Supplementary Figure Legends

Fig. S1. Prolonged ER stress induces necrotic cell death in apoptosis-deficient cells. (A) ER stress can induce non-apoptotic cell death. MEFs expressing Bcl-xL and the vector control were treated with thapsigargin (0.1 μM), tunicamycin (0.5 μg/ml) or brefeldin A (0.5 μg/ml). At the indicated time points cells were collected and cell death was measured by PI exclusion. Data are averages of triplicate treatments ± S.E.M. ($P < 0.01$ for all time points). (B) ER stress induces necrosis-like cell death. *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were treated with thapsigargin, tunicamycin or brefeldin A for 72 hours then photographed under a phase-contrast filter using a x32 objective. Note the plasma membrane swelling in treated cells. (C) HMGB1, a nuclear protein, is released into the extracellular environment in *bax*$^{-/-}$*bak*$^{-/-}$ MEFs upon ER stress treatment. *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were treated for 72 hours with thapsigargin (Thap), tunicamycin (Tuni), and brefeldin A (BFA). As a positive control for HMGB1 release, *bax*$^{-/-}$*bak*$^{-/-}$ cells were treated with MNNG (500 μM) for 30 min. MNNG-containing medium was washed away and cells were cultured in drug free media for 24 hours. Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells were treated with tunicamycin (0.5 μg/ml) for the indicated periods of time. Cell culture media and cell lysates were collected and probed for HMGB1 by immunoblotting.

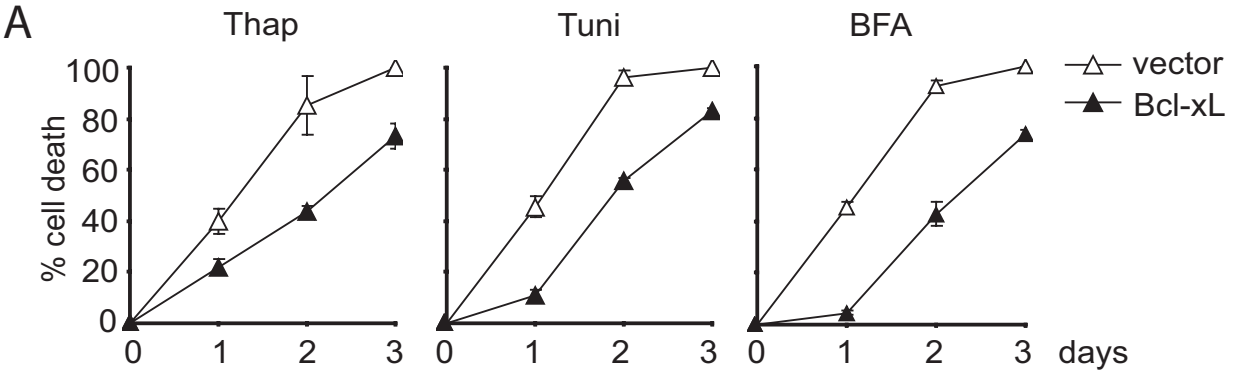
Fig. S2. ER stress induces autophagosome formation. (A) Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were treated with tunicamycin (0.5 μg/ml) for 48 hours. Cells were then fixed and observed with an electron microscope and representative images are shown. Note the chromatin condensation in wild-type cells treated for 48 hr, and accumulation of autophagosomes in *bax*$^{-/-}$*bak*$^{-/-}$ cells treated for 48 hrs. (B) Wild-type cells treated with tunicamycin for 48 h shows nucleosomal DNA laddering, while Bcl-xL expression blocks it. (C and D) Wild-type (C) or *bax*$^{-/-}$*bak*$^{-/-}$ (D) cells stably expressing LC3-GFP were treated for 8 hours with the indicated ER stress alone or in combination with 3MA (5 mM) or chloroquine (CQ, 10 μM) and analyzed by fluorescence microscopy. Formation of LC3-GFP puncta was observed in cells exposed to ER stress and this effect was reduced upon combined treatment with 3MA, whereas it was enhanced upon co-treatment with CQ.

Fig. S3. Autophagy is induced in *bax*$^{-/-}$*bak*$^{-/-}$ cells in response to ER stress. (A) Quantification of LC3-GFP puncta positive cells in wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells. Percentage of cells showing punctated LC3-GFP was determined and the values represent the mean ± S.E.M. of 3 countings ($P < 0.01$ for all data points). (B) Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were treated for the indicated times with the indicated ER stress. Thirty micrograms of total cell lysates was subjected to immunoblotting assay using antibodies against LC3 and CHOP. β-tubulin was probed as a control for equal loading. (C) Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells stably expressing LC3-GFP were treated with tunicamycin or thapsigargin in the absence or presence of the lysosomal protease inhibitors E-64-D (10 μg/ml) and pepstatin A (10 μg/ml). Twelve hours later, cells were fixed and observed under microscope. Note that the protease inhibitors enhanced the autophagosome formation induced by ER stress. (D) Wild-type and *bax*$^{-/-}$*bak*$^{-/-}$ cells were treated

with tunicamycin for 6 or 12 hours in the absence or presence of E-64-D and pepstatin A. Thirty micrograms of cell lysates were probed with an anti-LC3 antibody. Note the similar accumulation of LC3-II in the presence of the lysosomal protease inhibitors.

