

Expanded View Figures

Figure EV1. Sequence and *in vitro* MOMP activity of Bax mutants.

- A Bax sequence is shown with BH motifs highlighted by dashed lines above and helices identified by arrows below. The native cysteines (closed circle-indicated Cs) were changed to alanine to create the cysteine-null mutant. Single-cysteine Bax mutants were created from the cysteine-null mutant by individually replacing the red-colored residues with cysteine. Triangles indicate Gly¹⁰⁸, Gly¹⁷⁹, Thr¹⁸², and Ser¹⁸⁴ of Bax that were changed to glutamate, isoleucine, isoleucine, and valine in the G108E, G179I, T182I, and S184V mutants, respectively. The GxxxA (GVLTA) motif in $\alpha 9$ is in bold font.
- B The cytochrome c release from the Bax^{-/-}/Bak^{-/-} mitochondria-only sample (Mito only), and from the mitochondrial plus purified recombinant tBid protein (+tBid), the TNT reaction programmed by pSPUTK vector (+Vector) or wild-type Bax gene inserted after SP6 promoter in the vector (+Bax WT), or their combinations was measured in *n* independent replicates as indicated. The raw data shown as open bars are the means with the standard deviations (s.d.). A background release ~20% was observed in the mitochondria-only sample, which might be due to the mitochondria that were frozen and thawed once before used in the assay according to an established protocol (Yamaguchi *et al*, 2007). Addition of bacterial expressed and purified tBid protein increased the release slightly. The TNT mixture containing the vector plasmid, or the WT Bax plasmid that produced the WT Bax protein (see Appendix Fig S1) also increased the release slightly. While addition of both tBid protein and the vector-containing TNT mixture showed a marginally additive release, addition of both tBid protein and the WT Bax protein-producing TNT mixture resulted in a synergistic increase in cytochrome c release. After the “raw” release of “+Vector” control was subtracted from the “raw” releases of “+Vector, +tBid”, “+Bax WT”, and “+Bax WT, +tBid” samples, which all contained the vector (with or without the WT Bax coding region) and the TNT mixture, the “corrected” cytochrome releases were obtained and shown as hatched bars for the means \pm s.d.
- C Cytochrome c release by the wild-type (WT), cysteine-null (CO), or the indicated single-, double-, or triple-cysteine Bax mutants synthesized in the TNT reaction in the absence or presence of the tBid protein was measured in two independent replicates. The raw data were corrected as described in (B). The corrected data are shown as the dots with the lines for the averages. The amount of Bax protein that was bound to the mitochondria in each cytochrome c release assay above was determined and the results are shown in Appendix Fig S1.

