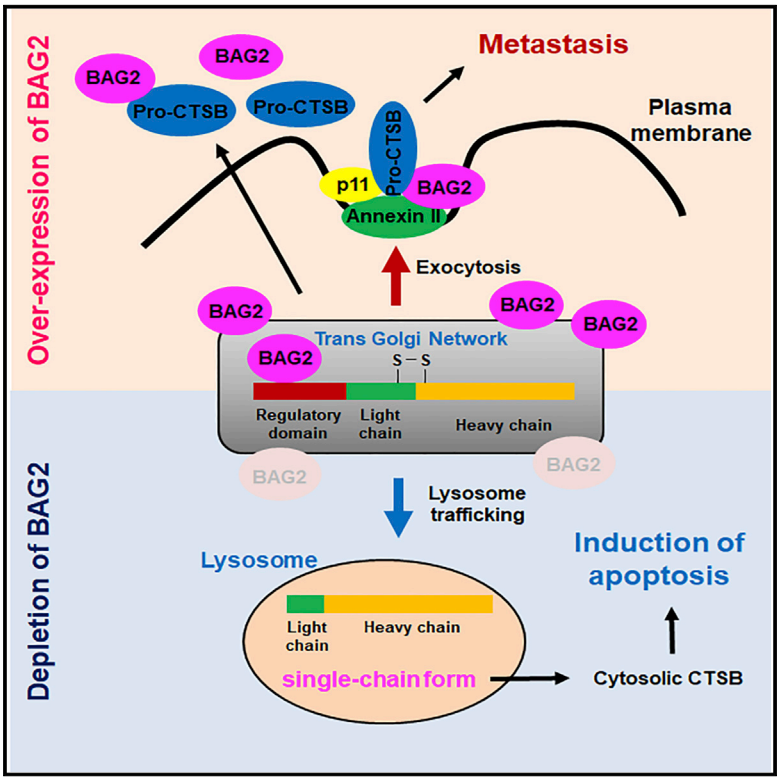
Co-chaperone BAG2 Determines the Pro-oncogenic Role of Cathepsin B in Triple-Negative Breast Cancer Cells

Graphical Abstract



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In Brief

The mechanisms controlling the pro- and anti-oncogenic roles of cathepsin B are unclear. Yang et al. find that BAG2 is a regulator of the dual functions of its client protein, CTSB, facilitating the progression of TNBC.

Highlights

- BAG2 overexpression in TNBC is strongly correlated with poor clinical outcomes
- BAG2 regulates auto-cleavage of pro-cathepsin B by binding to the propeptide region
- Silencing BAG2 induces apoptosis through increase of single-chain cathepsin B
- BAG2 controls the secretion of pro-CTSB through TGN38-positive vesicles

Data and Software Availability

GSE100878

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Co-chaperone BAG2 Determines the Pro-oncogenic Role of Cathepsin B in Triple-Negative Breast Cancer Cells

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SUMMARY

Triple-negative breast cancer (TNBC) is considered incurable with currently available treatments, highlighting the need for therapeutic targets and predictive biomarkers. Here, we report a unique role for Bcl-2-associated athanogene 2 (BAG2), which is significantly overexpressed in TNBC, in regulating the dual functions of cathepsin B as either a pro- or anti-oncogenic enzyme. Silencing BAG2 suppresses tumorigenesis and lung metastasis and induces apoptosis by increasing the intracellular mature form of cathepsin B, whereas BAG2 expression induces metastasis by blocking the auto-cleavage processing of pro-cathepsin B via interaction with the propeptide region. BAG2 regulates pro-cathepsin B/annexin II complex formation and facilitates the trafficking of pro-cathespin-B-containing TGN38-positive vesicles toward the cell periphery, leading to the secretion of pro-cathepsin B, which induces metastasis. Collectively, our results uncover BAG2 as a regulator of the oncogenic function of pro-cathepsin B and a potential diagnostic and therapeutic target that may reduce the burden of metastatic breast cancer.

INTRODUCTION

Among the molecular subtypes of breast cancer, triple-negative breast cancer (TNBC) is an extremely aggressive subtype that is correlated with a poor prognosis and high mortality rates despite systemic therapy; TNBC is also a heterogeneous disease compared to either luminal or HER2-enriched subtypes (Foulkes et al., 2010; Masuda et al., 2013). Although several clinical trials targeting molecules specific to TNBC, including poly ADP-ribose polymerase (PARP), epidermal growth factor receptor (EGFR), and Src tyrosine kinase, have been conducted, they have not led to significant improvements in the treatment of patients with TNBC (Crown et al., 2012; Hudis and Gianni, 2011). Thus, there is an urgent need to discover effective therapeutic targets that improve disease outcome and prognosis in patients with TNBC and serve to improve our understanding of the molecular basis for TNBC development, progression, and metastasis.

As a member of the Bcl-2-associated athanogene (BAG) domain family with anti-apoptotic activity, BAG2 has been described as a negative regulator of the chaperone-associated ubiquitin ligase C terminus of Hsc70-interacting protein (CHIP) that participates in the ubiquitin-mediated proteasomal degradation of misfolded substrate proteins (Arndt et al., 2005; Dai et al., 2005). The main effect of BAG2 on the control of protein quality through inhibition of CHIP activity is linked to neurodegenerative disorders and autosomal-recessive disorders through protein stabilization of chaperone clients, such as PINK1 and CFTR (Arndt et al., 2005; Qu et al., 2015). However, the role of BAG2 in cancer has not been well characterized, and its role in the regulation of tumorigenesis remains controversial. It has been reported that BAG2 expression is increased in proteasome-inhibitor-induced apoptosis, and BAG2 knockdown partially inhibits cell death in thyroid carcinoma cells exposed to the proteasome inhibitor MG132, suggesting that BAG2 may have pro-apoptotic activity (Wang et al., 2008). However, considerable attention has recently been paid to the findings that overexpression of BAG2, like BAG1 and BAG3, can promote tumorigenesis. BAG2 overexpressed in various cancer cells promotes nuclear accumulation of mutant p53 by directly

binding to mutant p53 and inhibit its degradation by E3 ligase MDM2, suggesting that BAG2 drives tumor growth by enhancing the function of the mutant p53 protein (Yue et al., 2015). Despite this findings, the precise role of BAG2 in cancer progression and metastasis has not been extensively investigated, but these data suggest an important gap in our knowledge, especially in the context of breast cancers.

Cathepsin B (CTSB), a lysosomal cysteine protease possessing both endo- and exo-peptidase activity, is considered to participate in protein turnover (Mort and Buttle, 1997). CTSB is synthesized as an inactive/immature pro-form enzyme (41/43 kDa), which is converted to the active single-chain form (31 kDa) or double-chain form (heavy chain, 25/26 kDa; light chain, 5 kDa) through proteolytic processing of a 62-amino-acid propeptide of the N terminus. Aberrant expression/activity of CTSB has often been linked to progression and metastasis of malignant tumors, especially in breast cancers (Joyce et al., 2004; Withana et al., 2012). The intracellular trafficking of CTSB is frequently altered in malignant tumors, resulting in the secretion of the pro-form of CTSB. Secreted pro-CTSB is activated by cathepsin D, elastase, and by itself in acidic tumor environments, leading to the activation of other proteases, such as urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMPs), consequently facilitating tumor progression and metastasis (Olson and Joyce, 2015). Moreover, it was reported that transgenic overexpression of human CTSB led to enhanced tumor growth and lung metastasis, whereas *Ctsb*-deficient mice showed delayed tumor progression and reduced metastatic colonies in the lung (Sevenich et al., 2011; Vasiljeva et al., 2008). However, it has also been recognized that mature CTSB is normally localized in lysosomes, where it functions in protein turnover as an initiator protease during lysosome-mediated autophagy/apoptosis, indicating a pro-apoptotic role for cytosolic CTSB (Bhoopathi et al., 2010; Foghsgaard et al., 2001). Although CTSB has been extensively studied in a wide variety of human cancers, the molecular basis regulating its dual functions as either a pro-oncogenic or pro-apoptotic enzyme is poorly understood. Furthermore, regulators that control the dual functions of CTSB in cancer progression remain to be identified.

Here, we show that BAG2 promotes cancer progression by regulating the dual function of CTSB in TNBC cells, implicating the potential for BAG2 to serve as a specific target for the treatment of TNBC.

RESULTS

Co-chaperone BAG2 Is Specifically Overexpressed in TNBC Cells

In order to identify molecular targets associated with TNBC progression, we initially performed transcriptome analysis using RNA sequencing in breast cancer cell lines classified as either the luminal subtype (MCF-7, T47D, and ZR-75B) or TNBC (MDA-MB-231 and Hs578T). We found that *BAG2* was specifically overexpressed in TNBC cells compared to luminal breast cancer cell lines (Figure 1A). RT-PCR and immunoblot analyses supported the RNA-sequencing data (Figure 1B). Furthermore, gene expression analysis of a public microarray dataset (GEO: GSE41313) in 52 breast cancer cell lines showed that expression of *BAG2* was significantly higher in TNBC cells than in luminal subtypes ($p < 0.0001$; Figure 1C) (Riaz et al., 2013). To examine this more precisely, we analyzed *BAG2* expression in different subtypes using RNA-sequencing datasets of breast cancer patients obtained from Genomic Data Commons (GDC). Notably, a significantly higher level of *BAG2* expression was observed in TNBC patients compared to normal-like, luminal A, luminal B, and HER2-enriched patients ($p < 0.0001$; Figure 1D). We next examined the expression level of BAG2 protein and mRNA in human primary breast tumor specimens by immunohistochemistry staining and qRT-PCR. Consistently, expression of BAG2 protein was remarkably elevated in the tumor compartments of TNBC tissues (Figure 1E), and BAG2 mRNA was significantly increased in TNBC tissues ($p = 0.0004$; Figure 1F). We also investigated whether other BAG protein family members were overexpressed in TNBC subtypes using The Cancer Genome Atlas (TCGA) datasets and public microarray datasets (GEO: GSE41313, GSE2034, and GSE5460) (Bos et al., 2009; Lu et al., 2008). Interestingly, only *BAG2* expression was significantly upregulated in TNBC cells and patients compared to the luminal subtypes (Figure S1). Next, to assess whether BAG2 expression is associated with breast cancer progression, we investigated the expression level of BAG2 in the MCF10A series of cell lines, which mimic different stages of breast cancer progression and comprise immortalized normal-like mammary epithelial cells (MCF10A) to highly metastatic breast cancer cells (MCF10CA1a). More strikingly, BAG2 mRNA and protein expression was increased in metastatic breast cancer cells (Figure 1G). Taken together, these results suggest that BAG2 is strongly associated with tumor progression of TNBC.

