Additional information

Supplementary Information

BRSK2 plays a role in autophagy and cancer cell growth and survival under nutrient deprivation stress via the PIK3C3 pathway

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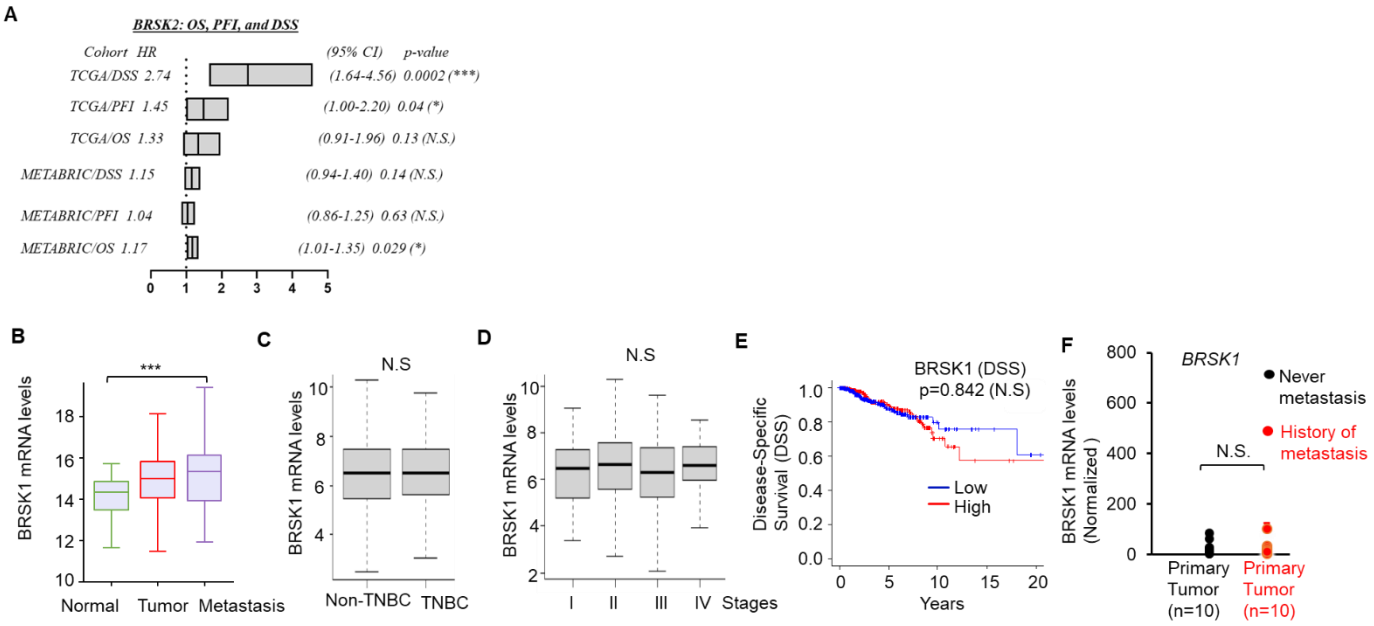


Figure S1. *BRSK2* expression score and association with patients' survival. (**A**) Patients with a high *BRSK2* expression score and association with survival in two breast cancer cohorts (TCGA and METABRIC). DSS, PFI, and O.S. rates in TCGA-BRCA and METABRIC cohorts, along with H.R. and their 95% confidence intervals (CI) and p-values, are shown. Note: Only TCGA-BRCA DSS and PFI data (p=0.0002, 0.04, respectively) and METABRIC OS data (p=0.029) are significant. (**B**) *BRSK1* levels are elevated in tumors of breast cancer patients vs. control breast tissues. Boxplots show elevated *BRSK1* transcript levels in tumors or metastatic tumors compared to adjacent normal tissues in the TCGA-BRCA cohort of breast cancer patients. One-way ANOVA, p<0.05, a significant difference. (**C**) Boxplots of *BRSK1* expression score by immunohistochemistry (IHC) determined subtype (TNBC vs. non-TNBC), N.S., not significant. (**D**) Boxplots of high expression of *BRSK1* mRNA score of tumors of different American Joint Committee on Cancer (AJCC) stages for TCGA-BRCA cohort. All boxplots are of the Tukey type, and the boxes depict medians and interquartile ranges. Student's t-test and One-way ANOVA, p<0.05, showed a significant difference. ***p<0.001. (**E**) Disease-specific survival (DSS) with higher *BRSK1* transcripts in the TCGA-BRCA cohort, as well as in the median-split patients, and the p-value is insignificant. (**F**) Primary tumors (never-metastasis, n=10) vs. age-matched primary tumors of patients with a history of metastasis (n=10)(Figure 1F)(42) were used for qPCR analysis for *BRSK1. GAPGH* is a housekeeping gene. Normalized *BRSK1* gene levels to *GAPDH* ($2^{\text{-delta}}$ CTx10,000) are shown. The Student's t-test, p = N.S., is not significant in the experimental triplicate.

