BAK α6 permits activation by BH3-only proteins and homooligomerization via the canonical hydrophobic groove

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BAK and BAX are the essential effectors of apoptosis because without them a cell is resistant to most apoptotic stimuli. BAK and BAX undergo conformation changes to homooligomerize then permeabilize the mitochondrial outer membrane during apoptosis. How BCL-2 homology 3 (BH3)-only proteins bind to activate BAK and BAX is unclear. We report that BH3-only proteins bind inactive full-length BAK at mitochondria and then dissociate following exposure of the BAK BH3 and BH4 domains before BAK homodimerization. Using a functional obstructive labeling approach, we show that activation of BAK involves important interactions of BH3-only proteins with both the canonical hydrophobic binding groove (α2–5) and α6 at the rear of BAK, with interaction at α6 promoting an open groove to receive a BH3-only protein. Once activated, how BAK homodimers multimerize to form the putative apoptotic pore is unknown. Obstructive labeling of BAK beyond the BH3 domain and hydrophobic groove did not inhibit multimerization and mitochondrial damage, indicating that critical protein–protein interfaces in BAK self-association are limited to the α2–5 homodimerization domain.

apoptosis | BAK | BAX | BH3-only protein | mitochondria

BAK and BAX are the pivotal effectors of intrinsic apoptosis, with one or the other being required for mitochondrial damage and cell death (1, 2). They are activated by interaction with BCL-2 homology 3 (BH3)-only proteins including BID and BIM (3). Structures show that peptides based on the BH3 domains of activator BH3-only proteins can bind directly to BAK and BAX via a hydrophobic groove (comprising α-helices 2–5) that is also shared with their prosurvival homologs (4–8). BAX has been proposed to have an additional activation site, distinct from the hydrophobic groove, at the rear of the molecule comprising α-helices 1 and 6 (9). Binding of stapled BH3 peptides at this site induced the dissociation of the BAX C-terminal transmembrane domain from the hydrophobic groove to facilitate mitochondrial localization (10, 11). Interaction of BH3-only proteins at this noncanonical site is not thought to be necessary for BAK activity because BAK is constitutively anchored in the mitochondrial outer membrane (MOM) via its transmembrane domain (12). However, recent reports that both BAX (13, 14) and BAK (15) are constantly "retrotranslocated" from the mitochondria to the cytosol suggest conserved mechanisms of activation for BAK and BAX.

Characterizing the interaction of BH3-only proteins with full-length BAK at mitochondria has proven difficult because the bound BH3-only protein dissociates during consequent BAK conformation changes, including exposure of the N-terminus (amino terminus) and BH3-domain (16–19) and dissociation of their α2–5 helices ("core") from their α6–8 helices ("latch") (6, 7) to facilitate self-association. Additionally, structural studies have been largely confined to truncated protein or peptides in the absence of a membrane where interactions between BH3-only proteins and BAK occur.

Once activated BAK and BAX self-associate to form pores that damage the MOM to release cytochrome *c* and other apoptotic factors. A key step is the formation of symmetrical BH3:groove homodimers (17, 20), which then form higher order oligomers including ring-like pores and aggregates (21, 22). However, how BAK and BAX homodimers multimerize is unknown. From linkage or proximity measurements based on electroparamagnetic resonance and double electron–electron resonance various secondary interfaces have been implicated to allow homodimers to associate (23–28). Alternatively, based on linkage analysis and mathematical modeling, random aggregation of homodimers has also been proposed (29).

Understanding the molecular details of BAK and BAX activation and oligomerization may facilitate the rational design of small molecules that can inhibit them and so inhibit pathological apoptosis. We find that cBID interacts with inactive BAK at mitochondria and dissociates following exposure of the BAK α2 (BH3) and α1 (BH4) domains. We also show that cBID promotes BAK activation by binding a site involving the BAK α6 to promote opening of the canonical hydrophobic groove. Blocking experiments also indicate that a stable protein–protein interface outside of the core BH3:groove dimerization domain is not essential for pore formation and MOM damage.

Results

BAK Conformation Change Destabilizes the cBID:BAK Interaction at Mitochondria. BH3-only proteins are thought to dissociate from BAK at mitochondria due to the induced conformation change

and/or self-association of BAK. Thus, we hypothesized that constraining BAK in its inactive conformation, thereby preventing conformation change and subsequent homooligomerization, would stabilize the cBID:BAK interaction at mitochondria. We thus induced disulphide linkage between the two native cysteines in BAK (C14 and C166, Fig. 1A). This tether impaired BAK conformation change, evidenced by decreased exposure of N-terminal and BH3 epitopes (Fig. 1B) and MOM permeabilization (Fig. 1C). As predicted, the tether stabilized the interaction with cBID at mitochondria (Fig. 1D). As controls, cBID failed to coimmunoprecipitate with either single-cysteine BAK mutant (C14S or C166S) that was not constrained by intramolecular disulphide linkage (Fig. 1D).

