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# Layered Mechanisms Regulating the Human Mitochondrial NAD+ Transporter SLC25A51

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## Abstract

SLC25A51 is the primary mitochondrial $NAD^+$ transporter in humans and controls many local reactions by mediating influx of oxidized $NAD^+$. Intriguingly, SLC25A51 lacks several key features compared to other members in the mitochondrial carrier family, thus its molecular mechanism has been unclear. A deeper understanding would shed light on the control of cellular respiration, the citric acid cycle, and free $NAD^+$ concentrations in mammalian mitochondria. This review discusses recent insights into the transport mechanism of SLC25A51, and in the process highlights a multitiered regulation that governs $NAD^+$ transport. The aspects regulating SLC25A51 import activity can be categorized as contributions from (1) structural characteristics of the transporter itself, (2) its microenvironment, and (3) distinctive properties of the transported ligand. These unique mechanisms further evoke compelling new ideas for modulating the activity of this transporter, as well as new mechanistic models for the mitochondrial carrier family.

### Keywords

SLC25A51; Oxidized Nicotinamide adenine dinucleotide ($NAD^+$); Mitochondrial transport; Mitochondrial Carrier Family (MCF)

SLC25A51 was recently identified in mammalian cells as a transporter that selectively imports oxidized $NAD^+$ into the mitochondrial matrix [1–3]. Corroborating data from multiple labs determined that it was required for maintaining mitochondrial $NAD^+$ levels in human cells and for countering turnover by local $NAD^+$ consuming enzymes [1–3]. SLC25A51 can be regarded as a regulator of the compartmentalized mitochondrial $NAD^+$ concentration because changes in its expression or activity are sufficient to either limit or increase matrix $NAD^+$ levels [1–3]. Thus far, aberrant SLC25A51 activity has been linked to blood cancers and solid tumors, as well as fatty liver disease, hypertriglyceridemia and DNA repair [4–8]. Misregulation of mitochondrial $NAD^+$ concentrations has context-specific consequences that include a decoupled and reduced $NAD^+$/NADH ratio and loss of activity for mitochondrial $NAD^+$-consuming enzymes [4–8].

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Competing Interests

The authors declare that they have no competing interests.

SLC25A51 is a member of the Mitochondrial Carrier Family (MCF) of transporters and it localizes within the inner mitochondrial membrane [1–3]. The majority of MCF members serve to transport specific ions, cofactors, amino acids, and other essential molecules across the inner membrane into the matrix [9–11]. A defining feature of an MCF member is a pseudo trisymmetrical arrangement of six transmembrane helices within the bilayer to create an interior core (Figure 1A) [12–14]. Each domain is comprised of two transmembrane helices (one kinked and one non-continuous) and a shorter connecting helix on the matrix side that is oriented in parallel to the length of the membrane (Figures 1A and 1B) [12–16].

The molecular characterization of individual mitochondrial carriers and determining how a specific carrier recognizes its targeted cargo has been limited. Experimental structures are currently available only for the bovine and fungal ADP/ATP carriers without their endogenous ligands, and for the recently published human UCP1 in its apo and inhibited state [12,17–22]. These examples notably represent the most abundant carriers within the MCF family [15,23], and efforts towards larger scale purifications of less abundant carriers such as SLC25A51 remain ongoing. A full characterization of any MCF carrier, furthermore, requires study of its dynamic conformations throughout the transport process. Ideally, this would be accomplished by experimental structures of its various conformations, in vitro binding and cellular assays, and recombinant uptake assays in proteoliposomes [24–31]. With limited examples of MCF structures and cognate ligands, it has not been straightforward to elucidate patterns and extrapolate predictions. Instead, as in the case of SLC25A51, site-directed mutagenesis, functional assays, and computational modeling have been fruitful for initially characterizing the mechanisms of many transporters.

In this review we discuss the modeled structural features of SLC25A51, and recent insights for how interactions with its surroundings and ligand impact the import of $NAD^+$. Published mutations that have been used to study SLC25A51 are listed in Table 1.

## STRUCTURAL FEATURES OF SLC25A51 THAT GOVERN TRANSPORT.

MCF carriers generally function as alternating access transporters with a mechanism that includes a cytoplasmic-open state (c-state) to allow access for the intended ligand [12,15,18–21]. The c-state is contrasted by a matrix-open state (m-state) with potential for binding a ligand to be exported [19,32]. Each of these two conformations has been structurally identified [12,17–21] and together define stable points in a repeating, alternating access mechanism that centers around a single ligand binding site (Figure 1C) [32–36]. A substrate-bound occluded transition is theorized [37] but such intermediate has not yet been experimentally captured.

The c-state in MCF carriers is stabilized by a planar interaction network (the matrix gate) among the odd-numbered helices (Figure 1D) [12,37]. These helices harbor the repeated motif PX[D/E]XX[K/R]XXXQ, and polar residues within this motif are responsible for creating up to three interhelical bonds (Figure 1D) [10]. Antipodally, to stabilize the complementary conformation of the m-state, an analogous cytoplasmic gate network is formed by interhelical bonding within repeated Y[D/E]XX[K/R] motifs in the even-numbered helices (Figure 1C and 1D) [10,19]. A canonical MCF gate consists of multiple

salt bridges and is further stabilized by bracing hydrogen bond interactions, which are either within the same plane or longitudinally adjacent by the distance of a helical turn (Figure 1D) [15]. This gate network is exemplified by the mitochondrial ADP/ATP carriers, members of the MCF family [10,12,18]. It is also present in Ndt1 and Ndt2, which are MCF $NAD^+$ transporters found in yeast, fungi, and plants (Figure 1D) [10]. It has been proposed that an optimized alternating transport cycle occurs when a carrier's cytoplasmic gate, matrix gate, and ligand binding have equivalent interaction energies [34]. Robust import of mitochondrial $NAD^+$ in yeast and plant is driven by the antiport of (d)AMP and (d)ADP [38,39]. This in turn couples the accumulation of AMP/ADP in the matrix with influx of $NAD^+$, which can drive an oxidative citric acid cycle in response to low ATP levels [40].

### Single Salt-bridge Gates.

Functional homologs of SLC25A51 in yeast and plants (*e.g.* *Sc*Ndt1, *Sc*Ndt2, *At*Ndt1 and *At*Ndt2) belong to the MCF family [38,39,41,42] but it is hard to predict whether their mechanisms extend to SLC25A51. These carriers have < 25% sequence identity with SLC25A51 and harbor amino acid differences at key sites [41]. SLC25A51's lack of close homology to other carriers—including the previously identified yeast and plant mitochondrial $NAD^+$ transporters—delayed its deorphanization as a mammalian mitochondrial $NAD^+$ transporter [41,43].

