Intracellular Localization Of The BCL-2 Family Member BOK And Functional Implications

N Echeverry[1], D Bachmann[1], F Ke[2,3], A Strasser[2,3], HU Simon[1] and T Kaufmann[*1]

[1]Institute of Pharmacology, University of Bern, Bern, Switzerland
[2]The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia
[3]Department of Medical Biology, The University of Melbourne, Melbourne, Australia

[*]Corresponding author: Thomas Kaufmann, University of Bern, Institute of Pharmacology, Friedbuehlstrasse 49, CH-3010 Bern, Switzerland. Tel: 0041 31 632 32 89; Fax: 0041 31 632 49 92; E-mail: thomas.kaufmann@pki.unibe.ch

Word count (Introduction, Results, Discussion): 3495

Running title: Localization and role of BOK on the ER and Golgi

Keywords: apoptosis, BOK, BAX/BAK, tail-anchor, Golgi, ER

Abbreviations: 4-OHT, 4-hydroxytamoxifen; BFA, brefeldin A; BH, BCL-2 homology; BOK, BCL-2-related ovarian killer; BIM, BCL-2-interacting mediator of cell death; ER, endoplasmic reticulum; GRP78 (BiP), glucose-regulated protein 78 (binding protein); GM130, Golgi matrix 130; MAM, mitochondria-associated membrane; IRE1α, inositol requiring kinase and endonuclease 1 alpha; MCL-1, myeloid-cell leukemia sequence 1; MOMP, mitochondrial outer membrane permeabilization; TMD, transmembrane domain.

Abstract

The pro-apoptotic BCL-2 family member BOK is widely expressed and resembles the multi-BH domain proteins BAX and BAK based on its amino acid sequence. The genomic region encoding BOK was reported to be frequently deleted in human cancer and it has therefore been hypothesized that BOK functions as a tumor suppressor. However, little is known about the molecular functions of BOK. We show that enforced expression of BOK activates the intrinsic (mitochondrial) apoptotic pathway in BAX/BAK-proficient cells but fails to kill cells lacking both BAX and BAK or sensitize them to cytotoxic insults. Interestingly, major portions of endogenous BOK are localized to and partially inserted into the membranes of the Golgi apparatus as well as the endoplasmic reticulum (ER) and associated membranes. The C-terminal transmembrane domain of BOK thereby constitutes a 'tail-anchor' specific for targeting to the Golgi and ER. Over-expression of full-length BOK causes early fragmentation of ER and Golgi compartments. A role for BOK on the Golgi apparatus and the ER is supported by an abnormal response of *Bok*-deficient cells to the Golgi/ER stressor brefeldin A. Based on these results, we propose that major functions of BOK are exerted at the Golgi and ER membranes and that BOK induces apoptosis in a manner dependent on BAX and BAK.

Introduction

The intrinsic (mitochondrial) apoptotic pathway is stringently controlled by the BCL-2 protein family, which is divided into three groups based on the ability to induce or antagonize apoptosis and the presence of up to four short conserved domains, termed BCL-2 homology domains (BH1-BH4) (1). The members of the pro-survival subgroup (BCL-2, MCL-1, BFL-1/A1, BCL-X_L and BCL-W) inhibit apoptosis and contain all four BH domains (BH1-BH4) (2). The pro-apoptotic members are divided into two subgroups, the multi-BH domain containing BAX-like family (BAX, BAK and BOK), and the BH3-only proteins (BIM, BID, NOXA, BAD, PUMA, BIK, HRK and BMF) (3). The latter contain only a BH3 domain, which is essential for apoptosis initiation upon activation in response to apoptotic stimuli (4). By a series of complex and still controversially discussed protein-protein interactions, activated BH3-only proteins transmit the apoptotic signal to BAX and BAK, which are essential for mitochondrial outer membrane permeabilization (MOMP) (5, 6). MOMP is commonly seen as the point-of-no-return in the intrinsic apoptotic pathway; it unleashes the activation of apoptotic caspases, which are responsible for the demolition of many vital cellular substrates and consequently the morphological changes observed during apoptosis (7, 8).

Most BCL-2 family members are tail-anchored to the mitochondrial outer membrane via a C-terminal alpha-helical transmembrane domain (TMD) that serves both as a targeting and anchoring device (2, 9). However, several members, including BCL-2, BAX and BAK, are also present on other organelles, most notably the endoplasmic reticulum (ER), where they have been reported to be involved in the regulation of calcium homeostasis, autophagy and ER stress responses (2, 10-15).

BOK (BCL-2-related ovarian killer) was identified in a yeast two-hybrid screen using MCL-1 as bait and was shown to selectively interact with MCL-1 and BFL-1/A1 but not with BCL-2 or BCL-X_L (16, 17). Among the BCL-2 family members, BOK shows highest

evolutionary conservation (18, 19). *Bok* expression was first reported to be restricted to reproductive tissues (16). However, we have recently shown that BOK can be detected in most tissues, albeit in several at only relatively low levels (20). The BOK protein was found to localize to mitochondria, the ER as well as to the nucleus (18, 21, 22).

Several studies, largely relying on protein over-expression, have shown that BOK can induce cell death; however, the exact mechanisms are poorly understood and may involve both caspase-dependent and -independent pathways (16, 17, 23). Based on the high sequence similarity of BOK with BAX and BAK (particularly in the BH1-3 domains) it has been assumed that BOK might function similar to BAX/BAK (16, 17, 20-22, 24). However, the high resistance of cells lacking both BAX and BAK towards possibly all intrinsic apoptotic stimuli (25, 26), together with detectable BOK levels in those cells, raises the question whether BOK can substitute for the function of BAX and BAK.

Using gene-targeting in ES cells, we have shown that mice develop normally in the absence of *Bok* and that young adult *Bok*$^{-/-}$ mice show no obvious abnormalities. Furthermore, *Bok*$^{-/-}$ hematopoietic cells responded normally to diverse classical apoptotic stimuli, such as cytokine deprivation or γ-irradiation (20). Interestingly, Beroukhim and colleagues have recently reported that the *BOK* gene is localized in a region of the genome that is frequently deleted in human cancer (27). It is therefore possible that BOK acts as a tumor suppressor. Loss of *Bok* did, however, not accelerate the development of pre-B/B-cell lymphoma in the *Eμ-Myc* transgenic mouse model (20). This result may indicate that *Bok* may play a role in tumor suppression in cells outside the lymphoid system, consistent with the observation that *Bok* expression in lymphocytes is low (20).

In this study, we investigated the subcellular localization of over-expressed and endogenous BOK and its potential to kill cells in the presence or absence of BAX/BAK. We found that substantial portions of BOK protein are present on the Golgi and ER

membranes. Over-expressed BOK was found to cause early fragmentation of Golgi and ER compartments upstream of caspase activation. Interestingly, BOK failed to kill *Bax*$^{-/-}$ *Bak*$^{-/-}$ doubly deficient cells and high BOK levels could not substitute for the combined loss of BAX and BAK in inducing apoptosis in response to stress stimuli. Finally, BOK-deficient cells responded abnormally to brefeldin A, a drug that impacts the Golgi/ER, supporting a role for BOK within these subcellular compartments. Taken together, these results indicate that BOK plays important roles on the membranes of the Golgi and ER and activates the intrinsic apoptotic pathway in a manner dependent on BAX and BAK.

Results

BOK-induced apoptosis is largely BAX/BAK-dependent

We have recently shown that BOK is widely expressed in the mouse ((20) and Supplementary Figure S1), therefore perhaps indicating that based on its resemblance to BAX and BAK it may play a role in apoptosis in many cell types. However, mice lacking both *Bax* and *Bak* present with severe phenotypic abnormalities in diverse tissues, such as epithelial, neuronal and hematopoietic ones. Moreover, cells derived from these mice are highly resistant towards possibly all intrinsic apoptotic stimuli (25, 28), even though they do express endogenous BOK. This may indicate that BOK does not exert a major role overlapping with those of BAX and BAK. To investigate the function of BOK further, and given that over-expression of BOK was previously reported to induce apoptosis (16-18, 21-23), we examined whether BOK-induced apoptosis depends on the presence of BAX/BAK.

Using an inducible expression system (29) or transient transfection, we first confirmed that BOK over-expression kills several non-transformed and transformed cells. As shown in Figure 1, SV40 large T antigen-immortalized mouse embryo fibroblasts (SV40 MEF) died upon induction of BOK, exhibiting classical features of apoptosis, including rounding

and detachment, plasma membrane blebbing, phosphatidylserine exposure, effector caspase activation and increased subG1 DNA content. Induction of EGFP expression did not kill these cells and 4-hydroxytamoxifen did not exert cytotoxicity. This shows that BOK over-expression can specifically kill these cells (Figure 1b and Supplementary Figure 2). Over-expressed BOK induced cytochrome c release and caspase-9 activation preceding caspase-3 processing (Figure 1e-h). Similarly, growth factor-dependent murine myeloid progenitors (FDM), the IL-3-dependent pro-B cell line BaF/3, human Jurkat T cell leukemia as well as HeLa cervical cancer derived cells were killed in response to BOK over-expression (Figure 2d, Supplementary Figure S3). This cell death could be inhibited by addition of the pan-caspase inhibitor Q-VD-OPh (Figures 1a, S3a and S3e). Importantly, BOK-induced cytochrome c release occurred upstream of caspase activation (Figure 1f). This indicates that BOK-induced cell killing proceeds via the intrinsic apoptotic pathway. BOK-induced killing was independent of the presence of endogenous BOK (Supplementary Figure S3f) and BOK over-expression killed MCL-1-deficient MEF significantly faster than WT cells, further supporting the notion that BOK activates the intrinsic apoptotic pathway (Figure 1a).

To investigate whether BOK-induced apoptosis was dependent on BAX and BAK, we over-expressed BOK in *Bax*$^{-/-}$*Bak*$^{-/-}$ MEF and FDM. Although BOK over-expression could efficiently kill WT MEF and FDM, this largely (MEF) or completely (FDM) failed to kill cells lacking both BAX and BAK (Figures 2a, b, d). In contrast, MEF or FDM lacking only BAX or BAK, respectively, were normally sensitive to BOK-induced killing (not shown). Importantly, BOK over-expression also failed to sensitize *Bax*$^{-/-}$*Bak*$^{-/-}$ MEF and FDM to classical apoptotic stimuli, including treatment with etoposide or staurosporine (Figure 2c and d).

We next examined which protein-protein interactions are critical for BOK over-expression-induced apoptosis. The D83G mutation in BAK was shown to prevent

homodimerization/-oligomerization and consequently abrogated its ability to kill *Bax*$^{-/-}$ *Bak*$^{-/-}$ MEF (30). Intriguingly, BAK(D83G) was still capable of killing HEK293T cells (30), as was the corresponding BAX(D68A) mutant (31), indicating that these mutants can activate endogenous BAX/BAK. We found that FLAG-(WT)BOK co-immunoprecipitated HA-(WT)BOK, but this interaction was disrupted by a point mutation of the conserved aspartate^{76} residue within its BH3 domain (Figure 2e). Interestingly, BOK(D76A) retained its full ability to kill WT and *Bok*$^{-/-}$ cells, indicating that BOK may trigger apoptosis as a monomer (Figure 2f). No significant interaction was found by co-immunoprecipitation analysis between WT BOK and pro- or anti-apoptotic BCL-2 family members (Figure 3). Collectively, these results show that BOK over-expression induces apoptosis in BAX/BAK-proficient but not in BAX/BAK DKO cells, indicating that BOK may act upstream of BAX and BAK in the intrinsic apoptotic pathway.

Over-expressed BOK localizes to the ER and Golgi apparatus

When we analyzed the subcellular localization of over-expressed FLAG- or EGFP-tagged BOK by confocal microscopy, we found that BOK only partially co-localized with mitochondrial markers but was also found on other intracellular membranes (Figure 4a and 5b). Using staining for organelle specific markers, we found that BOK was prominently associated with the membranes of the endoplasmic reticulum (ER) and its associated nuclear outer membrane (co-localization with calnexin) as well as with the Golgi apparatus (co-localization with cis-Golgi protein GM130 and trans-/medial-Golgi protein GalNAc-T2) (Figure 4a). In addition, over-expressed BOK showed vesicular localization patterns, particularly at later time points and upon blocking of apoptosis with Q-VD-OPh. This vesicular BOK partially co-localized with the early and recycle endosome markers RAB4 and RAB11, respectively, the late endosome marker RAB5a and was found in LysoTracker positive vesicles (acidic organelle marker) (Figure 4b and

Supplementary Figure S4). Interestingly, cells over-expressing BOK showed readily detectable changes in ER and Golgi morphology. The ER appeared swollen and fragmented (punctuated pattern) and the Golgi apparatus displayed weakened GM130 staining at early time points and a fragmented pattern at later time points. These alterations clearly depended on over-expression of full length BOK, since these organelles were not affected in cells over-expressing EGFP fused to the TMD of BOK only (Figure 5). BOK-induced disruption of Golgi and ER-structures occurred upstream of caspase activation as it was also seen in the presence of Q-VD-OPh (Figure 4c). Taken together, these results show that major portions of over-expressed BOK are found on the ER-, Golgi and associated membranes and BOK over-expression rapidly leads to the fragmentation of these organelles in a caspase-independent manner. BOK may thus play a role in the dismantling of these organelles during apoptosis.

The TMD of BOK is necessary and sufficient for Golgi and ER targeting

The putative TMD of BOK atypically contains two positively charged amino acids, arginine^{200} and lysine^{203}, weakening its hydrophobicity (Figure 5a). Similar to BCL-2, the net positive charge at the C-terminal end of BOK is +1, whereas others and we have shown that a net positive charge of ≥+2 is required for specific mitochondrial targeting (2, 11, 32, 33). To test whether the TMD of BOK is a functional tail-anchor, we generated a TMD deletion mutant. Over-expressed EGFP-BOKΔTMD lacked organelle-targeting potential and, accordingly, was diffusely distributed in the cytosol and nucleus (Figure 5b). To test whether the TMD of BOK is sufficient for organelle targeting, we fused the 35 (or 27, not shown) C-terminal amino acids of BOK, encompassing its TMD plus flanking residues, to the C-terminus of EGFP. EGFP-TMD(BOK) efficiently co-localized with Golgi- and ER-markers but not with mitochondrial markers (Figure 5c and d). These

results show that the TMD of BOK is both necessary and sufficient for intracellular targeting to ER- and Golgi-membranes.

Subcellular localization of endogenous BOK

We next analyzed the subcellular localization of endogenous BOK in MEF by confocal microscopy. This confirmed a strong association of BOK with the Golgi apparatus and the ER, whereas endogenous BOK only partially co-localized with mitochondrial markers (Figure 6). The localization of endogenous BOK on the ER resembled the pattern seen for BCL-2, which is known to reside on the ER/nuclear outer membrane (11, 34, 35). However, in contrast to BOK, BCL-2 showed no Golgi-specific staining. To further investigate the localization of BOK on the Golgi apparatus, we treated MEF with brefeldin A (BFA), a drug that leads to Golgi fragmentation and is widely used to investigate Golgi apparatus dynamics (36). BFA treated MEF showed a rapid fragmentation of the Golgi into GM130 positive vesicular structures largely staining positive for BOK (Figure 6d).

Subcellular fractionation of MEF followed by Western blotting revealed that a major portion of both over-expressed and endogenous BOK was present in the microsomal fraction positive for the Golgi marker GM130. BOK was further found in calnexin-positive ER-derived heavy membranes and in mitochondria-enriched fractions but not in cytosolic fractions (Figure 7a and b). In comparison, endogenous BAX and BAK, both of which were reported to also localize to ER membranes (37, 38), were found at only low levels in the microsomal fraction. The subcellular distribution of endogenous BOK was independently confirmed in freshly isolated mouse hepatocytes, a primary cell type that expresses relatively high amounts of BOK (Figure 7c).

To determine whether organelle-targeted BOK was integrally inserted into the membranes, we treated membrane fractions isolated from MEF and primary hepatocytes

with alkaline solution (11). Both endogenous and over-expressed BOK were fully inserted into heavy membranes, whereas a portion of BOK present on microsomes was only loosely attached (Figure 7d and e). Trypsin-treatment of isolated membrane fractions completely degraded endogenous BOK protein, demonstrating that the epitope-carrying N-terminus of BOK faces the cytosol (Figure 7f). This indicates that, similar to other BCL-2 family members, BOK may insert into organelle membranes with the bulk of the protein facing the cytosol. Furthermore, and in contrast to BAK, BAX or BCL-2, we found a significant portion of BOK in purified nuclear fractions (Figure 7g). Taken together, our microscopic and biochemical studies provide evidence that major portions of BOK localize to intracellular membranes other than the mitochondrial, in particular Golgi- and ER-membranes.

Bok-deficient cells respond abnormally to the Golgi/ER-stressing drug brefeldin A

As BOK was found on Golgi/ER membranes, we examined the response of WT and *Bok*-deficient cells to BFA, a drug that results in the collapse of the Golgi-complex into the ER, leading to ER stress and apoptosis (36, 39). Although BFA-induced Golgi-fragmentation occurred normally in the absence of BOK (Supplementary Figure S5), BFA treated $Bok^{-/-}$ cells (MEF, mast cells and myeloid progenitors) responded with an abnormal ER stress response (Figure 8). Interestingly, BFA-treated cells died slightly but significantly faster in the absence of BOK. The same cells responded normally to other apoptotic stresses (e.g. etoposide, staurosporine), even though a minor but significant acceleration in cell death was also observed for tunicamycin-treated $Bok^{-/-}$ mast cells (Supplementary Figure S6). Western blot analysis revealed a disturbed ER stress-response in $Bok^{-/-}$ MEF, as shown by defective upregulation of IRE1α and GRP78, and increase in active C-terminal Jun kinase (JNK), the BH3-only protein BIM, active caspase-3 and cleaved PARP. In conclusion, we observed an abnormal ER stress

response after BFA treatment in the absence of BOK, supporting a role for BOK within the Golgi and ER compartments.

