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CORRECTION

# Bok is a genuine multi-BH-domain protein that triggers apoptosis in the absence of Bax and Bak

**Stephanie Einsele-Scholz, Silke Malmsheimer, Katrin Bertram, Daniel Stehle, Janina Johänning, Marianne Manz, Peter T. Daniel, Bernhard F. Gillissen, Klaus Schulze-Osthoff and Frank Essmann**

There was an error published in *J. Cell Sci.* **129**, 2213–2223.

In Fig. 4A, a statement that duplicate α-tubulin blots are shown for Mcl-1 and Bok, and for caspase-3 and Bax, because the same membranes were probed for these proteins, was inadvertently omitted from the legend.

The authors apologise to the readers for any confusion that this error might have caused.



Journal of Cell Science

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RESEARCH ARTICLE

# Bok is a genuine multi-BH-domain protein that triggers apoptosis in the absence of Bax and Bak

**Stephanie Einsele-Scholz[1], Silke Malmsheimer[1], Katrin Bertram[1], Daniel Stehle[1], Janina Johänning[1], Marianne Manz[1], Peter T. Daniel[2,3], Bernhard F. Gillissen[2,3], Klaus Schulze-Osthoff[1,3,*] and Frank Essmann[1,3,*]**

## ABSTRACT

The pro-apoptotic multidomain Bcl-2 proteins Bax and Bak (also known as BAK1) are considered the gatekeepers of the intrinsic pathway of apoptosis by triggering the mitochondrial release of cytochrome *c*. The role of the third Bax- and Bak-homologous multidomain protein Bok, however, is still unresolved. As cells doubly deficient for Bax and Bak are largely resistant to various apoptotic stimuli, it has been proposed that Bok is either dispensable for apoptosis or that its role is dependent on Bax and Bak. Here, we demonstrate, in several cell systems, that Bok efficiently induces cytochrome *c* release and apoptosis even in the complete absence of both Bak and Bax. Moreover, modulation of endogenous Bok levels affects the apoptosis response. By RNA interference and targeted deletion of the *Bok* gene, we demonstrate that Bok can significantly influence the apoptotic response to chemotherapeutic drugs in ovarian carcinoma cells. Hence, our results not only establish Bok as a Bak- and Bax-independent apoptosis inducer, but also suggest a potential impact of Bok expression in ovarian cancer therapy.

**KEY WORDS: Apoptosis, Bok, Bcl-2-related ovarian killer, Matador, Ovarian cancer**

## INTRODUCTION

Apoptosis is an evolutionary conserved process of altruistic cellular suicide that can be induced by extra- and intra-cellular stress stimuli. Irrespective of the initiating upstream signal, apoptosis culminates in the activation of cell-death-specific cysteinyl aspartases, called caspases (Los et al., 1999). Caspase activation occurs in response to ligation of death receptors on the plasma membrane (extrinsic pathway) or following the release of cytochrome *c* from the mitochondrial intermembrane space (intrinsic pathway). The release of cytochrome *c* is regulated by members of the Bcl-2 family of proteins and decisively depends on the presence of multidomain proteins (MDPs) of the Bcl-2 family, that is, Bax or Bak (also known as BAK1) (Wei et al., 2001; Shamas-Din et al., 2013). The pro-apoptotic activity of Bax and Bak is counteracted by anti-apoptotic 'Bcl-2-like' members, such as Bcl-2, Bcl-$x_L$ (a splice variant encoded by *BCL2L1*), Bcl-w (also known as BCL2L2), Mcl-1 and A1 (also known as BCL2A1) (Chi et al., 2014). The anti-apoptotic Bcl-2 proteins can be inhibited in turn by a third subgroup, the Bcl-2 homology domain 3 (BH3)-only proteins, such as Bad, Bid, Bik (also known as Nbk), Bim (also known as BCL2L11), Noxa (also known as PMAIP1), Puma (also known as BBC3), Hrk and Bmf (Delbridge et al., 2016). Bcl-2 proteins form an intricate network that regulates apoptosis by direct protein interaction. The interaction of BH3-only proteins and anti-apoptotic proteins has been studied in great detail (Willis et al., 2007). Thus, it has been shown that Bim, Bid and Puma potently bind to each anti-apoptotic Bcl-2 protein, whereas other BH3-only proteins specifically interact with a subset of anti-apoptotic proteins. Certain BH3-only proteins, especially the caspase-cleaved truncated form of Bid and Puma, have been shown to directly interact and thereby activate Bax or Bak through induction of a conformational change and oligomerization (Kuwana et al., 2005; Du et al., 2011; Westphal et al., 2014). In addition, anti-apoptotic Bcl-2-like proteins differ in their binding specificity to Bax or Bak. Whereas Bax binds to Bcl-2 and Bcl-$x_L$, the homologous protein Bak preferentially binds to Mcl-1 and Bcl-$x_L$ (Gillissen et al., 2007).

It is widely accepted that mouse embryonic fibroblasts (MEFs) from mice that are null for both Bax and Bak (denoted $Bax^{-/-}/Bak^{-/-}$) or cells deficient for Bax and Bak, such as HCT116 $Bax^{-/-}/Bak^{-/-}$ cells, are largely resistant to a wide variety of pro-apoptotic stimuli (Wei et al., 2001; Wang and Youle, 2012). Consequently, the multidomain proteins Bax and Bak are considered indispensable for cytochrome *c* release and apoptosis induction through the intrinsic pathway. However, the fact that $Bax^{-/-}/Bak^{-/-}$ double-knockout mice can be born viable, although at a reduced rate (Lindsten et al., 2000), indicates that apoptosis during embryonic development of certain organs is dispensable, replaced by another mode of cell death or that alternative mechanisms compensate for the loss of Bax and Bak. Such a compensatory mechanism might be attributed to the third, largely unknown, multidomain protein Bcl-2-related ovarian killer (Bok) (Hsu et al., 1997; Inohara et al., 1998). Bok is evolutionary conserved and homologous to Bax and Bak (Zhang et al., 2000). Surprisingly, in contrast to the vast number of investigations on Bax and Bak, only limited work has been devoted to investigate the regulation and function of Bok. Bok is widely expressed, particularly in reproductive tissues, but its loss has apparently only minimal impact in mice (Ke et al., 2012). Even the combined deficiency of Bok in Bax or Bak single-knockout mice does not show additional phenotypic alterations as compared to the parental mouse strain (Ke et al., 2013).

Initially, Bok was assumed to function similarly to Bax and Bak, because it also forms clustered structures at the mitochondrial outer membrane that are associated with cytochrome *c* release (Gao et al., 2005). Because these experiments were performed in MCF7 cells, which are proficient in Bax and Bak (Neise et al., 2008), Bok might function as a BH3-only protein, for example, like Puma (Jabbour et al., 2008), and induce cytochrome *c* release indirectly by blocking

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**Received 5 October 2015; Accepted 11 April 2016**

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anti-apoptotic proteins or by directly activating Bax and Bak, which in turn mediate cytochrome *c* release. This is supported by experiments showing that Bok alone is insufficient for apoptosis induction in $Bax^{-/-}/Bak^{-/-}$ MEFs and that Bok depends on Bax and Bak to mediate release of cytochrome *c* (Echeverry et al., 2013). However, it should be considered that the inability of endogenous Bok to mediate apoptosis in the absence of Bax and Bak might be due to its low or even absent expression level in MEF $Bax^{-/-}/Bak^{-/-}$ cells (Echeverry et al., 2013; Llambi et al., 2016). In addition to mitochondria, a fraction of Bok is localized to the Golgi and ER, implying a potential role of Bok in ER-stress-induced apoptosis (Echeverry et al., 2013; Carpio et al., 2015; Llambi et al., 2016). Furthermore, in several human cancers, including ovarian and breast carcinoma, the genomic locus encoding Bok is frequently deleted, suggesting a potential role of Bok as a tumor suppressor, similar to Bax and Bak (Beroukhim et al., 2010).

Despite its early discovery, Bok is certainly the most poorly understood Bcl-2 protein and its physiological role remains enigmatic. We, therefore, set out to characterize the pro-apoptotic function of Bok and the relevance of Bok for apoptosis sensitivity towards anti-cancer drugs. We found that overexpression of Bok strongly induced apoptosis accompanied by oligomerization of Bax and Bak. Interestingly, in Bax- and Bak-deficient cells Bok expression also efficiently induced the release of cytochrome *c*, meaning that Bok is a genuine pro-apoptotic multidomain protein. Consequently, downregulation of endogenous Bok expression, as well as targeted disruption of the *Bok* gene, effectively reduced the apoptosis sensitivity of even Bax- and Bak-proficient cells. Hence, Bok is a relevant component of the Bcl-2 network and, therefore, a potential target for anti-cancer therapeutic approaches.

