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Review

Consequences of defective mitochondrial protein import: From targeting signals to pathology

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ABSTRACT

Cellular organelles are uniquely specialized membrane-bound structures that enable cells to organize and coordinate biochemical processes. Specifically, mitochondria are essential organelles for cellular metabolism, coordinating energy production, and connecting signaling networks for cellular homeostasis. 99% of mitochondrial proteins are encoded by nuclear genes that require precise and efficient translation and import into mitochondria for biological processes. This process is mediated by coordinated pathways involving the mitochondrial specific translocation complexes, chaperones, and specialized targeting routes. Tight regulation of these import mechanisms allows for proper protein localization, folding, and assembly. Disruptions in the mitochondrial protein import pathway compromise organelle homeostasis and activate proteostatic stress and quality control pathways. Such defects have been observed in a wide range of pathophysiological conditions, including cardiovascular disease, neurodegeneration, and cancer. The import defects destabilizing mitochondrial proteins can impair oxidative phosphorylation and metabolic signaling. In sum, defects to mitochondrial function can highlight a central role of mitochondrial protein import beyond maintaining cellular function and how defects at distinct stages of import contribute to disease, underscoring opportunities for therapeutic intervention targeting mitochondrial proteostasis.

1. Introduction

Energy production, orchestration of intra- and intercellular metabolic networks, and signaling pathways that sustain life are maintained by mitochondria (35804477 [1]). These functions rely on a finely tuned network of proteins that regulate mitochondrial import, processing, and assembly, critical processes that maintain cellular homeostasis (35804477 [1], 16860735 [2]). Despite representing only 7% of the total mammalian proteome, mitochondrial proteins play an indispensable role in sustaining cellular function and health (7219534 [3]). The 16.5 kb circular human mitochondrial genome encodes for 13 essential subunits of the electron transport chain (ETC), 22 tRNAs and 2 rRNAs needed for their translation (7219534 [3]). 99% of the mitochondrial proteome must be synthesized in the cytosol and imported into the organelle (34800366 [4], 9242927 [5]). Protein import depends on a highly coordinated system of translocases and chaperones. Together, these components ensure efficient trafficking, processing and assembly of mitochondrial proteins essential for cellular maintenance (Table 1).

Mitochondrial protein import is primarily driven by the translocase of the outer membrane (TOM) and the translocase of the inner membrane (TIM23) pathways. They are assisted by a network of chaperones that facilitate protein recognition, translocation, and folding (34800366 [4]). Mitochondrial proteins are recognized through diverse targeting signals, including N-terminal targeting sequences, internal motifs and transmembrane domain associated signals that are critical for directing proteins through the mitochondrial import machinery (9603986 [6]). On the mitochondrial surface, TOM receptor subunits recognize and initiate their translocation across the outer mitochondrial membrane

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Table 1
Human and yeast mitochondrial protein nomenclature.

Human name	Yeast name	Function	Complex
TOMM40	Tom40	Central pore of TOM complex	TOM complex
TOMM20	Tom20	Receptor- precursors with N-terminal sequence	TOM complex
TOMM70	Tom70	Receptor-hydrophobic/carrier precursors	TOM complex
TOMM22	Tom22	Central docking site	TOM complex
TOMM7	Tom7	Small TOM subunit; complex stability	TOM complex
TOMM6	Tom6	Small TOM subunit; complex stability/assembly	TOM complex
TOMM5	Tom5	Small TOM subunit; facilitates TOM → TIM transfer	TOM complex
TIMM23	Tim23	Core TIM23 pore subunit	TIM23 complex
TIMM17A/B	Tim17	Core TIM23 pore subunit; IM insertion	TIM23 complex
TIMM50	Tim50	IMS receptor; detects incoming targeting sequences	TIM23 complex
mtHsp70	Hsp70	Central ATPase of PAM; drives matrix import	PAM complex
GRPEL1	Mge1	Nucleotide exchange factor for mtHsp70	PAM complex
TIMM44	Tim44	Tethers mtHsp70 to TIM23 complex	PAM complex
TIMM16	Pam16/ Tim16	Recruits Pam18; part of Pam16-Pam18 heterodimer	PAM complex
TIMM14	Pam18/ Tim14	Stimulates mtHsp70 ATPase activity	PAM complex
TIMM22	Tim22	Pore subunit of TIM22 carrier pathway	TIM22 complex
TIMM9	Tim9	Small TIM chaperone; substrate delivery to TIM22	TIM22 complex
TIMM10	Tim10	Small TIM chaperone; pairs with TIMM9	TIM22 complex
DDP1	Tim8	Small TIM chaperone; pairs with TIMM13	TIM22 complex
TIMM13	Tim13	Small TIM chaperone; pairs with TIMM8A	TIM22 complex
MIA40	Mia40	Oxidoreductase receptor; forms disulfide bonds with IMS substrates	MIA pathway
ALR	Erv1	Sulfhydryl oxidase; reoxidizes MIA40	MIA pathway
SAMM50	Sam50/ Tob55	Core SAM complex subunit; β-barrel protein assembly in OM	SAM complex
VDAC1/2/3	Por1 (porin)	Voltage-dependent anion channel; β-barrel OM protein	SAM complex
MPPα/Mas2	Mas2/ α-MPP	Non-catalytic MPP subunit	Processing peptidase
MPPβ/Mas1	Mas1/ β-MPP	Catalytic MPP subunit; cleaves N-terminal presequences	Processing peptidase
XPNPEP3	Icp55	Removes single destabilizing residue after MPP cleavage	Processing peptidase
MIPEP	Oct1	Cleaves 8-aa segment from subset of precursors after MPP	Processing peptidase
IMMP1L	Imp1	Catalytic IMP complex subunit; cleaves IMS-targeted precursors	Processing peptidase
IMMP2L	Imp2/ Imp2p	Catalytic IMP complex subunit; cleaves IMS-targeted precursors	Processing peptidase
m-AAA	m-AAA	Matrix-AAA metalloprotease; degrades misfolded IM proteins	Quality control peptidase
YME1L	Yme1	IMS-AAA protease; quality control of IMS/IM proteins	Quality control peptidase
PARL	Pcp1/ Rbd1	Rhomboid protease in IM; cleaves PINK1, PGAM5	Quality control peptidase
PreP	Cym1	Matrix oligopeptidase; degrades presequences and short peptides	Oligopeptidases
Neurolysin	Prd1	Matrix oligopeptidase; degrades peptides up to 19 aa	Oligopeptidases
Hsp60	Hsp60	Mitochondrial matrix chaperonin; post-import protein folding	Chaperone
PDHA1	N/A	Pyruvate dehydrogenase E1α	
AGT	N/A	Alanine-glyoxylate transferase	
EHHADH	N/A	Peroxisomal fatty acid oxidation enzyme	
PINK1	N/A	Mitochondrial kinase; import/ processing sensor	
PGAM5	N/A	PARL substrate; modulates mitochondrial dynamics and cell death	
DIABLO/ SMAC	N/A	Pro-apoptotic IMP substrate	
Cytochrome *c*1	Cyt1	Complex III subunit; IMP substrate	
ANT1/ADT1	Aac1/2 (AAC)	ADP/ATP translocase; TIM22 pathway substrate	
COX17	Cox17	IMS copper chaperone; MIA pathway substrate with Cx9C motif	
NFS1	Nfs1	Iron-sulfur cluster biogenesis; Icp55/XPNPEP3 substrate	
BCL2	N/A	Anti-apoptotic OM protein; C-terminal hydrophobic anchor	
TIMM17A	Tim17	Core TIM23 subunit; no N-terminal targeting sequence	
ROMO1	Mgr2	TIM23 subunit processed by IMP independently of MPP	
N/A	Cox2	Yeast IMP substrate; cytochrome *c* oxidase subunit	
N/A	Mcr1	Yeast IMP substrate; NADH-cytochrome *b*5 reductase	
GPD2	Gut2	Glycerol-3-phosphate dehydrogenase; IMP substrate (Imp1p in yeast → Imp2p in mammals)	

through the Tom40 channel (10990453 [7], 10721992 [8]). Once translocated through TOM, proteins are subsequently guided by internal targeting sequences along specialized sorting pathways such as the TIM23, TIM22, SAM, and MIA pathways. These pathways ensure delivery of precursors to their proper mitochondrial sub-compartments (20457929 [9], 34446444 [10], 24407114 [11]). The precision of these targeting pathways is critical, as disruptions can compromise mitochondrial integrity and drive dysfunction (33314127 [12], 27810356 [13], 30190335 [14], 37129366 [15], 11875042 [16]).

