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Adenine Nucleotide Carrier Protein Dysfunction in Human Disease

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Abstract

Adenine nucleotide translocase is the prototypical member of the mitochondrial carrier protein family, primarily involved in ADP/ATP exchange across the inner mitochondrial membrane. Several carrier proteins evolutionarily related to ANT, including SLC25A24 and SLC25A25, are believed to promote the exchange of cytosolic ATP-Mg^{2+} with phosphate in the mitochondrial matrix. They allow a net accumulation of adenine nucleotides inside mitochondria, which is essential for mitochondrial biogenesis and cell growth. In the last two decades, mutations in the heart/muscle isoform 1 of ANT (ANT1) and the ATP-Mg^{2+} transporters have been found to cause a wide spectrum of human diseases by a recessive or dominant mechanism. Although loss-of-function recessive mutations cause a defect in oxidative phosphorylation and an increase in oxidative stress which drives the pathology, it is unclear how the dominant missense mutations in these proteins cause human diseases. In this review, we focus on how yeast was productively used as a model system for the understanding of these dominant diseases. We also describe the relationship between the structure and function of ANT and how this may relate to various pathologies. Particularly, mutations in Aac2, the yeast homolog of ANT, were recently found to clog the mitochondrial protein import pathway. This leads to mitochondrial Precursor Overaccumulation Stress (mPOS), characterized by the toxic accumulation of unimported mitochondrial proteins in the cytosol. We anticipate that in coming years, yeast will continue to serve as a useful model system for the mechanistic understanding of mitochondrial protein import clogging and related pathologies in humans.

1. Introduction.

Adenine nucleotide translocase (ANT) is a nuclear-encoded transmembrane protein, with a primary function in promoting ADP/ATP exchange across the inner mitochondrial membrane (IMM). Under physiological conditions, ANT imports ADP^{3-} into the mitochondrial matrix, which is coupled with the export of ATP^{4-} generated by oxidative phosphorylation (OXPHOS) into the intermembrane space (IMS). This ADP/ATP exchange process occurs at a 1:1 stoichiometry and is electrogenic in nature, mainly driven by the

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membrane potential of the IMM. ANT belongs to the mitochondrial carrier protein family called the solute carrier family SLC25. The SLC25 family consists of 53 members in humans, predominantly present on the IMM. These proteins participate in the transport of amino acids, nucleic acids, phosphate groups, and other metabolites across the IMM [1 2, 3]. ANT has four isoforms in humans, namely ANT1, ANT2, ANT3, and ANT4. ANT1 is predominantly expressed in the heart, skeletal muscle, and the central nervous system. ANT2 is expressed in the liver and proliferative tissues. ANT3 is ubiquitously expressed [4]. ANT4 is predominantly expressed in testis and germ cells, with low-level expression in brain and liver tissues [5, 6]. Of these, ANT1, encoded by *SLC25A4*, is the most well-studied isoform. Humans also express three additional mitochondrial nucleotide carriers that are evolutionarily related to ANT: SLC25A23 (also known as APC2 or SCaMC-3), SLC25A24 (APC1 or SCaMC-1), and SLC25A25 (APC3 or SCaMC-2) [7, 8]. In contrast to ANT, these carriers have an N-terminal extension of ~20 kDa that contains EF-hand Ca^{2+}-binding sites. They mediate Ca^{2+}-dependent import of cytosolic Mg^{2+}-ATP in exchange for matrix phosphate (Pi). These proteins are therefore also referred to as ATP-Mg/Pi Carrier (APC) or Short Calcium-binding Mitochondrial Carriers (SCaMC). They likely promote the net uptake of adenine nucleotide and may serve as a mechanism that can couple cytosolic calcium with mitochondrial biogenesis and/or metabolism, without direct entry of calcium into mitochondria.

Mitochondrial carrier proteins are highly conserved throughout evolution. In the budding yeast *Saccharomyces cerevisiae*, ANT is encoded by three AAC (ADP/ATP carrier) genes: *AAC1*, *AAC2* and *AAC3*. Under aerobic conditions, Aac2 is the major isoform expressed and Aac1 is poorly expressed. Aac3 is expressed only under anaerobic conditions, likely playing a role in the reversal of ATP (matrix)/ADP(cytosol) exchange to sustain mitochondrial biogenesis in the absence of ATP synthesis inside mitochondria. *S. cerevisiae* has only one Ca^{2+}-dependent mitochondrial carrier protein, named Sal1 (for Suppressor of *aac2*-lethality), which has been shown to functionally interact with Aac2 [9].

In the last two decades, a number of mutations have been discovered in mitochondrial carrier proteins including *ANT1*, *SLC25A24,* and *SLC25A25*, which are linked to a wide spectrum of human pathologies [10 11]. Loss of function mutations in ANT1 impair ADP/ATP exchange across the IMM. This stalls the electron transport chain, which is often manifested by cardiomyopathy and muscle dysfunction. However, in some cases human diseases are caused by dominant mutations in these carrier proteins. Understanding how the dominant mutations affect cellular function and tissue homeostasis has been challenging. Yeast has been a useful model for dissecting the role of dominant mutations. In this review, we focus on the use of yeast as a model system to improve our understanding of how mutant ANT1 and SCaMC proteins affect human health by mechanisms beyond adenine nucleotide transport.

2. Structure and function of adenine nucleotide translocase and related mitochondrial carrier proteins.

As a prototype of mitochondrial carrier proteins, ANT has been extensively studied in different organisms. ANT is made of three repeats of a ~100 amino acid sequence. Each of these repeats forms two transmembrane domains [12, 13]. Early studies showed that the ADP/ATP exchange activity is inhibited by several chemical agents such as the plant-derived carboxyatractyloside (CATR) and the bacteria-derived bongkrekic acid (BKA). CATR binds ANT from the cytosolic side, whereas BKA binds from the matrix side, therefore creating the 'cytosol-open' (or c-state) or in the 'matrix-open' (or m-state) conformation respectively [14–16]. These predicted conformations have lately been confirmed by crystal structure of bovine Ant1 and yeast Aac2, and the thermophilic fungus *Thermothelomyces thermophilus* Aac protein, complexed with CATR or BKA respectively [17–20] (Figure 1). ADP/ATP exchange by ANT has initially been proposed to occur by the simultaneous (sequential) mechanism of transport [21]. In this model, the nucleotide substrates form a ternary complex by binding to the carrier from either side of the membrane, e.g., ATP on the matrix and ADP on the cytosolic side. This is followed by conformational changes, promoting the movement and release of the substrates in opposite directions. This model was predicted based on the assumption that Ant1 functions as a homodimer. Briefly, one protomer is in c-state and the other in m-state, which bind the substrates simultaneously from the two sides of the membrane. Following substrate binding and conformational changes to the carrier, the two protomers release their bound substrate on the other side of the membrane, and the cycle repeats itself. However, a different model of transport, known as the ping-pong model, has gained increasing support for the ANT-catalyzed nucleotide transport process. In contrast to the sequential model, the ping-pong model predicts that one substrate binds to the carrier from one side of the membrane. After translocation through the membrane and substrate dissociation from the carrier on the other side, the counterion substrate is bound, which initiates the translocation process in the opposite direction. This mode of operation fits with the structural data showing that ANT1/Aac2 monomer is sufficient to form a substrate translocation path and that there is only one substrate binding site in an ANT monomer [18–20, 22–24].

