Expanded View Figures

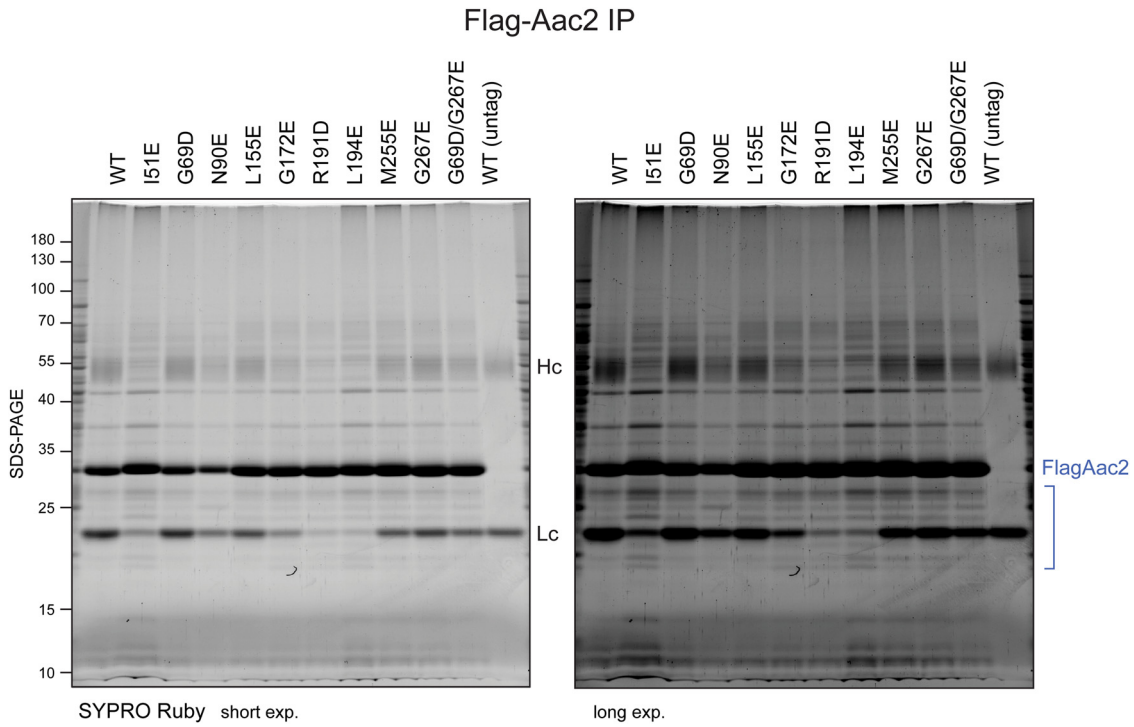

Figure EV1. Aac2 CL-binding mutants do not engage in aberrant protein interactions.

Isolated mitochondria were solubilized with 1.5% digitonin and subjected to FLAG immunoprecipitation. Co-purified extracts were resolved by 10–16% SDS-PAGE and resolved proteins detected by SYPRO Ruby staining.

