

Humanin induces conformational changes in the apoptosis regulator BAX and sequesters it into fibers, preventing mitochondrial outer-membrane permeabilization

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Running title: BAX sequestration into fibers by humanin

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## ABSTRACT

The mitochondrial, or intrinsic, apoptosis pathway is regulated mainly by members of the B-cell lymphoma 2 (BCL-2) protein family. BCL-2-associated X apoptosis regulator (BAX) plays a pivotal role in the initiation of mitochondria-mediated apoptosis as one of the factors causing mitochondrial outer-membrane permeabilization (MOMP). Of current interest are endogenous BAX ligands that inhibit its MOMP activity. Mitochondrial-derived peptides (MDPs) are a recently identified class of mitochondrial retrograde signaling molecules and are reported to be potent apoptosis inhibitors. Among them, humanin (HN), has been shown to suppress apoptosis by inhibiting BAX translocation to the mitochondrial outer membrane, but the molecular mechanism of this interaction is unknown. Here, using recombinant protein expression, along with light-scattering, CD, and fluorescence spectroscopy, we report that HN and BAX can form fibers together *in vitro*. Results from negative stain EM experiments suggest that BAX undergoes secondary and tertiary structural rearrangements and incorporates into the fibers, and that its membrane-associating C-terminal helix is

important for the fibrillation process. Additionally, HN mutations known to alter its anti-apoptotic activity affect fiber morphology. Our findings reveal for the first time a potential mechanism by which BAX can be sequestered by fibril formation, which can prevent it from initiating MOMP and committing the cell to apoptosis.

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Apoptosis is a programmed process by which cells can initiate their own death when prompted by some intracellular or extracellular stimuli. The mitochondrial pathway of apoptosis, or the intrinsic pathway, is regulated primarily by members of the B-cell lymphoma 2 (BCL-2) protein family. Some of these proteins stimulate the release of apoptotic factors into the cytosol from the mitochondria by causing mitochondrial outer membrane permeabilization (MOMP).(1, 2) Expression levels and activities of BCL-2 proteins are tightly controlled to maintain cell homeostasis while also remaining primed to affect MOMP. Factors that can enhance or inhibit MOMP via interfering with BCL-2 pathologies are of interest for disease states where apoptosis is misregulated, e.g., cancers,

neurological disorders, autoimmune diseases, and cardiovascular conditions.(3)

BCL-2 family members are classified by their pro-apoptotic, anti-apoptotic, or signal sensitizing functions and share regions of sequence homology called BCL-2 homology (BH) domains. The six anti-apoptotic and three pro-apoptotic BCL-2 proteins share up to four BH domains (BH1-4) and adopt similar globular folds: seven amphipathic  $\alpha$ -helices surrounding a single hydrophobic core helix and a ninth C-terminal helix with membrane-associating functions. This all-helical structure forms a characteristic hydrophobic groove along the surface of these proteins that can interact with BH3 domains from other members of the BCL-2 family.(4) Eight BH3-only proteins are signaling effectors for initiating apoptosis and are all intrinsically disordered with the exception of BH3 interacting-domain death agonist (BID).(5)

Subsequent to their activation, two proapoptotic BCL-2 proteins, BAX and Bak, congregate at the mitochondrial outer membrane (MOM) and oligomerize, which induces MOMP.(6) Both proteins exist in equilibrium between the MOM and cytosol; however, BAX is primarily in the cytosol while Bak is localized to membranes.(7) It is well established that translocation of BAX to the MOM is a prerequisite for apoptosis initiation and it is considered to be the final, irreversible trigger for apoptosis via the mitochondrial pathway.(8) This shift is modulated in several ways. Anti-apoptotic BCL-2 proteins can interact directly with BAX, preventing it from associating with the MOM,(9) or they can retro-translocate BAX from the MOM to the cytosol in an active process.(10–12) Additionally, pro-apoptotic BH3-only proteins directly or indirectly regulate the recruitment of BAX to the MOM and membrane permeabilization activity.(13–15) These can bind with BAX in the cytosol to affect translocation or they can bind to the MOM first and then recruit BAX to it.(16)

Among the multi-BH-domain BCL-2 proteins BAX has some unique properties due to its membrane-associating C-terminal helix ( $\alpha$ 9). Mutating any of  $\alpha$ 9's residues affects membrane association and truncating it by as few as five residues abolishes MOM translocation.(17) The NMR structure of BAX shows that  $\alpha$ 9 is sequestered and folds into the BH3 binding groove

where it acts as an autoinhibitor.(18) This is a major inactive conformation of BAX; however, FRET analysis in mouse embryonic fibroblast cells demonstrates that  $\alpha$ 9 can also be extended when monomeric BAX is still in the cytosol.(19) Helix  $\alpha$ 9 is also directly implicated in BAX's oligomerization and membrane permeabilization activity. When BAX is translocated to the MOM, the entire protein adopts a completely extended conformation that facilitates intermolecular contacts between BAX proteins at two sites,  $\alpha$ 9- $\alpha$ 9 and BH3-BH3.(19) These observations suggest that BAX's uniquely regulated BH3 binding groove and C-terminal helix are of great significance for its apoptotic function.

The present work was inspired by factors that are naturally produced by the mitochondria to regulate the effects of pro-apoptotic BCL-2 oligomerization on itself. Mitochondrially-derived peptides (MDP) are short proteins encoded by alternate small open reading frames within the region of the mitochondrial genome that is transcribed for the 12S and 16S rRNA subunits of the mitochondrial ribosome.(20, 21) MDPs play an essential role in age-related diseases and have been described as cytoprotective, metaboloprotective, and apoptotic regulators.(22–25) Humanin (HN), the first described MDP,(26) has been reported to have specific interactions with cytosolic BAX, inhibiting MOM translocation and therefore acting as a potent apoptosis antagonist.(27) Guo et. al. showed that expressing HN in neurons could attenuate apoptosis induced by staurosporine. Conversely, when they knocked down HN expression, cancer cells were sensitized to BAX-induced apoptosis.(28) Furthermore, HN was shown to interfere with the oligomerization of BAX and BID, preventing their interaction and associated membrane localization.(29–31)

A description of HN's specific mechanism of action against BAX currently does not exist and the present work was conducted to elucidate the molecular interactions between HN and BAX. Here, using a combination of quantitative spectroscopic methods, negative staining electron microscopy, and proteolytic digestion we investigated fibril formation and the concomitant sequestration of structurally reorganized BAX. The fibrillation process was rapid and complex. Both molecules are required for significant aggregation to occur and mature fibrils could be isolated from

the reactions. We showed that BAX is structurally part of the fiber lattice after undergoing secondary and tertiary structure conformational changes and that a portion of the protein is protected from digestion by trypsin.

We sought to further elucidate the interactions of HN with BAX by observing the effects of BAX mutations on formation of the fibers. Two BAX variants, namely the helix  $\alpha$ 9 deletion (BAX  $\Delta$ C) and the S184V mutants, were assessed for their HN fibril formation abilities. BAX with increased helix  $\alpha$ 9 exposure from the S184V mutation formed fiber more readily than wild type. Counterintuitively, the  $\alpha$ 9 deletion mutation, exposing the major BH3-binding groove, also showed an increase in fiber formation propensity relative to wild type BAX; albeit to a lesser degree than the S184V mutant. Taken together, those results allowed us to propose a novel BAX rearrangement mechanism involving both the helix  $\alpha$ 9 and the major BH3-binding groove upon HN binding, all while exposing the N-terminus. Our finding reveals for the first time a novel mechanism for BAX inhibition by sequestration into a stable fibril structure.

