Expanded View Figures

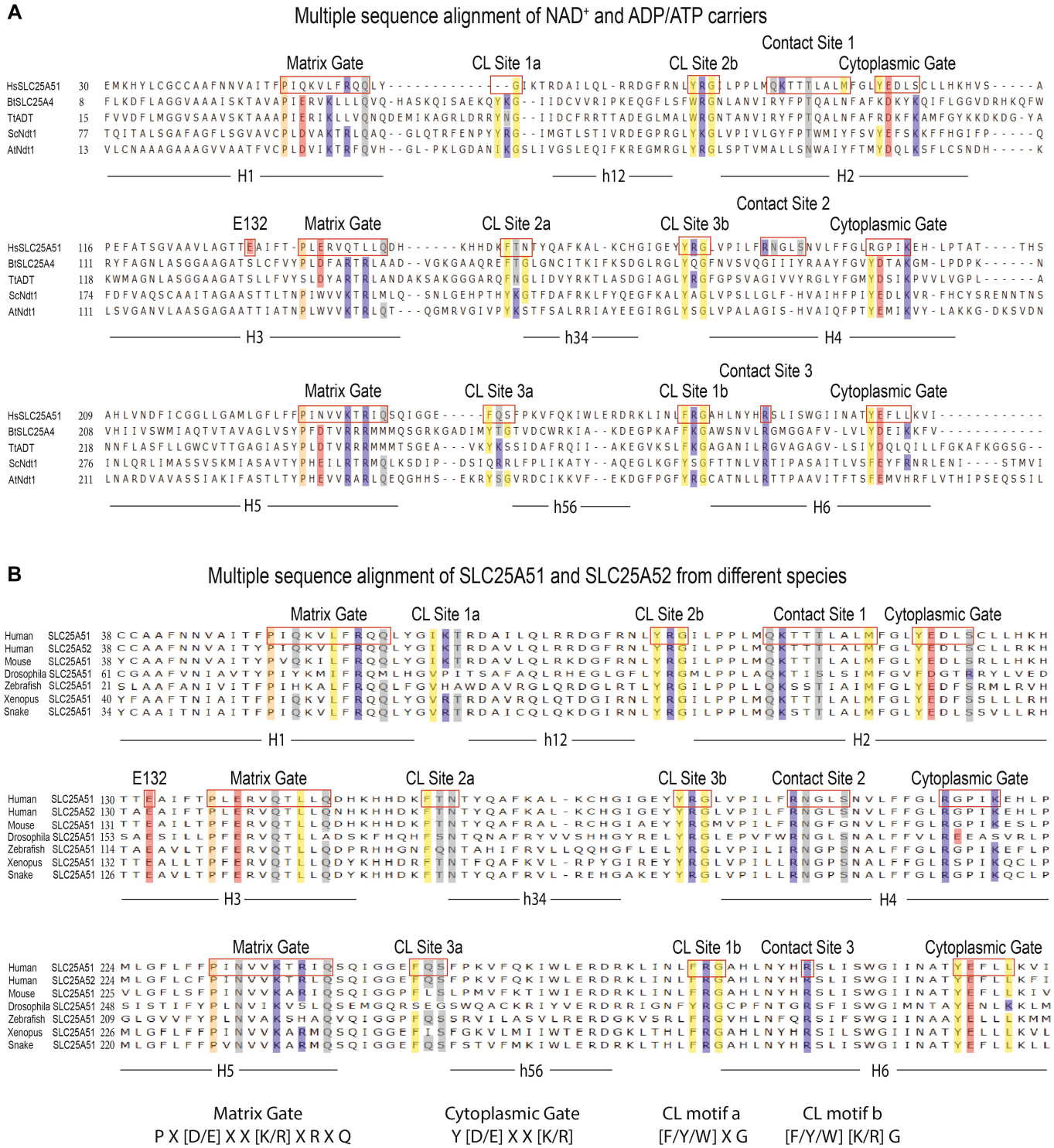

Figure EV1.