In addition to its function in promoting ADP/ATP exchange that supports oxidative phosphorylation (OXPHOS) and therefore mitochondrial respiration (the R function), the yeast Aac2 appears to play a role in mitochondrial biogenesis by a mechanism that is distinct from ADP/ATP exchange. The Chen laboratory found that the yeast Aac2 and Aac3 (but not Aac1) isoforms define a function essential for cell viability (the V function) even on a medium containing fermentable carbon sources[9]. Given that the R function is dispensable for cell survival under these conditions, this finding suggested that loss of V function likely affects a process essential for mitochondrial biogenesis. Interestingly, loss of the V function can be suppressed by the Ca^{2+}-dependent mitochondrial carrier Sal1 and the human SLC25A25 protein [25]. As SLC25A25 is assumed to mediate $ATP_{(cytosol)}/Pi_{(matrix)}$ exchange, it is possible that Aac2 functionally overlaps with Sal1 in promoting $ATP_{(cytosol)}/Pi_{(matrix)}$ exchange. This allows a net accumulation of adenine nucleotides inside mitochondria which may be essential for sustaining cell growth. The V function of Aac2 has been shown to

be required for mitochondrial protein synthesis and mitochondrial DNA maintenance which are known to be critical for the survival of cells lacking endogenous *AAC2*. It seems that Sal1 transports ATP-Mg^{2+}, suggesting that V function of Aac2 is also involved in ATP-Mg^{2+} uptake [26–28]. On the other hand, genetic studies showed that Aac2 variants mutated at the amino acids Arg253 and Arg254 have only a moderate reduction in V function. These two amino acids are critical for adenine nucleotide binding and translocation [20]. Thus, the possibility that the V function involves a process distinct from adenine nucleotide transport cannot be completely excluded. Another unanswered question is whether the mammalian ANT possesses the V function in addition to its role in promoting ADP/ATP exchange.

Several studies have shown that the yeast Aac2 physically interacts with respiratory supercomplexes and the TIM23 protein translocase, and that this association is evolutionarily conserved [29–31]. Loss of Aac2 in yeast decreases complex IV activity [29, 30, 32–34]. Loss of Aac2 activity has been shown to reduce the translation of mtDNA-encoded complex IV subunits [35]. This suggests that the yeast Aac2 may play a role in regulating oxidative phosphorylation either by affecting a mitochondrial process (e.g., adenine nucleotide homeostasis) or by directly affecting the biogenesis of the OXPHOS pathway through physical interactions.

Finally, ANT has also been proposed to play a role in the formation of the mitochondrial permeability transition pore (mPTP). Descriptions of the inner mitochondrial membrane's increased permeability to solutes gained traction in the late 1950s [36–39]. However, the concept of a proteinaceous pore that was activated by Ca^{2+} overloading in the mitochondria and led to mitochondrial swelling, rupture of the mitochondrial outer membrane and release of proapoptotic intermembrane space proteins into the cytosol was first described in the late 1970s and 1980s [40–42]. Observations such as ADP inhibiting mPTP, or ANT inhibitor atractyloside stimulating mPTP suggested that Ant1 may play a role in mediating the mPTP transition when present in a particular conformational state. However, the contribution of ANT to mPTP has remained an area of intrigue and controversy in the last few decades. An early study from the Wallace group found that ANT isoforms were not essential to induce mitochondrial permeability transition. They deleted two ANT isoforms in a mouse model and assayed for mPTP activation in mitochondria isolated from hepatocytes. The isolated mitochondria underwent permeability transition but had an increased calcium requirement for mPTP induction. This study concluded that ANT was not essential for mPTP activation but contributed to mPTP regulation [43]. In 2019, a similar study utilizing liver mitochondria from mice deficient in three of the four different isoforms of ANT (ANT1, ANT2, or ANT4) showed that loss of these three isoforms resulted in an increased requirement of Ca^{2+} ions to induce permeability transition in the IMM compared to mitochondria from wild-type mice [44]. Electrophysiological analysis using mitoplasts from mouse embryonic fibroblasts of Ant-triple-knockout mice showed no mitochondrial depolarization in the presence of calcium overload, unlike what would be expected if the mPTP was present. Some rare "pore-like" activity was detected in the mitochondria of these mutant mice but was insensitive to inhibition from ADP. This study concluded that the increased calcium requirement in the absence of the three ANT isoforms, as well as the minuscule electrophysiological signal in the mitochondria of ANT-deficient mutant mice in the presence of calcium overload implied that the ANTs constituted the major component of mPTP. Such contrasting interpretations

suggest that more work needs to be done in establishing the composition of mPTP and the mechanism of mitochondrial permeability transition, as researchers continue to appreciate that different proteins, ANTs included, may be acting in parallel with partial redundancy to help facilitate mPTP induction [45].

3. Biogenesis of adenine nucleotide translocase.

The majority of mitochondrial proteins, including ANT, are encoded by the nuclear genome and synthesized in the cytosol, before reaching their sub-compartmental destinations. The different mitochondrial protein import pathways have been extensively reviewed elsewhere [46]. Briefly, Tom40 in yeast or TOMM40 in mammals is the beta-barrel protein that forms a channel of the Translocase of the Outer Membrane (TOM) complex which allows for the translocation of mitochondrial proteins across the outer mitochondrial membrane (OMM). Almost all mitochondrial proteins traverse the TOM complex, apart from a few outer mitochondrial membrane proteins that utilize the Mitochondrial Import Machinery (MIM) complex instead. The proteins that traverse the TOM complex then go on to be substrates for one of the following downstream pathways. They utilize the mitochondrial Sorting and Assembly Machinery (SAM) complex if they are to be inserted into the OMM or the Mitochondrial Intermembrane Space Assembly (MIA) complex for intermembrane space localization. For proteins that have traversed TOM40 and need to be inserted into or cross the IMM, two distinct pathways exist. The Translocase of the Inner Membrane 23 (TIM23) pathway is responsible for importing matrix proteins as well as IMM proteins that contain a cleavable presequence motif. The Translocase of the Inner Membrane 22 (TIM22) pathway imports those IMM proteins that lack a presequence motif, such as ANT and the other SLC25 mitochondrial carrier proteins.

