Cell Metabolism
Perspective

The Protein Import Machinery of Mitochondria—A Regulatory Hub in Metabolism, Stress, and Disease

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http://dx.doi.org/10.1016/j.cmet.2014.01.010

Mitochondria fulfill central functions in bioenergetics, metabolism, and apoptosis. They import more than 1,000 different proteins from the cytosol. It had been assumed that the protein import machinery is constitutively active and not subject to detailed regulation. However, recent studies indicate that mitochondrial protein import is regulated at multiple levels connected to cellular metabolism, signaling, stress, and pathogenesis of diseases. Here, we discuss the molecular mechanisms of import regulation and their implications for mitochondrial homeostasis. The protein import activity can function as a sensor of mitochondrial fitness and provides a direct means of regulating biogenesis, composition, and turnover of the organelle.

Introduction

Mitochondria are essential cell organelles (Dolezal et al., 2006; Neupert and Herrmann, 2007; Chacinska et al., 2009). They are best known for their role as cellular powerhouses, which convert the energy derived from food into an electrochemical proton gradient across the inner membrane. The proton gradient drives the mitochondrial ATP synthase, thus providing large amounts of ATP for the cell. In addition, mitochondria fulfill central functions in the metabolism of amino acids and lipids and the biosynthesis of iron-sulfur clusters and heme (Lill, 2009; Schmidt et al., 2010; Hamza and Dailey, 2012). Mitochondria form a dynamic network that is continuously remodeled by fusion and fission. They are involved in the maintenance of cellular ion homeostasis, play a crucial role in apoptosis, and have been implicated in the pathogenesis of numerous diseases, in particular neurodegenerative disorders (Frederick and Shaw, 2007; Galluzzi et al., 2012; Nunnari and Suomalainen, 2012; Rugarli and Langer, 2012; Vafai and Mootha, 2012; Youle and van der Bliek, 2012; Andersen and Kornbluth, 2013; Shutt and McBride, 2013).

Mitochondria consist of two membranes, outer membrane and inner membrane, and two aqueous compartments, intermembrane space and matrix (Figure 1). Proteomic studies revealed that mitochondria contain more than 1,000 different proteins (Prokisch et al., 2004; Reinders et al., 2006; Pagliarini et al., 2008; Schmidt et al., 2010). Based on the endosymbiotic origin from a prokaryotic ancestor, mitochondria contain a complete genetic system and protein synthesis apparatus in the matrix; however, only ~1% of mitochondrial proteins are encoded by the mitochondrial genome (13 proteins in humans and 8 proteins in yeast). Nuclear genes code for ~99% of mitochondrial proteins. The proteins are synthesized as precursors on cytosolic ribosomes and are translocated into mitochondria by a multicomponent import machinery. The protein import machinery is essential for the viability of eukaryotic cells. Numerous studies on the targeting signals and import components have been reported (reviewed in Dolezal et al., 2006; Neupert and Herrmann, 2007; Endo and Yamano, 2010; Schmidt et al., 2010), yet for many years little has been known on the regulation of the import machinery. This led to the general assumption that the protein import machinery is constitutively active and not subject to detailed regulation.

Studies in recent years, however, indicated that different steps of mitochondrial protein import are regulated, suggesting a remarkable diversity of potential mechanisms. After an overview on the mitochondrial protein import machinery, we will discuss the regulatory processes at different stages of protein translocation into mitochondria. We propose that the mitochondrial protein import machinery plays a crucial role as regulatory hub under physiological and pathophysiological conditions. Whereas the basic mechanisms of mitochondrial protein import have been conserved from lower to higher eukaryotes (yeast to humans), regulatory processes may differ between different organisms and cell types. So far, many studies on the regulation of mitochondrial protein import have only been performed in a limited set of organisms. Here we discuss regulatory principles, yet it is important to emphasize that future studies will have to address which regulatory processes have been conserved in evolution and which processes are organism specific.

Protein Import Pathways into Mitochondria

The classical route of protein import into mitochondria is the presequence pathway (Neupert and Herrmann, 2007; Chacinska et al., 2009). This pathway is used by more than half of all mitochondrial proteins (Vögtle et al., 2009). The proteins are synthesized as precursors with cleavable amino-terminal extensions, termed presequences. The presequences form positively

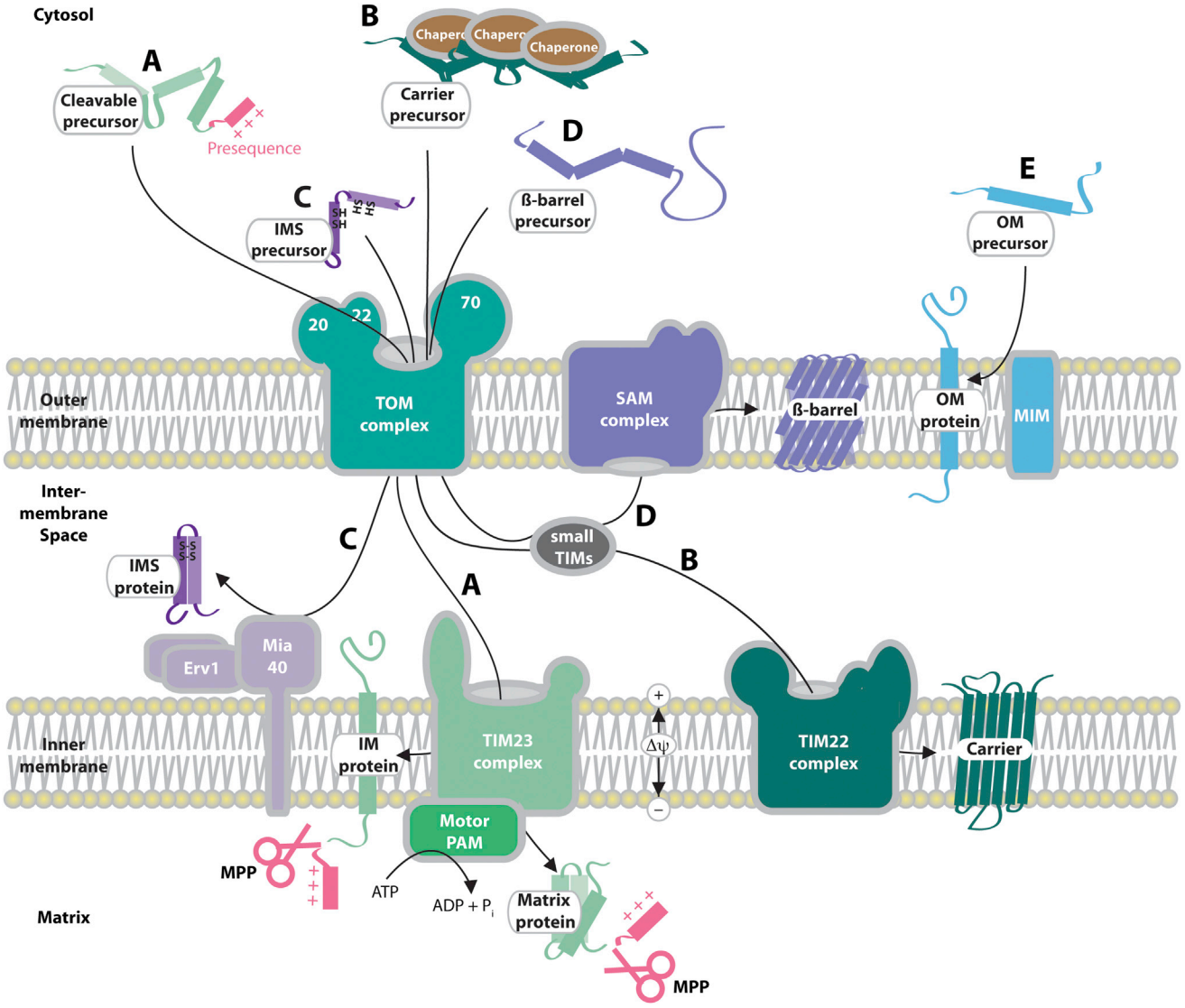

Figure 1. Protein Import Pathways of Mitochondria

Most mitochondrial proteins are synthesized as precursors in the cytosol and are imported by the translocase of the outer mitochondrial membrane (TOM complex).

(A) Presequence-carrying (cleavable) preproteins are transferred from TOM to the presequence translocase of the inner membrane (TIM23 complex), which is driven by the membrane potential ($\Delta\psi$). The proteins either are inserted into the inner membrane (IM) or are translocated into the matrix with the help of the presequence translocase-associated motor (PAM). The presequences are typically cleaved off by the mitochondrial processing peptidase (MPP).

(B) The noncleavable precursors of hydrophobic metabolite carriers are bound to molecular chaperones in the cytosol and transferred to the receptor Tom70. After translocation through the TOM channel, the precursors bind to small TIM chaperones in the intermembrane space and are membrane inserted by the $\Delta\psi$-dependent carrier translocase of the inner membrane (TIM22 complex).