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Figure EV1. Multiple sequence alignment of representative nucleotide carriers.

A Alignment of amino acid sequences for human SLC25A51 (*Hs*SLC25A51), bovine SLC25A4 (*Bt*SLC25A4), fungal ADT (*Tt*ADT), yeast Ndt1 (*Sc*Ndt1) and Arabidopsis Ndt1 (*At*Ndt1).

B Alignment of amino acid sequences for human SLC25A51 and SLC25A52 and SLC25A51 orthologues from mouse, drosophila, zebrafish, xenopus, and snake.

Data information: Select residues are highlighted by shading (hydrophobic, yellow; hydrophilic, gray; negatively charged, red; positively charged, blue; and proline kink residues, peach).

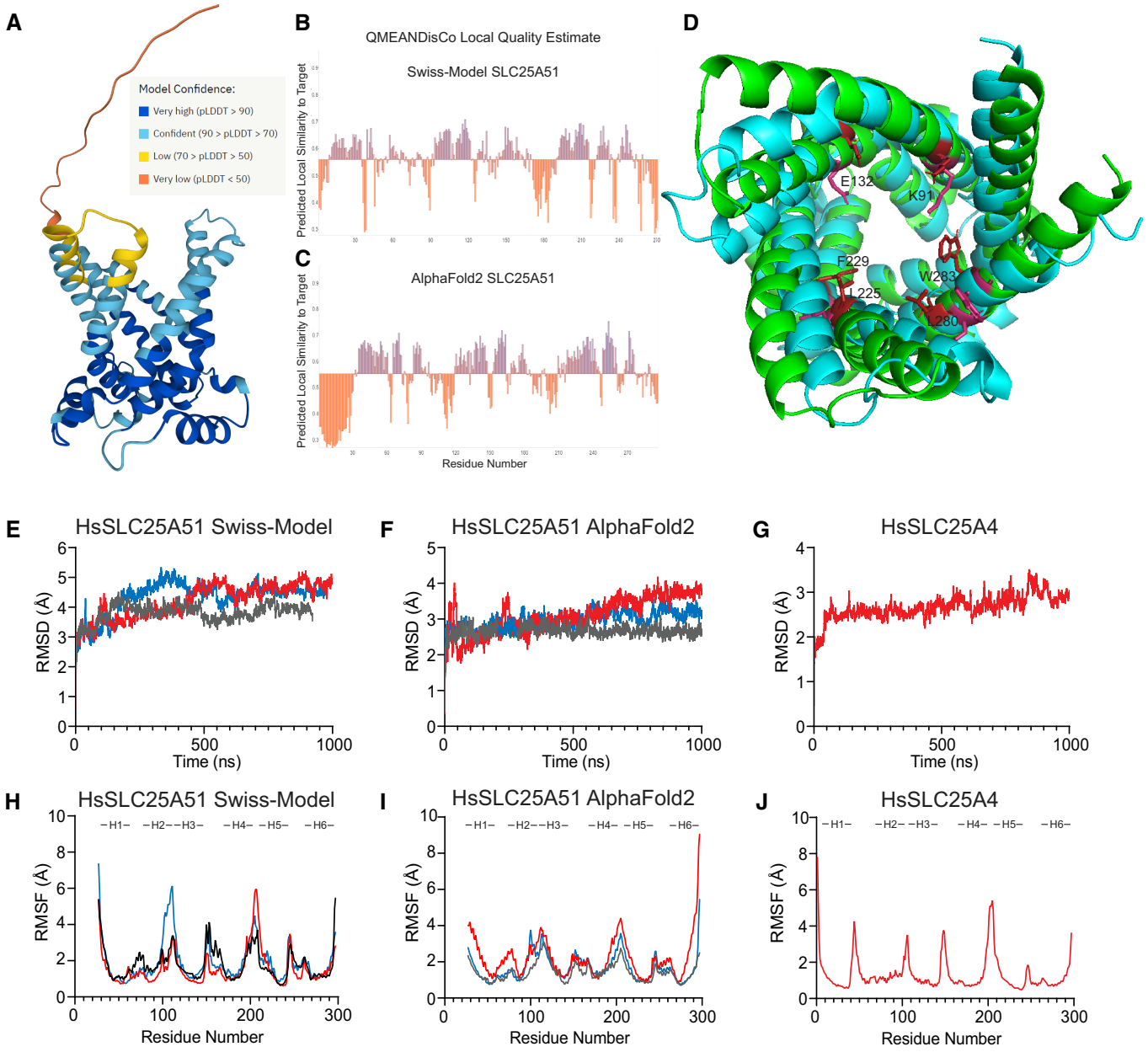

Figure EV2.

Figure EV2. Quality estimation using root mean square deviation and root mean square fluctuation of the simulations.

A Model confidence scores for AlphaFold2 structure of HsSLC25A51.
B, C QMEANDisCo Local Quality Estimate shows higher confidence for transmembrane helices and lower confidence for flexible loops.
D Alignment of Swiss-Model (Cyan) and AlphaFold2 (Green) structures. Key residues that were initially differentially positioned in the binding site are highlighted.
E RMSD of backbone atoms over time with the Apo Swiss-Model (amino acids 27–297), $n = 3$.
F RMSD of backbone atoms over time with the Apo AlphaFold2 (amino acids 27–297), $n = 3$.
G RMSD of backbone atoms over time with Apo SLC25A4, $n = 1$.
H RMSF of each residue with Apo Swiss-Model, $n = 3$.
I RMSF of each residue with Apo AlphaFold2, $n = 3$.
J RMSF of each residue with Apo SLC25A4, $n = 1$.

Source data are available online for this figure.

Figure EV3. Localization, expression, and function of SLC25A51 mutants.

A Immunofluorescence co-localization of CoxIV and ^{Flag}SLC25A51 variants transiently transfected in HeLa cells. Anti-Flag is shown in cyan, anti-CoxIV in red, and DAPI in blue.
B Relative abundance of ^{Flag}SLC25A51 mutants over wildtype from HeLa whole cell lysates normalized from Western blot data (24 h post-transfection). Data are shown in box and whisker format, with hinges at 25th and 75th percentiles, whiskers represent min and max and the line is the median, $n = 4$–7 biological replicates. One-sample two-sided *t*-test for significant difference from 1, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
C Membrane enrichment of *E. coli* cells expressing YFP control, wildtype SLC25A51 (amino acids 29–297) or the indicated mutant detected with anti-SLC25A51 Western blot; anti-TolC (membrane) and anti-DNAK (cytoplasmic), fractionation markers.