Defects in mitochondrial protein import contribute to a wide range of pathophysiological conditions, including cardiovascular disorders (35328774 [17], 23401503 [18], 16155110 [19]), neurodegenerative diseases (37129366 [15], 34944035 [20]), and cancer (31818360 [21], 30604908 [22]). When mitochondrial protein import fails at early stages, precursor proteins predominantly accumulate in the cytosol, where they can overwhelm cytosolic quality control systems and induce proteostatic stress (31195097 [23]). Mitochondrial proteostatic stress can then be resolved through proteolytic degradation, whereas sustained or excessive stress activates hierarchical stress responses including the unfolded protein response (UPR), and mitochondrial precursor over-accumulation stress (mPOS) impacting the cellular proteome (24353213 [24], 29090463 [25]). In contrast, when there is accumulation of defective proteins within the mitochondria, intramitochondrial folding, processing, or assembly is thereby impeded and is normally mitigated by resident mitochondrial proteases. In addition, mutations that impair precursor folding or disrupt recognition by the mitochondrial import machinery prevent efficient import, leading to cytosolic retention, degradation, or engagement of quality control pathways such as PINK1-mediated mitophagy. Such defects lead to destabilization of mitochondrial proteins, resulting in protein degradation or loss of function thereby impairing oxidative phosphorylation and metabolic signaling (35163221 [26], 34228161 [27]). Exploration of how failure at each stage of mitochondrial import contributes to disease will enhance our understanding of its pathological relevance and may

reveal new therapeutic targets. In this review, we highlight how mitochondrial protein import safeguards organelle integrity and cellular homeostasis. We further discuss how disruptions across distinct steps of the mitochondrial import pathway can initiate the onset and progression of human diseases, emphasizing the need to integrate mechanistic insights into translational strategies for mitochondrial targeted medicine (Graphical abstract).

2. Architecture for import: structural determinants of mitochondrial entry

Mitochondrial proteins rely on intrinsic sequence features that dictate their final destination (Fig. 1, Table 2). Approximately half of mitochondrial proteins carry a canonical N-terminal targeting sequence. These targeting sequences are positively charged amphipathic targeting sequences that direct mitochondrial proteins to the TOM complex in the outer membrane (OM) and subsequently through the TIMM23 translocase into the matrix or inner membrane (IM) (29270408 [28], 28701417 [29]). In the canonical import pathway, an N-terminal MTS directs precursor proteins through the TOM and the TIM23 complexes, a process that requires intact inner membrane potential ($\Delta\psi$), ATP-dependent motor activity and is frequently coupled to proteolytic processing by matrix peptidases (19703392 [30]). Beyond the canonical pathway, many proteins lack a cleavable N-terminal targeting sequence and instead rely on internal targeting information (iMTSs) or transmembrane domain-associated signals. These "non-canonical" precursors engage alternative import routes, such as TOMM70-dependent recognition and sorting through the TIM22 pathway. This mechanism is exemplified by metabolite carrier proteins, which rely on internal targeting signals for recognition and subsequently handed off to IMS chaperones, with hydrophobic transmembrane segments driving membrane insertion (32645990 [31]). Thus, intrinsic sequence and structural properties predetermine import route selection at the mitochondrial surface, representing an early yet critical tier of mitochondrial protein quality control. Delineating how these targeting sequences encode import specificity is essential for understanding mitochondrial proteostasis. Here, we describe how bona fide sequence features direct proteins to the mitochondria and other downstream organelles.

2.1. Mitochondrial targeting sequence (MTS)

N-terminal mitochondrial targeting sequence (MTS) directs the proteins into the mitochondria from the cytosol (Table 2). A typical MTS folds into an amphipathic α-helix whose structural properties allow for selective recognition by receptors on the mitochondrial surface (3396537 [32], 3015598 [33], 10721992 [8]). MTSs display remarkable sequence diversity, reflecting the broad functional and evolutionary demands of the mitochondrial proteome (9242927 [5], 3015598 [33]). This sequence diversity enables precise control over import efficiency, compartment selection, and precursor folding. Despite its diversity in sequence, MTSs share common physiochemical characteristics. The typical N-terminal MTS spans ~10–80 amino acids (40320395 [34], 23825690 [35]), typically has a net positive charge of +3 to +6 (40320395 [34], 10721992 [8], 23825690 [35]), and has hydrophobic residues that enable amphipathic helix formation (40320395 [34], 3396537 [32]). As a result, MTS targeting specificity depends on their overall chemical properties rather than a specific sequence motif. Whereas approximately 60% of mitochondrial proteins contain canonical targeting sequences, roughly 30% lack them entirely (19837041 [36]). This demonstrates that mitochondrial targeting relies on multiple classes of signals, not solely N-terminal targeting sequences.

2.2. Internal mitochondrial sequences (iMTS)

In the classic precursor import pathway, subsequent intramitochondrial localization is determined by additional sorting signals that act in concert with, or independently of the MTS. A well characterized example is, cytochrome b_2, a mitochondrial intermembrane space protein, which contains an N-terminal targeting sequence followed by a stop-transfer and sorting signal. These elements cooperate to mediate its import through the TOM-TIM23 pathway and subsequent release into the IMS (8508764 [37]). Upon reaching the mitochondrial surface, the precursor proteins rely on internal mitochondrial sorting sequences (iMTS) beyond the well-known MTS targeting sequences to specify their ultimate localization (10518522 [38]). Their destinations include the outer membrane (OM), intermembrane space (IMS), inner membrane (IM), or matrix. These internal features include hydrophobic transmembrane segments, β-hairpin motifs, or short linear elements that guide precursors into the TIM22, mitochondrial IMS import and assembly machinery (MIA), or sorting and assembly machinery (SAM) pathways (27345737 [39], 19703392 [30]). The TIM22, MIA, and SAM

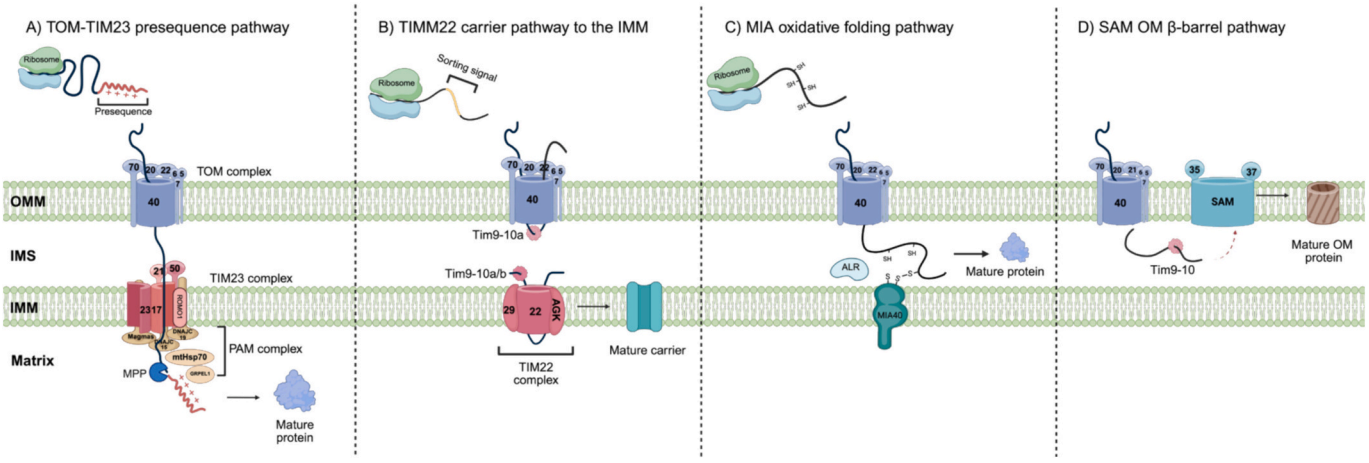

Fig. 1. Molecular pathways of mitochondrial protein targeting and import.
TOM = Translocase of the outer mitochondrial membrane, TIM = Translocase of the inner mitochondrial membrane, OMM = Outer mitochondrial membrane, IMS = Intermembrane space, IMM = Inner mitochondrial membrane, PAM = Presequence translocase-associated motor, MIA40 = Mitochondrial intermembrane space import and assembly protein 40, Erv1 = Essential for respiration and viability 1, SAM = Sorting and assembly machinery. All proteins have been noted in mammalian nomenclature.

Table 2
Architecture of mitochondrial targeting sequences (MTS) and internal mitochondrial targeting sequences (iMTS).

Targeting signal class	Targeting sequence features	Typical import pathway	Representative protein examples	Final localization
Classical MTS (presequence)	Cleavable N-terminal presequence, amphipathic α-helix	TOM-TIM23 pathway	PDHA1, PMPCB, HSPD1	Matrix
Presequence+ Stop-transfer sequence	Cleavable N-terminal presequence followed by a hydrophobic transmembrane domain	TOM-TIM23 with lateral release	PINK1	Inner membrane/ cytosol*
iMTS/non-cleavable signals	Multiple internal targeting elements	TOM-TIM23 pathway	TIMM50, IMMP2L	Inner membrane/IMS
Carrier targeting signals	C-terminal β-signal motif	TOM-SAM	TOMM40, VDAC	Outer membrane
Signal anchor sequences	Uncleaved N-terminal hydrophobic transmembrane domain	OM insertion	TOMM20, TOMM70	Outer membrane
MIA-dependent IMS targeting signals	Internal cysteine motifs	TOM-MIA40	COX17, TIMM9, TIMM10	IMS
Ectopic/cryptic/weak MTS (acquired)	Acquired amphipathic helices, mutation induced	TOM-TIM23	EHHADH, AGT	Matrix (mislocalized)
Bipartite signals	MTS+ downstream sorting signal	TOM-TIM23 with IMP	CYB2	IMS

MTS = Mitochondrial targeting sequence, iMTS = Internal mitochondrial targeting sequence, TOM = Translocase of the outer mitochondrial membrane, TIM = Translocase of the inner mitochondrial membrane, * = PINK1 is not a traditional IM targeted protein. It's targeting to mitochondria serves as a mitochondrial health sensor.

pathways insert IM carriers, oxidatively fold IMS proteins, and assemble β -barrel proteins in the OM, respectively highlighting its critical role in kickstarting the protein import process (19703392 [30], 23676665 [40], 28301740 [41]).

