2011

Distinct Domains of Bax are Involved in Mitochondrial Bioenergetics and Apoptosis

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DISTINCT DOMAINS OF BAX ARE INVOLVED IN MITOCHONDRIAL BIOENERGETICS AND APOPTOSIS

by

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B.S. Zhengzhou University, 1991

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
Orlando, Florida

Summer Term

2011

ABSTRACT

Apoptosis is essential for cellular homeostasis and is also a pathologic feature of various diseases. The balance between Bcl-2 family proteins determines whether a cell will live or die. Bax, a member of the BCL-2 family proteins, is a pro-apoptotic protein that exists in both a soluble, cytoplasmic form and a membrane-bound form. Upon apoptotic stimuli, Bax undergoes a conformational change and translocates to the mitochondria, initiating apoptotic events. However, little is known about whether Bax is involved in the regulation of mitochondrial function under non-apoptotic conditions, and how Bax binds to mitochondria to exert its activity. Here, we investigate the role of Bax in the regulation of mitochondrial function under non-apoptotic conditions and explore the molecular mechanisms for Bax binding mitochondria under apoptotic stimuli.

Using Bax-containing and Bax-deficient ($Bax^{-/-}$) HCT-116 cells, we examined Bax cellular localization and its effects on mitochondria bioenergetics, and also tested whether over-expression of full-length Bax in $Bax^{-/-}$ cells would recover mitochondrial metabolic activity. To determine the effects of Bax localization upon mitochondrial function, we measured citrate synthase activity and ATP generation. We showed that Bax localized to the outer and inner mitochondrial membranes in non-apoptotic cells, enabling the activity of citrate synthase and the generation of ATP. Loss of Bax led to impairment of respiring mitochondria morphology and reduced oxidative capacity, all of which was restored by expression of full-length or C-terminal-deleted Bax. These findings indicate that under non-apoptotic conditions, the constitutive expression of Bax is necessary for mitochondrial bioenergetics.

To determine the molecular mechanisms for Bax binding mitochondria under apoptotic stimuli, we previously performed in silico-mutagenesis and predicted that Lysines 189/190, in the C-terminal α9 helix, could regulate Bax binding to mitochondria. We demonstrated here that these lysines are the structural elements responsible for controlling how Bax interacts with the mitochondrial membrane. Expression of full-length Bax led to mitochondrial translocation and apoptosis, whereas deletion of the α9 helix resulted in cytosolic retention and dramatically reduced cell death. Mutation of the two lysine residues changed how Bax bound to mitochondrial membranes. We replicated the results achieved with full-length Bax by attaching the α9 helix of Bax to GFP or to a regulatory element, the degradation domain (DD), and induced apoptosis upon expression in cells. We demonstrated that the α9 helix alone promoted the mitochondrial translocation of Bax and increased apoptosis. These results indicate that the C-terminal α9 helix could be further studied for use in cancer therapies.

Overall, we have demonstrated that the constitutive expression of the inactive form of Bax in non-apoptotic cells is necessary for mitochondrial bioenergetics, and have identified the C-terminal α9 helix of Bax as the effector domain of apoptotic function.

ACKNOWLEDGMENTS

"Life is but a dream". This children's song packed with many meaning and inspiration to me. Doing this research project actualize one of my dreams.

I am heartily thankful to my advisor, Dr. Annette Khaled, who let me to realize my passion, full potential, true nature and to develop an understanding of the subject. This thesis would not have been possible without her encouragement, guidance and support from the very beginning to the end. It is a pleasure to thank my committee members Dr. Deborah A. Altomare and Dr. Zixi Cheng without their support this project would not have been quickly successful. I would like to thank Dr. Henry Daniell for his advice whenever I need. Special thanks also to all my lab mates, Dr. Kathleen Nemec, Rebecca Boohaker, Nuska Tschammer, Adina Loosley Carlson, Shannon Ruppert, Dr. Christina Kittipatarin, Dr. Mounir Chehtane for sharing the literature, abundantly helpful and invaluable assistance.

Lastly, deepest love and gratitude to my beloved families, my son Evan and my husband Jianli, for their understanding and endless love, they always been there during this journey.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ACRONYMS/ABBREVATIONS

AIF: Apoptosis inducing factor

ANT: Adenosine nucleotide translocator

Bax: BCL-2 associated protein

BCL-2: B-cell lymphoma 2 protein

BH: BCL-2 homology

BSA: Bovine serum albumin

DD: Degradation domain

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

dsRNA: Double stranded RNA

ECAR: Extracellular acidification rate

EGFP: Enhanced green fluorescent protein

ER: Endoplasmic reticulum

FACS: Fluorescence-activated cell sorter

FBS: Fetal bovine serum

FCCP: Carbonylcyanide-p-trifluoro-methoxyphenylhydrazone

HA: Hemaglutinin

HRP: Horseradish peroxidase

HSP60: Heat shock protein 60

IMM: Inner mitochondrial membrane

IMS: Intermembrane space

IRES: Internal ribosome entry site

NAO: 10-Nonyl Acridine Orange

OCR: Oxygen consumption rate

OMM: Outer mitochondrial membrane

PBS: Phosphate Buffer Solution

PCR: Polymer chain reaction

PTT: Proteo Tuner plasmid

PVDF: Polyvinylidene difluoride

RNA: Ribonucleic acid

SD: Standard deviation

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

3D: Three dimensional

VDAC: Voltage-dependent anion channel

WT: Wild type

CHAPTER 1: GENERAL INTRODUCTION

"Life is pleasant, death is peaceful. It's the transition that's troublesome." Isaac Asimov

Apoptosis is a cell death program that is critical for cellular and tissue homeostasis and is also a key pathologic feature of various diseases. Over the past decade, the role of mitochondria has been increasingly recognized as an important mediator for the apoptotic process, and release of mitochondria apoptotic factors is considered as the "point of no return" in apoptotic signaling [1-3]. Upon an apoptotic stress signal, the permeabilization of the outer mitochondrial membrane (OMM) allows the release of cytochrome *c* and other apoptotic proteins from the intermembrane space of mitochondria, leading to the activation of the caspase cascade and apoptotic cell death [2,3]. This process is in part regulated by the Bcl-2 family of proteins, which can be classified into three groups based on their structure and their role in apoptosis: (1) the pro-apoptotic proteins, such as Bax and Bak, which contain three Bcl-2 homology (BH) domains BH1, BH2, and BH3; (2) the anti-apoptotic proteins like Bcl-2, Bcl-X_L, Bcl-w and Mcl-1, which contain four homology domains BH1, BH2, BH3 and BH4; and (3) the BH3-only proteins such as Bid, Bim and Bad, which induce apoptosis by either activating pro-apoptotic proteins like Bax or by suppressing anti-apoptotic proteins like Bcl-2. Most of these proteins share one to four BH-domains and contain a C-terminal hydrophobic α-helix that is involved in their localization to either the OMM or endoplasmic reticulum (ER) membranes [4-7]. Bax

participates in the induction of apoptosis in response to a variety of stimuli, and Bax conformational changes and translocation into the OMM is recognized as a key event triggering mitochondria-dependent apoptosis [1, 3, 7].

Bax is a 21 KD pro-apoptotic protein of 192 amino acids comprising 9 alpha helices [8]. The hydrophobic α5-α6 hairpin helix is located in the central core surrounded by 7 amphipathic α-helices. NMR data on the 3-D structure of Bax shows that alpha helix 9 is situated within a hydrophobic groove. Generally, the localization of Bax is cytosolic in most healthy cells, although some mitochondria localization has been described [Putcha et al., 1999, 2002; Kaufmann et al., 2004]. Many functions of Bax can be attributed to specific domains. The BH3 domain is mostly found in the alpha 2 helix and has been shown to be involved in the hetero-dimerization with other Bcl-2 family proteins [9-11]. The hydrophobic helix 9 and helices 5/6 has been implicated in the activation, self-association and mitochondria membrane binding of Bax [11-14].

The translocation of Bax from the cytosol to the OMM is a critical event in apoptosis. Although the precise mechanism remains to be elucidated, two models of Bax activation have been proposed to explain the activation of Bax during apoptosis [7, 15]. Briefly, in the first model, upon apoptotic stimuli the BH3-only Bcl-2 family proteins interact with Bax, leading to conformational changes that result in the formation of oligomers and insertion into the OMM. In the second model, the conformational changes of Bax occurs in the cytosol leading to release of the C-terminal α9 helix from the hydrophobic grove, thereby exposing the previously hidden BH3 domain. Bax then inserts into the OMM either through its N-terminus [14,16,17] or its C-terminus [18-20]. Studies from our group and others showed that pH could induce a

conformational change in Bax, resulting in the C-terminal and BH3 domain being exposed, leading to mitochondria translocation [21, 22]. Others have shown that Bax can insert into membranes as a monomer, suggesting that the insertion step occurs prior to oligomerization [23]. How Bax translocates to the OMM is still a matter of debate.

Recently, it was suggested that Bax C-terminus is required for Bax translocation to mitochondrial during apoptosis [18-20]. Some reports suggest that the C-terminal anchor is not required for Bax translocation during apoptosis [14, 25]. Deletion of the C-terminal 22 amino acids failed to block recruitment of Bax to mitochondria during apoptosis [22]. While others studies showed that the C-terminus is needed to translocate Bax to mitochondria. As example, mutating proline 168, upstream of the C-terminus prevented Bax translocation during apoptosis and abolished its apoptotic activity (16, 19, 20). Despite these findings, whether the C-terminus of Bax is a membrane binding domain or a regulatory domain remains to be analyzed. We performed computational-mutagenesis studies to determine the potential amino acids of the C-terminus necessary for Bax membrane binding activity. Based on the differences in helical packing energy, we predicted that lysine 189 and lysine 190 at the C-terminus may be crucial for Bax membrane binding (Khaled A, unpublished data). However, this hypothesis has not been validated in a cell model system.

Under physiological conditions, Bax usually resides in the cytoplasm and translocates to mitochondria upon apoptotic stimuli. In its inactive form, Bax is also found in very low numbers in mitochondrial membranes [7, 8]. Thus, Bax localizes to mitochondria as its major site of action. Some studies have shown that Bax is involved in the formation of ion-conducting pores in the mitochondria membrane [26-28]. Bax may functionally interact with components of the

mitochondrial inner membrane, like the adenine nucleotide transporter (ANT), the mitochondrial F_0F_1 ATPase H^+ pump, and the outer membrane voltage-dependent anion channel [29-31]. Bax could also directly interact with the mitochondria channel Kv1.3 in lymphocytes [28]. Recently, Bax was shown to participate in mitochondrial reactive oxygen formation in non-apoptotic neuron cells [32, 33]. These findings imply that Bax's interaction with mitochondria may play an important role in regulation of mitochondrial function. However, the functional significance of the interaction of Bax with mitochondria under non-apoptotic conditions has not been determined. It is unknown whether the C-terminus of Bax is the critical region responsible for the function of non-apoptotic form of Bax.

Here we investigate the role of Bax in the regulation of mitochondrial function under non-apoptotic conditions and explore the molecular mechanisms for Bax translocation to mitochondria under apoptotic stimuli. Using $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells, we examined Bax cellular localization and its effects on mitochondrial bioenergetics in non-apoptotic cells. We further tested whether over-expression of full-length Bax in $Bax^{-/-}$ cells would restore mitochondrial metabolic activity. We also over-expressed a C-terminal truncated Bax (Bax-ΔCT) in $Bax^{-/-}$ cells to determine whether the C-terminal helix of Bax is crucial for mitochondrial bioenergetics. We showed that Bax localized to outer and inner mitochondrial membranes in non-apoptotic cells, affecting citrate synthase activity, intracellular ATP production and oxygen consumption. Loss of Bax led to impairment of mitochondria respiring morphology and oxidative capacity, all of which can be restored by expression of full-length Bax. We also found that expression of the C-terminal truncated Bax can also restore mitochondrial bioenergetics. These findings indicate that the constitutive expression of Bax in

non-apoptotic cells is associated with mitochondria and is necessary for mitochondrial bioenergetics.

To determine the molecular mechanisms for Bax targeting to mitochondria under apoptotic stimuli, we previously performed *in silico* mutagenesis and predicted that lysines 189/190, in the C-terminal α9 helix, could regulate the membrane binding activity of Bax. A series of *in vitro* mutagenesis were performed. Data presented here demonstrate that these lysines are the functional domain contribute to the mitochondrial translocation of Bax under apoptotic stimuli. Expression of full-length Bax led to mitochondrial translocation and apoptosis, whereas deletion of the α9 helix resulted in cytosolic retention, and no apoptosis. Mutation of the two lysine residues changed the localization of Bax from either the cytosol or mitochondria. We replicated the results achieved with full-length Bax by attaching just the last 20 residues of the α9 helix of Bax either to GFP or to a regulatory element: a degradation domain (DD), and induced apoptosis upon expression in cells. We demonstrated that the α9 helix alone promoted the mitochondrial translocation of either GFP or the DD and induced apoptosis. These results indicate that the C-terminal α9 helix alone is sufficient to induce cell death and therefore could be further studied for use in cancer therapies.

CHAPTER 2: BAX SUPPORTS THE MITOCHONDRIAL NETWORK, PROMOTING BIOENERGETICS IN NON-APOPTOTIC CELLS

Data presented here in part has been published at Am J Physiol Cell Physiol (February 2, 2011).

Introduction

As a pro-apoptotic protein, Bax has been established in participating in the induction of apoptosis in response to a variety of apoptotic stimuli [1-3, 9]. Under physiological conditions, Bax usually resides in the cytoplasm in most healthy cells, although a fraction of the cellular Bax pool is found to be associated with the outer mitochondria membrane [7, 8]. Over the past decades, most studies focused on how Bax translocates to mitochondria and triggers apoptosis, little is known about the functional significance of Bax in non-apoptotic cells. Recently, the essential role of Bax in non-apoptotic cells has emerged. It has been reported that Bax participates in mitochondrial production of reactive oxygen species in non-apoptotic sympathetic neuron cells [32, 33]. However, how Bax does this is unknown. Since Bax uses mitochondria as its major site of action, we postulated that Bax may interact with mitochondria and be involved in the regulation of mitochondria function. To test this possibility, mitochondrial bioenergetics were examined in Bax-containing ($Bax^{+/+}$) and Bax-deficient ($Bax^{-/-}$) HCT-116 cells. Our results showed that some of the cellular Bax localized to the outer and inner mitochondrial membranes in non-apoptotic cells and participated in citrate synthase activity, intracellular ATP production and oxygen consumption. Loss of Bax led to impairment of mitochondria respiration,

morphology and oxidative capacity, all of which could be restored by expression of full length Bax. These findings indicated that in non-apoptotic cells, the constitutive expression of Bax is associated with mitochondria and is necessary for mitochondrial bioenergetics.

Materials and Methods

Cell Lines and Reagents

$Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 colorectal cancer cell lines (a kind gift from Dr. Bert Vogelstein, John Hopkins University) were grown and maintained in McCoy's 5A Medium (Gibco), 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin. FCCP (carbonylcyanide-p-trifluoro-methoxyphenylhydrazone) (Sigma) was used at a concentration of 5 μM as described below. This was determined to be the optimal concentration after performing a titration experiment (FCCP 0-25 μM) and measuring effects on 100 cell viability and ATP production as described below.