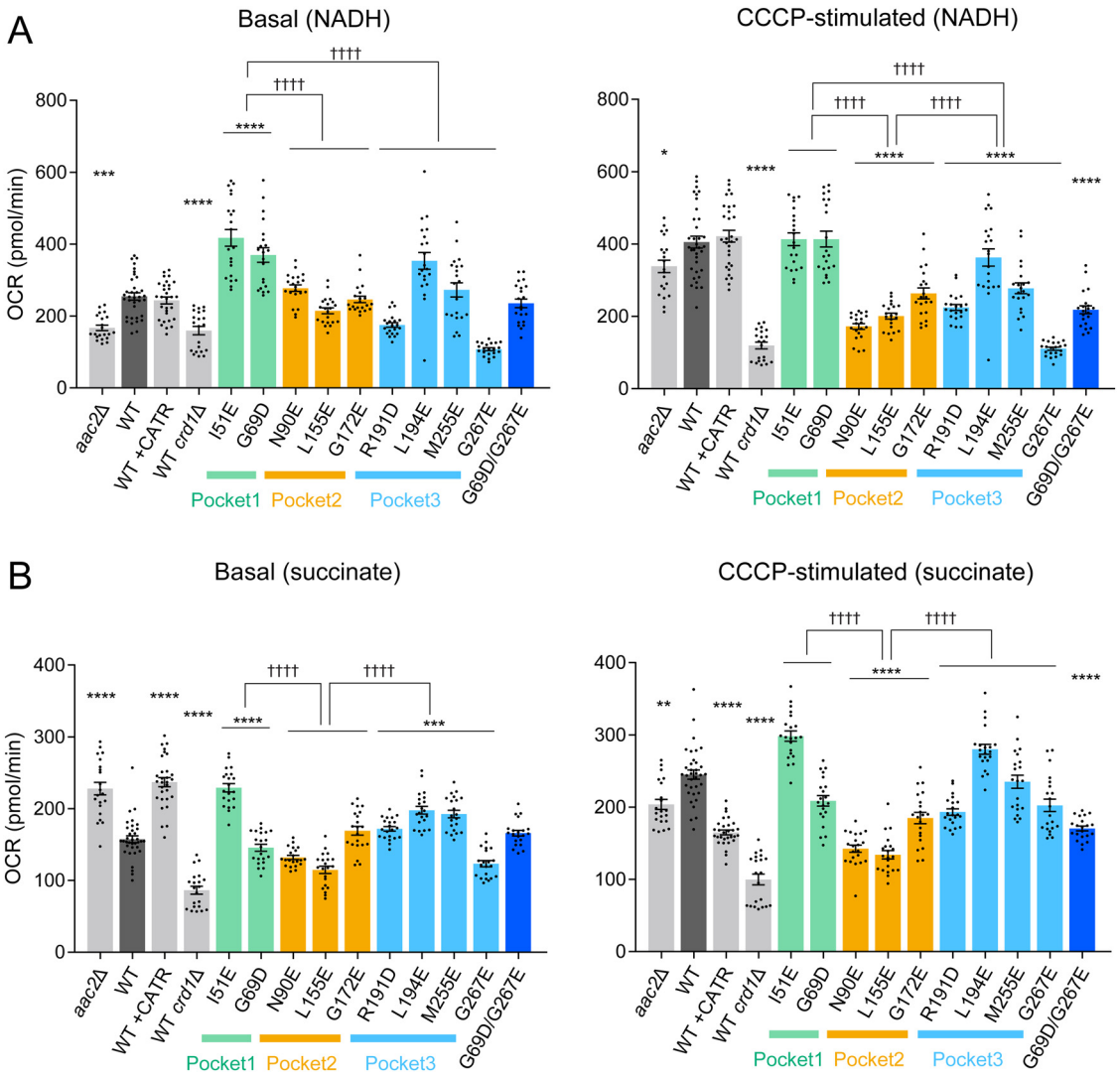

Figure EV2. Mitochondrial respiration of Aac2 CL-binding mutants.

Related to Fig. 4D–F, basal and CCCP-stimulated respirations of WT and mutant mitochondria in the presence of NADH (**A**) and succinate (**B**) were plotted as oxygen consumption rate (OCR) ($n = 21$–35, 3–5 biological replicates with 5–7 technical replicates). Mean with SEM. Significant differences obtained by two-way ANOVA followed by Tukey's multiple comparisons test are shown as * for comparison with WT and † for comparison between pockets; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

