## **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  $\dagger$  for comparison between pockets; \*p < 0.05, \*\*p < 0.01, \*\*\*\*p < 0.001.



## 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 < 0.001 (\*\*\*p < 0.001) (\*\*\*



## 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  $\dagger$  for comparison between pockets; \*p < 0.05, \*\*p < 0.001, \*\*\*p < 0.001.

	I51 G69	
yAac2	MSSNAQVKTPLPPAPAPKKESNFLIDFLMGGVSAAVAKTAASPIERVKLLIQNQDEMLKQGTLDRKYAGILDCFKRTATQE	
bANT1	MSDQALSFLKDFLAGGVAAAISKTAVAPIERVKLL <b>L</b> QVQHA-SKQISAEKQYK <b>G</b> IIDCVVRIPKEQ	
hANT1	MGDHAWSFLKDFLAGGVAAAVSKTAVAPIERVKLL <b>L</b> QVQHA-SKQISAEKQYK <b>G</b> IIDCVVRIPKEQ	
hANT2	MTDAAVSFAKDFLAGGVAAAISKTAVAPIERVKLL <b>L</b> QVQHA-SKQITADKQYK <b>G</b> IIDCVVRIPKEQ	
hANT3	MTEQAISFAKDFLAGGIAAAISKTAVAPIERVKLL <b>L</b> QVQHA-SKQIAADKQYK <b>G</b> IVDCIVRIPKEQ	
hANT4	MHREPAKKKAEKRLFDASSFGKDLLAGGVAAAVSKTAVAPIERVKLLLQVQAS-SKQISPEARYKGMVDCLVRIPREQ	
	N90 L155	
yAac2	GVISFWRGNTANVIRYFPTQALNFAFKDKIKAMFGFKKEEGYAKWFAGNLASGGAAGALSLLFVYSLDYARTRLAADSK	
bANT1	${\tt GFLSFWRG}{\bf N} {\tt LANVIRYFPTQALNFAFKDKYKQIFLGGVDRHKQFWRYFAGNLASGGAAGATSLCFVYPLDFARTR {\bf L} {\tt AADVG}$	
hANT1	${\tt GFLSFWRG}{\bf N} {\tt LANVIRYFPTQALNFAFKDKYKQLFLGGVDRHKQFWRYFAGNLASGGAAGATSLCFVYPLDFARTR {\bf L} {\tt AADVG}$	
hANT2	${\tt GVLSFWRG}{\bf N} {\tt LANVIRYFPTQALNFAFKDKYKQIFLGGVDKRTQFWLYFAGNLASGGAAGATSLCFVYPLDFARTR {\bf L} {\tt AADVG}$	
hANT3	GVLSFWRG <mark>N</mark> LANVIRYFPTQALNFAFKDKYKQIFLGGVDKHTQFWRYFAGNLASGGAAGATSLCFVYPLDFARTR <mark>L</mark> AADVG	
hANT4	${\tt GFFSFWRG}{\bf N} {\tt LANVIRYFPTQALNFAFKDKYKQLFMSGVNKEKQFWRWFLANLASGGAAGATSLCVVYPLDFARTR}{\bf L}{\tt GVDIG}$	
	<b>^</b>	
	G172 R191 L194 hANT1 L141	
yAac2	G172 R191 L194 hANTI L141 SSKKGGARQFN <mark>G</mark> LIDVYKKTLKSDGVAGLY <b>R</b> GF <b>L</b> PSVVGIVVYRGLYFGMYDSLKPLLLTGSLEGSFLASFLLGWVVTTGA	
<b>yAac2</b> bANT1	G172 R191 L194 hANTI L141 SSKKGGARQFN <mark>G</mark> LIDVYKKTLKSDGVAGLYRGFIPSVVGIVVYRGLYFGMYDSLKPLLLTGSLEGSFLASFLLGWVVTTGA KGAAQREFT <mark>G</mark> LGNCITKIFKSDGLRGLY <b>Q</b> GF <b>N</b> VSVQGIIIYRAAYFGVYDTAKGMLPD-PKNVHIIVSWMIAQTVTAVA	
yAac2 bANT1 hANT1	G172 R191 L194 hANTI L141 SSKKGGARQFNGLIDVYKKTLKSDGVAGLYRGFLPSVVGIVVYRGLYFGMYDSLKPLLLTGSLEGSFLASFLLGWVVTTGA KGAAQREFTGLGNCITKIFKSDGLRGLYQGFNVSVQGIIIYRAAYFGVYDTAKGMLPD-PKNVHIIVSWMIAQTVTAVA KGAAQREFHGLGDCIIKIFKSDGLRGLYQGFNVSVQGIIIYRAAYFGVYDTAKGMLPD-PKNVHIFVSWMIAQSVTAVA	
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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.