Plasmids, Mutagenesis, and Transfection

PCR-directed deletion of the C-terminus of Bax to generate the C-terminal truncated form of Bax (Bax-ΔCT) was performed using untagged primer sets and was confirmed by sequencing. BAX was PCR amplified from the template, pEGFP-Bax (a gift from Dr. Richard Youle, NINDS, NIH). To examine the transient expression of the DD-tagged full length (Bax-FL), DD-tagged Bax-ΔCT, DD-tagged C-terminal Bax peptides (DD-CT-Bax) constructs, bicistronic ProteoTuner vectors (CloneTech) containing these Bax inserts and green fluorescent protein (GFP) separated by an internal ribosome entry site (IRES) were transfected into $Bax^{+/+}$

and $Bax^{-/-}$ HCT-116 cells. Expression of the Bax constructs was induced upon the addition of Shield 1 (CloneTech) at a concentration of 1uM. The plasmid DNA was delivered at a concentration of 1µg/mL using the TransIT-LT1 transfection reagent (Mirus) following manufacturer's protocol. Transfection efficiency was determined microscopically by visualizing GFP expression after 18 hours, and ranged from 50-70%. Experiments were performed within defined timeframes to ensure optimal cell viability, prior to any induction of apoptosis.

Measurement of Oxygen Consumption and Extracellular Acidification Rates

$BAX^{+/+}$ and $Bax^{-/-}$ HCT-116 cells were cultured in a BD Oxygen Biosensor plate at a concentration of 50,000 cells per well. The biosensor plate contained an oxygen-sensitive dye embedded in a gas-permeable matrix. Based on the properties of this dye, oxygen quenches the ability of the dye to fluoresce. The dissolved oxygen in the medium is depleted by the cells metabolizing in the well, resulting in an increase in fluorescence. This allows a correlation of the rate of oxygen consumption to be made. The plate was read at 37ºC on an EnVision plate reader (PerkinElmer) using ex/em wavelengths of 485/630nm. Fluorescence intensity was measured every hour for 24 hours. For comparisons of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells were seeded at 40,000 cells/well in a 24 well Seahorse cell culture plate. Full-length and ΔCT Bax constructs were transfected into $Bax^{-/-}$ cells for measurement as well. The transfected cells were treated with shield 1 three hours prior to reading to induce expression of the protein. Measurements were taken on the Seahorse XF24 plate reader (Seahorse Bioscience). Values were normalized as changes relative to the initial reading.

Mitochondrial Staining, Fixed- and Live-Cell Imaging

For fixed cells, $Bax^{+/+}$ HCT-116 cells were plated onto coverslips coated with 10µg/mL laminin (Invitrogen). Cells were plated at a density of 50,000 cells/well and were fixed by methanol. The cells were then probed with anti-BAX (N-20; Santa Cruz) and anti-HSP60 (H-300; Santa Cruz) antibodies, followed by FITC and CY3 secondary antibodies. Images were scanned using the LSM 510 (Zeiss) with a 100x/1.4 plan-apochromat objective. The scanned image was processed using the Zen 2009 software (Zeiss). For live cell imaging, $BAX^{+/+}$ and $BAX^{-/-}$ HCT-116 cells were grown and plated in 24-well glass bottom dishes (MatTek) that had been pretreated with 1N HCl and coated with10ug/mL laminin. The cells were plated at 10,000 cells per well and allowed to grow over night. Bax-FL or Bax-ΔCT constructs were transfected using the TransIT-LT1 transfection reagent (Mirus) and expressed as previously described At 18 hours post-transfection, cells were incubated with 1uM MitoTracker Red 580 or 5µM 10-Nonyl Acridine Orange (NAO) (Molecular Probes) in McCoy's complete media for 30 minutes prior to imaging. Treatments with 5µM FCCP were done 15 minutes prior to imaging. Fluorescent images were acquired with the UltraView spinning disc confocal system (PerkinElmer) with AxioObserver.Z1 (Carl Zeiss) stand, and a Plan-Apochromat 63x/1.4 Oil DIC objective. Z-stack projections of the scanned images were generated and modified within the Volocity image processing program (PerkinElmer).

Sub-cellular Fractionation and Immunoblots

$Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells were plated in 75cm flasks and grown to 70-80% confluence. Cells were transiently transfected with the Bax-FL or Bax-ΔCT constructs and protein expressed as previously described. At 18 hours post transfection, the cells were lifted and

pelleted. The pellets were resuspended and lysed according to the protocol from the Mitochondrial Enrichment Kit (Pierce). Note that a low speed centrifugation step in the mitochondrial enrichment process ensures that only intact and unfragmented mitochondria are isolated. The enriched mitochondria were layered on an iodixanol gradient (6%, 10%, 15%, 20%, 23%, and 27%) and subjected to ultracentrifugation using an Optima L-100XP Ultracentrifuge for 2 hours at 145,000 rcf in a SW55.1 swing bucket rotor (Beckman Coulter). After centrifugation, each sample was unloaded in 500uL fractions using a fraction recovery system (Beckman Coulter), and washed in ice cold PBS twice (18,000 rcf, 30 minutes) to remove residual iodixanol. The pellets from each of these fractions were resuspended in 1x loading buffer and run on 8-16% Tris-glycine gradient gels (Invitrogen). The gels were transferred to PVDF membranes and probed with primary antibodies to Bax (N-20; Santa Cruz), Prohibitin (ABCAM), and GRP78 (Santa Cruz), followed by incubation with the appropriate HRP conjugated secondary antibodies (Santa Cruz, Cell Signaling) and developed by chemiluminescence (Pierce).

Proteinase K Digestion of Membrane Bound Proteins

$Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 mitochondria were isolated as above. The mitochondria were then treated with Proteinase K (Sigma) (10 µg/ml) for 5, 10, 15 or 20 minutes in order for the outer mitochondrial membrane associated proteins to be digested. The reaction was terminated with PMSF [35]. The mitochondria were then washed with isotonic buffer, pelleted, resuspended in 1X sample buffer and run on 12% acrylamide gels in parallel with untreated mitochondria. The gels were transferred to PVDF membranes which were then probed with Bax (N-20; Santa Cruz), as well as Bcl-XL (2762, Cell Signaling) primary antibodies, followed by

incubation with anti-mouse or anti-rabbit IRDye 800CW secondary antibodies (Licor). The membranes were imaged using the Licor Odyssey Infrared Imaging system. Densitometry measurements were made using Image J.

Measurement of ATP production and Mitochondrial Membrane Potential

Cells were seeded and treated in 24 well plates overnight. The cells were then lifted, counted and seeded in 96-black well flat-bottom plates at a density of 4,000-5,000 cells/well. To uncouple oxidative phosphorylation, 5μM FCCP was added to the cells two hours prior to analysis of ATP concentration. ATP levels were quantified using the ATPLite 1-Step assay kit (PerkinElmer). Luciferase activity, reported as Relative Luminescence Units (RLU), was measured on an EnVision (PerkinElmer) plate reader. The luminescence was normalized to the number of cells/well. Additionally a standard curve of known ATP concentrations was established to ensure that the experimental values were within an accurate range. Background signal was corrected for by subtracting the values of an empty plate. In addition, as the fluorescence of MitoTracker Red 580 (Invitrogen) is a result of oxidation of the compound within the mitochondrial matrix, changes in MitoTracker fluorescence intensity was used to assess the qualitative and quantitative efficiency of the electrochemical potential (the relative oxidative capability of actively respiring mitochondria [36]. Measurements of mitochondrial content, membrane potential, and viability were done by staining the cells with both ethidium bromide (Fisher Scientific) and NAO (6). Briefly, 500,000cells/sample was stained with 25ng/mL NAO and 40ng/mL ethidium bromide for 10 minutes. The cells were then washed 3 times with 0.1% BSA in PBS at 4ºC for 10 minutes at 1000g. The cells were read using the C6

flow cytometer (Accuri) using the FL1 and FL3 channels. Data was analyzed using FCSExpress (DeNovo).

Measurement of Citrate Synthase Activity

Isolated mitochondria from $Bax^{+/+}$and $Bax^{-/-}$ HCT-116 cells were assayed for citrate synthase activity. The citrate synthase enzyme catalyzes the reaction: CoA-SH + DTNB → TNB + CoA-S-S-TNB. The rate of TNB formation is linear and has a measurable absorbance at 412nm. The rate of TNB generation (μMol/ml/min) was measured by spectrophotometry (Beckman Coulter SD8000) for 200 seconds. Mitochondrial samples were run in Tris buffer to determine inner mitochondrial membrane integrity (reported as % ruptured mitochondria), or Tris buffer with Triton X-100 to determine citrate synthase activity.

Measurement of Mitochondrial DNA

DNA was isolated from $Bax^{+/+}$ and $Bax^{-/-}$ HCT116 cells using TRIZOL Reagent (Invitrogen) followed by ethanol precipitation. Resuspended DNA pellets were analyzed for nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) content. Primers for the detection of the nuclear housekeeping gene β-actin and for the mitochondrial DNA encoded ATPase (MTATP) genes were as follows: β-actin (forward): 5'-GAA ATC GTG CGT GAC ATC AAAG; (reverse): 5'-TGT AGT TTC ATG GAT GCC ACAG. MTATP (forward): 5'-AAT ATT AAA CAC AAA CTA CCA CCT ACC; (reverse): 5'- TGG TTC TCA GGG TTT GTT ATA [37]. The PCR was carried out in a 7500 Fast real-time PCR system (Applied Biosystems). All reactions were carried out in 20μl of total reaction volume containing 1μg/μl total DNA, appropriate primers and Fast SYBR Green Master Mix (Applied Biosystems). Each reaction

underwent a 30 s 90 ºC denaturation followed by 40 cycles of 30 s at 60 ºC then 3 s at 95 ºC. All samples were analyzed in triplicate. The relative amount of mtDNA (normalized to nuclear content) is represented as $2\Delta Ct$, calculated using the following equation: ΔCt = (CtmtDNA /CtnDNA) - (CtnDNA/CtnDNA), where Ct is the cycle threshold for each condition.

Statistics

All data in this study are expressed as means ± SEM. Statistical analysis of differences in data between the groups was determined using Prism for Windows, Version 5.02 (GraphPad Software Inc).

Results

Bax is essential in mitochondrial bioenergetics

To determine whether Bax has a previously unrecognized non-apoptotic activity, we examined mitochondrial structure, function and bioenergetics in $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 colon cancer cells. Mitotracker Red 580 fluorescence intensity was used as an indicator of oxidative capacity and to visualize actively respiring mitochondria [38]. Live-cell imaging was performed to ensure that fixatives would not disrupt MitoTracker uptake. As shown in Figure 1A, when Bax is present, we observed respiring mitochondria that displayed elongated and tubular shape with a loosely dispersed branching network. In contrast, when imaging of the $Bax^{-/-}$ cells, we observed that the respiring mitochondria were numerous but appeared less elongated due to the deficiency of Bax (Fig. 1B). To verify that the observed differences in functional morphology upon Mitotracker staining were not due to alterations in the fission/fusion process,

treatment with Mdivi 1, a chemical inhibitor of DRP1that prevents fission by blocking the early stages of DRP1 assembly, resulted in uniform changes in MitoTracker fluorescence that were independent of Bax (data not shown).

To determine whether the differences observed in Mitotracker staining (Figs. 1A, 1B) were caused by altered mitochondrial density induced by loss of Bax, we probed both $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells with N-nonyl acridine orange (NAO). NAO accumulates in mitochondria by binding to phospholipids, specifically cardiolipin [39, 40]. Hence NAO accumulation is independent on mitochondrial respiration and would directly show mitochondrial content. As shown in Figures 1A and 1B, NAO incorporation in mitochondria was similar in both $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells. This was confirmed by the measurement of total NAO fluorescence in each cell population (Fig. 1C). Hence, total mitochondrial density or content was independent of Bax expression. To confirm results from NAO staining, we perform a quantitative experiment by measuring total mitochondrial DNA (mtDNA) relative to nuclear DNA (nDNA). We found no detectable differences in mtDNA content between $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells (data not shown). To demonstrate that $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells were equally viable, we stained cells with NAO and ethidium bromide (EB). EB stains DNA only when the cell membrane is ruptured, as it is in cultured apoptotic cells. Representative data displayed in Fig. 1D revealed that there were no significant Bax-dependent differences in overall viability. The viability of cell lines used in all experiments reported herein was consistent with the data shown in Figure 1D. Together, these results suggest that the observed morphological differences detected by mitochondrial tracker staining were due more to a functional defect rather than decreased mitochondrial content.

To explore a possible functional defect upon BAX loss that was revealed by Mitotracker staining, we examined the electrochemical properties of the mitochondria in the two HCT-116 Bax variants using the uncoupling agent, FCCP. FCCP is a hydrophobic compound that carries protons across the IMM, releasing these protons into the matrix allowing for the dissipation of the chemiosmotic gradient. A titration of FCCP was previously performed to determine the optimal concentration of FCCP for use, that is, caused the least effect on cell viability and the most effect on ATP production (data not shown). When treated with FCCP, $Bax^{+/+}$ mitochondria displayed a stronger intensity of Mitotracker fluorescence compared to untreated cells (Fig. 2A, 2C). This was not observed in $Bax^{-/-}$ cells (Fig. 2B, 2C). Although relaxation of Mitotracker fluorescence quenching has been reported with FCCP, we do not think this artifact is the reason for the observation made with $Bax^{+/+}$ cells, since the increase in Mitotracker fluorescence was not repeated in $Bax^{-/-}$ cells. Quantification of the effect of FCCP treatment revealed a two fold increase in MitoTracker fluorescence in $Bax^{+/+}$ cells compared to untreated cells that was not observed with $Bax^{-/-}$ cells (Fig 2C). We propose that, in $Bax^{+/+}$ cells, the FCCP-mediated uncoupling of the electron transport system results in the rapid oxidation of matrix substrates, such as MitoTracker Red 580, which can be detected as an increase in MitoTracker fluorescence [39]. This does not occur in $Bax^{-/-}$ cells, suggesting that loss of Bax may cause a defect in mitochondrial oxidative capacity. $Bax^{-/-}$ cells also had significantly decreased levels of intracellular ATP as compared to $Bax^{+/+}$ cells (Fig. 2D). Furthermore, FCCP treatment resulted in a marked drop in ATP levels in $Bax^{+/+}$ cells, but not in $Bax^{-/-}$ cells (Fig. 2D). Note that a previous titration of FCCP concentrations correlated with ATP amounts detected, indicating that we were primarily assessing changes in ATP production (data not shown).

To determine whether the loss of ATP in $Bax^{-/-}$ cells was due to abnormal bioenergetics, we measured glycolysis and oxygen consumption using a Seahorse XF analyzer. Metabolic activity in $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells was assessed by plotting the oxygen consumption rate [OCR], a measure of mitochondrial respiration, against the extracellular acidification rate [ECAR], an indicator of glycolysis. Comparing changes that occur in aerobic and glycolytic metabolism, shown in Figure 2E, revealed that $Bax^{+/+}$ cells had increased oxygen consumption (OCR) and decreased glycolytic activity (ECAR) relative to $Bax^{-/-}$ cells; hence cells deficient in Bax were more dependent on glycolysis for their energy needs. A separate measure of oxygen consumption, using an alternative method, further confirmed these findings (Fig. 2F). Taken together, these results indicated that the decreased levels of ATP observed in $Bax^{-/-}$ cells were likely due to depressed respiration, suggesting that cells lacking Bax had a significant defect in oxidative capacity.

