Bik reduces hyperplastic cells by increasing Bak and activating DAPk1 to juxtapose ER and mitochondria

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Bik reduces hyperplastic epithelial cells by releasing calcium from endoplasmic reticulum stores and causing apoptosis, but the detailed mechanisms are not known. Here we report that Bik dissociates the Bak/Bcl-2 complex to enrich for ER-associated Bak and interacts with the kinase domain of DAPk1 to form Bik–DAPk1–ERK1/2–Bak complex. Bik also disrupts the Bcl2–IP3R interaction to cause ER Ca2+ release. The ER-associated Bak interacts with the kinase and calmodulin domains of DAPk1 to increase the contact sites of ER and mitochondria, and facilitate ER Ca2+ uptake by mitochondria. Although the Bik BH3 helix was sufficient to enrich for ER-Bak and elicit ER Ca2+ release, Bik-induced mitochondrial Ca2+ uptake is blocked with reduced Bak levels. Further, the Bik-derived peptide reduces allergen- and cigarette smoke-induced mucous cell hyperplasia in mice and in differentiated primary human airway epithelial cultures. Therefore, Bik peptides may have therapeutic potential in airway diseases associated with chronic mucous hypersecretion.

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FN-γ by activating STAT1 increases the susceptibility of cancer cells to apoptosis and plays an important role in the removal of hyperplastic epithelial cells to control chronic mucous secretions in bronchitic asthma or chronic bronchitis. IFN-γ sensitizes airway epithelial cells (AECs) to cell death by increasing expression of the Bcl-2 interacting killer (Bik) and blocking nuclear translocation of ERK1/2. Bik, being anchored in the endoplasmic reticulum (ER) initiates a Bak-dependent release of ER Ca2+ stores, resulting in DRP1-regulated mitochondrial fission and release of cytochrome c to initiate apoptosis. However, the physiological stimuli that enrich Bik at the ER mitochondria to elicit transfer ER Ca2+ to mitochondria. We show that Bik and other Bcl-2-related proteins also play a major role in regulating ER Ca2+ levels with diverse cellular functions such as cell proliferation and survival. While Ca2+ oscillations support cell survival in part by positively regulating mitochondrial metabolism, prolonged high-amplitude Ca2+ release into mitochondria via the inositol 1,4,5-trisphosphate receptors (IP3Rs) causes Ca2+ overload and apoptosis. The ER and mitochondria provide compartmentalized microenvironments, but these compartments communicate and exchange metabolites that ultimately determine the function of the cell. Proteins localized to the ER or mitochondria can determine sites of close contact also referred to as mitochondria-associated ER membrane. For example, mitofusin 2 (Mfn2) binds to ER derivatives of Mfn1 at specialized ER-mitochondrion contact sites and the mitochondrial outer membrane (MOM) fission protein, Fis1, makes contact with ER-localized BAP-3, suggesting that there is a bi-directional communication between the two organelles. The macromolecular complexes that facilitate ER/mitochondrion contact to determine between adaptive responses vs. proapoptotic signals have yet to be identified.

Other Bcl-2-related proteins also play a major role in regulating ER Ca2+ levels because enforced expression of Bik and Bax provokes ER Ca2+ release and Bik/Bax can localize to the ER and regulate ER calcium levels in the reticular lumen. In contrast, Bcl-2 overexpression prevents the reduction of ER Ca2+ concentrations by its BH4 domain binding the regulatory and coupling domain of the IP3R and inhibiting IP3-dependent channel opening.

In the present study, we identified the proteins that Bik assembles to initiate ER Ca2+ release and to facilitate efficient transfer to mitochondria. Bik increased Bak levels to enrich ER-associated Bik and facilitate the formation of the Bik-DAPk1-ERK1/2-Bak (BDEB) complex. We show that Bik is required for anchoring DAPk1 to the ER and increase the contact sites between ER and mitochondria to elicit transfer ER Ca2+ to mitochondria. Bik also disrupts Bcl-2 and IP3R interaction and causes ER-Ca2+ release. A double hydrocarbon-stapled (DHS) peptide modeled after the Bik BH3 helix does not include the ER-anchoring domain caused efficient Bak activation and cell death. Bik BH3 peptide restored cell death and reduced allergen- or cigarette smoke (CS)-induced epithelial and mucous cell hyperplasia in primary human AECs in culture and in vivo similar to the whole Bik protein when transgenically expressed in an inducible manner in airway epithelia of adult mice. Thus, Bik BH3 helix may be useful as a therapeutic agent to reduce mucous hypersecretion.

**Results**

**Bik enriches Bak at the ER to elicit ER Ca2+ release.** Bik is anchored on the ER membrane, promotes ER calcium release to cause mitochondrial apoptosis, and while Bik is constitutively anchored on the MOM, it can also be localized to the ER. As a phospho-protein, Bik gets activated by a conformational change in the N-terminus following dephosphorylation at tyrosine 108 (Y180). Bik increases Bak protein levels within the ER fraction, which was visualized by multimers in ER fractions of both Bik-WT- and Bik-Y108A-expressing cells, but more pronounced in Bik-Y108A-expressing cells. Confocal microscopy showed that in HAECS treated with IFN-γ (Supplementary Fig. 1k) or Ad-Bik (Supplementary Fig. 1l), the number of cells with activated Bak co-localized to the ER increased by 3–5-fold. Suppression of Bik using shRNA (Supplementary Fig. 1m) reduced the number of IFN-γ-treated cells with Bak co-localized to the ER (Fig. 1h). Together, these findings suggest that Bik mediates IFN-γ-induced Bak localization to the ER. Further confirmation for the translocation of Bak to the ER was derived from IFN-γ-treated HCT116 cells expressing fluorescently labeled F3YpetR-F3hBak. HCT116 cells were protected from IFN-γ (Supplementary Fig. 1n) or Ad-Bik (Supplementary Fig. 1o)-induced cell death. In addition, analysis of co-localization using the Manders’ coefficient showed that Bik enriched Bak cells expressing IFN-γ were resistant to IFN-γ treated HCT116 cells expressing F3YpetR-F3hBak to the ER (Fig. 1i).

