

Disease-Associated Genetic Variation in Human Mitochondrial Protein Import

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Mitochondrial dysfunction has consequences not only for cellular energy output but also for cellular signaling pathways. Mitochondrial dysfunction, often based on inherited gene variants, plays a role in devastating human conditions such as mitochondrial neuropathies, myopathies, cardiovascular disorders, and Parkinson and Alzheimer diseases. Of the proteins essential for mitochondrial function, more than 98% are encoded in the cell nucleus, translated in the cytoplasm, sorted based on the presence of encoded mitochondrial targeting sequences (MTSs), and imported to specific mitochondrial sub-compartments based on the integrated activity of a series of mitochondrial translocases, proteinases, and chaperones. This import process is typically dynamic; as cellular homeostasis is coordinated through communication between the mitochondria and the nucleus, many of the adaptive responses to stress depend on modulation of mitochondrial import. We here describe an emerging class of disease-linked gene variants that are found to impact the mitochondrial import machinery itself or to affect the proteins during their import into mitochondria. As a whole, this class of rare defects highlights the importance of correct trafficking of mitochondrial proteins in the cell and the potential implications of failed targeting on metabolism and energy production. The existence of this variant class could have importance beyond rare neuromuscular disorders, given an increasing body of evidence suggesting that aberrant mitochondrial function may impact cancer risk and therapeutic response.

Although best known for their role as the cellular powerhouse, mitochondria also have key functions as signaling hubs. Under conditions of altered substrate availability or changing environmental demands, reprogrammed signaling in mitochondria plays a crucial role in maintaining metabolic flexibility.^{1,2} Dynamic regulation of mitochondrial activity is essential under normal physiological conditions and is frequently altered in human disease.³ Mitochondrial failure leads to common devastating conditions such as mitochondrial neuropathies, myopathies, cardiovascular disorders, and Parkinson and Alzheimer diseases, all reflecting the high levels of energy production and consumption required in neuronal tissues, muscles, and the heart. Increasingly, mitochondrial defects have been linked to additional pathological states, including diabetes and cancer, for example when tumor cells adapt to a metastatic or a drug-resistant phenotype by reprogramming metabolism.⁴ Rather than solely reflecting the poor performance of dysfunctional mitochondria, these diseases may also involve pathological disease-associated mitochondrial signaling.⁵

In humans, the mitochondrial genome encodes 13 proteins involved in oxidative phosphorylation (OXPHOS), 22 mitochondrial (mt)-tRNAs that participate in translation, the 12S and 16S mitochondrial rRNAs, and mitochondrial-derived peptides (MDPs) such as humanin, MOTS-c, and SHLPs produced from short open reading frames within the 16S and 12S rDNA regions. In contrast, the nuclear genome encodes more than 98% of the mitochondrial proteome (~1,500 nuclear-encoded proteins). Because of the need to unfold and refold proteins as they cross mitochondrial membrane barriers and to promote

assembly of oligomeric complexes with mitochondrially encoded components, the import process is typically supported by chaperones and subject to tight regulation.

Mitochondrial signaling is bidirectional. In anterograde signaling, nuclear-encoded transcription factors and other proteins provide information regulating mitochondrial biogenesis and other functions. Retrograde signaling is often associated with mitochondrial distress, based on stimuli such as accumulation of reactive oxygen species (ROS), loss of membrane potential, or defects in mtDNA. Distressed mitochondria send signals to the nucleus that reconfigure the nuclear transcriptome⁶ and trigger cytosolic changes in translation, proteasomal assembly, and folding⁷ that together support cell adaptation or trigger cell death via reactive anterograde signaling (Figure 1). Therefore, besides being crucial for mitochondrial biogenesis, regulation of mitochondrial import is involved in the adaptive response to mitochondrial stress. For example, the UPR^{mt} (unfolded protein response associated with mitochondria) triggers expression of genes coding for proteases and chaperones that need to be imported when mitochondrial protein folding is defective⁸ or when mitochondrial translation is otherwise affected.⁹ The import machinery may be exploited to dispose of cytosolic protein aggregates resulting from misfolding.¹⁰ Import controls also govern the targeting of severely dysfunctional mitochondria to the autophagic system, for destruction via a PINK1-Parkin relay system. In this case, upon lethal loss of mitochondrial membrane potential or toxic ROS accumulation, PINK1 is redirected upon import from the inner to the outer mitochondrial membrane, where it recruits the E3 ubiquitin ligase Parkin, targeting the mitochondria

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<https://doi.org/10.1016/j.ajhg.2019.03.019>

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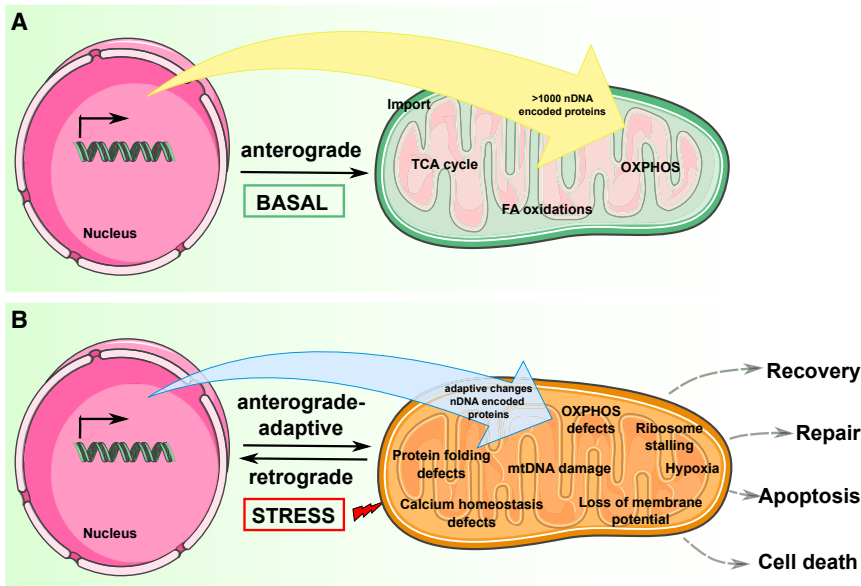


Figure 1. Anterograde and Retrograde Signaling in Mitonuclear Communication (A) Under basal conditions. (B) Under stress.

(*sdh1*)-deficient yeast strain transformed with wild-type or c.1660C>T *SDHA* cDNA.¹⁴

Causal defects linked to diseases of mitochondrial dysfunction have now been identified in ~300 of the ~1,200 nuclear genes that encode mitochondrial proteins, with a surge in discovery in recent years based on successful application of whole-exome or whole-genome sequencing.^{15,16} In 2015, it was proposed that the genetic variants that cause mitochondrial disorders could be classified in seven categories according to their impact on

for autophagosome engulfment.¹¹ Finally, separate from these complex signaling processes but also involving transport during stress response, is the process of inter-organelle coordination mediated by the direct redistribution of some nuclear or mitochondrial proteins between the two compartments.¹² Nuclear proteins with partial mitochondrial distribution are mainly estrogen, glucocorticoid, and thyroid hormone receptors or transcription factors (e.g., NF- κ B, CREB, p53, STAT3). These proteins translocate to mitochondria upon a stress stimulus or reside in mitochondria under steady-state conditions and are activated upon stress. Nuclear-encoded proteins that predominantly reside in the mitochondria under normal growth conditions but translocate to the nucleus in response to stress are mainly transcription factors with roles both in mitochondrial and nuclear DNAs (e.g., TFAM, ATFs), biosynthetic enzymes (e.g., CLK-1), or pro-apoptotic factors (e.g., AIF).¹² Although beyond the scope of this review, insights into the mechanisms of mitochondrial transport of nuclear receptors and transcription factors that differ from the canonical import linked to mitochondrial biosynthesis are detailed in Sepuri et al.¹³

Over the past several decades, a growing number of genetic disorders have been recognized as linked to some form of mitochondrial dysfunction. The first DNA variation in a nuclear gene responsible for a mitochondrial respiratory chain deficiency in humans was discovered in 1995 in two siblings from a consanguineous family diagnosed with Leigh syndrome (MIM: 256000), characterized by severe psychomotor regression usually in the first year of life and death within 2–3 years. These siblings were homozygous for the GenBank: NM_004168.3; c.1660C>T (p.Arg554Trp) variant (rs9809219) in *SDHA* (MIM: 600857), a subunit of the succinate dehydrogenase (SDH). The deleterious effect of this homozygous variant was confirmed by comparing the *SDHA* activity in an *SDHA*

specific cell processes: (1) oxidative phosphorylation, (2) mitochondrial DNA maintenance and gene expression, (3) mitochondrial quality control, (4) iron-sulfur cluster homeostasis, (5) mitochondrial dynamics of fission and fusion, (6) phospholipid metabolism, and (7) mitochondrial import.¹⁷ As more is learned about the intricacy of the various processes required for mitochondrial biogenesis and function, these categories are likely to evolve. For example, a recent review on membrane dynamics considered defects of membrane fusion as a subgroup of mtDNA maintenance defects.¹⁸ As discussed below, additional functional classes of genes may also significantly modify mitochondrial function.