Discussion

We show, in accordance with previous reports (16), that BOK over-expression can kill several cell lines tested. BOK activates the BCL-2-regulated (intrinsic) apoptotic pathway, as shown by characteristic morphological changes, cytochrome c release upstream of caspase-activation as well as caspase-9 and subsequent caspase-3 activation. Involvement of the BCL-2-regulated apoptotic pathway is supported by our observation that MEF lacking MCL-1 were significantly more sensitive to BOK-induced killing than WT controls. However, even though BOK shows amino acid sequence similarities to BAX and BAK, it is not known whether BOK is a genuine BAX-like protein that would be able to substitute for the combined loss of BAX and BAK in triggering apoptosis. Numerous studies have shown that *Bax*$^{-/-}$*Bak*$^{-/-}$ cells, including MEF or lymphocytes, are highly resistant to diverse apoptotic stimuli, even though they express detectable levels of endogenous BOK ((20) and Supplementary Figure S1). This strongly indicates that intrinsic apoptosis requires the presence of either BAX or BAK for its execution and hints at the inability of BOK to substitute for BAX/BAK function.

We tested the effect of BOK over-expression in SV40 MEF and FDM cells. Whereas WT FDM were susceptible to BOK-induced killing, *Bax*$^{-/-}$*Bak*$^{-/-}$ FDM were completely refractory to enforced BOK-expression. *Bax*$^{-/-}$*Bak*$^{-/-}$ MEF were also largely protected from BOK-induced killing, although a minor extent of cell death was observed. In contrast to FDM cells, the pan-caspase inhibitor Q-VD-OPh (used at 25 μM) only partially protected WT MEF from BOK-induced killing. The low extent of cell death seen in the absence of BAX/BAK could therefore be due to additional, caspase-independent death pathways, which, in fact, have been suggested in early reports on BOK (16, 17, 40). Importantly,

Bax$^{-/-}$*Bak*$^{-/-}$ MEF and FDM over-expressing BOK were still highly resistant to classical apoptotic stimuli. This indicates that BOK function cannot substitute for the combined loss of BAX and BAK and indicate that BOK may act upstream of BAX/BAK. This is in line with our observation that BOK over-expression increases the levels of the BH3-only proteins BIM and PUMA (Supplementary Figure S7). Alternatively, BOK may be activated by selective cell death stimuli that remain to be identified.

BOK has been reported to interact with MCL-1 and BFL-1/A1 but not with BCL-2 or BCL-X_L (16, 17). A BH3-domain deletion mutant of BOK was unable to interact with MCL-1 and BFL1/A1 but, surprisingly, still retained its killing activity (41). Although we did not detect interactions between BOK and other BCL-2 family members, we found that HA-BOK and FLAG-BOK co-immunoprecipitated in a BH3 domain-dependent manner, as the interaction was disrupted by the BOK(D76A) point mutation. This supports the idea that, similarly to BAX and BAK, BOK may form homodimers/-oligomers. Interestingly, however, BOK(D76A) retained its ability to kill cells, indicating that BOK may induce cell death as a monomer. This is in contrast to BAX and BAK, where loss of killing function seems to invariably correspond with failure to oligomerize (30, 42, 43).

We present evidence that the TMD of BOK is a targeting device both necessary and sufficient to target the protein to its intracellular destinations. Interestingly, the TMD of BOK has high affinity for Golgi and ER membranes. Accordingly, substantial portions of full-length BOK are localized on the ER and Golgi and associated membranes of the secretory system. Even though several BCL-2 family members, most notably BCL-2 but also BAX and BAK, were reported to bind to the ER/nuclear outer membranes where they exert specific functions (11, 34, 37, 44, 45), the Golgi targeting of BOK is of potential interest. Furthermore, whereas relatively minor fractions of BAX and BAK are detectable on the ER, a large portion of total BOK resides on the Golgi/ER. This indicates that BOK may exert specific functions on these organelles. Both BAX and BAK

were shown to interact with IRE1α at the ER and *Bax*$^{-/-}$*Bak*$^{-/-}$ cells showed defective IRE1α-signaling and increased cellular damage upon ER stress (46). We observed a similar defective ER stress response in *Bok*$^{-/-}$ cells, which was most pronounced after treatment with brefeldin A, a drug that strongly impacts on the Golgi/ER compartments. It will be interesting to uncover the functions that BOK exerts at these locations, both in healthy and in dying cells.

It is important to mention that our data do not exclude mitochondrial localization and function of BOK. In analogy to several BCL-2 family members localizing to multiple organelles, it is conceivable that BOK also localizes to mitochondria. Indeed, particularly when BOK was over-expressed, we found some co-localization with mitochondrial markers. Moreover, in subcellular fractionation experiments, BOK was also prominent in mitochondria-enriched fractions. However, these fractions were still significantly positive for the ER marker calnexin. Since the ER membranes interact with mitochondria through so-called mitochondria-associated membranes (MAM) (47), it is difficult to prepare MAM-free mitochondrial fractions and, hence, further investigation is needed to quantify the exact amount of mitochondrial BOK.

Irrespective of its subcellular localization, our data from *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO cells indicate that BOK cannot compensate for the combined loss of BAX and BAK in triggering MOMP and apoptosis. Instead, we show that BOK-induced apoptosis largely relies on the presence of BAX or BAK. BOK may therefore function upstream of BAX/BAK and may control the transmission of ER/Golgi-derived apoptotic signals to mitochondria. This hypothesis is supported by our observation that over-expressed BOK leads to the fragmentation of ER and Golgi upstream of caspase activation. However, when BOK is missing, the Golgi may be dismantled in conditions of stress in inappropriate manner, thereby causing increased ER stress and activation of BH3-only proteins, leading to apoptosis (48).

Little is known about the stress stimuli and mechanisms that lead to transcriptional or post-translational activation of BOK. In order to test whether BOK acts within the same pathway(s) as BAX and BAK or independently, triple knockout mice lacking *Bok*, *Bax* and *Bak* will have to be generated and their phenotype compared to those of *Bax*$^{-/-}$*Bak*$^{-/-}$ animals. Besides its cytoplasmic localization, we also found BOK in purified nuclear fractions. Given the existence of BOK-deficient mice, possible functions of BOK within the nucleus, including putative non-apoptotic roles (e.g. as reported for trophoblast cell proliferation (49)) can now be analyzed.

Finally, given that BOK has recently been identified as a candidate tumor suppressor (27) and increasing evidence emerges for non-apoptotic roles of BCL-2 family proteins, especially at non-mitochondrial sites, it will be interesting to test whether the putative tumor suppressor function of BOK is restricted to its pro-apoptotic function or whether it involves new functions yet to be discovered.

Materials and Methods

Mice and reagents

Mice used for the isolation of bone marrow, primary hepatocytes and embryonic fibroblasts were maintained under specific pathogen-free conditions in individually ventilated cages. The generation of *Bok*$^{-/-}$ mice on an inbred C57BL/6 genetic background using C57BL/6 derived ES cells was described (20). Animal experiments were reviewed and approved by the animal experimentation review board of the canton of Bern (41/08 and BE31/11).

High glucose Dulbecco's Modified Eagle Medium (DMEM, containing GlutaMAX™), RPMI 1640 medium (containing GlutaMAX™), William's E medium (containing GlutaMAX™), HEPES buffered saline solution (HBSS), trypsin solution 0.05% and

Penicillin/Streptomycin 100x stock solution were purchased from GIBCO (Life Technologies Europe, Zug, CH). Fetal calf serum (FCS, PAA Clone, endotoxin levels < 1EU/mL) and puromycin were purchased from PAA (PAA Laboratories GmbH, AT). 4-hydroxytamoxifen (4-OHT, #H7904), 2-mercaptoethanol, brefeldin A, dexamethasone and propidium iodide were purchased from Sigma-Aldrich Chemie GmbH (Buchs, CH). Q-VD-OPh was from SM Biochemicals (Anaheim CA, US). Staurosporine was purchased from Enzo LifeSciences AG (Lausen, CH). Recombinant His_6-tagged GFP-Annexin V was purified as described by Egger et al. (50).

Expression constructs and site-directed mutagenesis

Mouse *Bok* cDNA in the pCMV6 eukaryotic expression vector was purchased from ORIGENE (Rockville MD, US) with correct nucleotide sequence confirmed by DNA sequencing (Microsynth AG, Balgach, CH). Using a PCR based strategy employing the Pwo SuperYield polymerase (Roche Diagnostics AG, Rotkreuz, CH) a FLAG®-tag (DYKDDDDK) was added to the N-terminus of *Bok* and the resulting *FLAG-Bok* amplicon subcloned into the pcDNA3.1 expression vector using BamHI and EcoRI. Primer sequences: forward primer 5'-CTGGGATCCATGGACTACAAGGACGATGACGATAAGGAGGTGCTGCGGCGCTCTTC, reverse primer 5'-CTGGAATTCTCATCTCTCTGGCAACAACAGGAAG. Using the same strategy, the *Bok* C-terminal deletion mutant, FLAG-BokΔTMD, was generated using the reverse primer 5'-CTGGAATTCTCAGTGGGAGCGGAAGCCAGGATC. The pEGFP-TMD(BOK) construct was generated by PCR amplifying the transmembrane domain (consisting of its 35 C-terminal amino acids) encoding sequence of the *Bok* gene using the template pCMV6/Bok(mm) with the forward primer 5'-GATCTCGAGGTGGTCAGCACAGATC and the reverse primer 5'-gcagaattctcatctctctggcaac and subcloned into the pEGFP(C3) mammalian expression

vector using XhoI and EcoRI. FLAG-Bok and FLAG-BokΔTMD cDNA were subcloned from FLAG-Bok/pcDNA3.1 and FLAG-BokΔTMD/pcDNA3.1 into the pF-5xUAS-SV40-puro-GEV16 lentiviral vector using BamHI and XbaI/NheI or into the pEGFP(C3) expression vector using HinDIII and EcoRI. The Bok(D76A) single amino acid substitution was introduced into FLAG-Bok/pcDNA3.1 using QuikChange® Lightening Site-Directed Mutagenesis Kit (Agilent Technologies, Basel, CH) following the manufacturer's instructions; forward primer: 5'-CTGCGCTTGGGAGCTGAGCTGGAGCAG, reverse primer 5'-CTGCTCCAGCTCAGCTCCCAAGCGCAG. The coding sequences of all subcloned or mutated plasmids was verified by DNA sequencing (Microsynth AG, Balgach, CH).

Cell culture

Bax$^{-/-}$*Bak*$^{-/-}$ and matching wildtype SV40 LT-immortalized mouse embryo fibroblasts (MEF) were kindly provided by Dr. Christoph Borner (Freiburg, Germany). Primary C57BL/6 wildtype and *Bok*$^{-/-}$ MEF were isolated from 14.5-day-old embryos and immortalized with SV40 LT/pSG5. At least three independent lines, each derived from a different embryo were generated for each genotype. WT, *Bax*$^{-/-}$, *Bak*$^{-/-}$ and *Bax*$^{-/-}$*Bak*$^{-/-}$ FDM cells were kindly provided by Dr. Paul Ekert (Melbourne, AU). All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. HeLa cells were cultured in DMEM/GlutaMAX™ high glucose medium supplemented with 10% FCS and penicillin/streptomycin. SV40 immortalized MEF were cultured in DMEM/GlutaMAX™ high glucose medium supplemented with 5% FCS and penicillin/streptomycin.

Isolation and cultivation of primary hepatocytes

Primary mouse hepatocytes were isolated from 6- to 12-week-old mice by a collagenase perfusion based method as described in (51). The purity of isolated hepatocytes was

routinely >95%, as judged by cellular morphology of attached cells. Isolated hepatocytes were plated on gelatin-coated flasks in William's E medium supplemented with 10% FCS, 100 nM dexamethasone, 2 mM L-glutamine and 1% penicillin/streptomycin solution. Upon adherence of the cells (after 4 h), cells were washed and experiments immediately started in complete William's E medium (without dexamethasone) at 37°C under 5% CO_2.

Generation of SCF-^{cond}Hoxb8 cell lines and bone marrow-derived mast cells

See Online Supplementary Section

Transient transfection, immunofluorescence staining and confocal microscopy

HeLa cells were grown on 12-mm glass coverslips and transiently transfected with 0.4 µg of plasmid DNA and 1.6 µL Fugene HD transfection reagent (Roche Diagnostics, Rotkreuz, CH) in 24-well plates. SV40 immortalized MEF were transfected with 0.4 µg of plasmid DNA and 1.6 µL Metafectene Pro (Biontex Laboratories GmbH, DE). At various times after transfection, cells were stained with 370 nM MitoTracker® Deep Red 633 or 500 µM LysoTracker® Red DND-99 (Invitrogen), fixed for 10 min with 4% paraformaldehyde, permeabilized with 0.05% saponin for 5 min and treated with ice-cold acetone for 10 min. The cells were then incubated over night at 4°C with the following primary antibodies diluted in PBS containing 1% bovine serum albumin: mouse anti-FLAG® (clone M2, # F1804) and rabbit anti-calnexin (#C4731) from Sigma-Aldrich, rabbit anti-DYKDDDDK tag, rabbit anti-BOK (described in (20); adsorbed on PVDF membrane containing *Bok*$^{-/-}$ MEF lysate for IF applications), mouse anti-mouse BCL-2 (clone 10C4, BioLegend), mouse anti-cytochrome *c* (clone 6H2.B4) and mouse anti-GM130 (clone 35/GM130) from BD Biosciences. Secondary antibodies, Alexa Fluor 488- or 555-conjugated goat anti-mouse or goat anti-rabbit IgG (Life Technologies) antibodies

were added for 1 h at RT. Nuclear DNA was stained using 2 µg/mL Hoechst 33342 (Sigma-Aldrich). Coverslips were mounted using Glycergel (Dako) or Prolong Gold antifade reagent (Life Technologies). Images were acquired using a Zeiss LSM 510 laser confocal microscope with a 63x oil immersion objective and analyzed using the IMARIS software.

Quantification of cell death by flow cytometry

Cells were washed with Annexin V staining buffer (150 mM NaCl, 4 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 15 mM HEPES pH 7.2, 2% FCS and 10 mM NaN_3) and incubated with GFP-Annexin V diluted in staining buffer for at least 20 min on ice in the dark. Cells were then washed in Annexin V staining buffer and resuspended in 200 µL staining buffer. Propidium iodide was added to a final concentration of 2 µ g/mL and cells examined by flow cytometry using a FACS Calibur™. GFP-Annexin V- and propidium iodide negative cells were considered as viable cells.

Immunoprecipitation

$7x10^5$ HEK 293T cells were seeded in 6-well plates and transfected on the following day with a total of 2 µg DNA plus transfection reagent (FUGENE HD, Roche Diagnostics) at a ratio of 1:4. Cells were lysed in 300 µL IP lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, complete protease inhibitor cocktail (Roche Diagnostics) and 1 µg/ml pepstatin) for 15 min on ice and lysates cleared by centrifugation. Lysates were pre-cleared using 50 µL of a 50% slurry of Protein G sepharose beads (GE Healthcare) for 1 h at 4°C on a rotating wheel and then incubated with either mouse anti-FLAG (clone M2, Sigma-Aldrich) or rat anti-HA (clone 3F10, Roche Diagnostics) antibodies for 2 h on ice. 50 µL of protein G sepharose beads (50%

slurry) were added and the mixture incubated at 4°C for a minimum of 2 h on a rotating wheel. Beads were washed three times in IP lysis buffer and boiled for 5 min in 50 µL of 2x Lämmli buffer containing 100 mM DTT.

Subcellular fractionation

Subcellular fractionation was performed as described in (11). Briefly, a total of $1x10^7$ SV40 immortalized MEF or $5x10^6$ primary hepatocytes were harvested, washed twice in phosphate-buffered saline (PBS) and resuspended in MSH-MD buffer (210 mM mannitol, 70 mM sucrose, 20 mM HEPES, 1 mM EDTA, pH 7.4, $MgCl_2$, 2 mM DTT) supplemented with protease inhibitor cocktail (COMPLETE, Roche), 1 µg/mL pepstatin, 2 mM sodium orthovanadate and 50 mM sodium fluoride). Cells were mechanically lysed by passing them through a 23G (MEF) or 25G (hepatocytes) needle. Nuclei and cellular debris were removed by centrifugation at 500x g for 5 min. The post-nuclear supernatant (PNS) was centrifuged at 10'000 for 15 min, and the resulting crude mitochondrial pellet washed twice in PBS. To further purify mitochondria, crude mitochondria were laid on top of a 1-2 M linear sucrose gradient and ultracentrifuged at 50'000 x g for 90 min. Enriched mitochondria, which banded at a region corresponding to 1.4 M sucrose, and a visible band at about 1.0 M sucrose, containing ER-derived heavy membranes, were carefully collected and diluted to 0.25 M sucrose prior to centrifugation at 50'000 x g for 30 min. The resulting pellets were solubilized in hot H8 buffer (20 mM Tris/HCl pH 7.5, 2mM EGTA, 2mM EDTA and 1 % SDS, 1mM DTT, protease inhibitor cocktail (COMPLETE, Roche), 1 µg/mL pepstatin, 2 mM sodium orthovanadate and 50 mM sodium fluoride). Centrifugation of the post-mitochondrial supernatant (PMS) at 100'000 x g for 30 min yielded the microsomal pellet (P_{100}) and the cytosolic fraction (S_{100}). The microsome pellet was washed three times in MSH-MD buffer and lysed in hot H8 buffer. Nuclear fractionation was performed as described in Rosner et al. (52). Protein

concentration was measured using the Bradford assay. A total of 25 µg protein per fraction was separated on denaturing SDS-PAGE gels and analyzed by Western blotting.

