Expanded View Figures

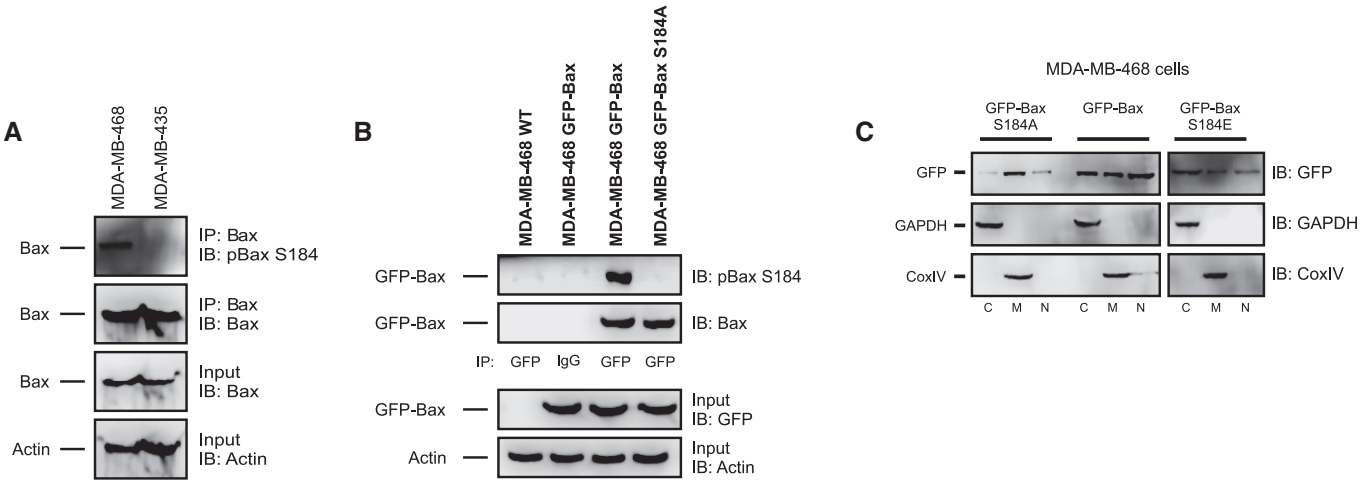

Figure EV1. Related to Fig 2: Bax is phosphorylated on residue S184.

A Top two panels: Phosphorylation of Bax S184 was evaluated using lysates from MDA-MB-435 and MDA-MB-468 cells by Western blotting with the indicated antibodies (IB) after immunoprecipitation with the indicated antibodies (IP). Lower two panels: 5–10% of the total lysates (input) were probed for Bax and actin as expression and loading controls, respectively, by Western blotting with the indicated antibodies (IB).

B Top two panels: Phosphorylation of Bax S184 was evaluated using lysates from GFP-Bax- and GFP-Bax S184A-expressing MDA-MB-468 cells by blotting with the antibodies indicated at the right after precipitation with the antibodies indicate below the panels. Lysates from untransfected cells (MDA-MB-468 WT) and IgG IP are used as negative controls. Lower panels: 5–10% of the total lysates were probed for GFP and actin as expression and loading controls, respectively.

C Localization of GFP-Bax constructs in MDA-MB-468 cells. MDA-MB-468 cells transiently expressing GFP-Bax S184A, GFP-Bax, or GFP-Bax S184E were lysed and separated into cytosolic (C), mitochondrial (M), or nuclear fractions (N) and then immunoblotted for GFP. GAPDH and CoxIV were immunoblotted for cytosolic and mitochondrial marker proteins, respectively.

Figure EV2. Related to Fig 3: Akt pathway inhibitors change the primed state of mitochondria, the localization of Akt, and the phosphorylation status of Bax S184.