To define the step in BAK activation at which cBID dissociates we used additional constraints between the α1–α2, α1–α6, and α5–α6 that we have shown impair defined steps in BAK activation to block cytochrome c release (7, 19) (Fig. 1E). We first confirmed that the induced intramolecular tethers did not alter the conformation (Fig. S1A) or oligomerization state (Fig. S1B) of BAK. Like the C14:C166 tether, tethering Y41 (α1) to A79 (α2) or A28C (α1/2loop) to L163C (α6) stabilized the interaction with cBID (Fig. 1F). We have previously shown that tethering V142C (α5) to F150C (α6) to constrain "core/latch" dissociation still shows evidence of cBID-induced N-terminal epitope exposure, indicating that cBID still interacts with the tethered form to induce early steps in BAK conformation change (19). However, in contrast with the α1:α2 and α1/2 loop:α6 tethers, tethering α5:6 failed to stabilize the cBID:BAK complex (Fig. 1F), placing α5/6 dissociation downstream of both cBID dissociation and separation of α1 and α2 (BH3). Because core/latch dissociation precedes dimerization, our data suggest that cBID dissociates from BAK as a consequence of activating conformation change, likely due to α1 (BH4) and/or α2 (BH3) exposure, rather than due to displacement of BH3-only proteins from the groove by a BAK BH3 domain during BAK homodimerization (Fig. 1G).

cBID Interacts with Soluble and Mitochondrial BAK at Both the Canonical Hydrophobic Groove and at α6. Whereas interactions of BAX with BH3-only proteins also occur at a distal site involving the α1 helix (30) and the α6 helix (9), interactions of BAK with BH3-only proteins seem restricted to the hydrophobic groove (11, 12). To investigate how cBID engages BAK at mitochondria we used an intermolecular disulphide-linkage approach to test whether cBID could link to regions in BAK other than the groove. To establish disulphide-linkage conditions we first used a functional semicytosolic mutant of BAK (BAK/BAXCS, where CS indicates C-segment) (31), because it could be at least partially activated by cBID in cytosolic fractions (Fig. S2 A and B), but its failure to undergo full activation and hence homooligomerize (Fig. S2A) allowed a stable interaction with cBID (Fig. S2C).

We introduced cysteine in the BID BH3 domain at position I83 or R84 of recombinant BID and confirmed they retained apoptotic activity (Fig. S3A). WT cBID (which lacks cysteine in the activating p15 fragment) and cBID I83C and R84C variants were incubated with cytosolic extracts from cells stably expressing BAK/BAXCS with cysteines engineered in either the groove (H99C or K113C) or α6 (R156C, D160C, or H164C). Disulphide linkage was induced with oxidant [copper (II) (1,10-phenanthroline)₃, CuPhe] and assessed on nonreducing SDS/PAGE following coimmunoprecipitation. Disulphide-linked cBID:BAK heterodimers were evident between the cBID R84C variant with cysteine at H99 in the BAK groove (Fig. 2A) and also at R156 and H164 in the BAK α6 (Fig. 2B). A G94E mutation in BID reduces its interaction with BCL-2 proteins (32) and, consistent with this, cBID G94E showed reduced ability to link with BAK/BAXCS at both sites (Fig. 2C and Fig. S3B).