Fig. S4. Multi-domain pro-apoptotic Bcl-2 proteins do not affect autophagy. *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were transfected with LC3-GFP together with DsRed-tagged Bax, or Bak-cb5 that localizes to the ER membrane. (A) Twenty-four hours later cells transfected with Bax were left untreated or treated with tunicamycin (0.5 μg/ml) for 8 hours. Cells were then observed under a Zeiss Axiovert fluorescence microscope. DsRed-Bax was localized in the cytosol and did not induce autophagy. Tunicamycin treatment induced Bax translocation to mitochondria, which did not affect the induction of autophagy. (B) Bak is targeted mainly to mitochondria. GFP-tagged Bak was transfected into MCF7 cells for 24 h, cells were immunostained for COX IV, a mitochondrial marker. Note the co-localization of Bak and COX IV. (C) ER-targeted Bak-cb5 failed to induce autophagy. DsRed-Bak-cb5 transfected *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were fixed and stained with DAPI to visualize nuclei. Note that Bak-cb5 induced nuclear condensation (arrow), and failed to induce autophagy. (D) Verification of apoptosis induction by DsRed-tagged Bax, Bak, and Bak-cb5. *bax*$^{-/-}$*bak*$^{-/-}$ MEFs expressing Bax, Bak, or Bak-cb5 were left untreated or treated with BFA (0.5 μg/ml) or tunicamycin (0.5 μg/ml) for 24 hours. Cell death was determined by DAPI exclusion.

Fig. S5. Inhibition of autophagy protects apoptosis-deficient MEFs from ER stress-mediated cell death, while promoting death in apoptosis-competent cells. (A) Cell morphology was assessed by phase-contrast microscopy in *bax*$^{-/-}$*bak*$^{-/-}$ MEFs after 3 days of treatment with ER stress alone or in the presence of 3MA. (B) 3MA promotes cell death in apoptosis-competent cells. Wild-type MEFs were treated for 24 hours with indicated ER stress alone or in combination with 3MA. Cell death was determined by PI exclusion. Results shown are averages of triplicate samples +/- S.E.M. (P<0.01 for all treatments). (C) Atg5 knockdown results in delayed cell death in *bax*$^{-/-}$*bak*$^{-/-}$ MEFs in response to ER stress. Atg5 was knocked down in *bax*$^{-/-}$*bak*$^{-/-}$ MEFs with an Atg5 hairpin. The cells were treated for 3-4 days with either thapsigargin or brefeldin A. Percent cell survival was determined by trypan blue exclusion and taken as an average of triplicate samples ± S.E.M. (P < 0.01 for all time points). Cell lysates from shAtg5 transfected *bax*$^{-/-}$*bak*$^{-/-}$ MEFs and *atg5*$^{-/-}$ MEFs were subjected to immunoblotting to show specific decreased Atg5 expression, n.s. is a non-specific band used as a control for equal loading. (D) *atg5*$^{+/+}$ and *atg5*$^{-/-}$ MEFs were treated for the indicated times with ER stress and cell death was measured by PI exclusion and indicated as an average of triplicate samples ± S.E.M. (P < 0.01 for all time points).


