Identification of an activation site in Bak and mitochondrial Bax triggered by antibodies

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During apoptosis, Bak and Bax are activated by BH3-only proteins binding to the α2-α5 hydrophobic groove; Bax is also activated via a rear pocket. Here we report that antibodies can directly activate Bak and mitochondrial Bax by binding to the α1-α2 loop. A monoclonal antibody (clone 7D10) binds close to α1 in non-activated Bak to induce conformational change, oligomerization, and cytochrome c release. Anti-FLAG antibodies also activate Bak containing a FLAG epitope close to α1. An antibody (clone 3C10) to the Bax α1-α2 loop activates mitochondrial Bax, but blocks translocation of cytosolic Bax. Tethers within Bak show that 7D10 binding directly extricates α1; a structural model of the 7D10 Fab bound to Bak reveals the formation of a cavity under α1. Our identification of the α1-α2 loop as an activation site in Bak paves the way to develop intrabodies or small molecules that directly and selectively regulate these proteins.
The commitment of cells to apoptotic cell death is determined by interactions between members of the Bcl-2 protein family on the mitochondrial outer membrane (MOM)\(^1,\)\(^2\). Members of this family contain one to four Bcl-2 homology (BH) domains, and are divided into three sub-clases: pro-survival members that contain the BH1-BH4 domains; pro-apoptotic BH3-only members; and pro-apoptotic Bak and Bax that also contain the BH1-BH4 domains.

A key step in apoptosis is the loss of MOM integrity, which requires Bak and Bax activation followed by their structural conversion into pore-forming oligomers\(^3\)–\(^5\). Both Bak and Bax contain nine a-helices, including a C-terminal transmembrane domain (\(\alpha 9\)), a buried BH3 domain (\(\alpha 2\)), as well as a hydrophobic surface groove (\(\alpha 2–\alpha 5\)) that can engage in interactions with other members of the Bcl-2 family. Whereas Bak is inherently mitochondrial, Bax is largely cytosolic with its \(\alpha 9\)-helix partly sequestered in the \(\alpha 2–\alpha 5\) groove\(^5\) until Bax accumulates on the MOM following an apoptotic stimulus\(^6\)–\(^8\).

Bak and Bax activation (that is, unfolding) are triggered when BH3-only proteins (for example, Bid or Bim) bind transiently to the \(\alpha 2–\alpha 5\) groove\(^8\)–\(^11\). In Bax, but not Bak, access of the activator to the \(\alpha 2–\alpha 5\) groove requires initial binding to a second site (rear pocket) between \(\alpha 1\) and \(\alpha 6\) to displace \(\alpha 9\) (refs 12–14). Bak may also be activated at sites other than the \(\alpha 2–\alpha 5\) groove, as several proteins reported to directly activate Bak appear to lack a BH3-like motif\(^15\)–\(^18\). Binding of BH3-only proteins to the Bak and Bax \(\alpha 2–\alpha 5\) groove initiates unfolding of \(\alpha 2\) followed by dissociation of both \(\alpha 1\) and the \(\alpha 6–\alpha 8\) latch\(^8\)–\(^9\),\(^19\). The unfolded proteins collapse onto the mitochondrial surface and dimerize via a reciprocal BH3 groove interaction to nucleate the oligomers thought to permeabilize the MOM\(^5\)–\(^20\)–\(^25\).

Here we report the proximal \(\alpha 1–\alpha 2\) loop as a second activation site in Bak and in mitochondrial Bax. This site can be targeted by antibodies to induce the same Bak and Bax homo-oligomerization and pore formation as that induced by BH3-only proteins. A structural model of the 7D10 Fab bound to Bak supports biochemical evidence that antibody binding to the \(\alpha 1–\alpha 2\) loop acts by directly dissociating \(\alpha 1\).

**Results**

**An antibody to Bak triggers mitochondrial permeabilization.**

While using antibodies to characterize Bak conformational changes triggered by tBid, we found that an anti-Bak antibody, clone 7D10, could trigger cytochrome c release from mitochondria expressing human Bak (hBak, Fig. 1a). During the incubation, Bak had become activated as shown by sensitivity to limited proteolysis (Fig. 1b; Supplementary Fig. 1a–c), and had oligomerized as shown by disulfide-linked dimers induced by addition of the oxidant copper phenanthroline (CuPhe, Fig. 1c). Two alternative antibodies, 8F8 and anti-FLAG, that bound Bak N-terminal to \(\alpha 1\), failed to activate Bak and FLAG-Bak, respectively (Fig. 1a–c; Supplementary Fig. 2a,b). These data demonstrate that an antibody can trigger Bak activation, oligomerization and mitochondrial cytochrome c release, and that the epitope recognized by 7D10 may be an important site for activating Bak.

