



Mechanistic Insights into the Conformational Regulation of Pro-Apoptotic BAX

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*Mechanistic Insights into the Conformational Regulation of Pro-Apoptotic
BAX*

presented by Noah Benjamin Bloch
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Mechanistic Insights into the Conformational Regulation of Pro-Apoptotic BAX

A dissertation presented

by

Noah Benjamin Bloch

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor in Philosophy

in the subject of

Biological Chemistry and Molecular Pharmacology

Harvard University

Cambridge, Massachusetts

December 2021

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Mechanistic Insights into the Conformational Regulation of Pro-Apoptotic BAX**ABSTRACT**

BCL-2 family proteins are apoptotic regulators that control the critical balance between cellular life and death at the level of the mitochondrion. BAX is a principal executioner of apoptosis, transforming from a latent cytosolic monomer into a lethal mitochondrial oligomer in response to cell stress. Because renegade BAX activation poses a grave risk to the cell, the architecture of BAX must ensure monomeric stability yet enable conformational transformation. The specific structural features that afford both stability and dynamic flexibility remain ill-defined and represent a critical control point of BAX regulation. In this dissertation, I identified a nexus of interactions involving discrete residues of BAX's core $\alpha 5$ helix, specifically amino acids 113-116, which are individually essential to maintaining the structural stability of monomeric BAX and are collectively required for higher order BAX assembly. Single alanine mutagenesis of residues 113-116 resulted in autoactive proteins that were capable of membrane permeabilization even in the absence of BH3-only ligand stimulation, which otherwise induces the activation of wild-type BAX. Analysis of conformational dynamics by hydrogen deuterium exchange mass spectrometry revealed regiospecific changes in BAX structure, providing a mechanism for mutant autoactivity. Further, combinatorial mutagenesis of the BAX 113-116 nexus revealed the collective role of these residues in stabilizing the dimeric form of BAX, which represents a key step of oligomeric self-assembly. The dual yet distinct roles of BAX residues 113-116 highlight the intricacy of BAX conformational regulation and opportunities for modulating BAX conformational dynamics for therapeutic benefit in diseases of deregulated apoptosis.

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For the blessed memory of my grandparents,

Israel “Sol” and Sabina Rosen

Max and Emily Bloch

CHAPTER 1

Introduction

Apoptosis and its Regulation by BCL-2 Family Proteins

Programmed Cell Death

Cells are the most basic unit of life, yet also the most basic unit of death. In the setting of acute and overwhelming physical or chemical stress, necrosis ensues, a less orchestrated and “messier” form of cell death. In contrast, sustained physiologic or pharmacologic stress causes apoptosis, a form of programmed cell death that is remarkably methodical and efficient. Just as cells serve a variety of functions in life, cell death can also function to protect tissues and enable the organism to survive in the face of threat¹. Apoptosis requires energy, and organisms encode a series of apoptosis signaling programs in their genetic material²⁻⁶. Thus, the same genetic blueprints that enable life encode the cellular pathways to death.

How does it benefit a cell to die, and why might there be different programs for death? In multicellular organisms with dedicated immune systems, cell death is generally classified as either immunogenic or non-immunogenic^{1,7,8}. If a virus has infected a cell, it not only benefits the organism for the infected cell to self-destruct, preventing further intracellular replication of the virus, but also to activate the immune system to attack the remainder of the circulating pathogen. Pyroptosis and necroptosis are just two examples in humans of genetically controlled death that result in the lysis of the plasma membrane and release of inflammatory molecules^{1,7,8}. Even single-celled organisms possess mechanisms of programmed cell death, which are also thought to prevent the spread of viruses as defense for the colony as whole^{6,9}.

Cell death can also benefit an organism by removing excess, stressed, or damaged cells without the need for inflammation that can damage nearby tissues^{4,10}. Apoptosis is the most common and best studied form of such a non-immunogenic mode of programmed cell death. The distinct morphological features of apoptosis provided scientists a visual basis for categorizing different forms of cell death. Described by Kerr, Wyllie, and Curie in 1972, apoptotic cells separate

from their neighbors and begin a process of compaction and condensation of cell materials into so called 'apoptotic bodies', which are promptly phagocytosed and remnant cell materials recycled¹¹. As an important distinction from necrosis and inflammatory cell death pathways, the plasma membrane remains intact during this process and cellular contents are not released into the environment^{1,7}. Apoptotic morphology had been observed throughout healthy adult tissues, where it was interpreted to be a homeostatic mechanism to control cell number in an organism. Indeed, the term apoptosis is derived from the Greek language and is translated as "falling of leaves from trees," in appreciation for the natural role of apoptosis in organism homeostasis¹¹. Cycles of mitosis occurring throughout the body are balanced by apoptosis and removal of cells in a delicately regulated equilibrium.

During development, cell death serves a critical function in determining the shape of organs, including lumens (the central cavities of certain organs), among other tissue specific functions^{10,12-15}. Thus, in addition to serving as a reactionary process to counterbalance stress or mitotic activity, the death of certain cells is predetermined from development through adulthood. The impact of apoptosis on cell count is readily apparent during development, with scientists having tracked individual cell fates. Even before the morphological classification of apoptosis, developmental biologists noticed that cells would die in a predictable manner, as first observed by 19th century scientist Carl Vogt and described in his studies of neuronal development in toads¹⁶. John Sulston and colleagues mapped out each specific instance of cell death, 131 in total, during the development of the nematode model organism *C. elegans*, as part of their series of seminal works tracing the entire cell lineage from single-celled egg to adult animal¹⁷⁻¹⁹. Equipped with the knowledge of which 131 cells are generated prior to programmed cell death, the laboratory of Robert Horvitz harnessed genetic methods of the nematode system to investigate the molecular mechanism(s) of cell death¹⁹. A single mutation in a gene called *ced-1* (cell death abnormal 1), resulted in the failure of worms to phagocytose apoptotic bodies and revealed the visual remnants

of cells that had undergone apoptosis²⁰. The Horvitz lab used this particular variant to identify genes that are required for the formation of apoptotic bodies, whereby a loss-of-function mutation that abrogated apoptotic body formation would identify genes required for apoptosis. The discovery of one such gene, *ced-3*, demonstrated for the first time that cell death was a genetic program requiring a specific protein's function^{19,21}. The Horvitz group soon found another gene that controlled cell death, called *ced-9*, but its function was instead to inhibit apoptosis²². The Horvitz lab continued to map the pathway in which *ced-3* initiates the cell death program upon activation by *ced-4*, a process antagonized by *ced-9* and relieved by *egl-1* inhibition of *ced-9*¹⁹. This first genetic mapping of the apoptotic pathway transformed our understanding of what began as a histological observation into a fundamental protein interaction mechanism that dictates cellular life and death.

The Discovery of the BCL-2 Family Proteins

Before Horvitz and colleagues mapped the apoptotic pathway out in worms, immunologists and oncologists were already busy studying a human gene named B-cell lymphoma 2 (*BCL2*). *BCL2* was cloned at the breakpoint of a translocation between chromosomes 14 and 18 (t14:18), a genetic defect found in most follicular B-cell lymphomas²³⁻²⁵. This translocation results in the overexpression of *BCL2* driven by the immunoglobulin heavy chain (IgH) gene enhancer²⁶. Unlike other known oncogenes at the time like *MYC*, *BCL2* did not promote proliferation, but rather prevented the death of lymphoid cells *in vitro* in the absence of growth factors^{27,28}. While Kerr, Wylie, and Curie posited that the rate of apoptosis can counteract tumor growth, the discovery that the *BCL2* oncogene actively suppressed cell death established blockade of apoptosis as a hallmark of cancer^{11,29,30}. Almost a decade later, the study of a chemically-induced leukemia resulted in the identification of another gene, *MCL1*, that promotes survival³¹. The homology between *MCL1* and *BCL2* provided scientists the earliest insight into the existence of a gene

family committed to apoptosis regulation³¹. Soon after, *ced-9* was cloned and found to be homologous to *BCL2*, an exciting discovery described to be Bob Horvitz's "aha!" moment^{19,32}. The death pathway first reported in worms shared a key component with an analogous signaling network in humans, highlighting the value of studying model organisms that provide robust and facile genetic manipulation.

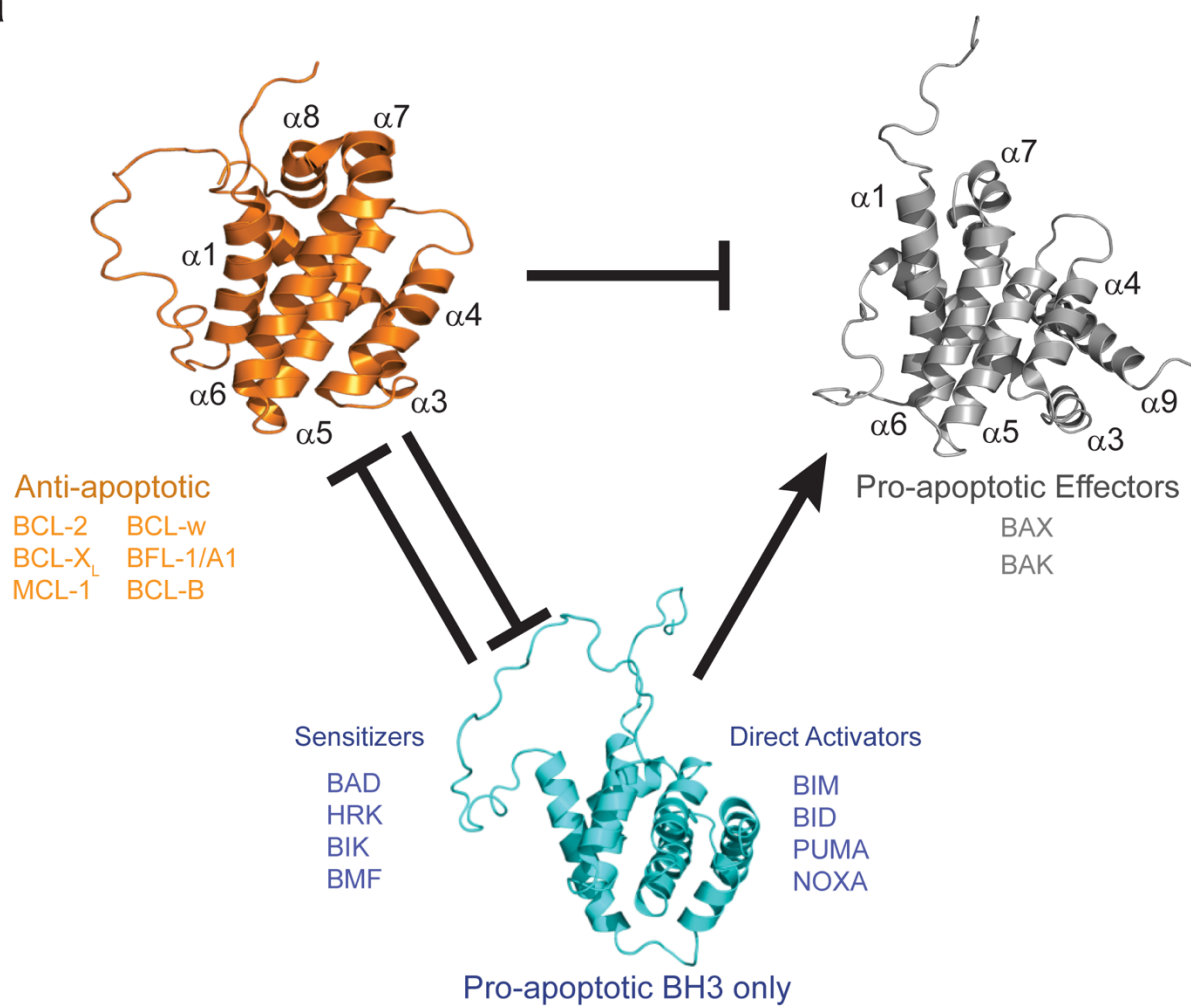
The homology between *BCL2* and the apoptosis inhibitor *ced-9* inspired the laboratory of Stanley Korsmeyer to hypothesize that humans likely possess effectors of apoptosis similar to *ced-3* or *ced-4*. To search for BCL-2 protein binding partners that could help explain the mechanism of regulation, the Korsmeyer group embarked on BCL-2 co-immunoprecipitation studies and identified BCL-2 associated X-protein (BAX) as a BCL-2 binding protein³³. While BAX was homologous to BCL-2, it unexpectedly enhanced, rather than inhibited, cell killing when overexpressed in cells challenged by IL-3 withdrawal. In addition to heterodimerizing with BCL-2, BAX was also observed to homodimerize with itself. Furthermore, it appeared that the balance between BCL-2 and BAX expression correlated with the cell's decision to live or die. Korsmeyer proposed a rheostat model for cell death, whereby BCL-2 heterodimerization with BAX competes with BAX homodimerization, and that BAX homodimerization was critical for initiating apoptotic cell death³³.

The rheostat model provided a new paradigm for this emerging family of BCL-2 proteins: protein-protein interactions between homologues determine the ultimate cellular outcome of life or death. Indeed, interaction screens and homologue searches revealed more and more members, resulting in BCL-2 family classification into three major categories (**Figure 1.1**)³⁴⁻³⁶. The first class consists of anti-apoptotic members, such as BCL-2, MCL-1, and BCL-X_L, which promote cellular survival. The second class is the pro-apoptotic effector proteins, BAX and BAK, which homo-oligomerize to initiate apoptosis. Interestingly, these two classes of proteins with diametrically opposing functions share four regions of sequence homology, called BCL-2

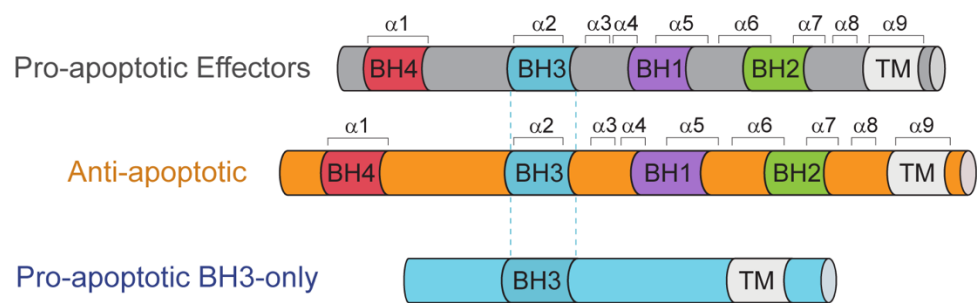
Figure 1.1 *The BCL-2 family interaction network.* (a) BCL-2 family proteins can be classified into three groups based on their structure and function. The anti-apoptotic BCL-2 family members (orange; represented by BCL-X_L, PDB: 1LXL) block the activation of the pro-apoptotic members (gray; represented by BAX, PDB: 1F16), which can otherwise permeabilize the mitochondrial outer membrane and initiate apoptosis. The BH3-only proteins (blue; represented by BID, PDB: 2BID) serve as modulators of this network by inhibiting the anti-apoptotic members, resulting in the derepression of BAX/BAK inhibition. A subset of the BH3-only proteins can also directly activate BAX/BAK and are also subject to inhibition by the anti-apoptotic proteins. The “multidomain” anti- and pro-apoptotic proteins share a similar nine-helical bundle structure composed of four regions of sequence homology called BCL-2 homology (BH) regions. (b) Sequence alignment of the three BCL-2 family subclasses showing the relative positioning of the BH motifs, α -helices, and the transmembrane domain (TM).

Figure 1.1 (Continued)

a



b



Homology (BH) domains, BH1-4³⁶. The third class of BCL-2 family proteins consists of the BH3-only subgroup, so named because they only share homology with the other members in the BH3 region. BH3-only proteins play pivotal pro-apoptotic roles by interacting with both classes of “multidomain” anti- and pro-apoptotic proteins³⁶. Together, the BCL-2 family regulates the mitochondrial pathway of apoptosis.

Mitochondrial Pathway of Apoptosis

The ultimate consequence of BAX/BAK activation and self-association is mitochondrial outer membrane permeabilization (MOMP), which is believed to be the “point of no return” for apoptosis induction. When the cell experiences internal distress, such as DNA damage, ER stress, oxidative stress, nutrient deprivation, or cytokine withdrawal, apoptosis is initiated through the intrinsic pathway (**Figure 1.2**)³⁷. BH3-only protein activity is heightened by transcriptional activation and/or post-translational modification^{5,38-44}. BH3-only activators, such as BID, BIM, PUMA, and NOXA, transiently bind to BAX/BAK, triggering a global conformational change that transforms BAX/BAK from inactive to activated states. A key structural feature of this process for BAX includes the release of its $\alpha 9$ helix, which results in translocation from the cytosol to the mitochondria (BAK is constitutively localized at the mitochondria) (**Figure 1.3**)^{5,44-48}. A critical consequence of BAX/BAK activation is exposure of the BH3 domain, which can alternatively be trapped by a ‘BH3-in-groove’ interaction involving a canonical pocket on anti-apoptotic members or enable BAX/BAK homo-oligomerization and MOMP^{45,49,50}. Activated monomeric conformers of BAX/BAK that become trapped by anti-apoptotic members can be liberated by the BH3-only proteins. BH3-only ‘sensitizers’, such as BAD and HRK, function solely to bind and inhibit anti-apoptotic proteins, whereas the BH3 ‘activators,’ such as BID and BIM, are capable of targeting anti-apoptotic proteins in addition to directly activating BAX/BAK^{5,36}. Once released, BAX/BAK homo-oligomerization and MOMP ensues, releasing cytochrome c from the mitochondria into the

Figure 1.2 *The intrinsic and extrinsic pathways of apoptosis.* The intrinsic pathway of apoptosis (right) is driven by cell stress, such as DNA damage, ER stress, and nutrient deprivation. The BCL-2 family controls the critical step of mitochondrial outer membrane permeabilization (MOMP), which releases cytochrome *c* and SMAC/DIABLO from the mitochondria into the cytosol. Soluble cytochrome *c* binds to the adaptor protein APAF-1, forming the apoptosome that cleaves and activates caspase-9, which can further cleave executioner caspases-3/7, allowing them to proteolytically dismantle their cellular substrates and execute apoptosis. Inhibitor of apoptosis proteins, such as XIAP, reside in the cytosol to inhibit caspases, but this inhibition is derepressed upon the mitochondrial release of SMAC/DIABLO, which blocks the IAPs. The extrinsic apoptotic pathway (left) is driven by the binding of death ligands to cell surface death receptors such as TRAIL-R and FAS, resulting in intracellular clustering of receptor components, such as the adaptor protein FAS-associated death domain protein (FADD), and pro-caspases-8/10. The formation of this complex results in the cleavage and activation of the initiator caspases-8/10, which can cleave executioner caspases to drive apoptosis. While the extrinsic pathway can operate independently from the mitochondria, death signal amplification can occur through crosstalk with the intrinsic pathway via caspase-8-mediated cleavage of BID to tBID, which then engages the BCL-2 family pathway, resulting in MOMP. Reprinted from reference³⁷ with permission.

Figure 1.2 (Continued)

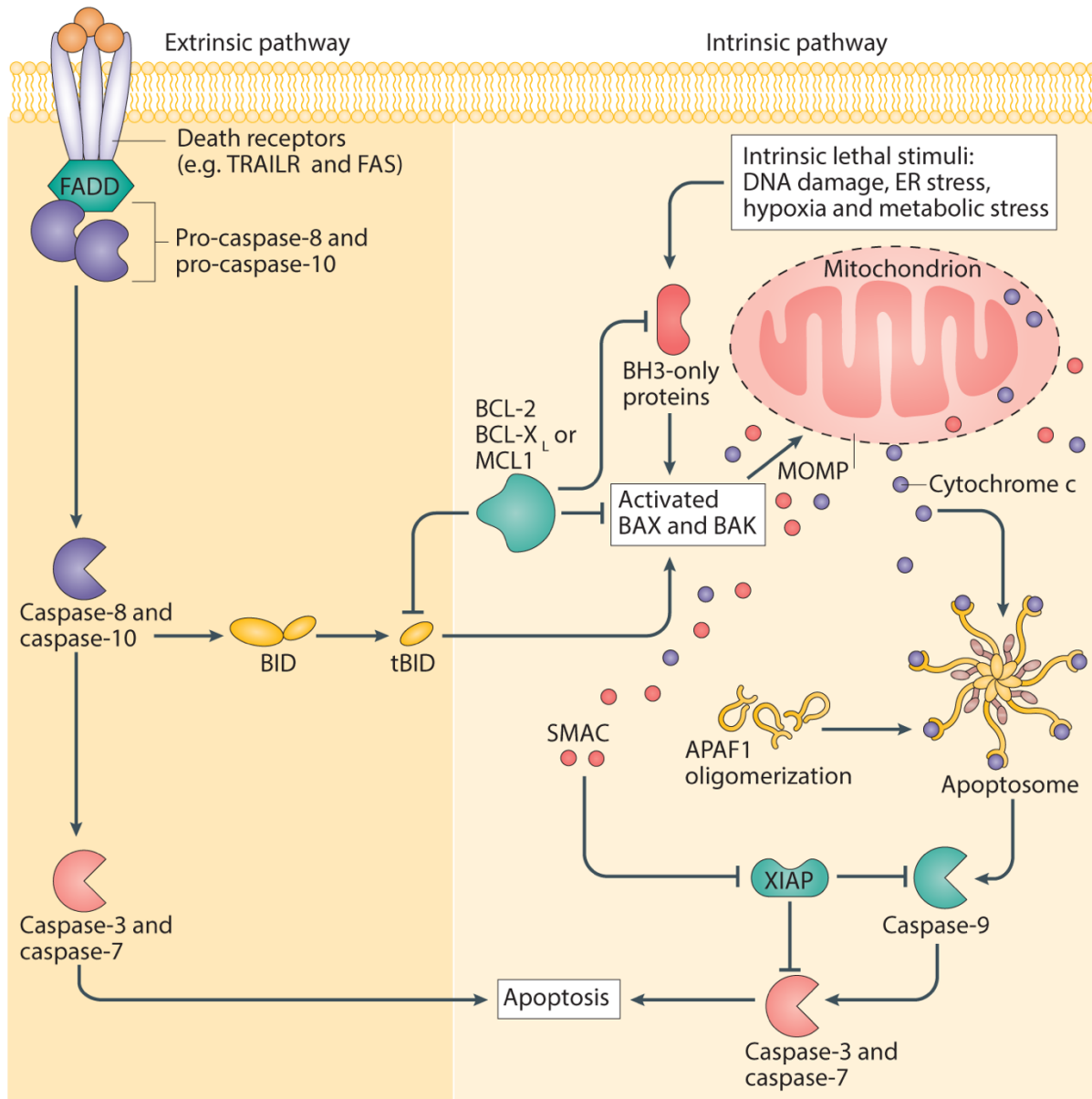
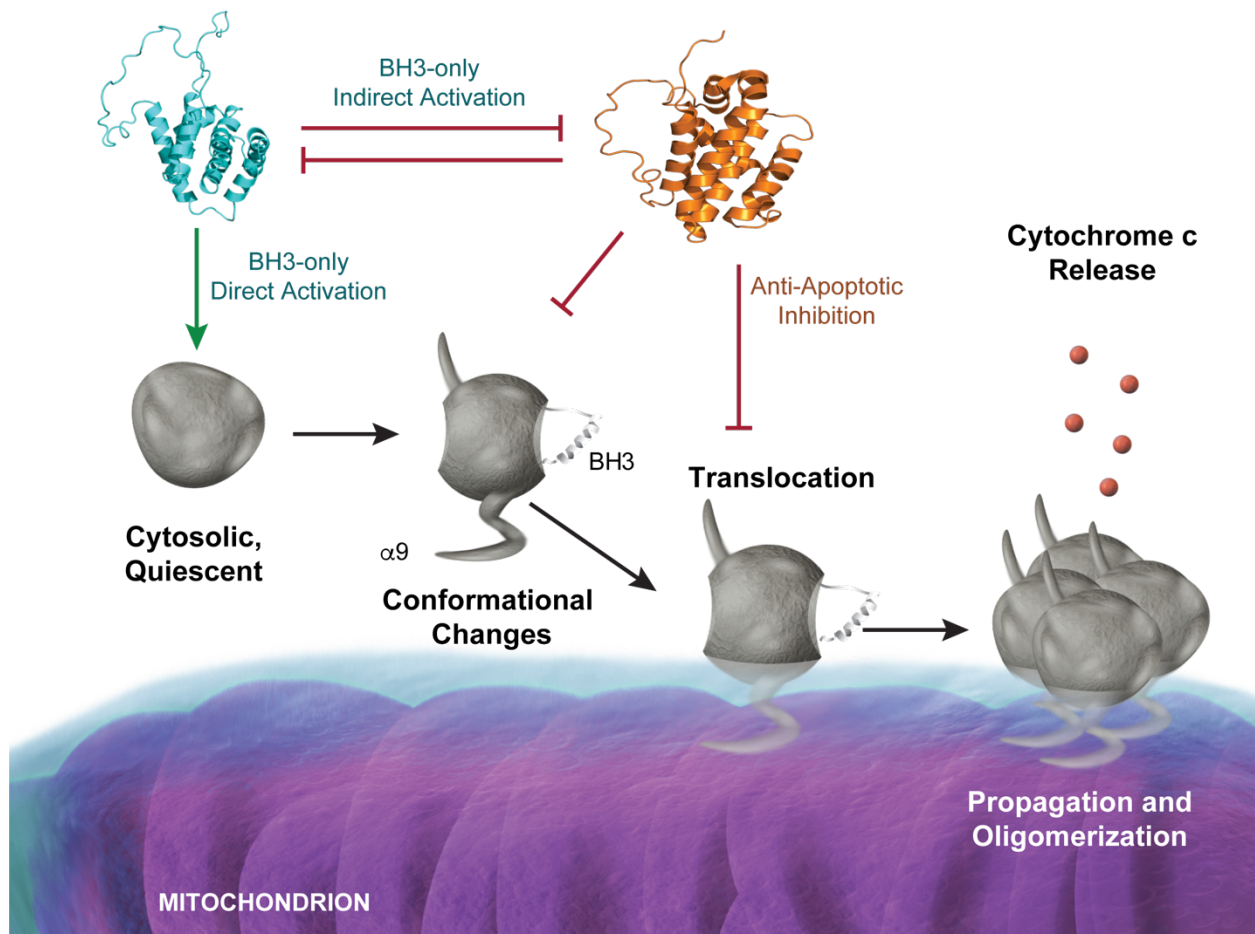


Figure 1.3 *Regulation of BAX-mediated mitochondrial apoptosis.* BAX (gray) transforms from an inactive cytoplasmic monomer into toxic homo-oligomers that permeabilize the mitochondrial outer membrane. Anti-apoptotic proteins (orange), such as BCL-2 and BCL-X_L, bind and sequester the critical BH3-helix of BAX, preventing apoptosis induction. The upstream BH3-only proteins (cyan) such as BIM, tBID, or PUMA can induce apoptosis by inhibiting the inhibitors or by directly activating the activators, such as BAX and BAK. Upon activation in the cytosol, BAX undergoes several conformational changes including the mobilization of the BH3 helix and the release of the $\alpha 9$ transmembrane helix. These structural changes induce BAX translocation from the cytosol to the mitochondrial outer membrane. Homo-oligomerization of BAX at the mitochondria leads to MOMP and release cytochrome *c*, thereby driving apoptosis.