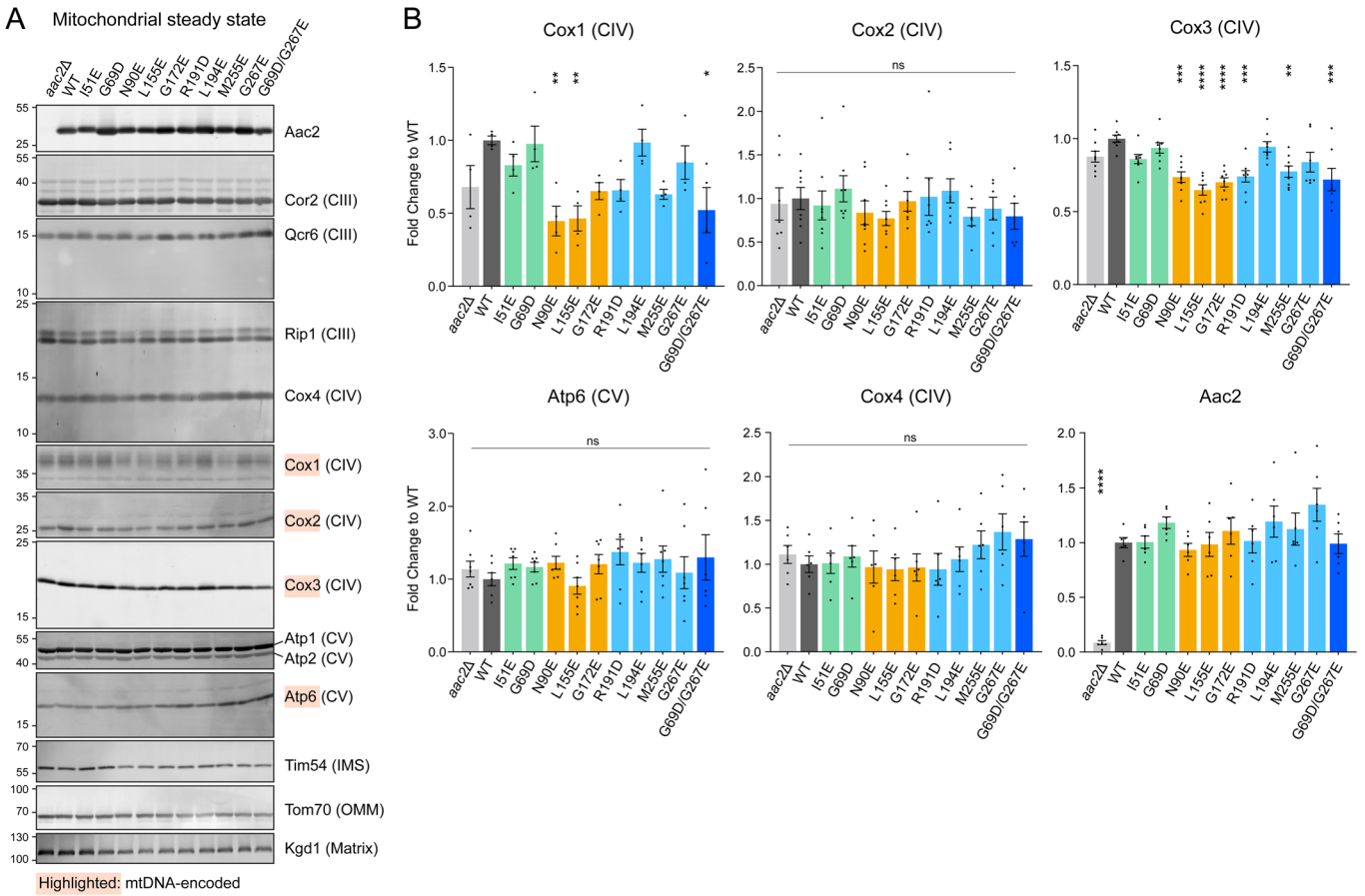

Figure EV3. The expression of respiratory complex subunits encoded in mitochondrial DNA is attenuated in Aac2 CL-binding mutants.

(**A**) Mitochondrial extracts were resolved by SDS-PAGE and immunoblotted for indicated proteins, including subunits of respiratory complexes III, IV, and V. (**B**) The expression of indicated respiratory complex subunits was quantified. Mean with SEM. Statistical differences were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ (vs. WT). Representative images from the replicates ($n = 4$–8, biological replicates) are shown.