Overexpression of BAG2 Is Strongly Correlated with Poor Clinical Outcomes

To address the functional significance of the association of BAG2 expression with clinicopathological parameters in breast cancer patients, we performed immunohistochemical staining of breast cancer tissue microarrays (TMAs) and scored according to the staining intensity (Figure 2A). Statistical analysis revealed the clinicopathologic relevance of high BAG2 expression in tumor grade ($p < 0.001$), ER or PR expression status ($p < 0.001$), HER2 overexpression status ($p = 0.021$), and the subtypes ($p < 0.001$) (Figure 2B; Table S1). Notably, BAG2 expression was strongly associated with TNBC (40 of 135 [29.7%]; $p < 0.001$) (Figure 2C). We further found that BAG2 overexpression was significantly correlated with poorer breast-cancer-specific survival (BSS; $p = 0.048$), recurrence-free survival (RFS; $p = 0.0359$) (Figure 2D), and distant metastasis-free survival (DMFS; $p = 0.02$; Figure S2A) in Kaplan-Meier analysis. Moreover, patients with lymph-node-positive breast cancer demonstrated a significant association between high BAG2 expression and unfavorable RFS ($p = 0.0046$; Figure 2E) and DMFS ($p = 0.0029$; Figure S2B), whereas no significant survival disadvantage was shown in patients with lymph-node-negative breast cancer. Consistent with our cohort studies, public meta-analyses using Kaplan-Meier Plotter software (Győrffy et al., 2014), which provides survival information in

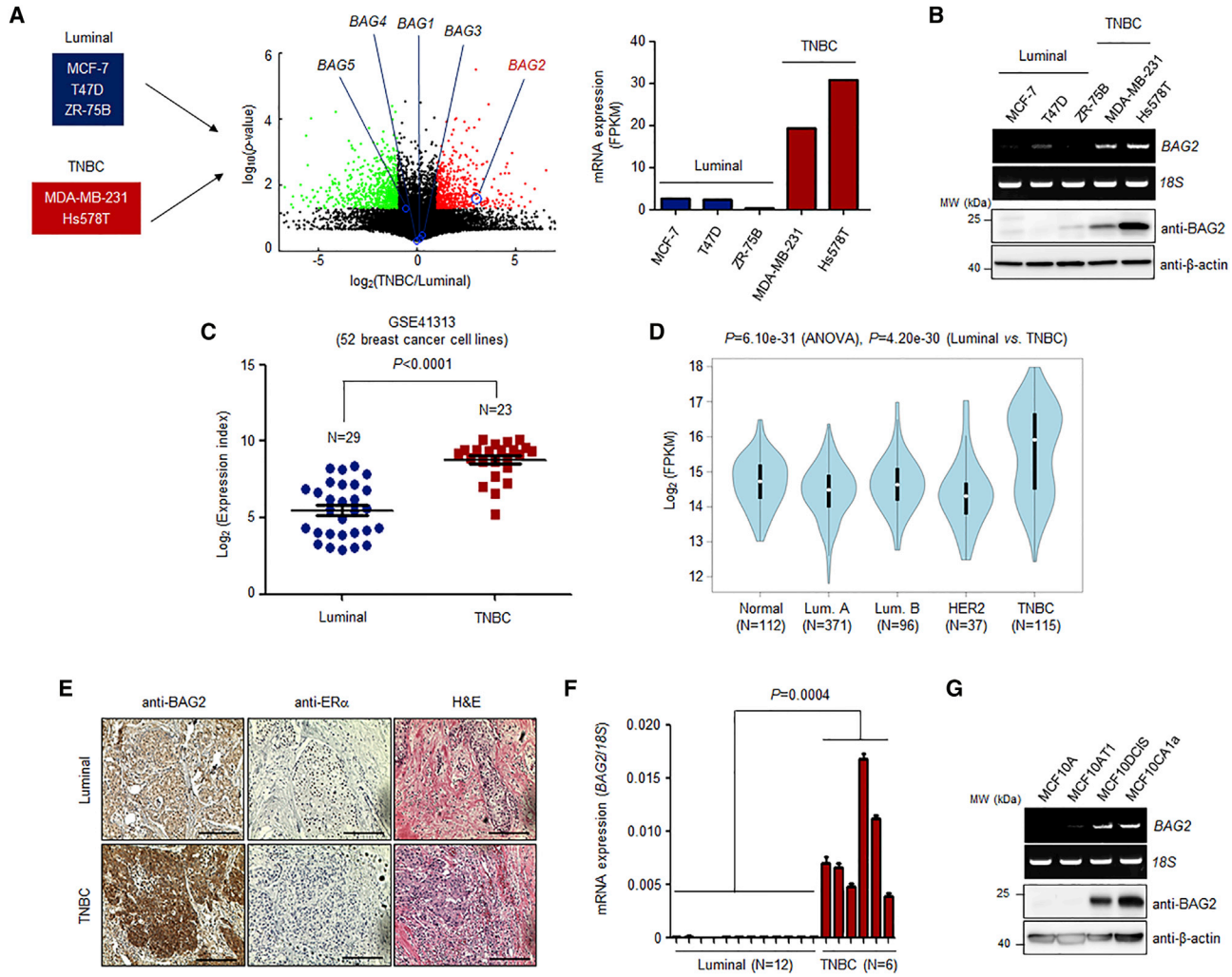


Figure 1. BAG2 Is Specifically Overexpressed in TNBC Cells and Primary TNBC Tissues

(A) Volcano plot of differential expression between luminal (MCF-7, T47D, and ZR-75B) and TNBC (MDA-MB-231 and Hs578T) cell lines using RNA sequencing.

(B) RT-PCR and immunoblot analysis of BAG2 mRNA and protein expression in luminal and TNBC cell lines.

(C) Scatter dot plots of *BAG2* expression in 52 breast cancer cell lines using published microarray datasets.

(D) Box plots of *BAG2* expression in different subtypes of breast cancer and normal tissues obtained from RNA-sequencing datasets of Genomic Data Commons (GDC).

(E) Histological analysis of BAG2 and ERα in luminal (top) and TNBC (bottom) breast cancer tissue.

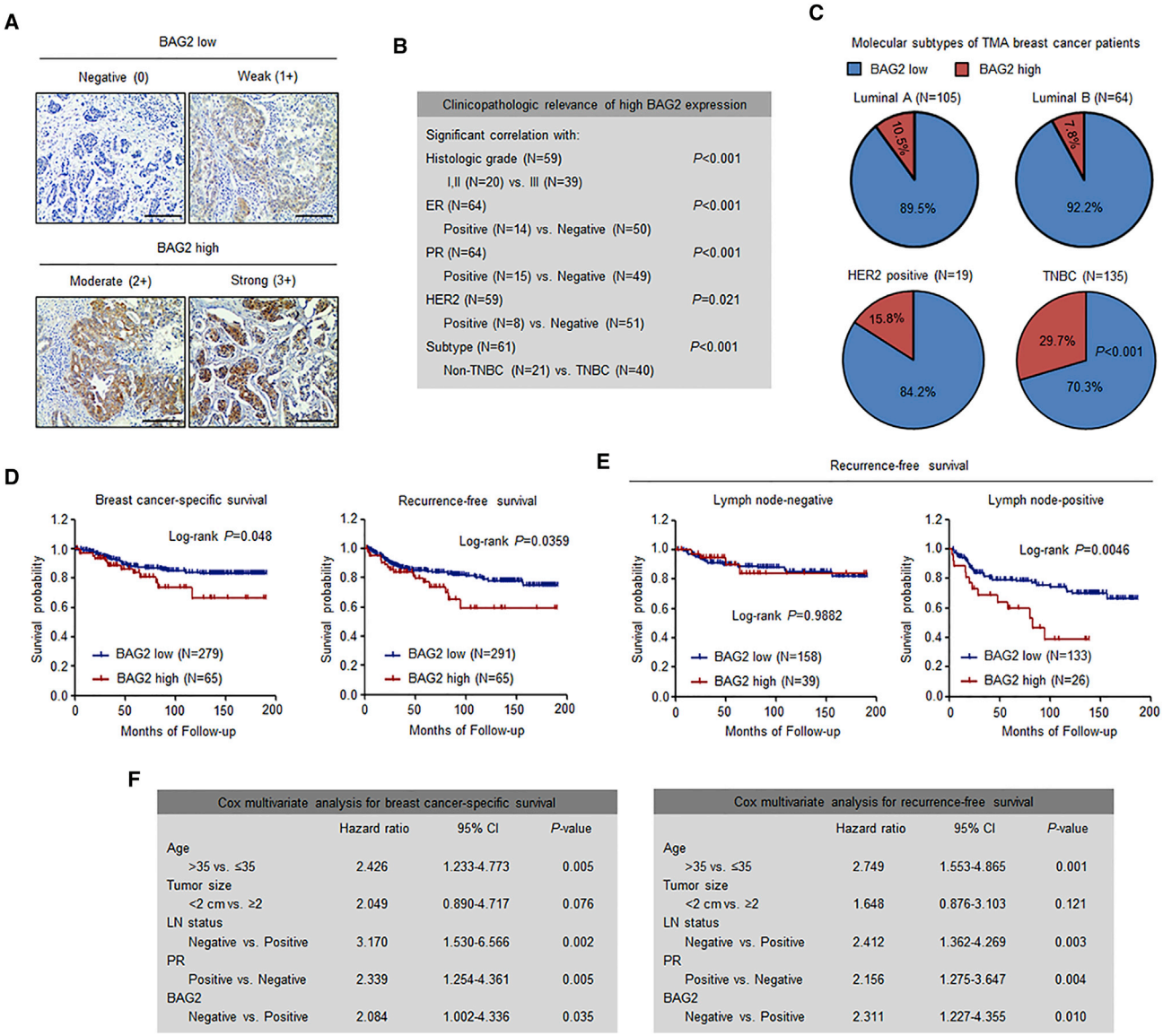
(F) qRT-PCR of BAG2 gene expression in 12 luminal tissues and 6 TNBC tissues.