## Results

### *BAX together with wild-type Humanin form fibers*

The interaction between BAX and HN was first interrogated using fluorescence spectroscopy. Scattering of the excitation beam reports on the aggregation propensity of the protein and peptide mixture, while the emission spectra provides information on potential conformational changes associated with their interaction. Addition of HN into a solution of BAX causes enhanced light scattering at a wavelength of 280 nm. (Fig. 1A) Individual datapoints are available in Table S1. It is worth noting that this scattering could be observed in a matter of seconds after the peptide was introduced to BAX. Increased scattering was not observed when HN was added into buffer alone or buffer was titrated into BAX. The scattering plot as a function of HN concentration shows significant aggregation formation between HN and BAX that continues to form beyond the equal molar ratio of the two. This hints at an aggregation mechanism that involves a single molecule of BAX and multiple HN peptides. A shift from 340 nm to 332 nm was observed in the maximum fluorescence emission spectra of BAX upon addition of HN,

indicating a conformational change in BAX. (Fig. S1)

Electron microscopy (EM) was used to probe the morphology of these aggregates directly. The aggregates were imaged as fibers and came in two major conformations: single fibers and bundles. (Fig. 1B) The latter contained multiple fibers organized in parallel and appeared to be highly ordered. These fibers were not observed on EM grids prepared from solutions containing BAX or HN alone. The fiber bundles could be separated from single fibers by centrifugation. Separation allowed imaging of the bundled fibers in much greater detail. (Fig. 1C) A statistical analysis of single fibers was performed to describe the average fiber dimensions. Images of the fibers were isolated from the background using difference of gaussians band-pass filtering,(32) and their dimensions were measured automatically using the ridge detection plugin for ImageJ/FIJI.(33) (Fig. S2) These fibers showed a relatively narrow distribution in width (Fig. S2E), while the length ranged from 10 nm to 250 nm with a maximum distribution around 40 nm (Fig. S2F). The average bundled fiber, however, is clearly much longer.

Conformational changes in BAX upon fibrillation with HN were further probed using circular dichroism (CD). (Fig. 1D) The CD spectrum of BAX alone is representative of  $\alpha$  helices with minima at 209 nm and 222 nm. A resuspension of purified fibers produces a CD spectrum with one minimum at 222 nm, characteristic of  $\beta$ -sheet structure.(34) These results demonstrate that HN induces structural rearrangements in BAX effecting a significant loss of its  $\alpha$ -helical structure and formation of  $\beta$ -sheet upon fibrillation. Additionally, the CD spectrum of HN peptide alone is characteristic of disordered protein, demonstrating that the protein and peptide are together required for  $\beta$ -sheet fibrillation. Their interaction instigates mutual conformational changes that result in fibers.

In order to investigate this phenomenon under ionizing conditions, we undertook a salt concentration study. Light scattering titrations in the presence of NaCl revealed a significant increase in the amount of scattered light over the standard reaction conditions. (Fig. S3A) Fibers were centrifuge purified as before; however, EM images of fibers purified from a no-salt reaction versus a

salt-containing reaction were quite different. The same bundled structures were observed in the reaction with no salt. (Fig. S3B) Fibers from the salt containing reaction produced only short fibers. There was no sign of the bundled structures and some fibers could be seen only loosely associating with each other. (Fig. S3C) Taken together, the observation that salt inhibits the formation of parallel bundles but also increases light scattering suggests that the BAX/HN interaction is enhanced by ionic conditions and that interactions between individual fibers might be mediated by electrostatic interactions.

#### *Fibers Structure is Altered by Functionally Important Humanin Mutants*

The fiber formation kinetics for two HN mutants were evaluated: C8A HN, showing reduced apoptotic inhibition; and S14G HN, exhibiting increased protective properties.(35) Light scattering experiments were conducted for the mutants and compared to the WT. (Fig. S4) A peptide derived from the BAX-binding domain of viral mitochondria-localized inhibitor of apoptosis (vMIA) was used as a control.(36) There was no statistically relevant variation in the titration scattering profile among the three HN peptides at these concentrations, and vMIA does not cause any increase in light scattering beyond forming a complex with BAX.

Both mutants present variation in fiber structure versus the WT. (Fig. 2A-F) The C8A mutant has a dense network with branching nodes of globular structures connecting fibers instead of the parallel bundles seen in the WT. (Fig. 2C) In addition, the fibers are non-uniform in their diameter and can appear thicker or thinner than the WT. (Fig. 2D) The S14G mutant produces similar structure to the WT, but there are fewer parallel bundles. (Fig. 2E) The fibers are also generally thicker than WT fibers. Bundled S14G fibers have a smaller diameter than fibers that are unassociated with other fibers. Single fibers could be found with diameters ranging from 15-50 nm. (Fig. 2F) No fibril structures were observed in samples containing vMIA. (Data not shown) It appears that sequence specific interactions between the peptide and BAX are required for fiber formation and their modification can alter fiber morphologies.

#### *BAX Helix $\alpha$ 9 Propagates Fibrillation*

To further investigate the role of BAX in fiber formation we used two BAX mutants, namely, BAX  $\Delta$ C and BAX S184V. In the first case, helix  $\alpha$ 9 was deleted (after residue Q171) to expose the major BH3 binding groove.(18) In the second case, a serine-to-valine substitution in position 184 (a phosphorylation site that regulates MOMP activity<sup>62</sup>) enhances helix  $\alpha$ 9 hydrophobicity, hence increasing  $\alpha$ 9 flexibility and MOM association.(17) Light scattering experiments were performed by titrations of WT HN peptide into WT BAX and each BAX mutant. Both BAX mutants featured enhanced fiber formation rates over WT BAX. (Fig. 3A) The individual datapoints for this graph are available in Tables S3A-C. BAX  $\Delta$ C only has a slight rate enhancement over WT BAX during the first part of the titration. After an excess amount of peptide has been titrated, this mixture results in comparable scattering to the WT BAX. Conversely, BAX S184V shows a significantly enhanced rate of fibrillation over the WT. It reaches an endpoint plateau early in the titrations with a scattering value close to the endpoints for the full WT and  $\Delta$ C titrations. This indicates that exposure of helix  $\alpha$ 9 can accelerate fibrillation. Since BAX  $\Delta$ C doesn't inhibit fiber formation; in fact, it is comparably better than the WT BAX, this indicates that the helix  $\alpha$ 9 is not a sole determinant for fiber formation. Exposing the BH3 groove or simply making the protein less stable overall may facilitate fiber formation.

#### *BAX is Incorporated into the Fibers*

To determine if there is a segment of BAX being incorporated into the fiber core, we performed a trypsin fragmentation assay on the fibers and with BAX alone as a control. After fibrillation was allowed to proceed for one hour, trypsin was added to the reactions and fragmentation products were analyzed after two hours of digestion. The total ion chromatogram (TIC) of a control digestion with BAX alone shows several peaks that could be assigned to six fragments in the N-terminal half of BAX. (Fig. 4A and Fig. S5) Fiber samples lose three of those six BAX fragments; the three most C-terminal of them. (Fig. 4D) A typical mass spectrum for an unprotected fragment in the BAX control sample is shown. (Fig. 4B) An additional peak (#12) appears in the fiber digestion that correlates to a fragment spanning residues 10-190 of BAX. (Fig. 4C and

4D). This is nearly the entire protein minus nine N-terminal and two C-terminal residues. Deconvoluted mass spectra for each peak showing fragment ion masses are available in Fig. S5. A summary of these data relating the observed and theoretical ions to the expected molecular weight is available in Supplementary Table 4. The full-length HN peptide and five fragments were also detected (Fig. S6). This digestion pattern implies that nearly all of BAX is protected by the fiber to some degree and that some portion of the N-terminus remains exposed. However, it isn't clear from these early digestion experiments what the exact conformation of BAX is within the fibers.