**The 7D10 antibody binds to the \(\alpha 1–\alpha 2\) loop of human Bak.**

7D10 is a rat monoclonal antibody raised against human BakAC25 (ref. 22). By peptide array we had defined \(^{51}\text{GVAAP}\) at the start of the \(\alpha 1–\alpha 2\) loop (Fig. 1d) as the minimal set of residues required for 7D10 binding, with G51 and P55 as particularly important residues within this sequence\(^19\). We then tested whether substituting each residue in this region (with cysteine) decreased 7D10 binding and activation. When tested by immunoprecipitation, G51 and P55 were confirmed to be important for 7D10 binding (Fig. 1e) and for 7D10-induced cytochrome c release (Fig. 1f). Curiously, 7D10 could immunoprecipitate BakP55C only after its activation by tBid (Fig. 1e), suggesting that cysteine at position 55 is permissive for binding of 7D10, but that proline at that position helps to orientate the epitope in non-activated Bak. The substitutions did not affect tBid-induced cytochrome c release (Fig. 1f), or Bak-mediated apoptosis in cells (Supplementary Fig. 3a), although we cannot exclude the possibility that variants may have slightly more or less function than wild-type Bak.

To test if \(^{51}\text{GVAAP}\) was sufficient for Bak activation by 7D10, four sets of substitutions were made in mouse Bak (mBak, Fig. 1g), which we had found was not activated by 7D10 (Fig. 1a). The substitutions did not affect Bak-mediated apoptosis in cells (Supplementary Fig. 3b). In mitochondria experiments, the GVAAP and AEGVAAP sequences were not sufficient for 7D10 to immunoprecipitate non-activated mBak or induce cytochrome c release, whereas the GAAAPAD sequence was sufficient (Fig. 1h,i). The importance of the aspartate residue was confirmed by the single substitution (N55D) allowing mBak binding and activation by 7D10 (Fig. 1h,i), and by the reverse substitution (D57N) in hBak inhibiting activation by 7D10 (Supplementary Fig. 4a–c). Thus, as discussed for the proline residue, this aspartate residue may help ‘present’ other epitope residues in non-activated hBak. In addition, both the hBak P55C and D57C variants were susceptible to proteasome K even before their activation (Supplementary Fig. 5a), suggesting that P55 and D57 structure the \(\alpha 1–\alpha 2\) loop in a way that allows recognition by 7D10 but not proteasome K. Further, D57 is not in contact with the antibody, as shown by the ability of D57C to disulfide bond (D57C&D57C linkage) between Bak molecules after 7D10 has bound and triggered Bak activation (Supplementary Fig. 5b,c). Similarly, the A49CA49C linkage after 7D10 activation indicates that the other end of the epitope is delimitated by A49 (Supplementary Fig. 5b,c). Thus, the functional 7D10 epitope in non-activated Bak is the \(^{51}\text{GVAAPAD}\) sequence, with P55 and D57 orientating other epitope residues (for example, G51) that directly contact the antibody.

**Antibody-triggered activation requires epitope close to \(\alpha 1\).** To investigate the mechanism of antibody-triggered Bak activation and in particular the relevance of the epitope being close to \(\alpha 1\), the FLAG epitope was placed in four positions in the \(\alpha 1–\alpha 2\) loop (Fig. 2a). Each variant retained pro-apoptotic function in cells (Supplementary Fig. 3c), and each variant could be immunoprecipitated with anti-FLAG antibody (Fig. 2b). Notably, antibody-triggered cytochrome c release occurred only if the FLAG epitope was N-terminal in the \(\alpha 1–\alpha 2\) loop (n-loop replacement or insertion; Fig. 2c). Moreover, when the 7D10 epitope was re-positioned 11 residues away from \(\alpha 1\), 7D10 could bind but could no longer activate Bak (n-loop insert, Fig. 2b,c). As \(\alpha 1\) dissociation is an important step in Bak activation by tBid\(^19\), the anti-FLAG and 7D10 antibodies may act by binding close to \(\alpha 1\) and directly destabilizing its many contacts with the remainder of Bak.

**7D10 binding to Bak does not directly target \(\alpha 2\).** To test which Bak conformational changes mediated 7D10-induced activation of Bak, we used disulfide tethers to restrain the various structural elements in Bak (Fig. 2d). Each tether was efficient and did not hinder 7D10 binding to Bak (Supplementary Fig. 6a,b). Three tethers (A28CL163C, Y41CA79C and V142CT148C) previously shown to block Bak activation by tBid\(^18\)–\(^23\) also blocked activation initiated by 7D10 (Fig. 2e), indicating that although
tBid and 7D10 bind to different sites on Bak (Fig. 1d), most of the subsequent unfolding events (illustrated in Supplementary Fig. 6c) are equivalent. A tether of the $\alpha_{1}-\alpha_{2}$ loop to the $\alpha_{5}-\alpha_{6}$ loop (E59C:T148C), similar to a tether (L45C:M137C) shown to block Bax activation$^{12}$, also blocked Bak activation by both tBid and 7D10 (Fig. 2e). This tether may prevent core/latch separation, although the tether is to the hinge region rather than to the latch ($\alpha_{6}-\alpha_{8}$) itself. In contrast, because the $\alpha_{1}-\alpha_{2}$ loop lies