Internal signals often become functionally accessible during translocation or while the precursor is maintained in a translocation-competent, partially unfolded state (9362487 [42], 9242927 [5], 27810356 [13]). Even subtle changes, like loss of a hydrophobic or charged residue, can influence import efficiency and final protein structure (10721992 [8]). Outer membrane proteins, such as TOMM40 or Voltage-Dependent Anion Channel (VDAC), and carrier proteins, such as ADP/ATP translocase 1 (ANT1) or phosphate carrier (PiC) lack N-terminal targeting sequences and instead use internal or C-terminal signals to interact with specialized machinery for membrane import (27810356 [13], 1534805 [43], 10369662 [44]). β-Barrel proteins like VDAC rely on β-hairpin motifs recognized by the SAM complex (34446444 [10]), whereas α-helical outer membrane proteins such as the anti-apoptotic protein, Bcl-2, use C-terminal hydrophobic segments for insertion (27810356 [13]). Some α-helical outer membrane proteins contain N-terminal signal-anchor helices paired with transmembrane segments that function as stop-transfer or membrane-anchoring elements (9242927 [5]).

Proteins targeted to the intermembrane space, are sorted utilizing the MIA oxidative folding pathway (35163221 [26]). IMS proteins, such as Cox17, contain a twin cysteine motif (CX_3C) which is recognized by MIA40 (24407114 [11]). MIA40 serves as a receptor and oxidoreductase that helps form disulfide bonds with the IMS protein's cysteine motif and help stabilize the IMS protein structure (15359280 [45], 24407114 [11]). IM proteins exhibit the greatest diversity in targeting and sorting information, particularly among metabolite carriers, which rely on multiple internal alpha-helical segments across a tripartite repeat architecture to mediate recognition by TOMM70 and routing through the TIM22 pathway (9242927 [5], 20457929 [9], 11867522 [46]). In contrast, TIMM17A, a core component of the TIM23 complex, lacks an N-terminal targeting sequence and contains internal hydrophobic segments for IM insertion (30190335 [14], 11867522 [46]), while metabolite carriers like ADP/ATP translocase 1 (ANT1), rely on hydrophobic helices that route them through the TIM22 pathway (11864609 [47], 16155110 [19]). Many carriers, also distribute their targeting information across multiple internal helices that are first recognized by Tom70 and cytosolic chaperones (29382700 [48], 9242927 [5]). Together, these internal signals ensure for accurate recognition by import machinery, precise localization within the mitochondria, and enable proper assembly and function (9242927 [5]).

2.3. New tools for targeting sequence identification

Both N-terminal and internal mitochondrial targeting sequences exhibit substantial diversity, united primarily by common physicochemical characteristics rather than conserved sequence motifs. Such diversity makes experimental characterization and identification difficult. However, recent computational tools have significantly improved predictive power. Resources such as MitoCarta3.0 provide curated, high-confidence mitochondrial proteomes for validating the accuracy of targeting predictions (26450961 [49], 33174596 [50]). Deep learning-based predictors, such as TargetP2.0, utilize neural network architectures to identify N-terminal sorting signals and to predict subcellular localization, including mitochondrial targeting sequences (39455219 [51], 31570514 [52]). TargetP2.0 was also able to demonstrate that mitochondrial targeting sequences are best identified using physiochemical features rather that conserved amino acid motifs (39455219 [51], 31570514 [52]). Other deep-learning models such as DeepMito extend this deep learning approach to predict sub-mitochondrial localization with finer annotation (31218353 [53]). Collectively, these tools have had a significant impact on the predictive modeling of mitochondrial targeting sequences, although important limitations remain. While these current models can reliably identify many mitochondrially targeted proteins, the prediction of dual-localized mitochondrial proteins remains a notable area for improvement. Dual-localized proteins make up approximately one-third of the mitochondrial proteome and not only localize to mitochondria, but in other organelles or cellular compartments, making their identification and annotation complex (21910249 [54]). As predictive modeling approaches continue to advance, so will the ability to detect dual localization. Ultimately, the combination of N-terminal signals, internal motifs, and protein conformational flexibility shapes how proteins interact with the mitochondrial import machinery, allowing precise trafficking and sub-mitochondrial sorting.

3. First destination to the central hub: mechanisms of mitochondrial protein trafficking

Import of nuclear-encoded proteins is essential for mitochondrial structure and function. Upon recognition of MTSs and internal targeting sequences, precursor proteins are guided through import machineries to reach their correct mitochondrial compartment. Mitochondrial proteins follow four primary import routes: 1) the presequence pathway directing proteins to the TIM23 import pathway 2) the TIM22 import pathway for IM metabolite carriers 3) the mitochondrial intermembrane space assembly (MIA) pathway for oxidative folding in the intermembrane space (IMS), and 4) the sorting and assembly machinery (SAM) pathway for β-Barrel outer membrane proteins (37696957 [55], 9430585 [56], 15359280 [45], 34446444 [10]). For precursors that have an N-terminal targeting sequence, the TIMM23 translocase is required. The translocase of the outer membrane (TOM) complex and the translocase of the inner membrane (TIM23) complex serve as the primary entry point for mitochondrial precursors into the organelle and its matrix (37696957 [55],

28301740 [41]). These multi-subunit complexes contain dedicated receptors, channels, and associated chaperones that ensure efficient and accurate import (37696957 [55]). Here, we outline how precursors engage with the TOM and TIM23 complex, focusing on the presequence pathway that directs roughly 60% of mitochondrial proteins to their matrix or IM destinations via a cleavable N-terminal targeting sequence (19837041 [36]).

3.1. Import through the TOM complex

Upon reaching the mitochondrial surface, mitochondrial precursor proteins are met with the TOM complex (37129366 [15]). The TOM complex consists of seven subunits: TOMM40, TOMM70, TOMM20, TOMM22, TOMM7, TOMM6, and TOMM5 (33083003 [57], 31740857 [58]). TOMM20 and TOMM70 act as receptor subunits, each recognizing distinct precursor features. TOMM20 primarily recognizes proteins with N-terminal targeting sequences (21087612 [59]), whereas TOMM70 interacts with hydrophobic carrier precursors containing internal targeting signals enriched with hydrophobic residues (11054285 [60]). Both receptors transfer precursor proteins to TOMM22, which functions as a central docking site and coordinates their insertion into the cytosolic face of the TOMM40 channel (21087612 [59]). Precursors containing targeting sequences then pass through TOMM40 in an unfolded, linear conformation, led by their N-terminal targeting sequence (25002531 [61]). The IMS domain of TOMM22 then mediates transfer to the TIM23 complex, facilitated by smaller TOMM components such as TOMM5 and TOMM6, which contribute to complex stability and assembly (2148157 [62]). This transfer is suggested to be driven by the “acid chain” or Brownian ratchet mechanism (29270408 [28]). In which, successive interactions between the positively charged MTS and negatively charged patches within the TOM complex bias precursor movement toward the intermembrane space as translocation proceeds (29270408 [28]). Precursors with targeting sequences are then handed off to the TIM23 complex proteins, where they take over recognition. Together, these subunits operate as a tightly coordinated network whose proper function is essential, demonstrating that the TOM complex is a central component of mitochondrial proteome assembly (2148157 [62], 2866845 [63], 10980201 [64]).

3.2. Translocation through the TIM23 complex

The TIM23 complex mediates the import and the translocation of targeting sequence containing proteins across or into the inner membrane through coordinated subunits resembling that of the TOM complex (8858146 [65], 10830167 [66]). TIMM23, along with TIMM17, forms the central translocation pore while TIMM50 functions as the IMS receptor that detects incoming targeting sequences (22065641 [67]). TIMM50 is an essential subunit of the TIM23 complex, interacting with the IMS-facing side of TIMM23, helping modulate channel activity. In the absence of a precursor, TIMM50 binding antagonizes premature channel activation, whereas the engagement of a precursor promotes channel opening in a $\Delta\psi$-dependent manner (22065641 [67]).

Upon precursor recognition, the TIM23 complex initiates translocation, an ATP-dependent process that distinguishes it from TOM-mediated import (8858146 [65]). TIMM23-mediated import requires both mitochondrial membrane potential ($\Delta\psi$) and energy derived from ATP hydrolysis (7914441 [68]). $\Delta\psi$, generated by proton pumping through the electron transport chain (ETC), provides the initial driving forces by pulling the positively charged targeting sequence through TIMM23 into the electronegative face of the matrix (7914441 [68], 19703392 [30]). Next, the energy from ATP hydrolysis powers the presequence translocase-associated motor (PAM) complex, actively drawing the precursor into the matrix (7914441 [68]). In yeast, the PAM complex contains five core components, mtHsp70, Mge1, TIMM44, and the Pam16-Pam18 heterodimer (36056001 [69], 19703392 [30]). Mitochondrial heat shock protein 70 (mtHsp70) serves as the central ATPase subunit of the PAM that hydrolyzes ATP to ADP, driving the import of matrix bound proteins (8408192 [70]). Mge1 is the nucleotide exchange factor (NEF) that promotes the release of ADP from mtHsp70 for new ATP to bind, resetting mtHsp70 for continued translocation (8918457 [71]). The Pam16 (TIMM16)-Pam18 (TIMM14) heterodimer, recruits Pam18 and stimulates the ATPase activity of mtHsp70, respectively (18003975 [72]).