Sodium carbonate extraction (alkaline treatment) and trypsin treatment

Crude mitochondrial (P_{10}) and microsomal (P_{100}) fractions from SV40 immortalized MEF or primary hepatocytes were prepared as described above, resuspended in 0.1 M sodium carbonate solution (pH 12) and incubated for 20 min on ice. After centrifugation at 20'000 x g for 15 min at 4°C, the supernatant (containing the alkali-extractable proteins) was titrated back to a neutral pH with 1 M HCl. The pellet (containing the alkali-resistant fraction) was washed three times in MSH-MD buffer and then solubilized in hot H8 buffer. 10 % of each fraction was used for a total lysate control. For trypsin treatments, crude mitochondrial and microsomal fractions were resuspended in complete MSH Buffer-MD and left on ice for 30 min with or without 0.1 mg/mL Trypsin. Protein concentration was measured using the Bradford assay. A total of 25 µg protein per fraction was separated on denaturing SDS-PAGE gels and analyzed by Western blotting.

Gel electrophoresis and immunoblotting

Total protein extracts were prepared in a lysis buffer containing 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1 % SDS, complemented with protease inhibitors (Roche COMPLETE protease inhibitor cocktail plus 1 µg/mL pepstatin) and phosphatase inhibitors (2 mM sodium orthovanadate and 50 mM sodium fluoride). Protein concentration was determined using a BCA assay (Thermo Scientific) and proteins were denatured by adding 4x reducing (100 mM DTT) Lämmli buffer and boiling the samples for 5 min. A total of 30 µg protein per extract was separated on denaturing SDS-PAGE

gels. Proteins were transferred on PVDF transfer membranes (0.45 µ m, Merck Millipore). For Western blotting, membranes were probed with the following primary antibodies: mouse anti-BCL-2 (clone 10C4, BioLegend); rabbit anti-GRP78/BIP and rat anti-BIM (clone 3C5) from Enzo LifeSciences; rabbit anti-PUMA (Pro-Sci, #3043); rabbit anti-calnexin (#C4731), mouse anti-tubulin (clone B-5-1-2) and mouse anti-FLAG® (M5) from Sigma-Aldrich; rabbit anti-caspase-3 (#9662), rabbit anti-active caspase-3 (#9661), rabbit anti-active caspase-7 (#9491), rabbit anti-IRE1α (clone 14C10), rabbit anti-JNK/SAPK(#9252) and mouse anti-phospho-JNK/SAPK (clone G9, #9255) from Cell Signaling Technology; rabbit anti-BAX (#sc-493) from Santa Cruz Biotechnologies; mouse anti-cytochrome c (clone 7h8.2C12) and mouse anti-PARP (clone C2-10) from BD Biosciences; mouse anti-porin (clone 89-173/016) from Calbiochem; mouse anti-GAPDH (clone 6C5) and rabbit anti-BAK (Bak NT) from Merck Millipore; rat anti-mouse MCL-1 (clone 14C11, kind gift from Dr. D. Huang, Melbourne, AU); rabbit polyclonal antibody raised against amino acids 19-32 of mouse BOK (described in (20)) and a rabbit monoclonal anti-BOK (RabMab BOK-1-5, raised against aa 19-32 of mouse Bok). Secondary antibodies specific to mouse, rat or rabbit IgG that had been coupled to horse radish peroxidase were obtained from Jackson ImmunoResearch Laboratories and signals were detected by enhanced chemiluminescence (ECL Western blotting substrate, Thermo Scientific and Luminata Forte, Merck Millipore) and detected on photosensitive film (ECL Hyperfilm, GE Healthcare).

Acknowledgments

This study was performed with the support of the Microscopy Imaging Center (MIC), University of Bern. We thank Grant Dewson (Melbourne, AU) for discussions and critical comments on the manuscript; Shida Yousefi (Bern, CH) for help with microscopy; John Silke (Melbourne, AU) for the lentiviral inducible expression system; Mario Tschan (Bern,

CH) for the CAD-G-Whiz lentiviral vector; Nadia Corazza and Anastasia Badmann (Bern, CH) for help with isolation of primary hepatocytes; Philippe Bouillet and David Huang (both Melbourne, AU) for *Mcl-1*-deficient MEF; David Huang, Lorraine O'Reilly, Paul Ekert (all Melbourne, AU), Christoph Borner (Freiburg, DE), Frank Essmann (Tübingen, DE), Julia Fernandez-Rodriguez (Gothenburg, SE), Philipp Berger (Villigen, CH), Georg Häcker (Freiburg, DE) for plasmids, cell lines, antibodies and reagents. This work was supported by grants and fellowships from the Swiss National Science Foundation (to TK, PP0033_119203), the Australian NHMRC (to AS, 1020363 and 1016701) and the Leukaemia Foundation of Australia (LFA to FK).

Supplementary Information

Supplementary information is available at Cell Death and Differentiation's website

References

1. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 2008 Jan; **9**(1)**:** 47-59.

2. Schinzel A, Kaufmann T, Borner C. Bcl-2 family members: integrators of survival and death signals in physiology and pathology [corrected]. *Biochimica et biophysica acta* 2004 Mar 1; **1644**(2-3)**:** 95-105.

3. Adams JM, Cory S. Life-or-death decisions by the Bcl-2 protein family. *Trends in Biochemical Sciences* 2001; **26:** 61-66.

4. Giam M, Huang DC, Bouillet P. BH3-only proteins and their roles in programmed cell death. *Oncogene* 2008 Dec; **27 Suppl 1:** S128-136.

5. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 family reunion. *Molecular cell* 2010 Feb 12; **37**(3)**:** 299-310.

6. Shamas-Din A, Brahmbhatt H, Leber B, Andrews DW. BH3-only proteins: Orchestrators of apoptosis. *Biochimica et biophysica acta* 2011 Apr; **1813**(4)**:** 508-520.

7. Pop C, Salvesen GS. Human caspases: activation, specificity, and regulation. *The Journal of biological chemistry* 2009 Aug 14; **284**(33)**:** 21777-21781.

8. Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol* 2004 Nov; **5**(11)**:** 897-907.

9. Borgese N, Fasana E. Targeting pathways of C-tail-anchored proteins. *Biochimica et biophysica acta* 2011 Mar; **1808**(3)**:** 937-946.

10. Szegezdi E, Macdonald DC, Ni Chonghaile T, Gupta S, Samali A. Bcl-2 family on guard at the ER. *Am J Physiol Cell Physiol* 2009 May; **296**(5)**:** C941-953.

11. Kaufmann T, Schlipf S, Sanz J, Neubert K, Stein R, Borner C. Characterization of the signal that directs Bcl-x_L, but not Bcl-2, to the mitochondrial outer membrane. *Journal of Cell Biology* 2003; **160:** 53-64.

12. Heath-Engel HM, Shore GC. Regulated targeting of Bax and Bak to intracellular membranes during apoptosis. *Cell death and differentiation* In Press May 19.

13. Heath-Engel HM, Chang NC, Shore GC. The endoplasmic reticulum in apoptosis and autophagy: role of the BCL-2 protein family. *Oncogene* 2008 Oct 27; **27**(50)**:** 6419-6433.

14. Rong YP, Aromolaran AS, Bultynck G, Zhong F, Li X, McColl K*, et al.* Targeting Bcl-2-IP3 receptor interaction to reverse Bcl-2's inhibition of apoptotic calcium signals. *Molecular cell* 2008 Jul 25; **31**(2)**:** 255-265.

15. Hacki J, Egger L, Monney L, Conus S, Rosse T, Fellay I*, et al.* Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. *Oncogene* 2000; **19**(19)**:** 2286-2295.

16. Hsu SY, Kaipia A, McGee E, Lomeli M, Hsueh AJ. Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. *Proceedings of the National Academy of Sciences of the United States of America* 1997; **94**(23)**:** 12401-12406.

17. Inohara N, Ekhterae D, Garcia I, Carrio R, Merino J, Merry A*, et al.* Mtd, a novel Bcl-2 family member activates apoptosis in the absence of heterodimerization with Bcl-2 and Bcl-x_L. *Journal of Biological Chemistry* 1998; **273**(15)**:** 8705-8710.

18. Gao S, Fu W, Durrenberger M, De Geyter C, Zhang H. Membrane translocation and oligomerization of hBok are triggered in response to apoptotic stimuli and Bnip3. *Cell Mol Life Sci* 2005 May; **62**(9)**:** 1015-1024.

19. Zhang H, Holzgreve W, De Geyter C. Evolutionarily conserved Bok proteins in the Bcl-2 family. *FEBS letters* 2000 Sep 1; **480**(2-3)**:** 311-313.

20. Ke F, Voss A, Kerr JB, O'Reilly LA, Tai L, Echeverry N*, et al.* BCL-2 family member BOK is widely expressed but its loss has only minimal impact in mice. *Cell Death Differ* 2012 Jun; **19**(6)**:** 915-925.

21. Bartholomeusz G, Wu Y, Ali Seyed M, Xia W, Kwong KY, Hortobagyi G*, et al.* Nuclear translocation of the pro-apoptotic Bcl-2 family member Bok induces apoptosis. *Mol Carcinog* 2006 Feb; **45**(2)**:** 73-83.

22. Yakovlev AG, Di Giovanni S, Wang G, Liu W, Stoica B, Faden AI. BOK and NOXA are essential mediators of p53-dependent apoptosis. *The Journal of biological chemistry* 2004 Jul 2; **279**(27)**:** 28367-28374.

23. Hsu SY, Hsueh AJ. A splicing variant of the Bcl-2 member Bok with a truncated BH3 domain induces apoptosis but does not dimerize with antiapoptotic Bcl-2 proteins in vitro. *The Journal of biological chemistry* 1998 Nov 13; **273**(46)**:** 30139-30146.

24. Rodriguez JM, Glozak MA, Ma Y, Cress WD. Bok, Bcl-2-related Ovarian Killer, Is Cell Cycle-regulated and Sensitizes to Stress-induced Apoptosis. *The Journal of biological chemistry* 2006 Aug 11; **281**(32)**:** 22729-22735.

25. Lindsten T, Ross AJ, King A, Zong W, Rathmell JC, Shiels HA*, et al.* The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. *Molecular cell* 2000; **6**(6)**:** 1389-1399.

26. Rathmell JC, Lindsten T, Zong W-X, Cinalli RM, Thompson CB. Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. *Nature Immunology* 2002; **3**(10)**:** 932-939.

27. Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J*, et al.* The landscape of somatic copy-number alteration across human cancers. *Nature* 2010 Feb 18; **463**(7283)**:** 899-905.

28. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ*, et al.* Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001; **292**(5517)**:** 727-730.

29. Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU*, et al.* IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell* 2007 Nov 16; **131**(4)**:** 682-693.

30. Dewson G, Kratina T, Sim HW, Puthalakath H, Adams JM, Colman PM*, et al.* To trigger apoptosis, Bak exposes its BH3 domain and homodimerizes via BH3:groove interactions. *Molecular cell* 2008 May 9; **30**(3)**:** 369-380.

31. Zha HB, Reed JC. Heterodimerization-independent functions of cell death regulatory proteins Bax and Bcl-2 in yeast and mammalian cells. *Journal of Biological Chemistry* 1997; **272**(50)**:** 31482-31488.

32. Kuroda R, Ikenoue T, Honsho M, Tsujimoto S, Mitoma JY, Ito A. Charged amino acids at the carboxyl-terminal portions determine the intracellular locations of two isoforms of cytochrome b5. *The Journal of biological chemistry* 1998; **273**(47)**:** 31097-31102.

33. Horie C, Suzuki H, Sakaguchi M, Mihara K. Characterization of Signal That Directs C-Tail-anchored Proteins to Mammalian Mitochondrial Outer Membrane. *Mol Biol Cell* 2002; **13**(5)**:** 1615-1625.

34. Lithgow T, van Driel R, Bertram JF, Strasser A. The protein product of the oncogene *bcl*-2 is a component of the nuclear envelope, the endoplasmic reticulum and the outer mitochondrial membrane. *Cell Growth and Differentiation* 1994; **5:** 411-417.

35. Gotow T, Shibata M, Kanamori S, Tokuno O, Ohsawa Y, Sato N*, et al.* Selective localization of Bcl-2 to the inner mitochondrial and smooth endoplasmic reticulum membranes in mammalian cells. *Cell death and differentiation* 2000 Jul; **7**(7)**:** 666-674.

36. Lisauskas T, Matula P, Claas C, Reusing S, Wiemann S, Erfle H*, et al.* Live-cell assays to identify regulators of ER-to-Golgi trafficking. *Traffic* 2012 Mar; **13**(3)**:** 416-432.

37. Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T*, et al.* BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. *Science* 2003; **300**(5616)**:** 135-139.

38. Zong WX, Li C, Hatzuvassiliou G, Lindsten T, Yu QC, Yuan J*, et al.* Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. *Journal of Cell Biology* 2003; **162**(1)**:** 59-69.

39. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* 2012 Feb; **13**(2)**:** 89-102.

40. Zhang H, Huang Q, Ke N, Matsuyama S, Hammock B, Godzik A*, et al. Drosophila* pro-apoptotic Bcl-2/Bax homologue reveals evolutionary conservation of cell death mechanisms. *Journal of Biological Chemistry* 2000; **275**(35)**:** 27303-27306.

41. Hsu SY, Hsueh AJW. A splicing variant of the Bcl-2 member Bok with a truncated BH3 domain induces apoptosis but does not dimerize with antiapoptotic Bcl-2 proteins *in vitro*. *Journal of Biological Chemistry* 1998; **273**(46)**:** 30139-30146.

42. Dewson G, Kratina T, Czabotar P, Day CL, Adams JM, Kluck RM. Bak activation for apoptosis involves oligomerization of dimers via their alpha6 helices. *Molecular cell* 2009 Nov 25; **36**(4)**:** 696-703.

43. Zha H, Reed JC. Heterodimerization-independent functions of cell death regulatory proteins Bax and Bcl-2 in yeast and mammalian cells. *J Biol Chem* 1997; **272:** 31482-31488.

44. He H, Lam M, McCormick TS, Distelhorst CW. Maintenance of Calcium homeostasis in the endoplasmic reticulum by Bcl-2. *Journal of Cell Biology* 1997; **138**(6)**:** 1219-1228.

45. Thomenius MJ, Wang NS, Reineks EZ, Wang Z, Distelhorst CW. Bcl-2 on the endoplasmic reticulum regulates Bax activity by binding to BH3 only proteins. *Journal of Biological Chemistry* 2003; **278**(8)**:** 6243-6250.

46. Hetz C, Bernasconi P, Fisher J, Lee AH, Bassik MC, Antonsson B*, et al.* Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1alpha. *Science* 2006 Apr 28; **312**(5773)**:** 572-576.

47. Rusinol AE, Cui Z, Chen MH, Vance JE. A unique mitochondria-associated membrane fraction from rat liver has a high capacity for lipid synthesis and contains pre-Golgi secretory proteins including nascent lipoproteins. *The Journal of biological chemistry* 1994 Nov 4; **269**(44)**:** 27494-27502.

48. Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND*, et al.* ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* 2007 Jun 29; **129**(7)**:** 1337-1349.

49. Ray JE, Garcia J, Jurisicova A, Caniggia I. Mtd/Bok takes a swing: proapoptotic Mtd/Bok regulates trophoblast cell proliferation during human placental development and in preeclampsia. *Cell death and differentiation* 2009 May; **17**(5)**:** 846-859.

50. Egger L, Schneider J, Rhême C, Tapernoux M, Häcki J, Borner C. Serine proteases mediate caspase-independent apoptosis and phagocytosis upstream of mitochondria. *Cell death and differentiation* 2003; **in press**.

51. Walter D, Schmich K, Vogel S, Pick R, Kaufmann T, Hochmuth FC*, et al.* Switch from type II to I Fas/CD95 death signaling on in vitro culturing of primary hepatocytes. *Hepatology* 2008 Dec; **48**(6)**:** 1942-1953.

52. Rosner M, Hengstschlager M. Cytoplasmic and nuclear distribution of the protein complexes mTORC1 and mTORC2: rapamycin triggers dephosphorylation and delocalization of the mTORC2 components rictor and sin1. *Human molecular genetics* 2008 Oct 1; **17**(19)**:** 2934-2948.

53. Grabenbauer M, Geerts WJ, Fernadez-Rodriguez J, Hoenger A, Koster AJ, Nilsson T. Correlative microscopy and electron tomography of GFP through photooxidation. *Nat Methods* 2005 Nov; **2**(11)**:** 857-862.

Figure Legends

Figure 1. Over-expressed BOK activates the intrinsic apoptotic pathway in SV40 immortalized MEF. (**a**) SV40 immortalized WT and *Mcl-1*$^{-/-}$ MEF/5xUAS-FLAG-BOK cells were treated as indicated with 4-OHT (0.1 μM) and Q-VD-OPh (25 μM) for 24 or 48 h and viability assessed by GFP-Annexin V/PI staining and flow cytometry. Data are presented as means +/- SD from three independent experiments. Induction of FLAG-BOK in SV40 immortalized WT (see **h**) and *Mcl-1*$^{-/-}$ MEF was controlled by Western blotting. (**b**) SV40 immortalized WT MEF/5xUAS-FLAG-BOK and WT MEF/5xUAS-EGFP cells were treated with 4-OHT (0.1 μM) for 24 or 48 h and viability assessed by PI staining and flow cytometry. Data represent means +/- SD from 6 (FLAG-BOK) and 5 (EGFP) independent experiments, respectively. (**c**) Wide field micrographs of WT MEF/5xUAS-FLAG-BOK cells induced for 48 h with 0.1 μM 4-OHT. White arrows indicate apoptotic cells characterized by plasma membrane blebbing, rounding and detachment). (**d**) Representative dot plots (n ≥ 6) of GFP-Annexin V/PI staining of MEF/5xUAS-FLAG-BOK cells with or without FLAG-BOK induction for 48 h. (**e**) Confocal micrographs of WT MEF transiently transfected with EGFP-BOK for 9 h and immunostained with an antibody against cytochrome c. (**f**) WT MEF were transfected with expression constructs for EGFP or FLAG-BOK, in the absence or presence of 20 μM Q-VD-OPh, for 9 h and percentages of EGFP- or FLAG-BOK positive cells with released cytochrome c determined by counting 100 cells for each condition. Data represent means +/- SD of 3 independent experiments. (**g**) WT MEF/5xUAS-FLAG-BOK cells were left untreated or treated with 0.1 μM 4-OHT for 14 h. Active caspases were covalently labeled with biotinylated X-VAD-fmk, pulled down with streptavidin-sepharose beads and Western blots probed for caspase-9 and caspase-3. Whereas active caspase-9 is pulled down, no signal for active caspase-3 could be detected at this specific time point. (**h**) WT MEF/5xUAS-FLAG-BOK cells were treated with 4-OHT (0.1

μM) for the indicated times and total protein lysates analyzed by Western blotting using anti-BOK and anti-caspase-3 antibodies. (**i**) Representative histograms (n ≥ 3) showing DNA content of WT MEF/5xUAS-FLAG-BOK with or without FLAG-BOK induction (t = 24 h). The cell population with fragmented genomic DNA is indicated as subG1. Significance values were determined by paired Student's t-Test. ***: p<0.005; **p<0.01; *: p<0.05.