At its gates, SLC25A51 has only one confirmed salt-bridge interaction at each end and was found to be significantly less dependent on brace interactions (Figure 1D) [41,44]. The strengths of the gate networks in SLC25A51 are balanced, nevertheless, suggesting it could be amenable for alternating transport. Accordingly, loss of the single E139-K236 salt-bridge at the matrix gate of SLC25A51 resulted in complete loss of activity, likely by destabilizing the c-state and preventing $NAD^+$ from accessing its binding site [44]. However surprisingly —in spite of computational support for a functional cytoplasmic gate formed by a salt bridge between K198 and E291—mutation of the cytoplasmic gate did not significantly impair mitochondrial $NAD^+$ import in intact cells [44]. The expectation would have been that mutation of the cytoplasmic gate would destabilize the m-state conformation and prevent access to an antiported molecule decreasing transport. This differed from observations with the fungal ADP/ATP carrier where disruption of the cytoplasmic gate reduced transport of ADP over a log-fold [19,32].

The retained ability to import $NAD^+$ in vitro suggests the capacity of SLC25A51 to function as a uniporter in the absence of an intact cytoplasmic gate (Figure 1C and 1D). It has been suggested that the weak gate in SLC25A51, comprised of a single salt-bridge, may have a lower energy barrier for spontaneous disruption and could further be influenced by its local environment [10,34]. It has been previously shown that yeast *Sc*Ndt1 has some uniport activity in vitro, but this was not seen with plant transporters *At*Ndt1 and *At*Ndt2 [38,39]. Whether uniport in SLC25A51 occurs under physiological scenarios still needs validation. Rather the data may hint instead at additional yet unknown drivers controlling SLC25A51 matrix gate opening.

### Transport of $NAD^+$ into mitochondria against electrochemical gradients.

An aspect to consider is that SLC25A51 must transport $NAD^+$ against both its concentration and electrical gradients. Free $NAD^+$ from the cytosol equilibrates with the mitochondrial intermembrane space through abundant porin complexes in the outer mitochondrial membrane. Steady-state free $NAD^+$ concentrations in the mitochondrial matrix measure higher compared to free cytosolic concentrations [45–49]. In respiring mitochondria, the anionic $NAD^+$ molecules must be transported from the more positively charged intermembrane space into the more negatively charged matrix. It is currently unknown how SLC25A51 overcomes these thermodynamic barriers. Without its reconstitution in vitro, it has not been formally established whether SLC25A51 requires ATP/GTP hydrolysis for transport activity, but SLC25A51, like most other MCF carriers, does not have such a catalytic domain. Rather, mechanisms typically used by MCF carriers include intramolecular coupling to an antiport or symport mechanism, regulatory domain coupling to a signaling pathway, or charge compensation of the transported molecule by the binding pocket.

Yeast and plant Ndt1 and Ndt2 mitochondrial $NAD^+$ transporters were determined to depend on antiport mechanisms using AMP or ADP molecules through reconstituted proteoliposome experiments [38,39], but so far the analogous experiment for SLC25A51 has been elusive. Being able to reconstitute SLC25A51 activity in vitro will be key for determining whether SLC25A51 similarly functions using antiported AMP, ADP or other molecules, or whether it uses another type of mechanism. This information will be insightful for understanding the physiological role of SLC25A51 and its emergence in the eukaryotic tree [41].

## IMPACT OF THE LOCAL ENVIRONMENT.

SLC25A51 resides within the inner mitochondrial membrane and thus is subjected to an environment that has a significant difference in pH and voltage across its membrane, as well as a distinctive membrane composition that includes the phospholipid cardiolipin.

### In consideration of pH.

Several MCF carriers use a proton coupling mechanism, where an $H^+$ ion is transported from the intermembrane space along its concentration gradient and used to drive co-transport of a co-substrate. Prominent examples include the human phosphate carrier, SLC25A3, and the aspartate/glutamate carriers, SLC25A12 and SLC25A13 [50,51]. Such a mechanism has been previously considered for SLC25A51 [10] due to a negatively charged residue in its ligand binding site [51]. However, mutation of all negative charges in the binding site of SLC25A51 did not fully abolish import of radiolabeled $NAD^+$ in an intact *E. coli* uptake system [44], as may be expected if proton-coupling is required for its activity. A specific dissipation of the pH gradient, which can be achieved with ionophores such as nigericin that rebalances the electrical gradient, could test whether a proton coupling mechanism is used by SLC25A51 [52–54].

### In consideration of voltage.

A charge compensation model has been proposed for MCF transporters to facilitate overcoming electrical barriers with charged ligands. This is intriguing to consider in light of SLC25A51 and its negatively charged ligand $NAD^+$ [9,15,55,56]. The ADP/ATP carriers import anionic substrates into the mitochondrial matrix despite the environment holding a negative voltage. In studies that monitored generated currents from bovine ADP/ATP carriers in proteoliposomes, it was found that transport of either ADP or ATP produced currents of opposite amplitudes. Although ATP and ADP are each anionic molecules, the transport of ADP (with a net charge of - 3) resulted in a net positive current [57,58]. It was speculated that this net positive current was critical to selectively facilitate import of ADP into the negative voltage matrix. The generated positive current with ADP was thought to arise from the net charge of the substrate and inward-facing charged residues in the transporter's binding site [57,58]. The theory became known as a charge-compensation model, where the charges from a transporter's pore residues "mask" those from the ligand, helping to make the ligand appear differently charged [9,15,55,56]. The $NAD^+$ molecule has a net charge of −1 and the ligand-binding site of SLC25A51 has a net charge of +2, thus there is potential for an analogous mechanism. One prediction is that the loss of membrane potential should then diminish the activity of SLC25A51. $NAD^+$ uptake assays along with the treatment with ionophores like valinomycin can be used to determine if membrane potential favors import of $NAD^+$ by SLC25A51 [54].

### Cardiolipin in the inner mitochondrial membrane.

Cardiolipin is an essential phospholipid in inner mitochondrial membranes [59,60], as well as a component of bacterial membranes [61,62]. It is composed of a glycerol backbone with phosphatidic acid groups on each end (Figure 2A) [63]. Thus, in addition to its glycerol backbone, a cardiolipin molecule has a total of four acyl chains and two phosphate groups (Figure 2A) [62–65]. There exists a large diversity of cardiolipin molecules arising from the variety of incorporated fatty acids of differing lengths and levels of saturation that likely represent the repertoire of fatty acids that are available to the cell. Nevertheless, the overall shape of this lipid is generally pyramidal with the phosphate groups toward the narrower end [66]. Established roles for cardiolipin include regulating mitochondrial morphology and the activity of the respiratory super-complex [67–74]. Additionally, cardiolipin molecule can directly bind specific MCF carriers impacting their activity and binding partners [12,17–21,26,44,75,76].