Among the categories of defects associated with mitochondrial disorder, the variants in genes involved in mitochondrial import are of particular interest because, as all nuclear-encoded proteins involved in these various mitochondrial functions have to be imported themselves, defects in mitochondrial import can potentially induce all classes of dysfunction, depending on which proteins experience faulty import. For example, while it could be argued that the disease-associated p.Val10Gly-iron-sulfur cluster assembly 1 (*ISCA1*) (MIM: 611006) acts by impairing the iron-sulfur clusters of metabolic and respiratory enzymes, the mechanistic basis for the defect is impaired import and protein stability.¹⁹ As another example, human syndromes arising from variants in genes encoding mt-tRNA-modifying proteins (e.g., *MTO1* [MIM: 614667], *GTPBP3* [MIM: 608536], *MTFMT* [MIM: 611766], and *TRIT1* [MIM: 617840]) were described as mt-translation disorders according to another simplified classification of mitochondrial diseases.²⁰ However, cellular and mouse models of *Mto1* deficiency both manifested an abnormal mitochondrial morphology, associated with aberrant trafficking of normally imported proteins. This import defect leads mitochondrial proteins

to aggregate and misfold in the cytoplasm, inducing a cytotoxic unfolding protein response.²¹ Rather than separating into classes, it may therefore be beneficial to look at the consequences of genetic defects in mitochondrial import more broadly.

Given the increasing number of diseases linked to defects in mitochondrial import, in this review, we focus on the mitochondrial import pathway and its signaling, particularly as this information impacts inherited disease-associated gene variants. After providing a basic overview of the current understanding of this pathway, we provide illustrative examples of disease-causing variants that highlight import defects that may underlie some general mechanisms at play in various inherited diseases. We also discuss the ways the findings may impact understanding of diseases such as cancer, in which altered mitochondrial bio-energetic processes are typical, and where changes in mitochondrial import are being linked to tumorigenesis. We conclude with some challenges faced by the variant curator trying to decipher the functional impact of a DNA variant coding for a protein that localizes to mitochondria, including examples of DNA variants that would not typically be classified as disease causing (as they are not rare in the general population) but are nevertheless associated with disease based on roles potentiating rare variants. An extended list of citations related to the work discussed herein is provided in the [Supplemental Data](#).

The Basic Machinery for Mitochondrial Import

The mitochondrial protein import pathway has recently been thoroughly reviewed.¹ [Figure 2](#) illustrates the key components of the mitochondrial import machinery, which we briefly present here to provide context for discussion of DNA-damaging variants. Mitochondrial proteins that are synthesized in the cytosol are initially recognized by the translocase of the outer membrane (TOM) complex, which mediates the initial entry through the outer mitochondrial membrane (OMM) into the intermembrane space (IMS). Its components include the pore forming β -barrel TOM40, which is membrane anchored with cytosol-exposed C-terminals that serve as receptors for the incoming complex components TOM20 and TOM70. The TOM complex also contains the architectural component TOM22, which provides further binding sites for many precursor proteins on both sides of the OMM. Subsequent sorting and transport of these proteins to their final destination (which may be the OMM, the IMS, the inner mitochondrial membrane [IMM], or the mitochondrial matrix) require the activity of additional complexes. Essential elements for appropriate localization of mitochondrial proteins include (1) a set of complexes mediating import of proteins to different regions of the mitochondria, (2) mitochondria-targeting sequences on the imported proteins, and (3) proteases that modify, and chaperones that sustain, proteins concomitant with their mitochondrial import.

Complexes Mediating Import of Proteins to Mitochondria

Insertion of Nuclear-Encoded Proteins into the OMM. After translocation through the TOM complex, proteins with β -barrel topology interact with the small translocases of inner membrane (TIM) chaperones (TIM8/13 and TIM9/10), which deliver clients to the topogenesis of mitochondrial outer membrane β -barrel proteins/sorting and assembly machinery (TOB/SAM) complex, which helps insert them in the OMM. Proteins transported by the TOB/SAM complex include the voltage-dependent anion channel (VDAC/Porin), the major channel for import of small hydrophilic molecules, and components of the TOM complex itself, such as TOM40 ([Figure 2A](#)). Of note, not all OMM proteins require the TOM and TOB/SAM complexes. For example, some α -helical OMM proteins, such as the outer membrane protein of 45 kDa (OM45), are directly inserted into the OMM by the mitochondrial import (MIM) complex, a 200 kDa oligomeric assembly of two small single-spanning outer membrane proteins, MIM1 and MIM2.

Insertion of Nuclear-Encoded Proteins into the IMM and IMS. The TIM22 and TIM23 complexes localize to the IMM, where their transport activity is driven by mitochondrial membrane potential. Besides their activity in transporting β -barrel proteins to the TOB/SAM complex, the small TIM chaperones also deliver hydrophobic proteins such as those of the mitochondrial carrier family (e.g., the SCL25 family) to TIM22 for insertion into the IMM. The TIM23 complex consists of the three essential components TIMM23, TIMM17A, and TIMM50. TIMM23 and TIMM17A are each tightly anchored in the membrane by four transmembrane helices, while TIMM50 is integrated in the membrane by a single helix, with an extended domain exposed in the IMS. Through dynamic associations with different partners, the TIM23 complex forms two non-identical complexes responsible for directing proteins to distinct compartments. The TIM23-SORT complex is used for protein insertion into the IMM. A subset of the nuclear-encoded mitochondrial proteins bearing a cleavage pre-sequence initially enter the matrix through TIM23-PAM (presequence translocase associated motor); some of these are subsequently redirected to the IMM through the oxidase assembly (OXA) translocase.

Small nuclear-encoded proteins with multiple cysteine residues enter the IMS after transport through TOM and are sorted by the mitochondrial intermembrane space assembly (MIA) complex for oxidative protein folding. The MIA complex consists of the oxidoreductase CHCHD4 (MIA40) and the flavin adenine dinucleotide (FAD)-dependent sulfhydryl oxidase ALR (augmenter of liver regeneration) encoded by *GFER* (MIM: 600924). Both factors form a disulfide relay system in which CHCHD4 functions as a receptor that transiently interacts with incoming polypeptides via disulfide bonds.

Delivery of Nuclear-Encoded Proteins to the Mitochondrial Matrix. Upon association with mtHSP70 and additional co-chaperones, the TIM23-PAM complex guides precursors with a cleavable N-terminal mitochondrial targeting signal

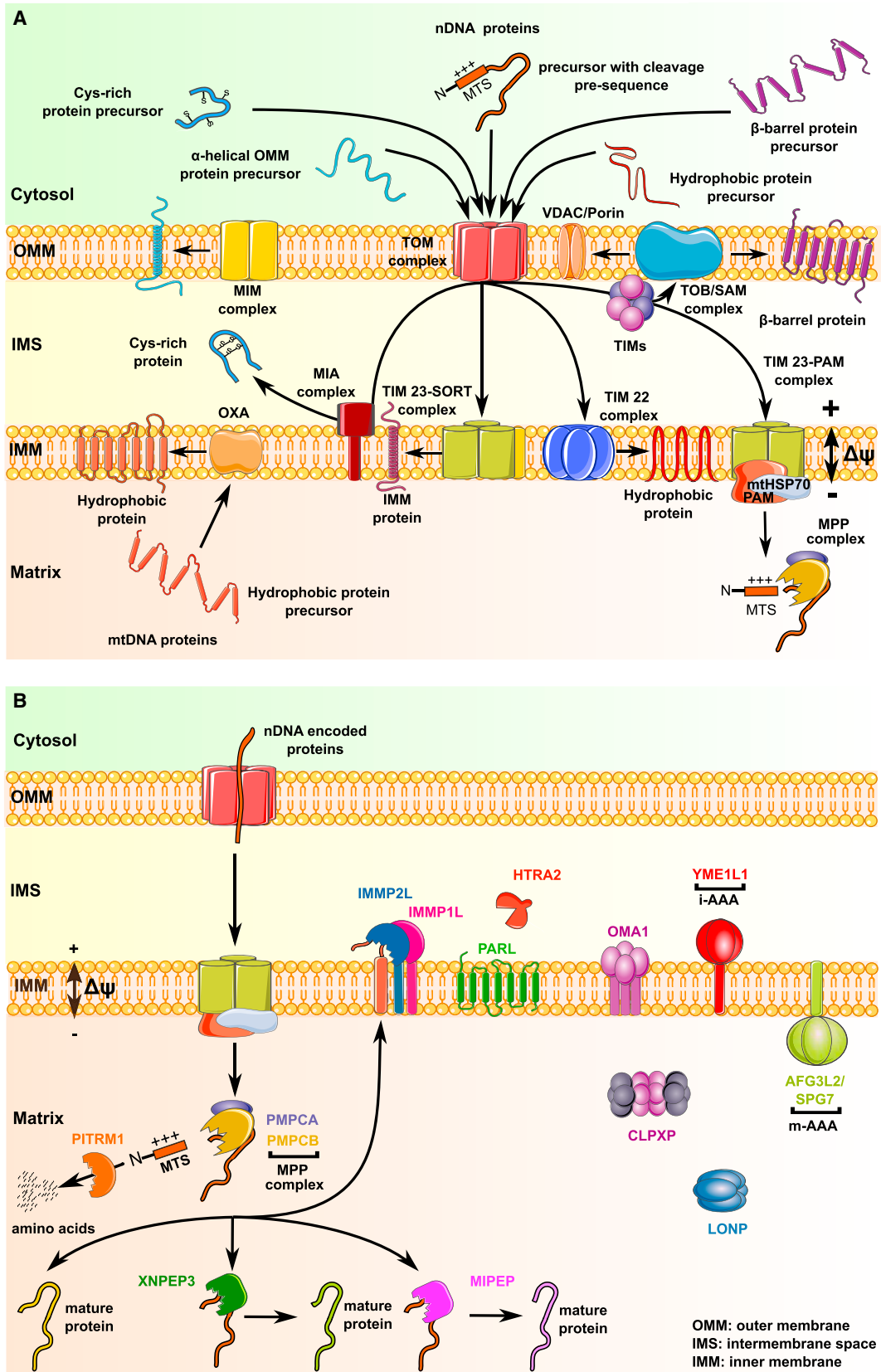


Figure 2. Overview of Proteins Involved in Mitochondrial Import, Protein Sorting, and Processing

(A) Individual proteins and protein complexes involved in import and sorting.