A MDA-MB-468 and ZR-75-1 cells were treated with 1 μM MK-2206, 0.4 μM A-443654, 25 μM LY294002, or 10 nM deguelin where indicated. Phosphorylation of Bax S184 was evaluated using lysates from the treated cells by Western blotting with the indicated antibodies (IB) after immunoprecipitation with the indicated antibodies (IP).

B S100 fractions from untreated cells (MDA-MB-468) or from cells treated with direct Akt inhibitors (MK-2206 [1 μM], A-443654 [0.4 μM]) or Akt pathway inhibitors (LY294002 [25 μM], deguelin [10 nM]) were isolated. Akt was immunodepleted by sequential immunoprecipitation in untreated S100 fractions, and the efficiency of immunodepletion was tested by immunoblot analysis. IgG was used as a negative control for immunodepletion experiments. The indicated S100 fractions were incubated with MDA-MB-435 mitochondrial preparations, and change in priming was assessed by using BH3 profiling. Responses to various BH3 peptides are shown. The phosphatase inhibitor cocktail PhosSTOP was used in all buffers. Cytochrome *c* release was determined by ELISA. Bars indicate the mean of three independent experiments (n = 3). Symbols indicate the mean of at least two technical replicates for each independent experiment. Two-way ANOVA was conducted on the influence of two independent variables (BH3 peptide, treatment) on cytochrome *c* release of isolated mitochondria from ABT-737-sensitive MDA-MB-435 cells that were treated with the S100 fraction isolated from ABT-737-resistant MDA-MB-468 or ZR-75-1 cells. Each treatment was statistically compared to control within each peptide group using *t*-tests with Bonferroni correction for multiple comparisons. See Appendix Table S2 for *P*-values.

C ABT-737-resistant MDA-MB-468 and ZR-75-1 and ABT-737-sensitive MDA-MB-435 and MCF-7 cells were treated with direct Akt inhibitors (MK-2206 [1 μM], A-443654 [0.4 μM]) or Akt pathway inhibitors (LY294002 [25 μM], deguelin [10 nM]), and mitochondrial and cytosolic fractions were immunoblotted for Akt. CoxIV was probed as a mitochondrial marker protein, and GAPDH was used as a cytosolic marker protein (M, mitochondrial fraction; C, cytosolic fraction).

D MDA-MB-435 cells were lysed and separated into cytosolic (C), mitochondrial (M), or nuclear fractions (N) and then immunoblotted for Bax. GAPDH and CoxIV were immunoblotted for cytosolic and mitochondrial marker proteins, respectively.

E Lack of expression of Bax and Bak in $Bax^{-/-}$ $Bak^{-/-}$ DKO MEFs was detected by immunoblotting with Bax and Bak antibodies. Actin was probed as a loading control. Bax Δ21 antibody was used for IB of Bax.

F Following kinase reaction with recombinant Akt, Bax was separated by SDS–PAGE. The resulting gels were stained with Pro-Q Diamond (stains phosphoproteins) or SYPRO Ruby (stains total protein) and imaged. Additionally, phosphorylation of recombinant Bax following kinase reaction by Akt was evaluated by immunoblotting with phosphoserine antibody. Bax was blotted as a loading control.

G Akt or ATP did not alter cytochrome *c* release triggered by tBid/Bax treatment when used alone. Mitochondria from $Bax^{-/-}$ $Bak^{-/-}$ DKO MEFs were directly exposed to Akt or ATP or after incubated Akt or ATP with tBid and Bax as designated. Cytochrome *c* release was determined by ELISA. Bars indicate the mean of three independent experiments (n = 3). Symbols indicate the mean of at least two technical replicates for each independent experiment.