Molecular dynamic simulations and analyses of solved structures of ADP/ATP and UCP1 carriers have demonstrated that cardiolipin lipids are bound to the exterior of these transporters at specific sites [12,17–21,77–79]. This revealed a cardiolipin binding motif in MCF carriers defined by bipartite sequences [F/Y/W]-X-G and [F/Y/W]-[K/R]-G [12,17–21,77–79]. The motifs are found at the interface between the domains of the transporter; more specifically, the first part of the sequence resides at the N-termini of the short matrix-residing helices and second part of the sequence is at the N-terminus of the even-numbered transmembrane helices (Figure 2B) [15,17,18]. This motif is present in all MCF carriers, including SLC25A51 where it is found at each interface [15,41,44]. Due to the location of these motifs, one of the proposed roles for cardiolipin is to bridge the domains

within SLC25A51 (Figure 2B). As found for other MCF carriers, this could be important for maintaining the structural integrity of SLC25A51 during conformational transitions between c- and m-states [17,19] or for maintaining its stability during assembly within larger macromolecular complexes [17,68,69].

Emerging data have indicated that in general the binding of cardiolipin is required for robust MCF transporter function. In support, disease alleles harboring specific mutations in the cardiolipin binding site for the ADP/ATP carrier have been identified [11,80,81] and introduction of cardiolipin-binding mutations prevented the transporter from attaining maximal efficiency [82]. For SLC25A51, while at this date no disease alleles have been reported in the Human Gene Mutation Database, the ClinVar database includes variant R82C (VCV002311085.1). Based on location of this SNP, it may affect cardiolipin binding and thus may indicate some variation in mitochondrial $NAD^+$ import among the population.

### The phosphate groups in cardiolipin bind to MCF carriers.

There are several distinct ways that cardiolipin lipids engage with the MCF carriers. First, the phosphate groups on cardiolipin interact with the peptide backbone specifically where there is positive dipole moment due to the convergence of the matrix and even-numbered transmembrane helices (Figure 2B and 2C) [12,17–21]. This overlaps with the positioning of the consensus motifs [12,17–21]. Additionally, many carriers including SLC25A51, UCP1 and AAC, have outward facing, positively charged side chain residues at these motifs that interact with the phosphate groups in cardiolipin (Figure 2D). In the solved crystal structures for the ADP/ATP and UCP1 carriers, nevertheless, cardiolipin molecules were found bound at consensus motif sites that lacked any positively charged residue indicating that this is not always necessary [12,17–21]. It has thus been challenging to pin down a general mechanism for how cardiolipin binds motifs in MCF transporters. This may need to be determined on a case-by-case basis and more data on cardiolipin binding with different MCF carriers will be informative. What is clear, nevertheless, is that cardiolipin's phosphate groups participate in electrostatic interaction with the transporter. Having the interaction driven by the phosphate groups in cardiolipin could explain how this interaction is conserved across tissues and organisms despite a diverse variety of fatty-acids incorporation into individual cardiolipin molecules [23,83,84].

### Cardiolipin is necessary for SLC25A51 function.

In the case of SLC25A51, each of its three cardiolipin-binding motifs has an externally facing arginine residue (site 1: R270; site 2: R82; site 3: R174). Molecular dynamic simulations performed on predicted models from Alpha-Fold and Swiss Model modeling indicated that the cardiolipin phosphates directly engage these positively charged side chains to mediate binding of the phospholipid to SLC25A51 [44]. Mutation of these positively-charged side chains diminished cardiolipin binding *in vitro* and resulted in a full loss of $NAD^+$ uptake activity [44]. Due to this dependency of SLC25A51 on cardiolipin, physiological changes in the availability of this particular lipid could impact the concentration of mitochondrial $NAD^+$ and may explain the effect that cardiolipin disorders such as Barth Syndrome have on mitochondrial $NAD^+$ metabolism [85]. The requirement for cardiolipin may even represent an additional route for how diet or lipid profiles can

influence mitochondrial $NAD^+$ metabolism [86]. Many human conditions such as aging, neurodegeneration, and cardiovascular disease have co-occurrence of cardiolipin imbalance, aberrant $NAD^+$ metabolism, and mitochondrial dysfunction [87–90].

Intriguingly, post translational modifications of the positively charged lysine or arginine residues in cardiolipin-binding motifs have been reported for MCF carriers. Acetylation, for example, has been found at this site in the phosphate carrier SLC25A3, the 2-oxoglutarate/malate carrier SLC25A11, the ADP/ATP carrier SLC25A5, and the aspartate/glutamate carrier SLC25A13 [91,92]. In UCP1, reversible succinylation was identited on K56, with the modification destabilizing the protein and perturbing its function [93]. The same study detected succinylation at K64 in one cardiolipin binding site of SLC25A51 [93]. In addition, phosphorylation of motif residue T65 has been reported for SLC25A51 [94–96]. Together the data suggest a broader use of cardiolipin binding to impact the activities of MCF carriers, and that this interaction has potential to be regulated by post-translational modifications and be attenuated in disease.

## Cardiolipin binds SLC25A51 asymmetrically.

Using molecular dynamic simulations, cardiolipin was observed to bind the positively charged residues in SLC25A51 in two distinct ways. In most cases, binding involved both phosphate groups of the cardiolipin, and each phosphate group engaged a neighboring helix to bridge the domains (Figure 2B). However, limited to “site 2”—represented by matrix helix 3/4 and transmembrane helix 2—a bound cardiolipin molecule could utilize only one of its phosphate groups to simultaneously bridge the two domains (Figure 2C). This is similar to a pose previously observed for UCP1 structures where cardiolipin could bridge adjacent domains with a single phosphate group [20,21].

One implication of using a single phosphate group is that it would physically shorten the bridged distance at that site (Figure 2C). Additionally, the cardiolipin bound by a single phosphate group would have a different binding energy at that site. If this occurred in cells, it would mean that one point of the triangularly shaped core could be pinched, and possibly result in an asymmetrical shape of the inner pore. This may be helpful for accommodating the asymmetrically shaped $NAD^+$ ligand or contribute to ensuring fit for intended ligands. The differential thermodynamics from binding with a single phosphate may be important for conferring flexibility during transitions between conformations. There is precedent to the concept that cardiolipin can influence the shape of an MCF transporter to promote activity. In molecular dynamics simulations of the *Thermothelomyces thermophila* ADP/ATP carrier (*Tt*AAC) in its m-state, the presence of bound cardiolipin increased the symmetry of the pore in simulations. This, in turn, supported the overall stability of the transporter and increased the rigidity of the ligand binding site to limit non-productive movement of the ligand [97]. Given that cardiolipin may bind distinctly at designated sites with SLC25A51, there is the possibility that specific sites have distinct roles. A recent study selectively blocked cardiolipin binding from individual sites in *Sc*AAC2, the yeast ADP/ATP carrier, and found that the extent of loss-of-function depended on which specific site was ablated [82].