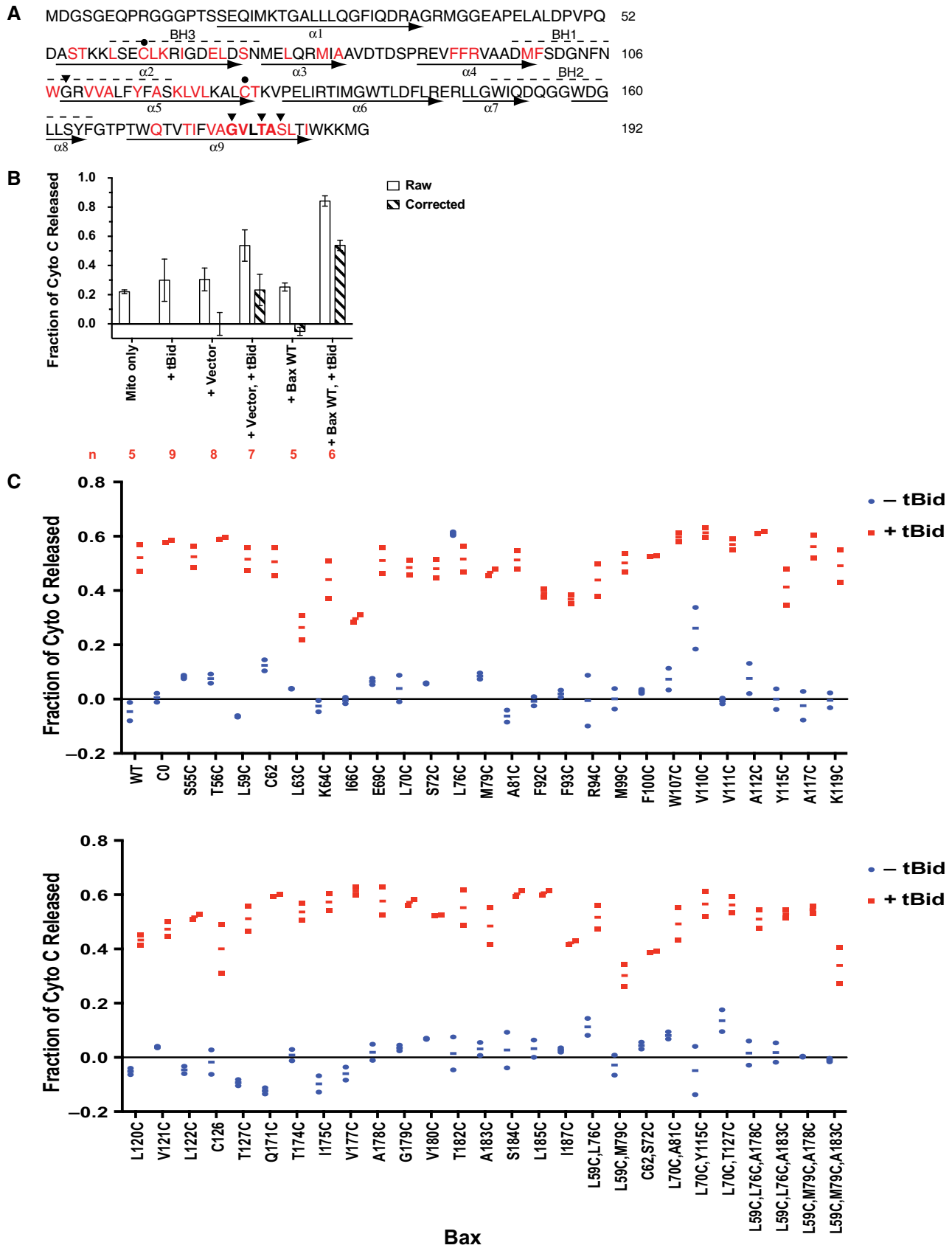


Figure EV1.

Figure EV2. Intracellular localization and apoptotic activity of Bax mutants.

Bax/Bak DKO BMK cells were transfected with either Venus or N-terminal Venus-Bax fusion constructs.

- A Expression of Venus-Bax fusion proteins. The average Venus intensity per cell was determined as an estimate of relative Bax expression.
- B Cell death induced by Venus-Bax constructs. The cells were treated with or without STS, imaged, and analyzed. Only Venus-positive cells were used to calculate the percent cell death using an automated image analysis routine. Small rounded up TMRE-negative cells were automatically classified as dead.
- C Colocalization of Venus-Bax constructs with mitochondria. Representative fluorescence images of cells show the localization of Venus-Bax proteins expressed in cells and were compared to the localization of Mitrotracker Red. Scale bar represents 10 μm .
- D Quantification of mitochondrial localization of Venus-Bax constructs. Pearson's correlation coefficient (PCC) was calculated using the Venus and Mitrotracker channels for each Venus-positive cell and averaged. Images of cells expressing Venus only were used as a control representing the PCC for a diffuse localization, and cells stained with Mitotracker Green were used as a control representing the PCC for the highest attainable mitochondrial colocalization.

Data information: In panels (A, B, D), bar graphs represent averages of three or more independent experiments, \pm s.d. For each independent experiment, a minimum of two wells were imaged with 15 images collected for each well. For (A, B), 150 or greater Venus-positive cells were analyzed. For (D), a minimum of 30 Venus-positive cells were analyzed.