The import of ANT and other carrier proteins through the TIM22 pathway has been well studied, but certain details remain to be clarified. The post-translational events in the cytosol, for example, are not well understood, although nascent carrier proteins are known to bind cytosolic chaperones such as HSP70 and HSP90 for delivery to the mitochondria [47]. Whether or not there is partial protein folding of carrier preproteins or whether they remain completely unfolded in the cytosol is unclear. The chaperones then bind to Tom70, which is loosely associated with the TOM complex, allowing the ATP-dependent insertion of the carrier proteins into the central Tom40 channel [47, 48]. Interestingly, the carrier preproteins are known to adopt a hairpin loop conformation for insertion through the TOM complex, such that the transmembrane α-helices are placed antiparallel to one another and the loops connecting the helices are inserted into the TOM complex pore first, before the N- or C-terminus [49, 50]. As discussed later in the article, this may be a crucial step in the biogenesis of carrier proteins, and mutations in these transmembrane α-helices that disrupt the helix-loop-helix formation could be detrimental for mitochondrial health and cell viability. After transition through the TOM complex, the carrier proteins are retrieved by hexameric chaperones composed of the small Tim proteins (Tim9, Tim10 and Tim12) [50–56]. Intriguingly, the residual secondary structure of the carrier preproteins may also be chaperoned by small Tim proteins [56]. The small Tim chaperones then deliver the proteins to the TIM22 complex on the IMM for final membrane-potential-dependent insertion into the IMM [57–62]. The TIM22 complex was shown to form two pores, suggesting a mechanism

in which presequenceless proteins are imported into the pore and then laterally released into the IMM [60]. However, recent structures of the human TIM22 complex lack a central pore [59]. The mechanism of membrane insertion of carrier proteins requires further study. After delivery to the IMM, cardiolipin plays an important role in the structural maturation and functional maintenance of ANT. A study based in yeast was able to determine that cardiolipin assists in the formation of ternary and quaternary Aac2 assemblies. Cardiolipin was also found to facilitate the interaction between Aac2 and the respiratory complexes III and IV [63].

4. Adenine Nucleotide Translocase and related nucleotide transporters in disease

Mutations in human ANT isoforms have been associated with various pathologies affecting different organ systems. Mutations in the *SLC25A4* gene (encoding ANT1) can lead to complete loss of nucleotide transport activity, which is in many cases manifested by hypertrophic cardiomyopathy and myopathy. Interestingly, missense mutations in *ANT1* have also been found to cause diseases including (1) autosomal dominant Progressive External Ophthalmoplegia (adPEO), (2) neuropsychiatric disturbances such as seizures, encephalopathies, and deafness; (3) musculoskeletal pathologies such as skeletal muscle dysfunction, and dystonia; and (4) cardiomyopathy [64–71]. For a comprehensive list of *ANT1* mutations, refer to Table 1.

4.1 Diseases associated with loss of ANT1 function

Wallace and coworkers established the first mitochondrial disease mouse model by knocking out the *SLC25A4* gene encoding ANT1 [72]. The *ANT1*-deficient mice displayed cardiac hypertrophy and mitochondrial myopathy, exercise intolerance and lactic acidosis. ANT1 loss causes respiratory deficiency. Mitochondrial amplification was observed, likely caused by compensatory transcriptional activation of OXPHOS genes [73]. Additionally, there was a reduction in mitophagy which suggested a mitophagy-related role for ANT1 [74]. Mitochondria isolated from skeletal muscle, heart, and brain of the ANT1-deficient mice have increased production of reactive oxygen species and accumulation of mtDNA rearrangements [75]. ROS detoxification enzyme such as manganese superoxide dismutase (Sod2) is increased in heart tissue but not in isolated heart mitochondria, suggesting that Sod2 is likely partially retained in the cytosol due to severe mitochondrial stress and reduced mitochondrial protein import efficiency. Subsequent studies from this group showed that loss of Ant1 also leads to dilated cardiomyopathy in mice that worsened with age [76]. Remarkably, loss-of-function *SLC25A4* mutations were later found to be pathogenic in humans, causing autosomal recessive myopathy and cardiomyopathy [77]. This strongly validated the pre-clinical work in *SLC25A4* knockout mice. The homozygous patients exhibit progressive myocardial thickening, hyperalaninemia, lactic acidosis, exercise intolerance, and persistent adrenergic activation. Mitochondrial amplification was also observed. Metabolic alterations have also been observed that include L-2 hydroxyglutaric aciduria and hyperlactatemia [70, 78]. Consistent with a critical role for ANT1 activity in cardiac health, Palmieri and colleagues showed that a patient homozygous for the *SLC25A4 (p.A123D)* developed hypertrophic cardiomyopathy [67]. Although this mutation eliminates

ADP/ATP exchange activity, subsequent studies in yeast showed that this is actually a gain-of-toxicity mutation affecting mitochondrial protein import (see below).

Another well-described cardiac pathology associated with ANT1-deficiency is Sengers syndrome. It is characterized by congenital cataracts, hypertrophic cardiomyopathy, and skeletal myopathy [79–81]. Early studies documented a severe depletion of Ant1 in heart and muscle tissues. However, no mutations were found in *ANT1*. Subsequent studies showed that Sengers syndrome is caused by mutations in the gene encoding acyl glycerol kinase or AGK [82–84]. AGK is also a subunit of the TIM22 complex present on the IMM which facilitates the import of mitochondrial carriers and other mitochondrial proteins that lack a cleavable presequence. Since ANT1 requires TIM22 for import [61], *AGK* mutations would be expected to prevent ANT1 from being inserted into the IMM thereby causing ANT1 deficiency. Given that the defective TIM22 complex is also expected to affect the import of IMM proteins other than ANT1, it is possible that factors other than ANT1 depletion (e.g., cytosolic proteostatic stress, see below) may also contribute to Sengers syndrome.

More recently, reduced ANT expression has been proposed to play a role in pulmonary diseases. In a genetic screen conducted on the amoeba *Dictyostelium discoideum*, Kliment and colleagues found that overexpression of ANT1 and ANT2 could suppress cigarette smoke toxicity [85]. Interestingly, both ANT1 and ANT2 were reduced by cigarette smoke in mice and in the lungs of COPD patients, suggesting a role for reduced ANT expression in the development of cigarette smoke-induced COPD. Although mechanistic details are less clear, it was proposed that reduced ANT1 expression limited mitochondrial metabolism and enhanced airway hydration through nucleotide transport via plasma membrane-associated ANT localization [85]. ANT1 may also be reduced in idiopathic pulmonary fibrosis (IPF), a chronic lung disease thought to be driven by cellular senescence [86]. Interestingly, knockdown of ANT1 could induce senescence in lung epithelial cells and global ANT1 knock-out increased senescence and fibrosis in two mouse models of IPF [87]. The mechanistic details linking ANT deficiency and cellular senescence in the lung will be an exciting area of future investigation.