D Membrane expression of wildtype SLC25A51 (amino acids 29–297), indicated variants, or YFP control in *E. coli* cells detected with anti-SLC25A51 Western blot; TolC, loading control.
E Radiolabeled NAD^+ uptake with time by *E. coli* cells expressing wildtype SLC25A51 (amino acids 29–297) or its N183Q mutant. Data are mean ± SD and $n = 3$–4 biological replicates.
F Anti-SLC25A51 Western blot of HEK293 wildtype, HEK293 SLC25A51 KO and HeLa cells transiently transfected with ^{Flag}SLC25A51 or empty vector control; HSP60, loading control. Red dots, endogenous SLC25A51; green dots, ectopically expressed ^{Flag}SLC25A51.
G, H Free mitochondrial NAD^+ levels measured using a ratiometric biosensor in HEK293 SLC25A51 KO cells co-expressing empty vector control, wildtype ^{Flag}SLC25A51, and indicated mutants. Measurements were taken at 48 h post-transfection; the dashed line indicates the baseline defined by the empty vector control, and red denotes data equivalent to wildtype. Data are shown in box and whisker format, with hinges at 25th and 75th percentiles, whiskers represent min and max and the line is the median, $n = 4$–6 biological replicates, ANOVA < 0.0001 with post-hoc Dunnett's Test compared to empty vector control $^{***}P < 0.001$. (*bottom*) Protein expression from HEK293 SLC25A51 KO cells transiently transfected with empty vector control, wildtype ^{Flag}SLC25A51 and the indicated variants detected using anti-Flag Western blot; HSP60, loading control.
I Membrane expression of wildtype SLC25A51 (amino acids 29–297), indicated variants or YFP control in *E. coli* cells using anti-SLC25A51 Western blot; TolC, loading control.
J Free mitochondrial NAD^+ levels measured using a ratiometric biosensor in HEK293 SLC25A51 KO cells expressing empty vector control ($n = 10$ biological replicates), wildtype ^{Flag}SLC25A51 ($n = 12$ biological replicates), and indicated mutants ($n = 4$ biological replicates). Measurements were taken at 48 h post-transfection; the dashed line indicates the baseline defined by the empty vector control, and red denotes data equivalent to wildtype. Data are shown in box and whisker format, with hinges at 25th and 75th percentiles, whiskers represent min and max and the line is the median, ANOVA < 0.0001 with post-hoc Dunnett's Test compared to empty vector control $^{***}P < 0.001$. (*bottom*) Protein expression from HEK293 SLC25A51 KO cells transiently transfected with empty vector control, wildtype ^{Flag}SLC25A51 and the indicated variants using anti-Flag Western blot; HSP60, loading control.

