Supporting Information

Materials and methods

Generation of caspase-6 variants

A synthetic *E. coli* codon-optimized His-tagged casp-6 gene coding for amino acids 24-293 (ΔN D179A) in pET11a vector (Stratagene) produced for earlier studies (*1*) was used for generating the variants. The mutations E53K, E63V were introduced in the caspase-6 ΔN D179A gene construct by Quikchange site-directed mutagenesis method (Stratgene). The E63V mutation was introduced into E53K ΔN D179A construct to make the E53K/E63V double mutant. The A132G / E135P (C7HB) and A132G / K133P (C3HB) variants were generated by simultaneously introducing, A132G, E135P and A132G, K133P mutations into the ΔN D179A construct.

Expression and purification

The caspase-6 variants in pET11a vectors were transformed into the BL21(DE3) T7 express strain of *E. coli* (NEB). The cultures were grown in 2xYT media with Amp (100 mg/L, Sigma-Aldrich) at 37°C until they reached OD_{600} = 0.6. The temperature was reduced to 20°C and cells were induced with 1 mM IPTG (Anatrace) to express soluble His-tagged protein. Cells were harvested after 18 hr for all other variants to ensure complete processing. Cell pellets stored at -20°C were freeze-thawed and lysed in a microfluidizer (Microfluidics, Inc.) in 300 mM NaCl, 2 mM imidazole, 5% glycerol, 50 mM Tris pH 8.6. Lysed cells were centrifuged at 17K rpm to remove cellular debris. The filtered supernatant was loaded onto a 5 ml HiTrap Ni-affinity column (GE Healthcare). The column was washed with 300 mM NaCl, 50 mM imidazole, 5% glycerol, 50 mM Tris pH 8.6 and the protein was eluted with 300 mM NaCl, 250 mM imidazole, 5% glycerol, 50 mM Tris pH 8.6. The eluted fraction was diluted by 5-fold into 2 mM DTT, 20 mM Tris pH 8.6 buffer to reduce the salt concentration. This protein sample was loaded onto a 5 ml Macro-Prep High Q column (Bio-Rad Laboratories, Inc.). The column was developed with a linear NaCl gradient and eluted in 120 mM NaCl, 2 mM DTT, and 20 mM Tris pH 8.6 buffer. The eluted protein was stored in -80°C in the above buffer conditions. The purity of the caspase-6 variants was analyzed by SDS-PAGE.

Activity Assays

For kinetic measurements of caspase activity, 100 nM freshly purified protein (within hours of purification and without ever being frozen, to prevent changes in cleavage pattern or activity) was assayed over the course of 7 minutes in a caspase-6 activity buffer containing 100 mM HEPES pH 7.5, 10% sucrose, 0.1% CHAPS, 5 mM DTT and 30 mM NaCl (*2*). For substrate titrations, a range of 0-500 μM fluorogenic substrate, VEID-AMC, (*N*-acetyl-Val-Glu-Ile-Asp-(7-amino-4-methylcoumarin), Enzo Lifesciences) Ex365/Em495, was added to initiate the reaction. Assays were performed in duplicates at 37°C in 100 μL volumes in 96-well microplate format using a Molecular Devices Spectramax M5 spectrophotometer. Initial velocities versus substrate concentration were fit to a rectangular hyperbola using GraphPad Prism (Graphpad Software) to determine kinetic parameters K_m and k_{cat}. For some of the mutants we observed an approximately 2-fold change in K_M. This modest change in K_M may be due to the contribution of the change in the equilibrium of the strand versus helical states. Enzyme concentrations were determined by active site titration with quantitative, inhibitor VEID-CHO (*N*-Acetyl-Val-Glu-Ile-Asp-Aldehyde, Enzo Lifesciences). Active site titrations were incubated over a period of 2 hours in 120 mM NaCl, 2mM DTT, 20 mM Tris pH 8.5 at nanomolar concentrations. Optimal labeling was observed when protein was added to VEID-CHO solvated in dimethylsulfoxide in 96-well V-bottom plates, sealed with tape, and incubated at room temperature in a final volume of 200 μL. 90 μL aliquots were transferred to black-well plates in duplicate, and assayed with 50 fold molar excess of substrate. The protein concentration was determined to be the lowest concentration at which full inhibition was observed.

Secondary structure prediction

Sequences of wild-type and variants ranging from residue 120-141 which include the 130's region and the loop above 130's region were analyzed for secondary structure using the expasy server using the Hierarchical Neural Network method (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_nn.html).