Figure 1.3 (Continued)



cytosol^{36,45,51-55}. Cytochrome c then binds to APAF-1 and promotes assembly of the apoptosome complex (**Figure 1.2**)^{55,56}. The apoptosome then processes the proteolytic maturation of pro-caspase-9 into caspase-9, an initiator caspase, which then cleaves pro-caspase-3 and pro-caspase-7 to activate the executioner caspases-3/7^{55,56}. The executioner caspases are cysteine proteases that hydrolyze the amide bonds of a diverse set of protein targets, resulting in the organized destruction of the cell in preparation for phagocytosis and clearance, as visualized histologically^{55,57}. For example, chromosomal condensation is achieved through caspase-3-mediated cleavage of the inhibitor of caspase-activated nuclease (ICAD), which liberates caspase-activated nuclease (CAD) to fragment DNA through internucleosomal cleavage⁵⁸.

The caspase cascade can also be triggered by the extrinsic pathway of apoptosis, which is initiated upon binding of ligands to extracellular death receptors, such as FAS (**Figure 1.2**)⁵⁹. Upon death receptor activation, intracellular adapter proteins, such as FAS-associated death domain protein (FADD), convert pro-caspase-8/10 into the initiator caspases-8/10, which can directly activate caspases-3/7^{44,59}. In some cell types, caspase-8 will also cleave the BH3-only protein BID to form activated truncated BID (tBID), which promotes MOMP⁶⁰. Expression of inhibitor of apoptosis proteins (IAPs) in these cells directly suppresses caspase activity, but the release of the mitochondrial protein SMAC (also known as DIABLO) upon MOMP allows SMAC to inhibit IAPs and reactivate caspases⁶¹. In this context, the extrinsic pathway of apoptosis is also subject to regulation by BCL-2 family proteins.

BCL-2 Family Proteins in Mammalian Physiology and Disease

Apoptosis serves a critical role in maintaining tissue homeostasis. At the genetic level, apoptosis naturally imposes a negative feedback loop in response to cell proliferation, as exemplified by the inclusion of pro-apoptotic genes *BAX*, *BID*, and *BIM* in the repertoire of targets that are

transcriptionally activated by the mitogen responsive transcription factor MYC^{5,62,63}. Organ systems that require continual proliferation and cell renewal also manifest heightened levels of cell death^{5,62}. For example, the mature hematopoietic system produces a remarkable $\sim 10^{11}$ neutrophils per day, each with a lifespan of ~ 5.4 days, resulting in continual neutrophil apoptosis on a daily basis⁶⁴⁻⁶⁶. Apoptosis is similarly important in regulating cell turnover during spermatogenesis and in the epithelium of the skin, liver, and intestines, where apoptosis is essential to clearing damaged cells^{5,67-72}.

The lymphoid and neuronal lineages are especially sensitive to regulation of apoptosis by the BCL-2 family^{5,62,73}. In mice, knockout of the gene encoding BCL-X_L is embryonic lethal, and these mice exhibit profound apoptosis in their developing brain and hematopoietic systems⁷⁴. Mice lacking *Bcl2* and *Bcl-w* are viable, but knockout of the former results in thymic and splenic involution due to increased apoptosis of developing lymphoid cells; knockout of the latter results in age-related sensory neuropathy, among other phenotypes^{75,76}. Conversely, a lack of apoptosis results in an excess of cells within these lineages. Deletion of the BH3-only protein *Bim* results in excess myeloid and lymphoid cells in mice and causes a fatal autoimmune kidney disease later in life⁴³. Interestingly, while *Bak* knockout mice appear developmentally normal, deletion of *Bax* causes lymphoid hyperplasia and an increase in the number of neurons, which has been linked to behavioral abnormalities⁷⁷⁻⁸⁰. Double knockout of *Bax/Bak* and triple knockout of BH3-only proteins *Bim/Bid/Bbc3* cause partial embryonic lethality (*Bbc3* encodes for PUMA)^{78,81}. Viable *Bax/Bak* double knockout mice have elevated neuronal and lymphoid cell counts, the former leading to unresponsiveness to auditory stimuli and seizures in response to external stress, and the latter resulting in increased lymphocytic infiltration and autoimmune antibodies that react with several tissue types^{78,82}.

Deregulation of apoptosis in humans causes a multitude of diseases that derive from either too many or too few cells. Immunodeficiency and neurodegenerative conditions such as

Alzheimer's and Parkinson's diseases result from excessive hematopoietic and neuronal cell death, respectively^{5,73,83-87}. Decreased cell death underlies autoimmune disorders and a host of human cancers^{5,11,86-88}. Indeed, suppression of apoptosis is a hallmark of cancer^{29,30}. During the course of tumorigenesis, cells experience a diversity of stresses that trigger apoptosis, yet also employ anti-apoptotic BCL-2 proteins to prevent or dampen the apoptotic response^{5,89}. Downregulation of certain BH3-only proteins at both transcriptional and post-translational levels in some cancers sever the connection between sensing cellular stress and inducing apoptosis activation^{5,89,90}. For instance, homozygous deletion of *BIM* has been observed in ~20 percent of mantle cell lymphomas and somatic copy number losses of *BBC3* has also been observed in many cancers^{91,92}. *BAX* and *BAK* are also subject to transcriptional regulation and are downregulated in certain cancer contexts, including chemoresistance⁹³⁻⁹⁵. Loss-of-function missense mutations in *BAX* have been characterized in hematologic malignancies, and frameshift mutations eliminating *BAX* entirely have been reported in colorectal cancer⁹⁶⁻⁹⁸. However, these and other inactivating mutations in *BAX* are not frequent occurrences, and combined loss of *BAX* and *BAK* is even more rare⁹⁸⁻¹⁰¹. Indeed, the apoptotic program is preserved in most cancers and accounts for the cell death that occurs in response to the vast majority of chemotherapeutic agents and precision therapies¹⁰². In the original description of apoptosis, Kerr and colleagues reported spontaneous apoptosis in malignant neoplasms and that apoptosis contributed to tumor shrinkage after chemotherapy treatment¹¹. The most potent and frequently observed mechanism of suppressing apoptosis results from overexpression of the anti-apoptotic members of the BCL-2 family^{90,92,100,101}. *MCL1* and *BCL2L1* (which encodes for BCL-X_L) are among the most amplified genes across human cancers⁹². Anti-apoptotic overexpression is distinctly advantageous for cancer by inhibiting both the BH3-only stress sensors and the pro-apoptotic multi-domain effectors *BAX/BAK*. Nevertheless, reliance on the anti-apoptotic reservoir for apoptotic suppression in cancer often leads to dependence on specific anti-apoptotic proteins, and thus

targeted inhibition can reactivate apoptosis^{90,103,104}. This vulnerability provides a therapeutic opportunity to restore the death pathway in cancer.

Therapeutic Reactivation of Apoptosis in Cancer

The mission of drugging BCL-2 family proteins has matured from hypothesis to proof-of-concept to FDA drug approval in humans, as exemplified by the selective BCL-2 inhibitor venetoclax (VENCLEXTA)¹⁰⁵. Venetoclax is a small molecule BH3 mimetic that binds to the canonical groove of BCL-2, freeing sequestered pro-apoptotic proteins to induce MOMP¹⁰⁶. Scientists at Abbot Labs first developed ABT-737, a potent inhibitor of BCL-2, BCL-X_L, and BCL-w, using fragment-based NMR screening of BCL-X_L¹⁰⁷. Extensive medicinal chemistry efforts led to the orally bioavailable ABT-263 (navitoclax), which was advanced to Phase 1 testing in patients with small cell lung cancer and lymphoid malignancies¹⁰⁸⁻¹¹⁰. On target inhibition of BCL-X_L in platelets, however, led to dose-limiting thrombocytopenia, hampering the clinical development of this compound and motivating the pursuit of BCL-2 selective agents, although ongoing trials continue to investigate the potential of navitoclax in the context of combination therapy^{110,111}. The BCL-2 selective agent, venetoclax, advanced by AbbVie was ultimately found to be more tolerable¹⁰⁶ and is currently approved for the treatment of chronic lymphocytic leukemia (CLL) and small lymphocytic leukemia (SLL), and as a combination treatment with azacytidine, decitabine, or low-dose cytarabine for acute myeloid leukemia (AML).

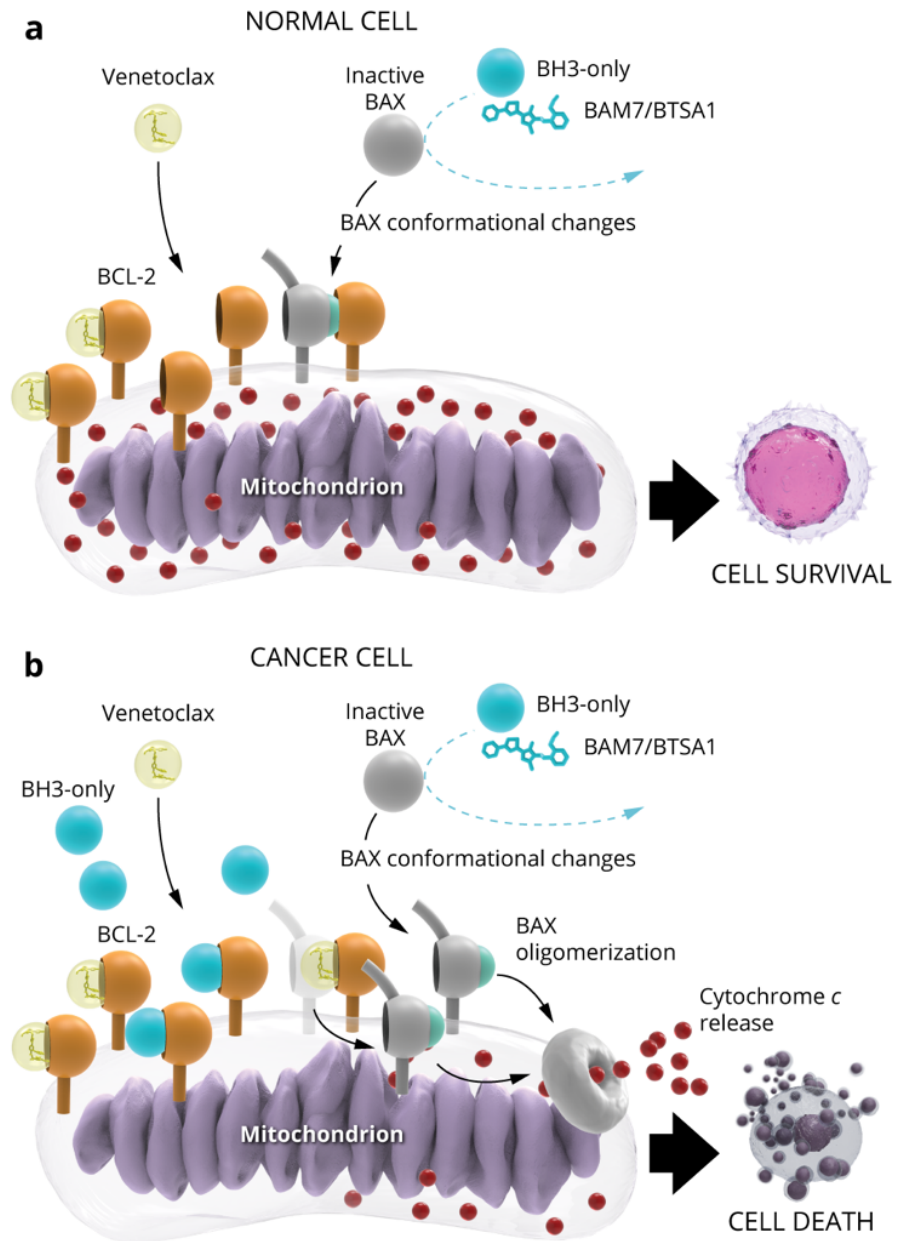
The dependencies of select cancers on BCL-2, and their vulnerability to its inhibition, can be detected by a technique called “BH3 profiling”^{112,113}. BH3 profiling involves treating permeabilized cells (or isolated mitochondria) with a panel of BH3 peptides and reporting the levels of induced MOMP^{113,114}. The power of this approach relies on the unique binding of anti-apoptotic proteins for discrete subsets of BH3-only proteins, whose peptide subcomponents can

thus inform the various anti-apoptotic protein dependences of particular cancer cells. For example, NOXA BH3 can induce MOMP of mitochondria/cells that are dependent on MCL-1 whereas BAD BH3 reports on BCL-2/BCL-X_L/BCL-w dependence; a response to HRK BH3 reports on selective BCL-X_L dependence in suppressing apoptosis^{113,114}. BH3 profiling can also be used to determine the overall susceptibility of a cell to apoptosis induction, or its level of “apoptotic priming” (a reflection of the apoptotic threshold), and has been used to predict sensitivity of cancer cells to chemotherapeutic agents *ex vivo*^{99,115}. Whereas cancer cells demonstrate a variable level of priming, normal cells are typically unprimed, accounting for the therapeutic window of chemotherapeutic agents, including BH3 mimetics (**Figure 1.4**)^{5,99}. For example, in response to chemotherapy and the resultant upregulation of BH3-only proteins, normal cells have a larger reservoir of unoccupied anti-apoptotic pockets to buffer BH3-only proteins, thereby preventing the activation of BAX/BAK. In cancer, however, anti-apoptotic proteins are often already bound by BH3-only proteins as a consequence of ongoing cellular stress; in this context, any further increases in BH3-only protein burden can overwhelm the remaining anti-apoptotic reserve and promptly trigger BAX/BAK activation and apoptosis^{5,99}. Indeed, the activator BH3-only protein BIM has been shown to be required for venetoclax efficacy in CLL and its liberation from BCL-2 results in triggering BAX/BAK^{116,117}.

Could direct activation of BAX/BAK serve as another strategy for tipping the apoptotic balance in cancer cells¹¹⁸? Conceptually, apoptotic priming should allow for a similar therapeutic window for direct BAX activator agents. Whereas healthy cells would be spared from direct BAX activation through sequestration of pharmacologically-activated conformers by the pool of unoccupied anti-apoptotic grooves, cancer cells would be unable to withstand elevated levels of activated BAX due to their relatively limited anti-apoptotic reservoir (**Figure 1.4**). The unique localization of BAX in the cytosol, and the requirement for a distinct triggering step to dislodge its auto-inhibitory $\alpha 9$ helix for mitochondrial translocation, provides a built-in grenade to

Figure 1.4 *Differences in apoptotic priming underly a therapeutic window for pharmacologic targeting of BCL-2 family proteins in cancer.* (a) In normal cells, in the absence of stress, the repertoire of anti-apoptotic proteins, such as BCL-2, are primarily unbound. An increase in BH3-mediated antagonism via the BH3-mimetic venetoclax, or BAX activation by BH3-only proteins or small molecule activators, is therefore tolerated and the cell survives. (b) In cancer cells, however, the reservoir of BCL-2 is already occupied by BH3-only proteins induced by the stress of oncogenesis. Further BH3-mediated antagonism beyond the inhibitory capacity of the anti-apoptotic reserve leads to activation of BAX, which can proceed unopposed to induce apoptosis. Adapted from reference¹¹⁸ with permission.

Figure 1.4 (Continued)



pharmacologically activate cellular suicide^{119,120}. In fact, certain cancer cells appear to enforce a cytosolic localization of BAX to avoid BAX translocation and MOMP. Relatively chemoresistant AML samples were found to have predominantly cytosolic BAX, and a comparison of BAX localization in normal liver tissue versus hepatocellular carcinoma showed relatively more cytosolic BAX in cancer tissue^{121,122}. Interestingly, AKT-mediated phosphorylation of BAX serine 184, which is located within the transmembrane domain, prevents BAX mitochondrial translocation in an ABT-737 resistant breast cancer cell line¹²³. Taken together, these data suggest that pharmacologic strategies capable of inducing the activation and mitochondrial translocation of BAX may be able to overcome this mechanism of resistance, either singly or in combination with AKT inhibition. BAX drug discovery efforts in the Walensky and Gavathiotis labs have led to the identification of a small molecule activator of BAX (BAM7), and its derivative, BAX trigger site activator 1 (BTSA1), which demonstrated promising pre-clinical efficacy in AML and an encouraging toxicology profile¹⁰⁰. Furthering our mechanistic understanding of the structure and function of BAX has the potential to expand the arsenal of pharmacologic strategies to activate BAX-mediated apoptosis in cancer, and perhaps other diseases of pathologic cellular excess.

BAX Structure and Function

BAX Activation Step 1: Triggering and Mitochondrial Translocation

BAX is a 21 kDa protein whose structure is similar to the other multi-BH domain BCL-2 family proteins: a globular bundle of 9 α -helices, 8 of which are wrapped around the central $\alpha 5$ ¹²⁴. The first structure of a BCL-2 family multi-BH domain protein, BCL-X_L lacking its C-terminal $\alpha 9$ helix, was solved by x-ray crystallography and NMR¹²⁵. Three BH regions were found in proximity to each other and formed a hydrophobic cleft. Residues previously known to be essential for hetero-

dimerization between BAX and BCL-X_L and necessary for BCL-X_L anti-apoptotic activity localized to this pocket (e.g., glycine 138)¹²⁶. The subsequent solution structure of BCL-X_L bound to a BAK BH3 peptide validated the role of this groove in sequestering BH3 helices and yielded the new paradigm of BH3-in-groove interactions as a mechanism for apoptosis regulation (**Figure 1.5**)⁵⁰. This canonical groove is composed of helices $\alpha 2$ - $\alpha 5$, containing the BH3 helix itself ($\alpha 2$) and the BH1 domain ($\alpha 4$ - $\alpha 5$). The heterodimeric BH3-in-groove structure also elucidated the mechanism for loss-of-function mutations in the BAX BH3 that prevented hetero-dimerization with anti-apoptotic members, namely mutagenesis of a conserved leucine (BAX L63; BAK L78)⁴⁹. Similar to anti-apoptotic proteins, mutations in BAX's groove abrogate association with select BH3-only proteins and self-association^{41,127,128}. Of note, BH3 mutations in BAX that prevent hetero-dimerization with BCL-2 also prevent BAX homo-oligomerization⁴⁹. Truncated forms of BAX lacking the C-terminus were shown to interact with BIM BH3 via the canonical groove and the crystal structure resembled the solution structure of BCL-X_L bound to BAK BH3 (**Figure 1.6a**)¹²⁹. Thus, the multi-domain members of the BCL-2 family act as receptors for BH3 helices, with the anti-apoptotic proteins functioning as decoy receptors to trap BH3s in the canonical groove. In contrast to anti-apoptotic heterodimeric BH3-in-groove interactions, binding of BH3-only proteins to the canonical groove of BAX/BAK promotes MOMP and the BH3 helices of BAX and BAK themselves uniquely enable homo-oligomerization as well^{127,128}.

When the structure of full-length BAX was first solved by NMR, the $\alpha 9$ transmembrane was observed to be lodged in the canonical pocket (**Figure 1.6b**)¹²⁴. Occlusion of this pocket suggested that initial engagement with BH3 helices must occur at a different site. Additionally, the sequestration of the transmembrane helix explained the cytosolic localization of inactive BAX, as well as how mutations in serine 184 (e.g., S184V) induced constitutive mitochondrial localization

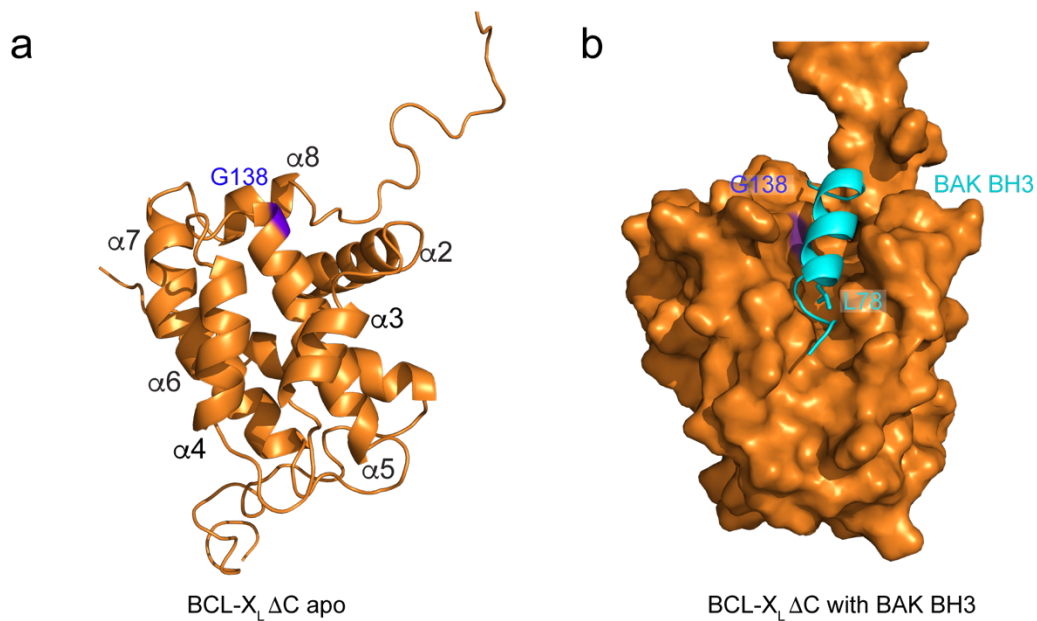
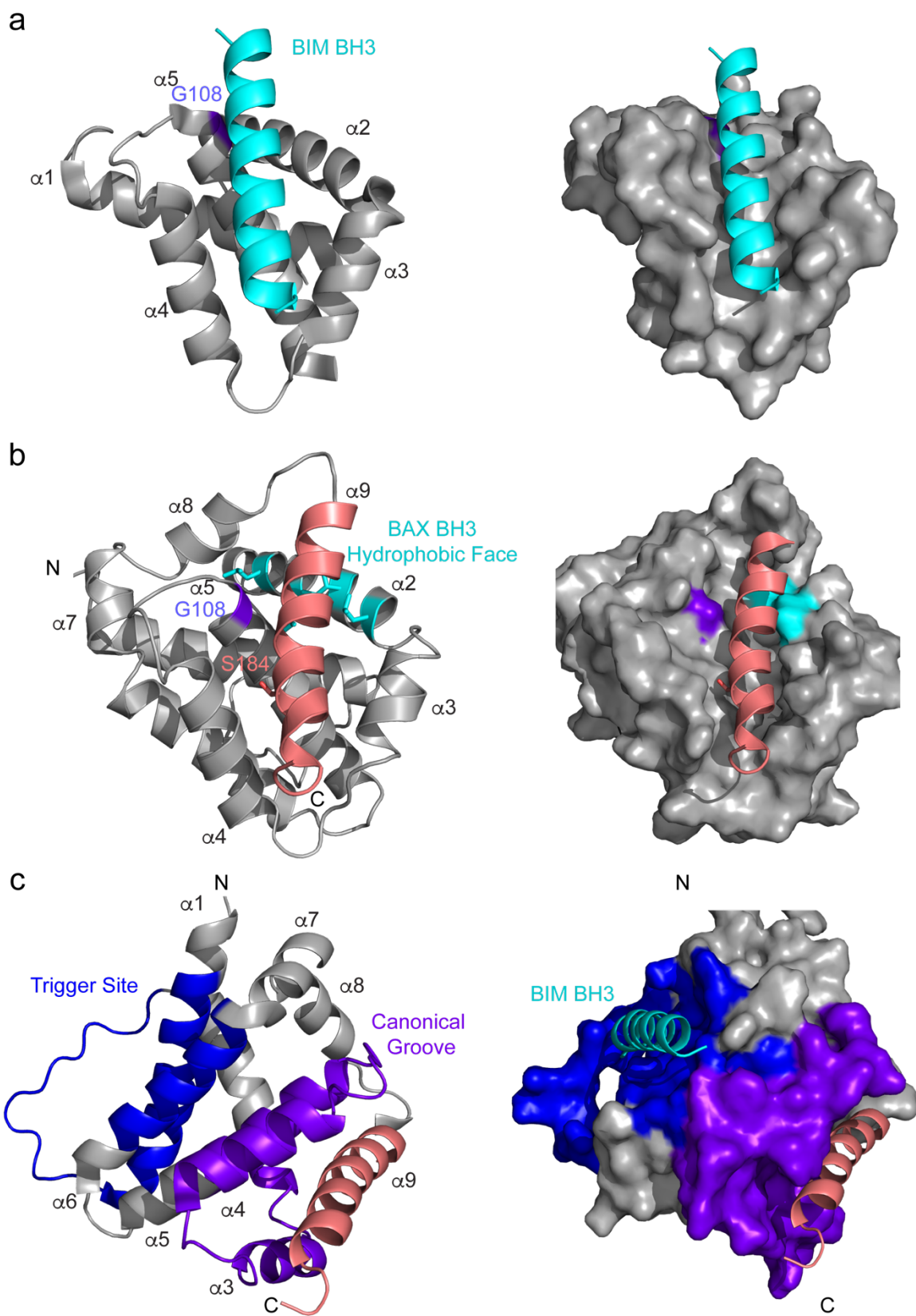


Figure 1.5 *Structural paradigm for BH3-in-groove interactions between BCL-2 family proteins.* Helices $\alpha 2$ - $\alpha 5$ of BCL-X_L form a groove in the (a) apo structure of BCL-X_L ΔC ($\alpha 1/\alpha 2$ loop removed for clarity, PDB: 1LXL) that binds (b) BH3 peptides (PDB: 1BXL). Mutagenesis of a conserved glycine (BCL-X_L G138, purple) in the groove and a conserved leucine in the BAK BH3 (BAK L78, cyan) abrogates binding interaction.