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**Figure 1**: Illustrations and data for the binding sites of tBid and 7D10 on Bak. (a) Schematic of Bak protein domain structure showing tBid and 7D10 binding sites. (b) Western blot analysis of Bak activation in the presence of tBid and 7D10. (c) Overlay of Western blot images showing the effect of tBid and 7D10 on Bak activation. (d) Diagram of Bak protein domain structure highlighting the $\alpha_{1}-\alpha_{2}$ loop and $\alpha_{5}-\alpha_{6}$ loop. (e) Western blot analysis of Bak activation in the presence of mutated Bak proteins. (f) Summary table of Western blot results. (g) Sequence alignment of $\alpha_{1}$-$\alpha_{2}$ loop of hBak and mBak. (h) Western blot analysis of Bak activation in the presence of mutated Bak proteins with IP: 7D10. (i) Western blot analysis of Bak activation in the presence of mutated Bak proteins with IP: tBid.
across z1, tethering the loop would greatly hinder z1 dissociation. The final tether, from the middle of the z1–z2 loop to z1, was designed to prevent the effect of antibody binding to the N-terminal end of the loop from being transduced to the C-terminal end of the loop and to z2 (H43C-M60C; Fig. 2d). In spite of this tether, 7D10 still activated Bak (Fig. 2e), further indicating that 7D10 binding directly impacts z1, and not z2.

The 7D10 Fab efficiently activates Bak. To test whether the bivalency or bulk (~150 kDa) of the 7D10 antibody might contribute to Bak activation, the 7D10 antibody fragment (Fab, ~50 kDa) was purified (Supplementary Fig. 7a–b). When Fab was incubated with mitochondria, Bak underwent activation and oligomerization (Supplementary Fig. 7c) and released cytochrome c with similar molar efficiency as the full antibody (Supplementary Fig. 7d). Thus, the bivalency and bulk of 7D10 are not essential for its activation of Bak.

The 7D10 Fab alters oocyte mitochondrial morphology. The 7D10 Fab was then tested in cells by microinjection into immature human oocytes. The 7D10 Fab, but not a control Fab, induced mitochondrial aggregation that progressed over 16 h (Supplementary Fig. 8a–c). The morphology was consistent with mitochondrial fusion, supported by a decrease in mitochondrial particle number and increase in size (Supplementary Fig. 8f,g). We also observed an apparent deformation of the plasma membrane creating a highly contoured surface compared with controls (Supplementary Fig. 8d,e). This is consistent with early stages of apoptosis seen in oocytes under other conditions. Importantly, changes in mitochondrial morphology were not apparent when the 7D10 Fab was injected into mouse oocytes (Supplementary Fig. 9a–c), consistent with an on target effect of the Fab on human Bak. Notably, the human oocytes appear not to have undergone MOMP, as TMRM was retained in the aggregated mitochondria (Supplementary Fig. 8a–c). Further, mitochondrial fission rather than fusion most often accompanies MOMP, although Bak and Bax may also contribute to mitochondrial fusion.

Mitochondrial Bax can be activated by antibody to z1–z2 loop. Due to the structural and functional similarities of Bak and Bax, we anticipated that Bax would also be activated by an antibody to its z1–z2 loop. This was first tested in a mitochondrial form of Bax (S184L), that like Bak, adopts a non-activated conformation in which z9 forms a transmembrane domain, and that converts to the same activated conformation as Bax in response to tBid. Because of its mitochondrial location, BaxS184L does not require initial binding of tBid to the rear pocket to extrude z9.

Structural model shows 7D10 binding causes cavities under z1. The mechanism of antibody-mediated Bak activation was further investigated by generating a molecular model of the 7D10 Fab bound to Bak (Fig. 4). We note that Bak is normally anchored in the outer membrane by z9 forming a transmembrane domain, a feature that may affect Bak activation. However, membranes were not incorporated in the MD simulations as the structure of membrane-bound Bak is not available. Models of Bak obtained from MD simulations were docked with homology-based models of 7D10: several models of both Bak and 7D10, representing different possible loop conformations of each, were cross-validated against one another in an attempt to account for flexibility in the uncomplexed proteins. The final model of the complex after molecular dynamics simulation (Fig. 4a) was also assessed for the effect of antibody binding on the structure of Bak (Fig. 4b,c).

The final model (Fig. 4a) has a large interaction surface (1,411 Å² and high shape (0.670) complementarity. Moreover, the interaction surface reflects our biochemical identification of the 7D10 epitope (Fig. 1d–i) and the availability of cysteine residues at either end of the epitope for linkage in 7D10-activated Bak oligomers (A94C and D57C; Supplementary Fig. 5d–e). Notably, the 7D10 epitope is flanked by CDR2 and CDR3 of the heavy chain. A much smaller contact forms between the light chain and the C terminus of z3; this interaction is dominated by weak van der Waals interactions, which may help explain why 7D10 can also bind and activate BaxS184L-GVAAPAD (Fig. 3b,c). Insertion of CDR3 in the groove underneath the z1/z2 loop region may contribute to activation, as both 7D10 and the M2 anti-FLAG antibody failed to activate Bak if the related epitope was positioned ~11 residues away from z1.