This approach was then used to map the transient interaction of cBID with BAK at mitochondria. cBID R84C did not detectably link at either the hydrophobic groove or α6 of BAK resident at
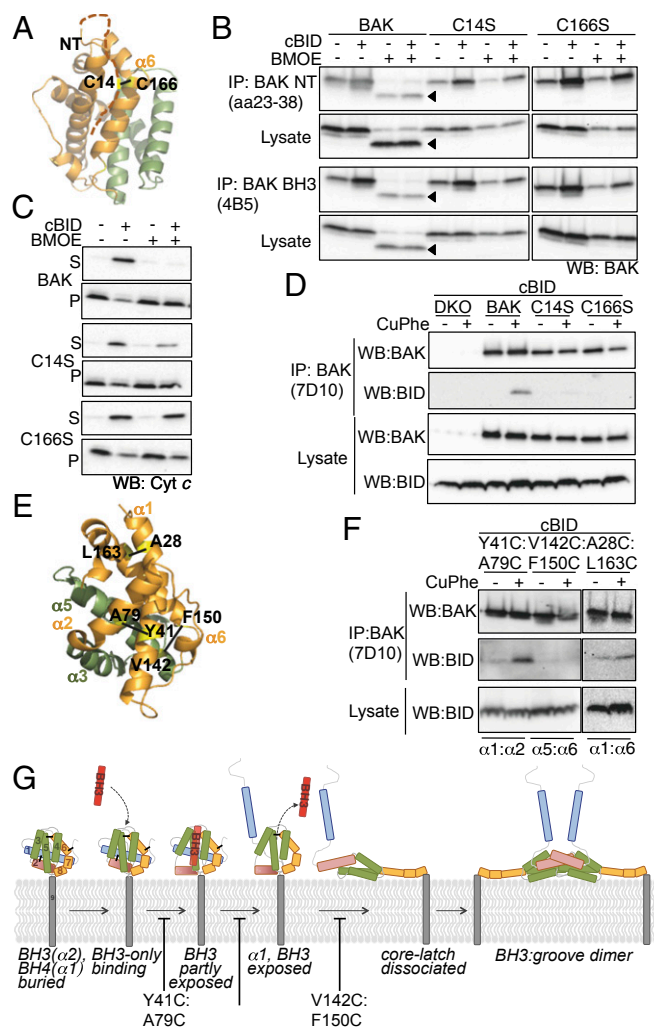
Fig. 1. Constraining events in BAK activation stabilize interaction with cBID. (A) Model of BAK C14:C166-induced disulphide linkage. N-terminal 20 amino acids are represented as a hashed line because the residues are absent from the crystal structure [Protein Data Bank (PDB) ID code 2IMS]. (B) An intramolecular tether constrains cBID-induced BAK conformation change. Membrane fractions from Bak⁻/⁻Bax⁻/⁻ mouse embryonic fibroblasts (MEFs) expressing BAK, BAK C14S, or BAK C166S were treated with bismaleimidoethane (BMOE, 0.5 mM) followed by recombinant cBID (100 nM, 30 °C, 30 min) before immunoprecipitation with conformation-specific BAK antibodies that recognize the N-terminus (amino acids 23–38) or BH3 domain (4B5). C14:C166 tether is indicated (arrowhead). (C) C14:C166 tether impairs cBID-induced BAK apoptotic function. Membrane fractions as in B were treated with the cysteine cross-linker BMOE (0.5 mM) then recombinant cBID (100 nM, 30 °C, 30 min) before fractionation into supernatant (S) and pellet (P) and immunoblotting for cytochrome c. (D) Constraining BAK conformation change stabilizes interaction with cBID. Membrane fractions as in B were treated with CuPhe to induce disulphide linkage followed by recombinant cBID (100 nM, 30 °C, 30 min) before immunoprecipitation with an antibody that recognizes both active and inactive BAK (7D10). (E) Cartoon representation of BAK (PDB ID code 2IMS) (4). The hydrophobic groove (green) and the residues mutated to cysteine (yellow) are indicated. (F) Intramolecular tethers constrain cBID interaction. Membrane fractions from Bak⁻/⁻Bax⁻/⁻ MEFs expressing the indicated BAK mutants were treated and analyzed as in D. (G) Schematic of the stepwise conformation change of BAK and BID interaction. All data are representative of at least three independent experiments.

mitochondria, because cBID R84C efficiently promoted BAK activation and homodimerization (Fig. 2D and Fig. S3C, BAK:BAK). However, hypomorphic cBID R84C/G94E, which failed to induce BAK homodimers, linked readily to both the groove (H99C) and
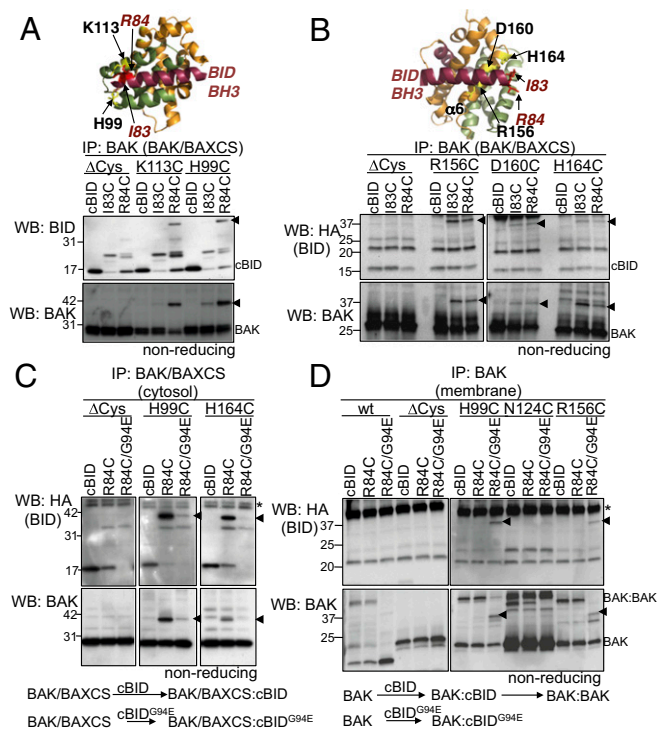
Fig. 2. cBID interacts with the α6 as well as the hydrophobic groove of BAK. (*A* and *B*) cBID binds the hydrophobic groove and α6 of BAK/BAXCS. Cytosolic fractions from *Bak⁻/⁻Bax⁻/⁻* MEFs expressing BAK/BAXCS mutants with cysteines at the indicated positions in the groove (*A*) or α6 (*B*) were incubated with the indicated HA-cBID variants (100 nM, 30 °C, 30 min) and disulphide-linked. BAK immunoprecipitations were run under nonreducing conditions and immunoblotted for BAK or BID. Note that the p15 fragment of cBID cysteine variants cross-linked to other proteins in the cytosol with likely linkage to cBID p7 fragment the most abundant. Note that in subsequent experiments in this figure we detected recombinant BID with an HA antibody. (*C*) Mutation in cBID BH3 reduces interaction at the groove and α6 of BAK/BAXCS. Cytosolic fractions as in *A* and *B* were incubated with the indicated HA-cBID variants (100 nM, 30 °C, 30 min), disulphide-linked, and analyzed as in *A*. (*D*) cBID interacts with the hydrophobic groove and α6 of mitochondrial BAK. Mitochondrial fractions from *Bak⁻/⁻Bax⁻/⁻* MEFs expressing the indicated BAK mutants were incubated with HA-cBID variants (100 nM, 30 °C, 15 min) and disulphide-linked. Membranes were solubilized with digitonin and BAK 7D10 immunoprecipitations were run under nonreducing conditions and immunoblotted for BAK or HA.