(B) Steps in processing of imported proteins, indicating localization of proteases in mitochondrial sub-compartments. See main text for detailed description of represented pathways.

(MTS) to the mitochondrial matrix, where mitochondrial processing proteases and peptidases remove and degrade the MTS, as discussed below.

Insertion of Mitochondria-Encoded Proteins into the IMM.

The membrane integral core subunits of the oxidative phosphorylation system are encoded by mtDNA. These typically hydrophobic proteins are synthesized in the mitochondrial matrix by mitochondrial ribosomes and exported into the IMM by the cytochrome oxidase assembly (OXA) translocase.

Mitochondrial Targeting Sequence (MTS)

The TOM40 and TIM23 complexes transport precursor proteins with positively charged N-terminal stretches which are present in precursor forms of soluble proteins targeted to the matrix, in the proteins targeted to the IMS, and in transmembrane-domain-bearing proteins targeted to the IMM. In this last case, the targeting signal is sometimes referred to as bipartite, as it consists of the MTS followed by a hydrophobic signal. Because this N-terminal signal gets cleaved from the proteins that reach the matrix, the generic term of pre-sequence has been widely used to describe it. However, the more precise designation as a mitochondrial targeting sequence (MTS) has been recommended as a possible source of higher reliability in data-mining analyses.²² The initial step of import is also driven by non-N-terminal signals distributed throughout imported proteins. These have been referred as internal MTS-like signals, as they share some sequence properties with the MTS. They mediate binding to TOM70 in a manner that prevents premature folding and aggregation, enhancing efficient import.²³

Initial low throughput sequence analyses of the N termini of mitochondrial precursor proteins based on Edman sequencing revealed four main features of the MTS: (1) a length of 20 to 40 amino acids, (2) an overall positive charge, (3) the ability to form an amphiphilic helix, and (4) in many cases, the presence of an arginine in position -2, -3, or -10 from the cleavage site, surrounded by specific flanking sequences.²⁴ In the genomic/proteomic era, this work has been greatly enhanced by global determination of the (mitochondrial) N-terminome.²⁵

An extensive survey of mitochondrial precursors has shown that the specific flanking sequences around arginine motifs, initially described as characteristic of the MTS, can be found in degenerate forms, with 15%–25% of experimentally defined cleavage sites not matching previously described motifs.²⁵ Approximately 30% of pre-sequences are now recognized as longer than 40 residues, with ability to form more than one helix.^{11,26} The great diversity of import sequences has made development of computational methods for prediction of mitochondrial localization challenging and sometimes controversial, as we discuss later in this review.^{11,27}

Transport-Associated Protein Processing

Import of nuclear proteins into the mitochondria is supported by their interaction with various chaperones and

often accompanied by transport-associated folding and processing. Components of the proteolytic system include the ubiquitin-proteasome system and a group of sub-compartment-specific proteases, which may have additional chaperone activities (Figure 2B). Of the nearly 600 proteases of the human degradome, about 30 localize to the mitochondrial matrix or are anchored in the IMM or OMM.²⁸ They fall into three classes: (1) processing peptidases for the maturation of the mitochondrial proteome, (2) quality control proteases for degradation of damaged or misfolded proteins and regulation of mitochondrial functions, and (3) oligopeptidases that further degrade residual peptides produced by action of the other two classes.

MTS-containing precursor proteins that enter the matrix are subject to maturation proteolytic steps that support appropriate protein stability. The most common step involves the cleavage of the MTS by the matrix-localized mitochondrial processing peptidase (MPP) complex (comprised of mitochondrial-processing peptidase subunits α and β , encoded by *PMPCA* [MIM: 613036] and *PMPCB* [MIM: 603131]). Additional processing by the mitochondrial intermediate peptidase (MIPEP/MIP/Oct1) which cleaves an octapeptide, or X-Prolyl aminopeptidase 3 (XPNPEP3/Icp55), which removes a single N-terminal amino acid, replace destabilizing N-terminal amino acids that would lead to degradation by amino acids associated with longer half-life.²⁹ Alternatively, after cleavage by MPP, the IMM protease subunits 1 and 2 (encoded by *IMMP1L* [MIM: 612323] and *IMMP2L* [MIM: 605977]) remove hydrophobic sorting signals for release to the IMS.

A thorough review of the mitochondrial quality control (QC) proteases and their crucial role as regulators of the integrity of the mitochondrial proteome and morphology and key players in apoptotic signaling and stress response has recently been provided.³⁰ For the present discussion, we note that the main players in mitochondrial QC (the i-AAA protease YME1L1, the m-AAA protease AFG3L2/SPG7, LONP, CLPXP [hetero-oligomer of CLPX and CLPP], HTRA2, OMA1, and PARL) are themselves clients of the import machinery. Therefore, any defect affecting import of these proteins could have more global damaging consequences.

Finally, the principal oligopeptidase in the matrix is pitrilysin metallopeptidase 1 (PITRM1). Known substrates include the cleaved MTS produced during the processing of precursor proteins and several forms of amyloid-beta peptide (A β) associated with Alzheimer disease.

Rare Disorders Caused by Aberrant Mitochondrial Import

A growing number of cases of defects in proteins involved in mitochondrial import have been associated with rare diseases. Some examples are presented here, organized by mitochondrial import process. An extended list of human genes with variants associated with defective import is presented as Table S1.

Pathological Genetic Variation Affecting the TIM and OXA Complexes

Rare variants in genes related to the TIM (translocases of inner membrane) and OXA complexes (see [Figure 2A](#)) may manifest as clinically severe childhood syndromes involving encephalopathy, 3-methylglutaconic aciduria (3-MGA-uria), and cardiomyopathy due to diverse activities of these complexes in oxidative phosphorylation, as well as mitochondrial functions involving and mediated by cardiolipin.

Several recent publications describe defects in *TIMM50*, *TIMM22*, and *OXA1L*. For example, homozygous missense variations in *TIMM50* (MIM: 607381) (GenBank: NM_001001563.3; c.649C>T [p.Arg217Trp] [rs1300848445] and c.755C>T [p.Thr252Met] [rs1244226820]) were reported in individuals who exhibited epileptic encephalopathy with 3-MGA-uria.³¹ In another report, compound heterozygous variants in *TIMM50* (c.335C>A [p.Ser112*] [rs35135520] and c.569G>C [p.Gly190Ala] [rs776019250]) were identified as disease associated in an infant with rapidly progressive severe encephalopathy. Here, the patient's fibroblasts presented low levels of *TIMM50* and other components of the TIM23 complex, lower mitochondrial membrane potential, and impaired TIM23-dependent protein import impacting mitochondrial oxidative phosphorylation.³² A case of compound heterozygous variants in *TIMM22* (MIM: 607251) (GenBank: NM_013337.3; c.75C>A [p.Tyr25*] [rs754537066] and c.97G>C [p.Val33Leu] [rs149879547]) was also recently identified in an individual exhibiting a neuromuscular phenotype associated with elevated lactate levels. Biochemical analyses of fibroblasts from this individual revealed reduced TIM22 protein levels, deficiency of TIM22 complex formation, a compromised oxidative capacity, and altered mitochondrial morphology.³³ Affecting a different part of the import machinery, biallelic disease-associated variants in *OXA1L* (MIM: 601066) (GenBank: NM_005015.3; c.500_507dup [p.Ser170Glnfs*18] and c.620G>T [p.Cys207Phe] [rs772751581]) were identified in an individual presenting with severe encephalopathy, hypotonia, and developmental delay in early childhood. These symptoms were associated with decreased production of *OXA1L* and subunits of mitochondrial oxidative phosphorylation complexes IV and V, and deficient activity of the respiratory chain.³⁴

It is possible that additional proteins that modulate TIM and OXA complex function will be identified. For example, in 2017, the mitochondrial acylglycerol kinase AGK was described as a component of the TIM22 complex, required for mitochondrial import of IMM transmembrane proteins, including members of the SLC25A family.^{35,36} AGK (MIM: 610345) defects are associated with Sengers syndrome (MIM: 212350) which is characterized by congenital cataracts, hypertrophic cardiomyopathy, mitochondrial myopathy, and lactic acidosis. Sengers syndrome has also been recognized as one of many mitochondrial DNA depletion syndromes (MTDPS10).³⁷ Recognizing that AGK is a

component of TIM22 provides the molecular basis for the decrease in SLC25A4 (also called ANT1; adenine nucleotide translocator 1) protein levels observed in the muscle tissues of two unrelated individuals with Sengers syndrome.³⁸ Hence, AGK is positioned to influence mtDNA abundance, at least in part by controlling SLC25A4 abundance, with AGK deficiency potentially contributing to mitochondrial depletion.