We further investigated the hypothesis that Bik accumulation on the ER may affect ER calcium release (Ca2+), IFN-γ treatment or Ad-Bik expression caused release of ER Ca2+ just 4–6 h after treatment (Fig. 1j). To determine whether Bik disrupts Bcl-2–IP3R interaction that is known to regulate the flux of ER Ca2+, we infected HAECS with Ad-Bik or Ad-Bik-L61G and immunoprecipitated the protein lysates with anti-IP3R or anti-Bcl2 antibodies. Expression of Bik-WT but not Bik-L61G disrupted the Bcl-2–IP3R interaction (Fig. 1k). Because Bak-deficient cells were resistant to IFN-γ- or Ad-Bik-induced cell death, we tested
whether deficiency of Bak also impairs Ad-Bik-induced ER [Ca²⁺]ᵢ efflux and [Ca²⁺]m accumulation. The number of cells with ER [Ca²⁺]ᵢ efflux and mitochondrial calcium [Ca²⁺]m (Supplementary Fig. 1p) accumulation was 6–7-fold higher in bak−/− compared with bak−/− MAECs when Bik was expressed, suggesting that ER-Bak is required for ER [Ca²⁺]ᵢ efflux.

Bak mediates resolution of hyperplastic epithelial cells. MAECs from bak−/− mice were significantly more resistant to 50 ng/ml murine IFN-γ over 24, 48, and 72 h of treatment (Fig. 2a) or Bik expression (Fig. 2b). Interestingly, even bak−/− MAECs were protected from Ad-Bik-induced cell death (Fig. 2b), suggesting that the dosage of Bak protein is important for IFN-γ and Bik-

Fig. 1 Bik activates and translocates Bak to the ER. HAECS were treated with 50 ng/ml human recombinant IFN-γ or medium alone as controls (a) or infected with 100 MOI Ad-Bik or Ad-BikL61G (b), and 24 h later protein lysates were analyzed for changes in the expression of Bak by western blotting. The fold change in Bak level was analyzed by densitometry. (c) MAECs from bik⁺/⁺ or bik−/− mice were treated with IFN-γ for 48 h and the levels of Bik and Bak protein levels analyzed by western blotting. (d) HAECS were transiently infected with retroviral vectors for shCtr or shBcl-2 and treated with 50 ng/ml IFN-γ or medium alone as control for 48 h or treated with 100 mM Mg132 for 24 h and protein lysates were analyzed for the expression level of Bak, Bcl-2, Bik and actin proteins by western blotting. (e) HAECS were infected with 100 MOI Ad-Bik or Ad-BikL61G and immunoprecipitates of protein lysates with anti-Bcl-2 antibody were probed for Bik and Bcl-2 levels by western blotting. (f) HAECS were infected with Ad-Bik or Ad-BikL61G and 24 h later analyzed for increases in active Bak using the Ab-1 antibody that is specific for activated Bak using FACS. (g) HCT116 cells retrovirally transduced with empty vector, BakWT or BakY108A were infected with 100 MOI Ad-Bik. The ER fractions were analyzed with anti-Bak antibodies. Calnexin was used as a marker for equal loading of the proteins. (h) Representative photomicrographs of HAECS stably expressing shCtr or shBik treated with 50 ng/ml IFN-γ for 48 h fixed with paraformaldehyde and immunostained for activated Bak and calnexin. Percent of cells with Bak localized to the ER was quantified by counting at least 200 cells for each experiment. Scale bar, 5 μm.

i Representative micrographs of HCT116bak−/− cells transfected with F3YpetR-F3hBak (green), followed by treatment with 50 ng/ml IFN-γ or media alone. Cells were stained with calnexin for ER (red) and confocal images of 3D reconstructions of ER and Bak mediates resolution of hyperplastic epithelial cells. MAECs from bak−/− mice were significantly more resistant to 50 ng/ml murine IFN-γ over 24, 48, and 72 h of treatment (Fig. 2a) or Bik expression (Fig. 2b). Interestingly, even bak−/− MAECs were protected from Ad-Bik-induced cell death (Fig. 2b), suggesting that the dosage of Bak protein is important for IFN-γ and Bik-
induced cell death. Further, expression of BakWT or BakY108A using a retroviral vector restored Ad-Bik-induced cell death of bak−/− (Fig. 2c) and bak+/− (Fig. 2d) MAECs, demonstrating that Bak is crucial for IFN-γ- and Bik-induced cell death pathway. IFN-γ (Supplementary Fig. 2a) and Ad-Bik (Supplementary Fig. 2b) caused significant increases in Annexin V positivity compared to the respective controls, confirming that this pathway induces apoptosis.

IFN-γ through Bik mediates the resolution of hyperplastic cells, when mice are exposed to allergen for a prolonged period. Therefore, we investigated the physiological relevance of Bak in vivo by testing whether this resolution process would be abrogated in bak−/− mice. Allergen-induced epithelial cell hyperplasia (ECH) remained significantly higher in bak+/− compared with bak+/+ mice after 15 days of allergen exposure (Fig. 2e, f). Furthermore, the number of mucous cells per millimeter of basal lamina (BL) was sustained in the lungs of bak+/− mice, while significantly reduced in bak+/+ mice (Fig. 2e, g), demonstrating that Bak is an obligatory protein for Bik-induced cell death in vivo.