Notable advances in mitochondrial proteostasis have refined our understanding of how nuclear-encoded proteins are translocated across mitochondrial membranes. Structural and mechanistic studies, including cryo-electron microscopy (cryo-EM) snapshots, suggest that TOM-TIM23 protein import is significantly more dynamic and modular than previously described (37696957 [55], 37344598 [73], 38087086 [74]). First, low-resolution cryo-EM successfully validated previously reported structures (376957 [55]). In addition, recent studies have revealed a third precursor binding interface, including a glutamine-rich region, within the Tom40 channel that may facilitate substrate passage and engagement during translocation (37696957 [55], 37344598 [73]). Identification of additional binding site further supports the broad spectrum of precursor proteins that are managed by TOMs. These models were further refined by high-resolution cryo-EM structures and AlphaFold2 predictions, which revealed additional details of the protein import mechanism. Rather than forming a single water-filled channel, Tim23 and Tim17 form distinct lipid-exposed cavities oriented in opposite directions (37344598 [73]). In this updated model, Tim17 seems to form the primary path for translocation, while Tim23 plays a more structural role in the complex. These findings vary from earlier models of Tim23 as a more static and instead support a model that is more dynamic, supporting both translocation and membrane insertion needs.

Collectively, these findings support the existing import pathways that exists within TIM23 but highlight the lack of linear and static process. The data presented to date clearly highlight a highly regulated pathways integrating membrane energetics, protein folding states and translocase plasticity.

3.3. The hidden patrol: TIM22, MIA40 and SAM

While most nuclear-encoded mitochondrial proteins are imported via the TOM-TIM23 presequence pathway, additional import pathways exist to accommodate proteins with distinct targeting signals and structural features. One major alternative pathway is the TIM22 inner membrane (IM) carrier pathway, which mediates the insertion of hydrophobic IM proteins containing multiple transmembrane segments. These proteins lack cleavable N-terminal presequences and rely on internal targeting signals such as those found in the ADP/ATP carrier, ANT1 (AAC in *S. cerevisiae*). After translocation through the TOM complex, precursor proteins are chaperoned across the IMS by small TIM chaperone complexes. The TIMM9-TIMM10 complex serves as the primary chaperone for metabolite carrier proteins to TIMM22 whereas the TIMM8-TIMM13 complex facilitates delivery of a more selective group of substrates. Recent structural studies have shown that the TIMM22 complex is comprised of the Tim22 core, Tim29, AGK, and other AGK associated factors that not only stabilize the complex but also assist in insertion. The TIM22 pathway occurs in coordination with the mitochondrial contact site and cristae organizing system (MICOS) which promotes contact between the OM and IM which is thought to help in import efficiency from the TOM to the TIM machinery (30626975 [75], 32901109 [76]).

An additional specialized route is the MIA pathway, which facilitates the import and oxidative folding in the IMS. Mia40, an oxidoreductase, acts as a receptor that binds small cysteine-rich precursor proteins through disulfide bond formation, promoting folding through oxidation of cysteine residues (19703392 [30]). Mia40 is then reoxidized by sulfhydryl oxidase, ALR in mammalian systems and Erv1 in yeast, allowing successive rounds of substrate import and folding within the

IMS. Finally, the sorting and assembly machinery (SAM) pathway mediates the biogenesis of beta barrel proteins in the outer membrane (OM) (19703392 [30]). Although these import routes deliver precursor proteins to their appropriate mitochondrial compartments, protein maturation extends well beyond membrane translocation. The second phase of translocation specializes in peptidases trimming, cleavage, or remodeling imported proteins to generate their active forms (22172993 [77]). These processing steps are mediated by distinct mitochondrial proteases such as the mitochondrial processing peptidase (MPP), inner membrane peptidases (Imp1/2), and AAA proteases, which are critical for proper import and folding, stability, and assembly into mitochondrial complexes (22172993 [77]). Thus, precursor processing represents an essential downstream stage of mitochondrial protein biogenesis.

4. Next destination: precursor processing pathways that shape the mitochondrial proteome

Once precursor proteins reach the mitochondrial matrix, they typically undergo additional processing before maturation (Fig. 2). Approximately, 40 out of 600 human proteases localize to mitochondria (32075415 [78]). Mitochondrial peptidases can be broadly grouped into three functional classes: 1) processing peptidases for maturation, which cleave N-terminal targeting sequences or other regulatory extensions (2684653 [79], 28821623 [80]) 2) quality control peptidases that degrade misfolded, stalled, or damaged proteins to maintain protein homeostasis and prevent proteotoxic stress (21138942 [81], 16839884 [82]) and 3) oligopeptidases that are responsible for the degradation of peptides generated by processing and quality-control proteases (16849325 [83], 29183787 [84]). Together, these proteolytic pathways complete the transition from imported precursor to mature functional protein, demonstrating that proteolysis is essential for establishing the mitochondrial proteome.

4.1. Peptidase processing

The major processing enzymes in mammals includes the MPP and secondary proteases, XPNPEP3 and MIPEP, which are orthologous to yeast proteases Icp55 and Oct1 respectively (19837041 [36]). Together, these enzymes ensure that precursor processing proceeds with the precision required for proper folding, stability, and activity. MPP is a well characterized, essential heterodimer composed of the conserved subunits Mas1 and Mas2 (8988249 [85], 9415998 [86]). Mas1/2 were originally identified in *S. cerevisiae* through early genetic analysis of mutants defective in precursor processing, revealing both subunits were necessary for presequence cleavage (3044780 [87], 3061808 [88]). MPP recognizes the N-terminal targeting presequences and catalyzes endoproteolytic cleavage at sites defined by conserved positional features rather than a strict consensus sequence (2684653 [79], 2967109 [89]). Canonical MPP cleavage sites are characterized by a conserved arginine residue at position −2 relative to the scissile bond, additional distal basic residues, and an aromatic or bulky hydrophobic residue at position +1, a configuration that promotes accurate positioning within the MPP active site (28122942 [90], 11470436 [91]). Cleavage by MPP is a crucial step in mitochondrial protein maturation, enabling folding and functional maturation of imported proteins (35163221 [26], 19837041 [36]).

Following the first step, additional stabilizing cuts are performed by secondary peptidases, Icp55 and Oct1. These matrix-localized enzymes act sequentially downstream of MPP, relying on its initial cleavage to refine precursor N-termini for distinct substrates (28122942 [90]). Icp55 removes a single destabilizing amino acid left by MPP, thereby stabilizing the N-terminus of many matrix proteins such as Mge1 and Nfs1 (19837041 [36]). Oct1, in contrast, cleaves an additional eight-amino-acid segment from a subset of precursors to generate a more stable and functional mature protein (28821623 [80]). While these enzymes are conserved, much of our mechanistic understanding is