3-MGA-Uria Is Associated with Multiple Factors Affecting Mitochondrial Import

Interactions between proteins and lipids regulate mitochondrial import. Elevated urinary excretion of 3-MGA (or 3-MGA-uria) is a hallmark of a genetically and phenotypically heterogeneous group of inherited metabolic syndromes. A long-standing OMIM Phenotypic Series (MIM: PS250950) classification scheme for this disorder describes nine different types of 3-MGA-uria ([Table S2A](#)). More recently, a new classification based on patho-mechanisms and genetics was proposed, with “primary” 3-MGA-uria associated with variants in *AUH* (required for leucine catabolism), multiple “secondary” subtypes of 3-MGA-uria associated with defective phospholipid remodeling (for defects in *TAZ* and *SERAC1*) or mitochondrial membrane associated disorder (for defects in *OPA3*, *DNAJC19*, and *TMEM70*), and the “not otherwise specified (NOS)” 3-MGA-uria for which no patho-mechanism has been identified.³⁹ Evaluation of the set of genes associated with this syndrome exemplifies the complexity of signaling that can impact mitochondrial import.

While the metabolic origin of 3-MGA in the urine, independent of a leucine catabolism defect, has long been unknown, it was recently proposed that the 3-MGA pathway functions as an overflow valve generating organic waste when acetyl-CoA exceeds utilization capacity.⁴⁰ Diversion of the intra-mitochondrial acetyl-CoA to organic waste occurs under conditions of reduced electron transport chain (ETC) activity. Reduced ETC is associated with the accumulation of NADH in the matrix space, which leads to inhibition of key enzymes of the TCA cycle, driving a compensatory increase in anaerobic glycolysis. As the ETC machinery is embedded in the IMM, its activity is highly susceptible to any perturbation of its lipid composition, with negative consequences for the import of mitochondrial matrix-targeted proteins, as import requires the electrochemical membrane potential generated by a functional ETC across the IMM. We hypothesize that these close relationships between control of lipid composition, and functionality of the import machinery indicate a common mechanism linking many of the genetic defects that result in 3-MGA-uria.

An example of IMM lipid perturbation is seen in individuals with Barth syndrome, associated with *TAZ* variants, who present with a characteristic lipid profile marked by decreased cardiolipin (CL) levels, mainly of (poly)unsaturated species, and elevated levels of the remodeling intermediate monolysocardiolipin. How these anomalies in

CL and other lipids in mitochondrial membranes impact ETC and lead to 3-MGA-uria is elaborated in Ikon and Ryan.⁴¹

Interestingly, we note many of the genes associated with secondary 3-MGA-uria (Table S2A) are linked to CL. CL comprises ~20% of the IMM, has a critical role in IMM dynamics, and associates with many IMM proteins, including those encoded by *TAZ*, *DNAJC19*, and *SERAC1*, as well as the prohibitins (PHB1 and 2), which function as protein and lipid scaffolds to ensure the integrity and functionality of the IMM. *TAZ* (MIM: 300394) encodes tafazzin, a phospholipid transacylase involved in CL remodeling, a process of sequential deacylation-reacylation/transacylation reactions that establish the final composition of the acyl chains of CL. *DNAJC19* (MIM: 608977) encodes a mitochondrial co-chaperone that associates with prohibitin to regulate CL remodeling by tafazzin. Phospholipid exchange also depends on activity of the *SERAC1* (MIM: 614725)-encoded phosphatidylglycerol remodeling protein found at the interface of mitochondria and endoplasmic reticula. In addition, analyses performed *in vivo*, in isolated mitochondria, and in model membrane systems demonstrate that the soluble receptor domain of TIMM50 interacts with membranes and with specific sites on the TIM23 channel in a manner that is directly modulated by CL.

Other genes associated with mitochondrial import have been linked to control of 3-MGA-uria and ETC. These include *AGK*, already described above as encoding a component of the TIM22 complex, and genes encoding components of complex V (ATP synthase), the complex V associated factor TMEM70 (MIM: 612418) and QIL1 encoded by *MICOS13* (MIM: 616658) (Table S2B). *AGK* exerts an essential function in defining membrane lipid composition, including CL content. CL is a component of the cristae in which complex V is embedded. TMEM70 and QIL1 are necessary for cristae formation. Globally, based on these various functional interactions, 3-MGA-uria emerges as linked to defects in the ultrastructure of the inner membrane in a manner that disrupts mitochondrial import.

Pathological Genetic Variation in the MIA Pathway

Amyotrophic lateral sclerosis (ALS) (MIM: PS105400) is a neurodegenerative disease affecting motor neurons in the brain and spinal cord, leading to muscle weakness. Familial ALS constitutes ~10% of case subjects. Variants in at least 15 different genes or loci have been identified as disease causing, with the most common in *SOD1* (MIM: 147450) and *C9orf72* (MIM: 614260). Variants in *CHCHD10* (MIM: 615903) also have been reported in families with autosomal-dominant ALS.⁴² *CHCHD10* encodes a soluble IMS-localized member of the coiled-coil-helix-coiled-coil-helix domain-containing protein family which participates in a system that stabilizes membrane curvature and forms contact sites between the IMM and the OMM. The disease-associated *CHCHD10* variant c.323A>C (p.Gln108Pro) (GenBank: NM_213720.2; rs889489701)

blocks CHCHD10 import to the IMS, thereby promoting its destruction.⁴² The dramatic reduction of CHCHD10 import, also observed upon CHCHD4 knockdown, supports the idea that import of MIA substrates into the IMS competes with their ubiquitin-dependent degradation in the cytosol.^{43,44}

In two additional examples, rare myopathies are caused by autosomal-recessive inheritance of components of the MIA pathway. In one example, an autosomal-recessive myopathy (MIM: 600924) is associated with an inherited variant in the core component of the MIA pathway ALR (GFER/ERV1).⁴⁵ Three children born to healthy consanguineous parents presented with progressive myopathy and partial combined respiratory-chain deficiency, congenital cataract, sensorineural hearing loss, and developmental delay. The homozygous c.581G>A (p.Arg194His) variant (GenBank: NM_005262.2; rs121908192) was found to affect ALR protein stability and FAD binding, causing reduced levels of several substrates of the disulfide relay.⁴⁶ In another example, compound heterozygosity of the *NDUFB10* (MIM: 603843) variants GenBank: NM_004548.3; c.207dup (p.Glu70*) and c.319T>A, (p.Cys107Ser) has been identified as a cause of fatal infantile lactic acidosis and cardiomyopathy.⁴⁷ *NDUFB10* encodes an accessory subunit of respiratory chain complex I that is a substrate of the MIA pathway. At autopsy, diminished NDUFB10 protein levels were observed, likely the result of the altered protein becoming a poor CHCHD4 substrate. Further, an *in vitro* assay indicated that the cysteine to serine substitution in position 107 resulted in impaired oxidation by CHCHD4. As with many mitochondrial diseases,⁴⁸ this variant had tissue-specific manifestation; while NDUFB10 and complex I activity were almost completely absent in muscle and heart, protein level and activity were almost normal in fibroblasts, implying that despite the variation, the protein could be imported and folded under certain environmental conditions (Figure 3A). For further reference, an extended discussion of disease-associated variants affecting the MIA machinery or its substrates is provided in Ikon and Ryan.⁴⁹

Pathological Genetic Variation Affecting the MTS

Genetic variants in the mitochondrial targeting sequences of proteins that are translated in the cell nucleus and then targeted to the mitochondria have been shown to manifest clinically as deficiencies in Krebs cycle components (pyruvate dehydrogenase and malonyl-CoA decarboxylase), and in one report, as leukodystrophy.

Pyruvate dehydrogenase complex (PDC) deficiency is one of the most common neurodegenerative disorders associated with abnormal mitochondrial metabolism. A genetically heterogeneous syndrome, PDC deficiency is characterized by progressive neurological and neuromuscular degeneration, lactic acidosis, and often death in childhood. Among the six different forms of PDC deficiency (MIM: PS312170), recessive disease is associated with defects in autosomally encoded subunits of PDC,