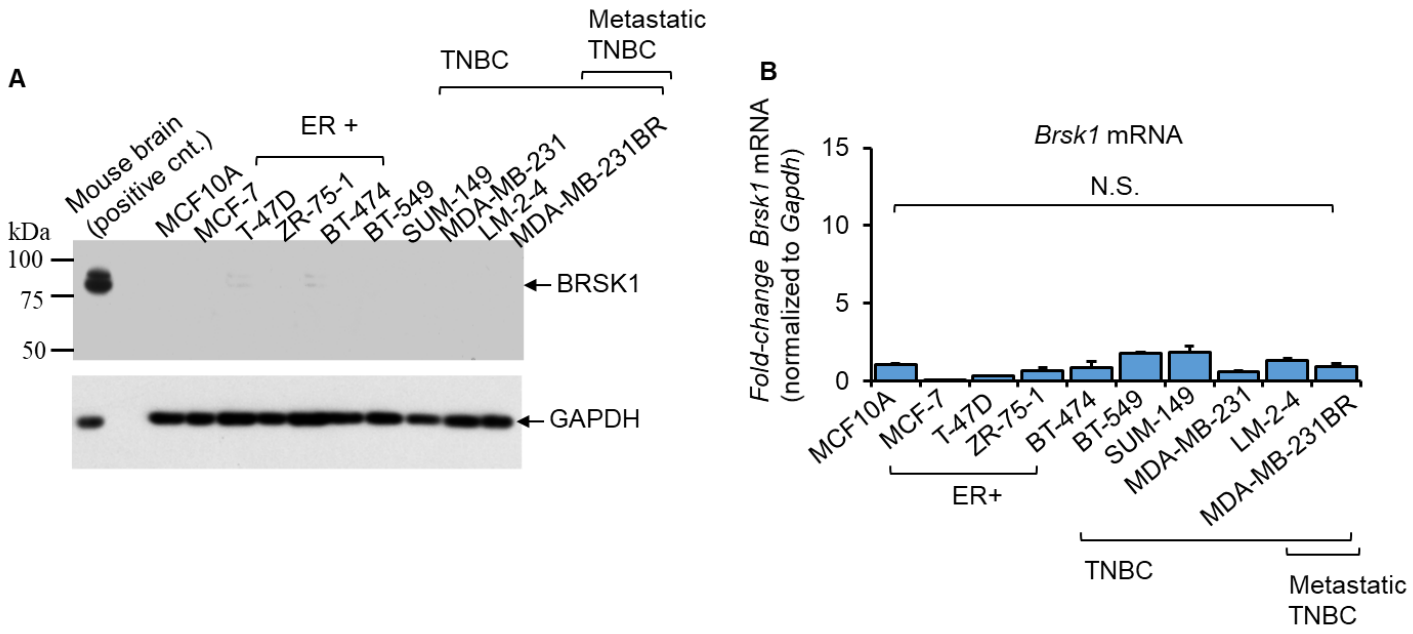


Figure S2. BRSK1 expression is silenced in breast cancer cells and benign tumor cells. (**A**) Total cell lysates from the duplicate culture in Figure 2 were used for Western blot analysis with antibodies against BRSK1 (**A**). A mouse whole-brain protein extract was used as a positive control for BRSK1 protein expression. GAPDH was used to show equal loading and transfer. N=3, representative blots were shown. The figure indicates the presence of the BRSK1 protein band (90 kDa) in the western blot of the positive control (**A**). cDNA from Figure 2 was used for SYBR-Green qPCR analysis for *BRSK1* transcript expression. *GAPDH* mRNA expression was used to normalize *BRSK1* transcript levels. Normalized transcript expression levels were calculated using the Delta CT method (n=3); data are means ±s.e. One-way ANOVA and Student's t-test, N.S. is not significant. **Note:** Normalized *BRSK1* mRNA levels are not significantly altered ($p<0.05$, cancer cells vs. MCF10A), whereas BRSK1 protein levels were silenced in human breast cancer cells, as well as in normal epithelial MCF10A cells. Please note that the blots were cropped before hybridization with the respective antibodies (Supplementary Figure S12).

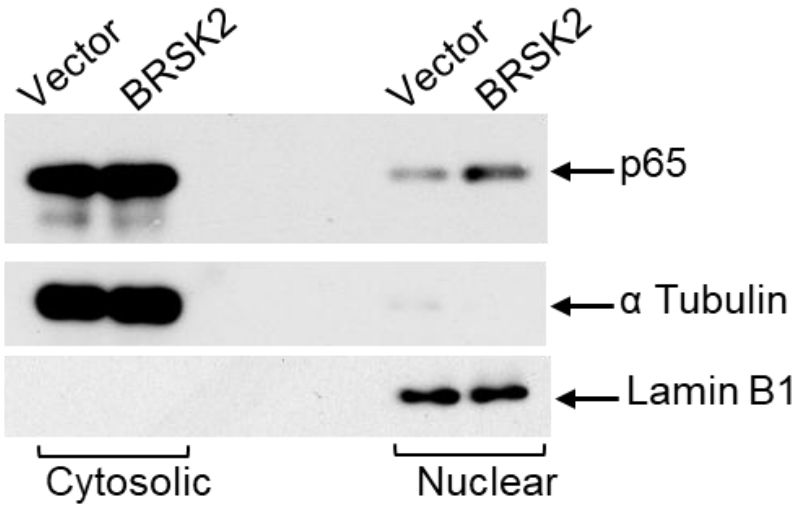


Figure S3. BRSK2 stable overexpression enhances p65-NF-κB nuclear translocation.
Human BRSK2-FLAG tagged MDA-MB-231 stable cell line was generated using pLentil-GIII-CMV-BRSK2-Flag-SV40-Puro Vector (abm's protocol as described on the website: https://www.abmgood.com/Lentivirus-Packaging-Systems.html, and stable transgene overexpression was validated by DNA sequencing, qPCR, and western blotting. Stable cells (empty vector and BRSK2-OE) were used for the isolation of nuclear and nuclei-free cytosolic fractions using established protocols. As indicated, equal amounts of nuclear and cytosolic proteins were used for Western blotting, using specific antibodies against the target proteins. Translocation of p65-NF-κB into the nucleus in response to stable BRSK2 expression vs. empty vector was observed in the MDA-MB-231 cell model. α-tubulin and Lamin B1 antibodies were used as markers for cytosolic and nuclear proteins, respectively. N=3, representative blots were shown. Scanned blots and fold intensities normalized to respective loading control vs. vector control were labeled (ImageJ). Please note that the blots were cropped before hybridization with the respective antibodies (Supplementary Figure S12).

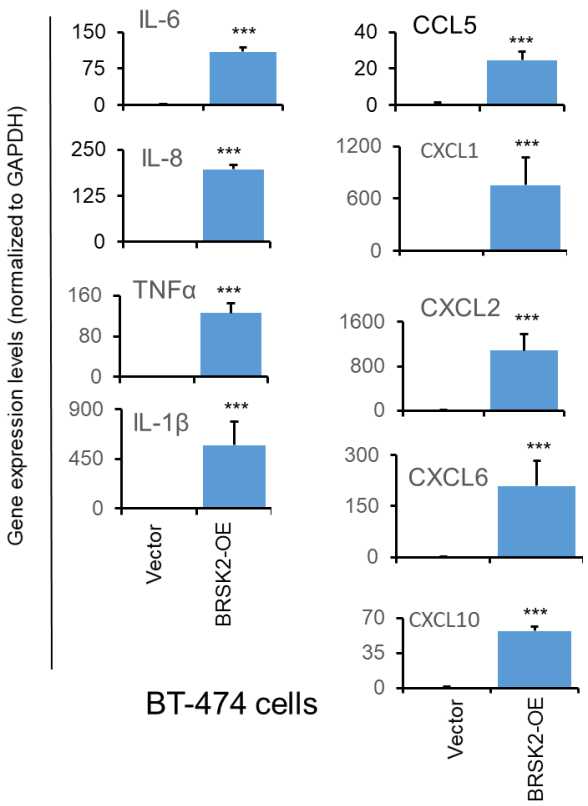


Figure S4. Ectopic BRSK2 expression in breast cancer cells elevates cytokine and chemokine metastasis mediator transcripts. BT-474 cells were transfected with the BRSK2 plasmid or empty vector for 48 h, and cells were processed for qPCR analysis with the indicated SYBR-Green primers. Gene levels were calculated using the delta-delta C.T. method, normalized to the housekeeping gene *GAPDH*. N=3, Student's t-test, ***p<0.001, a significant difference vs. empty vector control.