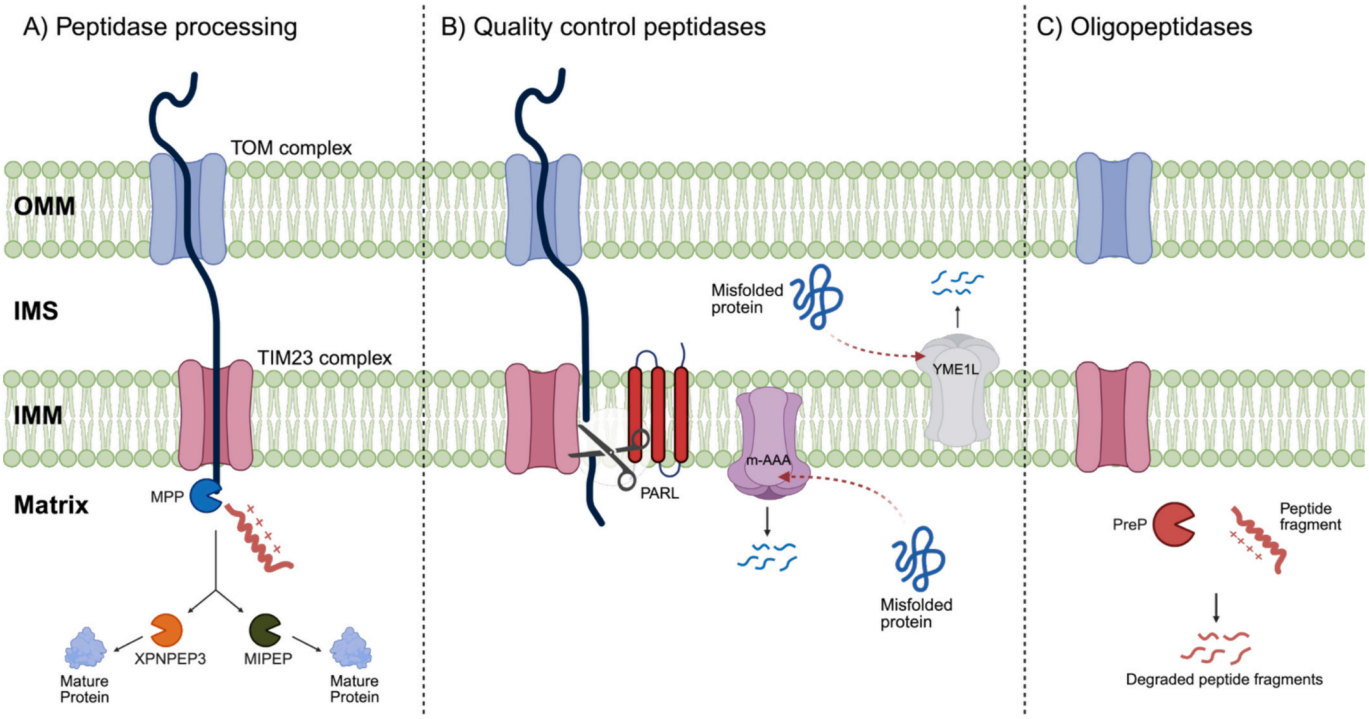

Fig. 2. Diverse roles of peptidases in protein maturation and processing.
TOM = Translocase of the outer mitochondrial membrane, TIM = Translocase of the inner mitochondrial membrane, OMM = Outer mitochondrial membrane, IMS = Intermembrane space, IMM = Inner mitochondrial membrane, MPP = Mitochondrial processing Peptidase, XPNPEP3 = X-prolyl aminopeptidase 3, MIPEP = Mitochondrial intermediate peptidase, PARL = PINK1/PGAM5-associated rhomboid-like protease, m-AAA = mitochondrial ATP-dependent AAA-type protease, YME1L = YME1-like 1 ATPase, PreP = Presequence peptidase. All proteins have been noted in mammalian nomenclature.

derived from yeast, and mammalian substrate specificity remains incompletely defined.

A subset of mitochondrial precursors destined for the IMS, contains a bipartite targeting sequence that mediate sequential translocation arrest and proteolytic processing. These proteins initially engage the TOM-TIM23 pathway through an N-terminal presequence and undergo MPP cleavage, after which a hydrophobic stop-transfer domain arrests translocation within the inner membrane (28821623 [80]). This arrest positions the precursor for a second processing event carried out by the inner membrane peptidase (IMP) complex, which is anchored in the inner membrane but cleaves substrates on the IMS side (28821623 [80]). The inner membrane peptidase (IMP) complex, composed of the conserved catalytic subunits Imp1 and Imp2 in yeast and their mammalian homologs, inner mitochondrial membrane peptidase subunit 1 and 2 (IMMP1L and IMMP2L) (35163221 [26]). IMP removes hydrophobic sorting signals from these precursors, releasing their mature domains into the IMS (28821623 [80], 35163221 [26]).

In yeast, classical IMP substrates include Cox2, Mcr1, Gut2, and cytochromes c_1 and b_2, whose bipartite targeting sequences and stop-transfer mediated sorting have been defined in foundational studies (16450175 [92], 15118906 [93], 1350514 [94]). To date, only a few mammalian IMP substrates have been validated such as cytochrome c_1, which contains a conserved Imp2p cleavage site motif and is processed by murine Imp2p, as well as glycerol-3-phosphate dehydrogenase precursor, which has shifted from Imp1p dependence in yeast to Imp2p dependence in mammals (16450175 [92]). More recently, Mgr2, a subunit of the TIMM23 complex that regulates inner membrane sorting and lateral release, was shown to be processed by IMP independently of MPP, with IMP removing carboxy-terminal targeting sequence (24287567 [95]). The pro-apoptotic protein DIABLO/Smac have been validated utilizing mitochondrial targeting assays and through IMP deletion assays in yeast models (15814844 [96]). Together, these distinct processing pathways highlight how multiple peptidases act in a compartment-specific manner to allow accurate protein maturation within the mitochondria, setting the stage for understanding how defects in these steps can disrupt mitochondrial function.

4.2. Quality control peptidases

Mitochondria also rely on ATP-dependent quality-control proteases to maintain protein homeostasis. These proteases remove misfolded or damaged proteins and regulate stability of IM components (22172993 [77]). Two major AAA proteases that carry out this function in the IM are the matrix-AAA and intermembrane space-AAA proteases, m-AAA and YME1L proteases in mammalian systems. The m-AAA protease, an ATP-dependent metalloprotease embedded in the inner membrane with catalytic domains oriented toward the matrix, where it recognizes and degrades misfolded or unassembled IM proteins (8861950 [97]). This complex also carries out additional proteolytic processing, a mechanism essential for mitochondrial ribosome assembly and for the activation specific precursor proteins through their maturation (8861950 [97]). Although originally characterized in yeast as m-AAA and Yme1, these functions are largely conserved in mammalian system, highlighting the importance of quality control peptidase activity for mitochondrial homeostasis.

Another key quality-control enzyme is PINK1/PGAM5-associated rhomboid-like protease, PARL, which is embedded in the inner membrane (20045481 [98]). PARL mediates regulated cleavage of substrates such as PTEN-induced kinase 1 (PINK1) and phosphoglycerate mutase family member 5 (PGAM5), positioning it at the intersection of several mitochondrial stress-response pathways such as inflammatory signaling, apoptosis, and mitophagy (22915595 [99], 21115803 [100]). PARL-mediated processing of PINK1 determines its fate, with cleavage by PARL resulting in rapid degradation of PINK1, or cleavage failure resulting in PINK1 accumulation on the OM (21115803 [100]). Cleavage of PGAM5, in contrast, influences mitochondrial dynamics and cell-death pathways, such as apoptosis, necroptosis, and pyroptosis, by modulating its activity toward downstream fission and stress-signaling factors (22265414 [101]). Through these substrate-specific cleavage events, PARL serves as a key enzyme at the center of mitochondrial dynamics, mitophagy and stress signaling (20045481 [98]). Quality-control peptidases like m-AAA and PARL are vital for proper mitochondrial function and prepare the matrix for downstream peptide clearance by oligopeptidases.

4.3. Oligopeptidases

Oligopeptidases primarily reside in the matrix, where most presequences accumulate following import (24043784 [102]). The accumulation of peptide fragments generated by MPP and other processing peptidases can lead to severe mitochondrial dysfunction ultimately affecting mitochondrial respiration and activate stress response (34356897 [103], 32632204 [104]). To prevent accumulation of these fragments, mitochondria rely on matrix oligopeptidases to rapidly clear peptides fragments before they become toxic (25176146 [105]). Most peptide fragments are derived from presequences, where they carry positively charged, amphipathic features that allow them to bind the MPP active site and compete with newly imported precursors, thereby blocking efficient processing (25176146 [105]). Their unique charges can also disturb the inner membrane potential, further impairing presequence-driven import (25176146 [105]). Efficient degradation of these peptides is therefore essential for maintaining both import capacity and matrix proteostasis.

In mammals, peptide fragment degradation is carried out primarily by the matrix oligopeptidases Presequence Peptidase (PreP) and neurolysin, homologs of the yeast enzymes Cym1 and Prd1, respectively (25176146 [105]). Both enzymes reside in the matrix and have complementary substrate preferences. PreP preferentially degrades presequences and other short peptides while neurolysin specializes in shorter peptide fragments up to 19 amino acids long (35383169 [106], 29183787 [84]). Together, they efficiently destroy the pool of presequences and processing fragments, preventing their accumulation and ensuring that MPP and other processing enzymes remain unobstructed (25176146 [105]). By removing these potentially toxic peptides, oligopeptidases complete the maturation process of imported proteins and maintain mitochondrial proteostasis.

In sum, maturation proteases, quality-control proteases, and oligopeptidases ensure that newly imported mitochondrial proteins acquire their correct N-termini, folded configuration, assemble into complexes, and avoid generating toxic intermediates (25176146 [105]). While much foundational knowledge is derived from yeast, new mammalian studies continue to reveal conserved mechanisms and disease-relevant differences (34356897 [103], 32632204 [104]). Understanding these pathways is critical as defects in the mitochondrial processing can compromise respiratory function, disrupt proteostasis, and contribute to diverse human pathologies.

5. Bump in the road: how mitochondrial proteins yield to pathology

Mitochondrial function depends on the precise targeting, import and proteostatic maturation of mitochondrial proteins. These processes are mediated by conserved translocase complexes and compartment-specific quality control and processing pathways that ensure precursors reach their correct mitochondrial destination and acquire functional conformations. Mitochondrial dysfunction has been recognized as a hallmark of neurological disease, with its earliest links emerging as far back as the 1980s (2830540 [107]). Mitochondrial import specifically has gained attention as a critical source of pathology given the pivotal role of mitochondrial import on overall cellular health (17300922 [108]) (Table 3).

One of the earliest studies to link defects in mitochondrial protein

Table 3
Human diseases associated with defects in mitochondrial protein targeting, import, processing, and folding.