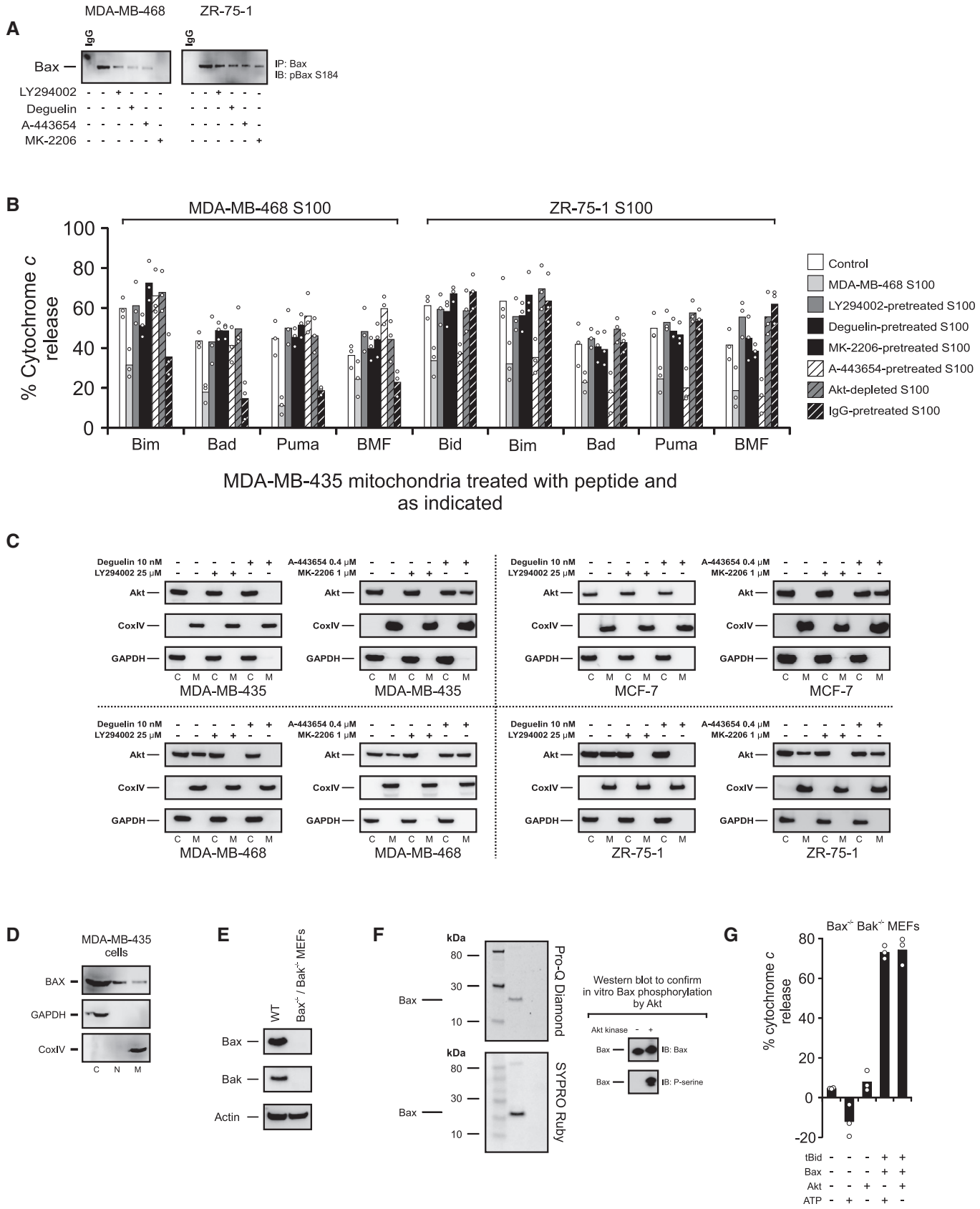
A
MDA-MB-468
ZR-75-1
IgG
Bax
IP: Bax
IB: pBax S184
LY294002
Deguelin
A-443654
MK-2206
B
MDA-MB-468 S100
ZR-75-1 S100
% Cytochrome c release
Bim
Bad
Puma
BMF
Bid
Control
MDA-MB-468 S100
LY294002-pretreated S100
Deguelin-pretreated S100
MK-2206-pretreated S100
A-443654-pretreated S100
Akt-depleted S100
IgG-pretreated S100
MDA-MB-435 mitochondria treated with peptide and as indicated
C
Deguelin 10 nM
LY294002 25 μM
A-443654 0.4 μM
MK-2206 1 μM
Akt
CoxIV
GAPDH
MDA-MB-435
MCF-7
MDA-MB-468
ZR-75-1
D
MDA-MB-435 cells
BAX
GAPDH
CoxIV
E
WT
Bax-/- / Bak-/- MEFs
Bax
Bak
Actin
F
kDa
Pro-Q Diamond
SYPRO Ruby
Western blot to confirm in vitro Bax phosphorylation by Akt
Akt kinase
IB: Bax
IB: P-serine
G
Bax-/- Bak-/- MEFs
% cytochrome c release
tBid
Bax
Akt
ATP