4.2 Diseases associated with ANT1 overexpression

Overexpression of wild-type ANT1 is linked to a form of skeletal myopathy called facioscapulohumeral dystrophy or FSHD. While FSHD has been described in the literature as being driven by under-repressed expression of the transcription factor DUX4 [88], *SLC25A4* occupies a nearby de-repressed genomic neighborhood and is also transcriptionally activated in FSHD [89]. In line with this, the ANT1 protein levels seem to be consistently upregulated in FSHD muscle [90, 91]. Recently, the Chen group showed that ANT1 overexpression indeed is sufficient to cause progressive muscle wasting in a mouse model [92]. The mechanism of how increased ANT1 may cause skeletal muscle atrophy is under study, but preliminary evidence suggests that it may involve ANT1-induced mitochondrial protein import stress and imbalanced protein synthesis and degradation in the cytosol. It is therefore possible that ANT1 overexpression contributes to FSHD pathogenesis.

Myocardial infarction is linked to high morbidity and exercise intolerance. The immune microenvironment of the heart plays an important role in supporting cardiac recovery from a myocardial infarction. Yergoz and colleagues discovered that ANT1 expression levels can modulate the immune response in the setting of myocardial ischemia. Using a rat model of ANT1 overexpression (ANT1-TG) and inducing myocardial infarction in these animals, the authors discovered that ANT1 overexpression in the heart was associated with greater anti-inflammatory signaling in the cardiac microenvironment relative to ANT1 wild-type hearts. The authors concluded that Ant1 overexpression in the rat heart is cardioprotective, by facilitating the cardiac immune microenvironment to secrete anti-inflammatory cytokines which allowed the heart to recover from the ischemic insult. They described that Ant1 overexpression was able to induce cardiomyocytes to secrete Hsp27 which acted as an inflammation-inhibiting signal for surrounding immune cells [93]. Another study also emphasized the cardioprotective effects of ANT1 overexpression in cardiomyocytes using the same rat model [94]. However, this study discovered that ANT1-mediated cardioprotection under settings of hypoxia was due to the increased activation of extracellular signal-regulated kinases ERK1 and ERK2, protein kinase B (AKT) and hypoxia-inducible factor 1 alpha (HIF1a) in Ant1-TG rat hearts relative to WT under hypoxia. These studies underscore the importance of ANT1 in maintaining cardiac health.

4.3 Diseases associated with dominant missense mutations in ANT1

Dominant missense mutations have been found in *SLC25A4* (*ANT1*) that cause autosomal dominant progressive external ophthalmoplegia (adPEO), manifested by adult or late onset muscle weakness (especially in eye muscles), exercise intolerance, sensory ataxia, hypertrophic cardiomyopathy, and myopathy. Multiple mtDNA deletions and mild defects in the respiratory complexes were detected in affected skeletal muscle. These mutations in *ANT1* include A114P, A90D, L98P, D104G, and V289M [65, 95–97]. While the *de novo* V289M mutation was initially detected in an adPEO patient, a subsequent study showed that the patient also carries a mutation in the *POLG1* gene encoding mitochondrial DNA polymerase γ, which is a known locus associated with multiple mtDNA deletions and adPEO [98]. The pathogenicity of the ANT1 p.V289M mutation is therefore unclear.

The clinical manifestations of diseases caused by missense mutations in *ANT1* are variable. In addition to adPEO, some develop other symptoms. For example, The A90D and L98P alleles are associated with neuropsychiatric conditions such as schizoaffective and bipolar disorders [68, 95]. The A114P mutation has been reported to be associated with dementia [99]. Interestingly, Thompson and colleagues reported two *de novo* missense mutations, namely R80H and R235G, that are linked to an infantile-onset mitochondrial disease in heterozygous patients, characterized by epileptic encephalopathy and skeletal muscle atrophy [69]. Additionally, the R80H mutation in ANT1 has been linked to a form of mitochondrial DNA depletion syndrome, characterized by severe hypotonia, respiratory insufficiency, and in some cases the development of hypertrophic cardiomyopathy [64]. Another recent study described a dominant *K33Q* mutation in ANT1 that presented with a childhood-onset mild skeletal myopathy without the existence of cardiomyopathy, encephalopathy, or ophthalmoplegia. The overall protein level of ANT1 was unaffected in patients' muscle biopsies. The study concluded that the mild clinical phenotype in the

presence of a dominant mutation may have been due to sufficient levels of the wild-type *SLC25A4* allele, as well as other ANT isoforms compensated for the mutant protein [100].

Collectively, dominant missense mutations are clinically distinct from recessive mutations in *SLC25A4*. They often display much more severe phenotypes compared to loss-of-function recessive mutations. Using a yeast model, it was found that these diseases involve a mechanism distinct from the loss of nucleotide transport activity, as discussed more in sections 6.3 and 6.4.

5. Calcium-dependent adenine nucleotide transporters and disease

As previously described, *SLC25A24* and *SLC25A25* encode calcium-dependent ATP-Mg^{2+}/Pi carrier proteins present on the IMM. Mutations in these two genes have been implicated in various pathologies linked to mitochondrial dysfunction. The heterozygous *de novo* R217H and R217C mutations in *SLC25A24* cause Gorlin-Chaudhry-Moss syndrome (GCMS) that manifests as coronal craniosynostosis, midface hypoplasia, hypertrichosis, microphthalmia, short stature, and short distal phalanges. Fibroblasts cultured from affected individuals showed signs of mitochondrial swelling, decreased ATP content in the mitochondrial matrix, and elevated mitochondrial membrane potential upon exposure to hydrogen peroxide when compared to fibroblasts from unaffected (control) individuals. Further, there was no reduction in mitochondrial DNA copy number in the fibroblasts derived from the affected individuals. Collectively, these observations suggest that *SLC25A24* mutations affect mitochondrial bioenergetics and cause increased sensitivity to oxidative stress [101]. Interestingly, a rare genetic condition called Fontaine syndrome has also been linked to the same mutations (R217C and R217H) in *SLC25A24* gene. This syndrome is characterized by premature aging, decreased fat and hair growth, bone dysplasia, distinctive facial features, and early death like that seen in progeria [102].

Another member of the calcium-dependent carrier family that has been implicated in human disease is *SLC25A25*. The Q349H mutation in *SLC25A25* was found to cause an autosomal dominant form of hereditary nephrolithiasis. The glutamine to histidine substitution in the protein was suggested to perturb a conserved polar interaction leading to structural instability in the mutant protein. Unsurprisingly, it was found that the mutant protein only had ~20% of the wild-type protein's ATP transport activity [103].

Interestingly, just as in dominant *ANT1* mutations, the dominant mutations in *SLC25A24* and *SLC25A25* described here all occur in the transmembrane α-helices. This may point to a particular vulnerability of these protein domains across the mitochondrial carriers. This idea is discussed in more detail in Section 6.4.