Protein	Import step affected	Mutation/alteration	Disease/condition	Mechanistic consequence	References
DDP1/TIMM8A	IMS chaperone-mediated import/ TIM23 biogenesis	C66W missense mutation altering DDP1-TIM13 complex formation	Mohr-Tranebjaerg syndrome (Deafness-dystonia syndrome (DFN-1))	Loss of DDP1-TIMM13 complex formation, reduction of Tim23 import, disruption of mitochondrial protein import	[124,125]
TOMM40	Outer membrane translocation	rs10524523, variable-length poly-T repeat polymorphism	Alzheimer's Disease, Ovarian cancer	Associated with earlier disease onset, increased ATP production and respiration, enhanced tumor growth	[126–129,151,154]
TIMM50	Inner membrane translocation	Homozygous-Gly372Ser, Arg217Trp, and Thr252Met; Heterozygous variants	Epilepsy, Severe intellectual disability, Seizures, Severe encephalopathy	Reduced TIM23 stability, impaired presequence import, decreased Δψ, elevated ROS, reduced OXPHOS	[14,66,123,130]
β-MPP	Presequence processing	Loss of function variants	Severe early-onset neurodegeneration	Impaired MPP activity, accumulation of unmodified proteins, defects in proteostasis, Fe-S cluster biogenesis	[138]
IMMP2L	Inner membrane proteolytic processing	Chromosomal breakpoints, intragenic deletions	Tourette syndrome, Autism spectrum disorders	Accumulation of unprocessed IM substrates, impaired respiration, oxidative stress apoptosis susceptibility	[139–145]
PINK1	Proteolytic processing	C92F, Q115L, R147H N-terminal mutations disrupting processing	Early-onset Parkinson's Disease	Accumulation of full-length PINK1, decrease in Δψ, elevated ROS, mitophagy dysregulation	[80,99]
Hsp60	Post-import folding	Val72Ile and reduced expression	Multisystem mitochondrial disorder, Spastic paraplegia-13	Destabilization of chaperonin complex; impaired matrix protein folding; abnormal mitochondria and reduced enzymatic activity	[149,150]
Pyruvate dehydrogenase E1α	MTS integrity and targeting	Arg23Pro in MTS	Pyruvate dehydrogenase deficiency	Reduced import efficiency and lactic acidosis	[110,111]
AGT	Cryptic MTS and targeting	Pro11Leu, Gly170Arg	PH1	Mistargeting from peroxisome to mitochondria, loss of peroxisomal glyoxylate detoxification, oxalate accumulation and kidney failure	[112–117]
EHHADH	Ectopic MTS and targeting	Disease-associated MTS-introducing mutations	FRTS3	Aberrant mitochondrial targeting of peroxisomal enzyme; disrupted mitochondrial fatty acid metabolism and respiration	[118–121]
TOMM20	Outer membrane recognition	rs7930, SNP in the 3′ untranslated region of the TOMM20 gene	Colorectal cancer	Increased import capacity, elevated ATP production, enhanced proliferation and migration	[21,153]
TIMM9	TIM22 carrier pathway	Overexpression	Multiple cancers	Correlates with mitochondrial metabolism, OXPHOS, and cell cycle gene expression; poor prognosis	[152]
AGK	TIMM22 carrier pathway	Loss of function	Sengers syndrome	Destabilization of TIMM22 complex formation	[131–133]
ANT1	TIMM22 carrier pathway	Missense mutations	adPEO/cardiomyopathy	Instability leads to mitochondrial dysfunction	[136,137]
Magmas	TIM23-associated import	Missense mutations	Spondylodysplastic dysplasia	Reduction in mitochondrial import, impacting mitochondrial proteostasis	[134,135]

MTS = Mitochondrial targeting sequence, iMTS = Internal mitochondrial targeting sequence, TOM = Translocase of the outer mitochondrial membrane, TIM = Translocase of the inner mitochondrial membrane, β-MPP = Mitochondrial processing peptidase subunit β, IMMP2L = Inner mitochondrial membrane peptidase subunit 2, AGT = Alanine-glyoxylate transferase, EHHADH = Enoyl-CoA hydratase/3-hydroxyacyl-coA dehydrogenase, PH1 = Primary hyperoxaluria type 1, FRTS3 = Fanconi renotubular syndrome 3.

import machinery to human disease demonstrated that mutations in deafness dystonia peptide 1 (DDP1)/translocase of the inner membrane 8a (TIMM8A) impair mitochondrial protein import (11875042 [16]). This work expanded the framework for understanding inherited mitochondrial diseases beyond respiratory chain deficiencies. Although many of these conditions are attributed to defects in oxidative phosphorylation (OXPHOS), emerging studies demonstrate that defects in mitochondrial protein import are equally disruptive (17300922 [108]). Since then, it has been identified that mitochondrial dysfunction underlies a broad spectrum of human diseases, including variety of rare genetic disorders, many of which manifest with complex phenotypes (31051112 [109]), cardiomyopathies, neurological disorders, metabolic disorders, and different cancers (17300922 [108], 24561263 [110]). In this section, we focus on disease-associated mutations in mitochondrial precursors and import pathway components to illustrate how defects at discrete stages of the import process disrupt mitochondrial proteostasis. Understanding how precursor targeting sequences engage dynamic import machineries beyond what static structural snapshots alone can reveal offers critical insight into dual-localized proteins, context-dependent targeting decisions, and the molecular basis of mitochondrial disease.

5.1. Mitochondrial targeting sequence as the early determinants of protein fate

The MTS represents a critical point of vulnerability in mitochondrial biology, serving as the first step in quality control. Emerging studies suggest that even subtle alterations in MTS composition, such as changes in charge distribution, hydrophobicity, or helix stability, can impair import efficiency and lead to protein mistargeting (17300922 [108]). These subtle sequence alterations can produce profound cellular consequences as several rare diseases arise directly from MTS mutations (17300922 [108]). The first documented human disease related to mitochondrial targeting was a specific form of pyruvate dehydrogenase (PDH) deficiency. Two male siblings with an X-linked mutation were the first to be identified as having a pathogenic variant in the MTS of the PDH E1α subunit (PDHA1) (7573035 [111]). Genetic evidence revealed substitution from Arginine (Arg) to Proline (Pro) in the 29-residue-long

MTS of PDHA1 was the driver of pathogenesis (7573035 [111]). This mutation results in pyruvate dehydrogenase deficiency, one of the most common causes of lactic acidosis in children (3123240 [112]). Studies have found that Arg-to-Pro substitution decreases import efficiency; although the exact mechanism is unknown, it is likely that it impairs helix stability and reduces the hydrophobic moment required for TOMM/TIMM engagement (7573035 [111]). Severe targeting error caused by a single point mutation trigger these disease phenotypes, which highlight how defects in mitochondrial protein import emphasize the vulnerability of mitochondrial homeostasis.

Disease can result when proteins are mistargeted due to the inappropriate presence or activation of a cryptic mitochondrial targeting sequence. A well-characterized example is alanine-glyoxylate transferase (AGT), a dual-localized liver-expressed enzyme that normally localizes to peroxisomes and catalyzes the conversion of glyoxylate to glycine (485171 [113]). In primary hyperoxaluria type 1 (PH1), mutations in the AGT gene, AGXT, alter peroxisomal targeting to mitochondrial targeting (1961759 [114]). The most common disease-associated variants include P11L, which generates a latent MTS at the N-terminus, and G170R, which destabilizes AGT folding. This destabilization leaves AGT primarily unfolded, allowing for recognition and import by mitochondrial import machinery (23229545 [115], 7559790 [116]). P11L alone is insufficient to cause disease, but in combination with G170R, it enhances mitochondrial mistargeting and contributes to pathology (23229545 [115]). P11L and G170R mutations favor recognition by the TOM/TIM complexes and reroute AGT to the mitochondrial matrix rather than to the peroxisome (20208150 [117]). Consequently, glyoxylate detoxification in the peroxisome is impaired, leading to excess oxalate production, calcium oxalate deposition, and progressive kidney failure (8592621 [118]).

Similarly, MTS mutations in enoyl-CoA hydratase/3-hydroxyacyl-coA dehydrogenase (EHHADH), a peroxisomal enzyme involved in fatty acid oxidation, have been implicated in renal Fanconi syndrome (34349672 [119]). EHHADH is canonically localized to peroxisomes, where it can act as a bifunctional enzyme catalyzing key steps in fatty acid oxidation (22534643 [120]). However, disease-associated mutations introduce an MTS, redirecting the protein to the mitochondrial matrix (24401050 [121]). Mistargeting of EHHADH results in mitochondrial accumulation of EHHADH, which interferes with fatty acid metabolism and respiration (24401050 [121], 27160910 [122]). In renal proximal tubule cells that are dependent on proper mitochondrial metabolism, these mutations result in energy deficiency and manifest as renal Fanconi syndrome (24401050 [121]). Fanconi renotubular syndrome 3 (FRTS3) is a disease that affects the kidneys proximal tubes, resulting in impaired reabsorption of solutes such as glucose and amino acids and progressive kidney dysfunction (24401050 [121]). Together these observations underscore how mutations affecting targeting sequences may be a key component in understanding mitochondrial dysfunction and metabolic disorders.