The differences in fiber morphology among HN mutants and tryptic digestion patterns of BAX mixed with HN, along with varying fibrillation kinetic profiles of BAX mutants suggest that BAX plays an integral role in formation of the fiber structure. To confirm that BAX is indeed tightly incorporated into the fibers rather than just acting as a nucleation site for HN fibrillation, a monoclonal antibody (2D2) specific to the N-terminal region (residues 3-16) of BAX and a gold-conjugated secondary antibody were used. BAX was preincubated with the primary antibody for one hour before HN was added to induce fibrillation. After an additional hour, the secondary antibody was added, and samples were incubated for one more hour. After centrifugation, EM samples were taken from the supernatant. Isolated fibers could be observed that were decorated with gold nanoparticles. (Fig. 5) These gold particles are distributed along the fibers rather than concentrated at the ends of them, indicating sequestration of BAX within the fibers rather than a nucleating event. No conclusion could be drawn based on the spatial arrangement of these gold particles, since only a fraction of BAX are labeled in this experiment. It is also interesting to note, since 2D2-bound BAX still reacts to form fiber, this confirms that the N-terminal region of BAX is exposed outside of the fiber.

## Discussion

There is a growing body of work describing small molecules that can activate BAX. Some of them target hot spots within the protein's BH3 binding sites. A screen focused on the major BH3 groove identified three small molecule BAX activators that inhibit S184 phosphorylation by Atk,

a known BAX-inactivating post-translational modification.(37, 38) Other screens have focused on the N-terminal trigger site, a small BH3-interacting pocket formed by helices  $\alpha$ 1 and  $\alpha$ 6 that has been described as a site for allosteric BAX activation and stimulating MOM recruitment.(39, 40) A lead compound was found to activate BAX-mediated MOMP and oligomerization activity.(41) Other compounds have been discovered that target the multi-site surface region proximal to the N-terminal trigger site that is ripe for allosterically regulating BAX.(42, 43) Many of these compounds' proposed interaction sites overlap with the BAX binding site for vMIA, a cytomegalovirus protein that ensures host cell survival and replication by specifically inhibiting a semi-activated form of BAX at the MOM.(36, 44) The overlap of this site with ligands that induce opposing BAX functions suggests that this multi-site surface area might be a region of structural susceptibility that is reinforced by vMIA but destabilized by BAX-activating compounds.(43)

Inhibitors of BAX MOMP activity are arguably just as important as activators but are identified less frequently. A screen using a vesicle release assay found small molecule BAX inhibitors that are believed to stabilize hydrophobic interactions of the core helix which inhibits BH3 triggering of the N-terminal site.(45) Other compounds have been identified that disrupt BAX oligomerization at the MOM and were found to protect neurons from BAX-induced apoptosis.(46) Despite confirmation of the existence of these BAX-interacting small molecules, no structures of BAX ligated with any of them have been published, presumably because of the protein's tendency to aggregate. Therefore, the molecular mechanisms of these compounds are poorly understood. Another promising avenue to study mechanisms for BAX inhibition is using peptides. The structure of BAX complexed with a peptide derived from the cytomegalovirus protein vMIA has been determined by NMR.(36) Additionally, peptides derived from Ku70, a protein involved in DNA double-strand break repair, were found to suppress BAX by inhibiting MOM translocation in a process independent of its repair functions.(47, 48)

Here we have described an unexpected and alternative regulation of BAX by fiber formation with one of its known endogenous peptide inhibitors, HN. The fibrillation process is rapid,

complex, and involves a significant structural rearrangement of BAX. BAX with increased helix  $\alpha$ 9 exposure from the S184V mutation forms fibers more readily than WT. Counterintuitively, our mutation that removes  $\alpha$ 9, thus exposing the major BH3-binding groove, also shows an increase in fiber formation propensity relative to wild type BAX; albeit less than the S184V mutant. The differential protease digestion profiles between BAX and BAX in the fiber with HN confirms the BAX conformational change associated with fiber formation as indicated by the shift in its fluorescence emission and CD spectra. We showed by EM that the morphology of the fibers formed between BAX and different HN variants are distinct. These results clearly show that the conformations of BAX as it is incorporated into these fibers are not alike.

HN is also known to be conformationally dynamic and these changes have important implications for the fibrillation process with BAX. CD and NMR spectroscopy have been used to investigate HN secondary structure propensity in various conditions. Multiple reports describe HN as generally disordered in aqueous solutions while gaining  $\alpha$ -helical structure in lipophilic environments. Conformational dynamics between the disordered and  $\alpha$ -helical states can change with HN mutations.(49–58) Some  $\beta$ -strand and  $\beta$ -sheet forming activity has also been described for HN with the C8A mutation disrupting this process, but C8A HN still forms disordered aggregations that are reversible with the monomer state.(59) WT HN and some mutant peptides substituted with D-serine at position 14 were shown to form  $\beta$ -sheet fibers as characterized by CD and ThT-binding assays.(60) Additionally, anti-parallel  $\beta$ -sheet structures have been reported when the peptide is interacting with membrane models and vesicles,(61–63) and some structures have been proposed for the interactions between HN and  $\beta$ -amyloid fibers.(64–66) HN could be modified to take advantage of its intrinsic  $\beta$ -sheet activity in a variety of ways. Mutations or incorporation of non-natural amino acids might increase fibrillation propensity with BAX, increase the peptide's half-life in the cytosol, or localize it to a specific cellular compartment, bearing in mind that these changes must not alter the inter- and intra-cellular trafficking of HN.

HN has been described interacting with BID in an  $\alpha$ -helical state by NMR. This was achieved using

a truncated mutant of the peptide that does not form aggregations when titrated with BID.(30) The mutant HN was reported to be interacting with BID with an  $\alpha$ -helical conformation in the hydrophobic groove analogous to the BH3 binding groove in BAX. This was proposed to be a potential mechanism by which HN disrupts BID-BH3 interactions with other BCL-2 proteins. Given our data concerning the kinetics of fiber formation related to  $\alpha$ 9 flexibility and BH3 groove exposure, it is likely that WT HN engages BAX in a similar manner to BID at the major BH3 groove. We further propose a hypothesis that the full-length WT HN induces a mutual conformational change between the two molecules after first engaging BAX in its BH3 binding groove, which results in the fibers described herein.

Considering these observations together, we propose a fibrillation mechanism where BAX and HN initially interact in a way that is mediated by  $\alpha$ 9 and the major BH3 groove. This interaction disrupts the hydrophobic core of BAX as it unfolds into an extended conformation that is incorporated into the growing fiber with the N-terminus of BAX exposed outside of it. We can't rule out the possibility of a molten globule transition state. Due to the rapid rate of fiber formation, however, studying these intermediate forms of the BAX and HN aggregates is quite challenging. Taking into account the CD spectrum, kinetics of aggregation, and protease protection, it appears that some portion of the BAX C-terminal region switches into  $\beta$ -strand structure and interdigitates with HN peptides in anti-parallel  $\beta$ -sheets to form the mature fibers. We further hypothesize that modulation of the anti-apoptotic activities of HN variants is related to the consequences of different BAX conformations within the fibers. One possible consequence is increasing the overall stability of BAX sequestration within the fiber which might be enhanced depending on its conformation in the fiber. The other consequence is the rate of incorporation of BAX into the fiber. For instance, the S14G mutant of HN, which has higher protective effect, either can hold BAX with much higher stability in the fiber or can incorporate a lot more BAX into the fiber, while the C8A mutant of HN, with its attenuated protective abilities, cannot sequester BAX at the same rates or as stably as the WT peptide.