Source data are available online for this figure.

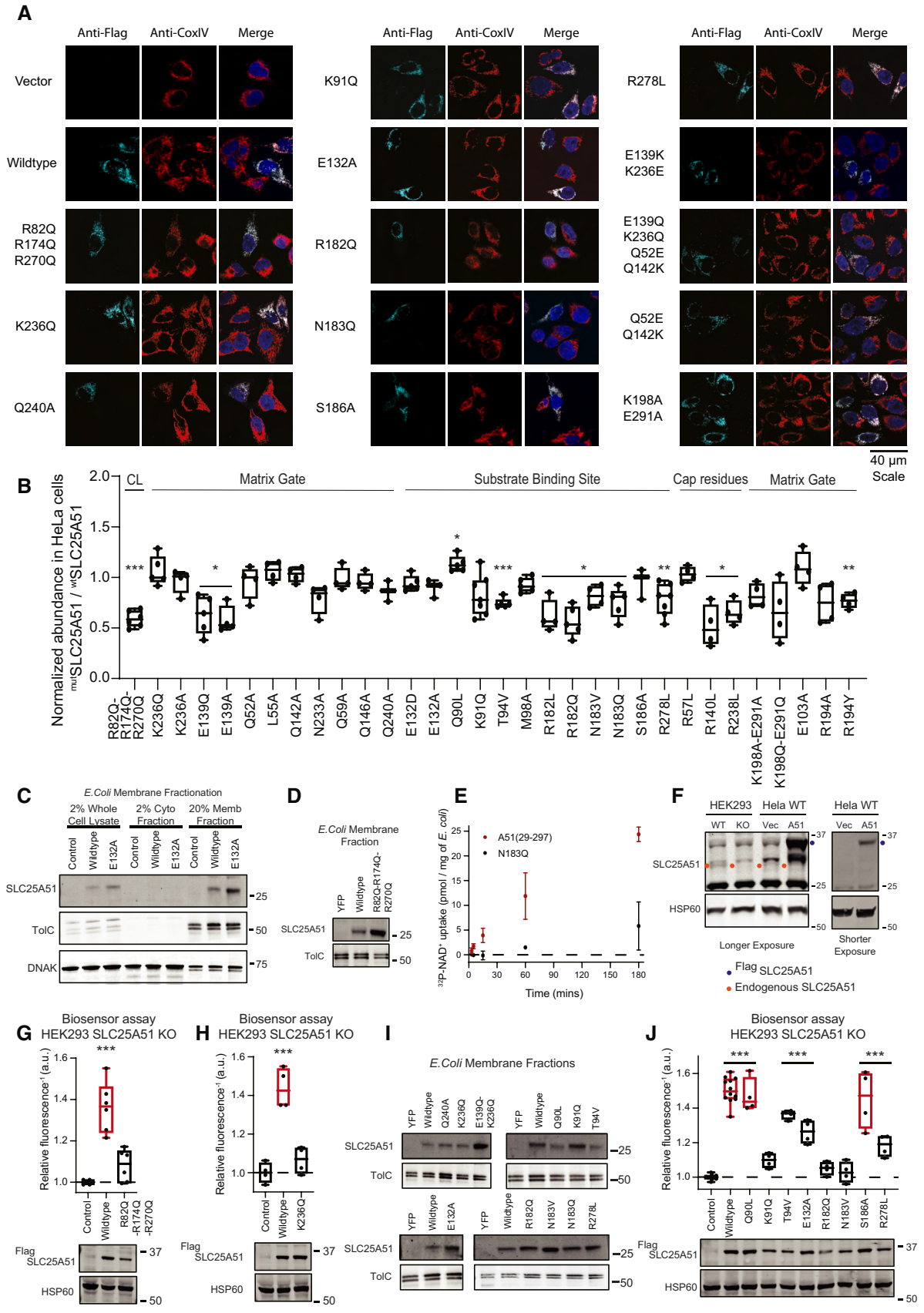

Figure EV3.