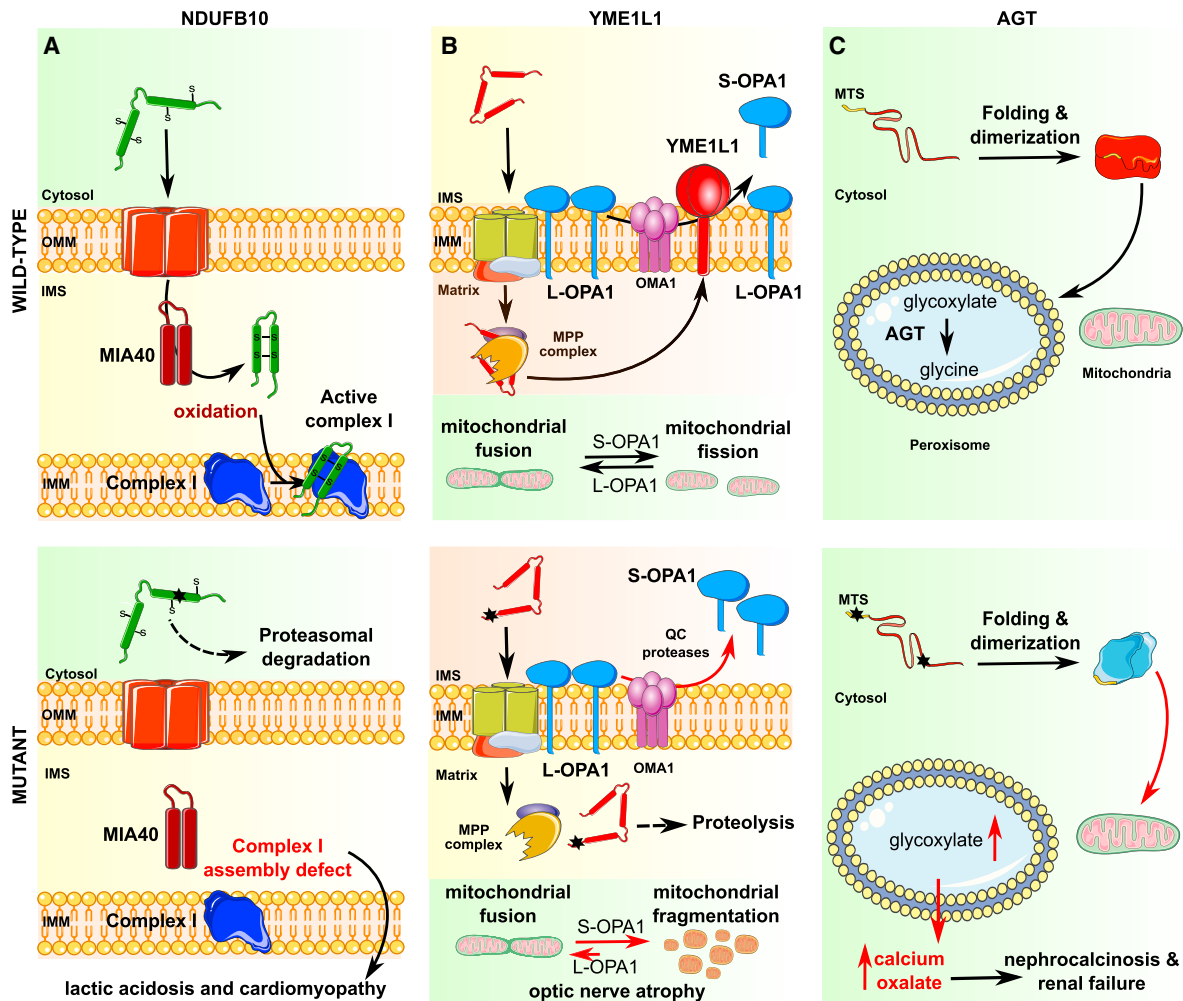


Figure 3. Schematic Representation of Pathogenic Signaling Associated with Disease-Associated Variants

(A) Effect of MIA pathway defects on maturation of NDUFB10 associated with lactic acidosis and cardiomyopathy.

(B) Deficient processing of YME1L1 by MPP upon import to the mitochondrial matrix causes defects in OPA1 cleavage, resulting in mitochondrial fragmentation and atrophy of optic nerves.

(C) Combination of genetic variants weakening AGT peroxisomal targeting and protein folding together lead to inappropriate localization to the mitochondria, causing deficient glyoxylate metabolism, nephrocalcinosis, and renal failure. For each example, top panel indicates wild-type function, and bottom panel illustrates defect associated with mitochondrial import variant. For full description of the variants, see text.

while the most common form arises from defects in *PDHA1* (MIM: 300502), located on the X chromosome.⁵⁰ A *PDHA1* variant, p.Arg10Pro (GenBank: NM_000284.3; c.29G>C [rs137853257]), was identified in a 10-month-old boy and his brother, both with pyruvate dehydrogenase deficiency, as well as in their more mildly affected mother. This was the first description of an amino acid substitution in a mitochondrial targeting sequence resulting in a human genetic disease.⁵¹ Defective import of proteins into the mitochondria was thought responsible for the reduced pyruvate dehydrogenase levels, as *in vitro* experiments indicated that the mitochondrial matrix protein ornithine transcarbamylase (OTC) was translocated at a reduced rate to the matrix when attached to the mutant p.Arg10Pro *PDHA1*-MTS.

Biallelic variants in the MTS of *MLCYD* (MIM: 606761), located in residues 1 to 39, cause an autosomal-recessive

form of malonyl-CoA decarboxylase deficiency (MIM: 248360). The resultant disease is characterized by malonic aciduria, developmental delay, seizure disorder, hypoglycemia, and cardiomyopathy. *MLYCD* catalyzes the decarboxylation of malonyl-CoA to acetyl-CoA, an important step in the breakdown of fatty acids. Immunocytochemistry performed on the fibroblasts of a *MLYCD*-deficient individual homozygous for the variant GenBank: NM_012213.2; c.8G>A (p.Gly3Asp) (rs121908081) revealed dense aggregates of *MLYCD* at the plasma membrane, indicative of mistargeting.⁵²

Leukodystrophies are a group of rare, progressive, metabolic, genetic diseases that impact the white matter of the central nervous system. A case of severe early-onset leukodystrophy with multiple defects of respiratory complexes and a severe impairment of lipoic acid synthesis has been linked to a homozygous DNA variant, GenBank:

NM_030940.3; c.29T>G (p.Val10Gly), in the MTS in the ISCA1 protein, a component of the mitochondrial Fe-S biogenesis machinery. This homozygous variant is predicted to reduce mitochondrial targeting, resulting in drastically reduced ISCA1 protein levels and the severe neurologic phenotype observed.¹⁹

The recognition that some mitochondrial proteins have internal MTS signals²³ may help explain the function of some previously defined variants. For example, Kobayashi et al. described the variant GenBank: NM_000274.3; c.268C>G (p.Gln90Glu) (rs121965060) in ornithine aminotransferase (OAT) (MIM: 613349) in a person with autosomal-recessive gyrate atrophy (MIM: 258870). Although far removed from the OAT N terminus, this variant was shown to obstruct the mitochondrial entry of the precursor, supporting that it was required in addition to the N-terminal signal sequence for mitochondrial entry.⁵³

Some MTS variants are associated with therapeutic response, rather than disease onset. *SOD2* (MIM: 147460) encodes the antioxidant enzyme that affords the major defense against ROS. The *SOD2* Val16Ala variant is reported in ClinVar (ID: 14751) as significant for drug response, as it affects the survival of individuals with breast cancer receiving cyclophosphamide-containing chemotherapy.⁵⁴ Amino acid position 16 in the MTS of *SOD2* was the first reported dimorphic site in an MTS (GenBank: NM_000636.3; c.47T>C [p.Val16Ala] [rs4880]) and has been much studied.⁵⁵ According to the gnomAD database, this variant is present in close to half of the individuals in all populations except East Asians, where it is observed at a frequency of 0.12. Notably, position 16 is located in a region of low evolutionary conservation. Although high prevalence and low conservation often causes dismissal of possible pathogenicity, there are a growing number of examples in which a disease-causing variant corresponds to the wild-type allele in another species,⁵⁶ emphasizing the need for caution in interpretation.

Pathological Genetic Variation Affecting Mitochondrial Proteases

Variants leading to changes in mitochondrial proteases have been identified in families with inherited forms of infantile neurodegeneration, Perrault syndrome (characterized by sensorineural hearing loss, additional neurological defects, and other symptoms), ocular ataxia, and cerebellar ataxia, reflecting the importance mitochondrial protease processing in neuronal tissues (see Table 1).

In one such example, biallelic variants in the gene encoding the protease PMPCB have been identified as a cause of early childhood neurodegeneration. Their presence caused defective proteolytic activity manifested by an accumulation of a processing intermediate of frataxin, a defined PMPCB substrate and iron chaperone involved in iron-sulfur cluster biogenesis and heme biosynthesis. This incomplete processing of frataxin, observed *in vivo* in mitochondria isolated from fibroblasts from the affected

individuals, led to a dysregulation of iron-sulfur cluster biogenesis that caused the neurological phenotype⁵⁷.

In another example, optic atrophy has been associated with biallelic variants in *YME1L1* (MIM: 607472) (GenBank: NM_014263.3; c.445C>T [p.Arg149Trp] [rs1057519312]). *YME1L1* is an IMM-associated quality control protease; pathogenic variation impairs *YME1L1* cleavage by MPP, causing *YME1L1* to undergo rapid proteolysis and reducing its steady-state level. Consequently, skin fibroblasts derived from affected individuals poorly process two substrates: PRELID1, a lipid transfer protein for phosphatidic acid in the IMS, and OPA1, a protein important for balancing fusion and fission in mitochondrial dynamics. After import to the mitochondria, the precursor OPA1 protein undergoes complex proteolytic processing by the mitochondrial QC proteases (giving rise to IMM-anchored L-OPA1 involved in fusion, which may be further cleaved to generate S-OPA1 involved in mitochondrial fission), making it vulnerable to processing defects (Figure 3B). The observed proliferation defects and mitochondrial network fragmentation in cells from affected family members are consistent with an abnormal processing of OPA1.²⁶ Genetic variations in *OPA1* are also a known cause of optic atrophy.⁵⁸

As a cautionary note, the complete understanding of the molecular functions of the proteases discussed here depends on the identification of their substrates/clients. However, these may be hard to discern, as the rules for substrate selection by the proteases are not well established. Although specific degron sequences have been identified for *YME1L1* and *AFG3L2* in the unstructured N termini of some substrates, the full spectrum of substrates remains poorly defined. Known substrates are used for functional variant validation although they may not be relevant for the cellular context or physiological conditions in which a new variant is identified (e.g., frataxin [FXN] or malate dehydrogenase [MDH2] are typically used to access the impact of variations in PMPCA^{59,60} or PMPCB,⁵⁷ and OPA1 for variations in *YME1L1*²⁶). Several known substrates are routinely tested as only some may be affected.^{57,60} In the case of ClpP, studies have demonstrated that the preference toward certain amino acids adjacent to the cleavage sites observed when using a tailored fluorogenic substrate library was not retained during proteolysis of endogenous substrates.