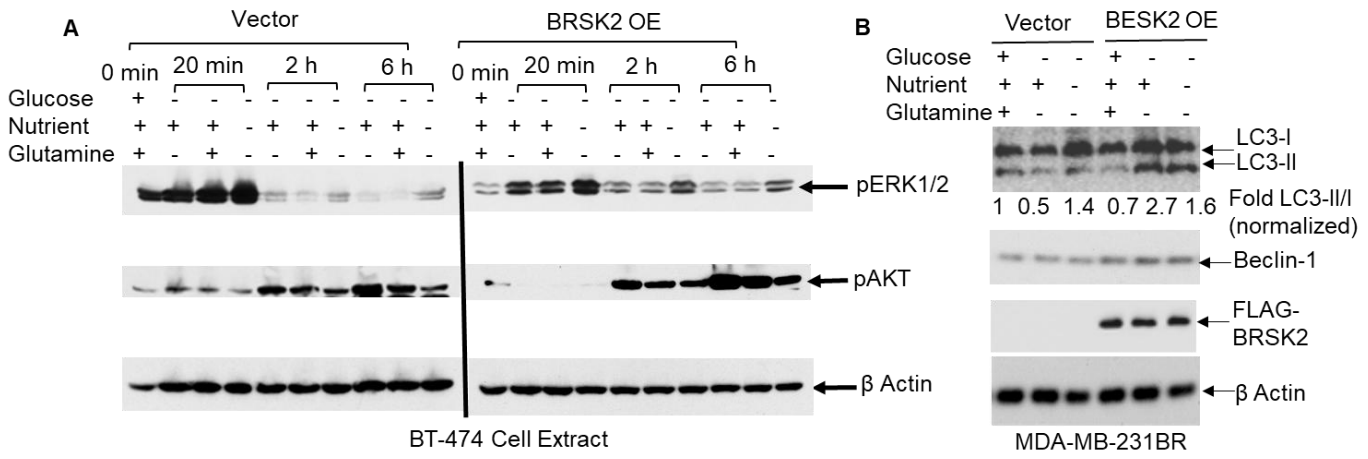


Figure S5. Ectopic BRSK2 expression in breast cancer cells elevates AKT and ERK1/2 activities in response to nutrient deprivation stress. (A) As indicated, equal amounts of protein extracts from each sample from the duplicate BT-474 cell extracts (**Figure 4A**) were used for western blot analysis with the cell signaling-related protein antibodies, including phospho-AKT (S473) and phospho-ERK1/2. FLAG-tagged BRSK2 expression in these samples is shown in Figure 4A. Anti-β-Actin antibody was used for equal loading and transfer. Blots were scanned, quantified, and normalized to the loading control (Image J), and fold intensities were shown compared to the control. N=3, representative blots were shown. **(B) BRSK2 overexpression in MDA-MB-231BR cells involves elevated autophagy in response to nutrient deprivation stress.** Human TNBC brain-metastatic variant MDA-MB-231BR cells were transfected with empty vector or BRSK2 overexpression plasmids for 48 h and subjected to exposure with various conditions with or without deprived energy sources medium (glucose, glutamine, or nutrients) for 6 h, as indicated. Total protein lysates were used for western blot analysis using the indicated antibodies, including LC3-I/II. Additionally, anti-FALG antibodies were used to detect the FLAG-tagged BRSK2 protein, and anti-β-actin antibodies were used to ensure equal loading and transfer. N=3, representative blots were shown. Fold LC3-II band intensities normalized to β-Actin vs. control vector-transfected cells were labeled (ImageJ). Please note that the blots were cropped before hybridization with the respective antibodies (Supplementary Figure S12).

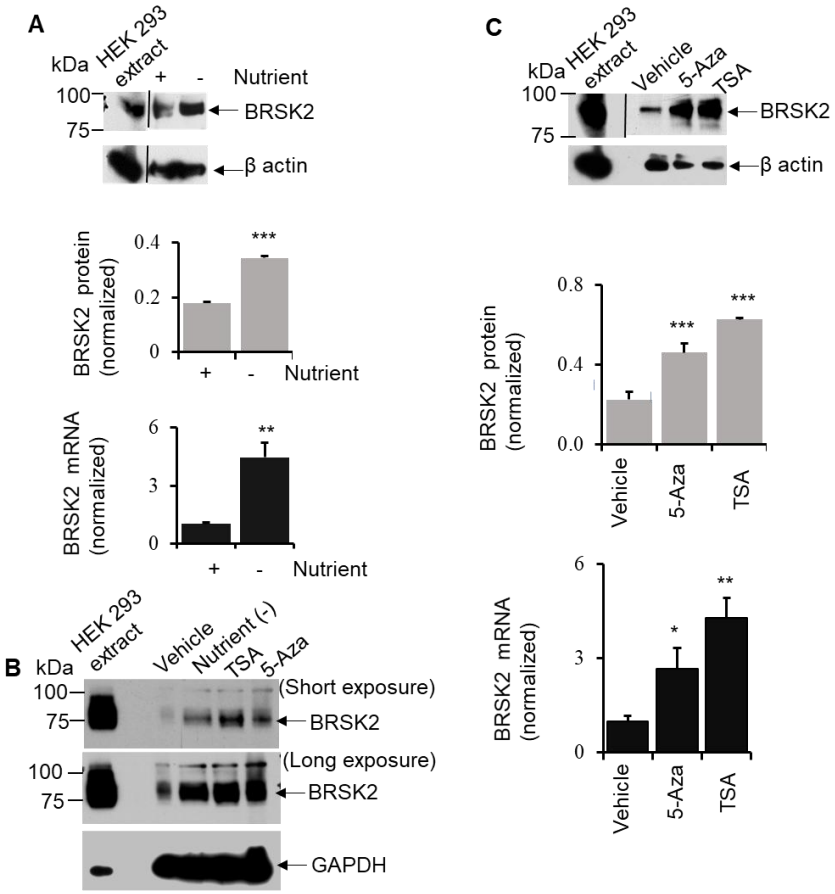


Figure S6. Nutrient deprivation-stress and epigenetic mechanisms regulate BRSK2 expression in breast cancer cells. (**A**) BT-474 cells were exposed to ± nutrient-free medium for 6 h, and total cell lysates were used for western blotting with BRSK2 antibodies, as indicated. β-Actin was used for equal loading and transfer. HEK293 cell extract was used for the BRSK2 protein positive control for the western blot analysis. (Middle) Densitometric BRSK2 protein bands were quantified and normalized to the loading control (n = 3). Student's t-test, ***p<0.001 (nutrient-free vs. control). (Bottom) Duplicate cultures of BT-474 cells were used for quantitative PCR (qPCR) analysis of the *BRSK2* gene. Gene levels were normalized to the housekeeping gene GAPDH (n = 3). Student's t-test was performed, **p<0.01 (nutrient-free vs. control). (**B**) ER+ MCF-7 cells were exposed to a nutrient-free medium or treated with 0.1 μM each of pan HDAC inhibitor Trichostatin A (TSA) or DNA methylation inhibitor 5-Azacytidine (5-AZA) for 24 h. Cell extracts were used for western blot analysis, as indicated. Short and long-exposure BRSK2 western blots were shown. N=3. (**C**) BT-474 cells were treated with vehicle, TSA, or 5-AZA, as indicated in (**B**), and cell extracts were used for western blot analysis. (Middle) The BRSK2 protein band was quantified and normalized; ***p < 0.001 (treatment vs. vehicle control). (Bottom) A duplicate culture was used for *BRSK2* gene qPCR analysis, and the results were normalized to the *GAPDH* housekeeping gene. N=3, *BRSK2* normalized mRNA levels are means ± s.e., one-way ANOVA, and post-hoc test for pair-wise comparison, *p < 0.05, ***p<0.001 vs. vehicle control, respectively. Please note that the blots were cropped before hybridization with the respective antibodies (Supplementary Figure S12).