Figure 1.6 *BH3 binding sites of BAX.* (a) BAX Δ C binds to BIM BH3 (cyan) via the canonical groove composed of α 2- α 5 (PDB: 4ZIE), shown as a ribbon on the left and as a surface representation on the right. Glycine 108 is highlighted in purple as an important BAX residue that regulates this interaction. (b) The canonical groove of BAX is occluded by the C-terminal α 9 transmembrane helix (salmon) in the inactive conformation (PDB: 1F16), shown as a ribbon on the left and as a surface representation on the right. Key residues that regulate the canonical groove (G108, purple) and its occupancy by α 9 (S184, salmon) are highlighted and shown as sticks. The functional, hydrophobic interaction face of the BAX BH3 region faces internally in the latent monomeric structure and is highlighted in cyan. (c) A model structure of an open-loop conformation of BAX (PDB: 2K7W) reveals a shallow groove composed of residues from α 1 and α 6 (blue) for BH3 interaction. This N-terminal trigger site (blue) lies opposite to the canonical groove (purple) that is occupied by α 9 (salmon). A calculated model structure of the interaction between BIM BH3 (cyan) and the BAX trigger site is shown on the right.

Figure 1.6 (Continued)

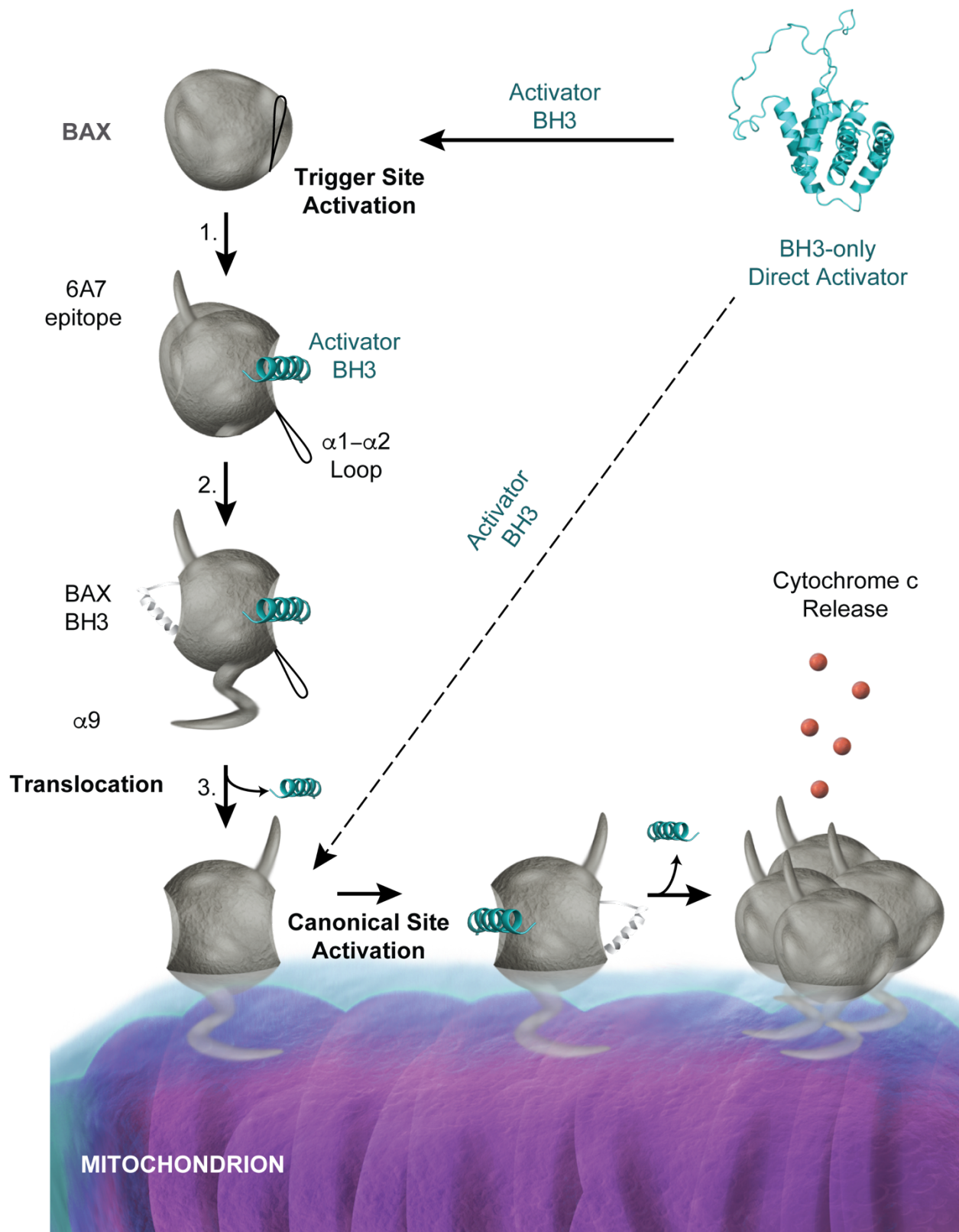


by preventing $\alpha 9$ -in-groove interaction^{124,130}. However, the NMR structure gave no indication as to how the wild-type $\alpha 9$ -helix is released to enable mitochondrial translocation¹²⁴. Another key observation was that BAX's own BH3 helix was buried internally, further raising the question of what mechanism enabled its release for either anti-apoptotic interaction or self-association^{49,124}. The structure of monomeric BAX suggested that a major conformational change must be required to induce BAX activation and exposure of its key functional helices, such as $\alpha 2$ and $\alpha 9$. Just how this could occur remained a mechanistic mystery.

Eight years after the Tjandra group solved the solution structure of monomeric BAX, the Walensky lab used NMR analyses to identify the binding site for a BIM BH3 helix that directly triggered the conformational activation of BAX, aptly named the 'trigger site' (**Figure 1.6c**)⁴⁷. Key to their discovery was the use of constrained helical peptides of the BIM BH3 motif, engineered to have sufficiently low affinity for BAX to perform heteronuclear single quantum coherence spectroscopy (HSQC) NMR analyses of this transient 'hit-and-run' interaction. The 'stapled' BIM BH3 peptide maintained its bioactive conformation and was able to directly bind BAX without inducing the rapid oligomerization that would otherwise preclude NMR analysis^{47,131}. Using paramagnetic relaxation enhancement NMR, the Walensky lab defined the interface between BIM BH3 and a groove composed of residues from $\alpha 1$ and $\alpha 6$ of BAX; a series of calculated model structures revealed that BIM BH3 interaction actually displaced a loop between $\alpha 1$ and $\alpha 2$, providing the first evidence of an induced conformational change in BAX upon BH3 triggering (**Figure 1.7**)⁴⁷. This finding further explained the mechanism by which an antibody, named 6A7, was able to selectively detect the activated conformer of BAX: upon BH3 triggering, displacement of the $\alpha 1/\alpha 2$ loop exposed the 6A7 epitope^{47,132}. Installing a disulfide tether between the $\alpha 1/\alpha 2$ loop and $\alpha 6$ did not fully prevent a stapled BIM BH3 peptide from binding, but did block downstream activation of BAX¹³¹. Dose- and time-dependent NMR analyses further showed that subsequent to BIM BH3-mediated mobilization of the $\alpha 1/\alpha 2$ loop, chemical shift changes within

Figure 1.7 *Activation of BAX by BH3-only Proteins.* Direct activator BH3-only proteins, such as BIM, trigger the activation of BAX by binding to the BAX trigger site and can propagate the signal by subsequent interaction at the canonical site. BH3 triggering interactions are transient, “hit and run” interactions resulting in BAX BH3 exposure. 1. BAX is a cytosolic protein and BIM BH3 binding at the BAX trigger site displaces the $\alpha 1/\alpha 2$ loop and exposes the 6A7 epitope. 2. These conformational changes are followed by the release of the BAX BH3 and the $\alpha 9$ transmembrane domain from the core of the protein. 3. Following conformational activation, BAX translocates to the mitochondrial outer membrane. Translocation exposes the canonical site, which is normally occluded by the $\alpha 9$ helix. The activated state of BAX at the mitochondria is proposed to be sustained by a second transient engagement of a BH3-only helix at the canonical site, ultimately leading to BAX homo-oligomerization and MOMP, which releases cytochrome c and other apoptogenic factors into the cytosol.

Figure 1.7 (Continued)



the $\alpha 9$ region occurred, pointing to an allosteric mechanism in which BH3 triggering at the N-terminal face of BAX resulted in $\alpha 9$ release at the C-terminal face¹³¹. This mechanism was confirmed by installing a disulfide bridge between $\alpha 9$ and $\alpha 5$ of the canonical groove, which blocked BAX mitochondrial translocation and cytochrome *c* release upon BH3 triggering¹³¹. Of note, chemical shift changes were also observed in internal residues of $\alpha 2$, $\alpha 4$, and $\alpha 5$, suggesting transmission of the BH3 signal from one face of BAX to the other through the core. Finally, and importantly, exposure of the BAX BH3, as detected by anti-BAX BH3 immunoprecipitation, was also detected in a dose-responsive fashion upon BIM BH3 triggering¹³¹.

The conformational interdependence of the N- and C-terminal faces of BAX was further demonstrated by the Cheng laboratory. They showed that HA-tagged BAX lacking its C-terminal helix (BAX ΔC) was able to pull-down GST-tagged $\alpha 9$, but further deletion of the N-terminus (BAX $\Delta N/\Delta C$) prevented this interaction¹²⁷. These data explained previous observations that implicated the BAX N-terminus as an auto-regulatory domain (named the Apoptotic Regulation of Targeting [ART] domain), whose deletion enhanced BAX mitochondrial targeting and activation^{133,134}. Consistent with allosteric sensing between the N- and C-terminal faces of BAX, HA-tagged BAX $\alpha 1$ was further shown to pull down BAX ΔN but not BAX $\Delta N/S184V$, whose C-terminus is constitutively dissociated from the protein core¹²⁷. Furthermore, mutants of BAX that are deficient in $\alpha 9$ mobilization demonstrate altered conformations of the $\alpha 1/\alpha 2$ loop¹³⁵. Taken together, these data implicate a connection between the N- and C-terminal faces of BAX, such that BH3 triggering at the N-terminal face induces allosteric release of $\alpha 9$ from the C-terminal face and represents a critical initiating component of the BAX activation pathway.

BAX Activation Step 2: Canonical BH3-in-Groove Binding and Homo-Oligomerization

Although direct BAX activation and mitochondrial translocation are required for the initiation of BAX activation, further structural changes are required for BAX-mediated MOMP. BAX proteins bearing S184 mutations bypass triggering and translocate to liposomes and mitochondria in the absence of BH3-only stimulation, but still require tBID to induce BAX-mediated MOMP^{123,127,135}. In addition, mutating glycine 108 within the canonical pocket of BAX to block homo-oligomerization prevents cytochrome *c* release but permits integration into the mitochondrial outer membrane¹³⁶. Double mutants that incorporate both gain- and loss-of functions (i.e. bypass triggering and disrupt BAX BH3 interaction), such as BAX S184V/G108E and BAX S184V/L63E, constitutively localize to the mitochondria but do not induce mitochondrial apoptosis¹²⁷. Thus, BAX self-association and oligomeric disruption of the mitochondrial outer membrane are discrete “execution phase” steps of the BAX activation pathway downstream of mitochondrial translocation.

The mechanism by which BAX inserts into the membrane, undergoes self-association, and then permeabilizes the mitochondrial membrane remains an active area of research. To date, there are no atomic resolution structures of full-length BAX (or BAK) in a membrane-bound or oligomeric conformation. Biochemical data suggests that activated monomers of BAX at the mitochondrial membrane can recruit inactive BAX monomers via a triggering interaction between the exposed BAX BH3 helix of the activated monomer and the N-terminal trigger site of an inactive monomer, analogous to BIM BH3 triggering of inactive BAX in the cytosol^{47,131,137}. Such a mechanism would serve to propagate BAX activation in an autocatalytic fashion^{47,137}. A second activating interaction between a BH3-only helix and the canonical groove of BAX has also been proposed (**Figure 1.7**)^{36,127,128}. Importantly, the BAX BH3 exposed state is the conformer of BAX that is alternatively trapped by BCL-2 family anti-apoptotic proteins or free to engage in homo-dimeric interaction that leads to higher order homo-oligomerization^{36,49,138}. Engagement of BAX with anti-apoptotic proteins can also result in the removal of BAX from the mitochondria. Indeed,

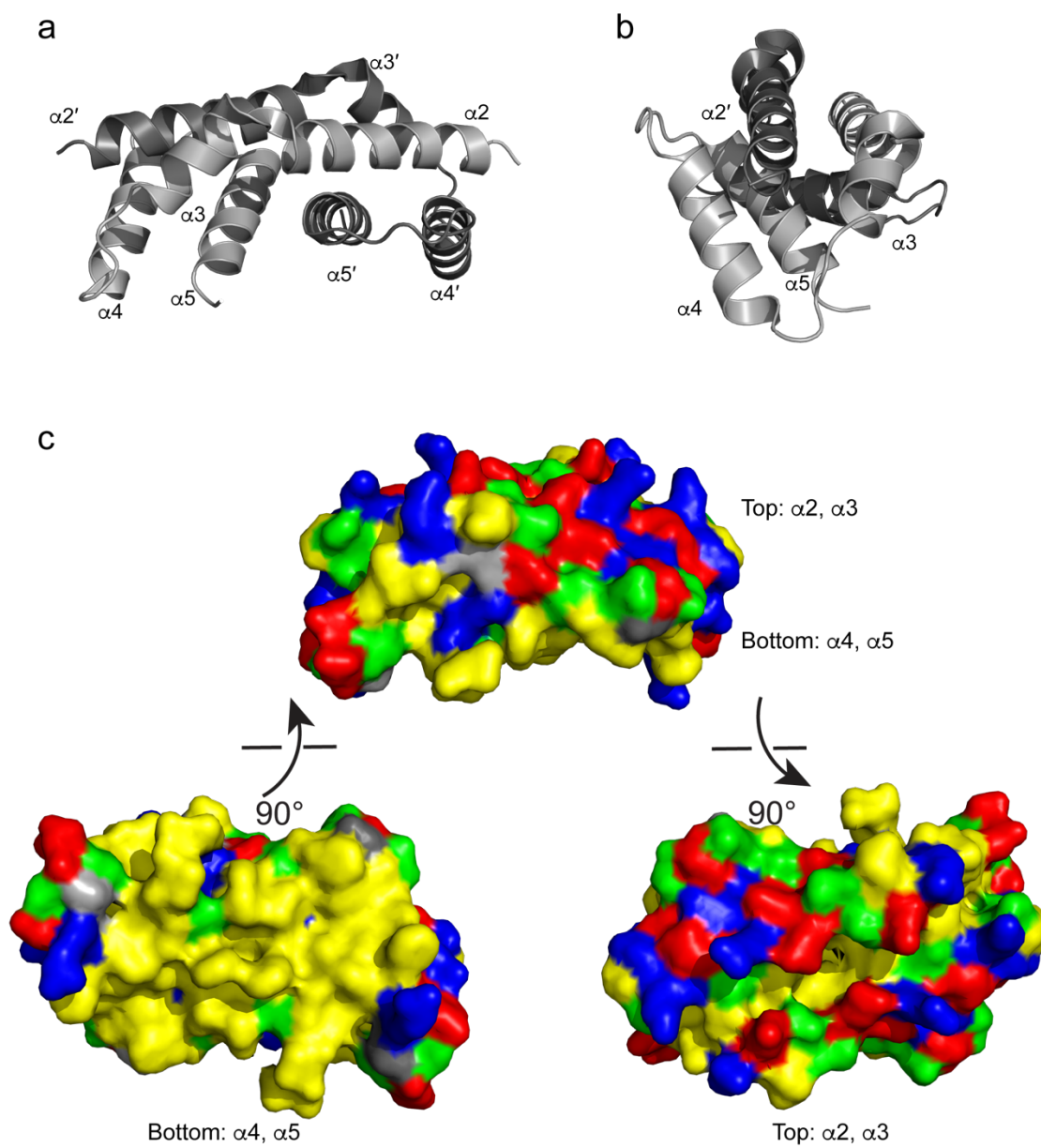
BCL-X_L has been shown to retro-translocate BAX from mitochondria to the cytosol, a potential mechanism for tipping the balance toward cell survival¹²⁰.

BAX is believed to be a homo-oligomer of BH3-in-groove BAX dimers, based on the structure of a dimeric BAX truncate comprised of the $\alpha 2$ - $\alpha 5$ region only (**Figure 1.8**).^{36,128,139} This model is further based on (1) crosslinking and spectroscopy studies of full-length BAX that provide pairwise distances between specific residues of the symmetric dimer¹³⁹⁻¹⁴¹, and (2) identification of $\alpha 2$ - $\alpha 5$ as a minimal, essential domain for homo-oligomerization and apoptosis induction in cells¹⁴². The crystal structure of a GFP-linked $\alpha 2$ - $\alpha 5$ BAX truncate, as solved by Czabotar and colleagues, suggested that $\alpha 4$ and $\alpha 5$ comprise a planar hydrophobic surface that engages the membrane, whereas $\alpha 2$ and $\alpha 3$ are cytosolically facing, in agreement with studies that monitored membrane engagement of specific residues in full-length BAX (**Figure 1.8c**)^{128,140,141,143}. The $\alpha 2$ - $\alpha 5$ portion of BAX is considered the core and helices $\alpha 6$ - $\alpha 8$ a latch that must separate from the core to enable self-association; consistent with this hypothesis, installing a disulfide tether between $\alpha 5$ and $\alpha 6$, which form a hairpin in the inactive form of BAX, blocked BAX-mediated cytochrome *c* release from isolated mitochondria¹²⁸.

In the absence of more definitive structural information on BAX self-assembly, particularly of the full-length protein at the membrane, the mechanism of oligomeric BAX-mediated MOMP remains unknown and controversial^{141,144,145}. Cross-linking studies suggest that residues of $\alpha 6$ and $\alpha 9$ may be required for higher order BAX homo-oligomerization, but the interplay between these residues and the $\alpha 2$ - $\alpha 5$ dimer is unclear^{140,146}. Although the oligomer-of-dimer model of BAX homo-oligomerization predicts that a second interface must exist, the molecular nature of this interaction is unknown and the heterogeneous nature of BAX self-association suggests that perhaps an alternate mode of self-assembly and membrane disruption may be involved^{147,148}. One study of BAK dimers revealed the formation of disordered clusters in the membrane and

Figure 1.8 *BAX forms symmetric BH3-in-groove dimers.* (a) A BAX $\alpha 2$ - $\alpha 5$ truncate forms a symmetric dimer in which (b) the BH3 ($\alpha 2$) of one monomer ($\alpha 2'$, dark grey) binds to the canonical groove ($\alpha 2$ - $\alpha 5$) of the other (light grey, PDB: 4BDU). (c) Surface representation of the symmetric dimer with residues colored according to hydrophobicity (yellow), hydrophilicity (green), or negative (red) or positive (blue) charge. The bottom of the dimer, $\alpha 4$ and $\alpha 5$ (bottom left), forms a hydrophobic face believed to interact with the mitochondrial membrane, whereas the top portion, $\alpha 2$ and $\alpha 3$ (bottom right), is more hydrophilic and may face the cytosol.

Figure 1.8 (Continued)



another identified a potential role of lipids in bridging BAK dimers^{149,150}. Some investigators have even suggested that higher-order homo-oligomerization may not be the functional species of BAX or BAK required for MOMP but instead a byproduct of membrane permeabilization¹⁵¹. The Czabotar group has recently suggested that the smallest unit of BAK required for MOMP is an $\alpha 2$ - $\alpha 8$ truncate, although what level of self-assembly is required in the mitochondria to achieve functional activity was not determined¹⁵². Thus, the execution phase of BAX/BAK activation remains a mechanistic mystery, yet holds promise to reveal the very basis of the cellular death program and opportunities to modulate BAX/BAK self-assembly and MOMP for therapeutic benefit in diseases of too many or too few cells.

Regulation and Pharmacologic Modulation of BAX Activation

The auto-inhibited monomeric conformation of BAX itself represents the first line of defense against renegade activation. The closed $\alpha 1/\alpha 2$ loop shields the BH3 trigger site and the $\alpha 9$ occludes the canonical groove. These two regulatory surfaces have been shown to interact, forming autoinhibited dimers in certain cell types, providing yet another layer of protection from unwanted BAX activation during homeostasis¹⁵³. Antibody or small molecule (e.g. eltrombopag) binding to the trigger site has been shown to prevent BH3-only interaction and thereby suppresses BAX activation¹⁵⁴⁻¹⁵⁶. Conversely, BAX can be activated at the trigger site by stapled BH3 peptides and small molecules identified through *in silico* screening (e.g., BAM7); BTSA1, a derivative of BAM7, has been shown to suppress AML in mice while sparing healthy tissues^{47,100,157,158}. Physiologic modulation of the $\alpha 9$ has also been observed, notably through AKT-mediated phosphorylation of serine 184, which prevents BAX association with the mitochondrial outer membrane¹²³. The canonical groove of BAK has been targeted by engineered high-affinity BIM BH3 peptides that block conformational activation,¹⁵⁹ a mechanism that could potentially also be

employed to disrupt downstream BAX activation. Such mechanisms of physiologic and pharmacologic modulation of BAX activation highlight the potential to target BAX, to either prevent or induce cell death, for therapeutic purposes.

To date, preliminary pharmacologic strategies to target BAX and BAK have focused on the BH3-binding grooves at the N-terminus for BAX and the C-terminus for BAK. However, the allosteric crosstalk between these two sites is a fascinating target of protein regulation, with important functional consequences. For example, an alternative mode of BCL-2 inhibition of BAX involves direct interaction at the junction of its $\alpha 2/\alpha 3$ and $\alpha 5/\alpha 6$ hairpins by the BH4 domain of BCL-2 (**Figure 1.9**)¹⁶⁰. This distinct BCL-2/BAX binding mode suppresses the conformational activation of monomeric BAX. Interestingly, at the adjacent side of the $\alpha 5/\alpha 6$ hairpin, the cytomegalovirus protein vMIA binds and stabilizes BAX, resulting in inhibition of pro-apoptotic function¹⁶¹. A small molecule fragment identified by an NMR screen was able to compete with vMIA at this site and served to sensitize BAX activation by interacting with the protein core, resulting in allosteric mobilization of the $\alpha 1$ - $\alpha 2$ region of BAX implicated in the initiating step of BAX activation¹⁶². A small molecule BAX activation inhibitor (BAI1) binds to an adjacent pocket formed by the confluence of the $\alpha 3/\alpha 4$ loop and $\alpha 5/\alpha 6$ hairpin, and also interacts with residues of the protein core, including F116 of $\alpha 5$; however, in this case, BAI1 interactions restrain the conformational activation of BAX upon BH3 triggering by stabilizing the protein core¹⁶³. Intriguingly, a cysteine residue at position 126 of the $\alpha 5/\alpha 6$ hairpin can react covalently with a distinct lipid of the mitochondrial outer membrane, namely 2-hexadecenal, sensitizing direct BAX activation by allosterically mobilizing $\alpha 1$ and the $\alpha 1/\alpha 2$ loop¹⁶⁴. These data highlight the critical role of the BAX core in mediating the transmission of ligand binding signals from one side of the protein to the other, with dramatic functional consequences for inhibition or activation of BAX.

Allosteric sensing and conformational modulation directly influence the BAX activation

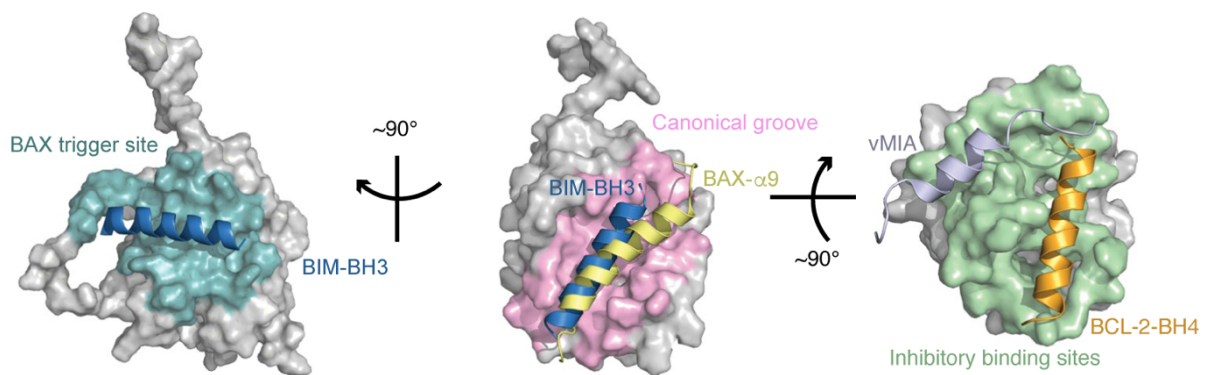


Figure 1.9 BAX is regulated at its trigger site, canonical groove, and a third site at the base of the protein. BAX engages BH3 helices (dark blue) at both its N-terminal trigger site (blue) and canonical groove (pink). Both sites are shielded in the latent conformer, with the $\alpha 1/\alpha 2$ loop overlying the N-terminal site and the $\alpha 9$ transmembrane helix (yellow) bound to the canonical groove. A distinct site on BAX (green), subjacent to the N-terminal trigger site, mediates inhibitory interactions by the BH4 region of BCL-2 (orange) and a helix of the cytomegalovirus vMIA protein (lavender). Reprinted from reference¹⁶³ with permission.

pathway and BAX-mediated MOMP. Nevertheless, how such physiologic and pharmacologic mechanisms are mediated by molecular interactions of the BAX core remain unknown. A unifying theme of BAX regulation is achieving exquisite control of the balance between monomeric stability and induction of the oligomeric self-assembly that leads to mitochondrial disruption and cellular destruction. Indeed, the focus of this thesis centers on the fundamental unanswered question, how does the structure of BAX enable both monomeric stability and striking conformational reorganization to ensure precise control over its lethal biochemical activity?