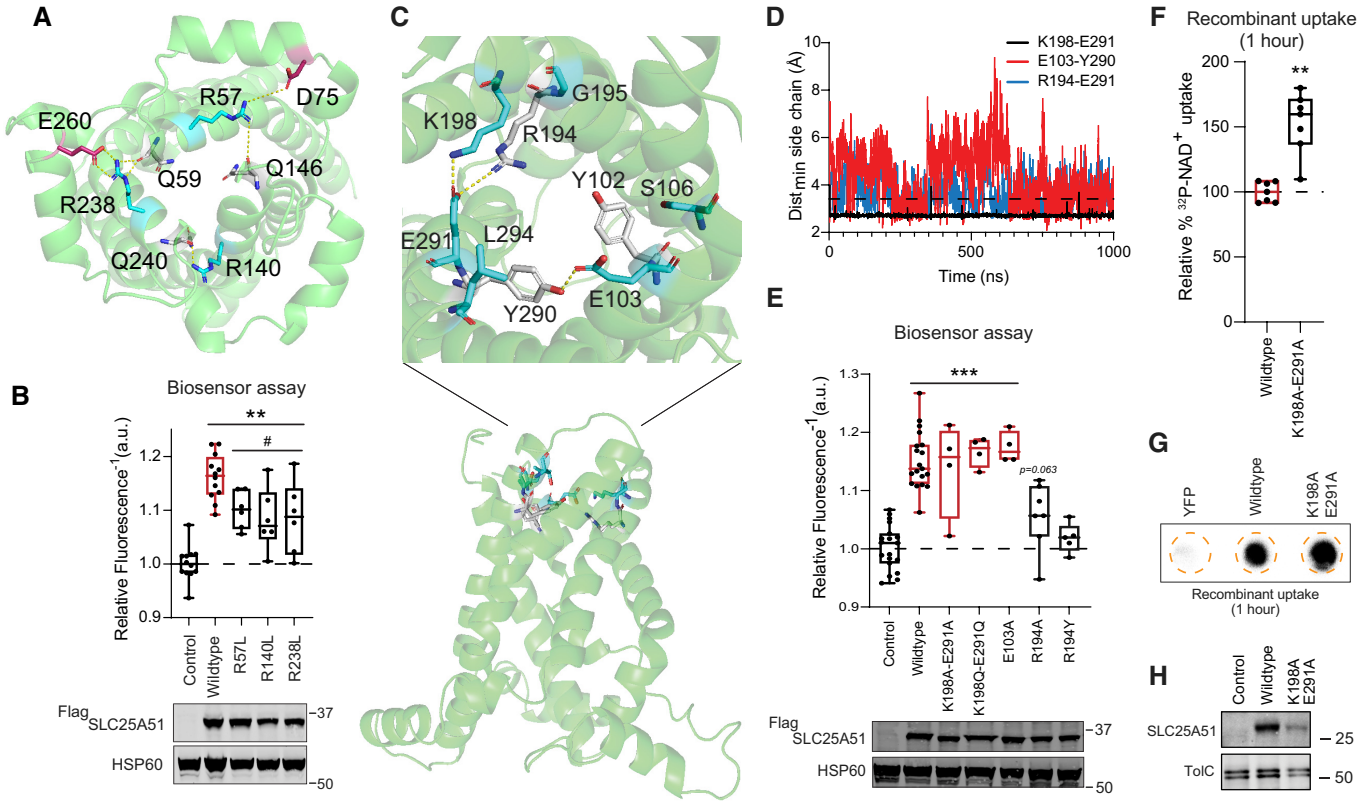

Figure EV4. The role of cap residues and cytoplasmic gate for SLC25A51 function.

A Relative positions of cap residues and their interactions (dashed lines) in a cartoon representation of SLC25A51.

B Free mitochondrial NAD^+ levels measured using a ratiometric biosensor in HeLa cells expressing empty vector control ($n = 12$ biological replicates), wildtype ^{Flag}SLC25A51 ($n = 12$ biological replicates), and indicated mutants ($n = 6$ biological replicates). Measurements were taken at 24 h post-transfection; the dashed line indicates the baseline defined by the empty vector control, and red denotes data equivalent to wildtype. Data are shown in box and whisker format, with hinges at 25th and 75th percentiles, whiskers represent min and max and the line is the median, ANOVA $P < 0.0001$, post-hoc Dunnett's test compared to empty vector control (**) and compared to wildtype ^{Flag}SLC25A51 (#), $^{**}P < 0.01$ and $^{\#}P < 0.05$. (*bottom*) Protein expression from HeLa cells transiently transfected with empty vector control, wildtype ^{Flag}SLC25A51 and the indicated variants using anti-Flag Western blot; HSP60, loading control.

C Relative position of the cytoplasmic gate in a cartoon representation of SLC25A51.

D Time evolution of distance between indicated side chains for putative gate interactions. Interaction cutoff at 3.5 Å is shown as a dashed horizontal line.