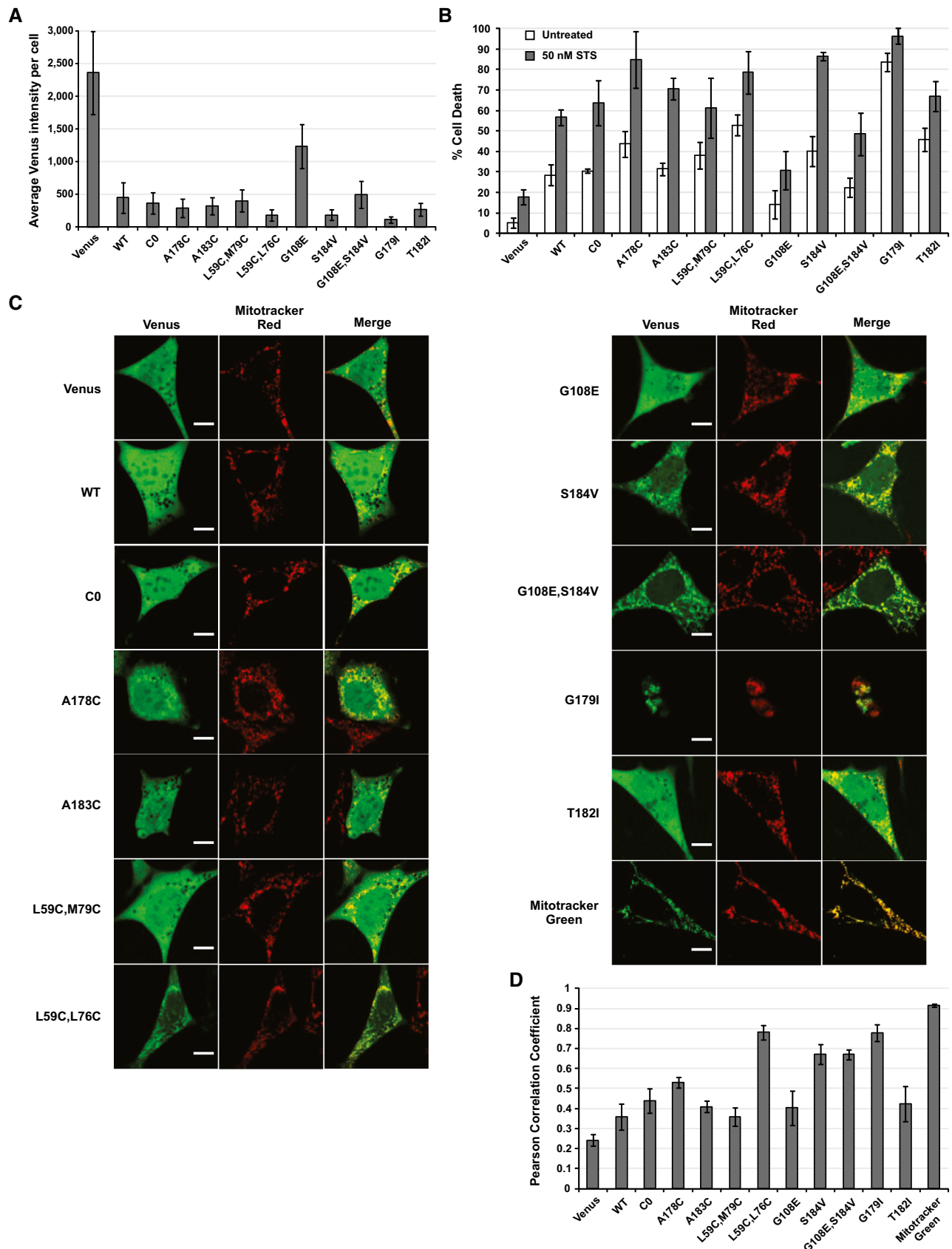


Figure EV2.