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CHAPTER 2

The Conformational Stability of
Pro-Apoptotic BAX is Dictated by
Discrete Residues of the Protein Core

Abstract

BAX is a pro-apoptotic member of the BCL-2 family, which regulates the balance between cellular life and death. During homeostasis, BAX predominantly resides in the cytosol as a latent monomer but, in response to stress, transforms into an oligomeric protein that permeabilizes the mitochondria, leading to apoptosis. Because renegade BAX activation poses a grave risk to the cell, the architecture of BAX must ensure monomeric stability yet enable conformational change upon stress signaling. The specific structural features that afford both stability and dynamic flexibility remain ill-defined and represent a critical control point of BAX regulation. We identify a nexus of interactions involving four residues of the BAX core $\alpha 5$ helix that are individually essential to maintaining the structure and latency of monomeric BAX and are collectively required for dimeric assembly. The dual yet distinct roles of these residues reveal the intricacy of BAX conformational regulation and opportunities for therapeutic modulation.

Attributions

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N.B.B. and L.D.W. conceived of and designed the study. N.B.B. produced the BAX proteins and performed all biochemical, mitochondrial, and cellular experiments, with assistance from M.S.P. and H.R.L. M.S.P. generated BAX-reconstituted MEFs. N.B.B. and T.E.W. performed the HDX MS analyses under the supervision of J.R.E.

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Introduction

BAX is a BCL-2 family protein whose killer function rids the body of damaged or unwanted cells¹. The constitutive expression of BAX ensures vigilant surveillance of cellular fidelity yet poses a grave risk to cellular survival if BAX is arbitrarily unleashed. Thus, exquisite conformational regulation is an essential feature of BAX physiology². The latent, cytosolic form of BAX is a 21 kDa monomer composed of a core hydrophobic helix ($\alpha 5$) surrounded by 8 additional α -helices³. The N- and C-terminal faces of the protein have auto-inhibitory features with the loop between $\alpha 1$ and $\alpha 2$ overlying a trigger site for BAX activation and the C-terminal $\alpha 9$ helix secured within a regulatory groove. Indeed, installing a disulfide tether that locks the $\alpha 1$ - $\alpha 2$ loop or C-terminal helix in place effectively prevents BAX activation⁴. A dimeric structure of full-length BAX further showed that the $\alpha 9$ surface of one monomer could engage the N-terminal face of another in a manner that shields the trigger site, resulting in a less activatable species⁵. Upon direct BAX activation at the trigger site by members of the “BH3-only” subclass of BCL-2 proteins, so named because they only bear the conserved BCL-2 homology 3 motif, the $\alpha 1$ - $\alpha 2$ loop is displaced, causing allosteric release of both the C-terminal helix for mitochondrial translocation and the BAX BH3 helix for propagation of activation and oligomerization.

NMR analyses have shown that conformational perturbations triggered by BH3 motifs, such as that of BIM, or small molecule BH3 mimetics, such as BAM7 and BTSA1, are transmitted through the protein's $\alpha 5$ core^{4,6,7}. A small molecule fragment, BIF-44, which binds at the junction of the $\alpha 3/\alpha 4$ and $\alpha 5/\alpha 6$ hairpins and sensitizes BAX activation by enhancing the conformational flexibility of the $\alpha 1$ - $\alpha 2$ region, also operates by an allosteric mechanism that reverberates through the hydrophobic $\alpha 5$ core⁸. In contrast, inhibitory peptides and molecules, such as the BCL-2 BH4 helix and BAI1, directly engage and stabilize portions of the BAX core, and effectively impede the N-terminal activation of BAX^{9,10}. Although generally implicated in conformational activation as a

conduit for allosteric regulation, the molecular mechanism by which the central BAX $\alpha 5$ helix alternatively restrains or enables BAX activation is unknown.

Once activated, BAX transforms from a cytosolic monomer into a mitochondrial membrane-embedded oligomer that permeabilizes the outer membrane, releasing a series of apoptogenic signaling factors that commits the cell to death. This execution-phase of BAX activation yields oligomeric forms that are, by definition, structurally distinct from the monomer and actuate the destruction of the mitochondrial membrane. A proposed dimeric building block of BAX oligomers, based on the structure of a complex between two BAX $\alpha 2$ - $\alpha 5$ truncates, provided an example of such structural reorganization¹¹. The inability of full-length BAX to form a dimeric species and induce mitochondrial apoptosis when $\alpha 5$ is disulfide tethered to $\alpha 6$ suggested that separation of $\alpha 6$ from $\alpha 5$ may represent another key step along the BAX activation pathway¹¹ and, notably, involves conformational alteration of the monomeric core.

To elucidate the molecular features of $\alpha 5$ that influence the conformational dynamics of BAX from the inside out, we analyzed its amino acid interaction network, which highlighted a striking nexus of interactions involving $\alpha 5$ residues 113-116. Structure-function studies, spanning alanine mutagenesis, biochemical and cellular analyses, and hydrogen-deuterium exchange mass spectrometry, revealed essential roles for each individual residue in preserving the conformational stability of monomeric BAX, yet a collective role of all four residues in stabilizing the proposed dimeric subcomponent of the BAX oligomer. Our findings implicate a strikingly focal region of BAX architecture with dual roles in regulating the monomeric and dimeric states of this critical arbiter of cell death.

Methods

Protein network analysis

Network models of BAX were generated using the NAPS server (<https://bioinf.iit.ac.in/NAPS/>) as unweighted atom pair contact networks with default parameters. Monomeric BAX was modeled using the NMR solution structure (PDB: 1F16) and dimeric BAX was modeled using the $\alpha 2$ - $\alpha 5$ truncate crystal structure (PDB: 4BDU, BAX amino acids 54-122). For the monomer-dimer comparison, the output of the network analysis for BAX amino acids 54-122 was analyzed and values normalized using Prism software (Graphpad).

Recombinant protein expression and purification

Recombinant full-length, wild-type BAX in the pTYB1 vector was expressed in *Escherichia coli* as previously reported^{3,8,12}. Point mutations were generated by PCR-based mutagenesis (Q5 Site-Directed Mutagenesis Kit, New England BioLabs) and confirmed by DNA sequencing. Transformed *E. coli* were cultured in LB medium containing carbenicillin (0.1 g/l), grown to an optical density (OD) of 0.6-0.8, and protein expression was induced by the addition of 1.0 mM IPTG at 30°C for 4 h. Bacterial pellets were resuspended in lysis buffer (20 mM Tris, 250 mM NaCl, pH 7.2) containing protease inhibitor tablets (Roche) and lysed over two passages through a microfluidizer (Microfluidics) on ice. The soluble fraction was isolated by centrifugation at 48,384 x g for 45 min at 4°C. BAX protein was purified by chitin affinity chromatography using chitin resin (NEB) on a gravity flow column. The intein and affinity tag were cleaved using 10 mg/mL dithiothreitol at 4°C for 12-36 h. The full-length, tagless protein was eluted, concentrated, and purified by SEC in fast protein liquid chromatography (FPLC) buffer (20 mM HEPES-KOH, 150 mM KCl, pH 7.2) using a Superdex S-75 (GE Healthcare) column on an FPLC system (AKTA Pure, GE Healthcare Life Sciences). Protein purity and identity was confirmed by western analysis using the 2D2 mouse monoclonal BAX antibody (Santa Cruz Biotechnology Cat# sc-20067; RRID: AB_626726; 1:200).

Recombinant full-length BCL-X_L (1-233) was cloned into the pTYB1 vector by HiFi DNA assembly (NEBuilder HiFi DNA Assembly Master Mix, NEB). BCL-X_LΔC (1-209) and BCL-X_LΔC G138A were then generated by PCR and PCR-based mutagenesis (Q5 Site-Directed Mutagenesis Kit, New England BioLabs), respectively, and confirmed by DNA sequencing. Expression and purification were performed as described above for BAX, with expression induced using 0.5 mM IPTG overnight at 16°C. Protein purity and identity was confirmed by western analysis using a mouse monoclonal anti-BCL-X_L (2H12) antibody (Abcam Cat# ab270253; RRID: AB_2536307; 1:1000).

BAX α2-α5 (53-128) truncates containing N-terminal His₆- and GFP-tags, and bearing C62S and C126S mutations, were cloned into pET28a by HiFi DNA Assembly (NEB). Point mutations were performed by PCR-based mutagenesis (Q5 Site-Directed Mutagenesis Kit, New England BioLabs) and confirmed by DNA sequencing. Transformed *E. coli* were grown and induced as described for BCL-X_LΔC except that kanamycin (0.05 g/l) was used in place of carbenicillin. Bacterial pellets were lysed in TBS (20 mM Tris, 150 mM NaCl, pH 8.0). Clarified lysate was applied to Ni-NTA agarose and washed with a gradual imidazole gradient before elution in TBS containing 200 mM imidazole. Eluted N-His₆-GFP-BAX α2-α5 was dialyzed overnight in TBS lacking imidazole, concentrated, and further purified by SEC using a Superdex S-200 (GE Healthcare) gel filtration system.

SEC analysis of BAX mutants

Purified monomeric BAX proteins were diluted to 5 μM in 200 μL of FPLC buffer (see above) and incubated at 25°C for 30 min before subjecting the sample to SEC analysis using a Superdex S-75 column. The data were analyzed using Microsoft Excel 2016 and Prism 8 (Graphpad) software.

Liposomal release assay

Large unilamellar vesicles (LUVs) encapsulating the fluorophore/quencher pair 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and p-xylene-bis-pyridinium bromide (DPX) were formed by liposome extrusion and SEC. Briefly, a lipid mixture that mimics the composition of the mitochondrial outer membrane was generated by dissolving a 48:28:10:10:4 molar ratio of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, dioleoyl phosphatidylserine, and tetraoleoyl cardiolipin (Avanti Polar Lipids) in chloroform. To produce lipid films, nitrogen gas was used to evaporate the chloroform and the lipids were dried under high vacuum overnight. For long-term storage, lipid films were maintained in a nitrogen atmosphere at -80°C. Lipid films were resuspended in 1 mL of liposomal release assay buffer (20 mM HEPES-KOH, 150 mM KCl, 5 mM MgCl₂, pH 7.2) containing the fluorophore/quencher pair ANTS (12.5 mM) and DPX (45 mM). Liposomes were formed by exposing the resuspended lipid mixture to five freeze/thaw cycles and then extruding the liposomes using a 100 nm polycarbonate membrane eleven times. Liposomes were then purified from unencapsulated ANTS and DPX using a Sepharose CL-2B size exclusion column (GE Healthcare). The liposomal release assay was performed using an M1000 Infinite (Tecan) equipped with i-control 1.11 software (Tecan). ANTS fluorescence (355 nm excitation, 540 nm emission, 20 nm slit width) was monitored every 60 s after the addition of recombinant BAX proteins (500 nM), with or without tBID (10 nM) or BCL-X_L proteins (10 μM). Maximal release was determined by the addition of Triton X-100 to a final concentration of 0.625% (v/v). Fraction release was calculated as $((F-F_0)/(F_{100}-F_0))$, where F is the observed fluorescence at a given time, and F₀ and F₁₀₀ represent baseline and maximal fluorescence, respectively. The data were analyzed using Microsoft Excel 2016 software and plotted with Prism 8 software (Graphpad).

Liposomal Translocation Assay

Liposomes (40 μ L) were incubated with (1) BAX proteins (1 μ M) treated with tBID (200 nM) for 15 min or (2) BCL-X_L proteins (1 μ M) for 45 min in liposomal release assay buffer at room temperature. Protein-containing solutions were then added to a Sepharose CL-2B (3 mL, GE Healthcare) size exclusion column equilibrated with liposomal release assay buffer and 14 equivalent fractions (250 μ L) were collected. Liposome-containing fractions were identified by adding 10% Triton X-100 to the fractions and measuring fluorescence associated with ANTS/DPX release. SDS PAGE followed by western blot analysis was conducted to identify BAX or BCL-X_L in the fractions using 2D2 mouse monoclonal BAX antibody (Santa Cruz Biotechnology Cat# sc-20067; RRID: AB_626726; 1:200) or mouse monoclonal anti-BCL-X_L (2H12) antibody (Abcam Cat# ab270253; RRID: AB_2536307; 1:1000), respectively.

Mitochondrial cytochrome c release assay

Mitochondria from the livers of *Alb-cre*^{pos}*Bax*^{ff}*Bak*^{-/-} mice were isolated and release assays performed as described previously¹³. Briefly, mitochondria (1 mg/mL) were incubated with recombinant BAX proteins (25 or 200 nM) in the presence or absence of tBID (5 or 40 nM, respectively), for 45 min at room temperature in experimental buffer (200 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES, pH7.4). Control wells contained mitochondria and buffer, with and without added 1% Triton X-100 (v/v). Following incubation, plates were centrifuged for 15 min at 2,055 x g and 50 μ L of supernatant was analyzed using the Rat/Mouse Cytochrome c Quantikine ELISA Kit (R&D Systems) according to the manufacturer's instructions using SoftMax Pro 7.0.2 software. Percentage cytochrome c released into the supernatant (%cytochrome c release) was calculated according to the following equation: %cytochrome c release = $[\text{cyto } c_{\text{sup}} - \text{cyto } c_{\text{buffer}}] / [\text{cyto } c_{\text{max}} - \text{cyto } c_{\text{buffer}}] \times 100$, where $\text{cyto } c_{\text{sup}} - \text{cyto } c_{\text{buffer}}$ and $\text{cyto } c_{\text{max}} - \text{cyto } c_{\text{buffer}}$ represent the amount of cytochrome c specifically released into the supernatant upon treatment with the indicated conditions or 1% (v/v) Triton X-100, respectively. The above animal-derived

materials (liver mitochondria) were obtained from *Alb-cre*^{pos} *Bax*^{ff} *Bak*^{-/-} mice in accordance with the guidelines and regulations set forth by the Institutional Animal Care and Use Committee of the Dana-Farber Cancer Institute and in compliance with approved study protocol #06-004. Animals were housed in microisolator cages at an ambient temperature of 68-79°F and humidity of 30-70%, with 12-hour light/dark cycles.

Retroviral transduction and cell culture

Human BAX constructs (wild-type, L113A, F114A, Y115A, and F116A) were cloned (Genewiz) into the pMIG II (pMSCV-IRES-GFP II) vector (Addgene #52107), and the presence of insert and the indicated mutations were confirmed by DNA sequencing. Transfection of the packaging cell line GPG-293 yielded amphotropic retroviral particles, which were collected by filtration and precipitation with PEG-it solution (System Biosciences), and then used to reconstitute *Bax*^{-/-} *Bak*^{-/-} MEFs with the indicated BAX mutants, as described^{12,14}. The reconstituted MEFs were sorted for GFP-positivity over two rounds of flow cytometry to ensure comparable levels of expression. Transduction was verified by BAX western analysis using the 2D2 antibody (Santa Cruz Biotechnology Cat# sc-20067; RRID: AB_626726; 1:200). Cells were maintained in Dulbecco's Modified Eagle Medium (GIBCO) with 10% FBS, 100 U/mL penicillin and streptomycin, and 2 mM glutamine. Cells were verified as mycoplasma-negative using the MycoAlert mycoplasma detection kit (Lonza Biologics).

Mitochondrial isolation from reconstituted MEFs

Bax^{-/-} *Bak*^{-/-} MEFs and those reconstituted with the indicated BAX proteins were cultured as described above and 2 x 10⁷ cells were harvested for fractionation. Cell pellets were resuspended in isolation kit buffers (ThermoFisher) and lysed by Dounce homogenization. Mitochondrial and cytosolic fractions were purified by differential centrifugation, performed in accordance with the manufacturer's protocol. Mitochondria were lysed in mitochondrial lysis buffer (25 mM Tris, 150

mM NaCl, 2% CHAPS, pH 7.2) and protein quantitation of the supernatant and cytosolic fractions was performed using the BCA assay (ThermoFisher). Samples were subjected to electrophoresis and western blotting using the 2D2 antibody (Santa Cruz Biotechnology Cat# sc-20067; RRID: AB_626726; 1:200). Western analysis using VDAC1 (Abcam cat #ab186321; 1:1000), LDH (Abcam cat #ab47010, RRID:AB_1952042; 1:1000), and actin (Cell Signaling Technology Cat# 5125, RRID:AB_1903890; 1:1000) antibodies was performed to verify mitochondrial and cytosolic fractions, and equal protein loading, respectively.

Cell viability assay

Bax^{-/-}*Bak*^{-/-} MEFs and those reconstituted with the indicated BAX proteins were cultured as described above and plated in 96-well plates (1.5 x 10³ cells per well) and allowed to adhere for 24 h. The cells were then treated with 10 μ M ABT-737 (Cayman Chemical) and the indicated concentration of S63845 (SelleckChem), or vehicle (0.05% and 0.1 % DMSO, respectively) for 24 h. Cell viability was measured by CellTiter-Glo assay (Promega) with luminescence read on a Spectramax M5 microplate reader equipped with SoftMax Pro 7.0.2 software.

Hydrogen deuterium exchange mass spectrometry

HDX MS analyses of BAX proteins were performed as described previously⁹, with details specific to the experiments conducted here provided in Supplementary Data 1, per the recommended format¹⁵. Briefly, BAX aliquots were freshly prepared in biological replicates prior to HDX MS analysis with stock concentrations in 20 mM HEPES, 150 mM KCl, 1 mM MgCl₂, pH 7.2, H₂O as follows: BAX WT: 22 μ M; BAX L113A: 29 or 26 μ M; BAX F114A: 29 or 18.6 μ M; BAX Y115A: 6.8 μ M; BAX F116A: 16 or 24 μ M. Deuterium labeling was initiated with an 18-fold dilution into D₂O buffer (20 mM HEPES, 150 mM KCl, 1 mM MgCl₂, pD 7.2, 99.9% D₂O). After each labeling time (10 seconds, 1 minute, 10 minutes) at 20 °C, the labeling reaction was quenched with the addition

of ice-cold quenching buffer (0.8 M guanidinium chloride, 0.8% [v/v] formic acid, pH 2.0, H₂O) and analyzed immediately using a Waters HDX system coupled to a Waters-G2Si mass spectrometer operated in HDMS^E mode. Deuterated and control samples were digested online at 15 °C, using an AffiPro Nepenthesin-2 column and peptides were trapped and desalted on a VanGuard Pre-Column trap for 3 minutes at 100 µL/min. Peptides were then eluted from the trap using a 5%–35% gradient of acetonitrile over 6 minutes at a flow rate of 100 µL/min, and separated using an ACQUITY UPLC HSS T3, 1.8 µm, 1.0 mm × 50 mm column with the main cooling chamber held at 0.0 ± 0.1 °C for the entire time of the measurements. Peptides were identified from replicate HDMS^E analyses (as detailed in Supplementary Data 1) of undeuterated control samples using PLGS 3.0.1 (Waters Corporation). Peptide masses were identified from searches using non-specific cleavage, no missed cleavages, no PTMs, a minimum of 250 ion counts for low energy threshold, and a 50 ion count threshold for their fragment ions, with no set minimum peptide length. No false discovery rate (FDR) control was performed. The peptides identified in PLGS (excluding all neutral loss and in-source fragmentation identifications) were then analyzed in DynamX 3.0 (Waters Corporation) implementing a minimum products per amino acid cut-off of 0.25, at least 2 consecutive product ions (see Supplementary Data 1). A database containing only the sequence from human BAX (UniprotKB Q07812) was used with no cleavage specificity and no PTMs considered to refine the peptide list. Those peptides meeting the filtering criteria to this point were further processed by DynamX 3.0 (Waters Corporation). The relative amount of deuterium in each peptide was determined using DynamX 3.0 by subtracting the centroid mass of the undeuterated form of each peptide from the deuterated form, at each time point, for each condition. These deuterium uptake values were used to generate all uptake graphs and difference maps. The error of determining the average deuterium incorporation for each peptide was at or below ± 0.25 Da. Deuterium levels were not corrected for back exchange and thus reported as relative¹⁶.

Anti-BAX BH3 Immunoprecipitation

BAX proteins (9 μ M) were prepared in a 20 μ L solution of PBS. After equilibrating for 15 min at room temperature, 10 μ L of each sample was added to a 3% BSA in PBS solution (250 μ L) containing 15 μ L BAX BH3 antibody (Abcepta Cat# AP1302a; RRID:AB_2227716) for 1 hr at 4°C. Preclarified Pierce Protein A/G agarose beads (50 μ L) (ThermoFisher) were then added to the BAX protein/antibody mixture and incubated for an additional 2 hr at 4°C. The agarose beads were spun down, washed three times with 1 mL of 3% BSA in PBS, resuspended in 50 μ L of SDS-sample buffer and boiled for 5 min. Inputs (2%) and immunoprecipitation samples (20 μ L) were subjected to SDS PAGE and western blot analysis was conducted using 2D2 mouse monoclonal BAX antibody (Santa Cruz Biotechnology Cat# sc-20067; RRID: AB_626726; 1:200).

Results

Network Analysis Identifies Amino Acids 113-116 of the BAX α 5 Core as a Nexus of Circumferential Interaction.

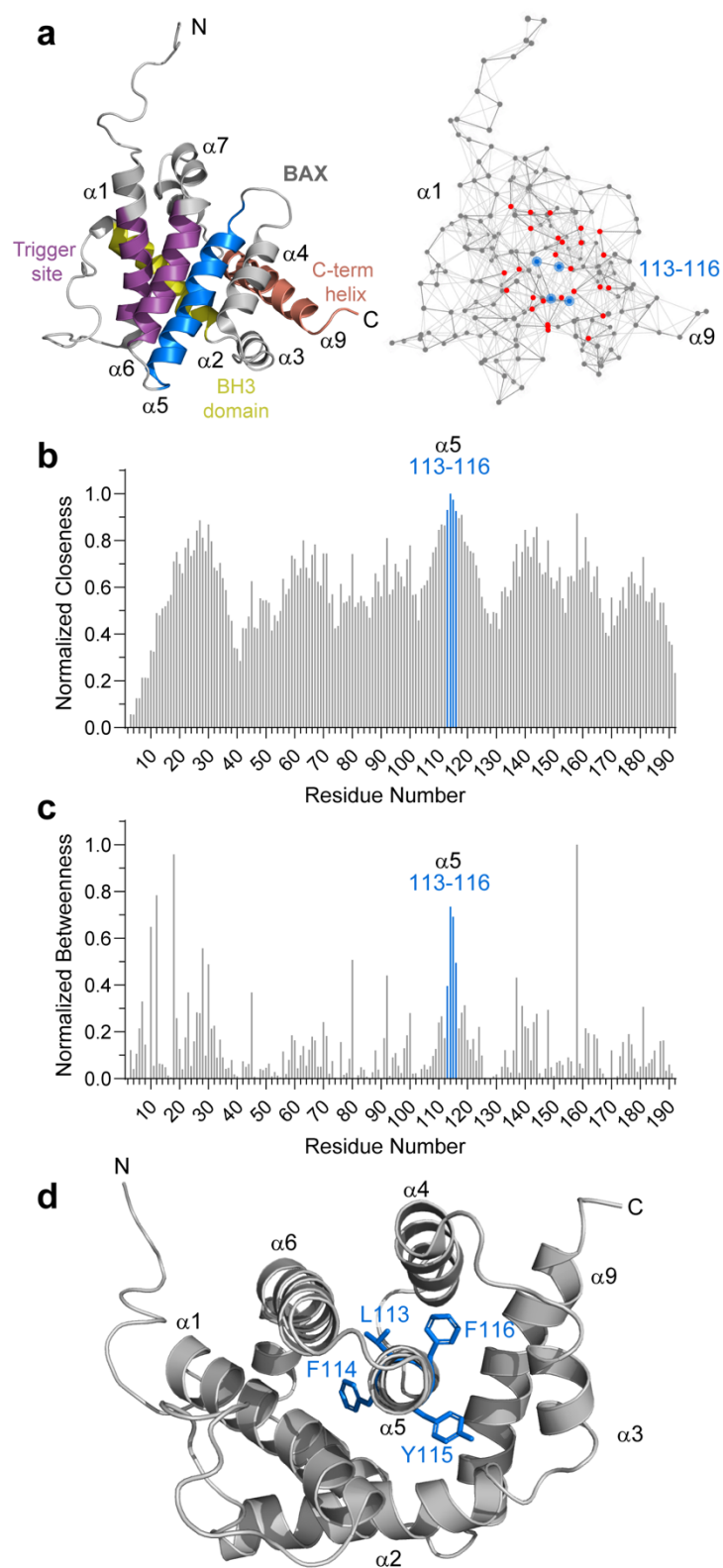
To investigate how the allosteric nature of BAX conformational regulation could be mediated by the α 5 core, we took an unbiased computational approach based on an analysis of BAX as a network of interactions between each of its amino acid residues (**Figure 2.1a**). Specifically, we used the Network Analysis of Protein Structures (NAPS) algorithm¹⁷, which has been widely applied to generate hypotheses about how protein structure influences protein function¹⁸⁻²⁵. The mathematical parameters of centrality, closeness and betweenness, provide a quantitative framework for the network analysis. Whereas closeness reflects the inverse distance of all shortest paths to a particular residue, betweenness represents the number of shortest bridges incorporating that amino acid. Thus, an amino acid with a high closeness value indicates the network proximity to neighboring residues and that with a high betweenness value signifies a high degree of engagement in shortest path interactions. Strikingly, amino acid F114 of α 5 demonstrated the highest closeness value among all BAX residues, and the 113-116 quartet of residues was the highest scoring cluster of consecutive residues for both closeness and betweenness within the BAX network (**Figure 2.1b-c**). Mapping these residues onto the BAX structure revealed that they comprise a complete helical turn of α 5 and radiate outward to contact a series of hydrophobic amino acids from every other α -helix in BAX (**Figure 2.1d, 2.2**). These data implicated α 5 residues 113-116 as a potential control point for the conformational regulation of BAX.