Secondary structure and stability measurements by circular dichroism

Active-site liganded caspase-6 variants were prepared by 2-hour room temperature incubation with 3 molar equivalents of VEID-CHO (*N*-Acetyl-Val-Glu-Ile-Asp-aldehyde, Enzo Lifesciences) at a concentration of 6-12 μM in 120 mM NaCl, 2mM DTT, 20 mM Tris, pH 8.6

buffer. Caspase-7, -3 samples were prepared in a similar fashion by incubating with DEVD-CHO (*N*-Acetyl-Asp-Glu-Val-Asp-aldehyde, Enzo Lifesciences). To ensure complete binding of active-site ligand to the protein, the protein was assayed with 50 molar excess of substrate VEID-AMC for caspase-6 and DEVD-AMC (*N*-acetyl- Asp-Glu-Val-Asp -AMC (7-amino-4-methylcoumarin)) for caspase-7, -3. A 95-97 % inhibition was observed indicating that the protein was fully occupied with the substrate mimic. Liganded and apo proteins were then buffer exchanged 6 times (7-fold dilution each time) into 10 mM Phosphate buffer, pH 7.5 with 120 mM NaCl using Vivaspin 500, 3K MWCO membrane concentrators (Sartorium Stedim Biotech) for repeated dilution and buffer exchange. After buffer exchange the final concentration of DTT was ~ 10 nM. A protein concentration of ~6 μM for caspase-6, -7 and ~4 μM for caspase-3 as assessed by absorbance at 280 nm (Nanodrop 2000C spectrophotometer) were used for analysis. A similar procedure was followed to prepare sample for apo protein without the active-site ligand.

Thermal denaturation of caspase-6 variants was monitored by loss of circular dichroism signal at 222 nm over a range of 12-90°C and CD spectra (250-190 nm) were measured on a J-720 circular dichroism spectrometer (Jasco) with a peltier controller. The temperature was increased at a rate of 1° per minute. We found that his rate of temperature increase is appropriate for this analysis as we have found that 1 minute is sufficient to fully unfold caspase-6 in 8 M urea (data not shown). The CD spectra ranging from 190 - 270nm were collected for the apo and ligand-bound forms before and after thermal denaturation. All data were collected in duplicates on separate days. The thermal denaturation data was fit using Origin Software (OriginLab) using sigmoid fit to determine the melting temperature.

Figures

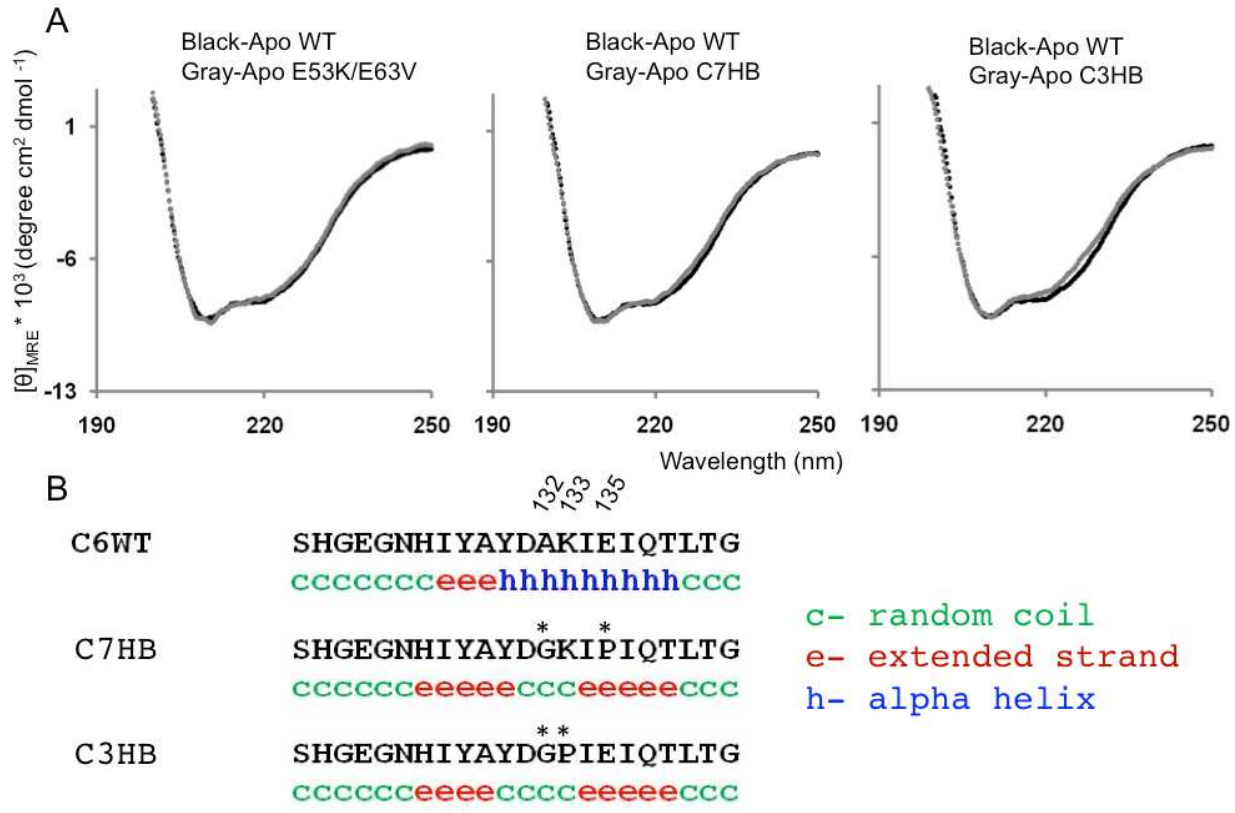

Figure S1. A. Overlay of CD spectra of wild-type (WT) caspase-6 against caspase-6 network-disrupting and helix-breaking variants. B. Secondary structure prediction of the 130's region in wild-type, C7HB and C3HB helix-breaking variants.