A
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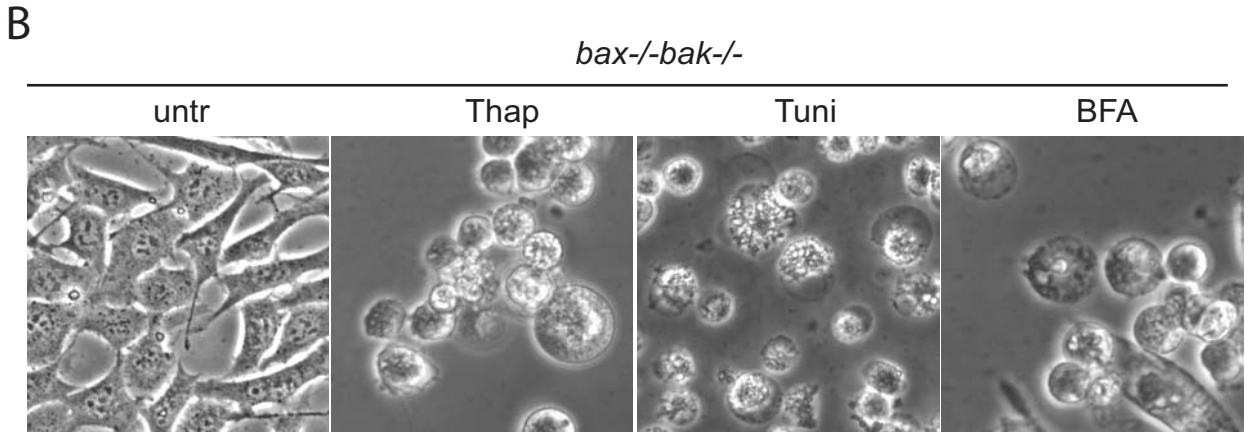
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Thap
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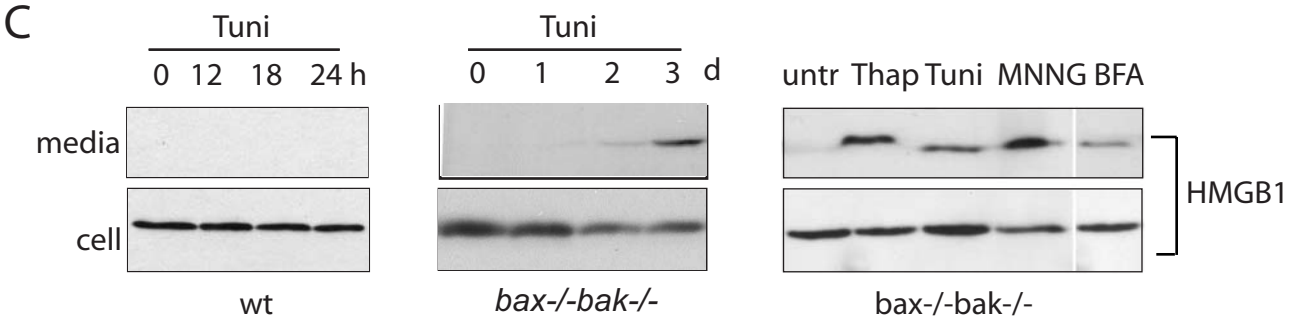
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media
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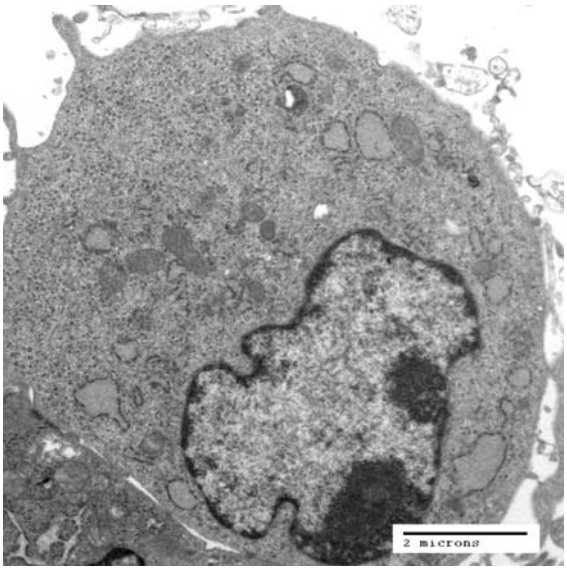
bax-/-bak-/-