Figure EV2.

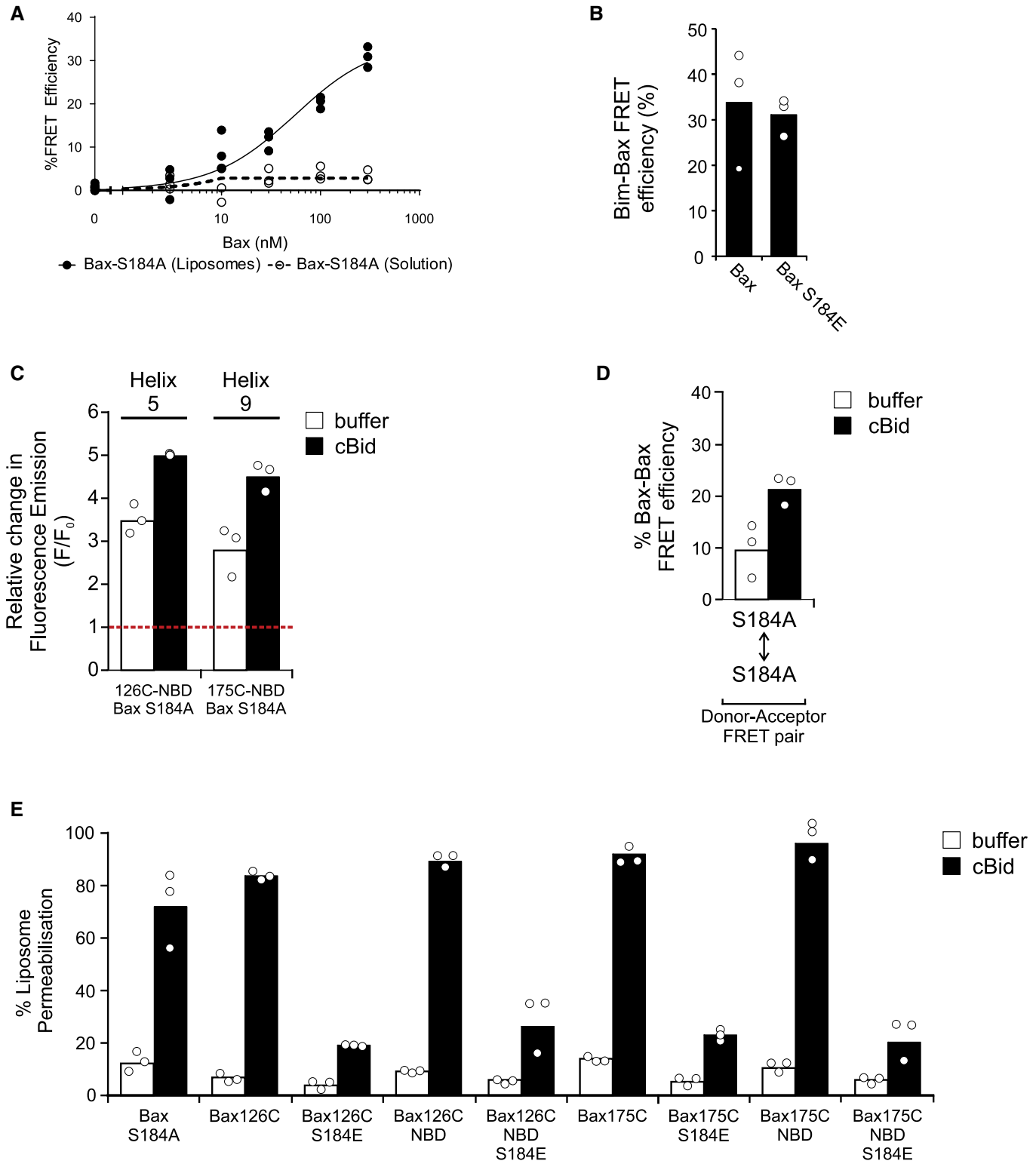

Figure EV3.

Figure EV3. Related to Fig 4: Bax S184A is a functional Bax mutant that spontaneously inserts into membranes.

A The apparent affinity between cBid and Bax S184A was measured with liposomes and in solution using FRET. Samples containing liposomes were incubated with 20 nM cBid 126C-DAC, and Bax-126C-NBD S184A was added as indicated. FRET efficiency was calculated at 2-h endpoint. Symbols indicate the mean of at least two technical replicates for each independent experiment (n = 3). These data were fit to an equation that describes the equilibrium binding between two proteins (lines; see Materials and Methods).

B The interaction between Bim and Bax was measured by FRET. Samples containing liposomes were incubated with 20 nM BIM 41C-DAC and 100 nM of Bax175C-NBD or Bax 175C-NBD S184E. FRET efficiency was calculated at 2-h endpoint. Bars indicate the mean of three independent experiments (n = 3). Symbols indicate the mean of at least two technical replicates for each independent experiment.

C Insertion of alpha-helices 5 and 9 of Bax S184A into the liposome bilayer was measured using the environment-sensitive dye NBD covalently attached to cysteine residues at the indicated locations in Bax S184A. The relative change of NBD emission (F/F_0) was calculated at a 1-h endpoint with 20 nM cBid (cBid) or buffer as a control (buffer) in the presence of liposomes. Bars indicate the mean of three independent experiments (n = 3). Symbols indicate the mean of at least two technical replicates for each independent experiment. The red dotted line indicates no change in fluorescence (F/F_0 = 1).