5.2. *Defects in precursor engagement and translocation*

The TOM and TIM23 complexes govern recognition and translocation of mitochondrial proteins, and the integrity of the mitochondrial proteome depends heavily on their proper function. Given their critical role, mutations, downregulation, and structural perturbations in TOM and TIM complexes have been associated with a growing number of human diseases (17300922 [108]). Defects affecting precursor recognition, translocation, or transfer between TOM and TIM machinery compromise respiratory function, metabolic homeostasis, and proteostasis (30405116 [123], 38828998 [124]), collectively contributing to a wide spectrum of mitochondrial dysfunction–associated disorders.

Notably, one of the first human diseases directly linked to defects in mitochondrial protein import was Mohr-Tranebjaerg syndrome (10051608 [125]). Also known as deafness-dystonia syndrome (MTS/DFN-1), this recessive, X-linked, neurodegenerative disorder is caused by mutations in deafness/dystonia protein 1/translocase of mitochondrial inner membrane 8a (DDP1/TIMM8a) (8841189 [126]). One disease-associated mutation, C66W, disrupts formation of the DDP1/TIMM8a-TIMM13 complex. This formation is required for the import of Tim23 (8841189 [126]). Loss of this complex leads to reduced Tim23p import and impaired mitochondrial protein translocation, ultimately resulting in Mohr-Tranebjaerg syndrome. These findings provided early evidence that disruptions in mitochondrial protein import machinery had the potential to drive human disease.

TOMM40, the central β-barrel pore of the outer membrane, is not only essential for life but has also been implicated in several mitochondria-related pathologies (2250717 [127], 25745640 [128], 28768149 [129]). rs10524523, a variable-length poly-T repeat polymorphism in the TOMM40 gene was identified through phylogenetic haplotype analysis of the TOMM40–APOE locus in Caucasian patients (20029386 [130]). Longer poly-T alleles, particularly on APOE ε3 haplotypes, are associated with significantly earlier disease onset compared to shorter repeats. These findings suggest that structural variation in TOMM40 contributes to Alzheimer's risk beyond the effects of APOE alone. Given that TOMM40 encodes the pore-forming subunit of the TOM complex, this work suggests a potential connection between mitochondrial protein import efficiency and neurodegeneration, underscoring the importance of considering mitochondrial pathways in studying Alzheimer's disease.

Downstream of TOM, mutations in TIM23 subunits have been implicated in human disease. In the TIMM50 gene, siblings with a homozygous Gly372Ser mutation were first discovered to have intractable epilepsy and developmental delay (27573165 [131]). Subsequent studies identified siblings from two unrelated families were found to have two homozygous mutations, Arg217Trp and Thr252Met, in the TIMM50 gene, resulting in severe intellectual disability and seizures (27573165 [131]). Heterozygous variants in TIMM50 were also identified in infants with severe encephalopathy (30190335 [14]). These observations are further supported by in vitro mechanistic studies that illustrate the impact of TIMM50 dysfunction. Patient-derived fibroblast analysis of TIMM50 deficiency led to marked reductions in TIM23 complex subunits, decreased mitochondrial membrane potential, and impaired import of presequence-containing precursors, confirming a direct defect in the TIMM23 pathway. These cellular defects were accompanied by reduced OXPHOS complex abundance noted by lower respiration, and elevated reactive oxygen species (ROS) levels, linking disrupted TIMM23-mediated import to metabolic failure. Together, these data support TIMM50 as a critical determinant of mitochondrial homeostasis, with loss-of-function variants causing profound neurological disease.

An alternative transmembrane insertion pathway via TIMM22 contributes to wide range of human diseases. Acylglycerol kinase (AGK), a multifunctional component in the TIM22 complex plays a critical role. Although AGK is well known for its role as a mitochondrial lipid kinase, it also plays a critical structural role in the import and membrane insertion of metabolite carrier proteins, including ANT1 (SLC25A4) (33476211 [132]). Loss-of-function mutations in AGK cause Sengers syndrome, a rare autosomal recessive mitochondrial disorder characterized by congenital cataracts, cardiomyopathy, and lactic acidosis (1168700 [133]). At the molecular level, AGK deficiency disrupts mitochondrial function in two distinct mechanisms. The first is through impaired lipid metabolism and the second is through defective TIMM22-dependente insertion of essential inner membrane carrier proteins. The resulting loss of key metabolite transporters compromises proteostasis, membrane integrity, and energy production, thus driving disease pathology (33476211 [132], 28712726 [134]).

Inner membrane import can also be disrupted by defects in the TIM23-associated PAM complex. One critical component of this import machinery is Magmas (mitochondrial associated granulocyte-macrophage colony-stimulating factor signaling protein), the human ortholog of Pam16 in *S. cerevisiae*. Initial studies found that Magmas

localizes to the IMM and is a regulatory subunit of the PAM complex with similar function as its yeast ortholog. Mechanistic studies revealed that Magmas forms a crucial subcomplex with the J-protein Pam18, the yeast ortholog of DnaJC19, allowing for proper tethering of the PAM complex to the TIM23 channel (20052669 [135]). This interaction is critical for maintaining proper protein import and mitochondrial proteostasis. This suggests that this interaction may be conserved in mammalian cells and thereby contribute to pathophysiology. Thus, mutations in Magmas destabilizes the PAM complex thus impairing protein translocation and decreases cell proliferation and viability (20052669 [135]). Physiologically, the Magmas homozygous missense mutations in MAGMAS gene results in severe spondylodysplastic dysplasia, an early-onset developmental disorder characterized by skeletal abnormalities and impaired growth (24786642 [136]). These findings directly link defects in PAM complex assembly to human pathology, underscoring the essential role of TIM23-mediated import.

Another instance of import-associated dysfunction involves the ATP/ADP carrier protein adenine nucleotide translocase (ANT1), also known as AAC in *S. cerevisiae*. Although initially characterized in yeast, these findings are highly relevant to mammalian systems. Missense mutations in ANT1 can impair its import and membrane insertion into the mitochondria (37449547 [137]). Stalling then results in translocase "clogging", interfering with the import of other mitochondrially targeted proteins, triggering their accumulation in the cytosol and mPOS. ANT1 mutations have been associated to autosomal dominant progressive external ophthalmoplegia with mitochondrial DNA deletions 2 (AdPEO2) and mitochondrial DNA depletion syndrome (MTDPS 12B and 12A) (32340404 [138]). These disorders are characterized by impairments respiratory chain and OXPHOS function highlighting that disruptions in protein import can cause widespread cellular dysfunction in addition to mitochondria.

5.3. Pathogenic disruption of precursor processing and maturation

Many unmodified proteins require cleavage by mitochondrial processing peptidases, and defects in these enzymes can also be pathogenic. Variants in mitochondrial processing peptidase subunit β (PMPCB), the catalytic subunit of the mitochondrial processing peptidase (MPP), were identified in children with severe early-onset neurodegeneration (29576218 [139]). Patient-derived fibroblasts and iPSC-derived neuronal precursors with PMPCB mutations demonstrated impaired MPP activity, evidenced by accumulation of intermediate frataxin and widespread defects in preprotein maturation (29576218 [139]). Parallel yeast models with analogous mutations also demonstrated these processing defects and revealed early impairment of mitochondrial proteostasis and iron–sulfur cluster biogenesis (29576218 [139]).

Beyond presequence removal by MPP, additional proteolytic processing occurs at the inner mitochondrial membrane and disruption at this level has also been linked to human disease. IMMP2L, the mammalian homolog of the yeast inner-membrane peptidase Imp2, has been linked to neurodevelopmental disorders such as Tourette syndrome (11254443 [140], 21386874 [141], 24549057 [142]). Chromosomal breakpoints and intragenic deletions disrupting IMMP2L have been identified in individuals with Tourette syndrome and autism spectrum disorders (21386874 [141], 24549057 [142]). To assess causality, suppression of IMMP2L was modeled in mammalian cells after IMMP2L depletion using shRNAs (29808012 [143]). Unprocessed proteins and intermediate forms of IMMP2L substrates accumulated in the matrix, demonstrating that mitochondrial inner-membrane protein processing were defective (29808012 [143]). This accumulation was associated with altered cellular stress-response pathways, such as activation of oxidative stress responses and susceptibility to cell death (29808012 [143]). Additional mouse studies also identified that loss of IMMP2L impairs the maturation of select inner-membrane substrates, like cytochrome c_1 (CYC1) a subunit of complex III, and compromises mitochondrial respiration (18094351 [144], 21824519 [145]). Together, these findings show that disruption of mitochondrial presequence and inner-membrane processing pathways directly compromises mitochondrial function, creating neuronal vulnerability that can contribute to both neurodevelopmental and neurodegenerative disease (18094351 [144], 21824519 [145], 37761857 [146]).