(G) RT-PCR and immunoblot analysis of BAG2 mRNA and protein expression in MCF10A-derived cell lines representing degrees of metastatic potential.

more than 3,000 breast cancer patients, also revealed that patients with high *BAG2* expression had shorter survival times compared to those with low *BAG2* expression (Figures S2D–S2F). In addition, Cox multivariate regression analysis exhibited significantly increased hazard ratio (HR) in patients with high BAG2 expression for predicting BSS (p = 0.035; HR = 2.084; 95% confidence interval [CI], 1.002–4.4336), RFS (p = 0.01; HR = 2.311; 95% CI, 1.227–4.355) (Figure 2F), and DMFS (p = 0.012; HR = 2.362; 95% CI, 1.209–4.614) (Figure S2C), suggesting that BAG2 overexpression is an independent prognostic factor for breast cancer patients' chances of survival. Taken together, these findings indicate that BAG2 is strongly correlated with poor clinical outcomes and may serve as a positive predictive factor for the risk of mortality in breast cancers.

BAG2 Facilitates Tumor Growth and Lung Metastasis in TNBC Cells

To identify BAG2-associated pathways in breast cancers, we first performed gene set enrichment analysis (GSEA) on the TCGA breast cancer patient samples (N = 1,166). GSEA analysis revealed that high *BAG2* expression was positively associated with the genes involved in pathways driving breast cancer progression, such as cell proliferation (false discovery rate [FDR] = 0.006; p < 0.001) and cell migration



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Cox multivariate analysis for breast cancer-specific survival

	Hazard ratio	95% CI	*P*-value
Age			
>35 vs. ≤35	2.426	1.233-4.773	0.005
Tumor size			
<2 cm vs. ≥2	2.049	0.890-4.717	0.076
LN status			
Negative vs. Positive	3.170	1.530-6.566	0.002
PR			
Positive vs. Negative	2.339	1.254-4.361	0.005
BAG2			
Negative vs. Positive	2.084	1.002-4.336	0.035

Cox multivariate analysis for recurrence-free survival

	Hazard ratio	95% CI	*P*-value
Age			
>35 vs. ≤35	2.749	1.553-4.865	0.001
Tumor size			
<2 cm vs. ≥2	1.648	0.876-3.103	0.121
LN status			
Negative vs. Positive	2.412	1.362-4.269	0.003
PR			
Positive vs. Negative	2.156	1.275-3.647	0.004
BAG2			
Negative vs. Positive	2.311	1.227-4.355	0.010

Figure 2. Overexpression of BAG2 Has Clinical Relevance and Prognostic Value

(A) Representative images of IHC staining of BAG2 on paraffin-embedded TMAs of breast cancer tissues.

(B) Associations between high BAG2 expression and clinical parameters.

(C) Pie charts showing the percentages of low and high BAG2 expression in patients with different breast cancer subtypes.

(D) Kaplan-Meier analysis showing breast-cancer-specific survival and recurrence-free survival depending on BAG2 expression level.

(E) Kaplan-Meier analysis showing the association between BAG2 expression and recurrence-free survival according to the lymph node status.

(F) Cox proportional hazards regression model.

(FDR < 0.0001; p < 0.001) (Figure 3A). Because BAG2 expression was specifically increased in TNBC cells, we generated *BAG2*-depleted TNBC cells (Hs578T, MDA-MB-231, and MCF10CA1a) to verify the physiological function of BAG2 in the cancer progression of TNBC cells *in vitro* and *in vivo* (Figure S3A). BAG2 knockdown demonstrated anti-proliferative effect in all three TNBC cell lines (Figures 3B and S3B). We further examined whether depletion of BAG2 affected the tumorigenic capacity of TNBC cells *in vivo* through subcutaneous injection of three different TNBC cells into the flank of immunodeficient mice. Knockdown of BAG2 decreased the ability of TNBC cells to form tumors, indicating that BAG2 is required to reinforce tumorigenicity *in vivo* (Figures 3C and 3D).

As transforming potential is generally associated with enhanced invasion capability for metastasis of tumor cells, we next investigated cell invasion *in vitro* in BAG2-depleted TNBC cells. Cell invasion was significantly reduced by BAG2 knockdown (Figure S3C). We further observed that tail vein injection of BAG2-depleted MDA-MB-231 and MCF10CA1a cells with a luciferase reporter resulted in a significant reduction of lung

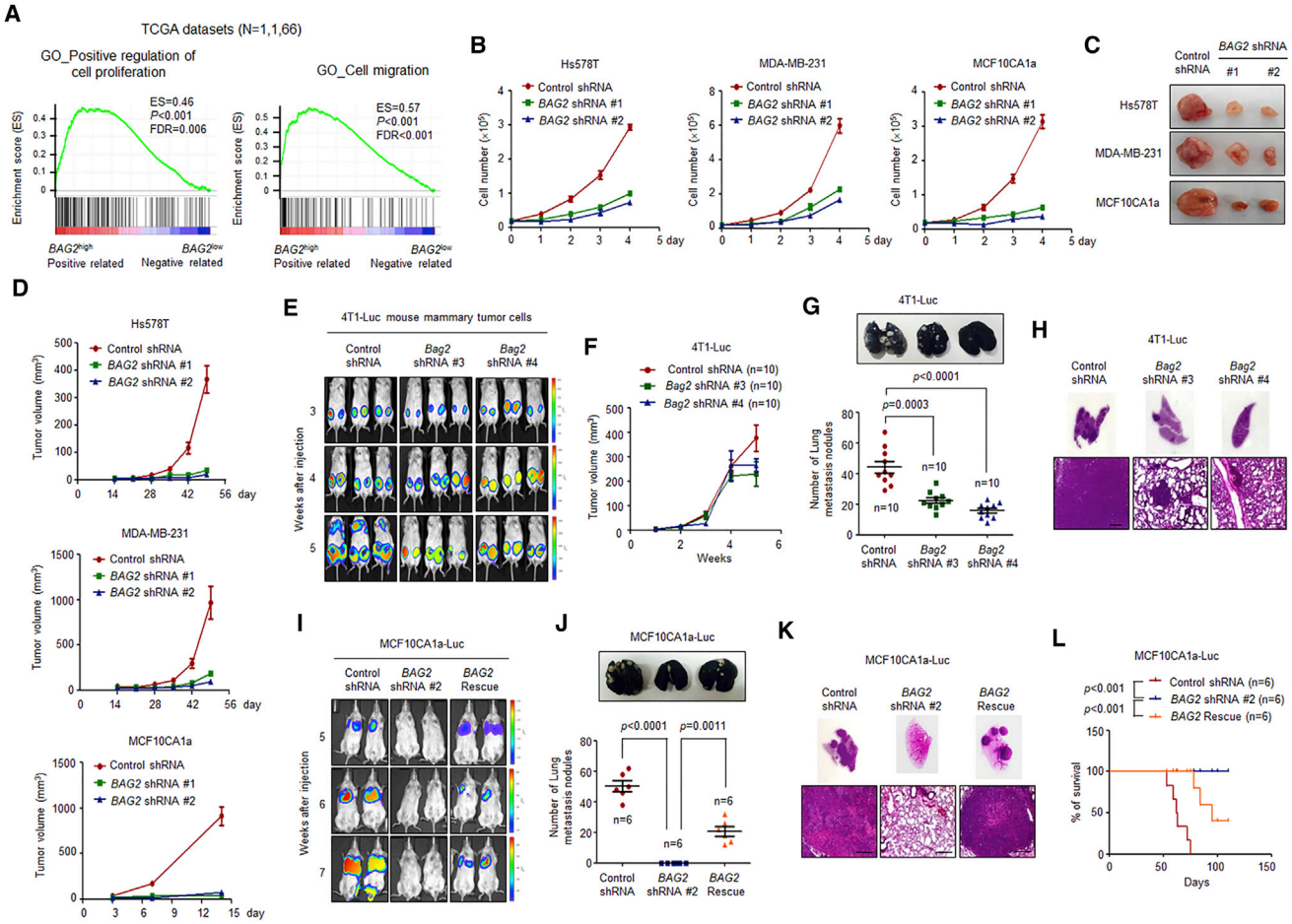

Figure 3. Depletion of BAG2 Significantly Suppresses the Tumorigenicity and Lung Metastasis of TNBC Cells

(A) Identification of gene sets enriched by GSEA in breast cancer patients with *BAG2*^{high} versus *BAG2*^{low} obtained from TCGA.

(B) Cell doublings of BAG2-depleted TNBC cells.

(C and D) Tumor formation (C) and growth (D) of control and BAG2-deficient TNBC cells subcutaneously injected into the flanks of non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (n = 6).

(E) Representative biolayer interferometry (BLI) imaging of BALB/c mice (n = 10) showing primary tumors and spontaneous lung metastases generated by control or BAG2-depleted 4T1-Luc cells.

(F) Tumor volume curves showing primary tumor growth at 5 weeks postinjection.

(G) Representative whole-lung image stained with India ink showing metastatic nodules (top) and scatterplot on the number of spontaneous lung metastatic nodules (bottom).

(H) Representative image of H&E staining of lungs showing spontaneous lung metastatic nodules.

(I) Representative BLI imaging of NOD/SCID mice (n = 6) showing lung metastasis from control and BAG2-depleted MCF10CA1a-Luc cells along with BAG2 rescue.

(J) Representative image of lungs stained with India ink.

(K) Representative image of H&E staining of lungs showing metastatic nodules.