(C) Cysteine-rich proteins destined for the intermembrane space (IMS) are translocated through the TOM channel in a reduced conformation and imported by the mitochondrial IMS import and assembly (MIA) machinery. Mia40 functions as precursor receptor and oxidoreductase in the IMS, promoting the insertion of disulfide bonds into the imported proteins. The sulfhydryl oxidase Erv1 reoxidizes Mia40 for further rounds of oxidative protein import and folding.

(D) The precursors of outer membrane β-barrel proteins are imported by the TOM complex and small TIM chaperones and are inserted into the outer membrane by the sorting and assembly machinery (SAM complex).

(E) Outer membrane (OM) proteins with α-helical transmembrane segments are inserted into the membrane by import pathways that have only been partially characterized. Shown is an import pathway via the mitochondrial import (MIM) complex.

charged amphipathic α helices and are recognized by receptors of the translocase of the outer mitochondrial membrane (TOM complex) (Figure 1A) (Mayer et al., 1995; Brix et al., 1997; van Wilpe et al., 1999; Abe et al., 2000; Meisinger et al., 2001; Saitoh et al., 2007). Upon translocation through the TOM channel, the cleavable preproteins are transferred to the presequence translocase of the inner membrane (TIM23 complex). The membrane potential across the inner membrane ($\Delta\psi$, negative on the matrix side) exerts an electrophoretic effect on the positively charged presequences (Martin et al., 1991). The presequence translocase-associated motor (PAM) with the ATP-dependent heat-shock protein 70 (mtHsp70) drives preprotein translocation into

the matrix (Chacinska et al., 2005; Mapa et al., 2010). Here the presequences are typically cleaved off by the mitochondrial processing peptidase (MPP). Some cleavable preproteins contain a hydrophobic segment behind the presequence, leading to arrest of translocation in the TIM23 complex and lateral release of the protein into the inner membrane (Glick et al., 1992; Chacinska et al., 2005; Meier et al., 2005). In an alternative sorting route, some cleavable preproteins destined for the inner membrane are fully or partially translocated into the matrix, followed by insertion into the inner membrane by the OXA export machinery, which has been conserved from bacteria to mitochondria ("conservative sorting") (He and Fox, 1997; Hell et al., 1998; Meier et al., 2005; Bohnert et al., 2010).

Hydrophobic inner membrane proteins that are synthesized without a presequence use a different import pathway (Endres et al., 1999; Curran et al., 2002; Rehling et al., 2003). The members of the large metabolite carrier family are major substrates of the so-called carrier pathway (Figure 1B). Carrier precursors contain internal targeting signals in the mature protein part (Brix et al., 1999). Like cleavable precursors, they use the TOM complex, but are then transported by different machineries, the small TIM chaperones in the intermembrane space and the carrier translocase of the inner membrane (TIM22 complex) (Endres et al., 1999; Curran et al., 2002; Rehling et al., 2003). Inner membrane insertion is driven by $\Delta\psi$ (Rehling et al., 2003).

A third mitochondrial protein import pathway is used by cysteine-rich intermembrane space proteins (Chacinska et al., 2004; Naoé et al., 2004; Banci et al., 2009; Bien et al., 2010; Vögtle et al., 2012). The intermembrane space receptor Mia40 is the central component of the mitochondrial intermembrane space import and assembly (MIA) machinery (Figure 1C). The precursors are translocated through the TOM complex in a reduced, unfolded conformation. Mia40 forms a transient disulfide bond with the incoming precursor and functions as oxidoreductase that inserts two or more disulfide bonds into the imported protein. The protein is thus stably folded, preventing its retrotranslocation into the cytosol. The sulfhydryl oxidase Erv1 (essential for respiration and viability) oxidizes reduced Mia40, thus enabling new rounds of precursor import and oxidation.

The mitochondrial outer membrane contains two major classes of integral membrane proteins: proteins with α-helical transmembrane segments and β-barrel proteins. The precursors of β-barrel proteins are imported by the TOM complex, bind to small TIM chaperones of the intermembrane space, and are inserted into the outer membrane by the sorting and assembly machinery (SAM complex, also termed TOB complex) (Figure 1D) (Paschen et al., 2003; Wiedemann et al., 2003; Gentle et al., 2004; Kutik et al., 2008; Qiu et al., 2013). The import of α-helical outer membrane proteins is only understood in part. Several import routes have been described, including an import pathway via the mitochondrial import (MIM) complex (Figure 1E) (Becker et al., 2011; Dimmer et al., 2012). Whereas the TOM complex is the main mitochondrial protein entry gate used by most classes of precursor proteins, some α-helical outer membrane proteins likely bypass the TOM channel (Otera et al., 2007; Meineke et al., 2008; Chacinska et al., 2009; Krumpe et al., 2012).

Regulatory Processes Acting at Cytosolic Precursors of Mitochondrial Proteins

Two properties of cytosolic precursor proteins are crucial for import into mitochondria. (1) The targeting signals of the precursors have to be accessible to organellar receptors. Modification of a targeting signal by posttranslational modification or masking of a signal by binding partners can promote or inhibit import into an organelle. (2) The protein import channels of mitochondria are so narrow that folded preproteins cannot be imported. Thus preproteins should be in a loosely folded state or have to be unfolded during the import process. Stable folding of preprotein domains in the cytosol impairs protein import.

Regulation at the level of cytosolic preproteins has only been observed for the import of a small subset of mitochondrial proteins so far. The reason is that these regulatory processes are specific for one preprotein or a closely related group of preproteins. We will not list all individual preproteins but will discuss characteristic cases to exemplify the regulatory mechanisms and show how the import of individual preproteins can be controlled under different physiological conditions. Regulation at the level of preproteins is of particular importance for proteins with a dual localization in the cell (for a detailed review of dual targeting, see Yogev and Pines, 2011).

Import Regulation by Binding of Metabolites or Partner Proteins to Preproteins

Binding of a metabolite to a precursor protein can represent a direct means of import regulation (Figure 2A, condition 1). A characteristic example is the import of 5-aminolevulinate synthase, a mitochondrial matrix protein that catalyzes the first step of heme biosynthesis (Hamza and Dailey, 2012). The precursor contains heme binding motifs in its amino-terminal region, including the presequence (Dailey et al., 2005). Binding of heme to the precursor inhibits its import into mitochondria, likely by impairing recognition of the precursor protein by TOM receptors (Lathrop and Timko, 1993; González-Domínguez et al., 2001; Munakata et al., 2004; Dailey et al., 2005). Thus the biosynthetic pathway is regulated by a feedback inhibition of mitochondrial import of a crucial enzyme, providing an efficient and precursor-specific means of import regulation dependent on the metabolic situation.

Binding of precursor proteins to specific partner proteins in the cytosol can positively or negatively affect mitochondrial import (Figure 2A, condition 2). (1) The yeast DNA repair enzyme Apn1 (apurinic/apyrimidinic endonuclease) contains a carboxy-terminal nuclear localization signal and a putative (weak) amino-terminal mitochondrial targeting signal. Apn1 is mainly found in the nucleus but upon interaction with the protein Pir1 (protein containing internal repeats) is also translocated into mitochondria. Pir1 interacts with the carboxy-terminal region of Apn1, likely masking the nuclear localization signal and promoting import into mitochondria (Vongsamphanh et al., 2001). (2) Glutamyl-tRNA synthetase is located in the cytosol and mitochondria. Its translocation into mitochondria is prevented by binding to Arc1 (Acyl-RNA-complex) that serves as a cytosolic retention platform. When yeast cells shift from fermentation to respiration, the levels of Arc1 are decreased and thus mitochondrial import of glutamyl-tRNA synthetase is increased (Frechin et al., 2009). Taken together, binding to nonmitochondrial proteins can stimulate or inhibit the mitochondrial import of proteins

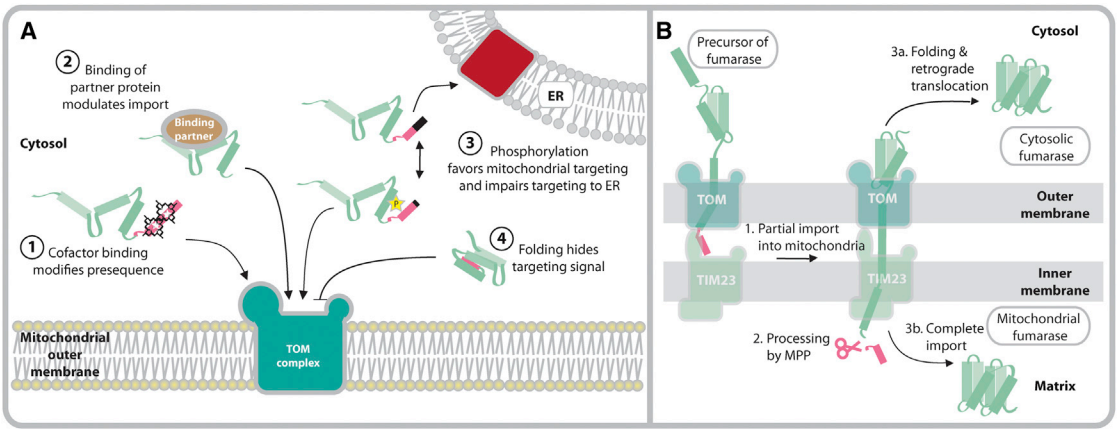

Figure 2. Regulation of Cytosolic Precursors of Mitochondrial Proteins

(A) The import of a subset of mitochondrial precursor proteins can be positively or negatively regulated by precursor-specific reactions in the cytosol. (1) Binding of ligands/metabolites can inhibit mitochondrial import. (2) Binding of precursors to partner proteins can stimulate or inhibit import into mitochondria. (3) Phosphorylation of precursors in the vicinity of targeting signals can modulate dual targeting to the endoplasmic reticulum (ER) and mitochondria. (4) Precursor folding can mask the targeting signal.