α6 (R156C) of mitochondrial BAK (Fig. 2*D* and Fig. S3*C*). No linkage was observed with WT BAK (C14 and C166), BAKΔCys, or BAK with a cysteine at N124 (Fig. 2*D*). The ability of cBID R84C/G94E to link at both the groove and α6 was competitively inhibited by preincubation with a cBID G94E/Cys null variant (Fig. S3*E*), supporting that the linkage profile reported genuine protein–protein interactions.

Thus, cBID binds BAK at mitochondria to promote its conformation change and consequently dissociates to facilitate BAK homooligomerization. In contrast, the hypomorphic cBID G94E variant binds BAK but does not induce activating conformation change and homooligomerization, and consequently its interaction with BAK at mitochondria persists, allowing it to disulphide-link at both the groove and α6 (Fig. 2*D* and Fig. S3*C*). The inability of cBID G94E to promote BAK activation was potentially due to its limited affinity. Alternatively, interactions between the C-terminal end of the BID BH3 domain with the BAK hydrophobic groove may be key in initiating BAK conformation change.

Obstructing the Hydrophobic Groove or α6 Inhibits BAK Activation. To map the functionally relevant activation site(s) on mitochondrial BAK we labeled single cysteines engineered at various positions throughout BAK with cysteine-reactive 5-kDa PEG-maleimide and tested the effect on BAK-mediated cytochrome *c* release. Labeling of BAK cysteine variants was confirmed by retarded migration on reducing SDS/PAGE (Fig. S4 *A* and *B*). Ability to label each variant before activation correlated with the solvent exposure of residues in the structure of BAK (4). For example, residues in the buried face of the α2 (M71C) and in the core α5 helix (e.g., A130C, L132C, R137C, L138C, and A139C) were resistant to labeling (Fig. 3*B*, Figs. S4*C* and S5, and Table S1). Additionally, labeling was consistent with known changes in BAK conformation during apoptosis as indicated by the ability of M71C in the BAK BH3 domain to label only following its cBID-induced exposure (Fig. S4*C*). PEG-maleimide treatment had no impact on cytochrome *c* release nonspecifically or by labeling an alternative target, becasue PEG-maleimide treatment of WT BAK (labeled on both C14 and C166) and BAK ΔCys did not impair MOM permeabilization (Fig. 3*A*). Labeling of the majority of positions throughout BAK did not impair cytochrome *c* release and, additionally, labeling did not induce cytochrome *c* release (Fig. 3 *A* and *B* and Fig. S5). Labeling at residue Y89, which would be expected to obscure the hydrophobic groove (4), significantly impaired BAK-mediated cytochrome *c* release in response to 100 nM cBID (Fig. 3 *A* and *B*, Fig. S5*A*, and Table S1), confirming the groove's important role in BAK apoptotic function (20, 33).

Given the flexibility of the linear PEG-maleimide molecule, we reasoned that an inhibitory effect of the label on cBID-induced BAK activation might be overcome by the high concentrations (100 nM) of cBID used in these initial experiments. Thus, we tested whether an inhibitory effect of PEG-maleimide label might be revealed by a lower (threshold) concentration of cBID (1 nM, Fig. 3*C* and Fig. S6*A*). As expected, labeling Y89C inhibited BAK function (Fig. 3*C*). Labeling at most other positions did not impair BAK activity in response to 1 nM cBID (Fig. S6*B*), but labeling at three positions in the BAK α6 reduced BAK apoptotic function (Fig. 3*C* and Table S1). Simultaneous labeling at two positions in α6 (D160 and H164) blocked BAK activity even when induced by high concentrations of cBID (Fig. 3*D*). BAK activity induced by the BH3 domain of either BIM or PUMA was also impaired by α6 labeling (Fig. S6*C*), suggesting a conserved mechanism.