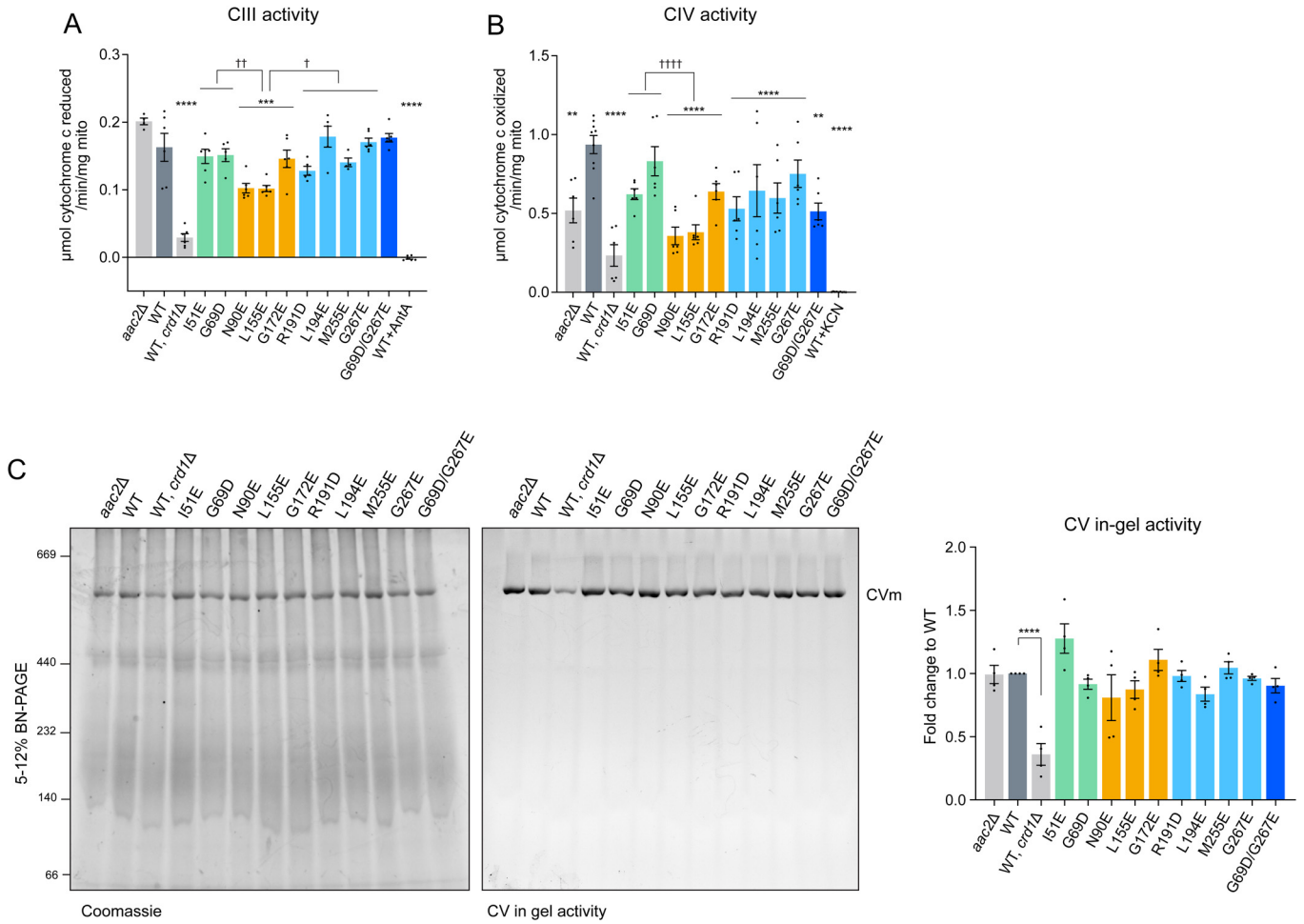

Figure EV4. Activities of respiratory complexes III, IV, and V of CL-binding mutants.