## RESULTS

### Overexpression of Bok activates Bax and Bak and induces cytochrome *c* release

It has been previously shown that, in MCF7 cells, overexpressed EGFP–Bok displays clustered signals at the mitochondria that are associated with the release of cytochrome *c* from the mitochondrial intermembrane space (Gao et al., 2005). Because MCF7 cells express both Bax and Bak (Neise et al., 2008), the release of cytochrome *c* might be mediated either directly by Bok or indirectly by Bok-induced activation and oligomerization of Bax and/or Bak. In order to investigate these possibilities in further detail, we transfected MCF7 cells with pEGFP-Bok and performed immunofluorescence microscopy, detecting active Bax and Bak with conformation-specific antibodies, in parallel with detection of the mitochondrial marker Tom20. As a control, we also transfected MCF7 cells with pEGFP-Bax or pEGFP-Bak. Immunofluorescence microscopy showed that clustered EGFP signals of either protein coincided with the detection of active conformations of endogenous Bax and Bak (Fig. 1A,B). Interestingly, Bax and Bak mainly colocalized, whereas EGFP–Bok colocalized with active Bax or active Bak to a lower extent. Immunoblot analysis, however, verified that Bok was clearly detectable in mitochondria-enriched subcellular fractions in transfected MCF7 and HEK293 cells (Fig. S1A). Moreover, co-staining for the ER marker calnexin revealed that Bok was also partially localized at the ER (Fig. S1B), consistent with the reported broader localization of Bok at intracellular membranes (Echeverry et al., 2013). Next, we again transfected MCF7 cells with pEGFP-Bax, -Bak or -Bok and detected active Bax or Bak in parallel to cytochrome *c*. As already indicated by the clustering of endogenous Bax and Bak in response to overexpressed Bax, Bak and Bok, we found that cytochrome *c* was readily released from the mitochondria in MCF7 cells with clustered Bax, Bak and also Bok (Fig. 1C,D).

### Overexpression of Bok induces cell death in the combined absence of Bax and Bak

Having shown that overexpression of Bok is accompanied by activation of Bax and Bak as well as by the release of cytochrome *c*, we next investigated whether Bax and Bak are necessary for Bok-mediated mitochondrial alterations. To this end, we transfected wild-type and $Bax^{-/-}/Bak^{-/-}$ HCT116 cells (denoted HCT116/wt and HCT116/$Bax^{-/-}/Bak^{-/-}$, respectively) (Wang and Youle, 2012) with pEGFP-Bok or the pEGFP empty control and subsequently used fluorescence-activated cell sorting (FACS) analysis to assess cell death induction in EGFP-expressing cells by Sytox Red staining. Overexpression of EGFP–Bok was clearly associated with cell death induction both in HCT116/wt and HCT116/$Bax^{-/-}/Bak^{-/-}$ cells. Despite similar levels of Bok expression, as revealed by FACS analysis, Bok induced cell death slightly less efficiently in HCT116/$Bax^{-/-}/Bak^{-/-}$ (24 h, 24%; 48 h, 40%) as compared to HCT116/wt (24 h, 28%; 48 h, 55%) cells (Fig. 2A). To exclude the possibility that the weaker cell death induction in HCT116/$Bax^{-/-}/Bak^{-/-}$ cells was due to enhanced expression of anti-apoptotic proteins, we performed immunoblot analysis verifying a largely similar expression of anti-apoptotic proteins in HCT116/$Bax^{-/-}/Bak^{-/-}$ and HCT116/wt cells (Fig. 2B).

Although overexpression of Bok efficiently induced cell death in HCT116/$Bax^{-/-}/Bak^{-/-}$ cells, we wanted to confirm Bax- and Bak-independent cell death in another cellular system. Experiments in MEFs from $Bax^{-/-}/Bak^{-/-}$ mice demonstrated cytochrome *c* release upon Bok expression (Fig. S1C); however, in our hands the cells showed a poor transfection efficiency. We therefore used baby mouse kidney (BMK) cells from $Bax^{-/-}/Bak^{-/-}$ mice (Degenhardt et al., 2002). These cells also are deficient for Bax and Bak but show high expression of the anti-apoptotic proteins Bcl-2 and Mcl-1 (Fig. 2B) and are more efficiently transfected. Cell death induced by overexpression of EGFP-tagged Bax, Bak or Bok was quantified by measuring lactate dehydrogenase (LDH) release (Fig. 2C). In accordance with results from HCT116/$Bax^{-/-}/Bak^{-/-}$ cells, BMK/$Bax^{-/-}/Bak^{-/-}$ cells also readily died upon overexpression of any of the multidomain proteins, including Bok.

### Bok induces apoptosis in the absence of Bax and Bak

We showed above that Bok expression efficiently induces cell death in the absence of Bax and Bak both in human HCT116 cells and in murine BMK cells. We next analyzed cell death induction in BMK cells after transfection with pEGFP-Bax, -Bak and -Bok by FACS analysis of EGFP-positive cells in more detail. Flow cytometric analysis of Sytox-Red-stained cells revealed that, at 24 h post transfection, pEGFP-Bok induced cell death in 22% of the EGFP-positive cells, which was lower as compared to that induced by EGFP–Bak (42%) and EGFP–Bax (>55%) (Fig. 3A). After 48 h, the proportion of EGFP–Bax- or EGFP–Bak-expressing Sytox-Red-positive cells was similar (~55%) and the number of Sytox-Red-positive, EGFP-Bok-expressing BMK cells was 33%. In each case, the caspase inhibitor Q-VD-OPh significantly reduced the number of EGFP-expressing Sytox-Red-positive cells, indicating caspase-dependent (i.e. apoptotic) cell death (Fig. 3A).

In an analogous set of experiments, we investigated exposure of phosphatidylserine at the outer leaflet of the plasma membrane, a common marker of apoptosis. Cells were transfected with the respective expression constructs and, after staining with annexin V, were analyzed by flow cytometry (Koopman et al., 1994). These

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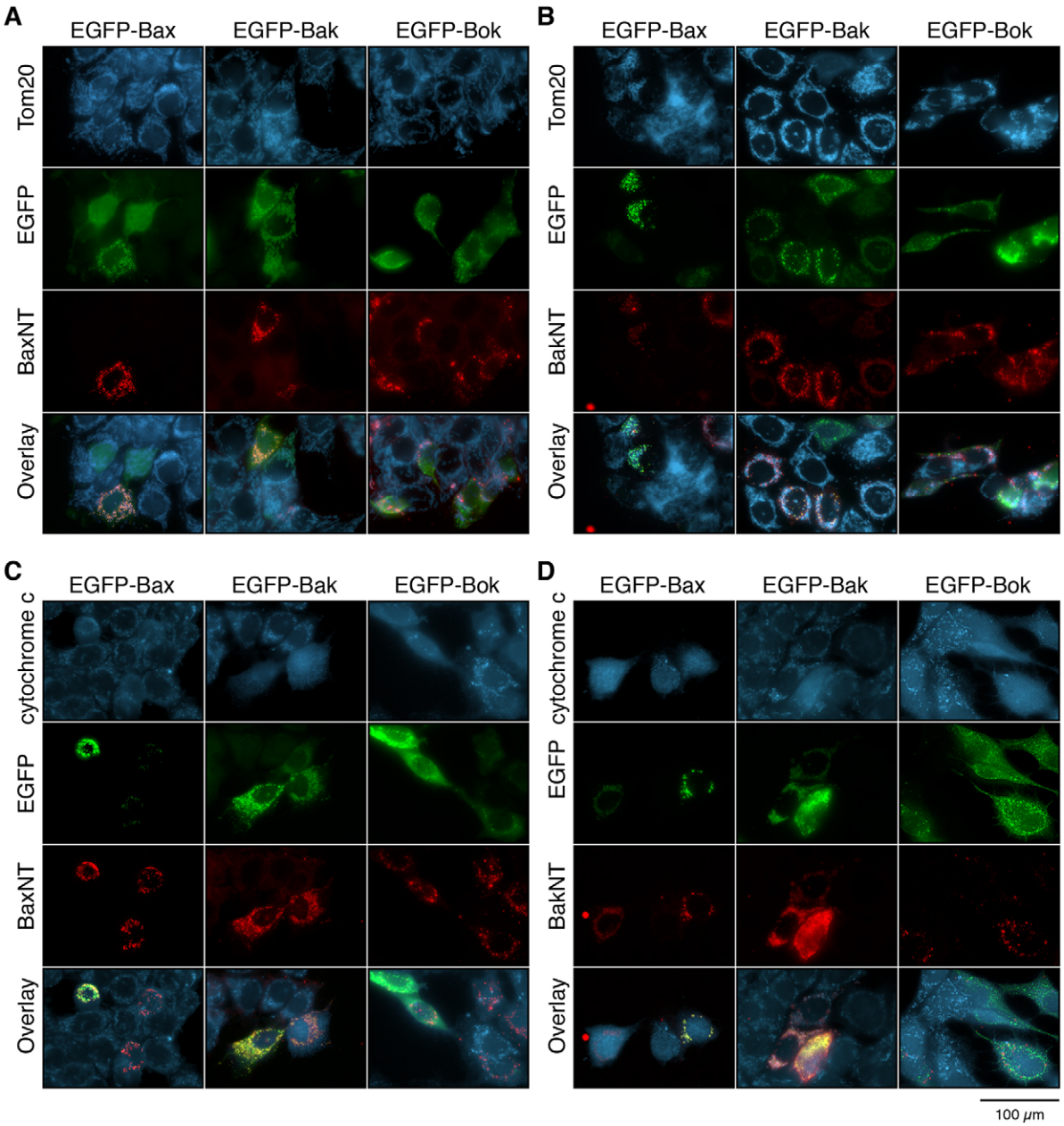


**Fig. 1. EGFP–Bok forms clusters at mitochondria and triggers activation of Bax and Bak and cytochrome *c* release.** MCF7 cells were transfected with plasmids for the expression of EGFP fusion proteins of Bax, Bak and Bok. After 16 h, cells were analyzed by immunofluorescence microscopy for the mitochondrial marker Tom20 (A,B), cytochrome *c* (C,D) and the respective EGFP fusion proteins. The active forms of Bax (A,C) and Bak (B,D) were detected by conformation-specific antibodies against the N-terminus (NT) of the proteins.

analyses showed that overexpression of any of the multidomain proteins (i.e. EGFP-tagged Bax, Bak and Bok), induced phosphatidylserine exposure at 24 h and 48 h post transfection (Fig. 3B), which was blocked by the caspase inhibitor Q-VD-OPh. In line with the Sytox Red staining, expression of Bax and Bak resulted in an increased number of annexin-V-positive BMK cells as compared to the expression of Bok. A more detailed analysis revealed that indeed, at similar intensities of the EGFP signal, apoptosis induction was stronger in Bax- and Bak- than in Bok-expressing cells (Fig. S2).