### Does cardiolipin promote the cytoplasmic open state to facilitate $NAD^+$ transport?

An emerging theme from the studies of fungal ADP/ATP carriers (*Mt*AAC and *Tt*AAC) and human UCP1 is the coincidence between the presence of cardiolipin and evidence for the transporters in a c-state conformation [19–21,32,98]. In the presence of cardiolipin, UCP1 stably retained its c-state in cryo-EM structures without aid of any stabilizing small molecules (PDB: 8HBV) [21], and purified *Mt*AAC and *Tt*AAC readily bound to small molecule inhibitor carboxyatractyloside indicating that these carriers were stably isolated in their c-state [19,32,98]. In contrast, addition of ADP was required to promote the m-state conformation for both *Mt*AAC and *Tt*AAC, as indicated by efficient *in-vitro* binding of bongkrekic acid [19,32,98]. Further, it was shown that in the absence of cardiolipin, the yeast ADP/ATP carrier *Sc*AAC2 preferred adoption of the m-state [68]. Together, the data suggest that the binding of cardiolipin could favor the c-state of these carriers in a general way.

The positioning of the cardiolipin binding motifs on the transporters indicate that the lipid may exert a direct function within the inner leaflet (Figure 2B). One model is that this may help neutralize the positive dipoles from the helical segments so that they can physically come together (Figure 2B and 2C). Additionally, the natural cone shape of the lipid and its positioning with its acyl groups upward radiating from the transporter-bound phosphate groups may contribute to the stabilization of the transporter's basket shape in the c-state. Relatedly, when the ADP/ATP carrier (PDB: 6GCI) is in its m-state, the distance that cardiolipin would need to span at specific sites between domains is wider than the c-state (PDB: 1OKC) (Figure 2E, left) [12,19,97]. Modeling SLC25A51 also indicated similar physical challenges for cardiolipin binding at all three sites (Figure 2E, right). Overall, this suggests that cardiolipin may increase the probability of the carrier to be in the c-state relative to m-state. With relatively weaker single salt bridge gates, it is possible that SLC25A51 is especially dependent on cardiolipin for stabilization of its c-state and this is consistent with loss of $NAD^+$ uptake activity and diminished cardiolipin binding [44].

## ROLE OF THE $NAD^+$ LIGAND IN TRANSPORT

### Initial engagement of the ligand in the pocket.

A series of elegant studies have revealed that a common feature among MCF carriers is a centrally-located ligand binding pocket [9,10,44,50,56,97–105]. While the individual residues in the pocket will differ depending on the carrier, there are some similarities in the position and role for critical residues among the purine nucleotide carriers. For example, in SLC25A51, molecular dynamic simulations and functional assays demonstrated that specific positively charged residues K91, R182 and R278 were critical for engaging the phosphate groups of $NAD^+$ (Figure 3A) [2,44]. The role and position of K91 and R278 aligned with residues previously identified in ADP/ATP carriers to interact with the phosphate groups within the ADP and ATP structures [44,98].

Acidic residue, E132, is conserved in the ligand-binding pocket among all SLC25A51 homologues [41,44]. It is the sole negatively charged residue in the substrate binding plane and it is located away from the positive charges that engage the phosphates on $NAD^+$. The

negative charge on E132 was found to be required for SLC25A51 function, as E132D but not E132A could substitute for its function [44]. Previously, the role of E132 was speculated to co-transport a proton with the substrate [10,51]. Recent molecular dynamic simulations revealed instead that E132 electrostatically engages with the positively charged nicotinamide ring of $NAD^+$ (Figure 3A). The combination of the nicotinamide ring being attracted to E132 and repulsed from the positively charged residues in the pocket works to position the nicotinamide ring. Once the nicotinamide ring is oriented, the rest of the $NAD^+$ molecule engages its contact sites in the pocket through additional polar interactions, including the ligand's phosphate groups with the positively charged residues (Figure 3A) [44].

### Distinguishing between $NAD^+$ and NADH.

A major structural difference between oxidized $NAD^+$ and reduced NADH molecules is the positive charge of the nicotinamide ring in the oxidized form (Figure 3B). An electrostatic interaction between the negative charges on the transporter and the positively charged nicotinamide ring represents a structural feature to favor $NAD^+$ import over NADH.

Loss of the negative charge in the binding pocket of SLC25A51 from E132 diminished transport activity in a recombinant *E.coli* uptake system, but the mutation did not completely ablate import [44]. Instead, the data indicated that what was lost was the transporter's ability to distinguish between $NAD^+$ and NADH when each was presented together at equimolar concentrations [44]. When E132 was left intact as a negatively charged residue in the binding pocket, introduction of unlabeled $NAD^+$ blocked uptake of tracer $^{32}$P-$NAD^+$ in direct competition assays. Unlabeled NADH at the same concentration was unable to compete for uptake at 100 μM and 250 μM. However, unlabeled NADH gained the ability to compete at these concentrations when the charge on E132 was lost [44]. There is precedent for single amino acid substitutions impacting substrate specificity in MCF carriers [31]. Mutation of a key arginine residue in the pocket of the yeast ADP/ATP carrier *Sc*AAC2 resulted in lost ability to homo-exchange ATP while retaining the homo-exchange for ADP [106]. Similarly, mutation at position S245 in the yeast ADP/ATP carrier, *Sc*AAC2p, broadened substrate selectivity enabling the transport of TTP and UTP in addition to canonical substrates [107]. Moreover, for the two human isoforms of the ornithine carriers ORC1 and ORC2, substrate specificity can be modulated by a single amino acid substitution in its pocket [108].