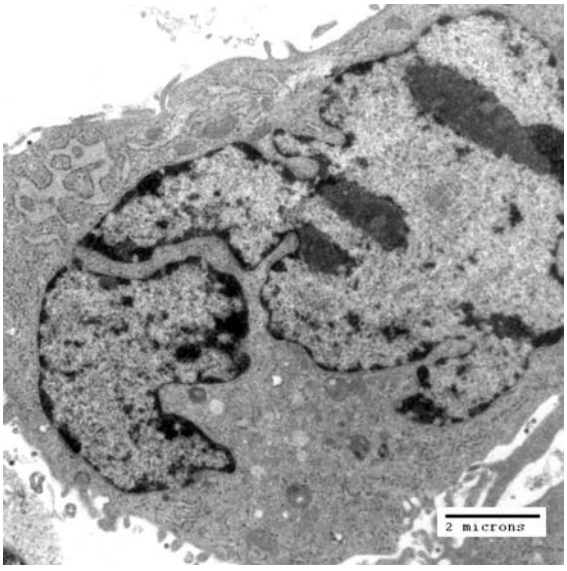
A

wild-type

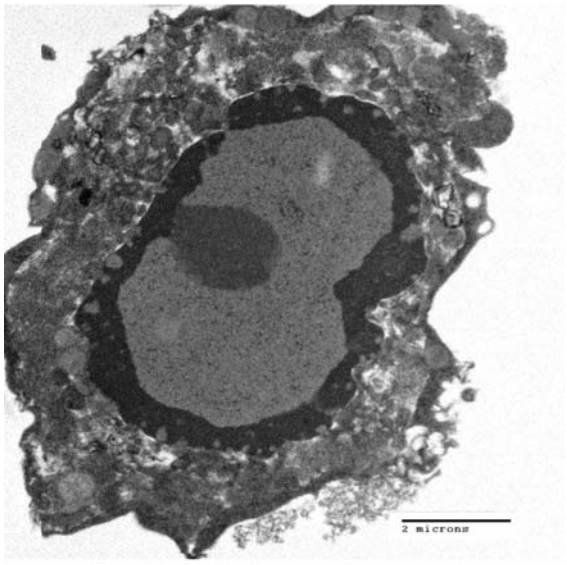
bax-/-bak-/-

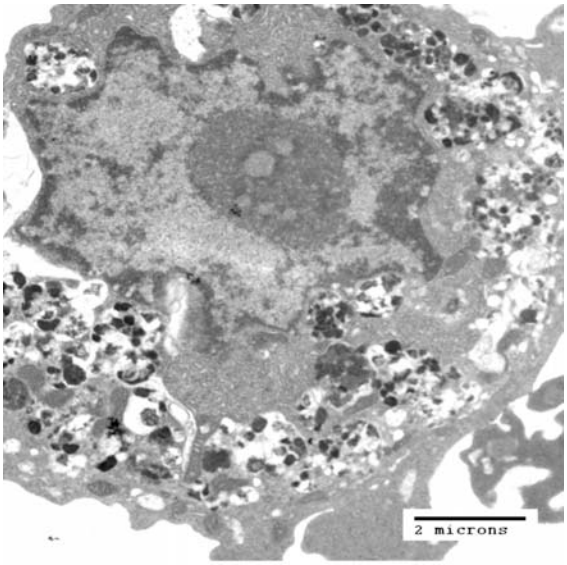
untr





tuni
48 hr

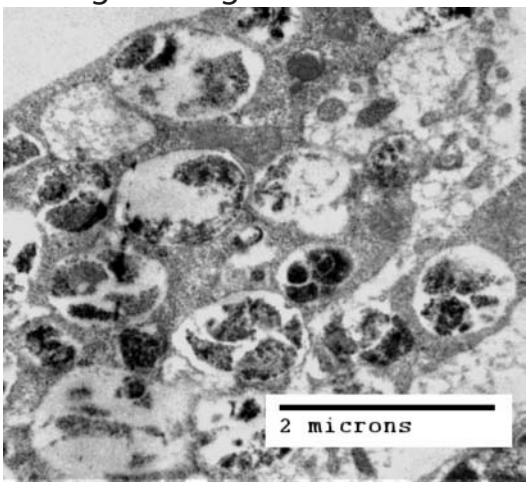


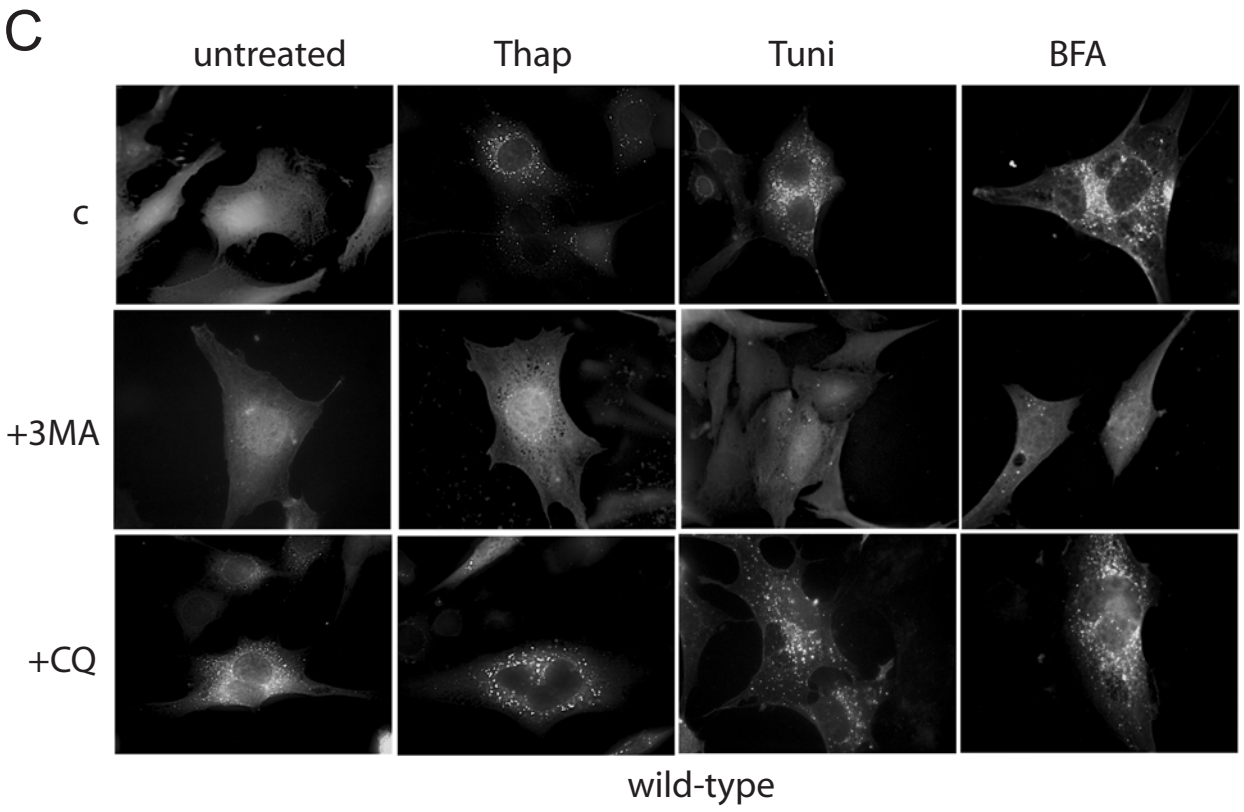


B

wt Bcl-xL

bax-/-bak-/- Tuni 48 hr
higher magnificantion

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untreated