Figure 2. BOK-induced apoptosis is largely BAX/BAK dependent. (**a**) FLAG-BOK was induced in SV40 immortalized WT and *Bax*$^{-/-}$*Bak*$^{-/-}$ MEF/5xUAS-FLAG-BOK cells by addition of 0.1 μM 4-OHT. Cell death was determined after 48 h by flow cytometric analysis (after staining with GFP-AnnexinV and PI). Data are presented as means +/- SD from ≥3 independent experiments. (**b**) WT and *Bax*$^{-/-}$*Bak*$^{-/-}$ SV40 MEF were transduced with CAD-G-Whiz empty vector (CGW) or CGW/FLAG-BOK for 40 h and apoptosis of EGFP-positive cells measured by flow cytometry (Cherry-Annexin V positive cells). Data are presented as means +/- SD from 3 independent experiments. FLAG-BOK expression and correct genotypes were verified by Western blotting, using antibodies to BOK, BAX and BAK. (**c**) FLAG-BOK was induced in *Bax*$^{-/-}$*Bak*$^{-/-}$ MEF/5xUAS-FLAG-BOK cells for 24 h, followed by treatment with 10 nM staurosporine or 10 μg/mL etoposide for an additional 24 h. Cell death was quantified as described in (**a**). WT SV40 MEF treated with the same concentration of drugs for 24 h are shown as a control. Data represent means +/- SD from 3 independent experiments. (**d**) Survival of WT and *Bax*$^{-/-}$*Bak*$^{-/-}$ FDM/5xUAS-FLAG-BOK cells with or without induction of FLAG-BOK expression (upper panel). Surviving cells were determined by flow cytometry and data represent GFP-Annexin V/PI double-negative cells. Data represent means +/- SD from 3 independent experiments. Lower panel: FLAG-BOK was induced for 8 h in *Bax*$^{-/-}$ *Bak*$^{-/-}$ FDM/5xUAS-FLAG-BOK cells, followed by treatment with 100 nM staurosporine

for an additional 24 h. Data represent means +/- SD from ≥3 independent experiments. FLAG-BOK expression and correct genotypes were verified by Western blotting, using antibodies to FLAG, BOK (RabMab BOK-1-5), BAX and BAK. (**e**) Co-immunoprecipitation assay in HEK 293T cells transiently co-transfected for 14 h with HA-BOK/pcDNA3 and FLAG-BOK/pcDNA3 or FLAG-BOK(D76A)/pcDNA3. Anti-FLAG and non-specific mouse IgG1 (isotype control) immunoprecipitates were probed by Western blotting using HA- and FLAG-specific antibodies. (**f**) Viability of WT and *Bok*$^{-/-}$ SV40 MEF upon induction of FLAG-BOK(D76A) was determined by GFP-Annexin V/PI negativity by flow cytometry. Induction of FLAG-BOK(D76A) was controlled by Western blotting using anti-BOK antibody. Data are presented as means +/- SD of ≥ three independent experiments. Significance was determined by Student's t-Test (***p<0.005, **p<0.01 and *p<0.05; n.s.: not significant).

Figure 3. BOK does not co-immunoprecipitate with other BCL-2 family members. (**a**) HA-BOK or HA-BAK were transiently co-expressed with FLAG-tagged MCL-1, BCL-X_L, BCL-2 or BCL-W in HEK 293T cells. Immunoprecipitates (C: protein G sepharose beads only; F: anti-FLAG; H: anti-HA) were probed by Western blotting with anti-FLAG and anti-HA antibodies. (**b**) HA-BOK was transiently co-transfected with FLAG-BAX or FLAG-BAK. Anti-FLAG immunoprecipitates were probed by Western blot using anti-HA and anti-FLAG antibodies.

Figure 4. Over-expressed BOK mainly localizes to membranes of the endoplasmic reticulum, Golgi apparatus and associated membranes. (**a**) Representative confocal scanning micrographs of HeLa cells transiently transfected with FLAG-BOK/pcDNA3 for 18 h and stained with organelle-specific antibodies against calnexin (endoplasmic reticulum) or GM130 (Golgi matrix), MitoTracker® Deep Red 633 (mitochondria),

GalNAc-T2-CFP (trans and medial Golgi) and Hoechst 33342 (nuclei). (**b**) HeLa cells were transfected with pEGFP-BOK for 8 h. Images were taken at real time. EGFP-BOK is found in vesicular patterns and partially co-localizes with the acidic organelle marker LysoTracker® (acidic organelles), as indicated by white arrows. FLAG-BOK and GFP-RAB4Q67L-GFP (constitutive active mutant, endosomal marker) were co-expressed in HeLa cells for 22 h. Partial co-localization is indicated by white arrows and in the magnified inlet. (**c**) HeLa cells stably expressing GaLNAc-T2-CFP (specific marker for trans and medial Golgi (53)) were transfected with FLAG-BOK/pcDNA3 for 21 h in the presence of 25 μ M Q-VD-OPh. Cells were stained with antibodies to calnexin and MitoTracker® Deep Red 633. 40 Z-stacks were acquired and GaLNaC-T2-CFP channel iso-surface 3D Golgi reconstruction was performed using the IMARIS software. Cells expressing FLAG-BOK (white arrows) show weakened GalNAc-T2-CFP signal intensity, correlating with increased Golgi dissociation and fragmentation compared to non-transfected cells.

Figure 5. The transmembrane domain of BOK is necessary and sufficient for membrane targeting. (**a**) Amino acid sequences of the C-terminal transmembrane domains (TMD) of BOK, BCL-2, BCL-X, BAX and BAK. Positively charged amino acids are printed in bold. (**b**) Confocal scanning micrographs of HeLa cells and SV40 immortalized *Bok*$^{-/-}$ MEF transfected with pEGFP-BOKΔTMD for 18 and 8 h, respectively, and stained as indicated. pEGFP-BOKΔTMD shows cytosolic and nuclear localization (Hoechst 33342, blue). EGFP-(WT)BOK is shown as control. (**c**) HeLa and HeLa/GalNAc-T2-CFP cells were transfected with pEGFP-TMD(BOK) for 24 h and stained with antibodies to calnexin (ER), MitoTracker® Deep Red 633 (mitochondria) and Hoechst 33342 (DNA). EGFP-TMD(BOK) localizes extensively to Golgi membranes and ER membranes. (**d**) Confocal micrographs of SV40 immortalized WT MEF

transiently transfected with GFP-TMD(BOK) for 24 h and stained with the indicated organelle markers.

Figure 6. Endogenous BOK strongly associates with the Golgi and ER compartments. (**a-c**) Confocal scanning micrographs of SV40 immortalized WT MEF stained with Hoechst 33342 (nuclei) and antibodies against BOK, GM130 (cis-Golgi marker), BCL-2 and cytochrome *c*. (**d**) WT SV40 MEF were treated with 0.3 μg/mL brefeldin A for 15 h and stained with anti-BOK and anti-GM130 antibodies and Hoechst 33342. (**e**) SV40 immortalized *Bok*$^{-/-}$ MEF were stained with antibodies to BOK and Hoechst 33342.

Figure 7. Analysis of the localization of over-expressed and endogenous BOK by subcellular fractionation and membrane insertion assays. Anti-BOK Western blots of subcellular fractions prepared from SV40 immortalized WT MEF/5xUAS-FLAG-BOK cells after FLAG-BOK induction with 0.1 μM 4-OHT for 14 h (**a**), SV40 WT MEF (**b**) and primary hepatocytes freshly isolated from young adult C57BL/6 (WT) mice (**c**). Membranes were re-probed with antibodies to mouse BCL-2, MCL-1, BAX and BAK for comparison. Re-probing of the membranes with antibodies to Porin or cytochrome C (mitochondria), calnexin (ER), GM130 (Golgi) and GAPDH (cytosol) served as controls for the purity of the subcellular fractions. Heavy (P10) and light (P100) membrane fractions from SV40 WT MEF (**d**) and mouse primary hepatocytes (**e**) were extracted directly with detergent (total) or first treated with sodium carbonate solution (pH 12) to remove loosely attached membrane proteins. Remaining membrane proteins were lysed in detergent and all fractions analyzed by Western blotting for endogenous BOK and indicated proteins that served as controls for integrally inserted membrane proteins

(Porin, BAK, BCL-2), or loosely attached and soluble proteins (BAX, GAPDH). BiP and PDI are luminal ER proteins and probing for them served as a control for vesicle integrity. (**f**) Heavy and light membranes were prepared from SV40 WT MEF and membrane proteins facing the cytosol, including endogenous BOK, were digested with 0.01% trypsin. (**g**) Purified nuclear and cytoplasmic fractions were prepared from SV40 WT MEF. A large portion of endogenous BOK was found in the purified nuclear fraction. The purity of the fractions was controlled by re-probing the membrane with antibodies against Histone H3 and PARP (nuclear) as well as Tubulin and GAPDH (cytoplasm). In contrast to BOK, no endogenous BCL-2, BAX or BAK could be detected in the nuclear fraction.

Figure 8. BOK-deficient cells show an atypical response to treatment with brefeldin A. (**a**) The survival of SV40 immortalized WT, *Bok*$^{-/-}$ and *Bax*$^{-/-}$*Bak*$^{-/-}$ MEF treated with the indicated doses of brefeldin A for 24 h was quantified by flow cytometry (GFP-Annexin V/PI negative cells). (**b**) Time kinetics of cell survival of SV40 WT and *Bok*$^{-/-}$ MEF treated with 0.5 µg/mL brefeldin A with or without 25 µM Q-VD-OPh. (**c**) WT and *Bok*$^{-/-}$ SCF-^{cond}Hoxb8 immortalized myeloid progenitors were treated with the indicated doses of brefeldin A for 18 h. Cell survival was determined by GFP-Annexin V/PI staining followed by flow cytometric analysis. (**d**) Brefeldin A dose response curves (treatment for 24 h) of *ex vivo* generated bone marrow-derived mast cells (BMMC) derived from WT or *Bok*$^{-/-}$ mice. (**e**) Western blot analysis of protein lysates prepared from SV40 WT and *Bok*$^{-/-}$ MEF that had been treated with 3 µg/mL brefeldin A for the indicated times. Data are representative of 3 independent experiments using different SV40 immortalized MEF lines. All quantitative data (dose responses and time kinetics) are presented as means +/- SD of at least five (MEF) or three (BMMC, SCF-^{cond}Hoxb8) independent experiments, respectively. Three independent lines (each derived from a

different embryo) were used for SV40 immortalized WT and *Bok*$^{-/-}$ MEF, respectively. Significance was determined by unpaired Student's t-Test (***p<0.005, **p<0.01 and *p<0.05).

Contributions:

TK designed the study and wrote the manuscript.

NE designed and performed research and wrote the manuscript.

DB performed research.

FK, AS and HUS provided crucial reagents, intellectual input and helped to revise the manuscript.

Conflict of interest: the authors declare no conflict of interest

ONLINE SUPPLEMENTARY INFORMATION

Supplementary Material And Methods

Production of lentiviral particles and transduction of cell lines

The coding sequence of the gene of interest was cloned into the 4-OHT-inducible lentiviral vector pF-5xUAS-SV40-puro-GEV16 (1) via BamHI and NheI. pMD2.VSV-G (envelope coding sequence):pCMVδR8.2 (packaging elements):pF-5xUAS-GI-Sv40puro-Gev16 (gene of interest) were co-transfected (total of 5 µg of DNA) at a ratio of 2:5:3 in 10-cm tissue culture dishes using FUGENE HD transfection reagent (Roche Diagnostics, Rotkreuz, CH). Lentiviral particles were harvested from the medium 24 and 36 h later, pooled and passed through a 0.2 µm filter and used fresh for infection or stored in aliquots at -80°C. Target cell lines were transduced in the presence of 8 µg/mL polybrene by spin infection (500x g, 90 min, 30°C). Successfully transduced cells were selected with puromycin for a minimum of three weeks. Induction of expression of the gene of interest was achieved by addition of 100 nM 4-OHT.

Generation of SCF-dependent conditional Hoxb8-immortalized neutrophil/ macrophage progenitor lines

The protocol is based on the method described by Wang et al. (2), with the major modification of using a different inducible expression system (1). In this lentivirus based expression system, untagged Hoxb8, which is under the control of the 5xUAS yeast promoter, is induced by constitutively expressed GEV16 transcription factor (GAL4-DBD_ER-LBD T2 mutant_VP16 TD) upon addition of 4-OHT (1, 3). Bone marrow-derived hematopoietic progenitors were isolated from sacrificed WT or *Bok*$^{-/-}$ mice by magnetic bead based depletion of cells expressing lineage specific surface markers (BD IMag^{TM} mouse hematopoietic progenitor cell enrichment set-DM, BD Biosciences)

following the manufacturer's instructions. $5x10^5$ lineage marker$^+$ depleted cells were incubated for 36 h in complete RPMI Medium (RPMI 1640/GlutaMAX™ supplemented with 10% FCS, 1% penicillin/streptomycin solution and 50 µM 2-mercaptoethanol). Cells were then transduced with pF-5xUAS-Hoxb8(mm)-SV40-puro-GEV16 lentiviral particles (see above) by spin-infection for 2 h at 1'000 rpm at 30°C in the presence of 8 µg/mL polybrene. Cells were subsequently grown in complete RPMI medium supplemented with murine stem cell factor, SCF (added as 10% of CHO/SCF(mm)-conditioned supernatant) and 100 nM 4-OHT to switch on Hoxb8 expression. Successfully transduced cells were selected in 1.0 µg/mL puromycin for 4 weeks, resulting in the outgrowth of SCF-dependent conditional Hoxb8-immortalized myeloid progenitor cell lines, termed SCF-^{cond}Hoxb8.

***In vitro* growth of bone marrow-derived mast cells**

Bone marrow-derived mast cells (BMMC) were generated as described by Ekoff et al. (4). Briefly, total bone marrow was harvested from the femora of sacrificed mice and cultured in complete RPMI medium (see above) supplemented with 10% of WEHI-3B conditioned medium (as a source of murine IL-3). Cells were re-plated weekly for 3 weeks to dispose of adherent cells. In the fourth week of culture, murine stem cell factor (SCF) was added in the form of 10% of CHO/SCF(mm)-conditioned medium. A highly enriched population of murine mast cells was obtained, as judged by surface marker expression of c-KIT(CD117) and the high affinity receptor for immunoglobulin E, FcεRI. Cells were maintained in IL-3 and SCF-containing medium and all experiments were performed within 4 weeks after obtainment of enriched BMMC cultures.

References:

1. Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU*, et al.* IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell* 2007 Nov 16; **131**(4)**:** 682-693.

2. Wang GG, Calvo KR, Pasillas MP, Sykes DB, Hacker H, Kamps MP. Quantitative production of macrophages or neutrophils ex vivo using conditional Hoxb8. *Nat Methods* 2006 Apr; **3**(4)**:** 287-293.

3. Callus BA, Ekert PG, Heraud JE, Jabbour AM, Kotevski A, Vince JE*, et al.* Cytoplasmic p53 is not required for PUMA-induced apoptosis. *Cell death and differentiation* 2008 Jan; **15**(1)**:** 213-215; author reply 215-216.

4. Ekoff M, Strasser A, Nilsson G. Fc{epsilon}RI Aggregation Promotes Survival of Connective Tissue-Like Mast Cells but Not Mucosal-Like Mast Cells. *J Immunol* 2007 Apr 1; **178**(7)**:** 4177-4183.

Figure Legends To Supplementary Figures

Supplementary Figure S1. BOK is widely expressed in mice (**a**) Quantitative RT-PCR (qPCR) analysis of *Bok* mRNA levels in different mouse organs (mixed gender, 6-12 weeks old): small intestine, colon, stomach, spleen, thymus, heart, kidney, liver, lung, pancreas, cerebellum, brain, ovaries, uterus (n=3) and hippocampus, caecum, duodenum, bone marrow, lymph nodes and placenta (n=2). *Hprt* served as the reference gene and values were normalized to *Bok* levels in the uterus. (**b**) BOK immunoblots of tissue lysates derived from C57BL/6 WT mice. Brain and pancreatic lysates from *Bok*$^{-/-}$ mice served as specificity control for the BOK antibody. Re-probing of the membrane with a GAPDH-specific antibody served as loading control.

Supplementary Figure S2. 4-hydroxytamoxifen has no intrinsic cytotoxicity at the concentrations used. SV40-immortalized WT MEF and FDM were treated with various

concentrations of 4-hydroxytamoxifen (4-OHT) and cellular viability determined over time by FACS analysis (GFP-AnnexinV/PI exclusion). No cytotoxicity was detectable at 0.1 μM 4-OHT, the concentration used to induce protein expression, or even at a ten-fold higher concentration of 1.0 μ M. Data are presented as means +/- SD from 3 independent experiments.