(L) Kaplan-Meier survival curves of mice from (I).

metastasis and prolonged survival in immunodeficient mice (Figures S3D–S3I). Consistent with previous observations, we found that Bag2 knockdown decreased invasiveness of 4T1-Luc cells, which are highly metastatic murine breast cancer cells (Figures S3J and S3K). Moreover, orthotopic transplantation of Bag2-deficient 4T1-Luc cells into the mammary fat pad resulted in a significant inhibition of primary tumor growth as well as spontaneous lung metastasis (Figures 3E–3H). To ensure the possibility that BAG2 may directly influence lung metastasis of TNBC cells, we rescued BAG2-depleted MCF10CA1a-Luc cells via stable overexpression of *BAG2*-specific small hairpin RNA (shRNA)-resistant Myc-tagged BAG2 (Figures S3L). Interestingly, BAG2-rescued cells significantly restored attenuated invasion capability of BAG2-depleted cells to the control levels (Figure S3M). In accordance with this observation, the re-introduction of BAG2 prominently rescued BAG2 knockdown effects of decreased lung metastasis and prolonged survival of mice (Figures 3I–3L). Taken together, these *in vitro* and *in vivo* results

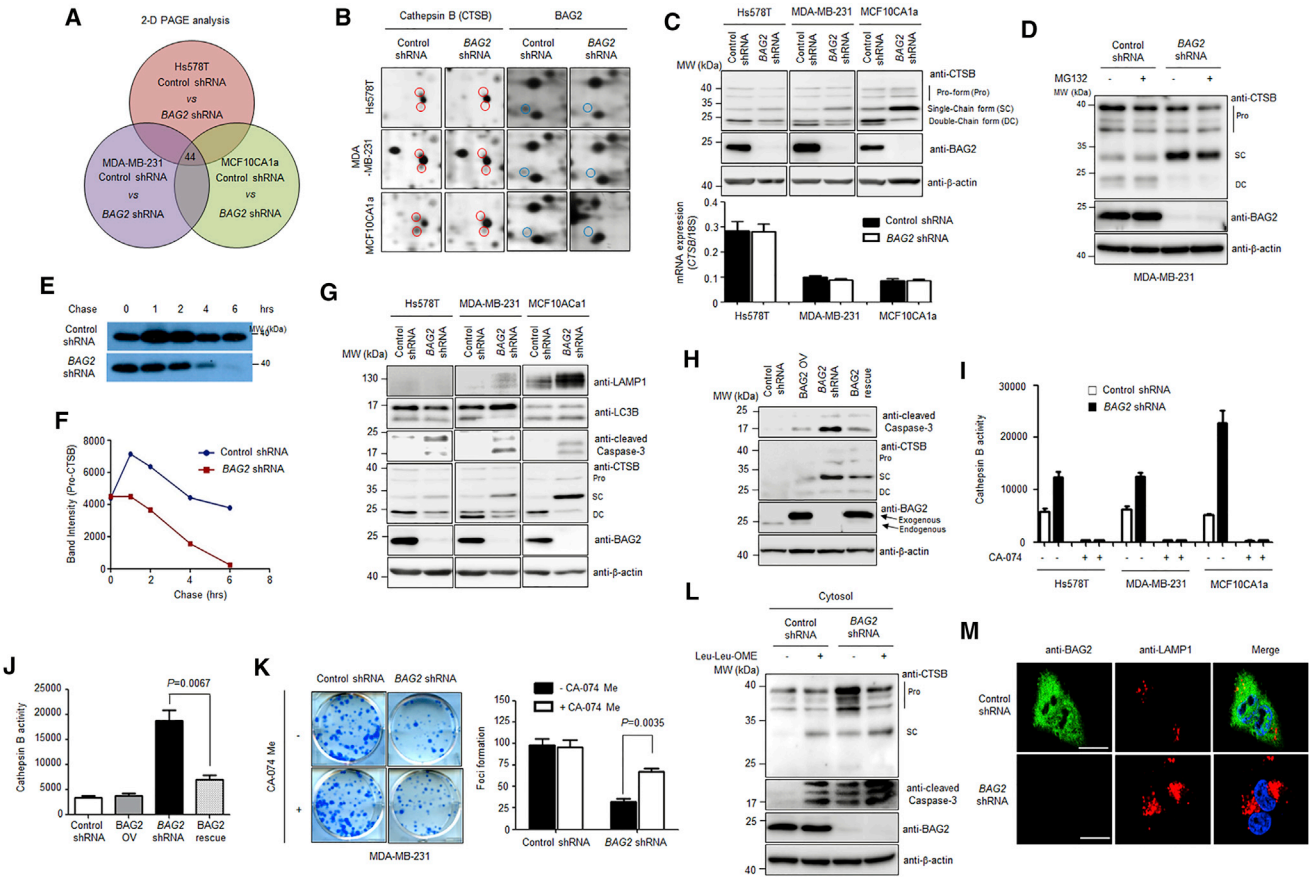

Figure 4. Depletion of BAG2 Induces Lysosomal-Mediated Cell Death by Releasing the Single-Chain Form of CTSB into the Cytosol and Impairing Lysosome Biogenesis

(A) Venn diagram showing the number of downregulated spots by BAG2 knockdown at the intersection of three different TNBC cell lines through 2-DE analysis.
(B) Magnified 2-DE gel image displaying the spots of CTSB (double-chain form; red) and BAG2 (blue) proteins.
(C) Immunoblot analysis (top) and qRT-PCR (bottom) of CTSB and BAG2 in control and BAG2-depleted TNBC cells.
(D) Immunoblot analysis of MDA-MB-231 cells treated with MG132.
(E) Pro-CTSB biogenesis in control and BAG2-depleted MDA-MB-231 cells.
(F) Densitometry of the pro-CTSB bands in (E) using ImageJ software.
(G) Immunoblot analysis of control and BAG2-depleted TNBC cells.
(H) Immunoblot analysis of MCF10CA1a-Luc cells stably expressing control shRNA, BAG2 protein, *BAG2*-specific shRNA, or BAG2 rescue.
(I) CTSB activity of control and BAG2-depleted TNBC cells.
(J) CTSB activity of each fraction eluted from (H).
(K) Focus-forming assay of BAG2-depleted TNBC cells.
(L) Immunoblot analysis of control and BAG2-deficient cells treated with or without Leu-Leu-OME.
(M) Immunofluorescence analysis of endogenous BAG2 (green) and LAMP-1 (red) in control and BAG2-depleted MDA-MB-231 cells.

strongly suggest that BAG2 is critical for tumorigenesis and metastatic progression of TNBC.

Knockdown of BAG2 Induces Cell Death by Impairing CTSB Activation

In order to assess the molecular mechanism behind BAG2-mediated TNBC progression and to ascertain the BAG2 client proteins, we performed 2-dimensional electrophoresis (2-DE) analysis using control and BAG2-depleted TNBC cells. Through comparative analysis, we identified 44 downregulated spots by BAG2 knockdown at the intersection of three different TNBC cells (Figures 4A). Through mass spectrometric analysis, the double-chain (DC) form of cathepsin B (CTSB) was identified as the highest-ranked downregulated protein (Figures 4B). First, we found that BAG2 knockdown decreased protein expression of DC CTSB without affecting mRNA expression (Figure 4C). Most interestingly, among the pro- (41/43 kDa), single-chain (SC; 31 kDa), and DC (24/25 kDa) forms of CTSB, SC CTSB was markedly increased by BAG2 knockdown, whereas DC CTSB was decreased. We further examined whether the decrease of DC CTSB by BAG2 knockdown was induced by proteasomal degradation. MG132 treatment did not increase protein stability of DC CTSB in both control and BAG2-depleted MDA-MB-231 cells, indicating that BAG2 may be associated

with the cleavage processing of pro-CTSB rather than the proteasomal degradation (Figure 4D). Because pro-CTSB is converted into the mature SC and DC forms through rapid biogenesis, we speculated that immunoblotting analysis might not be able to detect BAG2-knockdown-induced changes in the total expression of pro-CTSB. Therefore, we evaluated early events in pro-CTSB biogenesis by pulse-chase experiments. Indeed, BAG2 depletion markedly decreased the level of labeled pro-CTSB in a time-dependent manner compared to control cells, implying the rapid conversion of pro-CTSB into mature SC CTSB (Figures 4E and 4F). Furthermore, we investigated whether BAG2 knockdown affected cleavage processing of other lysosomal enzymes such as cathepsin D and legumain. Interestingly, mature form of cathepsin D, which is active, was decreased by BAG2 depletion in Hs578T and MCF10CA1a cells but increased in MDA-MB-231 cells. However, depletion of BAG2 did not influence the cleavage processing of legumain, suggesting that BAG2 may specifically regulate pro-CTSB cleavage processing in TNBC cells, at least among lysosomal enzymes (Figure S4A). In addition, it has been known that BAG2 modulates Hsp70/Hsc70- and Hsp90-mediated chaperone expression/activity through direct interaction (Arndt et al., 2005). Unlike BAG2, depletion of Hsc70/Hsp90 did not influence the SC form of CTSB, indicating that BAG2-mediated cleavage of pro-CTSB may be independent of Hsp70/Hsc70- and Hsp90-mediated chaperone expression (Figures S4C–S4E).

We next investigated whether BAG2 knockdown affected lysosomal-mediated cell death by increasing intracellular mature SC CTSB. Interestingly, BAG2 knockdown increased expression of cleaved caspase-3, a pivotal mediator of apoptosis, without affecting LC3B expression, a marker of autophagy induction (Figure 4G). This was further corroborated by the finding that re-introduction of BAG2 to BAG2-deficient MCF10CA1a cells significantly reduced the expression of active casepase-3 and mature SC CTSB (Figure 4H). Because the activity of lysosomal mature CTSB plays a role in the activation of caspase-3, we next tested the activity of CTSB by using synthetic Arg-Arg, a substrate sequence for mature CTSB. CTSB activity was significantly increased by BAG2 knockdown (Figure 4I), and intracellular active CTSB was localized throughout the cytoplasm of BAG2-deficient cells compared to control cells (Figure S5A). Furthermore, BAG2 regulation of CTSB activity was further confirmed by observing that increased CTSB activity by BAG2 knockdown was significantly attenuated in BAG2-rescued cells (Figure 4J). Considering that both mature SC and DC forms of CTSB generally possess proteolytic activity, we examined intracellular CTSB activity using the fractions obtained from size exclusion chromatography to determine the active form of mature CTSB in BAG2-deficient cells. Indeed, in control cells, fractions 7–8, in which the DC form was detected, exhibited lower CTSB activity than fractions 9–11, in which the SC form was observed, suggesting that the DC form of CTSB is less active than the SC form (Figures S5B and S5C). We next investigated foci formation of MDA-MB-231 cells using CA-074 Me, a specific cell-permeable inhibitor of active CTSB, to see whether BAG2-knockdown-mediated activation of SC CTSB directly induces inhibition of cell growth. Reduction of foci formation by BAG2 depletion was moderately rescued by CA-074 Me in BAG2-deficient cells (Figure 4K). Furthermore, to examine whether BAG2 depletion induces the release of active SC CTSB into the cytosol through lysosomal leakage, we examined the cytosolic active SC CTSB using Leu-Leu-OME, a lysosomotropic agent. The increase in expression levels of cytosolic SC CTSB and cleaved caspase-3 after BAG2 knockdown was similar to that produced by Leu-Leu-OME in control cells (Figure 4L). Also, foci formation was decreased by Leu-Leu-OME in both control and BAG2-deficient cells (Figure S6A). Collectively, these results indicate that BAG2 may maintain TNBC cell survival by inhibiting the release of active SC CTSB into the cytosol and subsequent caspase-3-mediated apoptosis through blockage of lysosomal leakage.