In conclusion, here we show that BAX and HN can interact to form fibers. There are some hints of molecular details of this interaction such as the involvement of helix  $\alpha$ 9, BH3 groove, or the exposed N-terminal of BAX, and we propose that BAX is fully incorporated into the fiber in an extended conformation. This process represents a novel structural mechanism for BAX inhibition by sequestration into the fibril superstructure which effectively prevents BAX from being activated to initiate MOMP and apoptosis. This alternative inhibition mechanism offers a new concept for therapeutic strategies that target BCL-2 proteins and regulate apoptosis in a diverse range of diseases.

## Experimental procedures

### *BAX expression and purification*

Protein expression was performed as described previously.(67) *E. coli* cell pellets were resuspended and homogenized in TEN buffer (0.1 M Tris-Cl pH 8.0, 10 mM EDTA, and 1.0 mM NaCl) with Benzonase® nuclease (Merck KGaA, Darmstadt, Germany) and cComplete™ protease inhibitor cocktail (Roche, Basel, Switzerland). Insoluble material was pelleted by ultracentrifugation for 40 minutes at 185,500 x g and 4 °C. A 20 mL chitin column was prepared by washing the resin (New England Biolabs, Ipswich, MA, USA) with 10 column volumes (CV) of water and then equilibrating with 15 CV of TEN buffer. The supernatant was passed over the column, immobilizing BAX. Self-cleavage of the intein inserted between BAX and its chitin binding protein (CBP) fusion partner was induced by percolating a solution of TEN buffer with 50 mM DTT into the resin and allowing the resin to incubate in this solution for about 40 hours at 4 °C. After cleavage, the BAX/DTT-containing eluate was collected and buffer exchanged into 20 mM Tris, pH 8.0 using a PD-10 column (GE Healthcare, Chicago, IL, USA). The protein was further purified by ion-exchange chromatography using a 5 mL HiTrap® 5 Q HP column (GE Healthcare) with a linear anion salt gradient up to 800 mM NaCl over 20 CV. The BAX-containing fractions were pooled, concentrated, and purified by size exclusion chromatography (Sephadex G-75, GE Healthcare) using 20 mM sodium acetate pH 6.3, 150 mM NaCl, 1 mM DTT, and 0.1 mM EDTA buffer.

BAX-containing fractions were pooled, concentrated, and exchanged into storage buffer (20 mM sodium acetate, pH 6.3 and 0.1% sodium azide) by filter centrifugation. Purified protein was stored at -20 or -80 °C.

### *Engineering Expression Plasmids for BAX Mutants*

The serine in position 184 of hBAX was mutated to a valine using a QuikChange® II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara CA) and two custom primers: 5'-GGA GTG CTC ACC GCC GTG CTC ACC ATC TGG AA-3' and its reverse complement strand (Eurofins Genomics, Louisville, KY, USA). The cDNA for hBAX in a pTYB1 vector (New England Biolabs®, Ipswich, MA, USA) was used as a template. The entire coding region of the fusion protein was verified by sequencing (Macrogen USA, Rockville, MD, USA). The 1-171 hBAX mutant was produced by deletion using a Q5 Site-Directed Mutagenesis Kit (New England Biolabs®) and two custom primers 5'-CTG CCA CGT GGG CGT CCC-3' and 5'-TGC TTT GCC AAG GGT ACC AAT GTT TTA ATG G-3' (Eurofins Genomics, Louisville, KY, USA); hybridizing to the redesigned hBAX C-terminus DNA sequence and the N-terminus sequence of the intein tag, respectively. The reliability of the entire coding sequence was verified by sequencing (Macrogen USA).

### *Fiber Formation and Purification*

Solid HN peptides (Anaspec, Fremont, CA, USA) were pre-dissolved in reaction buffer (20mM sodium acetate, pH 6.3) to 1 mM stock. Fibers were produced by preparing solutions of BAX and HN in the reaction buffer. Fibers to be purified for electron microscopy were made by combining 5  $\mu$ M BAX with 50  $\mu$ M HN in the reaction buffer in a volume of 100  $\mu$ L and allowing the reaction to proceed at room temperature (RT) for 1 hour. After the incubation period, fibers were pelleted by centrifugation for 15 minutes at 21,000 x g and RT. The pellets were further purified by successive washes in 1 mL reaction buffer and then 1 mL of ultrapure water. Pelleted fibers were resuspended a final time in 100  $\mu$ L of water, and this final solution was used to prepare samples for electron microscopy.

### *Light Scattering and Fluorescence Spectroscopy*

A QuantaMaster<sup>TM</sup> 8000 Series spectrofluorometer (PTI, Birmingham, NJ, USA) with an excitation wavelength of 280nm and a vertical polarizer was used. Emission wavelengths were recorded for 340 nm at 54.70° and 280nm with a vertical polarizer. All slit widths were set to 1 mm. For emission scanning experiments, the emission wavelengths were recorded from 290 to 470 nm. For titration experiments, the number of photons at each titration point were averaged from continuous measurement over one minute at a rate of one measurement per second using the PTI FelixGX software (Horiba, Kyoto, Japan). All titrations were carried out, in triplicate, at 24 °C in quartz cells with a reaction volume of 200µL. Solutions containing 5 µM BAX were titrated with 1 mM HN up to a final HN concentration of 50 µM in a buffer of 20 mM sodium acetate pH 6.3 at 24 °C, a 5% volume increase. For the titrations with mutant proteins in Fig. 3A, each BAX stock solution was ultracentrifuged at 69,500 x g for 45 minutes to remove any potential aggregates immediately before performing titrations.

### *Circular Dichroism*

CD spectra were recorded from 200 nm to 260 nm on a Jasco J-715 spectropolarimeter (Jasco Analytical Instruments, Easton, MD, USA) equipped with a Jasco PTC-348WI temperature controller. Samples were measured in volumes of 200 µL using a 1 mm cuvette at 24 °C. Each experiment is reported as an average of 3 accumulated scans and spectra were baseline corrected by subtraction of a spectrum collected on the reaction buffer. Scans were collected in continuous mode at a rate of 50 nm per minute with 1 nm bandwidth. For this experiment a highly concentrated solution of the fibers was required to measure a spectrum with reasonable signal to noise. To achieve this fibers were purified as described in Fiber Fomation and Purification, but a fibrillation reaction with ten times the amount of starting material was used containing 50 µM of BAX and 500 µM of HN. The concentrations of control experiments with 25µM BAX or 50 µM HN only were chosen to accurately report on their CD spectra with good signal to noise. Data were plotted concurrently with two y-axes to accommodate the

ellipticity differences between the two control samples.