DAPk1 assembles Bak and ERK1/2 to mediate Bik-induced death. Bik interacts with and blocks nuclear localization of ERK1/2 to promote cell death30 and ERK1/2 promotes the apoptotic activity of DAPk1 by interacting with the death domain31. Therefore, we tested the hypothesis that Bik forms a complex with DAPk1 and ERK1/2. We expressed HA-tagged Bik or Bik161G in HAECS and detected both Bik and DAPk1 in the immunoprecipitates with anti-p-ERK1/2 antibodies in cells expressing Bik but not Bik161G (Fig. 3a). The site of interaction of Bik with DAPk1 was further examined by immunoprecipitation with anti-Flag antibodies of protein lysates from 293T cells that were transfected with Flag-DAPk1 and infected with adenoviral vectors expressing

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**Fig. 2** Bak mediates IFN-γ- or Bik-induced cell death and resolution of ECH and MCM during prolonged exposure to allergen. **a** MAECs isolated from bak−/− and bak+/− mice treated with 50ng/ml murine recombinant IFN-γ or left untreated as controls and viable cells quantified 24, 48, and 72 h later with Trypan blue. Protein lysates were analyzed for the expression level of Bak by western blot. **b** MAECs from bak+/−, bak−/−, and bak+/− mice were infected with 100 MOI Ad-Bik or Ad-BikL61G, and cell viability was quantified 24 h later. **c** MAECs from bak−/− mice were infected with empty vector or retroviral expression vector for BakWT or mutant constitutively activation competent form of Bak (BakY108A). Cells were collected and quantified by trypan blue exclusion assay and protein lysates were analyzed by western blot. **d** MAECs from bak+/− mice were infected with empty vector and retroviral expression vectors for mutant constitutively activation competent form (BakY108A) of Bak. And 48 h later, cells were infected with 100 MOI Ad-Bik for 24 h. Cells were collected and quantified by trypan blue exclusion assay and protein lysates were analyzed by western blotting. **e** Representative micrographs of bak+/+ and bak−/− mice that were immunized with ovalbumin/alum on d 1 and 7, and were exposed to ovalbumin aerosols for 15 d. After killing, the lungs of each mouse were harvested, and the epithelial cells/mm of basal lamina in bak+/+ mice but not in bak−/− mice at 15 d of exposure. Differences between two groups were assessed for significance by Student’s t test. ANOVA was used to perform pair-wise comparison of the data from more than two groups followed by Fisher least significant difference test. Error bars indicate ± SEM; (n = 8 mice per group). * = P < 0.05; ** = P < 0.01
DAPK1K42A and 48 h later infected with 100 MOI HA-Ad-Bik. Protein lysates were immunoprecipitated using α immunoprecipitated using expressing empty vector or Bcl-2b5 together with plasmids expressing Flag-DAPk1 and 48 h later infected with 100 MOI HA-Ad-Bik. Protein lysates were assessed for signi... was analyzed by trypan blue exclusion. 293T cells were transfected with Flag-DAPk1 or Flag-DAPK1K42A and 48 h later infected with 100 MOI HA-Ad-Bik or HA-Ad-BikL61G. After 24 h, protein lysates were immunoprecipitated with α-Flag antibodies and the FT and IP were probed for activated Bak, HA, and ERK1/2 by western blotting. 293T cells were transfected with various GFP-tagged DAPk1 deletions constructs, infected with HA-Ad-Bik, and subjected to immunoprecipitation using anti-HA or anti-GFP antibodies. The flow through (FT) and the IPs were probed for GFP, HA, activated Bak, or anti-active Bak (Abi) antibodies. The FT and IPs were probed for activated Bak, HA, DAPK1, and p-ERK1/2 by western blotting. Mutation of Lys42 in the kinase domain of DAPk1 diminishes Bik or shDAPk1 were infected with HA-Ad-Bik and immunoprecipitated using anti-pERK1/2 antibodies. The FT and IPs were probed for activated Bak, HA-Bik, and p-ERK1/2 by western blotting. Mutation of Lys42 in the kinase domain of DAPk1 diminishes Bik or shDAPk1 were infected with HA-Ad-Bik and immunoprecipitated using anti-pERK1/2 antibodies. The FT and IPs were probed for activated Bak, HA-Bik, and p-ERK1/2 by western blotting.

**Fig. 3** DAPk1 facilitates Bik-induced cell death by forming a complex with Bik, ERK1/2 and Bak. 

a) Protein lysates from HAECs 24 h after infection with HA-Ad-Bik or HA-Ad-BikL61G were immunoprecipitated with anti-pERK1/2 antibodies. The flow through (FT) and the immunoprecipitates (IPs) were probed with antibodies to HA, DAPK1, p-ERK1/2, and ERK1/2 by western blotting. 

b) 293T cells were transfected with Flag-DAPk1 and 48 h later infected with 100 MOI HA-Ad-Bik or HA-Ad-BikL61G. After 24 h, protein lysates were immunoprecipitated with α-Flag antibodies and the FT and IP were probed for activated Bak, HA, and ERK1/2 by western blotting. 

c) 293T cells transfected with various GFP-tagged DAPk1 deletions constructs, infected with HA-Ad-Bik, and subjected to immunoprecipitation using anti-HA or anti-GFP antibodies. The flow through (FT) and the IPs were probed for GFP, HA, activated Bak.

d) Mutation of Lys42 in the kinase domain of DAPk1 diminishes Bik-Bak-DAPk1-ERK1/2 interaction. 293T cells were transfected with Flag-DAPk1 or Flag-DAPK1K42A and 48 h later infected with 100 MOI HA-Ad-Bik. Protein lysates were immunoprecipitated using α-Flag antibodies and the FT and IP were probed for Flag, HA, Bak, and ERK1/2 by western blotting. 

e) Cell viability of 293T cells transfected with Flag-DAPk1 or Flag-DAPK1K42A was analyzed by trypan blue assay. 

f) Knockdown of DAPk1 suppresses the interaction between Bak, Bik, and ERK1/2. Protein lysates from HAECs stably expressing shCtr or shDAPk1 were infected with HA-Ad-Bik and immunoprecipitated using anti-pERK1/2 or anti-active Bak (Abi) antibodies. The FT and IPs were probed for activated Bak, HA, DAPK1, and p-ERK1/2 by western blotting. 