Notably, supraphysiological concentrations of NADH at the millimolar range block SLC25A51 activity in vitro [1,2]. This indicates that NADH can "fit" in the pocket sufficiently well to competitively block $NAD^+$ from binding, and while the charge in SLC25A51's pocket helps to distinguish the molecules, it is not the full story. The critical aspect is that SLC25A51 will likely encounter the oxidized $NAD^+$ molecule more frequently than reduced NADH in cells. This is because the cytosolic concentration of free $NAD^+$ is typically ~500–1000 fold higher than free NADH [46–48,109,110]. Cytosolic free $NAD^+$ concentrations in human cells have been corroborated by multiple groups to be 50–100 μM [45,49]. Cytosolic free NADH in human cells has been measured by multiple groups as 70–100 nM [47,48]. Thus, the higher concentration of $NAD^+$ encountered by SLC25A51—in combination with the charge discrimination conferred by E132—means that SLC25A51

functions in practice to import $NAD^+$ into mammalian mitochondria. The physiological roles for SLC25A51-dependent preferential import of oxidized $NAD^+$ is a current area of active research. One recently reported role for SLC25A51 is its transcriptional regulation in liver to support mitochondrial fatty acid oxidation in response to feed/fast cycles [4], and upregulation of SLC25A51 has been also shown as a mechanism to decouple the mitochondrial $NAD^+$/NADH redox state in AML cells [6].

## The $NAD^+$ ligand initiates its transport by disrupting the matrix gate.

After recognition of oxidized $NAD^+$ in the binding pocket of SLC25A51, the next step in import of the cognate ligand would be a linked disruption of the matrix gate to create a physical opening in the transporter, to destabilize the c-state, and to initiate transition to the m-state. Two possible mechanisms have been speculated in this family for disruption of the matrix gate, including involvement of a positive residue in the transporter's pore (predicted for keto-acid and amino-acid carriers) or via the ligand (predicted for the ADP/ATP carriers) [56].

The matrix gate in SLC25A51 is comprised of a single interdomain ionic interaction—an E139-K236 salt bridge [44]—rather than the more elaborate interhelical network of polar interactions as found in other MCF carriers. Residue R182 in SLC25A51 is at an analogously positioned location as the predicted disrupting arginine in keto-acid and amino acid carriers. However, in the case of SLC25A51, this residue is located far away from the single ionic interaction that forms the gate (Figure 3A). Moreover, as observed in simulations, bound $NAD^+$ would block R182 from accessing the E139-K236 salt bridge (Figure 3A). Thus, R182 is unlikely to be part of the mechanism for SLC25A51 gate opening, however this needs to be validated experimentally.

Molecular dynamic simulations of SLC25A51 instead identified a model where the distinctive positive charge on the nicotinamide ring of $NAD^+$ is directly used to initiate opening of the matrix gate (Figure 3C) [44]. After positioning of $NAD^+$ in the binding pocket of SLC25A51, the positive charge in the nicotinamide ring of $NAD^+$ can shuttle from binding pocket residue E132 to gate residue E139 [44]. Residue E139 is located directly below E132 (Figure 3C). Parallelly, the phosphate groups in the $NAD^+$ ligand then engage the other half of the gate via K236 (Figure 3C). This indicated that bound $NAD^+$ can initiate a transient and dynamic disruption of the salt-bridge gate [44].

A ligand-initiated gate opening would depend on alignment of the negative charges in the binding pocket and the gate. In support of this mechanism, switching the orientation of the gate residues or transposing the location of the single gate in SLC25A51 both resulted in loss of uptake function [44]. Moreover, introduction of an additional ionic interaction into the gate resulting in retained but dampened activity, indicating that opening of the gate led to initiation of transport [44].

Having the gate-opening in SLC25A51 rely on its ligand achieves several interesting consequences. It can serve as an addition layer to distinguish $NAD^+$ from NADH due to dependency on the charge. Additionally, it ensures that the opening of a weakly stabilized gate without braces predominantly occurs when it can be productive, *i.e.* when

its cognate ligand is correctly positioned. Lastly, this model implies critical roles for local concentrations of cytosolic free $NAD^+$ in its own transport and for expression levels of the SLC25A51 transporter.

## State of the field

Current work on SLC25A51 has relied on computational predictions that were validated by functional assays to elucidate substrate preference and structural features of SLC25A51 [2,44,111]. These approaches have yielded meaningful insight into the mechanism and role of this transporter. Nevertheless, the data await future testing with experimentally solved structures. The work thus far has formally depended on the inference that SLC25A51 shares structural homology with other MCF transporters. Due to divergence at key residues in SLC25A51's sequence, an experimental determination of its structure will be required to confirm its exact mechanism. Moreover, several challenges in purifying recombinant SLC25A51 will need to be addressed to move the field forward. This specific transporter has relatively lower expression levels, it is unstable when taken out of its environment, and it is dynamic. Many biophysical approaches have been elusive at the time of this review. This includes experimentally capturing its conformations, reconstituting its activity in vitro, single-molecule studies, and developing antibodies or small-molecule modulators with high-affinity and high specificity.

A deeper understanding of SLC25A51's mechanism would elucidate both intrinsic and extrinsic approaches to modulate its function. These can be developed to improve human health, bioengineering, environmental engineering, industrial synthesis, and the treatment of diseases.

## Conclusion

Multiple mechanisms contribute to the regulation of SLC25A51-dependent import of $NAD^+$ into the mitochondrial matrix. In addition to intrinsic structural characteristics of the transporter, SLC25A51 is dependent on specific binding of cardiolipin, as well as a productive fit of its cognate $NAD^+$ in its pocket to initiate transport (Figure 4). Together this implicates multiple intrinsic, extrinsic, and signaling inputs contributing to $NAD^+$ concentrations in mitochondria. These inputs represent new avenues for impacting citric acid cycle flux and respiration in human cells.

## Perspectives section

**Importance of the field—**Elucidating mechanisms for SLC25A51-dependent control of mitochondrial $NAD^+$ fills gaps in our understanding of central carbon metabolism as well as reveals the interconnectivity among signaling and post-translational modification of mitochondrial proteins, lipid metabolism, cytosolic $NAD^+$, the citric acid cycle, and cellular respiration.

**Summary of the current thinking.—**The characterization of an $NAD^+$ binding pocket in an MCF transporter has revealed mechanisms that are unique to SLC25A51, as well as other types of regulation that may apply broadly to the family. The $NAD^+$ import activity of SLC25A51 is controlled by both intrinsic structural characteristics of the transporter, as

well as extrinsic factors such as the composition of the lipid bilayer and cytosolic ligand concentrations.

**Future Directions—**One major conceptual gap that remains is to elucidate that the driving force for SLC25A51-dependent import of $NAD^+$ across its electrochemical gradient. The continued characterization of MCF transporters, nevertheless, is expected to reveal both unique and shared mechanisms. In turn, the data will provide insight and opportunities to modulate activity for research and interventions, as well as to better understand their roles in physiology and in disease contexts.