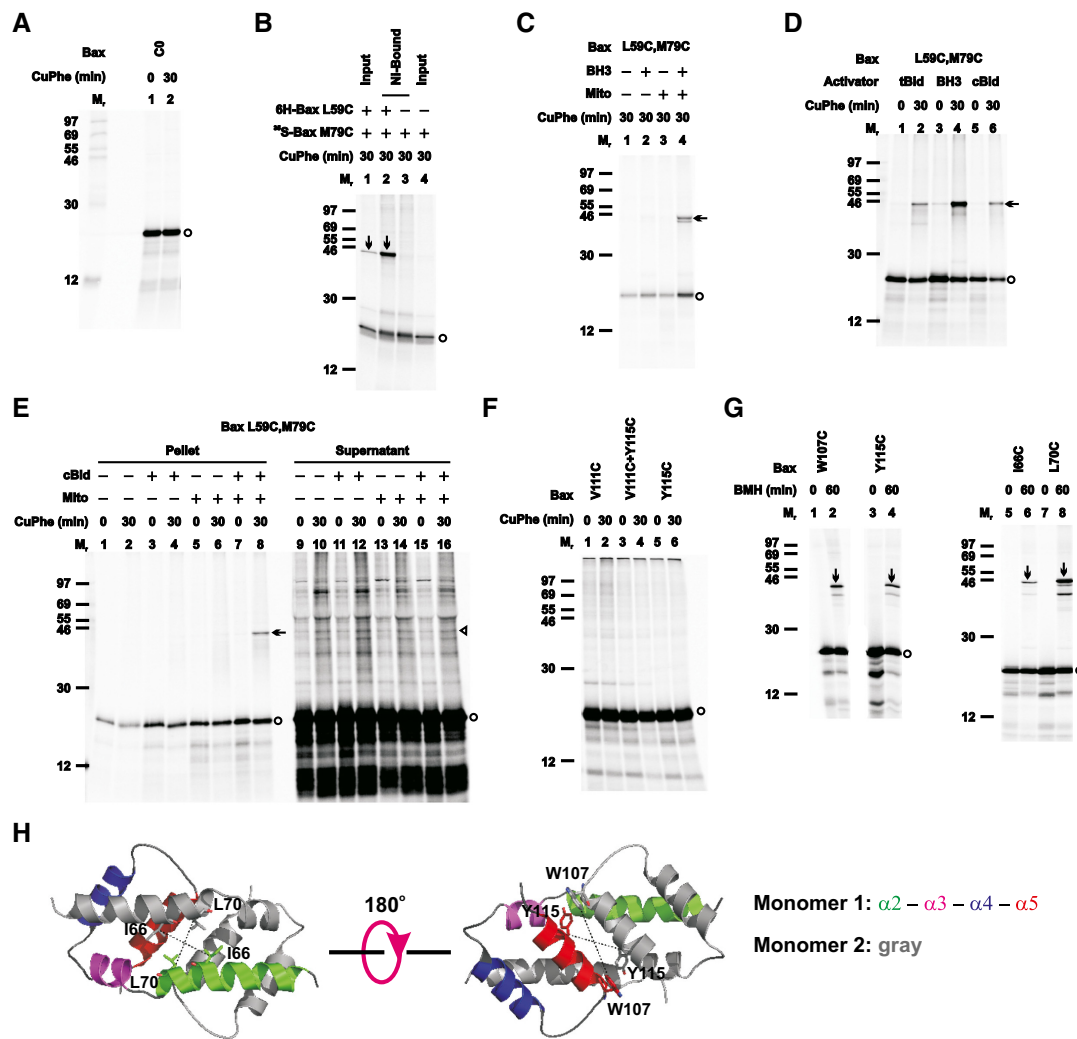


Figure EV3. Control disulfide-crosslinking data for the BH3-in-groove dimer interface, related to Fig 1.

A The oxidized mitochondria with radioactive cysteine-null Bax protein (C0) were prepared and analyzed as in Fig 1B.

B The *in vitro* synthesized [³⁵S]Met-labeled single-cysteine Bax protein (³⁵S-Bax M79C) was activated and targeted to the Bax^{-/-}/Bak^{-/-} mitochondria in the absence or presence of the *in vitro* synthesized non-radioactive hexahistidine-tagged single-cysteine Bax protein (6H-Bax L59C). The resulting mitochondria were oxidized, and an aliquot (4 μl) was saved as input. The remaining sample (56 μl) was solubilized with buffer A (the high salt buffer plus 1% Triton X-100 and 2.5 mM imidazole), and incubated with 15 μl of 50% Ni-NTA agarose suspension. After washing the beads with the buffer A and phosphate-buffered saline (pH 7.5), the Ni-bound proteins were eluted with the non-reducing SDS sample buffer plus the quenching buffer, and analyzed alongside with the input, as in Fig 1B.