6. Yeast as a model system for the understanding of ANT-induced mitochondrial diseases

6.1. Yeast models of missense mutations.

S. cerevisiae provides a genetically amenable system to study mitochondrial dysfunction due to the presence of conserved biomolecular pathways between yeast and humans coupled

with a smaller genome size (approximately 6,000 protein-coding genes in yeast compared to approximately 20,000-25,000 in humans). Complete loss of mitochondrial respiration does not inhibit cell growth on fermentable carbon sources, allowing deep molecular and biochemical characterization of cells deficient in oxidative phosphorylation. Early studies in yeast have played a critical role in establishing the biochemical properties of ANT and dissecting its role in mitochondrial respiration and biogenesis. In more recent years, yeast has also provided a powerful system for the understanding of dominant diseases caused by mutant ANT.

Several studies have characterized the dominant missense mutations causing adPEO, by introducing equivalent mutations into the yeast Aac2 protein. These mutations include ANT1^{L98P}, ANT1^{A90D}, ANT1^{A114P} and ANT1^{V289M}, equivalent to Aac2^{M114P}, Aac2^{A106D}, Aac2^{A128P} and Aac2^{S303M}. The remaining adPEO-related dominant missense mutation ANT1^{D104G} does not map to yeast Aac2 but chimeric complementation studies have successfully reported its pathologic nature in a yeast model (Figure 2). Numerous studies have shown that these mutant *aac2* alleles have either a reduced or complete loss of activity in supporting respiratory growth on non-fermentable carbon sources in haploid cells disrupted of the endogenous *AAC2* [32, 65, 67]. It was also found that when the wild-type and mutant *aac2* were co-expressed in heteroallelic haploid cells, the level of mitochondrial respiration remained below wild-type levels [32]. A similar phenomenon was observed with *aac2*^{R96H} and *aac2*^{R252G}, mimicking the early onset pathogenic *ANT1*^{R80H} and *ANT1*^{R235G} alleles in humans [104]. These suggested a dominant effect of the mutant Aac2 protein, consistent with the dominant nature of diseases caused by mutant ANT1. Finally, it was also found that expression of the catalytically dead Aac2^{A137D} (equivalent to the cardiomyopathy-related mutant ANT1^{A123D}) reduces complex IV activity, to an extent that is greater than that caused by a null *aac2* allele [35]. Taken together, these results suggest a gain of toxicity by the mutant protein that may globally affect mitochondrial function. Another relevant note is that the mutant Aac2 variants seem to have altered nucleotide transport activities. Using *in vitro* reconstituted proteoliposomes, it was found that Aac2^{A128P}, Aac2^{M114P} and Aac2^{S303M} seem to preferentially import ATP over ADP. This was proposed to cause ATP/ADP imbalance and increased dATP level, which in turn affects the accuracy of mtDNA replication [32]. In fact, the presence of multiple mtDNA deletions is a hallmark of adPEO [65, 105], although whether moderate levels of mtDNA deletions are the main pathogenic factor remains unclear.

In the last two decades, studies from the Chen laboratory showed that expression of yeast *aac2* alleles resembling the human pathogenic *SLC25A4* mutations exhibit severe dominant effects that strongly inhibit cell growth even on fermentable carbon sources [106, 107]. Yeast cells co-expressing the mutant *aac2* alleles and the wild-type *AAC2* have reduced cellular respiration, low membrane potential, increased mtDNA instability and complete inhibition of cell growth on complete medium with fermentable carbon sources. This phenotype is particularly strong in yeast strains of non-W303 background and when cells are grown at low temperatures. It is currently unclear why strains in the W303 background are less susceptible to the expression of mutant Aac2. Nor is it known why low temperature accentuates the effect of the mutant Aac2 in inhibiting cell growth. Double mutants combining *aac2*^{A128P} with mutations in the Arg252-254 triplet were generated [107]. Although

the arginine mutations are known to completely mitigate adenine transport function, they negligibly affected the inhibition of cell growth by *aac2*^{A128P} in cells expressing the double mutant alleles. Cellular damage caused by mutant Aac2 is therefore driven by some unknown gain-of-function toxic mechanism independent of nucleotide transport and oxidative phosphorylation. This is consistent with clinical observations described above, that dominant ANT1-induced syndromes often have clinical features absent in recessive loss-of-function ANT1 disease.

6.2. Cellular proteostasis and mitochondrial precursor overaccumulation stress (mPOS).

To understand the mechanism by which the "pathogenic" variants of Aac2 cause the OXPHOS-independent inhibition of cell growth, the Chen laboratory screened for conditions that suppress Aac2-mediated cell toxicity. An early study showed that genetic manipulations that reduce cytosolic protein synthesis robustly suppressed cell growth inhibition caused by Aac2^{A128P} [108]. These include the disruption of *RPL6B*, *REI1,* and *SCH9*. *RPL6B* encodes a component of the 60S ribosomal subunit. *REI1* is involved in ribosomal biogenesis. *SCH9* encodes a protein kinase in the TOR signaling pathway. These observations suggested that expression of mutant Aac2 causes proteostatic stress in mitochondria, which is alleviated by reducing global protein synthesis and therefore protein loading to mitochondria. Another possibility is that expression of mutant Aac2 causes proteostatic stress in the cytosol, which is suppressed by reduced protein synthesis.

When screening for yeast genes that suppress cell lethality caused by the expression of the *aac2*^{A128P} allele, the Chen lab discovered that genes involved in reducing cytosolic protein synthesis and those that promote cytosolic protein quality control were the most enriched [109]. This observation suggested that a form of mitochondria-induced proteostatic stress was being relayed to the cytosol. Further proteomic analysis revealed the presence of unimported mitochondrial precursor proteins accumulating in the cytosol. This piece of critical evidence suggested that the overexpression of mutant Aac2 protein somehow prevents other mitochondrial proteins from getting imported, resulting in their accumulation in the cytosol. This accumulation was found to result in cytosolic aggregation of mitochondrial proteins as well as synergistic toxicity with ectopic expression of the aggregation-prone cytosolic protein Huntingtin. Co-aggregation of cytosolic mitochondrial precursors and pathogenic aggregation-prone cytosolic proteins was later independently replicated [110]. Intriguingly, stimulation of mitochondrial protein import was also found to mitigate toxicity driven by pathogenic cytosolic protein aggregation, providing further evidence of mitochondrial precursor-associated toxicity in the cytosol [111]. The cytosolic proteostatic stress imposed by unimported mitochondrial proteins was termed mitochondrial precursor overaccumulation stress or mPOS [109]. The discovery of mPOS raised the possibility that protein import defects and cytosolic proteostatic stress could underlie dominant ANT1-induced disease [112].