(B) Cytosolic and mitochondrial fumarases are derived from the same presequence-carrying preprotein. The precursor is partially imported by the TOM and TIM23 complexes of the mitochondrial membranes and the presequence is removed by the mitochondrial processing peptidase (MPP). Folding of the preprotein promotes retrograde translocation of more than half of the molecules into the cytosol, whereas the other molecules are completely imported into mitochondria.

with dual localization, depending on the specific binding properties (masking of nonmitochondrial targeting signal, retention in the cytosol).

Import Regulation by Covalent Modification or Cleavage of Precursor Proteins

Phosphorylation of precursor proteins can alter the import of a subset of mitochondrial proteins (De Rasmo et al., 2008; Avadhani et al., 2011; Rao et al., 2012). Characteristic examples are isoenzymes of cytochrome P450 monooxygenases that have a dual localization in mitochondria and the endoplasmic reticulum (ER) (Figure 2A, condition 3) (Avadhani et al., 2011). The proteins carry an amino-terminal ER targeting and anchoring signal followed by a cryptic mitochondrial targeting signal. Phosphorylation of amino acid residues in the vicinity of the mitochondrial targeting signal by protein kinase A (PKA) or protein kinase C (PKC) favors import into mitochondria. Depending on the isoenzyme, different mechanisms are discussed. Phosphorylation can increase the affinity for the mitochondrial protein import machinery (TOM, TIM23, mitochondrial Hsp70) or decrease the affinity for the signal recognition particle (SRP) and thus impair targeting to the ER (Robin et al., 2002; Avadhani et al., 2011).

Proteolytic cleavage in the cytosol and attachment of a fatty acid moiety are further examples that have been found to regulate mitochondrial targeting of individual precursor proteins in mammalian systems. Cleavage of a specific cytochrome P450 isoenzyme by a cytosolic protease removes the amino-terminal ER signal sequence and thus exposes the cryptic mitochondrial targeting signal (Boopathi et al., 2008). Modification of a dually targeted protein by myristoylation impairs its interaction with SRP and thus favors targeting to mitochondria (Colombo et al., 2005).

Regulation of Precursor Sorting by Protein Folding Before or During Import into Mitochondria

Folding of precursor proteins on the cytosolic side can inhibit mitochondrial import by two mechanisms. (1) Masking of the targeting signal: The major adenylate kinase of yeast is mainly located in the cytosol, yet a small percentage of the molecules reside in the mitochondrial intermembrane space. Folding of the enzyme in the cytosol masks the mitochondrial targeting signal and prevents import into mitochondria (Figure 2A, condition 4). The dual localization of the enzyme is thus determined by a competition between cytosolic folding and translocation into mitochondria (Strobel et al., 2002). Since the enzyme folds rapidly, only a small fraction of newly synthesized precursors can enter the mitochondrial intermembrane space. (2) Reverse translocation: A series of elegant studies revealed a remarkable sorting mechanism for yeast fumarase (Yogev and Pines, 2011) (Figure 2B). Cytosolic and mitochondrial fumarase isoenzymes are identical, and are both derived from the same presequence-carrying preprotein. All precursors are partially imported into mitochondria such that the presequence can be cleaved off by MPP in the matrix. Rapid folding of fumarase promotes retrograde translocation of more than half of the processed proteins into the cytosol, whereas the remaining proteins are fully imported into mitochondria (Sass et al., 2003). The distribution of fumarase between cytosol and mitochondria thus depends on a competition between the rate of fumarase folding on the cytosolic side and the activity of the mitochondrial protein import machinery. It has been observed that sorting of fumarase is regulated by the activity of the glyoxylate shunt. Metabolites of the glyoxylate shunt likely affect targeting and distribution of fumarase (Regev-Rudzki et al., 2009), though the exact molecular mechanism has not been elucidated.

Regulation of Mitochondrial Protein Entry Gate by Cytosolic Kinases

Regulation at the level of cytosolic precursor proteins is specific for individual proteins and therefore affects the import of only a small number of proteins. To our knowledge these processes are not relevant for the majority of mitochondrial proteins. Do

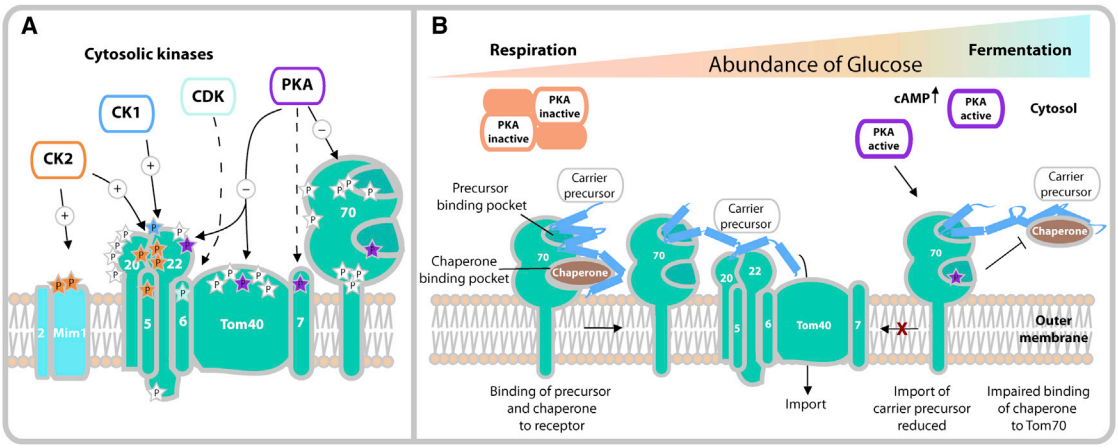

Figure 3. Regulation of TOM Complex by Cytosolic Kinases

(A) All subunits of the translocase of the outer mitochondrial membrane (TOM complex) are phosphorylated by cytosolic kinases (phosphorylated amino acid residues are indicated by stars with P). Casein kinase 1 (CK1) stimulates the assembly of Tom22 into the TOM complex. Casein kinase 2 (CK2) stimulates the biogenesis of Tom22 as well as the mitochondrial import protein 1 (Mim1). Protein kinase A (PKA) inhibits the biogenesis of Tom22 and Tom40, and inhibits the activity of Tom70 (see B). Cyclin-dependent kinases (CDK) are possibly involved in regulation of TOM.

(B) Metabolic shift-induced regulation of the receptor Tom70 by PKA. Carrier precursors bind to cytosolic chaperones (Hsp70 and/or Hsp90). Tom70 has two binding pockets, one for the precursor and one for the accompanying chaperone (shown on the left). When glucose is added to yeast cells (fermentable conditions), the levels of intracellular cAMP are increased and PKA is activated (shown on the right). PKA phosphorylates a serine of Tom70 in vicinity of the chaperone binding pocket, thus impairing chaperone binding to Tom70 and carrier import into mitochondria.

mitochondria possess mechanisms to directly regulate the import of a large number of precursor proteins? Since most precursor proteins are imported by the TOM complex, the TOM machinery would represent an ideal target for posttranslational regulation of mitochondrial biogenesis. Recent studies in baker's yeast indeed revealed that the TOM complex is embedded in a regulatory network of cytosolic kinases (Figure 3A).

Casein Kinase 2 Stimulates TOM Biogenesis and Protein Import

The TOM complex contains three receptor proteins: Tom20, Tom22, and Tom70 (Figure 1). Tom22 is the central receptor. In cooperation with Tom20, it plays a major role in the import of presequence-carrying preproteins into mitochondria but is also involved in the import of further classes of preproteins (van Wilpe et al., 1999; Neupert and Herrmann, 2007; Yamano et al., 2008). Tom70 is mainly required for the import of noncleavable hydrophobic precursors like the metabolite carriers of the inner membrane (Brix et al., 1997, 1999; Young et al., 2003).