D Bax S184A oligomerization was measured by FRET between Bax monomers. Samples containing liposomes were incubated with 10 nM DAC (donor)-labeled Bax S184A and 100 nM of NBD (acceptor)-labeled Bax S184A in the absence (buffer) or presence of 20 nM cBid. FRET efficiency was calculated at 2-h endpoint. Bars indicate the mean of three independent experiments (n = 3). Symbols indicate the mean of at least two technical replicates for each independent experiment.

E The liposome permeabilization activity of Bax S184A or single cysteine mutants of Bax and Bax S184E (labeled and unlabeled) was measured using an ANTS/DPX release assay. ANTS/DPX liposomes were incubated with 100 nM of the indicated Bax in the absence (buffer) or presence of 20 nM cBid. Bars indicate the mean of three independent experiments (n = 3). Symbols indicate the mean of at least two technical replicates for each independent experiment.

Figure EV4. Related to Fig 5: mCerulean3-Bax-S184E protects cells from apoptosis but Bax S184A does not.

A Liposome permeabilization was measured as an increase in fluorescence due to release of ANTS/DPX from liposomes. Fluorescence was normalized using samples not containing Bax S184A. Samples were incubated with 50 nM WT Bax, 20 nM cBid (black circles) or Bim (red squares), and the indicated concentration of Bax S184A. Symbols indicate the mean of at least two technical replicates for each independent experiment (n = 3). These data were fit to a simple exponential decay (lines).