6.3. Mitochondrial protein import clogging as a mechanism for mPOS

What is the molecular mechanism of mutant Aac2-induced protein import defects? Possible contributors include reduction in membrane potential [107], mutant Aac2 misfolding and resultant proteostatic stress on the inner mitochondrial membrane causing destabilization of the TIM22 complex [113, 114], and/or additional mechanisms. Interestingly, our published and

unpublished work showed that expression of mutant Ant1 in human cells robustly induced mPOS without reducing membrane potential or destabilizing the TIM22 complex, arguing against these processes as primary drivers of mPOS [115]. Subsequent studies uncovered that pathogenic Aac2 and Ant1 proteins directly clog the mitochondrial protein import translocase channels, providing a direct mechanistic explanation for mPOS [116]. In both yeast and human cells, mutant Aac2/ANT1 proteins had increased association with the protein translocase channels, which was consistent with proteomic evidence of mPOS in the cytosol. Most strikingly, mutant Aac2 (particularly Aac2 containing two dominant mutations) was unable to efficiently traverse the TOM complex when imported in vitro into wild-type, fully energized yeast mitochondria [116]. This finding demonstrated that the biophysical properties of the mutant Aac2, as opposed to secondary mitochondrial damage, are responsible for the clogging phenotype. Clogging at the TOM complex is sufficient to obstruct protein traffic into the mitochondria, leading to mPOS.

The specific import step that is impaired in handling mutant Aac2/Ant1 is still unclear, though the data suggest it involves the TOM complex. Three non-mutually exclusive possibilities exist. First, it is possible that the substitutions placing proline and aspartic into the transmembrane α-helices disrupt the “hairpin loop” structure that forms as carrier proteins are inserted into the TOM complex. This could generate steric hindrance such that the new width of the hairpin loop cannot efficiently travel through the Tom40 pore. Second, it is possible that the interactions between the carrier preprotein and the inner surface of the Tom40 pore are disrupted by the substitutions. Finally, it could be that association with the small Tim chaperones is disrupted by the mutations, effectively “stranding” the carrier preprotein at the exit site of the TOM complex. This possibility is supported by the observation that carriers display residual secondary structure as they are chaperoned by the small Tim chaperones [56]. Future work is required to decipher between these possibilities.

Clogging of the TOM complex proved to be a conserved gain-of-toxicity mechanism, as mutant ANT1 proteins were also found to associate with the TOM complex and cause mPOS in human cells [116]. Importantly, as noted above, this was not associated with reduced membrane potential, suggesting that mutant ANT1 directly obstructs protein traffic to cause mPOS. Most importantly, protein import clogging and subsequent mPOS was validated *in vivo* in a mouse model expressing a “super-clogger” ANT1 variant containing two missense mutations (A123D and A137D). Clogging and mPOS were associated with development of a mitochondrial myopathy phenotype in the mice, reminiscent of adPEO patient myopathy [66, 67, 116]. It is therefore likely that protein import clogging and mPOS are at least partial contributors to the pathophysiology of adPEO, despite the “clogger mice” not directly genocopying adPEO patients (Figure 3).

More generally, the clustering of clogging-associated adPEO mutations in the transmembrane α-helices of *SLC25A4* suggest that the fidelity of these protein domains is essential to prevent clogging. Moreover, that pathogenic mutations in *SLC25A24* and *SLC25A25* are also found in transmembrane α-helices [101–103] raising the possibility that protein import clogging contributes to these syndromes as well.

7. Concluding remarks and future directions

In the last few decades, yeast proved to be an essential model system for the study of mitochondrial carrier proteins in disease. Particularly, it enabled the discovery that the pathogenic missense mutations in ANT1 cause toxic clogging of the mitochondrial protein import pathway. It provided a striking example that a mutant mitochondrial protein can become highly toxic, and that the mitochondrial protein import pathway is vulnerable to clogging by mutant substrate proteins. Furthermore, these studies in yeast also led to the discovery of the mPOS mechanism that could have far-reaching implications for diseases beyond those caused by mutant ANT [112, 116, 117]. These studies are now opening new areas of investigation on the compatibility between the protein import pathway and substrate proteins for successful protein translocation, and on how the loss of this compatibility causes human diseases.

From a clinical perspective, an important question is whether anti-clogging and anti-mPOS mechanisms exist that can be used to treat diseases caused by mitochondrial protein import clogging and subsequent mPOS in the cytosol. Indeed, recent studies have shown that yeast has multiple levels of quality control mechanisms to circumvent mitochondrial protein import stress. These quality control mechanisms are spatiotemporally separated, with some acting in different mitochondrial subcompartments, some at the cytosol-mitochondria interface, and some within the cytosol. Mitochondrial Compromised import Response (MitoCPR) [118] and Mitochondrial Protein Translocation-associated Degradation (MitoTAD) [119] are activated to degrade unimported mitochondrial proteins on the mitochondrial surface. Becker and colleagues utilized a comprehensive approach to map the mitochondrial complexome or MitCOM which revealed that Rsp5 could be the E3 ligase that acts on the OMM to sense stalled preproteins and ubiquitinate them [120]. In the cytosol, the Unfolded Protein Response activated by the mistargeting of proteins (UPR^{am}) [121] mechanism is activated to stimulate proteasomal function to degrade unimported mitochondrial proteins in the cytosol. Many nuclear-encoded mitochondrial proteins are imported co-translationally and cytosolic ribosomes are directly associated with the OMM in the vicinity of the TOM complex to allow for the nascently-forming polypeptides to be threaded across the TOM40 channel. When import is disturbed, these OMM-associated ribosomes are disassembled and their partially generated polypeptide chains degraded by the ribosome quality control (RQC) pathway [122, 123]. More recently, it was found that small heat shock proteins (sHSPs) are imported from the cytosol into the IMS where they act as cochaperones to prevent misfolding of preproteins being translocated across the TIM22 and TIM23 complexes, as well as maintaining the properly folded states of other resident IMS proteins [124]. In coming years, it would be interesting to learn whether these pathways are conserved in humans and whether they play a role in preventing mitochondrial protein import caused by mutant ANT.