Bax associates with mitochondria and contributes to ATP production

Since we showed a non-apoptotic function for Bax in the regulation of mitochondrial metabolism, immunofluorescence was performed to determine whether Bax localizes to mitochondria in the absence of apoptosis. As shown in Figure 3A, a partial overlay of Bax with mitochondria (HSP-60 staining for mitochondrial marker) was observed in $Bax^{+/+}$ HCT-116 cells. To determine the interaction of Bax with mitochondria under standard growth conditions, ultra centrifugation of enriched mitochondria was performed to examine the localization of Bax. Cell lysates were prepared from cells that had the same viability. The enriched mitochondrial lysate was layered on an iodixanol gradient and subjected to ultracentrifugation as described in methods. Separation of organelles was based on density, which is a function of the protein-lipid

ratio of the organelles. Western blot analysis of the gradient fractions for Prohibitin (mitochondrial content) (Fig. 3B) showed that the distribution of the mitochondria between $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells was similar (across the 20-23% fractions). Approximately 7% of the total endogenous Bax was detected in the non-apoptotic $Bax^{+/+}$ cells associated with the mitochondrial fractions, while no Bax was detected in $Bax^{-/-}$ cells (Fig. 3B).

To determine whether Bax was loosely associated with mitochondria (i.e. bound to the outer mitochondrial membrane (OMM)) or whether it was sequestered within an inner membrane compartment (IMM). Enriched mitochondrial lysates were treated with Proteinase K for 5 to 20 minutes. Digested lysates were analyzed by SDS-PAGE and immunoblotted with antibodies to detect Bax and, as a control, BCL-XL. BCL-XL is mainly an OMM localized protein and sensitive to protease digestion. We found that treatment with Proteinase K resulted in 50-60% lysis of Bax, compared to BCL-XL, in which 77-100% of the protein was lysed (Fig. 3C). Although in the same sample there was less BCL-XL protein detected compared to Bax, an increase in the amount of BCL-XL digested occurred over time, while Bax was mostly digested within 5 minutes. These results suggest that a significant amount of Bax was sequestered within the mitochondria and not accessible to be digested by the protease. Hence Bax has both outer and inner mitochondrial localization, supporting its proposed role in mediating mitochondrial respiration. The ability of Bax to regulate mitochondrial metabolism was further demonstrated by measurements of citrate synthase activity, which localized to the mitochondrial matrix and is one of the first enzymes of the tricarboxylic cycle. As shown in Figure 3D, $Bax^{-/-}$ cells had reduced levels of citrate synthase activity in comparison to $Bax^{+/+}$ cells.

Expression of full-length Bax restores mitochondrial metabolic activity in Bax$^{-/-}$ cells

Since cells lacking of Bax displayed impaired mitochondrial metabolic activity, we determined whether restoring Bax to deficient cells would rescue mitochondrial metabolic activity. Over-expression of full-length Bax (Bax-FL) in Bax$^{-/-}$ cells resulted in recovery of respiring mitochondria morphology as observed in Bax$^{+/+}$ cells (Fig. 4A). Western blot analysis of density gradients showed that ~2.5% of the total expressed protein associated with mitochondrial fractions (Fig. 4B). We also observed a three-fold increase in ATP production upon the expression of Bax-FL, whereas this increase in ATP production was inhibited by treatment with FCCP (Fig. 4C), suggesting that that Bax-FL could rescue ATP production through oxidative phosphorylation. ECAR/OCR measurements further confirmed that expression of Bax-FL could increase oxygen consumption and reduce glycolytic activity, indicating that Bax was a critical regulator of these activities (Fig. 4F). To determine the contribution of the C-terminal helix of Bax to mitochondrial bioenergetics, over-expression of C-terminal truncated Bax (Bax-ΔCT) in Bax$^{-/-}$ cells was performed. As shown in Figure 4D, expression of Bax-ΔCT did not restore the respiring mitochondrial morphology seen in the presence of Bax, suggesting that the C-terminal domain of Bax is essential to regulate mitochondrial metabolic activity by Bax. The removal of this C-terminal helix also resulted in a scattered localization of Bax-ΔCT, which was supported by the altered banding pattern in the gradient fractions (Fig. 4E). Even though mitochondrial targeting was deregulated in this mutant, Bax-ΔCT was found in the mitochondrial fractions (~9%, Fig. 4E), and was able to restore ATP production as effectively as Bax-FL (Fig. 4E). This was further demonstrated by measurements ECAR/OCR, which showed

that expression of Bax-ΔCT was able to restore metabolic balance in $Bax^{-/-}$ cells, almost the same levels as Bax-FL (Fig. 4F).

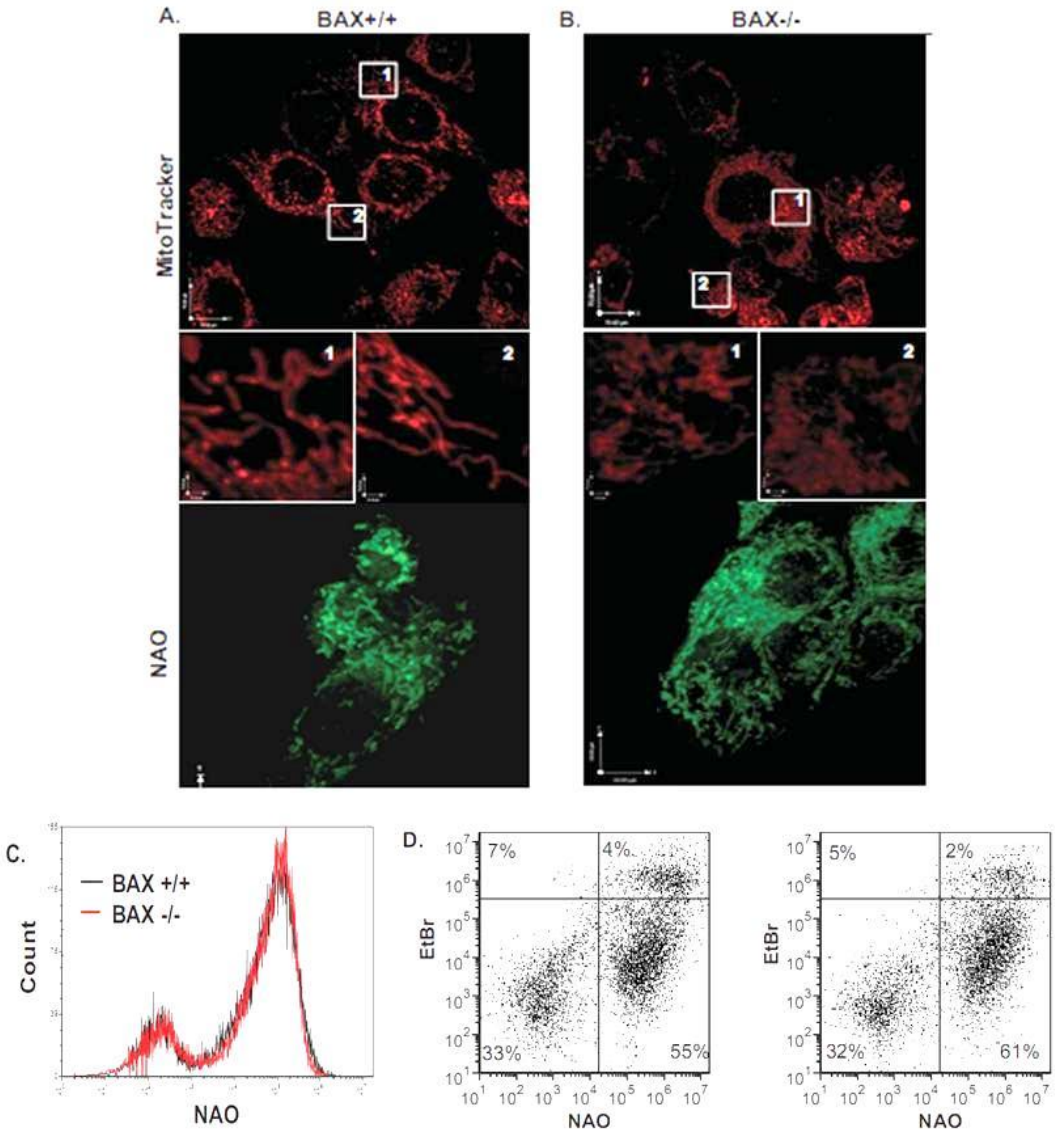

Figure 1. Loss of Bax leads to altered respiring mitochondria morphology.

Bax+/+ HCT-116 cells (A) and Bax-/- HCT-116 cells (B) were treated with MitoTracker Red 580 or NAO prior to imaging as described in Methods. Live cell images were obtained using the UltraView (PerkinElmer) spinning disc confocal system. Images are representative of three replicates per condition. Insets are 10X magnification of the two highlighted areas in each field. (C) and (D) To assess mitochondrial density (C) and viability (D), 500,000 cells/sample were stained with NAO (C) or NAO and ethidium bromide (EtBr) (D) for 10 minutes. Fluorescence output was read using the FL1 and FL3 channels on a C6 flow cytometer (Accuri). Each experiment was performed in triplicate for each cell line variant. (contributed by R. Boohaker)

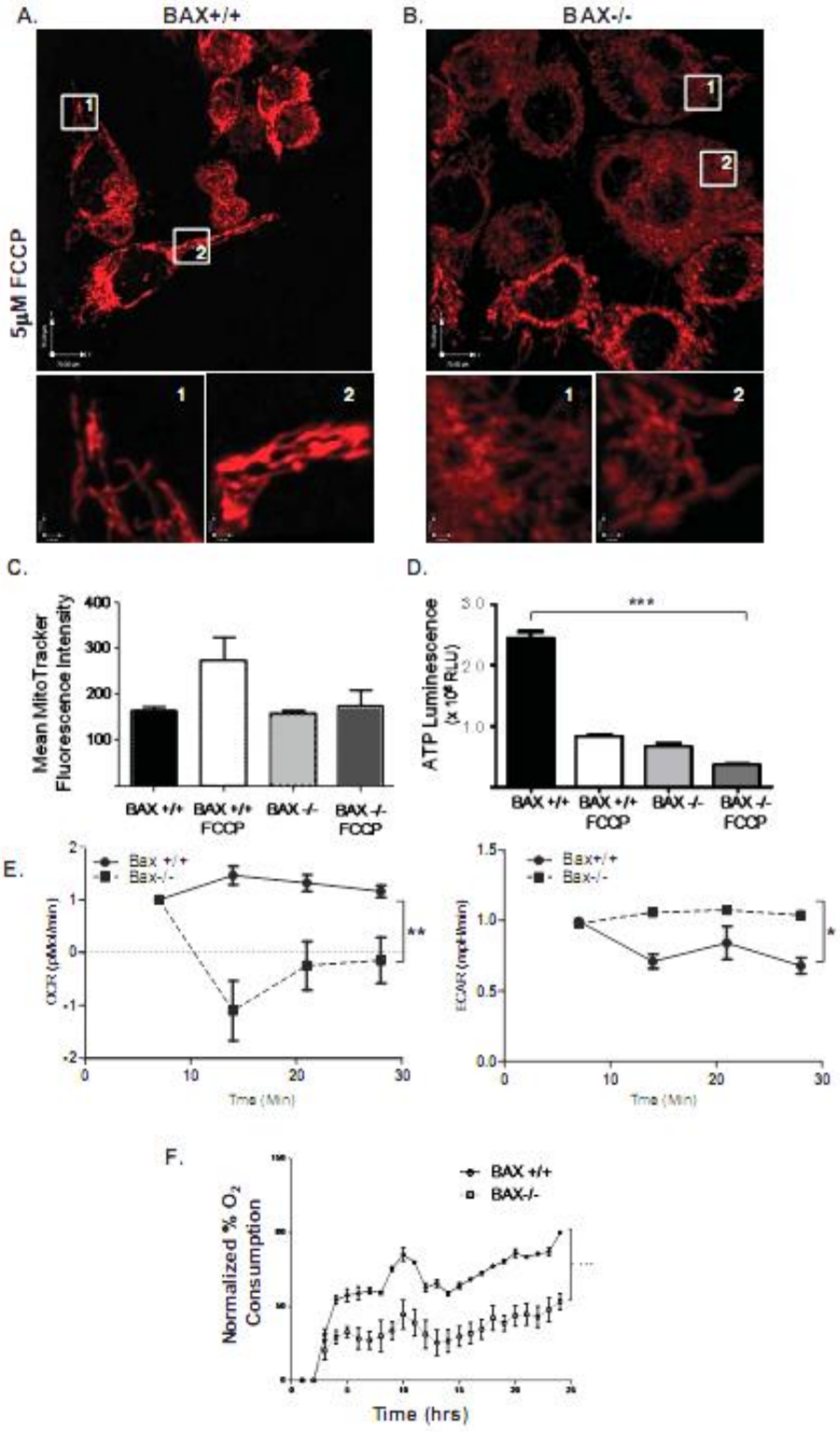
A.
BAX+/+
B.
BAX-/-
5μM FCCP
1
2
C.
Mean MitoTracker Fluorescence Intensity
400
300
200
100
BAX +/+
BAX +/+ FCCP
BAX -/-
BAX -/- FCCP
D.
ATP Luminescence (x 10⁶ RLU)
3.0
2.0
1.0
***
E.
Bax +/+
Bax-/-
OCR (pMol/min)
ECAR (mpH/min)
Time (Min)
**
*
F.
BAX +/+
BAX-/-
Normalized % O2 Consumption
Time (hrs)

Figure 2. Loss of Bax leads to reduced mitochondrial oxidative capacity.