**Figure 1 | The 7D10 antibody triggers mitochondrial outer membrane permeabilization by binding to the z1–z2 loop of human Bak. (a) The 7D10 antibody induces cytochrome c release. Membrane fractions from Bak−/− Bax−/− MEFs, those cells expressing human Bak (hBak), or Bax−/− MEFs, were incubated with tBid or with the 7D10 or 8F8 antibodies. Supernatant (Sup) and pellet (Mito) fractions were assessed for cytochrome c release. (b) 7D10 triggers Bak conformational change as indicated by susceptibility to proteinase K. Incubations from a were treated with proteinase K and immunoblotted for Bak. Note that 7D10 binding at the loop masks a cleavage site (lane 4, Supplementary Fig. 1a), and that uncleaved Bak and light chain co-migrate. (c) 7D10 triggers Bak oligomerization. Incubations from a were treated with oxidant (CuPhe) to induce disulfide bond formation and immunoblotted for Bak. M, monomer; M2, intramolecular linked monomers; D, intramolecular linked dimers. (d) The 7D10 trigger site in Bak is distinct from the canonical BH3-only trigger site. Cartoon representation of BAK19ACA25 (2IMT, white) highlighting the z1–z2 loop (blue), and z3 and z4 of the hydrophobic groove (green). (e) Mutation of Bak G51 or P55 inhibits binding by 7D10. Membrane fractions from Bak−/− Bax−/− MEFs expressing the indicated hBak variants were incubated with or without tBid followed by immunoprecipitation with 7D10 and immunoblotting for Bak. IP, immunoprecipitated; UB, unbound; #, light chain. (f) Mutation of Bak G51 or P55 prevent Bak activation and cytochrome c release by 7D10. Membrane fractions from c were incubated with tBid or 7D10 and assessed for cytochrome c release. (g) Substitutions in mouse Bak to generate the 7D10 epitope. (h) The GVAAPAD sequence allows binding of 7D10 to mouse Bak. Membrane fractions from Bax−/− MEFs (mBak cells), or Bax−/− Bax−/− MEFs expressing hBak or the indicated mBak variants, were incubated with or without tBid followed by immunoprecipitation with 7D10 and immunoblotting for Bak. (i) The GVAAPAD sequence allows 7D10 to activate mouse Bak and release cytochrome c. Membrane fractions from h were incubated with tBid or 7D10 and assessed for cytochrome c release. In a–c, f, h and i data are representative of three independent experiments.
Molecular dynamics simulations of non-activated Bak indicated significant flexibility specifically in the region containing the 7D10 epitope and α2 (Fig. 4b, left), consistent with the high B-factors observed in this region in X-ray structures of Bak (2IMT, 2JCN, 2YV6)\textsuperscript{36,37}. Binding of 7D10 to the epitope resulted in reduced flexibility in both regions (Fig. 4b, right), consistent with 7D10 binding to a preferred conformation. Insertion of the CDR3 loop appears to pry α1 from α2, evidenced by the creation of cavities between these two helices (Fig. 4c, right). In contrast, in unbound Bak only a small cavity is observed between α5 and the α3–α4 loop (Fig. 4c, left), again consistent with the high B-factors in X-ray structures. This weakening of the association of α1 likely represents the initial destabilizing event leading to α1 dissociation from the remainder of Bak (see diagram in Supplementary Fig. 10). Following this event, exposure of the N terminus allows high-affinity interaction of the α1–α2 loop with the heavy chain to persist without hindering formation of BH3-groove dimers (see diagram in Supplementary Fig. 10).

Discussion
We report here that certain antibodies can bind and activate Bak and mitochondrial Bax to induce cytochrome c release. Anti-Bak antibodies required the epitope to be at the start of the α1–α2 loop, and acted by directly dissociating the α1-helix. As antibodies have not been reported to activate either Bak or Bax, nor has the epitope been targeted as a potential site for drug inhibition, our findings provide novel opportunities to develop therapeutics that directly activate Bak, and perhaps Bax.

The activation site we have identified at the start of the α1–α2 loop is remote from two sites targeted by BH3-only proteins. The α2–α5 hydrophobic groove in Bak and mitochondrial Bax is engaged by BH3-only proteins to release α1 and un latch α6–α8 (refs 8–11,19). A second site at the α1–α6 rear pocket in Bak (but not Bax) is initially engaged by BH3-only proteins to release α9 from the groove\textsuperscript{12,13}. The section of the α1–α2 loop bound by three activating antibodies, 7D10, anti-FLAG and 3C10, lies between these two sites.