Single Alanine Mutagenesis of Amino Acids 113-116 Induces Ligand-Independent BAX Activation

To assess the influence of amino acids 113-116 on the conformational stability and functional activity of BAX, we generated recombinant full-length BAX proteins bearing L113A, F114A,

Figure 2.1. *Interaction centrality of core $\alpha 5$ residues 113-116 in the BAX protein network.* (a) Structure of monomeric BAX (PDB: 1F16) highlighting regions implicated in BAX activation, including the $\alpha 1/\alpha 6$ trigger site (purple), BH3 domain ($\alpha 2$, yellow), C-terminal transmembrane helix ($\alpha 9$, salmon), and the $\alpha 5$ hydrophobic core helix (blue). To the right is a representation of the BAX amino acid interaction network, with residues 113-116 and their network of interacting residues highlighted in blue and red, respectively. (b-c) Network centrality analysis of BAX residues plotted as normalized closeness (b) and betweenness (c). Amino acids 113-116, highlighted in blue, demonstrate the highest closeness values of all BAX residues (b) and the highest betweenness values for a consecutive cluster of BAX residues. (d) A view of monomeric BAX down the barrel of $\alpha 5$ demonstrating the radial distribution of residues 113-116 along a single α -helical turn and their collective engagement with every other α -helix of the BAX structure.

Figure 2.1 (Continued)



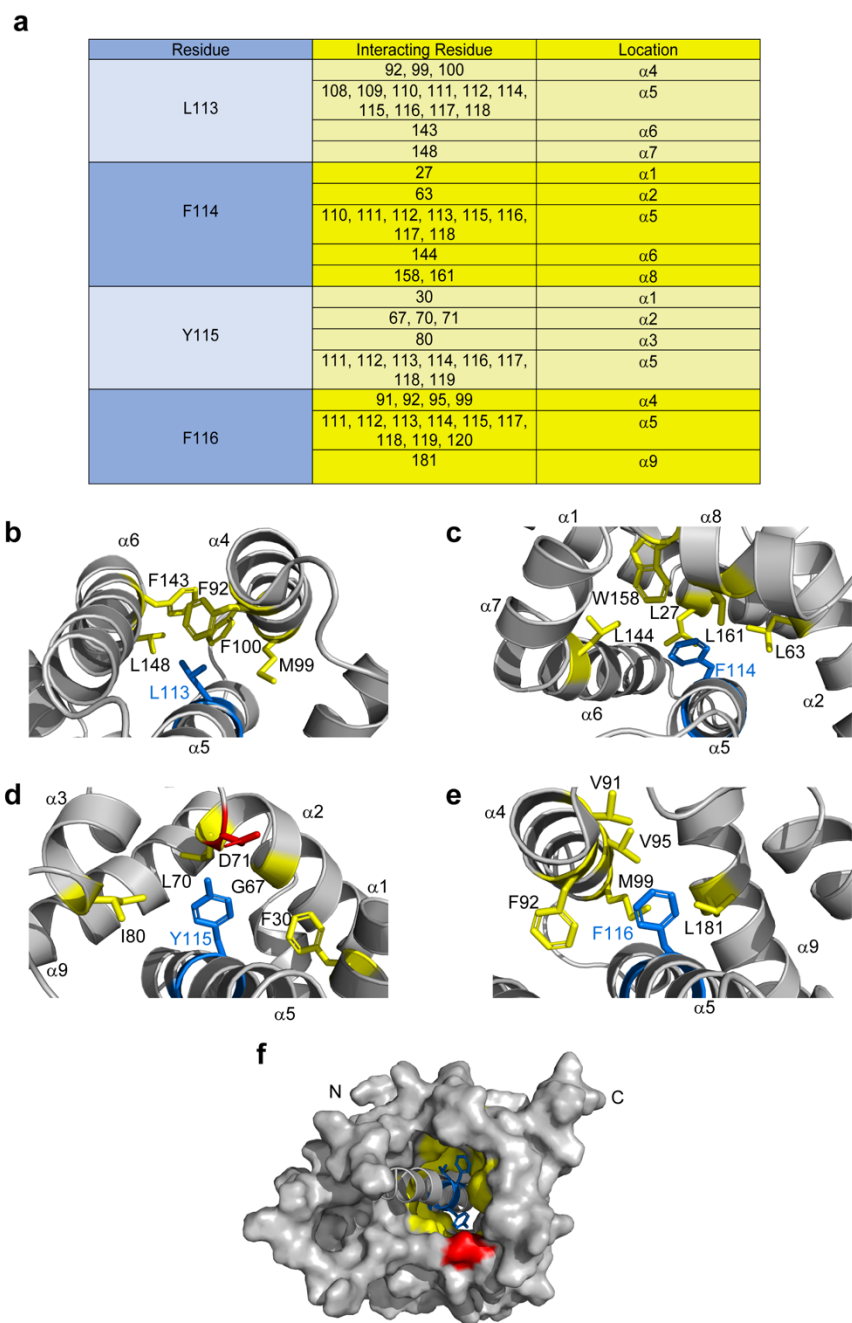


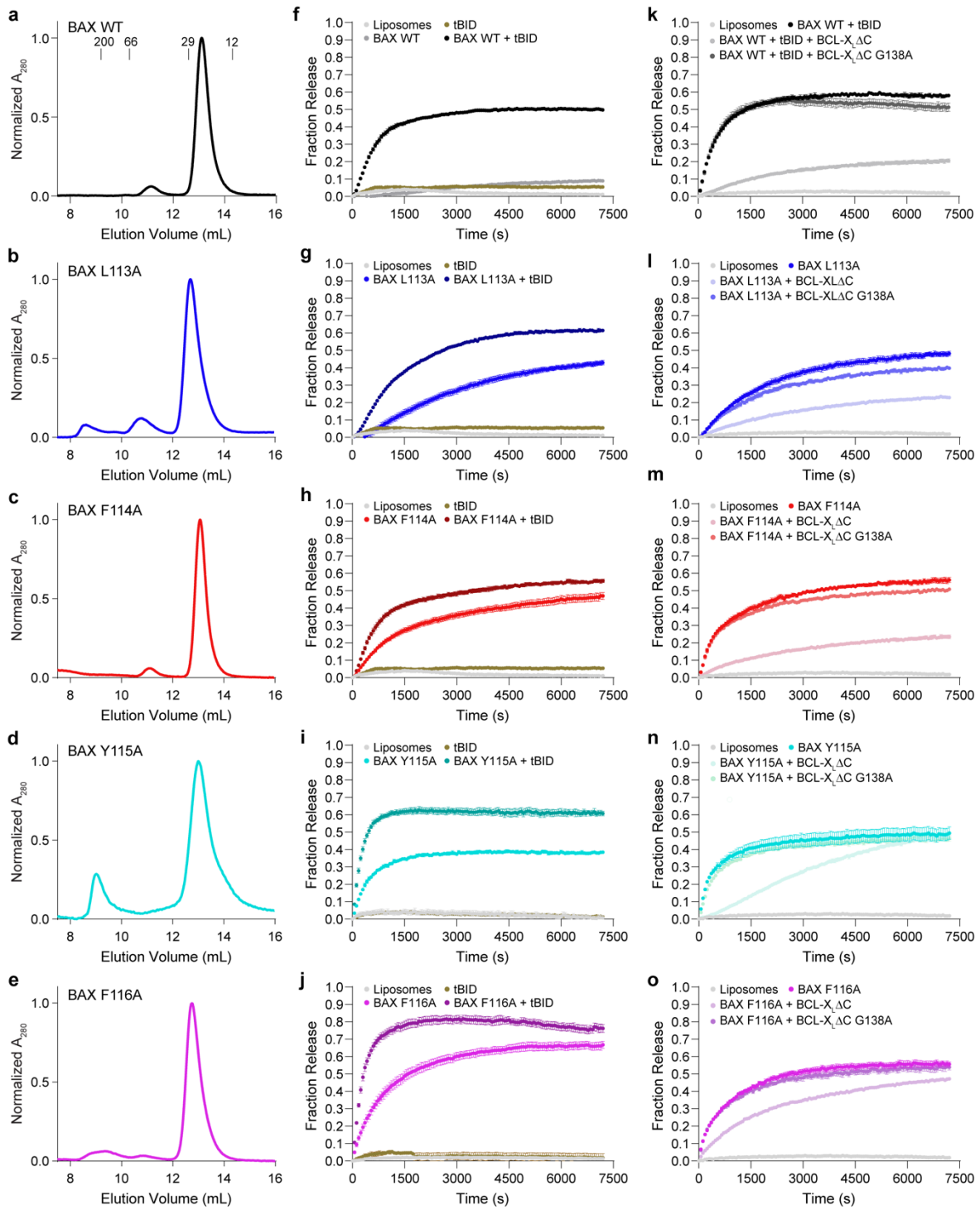
Figure 2.2 Network of residues engaged by BAX amino acids 113-116 of the $\alpha 5$ core. (a) Tabulated listing of BAX residues engaged by BAX $\alpha 5$ residues 113-116. (b-e) The network of amino acids that interact with residues 113-116 (blue) of the BAX $\alpha 5$ core through hydrophobic (yellow) or hydrogen bonding (red) contacts, as shown in ribbon diagrams for each of the four BAX $\alpha 5$ residues (b-e) and as an overall surface view (f).

Y115A, or F116A single point mutants. In each case, the predominant species generated in solution, as detected by size exclusion chromatography (SEC), was monomeric BAX protein (**Figure 2.3a-e**). We then incubated each BAX construct with liposomes loaded with the fluorophore/quencher pair ANTS/DPX and monitored membrane permeabilization, as quantitated by time-dependent liposomal release of fluorophore, in the presence and absence of the direct BAX activator BH3-only protein, tBID^{13,26}. Whereas incubation of liposomes with wild-type BAX had little to no membrane permeabilization effect, robust time-dependent release was observed upon tBID treatment, consistent with direct BH3-triggered BAX activation and functional BAX poration (**Figure 2.3f**). In contrast, and in each case, single alanine mutagenesis of residues 113, 114, 115, or 116 resulted in BAX-mediated liposomal release in the absence of a BH3 ligand, with BAX F116A showing the highest level of autoactivation in the presence of a membrane environment (**Figure 2.3g-j**). Addition of tBID enhanced activation of the BAX mutants even further, indicating that the direct, BH3-triggering mechanism was still intact (**Figure 2.3g-j**). For each mutant, and especially F116A, tBID treatment resulted in a maximal level of liposomal release exceeding that of the wild-type BAX protein, with enhanced release kinetics especially evident for the Y115A and F116A mutants (**Figure 2.3f-j**). We confirmed that alanine mutagenesis had no disruptive effect on liposomal membrane engagement as evidenced by equivalent tBID-induced BAX translocation (**Figure 2.4**).

To further confirm that the BAX alanine mutants were operating via the canonical conformational-activation pathway⁴, we tested the capacity of anti-apoptotic BCL-X_LΔC to impair BAX activity. We first demonstrated that our recombinant BCL-X_LΔC construct was functional and that its inhibitory activity was specifically dependent upon an intact canonical pocket, which is required to capture and neutralize pro-apoptotic BH3 domains²⁷. Our positive control experiments showed that tBID-triggered activation of wild-type BAX was effectively suppressed by BCL-X_LΔC and that G138A mutagenesis within the BCL-X_LΔC pocket abrogated its neutralizing activity

Figure 2.3 *Single alanine mutations of the 113-116 nexus cause BAX autoactivation and membrane poration.* (a-e) SEC profiles of full-length, recombinant wild-type (a), L113A (b), F114A (c), Y115A (d), and F116A (e) BAX proteins (5 μ M). SEC experiments were repeated twice using independent preparations of protein with similar results. (f-j) Liposomal release of ANTS/DPX by wild-type (f), L113A (g), F114A (h), Y115A (i), and F116A (j) BAX proteins (500 nM) in the presence and absence of the BH3-only activator tBID (10 nM). (k) Control experiment documenting the capacity of BCL-X_L Δ C (10 μ M) to inhibit tBID-induced, BAX-mediated liposomal release, whereas G138A point mutagenesis blocks the inhibitory activity of BCL-X_L Δ C. (l-o) Effect of BCL-X_L Δ C and BCL-X_L Δ C G138A (10 μ M) on the liposomal release autoactivity of L113A (l), F114A (m), Y115A (n), and F116A (o) BAX proteins (500 nM). Data are mean $\pm$ s.e.m. for liposomal release assays performed in technical quadruplicate and conducted twice using independent preparations of liposomes and proteins with similar results.

Figure2.3 (Continued)



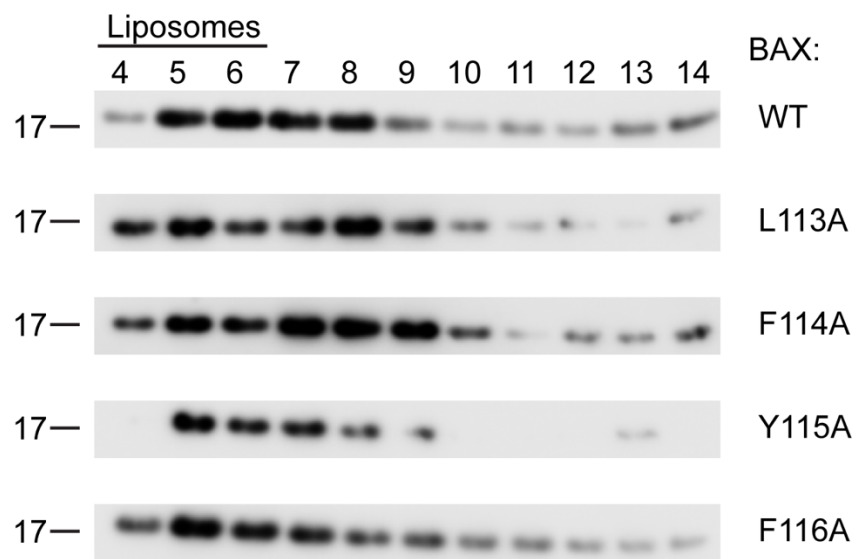


Figure 2.4 *Liposomal translocation of tBID-triggered BAX mutants.* tBID uniformly induces translocation of recombinant full-length BAX and its L113, F114, Y115, and F116 alanine mutants to the liposomal fractions, as demonstrated by BAX western analysis of fractions after 15 min incubation.

(**Figure 2.3k**). For each BAX mutant, co-incubation with BCL-X_LΔC impaired autoactivation, whereas BCL-X_LΔC G138A had no such effect (**Figure 2.3l-o**). Whereas the efficacy of BCL-X_LΔC inhibition of the L113A and F114A mutants was similar to that observed for suppression of tBID-triggered wild-type BAX (**Figure 2.3k-m**), the autoactivity of the BAX Y115A and F116A variants was only partially blocked (**Figure 2.3n-o**), suggesting that the latter mutants are more active, as also evidenced by the shorter time to reach maximal liposomal release in the presence and absence of tBID (**Figure 2.3i-j**). To confirm that incomplete inhibition of BAX Y115A and F116A was due to a kinetics disadvantage of BCL-X_LΔC, we subjected the most active variant, BAX F116A, to incubation with full-length BCL-X_L, which bears the C-terminal membrane-insertion helix. In contrast to BCL-X_LΔC, full-length BCL-X_L is independently capable of partitioning to liposomal membranes (**Figure 2.5a**) and is indeed more effective at suppressing release by BAX F116A autoactivation in the presence of liposomes (**Figure 2.3o, 2.5b**). Taken together, these data reveal that just a single point mutation within the 113-116 amino acid nexus of the BAX α5 core causes autoactivation in the presence of liposomal membranes. The observed activity can be enhanced by canonical BH3-triggering and suppressed by canonical anti-apoptotic BH3-in-groove capture, consistent with an on-pathway mechanism of action.

Functional Consequences of Individual BAX 113-116 Mutagenesis in Mitochondria and Cells

To evaluate our findings in a physiologic context, we first examined the influence of each single alanine mutant on mitochondrial outer membrane permeabilization, as assessed by BAX-mediated cytochrome *c* release from liver mitochondria isolated and purified from *Alb-cre^{pos}Bax^{fl/f}Bak^{-/-}* mice⁴. In contrast to the reductionist liposomal release assay, which contains only the core components for biochemical analysis, namely membrane, BAX, a BH3 trigger, and an anti-apoptotic protein, isolated mitochondria contain the full spectrum of proteins and lipids of the native organelle, adding complexity to the experimental system. Consistent with the liposomal

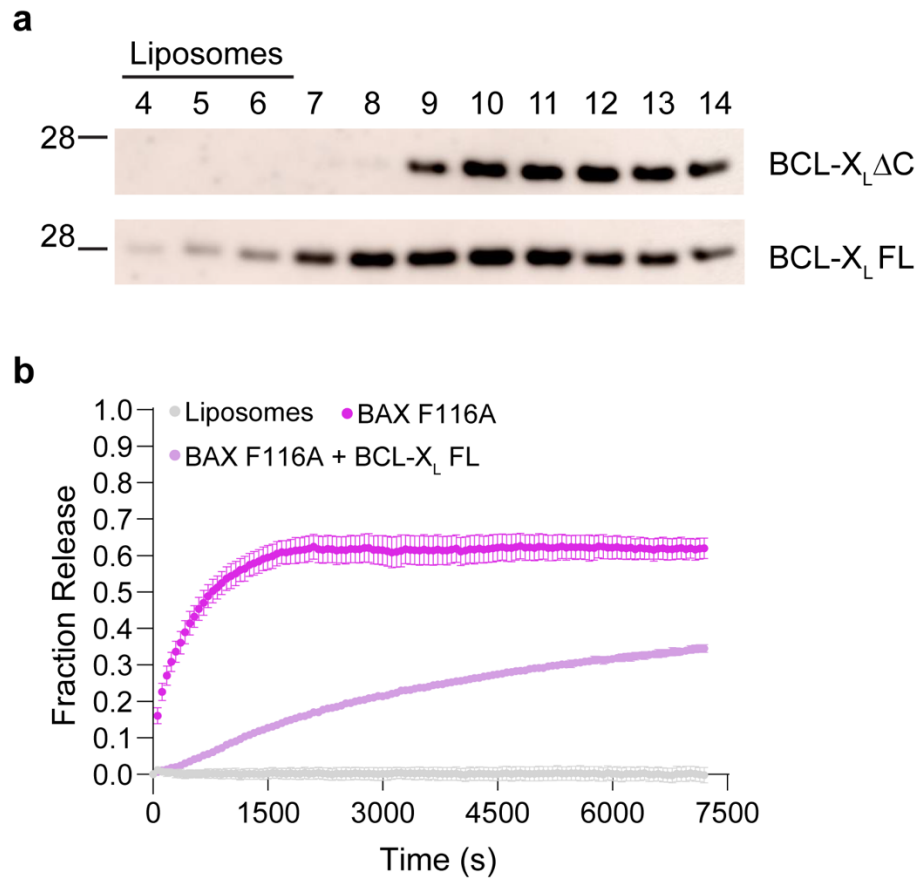


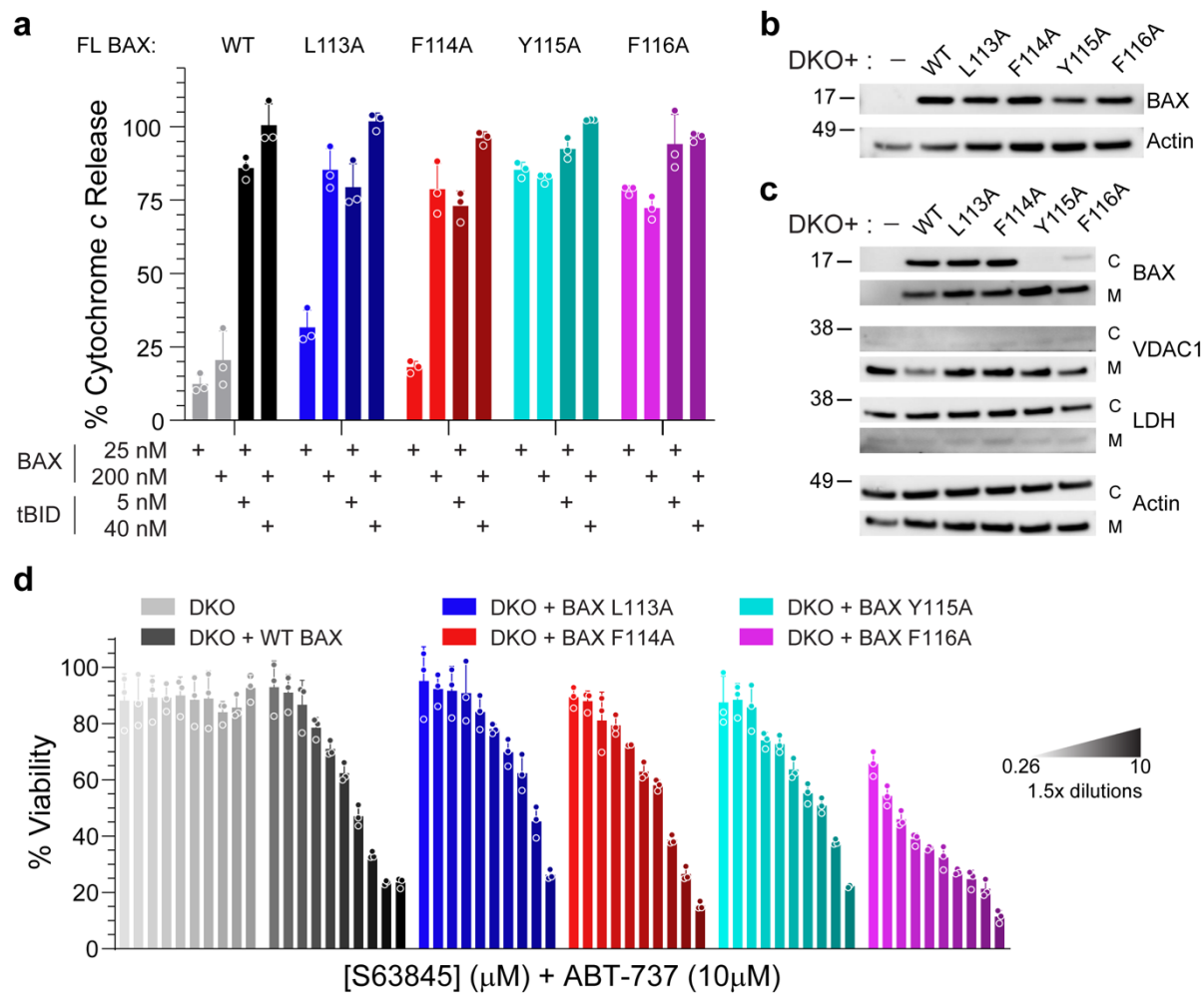
Figure 2.5 Liposomal partitioning of full-length BCL- X_L and its suppression of BAX F116A. (a) Whereas BCL- $X_L\Delta C$ remains fully in the aqueous phase, full-length BCL- X_L (BCL- X_L FL) independently partitions, in part, to liposomal membranes. (b) Compared to BCL- $X_L\Delta C$ (**Figure 2.3o**), BCL- X_L FL is more effective at suppressing BAX F116A-mediated liposomal release.

release data (**Figure 2.3g-j**), mitochondrial treatment with the individual BAX alanine mutants induced cytochrome *c* release in the absence of a BH3 stimulus (**Figure 2.6a**). Again, the BAX Y115A and F116A variants were the most active, and in the mitochondrial context equally active, inducing near maximal cytochrome *c* release at 25 nM dosing, whereas the BAX L113A and F114A proteins achieved a similar level of activity at 200 nM dosing (**Figure 2.6a**). Wild-type BAX exhibited little to no autoactivation at either protein dose and required triggering with tBID to induce cytochrome *c* release. tBID enhanced BAX-mediated cytochrome *c* release for the alanine mutants as well and was most evident for the relatively less autoactive L113A and F114A variants, particularly at the 25 nM dosing level. All BAX variants achieved complete cytochrome *c* release at the maximal tBID (40 nM) and BAX (200 nM) dosing applied, again indicating that the predominant impact of mutagenesis involved initiation of BAX activation rather than execution.