Thap
Tuni
BFA
c
+3MA
+CQ
wild-type
D
untreated
Thap
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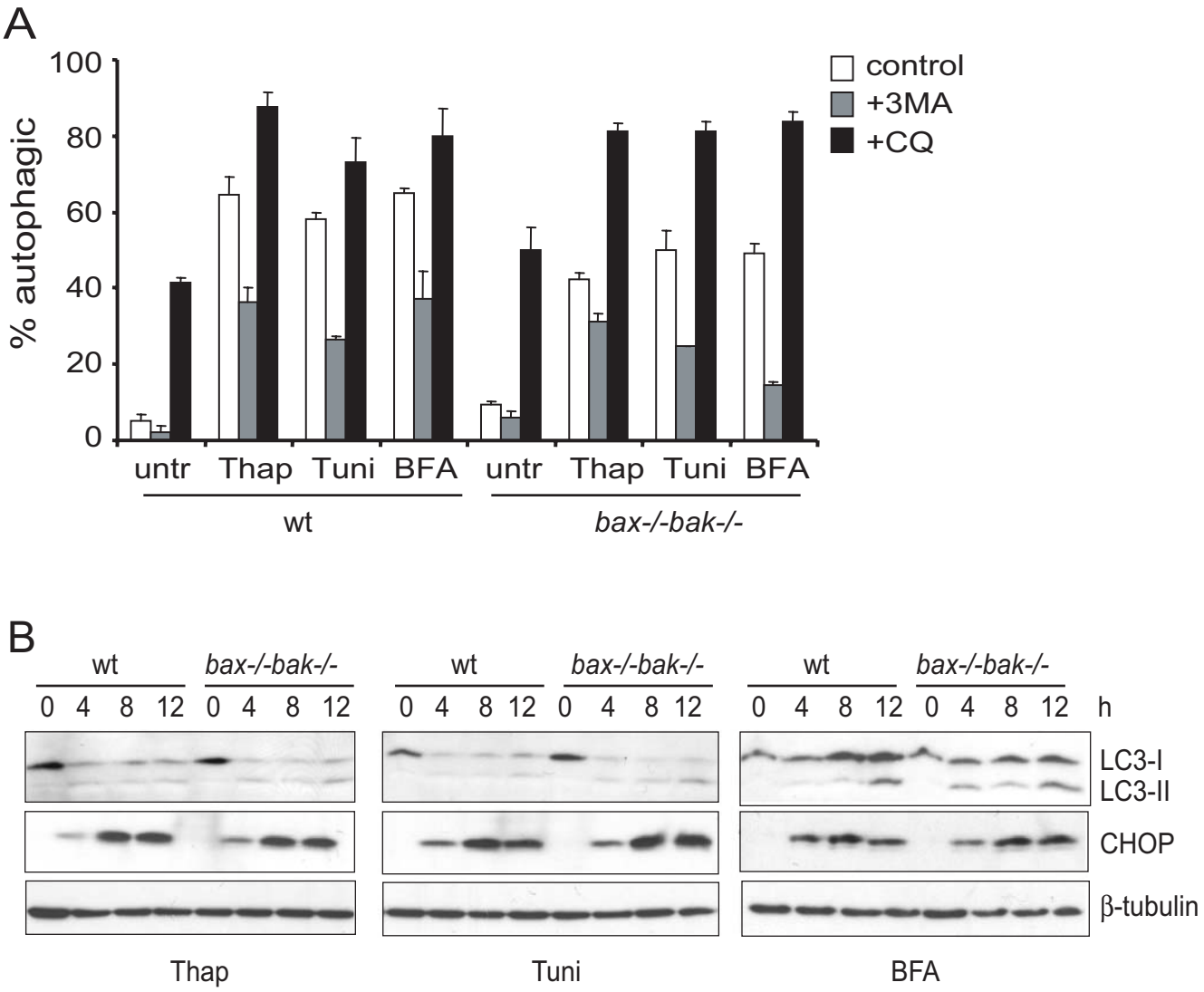
c
+3MA
+CQ
bax-/-bak-/-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control
+3MA
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Tuni
BFA
wt
bax-/-bak-/-
B
wt
bax-/-bak-/-