Bax+/+ HCT-116 cells (A) and Bax-/- HCT-116 cells (B) were treated with FCCP in McCoy's complete media for two hours prior to imaging. Live cell images were obtained using the UltraView spinning disc confocal system. The stand was a Zeiss AxioObserver Z.1 with a Plan-Apochromat 63x/1.4 Oil DIC objective. Insets are 10X magnification of the two highlighted areas in each field. Images are representative of three separate experiments with two replicates per condition. (C) Calculation of Mitotracker fluorescence intensity was done with Velocity software by averaging the mean intensity of the pixels in each fluorescent cell. (D) ATP concentration was assessed by measuring the activity of Luciferase as relative luminescence units (RLU). (E) OCR and ECAR data obtained from the Seahorse XF24 Analyzer was calculated based on the oxygen consumption and acidification rates of 40,000 cells per well. The values were pooled from three separate experiments and normalized to changes from the initial rate, n=9. (F) Oxygen consumption in both Bax+/+ and Bax-/- cells was measured over time as a function of increased fluorescence. Fluorescence, oxygen biosensor, and ATP data are representative of n=6. The values for the ATP, OCR and ECAR assays were normalized to cell number per well, and statistics were done using One-way ANOVA with Dunnett's post-test anlysis. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. (contributed by R. Bohhaker)

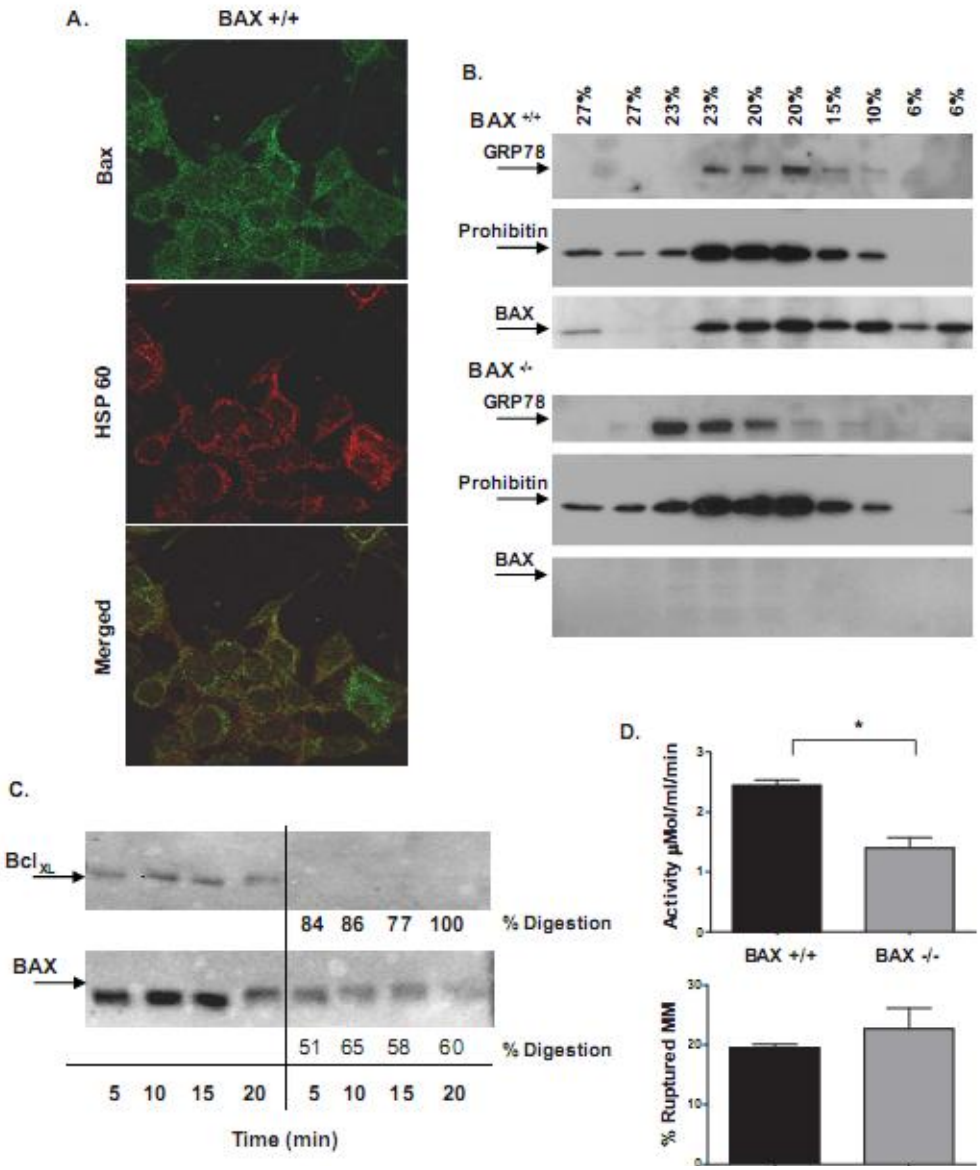

Figure 3. Bax associates with mitochondria in non-apoptotic cells resulting in reduced citrate synthesis activity.

(A) Bax+/+ HCT-116 cells were fixed and stained with the indicated fluorochrome-conjugated antibody as described in Methods. Confocal microscopy of cells shows endogenous Bax (green), the mitochondrial protein, HSP60 (red), and the merged field (yellow) indicating co-localization of Bax with HSP60. Images were acquired with LSM 510 using 100 x/1.4 Oil DIC objectives and are representative of ten different images scanned from three separate experiments. (B) Enriched mitochondrial lysates of membrane-bound proteins were prepared from both Bax+/+

and Bax-/- HCT-116 cells and subjected to differential ultracentrifugation as described in methods. Gradient fractions collected were analyzed by SDS-PAGE and membranes probed for the presence of Bax, prohibitin (mitochondria), and GRP78 (ER). Each blot shown is representative of three independent experiments. (C) Western blot analysis of mitochondria isolated from BAX+/+ cells shows the effects of Proteinase K digestion on mitochondrial membrane proteins over time. The extent of the digestion was calculated based on the percent change in densitometry between the treated and untreated samples. (D) The assessment of citrate synthase activity was determined by the rate of formation of thionitrobenzoic acid as described in methods. The rate of activity is shown as μMol/ml/min. Background levels of citrate synthase activity in spontaneously ruptured mitochondria are included as controls. The data encompasses two separate experiments for a total of n=4 per sample. Statistics were calculated using unpaired Students t-test. *$P < 0.05$. BCL-XL is mainly an OMM localized protein and sensitive to protease digestion. (parts contributed by R. Boohaker and K. Nemec)

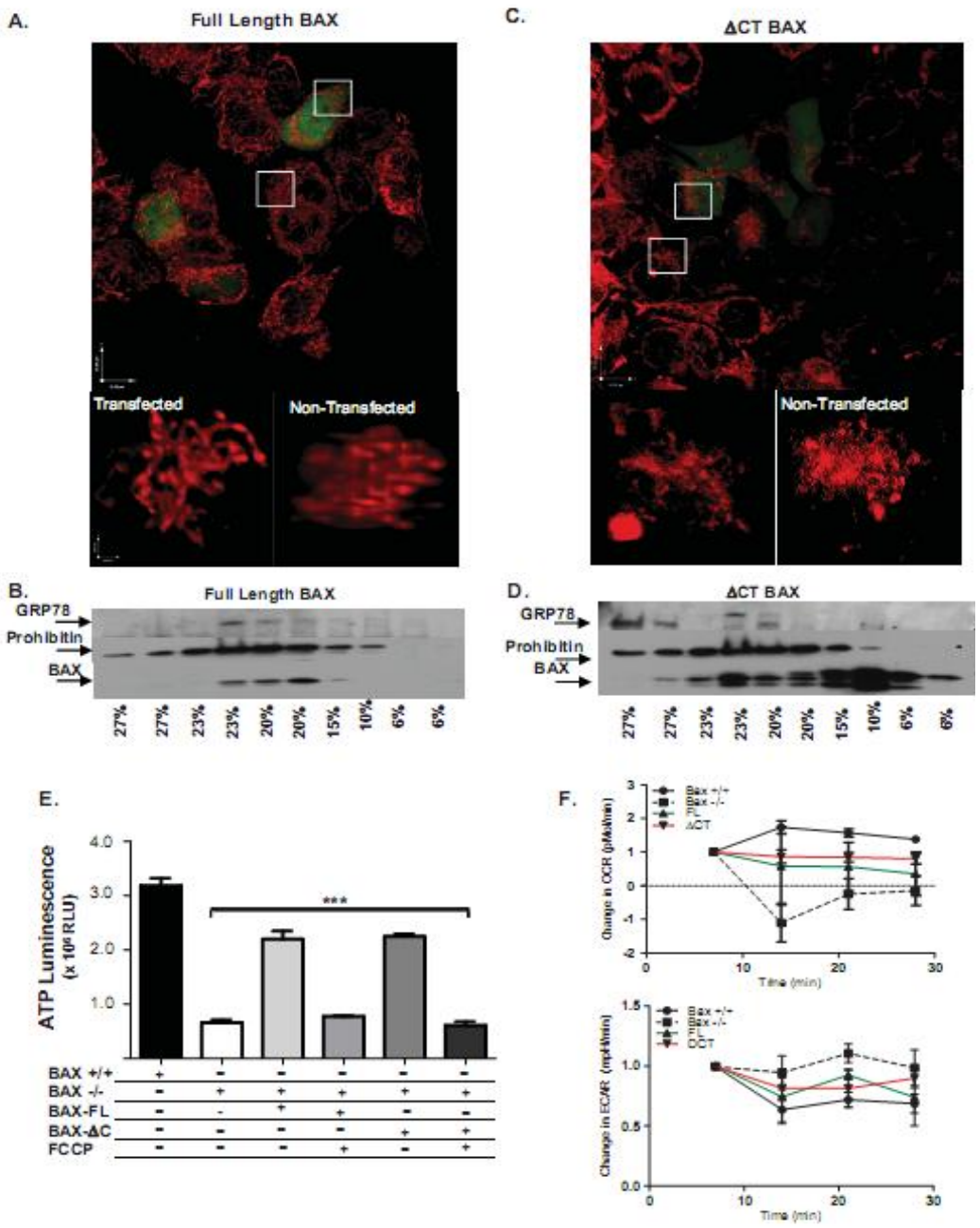

Figure 4. Expression of full-Length Bax restores mitochondrial association and ATP production in Bax$^{-/-}$ cells.

Bax$^{-/-}$ 716 HCT-116 cells were transfected with a ProteoTuner bi-cistronic vector expressing either full-length (FL) Bax (A, B) or Bax-ΔCT(C, D) and GFP(A, C) Cells were grown on MatTek plates as described in Methods, and transiently transfected (efficiency 60-70%). Shield

1 was added to induce expression. Transfected cells were imaged based on GFP expression, and MitoTracker Red was used for mitochondria visualization. Live cell images were obtained using the UltraView spinning disc confocal system. The stand was a Zeiss AxioObserver Z.1 with a Plan-Apochromat 63x/1.4 Oil DIC objective with a resolution of 0.124μm. Insets were acquired at 10 X magnifications. Post-acquisition processing was done using Velocity software. (B, D) Bax-/-HCT-116 cells were transfected with either Bax-FL or Bax-ΔCT as described above. Mitochondrial enriched lysates were subjected to differential ultracentrifugation and SDS-PAGE as described in methods. Membranes were probed with antibodies for Bax, prohibitin (mitochondria) and GRP78 (ER). Images are representative of three independent experiments. (E) Bax+/+ and Bax-/- HCT-116 cells transiently transfected with Bax-FL or Bax-ΔCT (ΔC) were treated with and without FCCP and analyzed for changes in ATP levels as described in Methods. (F) Bax+/+ HCT-116 cells, Bax-/-HCT-116 cells, and Bax-/- HCT-116 cells, transiently transfected with Bax-FL (FL) or Bax-ΔCT (ΔCT or DCT,) were analyzed for changes in OCR and ECAR as in methods. One-way ANOVA was used for statistical analysis. Microscopy and ATP data is representative of two experiments each with three replicates. OCR and ECAR data are representative of two experiments with a total of six replicates. Statistical results for OCR and ECAR assays are *p <0.05for Bax-/- vs. Bax-FL and Bax-/- vs. Bax-ΔCT. *** $p < 0.001$ for the ATP assay. (parts contributed by R. Boohaker)

Discussion

The Bcl-2 family proteins that tightly control the mitochondrial pathway of apoptosis have been classified into pro- and anti-apoptotic members [7,9]. Among which Bax is a pro-apoptotic protein, and its activation is a highly regulated, multi-step process that involves Bax conformational changes, mitochondrial translocation and oligomerization, and ultimately leading to mitochondrial dysfunction and apoptotic cell death [1-3,7]. Although the precise mechanisms remain unclear, it is generally accepted that Bax conformational changes and translocation to the mitochondria are critical events for Bax to exert it apoptotic activity upon apoptosis stimuli [7,9]. In dying cells the accumulations of activated Bax at the mitochondrial outer membrane trigger the release of apoptotic mediators, such as cytochrome c, from the intermitochondrial membrane space. Thus, Bax localizes to and uses mitochondria as its major site of action. In addition to its apoptotic form, Bax is also constitutively expressed in non-apoptotic cells and resides in a soluble, cytosolic form or associated with mitochondria [7, 8]. Several studies have demonstrated that this non-apoptotic form of Bax may be involved in mitochondrial function such as reactive oxygen formation and ion channel conduction [32, 33]. However, relatively little is known about the direct role that the inactive form of Bax plays in regulation of mitochondrial function.

In the present study, we assessed the direct role of non-apoptotic Bax in mitochondrial function in non-apoptotic cells by using the $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cell model system. The results showed that Bax localized to outer and inner mitochondrial membranes in non-apoptotic cells and participated in citrate synthase activity, intracellular ATP and oxygen consumption.

Loss of Bax led to impairment of mitochondria respiring morphology and oxidative capacity, all of which could be restored by expression of full-length Bax. These findings indicated that under normal conditions, the constitutive expression of non-apoptotic Bax is associated with mitochondria and is necessary for mitochondrial bioenergetics.

Under non-apoptotic conditions, Bax is present in a soluble, cytosolic form or associated with mitochondria as a monomer. A small amount of "active" (non-apoptotic) BAX associated with healthy mitochondria may contribute to the regulation of functional architecture and bioenergetics of mitochondria. This leads to the hypothesis that BAX associated with mitochondria under non-lethal conditions could be restricted by the lipid and protein composition of the organelle membrane [41, 42]. Apoptosis induces changes in mitochondria that could help recruit Bax, while, in the absence of apoptosis the outer mitochondrial membrane environment is less likely to support the translocation of Bax. In mitochondria from healthy cells, a few BAX monomers or dimers may insert into the mitochondrial membranes to form small pores, but this process needs to be tightly regulated. Bax may also functionally associate with components of the mitochondrial inner membrane, like the adenine nucleotide transporter (ANT), the mitochondrial F_0F_1 ATPase H^+ pump, and the outer membrane voltage-dependent anion channel (VDAC) [29-31]. Thus, it is more feasible for Bax to be interacting with existing mitochondrial proteins. This possibility is supported by our findings that a portion of Bax was sequestered within the mitochondria, perhaps interacting with an IMM protein. Hence, the amount of Bax associated with mitochondria in non-apoptotic cells would be constrained by the availability of the binding partner(s), although most were discovered in the context of an apoptotic scenario [29-31]. In addition to ANT and VDAC, many other proteins are known to interact with Bax,

including factors that mediate changes in the mitochondrial network such as fission or fusion proteins like Drp1[43], Mfn-2 [44] or endophilin B1 (Bif-1) [45] as well as other regulatory proteins such as cyclophilin D or Ku70[46]. Although this may seem speculative, the excess of circumstantial evidence for the interaction of Bax with healthy mitochondria highlights a real need for further study to determine whether these proteins act to facilitate Bax's role in mitochondrial bioenergetics.

A recent proteome-wide quantification of proteins analysis conducted in Bax-containing and Bax-deficient HCT-116 cells shows that a number of mitochondrial proteins, like VDAC, were found to be down regulated significantly upon loss of Bax [48]. Two enzymes essential for glucose metabolism and ATP production were also decreased in Bax deficient cells: Glucose-6-phosphate isomerase was reduced over 10-fold and citrate synthase was reduced over 7-fold. Consistent with this, our results showed reduced citrate synthase activity in $Bax^{-/-}$ HCT-116 cells. Loss of critical metabolic components exacerbates the inability of Bax deficient cells to produce energy from glucose. However, the impairment of citrate synthase activity in the $Bax^{-/-}$ cells was restored by the reintroduction of Bax into these cells with dramatically increased respiration and ATP production. These data strongly supports the concept that Bax plays an important homeostatic role in supporting growth and metabolism.

In conclusion, we have shown that Bax localized to outer and inner mitochondrial membranes in non-apoptotic cells and participated in citrate synthase activity, intracellular ATP production and oxygen consumption. Loss of Bax led to impairment of mitochondria respiring morphology and oxidative capacity, all of which can be restored by expression of full-length Bax. Bax C-terminal deletion can also restore mitochondrial bioenergetics. These findings

indicate that the constitutive expression of inactive form Bax is associated with mitochondria and is necessary for mitochondrial bioenergetics.

CHAPTER 3: THE C-TERMINAL α9-HELIX OF BAX IS THE EFFECTOR DOMAIN OF APOPTOTIC FUNCTION

Introduction

Bax participates in the induction of apoptosis in response to a variety of apoptotic signals [1]. Although the precise mechanism responsible for its apoptotic activity remains undefined, Bax conformational changes and translocation into the mitochondria are recognized as the key events triggering mitochondria-dependent apoptosis. Once inserted into the mitochondrial membrane, Bax undergoes conformational changes, leading to membrane destabilization. This leads to the release of cytochrome c, SMAC/DIABLO, and Htra2/Om from the mitochondrial intermembrane space into the cytoplasm where they amplify the caspase cascade, resulting in apoptotic cell death. Although the pro-apoptotic activity of Bax is well-established, how Bax translocates to mitochondria and triggers apoptosis remains unclear.