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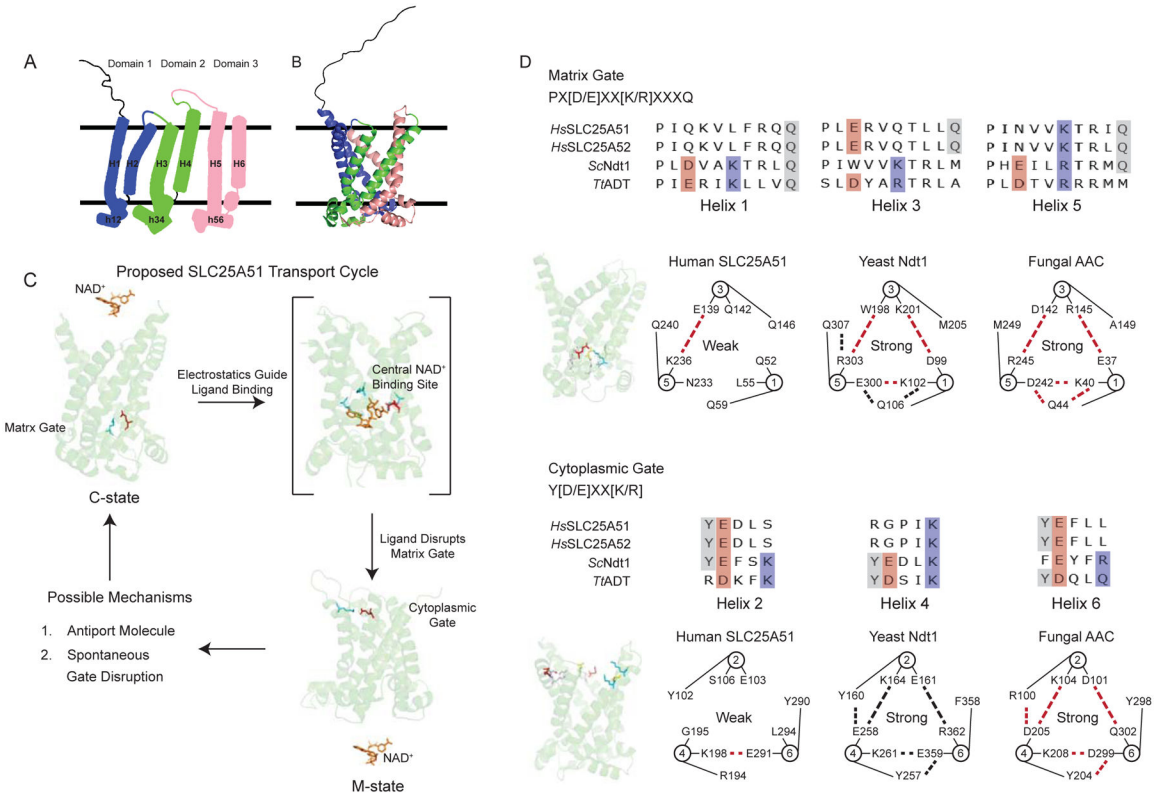


**Figure 1. Structural Features of SLC25A51 that Govern Mitochondrial NAD$^+$ Import.**

A. Predicted topology of SLC25A51 generated with Protein Imager [112]. Domains are delineated by color and numbered according to convention [15].

B. An AlphaFold2 predicted structure of SLC25A51 in cartoon representation with the repeated domains delineated by color [113].

C. Proposed conformations of SLC25A51 during its transport cycle of NAD$^+$(orange); positively charged side chains (cyan), negatively charged side chains (red).

D. Graphical representation and multiple sequence alignment of the matrix and cytoplasmic gates in mitochondrial NAD$^+$ transporters *Hs*SLC25A51 (UniProt: Q9H1U9), *Hs*SLC25A52 (UniProt: Q3SY17) and *Sc*Ndt1 (UniProt: P40556) and the fungal ADP/ATP carrier *Tt*ADT (UniProt: G2QNH0). In gate representations, helix numbers are shown in circles and dashed lines indicate polar interactions; red, experimentally supported for the transporter or its homologue [10,12,18,19,32,44,114]. Positively charged residues (cyan), negatively charged residues (red), hydrophobic residues (yellow) and polar residues (gray).