h) HAECs stably expressing shCtr or shDAPk1 were infected with 100 MOI of Ad-Bik in the presence of 20 µM pan-caspase inhibitor Q-VD-Oph for 18 h, fixed and stained for cytochrome c, and the percentage of cells with cytochrome c release was analyzed by fluorescent microscopy. 

i) Cell lysates from HAECs stably expressing shCtr or shDAPk1 and treated with 50 ng/ml IFN-γ or 100 MOI Ad-Bik were analyzed for cleaved caspase 3 by immunoblotting. 

j) HAECs stably expressing shCtr or shDAPk1 were analyzed for knockdown of DAPk1 by western blots. Cell viability was determined by trypan blue exclusion assay 24 h after infection with 100 MOI Ad-Bik. 

k) 293T cells were transfected with plasmds expressing empty vector (EV) or ER-targeted Bcl-2 (Bcl-2s5). Forty-eight hours later, cells were infected with 100 MOI Ad-Bik and protein lysates were analyzed for the expression of Bcl-2, Bak, and Bik. Cell viability was determined by trypan blue exclusion. 

l) 293T cells were transfected with plasmids expressing empty vector or Bcl-2s5 together with plasmids expressing Flag-DAPk1 and 48 h later infected with 100 MOI HA-Ad-Bik. Protein lysates were immunoprecipitated using α-Flag antibodies and the FT and IP were probed for Flag-DAPk1, HA-Bik, Bak and ERK1/2 by western blotting. Differences between two groups were assessed for significance by Student’s t test. ANOVA was used to perform pair-wise comparison of the data from more than two groups followed by Fisher least significant difference test. Error bars indicate ± SEM, n = 5; * P < 0.05, ** P < 0.01.
DAPk1 tethers ER to mitochondria to facilitate Ca$^{2+}$ release. The efflux of ER calcium ([Ca$^{2+}$]$_i$) and elevation of mitochondrial Ca$^{2+}$ ([Ca$^{2+}$]$_m$) were analyzed using a fluorescent-based ER and mitochondrial calcium indicators, respectively. Bik expression caused the release of ER Ca$^{2+}$ (Fig. 4a, Supplementary Fig. 4a), and increase of mitochondrial [Ca$^{2+}$]$_m$ (Fig. 4b), which were diminished significantly when DAPk1 levels were suppressed in shDAPk1 cells. Further, suppression of Bik using shRNA inhibited IFN-γ (Supplementary Fig. 4b) or Ad-Bik (Supplementary Fig. 4c), d-induced ER-Ca$^{2+}$ release and mitochondrial Ca$^{2+}$ accumulation (Supplementary Fig. 4e).

Ca$^{2+}$ transmission from the ER to mitochondria occurs at contact sites between the two organelles. However, little is known about how physiological stimuli control the distance between ER and mitochondria. Electron microscopy analyses confirmed that treatment of HAECs with IFN-γ or Ad-Bik significantly increased ER-mitochondrial contact. Compared to untreated controls, treatment of cells with IFN-γ for 16 h shortened the mean ER-mitochondria distance from 35.42 ± 1.37 to 18.62 ± 0.85 and increased the percentage of mitochondria that are in close contact with the ER by >3-fold (Fig. 4c, Table 1). Similar results were observed when cells were infected with Ad-Bik compared with cells infected with Ad-Bik$_{L61G}$ (Fig. 4d, Table 1). Because DAPk1 is a large protein and was essential for Bik-induced Bak co-localization to the ER and increased ER [Ca$^{2+}$]$_i$, efflux, we hypothesized that DAPk1 may be involved in ER–mitochondria contact to facilitate the transfer of death signals. HAECs stably expressing shDAPk1 or shCtr treated with IFN-γ or expressing HA-Bik or HA-Bik$_{L61G}$ were immunostained for mitochondria and ER using anti–Cox IV and anti-calnexin antibodies, respectively. Images separated by 0.2 μm to span a total of 10 μm were acquired within 1.1 s to minimize reconstruction artifacts caused by the movement of mitochondria and/or ER. Rotation of the reconstruction on the y-axis corroborated that areas of overlap represent juxtaposition of organelles. Areas of ER-mitochondria contact were increased by Bik expression (Fig. 4e) or IFN-γ treatment (Supplementary Fig. 4f) compared to the respective controls. However, the IFN-γ- or Ad-Bik-induced remodeling of ER-mitochondria contact was disturbed in shDAPk1 cells but not in shCtr cells. Also, deficiency of Bak diminished Ad-Bik-induced ER-mitochondrial contact (Fig. 4f), suggesting that ER-Bak anchors DAPk1 to the ER to ultimately tether ER and mitochondria.

Bik BH3 domain alone is sufficient to enrich for ER-Bak. To determine whether Bik expression in the airways reduces allergen- or CS-induced ECH and mucous cell metaplasia (MCM), we generated transgenic mice that conditionally induce Bik expression in the respiratory epithelium utilizing the reverse tetracycline transactivator (rtTA) expressed under the control of CCSP promoter. When doxycycline was administered intranasally, the transgene was activated in the CCSP-rtTA/TetOBik mice but not in CCSP-rtTA-only littermates (Fig. 5a, b). Mice were immunized with ovalbumin/Alum and exposed to ovalbumin (OVA) aerosols for 5 days and on the following 2 days instilled with doxycycline. ECH (Fig. 5c) and MCM (Fig. 5d) were significantly reduced in CCSP-rtTA/TetOBik compared to CCSP-rtTA controls. Similarly, when mice were exposed to CS for 3 weeks and instilled with Dox for two consecutive days, ECH (Fig. 5e) and MCM (Fig. 5f) were reduced significantly in CCSP-rtTA/TetOBik compared to CCSP-rtTA controls. Increased transgenic expression of Bik in the airways also caused significant increases in TUNEL positivity compared to controls (Fig. 5g), suggesting that targeted expression of Bik is sufficient to reduce allergen- or CS-induced mucous cells in models of asthma and chronic bronchitis by inducing cell death of hyperplastic cells.