Although phosphatidylserine exposure at the cell surface is an early marker of apoptosis, it occurs downstream of caspase activation. Caspase activation in intrinsic apoptosis signaling is induced by the mitochondrial release of cytochrome *c* and is associated with loss of the mitochondrial membrane potential ($\Delta\Psi_m$). To investigate whether the mitochondrial transmembrane potential declines in response to overexpression of EGFP–Bok, we used TMRE staining and flow cytometric analysis. Overexpression of EGFP–Bok, similar to EGFP–Bax and EGFP–Bak, was accompanied by low TMRE fluorescence, indicating a reduction of $\Delta\Psi_m$ (Fig. 3C). Bok expression in the presence of the caspase inhibitor Q-VD-OPh reduced the number of EGFP-positive cells with low $\Delta\Psi_m$ (Fig. 3C), which is consistent with a caspase dependency for the loss $\Delta\Psi_m$ (Ricci et al., 2003).

In addition to flow cytometric analyses, Bok-mediated apoptosis induction was confirmed by analysis of caspase-3 and lamin A/C cleavage. Immunoblot analysis revealed processing of pro-caspase-

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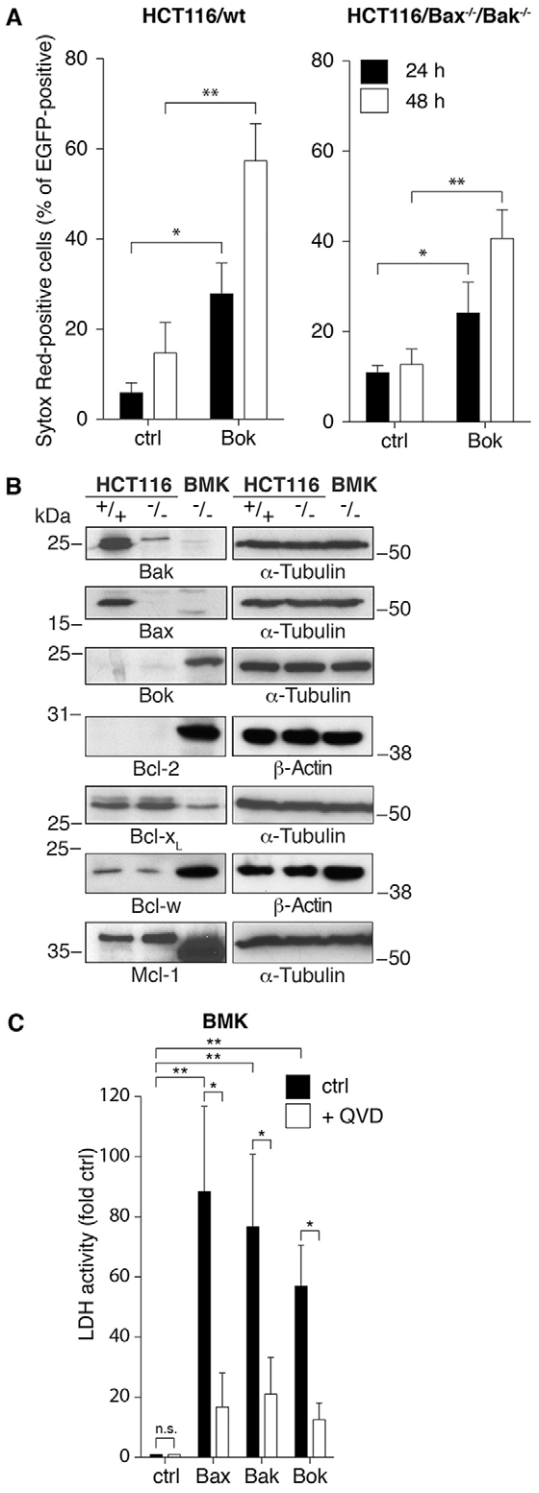


**Fig. 2. Bok induces cell death in the absence of Bax and Bak.** (A) HCT116/wt and HCT116/Bax$^{-/-}$/Bak$^{-/-}$ were transfected with expression plasmids for EGFP (ctrl) or EGFP–Bok. At 24 or 48 h after transfection, EGFP-positive dead cells were detected by flow cytometric analysis of Sytox Red staining. (B) Immunoblot analysis confirms Bax and Bak knockout in HCT116/Bax$^{-/-}$/Bak$^{-/-}$ (–/–) and BMK cells and expression of anti-apoptotic proteins Bcl-2, Bcl-x$_L$, Bcl-w and Mcl-1. (C) Overexpression of EGFP–Bax, EGFP–Bak and EGFP–Bok induces LDH release in BMK cells that is blocked by the caspase inhibitor Q-VD-OPh (QVD). Data indicate the fold release of LDH into supernatants relative to the EGFP-only control. Results in panels A and C show the mean±s.d. from three independent experiments. *$P$≤0.05, **$P$≤0.01; n.s., not significant (unpaired two-tailed Student's $t$-test).

3 to the p17 subunit and cleavage of the caspase-6 substrate lamin A/C in response to EGFP-tagged Bok, as well as Bak and Bax, expression, which was efficiently blocked by Q-VD-OPh (Fig. 3D). Finally, in line with the results in HCT116 cells, BMK cells overexpressing EGFP-tagged Bax, Bak and also Bok displayed mitochondrial release of cytochrome *c*, as assessed by immunofluorescence microscopy (Fig. 3E) and flow cytometry (Fig. 3F). Hence, Bok expression in Bax$^{-/-}$/Bak$^{-/-}$ BMK cells induced each apoptosis-associated hallmark that has been ascribed to the activity of the pro-apoptotic multidomain proteins Bax and Bak. Apoptotic alterations, such as caspase activation, in the absence of Bax and Bak were not only observed upon expression of EGFP-tagged Bok, but also when a Bok version with a smaller Flag tag was used (Fig. S3).

### Bok knockdown reduces the apoptosis sensitivity of ovarian carcinoma cells

Having established that there is a Bax- and Bak-independent pro-apoptotic function of Bok in overexpression experiments, we next analyzed whether Bok is functionally relevant for apoptosis induction. For these analyses, we chose ovarian cancer cell lines that revealed significant endogenous expression of Bok. As shown by immunoblot analysis, all cell lines (OVCAR-3, -4, -8) expressed Bok as well as Bax, Bak and caspase-3 (Fig. 4A). The cell lines also expressed the anti-apoptotic proteins Bcl-2, Bcl-x$_L$ and Mcl-1, although in varying amounts.

In order to analyze a role of Bok in apoptosis sensitivity, we performed small interfering RNA (siRNA)-mediated Bok knockdown (siBok) and subsequently induced apoptosis by drug treatment with *cis*-diaminodichloroplatinum(II) (*cis*Pt, 40 µM), taxol (0.1 µM) or camptothecin (1.0 µM). At 48 h after transfection with Bok-specific or control siRNAs, cells were exposed to the respective drugs for 24 h, before caspase (DEVDase) activity was assessed in cell lysates by a luminometric substrate assay. DEVDase activity induced by *cis*Pt and taxol was reduced by ∼20% in siBok-transfected OVCAR-3 and -4 cells as compared to control-siRNA-transfected cells (Fig. 4B). Knockdown of Bok reduced the DEVDase activity in response to camptothecin treatment even more efficiently, by almost 30% in OVCAR-3 and 35% in OVCAR-4 cells (Fig. 4B). Reduction of drug-induced caspase activation upon Bok knockdown was most pronounced in OVCAR-8 cells, which showed 35% (*cis*Pt), 36% (taxol) and 32% (camptothecin) less DEVDase activity after incubation with the chemotherapeutic drugs (Fig. 4B).

Furthermore, we compared the effect of Bok knockdown with that of Bax and Bak in OVCAR-8 cells. Immunoblot analysis confirmed an efficient knockdown of each pro-apoptotic multidomain protein (Fig. 4C, upper panel) and revealed concomitantly reduced activation-associated cleavage of pro-caspase-3 after incubation with *cis*Pt, taxol or camptothecin (Fig. 4C, lower panel). Whereas the reduction of caspase-3 cleavage in camptothecin- and taxol-treated OVCAR-8 cells upon the knockdown of Bok, Bak and Bax was similar, *cis*Pt-induced caspase-3 processing was efficiently reduced by the knockdown of Bok or Bak and slightly by the knockdown of Bax (Fig. 4C, lower panel). In line with reduced caspase-3 processing, lysates from OVCAR-8 cells transfected with siRNA against Bak and Bok (siBak and siBok, respectively) also showed reduced DEVDase activity in response to drug treatment (Fig. 4D). As siBok and siBak individually protected cells from drug-induced cell death, we performed simultaneous knockdown of both Bok and Bak, and analyzed caspase activation in response to drug treatment. The