The cytosolic casein kinase 2 (CK2) phosphorylates Tom22 at two specific serine residues (Figure 3A). Upon inactivation of CK2 or replacement of the two serines, the mitochondrial levels of Tom22 are strongly decreased (Schmidt et al., 2011). How does CK2 regulate the levels of Tom22? All TOM proteins are encoded by nuclear genes and are synthesized on cytosolic ribosomes. The TOM precursors, including the precursor of Tom22, have to be imported into the mitochondrial outer membrane. CK2 phosphorylates the Tom22 precursor in the cytosol and stimulates its import into mitochondria. Tom20 has been shown to function as receptor for the precursor of Tom22 (Keil and Pfanner, 1993). Phosphorylation of Tom22 by CK2 stimulates its binding to Tom20 and thus promotes its import (Schmidt et al., 2011).

CK2 also promotes the biogenesis of Tom20 and Tom70. The molecular mechanism involves phosphorylation of Mim1, the main component of the MIM complex of the outer membrane (Figure 3A). Mim1 is required for the insertion of several α-helical outer membrane proteins including the precursors of Tom20 and Tom70 (Becker et al., 2008; Hulett et al., 2008). When CK2 is inactivated, the levels of Mim1 are decreased, and consequently also the levels of Tom20 and Tom70 will be decreased (Schmidt et al., 2011).

Thus CK2 controls the biogenesis of all three mitochondrial protein import receptors by phosphorylation of two key components, Tom22 and Mim1. The strongly reduced levels of TOM receptors in *ck2* mutant cells lead to a general impairment of mitochondrial protein import. CK2 is a major stimulatory factor for the biogenesis of the TOM complex and protein import into mitochondria. Whereas numerous cellular substrates of CK2 are known, little is known about the upstream regulation of CK2 itself (Meggio and Pinna, 2003). The kinase is constitutively active and may be regulated by changes in its intracellular location or by partner proteins (Poole et al., 2005). The activity of CK2 is increased in rapidly growing cells that require a high activity of mitochondria, though the exact mechanism has not been elucidated.

Metabolic Switch from Respiratory to Fermentable Conditions Involves Protein Kinase A-Mediated Inhibition of TOM

PKA is activated by increased levels of cAMP in cells, leading to a dissociation of the inhibitory PKA subunits from the catalytic subunits (Zaman et al., 2008). In yeast, PKA is activated when glucose is added to cells, leading to fermentable conditions where a lower activity of mitochondria is required. PKA inhibits mitochondrial protein import by two mechanisms and thus provides a rapid means for decreasing mitochondrial biogenesis under yeast fermentation (Figure 3A). (1) PKA phosphorylates the precursor of Tom40, the channel-forming subunit of the TOM complex, in the cytosol and thus impairs its import into

mitochondria (Rao et al., 2012). Phosphorylation of Tom40 inhibits an early stage of translocation of the precursor into mitochondria. Phosphorylated Tom40 precursor remains bound to the mitochondrial surface in a nonfunctional form, whereas nonphosphorylated Tom40 is imported and assembled into the TOM complex. Similarly, PKA phosphorylates the cytosolic precursor of Tom22 and impairs import of the precursor into mitochondria (Gerbeth et al., 2013b). (2) PKA phosphorylates Tom70 on the mitochondrial surface and inhibits its receptor activity (Schmidt et al., 2011). Cytosolic chaperones of the heat-shock protein 70 and 90 families (Hsp70 and Hsp90) bind to the hydrophobic carrier precursors and prevent their misfolding and aggregation in the cytosol (Young et al., 2003). Tom70 carries two distinct binding sites, one for the precursor protein and one for the accompanying chaperone (Figure 3B) (Li et al., 2009). PKA selectively phosphorylates a serine in close vicinity of the chaperone binding site of Tom70 and thus impairs binding of the chaperone to Tom70. Thereby PKA disturbs the reaction cycle of Tom70 with the chaperone-bound precursor and delays the import of metabolite carriers into mitochondria.

Network of Stimulatory and Inhibitory Kinases Acts on TOM Receptors, Channel, and Assembly Factors

It is likely that additional cytosolic kinases regulate the biogenesis and function of the TOM complex (Figure 3A). Recent analyses suggest that at least two further kinases act on TOM components (Rao et al., 2011; Schmidt et al., 2011; Gerbeth et al., 2013a, 2013b). Casein kinase 1 (CK1), which plays a role in glucose-induced signal transduction, phosphorylates mitochondria-bound Tom22 and stimulates its assembly into the TOM complex (Gerbeth et al., 2013b). So far, CK1 has been known to function at the plasma membrane, yet upon growth of yeast on glucose a fraction of CK1 molecules is transferred to the mitochondrial surface, where CK1 may phosphorylate additional substrates. Cyclin-dependent kinases (CDKs), which are crucial for cell-cycle regulation, can phosphorylate the small assembly factor Tom6. CK2 and PKA likely have additional targets at the TOM complex (Rao et al., 2011; Schmidt et al., 2011) and thus may control biogenesis and function of additional TOM subunits (Figure 3A).

The function as main protein entry gate of mitochondria renders the TOM complex an ideal substrate for regulation by the cytosolic kinase network. TOM receptors are directly accessible to cytosolic kinases as well as to phosphatases, and thus cycles of phosphorylation and dephosphorylation will be possible. Such a mechanism likely applies to the receptor Tom70 during import of metabolite carriers. In addition, since all TOM proteins are synthesized as precursors in the cytosol, their biogenesis can be directly controlled at an early stage by phosphorylation/dephosphorylation, and thus the amount inserted into the outer membrane can be regulated. The biogenesis of Tom22 reveals a remarkable complexity of TOM regulation, as import and assembly of this receptor are controlled by three kinases (CK1, CK2, and PKA). We propose that multiple cytosolic signaling pathways converge at the TOM complex and control biogenesis and activity of TOM receptors (Tom20, Tom22, and Tom70), import channel (Tom40), and assembly/biogenesis factors (Mim1, Tom6). To date, regulation of the TOM complex by phosphorylation has been mainly studied in yeast, and thus the physiological implications for protein import into mammalian mitochondria have not been defined. Regulation of the mitochondrial protein entry gate may represent an attractive system for controlling mitochondrial activity (energy metabolism) in different cell types of higher eukaryotes or even in different intracellular locations of the same cell, e.g., in neurons.

Protein Import Activity as Sensor of Mitochondrial Stress and Dysfunction

The protein import machinery is intimately connected to the energetic state of mitochondria. Both pathways of preprotein translocation into and across the mitochondrial inner membrane, the presequence pathway via the TIM23 complex and the carrier pathway via the TIM22 complex, require the membrane potential $\Delta\psi$ (Figures 1A and 1B). $\Delta\psi$ plays a dual role in protein import. It exerts an electrophoretic effect on positively charged segments of preproteins and thus generates an import-driving force, in particular on presequences (Martin et al., 1991; Huang et al., 2002). In addition, $\Delta\psi$ activates the channel-forming proteins Tim23 and Tim22 (Truscott et al., 2001; Kovermann et al., 2002; Malhotra et al., 2013). Hence, the magnitude of $\Delta\psi$ is a critical determinant for the efficiency of inner membrane protein translocation (Martin et al., 1991; Huang et al., 2002; Rehling et al., 2003; van der Laan et al., 2006; Krayl et al., 2007).

Whereas preprotein insertion into the inner membrane can be driven by $\Delta\psi$ alone (van der Laan et al., 2007), preprotein translocation into the matrix additionally requires ATP to drive the molecular chaperone mtHsp70, the central component of the import motor PAM (Figure 4A). mtHsp70 molecules bind to preproteins in transit and generate an import-driving activity on the polypeptide chains (Krayl et al., 2007; Neupert and Herrmann, 2007; Chacinska et al., 2009). mtHsp70 thereby promotes the unfolding of preprotein domains that are still located on the cytosolic side.

The TIM23 complex physically interacts with the $\Delta\psi$-generating respiratory chain and the ATP-driven import motor in an alternating manner (van der Laan et al., 2006, 2007; Wiedemann et al., 2007). (1) The TIM23 subunits Tim21 and Mgr2 connect the translocase with a respiratory chain supercomplex consisting of complex III (bc_1-complex) and complex IV (cytochrome *c* oxidase) and thus stimulate the $\Delta\psi$-dependent insertion of inner membrane proteins (Figure 4A) (van der Laan et al., 2006; Gebert et al., 2012). (2) Several membrane-bound cochaperones associate with the TIM23 complex and direct mtHsp70 to the protein import channel (Figure 4A) (D'Silva et al., 2003; Chacinska et al., 2005; Neupert and Herrmann, 2007; Hutu et al., 2008; Mapa et al., 2010). (3) Additionally, the TIM23 complex interacts with the TOM complex during preprotein translocation from the outer membrane to the inner membrane (Chacinska et al., 2005, 2010). Taken together, the presequence translocase functions at a central junction of the presequence import pathway. Impairment of respiratory chain activity, reduction of ATP levels in the matrix, or an increased occupancy of mtHsp70 by misfolded proteins will directly affect the import-driving activity of the translocase. The protein import activity of mitochondria is thus a sensitive indicator of their energetic state and fitness.