To assess the impact of alanine mutagenesis of the BAX 113-116 residues in a cellular context, we reconstituted *Bax*^{-/-}*Bak*^{-/-} mouse embryonic fibroblasts (MEFs) with wild-type BAX or the individual mutants, selecting for live cells that expressed equivalent levels of BAX protein (**Figure 2.6b**). In comparison to the isolated mitochondrial experiments, the cellular context adds even further complexity to the analysis given the presence of the full spectrum of regulatory BCL-2 family and other proteins within the cytosol and cellular organelles. We first examined how the BAX proteins partitioned between cytosolic and mitochondrial fractions under baseline conditions, in the absence of a stress stimulus. Intriguingly, BAX L113A and BAX F114A proteins were identified in relatively equal proportions between the two fractions, whereas BAX Y115A and F116A were almost exclusively found in the mitochondrial fraction, consistent with an increased propensity for mitochondrial translocation – a feature of autoactivation (**Figure 2.6c**). To measure comparative pro-apoptotic activity of the reconstituted BAX proteins, we treated the individual cell lines with small molecules that selectively target a discrete spectrum of anti-apoptotic proteins, blocking MCL-1 with S63845²⁸ and BCL-2/BCL-X_L with ABT-737²⁹, thereby derepressing native BH3-only protein activators of BAX and disrupting inhibitory interactions between the targeted

Figure 2.6 *Effect of BAX 113-116 alanine mutagenesis on mitochondrial apoptosis, intracellular localization, cell viability.* (a) Cytochrome *c* release from liver mitochondria purified from *Alb-cre^{pos}Bax^{fl/fl}Bak^{-/-}* mice. Mitochondria were treated with the indicated concentrations of full-length (FL) BAX proteins in the presence or absence of tBID, and cytochrome *c* released into the supernatant was measured by ELISA. Data are mean $\pm$ s.d. for cytochrome *c* release assays performed in technical triplicate and conducted twice using independent preparations of proteins and mitochondria with similar results. (b) Western analysis of lysates from *Bax^{-/-}Bak^{-/-}* MEFs reconstituted with wild-type BAX or the indicated alanine mutants, using BAX and actin antibodies. The analysis was performed twice using independent preparations of proteins with similar results. (c) Western analysis of cytosolic (C) and mitochondrial (M) fractions from *Bax^{-/-}Bak^{-/-}* MEFs with wild-type BAX or the indicated alanine mutants, using BAX, VDAC1, LDH and actin antibodies. The experiment was performed twice using independent cell cultures and fractionation with similar results. (d) Cell viability of *Bax^{-/-}Bak^{-/-}* MEFs reconstituted with wild-type BAX or the indicated alanine mutants and co-treated with ABT-737 (10 μ M) and the indicated doses of S63845 for 24 h, as measured by CellTiter-Glo. Data are mean $\pm$ s.d. for viability experiments performed in technical triplicate and conducted twice using independent cell cultures and treatments with similar results.

Figure 2.6 (Continued)



anti-apoptotic proteins and reconstituted BAX. As a measure of “end stage” BAX activity, cell viability was impaired across all cell lines except for the *Bax*^{-/-}*Bak*^{-/-} MEFs, validating that each reconstituted BAX protein - irrespective of BAX 113-116 point mutagenesis that individually influence the afferent step of conformational activation of monomeric BAX - can effectively execute its pro-apoptotic function (**Figure 2.6d**). Although BAX Y115A and F116A were equally hyperactive in inducing cytochrome *c* release from isolated mitochondria and showed similar mitochondrial auto-translocation in cells, MEFs reconstituted with BAX F116A were the most sensitive to anti-apoptotic inhibition (**Figure 2.6d**), consistent with this particular mutant having the fastest kinetics and highest absolute level of liposomal release activity in response to BH3-triggering (**Figure 2.3j**).

Each Residue of the 113-116 Nexus Plays a Regiospecific Role in Stabilizing the Structure of Monomeric BAX

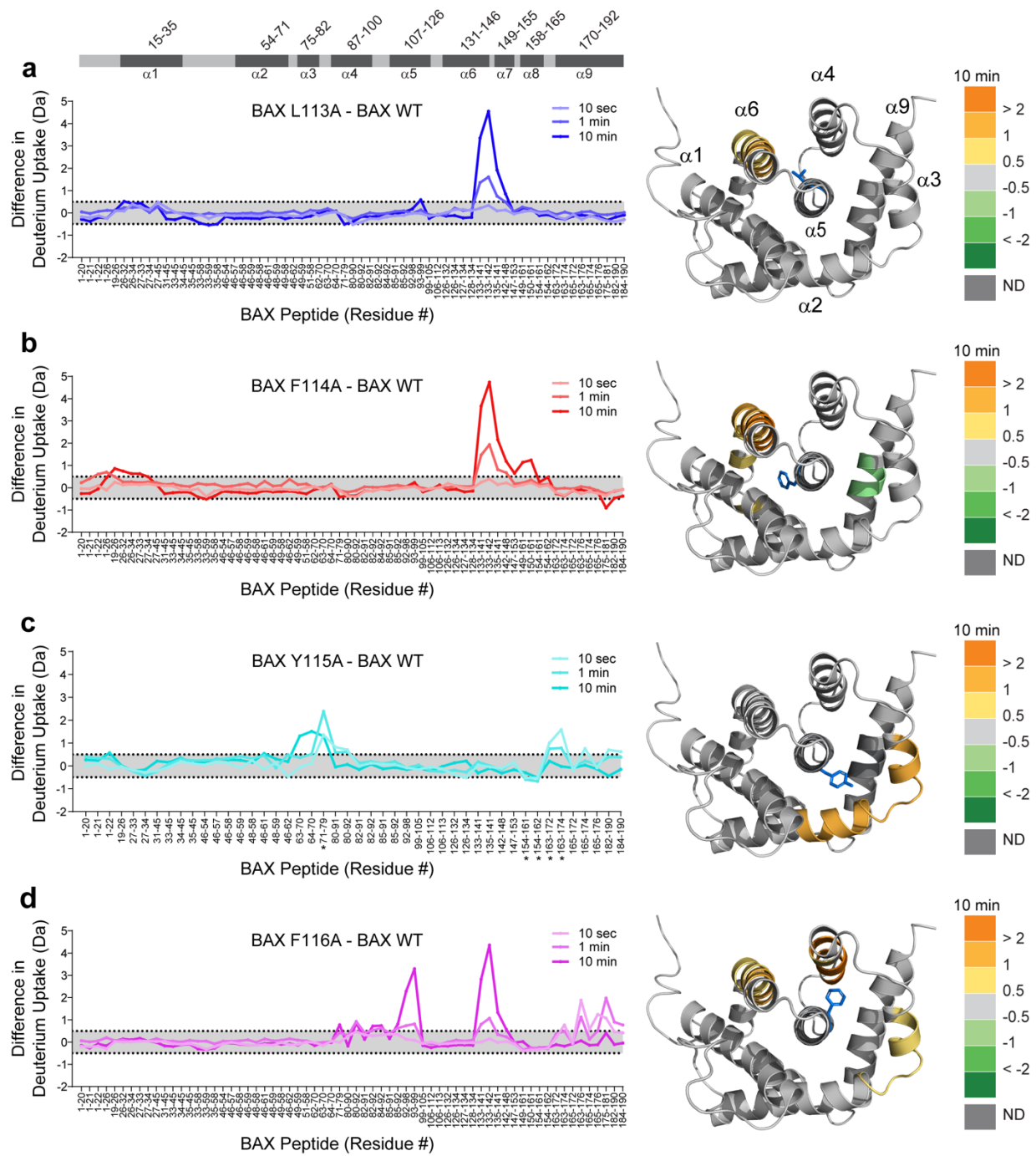
To investigate the structural basis for the biochemical and cellular phenotype upon single alanine mutagenesis of the 113-116 nexus, we turned to hydrogen-deuterium exchange mass spectrometry (HDX MS) analysis, which has the capacity to assess conformational dynamics³⁰. HDX MS probes protein structure by measuring deuterium incorporation at backbone amide hydrogens. When diluted into deuterium buffer, backbone hydrogens of flexible and/or exposed protein regions rapidly exchange with deuterium. In contrast, buried domains and/or those regions that contain hydrogen bonding of backbone amide hydrogens (such as in α -helices), or that are engaged in ligand interactions, demonstrate slowed or suppressed deuterium exchange. Here, we compared the HDX MS profile of full-length recombinant BAX in solution with that of the BAX L113A, F114A, Y115A, and F116A single point mutants. In each case, we observed striking, regiospecific consequences of replacing the bulky hydrophobic residue with alanine. Specifically, the L113A mutant showed progressive deprotection over time of $\alpha 6$, which is directly engaged by

L113 and implicated in both the mechanism of BH3 triggering¹² and membrane interaction by its conserved arginine residues³¹ (**Figure 2.7a**). For F114A, the location of increased deuterium exchange shifts to portions of $\alpha 1$, $\alpha 6$ and $\alpha 7$, which are in direct contact with F114, in addition to allosteric protection of the very portion of $\alpha 9$ located at the opposite surface of $\alpha 5$ relative to F114 (**Figure 2.7b**). Y115A mutagenesis caused deprotection of the distal half of $\alpha 2$ and the entirety of $\alpha 3$, to which Y115 points (**Figure 2.7c**). In contrast to L113A and F114A, Y115A mutagenesis induced rapid and transient deprotection of the C-terminal $\alpha 9$ helix, as evidenced by increased deuterium exchange at the earliest labeling time point (**Figure 2.7c, 2.8a**). F116A mutagenesis also exhibited early deprotection of $\alpha 9$, in addition to progressive deprotection over time of the most distal residue of $\alpha 2$, in addition to $\alpha 3$, $\alpha 4$ and $\alpha 6$ (**Figure 2.7d, 2.8b**). Among the four constructs, F116A mutagenesis induced the greatest conformational loosening of the BAX structure, as demonstrated by increased deuterium exchange across portions of helices $\alpha 2$, $\alpha 3$, $\alpha 4$, and $\alpha 6$ (**Figure 2.7d**).

These HDX MS data provide a series of mechanistic insights into the relative roles of residues 113-116 in both girding the monomeric structure of BAX and regulating its conformational dynamics. First, the sequential radial deprotection of BAX α -helices that directly face each of the four core $\alpha 5$ residues underscores the critical infrastructural role these residues play in stabilizing monomeric BAX (**Figure 2.7a-d**). Second, the differential regions of deprotection among the four mutants provide a structural explanation for the degree of autoactivation observed in membrane release assays and the differences in mitochondrial partitioning upon reconstitution of the BAX mutants in cells. For example, BAX Y115A and F116A, which were observed to have heightened kinetics of autoactivation in liposomal release assays (**Figure 2.3i-j**) and a lower dosing threshold for autoinduction of mitochondrial cytochrome *c* release (**Figure 2.6a**) are the two mutants that show (1) similar deprotection of the distal portion

Figure 2.7 *HDX MS reveals regiospecific changes in conformational dynamics upon single alanine mutagenesis of BAX 113-116.* (a-d) Deuterium difference plots showing the relative deuterium incorporation of BAX L113A (a), F114A (b), Y115A (c), and F116A (d) minus the relative deuterium incorporation of BAX wild-type, as measured at 10 sec, 1 min, and 10 min of deuterium labeling. Regions of deprotection (orange) and protection (green) above the 0.5 Da significance threshold at 10 min labeling are mapped onto the solution structure of BAX (PDB: 1F16), with the respective mutated residue colored in blue. HDX MS experiments were performed at least twice using independent preparations of BAX proteins. The asterisk in (c) marks 5 of 315 peptides in the study (63 unique peptides were followed per BAX protein) that display minor EX1 kinetic signatures (bimodal exchange patterns reflective of correlated/cooperative unfolding) at only the earliest time point; all other peptides display EX2 kinetics (uncorrelated local dynamics producing a binomial exchange profile).

Figure 2.7 (Continued)



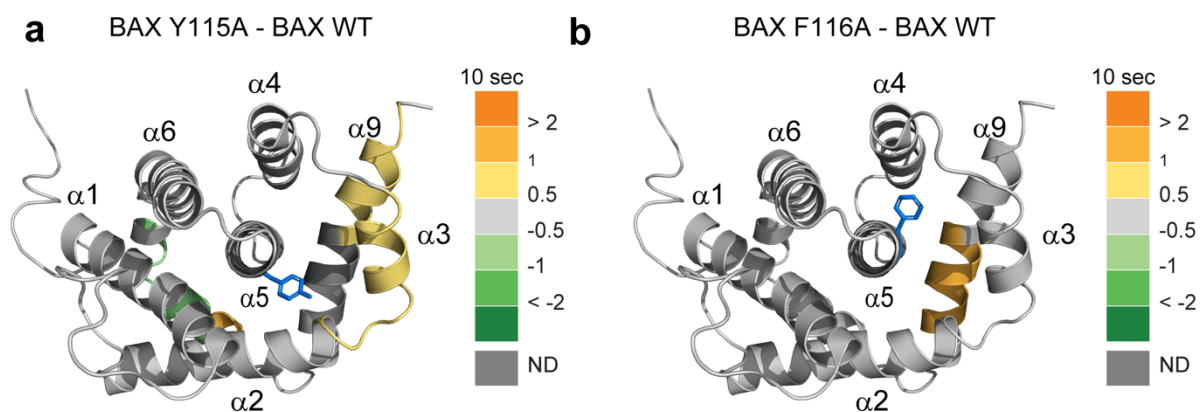


Figure 2.8 HDX MS analysis of BAX Y115A and BAX F116A reveals changes in $\alpha 9$ conformational dynamics. (a-b) Regions of relative deuterium deprotection (orange) and protection (green) above the 0.5 Da significance threshold at 10 sec of deuterium labeling are mapped onto the solution structure of BAX (PDB 1F16) for Y115A (a) and F116A (b), with the respective mutated residue colored in blue. HDX MS experiments were performed at least twice using independent preparations of BAX proteins.

of $\alpha 2$, the $\alpha 2$ - $\alpha 3$ hinge, and $\alpha 3$ (**Figure 2.7c-d**), the very region of BAX containing the critical BH3 helix that is essential to the propagation of BAX activation and oligomerization³² and (2) early exposure of $\alpha 9$, whose release from the canonical binding pocket drives mitochondrial translocation^{3,4}. Indeed, comparative anti-BAX BH3 pull-down of the four alanine mutants demonstrated selective immunoprecipitation of Y115A and F116A (**Figure 2.9**), corroborating the HDX MS results that revealed conformational deprotection of the $\alpha 2$ - $\alpha 3$ region. Of the four mutants, F116A mutagenesis results in the fastest kinetics and highest absolute level of liposomal release activity, both alone and in response to a BH3 stimulus (**Figure 2.3i**). Upon targeted inhibition of anti-apoptotic proteins in *Bax*^{-/-}*Bak*^{-/-} MEFs reconstituted with the BAX mutants, F116A mutagenesis also has the most detrimental effect on cell viability (**Figure 2.6d**). Consistent with these functional data, BAX F116A is the mutant that shows deprotection across the most α -helices by HDX MS (**Figure 2.7d**). Finally, the two mutants with relatively lower yet clearly evident propensity for autoactivation, L113A and F114A, share the common feature of $\alpha 6$ deprotection (**Figure 2.7a-b**), which is also observed for F116A (**Figure 2.7d**). As both a component of the BH3 trigger site and harboring critical arginine residues involved in mitochondrial membrane interaction and poration, $\alpha 6$ has important and separable functions in the initiation and execution phases of BAX activation³¹. Taken together, the HDX MS analysis reveals how the nexus of BAX 113-116 interactions at the $\alpha 5$ core stabilize the monomeric state and further demonstrates how disruption of these interactions, whether by alanine mutagenesis, direct BH3 triggering^{4,12}, or pharmacologic modulation⁶⁻⁸, can facilitate the transformation of latent BAX into a membrane-disruptive oligomer.

Distinguishing Roles for BAX 113-116 in Monomeric Versus Oligomeric Stability

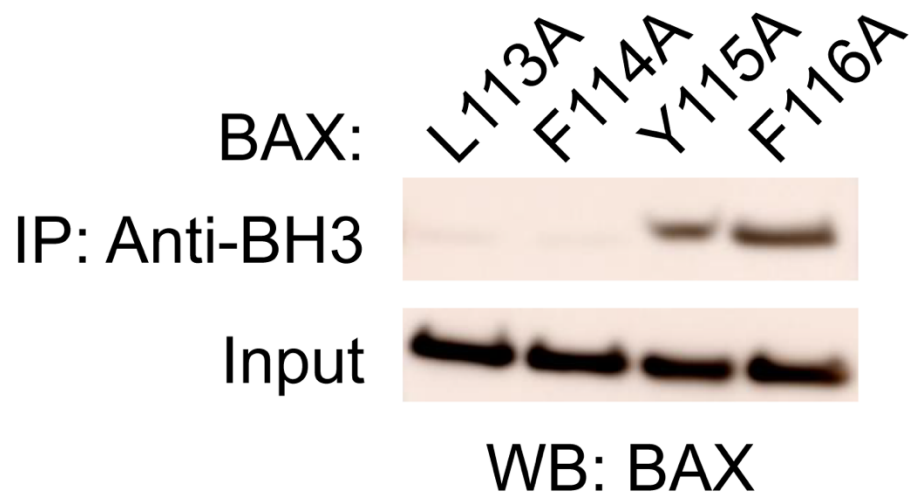


Figure 2.9 *Comparative immunoprecipitation of BAX alanine mutants by a BAX BH3 antibody.*

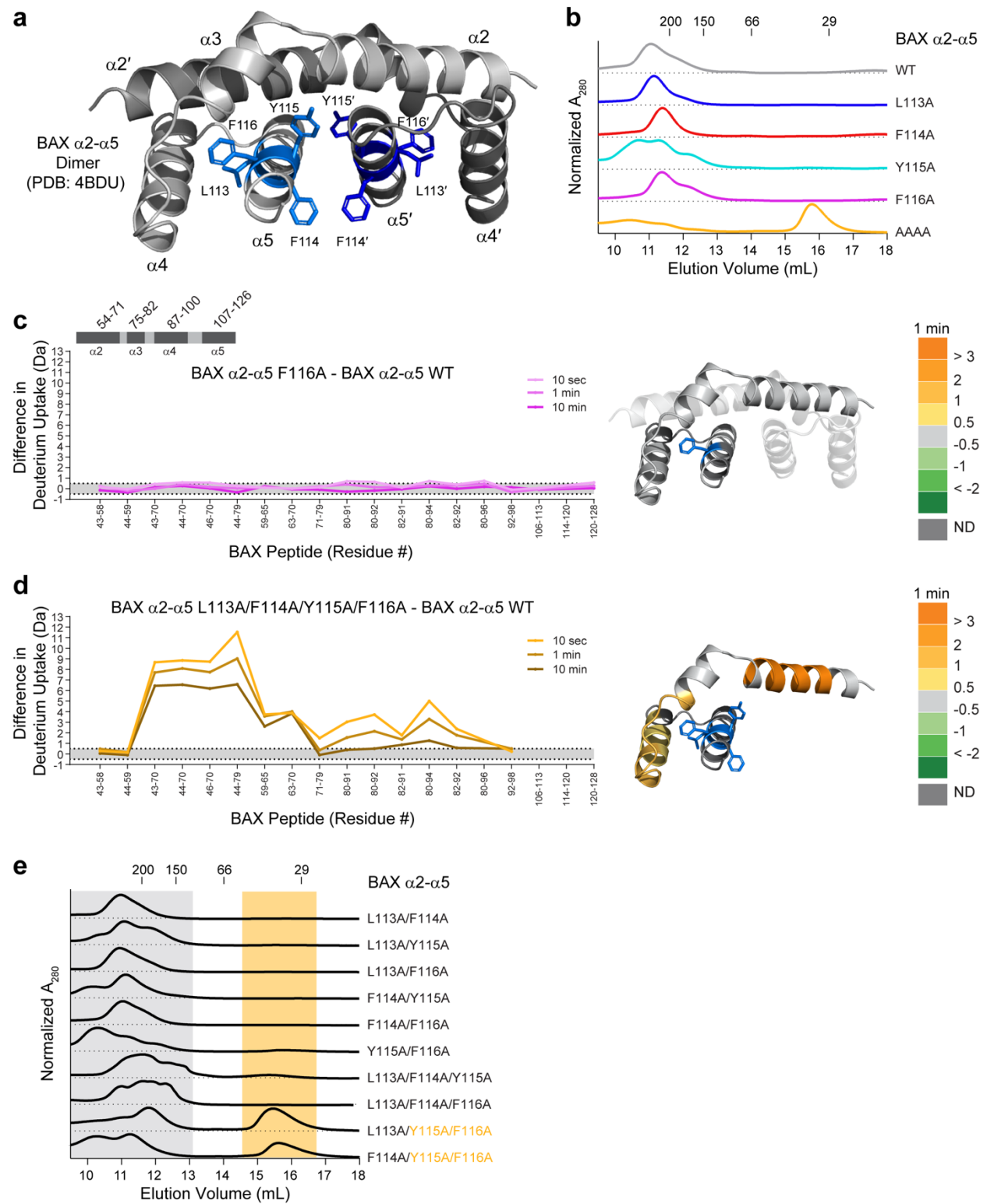
The indicated single alanine BAX mutants were incubated with a BAX BH3 antibody, followed by immunoprecipitation, electrophoresis, and detection by BAX western analysis. BAX Y115A and BAX F116A, but not BAX L113A and F114A, were immunoprecipitated by the BAX BH3 antibody, consistent with conformational deprotection of the $\alpha 2$ - $\alpha 3$ region in the two mutants, as detected by HDX MS (**Figure 2.7c-d**).

Intriguingly, a prior cellular study showed that quadruple mutagenesis of BAX 113-116 in the context of the full-length protein abrogated the pro-apoptotic activity of BAX, consistent with a defect in the execution phase of BAX activation, and homo-oligomerization in particular³³. A dimeric species of BAX $\alpha 2$ - $\alpha 5$ was proposed to function as an essential subunit of homo-oligomerization³³ and a crystal structure of the dimeric truncate (GFP-BAX $\alpha 2$ - $\alpha 5$) was subsequently solved¹¹. Most recently, NMR analysis of this dimeric species (tagless) in bicelles delineated portions of BAX $\alpha 2$ - $\alpha 5$ that can directly engage membrane lipids³⁴. The specific amino acid interactions of BAX 113-116 are distinct in the latent, full-length monomer and the dimeric BAX $\alpha 2$ - $\alpha 5$ complex (**Fig 2.2d, 2.10a**). To dissect the specific roles of BAX residues 113-116 in monomeric versus dimeric stability, we generated BAX $\alpha 2$ - $\alpha 5$ truncates bearing the individual alanine mutants and the quadruple mutant, and evaluated dimeric assembly by SEC. We strikingly found that the individual alanine mutants had no disruptive effect on dimeric assembly (observed as a GFP-mediated dimer/oligomer of BAX $\alpha 2$ - $\alpha 5$ dimers¹¹), whereas quadruple mutagenesis completely disrupted formation of the complex (**Figure 2.10b**), as observed in the context of full-length BAX in cells³³. To examine and compare the conformational consequences of individual versus collective alanine mutagenesis in the dimeric context, we performed HDX MS analyses of GFP-BAX $\alpha 2$ - $\alpha 5$ and its F116A and quadruple alanine mutants. Whereas single F116A mutagenesis induced the most dramatic conformational consequences in monomeric full-length BAX, no effect was observed in the context of the BAX $\alpha 2$ - $\alpha 5$ dimer (**Figure 2.10c**), consistent with the SEC analysis (**Figure 2.10b**). Instead, quadruple mutagenesis induced marked conformational deprotection in BAX $\alpha 2$ - $\alpha 5$ (**Figure 2.10d**), consistent with monomeric exposure of previously buried motifs due to blockade of dimer formation.

Finally, to discern the relative contributions of each residue of the 113-116 nexus to formation of the core BAX $\alpha 2$ - $\alpha 5$ dimer, we undertook a comprehensive mutational analysis, generating the 6 combinations of double mutants and 4 combinations of triple mutants

Figure 2.10 *Collective rather than singular mutagenesis of the BAX 113-116 nexus is required to disrupt the conformational stability of the BAX α 2- α 5 dimer.* (a) Crystal structure of dimeric GFP-BAX α 2- α 5 (PDB: 4BDU) with the N-terminal GFP-tag removed for clarity. The positions of residues 113-116 are shown in blue. (b) SEC of the indicated wild-type and single alanine and quadruple alanine (AAAA) mutant forms of GFP-BAX α 2- α 5. The dimeric complex of GFP-BAX α 2- α 5 is observed as a dimer/oligomer of dimers (e.g. tetramerization mediated by GFP). The experiment was performed twice using independent preparations of proteins with similar results. (c-d) Deuterium difference plots showing the relative deuterium incorporation of (c) BAX α 2- α 5 F116A and (d) BAX α 2- α 5 L113A/F114A/Y115A/F116A minus the relative deuterium incorporation of wild-type BAX α 2- α 5, as measured at 10 sec, 1 min, and 10 min of deuterium labeling. Regions of deprotection (orange) and protection (green) above the 0.5 Da significance threshold at 1 min labeling are mapped onto the crystal structure (PDB: 4BDU) of the BAX α 2- α 5 dimer for (c) and a monomeric subunit for (d), with the respective mutated residue(s) colored in blue. HDX MS experiments were performed at least twice using independent preparations of BAX proteins. All peptides display EX2 kinetics (uncorrelated local dynamics producing a binomial exchange profile). (e) SEC profiles of the indicated double and triple alanine mutant forms of GFP-BAX α 2- α 5. Elution positions of wild-type dimer and quadruple-mutant monomer are shaded in grey and gold, respectively, as references.