B Western blot of cell lysates of the indicated cell lines (top) either untransfected or stably expressing mC3-Bax. Constructs were analyzed by gel electrophoresis followed by Western blot using the antibodies indicated on the right. Bax (#2772; Cell Signaling) was used for IB of Bax.

C WT BMK or $Bax^{-/-}$ $Bak^{-/-}$ DKO BMK cells that are either untransfected or stably expressing the indicated mCerulean3 (mC3) Bax constructs were treated with increasing concentrations of the indicated cell death stimuli for 24 h. Cells treated with TNF-alpha were also treated with 5 μg/ml cycloheximide. Cells stained with DRAQ5, Annexin V conjugated to Alexa 488, and TMRE were imaged on an Opera Phenix automated confocal microscope. Intensity of Annexin V and TMRE staining was measured for > 500 cells per independent experiment. A threshold was used to automatically determine Annexin V-positive and TMRE-negative cells. Percent apoptosis was calculated by taking the total number of Annexin V–Alexa 488-positive and TMRE-negative cells and dividing by the total number of cells. Each point represents the mean % apoptosis calculated from four independent experiments (n = 4) each with two technical replicates. Shown is mean ± SEM.

D, E The indicated cell lines (left) were treated with increasing doses of STS (0–100 nM) and 10 μM of caspase inhibitor (Q-VD-OPH) for 20 h, stained with DRAQ5 (DNA), and imaged using an Opera Phenix automated confocal microscope (D, representative images; and E, image analysis). (D) Representative images (63× water-immersion objective) of cells treated with caspase inhibitor (10 μM Q-VD-OPH) and either DMSO or 50 nM STS are shown. Images shown are cropped, highlighting single cells to aid in visual discrimination of localization. Scale bars: 20 μm. (E) Cells were imaged as representative images in EV4D above except with a 40× water-immersion lens. The percent of cells with a punctate mCerulean3 localization pattern was quantified based on automated assessment of intensity fluctuations for cells expressing the indicated mC3-Bax construct in the absence (DMSO control, open symbols) or presence of increasing concentrations of STS (closed symbols). Symbols indicate the mean of at least two technical replicates (≈400–1,600 cells per condition) for each independent experiment (n = 3). These data were fit to a simple log(dose) vs. response model with a hill slope of 1 (lines). Shown is mean ± SEM.