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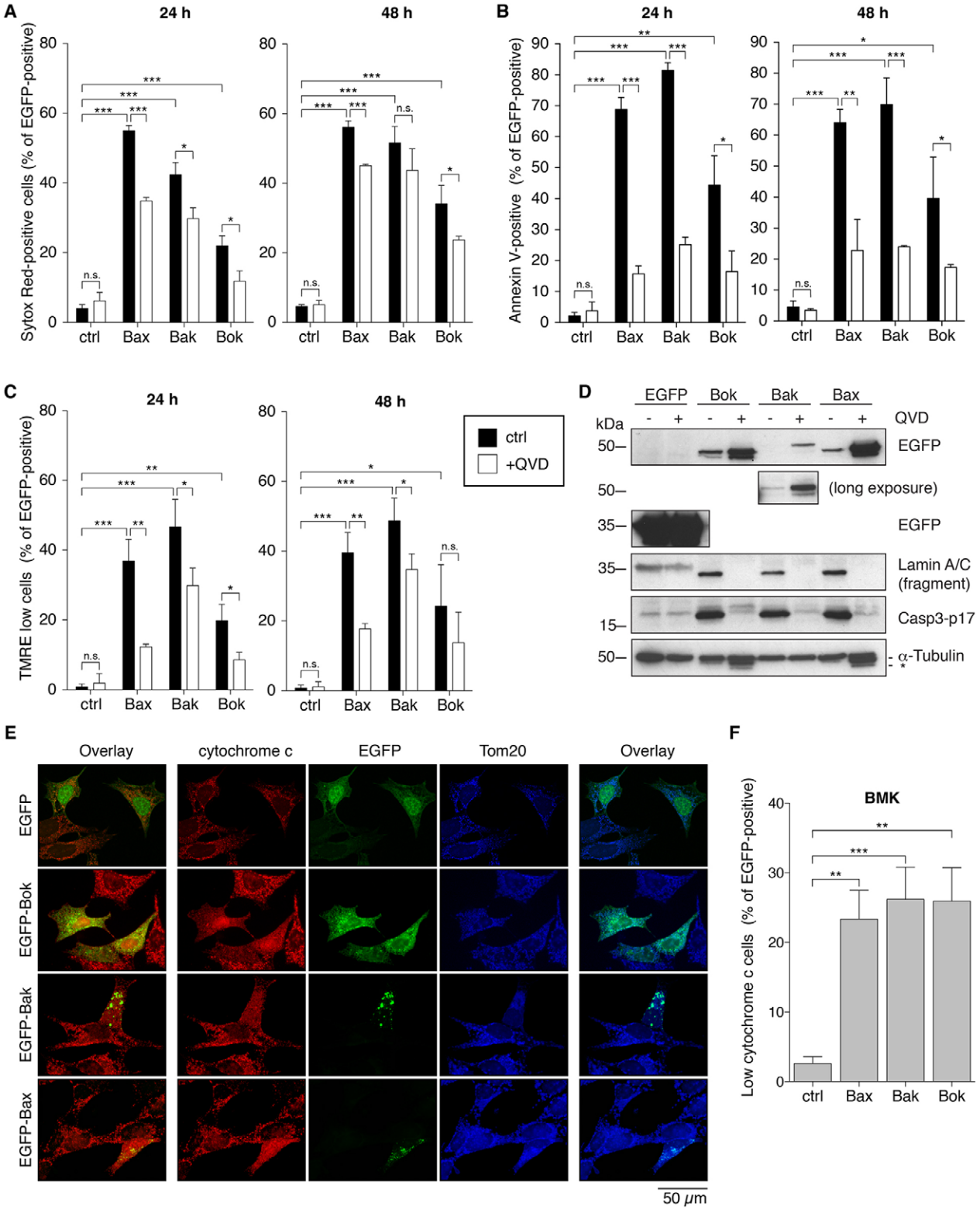


**Fig. 3. Overexpressed EGFP–Bok induces apoptosis in Bax and Bak doubly deficient BMK cells.** BMK cells were transfected with expression constructs for EGFP (ctrl), EGFP–Bax, EGFP–Bak or EGFP–Bok and cultured for 24 h or 48 h in the presence or absence of Q-VD-OPh (QVD). Cells were analyzed for Sytox Red (A), annexin V (B) or TMRE (C) staining in the EGFP-positive cell population. (D) Immunoblot analysis of EGFP–Bax-, EGFP–Bak- and EGFP–Bok-expressing cells reveals activation-associated cleavage of pro-caspase-3 and cleavage of the caspase-6 substrate lamin A/C (asterisk indicates EGFP signal). (E) BMK cells transfected with pEGFP-Bax, -Bak or -Bok were stained for the mitochondrial protein Tom20 (blue) and cytochrome *c* (red). Immunofluorescence microscopy shows cytosolic cytochrome *c* in cells with clustered EGFP signals of the Bax, Bak and Bok proteins. (F) Flow cytometric analysis of transfected BMK cells shows reduced cytochrome *c* signal intensity in EGFP-tagged cells expressing Bax, Bak and Bok as compared to EGFP-expressing control cells. Results in panels A–C, F show the mean±s.d. from three or more independent experiments. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$; n.s., not significant (unpaired two-tailed Student's *t*-test).



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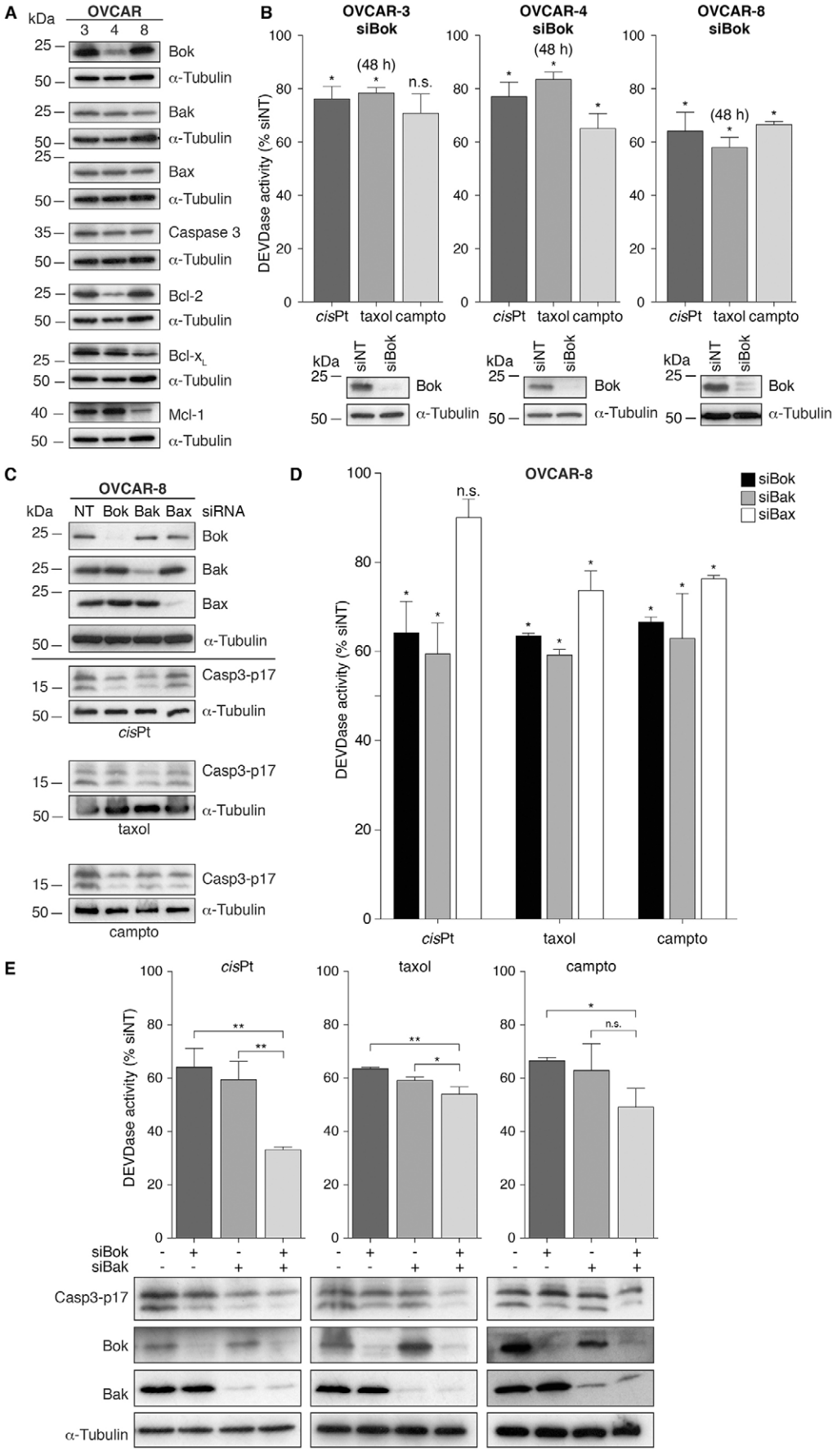


**Fig. 4. Knockdown of Bok reduces drug-induced apoptosis in ovarian carcinoma cells.** (A) Immunoblot analyses demonstrating expression of the pro-apoptotic multidomain proteins Bax, Bak and Bok, the anti-apoptotic proteins Bcl-2, Bcl-$x_L$ and Mcl-1, and pro-caspase-3 in OVCAR-3, OVCAR-4 and OVCAR-8 cells. (B) Bok knockdown results in reduced caspase activity. Bok was knocked down in OVCAR-3, OVCAR-4 and OVCAR-8 cells, before cells were cultured in the presence of *cis*Pt (24 h), taxol (48 h) or camptothecin (24 h). Caspase (i.e. DEVDase activity) was analyzed by the CaspaseGlo 3/7 assay and is given as percentage of DEVDase activity from cells transfected with a non-targeting control siRNA (siNT). Knockdown of Bok was verified by immunoblot analysis. (C,D) Bok, Bak and Bax were knocked down in OVCAR-8 cells and cells were incubated with *cis*Pt, taxol or camptothecin. Knockdown of Bok and Bak, and to a minor extent of Bax, is associated with reduced caspase-3 cleavage (C) and reduced DEVDase activity (D) after incubation with the cytotoxic drugs. (E) Combined knockdown of Bok and Bak enhances inhibition of DEVDase activity in OVCAR-8 cells in response to *cis*Pt (left panel) and to a lesser extent to taxol (middle panel) and camptothecin (right panel). Knockdown of Bok and Bak and cleavage of pro-caspase-3 was analyzed by immunoblotting (lower panels). Downregulation of Bok and Bak expression resulted in reduced pro-caspase-3 processing. Results in panels B, D and E show the mean± s.d. from at least three experiments. *$P \leq 0.05$, **$P \leq 0.01$; n.s., non-significant (unpaired two-tailed Student's *t*-test).