C The radioactive double-cysteine Bax protein was incubated in the absence or presence of Bax BH3 peptide (BH3), the Bax^{-/-}/Bak^{-/-} mitochondria (mito), or both. The “mitochondrial” fraction was isolated, oxidized, and analyzed as in Fig 1B.

D The radioactive double-cysteine Bax protein was activated by Bax BH3 peptide (BH3), tBid or cBid protein, targeted to the mitochondria, oxidized, and analyzed as in Fig 1B.

E The radioactive double-cysteine Bax protein was incubated in the absence or presence of cBid protein, the Bax^{-/-}/Bak^{-/-} mitochondria (mito), or both. The “mitochondrial” pellet was separated from the “post-mitochondrial” supernatant. Both fractions were oxidized and analyzed as in Fig 1B.

F The oxidized mitochondria with the radioactive single-cysteine Bax protein pair were prepared and analyzed as in Fig 1B.

G The mitochondria with the radioactive single-cysteine Bax proteins were prepared as in Fig 1B, and crosslinked by 100 μM bismaleimidoethane (BMH). After 60 min, the reactions were stopped by the addition of 50 mM mercaptoethanol. The products were analyzed by reducing SDS-PAGE and phosphorimaging. For the “0 min” control, mercaptoethanol was added before the addition of BMH.

H The structure of the BH3-in-groove dimer is shown with the residue pairs replaced with cysteine pairs in (G) presented in stick form, and their β-carbon atoms linked by dashed lines with the distances ranging from 8.5 to 18.1 Å.

Data information: In (A–G), the protein standards are indicated on the side of phosphor images by their M_r, the Bax monomers by open circles, the disulfide- or BMH-linked Bax dimers by arrows, and non-disulfide-linked products in the supernatant of (E) that have the same M_r as the disulfide-linked Bax dimer in the pellet are indicated by open triangle. n = 2.