Induction of Mitochondrial Stress Response via Reduced Protein Import Activity

A number of conditions lead to a mitochondrial stress response or unfolded protein response (UPR^{mt}), including accumulation of

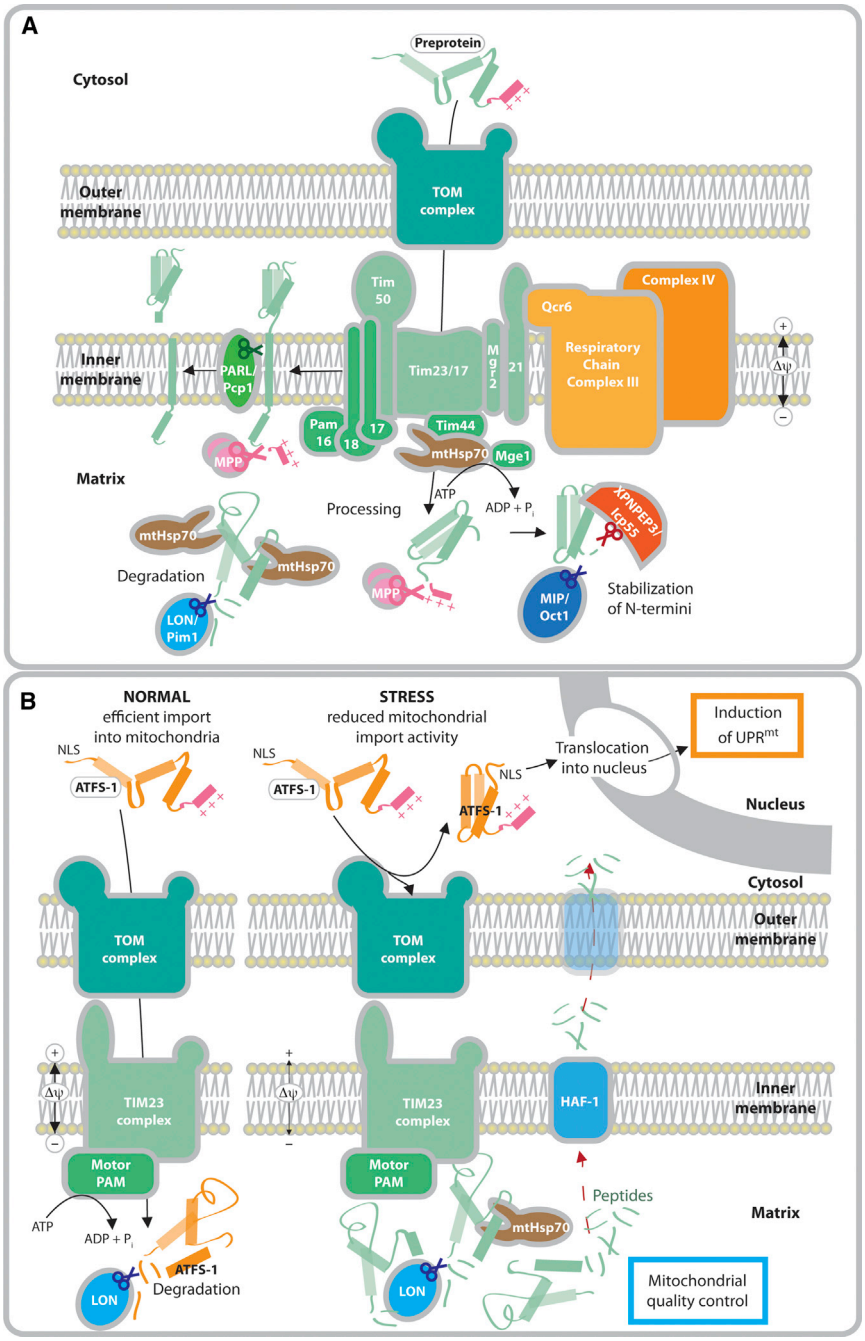

Figure 4. Mitochondrial Quality Control and Stress Response

(A) Import and quality control of cleavable preproteins. The TIM23 complex cooperates with several machineries: the TOM complex, a supercomplex consisting of the respiratory chain complexes III and IV, and the presequence translocase-associated motor (PAM) with the central chaperone mtHsp70. Several proteases/peptidases involved in processing, quality control, and/or degradation of imported proteins are shown, including mitochondrial processing peptidase (MPP), intermediate cleaving peptidase (XPNPEP3/Icp55), mitochondrial intermediate peptidase (MIP/Oct1), mitochondrial rhomboid protease (PARL/Pcp1), and LON/Pim1 protease.

(B) The transcription factor ATFS-1 contains dual targeting information, a mitochondrial targeting signal at the amino terminus, and a nuclear localization signal (NLS). In normal cells, ATFS-1 is efficiently imported into mitochondria and degraded by the Lon protease in the matrix. When under stress conditions the protein import activity of mitochondria is reduced (due to lower $\Delta\psi$, impaired mtHsp70 activity, or peptides exported by the peptide transporter HAF-1), some ATFS-1 molecules accumulate in the cytosol and can be imported into the nucleus, leading to induction of an unfolded protein response (UPR^{mt}).

misfolded proteins in the matrix, decreased activity of the respiratory chain, and generation of high levels of reactive oxygen species (ROS) (Haynes and Ron, 2010). By retrograde signaling to the nucleus, the expression of numerous genes encoding mitochondrial chaperones, proteases, import components, morphology factors, and metabolic enzymes is induced, leading to a protective response during mitochondrial dysfunction (for reviews of mitochondrial retrograde signaling, see Liu and Butow, 2006; Ryan and Hoogenraad, 2007; Haynes et al., 2013).

A recent study in the worm *Caenorhabditis elegans* revealed an elegant mechanism how the protein import machinery can play a crucial role in the mitochondrial stress (unfolded protein) response (Figure 4B). The activating transcription factor associated with stress-1 (ATFS-1) contains a mitochondrial targeting signal (Nargund et al., 2012). Under normal conditions (fully active mitochondria), ATFS-1 is efficiently imported into the mitochondrial matrix and degraded by the Lon protease. When under stress conditions the mitochondrial protein import activity is impaired, a fraction of ATFS-1 molecules is not imported into mitochondria. Since ATFS-1 also contains a nuclear localization signal, molecules that are not imported into mitochondria can be translocated into the nucleus and induce the UPR^{mt} (Nargund et al., 2012). A reduction of the mitochondrial protein import activity can be caused by an impairment of the TOM, TIM23 or import motor (chaperone) machineries, reduction of $\Delta\psi$ (impaired respiratory chain activity), or an increased peptide efflux via the peptide transporter HAF-1. The latter mechanism involves a degradation of unfolded or misfolded proteins by the protein quality control system in the matrix, followed by export of peptides that impair the protein import activity of mitochondria (by an unknown mechanism).

These findings demonstrate that the protein import machinery can function as sensor for mitochondrial dysfunction and trigger a mitochondrial stress response.