To test whether labeling impaired cBID-induced conformation change we performed blue native (BN)-PAGE in combination with gel shift with a conformation-specific antibody (amino acids 23–38). As expected, unlabeled BAK gel-shifted with the antibody only after activation with cBID (Fig. 3*E*, indicated by the loss of the dimer in lane 5 compared with lane 2), and treatment with PEG-maleimide did not perturb complete antibody gel shift following activation of BAK ΔCys or G4C (Fig. 3*E*, compare lanes 5 and 6). Labeling of Y89C with PEG-maleimide to obscure the hydrophobic groove reduced, but did not completely block, gel shift with the conformation-specific antibody in response to both 1 and 100 nM cBID (Fig. 3*E*, compare lanes 5 and 6). Labeling of R156C and H164C in α6 likewise reduced gel shift of BAK induced by 1 nM cBID (Fig. 3 *E* and *F*). Labeling of BAK G4C did not impair dimerization, as evidenced by labeled dimers and the lack of labeled monomer after cBID treatment (Fig. 3*E*, lane 3). In contrast, clear retention of labeled monomeric BAK R156C and BAK H164C variants further supports that labeling of α6 impairs cBID-induced activation. That obstructing the α6 perturbs BAK activation rather than homo-oligomerization is consistent with the effect of α6 labeling's being overcome by high concentrations of cBID.

Labeling of the BAK α6 Inhibits cBID-Induced Opening of the BAK Groove. The crystal structure of inactive BAK revealed that the side chains from R88 and Y89 partially occlude the hydrophobic groove and suggested that the groove needed to open to present a binding site for BH3-only proteins (4). Consistent with this, in the structure of BAK with a BID BH3 peptide bound in its hydrophobic groove, the groove is more open and thereby increases
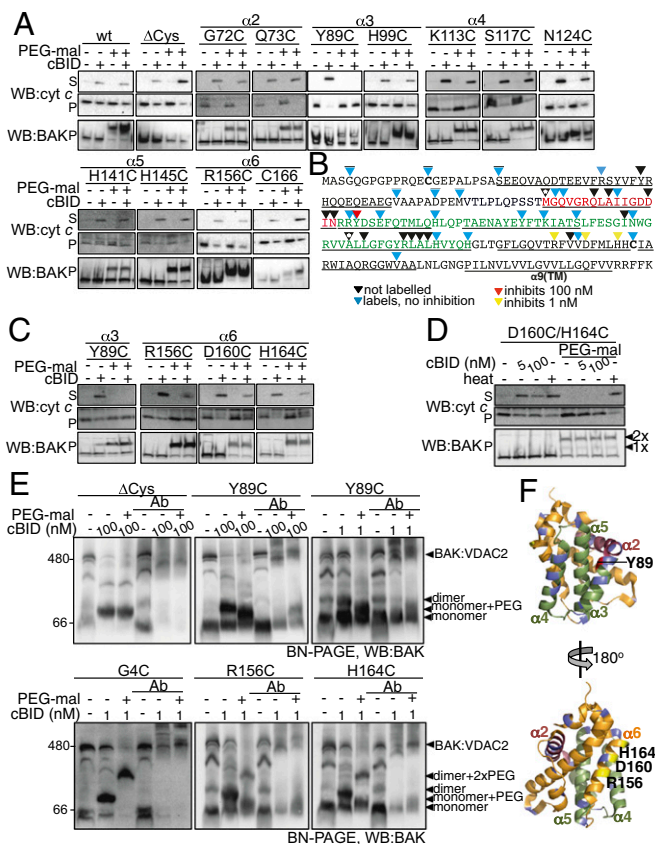
Fig. 3. BAK hydrophobic groove and α6 site are important for BAK apoptotic activity. (*A* and *B*) Obstructive labeling of the groove abrogates BAK-mediated MOM permeabilization. *Bak⁻/⁻Bax⁻/⁻* MEFs expressing the indicated BAK mutants were labeled with PEG-maleimide (5 kDa) before treatment with cBID (100 nM, 30 °C, 30 min) and supernatant (S) and membrane (P) fractions were immunoblotted for cytochrome *c* and membrane fractions immunoblotted for BAK. PEG-maleimide labeling and its effect on BAK-mediated cytochrome *c* release is shown in *B*. A labeled residue was defined as showing ≥50% labeled form on SDS/PAGE. Asterisks indicate residues that do not label in inactive BAK but label after activation with cBID. Endogenous cysteines (C14 and C166) in BAK are indicated in bold. The α2/BH3 domain (red), α3–5/hydrophobic groove (green), and α-helices (underlined) are indicated. (*C*) Obstructive labeling of the BAK α6 impairs apoptotic function in response to limiting cBID concentration. *Bak⁻/⁻Bax⁻/⁻* MEFs expressing the indicated BAK mutants were treated as in *A* except they were treated with 1 nM cBID. (*D*) Double labeling of the BAK α6 impairs apoptotic function in response to high concentrations of cBID. Membranes from *Bak⁻/⁻Bax⁻/⁻* MEFs expressing FLAG-BAK D160C/H164C were treated with cBID (5 or 100 nM) or heated at 43 °C and assessed for cytochrome *c* release. (*E*) Obstructive labeling of the groove and α6 inhibits cBID-induced conformation change. Membrane fractions from *Bak⁻/⁻Bax⁻/⁻* MEFs expressing the indicated BAK mutants were incubated with cBID and PEG-maleimide as indicated, followed by incubation with the conformation-specific antibody BAK aa23-38 and BN-PAGE. (*F*) Labeling of groove and α6 residues inhibits BAK activity. Cartoon representation of the structure of inactive BAK (PDB ID code 2IMS) showing the groove (green) and α2 (BH3 domain, raspberry). Residues that when labeled with PEG-maleimide inhibited cBID-induced BAK activation at 1 nM (yellow) or 100 nM (red) or did not inhibit BAK activation (blue) are indicated. All data are representative of at least two independent experiments.