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simultaneous knockdown of Bok and Bak led to an enhanced inhibition of *cis*Pt-induced DEVDase activity and caspase-3 cleavage (Fig. 4E).

### Bok knockout diminishes apoptosis induction by cytostatic drugs in OVCAR-8 cells

The previous knockdown experiments support a functional relevance of Bok for apoptosis sensitivity of ovarian carcinoma cells. We next sought to confirm these results in a suitable knockout rather than knockdown system. To this end, we employed transcription-activator-like effector nuclease (TALEN) technology and co-transfected OVCAR-8 cells with vectors targeting exon 1 of the *Bok* gene and a DNA-cleavage-sensitive reporter construct that allowed the enrichment of gene-modified cells (Sun and Zhao, 2013). After transfection with both plasmids, individual cells that simultaneously expressed GFP and RFP were FACS-sorted into a 96-well plate. We chose three clonal cell lines devoid of Bok expression as detected by immunoblot analysis (Fig. 5A). Moreover, DNA sequence analysis confirmed frameshift mutations in each clonal cell line, resulting in a stop codon in the TALEN-targeted exon of *Bok* (Fig. S4). In contrast to Bok, the expression level of other apoptosis-relevant proteins (i.e. Bak, Bax, Bcl-$x_L$, Bcl-2 and Mcl-1) remained unaltered in the three cell clones as compared to the parental cell line (Fig. 5A), thereby excluding off-target effects.

We next compared the sensitivity towards drug-induced apoptosis of the generated OVCAR-8 Bok$^{-/-}$ cells with the parental cell line. As expected, each of the three individual OVCAR-8 Bok$^{-/-}$ cell lines showed reduced DEVDase activity and caspase-3 cleavage after incubation with *cis*Pt, taxol and camptothecin as compared to parental OVCAR-8 cells (Fig. 5B). Because expression of anti-apoptotic proteins was not enhanced and expression of Bax and Bak was also not affected, the reduced sensitivity of OVCAR-8 Bok$^{-/-}$ cells can be attributed to the absence of Bok. In addition, upon drug treatment, the Bok deficiency of OVCAR-8 cells resulted in a significant reduction of annexin-V-positive early apoptotic cells as well as secondary necrotic cells (Fig. 5C). A reduced drug sensitivity upon Bok ablation was not restricted to ovarian cancer cells, but also observed in SH-SY5Y neuroblastoma cells following siRNA-mediated knockdown of Bok (Fig. 5D).

## DISCUSSION

Members of the Bcl-2 family are crucial regulators of the intrinsic apoptosis pathway that act by controlling the mitochondrial release of cytochrome *c* (Czabotar et al., 2014; Moldoveanu et al., 2014). Research over the past 30 years has unveiled a complex interaction-based network of Bcl-2 proteins (Chi et al., 2014; Delbridge et al., 2016). Whereas many BH3-only proteins merely inhibit anti-apoptotic Bcl-2 proteins, distinct BH3-only proteins catalyze the recruitment and insertion of Bax or Bak into the outer mitochondrial membrane. The Bax and Bak homolog Bok has largely slipped attention, most likely due to its low expression level in commonly used cell lines as compared to Bax and Bak. Additionally, the Bok-knockout mouse strain generated by Ke et al. does not present an overt phenotype and even the phenotypes of Bok and Bax, and Bok and Bak double-knockout strains do not significantly differ from Bax or Bak single-knockout mice, allegedly underlining an insignificant role for Bok (Ke et al., 2012, 2013).

In line with the homology of Bok to Bax and Bak, we found that enforced Bok expression was able to induce classical apoptosis hallmarks associated with cytochrome *c* release. Interestingly, Bok expression also triggered mitochondrial clustering of Bax and Bak signals reflecting their oligomerization and activation state. There are several possibilities to explain the downstream Bax and Bak activation. For instance, through its own conserved BH3 domain, Bok might act itself like a BH3-only protein and directly activate Bax and Bak or neutralize anti-apoptotic Bcl-2 proteins, leading to BH3-only protein release, and Bax and Bak activation. In addition, Bok might form heterodimers with Bax and/or Bak, although previous studies failed to demonstrate a direct interaction of Bok with other multidomain proteins (Carpio et al., 2015).

At first view, Bok-mediated downstream activation of Bak and Bax is consistent with earlier assumptions claiming that Bok-induced apoptosis is dependent on Bax and Bak. Thus, it has been reported that enforced expression of Bok activates the intrinsic apoptotic pathway in Bax- and Bak-proficient cells, but fails to kill cells lacking both Bax and Bak or sensitize them to cytotoxic insults (Echeverry et al., 2013). In line with this report, we also detected a certain portion of Bok not only at mitochondria, but also at the ER, as revealed by co-localization with the ER marker calnexin. It has been recently suggested that Bok might exert a selective role in ER-stress-induced Bax and Bak activation, and promotion of mitochondrial apoptosis, as Bok$^{-/-}$ cells have been found to be defective in their response to ER stress stimuli (Carpio et al., 2015). The contribution of Bok to ER-stress-induced apoptosis, however, remains controversial, as other studies have detected no differences of Bok-deficient cells in their sensitivity to ER stress or have even reported that brefeldin-A-treated cells died faster in the absence of Bok (Fernandez-Marrero et al., 2016; Echeverry et al., 2013). A recent study that appeared during revision of this manuscript further suggested that Bok plays a selective role for apoptosis in response to proteasome inhibition or ER-associated protein degradation (ERAD) dysfunction (Llambi et al., 2016).

In several cellular systems, we clearly demonstrate a pro-apoptotic role for Bok. A functional role of Bok is not only evident in overexpression experiments, but also by our finding that siRNA-mediated knockdown of endogenous Bok in three different ovarian cancer cell lines, as well as in neuroblastoma cells, could significantly protect against apoptosis induced by different chemotherapeutic drugs. The RNA interference or overexpression experiments do certainly not exclude that Bok, through its own BH3 domain, might act in a similar manner as classical BH3-only proteins. However, our experiments in MEFs, HCT116 and BMK cells clearly reveal that Bok can act as a bona fide multidomain protein and induce apoptosis in the absence of Bak and Bax, a finding supported by the recent study of Llambi et al. (2016). Moreover, TALEN-mediated *Bok* knockout in ovarian cancer cells lowered the sensitivity to drug-induced apoptosis. These results seem to contradict previous findings that failed to detect altered apoptosis sensitivity in the absence of Bok (Ke et al., 2012; Echeverry et al., 2013). In contrast to the ovarian cancer cells or SH-SY5Y neuroblastoma cells with a strong Bok expression, however, previous studies employed MEFs or lymphocytes with rather low endogenous Bok levels, whose downregulation might not affect the apoptotic response.

The *Bok* gene locus is frequently deleted in human cancer (Beroukhim et al., 2010). This finding, as well as our result that reduced or absent expression of Bok affects apoptosis sensitivity in response to conventional chemotherapeutic drugs, suggest, at least in ovarian cancer, that Bok expression might be relevant for cancer therapy. The proteins Bax and Bak show a specific yet overlapping interaction pattern, with Bax preferentially binding to Bcl-2 and Bcl-$x_L$, whereas Bak primarily interacts with Bcl-$x_L$ and Mcl-1