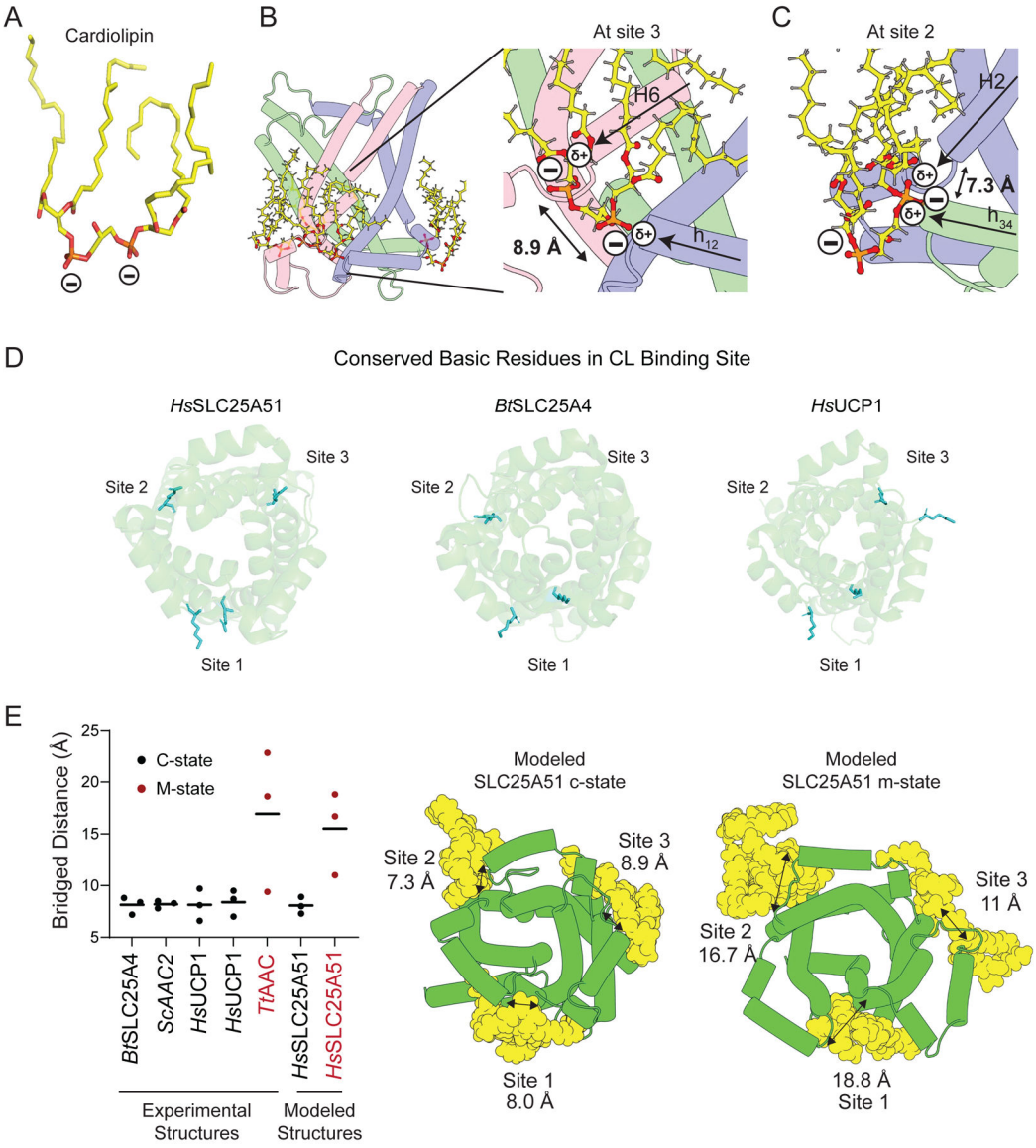


**Figure 2. Cardiolipin in the Inner Mitochondrial Membrane Regulates SLC25A51 Import Activity.**

A. Structure of a cardiolipin molecule; carbon atoms (yellow), phosphate atoms (orange), oxygen atoms (red).

B. Cardiolipin binding at site 3 of SLC25A51 represents a typical formation that utilizes the two phosphate groups to bridge protein domains 2 and 3. Black arrows indicate the direction of the helical dipoles towards their positive poles (δ+).

C. Site 2 of SLC25A51 can use one phosphate group of cardiolipin to bridge protein domains 2 and 1. Black arrows indicate the direction of the helical dipoles towards their positive poles (δ+).

D. Conserved basic residues (cyan) in the cardiolipin binding motifs in *Hs*SLC25A51, *Bt*SLC25A4 (PDB ID: 1OKC) and *Hs*UCP1 (PDB ID: 8HBV).

E. *(Left)* Measurement of distances from available experimental and homology structures of MCF carriers in their c-state (black) and m-state (red). Data is shown as individual

points for each cardiolipin binding site in the structure, with a line at the mean. *(Right)* Representatives distances cardiolipin would need to bind when SLC25A51 is either in its c-state or m-state conformation. Measurement is between the $C_\alpha$ atoms of the residues present at the conserved glycine position in the [F/Y/W]-X-G and [F/Y/W]-[K/R]-G motifs.

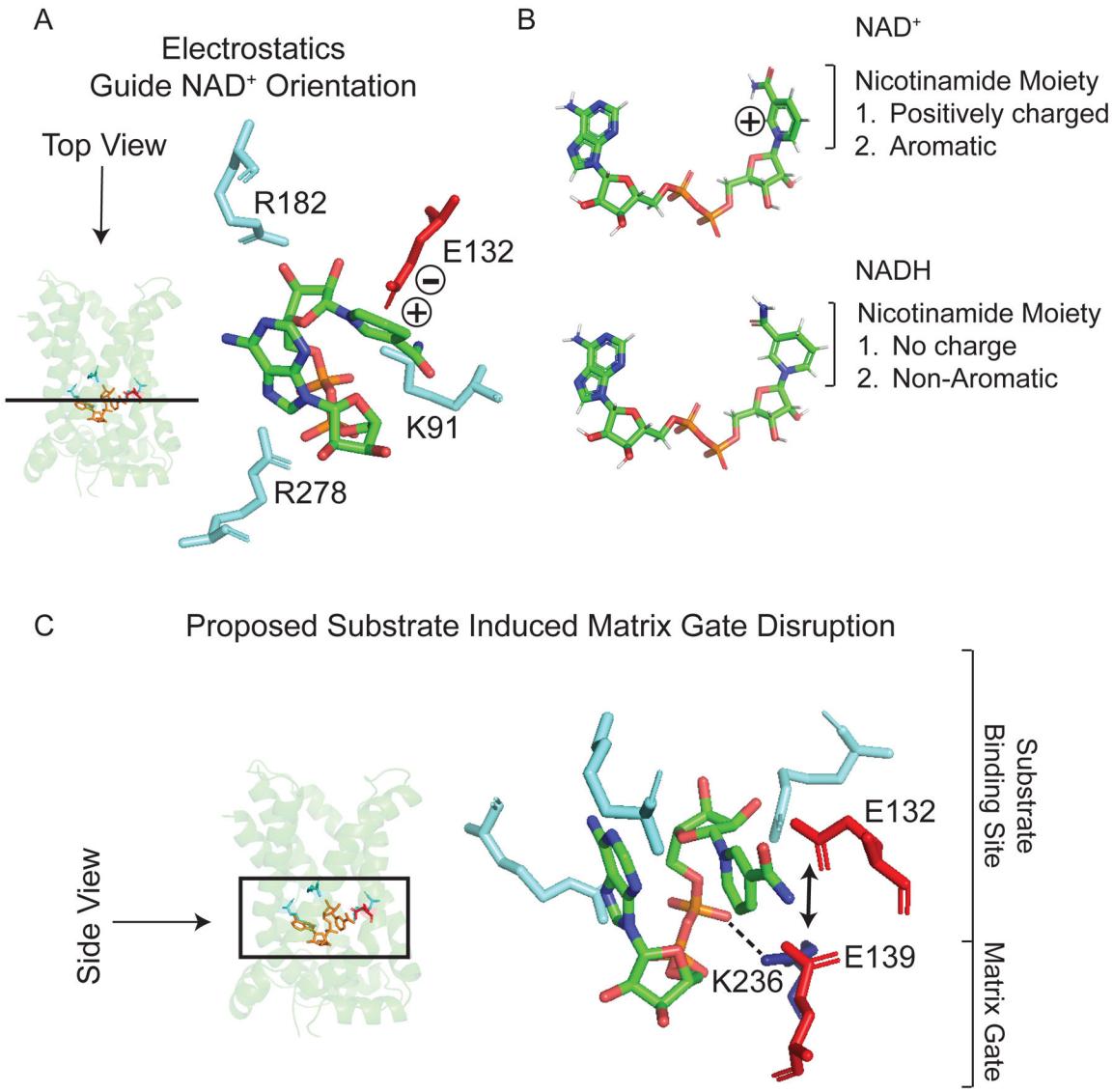


**Figure 3: Electrostatic Interactions Guide Binding of NAD$^+$ and Ligand-Initiation Gate Disruption.**

A. A positive charge on the nicotinamide ring of NAD$^+$ orients the molecule away from positively charged residues and towards E132 in the binding site.