### *Negative Stain Electron Microscopy*

Images were collected on a JEOL JEM 1200EX transmission electron microscope (accelerating voltage 80 keV) (JEOL Ltd., Akishima, Tokyo, Japan) equipped with an AMT XR-60 digital camera (Advanced Microscopy Techniques, Woburn, MA, USA). Uranyl formate (UF) applied as a 1% solution at pH 4.5 was used as a contrasting agent. The UF solution was prepared by dissolving UF salt to 1% w/v in boiling water and then titrating in 5M NaOH in until the yellow color deepened and the final pH measured about 4.5 by litmus test. Holey Formvar EM grids (Electron Microscopy Sciences, Hatfield, PA, USA) were ionized by glow discharge (Pelco easiGlow<sup>TM</sup>, Ted Pella Inc, Redding, CA, USA) for 30 seconds and 5 µL of fiber solution, prepared as described in Fiber Formation and Purification, were allowed to adhere to the grid surface for 2 minutes. Grids were prepared with 5 µL of 1:50 or 1:100 dilutions of the purified fiber solutions. Excess liquid was removed using the side blot method, and the grids were washed by passing them through three drops of water and reblotted. 5 µL of 1% UF was applied for 2 minutes and blotted away; the staining was then repeated a second time. After the final blot the grids were allowed to dry overnight resting on filter paper in the dark.

For the secondary antibody gold-labeling experiments, BAX was diluted into the 2D2 primary antibody solution (ab77566, Abcam, Cambridge, United Kingdom) to a final concentration of 5 µM with a volume of 100 µL. The BAX/antibody interaction was then allowed to preincubate at RT for two hours with gentle agitation. 50 µM HN was added and fibrillation was allowed to proceed for one hour. The reaction was centrifuged to remove large aggregations and gelatin from the antibody solution. The 12 nm gold-conjugated secondary antibody (ab105286, Abcam) was diluted 1:5 in water and 100 µL of this was added to the primary antibody-labeled fibers. This secondary antibody labeling was allowed to proceed at RT for one hour with gentle agitation. The reaction was centrifuged a final time and dilutions of the supernatant were applied to EM grids as previously described.

### *Analysis of Fiber Dimensions*

EM images were first processed using the GDSC difference of gaussians band-pass filter plugin for ImageJ with  $\sigma_1$  and  $\sigma_2$  values of 20 and 10 pixels, respectively. This produced a black background with the fibers highlighted in white. Following this, the ridge detection plugin was used to automatically detect and measure the fibers using a  $\sigma$  value of 7 pixels. The data were manually trimmed to remove datapoints from fibers that were too overlapped to resolve their path or if they intersected with the edge of a frame. 281 fibers in total were measured to produce the distribution histograms. Sample images from the dataset are provided in Figure S2.

It is important to note that ridge detection algorithm used in the analysis consistently identified the extreme inside edge of the fibers, ignoring the dark stained area. This resulted in underestimation of the measurements by roughly 4 nm compared to measurements taken manually.

### *Trypsin Digestions*

Fibrillation reactions were prepared in a 20 mM Na-acetate buffer at pH 6.3 with 20  $\mu\text{L}$  reaction volumes using 20  $\mu\text{M}$  of BAX and 200  $\mu\text{M}$  HN. Fibrillation was allowed to proceed for 1 hour at RT with gentle agitation. After this, 1  $\mu\text{L}$  of trypsin (Trypsin Gold MS Grade, Promega, Madison, WI, USA) was added to a final

concentration of 50 ng/ $\mu\text{L}$ . Digestions were incubated for 2 hours at 37 °C in a 300-rpm shaking incubator and were quenched by addition of 2.2  $\mu\text{L}$  of 10 % TFA.

### *HPLC-Mass Spectrometry*

To create samples for peptide separation and mass determinations, 10  $\mu\text{L}$  of the quenched digestion was combined with 40  $\mu\text{L}$  of a mixture of 5% acetonitrile and 0.05% TFA in water. 5  $\mu\text{L}$  of the sample was injected into an Agilent 1260 series high pressure liquid chromatography system equipped with an autosampler maintained to 4 °C and a capillary pump system. Peptides were eluted from a reverse phase C18 HPLC column (Zorbax 300SB-C18, 1.0 x 50 mm, 3.5 $\mu\text{m}$  particle size, Agilent Technologies, Wilmington, DE, USA) with a flow rate of 20  $\mu\text{l}$  per minute at a gradient profile of 0 to 50 percent acetonitrile at 1 percent per minute. Electrospray mass spectrometry was performed on an Agilent 6530C accurate mass quadrupole-time of flight system (Agilent Technologies, Wilmington, DE, USA) equipped with double electrospray ionization (ESI) source and Q-TOF analyzer. Mass spectra were obtained at positive polarity in the range of 100-2500 m/z. Mass spectra were analyzed using the Agilent software MassHunter version B. 06. And matched to fragment digestions predicted using GPMAW (ver. 12, Lighthouse Data, Odense, Denmark).

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**Author contributions:** DLM, DWK, SJ, and NT designed and performed the experiments and analyzed the data. MPS planned and carried out mutagenesis. YH performed *E. coli* expressions while DLM purified BAX proteins. CKEB assisted with EM experiments and data collection. DYL assisted with MS experiments and data collection. DLM and NT wrote the manuscript in consultation with DWK, SJ, MPS, YH, CKEB, and DYL.

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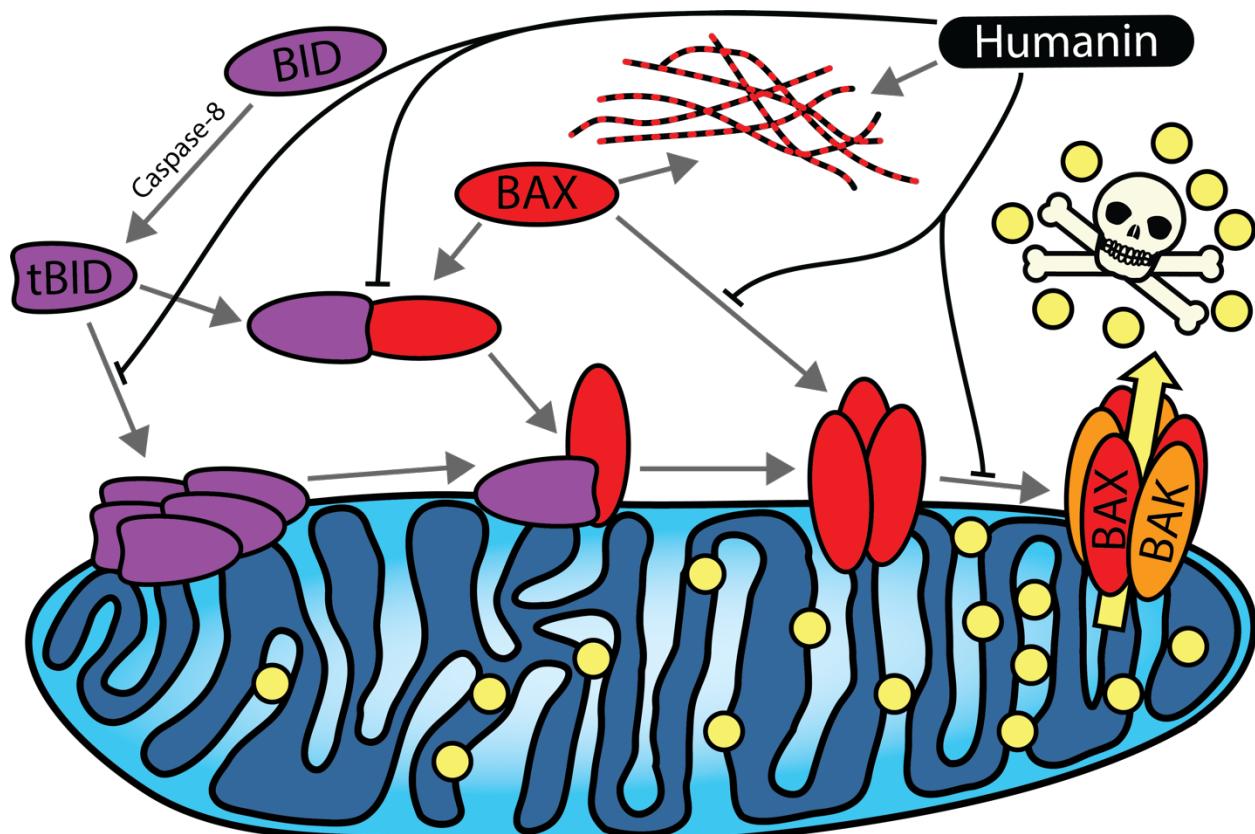
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**FOOTNOTES**