Supplementary Figure S3. Over-expressed BOK induces apoptosis in various cell lines. (**a**) FLAG-BOK was induced in WT FDM/5xUAS-FLAG-BOK cells by addition of 0.1 μM 4-OHT, in the presence or absence of 25 μM Q-VD-OPh, and cell viability was determined by flow cytometry (GFP-Annexin V/PI exclusion). Anti-FLAG immunoblot of untreated and 4-OHT treated cells shows induction of FLAG-BOK at 24 h. Data are presented as means +/- SD from three independent experiments. (**b**) FLAG-BOK was induced as described in (**a**) in BAF/3-5xUAS-FLAG-BOK cells. Cell viability was measured at 24 and 48 h and caspase-3-/-7 activity was quantified by using the fluorogenic caspase activity assay at 24 h using DEVD-AMC as a substrate. BAF/3 cells deprived of IL-3 for 15 h served as a positive control for caspase-3 activation. Anti-FLAG and anti-caspase-3 immunoblots of BAF/3 cell lysates after 0, 16, 24, 36 and 48 h of FLAG-BOK induction. Viability data are presented as means +/- SD from three independent experiments. DEVDase data are presented as means from 2 independent experiments. (**c**) Viability assay of Jurkat/5xUAS-FLAG-BOK cells after 0, 24, 48 and 72 h of FLAG-BOK induction. Induction of FLAG-BOK was verified by Western blot analysis using anti-FLAG specific antibody. Data are presented as means +/- SD from three independent experiments. (**d**) Confocal scanning micrographs of HeLa cells transiently transfected with a pEGFP-BOK expression construct for 12 h and stained with anti-cytochrome c antibody and Hoechst 33342 (nuclei). (**e**) Confocal scanning micrographs of HeLa cells transiently transfected with a FLAG-BOK/pcDNA3 expression construct for

18 h in the presence or absence of 25 μM Q-VD-OPh. Cells were stained with antibodies to FLAG and active caspase-3 specific and Hoechst 33342 dye. (**f**) FLAG-BOK was induced for 24 or 48 h in WT and *Bok*$^{-/-}$ SV40-immortalized MEF/5xUAS-FLAG-BOK cells and viability measured by flow cytometry (GFP-Annexin V/PI exclusion). Quantitative data are presented as means +/- SD of at least three independent experiments.

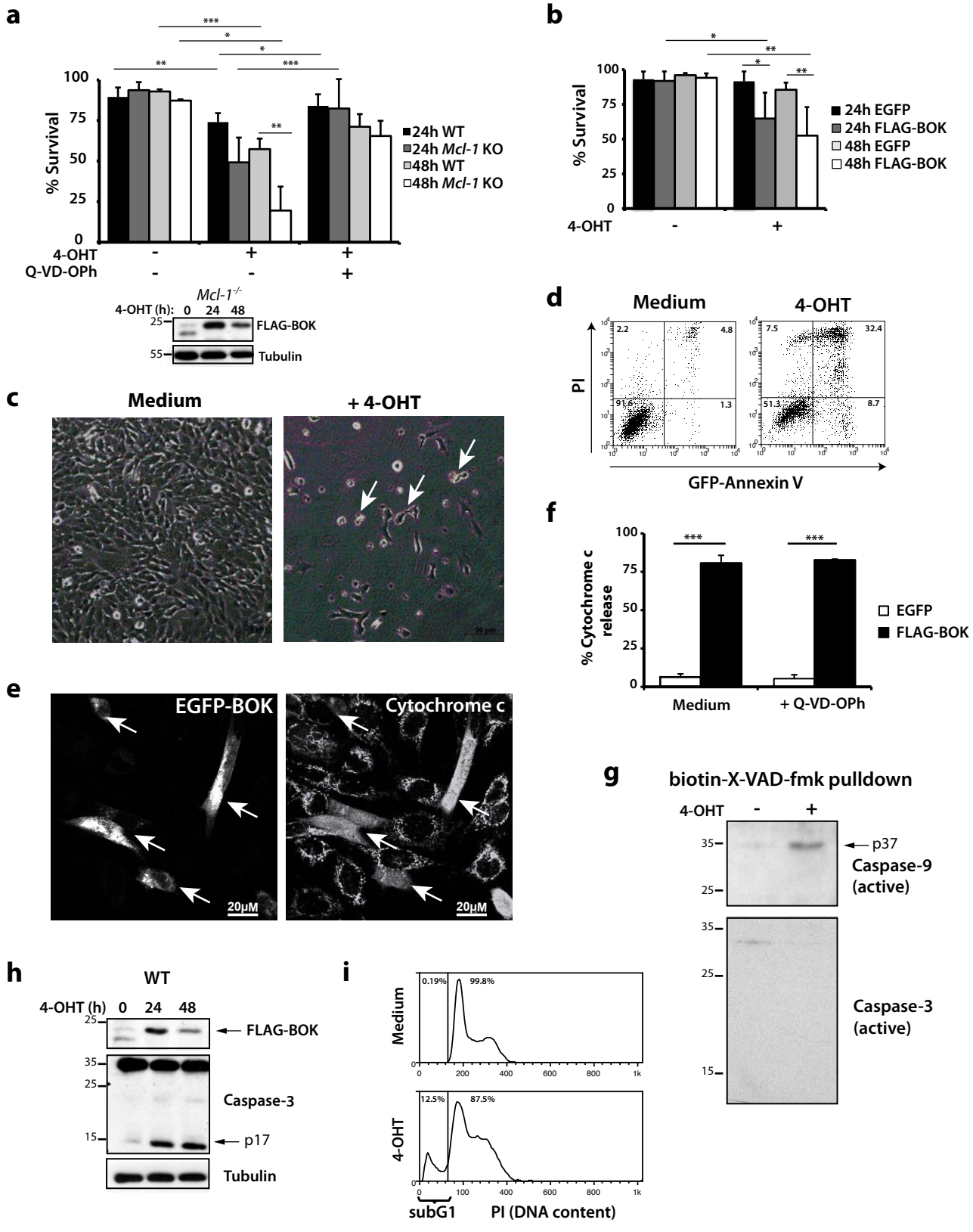
Supplementary Figure S4. BOK present on vesicular structures partially co-localizes with early and late endosomal markers. Confocal scanning micrographs of HeLa cells treated with 25 μM Q-VD-OPh and co-transfected for 21 h with expression constructs for FLAG-BOK/pcDNA3 (red) and early endosome/recycle marker RAB4Q67L-GFP (**a**), late endosome marker RAB5aQ97L-GFP (**b**), late endosome/lysosome marker RAB7Q67L-GFP (**c**) and early endosome marker RAB11Q70L-YFP (**d**). Constitutive active RAB mutants were used. Vesicular BOK partially co-localizes with the early endosome markers RAB4, RAB11 and with the late endosome marker RAB5a but not with the late endosome and lysosome marker RAB7 as indicated by the white arrows.

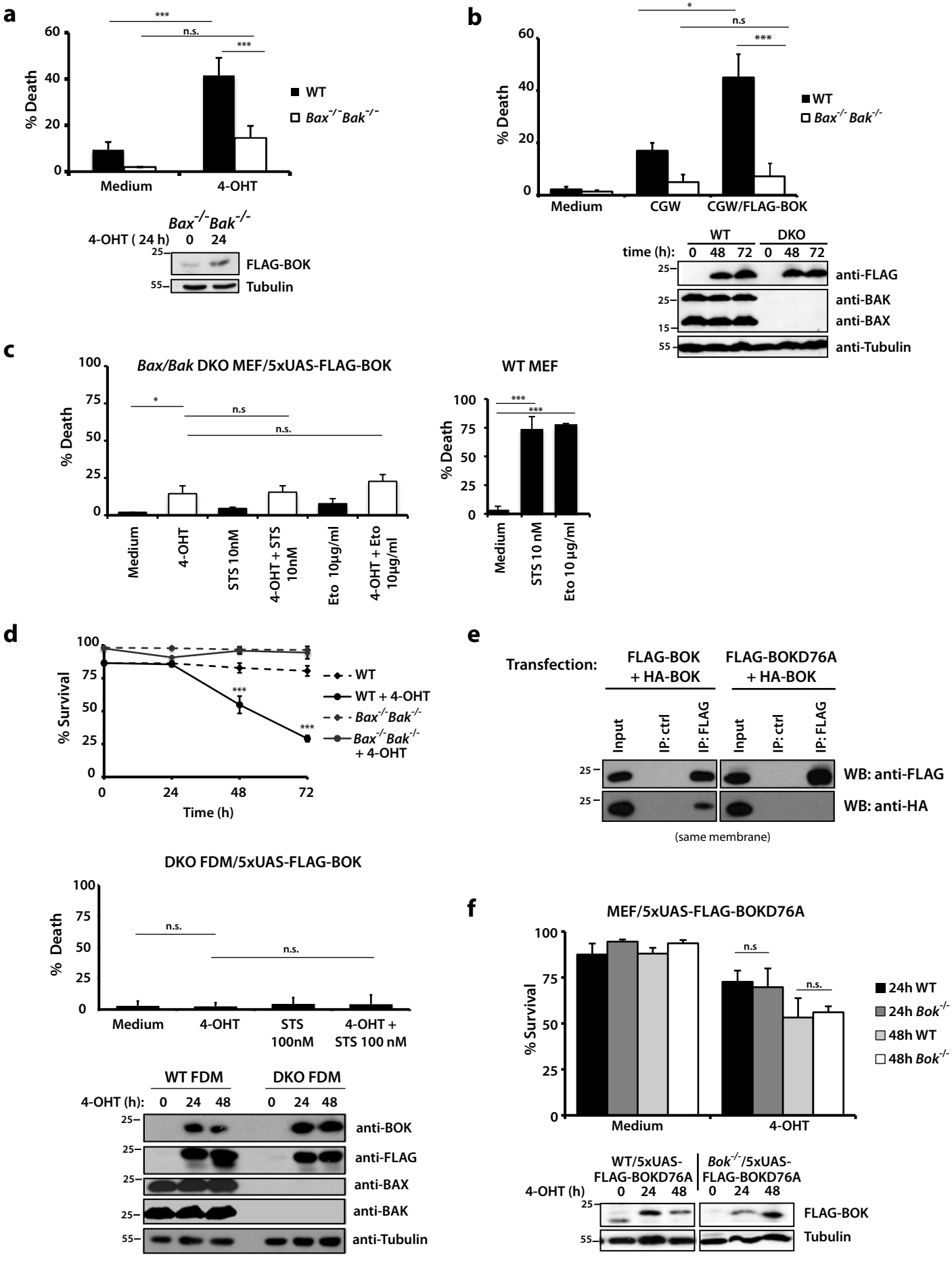
Supplementary Figure S5. Brefeldin A-induced Golgi fragmentation is independent of BOK. Confocal scanning micrographs of SV40-immortalized WT and *Bok*$^{-/-}$ MEF treated for the indicated times with brefeldin A (BFA, 5 μg/mL) and stained with an antibody against the cis-Golgi matrix protein GM130 (green). Nuclear DNA was stained with Hoechst 33342 dye (blue).

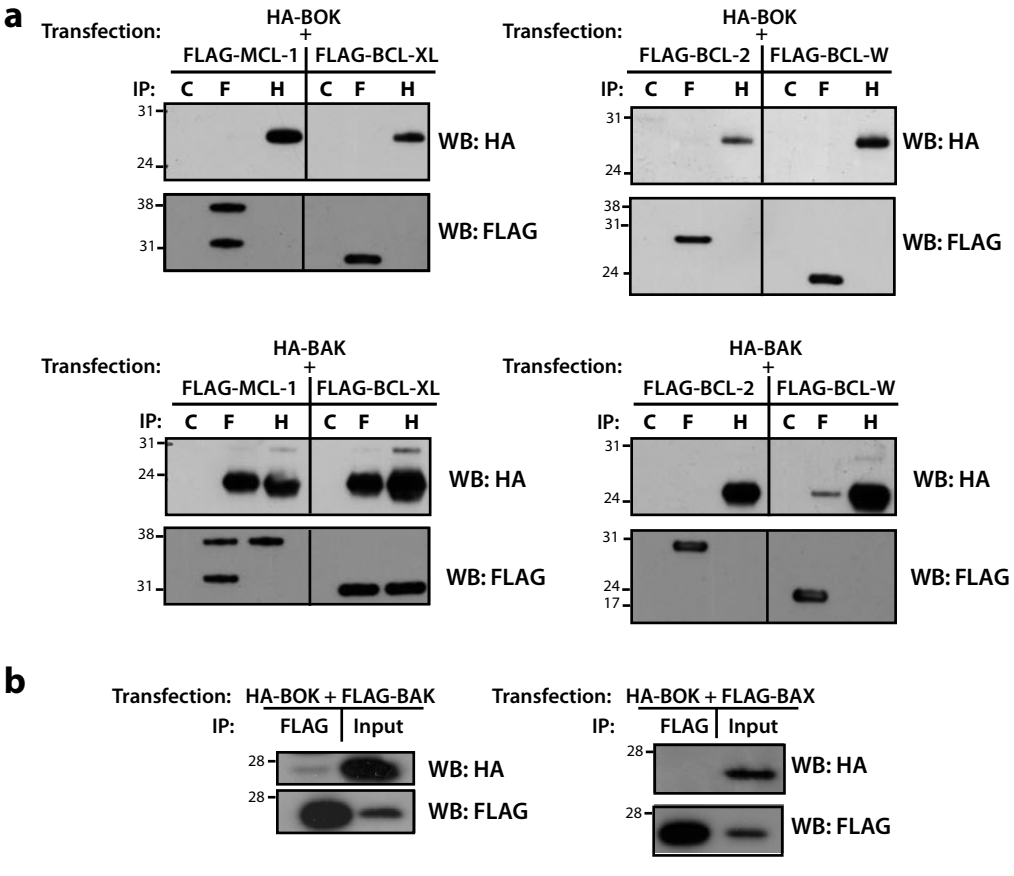
Supplementary Figure S6. BOK-deficient cells respond normally to classical apoptotic stimuli. (**a**) SV40-immortalized WT and *Bok*$^{-/-}$ MEF were treated with the

indicated doses of tunicamycin, thapsigargin or etoposide for 24 h (dose responses). Cell survival was also examined over a period of 40 h for treatment with 0.1 µg/mL tunicamycin, in the presence or absence of 25 µM Q-VD-OPh. (**b**) WT and *Bok*$^{-/-}$ bone marrow-derived mast cells (BMMC) were treated with various doses of tunicamycin, thapsigargin, etoposide or staurosporine for 24 h. Apoptosis was also induced by withdrawal of IL-3 for 24 or 48 h. (**c**) WT and *Bok*$^{-/-}$ SCF^{-cond}Hoxb8 immortalized myeloid progenitors were treated with tunicamycin, thapsigargin or etoposide for 18 h. Cell survival data (**a-c**) are shown as percentage of GFP-Annexin V/PI double-negative cells and presented as means +/- SD of at least three independent experiments. Significance was determined by unpaired student's t-Test (**p<0.01).

Supplementary Figure S7. Enforced expression of FLAG-BOK leads to upregulation of the BH3-only proteins BIM and PUMA. SV40-immortalized WT MEF/5xUAS-FLAG-BOK (**a**) or WT FDM/5xUAS-FLAG-BOK (**b**) cells were treated with 0.1 µM 4-OHT to induce FLAG-BOK expression. Total protein lysates were prepared at the indicated times and analyzed by Western blotting using antibodies against FLAG, BOK, BIM and PUMA. Re-probing of the membrane with an anti-Tubulin antibody served as loading control.