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bax-/-bak-/-
wt
bax-/-bak-/-
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0 4 8 12 0 4 8 12
0 4 8 12 0 4 8 12
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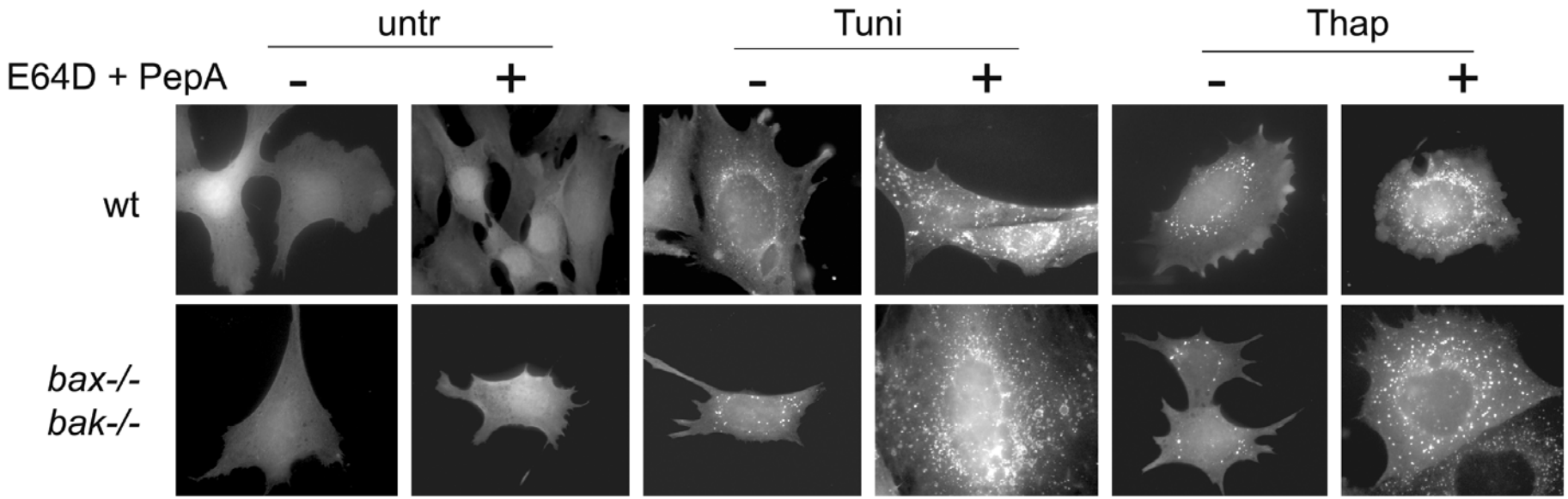
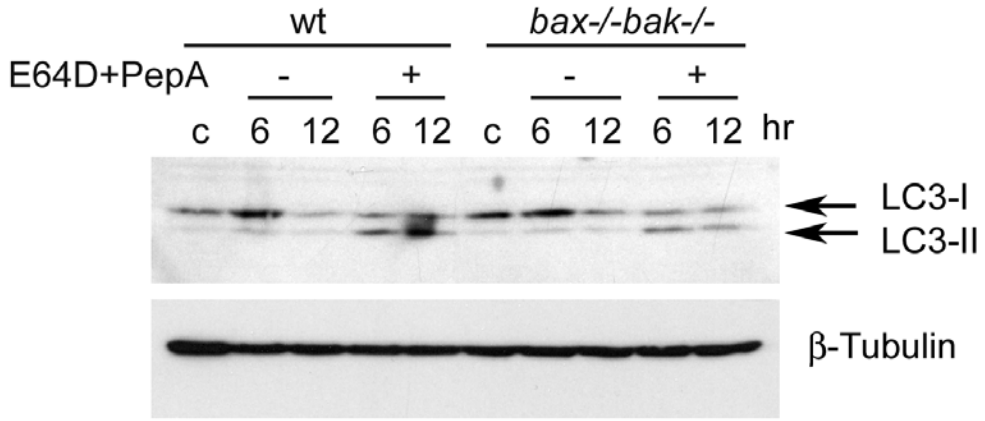
LC3-I
LC3-II
CHOP
β-tubulin
Thap
Tuni
BFA