As pro-CTSB is activated by auto-catalytic processing in an acidic environment, we next tested whether BAG2-regulated auto-catalytic processing of pro-CTSB was dependent on intracellular lysosomal acidification. BAG2 depletion created an enhanced acidic environment, as shown by increased acridine-orange-induced red fluorescence staining under exciting blue light (Figure S6B), and bafilomycin A1, an inhibitor of vacuolar H^+-ATPase, suppressed the BAG2-depletion-mediated increase in SC CTSB (Figure S6C). Interestingly, depletion of BAG2 caused a significant increase in lysosomal-associated membrane protein 1 (LAMP-1) along with SC CTSB (Figures 4G). Consistent with this observation, confocal microscopy imaging showed that BAG2 knockdown dramatically increased the abundance of LAMP-1-positive lysosomes and induced the accumulation of LAMP-1 around the perinuclear region, indicating that BAG2 depletion may inhibit TNBC cell growth by impairing lysosome biogenesis (Figures 4M and S6D). Taken together, our findings suggest that knockdown of BAG2 induces apoptosis through cytosolic release of increased amounts of the mature SC form of CTSB and impairs lysosome biogenesis, thereby positioning BAG2 as an important negative regulator in intracellular active SC-CTSB-mediated apoptosis in TNBC cells.

BAG2 Prevents Auto-cleavage of Pro-CTSB by Binding to the Propeptide Region

We next sought to elucidate the underlying mechanism by which BAG2 regulates the auto-cleavage processing of pro-CTSB. To investigate whether BAG2 interacts with pro-CTSB, we first performed an *in situ* proximity ligation assay (PLA). Confocal imaging showed that BAG2 interacted with CTSB in the cytoplasm (Figure 5A). Because the CTSB antibody recognizes both pro-CTSB and the mature forms of CTSB (SC and DC), we also performed size exclusion chromatography and an immunoprecipitation assay to identify the specific form of CTSB to which BAG2 bound. Size exclusion chromatography revealed that BAG2 and pro-CTSB remained in the same fraction (sample 5 and 6), whereas the SC or DC forms did not, suggesting that BAG2 and pro-CTSB co-exist in the same complexes (Figure 5B). Furthermore, anti-BAG2 immunocomplexes demonstrated an endogenous interaction with pro-CTSB, but not with SC, DC CTSB (Figure 5C), and other lysosomal cathepsin family members, such as cathepsin D (Figure S4B). Considering that pro-CTSB contains the propeptide region (PPR), which includes the cleavage site for its conversion to the SC CTSB, we assumed that BAG2 might interact with the PPR of pro-CTSB

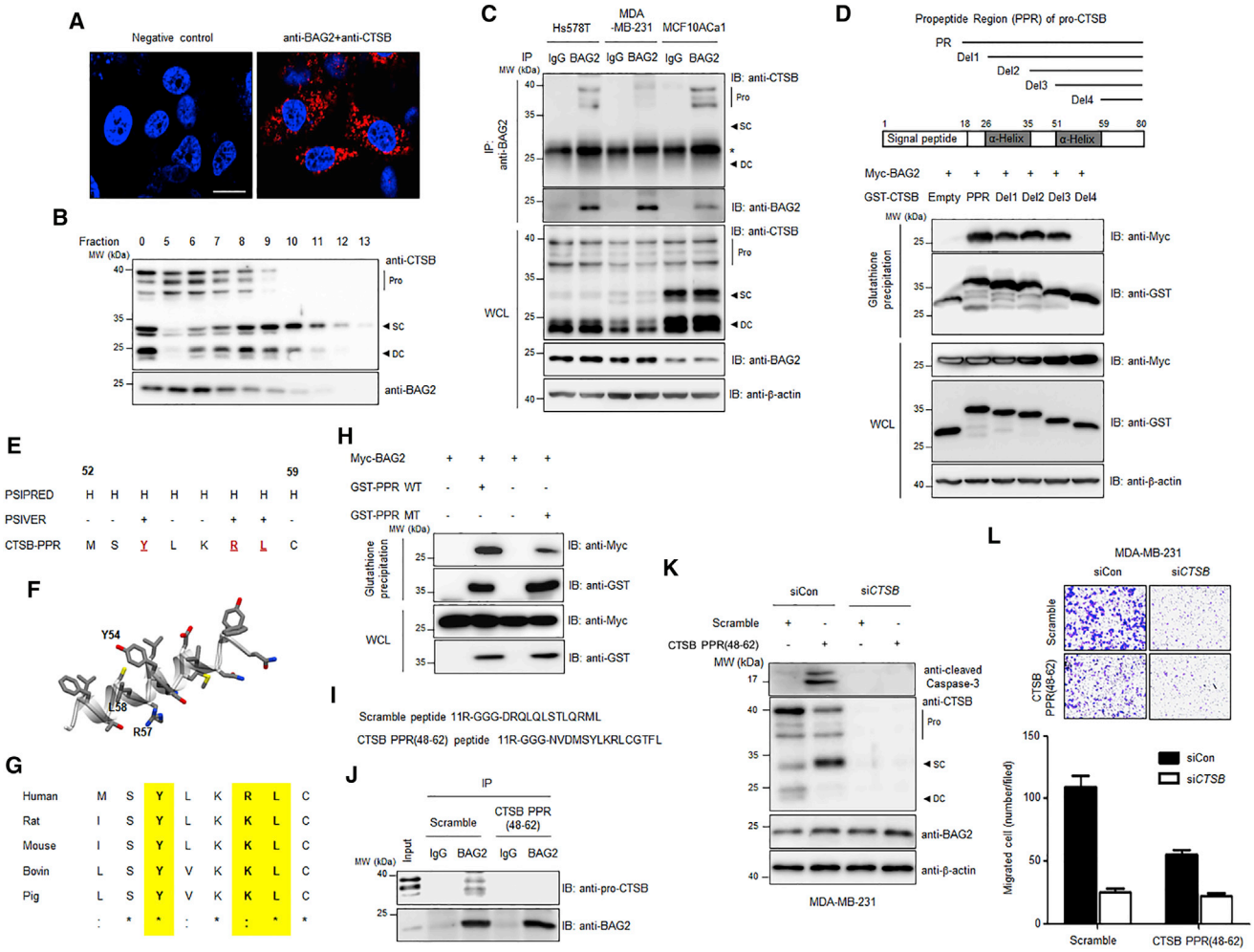

Figure 5. BAG2 Regulates Auto-cleavage of Pro-CTSB by Binding to the Propeptide Region

(A) Confocal image of *in situ* PLA showing the interaction (red) between BAG2 and CTSB in MDA-MB-231 cells.
(B) Size distribution of CTSB following size exclusion chromatography.
(C) Immunoprecipitation assay showing the endogenous interaction between BAG2 and CTSB in TNBC cells.
(D) GST pull-down assay of Myc-tagged BAG2 bound to glutathione S-transferase (GST)-fused deletion mutants of the propeptide region of pro-CTSB.
(E) The predicted interaction sites determined by PSIVER software.
(F) The putative BAG2-binding sites (Y54, R57, and L58) on a predicted three-dimensional structure of CTSB PPR.
(G) The conserved sequence in CTSB PPR among different mammalian species.
(H) Immunoprecipitation assay showing BAG2-binding region in CTSB PPR.
(I) Peptide sequences of scramble or CTSB PPR (48–62).
(J) Immunoprecipitation assay showing the inhibitory effect of CTSB PPR (48–62) peptide.
(K) Immunoblot analysis of MDA-MB-231 cells treated with CTSB PPR (48–62) peptide.
(L) Transwell migration assay showing the inhibitory effect of cell migration of CTSB PPR (48–62) peptide in MDA-MB-231 cells.

to regulate cleavage processing. Indeed, an immunoprecipitation assay showed that BAG2 specifically bound to the PPR of pro-CTSB (Figures S7A and S7B). We further identified that residues 51–58 in the second alpha helix of the PPR, which contains the intermediate starting site of auto-cleavage processing, interacted with BAG2, whereas residues 59–80 in the PPR, which include the major cleavage site of pro-CTSB, failed to interact with BAG2 (Figure 5D). Moreover, we found that pro-CTSB bound to the BAG domain of BAG2, which harbors the dual functions of nucleotide exchange and client binding, and the coiled-coil domain of BAG2 failed to interact with pro-CTSB (Figure S7C). In parallel with this approach, we attempted to predict the specific protein-binding sites in the PPR of pro-CTSB using PSIVER software (Murakami and Mizuguchi, 2010). We identified a cluster of three candidate binding residues (Y54, R57, and L58), which were oriented in the same direction, displayed the highest score ($\geq$0.56) (Figures 5E and 5F), and are highly conserved in different mammalian species (Figure 5G). Interestingly, the mutation in the PPR of pro-CTSB markedly reduced its interaction with BAG2, indicating that the Y54,

R57, and L58 residues are critical for the interaction between BAG2 and pro-CTSB (Figure 5H). Next, we investigated the possibility of a cell-penetrating peptide of CTSB PPR as a dominant-negative inhibitor targeting the interaction between BAG2 and pro-CTSB (Figure 5I). Indeed, immunoprecipitation experiments revealed that the peptide CTSB PPR (48–62) completely inhibited the complex formation of endogenous BAG2 with pro-CTSB (Figure 5J). In addition, treatment of the peptide CTSB PPR (48–62) markedly induced not only an increase in active caspase-3 and SC CTSB but also a decrease in the ability of MDA-MB-231 cells to migrate (Figures 5K and 5L). Taken together, these results suggest that BAG2 prevents auto-cleavage processing of pro-CTSB through direct interaction with the PPR of pro-CTSB, eventually inhibiting the conversion of CTSB from the immature pro-form to the mature SC form.