E Free mitochondrial NAD^+ levels measured using a ratiometric biosensor in HeLa cells expressing empty vector control ($n = 19$ biological replicates), wildtype ^{Flag}SLC25A51 ($n = 19$ biological replicates), and indicated mutants ($n = 4$–7 biological replicates). Measurements taken at 24 h post-transfection; empty vector control baseline is shown as dashed line and wildtype activity and equivalent is in red color. Data are shown in box and whisker format, with hinges at 25th and 75th percentiles, whiskers represent min and max and the line is the median, ANOVA $P < 0.0001$, post-hoc Dunnett's test compared to empty vector control, $^{***}P < 0.001$. (*bottom*) Protein expression from HeLa cells transiently transfected with empty vector control, wildtype ^{Flag}SLC25A51 and the indicated variants using anti-Flag Western blot; HSP60, loading control.

F Quantitation of uptake of ^{32}P-NAD^+ after 1 h by *E. coli* cells expressing wildtype SLC25A51 (amino acids 29–297) and the indicated mutant; wildtype activity and equivalent is in red color and the wildtype baseline is shown as dashed line. Data are shown in box and whisker format, with hinges at 25th and 75th percentiles, whiskers represent min and max and the line is the median, $n = 7$ biological replicates, unpaired *t*-test $^{***}P < 0.001$.

G Representative phosphorimage of retained ^{32}P-NAD^+ after 1 h by *E. coli* cells expressing wildtype SLC25A51 (amino acids 29–297), indicated variant, or YFP control.

H Membrane expression of wildtype SLC25A51 (amino acids 29–297), its indicated variant or YFP control in *E. coli* cells using anti-SLC25A51 Western blot; TolC, loading control.

Source data are available online for this figure.