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Abbreviations:

AAC2	ADP/ATP Carrier protein 2
adPEO	Autosomal Dominant Progressive External Ophthalmoplegia
AGC	Aspartate Glutamate Carrier
AGK	acyl glycerol kinase
AKT	alpha-serine/threonine kinase
ANT1	Adenine Nucleotide Translocase
BKA	Bongkrekic acid
CaMC	Calcium-dependent Mitochondrial Carrier
CATR	Carboxyatractyloside
COPD	Chronic Obstructive Pulmonary Disease
ERK family	Extracellular signal-regulated kinases
FCCP	carbonylcyanide-p-trifluoromethoxyphenylhydrazone
FSHD	Facioscapulohumeral Muscular Dystrophy (FSHD)
HIF1-alpha	Hypoxia Inducible Factor 1
HSP family	Heat Shock Protein family
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IPF	Idiopathic Pulmonary Fibrosis
MIA complex	Mitochondrial Intermembrane space Assembly complex
miDaS	Mitochondrial-Dysfunction associated Senescence
MIM complex	Mitochondrial Import complex for outer membrane proteins
mPOS	mitochondrial precursor overaccumulation stress
mPTP	mitochondrial permeability transition pore
mtDNA	Mitochondrial DNA
MTDPS12A	mitochondrial DNA depletion syndrome 12A
OMM	Outer mitochondrial membrane

PKB	Protein Kinase B
Sal1	Suppressor of Aac2 Lethality
SAM complex	Sorting and Assembly Machinery complex
SCaMC	Short Calcium-binding Mitochondrial Carrier
SOD2	superoxide dismutase 2
TIM complex	Translocase complex of the Inner Membrane
TOM complex	Translocase complex of the Outer Membrane
VDAC	Voltage-dependent Anion Channel

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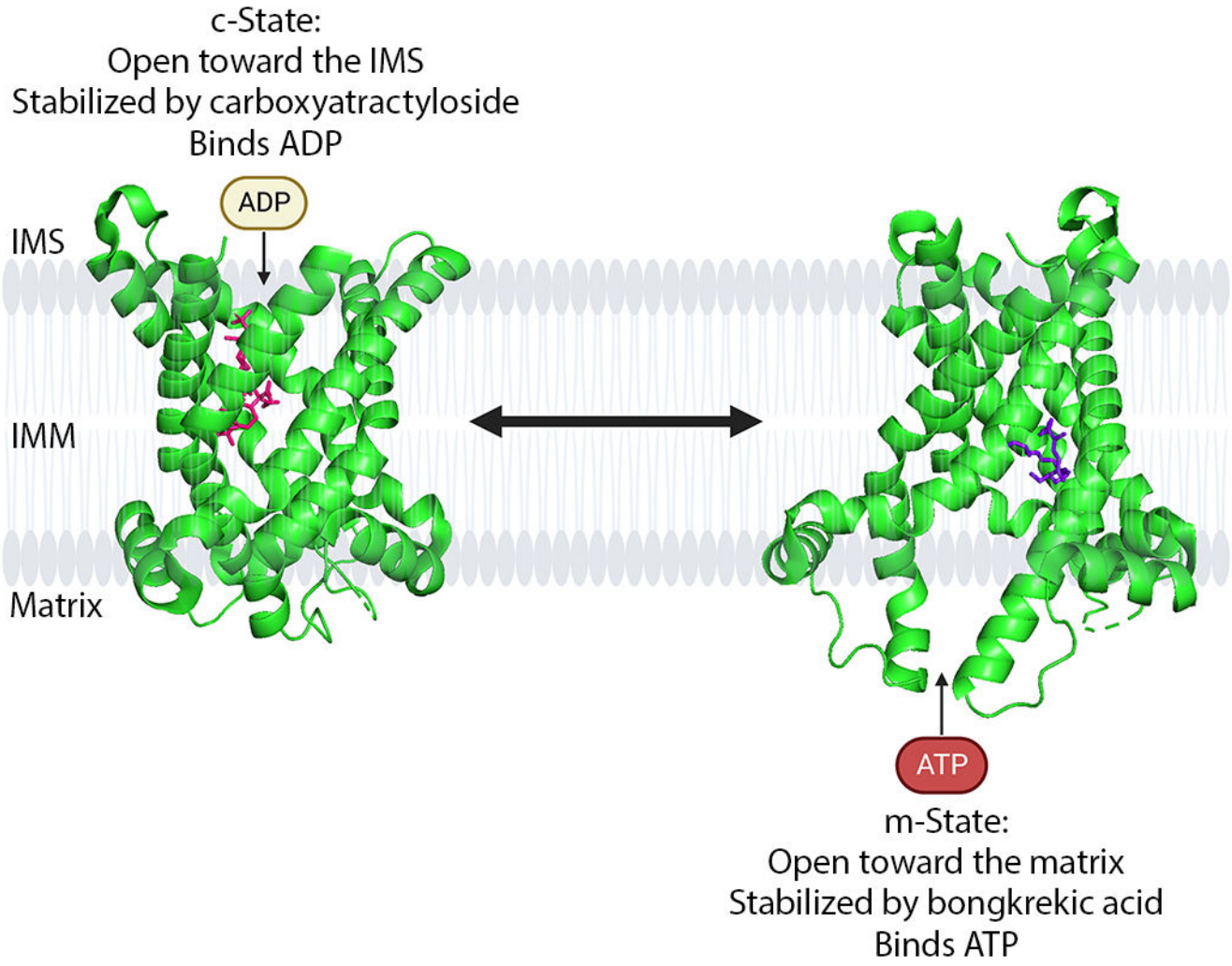

Figure 1. Adenine Nucleotide Translocase exists in distinct conformation states when bound to different substrates.

The structures of *S. cerevisiae* Aac2p (left, PDB ID: 4C9H) and *T. thermophilus* Aac (right, PDB ID: 6GCI) were determined using X-ray crystallography [18, 19]. Aac2p structure was determined when it was bound to the inhibitor carboxyatractyloside (shown in pink), which stabilizes the protein in the cytosol-facing c-state. Aac structure was determined while bound to the inhibitor bongkrekic acid (shown in purple), which stabilizes the protein in the matrix-facing m-state. This figure was generated using assistance from Pymol and Biorender.com.