Rare Pathologic Genetic Variation Leading to Protein Mistargeting to the Mitochondria

MTSs bear similarity to other targeting signals, including peroxisomal targeting signal type 2 (PTS2). PTS2 mediates the import of a fraction of soluble proteins to the peroxisome. Osumi et al. first showed, by site-directed mutagenesis, that the replacement of His17 in the N-terminal signal peptide of rat peroxisomal 3-ketoacyl-CoA thiolase encoded by *Acaa1a* by Arg, Lys, Leu, or Val results in mitochondrial localization of this protein.⁶¹

AGXT (MIM: 604285) and *EHHADH* (MIM: 607037) are two examples of genes encoding proteins normally

Table 1. Reported Disease-Causing Variants Associated with Defects in Mitochondrial Proteases

Gene, Function (RefSeq#) ^a	Variants Effect on DNA and Protein Sequence (Linked SNPs)	Clinical Phenotype (OMIM#)	Evidence for Pathogenicity	Ref.
<i>PMPCA</i> , presequence cleavage (NM_015160.2)	1. c.[1129G>A];[1129G>A] ^b , p.[Ala377Thr];[Ala377Thr]; (rs753611141) 2. c.[287C>T];[1543G>A], p.[Ser96Leu];[Gly515Arg]; (rs869025292); (rs869025293)	cerebellar ataxia (MIM: 213200)	Allelic frequency: 0% ^c p.Ala377Thr associated with deficient MPP activity in patient-derived cell lines; predicted to cause a catalytic activity or substrate binding defect p.Ser96Leu and p.Gly515Arg in structurally conserved domains	Jobling et al. ⁵⁹
<i>PMPCB</i> , presequence cleavage (NM_004279.2)	1. c.[523C>T];[601G>C], p.[Arg175Cys];[Ala201Pro]; (rs145596167);(rs146343535) 2. c.[524G>A];[530T>G], p.[Arg175His];[Val177Gly]; (rs200188353); (rs1436866272) 3. c.[1265T>C];[1265T>C], p.[Ile422Thr];[Ile422Thr]; (rs1461200360)	infantile neurodegeneration (MIM: 29576218)	p.Arg175Cys, p. Ala201Pro, and p.Ile422Thr decrease protein level and activity in fibroblast and/or hiPSCs/NESs patient cell lines, and severe growth and/or MPP processing defects in yeast complementation. p.Arg175His, p.Ala201Pro, p.Ile422Thr, and p.Val177Gly predicted to cause protein stability, active site, and/or dimerization defects	Vögtle et al. ⁵⁷
<i>MIPEP</i> , presequence trimming (NM_005932.3)	1. c.[212T>A];[1745T>G], p.[Leu71Gln]; [Leu582Arg]; (rs10575118740); (rs1057518739) 2. c.[916C>T];[1804G>T], p.[Leu306Phe];[Glu602*]; (rs143912947); (rs114638163) 3. c.[1027A>G];[1027A>G], p.[Lys343Glu];[Lys343Glu]; (rs1057518741) 4. c.[1534C>G]; + ch13: 1.4 Mb CNV_del, p.[His512Asp]	left ventricular non-compaction (MIM: 602241)	Allelic frequency: 0% ^d for all variants except p.Leu306Phe = 8.2×10^{-6} , p. pHis512Asp = 3.2×10^{-5} p.Leu71Gln and p.Lys343Glu and orthologous yeast mutants have decreased enzymatic activity p.Leu71Gln, p.Leu582Arg, p.Leu306Phe, p.Lys343Glu, and p.His512Asp predicted pathogenic	Eldomery et al. ¹⁰⁵
<i>XPNPEP3</i> , presequence trimming (NM_022098.3)	1. c.[1357G>T]; [1357G>T], p.[Gly453Cys];[Gly453Cys]; (rs267607179) ^e 2. c.[931_934del];[931_934del], p.[Asn311Leufs*5];[(Asn311Leufs*5)] ^f	nephronophthisis-like nephropathy-1 (MIM: 613159)	Allelic frequency: 0% ^g p.Gly453Cys has been showed to cause a splicing defect on patient's mRNA	O'Toole et al. ¹⁰⁶
<i>PITRM1</i> , post cleavage MTS degradation (NM_014889.3)	c.[548G>A];[548G>A], p.[Arg183Gln];[Arg183Gln]; (rs1249144069)	mental retardation, spinocerebellar ataxia, cognitive decline and psychosis	Allelic frequency: 0% ^h Protein instability, impaired cell proliferation, and mitochondrial function have been detected on patients' fibroblasts and <i>in vitro</i> functional assay	Brunetti et al. ¹⁰⁷
<i>HTRA2</i> , intermembrane space protease (NM_013247.4)	1. c.[1211G>A];[1211G>A], p.[Arg404Gln];[Arg404Gln]; (rs767006508) 2. c.[1316_1320del];[1316_1320del], p.[Ala439Aspfs*15];[Ala439Aspfs*15]; (rs1057519080)	3-methylglutaronic aciduria type VIII, MGCA8 (MIM: 617248)	Allelic frequency: 0% ³ Both variants cause complete absence of the protein; fibroblasts with p.Arg404Gln have impaired cell growth	Mandel et al. ¹⁰⁸
<i>LONP1</i> , matrix protease/chaperone (NM_004793.3)	1. c.[2245C>T];[2300G>A], p.[Pro749Ser];[Gly767Glu] ^f 2. c.[1427A>C];[2245C>T], p.[Glu476Ala];[Pro749Ser] 3. c.[2009C>T];[2780_2782del], p.[Ala670Val];[Ile927del] ^f 4. c.[2014C>T];[2014C>T], p.[(Arg672Cys)];[(Arg672Cys)] ^f 5. c.[2009C>T];[2009C>T], p.[(Ala670Val)];[(Ala670Val)] ^f 6. c.[1392G>A]; [2036G>A], p.[(Trp464*)];[(Arg679His)]; (rs549574673)	cerebral, ocular, dental, auricular, skeletal anomalies (CODAS) syndrome (MIM:600373)	Allelic frequency: 0% ^c All variants except p.Arg679His predicted as pathogenic and to cause structural defects	Dikoglu et al. ¹⁰⁹
<i>CLPP</i> , matrix protease/chaperone (NM_006012.2)	1. c.[433A>C];[433A>C], p.[Thr145Pro];[Thr145Pro]; (rs398123033) 2. c.[440G>C];[440G>C], p.[Cys147Ser];[Cys147Ser]; (rs398123034) 3. c.[270+4A>G];[270+4A>G]; (rs398123035)	Perrault syndrome (MIM: 23541340)	Allelic frequency: 0% ⁱ p.Thr145Pro and p.Cys147Ser predicted as pathogenic and to cause folding defect c.270+4A>G caused splicing defect <i>in vitro</i>	Jenkinson et al. ¹¹⁰

(Continued on next page)

Table 1. Continued

Gene, Function (RefSeq#) ^a	Variation Effect on DNA and Protein Sequence (Linked SNPs)	Clinical Phenotype (OMIM#)	Evidence for Pathogenicity	Ref.
<i>YME1L1</i> , i-AAA-protease (NM_014263.3)	c.[445C>T];[445C>T], p.[Arg149Trp];[Arg149Trp]; (rs1057519312)	optic atrophy-11 (MIM: 617302)	Allelic frequency: 0% ^j Variant causes protein degradation, abnormal substrate processing, and other functional defects	Hartmann et al. ²⁶

Abbreviations: hiPSC, human induced pluripotent stem cells; MPP, mitochondrial processing peptidase; NES, neuroepithelial stem cells.

^aAll the identified DNA variants cosegregate with disease phenotype.

^bFounder mutation in a Lebanese Christian Maronite population.

^cAllelic frequency is based on control group from ExAC.

^dAllelic frequency is based on control groups from ExAC and Baylor-CMG exome.

^eFounder mutation in a Northern Finnish population.

^fdbSNP ID not applicable.

^gAllelic frequency is based on in-house control database, ethnicity or geographic-matched controls.

^hAllelic frequency is based on 1000 Genomes.

ⁱAllelic frequency is based on NHLBI Exome Sequencing Project (ESP) Exome Variant Server.