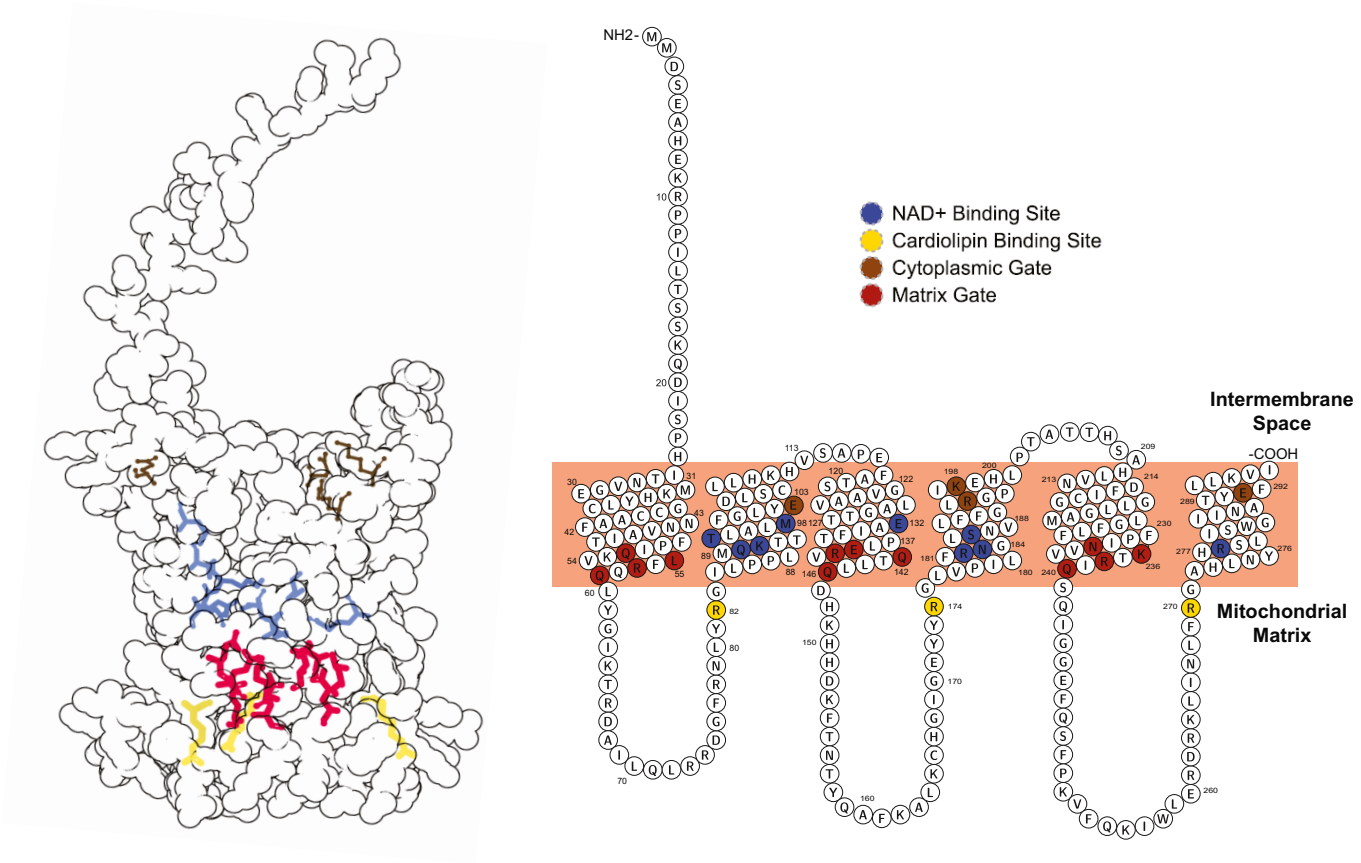

Figure EV5. Summary of the SLC25A51 residues mutated in this study.

The mutations studied in this work are highlighted in a tertiary structure representation (Left) generated using Protein Imager (Tomasello *et al*, 2020) and a modified primary structure representation (Right) generated using Protter (Omasits *et al*, 2014).