the solvent exposure of residues at the base of the groove including A130 (4, 33) (Fig. S7). Accordingly, we found that although BAK A130C was not efficiently labeled before activation, its labeling was encouraged by cBID (Fig. 4 *A* and *B*, *during*). Labeling of A130C was reduced after cBID had induced BAK homooligomerization (*post*, Fig. 4*A*), consistent with its burial beneath a bound BAK BH3 domain in the BAK BH3:groove homodimer (7). We hypothesized that binding of cBID to the

α6 activation site may promote opening of the hydrophobic groove to facilitate BID BH3 domain binding. Consistent with this, labeling α6 at either R156 or H164 inhibited the PEG-maleimide labeling of A130C induced by cBID (Fig. 4*C*). In contrast, both cysteines could be efficiently labeled when BAK was activated by heat (Fig. 4*C*). This suggests that binding of cBID to the α6 site promotes exposure or opening of the groove to allow BH3-only protein binding and BAK activation (Fig. 4*D*).

Labeling of BAK Homodimers Does Not Interfere with Apoptotic Activity. Obstructive labeling at the groove could affect BAK-mediated cytochrome *c* release either by inhibiting cBID-induced conformation change or by blocking downstream BAK homo-oligomerization (Fig. 5*A*). To assess the influence of labeling on BAK oligomerization downstream of cBID-induced activation, we tested the effect of labeling on heat-induced cytochrome *c* release that is independent of BH3-only proteins (34). Labeling in the canonical hydrophobic groove (Y89) impaired BAK-mediated MOM permeabilization in response to heat, supporting the important role of the groove not only in BAK activation but also in homodimerization (20) (Fig. 5*B*). As expected, those mutants whose labeling did not inhibit cytochrome *c* release induced by cBID also did not inhibit heat-induced cytochrome *c* release (Fig. 5*B* and Fig. S5). Additionally, double labeling of the α6 did not impair BAK activity when induced by heat (Fig. 3*D*).

Consistent with the proposed important role for the hydrophobic groove in the homodimerization, BN-PAGE revealed that labeling of Y89C and A130C prevented homodimerization in response to heat as well as in response to cBID (Figs. 3*E* and 5*C*). The effect of labeling on dimerization of BAK Y89C was more pronounced than on dimerization of R156C and H164C (Fig. 3*E*, lane 3; note the labeled dimers in R156C and H164C compared with their absence in Y89C). This likely reflects the role of the hydrophobic groove in both activation and homodimerization, whereas the role of α6 is restricted to activation.