Hydrocarbon-stapled peptide, referred to as stabilized alpha-helix of Bcl-2 domains (SAHBs) were previously described to stabilize the helical conformation and to improve entry into
cells. To explore the therapeutic potentials of Bik BH3 peptides, we generated a DHS BikWT BH3 helix (aa 57–71) and a control peptide with the conserved leucine within the BH3 domain mutated to glycine (BikL61G). Four peptides with either a single staple (substitution of Ser63 and Glu67 to amino acids to (S)-2-(2'-penetyl) Ala residues) or double staple (substitution of Asp55, Leu59, Glu67, and Ser71) and the respective controls with mutation of Leu61 to Gly were synthesized. In addition, one peptide with scrambled amino acid sequence was prepared as further control (Supplementary Fig. 5a). The peptides were labeled with the fluorescent tag carboxyfluorescein (FAM) to monitor uptake into cells. We screened these peptides for cytotoxicity in four airway epithelial cell lines (AALEB, N3, H1975, and HBEC2 cells) (Supplementary Fig. 5a). We found that the (DHS) BikWT peptides were most effective compared to the scrambled and BikL61G peptide controls. Treatment of cells with 5μM of BikWT peptides was sufficient to cause similar cytotoxicity to 100 MOI Ad-Bik in AALEB cells (Supplementary Fig. 5b) and in primary human AECs from five donors (Supplementary Fig. 5c). The human AECs showed similar uptake of peptides when treated with 5μM DHS BikWT or BikL61G BH3 peptides (Fig. 6a, Supplementary Fig. 5d). Similar to

Fig. 4 Bik facilitates ER Ca2+ release and enhances through DAPk1 the proximity of ER and mitochondria. HAEcs stably expressing shCtr or shDAPk1 were infected with Ad-Bik and at the indicated time points fixed and stained with Ca2+-flux indicator, Fluo-4 (green) (a) or with [Ca2+]i, indicator, Rhod-2 (red) (b) and counterstained for nuclei with DAPI (blue). The percentage of cells positive for Fluo-4 or Rhod-2 were quantified. Graphs show mean ± SEM of quantified cells. c, d Transmission electron microscopy of HAEcs treated with medium alone or 50 ng/ml IFN-γ (c) or infected with 100 MOI Ad-Bik or Ad-BikL61G (d) for 16 h. The bar graphs show the mean ER-OMM distances and the percentage of mitochondria with tight contact with the ER. White arrows show contact sites of ER and mitochondria. e Representative micrographs of HAEcs stably expressing shCtr and shDAPk1, and infected with 100 MOI Ad-Bik or Ad-BikL61G. Cells were stained with anti-CoxIV for mitochondria (green), with anti-calnexin for ER (red), and with DAPI for nuclei staining (blue). Confoecal images of 3D reconstructions of ER and mitochondria were acquired. Scale bars, 5 μm. ER-mitochondria contacts were quantified by Manders’ coefficient, means and SEM (n = 5, at least 40 cells per experiment) of the morphometric data. Differences between two groups were assessed for significance by Student’s t test. ANOVA was used to perform pair-wise comparison of the data from more than two groups followed by Fisher least significant difference test. Error bars indicate ± SEM, (n = 5, with > 200 cells analyzed per condition); * P < 0.05, ** P < 0.01
the endogenous Bik protein FAM- labeled DHS Bik\(^{WT}\) compared to DHS Bik\(^{L61G}\) peptide increased Bak protein levels (Fig. 6b), activated Bak (Supplementary Fig. 5e), diminished interaction of Bak and Bcl-2 (Fig. 6c), and impaired cell viability (Fig. 6d). While wild-type and \(bik^{-/-}\) MAECs were sensitive to FAM-Bik\(^{WT}\) treatment, \(bak^{-/-}\) MAECs were protected from FAM-Bik\(^{WT}\) induced cell death (Fig. 6e).

The therapeutic role of FAM-Bik\(^{WT}\) peptide in reducing allergen- or CS-induced hyperplastic epithelial and mucous cells was tested in mouse models of disease. Sensitized mice that were exposed to OVA aerosols for 5 days were intranasally instilled with 5\(\mu\)M FAM-Bik\(^{WT}\) or FAM-Bik\(^{L61G}\) peptides on two consecutive days. Allergen-induced ECH and MCM (Fig. 6f) were reduced significantly in the lung tissues of mice instilled with FAM-Bik\(^{WT}\) compared to FAM-Bik\(^{L61G}\). In addition, co-localization of ER and Bak were detected in 7% of cells when mice were treated with FAM-Bik\(^{WT}\) and in 2.5% in mice treated with FAM-Bik\(^{L61G}\) peptides (Fig. 6g). TUNEL positivity was significantly increased in the airways of mice instilled with FAM-Bik\(^{WT}\) compared to FAM-Bik\(^{L61G}\) (Fig. 6h). Importantly, TUNEL positivity was restricted to the airways. FAM-Bik\(^{WT}\) showed similar results in mice exposed to CS for 3 weeks (Supplementary Fig. 5f, g). To test the efficacy of these peptides in human models, we differentiated primary HAECs obtained from five donors in air-liquid-interface cultures over 21 days and treated them with IFN-\(\gamma\)- and Bik-induced cell death pathway was demonstrated by restoring Bak\(^{WT}\) or Bak\(^{Y108A}\) in HCT116\(\text{bam}^{-/-}\)-cells. Bak is a phospho-protein and its activation involves a series of conformational changes, including exposure of the occluded N-terminal epitopes\(^{27, 43}\) followed by the formation of homo-oligomeric complexes that permeabilize the MOM\(^{43, 44}\). Such conformational changes and oligomerization require dephosphorylation of Bak at Tyr108 (Y108) and Ser117\(^{46}\). Further, when Bak undergoes the N-terminal conformational change, the BH3 domain is exposed\(^{35}\) that may promote the oligomerization of reticular Bak\(^{13}\). Interestingly, Bik expression was required to elicit Bak oligomerization even when Bak\(^{Y108A}\) was expressed, suggesting that inactivation of Bcl-2 may be a crucial trigger for Bak to form oligomers. The oligomerized Bak anchors DAPK1 to the ER and provides the means for the released ER Ca\(^{2+}\) to enter mitochondria that are in close proximity.