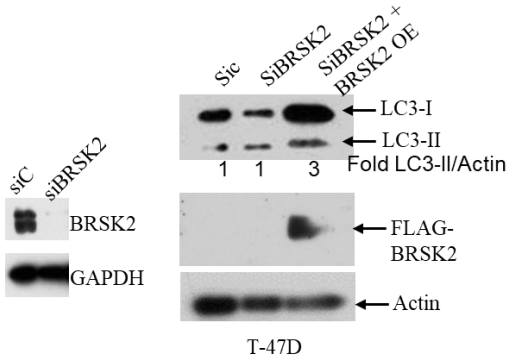


Figure S7. Ectopic overexpression of BRSK2 rescued LC3 in BRSK2 knockdown Cells. T-47D cells with siRNA targeted to BRSK2, compared to siControl, showed rescued BRSK2 levels after ectopic overexpression of BRSK2. Equal amounts of total protein were used for Western blotting, using antibodies against LC3 and FLAG-BRSK2 proteins. β-Actin was used for equal loading and transfer. N=3, and representative blots are shown. Please note that the blots were cropped before hybridization with the respective antibodies (Supplementary Figure S12).

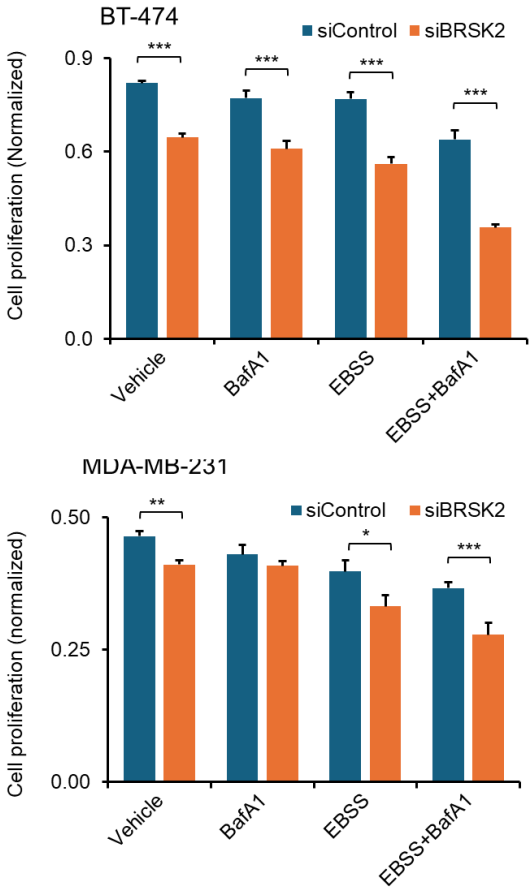


Figure S8. Knockdown of BRSK2 with siBRSK2 reduces cell proliferation. An equal number of cells were transfected with siBRSK2 #1 for 24 hours and treated with either -/+ BafA1 (100 nM) in full serum or -/+ EBSS for an additional 24 hours. Cell proliferation was measured using WST-8, and background values were subtracted. N=4, ANOVA ($p<0.001$) and post-hoc t-test, ***$p<0.001$, **$p<0.01$, or *$p<0.05$ vs. respective control.

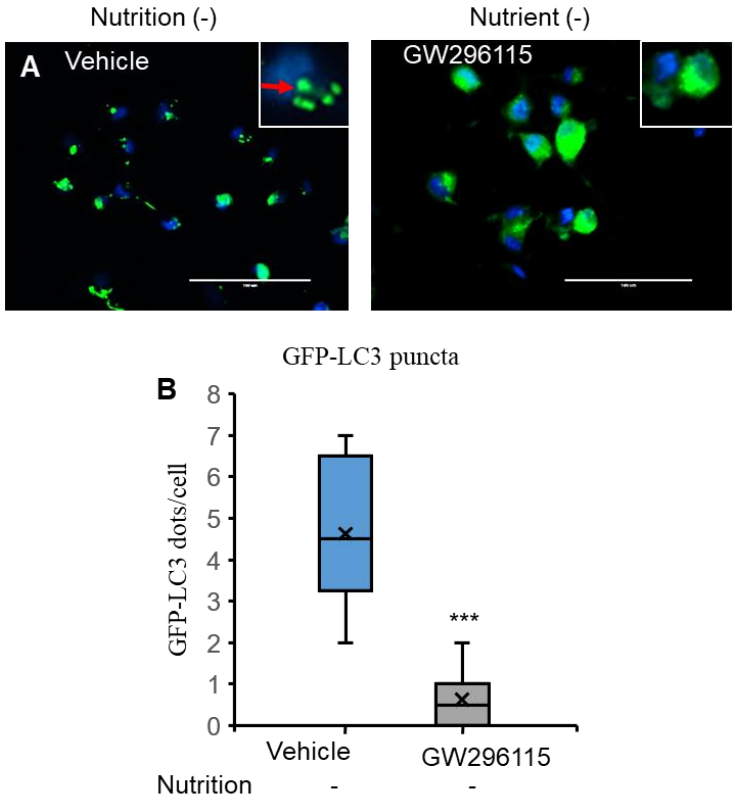


Figure S9. GW296115 inhibits autophagy in breast cancer cells in response to nutrient deprivation. (**A**) The GFP-LC3-transfected MDA-MB-231 cells were pre-incubated with vehicle or GW296115 (2 μM) for 18 hours and then exposed to ± nutrient-free medium for 6 hours, followed by GFP immunofluorescence staining. Representative inverted fluorescence microscopic images, n=10, scale bar 100 μm, red arrows on blowout image indicating autophagy GFP-LC3 puncta staining (n=20 cells). (**B**) Box plots are the number of dots/cells from at least n=10 counted, Student's t-test, ***p<0.001 (GW296115 vs. vehicle control).