Figure 2.10 (Continued)



(**Figure 2.10e**). Of note, the BAX $\alpha 2$ - $\alpha 5$ system was especially useful for this comparative analysis in solution owing to the inability to generate the quadruple mutant construct in full-length form. Like the individual alanine mutants (**Figure 2.10b**), all variants bearing double alanine mutations maintained the capacity to self-assemble. The triple BAX $\alpha 2$ - $\alpha 5$ mutants L113A/F114A/Y115A and L113A/F114A/F116A also eluted as a complex, indicating that retention of native residues Y115 or F116 is sufficient to preserve dimerization and thus rescue quadruple alanine mutagenesis. Interestingly, when Y115 and F116 are mutated in the context of a triple alanine mutation, specifically L113A/Y115A/F116A and F114A/Y115A/F116A, dimerization is partially impaired, as demonstrated by the presence of a monomeric species (**Figure 2.10e**). These data highlight (1) key combinatorial roles of residues Y115 and F116 in BAX self-association; (2) the capacity of L113 or F114 to maintain at least partial self-association of the triple mutant bearing Y115A and F116A mutations; and (3) L113 and F114 in combination can completely overcome Y115A/F116A mutagenesis in the context of the double mutant. These findings reinforce the conclusion that each of the 113-116 residues can independently influence the stability of monomeric BAX, but this nexus of residues functions collectively to regulate the formation and stability of the core dimeric species of BAX $\alpha 2$ - $\alpha 5$. Indeed, comparative network analysis demonstrates that in the dimeric assembly, a series of residues in the $\alpha 2$ helix and proximal $\alpha 3$ have substantially increased closeness and betweenness values, reflecting additional reinforcement of the dimeric assembly compared to monomeric BAX (**Figure 2.11, 2.12**). Correspondingly, we observe that dimeric assembly persists despite single alanine mutagenesis of residues 113-116 and instead quadruple mutagenesis is required for complete complex disruption, highlighting the distinct roles of these key residues and their interactions in regulating the conformational dynamics of monomeric versus oligomeric BAX.

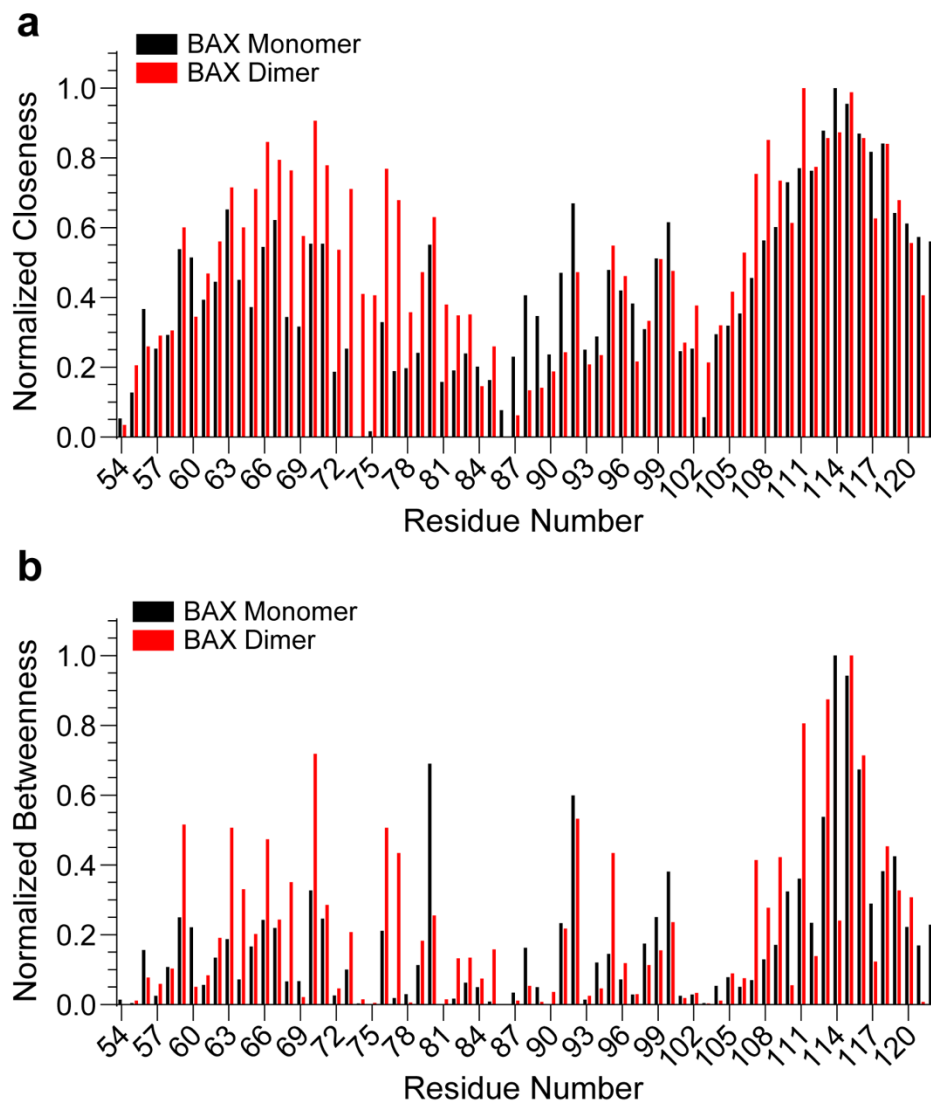


Figure 2.11 *Comparative network analyses of monomeric and dimeric BAX. (a-b) Network analysis of BAX residues 54-122 ($\alpha 2$ - $\alpha 5$) plotted as normalized closeness (a) and betweenness (b) for the monomer versus dimer.*

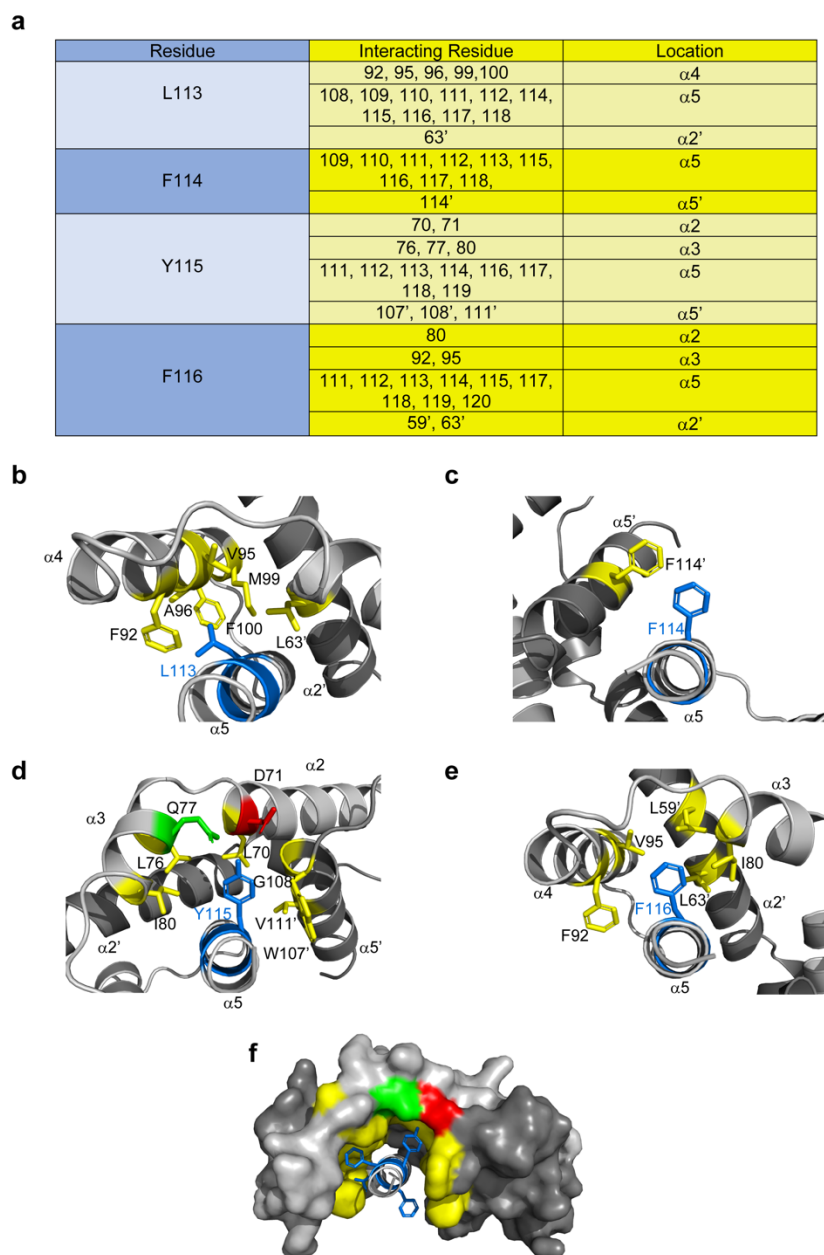


Figure 2.12 Network of residues engaged by BAX amino acids 113-116 of the BAX $\alpha 2$ - $\alpha 5$ dimer.

(a) Tabulated listing of BAX $\alpha 2$ - $\alpha 5$ residues engaged by BAX $\alpha 5$ residues 113-116. (b-e) The network of amino acids that interact with residues 113-116 (blue) of the BAX $\alpha 5$ core through hydrophobic (yellow) or hydrogen bonding (red, green) contacts, as shown in ribbon diagrams for each of the four BAX $\alpha 5$ residues (b-e) and as an overall surface view (f).

Discussion

BAX is a relatively small protein of only 21 kDa in size yet has the capacity to transform into a higher order species that is responsible for cellular self-destruction. Given its lethal potential, BAX is subject to a remarkable degree of regulation. Such physiologic mechanisms include auto-inhibitory interactions at the N- and C-terminal faces of BAX^{3,4} and targeted inhibition by anti-apoptotic members of the BCL-2 family^{1,9,27} and other proteins, such as the cytomegalovirus vMIA protein³⁵. BAX activation can occur directly, through triggering interactions between select BH3-only proteins and a binding site formed by the confluence of $\alpha 1$ and $\alpha 6$ residues at the N-terminal face¹², and indirectly, by BH3-only protein displacement of activated BAX from neutralizing heterodimeric complexes with anti-apoptotic proteins^{36,37}. BAX can also be activated by heat³⁸, highlighting that a discrete energetic threshold exists that, when breached, can transform an otherwise stabilized architecture into a volatile one. In contrast to heat-mediated protein denaturation and loss-of-function, enhancing the structural flexibility of BAX results in a dramatic conformational change that underlies a gain-of-function, namely higher order oligomerization and permeabilization of the mitochondria.

A series of structure-function studies have demonstrated the critical role of allostery in mediating the conformational regulation of BAX^{3,4,39,40}. For example, biochemical and structural analyses using full-length recombinant BAX protein revealed that BH3 ligands capable of displacing the $\alpha 1$ - $\alpha 2$ loop from the N-terminal surface, can initiate BAX activation by allosteric release of $\alpha 9$ ⁴. In contrast, synthetic tethering of the $\alpha 1$ - $\alpha 2$ loop or $\alpha 9$ to its respective N- and C-terminal grooves effectively restrains monomeric BAX⁴. The discovery and structure-function analyses of additional BAX-interacting ligands have further reinforced the allostery theme of BAX regulation. A small molecule that binds to the confluence of the $\alpha 3/\alpha 4$ and $\alpha 5/\alpha 6$ hairpins sensitizes BAX activation by allosteric mobilization of the $\alpha 1$ - $\alpha 2$ loop and $\alpha 2$ at the opposite side of the protein⁸. Non-enzymatic covalent derivatization of C126 by the mitochondrial lipid

electrophile, 2-hexadecenal, induces allosteric chemical shift perturbations at both the $\alpha 1$ - $\alpha 2$ loop and $\alpha 9$, as observed by NMR analysis, and enhances BAX activity⁴¹. In contrast, inhibitors such as the BCL-2 BH4 domain, which engages BAX at a groove formed by the confluence of the $\alpha 2/\alpha 3$ and $\alpha 5/\alpha 6$ hairpins, allosterically restrain $\alpha 1$ and the $\alpha 1$ - $\alpha 2$ loop, and thereby impair BAX activation⁹. Just recently, eltrompobag, a structural analog of the BAX-activating molecules BAM7⁶ and BTSA1⁷, was shown to engage the $\alpha 1/\alpha 6$ trigger site yet inhibit rather than activate BAX by stabilizing key regions of the BAX architecture, including the $\alpha 1$ - $\alpha 2$ loop and $\alpha 9$ ⁴². All of these studies focus on how “external forces” drive the conformational dynamics of BAX, but how are these mechanisms mediated from within?

Here, we find that a nexus of hydrophobic residues that radiate outward from the central portion of the $\alpha 5$ core provides critical stabilizing interactions to monomeric BAX. Mutating any single residue of the 113-116 region leads to heightened BAX autoactivation in the membrane environment, with the two most active mutations, Y115A and F116A, mobilizing both $\alpha 2$ and $\alpha 9$, two domains especially critical to BAX function. By nature of the very location of the four residues and their interactions with each surrounding BAX helix, natural protein ligands that engage their respective binding sites on the BAX surface can directly influence, positively or negatively, the stability of this critical network and thus the threshold for conformational activation. Correspondingly, peptide^{9,13,35,42} and small molecule^{6,7,10,42} mimetics of these interacting motifs can disrupt or reinforce this nexus for potential therapeutic benefit in diseases of pathologic cellular excess or deficiency.

Another unique feature of the BAX 113-116 nexus is that it subserves two distinct roles along the BAX activation pathway. In addition to having singular and independent roles in stabilizing monomeric BAX, amino acids 113-116 function collectively, rather than individually, to stabilize a dimeric conformation of BAX that may represent a key subunit of higher order oligomers. Distinct and step-specific roles of discrete residues along the BAX activation continuum has emerged as

a remarkable feature of this pro-apoptotic mechanism. For example, we recently demonstrated that mutagenesis of the $\alpha 6$ surface residue I133 disrupted BH3 binding to the trigger site and thus the initiation of BAX activation but had no adverse effect on the execution phase steps of mitochondrial translocation, self-association, and membrane poration³¹. In contrast, mutagenesis of R134, a surface residue immediately adjacent to I133 and also a component of the N-terminal trigger site had no effect on BH3-mediated initiation of BAX activation and instead impaired membrane tropism and oligomeric poration³¹. Here we find that, depending on the stage-specific structural context of monomer vs. dimer, BAX $\alpha 5$ core residues 113-116 and their network of hydrophobic interactions either have individual or cumulative functional importance.

Protein hydrophobic cores can often play a key role in protein assembly and allosteric function. For example, protein kinases rely on allosteric switches to confer on-off enzymatic function. Their prototypical architecture involves two core hydrophobic spines that mediate signal transduction between distal sites⁴³. Disruption of these central nodes of communication by point mutagenesis typically leads to loss of enzymatic function^{43,44}. In contrast, loosening of the hydrophobic interactions within the core of monomeric BAX by point mutagenesis activates protein function in the membrane environment, leading to oligomeric assembly and poration. Transient exposure of hydrophobic BAX surfaces drives this structural transformation, as evidenced by HDX MS studies that revealed initial deprotection of the critical BAX $\alpha 2$ and $\alpha 9$ regions upon BAX activation, followed by prompt protection as oligomerization ensues^{9,31,41}. An alternative mechanism for regulated protein oligomerization upon hydrophobic domain exposure is exemplified by gasdermin, whose N-terminal pore-forming monomer is auto-inhibited by a C-terminal region until cleaved by inflammatory caspases, which leads to dissociation of the C-terminal fragment and N-terminal protein self-association and membrane poration^{45,46}. Whereas stress conditions, such as inflammation, temperature elevation, or nutrient or oxygen deprivation, typically lead to protein dysfunction, it is fitting that BAX destabilization instead induces protein

activation, so that it can execute its mitochondrial permeabilization and cell-killing functions. Indeed, we find that disrupting key interactions of the BAX $\alpha 5$ core is tantamount to “pulling the pin” of the cell’s pro-apoptotic grenade.

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CHAPTER 3

Conclusions and Future Directions

Discussion of Results

BAX is Restrained by its Hydrophobic Core

Apoptosis is an essential cell death program during development and for the maintenance of organismal health. Turnover of cells allows tissues to continually replenish their functional cell types through proliferation of stem cells¹⁻³. Stressed and damaged cells must be removed lest they undergo oncogenic transformation that can be lethal to the organism. Yet, this powerful capacity to commit a cell to death must be kept in check to prevent uncontrolled cell loss and atrophy of tissues. BCL-2 family proteins serve key roles as gatekeepers of apoptosis. The BH3-only proteins are responsive to stress signaling and call into action the effector proteins BAX and BAK, which permeabilize the mitochondria to release apoptogenic factors⁴. The anti-apoptotic proteins act as guardians to prevent unwanted cellular destruction by restraining both the BH3-only proteins and BAX/BAK.

For BAX, another layer of regulation involves its cellular localization in the cytosol. Latent BAX is autoinhibited by its transmembrane $\alpha 9$ helix, which lies in the canonical BH3-binding groove of BAX, thus also blocking the site from interaction with BH3 helices⁵. To release its $\alpha 9$, BAX must first be activated at the N-terminal trigger site composed of residues from $\alpha 1$ and $\alpha 6$, which is regulated by the $\alpha 1/\alpha 2$ loop that is typically overlying the N-terminal face of BAX^{6,7}. Crosstalk between the trigger site at the N-terminal face of BAX and $\alpha 9$ at the C-terminal face is believed to occur through the core of the protein. Subjacent to these sites, the “base” of the BAX protein serves as an access point for allosteric modulation that can alternatively inhibit or enhance BAX activation⁸.

Whereas disrupting allosteric communication can often lead to loss of protein function^{9,10}, here we find that alanine mutagenesis of BAX core hydrophobic residues L113, F114, Y115, and F116, causes autoactivation, a gain of function (**Chapter 2**). The single alanine mutants of BAX, BAX L113A, F114A, Y115A, and F116A, permeabilized liposomes and mitochondria even in the

absence of a BH3 ligand, such as the BH3-only protein tBID that is required for wild-type BAX function. Nevertheless, tBID was able to further enhance BAX-mediated permeabilization by these mutants, suggesting that the physiologic triggering mechanism remained intact and that the overall fold of these proteins was preserved, which was further supported by their elution as monomers by size exclusion chromatography. Additionally, permeabilization by autoactive BAX mutants could be inhibited by BCL-X_L Δ C, indicating an on-pathway mechanism. MEFs lacking both pro-apoptotic effectors BAX and BAK reconstituted with each mutant form of BAX were able to execute a BAX-dependent apoptotic program upon treatment with a combination of small molecule inhibitors of BCL-2/BCL-X_L/BCL-w and MCL-1. Furthermore, cells expressing BAX F116A, the most autoactive mutant in biochemical assays, were most sensitive to apoptosis activation in response to pharmacologic anti-apoptotic inhibition. In addition, MEFs reconstituted with BAX Y115A and F116A showed enhanced conformational activation during cellular homeostasis, as evidenced by their constitutive localization at the mitochondria.

Residues L113, F114, Y115, and F116 comprise a helical turn of the central α 5 helix of BAX and collectively contact every other helix of BAX. These inter-residue interactions maintain BAX stability, whereas alanine mutagenesis of any one of these four residues increases the conformational dynamics of the region directly facing the residue, as evidenced by hydrogen deuterium exchange mass spectrometry (HDX MS) that showed a regiospecific increase in deuterium uptake compared to wild-type BAX. For BAX L113A and F114A, the peptides that exhibited the most deprotection were in α 6. This helix is a critical component of the BH3 trigger site and contributes to BAX's interaction with the mitochondrial outer membrane^{6,11,12}. Increased dynamics of α 6 may reflect its separation from α 5, consistent with the proposed requirement that the hairpin formed by these two helices comes apart during BAX activation¹³. An increase in deuterium uptake in peptides corresponding to α 6 was also observed in BAX F116A, which demonstrated additional regions of deprotection, including α 3 and the distal portion of α 2, seen

upon Y115A point mutagenesis as well. Thus, BAX Y115A and F116A show increased conformational flexibility in the BH3 region, which was confirmed by the unique accessibility of these mutants to anti-BH3 immunoprecipitation. Exposure of the BH3 motif is required for BAX activation and provides a mechanistic explanation for why the BAX Y115A and F116A mutants are the most autoactive. At shorter durations of deuterium incubation, BAX Y115A and F116A demonstrated increased deuterium uptake in $\alpha 9$, which is mobilized from the canonical groove upon initiation of BAX activation for mitochondrial translocation.

Given the ubiquitous expression of BAX, renegade activation is a clear and present danger to organism homeostasis¹⁴. In addition to protein interaction mechanisms that can prevent or block BAX activation, here we find that the identified nexus of BAX $\alpha 5$ residues provided another critical layer of BAX regulation, *from within*. Indeed, these hydrophobic interactions are central to the molecular infrastructure that stabilizes the latent BAX monomer. Yet, the reliance on focal hydrophobic contacts for BAX stability provides a relatively low energetic threshold for BAX activation¹⁵. Indeed, heat activates BAX *in vitro* at 37°C, well below BAX's melting temperature (>70°C)¹⁵⁻¹⁸. The hydrophobic core of a protein is considered critical to its structure and by extension its function, such that a failure to preserve the integrity of the hydrophobic core results in loss-of-function. However, BAX functions to kill the cell in response to stress and thus its very structure leverages protein unfolding to carry out its pro-apoptotic duty – yielding gain-of-function rather than loss-of-function biological activity.

Mechanisms of Mutant BAX Autoactivity

An interesting element to the autoactive nature of BAX mutants L113A, F114A, Y115A, and F116A is their dependence on membrane interaction for complete activation. That is, in solution the BAX mutants remain monomeric, whereas several previously described BAX mutants (discussed below) and wild-type BAX treated with stapled BIM peptides oligomerize in solution in

the absence of membrane^{6,19}. The kinetic characteristics of BAX alanine-mutant liposomal release, including the enhanced response to tBID, suggests that these mutants remain in at least a partially restrained configuration, which lies upstream of a conformation that enables self-association and membrane permeabilization. BAX Y115A and F116A demonstrate increased conformational dynamics in their $\alpha 9$ transmembrane helix, which explains their propensity to auto-translocate to membranes in cells. Constitutive exposure of the BAX BH3 in these mutants further sheds light on how they bypass traditional activation at the N-terminal trigger site. The relatively decreased autoactivity of BAX L113A and BAX F114A likely derives from persistent shielding of BAX BH3 and instead conformational alteration of $\alpha 6$, a region of BAX implicated in both triggering and membrane interaction^{17,20}. Select residues of $\alpha 6$, in the context of oligomeric BAX, are buried in the membrane and face internally in the latent, monomeric structure^{11,12}. It is plausible that increased exposure of these residues drives translocation of BAX L113A and F114A *in vitro* and that stabilization of these residues in an externally facing conformation in the membrane allosterically promotes further conformational changes characteristic of BAX activation. Whether mutagenesis of $\alpha 6$ residues in the context of BAX L113A or F114A results in loss of oligomeric function would test this mechanistic model. Probing BAX-membrane interactions in this manner, and the consequences for oligomeric function, would further advance our understanding of the critical interplay between activated conformers of BAX, self-association, and membrane permeabilization. Indeed, key roles for lipid interactions with BAX and BAK in apoptotic function are emerging^{11,21-25}.

Mutagenesis at other BAX sites has also yielded autoactivation, but with distinct structural and functional consequences. For example, S184 mutagenesis directly influences exposure of $\alpha 9$ (**Figure 3.1a**). BAX mutants S184A/V/L auto-translocate to liposomes and mitochondria and have been useful for probing events downstream of mitochondrial translocation²⁶⁻²⁹. Importantly, these mutants do not permeabilize liposomes or isolated mitochondria *in vitro* upon translocation,

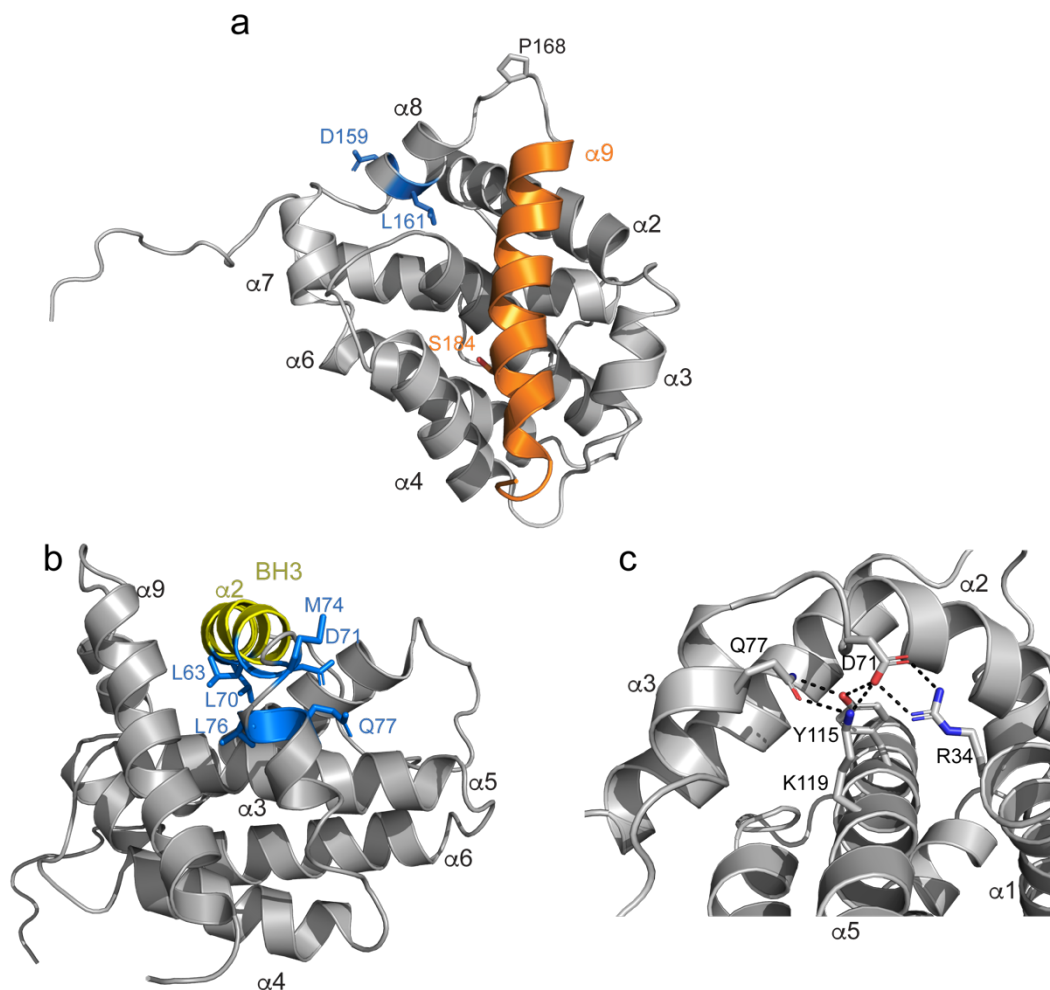


Figure 3.1 *Structural mapping of BAX mutations that lead to autoactivity.* (a) S184 mutagenesis of $\alpha 9$ (orange) results in constitutive $\alpha 9$ displacement and BAX auto-translocation to membranes. Residues D159 and L161 (blue) reside in $\alpha 8$ and mutant BAX proteins D159C and L161P exhibit autoactivity. Residue P168 is shown in grey for reference and functions as a regulator of BAX $\alpha 9$ exposure, but mutagenesis disrupts rather than induces BAX translocation. (b) Residues in the BH3 ($\alpha 2$, yellow) and nearby $\alpha 3$, including L63, L70, D71, M74, L76, and Q77 (blue), regulate BAX stability. (c) Molecular dynamics simulations (last frame; 100 ns) reveal an extensive hydrogen bonding network that regulates BAX stability and involves D71, Q77, and Y115, as well as other residues whose mutagenesis does not result in autoactivation, R34 and K119.

nor induce apoptosis in cells in the absence of a BH3-only ligand; however, cells expressing BAX S184A are more sensitive to pharmacologic inhibition of anti-apoptotic BCL-2 family proteins, and cells expressing BAX S184V are more sensitive to treatment with staurosporine or etoposide^{18,27,29}. Mutations in $\alpha 8$, including BAX D159C and L161P, also cause autoactivation. BAX D159C permeabilizes isolated mitochondria in the absence of a BH3 stimulus, whereas BAX L161P expression directly induces apoptosis in cells^{30,31}. The exact structure-function mechanism of autoactivity of these mutants has not been explored, other than the melting temperature of BAX D159C shown to be 5 degrees lower than that of wild-type BAX, indicative of a lower energetic threshold for destabilization of the protein core³⁰. The proximity of residues 159 and 161 to $\alpha 9$ raises the possibility that direct mobilization of $\alpha 9$ due to structural incompatibility could be involved. A local role for the $\alpha 8$ hinge in regulating $\alpha 9$ has been demonstrated by P168 mutagenesis, whereby BAX P168G and P168A fail to translocate to mitochondria³².