C


untr
Tuni
Thap
E64D + PepA
-
+
-
+
-
+
wt
bax-/-
bak-/-

D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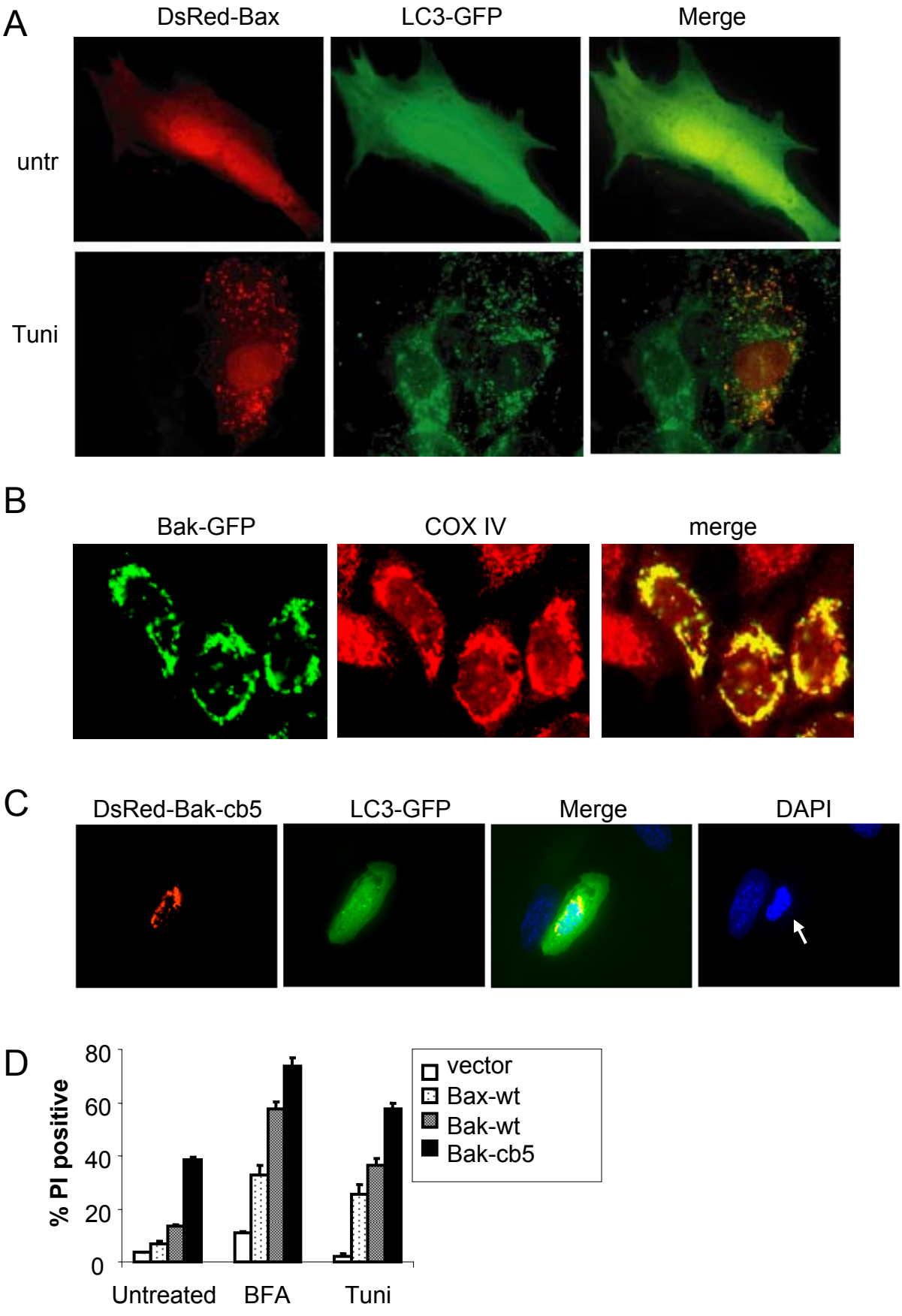
-
+
-
+
c 6 12 6 12 c 6 12 6 12 hr
LC3-I
LC3-II
β-Tubulin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DsRed-Bax
LC3-GFP
Merge
untr
Tuni
B
Bak-GFP
COX IV
merge
C
DsRed-Bak-cb5
LC3-GFP
Merge
DAPI
D
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60
40
20
0
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BFA
Tuni
vector
Bax-wt