The Bik BH3 domain is sufficient for efficient heterodimerization with Bcl-2 but this heterodimerization is not sufficient to cause death in MCF-7 cells\(^{46}\). Our studies show that the Bik BH3 domain dissociates Bak from the Bcl-2/Bak complex, activates and increases the ER-localized Bak levels by > 5-fold. That Bik dissociates Bak from Bcl-2 suggests that the BH3 domain of Bik has a higher affinity to Bcl-2 than to Bak. More interestingly, the dissociation of Bak from Bcl-2 led to stabilization and increase in Bak levels, indicating that the heterocomplex, Bak/Bcl-2, is degraded by the proteasomal pathway at a higher rate than the Bak monomer. Further, while blocking proteasomal degradation with MG-132 increases Bak levels, suppression of Bcl-2 expression further enhances stability of Bak, suggesting that Bcl-2 may promote Bak degradation by mechanisms that do not involve the proteasome. The underlying mechanisms for this difference in degradation need further investigation.

The Bik BH3 domain also modifies the conformation of DAPK1 to facilitate the binding to the other proteins because mutation of Lys42 that is crucial for Bik interaction with DAPK1 also disrupts the complex formation and cell death. Because the BDEB complex is no longer formed when expression of DAPK1 is suppressed, it is likely that DAPK1 may serve as a scaffold, whereby Bik binds to the kinase domain and activated Bak binds to the kinase and CaM domains of DAPK1. DAPK1 sequesters ERK1/2 in the cytoplasm by interacting with ERK through its death domain to promote the proapoptotic function of DAPK1\(^{31}\). Similarly, Bik mediates IFN-\(\gamma\)-induced cell death in the AECs by interacting with and sequestering ERK1/2 in the cytosol\(^{28}\). However, deletion of the death domain of DAPK1 did not affect the

### Table 1 Measurements of ER-mitochondria interface

|            | ER-mito distance (nm) | Contacts ≤ 15 nm (%) |
|------------|-----------------------|----------------------|
| Untreated  | 35.42 ± 1.32 (n = 132)| 12.26 ± 2.47 (n = 132) |
| IFN-\(\gamma\) | 18.62 ± 0.85 (n = 128)| 37.54 ± 1.1 (n = 163) |
| Ad-Bik\(^{L61G}\) | 37.42 ± 1.2 (n = 163)| 16.3 ± 1.6 (n = 163) |
| Ad-Bik    | 19.93 ± 0.69 (n = 161)| 35.77 ± 1.34 (n = 161) |
interaction of DAPk1 with Bik or Bak, suggesting that interactions of DAPk1 with Bik and Bak occurs independent of ERK–DAPk1 interaction. At present, the only evidence for Bik and DAPk1 interaction is based on co-immunoprecipitation of over-expressed Bik and future studies need to validate this finding using other approaches such as fluorescent polarization assays. These studies would confirm the possibility that DAPk1 may have a Bcl-2-like binding groove in the kinase domain.

Disturbances in cellular \(\text{Ca}^{2+}\) homeostasis, such as cytosolic \(\text{Ca}^{2+}\) overload, ER \(\text{Ca}^{2+}\) depletion, and mitochondrial \(\text{Ca}^{2+}\) overload can lead to increased mitochondrial permeability and cytochrome c release\(^{15}\). On the basis of previous studies\(^{11, 12}\), we have to assume that ER-\(\text{Ca}^{2+}\) precedes the accumulation of mitochondrial \(\text{Ca}^{2+}\). Our studies also support the idea that ER-Bak is the main driver of ER-\(\text{Ca}^{2+}\) release that results in mitochondrial \(\text{Ca}^{2+}\) accumulation. We found that Bik not only dissociating Bcl-2/Bak complex and activating Bak. The fact that the ER anchoring domain of Bik was not required for Bak activation and cell death is further supporting evidence that ER Bik but not Bik is responsible for anchoring DAPk1 to the ER. The resulting frequency of contact sites between ER and mitochondria determine extent of MOMP disruption and cytochrome c release that ultimately activates downstream caspases.

Our studies are the first to identify DAPk1 as an ER mitochondrial tethering protein to facilitate IFN-\(\gamma\) and Bik-induced ER \(\text{Ca}^{2+}\) release and caspase activation. Future studies will address the involvement of proteins known to attach DAPk1 to mitochondria. As a large protein, DAPk1 interacts with numerous proteins, including ZIPK\(^{52}\); HSP90, CHIP, and DIP\(^{53}\), \(^{54}\). DIP localizes to the mitochondria to cause caspase-dependent cell death\(^{55}\), therefore, it is possible that any of these mitochondrial proteins binds to DAPk1 to help tether ER to mitochondria.

The Bik BH3 SAHB peptide was sufficient to trigger cell death in a pathway similar to the whole Bik protein that was expressed in AECs of adult mice using transgenic approach. Intranasal delivery of Bik\(^{\text{WT}}\), but not Bik\(^{L61G}\) peptides, caused cell death restricted to the airways and resolved allergen- or CS-induced MCM and ECH. Bik peptide increases the level of Bak protein by displacing Bak from Bcl-2. Bik peptides also caused bik to localize with the ER and increase TUNEL positivity in the airways of mice in vivo. These findings support the fact that stapled Bik peptides cause cell death in the AECs by the same pathway as the Bik protein and that small peptides derived from Bik BH3 domain may be used to restore Bik function and control allergen- or CS-induced ECH and MCM.