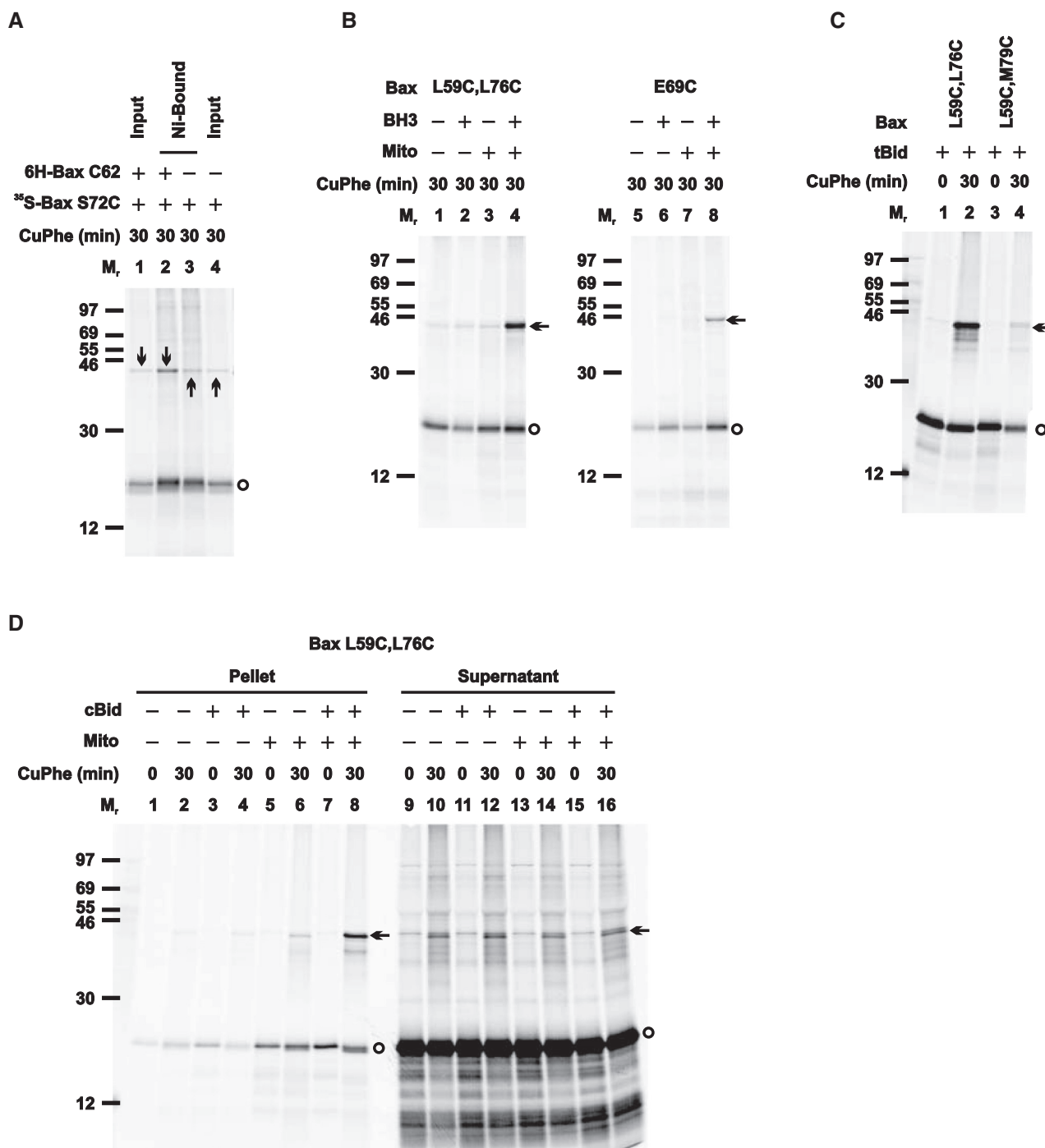


Figure EV4. Control disulfide-crosslinking data for the helices $\alpha 2$ - $\alpha 3$ - $\alpha 4$ dimer interface, related to Fig 3.

A The Ni-bound proteins from the oxidized mitochondria containing the non-radioactive 6H-Bax C62 and/or the radioactive ³⁵S-Bax S72C were prepared and analyzed alongside with the inputs, as in Fig EV3B.

B The radioactive single- or double-cysteine Bax protein was incubated in the absence or presence of Bax BH3 peptide, the Bax^{-/-}/Bak^{-/-} mitochondria, or both. The "mitochondrial" fraction was isolated, oxidized, and analyzed as in Fig 1B.

C The radioactive double-cysteine Bax protein was activated by tBid protein, targeted to the mitochondria, oxidized and analyzed as in Fig 1B.

D The radioactive double-cysteine Bax protein was incubated in the absence or presence of cBid protein, the Bax^{-/-}/Bak^{-/-} mitochondria (mito), or both. The "mitochondrial" pellet was separated from the "post-mitochondrial" supernatant. Both fractions were oxidized and analyzed as in Fig 1B.

Data information: In (A–D), the protein standards are indicated on the side of phosphor images by their M_r, the Bax monomers by open circles, and the disulfide-linked Bax dimers by arrows. *n* = 2.

Figure EV5. Control disulfide-crosslinking data for the helix $\alpha 9$ dimer interface, related to Fig 6; and for the G179I mutants, related to Fig 10.

- A The Ni-bound proteins from the oxidized mitochondria containing the non-radioactive 6H-tagged and/or the [35 S]Met-labeled single-cysteine Bax protein were prepared and analyzed alongside with the inputs, as in Fig EV3B.
- B The radioactive single-cysteine Bax protein was incubated in the absence or presence of Bax BH3 peptide, the Bax $^{-/-}$ /Bak $^{-/-}$ mitochondria, or both. The “mitochondrial” fraction was isolated, oxidized, and analyzed as in Fig 1B.
- C The radioactive single-cysteine Bax protein was activated by tBid protein, targeted to the mitochondria, oxidized, and analyzed as in Fig 1B.
- D, E The radioactive single-cysteine Bax protein (D) or double-cysteine Bax protein with G179I mutation (E) was incubated in the absence or presence of cBid protein, the Bax $^{-/-}$ /Bak $^{-/-}$ mitochondria (mito), or both. The “mitochondrial” pellet was separated from the “post-mitochondrial” supernatant. Both fractions were oxidized and analyzed as in Fig 1B.