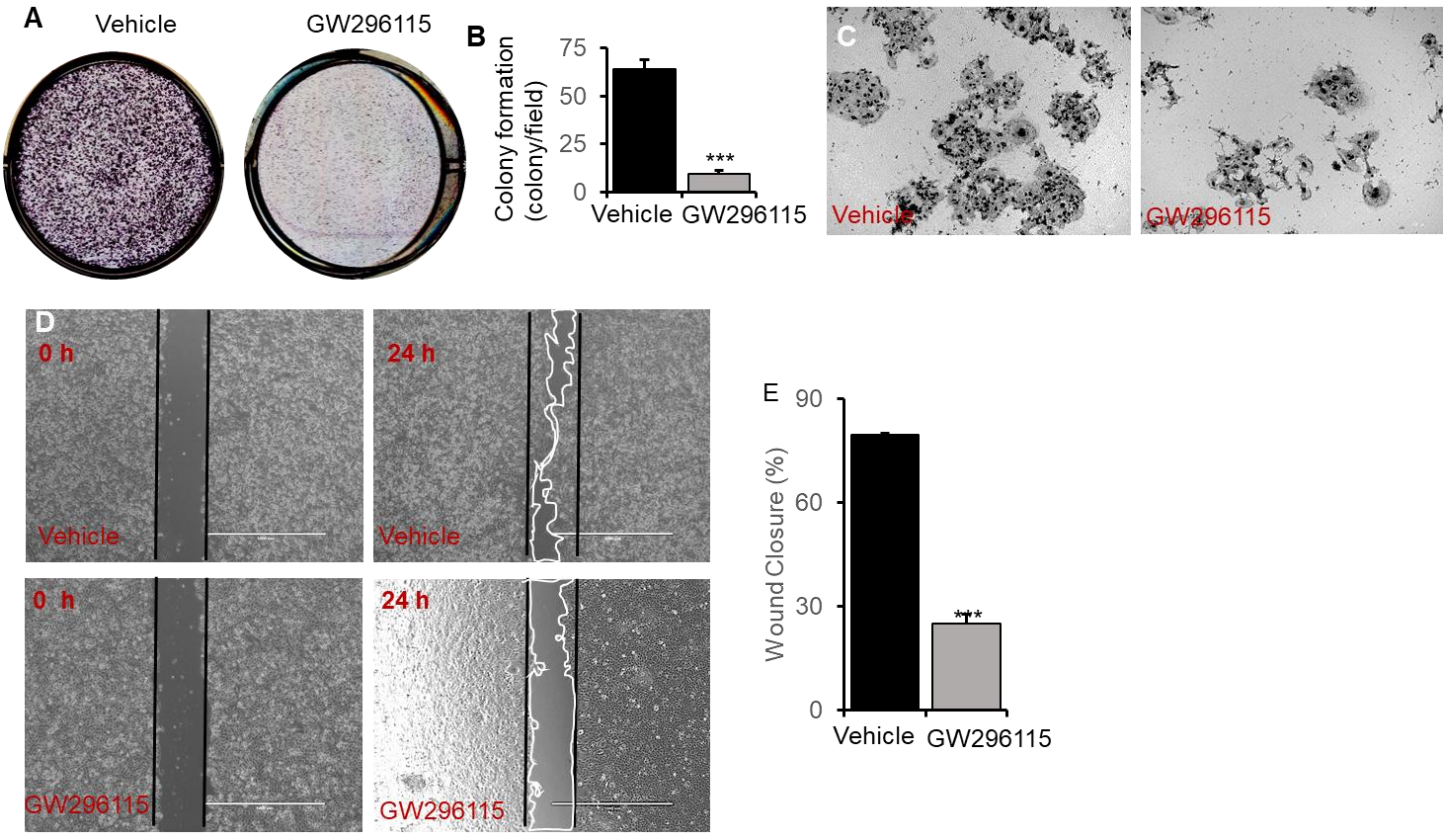


Figure S10. BRSK2 inhibition with GW296115 in breast cancer cells reduces colony formation and cell migration. (**A**) The colony formation ability of vehicle- or GW296115 (3 µM)- treated MDA-MB-231 cells was assessed by clonogenic assay after 7 days. N=3, representative light microscopy images of crystal violet-stained colonies from each group were shown. (**B**) A Histogram of the number of forming colonies/fields in each group was shown. N=7 field/group, 4x magnification, cells>50/colony counted, Student's t-test, ***p<0.001 (GW296115 vs. vehicle control). (**C**) Representative colony formation image/group, n=5 field (ZOE Fluorescent Cell Imager, Bio-rad, scale bar 100 µm). (**D**, **E**) Cell migration determined by the wound healing assay. (**D**) Optical microscope images presenting scratch area covered by the MDA-MB-231 cells 24 h after the scratching (representative images, N=5 independent samples for vehicle and 3 µM GW296115) or (**E**) Quantitative evaluation of wound closure (%) is expressed as the percentage of the wound area covered after 24 hours (N=5, Student's t-test, ***p<0.001, GW296115 vs. vehicle for 24 h, 0 h wound area just after scratching and 24 h wound area 24 h after scratching and measured and calculated by Image J). Similar reduced colony formation and cell migration data (GW296115 vs. vehicle) were obtained in other breast cancer cells, including BT-474, BT-549, and 4T1 cells.