Journal of Cell Science (2016) 129, 2213-2223 doi:10.1242/jcs.181727

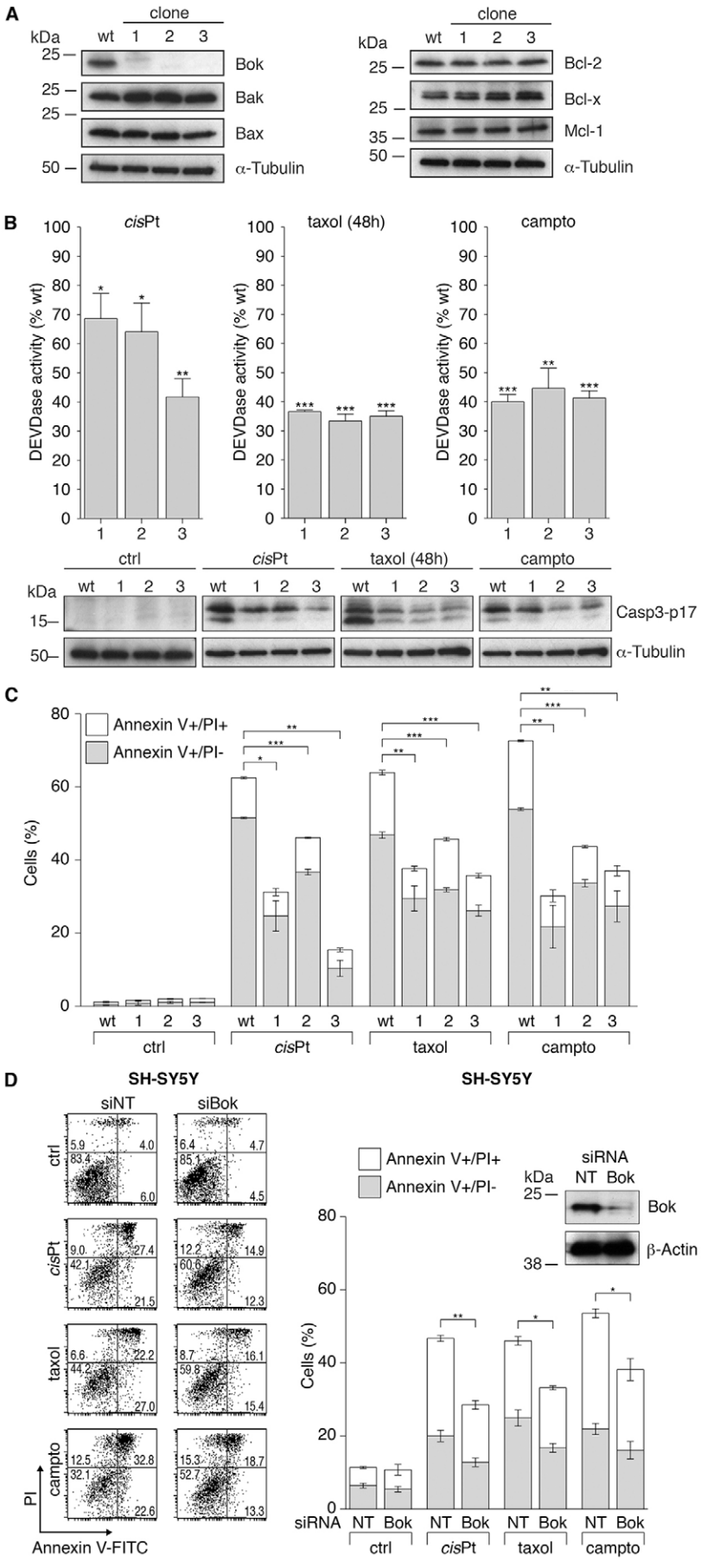


**Fig. 5. Inhibition of Bok expression attenuates sensitivity of OVCAR-8 and SH-SY5Y cells to drug-induced apoptosis.** (A) Western blot analysis confirms TALEN-mediated loss of Bok expression in three OVCAR-8 Bok-knockout clones, whereas expression of Bax, Bak Bcl-2, Bcl-$x_L$ and Mcl-1 was similar to that in parental wild-type OVCAR-8 cells (wt). (B) Induction of DEVDase activity (upper panels) and pro-caspase-3 cleavage (lower panels) by treatment with *cis*Pt (24 h), taxol (48 h) and camptothecin (24 h) is markedly reduced in Bok-knockout cell lines. (C) OVCAR-8/wt and Bok-knockout clones were incubated in the presence of *cis*Pt (80 µM), taxol (0.1 µM) or camptothecin (1.0 µM) for 48 h. ctrl, cells incubated with with 0.1% DMSO. Cells were stained with annexin-V–FITC and propidium iodide (PI) and analyzed by flow cytometry. (D) SH-SY5Y cells were transfected with Bok-specific or a non-targeting siRNA control (siNT) before incubation in the presence of *cis*Pt (20 µM), taxol (0.05 µM) or camptothecin (0.5 µM) for 24 h. Flow cytometric analysis of annexin V and propidium iodide staining shows that Bok knockdown (upper panels) results in a reduced number of annexin-V-positive and annexin V and propidium iodide double-positive SH-SY5Y cells. Representative dot blots are shown on the left. Efficient Bok knockdown was verified by immunoblot analysis. Results in panels B,C and D show the mean±s.d. from at least three independent experiments. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ (unpaired two-tailed Student's *t*-test).

(Willis et al., 2005; Llambi et al., 2011). Hence, small-molecule drugs such as ABT-737, ABT-263 and ABT-199, which bind to and inhibit the anti-apoptotic activity of Bcl-2, or A-1210477, which targets Mcl-1, are highly promising drugs in cancers with Bcl-2 or Mcl-1 overexpression (Leverson et al., 2015). Mcl-1 and also A1 have been shown to interact with Bok and to prevent Bok-induced apoptosis after overexpression (Hsu et al., 1997; Inohara et al., 1998, and data not shown). In addition, Bok overexpression has been found to kill Mcl-1-deficient MEFs significantly faster than wild-type cells (Echeverry et al., 2013). Therefore, the Mcl-1 inhibitor A-1210477 or an A1 inhibitor might sensitize Bok-proficient tumors to apoptosis induction. Intriguingly, as we show an autonomous function of Bok for cytochrome *c* release and apoptosis in the absence of Bax and Bak, such drugs might be even effective in Bax- and/or Bak-deficient tumor cells. In contrast to previous reports, Green and colleagues recently proposed that Bok-induced apoptosis is not inhibited by anti-apoptotic Bcl-2 proteins, although a potential antagonistic effect of A1 had not been explored (Llambi et al., 2016). Moreover, the authors proposed that Bok does not interact with BH3 proteins, but had an autonomous role in apoptosis induction, although only a limited number of BH3-only proteins were tested in that study. Furthermore, because the interaction of Bok with BH3-only proteins or BH3 peptides has been mostly studied *in vitro*, either by following the permeabilization of unilamellar vesicles or by measuring surface plasmon resonance, the exact positioning of Bok within the Bcl-2 network warrants further investigation.

The detailed mechanism of how multidomain Bcl-2 proteins cause mitochondrial outer membrane permeabilization (MOMP) to trigger cytochrome *c* release is still under debate. Here, we have established Bok not only as a modulator of apoptosis sensitivity but, moreover, as a bona fide pro-apoptotic multidomain protein that autonomously mediates MOMP and cytochrome *c* release. Future studies are required to shed further light into several aspects of Bok biology, including its position in the Bcl-2 network, its functional difference to the Bax and Bak homologs, its putative tumor suppressor function and its suitability for targeted cancer therapy.

## MATERIALS AND METHODS

### Cell culture

The ovarian cancer cell lines OVCAR-3, -4 and -8 as well as HCT116, and MCF7 cells were from the authenticated NCI60 panel of cancer cell lines and obtained from the NCI Developmental Therapeutics Program. OVCAR-3, OVCAR-8 and OVCAR-8 Bok$^{-/-}$ cells were maintained in RPMI-1640 medium (Sigma), supplemented with 10% fetal calf serum (FCS; PAA Laboratories) and antibiotics (MycoZapPlus-CL; Lonza). OVCAR-3 cells additionally received 2 mM glutamine (Life Technologies), whereas OVCAR-4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% tetracycline-free FCS (Life Technologies), 1× MEM non-essential amino acids (PAA Laboratories) and antibiotics. Primary baby mouse kidney (BMK) epithelial cells from Bax$^{-/-}$/Bak$^{-/-}$ mice (kindly provided by David Andrews, Department of Biochemistry, Sunnybrook Research Institute, University of Toronto, ON, Canada), authenticated Bax$^{-/-}$/Bak$^{-/-}$ murine embryonic fibroblasts, HEK293 cells and SH-SY5Y neuroblastoma cells (Janssen et al., 2007) were obtained from the ATCC and maintained in DMEM supplemented with 10% FCS, 1× MEM non-essential amino acids and antibiotics. HCT116/wt and HCT116/Bax$^{-/-}$/Bak$^{-/-}$ cells (kindly provided by Richard Youle, Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, Bethesda, MD) were cultured in McCoy's 5A medium (Life Technologies) with 10% FCS and antibiotics. All cells were cultured at 37°C in a humidified 5% $CO_2$ atmosphere. Bax and Bak deficiency of the Bax- and Bak-deficient MEFs, BMK and HCT116 cells was verified by immunoblot analysis. Cells were routinely tested for contamination.

### Transfection and drug treatment

BMK cells ($3\times10^5$ cells/well) were seeded 24 h before transfection in six-well plates. Transfection was performed with FuGENE 6 (Promega, Mannheim, Germany) according to the manufacturer's protocol using 3 µl FuGENE 6 and 1 µg of the following plasmid DNAs: pEGFP-C1 empty vector, pEGFP-C1-Bok, pEGFP-C1-Bak and pEGFP-C1-Bax. At 24 h and 48 h after transfection cells were stained and analyzed by flow cytometry. OVCAR-8/wt and OVCAR-8/Bok$^{-/-}$ cell lines were seeded in six-well plates at a density of $2\times10^5$ cells/well. After 24 h, the following cytostatic agents were added to the cells: cisplatin (40 µM), taxol (0.1 µM) and camptothecin (1 µM). After an additional 24 h, or 48 h for taxol, cells were analyzed by flow cytometry.

### Knockdown, induction of cell death and caspase activity assay

OVCAR-8, OVCAR-4, OVCAR-3 or SH-SY5Y cells were seeded 24 h before transfection in six-well plates at a density of $1\times10^5$ cells/well. For siRNA transfection Bok, Bak and Bax ON-TARGET Plus Smartpool siRNAs or non-targeted (NT) Smartpool ON-TARGET plus control siRNA (GE Healthcare, Munich, Germany) were delivered using Dharmafect I reagent (GE Healthcare) according to the manufacturer's protocol. At 48 h after transfection, cells were treated with the indicated chemotherapeutic drugs. After an additional 24 h, or 48 h for taxol after, cells were collected by scraping, washed with PBS and resuspended in 1 ml PBS. Aliquots of the cell suspension were used to assess cell death by annexin V and propidium iodide staining or caspase-3 and -7 activity in the Caspase-Glo 3/7 assay (Promega) as described previously (Gillissen et al., 2013; Volkmann et al., 2007). Successful knockdown was verified by immunoblot analysis.