Recently, specific regions in the C terminus of Bax have been found to be critical in the regulation of Bax binding to mitochondria [1, 8, 13, 48]. In some studies, the C-terminus has been considered to be required for Bax binding to mitochondria and represents a transmembrane domain to facilitate translocation and insertion of Bax to the outer membrane of mitochondria [12, 13, 18, 49, 50]. Other reports suggest that the C-terminal anchor is not required for Bax translocation during apoptosis [14, 22, 25]. In these latter studies, deletion of the C-terminal 22 amino acids failed to block recruitment of Bax to mitochondria during apoptosis [22]. Thus, whether the C-terminus of Bax is a membrane binding domain or a regulatory domain remains to

be analyzed. Recently, we performed computational-mutagenesis to identify possible structural elements of the C-terminal necessary for Bax membrane binding activity. Based on variations in helical packing energy, we predicted that lysines 189/190, in the α9 helix at the C-terminal, may be crucial for Bax membrane insertion (Khaled A, unpublished data). Therefore, the purpose of the present study was to determine whether lysines189/190 at the C-terminal helix are the structural elements that mediate the binding of Bax to mitochondria and regulate its apoptotic activity. Data presented here demonstrated that the C-terminal α9-helix is the functional domain responsible for the apoptotic function of Bax. Expression of full-length Bax (Bax-FL) led to mitochondrial translocation and apoptosis, whereas deletion of the α9 helix resulted in cytosolic retention and no apoptosis. Mutation of the two lysine residues changed localization of Bax to mitochondrial membranes. These results were replicated by attaching the last 20 amino acids of the α9 helix of Bax to either GFP or to a regulatory element, a degradation domain (DD). We demonstrated that the α9 helix alone promoted the mitochondrial translocation of either GFP or the DD and increased apoptosis. These results indicated that the C-terminal α9 helix of Bax alone is sufficient to promote cell death.

Materials and Methods

Cell lines and Reagents

The Flp-In T-REx-293 cell line (Invitrogen) was derived from 293 human embryonic kidney cells and stably expresses the *lacZ*-Zeocin fusion gene and the Tet repressor. The 293 cell line was maintained in D-MEM (high glucose), 10% FBS (tetracycline-reduced), 2 mM L-

glutamine and 1% Penicillin-Streptomycin (DMEM complete medium). The HCT116 $Bax^{-/-}$ and $Bax^{+/+}$ colorectal cancer cell lines [51] (a kind gift from Dr. Bert Vogelstein, John Hopkins University) were maintained in McCoy's 5A media, 10% FBS and 1% Penicillin-Streptomycin (McCoy's complete medium).

Plasmids, Mutagenesis and Transfection

PCR-directed mutagenesis of the C-terminus of Bax was performed using HA-tagged primer sets (Table 1), with mutations incorporated within the primer sequences. For inducible expression of Bax wild-type (WT) and mutant proteins, we employed the Flp-In T-REx System (Invitrogen). Bax constructs were amplified by PCR from the template, pEGFP-Bax (a gift from Dr. Richard Youle, NINDS, NIH), digested with EcoRV and cloned into the plasmid, pcDNA5/ FRT/TO, which undergoes recombinase-mediated DNA recombination at the Flp Recombination Target (FRT) site when co-expressed with the pOG44 plasmid that constitutively expresses the Flp recombinase. All constructs were confirmed by sequencing. The pOG44 plasmid and the pcDNA5/FRT/TO vectors were co-transfected into the Flp-In T-REx 293 cell line, at a ratio of 9:1, using Fugene transfection reagent (Roche) following manufacturer's protocol. Stable Flp-In T-REx expression cell lines were then selected for Blasticidin resistance (10μg/ml), Hygromycin resistance (100μg/ml) and Zeocin sensitivity (200μg/ml). Bax expression in 293 cells was induced by the addition of tetracycline (1μg/ml) and cells were assayed for the mitochondrial translocation of Bax by immunoblotting as described below.

To generate the EGFP fusion C-terminus (amino acids 168-192) of Bax (EGFP-Bax-CT) constructs, primers incorporating the C-terminus of Bax (Table 1) were used in a PCR to amplify EGFP from the template pEGFP (Clontech), and the PCR insert was cloned into

pcDNA5/FRT/TO as previously described. $Bax^{-/-}$HCT-116 cells were transiently transfected and cells assayed microscopically for EGFP expression 48 hours later.

To generate the DD-tagged Bax C-terminus (amino acids 173-192) wild-type (WT) or EE, LL, and RR mutations, primers were annealed and ligated into the ProteoTuner vector digested by EcoRI and BamHI. To generate the DD-tagged full-length WT Bax and ΔCT-Bax, the PCR product of Bax was digested with XhoI and EcoRI restriction endonucleases and ligated into the ProteoTuner vector. The ProteoTuner IRES2 system also has the marker protein GFP downstream to the internal ribosome entry sequence (IRES) and can be translated independently of the DD-tagged Bax protein.

Mitochondrial Translocation Assay and Immunoblotting

To examine the mitochondrial translocation of Bax, HA-tagged full-length Bax and its mutants were transfected into HEK293 cells and expression of Bax was induced by addition of 1μg/ml tetracycline for 24 hrs. To further determine the Bax C-terminal mutations on Bax mitochondria translocation, EGFP-CT-Bax or Bax-ΔCT was constitutively expressed in $Bax^{-/-}$ HCT116 cells for 24 hrs. We also examined whether DD-tagged Bax C-terminal peptide and its mutants could affect Bax mitochondria translocation. DD-tagged FL-Bax and DD-tagged CT-Bax as well as it mutants (EE, LL and RR) were transfected into $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells for 24 hrs, then expression of these DD-tagged constructs were induced by the addition of shield for 4 hrs. The cells were then lysed and mitochondrial and cytosolic proteins were isolated using a mitochondrial enrichment kit (Pierce) according to the manufactor's instructions. The final mitochondrial pellet was washed in 1M NaCl (in place of carbonate (pH 11) buffers) to remove any proteins peripherally associated with mitochondria. Mitochondrial and cytosolic protein

fractions were loaded on 12-15% SDS-PAGE gels, transferred to PVDF membranes and probed with the appropriate primary antibodies. For detection of HA-tagged Bax, an anti-HA mouse monoclonal antibody was used (16B12, Covance). Anti DD monoclonal antibody (631073, Clontech) was used to probe for DD-Bax. Antibodies for the loading controls and for mitochondrial and cytosolic contents were: endogenous Bax (N-20 Santa Cruz), prohibitin (Ab-2, Fitzgerald) and p38 MAP kinase (MAPK) (C20, Santa Cruz). A mouse polyclonal antibody was used for detection of EGFP (A.v. antibody, Clontech). Appropriate secondary rabbit or mouse antibodies conjugated to horseradish peroxidase (HRP) and enhanced chemiluminescence kit (Pierce) was used for visualization following the manufacturer's protocol.

Fixed- and Live-Cell Imaging

To determine whether expression of the C-terminal of Bax mutations affect the apoptotic activity of Bax, $Bax^{-/-}$ HCT-116 cells were grown on coverslips coated with 200 μg/ml poly-L-lysine (Sigma) and co-transfected with both EGFP and one of the HA-tagged Bax mutant constructs using Mirus LT-1 reagent following manufacturer's protocol. After 48 hours, apoptotic cell death was assessed by loss of EGFP-expressing cells. Images were acquired using a Nikon Eclipse E1000 microscope (10X objective) with a SPOT RT camera.

To determine whether expression of DD-tagged Bax C-terminal peptides co-localizes with mitochondria, $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells were cultured for 24 hours on coverslips pretreated with laminin. The DD-Bax C-terminal WT peptide and DD-Bax C-terminal EE, LL, RR mutations constructs were transfected into the cells using Mirus LT-1 reagent according manufacturer's protocol. After 24 hrs, expression of the transfected peptides was induced by the

addition of shield 1 for 4 hrs. Cells were fixed with 2% w/v formaldehyde/PBS for 15 minutes and permeabilized using 0.05% Triton X-100/PBS for 15 minutes. After washing, cells were incubated with primary antibodies HSP60 (H-300, Santa Cruz) and DD monoclonal antibody for 1 hour at room temperature, followed by incubation with secondary anti-rabbit-Cy3 (81-6115, Invitrogen) and anti-mouse-Texas red (715076020, Jackson Immunoresearch) for 30 minutes. After the final wash, cells were mounted with gel/mount medium (Mo1, Biomeda) and images were acquired with UltraView (PerkinElmer) microscopy with a plan-apochromat 63 X /1.4 oil objective. The scanned images were processed and Pearson's correlation coefficients determined using Velocity Version 5.5 (Perkin Elmer). Analysis of the images was completed through Prism 5 (Graphpad) software.

$Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells were cultured in 24-well plates (MatTek Corporation) in McCoy's complete medium. Cells were transfected with the GFP-tagged C-terminal wild type (KK), EE and LL mutants of Bax constructs into both $Bax^{+/+}$ and $Bax^{-/-}$ HCT116 cells using the TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's protocol. After transfection for two hours, time-lapse movies were made for 12 hrs using the UltraView (PerkinElmer) spinning disc confocal system with AxioObserver.Z1 stand, and a Plan-Apochromat 10x objective. Snapshots were taken as indicated in the images.

The constructs of DD-Bax-C-terminal-WT, DD-Bax-CT-EE, DD-Bax-CT-LL, DD-Bax-CT-RR were delivered as mentioned above for 48 hours. Cells were incubated with 1nM MitoTracker Red 580 in McCoy's complete media for 30 minutes at 37 °C prior to imaging. Time-lapse movies were recorded for 5 hrs using the UltraView spinning disc confocal system (PerkinElmer) with AxioObserver.Z1 (Carl Zeiss) stand equipped with a Plan-Apochromat 63x

Oil DIC objective, in a humidity and temperature-controlled chamber (LiveCell). Z-stack projections of the scanned images were generated within the imaging program, Volocity Version 5.5 (PerkinElmer). Post-acquisition snapshots were taken from time-lapse movies.at 0, 3 and 5hrs.

Detection of apoptotic cells by flow cytometry

$Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells were plated in 6-well plates, and cells transfected with DD-Bax C-terminal WT, EE, LL, or RR mutants for 24 hrs, followed by treatment with Shield 1 to induce peptide expression for an additional 24 hrs. Cells were collected at a final concentration of 1 x 10^6 cells/ml in HBSS. Cells were then stained using the Violet Ratiometric Membrane Asymmetry Probe/Dead Cell Apoptosis Kit (A35137, Invitrogen) according the manufacturer's protocol by adding F2N12S solution and SYTOX® AADvanced™ dead cell stain solution to each sample. Analytes were incubated at room temperature for 5 minutes, and then analyzed using the BD FACSAria II flow cytometer. F2N12S was visualized with a laser source at 405nm and emissions collected at 530nm and 585nm, and excitation of SYTOX® AADvanced™ visualized at 488nm and emissions collected at 695nm.

Statistical Analysis

Data were presented as mean ± SD. Statistical analysis was determined using Prism for Windows, Version 5.02 (GraphPad). A *p* value under 0.05 was considered a significant difference.

Results

The C-terminal α9 helix is critical for Bax intracellular localization and apoptotic activity

To verify our predictions derived from our computational studies, we performed a series of mutagenesis experiments targeting the C-terminal of Bax, as summarized in Table 2. We generated wild-type (WT) and N- or C-terminal deleted Bax mutants as well as the C-terminal Lys189/190 mutants. The inducible expression of Bax mutants in 293 cells, upon addition of tetracycline, was examined by immunoblot. In this cell model system, the inducible low expression of Bax in these cells did not cause apoptosis. p38 MAPK and prohibitin were used as indicators for cytosolic and mitochondrial content, respectively. Expression of WT Bax was found both in cytosolic and mitochondrial extracts. The N-terminal deleted Bax lacking residues 1-19 (Bax-ΔNT), was localized primarily to mitochondria, while the C-terminal deleted Bax lacking residues 173-192 (Bax-ΔCT), was retained in the cytosol (Fig. 5, Table 2). To examine whether expression of these Bax constructs would induce apoptosis, we constitutively co-expressed WT Bax or the deletion mutants along with GFP in a Bax deficient cell line ($Bax^{-/-}$ HCT116) [51]. As shown in Figure 6, like Bax-ΔNT, expression of WT Bax induced apoptosis in $Bax^{-/-}$ HCT116 cells, as evidenced by the loss of GFP^{+} cells, whereas Bax-ΔCT remained cytosolic and did not induce apoptosis, as evidences by the presence of GFP^{+} cells. These results show that an intact C-terminus of Bax causes apoptosis, while the N-terminus promotes cytosolic retention.

Substitution of Lys189 and/or Lys190 with negatively charged residues, Asp or Glu, resulted in the cytosolic retention of Bax (Bax-DD, Bax-EE, and Bax-EK) in 293 cells, and no

apoptosis was observed in BAX$^{-/-}$ HCT116 cells (Table 2, Figs. 5 and 6). Whereas, substitution of Lys189 and/or Lys190 with another positively charged residue, Arg (Bax-RR) led to mitochondrial localization and apoptosis (Table 2, Figs. 5 and 6). Substitution of Lys 189 and Lys190 with a polar amino acid, like Gln (Bax-QQ), led to more Bax in the cytosol and less cell death, when compared WTBax and Bax-RR, but more mitochondrial Bax and cell death was observed when compared to Bax-ΔCT and Bax-EE (Table 2, Figs. 5and 6). Substitution of Lys189 and/or Lys190 with the hydrophobic residue leucine (Bax-LL) resulted in mitochondrial binding and apoptosis (Figs. 5 and 6, Table 2). Loss of Lys189 (Bax-EK) also rendered Bax to be mainly cytosolic, which was not observed by loss of Lys190 (Bax-KMGK) (Figs. 5 and 6, Table 2). These data suggest that Lys 189/190 was essential for both Bax binding to mitochondria and its apoptotic activity.

The C- terminus Lys 189/190 of Bax is involved in Bax binding to mitochondria

To determine whether the C-terminus Lys 189/190 is critical for Bax binding to mitochondria, we attached the C-terminal α9 helix of Bax (CT) (amino acids 168-192) to EGFP (EGFP-CT) and examined the subcellular localization in Bax$^{-/-}$ HCT-116 cells. Moreover, mutant C-terminal constructs, CT-EE and CT-LL, were also attached EGFP for evaluation. The constructs were constitutively expressed in Bax$^{-/-}$ HCT-116 cells. After 24 hours, Bax C-terminal tagged EGFP was assayed by immunoblotting with an anti-EGFP antibody in both mitochondrial and cytosolic lysates. As shown in Figure 7, a heavy band of EGFP was observed in cytosolic lysates but not in mitochondrial lysates. Both EGFP-CT-WT) and EGFP-CT-LL expression displayed translocation from cytosol to mitochondria, as evidenced by the increased aggregates of EGFP in the mitochondrial extracts. EGFP-CT-EE was located in the cytosolic but

not in the mitochondrial fractions, as evidenced by the decreased aggregates of EGFP in the mitochondrial extracts. These data further confirmed that Lys 189/190 in the C-terminal of Bax are involved in Bax binding to mitochondria.