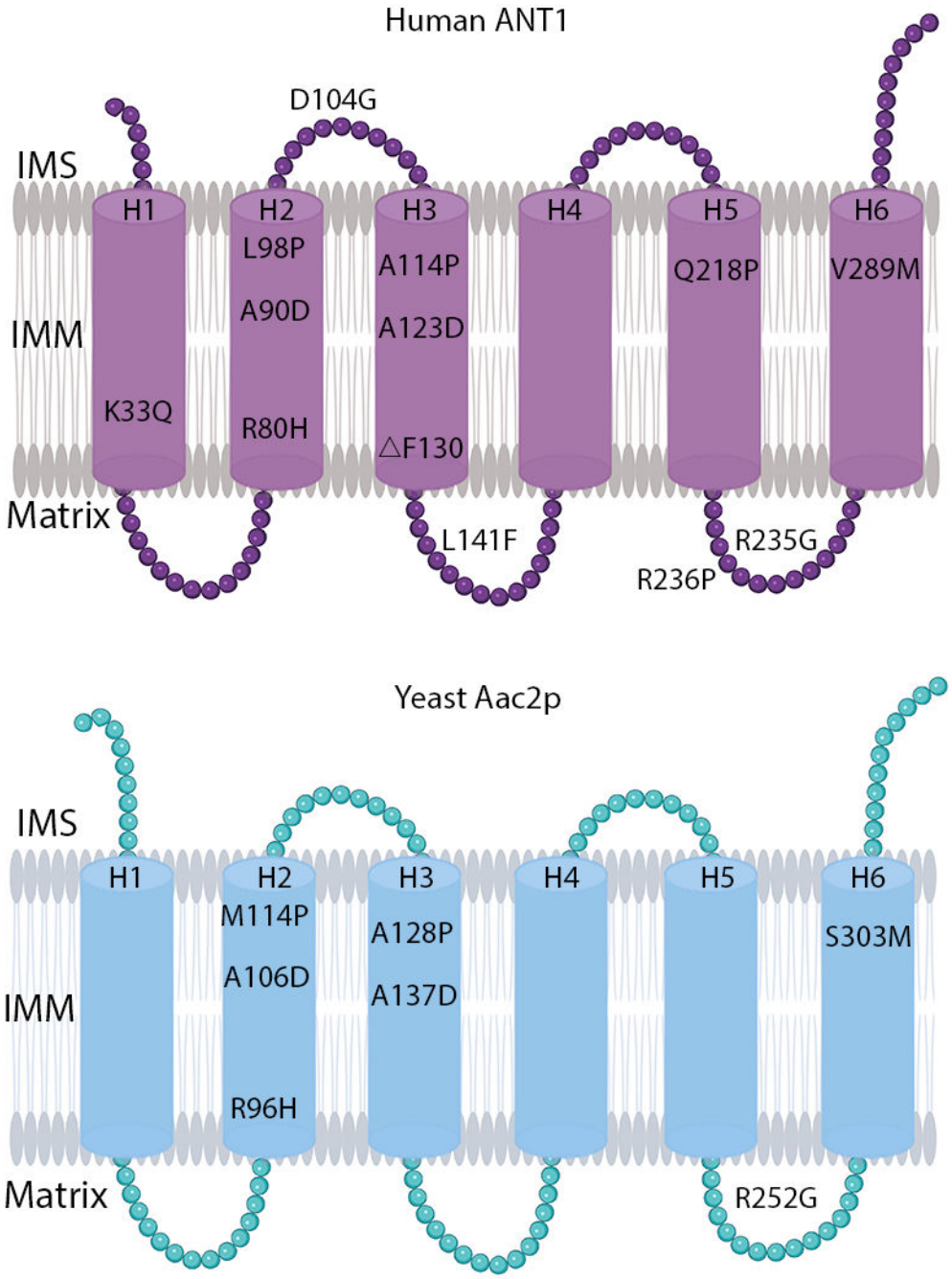

Figure 2. Projected sites of pathogenic *ANT1* mutations (top panel) and their equivalents in the yeast Aac2 protein (bottom panel).

The majority of ANT1 mutations with associated clinical phenotypes tend to cluster in the transmembrane alpha helices. H1-H6: α-helices 1 through 6. This figure was generated using Biorender.com.

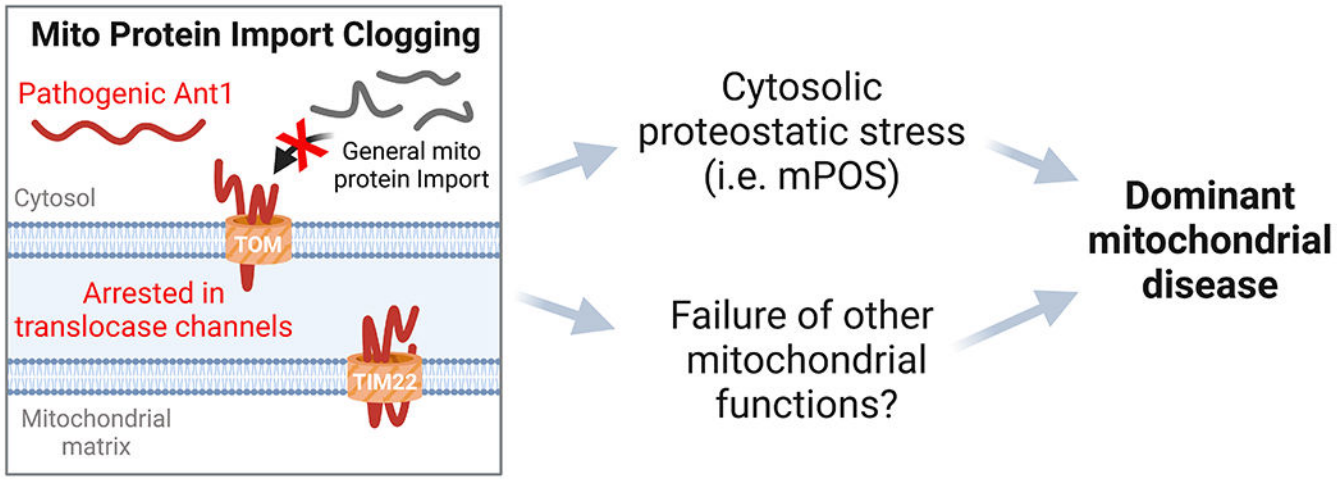

Figure 3. Mitochondrial protein import clogging as a mechanism for ANT1 dominant disease.
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Table 1.

Pathogenic mutations in *ANT1*.

List of mutations found in human *ANT1* with information on the mutation type, disease associations, and whether the mutation is conserved in yeast *AAC2*.

Mutation Genotype	Mutation Type	Disease association(s)	Equivalent amino acid in yeast Aac2	References
A114P	Missense; dominant	adPEO	A128	65, 66
V289M	Missense; dominant	adPEO	S303	65, 66
A90D	Missense; dominant	adPEO, schizoaffective disorder	A106	95, 107
D104G	Missense; dominant	adPEO	Not conserved in yeast	96, 125
K33Q	Missense; dominant	Mild myopathy	K48	100
L98P	Missense; dominant	adPEO, bipolar disorder	M114	68, 97
R80H	Missense; dominant	Mitochondrial DNA Depletion syndrome (MTDPS-12A); epileptic encephalopathy; early onset mitochondrial disease	R96	69
R235G	Missense; dominant	early-onset mitochondrial disease, skeletal muscle wasting	R252	69
A123D	Recessive; LOF and gain of toxicity	Hypertrophic cardiomyopathy (HCM) and skeletal myopathy	A137	67
Ant1 loss	null; recessive	Congenital cataracts, lactic acidosis, HCM, myopathy	N/A	126
Q218P	Recessive; missense	cardiomyopathy, muscle weakness	W235	78
R236P	Recessive; missense	Myopathy, mitochondrial aggregations, L-2-hydroxyglutaric aciduria, cardiomyopathy, lactic acidosis	R253	78
L141F	Recessive; missense	mitochondrial myopathy, hyperlactatemia, cardiomyopathy	L155	70
ΔPhe130	Recessive; deletion	mitochondrial myopathy, hyperlactatemia, cardiomyopathy	F144	70