Bak-wt
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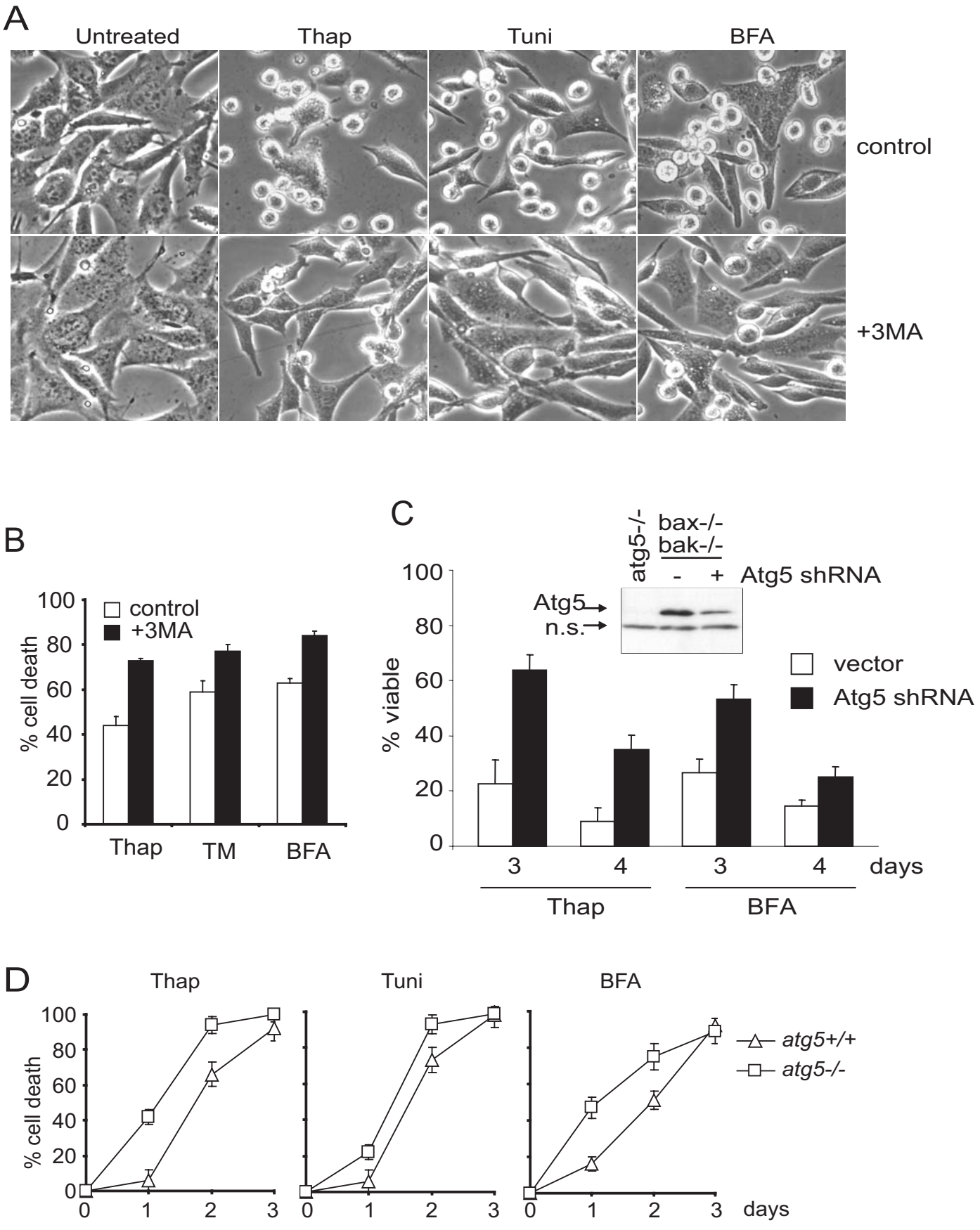
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Untreated
Thap
Tuni
BFA
control
+3MA
B
control
+3MA
% cell death
100
80
60
40
20
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Thap
TM
BFA
C
atg5-/-
bax-/-
bak-/-
- + Atg5 shRNA
Atg5
n.s.
% viable
vector
Atg5 shRNA
3 4 3 4 days
Thap
BFA
D
Thap
Tuni
BFA
% cell death
atg5+/+
atg5-/-
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