The C- terminus Lys 189/190 of Bax is critical for Bax apoptotic activity

Since Lys 189/190 mutants displayed different intracellular localization (Fig. 7), we determined whether these mutants altered the apoptotic process in the presence and absence of endogenous Bax using $Bax^{+/+}$ HCT-116 and $Bax^{-/-}$ HCT-116 cells. Apoptosis was detected by live cell imaging to examine any morphological changes over time after transfection with EGFP-tagged Bax C-terminal and its mutants. As shown in Figure 8, expression of EGFP-CT-WT in $Bax^{+/+}$ HCT-116 cells quickly caused apoptotic cell death, whereas expression of both EGFP-CT-EE and EGFP-CT-LL did not induce apoptosis within the same time period. However, expression of EGFP-CT-WT in $Bax^{-/-}$ HCT-116 cell, or either EGFP-tagged CT-EE, or LL mutants, did not cause cell death within the same time period. These results suggest that Lys 189/190 is crucial for Bax apoptotic activity and also indicates that the apoptotic activity of EGFP-tagged CT-WT might be correlated and depend upon the levels of endogenous Bax.

Expression of the C-terminal α9 helix of Bax induces the translocation of Bax to mitochondria

To further determine whether expression of the C-terminal α9 helix of Bax induces the translocation of Bax to mitochondria, we examined the effects of induction of DD-tagged full-length Bax (DD-FL-Bax) and DD-tagged C-terminal α9 helix (amino acids 173-192) (DD-CT-Bax) WT and EE, LL, RR mutations. Mitochondrial translocation of the DD-CT Bax constructs

was examined in HCT-116 cells. To induce expression, Shield 1 was added for after to cells that had been transfected with constructs for 24 hours. Induction time was 4 hours. Both the cytosolic and mitochondrial proteins were isolated from non-apoptotic HCT116 cells, and the expression of DD-tagged Bax peptides and endogenous Bax were determined by immunoblots with either anti-DD or anti-Bax antibodies. As shown in Figure 9, most of DD-tagged full-length Bax was found in cytosolic extracts, and a substantial fraction of DD-FL-Bax was also found in mitochondria extracts. Expression of DD-CT-WT Bax peptides or DD-tagged mutant peptides (EE, LL and RR) was observed all in the mitochondrial extracts; only DD-tagged LL was found a very faint band in the cytosolic extracts. More interestingly, expression of DD-tagged C-terminal Bax activated endogenous Bax, which was observed by translocation into mitochondria. These results were further confirmed by immunofluorescence, double-staining with anti-DD and anti-HSP60 (mitochondrial content) antibodies (Fig.10A). Expression of DD-CT-WT and mutants (EE, LL and RR) localized to mitochondria as evidenced by double-positive staining. There was no significant difference in the co-localization of DD-tagged peptides to mitochondria between $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells (Fig.10B). These data suggest that expression of C-terminal α9 helix of Bax alone can bind to mitochondria.

Expression of the C-terminal α9 helix of Bax induces apoptotic cell death

To determine whether the C-terminal α9 helix of Bax could mimic the apoptotic activity of the full-length Bax, expression of DD-tagged Bax-FL, the C-terminal α9 helix of Bax and its mutant peptides was induced in $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells as described in Methods. Cells were transfected for 24 hours. To image mitochondria, the cells were incubated 30 minutes with 1nM MitoTracker Red 580. Cells were imaged before adding shield 1 and 5 hours after adding

shield 1. As shown in Figure 11, expression of DD-CT-WT caused apoptotic cell death in both $Bax^{+/+}$ HCT-116 and $Bax^{-/-}$ HCT-116 cells within 5 hours. However, expression of DD-tagged CT mutant peptides, like EE and LL, RR did not cause apoptosis in either $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells within the same time period. The apoptotic activity of DD-tagged CT-WT was further examined by flow cytometry analysis. Representative cytometric analytical data are shown in Figure 11 and Table 3. Cells were examined 24 hours after addition shield 1. Parameters assessed were early apoptotic indicators (DNA fragmentation and membrane instability). From previous data (Figure 11), cells expressing high levels of DD-tagged WT peptide died within 5 hours after induction in $Bax^{+/+}$ cells. Whereas 24 hours after induction, cells, mostly expressing low levels of DD-tagged CT-WT peptide, showed less DNA fragmentation and membrane destabilization. Cells expressing heterogeneous levels of DD-tagged EE showed less DNA fragmentation and membrane instability. Cells expressing DD-tagged LL showed increased DNA fragmentation but less membrane destabilization. Cells expressing heterogeneous levels of DD-tagged RR showed both increased DNA fragmentation and membrane destabilization. These data suggest that DD-tagged CT-WT has significant cell toxicity and kills cells quickly, while DD-tagged RR has the potential to kill cells but may take longer. DD-tagged LL and EE displayed the least cell toxicity. These results showed the possible cytotoxic effects of Bax binding to mitochondria, as evidenced by immunoblots shown in Figure 9. Taken together, these data suggest that the C-terminal α9 helix of Bax may activate endogenous Bax, thereby enhancing Bax apoptotic activity in $Bax^{+/+}$ HCT-116 cells, and that Lys 189/190 is instrumental for the apoptotic activity of the C-terminal α9 helix of Bax.

Expression of the C-terminal of Bax on ATP production in HCT-116 cells

Since we had shown that expression of the full-length Bax can restore ATP production in $Bax^{-/-}$ HCT-116 cells, we examined whether the C-terminal α9 helix of Bax could mimic the activity of the full-length Bax on ATP production. After transfection of DD-tagged C-terminal α9 helix of Bax constructs for 24 hours, expression of DD-CT-WT of Bax peptides was induced by shield 1 for 0, 2, 4, 6, and 8 hours in $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells. The intracellular ATP production was examined as described in the Methods in Chapter 2. As shown in Figure 13, the basal levels of ATP in non-transfected $Bax^{+/+}$ controls is higher than that in the $Bax^{-/-}$ controls. Expression of the DD-tagged C-terminal α9 helix of Bax (WT or mutants) had no significant effects on ATP production in both $Bax^{+/+}$ HCT-116 cells and $Bax^{-/-}$ HCT-116 cells. These results suggest that the C-terminal α9 helix of Bax may be not involved in the regulation of mitochondrial bioenergetics in non-apoptotic cells.

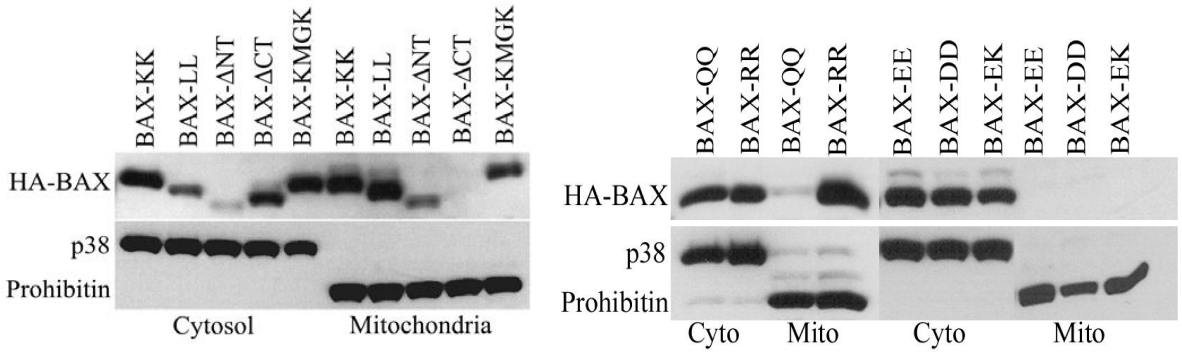

Figure 5. Mutation of Lys 189/190 at C-terminal of Bax alters intracellular localization.

Mitochondrial translocation of HA-tagged Bax mutants was examined by western blot. Bax was inducibly expressed in 293 cells using the Flp-In T-Rex expression system. After 24 hours of induction with tetracycline, cells were lysed and mitochondrial and cytosolic lysates prepared. Expression of induced HA-tagged Bax was assayed by immunoblotting with an anti-HA antibody. As controls for loading and subcellular fractionation, p38 MAPK was blotted for cytosolic content and Prohibitin was blotted for mitochondrial content. HA-tagged Bax-KK indicates HA-tagged Bax wild type. Data are representative of five independent assays.

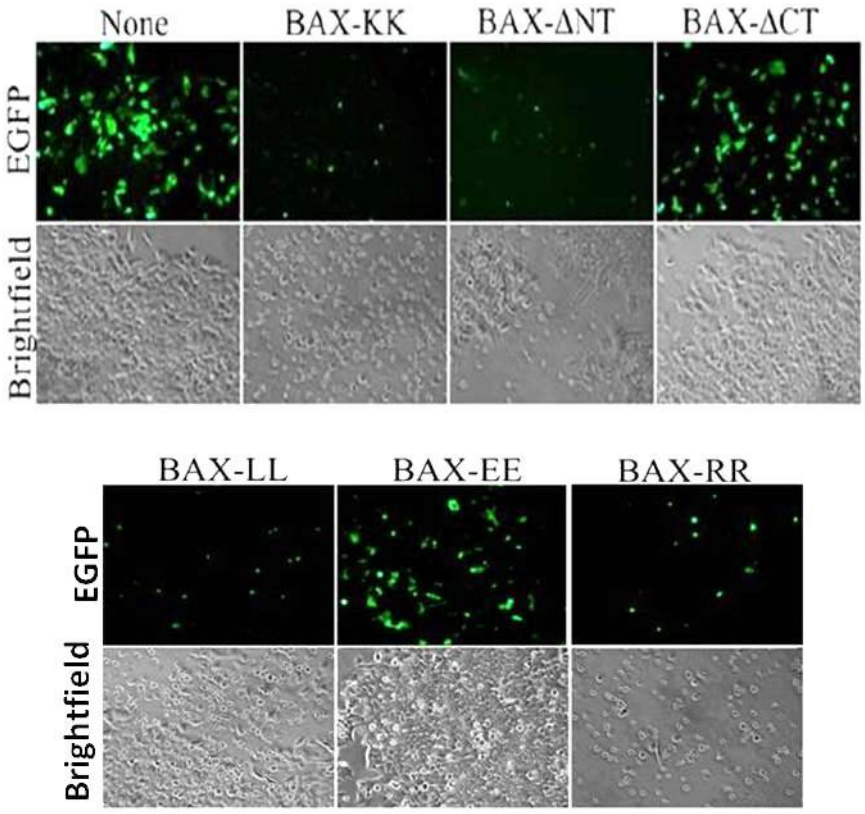

Figure 6. Mutation of Lys 189/190 at C-terminus of Bax alters apoptotic activity of Bax.

Apoptosis was assessed by loss of EGFP expressing cells. HA-tagged Bax mutants were transiently co-expressed with EGFP in $Bax^{-/-}$ HCT116 cells. After 48 hours, HCT116 cells were microscopically observed for loss of EGFP+ cells. Images were obtained using a Nikon Eclipse E1000 microscope 10X objective with a SPOT RT camera.

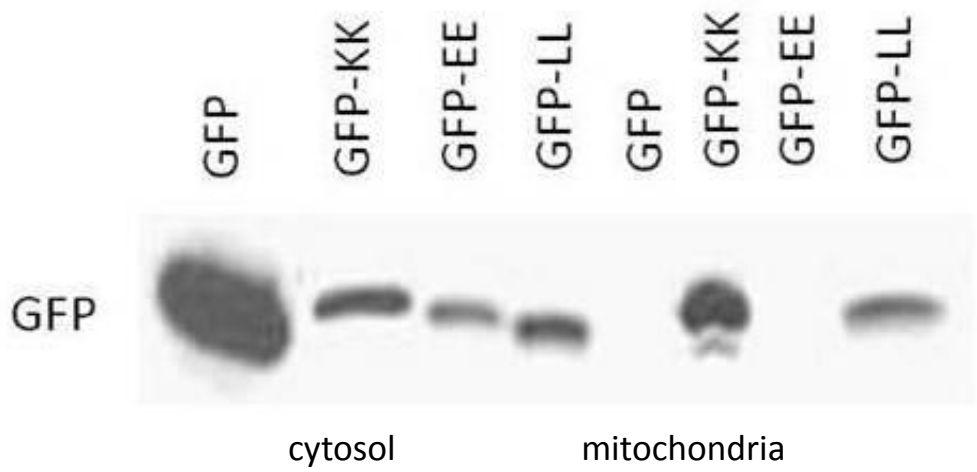

Figure 7. EGFP tagged C- terminus Lys 189/190 mutation of Bax alters intracellular localization.

Mitochondrial translocation of EGFP-tagged Bax C-terminus mutants was examined by western blot. Bax was inducibly expressed in $Bax^{-/-}$HCT116 cells using the Flp-In T-Rex expression system. After 24 hours of induction with tetracycline, cells were lysed and mitochondrial and cytosolic lysates prepared. Induced EGFP-tagged Bax peptides were probed with an anti-GFP antibody. GFP-KK indicates EGFP-tagged wild type Bax. Data are representative of five independent assays.

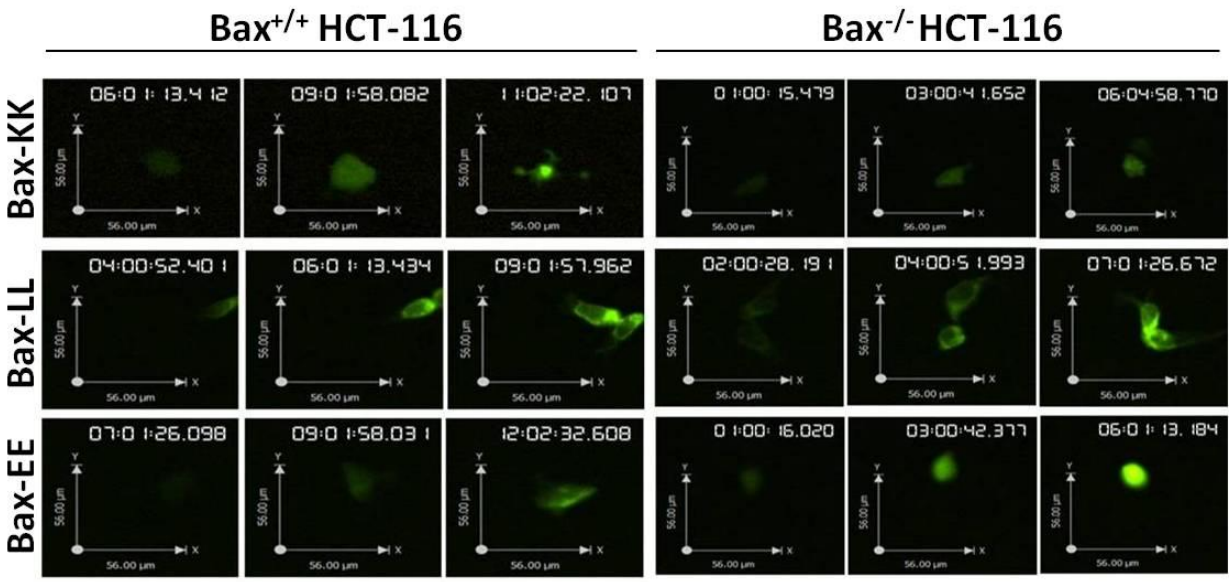

Figure 8. GFP tagged C-terminal peptide of Bax, but not mutant peptides, induced cell death in HCT-116 cells.

Cells were transfected with the GFP-tagged C-terminal wild type (KK), EE and LL mutants of Bax constructs into both $Bax^{+/+}$ and $Bax^{-/-}$ HCT116 cells using the TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's protocol. Time-lapse movies were made by the UltraView spinning disc confocal system with AxioObserver.Z1 stand, and a Plan-Apochromat 63x Oil objective.