Data information: In (A–E), the protein standards are indicated on the side of phosphor images by their M_r , the Bax monomers by open circles, and the disulfide-linked Bax dimers by arrows. $n = 2$.

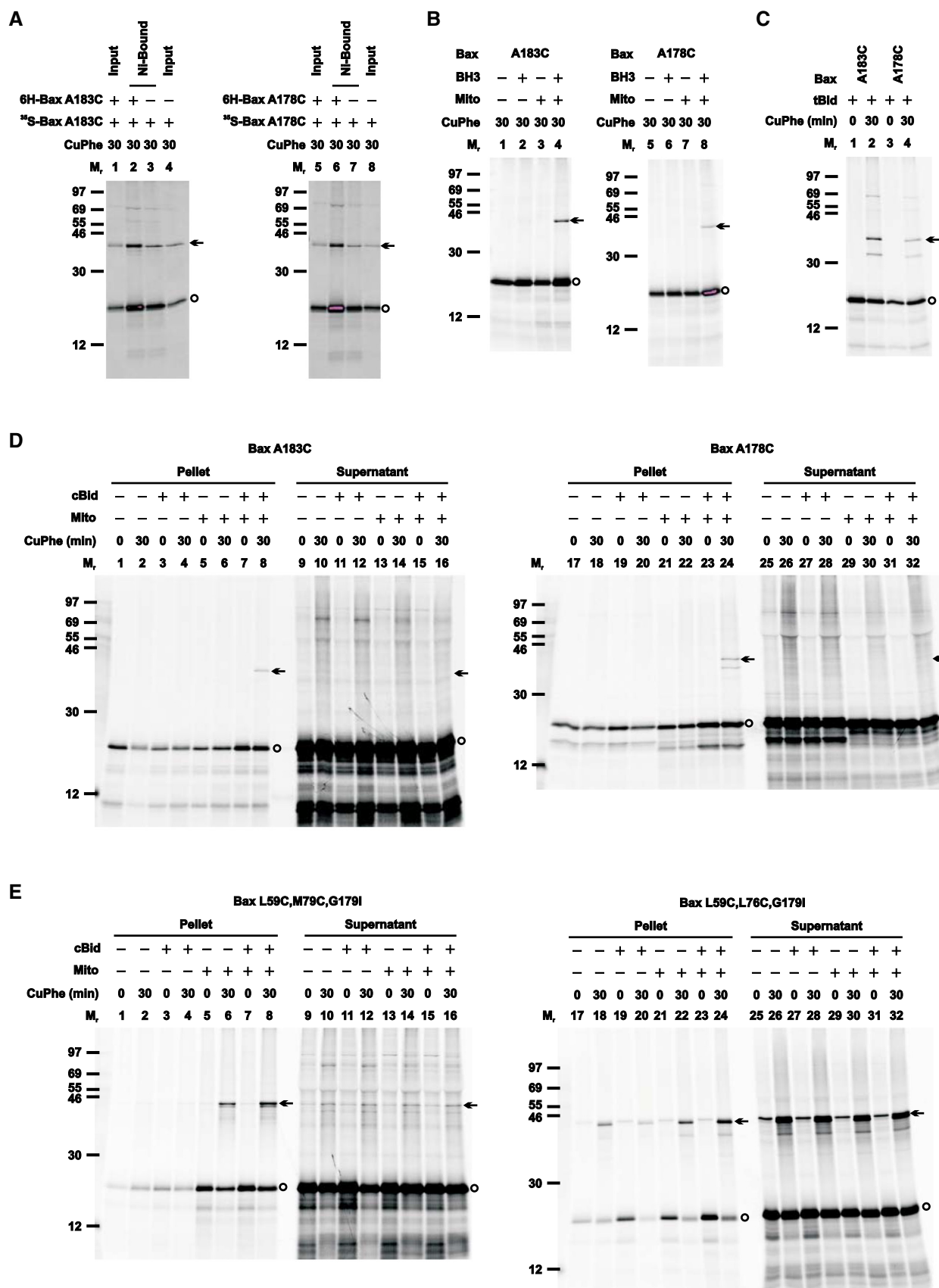


Figure EV5.