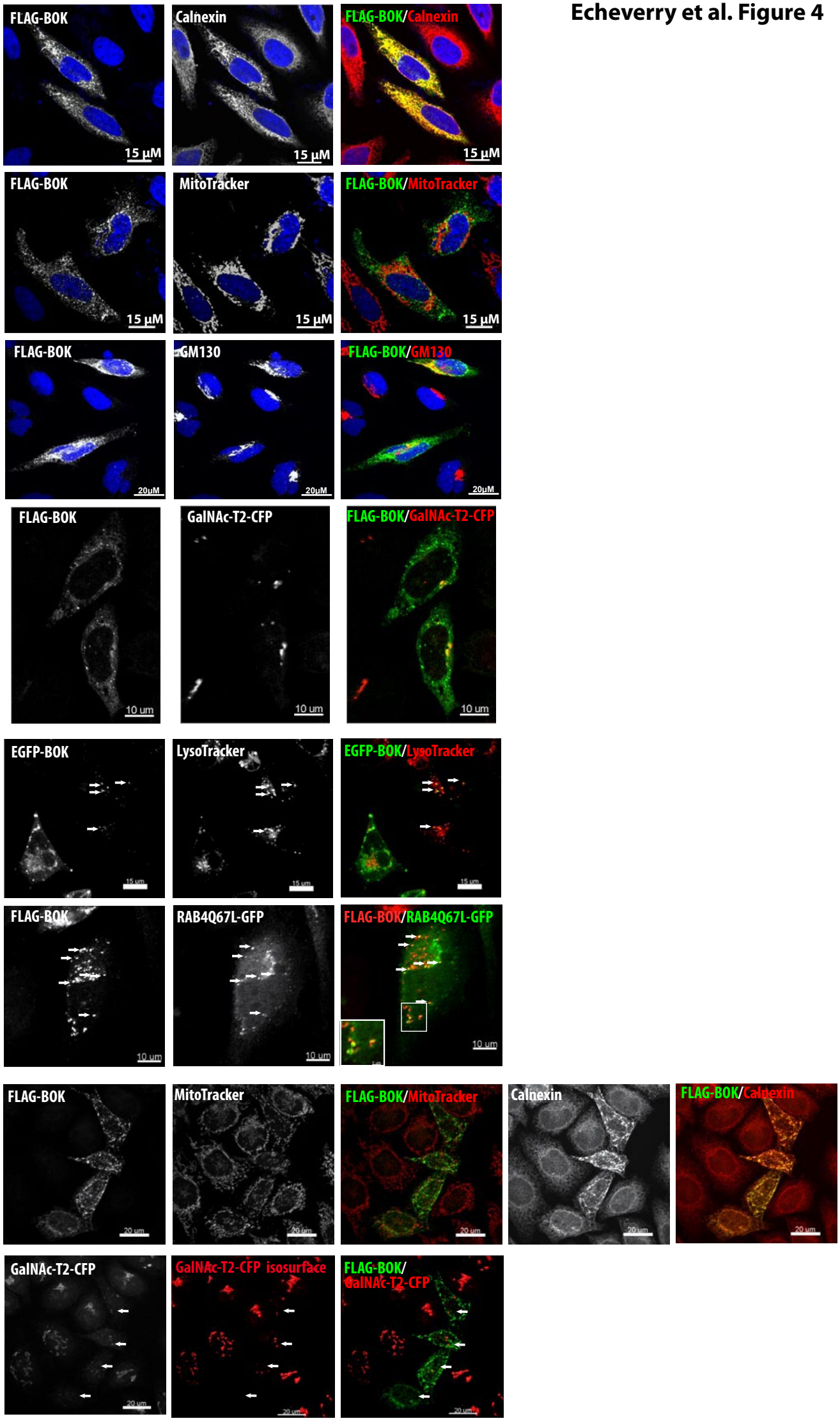

a
% Survival
24h WT
24h Mcl-1 KO
48h WT
48h Mcl-1 KO
4-OHT
Q-VD-OPh
Mcl-1-/-
4-OHT (h): 0 24 48
FLAG-BOK
Tubulin
b
% Survival
24h EGFP
24h FLAG-BOK
48h EGFP
48h FLAG-BOK
4-OHT
c
Medium
+ 4-OHT
d
Medium
4-OHT
PI
GFP-Annexin V
e
EGFP-BOK
Cytochrome c
20μM
f
% Cytochrome c release
EGFP
FLAG-BOK
Medium
+ Q-VD-OPh
g
biotin-X-VAD-fmk pulldown
4-OHT
p37
Caspase-9 (active)
Caspase-3 (active)
h
WT
4-OHT (h) 0 24 48
FLAG-BOK
Caspase-3
p17
Tubulin
i
Medium
4-OHT
0.19%
99.8%
12.5%
87.5%
subG1
PI (DNA content)

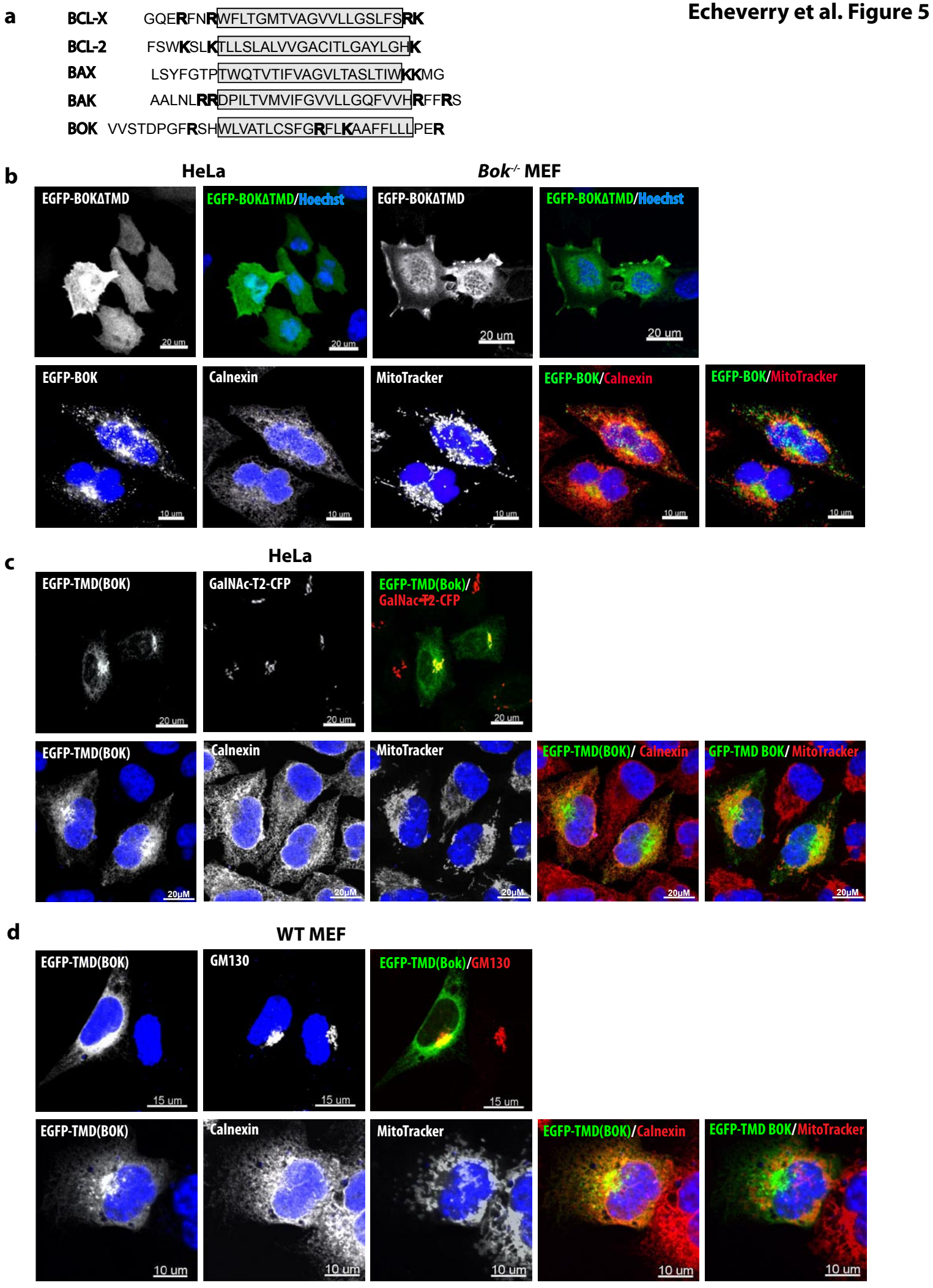

a
% Death
WT
Bax-/-Bak-/-
Medium
4-OHT
Bax-/-Bak-/-
4-OHT (24 h) 0 24
FLAG-BOK
Tubulin
b
% Death
WT
Bax-/- Bak-/-
Medium
CGW
CGW/FLAG-BOK
WT
DKO
time (h): 0 48 72 0 48 72
anti-FLAG
anti-BAK
anti-BAX
anti-Tubulin
c
Bax/Bak DKO MEF/5xUAS-FLAG-BOK
% Death
Medium
4-OHT
STS 10nM
4-OHT + STS 10nM
Eto 10µg/ml
4-OHT + Eto 10µg/ml
WT MEF
% Death
Medium
STS 10 nM
Eto 10 µg/ml
d
% Survival
Time (h)
- WT
WT + 4-OHT
- Bax-/-Bak-/-
Bax-/-Bak-/- + 4-OHT
DKO FDM/5xUAS-FLAG-BOK
% Death
Medium
4-OHT
STS 100nM
4-OHT + STS 100 nM
WT FDM
DKO FDM
4-OHT (h): 0 24 48 0 24 48
anti-BOK
anti-FLAG
anti-BAX
anti-BAK
anti-Tubulin
e
Transfection:
FLAG-BOK + HA-BOK
FLAG-BOKD76A + HA-BOK
Input
IP: ctrl
IP: FLAG
WB: anti-FLAG
WB: anti-HA
(same membrane)
f
MEF/5xUAS-FLAG-BOKD76A
% Survival
Medium
4-OHT
24h WT
24h Bok-/-
48h WT
48h Bok-/-
WT/5xUAS-FLAG-BOKD76A
Bok-/-/5xUAS-FLAG-BOKD76A
4-OHT (h) 0 24 48 0 24 48
FLAG-BOK
Tubulin

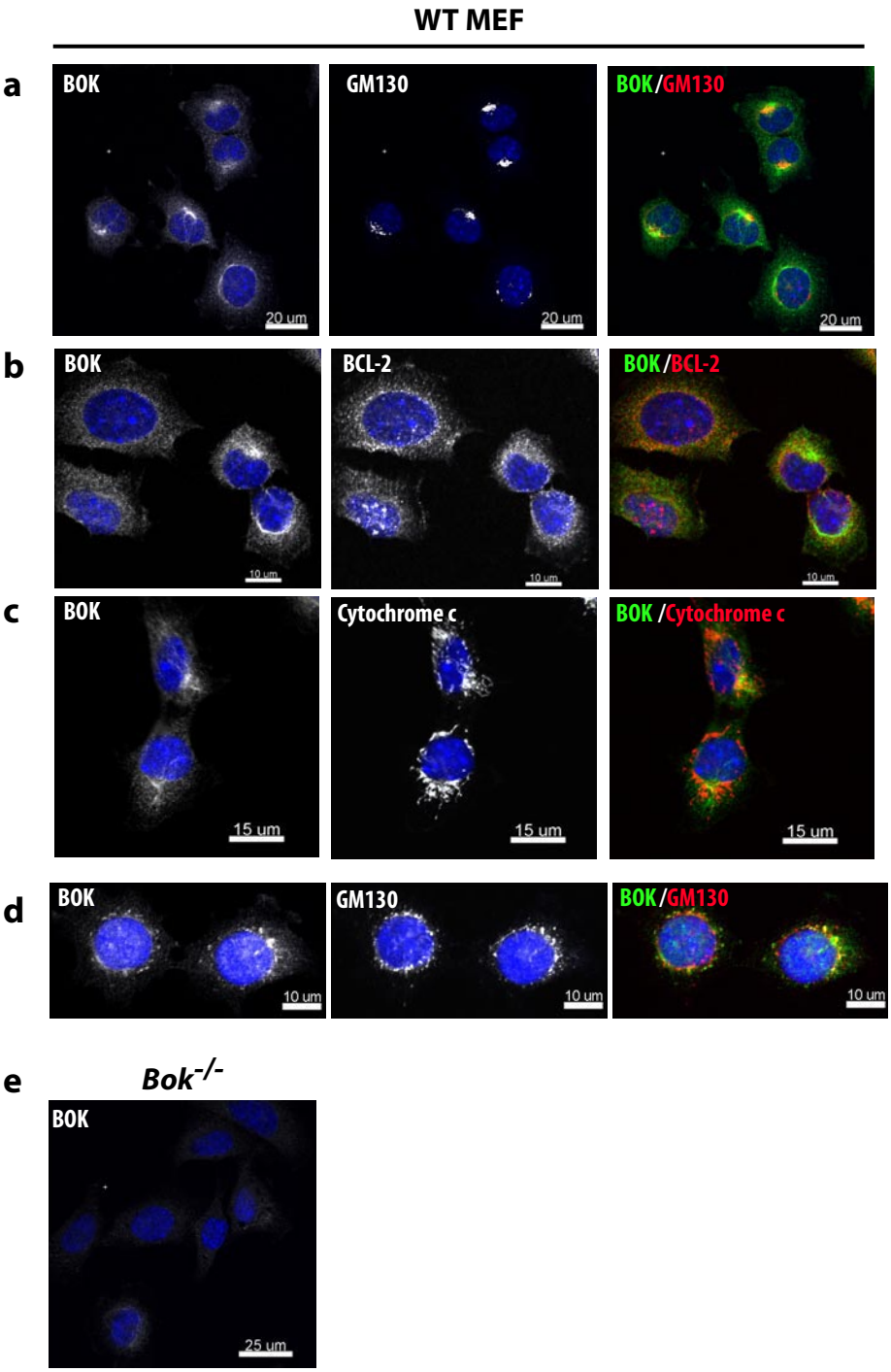

a
Transfection:
HA-BOK
+
FLAG-MCL-1
FLAG-BCL-XL
IP: C F H C F H
31
24
WB: HA
38
31
WB: FLAG
Transfection:
HA-BOK
+
FLAG-BCL-2
FLAG-BCL-W
IP: C F H C F H
31
24
WB: HA
38
31
24
WB: FLAG
Transfection:
HA-BAK
+
FLAG-MCL-1
FLAG-BCL-XL
IP: C F H C F H
31
24
WB: HA
38
31
WB: FLAG
Transfection:
HA-BAK
+
FLAG-BCL-2
FLAG-BCL-W
IP: C F H C F H
31
24
WB: HA
31
24
17
WB: FLAG

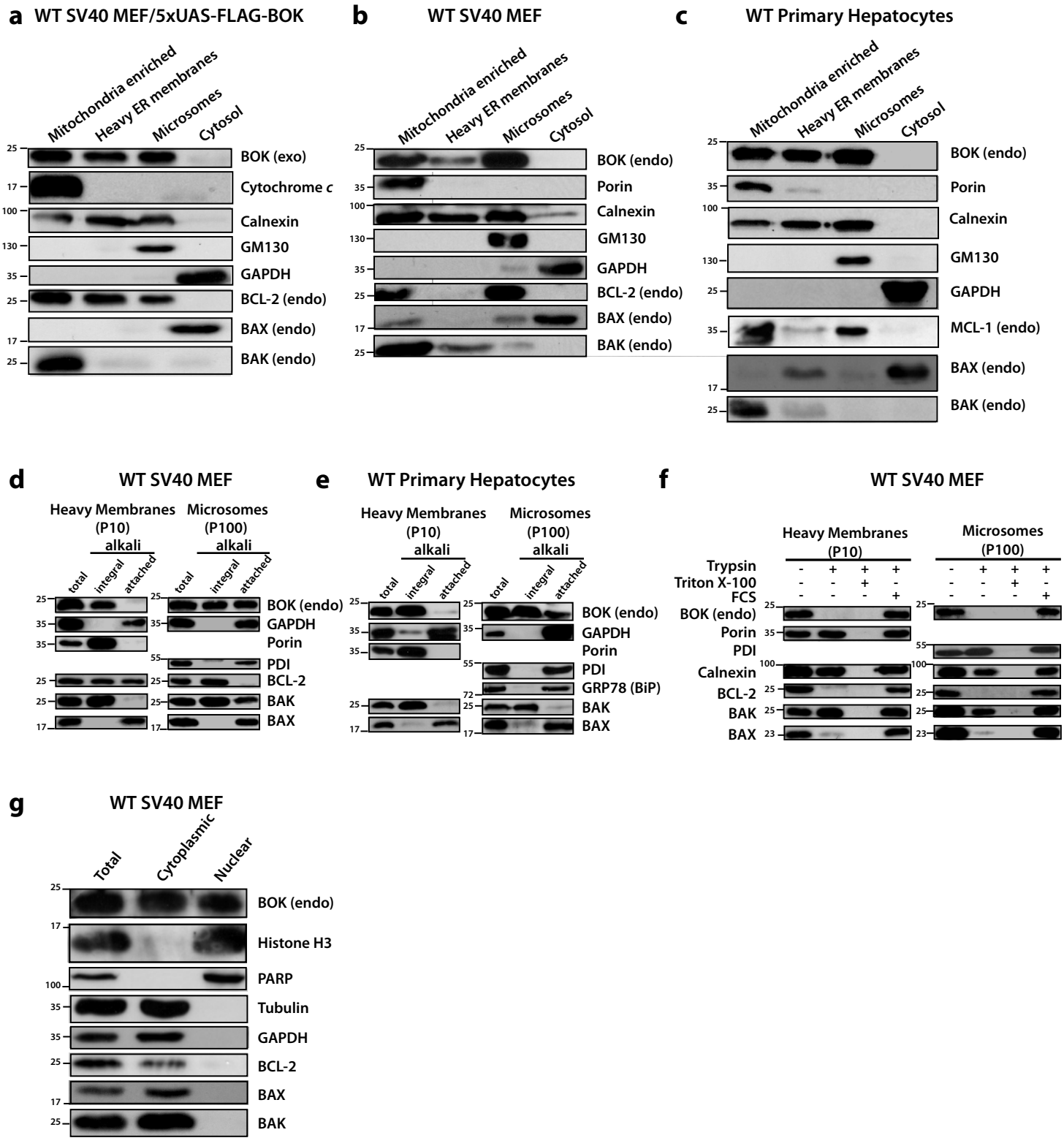
b
Transfection: HA-BOK + FLAG-BAK
IP: FLAG Input
28
WB: HA
28
WB: FLAG
Transfection: HA-BOK + FLAG-BAX
IP: FLAG Input
28
WB: HA
28
WB: FLAG