^jAllelic frequency is based on ExAC, 1000 Genomes, NHLBI Exome Sequencing Project (ESP) Exome Variant Server, dbSNP.

destined to the peroxisomes for which disease-causing mislocalization in the mitochondrial matrix has been reported. In rare individuals, mislocalization of the protein products of AGXT and EHHADH to the mitochondrial matrix rather than the peroxisome can cause severe renal disease. Primary hyperoxaluria type I (PH1) (MIM: 259900) is an autosomal-recessive disorder of the glyoxylate metabolism leading to overproduction of oxalate, which manifests as renal stones and/or nephrocalcinosis. PH1 is caused by mutations in *AGTX*, which encodes the hepatic, peroxisomal alanine-glyoxylate aminotransferase (AGT). Mitochondrial targeting of AGTX occurs in persons homozygous for the variant c.32C>T (p.Pro11Leu) (GenBank: NM_000030.2, rs34116584) but, as described below, this variant is ineffective by itself. Mistargeting of AGT leads to poor efficiency of glyoxylate detoxification and accumulation of calcium oxalate in various bodily tissues, especially the kidney, leading to renal failure⁶² (Figure 3C), although at this time conflicting interpretations of the pathogenicity of this variant are found in ClinVar (ID: 5641). The relatively common p.Pro11Leu variant is reported in the gnomAD database at an allelic frequency of ~0.15 in the general population (ranging from ~0.20 in Europeans to ~0.0004 in East Asians), and hence is clearly not sufficient as a monogenic source of dysfunction. A possible molecular explanation for the relationship of this specific variant to control of mitochondrial import and disease lies in the fact that the leucine residue introduces a weak, non-cleavable putative mitochondrial targeting sequence at the N terminus of the molecule (MASHKLLVTP_LKALLKPLSC). Because the dimerization of AGT that rapidly follows its folding results in a tight folded structure with a buried N terminus, incompatible with mitochondrial import, the variant is functionally ineffective by itself. However, present in *cis* with rare variants that disrupt the folding/dimerization process and expose the N terminus (e.g., c.508G>A [p.Gly170Arg], rs121908529; c.731T>C [p.Ile244Thr], rs121908525; c.454T>A [p.Phe152Ile], rs121908524, and c.121G>A [p.Gly41Arg], rs121908523), this variant allows preferen-

tial mitochondrial import.⁶³ Mild translation inhibition with emetine was recently found to correct the mislocalization of the mutant protein Pro11Leu;Gly170Arg, likely because slowing translation improved folding.⁶⁴

EHHADH encodes one of the four enzymes of the peroxisomal β -oxidation pathway and has also been linked to disease due to mitochondrial mistargeting. *EHHADH* is a bifunctional protein; its N-terminal region contains enoyl-CoA hydratase activity while its C-terminal region contains 3-hydroxyacyl-CoA dehydrogenase activity. The *EHHADH* variant GenBank: NM_001966.3; c.7G>A (p.Glu3Lys) (rs398124646) has been associated with a familial case of autosomal-dominant renal Fanconi syndrome (MIM: 615605),⁶⁵ manifesting as rickets, impaired growth, glucosuria, generalized aminoaciduria, phosphaturia, metabolic acidosis, and low-molecular-weight proteinuria. This variant generates a *de novo* MTS which is cleaved upon import to the mitochondria. The neoprotein is then incorporated into mitochondrial trifunctional protein (MTP), an IMM-bound hetero-octamer encoded by *HADHA* (MIM: 600890) and *HADHB* (MIM: 143450), which catalyze the final steps of mitochondrial long-chain fatty acid β -oxidation. The improper incorporation of the *EHHADH* protein resulting from the DNA variation impairs the normal catalytic process for fatty acid β -oxidation and also impairs mitochondrial respiration due to the coupling of these processes by MTP.⁶⁶

Tail-anchored (TA) proteins are also aberrantly targeted to the mitochondria. This unique class of functionally diverse membrane proteins is defined by their single C-terminal transmembrane domain and their ability to insert post-translationally into specific organelles (including endoplasmic reticulum [ER], mitochondria, and peroxisomes) with N termini exposed to cytoplasm and C termini within the organelle. Mislocalized tail-anchored (TA) proteins of the OMM are cleared by the quality control pathway involving the conserved eukaryotic protein Msp1 (ATAD1/Thorase in humans),⁶⁷ a signal-anchored membrane protein comprising an N-terminal transmembrane domain that tethers its soluble ATPase domain to

the cytosolic face of the OMM and peroxisome. ATAD1 is also known as a mediator of recycling of the glutamate-gated channels α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors, thereby regulating synaptic plasticity and learning and memory.⁶⁸ Several recent studies have reported lethal encephalopathy cases with defects in AMPAR recycling linked to *ATAD1* (MIM: 614452) variants.^{69,70} It is interesting to note that Msp1 also is involved in the mitoCPR pathway in yeast. This pathway resolves mitochondrial import defects occurring at the intermembrane level;⁷¹ Msp1 mediates clearance and proteasomal degradation of unimported precursors from the mitochondrial surface. The recognition that Msp1/MitoCPR can reduce mitochondrial mistargeting in yeast suggests that further studies of its human homolog ATAD1 could elucidate similar or expanded functionality in mammalian cells.⁷²

A final example of protein mistargeting to the mitochondria involves the variant GenBank: NM_007262.4; c.497T>C (p.Leu166Pro) variant (rs28938172) in *DJ-1/PARK7* (MIM: 602533). This variant has been linked to early-onset familial Parkinson disease and appears to cause mistargeting of the normally cytoplasmic localized protein to the mitochondria. In the mitochondria, DJ-1/PARK7 impairs cell growth by differentially regulating mitochondrial Bax/Bcl-XL functions. The variation also reduces stability of DJ-1 by allowing it to directly bind to the mitochondrial serine protease HTRA2.⁷³

Deregulated Mitochondrial Import in Cancer

As discussed in the preceding sections, most of the genetic diseases associated with defects of mitochondrial import involve neurologic and neuromuscular degeneration. As with neurodegenerative diseases, the physiologic changes that accompany and support cancer involve altered abundance of reactive oxygen species (ROS), altered control of protein production and degradation, and altered mitochondrial activity, albeit with some changes being opposite to those seen in neurodegenerative disease. For example, most cancer cells preferentially use glycolysis as an energy source (the Warburg effect), while neuronal cells bearing genetic variants associated with neurodegeneration upregulate proteins required for oxidative phosphorylation, in what has sometimes been termed an inverse Warburg effect.⁷⁴ These relationships have raised the interesting possibility that inherited damaging genetic variants affecting import of proteins required for normal mitochondrial activity may in some cases reduce the risk of some forms of cancer through alteration of necessary energy resources in the cancer cell.⁷⁵ Conversely, the enhanced production or activity of proteins promoting mitochondrial import, and thereby supporting mitochondrial biogenesis or function, may characterize some tumors with particularly high energy needs.

While the relative rarity of known disease-associated variants affecting the import machinery or import signals makes it difficult to assess the impact of such variants on

cancer incidence or other clinical correlates of cancer, studies of tumors have revealed intriguing patterns of changes affecting subsets of mitoproteases,²⁸ translocases,⁷⁶ and mitochondrial chaperones.⁷⁷ Although no global analyses have been reported, multiple components of the IMM and OMM import complexes have been shown to be present at elevated protein levels in some tumor types; for example, aberrant overproduction of MIA40 has been seen in multiple forms of cancer and has been associated with poor prognosis. Similarly, the viral oncogenic VacA protein of *Helicobacter pylori* has also been shown to rapidly induce production of mitochondrial translocases, concomitant with the progression of gastric lesions to a highly malignant state, suggesting a direct pathogenic role of upregulation of mitochondrial components. In some cases, proteins typically thought to serve as elements of oncogenic signaling cascades are emerging as regulators of mitochondrial import. For example, phosphorylation of the chaperone HSP70 by AKT1 was shown to regulate the mitochondrial import of SOD2, thereby affecting cellular redox balance.⁷⁸ We note that this finding emphasizes the difficulty in exactly defining the import machinery as it uses many accessory components in addition to the core components.

The studies mentioned above demonstrating elevated levels of proteins associated with mitochondrial import in cancer align with a growing number of studies that emphasize that mitochondrial import of proteins normally thought of as cytoplasmic or nuclear may contribute to tumor growth (e.g., MDM2⁷⁹). Moreover, a substantial literature addresses the roles of mitochondrial proteases such as HTRA2 and LONP in cancer (reviewed in Quiros et al.²⁸). For example, heterozygous LONP deletion in mouse models for colorectal and skin cancer has been shown to attenuate tumor formation; elevated LONP in human specimens is associated with poor prognosis for these cancers;⁸⁰ and *H. pylori* infection was also shown to strongly induce LONP protein production in gastric cancer. In each of these examples, increased LONP activity was also associated with increased glycolysis.

Certainly, the pattern of mitochondrial protein production observed in cancer is relevant to expected requirements for metabolic reprogramming and mitochondrial biogenesis. However, we note, for the examples cited (and numerous other emerging examples), the degree to which these phenotypes are dependent on amplification of normal function of abnormally increased levels of proteins involved in mitochondrial import and related quality control, versus neomorphic activities remains to be established. From the point of view of the geneticist, these examples suggest that it may be of interest to determine whether known polymorphisms or rare DNA variants are associated with elevated levels of proteins of the import machinery, as these may emerge as relevant to elevated cancer risk.