Another area of BAX mutagenesis that causes autoactivity involves the BH3 domain ($\alpha 2$) and the adjacent $\alpha 3$ (**Figure 3.1b**). BAX L63E auto-translocates to mitochondria, but because this mutation disrupts the universally conserved leucine, this mutant fails to self-associate and thus causes a loss-of-function phenotype²⁷. Other BH3 mutants, such as BAX L70A, D71A, M74D/E, L76C, Q77P, display autoactivity that manifests in cellular apoptosis or cytochrome *c* release from isolated mitochondria^{19,31,33,34}. Mobilization of the BAX BH3 motif promotes BAX activation, given its critical role in triggering inactive monomers and participating in dimer formation, which is believed to be a key step of higher order oligomerization. For example, recombinant BAX L70A and D71A proteins constitutively oligomerize in solution¹⁹. Curiously, substitution of D71 with asparagine (BAX D71N) also resulted in autoactivation, whereas substitution with glutamate (preserving its charge and hydrogen bonding characteristics) or double alanine mutation of nearby positively charged residues (BAX R34A/K119A) results in wild-type behavior, suggesting that the charge of this residue is important for BAX stability, but not

necessarily for salt-bridge interactions with the two adjacent positively charged residues¹⁹. Rather, it is plausible that autoactivating D71N mutagenesis alters hydrogen bonding in a manner that could disrupt interaction with Q77 and Y115, two other residues that yield autoactive BAX upon mutagenesis. Preliminary molecular dynamics simulations suggest that a more extensive hydrogen bonding network among D71, Y115, Q77, R34, and K119 could exist and regulate BAX dynamics (**Figure 3.1c**). Whereas mutagenesis of Y115 to alanine results in the loss of hydrophobic bulk, in addition to hydrogen bonding capability, BAX Y115F mutagenesis is predicted to preserve the hydrophobic character but lose hydrogen bonding capacity. If BAX Y115F was found to be autoactive, even if less autoactive than Y115A, a role for hydrogen bonding in this region in maintaining BAX stability would be confirmed. Specific roles of inter-residue interactions could be further validated by installing disulfide tethers, generating a controllable redox switch to probe the relevance of particular linkages of the intramolecular network⁷. Interestingly, the double alanine mutant, BAX L70A/D71A, was found to require BH3-only triggering in the cellular context, although *in vitro* studies were not performed³⁵. Importantly, we find that a combination of *in vitro* and *in cellulo* studies are useful to dissect BAX biochemistry, as mechanistic features evident in a reductionist system may be overshadowed by the complexity of the cellular environment. Indeed, we found that our nexus of alanine mutants were capable of sufficient autoactivation *in vitro* to induce membrane permeabilization, but that pharmacologic stimulus was required to trigger cellular apoptosis, even for the Y115A and F116A mutants that underwent constitutive mitochondrial translocation. Taken together, mutagenesis studies continue to provide mechanistic insight into how the architecture of BAX regulates its critical pro-apoptotic function and the series of steps along the pathway from latency to oligomeric membrane permeabilization.

BAX Residues 113-116 are Critical for BAX Homo-Oligomerization and are Evolutionarily Conserved

In addition to their individual roles in maintaining the structural stability of monomeric BAX, residues 113-116 are collectively required for BAX homo-oligomerization. We found that quadruple alanine mutation of these residues abrogated dimerization of a recombinant, GFP-fusion protein containing the $\alpha 2$ - $\alpha 5$ portion of BAX, consistent with disruption of oligomerization and apoptosis upon expression of the full-length protein in cells upon quadruple of mutagenesis of the corresponding residues³¹. Furthermore, we determined that adding back native residues Y115 (BAX L113A/F114A/F116A) or F116 (BAX L113A/F114A/Y115A) was sufficient to restore BAX self-association of GFP-BAX $\alpha 2$ - $\alpha 5$, and that the combination of native L113 and F114 could enforce BAX self-association even if both Y115 and F116 were mutated. The distinct requirements for stabilization of the monomer versus dimer derive from differences in their interaction networks (**Chapter 2, Figure 2.11**). Interestingly, residues Y115 and F116 are buried, and stabilize the outgoing intra-monomeric BH3; F116 also interacts with the incoming inter-monomeric BH3. Thus, the heightened interactions of Y115 and F116, as compared to L113 and F114 that are predominantly solvent exposed in the dimer and make less intra-dimeric contacts, likely explains the why restoring Y115 or F116 can rescue at least partial self-association of the dimer.

The importance of the 113-116 nexus of residues in both stabilizing the distinct monomeric and dimeric conformations of BAX is underscored by sequence conservation across species. An alignment of a curated database of 440 BAX-like sequences revealed high conservation of residues 113 to 116 in the consensus sequence: L113 (92%), F114 (98%), Y115 (71%), F116 (75%) (**Figure 3.2a**)^{36,37}. Interestingly, the two residues whose mutagenesis had the most impact on both monomeric stability and dimeric assembly of recombinant human BAX, Y115 and F116, are less conserved than their N-terminal counterparts L113 and F114. However, it is likely that substitution of Y with H (24%) implicates hydrogen bonding in the functionality of this residue, whereas substitution of F with L (24%) points to a key role for hydrophobic bulk (**Figure 3.2b**). Of

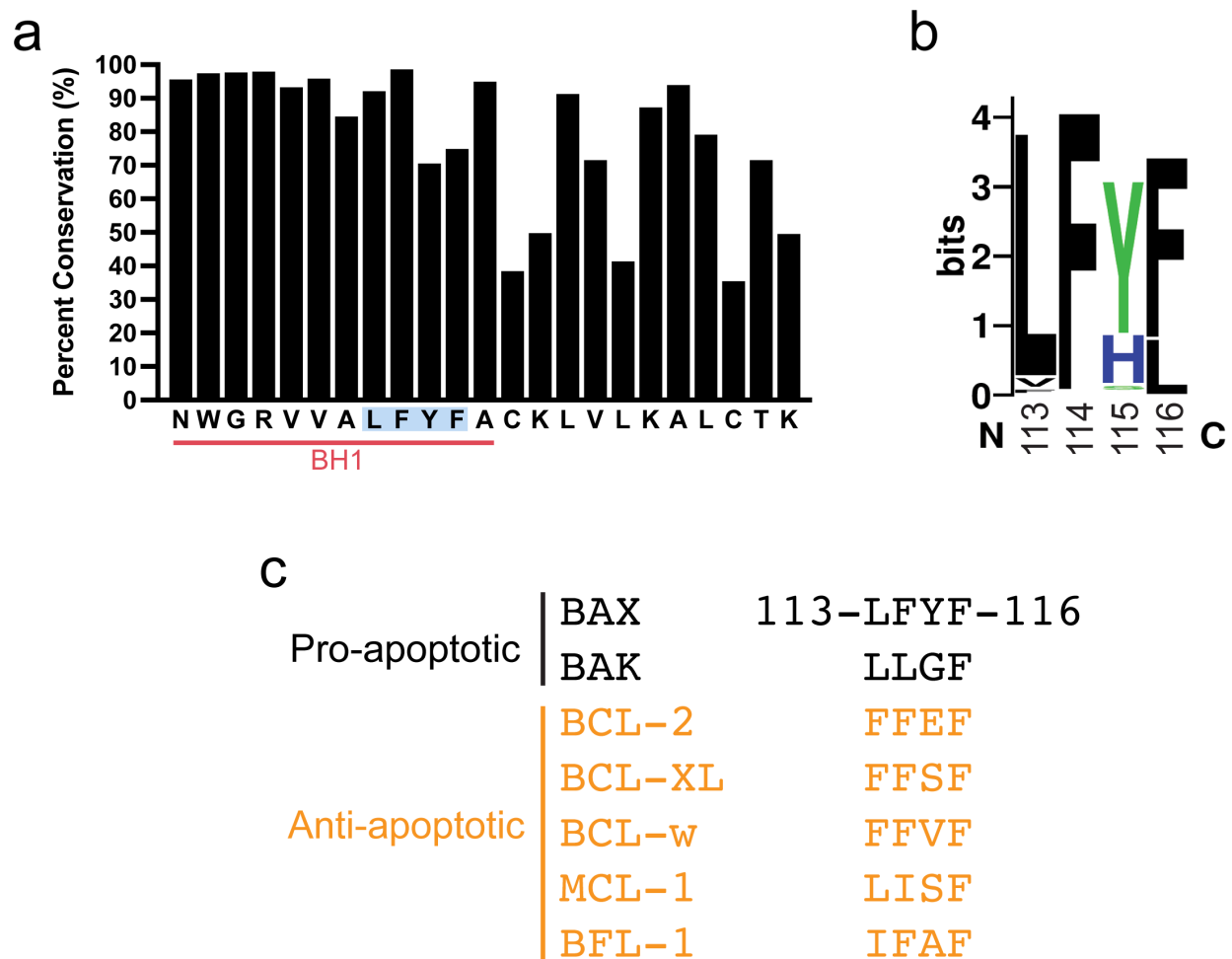


Figure 3.2 *BAX residues 113-116 are evolutionarily conserved.* (a) Conservation of BAX $\alpha 5$ consensus sequence across 440 BAX-like species. The BH1 motif is underlined in red and the 113-116 nexus highlighted in blue. (b) Amino acids corresponding to human BAX residues 113-116 are hydrophobic, except at position 115, which is represented by polar amino acids, including tyrosine (71%), histidine (24%), and serine (3%). (c) Comparison of homologous sequences among the human multi-domain members of the BCL-2 family proteins at positions corresponding to human BAX residues 113-116.

note, residues of 113-116 of BAX are found in one of the conserved BH regions of BAX, namely BH1. Sequence alignment of this nexus across exemplary BCL-2 family proteins demonstrates analogous hydrophobic residues in positions 1, 2, and 4, with the third position most variable, perhaps suggesting isoform-specific requirements for inter-residue compatibility within the structural fold, which could have implications for pro- and anti-apoptotic functionality. Intriguingly, George and colleagues reported that transplantation of the BAX $\alpha 5$ sequence into BCL-X_L conferred apoptotic activity³¹. When BAX 113-116 were collectively mutated to alanine in this context, apoptosis induction was again lost. Taken together, these data suggest that $\alpha 5$ in general and residues 113-116 in particular play a key role in pro-apoptotic functionality.

Future Directions: Alanine Mutants of BAX Residues 113-116 as Tools for Dissecting BAX Structure, Function, and Therapeutic Modulation

Combinatorial Mutagenesis

BAX activation is a multi-step process that involves BH3 triggering, conformational activation, membrane translocation, self-association, and membrane permeabilization²⁷. Given the striking structural gymnastics that takes place between the latent cytosolic monomer and lethal mitochondrial oligomer, how the residues and substructures of BAX mediate this conformational transformation remains a fascinating area for structure-function dissection. By harnessing interaction network analysis and alanine mutagenesis, we discovered that a discrete nexus of residues plays dual roles in monomeric and dimeric stabilization based on the distinct interaction networks of the two structures. The development of a recombinant, homogeneous BAX oligomer by detergent treatment has enabled residue-specific functional comparisons between monomeric activation (“initiation”) and downstream membrane permeabilization (“execution”); for example, I133 of $\alpha 6$ was shown to be essential for BH3 triggering but dispensable for execution, whereas nearest neighbor R134 is dispensable for BH3 triggering but participates in membrane tropism

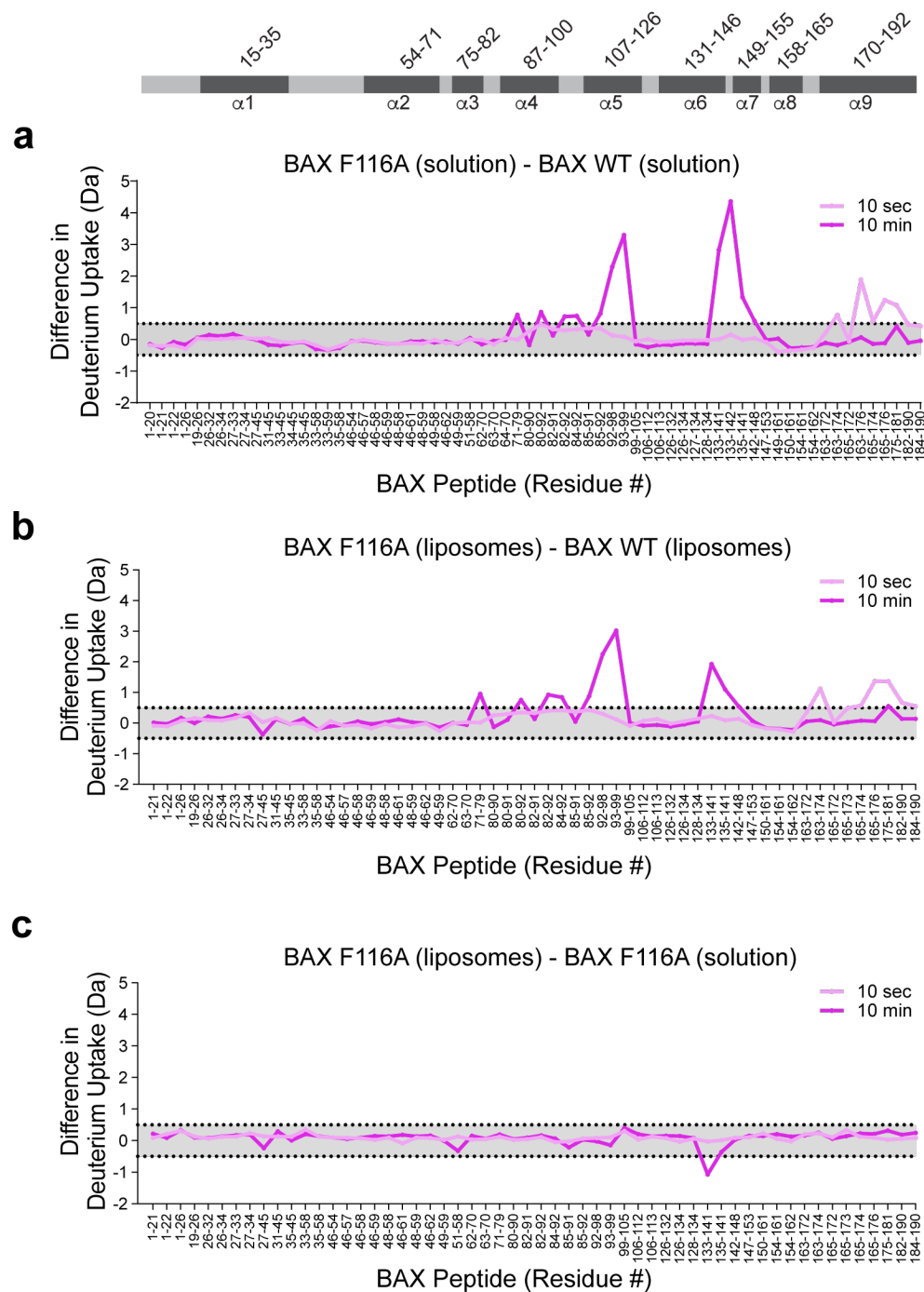
and permeabilization¹¹. The autoactive alanine mutants characterized here (113-116) add to the toolbox by enabling the isolation and characterization of BAX-activation steps downstream of BH3 triggering, particularly because these single mutants are partially activated. Combinatorial mutagenesis studies that incorporate these mutants into BAX monomers and oligomers may elucidate residues and mechanisms that alternatively agonize or restrain their autoactivity, and further explain the variability in autoactivity observed upon single mutagenesis of these residues.

Conformational Dynamics of BAX Mutants at the Membrane

An interesting next step of BAX 113-116 analysis would be evaluating the impact of alanine mutagenesis on BAX conformation in the presence of membrane. The Walensky and Engen laboratories have extensively monitored BH3-triggered monomeric BAX activation and oligomeric BAX in solution and in the membrane environment, revealing new insights into conformational dynamics and the influence of the membrane^{11,17,20,23}. Given the unique autoactivated monomeric structures observed by HDX MS in solution, monitoring the impact of added liposomes would allow for characterization of conformational dynamics in the absence of triggering ligands. We piloted this approach for BAX F116A and noted that, in the presence of liposomes, there was a relative decrease in $\alpha 6$ deprotection, with $\alpha 4$ deprotection remaining approximately the same in both conditions (**Figure 3.3**). Consistent with an identified role for $\alpha 6$ in membrane interaction, the decrease in $\alpha 6$ deprotection observed for BAX F116A in the liposomal context likely reflects protection via liposomal membrane engagement^{11,12,30}. In contrast, $\alpha 4$ may remain in a similarly exposed or conformationally dynamic state, whether in solution or in the membrane environment. Thus, comparing the deuterium exchange profiles of BAX 113-116 mutants in solution vs. the membrane environment over time has the potential to identify sequential changes in BAX structure upon engagement of the membrane, revealing new insights into this dynamic process.

Figure 3.3 *HDX MS identifies regiospecific conformational changes of BAX F116A in solution and in the membrane environment.* Deuterium difference plots showing the relative deuterium incorporation of BAX F116A minus that of wild-type BAX in solution (a) and upon incubation with liposomes (b), as measured after 10 sec and 10 min of deuterium labeling. (c) Deuterium difference plots showing the relative deuterium incorporation of BAX F116A in solution minus the relative deuterium incorporation of BAX F116A upon incubation with liposomes. The 0.5 Da significance threshold is highlighted in gray. HDX MS experiments were performed at least twice using independent preparations of BAX proteins. Experiments were conducted by N.B.B. and T.E.W., under the supervision of J.R.E.

Figure 3.3 (Continued)



Heterodimeric Structures of BAX and Anti-Apoptotic Proteins

The conformation that BAX adopts at the membrane exposes BAX to inhibition by the anti-apoptotic members of the BCL-2 family. Our current structural understanding of this inhibitory hetero-dimer is informed by peptide-protein interactions, including pro-apoptotic BH3 peptides in the canonical groove of anti-apoptotic proteins and BCL-2 BH4 peptides bound to a distinctive surface groove on BAX^{17,38,39}. How full-length pro- and anti-apoptotic proteins interact in solution or at the membrane is unknown due to the absence of any structures of the heterodimeric complexes. Another technical challenge arises from the need to induce the activated conformer of BAX for anti-apoptotic interaction by use of BH3 ligands, which themselves can target the anti-apoptotic grooves, complicating heterodimeric assembly. The auto-active mutants of full-length BAX generated here, particularly those with mobilized BH3 helices, eliminate the need for BH3 triggering and thus may be useful in generating heterodimers for structure-function analyses.

Analyses of BAX-mediated Apoptosis Induction

Further studies of BAX mutants in the cellular context can also provide new insights into the influence of the proteome, cell type, and stimulus on BAX-mediated apoptosis. Here, we performed retroviral reconstitution of BAX/BAK-deficient MEFS with BAX alanine mutants to assess their intracellular localization and comparative effects on pharmacologic induction of apoptosis. The reconstituted cells obtained did not undergo spontaneous apoptosis, by definition, because of the selection method. Thus, a useful next step would be to repeat these studies using an inducible expression system, which could reveal acute pro-apoptotic effects for the individual mutants upon dose-dependent expression. Such studies as well as further use of the stable cell lines already generated could facilitate investigations into (1) why BAX F116A-reconstituted MEFs are more susceptible to pharmacologic apoptosis induction compared to other mutants, (2) what influence BAX Y115 and BAX F116 auto-translocation could have on mitochondrial morphology or energetics, and (3) what protein interaction partners may be involved in restraining the various

mutants, including BAX L113A and F114A in the cytosol and BAX Y115A and BAX F116A at the mitochondria, as investigated by immunoprecipitation and proteomic studies. Finally, reconstitution of diverse cells, including cancer cells, would determine whether the behavior of such mutants is differentially regulated by cell type, cell state, and the diversity of stress stimuli.

Dissecting the Mechanisms of Therapeutic Modulation of BAX

Given its central role in dictating whether a cell lives or dies, BAX is a high impact target for therapeutic modulation, particularly in the cancer field where pharmacologic triggering of BAX has the potential to restore apoptosis. Importantly, BAX is rarely mutated or deleted in cancer: only 90 out of 10,294 (0.87%) samples in the Genomic Data Commons database demonstrate mutations in *BAX* and 625 out of 10,615 (5.89%) samples have *BAX* copy number loss (release 31.0, <https://portal.gdc.cancer.gov/>). In some cancer contexts, *BAX* is unexpectedly overexpressed and exhibits copy number gains, such as acute myeloid leukemia (AML) and breast cancer^{40,41}. Indirect activation of BAX through pharmacologic targeting of BCL-2 by the small molecule inhibitor venetoclax, which can release sequestered BAX, is clinically effective in a variety of human cancers, including chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), and AML^{42,43}. BAX can be directly activated by stapled BH3 peptides^{44,45} and small molecules^{46,47}, including BAX activator molecule 7 (BAM7) and an analog, BAX trigger site activator 1 (BTSA1); the latter compound has been shown to suppress AML in a mouse model, sparing normal tissues⁴⁷. The network of stabilizing interactions involving BAX residues 113-116 may provide insight into alternative approaches to pharmacologic BAX activation, such as by disrupting key binding modes of the protein core.

Pharmacologic inhibition of BAX also has the potential for therapeutic benefit, in this case by preventing unwanted cell death, such as in neurodegenerative diseases and ischemia-reperfusion injury following stroke or myocardial infarction^{1,8,48,49}. A small molecule BAX inhibitor, BAX allosteric inhibitor 1 (BAI1), was recently shown to protect cardiomyocytes in zebrafish and

in mice from doxorubicin-induced death⁴¹. Importantly, BAI1 did not diminish doxorubicin efficacy in multiple mouse cancer models, apparently due to increased BAX expression and apoptotic priming in cancer cells compared to cardiomyocytes. BAI1 is believed to stabilize BAX by binding to a discrete, hydrophobic pocket formed by select residues of $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\alpha 6$, including F116, which experienced significant chemical shift perturbations upon BAI1 titration in HSQC NMR analyses of BAX⁴⁸. In addition to BAI1, a stapled BH4 peptide¹⁷, the vMIA protein of cytomegalovirus⁵⁰, and the thrombopoietin agonist eltrombopag⁵¹ have been shown to engage BAX at distinct locations to block its pro-apoptotic function. Whether or not these inhibitors can also block the autoactive mutants of BAX characterized here could inform their mechanisms of inhibition, which is likely to involve local and/or allosteric effects on stabilizing the BAX structure.

Finally, the autoactive properties of BAX alanine mutants 113-116 could be of tremendous strategic benefit in the design of BAX-targeted drug discovery campaigns. For example, small molecule screening of individual BAX mutants could reveal molecules that selectively activate or inhibit particular autoactive conformers. Screening could be performed using the liposomal release assay that was employed to identify inhibitors of tBID-induced BAX activation^{48,52}. Given the distinctive conformations of BAX L113A, F114A, Y115A, and F116A mutants, the small molecule activators and inhibitors that emerge are likely to have diverse mechanisms of action, and thereby expand the breadth of compositions of matter for development as pharmacologic modulators of BAX.

Final Thoughts

The overall stability of BAX determines whether a cell lives or dies. Structurally stable BAX is inactive and lies dormant in the cytosol, whereas conformationally active BAX translocates to the mitochondrial, self-associates, and destroys the cell's power plants. Thus, the biochemical and structural underpinnings of BAX's spectrum of conformations is of critical importance to cell survival, tissue homeostasis, overall health of the organism, and the development of therapeutics

to modulate BAX to either promote or block cell death in human disease. This thesis research determined that residues 113-116 play central roles, literally and figuratively, in both monomeric and dimeric BAX architecture, and thus contribute significantly to BCL-2 family regulation of cellular life and death.

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