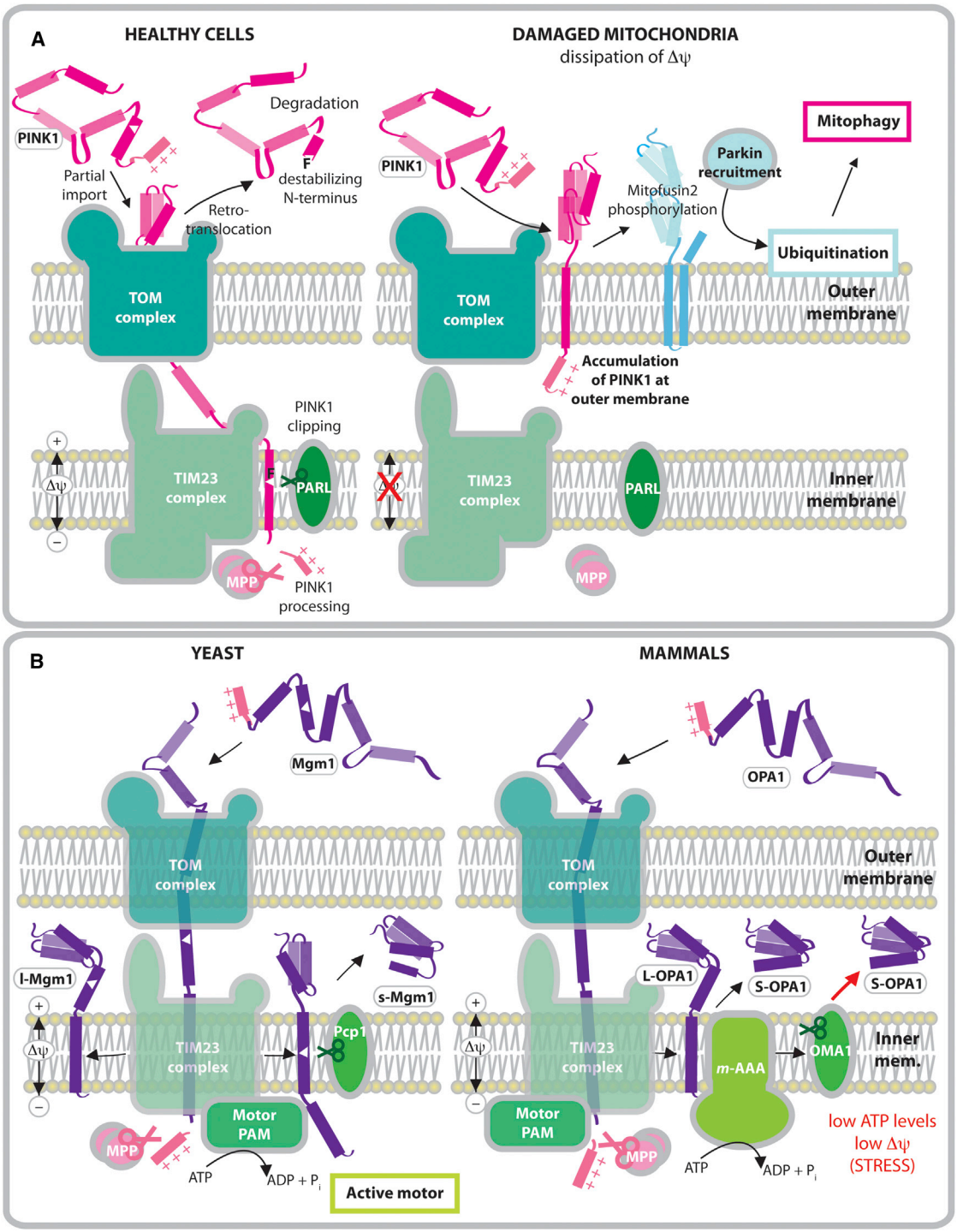

Figure 5. Mitochondrial Dynamics and Disease

(A) In healthy cells, the kinase PINK1 is partially imported into mitochondria in a membrane potential ($\Delta\psi$)-dependent manner and processed by the inner membrane rhomboid protease PARL, which cleaves within the transmembrane segment and generates a destabilizing N terminus, followed by retro-translocation of cleaved PINK1 into the cytosol and degradation by the ubiquitin-proteasome system (different views have been reported if PINK1 is first processed by MPP or not; Greene et al., 2012; Kato et al., 2013; Yamano and Youle, 2013). Dissipation of $\Delta\psi$ in damaged mitochondria leads to an accumulation of unprocessed PINK1 at the TOM complex and the recruitment of the ubiquitin ligase Parkin to mitochondria. Mitofusin 2 is phosphorylated by PINK1 and likely functions as receptor for Parkin. Parkin mediates ubiquitination of mitochondrial outer membrane proteins (including mitofusins), leading to a degradation of damaged mitochondria by mitophagy. Mutations of PINK1 or Parkin have been observed in monogenic cases of Parkinson's disease.

(B) The inner membrane fusion protein OPA1/Mgm1 is present in long and short isoforms. A balanced formation of the isoforms is a prerequisite for the proper function of OPA1/Mgm1. The precursor of OPA1/Mgm1 is imported by the TOM and TIM23 complexes. A hydrophobic segment of the precursor arrests translocation in the inner membrane, and the amino-terminal targeting signal is cleaved by MPP, generating the long isoforms. In yeast mitochondria, the import

(legend continued on next page)

Regulation of PINK1/Parkin-Induced Mitophagy by the Activity of the Mitochondrial Protein Import Machinery

The mitochondrial kinase PTEN-induced putative kinase 1 (PINK1) and the mainly cytosolic Parkin, an E3 ubiquitin ligase, have been linked to familial cases of Parkinson's disease. PINK1 and Parkin function as part of a mitochondrial quality control pathway that is likely impaired in Parkinson's disease (Park et al., 2006; Narendra and Youle, 2011; Exner et al., 2012; Corti and Brice, 2013).

Studies in recent years revealed a crucial role of the mitochondrial protein import machinery for the function of PINK1 and Parkin. PINK1 carries an amino-terminal mitochondrial targeting signal. In healthy cells, PINK1 is partially imported into mitochondria via the TOM and TIM23 complexes in a $\Delta\psi$-dependent manner and proteolytically processed by the inner membrane rhomboid protease PARL (presenilin-associated rhomboid-like protein) (Figure 5A) (Jin et al., 2010). PARL cleaves within the transmembrane segment of PINK1 and generates an amino terminus with a destabilizing amino acid residue. Cleaved PINK1 is then retro-translocated to the cytosol, where the amino-terminal region is recognized by ubiquitin ligases, leading to degradation of PINK1 by the proteasome (Yamano and Youle, 2013).

When mitochondria are damaged and their $\Delta\psi$ is dissipated, PINK1 cannot be translocated to the inner membrane anymore and is not processed. Thus PINK1 retains its transmembrane segment and accumulates at the outer membrane in dynamic association with the TOM complex (Figure 5A) (Lazarou et al., 2012). Outer-membrane-located PINK1 promotes the recruitment of Parkin to mitochondria, resulting in ubiquitination of a number of mitochondrial proteins. Together with additional factors, the Parkin-mediated ubiquitination leads to the removal of damaged mitochondria by autophagy (mitophagy). The exact molecular mechanism of the PINK1/Parkin-mediated quality control system is currently under discussion. An attractive model involving the fusion protein mitofusin 2 was presented by Chen and Dorn (2013). They reported that PINK1 phosphorylates mitofusin 2 at the mitochondrial outer membrane, stimulating its function as mitochondrial receptor for Parkin. Mitofusin 2 may thus recruit Parkin to damaged mitochondria.

An important question is how the distinction between a mild dysfunction of mitochondria (leading to a protective unfolded protein response like the ATFS-1 pathway) and a strong dysfunction (leading to removal of damaged mitochondria via PINK1/Parkin-induced mitophagy) is controlled and regulated (Vögtle and Meisinger, 2012; Haynes et al., 2013). The magnitude of the inner membrane potential and thus the efficiency of preprotein import may be a crucial sensor for mitochondrial fitness, permitting a differentiation between mild defects (partial reduction of $\Delta\psi$) and damage of mitochondria (strong reduction or dissipation of $\Delta\psi$).

Preprotein Processing Influences Mitochondrial Dynamics and Quality Control

Most preproteins imported by the presequence pathway are proteolytically processed at their amino termini in order to remove the mitochondrial targeting signal. The first cleavage is typically performed by MPP in the matrix; however, a number of additional processing enzymes that can exert regulatory effects on protein activity and quality control have been found in the mitochondrial inner membrane and matrix (Figure 4A) (for review on mitochondrial processing enzymes, see Mossmann et al., 2012; Teixeira and Glaser, 2013).

Differential Processing of Inner Membrane Fusion Protein in Dependence on Protein Import Activity and Stress Conditions

The fusion protein OPA1 (optic atrophy) of the mitochondrial inner membrane is an example of how protein import and processing are connected to mitochondrial membrane dynamics and morphology. OPA1, also termed mitochondrial genome maintenance (Mgm1) in yeast, is synthesized as precursor in the cytosol and imported by the TOM and TIM23 complexes such that the amino-terminal presequence can be removed by MPP (Figure 5B). A hydrophobic segment following the matrix targeting signal arrests translocation in the inner membrane, and OPA1/Mgm1 is laterally released into the inner membrane (Herlan et al., 2004; Ishihara et al., 2006). To be functional in inner membrane fusion, OPA1/Mgm1 has to be present in long and short isoforms (Song et al., 2007; Ehses et al., 2009). The long isoforms are anchored to the inner membrane by the hydrophobic segment, whereas the short isoforms are generated by a second proteolytic cleavage and released to the intermembrane space. The balanced formation of both long and short isoforms is required for efficient inner membrane fusion (Song et al., 2007; DeVay et al., 2009; Hoppins and Nunnari, 2009).