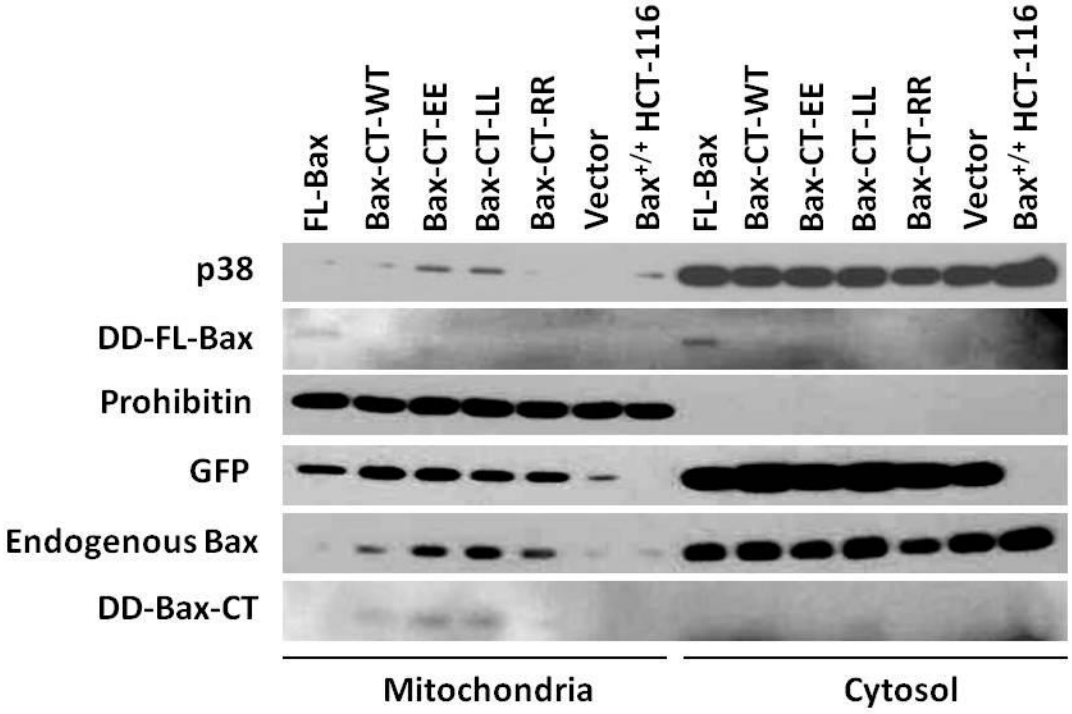

Figure 9. Intracellular localization of DD-tagged full-length and DD-tagged C-terminus Lys 189/190 mutants.

The intracellular localization of DD-tagged Bax full-length (FL-Bax) and C-terminus mutants was examined by western blot. Bax was inducibly expressed in $Bax^{+/+}$HCT-116 cells by adding the ligand Shield 1. After 4 hours of induction, cells were lysed and mitochondrial and cytosolic lysates were extracted. Expression of induced DD-tagged Bax peptides was assayed by immunoblotting with an anti-DD antibody. Endogenous Bax were probed with anti-Bax antibody. p38 MAPK and prohibitin were probed as loading controls for cytosolic and mitochondrial content, respectively. Data are representative of two independent assays.

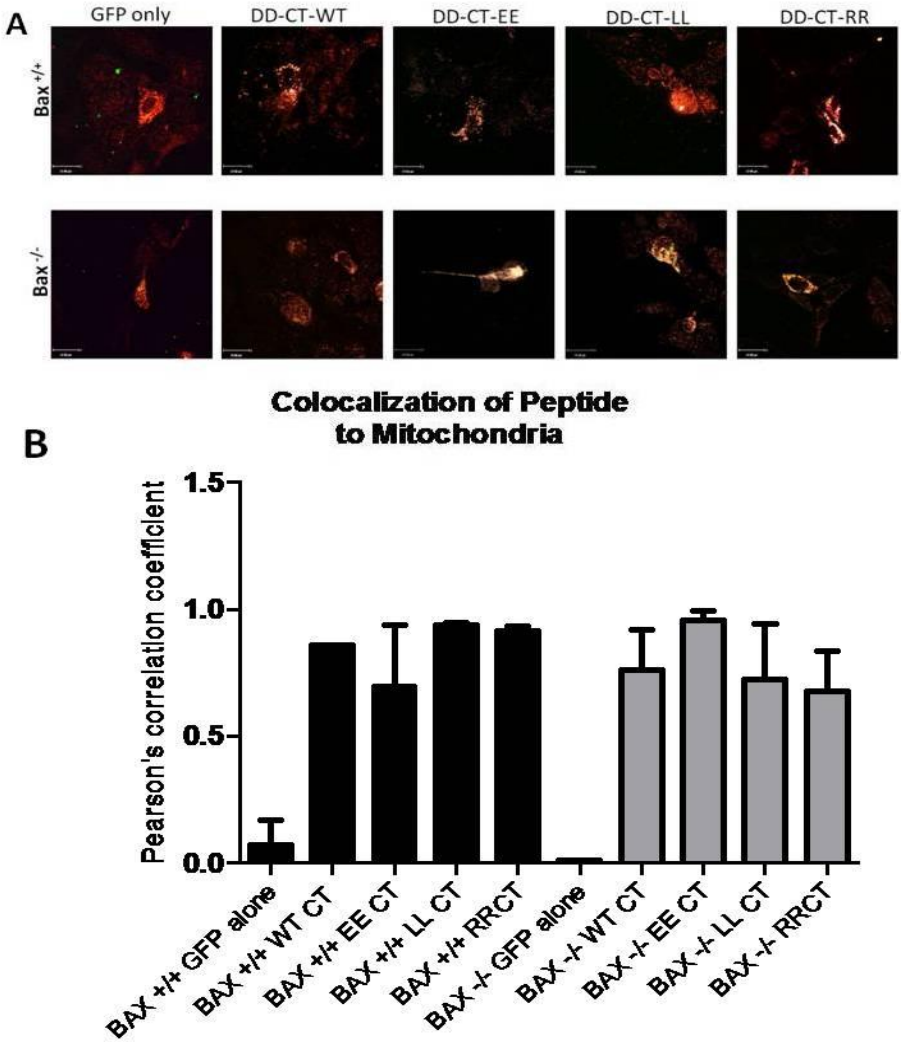

Figure 10. Detection of intracellular localization of DD-tagged C-terminus Lys 189/190 mutants by immunofluorescent staining.

$Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells were cultured for 24 hours. The DD-tagged Bax C-terminal WT peptide and DD-tagged Bax C-terminal EE, LL, RR mutants constructs were transfected into the cells for 24 hours, followed by treatment with shield 1 for 4 hours. Cells were fixed and double-stained with primary antibodies for HSP60 and DD, followed by incubated with secondary anti-rabbit-Cy3 and anti-mouse-Texas red as described in methods. Representative images are shown in (A). The bar graphs (B) indicated the quantitative assay of colocalization of DD-tagged peptides to mitochondria. The measurements are represented by three samples. Statistical analysis was performed with ANOVA and there is no statistically significant difference. (contributed by R. Boohaker)

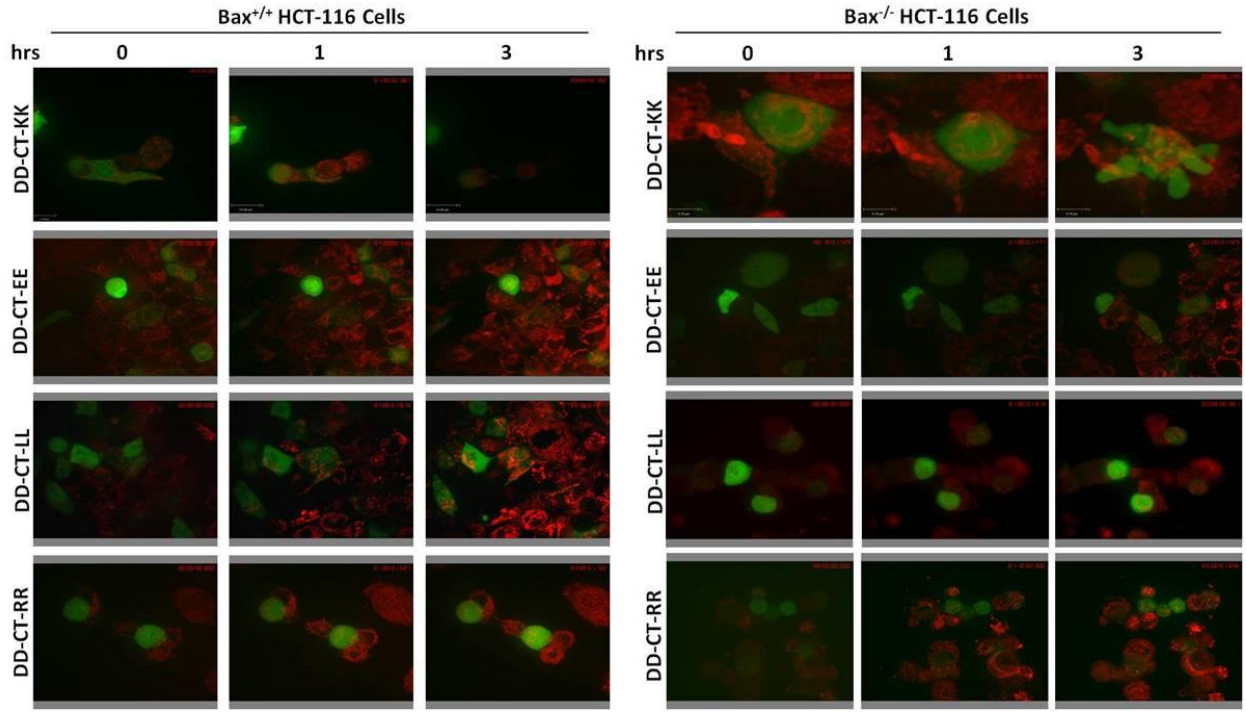

Figure 11. Expression of DD-tagged Bax C-terminal peptides induced cell death in HCT-116 cells.

DD-tagged C-terminal wild type (KK) and EE, LL, RR mutants of Bax were transfected into both $Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells using the TransIT-LT1 transfection reagent at described in the methods. After adding shield 1 to induce the Bax peptide expression, the time-lapse movies were acquired with the UltraView spinning disc confocal system over 5 hours. Snapshots were taken at indicated time points. Data are representative of four independent images.

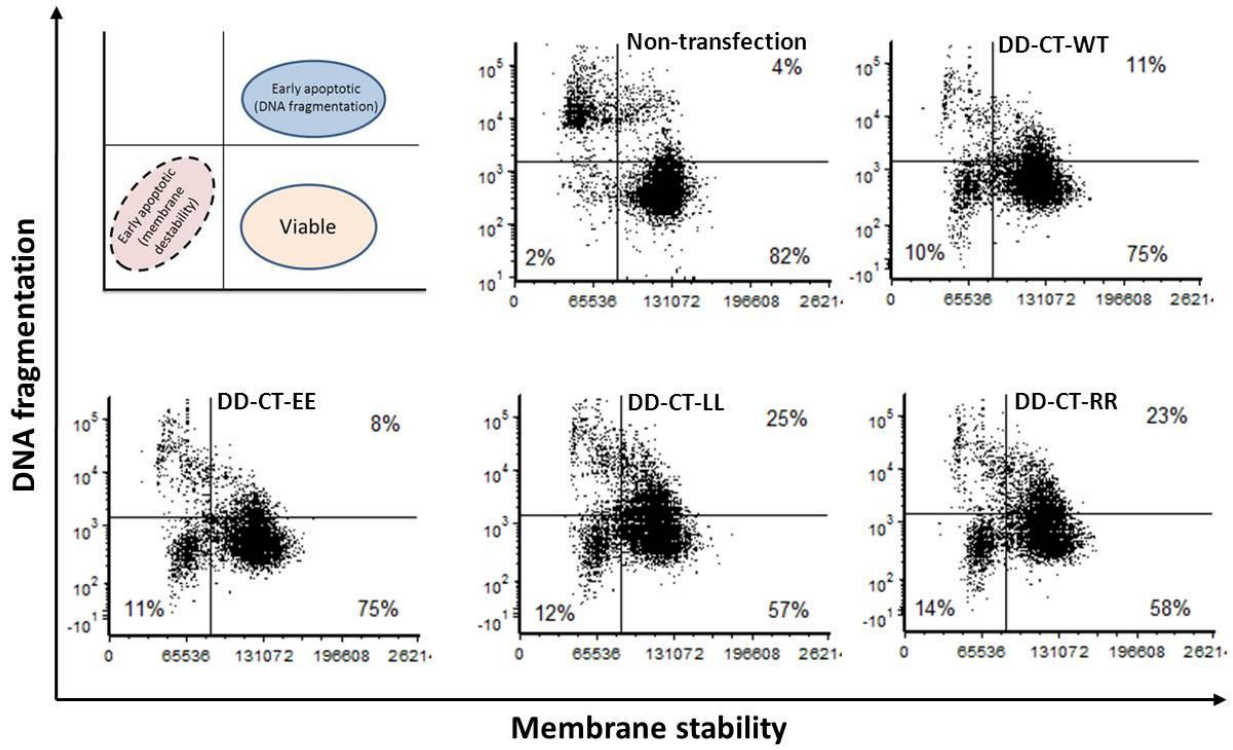

Figure 12. Detection of cell death by flow cytometry.

DD-tagged Bax C- terminus wild type (DD-CT-KK) ant its mutants (DD-CT-EE, DD-CT-LL and DD-CT-RR) were transfected into the $Bax^{+/+}$ HCT-116 cells using the TransIT-LT1 transfection reagent at described in the Methods. After induction with shield 1 for 24 hours, cells ($1x10^6$) were harvested and stained with F2N12S and SYTOX® AADvanced™ solutions as described in methods. Samples were analyzed on BD FACSAria II flow cytometer. Data are representative of two independent assays.

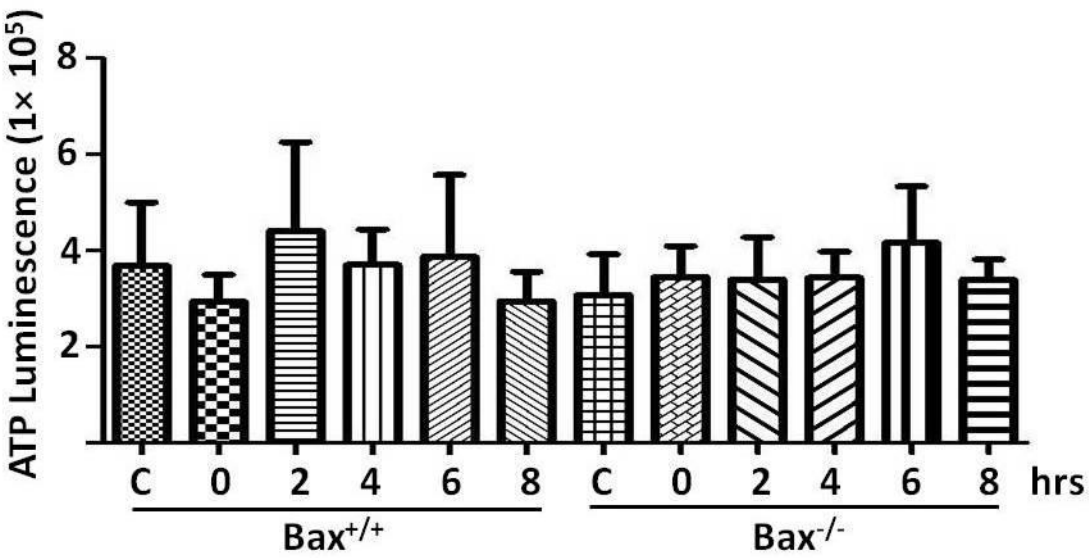

Figure 13. Expression of C-terminal Bax on ATP production in $Bax^{+/+}$and $Bax^{-/-}$ HCT-116 cells.