Several studies have shown that secretory cells are the cells that proliferate following injury to the epithelium\(^{36-38}\). The cell death
caused by Bik BH3 peptides in vivo may be restricted to hyperplastic mucus cells of the airways as most of the epithelial cells remain unharmed even when Bik is expressed. This removal process may also occur rapidly, supporting the observation that the percentage of TUNEL-positive cells and cells with ER-Bak co-localization in the ER were significantly reduced in the airways of mice instilled with FAM-BikWT compared to FAM-BikL61G. Paraffin-embedded lung tissues from mice exposed to ovalbumin and instilled with 5 μM FAM-BikWT or FAM-BikL61G for 48 h were stained with anti-calcnexin and anti-Bak antibodies, and the percentage of cells in the airways with Bak co-localized to the ER were compared after confocal microscopy.

Methods

Mice. Male-specific pathogen-free wild-type C57BL/6 and bak−/− mice on C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in isolated cages under specific pathogen-free conditions. After a 14-day quarantine period, mice were acclimated for 8 days and entered into the experimental protocol at 8–10 weeks of age. The bik−/− mice on C57BL/6 backbone were provided by Andreas Strasser (Walter and Eliza Hall Institute, Melbourne, Australia). bik−/− with bik+/− littermates were bred from the respective
heterozygote mice at the Lovelace Respiratory Research Institute under specific pathogen-free conditions and genotyped

Transgenic mice that conditionally express Bik in the respiratory epithelium were generated in our lab utilizing the reverse tetracycline-controlled transactivator (rtTA) expressed under the control of CCSP promoter

Mouse virus stocks were provided by S. Randell (University of North Carolina at Chapel Hill, Chapel Hill, NC)

Wild-type and HCT116 Δfl–/–Δfl–/– double knockout cells were kindly provided by Dr. Richard Youle, National Institute on Aging (National Institutes of Health, Baltimore, MD)

Mice were exposed to 250 mg/m3 CS or intranasally instilled urane and intranasally instilled

For Bik and BikL61G were provided by G. Shore (McGill University, Montreal, Canada)

Because all of our studies showed that the BH-3 domain is the active site, we designed several peptides that comprise the BH-3 domain. Previous studies have demonstrated that modification of these peptides by formation of hydrocarbons–staples, referred to as stabilized alpha-helix of Bcl-2 domains (SABHs), stabilizes helical conformation and increases stability for Bim

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Bik constructs (pcDhA F3CypetR (G4S)4–F3hBak, hBak-wt (1–183) F3Cypr, or F3CypetR F3hBak) were generated by PCR and standard cloning procedures. Briefly, the sequence of wild-type Bik was amplified with specific primers flanked by EcoRI and XhoI restriction sites to allow the cloning into a mammalian expression vector in a frame with triple FLAG-tagged Cypet (G4S)

To further characterize the interaction with DAPk1, we designed several peptides that comprise the BH-3 domain. Previous studies have demonstrated that modification of these peptides by formation of hydrocarbons–staples, referred to as stabilized alpha-helix of Bcl-2 domains (SABHs), stabilizes helical conformation and increases stability for Bim

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Protein cross-linking. Mitochondrial or ER fractions were incubated with the 5 mM m-coupling agent, BMH (Pierce Chemical Co.) for 30 min at room temperature. After incubation, the reaction was stopped by adding 5 mM DTT for 15 min, samples were diluted with SDS sample buffer, heated to 95 °C for 5 min, separated on 12% (wt/vol) SDS-PAGE, transferred to nitrocellulose, and probed with anti-Bak antibodies.

Immunoprecipitation. Immunoprecipitation from protein preparations was performed using the Pierce Crosslink Immunoprecipitation Kit (Pierce Technology, Rockford, IL) according to the instruction of manufacturer. Briefly, cell lysates were incubated with antibody cross-linked Pierce Protein A/G Plus Agarose overnight at 4 °C. The immunoprecipitates were eluted and subjected to Western blot analysis.

Detection of intracellular Ca2+. Cells were washed three times with HBSS medium (Invitrogen, Grand Island, NY) and incubated in 200 μM Fluo-4 AM (Life Technologies Inc, Eugene, OR) or 10 μM Rhod-2 AM (Life Technologies Inc., Eugene, OR) as an indicator of Ca2+ in the cytosol ([Ca2+]i) or mitochondria ([Ca2+]m), respectively, at 37 °C for 45 min. Cells were washed three times with HBSS, fixed with 3% paraformaldehyde, mounted with DAPI-containing Fluormount-G (SouthernBiotec, Birmingham, AL) for nuclear staining, and analyzed with a fluorescence microscope.

For calcium release assays using fluorimeter, intracellular calcium was detected by staining cells with Fluo-4 dye (Invitrogen) and a single-excitation (490 nm) fluorophore for which emission intensity (510 nm) that is directly proportional to the level of bound calcium. Alternatively, a lipophilic calcium ester, the fluorescent derivative of Fluo-4 was used for staining cells as the derivative can cross the plasma membrane and following the cleavage of AM moiety by endogenous esterases the charged dye remains trapped within the cell. Fluorescence was measured at excitation/emission of 485/535 using a Fluoroscan Ascent plate reader (Labsystems) and the relative fluorescent units (RFU) were calculated.

Western blot analysis. Protein lysates for western blot were prepared by lysing cells in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 5 mM EDTA) supplemented with the protease inhibitors PMSF (1 mM), pepstatin (10 μg/ml), aprotinin (2 μg/ml), and benzamidine (2 μg/ml). Protein concentration was determined by BCA kit (Pierce; Rockford, IL). A total of 40 Western blot analysis (2 μECL, PerkinElmer, Inc., MA) 60. The following antibodies were used: Rabbit anti-Bak antibodies (Cat.#ab104124), rabbit anti-calnexin (Cat.#ab13575) antibodies were obtained from Abcam Inc. (Cambridge, MA) and used at 1:1000 dilutions. Mouse anti-Bak (Cat.#F3165) from Sigma-Aldrich Inc. (St. Louis, MO) was used at 1:500 dilution, mouse anti-Flag antibody (Cat.#F3165) from Sigma-Aldrich Inc. (St. Louis, MO) was used at 10 μg/ml concentration, rabbit anti-IP3R antibody (Cat.#ABS2129) from Calbiochem (EMD Millipore Corporation, Billerica, MA) and used at 1:1000 dilution. Unscorched scans of blots are provided as supplementary figures in the Supplementary Figs. 6–11.