B. Structural differences between NAD$^+$ and NADH.

C. NAD$^+$ can disrupt the E139-K236 ionic bond in the matrix gate. The nicotinamide ring may be channeled from E132 to E139 and the phosphate groups in NAD$^+$ can then engage K236.

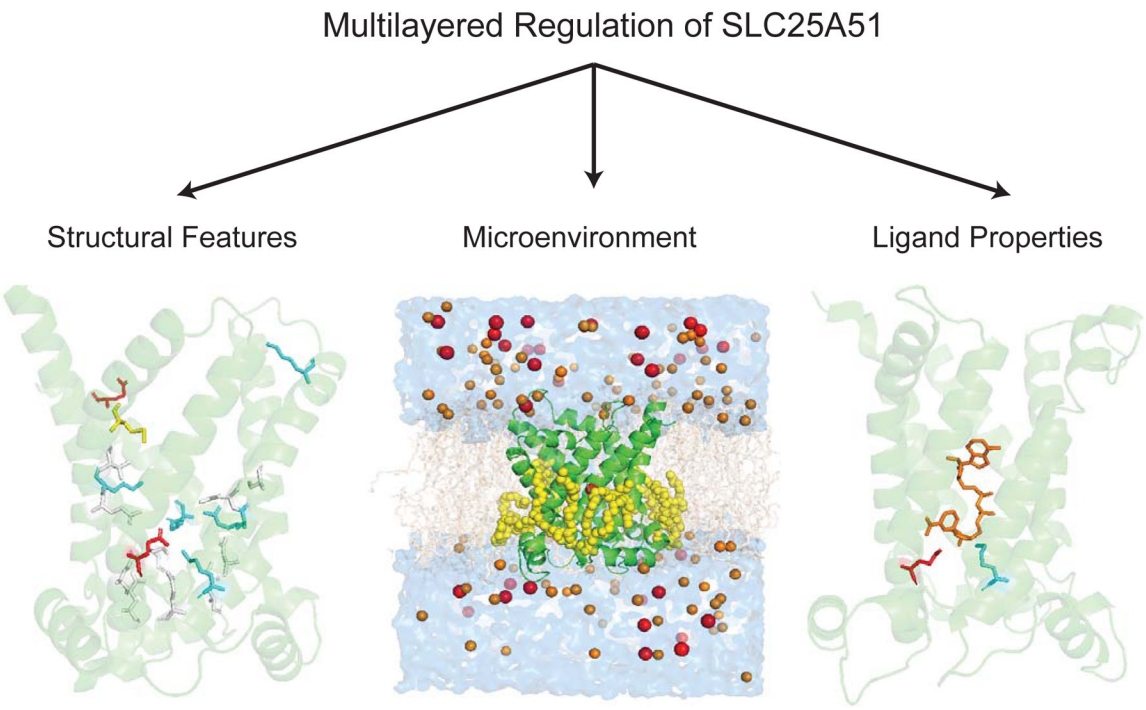


**Figure 4. Multi-layered regulation of SLC25A51.**

The $NAD^+$ import activity of SLC25A51 is impacted by intrinsic structural features in the transporter, the local composition of the inner mitochondrial membrane, and characteristic properties of its cognate ligand.

**Table 1.**

Studied Point Mutations in Human SLC25A51.

| Mutations | Predicted Function | Relative Activity | Relative stability | Reference |
|---|---|---|---|---|
| **Matrix Gate Px[D/E]xx[K/R]xRxQ** | | | | |
| Q52A | Putative hydrogen bond | ++ | **** | [44] |
| L55A | Unknown function | ++ | **** | [44] |
| E139Q | Salt Bridge | - | ** | [44] |
| E139A | Salt Bridge | - | ** | [44] |
| Q142A | Putative hydrogen bond | ++ | **** | [44] |
| N233A | Unknown function | ++ | **** | [44] |
| K236Q | Salt Bridge | - | **** | [44] |
| K236A | Salt Bridge | - | **** | [44] |
| E139Q-K236Q | Salt Bridge | - | **** | [44] |
| R57L | Cap residue | + | **** | [44] |
| R140L | Cap residue | + | ** | [44] |
| R238L | Cap residue | + | ** | [44] |
| Q59A | Brace residues | ++ | **** | [44] |
| Q149A | Brace residues | ++ | **** | [44] |
| Q240A | Brace residues | ++ | **** | [44] |
| Q52E-Q142K | Extra salt bridge | + | **** | [44] |
| E139K-K236E | Reversed salt bridge | - | ** | [44] |
| Q52E-E139Q-Q142K-K236Q | Shifted salt bridge | - | ** | [44] |
| **Cytoplasmic Gate Y[D/E]xx[K/R]** | | | | |
| E103A | Salt bridge/Hydrogen bond | ++ | **** | [44] |
| R194A | Brace | - | **** | [44] |
| R194Y | Brace | - | *** | [44] |
| K198A-E291A | Salt Bridge | ++ | **** | [44] |
| K198Q-E291Q | Salt Bridge | ++ | **** | [44] |
| **Cardiolipin Binding Site** | | | | |
| R82Q-R174Q-R270Q | Cardiolipin binding | - | ** | [44] |
| **$NAD^+$binding site** | | | | |
| Q90L | Contact site 1 | ++ | **** | [44] |
| Q90A | Contact site 1 | + + | **** | [2] |
| K91Q | Contact site 1 | - | **** | [44] |
| K91A | Contact site 1 | - | **** | [2] |
| T94V | Contact site 1 | + | *** | [44] |
| M98A | Contact site 1 | ++ | **** | [44] |
| E132A | Helix 3 | + | **** | [44] |
| E132D | Helix 3 | ++ | **** | [44] |
| R182Q | Contact site 2 | - | ** | [44] |

| Mutations | Predicted Function | Relative Activity | Relative stability | Reference |
|---|---|---|---|---|
| R182L | Contact site 2 | - | ** | [44] |
| R182A | Contact site 2 | - | * | [2] |
| N183V | Contact site 2 | - | *** | [44] |
| N183Q | Contact site 2 | - | *** | [44] |
| S186A | Contact site 2 | ++ | **** | [44] |
| R278L | Contact site 3 | - | *** | [44] |
| R278A | Contact site 3 | - | **** | [2] |
| W283A | Helix 6 | ++ | * | [2] |

Relative Activity: ++ similar to wildtype, + partially impaired and - not functional; expression in mammalian cells, **** similar to wildtype, *** >0.75 × wildtype, ** >0.5 × wildtype and * <0.5 × wildtype.