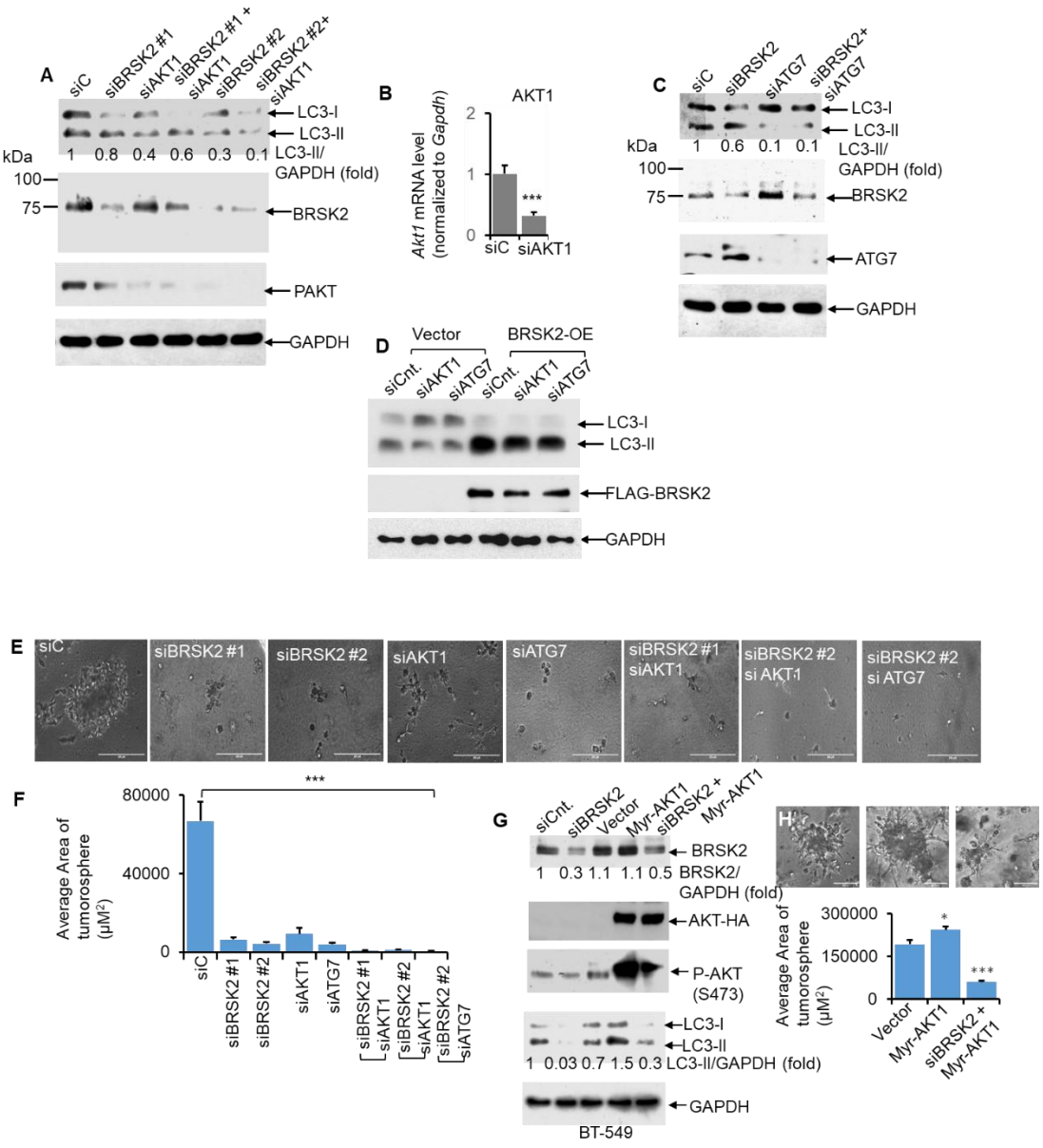
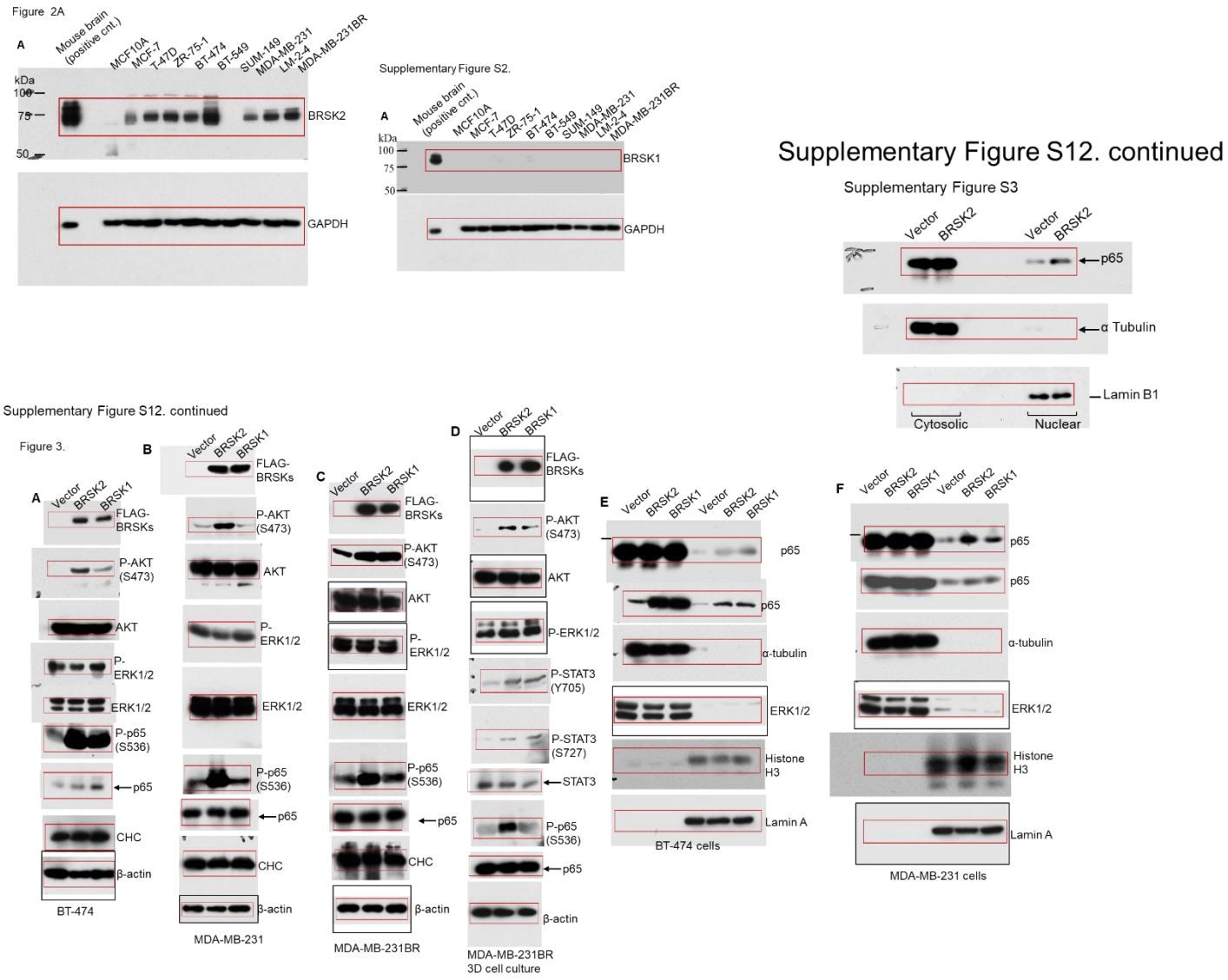


Figure S11. Downregulation of BRSK2 and AKT with siRNAs reduced autophagy and TNBC 3D cell growth. (**A**) BT-549 cells were treated with siRNA targeted to BRSK2 #1, siBRSK2 #2, or siAKT1 individually or in combination (siAKT1 and siBRSK2 #2) compared to siControl for 48 hours, and total extracts were prepared. Equal amounts of total protein were used for Western blotting, using antibodies against LC3, BRSK2, and GAPDH (loading control). N=3, and representative blots are shown. Fold LC3-II band intensities normalized to β-Actin vs. control vector-transfected cells were labeled (Image J). (**B**) Total RNA was isolated separately from duplicate cells (siControl and siAKT1) and treated with DNase. cDNA was prepared from total RNA and used for SYBR-Green qPCR analysis for *BRSK2* transcript expression. *GAPDH* mRNA expression was used to normalize *AKT1* transcript levels. Normalized transcript expression levels were calculated using the Delta CT method (n=3); data are means ±s.e. Student's t-test, and ***p<0.001. (**C**) In a separate experiment with BT-549 cells, siATG7 and siBRSK2 #2, individually or together, transfected for 48 h vs. siControl cells, were used for total

protein extraction and western blot analysis with the indicated antibodies. GAPDH was used as a loading control and transfer standard. N=3, representative blots were shown. Fold LC3-II band intensities normalized to GAPDH vs. control vector-transfected cells were labeled (Image J). (**D**) BT549 cells were transfected with siAKT1, siATG7, or siControl for 24h and further transfected with vecor or BRSK2 plasmids for another 24h. Total proteins were used for western blot analysis, as indicated. N=3. (**E**) As indicated, Duplicate cells from (**A and B**) were used for 3D cell growth for 72 h in Matrigel matrix. Representative light microscopic images (n=10), scale bar 250 µm. (**F**) Quantification of spheroid areas (average area of tumorosphere, μM^2, Image J), n=20, ANOVA and post-hoc t-test, ***p<0.001 vs. siControl. (**G and H**) BT-549 cells transfected with siBRSK2 #2 or siControl, after 24h, siBRSK2 cells were transfected with the constitutively active myristoylated-AKT1- AKT1 (Myr-AKT1) plasmid DNA (Addgene #1036) for another 24h along with empty vector, as indicated. Cell extracts were used for Western blotting with the indicated antibodies, and GAPDH was used as a loading control (**G**). N=3, representative blots were shown. Blots, including the LC3-II band, were scanned quantitatively and normalized to GAPDH; the fold change was indicated and compared to the control (ImageJ). (**H**) Duplicate cells from (**H, top**) were used for 3D cell growth, as mentioned above. Representative light microscopic images (n=5), scale bar 250 µm. (**H, bottom**) Quantification of spheroid areas (average area of tumorosphere, μM^2, Image J), n=10, ANOVA and post-hoc t-test, *p<0.05 or ***p<0.001 vs. vector. Note that similar data were obtained for MDA-MB-231 cells. Please note that the blots were cropped before hybridization with the respective antibodies (Supplementary Figure S12).