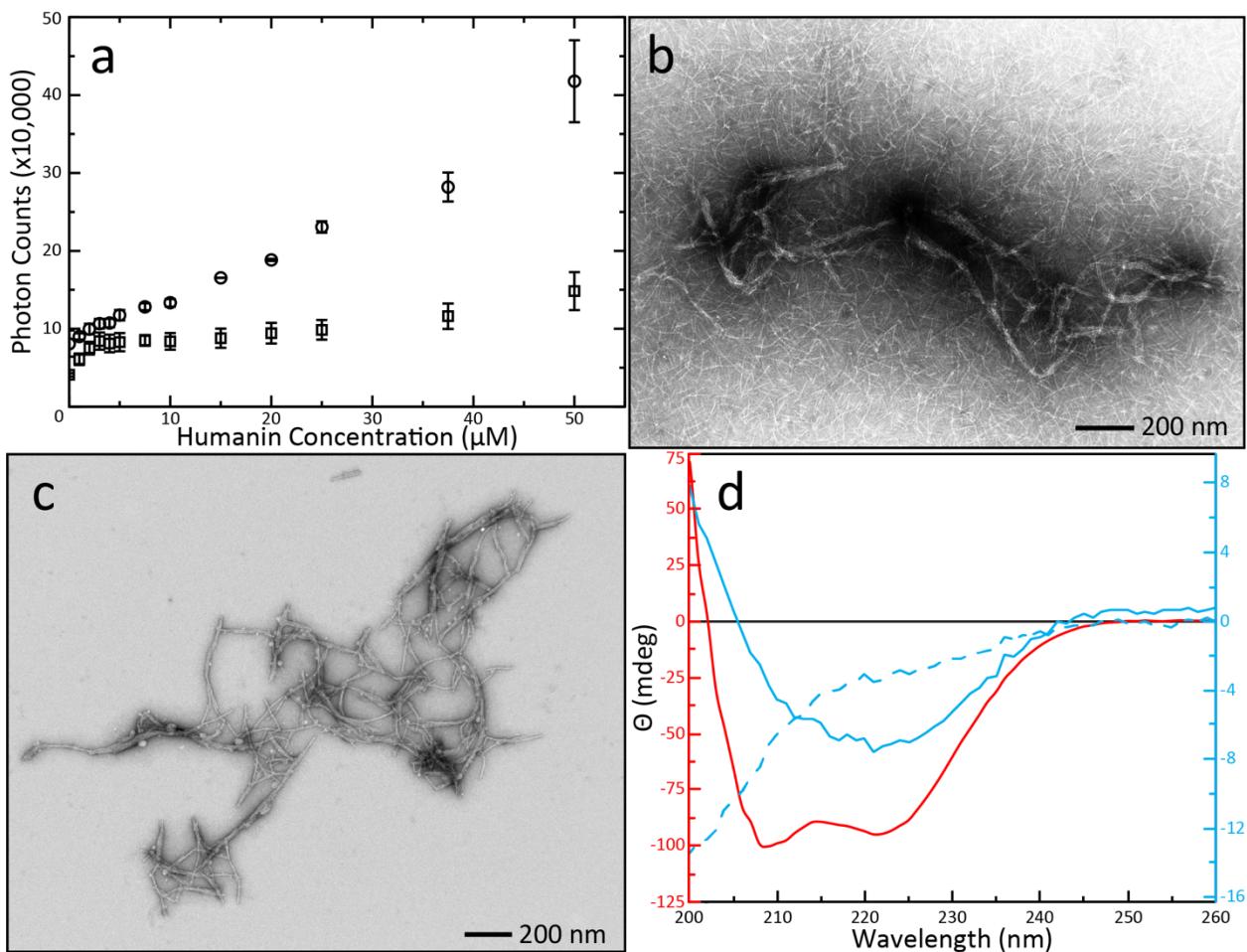
These investigations were supported by the Intramural Research Programs of the National Heart, Lung, and Blood Institute (NHLBI) of the NIH to NT.

Research Collaboratory for Structural Bioinformatics Protein Databank = PDB # 1F16

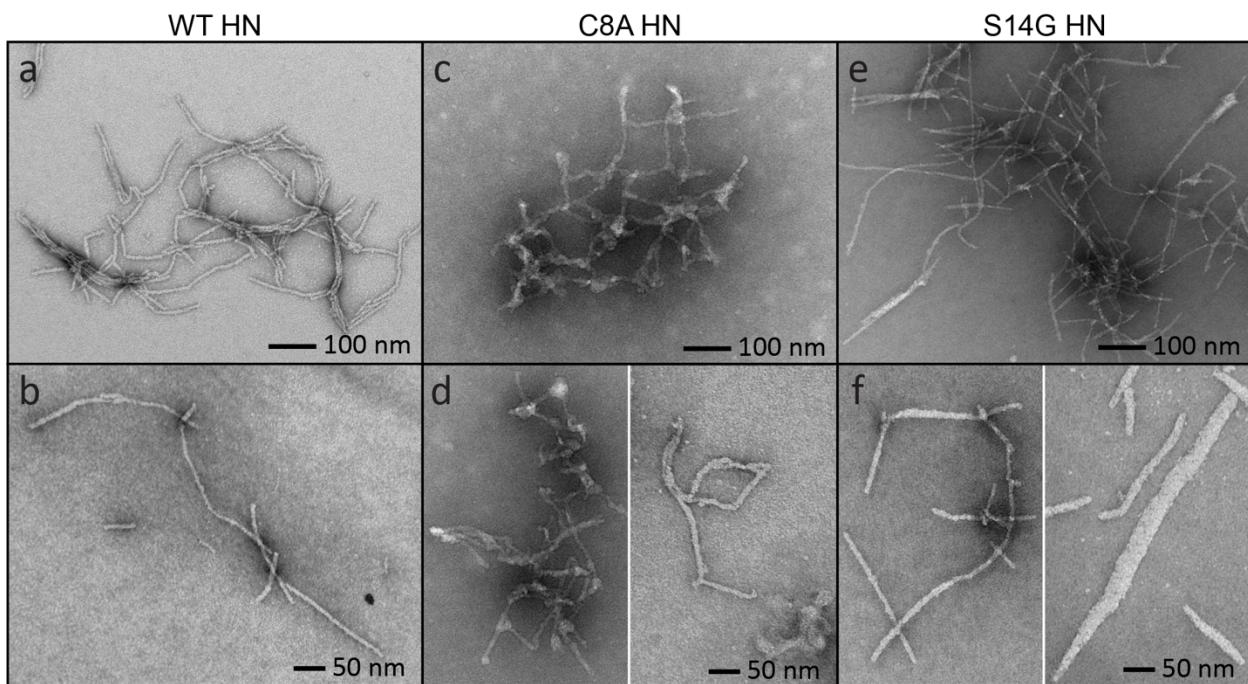
The abbreviations used are: MOMP, mitochondrial outer membrane permeabilization; MDP, mitochondrial derived peptide; HN, humanin; BCL-2, B-cell lymphoma 2; BH, BCL-2 homology; EM, electron microscopy; CD, circular dichroism; vMIA, viral mitochondria-localized inhibitor of apoptosis; TIC, total ion chromatogram