(**A**) Complex III activity in 0.5% (w/v) DDM-solubilized mitochondria ($n = 4$–6, biological replicates). (**B**) Complex IV activity in 0.5% (w/v) DDM-solubilized mitochondria ($n = 6$–9, biological replicates). (**C**) Complex V in-gel activity assay. Mitochondria were solubilized in 1% (w/v) DDM, resolved by 5–12% blue native-PAGE, and incubated with the substrate ($n = 5$, biological replicates). Mean with SEM. Significant differences obtained by two-way ANOVA followed by Tukey's multiple comparisons test are shown as * for comparison with WT and † for comparison between pockets; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

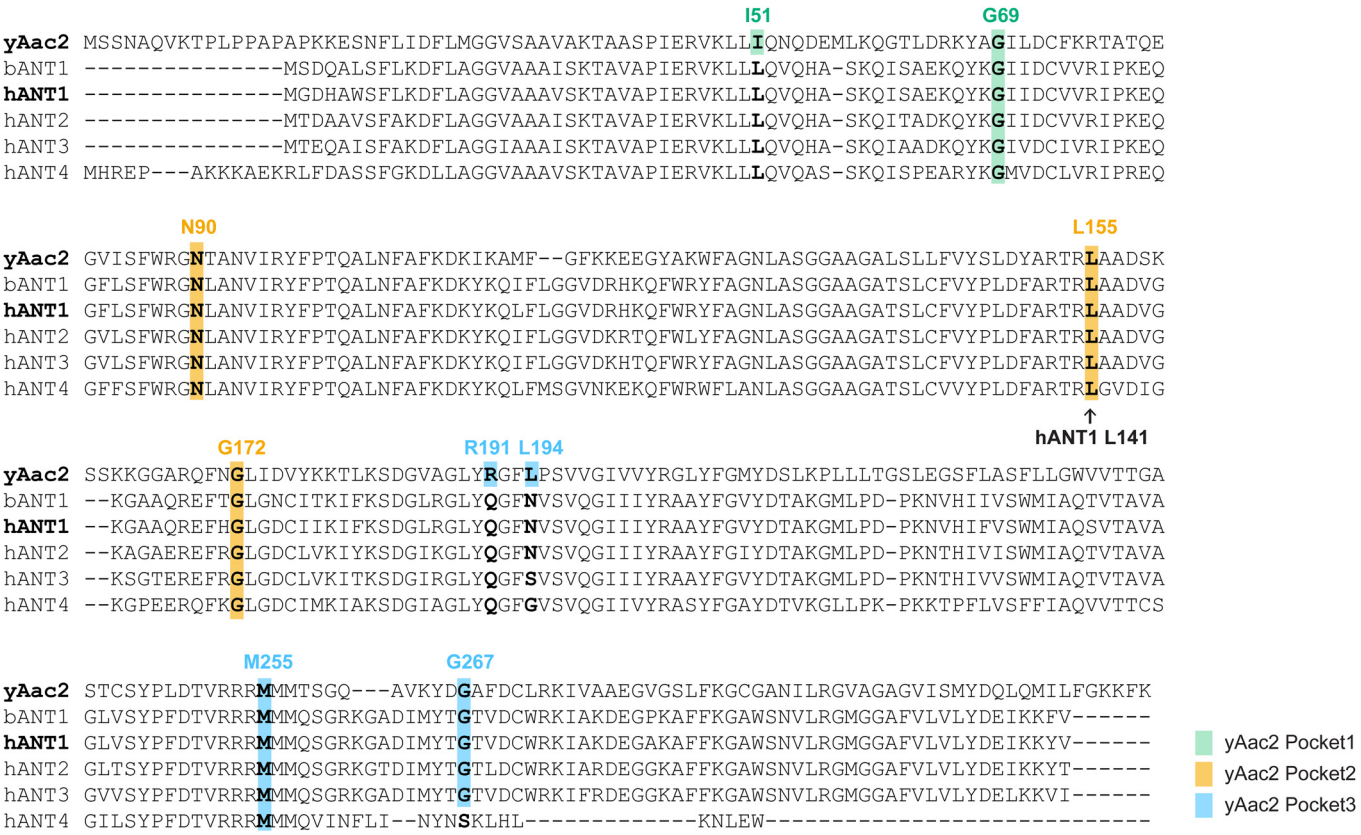

Figure EV5. CL-binding sites are conserved across species.

Amino acid sequence alignment of yeast Aac2, bovine ANT1, and human ANT isoforms. The residues designed for the Aac2 CL-binding mutants are highlighted as indicated.