### Generation of OVCAR-8/Bok$^{-/-}$ cell lines using TALEN constructs

TALEN constructs targeting human Bok and a corresponding mRFP or GFP reporter construct were obtained from ToolGen Genome Engineering (Seoul, Korea). OVCAR-8 cells were seeded 1 day before transfection in six-well plates at a density of $2\times10^5$ cells/well. Transfection was performed at a DNA:FuGENE 6 ratio of 1:3 using 1 µg of the plasmids Human-BOK_TALEN_L1, Human-BOK_TALEN_R1 and Human-BOK-RG2S1. At 40 h after transfection cells were trypsinized, washed and resuspended in PBS. With fluorescence-activating cell sorting, GFP-positive cells were sorted as single cells in 96-well plates. Three clonal cell lines (clone 1, 2 and 3) were used for further experiments and verified for loss of Bok expression by immunoblotting and DNA sequence analyses.

### Immunoblot analysis

Immunoblot analyses were performed as described previously (Essmann et al., 2003). Cells were harvested by scraping, washed with ice-cold PBS and resuspended with lysis buffer (1% Triton X-100, 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$ and 5 mM EDTA, pH 8.0) supplemented with Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, Bonn, Germany). Protein concentrations were determined using the BCA assay kit (Thermo Fisher Scientific). Protein lysates were mixed with 5× Laemmli buffer (300 mM Tris-HCl pH 6.8, 40% glycerol, 4% SDS and 3% 2-mercaptoethanol) and heated for 10 min at 95°C. Then, 15–30 µg of proteins were separated on SDS-PAGE gels at 120 V. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (Amersham, GE Healthcare) by tank blotting (90 V, 2 h). Membranes were blocked in blocking buffer (5% BSA, 0.1% Tween-20 in PBS) for 1 h, followed by an overnight incubation with primary antibodies (1:1000) in blocking buffer at 4°C. Primary antibodies used were rabbit anti-Bak (NT, 06-536) and anti-Bax (NT, 06-499; both from Merck Millipore), mouse anti-Bcl-2 (clone C-2) and anti-GFP (clone B-2, both from Santa Cruz Biotechnology), mouse anti-Bcl-$x_L$ (clone 44) and anti-Mcl-1 (clone 22; both from Becton Dickinson); goat anti-caspase-3 (AF-605-NA; R&D Systems), rabbit anti-Bcl-w (clone 31H4), rabbit anti-caspase-3 (clone 8G10), rabbit anti-lamin A/C (clone 2032) and rabbit anti-Mcl-1

(clone D35A5; all from Cell Signaling Technology); mouse anti-α-tubulin (clone DM1A; Sigma) and monoclonal rabbit anti-Bok (1:500, BOK-1-5; Echeverry et al., 2013; a kind gift from Thomas Kaufmann, Institute of Pharmacology, University of Bern, Bern, Switzerland). Secondary antibodies coupled to horseradish peroxidase (1:10,000; Promega) and ECL reagents were used to detect proteins by chemoluminescence.

### Immunofluorescence microscopy

Cells were seeded on coverslips in 12-well plates at 48 h before transfection using 1.5 µg plasmid DNA and 4.5 µl FuGENE 6 in 100 µl OptiMEM. After 16 h, cells were fixed with 4% formaldehyde for 30 min on ice, washed with PBS, and followed by incubation for 1 h in blocking buffer (4% BSA and 0.05% saponin in PBS) at room temperature. The cells were then incubated overnight at 4°C with the following primary antibodies diluted 1:500 in blocking buffer: rabbit anti-Tom20 (sc-11415; Santa Cruz Biotechnology), mouse anti-cytochrome *c* (clone 6H2.B4), mouse anti-Tom20 (clone 29, BD Biosciences) and conformation-specific antibodies against Bak and Bax (NT, 06-536 and 06-499; Merck Millipore). After washing the cells in PBS and twice in blocking buffer, secondary antibodies (Alexa-Fluor-568 or -405-conjugated goat anti-mouse-IgG or anti-rabbit-IgG; 1:500 in PBS; Life Technologies) were added for 2 h at room temperature. The cells were washed twice in PBS and incubated in PBS containing 1 µg/ml 4′,6-diamidino-2-phenylindol (DAPI; Life Technologies) for 2 min. Coverslips were washed in PBS and mounted in fluorescence-mounting medium (DAKO). Images were taken using a Leica DMI6000 fluorescence microscope with a 63× oil immersion objective, and processed with Leica DFC365FX and MetaMorph Software (Leica, Wetzlar, Germany).

### Flow cytometric analyses

For TMRE staining (Sohn et al., 2006), cell supernatant was collected, cells were trypsinized, re-combined with supernatant and washed with RPMI without Phenol Red (Life Technologies) supplemented with 2% FCS. Cells were then incubated in RPMI with 2% FCS containing 400 nM TMRE (Abcam) for 30 min at 37°C. After washing in cold PBS, cells were resuspended in PBS containing 0.2% BSA and analyzed using a LSRII flow cytometer (BD Biosciences). For annexin V staining, cells were harvested as described above, washed twice in ice-cold PBS, resuspended in annexin-V-binding buffer and incubated with 5 µl of APC- or FITC-coupled annexin V (BD Biosciences) for 15 min at room temperature in the dark. Annexin V and propidium iodide staining was performed as described previously (Gillissen et al., 2013). For Sytox Red staining, culture supernatant was collected, cells were washed in Hank's Balanced Salt Solution (Life Technologies), trypsinized and combined with supernatant. After washing cells were resuspended in HBSS containing 6 nM Sytox Red Dead Cell Stain (Life Technologies) and incubated for 15 min at room temperature in the dark. Flow cytometric quantification of cytochrome *c* release was carried out as described previously (Waterhouse and Trapani, 2003; Janssen et al., 2009). EGFP-positive cells were analyzed by flow cytometry.

### Statistical analysis

Statistical significance of data was assessed by unpaired two-tailed Student's *t*-test using GraphPad Prism 5.0f software (GraphPad Software Inc., La Jolla, CA, USA). *P* values of <0.05 were considered significant.

#### Acknowledgements

We thank D. Andrews, E. White and R. Youle for cell lines, T. Kaufmann for the Bok antibody and Antje Richter, Anja Richter and M. Grimm for expert technical assistance.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

S.E.-S. designed and performed experiments, analyzed and interpreted data and participated in writing the manuscript. S.M., K.B., D.S., J.J., and M.M. performed experiments. P.T.D. and B.F.G. participated in conceptual design and wrote the manuscript. F.E. and K.S.-O. designed experiments, analyzed and interpreted data, and wrote the manuscript.

#### Funding

This work was supported by grants from the Deutsche Forschungsgemeinschaft [grant numbers SFB685 to K.S.-O., GRK1302 to K.S.-O.]; and the Deutsche Krebshilfe [grant number 109894 to F.E.].

#### Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.181727/-/DC1

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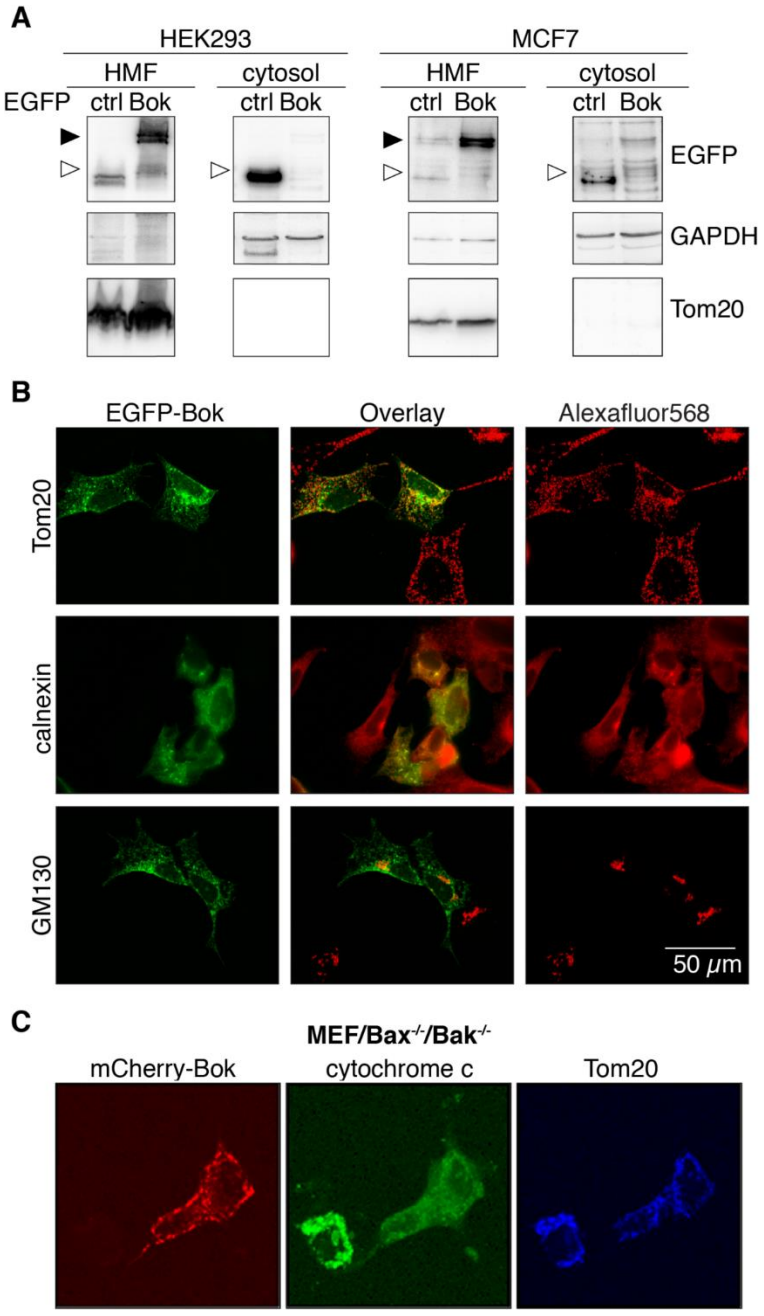