When PEG-maleimide labeling was performed after cBID-induced activation, dimers of BAK Y89C were largely resistant to labeling (Fig. 5*C*), indicating that Y89C is buried in dimerized
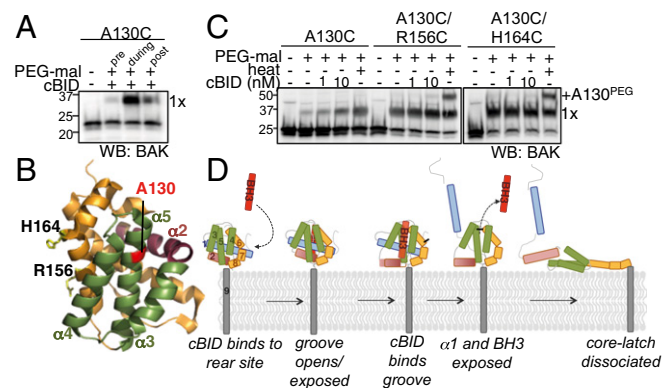
Fig. 4. Labeling the α6 inhibits cBID-induced exposure of the hydrophobic groove. (*A*) Labeling reveals transient exposure of the α5 groove residue A130 during activation. Membranes from *Bak⁻/⁻Bax⁻/⁻* MEFs expressing the BAK A130C were treated with PEG-maleimide before treatment with cBID (100 nM, 30 °C, 30 min) (*pre*), in the presence of cBID (*during*), or after cBID treatment (*post*) and immunoblotted for BAK. (*B*) A130 localizes to the base of the hydrophobic groove in inactive BAK. A130, R156, and H164 are indicated on a cartoon representation of the structure of inactive BAK (PDB 2IMS). (*C*) Labeling of α6 residues impairs induced exposure of A130C. Membranes from *Bak⁻/⁻Bax⁻/⁻* MEFs expressing BAK variants were pretreated with PEG-maleimide without quenching before treatment with cBID (30 °C, 30 min) or heating at 43 °C (30 min) and immunoblotted for BAK. (*D*) Obstructive labeling informs the stepwise activation of BAK at mitochondria.
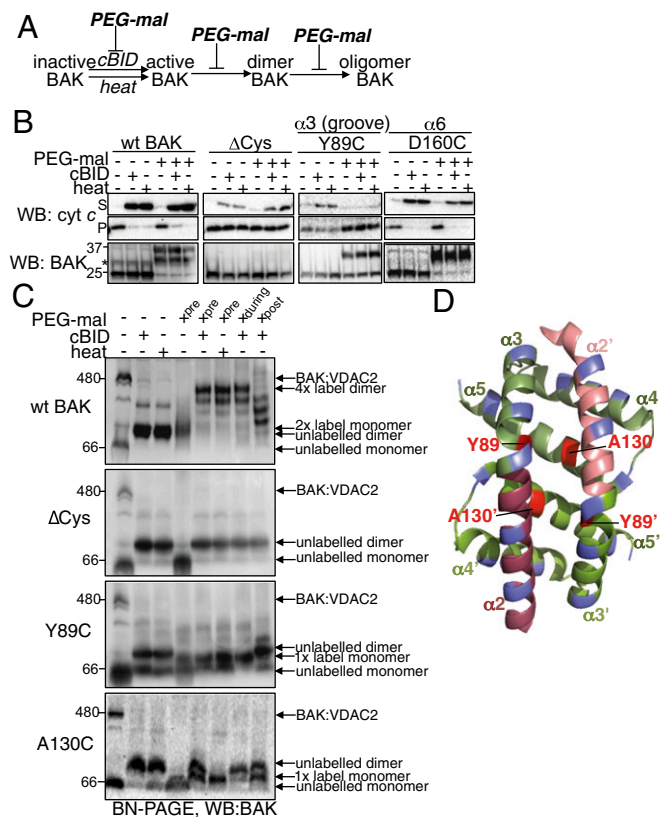
Fig. 5. Labeling of the groove impairs BAK homooligomerization and apoptotic function. (*A*) Obstructive labeling can impair distinct steps in BAK activation and oligomerization. (*B*) Labeling of the groove impairs BAK apoptotic function independent of interaction with BH3-only proteins. Membranes from *Bak*⁻/⁻*Bax*⁻/⁻ MEFs expressing the indicated BAK mutants were treated with PEG-maleimide before treatment with cBID (100 nM, 30 °C, 30 min) or heated at 43 °C for 30 min. Supernatant (S) and membrane (P) fractions were immunoblotted for cytochrome *c* and membrane fractions for BAK. (*C*) Labeling of the groove impairs BAK homooligomerization. Mitochondrial fractions from *Bak*⁻/⁻*Bax*⁻/⁻ MEFs expressing the indicated BAK variants were treated with PEG-maleimide before treatment with cBID (100 nM, 30 °C, 30 min), in the presence of cBID (*during*), or after cBID treatment (*post*) or alternatively heated at 43 °C for 30 min and assessed on BN-PAGE. (*D*) Labeling of residues on the periphery of the BAK core homodimer does not impair apoptotic function. Cartoon representation of the BAK α2–5 core BH3:groove homodimer (PDB ID code 4U2V) (7). Residues that label and impair BAK function (red) and residues that label but do not affect BAK function (blue) are indicated. Data are representative of at least three independent experiments.

BAK (Fig. 5*D*). Both C14 and C166 were efficiently labeled in WT BAK but did not impair oligomerization (Fig. 5*C*). However, once activated and oligomerized these positions in BAK became more resistant to labeling, as indicated by the marked increase in 1× and 2× labeled dimer species when labeled after oligomerization in comparison with the predominant 4× prelabeled species (Fig. 5*C*, compare lanes 5 and 8). This indicates that both the N termini (C14) and α6 (C166) become buried once BAK is oligomerized. Thus, taken together, labeling a single position in the groove with PEG-maleimide was sufficient to block homodimerization and MOM permeabilization, yet labeling of each position tested outside of the groove failed to do so (Fig. 5*D* and Fig. S5).