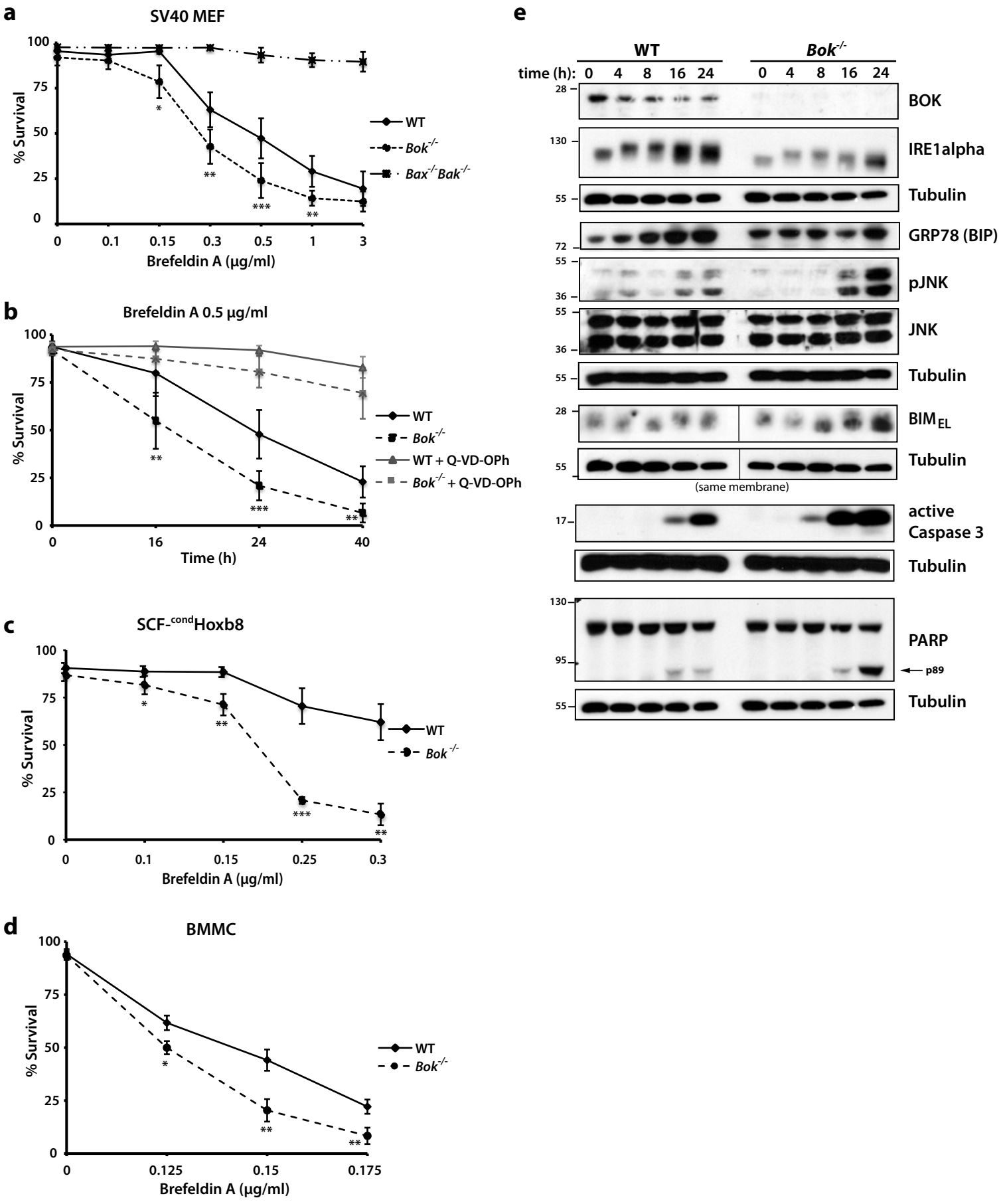
Echeverry et al. Figure 4

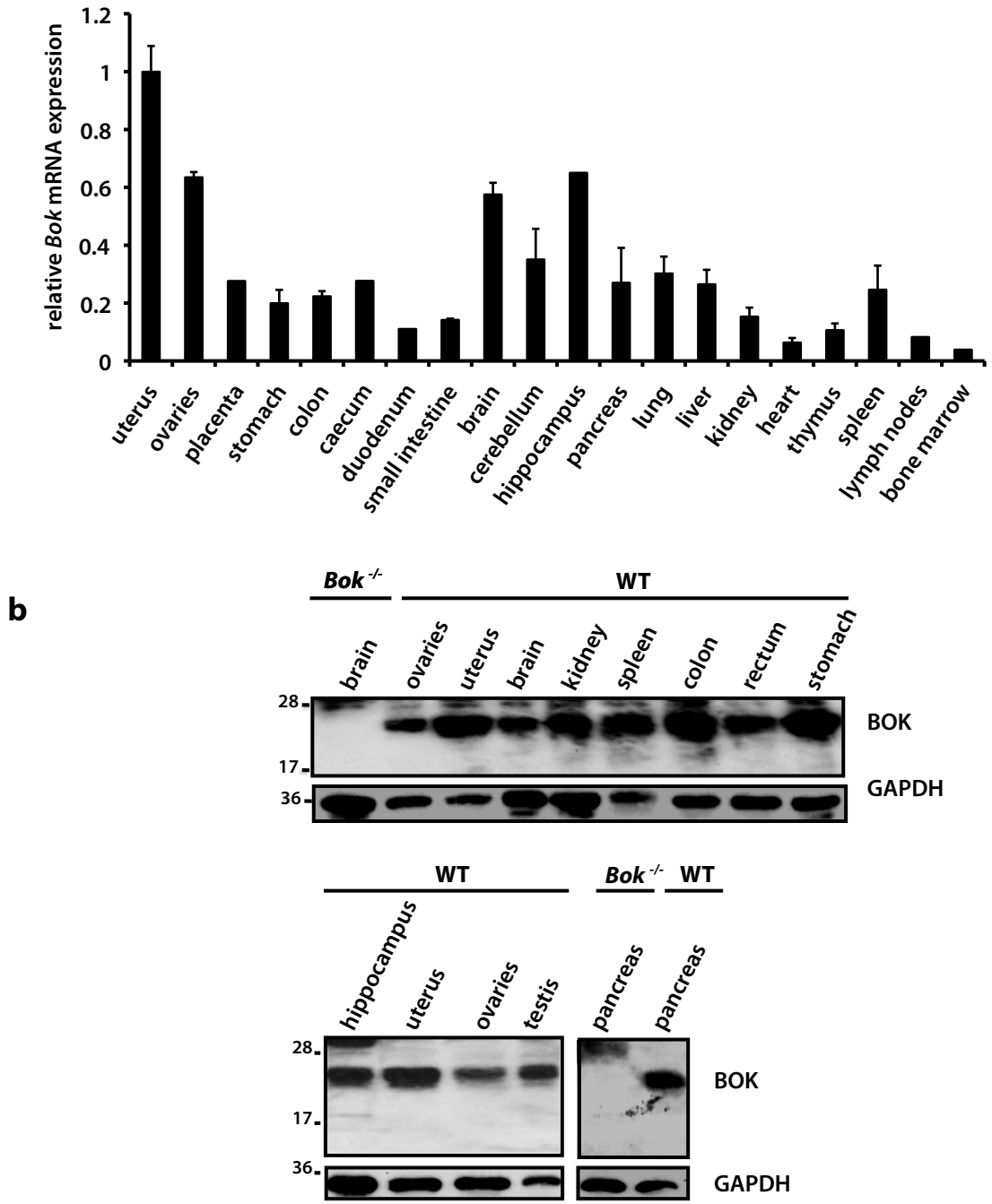



a
BCL-X GQERFNRWFLTGMTVAGVVLLGSLFSRK
BCL-2 FSWKSLKTLLSLALVVGACITLGAYLGHK
BAX LSYFGTPTWQTVTIFVAGVLTASLTIWKKMG
BAK AALNLRRDPILTVMVIFGVVLLGQFVVHRFFRS
BOK VVSTDPGFRSHWLVATLCSFGRFLKAAFFLLLPERR
b
HeLa
Bok-/- MEF
EGFP-BOKΔTMD
EGFP-BOKΔTMD/Hoechst
EGFP-BOKΔTMD
EGFP-BOKΔTMD/Hoechst
20 um
EGFP-BOK
Calnexin
MitoTracker
EGFP-BOK/Calnexin
EGFP-BOK/MitoTracker
10 um
c
HeLa
EGFP-TMD(BOK)
GalNAc-T2-CFP
EGFP-TMD(Bok)/ GalNac-T2-CFP
20 um
EGFP-TMD(BOK)
Calnexin
MitoTracker
EGFP-TMD(BOK)/ Calnexin
GFP-TMD BOK/ MitoTracker
20μM
d
WT MEF
EGFP-TMD(BOK)
GM130
EGFP-TMD(Bok)/GM130
15 um
EGFP-TMD(BOK)
Calnexin
MitoTracker
EGFP-TMD(BOK)/Calnexin
EGFP-TMD BOK/MitoTracker
10 um

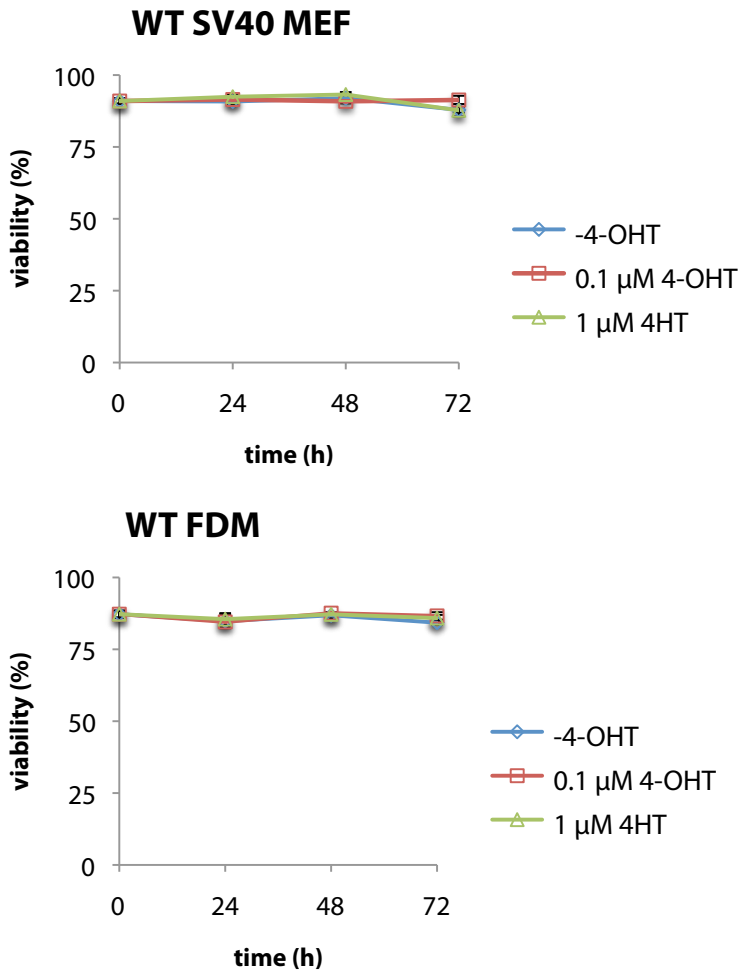

WT MEF
a
BOK
GM130
BOK/GM130
20 um
20 um
20 um
b
BOK
BCL-2
BOK/BCL-2
10 um
10 um
10 um
c
BOK
Cytochrome c
BOK /Cytochrome c
15 um
15 um
15 um
d
BOK
GM130
BOK/GM130
10 um
10 um
10 um
e
Bok-/-
BOK
25 um


a
WT SV40 MEF/5xUAS-FLAG-BOK
Mitochondria enriched
Heavy ER membranes
Microsomes
Cytosol
BOK (exo)
Cytochrome c
Calnexin
GM130
GAPDH
BCL-2 (endo)
BAX (endo)
BAK (endo)
b
WT SV40 MEF
Mitochondria enriched
Heavy ER membranes
Microsomes
Cytosol
BOK (endo)
Porin
Calnexin
GM130
GAPDH
BCL-2 (endo)
BAX (endo)
BAK (endo)
c
WT Primary Hepatocytes
Mitochondria enriched
Heavy ER membranes
Microsomes
Cytosol
BOK (endo)
Porin
Calnexin
GM130
GAPDH
MCL-1 (endo)
BAX (endo)
BAK (endo)
d
WT SV40 MEF
Heavy Membranes (P10)
Microsomes (P100)
alkali
total
integral
attached
BOK (endo)
GAPDH
Porin
PDI
BCL-2
BAK
BAX
e
WT Primary Hepatocytes
Heavy Membranes (P10)
Microsomes (P100)
alkali
total
integral
attached
BOK (endo)
GAPDH
Porin
PDI
GRP78 (BiP)
BAK
BAX
f
WT SV40 MEF
Heavy Membranes (P10)
Microsomes (P100)
Trypsin
Triton X-100
FCS
BOK (endo)
Porin
PDI
Calnexin
BCL-2
BAK
BAX
g
WT SV40 MEF
Total
Cytoplasmic
Nuclear
BOK (endo)
Histone H3
PARP
Tubulin
GAPDH
BCL-2
BAX
BAK

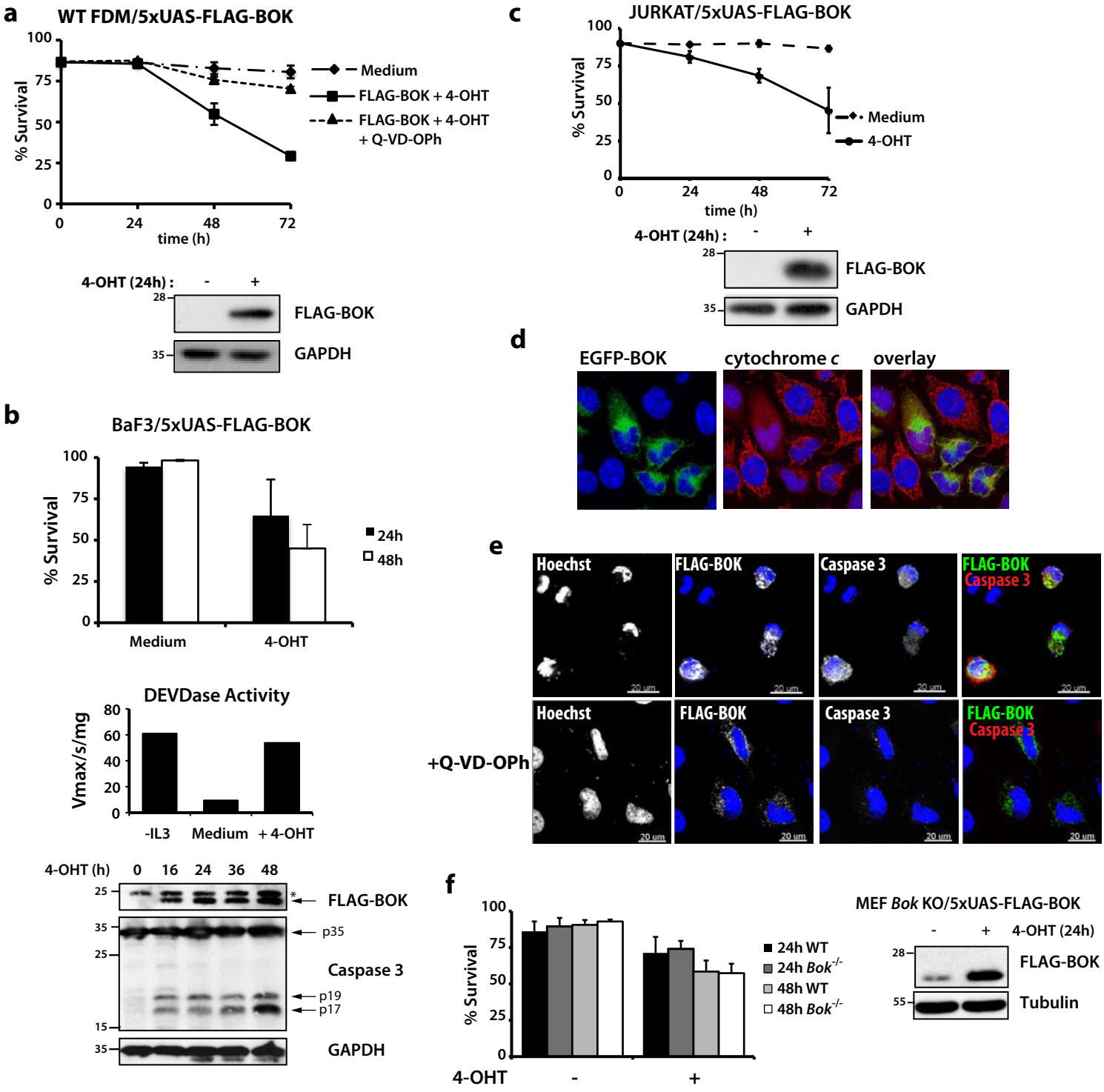

a
SV40 MEF
% Survival
Brefeldin A (µg/ml)
WT
Bok-/-
Bax-/-Bak-/-
b
Brefeldin A 0.5 µg/ml
% Survival
Time (h)
WT
Bok-/-
WT + Q-VD-OPh
Bok-/- + Q-VD-OPh
c
SCF-condHoxb8
% Survival
Brefeldin A (µg/ml)
WT
Bok-/-
d
BMMC
% Survival
Brefeldin A (µg/ml)
WT
Bok-/-
e
WT
Bok-/-
time (h): 0 4 8 16 24 0 4 8 16 24
BOK
IRE1alpha
Tubulin
GRP78 (BIP)
pJNK
JNK
Tubulin
BIM EL
Tubulin
(same membrane)
active Caspase 3
Tubulin
PARP
p89
Tubulin