**Fig. S1: Bok localizes to mitochondria and the ER.** (A) HEK293 and MCF7 cells were transfected with expression constructs for EGFP or EGFP-Bok, before cell lysates were separated by centrifugation (10,000*g*) in a cytosolic and a mitochondria-enriched heavy membrane fraction (HMF). Western blot reveals predominant localization of EGFP in GAPDH-containing cytosolic fractions, whereas EGFP-Bok is enriched in Tom20-containing mitochondrial fractions. (B) Immunofluorescence microscopy shows that EGFP-Bok partially localizes at mitochondria (Tom20) and ER (calnexin) and only marginally at the Golgi apparatus (GM130). (C) Expression of mCherry-Bok mediates release of cytochrome c from mitochondria (Tom20) in Bax/Bak-deficient murine embryonic fibroblasts (MEF) as shown by immunofluorescence microscopy.

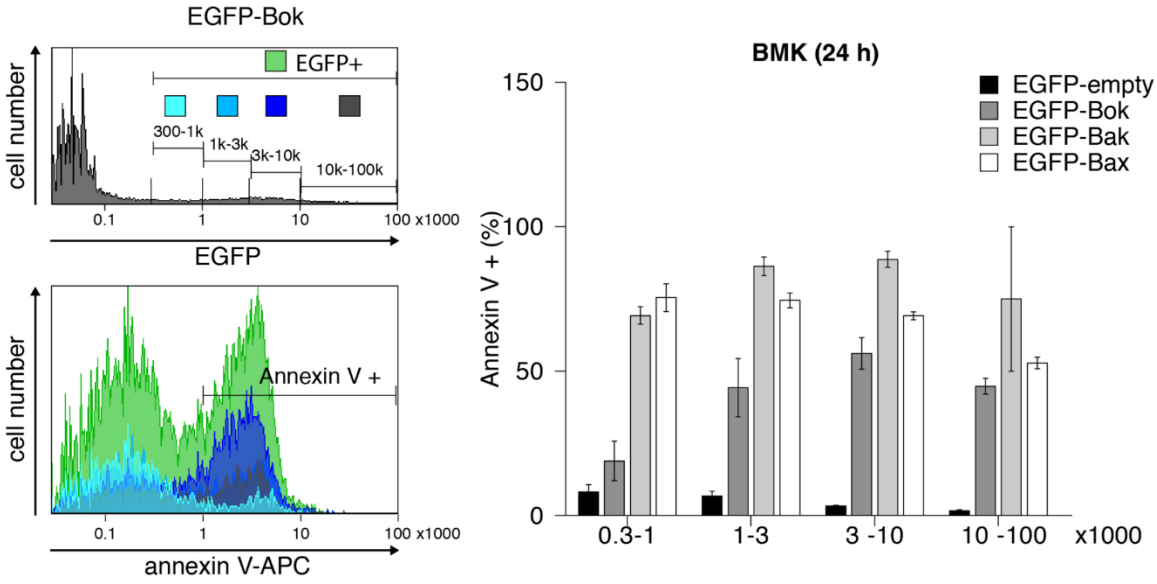


**Fig. S2: Bok induces cells death less potently than Bax and Bak in BMK cells.** BMK cells were transfected with expression vectors for EGFP or EGFP fusion proteins of Bax, Bak and Bok, and apoptosis was measured by flow cytometric annexin V staining. As indicated by the gating for the EGFP signal, annexin V staining was assessed in four cell populations with different levels of EGFP expression (left panel). Results (right panel) show that at different ranges of similar EGFP signal intensity the number of annexin V-positive cells is lower in EGFP-Bok expressing cells as compared to EGFP-Bak and -Bax expressing cells.

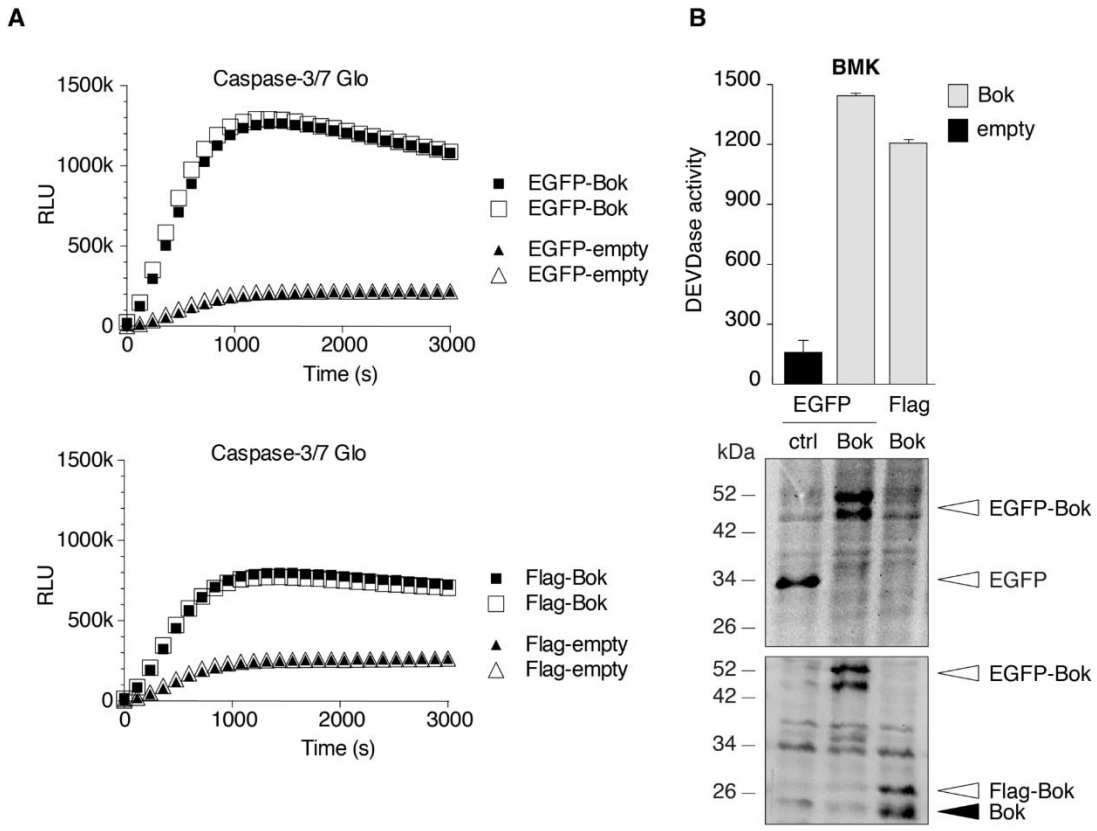


**Fig. S3: Expression of EGFP-Bok and FLAG-Bok mediates enhanced DEVDase activity in Bax/Bak-deficient BMK cells.** BMK cells were transfected with vectors for the expression of EGFP-Bok, FLAG-Bok or the respective vector controls. (A) As measured by the kinetics of cleavage of a luminometric DEVD substrate in the Caspase 3/7 Glo assay, extracts from EGFP-Bok and Flag-Bok expressing cells show enhanced cleavage of a luminometric DEVD substrate as compared to controls. (B) DEVDase activity normalized for protein content in extracts from cells expressing EGFP-Bok or FLAG-Bok (lower panel). RLU: relative light units.

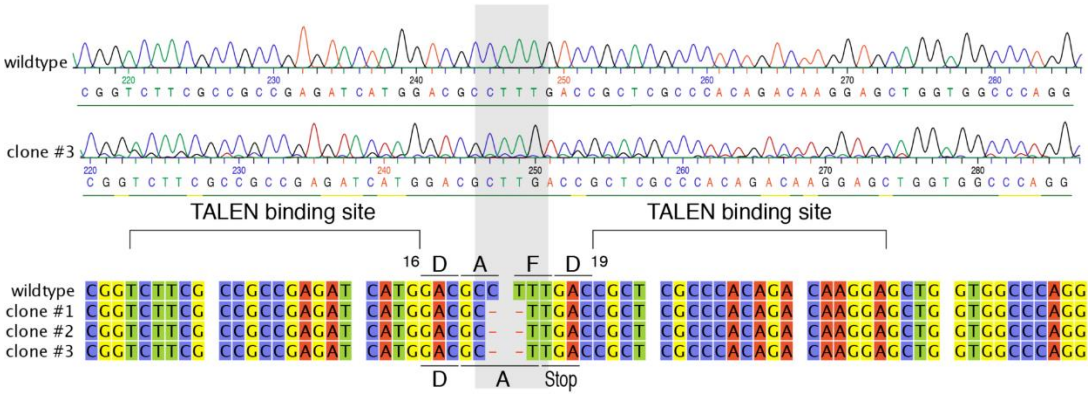


**Fig. S4: Verification of the TALEN-mediated Bok knockout by DNA sequence analysis.** Sequencing of genomic DNA from OVCAR-8 wildtype and the three OVCAR-8/Bok$^{-/-}$ cell clones shows a premature stop codon at the TALEN target site.