Herlan et al. (2004) elucidated the mechanism of Mgm1 processing in yeast mitochondria. The ATP-dependent activity of the import motor PAM drives the precursor further into the matrix such that a second hydrophobic segment can be cleaved within the inner membrane by the rhomboid protease Pcp1 (Figure 5B). This second cleavage generates the short isoform that is released to the intermembrane space. The ATP level in the mitochondrial matrix may thus control the formation of short Mgm1 via the activity of the import motor, providing a link between the energetic state of mitochondria and regulation of their morphology.

The principles of OPA1 processing in mammals are related to that in yeast; however, different proteases are involved (Figure 5B). The inner-membrane-bound ATPase associated with diverse cellular activities (*m*-AAA) protease forms a large oligomer with ATPase domains in the matrix. Though the exact mechanisms are still under debate, the *m*-AAA protease likely is important for the balanced formation of long and short isoforms of OPA1 (Ehses et al., 2009). The *m*-AAA protease may exert a membrane dislocation activity on the OPA1 precursor in an ATP-dependent manner and regulate its differential processing. However, an additional inner-membrane-bound protease OMA1, which functions in an ATP-independent manner, can convert long OPA1 isoforms into short isoforms, in particular under stress conditions like low ATP or reduced membrane

motor PAM drives the Mgm1 precursor further toward the matrix such that a second hydrophobic segment is cleaved by the inner membrane rhomboid protease Pcp1, generating the short isoform (s-Mgm1). In mammals, the *m*-AAA protease is likely responsible for the balanced formation of long (L) and short (S) isoforms of OPA1. A further protease, OMA1, can convert long isoforms into short isoforms in particular under stress conditions, leading to an impairment of mitochondrial fusion and thus to fragmentation of mitochondria.

potential, resulting in a loss of long isoforms (Ehses et al., 2009). Under stress, mitochondrial fusion will thus be impaired, leading to a fragmentation of mitochondria and the possibility to selectively remove damaged mitochondria. Mutations in OPA1 and *m*-AAA subunits lead to neurodegenerative and metabolic disorders (Atorino et al., 2003; Zanna et al., 2008; Ehses et al., 2009; Quirós et al., 2012), underscoring the importance of these components for the correct functioning of mitochondria.

Quality Control of Imported Proteins by Specific Processing of Mitochondrial Targeting Sequences

Studies in bacteria and the eukaryotic cytosol revealed the existence of an N-end rule pathway of regulated proteolysis (Mogk et al., 2007; Varshavsky, 2011). The N-end rule links the amino-terminal amino acid residue to the half-life of proteins. Destabilizing amino acid residues at the amino terminus favor a rapid degradation of proteins, whereas proteins with stabilizing amino acid residues at the amino terminus are considerably more stable. Recent studies revealed that an N-end rule also applies to proteins imported into mitochondria (Vögtle et al., 2009, 2011). Preproteins imported into the matrix are cleaved by MPP, leading to a large variety of different amino termini of the cleaved proteins, including many proteins with destabilizing amino termini. This situation would lead to a high instability of the mitochondrial proteome, since a considerable fraction of imported proteins would be rapidly degraded. How is the stability of the mitochondrial proteome achieved as the majority of mature mitochondrial proteins carry stabilizing amino termini in vivo? It was observed that two peptidases can perform a second cleavage of MPP-processed preproteins in the matrix. The yeast aminopeptidase intermediate cleaving peptidase of 55 kDa (Icp55) removes single destabilizing amino acid residues after the cleavage by MPP and typically generates mature proteins with a stabilizing amino terminus (Figure 4A) (Vögtle et al., 2009; Naamati et al., 2009). The mitochondrial intermediate peptidase (MIP), also termed octapeptidyl aminopeptidase (Oct1), removes an octapeptide from a subset of MPP-processed preproteins (Gakh et al., 2002). Thereby MIP/Oct1 removes destabilizing amino-terminal amino acid residues and, like Icp55, typically generates mature proteins with stable amino termini (Vögtle et al., 2011).

Icp55 and MIP/Oct1 thus prevent a premature degradation of mitochondrial proteins and are major players of a quality control system that regulates protein homeostasis of mitochondria (Vögtle et al., 2009, 2011; Anand et al., 2013; Shutt and McBride, 2013). The mitochondrial proteases that are responsible for degrading proteins with destabilizing amino termini have not been identified so far.

In humans, Icp55 is termed aminopeptidase 3 (APP3 or XPNPEP3) (Figure 4A). Mutations in the *XPNPEP3* gene can lead to cystic kidney disease with ciliary dysfunction (O'Toole et al., 2010), suggesting a so-far-unknown connection between mitochondrial function and cilia. Future studies will have to address the molecular role of human APP3/XPNPEP3 in mitochondrial protein homeostasis.

Mitochondrial Protein Import Machinery as Regulatory Hub: A Model

Mitochondria are of central importance for the functioning of cells under physiological and pathophysiological conditions. Therefore mitochondrial biogenesis, activity, and turnover are deeply embedded in the signaling network of cells (Liu and Butow, 2006; Ryan and Hoogenraad, 2007; Galluzzi et al., 2012; Nunnari and Suomalainen, 2012; Youle and van der Bliek, 2012; Shutt and McBride, 2013). Whereas regulation of mitochondrial biogenesis at the level of gene expression has been well established (Santangelo, 2006; Scarpulla, 2006; Hock and Kralli, 2009), less has been known about regulation of the protein import machinery on a posttranslational level. As discussed here, recent studies revealed a remarkable diversity of mechanisms for regulation of mitochondrial protein import and processing. Importantly, the protein import machinery is not only a target of regulatory processes but also functions as sensor for the activity and quality of mitochondria.

We propose that the mitochondrial protein import machinery plays an important role as regulatory hub in metabolism, stress response, and pathogenesis of diseases. The concept includes two major aspects. (1) Sensor: The protein import activity serves as sensor for the fitness and quality of mitochondria, determined by the energetic state ($\Delta\psi$, ATP levels) and protein homeostasis of mitochondria. This is mainly achieved by the $\Delta\psi$ dependence of preprotein translocation, the direct coupling of presequence translocase and respiratory chain, and the essential role of the ATP-dependent chaperone mtHsp70 in protein import and folding in the matrix. Impairment of protein import triggers pathways that can induce a mitochondrial stress response or in case of severe damage, a removal of mitochondria by mitophagy. (2) Target: Controlling the assembly and activity of the protein import machinery provides a direct means of regulating the biogenesis of mitochondrial proteins. Depending on the targeting signals of precursors and the intramitochondrial destination of the proteins, different classes of proteins are differentially regulated: critical determinants are the activities of TOM receptors, the $\Delta\psi$-dependent presequence translocase, the ATP-driven import motor, and the activity of processing enzymes. Cytosolic signaling pathways targeting the TOM machinery and changes of the energetic state of mitochondria can lead to a rapid modulation of the protein import efficiency, and thus the protein content of mitochondria can be adapted under physiological and pathophysiological conditions. In addition, a specific regulation of individual precursor proteins is achieved by the modification of precursor proteins themselves (binding of ligands/metabolites, covalent modification, and processing of preproteins). Thus, protein composition, activity, and morphology of mitochondria can be actively controlled.

The original view of a constitutively active and not regulated mitochondrial protein import apparatus has to be changed radically. Upon uptake of the prokaryotic ancestor by a primordial eukaryotic cell about 1.5 billion years ago, the developing mitochondria were more and more integrated into numerous cellular activities, and thus many machineries and systems evolved in parallel. This includes the mitochondrial respiratory chain, metabolic processes, signaling pathways, machineries responsible for mitochondrial morphology and membrane dynamics, apoptotic processes, and, as central system, the essential protein import machinery. These machineries and pathways did not develop independently, but the close spatial and functional relationship led to a coevolution and intensive crosstalk (Becker et al., 2012). The protein import machinery is deeply integrated

into a network of mitochondrial activities, biogenesis, and quality control, representing an ideal system for sensing mitochondrial fitness and regulating its biogenesis.

Perspectives

The analysis of regulatory processes connected to mitochondrial protein import bears the potential for many more exciting findings. We speculate that the following topics may be of particular importance for future research on the protein import machinery and its regulation.

Presequence and Carrier Pathways

Although most regulatory processes studied so far target the TOM complex or the presequence pathway, even here many aspects are unknown. The phosphatases responsible for dephosphorylation of TOM proteins have not been identified. It is likely that additional signaling pathways will target TOM and further mitochondrial translocases. Proteomic studies revealed many phosphorylated intramitochondrial proteins (Reinders et al., 2007; Deng et al., 2011; Grimsrud et al., 2012) and a number of intramitochondrial kinases and phosphatases of unknown function (for review, see Rao et al., 2011), suggesting the existence of not-yet-defined intramitochondrial signaling pathways. The coordination of assembly of imported proteins and mitochondria-synthesized proteins into respiratory chain complexes (Mick et al., 2011, 2012) is a highly complex process, and its regulation is a major topic of current and future research.