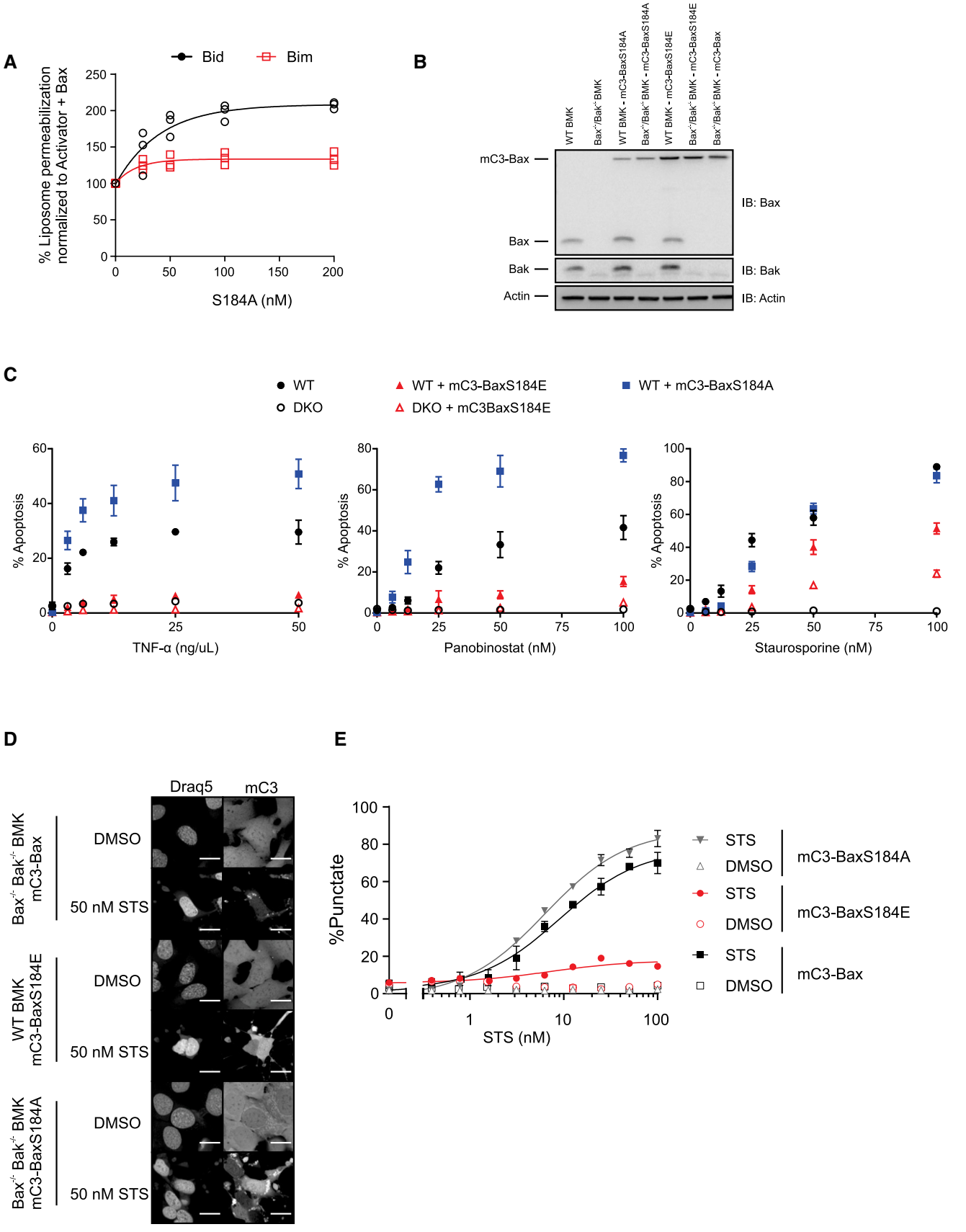

Figure EV4.

Figure EV5. Related to Fig 6: Overexpression of Bax is countered by Akt pathway activation in breast invasive carcinoma.

A PTEN, Akt, pAkt S473, and pAkt T308 levels were evaluated using lysates from the indicated primary ovarian cancer cells by Western blotting with the indicated antibodies (IB) after immunoprecipitation with the indicated antibodies (IP).

B TCGA database of invasive breast carcinoma was probed for significant associations between the row heading (e.g., *BAX* CNA gain) and copy number (CNA) gain/loss (red/blue) or mRNA up/downregulation (red/blue) of the indicated genes (*AKT1*, *AKT2*, and *PTEN*). Association strength is proportional to $-\log(P\text{-value})$, reflected in the radii of the circles. Black circles indicates non-significant association (P-value cutoff = 0.02). The data used to generate this figure are located in Appendix Table S3.

C Each circle represents the total number of patients with invasive breast carcinoma from the TCGA database, with either high *BAX* or *BAK1* (higher than the mean, left) or low *BAX* or *BAK1* (lower than the mean, right) copy number. Within each circle, the proportion of patients with upregulated Akt pathway (red) is compared to the proportion of patients without upregulated Akt pathway (blue). Upregulated Akt pathway is defined as an increase in *AKT1* or *AKT2* or *PIK3CA* or decrease in *PTEN* copy number.