An intriguing finding was the ability of the 3C10 antibody to trigger cytochrome c release via mitochondrial but not cytosolic Bak, whereas tBid released cytochrome c via both forms. A partial explanation derived from the ability of 3C10 to block Bax translocation triggered by tBid. Indeed, a recent study reported that Bax translocation triggered by tBid can also be blocked by a synthetic Fab that binds residues in α1, α6 and the α1–α2 loop\textsuperscript{35}. The blocking effect of that Fab was attributed to the Fab obstructing tBid from binding to the rear pocket, or suppressing

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Figure 2 | Antibody-triggered Bak activation requires proximity of the epitope to α1. (a) The FLAG epitope at four positions in the Bak α1–α2 loop. The FLAG epitope (red) and glycine residues (underlined) added to optimize epitope presentation are highlighted. (b) The FLAG epitopes allow recognition by anti-FLAG antibody. Membrane fractions from Bak−/−/Box−/− MEFs expressing the indicated hBak variants were immunoprecipitated with the 7D10 or anti-FLAG antibodies and immunoblotted for Bak. IP: immunoprecipitated; UB: unbound. (c) Only epitopes positioned next to α1 allow cytochrome c release by related antibodies. Membrane fractions from b were incubated with tBid, 7D10 or anti-FLAG antibody and assessed for cytochrome c release. (d) Disulfide tethers introduced at five positions in Bak. Cartoon representation of BakΔN19ΔC25 (2IMT, white) highlighting the α1–α2 loop (blue) and cysteine substitutions (red) for each disulfide tether. (e) A tether between the 7D10 epitope and α2 indicates 7D10 does not act directly on α2. Membrane fractions from Bak−/−/Box−/− MEFs expressing the indicated double cysteine variants were pre-incubated with CuPhe to induce a tether (T; Supplementary Fig. 6a), and then incubated with tBid or 7D10 and assessed for cytochrome c release. Note that the H43C:M60C tether lies between the 7D10 epitope and α2, but does not block 7D10 activation of Bak. In b, c and e data are representative of three independent experiments.
conformational changes in the N-terminal surface. As the rear pocket does not seem to be involved in antibody-triggered activation of mitochondrial Bax (or Bak), 3C10 may be unable to activate cytosolic Bax because it suppresses release of α2 from the Bax groove, or because it more directly prevents Bax from associating with the mitochondrial outer membrane.

While BH3-only proteins do not bind the α1–α2 loop, several non-Bcl-2 proteins reported to directly activate Bak or Bax, may do so. These include Bif-1, αβ amyloid oligomers, protein disulfide isomerases and p53 (refs 15,18,38–40), although p53 has recently been reported to bind to the Bak N–α1 region41. As these proteins lack well-defined BH3 domains, they may bind to the α1–α2 loop, or to a region nearby, to dislodge the α1 helix. It is also possible that proteins may bind to this region to inhibit Bak and Bax activation, as proposed for the Bcl-2 BH4 fragment42, and act in a similar way to tethering the α1–α2 loop within Bak (Fig. 2d,e) or within Bax12.

Bak conformational changes induced by antibody (see diagram in Supplementary Fig. 10) are similar to those induced by BH3-only proteins (see diagram in Supplementary Fig. 6c), as both agents induced similar Bak conformational change and oligomerization, and cytochrome c release initiated by either agent was blocked by each of four tethers within Bak. However, the initial unfolding events triggered by both agents were different, consistent with distinct binding sites on Bak. tBid binding first initiates movement of α2 (Supplementary Fig. 6c)8,19, consistent with binding to the α2–α5 groove. In contrast, 7D10 binding initially triggers α1 dissociation, as 7D10 and FLAG epitopes allowed activation only when placed proximal to α1, and tethering the loop to α1 did not block 7D10-triggered Bak activation. Moreover, molecular dynamics of a 7D10:Bak structural model caused extrication of α1 from α2 to form cavities between the two helices.

**Figure 3 | Bax at mitochondria can also be activated by an antibody to the α1–α2 loop.** (a) Substitutions in mitochondria-targeted Bax to generate the 7D10 epitope. The α1–α2 loop in BaxS184L highlighting substitutions (red). (b) The 7D10 epitope allows 7D10 to bind BaxS184L and trigger cytochrome c release. Membrane fractions from Bak−/−/Bax−/− MEFs expressing either of the BaxS184L variants were incubated with tBid or 7D10, and tested for 7D10 immunoprecipitation of Bax (b) and for cytochrome c release (c). (d) The 3C10 epitope maps to the Bax α1–α2 loop, close to α1. Immunoreactivity of the rat monoclonal antibody, clone 3C10, towards biotinylated human Bax peptides as determined by enzyme-linked immunosorbent assay. X axis indicates Bax residue number or control reactions. Mean and s.d. of three independent experiments. (e) 3C10 binds non-activated BaxS184L. Membrane fractions from Bak−/−/Bax−/− MEFs expressing BaxS184L were immunoprecipitated with the 3C10 or anti-FLAG (negative control) antibodies and immunoblotted for Bax. (f) 3C10 activates and oligomerizes BaxS184L to release cytochrome c. Membrane fractions from e were incubated with tBid, 3C10 or anti-FLAG and assessed for disulphide-linked dimers (D, upper panel) and for cytochrome c release (lower panel). Note that after CuPhe addition, the monomer (M) appears to link to very high-order species. (g) 3C10 binds WT Bax. Lysates of Bak−/−/Bax−/− MEFs expressing Bax (Exp.) or of Bak−/−/Bax−/− membranes reconstituted with recombinant Bax (100 nM, Rec.) were immunoprecipitated with 3C10 and immunoblotted for Bax. (h) 3C10 fails to trigger WT Bax translocation to mitochondria and release of cytochrome c. Permeabilized Bak−/−/Bax−/− MEFs expressing hBax (Exp.) or Bak−/−/Bax−/− MEF membranes reconstituted with recombinant Bax (50 nM, Rec.) were incubated with tBid or 3C10. Supernatant (Sup) and pellet (Mito) fractions were immunoblotted for Bax, VDAC or cytochrome c. (#, light chain). (i) 3C10 blocks Bax translocation and integration and cytochrome c release triggered by tBid. Bak−/−/mouse liver mitochondria were incubated as in h, but an additional sample also contained both 3C10 and tBid. Samples were assessed for Bax membrane integration (i), or for cytochrome c release (j) as in h. In b, c and e–j data are representative of three independent experiments.
As either Bak or Bax is essential for mitochondrial apoptosis, developing agents that directly trigger their activation is of great interest\textsuperscript{43,44}. Developing anti-cancer agents that target the Bak loop may complement BH3-mimetics that target the Bcl-2 prosurvival proteins\textsuperscript{1,45}. While BH3-mimetics release endogenous BH3-only proteins that activate both Bak and Bax\textsuperscript{46–48}, the 7D10 antibody, and other activators that might directly dislodge α1, are likely to specifically bind Bak due to the lack of sequence homology in the loop regions. This specificity also implies that this class of activators cannot be sequestered by prosurvival Bcl-2 proteins such as Bcl-x\textsubscript{L} and Mcl-1, and could bypass resistance caused by those proteins.