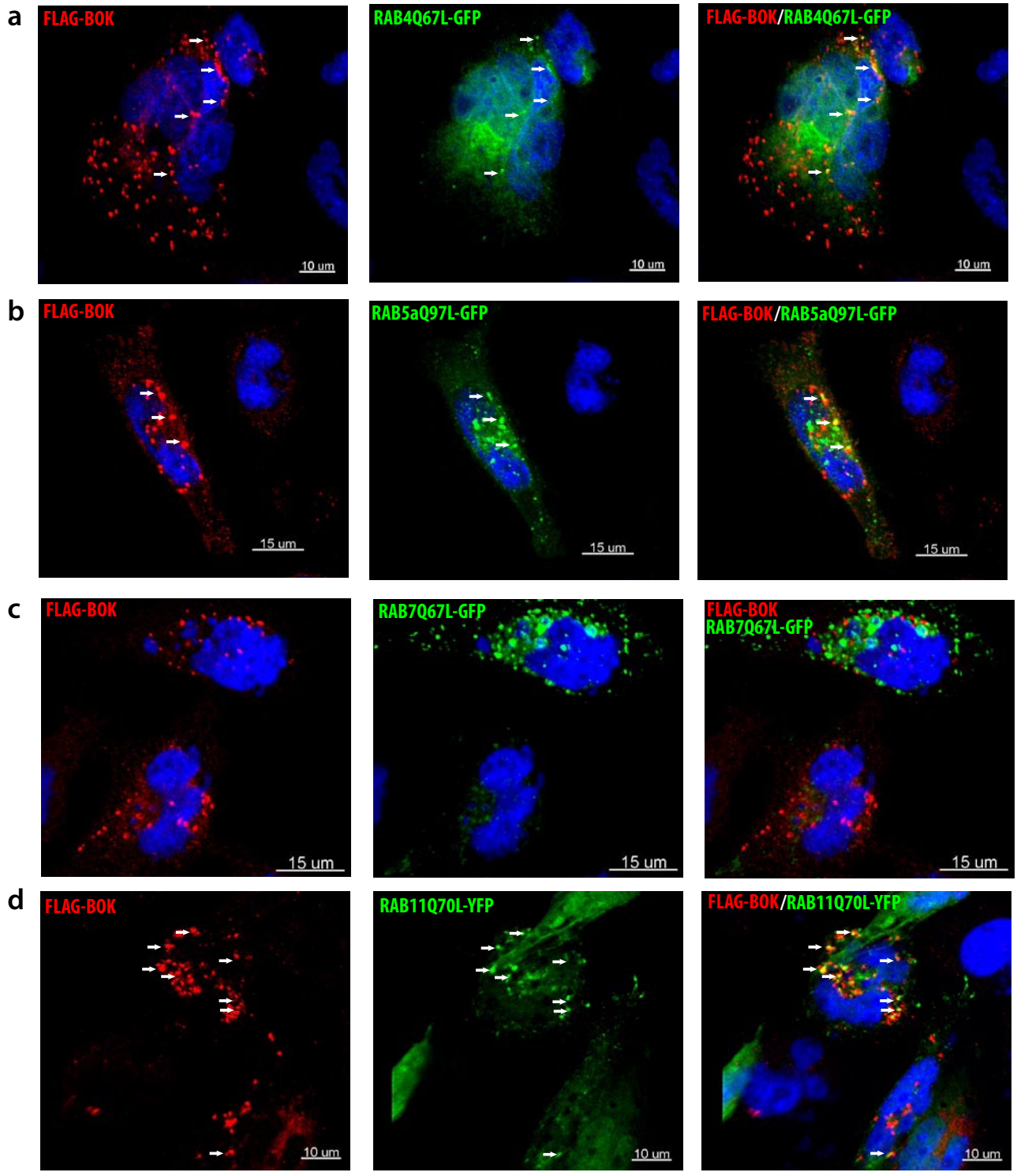
Supplementary Figure S1. BOK is widely expressed in mice. (**a**) Quantitative RT-PCR (qPCR) of *Bok* mRNA levels in different mouse organs (mixed gender, 6-12 weeks old mice): small intestine, colon, stomach, spleen, thymus, heart, kidney, liver, lung, pancreas, cerebellum, brain, ovaries, uterus (n=3) and hippocampus, caecum, duodenum, bone marrow, lymph nodes and placenta (n=2). *Hprt* served as reference gene and values were normalized to *Bok* levels in the uterus. (**b**) BOK immunoblots of tissue lysates derived from C57BL/6 WT mice. Brain and pancreatic lysates from *Bok-/-* mice served as specificity control for the BOK antibody. Re-probing of the membrane with a GAPDH-specific antibody served as loading control.



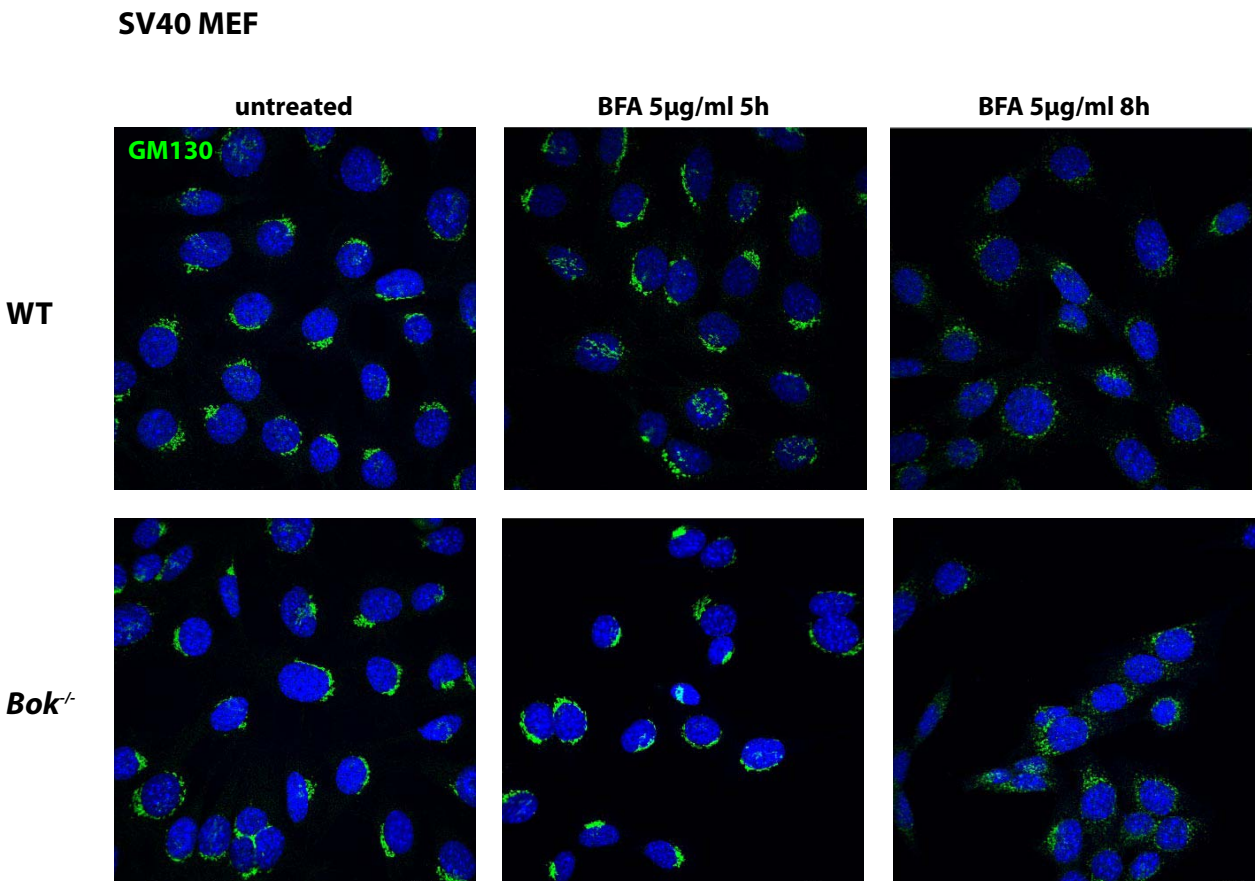
Supplementary Figure S2. 4-hydroxytamoxifen has no intrinsic cytotoxicity at the concentrations used. SV40-immortalized WT MEF and FDM were treated with various concentrations of 4-hydroxytamoxifen (4-OHT) and cellular viability determined over time by FACS analysis (GFP-AnnexinV/PI exclusion). No cytotoxicity was detectable at 0.1 µM 4-OHT, the concentration used to induce protein expression, or even at a ten-fold higher concentration of 1.0 µM. Data are presented as means +/- SD from 3 independent experiments.



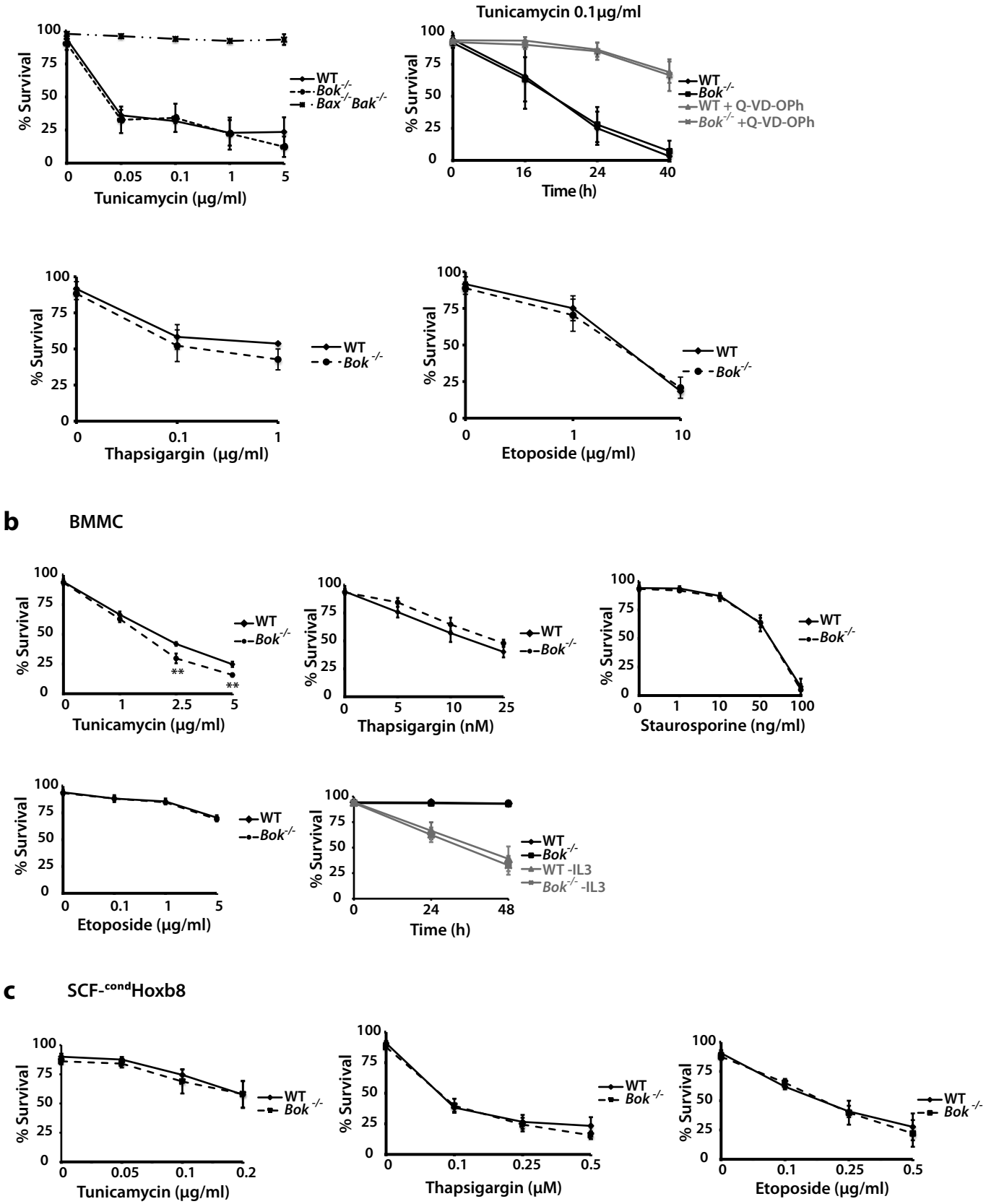
Supplementary Figure S3. Over-expressed BOK induces apoptosis in various cell lines. (**a**) FLAG-BOK was induced in WT FDM/5xUAS-FLAG-BOK cells by addition of 0.1 μM 4-OHT, in the presence or absence of 25 μM Q-VD-OPh, and cell viability was determined by flow cytometry (GFP-Annexin V/PI exclusion). Anti-FLAG immunoblot of untreated and 4-OHT treated cells shows induction of FLAG-BOK at 24 h. Data are presented as means +/- SD from three independent experiments. (**b**) FLAG-BOK was induced as described in (**a**) in BAF/3-5xUAS-FLAG-BOK cells. Cell viability was measured at 24 and 48 h and caspase-3-/-7 activity was quantified by using the fluorogenic caspase activity assay at 24 h using DEVD-AMC as a substrate. BAF/3 cells deprived of IL-3 for 15 h served as a positive control for caspase-3 activation. Anti-FLAG and anti-caspase-3 immunoblots of BAF/3 cell lysates after 0, 16, 24, 36 and 48 h of FLAG-BOK induction. Viability data are presented as means +/- SD from three independent experiments. DEVDase data are presented as means from 2 independent experiments. (**c**) Viability assay of Jurkat/5xUAS-FLAG-BOK cells after 0, 24, 48 and 72 h of FLAG-BOK induction. Induction of FLAG-BOK was verified by Western blot analysis using anti-FLAG specific antibody. Data are presented as means +/- SD from three independent experiments. (**d**) Confocal scanning micrographs of HeLa cells transiently transfected with a pEGFP-BOK expression construct for 12 h and stained with anti-cytochrome c antibody and Hoechst 33342 (nuclei). (**e**) Confocal scanning micrographs of HeLa cells transiently transfected with a FLAG-BOK/pcDNA3 expression construct for 18 h in the presence or absence of 25 μM Q-VD-OPh. Cells were stained with antibodies to FLAG and active caspase-3 specific and Hoechst 33342 dye. (**f**) FLAG-BOK was induced for 24 or 48 h in WT and Bok-/- SV40 immortalized MEF/5xUAS-FLAG-BOK cells and viability measured by flow cytometry (GFP-Annexin V/PI exclusion). Quantitative data are presented as means +/- SD of at least three independent experiments.



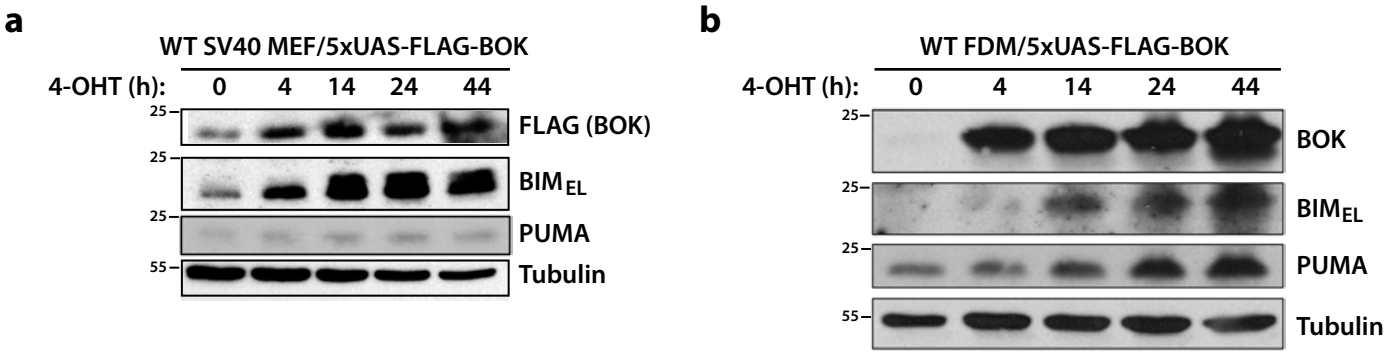
Supplementary Figure S4. BOK present on vesicular structures partially co-localizes with early and late endosomal markers. Confocal scanning micrographs of HeLa cells treated with 25 μM Q-VD-OPh and co-transfected for 21 h with expression constructs for FLAG-BOK/pcDNA3 (red) and early endosome/recycle marker RAB4Q67L-GFP (**a**), late endosome marker RAB5aQ97L-GFP (**b**), late endosome/lysosome marker RAB7Q67L-GFP (**c**) and early endosome marker RAB11Q70L-YFP (**d**). Constitutive active RAB mutants were used. Vesicular BOK partially co-localizes with the early endosome markers RAB4, RAB11 and with the late endosome marker RAB5a but not with the late endosome and lysosome marker RAB7 as indicated by the white arrows.



Supplementary Figure S5. Brefeldin A-induced Golgi fragmentation is independent of BOK. Confocal scanning micrographs of SV40 immortalized WT and Bok-/- MEF treated for the indicated times with brefeldin A (BFA, 5 µg/mL) and stained with an antibody against the cis-Golgi matrix protein GM130 (green). Nuclear DNA was stained with Hoechst 33342 dye (blue).



Supplementary Figure S6. BOK-deficient cells respond normally to classical apoptotic stimuli. (**a**) SV40 immortalized WT and Bok-/- MEF were treated with the indicated doses of tunicamycin, thapsigargin or etoposide for 24 h (dose responses). Cell survival was also examined over a period of 40 h for treatment with 0.1 µg/mL tunicamycin, in the presence or absence of 25 µM Q-VD-OPh. (**b**) WT and Bok-/- bone marrow derived mast cells (BMMC) were treated with various doses of tunicamycin, thapsigargin, etoposide or staurosporine for 24 h. Apoptosis was also induced by withdrawal of IL-3 for 24 or 48 h. (**c**) WT and Bok-/- SCF-condHoxb8 immortalized myeloid progenitors were treated with tunicamycin, thapsigargin or etoposide for 18 h. Cell survival data (a-c) are shown as percentage of GFP-Annexin V/PI double-negative cells and presented as means +/- SD of at least three independent experiments. Significance was determined by unpaired student's t-Test (**p<0.01).



Supplementary Figure S7. Enforced expression of FLAG-BOK leads to upregulation of the BH3-only proteins BIM and PUMA. SV40-immortalized WT MEF/5xUAS-FLAG-BOK (**a**) or WT FDM/5xUAS-FLAG-BOK (**b**) cells were treated with 0.1 μM 4-OHT to induce FLAG-BOK expression. Total protein lysates were prepared at the indicated times and analyzed by Western blotting using antibodies against FLAG, BOK, BIM and PUMA. Re-probing of the membrane with an anti-Tubulin antibody served as loading control.