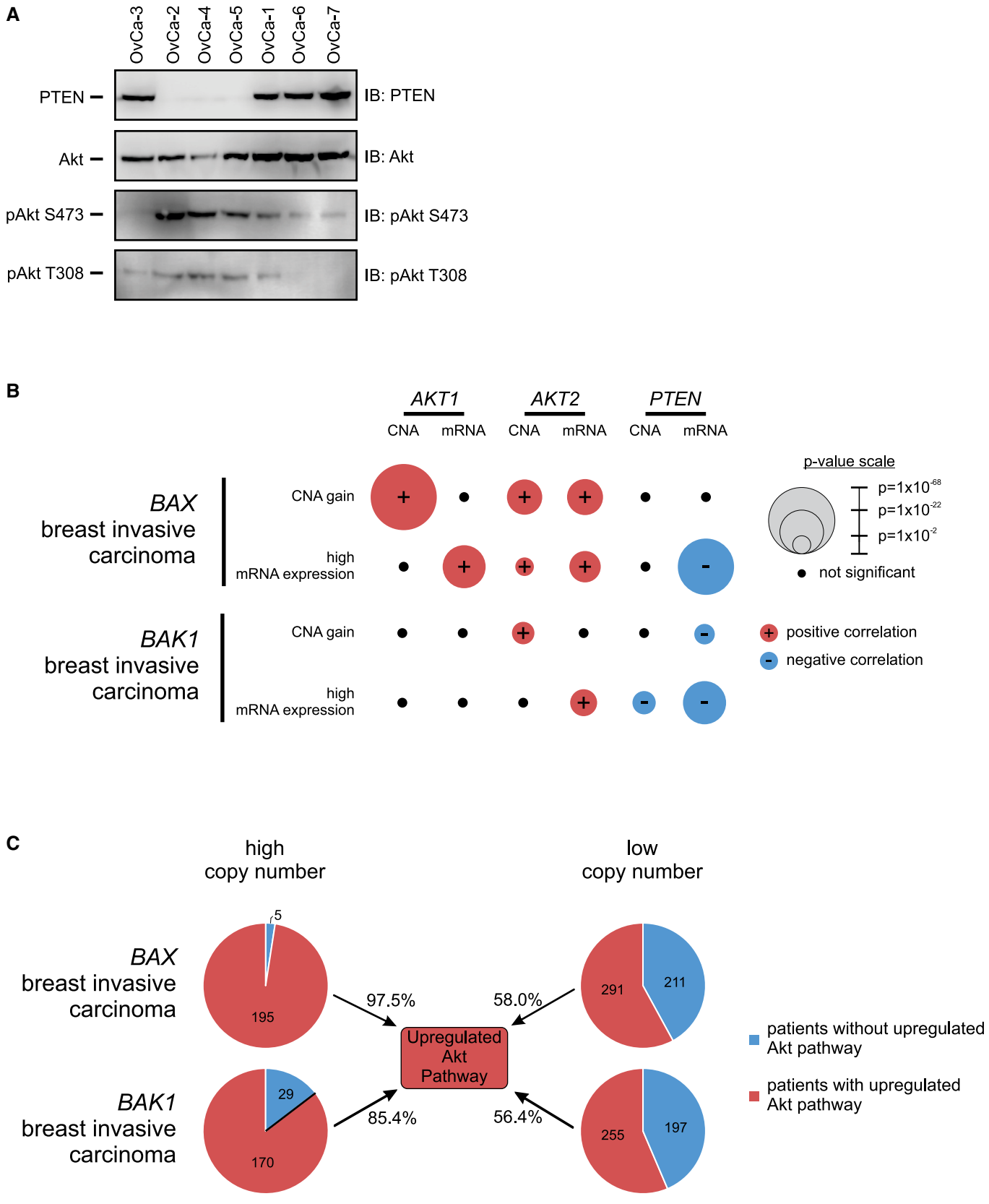

Figure EV5.