It remains to be demonstrated that the 7D10 Fab-induced changes in oocyte mitochondrial morphology are due to Bak conformational change. There are very few studies investigating apoptosis in oocytes at the immature fully grown germinal vesicle stage we have used. It is well known that early oocytes in primordial follicles undergo a TAP63-mediated apoptosis in response to DNA damage\textsuperscript{49} but downregulation of TAP63 on the initiation of oocyte growth is thought to make fully grown oocytes, such as those used here, more refractory to apoptosis\textsuperscript{50}. Some evidence of oocyte fragmentation to form apoptotic bodies has been reported when mature ovulated oocytes are allowed to age \textit{in vitro} but the outward signs of apoptosis remain controversial at these mature stages\textsuperscript{51,52}. The limited availability of healthy human oocytes for these studies is a practical limitation on our ability to formally prove the association of mitochondrial morphology with Bak conformational change.

In conclusion, we have identified the proximal α1–α2 loop as a second activation site in Bak and in mitochondrial Bax. This site

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**Figure 4** | A structural model indicates that 7D10 binds under the α1/loop region to generate cavities underneath α1. (a) Final model of the Bak-7D10 complex following docking and MD simulations. Bak is shown as cartoon (white), with α1 (orange), the α1–α2 loop (blue) and α2 (red) highlighted. The 7D10 variable regions are shown as cartoon, with heavy chain (teal) and light chain (cyan) as indicated. Note that the 7D10 epitope is clasped by the CDR2 and CDR3 of the heavy chain (upper insert), with CDR3 inserting in a groove underneath the α1/loop region (lower insert; V\textsubscript{L} omitted for clarity). (b) Flexibility of Bak before and after 7D10 binding from molecular dynamics simulations, displayed in putty format. An ensemble of all structures generated throughout the MD simulation show that before antibody binding (left), the 7D10 epitope and α3 are flexible. Antibody binding (right) selects for a conformation of the epitope belonging to this ensemble of structures with similar energy. Colours as in a. (c) Molecular dynamics indicate new cavities form between α1 and α2 after 7D10 binds to Bak. The ensemble of all cavities identified throughout the MD simulation is represented as a purple surface, with other colours as in a.
can be targeted by antibodies to directly dissociate Bak, and as a result induce the same Bak oligomerization and pore formation that is induced by BH3-only proteins. This site may allow the development of novel therapeutics such as intrabodies based on 7D10, and possibly small molecules, that pray 1 from 12. Unlike the BH3-mimetics, such agents would specifically activate either Bak or Bax, and, as they would not be sequestered by prosurvival Bcl-2 proteins, they may bypass resistance due to those proteins. Conversely, agents that bind the 1/loop region and prevent 1 dissociation may provide novel anti-apoptotic agents.

Methods

Cloning and expression of Bak and Bax variants. Bak and Bax mutants were generated by site-directed mutagenesis and overlap extension PCR using Phusion High-Fidelity Master Mix (New England BioLabs). The two BaxS184L variants also contain two cysteine substitutions in the BH3 domain (S55C and groove (R94C)) to monitor BH3-groove interaction after Bak activation and oligomerization52. Primer sequences are listed in Supplementary Table 1. PCR products were digested with EcoRI and Xhol, and cloned into the pMX-IRES-GFP retroviral expression vector, and the introduced mutations confirmed by DNA sequencing. The variants were then stably transfected in SV40-immortalized Bax−−/−/Bax−/− mouse embryonic fibroblasts (MEFs), and were a gift from Professor David Huang. MEFs were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 0.1 mML-asparagine and 55 mM 2-mercaptoethanol. All cultures were maintained at 37 °C in a humidified 10% CO2 incubator.