Supplementary Figure S12.



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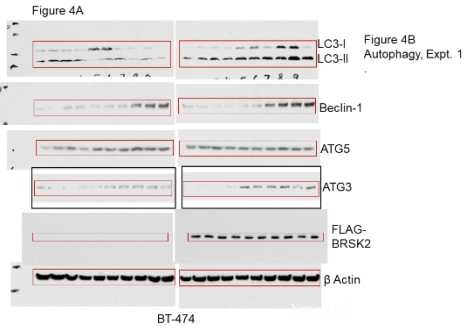

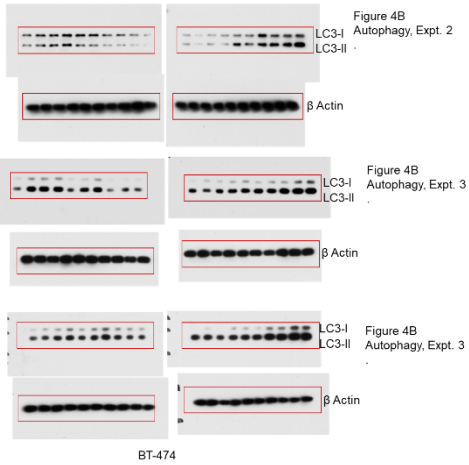

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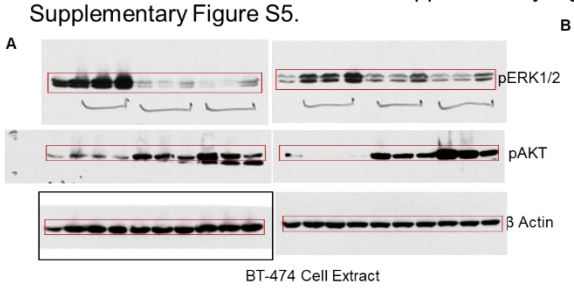
Supplementary Figure S5.



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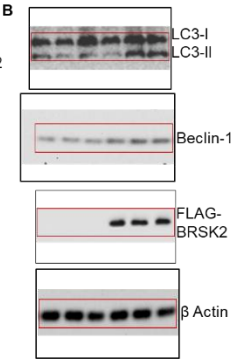

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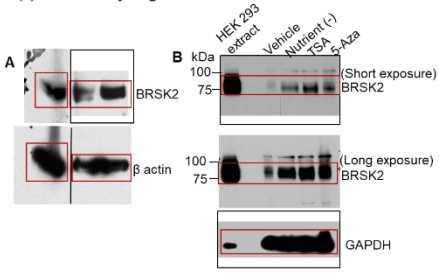

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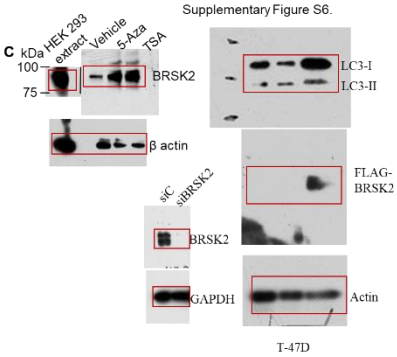

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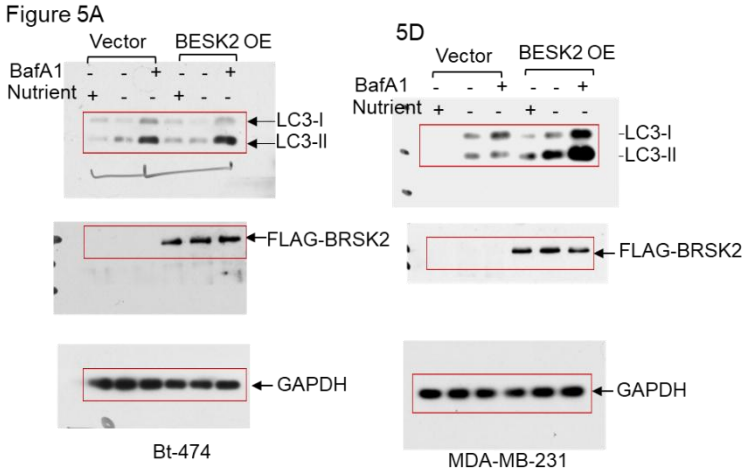

Figure 6

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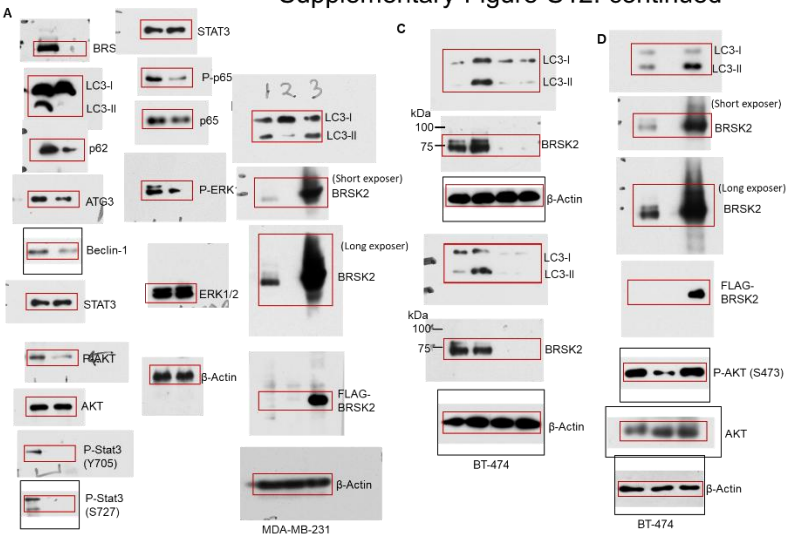

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Supplementary Figure S7

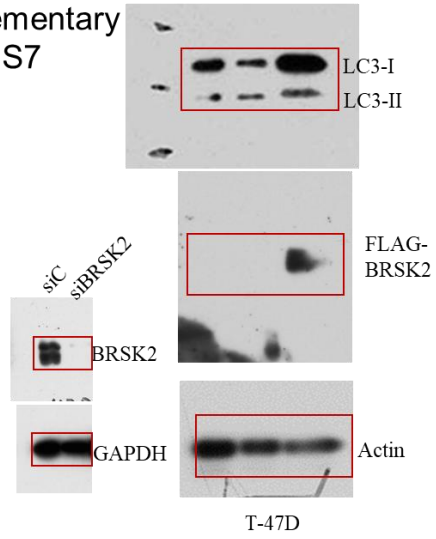

Figure 7

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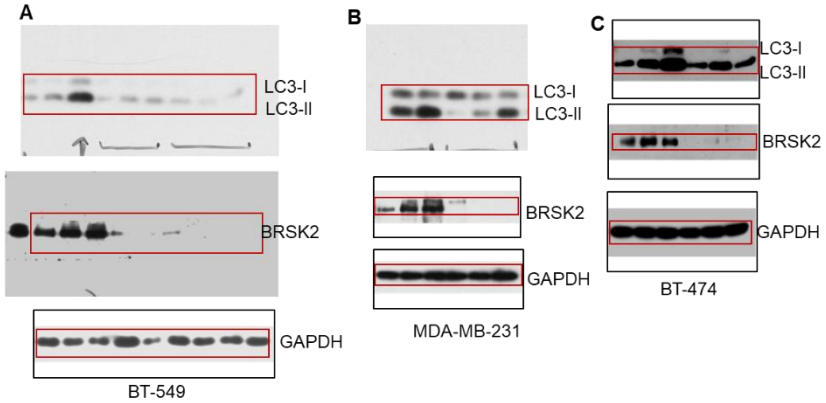