$Bax^{+/+}$ and $Bax^{-/-}$ HCT-116 cells were transiently transfected with DD-tagged Bax-CT-WT for 24 hrs, then cells were treated with shield 1 for 0, 2, 4, 6, 8 hours. Changes in ATP levels were measured as described in methods. There was no statistically significant difference in ATP levels at each time points.

Table 1. Lists of primer pairs

BAX mutant	Forward Primer	*Reverse Primer*
BAX-KK	5'-GGATCACTCTCGGCCTG GACACCATGGGGATGTACCCATACGATGTTCCAG ATTACGCTGACGGGTCCGGGGAGCAG-3'	5'-CGTCGACTGCAGAATTCT CAGCCCATCTTCTTCCAGATGGTGAGCGAGG-3'
BAX-ΔNT (1-19)	5'-CCGGGGAGCAGCCCCATA TGTACCCATACGATGTTCCAGATTACGCTATGAAG ACAGGGGCCCTTTTGC-3	5'-CGTCGACTGCAGAATTCT CAGCCCATCTTCTTCCAGATGGTGAGCGAGG-3'
BAX-ΔCT (173-192)	5'-GGATCACTCTCGGCCTG GACACCATGGGGATGTACCCATACGATGTTCCAG ATTACGCTGACGGGTCCGGGGAGCAG-3'	5'-CGTCGACTGCAGAATTCT CAGGTCTGCCACGTGGGCGTCCAAAG-3'
BAX-LL	5'-GGATCACTCTCGGCCTG GACACCATGGGGATGTACCCATACGATGTTCCAG ATTACGCTGACGGGTCCGGGGAGCAG-3'	5'-CGTCGACTG CAGAATTCTC AGCCCATGAGGAGCCAGATGGTGAGCGAGG-3'
BAX-DD	5'-GGATCACTCTCGGCCTG GACACCATGGGGATGTACCCATACGATGTTCCAG ATTACGCTGACGGGTCCGGGGAGCAG-3'	5'-CGTCGACTGCAGAATTCTC AGCCCATGTCGTCCCAGATGGTGAGCGAGG-3'
BAX-EE	5'-GGATCACTCTCGGCCTG GACACCATGGGGATGTACCCATACGATGTTCCAG ATTACGCTGACGGGTCCGGGGAGCAG-3'	5'-CGTCGACTGCAGAATTCTC AGCCCATCTCCTCCCAGATGGTGAGCGAGG-3'
BAX-RR	5'-GGATCACTCTCGGCCTG GACACCATGGGGATGTACCCATACGATGTTCCAG ATTACGCTGACGGGTCCGGGGAGCAG-3'	5'CGTCGATCTCAGCCCATTCGTCGCCAGATGGTG AGCGAGG-3'
BAX-QQ	5'-GGATCACTCTCGGCCTG GACACCATGGGGATGTACCCATACGATGTTCCAG ATTACGCTGACGGGTCCGGGGAGCAG-3'	5'-CGTCGACTGCAGAATTC TCAGCCCATCTGCTGCCAGATGGTGAGCGAGG-3'
BAX-KMGK	5'-GGATCACTCTCGGCCTG GACACCATGGGGATGTACCCATACGATGTTCCAG ATTACGCTGACGGGTCCGGGGAGCAG-3'	5'CGTCGACTGCAGAATTCTC ACTTCCCCATCTTCCAGATGGTGAGCGAGG-3
BAX-EK	5'-GGATCACTCTCGGCCTG GACACCATGGGGATGTACCCATACGATGTTCCAG ATTACGCTGACGGGTCCGGGGAGCAG-3'	5'-CGTCGACTGCAGAATTCTC AGCCCATCTTCTCCCAGATGGTGAGCGAGG-3'
GFP-CT-WT	5'-GGATCACTCTCGGCCTGGACGA GGATATCATGGTGAGCAAG-3'	5'-CGTCGACTGCAGATATCTCAGCCCATCTTCTTC CAGATGGTGAGCGAGGCGGTGAGCACTCCCGCCA CAAAGATGGTCACGGTGTTATCTAGATC-3'
GFP-CT-EE	5'-GGATCACTCTCGGCCTGGACGA GGATATCATGGTGAGCAAG-3'-	5'-CGTCGACTGCAGATATCTCAGCCCATCTCCTCC CAGATGGTGAGCGAGGCGGTGAGCACTCCCGCCA CAAAGATGGTCACGGTGTTATCTAGATC-3'
GFP-CT-RR	5'-GGATCACTCTCGGCCTGGACGA GGATATCATGGTGAGCAAG-3'	5'-CGTCGACTGCAGATATCTCAGCCCATGAGGAGC CAGATGGTGAGCGAGGCGGTGAGCACTCCCGCCA CAAAGATGGTCACGGTGTTATCTAGATC-3'
DD-CT-WT	5'-AATTCTGTGACCATCTTTGTGGCG GGAGTGCTCACCGCCTCGCTCACCATCTGGAAGA AGATGGGCTGA-3'	5'-GATCTCAGCCCATCTTCTTCCAGA TGGTGAGCGAGGCGGTGAGCACTCCCGCCACAAA GATGGTCACAG-3'
DD-CT-EE	5'-AATTCTGTGACCATCTTTGTGGCG GGAGTGCTCACCGCCTCGCTCACCATCTGGGAGG AGATGGGCTGA-3'	5'-GATCTCAGCCCATCTCCTCCCAGA TGGTGAGCGAGGCGGTGAGCACTCCCGCCACAAA GATGGTCACAG-3'
DD-CT-LL	5'-AATTCTGTGACCATCTTTGTGGCG GGAGTGCTCACCGCCTCGCTCACCATCTGGCTCCT CATGGGCTGA-3'	5'-GATCTCAGCCCATGAGGAGCCAGA TGGTGAGCGAGGCGGTGAGCACTCCCGCCACAAA GATGGTCACAG-3'
DD-CT-RR	5'-AATTCTGTGACCATCTTTGTGGCG GGAGTGCTCACCGCCTCGCTCACCATCTGGCGAC GAATGGGCTGA-3'	5'-GATCTCAGCCCATTCGTCGCCAGA TGGTGAGCGAGGCGGTGAGCACTCCCGCCACAAA GATGGTCACAG-3'

Table 2. Lys189/190 mutations on Bax intracellular localization

	189 190 191 192	Cellular Localization
Wild type	KKMG	cytosol
(+) to hydrophobic	LKMG	Both(m)
(+) to hydrophobic	KLMG	mitochondria
(+) to (-) charge reversal	EKMG	cytosol
(+) to (-) charge reversal	KEMG	Both (c)
(++) to hydrophobic	LLMG	mitochondria
(++) to polar	QQMG	Both (c)
(+) to (-) charge reversal	DDMG	cytosol
(+) to (-) charge reversal	EEMG	cytosol
Increased (+) charge, length	RRMG	mitochondria
Rearrangement of (++)	KMGK	both
Deletion of (+)	-KMG	both
Deletion of (+), rearrangement	-MGK	cytosol

Table 3. Effects of DD-tagged Bax-CT and its mutants on cell vability

	$Bax^{+/+}$ HCT-116				
	DD-CT-WT	DD-CT-EE	DD-CT-LL	DD-CT-RR	Non-transfection
Early apoptotic cells (DNA fragment)	11%	8%	25%	23%	4%
Early apoptotic cells (Membrane destabilization)	10%	11%	12%	14%	2%
Live cells	75%	75%	57%	58%	82%

Discussion

As an important pro-apoptotic protein, Bax is involved in the pathology of a number of diseases and is increasingly considered as a potential therapeutic target. Abounding evidence has demonstrated that translocation of Bax to the mitochondria, permeabilization of the mitochondrial outer membrane and release of cytochrome c are key events in apoptosis [1-3]. Bax is a member of the BCL-2 family of apoptotic modulators that exists either in a soluble cytoplasmic form or a membrane-bound form. Today, growing evidence indicates that upon apoptotic stimuli, Bax undergoes a conformational change, exposing its C-terminal α9 helix, and translocates to mitochondria, leading to apoptosis [8, 18-20]. It is unknown, however, whether the α9 helix of Bax is a membrane binding domain or a regulatory domain. In our previous computational mutagenesis studies, we predicted that Lys 189/190 at the C-terminal α9 helix of Bax may play a critical role in the regulation of mitochondrial membrane binding activity of Bax in response to apoptotic stimuli (Khaled A, unpublished data). In the present study, using a mutagenesis strategy we identified that positively charged lysines 189/190, located at the distal end of the C-terminal α9 helix of Bax, are critical for the apoptotic activity of Bax, and these two lysines might define how Bax interacts with mitochondrial membranes. We showed that expression of full-length Bax (Bax-FL) led to mitochondrial translocation and apoptosis, whereas deletion of the C-terminal α9 helix resulted in cytosolic retention and no apoptosis. Mutation of the two lysine residues changed how Bax bound to mitochondrial membranes. We replicated the results achieved with full-length Bax by attaching the α9 helix of Bax to EGFP or to a regulatory element, a degradation domain (DD), and induced apoptosis upon expression in cells, whereas

these effects were impaired by mutation of Lys 189/190. These results indicated that the two lysines in the α9 helix are critical for the apoptotic activity of Bax.

Bax translocation from the cytosol to the mitochondria is a critical event of apoptosis. Currently, Bax activation by direct interaction with BH3-proteins has been suggested [50]. However, this working model cannot explain how Bax moves to mitochondria in cells from mice deficient in BIM, BAD or PUMA, suggesting that translocation of Bax can occur independently of such BH3-only proteins [53]. Thus, it was recently suggested that the conformational change of Bax occurs in the cytosol leading to release of the C-terminal α9 helix from the hydrophobic grove, thereby exposing the previously hidden BH3 domain and then Bax anchors into the OMM through its N-terminus [14] or its C-terminus [19]. Our recent studies showed that the increase in intercellular pH could induce a conformation change in Bax, resulting in the C-terminal being exposed, leading to Bax mitochondria translocation [21]. In non-apoptotic cells, the C-terminal helix 9 occupies the hydrophobic groove of Bax. It is possible that the conformational change that occurs upon an apoptotic stimulus may expose the membrane binding domains, since the C-terminus has been proposed to mediate the mitochondrial insertion of Bax. In contrast, studies demonstrated that the N-terminal α1 helix domain, not the C-terminal, is essential for Bax insertion and function [14, 22, 54]. To understanding the role of the C-terminal of Bax, mutation of the C-terminal (Bax-ΔCT) was performed and we found that expression of Bax-ΔCT retained Bax in the cytosol and abolished apoptosis in $Bax^{-/-}$ HCT-116 cells. However, mutation of the N-terminal (Bax-ΔNT) caused Bax to move to mitochondria and cell death. These data suggest that the α9 helix plays an essential role in the translocation of Bax to mitochondria. The crucial role of α9 helix was investigated by analyzing different substitutions at Lys 189/190, the positive

charged residues in α9 helix. Data presented here clearly demonstrated that the C-terminal α9 helix can mediate insertion of Bax into mitochondria. We also identified Lys 189/190 as critical for the apoptotic activity of Bax. We replicated the results achieved with full-length Bax by attaching the α9 helix of Bax to EGFP or to a regulatory element, a degradation domain (DD), and induced the translocation of Bax to mitochondria and induced apoptosis upon expression in cells. Our conclusions are based on results derived from computational structural studies, targeted mutagenesis studies, and analysis through live cell imaging, flow cytometry, and immunoblotting.

Work done by others have shown that the modification of C-terminal residues by phosphorylation could be a means to regulate the mitochondrial translocation of Bax. For example mutation of serine 184 in the full length protein [56] or a C-terminal peptide [57] altered the localization and membrane-binding functions of Bax protein or peptide. Substitution of Ser184 with Asp, Gln or Lys leads to a protein exclusively cytosolic, whereas the deletion of Ser184 or substitution with Val or Ala causes to a protein exclusively localized to the mitochondria [58]. Mutation of threonine likewise did the same [55], suggesting that the C-terminus of Bax was a regulatory domain. We found that mutation of the two lysines significantly affected the apoptotic activity of the C-terminal of Bax. Mutation of these two lysines altered Bax intracellular localization. Expression of the C-terminal Bax mutants may activate the endogenous Bax by moving it to mitochondria. Recently, it was reported that the Bax C-terminus was essential for Bax translocation to mitochondria but was not the only binding signal [59]. Therefore, it will be interesting to examine how the C-terminal mutants affect the intracellular localization of endogenous Bax.

In summary, our results suggest that the C-terminal α9 helix of Bax serves as a mitochondrial membrane binding domain and that Lys 189/190 are critical for the apoptotic activity of Bax. This study also suggests that expression of the C-terminal α9 helix is sufficient to induce apoptotic cell death, indicating the C-terminal α9 helix may be developed as a novel agent for cytotoxic therapy.

CHAPTER 4: CONCLUSIONS

Apoptosis is essential for tissue development and homeostasis, and is involved in many disease pathologies. The Bcl-2 family of proteins is a major regulator of apoptosis and includes both pro- and anti-apoptotic proteins. The balance between these proteins defines whether a cell will live or die. Bax is a well-known pro-apoptotic Bcl-2 family protein which participates in the induction of apoptosis in response to a variety of apoptotic signals. Mostly Bax resides in the cytoplasm of non-apoptotic cells, and a minor fraction of Bax is associated with the mitochondrial membrane. Multiple mechanisms have been suggested to explain how Bax induces apoptosis. The translocation of Bax to mitochondria is considered as a principal trigger of apoptosis, although how Bax translocates to mitochondria remains unclear. Recently, it has been shown that Bax can affect mitochondrial reactive oxygen species (ROS) production in non-apoptotic neurons, suggesting that Bax localizes to and uses mitochondria as its major site of action, and has dual function in regulation of cell fate.

The results presented here demonstrate that Bax has a dual property of action: (1) regulation of mitochondrial bioenergetics in non-apoptotic cells, and (2) triggering apoptosis by translocation to mitochondria via its C-terminal α9 helix. Our results, however, suggest that the C-terminal α9 helix domain is not involve in or regulates mitochondrial bioenergetics, and that the Lys189/190 at the C-terminal α9 helix is the structural element responsible for the mitochondrial membrane binding activity of Bax. We further demonstrate that the C-terminal α9 helix can mimic the function of the full-length Bax, binding to mitochondrial membranes and inducing apoptosis. These results suggest two possible mechanisms to explain the apoptotic

activity of the C-terminal peptides. One mechanism could be that the peptides directly bind to mitochondria, disrupt the membrane and cause the release of cytochrome C. Alternatively, the C-terminal peptides could bind to and activate cytoplasmic Bax, which translocates to the mitochondria and causes the release of cytochrome C. Thus, development of a novel agent based on the C-terminal of Bax may overcome resistance to cancer chemotherapy and could be used as therapeutic strategy for treatment of cancer. On the other hand, Bax C-terminal mutate peptides (like Bax-CT-EE) may counteract apoptosis and protect cells from damage that leads to neurodegeneration and cardiovascular diseases.

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