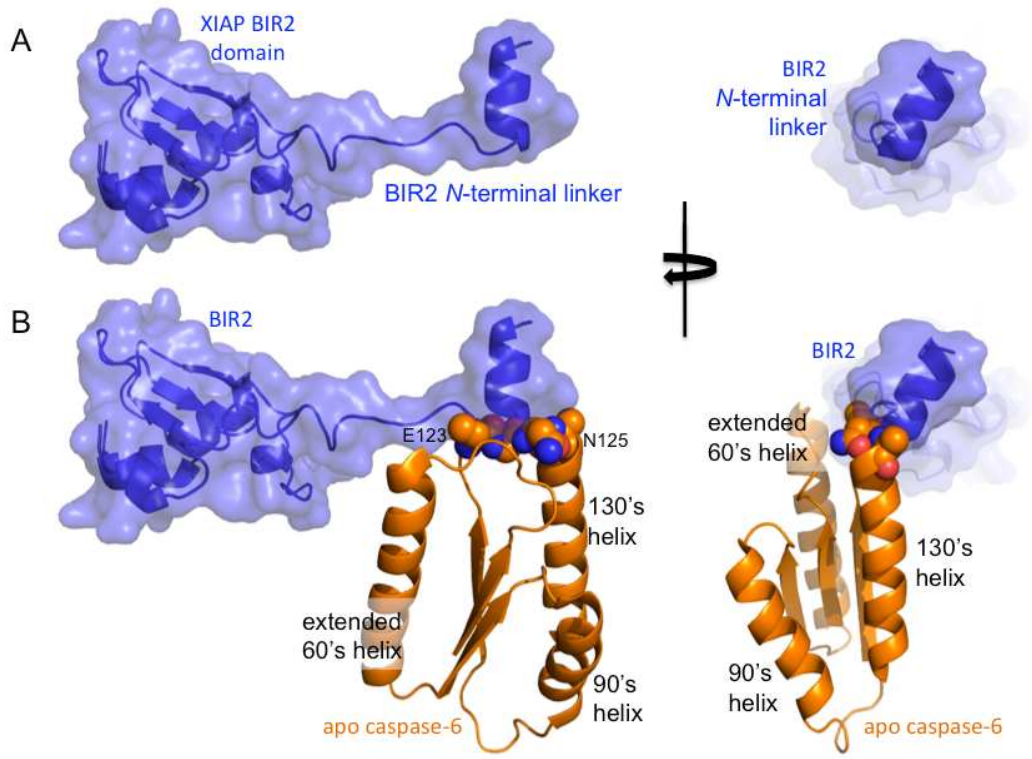

Figure S2. Mature caspase-6 (extended helical conformation) is incompatible with binding of the Inhibitor of Apoptosis Protein (IAP) family. A. The substrate binding grooves of executioner caspases such as caspase-3 and -7 bind to the X-linked Inhibitor of Apoptosis Protein (XIAP, blue) by recognition of the *N*-terminal linker in the BIR2 domain (*3*). B. Superposition of caspase-6 (orange, PDB ID 3K7E) with caspase-3 bound to XIAP (blue, PDB ID 1I03) enabled us to build a meaningful model of the potential interaction of caspase-6 with XIAP. Caspase-3 and parts of caspase-6 have been excluded from the figure for clarity. This model demonstrates that the extended helical conformation of caspase-6 is incompatible with binding to XIAP due to steric clashes with the *N*-terminal linker of BIR2 and caspase-6 residues 123-125 at the top of the 130's helix. This model suggests a mechanism by which the IAP family of apoptosis inhibitors selectively inhibit caspase-3 and-7 but not caspase-6.

References:

1. Vaidya, S., Velazquez-Delgado, E. M., Abbruzzese, G., and Hardy, J. A. (2010) Substrate-Induced Conformational Changes Occur in All Cleaved Forms of Caspase-6, *J Mol Biol*.
2. Stennicke, H. R., and Salvesen, G. S. (1997) Biochemical Characteristics of Caspases-3,-6,-7, and-8, *Journal of Biological Chemistry 272*, 25719-25723.
3. Riedl, S. J., Renatus, M., Schwarzenbacher, R., Zhou, Q., Sun, C., Fesik, S. W., Liddington, R. C., and Salvesen, G. S. (2001) Structural basis for the inhibition of caspase-3 by XIAP, *Cell 104*, 791-800.