Cell death assays. For each cell line expressing a Bak or Bax variant, 20,000 and 80,000 cells were seeded in a six-well plate and on the following day wells seeded with 80,000 cells were incubated with 10 μM etoposide for 24 h to induce cell death. Cells were collected and resuspended in KDS-BSS buffer and 5 μg ml−1 propidium iodide (PI), and cell death (%PI-positive) assessed by flow cytometry.

Preparation of mitochondrial fractions. Mitochondria-enriched membrane fractions were generated by resuspending MEFs at 1 × 107 ml−1 in MELB buffer (93.5 mM sucrose, 20 mM HEPES, pH 7.4, 2.5 mM MgCl2 and 100 mM KC1) supplemented with Complete Protease Inhibitor cocktail (Roche) and 0.025% w/v digitonin to permeabilize the cell membrane. Cells were left on ice for 10 min and centrifuged at 13,000 g for 5 min to separate the supernatant (cytosolic) and pellet (mitochondria-enriched membrane) fractions, and the membrane fractions resuspended in MELB buffer with protease inhibitors.

Mitochondrial incubations and cytochrome c release assays. For activation of Bak or BaxS184L and mitochondrial cytochrome c release, 50 μl of membrane fractions were incubated with 100 mM n-capas8-8-cleaved human Bid (B1B)53, or with the indicated antibody (5 μg) for 30 min at 30 °C. Activation of WT Bak expressed in cells or as recombinant protein9 (Fig. 3h,i) is described in the figure legends. To monitor cytochrome c release from mitochondria, reactions were spun at 13,000 g and the supernatant and pellet fractions immunoblotted for cytochrome c.

Bak activation assessed by limited proteolysis. Bak conformational change was assessed by limited proteolysis9. Briefly, 50 μl of membrane fractions resuspended in MELB buffer with 4 μg ml−1 pepstatin A (Sigma) were pre-chilled to 0 °C then incubated with 30 μg ml−1 proteinase K (Roche) or trypsin (Sigma) on ice for 20 min, or 0.06 μU ml−1 enterokinase (Merck Millipore) at room temperature for 2 h. The reactions were stopped by addition of 1 mM phenylmethylsulfonyl fluoride (PMFS) and immunoblotted with antibodies to the Bak BH3 domain (4B5).

Disulfide bond formation in Bak. Bak conformational change and oligomerization was assessed by inducing disulfide bonds within or between Bak molecules. Membrane fractions were exposed to the red catalyst copper (II) (1,10-phenanthroline), (CuPhe) diluted 100-fold from a stock containing 30 mM CuSO4 and 100 mM 1,10-phenanthroline in 4 ml waterethanol54. Reactions were incubated for 30 min on ice and then re-suspended in SDS sample buffer containing 20 mM EDTA after analysis by non-reducing SDS–PAGE and immunoblotting. Where indicated, CuPhe was also used to ‘teether’ non-activated Bak by forming disulfide bonds between cysteine residues55. Briefly, fractions were incubated with CuPhe (1 μM) for 5 min on ice followed by quenching with 20 mM N-ethylmaleimide (NEM) for 10 min at room temperature.

Immunoprecipitation. Briefly, whole cell or membrane fractions from 3–5 × 106 cells were solubilized with 1% w/v digitonin in lysis buffer (20 mM Tris, 150 mM NaCl, 1.5 mM MgCl2, 1 mg ETGA, 10% glycerol, pH 7.4) for 1 h on ice. Lysates were centrifuged at 13,000 g for 5 min and pre-cleared with 50 μl Protein G sepharose (Amersham Biosciences). Pre-cleared supernatant was then incubated with antibody (4 μg) and Protein G sepharose. Unbound proteins were collected and the resin washed with lysis buffer containing up to 0.1% w/v digitonin. The immunoprecipitated proteins (IP) were eluted by boiling in sample buffer, and together with unbound (UB) and total lysates (input), were analysed by immunoblotting. Several rat monoclonal antibodies generated in-house were used for immunoprecipitation, including clones 8F8 and 7D10 that recognize Bak9,20, and clone 3C10 that recognizes Bax (Fig. 3d). Other antibodies include mouse monoclonal anti-FLAG (M2; Sigma) and several anti-Bak antibodies described in Supplementary Fig. 2.