Kuhn et al. (2009) reported an involvement of calcium and calmodulin in transport of cleavable and noncleavable proteins into and across the inner membrane of plant mitochondria. Though the mechanism has not been elucidated, this observation points to an additional regulatory process. Calcium/calmodulin did not affect protein import into yeast mitochondria, supporting the view that regulatory processes can function in a species-specific or cell-type-specific manner. PKA represents a further example, since activation of PKA by cAMP can lead to stimulatory or inhibitory effects on mitochondrial protein import, depending on the organism analyzed (De Rasmo et al., 2008; Avadhani et al., 2011; Schmidt et al., 2011; Rao et al., 2012). A study by Matthews et al. (2010) indicated that the magnitude of the mitochondrial membrane potential is a crucial determinant for the differential sorting of glutamine synthetase in liver and brain cells of chicken. This protein carries a weak mitochondrial targeting signal. Hepatocyte mitochondria generate a high $\Delta\psi$ that drives import of glutamine synthetase into mitochondria, whereas the $\Delta\psi$ of astrocyte mitochondria is lower and thus glutamine synthetase remains in the cytosol. The TIM23 complex of metazoans contains two Tim17 homologs with a differential tissue distribution, Tim17A and Tim17B. Rainbolt et al. (2013) showed that stress-regulated translational attenuation leads to a decrease of Tim17A levels via two mechanisms, reduced biogenesis and enhanced degradation of Tim17A. This results in decreased protein import and promotes a mitochondrial stress response. Regulation of translocases in a tissue-specific manner may represent an important mechanism for a differential adaptation of mitochondrial import and protein homeostasis under stress conditions.

Protein Sorting to Intermembrane Space

The MIA pathway translocating cysteine-rich proteins into the intermembrane space may act as a putative sensor and target for regulatory processes. MIA functions in a redox-regulated manner, and thus its activity is closely connected to the redox conditions in mitochondria as well as in the cytosol (Tienson et al., 2009; Bien et al., 2010; Kojer et al., 2012). The import of MIA substrates like the small TIM chaperones depends on a reduced state of the cytosolic precursor proteins, which is maintained by the cytosolic thioredoxin system (Durigon et al., 2012). Thioredoxins and thioredoxin reductases have recently been found to be located in the mitochondrial intermembrane space as well (Vögtle et al., 2012). Thus changes in the cytosolic and mitochondrial redox states likely regulate the biogenesis of MIA-dependent precursor proteins. A recent study revealed that a fraction of cysteine-rich proteins are ubiquitinated in the cytosol and degraded by the proteasome (Bragoszewski et al., 2013), representing an additional early system for regulating import of MIA substrates. The regulation of mitochondrial protein import pathways by covalent protein modifications different from phosphorylation is largely unexplored and will represent an important topic for future studies.

Protein Sorting to Outer Membrane and Connection to Membrane Contact Sites

The SAM complex of the outer membrane shares a subunit with the ER-mitochondria encounter structure (ERMES) that physically connects ER and mitochondria in yeast (Kornmann et al., 2009). The mitochondrial distribution and morphology protein Mdm10 associates with both SAM and ERMES (Meisinger et al., 2004) and, by mutant analysis, has been shown to play distinct roles in mitochondrial protein assembly, interorganellar contact site formation, and organellar morphology. Remarkably, Tom7 shows a dual localization. Tom7 is not only a TOM subunit involved in assembly of the translocase but also interacts with Mdm10 and controls its distribution between SAM and ERMES, as deletion of Tom7 shifts the distribution of Mdm10 toward the SAM complex (Meisinger et al., 2006; Yamano et al., 2010). Regulation of Tom7 amount and function as well as the distribution of Mdm10 and Tom7 would represent attractive possibilities for connecting protein assembly in the mitochondrial outer membrane with the proposed ERMES functions in calcium homeostasis, lipid transfer, and membrane dynamics (Kornmann et al., 2009; Kornmann and Walter, 2010; Voss et al., 2012; Murley et al., 2013). In mammals, mitofusin 2, which may function as mitochondrial receptor for the recruitment of Parkin (Chen and Dorn, 2013), has also been found to mediate ER-mitochondria interactions and to promote calcium uptake by mitochondria (de Brito and Scorrano, 2008). The recently identified mitochondrial inner membrane organizing system (MINOS, also termed MICOS or MitOS), which is crucial for the maintenance of inner membrane cristae organization, is embedded in a network of interactions with protein translocases, including TOM, SAM, and MIA (Harner et al., 2011; Hoppins et al., 2011; von der Malsburg et al., 2011; Bohnert et al., 2012), providing a dynamic link between protein import, mitochondrial membrane dynamics, and membrane contact sites. The molecular mechanisms of the dynamic interactions between protein machineries of the mitochondrial outer and inner membranes are largely unexplored; we envisage that they will represent important subjects for studies on the regulation of mitochondrial biogenesis and membrane dynamics.

Cotranslational Import and Targeting of mRNAs to Mitochondria

Most in vitro systems used to study mitochondrial protein import operate in a posttranslational manner, i.e., import completely synthesized precursor proteins. It is a longstanding and unresolved debate if and which fraction of mitochondrial precursor proteins are imported in a cotranslational manner in vivo. Cotranslational import is likely of considerably higher importance than previously assumed (Verner, 1993; Quenault et al., 2011). For example, fumarase, which we discussed with regard to its dual localization, cannot be imported into mitochondria posttranslationally, suggesting a translation-coupled (cotranslational) translocation mechanism (Yogev et al., 2007). Regulation between co- and posttranslational transport may represent a further mechanism of import regulation.

Connected to this question, evidence has been presented that mRNAs can be targeted to mitochondria, favoring the synthesis of a number of precursor proteins close to the mitochondrial import sites (Marc et al., 2002). Different views on the molecular mechanism are discussed, ranging from specific targeting of mRNAs via elements in the untranslated regions (Saint-Georges et al., 2008) to the interaction of translating polyribosomes with the TOM machinery. In the latter case, the amino-terminal targeting sequences of nascent polypeptide chains interact with TOM receptors, leading to an association of polyribosomes including mRNA with mitochondria (Eliyahu et al., 2010). Regulation of mRNA targeting to mitochondria will represent an early mechanism of controlling preprotein import (Quenault et al., 2011).

Mitochondrial Protein Import and Disease

Mitochondrial research is of increasing importance for the molecular understanding of numerous diseases, in particular of neurodegenerative disorders. The well-established connection between the pathogenesis of Parkinson's disease and mitochondrial protein import has been discussed above. Several observations point to a possible connection of mitochondrial protein import with the pathogenesis of Alzheimer's disease, though a direct role of mitochondria has not been demonstrated so far. The amyloid-β peptide (Aβ), which is generated from the amyloid precursor protein (APP), was found to be imported into mitochondria by the TOM complex, to impair respiratory activity, and to enhance ROS generation and fragmentation of mitochondria (Hansson Petersen et al., 2008; Ittner and Götz, 2011; Itoh et al., 2013). An accumulation of APP in the TOM and TIM23 import channels has also been reported (Devi et al., 2006). The molecular mechanisms of how mitochondrial activity and dynamics may be altered by Aβ (and possibly APP) and how mitochondrial alterations may impact on the pathogenesis of Alzheimer's disease await further analysis.

It is tempting to speculate that regulatory changes in mitochondrial protein import may be involved in tumor development. Cancer cells can shift their metabolism from respiration toward glycolysis (Warburg effect) (Warburg, 1956; Frezza and Gottlieb, 2009; Diaz-Ruiz et al., 2011; Nunnari and Suomalainen, 2012). A glucose-induced downregulation of import of metabolite carriers into mitochondria may represent one of the possible mechanisms during metabolic shift to glycolysis. Such a mechanism has been shown for the carrier receptor Tom70 in yeast mitochondria (Schmidt et al., 2011). A detailed analysis of regulation of mitochondrial preprotein translocases in healthy mammalian cells as well as in cancer cells will represent an important task for the future.

Conclusion

In summary, the concept of the "mitochondrial protein import machinery as regulatory hub" will promote a rapidly developing field of interdisciplinary research, ranging from studies on molecular mechanisms to the analysis of mitochondrial diseases. In addition to identifying distinct regulatory mechanisms, a major challenge will be to define the interactions between different machineries and regulatory processes, including signaling networks, preprotein translocases, bioenergetic complexes, and machineries regulating mitochondrial membrane dynamics and contact sites, in order to understand the integrative system controlling mitochondrial biogenesis and fitness.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft, Excellence Initiative of the German Federal & State Governments (EXC 294 BIOSS; GSC-4 Spemann Graduate School), Sonderforschungsbereich 746, Bundesministerium für Bildung und Forschung, Trinationales Graduiertenkolleg GRK 1478, and the Ministerium für Innovation, Wissenschaft, und Forschung des Landes Nordrhein-Westfalen.

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