Figure 8

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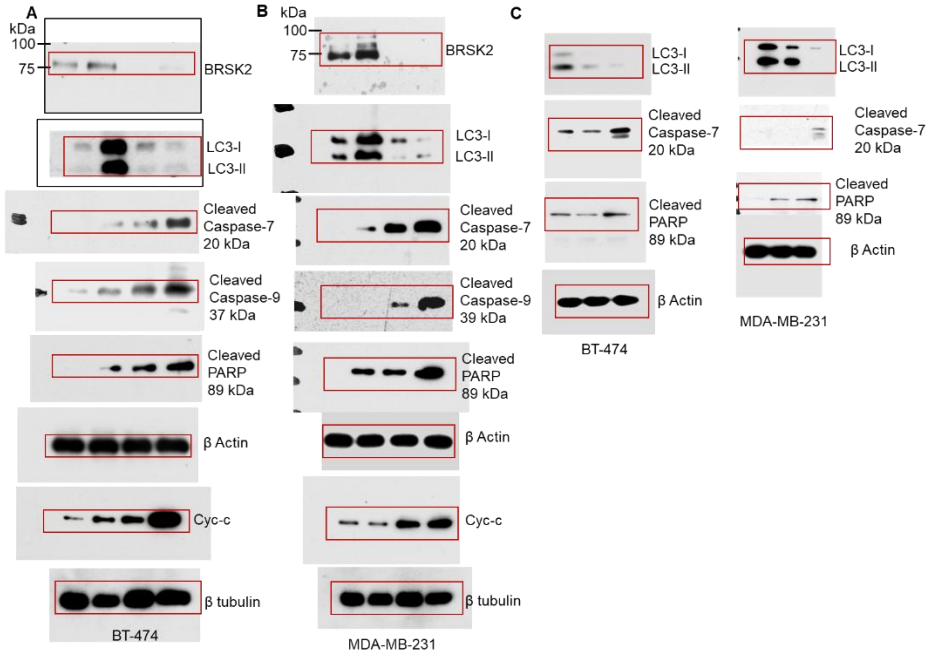

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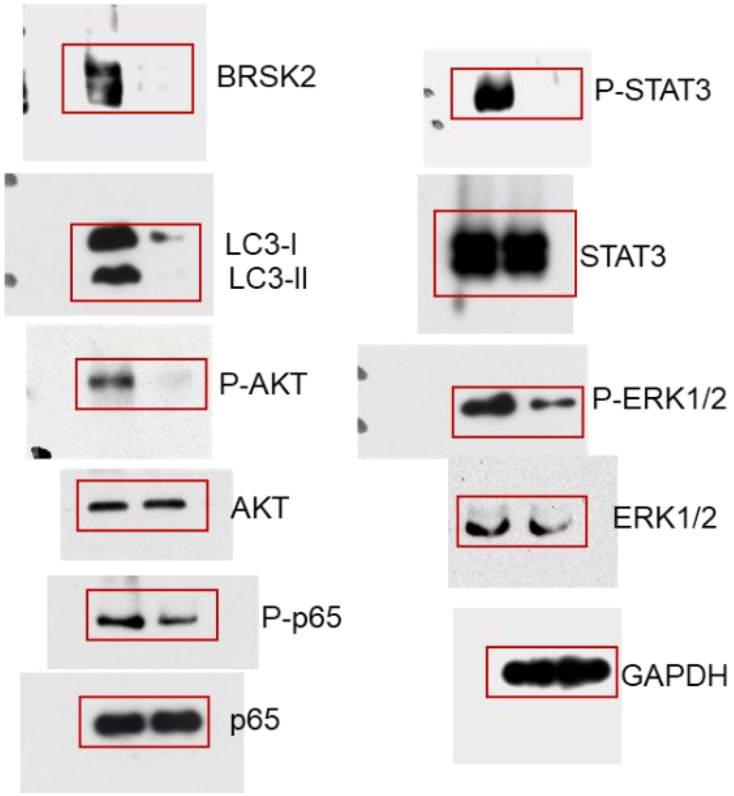
Figure 9A



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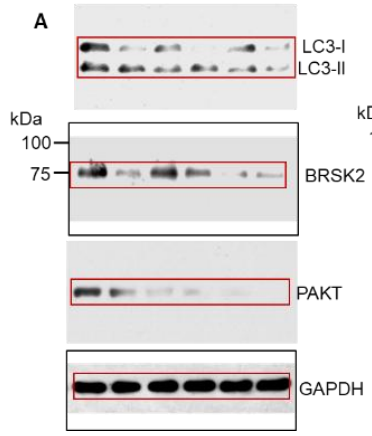

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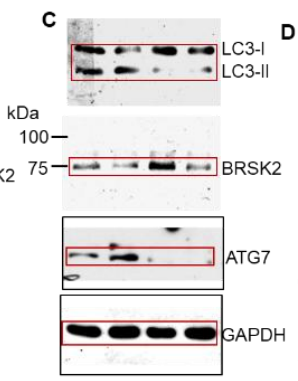
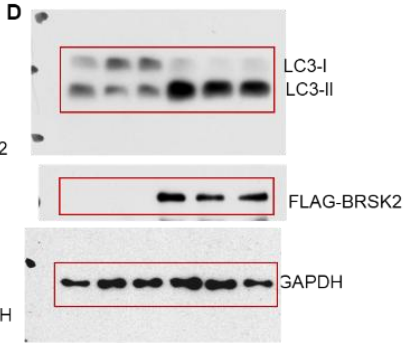




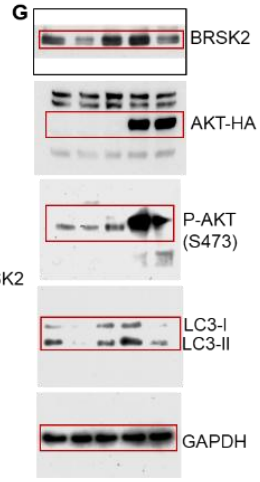
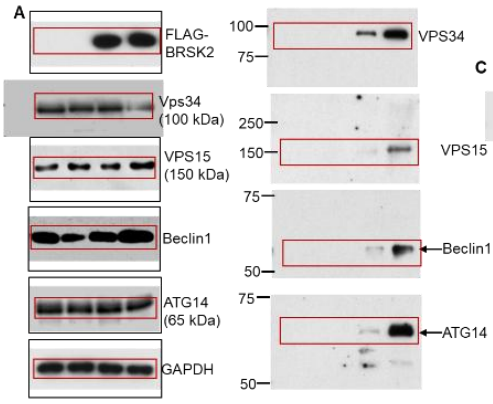


Figure 10



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