Discussion
Our data indicate that BH3-only proteins like cBID interact with full-length BAK at mitochondria via the canonical hydrophobic groove comprising α2–5 and also at a site on the opposite face of

BAK comprising α6, indicating that the BAK and BAX activation mechanisms are conserved (11, 33). A previous report failed to find evidence of an interaction between BH3 peptides and the putative rear site in a recombinant mutant BAK protein in solution (12). This difference led to the proposition that this noncanonical site was restricted to BAX to promote its translocation to the MOM, with the mitochondrial localization of BAK precluding the need for activation via the noncanonical site. Our findings with full-length BAK at mitochondria suggest that the consequence of BH3-only protein binding at the noncanonical site is likely the same for BAK as it is for BAX: it promotes opening or accessibility of the hydrophobic groove. In BAK that resides at mitochondria this exposes a reactive groove for BH3-only protein binding, whereas in cytosolic BAX it additionally serves to encourage dissociation of the transmembrane anchor from the hydrophobic groove and promote mitochondrial localization. The increased accessibility of the groove may be due to induced conformation change to allosterically open the hydrophobic groove. Alternatively, because both BAK and BAX interact with VDAC2 at the MOM (35–37), binding of BH3-only proteins to the α6 site in BAK or BAX may be an important step in dissociating them from VDAC2 during apoptosis. Recent evidence that the distinct subcellular localization of BAK and BAX is a consequence of their relative rates of retrotranslocation to the cytosol argues against qualitatively different modes of BAK and BAX activation (13, 15). Although our linkage and blocking data support a two-site mechanism of BAK activation, it remains possible that labeling the BAK α6 indirectly impairs a key event in BAK activation induced by groove binding that precedes homodimerization.

Structural information regarding regions beyond the core dimerization domain of α2–α5 in dimerized BAK and BAX is lacking. PEG-maleimide labeling was consistent with the known structures of inactive (4) and homodimeric BAK (7). This includes residues of BAK that become exposed during activation such as those in the α2/BH3 domain and base of the hydrophobic groove. Additionally, Y89C's inability to be labeled in the homodimer form is consistent with its burial in a BH3:groove homodimer (7), providing further support that full-length BAK adopts a symmetric BH3:groove homodimer in the MOM. Thus, PEG-maleimide labeling is an efficient way to interrogate the conformations of full-length BAK and BAX in the MOM while also informing functional relevance.

BAK and BAX homodimers have been proposed to multimerize via secondary interfaces including α1, α3/4, α4/5, and α6 to form the oligomers necessary for MOM permeabilization (7, 23–25, 27, 28, 38, 39). However, labeling of residues in these implicated secondary interfaces failed to significantly affect MOM permeabilization, suggesting that these putative interfaces are not necessary for BAK multimerization and higher order pore formation. We have previously shown that mutation of the BAK α6 abrogated higher order complex formation and apoptotic function downstream of BH3:groove homodimerization, indicating that BAK BH3:groove homodimers are not sufficient to mediate MOM permeabilization and cause cell death (38), while also implicating the α6 in higher-order pore formation. However, even dual labeling of α6 residues failed to significantly inhibit BAK-mediated cytochrome *c* release in response to heat, suggesting that mutation of α6 may inhibit apoptotic function by impairing events that lead to the aggregation of dimers such as conformation change and/or membrane integration following BH3:groove dimerization. The lack of a putative secondary protein interface that mediates BAK oligomerization suggests that BAK (and by homology likely also BAX) homodimers may aggregate in a disordered fashion, as implicated by our mathematical modeling of BAK oligomers based on disulphide-linkage constraints (29). Additionally, our data are consistent with the notion that homodimers associate with the phospholipid of the MOM to form a proteolipidic pore (21, 40, 41). We have reported that BAX and BAK homodimers expose a hydrophobic face comprising

aromatic residues in α4 and α5 that may promote interaction with the lipids of the MOM (6, 7, 42). Biophysical studies showed evidence of proteolipidic pore formation by BAX and BAK (21, 40, 43), and our earlier studies indicating that the higher order oligomeric pore is heterogeneous and unstable once removed from the MOM (23, 38) are consistent with the characteristics of proteolipidic pores.

Experimental Procedures

Cell Culture and Retroviral Infection. SV40-transformed *Bak⁻/⁻Bax⁻/⁻* mouse embryonic fibroblasts were generated, passaged, and retrovirally transduced with BAK expressions constructs as described (20). Details are provided in *SI Experimental Procedures*.

Subcellular Fractionation, Cytochrome *c* Release, Cysteine Linkage Analysis and PEG-Maleimide Labeling, Immunoprecipitation, SDS/PAGE, and BN-PAGE. Procedures were performed essentially as described (20). For PEG-maleimide labeling, mitochondria-enriched membrane fractions were resuspended in fractionation buffer and treated with methoxy PEG-maleimide (5 kDa, PLS-234; Creative PEGworks). Details are provided in *SI Experimental Procedures*.

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