BAG2 Interacts with the Pro-CTSB/Annexin II Complex and Regulates Pro-CTSB Secretion on the Surface of TNBC Cells

Studies have shown that the interaction between pro-CTSB and the annexin II tetramer on the surface of tumor cell plays an important role in extracellular proteolysis, which promotes tumor progression and metastasis (Mai et al., 2000; Olson and Joyce, 2015). We hypothesized that BAG2 might control the activation of pro-CTSB on the cell surface of TNBC cells by regulating the pro-CTSB/annexin II complex. To test our hypothesis, we initially analyzed the biotinylated cell surface fractions by immunoblotting. BAG2 knockdown led to a significant reduction in pro-CTSB expression on the membrane and cell surface (Figure 6A). Most interestingly, BAG2, pro-CTSB, and SC CTSB were all localized on the cell surface. Thus, we examined whether BAG2 bound to the cell-surface complex of pro-CTSB/annexin II. Anti-CTSB immunocomplexes demonstrated an endogenous interaction with BAG2 along with annexin II (Figure 6B), and the PPR of CTSB was responsible for the complex formation with BAG2 and annexin II, as well as S100A10, a light chain of annexin II (Figures 6C–6F). Interestingly, BAG2 regulated the complex formation of pro-CTSB and annexin II on the cell surface, which may ultimately facilitate the activation of pro-CTSB (Figure 6G). Next, to further determine whether BAG2 regulates pro-CTSB at the surface of TNBC cells, we treated MDA-MB-231 cells with Versene solution to disrupt Ca^{2+}-dependent annexin II. Interestingly, the Versene-wash fractions of control cells contained pro-CTSB, annexin II, and BAG2, whereas BAG2 knockdown significantly reduced the level of detached pro-CTSB from the cell surface, but not that of annexin II, suggesting that BAG2 may regulate the secretion of pro-CTSB (Figure 6H). Secreted CTSB activates MMPs by inducing their cleavage, and MMPs in turn degrade components of the extracellular matrix (ECM). We observed decreased MMP-2 activity but unaffected protein levels of endogenous MMP-2 after BAG2 knockdown (Figure 6I). Interestingly, immunoblotting using concentrated supernatants demonstrated that only pro-CTSB was detected in the supernatants of control TNBC cells, whereas BAG2 depletion substantially reduced the expression level of pro-CTSB in the supernatants of TNBC cells (Figure 6J). More strikingly, increased extracellular levels of both BAG2 and pro-CTSB were observed when BAG2 was overexpressed in MCF10AT1 cells, which express low levels of BAG2 mRNA and protein (Figure 6K), and serum levels of BAG2 were significantly higher in patients with breast cancer than in healthy volunteers ($p = 0.0013$), suggesting that BAG2 secretion may also serve as a predictive factor in breast cancer (Figure 6L). Taken together, these results indicate that BAG2 regulates not only the interaction between pro-CTSB and annexin II tetramers on the TNBC cell surface but also pro-CTSB secretion, which induces the degradation of ECM components by activating extracellular proteases, eventually contributing to the promotion of cancer metastasis.

BAG2 Controls Pro-CTSB Trafficking through the *trans*-Golgi Network

Considering that pro-CTSB is rapidly secreted from the cell via the secretory pathway, which is mainly associated with the *trans*-Golgi network (TGN) (Linke et al., 2002), we initially investigated the distribution of pro-CTSB in subcellular organelles. BAG2 and pro-CTSB were abundant in the Golgi/ER fraction in control cells, whereas depletion of BAG2 led to increased lysosomal distribution of the SC form of CTSB and LAMP-1 (Figure 7A). Most intriguingly, BAG2 knockdown led to significantly elevated Golgi/ER distribution of pro-CTSB and TGN38, a TGN marker, without affecting the expression of PDI-1, an ER marker, and GM130, a *cis*-Golgi marker, suggesting that extracellular trafficking of both pro-CTSB and TGN38 might be interrupted by the depletion of BAG2. This was further supported by an immunofluorescence assay showing that TGN38 was broadly co-localized with CTSB and BAG2 in the cytoplasm of control cells, whereas BAG2 depletion markedly induced retrograde movement of both CTSB and TGN38 from the cell periphery to the perinuclear region (Figure 7B). Considering that BAG2 may regulate anterograde trafficking of TGN38-positive vesicles, which contain pro-CTSB, toward the cell periphery and that Golgi extension reflects TGN38 trafficking to promote vesicular release, we hypothesized that BAG2-mediated secretion of pro-CTSB may be controlled by the secretory capacity of TGN38-positive vesicles. Therefore, we investigated Golgi vesicular release by tracking TGN38-positive vesicles enclosing pro-CTSB in the cytoplasm through a Golgi exit assay. Interestingly, BAG2 depletion significantly reduced the trafficking of pro-CTSB-containing TGN38-positive vesicles from the Golgi toward the cell periphery (Figure 7C), consistent with previous results that pro-CTSB was not detected on the cell membrane or extracellular space upon BAG2 depletion as a consequence of impaired TGN function. Taken together, our findings suggest that BAG2 facilitates the secretion of pro-CTSB through TGN38-positive vesicles, thereby promoting the metastatic potential of TNBC cells by enhancing the secretory capacity of the Golgi (Figure 7D).

DISCUSSION

In this study, our findings suggest a unique role for BAG2 as a crucial regulator of the dual functions of its client protein, CTSB, facilitating the progression of TNBC. BAG2 is highly expressed in human TNBC cell lines and TNBC patient cohorts and is closely associated with recurrence- and metastasis-free

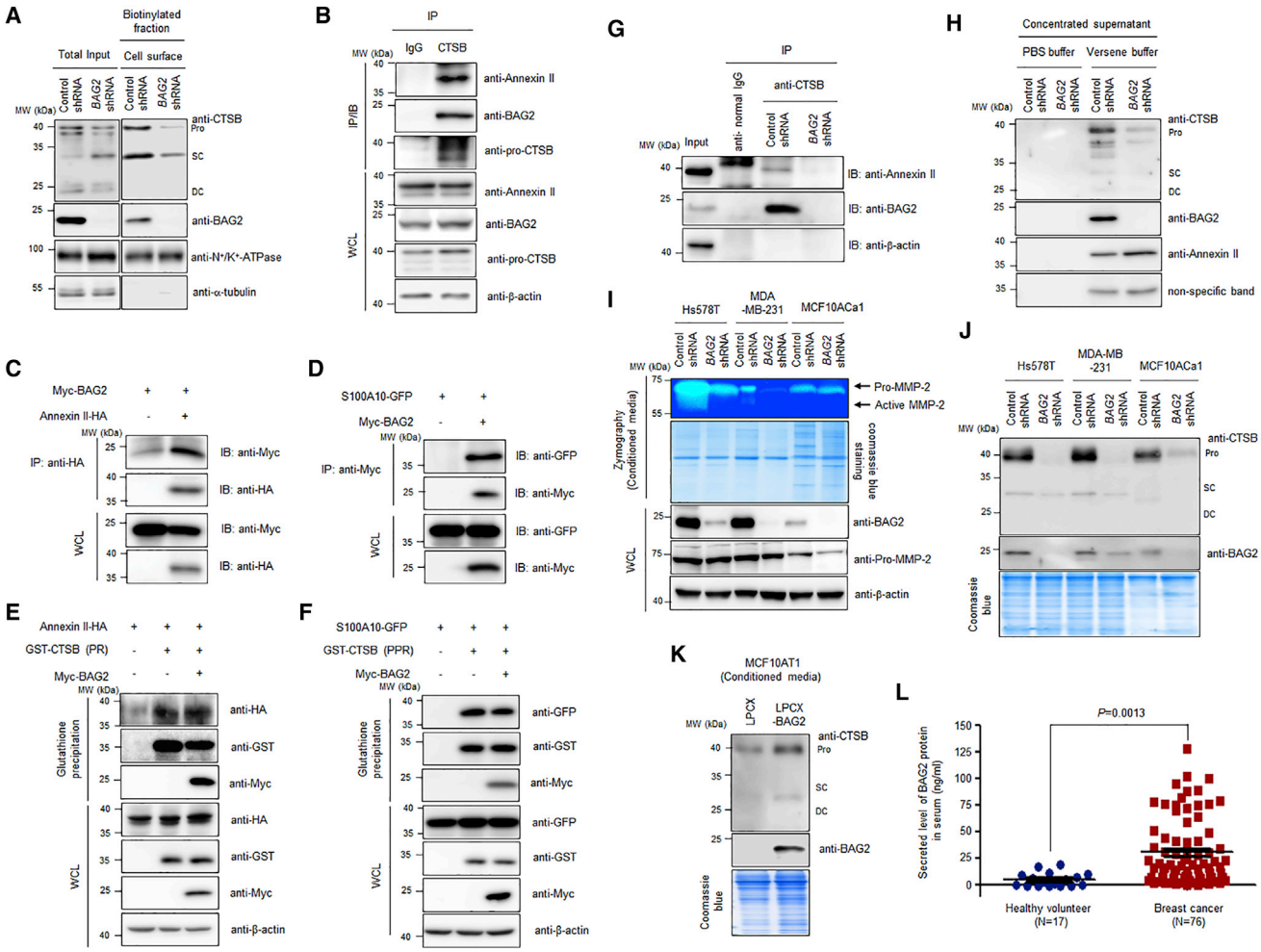

Figure 6. BAG2 Interacts with the Pro-CTSB/Annexin II Complex on the Surface of TNBC Cells

(A) Immunoblot analysis showing biotinylated plasma membrane fraction in control and BAG2-depleted MDA-MB-231.

(B) Immunoprecipitation assay for endogenous interaction between BAG2 and annexin II along with pro-CTSB in MDA-MB-231 cells.

(C and D) Immunoprecipitation assay to verify the interaction region between BAG2 and annexin II (C) or S100A10 (D).

(E and F) GST pull-down assay of Myc-tagged BAG2 and C-terminal hemagglutinin (HA)-tagged annexin II (E) or GFP-tagged S100A10 (F) bound to the GST-fused propeptide region (PPR) of pro-CTSB.

(G) Immunoprecipitation assay for endogenous interaction between CTSB and annexin II in control and BAG2-depleted MDA-MB-231 cells.

(H) Immunoblot analysis showing CTSB, BAG2, and annexin II bound to the cell surface in a Ca^{2+}-dependent manner.

(I) The supernatants were analyzed by gelatin zymography for MMP-2 activation.

(J) Immunoblot analysis showing secreted pro-CTSB and BAG2 in conditioned media of control and BAG2-depleted TNBC cells.

(K) Immunoblot analysis showing secreted pro-CTSB and BAG2 in conditioned media of control and BAG2-overexpressed MCF10AT1 cells.

(L) ELISA analysis showing BAG2 protein levels in the serums of healthy volunteers (n = 17) and malignant breast cancer patients (n = 76). p values were calculated by unpaired two-tailed Student's t tests. Data represent means ± SD of three independent experiments.

survival, implying that patients with high BAG2 expression are at risk of developing recurrence and metastatic disease. Mechanistically, BAG2 has been shown to impair auto-cleavage processing of pro-CTSB and facilitate the secretion of pro-CTSB through TGN-positive vesicles, eventually resulting in enhanced cell death and attenuated metastasis.