PTEN-induced kinase 1 (PINK1) is key regulator of mitochondrial homeostasis that functions as a sensor for mitochondrial integrity. Under normal conditions, PINK1 is imported into mitochondria via the TOM-TIM23 pathway and sequentially processed by MPP and PARL. Cleavage of PINK1 results in the removal of the transmembrane domain, which serves as an anchor, promoting rapid PINK1 turnover in the cytosol. In contrast, loss of Δψ or other mitochondrial dysfunction disrupts the import and processing of the PINK1 precursor, resulting in its accumulation on the OM and activation of downstream mitophagy pathways (21138942 [81]). Mutations in PINK1 have been causally linked to early-onset Parkinson's Disease, with most pathogenic mutations characterized affecting the C-terminal region of PINK1, noting a loss of function mutation (1138942 [81]). However, emerging studies have demonstrated that when N-terminal region is mutated, this dictates mitochondrial targeting and processing, which can also contribute to disease pathogenesis (21138942 [81]). Missense mutations associated with early-onset Parkinson's Disease (PD) patients, C92F, Q115L, and R147H, have demonstrated an increase in full-length PINK1 levels, a decrease in Δψ and an increase in reactive oxygen species (ROS), a similar phenotype to that of PINK1 KO cell lines (21138942 [81]). Although a direct connection between N-terminal mutations and early-onset PD remains unclear, work to date highlight yet another example of how disruptions in mitochondrial protein import and processing greater implications in human disease than previously thought. Notably, several studies have shown that aggregation-prone proteins with connections to neurodegenerative disorders, including alpha-synuclein and amyloid beta, have been shown to impair mitochondrial protein import (24836077 [147], 16943564 [148], 27280685 [149]). Together, these findings highlight the vulnerability of mitochondrial import and specifically, how mutations to the MTS can trigger devastating metabolic disease.

5.4. Failures in mitochondrial folding and proteostasis

Following protein import, proteins must fold into their native conformations to become fully functional. Mutations in Hsp60, a central mitochondrial matrix chaperonin responsible for post-import folding, have been directly linked to human disease (18400758 [150], 11898127 [151]). Reduced Hsp60 levels were identified in patients with multisystem mitochondrial disorder and were shown, using patient derived cells, decreased mitochondrial abundance, structurally abnormal mitochondria and severely diminished enzymatic activity. All these effects being consistent with a folding defect of matrix proteins (18400758 [150]). In addition, a point mutation in HSPD1, encoding Hsp60 (V72I), causes spastic paraplegia-13, a hereditary neurodegenerative disorder (11898127 [151]). Biochemical analysis demonstrated that the mutation destabilizes the Hsp60 chaperonin complex, impairs folding of matrix proteins immediately after import, and causes mitochondrial dysfunction in neuronal cells (18400758 [150]). These studies highlight that even when targeting and processing are intact, disruptions in protein folding can ultimately compromise mitochondrial maintenance and neuronal function.

5.5. Adaptive rewiring in cancer

Although neurodegenerative disorders have historically dominated research on mitochondria-associated disease, cancer has been notably recognized as another pathology influenced by mitochondrial dysfunction (33314127 [12]). In recent years, accumulating evidence suggests that alterations in components of both the TOMM and TIMM complexes are found in a wide range of cancer types (33314127 [12]).

Overexpression of TOMM20 and TOMM40 has been linked to promoting tumor cell proliferation and invasion in colorectal and ovarian cancers, respectively (31818360 [21], 32456076 [152]). Additionally, TIMM9, a component of the TIMM22 carrier import pathway, is being explored as a potential biomarker given its elevated levels in multiple cancers (39232081 [153]). Although the relationship between mitochondrial import and cancer remains poorly understood, the observed patterns highlight a potentially important link.

A population-based study identified a single-nucleotide polymorphism (SNP), rs7930, in the 3′ untranslated region of the TOMM20 gene (27853382 [154]). Individuals with the rs7930 AG genotype exhibited a 1.721-fold increased risk of colorectal cancer compared to those with the AA genotype (27853382 [154]). In addition, TOMM20 expression is significantly elevated in colorectal tumors compared with normal tissue, as shown in The Cancer Genome Atlas (TCGA) (31818360 [21]). Not to a surprise, TOMM20 overexpression studies in vitro indeed enhanced cancer cell proliferation and migration (31818360 [21]) and its pro-proliferative properties were muted following the knockdown of TOMM20 (31818360 [21]). As expected, xenograft mouse models revealed that loss of TOMM20 reduced tumor growth (31818360 [21]). Mechanistically, TOMM20 overexpression was associated with increased ATP production, suggesting that enhanced protein import may support the metabolic demands of tumor cells.

A similar association between mitochondrial import and tumor progression has been described for TOMM40. In this study, TOMM40 expression was significantly elevated in ovarian tumor samples compared with normal ovarian tissue (32456076 [152]). Additionally, higher expression was correlated with reduced disease-free survival (32456076 [152]). In vitro analysis showed that increased TOMM40 expression supports ovarian cancer cell proliferation by various growth assays (32456076 [152]). Subsequent siRNA-mediated knockdown of TOMM40 reduced tumor cell growth in vitro (32456076 [152]). TOMM40 suppression also reduced tumor burden in in vivo xenograft mouse models, directly linking TOMM40 expression to tumor growth (32456076 [152]). Increased TOMM40 expression was further associated with elevated intracellular ATP levels and changes in mitochondrial membrane potential and ROS, suggesting enhanced mitochondrial activity and altered energy metabolism (32456076 [152], 39060752 [155]). These findings identify TOMM40 as a pro-tumorigenic component in ovarian cancer.

Additional evidence linking mitochondrial import to cancer comes from analysis of TIMM subunits, including TIMM9 (39232081 [153]). TIMM9 has been identified as upregulated and proposed as a pan-cancer biomarker (39232081 [153]). Bulk and single-cell transcriptomic datasets have shown that elevated TIMM9 expression strongly correlated with transcripts related to tumorigenesis, metastatic potential, and poor clinical prognosis across diverse cancer types (39232081 [153]). Transcriptomic analysis demonstrated that TIMM9 expression was enriched in malignant cell populations from cancers, including cervical squamous cell carcinoma (CESC), kidney renal clear cell carcinoma (ccRCC), cholangiocarcinoma (CHOL), liver hepatocellular carcinoma (LIHC), esophageal carcinoma (ESCA), non-small cell lung cancer (NSCLC), and prostate adenocarcinoma (PRAD) cell lines (39232081 [153]). Pathway enrichment analysis further associated increased TIMM9 expression with mitochondrial metabolism, OXPHOS, and cell cycle regulation. While the mechanistic role of TIMM9 in mitochondrial import dysregulation during cancer remains unclear, its consistent overexpression across diverse tumor types underscores mitochondrial import as an emerging and understudied axis of cancer biology.

5.6. Tools for identifying pathogenic mutations associated with human disease

There are numerous pathogenic mutations in proteins that disrupt their roles in mitochondrial protein import. Although several representative examples are highlighted here, the rapidly growing catalog of pathogenic variants underscores the importance of using computational resources for the most current information. Publicly available databases such as ClinVar provide centralized and continuously updated database of human genetic variants and their associated phenotypes (24234437 [156]). Submissions from clinical laboratories and research studies make ClinVar a powerful tool to identify clinically relevant variants linked to defects in mitochondrial import pathways. In addition, MTSViewer is a specialized resource designed to analyze mitochondrially targeted proteins with an N-terminal targeting sequence (37093842 [157]). MTSviewer combines data from mitochondrial proteome databases, variant databases such as ClinVar and GnomAD, and predictive algorithms to identify targeting sequences and functional consequences. This platform allows users to interrogate variation in the MTS region of a protein of interest and understand how this may contribute to disease phenotypes in a single resource. Together these resources provide frameworks for understanding genetic variation in mitochondrial import pathways and how these disruptions contribute to human disease.

6. Conclusion

Mitochondria are indispensable organelles that rely on the accurate delivery, import, and processing of nuclear-encoded proteins. Each component of import machinery requires precise regulation for maintaining organelle integrity, including structure of mitochondrial membranes, proper folding of proteins, and assembly of respiratory complexes (35804477 [1], 16860735 [2], 34800366 [4]). Disruptions in these processes, such as defective precursor targeting, inefficient translocation, or impaired proteolytic processing, can compromise oxidative phosphorylation, metabolic signaling, and overall organelle homeostasis (33314127 [12], 37129366 [15], 35163221 [26]).

Increasing evidence implicates mitochondrial import dysfunction in a wide variety of human diseases, including neurological disorders, metabolic disease, and even cancer. However, directly linking specific import defects to these pathologies remains difficult due to the complexity of mitochondrial networks, compensatory pathways, and the complex nature of these diseases (33314127 [12]). Additionally, because import pathways are highly interconnected with other mitochondrial quality control mechanisms, defining the precise mechanistic consequences of specific defects remains challenging (24353213 [24], 37129366 [15]). As a result, much of the current evidence is correlative, relying on observations of altered import components or accumulation of unprocessed precursors in disease tissues. Continued mechanistic investigation is essential to pinpoint causal relationships, identify vulnerabilities within the import system, and reveal potential therapeutic targets. Advancing our understanding of these processes will not only deepen fundamental insights into mitochondrial biology but also create opportunities for diagnostics and interventions to mitigate diseases rooted in import dysfunction. Together, these findings highlight mitochondrial protein import as a critical hub of cellular health whose perturbation may represent a unifying mechanism across diverse pathologies.

CRediT authorship contribution statement

Maya Cielo Cornejo: Writing – review & editing, Writing – original draft, Visualization, Investigation, Funding acquisition, Data curation, Conceptualization. **Sandy Che-Eun S. Lee:** Writing – review & editing, Visualization, Project administration, Investigation, Funding acquisition, Conceptualization. **Shaya Smith:** Visualization, Data curation. **Carla M. Koehler:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Carla M. Koehler reports financial support was provided by University of California Los Angeles. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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