Immunofluorescence. For cytometry, cells were grown on Lab-Tek II eight-chamber slides (Nalge Nunc International, Rochester, NY) and after treatments were fixed using 3% paraformaldehyde with 3% sucrose in PBS and processed for immunostaining. Briefly, after antigen retrieval, sections were incubated with a 1:500 dilution of anti-Bak antibodies at 4 °C overnight. For mitochondrial and ER co-localization studies, cells were incubated with Alexa Fluor 488 goat anti-rabbit (Invitrogen, Inc.) antibody or Alexa Fluor 568-labeled goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:1000 dilution and mounted with DAPI-containing Fluormount-G (SouthernBiotec, Birmingham, AL) for nuclear staining. Immunofluorescence was imaged using Axiosplan 2 microscope (Carl Zeiss, Inc., Thornwood, NY) with a Plan-Neofluor 403/0.75 air objective and a charge-coupled device camera (Hamamatsu Photonics, Hamamatsu, Japan) with the acquisition software Slidebook 6.0 (Intelligent Imaging Innovation, Denver, CO).

Flow cytometry. To analyze for Bak activation, cells were trypsinized, washed with PBS, fixed in 0.25% paraformaldehyde, and permeabilized with 0.01% saponin/PBS before the incubation with anti-human Bak monoclonal primary antibody (Clone Ab-1, A033; Calbiochem) for 30 min at 4 °C. Cells were then washed and incubated with Alexa467-labeled goat anti-mouse (Invitrogen Inc.) antibody for 30 min at 4 °C. Cells were washed and resuspended in 0.2% BSA/PBS for analysis with a BD FACSCount™ Flow Cytometer (BD Biosciences). Fluorescence was acquired using logarithmic amplifiers. Approximately, 10,000 cells were analyzed per sample. Flow cytometric results were quantified by manipulating the raw data as described (Griffiths, 1999) using FlowJo analysis software (TreeStar Inc.). Briefly, cells exhibiting a light scatter profile associated with apoptotic cells were gated out and the median Bak-associated fluorescence was determined by subtracting the median fluorescence of the mouse IgG control from each test sample. The median value was then multiplied by the percentage of Bak-positive cells as determined by the Alexa 467 control to give the Ab-1 Bak-specific fluorescence of each sample.

Early and late apoptotic cells were quantified by fixing collected cells in 0.25% paraformaldehyde and incubating them with Annexin V-FITC conjugate (BD Biosciences Inc., San Jose, CA) and propidium iodide (PI, Sigma-Aldrich Inc., St. Louis, MO) for 30 min at 4 °C. After washing, resuspended cells in 0.2% BSA/PBS –100) from BioVision Inc. (Milpitas, CA) and used at 1:250 dilution, mouse anti-DAPK1 antibody (Cat.#610290) from BD Biosciences (BD Biosciences Inc., San Jose, CA). Flow cytometric results were quantified by manipulating the raw data as described (27) using FlowJo analysis software (Tree Star Inc., Ashland, OR). Detection of Annexin V-positive (AnnV+/PI−) was used as a marker for early apoptotic cells, propidium iodide-positive (AnnV−/PI+) was used as necrotic cell marker, the double-positive (AnnV+/PI+) cells represented late apoptotic cells and double-negative (AnnV−/PI−) cells represented viable cells.

ER-mitochondria co-localization. The ER and mitochondria were immunostained in fixed cells using rabbit anti-calnexin (Abcam Inc., Cambridge, MA) and mouse anti-cyclo-oxygenase IV antibodies (CoxIV, Cell Signaling Technology Inc., Beverly, MA), respectively, followed by detection with Alexa-546 or -467 conjugated respective secondary antibodies (Invitrogen). The cells were then mounted with Fluormount-G containing DAPI (for nuclear staining). The confocal fields were acquired every 0.2 μm along the z-axis (for a total of 20–30 images) with a 100X objective using a Zeiss 510 Meta confocal system (CarlZeiss Inc.). For ER-mitochondria interaction analysis, stacks were automatically thresholded using ImageJ, 3D reconstructed and surface rendered. Interactions were quantified by Manders’ colocalization coefficient as described (13) after the background was subtracted using BG subtraction function (ImageJ).

Transmission electron microscopy. HAECs were fixed with 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at 4 °C. Cells were subsequently postfixed with 1% osmium tetroxide in PBS, infiltrated with L-1216 research grade Epon, and embedded on the culture dish, an appropriate sized block face trimmed, and 70-nm ultrathin sections cut with a Diatome diamond knife in an MT5000 ultramicrotome (Sorvall Instruments Div., Newton, CT). Sections were picked up on parlodion-coated copper EMi grids, tanned with uranyl acetate and lead citrate, and photographed using a JEOL JEM 1400 transmission electron microscope (JEOL, Peabody, MA) at 120 kV.

Statistical analysis. The data were analyzed using statistical analysis software (Statistical Analysis Software Institute). Grouped results from at least three different sets of experiments were expressed as mean with SEM, and differences between groups were assessed for significance by Student’s t test when the data were available in only two groups. When the data were available in more than two groups, analysis of variance (ANOVA) was used to perform pair-wise comparisons. When significant main effects were detected (P < 0.05), Fisher least significant difference test was used to determine the differences between groups. A P value of < 0.05 was considered to indicate statistical significance.

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Author contributions

Y.T. conceived the idea; Y.T. and Y.A.M. designed the research; Y.A.M., I.L.-B., N.L., M.G.W., H.S.C. performed research and analyzed the data; A.M.K.C. facilitated the electron microscopy studies; Y.T. and Y.A.M. wrote the manuscript with input from all authors.

Additional information

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