Although it is recognized that BAG2 is predominantly localized in the cytoplasm, a recent study reported that BAG2 binds to mutant p53 proteins and promotes their translocation from the cytoplasm to the nucleus in various cancer cells (Yue et al., 2015). Considering that mutant p53 is the most frequent genetic alteration found in breast cancer and is positively associated with more aggressive breast cancers, it is possible that BAG2 may be predominantly localized in the nucleus of TNBC cells. Unexpectedly, BAG2 was mainly localized in the cytoplasm, regardless of either breast cancer subtype or mutant p53 status, as demonstrated by subcellular fractionation and confocal imaging (data not shown). Therefore, our findings suggest that a cytoplasmic function of BAG2 overexpressed in TNBC cells promotes tumorigenesis and metastasis, regardless of mutant p53 status.

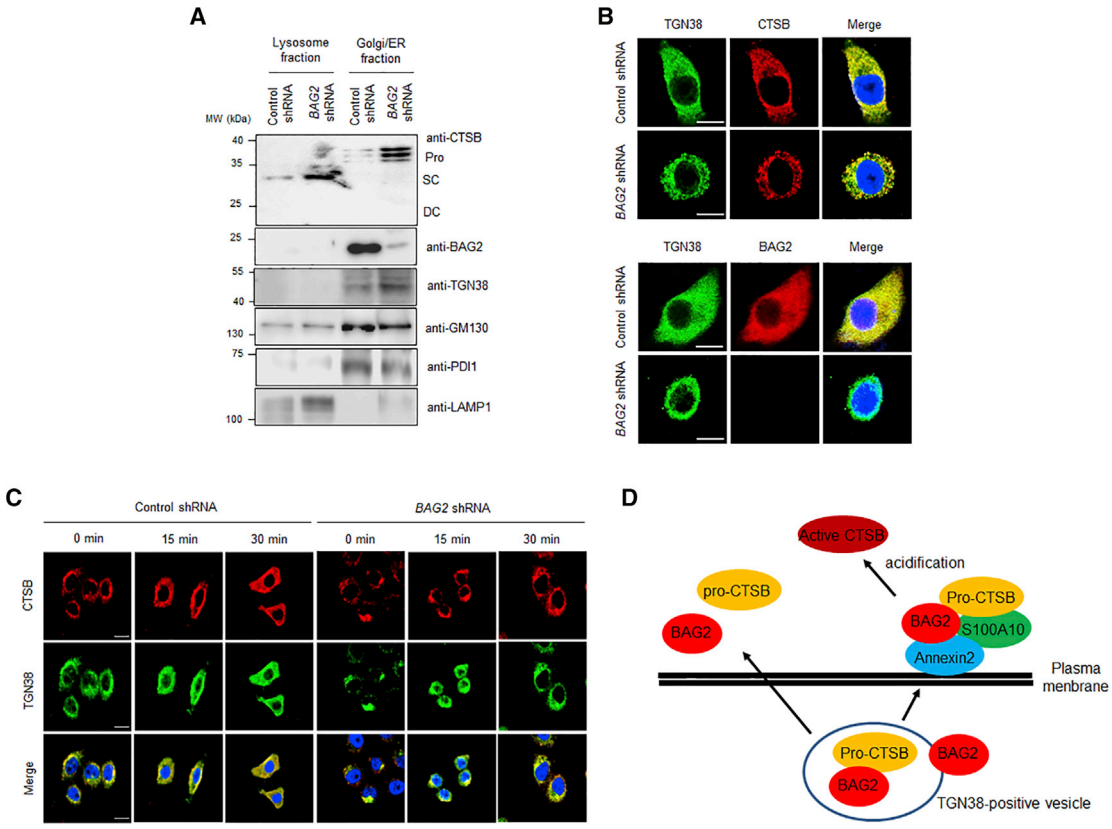

Figure 7. BAG2 Facilitates Pro-CTSB Secretion through Regulation of TGN Trafficking

(A) Immunoblot analysis showing lysosome and Golgi/ER fractions of control and BAG2-depleted MDA-MB-231 cells.

(B) Immunofluorescence analysis of endogenous TGN38, CTSB, and BAG2.

(C) Golgi exit assay of control and BAG2-depleted MDA-MB-231 cells.

(D) Schematic diagram of pro-CTSB trafficking regulated by BAG2.

Based on proteomic analysis, we identified CTSB as a BAG2 client protein. Many studies have shown that redistribution of CTSB in cancer cells can affect the ECM directly by causing its proteolytic degradation or indirectly by activating other extracellular-matrix-degrading proteases, such as uPA and MMPs in acidic tumor environments, eventually facilitating tumor progression and metastasis (Gondi and Rao, 2013). On the contrary, recent studies have also reported that CTSB contributes to the induction of autophagy/apoptosis, linking the pro-apoptotic role of cytosolic CTSB as an initiating protease to the activation of Bid or caspases in lysosome-mediated cell death (Chwieralski et al., 2006). Therefore, considering these likely context-dependent dual roles of CTSB as either a tumor promoter or tumor suppressor, the identification of regulators and the specific mechanisms controlling the dual functions of CTSB is crucial. In this study, we identified BAG2 as a regulator of the dual functions of CTSB. First, we found that BAG2 depletion markedly increased the active/mature SC form of CTSB, whereas it induced a decrease in the pro- and DC forms of CTSB. However, given that either the DC form or both the SC and DC forms of CTSB are active in purified CTSB (Moin et al., 1992), the relative activity of the two forms of CTSB may be observed in a context-dependent manner.

In addition, we demonstrated that BAG2 knockdown led to the release of lysosomal active SC CTSB into the cytosol and a subsequent increase in executioner caspase-3-mediated apoptosis, indicating that BAG2 may prevent the growth inhibition of TNBC cells by protecting the conversion of inactive pro-CTSB to the active SC form. Besides the pro-apoptotic role of intracellular mature SC CTSB, pro-CTSB is constitutively secreted into the extracellular space in malignant tumors. This secreted pro-CTSB can be activated by other membrane-associated proteases and is bound on the tumor cell surface; subsequently, membrane-associated pro-CTSB can be activated by other soluble proteases, thereby degrading ECM components and facilitating metastasis (Gondi and Rao, 2013; Mohamed and Sloane, 2006). It is also known that the binding of pro-CTSB to the annexin II tetramer, which is a Ca^{2+}-dependent phospholipid-binding protein that is known to be upregulated on the surface of tumor cells, results in the activation of pro-CTSB on the tumor cell surface (Mai et al., 2000). In this regard, we confirmed that BAG2 interacted with annexin II along with the PPR of pro-CTSB and that its complex was present on the cell surface in a Ca^{2+}-dependent manner, suggesting that BAG2 may mediate a link between membrane association and activation of pro-CTSB. Furthermore, localization of CTSB to the cell

surface has been associated with Ras mutations (Cavallo-Medved et al., 2003). However, considering that BAG2 was under-expressed in Ras-mutated T47D and MCF10AT1 cells, which are nonaggressive breast cancer cells, the expression level of BAG2 does not seem to be dependent on mutant Ras status in breast cancer cells.

Moreover, our findings raise questions regarding how BAG2 regulates the trafficking of pro-CTSB to the cell periphery. Previous studies have indicated that the secretion of the pro-form as well as the SC form of CTSB appears to be facilitated by lysosomal exocytosis (Keppler and Sloane, 1996; Tu et al., 2008). However, our results suggest that BAG2 may facilitate trafficking of pro-CTSB through TGN38-positive vesicles rather than by lysosomal exocytosis. Indeed, BAG2-depleted TNBC cells demonstrated concentrated distribution of pro-CTSB and TGN38 at the perinuclear region, whereas pro-CTSB was more diffusely co-localized with TGN38 toward the cell periphery in the control cells. This observation was further supported by a Golgi exit assay, corroborating the presence of TGN38-positive vesicles that enclose pro-CTSB during its trafficking from the TGN. Therefore, BAG2 is responsible for the localization and activation of pro-CTSB on the external cell surface of TNBC cells during tumor metastasis. It also was reported that annexin II mediates plasma membrane fusion of TGN38-positive vesicles via its interaction with soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) in the regulation of lung surfactant secretion (Dassah et al., 2014; Wang et al., 2007). In this regard, it is possible that BAG2 may participate in the regulated exocytosis of pro-CTSB through interactions with SNAREs, because BAG2 binds to annexin II and regulates trafficking of TGN38-positive vesicles containing pro-CTSB to the plasma membrane. However, considering that BAG2 depletion did not inhibit translocation of annexin II on the cell surface, the ability of BAG2 to control trafficking of TGN38-positive vesicles containing pro-CTSB seems to be independent of annexin-II-mediated exocytosis.

Our findings also suggest that BAG2 prevents the conversion of the immature pro-form of CTSB to the mature SC form through its interaction with pro-CTSB at the PPR of the N terminus. This PPR has been shown to inhibit the enzyme activity of CTSB by masking the active-site cleft, which is required for substrate hydrolysis (Podobnik et al., 1997). In addition, the two-turn α helix (Met-P51 to Cyc-P58) of the PPR sits on top of the active site and includes the intermediate starting site (Arg-P56 to Leu-P57) for auto-catalytic processing, although it is unclear whether propeptide removal is accomplished in a single step or through one or more intermediates (Chagas et al., 1996; Quraishi and Storer, 2001). Interestingly, we found that that residues 51–58 of the two-turn α helix of the propeptide region of pro-CTSB is responsible for the interaction with the BAG domain of BAG2, but the well-established cleavage sites (Met-P72 to Phe-P73) of pro-CTSB near the pro/mature junction (Gly-P59 to Lys-P78) is not involved in this interaction. Altogether, BAG2 seems to play a negative role in the pro-apoptotic activation of intracellular mature CTSB by inhibiting auto-proteolytic processing of pro-CTSB through its interaction with pro-CTSB at the intermediate sites of the PPR.

In conclusion, our results present a mechanism by which BAG2 regulates the dual functions of CTSB by controlling the auto-cleavage processing of CTSB, thereby exerting a unique function in TNBC progression. In addition, given that the oncogenic function of CTSB is strongly linked to breast cancer progression, our findings highlight BAG2 as a promising and therapeutic target for TNBC.

EXPERIMENTAL PROCEDURES

Cell cultures, reagents, antibodies, and transfections are described in detail in Supplemental Experimental Procedures.

Public Transcriptomic Data Analysis

We obtained two types of transcriptomic data (RNA-sequencing datasets) from the GDC Data Portal (https://portal.gdc.cancer.gov). We used fragment per kilobase exon model per million mapped reads (FPKM) values normalized by upper quartile (UQ) normalization for RNA sequencing. One-way ANOVA was performed by comparing RNA-sequencing data of the four subtypes of breast cancer (luminal A, luminal B, HER2 positive, and TNBC) for five BAG family genes (*BAG1*, *BAG2*, *BAG3*, *BAG4*, and *BAG5*). The public microarray datasets (GEO: GSE41313, GSE2034, GSE5460, and GSE26971) were downloaded from NCBI's GEO.

DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA-sequencing data reported in this paper is Gene Expression Omnibus (GEO): GSE100878.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.11.026.

AUTHOR CONTRIBUTIONS

K.-M.Y. designed and conceptualized the research, did the experimental work, analyzed data, and wrote the manuscript. E.B. did the animal experiments. S.G.A., A.O., and J.J. statistically analyzed the clinical data of breast cancer patients of Gangnam Severance Hospital. J.K. analyzed the TCGA datasets. K.P., Y.P., J.P., J.L., B.P., and Y.J. provided technical assistance. S.T. and S.H.P. participated in the study design and coordinated the study, S.-J.K. designed and conceptualized the research, supervised the experimental work, analyzed data, and wrote the manuscript.

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REFERENCES

Arndt, V., Daniel, C., Nastainczyk, W., Alberti, S., and Höhfeld, J. (2005). BAG-2 acts as an inhibitor of the chaperone-associated ubiquitin ligase CHIP. Mol. Biol. Cell *16*, 5891–5900.

Bhoopathi, P., Chetty, C., Gujrati, M., Dinh, D.H., Rao, J.S., and Lakka, S. (2010). Cathepsin B facilitates autophagy-mediated apoptosis in SPARC overexpressed primitive neuroectodermal tumor cells. Cell Death Differ. *17*, 1529–1539.

Bos, P.D., Zhang, X.H., Nadal, C., Shu, W., Gomis, R.R., Nguyen, D.X., Minn, A.J., van de Vijver, M.J., Gerald, W.L., Foekens, J.A., and Massagué, J. (2009). Genes that mediate breast cancer metastasis to the brain. Nature *459*, 1005–1009.

Cavallo-Medved, D., Dosescu, J., Linebaugh, B.E., Sameni, M., Rudy, D., and Sloane, B.F. (2003). Mutant K-ras regulates cathepsin B localization on the surface of human colorectal carcinoma cells. Neoplasia *5*, 507–519.

Chagas, J.R., Ferrer-Di Martino, M., Gauthier, F., and Lalmanach, G. (1996). Inhibition of cathepsin B by its propeptide: use of overlapping peptides to identify a critical segment. FEBS Lett. *392*, 233–236.

Chwieralski, C.E., Welte, T., and Bühling, F. (2006). Cathepsin-regulated apoptosis. Apoptosis *11*, 143–149.

Crown, J., O'Shaughnessy, J., and Gullo, G. (2012). Emerging targeted therapies in triple-negative breast cancer. Ann. Oncol. *23* (*Suppl 6*), vi56–vi65.

Dai, Q., Qian, S.B., Li, H.H., McDonough, H., Borchers, C., Huang, D., Takayama, S., Younger, J.M., Ren, H.Y., Cyr, D.M., and Patterson, C. (2005). Regulation of the cytoplasmic quality control protein degradation pathway by BAG2. J. Biol. Chem. *280*, 38673–38681.

Dassah, M., Almeida, D., Hahn, R., Bonaldo, P., Worgall, S., and Hajjar, K.A. (2014). Annexin A2 mediates secretion of collagen VI, pulmonary elasticity and apoptosis of bronchial epithelial cells. J. Cell Sci. *127*, 828–844.

Foghsgaard, L., Wissing, D., Mauch, D., Lademann, U., Bastholm, L., Boes, M., Elling, F., Leist, M., and Jäättelä, M. (2001). Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. J. Cell Biol. *153*, 999–1010.

Foulkes, W.D., Smith, I.E., and Reis-Filho, J.S. (2010). Triple-negative breast cancer. N. Engl. J. Med. *363*, 1938–1948.

Gondi, C.S., and Rao, J.S. (2013). Cathepsin B as a cancer target. Expert Opin. Ther. Targets *17*, 281–291.

Győrffy, B., Bottai, G., Lehmann-Che, J., Kéri, G., Orfi, L., Iwamoto, T., Desmedt, C., Bianchini, G., Turner, N.C., de Thè, H., et al. (2014). TP53 mutation-correlated genes predict the risk of tumor relapse and identify MPS1 as a potential therapeutic kinase in TP53-mutated breast cancers. Mol. Oncol. *8*, 508–519.

Hudis, C.A., and Gianni, L. (2011). Triple-negative breast cancer: an unmet medical need. Oncologist *16* (*Suppl 1*), 1–11.

Joyce, J.A., Baruch, A., Chehade, K., Meyer-Morse, N., Giraudo, E., Tsai, F.Y., Greenbaum, D.C., Hager, J.H., Bogyo, M., and Hanahan, D. (2004). Cathepsin cysteine proteases are effectors of invasive growth and angiogenesis during multistage tumorigenesis. Cancer Cell *5*, 443–453.

Keppler, D., and Sloane, B.F. (1996). Cathepsin B: multiple enzyme forms from a single gene and their relation to cancer. Enzyme Protein *49*, 94–105.

Linke, M., Herzog, V., and Brix, K. (2002). Trafficking of lysosomal cathepsin B-green fluorescent protein to the surface of thyroid epithelial cells involves the endosomal/lysosomal compartment. J. Cell Sci. *115*, 4877–4889.

Lu, X., Lu, X., Wang, Z.C., Iglehart, J.D., Zhang, X., and Richardson, A.L. (2008). Predicting features of breast cancer with gene expression patterns. Breast Cancer Res. Treat. *108*, 191–201.

Mai, J., Finley, R.L., Jr., Waisman, D.M., and Sloane, B.F. (2000). Human procathepsin B interacts with the annexin II tetramer on the surface of tumor cells. J. Biol. Chem. *275*, 12806–12812.

Masuda, H., Baggerly, K.A., Wang, Y., Zhang, Y., Gonzalez-Angulo, A.M., Meric-Bernstam, F., Valero, V., Lehmann, B.D., Pietenpol, J.A., Hortobagyi, G.N., et al. (2013). Differential response to neoadjuvant chemotherapy among 7 triple-negative breast cancer molecular subtypes. Clin. Cancer Res. *19*, 5533–5540.

Mohamed, M.M., and Sloane, B.F. (2006). Cysteine cathepsins: multifunctional enzymes in cancer. Nat. Rev. Cancer *6*, 764–775.

Moin, K., Day, N.A., Sameni, M., Hasnain, S., Hirama, T., and Sloane, B.F. (1992). Human tumour cathepsin B. Comparison with normal liver cathepsin B. Biochem. J. *285*, 427–434.

Mort, J.S., and Buttle, D.J. (1997). Cathepsin B. Int. J. Biochem. Cell Biol. *29*, 715–720.

Murakami, Y., and Mizuguchi, K. (2010). Applying the naïve Bayes classifier with kernel density estimation to the prediction of protein-protein interaction sites. Bioinformatics *26*, 1841–1848.

Olson, O.C., and Joyce, J.A. (2015). Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. Nat. Rev. Cancer *15*, 712–729.

Podobnik, M., Kuhelj, R., Turk, V., and Turk, D. (1997). Crystal structure of the wild-type human procathepsin B at 2.5 A resolution reveals the native active site of a papain-like cysteine protease zymogen. J. Mol. Biol. *271*, 774–788.

Qu, D., Hage, A., Don-Carolis, K., Huang, E., Joselin, A., Safarpour, F., Marcogliese, P.C., Rousseaux, M.W., Hewitt, S.J., Huang, T., et al. (2015). BAG2 gene-mediated regulation of PINK1 protein is critical for mitochondrial translocation of PARKIN and neuronal survival. J. Biol. Chem. *290*, 30441–30452.

Quraishi, O., and Storer, A.C. (2001). Identification of internal autoproteolytic cleavage sites within the prosegments of recombinant procathepsin B and procathepsin S. Contribution of a plausible unimolecular autoproteolytic event for the processing of zymogens belonging to the papain family. J. Biol. Chem. *276*, 8118–8124.

Riaz, M., van Jaarsveld, M.T., Hollestelle, A., Prager-van der Smissen, W.J., Heine, A.A., Boersma, A.W., Liu, J., Helmijr, J., Ozturk, B., Smid, M., et al. (2013). miRNA expression profiling of 51 human breast cancer cell lines reveals subtype and driver mutation-specific miRNAs. Breast Cancer Res. *15*, R33.

Sevenich, L., Werner, F., Gajda, M., Schurigt, U., Sieber, C., Müller, S., Follo, M., Peters, C., and Reinheckel, T. (2011). Transgenic expression of human cathepsin B promotes progression and metastasis of polyoma-middle-T-induced breast cancer in mice. Oncogene *30*, 54–64.

Tu, C., Ortega-Cava, C.F., Chen, G., Fernandes, N.D., Cavallo-Medved, D., Sloane, B.F., Band, V., and Band, H. (2008). Lysosomal cathepsin B participates in the podosome-mediated extracellular matrix degradation and invasion via secreted lysosomes in v-Src fibroblasts. Cancer Res. *68*, 9147–9156.

Vasiljeva, O., Korovin, M., Gajda, M., Brodoefel, H., Bojic, L., Krüger, A., Schurigt, U., Sevenich, L., Turk, B., Peters, C., and Reinheckel, T. (2008). Reduced tumour cell proliferation and delayed development of high-grade mammary carcinomas in cathepsin B-deficient mice. Oncogene *27*, 4191–4199.

Wang, P., Chintagari, N.R., Gou, D., Su, L., and Liu, L. (2007). Physical and functional interactions of SNAP-23 with annexin A2. Am. J. Respir. Cell Mol. Biol. *37*, 467–476.

Wang, H.Q., Zhang, H.Y., Hao, F.J., Meng, X., Guan, Y., and Du, Z.X. (2008). Induction of BAG2 protein during proteasome inhibitor-induced apoptosis in thyroid carcinoma cells. Br. J. Pharmacol. *155*, 655–660.

Withana, N.P., Blum, G., Sameni, M., Slaney, C., Anbalagan, A., Olive, M.B., Bidwell, B.N., Edgington, L., Wang, L., Moin, K., et al. (2012). Cathepsin B inhibition limits bone metastasis in breast cancer. Cancer Res. *72*, 1199–1209.

Yue, X., Zhao, Y., Liu, J., Zhang, C., Yu, H., Wang, J., Zheng, T., Liu, L., Li, J., Feng, Z., and Hu, W. (2015). BAG2 promotes tumorigenesis through enhancing mutant p53 protein levels and function. eLife *4*, 08401.