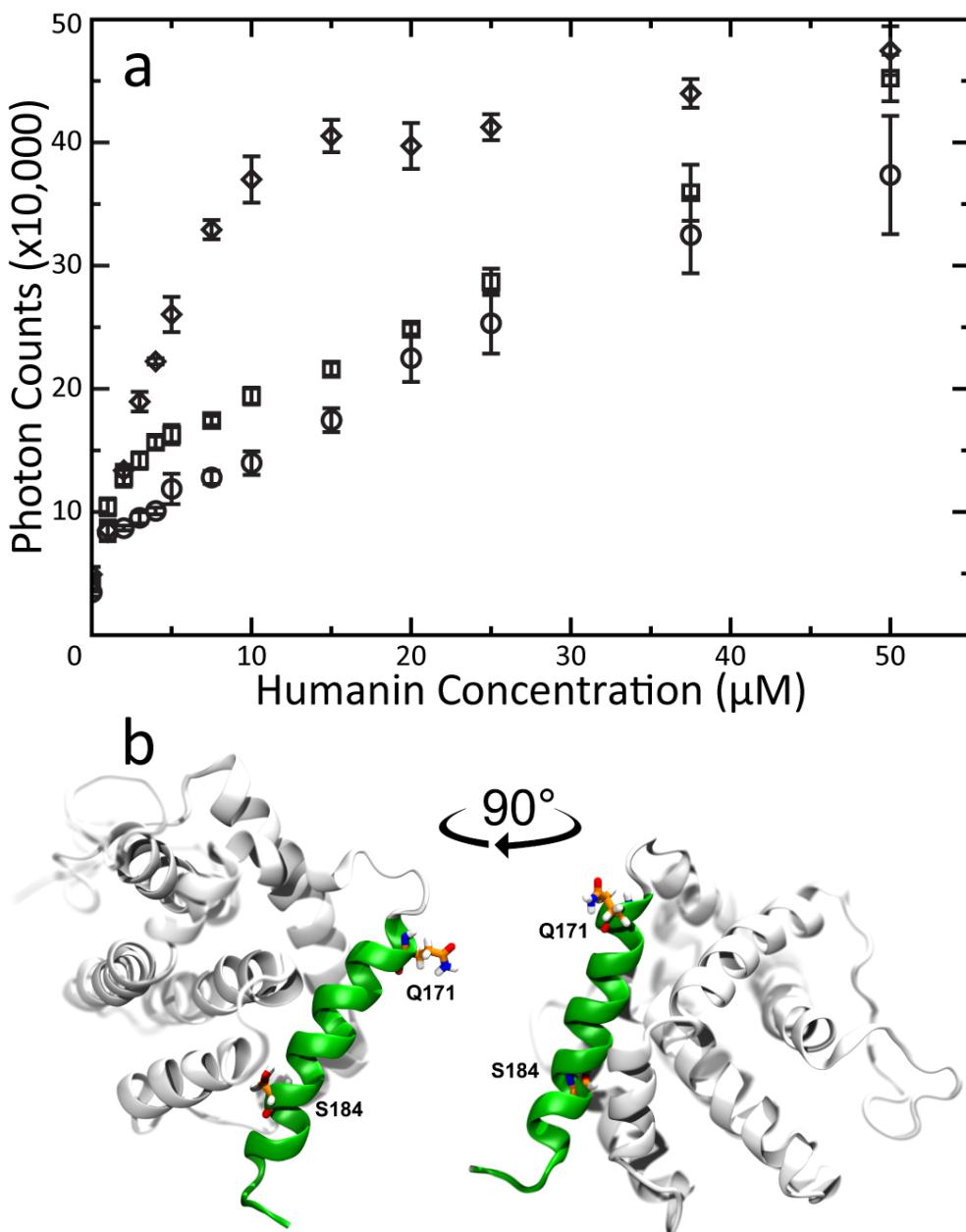
Graphical Abstract adapted from Ref #31, Figure 5



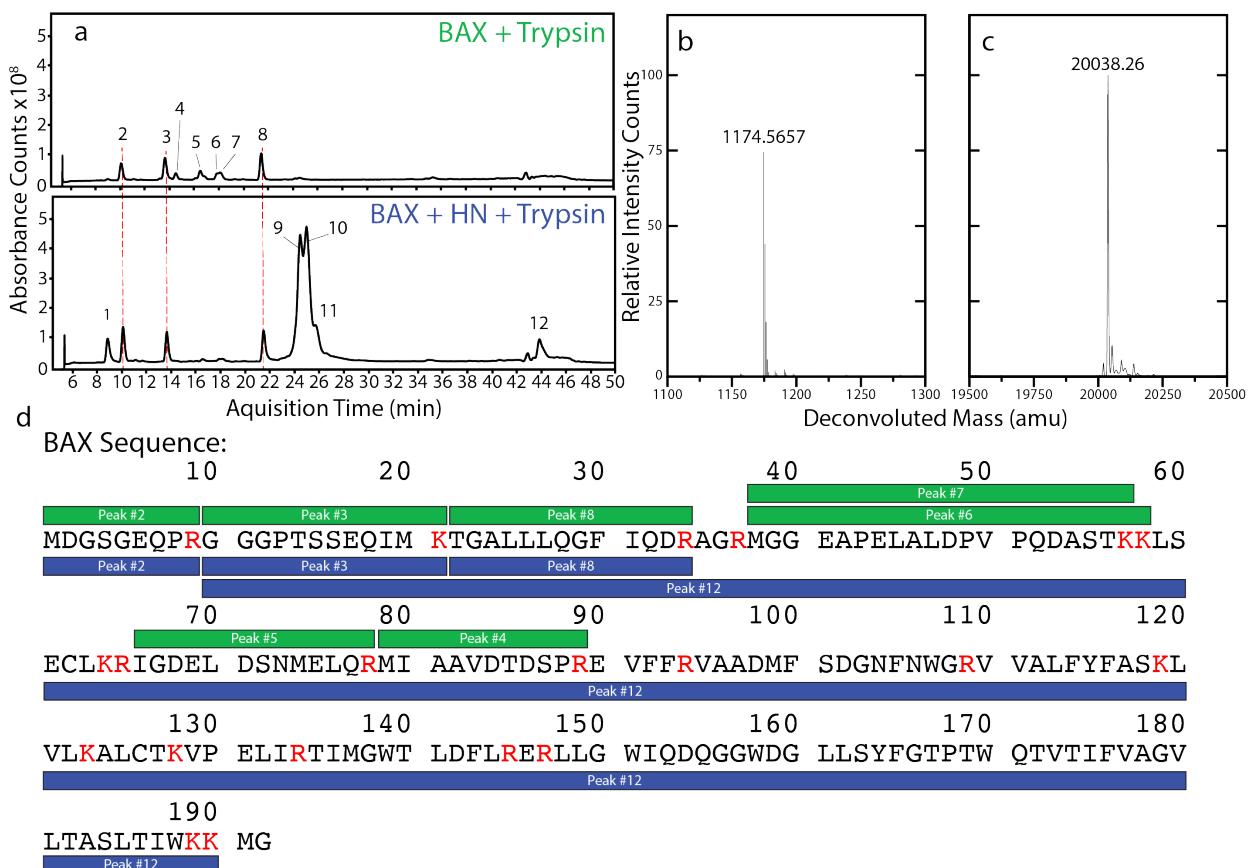
**Figure 1. Formation of HN aggregations, electron microscopy imaging of novel fibers, and secondary structure characterization** (A) Increase of scattered photons from a 280 nm laser caused by aggregations in solution as a function of HN concentration in the presence (circle) or absence (square) of 5  $\mu\text{M}$  BAX. Error bars were calculated from the standard deviation of three replicate titrations. Control titrations of buffer alone into BAX did not show increased light scattering. (B) EM images of aggregations revealed multifibrous superstructures with a background of single fibers. The contrast in this image was enhanced with 0.5% pixel saturation by histogram stretching. (C) EM of centrifuge purified fibers. The expanded view shows fibers stacked against each other forming bundles with some individual fibers associating with multiple bundles. Fibers can also appear alone or associated in non-parallel configurations but are not branching. This image is unaltered from the original data collection. (D) CD spectra of centrifuge purified fibers and control samples. Data are displayed with multiple y-axes as described in the Methods section and spectra are color-coordinated with their axis. Spectra were produced from three accumulated scans on a single sample. Fibers separated from a solution of 50  $\mu\text{M}$  BAX and 500  $\mu\text{M}$  HN (solid blue line) produced a spectrum with a single valley at 222 nm indicating the presence of predominantly anti-parallel  $\beta$ -sheets. A control spectrum of 25  $\mu\text{M}$  BAX (solid red line) yielded two minima showing the globular  $\alpha$ -helical BAX structure while the 50  $\mu\text{M}$  HN spectrum (dashed blue line) reflected disordered conformation.