SDS-PAGE and western blotting. Samples were resolved by SDS–PAGE (Bio-Rad), and transferred to 0.22 μm nitrocellulose or polyvinylidene difluoride membranes. Primary antibodies used were rabbit polyclonal anti-Bak aa23–38 (1:5,000; #B8897; Sigma), rat monoclonal anti-Bak that recognizes Bak aa82–86 (1:2,000, clone 4BS, R. Kluck), rat monoclonal anti-Bak that recognizes Bak aa51–55 (1:2,000, clone 7D10, D.C. Huang), rat monoclonal anti-Bax that recognizes Bak aa12–24 (1:5,000, clone 49P9, D.C. Huang), rabbit polyclonal anti-Bak NT (1:1,000, #ABCl1; Millipore), mouse monoclonal anti-cytocrome c (1:2,000, #556433; BD Pharmingen) and mouse monoclonal anti-VDAC (1:2,000, #AB10527, Merck Millipore). Detection was achieved using horseradish peroxidase (HRP)-conjugated anti-rabbit (1:5,000, #4010-05, Southern Biotech), anti-rat (1:1,000, #3010-05, Southern Biotech) and anti-mouse (1:1,000, #1010-05, Southern Biotech) secondary antibodies. To avoid signals from antibody light chains in western blots, heavy chain-specific HRP-conjugated goat anti-rabbit IgG (1:5,000, #4014-05; Southern Biotech) and goat anti-rat IgG (1:5,000, #3030-05; Southern Biotech) were also used. To identify Fab fragments, HRP-conjugated goat anti-rat light chain-specific IgG (1:2,000, #112-035-175; Jackson ImmunoResearch) was used. Proteins were visualized by Luminata Forte Western HRP substrate (#WBLUF0500, Millipore) on a ChemiDoc XRS + System, and images processed with ImageLab Software (Bio-Rad). Uncropped images from Figs 1–3 are shown in Supplementary Figs 11–13.

Bax membrane integration. To assess Bax membrane integration, membrane fractions were subjected to carbonate extraction. Mitochondria isolated from 2Bak−−/− mouse liver were incubated for 2 h at 37 °C with 50 mM recombinant Bax, and with B1B and 3C10 as indicated. The incubations were centrifuged at 13,000 g for 5 min to obtain cytosolic and pellet fractions. The pellet was re-suspended in 0.1 M Na2CO3 (pH 11.5) and incubated on ice for 20 min. pH was neutralized with an equal volume of 0.1 M HCl and the sample incubated at 22 °C for 5 min. Samples were then supplemented with nuclease buffer from a 10 × stock (400 mM Tris, 100 mM MgSO4, 10 mM CaCl2 and 1 U DNase, before being centrifuged at 13,000 g for 10 min, and supernatant (peripherally attached) and pellet (membrane inserted) fractions immunoblotted for Bax.

Generation and purification of 7D10 Fab. The 7D10 antibody was cleaved with papain to generate the Fab fragment. Papain (Sigma) at 1 mg ml−1 was solubilized and activated in 10 mM cysteine and 20 mM EDTA in PBS for 10 min on ice. Following 10-fold dilution in PBS, papain was added to the 7D10 antibody at a ratio of 1:20 and incubated at 37 °C followed by inactivation with 30 mM iodoacetamide (Sigma). Papain-cleaved antibody was dialysed overnight against 20 mM Tris-HCl buffer (pH 7.5). Fab fragments were then purified by the protein A-Sepharose affinity column, 1 ml of 200 mM NaCl, pH 4.5 and applied to a Mono S column equilibrated in Buffer A. A linear 20 ml gradient with Buffer B (10 mM acetic acid, pH 4.5) and applied to a Mono S column equilibrated in Buffer A. A linear 20 ml gradient with Buffer B (10 mM acetic acid, pH 4.5) was used to separate Fab and Fc in 0.5 ml fractions.

Microinjection of 7D10 Fab. Human germinal vesicle (GV) stage oocytes were collected from Monash IVF Australia under ethics approval number CF13/2664-2013001409 provided by the Monash University Human Research Ethics Committee. Oocytes were injected in M2 media (Sigma-Aldrich) containing 20 mM triethanolamine and 10 μM 3-isobutyl-1-methyl-xanthine (IBMX, Sigma-Aldrich) at 37 °C, for 30 min. Oocytes were then either microinjected9 with Fab from 7D10, or with control Fab from the rat monoclonal 9E10 anti-c-Myc antibody (kindly provided by J. Menting), and imaged live using confocal microscopy (Leica SP8) every 10 min for 3 h.
Image analysis. Accurately scaled single slice oocyte images at 3 h post injection were converted using ImageJ to 8-bit then a threshold was applied accordingly to each image. An ROI around each oocyte was then used to analyse the number and size of particles.

Molecular dynamics and protein–protein docking. Computational docking of Bak and 7D10 was performed using structures of 7D10 obtained from the RosettaAntibody3 online server58, and structures of Bak obtained from molecular dynamics (MD) simulations. The sequence of 7D10 is available from International Patent Application No. PCT/AU2015/000290. Flexibility (beyond just side-chain movement) was incorporated in the docking by considering multiple structures of both Bak and 7D10. Molecular dynamics simulations used the GROMACS (v5.0.4) package of programs59 with the OPLS-aa force field60. Ionizable residues were assumed to be in their charged state. Each molecule was solvated in a water box extending 10 Å beyond all atoms; sodium and chloride ions were added to neutralize the system (including water) confined between the two plates with a size of particles.

Data availability. The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files.

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Author contributions

S.I., K.A., A.E.A., W.S.Y. and N.A.S. designed and performed experiments, D.C.S.H. generated the 7D10 antibody, J.C., B.J.S. and G.D. designed experiments, and R.M.K. supervised the project. D.C.S.H., J.C., B.J.S. and G.D. designed experiments and supervised the project.

Additional information

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