Finally, it is possible that underlying or induced variance in mitochondrial import capacity may influence efficacy of

cancer therapy.⁸¹ Based on the recognition that tumors differ significantly from untransformed cells in utilization of oxidative phosphorylation, some therapeutic approaches may act by selectively inhibiting energy production in cancer cells (for example, through reduced mitochondrial import, discussed in Zhu et al.⁸¹). The natural product stendomycin, long used as an antifungal, was recently identified as an inhibitor of the TIM23-dependent import machinery⁸² and may be useful in this regard. Conversely, some existing therapies may in part function via interaction with the mitochondrial import machinery. For example, the multikinase inhibitor sorafenib was found to unexpectedly cause inhibition of complex II/III of the mitochondrial electron transport chain. This activity was due to sorafenib stabilization of PINK1 on the mitochondrial outer membrane, and recruitment of Parkin,⁸³ associated with changes in import. In another example, reduced levels of proteins required for mitochondrial import (TOM40, TIM23, and ALR) were selectively cytotoxic in acute myeloid leukemia (AML) and in stem cells, in contrast to non-transformed hematopoietic cells, reflecting the greater OXPHOS requirements of AML.⁸⁴ Overall, these factors reinforce the clinical value of gaining insights into mitochondrial function through consideration of the features of monogenic diseases involving mitochondrial import.

Diagnosis and Potential Implications of Altered Mitochondrial Import and Targeting

In the diagnosis of rare disorders such as many of those described here, traditional approaches to diagnosis through careful phenotypic characterization and tissue biopsy are being replaced by a “genotype first” approach to diagnosis by way of reverse phenotyping. With growing use and availability of NGS-based exome and whole-genome testing and array technologies, it is likely that an increasing number of variants within mitochondrial genes and gene products associated with mitochondrial function, transport, and targeting will be identified across a wide range of human diseases including common diseases like cardiovascular disease and cancer. While at present many of the examples we discuss are supported only by limited numbers of case studies, we anticipate the number of observed cases will rapidly increase. The examples cited above make it clear that the process of mitochondrial targeting is vulnerable at numerous steps and that targeting defects may explain at least some common features of otherwise distinct genetic disorders.

The scarcity of established pathogenic variants in genes relevant for mitochondrial import has been proposed to reflect their essentiality and intolerance to variation.^{85,86} Indeed, only ~5% of known mitochondrial disorders are autosomal dominant (AD) (see Table S1).⁸⁷ Moreover, genetic and other high-throughput screens in yeast^{47,88,89} and targeted studies performed in mice and in other model organisms (e.g., Sokol et al.⁷⁶) have since demonstrated the essentiality of a number of the genes of the import ma-

chinery. Further, although lethality is typically associated with homozygous loss, compatible with a recessive phenotype, some of these genes are at least partially haploinsufficient. For example, while homozygous mutant mice in *Lonp1* or *Tmem70* are not viable (Table S1), *Lonp1*^{+/-} and *Tmem70*^{+/-} heterozygous mice display altered susceptibility to some forms of cancer and mild deterioration of heart function, respectively. Ongoing efforts to identify genes essential in humans by observing intolerance to loss-of-function variants across large numbers of sequenced genomes⁹⁰ will provide clarity as to whether genes involved in mitochondrial import are unusually sensitive to function-disrupting gene variants.

A number of variant databases are available to assist clinical researchers address disorders associated with mitochondrial import. Some have general scope (e.g., the Mitochondrial Disease Sequence Data Resource consortium [MSeqDR]), while others focus on a specific gene (e.g., *POLG*). The availability of public databases including NCBI LitVar (which automatically extracts variant data from PubMed articles), NCBI ClinVar (which reports of the relationships among human variations and phenotypes), and gnomAD (providing allele frequencies in the context of variance among ethnic groups, based on exome data from >120,000 individuals and whole-genome data from >15,000 individuals), can help in identifying disease-associated variants. This analysis suffers the general pitfalls involving any variant curation such as the unfortunate bias in databases toward populations of European descent and the limitations of self-reported race, ethnicity, and genetic ancestry⁹¹ that may lead to worse clinical care and outcome for under-represented populations but also variant pathogenicity misclassifications.⁹²

For a significant number of variants, these methods can be complemented by computational approaches to predict the consequences of an amino-acid replacement. For the cases of mitochondrial targeting defects, algorithms that predict the effect of amino acid substitutions can be useful in cases where a variant affects a folded domain of a protein with a high degree of evolutionary conservation and well-defined structure-function relationship. However, for variants affecting the MTS, these programs can have limited utility, and there is clear need for experimental validation of predictions. Similar to other targeting signals that are involved in promiscuous recognition by a common machinery (e.g., in the secretory pathway),⁹³ MTS may be conserved between species in terms of general structural properties (such as the ability to form an α -helix) but not in primary amino acid sequences.²⁵ This low conservation has practical implications for assessment of gene variants potentially associated with genetic disease, as low conservation may result in poor scores by some of the most frequently used pathogenicity predictor programs (e.g., SIFT and PolyPhen), which are based on the principle that evolutionary constrained regions are of critical importance for protein function. This may explain why only 0.9% of disease variations reported in UniProt fall within

annotated sequences or motifs targeting signals for nuclear import or export, mitochondrial localization, or the secretory pathway.⁹⁴ A number of groups have proposed specific structural features and lists of MTS sequences, but for many proteins, these sequences remain cryptic. Hence, empirical investigations that yield a mechanistic understanding of how specific variants relate to biology is highly desirable, particularly for suspected targeting variants (see discussion in Gonzalez-Sanchez et al.⁹⁵). As pointed out in a recent review of mechanisms by which coding sequence variations disrupt a protein function, particularly common defects include folding disruption, subcellular localization alteration, and rewiring of an interaction network.⁹⁴ As illustrated above, all three classes of defect pertain to variants of proteins involved in mitochondrial import.

An issue unique to mitochondrial dysfunction, and potentially important in assessment of inherited variants, is the involvement of both the nuclear and the mitochondrial genomes in the mitochondrial proteome. The human population is subdivided into a small number of mitochondrial haplogroups created by variations in the mtDNA that have accumulated throughout human history. Cases of incompatibility between mitochondrial and nuclear genomes have been reported in various organisms (e.g., in *Drosophila*). In humans, divergent patterns of mitochondrial and nuclear ancestry are associated with the risk for preterm birth and it has been suggested that deleterious interactions between the mitochondrial and nuclear genomes could pose a problem for mitochondrial replacement therapy (MRT) (reviewed in Eyre-Walker⁹⁶). Examples of nuclear modifier genes that would explain the variable penetrance of disorders caused by mitochondrial variants have been presented.^{97,98} Conversely, it was recently shown that the severity of the cardiomyopathy caused by variations in *ANT1* can be modulated by the presence of otherwise mild mtDNA variants.⁹⁹ Studies on the potential effects of nuclear polymorphism-mitochondrial haplotype interactions are emerging.¹⁰⁰

Finally, it may be possible to develop therapies for mitochondrial diseases or diseases that are in part driven by aberrant mitochondrial function or energy production based on targeting the mitochondrial import and transport processes. A number of clinical trials are ongoing or planned for treatment of mitochondrial diseases (discussed in El-Hattab et al.¹⁰¹ and Hirano et al.¹⁰²); for example, use of elamipretide (MTP 131) for cardiolipin protection from oxidation and stabilization is proposed in cases of mitochondrial myopathy. Pyridoxine is being commonly used for treatment of PH1; *in vitro*, pyridoxine has been shown to increase AGT levels and enhance peroxisomal localization of some common disease-associated variants;¹⁰³ conversely, other compounds, such as dequalinium chloride, block AGT import into mitochondria.¹⁰⁴ Extensions and variations of such approaches to target mitochondrial function offer future promise for treatment of the rare disorders described here and more common conditions that

may, in part, be susceptible to therapies targeting mitochondrial protein trafficking.

Supplemental Data

Supplemental Data can be found online at <https://doi.org/10.1016/j.ajhg.2019.03.019>.

Acknowledgments

The authors were supported by NIH R01 DK108195 (to E.A.G.) and by NIH Core Grant CA006927 (to Fox Chase Cancer Center). The authors would like to thank the Genome Aggregation Database (gnomAD) and the groups that provided exome and genome variant data to this resource. A full list of contributing groups can be found at <https://gnomad.broadinstitute.org/about>.

Web Resources

1000 Genomes, <http://www.internationalgenome.org/1000-genomes-browsers>
Baylor-Hopkins Center for Mendelian Genomics, <https://mendeliangenomics.org/>
ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>
dbSNP (build 138), <https://www.ncbi.nlm.nih.gov/projects/SNP/>
ExAC Browser, <http://exac.broadinstitute.org/>
GenBank, <https://www.ncbi.nlm.nih.gov/genbank/>
gnomAD Browser, <https://gnomad.broadinstitute.org/>
Human DNA Polymerase Gamma Mutation Database, <https://tools.niehs.nih.gov/polg/>
International Mouse Phenotyping Consortium, <http://www.mousephenotype.org/data/genes/>
LitVar, <https://www.ncbi.nlm.nih.gov/CBBresearch/Lu/Demo/LitVar/>
Mouse Genome Informatics, <http://www.informatics.jax.org/>
MSeqDR, <https://mseqdr.org/>
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, <http://evs.gs.washington.edu/EVS/>
OMIM, <http://www.omim.org/>
PubMed, <http://www.ncbi.nlm.nih.gov/PubMed/>

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