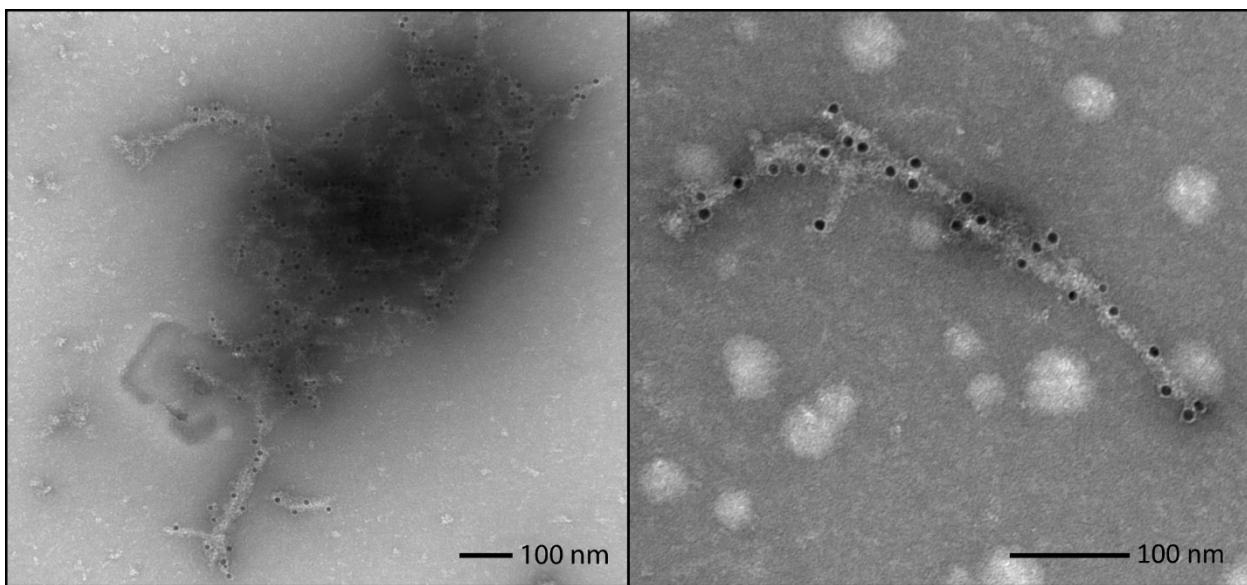
**Figure 2. Fiber formation of mutant peptides** (A) Control samples of the WT network and (B) single WT fibers are imaged as before. (C) The C8A mutant produced shorter, more irregularly shaped fibers and showed clear evidence of branching. Their diameters are not uniform, and the fibers form into a dense network. (D) The C8A mutant cannot form into single fibers. There is always some branching or fibers are attached by globular nodes. (E) The S14G mutant has similar networking properties to the WT, except there are fewer fibers running in parallel. (F) Single S14G fibers can appear as seen in the WT, but their diameters are larger on average ranging from 15 to 50 nm.



**Figure 3. Solvent-exposed  $\alpha$ 9 propagates fiber formation** (A) Light scattering titrations of HN into 5  $\mu$ M solutions of either WT (circle), BAX  $\Delta$ C (square) or S184V (diamond) mutant BAX. Error bars were calculated from the standard deviation of three replicate titrations. BAX  $\Delta$ C fibrillation has a slight rate enhancement during the sub-stoichiometric phase of the titration but eventually averages into the WT BAX curve. In contrast BAX S184V, with its flexible  $\alpha$ 9 helix, shows a greatly enhanced fiber formation rate. (B) The NMR structure of BAX (PDB 1F16) highlighting the changes made by mutations. Residues Q171 and S184 are rendered in sticks. Q171 is the final residue in the C-terminal truncated mutant. Helix  $\alpha$ 9 is rendered in green. BAX models were created using Visual Molecular Dynamics.(68)



**Figure 4. Fibers protect BAX from proteolytic digestion by trypsin** (A) Total Ion Chromatograms for BAX digested with trypsin (above) and for the fiber digested under the same conditions (below). The fibers formed protect some part of BAX from digestion as evidenced by several peaks missing with the fiber samples. (B) The deconvoluted MS for Peak #4 showing a typical mass intensity for a fragment that only appears in the control sample. (C) The deconvoluted MS for Peak #12 showing the correct mass for a segment of BAX that is incorporated into the fiber. (D) Fragments identified in BAX-only control digestions (green) and digestions with fibers (blue) overlaid with the sequence of WT BAX. Residues labeled red precede trypsin cleavage sites. In the control digestions the N-terminal half of BAX can be detected in 6 fragments. When a fiber sample is digested, the 3 fragments closer to the C-terminal end of the protein are no longer observed and a new peak appears corresponding a fragment that is nearly the full length of the protein.



**Figure 5. BAX-specific antibody labeling of fibers** Antibody labeling of Bax/HN fibers with a BAX-specific antibody (2D2) targeted to the protein's N-terminus. Secondary antibodies conjugated to 12 nm gold particles indicate points along the fibers where Bax has been integrated into them. EM images illustrate examples of BAX sequestration in networked bundles (left) and single fibers (right). These pictures were unaltered from the original data collection.

**Humanin induces conformational changes in the apoptosis regulator BAX and sequesters it into fibers, preventing mitochondrial outer-membrane permeabilization**

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